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Comparative toxicity of imidacloprid, of its commercial liquid formulation and of diazinon to a non-target arthropod, the microcrustacean *Daphnia magna*

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Abstract

Imidacloprid (IMI) is at the moment the insecticide with the world's fastest growing sales and is considered possible replacement for the widely used organophosphorus pesticide, diazinon, which is subject to phased revocation in many countries. In this study, biochemical, reproductive and survival parameters of the water flea (*Daphnia magna*) after chronic exposure to IMI, its commercial liquid formulation Confidor SL 200 and diazinon are presented and compared. According to the lowest observed effect concentrations, diazinon is more toxic to the reproduction of *D. magna* than IMI and Confidor SL 200, which exert similar toxicity. The same was observed for the survival, except that Confidor SL 200 is more toxic than IMI. In polluted aquatic environments, the actual levels of diazinon are potentially chronically hazardous to the reproduction of *D. magna* (risk quotient > 1). According to very few measured environmental levels of IMI, the latter is not expected to be chronically hazardous, unless it is accidentally spilled in a small pond. In such case, the predicted concentrations of IMI would present a potential chronic risk to *D. magna*, and a potential acute risk to other aquatic invertebrates. In the future, higher environmental levels of IMI are expected due to its increasing use and physico-chemical properties. The literature survey summarized in this work suggests that further ecotoxicological studies with a broader spectrum of aquatic organisms are needed before IMI is classified as safer than currently applied pesticides.

Keywords: Confidor SL 200; Cholinesterase; Catalase; Glutathione S-transferase

1. Introduction

The insecticide imidacloprid [1-(6-chloro-3-pyridylmethyl)-*N*-nitro-imidazolidin-2-ylideneamine] (IMI) has been increasingly used since 1991 (Elbert et al., 1991) and belongs to the fastest growing group of insecticides introduced to the market, referred to as neonicotinoids (Tomizawa and Casida, 2003). It acts as an agonist of the postsynaptic nicotinic acetylcholine receptors (Matsuda et al., 2001), disrupting the normal neural processes, and is used mainly to control sucking insects on crops (Tomlin, 1997; Tomizawa and Casida, 2005). IMI is a potential groundwater and surface water contaminant (PAN Pesticides database, 2006), because it can leach and runoff from soil and crops (Felsot et al., 1998; Gonzalez-Pradas et al., 1999; Armbrust and Peeler, 2002; Gupta et al., 2002; Fossen, 2006). Additionally, it may enter water bodies from spray drift or accidental spills, leading to local point-source contaminations.

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IMI is considered a possible replacement for urban uses of diazinon (TDC Environmental, 2003), one of the most used insecticides in the last 50 years. Namely, diazinon is currently subject to phased revocation in USA (US EPA, 2004), European Union and Australia (APVMA, 2003), because unacceptable risk to agricultural workers and environment was proved. As a result, the annual use of diazinon has already declined, for instance in USA (California) by 65% in the years 1994–2004 (California DPR, 2004).

To regulate the impacts of IMI on aquatic ecosystems, its toxicological profile needs to be thoroughly established. Until now, the toxicity of IMI to aquatic invertebrates has rarely been assessed and very few monitoring studies of this insecticide have been performed in aquatic environments (Table 1). This is due to the former belief that the compound is relatively immobile in soil and does not leach to groundwater (Bayer technical information for Confidor®, 2000; Krohn and Hellpointner, 2002).

A variety of standard toxicity tests are available for testing the toxicity of chemicals present in aquatic environment. Standard acute (ISO 6341:1996) and chronic (ISO 10706: 2000) toxicity test with the water flea *Daphnia magna* are among the most used, where immobility and reproduction are monitored, respectively. In the case of low concentrations of chemicals, biochemical biomarkers are generally considered a more sensitive and sometimes more specific measure of toxic exposure and effect than the survival, however this approach is not standardised yet (Adams, 2002).

Among the most commonly analyzed biochemical biomarkers are the activities of cholinesterases (ChE), glutathione S-transferase (GST) and catalase (CAT). The inhibition of ChE by organophosphorus and carbamate pesticides results in overaccumulation of the neurotransmitter and, as a consequence, prolonged electrical activity at nerve endings (Chambers, 1992). GST catalyses the conjugation of glutathione with xenobiotics, including organophosphorus pesticides (Booth and O'Halloran, 2001), and the cytotoxic aldehydes produced during lipid peroxidation (Halliwell and Gutteridge, 1999). Catalase decomposes the hydrogen peroxide extensively formed during oxidative stress (Halliwell and Gutteridge, 1999). Protein content in *D. magna* is also used as a biomarker of chronic chemical exposure (Knowles and McKee, 1987), and reflects the entire physiological state of the organism (Printes and Callaghan, 2003).

In this study, chronic effects of IMI on different biochemical, reproductive, and survival parameters of *D. magna* were determined. Chronic effects of IMI on *D. magna* have rarely been evaluated; only one publicly inaccessible study describing the effects of IMI on the reproduction of *D. magna* (Young and Blakemore, 1990) has been conducted so far. The hazards of chemicals were compared using risk quotients (RQ); e.g. the ratio between the estimated/detected environmental concentrations divided by chronic toxicity values (21 d LOEC; the lowest observed exposure concentration that produces a statistically different response from the control response after 21 d) (US EPA, 2004). The chemical was considered potentially chronically hazardous if $RQ > 1$, and acutely hazardous when $RQ > 0.5$. Higher RQ value corresponds to the higher potential risk (US EPA, 2004). The toxicity data of IMI were compared with its commercial liquid formulation (Confidor SL 200; containing 200 g/l of IMI in solvents) and with diazinon.

The aims of this work were: (1) to assess the chronic effects of IMI on biochemical, reproductive, and survival parameters in a non-target arthropod, *D. magna,* and (2) to compare its effects with its commercial liquid formulation Confidor SL 200 and with the organophosphorus pesticide diazinon. The comprehensive literature data on physico-chemical properties and environmental fate of IMI and diazinon and their toxicities to aquatic organisms are provided. The environmental risks of IMI and diazinon based on the actual and expected environmental concentrations are discussed.

2. Materials and methods

2.1. Chemicals

The following chemicals were purchased from Sigma (Germany): dibasic and monobasic potassium phosphate, hydrogen peroxide (30%), 1-chloro-2,4-dinitrobenzene, L-glutathione (reduced form), 5,5-dithiobis-2-nitrobenzoic acid, sodium hydrogen carbonate, acetylthiocholine chloride, and ethylenediaminetetraacetic acid. BCA Protein Assay Reagents A and B were purchased from Pierce (USA). Diazinon and 1-methyl-2-pyrrolidone were provided by Pestanal, Riedel-de Haën (Seelze, Germany); imidacloprid, Confidor SL 200 by Bayer CropScience AG (Monheim, Germany), and dimethylsulfoxide by Merck (Darmstadt, Germany). All chemicals were of the highest commercially available grade, typically 99% or higher.

2.2. Chronic toxicity test with D. magna Straus 1820 (water flea)

Water fleas (*D. magna* Straus 1820) were obtained from the Institut für Wasser, Boden und Lufthygiene des Umweltbundesamtes (Berlin, Germany). They were cultured in 2.5 l of modified M4 media (Kühn et al., 1989) at 21 ± 1 °C and 16:8 h light/dark regime (1800 lux) with a diet of the algae *Desmodesmus subspicatus* Chodat 1926 corresponding to 0.13 mg carbon/daphnia per day.

Our laboratory is accredited according to ISO 17025:1999 for standard acute testing with *D. magna*. Chronic toxicity to daphnids was evaluated using a semi-static exposure system under the same conditions as culturing (ISO 10706: 2000). Individual daphnids less than 24 h old were placed in 50 ml of test solution; 10 test containers per each concentration and a control were prepared. Chronic tests for each chemical were repeated up to three

Table 1
Properties of IMI and diazinon

	Diazinon	Ref. no.	IMI	Ref. no.
First introduced commercially	1952; J.R. Geigy S.A. (Novartis Crop Protection AG)	28	1991; Bayer AG and Nihon Tokushu Noyaku Seizo KK	28
Physico-chemical properties				
Water solubility at 20 °C (mg l^{-1})	60	28	(a) 610	(a) 28
			(b) 514	(b) 11
K_{oc} (soil organic carbon–water partitioning coefficient)	(a) 1589 (20 °C)	(a) 19	(a) 210 (20 °C)	(a) 19
	(b) 1520	(b) 26	(b) 249–268	(b) 20
	(c) 851 ± 180	(c) 6	(c) 109–411 (20 °C)	(c) 16
Log K_{ow} (octanol–water partition coefficient)	(a) 3.14 (20 °C)	(a) 28	(a) 0.57 (22 °C)	(a) 28
	(b) 3.3 (25 °C)	(b) 26	(b) 0.92 (20 °C)	(b) 19
	(c) 3.81 (20 °C)	(c) 19	(c) 0.589 (22 °C)	(c) 14
Average application rate (kg of active ingredient ha^{-1})	(a) 3.0–3.1 (orchard)	(a) 5	(a) 0.3–0.5 (soil)	(a) 24
	(b) 0.5 (foliar); 4 (soil); 1–3 (fruit)	(b) 30		
Environmental fate				
Detected aquatic concentrations (μg l^{-1})	(a) 3.34 (Salinas river, California, USA)	(a) 3	(a) 1.6 (sea Wilapa Bay, USA)	(a) 8
	(b) 6.8 (Sacramento river watershed, USA)	(b) 13	(b) 1 (surface water, Florida, USA)	(b) 22
	(c) 0.775 (Greece rivers, EU)	(c) 15	(c) 14 (Lake Wales Ridge, USA)	(c) 31
	(d) 24.6 (Vicinity of pesticide factory, Egypt)	(d) 1	(d) 6.7 (ground water, New York, USA)	(d) 11
Estimated aquatic concentrations	8.89–429 μg l^{-1} (depends on the type of application on the crop)	30	(a) 36.04 μg l^{-1} (acute surface water exposure); 17.24 μg l^{-1} (chronic surface water exposure)	(a) 11
			(b) 22 μg l^{-1} (accidental direct spray in a pond or stream); 1.8–7.3 mg l^{-1} (accidental spill in a small pond)	(b) 24
Aqueous photolysis DT_{50}	140 d	26	(a) 3 h (simulated sunlight, 30 °C)	(a) 14
			(b) 1.2 h (d H_2O, $\lambda = 290$ nm, 24 °C)	(b) 18
			(c) 0.7 h (d H_2O, $\lambda = 280$ nm); 2.1 h (Confidor; d H_2O, $\lambda = 280$ nm)	(c) 32
			(d) 1 h (d H_2O, simulated sunlight)	(d) 16
Hydrolysis DT_{50} (d)	(a) 12 (pH 5.0); 138 (pH 7.0); 77 (pH 7.7)	(a) 30	(a) 168 (26 °C, pH 4.7, 7.7, 9.0)	(a) 14
	(b) 3 (natural water pH 9.0, 12 h photoperiod)	(b) 10	(b) 90 (20 °C, pH 3, 5, 7)	(b) 36
	(c) 5, highly depends on pH	(c) 26	(c) >30	(c) 11
			(d) 37.5 (Confidor 200 SL); 41 (Gaucho 70 WS) (pH 7.0, 30 °C)	(d) 23
Soil photolysis DT_{50} (d)	5	26	(a) 39	(a) 16
			(b) 38.9	(b) 11
Soil anaerobic DT_{50} (d)	17	26	27.1	11
Soil aerobic DT_{50} (d)	39	26	(a) 156	(a) 16
			(b) 997	(b) 11
Field dissipation DT_{50} (d)	(a) 5.4–27 (lower value in moist, irradiated sandy soil)	(a) 12	(a) 190 (no vegetation), 45 (vegetation); 180 (sandy and silt loam)	(a) 20
	(b) 7–87.5 (lower value in non-sterile sandy loam)	(b) 33	(b) 74–156 (20 °C, bare soil); 30–160 (sediments)	(b) 16
	(c) 5–20	(c) 30	(c) 27–229	(c) 11
	(d) 3–13	(d) 26		
Toxicity				
WHO classification	II = moderately hazardous	34	II = moderately hazardous	34
Fish: (1) Rainbow trout *Oncorhynchus mykiss*	(1a) LC_{50} (96 h) = 90–400 μg l^{-1}	(1a) 29	(1a) LC_{50} (96 h) = 211 mg l^{-1}	(1a) 11
	(1b) LC_{50} (96 h) = 20 μg l^{-1}	(1b) 26	(1b) LC_{50} (96 h) >83 mg l^{-1}	(1b) 24
	(1c) LC_{50} (96 h) = 90–400 μg l^{-1}	(1c) 7	-LC_{50} (96 h) = 211 mg l^{-1}	
			-LOLC (96 h) = 64 mg l^{-1}	
			-LOLC (96 h) = 281 mg l^{-1}	
(2) Bluegill *Lepomis machrochirus*	(2a) LC_{50} (96 h) = 136 μg l^{-1}; 168 μg l^{-1}; 460 μg l^{-1}	(2a) 29	(2) LC_{50} (96 h) >105 mg l^{-1}	(2) 24
			-LOLC (96 h) = 42 mg l^{-1}	
	(2b) LC_{50} (96 h) = 120–670 μg l^{-1}	(2b) 7		
(3) Zebrafish *Danio rerio*	(3) LC_{50} (96 h) = 10 mg l^{-1}	(3) 17	(3) LC_{50} (96 h) = 241 mg l^{-1}	(3) 21

(*continued on next page*)

Table 1 (*continued*)

	Diazinon	Ref. no.	IMI	Ref. no.
Aquatic invertebrates: (1) Water flea *D. magna*	(1a) LC_{50} (48 h) = 0.96 μg l^{-1} (1b) LC_{50} (48 h) = 0.83; 1.1 μg l^{-1} (1c) EC_{50} (48 h) = 0.9 μg l^{-1} (1d) $NOEC_{repr.}$ (21 d) = 5 μg l^{-1} (1f) $LOEC_{repr.}$ (21 d) = 0.15–0.25 μg l^{-1}	(1a) 28 (1b) 29 (1c) 17 (1d) 27 (1f) 9	(1a) LC_{50} (48 h) = 85 mg l^{-1} (1b) LC_{50} (48 h) = 10.4 mg l^{-1} (1c) EC_{50} (48 h) = 56.6 mg l^{-1} (1d) $LOEC_{repr.}$ (21 d) = 7.3 mg l^{-1} (1f) $LOEC_{repr.}$ (21 d) = 2.5 mg l^{-1}	(1a) 11 (1b) 25 (1c) 21 (1d) 35 (1f) 27
(2) Amphipod *Hyalella azteca*	(2a) LC_{50} (96 h) = 6.51 μg l^{-1} (2b) LC_{50} (96 h) = 4.3 μg l^{-1}	(2a) 4 (2b) 2	(2) LC_{50} (96 h) (juveniles) = 0.526 mg l^{-1} –LC_{50} (96 h) (14–21 d old) = 51.8 mg l^{-1} –LC_{50} (96 h) (7–21 d old) = 94.8 mg l^{-1} –$LOEC_{immobility}$ (96 h) (juveniles) = 0.00097 mg l^{-1} –LOLC (96 h) (14–21 d old) = 43.8 mg l^{-1} –$NOEC_{immobility}$ (96 h) (7–21 d old) = 94.8 mg l^{-1}	(2) 24
(3) Midge *Chironomus tentans*	(3) LC_{50} (96 h) = 10.7 μg l^{-1}	(3) 4	(3) LC_{50} (96 h) (2nd instar) = 0.0105 mg l^{-1}; –LOLC (96 h) (2nd instar) = 0.00339 mg l^{-1}	(3) 24

Abbreviations: DT_{50} (half life); EC_{50} (median effective concentration for immobility); LC_{50} (median lethal concentration); $LOEC_{repr./immobility}$ (lowest observed effect concentration for reproduction/immobility); LOLC (lowest observed lethal concentration); $NOEC_{repr./immobility}$ (no observed effect concentration for reproduction/immobility); OC (organic carbon).

References: Abdel-Halim et al., 2006 (1); Anderson and Lydy, 2002 (2), Anderson et al., 2003 (3); Ankley and Collyard, 1995 (4); Cobb et al., 2000 (5); Cooke et al., 2004 (6); Eisler, 1986 (7); Felsot and Ruppert, 2002 (8); Fernandez-Casalderrey et al., 1995 (9); Ferrando et al., 1992 (10); Fossen, 2006 (11); Graebing and Chib, 2004 (12); Hall, 2003 (13); Kagabu and Medej, 1995 (14); Konstantinou et al., 2006 (15); Krohn and Hellpointner, 2002 (16); Lee et al., 1993 (17); Moza et al., 1998 (18); Nemeth-Konda et al., 2002 (19); Oi, 1999 (20); Our laboratory, unpublished (21); Pfeuffer and Matson, 2001 (22); Sarkar et al., 1999 (23); SERA, 2005 (24); Song et al., 1997 (25); TDC Environmental, 2003 (26); This study (27); Tomlin, 1997 (28); US EPA, 1999 (29); US EPA, 2004 (30); US Geological Survey, 2003 (31); Wamhoff and Schneider, 1999 (32); Watanabe and Grismer, 2001 (33); WHO, 2005 (34); Young and Blakemore, 1990 (35); Zheng and Liu, 1999 (36).

times. The survived initial daphnids were transferred into freshly prepared test solutions three times per week. The animals were fed daily a diet of *Desmodesmus subspicatus* (0.13 mg carbon/daphnia per day) and the newly born neonates were counted and removed. The criteria used to evaluate reproduction after 21 d were the number of neonates per adult, the average brood size per adult, the number of broods per adult, and the time to the first reproduction. The mortality of the daphnids during 21 d was also monitored.

The following concentrations of IMI: 0, 0.625, 1.25, 2.5, 5, 10, 20, 40 mg l^{-1} and diazinon: 0, 0.0753, 0.165, 0.312, 0.625, 1.25, 2.5, 5, 8 μg l^{-1} were tested. Confidor SL 200 was diluted in distilled water to obtain the following solutions: 0, 0.000625, 0.00125, 0.0025, 0.005, 0.01, 0.02% (v/v); which contained 0, 1.25, 2.5, 5, 10, 20, 40 mg l^{-1} of IMI, respectively. The toxicity of solvents incorporated in Confidor SL 200 (a solution consisting of 38.4% of dimethylsulfoxide, 37.5% of 1-methyl-2-pyrrolidone and 24.1% of distilled water in place of IMI) was tested to exclude the possible toxic effect. The concentration of this negative control was equivalent to the highest concentration of Confidor SL 200 used in the tests (0.02%; v/v).

2.3. Monitoring of the stability of test chemicals during the tests

The test media were changed every two days. Prior to toxicity tests, the stabilities of IMI and diazinon in the test solution were checked. The test solutions were exposed separately to the same experimental conditions as the toxicity tests and the concentrations of the specific chemicals were measured at the outset and after two days.

Diazinon solution (10 ml) was extracted with three portions of ethyl acetate (25, 20 and 10 ml) with the addition of 50 ml of a 10% aqueous solution of sodium hydrogen carbonate (Bavcon et al., 2003). The solvent was evaporated, and the residue redissolved in 1 ml of ethyl acetate and analysed by gas chromatography (HP 6890, Germany) with a flame ionization detector. Extraction of IMI was performed on initially preconditioned Strata C18-E columns (Phenomenex, USA) with 5 ml of methanol and 5 ml of distilled water (Baskaran et al., 1997). 1 ml of IMI solution was added to the column, and afterwards eluted with 2 ml of methanol. The solvent was evaporated and dried IMI was dissolved in 1 ml of acetonitrile–water (20:80, v/v) solution. The samples were analyzed on Agilent 100 Series liquid chromatograph (Germany) equipped with DAD detector on Zorbax C8 column.

Our experiments showed no changes in concentrations of IMI and diazinon in test solutions during two days of exposure to the same experimental conditions as in the toxicity tests. No degradation products of diazinon were detected. The actual exposure concentrations of both chemicals did not differ by more than 20% from the nominal or initial concentrations. Therefore the results are given in nominal concentrations, as suggested by ISO 10706: 2000.

2.4. Determination of enzyme activities

For each experiment, 10 test containers per each concentration of the chemical were prepared. After 21 d in presence of the chemicals, five adult daphnids per concentration were combined into one enzyme sample, thus two samples were prepared for each concentration. Since each experiment was repeated three times, a total of six samples per concentration were prepared. Prior to homogenization, excess chemical was removed from the surface of the animals by rinsing three times with 2 ml of 50 mM phosphate buffer pH 7.0 combined with 5 mM ethylenediaminetetraacetic acid (Jemec et al., 2007). The animals were then homogenized for 3 min in 0.8 ml of 50 mM phosphate buffer pH 7.0, using a glass–glass Elvehjem–Potter homogenizer. The homogenate was centrifuged for 25 min at 15 000*g* and 4 °C. Enzyme activities were measured on freshly prepared supernatants.

ChE activity was determined according to Ellman et al. (1961), using microtiter plates (Bio-Tek® Instruments, USA; PowerWave™ XS) as described by Jemec et al. (2007). The reaction mixture was prepared in 100 mM of potassium phosphate buffer pH 7.3 containing acetylthiocholine chloride and 5,5′-dithiobis-2-nitrobenzoic acid in the final concentrations of 1 mM and 0.5 mM, respectively. 100 µl of protein supernatant were added to start the reaction, which was followed spectrophotometrically at 412 nm and 25 °C for 15 min.

GST activity was measured on microtiter plates (Bio-Tek® Instruments, USA; PowerWave™ XS) (Habig et al., 1974; Jemec et al., 2007). 1-Chloro-2,4-dinitrobenzene was dissolved in ethanol to obtain a 50 mM solution, which was afterwards diluted with 100 mM potassium phosphate buffer pH 6.5 to the final concentration of 4 mM. This solution was used to prepare a reaction mixture containing 1 mM of 1-chloro-2,4-dinitrobenzene and 1 mM of reduced glutathione. 50 µl of protein supernatant were added to start the reaction, which was followed spectrophotometrically at 340 nm and 25 °C for 3 min.

CAT activity was determined according to Aebi (1984). 50 µl of protein supernatant were combined with 750 µl of hydrogen peroxide solution (10.8 mM) prepared in 50 mM potassium phosphate buffer pH 7.0. The final concentration of hydrogen peroxide was 10 mM. The reaction was followed spectrophotometrically for 3 min at 25 °C and 240 nm on a Shimadzu UV-2101PC spectrophotometer (Japan).

Protein concentration was measured using a BCA™ Protein Assay Kit, a modification of the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

2.5. Interpretation of enzyme activities

Enzyme activities were expressed in enzyme units (EU) per one adult daphnia. Specific enzyme activities with protein content as a standard reference were also calculated for purposes of comparison. One EU was determined as the amount of ChE that hydrolyses 0.01 nmoles of acetylthiocholine min^{-1} ($\varepsilon_{412} = 13600\ M^{-1}\ cm^{-1}$), the amount of CAT that degrades 1 µmole of hydrogen peroxide min^{-1} ($\varepsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$), and the amount of GST that conjugates 1 nmole of reduced glutathione min^{-1} ($\varepsilon_{340} = 9600\ M^{-1}\ cm^{-1}$). These enzyme units were chosen to facilitate the graphical comparison of all enzyme activities for each chemical.

2.6. Data analysis

The 21 d LOEC values (e.g. the lowest observed effect concentration that produces a statistically different response from the control response after 21 d) were determined by one-way analysis of variance (ANOVA; $P < 0.05$), and the Games–Howell post-hoc test for biochemical parameters and Dunnett's test for reproduction data, using SPSS for Windows 8.0 (SPSS Inc., USA). The LOLC value for mortality was determined as the lowest observed lethal concentration that causes mortality higher than 20% as allowed for control organisms by the ISO standard (ISO 10706: 2000). The results for IMI and Confidor SL 200 were fitted to sigmoid curves to calculate the slopes using the GOSA Software (www.bio-log.biz, France). The values for unexposed control animals were not included in the data fitting, but they are shown on graphs for comparison.

2.7. Calculation of risk quotients (RQ) of tested chemicals

Risk quotients (RQ) for all tested chemicals were calculated as a ratio between the estimated/detected environmental concentrations divided by the LOEC for biochemical parameters and reproduction, and LOLC for survival determined in this study. For the comparison of the hazards of diazinon and IMI to different species of freshwater invertebrates and vertebrates, RQ were calculated using LC_{50} (96 h) values based on literature data.

Only four monitoring studies are at the moment available on environmental levels of IMI (Table 1). To determine RQ values for IMI, the lowest (1 $\mu g\ l^{-1}$), and the highest (14 $\mu g\ l^{-1}$) measured values, estimated chronic value in surface waters (17.24 $\mu g\ l^{-1}$), and estimated worse-case scenario level of accidental spill in a small pond (7300 $\mu g\ l^{-1}$) were used (Table 1). On the other hand, diazinon has been extensively monitored. The lowest (0.775 $\mu g\ l^{-1}$), and the highest (24.6 $\mu g\ l^{-1}$) recently reported values in the literature, and the estimated value in surface waters (429 $\mu g\ l^{-1}$) were used for calculation (Table 1).

3. Results

3.1. Chronic toxicity tests

In standard chronic toxicity tests with *D. magna*, reproduction and mortality of adult daphnids were assessed.

Table 2
21 d LOEC (lowest observed effect concentration) values for biochemical and reproduction data, and 21 d LOLC (lowest observed lethal concentration) values for mortality data

Chemical	Endpoint								
	Number of neonates per adult	Brood size	Days to first brood	Number of broods per adult	Mortality	GST	CAT	ChE	Total protein content
IMI (mg l^{-1})	2.5	5	5	10	40	2.5	5	10	1.25
Confidor SL 200 (in mg l^{-1} of IMI)	5	5	5	10	10	5	2.5	5	2.5
Diazinon (μg l^{-1})	5 < (L)OEC < 8	5 < (L)OEC < 8	5 < (L)OEC < 8	5 < (L)OEC < 8	8[a]	5 < (L)OEC < 8	N.d.[b]	5 < (L)OEC < 8	5 < (L)OEC < 8

The following concentrations of analytical grade IMI: 0, 0.625, 1.25, 2.5, 5, 10, 20, 40 mg l^{-1}; IMI formulated as Confidor SL 200: 0, 1.25, 2.5, 5, 10, 20, 40 mg l^{-1} of IMI, and diazinon: 0, 0.0753, 0.165, 0.312, 0.625, 1.25, 2.5, 5, 8 μg l^{-1} were tested.

[a] 100% mortality of adult daphnids was observed at this concentration. At lower tested concentration (5 μg/l), no statistically significant mortality was detected (≤20%).

[b] LOEC could not be determined due to insignificant trend.

These data for all chemicals are shown in Table 2, columns 2–6. The negative control (solvent mixture commercially used for the preparation of Confidor SL 200) did not have any adverse effects on *D. magna* at the highest tested concentration of this chemical (0.02%; v/v).

Tested concentrations of IMI and Confidor SL 200 have similar impacts on the reproduction of *D. magna* (21 d LOEC = 2.5–10 mg l^{-1} for different reproduction parameters), but Confidor SL 200 (21 d LOLC = 10 mg l^{-1} of IMI) affected their survival at lower concentrations than IMI (21 d LOLC = 40 mg l^{-1}) (Table 2, Fig. 1).

Up to 5 μg l^{-1} of diazinon, the reproduction of daphnids was not affected. At this concentration the mortality was 20%. At the next tested concentration of diazinon (8 μg l^{-1}), the 100% mortality of daphnids was observed (Table 2).

3.2. Enzyme activities

In this study, the results of enzyme activities are expressed per animal and not per protein content, since the changes in protein content were observed as a result of exposure to the chemicals. The activities of all analyzed enzymes and the protein content in animals exposed to increasing concentrations of IMI and Confidor SL 200 decreased significantly (Figs. 2a and b, Table 2).

In the experiments with diazinon, protein content of daphnids, ChE and GST activities did not change at any of the concentrations tested (up to 5 μg l^{-1}). Contrary to other analysed enzymes, CAT activities significantly decreased at 0.312, 0.625 and 1.25 μg l^{-1} of diazinon, but not at the highest concentrations 2.5 and 5 μg l^{-1} (Fig. 2c, Table 2).

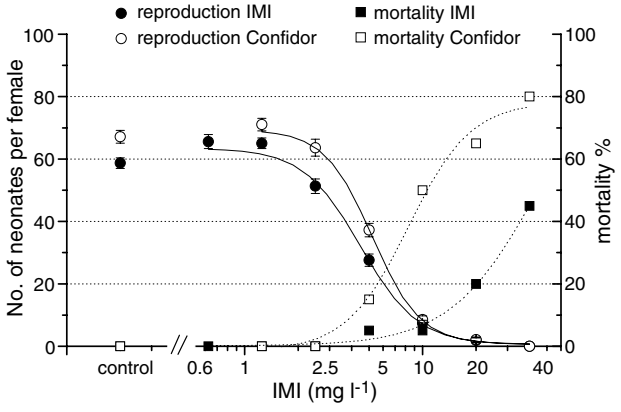

Fig. 1. Effects of IMI and Confidor SL 200 on the reproduction (number of neonates per female) and survival of *D. magna.* Data for reproduction are shown as mean of six replicates ± standard error of mean, and for survival as mean of three replicates. Data were fitted using sigmoid curves with the following slopes: −2.36 ± 0.42 and 1.81 ± 1.52 for the reproduction and survival of IMI, respectively, and −2.88 ± 1.00 and 2.63 ± 1.36 for the reproduction and survival of Confidor SL 200, respectively (95% confidence interval). The values for control unexposed animals were not included when fitting the data, but they are shown on graphs for comparison.

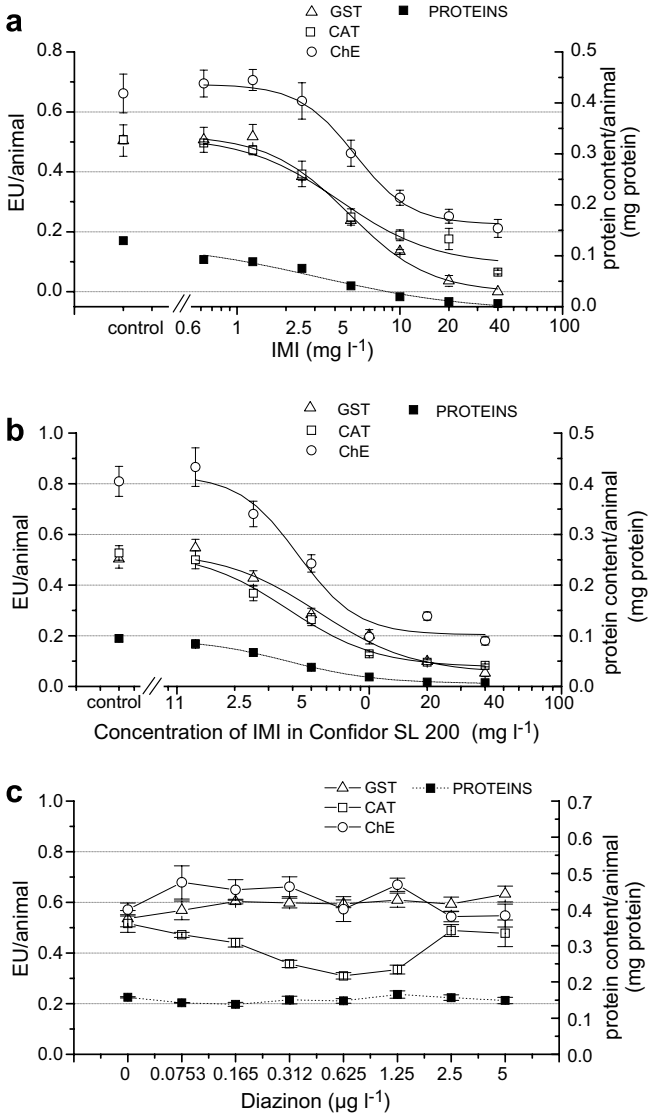

Fig. 2. GST, CAT, ChE activities and protein content in *D. magna* exposed to IMI (2a), Confidor SL 200 (2b), and diazinon (2c) (mean of six replicates ± standard error of mean). Data for daphnids exposed to IMI and Confidor SL 200 were fitted using sigmoid curves. The values for control unexposed animals were not included when fitting the data, but they are shown on graphs for comparison. The slopes of the sigmoid curves for the GST, CAT, ChE activities and protein content in daphnids exposed to IMI were: -1.39 ± 1.39, -1.32 ± 1.56, -2.24 ± 2.28 and -2.44 ± 1.22, respectively, and in the case of Confidor SL 200: -0.98 ± 3.31, -1.12 ± 3.69, -1.68 ± 3.656 and -2.00 ± 3.25, respectively (95% confidence interval). Enzyme units (EU) were defined as: the amount of ChE that hydrolyses 0.01 nmoles of acetylthiocholine min^{-1}, the amount of CAT that degrades 1 µmole of hydrogen peroxide min^{-1}, and the amount of GST that conjugates 1 nmole of reduced glutathione min^{-1}.

To point out the importance of careful interpretation of enzyme activities, in case the protein content is changed during the exposure, specific enzyme activities per protein content were also calculated. In this case, CAT, GST and ChE activities increased significantly when exposed to IMI and Confidor SL 200. In the case of diazinon, enzyme activities were the same when calculated per animal or per protein content, since the protein content in this case did not change (not shown).

3.3. Risk quotients of tested chemicals

RQ values were calculated on the basis of recently detected and predicted aquatic levels of the chemicals tested, and on chronic toxicity data on *D. magna* gained in this work. These data show that only actual measured environmental levels of diazinon have RQ values higher than one, indicating them as potentially chronically hazardous to the reproduction of *D. magna* (Table 3), while RQ values for Confidor SL 200 and IMI are lower than one. In the case of an accidental spill, estimated concentrations of IMI and Confidor SL 200 would pose a serious chronic risk to the reproduction and selected enzyme activities of *D. magna* (RQ > 1).

Based on recent literature data, diazinon has higher RQ values for aquatic organisms than IMI, but in general both chemicals are more harmful to aquatic invertebrates than fish (Table 4). Actual measurements of diazinon levels in the environment show that this insecticide is more hazardous to aquatic invertebrates (the highest calculated RQ = 117) than IMI (the highest calculated RQ = 1.4). However, the risk of estimated concentrations of IMI to aquatic invertebrates in the case of an accidental spill (the highest calculated RQ = 695.2) is very high (Table 4).

4. Discussion

In this study, chronic effects of imidacloprid, its commercial liquid formulation Confidor SL 200 and the organophosphorus pesticide diazinon on different biochemical, reproductive, and survival parameters in *D. magna* were assessed and compared.

Enzyme activities were expressed per animal and not per protein amount, because significant changes of the latter were found in daphnids exposed to IMI and Confidor SL 200. This suggests that increasing concentrations of these chemicals affected not only the investigated enzymes, but proteins in general. Consequently, enzyme activities expressed per protein content differ from those expressed per animal, implying that cautious interpretation of enzyme activities is needed in toxicity experiments. Similar point was raised by Printes and Callaghan (2003).

The activities of ChE, GST and CAT in control adult daphnids (22 d old) expressed per protein content were: 0.61 ± 0.043 nmol min^{-1} mg^{-1} protein; 87.26 ± 6.67 nmol min^{-1} mg^{-1} protein and 84.28 ± 4.84 µmol min^{-1} mg^{-1} protein, respectively. These values are lower than those previously recorded in juvenile daphnids (2.5–62.3 nmol min^{-1} mg^{-1} protein for ChE (Guilhermino et al., 1996; Diamantino et al., 2000; Barata et al., 2001), 250 nmol min^{-1} mg^{-1} protein for GST (Barata et al., 2005), and 250 µmol min^{-1} mg^{-1} protein for CAT (Barata et al., 2005)). This is in agreement with Printes and Callaghan (2003) who observed significantly lower ChE activity in 14–21 d old

Table 3
Calculated risk quotients (RQ) of tested chemicals for *D. magna* (based on 21 d LOEC (lowest observed effect concentration) for biochemical parameters (bio. param.), reproduction, and 21 d LOLC (lowest observed lethal concentration) for survival)

Chemical	Exposure concentration ($\mu g\,l^{-1}$)[b]	$RQ_{bio.\ param.}$	$RQ_{reproduction}$	$RQ_{survival}$
IMI	(a) Highest detected: 14	(a) 0.0112	(a) 0.0056	(a) 0.00035
	(b) Lowest detected: 1	(b) 0.0008	(b) 0.0004	(b) 0.000025
	(c) Estimated (chronic surface water): 17.24	(c) 0.0138	(c) 0.0069	(c) 0.00043
	(d) Estimated (accidental spill): 7300	(d) 5.8[a]	(d) 3[a]	(d) 0.183
IMI in Confidor	(a) Highest detected: 14	(a) 0.0056	(a) 0.0028	(a) 0.0014
	(b) Lowest detected: 1	(b) 0.0004	(b) 0.0002	(b) 0.0001
	(c) Estimated (chronic surface water): 17.24	(c) 0.0069	(c) 0.00345	(c) 0.00172
	(d) Estimated (accidental spill): 7300	(d) 3[a]	(d) 1.46[a]	(d) 0.73
Diazinon	(a) Highest detected: 24.6	(a) >3.1[a]	(a) >3.1[a]	(a) 3.1[a]
	(b) Lowest detected: 0.775	(b) >0.097	(b) >0.097	(b) 0.097
	(c) Estimated: 429	(c) >53.6[a]	(c) >53.6[a]	(c) 53.6[a]

[a] Potentially chronically hazardous to *D. magna* (RQ > 1) (US EPA, 2004).
[b] Please refer to Table 1 for references on exposure concentrations.

Table 4
Calculated risk quotients (RQ) of diazinon and IMI for freshwater invertebrates and vertebrates (fish) (based on LC_{50} (96 h))

Chemical	Exposure concentration ($\mu g\,l^{-1}$)[b]	RQ: Aquatic invertebrates[b]	RQ: Vertebrates (fish)[b]
IMI	(a) Highest detected: 14	0.0266–1.4[a]	0.000058–0.000168
	(b) Lowest detected: 1	0.0019–0.095	0.0000041–0.000012
	(d) Estimated (accidental spill): 7300	13.8[a]–695.2[a]	0.031–0.0879
Diazinon	(a) Highest detected: 24.6	0.145–117[a]	0.0025–1.23[a]
	(b) Lowest detected: 0.775	0.0046–3.7[a]	0.000077–0.0387
	(c) Estimated: 429	2.52[a]–2043[a]	0.0429–21.45[a]

[a] Potentially acutely hazardous to selected aquatic organisms (RQ > 0.5) (US EPA, 2004).
[b] Please refer to Table 1 and TDC Environmental (2003) for references on exposure concentrations and LC_{50} (96 h) data, respectively.

daphnids (0.5 nmol min^{-1} mg^{-1} protein) compared to 1–2 d old juveniles (2.5 nmol min^{-1} mg^{-1} protein), and with our previous study (Jemec et al., 2007), where the activities of juvenile daphnids were significantly higher than the values published here for the adult ones. This apparent inverse relationship between the age and enzyme activity was related to an increase in total protein of the animals during aging, which is not proportional to the increase in the rate of substrate hydrolysis (Printes and Callaghan, 2003).

Our results indicate that tested concentrations of IMI and Confidor SL 200 have similar impacts on the reproduction of *D. magna*, but Confidor SL 200 affected survival at lower concentrations than IMI, possibly due to the synergism between the solvents and IMI. The same was noticed for biochemical parameters, where Confidor SL 200 was slightly more toxic than IMI (Table 2). The LOECs (2.5 mg l^{-1}) for the number of neonates per adult exposed to IMI are similar to those reported by Young and Blakemore (1990), who found the LOEC for reproduction at 7.3 mg l^{-1}.

The activities of all enzymes exposed to increasing concentrations of IMI and Confidor SL 200 were significantly decreased in this study. No data on the chronic effect of IMI on ChE, GST and CAT activities in daphnids are available in the literature. Only one study by Capowiez et al. (2003) showed no acute effects on ChE and GST activities in earthworms exposed up to 1 mg l^{-1} of IMI. The sensitivities of enzymes and reproduction end-points of animals exposed to IMI in our study are similar (e.g. similar LOEC). This suggests that the decrease of enzyme activities in this case is probably not an early, sensitive biomarker of stress, but reflects a generally impaired physiological state of an organism.

In animals exposed to diazinon, no effects on the reproduction and survival of daphnids up to 5 $\mu g\,l^{-1}$ of diazinon were observed. However, already at 8 $\mu g\,l^{-1}$, 100% mortality was determined. Published data on the LOEC values for the reproduction of *D. magna* exposed to diazinon are very inconsistent. Fernandez-Casalderrey et al. (1995) reported LOEC values for the reproduction in the range of 0.15–0.25 $\mu g\,l^{-1}$, while Sanchez et al. (1998) found significantly lower LOEC values for the same endpoint performed in similar experimental setup (0.00005–0.0005 $\mu g\,l^{-1}$ of diazinon). Our higher LOEC values for the reproduction of daphnids might be explained by differences in daphnid clones, and experimental setup.

ChE activity was reported to be inhibited in daphnids exposed to organophosphates (Day and Scott, 1990; Gälli et al., 1994), but no study has been performed on the effects of organophosphorus pesticide diazinon on ChE activity in *D. magna* yet. Inhibition of ChE activity was found in other organisms exposed to diazinon, for example in the

white shrimp *Litopenaeus vannamei* exposed to 12 μg l^{-1} of diazinon for 7 d (Gallindo-Reyes et al., 2000), earthworm *Aporrectodea caliginosa* exposed to diazinon at 12 mg kg^{-1} (dry weight of soil) (Booth and O'Halloran, 2001) and isopod *Porcellio scaber* at 5 μg g^{-1} of leaf (Stanek et al., 2006). However, this paper and our previous work (Jemec et al., 2007) indicate that ChE activity does not change in daphnids acutely and chronically exposed up to 5 μg l^{-1} of diazinon. The differences in the changes of ChE activity after diazinon exposure can be explained by species-specific biotransformation and detoxification mechanisms of diazinon to a more potent diazoxon (Keizer et al., 1995). The induction of GST activity in diazinon-treated organisms was expected, because GST is able to detoxify this insecticide (Chambers, 1992), but in this study no GST induction was detected when animals were exposed up to 5 μg l^{-1} of diazinon. The same observation was reported in our previous paper, where no changes of GST activity were observed in daphnids acutely exposed up to 7 μg l^{-1} of diazinon (Jemec et al., 2007). No other studies on chronic effects of diazinon on GST activity in daphnids have previously been published.

There are very few data on environmental levels of IMI (only four studies in USA), due to its irregular monitoring in aquatic environment. Based on our results, the levels of IMI in freshwaters that have been detected so far (1–14 μg l^{-1}), are not expected to be chronically hazardous to the reproduction and survival of *D. magna* (RQ < 1), however the same data are reported to pose potential acute risk to some other aquatic invertebrates (RQ = 1.4). In comparison to diazinon, actual aquatic levels of IMI are less hazardous (higher RQ) to aquatic invertebrates, thus IMI is considered a possible replacement for diazinon (US EPA, 2004). However, in the case of accidental spill, estimated concentrations of IMI can also pose a potential chronic risk to the reproduction of *D. magna* (RQ = 3), and acute risk to other aquatic invertebrates (the highest calculated RQ = 695.2). Additionally, due to the increasing use of IMI, one might expect significantly higher aquatic levels in the future. IMI also has more physico-chemical properties that would favour its appearance in surface waters when compared to diazinon (Table 1). It has higher water solubility, lower octanol–water partition coefficient (K_{oc}), lower potential for sorption on soil (K_{ow}), and is more stable to hydrolysis and soil degradation. Due to these characteristics, IMI is quite mobile in the environment and stable on application sites, and it is very likely to be washed off the application sites, especially off impervious surfaces (Oi, 1999). It degrades relatively quickly by aqueous photolysis, but such decomposition can only occur at the surface of well-sunlit waters (TDC Environmental, 2003; Fossen, 2006).

The toxicity of IMI is supposed to be very highly specific towards insects in comparison to mammals, due to specific binding to the postsynaptic nicotinic acetylcholine receptors (nAChR) of insects (Tomizawa and Casida, 2003). However, limited attention was paid to binding affinity of IMI to the nAChRs of other arthropods or more generally invertebrates. Additionally, the toxicity is not solely the result of binding between the ligand and the receptor, but depends on many activities in the organism, such as the metabolism of the chemical or its interactions with cell components. It was shown that the toxicity of IMI towards aquatic invertebrates varies, with *D. magna* being less sensitive than others, for instance amphipod *Hyalella azteca* or midge *Chironomus tentans,* and having acute LC_{50} values in the same concentration range as fish (Table 1, Table 4). This suggests that the toxicity of IMI is species-specific and may not easily be extrapolated to other organisms. Relevant toxicity data could be obtained only when toxicity is tested with organisms belonging to different taxonomic groups and trophic levels.

In conclusion, according to LOEC values, diazinon is more toxic to the reproduction of *D. magna* than IMI and Confidor SL 200, which show similar toxicity. The same was observed for the survival, except that commercial formulation (Confidor SL 200) is more toxic than pure grade IMI. The actual aquatic levels of diazinon are potentially chronically hazardous to the reproduction of *D. magna* (RQ > 1), while recently detected concentrations of IMI are not. Higher environmental levels of IMI are expected in the future due to its increasing application and higher risk to aquatic organisms is anticipated. Additionally, we have shown that in case IMI was accidentally spilled in a small pond, its predicted environmental concentrations would chronically affect less sensitive organisms like *D. magna* and acutely affect other, more sensitive aquatic invertebrates. Toxicity data on IMI presented so far indicate that IMI is highly species-specific, therefore further (eco)toxicological studies have to be performed with organisms belonging to different taxonomic groups, trophic levels and habitats.

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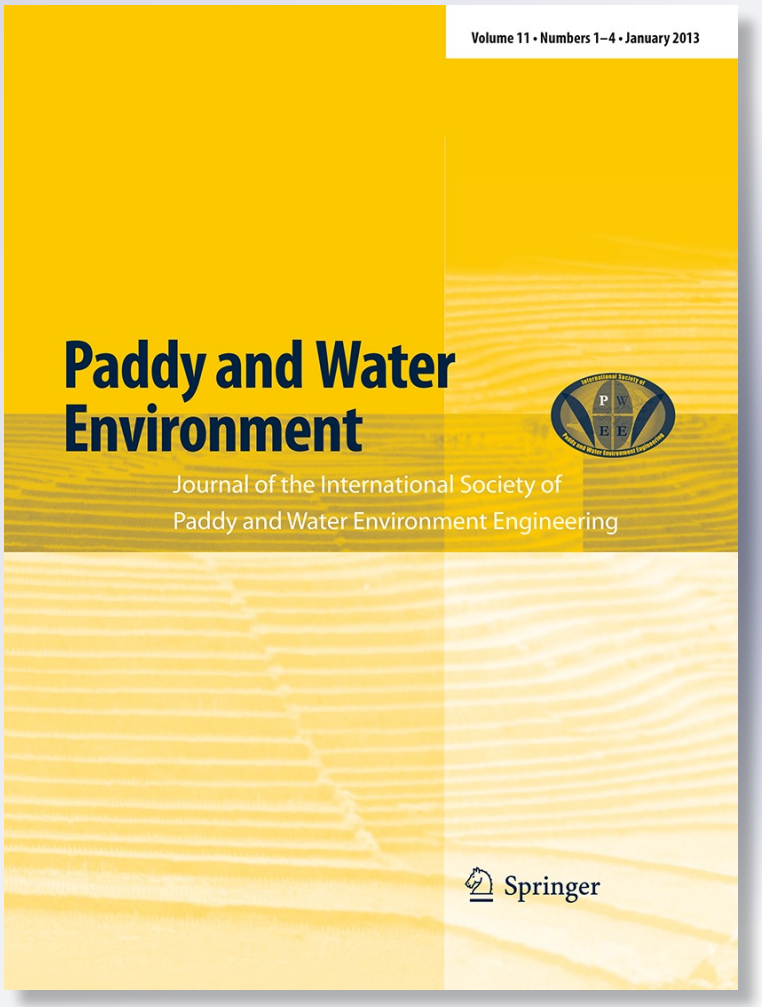

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ARTICLE

Effect of imidacloprid and fipronil pesticide application on *Sympetrum infuscatum* (Libellulidae: Odonata) larvae and adults

Hiroshi Jinguji · Dang Quoc Thuyet · Tetsuyuki Uéda · Hirozumi Watanabe

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Abstract The effect of imidacloprid and fipronil on *Sympetrum infuscatum* larvae and adults during the rice cultivation period was monitored using an experimental micro-paddy lysimeter (MPL) system. Twenty-two hatched larvae were laid on the soil surface of each MPL. MPLs were treated with imidacloprid, fipronil, and the control MPL was left untreated. The pesticide concentration, *S. infuscatum* larval and adult populations, and larval emergence time were monitored in each MPL. The maximum imidacloprid and fipronil concentration in paddy water was 52.8 μg/l at 1 day, and 1.3 μg/l at 6 h, respectively, after the pesticide application. Both pesticides dissipated quickly in paddy water, with half-lives of 8.8 and 5.4 days for imidacloprid and fipronil, respectively. The absence of *S. infuscatum* larvae and exuviae in the fipronil-treated MPL was remarkable. The larval survival decreased to 63.6 ± 18.2, 15.2 ± 2.6, and 0% in the control, imidacloprid-treated, and fipronil-treated MPLs, respectively, by 9 days after pesticide application. Emergence in the imidacloprid-treated MPL was also significantly lower than that in the control MPL. The observed decrease in the abundances of *S. infuscatum* larvae and adults in MPLs seems to be both directly and indirectly associated with nursery-box application of fipronil and imidacloprid.

Keywords Imidacloprid · Fipronil · Sympetrum infuscatum · Micro paddy lysimeter

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Introduction

Maintaining biodiversity in agricultural environments is important for agronomic sustainability (Swift and Anderson 1994; Matson et al. 1997). Both aquatic and terrestrial components of rice paddy fields typically support high levels of biodiversity, which is essential for agricultural productivity (Cohen et al. 1994; Schoenly et al. 1998). Many studies have shown that the impact of pests in rice paddy fields is often reduced to negligible levels when predator communities are conserved through reducing the use of pesticides (Way and Heong 1994; Settle et al. 1996; Schoenly et al. 1998).

About 20 species of dragonflies belonging to the genus *Sympetrum* have been identified in Japan, most of which utilize rice fields during some portion of their life cycle. *Sympetrum* spp. larvae and adults are considered useful insects because they prey on harmful insects in rice paddy fields. *Sympetrum infuscatum* has a wide distribution, and is commonly found in rice paddies in Japan, Korea, and China (Sugimura et al. 1999; Han et al. 2010). *S. infuscatum* is one of the most effective predators of pests that infest rice, in part because their density in rice fields increases through the growing season (Nakano et al. 1977). *S. infuscatum* deposits its eggs on the soil surface of rice fields before harvest. Eggs overwinter on the soil surface and hatch immediately upon filling the paddies with water in the spring. Larvae develop to imagoes in approximately

2 months. After emergence, the adults enter the forests near the paddies, where they remain throughout the sexually immature stages, and after maturation, they return to the paddies for oviposition (Watanabe et al. 2005). The presence of *S. infuscatum* in rice paddy fields is limited during the egg, larval, and adult stages.

The use of nursery-boxes for rice cultivation is popular in Japan and East Asia. The application of pesticides to nursery-boxes before transplantation to protect rice plants from pests during the early growth stage has been practiced in Japan since the 1970s (Asaka et al. 1978). Insecticides are applied to the nursery-box either immediately before transplanting or at sowing (Thuyet et al. 2011b), depending on farmer practice and target pests.

Imidacloprid (1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylidene-amine) and fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfinyl)-1H-pyrazole-3-carbonitrile) are systemic insecticides that have been widely used worldwide for broad spectrum insect control (Liu et al. 2002; Aajoud et al. 2003). Imidacloprid has a low mammalian toxicity but is highly effective as an insecticide (Fossen 2006), while fipronil has a high efficacy, even at low field application rates (Aajoud et al. 2003). These advantages have contributed to the rise in popularity of imidacloprid and fipronil as insecticides for use in rice cultivation in Japan, particularly for nursery-box application. While imidacloprid and fipronil use has increased in recent years, populations of *Sympetrum* spp. have declined (Uéda 2008a, b), yet there have been few reports on the cause of this decline or on the ecological impacts of rice pesticides on *Sympetrum* spp. larvae inhabiting rice paddies. Thus, an ecotoxicological assessment of the effect of pesticides on *Sympetrum* spp., particularly *S. infuscatum*, is necessary for agronomic sustainability. The hatching speed rate of *S. infuscatum* eggs tends to increase proportionately with increases in water temperature (Jinguji et al. 2010). Thus, larvae that hatch immediately after irrigation water is flooded into a paddy might be exposed to concentrations of pesticide that impact their survival.

Field ecotoxicological assessments are important for clarifying the environmental impact of pesticides. However, such assessments are usually expensive and labor intensive. The micro-paddy lysimeter (MPL) was developed as an alternative portable ecotoxicological testing system, and has proven to be an effective and convenient tool for simulating solute transport in paddy environments (Thuyet et al. 2010a). The MPL system has also been used to evaluate the behavior of broadcast granular herbicides (Nhung et al. 2009) and nursery-box-applied granular insecticides in rice paddies (Thuyet et al. 2012). In this study, an experimental MPL system was employed to evaluate the effects of rice nursery-box-applied imidacloprid and fipronil insecticides on *S. infuscatum* from larvae to emergence under a typical rice paddy water management scenario. The evaluation was based on the monitoring of pesticide concentrations in paddy water and the survival of larvae and adult *S. infuscatum*.

Materials and methods

MPL experiment

The MPLs used in this study (350 × 500 × 300 mm^3) are shown in Fig. 1. To eliminate any effects due to residual insecticides from previous applications, soil was taken from a rice paddy to which no insecticide or chemical fertilizer had been applied during the previous 2 years. The MPL was packed to a depth of 26 cm with undisturbed paddy soil. The water balance components in the lysimeter such as irrigation, percolation, and surface drainage were adjusted to simulate actual conditions used in water management in typical paddies in northern Japan. Groundwater was initially added to the MPL as a source of irrigation water to a depth of approximately 5 cm. After recording the daily evapotranspiration (ET), appropriate volumes (depth) of percolation, and surface runoff were discharged at once daily from drain pipes installed on the side and the bottom of MPL. The designed percolation and irrigation rates were set to about 10 and 20 mm/day, respectively. Experiments were conducted outdoors, and the MPLs were protected from precipitation by placing them under the eaves of the laboratory building.

Imidacloprid and fipronil were applied as the commercial granular formulations Admire® Box Granule (2% imidacloprid; Bayer Cropscience K.K., Japan) and Prince® (1.0% fipronil, BASF Agro Ltd.), respectively, to nursery-boxes containing 32 day-old rice seedlings (*Oryza sativa*

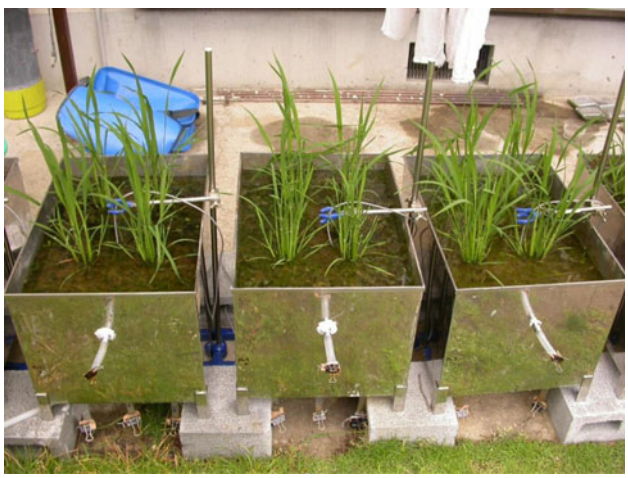

Fig. 1 Layout of micro-paddy lysimeter

cv. var. Hitomebore). The application rate for both imidacloprid and fipronil was 50 g of granules per nursery-box, as recommended for field use. The pesticide product was first applied homogeneously over the rice seedlings. Immediately after pesticide application, four rice seedlings to which pesticides had been applied were transplanted by hand on 19 May 2008 to a depth of approximately 3 cm in each MPL with a spacing of 12 × 20 cm to achieve the recommended densities. Three treatments with three replicates for each treatment were set in MPLs: imidacloprid treatment, fipronil treatment, and a control without insecticide application were applied. Entire experiment lasted until 1 August 2008 for final observation on emerged adults.

Chemicals and materials

Imidacloprid and fipronil standards (>99% purity) and analytical grade solvents used for chemical analyses were purchased from Wako Pure Chemical Industries (Osaka, Japan). Water was produced using a Milli-Q Water Purification System (Millipore, Billerica, MA, USA). The solid-phase extraction cartridges used for water extraction were Supelclean ENVI-18 (500 mg/6 ml; Supelco, MA, USA).

Water sampling

Paddy water samples were taken at 6 h, 12 h, 24 h, 48 h, 7 days , 14 days, and 30 days after transplantation (DAT). At each sampling, five 100 ml samples of paddy water taken from five randomly selected locations were mixed to form one composite water sample for each MPL. The samples were then frozen until chemical analysis.

Chemical analysis of water samples

Water samples for fipronil analysis were thawed at ambient temperature and filtered through 1.2 μm glass-fiber filters (GF/C, Whatman, UK) before extraction. Each water sample was analyzed using a solid-phase ENVI C18 Superclean cartridge. Each cartridge was preconditioned with 5 ml of acetonitrile and then washed with 5 ml of water. 500 ml of each water sample was passed through the cartridge at a flow rate of 3 ml/min without allowing the cartridge bed to dry; the eluate was discarded. Chemicals adsorbed to the cartridge were eluted with 6.0 ml of acetonitrile and the eluate was evaporated under vacuum. The resulting residue was redissolved in 1 ml of acetonitrile/water (20:80, v/v) and the sample was filtered through a 0.2 μm syringe filter (Whatman, Maidstone, UK) and kept at 4°C for HPLC analysis.

HPLC analyses were performed on a Shimadzu HPLC System consisting of an LC20AD Separations Module and an SPD-M20A photodiode array detector. The system was controlled using LCSolution software. The analytical column was a Shimadzu C-18 (150 mm × 4.6 mm × 4.6 μm particle size; Shimadzu Corporation, Kyoto, Japan) which was kept at 40°C during analytical runs. The detector operated at a fixed wavelength of 280 nm. The pump was set in isocratic mode at a flow rate of 1 ml/min with the mobile phase consisting of acetonitrile/water (60:40, v/v). The volume of the sample injection was 20 μl. The detection limit and recovery for fipronil were 0.05 μg/l and 93.0 ± 4.6% ($n = 3$), respectively.

Water samples containing imidacloprid were analyzed similarly by following the method of Thuyet et al. (2011b). The detection limit and recovery for imidacloprid were 0.05 μg/l and 90.0 ± 5.6% ($n = 3$), respectively.

Egg collection and larvae rearing

Collection of eggs from sexually mature *S. infuscatum* females was carried out in a paddy field at Miyagi University in the Miyagi Prefecture, Japan (38°13′N, 140°49′E). Eleven females were captured while ovipositing. A total of 2,018 eggs were collected by holding each insect's wings and dipping the tip of the abdomen into a dry glass tube. All eggs were combined at the end of collection. The eggs were then divided into water-permeable packs (50 eggs per pack) containing soil that had been oven-dried at 110°C for 24 h. These packs were placed on the surface of a paddy at the Miyagi University farm on 30 September 2007 and left to overwinter in order to allow the eggs to complete diapause under natural conditions. The packs were removed from the paddy and transported to the laboratory on 15 May 2008, and 50 eggs and the soil in each pack was transferred into a square plastic tray ($L = 10$ cm, $W = 10$ cm, $H = 3$ cm). The eggs were then covered with distilled water to a depth of 2 cm and the trays were maintained in an incubator (GC351, Sanyo, Japan) at 23°C with a photoperiod of 14L:10D (relative light intensity = 3,000 lux). Beginning on 16 May 2008, the eggs were examined daily under a binocular microscope (SZ60, Olympus, Japan) at 30× magnification. Newly hatched larvae were counted and removed.

Larvae sampling

Immediately after rice seedlings were transplanted into each MPL, 22 *S. infuscatum* larvae that had been reared in the incubator for 4 days were placed in the center of each MPL by a pipette in order to prevent larvae from directly touching the insecticide granules on the surface of the transplanted rice seedlings. Quantitative sampling of larvae was conducted at 9 DAT and then every 7 days until 52 DAT for a total of seven times. In the first two sampling

events, 5 cm-tall stainless steel cores were driven about 1 cm into the soil at five randomly chosen spots in the MPL. Next, the water and about 1 cm of the surface soil were collected from inside the core using a pipette. The number of larvae in the water and soil samples was counted in a Petri dish under a binocular microscope, and the number of larvae per unit area in the MPL was extrapolated from observations of the core samples. From the second sampling event, the number of larvae in each MPL was visually confirmed. Survival here was defined as a percentage of living larvae relative to initial released larvae in MPL and it was calculated from the following equation:

$$\text{Survival}\,(\%) = 100 \times n/N \quad (1)$$

where N represents the initial number of larvae ($N = 22$) and n represents the number of surviving larvae at each weekly sampling.

Exuviae and adult sampling

Exuviae and adults were collected by covering each MPL with a net and the collected specimens were then stored individually in paraffin paper. The day of emergence, the condition of each adult, and the number of dead adults observed during emergence were recorded. Exuviae and adult sampling was conducted every day from 50 to 70 DAT.

Statistical analyses

One-way ANOVA was followed by a multiple comparison test (Tukey HSD post hoc test) to determine whether treatment results were significantly different. All statistical analyses were performed using SPSS statistics software (Ver. 18.0, SPSS Institute Inc., Japan). Survival data were arcsine transformed and analyzed using ANOVA.

Results

MPL conditions

The paddy water temperature was similar among the MPLs and was dependent on weather patterns. Although the MPL was slightly shaded in the morning time, the paddy water temperature remained at 18.5 ± 3.7°C during the first month; however, it increased to 21.5 ± 2.5°C toward the end of the rainy season in late June. During the emergence period in mid-July the paddy water temperature was 22.3 ± 2.8°C and remained at or near this temperature throughout most of the summer. The ET was similar among the MPLs and ranged from 5 to 8 mm/day. Electrical conductivity (EC) of MPL paddy water during the trial was generally low. Mean EC were 105.1 μS cm^{-1} and ranged from 72 to 124 μS cm^{-1}.

Dissipation of fipronil and imidacloprid in paddy water

The dissipation curve for fipronil in MPL water is shown in Fig. 2. Fipronil reached a maximum concentration of 1.3 μg/l at 6 h after the seedlings were transplanted, and then dissipated exponentially to <0.5 μg/l by 7 DAT. Fipronil remained in this concentration range until the end of the monitoring period. During the first 7 days of the monitoring period, the fipronil DT50 value in paddy water was determined to be 5.4 days ($r^2 = 0.92$).

The dissipation curve for imidacloprid in MPL water is shown in Fig. 3. Imidacloprid reached a maximum concentration of 52.8 μg/l at 1 DAT. The initial imidacloprid concentration was approximately 50 times higher than that of fipronil. The imidacloprid concentration decreased to 13.2 μg/l by 14 DAT and dropped to 4.9 μg/l after 1 month. During the first 14 days of the monitoring period, the imidacloprid DT50 value in paddy water was determined to be 8.8 days ($r^2 = 0.98$).

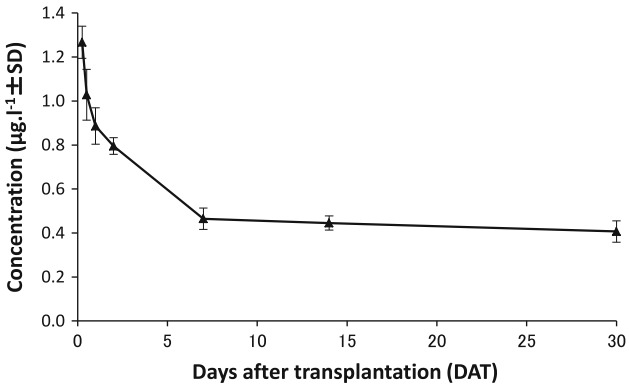

Fig. 2 Dissipation of fipronil in MPL surface water

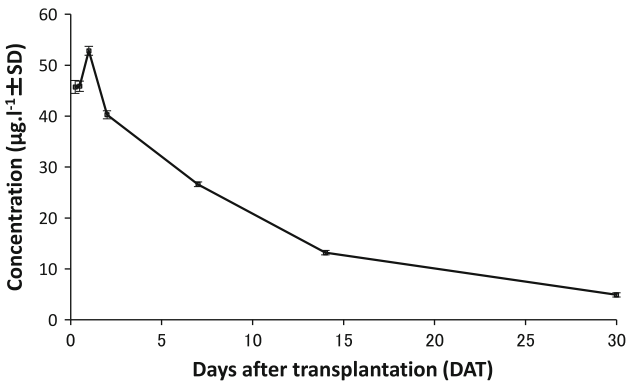

Fig. 3 Dissipation of imidacloprid in MPL surface water

Larvae survival

Figure 4 shows the survival of *S. infuscatum* larvae during the rice cultivation season for the three MPL treatments. Survival decreased sharply immediately after transplantation, especially in the fipronil-treated MPL. By 9 DAT, the larvae survival dropped to 63.6 ± 18.2, 15.2 ± 2.6, and 0% in the control, imidacloprid-, and fipronil-treated MPLs, respectively. After 9 DAT, the larvae survival in the imidacloprid-treated MPL was significantly lower than in the control MPL (ANOVA, $P < 0.01$, $P < 0.001$). At 9 DAT, no larvae were observed during the remainder of the experimental period in the fipronil-treated MPL. Note that the variation of estimated survival rate was large and the temporal increase in mean survival rate at 30 DAT in the control MPL may be the result of heterogeneous distribution of larvae in MPL. By 52 DAT, the larvae survival decreased to 12.1 and 53% in the imidacloprid-treated and controls MPLs, respectively.

Successful emergence and daily emergence patterns

Figure 5 shows the mean emergence percentage of successful *S. infuscatum* emergence in the MPLs. No exuviae or adults were captured in the fipronil-treated MPL. The mean emergence percentage in the control MPL was 66.7 ± 2.6, 12.1 ± 2.6% in the imidacloprid-treated MPL, and 0% in the fipronil-treated MPL. The emergence percentage in the imidacloprid-treated MPL was significantly lower than it was in the control (ANOVA, $P < 0.05$). The proportion of larvae that did not emerge into adults was 1.3% in the imidacloprid-treated MPL.

Figure 6 shows the daily emergence pattern in the MPLs during the experimental period. Emergence began on 17 July (55 DAT) in the control MPL and on 21 July (58 DAT) in the imidacloprid-treated MPL. The mean total period of emergence in the control and imidacloprid-treated MPLs was 14.7 and 8.7 days, respectively.

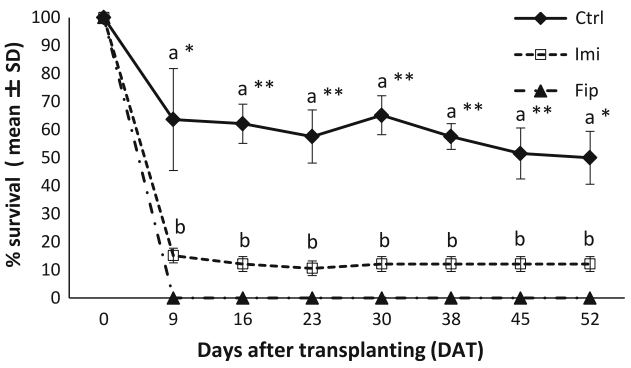

Fig. 4 Survival of *S.infuscatum* larvae during the rice cultivation season in *Imi* imidacloprid-treated, *Fip* Fipronil-treated, and *Ctrl* control MPLs. Both treatments started on the day of planting. *Letters* denote significant differences compared to the control (*$P < 0.01$, **$P < 0.001$). *Error bars* indicate standard deviation

Discussion

Dissipation of fipronil and imidacloprid in paddy water

Fipronil appeared at a low range of concentrations from about 1.3 µg/l to about 0.5 µg/l for the first week and became relatively stable afterwards (Fig. 2). The maximum mass of fipronil in paddy water peaked at 1 DAT and accounted for 0.43% of the applied mass. Fipronil is very sensitive to sunlight, and its photolysis half-life has been reported to be 0.33 days (Gunasekara et al. 2007), and 1.5 days (Thuyet et al. 2011a). In an actual rice paddy field monitoring study, Thuyet et al. (2010b) found the maximum concentration of fipronil was 2.5 µg/l at 1 DAT, and it dissipated quickly in paddy water, with a DT50 of 0.9 days.

Imidacloprid tended to dissipate in a manner similar to reported paddy field experiments. The initial concentration of imidacloprid in paddy water was varied depending on

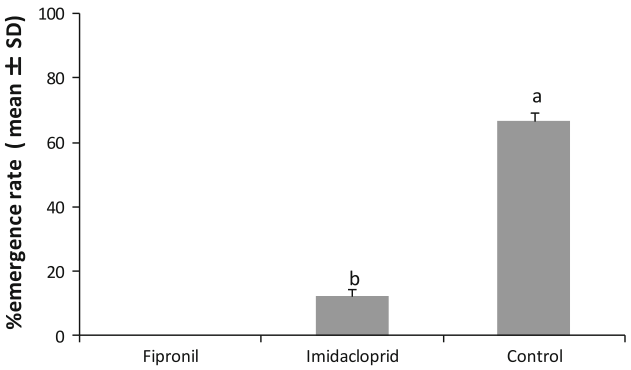

Fig. 5 *S. infuscatum* successful emergence rate. *Letters* denote significant difference compared to control ($P < 0.05$)

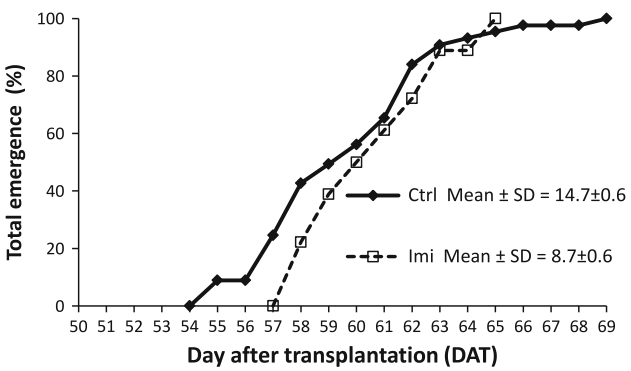

Fig. 6 Emergence pattern of *S. infuscatum* during the rice cultivation season in *Imi* imidacloprid-treated and *Ctrl* control lysimeters

experiments and sample time, e.g., 58.6 μg/l at 1 DAT (Phong et al. 2009), 30.2 μg/l at 0.5 DAT (Thuyet et al. 2011b), and 240 μg/l at 2 h after transplantation (Sanchez-Bayo and Goka 2006a) with a similar application rate, however, it was around 1.0 μg/l after 1 month (Sanchez-Bayo and Goka 2006a). DT50 of imidacloprid was almost similar to previously reported values of, 1.9–2.0 days (Phong et al. 2009), 2.0 days (Thuyet et al. 2011b), and 4 days (Sanchez-Bayo and Goka 2006a). As is the case with fipronil, imidacloprid is reportedly sensitive to photodegradation, its half-life in paddy water exposed to natural sunlight in October being reduced to 1.0 day (Thuyet et al. 2010a).

The DT50 of fipronil and imidacloprid in this study was longer than those reported in other studies, probably due to differences in experimental variables such as water, soil properties, and solar radiation intensity. Fate of insecticides in rice paddy may highly depend upon environmental factors as well as management factors (Thuyet et al. 2011b). The field dissipation of both compounds encompasses three major fate processes such as photolysis, biochemical degradation, and soil/water partitioning. The latter process could be more prevalent in the case of fipronil due to its higher adsorption properties (Gunasekara et al. 2007). Considering the micro habitat (water and soil) of larvae, monitoring of concentrations in paddy soil surface in addition to paddy water may give realistic assessment on their toxicological response although the data for paddy soil is not available in this study. The period of insecticide monitoring were conducted for 30 days by assuming the effects in later period is negligible, however, the appropriate period of chemical monitoring may be depend on the sensitivities of test species.

Effect of fipronil and imidacloprid on *S. infuscatum*

Our data indicate that nursery-box-application of fipronil and imidacloprid cause significant mortality to *S. infuscatum* larvae. Fipronil at ppb levels (0.4–1.3 μg/l; Fig. 2) was found to be very toxic to young *S. infuscatum* larvae. *S. infuscatum* larvae were completely eliminated in the fipronil-treated MPL. The survival of *S. infuscatum* decreased to 0% in the first 9 days after pesticide application. No adults or exuviae were captured during the experimental period. Jinguji et al. (2009) reported a similar early decline in the larvae of *S. frequens* in MPLs; they demonstrated that *S. frequens* larvae were eliminated by 14 DAT in fipronil-treated lysimeters. In addition, no exuviae or adults were observed during their experiment. These results suggest that application of fipronil may be the cause of early decline of larvae and may lead to the extinction of *S. infuscatum* and *S. frequens* in treated rice paddies. Fipronil is highly toxic to many aquatic species even at low concentrations (Gunasekara et al. 2007) and has been reported to be highly effective against several mosquito species. Its reported 24 h LC_{90} and 24 h LC_{50} for larvae of the mosquito *Culex quinquefasciatus* are 0.90 and 0.35 μg/l, respectively (Ali et al. 1999). Moreover, the 24 and 48 h LC_{50s} for larvae of the mosquito *Aedes aegypti* are 24.8 nM (~11.7 μg/l) and 15.1 nM (~7.14 μg/l), respectively (Aajoud et al. 2003). Fipronil is also highly toxic to midges (*Chironomus tepperi*), which are common pests in rice fields; the LC_{50} and LC_{90} values for midges are 0.43 and 1.05 μg/l, respectively (Stevens et al. 1998).

In this study, the maximum fipronil concentration of 1.3 μg/l, which correspond to 0.43% of the applied mass, was observed at 1 DAT, after which the concentration decreased exponentially. The half-life of fipronil in our study was 5.4 days. The fipronil concentration decreased to less than 0.5 μg/l by 7 DAT and remained in this range until the end of the monitoring period. A second stadium *S. infuscatum* larva needs around 30 days to develop into a six stadium larva (Jinguji and Tsuyuzaki 2008). Thus, it is reasonable to suggest that released second stadium larvae were exposed to fipronil concentrations of 0.4–1.5 μg/l, which are close to the LC_{50} for *C. quinquefasciatus* and the LC_{90} for *C. tepperi*. It is certain that *S. infuscatum* larvae are sensitive to fipronil concentrations in this range as are *C. quinquefasciatus* and *C. tepperi*, and that this sensitivity is responsible for the sharp decline in the number of individual larvae observed just after rice transplantation.

Imidacloprid is reportedly less toxic to young *S. frequens* larvae than fipronil (Jinguji et al. 2009). Jinguji et al. (2009) reported a 60% survival for *S. frequens* larvae at 9 DAT in an imidacloprid-treated MPL. In this study, the survival of *S. infuscatum* larvae decreased to 15.2% by 9 DAT, and the mean emergence percentage was 12.1%. These results suggest that *S. infuscatum* is more susceptible to imidacloprid than is *S. frequens*. The diet of many odonates, although encompassing many insect taxa, consists predominantly of small *Diptera*, among which *Chironomidae* and *Culicidae* are well-represented (Corbet 1999). Zooplankton and midges are important because they serve as food sources during the early stages of larval development. Sanchez-Bayo and Goka (2006a) found that zooplankton species were absent from imidacloprid-treated rice fields during the first 2 months following application, when the concentration of imidacloprid was greater than 1 μg/l, and the recovery of zooplankton populations was slow and never returned to the levels found in untreated fields. A similar effect was observed in this study. Many zooplankton and midges were observed in the paddy water of the control MPL; however, crystal-clear water indicative of few zooplankton or midges was observed in the imidacloprid-treated MPL. This suggests that populations of zooplankton and midges might have been impacted by

imidacloprid. As an indirect effect of imidacloprid application, the lack of zooplankton and midges might have caused a delay and shortened the emergence period in the imidacloprid-treated MPL. Delay of emergence for larvae makes them more vulnerable to drying by the mid-summer due to drainage. Moreover, lack of food due to the use of imidacloprid is likely to cause incomplete emergence. Toxicological data suggest that imidacloprid is not very toxic to fish or Daphnia, but it is very toxic to chironomids and all other crustaceans, particularly ostracods, amphipods, and crayfish (Stoughton et al. 2008; Overmyer et al. 2005; Sanchez-Bayo and Goka 2006b). The 48 h LC_{50} for *Daphnia magna* is reportedly 17–85 mg/l (Song et al. 1997; Iwaya and Kagabu 1998), whereas imidacloprid concentrations in rice paddies have been reported to be quite low: 240 μg/l at 2 h after transplantation (Sanchez-Bayo and Goka 2006a), 30.2 μg/l at 0.5 DAT (Thuyet et al. 2011b), and 58.6 μg/l at 1 DAT (Phong et al. 2009). However, the absence of typical paddy ostracods and other microcrustaceans from imidacloprid-treated fields (Sanchez-Bayo and Goka 2006a) is relevant to the survival of *S. infuscatum* larvae, which may be indirectly affected in imidacroprid-treated rice paddies. Susceptibility to imidacloprid appears to differ among aquatic species; while some organisms are relatively unimpacted, *S. infuscatum* larvae are highly susceptible to low imidacloprid concentrations.

Conclusion

We investigated the effect of nursery-box-applied fipronil and imidacloprid pesticides on *S. infuscatum* in rice paddies using MPLs. Fipronil completely eliminated young *S. infuscatum* larvae at concentrations of 0.4–1.3 μg/l (ppb levels) in the first 9 DAT. The effect of imidacloprid on larvae right after hatching was not as great as that of fipronil, however, the impact of imidacloprid was not negligible, as indicated by the low survival during emergence as compared to the control. Imidacloprid is likely to produce an indirect effect by diminishing prey availability. Therefore, growers should be aware that when nursery-box-applied pesticides are used in rice paddies, *Sympetrum* larvae will be exposed to pesticide immediately after hatching upon transplantation of the rice seedlings. Decreases in the abundance of *S. infuscatum* larvae and adults appear to be both directly and indirectly associated with nursery-box application of fipronil and imidacloprid in MPLs. Our research has demonstrated an applicability and usefulness of MPL for ecotoxicological assessments of nursery-box-applied pesticides for rice paddy field ecosystems. Such microcosm-based approaches establish a relevant context for faunal assessments, and complement traditional experimental methods, including laboratory toxicology studies.

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Letter

Quantum yields for direct photolysis of neonicotinoid insecticides in water: Implications for exposure to non-target aquatic organisms

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Quantum yields for direct photolysis of neonicotinoid insecticides in water: Implications for exposure to non-target aquatic organisms

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ABSTRACT

Environmental fate processes of neonicotinoid insecticides are of significant interest, given the serious threats these chemicals can pose to non-target organisms such as pollinators (e.g., bees). Direct photolysis was investigated using a laboratory photoreactor approximating full-spectrum sunlight to predict the aquatic fate of neonicotinoids. Quantum yields (ϕ_c) were 0.019±0.001, 0.013±0.001, 0.0092±0.0005, 0.0022±0.0003 and 0.0013±0.0002 for thiamethoxam, clothianidin, imidacloprid, acetamiprid and thiacloprid, respectively. Based on these values, estimated half-lives were 0.2-1.5 days for different seasons in surface waters at temperate latitudes for thiamethoxam, consistent with the 0.98 day half-life observed experimentally outdoors at Winnipeg, Manitoba, Canada (50°N) for thiamethoxam in summer. Light attenuation through shallow clear surface waters (e.g., by natural organic matter) indicated that photolysis of thiamethoxam at depths greater than 8 cm was negligible, which may help explain reports of their environmental persistence.

INTRODUCTION

Neonicotinoid insecticides are widely used in agriculture to protect against a variety of pests such as whiteflies, beetles and termites.[1] Neonicotinoids act by selectively binding with the nicotinic acetylcholine receptor to disrupt neural transmission.[2] They are applied as seed coatings or as foliar sprays, and released via seed leaching, spray drift, surface run-off, and wind or animal-mediated dispersal of contaminated pollen and nectar from treated plants.[3] Consequently, they are widely detected in environmental media such as plants,[4] soil and water.[5] Neonicotinoids are of great environmental concern because they exhibit adverse effects on pollinators (e.g., bees),[1,6,7] non-target invertebrates,[8] vertebrates[9,10] and even humans.[11] Furthermore, the controversial nature of neonicotinoids and their possible link to major bee die-offs globally has increased pressure to phase out this class of insecticides,[12] as exemplified by a recent two-year moratorium in Europe.[13, 14] However, it is not clear if neonicotinoids are responsible for declines in bees, or if other variables are in play, as pollinating species can experience complex and confounding environmental stressors. That, combined with the paucity in data characterizing exposure via realistic field studies, makes it difficult to isolate the issue just to neonicotinoids.[12,14] It is thus essential to understand the environmental processes controlling the fate of neonicotinoids to inform better decisions relating regulations of these insecticides.

Direct photolysis is an important factor affecting the environmental fate of many organic contaminants, including neonicotinoids.[15,16] The quantum yield (ϕ_c) is a characteristic parameter defining how efficiently a compound degrades upon absorption of a photon,[15,17] facilitating the modeling and prediction of direct photolysis rate constants (k_p) and half-lives ($t_{1/2}$).[17] Although the photodegradation of neonicotinoids using either laboratory photoreactors or natural sunlight has been investigated,[16,18-20] to our knowledge there exists no peer-reviewed literature reporting ϕ_c values under environmentally relevant conditions (i.e. λ>290 nm). Some internal reports,[21-29] including those from the European Commission [21-24] and the U.N. Food and Agriculture Organization (FAO),[25-28] report ϕ_c

values for acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam. However the experimental conditions and reliability of these values are unknown, and independent verification is required. For example, the *p*-nitroanisole (1×10^{-5} M)/pyridine (2.5×10^{-3} M) actinometer system, which could not provide a similar half-life of all target compounds (from 3.5 min to 254 h) and consequently correct photon flux monitoring, was used for the determination of ϕ_c of several neonicotinoids in various unpublished phototransformation experiments.[29]

Thus, our objective was to determine ϕ_c values of the frequently used neonicotinoid insecticides thiamethoxam, clothianidin, imidacloprid, acetamiprid, and thiacloprid in water with a laboratory photoreactor under environmentally relevant light conditions. These results were further evaluated (for thiamethoxam only) under natural sunlight to investigate indirect photolysis and biotic degradation processes, as well as effects of light attenuation through the water column. This will allow prediction of the persistence of these chemicals in surface waters, and aid in ascertaining exposure levels to vulnerable non-target species (e.g., pollinators).

MATERIALS AND METHODS

/Details on chemicals and reagents used, including structures and basic physical-chemical parameters of the studied neonicotinoids, are listed in Supporting Information (SI). All irradiations were performed using a Rayonet Merry-Go-Round Photochemical Reactor (model RPR-100, The Southern New England Ultraviolet Company, Branford, CT). The photoreactor had 16 medium-pressure mercury lamps with spectral emission ranging from 250 to 400 nm, centered at 300 nm (Figure S1 and Table S2).[30] Cylindrical Pyrex tubes (50mL) which filtered wavelengths <290 nm were used as irradiation vessels.

The *p*-nitroanisole/pyridine and *p*-nitroacetophenone/pyridine actinometer systems[31] were used to monitor photon flux in the photoreactor, with 4.6×10^{-5} M *p*-nitroanisole and 0.01 M pyridine for ϕ_c

85 determination of thiamethoxam, clothianidin and imidacloprid, while 6.0×10^{-5} M *p*-nitroacetophenone
86 and 0.01 M pyridine were used for experiments with acetamiprid, thiacloprid and the outdoor
87 experiment of thiamethoxam. Actinometers were included in all irradiation and dark experiments.

88 Triplicate laboratory irradiations (10 mg/L, high concentrations were used to facilitate
89 photoproduct identification) were conducted with 40 mL solutions of each individual insecticide in 50
90 mM borate buffer at pH 7.4 in Pyrex tubes. Given the pK_a values for these five neonicotinoids are well
91 above or below (>2 pH units) this pH (Table S1),[32] each compound is present only as a single species
92 during the duration of these experiments. Dark experiments were carried out in an oven that matched
93 the maximum temperature (45°C) and time reached in the photoreactor.[30] Experiments were performed
94 in triplicate over 45 min for thiamethoxam, clothianidin and imidacloprid, and 36 h for acetamiprid and
95 thiacloprid. Chemical concentrations were determined using high performance liquid chromatography
96 (HPLC) with diode array detection, while photoproducts were measured using HPLC tandem mass
97 spectrometry and time-of-flight high resolution mass spectrometry (QTOF) as detailed in SI.

98 Detailed methods for calculating molar absorptivity and ϕ_c (290-360 nm) using our actinometers,
99 as well as natural sunlight estimations (SI) were published previously.[17,30] The solar irradiance
100 parameter (L_λ) used for the $t_{1/2}$ estimation of neonicotinoids under sunlight was obtained from the
101 literature.[33]

102 Degradation of thiamethoxam under natural sunlight conditions was assessed at the Prairie
103 Wetland Research Facility at the University of Manitoba in July 2014. Details of this facility are
104 published elsewhere.[34] Briefly, sealed Pyrex tubes containing thiamethoxam and nanopure water were
105 deployed in three randomly-selected 3500 L mesocosms containing approximately 2000 L of water,
106 natural uncontaminated sediments, macrophytes, and invertebrates typical of Canadian Prairie wetlands,
107 at different depths (0 cm, 8 cm, 18 cm and 28 cm). Photon flux was measured using *p*-
108 nitroacetophenone/pyridine at these depths, along with dark controls as above. In order to clarify

whether other environmental degradation processes such as non-photolytic abiotic transformation (e.g., hydrolysis), microbial biotransformation and indirect photolysis were involved in removing thiamethoxam during the experiment, other control tubes were deployed in triplicate (see SI for details).

RESULTS AND DISCUSSION

Photolysis kinetics and quantum yields

The photolysis of neonicotinoid insecticides, which absorb photoreactive light (Figure S2) followed pseudo-first-order kinetics (Figure 1). No loss of these insecticides was observed in the dark (Figure 1). Imidacloprid, clothianidin, thiamethoxam, acetamiprid, and thiacloprid exhibited direct photolysis half-lives of 12±0.4 min, 12±1.1 min, 22±1.3 min, 26±1.0 h and 42±1.6 h, respectively (Figure 1). Direct photolysis ϕ_c were calculated as 0.019±0.001, 0.013±0.001, 0.0092±0.0005, 0.0022±0.0003 and 0.0013±0.0002 (290-360nm) for thiamethoxam, clothianidin, imidacloprid, acetamiprid and thiacloprid, respectively (Figure 1). The averaged photon flux of the photoreactor ranged from 8.8×10^{14} to 1.1×10^{15} photons×cm^{-2} sec^{-1} over the course of the entire experiment. The half-life for thiamethoxam under natural sunlight (300-360nm) at the surface of the mesocosm water was 0.98±0.03 days (Table 1 and Figure S3A). In comparison with the surface water photodegradation, the light flux decreased 89% to 7.9×10^{13} and 98% to 1.1×10^{13} photons×cm^{-2} sec^{-1} at depths of 8 cm and 18 cm, respectively.

Given the paucity of data existing for published neonicotinoid quantum yields, it was necessary to rely on the few unpublished values from technical documents to place our results in context. The ϕ_c of direct photolysis of thiamethoxam in water was reported as 0.013±0.002 in an unpublished European Commission document (experimental conditions unknown),[21] similar to our result (0.019). The half-life of thiamethoxam was predicted to be 0.20-1.5 days at 50°N (Table 1) for different seasons based on the measured ϕ_c in the present study, which corresponds well with our measured half-life. Discrepancies

are possibly due to specific weather conditions (e.g., cloudy) during the outdoor irradiations. The very similar half-life in poisoned tubes in mesocosms of 1.1±0.2 days (Figure S3B) indicates that direct photolysis dominated transformation processes for thiamethoxam. However, screening of UV light in the mesocosm water column (e.g., by natural organic matter; total organic carbon was measured as 16.5±3.1 mg/L in the present study) resulted in considerably longer half-lives at depth.[35] The pseudo-first order rate constant for thiamethoxam in tubes at the surface (0.71±0.02 d^{-1}) of the mesocosm tanks decreased to 0.02±0.008 d^{-1} and 0.01±0.003 d^{-1}, respectively, at 8 and 18 cm depth (Figure S4). In contrast, FAO[25] reported a photolytic $t_{1/2}$ for thiamethoxam of 2.3-3.1 days in phosphate buffered aqueous solutions (pH=5) using xenon arc light irradiation. That report noted that samples were exposed to light for 12 h at an average intensity of 410 W/m^2 per day followed by 12 h dark intervals with a total reaction time for 30 days.[25] Moreover, Bonmatin et al.[32] estimated that the aqueous photolysis $t_{1/2}$ of thiamethoxam under sunlight at pH 7 to be 2.7 days. However, details of experimental conditions were not clear. Experimental designs that were inconsistent (e.g., different light sources) and/or problematic (e.g., involvement of cosolvent and inappropriate actinometer) may help explain the variability in ϕ_c and $t_{1/2}$ reported throughout the peer-reviewed and grey literature for environmental contaminants, a topic that has been fully reviewed previously.[15]

An outdoor sunlight experiment conducted in March 2012 in Zürich (47° N latitude) reported a ϕ_c = 0.0073 and $t_{1/2}$ = 3.3 h for clothianidin,[29] similar to the ϕ_c value reported by European Commission (0.014)[22] and measured in the present study (0.013±0.001). The outdoor $t_{1/2}$ of clothianidin was predicted as 0.35-3.3 days for different seasons at 50° N latitude based on our measured ϕ_c. FAO[26] reported a $t_{1/2}$ of 0.6 days of summer solar exposure for clothianidin at Phoenix, Arizona (33°N latitude).

Studies by Redlich et al.[36] report laboratory measured ϕ_c values for imidacloprid determined at wavelengths <290 nm, which are not environmentally relevant. Von Gunten[29] conducted quantum yield

measurements for imidacloprid under natural sunlight in March 2012 in Zürich and observed a ϕ_c = 0.0055 and $t_{1/2}$ = 2 h, comparable with our results (ϕ_c = 0.0092; Figure1 and Table 1). The environmental $t_{1/2}$ of imidacloprid in surface waters at 50°N latitude was calculated as 4.2 h at the equinox,[27] whereas our estimation was 0.36 d (8.6 h) and 0.83 d (19.9 h) in spring and autumn, respectively.

Again, good agreement is observed when comparing our results to those of von Gunten[29] who reported quantum yields from outdoor sunlight experiments (March 2012 in Zürich). Von Gunten[29] observed a ϕ_c = 0.0046 and $t_{1/2}$ = 254 h for acetamiprid under natural sunlight, which agree reasonably well with our values: ϕ_c = 0.0022 and $t_{1/2}$ (predicted) = 9.7 days in summer (232 h). In contrast, the study by the European Commission reported the ϕ_c of acetamiprid as 0.10 at λ>290 nm (experiment condition unknown), [23] which was much higher than our results (0.0022) and those values from von Gunten (0.0046).[29] However, the $t_{1/2}$ determined in this European Commission report (34 days under xenon lamp, irradiation: 12 hours/day) [23] was comparable with our estimation (9.7-68 days in different seasons), again pointing to experimental inconsistencies surrounding quantum yield determinations

The FAO[28] and European Commission[24] reported the ϕ_c of thiacloprid as 0.00035 and estimated an 80 days $t_{1/2}$ with simulated sunlight and 324 days under natural sunlight at Phoenix. Their ϕ_c was lower than our measured number (0.0013±0.0002) and the $t_{1/2}$ was higher than our results (8.8-60 days), but the reasons were not clear.

Photoproduct identification

It was evident from the HPLC-MS/MS analysis that the irradiations generated photoproducts and the abundance of these products increased with reaction time (Figure S5-S9). The mass spectra from these total ion chromatograms (TIC) were used to identify potential photoproducts, with further analysis, structural elucidation, and confirmation done using QTOF. It should be noted that chromatographic separation of photoproducts was neither attempted nor necessary for the purposes of

this study, and thus, single chromatographic peaks observed in Figures S5-S9 may represent multiple photoproducts. Two photoproducts of thiamethoxam were identified, corresponding to *m/z* 247.0417 and 168.0767 (Table S3, Figures S10 and S11). Both of these masses and proposed structures correspond to major photoproducts previously identified for thiamethoxam.[20] Two photoproducts were identified for clothianidin, *m/z* 206.0149 (Figure S12) and *m/z* 205.0307 (Figure S13), both previously reported by Gong et al.,[18] however the proposed structure for *m/z* 206 in the current study differs. QTOF evidence from the fragmentation pattern of the *m/z* 206 ion supports our proposed structure (Figure S12). Please see SI for further details. Three major photoproducts were identified for imidacloprid, two of which are strongly supported by the literature and a third that has not been previously reported (Table S3). Photoproducts *m/z* 212.0586 and 211.0741 and their corresponding structures (Figures S14 and S15) have been observed multiple times in the literature.[36-39] The imidacloprid photoproduct *m/z* 189.0769 was observed for the first time in the present study (Figure S16). However, it is not clear what the structure of this observed ion is.

Photoproduct identification for both acetamiprid and thiacloprid was markedly more challenging than the other neonicotinoids, likely because of their relatively recalcitrant nature towards photolysis. Acetamiprid showed a photoproduct at *m/z* 205.1081 that corresponded to a logical structure shown in Figure S17. Alternatively, the structure of the thiacloprid photoproduct at *m/z* 235.0646 could not be confidently determined. Three plausible, very similar structures are proposed in Figure S18. Both of these photoproduct masses have not been previously reported in the literature. It should be noted that masses for acetamiprid and thiacloprid were observed in the irradiated samples at exactly 4 mass units greater than the parent masses, 227.0905 and 257.0469, respectively (Figures S17 and S18). No plausible chemical formula information was generated from the QTOF software and thus structure elucidation was not attempted, however this may warrant further investigation.

IMPLICATIONS

Thiamethoxam, clothianidin and imidacloprid will quickly undergo direct photolysis in surface waters, resulting in decreased exposure of non-target organisms consuming or exposed to water at these depths. However, light screening in waters can rapidly decrease photodegradation, as evidenced by the significant light attenuation observed in our deployments in mesocosm waters, which were clear in appearance. While those experiments were for thiamethoxam only, it is very likely that other neonicotinoids would be similarly affected. This would increase exposure of biota to these chemicals, and may help to explain their observed persistence in shallow surface waters.[5,32] In any event, acetamiprid and thiacloprid are relatively stable toward sunlight degradation in aquatic systems.

ASSOCIATED CONTENT

--Supporting information

Supporting Information available: details of experimental work, Tables S1-S3 and Figures S1-S18. This material is available free of charge via the Internet at http://pubs.acs.org.

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--Notes

The authors declare no competing financial interest.

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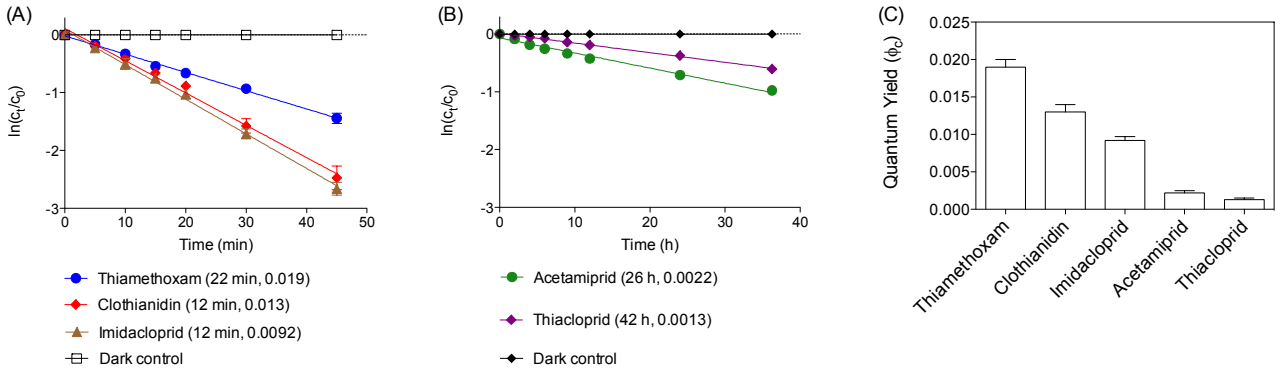
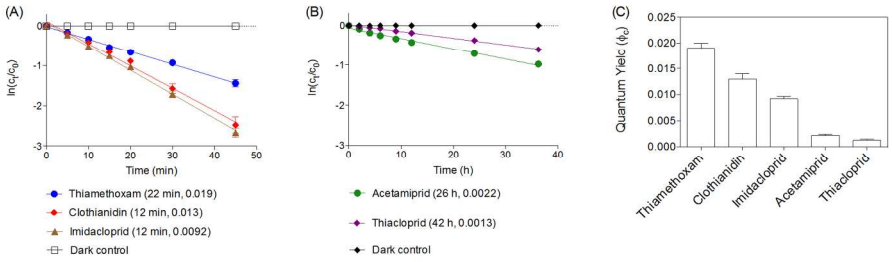


Figure 1 First-order direct photolysis (A and B) and quantum yield (ϕ_c) (C) of neonicotinoid insecticides in water for irradiations in a laboratory photoreactor. Error bars in (A) and (B) represent standard deviations (SD) of the mean. The correlation coefficients (r^2) for the pseudo first-order kinetic plots ranged from 0.982-0.994. Average half-lives and ϕ_c of each neonicotinoid insecticide are shown in brackets in the legend. Errors in (C) were calculated through error propagation.

348 **Table 1** Estimated photolysis rate constants (k_{dcE}) ($days^{-1}$) and half-lives ($t_{(1/2)E}$) (days) for
349 neonicotinoid insecticides in surface water at 50° N latitude for spring, summer, autumn and winter by
350 sunlight on clear days.

Compounds	Spring		Summer		Autumn		Winter	
	k_{dcE}	$t_{(1/2)E}$	k_{dcE}	$t_{(1/2)E}$	k_{dcE}	$t_{(1/2)E}$	k_{dcE}	$t_{(1/2)E}$
Thiamethoxam	2.17	0.32	3.46 (0.71)[a]	0.20 (0.98)[a]	1.10	0.63	0.46	1.49
Clothianidin	1.31	0.53	1.98	0.35	0.56	1.23	0.21	3.31
Imidacloprid	1.94	0.36	2.93	0.24	0.84	0.83	0.31	2.22
Acetamiprid	0.04	16.5	0.07	9.67	0.02	29.7	0.01	67.9
Thiacloprid	0.05	14.3	0.08	8.75	0.03	26.6	0.01	60.3

351 [a] Numbers in brackets were measured under natural sunlight in Winnipeg, Manitoba, Canada in July
352 2014.



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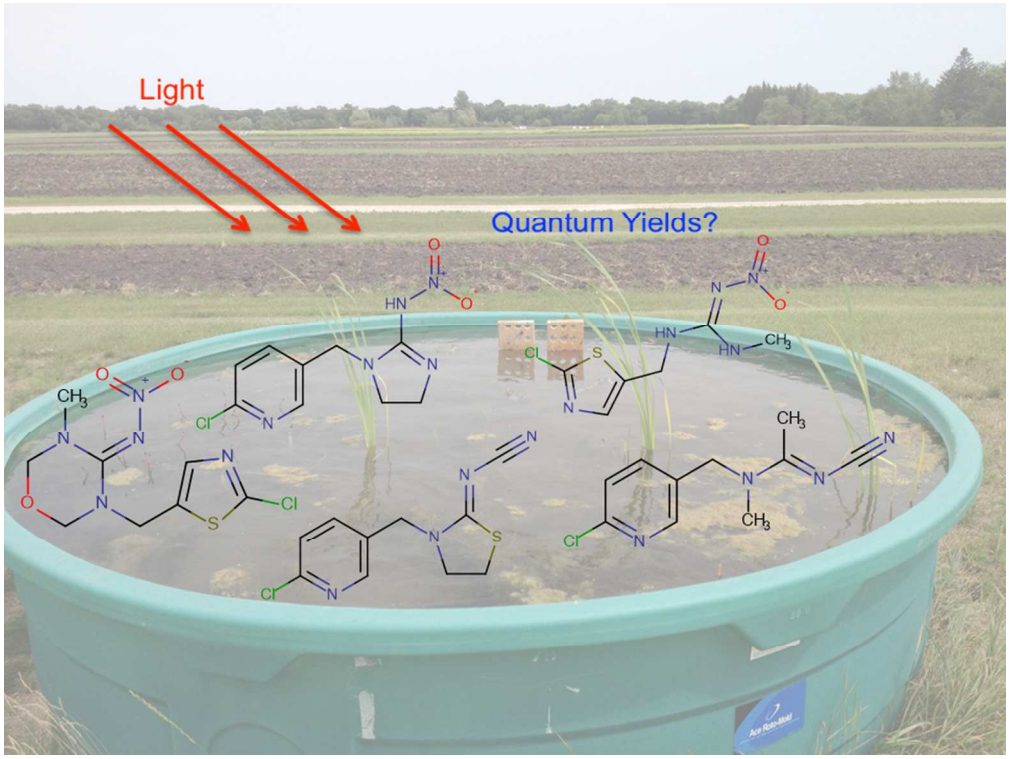

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Effects of Exposing Two Non-Target Crustacean Species, *Asellus aquaticus* L., and *Gammarus fossarum* Koch., to Atrazine and Imidacloprid

Simon Lukančič · Uroš Žibrat · Tadej Mezek · Andreja Jerebic · Tatjana Simčič · Anton Brancelj

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Abstract The physiological responses of two freshwater crustaceans, *Asellus aquaticus* L. and *Gammarus fossarum* Koch., following in vitro exposure to two pesticides were measured. Both species responded to short-term exposure with elevated levels of Respiration (R) and/or lower levels of Electron Transport System (ETS) activity. 1 h exposure to concentrations of up to 10 mg L^{-1} showed an effect in both test species. Laboratory tests confirmed that *G. fossarum* is more sensitive to short-term pesticide exposure than *A. aquaticus*. ETS/R ratio may be used as a quick predictor of effects on organisms exposed to pesticides.

Keywords Pesticide stress assessment · Non-target species

The majority of pesticides are designed to be used in a terrestrial environment, however substantial amounts end up in aquatic ecosystems, in either surface or groundwater (Fernandez-Alba et al. 2002). Their effects on aquatic ecosystems may arise from chronic exposure (long-term and low concentrations), as well as from short-term exposure to high concentrations that can result from accidents, improper use, or run-off from treated fields. Non-target animal populations can be affected and some need more than 6 months for their abundance to recover after pesticides run-off that end up in streams (Liess and Schultz 1999). On several occasions it has been suggested that a broader spectrum of aquatic test animals should be used before newer pesticides (like imidacloprid) can be classified as being safer than those currently applied (Munn and Gillom 2001; Jemec et al. 2007). Crustaceans are frequently used as bioindicators in aquatic toxicity tests due to their prolific breeding, high abundance in nature and sensitivity to anthropogenic toxic compounds in water bodies which they inhabit (Fernandez-Alba et al. 2002). Furthermore, the presence of toxic compounds can be found in their tissues long after exposure, thus external influences can be monitored and spotted after incidents. Song et al. (1997) reported that imidacloprid can be used safely with regard to freshwater arthropods, although it was already known that some aquatic arthropods can be even more susceptible to imidacloprid than *D. magna* (Fernandez-Alba et al. 2002; Jemec et al. 2007). The main disadvantage regarding toxicity tests in *Daphnia* is that their reproduction is based on parthenogenesis, which produces genetically identical offspring. Toxicity tests for *D. magna* therefore offer a limited insight into intraspecific responses on toxic substances.

Selected new species–water louse, *Asellus aquaticus* L. and stream scud, *Gammarus fossarum* were chosen for toxicity tests in relation to their differences in habitat preference. Since *D. magna* is a pond/pelagic species, we selected two, which in addition to lentic can also be found in lotic ecosystems. Due to prevailing sexual reproduction their genetic variability is relatively higher than in *D. magna*, therefore they offer better insight into interspecific responses (Sket et al. 2003). The purpose of our study was to test the both species for differences in stress responses as a function of habitat preference.

Materials and Methods

Specimens of *A. aquaticus* were collected from Zadnji kraj on intermittent Lake Cerkniško Jezero (central Slovenia)

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with low or no rural activity. *G. fossarum* was collected from a small permanent karstic spring located near the village Duplje (northern Slovenia). The catchment area has low human activity. Chemical water quality analysis (ion chromatography, alkalinity, pH, oxygen content and saturation) were performed on samples from both sampling sites and showed no pollution. Animals were transported to the laboratory in a cool box. Up to one day prior to experiments test animals were kept in a laboratory at 10°C, with 12/12 h day/night cycle, in water from the sampling location, which was partly (at two-day intervals) replaced by synthetic water (ISO-standard 6341 1996). Animals were fed on biofilm grown on leaves of black alder (*Alnus glutinosa* L.) infected by bacteria and mould.

Sub-lethal toxicity was studied with standard toxicity tests. The effective and lethal dose concentrations, LC_{50} 48 h and EC_{50} 24 h, were determined as the concentration at which 5 animals out of 10 were paralyzed (only respiration movement was left) (EC_{50} 24 h) or died (no movement at all) (LC_{50} 48 h) (Clesceri et al. 1998). Another set of test animals were later exposed for 1 h (Cold and Forbes 2004) to the same concentrations of two selected toxic compounds prior to respiration measurements.

Atrazine (i.e., 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) was obtained from Riedel-de Haën, 35702 Pestnal® as analytical standard of technical grade 99.9% ($M = 215.69$ g mol^{-1}). A stock solution was prepared at a concentration of 100 mg L^{-1} in methanol (technical grade (GC) = 99.8%), thus the initial concentration for the first exposure solution did not exceed 0.1% of methanol. For the wide range finding toxicity test (WRFTT) nominal test concentrations of 0.01, 0.1, 1.0, 10 and 100 mg L^{-1} of atrazine were used. The highest concentration used, 100 mg L^{-1}, contained 0.1% of methanol. A negative control solution containing 0.1% of methanol was used to check for mortality caused by solvent phase. For the definitive acute toxicity test (DATT), nominal concentrations 0.3, 1, 3, 10 and 30 mg L^{-1} of atrazine were used. The numbers of animals affected by each concentration and their mortality was monitored every 6–12 h, from which effective and lethal concentrations were calculated. Selected 1 h exposure concentrations for *A. aquaticus* were 5 and 10 mg L^{-1} and, for *G. fossarum*, 1, 3 and 10 mg L^{-1} of atrazine (Tables 2, 3).

Stock solutions of imidacloprid (1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazol idinimine), as the original Confidor SL 200, were prepared in bi-distilled water containing 200 g L^{-1} of imidacloprid. This aqueous soluble concentrate was obtained from Pinus d.d. (Bayer CS d.o.o., Slovenia) and stored at 4°C.

For WRFTT, nominal test concentrations 0.01, 0.1, 1, 10 and 100 mg L^{-1}, while for DATT they were 1, 3, 10, 30, 100 mg L^{-1} of imidacloprid. Effective and lethal concentrations were calculated from data gained by monitoring the effects every 6–12 h. Selected 1 h exposure concentrations for both test species were 0.01, 0.1, 1.0 and 10 mg L^{-1} of imidacloprid (Tables 4, 5).

Prior to the experiment, animals were kept in synthetic water at 10°C. After 24 h of fasting, animals were exposed to water containing selected concentration of pesticides for 1 h according to Cold and Forbes (2004). Animals were transferred to pesticide-free and oxygen-rich water immediately after termination of exposure to pesticides. For the respiration measurements the test chambers were completely darkened. A control experiment was run with a set of animals that was not exposed to pesticide, but for 1 h to synthetic water only (in the results referred to as Control).

Respiration was measured with a microrespirometer Presens OXY-4 oxygen meter (PreSens GmbH, Regensburg, Germany) with polymer optical fibres inserted airtight into three flow-through test chambers, positioned parallel to each other. The microrespirometer consists of a water tank with aerated (=air saturated) water. This was connected with a Viton tube to a flow-through test chamber with oxygen sensor measuring the concentration of oxygen entering the chamber. After the chamber, a tube connection splits into three parallel tubes, each connected to a test chamber (5 mm in diameter and 25 mm long glass tubes) containing an individual test animal and equipped at the outflow by another oxygen sensor. A peristaltic pump on the end of the system creates negative pressure to produce a flow of water out of all three test chambers (with approx. rate of 5 mL h^{-1}). The oxygen concentrations on entering and leaving each test chamber were recorded on-line by PC (at five second intervals) and the drop of oxygen concentration was recalculated for each test chamber separately. Respiration was measured in the water reservoir at a constant temperature of 10.0 ± 0.1°C.

ETS activity was measured using the method designed by Packard in 1971 and improved by G-Tóth in 1993, followed exact details on the method as described by Simčič and Brancelj (2003).

OpenOffice Calc was used for sorting and calculating primary data from all experiments. Data sets from each experiment were analyzed in computer programs Sigma Stat 3.5 (SYSTAT) and JMP 7 (SAS). For ETS/R ratios at the start, basic descriptive statistics were extracted; if normality test and equal variance test passed, ANOVA or MANOVA tests were performed. When normality tests failed, nonparametric tests on ranks were used (Kruskal–Wallis One Way Analysis of Variance, Mann–Whitney Rank Sum Test). Additional tests (Dunn's Method and Holm–Sidak method) were performed in order to establish differences between groups that were on the margin of significance.

Results and Discussion

LC_{50} (48 h) and EC_{50} (24 h) for atrazine are compared in Table 1 for *A. aquaticus* and *G. fossarum*. Respiration (R) in *A. aquaticus* shows that animals in group 3 (exposed to 10 mg L^{-1} atrazine for 1 h) have significantly higher R (ca. 2.5-fold) than the control (ANOVA, $p < 0.001$). No statistical changes in ETS activity were observed on 1 h exposure to atrazine (ANOVA, $p > 0.05$) (Table 2). In *G. fossarum* the values of R were significantly higher, by ca. 1.3-fold in groups 3 and 4 (concentrations of 3 and 10 mg L^{-1}, ANOVA, $p < 0.01$), while ETS activity did not change at higher concentrations of atrazine (Table 3). The ETS/R ratio was significantly higher than the control value in *A. aquaticus* in group 3 for 10 mg L^{-1} of atrazine, (ANOVA, $p < 0.001$), but not at lower concentration (5 mg L^{-1} of atrazine) (Fig. 1a). In *G. fossarum* ETS/R ratios from all tested groups exposed to atrazine (1, 3 and 10 mg L^{-1}) differ significantly from those for the control group (ANOVA, $p < 0.05$) (Fig. 1b). The EC_{50} values obtained here for *A. aquaticus* at 17.5 mg L^{-1} and for *G. fossarum* at 6 mg L^{-1}, (24 h, at 10°C) are comparable to those reported by Munn and Gillom (2001) who defined atrazine effective concentrations EC_{50} (24 h) for *Gammarus pulex, G. italicus, Daphnia pulex* and *Hyalea azteca* of 14.9, 10.1, 41.5, 14.7 mg L^{-1}, respectively. Although the temperature at which their experiments were performed was not quoted. Our values of LC_{50} (48 h) were 42.5 mg L^{-1} of atrazine for *A. aquaticus* and 7.5 mg L^{-1} of atrazine for *G. fossarum*, which can be compared with those of Pantani et al. (1997) for LC_{50} (96 h), 10.1 mg L^{-1} of atrazine for *Gammarus italicus* and 3.3 mg L^{-1} of atrazine for *Echinogammaus tibaldii*. The reported time intervals, (96 h), make it difficult to compare and evaluate these results with our LC_{50} (48 h), but the concentrations are of a similar order of magnitude.

Acute 1 h exposure of *A. aquaticus* to atrazine at a concentration of 10 mg L^{-1} (group 3) resulted in significantly higher R but similar ETS activity, leading to a significantly lower ETS/R ratio (Table 2; Fig. 1a). No significant differences in R and ETS/R were observed exposure to lower concentrations (i.e. 5 mg L^{-1} or less). A low ETS/R ratio at high concentrations of pesticides indicates stress conditions in test animals. In *G. fossarum*, the ETS/R ratio for all exposed groups was significantly lower due to higher respiration, indicating higher sensitivity of *G. fossarum* to the pesticide than *A. aquaticus*. Respiration was significantly increased in *G. fossarum* in group 3 (3 mg L^{-1}) and group 4 (10 mg L^{-1}). Short term exposure of both *G. fossarum* and *A. aquaticus* to atrazine did not affect ETS activity, as was also observed in the experiment with imidacloprid (compare 4.2). Values of ETS in the atrazine experiment were similar to those obtained from other experiments where no stress-induced chemicals were used (Simčič and Brancelj 2003; Simčič et al. 2005).

Table 1 Lethal and effective concentrations

		A. aquaticus [mg L^{-1}]	*G. fossarum* [mg L^{-1}]
Atrazine	LC_{50} (48 h)	42.5	7.5
	EC_{50} (24 h)	17.5	6
Imidacloprid	LC_{50} (48 h)	8.5	0.8
	EC_{50} (24 h)	0.8	0.07

Table 2 R and ETS activity in specimens of *Asellus aquaticus* exposed for 1 h to different concentrations of atrazine

Treatment	Group 1		Group 2		Group 3	
	Control	SD	5 mg L^{-1}	SD	10 mg L^{-1}	SD
N	9		10		9	
WW (mg)	11.8	3.3	9.9	3.4	13.7	3.9
R (μL O_2 mg^{-1} h^{-1})	0.054	0.016	0.056	0.021	0.133***	0.034
ETS (μL O_2 mg^{-1} h^{-1})	0.478	0.096	0.468	0.060	0.526	0.063

*** $p < 0.001$

Table 3 R and ETS activity in specimens of *Gammarus fossarum* exposed for 1 h to different concentrations of atrazine

Treatment	Group 1		Group 2		Group 3		Group 4	
	Control	SD	1 mg L^{-1}	SD	3 mg L^{-1}	SD	10 mg L^{-1}	SD
N	18		21		22		18	
WW (mg)	16.3	3.5	14.8	3.4	16.2	3.8	14.1	4.2
R (μL O_2 mg^{-1} h^{-1})	0.074	0.018	0.087	0.024	0.097**	0.032	0.094**	0.018
ETS (μL O_2 mg^{-1} h^{-1})	0.478	0.065	0.424	0.094	0.435	0.073	0.458	0.092

** $p < 0.01$

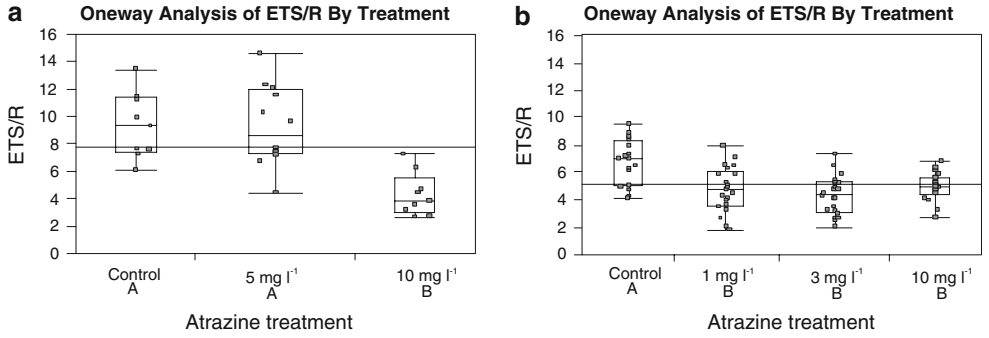

Fig. 1 Box and Whiskers plots of ETS/R ratio for *Asellus aquaticus* (Fig. 1a) and *Gammarus fossarum* (Fig. 1b) exposed for 1 h to various atrazine concentrations at 10°C. Dots indicate results from individual animals, boxes indicate mean ± 1st quartile, and the horizontal line the mean of all the groups. On the *x axis* is treatment with different concentrations of atrazine, (1a: n = 9; 10; 9 and 1b: n = 18; 21; 22; 18). Results of pair-wise comparisons are indicated below the graph, the different letter below data groups meaning that groups differ significantly ($p < 0.001$)

A. aquaticus was shown to be less sensitive than *G. fossarum*, based on the higher lethal and effective dose concentrations for the former, assuming that the animals were taken from a "pesticide free" environment. Moreover, the larger decrease in ETS/R values in *G. fossarum* is also in accordance with reports of other authors like Graça et al. (1994) and Maltby (1995) who found that *Gammarus* species were more sensitive than *A. aquaticus*.

LC_{50} (48 h) and EC_{50} (24 h) for imidacloprid exposure of *A. aquaticus* and *G. fossarum* are in Table 1. Respiration (R) in *A. aquaticus* exposed to high short-term concentrations of imidacloprid (1 and 10 mg L^{-1}) was significantly higher by ca. 1.2-fold, compared to the control (ANOVA, $p < 0.01$). In contrast to R, ETS activity values in all groups were similar, except in group 5 where it was 1.4-fold lower (exposed to 10 mg L^{-1}, ANOVA, $p < 0.001$) (Table 4).

In *G. fossarum* the values of R and ETS did not differ significantly for most of the groups, except for ETS (1.4-fold) in group 4 (10 mg L^{-1}, Kruskal–Wallis, $H = 19.721$, $p < 0.05$) (Table 5). Higher concentrations of imidacloprid are correlated with lower mean ETS/R ratio for both *A. aquaticus* (Kruskal–Wallis, $H = 51.053$, $p < 0.05$) (Fig. 2a) and *G. fossarum* (ANOVA, $p < 0.001$) (Fig. 2b). Effective concentrations (EC_{50} (24 h)) determined for our test animals (0.8 mg L^{-1} for *A. aquaticus* and 0.07 mg L^{-1} for *G. fossarum*) for imidacloprid are approximately one magnitude greater for *A. aquaticus* than for *G. fossarum*, the value for the latter being in agreement with that reported by Kreutzweiser et al. (2007). These authors also found high mortality of aquatic insects at a concentration higher than 0.13 mg L^{-1}, and significant inhibition of feeding at concentrations above 0.012 mg L^{-1} for imidacloprid at 20 ± 3°C. LC_{50} (48 h) concentrations in the more tolerant *A. aquaticus*, which is a common inhabitant of stagnant water where oxygen concentrations can be low, were found to be similar to those reported by Song et al. (1997) for *Daphnia magna.* The latter authors determined the acute toxicity test concentration of LC_{50} (48 h) at 27°C to be 10.4 mg L^{-1}, i.e. one magnitude higher than in the more sensitive *G. fossarum* (1 mg L^{-1}), which is a typical inhabitant of running water, rich in oxygen. The reported concentrations could not be directly compared with that for *Artemia* sp. (LC_{50} (48 h) = 361.2 mg L^{-1}), which is a common inhabitant of hypersaline salt ponds. At lower temperature (20°C) the effects were tested only for *Daphnia magna* (Song et al. 1997) and are higher, the LC_{50} (48 h) concentration being 17.4 mg L^{-1}. However, our LC_{50} (48 h) values, measured at 10°C, are lower than those reported by Song et al. (1997) and Sánchez-Bayo and Goka (2006) who

Table 4 R and ETS activity in specimens of *Asellus aquaticus* exposed for 1 h to different concentrations of imidacloprid

	Group 1		Group 2		Group 3		Group 4		Group 5	
Treatment	Control	SD	0.01 mg L^{-1}	SD	0.1 mg L^{-1}	SD	1 mg L^{-1}	SD	10 mg L^{-1}	SD
N	9		24		30		26		30	
WW (mg)	15.7	3.0	21.8	4.1	16.9	2.8	22.3	4.3	19.7	2.9
R (µL O_2 mg^{-1} h^{-1})	0.141	0.044	0.148	0.033	0.131	0.030	0.192**	0.044	0.169**	0.031
ETS (µL O_2 mg^{-1} h^{-1})	0.296	0.042	0.291	0.046	0.273	0.045	0.281	0.047	0.208***	0.031

** $p < 0.01$

*** $p < 0.001$

Table 5 R and ETS activity in specimens of *Gammarus fossarum* exposed for 1 h to different concentrations of imidacloprid

	Group 1		Group 2		Group 3		Group 4	
Treatment	Control	SD	0.01 mg L^{-1}	SD	0.1 mg L^{-1}	SD	10 mg L^{-1}	SD
N	11		25		21		18	
WW (mg)	14.4	4.3	22.7	3.9	22.3	4.5	15.8	3.2
R (μL O_2 mg^{-1} h^{-1})	0.089	0.021	0.102	0.034	0.133	0.032	0.113	0.039
ETS (μL O_2 mg^{-1} h^{-1})	0.396	0.104	0.354	0.069	0.353	0.082	0.271**	0.071

** $p < 0.01$

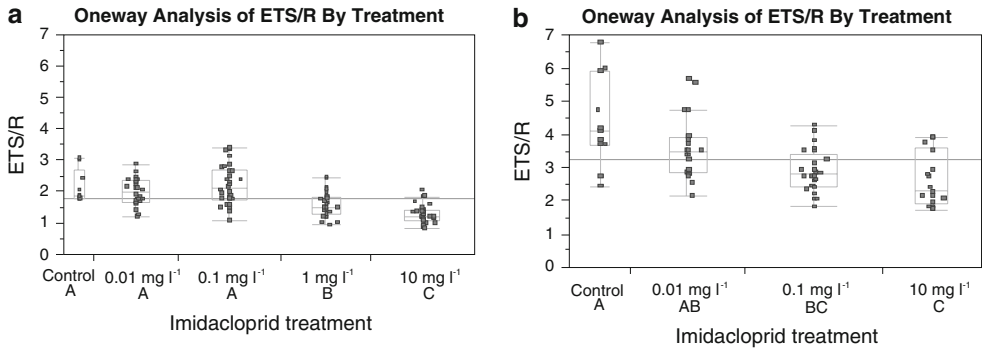

Fig. 2 Box and Whiskers plots for ETS/R ratio for *Asellus aquaticus* (2a) and *Gammarus fossarum* (2b) exposed for 1 h to different imidacloprid sublethal concentrations at 10°C. Dots indicate the results from individual animals, boxes indicate means ± 1st quartile, and the horizontal line is the mean of all the groups. On the *x axis* are treatments with different concentrations of imidacloprid (2a: n = 9; 24; 30; 26; 30 and 2b: n = 11; 25; 21; 18). Results of pair-wise comparisons are indicated below the graph, the different letter below data group means groups differ significantly ($p < 0.001$)

obtained values from 65 up to 133 mg L^{-1} for *Daphnia* sp. Jemec et al. (2007) determined LC_{50} (48 h) (as LOLC–lowest observed lethal concentration) for *D. magna* of 10 mg L^{-1} for imidacloprid (product Confidor SL 200), which is the same as LC_{50} (48 h) for our less susceptible species *A. aquaticus.*

All these comparisons of values for different animals as well as for the same animals indicate that correct experimental temperature was selected and that results can be compared to results from other authors. The significant decrease in ETS/R ratio in *A. aquaticus* after 1 h exposure in group 4 (1 mg L^{-1}) and group 5 (10 mg L^{-1} of imidacloprid) (Fig. 2a) was the result of a combination of higher respiration and lower ETS activity than those for the control (Table 4). Thus, imidacloprid influences not only respiration but also ETS activity. Choi et al. (2001) reported a similar decrease of ETS activity in *Chironomus riparius* exposed to high concentration of fenitrothion. This effect is a consequence of different processes, including oxidative stress. Glutathione peroxidase activity was decreased and, since the enzyme is involved in the reduction of lipid hydroperoxide, a decrease of its activity may enhance the peroxidation of cells and membranes. Partial damage to the inner mitochondria membrane by lipid peroxidation may impair the function of ETS and reduce its activity which took place in mitochondrial membranes only. We found similar effects of pesticides on enzymatic activity in *G. fossarum*. The lower ETS/R ratio in groups 3 and 4 in *G. fossarum* (0.1 mg L^{-1} and 10 mg L^{-1} of imidacloprid) is due to reduction of ETS activity, which was significantly lower in group 4 (10 mg L^{-1} of imidacloprid) and slightly decreased in the other two exposed groups 2 and 3 (Table 5; Fig. 2b). Respiration values for both species stayed relatively unchanged compared to control in groups exposed to lower concentrations but were significantly higher in *A. aquaticus* exposed to higher concentrations (1 and 10 mg L^{-1}). At the same time, even short exposure to high concentrations (10 mg L^{-1}) partly inactivates/destroys the ETS in both tested species. The results with imidacloprid indicate that *G. fossarum* from running water is more affected by short-term higher concentrations of imidacloprid than is *A. aquaticus,* a common inhabitant of standing water (ponds, lakes). *G. fossarum* reacts with a lower ETS/R, even at very low concentrations of pesticide (i.e. 0.01 mg L^{-1}), while *A. aquaticus* reacts only to concentrations that are at least two orders of magnitudes higher. In both sets of experiments *A. aquaticus* was shown to be less sensitive to atrazine and imidacloprid than *G. fossarum.*

Water louse prefers standing or slow flowing waters, retention time of both pesticides is therefore longer than in fast flowing waters, which stream scud prefers. Therefore water louse developed a relatively higher resistance to allochtonous substances.

This combination of measurements of R and ETS activity, provides a good assessment of stress after exposure of animals to pesticides for short periods of time. Stress in exposed animals is normally shown as a reduction in ETS/R ratio, which drops to a value close to 1 when the animal is highly affected. The ETS/R ratios are high in normal conditions; normally well above 2–4 (as indicated in the control group). Values close to 1 indicate that the animal is using 100% of its respiratory potential and that the whole enzyme system is exploited. Hypothetically, R should increase under stress conditions but ETS should stay at the same level under short exposure times. This would be reflected in decreased ETS/R values. Some pesticides at higher concentrations, not only increase the demand on energy in animals, but actually destroy the energy production system. Those types of pesticide that affect animals on both levels (ETS & R) are thus harmful also for non-target organisms, even at low concentrations. Decrease in ETS activity is reflected in reduced ETS/R ratio, which indicates greater stress on test animals. Such effects of pesticides on a biochemical level could not be detected by standard toxicity tests.

Both tested animals, *A. aquaticus* and *G. fossarum*, are very susceptible to short-term atrazine and imidacloprid exposure. *A. aquaticus* shows significant effects at concentrations of 10 mg L^{-1} or more of atrazine and higher than 1 mg L^{-1} for imidacloprid. The more sensitive *G. fossarum* shows significant effects at concentrations higher than 1 mg L^{-1} of atrazine and higher than 0.1 mg L^{-1} of imidacloprid. Elevated levels of R and diminution of ETS activity result in a lower ETS/R ratio, which we propose in this article to be an indicator of stress. Using this method, maximum permissible levels of toxic compounds in water bodies can be determined more accurately. With such more reliable data, better environmental policies and industrial discharge regulations can be applied.

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Comparative toxicity of imidacloprid and its transformation product 6-chloronicotinic acid to non-target aquatic organisms: Microalgae *Desmodesmus subspicatus* and amphipod *Gammarus fossarum*

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ABSTRACT

Neonicotinoids are widely applied pesticides due to their higher affinity for insect nicotinic acetylcholine receptors. These compounds are extensively applied to control pest insects in different agricultural crops; however they can also affect non-target invertebrates. Little is known about the toxicity effects of their transformation products on aquatic non-target organisms. Oxidative stress responses and behavioural changes in the crustacean amphipod *Gammarus fossarum* were investigated as well as the growth rate in freshwater algae *Desmodesmus subspicatus* after 96 h exposure to imidacloprid, its commercial formulation Confidor 200SL and its transformation product 6-chloronicotinic acid. Algal growth has shown significant sensitivity to Confidor 200SL and 6-chloronicotinic acid when compared to imidacloprid. In the case of amphipods, low doses of imidacloprid (102.2 $\mu g\,L^{-1}$) were sufficient to induce lipid peroxidation, while Confidor 200SL induced increased catalase activity (511.3 $\mu g\,L^{-1}$) and lipid peroxidation (255.6 $\mu g\,L^{-1}$). 6-Chloronicotinic acid altered significantly only antioxidant mechanisms (catalase activity) without changing lipid peroxidation levels. These different biochemical responses are helpful to understand the mechanism of imidacloprid and 6-chloronicotinic acid-induced oxidative stress. In addition, obtained data demonstrate potential harmful effects of neonicotinoid-based pesticides on non-target aquatic organisms.

1. Introduction

Pesticide producers are continuously replacing older generation pesticides with an array of newly developed pesticides. These products are characterised by selective action on target organisms in order to reduce their possible unwanted effects. One of these representatives is imidacloprid [IMI; 1-[(6-chloro-3 pyridynil) methyl]-*N*-nitro-2-imidazolidinimine] a nicotine-derived compound (neonicotinoid) with a large potential distribution due to its agonistic action on insect nicotinic acetylcholine receptors and its selective toxicity to insects over vertebrates [1].

Legislations related to the placement of pesticides on market focus only on parent compounds rather than formulations or transformation products. It is important to notice that the commercial formulations of IMI (such as Confidor 200SL, Admire, Merit and Gaucho) [2] are the ones applied in the environment with relevant soil (50–320 $g\,ha^{-1}$) and foliar concentrations (73–150 $mg\,L^{-1}$). These frequently used commercial mixtures of IMI contain co-formulants and other solvents that could modify its toxicity and bioavailability. Recent evaluation of the data relative to different formulations noted high levels of IMI in leaves and in blossoms of treated plants, and increases in residue levels over time [3]. Data indicated that the use of these IMI-formulations on annual basis may be at the end cumulative. Due to recent findings certain commercial products within the class of neonicotinoids (containing active ingredient IMI) were placed under re-evaluation and need further studies [3]. In addition, no particular control or monitoring over the presence of IMI transformation products is performed. The primary IMI breakdown products in soil are: imidacloprid urea, 6-hydroxynicotinic acid and 6-chloronicotinic acid (6CNA) [4]. 6CNA is one of the final transformation products of IMI and due to its high water solubility (2 $g\,L^{-1}$) it may leach from soil into the aquatic environment. Furthermore, IMI persistence in soil is affected by various factors such as temperature, organic matter, cropping and its solubility of 0.51 $g\,L^{-1}$. It can contaminate surface and ground water by runoff or leach from agricultural areas and lead to pulse-pesticide or localised contaminations [5,6].

Detected aquatic concentration indicate measured levels of IMI going from 14 $\mu g\,L^{-1}$ up to 0.3 $mg\,L^{-1}$ for surface waters [7,8], while the estimated concentrations for accidental spills reach high values going from 1.8 up to 7.3 $mg\,L^{-1}$ [9]. Different studies are

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referring to the persistence of IMI in the aquatic environment and its toxicity to different non-target aquatic invertebrates. These investigations span from single species toxicity tests in laboratory [10–13] to complete indoor/outdoor stream mesocosms studies under constant exposure [14] and short-pulse exposure conditions [15,16]. The authors observed generally modifications in survival, behaviour and population growth rate; while some of them evaluated biochemical alterations at molecular level and compared the toxic effects of pure compound IMI and its formulated version [11,12]. Although IMI is continuously studied, fewer investigations have been conducted on its transformation products. There is a single study relative to the toxicity of 6CNA on aquatic invertebrates performed on midge *Chironomus tentans* with LC_{50} (96 h) higher than 1 mg L^{-1} [17] which warrants expanding our knowledge in this field. In addition, major part of studies on IMI's transformation products are performed mainly on common honey bee *Apis mellifera* [18,19]. To this end, it is necessary to investigate their effects on freshwater biota, especially on non-target aquatic organisms.

Among the potential non-target organisms, unicellular green algae are commonly used for toxicity tests [20]. Any adverse impact on algae is likely to affect organisms at higher trophic levels and may have important consequences for the health status of the whole aquatic ecosystem [21]. In addition, aquatic non-target crustaceans of the genus *Gammarus* are frequently used in ecotoxicological studies [22–24]. They play a major role in leaf litter breakdown and are important for material transfer in the food web [25,26]. In this study *Gammarus fossarum* Koch, 1835 was used as a model organism considering its sensitiveness to several environmental pollutants. Furthermore, crustaceans as well as insects belong to arthropods and due to this crustacean-insect relationship they could present a potentially suitable non-target group for neonicotinoid testing [27].

Different classes of pesticides may be related to enhanced production of reactive oxygen species (ROS) which could contribute to the toxicity of these compounds [28]. Basic cellular metabolism in aerobic organisms involves the production of oxygen free radicals and non-radical ROS [29]. The imbalance between the generation and the neutralisation of ROS by antioxidant mechanisms within an organism generates the oxidative stress [30,31]. In order to have a better understanding of the toxic action of these compounds the involvement of induced ROS production was investigated by measurement of oxidative stress biomarkers such as antioxidant enzyme catalase (CAT), detoxifying enzyme glutathione-*S*-transferase (GST) and the levels of lipid peroxidation (LP) damage [28,32,33].

The aim of the study was to provide additional information on the possible toxic effects of IMI, its commercial formulation Confidor 200SL and its transformation product 6CNA on non-target aquatic organisms. For this reason physiological/biochemical biomarkers, mortality and behavioural alterations on the amphipods (acute toxicity) were evaluated as well as the growth rate of microalgae (chronic toxicity). Antioxidant defence system alterations and lipid peroxidative damage to cell membrane were studied because of their potential to serve as useful biochemical biomarkers that could be applied in environmental monitoring programmes.

2. Materials and methods

2.1. Chemicals

Imidacloprid (IMI) was purchased as the Pestanal® grade chemical (99.8% purity; Sigma–Aldrich, UK), and as a commercial formulation known as Confidor 200SL (200 g L^{-1} of active ingredient (a. i.) IMI, Bayer Crop Science Slovenia, Ljubljana, Slovenia) and 6CNA was obtained as pure compound (97%) from Fluka (Sigma–Aldrich, Switzerland). The following chemicals were all obtained from Sigma Aldrich: acetonitrile CHROMASOLV® for HPLC grade, dibasic and monobasic potassium and sodium phosphate, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), acetylthiocholine iodide, 1-chloro-2,4-dinitrobenzene (CDNB), L-glutathione (reduced form), hydrogen peroxide (30%), bovine serum albumin (BSA), Bradford reagent, trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylhydroxytoluene (BTH), 96% ethanol, 1-butanol, hydrochloric acid (37%), dimethyl sulfoxide (DMSO). Potassium hydrogen phthalate was purchased from Alfa Aesar GmbH (Karlsruhe, Germany). Acetic acid glacial 100% p.a. was provided from Merck (Darmstadt, Germany). All chemicals were of the highest commercially available grade.

2.2. Stability study of tested chemicals during experimental trial

To ensure reliable toxicity data, the stability of IMI and 6CNA was checked. The exposure was confirmed measuring the concentrations of the specific chemicals at the beginning and end of the experimental trial, under the same condition as toxicity tests (described in Sections 2.4.1 and 2.4.2). IMI and 6CNA samples were taken in duplicates and all determinations were performed in four experiments.

IMI and 6CNA were extracted with the use of the miVac centrifugal concentrator Modular Series (Genevac). The water was evaporated (under maintained vacuum conditions at 70 °C for approximately 200 min). The dried leftover was re-dissolved in 500 μL of double deionised H_2O. Previous procedure was applied for samples with lower concentration of chemicals (used for tests with amphipods), while samples with higher concentration of chemicals (used for tests with algae) were analysed immediately without pre-concentration treatment. All prepared samples were stored in glass vials under dark at 4 °C until subjected to HPLC-DAD (UV–Vis). For quantification purposes, calibration curves were prepared. The r^2 value of the regression line for IMI was 0.9999 and for 6CNA was 0.9996.

2.2.1. HPLC-DAD analysis

Aqueous solutions of IMI and 6CNA were analysed by HPLC-DAD (UV–Vis) consisting of an Agilent 1100 Series chromatograph, coupled with a DAD detector operating in the UV–Vis range The separation was achieved using a Zorbax C8 column (250 mm × 4.6 mm) filled with a stationary phase Chromasil 100 (pore size 5 μm, end-capped) produced by BIA Separations d.o.o., Slovenia. The column thermostat was maintained at 25 °C and injection volume was 75 μL.

According to Žabar et al. methods for IMI [34] and 6CNA [35] detection were applied. For IMI detection the eluents consisted of 30% acetonitrile (A) and 70% acetic acid 0.75% v/v (B); isocratic elution; flow rate was 1 mL min^{-1}. The wavelength was 270 nm and the retention time was 8.9 min. While for the 6CNA detection the eluents consisted of acetonitrile (A) and acetic acid 1.5% v/v (B); flow rate was 1 mL min^{-1}. The gradient elution was as follows: 0–16 min 15% A; 16–20 min 70% A. The wavelength was 242 nm and the retention time for 6CNA was 13.2 min.

2.3. Test organisms

Desmodesmus subspicatus (Chodat) Hegewald *et* Schmidt (formerly *Scenesdesmus subspicatus*, CCAP 276/22) was kindly provided by the Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany. Microalgae were grown in a medium recommended by standard guidelines for freshwater algal growth inhibition test [36].

G. fossarum were collected in April–July 2011 using a net (by the kick sampling method) from the stream Vogršček (Slovenia). The

sampling site is in the lower Vipava Valley in Goriška region of Slovenia (45°90′ N; 13°70′ E). It is a small waterbed free of industrial, agricultural contamination or human activities and it can be considered unpolluted. This site has a good water quality according to ARSO data record (Agencija Republike Slovenije za okolje – Slovenian Agency for Environment; http://www.arso.gov.si/vode/poro%C4%8Dila%20in%20publikacije/povrsinske_letna.html) and high densities of gammarids are found.

All water samples from the sampling site and during the experimental trial were monitored for temperature, pH, conductivity, oxygen concentration and saturation with a multi-meter WTW 350i (with microelectrode replacements for small volumes). In addition, total organic carbon and total nitrogen (TOC and TN) were measured in water samples from the sampling site with a TOC Analitik Jena multi N/C 3100, calibrated with potassium hydrogen phthalate. Before being processed for the TOC and TN analyses, samples were acidified to pH 2–3 with hydrochloric acid.

Gammarids were kept during an acclimatisation period of at least 14 days in a 20-L glass aquarium supplied with thoroughly aerated original stream water. An 8/16 h light/dark natural photoperiod was maintained with the temperature at 12 ± 2 °C in a temperature and humidity controlled chamber and regular water renewal every two days. Animals were fed *ad libitum* twice a week using a pinch of dry food (e.g. TetraMina® flakes) or raw peas.

2.4. Experimental procedures

2.4.1. Algae toxicity test

Chronic toxicity of pesticides was conducted in 96 microwell plate. The algal inoculum was taken from an exponentially growing pre-culture and added into 25 mL of growth media in order to obtain an initial cell density of 10^4 cells mL^{-1}. Final volume of each well was 200 μL. Serial dilutions of tested pesticides were made in culture medium. Six replicates of controls (untreated) and three replicates of each test concentration were applied. All the plates with cover, control and treatments, were incubated for four days (96 h) at a temperature of 23 ± 1 °C and light intensity of 1100 lux. Algal growth was detected fluorometrically in intervals of 24 h over a period of 96 h in order to achieve a virtual kinetic data distribution. Analyses of chlorophyll fluorescence were performed by a Tecan Infinite® 200 PRO (Männedorf, Switzerland). Measurements were conducted using fluorescence excitation of 440 nm and by an emission of 680 nm. Before reading, tested microplates were shaken for 30 s at 100 rpm. Average of specific growth rates were calculated and subsequently used for calculation of percentage inhibition in comparison to control [37]. IC_{50} at 96 h (inhibition concentration that cause 50% inhibition of algal growth) was estimated for tested compounds using linear regression analysis [38].

Solution of 1 M IMI and 6CNA was prepared in DMSO. Afterwards, a 10 mM (2.55 g L^{-1} for IMI and 1.57 g L^{-1} for 6CNA) stock solution was prepared by the addition of IMI and 6CNA (1 M) or Confidor 200SL to standard algal medium, with constant mixing until complete dissolving. The test solutions were prepared by adding an appropriate volume of the stock solution in the algal medium to achieve final concentration. The following range of equal molar concentrations was prepared for all tested compounds: 7.6; 25.6; 51.1; 127.8 and 255.6 mg L^{-1} for IMI and 4.7; 15.7; 31.5; 78.7 and 157.5 mg L^{-1} for 6CNA. For Confidor 200SL the final concentrations were corresponding to 0.003–0.12% (v/v) which contained 7.6–255.6 mg L^{-1} of a. i. IMI. Lower concentrations of IMI than those monitored in this experimental trial were already tested on *D. subspicatus* and showed no effect on algal growth up to 10 mg L^{-1} and due to this fact were excluded [39]. The toxicity of co-formulants incorporated in Confidor SL 200 (as negative control – a solution consisting of 38.4% of dimethylsulfoxide, 37.5% of 1-methyl-2-pyrrolidone and 24.1% of double deionised water in place of IMI) was tested. In addition, as an internal quality control, the bioassays were also performed on the reference chemical potassium dichromate (positive control – $K_2Cr_2O_7$; 0.1–30 mg L^{-1}) [36].

2.4.2. Amphipods toxicity test

Gammarids were exposed for 24 h (acute toxicity) to equal molar concentrations of IMI, Confidor 200SL and 6CNA for better comparison. A short exposure period sufficient to promote early alterations (24 h) was used also to mimic runoff-related pulse exposures to pesticides [40,41]. The peak pesticide concentrations usually persist for about 24 h. Furthermore, *G. fossarum* from running water is greatly affected by short-term higher concentration of IMI [12]. Sub-lethal exposure concentrations were based on previously determined acute LC_{50} (48 h) and EC_{50} (24 h) values for IMI of 0.8 and 0.07 mg L^{-1} [12].

Solution of 1 M IMI and 6CNA was prepared in DMSO. Afterwards, a 10 mM (2.55 g L^{-1} for IMI and 1.57 g L^{-1} for 6CNA) stock solution was prepared by the addition of IMI and 6CNA (1 M) or Confidor 200SL to distilled water, with constant mixing until complete dissolving. The test solutions were prepared by adding an appropriate volume of the stock solution in the original stream water to achieve final concentration. The following range of concentrations was prepared for all tested compounds: 6.3; 12.7; 25.5; 51.1; 102.2; 153.3; 204.5; 255.6 and 511.3 μg L^{-1} for IMI and 3.9; 7.8; 15.7; 31.4; 62.8; 94.6; 126. 2; 157.7 and 315.5 μg L^{-1} for 6CNA. For Confidor 200SL the final concentrations were corresponding to 0.000003–0.0002% (v/v) which contained 6.3–511.3 μg L^{-1} of a. i. IMI. The tested concentrations of the negative control (co-formulants only) in case of amphipods were equivalent to concentrations of Confidor SL 200 used in the tests.

The experimental trial was performed using adult male specimens. After sex determination, total body length [42] and total wet weight was measured (animal were dried between two sheets of filter paper before being weighted). Fifty individuals per exposure concentration were used for every tested compound. Plastic Petri dishes (100 mm × 20 mm; 20 mL volume) covered in order to reduce water evaporation were used for exposure experiments. The bioassays were conducted in darkness, in a temperature and humidity-controlled chamber (12 ± 2 °C; 60% humidity). After a 24 h exposure period, immobility or moulting and mortality were observed. Live/dead organisms were determined by gently poking and observing movement of appendages. Organisms were counted as dead if none of the appendices were moving after poking for three times. Inactive/paralysed animals were identified when only respiration movements were left [43]. Moulted animals were counted based on the presence of the entire old *exuvia* in the exposure vessel (moulted amphipods were not used for biochemical parameters analyses). For each biochemical assay 10 randomly selected gammarids per concentration (from fifty individuals) were processed using whole-body homogenates due to their small body size.

2.5. Biochemical biomarker assays

Prior to individual homogenisation, excess chemicals present on the animals' surface were rinsed several times according to Jemec [11]. Whole-body specimens were homogenised in 500 μL of ice-cold phosphate buffer (pH 7.0) for 3 min using a glass–glass Elvehjem–Potter homogeniser. The homogenate was sonicated on ice (5–10 s) and centrifuged for 15 min at 3000 rpm and 4 °C. Freshly prepared clear supernatant was collected and kept on ice to be used for enzyme activities measurements.

Activity of acethylcholinesterase (AChE) was determined using DTNB and acetylthiocoline iodide as substrate according to Ellman et al. [44]. The reaction was followed on a Perkin Elmer Lambda 35

UV/VIS spectrophotometer at 412 nm for 8 min. AChE activity is expressed as μmol of substrate hydrolysed per minute per mg protein (ε = 13 600 L cm^{-1} mol^{-1} for DTNB).

CAT activity was determined according to the method of Jamnik and Raspor [45] by measuring the decrease in absorbance on spectrophotometer at 240 nm for 2 min due to the decomposition of H_2O_2 (ε = 40 L cm^{-1} mol^{-1}). The specific activity of CAT was expressed as μmol of H_2O_2 reduced per minute per mg protein.

GST activity was determined according to the protocol of Habig et al. [46]. The method is based on determination of the conjugated product dinitrophenyl-thioether at 340 nm produced from CDNB used as an artificial substrate and reduced glutathione. Values expressed as nmol of reduced glutathione and CDNB conjugate formed per min per mg protein (ε = 9600 L cm^{-1} mol^{-1} for CDNB).

All the data relative to the enzymatic activity are normalised to the total protein content based on the method of Bradford [47].

LP was estimated *in vitro* after the formation of malondialdehyde (MDA), a major by-product of lipid peroxidation that reacts with thiobarbituric acid [48], with slight modifications. Whole-body gammarids were rinsed, as described previously and homogenised individually in TCA–TBA–BTH reagent [15% (w/v) TCA, 0.37% (w/v) TBA, 1 M HCl, and 0.01% BTH]. Samples were incubated at 90 °C for 30 min, then chilled at room temperature, added 1.2 mL of 1-butanol and centrifuged at 12,000 rpm for 10 min. Absorbance of the supernatant was measured at 535 and 600 nm, the final one to correct the non-specific turbidity. Before the heating step, absorbance was measured at 280 nm for total protein concentration. These absorbance values of protein content were used to properly normalise absorbance values obtained for LP.

2.6. Statistical analyses

All statistical tests were performed using STATISTICA 7 StatSoft software. Results from each exposure trial are presented in graphs as mean ± standard error (SE). Statistical comparisons were conducted between control and exposure data using the Student's *t*-test or the Mann–Whitney rank sum test after the software direct choice of parametric or nonparametric data, respectively. In addition, multiple comparisons were analysed with the One-way ANOVA and Tukey post-test. $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*) were accepted as levels of statistical significance and shown in graphical representations.

3. Results

3.1. Water quality parameters and stability study

Water quality parameters were measured for all water samples from the sampling site and during toxicity tests. No significant changes were observed across the whole experimental trial (n = 10). Mean values were as follows: pH 7.9 ± 0.1, temperature 14.7 ± 0.3 °C and water conductivity of 378.3 ± 21.7 μScm^{-1}. The water had average oxygen concentration of 9.8 ± 0.2 mg L^{-1} and saturation of 95.8 ± 2.3%. Mean values of TOC and TN at the water source location were 8.7 ± 0.1 and 0 mg L^{-1}, respectively. Moreover, dissolved oxygen concentration during whole experimental trial was between 70% and 80% of saturation. These were all acceptable conditions for toxicity test [49].

Our experiments showed no significant changes in concentration of IMI and 6CNA in test solutions during 24 h (amphipods) and 96 h (algae) exposure (Table 1). The actual exposure concentrations of both chemicals did not differ by more than 3.4 ± 0.3% (for concentrations in tests with amphipods) and by 15.8 ± 0.4% (for concentrations in tests with algae) from the initial concentrations. IMI and 6CNA concentrations were consistent over time in all tests. Therefore the results are given in nominal concentrations, as suggested by ISO 10706 [50].

3.2. Algae toxicity test

Algal chronic toxicity revealed a high toxic potential of 6CNA at the highest concentration (Fig. 1C). 6CNA induced some perceivable alterations in algae growth, causing slight and temporary inhibition effects at lower doses (4.7 and 15.7 mg L^{-1}) already after 24 h compared to control ($p < 0.05$) (Fig. 1C). The highest dose of 6CNA extensively suppressed the algal growth. 6CNA induced acidification of the algal medium (pH up to 5.5 ± 0.1; n = 3). In all other groups, pH did not deviate significantly from the initial values as in the case of 6CNA at the highest dose. Overall 6CNA effects were stimulatory on algae growth. Major stimulatory effect of 6CNA was observed at 31.5 mg L^{-1} (48 h) reaching 176.4 ± 3.4% and stayed significantly increased also after 72 h compared to control ($p < 0.001$) (Fig. 1C). It was not possible to calculate the IC_{50} value for IMI due to its low inhibitory effects within the entire range of tested concentrations (Fig. 1A). Furthermore, the toxicity of Confidor 200SL ranged from 27.9% up to 49.72% (Fig. 1B). Inhibition of algal growth was significant at 127.8 and 255.6 mg L^{-1} compared to control ($p < 0.01$). Higher toxicity of Confidor 200SL was possibly induced by the co-formulants present in the commercial formulation which contributed as a major part to toxicity for algae. The co-formulants alone induced a significant inhibition of 82.3% and 89.7% (at 0.06 and 0.12%; v/v) compared to control ($p < 0.001$) (Fig. 1B).

3.3. Amphipods toxicity test

3.3.1. Survival rate and behavioural alterations

After 24 h of acute toxicity test, monitored in all groups were: (1) the number of dead amphipods (mortality) and (2) the number

Table 1
Mean ± standard error detected concentrations expressed as μg L^{-1} and mg L^{-1} of IMI and 6CNA in aqueous samples for the 24 h *G. fossarum* and 96 h *D. subspicatus* static toxicity tests (n = 3).

Nominal concentration μ L^{-1}	Dark T = 22 °C [μ L^{-1}]		Nominal concentration (mg L^{-1})	Light T = 22 °C [mg L^{-1}]	
IMI	0 h	24 h	IMI	0 h	96 h
102.2	105.5 ± 2.5	99.7 ± 0.7	7.6	7.5 ± 0.1	6.4 ± 0.1
153.3	154.7 ± 0.7	148.5 ± 1.4	25.6	26.3 ± 0.5	21.9 ±0.5
204.5	203.9 ± 1.8	198.1 ± 0.5	51.1	51.4 ± 1.2	44 ± 1.4
255.6	254.2± 1. 6	250.8 ± 0.4	127.8	127.4 ± 0.7	103.6 ± 2.2
511.3	511.7 ± 0.18	481.2 ± 0.6	255.6	255.1 ± 0.8	240.4 ± 2.9
6CNA	0 h	24 h	6CNA	0 h	96 h
62.8	62.4 ± 0.5	63.3 ± 0.9	4.7	4.5 ± 0.1	4.1 ± 0.1
94.6	93.5 ± 0.8	92 ± 0.8	15.7	14.8 ± 0.5	14 ± 0.2
126.2	127.3 ± 0.4	120 ± 0.9	31.5	29.9 ± 0.9	28.7 ± 0.6
157.7	157.4 ± 0.9	152.6 ± 1.1	78.7	77.1 ± 1.3	71.1 ± 0.8
315.5	315.7 ± 0.3	310.1 ± 1.2	157.5	156.1 ± 0.8	122.3 ± 2.6

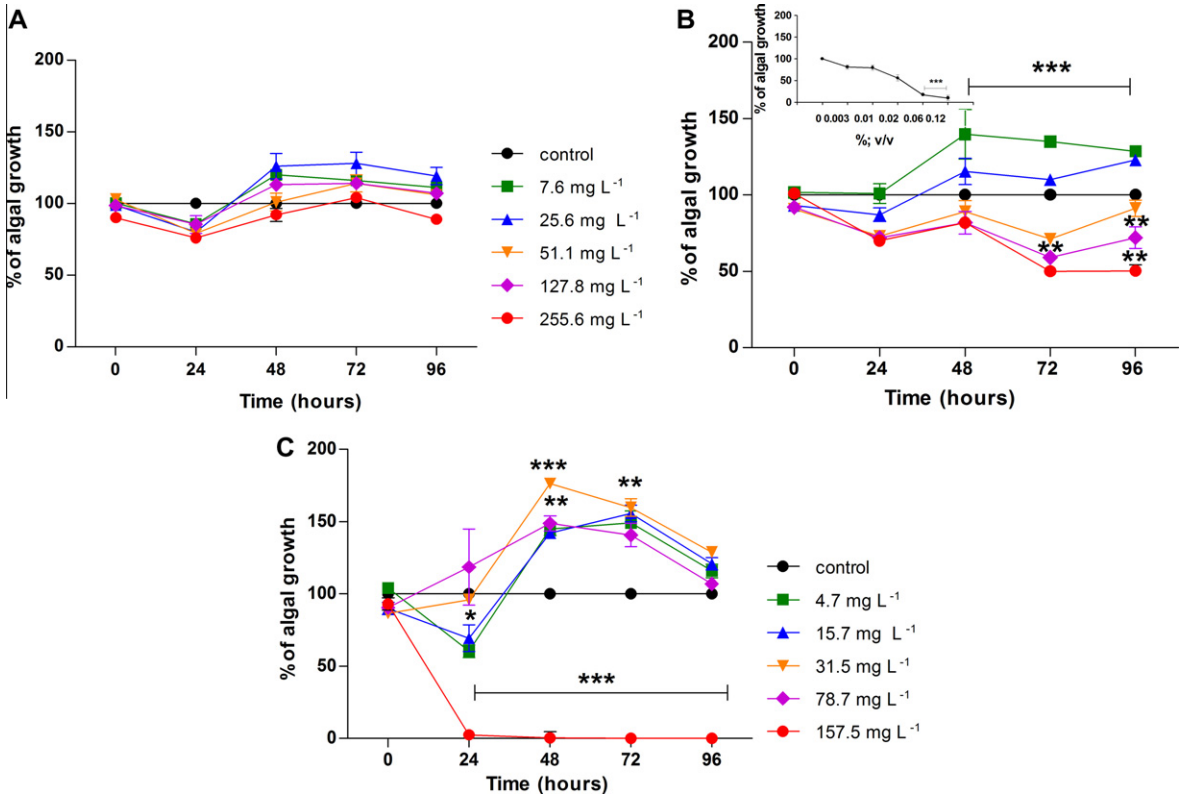

Fig. 1. *D. subspicatus* % of algal growth compared to control after exposure to IMI (A) Confidor 200SL (B) and 6CNA (C) at 24, 48, 72 and 96 h. The inside graph represents exposure to negative control-co-formulants only. Data are reported as mean ± standard error ($n = 3$). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

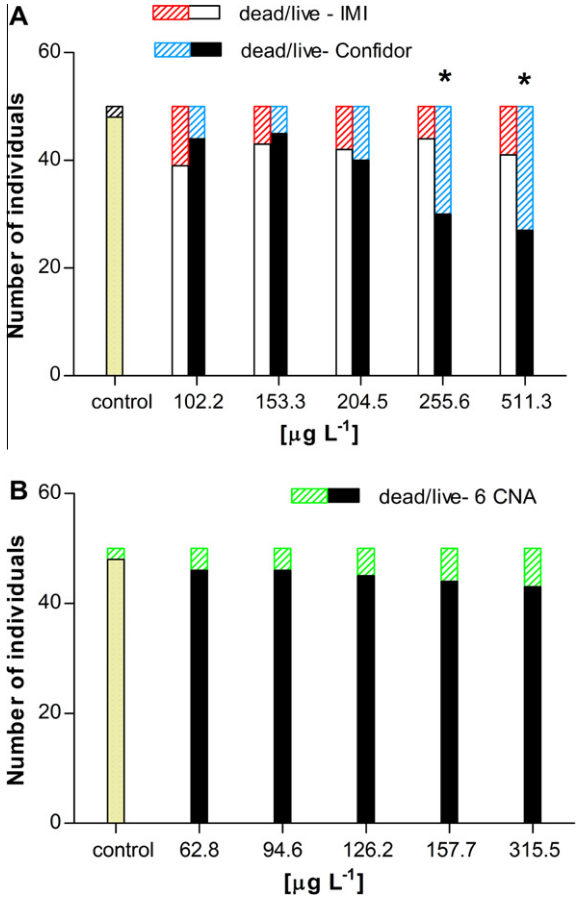

Fig. 2. Mortality rate of *G. fossarum* after 24 h of exposure to IMI or Confidor 200SL (A) and 6CNA (B). ($n = 50$). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

of immobile/paralysed or recently moulted amphipods. Only male adult specimens were used for laboratory tests. All specimens presented a mean total body length of 12.35 ± 0.25 mm and mean weight of 0.029 ± 0.002 g. Individuals which sex was not possible to determine were classified as juveniles and not used for this research.

The negative control (co-formulants mixture) did not have any adverse effects on *G. fossarum* at all tested concentrations (data not presented). Due to this fact, all values were compared to control (only stream water). Furthermore, concentrations of all tested compounds lower than 102.2 µg L^{-1} for IMI and 62.8 µg L^{-1} for 6CNA did not induce significant effects compared to control (data not shown).

Average mortality in control groups was between 2.2 ± 1.1 and 4.3 ± 1.9% in all bioassays. Our data demonstrated slight toxicity of IMI with minor changes in mortality rate (Fig. 2A). IMI induced only 22.3% ± 5.09 of dead organisms at 102.2 µg L^{-1}. Commercial formulation Confidor 200SL demonstrated an increased effect on mortality, especially at higher concentrations. Percentages of dead organisms at 255.6 and 511.3 µg L^{-1} of a. i. reached 40 ± 5.7% and 45.5 ± 7.3%, respectively (Fig. 2A). This increased mortality was significant for the both concentrations ($p < 0.05$). On the contrary 6CNA showed an overall low toxicity, ranging from 8.6 ± 1.9% up to 14.1 ± 1.1% (at 62.8 and 315.5 µg L^{-1}, respectively; Fig. 2B).

At 511.3 µg L^{-1} of IMI and Confidor 200SL was present a high number of inactive animals with only respiration movements. These values were of 76.6 ± 6.6% for IMI and of 90 ± 5.7% for Confidor 200SL ($p < 0.001$; compared to control) (Table 2). It is also interesting to report the number of animals that underwent moult (leaving the entire old *exuvia*) after treatment with tested compounds, apparently stimulatory effect on moult processes was due to the action of transformation product 6CNA. Number of moulted amphipods after 24 h exposure to 6CNA at 315.5 µg L^{-1} was of 56.6 ± 3.3% ($p < 0.001$) (Table 2). Number of moulted

Table 2
Number of immobile/paralysed, hyperactive and moulted individuals of *G. fossarum* (% of total treated animals) exposed to IMI, Confidor 200SL and 6CNA for 24 h. Data are expressed as mean ± standard error ($n = 30$).

Nominal concentration (Vg L^{-1})	Immobile/paralysed individuals	Hyperactive individuals	Moulted individuals
IMI			
Control	None		None
102.2	16.6 ± 3.3**	None for all groups	10 ± 5.7
153.3	16.6 ± 8.8		13.3 ± 3.3
204.5	13.3 ± 3.3		23.3 ± 8.8
255.6	43.3 ± 3.3***		26.6 ± 3.3**
511.3	76.6 ± 6.6***		23.3 ± 3.3**
Confidor 200SL			
Control	None		None
102.2	23.3 ± 3.3**	None for all groups	6.6 ± 3.3
153.3	33.3 ± 3.3**		13.3 ± 3.3
204.5	46.6 ± 14.5**		13.3 ± 8.8
255.6	56.6 ± 3.3***		10 ± 0
511.3	90 ± 5.7***		13.3 ± 3.33
6CNA			
Control		None	None
62.8	None for all groups	16.6 ± 3.3**	20 ± 5.7**
94.6		23.3 ± 3.3**	33.3 ± 3.3**
126.2		43.3 ± 3.3***	43.3 ± 12**
157.7		43.3 ± 3.3***	46.6 ± 3.3***
315.5		80 ± 5.7***	56.6 ± 3.3***

** $p < 0.01$.
*** $p < 0.001$.

animals was minor after 24 h of exposure to IMI and Confidor 200SL at 511.3 μg L^{-1} (23.3 ± 3.3% and 13.3 ± 3.3%, respectively; $p > 0.05$). 6CNA seemed to induce overall hyperactivity and rapid swimming (with numerous sideways and back-and-forth movements) which affected 80 ± 5.7% of total treated gammarids at 315.5 μg L^{-1} 6CNA (compared to control; $p < 0.001$). Numbers of counted individuals which presented the described behavioural characteristic are summarized in Table 2. It is important to emphasise that this data need further quantification with technologies that allow a more detailed analyses and recording of behavioural patterns.

3.3.2. Effects on enzyme activities and lipid peroxidation

Results of enzyme activities were expressed per protein content, since changes in the protein were not significant as a result of 24 h exposure to all tested compounds.

In this study a possible indirect effect of IMI on AChE activity in neonicotinoid exposed gammarids was tested as a biomarker of the cholinergic system. *G. fossarum* exposed to IMI displayed no significant changes of AChE activity at all concentrations (data not presented in graph). The AChE values at all exposure concentrations of IMI ranged between 70.6 ± 7.8 and 78.2 ± 11.6 μmol/min/mg proteins ($p > 0.05$; compared to control). CAT activity was not modified after IMI exposure (Fig. 3A). The values ranged between 22.04 ± 1.5 μmol/min/mg protein for control and 28.4 ± 8.6 μmol/min/mg protein at 255.6 μg L^{-1}. Commercial formulation induced a moderate change in CAT at 511.3 μg L^{-1} a. i. going up to 48.06 ± 9.7 μmol/min/mg protein compared to control ($p < 0.05$). Values of CAT activity in the case of exposure to 6CNA reached 48.9 ± 6.7 μmol/min/mg protein already at 157.7 μg L^{-1} ($p < 0.001$) (Fig. 3B). After exposure to Confidor 200SL two different outcomes for GST activity at 255.6 and 511.3 μg L^{-1} were evident (Fig. 4A). At 255.6 μg L^{-1} was present an observable, but statistically not significant decrease in GST activity ($p = 0.053$). The values of GST went from control values of 419.1 ± 101.8 nmol/min/mg protein

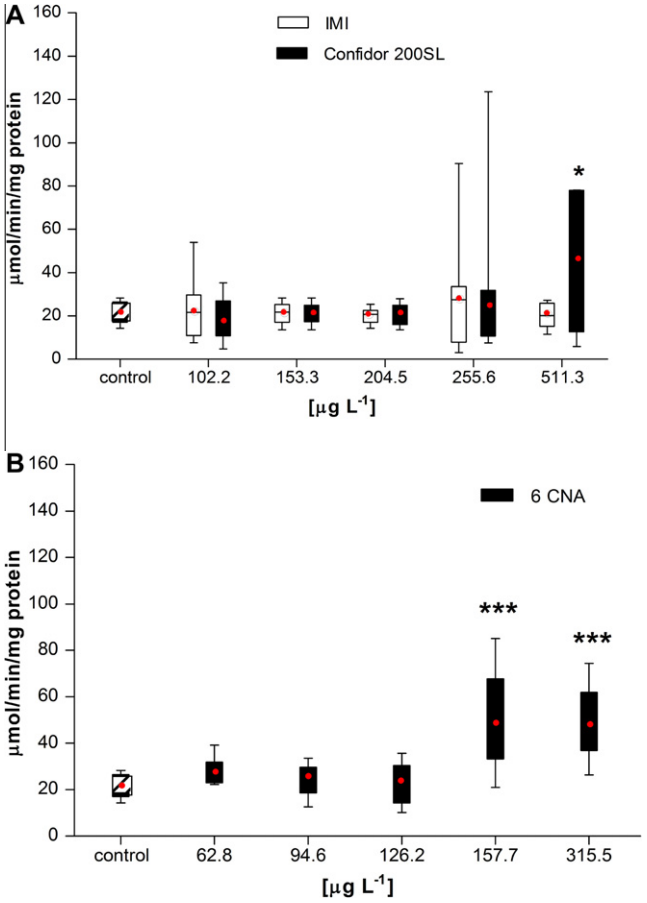

Fig. 3. Whole-body CAT activity (μmol/min/mg protein) of *G. fossarum* measured after 24 h of exposure to IMI or Confidor 200SL (A) and 6CNA (B). The boxes contain 75% of all readings, the symbols represent minimum and maximum values (⊥) and the mean value (●). ($n = 10$). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

to 286.8 ± 92.71 nmol/min/mg protein at 255.6 μg L^{-1}. Higher concentration of Confidor 200SL (511.3 μg L^{-1} of a. i.) induced an increase of GST activity up to 831.4 ± 117.2 nmol/min/mg protein ($p < 0.05$). IMI and 6CNA exposure provoked no significant changes in GST activity compared to control ($p > 0.05$) (Fig. 4A and B, respectively). IMI induced at 102.2 μg L^{-1} an increase in lipid peroxidation (LP) levels (Fig. 5A). This increase was 2.7-fold higher in contrast to the control group ($p < 0.01$). On contrary, Confidor 200SL induced significant rise of thiobarbituric acid reactive substances (TBARS) only at higher dose (255.6 μg L^{-1} of a. i.; $p < 0.05$). This increase was lower than the significant peak induced by IMI at 102.2 μg L^{-1} (Fig. 5A). No significant effect of 6CNA on LP increase was noted after 24 h at all concentrations (Fig. 5B). However, it was detected a significant decrease of LP values at 315.5 μg L^{-1} ($p < 0.001$).

4. Discussion

Chronic testing was performed on freshwater microalgae *D. subspicatus*. Generally, it appears that algae are some orders of magnitude less sensitive to IMI than arthropod species and exhibiting no effects of IMI on their growth rate [36,51]. Tišler et al. [38] determined for *D. subspicatus* an IC_{50} (72 h) for IMI a. i. at 389 mg L^{-1} (in comparison highest applied concentration in this study was 255 mg L^{-1}). Data presented in this research confirmed the same action of IMI as pure compound causing no significant adverse effects on algal growth. On the contrary, Confidor 200SL was highly toxic to algae due to the presence of co-formulants which

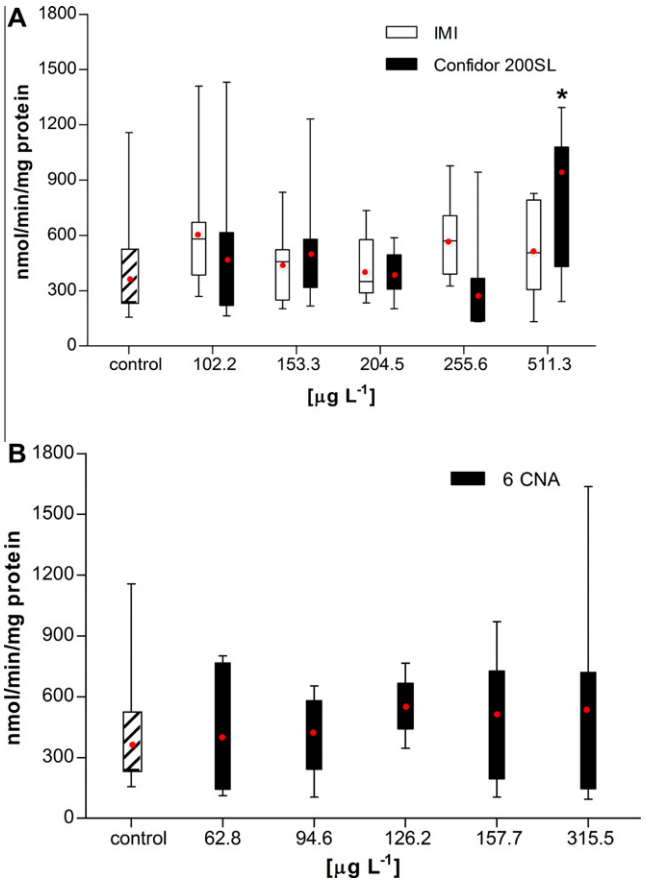

Fig. 4. Whole-body GST activity (nmol/min/mg protein) of *G. fossarum* measured after 24 h of exposure to IMI or Confidor 200SL (A) and 6CNA (B). The boxes contain 75% of all readings, the symbols represent minimum and maximum values (⊥) and the mean value (●). ($n = 10$). $p < 0.001$ (∗∗∗), $p < 0.01$ (∗∗), and $p < 0.05$ (∗).

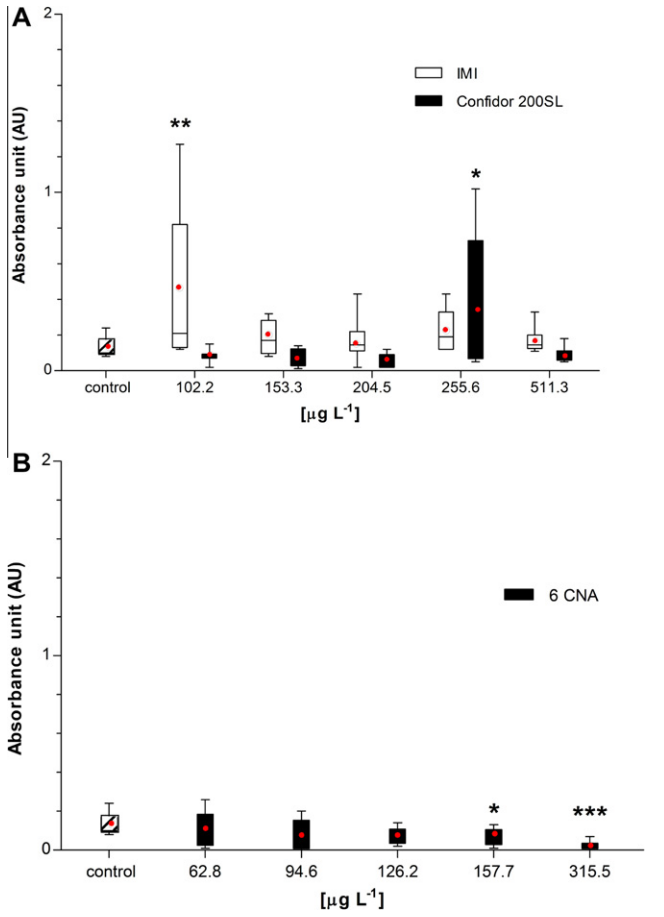

Fig. 5. Whole-body LP of *G. fossarum* (expressed in absorbance units of TBARS products) measured after 24 h of exposure IMI or Confidor 200SL (A) and 6CNA (B). The boxes contain 75% of all readings, the symbols represent minimum and maximum values (⊥) and the mean value (●). ($n = 10$). $p < 0.001$ (∗∗∗), $p < 0.01$ (∗∗), and $p < 0.05$ (∗).

started to inhibit their growth already at 0.003 v/v%. On the other hand, 6CNA induced algal growth and proliferation after a 96 h period of exposure at all concentrations, while at the highest dose (157.5 mg L^{-1}) already after 24 h induced a significant inhibition and algae death. Presumably the algal growth was inhibited because of the dissociation of the carboxylic group present in 6CNA [52]. This issue induced acidic changes in pH of the algal media and adversely influenced the sensitive microalgae. 6CNA is a final transformation product formed in environment that does not act as nicotinic agonist but may also contribute to the toxicity effects [18]. 6CNA contains the 6-chloropyridinyl moiety and based on its structural/chemical consideration may be of toxicological significance. This transformation product is included in the tolerances established for the IMI residues, although should be considered on its own in order to recognise additional IMI-toxicity effects. Algae as primary producers contribute substantially to aquatic habitats and their sensitivity to Confidor 200SL and 6CNA found in this study could cause environmental problems.

Acute toxicity (24 h) of IMI and 6CNA was evaluated on the freshwater amphipod *G. fossarum*. After exposure to the highest dose of IMI (511.3 μg L^{-1}) and 6CNA (315.5 μg L^{-1}), an overall low mortality was noticed. Most significant effect, as in algae, was observed in case of Confidor 200SL. Increased mortality induced by Confidor 200SL supports the idea that major side effects could be caused by additives such as dimethyl sulfoxide (DMSO) and *N*-methylpyrrolidone (NMP). These co-formulants mixture alone induced no toxicity in amphipods, while the combined action of IMI and co-formulants increased the toxicity of the commercial formulation. In the case of another amphipod crustacean *Hyalella azteca*, Stoughton et al. [15] confirmed its higher sensitivity to formulated product than to technical IMI. Also, other tests have shown formulated pesticides to be more toxic to aquatic organisms [11,37,53]. These supplementary substances in commercial formulations often represent the highest proportion in pesticide mixtures, so even a minor concern regarding their toxicity and possible synergistic effects with other ingredients should be considered [54,55]. Additional studies will be needed to address the potential effect of additives, but such studies are not easily feasible since identity and quantity of other ingredients is most often regarded as confidential information and therefore rarely revealed in easily accessed literature or product labels.

Neonicotinoids are agonist of nAChRs [1] and do not exert a direct inhibition of the AChE activity as for example organophosphates. In our study we tested possible indirect inhibitory effects on freshwater amphipods exposed to neonicotinoids. This measurement was also performed on gills of neonicotinoid exposed mussels and showed an interesting outcome with 'U-shape dynamics' of AChE activity [56]. In this study Dondero et al. observed significant inhibition at the lowest and at the intermediate tested concentration. On contrary, in our case the outcome of IMI effect on AChE activity presented no indirect effect or changes at all exposure concentrations compared to control group.

It is well-known that pesticides can induce oxidative stress by the generation of ROS, which can induce oxidant-mediated effects (such as increased activities of antioxidant enzymes) and oxidant-mediated toxicities (such as oxidation of lipids) [57]. Only a few

previously published data are available regarding the IMI-induced oxidative stress and these merely relate to mammalian model organisms. These studies showed a slight increase in intracellular ROS and nitric oxide production after IMI exposure [58,59]. A study of Lukančič et al. on *G. fossarum* demonstrated that IMI influenced not only the respiration but also the electron transport system (ETS) activity [12]. This effect was a consequence of different processes, including oxidative stress. Partial damage to the inner mitochondrial membrane by lipid peroxidation possibly impaired the function of ETS. For better understanding of ROS involvement in the toxicity mechanisms of neonicotinoids, antioxidant enzyme activity, detoxifying GST mechanism and lipid peroxidative damage were monitored in amphipods. In this study, CAT activity after Confidor 200SL and 6CNA exposure at highest doses was significantly increased and indicated action of the protection mechanisms involved in cellular repair processes. El-Gendy et al. [60] reported a similar increase of CAT after neonicotinoid exposure, but again only in IMI-treated mice. Enhanced GST activity after Confidor 200SL exposure reflects the detoxification processes in treated gammarids and this induction may be due to the glutathione dependent enzyme system that provides major protection against xenobiotic agents. A recent study on the mosquito, *Aedes aegypti*, demonstrated that exposure to IMI increased glutathione transferase mRNA levels as well as other genes coding for antioxidant proteins [61]. In addition was also noticed a slight decrease in antioxidant enzyme GST after exposure to Confidor 200SL (at 255.6 $\mu g\ L^{-1}$ of a. i.). This decrease of the GST activity, although not significant was evident with 1.5-fold lowered GST activity at 255.6 $\mu g\ L^{-1}$ of a.i. in Confidor 200SL. This decrease could be interpreted as being overwhelmed by conspicuous ROS production. An additional explanation of enzyme's indirect inhibition is related to their binding with ROS produced also during pesticide metabolism. Metabolism of IMI involves many processes of hydroxylation, i.e. the hydroxylation of the imidazolidine ring at position 4 or 5 leading to the formation of hydroxylated compounds and subsequent loss of important amounts of hydroxyl radicals [62]. Concurrently, with slightly diminished GST activity increased lipid peroxidation levels occurred (at the same exposure concentration of Confidor 200SL). IMI and Confidor 200SL exposure provoked an increase of LP in amphipods. During IMI exposure LP increase occurred at 102.2 $\mu g\ L^{-1}$ and was represented by a similar-to-hormetic effect. This increase was induced at lower concentrations of IMI and not at higher doses as expected. On the other hand, Confidor 200SL induced an increase of TBARS products, which was highest at 255.6 $\mu g\ L^{-1}$ of a. i. Higher TBARS levels at 255.6 $\mu g\ L^{-1}$ suggested that exposure to Confidor 200SL resulted in a different time-course of cellular ROS generation or in a possible direct lipid oxidation due to the interactive action of co-formulants and IMI. It is important to notice potentially different toxicity pathways or time-course effects of the parent compound and its transformation product that were observed during this study. After a 24 h exposure 6CNA provoked strong induction of antioxidant enzyme CAT, while its effect was completely absent on the LP, probably due to highly active CAT. On the contrary, Confidor 200SL altered all parameters confirming its higher toxicity compared to active ingredient.

Behaviour is considered as a useful tool in ecotoxicology since is one of the early warning indicators of toxicant stress [14]. During experimental pesticide exposure analysed individual biochemical biomarkers should be linked to behavioural responses whenever this is possible [63]. In this study individuals with modified behaviour were counted. During exposure, animals treated with 511.3 $\mu g\ L^{-1}$ (IMI) exhibited an increase in immobility and inactivity that can be a direct IMI effect on neuro-muscular acetylcholine receptors provoking impairment of locomotion and food filtration, with consequent animal starvation and difficulties in ventilation [64]. Alternatively, 6CNA at the highest dose induced rapid movements and animal hyperactivity, as well as disorientation. This disoriented behaviour was also shown in non-target organisms, such as *Apis mellifera*. Honey bees treated with IMI were confused and failed to return to their homing site [65]. Hyperactivity in swimming may also be linked to an avoidance response towards present chemicals [66]. Interestingly, short-term 6CNA exposure stimulated amphipods moulting processes. Moulting is an essential physiological process for crustaceans controlled by the neuroendocrine system, on which different toxicants, such as pesticides, can act [67]. Moreover, moulted or recently moulted animals could be more susceptible to pesticide action.

This research confirmed the importance of testing commercial formulations of IMI and IMI's transformation products as they interfere with pure compound safety characteristics. Our present results show that commercial formulation of IMI and its by-product 6CNA exert oxidative stress in freshwater amphipods as well as negative effects on algae growth. The induction of CAT, GST and LP levels demonstrates that exposure of *G. fossarum* to Confidor 200SL leads to peroxidation of membrane lipids and triggers antioxidant and detoxifying cellular mechanisms. Amphipods exposed to 6CNA experienced mainly the activation of catalase scavenging protection mechanism. In general, the major toxic effects were due to the commercial formulation Confidor 200SL both in case of algae and amphipods. This issue is relevant as these marketed mixtures are the one applied directly in the environment and should be further monitored.

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RESEARCH ARTICLE

Effects of clothianidin on aquatic communities: Evaluating the impacts of lethal and sublethal exposure to neonicotinoids

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Abstract

The widespread usage of neonicotinoid insecticides has sparked concern over their effects on non-target organisms. While research has largely focused on terrestrial systems, the low soil binding and high water solubility of neonicotinoids, paired with their extensive use on the landscape, puts aquatic environments at high risk for contamination via runoff events. We assessed the potential threat of these compounds to wetland communities using a combination of field surveys and experimental exposures including concentrations that are representative of what invertebrates experience in the field. In laboratory toxicity experiments, LC_{50} values ranged from 0.002 ppm to 1.2 ppm for aquatic invertebrates exposed to clothianidin. However, freshwater snails and amphibian larvae showed high tolerance to the chemical with no mortality observed at the highest dissolvable concentration of the insecticide. We also observed behavioral effects of clothianidin. Water bugs, *Belostoma flumineum*, displayed a dose-dependent reduction in feeding rate following exposure to clothianidin. Similarly, crayfish, *Orconectes propinquus*, exhibited reduced responsiveness to stimulus with increasing clothianidin concentration. Using a semi-natural mesocosm experiment, we manipulated clothianidin concentration (0.6, 5, and 352 ppb) and the presence of predatory invertebrates to explore community-level effects. We observed high invertebrate predator mortality with increases in clothianidin concentration. With increased predator mortality, prey survival increased by 50% at the highest clothianidin concentration. Thus, clothianidin contamination can result in a top-down trophic cascade in a community dominated by invertebrate predators. In our Indiana field study, we detected clothianidin (max = 176 ppb), imidacloprid (max = 141 ppb), and acetamiprid (max = 7 ppb) in soil samples. In water samples, we detected clothianidin (max = 0.67 ppb), imidacloprid (max = 0.18 ppb), and thiamethoxam (max = 2,568 ppb). Neonicotinoids were detected in >56% of soil samples and >90% of the water samples, which reflects a growing understanding that neonicotinoids are ubiquitous environmental contaminants. Collectively, our results underscore the need for additional research into the effects of neonicotinoids on aquatic communities and ecosystems.

Introduction

Neonicotinoid insecticides, which account for 26% of the global insecticide market, have recently become the most widely used insecticide class worldwide [1]. Developed in the 1980s, neonicotinoids first came into regular use with imidacloprid starting in the early 1990s. Since that time additional active ingredients have been developed and classified into three groups: *N*-nitroguanidines (imidacloprid, thiamethoxam, clothianidin, dinotefuran), nitromethylenes (nitenpyram), and *N*-cyanoamidines (acetamiprid and thiacloprid) [2]. Currently, thiamethoxam and its breakdown product clothianidin dominate usage in North American cropping systems [3]. The increasing usage of neonicotinoids has been fueled by their relatively low toxicity to vertebrate species [4]. Neonicotinoids target the post-synaptic nicotinic acetylcholine receptor, causing paralysis and death. Because neonicotinoids bind more strongly to insect receptors than vertebrate receptors and invertebrates have a higher ratio of nicotinic receptors, they generally have low toxicity to vertebrate species [4]. A key driver of rapid neonicotinoid adoption in North America is the ability to apply them prophylactically as a seed dressing to some of the most widely grown annual crops [3]. As seeds germinate, the insecticide is incorporated into the plant and distributed systemically during growth. This process is facilitated by the high water solubility of neonicotinoids [4]. Although neonicotinoids can be used as spray applications, approximately 60% of applications are as seed dressings [2]. The prophylactic application of neonicotinoids to virtually all seeds of corn, soybeans and other annual crops without prior knowledge of the season's pest populations has raised concern over the environmental risks associated with their use [3].

Only a small fraction of neonicotinoid active ingredient applied to seeds is taken up by plants. For example, in a container study, less than 20% of the imidacloprid applied to corn seeds was later found in the plant, the remainder presumably retained in soils and water [5]. These findings raise questions about environmental fate, as neonicotinoids generally have exceptionally high water solubility values; clothianidin and thiamethoxam, the two compounds used most frequently in our study area in the Midwestern US [3], have solubility values of 0.327 g L^{-1} and 4.1 g L^{-1}, respectively [6,7]. While the high water solubility and low soil binding by neonicotinoids facilitates translocation by plants, it can lead to significant leaching into ground water, streams, and ponds. For example, imidacloprid was detected in 89% surface water samples (n = 75) in California [8]. Similarly, the Washington State Departments of Agriculture and Ecology have detected imidacloprid (max = 0.705 ppb, mean = 0.06 ppb) during monitoring studies of salmon-bearing rivers and streams [9]. Additionally, at least one of four different neonicotinoid compounds (clothianidin, thiamethoxam, acetamiprid, imidacloprid) were found in 16 to 91% of water and sediment samples in the Canadian Prairie Pothole Region, dependent on time of sampling [10]. In a review of 29 studies from nine countries, neonicotinoids were common contaminants of surface waters [11].

Given the frequency of detection of neonicotinoids in aquatic systems, many recent studies have explored the potential lethal and sublethal effects of neonicotinoids on aquatic species (reviewed in [12,13]). Aquatic insects are generally more sensitive to neonicotinoids compared to other aquatic species (e.g., mollusks, crustaceans, fish), which is not surprising given their mode of action [13]. In addition to their effects on mortality, neonicotinoids have been shown to reduce feeding rates, movement, fecundity, developmental rates, and growth in aquatic insects [14–21]. However, the majority of this research has focused on imidacloprid, which was the first widely applied neonicotinoid and is rarely used in modern row crop agriculture production systems. There is a dearth of information on the toxicological effects of the neonicotinoids that are most commonly used presently, including thiamethoxam and its metabolite clothianidin [13].

While laboratory experiments can provide a wealth of information on the effects of pesticides on individuals and populations, community-level experiments can broaden our perspective of how natural systems are likely to respond to these common stressors, including neonicotinoids [22–27]. Ecological communities are complex systems composed of species representing different trophic levels and functional groups that directly and indirectly interact. Direct interactions including competition, predation, and parasitism have routinely been explored in ecological research [28]. Moreover, there is increasing focus on how these direct interactions can indirectly influence other species within communities (e.g., trophic cascades; [29]). Indeed, indirect interactions within communities are mediated by a combination of changes in species abundance and changes in species traits (e.g., behavior). Because neonicotinoids are designed to target insects, they should have predictable direct effects (e.g., mortality) and more difficult to quantify sublethal effects (e.g., reduced foraging and activity) on predatory invertebrates [23,30]. Broadly, macroinvertebrates represent a significant component of the biodiversity in many freshwater water systems (e.g., ponds, wetlands, streams; [31]). Moreover, insects are a dominant predatory guild in lentic systems that lack fish [32]. Using basic food web theory, we would predict that the elimination of predatory insects or reductions in their foraging activity in a system will lead to a "top-down" effect that indirectly increases the abundance of prey species [33–35]. By integrating research across multiple ecological scales (e.g., individuals, populations, communities), we can develop a broader understanding of how neonicotinoids can influence community structure and function.

We combined laboratory and mesocosm experiments with field surveys to assess the potential effects of neonicotinoids on wetland species. Our experiments focused on the neonicotinoid clothianidin, which is a breakdown product of the widely used neonicotinoid thiamethoxam but also used as an active ingredient. In fact, within the last decade, clothianidin has become the dominant neonicotinoid used in North America for many applications. It is registered for use as a foliar insecticide and as a seed treatment for most annual crops [2,3]. Given the shift from imidacloprid to thiamethoxam and clothianidin as the dominant neonicotinoid active ingredients used in agriculture, there is a need to evaluate the risk that these compounds pose to natural systems. To date, clothianidin toxicity testing for aquatic species has been limited to a small number of aquatic invertebrates (e.g., *Chironomus riparius*, *Mysidopsis bahia*, *Daphnia*), with LC_{50} estimates ranging from 0.022 ppm to 119 ppm [36,37]. Given the broad diversity of species, particularly invertebrates, that inhabit aquatic systems, there is a need for studies that expand beyond traditional model species. Moreover, the sublethal effects of clothianidin on aquatic taxa and the community-level implications of typical exposures are largely unknown. In order to provide a baseline for further work in aquatic systems, our experimental objectives were to assess the lethal and sublethal effects of clothianidin to common wetland invertebrate (e.g., snails, insects, crustaceans) and vertebrate (i.e. amphibian) species in the Upper Midwestern United States (Indiana), where the use of clothianidin and thiamethoxam is as intensive as any region in the country [38]. To assess lethal effects, we conducted toxicity assays (i.e. 48 h LC_{50} tests). Additionally, we examined the sublethal effects of clothianidin exposure on movement and foraging activity (i.e. predation rates). Building upon results of our laboratory experiments, we conducted a mesocosm experiment to examine the effects of clothianidin on aquatic communities with different trophic structures (i.e. presence or absence of invertebrate predators). Finally, we used a field survey to collect weekly soil and water samples across multiple sites in central Indiana to determine the presence and environmental range of neonicotinoids on the landscape.

Methods

LC_{50} tests

We examined the toxicity of the neonicotinoid clothianidin to 10 aquatic macroinvertebrates and three larval anuran species using 48 hr LC_{50} (lethal concentration to 50% of organisms exposed) tests. The scale of our tests and volumes of water required precluded us from using technical grade active ingredient due to cost, and we used a formulated product, Arena 0.25% granules (Valent Corp., Walnut Creek, CA), to formulate our concentration regimes. Given that we used a commercial formulation of clothianidin, we cannot separate effects of the active ingredient from those of inert ingredients. Information on each species, including number of individuals used, is included in Table 1. All species were collected from ponds located near the Purdue Wildlife Area (PWA), Aquatic Research Lab, and Martell Forest in West Lafayette, IN U.S.A. between May and July of 2014 and 2015. After collection, the species were housed indoors at the PWA-Animal Care Facility under a 14:10-h light:dark cycle for no longer than 48 h prior to experimental use. Animals were housed individually in 1-L plastic containers filled with 0.5 L of UV-sterilized, filtered well water.

We conducted individual LC_{50} tests for each species. Because the species differed in body size, we varied the size of our experimental units (10–1000 mL glass containers; Table 1). A single individual was placed into each experimental unit for the tests. Because little was known regarding the toxicity of clothianidin, we first conducted range-finding studies to determine lethal concentrations for each species. Based on these studies, we selected 6 to 10 nominal concentrations for each species and each concentration was replicated 4 to 15 times based on the availability of organisms (Table 1). In accordance with standard toxicity protocols, we did not feed individuals during the 48-h tests [39]. Tests were conducted under a 14:10-h light:dark cycle.

We prepared a stock solution of 300 ppm clothianidin using Arena 0.25% granules mixed with filtered, ultraviolet-irradiated well water. The solution was filtered using Whatman GF/C filters (90 mm) and stored in glass amber jugs for no more than 1 h before addition to the experimental units. To achieve the desired nominal concentrations, we used micropipettes to add stock solution to each container. Due to the small volume used in the experiments for the damselfly nymphs and beetle larvae, we premixed concentrations using a serial dilution for increased accuracy. We stirred the water in each experimental unit prior to the addition of the animals. To quantify the insecticide concentration, a mock stock solution was prepared in a glass amber jar and immediately taken for chemical analysis to determine preparation accuracy. The experimental units were monitored for mortality every 4 h for 48 h. We performed a probit analysis using SPSS software to determine LC_{50} values and 95% confidence intervals.

Sublethal experiments with tadpoles

We conducted a laboratory experiment to explore the potential sublethal effects of clothianidin exposure on tadpole behavior (i.e. activity). The focal species was the northern leopard frog, *Lithobates pipiens*. The experiment consisted of a no-insecticide control or exposure to three concentrations of clothianidin (0.25 ppm, 0.5 ppm, or 1 ppm). All stock solutions for the experiments were prepared as described for the LC_{50} tests. Each treatment was replicated five times for a total of 20 experimental units. Our experimental units were 10-L plastic tubs filled with 2 L of UV-sterilized, filtered well water. We added 10 tadpoles to each experimental unit following the addition of the insecticide. Our behavioral observations were conducted by scan sampling [40]. For each tub, we recorded the number of individuals that were active (e.g., tail movement, movement through the water column). We conducted observations 30 min post-

Table 1. Species and their respective experimental units and dosage concentrations. A single individual was assigned to each replicate.

Species	Order	Trophic position	Container Volume (mL)	Replicates	Nominal concentrations (ppm)
Graphoderus fascicollis	Coleoptera	Predator	10	10	0, 0.001, 0.010, 0.25, 0.50, 0.100
Anax junius	Odonata	Predator	500	4	0, 0.5, 1, 5, 10, 20
Lestes unguiculatus	Odonata	Predator	10	10	0, 0.5, 1, 3, 5, 10
Plathemis lydia	Odonata	Predator	500	10	0, 0.05, 0.5, 1, 10, 50
Belostoma flumineum	Hemiptera	Predator	110	8	0, 0.010, 0.050, 0.100, .250, 0.500
Hesperocorixa atopodonta	Hemiptera	Herbivore	100	10	0, 0.01, 0.025, 0.05, 0.1, 0.3
Notonecta undulata	Hemiptera	Predator	100	10	0, 0.01, 0.025, 0.05, 0.1, 0.3
Orconectes propinquus	Decapoda	Predator	1000	10	0, 0.5, 0.15, 0.3, 0.5, 0.6, 0.9, 1, 5, 20
Physa acuta	Pulmonata	Herbivore	500	5	0, 327
Helisoma trivolvis	Pulmonata	Herbivore	500	5	0, 327
Hyla versicolor	Anura	Herbivore	500	5	0, 327
Lithobates clamitans	Anura	Herbivore	500	5	0, 327
Lithobates pipiens	Anura	Herbivore	500	5	0, 327

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dosage, 1 h post-dosage, and then every 12 h for 48 h. For each set of observations, we conducted five scan samples for each tub and calculated the mean activity as our response variable. We used repeated-measures analysis of variance (ANOVA) to assess treatment effects over time using SPSS. We conducted mean comparisons using Bonferroni correction.

Sublethal experiments with predators

We conducted laboratory experiments to explore the sublethal effects of clothianidin exposure on predator behavior (i.e. response to stimulus) and predator-prey interactions (i.e. predation rates). The focal predator species in these experiments were crayfish, *Orconectes propinquus*, and water bugs, *Belostoma flumineum*. All stock solutions for the experiments were prepared as described for the LC_{50} tests.

The feeding rate experiments consisted of a no-insecticide control or exposure to three concentrations of clothianidin. For crayfish, the three insecticide concentrations were 0.05 ppm, 0.1 ppm, and 0.2 ppm while the three concentrations for water bugs were 0.01 ppm, 0.05 ppm, and 0.1 ppm. The experimental units were 10-L tubs filled with 2 L of UV-sterilized, filtered well water. We added 10 snails (*Physa acuta*) and introduced a single predator to each experimental unit. Clothianidin was added to the tubs immediately prior to predator addition. The water was stirred to equally distribute the insecticide. We replicated each treatment six times for the water bug experiment and 10 times for the crayfish experiment, resulting in 24 and 40 total units, respectively. We checked twice daily for the number of snails consumed and removed dead snails from the tubs. The experiment was terminated after 4 d for the water bugs and 8 d for the crayfish. Our response was the total number of snails consumed in each experimental unit at the end of the experiment. We used ANOVA to assess the effects of clothianidin on prey consumption using SPSS. We conducted mean comparisons using Bonferroni correction.

We also examined the effects of clothianidin exposure on crayfish behavior. We used the same experimental design described above with the exception that the experimental units were 2-L container filled with 1 L of water. Stimuli were introduced by approaching experimental units, then touching the center of the cephalothorax using a disposable transfer pipette. A reaction was measured as either an escape movement away from stimulus, or aggressive stance towards the stimulus. This was performed 1 h post exposure, then every 24 h for 7 d (n = 8

total observations per individual). At the end of the experiment, we calculated the proportion of observations with responses to the stimulus as the response variable. We used ANOVA to assess the effect of clothianidin exposure on stimulus response. We conducted mean comparisons using Bonferroni correction.

Mesocosm experiment

We investigated the potential interactive effects of clothianidin and predation on aquatic communities using a semi-natural mesocosm experiment. The herbivore trophic level consisted of amphibian larvae, freshwater snails, and zooplankton. The predator trophic level consisted of larval dragonflies (*Anax junius*), water bugs (*Belostoma flumineum*), backswimmers (*Notonecta undulata*), and crayfish (*Orchonectes propinquus*). Dragonflies, water bugs, and crayfish were selected because they will consume tadpoles and snails while backswimmers were selected because they will consume zooplankton. Based on previous research with imidacloprid [11,13,41], we expected the herbivores to be tolerant of clothianidin but the predatory insects and crayfish to be sensitive to it. Thus, we predicted that clothianidin exposure would have negative effects on predator survival and behavior, which would indirectly benefit herbivore survival and growth. Moreover, we expected sublethal effects on predator behavior to be the main driver of effects on herbivore responses at the low clothianidin concentration and lethal effects to dominate at the high clothianidin concentration.

The experiment was conducted at the PWA in July 2014. We used a complete randomized factorial design consisting of two predator treatments (presence or absence of invertebrate predators) crossed with three nominal concentrations of clothianidin (0, 10, or 500 ppb). The 10 ppb treatment was selected to reflect clothianidin concentrations that have been detected in water samples near agricultural fields [42] and expected to be sublethal to invertebrates. The 500 ppb treatment was selected to represent a worst-case scenario that would be potentially lethal to predatory invertebrates. We replicated the six treatments nine times for a total of 54 experimental units. Our experimental units were 1200-L cattle tanks located in an open field with no tree cover.

Between 17 and 19 June, we filled each tank with 595 L of well water and then covered the tank with 70% shade cloth to prevent unwanted colonization of insects and amphibians. On 22 June, we added 20 g of commercial rabbit chow (Small World Complete Rabbit Feed) and 200 g of dry leaf litter (primarily *Quercus* spp.) to provide an initial nutrient source and refuges, respectively. Additionally, we collected pond water from a local pond, removed all unwanted macroinvertebrates, and added a 500-mL sample from the mixture to each tank. This sample provided in initial source of algae (periphyton and phytoplankton) for the tanks. On 30 June, we placed two 10 x 10 cm clay tiles (oriented vertically and facing north) in each tank. After allowing seven days for algal populations to develop, we seeded each tank with a zooplankton assemblage gathered from previously established mesocosms at our facility.

We assembled aquatic communities that are common across wetlands in our region [43,44]. Our base community (no-predator treatments) consisted of two species of larval amphibians (northern leopard frogs, *Lithobates pipiens*, and green frogs, *L. clamitans*) and two species of freshwater snails (*Helisoma trivolvis* and *Physa acuta*). We collected eight egg masses of northern leopard frogs from a local pond and reared the hatchlings in 100-L culture pools filled with 70 L of well water covered with 70% shade cloth. Tadpoles were fed rabbit chow until used in the experiment. We collected green frog tadpoles from a nearby wetland on 4 July for use in the experiment. On 7 July, we added 20 northern leopard frog tadpoles and 10 green frog tadpoles to each tank. The snail species were also collected from local ponds between 30 June and 4 July. On 7 July, we added 30 individuals of each snail species to each tank. Our

predator species consisted of water bugs (*B. flumineum*; n = 5), backswimmers (*N. undulate;* n = 5), dragonfly larvae (*A. junius;* n = 2), and crayfish (*O. propinquus;* n = 10) collected from local ponds and reared in the laboratory until used in the experiment. The densities of all species were within the range found in wetlands [43,44]. The predators were added to the tanks on 7 July after the addition of the prey species.

The tanks were dosed on 7 July with 18.4 and 921 mL of clothianidin stock solution (323 ppm) to achieve nominal concentrations of 10 and 500 ppb, respectively. The water in each tank was gently agitated with a metal rod to distribute the insecticide throughout the tank. A 200-mL sample was immediately collected from five randomly selected tanks in each treatment. The five samples were mixed together and a 200-mL sample of the pooled sample was removed for chemical analysis to determine the actual concentrations achieved in the treatments (S1 Table). At day 0, actual concentrations were 5 ppb and 352 ppb for the 10 and 500 ppb treatments, respectively. We also note that clothianidin was detected in our well water; the clothianidin concentration in our control tanks was 0.6 ppb. Given that the actual concentrations were less than our nominal concentrations, we will refer to the actual concentrations below. Additionally, we collected water samples on day 21 of the experiment to assess degradation of clothianidin over time; concentrations were 0.3, 1.5, and 77.6 ppb for the 0, 10, and 500 ppb treatments, respectively. A mock solution was also made to determine accuracy of stock solutions. All samples were stored in glass amber jars and analyzed within 24 h of collection at the Purdue University Bindley Bioscience Lab using a triple quadrupole (QQQ) liquid chromatography/mass spectrometer (LC/MS).

During the experiment, we measured pH, temperature, conductivity, periphyton biomass, phytoplankton (Chlorophyll *a*), and zooplankton abundance. Sampling methods and results are presented in S1 Appendix, S2 and S3 Tables, and S1 and S2 Figs. The experiment was taken down 21 d post insecticide exposure. Upon termination, we removed all of the amphibians, snails, and predators from the tanks. Individuals were euthanized and then preserved in 10% formalin (amphibians and snails) or 70% ethanol (predators). For each tank, we determined the number of surviving individuals for each species.

Predator mortality in our mesocosm experiment did not meet the assumptions of parametric analyses. Thus, we used a Kruskal-Wallis test to determine the effect of clothianidin on overall predator mortality and the mortality of each predator species. We used generalized linear models (GLM) to test for the effects of predators, clothianidin, and the predator*clothianidin interaction on overall prey mortality and the mortality of each prey species. For significant univariate effects, we conducted mean comparisons using Bonferroni correction.

Field survey

We conducted field surveys to determine neonicotinoid concentrations in soil and water samples from multiple sites in Tippecanoe Co., Indiana (Fig 1). We tested for the most commonly used neonicotinoids in our area (acetamiprid, clothianidin, imidacloprid, and thiamethoxam). TPAC, Box, and Marshall were agricultural sites whereas Martell Forest served as a reference site. However, we note that Martell Forest is embedded within an agricultural landscape. Each of these four sites has an associated stream or ditch that served as a location for our water samples. The PWA was selected because it contains wetland areas that would allow us to assess neonicotinoid concentrations in lentic water bodies, including sites that served as sources for our experimental animals. We conducted soil and water sampling at Martell Forest, TPAC, Box, and Marshall. Sampling was performed at each site two weeks prior to planting and weekly from two through eight weeks post-planting. For the two sites at the PWA, we only conducted water sampling.

Fig 1. Map of field sites and sampling locations in Tippecanoe Co., Indiana, U.S.A. For each site, the location of water (W) and/or soil (S) samples is indicated. Our study sites were the Purdue Wildlife Area (PWA East Pond [40.452261˚, -87.055185˚] and PWA West Pond [40.450746˚, -87.052397˚]), Martell Forest (40.435215˚, -87.029180˚), Throck Morton Purdue Agricultural Center (TPAC, [40.295857˚, -86.899099˚]), and the Purdue Animal Farm (Box [40.503325˚, -87.026892˚] and Marshall [40.492395˚, -87.014538˚]).

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For water samples, we randomly collected 100-mL samples from three different locations. The three samples were pooled together into a 500-mL amber Nalgene bottle and frozen to prevent degradation of compounds until processing. Once thawed, we removed 3 replicate samples of 20 mL from each bottle for analysis. The samples were collected in amber vials to determine neonicotinoid concentrations. The samples were first mixed with 10 μL of a 1–10 ng/μL analytical grade standards, then poured through *Oasis Waters* SPE cartridges, with 3 mL of acetonitrile used to elute the sample prior to measurement. We then used QQQ LC/MS to determine neonicotinoid concentrations. For each sample, the reported analytical results are the mean of the three replicate measurements (see S1 Appendix for concentration determination).

Soil samples were collected from five randomly chosen locations in the fields near the water collection sites. Soil cores were taken with the top six inches of topsoil removed. The five samples were mixed together to form a single sample and held in opaque paper bags and frozen prior to analysis. In order to extract the neonicotinoids from soil, 5 g of soil was added to a 50-mL centrifuge tube along with 10 μL of a 1-10ng/μL analytical grade standards, 5 mL ddH_2O, 10mL CAN + 1%HOAC, in addition to 1 g of NaCl and 4 g $MgSO_4$. The mixture was hand shaken vigorously for 1 min, and centrifuged at 4,000 rpm for 5 min. Following centrifugation, 1 mL of the supernatant was transferred to a *Quechers* dSPE Tube containing PSA and $MgSO_4$, vortexed for 1 min followed by 5 min of centrifugation at 15,000 rpm. The resulting supernatant was transferred to a microcentrifuge tube and dried in a SpeedVac concentrator prior to analysis using QQQ mass spectrometry. Reported analytical results are the mean of three replicate measurements from each sample (see S1 Appendix for concentration determination).

Ethics statement

The Purdue Institutional Animal Care and Use Committee (IACUC) approved all animal husbandry and euthanasia procedures (protocol #1304000846). Field permits for collecting animals were provided by the Indiana Department of Natural Resources, Division of Fish and Wildlife.

Results

LC_{50} tests

Our LC_{50} experiments revealed dramatic differences (several orders of magnitude) in the toxicity of clothianidin to aquatic invertebrates and vertebrates (Table 2, Fig 2). Survival curves are presented in S3 and S4 Figs. In general, predatory invertebrates displayed high sensitivity. Additionally, species within the same order (i.e. Hemiptera, Odonata) tended to cluster in their LC_{50} values. Moreover, the hemipterans were more sensitive than the odonates to clothianidin. The single member of the Coleoptera (*Graphoderus*) had the highest sensitivity to the insecticide. For larvae of the three amphibian species (*L. pipiens*, *L. clamitans*, and *H. versicolor*) and the two snail species (*P. acuta*, *H. trivolvis*), we were unable to calculate LC_{50} values because there was no mortality at the saturation point of formulated clothianidin (Arena) in water (~327 ppm).

Sublethal experiments

There was no evidence that clothianidin influenced tadpole behavior (data not shown). While there was a significant effect of elapsed time on tadpole activity ($F_{3,48} = 5.6$, $P = 0.002$), there was no effect of clothianidin ($F_{3,16} = 1.8$, $P = 0.197$) or time*clothianidin interaction ($F_{9,48} = 1.3$, $P = 0.283$). In the predation trials, we found that clothianidin exposure reduced the consumption of prey by water bugs in a dose-dependent manner ($F_{3,20} = 5.86$, $P = 0.005$; Fig 3). At the highest clothianidin concentration (0.1 ppm), there was a 62% reduction in prey consumption compared to the control. In contrast, clothianidin exposure did not influence prey consumption in crayfish ($F_{3,35} = 0.89$, $P = 0.445$; Fig 4A). However, we did detect a significant dose-dependent effect on their response to stimuli ($F_{3,34} = 14.23$, $P = <0.001$; Fig 4B). For example, at the highest clothianidin concentration (0.2 ppm), there was a 70% reduction in stimulus response compared to the control.

Table 2. LC_{50} values and associated 95% confidence intervals for the invertebrate species that experienced mortality when exposed to clothianidin.

Species	$LC50_{48\text{-}h}$ (ppm)	95% confidence limit	
		Lower	Upper
Lestes unguiculatus	1.245	0.572	2.11
Anax junius	1	a	a
Plathemis lydia	0.865	0.306	2.133
Orchonectes propinquus	0.805	0.509	1.462
Belostoma flumineum	0.079	0.052	0.107
Notonecta undulata	0.059	0.035	0.107
Hesperocorixa atopodonta	0.056	0.039	0.082
Graphoderus fascicollis	0.002	0.001	0.005

[a] = The 95% CI could not be calculated because the treatments resulted in 0, 50 or 100% mortality.

https://doi.org/10.1371/journal.pone.0174171.t002

Mesocosm experiment

Clothianidin exposure had significant effects on the total mortality of invertebrate predators and the mortality of each species ($\chi^2 > 7.8$, $P \leq 0.02$; S4 Table, Fig 5). Across all predator species, predator mortality increased by 52% with 352 ppb of clothianidin compared to 0.6 ppb (P = 0.011). However, there was no difference between 0.6 ppb and 5 ppb or between 5 ppb and 352 ppb ($P \geq 0.071$). When examining the individual predator species, we found that *Notonecta* had high mortality in all treatments. However, there was still a significant increase in mortality in the 352 ppb treatment compared to the 0 ppb treatment (P = 0.005). For *Anax*, mortality was highest at 5 ppb but 50% lower at 0 ppb (P = 0.013) and 80% lower at 352 ppb

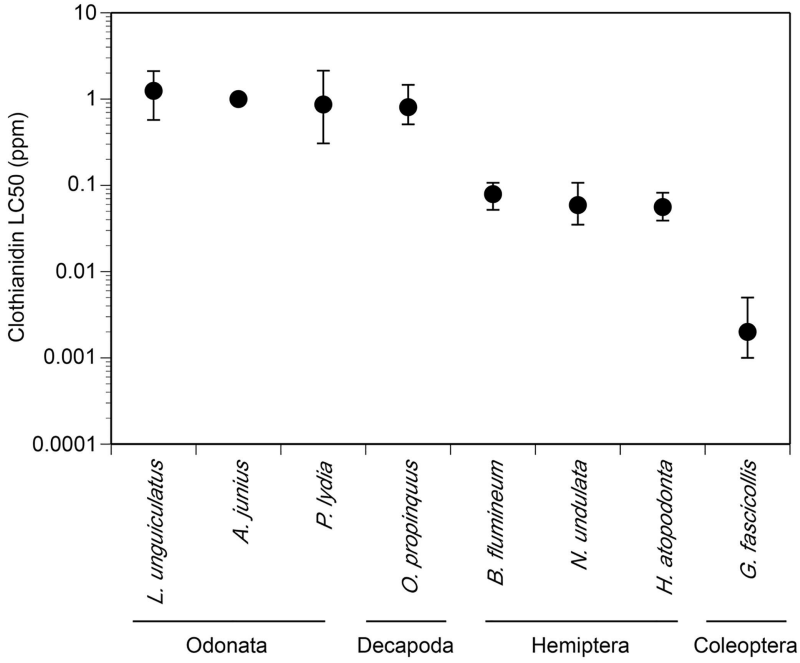

Fig 2. LC_{50} values with 95% confidence intervals for select aquatic macroinvertebrates. The 95% CI for *Anax* could not be calculated because the treatments resulted in 0, 50 or 100% mortality.

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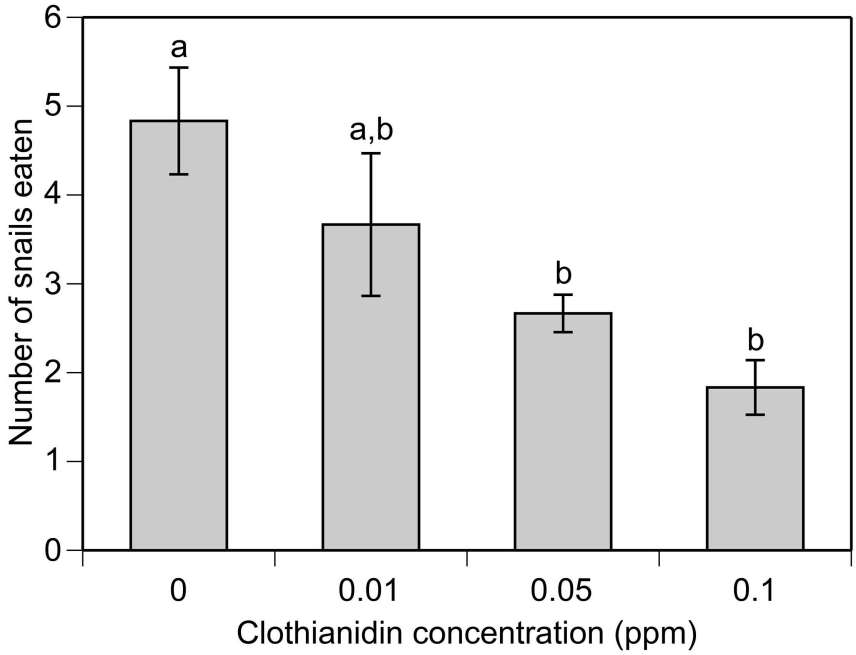

Fig 3. Number of snail prey consumed by water bugs exposed to different clothianidin concentrations. Treatments sharing letters are not significantly different from each other based on pairwise comparisons (Bonferroni corrected P > 0.05). Data are means ± 1 SE.

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(P = 0.001), with no differences between 0.6 ppb and 352 ppb (P = 0.843). For *Orconectes* and *Belostoma*, we found no significant difference between 0.6 ppb and 5 ppb ($P \geq 0.056$). However, mortality was greater in the 352 ppb treatment compared to the 0.6 ppb and 5 ppb treatments ($P \leq 0.026$).

There were significant effects of predators, clothianidin, and their interaction on overall prey mortality and the mortality of individual prey species (Fig 6, Table 3). Averaged across the clothianidin treatments, overall prey mortality and the mortality of individual prey species was 9 to 57% higher in the predator treatments compared to the no-predator treatments. In contrast, clothianidin exposure decreased overall prey mortality and the mortality of individual prey species with the exception of *L. clamitans*. Averaged across predator treatments, prey mortality was 10 to 25% lower at 352 ppb of clothianidin compared to 0.6 ppb. Lastly, we only observed an interactive effect of predators and clothianidin on overall prey mortality and the mortality of *P. acuta*. For both response variables, mortality was relatively low across the clothianidin concentrations in the no-predator treatment. However, mortality in the predator treatment was lower in the 352 ppb treatment compared to the 0.6 ppb and 5 ppb.

Field survey

We detected the neonicotinoids acetamiprid, imidacloprid, and clothianidin in 56%, 78%, and 81% of our soil samples, respectively (n = 32 total samples per chemical; Fig 7). The mean concentration of acetamiprid, imidacloprid, and clothianidin across all sites and sampling periods was 2.8, 22.0, and 24.2 ppb, respectively. The maximum concentration of clothianidin, imidacloprid, and acetamiprid across all sites and sample periods was 176, 141, and 7 ppb, respectively. Peak concentrations tended to occur 4 weeks post planting (S5 Table).

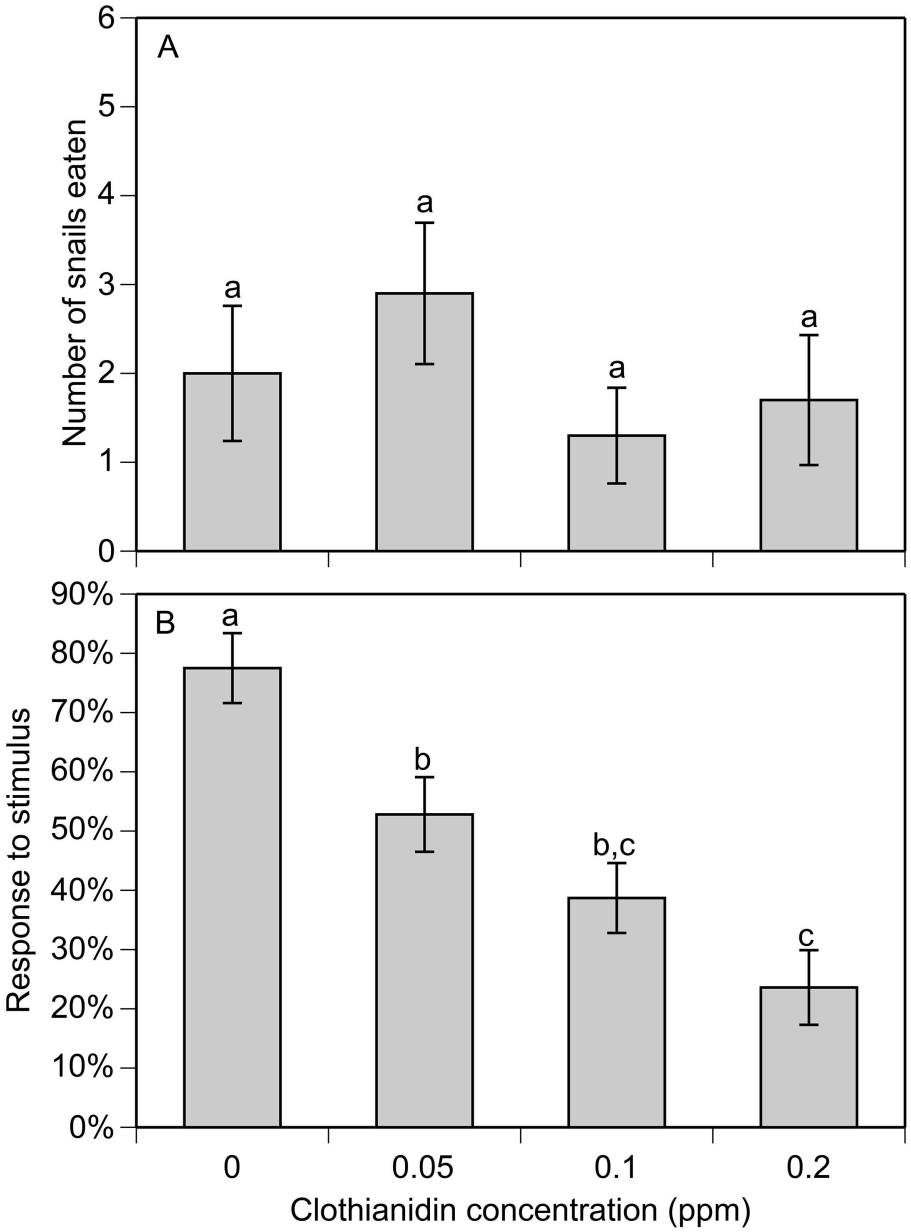

Fig 4. Number of snail prey consumed by crayfish (A) and the percentage of responses to stimulus for crayfish (B). Treatments sharing letters are not significantly different from each other based on pairwise comparisons (Bonferroni corrected P > 0.05). Data are means ± 1 SE.

https://doi.org/10.1371/journal.pone.0174171.g004

We detected the neonicotinoids clothianidin, imidacloprid, and thiamethoxam in 96%, 90%, and 98% of our water samples, respectively (n = 48 total samples per chemical; Fig 8). The mean concentration of clothianidin, imidacloprid, and thiamethoxam across all sites and sample periods was 0.10, 0.02, and 302 ppb, respectively. The maximum concentration of clothianidin, imidacloprid, and thiamethoxam was 0.67 ppb, 0.18 ppb, and 2,568 ppb, respectively. In general, concentrations tended to peak 5 to 7 weeks post planting (S6 Table).

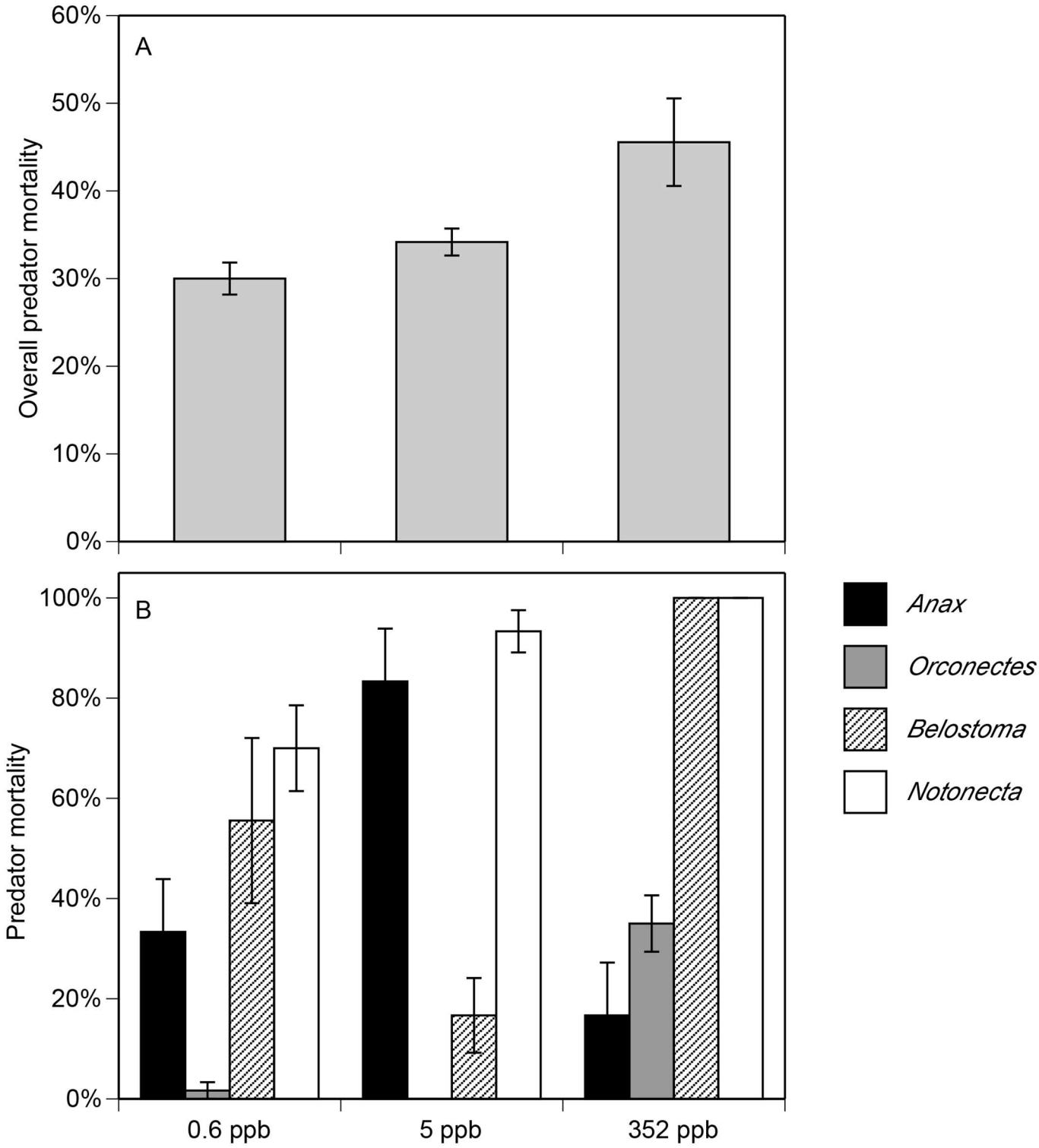

Fig 5. Total predator mortality (A) and the mortality of each predator species (B) following exposure to different clothianidin concentrations. Clothianidin concentrations represent actual concentrations measured in the tanks following addition of Arena granules. Data are means ± 1 SE.

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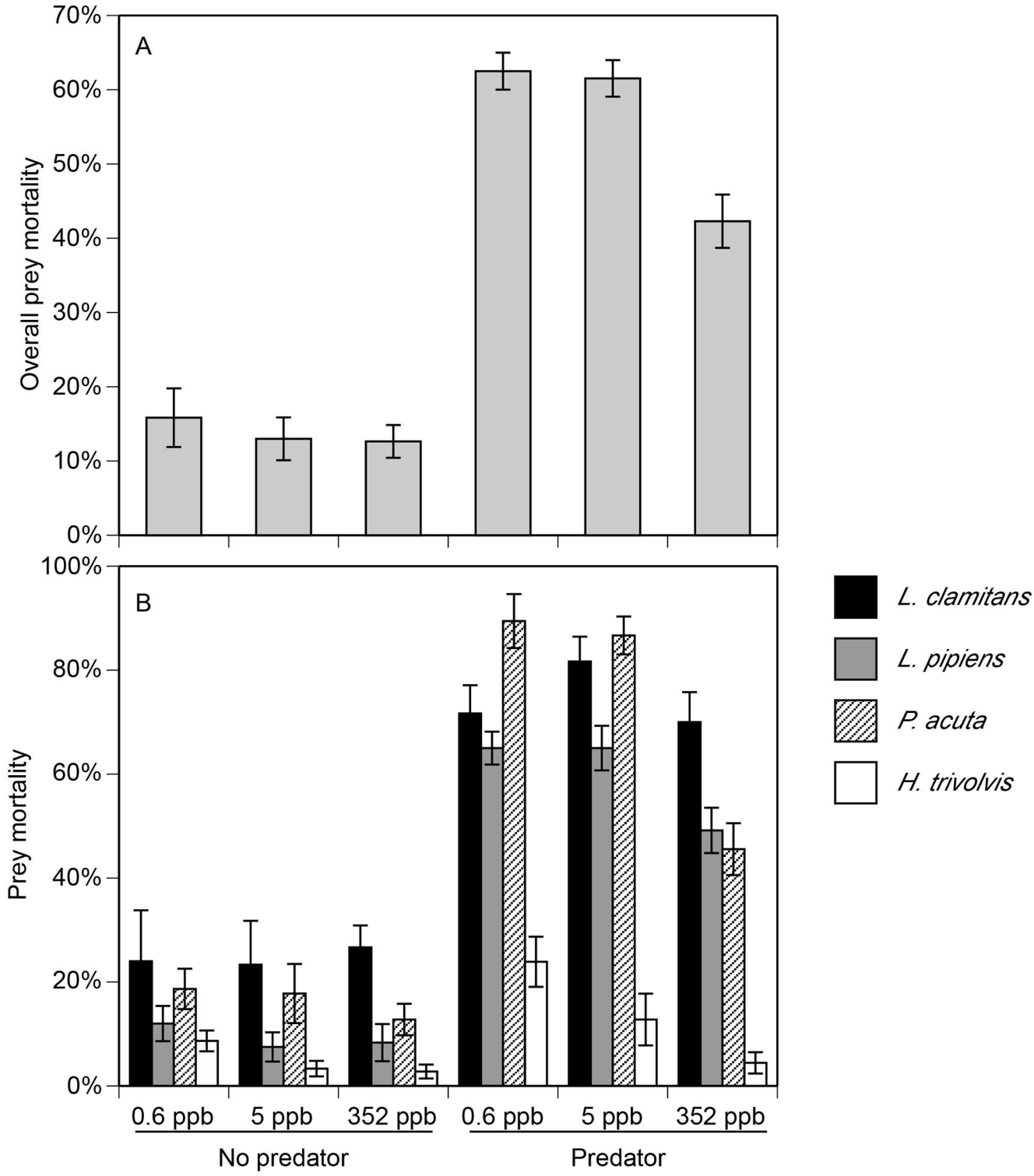

Fig 6. Total prey mortality (A) and the mortality of each predator species (B) following exposure to different clothianidin concentrations and predator environments. Clothianidin concentrations represent actual concentrations measured in the tanks following addition of Arena granules. Data are means ± 1 SE.

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Table 3. The results of ANOVAs on the mortality of all prey species combined and each individual species when exposed to a factorial combination of predators and clothianidin concentration. Bold P-values are significant at P < 0.05.

Source	d.f.	Prey total		*L. pipiens*		*L. clamitans*		*P. acuta*		*H. trivolvis*	
		F	P	F	P	F	P	F	P	F	P
Predator	1,29	299.3	**<0.001**	282.9	**<0.001**	87.5	**<0.001**	238.0	**<0.001**	11.0	**0.002**
Insecticide	2,29	9.2	**<0.001**	3.9	**0.032**	0.3	0.734	18.7	**<0.001**	7.7	**0.002**
Interaction	2,29	6.4	**0.005**	2.8	0.075	0.7	0.494	11.2	**0.001**	2.2	0.131

https://doi.org/10.1371/journal.pone.0174171.t003

Discussion

Neonicotinoids pose a risk to aquatic systems due to their low soil binding, high soil persistence, and high water solubility [45]. Using controlled laboratory experiments, we documented that the neonicotinoid clothianidin has lethal and sublethal effects on wetland invertebrates at field relevant concentrations. Using a community-level mesocosm experiment, we found that clothianidin can reduce the abundance of predatory invertebrates, which indirectly benefits clothianidin-tolerant herbivores in the community. Additionally, we detected four neonicotinoids in the vast majority of soil and water samples at field sites in close proximity to agricultural lands.

Despite the increasing usage of neonicotinoids, toxicity tests with aquatic species have largely focused on the older neonicotinoid imidacloprid [13,46–49]. We found wide variation in the toxicity of clothianidin to the wetland species tested. The most sensitive species was *Graphoderus fascicollis* (Coleoptera) with a LC_{50} value of 0.002 ppm. The current U.S. EPA Aquatic Life Benchmark for clothianidin (acute exposure) and freshwater invertebrates is 0.011 ppm. Yet, clothianidin has been detected in field samples as high as 0.043 ppm [42]. Given that the sensitivity of *G. fascicollis* was an order of magnitude lower than the benchmark, future research should consider including species beyond the typical toxicological models (e.g., *Chironomus riparius*, *Mysidopsis bahia*, *Daphnia* spp.) in neonicotinoid risk assessment [50]. For example, the acute toxicity of *Daphnia magna*, a common model for aquatic toxicology, to clothianidin is 67 ppm [37], suggesting that they are remarkably tolerant compared to other invertebrates. Indeed, cladocerans in general tend to display higher tolerance than other aquatic arthropods to neonicotinoids [11,50,51]. We also found that species from the same order displayed similar levels of sensitivity to clothianidin; the odonates had LC_{50} values around 1 ppm while the hemipterans had LC_{50} values around 0.06 ppm. Previous studies have observed phylogenetic relatedness as a predictive factor for toxicity among related species for other contaminants (e.g. endosulfan, zinc, *Bacillus thuringiensis* toxin) [52–54]. Our results provide support for the notion that phylogenetic relatedness may be useful for predicting toxicity of clothianidin and possibly other neonicotinoids in aquatic invertebrates. It is also important to note that several neonicotinoids including clothianidin were detected at our wetland sites, which served as sources for several of our experimental animals. Recent research has demonstrated that non-target aquatic species can evolve tolerance to insecticides (e.g., carbaryl; [55–57]). Thus, our toxicity values could be underestimates of toxicity for populations without a history of neonicotinoid exposure. However, given the widespread neonicotinoid contamination of surface waters in North America [8–10], our results are representative of real-world scenarios.

We also tested two snail species (*H. trivolvis* and *P. acuta*) and three amphibian species (*H. versicolor*, *L. pipiens*, *L. clamitans*) for their sensitivity to clothianidin. These species displayed high tolerance to the chemical and no individuals died at the highest dissolvable concentration tested (327 ppm). In general, freshwater snails appear to be highly tolerant to a diverse array of

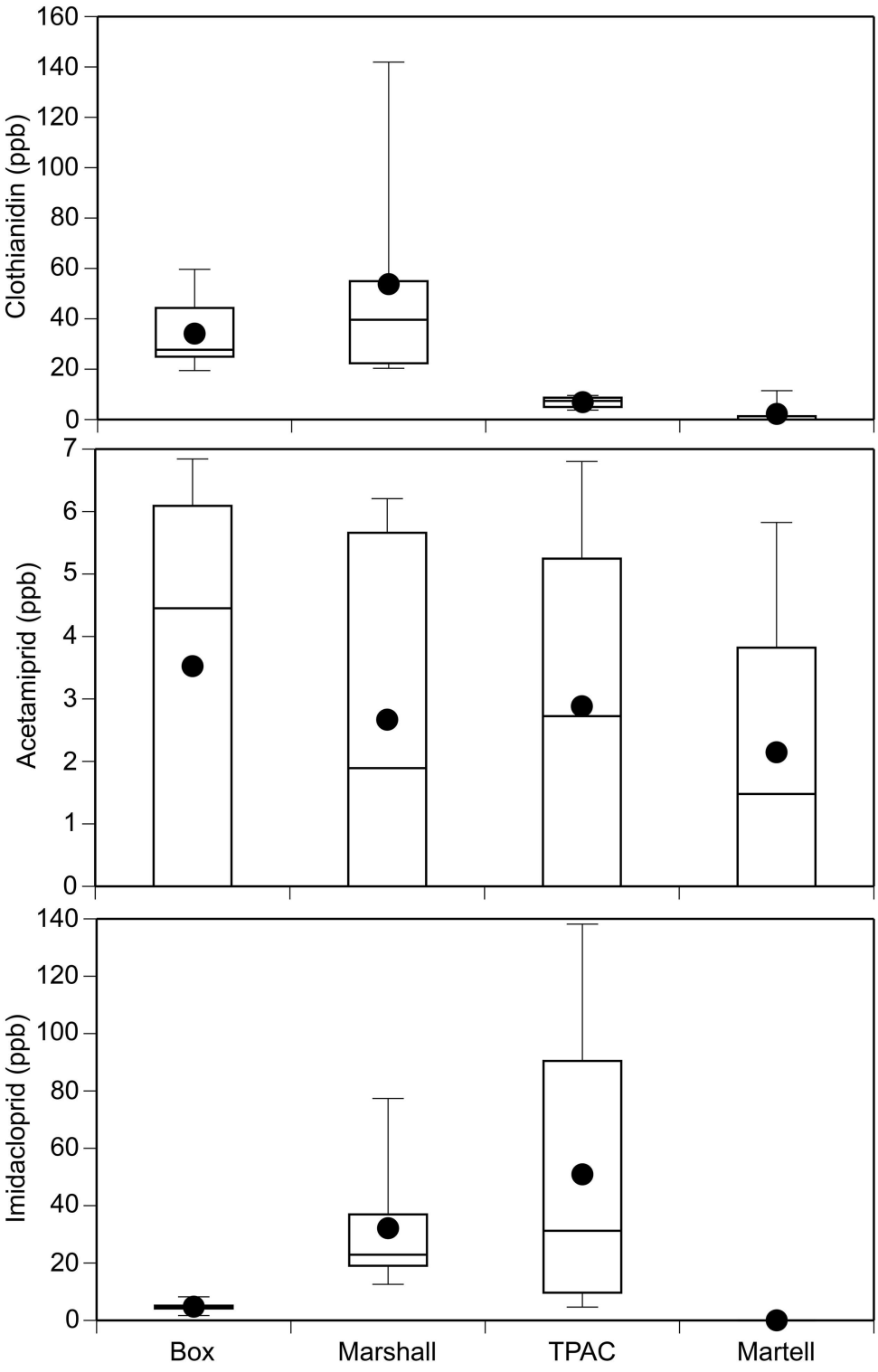

Fig 7. Boxplots of clothianidin, imidacloprid, and acetamiprid concentrations (ppb) detected in soil samples at four sites in Tippecanoe County, Indiana. Data includes samples taken throughout the growing season.

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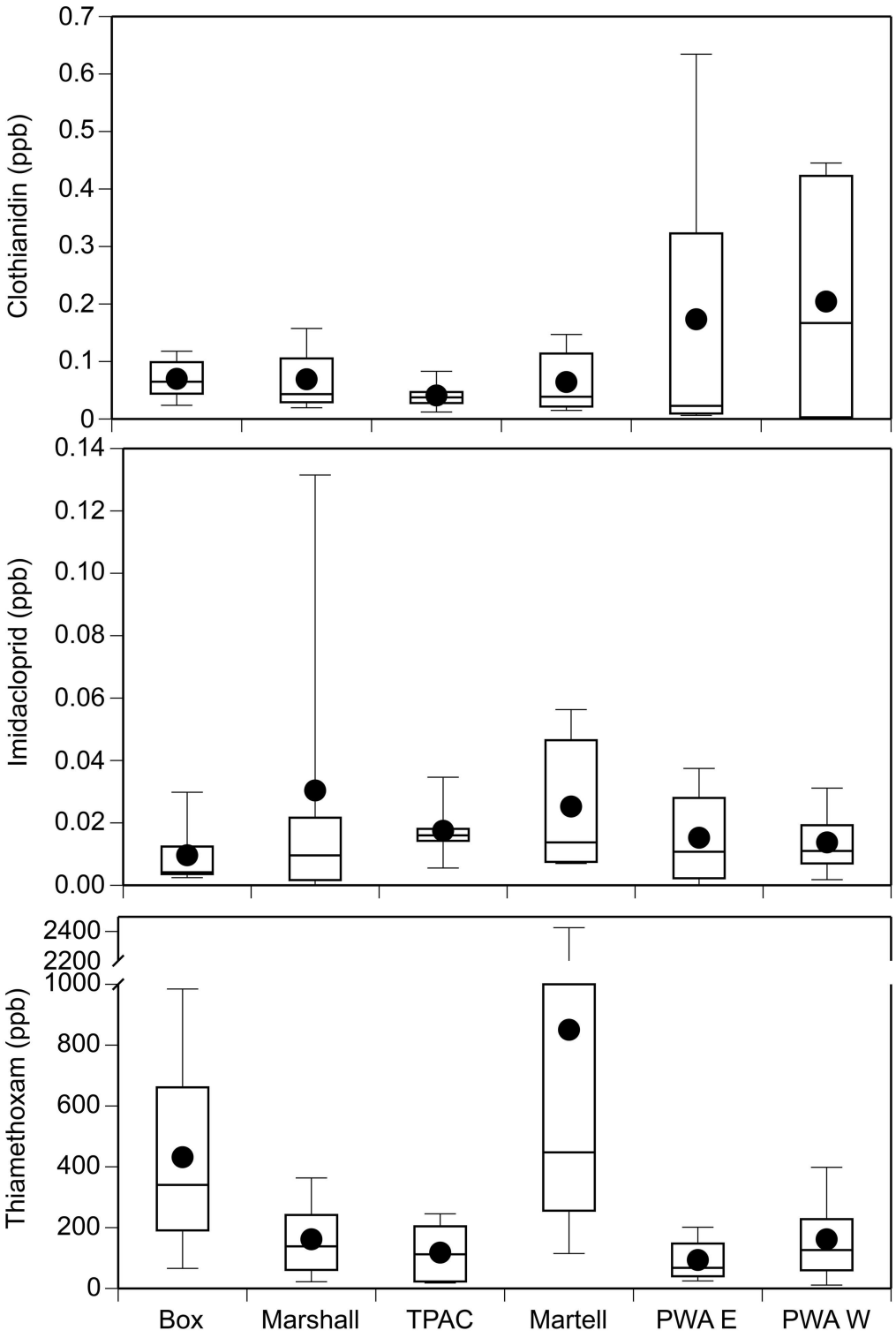

Fig 8. Boxplots of clothianidin, imidacloprid, and thiamethoxam concentrations (ppb) detected in water samples at six sites in Tippecanoe County, Indiana. Data includes samples taken throughout the growing season.

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insecticides and herbicides [34,58–60]. It was also not surprising that tadpoles were tolerant to clothianidin; neonicotinoids generally have low toxicity in vertebrates [49].

In addition to direct lethal effects, neonicotinoids have been shown to cause a diverse range of sublethal effects on aquatic organisms including effects on feeding, movement, immunity, growth, and development [14–21,48,61]. Using a subset of the species from the LC_{50} tests, we found that sublethal clothianidin concentrations can alter behavior and foraging of predatory invertebrates but not tadpoles. For water bugs, we found that clothianidin reduced the consumption of snails with a ~62% reduction at the highest tested clothianidin concentration (0.1 ppm). In the case of crayfish, we did not observe a similar effect on snail consumption. However, we did observe a reduction in the response to external stimuli (i.e. physical agitation). At the highest concentration of clothianidin (0.2 ppm), crayfish were 70% less responsive compared to the control. For both species, we observed behavioral effects at 0.05 ppm, which is within the range of concentrations detected in water samples taken from agricultural areas [42]. Collectively, these results demonstrate that clothianidin can have sublethal effects on the behavior of aquatic invertebrates, at environmentally relevant concentrations, and provide the basis for future work that investigates potentially important sublethal behavioral effects.

While laboratory experiments documenting the toxicity of neonicotinoids are a critical step in ecotoxicology, there is a need for research that explores the community-level and ecosystem-level consequences of exposure, especially in aquatic systems. Community-level experiments have been conducted with the earliest neonicotinoids, imidacloprid and thiacloprid [15,23,27,62,63], but there is no similar work to report using clothianidin. Thus, we conducted a semi-natural mesocosm experiment to explore the community-level effects of clothianidin exposure. As expected, there was a 52% increase in predator mortality when exposed to 500 ppb clothianidin compared to the control. There was no effect on mortality at 5 ppb. However, there were differences in the response of each predator species to clothianidin. For instance, water bugs and backswimmers were the most heavily affected; 100% mortality occurred in the 352 ppb treatment. In contrast, crayfish displayed much higher tolerance to the insecticide with only 35% mortality at the highest concentration. Dragonfly larvae experienced over 80% mortality at 5 ppb but just 15% mortality at 500 ppb. However, we note that our sample size for dragonfly larvae (n = 2 per tank) was relatively low. Although our experiment included a dose (352 ppb) that was beyond what organisms typically encounter in the field, they collectively reaffirm our predictions regarding the lethal effects of clothianidin at different concentrations, which can be useful in assessing does-response relationships. Lethal effects are admittedly a coarse measurement of insecticide effects, but they provide a foundation for experiments investigating population-level effects upon key sublethal parameters such as growth, feeding and reproduction.

In general, overall prey mortality followed our *a priori* predictions. In the absence of predators, prey mortality was low across clothianidin treatments (between 2% and 25%), which was consistent with our toxicity trials with tadpoles and snails. In treatments containing predators, prey mortality was dependent on the level of clothianidin; there was less prey mortality at 352 ppb clothianidin compared to the control and 5 ppb clothianidin treatments. This indirect effect of clothianidin was likely mediated by a combination of direct lethal effects on the predators and sublethal effects on predator foraging behavior. While water bugs were eliminated from the tanks at 352 ppb, a large proportion of the crayfish and dragonfly larvae remained. Thus, the increase in prey survival at 352 ppb was likely mediated by direct mortality of water bugs and sublethal effects on crayfish and dragonfly larvae foraging. In contrast to the 352 ppb treatment, we did not observe significant changes in prey mortality at 5 ppb. Although dragonfly larvae experienced increased mortality in this treatment, the presence of water bugs and crayfish appeared to compensate for the loss of this predator. Moreover, these results suggest

that there were no sublethal effects on predator foraging at 5 ppb. Our data suggest that prey species embedded within communities containing invertebrate predators can benefit from neonicotinoid exposure; these results are not exclusive to neonicotinoids. Ecotoxicology experiments using communities have observed an increase in herbivore survival as a result of predator elimination across a diversity of chemicals including neonicotinoids [21,27,33,48,64–67]. Zooplankton were the only group that were largely unaffected by our treatments. Acute toxicity tests have generally demonstrated that many daphnid, cladoceran, and crustacean species have high tolerance for neonicotinoids [37,50,61]. Moreover, the main zooplankton predator (the backswimmer *N. undulata*) exhibited low survival across all treatments, which minimized predator effects on their populations.

Over the course of the 2015 growing season, we monitored water and soil from sites in Tippecanoe County, Indiana that were located near corn and soybean crops to capture the seasonal variation of potential neonicotinoid exposure levels. Clothianidin, imidacloprid, and acetamiprid were detected in soil samples while clothianidin, imidacloprid, and thiamethoxam were detected in water samples. There was broad variation in the detected clothianidin concentration (0 to 176 ppb) in our soil samples. Likewise, the mean and maximum concentration of clothianidin in our water samples was 0.10 ppb and 0.67 ppb, respectively. While imidacloprid has been the focus of most field studies, there are a growing number of studies that have expanded to include clothianidin especially in surface waters [10,13,42,68–71]. Hladik et al. [68] detected levels of clothianidin as high as 0.0257 ppb in the midwestern U.S., and higher concentrations up to 3.1 ppb were found in the prairie pothole region of Canada [10]. However, Schaafsma et al. [42] detected up to 43 ppb of clothianidin in standing water within agricultural fields in Canada. Interestingly, we detected acetamiprid in soil samples but not water samples while the reverse was observed for thiamethoxam. Given that the concentration of acetamiprid in the soil samples was relatively low, it is possible that this insecticide degraded below detectability for our water samples. We detected high concentrations of thiamethoxam in our water samples (mean = 302 ppb, maximum = 2,568 ppb), which is likely due in part to the very high water solubility of this compound [72]. The concentrations we report here are significantly higher than the U.S. EPA Aquatic Life Benchmark (acute exposure) for freshwater invertebrates (17.5 ppb). Interestingly, this insecticide was not detected in our soil samples. For thiamethoxam that is not washed into surface waters, it is possible that soil microorganisms degrade the chemical to its metabolite clothianidin. This may explain the wide range of clothianidin concentrations detected in our soil samples. Moreover, because clothianidin is the toxic metabolite of thiamethoxam, our results suggest that the actual clothianidin concentrations that organisms will encounter is likely to be underestimated by focusing on clothianidin concentrations alone. However, more research is needed to determine the factors contributing to these field concentrations in our study area. Overall, we detected neonicotinoids in >90% of our water samples. Thus, our study adds to the growing evidence that neonicotinoids are ubiquitous contaminants in surface waters [8,11,42,68].

Benthic invertebrates play an important role in energy flow and nutrient cycling in aquatic systems [73]. Consequently, chemical contaminants that enter these systems have the potential to alter community structure and ecosystem function. Our results demonstrate that the neonicotinoid clothianidin can have lethal and sublethal effects on aquatic invertebrates. While more work examining other neonicotinoids is necessary to assess generality, our work combined with existing studies suggest that the most widely used compounds in this insecticide class have the potential to significantly alter aquatic communities, highlighting the need for more research into the community- and ecosystem-level consequences of exposure [74].

Supporting information

S1 Appendix. Supplemental methods and results for the mesocosm experiment.
(PDF)

S1 Fig. Phytoplankton measurements for the two sampling periods (days 11 and 18 of the experiment).
(PDF)

S2 Fig. pH measurements for the two sampling periods (days 11 and 18 of the experiment).
(PDF)

S3 Fig. Survival curves for (A) *Lestes unguiculatus*, (B) *Anax junius*, (C) *Plathemis lydia*, and (D) *Orconectes propinquus* in the 48 hr LC_{50} tests.
(PDF)

S4 Fig. Survival curves for (A) *Belostoma flumineum*, (B) *Notonecta undulata*, (C) *Hesperocorixa atopodonta*, and (D) *Graphoderus fascicollis* in the 48 hr LC_{50} tests.
(PDF)

S1 Table. QQQ mass spectrometry measurements of clothianidin over time in the three insecticide treatments from the mesocosm experiment.
(PDF)

S2 Table. Results of repeated-measures MANOVA on the effects of predators and clothianidin concentration on periphyton, phytoplankton, and zooplankton on the two sample dates.
(PDF)

S3 Table. Results of repeated-measures MANOVA on the effects of predators and clothianidin concentration on temperature, conductivity, and pH on the two sample dates.
(PDF)

S4 Table. The results of analyses on the survival and biomass of all predator species combined and each individual species when exposed to different levels of clothianidin.
(PDF)

S5 Table. Mean concentrations (ppb) of neonicotinoids detected in soil samples at four sites in Tippecanoe County, IN over the 2015 planting season.
(PDF)

S6 Table. Mean concentrations (ppb) of neonicotinoids detected in water samples at six sites in Tippecanoe County, IN over the 2015 planting season.
(PDF)

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Formal analysis: JCM JTH.

Funding acquisition: MSS CHK JTH.

Investigation: JCM JH.

Methodology: JCM JH MSS CHK JTH.

Project administration: JTH.

Visualization: JCM JH JTH.

Writing – original draft: JCM JTH.

Writing – review & editing: JCM JH MSS CHK JTH.

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Macroinvertebrate community response to repeated short-term pulses of the insecticide imidacloprid

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ABSTRACT

Small streams in agricultural landscape can experience short and repeated pulses of fluctuating pesticide concentrations. A single pesticide pulse may not have adverse effects on macrozoobenthos species but repeated pulses may have, especially if the organisms have not yet fully recovered when the second pesticide pulse occurs. Against this background, a comprehensive indoor stream mesocosm study was carried out in order to evaluate the cumulative effects of repeated insecticide pulses on a macrozoobenthos community. Weekly 12 h pulses of 12 μg/L of the insecticide imidacloprid were set 3 times in 4 stream mesocosms in 2 series, one in spring and one in summer. Another 4 mesocosms served as controls. Prior to each pulse series, the mesocosms were stocked with macroinvertebrates from an uncontaminated reference stream using straw bags as attraction devices. The straw bag method proved suitable for establishing a functional macroinvertebrate community in the stream mesocosms. The caddisfly species *Neureclipsis* sp. reacted immediately and most sensitively after a single imidacloprid pulse whilst insect larvae such as ephemerids and dipteran larvae were negatively affected only after repeated imidacloprid pulses. Effects on insect larvae were more pronounced in the summer series most likely due to increased temperature. Abundance was a less sensitive endpoint than sublethal endpoints such as emergence. The results of the study underline that pulse effects are driven by a number of variables like pulse height, pulse duration, number of pulses, time in between pulses and by the species and live stage specific ability of temperature dependent detoxification which all should be taken into account in the risk assessment of pesticides.

1. Introduction

By the end of 2010, the new EU (European Union) pesticide directive 2009/128/EC became effective (EC, 2009; http://www.eppo.org/PPPRODUCTS/information/2009_0128_EU-e.pdf). As in the former directive, pesticide risk assessment is based on the toxicity/exposure ratio calculations in order to protect non-target organisms. For estimating the predicted environmental concentration (PEC) of pesticides, which may reach surface waters via run-off, spray drift, or drainage after intended pesticide application, a standard model aquatic ecosystem of 0.3 m depth and 1 m width is used as supposed worst case scenario. This scenario simulates little ponds and ditch-like water bodies adjacent to the field rather than creeks or small streams which are more typical for agricultural landscapes (Kreuger and Brink, 1988; Cooper, 1993). However, negative effects of pesticide pulses on aquatic organisms in lentic water bodies may turn out less pronounced in streams compared to ponds due to steady dilution by flow and the resulting shorter exposure time. In the FOCUS scenario for surface waters (FOCUS, 2001), models were developed to assess a more realistic exposure scenario for different stream types in Europe. The FOCUS surface water scenarios are a set of ten standard combinations of weather, soil and cropping data, and water bodies, which represent the entire range of agriculture in the EU for concentration estimates in step 3 EU-level assessment.

Whilst repeated applications in the same crop or field are assessed under FOCUS, the cumulative risk caused by the application of pesticide mixtures or repeated pesticide applications caused by different farmers in the same catchment area are not considered by both FOCUS and the new directive pesticide 2009/128/EC. In particular, exposed stream sections may experience repeated pesticide

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pulses, which may be of short duration but with high pesticide concentrations (Liess, 1994; Kreuger, 1998; Reinert et al., 2002; Schulz, 2008). A single short-term pulse may not have adverse effects on certain species but repeated short pulses may have, especially if the toxicokinetic and elimination process of the pesticide body burden takes longer than the time interval between pulses (McCarty and Mackay, 1993; Escher and Hermens, 2002; Ashauer et al., 2010a). After a single pulse, an organism may not die, but may experience reduced health, which is hard to detect with routine sampling methods. If the organism has not yet fully recovered whilst the second pesticide pulse occurs, an amplification of negative effects on health is likely (Ashauer et al., 2010a). Several models (e.g. toxicokinetic, toxicodynamic) have been developed so far to explain and predict effects of pulses or fluctuating toxicant concentrations on the individual level (McCarty and Mackay, 1993; Reinert et al., 2002; Escher and Hermens, 2004; Ashauer et al., 2007, 2010a; Jager et al., 2011). However, models still need experimental validation especially if it is intended to extrapolate to the community level including complex interactions and indirect effects. In this context, stream mesocosms are a good tool to study effects at the community level (Kosinski, 1989; Brock et al., 2010).

In 2009, the Federal Environment Agency of Germany conducted a stream mesocosm study in order to investigate the effects of short and repeated pulses of the insecticide imidacloprid on macroinvertebrate communities. Imidacloprid was chosen as reference substance since it is repeatedly applied to various crops in the course of the vegetation period (EC, 2008; BVL, 2011), it has a high potential for run-off due to its high water solubility (Armbrust and Peeler, 2002; CCME, 2007), and induces macroinvertebrate drift (Beketov and Liess, 2008). The neonicotinoid imidacloprid is applied as a soil and foliage treatment, and as seed dressing depending on crop (Tomlin, 2000). Imidacloprid acts by disrupting nicotinic acetylcholine receptors in the insect central nervous system (Buckingham et al., 1997; Fossen, 2006). It is highly toxic to midges (Overmyer et al., 2005; CCME, 2007; Stoughton et al., 2008) and mayflies (Alexander et al., 2007). It is mainly used against sucking insects and applied on fruit and vegetables but is also used for lawns and gardens, in stables, and against pet lice (CCME, 2007). Fossen (2006) estimated concentrations up to 36 μg/L for acute surface water exposure. However, a maximum concentration of 320 μg/L imidacloprid was measured in surface waters (Jemec et al., 2007; Tennekes, 2010a) and up to 6.7 μg/L in ground water (Fossen, 2006).

Two application series (spring and summer) of 3 successive 12 h pulses of imidacloprid were run in highly controlled indoor stream mesocosms (Mohr et al., 2005) to simulate repeated imidacloprid application. The objectives were to investigate (i) if repeated short term low-level insecticide pulses may have cumulative effects on macroinvertebrate abundance and emergence, (ii) if the effects in a stream model ecosystem differ from those obtained in existing static mesocosm tests (iii) if a risk assessment based on results from pond mesocosms is also protective for a stream scenario, and (iv) if there is a difference in effects between a spring and a summer community. Effects on summer populations may be different due to higher temperature and different community structure (Brock et al., 2010). For this purpose, macrozoobenthos communities from a reference site were transferred to the mesocosms by using straw bags as attraction devices. This method was evaluated for its suitability in mesocosm studies concerning stocking success and synchronism of macroinvertebrate development in the stream mesocosms after stocking. In this paper, only effects on community structure and insect emergence under stress of insecticide pulses are reported. A second paper will address effects of imidacloprid pulses on macroinvertebrate behaviour and drift response (Rüdiger Berghahn, Umweltbundesamt, personal communication).

2. Methods

2.1. Experimental design

Eight indoor stream mesocosms were equipped with sediment, water, macrophytes, plankton and macroinvertebrates in order to investigate effects of insecticide pulses. Macroinvertebrates were introduced from a reference stream into the mesocosms using straw bags as attraction and transport devices. The mesocosms were stocked with macroinvertebrates in spring and re-stocked in summer, respectively, prior to the start of the two pulse series (spring and summer, Fig. 1).

Each pulse series consisted of three successive 12 h pulses 1 week apart with the insecticide imidacloprid and was conducted in 4 stream mesocosms. Four further mesocosms served as controls. For all six 12 h pulses, the same concentration of 12 μg/L was chosen (Fig. 1). This concentration is within the range of measured environmental concentrations of imidacloprid in surface waters (CCME, 2007) and in line with predicted environmental concentrations from the prospective regulatory risk assessment. According to FOCUS STEP3 and 4, the highest global maximum PEC is 8 μg active substance/L after correct use of a product containing 200 g/L imidacloprid on apple trees using the FOCUS scenario R3 stream (Bologna, Italy; FOCUS, 2001). This scenario was chosen in order to simulate a stream in agricultural landscape, which experiences pesticide entries via run-off or spray drift events. For the experiment at hand, the scenario was extended by the assumption that the same active substance is applied on several fields within a time slot of 3 weeks, resulting in successive exposure peaks in the model stream. The reference insecticide imidacloprid can be applied during the whole season depending on pest and crop (CCME, 2007; BVL, 2011).

2.2. Stream mesocosms

The research was carried out in indoor stream mesocosms, which are part of the artificial pond and stream system of the German Federal Environment Agency (UBA, 2011, http://www.umweltbundesamt.de/wasser-und-gewaesserschutz-e/fsa/index.htm). Eight 75 m long and 1 m wide stream mesocosms were filled with washed sand from a gravel pit and fine sediment from an uncontaminated lake (Schmachter See, Mecklenburg Western Pommerania, Germany) up to a height of 0.2 m. Then, the mesocosms were filled with a mixture of well water and deionised water up to a water depth of 0.2 m. Four pool sections of 300 cm length and 120 cm width were stocked with the macrophyte species *Sparganium erectum*. Fluorescent tubes (OSRAM LF72) provided light in the pools for 14 h/d at a mean light intensity of 7000 lx (~120 $\mu E/m^2$ s) at the water surface. Riffle sections were only indirectly illuminated by diffuse natural light in order to simulate shaded areas. The stream mesocosms were operated in a circular mode at a current velocity of 0.1 m/s. For more information and technical details of the stream mesocosm system see Mohr et al. (2005, 2007).

2.3. Reference stream

Prerequisite for the choice of the reference stream was that the stream had to be more or less unpolluted and morphologically similar to the stream mesocosms concerning width, sediment characteristics, water depth, and water temperature regime. The choice was made for the sublacustrine reference creek Barolder Fließ (Brandenburg, Germany), since some sections were sand dominated, macrophyte rich, partly shaded and the water temperature in summer may reach up to 25 °C (Hensel and Kiel, 2006). According to the German water quality categories (Berghahn,

1997) of the Länderarbeitsgemeinschaft Wasser und Abwasser (LAWA) The Barolder Fließ is classified I-II (oligo- to slightly β-mesosaprobic) and its structure graded category III (moderately modified, LAWA, 2000) by the environmental authorities of the federal state of Brandenburg. The section, in which the straw bags were placed, was 400 m downstream of the effluent of Lake Mochow and was in the vicinity of hay meadows and farmland.

2.4. Colonisation period and transfer of straw bags to the stream mesocosms

Polyethylene bags (30 cm × 20 cm, mesh opening 6 mm) filled with 100 g of loose organic straw (triticale, Fa. Maerkische Biofutter) served as attraction devices for macrozoobenthos. Prior to the filling of the bags, the straw was pressed in order to break and crease long straws.

In total, 154–158 straw bags tied to bamboo sticks were exposed in the Barolder Fließ in late March and again in late May 2009. The bags were evenly distributed over a 100 m long section of the creek. After 2 weeks of exposure the straw bags were removed from the stream bed using a fine meshed dip net (mesh opening 300 μm). Then, each bag was put separately in a fine meshed cover bag and transported cool and humid but not wet (Engelhardt et al., 2008) to the experimental site, since a pilot study had revealed that mortality is <0.5% under these conditions (unpublished data). Transport duration to the experimental site was about 2 h. Eight bags were immediately fixed in 96% ethanol for determination of both the colonisation success and the estimation of initial abundance of macroinvertebrates in the mesocosms. Later the straw in these bags was carefully rinsed with tap water and the remaining macrozoobenthos was stored in 80% ethanol. For macrozoobenthos stocking, the remaining 144 straw bags were evenly distributed over the artificial streams (18 bags per stream).

On the next day, 80 cm wide and ×10 cm deep cross-drains were formed with a wooden plank at 1 m intervals on the bottom of each stream. The straw bags were opened and emptied to allow the loose straw to evenly disperse over the sediment area in the stream mesocosms. Thus, the straw accumulated in the cross-drains and produced a 3rd stratum besides the mesocosm walls and sand bottom, which was akin to foliage and vegetation.

2.5. Simulation of insecticide pulses

For pulse simulation, 1 treatment and 1 control stream per day were dosed simultaneously in the early evening (8 pm CET) employing multi-channel tube pumps. In the treatments, the imidacloprid stock solution was mixed by strong aeration 1 m downstream of the tube pump inlet. The controls were pulsed with the fluorescent nontoxic tracer substance uranine only (Peeters et al., 1996). The tracer was used in order to check complete mixing of imidacloprid in the stream mesocosms by means of a SCUFA fluorometer. The next morning, after 12 h, both streams were flushed with 30 m^3 of water of similar quality and temperature as in the streams in order to completely remove the contaminated water body. Fine meshed nets (mesh size 1 mm) prevented the fauna from passing into the effluent. The entire dosing and flushing procedure was repeated with the remaining pairs of streams on the 3 following days. The scenario for each stream was 1 pulse per week for 3 weeks in 2 series (spring and summer, Fig. 1). Contaminated water was pumped into the sun-exposed outdoor stream mesocosms and stored for discharging until complete disappearance of imidacloprid in water by photo-degradation.

2.6. Sampling and macroinvertebrate determination

Within the investigation period of 11 weeks, quantitative emergence and benthos samples were taken at 10 occasions. More than 5% of the water surface of each mesocosm at 4 different locations was covered with 4 emergence traps of 1 m^2 and 1.4 mm mesh opening, each of which was equipped at the open top with a dimly lit plastic beaker filled with a mixture of ethanol, distilled water, glycerine, acetic acid, and detergent. The emergence traps were emptied and refilled with fixation solution once a week.

Benthos sampling consisted of 5 wall samples, 5 sand samples, and 5 straw samples per sampling date and stream mesocosm, which were pooled for each stratum and each stream separately. Samples were taken once a week on 5 occasions after the first and after the second stocking series. The walls of the stream mesocosms were randomly scraped at 5 positions with a modified kick-sampler (opening: 30 cm) and the catch fixed in 80% ethanol. Prior to the weekly bottom sampling, the % straw coverage was mapped for each 30 cm stretch of the sand covered part in the stream mesocosms in order to determine the sand to straw area relation for determination of total abundance.

The screw pumps were stopped prior to benthos sampling. Sand and straw samples were then taken by means of a Plexiglass tube of 18.7 cm inner diameter, which was plunged into the sediment. After collecting the enclosed straw and stirring up the upper sediment layer of 1 cm by hand, the supernatant water was immediately siphoned and filtered through a 500 μm net. For this suction sampling, an especially designed low-pressure device operated by a double stroke hand piston pump (2 × 3000 cm^3 Sun & Sea, Simex Sport, Nettetal-Kaldenkirchen, Germany) was used. The residual water in the plexiglass tube was sampled with a spoon net (mesh opening 400 μm) for larger animals and remains of straw. In test trials, this method had proven to quantitatively extract both infauna and epifauna, since the smaller animals were unable to resist the suck and large positive rheotactic animals like adult gammarids could easily be detected and caught in the residual water of the plexiglass tube with the spoon net. The straw pool samples were fixed in 96% ethanol since there was remaining water in the straw. All other samples were fixed in 80% ethanol and stored for species determination and counting under the stereoscope.

Prior to straw sample analysis, the major part of the straw was removed by carefully rinsing the animals out of the samples with tap water. Only for the fixed straw bags exposed in the Barolder Fließ, fixed samples were divided with the help of a sample divider (Meier et al., 2006) into one fifth to one sixth of the sample. All macroinvertebrate samples were counted under a stereoscope (Zeiss, Jena). Each specimen was identified to the species level if possible, or to genus or family level.

After the 1st imidacloprid pulse of the summer application series, before and after flushing the streams with uncontaminated water, noticeably fewer gammarids were observed in the sand areas of the treatments as compared to the control systems. For that reason, live counts of larger gammarids in distinct sandy reference areas of the mesocosms were conducted repeatedly until the end of the experiment. In addition, the large visible trumpet shaped filtration nets produced by the trichopteran *Neureclipsis* sp. were counted prior to the second application pulse series directly after new stocking with macroinvertebrates in the summer.

2.7. Imidacloprid analysis

Water samples for imidacloprid analysis were taken after complete mixing of the water body 11.5 h after setting the pulses. The efficiency of flushing was checked with water samples, which were taken 1 d after flushing out the contaminated water.

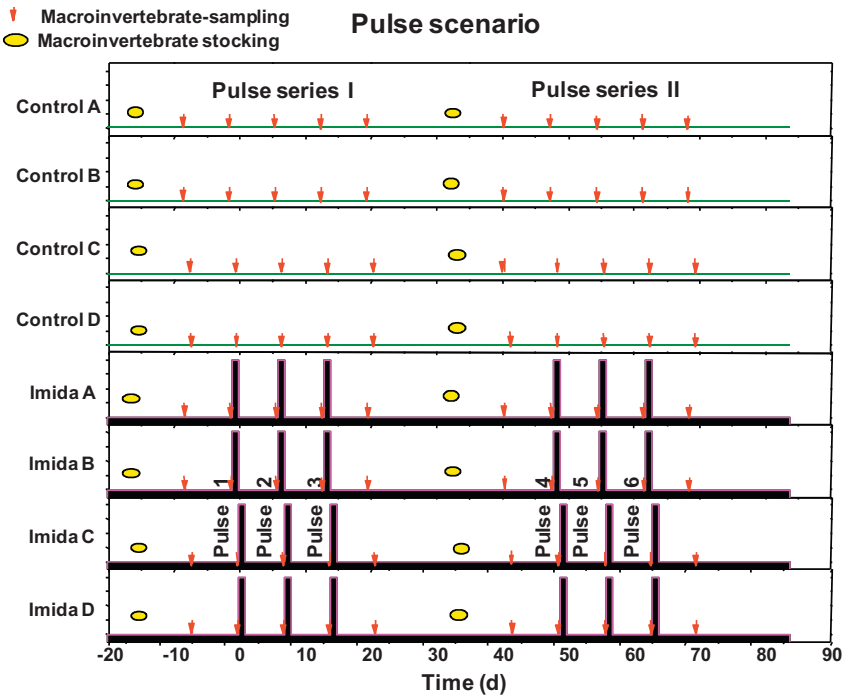

Fig. 1. Imidacloprid pulse scenario and sampling scheme. Imida: imidacloprid treatments.

Imidacoprid was extracted from water samples using a solid phase extraction (SPE) column of modified polystyrene–divinylbenzene resin (ENV + 200 mg/6 mL, IST Biotage). Derivatisation of imidacloprid for gas chromatographic-mass spectrometric (GC–MS) analysis was performed with pentafluorobenzoyl chloride (CAS-No. 2251-50-5) instead of hepta-fluorobutyric anhydride and pyridine as has been reported by MacDonald and Meyer (1998). Imidacloprid-D4 was used as internal standard, squalane (CAS-No. 111-01-3) and dibromo octafluoro biphenyl (DBOFB, CAS-No. 10386-84-2) served as volume control standards. Analysis of the derivatised extracts was performed using a GC–MS system HP 6890/5973 (Hewlett-Packard) equipped with a split–splitless injector (250 °C), a 50 m capillary column, i.d. 0.25 mm coated with a 0.25 μm film of 95% polydimethyl siloxane and 5% of phenyl siloxane (CP Sil 8 CB, Varian). The transfer line was at 280 °C, carrier gas was helium in the constant flow mode. The mass chromatograms *m*/*z* 405, 407, and 212 for imidacloprid, and these of 409, 411, and 216 for imidacloprid-D4 were monitored in the selected ion mode (SIM).

2.8. Statistical analysis

The principal response curves (PRC) ordination technique was used to evaluate the effects of the imidacloprid pulses on the macroinvertebrate community in the stream mesocosms (Van den Brink and Ter Braak, 1998, 1999). Imidacloprid treatment effects are expressed as deviations from the control, so that the control becomes a straight line over time (see Fig. 8). The canonical coefficients express the part of the variance in community structure, which can be attributed to treatment (shown on the *y*-axis, see Fig. 8). By plotting the community-level multivariate response against time (*x*-axis), treatment effects are separated from temporal changes in community structure. Calculated species weights can be interpreted as the affinity of the taxon to the principal response curve. In the redundancy analysis for each sampling date the data were permutated to test the statistical significance of the treatment effect on the species composition for each sampling day (Monte Carlo permutation test). For general concepts of PRC analysis and Monte Carlo permutations see Van den Brink and Ter Braak (1999). The analysis was performed using CANOCO for Windows (Biometris, version 4.5).

The counts per taxon in the pool samples from the 3 different strata of the weekly population census were related to the area of the corresponding stratum. Due to reduction of straw by sampling and decomposition, the area covered by sand and straw had to be newly determined by mapping prior to each benthos sampling date. Differences between treatments and controls were tested for significance at the 5% level with the nonparametric median test, which tests the null hypothesis that the medians of the populations from which two samples are drawn are identical.

3. Results

3.1. Colonisation success of the straw bags and the stream mesocosms

In spring, mean colonisation per straw bag was 2432 ± 419 individuals with dipterans being the dominant group followed by crustaceans. Crustaceans, however, were dominant in the bags after the summer colonisation period with more than 50% of the mean total individuals of 4921 ± 542 (Fig. 2). Variation in the colonisation success between bags was relatively low for highly abundant taxa and as expected higher in rare taxa (Fig. 2). Coefficients of variation (CV) were 40% and 31% for crustaceans, 33% and 43% for ephemerids, 30% and 56% for trichopterans, as well as 14% and 32% for dipterans in spring and summer, respectively. In case of rare taxa CV exceeded 300%. All functional groups were present (Fig. 3). The communities changed from collector gatherer dominance in spring to shredder dominance in summer with over 50% of the total abundance each (Fig. 3). The percentage of predators was about 10% in both spring and summer colonisation period.

At the beginning of the experiment, 18 bags with about 2400 individuals per stream mesocosm were introduced, which resulted in an initial abundance of about 1000 individuals/m^2.

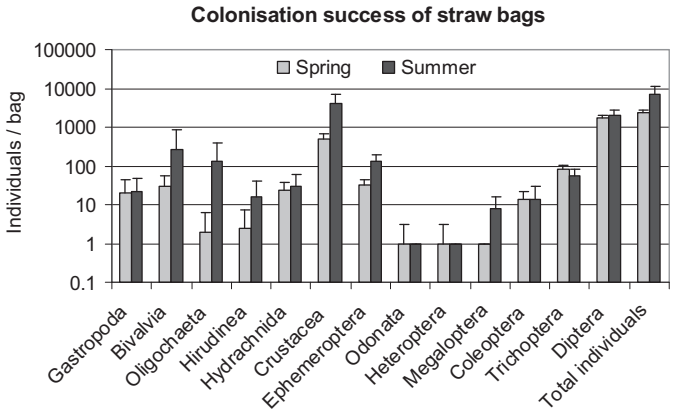

Fig. 2. Mean colonisation success of the straw bags with macroinvertebrates during spring and summer exposure in the reference stream ($n = 10$ and 12). Bars indicate standard deviation.

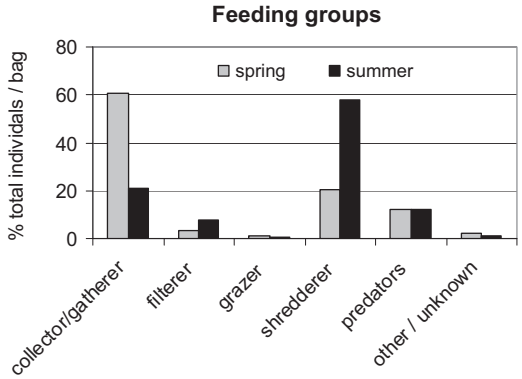

Fig. 3. Occurrence of macroinvertebrate functional feeding groups in the straw bags during spring and summer exposure.

During the second stocking in summer twice the amount of macroinvertebrates was introduced (Fig. 4). Overall, a total of 48 taxa were identified in the stream mesocosms. Species-richest group were dipterans with at least 9 taxa (depth of identification to subfamily level) and trichopterans with 7 taxa.

The first macroinvertebrate sampling after the straw bag introduction revealed a population increase for gammarids by a factor 4.5 and 1.3, respectively. In contrast, populations of insect larvae decreased by a factor 3 and 3.6 respectively (Fig. 4). Population

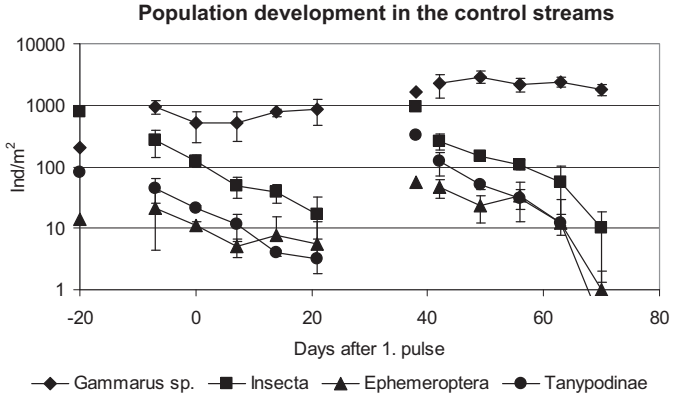

Fig. 4. Population dynamics of different macroinvertebrate groups in the control stream mesocosms ($n = 4$). Symbols, which are not linked, indicate the abundance of introduced macroinvertebrates via stocking from the straw bags. Bars indicate standard deviation.

Table 1
Mean imidacloprid concentrations ($n = 4$) in water during the 12 h pulse and 1 d after rinsing with uncontaminated water.

Pulse	Imidacloprid (μg/L)	
	During pulse	After pulse
P1	12.02 ± 0.49	0.08 ± 0.020
P2	12.09 ± 0.61	0.10 ± 0.004
P3	11.58 ± 0.54	0.07 ± 0.007
P4	11.25 ± 0.75	0.08 ± 0.005
P5	11.12 ± 0.68	0.08 ± 0.007
P6	11.42 ± 0.69	0.08 ± 0.017

dynamics in the 4 control streams were relatively synchronous (Fig. 4). The CV ranged from 14% to 50% for gammarids (mean 31%), 16% to 141% (mean 74%) for ephemerids, from 6% to 102% (mean 37%) for Tanypodinae and from 9% to 89% (mean 46%) for total insects. Higher CVs were generally found for taxa with decreasing abundance such as Tanypodinae and especially ephemerids.

3.2. *Effects of imidacloprid pulses*

It was intended to dose the stream mesocosms with 12 μg/L imidacloprid. This concentration was achieved in the first series (P1–P3), but concentrations in the second series were slightly lower (Table 1). One day after rinsing out the contaminated water, imidacloprid concentrations were 0.1 μg/L at most (Table 1). The measured low concentrations of the tracer uranine confirmed these findings.

The principle response curve analysis of the macroinvertebrate abundance data was not significant ($p = 0.78$) and showed only very weak treatment effects (variance of treatment: 5.2% of total variance; data not shown). Nevertheless, the species weights (b_k values) representing the affinity of species to the PRC, identified taxa, which may have been affected by the imidacloprid pulses. Bk values were highest for Tanypodinae and for *Baetis* sp. (BK = 2.74 and 1.64). Indeed, significant differences between controls and treatments were found for Tanypodinae at the end of the second pulse series (Fig. 5d). Abundance data indicated stronger effects in the second series of 3 pulses at higher water temperatures.

In general, the number of taxa decreased over time in both controls and pulse treatments of both pulse series (Fig. 5a). This species loss was mainly due to the emergence of dipterans, which formed the dominant group in the mesocosms (Fig. 5c and d). Non-emerging macroinvertebrates such as gammarids increased in the course of the study or the abundance remained constant (Fig. 5b). On the basis of the population count data alone, no pulse effects on taxa numbers and gammarid abundance were evident in both pulse series. Development was rather synchronous in all 8 stream mesocosms (Fig. 5b).

Live counts revealed considerable changes in abundance of large gammarids directly after the 5th pulse, with 374 ± 127 ind/m^2 in the controls and 16 ± 10 ind/m^2 in the pulsed stream mesocosms (Fig. 6). Just before the 6th pulse, the numbers in the treatments had increased again and almost reached the level of the controls. The same pattern was observed after the 6th pulse (Fig. 6). The live count results for large gammarids were in good accordance with the abundance data obtained from the sampling of straw, sand and wall (day 70 control–live counts: 495 ± 144 large gammarids/m^2, control–abundance data: 409 ± 58 large gammarids/m^2).

Regardless of the slightly lower number of *Neureclipsis* nets in the treated streams compared to the controls prior to the 4th pulse, the strong decline of nets in the treated mesocosms directly after the 4th pulse was evident (Fig. 7). The animals have to take care of the fragile nets constantly. An abandoned net would quickly be destroyed by drifting organisms or detritus. Therefore one net

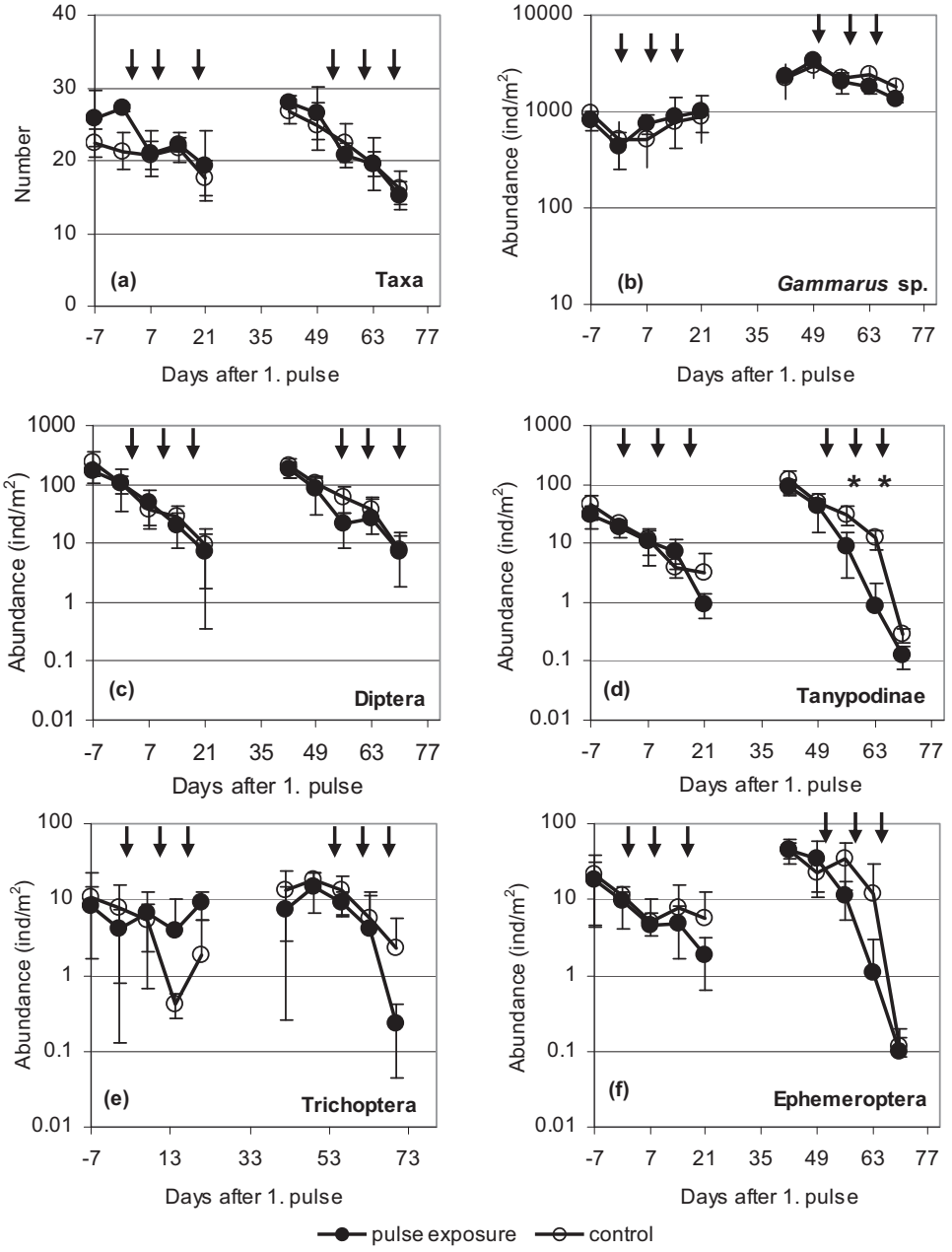

Fig. 5. Response of the macrozoobenthos taxa in the controls and treatments ($n = 4$) over the experimental period. All data in log scale except 1(a). (a) Number of taxa during the investigation time, (b) dynamics of gammarids, (c) dipterans, (d) Tanypodinae indet., (e) trichoterans, and (f) ephemerids. Asterisks indicate significance ($p < 0.05$). Arrows indicate date of pulses. Bars indicate standard deviation.

can be equated to one animal (Hünken and Mutz, 2007). In contrast to the large gammarids, recovery, which would in this case have been indicated by the re-building of nets, was not observed (Figs. 6 and 7).

Overall, the amplitude of the PRC curve (cdt values) for emerged insects was very low indicating only slight effects at the population level (Fig. 8). Nevertheless, the PRC analysis revealed significant differences in emergence between controls and treatments after the 4–6th imidacloprid pulse. A similar pattern was observed for the first pulse series but differences were not significant. The b_k values indicate negative effects of imidacloprid on Tanypodinae indet., Tanytarsini indet., and Baetidae indet., whilst a positive effect was detected for Ortocladiinae indet. (negative b_k value).

Accordingly, emergence of these taxa was significantly higher in the controls than in the treatments (Fig. 9). Ephemerids showed the strongest effects (Fig. 9d) with no emergence in the treatments during the first pulse series and significantly reduced emergence from 4th pulse on. For both, ephemerids and Tanypodinae no emergence on day 70 in the treated streams occurred whereas it was high in the controls.

4. Discussion

4.1. Imidacloprid effects on the stream macroinvertebrate community

Repeated pulses of environmentally relevant concentrations of imidacloprid had adverse effects on the macroinvertebrate community in the stream mesocosms. Especially for the endpoint emergence, insect larvae such as Tanypodinae, Tanytarsini, Ephemeroptera (mainly *Caenis* sp. and *Baetis* sp.), and the caddisfly *Neureclipsis* sp. reacted sensitively to the repeated imidacloprid

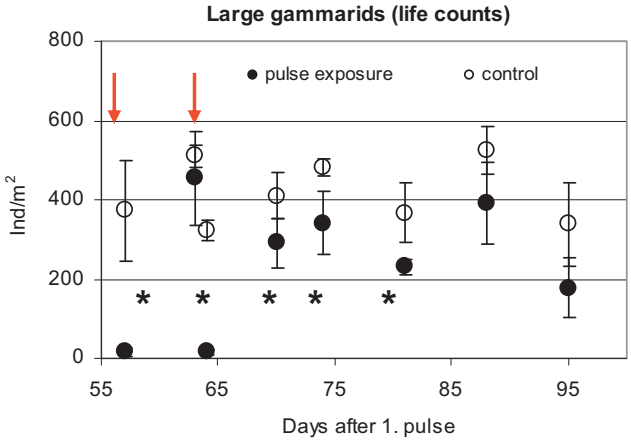

Fig. 6. Life counts of large gammarids (adults) on the sediment after the 4th pulse in the control and pulse exposure treatments till end of investigation period ($n = 4$). Arrows indicate date of pulses. Asterisks indicate significance (median test, $p < 0.05$). Bars indicate standard deviation.

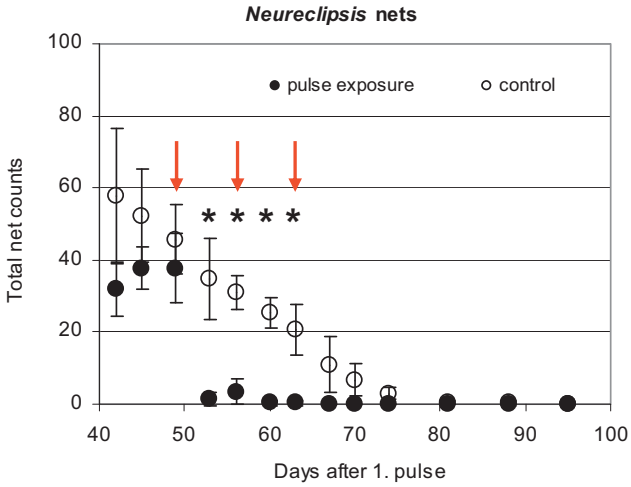

Fig. 7. Total net counts of the trichopteran *Neureclipsis* sp. in the control and pulse exposure treatments for the summer pulse series till the end of the investigation period ($n = 4$). Arrows indicate date of pulses. Bars indicate standard deviation.

pulses. *Neureclipsis* sp. was the most sensitive organism in the stream mesocosms. The silky, fragile nets of *Neureclipsis* sp. are essential for food up take (Petersen et al., 1984) and the lack of net rebuilding directly after the pulse of imidacloprid most likely indicated the death of *Neureclipsis* larvae. The larvae could also have emerged earlier but emergence data did not show such an effect. *Neureclipsis* sp. is a holarctic passive filter feeder and can play a major role in some ecosystems reaching densities >1000 animals/m^2 (Hünken and Mutz, 2007). It is adapted to slow currents and builds its net on rooted aquatic vegetation (Hoffsten, 1999; Hünken and Mutz, 2007). The species is bivoltine and therefore has some potential for recolonisation. However, as imidacloprid can be applied over the whole vegetation period and short term effects were very strong, this species is most likely endangered at environmentally relevant imidacloprid concentrations.

The fact that mayflies, dipterans, and caddisflies were the most sensitive species in this study is also underlined by laboratory findings. In laboratory toxicity tests, the most sensitive species to imidacloprid was the mayfly *Epeorus longimanus* (late instars) with an EC_{50} (96 h) of 0.65 μg/L (Alexander et al., 2007). For the midge *Chironomus tentans* a LOEC of 1.24 μg/L active ingredient (ai; CCME, 2007) and an EC_{50} (96 h) of 5.75 μg/L ai was found (Stoughton et al., 2008). EC_{50} values were in same range for larvae of the black fly *Simulium vittatum* (EC_{50} (48 h): 6.75–9.54 μg/L ai; Overmyer et al., 2005) and for the oligochaete *Lumbriculus variegates* (EC_{50} (96 h, immobility): 6.2 μg/L; Alexander et al., 2007). As also shown in this study for *Gammarus roeseli*, – the dominant species in this mesocosm study – amphipods were more tolerant to imidacloprid. For *Hyallela azteca* an EC_{50} (96 h) of 65.43 μg/L (Stoughton et al., 2008) and for *Gammarus pulex* a LC_{50} (96 h) of 270 μg/L (Beketov and Liess, 2008) have been found. The gammarid species *G. roeseli* was more sensitive to imidacloprid with an EC_{50} (96 h) of 29 μg/L for adults tested in mesocosm stream water; R. Boettger, Umweltbundesamt, personal communication).

4.2. Influence of pulse heights and duration on macroinvertebrates

In acute laboratory tests, organisms are exposed to constant pesticide concentrations by means of a flow-through or a semi-static system. In many cases, this exposure scenario does not represent realistic field situations especially for stream organisms. Under natural conditions, stream macroinvertebrate communities experience in general short sequential pesticide pulses with fluctuating

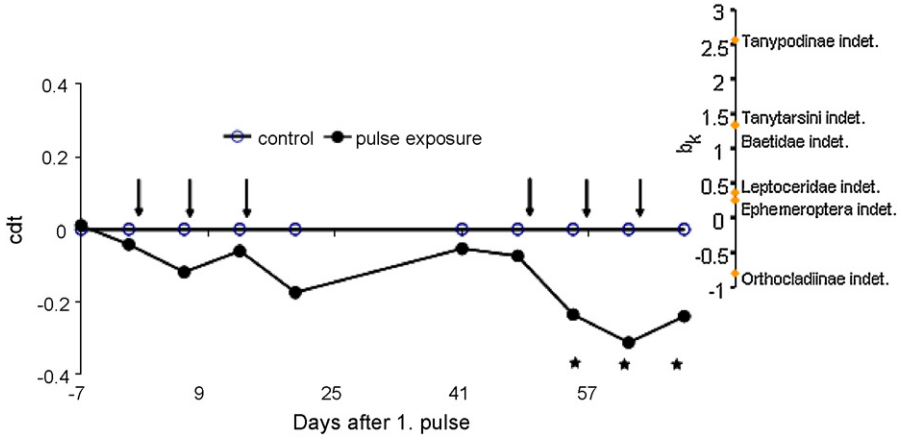

Fig. 8. Principal response curve (PRC) diagram showing the effects of imidacloprid pulses on emergent insects over time. Date of the 6 pulses is indicated by arrows. Of the variance, 46.1% is attributed to the sampling date, 37.1% of the replicates, and 16.8 of the pulse treatment ($p = 0.054$), of which 60% is displayed on the vertical axis (cdt; $p = 0.022$). The species weights (b_k values) represent the affinity of the species/taxa to the PRC. Negative values indicate an increase in abundance whilst positive weights indicate a decrease in abundance. Only species with a b_k value of <−0.5 and >0.5 are shown in the diagram. Asteriks indicate significant differences between controls and pulse treatments at $p < 0.05$ via permutation test for each sampling date. Arrows indicate date of pulses.

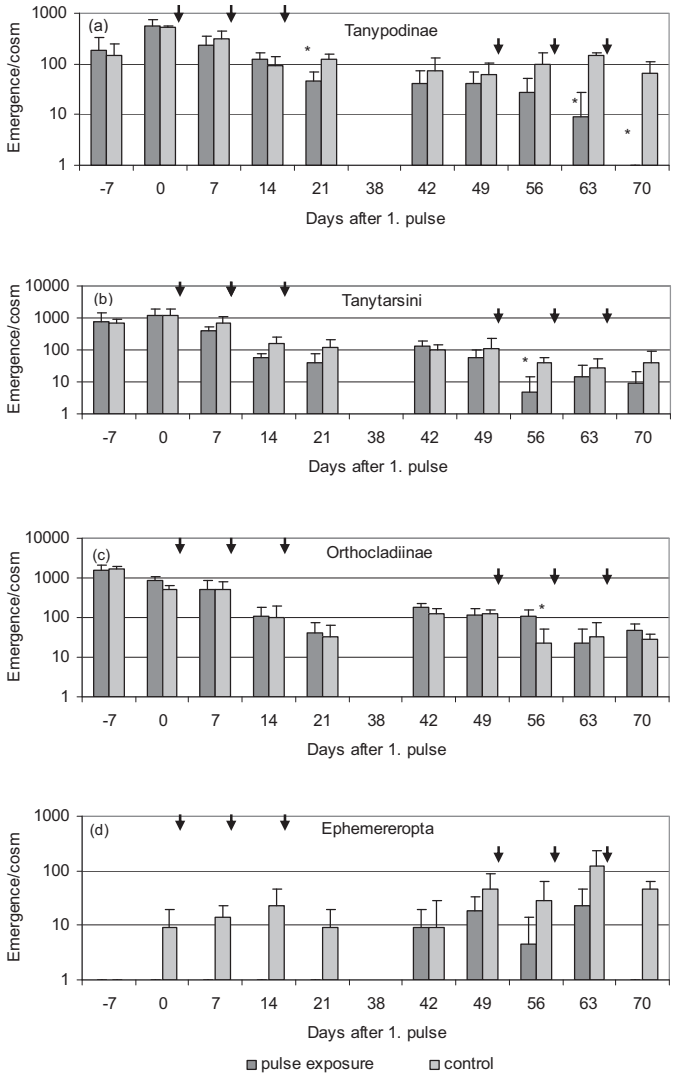

Fig. 9. Emergence per stream mesocosm (controls and pulse exposure; $n = 4$) during the investigation time for (a) Tanypodinae indet., (b) Tanytarsini indet., (c) Orthocladiinae indet., and (d) Ephemerotera indet. Asterisks indicate significance (median test, $p < 0.05$). Arrows indicate date of pulses. Bars indicate standard deviation.

pesticide concentrations. These sporadic pollution pulses may not be acutely toxic to aquatic animals, but harmful effects may become apparent long after poisoning (Handy, 1994). It is therefore of utmost importance to look at the long term detrimental effects of toxicants. According to the Haber's rule, toxicity depends on the product of concentration and time (Hommen et al., 2010a). For example a 2 d exposure at 2 μg/L of a toxicant would cause the same effects as a 1 d exposure at 4 μg/L and a stronger effect than 1 d exposure at 2 μg/L (Reinert et al., 2002). A relationship between concentration and time for imidacloprid has indeed been reported from other laboratory and mesocosm experiments. Alexander et al. (2007) found the EC_{50} of 2.1 μg/L imidacloprid for mayflies to be 3.2 times higher after 24 h exposure as compared to 96 h exposure. Stoughton et al. (2008) also reported lower NOECs with increasing exposure time for *C. tentans* for the imidacloprid formulation Admire®. Tennekes (2010b) found that a longer exposure time to very low toxicant levels even reinforced the effects of imidacloprid. Pestana et al. (2009a) exposed a stream macroinvertebrate community in microcosms to repeated 24 h pulses of imidacloprid (7 days interval) and found significant effects on ephemerids and total insect abundance at a concentration of 17.6 μg/L imidacloprid. In the study at hand, the imidacloprid pulse concentration was lower and pulse duration was shorter compared to the study of Pestana et al. (2009a). Maybe for that reason the effects on insect larvae abundance except for *Neureclipsis* were less pronounced.

4.3. Recovery potential of macroinvertebrates

Organisms, which experience repeated pulses of a pesticide, may recover depending on both their capacity to eliminate a substance in the detoxification process and the time interval between pulses (Reinert et al., 2002). If the elimination time exceeds the time between two pulses, the individual toxic effects will most likely cumulate (Dautermann, 1994). Ashauer et al. (2010b) measured a relatively long elimination time of 95% imidacloprid in *G. pulex*, which amounted to 11.2 d after a single 24 h exposure. The repeated pulses after 7 days in this study are therefore likely to have had a cumulative effect on the gammarids. Nevertheless, *G. roeseli* abundance did not decrease even after the 6th imidacloprid pulse, which might be attributed to the complete recovery of *G. roeseli* from one to the next imidacloprid pulse owing the shorter exposure time of only 12 h and the low pulse concentration of 12 μg/L as compared to theEC_{50} (96 h) of 29 μg/L for *G. roeseli* (R. Boettger, Umweltbundesamt, personal communication). This was also reflected in the direct observations: only few *G. roeseli* were visible on the sediment directly after an imidacloprid pulse, but before the next pulse the numbers were higher than before and almost at the level of the controls. In response to the pulse, the adult *G. roeseli* first seemed to have sought shelter in the straw from current and potential predators. After having recovered they left the straw area again, which indicates the detoxification process to have been shorter than 7 days. Behaviour investigations with *G. roeseli* employing a freshwater biomonitor MFB underlined these findings (Rüdiger Berghahn, Umweltbundesamt, personal communication). In contrast, the decrease in dipteran and ephemerid larvae became more pronounced after the 2nd and 3rd pulse for both pulse series, which may suggest that the elimination time for insect larvae exceeded 7 days and internal imidacloprid concentrations increased over time resulting in more pronounced effects. This interpretation is also supported by the results of the microcosm study of Pestana et al. (2009a). Rubach et al. (2010) also found longer elimination times for two dipterans, 1 ephemerid and 1 trichopteran species by a factor 2–16 as compared to gammarids for the insecticide chlorpyrifos.

4.4. Sublethal effects of imidacloprid on macroinvertebrates

Pestana et al. (2009b) investigated sublethal effects on the midge larvae *Chironomus riparius* and the caddisfly larvae *Sericostoma vittatum* with and without additional predator stress. At concentrations <10 μg/L imidacloprid affected growth, feeding and respiration rates, burrowing behaviour and emergence of the tested organisms. In some cases, presence of predator kairomones increased the effects. Delayed effects and incomplete moulting of crustaceans were also reported (Song et al., 1997). Alexander et al. (2008) reported sublethal effects on mayflies at very low concentrations and observed reduced growth of head and thorax in male *Epeorus* and *Baetis* at concentrations as low as 0.1 μg/L after a 12 h imidacloprid pulse. In general, sublethal endpoints were better indicators for adverse effects than abundance data.

In study at hand, *Gammarus* behaviour (live counts as well as organism drift, Rüdiger Berghahn, Umweltbundesamt, personal communication), net counts of *Neureclipsis* sp. and insect emergence were good indicators for adverse effects. Furthermore, the PRC analyse was not significant for the abundance but for the emergence data. Emergence of chironomid species as well as ephemerids was significantly lower than in the control treatments, which is

in accordance with the findings of Alexander et al. (2008) for ephemerids.

Sublethal effects like behavioural and metabolic changes, enzyme reactions, and fluorescence markers have frequently been reported to be sensitive endpoints for toxicant effects on organisms (Hyne and Maher, 2003; Brain and Cedergreen, 2009; Amiard-Triquet, 2009). However, in EU risk assessment the subjects of protection are populations, communities, and ecosystems (Hommen et al., 2010b). Effects on behaviour and biomarkers should therefore clearly be linked to this level (Forbes et al., 2006; Amiard-Triquet, 2009). Mesocosm studies have the potential to combine sublethal effects and community response for long experimental periods. If the focus of effect studies in stream systems were exclusively on community changes, subtle or chronic biological effects that may result in irreversible long-term changes could occur without being noticed in the apparently healthy ecosystems (Hyne and Maher, 2003). Indeed, at the beginning of this mesocosm study, abundance data did not reflect effects as indicated by sublethal endpoints, but became more linked and apparent by the end of each pulse series. These findings are in line with the statement of Liess et al. (2006) that short pulse exposures may add up to long-term alterations of population structures even at sublethal concentrations. Furthermore, they underline the suitability of behavioural, structural, and physiological parameters to provide both evidence of exposure to one or more pollutants and indications for possible long-term chronic effects (Amiard-Triquet, 2009).

4.5. Seasonal differences of imidacloprid effects

The multivariate PRC analysis indicated slightly stronger effects on the stream mesocosm community after the 3 pulses in the summer than in the spring series. The remaining organisms exposed to the spring pulse series may have experienced cumulative toxic effects during the summer pulse series or more sensitive species and more sensitive developmental stages of organisms may have been transferred into the mesocosms during re-stocking prior to the summer pulse series. A further explanation for the increased summer sensitivities of the macroinvertebrate communities could be the slightly increased water temperature (spring: mean 15.7 °C; summer: mean 17.5 °C). Stream organisms are generally acclimatised to changing environmental conditions such as temperature change (Allan, 1995), but temperature can also be an additional stressor for organisms if the water temperature exceeds the species-specific threshold levels (Cairns et al., 1975; Howe et al., 1994; Heugens et al., 2006; Daam and van den Brink, 2010). However, in their review Cairns et al. (1975) emphasised that changes in detoxification and excretory rates may also reduce or even suppress temperature effects on chemical uptake in aquatic organisms. In contrast to our study, Van Wijngaarden et al. (2006) did not detect strong differences between spring and late summer macroinvertebrate community of ditches after application of the pyrethroid lambda-cyhalothrin. The temperature difference between the spring and late summer application was, however, only marginal.

In a complex ecosystem, it is most likely that a combination of several natural stressors such as e.g. temperature, food limitation, increased predation pressure, or intraspecific competition may have led to the increased sensitivity of the summer macroinvertebrate community in this study.

4.6. Implications for risk assessment

In higher tier risk assessment of plant protection products, pond mesocosm studies are often conducted with a view to increase the regulatory acceptable concentration (RAC) by simulating a more realistic exposure scenario and by investigating effects on model communities. An important question in this context is to what extent results from a pond mesocosm study are representative for stream scenarios as there may be considerable differences in exposure and community structure between the two systems. A comparison of results and implications from a pond mesocosm study with the stream pulse scenario in this study may help to answer this question.

In this stream mesocosm study the effects of one 12 μg/L pulse of imidacloprid on insect larvae such as chironomids and ephemerids were far less pronounced than in pond mesocosms, which had been pulsed once with 10.7 μg/L imidacloprid (CCME, 2007, unpublished protected data from the national authorisation procedure of a plant protection product containing imidacloprid). From this pond mesocosm study, a NOEC of 0.6 μg/L imidacloprid was derived. One might expect similar results for the organisms exposed to one imidacloprid pulse in the stream mesocosms (i.e., no statistical significant effect) provided that the time weighted average concentrations (TWA) in both systems are similar.

For comparison, the duration of 1 application series with 3 pulses in this study, namely 21 days was chosen for TWA calculation. The resulting TWA was almost the same for both systems: 0.28 μg/L imidacloprid in the pond study and 0.29 μg/L for only one pulse in the stream study. With regard to all 3 pulses, the TWA in the stream study would amount to 0.85 μg/L. If the pond results were translated to lotic systems, a single 12 h pulse of 12 μg/L in a stream should in theory not entail significant effects. However, negative effects were evident in the study at hand, namely the complete extinction of *Neureclipsis* after the first pulse in the second summer series and the absence of ephemerid emergence during the first pulse series. Consequently, the idea that negative effects of pesticide pulses on aquatic organisms are less pronounced in streams compared to ponds is not supported. The more sensitive response of the lotic systems may in part be due to the different species composition. Even more important was most likely the inclusion of more sensitive sublethal endpoints in this study, which seem to be in particular indicative for neurotoxic insecticides like imidacloprid and are much more difficult to test in lentic systems in a comparable way. The fact that *Neureclipsis* sp. failed to maintain its nets under imidacloprid pulse conditions may have resulted from strong effects on health. Anyway, the main findings 'extinction' and 'absence of emergence' underline the integrative nature of mesocosm studies in observing the influence of effects on life history traits at the population and community level.

4.7. Evaluation of the straw method

A prerequisite in conducting effect studies in mesocosms is to establish a functional community encompassing sensitive species and a synchronous development of organisms between the mesocosms (Campbell et al., 1999; de Jong et al., 2008). Straw bags proved to be both ideal attraction and transfer devices for macroinvertebrates from reference streams to indoor mesocosms and substrate in the mesocosms providing food and shelter substrate for the fauna. With the straw bags all important functional groups were introduced into the stream mesocosms. In general, collectors/gatherers are the most abundant stream macroinvertebrates (e.g. chironomids; Wallace and Webster, 1996), which was also the case in this study during the colonisation period in spring. However, with the re-stocking in summer a shift to shredders became evident. This increase in shredding organisms (mainly gammarids) reflected natural stream situations. Mortensen (1982) found that the mean annual population densities of *G. pulex* in a small stream in Denmark varied from 500/m^2 in early May to 5500/m^2 in September. Indeed, the presence of gammarids in the straw bags increased from spring to summer by a factor of 8.

In this study, straw was used as food source and shelter for macroinvertebrates whereas in the field leaves of the trees along the riverbank are typical allochtonous food substrates for many stream invertebrates (Wallace and Webster, 1996). It is known that especially alder leaves are a good food source for several macroinvertebrate species due to their favourable carbon to nitrogen relationship (Irons et al., 1988; Motomori et al., 2001) and therefore they would be also a good food source in stream mesocosm studies. However, alder leaves are only available in autumn, difficult to collect from the ground, and very laborious to pick from trees. As more than 30 kg of organic straw was used in this study, the gathering of high amounts of leaves needed in uncontaminated quality for mesocosm studies over the whole investigation period is rather challenging.

The use of straw as attraction device, food source and shelter for mesocosm studies is new. Pilot experiments in microcosms had proven the suitability of straw (unpublished data) as attraction device, but the suitability of straw as food source still has to be clarified. As part of this mesocosm study (R. Boettger, Umweltbundesamt, personal communication) compared the suitability of straw and alder leaves as food for in situ caged *G. roeseli*. Straw proved to be an adequate substitute for alder leaves as food substrate for gammarids. Reproduction rates were almost identical with both food sources. Furthermore, data revealed that the degradation rate was significantly lower for straw than for alder leaves (R. Boettger, Umweltbundesamt, personal communication). This indicates that gammarids rather graze the aufwuchs on the straw than shred the substrate. The low degradation time of straw is a further advantage since it reduces the restocking effort for fresh straw. Grazed straw can be re-colonised with aufwuchs whereas the breakdown in alder leaves is just too fast and reduces experimental control.

4.8. Suitability of the experimental design for effect studies

The transfer of macroinvertebrate communities from streams to mesocosms by means of attraction devices proved to be a good method to investigate effects of insecticide pulses. The highly controlled stocking resulted in synchronous development of macroinvertebrates in the different mesocosms (Figs. 4 and 5). The macroinvertebrate density can easily be controlled by increasing or decreasing the amount of straw bags. In this study, 18 straw bags for each stream allowed for densities, which were similar to the ones in leaf litter sections of the reference stream Barolder Fließ in May 2005 (Hensel and Kiel, 2007) using AQEM (2002) sampling methods. During this study, the densities of 150 ind/g leaf litter were similar or only by a factor of 2 lower in the stream mesocosms (70–140 ind/g straw) during this study.

In this study, the mesocosms were stocked twice with macroinvertebrates in order to simulate spring and summer communities. Re-stocking with new invertebrates can also be seen as re-colonisation of the contaminated site from up-stream sections. The re-stocking idea was previously proposed by Campbell et al. (1999) for the evaluation of recovery potential in mesocosm studies. Caquet et al. (2007) found that external recovery is highly important for recovery processes of insect populations. Therefore, isolated ecosystems are likely to display post-treatment insect recovery very differently from highly connected ones. In general, streams can be seen as "highly connected ecosystems". However, recovery of sensitive taxa seems to be restricted to uncontaminated stretches in the headwaters (Liess and von der Ohe, 2005) and hence, for agricultural streams with intense landuse, recovery may be only limited. In this way, stream mesocosms whether in- or outdoors are highly isolated systems unless they are not directly connected with a natural stream as bypass system. In any mesocosm study, in which the systems are isolated and re-colonisation is only depending on the amount of insects emerging from control systems, recovery effects should always be evaluated with caution.

4.9. Sampling methods

In this study, all potential habitats (sand, straw, and wall) were sampled to estimate the macrozoobenthos standing stock in each mesocosm. Unfortunately, there are no guidance documents for macrozoobenthos sampling in mesocosm studies (OECD, 2006). One way to sample macroinvertebrates in mesocosm studies is to use artificial substrates or substrate samplers (Belanger et al., 2004; Wong et al., 2004; Caquet et al., 2007; Brock et al., 2009). In contrast to traps or artificial substrate samplers, direct substrate sampling allows for estimates of the total standing stock of the system and ind/m^2 as a measure for comparison with other studies and ecosystems.

In the OECD guidance document for freshwater lentic field experiments (OECD, 2006), advice is given that sampling should not signtificantly alter community structure and level of standing stocks. Beketov et al. (2008) and Brock et al. (2009) evaluated macroinvertebrate samples by counting living organisms. Counting benthos alive may reduce potentially negative sampling effects, but requires relatively clean samples with low macroinvertebrate densities. In this study, it was impossible to count the often more than 1000 individuals in just one straw sample alive. Nevertheless, the mean amount of animals removed from the systems by sampling was considered negligible.

5. Conclusions

This study demonstrated that stream mesocosms are a suitable tool for the detection of subtle and chronic biological effects of pesticide pulses by combining sublethal endpoints such as live counts, presence of caddisflies nets, and insect emergence with community level endpoints.

When comparing lotic and a lentic test systems, this study showed that the sensitivity of mesocosm test systems can vary considerably, depending on species composition, endpoints and sampling methods. This should be considered in the risk assessment in terms of estimating the level of remaining uncertainty. A regulatory acceptable concentration derived from a pond study may be safe for stream organisms as long as only peak concentrations are regarded. However, the extrapolation between different complex test systems on the basis of time weighted average concentrations (i.e., inclusion of pulse duration in addition to pulse height) may be misleading as demonstrated in this study since it failed to predict the results obtained from the stream mesocosms.

In concert with other experiments, this study revealed that different macroinvertebrate species and developmental stages such as juvenile gammarids reacted differently on imidacloprid pulses, which may be linked to their different elimination and recovery potential. Consequently, NOECs determined on the basis of single pulses may not be extrapolated to NOECs obtained from multiple/repeated pulses without detailed knowledge of the specific time needed for elimination and recovery. In fact, toxicokinetics should much more be taken into account in aquatic toxicology (Jager, 2011). These data are needed to feed models, which may help to estimate the risk of repeated pesticide applications for aquatic organisms, in particular for uni- and semivoltine insect larvae. However, deriving representative datasets for the spectrum of taxa to be expected in the field (i.e., to be considered in the risk assessment) remains to be a challenge.

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Derivation of an aquatic benchmark for invertebrates potentially exposed to imidacloprid

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Water quality benchmarks are developed by many jurisdictions worldwide with the general goal of identifying concentrations that protect aquatic communities. Imidacloprid is a widely-used neonicotinoid insecticide for which benchmark values vary widely between North America and Europe. For example, the European Food Safety Authority (EFSA) and Dutch National Institute for Public Health and the Environment (RIVM) recently established chronic water quality benchmarks for imidacloprid of 0.009 and 0.0083 µg/L, respectively. In Canada and the United States (US), however, the current chronic water quality benchmarks – termed aquatic life benchmark by the United States Environmental Protection Agency (US EPA) – for freshwater biota are orders of magnitude higher, i.e., 0.23 and 1.05 µg/L, respectively. Historically, aquatic benchmarks for imidacloprid have been derived for invertebrates because they are the most sensitive aquatic receptors. To date, derivation of water quality benchmarks for imidacloprid have relied on the results of laboratory-based toxicity tests on single invertebrate species. Such tests do not account for environmental factors affecting bioavailability and toxicity or species interactions and potential for recovery. Microcosm, mesocosm and field studies are available for aquatic invertebrate communities exposed to imidacloprid. These higher tier studies are more representative of the natural environment and can be used to derive a chronic benchmark for imidacloprid. A water quality benchmark based on the results of higher tier studies is protective of freshwater invertebrate communities without the uncertainty associated with extrapolating from laboratory studies to field conditions. We used the results of higher tier studies to derive a chronic water quality benchmark for imidacloprid as follows: (1) for each taxon (family, subfamily or class depending on the study), we determined the most sensitive 21-day No Observed Effects Concentration (NOEC), (2) we fit the taxon NOECs to five distributions and determined the best-fit distribution, and (3) we determined the HC5

from the best-fit distribution. The higher tier chronic HC5 for imidacloprid is 1.01 µg/L, which is close to the current US EPA chronic aquatic life benchmark of 1.05 µg/L.

Derivation of an Aquatic Benchmark for Invertebrates Potentially Exposed to Imidacloprid

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ABSTRACT

Water quality benchmarks are developed by many jurisdictions worldwide with the general goal of identifying concentrations that protect aquatic communities. Imidacloprid is a widely-used neonicotinoid insecticide for which benchmark values vary widely between North America and Europe. For example, the European Food Safety Authority (EFSA) and Dutch National Institute for Public Health and the Environment (RIVM) recently established chronic water quality benchmarks for imidacloprid of 0.009 and 0.0083 µg/L, respectively. In Canada and the United States (US), however, the current chronic water quality benchmarks – termed aquatic life benchmark by the United States Environmental Protection Agency (US EPA) – for freshwater biota are orders of magnitude higher, i.e., 0.23 and 1.05 µg/L, respectively. Historically, aquatic benchmarks for imidacloprid have been derived for invertebrates because they are the most sensitive aquatic receptors. To date, derivation of water quality benchmarks for imidacloprid have relied on the results of laboratory-based toxicity tests on single invertebrate species. Such tests do not account for environmental factors affecting bioavailability and toxicity or species interactions and potential for recovery. Microcosm, mesocosm and field studies are available for aquatic invertebrate communities exposed to imidacloprid. These higher tier studies are more representative of the natural environment and can be used to derive a chronic benchmark for imidacloprid. A water quality benchmark based on the results of higher tier studies is protective of freshwater invertebrate communities without the uncertainty associated with extrapolating from laboratory studies to field conditions. We used the results of higher tier studies to derive a chronic water quality benchmark for imidacloprid as follows: (1) for each taxon (family, subfamily or class depending on the study), we determined the most sensitive 21-day No Observed Effects Concentration (NOEC), (2) we fit the taxon NOECs to five distributions and determined the best-fit distribution, and (3) we determined the HC5 from the best-fit distribution. The higher tier chronic HC5 for imidacloprid is 1.01 µg/L, which is close to the current US EPA chronic aquatic life benchmark of 1.05 µg/L.

INTRODUCTION

Imidacloprid is a neonicotinoid insecticide used in agriculture to control a variety of pest insects, including aphids, Japanese beetles, lacebugs, leafhoppers, thrips, and others. It is widely used in row crops (e.g., cotton, potatoes), greenhouse vegetables, vine crops, citrus, stone fruit and pome orchards, bush berries, and tree nuts. Imidacloprid acts as a contact insecticide when applied to foliage or soil and is also systematically translocated through plants.

Imidacloprid is highly toxic to some classes of aquatic invertebrates including midges, mysids and mayflies (Gagliano, 1991; Ward, 1991; Roessink et al., 2013). As a result, various jurisdictions have based their water quality benchmarks for imidacloprid on the results of laboratory toxicity tests conducted with aquatic invertebrates.

Current chronic benchmarks that have the general goal of protecting freshwater aquatic biota vary widely despite all being based on laboratory toxicity data. The European Food Safety Authority (EFSA, 2014) recently established water quality benchmarks, known as Regulatory Acceptable Concentrations (RACs), for the European Union. The chronic RAC is 0.009 µg/L. In 2013, the Dutch National Institute for Public Health and the Environment (RIVM) revised their chronic water quality standard for imidacloprid to 0.0083 µg/L (RIVM, 2013). In Canada and the United States, however, the current chronic water quality benchmarks for freshwater biota are orders of magnitude higher, i.e., 0.23 and 1.05 µg/L, respectively (CCME, 2007; EPA, 2016). Using a species sensitivity distribution approach with laboratory toxicity data, Morrissey et al. (2015) recommended that concentrations of imidacloprid and other neonicotinoids need to be below 0.035 µg/L "to avoid lasting effects on aquatic invertebrate communities".

To date, chronic water quality benchmarks for imidacloprid have relied on laboratory toxicity tests conducted with single species. Laboratory studies generally follow strict regulatory guidelines and are performed under controlled conditions. However, laboratory conditions are not reflective of the real world. Higher tier studies (e.g., microcosms, mesocosms and field studies; hereafter "cosm" studies) are specifically designed to have exposure conditions that are representative of natural freshwater environments and consider species interactions, species recovery and other ecological factors. Additionally, higher tier studies can be designed to evaluate community-level effects, which is consistent with the protection goal of the water quality benchmark.

The objective of this paper was to use the best available, higher-tier toxicity data to develop a chronic water quality benchmark for imidacloprid that is protective of freshwater invertebrate communities.

Data relevance and data quality are critical aspects of deriving a water quality benchmark (Breton, 2014; Knopper et al., 2014). To ensure a scientifically defensible water quality benchmark for imidacloprid, we developed a data evaluation rubric to determine which higher tier cosm studies were acceptable, supplemental or unacceptable. Only acceptable studies were used in benchmark derivation.

METHODS

Data Evaluation

A data evaluation rubric was developed to assess the relevance and quality of aquatic invertebrate toxicity studies that have been conducted for imidacloprid. A total of 31 higher tier cosm studies were found and evaluated. Studies were obtained from the primary literature, registrant-sponsored studies following guidelines for Good Laboratory Practice (GLP), EPA's EcoTox database, existing water quality guideline documents, and grey literature studies. The study evaluation rubrics and evaluation results can be found in the Supplemental Information accompanying Whitfield-Aslund et al. (2016).

All studies were first evaluated for relevance and utility. Data relevance was assessed using five criteria: (1) Was the study community/ecosystem relevant (e.g., includes freshwater invertebrates)?; (2) Was imidacloprid the only active ingredient to which test organisms were exposed?; (3) Were test endpoints relevant to the population (e.g., mortality, growth or reproduction) or community level (e.g., richness, productivity) of organization?; (4) Was the exposure route relevant to what is expected in the environment?; and (5) Was the exposure duration consistent with potential chronic exposures in the field? For a study to be considered relevant, each relevance question had to be answered with a "yes", otherwise the study was deemed irrelevant and not considered further.

Relevant studies were further evaluated for data quality. The data quality evaluation focused on objectivity, clarity and transparency, and integrity. Data quality questions were weighted using a scoring rubric, whereby answers were scored from 0 (poor) to 3 (excellent). Questions that could be answered simply with a "yes" or "no" (e.g., was a concentration-response relationship observed?) were weighted lower in the overall study score and were given a 0 for "no" or 1 for "yes". The maximum score was 29 for cosm studies. Studies that scored 29-23 were rated as acceptable. Such studies followed scientifically-defensible guidelines, were considered relevant, and provided sufficient detail to fully reproduce the study. Supplemental (scored 22-13) and unacceptable (12-0) studies provided fewer details, had performance issues, and/or did not follow internationally recognized guidelines or scientifically-defensible protocols. Only acceptable studies were used for derivation of the higher tier chronic benchmark.

Chronic Benchmark Using Higher Tier Cosm Toxicity Data

The HC5 from a taxon sensitivity distribution (TSD) was used as the basis for the cosm-based chronic benchmark for imidacloprid. This approach is broadly consistent with that used by the United States Environmental Protection Agency (US EPA) in deriving water quality criteria (Stephan et al., 1985). Water quality criteria derived by the US EPA generally aim to protect 95% or more of aquatic biota (Stephan et al., 1985). The lowest NOEC was determined for each taxon, generally at the family or subfamily level of organization because NOECs were typically not available for species or genera. If multiple studies with acceptable endpoints were available for a taxon, the geometric mean was calculated. Ten cosm studies were found to be acceptable (Table 1). However, four of the acceptable studies only reported effects on overall invertebrate abundance and not taxon-specific endpoints (Hayasaka, 2012a,b; Kreutzweiser et al., 2009) or

only reported endpoints for macrophytes and periphyton (Heimbach & Hendel, 2001). Thus, these studies could not be used to derive a water quality benchmark for aquatic invertebrates. The remaining acceptable cosm studies had varying exposure concentrations over time due to single or multiple applications, varying application intervals, and temporal decline following application as expected in the natural environment. Studies with a single imidacloprid application were conducted by Kreutzweiser et al. (2007, 2008). Studies with two applications and a 21-day retreatment interval were conducted by Ratte & Memmert (2003), Roessink et al. (2015), and Roessink & Hartgers (2014). The other exposure regime included four applications with a 14-day retreatment interval (Moring et al., 1992). Additionally, by extending the observation period beyond the final imidacloprid treatment, several cosm studies determined the potential for recovery of aquatic invertebrate populations (e.g., Moring et al., 1992; Ratte and Memmert, 2003). However, we did not consider recovery in selecting taxon NOECs for benchmark derivation.

To ensure that cosm-based NOECs were comparable, time-weighted average concentration estimates were determined for the reported no effect treatments. This approach helped to standardize results between different studies with varying exposure regimes. Time-weighted average concentration estimates were calculated using the degradation half-life (DT50) of 11.6 days reported by Roessink et al. (2015). Using this DT50 and assuming first-order elimination kinetics, time-weighted average concentrations were determined by averaging the daily estimated imidacloprid concentrations from the day of the first application to 21 days following the final application. The calculation period was limited to 21 days post final application as this duration corresponded to the most common application interval in the higher tier studies with multiple applications. Additionally, a consistent cutoff was required to ensure that exposure estimates were not severely underestimated in studies that had very long durations. The resulting time-weighted NOECs are reported in Table 1. The time-weighted NOECs include a range of population and community-relevant endpoints including density, abundance, emergence, mortality, and feeding rate. Unbounded data points (i.e., > or < values) were excluded.

If family or subfamily NOECs were not reported for a taxon, the data were grouped by subclass (e.g., Copepoda). Once grouped, a geometric mean of the lowest time-weighted NOEC from each study for each taxonomic group was calculated (Table 1). If only one study was available for a taxon, the lowest NOEC was used. SSD Master v3.0 software (Rodney et al., 2013) was used to derive the taxon sensitivity distribution (TSD). SSD Master fits up to five non-linear regression models (normal, logistic, extreme value, Weibull, and Gumbel) in log or arithmetic space to establish the best-fitting cumulative distribution function (CDF). Model fit was evaluated using the Anderson-Darling (AD) goodness-of-fit test statistic (A^2) and various graphical plots of model residuals to determine the best fit distribution for the TSD.

Table 1 **Data used to derive the chronic taxon sensitivity distribution (TSD) using results from cosm studies for imidacloprid.**

Taxon (Family, Subfamily, Subclass)	*NOEC (µg/L)*	*Geometric Mean NOEC (µg/L)*	*Time-weighted Average NOEC (µg/L)*	*Reference*
Baetidae	0.6	0.816	0.581	Ratte & Memmert, 2003
	2			Moring et al., 1992
	1.52			Roessink and Hartgers, 2014

Table 1 **Data used to derive the chronic taxon sensitivity distribution (TSD) using results from cosm studies for imidacloprid.**

Taxon (Family, Subfamily, Subclass)	*NOEC (µg/L)*	*Geometric Mean NOEC (µg/L)*	*Time-weighted Average NOEC (µg/L)*	*Reference*
	0.243			Roessink et al., 2015
Chironominae	0.6	1.90	1.48	Ratte & Memmert, 2003
	6			Moring et al., 1992
Caenidae	2	2	1.87	Moring et al., 1992
Hydrophilidae	2	2	1.87	Moring et al., 1992
Hydroptilidae	2	2	1.87	Moring et al., 1992
Chaoboridae	3.8	3.8	2.47	Ratte & Memmert, 2003
Naididae	3.8	3.8	2.47	Ratte & Memmert, 2003
Orthocladiinae	3.8	3.8	2.47	Ratte & Memmert, 2003
Copepoda	6	7.51	5.85	Moring et al., 1992
	9.4			Ratte & Memmert, 2003
Daphniidae	9.4	9.4	6.12	Ratte & Memmert, 2003
Glossiphoniidae	9.4	9.4	6.12	Ratte & Memmert, 2003
Planorbidae	9.4	9.4	6.12	Ratte & Memmert, 2003
Tipulidae	12	12	6.84	Kreutzweiser et al., 2007
Tanypodinae	20	13.7	10.7	Moring et al., 1992
	9.4			Ratte & Memmert, 2003
Pteronarcyidae	12	24	13.7	Kreutzweiser et al., 2007
	48			Kreutzweiser et al., 2008

RESULTS

The cosm-based chronic TSD was fit to time-weighted NOECs representing 15 taxa. Time-weighted average effects concentrations ranged from 0.581 to 13.7 µg/L (Table 1). The Gumbel distribution in log space (Equation 1) was the best-fitting model.

$$f(x) = e^{-e^{\frac{(\mu - x)}{b}}} \qquad \textbf{Equation 1}$$

where, $f(x)$ = proportion of taxa affected, x = log concentration (µg/L), μ = location parameter, and s = scale parameter (always positive). The AD goodness-of-fit test statistic ($A^2 = 0.612$, $p > 0.05$) indicated good model fit as confirmed by visual inspection of the residuals and the distribution and the data (Figure 1).

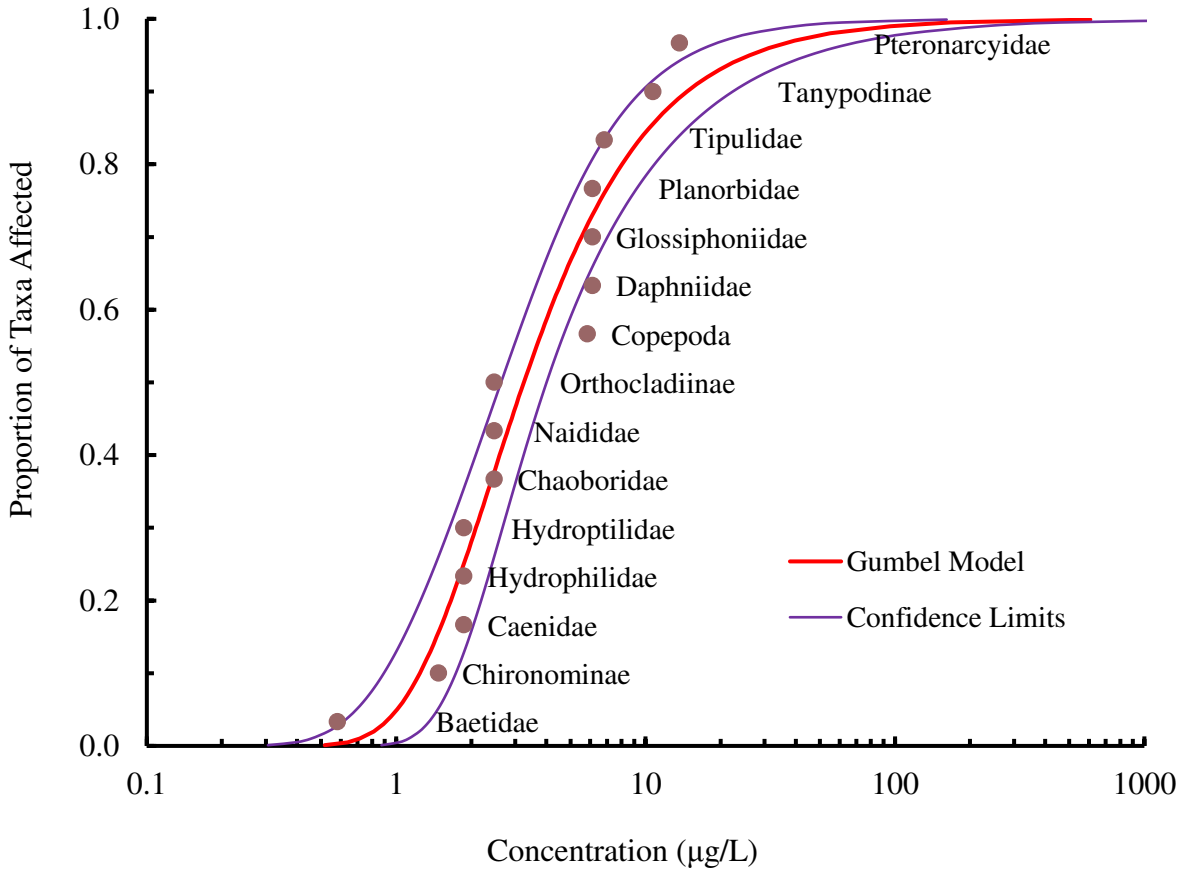

Figure 1 **Chronic taxon sensitivity distribution (TSD) for imidacloprid with 95% confidence limits for family, subfamily and subclass level data extracted from cosm studies.**

The fitted location and scale parameters were 3.38 and 0.347, respectively, for chronic toxicity data reported in log ng/L (the results were subsequently converted to µg/L). The HC5 was 1.01 µg/L, with approximate 95% confidence limits of 0.692 and 1.47 µg/L.

DISCUSSION

In this paper, we derived a chronic water quality benchmark for imidacloprid using the best available data from higher tier cosm studies. The studies underwent detailed evaluations for relevance and quality (see supplemental information in Whitfield-Aslund et al., 2016 for evaluations), and only data of acceptable quality were used to derive the water quality benchmark.

Although a laboratory-based water quality benchmark for imidacloprid can consider a broad range of taxa through the use of the species sensitivity distribution (e.g., Morrissey et al. 2015), it does not account for the more realistic environmental conditions that occur outside the laboratory, reduced fitness due to stress from laboratory confinement, or indirect effects including changes in food, habitat availability, and interspecies interactions. Mesocosm, semi-

field and field studies explicitly account for many of these factors and generally provide data that match the goal of protection of the aquatic invertebrate community. Further, concentrations of imidacloprid are temporally variable in the environment, as they were in the cosm studies, but not in standard toxicity tests conducted in the laboratory. Given the limitations of laboratory-based water quality benchmarks with regard to extrapolating to natural aquatic invertebrate communities, we recommend adopting the chronic water quality benchmark for imidacloprid derived using the higher tier toxicity data from acceptable cosm studies, i.e., 1.01 µg/L. In the discussion that follows, we provide further rationale for this recommendation.

Adverse effects observed in laboratory studies with singles aquatic invertebrate species are not necessarily translated to the community level of organization because adverse effects to one or a few sensitive species may be offset by increases in functionally similar but more tolerant species (Rosenfeld, 2002). Thus, overall community structure and function are not necessarily affected by adverse effects to one or a few sensitive species. In short, the effects of a pesticide such as imidacloprid are not, as a rule, transmitted to higher levels of organization. This statement is one of the foundations of hierarchy theory as proposed by Allen & Starr (1982). There are many examples of aquatic invertebrate communities exhibiting functional redundancy or compensation (e.g., Boersma et al., 2014; Schriever & Lytle, 2016). At some level, all species are unique, but overlap in resource use is common in freshwater food webs (Ehrlich & Walker, 1998). Thus, there are often multiple species present for each of the major functional roles of aquatic invertebrates in freshwater ecosystems, e.g., leaf shredders, suspension feeders, scrapers, detritivores and others that are critical to overall production, nutrient cycling, decomposition and energy flow (Covich et al., 1999). In highly stressed aquatic ecosystems, e.g., those with low functional richness and functional redundancy, the loss of a taxon is likely to have a greater impact on community functioning than in less stressed systems (Suarez et al., 2016). Thus, there are limits to the role that functional redundancy plays in preserving community structure and function. Functional redundancy likely partially explains why the overall aquatic invertebrate community is more resilient to imidacloprid exposure in cosm studies than would be predicted by laboratory studies on single species (Whitfield-Aslund et al., 2016).

Rather than assuming exposure to a constant concentration of imidacloprid, the higher tier cosm studies accounted for varying exposure concentrations over time due to multiple applications, varying application intervals, and temporal decline following application as expected in the natural environment. Cosm studies also had more realistic exposure conditions by, for example, including sediment (Moring et al., 1992; Ratte & Memmert, 2003; Roessink and Hartgers, 2014; Roessink et al., 2015), and carrying out the studies in open air environments with natural lighting and weather fluctuations (Moring et al., 1992; Ratte & Memmert, 2003). Some of these factors may have reduced bioavailability and/or toxicity, e.g., declining concentrations allow for detoxification. In all likelihood, functional redundancy and more realistic peak exposure conditions both contributed to the cosm-based chronic benchmark of 1.01 µg/L for imidacloprid being much higher than the laboratory-based chronic benchmarks derived by EFSA (2014), RIVM (2013) and Morrissey et al. (2015).

The cosm-based chronic benchmark for imidacloprid is conservative because the NOECs used in the benchmark derivation did not consider that many aquatic invertebrates are capable of rapid recovery following cessation of exposure. For example, in Moring et al. (1992), the test system was observed for three months following the final application of imidacloprid. Although a number of macroinvertebrate families (e.g., Baetidae, Caenidae, Hydroptilidae, Hydrophilidae, and Libellulidae) experienced declines in abundance during exposure to the treatment with an initial concentration of 6 µg/L, full recovery of all taxa was observed within eight weeks of the final treatment. During the exposure period, the most sensitive NOEC in this study was an initial concentration of 2 µg/L (time-weighted average concentration = 1.87 µg/L); the corresponding time-weighted NOEC was used in our benchmark derivation (Table 1). Moring et al. (1992), however, recommended that the next highest treatment (initial treatment concentration = 6 µg/L) be adopted as the regulatory NOEC because effects were transient in this treatment and recovery occurred after exposure ceased. Similar results were observed by Ratte & Memmert (2003), who noted complete recovery of Baetidae and Chironominae within eight weeks of the last application. Had recovery been considered in this study the most sensitive initial concentration NOEC of 0.6 µg/L (Table 1) would have increased to ≥9.4 µg/L.

CONCLUSIONS

Higher-tier studies (i.e., mesocosm, microcosm and field studies) should be used when available to derive water quality benchmarks because they offer a level of realism not attainable with standard laboratory toxicity tests. We derived a chronic cosm-based benchmark for imidacloprid for the protection of freshwater invertebrates using relevant and high quality toxicity data. The cosm-based water quality benchmark (1.01 µg/L) supports the current US EPA chronic aquatic life benchmark (1.05 µg/l) as being protective of aquatic invertebrate communities. Although the cosm-based benchmark is higher than the laboratory-based benchmarks adopted in Europe and Canada for imidacloprid, our benchmark accounts for potential effects under more realistic conditions. Functional redundancy and the more realistic exposure conditions used in cosm studies likely explain this difference.

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The Insecticide Imidacloprid Causes Mortality of the Freshwater Amphipod *Gammarus pulex* by Interfering with Feeding Behavior

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Abstract

If an organism does not feed, it dies of starvation. Even though some insecticides which are used to control pests in agriculture can interfere with feeding behavior of insects and other invertebrates, the link from chemical exposure via affected feeding activity to impaired life history traits, such as survival, has not received much attention in ecotoxicology. One of these insecticides is the neonicotinoid imidacloprid, a neurotoxic substance acting specifically on the insect nervous system. We show that imidacloprid has the potential to indirectly cause lethality in aquatic invertebrate populations at low, sublethal concentrations by impairing movements and thus feeding. We investigated feeding activity, lipid content, immobility, and survival of the aquatic arthropod *Gammarus pulex* under exposure to imidacloprid. We performed experiments with 14 and 21 days duration, both including two treatments with two high, one day pulses of imidacloprid and one treatment with a low, constant concentration. Feeding of *G. pulex* as well as lipid content were significantly reduced under exposure to the low, constant imidacloprid concentration (15 µg/L). Organisms were not able to move and feed – and this caused high mortality after 14 days of constant exposure. In contrast, feeding and lipid content were not affected by repeated imidacloprid pulses. In these treatments, animals were mostly immobilized during the chemical pulses but did recover relatively fast after transfer to clean water. We also performed a starvation experiment without exposure to imidacloprid which showed that starvation alone does not explain the mortality in the constant imidacloprid exposure. Using a multiple stressor toxicokinetic-toxicodynamic modeling approach, we showed that both starvation and other toxic effects of imidacloprid play a role for determining mortality in constant exposure to the insecticide.

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Introduction

To protect crops and seeds from pests, about 3 billion tons of pesticides are applied annually to fields worldwide [1]. A fraction of this reaches other environmental compartments such as surface waters via runoff, spray drift and leaching. One of the world's best-selling insecticide is imidacloprid, 1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine, which belongs to the chemical group of neonicotinoid insecticides [2]. Neonicotinoids have selective toxicity for insects and act by binding to the nicotinic acetylcholine (ACh) receptors in the receiving nerve cells of the central nervous system [3,4]. Mammals have lower numbers of nicotinic receptors with high affinity to neonicotinoids, which is why the toxicity of these insecticides is low in mammals [5]. Imidacloprid has a relatively high water solubility (610 mg/L in 20°C H_2O; log $K_{ow} = 0.57$) and therefore, a great potential to reach water bodies. Accordingly, several studies have reported the occurrence of imidacloprid in surface waters [6,7] where it may affect non-target organisms such as *Gammarus pulex* (Crustacea, Amphipoda, Gammaridae). The concentrations of imidacloprid in surface waters in Sweden reported by Kreuger and coworkers (max. 15 µg/L) [7] are below lethal acute toxicity levels in *G. pulex* (50% of the test individuals die after constant exposure to 270 µg/L for 4 days [8]). However, the lower concentrations found in water bodies might cause sublethal effects.

In aquatic environments pesticide contamination generally occurs in pulses due to fluctuation in rainfall, seasonal application of pesticides, and accidents [9,10]. Because neonicotinoids lack ester bonds and thus cannot be hydrolyzed by ACh esterase, also temporary exposure to these insecticides can generate sustained activation in receptors and cause long lasting effects. However, it has been shown that imidacloprid can be dissociated (dissociation constant 0.419 min^{-1}) and removed from ACh receptors by ACh and other ligands [11]. Therefore, it is possible that organisms recover between imidacloprid pulses. On the other hand the elimination of imidacloprid in *G. pulex* is very slow [12] and the substance is not biotransformed [13]. Thus, one could also expect cumulative effects from subsequent exposure events. Imidacloprid has not shown cumulative effects on *Gammarus roeseli* survival after repeated pulses of the insecticide [14]. However, a cumulative sublethal effect (increased drifting) has been reported [15].

By binding to the ACh receptors and interfering with nerve impulses, imidacloprid causes twitching, cramps and muscle weakness. Therefore, it impairs invertebrate movements and can lead to starvation and death via dysfunctional feeding behavior. It has been shown previously that imidacloprid inhibits feeding of many non-target aquatic species [16–18]. However, the connection between impaired feeding and mortality via starvation has not been further investigated, in spite of the importance of conserving populations of aquatic shredding invertebrates like *Gammarus pulex*.

We studied the effects of imidacloprid on feeding rate, lipid content, immobility and survival of *G. pulex* in 14-day and 21-day long experiments. As exposures in aquatic environments generally occurs in pulses, we exposed the animals to two high imidacloprid concentrations. To further study the impact of imidacloprid under low constant exposure, we also exposed *G. pulex* to the time weighted average concentration, which was 15 µg/L. Using the time weighted average concentration allowed us to compare effects of different exposure patterns while still employing the same overall dose (time × concentration). We chose the low concentrations for the constant treatments [8], because we hypothesized that starvation causes death in constant treatments due to impaired movements. In the pulsed treatments, however, feeding activity might recover between chemical pulses and thus starvation would not play a big role in determining mortality. In contrast, we can observe other toxic effects of imidacloprid, e.g. direct mortality, in response to high imidacloprid peaks. Thus, there might be different mechanisms behind mortality in pulsed and constant treatments. To investigate this, we used toxicokinetic-toxicodynamic (TKTD) modeling to analyze the survival data and tested whether fitted model parameters indicate different effect mechanisms in the pulsed and constant exposures. We performed also a starvation experiment, without adding imidacloprid, to further investigate the effect of starvation on survival and to test if using a calibrated starvation model would predict survival in our constant imidacloprid treatments. The chemical effect and the starvation models were also combined to develop a multiple stressor model which again was tested by simulating survival in constant exposure treatments.

Materials and Methods

Test Animals and Chemicals

Gammarus pulex is an important invertebrate species in lentic waters for e.g. decomposition of organic material and nutrient cycling [19]. The *G. pulex* test individuals in our study were collected from a small headwater stream in the Itziker Ried, Switzerland (E 702150, N 2360850). No permission to collecting was required as *G. pulex* is not an endangered species and the site is located on public land. The test animals were maintained for 5–7 days prior to the experiments in a large aquarium in a temperature controlled room (13°C, 12:12 light:dark photoperiod) and were fed with horse chest-nut (*Aesculus hippocastanum*) leaves which were inoculated with the fungi *Cladosporium herbarum* for at least 10 days [20]. The water in the aquarium was preaerated artificial pond water (APW, Table S1 in File S1).

^{14}C-labelled imidacloprid (radiochemical purity 96.97%) was purchased from the Institute of Isotopes Co., Ltd. Budapest, Hungary and unlabeled material (chemical purity 99.9%) from Sigma-Aldrich. A mixture of both was dissolved in acetone and used for dosing.

Imidacloprid Experiments

A 14-d and a 21-d toxicity experiment including three treatments plus controls in each were conducted. Two of the treatments (A, B) included two 1-day imidacloprid pulses with differing recovery time between pulses in uncontaminated APW. In another treatment (C), the concentration was maintained constant (15 µg/L, 0.06 µmol/L in both experiments) but the overall dose was the same over time as in the pulsed treatments (i.e. time-weighted average concentration). All treatments included 7 replicate beakers, one plain and one solvent control beaker. Each 600 mL Pyrex beaker contained 500 ml of APW and 5 leaf discs (diameter of 20 mm, *Aesculus hippocastanum* leaves inoculated with *Cladosporium herbarum*). Ten *G. pulex* were placed in each beaker a day prior to the experiments. The beakers were covered with parafilm and kept in a climate chamber (13°C, 12:12 light:dark photoperiod). The beakers were spiked individually and after spiking, test solutions were stirred with a glass rod and 1 mL samples were taken from the solution to quantify the initial chemical concentration in medium. Ten mL of Ecoscint A scintillation cocktail (Chemie Brunschwig, Switzerland) were added to the samples and activities were counted using a liquid scintillation counter (LSC, Tri-Carb 2200CA, Packard, USA). Samples to determine imidacloprid concentrations in water were taken throughout the experiments (see time points and concentrations in Tables S2 and 3 in File S1). Only the total radioactivity in the aqueous samples was measured and therefore imidacloprid could not be differentiated from its possible breakdown products. For example, organisms might be exposed to the breakdown products of imidacloprid rather than the parent compound due to fast photolysis of imidacloprid in aqueous medium with a half-life of 1.2 h at 290 nm irradiation [21]. However, it is also shown that wavelength has a great impact on the photolysis and already in 365 nm, the half-life is extended to 18 h [22]. The wavelengths in our experiments resemble those of day light ranging from 380–730 nm (relative intensity being the highest in 580 nm). Thus under the conditions of our experiments, imidacloprid most likely is less susceptible to photolysis and in an earlier study no breakdown products of imidacloprid were observed in *G.pulex* samples [13]. The test solution was changed at least every 5 days. Always during water change and every time when eaten leaf discs were observed, they were replaced by new ones. Water pH, conductivity and oxygen concentration were measured in exposed and non-exposed conditions during experiments (see in more detail in Tables S4 and S5 in File S1).

In the 14-day experiment mortality, immobility and consumption of leaf discs were observed. In addition, internal concentrations of imidacloprid in *G. pulex* were measured. The pulsed treatments (A, B) had 4 (A) and 8 (B) days between 1-day pulses (concentration 90 µg/L = 0.35 µmol/L) and individuals in treatment C were exposed constantly to a concentration of 15 µg/L (0.06 µmol/L). Immobility was defined as incapability of moving after ten gentle prods with a glass rod. Immobile individuals were taken out of the beakers and frozen until analysis of internal concentrations. In addition, mobile individuals were sampled for analysis of internal concentrations at the end of the experiment (A and B: $n = 21$ per treatment, C: $n = 10$) and during the experiment from additional beakers of the treatments A and B. These supplemental beakers were not used for observation of survival, immobility and consumption of leaf discs. See detailed sampling times and internal concentrations in Tables S6, S7, S8 in File S1.

Sample processing and quantification of radioactivity in *G. pulex* were measured similarly to Ashauer and co-workers [12]. In short, individuals were plotted dry with tissue paper, weighed in preweighed glass vials and frozen in −20°C. For analysis, 3 mL of the tissue solubilizer Soluene-350 (Perkin Elmer, USA) was added to vials. Vials were placed in a water bath (60°C, 24 h) and after cooling down, 15 mL of scintillation liquid Hionic Fluor (Perkin

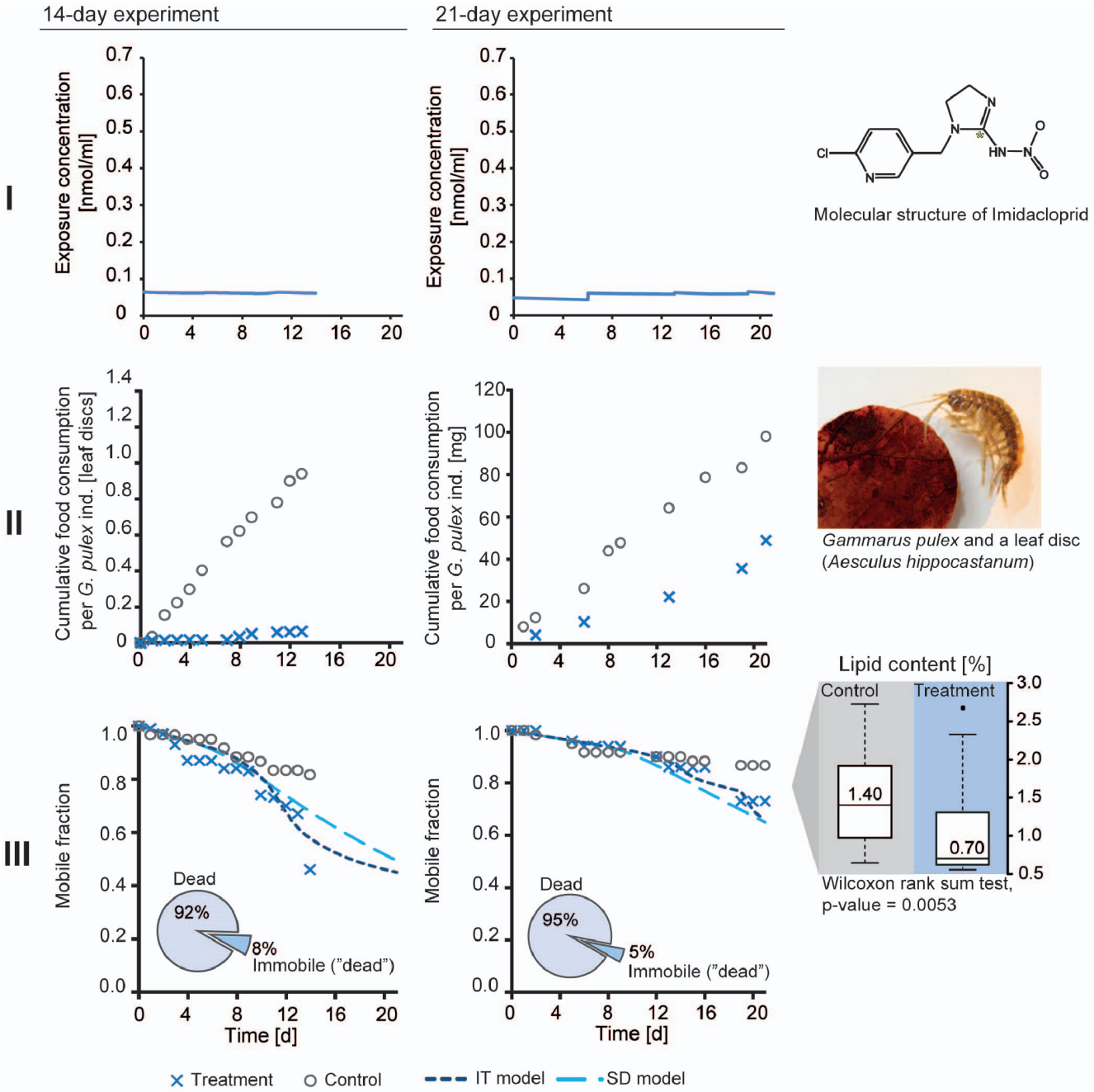


Figure 1. Feeding, lipid content, and survival of *Gammarus pulex* under constant exposure to imidacloprid. Imidacloprid concentrations in medium (I), cumulative food consumption (II), mobile fraction of individuals, and lipid content (% of total wet weight) of *Gammarus pulex* (III) in the constant treatments (C) and controls of 14-day and 21-day experiments. Pie charts show the percentage of dead and immobile individuals amongst those removed from the beakers (non-mobile individuals = immobile+dead).
doi:10.1371/journal.pone.0062472.g001

Elmer, USA) was added. Radioactivity was counted using a liquid scintillation counter; color quenching and efficiency were corrected using external standards and background activity (i.e. activity in control samples was subtracted from counts of the samples).

The second, 21-day long experiment included also observations of survival, immobility and food consumption. In addition, lipid content was measured from immobile individuals, which were sampled and frozen any time they were observed as well as from mobile individuals sampled at the end of the experiment. The pulsed treatments (A, B) had 4 (A) and 11 (B) days between 1-day pulses (concentration 140 µg/L = 0.59 µmol/L) and individuals in treatment C were exposed constantly to a concentration of 0.06 µmol/L.

Feeding Rate

In the 14-day experiment, food consumption was measured as the number of leaf discs consumed by *G. pulex* individuals in each beaker. Every time when a leaf disc was fully eaten, it was replaced by a new leaf disc and the exchange was noted. This way of measuring was not based on the mass of leaf discs but only on the

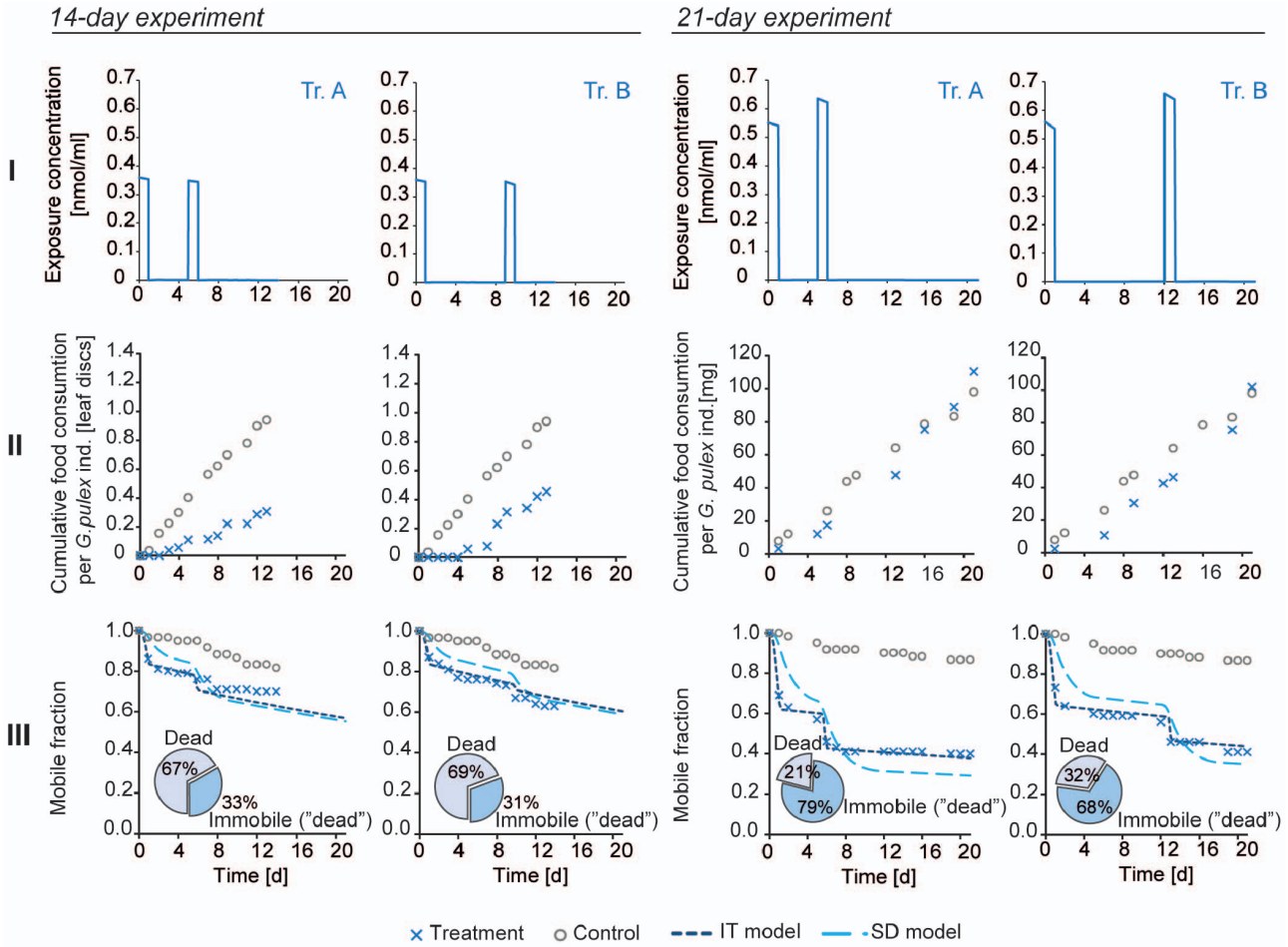


Figure 2. Feeding and survival of *Gammarus pulex* under pulsed exposure to imidacloprid. Imidacloprid concentrations in medium (I), cumulative food consumption (II), and mobile fraction of *Gammarus pulex* (III) in the pulsed treatments (A, B) and controls of 14-day and 21-day experiments. Pie charts show the percentage of dead and immobile individuals amongst those removed from beakers (non-mobile individuals = immobile+dead).
doi:10.1371/journal.pone.0062472.g002

Table 1. Feeding activity of *Gammarus pulex* under constant (treatment C) or pulsed (treatments A and B) exposure to imidacloprid.

				Statistics (*p*-value)	
Experiment	**Treatment**	**Median cumulative feeding**	**Units**	**Wilcoxon test[4]**	**Kruskal-Wallis test[5]**
14-day	Control	0.929	leaf discs/*G.pulex*		0.0010
14-day	A (pulsed)[1]	0.286	leaf discs/*G.pulex*	0.010	
14-day	B (pulsed)[2]	0.500	leaf discs/*G.pulex*	0.031	
14-day	C (constant)	0.000	leaf discs/*G.pulex*	0.003	
21-day	Control	103.9	mg/*G.pulex*		0.0023
21-day	A (pulsed)[1]	114.2	mg/*G.pulex*	0.234	
21-day	B (pulsed)[3]	104.4	mg/*G.pulex*	0.731	
21-day	C (constant)	44.75	mg/*G.pulex*	0.002	

[1]Pulsed treatment with a short interval in uncontaminated water between imidacloprid pulses (4 days).
[2]Pulsed treatment with a long interval in uncontaminated water between imidacloprid pulses (8 days).
[3]Pulsed treatment with a long interval in uncontaminated water between imidacloprid pulses (11 days).
[4]Between control and treatment.
[5]Among all treatments within one experiment.
doi:10.1371/journal.pone.0062472.t001

number of same sized discs. During the 21-day experiment food consumption was measured as the mass of leaf material. Wet weight of the leaf discs of each beaker was measured before providing them to the test organisms and when removing the rest of them from the beakers. Food consumption is given as cumulative amount consumed over time (either amount of leaf discs (14-day experiment) or mg (21-day experiment)). The food consumption was divided by the number of mobile organisms in the respective beaker (amount consumed/*G. pulex*). Statistical testing to compare feeding among treatments was performed for cumulative food consumption at the end of the experiments using the non-parametric Kruskal-Wallis test and further pairwise testing with the Wilcoxon rank sum test. The assumption of normality could not be tested due to the too small sample size (i.e. number of replicate beakers, 7 per treatment) and thus a normal distribution of the data could not been assumed and one-way analysis of variance could not been used. Analyses were performed using the software R (www.r-project.org).

Lipid Content

The lipid content was analysed from immobile *G. pulex* sampled during the 21-day experiment (treatment A: 33 samples, treatment B: 28 samples, treatment C: 1 sample) and mobile individuals sampled at the end of the experiment ($n = 21$ for each treatment, total of 30 for controls: 5 from plain and 5 from solvent control beaker of each treatment). A gravimetric method was used to determine the lipid content according to Kretschmann and coworkers [23]. In short: Extraction was done using H_2O, i-PrOH, and cyclohexane (11:8:10) in 2 mL Eppendorf tubes. 300 mg of zirconia/silica beads (Ø 0.5 mm, BioSpec Products, Bartlesville, OK, USA) was added to iPrOH/cyclohexane solution together with the sample and FastPrep® FP120 Bio 101 (Savant Instruments, Inc., NY, USA) was used to break down the tissues of *G. pulex*. Nanopure water was added to samples. A water content of 77% of *G. pulex* wet weight was assumed to achieve a 11:8:10 ratio of H_2O, i-PrOH, and cyclohexane. Then, samples were vortexed, centrifuged (20 min, 450 g, 20°C) (Centrifuge 5417R, Vaudaux-Eppendorf AG, Schönenbuch/Basel, Switzerland) and the organic phases were separated. Volumes of 435 µL of cyclohexane and 65 µL of iPrOH were added once more, and after vortexing, centrifugation and separation of the organic phase, the solvents of the combined organic phase aliquots were evaporated under nitrogen flow and extracts were dried at 60°C for 14 hours. The remaining phases, i.e. the lipids, were weighted. The weight of lipids was divided by total wet weight to obtain the lipid content as a percentage. Because the lipid content in treatment C did not follow a normal distribution (see Figure 1 and Figure S1, raw data are provided in Table S11 in File S1), the differences among treatments at the end of experiment were compared by the Kruskal-Wallis rank sum test and further pairwise testing (control-treatment) using the Wilcoxon rank sum test. The software R was used for the analysis.

Chemical Stress Modeling

The survival/mobility data of the imidacloprid experiments was analyzed using the toxicokinetic-toxicodynamic (TKTD) model GUTS (General Unified Threshold model for Survival) published by Jager and co-workers [24]. The model was implemented in the software ModelMaker 4 (Cherwell Scientific Ltd., Oxford, UK). The fraction of mobile animals over time was used to calibrate the model. Note that immobile animals were removed from the experiment, allowing us to apply the same assumptions about error structure to our immobility data as to survival data and to use GUTS for modeling the mobility/survival data. As we suspect that constant low concentration of imidacloprid (treatments C) might have a different mechanism for survival/mobility than the pulsed treatments (A and B), the TKTD model was calibrated separately using the constant treatments C of both experiments together and the pulsed treatments (A, B) of both experiments together. In addition, the model was fitted to all data in order to compare the parameter estimates with those of the separately fitted data sets. The parameter estimates from the fit to all data were used as initial values for fitting the model separately to pulsed and constant treatments.

Imidacloprid is not biotransformed in *G. pulex* [13]. Therefore, a one-compartment toxicokinetic model (Eqn 1) was used to simulate the internal concentration of imidacloprid.

$$\frac{\mathrm{d}C_{\mathrm{int}}(t)}{\mathrm{d}t} = C_{\mathrm{ext}}(t)\cdot k_{\mathrm{in}} - C_{\mathrm{int}}(t)\cdot k_{\mathrm{out}} \quad (1)$$

where C_{int} (t) is the internal imidacloprid concentration in organisms [µmol/kg], C_{ext} (t) is the concentration in water [µmol/L], k_{in} is the uptake rate constant [$\mathrm{L\cdot kg^{-1}\cdot d^{-1}}$], k_{out} is the elimination rate constant [1/d] and t is time [d]. Uptake and elimination rate constants were estimated by Ashauer and co-workers (k_{in}: 1.96 [$\mathrm{L\cdot kg^{-1}\cdot d^{-1}}$]; k_{out}: 0.267 [1/d]) [12]. We validated this TK model by comparing its predicted internal concentrations with measured internal concentrations in our first experiment.

Survival modeling was based on the GUTS model and the modeling is similar to our previous study with *G. pulex* and propiconazole [25]. However, here least squares optimization together with the Marquardt algorithm was used to fit models to the data. Confidence intervals (95%) were calculated from standard errors as described in Motulsky & Christopoulos 2003 [26]. Fitting was also performed by maximizing the log-likelihood function, which maximizes the likelihood of yielding the parameter set which best describes the number of death events between time intervals (see Table S14 in File S1). In this study, the cumulative fraction of survivors over time was better described by the parameters found via least squares optimization.

Two models, either assuming stochastic death (SD) or individual tolerance distribution (IT) were used separately to test which hypothesis of death applies for imidacloprid. SD models have one value for the threshold of survival and after exceeding it, an organism has an increased probability to die. In contrast, according to IT models the threshold is distributed within the population and death is instantaneous after exceeding the individual threshold. To date, it is not known which of the two hypotheses describes our data better (see also discussion in [24] and [25]). Therefore, both models, SD and IT, were calibrated and the goodness of fit values were compared.

Equation 1 was used to simulate the internal concentrations (C_{int}) in the survival model for both, GUTS-SD and GUTS-IT. The implementation of the stochastic death model (GUTS-SD) is given in Eqns 2 to 5. Eqns (2) and (3) were used to calculate the cumulative hazard at time t ($H(t)$).

$$\frac{\mathrm{d}D^*(t)}{\mathrm{d}t} = k_{\mathrm{d}}\cdot(C_{\mathrm{int}}(t) - D^*(t)) \quad (2)$$

$$\frac{\mathrm{d}H(t)}{\mathrm{d}t} = k_{\mathrm{k}}\cdot \max\left(D^*(t) - z, 0\right) + h_{\mathrm{b}}(t) \quad (3)$$

where D^* (t) is the scaled damage [µmol/kg], k_{d} is the damage recovery [1/d], k_{k} is the killing rate [$\mathrm{kg\cdot \mu mol^{-1}\cdot d^{-1}}$], H (t) is the

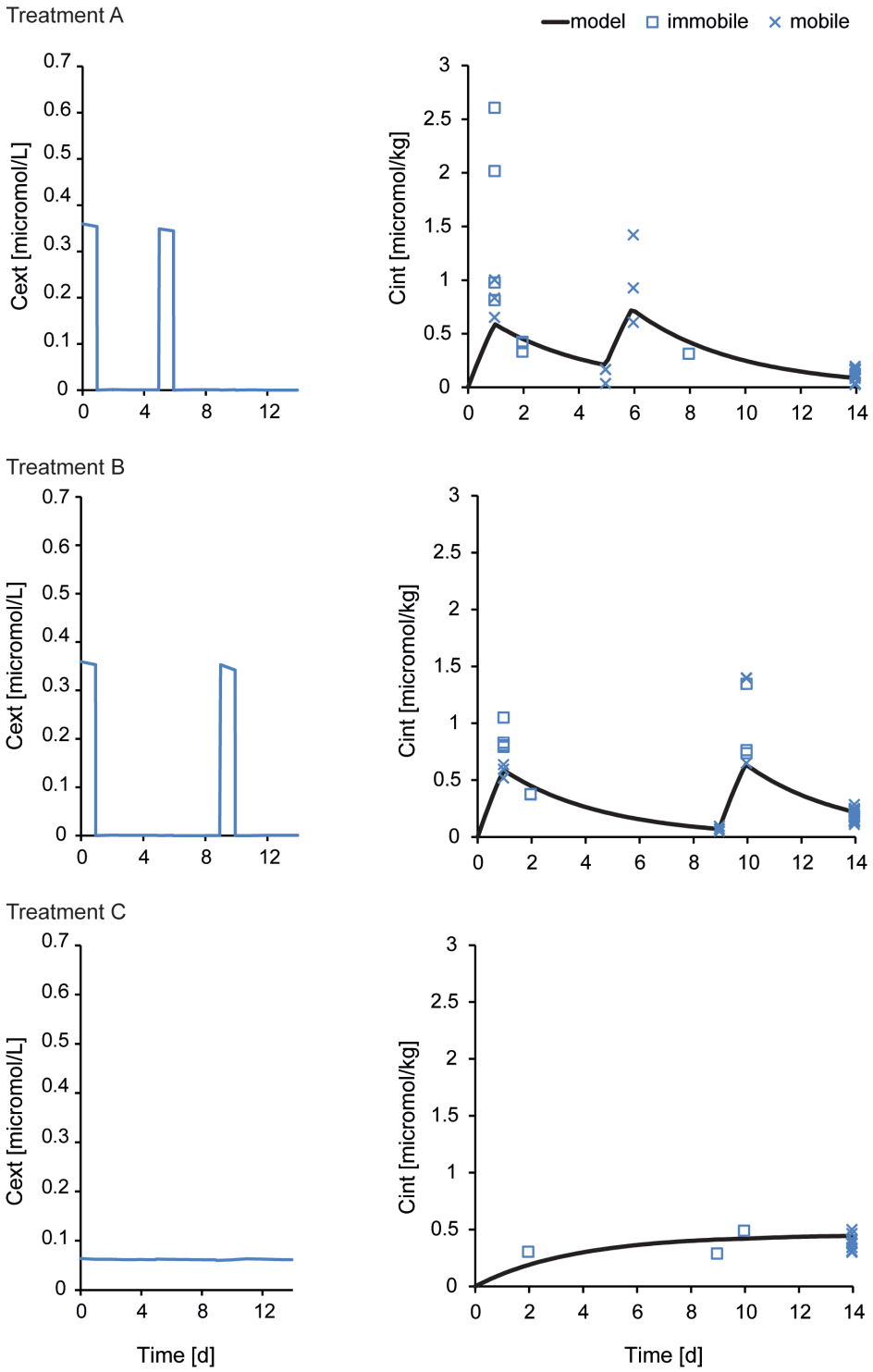


Figure 3. Toxicokinetic model validation. Internal concentrations were measured from immobile individuals in 14-day experiment (open squares) and from mobile individuals in additional beakers which were not used for observing mortality (crosses). These values are plotted with predictions of internal concentration (black line) by a previously published and calibrated toxicokinetic model [1].
doi:10.1371/journal.pone.0062472.g003

cumulative hazard of an individual [−], z is the threshold for effects [µmol/kg], h_b is the background hazard rate [1/d] (Eqn 4) and the 'max' function selects the maximum of either 0 or (D^*(t) − z). The background hazard rate h_b was obtained by fitting Eqn 4 to survival data of plain and solvent controls combined.

$$S_b = e^{-h_b * t} \quad (4)$$

where Sb is the background survival probability [−] describing survival in unexposed conditions.

Table 2. Mean percentage error (%) of individual tolerance (IT) and stochastic death (SD) model when pulsed (PT), constant (CT), or all data was used for calibration of the survival model for *Gammarus pulex* exposed to imidacloprid.

		Pulsed treatments (PT)				Constant treatments (CT)	
Model	**Calibration data**	**Tr A [1](14-day)**	**Tr B [2](14-day)**	**Tr A [1](21-day)**	**Tr B [2](21-day)**	**Tr C(14-day)**	**Tr C(21-day)**
IT	PT alone/CT alone	5.0	3.5	3.4	3.9	5.4	2.6
	All data	5.4	3.1	3.3	3.8	5.4	4.7
SD	PT alone/CT alone	8.3	7.2	23.6	13.4	5.7	4.0
	All data	7.3	8.3	22.7	13.4	5.1	5.0

[1]Pulsed treatment with a short interval in uncontaminated water between imidacloprid pulses.
[2]Pulsed treatment with a long interval in uncontaminated water between imidacloprid pulses.
doi:10.1371/journal.pone.0062472.t002

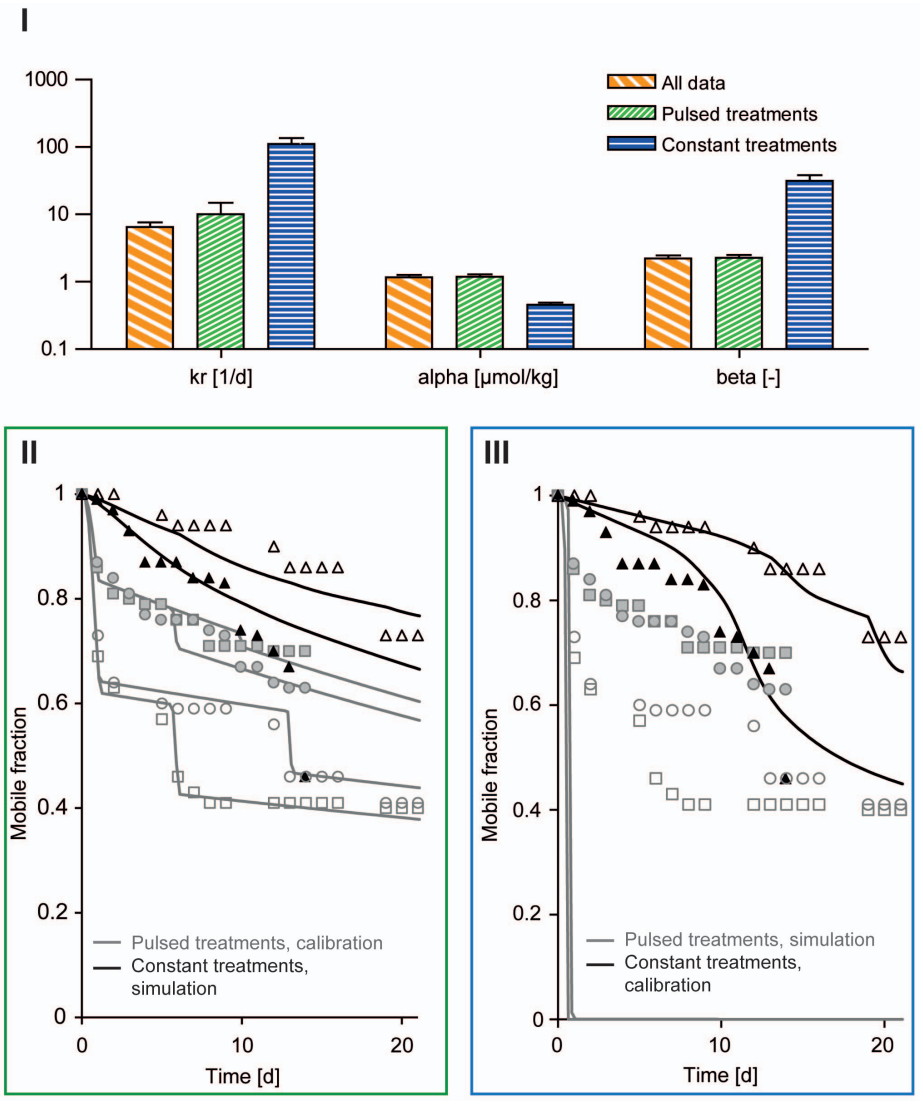
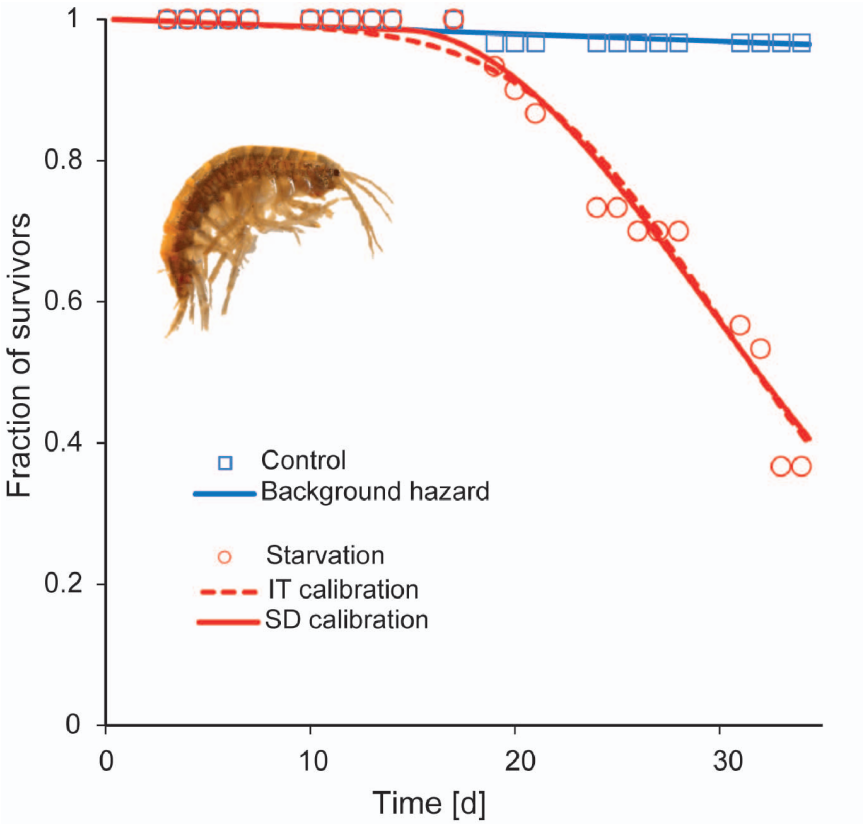


Figure 4. Parameter estimates and fraction of mobile animals simulated with the individual tolerance distribution model. Parameter estimates of individual tolerance models calibrated with all data, pulsed data only, and constant treatments only (I). Calibration to pulsed treatments (gray lines and gray symbols) and using these parameters to simulate the fraction of mobile animals in the constant scenario (black lines) are shown in the green box (II). Vice versa, calibration to constant treatments (black lines and black symbols) and using these parameters to simulate the fraction of mobile animals in the pulsed scenario (grey lines) are shown in the blue box (III). Symbols represent the data: black triangles are the mobile fraction in constant treatments (C), gray squares are data from pulsed treatments A and gray circles are from pulsed treatments B. Closed symbols are data from 14-day experiment and open symbols from 21-day experiment.
doi:10.1371/journal.pone.0062472.g004



	Parameters IT			Parameters SD		
k_r [1/d]	0.00015	±	0.01060	0.0008	±	0.0030
alpha [-]	0.00462	±	0.33559	-	±	-
beta [-]	5.33700	±	2.55914	-	±	-
k_k [1/d]	-	±	-	5.4062	±	18.262
z [-]	-	±	-	0.0116	±	0.0444

Figure 5. Calibration of the starvation model. The table shows the calibrated parameter values and their standard deviation.
doi:10.1371/journal.pone.0062472.g005

Once the cumulative hazard $H(t)$ is obtained, the survival probability, S (t) [−], was calculated using Eqn 5.

$$S(t) = e^{-H(t)} \tag{5}$$

The model that assumes the threshold for death to be drawn from an individual tolerance distribution (GUTS-IT model) is presented in Eqns 6 and 7. The IT model uses the same dose metric, scaled damage D^*, as the SD model (Eqn 2). Cumulative threshold distributions are based on a log-logistic cumulative distribution function (Eqn 6). The resulting survival probability is given by Eqn 7.

$$F(t) = \frac{1}{1 + \left(\max_{0<\tau<t} D^*(\tau) \Big/ \alpha\right)^{-\beta}} \tag{6}$$

$$S(t) = (1 - F(t)) \cdot e^{-h_b * t} \tag{7}$$

where F(t) is the log-logistic cumulative distribution function for the threshold [−], α is the median of the distribution [µmol/kg], β determines the width of the distribution [−] and the 'max' function selects the largest value of the dose metric D^* that occurred until time t.

To compare the goodness of fit among models and calibration data sets, the mean percentage error (MPE) was calculated (Eqn 8) [25]. The MPE was calculated for each treatment separately and therefore for each treatment the goodness of fit could be compared between a) stochastic death and individual tolerance models and b) models calibrated with different data sets (i.e. pulsed or constant exposures).

$$MPE = \frac{1}{n} \sum \frac{|S_{\text{obs}} - S_{\text{model}}|}{S_{\text{model}}} \cdot 100 \tag{8}$$

where MPE is the mean percentage error [%] of the fraction of

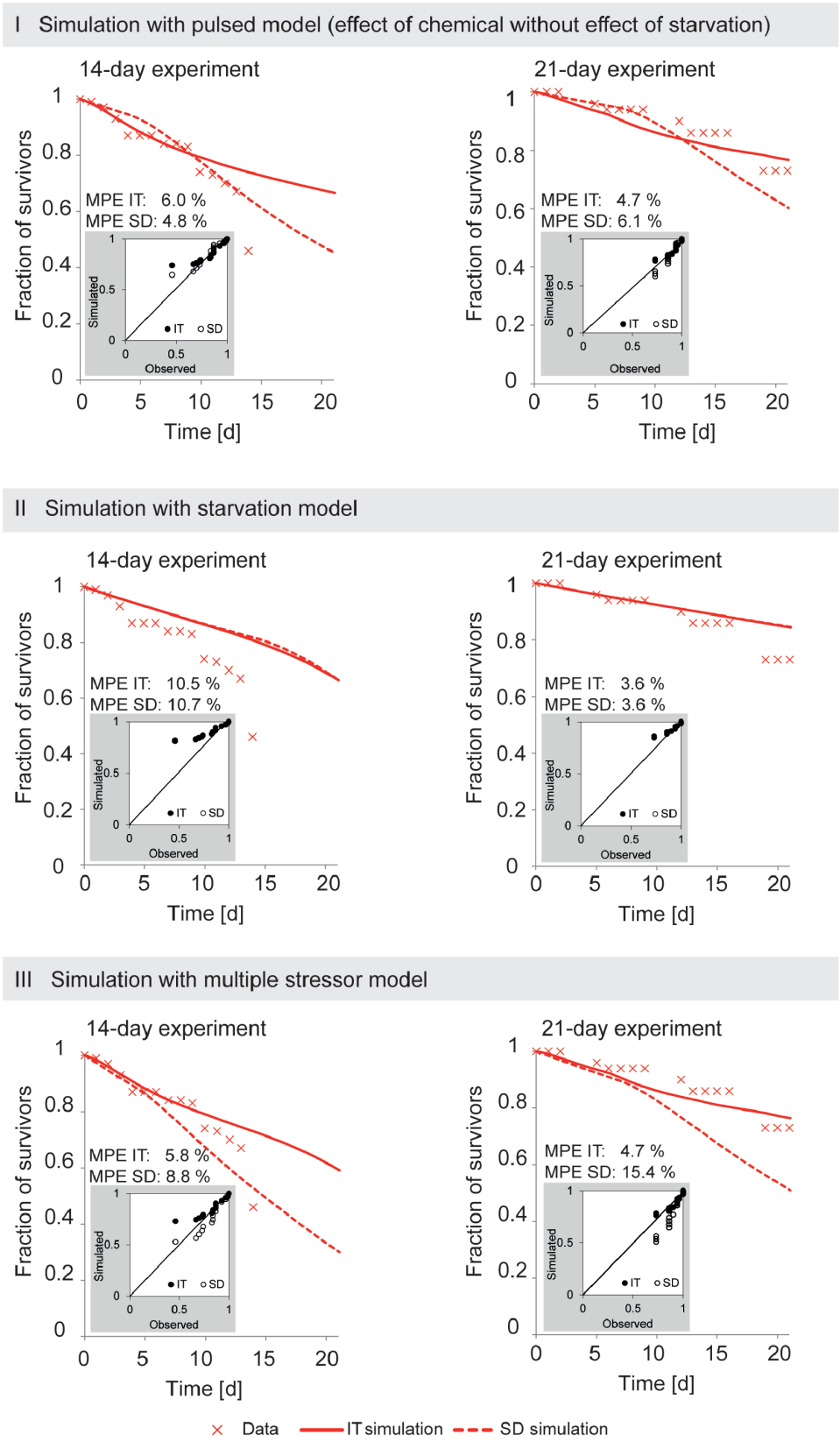


Figure 6. Simulation of survival of *Gammarus pulex* in constant imidacloprid exposure according to the chemical stress model (I), starvation model (II), and multiple stressor model (III). In the starvation model (II), lack of food (*LF*) for the 14-day experiment was set to 1.0 and for the 21-day experiment 0.5 due to differences in feeding activity (no feeding in 14-day experiment, ca. 50% reduced feeding in the 21-day experiment). The chemical stress model was GUTS calibrated with pulsed toxicity data sets.
doi:10.1371/journal.pone.0062472.g006

survivors, S_{obs} is the observed fraction of survivors [−], S_{model} is the model prediction of the fraction of survivors [−] and n is the number of data points used in the calculation.

Starvation Experiment and Modeling

As we hypothesise that organisms in the constant imidacloprid exposure die due to impaired movements leading to starvation, an experiment studying the effect of starvation, without exposure to imidacloprid, on survival of *G. pulex* was conducted. There were 30 replicates for both, the control and the starvation treatment. In the control group, one leaf disc was provided for food and more was given when the disc was eaten. In the starvation treatment, no food was given. *G. pulex* were placed individually in 100 mL beakers in order to prevent cannibalistic behavior of the test animals, which is more likely without leaf discs. Mortality was monitored for 34 days, i.e. long enough to observe mortality of at least half of the animals. Experimental water was APW (Table S1 in File S1) which was renewed weekly.

The mortality observed in this experiment was compared with mortality under the low, constant exposure to imidacloprid. For easier comparison, modified versions of the TKTD models described above (SD and IT) were calibrated using the starvation data and the survival in the constant imidacloprid treatments was predicted using this new starvation model. Instead of using chemical internal concentration causing the scaled damage D^* (t), a new concept, lack of food (*LF*), leading to the damage (Eqn 9) was introduced. In other words, the dose metric is *LF*, defined as the relative lack of food compared to control conditions ($LF = 1$ - (available food/food available in control)). Thus the survival model for starvation consists of equations identical to Eqns 3–7, Eqn 2 being replaced by Eqn 9 (*LF* replaces $C_{int}(t)$), which also leads to different dimensions of the model parameters. The *LF* was set to 1 when calibrating the model with starvation data as well as simulating the survival in the constant imidacloprid treatment in the 14-day experiment where hardly any food consumption was observed. When simulating the survival in 21-day imidacloprid experiment, the *LF* was set to 0.5 as food consumption in this experiment appeared to be only partially inhibited (the feeding activity was approximately half of control levels, see Figure 1).

$$\frac{dD^*(t)}{dt} = k_d \cdot (LF(t) - D^*(t)) \quad (9)$$

where D (t) describes the damage caused by lack of food [−], k_d is damage recovery rate [1/d] and LF (t) is lack of food [−]. Units of the following parameters in Eqns 3–7 were therefore different from described above: k_k [1/d], z [−] and α [−]. The background hazard rate was calibrated for each experiment separately.

Multiple Stressor Modeling

The starvation model was combined with the chemical stress model and the survival in constant imidacloprid exposure was simulated in order to test whether survival is determined by both, the effect of starvation and other toxic effects of imidacloprid. The chemical stress models were the GUTS models described above (Eqns 1–7) calibrated with the data from the pulsed toxicity treatments alone. In this model the effect of starvation is excluded from other chemical effects because we can assume that in the pulsed toxicity treatments starvation does not play a role due to possible recovery of the movements and feeding between chemical pulses. To implement the multiple stressor model, equations for both, chemical stress (SD: Eqns 1–4; IT: Eqns 1–2, 6) and starvation (SD: Eqns 9, 3–4; IT Eqns 9, 6) were followed. Then the cumulative hazards H (t) of both chemical and starvation stress were added (SD model, Eqn 10) or both cumulative distribution functions for the threshold F (t) were subtracted (IT model, Eqn 11) to predict survival in the constant imidacloprid treatments where both processes likely play role.

$$S(t) = e^{-(H\_chemical(t) + H\_starvation(t))} \quad (10)$$

$$S(t) = (1 - F_{chemical}(t) - F_{starvation}(t)) \cdot e^{-h_b * t} \quad (11)$$

Results and Discussion

Feeding Rate

Inhibition of feeding by imidacloprid has been observed in many invertebrate species [16–18,27–29]. We observed that feeding of *Gammarus pulex* was heavily inhibited by imidacloprid in the constant treatment of the 14-day experiment (Figure 1, Table 1, Table S9 in File S1) while in the pulsed treatments the effect was not as strong (Figure 2, Table 1, Table S9 in File S1). However, in this experiment, the method of measuring feeding activity was only semi-quantitative because it was based on the number of same-sized, however undefined by their mass, leaf discs consumed by *G. pulex* individuals. Thus, we focused on the 21-day experiment to draw conclusions with regards to the effects of imidacloprid on feeding activity. In the 21-day experiment, feeding activity was measured as the mass of leaf material eaten [mg]. There the effect on feeding in the constant treatment was again evident, however, not as strong as in the 14-day experiment (Figure 1, Table 1, Table S10 in File S1). In the pulsed treatments, no effects were observed on feeding activity - the organisms started to feed roughly 2 days after they were transferred to uncontaminated media (Figure 2, Table 1, Table S10 in File S1). This finding is in agreement with the fact that imidacloprid binding to ACh receptors in insect membranes is reversible, i.e. it can be dissociated as well as removed by ACh and other ligands [11,30,31]. However, in spite of the fairly fast dissociation of imidacloprid from the ACh receptors (0.419 min^{-1} [11]), the elimination of imidacloprid has been shown to be slow in *G. pulex* [12] and can be associated with the receptors again. The internal concentrations in our experiments decrease relatively fast (Figure 3) and 2 days after a pulse around 60% of imidacloprid is left inside the organisms. Once feeding activity is recovered in 2 days, it seems that the internal concentrations during these days fall below the threshold of preventing animals from feeding.

The ability to recover from imidacloprid pulses has been observed also by other authors [17,18,32]. Alexander and coworkers [17] observed that both mayfly (*Epeorus longimanus*) larvae and the oligochaete worm (*Lumbriculus variegatus*) could recover from 1-day exposure to imidacloprid in 4 days and the recovery potential was concentration dependent [17]. Concentration dependency was not observed in this study – in fact in the 21-day experiment where we had higher imidacloprid pulses, the feeding rate was not different from the controls while in the 14-day experiment we observed an effect. However, this observation could be caused by differences in the measurement method (number of leaf discs versus mg) and variation in organism fitness between the experiments, which can be seen for example in the control mortality (Figure 1).

Lipid Content

We observed reduced lipid content in *G. pulex* after exposing them constantly to imidacloprid for 21 days (21-day experiment, Figure 1). In the pulsed treatments, lipid content was not different from that of the controls (medians of A: 1.36%, B: 1.45%, Figure S1). When comparing among all treatments using the Kruskal-Wallis rank sum test, the *p*-value of 0.017 indicated significant differences and the pairwise comparisons showed that the constant treatment was responsible for the difference (Wilcoxon rank sum test between control and C: $p = 0.0053$). The differences in lipid content between pulsed treatments and controls were not significant (A: $p = 0.4646$; B: $p = 0.6834$). The decreased lipid content was observed in the same treatment where the feeding was inhibited (C). Therefore, we can conclude that lipid content is affected by a decreased feeding rate and might imply starvation of *G. pulex* in the presence of imidacloprid.

Mortality and Immobility due to Imidacloprid

We observed a sudden drop in survival in the end of the constant treatments (C), especially in the 14-day experiment (Figure 1, part III, Tables S12 and S13 in File S1). The percentage of dead out of all immobile individuals (dead+only immobile) was high in the constant treatments. In fact, we did not observe many individuals classified as immobile in the constant treatment (i.e. 14-day experiment: 3 out of 70, 21-day experiment: 1 out of 70). However, almost all the "mobile" animals were close to the limit of immobility, they were passive and could not move in a normal way. Similar behavior has been observed before in *Gammarus roeseli* exposed to 12 µg/L imidacloprid [15] which is close to the concentrations in our constant treatments. This effect of imidacloprid decreased the feeding rate and might have caused starvation in our experiments. In our treatments with high imidacloprid pulses, the organisms were mostly immobile (Figures 1 and 2), thus even in the highest concentration that we used (0.59 µmol/L), the acute lethal toxicity was not reached within one day. There were no differences in the mobile fraction at the end of both experiments between treatments with short (A) and long (B) recovery time between pulses, also not in feeding activity and lipid content in the 21-day experiment (Figure 2, Figure S1). This indicates that organisms recovered fast from imidacloprid exposure between the pulses, even in the treatments A which had short intervals between pulses. Calculated 95% organism recovery times were 12.7 and 12.3 days according to IT and SD models. Organism recovery times were calculated as the time when the modeled internal damage has dropped to 5% from the maximum in a pre-defined exposure scenario [25,33,34]. Note that organism recovery refers to the recovery of the underlying damage that causes effects on survival until it reaches levels far below those causing mortality. Thus, the observed fast recovery with regards to mortality after the pulses does not necessarily conflict with organism recovery times of 12 to 13 days.

We observed more mortality in the constant treatment of the 14-day experiment than in the constant treatment of the 21-day experiment, even though the exposure concentration was the same and the animals were exposed longer in the 21-day experiment. This variation in our results was also seen in the background mortality: during the 14-day experiment, mortality in the controls was much higher (Figure 1). This observation can be explained by organism fitness, which varies when we collect animals from the field for each experiment. For instance, season has been shown to influence the condition of gammarids [35–37]. One important seasonal factor is food availability. After leaf fall during autumn, organisms have shown to have better lipid reserves during winter (maximum for males in November and for females in January) while during summer lipid reserves are the lowest due to scarcity of food [35]. This can make summer populations more sensitive to toxicants [37], especially to imidacloprid which interferes with feeding behavior.

We conducted the experiments in November (14-day experiment) and February (21-day experiment) – both are months of the season where we expect high lipid contents according to Stroda and Cossu-Leguille [35]. We can hypothesize that in November, when we conducted the 14-day experiment resulting in high mortality in the constant treatment, the organisms had not had enough time to build up and store lipids after the leaf fall. Thus, in our 21-day experiment, organisms possibly had better lipid reserves, which might be why less individuals died than in the 14-day experiment. An alternative explanation could be pre-exposure of the animals to pollutants in the field before collection, which is more likely in November and the weeks before. Although the collection site is in a headwater stream with low probability for pollution, it cannot be ruled out that some exposure occurred, for example to pesticides applied in autumn and transported via runoff in autumn rains. Independent of the possible causes, the variance in background mortality among experiments was corrected in our survival models by using experiment specific background hazard rates (Eqn 4, 14-day experiment: 0.0144 [1/d]; 21-day experiment: 0.0079 [1/d]).

From our survival models, stochastic death (SD) and individual tolerance distribution (IT), the IT model fitted better to the data (Table 2). The mean percentage error (MPE) in the treatments was always below 5.4% in the IT models (mean 4.3±1% when all data was used for calibration) while in the SD models the maximum error was as high as 22.7%, with a mean of 10.3±6.8% when all data was used for calibration. This would imply that the data provided here supports the individual tolerance distribution hypothesis, which assumes that organisms have individual effect doses which are distributed within a population. A possible interpretation is the variability of lipids and other energy reserves within the population. Similar and opposing findings, i.e. studies where the SD hypothesis described the data better, have been published earlier [34,38–40]. The applicability of either extreme hypothesis seems to be chemical and species specific. Because our data supports the IT theory, we use the IT model results to compare survival among pulsed and constant treatments. The toxicokinetic sub-model calibrated by Ashauer and coworkers [12] predicted well the internal concentrations, which were measured in the 14-day experiment (i.e. model validation, see Figure 3).

Parameters differed when the models were calibrated with either pulsed or constant survival/immobility data. In Figure 4, parameter estimates of the damage recovery rate (k_r), the median of the threshold distribution (*alpha*), and the width of the distribution (*beta*) for pulsed treatments, constant treatments and all data are illustrated. The biggest differences between constant and pulsed calibration data can be seen in the values of the parameter *beta*, which was extremely high for the constant data compared to the pulsed data. The value of *beta* determines the width of the threshold distribution – the higher the value is, the narrower is the distribution and the steeper is the drop in the fraction of survival/mobility close to the median value *alpha*. This would imply that the individuals in a population react fairly similarly to starvation, or the damage describing decreasing lipid content.

However, parameter estimates should not be over-interpreted because they are linked to each other (parameter co-variation). To compare among calibration data sets in a more reliable manner, the whole set of parameters can be used to simulate the survival in another exposure type (i.e. from calibration to the constant data

set to simulate survival in the pulsed scenario and vice versa). When the pulsed calibration data was used, mobility in the constant scenario was rather well predicted with MPE of 5.5±9.5% (14-day experiment) and 4.4±2.4% (21-day experiment) (Figure 4, II). However, the high mortality in the end of constant treatments was not captured (i.e. the effect of starvation). When the constant calibration data was used, the mobility in the pulsed scenario could not be predicted well; the mobile fraction went directly to zero in all pulsed treatments (Figure 4, III).

This simulation result indicates that there are different processes, described by very different model parameters, governing the mobility under long, constant exposure to imidacloprid vs. the pulsed exposure scenario. The toxic processes in the constant treatments can be related to a low degree of imidacloprid binding in nicotinic receptors making organisms passive and causing death via impaired movements and starvation. In the pulsed treatments other toxic effects from higher degree of binding to the target sites play also a role. This can be seen also as a difference between the adverse effects of imidacloprid in pulsed treatments, where imidacloprid immobilizes the organisms immediately, and more chronic effects like starvation which appears as a result from impaired movements in long constant exposure. However, we cannot rule out the possibility that the differences in model parameters can also be caused by differing proportions of dead and immobile individuals among treatment types (the model treats them equally, because immobile individuals were removed). Almost only mortality was observed in the constant treatments while there were much more immobile individuals than dead ones in the pulsed treatments (pie charts in Figures 1 and 2, part III).

Mortality in the Starvation Experiment

In one of the target organisms, the tobacco whitefly (*Bemisia tabaci*), imidacloprid has been shown to cause starvation [41,42]. Even though the exposure route is partly different for aquatic species (oral uptake alone for the tobacco whitefly versus oral uptake and diffusion from water for *G. pulex*), low concentrations of imidacloprid can cause death of non-target amphipods in aquatic environments via behavioral changes which prevent the organisms from feeding. To investigate whether other effects of imidacloprid than starvation play a role in the constant exposures, we performed a pure starvation experiment where no imidacloprid was added. We calibrated the TKTD models with food limitation as dose metric and the models were then used to simulate mortality in the constant imidacloprid treatments. Results of the starvation experiment and calibration of the models are shown in Figure 5. The simulation of survival in the constant lack of food conditions, representing our constant imidacloprid exposure, showed rather poor agreement with the measured values, especially for the 14-day experiment (Figure 6, part II). However it must be noted, that this prediction is based only on starvation stress. As cholinergic neurotransmission has been suspected to have a central role in neurotransmission in invertebrate central nervous system [43], likely also other pathways of toxicity, e.g. failure of respiration, than starvation through impaired movements occur in imidacloprid exposure.

Survival in Multiple Stress Conditions

We developed a multiple stress model, combining the effects of starvation and the other toxic pathways of the chemical, for simulating survival in the constant imidacloprid exposure. The SD model for multiple stressors did not predict survival well when we compare mean percentage error (MPE) values between three different model types: The 14-day experiment was better predicted by the chemical stress model and the 21-day experiment by the starvation model (Figure 6). However, as discussed above, the IT model had a better fit to the data (Figure 2, Table 2) and therefore we should focus on comparing results given by this model (see solid line in Figure 6). The agreement of the simulation with the data increased with the multiple stress model, when compared to simulations using either of the individual stressor models (i.e. chemical stress and starvation) alone especially in the case of the 14-day experiment (Figure 6). Using the multiple stress model did improve the predictive power, however, one pattern in the data, i.e. the sudden drop in survival at the end of the experiments, was not predicted quantitatively.

Conclusions

Organisms in nature are facing multiple stress conditions, natural and anthropogenic. We have investigated the effects of the insecticide imidacloprid in *Gammarus pulex* and showed that multiple stress pathways might influence organism survival, rather than just a single mechanism of toxicity or stress pathway alone. By binding to acetylcholine receptors, imidacloprid impairs invertebrate movements and feeding behavior and therefore, in addition to other pathways of toxicity, starvation can influence organism survival if the exposure lasts longer than the duration of standard toxicity tests. Another noteworthy point of our findings is that the effect of reduced feeding on invertebrate survival is affected by food availability and initial nutritional status of organisms which vary along with the season. Nevertheless, in aquatic systems the exposure times are usually short due to water flow and dilution. We showed that in these conditions, organism movements and feeding can recover fast after the exposure and therefore the stress from starvation is reduced, however, other toxic pathways can still affect organism survival.

Supporting Information

Figure S1 Lipid content [%] of *Gammarus pulex* at the end of the second experiment in control and treatments A, B and C. Green color of the box denotes pulsed treatments (Tr A and B) and red color constant treatment (Tr C). The numbers are the median values represented by the black line in boxes.
(TIF)

File S1 Supporting Tables. Table S1. Composition of artificial pond water (APW) and stock solutions. **Table S2.** Imidacloprid concentrations in water in 14-day experiment. **Table S3.** Imidacloprid concentrations in water in 21-day experiment. **Table S4.** Water characteristics in 14-day experiment. **Table S5.** Water characteristics in 21-day experiment. **Table S6.** Data on internal concentrations in 14-day experiment: Tr A. **Table S7.** Data on internal concentrations in 14-day experiment: Tr B. **Table S8.** Data on internal concentrations in 14-day experiment: Tr C. **Table S9.** Cumulative food consumption (leaf discs/*G. pulex* individual) in 14-day experiment. Value is corrected with number of mobile individuals in the beaker. **Table S10.** Cumulative food consumption (mg/*G. pulex* individual) in 21-day experiment. Nm denotes "not measured" in that time point. This is taken into account in the value in the next time point. **Table S11.** Lipid content of immobile *Gammarus pulex* individuals in the 21-day experiment and mobile individuals in the end of experiment. **Table S12.** Number of mobile and immobile individuals in the 14-day experiment. Cint A and Cint B denotes beakers which were used to sample mobile individuals and were not used for survival modeling. **Table S13.** Number of mobile and immobile individuals in the 21-day experiment. **Table S14.** Parameter estimates for different calibration data sets. Parameter estimates of stochastic death (SD) and individual tolerance

distribution (IT) models based on fit using log-likelihood function or ordinary least squares fit.
(DOCX)

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Author Contributions

Conceived and designed the experiments: AMN AH KS RA. Performed the experiments: AMN AH. Analyzed the data: AMN AH RA. Contributed reagents/materials/analysis tools: AMN AH KS RA. Wrote the paper: AMN AH KS RA.

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Review

The biology of insecticidal activity and resistance

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ABSTRACT

Identifying insecticide resistance mechanisms is paramount for pest insect control, as the understandings that underpin insect control strategies must provide ways of detecting and managing resistance. Insecticide resistance studies rely heavily on detailed biochemical and genetic analyses. Although there have been many successes, there are also many examples of resistance that still challenge us. As a precursor to rational pest insect control, the biology of the insect, within the contexts of insecticide modes of action and insecticide metabolism, must be well understood. It makes sense to initiate this research in the best model insect system, *Drosophila melanogaster*, and translate these findings and methodologies to other insects. Here we explore the usefulness of the *D. melanogaster* model in studying metabolic-based insecticide resistances, target-site mediated resistances and identifying novel insecticide targets, whilst highlighting the importance of having a more complete understanding of insect biology for insecticide studies.

1. Introduction

Insecticides continue to be key weapons for the control of insect pests that threaten agriculture and vector disease. However, despite intensive ongoing research, there are a limited number of commercially available insecticide chemistries that target an even smaller number of insect proteins. Whilst preserving the efficacy of these insecticides and the integrity of targets is vital, resistance evolving due to strong selection imposed by widespread insecticide usage compromises insect pest control.

Insecticide resistance is a genetic phenomenon, with mutations affecting insecticide target proteins and metabolism being the most commonly described (ffrench-Constant et al., 2004; Li et al., 2007). Studying the molecular bases of target-site mediated insecticide resistances provides useful information on how specific insecticides exert their lethal effects, information often transferable between insect species. Studying metabolic-based insecticide resistance offers valuable insights into how insecticides are inactivated before reaching their molecular target within the insect. Thus describing the molecular basis of insecticide resistance is important. It opens windows of understanding that may improve future pest control. However, these windows are not large enough or sufficient in number to provide a comprehensive understanding of the complex biology relevant for sustainable rational insect pest control. For example, the targets for some insecticides have not even been identified. Where targets have been identified, in some cases their native functions have only been rudimentarily characterised. Similarly, although the molecular bases of many metabolic resistances have been studied, little is known about where insecticides are metabolised or the specific metabolic pathways involved.

In studying other complex biological processes geneticists have used model organisms to systematically mutagenise and manipulate most genes involved in a given process. By way of contrast, in considering insecticide targets and metabolism there is a preponderance of research with field-derived resistant variants that define a small proportion of the genes involved. Other reviewers have noted how the powerful insect genetic model *Drosophila melanogaster* can be used to investigate insecticide resistance and underscore the relevance of research in this model to pest systems (ffrench-Constant et al., 1992, 2004; Morton, 1993; Schneider, 2000; Wilson, 1988, 2001). Although *D. melanogaster* is generally not considered an insect pest, it is exposed to insecticides and resistances have arisen (Daborn et al., 2002; ffrench-Constant et al., 1993; Wilson and Cain, 1997). The ease of lab culture, the availability of an ever accumulating array of genetic resources assembled through over a century of research by a large global community of researchers (Drysdale, 2008), a complete genome sequence (Adams et al., 2000) and the capacity to precisely manipulate the genome (Rubin and Spradling, 1982; Spradling and Rubin, 1982) represent key advantages of using *D. melanogaster* as a model. This paper does not set out to review the resistance

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literature. It describes ways in which technologies available in *D. melanogaster* permit the systematic analysis of the biology of insecticide targets and metabolism, providing opportunities for rational insect pest control. Such control will require a detailed understanding of how insecticides are metabolised and new generation insecticides directed against precisely defined targets. Nicotinic acetylcholine receptors are used as a primary example. These receptors are the targets of current generation insecticides (neonicotinoids and spinosyns), but given their complexity can be exploited for future rational insecticide design.

2. *D. melanogaster* as a model for investigating insecticide targets and target site resistance

2.1. Insecticides targeting the nervous system

2.1.1. Conservation is key

D. melanogaster has proven suitable for identifying several insecticide targets. Where insecticide targets are known in both *D. melanogaster* and one or more pests, there are striking similarities in the target and also the nature of the mutations found to confer resistance (Fig. 1). Given the large evolutionary distance between *D. melanogaster* and many insect pests, it is clear that the targets for many widely used insecticides are conserved. This conservation is a double-edged sword. Insecticides directed against highly conserved targets kill both pest and beneficial insects producing collateral damage to the environment. While the conservation of targets is a problem in the field, it is a benefit in the laboratory. By identifying molecular bases of resistances, *D. melanogaster* can be used to identify the targets of insecticides in those cases where they are not known.

A GABA gated chloride channel allele, *Resistance to dieldrin* (*Rdl*), was one of the first target site resistances characterised to a molecular resolution. It exemplifies a direct translation of understandings gained from the model to a range of pest insects in the field. The discovery of the candidate receptor was made in field-isolated *D. melanogaster* resistant to the cyclodiene insecticide, dieldrin (ffrench-Constant et al., 1991). Further work using electrophysiology on cell expression systems demonstrated the mechanism of resistance to be an amino acid substitution (A301S) which resides within the M2 (pore forming) domain of the GABA receptor causing both a change in dieldrin binding affinity and destabilisation of the cyclodiene favoured conformation through alteration of channel desensitisation rates (ffrench-Constant et al., 1993; Zhang et al., 1994). The A301S (or an alternative, A301G) substitution has subsequently been found in *Rdl* orthologs of resistant strains of many pest species (ffrench-Constant et al., 2004; Thompson et al., 1993) (Fig. 1A). The highly conserved nature of the mutation allowed PCR assays to be developed for rapid identification of *Rdl* alleles in field populations (ffrench-Constant, 1994). Several techniques uniquely available in *D. melanogaster* at the time, including cytologically defined chromosome deficiency libraries and *Drosophila* germ-line transformation techniques, played critical roles in the discovery of the molecular lesion in *Rdl* (ffrench-Constant et al., 1991).

2.1.2. Neural targets and redundancy

The *Rdl* example highlights the exquisite precision of adaptation to strong insecticide selective pressure. Given that loss of *Rdl*

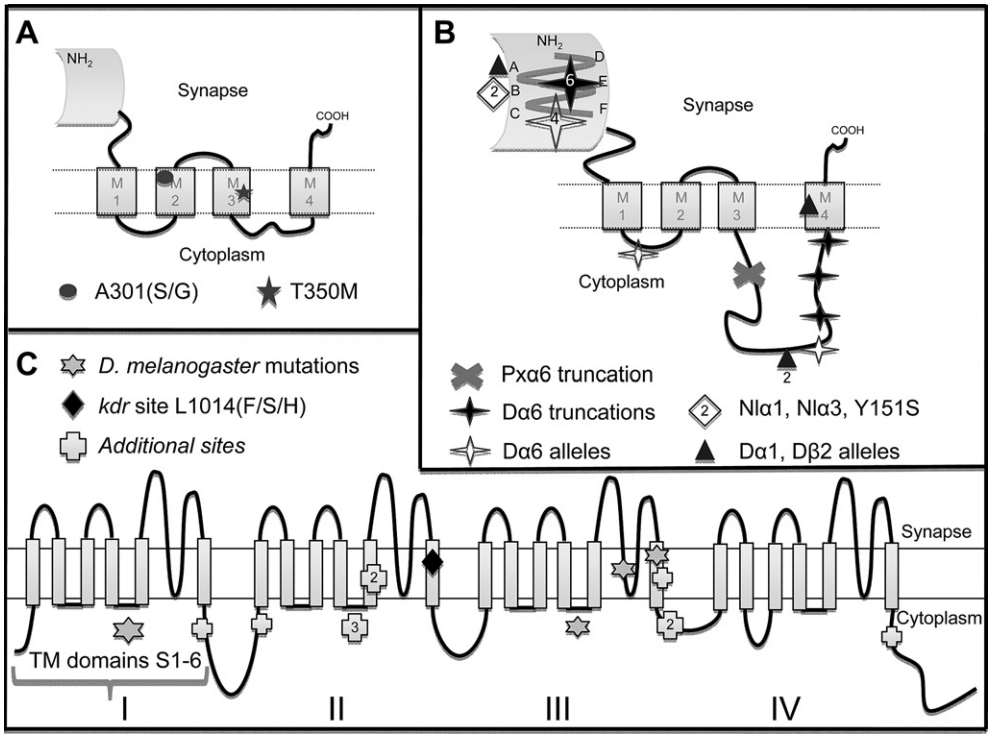

Fig. 1. Relative locations of characterised resistance mutations on insect ligand-gated and voltage-gated ion channels. A: The RDL protein has an A301S or A301G substitution in transmembrane domain 2 which lines the ion channel pore in the assembled pentameric receptor. An additional T350M mutation contributes to resistance to a phenylpyrazole insecticide, fipronil (Le Goff et al., 2005). B: nAChR subunit mutations that confer neonicotinoid and spinosyn resistance are dominated by allosteric or loss of function alleles (Baxter et al., 2010; Liu et al., 2005; Perry et al., 2008, 2007; Watson et al., 2010). The two alleles within the ligand-binding pocket in *N. lugens* show decreased neonicotinoid binding (Liu et al., 2006). C: Sites of amino acid substitutions on the Para-like voltage-gated ion channel. The α-subunit sodium channel depicted contains 4 domains (I–IV) consisting of 6 transmembrane motifs (S1–6), all linked in the one protein. The *kdr* allele confers resistance to DDT and pyrethroids. Additional mutations can enhance resistance to particular pyrethroid compounds. Interestingly the mutations of *D. melanogaster* have been detected in orthologous regions but in different domains to those found in pests (Soderlund and Knipple, 2003).

function is lethal, dieldrin resistance could only be afforded by an amino acid replacement hindering dieldrin binding while retaining receptor function. A301S and A301G are the only amino acid substitutions within RDL that could fulfil this demanding evolutionary brief (ffrench-Constant et al., 2000). While there is evidence of a temperature-dependent paralytic phenotype associated with the *Rdl* allele in *D. melanogaster*, this does not seem to translate into a significant fitness affect impacting allele frequencies (ffrench-Constant, 1994). By way of contrast, there are significant fitness costs associated with the A301S replacement in the Australian sheep blowfly, *Lucilia cuprina* (Mckenzie, 1990). Differences in the fitness impact of A301S in *D. melanogaster* and *L. cuprina* are likely to be due to a difference in RDL between the two species. Another layer of complexity is the reported duplication of *Rdl* in Lepidoptera, of which *Bombyx mori*, *Heliothis virescens* and *Helicoverpa armigera* appear to have three paralogs, while *Plutella xylostella* and *Myzus persicae* have at least two paralogs (Anthony et al., 1998; Yuan et al., 2010). In the case of *M. persicae*, although the A301S allele is fixed in one paralog, the resistance phenotype is associated with the presence of an A301G substitution, suggesting that these genes have been co-opted into distinct functions in the insects (Anthony et al., 1998).

Nicotinic acetylcholine receptors (nAChRs) are a class of the ligand-gated ion channel superfamily. Ten nAChR subunit genes have been described in *D. melanogaster*, seven α-like and three β-like (Sattelle et al., 2005). The discriminating feature between α and β is the presence of a YXCC motif in the α subunit that contributes to the acetylcholine binding. The functional nAChR pentamer can consist of either α-subunits or a combination of α and β subunits. The N-terminal ligand binding region contains six loops (A–F) which form the acetylcholine binding pocket between adjacent α/α and α/β subunits. nAChRs have been targeted by at least two insecticide classes, spinosyns and neonicotinoids. Spinosyns, a highly valued class of biopesticide, are macrocyclic lactone fermentation products of *Saccharopolyspora spinosa* that are widely used in the control of Lepidoptera, Coleoptera and Diptera (Thompson et al., 2000). Evidence from resistant insects suggests binding to different nAChR subunits for spinosyns when compared to neonicotinoids (Perry et al., 2008; Perry et al., 2007; Salgado and Saar, 2004; Watson et al., 2010). Dα6 was shown to be the target of spinosyns in *D. melanogaster* on the basis of high level resistance being observed in a gene disruption mutant (Perry et al., 2007) and in EMS induced mutants (Watson et al., 2010). It has also been demonstrated that disruptions in the *P. xylostella* (diamondback moth) *Pxα6* gene confer spinosad resistance, the mechanism being a truncated product (Baxter et al., 2010) (Fig. 1B).

The widely used neonicotinoid class of insecticide exhibits a high level of specificity for invertebrate pest nAChRs over vertebrate nAChRs (Tomizawa et al., 2000), meaning that neonicotinoids can be safely used as oral protective agents for domestic pets as well as for the field control of agricultural pests. Imidacloprid alone captured over $1bn of the market in 2008 (2010 Bayer Annual Report, www.annualreport2010.Bayer.com). In contrast to spinosyns, reported neonicotinoid resistance alleles implicate multiple nAChR subunits. In a resistant strain of *Nilaparvata lugens* (brown planthopper) a Y151S amino acid substitution was discovered in two different nicotinic acetylcholine receptor α-subunits, *Nlα1* and *Nlα3* (Liu et al., 2005) (Fig. 1B). This amino acid substitution reduces imidacloprid binding in heterologous expression experiments (Liu et al., 2005). Findings in *D. melanogaster* also demonstrate that two nAChR genes confer resistance when mutated. Only one of these mutations is in a gene orthologous to *Nlα1* (*dα1*). The other is a β-subunit, *dβ2* (Fig. 1B). Further, the mutations in *D. melanogaster* result in a drastic, if not complete, ablation of function compared with the specific substitutions found in *N. lugens* (Perry et al., 2008).

Resistance to both spinosyns (*dα6*) and neonicotinoids (*dα1*, *dβ2*) is associated with loss of subunit function. This has implications when considering the potential for resistance evolution in the field. As there are many more mutations that can lead to a loss of target gene function compared to those that specifically reduce the binding of the insecticide to the target protein, it is predicted that resistance to spinosyn and neonicotinoid insecticides will arise reasonably frequently in field populations. The only mitigating factor is that these resistances are largely recessive which, in a field setting, would slow their increase in frequency. Recessive resistances to spinosyn and neonicotinoids due to target insensitivity have been observed in several pest insect species (Bielza et al., 2007; Shono and Scott, 2003; Wang et al., 2009; Wyss et al., 2003). To date the only characterised cases are the truncated *Pxα6* conferring resistance to spinosyn in *P. xylostella* (Baxter et al., 2010) and the neonicotinoid resistant, ligand binding region substitutions in *N. lugens* (Liu et al., 2005).

That loss of function mutations in three different *D. melanogaster* genes leads to viable resistant strains highlights a surprising level of functional redundancy among the nAChR subunits. This finding is instructive in considering insecticide design. Based on the effectiveness of spinosyns and neonicotinoids, the nAChRs can be excellent targets. However, in terms of achieving sustainable control, the insecticide should target a non-redundant subunit or a non-redundant set of subunits. In order to identify these subunits the redundancy rules need to be investigated and understood. Such investigation would contribute much to the understanding of functional options that exist in the insect nervous system and might allow further exploitation of these useful targets.

2.1.3. Expression studies

Addressing a number of important questions requires functional expression of target proteins in cell culture systems. These include determining the specific subunits and the amino acids within these subunits that bind insecticides, as well as identifying other proteins that are associated with the target protein. Functional expression of insect nAChRs has been a major hurdle. In combination with vertebrate β subunits some insect nAChR subunits are capable of forming heterogeneous receptors that respond to ligands, including insecticides. Until recently it has not been possible to form native *D. melanogaster* channels in any of the expression systems tested (Watson et al., 2010). This means it has been difficult to characterise relevant subunit combinations for insect nAChRs and assess their specific binding affinities and pharmacological properties. In the absence of a functional expression system other approaches have been employed. Hence, analyses of insecticide binding focus on subunit expression in combination with chick or rat β2 subunits, co-immunoprecipitation, or whole membrane binding studies (reviewed in (Millar and Lansdell, 2010). This highlights how limiting it can be to tackle a difficult question such as mode of action without the necessary biological knowledge. In the pursuit of understanding neonicotinoid targets in *N. lugens*, native subunit combinations have been identified for cloned subunits using co-immunoprecipitation (Li et al., 2010). This research suggests that neonicotinoid sensitive nAChR may involve similar subunit combinations in *N. lugens* and *D. melanogaster* although the complete picture is far from clear (Li et al., 2010). The work is restricted by the lack of genome sequence data from this non-model organism, meaning that the entire complement of nAChR subunits is still unknown.

Recently, hurdles preventing the functional expression of insect nAChRs may have been overcome, with the expression of a receptor combining Dα6 and Dα5 using the *C. elegans* chaperone protein RIC3 (Watson et al., 2010). This receptor was capable of binding to spinosynA. It remains to be seen whether RIC3 or other accessory

proteins will be required to facilitate the expression of other insect nAChRs.

2.2. Utilising Drosophila to investigate targets

2.2.1. Generating target site resistance

Mutagenesis can be used to increase the variety of insecticide resistant mutants in genes encoding targets. These mutations can be valuable for structure-function analysis. For example it has been possible to identify insecticide targets using mutants recovered following mutagenesis and selection with neonicotinoids, spinosyns and the insect growth regulator methoprene (Perry et al., 2008; Shemshedini and Wilson, 1990; Watson et al., 2010). Mutagenesis comes with a number of advantages. It is possible to generate mutants that would allow the nature of target site resistance to be determined before resistance arises in the field. Beyond this, it is possible to mutagenise to saturation, isolating a wider spectrum of resistant mutations as a resource for the study of the target and its association with the insecticide. In those cases where more than one target is involved (e.g. neonicotinoids), it provides the opportunity to pursue targets individually. While mutagenesis has been successfully pursued in pests and often correlates well with field resistance cases (Smyth et al., 1992), issues regarding creation of resistant variants in the laboratory means this is not generally practical. A second advantage of mutagenesis in the laboratory is the control over genetic background. In comparing any two field-derived strains there can be varying background susceptibilities, unknown histories of insecticide exposure and multiple resistance factors present. Mutagenesis can be performed in a standard genetic background and generates a limited number of mutations making it easier to associate resistance with a particular mutation found in the resistant mutant and not in the strain from which it was derived. The contribution of individual genes to the resistance phenotype can then be measured with precision. Beyond resistance considerations, mutants can be used for fitness assessments. Ascertaining whether the target is essential to insect survival is important for understanding whether such resistant alleles could increase in frequency in pest populations.

Given that many newer insecticides target the ligand gated ion channel receptors, the availability of mutants for the receptor subunit genes facilitates screens for resistance before these compounds are deployed in the field. A sulfoximine insecticide, Sulfoxaflor, which targets nAChRs with a distinct mode of action from neonicotinoids (Babcock et al., 2010) has been tested on these mutants and no cross-resistance was observed (Perry and Sparks, personal communication). Thus resistant mutants can provide a powerful tool with useful applications at both the discovery and the development stages of novel compounds in determining overlapping modes of action. Another practical application is in the assessment of second-generation compounds derived from insecticides that have already been used in the field, as cross-resistance issues are more likely to arise. Neonicotinoid resistant mutants isolated on the basis of resistance to nitenpyram were shown to be cross-resistant to other neonicotinoids such as imidacloprid and thiamethoxam (Perry et al., 2008). Screening for cross-resistance provides valuable information about mode of action, resistance, as well as a guide to rotation strategies for integrated pest management.

2.2.2. The use of mapping and sequencing technologies to identify the resistance gene

Resistant mutants can be readily mapped to a genomic interval of less than 5Mb using an appropriate selection of visible phenotypic markers (Bogwitz et al., 2005; Chen et al., 2006; Daborn et al., 2001). The location of the gene can be more closely defined using *P*-element mapping strategies (Zhai et al., 2003). Having genetically mapped the resistant gene, the use of next-generation sequencing is ideal to overcome the hurdle of identifying the molecular lesion. This is likely to become the method of choice with reasonable coverage of *Drosophila* genomes (~24×) now available for less than $3000(USD). Already this technology has been combined with EMS mutagenesis and verified with complementation assays to identify a gene involved in egg-shell morphology (Blumenstiel et al., 2009). There have also been successful examples with this technique from another model organism, *C. elegans* (Shen et al., 2008).

2.2.3. The use of loss of function analysis to identify targets

The availability of a large collection of deficiency strains, each of which is stably heterozygous for large genomic deletions, has played a role in identifying the targets for three different insecticide classes in *D. melanogaster*. In the case of dieldrin resistance, two overlapping deficiencies were used to narrow down the region encompassing *Rdl* (ffrench-Constant et al., 1991). For neonicotinoid resistance, nAChR deficiencies were used to screen for candidate subunits on which EMS mutagenesis and selection was then performed allowing the isolation of recessive alleles in the *dα1* and *dβ2* genes (Perry et al., 2008). Spinosyn resistance was a more straightforward case where screening nAChR subunit deficiencies led to the discovery of a highly resistant strain (Perry et al., 2007). This deficiency has since been used to perform mutagenesis and isolate further *dα6* alleles (Watson et al., 2010).

RNAi lines are a convenient large-scale genetic screening tool, with a line available for the silencing of almost each *Drosophila* gene. Different enhancer driver lines can be used in conjunction with the GAL4/UAS system to produce a partial loss of function due to reduced mRNA level for any given gene in a particular tissue/life stage or ubiquitously throughout development (refer to Fig. 2). High-throughput resistance screens for potential targets can be conducted. If a candidate target has been identified by other means then this resource can be used to provide supporting evidence. This approach has been successfully tested for both nitenpyram and spinosad (Mitchell, Ali, Batterham and Perry, unpublished).

2.2.4. Analysing the target gene

Having identified a target gene it becomes important to understand the biological function of the target, how that function is

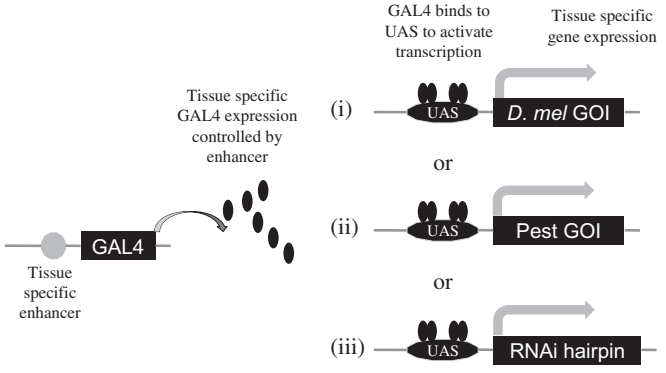

Fig. 2. GAL4-activated gene expression in *D. melanogaster* can be used to investigate both target site and metabolic insecticide resistance. A transgenic fly strain is made using a construct containing the yeast GAL4 gene downstream of a *Drosophila* tissue specific enhancer sequence. In this fly strain, GAL4 will be expressed in a tissue and temporal specific manner, governed by the enhancer sequence. Many different GAL4 strains are available, expressing GAL4 in different tissues, for example the midgut, the Malpighian tubules, or the nervous system. A second transgenic fly strain is made, containing a GAL4 specific upstream activation sequence (UAS) upstream of either (i) a *Drosophila* gene of interest (GOI), (ii) a pest species GOI, or (iii) an RNAi hairpin sequence for gene silencing. When the two strains are crossed the GAL4 and the UAS will both be present. GAL4 protein binds to the UAS and activates transcription of the GOI in the specific tissue(s) where GAL4 is present.

perturbed by insecticide binding, the range of target site mutations that might afford resistance and other impacts of resistance on the organism, including fitness. Contemporary genetic methods can be used to generate the allelic variation useful in addressing each of these questions. Beyond the resources that may be available in *Drosophila* stock centres, a series of random point mutations can be produced in any given gene using the tilling approach (Cooper et al., 2008). There are ongoing efforts to isolate insertion mutants for every gene in the *D. melanogaster* genome with these mutants being made publically available. A collection of strains each deleted for a small number of genes provides excellent coverage of the genome (Ryder et al., 2007). Strategies using the mobilization of transposable elements such as *P* and *Minos*, and the FLP/FRT recombination system provide the capacity to isolate deletions of single genes of interest (Golic and Golic, 1996; Metaxakis et al., 2005; Voelker et al., 1984). The sophistication and precision with which genes can be manipulated has increased with the innovation of targeted gene deletion and replacement utilising homologous recombination and the attB/φC31 integrase system (Bischof et al., 2007; Gong and Golic, 2003; Groth et al., 2004; Huang et al., 2008; Rong and Golic, 2000; Venken et al., 2006). Specific changes engineered into a copy of a gene can be inserted into the genome to replace the deleted genomic copy (Venken et al., 2009). The engineered copy of the gene is then expressed in the fly in the normal pattern for that gene, allowing the impact of the introduced mutations to be assessed. That these manipulations are performed in a controlled genetic background means that different introduced alleles can be examines for their impact on resistance, electrophysiology or fitness. Beyond creating mutant alleles it is possible to put tags into genes (e.g. GFP) that will allow the expression of target proteins to be followed throughout development. While these approaches were first developed for small genes, similar manipulations can be performed for genomic regions of up to 150 kb in length (Venken et al., 2009), particularly useful in the analysis of some of the large genes that encode receptors in the nervous system.

2.3. Investigating pest target proteins in Drosophila

D. melanogaster insecticide targets can be readily identified and analysed but it is essential that the findings are extended to the corresponding targets from pests. Many of the requisite technologies are not available in pest systems, or are less finely tuned, making it easier to express and manipulate pest genes in *D. melanogaster*. The GAL4/UAS expression system (Brand and Perrimon, 1993) can be fine-tuned to allow appropriate tissue specific expression of introduced pest genes (Fig. 2). The first step along this path is to show that the pest gene is functional in *D. melanogaster*. As a proof of principle we have investigated *α6 nAChR subunit orthologues*. *dα6* loss of function mutants are resistant to spinosad (Perry et al., 2007). When *α6* orthologues from *L. cuprina* and *Musca domestica* were individually expressed in a *D. melanogaster dα6* loss of function mutant, sensitivity to spinosyn was rescued (Perry and Batterham, unpubl). These experiments show that the pest receptor subunits from these two species function in *D. melanogaster*. In addition, the susceptibility of the flies to spinosyn indicates that the α6 receptor subunit of these two species is a potential spinosyn target. The degree of evolutionary sequence conservation required for *D. melanogaster* to be useful in studying pest targets is likely to require case-by-case evaluation. When it comes to the neural targets, other factors such as interacting proteins may be important. The increasing availability of pest insect genome sequences will allow many candidate target genes to be tested. In those cases where pest genes are successful expressed, the *D. melanogaster* system offers a wide range of options for functional dissection.

2.4. Capacity for insecticide design against new and existing targets

2.4.1. Enabling the insecticide design process

If the insecticides of the future are to be more effective, lower in environmental impact and less easily overcome by evolved resistances, then they need to be designed based on a detailed understanding of their targets. Increased knowledge of the MOA of compounds has certainly led to more strategic approaches. Neonicotinoids such as imidacloprid arose from structure activity studies on 3-Pyridylmethylamine (Tomizawa and Casida, 2003) and nithiazine, a compound previously found to be a highly potent insecticide, but unfortunately not stable enough for crop use due to photo-instability (Soloway et al., 1979). Other neonicotinoids were developed again through comparison of different side-chain groups and their insecticidal activity. However, none of this development took place in conjunction with a detailed knowledge of the nAChR subunits or the domains within those subunits to which the various insecticides bind. The availability of such knowledge would allow a broader range of new insecticides to be considered for these existing targets as chemists could design better compounds if information on the precise binding pocket existed.

Current insecticides could be used to broaden our biological understanding of the nervous system and point to new targets. For example, the identification and functional analysis of neural receptor accessory proteins could be a useful starting point. These proteins are already of great interest in vertebrate model systems due to the association of a number of human drugs and pathologies with neural receptors such as the α4β2 and α7-like nAChR and GABA gated chloride channels. Recent work has demonstrated the complex nature of the vertebrate α7 nAChR at the postsynaptic membrane using proteomics to describe 55 proteins that are present in wild-type samples of carbachol-sensitive α-bungarotoxin binding complexes, but absent in nAChR subunit α7 knockout mice samples (Paulo et al., 2009). Many other proteins have been identified that play critical roles in trafficking, assembly, clustering and function of nAChRs (Jones et al., 2010). It is likely that a number of these will be conserved in function in *D. melanogaster* and other insects, providing a new set of potential targets to affect the nervous system. Insecticides targeting proteins involved in modifying the pharmacology of the receptor may be successful compounds in their own right, but may also perform well as synergists that sensitise insects to other chemicals, including current ones, lowering doses required and increasing pest specificity.

2.4.2. In vivo systems

Toxicological analysis examines the accumulated impact of an insecticide on an insect. Electrophyisiological analysis offers a path to understanding the direct impact on the nervous system. In *Drosophila* there are well-established paradigms for studying responses of the nervous system to xenobiotics. A prime example is the giant fibre system (GFS), one of the largest and best characterised neural circuits in the fly (King and Wyman, 1980; Tanouye and Wyman, 1980). Its electrophysiological responses have been extensively studied and a loss of escape response behavioural phenotype has been associated with knockout of *dα7* (an ortholog of vertebrate α7-like nAChR subunits) (Fayyazuddin et al., 2006). Building on this knowledge (Mejia et al., 2010) have begun testing conotoxins and other molecules for both pharmacological and insecticidal potential by pairing the application of the compound with electrophysiology to assess the quality of responses in the GFS. It has a variety of cholinergic, glutamatergic and voltage gated channels allowing multiple potential targets to be tested simultaneously. This line of investigation is likely to uncover compounds with excellent insecticidal properties and also hints at the possibilities other neural systems may have to offer with further study.

2.4.3. In silico systems

With recent advances in structural biology it has become possible to create models for complex insecticide targets such as the ligand-gated ion channels. These models can be used to predict (i) the amino acids to which current insecticides bind and (ii) which amino acid replacements would lead to resistance. These predictions can be tested *in vivo* using methods identified in section 2.2.4. This approach has been applied to the neonicotinoids and the nAChRs helping to characterise the role of residues in the binding pocket, the action of the compound on the receptor and the selectivity for invertebrate receptors (Ihara et al., 2008; Talley et al., 2008). It is possible to computationally test the likelihood that any given known chemical, among the millions now known, could bind to a given target if its structure can be modelled (Pierri et al., 2010). The candidate compounds identified by this 'fast docking' procedure could be used in toxicological studies with *D. melanogaster* and pest insects. This method has the potential to be transformational but a chequered past has hampered its use (Shoichet, 2004). By searching for chemicals that bind to functional domains that are diverged from their counterparts in non-target species, ranging from insects to vertebrates, low environmental impact insecticides might be developed. Alternatively, the likelihood of target site resistance could be minimized if docking was utilised to identify chemicals that bound to a functional domain shared by multiple related targets.

The rational design of insecticides can afford more sustainable control over insect pests. The frequency and rapidity with which high-level target site resistances have evolved speaks loudly that there must be a better way. Regardless of whether insecticides of the future are discovered or designed, an enhanced understanding of insect metabolism enzymes and pathways is required to minimize another major resistance risk factor, insecticide detoxification.

2.5. Metabolic based insecticide resistance

To exert its lethal effect, an insecticide must first reach its molecular target. The process by which insecticides reach their targets is not well characterised. Insecticide uptake and efflux mechanisms are not well defined. Although there is evidence that ABC transporters such as the *Multidrug Resistant-associated Protein* (*dMRP*) can affect insecticide resistance levels by the active transport of insecticides out of cells (Aurade et al., 2006; Lanning et al., 1996), here we concentrate on insecticide detoxification enzymes. Through insecticide resistance studies, a number of enzymes with insecticide metabolising properties have been identified (Li et al., 2007). These enzymes are collectively known as detoxification enzymes, and are encoded by members of the cytochrome P450 (P450), glutathione S-transferase (GST), carboxylesterase (COE) and UDT-glycosyltransferase (UGT) multi-gene families. In *D. melanogaster*, 196 detoxification genes have been identified (89 P450, 39 GST, 35 COE and 33UGT) (Low et al., 2007; Luque and O'Reilly, 2002; Ranson et al., 2002; Tijet et al., 2001). Comparable numbers are found in most insects with sequenced genomes (Claudianos et al., 2006; Lee et al., 2010; Nene et al., 2007; Ranson et al., 2002; Strode et al., 2008). Members from all four detoxification gene families are found in a variety of organisms, suggesting they have ancient origins.

Before discussing the roles of detoxification enzymes in insecticide resistance, it is important to distinguish resistance from insecticide metabolism. Insecticide metabolism is a biochemical process whereby insecticides are broken down into non-toxic forms. It involves metabolic pathways that almost certainly contain multiple enzymes. The insecticide metabolism paradigm comes from mammalian drug metabolism literature, where detoxification enzymes are classified as Phase I or Phase II (Williams, 1959). Phase I enzymes, for example P450 and COE enzymes, perform oxidation, reduction or hydrolysis reactions, often the first step/s of detoxification. Some P450s can also be involved in the activation of insecticides, chemically modifying insecticides to biologically active forms, as is the case for some organophosphorus insecticides. Although P450s are best known for their ability to carry out oxidation reactions, many P450s have the ability to catalyse a wide range of chemical reactions (Mansuy, 1998). One human P450, CYP3A4, is capable of metabolising over half of all known therapeutic drugs (Luo et al., 2004). COE enzymes catalyse the hydrolysis of an ester group to its component alcohol and acid, and can act on a diverse range of carboxylic, thio-, phospho-, and other ester substrates. The genomics and functions of insect esterases has recently been reviewed (Oakeshott et al., 2005). The products of Phase I reactions often become substrates for Phase II enzymes such as GST and UGT enzymes that add glutathione and glycosyl groups, respectively, aiding the export of the compound from the cell. Insecticide metabolism occurs in all insects, regardless of insecticide resistance status, and is likely to involve a multistep pathway. The metabolism of insecticides can be complex. For many insecticides, different metabolic breakdown products are possible. A level of functional redundancy may also exist, with different enzymes being capable of producing the same metabolites, as exemplified for DDT metabolism in Diptera (Fig. 3).

Insecticide resistance is a genetic phenomenon, defined by insect survival at insecticide concentrations that are lethal to susceptible insects (McKenzie, 1996). Genetic changes leading to metabolic based insecticide resistance can arise via a number of different ways. Mutations resulting in the production of more detoxification enzyme, either by gene amplification or gene duplication events, mutations increasing gene transcription, or mutations altering the tissue specificity or timing of gene expression are commonly documented (Daborn et al., 2002; Field et al., 1999; Hemingway, 2000; Schmidt et al., 2010). Another class of mutants are those that change the kinetics or substrate specificity of detoxification enzymes, arising via point mutations in the coding regions of genes (Claudianos et al., 1999; Newcomb et al., 1997). Our understanding of the available evolutionary options for insecticide resistance is superficial, as studies are typically confined to variation arising in natural populations. Due to the limitations of genetic

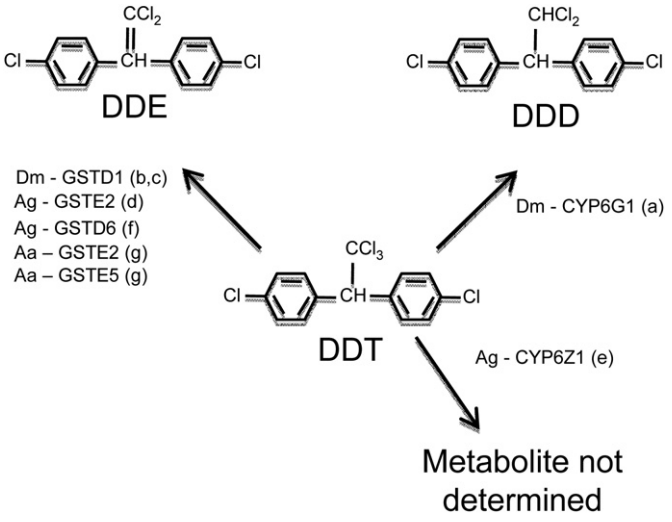

Fig. 3. Metabolism of the insecticide DDT, and metabolites detected by different insect enzymes as determined by *in vitro* expression studies. Dm = *Drosophila melanogaster*, Ag = *Anopheles gambiae*. Aa = *Aedes aegypti*. (a) (Joussen et al., 2007), (b) (Tang and Tu, 1994) (c) (Low et al., 2010), (d) (Wang et al., 2008), (e) (Chiu et al., 2008), (f) (Ranson et al., 1997), (g) (Lumjuan et al., 2011).

techniques, single gene responses are most commonly studied. Given the large number of different genes encoding enzymes with the potential to be involved in insecticide metabolism and therefore resistance, and our ignorance of underlying metabolic pathways, identifying the precise enzymes involved in these processes is challenging. For many examples of metabolic resistance strong evidence of the exact molecular mechanism of resistance does not exist. The following section describes the processes involved in determining the bases of metabolic resistances and the role *D. melanogaster* can play in this.

2.6. Approaches for characterising metabolic based insecticide resistance

2.6.1. Establishing the resistance phenotype is metabolic

Initial testing for the involvement of detoxification mechanisms in insecticide resistance is often performed by co-administration of the insecticide and a synergist, for example piperonyl butoxide (PBO) which inhibits the activity of P450 enzymes, or diethyl maleate (DEM) which inhibits the activity of GST enzymes. A reduction in insecticide concentration needed to kill an insect due to the administration of a synergist implies the involvement of a particular class of enzyme in resistance (Liu and Yue, 2000; Pasay et al., 2009; Sanchez-Arroyo et al., 2001). It fails to identify the individual enzyme responsible for resistance however. Other approaches that directly measure enzyme activity using diagnostic substrates, for example methoxy-resorufin ether (MROD) and ethoxy-resorufin ether (EROD) for P450s suffer from similar limitations.

2.6.2. Approaches to identifying candidate genes

More specific investigations of metabolic-based insecticide resistance are directed towards identifying the individual genes responsible for resistance. By comparing expression levels of individual detoxification genes between resistant and susceptible or laboratory reared strains, genes with elevated expression in resistant strains are identified (Pittendrigh et al., 1997; Ranasinghe and Hobbs, 1998; Scharf et al., 2001; Tomita and Scott, 1995; Yang et al., 2006; Zhu and Snodgrass, 2003). Where DNA sequence information is available, microarrays representing detoxification gene or total gene complements have been used to detect transcript level differences between resistant and susceptible strains. This is an important technological advance, helpful in identifying candidate genes. For example, *Gste2 Cyp6z1*, *Cyp6m2* and *Cyp6p3* from *An. gambiae* (David et al., 2005; Djouaka et al., 2008), *Cyp6g1*, *Cyp12d1* and *Cyp6w1* from *D. melanogaster* (Daborn et al., 2002; Pedra et al., 2004) and *Cyp6g1* and *Gsts1* from *Drosophila simulans* (Le Goff et al., 2003) were identified using this approach. However, as variation in transcript levels for over 10% of genes in the genome exists between any two given strains (Gibson and Weir, 2005), the elevated expression of a gene in a resistant strain is only preliminary evidence for its role in resistance. Corroborating evidence via genetic mapping of resistance, metabolism studies, RNAi, *in vivo* expression studies, or, as a matter of best practice, a combination of these approaches should be obtained.

2.6.3. Validating metabolic resistance mechanisms

In species with well characterised genomes that are amenable to crossing, such as *D. melanogaster*, genetic mapping can be used to identify or validate resistance candidates. Resistance will map to the genetic cause of resistance. Therefore, for cis-acting mutations, resistance will map to the structural gene involved. Mapping approaches have been successful in identifying resistance-conferring detoxification genes in *D. melanogaster* and *An. gambiae* (Bogwitz et al., 2005; Daborn et al., 2001; Ranson et al., 2000; Zhu et al., 2010).

To verify a given enzyme is capable of insecticide metabolism, expression in a heterologous system is often used. *E. coli*, baculovirus, yeast and tobacco cell systems have all been useful for expressing detoxification enzymes to study insecticide metabolism providing information on substrates, enzyme kinetics and the metabolites produced (Amichot et al., 2004; Andersen et al., 1994; Campbell et al., 1998; Dunkov et al., 1997; Guzov et al., 1998; Joussen et al., 2007; Karunker et al., 2009; Ranson et al., 1997; Sabourault et al., 2001; Stevenson et al., 2011; Zhu et al., 2010). Expression systems for insect P450s have been reviewed previously (Feyereisen, 2005).

In a limited number of examples, RNAi has been used to reduce the expression level of candidate detoxification genes. The role of *CYP6BG1* in a permethrin resistant strain of *P. xylostella* was validated by feeding dsRNA targeting *CYP6BG1*. A significant decrease in both *CYP6BG1* transcript level and permethrin resistance was observed (Bautista et al., 2009). Similarly, targeting *CYP6BQ9* by injection of dsRNA into the deltamethrin resistant QTC279 strain of *Tribolium castaneum* confirmed the role of this gene in deltamethrin resistance (Zhu et al., 2010). In Diptera, including *Drosophila*, the RNAi response is cell-autonomous, meaning that dsRNA does not effectively travel between cells, making feeding and injection methods of delivering dsRNA ineffective (Huvenne and Smagghe, 2010). Transgenic expression of dsRNA using the GAL4/UAS system is the most efficient means of achieving gene knockdown in *D. melanogaster* (Kennerdell and Carthew, 2000). Using the GAL4/UAS system, tissue specificity of gene silencing can be achieved (Brand and Perrimon, 1993; Duffy, 2002) (Fig. 2). So far, there is only one published example specifically using RNAi in *D. melanogaster* to investigate insecticide resistance. Knockdown of *D. melanogaster Cyp6g1* specifically in the Malpighian tubules of the adult resulted in a reduction of P450 activity (McCart and ffrench-Constant, 2008). However, no increase in susceptibility to DDT could be detected in a susceptible strain. RNAi knockdown of *Cyp6g1* in insecticide resistant strains over-expressing *Cyp6g1* is yet to be conducted. Given RNAi strains exist for most *D. melanogaster* genes (Dietzl et al., 2007), systematic RNAi of detoxification genes in *D. melanogaster*, combined with insecticide resistance or metabolism studies may be a useful approach in the future. In addition, as RNAi technology in other insect species besides *Drosophila* is becoming more routine (Belles, 2010), further use of this approach to validate resistance genes can be expected.

Strong evidence for genes being involved in resistance can also be achieved by transgenic approaches using the GAL4/UAS system in *D. melanogaster* (Fig. 2). The GAL4/UAS system provides temporal and spatial control of gene expression. Over-expression of *Cyp6g1* in the larval midgut, Malpighian tubules and fat body (Chung et al., 2007), inducible expression of *Cyp6g1* in all tissues of the adult via heat shock (Daborn et al., 2002) and specific expression of *Cyp6g1* in the adult Malpighian tubules (Yang et al., 2007) have all been used to validate the role of *Cyp6g1* in insecticide resistance. Resistance roles for other *D. melanogaster* P450s have been investigated by transgenic expression studies (Bogwitz et al., 2005; Daborn et al., 2007). Transgenic expression of *Cyp12d1* confers resistance to DDT, while transgenic expression of *Cyp6g2*, the closest paralog to *Cyp6g1*, confers resistance to nitenpyram (Daborn et al., 2007). Interestingly, using the same approach, a role for *Cyp6a2* in DDT resistance could not be validated, even when specific alleles thought to be important for DDT metabolism (Amichot et al., 2004) were expressed (Daborn, Yang, Lumb, and Batterham, unpublished data).

Resistance gene candidates from pest species have also been transgenically expressed in *D. melanogaster*. Ectopic expression of *CYP6BQ9* from *T. castaneum* specifically in the *D. melanogaster* brain confers deltamethrin resistance (Zhu et al., 2010). Likewise, *Rop-1*

and *Cyp6g3* from *L. cuprina*, *CYP6CM1* from the whitefly *Bemisia tabaci*, and *Gste2* from *An. gambiae* have all been ectopically expressed in *D. melanogaster* and confer insecticide resistances (Daborn, Lumb and Batterham, unpublished data). The wide variety of detoxification enzyme families, and diversity of species used, indicate that ectopic expression in *D. melanogaster* is a robust approach for validating candidate resistance genes. The ability to ectopically express multiple detoxification genes in the same individual fly is also possible, which may become important for investigating multi-factorial metabolic based resistances.

2.7. Using D. melanogaster to study metabolism

The previous section concentrated on the genetics of metabolic-based insecticide resistance, with the emphasis on validating candidate genes for insecticide resistance. Questions relating to how insecticides move within the insect, how and where insecticides are metabolised, and how this relates to the underlying biology of insect metabolism also need to be addressed. Combining this with a systematic approach for studying individual detoxification genes, achievable in *D. melanogaster*, would provide a more complete understanding of insecticide metabolism.

Although there is evidence of insecticide metabolism in other tissues (Korytko and Scott, 1998; Zhu et al., 2010), there is building evidence that the key tissues for the metabolism of most compounds are the midgut, the Malpighian tubules and the fat body. Recent large-scale transcript sequencing projects and microarray studies identify a large number of detoxification genes expressed in these tissues (Li et al., 2008; Mittapalli et al., 2010; Neira Oviedo et al., 2008; Pauchet et al., 2009, 2010; Wang et al., 2004). Thus the tissue specificity of gene expression is an important aspect to identifying those enzymes with the potential to metabolise insecticides. In *D. melanogaster*, studies using GFP reporter constructs have been useful in characterising the tissue expression patterns of individual P450s, including *Cyp6g1* and *Cyp6a2*, with expression of *Cyp6g1* in the larval midgut, fatbody and Malpighian tubules (Chung et al., 2007), and expression of *Cyp6a2* detectable in these tissues as well as the larval epidermis, hindgut, skeletal muscles and nervous system (Giraudo et al., 2010). Although not possessing the same sensitivity, another technique for studying the tissue specificity of gene expression is *in situ* hybridisation. Of the 85 P450s present in *D. melanogaster*, 35 were detected in third instar larvae in the midgut, Malpighian tubules, fat body or combinations thereof (Chung et al., 2009) (Fig. 4). Specificity of expression within the midgut and Malpighian tubules was observed, with P450 expression detected in distinct regions within both tissues (Chung et al., 2009) (Fig. 4). For the midgut, functions of different regions are not well defined, and many regions are not morphologically distinct (Nakagoshi, 2005). It is hoped that the construction and use of midgut region specific reporter constructs in transgenic *D. melanogaster* will help in the physical dissection of midgut regions for transcriptomic analyses. Identifying detoxification genes that are co-expressed in particular regions may help in defining detoxification pathways. The genetic dissection of midgut regions using region specific GAL4 enhancer strains and RNAi could help in defining gene function within specific regions.

Many insect detoxification gene family members are rapidly evolving. Given this, caution must be exercised in extrapolating findings of gene function in one species to its ortholog in another. Within the cytochrome P450 family, for example, few orthologs are identifiable between *Drosophila* and mosquitoes, and those that are, are involved in important endogenous functions (Chung et al., 2009; Ranson et al., 2002; Rewitz et al., 2007). Similar observations have been made for mammalian P450s, with P450 genes categorised as being either phylogenetically stable or unstable (Thomas, 2007). The stable genes are characterised by few or no gene duplications or gene losses between species compared, and these usually encode P450s with endogenous substrates. By

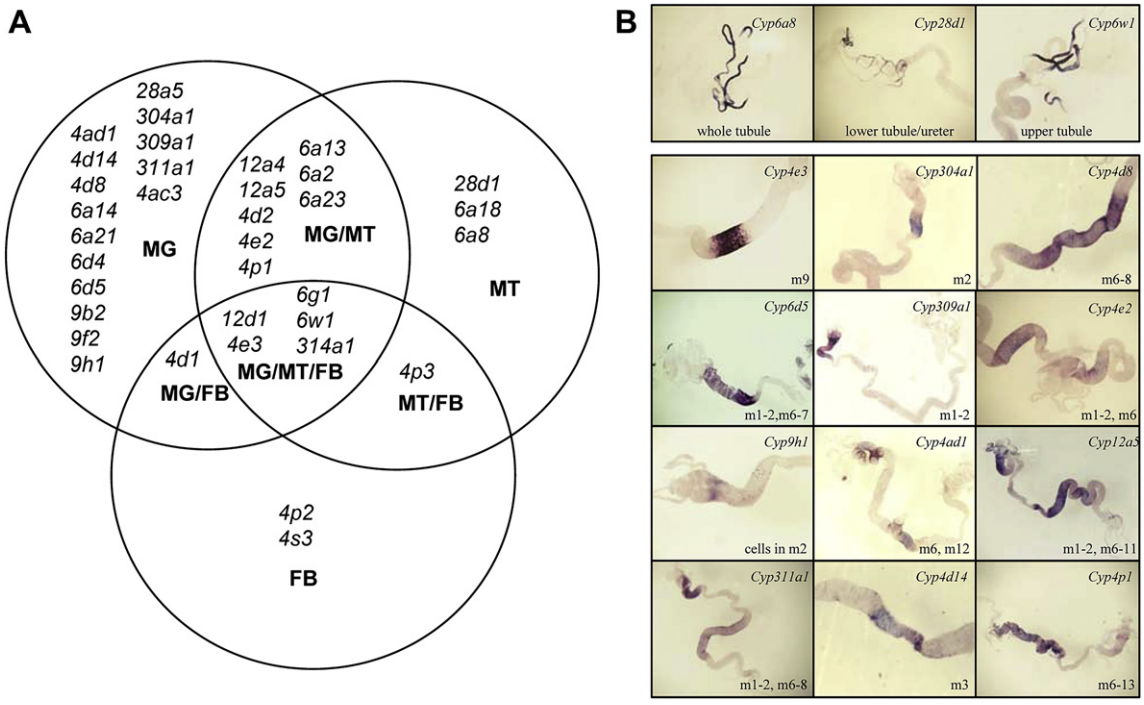

Fig. 4. Cytochrome P450 expression patterns in *D. melanogaster*. A. Venn diagram representing unique and overlapping tissue expression of P450 genes expressed in the three key metabolic tissues of third instar larvae. FB, fat body; MG, midgut; MT, Malpighian tubules. B. Cytochrome P450s expressed in the midgut and Malpighian tubules are detected in specific compartments by *in situ* hybridisation. (Chung et al., 2009).

contrast, phylogenetically unstable genes are characterised by frequent gene duplications and losses among even closely related species, with unstable P450s hypothesized to be involved in the metabolism of foreign compounds such as toxins (Thomas, 2007). Similar analyses have been conducted for P450s in a number of *Drosophila* species (Gramzow, Good and Robin, unpublished), and with ever increasing amounts of insect genome sequence data becoming available, the approach can be used in comparisons of P450s in other insect species (Feyereisen, 2010). Combining phylogenetic analyses with tissue expression patterns (Chung et al., 2009) in *Drosophila* has been useful in categorising genes as candidates for insecticide metabolism. Three rapidly evolving P450s involved in insecticide resistance in *D. melanogaster*, *Cyp6g1*, *Cyp12d1* and *Cyp6w1* are all expressed in the midgut, Malpighian tubules and fat body (Chung et al., 2009) (Fig. 4). Incidentally, all three genes are inducible by chemical challenges, another common feature of many P450s causally linked to insecticide resistance (Giraudo et al., 2010). Despite the rapid evolution of many detoxification genes between insects, approaches such as these could be taken in pest species in the future to help in the identification of candidates.

Advances in determining fine scale structures of some insect detoxification enzymes by crystallography and NMR techniques, *in silico* modelling and docking of insecticides to enzyme structures, are increasing our understanding of how chemical insecticides are metabolised (Baudry et al., 2003; Low et al., 2010). Although there is still a long way before the computational prediction of insecticide metabolism, as in drug metabolism in mammals (de Groot, 2006), it is hoped that prediction of metabolism may help in the design of insecticides that are less susceptible to metabolic attack.

3. Conclusions

When early insecticides such as DDT were first deployed to control insect pests, the capacity to understand the target, relevant metabolic pathways and options for resistance did not exist. For many years the lack of genomic tools in pest organisms made it difficult to identify resistance genes. Through necessity, responses to the problems of insecticide resistance were reactive, not proactive. Resistance mechanisms could not be anticipated. It is therefore not surprising that the control of many insect pests have been under constant threat with thousands of cases of resistance now documented. Rapid technological advances in genomics, metabolomics and structural biology have ushered in an era where the capacity for rational control is rising. There are two key elements to a rational control strategy using insecticides:-

1. Insecticides would be designed against targets for which the insecticide binding site has been defined down to the point of knowing the precise amino acids involved. The capacity for target-site resistance to evolve would be evaluated. Design would take into account the potential for functional redundancy; situations where loss of function variants are viable and resistant (e.g. spinosyn and neonicotinoid resistance) need to be avoided. As far as is possible, insecticides should be pest specific, allowing the parallel use of biological control agents in an Integrated Pest Management strategy.
2. Metabolism of the insecticide by the pest insect would be studied in detail. In particular it would be important to identify any enzymes that, if over-expressed, could confer resistance.

In this paper the contribution that *D. melanogaster* can make to a deeper understanding of targets and metabolism has been described. Given the degree of evolutionary conservation observed for existing targets, lessons learned from *D. melanogaster* can be used to direct research on corresponding pest targets. The capacity to functionally express pest target genes in *D. melanogaster* means that hypotheses concerning resistance and the interaction between insecticides and targets can be tested *in vivo*. Target site resistances identified in *D. melanogaster* appear to be useful in predicting the bases of field-based resistances in pest systems. Insecticide metabolism remains something of a *black box* process. The power of tools available in the *D. melanogaster* system will, in time, allow a detailed description of how one insect metabolises insecticides. Given the high degree of amino acid sequence divergence among insects for metabolic genes it is more likely that lessons learned from *D. melanogaster* will guide pest research rather than providing a detailed knowledge of pest systems. But again, the ability to express pest genes in *D. melanogaster* will be very useful. Anticipating metabolic resistance before it evolves in the field will be difficult. Without having a capacity to predict the substrate specificity of metabolic enzymes from their amino acid sequences it is difficult to identify the enzymes that pose the greatest resistance threat. It is likely that the over-expression of any given metabolic gene can be tested in *D. melanogaster*, but for any given pest species there are many candidates that would need to be tested.

The *D. melanogaster* system does not offer a panacea. Research into this model and the major insect pests needs to proceed in parallel, but it is certain that research in *D. melanogaster* will accelerate progress in improving the way in which insecticides are used to control insect pests. The battle with insect pests using insecticides such as DDT was once fought in the dark – neither the targets against which the insecticide weapons were directed or the metabolic systems that would defend them were illuminated. Going forward, a rich understanding of the relevant biology will help with these battles.

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Fear and loathing in the benthos: Responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk

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ABSTRACT

The influence of interactions between pesticide exposure and perceived predation risk on the lethal and sub-lethal responses of two aquatic insects was investigated using the pesticide imidacloprid, and a combination of predator-release kairomones from trout and alarm substances from conspecifics. Laboratory experiments examined feeding and respiration rates of the caddisfly *Sericostoma vittatum* as well as the growth, emergence and respiration rates of the midge *Chironomus riparius*, exposed to sub-lethal concentrations of imidacloprid. The effects of the two stressors on burrowing behaviour of both species were also assessed. The results show significant effects of environmentally relevant concentrations of imidacloprid on all endpoints studied. Perceived predation risk also elicited sub-lethal effects in *C. riparius* and *S. vittatum*, the latter species being less responsive to predation cues. The effects of simultaneous exposure to both types of stressors were assessed using two different approaches: analysis of variance and conceptual models [concentration addition (CA) and independent action (IA)] normally used for the evaluation of contaminant mixture exposure. Both statistical approaches showed no significant interactions on responses in simultaneous exposures in the majority of parameters assessed with only a signification deviation from the reference CA and IA models being found for *C. riparius* respiration data contrary to the ANOVA results.

Exposure to imidacloprid also compromised antipredator behavioural responses of both insect species, with potential negative consequences in terms of mortality from predation in the field.

The results obtained demonstrate that natural and anthropogenic stressors can be treated within the same framework providing compatible data for modelling. For an improved interpretation of ecological effects it will be important to expand the mechanistic study of effects of combined exposure to pesticides and perceived predation risk by measuring different endpoints over a wider range of pesticide concentrations.

1. Introduction

Macroinvertebrates that live in streams and rivers adjacent to agricultural areas are subjected to episodic and continuous inputs of pesticides which contribute to the contamination of inland waters and subsequent loss of biodiversity (Tilman et al., 2001). Under natural conditions, pesticides act in concert with other abiotic and biotic stressors to exert negative effects on aquatic ecosystems which are determined by stressor interactions. Nevertheless, stress ecology studies tend to focus on the effects of single factors, and often ignore the reality of stressor interactions (Relyea and Hoverman, 2006). We need a better understanding of how multiple stressors interact with each other, to facilitate the assessment of possible additive or more-than-additive effects, and thus better inform risk assessment practices (Van Straalen, 2003; Relyea and Hoverman, 2006). The effects of pesticides are typically assessed with classical laboratory ecotoxicological studies which are designed to generate information to be used to determine water quality criteria and safe concentrations of pollutants (Walker et al., 2001). The goal of increasing the ecological relevance in toxicity studies is being achieved with respect to several abiotic factors such as temperature, pH, water chemistry (Heugens et al., 2001), but ecotoxicologists tend to exclude biotic stressors such as competition or predation from their investigations. This will limit the extrapolation of laboratory results to effects on populations in the field because the effects of pesticides may be underestimated if possible interactions with natural

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stressors are not taken into consideration (Hanazato, 2001; Relyea, 2005).

Predation, an important and pervasive form of biotic stress in natural ecosystems, is a major driver of population and community dynamics. In addition to the direct lethal effects arising from prey consumption, predators, simply by their presence within a system, can affect prey abundance, morphology, physiology, life-history and behaviour (Lima and Dill, 1990; Kats and Dill, 1998; Lass and Spaak, 2003). Prey organisms exhibit a variety of morphological, physiological, behavioural and life-history adaptations as defences against predation (Kats and Dill, 1998). These antipredator responses generally have associated fitness costs and therefore are only deployed when prey organisms perceive the risk of predation, and thus only when a clear benefit is gained from the expression of the defensive trait (DeWitt et al., 1998).

Recent studies have illustrated the proximate mechanisms involved in predator–prey interactions, focusing on mechanisms of assessing risk (Kats and Dill, 1998). In aquatic environments, chemical cues are important stimuli that mediate antipredator responses (Dicke and Grostal, 2001), in combination with visual, mechanical and auditory cues. Infochemicals such as predator-release kairomones or alarm substances from disturbed or damaged conspecifics allow prey organisms to assess the level of risk and to deploy effective antipredator responses.

Ecological theory predicts that when sensing situations of predation risk, aquatic insects engage in behaviourally and physiologically based adaptive responses that are translated in a trade-off between the fitness benefits of energy gain and the risks of being eaten by predators (Benard, 2004).

Avoidance behaviour can lead to a decrease in foraging activity and feeding and thus, prey organisms' decisions reflect a trade-off between energy gain with consequences to growth and/or development rates, and mortality risk (Abrams and Rowe, 1996; McPeek and Peckarsky, 1998). The presence of predators has also been shown to place higher metabolic demands on prey organisms, manifested in elevated rates of oxygen consumption, reflecting an increased cost of vigilance under predation risk (Bengtsson, 1982; Beckerman et al., 2007). Thus, in the presence of predators like fish, the energy budget of aquatic insect larvae can be reduced, leading to delayed maturity and/or smaller size at maturity (Ball and Baker, 1996; Peckarsky et al., 2002; Dahl and Peckarsky, 2003a; Benard, 2004; Holker and Stief, 2005).

The combined effects of contaminants and predation risk have received little attention from ecologists and ecotoxicologists alike. Nevertheless, combined effects of pollutants and predators may be important if the uptake or effect of the pollutant is altered by the presence of predators, or if pollutants compromise normal antipredator responses. Pesticide contamination has been shown to disturb predator–prey interactions (Preston et al., 1999; Lefcort et al., 2000; Schulz and Dabrowski, 2001) and the detrimental effects of pesticides can even be influenced or modified solely by the perception of risk from predation, due to combined physiological or behavioural stresses (Relyea, 2003; Campero et al., 2007)

The purpose of this study was to examine the responses of the midge *Chironomus riparius* Meigen and the caddisfly *Sericostoma vittatum* Rambur, to imidacloprid, under different levels of perceived predation risk simulated using a combination of chemical cues from brown trout and alarm substances from conspecifics.

Chironomids are an ecologically important group within freshwater ecosystems which often dominates the benthic communities of lotic and lentic environments in terms of numbers and biomass (Merrit and Cummins, 1996). Chironomids have been used as model organisms for sediment toxicity studies (Faria et al., 2006; Stoughton et al., 2008). As a major component of fish diets, they have also been used to investigate predator impacts on invertebrate communities and changes in activity (Holker and Stief, 2005), growth and development (Ball and Baker, 1996; Noonburg and Nisbet, 2005) have been shown for chironomids in response to nonlethal cues indicating the presence of predators.

The caddisfly *S. vittatum*, (Trichoptera: Sericostomatidae) is an endemic species of the Iberian Peninsula, playing a key role in the fragmentation of allochthonous organic matter of streams in central Portugal (Feio and Graça, 2000). Caddisflies have previously been used in ecotoxicological studies (Schulz and Liess, 2000), and responses induced by chemical cues from predators have also been demonstrated (Kuhara et al., 2001; Wissinger et al., 2006).

In this study brown trout, *Salmo trutta* Linnaeus was chosen as a model vertebrate predator. Brown trout are native to Europe, and prefer cold, well-oxygenated upland waters. They are mainly diurnal, feeding on aquatic and terrestrial insects, crustaceans and small fish. Chironomids and caddisflies are two of the most important items in the diet of brown trout (Penczak and Formigo, 2000) and several studies have previously demonstrated antipredator responses induced by chemical cues from trout in several different insect species (Huryn and Chivers, 1999; Dahl and Peckarsky, 2002; Peckarsky et al., 2002).

Imidacloprid was chosen as a model pesticide because it is a relatively new pest control substance, which is generating increasing concern on its impacts on natural ecosystems (Matsuda et al., 2001, Jemec et al., 2007). Imidacloprid is a systemic insecticide, belonging to a class of chloronicotinyl insecticides, acting on the nicotinic acetylcholine receptors (nAChRs) which are common to many invertebrate taxa, and insects in particular (Tomizawa and Casida, 2003). Due to its relatively high solubility (~510 mg/L) and mobility in soils it has the potential to enter streams by dissolved runoff and leach to ground water (Gupta et al., 2002). Imidacloprid has been found in inland waters at concentrations ranging from 0.2 to 12 μg/L (CCME, 2007; Jemec et al., 2007).

Our main objective was to investigate possible interactions between imidacloprid and perceived predation risk. This was performed employing standard tests currently used in pesticide hazard assessments in order to improve our knowledge of the potential effects of pesticide exposure on macroinvertebrate populations under field conditions, where predators are generally present. We focused on the model species *C. riparius* and the caddisfly shredder *S. vittatum* using lethal as well as several sub-lethal parameters (feeding, growth, respiration, behaviour). For the assessment of the combined effects of the two stressors we employed and compared the results of two different approaches: traditional analysis of variance and conceptual models (CA and IA) normally used for the evaluation of contaminant mixture exposure.

2. Material and methods

2.1. Animals

C. riparius larvae were obtained from laboratory cultures at the University of Aveiro, Portugal and had been maintained for more than five years in standard conditions, at 20 °C, in a light–dark cycle of 16–8 h, in hard water ASTM and fed twice a week with macerated fish food, Tetramin®. *S. vittatum* larvae were collected from Ribeira S. João, Serra da Lousã, Central Portugal (40°06′N, 8°14′W), using a hand net. Organisms were acclimated for two weeks to laboratory conditions: 20 °C, light–dark cycle of 14:10 h, in aerated artificial pond water (APW) (Naylor et al., 1989) and were fed *ad libitum* with unconditioned alder (*Alnus glutinosa*) leaves.

Young brown trout (*Salmo trutta* L.) were obtained from a fish farm and transferred to laboratory where they were kept at 17 ± 1 °C in 60 L plastic tanks with aerated APW and fed every day with commercial fish food. After one month we selected healthy fish to be used in the preparation of the fish chemical cues.

2.2. Preparation of predatory chemical cues

Ten young brown trout (12–15 cm) were held in 10 L aerated APW for 24 h. After that period, the water, containing trout exudates, was filtered (0.45 μm Whatman acetate cellulose filter), frozen at −20 °C and thawed as necessary. Fish were not fed for 3 days before this 24 h period to reduce the level of ammonia and faeces in the exudate water (final concentration of 10 fish L^{-1} was 2.45 mg NH_3 L^{-1}). For the production of alarm substances we macerated 5 *S. vittatum* larvae (6–8 mg dry weight) or 50 *C. riparius* 6-day-old larvae in 100 mL of APW. These solutions were filtered (0.45 μm Whatman acetate cellulose filter) and frozen at −20 °C.

The stock solutions of chemical cues were used to prepare the treatments described below as "low predation risk" and "high predation risk". The concentrations of fish used in treatments were comparable to low and high densities of brown trout found in a long term study in the British Lake District (Elliott, 1994). Likewise, the concentrations of alarm substances, although somewhat arbitrary, were chosen based on the number of prey (chironomids and caddisflies) actively consumed by a single trout in 15 min in our tanks in the laboratory:

For *S. vittatum*:

- No risk = APW with no cues added
- Low predation risk = 0.02 fish L^{-1} + 0.08 macerated caddis larvae L^{-1}
- High predation risk = 0.1 fish L^{-1} + 0.4 macerated caddis larvae L^{-1}

For *C. riparius*:

- No risk = APW with no cues added
- Low predation risk = 0.02 fish L^{-1} + 0.4 macerated chironomid larvae L^{-1}
- High predation risk = 0.1 fish L^{-1} + 2 macerated chironomid larvae L^{-1}

2.3. Test chemical

Imidacloprid (Confidor® 200 SL) was purchased from Bayer CropScience AG (Monheim, Germany) and was used to prepare the appropriate stock solutions of imidacloprid with distilled water. Confidor® 200 SL, contains 200 gr L^{-1} of imidacloprid (CAS no.: 138261-41-3) as the active ingredient and N-metil-2-pirrolidone (CAS no. 872-50-4) as adjuvant, was used in all experiments. Freshwater half-life of imidacloprid can vary (4–28 days under exposure to sunlight) being dependent on formulation, pH, microbial communities and temperature (CCME, 2007).

Chemical analyses of the imidacloprid samples were conducted at the Terracon laboratory (Jütterborg, Germany) on a HPLC-PDA-System equipped with 2 HPLC pumps Model LC-10ADvp, Autosampler SIL-10ADvp, column oven CTO-10ASvp, and a photodiodenarray-detector (PDA) SPD-M10Avp (Shimadzu, Japan). Samples with high imidacloprid concentrations were diluted with deionised water while samples with lower concentrations of imidacloprid were extracted from 100–200 mL (flow: 0.5 mL min^{-1}) of water samples using solid phase extraction (SPE cartridges Supelclean ENVI-18 (100 mg mL^{-1}), Supelco, Schnelldorf, Germany) using acetonitril (1.1) for elution. 10 μl acetonitril-extracts were then injected (0.4 mL min^{-1}) in the chromatography column (LUNA C18, Phenomenex, Aschaffenburg, Germany) using water, 0.1% formic acid and acetonitril (1.1) as eluents. Readings were taken at 270 nm with a limit of quantification of 0.1 μg L^{-1}

The concentrations determined for stock solutions were 1666 μg L^{-1} for *C. riparius* tests and 7840 μg L^{-1} for the experiments with *S. vittatum*. Stock solutions were stored at 4 °C protected from light. Tests solutions were prepared by adding an appropriate amount of stock solution in APW. The concentrations presented in graphs are nominal concentrations at day 0, taking into account the actual concentrations of stock solutions.

During chronic experiments (see below), the concentrations of imidacloprid were also measured at the end of day 2, i.e. before partial renewal of medium, including in treatments with high levels of predatory cues to control the effects of fish conditioned water on pesticide degradation.

2.4. Acute toxicity experiments

Acute lethality was estimated to gauge the inherent sensitivity of each species to the pesticide, and also to establish a range of sub-lethal concentrations to be used in the feeding and respiration experiments. Test solutions of imidacloprid (Confidor®) were prepared in APW. There were ten replicates with one organism each per treatment in of *S.vittatum* acute tests and 5 replicates with 25 larvae (second stage, six days old) for *C. riparius*. Organisms were exposed in glass vials containing 150 mL of pesticide solutions and no food. After 96 h exposure, mortality was determined by mechanical stimulation, with animals that failed to show any response being considered as dead. The mortality assessments for both species were also conducted in the presence of predator exudates (high predation risk only), to find out if the predator's chemical cues altered the lethal sensitivity of *S. vittatum* and *C. riparius* to imidacloprid.

2.5. S. vittatum feeding experiments

An experimental design for the feeding experiments was adapted from studies with *Gammarus pulex* (Naylor et al., 1989). We used a full factorial design where pesticide exposure was tested in combination with different levels of predation risk. Thus, for every predation risk level (no risk, low and high) we tested three concentrations of pesticide: 1.9, 3.9, and 7.8 μg/L of imidacloprid plus the control treatment.

We used ten replicates with one animal per replicate in all experiments. The animals were allocated to individual glass vessels, containing a 1 cm layer of inorganic fine sediment (<1 mm), 150 mL of experimental medium and food in the form of five alder leaf discs. Alder leaf discs used in each replicate for the feeding experiments were autoclaved and then dried at 60 °C for 4 days and weighed. They were soaked in APW for 96 h prior to use. After 6 days animals and the respective remaining food (leaf discs set) were removed, dried at 60 °C for 4 days and reweighed. Feeding rate was calculated as the difference between the initial and final leaf disc dry mass (mg) and divided by the dry mass of organisms (mg) and elapsed time (days). Five control cages per treatment with leaf discs but no animals were used to correct for weight change due to factors other than feeding. Every 48 h, 100 mL of the solution (imidacloprid and chemical cues) was renewed. During the experiment all replicates were examined daily. All tests were conducted at 20 ± 1 °C with a photoperiod of 14 h light: 10 h dark.

2.6. C. riparius growth experiment

We performed our experiment as a sediment–water chironomid toxicity test according to the OECD guideline (OECD, 2001). The experiment was performed with 200 mL glass vials (10 replicates per treatment) with five larvae (first stage, three days old) per replicate. Each replicate contained a 1 cm layer of inorganic fine sediment (<1 mm), and 150 mL of experimental medium. Food (Tetramin®) was provided every other day at a ration of 0.5 mg/larvae/day). Survival, head capsule width, total length and time to emergence were the response parameters measured. Every 48 h, 100 mL of the test solution (imidacloprid and chemi-

cal cues) was renewed. 0.4, 1.2 and 3.7 μg/L of imidacloprid was used in combination with different predator treatments: no risk (i.e. no chemical cues added), low and high predation risk. Five replicates (25 organisms) were used to measure growth and 5 replicates to measure emergence. During the experiment all replicates were examined daily. All tests were conducted at 20 ± 1 °C with a photoperiod of 14 h light: 10 h dark. Growth was estimated by measuring the total length and head capsule width of each larva at day 10 with a stereo microscope (MS5, Leica Microsystems, Houston, USA) fitted with a calibrated eye-piece micrometer.

2.7. Measurement of oxygen consumption

Oxygen consumption was determined by simple static respirometry, using larvae held for 24 h in 50 mL gastight syringes (Hamilton, USA). To measure oxygen consumption in *C. riparius*, we employed three syringes per treatment, each holding five 6-day-old larvae; for *S. vittatum* we used five replicates each holding three organisms of approximately similar size. Syringes were filled with the appropriate test solutions and organisms, the air remaining was expelled from each syringe and they were left in the dark in a water bath (20 °C). After 2 h, initial oxygen concentrations were measured with an oxygen meter (model 782, with an oxygen electrode model 1302, Strathkelvin Instruments, Glasgow). Samples were taken with a 0.5 mL gastight syringe and the test solutions were injected manually into the electrode chamber (volume = 70 μL) at a constant rate (0.5 mL min^{-1}). Readings were taken after 1 min. After 22 h, the final oxygen concentrations were measured in the same way. In the case of *S. vittatum*, larvae were dried (with no case) for 4 days at 60 °C and weighed. Oxygen consumption was determined by the differences in the oxygen content of water before (T_0 = 2 h) and after (T_{final} = 24 h) the exposure period, and the respiration rate was expressed as μg oxygen consumed per mg of organism per hour. For *C. riparius* respirometry we followed the same protocol, but the respiration rate was expressed as μg oxygen consumed per organism per hour. For each treatment we used five blank controls (syringes with no organisms) to correct for the ambient oxygen depletions due to factors other than organism respiration. We observed that predation cues affected background depletion of O_2 in both experiments (ca. 13, 16 and 20% for control, low and high predation risk treatments, respectively) reflecting the effects of fish water on microbial oxygen consumption. These positive controls were used as correction factors in the appropriate treatments to exclude effects of differential microbial respiration across predation risk treatments. We used a full factorial design where pesticide exposure was tested in combination with all the different levels of predation risk. Thus, for every predation risk level (no risk, low and high) we tested three concentrations of imidacloprid: 1.9, 3.9 and 7.8 μg/L plus the control treatment for *S. vittatum* and 0.4, 1.2 and 3.7 μg/L plus the control treatment for *C. riparius*. Due to the limited number of syringes available, these experiments were performed in two runs over two consecutive days for *C. riparius* and three runs over three consecutive days for *S. vittatum* in a randomised block design.

2.8. Behavioural endpoints

The effects of imidacloprid and perceived risk of predation on burrowing behaviour of insect larvae were assessed at day 6 (for *C. riparius*) and day 3 (*S. vittatum*). The observations were conducted for a 15 min period in each treatment assessing the percentage of live organisms visible on top of sediment or drifting in the water column relatively to the initial number of organisms.

2.9. Statistical analysis

EC50 values for mortality (measured as immobilization) were calculated using the probit method (Minitab, 2000). For all other experiments, two-way ANOVAs were performed using imidacloprid concentrations and chemical cues as treatments. For respiration rates, day of measurement was blocked as a random factor. Whenever significant differences were observed Dunnett post hoc test was used for multiple comparisons to determine which treatments were significantly different from the control. Data from *S. vittatum* feeding experiments were log (x + 1) transformed to stabilise variances across treatments (Zar, 1996). The behavioural data was also Arcsine ($\sqrt{x}$) transformed. All statistical analyses were performed using the Minitab 13.0 statistical package (Minitab, 2000).

To address the responses to the combination of the two stressors, the observed effects on *S. vittatum* feeding and on growth (length and head width), and respiration of *C. riparius* were compared to the expected effects of stressors combinations calculated from effects of single compound exposures. This procedure is usually based on already described conceptual models: concentration addition (CA) and independent action (IA) (Backhaus et al., 2004; Jonker et al., 2005; Ferreira et al., 2008). Both concepts allow us to calculate expected mixture toxicity on the basis of known individual toxicities of the mixture components taking into consideration the chemicals' pharmacological mode of action. However, it has been suggested to consider the ecotoxicological mode of action of chemicals when considering organismal responses such as effects on physiological (e.g. feeding) and life-history traits (Barata et al., 2007).

Although the mechanism of action of predatory cues is unknown; imidacloprid and predation risk can impair food acquisition and affect respiration rates due to effects on behaviour of insects. Although we can consider that they share a common ecotoxicological mode of action (sensu Barata and Baird, 2000) both conceptual models were applied to our data.

For the IA conceptual model the fit to our data was made using Eq. (1):

$$Y = \mu \max \prod_{i=1}^{n} q_i(C_i) \quad (1)$$

(Ferreira et al., 2008)

where Y denotes the biological response, C_i is the concentration of chemical i in the mixture, $q_i(C_i)$ the probability of non-response, μ_{max} the control response for the selected endpoint and $\prod$ the multiplication function.

For the CA model fit, Eq. (2) was applied, where C_i is the concentration used for stressor i in the mixture and EC_{xi} is the effect concentration of stressor i that produces the same effect (x%) as the whole mixture.

$$\sum_{i=1}^{n} C_i/EC_i = 1 \quad (2)$$

(Ferreira et al., 2008)

The procedure analysis suggested by Jonker et al. (2005) was used here for the analysis of feeding of *S. vittatum* as well as oxygen consumption and growth (head capsule width) of *C. riparius* since it permitted significance testing of model fit for both the IA model and CA model and also because the analysis takes into account different nonlinear concentration–response characteristics of stressors (Jonker et al., 2005).

This procedure allows the evaluation of deviations from reference models such as synergism, antagonism as well as concentration-ratio and concentration level deviations. Concentration level dependent deviation means that the "deviation from

either reference model at low dose levels is different from the deviation at high dose levels. For instance, antagonism may be observed at low dose levels and synergism at high dose levels" (Jonker et al., 2005).

Concentration-ratio dependent deviations arises when "the deviation from either reference model depends on the composition of the mixture. In the case of two substances, antagonism can be observed where the toxicity of the mixture is caused mainly by toxicant 1, whereas synergism can be observed where the toxicity is caused mainly by toxicant 2" (Jonker et al., 2005).

These deviations from the reference models were obtained by the addition of two parameters (a and b) and are tested within a nested framework, see Jonker et al. (2005). For reference models and their deviations the fitting process was conducted through a series of interactions performed in a spreadsheet environment using the built-in solver function (using the initial μ_{max} as the average response in control treatment). The models are fitted to the data using a maximum-likelihood method. The best fit is chosen using a Chi-square test which minimizes the objective function based on the log likelihood. The biological interpretation of these additional deviation parameters is described in Table 3. Here, average data from *S. vittatum* feeding and *C. riparius* growth experiments was used as a preliminary approach (Jonker et al., 2005) because the raw dataset showed a poor fit to the models. The complete dataset from *C. riparius* respiration experiments was used in the analysis of joint effects of the stressors tested. Here, data was transformed ($1/x$); μ_{max} being the reciprocal of the average response in the control treatment.

A different approach using the IA reference model was followed to calculate the predicted responses of both insect species when exposed to both stressors simultaneously. Estimated mean values were obtained directly from Eq. (1), using the maximum response from the dataset. Additive effects estimated by the reference IA model were compared to the average values observed and their respective 95% confidence intervals to infer significant antagonism or synergism for each mixture treatment. This approach was applied to compare results in terms of the sensitivity of the different modelling approaches and was also used to calculate the predicted responses of the mixture on the respiration rates of *S. vittatum*. For this latter parameter, the approach described by Jonker et al. (2005) was not applicable, as it relies on dose response curves which cannot be estimated if the stressors in the mixture cause contrasting effects (stimulation vs. inhibition), as was observed for *S. vittatum* respiration.

3. Results

3.1. C. riparius

The imidacloprid 96-h EC50 (95% CI) for *C. riparius* was 12.94 μg/L (9.74–18.22) with no predator cues and 14.06 μg/L (10.74–20.18) when simultaneously exposed to high concentration of predation cues. Although acute tests were conducted without sediment, no indication of cannibalism was noted and no mortality was observed in the control treatments in both experiments.

In the 10-day growth experiment, mortality was below 10% except in treatments with highest concentrations of imidacloprid, where mortalities of 80–100% were observed. These treatments were excluded from analysis of growth, behaviour and emergence. The concentrations of imidacloprid measured at the end of day 2, i.e. before partial renewal of medium, were 96% of initial concentrations for the lower concentration, 64% for the medium concentration and 40% for the highest of initial concentrations. The degradation of pesticide increased along the experimental period probably as a result of bacterial growth in vials due to continuous food addition which can greatly increase the degradation rate of imidacloprid (CCME, 2007). The analysis of imidacloprid concentrations on treatments with high levels of predation risk revealed no effect of chemical predatory cues on degradation of the pesticide.

Table 1
Two-Way ANOVA results for C. riparius experiments.

Factor	df	*F*	*P-value*	LOEC for IMI
C. riparius				
Total length				
[IMI]	2	5.20	0.010	1.2 μg/L
Predation cues	2	3.77	0.033	
[IMI] × predation cues	4	0.58	0.682	
Head capsule width				
[IMI]	2	9.19	0.001	1.2 μg/L
Predation cues	2	6.87	0.003	
[IMI] × predation cues	4	0.07	0.991	
Burrowing behaviour				
[IMI]	3	77.51	<0.001	3.7 μg/L
Predation cues	2	2.22	0.113	
[IMI] × predation cues	6	0.98	0.442	
Development rate				
[IMI]	2	22.86	<0.001	0.4 μg/L
Predation cues	2	9.03	0.001	
[IMI] × predation cues	4	0.19	0.941	
Emergence ratio				
[IMI]	2	4.62	0.016	1.2 μg/L
Predation cues	2	0.03	0.971	
[IMI] × predation cues	4	0.12	0.975	
Respiration				
[IMI]	3	61.79	<0.001	0.4 μg/L
Predation cues	2	9.62	0.001	
[IMI] × predation cues	6	0.72	0.677	

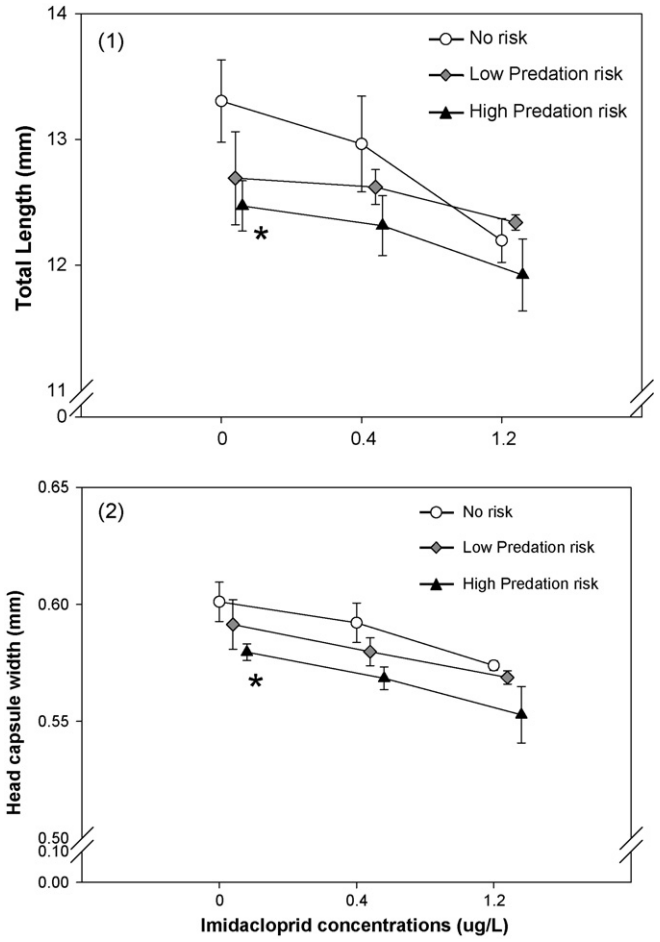
IMI—imidacloprid.

There was a reduction in *C. riparius* growth when exposed to sub-lethal concentrations of imidacloprid (Table 1, Figs. 1 and 2). NOECs and LOECs for imidacloprid were 0.4 and 1.2 μg/L, respectively. Growth of *C. riparius* larvae was also significantly affected by the presence of high levels of predator cue, with a decrease in the total length and head capsule width being observed. Emergence results revealed a significant reduction, relative to controls, in emergence ratio for larvae exposed to 1.2 μg/L imidacloprid (Table 1, Fig. 3), and a significant delay in time-to-emergence when larvae were exposed to 0.4 μg/L and to high levels of predation cue (Table 1, Fig. 4).

In the respirometry experiments, mortality was always below 10%. *C. riparius* showed a significant increase in their respiration rates when exposed to sub-lethal concentrations of imidacloprid (Table 1, Fig. 5), with LOEC values for imidacloprid of 1.2 μg/L.

Predation cues also significantly increased oxygen consumption in *C. riparius* (Table 1, Fig. 5). No significant effects was observed for day of measurement (ANOVA $F_{1,38} = 0.72$, $P = 0.405$). After six days of exposure to imidacloprid, *C. riparius* burrowing behaviour was also affected. A higher percentage of larvae were found on top of sediment (or in the water column) as a result of exposure to 3.7 μg/L of imidacloprid (Table 1). No statistical differences in the burrowing behaviour of *C. riparius* larvae were noted under exposure to varying concentrations of predation cues (Table 1, Fig. 6).

Concerning the prediction of joint effects of both stressors no statistically significant interaction of imidacloprid and predation cues were detected by analysis of variance for any of the parameters tested for *C. riparius* thus indicating additivity of effects (Table 1). The application of conceptual models held similar results. However, data from respiration measurements did not show a good fit to the CA reference model (SS = 47.9215; $r^2 = 0.420$; $P = 0.001$) but a significant concentration-ratio dependent deviation was found (SS = 18.8944; $r^2 = 0.772$; $P < 0.001$; $a = 0.821$; $b = -5.695$) meaning that synergism is observed where the toxicity of the mixture is caused mainly by imidacloprid (see Table 3). The shift between syn-



Figs. 1 and 2. Growth measurements of *C. riparius* under exposure to a gradient of imidacloprid concentrations and different levels of perceived risk of predation. Total length (1) and head capsule width (2) (mean ± SE) at day 10. Asterisks denote predation risk treatments that are significantly different ($P < 0.05$) compared to the control treatment (no predation).

ergism to antagonism occurs when [cues] = 0.2 [IMI], means that for the concentrations ratios used here, antagonism is only observed in the treatments with the lowest imidacloprid concentration and the highest predation risk. Using the IA approach data also showed a

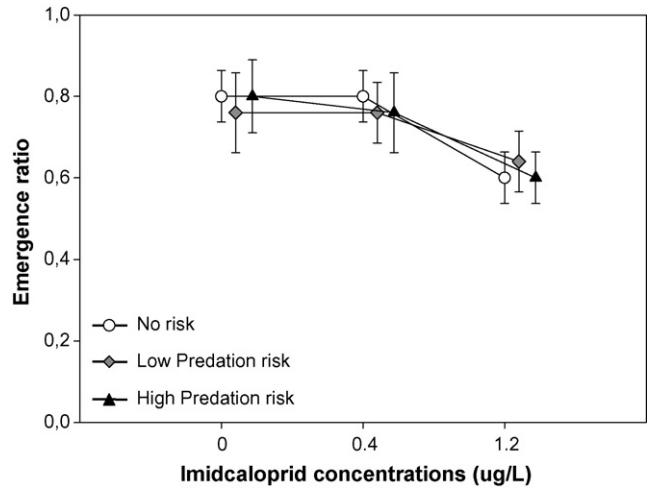


Fig. 3. Mean emergence ratio (mean ± SE) of *C. riparius* under exposure to a gradient of imidacloprid concentrations and different levels of perceived risk of predation.

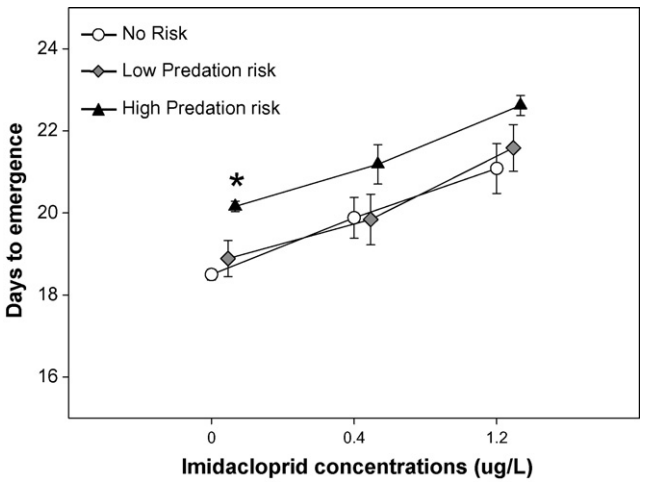


Fig. 4. Development time (1/development rate) (mean ± SE) of *C. riparius* under exposure to a gradient of imidacloprid concentrations and different levels of perceived risk of predation. Asterisks denote predation risk treatments that are significantly different ($P < 0.05$) compared to the control treatment (no predation).

weak fit to the reference model (SS = 43.9189; $r^2 = 0.469$; $P < 0.001$) also with a significant concentration-ratio dependent deviation (SS = 19.1956; $r^2 = 0.768$, χ^2 test, $P < 0.001$; $a = 1.769$; $b = -1294$) where synergism is observed where the toxicity of the mixture is caused mainly by imidacloprid. The ratio at which synergism shifted to antagonism was not possible to calculate, means that the CA approach is probably more suitable for this type of data.

For *C. riparius* respiration data, the comparison between the observed data and the mean estimated responses calculated directly using the IA reference model for each combination treatment, revealed a significant deviation from additivity (synergism) for the treatment with high concentrations of both stressors (Fig. 10C).

Concerning the prediction of joint effects of both stressors on *C. riparius* growth, average data (head capsule width measurements) adequately fitted the IA reference model (SS < 0.0001; $r^2 = 0.966$; $P < 0.05$) and also the CA approach (SS < 0.0001; $r^2 = 0.951$, $P < 0.05$) with no significant deviations found. Additive effects of imidacloprid and perceived predation risk on head capsule width of *C. riparius* were also observed in the comparison between

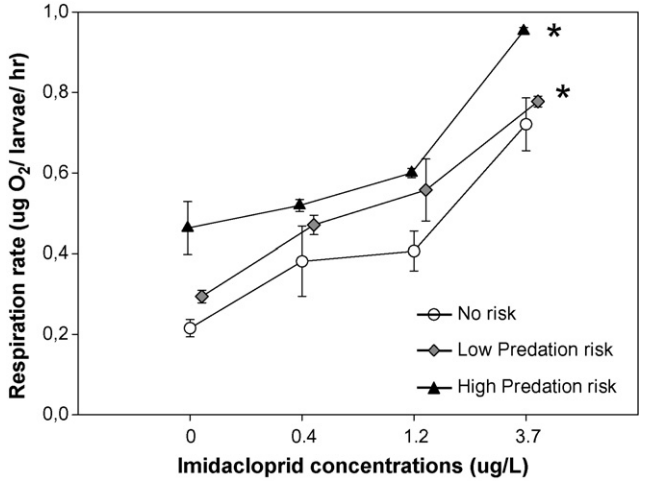


Fig. 5. Respiration rate (mean ± SE) of *C. riparius* under exposure to a gradient of imidacloprid concentrations and different levels of perceived risk of predation. Asterisks denote predation risk treatments that are significantly different ($P < 0.05$) compared to the control treatment (no predation).

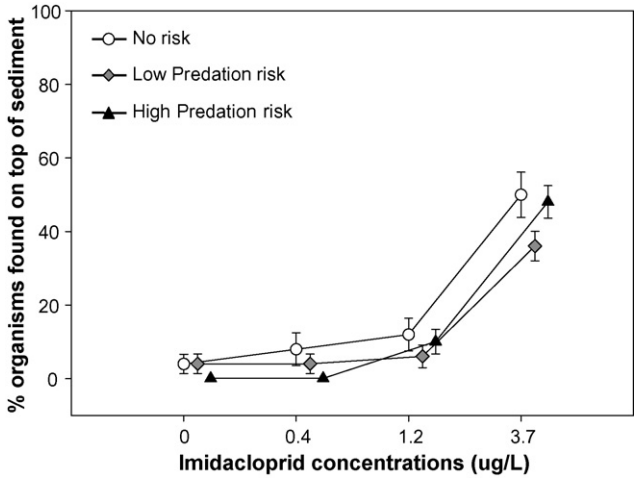


Fig. 6. *C. riparius* burrowing behaviour under exposure to a gradient of imidacloprid concentrations and different levels of perceived risk of predation. The observations of number of larvae visible on top of sediment (mean ± SE), were taken at day 6 for a 15 min period for each treatment and dead organisms were excluded from the analysis.

the observed data and the mean estimated responses calculated directly using the IA reference model for each mixture treatment (Fig. 10D). These results are also in concordance with the results from analysis of variance where no significant interaction between stressors was found (Table 1).

3.2. *S. vittatum*

The imidacloprid 96-h EC50 (95% CI) for *S. vittatum* was 47.22 μg/L (34.17–70.74) with no predator cues and 35.86 μg/L (25.47–52.15) when simultaneously exposed to high concentration of predation cues.

In the feeding and respiration experiments, observed mortality was always below 10% with the exception of the feeding experiment treatment of 1.9 μg/L + high level of predation cues, in which mortality was 20%. The concentrations of imidacloprid at the end of day 2, i.e. before partial renewal of medium, were checked and the level corresponded to 66–63% of initial concentrations. The same relative concentrations were measured at the end of the experimental period. The analysis of imidacloprid concentrations on treatments with high levels of predation risk revealed no effect of chemical predatory cues on degradation of the pesticide.

Percentage of leaf weight loss in the replicates without organisms was low: 2.82% ± 2.28 (SD) with no significant differences or trend observed in control replicates across treatments revealing no effects of imidacloprid or predation cues on microbial decomposition of leaves. Nevertheless, leaf weight loss in these control replicates was used as correction factors in each respective experimental treatment.

The feeding rate of *S. vittatum* was significantly reduced under exposure to sub-lethal concentrations of imidacloprid (Table 2 Fig. 7). Feeding-related NOEC and LOEC values for imidacloprid were 1.9 and 3.9 μg/L, respectively.

S. vittatum showed reductions in oxygen consumption when exposed to sub-lethal concentrations of imidacloprid, but in contrast, significant increases in respiration rates were observed under exposure to predation cues (Table 2 Fig. 8f) NOEC and LOEC values for imidacloprid were 3.9 and 7.8 μg/L, respectively. No significant effects were observed for day of measurement (ANOVA $F_{1,64} = 0.45$, $P = 0.640$).

Sub-lethal concentrations of the insecticide also affected the burrowing behaviour of *S. vittatum* with a significantly higher percentage of larvae found on top of the sediment as a result of exposure to the highest concentration of imidacloprid (Table 2, Fig. 9). No statistical differences were observed in the burrowing behaviour of *S. vittatum* larvae under exposure to different levels of

Table 2
Two-Way ANOVA results for *S. vittatum* experiments.

Factor	df	*F*	*p*	LOEC
S. vittatum				
Feeding				
[IMI]	3	22.90	<0.001	3.9 μg/L
Predation cues	2	0.70	0.501	
[IMI] × predation cues	6	0.64	0.701	
Respiration				
[IMI]	3	10.99	<0.001	3.9 μg/L
Predation cues	2	4.26	0.020	
[IMI] × predation cues	6	1.16	0.342	
Burrowing behaviour				
[IMI]	3	11.89	<0.001	7.8 μg/L
Predation cues	2	1.02	0.363	
[IMI] × predation cues	6	0.15	0.989	

IMI—imidacloprid.

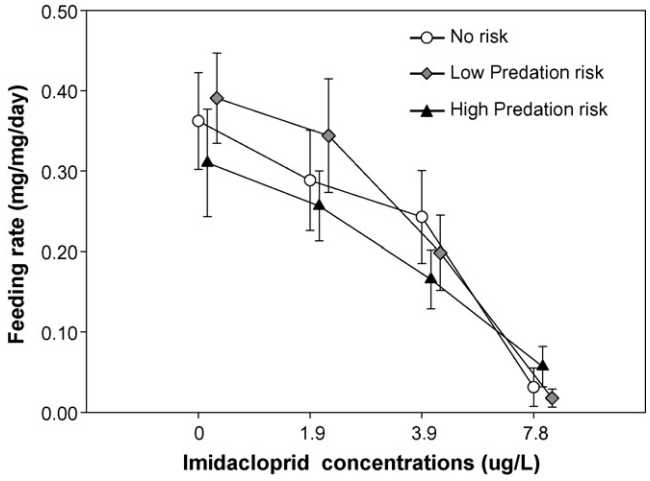


Fig. 7. Feeding rate (mean ± SE) of *S. vittatum* under exposure to a gradient of imidacloprid concentrations and different levels of perceived risk of predation.

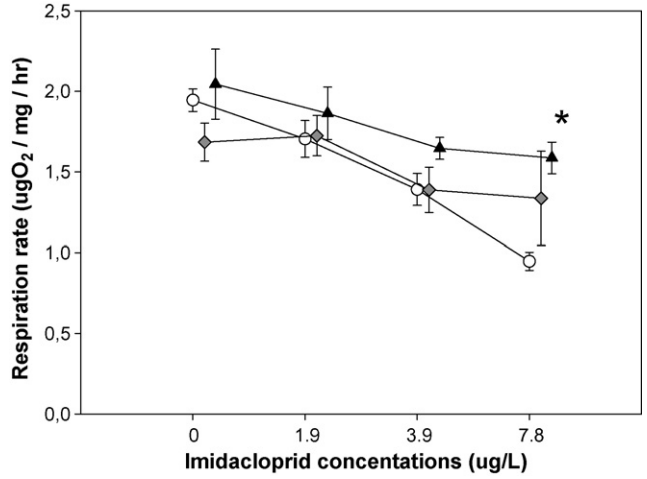


Fig. 8. Respiration rate (mean ± SE) of *S. vittatum* under exposure to a gradient of imidacloprid concentrations and different levels of perceived risk of predation. Asterisks denote predation risk treatments that are significantly different ($P < 0.05$), compared to the control treatment (no predation).

Table 3
Interpretation of additional parameters substituted into the concentration addition (CA) and independent action (IA) reference models that define the functional form of the deviation pattern adapted from Jonker et al., 2005.

Deviation pattern	Parameter *a* (CA and IA)	Parameter *b* (CA)	Parameter *b* (IA)
Synergism/antagonism	$a > 0$: antagonism $a < 0$: synergism		
Dose-ratio dependent (DR)	$a > 0$: antagonism except for those mixture ratios where negative b value indicate synergism $a < 0$: synergism except for those mixture ratios where positive b value indicate antagonism	$b_i > 0$: antagonism where the effect of the mixture is caused mainly by toxicant *i* $b_i < 0$: synergism where the effect of the mixture is caused mainly by toxicant *i*	
Dose-level dependent (DL)	$a > 0$: antagonism low dose level and synergism high dose level $a < 0$: synergism low dose level and antagonism high dose level	$b_{DL} > 1$: change at lower EC50 level $b_{DL} = 1$: change at EC50 level $0 < b_{DL} < 1$: change at higher EC50 level $b_{DL} < 1$: No change but the magnitude of S/A is DL dependent	$b_{DL} > 2$: change at lower EC50 level $b_{DL} = 2$: change at EC50 level $1 < b_{DL} < 2$: change at higher EC50 level $b_{DL} < 1$: No change but the magnitude of S/A is effect level dependent

predation cues (Table 2). Nevertheless, results indicated that caddis larvae exposed to predation cues appeared to remain hidden in the sediment for longer periods of time since more larvae were observed on top of the sediment in the treatments where no cues were added.

Concerning the prediction of joint effects of both stressors, average data from feeding experiment with *S. vittatum* adequately fitted the IA reference model (SS = 0.0093; $r^2 = 0.949$; $P < 0.001$) and also the CA approach (SS = 0.0094; $r^2 = 0.948$, $P < 0.001$) with no significant deviations found. Additive effects of imidacloprid and perceived predation risk on feeding rates of *S. vittatum* were also observed in the comparison between the observed data and the mean estimated responses calculated directly using the IA reference model for each mixture treatment (Fig. 10A). These results are also in concordance with the results from analysis of variance where no significant interaction between stressors was found (Table 2).

For *S. vittatum* respiration data the analysis of variance could not detect a significant interaction between stressors (Table 2), but the average values observed were higher than the estimated responses calculated directly using the IA in all treatments (Fig. 10B). A significant deviation from additivity was found in the treatment with the highest concentrations of both stressors in the comparison between the observed data and the mean estimated responses (Fig. 10B).

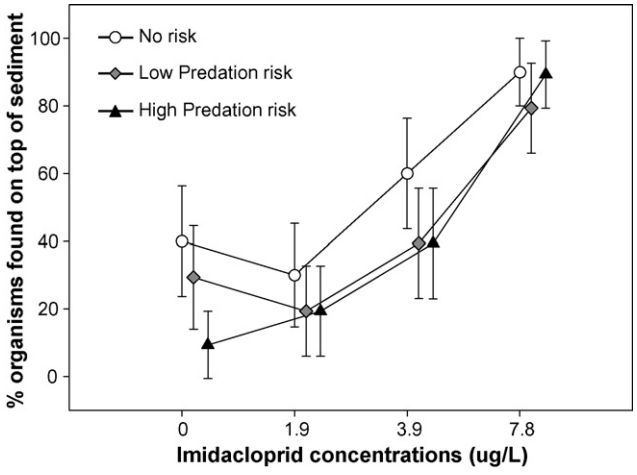


Fig. 9. *S. vittatum* burrowing behaviour under exposure to a gradient of imidacloprid concentrations and different levels of perceived risk of predation. The observations of number of larvae visible on top of sediment (mean ± SE), were taken at day 3 for a 15 min period for each treatment. Dead organisms were excluded from the analysis.

4. Discussion

Since inland waters commonly exhibit at least some degree of pesticide contamination, and since predation has a major impact on prey communities, populations and individual traits, it is prudent to study the effects of pesticides and predation to aquatic biota and the consequences of their interaction. Assessing the effects of pesticides in organisms facing different levels of predation risk is important to reduce the uncertainty surrounding pesticide effects on natural populations and communities and thus facilitate the extrapolation of laboratory ecotoxicological test results to natural ecosystems (Hanazato, 2001, Relyea et al., 2005).

In the presence of predators like fish, the energy budget of aquatic insect larvae can be affected with possible important consequences in terms of growth and reproduction (Ball and Baker, 1996; Peckarsky et al., 2002; Dahl and Peckarsky, 2003a; Benard, 2004; Holker and Stief, 2005). The energy budget of organisms has also been used in ecotoxicology to assess the sub-lethal effects of different types of contaminants (Maltby, 1999; De Coen and Janssen, 2003). Low levels of contaminants have been previously shown to cause alterations in feeding and respiration rates of many species with potential consequences for growth, reproduction and survival (Widdows, 1985; Soucek, 2006). In turn, such effects can propagate through higher levels of organisation, to populations, communities and ecosystems (Maltby, 1999)

Imidacloprid is a systemic insecticide that has been previously shown to have potentially harmful effects on aquatic non-target insects (Alexander et al., 2007; Stoughton et al., 2008). Our results support these previous studies, showing that imidacloprid is acutely toxic to *C. riparius* and *S. vittatum* at low concentrations. Although the acute tests performed did not include sediment, the EC_{50} (immobilization) found for both species was within the range of imidacloprid lethal concentrations observed for other insect species (Song et al., 1997; Alexander et al., 2007; Stoughton et al., 2008) and near concentrations measured in natural systems in the case of *C. riparius*. *S. vittatum* showed a relatively higher tolerance to imidacloprid than *C. riparius.*

Low imidacloprid levels have been shown to induce several behavioural responses such as reduced activity and uncontrolled muscular contractions, which can limit foraging activity of aquatic insects and consequently impair feeding and growth (Alexander et al., 2007). This was also the case for the species tested here. Growth, development rates and emergence ratio of *C. riparius* were significantly reduced by exposure to low concentrations of imidacloprid. *S. vittatum* feeding rates were also significantly reduced with exposure to sub-lethal concentrations of imidacloprid.

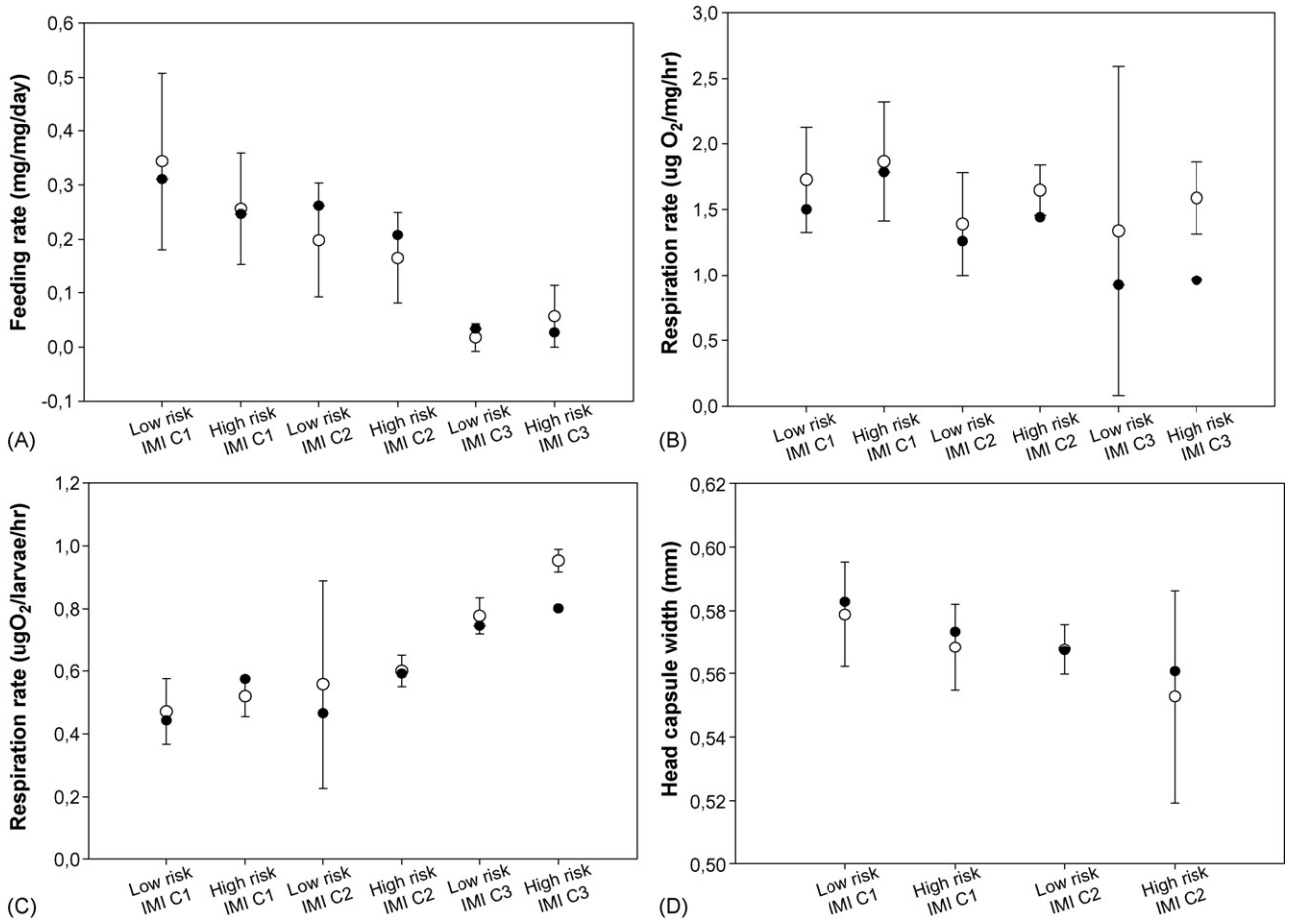


Fig. 10. Effects of combined exposures to imidacloprid and different levels of perceived risk of predation on *S. vittatum* feeding (A) and respiration rate (B), and on *C. riparius* respiration rate (C) and growth (head width) (D). Empty symbols denote observed responses (mean and 95% confidence intervals) and filled symbols represent effects predicted by independent action reference model.

The respiration of insect larvae was also affected by imidacloprid, and we observed increased respiratory rates in *C. riparius*, likely a reflection of uncontrolled muscular activity, which increases energy costs. Curiously, imidacloprid exposure induced a reduction in the respiratory rates of *S. vittatum*. We hypothesize that the different responses in terms of respiration patterns were due to different behavioural responses of *C. riparius* and *S. vittatum* larvae when exposed to imidacloprid in the respirometers (gastight syringes). It is important to reinforce also that respiratory experiments were done with no food, no sediment and only for a 24 h exposure period. In response to the lack of sediment, larvae of each species respond differently: *S. vittatum* larvae showed low locomotor activity, whereas *C. riparius* larvae increased activity by continuously swimming in the water column. Furthermore, in these respirometry experiments, we exposed *S. vittatum* larvae with their cases but chironomids were exposed without their tubes. Cased caddisflies use body undulations to pump water through their cases to promote efficient ventilation by restricting and directing water flow (Merrit and Cummins, 1996). Since we failed to observe uncontrolled muscular contractions in *S. vittatum* exposed to the imidacloprid concentrations tested, we hypothesize that reduced respiration rates with increasing insecticide concentration might be due to a disruption of these ventilatory movements together with decreased locomotor activity. Nevertheless, the measured respiration rates in the control treatment are in the range of those observed before for this species in laboratory flow-through respirometry experiments (Feio and Graça, 2000). *C. riparius* larvae on the other hand, having no sediment to burrow into, swam continuously, which, together with continuous stimulation of the nervous systems (mild tremors) caused by exposure to imidacloprid, led to higher respiration rates reflecting increased metabolic costs. Thus, differing behavioural responses of the two species in response to different concentrations of imidacloprid and the lack of sediment substrate might explain the apparent contradictory effects of imidacloprid on respiration of *C. riparius* and *S. vittatum*.

C. riparius responded to predation cues in a concentration-dependent manner, i.e responses were stronger when higher concentration of chemical cues from predators and macerated conspecifics were present. This implies that *C. riparius* could detect cue concentrations, leading to perceived differences in levels of predation risk. This is in accordance with empirical results showing that prey species adjust the level of responses to the perceived level of predation risk (McIntosh and Peckarsky, 2004; Holker and Stief, 2005). The combination of chemical cues from predators, or kairomones, with alarm substances from alarmed, injured or dead conspecifics has also been shown to produce a stronger response and to be a more reliable cue in terms of assessment of threat and level of risk to different prey species (Schoeppner and Relyea, 2005; Laforsch et al., 2006; Beketov and Liess, 2007).

C. riparius were significantly smaller, and emergence was delayed after 10 days of exposure to high levels of predation cues. As previously shown in chironomids, reductions in activity and the consequent decrease in time spent foraging can impair growth and development rates under high levels of predation risk (Noonburg and Nisbet, 2005). Our results support the view that elevated respiration rates arising from increased awareness of risky situations

is a sensitive response to the presence of predators. The trade-off between energy gain and mortality risk is thus apparent in *C. riparius* under the risk of predation simulated by exposure to non-lethal cues, kairomones from trout combined with alarm substances from conspecifics.

S. vittatum feeding rates observed under different levels of perceived predation risk were not statistically different from the control (no chemical cues added), although a non-significant decrease in feeding rates was noted when larvae were exposed to high concentrations of predatory chemical cues. Lower feeding rates observed in organisms exposed to high perceived risk of predation were possibly a consequence of reduced activity, also observed as a response to chemical cues from fish in other cased caddisfly species (Kuhara et al., 2001). The lack of statistically significant results here could indicate a lower responsiveness to presence of predators in this species. A lack of responses to non-lethal cues from fish has been observed before for caddisflies species (Kohler and McPeek, 1989; Lefcort et al., 2000), and could be a consequence of their defensive armouring. The mineral cases of cased caddisflies constitute an effective antipredator defence, and can be considered as armour that not only camouflages larvae but also increases the handling time for predators (Kuhara et al., 1999, Boyero et al., 2006). Furthermore, they show low locomotor activity and are normally found hidden under the sediment or organic material, and are usually more active at night, all of which can be considered behavioural adaptations to avoid predators. This lifestyle may explain the weaker feeding response of caddisflies when under fish predation risk (Kuhara et al., 2001).

In our experiments with *S. vittatum,* respiration was a more sensitive measure of effects of the predatory chemical cues than feeding. Respiration rates of *S. vittatum* larvae increased under high levels of perceived predation threat and as for chironomids, we hypothesise that this response is due to the increased alertness exhibited by organisms under risky situations (Beckerman et al., 2007). Higher nocturnal respiration rates of *S. vittatum* were previously shown to be as consequence of higher activity shown by larvae at dark possibly to avoid competitors and predators (Feio and Graça, 2000). Here, the respiratory experiments were performed with no light so it remains unclear if *S. vittatum* shows stronger behavioural responses to chemical cues from predators during the day.

Although we did not measure growth or emergence in *S. vittatum*, it is likely that respiration (as an indication of metabolism) and feeding results could be used cautiously to infer potential growth impairment in *S. vittatum*. However, it should be kept in mind that other factors such changes in assimilation efficiency could complicate this interpretation (Stoks, 2001).

We failed to observe any significant interaction in the combined exposures for most parameters evaluated in these two species. However, in the sub-lethal exposures, significant deviations from the conceptual models were found for *C. riparius* respiration rates in treatments with the highest imidacloprid concentrations. This reveals that exposure to this insecticide can increase the metabolic costs of antipredator responses of insect larvae. For *S. vittatum*, the lack of statistically significant results, in the case of feeding response to predation cues, and contrasting responses to different stressors (in the case of respiration), complicate our interpretation of the interaction between these major stressor classes.

The assessment of joint effects of these stressors with conceptual models generally agreed with results of analysis of variance which showed no significant interaction between the two stressors. Although only additive effects were observed for growth and development rates of chironomids, it is possible that effects of imidacloprid under predation pressure could be stronger in terms of reproductive fitness of insects through reductions of size at emergence that although not measured here, have been observed in aquatic species in response to perceived risk of predation (Peckarsky et al., 2001) and also to sub-lethal concentrations of imidacloprid (Alexander et al., 2008).

Moreover it was demonstrated how mathematical approaches usually used to assess effects of chemical mixtures can be employed to predict the joint effects of these two different stressors. The fact that the models used here, based on concentration response curves, detected deviation patterns from additivity that are not limited to just synergism or antagonism (such as dose ratio dependent deviation for *C. riparius* respiration) is an indication that the effects of pesticides under different levels of perceived risk of predation can change considerably according to the level of both stressors and also their ratio in the mixture. A better calibration of models is also necessary with the assessments of responses to more concentrations of each stressor and of their combinations.

With the ecotoxicological mode of action in mind, our results suggest that concentration addition and independent action conceptual approaches can be applied to different parameters measured at the organism level to predict the responses to combinations of pesticides and biotic natural stressors.

Reductions in activity leading to reduced food intake mediate the effects of perceived predation risk on larvae growth in insects with complex life cycles and that do not feed as adults (Ball and Baker, 1996) Here, *C. riparius* data showed these behavioural mediated life-history changes with an increase in predation risk resulting in slower growth and also slower development rates. Because the toxicity of imidacloprid is also manifested through feeding impairments, these two stressors share a common ecotoxicological mode of action being responses mediated through behaviour (Ball and Baker, 1996). However other insects species have shown to increase development rates at the cost of size at maturity in response to fish predation risk (Dahl and Peckarsky, 2003b) suggesting a possible physiological basis for the antipredator response. Because sub-lethal concentrations of pesticides have also been shown to affect the size of emergent insects (Alexander et al., 2008), it is important to expand the mechanistic study of effects of combined exposure to pesticides and perceived predation risk by measuring different endpoints (digestive physiology, energetic reserves, size at maturity, fecundity) over a wider range of pesticide concentrations.

To better understand and evaluate the effects of predation risk on insect population dynamics it is also crucial to consider other environmental variables such as food deficiency or time stress that can change the magnitude of predator sub-lethal effects (Stoks et al., 2005; Beketov and Liess, 2007). Moreover, the use of *C. riparius* laboratory cultures with probable high levels of inbreeding and thus possibly genetically impoverished (Nowak et al., 2008), calls for the evaluation of imidacloprid and predation cues effects on natural populations of *C. riparius* thus accounting for intraspecific variation in life-history traits caused by a differential response to the different stressors.

The results presented above provide evidence that pesticide contamination can have enhanced negative effects on aquatic insects under the perceived presence of predators. Predator-avoidance behaviour by aquatic insect larvae can result in reductions in time spent feeding, with consequences for rates of growth and development (McPeek and Peckarsky, 1998). Behaviour that results in reduced growth and/or changes in development time may be particularly important for insects with short adult stages such as *C. riparius* and *S. vittatum* because fecundity is determined by the size of larva upon metamorphosis (Peckarsky et al., 2001). If we consider that many of these organisms under predation pressure can also be exposed to pesticides and that sub-lethal concentrations of pesticides can also affect energy intake or expenditure, it is clear that further reductions in growth rate and/or size upon emergence could have significant consequences for reproductive success and thus influence population dynamics and viability.

Our results further suggest the possibility for additional indirect effects of pesticides on susceptibility to predation: since imidacloprid significantly impairs the burrowing behaviour of larvae of both species, this in itself could further increase the risk of mortality from fish predation (Schulz and Dabrowski, 2001).

5. Conclusion

In this study we have assessed the effects of sub-lethal concentrations of imidacloprid on two insect species under different levels of predation risk. The experimental designs employed closely matched methods commonly used in ecotoxicological hazard assessment and clearly demonstrated responses of *C. riparius* and *S. vittatum* to environmental relevant concentration of imidacloprid (Confidor®). The responses were in accordance with the neuromuscular impairment observed with exposure to neonicotinoids which is translated in depressions of feeding and growth. Behaviour was also affected by exposure to imidacloprid and contradictory responses comparing the two species were observed for respiration rates.

Sub-lethal effects of perceived predation risk were also observed in both species and were found to occur even under exposure to imidacloprid.

Analysis of variance and conceptual models normally used for the evaluation of contaminant mixture exposure showed significant effects of imidacloprid in most of the parameters analysed and showed a relatively good agreement to the combined effects of both stressors. No interaction and thus only additive effects were found for most parameters. The conceptual models used have nevertheless identified significant deviations from additivity concerning respiration of both species.

Previous studies (Barata and Baird, 2000; Barata et al., 2007) have indicated the need for a paradigm shift in ecological risk assessment, moving away from narrow, toxicological definitions of stressor modes of action towards broader, more encompassing definitions of ecologically relevant stressor effects. The results discussed above further advance this approach by demonstrating that 'natural stressors' such as predation and pesticides can be considered 'similarly-acting' in an ecological sense and thus can be studied employing similar experimental approaches. Moreover, treating natural and anthropogenic stressors within the same framework can yield compatible data for modelling, allowing improved interpretation of ecological effects within a broader ecosystem context.

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Effects of neonicotinoids and fipronil on non-target invertebrates

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Abstract We assessed the state of knowledge regarding the effects of large-scale pollution with neonicotinoid insecticides and fipronil on non-target invertebrate species of terrestrial, freshwater and marine environments. A large section of the assessment is dedicated to the state of knowledge on sublethal effects on honeybees (*Apis mellifera*) because this important pollinator is the most studied non-target invertebrate species. Lepidoptera (butterflies and moths), Lumbricidae (earthworms), Apoidae sensu lato (bumblebees, solitary bees) and the section "other invertebrates" review available studies on the other terrestrial species. The sections on freshwater and marine species are rather short as little is known so far about the impact of neonicotinoid insecticides and fipronil on the diverse invertebrate fauna of these widely exposed habitats.

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For terrestrial and aquatic invertebrate species, the known effects of neonicotinoid pesticides and fipronil are described ranging from organismal toxicology and behavioural effects to population-level effects. For earthworms, freshwater and marine species, the relation of findings to regulatory risk assessment is described. Neonicotinoid insecticides exhibit very high toxicity to a wide range of invertebrates, particularly insects, and field-realistic exposure is likely to result in both lethal and a broad range of important sublethal impacts. There is a major knowledge gap regarding impacts on the grand majority of invertebrates, many of which perform essential roles enabling healthy ecosystem functioning. The data on the few non-target species on which field tests have been performed are limited by major flaws in the outdated test protocols. Despite large knowledge gaps and uncertainties, enough knowledge exists to conclude that existing levels of pollution with neonicotinoids and fipronil resulting from presently authorized uses frequently exceed the lowest observed adverse effect concentrations and are thus likely to have large-scale and wide ranging negative biological and ecological impacts on a wide range of non-target invertebrates in terrestrial, aquatic, marine and benthic habitats.

Keywords Pesticides · Neonicotinoids · Fipronil · Non-target species · Invertebrates · Honeybee · Earthworms · Butterflies · Freshwater habitat · Marine habitat

Introduction

Neonicotinoids and fipronil are relatively new, widely used, systemic compounds designed as plant protection products to kill insects which cause damage to crops. They are also used in veterinary medicine to control parasites such as fleas, ticks and worms on domesticated animals and as pesticides to control non-agricultural pests. Other papers in this special issue have shown that neonicotinoid insecticides and fipronil are presently used on a very large scale (e.g. Simon-Delso et al. 2014, this issue), are highly persistent in soils, tend to accumulate in soils and sediments, have a high runoff and leaching potential to surface and groundwater and have been detected frequently in the global environment (Bonmatin et al. 2014, this issue). Effects of exposure to the large-scale pollution with these neurotoxic chemicals on non-target insects and possibly other invertebrates can be expected as identified for other insecticides. However, for the majority of insect and other invertebrate species that are likely to be exposed to neonicotinoids and fipronil in agricultural or (semi)natural ecosystems, no or very little information is available about the impact of these pesticides on their biology. Here we assess the present state of knowledge on effects on terrestrial and aquatic invertebrates.

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Terrestrial invertebrates

Honeybees

Many studies have focused on investigating the effects of neonicotinoids and fipronil on honeybees (*Apis mellifera*). Apart from its cultural and honey production value, the honeybee is the most tractable pollinator species and critical for the production of many of the world's most important crops (Klein et al. 2007; Breeze et al. 2011). Losses of honeybees are generally measured as winter loss on national to regional level, and indications are that honeybee populations undergo high losses in many parts of the world (Oldroyd 2007; Stokstad 2007; van Engelsdorp and Meixner 2010; Van der Zee et al. 2012a, b).

No single cause for high losses has been identified, and high losses are associated with multiple factors including pesticides, habitat loss, pathogens, parasites and environmental factors (Decourtye et al. 2010; Mani et al. 2010; Neumann and Carreck 2010; Kluser et al. 2011). Apart from direct biotic and abiotic factors, changes in honeybee populations also depend on the economic value of honeybees and thus on human effort (Aizen and Harder 2009; Mani et al. 2010). Neonicotinoids are among the most used insecticides worldwide and are thus prime targets for investigating possible relationships with high honeybee losses.

Acute and chronic lethal toxicity to honeybees

Neonicotinoids and fipronil show high acute toxicity to honeybees (Table 1). The neonicotinoid family includes imidacloprid, clothianidin and thiamethoxam (the latter is metabolized to clothianidin in the plant and in the insect). Imidacloprid, clothianidin and thiamethoxam belong to the nitro-containing neonicotinoids, a group that is generally more toxic than the cyano-containing neonicotinoids, which includes acetamiprid and thiacloprid. Although neonicotinoids

Table 1 Toxicity of insecticides to honeybees, compared to DDT. Dose used is given in gram per hectare, median lethal dose (LD_{50}) is given in nanogram per bee. The final column expresses toxicity relative to DDT (DDT is 1). Source: Bonmatin (2011)

Pesticide	®Example	Main use	Typical dose (g/ha)	Acute LD_{50} (ng/bee)	Ratio of LD_{50} as compared to DDT
DDT	Dinocide	Insecticide	200–600	27,000	1
Thiacloprid	Proteus	Insecticide	62.5	12,600	2.1
Amitraz	Apivar	Acaricide	–	12,000	2.3
Acetamiprid	Supreme	Insecticide	30–150	7,100	3.8
Coumaphos	Perizin	Acaricide	–	3,000	9
Methiocarb	Mesurol	Insecticide	150–2,200	230	117
Tau-fluvalinate	Apistan	Acaricide	–	200	135
Carbofuran	Curater	Insecticide	600	160	169
Λ-cyhalotrin	Karate	Insecticide	150	38	711
Thiametoxam	Cruiser	Insecticide	69	5	5,400
Fipronil	Regent	Insecticide	50	4.2	6,475
Imidacloprid	Gaucho	Insecticide	75	3.7	7,297
Clothianidin	Poncho	Insecticide	50	2.5	10,800
Deltamethrin	Decis	Insecticide	7.5	2.5	10,800

are applied as foliar insecticides with possible direct exposure risks to honeybees, a large part of neonicotinoid use consists of seed coating or root drench application. Fipronil belongs to the phenylpyrazole family of pesticides and, like the neonicotinoids, has systemic properties (Simon-Delso et al. 2014).

Given that neonicotinoids and fipronil act systemically in plants, oral lethal doses for honeybees have been extensively studied for these compounds. Unlike many older classes of insecticides, neonicotinoids may be more toxic when ingested (Suchail et al. 2001; Iwasa et al. 2004). The level of neonicotinoids and fipronil that honeybees are exposed to in the nectar and pollen of treated plants varies greatly, although there are trends based upon application method. Generally, soil drenches and foliar application result in higher concentrations of the active compounds in plants than seed treatments, with the latter application used in large, annual cropping systems like grain crops, cotton and oilseed crops.

In practice, the honeybee lethal dose 50 (LD_{50}) for these pesticides varies for a wide range of biotic and abiotic conditions. The LD_{50} of imidacloprid, for example, has shown values between 3.7 and 40.9, 40 and 60, 49 and 102 and 490 ng/bee (Nauen et al. 2001; Schmuck et al. 2001; Suchail et al. 2001; DEFRA 2007, 2009). This variation, of a factor 100 (5–500 ng/bee), has been observed not only between colonies but also among bees taken from a single colony. A major component of this observed variation likely stems from the discrepancy in the contact and oral toxicities of these compounds, with contact lethal doses generally being higher than oral lethal doses. However, contact with the floral parts is frequent when the bees visit flowers, and this is different from the topical application used in laboratory conditions.

Other sources of variability may be attributed to differences in environmental conditions during testing as well as any inherent differences in the condition of the tested bees themselves. For example, data have shown that measured LD_{50} values for bees vary with temperature (Medrzycki et al. 2011), the age of bees (Schmuck 2004; Medrzycki et al. 2011), the honeybee subspecies tested (Suchail et al. 2000), the pattern of exposure (Illarionov 1991; Belzunces 2006) and prior exposure of bees to pesticides (Belzunces 2006). Given the large variability of honeybee toxicity data, it has been suggested that LD_{50} values should only be used to compare levels of toxicity among pesticides rather than drawing conclusions about the risk of mortality posed to honeybees via environmental exposure to pesticides (Belzunces 2006).

Oral subchronic exposure to imidacloprid and six of its metabolites induced a high toxicity at concentrations of 0.1, 1 and 10 ppb (part per billion) or ng/g, whereas the metabolites olefin-imidacloprid and 5-OH-imidacloprid were toxic in acute exposure. The main feature of subchronic toxicity is the absence of a clear dose–effect relationship that can account for a maximum effect of the lowest concentration due to the existence of multiple molecular targets, as has been demonstrated in the honeybee (Déglise et al. 2002; Thany et al. 2003; Thany and Gauthier 2005; Barbara et al. 2008; Gauthier 2010; Dupuis et al. 2011; Bordereau-Dubois et al. 2012). The absence of clear dose–effect relationships has also been observed in other studies, at higher concentrations (Schmuck 2004).

Existence of non-monotonic dose–response relations implies that some chemicals, including neonicotinoids,

have unexpected and potent effects at (very) low doses. These non-linear and often non-intuitive patterns are due to the complex interplay of receptor binding and gene reprogramming effects of such substances and can generate unexpected dose–response relationships, many of which are still being mapped out (Fagin 2012; Charpentier et al. 2014). This poses major challenges to risk assessment based on the classical log-probit model.

As previously reviewed by van der Sluijs et al. (2013), there are no standardised protocols for measuring chronic lethal effects. In traditional risk assessment of pesticides, they are usually expressed in three ways: LD_{50}: the dose at which 50 % of the exposed honeybees die (usually within a 10 day time span); no observed effect concentration (NOEC): the highest concentration of a pesticide producing no observed effect; and lowest observed effect concentration (LOEC): the lowest concentration of a pesticide producing an observed effect.

For imidacloprid, including its neurotoxic metabolites, lethal toxicity can increase up to 100,000 times compared to acute toxicity when the exposure is extended in time (Suchail et al. 2001). There has been some controversy on the findings of that study, which are discussed in detail by Maxim and Van der Sluijs (2007, 2013). However, the key finding that exposure time amplifies the toxicity of imidacloprid is consistent with later findings, implying that the standard 10 day chronic toxicity test for bees is far too short for testing neonicotinoids and fipronil, given their persistence and hence the likely chronic exposure of bees under field conditions. Indeed, honeybees fed with 10^{-1} of the LC_{50} of thiamethoxam showed a 41.2 % reduction of life span (Oliveira et al. 2013). Recent studies have shown that chronic toxicity of neonicotinoids can more adequately be expressed by time to 50 % mortality instead of by the 10 day LD_{50} (Sánchez-Bayo 2009; Maus and Nauen 2010; Tennekes 2010; Tennekes 2011; Tennekes and Sánchez-Bayo 2012; Mason et al. 2013; Rondeau et al. 2014). There is a linear relation between the logarithm of the daily dose and the logarithm of the time to 50 % mortality (Tennekes 2010, 2011; Tennekes and Sánchez-Bayo 2012; Tennekes and Sánchez-Bayo 2013; Rondeau et al. 2014). Sanchez-Bayo and Goka (2014) demonstrated that field-realistic residues of neonicotinoid insecticides in pollen pose high risk to honeybees and bumblebees, whilst in the field synergisms with ergosterol inhibiting fungicides will further amplify these risks. They found that imidacloprid poses the highest risk to bumblebees (31.8–49 % probability to reach the median lethal cumulative dose after 2 days feeding on field-realistic dose in pollen) and thiamethoxam the highest risk to honeybees (3.7–29.6 % probability to reach median lethal cumulative dose). In experiments with honeybee colonies, similar, long-term chronic effects have been found with typical times of 80–120 days for 1 ppm dinotefuran and 400 ppb clothianidin (Yamada et al. 2012). Note that these studies used concentrations that are on the uppermost limit of the currently reported ranges of concentrations found in pollen and nectar in the field. However, such data are sparse and limited to a few crops only, so it cannot yet be concluded whether such concentrations are rare or common in the field—the question of "field-relevant dose" is not yet fully resolved, and it is likely that there is a wide range in these values over space and time (Van der Sluijs et al. 2013).

Field and laboratory studies attempting to test field-realistic lethal doses have shown variable, often conflicting, results. In one study, chronic oral and contact exposure during 10–11 days to 1 μg/bee of acetamiprid and 1,000 μg/bee of thiamethoxam caused no significant worker mortality (Aliouane et al. 2009). Conversely, laboratory studies using imidacloprid showed high worker mortality when honeybees consumed contaminated pollen (40 ppb) (Decourtye et al. 2003, 2005) and contaminated sugar syrup (0.1, 1.0 and 10 ppb) (Suchail et al. 2001). These results were contrary to those of field studies performed by Schmuck et al. (2001), who reported no increased worker mortality when colonies were exposed to sunflower nectar contaminated with imidacloprid at rates from 2.0 to 20 μg/kg. Faucon et al. (2005) also found no worker mortality in a field study of honeybees fed imidacloprid in sugar syrup. A meta-analysis by Cresswell (2011) concluded that oral exposure to imidacloprid at realistic field concentrations did not result in worker mortality, although a subsequent study by Yamada et al. (2012) feeding a range of dinotefuran (1–10 ppm) and clothianidin (0.4–4 ppm) concentrations demonstrated colony failure within 104 days in each case, suggesting that detection of colony-level effects may require longer post-exposure observation.

Field studies to investigate the exposure of bees to pesticides face major difficulties. For the analysis of very low concentrations of compounds present in pollen, nectar, bees or other matrices, appropriate methods that meet validity criteria of quantitative analysis have to be developed. Pilling et al. (2013) exposed bees to thiamethoxam-treated maize and oilseed rape but were not able to quantify concentrations lower than 1 ppb, although this may be a result of the authors using a lower seed treatment application than is used in normal agricultural practice. Even though both treatment and control colonies experienced relatively high losses (mostly queens laying only drone brood) and the authors were unable to undertake any statistical analysis due to a lack of replication, they wrongly concluded that there is a low risk to honeybees from exposure to treated maize and oilseed rape.

Also, in terms of activity and feeding behaviour, bees might not be foraging on treated crops in (exactly) the same way as they would do on untreated crops (Colin et al. 2004). Furthermore, comparison of treated and control areas can be totally flawed because control fields might not be "clean" but treated with other pesticides,

including insecticides. The recent study of Pilling and coworkers on thiamethoxam (Pilling et al. 2013) is illustrative for this case as it did not provide information about the treatment status of the control plots.

For mass-dying of bees in spring near corn fields during sowing of neonicotinoid-treated seeds, there now is a one to one proven causal link. Acute intoxication occurs through exposure to the dust cloud around the pneumatic sowing machines during foraging flights to adjacent forests (providing honeydew) or nearby flowering fields (Apenet 2010; Girolami et al. 2012; Tapparo et al. 2012; Krupke et al. 2012; Pochi et al. 2012; Tapparo et al. 2012). In these cases, dead bees have typically been found to have high levels of seed treatment neonicotinoids on, or in, their bodies. Such mass colony losses during corn sowing have been documented in Italy, Germany, Austria, Slovenia, the USA and Canada (Gross 2008; Krupke et al. 2012; Sgolastra et al. 2012; Tapparo et al. 2012). In response to the incidents, the adherence of the seed coating has been improved owing to better regulations, and an improved sowing technique has recently become compulsory throughout Europe (European Commission 2010). However, despite the deployment of air deflectors in the drilling machines and improved seed coating techniques, emissions are still substantial and the dust cloud remains acutely toxic to bees (Biocca et al. 2011; Marzaro et al. 2011; Girolami et al. 2012; Tapparo et al. 2012; Sgolastra et al. 2012).

Acute lethal effects of neonicotinoids dispersed as particles in the air seem to be promoted by high environmental humidity (Girolami et al. 2012). Honeybees also transport toxic dust particles on their bodies into the hive (Girolami et al. 2012). Sunny and warm days also seem to favour the dispersal of active substances (Greatti et al. 2003).

Sublethal effects on honeybees

Effects on activity, locomotion, metabolism and ontogenetic development Imidacloprid, thiamethoxam and clothianidin have been shown to rapidly induce flight muscle paralysis in honeybees exposed to guttation drops containing these substances, resulting in the cessation of wing movements (Girolami et al. 2009). Imidacloprid further impairs the mobility of bees, as reflected by decreases in running and walking and increases in the time that exposed bees remain stationary (Medrzycki et al. 2003). However, when exposed to subchronic doses of neonicotinoids, decreases in locomotion were not observed in honeybees and bumblebees by Cresswell et al. (2012b).

Ontogenetic development is a crucial period that determines the physiological and functional integrity of adult individuals. Thus, in addition to the effects on adults, neonicotinoids may act on larval development with consequences for the adult stage. Adult honeybees exposed to imidacloprid during the larval stage exhibit impairment of olfactory associative behaviour (Yang et al. 2012). This could be due to altered neural development. Impairments in mushroom body development in the bee brain and the walking behaviour of honeybee workers have been observed in individuals exposed to imidacloprid during the larval period (Tomé et al. 2012). Effects on adult bees exposed during the larval stage could also be attributed to the induction of cell death by imidacloprid in larvae (Gregorc and Ellis 2011). In the early stages of adult life, after emergence, imidacloprid can disrupt the development of hypopharyngeal glands by decreasing the size of the acini and by increasing the expression of hsp70 and hsp90 (Smodis Skerl et al. 2009; Hatjina et al. 2013). Derecka et al. (2013) provided beehives in the field for 15 days with syrup tainted with 2 μg/l imidaclopid. They found that these levels of imidacloprid, at the low end of the field-realistic range, significantly impact energy metabolism in worker bee larvae.

Impacts of pesticides on metabolism may affect the detoxifying, intermediary and energetic metabolism pathways. Imidacloprid impairs brain metabolism in the honeybee which results in an increase of cytochrome oxidase in mushroom bodies (Decourtye et al. 2004a, b).

Effects on behaviour, learning and memory Optimal function of the honeybee nervous system is critical to individual and colony functioning (Desneux et al. 2007; Thompson and Maus 2007). Increasing levels of research effort have been devoted to developing an improved understanding of how sublethal exposure to neonicotinoids and fipronil may affect the honeybee nervous system. There is evidence that sublethal exposure can affect learning, memory and orientation in honeybees.

Laboratory experiments administering a single dose of imidacloprid demonstrated that learning was altered (Guez et al. 2001; Lambin et al. 2001), and exposure to chronic sublethal doses has demonstrated that learning and foraging are impaired by imidacloprid and fipronil (Decourtye et al. 2003). Furthermore, thiamethoxam has been shown to decrease memory capacity (Aliouane et al. 2009). The methodologies and doses varied in these laboratory tests, but all used concentrations above 20 ppb; this is towards the upper end of concentrations found in most field situations. These concentrations would not be expected to be found in pollen or nectar following seed treatment applications, but have been found in cucurbit flowers following soil drench applications (Dively and Hooks 2010). Field experiments offer the potential for powerful tests; however, results have been mixed, and many studies focus on honeybee orientation to and from a feeding source. A study that trained honeybee foragers to a sugar syrup reward in a complex maze demonstrated that 38 % of bees found the food source following ingestion of 3 ng/bee of

thiamethoxam, compared with 61 % in an unexposed control group (Decourtye and Devillers 2010). A series of studies training foragers to orient to a sugar feeder found that foragers were unable to return to the hive after ingesting imidacloprid at concentrations ranging from 100 to 1,000 ppb (Bortolotti et al. 2003; Ramirez-Romero et al. 2005; Yang et al. 2008). In contrast, other semi-field studies have shown no effects upon foraging or survivorship following exposure to canola, maize and sunflowers grown from neonicotinoid-treated seeds (Schmuck et al. 2001; Cutler and Scott-Dupree 2007; Nguyen et al. 2009). Possible explanations for these conflicting results may be that when given a range of foraging opportunities, honeybees may reduce foraging visits to food sources containing pesticides (Mayer and Lunden 1997; Colin et al. 2004), or that neonicotinoids do not have effects on colonies in the exposure regimes tested here.

Recently, Henry et al. (2012a, b) described the results of innovative field experiments using radio frequency identification (RFID) tags to determine the colony-level effects of orientation impairment upon foragers fed a sublethal dose of imidacloprid (1.42 ng in 20 μl of sucrose syrup). In two separate experiments, treated foragers failed to return to the colony at rates of 10.2 and 31.6 %, relative to untreated foragers feeding upon the same flowering plants. A higher risk of not returning was associated with the more difficult orientation tasks. Using these forager loss rates, the researchers modelled the colony-level effects and found significant, largely consistent deviations from normal colony growth rates, in some cases to levels that may put the colony at risk of collapse. A subsequent suggestion by Cresswell and Thompson (2012) to alter the simulation slightly to reflect the period when seed-treated crops are flowering demonstrated that the risk of collapse was no longer evident. However, a follow-up calculation by Henry et al. (2012a) using a larger dataset that incorporated a range of empirically derived colony growth estimates revealed even higher deviations from normal than the original work: a more serious negative outcome for colonies. The variable outcomes based upon model assumptions reflect uncertainties that have plagued honeybee researchers and further underscore the importance of ensuring that models are robust and represent a range of scenarios. The key contribution of this work was the demonstration that sublethal doses can impose a stressor (i.e. non-returning foragers) that can have significant negative outcomes on a colony level.

Learning and memory represent fundamental functions involved in the interaction of individuals with their environment and are critical in enabling bees to respond to the requirements of the colony throughout their life. Imidacloprid impairs learning and olfactory performance via both acute and chronic exposure pathways, and summer bees appear more sensitive than winter bees (Decourtye et al. 2003). These effects are observed not only in the laboratory but also in semi-field conditions, and bees do not recover after exposure ceases. Results obtained with acetamiprid and thiamethoxam showed that the action of neonicotinoids depends on the level/degree of exposure and cannot be generalized to structurally related compounds. Unlike contact exposure, oral exposure of acetamiprid resulted in an impairing of long-term retention of olfactory learning (El Hassani et al. 2008). Conversely, for thiamethoxam, subchronic exposure, but not acute exposure, elicited a decrease of olfactory memory and an impairment of learning performance (El Hassani et al. 2008; Aliouane et al. 2009).

Neonicotinoids have specific routes of metabolism in insects, particularly in the honeybee, that lead to complex influences on learning and memory processes. Imidacloprid and thiamethoxam are metabolized into toxic metabolites that may potentially bind to different honeybee nicotinic acetylcholine receptors (Nauen et al. 2001; Suchail et al. 2001, 2004a; Nauen et al. 2003; Ford and Casida 2006; Benzidane et al. 2010; Casida 2011). The metabolism of acetamiprid results in the appearance of different metabolites in the honeybee, among which 6-chloronicotinic acid is toxic in chronic exposure but not in acute exposure and remains stable for at least 72 h, especially in the head and the thorax (Suchail et al. 2001, 2004a; Brunet et al. 2005). Considering the presence of multiple active metabolites over time, it is very difficult to ascertain what steps of the memory process (acquisition, consolidation or retrieval) are affected by imidacloprid, acetamiprid, thiamethoxam or their metabolites.

Habituation may be defined as "a form of learning that consists in the gradual and relatively prolonged decrease of the intensity or the frequency of a response following the repeated or prolonged stimulus responsible for eliciting such a response" (Braun and Bicker 1992; Epstein et al. 2011a, b; Belzunces et al. 2012). Habituation can be regarded as an important adaptive behaviour because it allows individuals to minimize their response and, therefore, their energy investment, towards unimportant stimuli. The neonicotinoid imidacloprid alters patterns of habituation in honeybees following contact exposure to a sublethal dose (Guez et al. 2001; Lambin et al. 2001). Imidacloprid-induced changes in habituation appear to vary depending on the age of bees and time after exposure. Furthermore, these changes in habituation may be due to factors such as differential sensitivity of different nicotinic acetylcholine receptors (nAChRs) to imidacloprid (Déglise et al. 2002; Thany et al. 2003; Thany and Gauthier 2005; Barbara et al. 2008; Gauthier 2010; Dupuis et al. 2011; Bordereau-Dubois et al. 2012; Farooqui 2013), or the accumulation of imidacloprid metabolites like olefin and 5-hydroxy-imidacloprid, which can delay or accelerate habituation, respectively (Guez et al. 2001, 2003).

Olfaction and taste are very important physiological senses for honeybees (Detzel and Wink 1993; Giurfa 1993; Balderrama et al. 1996; Goulson et al. 2001; Reinhard et al.

2004; Gawleta et al. 2005; Couvillon et al. 2010; Maisonnasse et al. 2010; Kather et al. 2011). The effects of neonicotinoids on gustation can be explored by studying the modulation of the gustatory threshold, which can be defined as the lowest concentration of a sucrose solution applied to the antenna that triggers a feeding response. Different active compounds have been shown to induce dissimilar effects on gustation in honeybees. For example, fipronil increases the gustatory threshold of bees subjected to contact exposure (El Hassani et al. 2005). Whilst similar results were found for imidacloprid, acetamiprid decreases the threshold of bees that are exposed orally, but not topically (El Hassani et al. 2009). Thiamethoxam elicits a decrease in honeybee responsiveness to sucrose, and exposure to acetamiprid increases the responsiveness of honeybees to water regardless of exposure route (El Hassani et al. 2008; Aliouane et al. 2009).

The discrepancy in the effects observed could be explained in part by neonicotinoid metabolism that induced the appearance of toxic metabolites (Suchail et al. 2004a, b; Brunet et al. 2005) and by the existence of different nAChRs that are either sensitive and resistant to particular neonicotinoids (Déglise et al. 2002; Thany et al. 2003; Thany and Gauthier 2005; Barbara et al. 2008; Gauthier 2010; Dupuis et al. 2011; Bordereau-Dubois et al. 2012). Although it has been demonstrated in pollinating flies and in beetles, the repellent effect of imidacloprid and other neonicotinoids has not been investigated in the honeybee (Easton and Goulson 2013).

Accurate navigation is essential for efficient foraging and, hence, for colony health and survival. Neonicotinoids and fipronil may impair navigation in different ways. Sublethal exposure of honeybees to clothianidin and imidacloprid elicits a decrease in foraging activity and induces longer foraging flights (Schneider et al. 2012). Thiamethoxam induces high mortality by causing failure in the homing behaviour of foraging bees, leading to large losses of foragers from the colony (Henry et al. 2012a, b). Although this effect has been demonstrated for the pyrethroid deltamethrin for almost 20 years (Vandame et al. 1995), impacts on the homing behaviour of foraging bees continue to be left out of the assessment process for pesticide registration.

Proper foraging behaviour is essential for both individual bees and the colony as a whole because it determines the availability of food (stores) and, consequently, the survival of the colony. Exposure to imidacloprid, clothianidin and fipronil can lead to reductions in the proportion of active bees in the hive and, furthermore, initiate behaviours that can reduce the efficiency of foraging flights. For example, exposed individuals may spend longer periods of time at a food source, decrease the frequency of visits, increase the time between foraging trips, engage in longer foraging flights, reduce foraging distances, exhibit problems revisiting the same feeding site or exhibit reductions in visual learning capacities (Nielsen et al. 2000; Morandin and Winston 2003; Colin et al. 2004; Ramirez-Romero et al. 2005; Yang et al. 2008; Han et al. 2010; Schneider et al. 2012; Teeters et al. 2012). Fischer et al. (2014) exposed adult honeybees to sublethal doses of imidacloprid (7.5 and 11.25 ng/bee), clothianidin (2.5 ng/bee) and thiacloprid (1.25 μg/bee) and subsequently tracked the flight paths of individual bees with harmonic radar. The rate of successful return was significantly lower in treated bees, the probability of a correct turn at a salient landscape structure was reduced and less directed flights during homing flights were performed. These findings show that sublethal doses of these three neonicotinoids either block the retrieval of exploratory navigation memory or alter this form of navigation memory. Reproduction and colony development may be regarded as integrative endpoints for assessing the final impacts of pesticides on bees as both are a compulsory condition of social insect physiology.

Neonicotinoids such as thiacloprid, thiamethoxam and imidacloprid decrease brood production, larval eclosion, colony growth rate and the number of queens reared in bumblebees (Tasei et al. 2000; Mommaerts et al. 2010; Whitehorn et al. 2012). Studies suggest that the reduction in brood production may be associated with a reduction in pollen and sugar consumption by adult bees (Laycock et al. 2012a, b). The rearing of honeybees on brood comb containing high levels of pesticide residues results in delayed larval development and emergence and shortened adult longevity (Wu et al. 2011). Since the brood combs in the latter study contained five neonicotinoids at relatively high concentrations, it is difficult to ascribe the observed effects to any one pesticide, or pesticide class. An epidemiological study involving Hill's criteria (minimal conditions that prove evidence of a causal relationship) revealed conflicting results on the involvement of dietary traces of neonicotinoids in the decline of honeybee populations (Cresswell et al. 2012a) and could not establish a causal link between observations of bee decline and neonicotinoid use rates.

Interaction with pathogens

Detrimental effects of pesticides might be increased in combination with other environmental stress agents (Mason et al. 2013). Specific pathogens and parasites are ancestral companions of (some) honeybee populations, and accidental movement of parasites and pathogens by man has exposed both honeybees and wild bees to non-native enemies to which they may have reduced resistance (e.g. Goulson 2003; Graystock et al. 2013a, b). Imidacloprid can act synergistically with the pathogen *Nosema* spp. by increasing *Nosema*-induced mortality (Alaux et al. 2010). It affects social immunity and so increases the number of *Nosema* spores in the guts of bees from imidacloprid-exposed colonies exposed in cage studies (Pettis et al. 2012). Sequential exposure to *Nosema ceranae* can sensitize bees to thiacloprid by eliciting potentiation that

leads to high mortality rates, a feature shared with fipronil (Vidau et al. 2011; Aufauvre et al. 2012). Similarly, other experiments with fipronil and *N. ceranae* have demonstrated reciprocal sensitization (Aufauvre et al. 2012). Furthermore, exposure to pesticides during embryonic and post-embryonic development may alter the susceptibility of adult bees to pathogens. For example, adult honeybees reared in brood combs containing high levels of pesticide residues exhibit higher levels of infection by *N. ceranae* and higher levels of *Nosema* spores (Wu et al. 2012).

Di Prisco et al. (2013) demonstrated that clothianidin negatively modulates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB, a protein involved in DNA transcription) immune signaling in insects and adversely affects honeybee antiviral defences controlled by this transcription factor. They identified a negative modulator of NF-κB activation specific for insects. Exposure to clothianidin, by enhancing the transcription of the gene encoding this inhibitor, reduces immune defences and promotes the replication of the deformed wing virus present in honeybees. Similar immunosuppression was found to be induced by imidacloprid. The occurrence of this insecticide-induced viral proliferation at sublethal doses that are well within field-realistic concentrations suggests that the studied neonicotinoids are likely to have a negative effect under field conditions.

Synergistic effects with other pesticides

In agricultural ecosystems, honeybees are seldom exposed to only a single pesticide. Combined exposures could be of high concern because they can elicit synergies and potentiations. For example, thiacloprid acts synergistically with ergosterol biosynthesis inhibitor (EBI) fungicides in bees exposed in laboratory conditions but not in tunnel conditions (Schmuck et al. 2003).

Analyses of honeybees and colony contents indicate that honeybees are indeed frequently exposed to multiple pesticides simultaneously (Mullin et al. 2010; Krupke et al. 2012; Paradis et al. 2013). However, the study of pesticide mixtures can be challenging (Lydy et al. 2004), and there is a paucity of information in the literature regarding the mixtures encountered by honeybees. Triazole fungicides have been found in pollen collected from colonies (Krupke et al. 2012) and have been shown to synergize toxicity of some neonicotinoids (thiacloprid and acetamiprid) up to 559-fold in the laboratory, although the same results have not been shown in semi-field studies (Schmuck et al. 2003). Piperonyl butoxide also has been found in pollen and has been shown to synergize toxicity of some neonicotinoids (thiacloprid and acetamiprid) up to 244-fold in the laboratory (Iwasa et al. 2004). Despite the challenges associated with this type of research, this is a clear research gap that should be addressed in the future, given that honeybees rarely encounter only a single pesticide during foraging and/or in the hive.

Toxicity to bumblebees and solitary bees

Bumblebees (genus *Bombus*) are primitive social bees. Colonies start from overwintering queens, build up to a few hundred adult workers and break down when new queens and males are produced. A small number of bumblebee species are commercially reared for pollination, but many of the non-managed bumblebees also contribute substantially to crop pollination (Chagnon et al. 1993; Bosch and Kemp 2006; Greenleaf and Kremen 2006; Goulson 2010). Solitary bees that are also commonly managed in agricultural settings include the alfalfa leafcutter bee (*Megachile rotundata*), alkali bees (*Nomia melanderi*), blue orchard bees (*Osmia lignaria*) and Japanese horn-faced bees (*Osmia cornifrons*). *M. rotundata* is the major pollinator of alfalfa, which is grown as a high value livestock feed in North America. It is often considered a domesticated species, although populations frequently occur naturally. This species contributed US$5.26 billion to the value of alfalfa hay in 2009 (Calderone 2012). In addition to managed bees, there are more than 20,000 species of wild bees in the world, many of which contribute to crop pollination, and all of them contribute to pollination of wild flowers.

There are few long-term population-level studies involving bumblebees and other bee species, and in many cases, the impacts of pesticide exposure and dosage are unclear. These species differ from honeybees in that they generally exhibit smaller foraging ranges and often prefer to nest in the ground. Therefore, populations located near agricultural operations and associated pesticide applications may have fewer alternative options for food and habitat resources. Furthermore, ground-nesting species may face additional exposure risks (i.e. pesticide-contaminated soil) that are not encountered by honeybees, but which remain to be evaluated. Finally, whilst bumblebees tend to be bigger, solitary bees are often smaller than honeybees; thus, these species likely receive a different dose relative to their body weight than honeybees do.

Likely levels of exposure of wild bee species are poorly understood. Whilst neonicotinoid levels have been quantified in the nectar and pollen of various crop plant species (Cresswell 2011; Anon 2012), the degree to which wild bees utilize these resources has not been measured, and furthermore, basic values of toxicity, such as LD_{50} and LC_{50}, are completely lacking for the vast majority of these species. The few studies that do exist have employed a range of methods with conflicting results so that drawing general conclusions is difficult at this stage. Moreover, these studies are criticised for low sample size, which limits power to detect effects and/or highly unnatural laboratory conditions.

It is clear that neonicotinoids and fipronil are highly toxic to all bee species tested so far, which in addition to honeybees

includes various *Bombus* species, several social stingless bee species and the solitary species *O. lignaria* and *M. rotundata* (Scott-Dupree et al. 2009; Valdovinos-Núñez et al. 2009; Gradish et al. 2010; Mommaerts et al. 2010; Tomé et al. 2012). Cresswell et al. (2012a, b) demonstrated that bumblebees exhibit sublethal responses to imidacloprid at 10 ppb, whilst honeybees were unaffected at this concentration. Scott-Dupree et al. (2009) found that *O. lignaria* is more sensitive to both clothianidin and imidacloprid than *Bombus impatiens*, with *M. rotundata* more sensitive still. Stark et al. (1995) found no difference in the 24 h contact LD_{50} of imidacloprid between honeybees and the solitary bee species *M. rotundata* and *N. melanderi*. Scott-Dupree et al. (2009) demonstrated that *B. impatiens* individuals were more tolerant of thiamethoxam and clothianidin than *O. lignaria* and *M. rotundata*. However, the orchard bee *O. lignaria* exhibits delayed hatching and development when fed imidacloprid at rates from 30 to 300 μg/kg (Abbott et al. 2008). Arena and Sgolastra (2014) compared the acute toxicity of numerous pesticides and found that *Scaptotrigona postica* and *M. rotundata* were more sensitive than honeybees to fipronil, whilst *N. melanderi* was more tolerant. Together, these results suggest that "other" bees may be at least as sensitive, if not more sensitive, to neonicotinoids than honeybees, although more work is clearly needed.

A number of studies have used queenless micro-colonies of bumblebees (containing only workers) to examine the sublethal effects of cumulative neonicotinoid exposure to low, field-realistic doses. Several have found no detectable effects; for example, Tasei et al. (2000) exposed *Bombus terrestris* micro-colonies to 6–25 ppb of imidacloprid and found no significant response. Similarly, Franklin et al. (2004) exposed *B. impatiens* to concentrations of up to 36 ppb of clothianidin without detecting an impact (see also Morandin and Winston 2003). Most recently, Laycock et al. (2012a, b) exposed micro-colonies of *B. terrestris* to a range of concentrations of imidacloprid (0–125 μg/l) and detected a 30 % reduction in fecundity at doses as low as 1 ppb. In the only comparable work on other bee species, Abbott et al. (2008) injected concentrations of up to 300 ppb of neonicotinoids into pollen stores of *O. lignaria* and *M. rotundata* with no measurable impact on larval development.

Interestingly, negative effects seem to be detected more frequently and at lower concentrations when bees have to forage at a distance, even when the distances are tiny. Mommaerts et al. (2010) found no impact of imidacloprid exposure on micro-colonies of *B. terrestris* at field-realistic concentrations when food was provided in the nest, but when workers had to walk just 20 cm down a tube to gather food they found significant sublethal effects on foraging activity, with a median sublethal effect concentration (EC_{50}) of just 3.7 ppb. The same researchers also studied queenright colonies foraging in a glasshouse where food was 3 m from their nest and found that ingestion of 20 ppb of imidacloprid caused significant worker mortality, including bees dying at the feeder. Significant mortality was also observed at 10 ppb, but not at 2 ppb. This may explain why some lab studies have failed to find effects.

With impacts more pronounced when bees have to leave the colony, one might predict more marked effects when bees are foraging naturally, travelling kilometres across the landscape (Knight et al. 2005; Osborne et al. 2008). Only four studies have examined impacts of exposure to neonicotinoids on non-*Apis* bees when free-flying in the landscape. Tasei et al. (2001) placed *Bombus lucorum* colonies in the field for 9 days, either adjacent to an imidacloprid-treated field or a control field of sunflowers. During this time, 54 % more of the foragers from the ten imidacloprid-exposed colonies failed to return compared to the ten control colonies; however, this difference was not statistically significant as sample sizes were very small. After 9 days, the colonies were returned to the lab and fed ad libitum. Treated colonies grew more slowly but the difference was not significant. Gill et al. (2012) provided *B. terrestris* colonies with feeders containing 10 ppb of imidacloprid in sugared water whilst simultaneously allowing bees freedom to forage outside the nest. Bees exposed to imidacloprid brought back pollen less often and tended to bring back smaller loads, compared to control bees. Feltham et al. (2014) simulated exposure of queenright *B. terrestris* colonies to a crop of flowering oilseed rape, providing them with sugared water and pollen containing 0.7 and 6 ppb of imidacloprid, respectively, for 2 weeks. They found a 57 % reduction in the mass of pollen brought back to colonies, which persisted for at least 4 weeks after treatment ceased. Only one study to date has attempted to examine the effects of exposure to neonicotinoids on colony-level development of bumblebees under field conditions; Whitehorn et al. (2012) used the same field-realistic doses as Feltham et al. (2014) and then allowed colonies to develop naturally in the field. They recorded significantly reduced nest growth and an 85 % decrease in queen production in imidacloprid-exposed colonies compared to control colonies. This reduction in colony performance is likely due to a combination of factors such as reduced pollen input (as demonstrated by Gill et al. 2012 and Feltham et al. 2014) and perhaps impaired fecundity of queens (following Laycock et al. 2012a, b). In an 11 week greenhouse study, caged queenright colonies of *B. impatiens* were fed treatments of 0, 10, 20, 50 and 100 ppb of imidacloprid, respectively, and clothianidin in sugar syrup (50%) (Scholer and Krischik 2014). At 6 weeks, queen mortality was significantly higher in 50 and 100 ppb and by 11 weeks in 20–100 ppb neonicotinyl-treated colonies. Starting at 20 ppb, there is a statistically significant reduction in queen survival (37 % for imidacloprid, 56 %

for clothianidin), worker movement, colony consumption and colony weight compared to 0 ppb treatments. At 10 ppb imidacloprid and 50 ppb clothianidin, fewer males were produced (Scholer and Krischik 2014).

Bryden et al. (2013) conceived a model to simulate bumblebee colony development to assess the colony-level impacts of well-known sublethal effects on individuals. Their study shows that bumblebee colonies fail when exposed to sustained sublethal levels of pesticide. This is explained by impairment of colony function. Social bee colonies have a positive density dependence, and they are subject to an Allee effect. There is a critical stress level for the success of a colony such that a small increase in the level of stress can make the difference between failure and success.

It seems likely that intoxicated bees are fully able to gather food when it is presented to them within the nest, but when bees have to navigate over realistic distances to extract nectar and pollen from complex, patchily distributed flowers, the effects of intoxication become evident. Studies have focused mainly on behavioural effects in adult bees shortly after exposure to neonicotinoids, but there is evidence from both honeybees (Yang et al. 2012) and stingless bees (Tomé et al. 2012) that exposure during larval stages can impair development of the central nervous system and, hence, result in reduced adult performance several weeks after colony exposure. Therefore, the implications for risk assessment are clear; lab trials, and even trials where colonies are placed immediately adjacent to treated crops, are not appropriate for detecting these impacts. Similarly, experiments need to run for many weeks to examine the long-term effects of exposure on bee health.

The existing toxicological data suggests that impacts on diverse bee taxa are broadly similar at the level of the individual bee, with some evidence that bumblebees and solitary bees may be more susceptible than honeybees. It is clear that field-realistic doses of neonicotinoids can have a range of significant detrimental effects on larval development, adult fecundity, adult foraging behaviour and colony performance in social species. However, the effects of neonicotinoids on the vast majority of bee species have not been examined, and caution is necessary when extrapolating from social to solitary species. No studies have evaluated the impacts of neonicotinoids on solitary species under field conditions. It might plausibly be argued that the large colony size exhibited by honeybees and some stingless bees could buffer these species against reductions in foraging performance, as well as any navigational errors on the part of workers; however, this is unlikely to be the case for either bumblebee colonies, which have just a few hundred workers at most, or solitary bees, where a single female has sole responsibility for provisioning of offspring. Thus, impacts at the population level may be inversely related to levels of sociality. This possibility awaits experimental investigation.

Butterflies and moths (Lepidoptera)

Among agricultural practices, pesticide use is known to impact butterflies and moths; however, based on observational field data, it is difficult to distinguish the impacts of pesticides from other agricultural customs, such as fertilizer application or landscape simplification, e.g. by removal of hedgerows (Geiger et al. 2010). In the case of butterflies or moths that inhabit structures adjacent to areas where pesticides are applied via aerial spraying, indirect effects of drift from spraying may pose risks both during and after applications (Sinha et al. 1990). In the 1980s for example, helicopter application of pesticides in vineyards of the Mosel Valley in Germany nearly led to the extinction of an isolated population of the Apollo butterfly (*Parnassius apollo*) which was restricted to adjacent rocky slopes (Kinkler et al. 1987; Richarz et al. 1989; Schmidt 1997). In Northern Italy, butterfly communities in natural grasslands have suffered drastic declines downwind of intensively sprayed orchards, leading to the disappearance of all but the most generalist species (Tarmann 2009). Furthermore, spray applications of pesticides may alter soil quality (Freemark and Boutin 1995) and thereby indirectly affect the larvae and pupae of moth species residing in the upper layers of the soil surface during the spring.

Contrary to other non-target species (e.g. bees, birds, spiders, ground beetles), very few comparative pesticide sensitivity tests have been carried out for butterflies and moths. This is surprising given the significant role these insects play for conservation programs. One such study conducted by Brittain et al. (2010b) evaluated the impact of pesticides on various groups of pollinators. When comparing intensively managed systems (high pesticide application rates) with less intensively managed systems (fewer pesticide applications), the authors demonstrated that fewer bumblebee and butterfly species were observed in intensively managed habitat patches. The study also demonstrated that wild bees have higher pesticide-related risks than butterflies (Brittain et al. 2010b).

Moreover, studies by Feber et al. (1997) and Rundlöf et al. (2008) have demonstrated negative impacts of pesticides on butterflies. Both studies evaluated the impacts of organic versus conventional agriculture on butterfly populations. In each case, organic farms were found to host greater numbers and species of butterflies. This response was likely due in part to reduced applications of herbicides in organic systems, as herbicides reduce the abundance of host and nectar plants that are crucial for the survival of larvae as well as adults (Boggs 2003). In contrast, similar studies comparing Lepidopteran communities between organic and conventional agriculture systems found no differences in the number or species richness of butterflies (Weibull et al. 2000 and Brittain et al. 2010a). In the case of these studies, characteristics of the surrounding landscape such as the absence of specific vegetation

elements (e.g. hedgerows or floral nectar sources) at both the local and regional scales, or the broad scale application of pesticides, may have influenced the outcome of the findings.

In contrast to the few ecological and ecotoxicological studies on the direct and indirect impacts of pesticides on non-target Lepidoptera, numerous results are available on the impacts of pesticides on the butterfly and moth species that are regarded as agricultural pests during the larval stage (Haynes 1988; Davis et al. 1991a, b, 1993; Liang et al. 2003). The impacts of systemic pesticides on Lepidoptera have been investigated for some 32 pest species of moths in nine different families (Table 2). This represents a tiny fraction of the estimated 200,000 Lepidoptera species. The results demonstrate considerable variation in the impact of pesticides on different species of Lepidoptera. For example, Doffou et al. (2011a, b) noted that the susceptibility of two cotton pests, *Pectinophora gossypiella* (Gelechiidae) and *Cryptophlebia leucotreta* (Tortricidae), to acetamiprid differs almost 3-fold (LD_{50}=11,049 and 3,798 ppm, respectively). First instar *Cydia pomonella* caterpillars (Tortricidae) are more than 100 times as sensitive as final fifth instar caterpillars, with an LC_{50}/LC_{90} of 0.84/1.83 and 114.78/462.11 ppm, respectively (Stara and Kocourek 2007a, b).

Not surprisingly, different neonicotinoid compounds vary in toxicity. Thiacloprid and acetamiprid for example are recorded to have stronger effects on the survival of *Phyllonorycter ringoniella* than all other neonicotinoid substances (Funayama and Ohsumi 2007a, b). Acetamiprid has been shown to be more toxic than thiacloprid in several studies, but the degree of difference varies greatly. For example, a study by Cichon et al. (2013) found thiacloprid to be two times as toxic to *C. pomonella* as acetamiprid (LC_{99}/LC_{50}= 1.55/0.17 vs 0.71/0.08 ppm, respectively), whilst Magalhaes and Walgenbach (2011) recorded a 60-fold difference in the sensitivity of the same species to these compounds (LC_{50}= 1.06 and 65.63 ppm, respectively).

Many studies have documented systemic pesticide resistance in Lepidoptera; for example, *Phtorimaea operculella* has been found to be resistant to fipronil (Doğramacı and Tingey 2007), *Spodoptera litura* to both fipronil and imidacloprid (Huang et al. 2006a, b; Ahmad et al. 2008; Abbas et al. 2012), *C. pomonella* to acetamiprid and thiacloprid (Cichon et al. 2013; Knight 2010; Stara and Kocourek 2007a, b), and *Plutella xylostella* to acetamiprid (Ninsin et al. 2000a, b). In the latter field study from Japan, an almost 10-fold higher dosage was required to reach the same lethal concentration ($LC_{50/95}$=315/2,020 compared to 35.1/137 ppm in susceptible laboratory colonies). Applications of such high concentrations may further increase negative impacts on non-target species of insects. Even low sublethal doses can have severe impacts on Lepidoptera populations. In a study on *Helicoverpa armigera* by Ahmad et al. (2013), a 16th of the LC_{50} of imidacloprid (5.38 ppm) increased the next generation survival rate by a factor of 4 (i.e. equivalent to LC_{10}) compared to a treatment with the LC_{50} dose. Sublethal effects included a significant reduction in the survival and fecundity as well as increased mortality in the first and subsequent generations. Asaro and Creighton (2011a, b) noted that loblolly pines appeared to be protected from the Nantucket pine tip moth (*Rhyacionia frustrana*) even 1 year after treatment, and the treatment effect apparently was not confined to the target pest species, but extended to further non-target insect species.

There is a clear need for studies on the impact of pesticides on butterflies and moths and in particular those species that are not agricultural pests, but which commonly inhabit managed landscapes. Extensive studies on the direct and indirect effects of pesticides on these non-target groups are urgently needed on different geographical scales and across long time periods (Aebischer 1990) and which include all developmental stages of butterflies and moths (i.e. egg, larva, pupa, adult). It is of paramount importance to include varying intensities of pesticide applications, their persistence and their interplay with biotic and abiotic factors (Longley and Sotherton 1997; Brittain et al. 2010b).

Other invertebrates

This section will review the studies on neonicotinoids and non-target organisms, in particular the predatory invertebrates of natural pest species. Biological pest control plays an important role in integrated pest management (Byrne and Toscano 2007; Peck and Olmstead 2010; Prabhaker et al. 2011; Khani et al. 2012) with studies suggesting that predators may contribute to the similarity in crop yields between non-treated and pesticide-treated fields (Albajes et al. 2003; Seagraves and Lundgren 2012).

Routes of exposure

Non-target organisms can be exposed to neonicotinoid pesticides in a variety of ways. Predatory invertebrates may become contaminated by consuming pests such as leafhoppers or aphids that feed on treated crops (Albajes et al. 2003; Papachristos and Milonas 2008; Moser and Obrycki 2009; Prabhaker et al. 2011; Khani et al. 2012). Direct contamination through the diet can also be a problem for other beneficial plant-feeding invertebrates (Dilling et al. 2009; Girolami et al. 2009; Moser and Obrycki 2009; Prabhaker et al. 2011; Khani et al. 2012). For example, several species of hoverfly and parasitoid wasps attack agricultural pests, but also subsidise their diet with nectar. Therefore, these insects can be affected by neonicotinoids, which are translocated into the nectar and pollen of treated crop plants (Stapel et al. 2000; Krischik et al. 2007).

Table 2 Studies on the effects of systemic pesticides in Lepidoptera

Family	Species	Host	Imidacloprid	Thiamethoxam	Clothianidin	Acetamiprid	Thiacloprid	Dinotefuran	Fipronil
Gelechiidae	*Pectinophora gossypiella*	Cotton				Doffou et al. (2011a, b)			
Gelechiidae	*Phthorimaea operculella*	Potato	Symington (2003)				Saour (2008)		Dogramaci and Tingey (2008)
Gracillariidae	*Cameraria ohridella*	Horse chestnut tree	Stygar et al. (2013)						
Gracillariidae	*Phyllocnistis citrella*	Citrus	Villanueva-Jimenez and Hoy (1998), Setamou et al. (2010)						
Gracillariidae	*Phyllonorycter ringoniella*	Apple	Funayama and Ohsumi (2007a, b)	Funayama and Ohsumi (2007a, b)	Funayama and Ohsumi (2007a, b)	Funayama and Ohsumi (2007a, b)	Funayama and Ohsumi (2007a, b)	Funayama and Ohsumi (2007a, b)	
Lyonetiidae	*Leucoptera coffeella*	Coffee		Diez-Rodrijguez et al. (2006)					
Noctuidae	*Agrotis ipsilon*	Corn and various crops			Kullik et al. (2011a)				
Noctuidae	*Helicoverpa armigera*	Various crops	Ahmad et al. (2013)						
Noctuidae	*Helicoverpa zea*	Cotton	Kilpatrick et al. (2005)	Kilpatrick et al. (2005)		Kilpatrick et al. (2005)			
Noctuidae	*Heliothis virescens*	Tobacco							Pedibhotla et al. (1999)
Noctuidae	*Lacanobia subjuncta*	Apple and various fruits		Brunner et al. (2005)	Brunner et al. (2005)	Brunner et al. (2005)			
Noctuidae	*Sesamia inferens*	Rice							Fang et al. (2008)
Noctuidae	*Spilarctia obliqua*	Polyphagous	Ansari et al. (2012)						
Noctuidae	*Spodoptera litura*	Polyphagous	Abbas et al. (2012)						Ahmad et al. (2008), Huang et al. (2006a, b)
Psychidae	*Thyridophteryx ephemeraeformis*	Thuja and other ornamental plants			Rhainds and Sadof (2009)			Rhainds and Sadof (2009)	
Pyralidae	*Acrobasis vaccinii*	Blueberry				Wise et al. (2010)	Wise et al. (2010)		
Pyralidae	*Cactoblastis cactorum*	Opuntia	Bloem et al. (2005)						
Pyralidae	*Chilo infuscatellus*	Sugarcane							Mann et al. (2009)
Pyralidae	*Chilo suppressalis*	Rice	Yu et al. (2007a, b)						Fang et al. (2008), He et al. (2013), Chen and Klein (2012), Cheng et al. (2010), He et al. (2007, 2008), Li et al. (2007)
Pyralidae	*Ostrinia nubilalis*	Stored grain	Yue et al. (2003)	Yu et al. (2007a, b)					Durham et al. (2001, 2002), Siegfried et al. (1999)
Pyralidae	*Plodia interpunctella*	Stored grain	Yue et al. (2003)	Yue et al. (2003)					
Pyralidae	*Tryporyza incertulas*	Rice	Wang et al. (2005)						
Sesiidae	*Pennisetia marginata*	Raspberry	McKern et al. (2007)						

Table 2 (continued)

Family	Species	Host	Imidacloprid	Thiamethoxam	Clothianidin	Acetamiprid	Thiacloprid	Dinotefuran	Fipronil
Tortricidae	*Choristoneura rosaceana*	Apple		Brunner et al. (2005)	Brunner et al. (2005)	Brunner et al. (2005), Dunley et al. (2006)			
Tortricidae	*Cryptophlebia leucotreta*	Cotton				Doffou et al. (2011a, b)			
Tortricidae	*Cydia pomonella*	Apple		Brunner et al. (2005)	Brunner et al. (2005)	Brunner et al. (2005), Cichon et al. (2013), Knight (2010), Magalhaes and Walgenbach (2011), Mota-Sanchez et al. (2008)	Cichon et al. (2013), Magalhaes and Walgenbach (2011), Stara and Kocourek (2007), Voudouris et al. (2011), Reyes et al. (2007)		
Tortricidae	*Epiphyas postvittana*	Trees	Taverner et al. (2012)				Taverner et al. (2011, 2012)		
Tortricidae	*Grapholita lobarzewskii*	Apples	Charmillot et al. (2007)				Charmillot et al. (2007)		
Tortricidae	*Grapholita molesta*	Apple		Jones et al. (2012)		Magalhaes and Walgenbach (2011), Jones et al. (2010)	Magalhaes and Walgenbach (2011)		
Tortricidae	*Pandemis pyrusana*	Apple		Brunner et al. (2005)	Brunner et al. (2005)	Brunner et al. (2005), Dunley et al. (2006)			
Tortricidae	*Rhyacionia frustrana*	Pine trees	Asaro and Creighton (2011a, b)						Asaro and Creighton (2011)
Yponomeutidae	*Plutella xylostella*	Cabbage	Hill and Foster (2000)			Ninsin et al. (2000a, b), Sayyed and Crickmore (2007), Ninsin and Tanaka (2005), Ninsin (2004a, b), Ninsin and Miyata (2003)			Li et al. (2006), Sayyed and Wright (2004), Shi et al. (2004), Zhou et al. (2011)

Other routes of exposure include contact with treated surfaces, exposure to sprays or consumption of guttation droplets (Papachristos and Milonas 2008; Prabhaker et al. 2011; Khani et al. 2012). For example, neonicotinoid soil drenches or injections have been found to adversely affect the development of Lepidoptera larvae pupating within the soil (Dilling et al. 2009), whilst soil drenches have been found to significantly lower the overall abundance of insect species and species richness. In one study, imidacloprid was used on eastern hemlock (*Tsuga canadensis*) to effectively control the hemlock woolly adelgid (*Adelges tsugae*); however, the abundance of non-target detrivorous, fungivorous and phytophagous invertebrates was significantly lower in soil drench and injection treatments, when compared to untreated plots (Dilling et al. 2009).

Parasitoid wasps such as *Gonatocerus ashmeadi* can come into contact with neonicotinoids when emerging from the eggs of its host. One such host, the glassy-winged sharpshooter (*Homalodisca itripennis*), a common agricultural pest of many different crops, lays its eggs on the underside of leaves, beneath the epidermal layer. If eggs are laid on neonicotinoid-treated plants, *G. ashmeadi* nymphs may be exposed to toxins when they emerge from the egg and chew through the leaf to get to the surface (Byrne and Toscano 2007).

A 3 year study by Peck (2009) found that when imidacloprid was used as a lawn treatment to target neonate white grubs (Coleoptera: Scarabaeidae), it exhibited cumulative detrimental effects on the abundance of Hexapods, Collembola, Thysanoptera and Coleoptera adults, which were suppressed by 54–62 % overall throughout the course of the study. Population numbers of non-target organisms can also be indirectly affected by a reduction in prey or host species (Byrne and Toscano 2007; Dilling et al. 2009).

Diptera

Of the Diptera, the genus *Drosophila* provides well-known and convenient model species for toxicity testing. Mechanisms of resistance to imidacloprid and its metabolism have been studied in *Drosophila melanogaster*. Particularly, cytochrome P450 monooxygenases (CYPs) are involved, as is the case in mosquitoes (Riaz et al. 2013). According to Kalajdzic et al. (2012), three P450 genes (Cyp4p2, Cyp6a2 and Cyp6g1) located on the 2R chromosome were highly up-regulated in imidacloprid-resistant flies. However, the same authors did not find that imidacloprid induced expression of Cyp6g1 and Cyp6a2 (Kalajdzic et al. 2013). More recently, it has been shown that imidacloprid was metabolized to eight derivatives in *D. melanogaster*. In this process, only the P450 Cyp6g1 was involved in the enhanced metabolism in vivo (Hoi et al. 2014). Direct toxicity (LC_{50}) has been determined for various *D. melanogaster* strains. For instance, the toxicity of several neonicotinoids and butene-fipronil was evaluated (Arain et al. 2014) with neonicotinoids being less toxic than butene-fipronil. It was suggested that differences exist between adults and larvae. Acute LC_{50} values can be compared to LC_{50} measured after chronic exposure, within two studies. With a mutant strain, Frantzios et al. (2008) found a decrease by a factor of 2 for adult flies (acute vs chronic) and a factor of 3 for larvae. Very recently, Charpentier and co-workers have distinguished between male and female flies, from a field strain (Charpentier et al. 2014). Here, the chronic LC_{50} was 29 times lower than the acute LC_{50} for males; it was 172 times lower for females and 52 times lower for larvae. Additionally, this study demonstrated that a significant increase of mortality (27–28 %), with a V-shape, was occurring at concentrations 1,100 and 4,600 times lower than the chronic LC_{50} for males and females, respectively. Other parameters that are crucial for reproduction were tested (mating and fecundity). The LOEC was determined at a concentration that is 3,300,000 and more than 7,900,000 times lower than the acute LC_{50} for females and males, respectively. These data can be linked to data concerning mortalities observed by chronic exposure of bees at very low concentrations.

Hymenoptera (excluding bees)

A few studies have investigated the effect of neonicotinoid pesticides on parasitic wasps used as biological control agents. Stapel et al. (2000) found that the parasitoid wasp *Microplitis croceipes* had significantly reduced foraging ability and longevity after feeding on extrafloral nectar of cotton (*Gossypium hirsutum*) treated with imidacloprid. Prabhaker et al. (2007) give acute toxicity for two different exposure times for the parasitic wasp species *Eretmocerus eremicus*, *Encarsia formosa*, *Aphytis melinus* and *G. ashmeadi* (Table 3).

Table 3 Acute neonicotinoid toxicity for different Hymenoptera species (Prabhaker et al. 2007)

Species	48 h exposure time mg (AI)/ml		24 h exposure time mg (AI)/ml
	Acetemiprid	Thiamethoxam	Imidacloprid
Eretmocerus eremicus	108.27	1.01	1.93
Encarsia formosa	12.02	0.397	0.980
Gonatocerus ashmeadi	0.134	1.44	2.63
Aphytis melinus	0.005	0.105 (24 h exposure time)	0.246

In another study, *Anagyrus pseudococci* (a nectar-feeding wasp) was fed using buckwheat (*Fagopyrum esculentum*) flowers that had been exposed to imidacloprid as a soil treatment, applied at the label rate. Only 38 % of the wasps survived after 1 day, compared to 98 % fed on untreated flowers. This decreased to 0 % survivorship after 7 days for treated flowers, compared to 57 % on the untreated flowers (Krischik et al. 2007).

As stated in the section on exposure routes, exposure to imidacloprid did not affect mortality of *G. ashmeadi* (a parasitoid wasp) during development within its host, and the adults were sensitive during emergence from the host egg. When mortality was assessed within 48 h of emergence, the LC_{50} for the parasitoid was 66 ng of imidacloprid per cm^2 leaf (Byrne and Toscano 2007).

Neonicotinoids are commonly used in household products as highly concentrated bait formulations to control ants (Rust et al. 2004; Jeschke et al. 2010); however, the use of agrochemical products at less concentrated doses is an issue for non-target ants. For the leafcutter ant *Acromyrmex subterraneus subterraneus*, Galvanho et al. (2013) found that sublethal doses of imidacloprid reduced grooming behaviour. Grooming behaviour in this ant is a defence against pathogenic fungi like *Beauveria* species. Barbieri et al. (2013) recently discovered that interactions between different ant species may be negatively affected using sublethal doses of neonicotinoids. In interspecific interactions, individuals of a native ant species (*Monomorium antarcticum*) lowered their aggression towards an invasive ant species (*Linepithema humile*) although survival was not affected. Exposed individuals of *L. humile* displayed an increase in aggression with the outcome that the probability of survival was reduced.

Hemiptera

Whilst many Hemiptera are acknowledged as being problematic agricultural pests, a number are important predators of these pests, although they do also feed on some plant tissues, which would be contaminated by neonicotinoids (Prabhaker et al. 2011). Table 4 shows LC_{50} rates for different Hemiptera species.

Neuroptera

It is not only the agricultural use of neonicotinoids that affects beneficial invertebrates. In one study, Marathon 1 % G, a product for amateur use on flowers containing imidacloprid, had been found to affect lacewings (*Chrysopa* spp.) when used at the label rate. Survival rates on untreated flowers were found to be 79 % for adults, compared to 14 % on treated flowers over a 10 day period (Rogers et al. 2007).

Coleoptera

A number of studies have looked into the effects of neonicotinoids on various taxa of Coleoptera such as Histeridae (Hister beetles) (Kunkel et al. 1999), Carabidae (ground beetles) (Kunkel et al. 2001; Mullin et al. 2010) and Coccinellidae (ladybird beetles) (Smith and Krischick 1999; Youn et al. 2003; Lucas et al. 2004; Papachristos and Milonas 2008; Moser and Obrycki 2009; Eisenback et al. 2010; Khani et al. 2012).

Some Coleoptera, notably in the carabid and staphyliniid families, are voracious predators and are a vital aspect of integrated pest management. For example, although the provision of beetle banks as nesting habitat takes land out of crop production, in wheat (*Triticum aestivum*) fields, any losses have been found to be more than offset by savings from a reduced need for aphid-controlling pesticides (Landis et al. 2000).

Many of these beetle groups are undergoing rapid declines. In the UK, three quarters of carabid species have reduced in numbers, half of which have been undergoing population declines of more than 30 %, although the reason for these considerable declines are unknown (Brooks et al. 2012). These groups have been particularly useful as bioindicators, due to their sensitivity to habitat changes especially in agricultural environments (Kromp 1999; Lee et al. 2001). In the EU Draft Assessment Report for imidacloprid, acute toxicity tests were

Table 4 LC_{50} rates for different Hemiptera species

Species	Chemical	LC_{50} residual contact (mg AI/l)		
		Nymphs	Adults	Reference
Orius Laevigatus	Imidacloprid	0.04	0.3	Delbeke et al. (1997)
Hyaliodes vitripennis	Thiacloprid	1.5	0.3	Bostanian et al. (2005)
Hyaliodes vitripennis	Thiamethoxam	1.43	0.5	Bostanian et al. (2005)
Geocoris punctipes	Imidacloprid		5,180	Prabhaker et al. (2011)
	Thiamethoxam		2,170	
Orius insidiosus	Imidacloprid		2,780	
	Thiamethoxam		1,670	

undertaken on the carabid beetle *Poecilus cupreus*, finding the larvae to be highly sensitive. Despite the rapporteur Member State deeming that the concentrations tested were too high for it to conclude no risk to carabids for use on sugar beet, there was no indication of further research required (EFSA 2006).

When exposed to turf plots treated with imidacloprid, the carabid beetle *Harpalus pennsylvanicus* displayed a range of neurotoxic problems including paralysis, impaired walking and excessive grooming. These abnormal behaviours then rendered the individuals vulnerable to predation from ants (Kunkel et al. 2001). A study by Mullin et al. (2010) exposed 18 different carabid species to corn seedlings treated to field-relevant doses of either imidacloprid, thiamethoxam or clothianidin. Nearly 100 % mortality was observed for all species over 4 days.

Coccinellids predators are well known for their ability to control common pests, both in agricultural and domestic environments. In soil treatments of imidacloprid, reduced mobility and delayed reproduction have been found in pollen-feeding species such as *Coleomegilla maculata* (Smith and Krischick 1999), whilst egg production and oviposition periods of the Mealybug destroyer (*Cryptolaemus montrouzieri*) (Khani et al. 2012) and *Hippodamia undecimnotata* (Papachristos and Milonas 2008) were significantly reduced. Table 5 shows available acute toxicity for some coccinellid species.

Harmonia axyridis (harlequin ladybird) larvae were exposed to corn seedlings grown from seeds treated with the label recommended doses of either thiamethoxam or clothianidin. Seventy-two percent of the larvae exhibited neurotoxic symptoms such as trembling, paralysis and loss of coordination, with only 7 % recovery from the poisoning (Moser and Obrycki 2009).

Arachnida

In addition to crop protection, applications of neonicotinoid insecticides in veterinary medicine have expanded. Imidacloprid is applied to domestic pets as a spot-on formulation against ear mites (*Otodectes cynotis*) (Jeschke et al. 2010). However, studies on mites have found a positive effect on population numbers. Zeng and Wang (2010) found that sublethal doses of imidacloprid (determined for the green peach aphid (*Myzus persicae*)) significantly increased the hatch rate of eggs and pre-adult survivorship of the carmine spider mite (*Tetranychus cinnabarinus*). James and Price (2002) also found that imidacloprid increased egg production by 23–26 % in two-spotted spider mites (*Tetranychus urticae*) in the laboratory. Another study found that fecundity of this species was slightly elevated when treated with thiamethoxam (Smith et al. 2013).

Szczepaniec et al. (2013) discovered that the application of neonicotinoids supressed expression of plant defence genes when applied to cotton and tomato plants. These genes alter the levels of phytohormones and decrease the plant's resistance to spider mites (*T. urticae*). When mites were added to the crops, population growth increased from 30 to over 100 % on neonicotinoid-treated plants in the greenhouse and up to 200 % in the field experiment. This study was prompted after the same author had investigated an outbreak of *T. urticae* in New York City, USA. In an attempt to eradicate the emerald ash borer beetle (*Agrillus planipennis*) from Central Park, imidacloprid was applied to trees as a soil drench and trunk injections. This resulted in an outbreak of *T. urticae* on elms due to the natural predators being poisoned through ingestion of prey exposed to imidacloprid, combined with fecundity elevation in the mites themselves (Szczepaniec et al. 2011).

Another study found that thiamethoxam and imidacloprid treatments significantly increased two-spotted spider mite (*T. urticae*) densities on cotton plants when compared to the untreated controls (Smith et al. 2013). This study suggested that the increased usage of neonicotinoids could explain the recent infestation increases of two-spotted spider mite occurring in various crops across the mid-south of the USA.

Table 5 Acute neonicotinoid toxicity for different Coccinellid species

Species	Chemical	LD_{50} (ng AI per beetle)	LC_{50} (μg AI/ml)	Reference
Sasajiscymnus tsugae	Imidacloprid	0.71		Eisenback et al. (2010)
Harmonia axyridis	Imidacloprid		364	Youn et al. (2003)
Harmonia variegata	Thiamethoxam		788.55	Rahmani et al. (2013)
Cryptolaemus montrouzieri	Imidacloprid		17.25–23.9	Khani et al. (2012)
Coccinella undecimpunctata	Imidacloprid		34.2	Ahmad et al. (2011)
Coccinella undecimpunctata	Acetamiprid		93.5	Ahmad et al. (2011)
Coleomegilla maculata—adult	Imidacloprid	0.074		Lucas et al. (2004)
Coleomegilla maculata—larvae	Imidacloprid	0.034		Lucas et al. (2004)

Earthworms (Lumbricidae)

Earthworms are vitally important members of the soil fauna, especially in agricultural soils where they can constitute up to 80 % of total soil animal biomass (Luo et al. 1999). They play critical roles in the development and maintenance of soil physical, chemical and biological properties (Lee 1985). Their activities improve soil structure by increasing porosity and aeration, facilitating the formation of aggregates and reducing compaction (Edwards and Bohlen 1996; Mostert et al. 2000). Soil fertility is enhanced by earthworm effects on biogeochemical cycling (Coleman and Ingham 1988; Bartlett et al. 2010), the modification of microbial biomass and activity (Sheehan et al. 2008), breakdown of plant litter (Knollengberg et al. 1985) and the mixing of litter with soil (Wang et al. 2012a).

Neonicotinoid and other systemic insecticides can pose a risk of harm to earthworm survival and behaviour, potentially disrupting soil development and maintenance processes. The same neural pathways that allow neonicotinoids to act against invertebrate pests (Elbert et al. 1991) are also present in earthworms (Volkov et al. 2007). Thus, when neonicotinoids are applied for the protection of agricultural and horticultural crops, earthworms can be exposed by direct contact with the applied granules or seeds, or with contaminated soil or water. Moreover, their feeding activities may result in ingestion of contaminated soil and organic particles (e.g. Wang et al. 2012b). Foliar residues in plant litter after systemic uptake from soils or from direct plant injections also pose a risk to litter-feeding earthworms that consume the contaminated plant litter (e.g. Kreutzweiser et al. 2009).

Neonicotinoids can persist and move in soils thereby increasing the likelihood that earthworms will be exposed for extended periods of time. Laboratory and field trials with neonicotinoids have demonstrated that their half-life in soils varies depending on soil conditions but can range from several weeks to several years (Cox et al. 1997; Sarkar et al. 2001; Cox et al. 2004; Bonmatin et al. 2005; Fossen 2006; Gupta and Gajbhiye 2007; Goulson 2003). Imidacloprid is the most widely used neonicotinoid, and its adsorption to soils is increased by moisture and organic matter content (Broznic et al. 2012), resulting in increased imidacloprid concentrations in organic-rich soils compared to low-organic soils (Knoepp et al. 2012). Earthworms generally prefer moist, organic-rich soils. When soil organic carbon content is low, the high solubility of imidacloprid renders it mobile and it is readily moved through soils (Broznic et al. 2012; Knoepp et al. 2012; Kurwadkar et al. 2013), thereby increasing the likelihood that earthworms could be exposed to the pesticide in soils outside the direct area of application.

Effects on survival

Neonicotinoids can be highly toxic to earthworms. However, reported median lethal concentrations (LC_{50}) were variable depending on the particular insecticide, test conditions, route of exposure and duration (Table 6). In 13 separate studies, the reported LC_{50} ranged from 1.5 to 25.5 ppm, with a mean of 5.8 and median of 3.7 ppm. In seven studies that reported lowest concentrations at which effects on survival were measureable, those lowest effective concentrations ranged from 0.7 to 25 ppm, with a mean of 4.7 and median of 1.0 ppm. *Eisenia fetida* was the most common test species in these survival studies and represented the range of reported lethal concentrations, giving little indication from among these studies that other species were more sensitive than *E. fetida*.

When compared to other common insecticides, neonicotinoids tend to be among the most toxic to earthworms. Wang et al. (2012a) tested the acute toxicities of 24 insecticides to *E. fetida* and found that the neonicotinoids were the most toxic in soil bioassays and that acetamiprid and imidacloprid in particular were the two most toxic insecticides overall. They also reported that a contact toxicity bioassay demonstrated that the neonicotinoids were extremely toxic by a contact route of exposure (LC_{50} of 0.0088 to 0.45 μg cm^{-2}), although the units of contact toxicity concentration were difficult to compare to standard lethal concentrations. Across a broader range of 45 pesticides, Wang et al. (2012b) found that in soil bioasssays, the neonicotinoid insecticide, clothianidin, was the most toxic pesticide to *E. fetida*. Alves et al. (2013) compared three insecticides used for seed treatment and reported that imidacloprid was the most toxic to earthworms. In soil bioassays with five different insecticides, Mostert et al. (2002) found that imidacloprid was the second most toxic (behind carbaryl) to earthworms. We found only two studies that reported the toxicity of fipronil, another common, agricultural systemic insecticide, and both found it to be substantially (at least 100 times) less lethal to earthworms than the neonicotinoids (Mostert et al. 2002; Alves et al. 2013).

Effects on reproduction

Only a few studies tested sublethal effects of neonicotinoids on earthworm reproduction, but it is apparent that reductions in fecundity can occur at low concentrations (Table 6). Baylay et al. (2012) reported EC_{50}s for imidacloprid and thiacloprid against cocoon production by *Lumbricus rubellus* of 1.5 and 1.3 ppm, respectively, whilst Gomez-Eyles et al. (2009) found similar EC_{50}s for the same two insecticides at 1.4 and 0.9 ppm for *E. fetida*. The latter study also reported measurable reductions in cocoon production at 0.3 ppm of thiacloprid. Alves et al. (2013) reported an EC_{50} for reproduction effects of imidacloprid on *Eisenia andrei* of 4 ppm with measureable

Table 6 Impacts of neonicotinoids and fipronil on earthworms. The impact rating scale is as follows: ––, large decrease; –, moderate decrease; 0, little or no measurable effect (where little is either a small or a brief change); +, moderate increase; and ++, large increase. Endpoints are listed together, separated by a semi-colon, for studies that examined multiple measurement endpoints. Lowest effective concentration is the lowest concentration at which a significant effect was reported, not necessarily the mathematically modelled lowest effective concentration

Taxa	Insecticides	Location	Measurement endpoint	Impact	LC/EC_{50}	Lowest effective concentration	Reference
Eisenia fetida	Imidacloprid	China	Contact toxicity survival; soil toxicity survival	–; –	LC_{50}=0.027 μg cm^{-2}; LC_{50}=2.82 ppm		Wang et al. (2012a)
Eisenia fetida	Imidacloprid	France	Survival; biochemical (hsp70); avoidance	–; –; ++		0.66; 0.66; 0.2 ppm	Dittbrenner et al. (2012)
Eisenia fetida	Imidacloprid	France	Survival; body mass	–; –		0.66; 0.2 ppm	Dittbrenner et al. (2011a)
Eisenia fetida	Imidacloprid	UK	Cocoon production; weight change	–; ––	EC_{50}=1.41; EC_{50}=2.77 ppm		Gomez-Eyles et al. (2009)
Eisenia fetida	Imidacloprid	China	Survival	–	LC_{50}=2.30 ppm		Zang et al. (2000)
Eisenia fetida	Imidacloprid	China	Survival	–	LC_{50}=2.30 ppm	1 ppm	Luo et al. (1999)
Eisenia fetida	Imidacloprid	Canada	Survival; weight loss	–; ––		25; 14 ppm	Kreutzweiser et al. (2008b)
Eisenia fetida	Fipronil	Brazil	Survival; reproduction; avoidance	0; –; +		>1,000; 62; >10 ppm	Alves et al. (2013)
Eisenia fetida	Clothianidin	China	Contact toxicity survival; soil toxicity survival	–; ––	LC_{50}=0.28 μg cm^{-2}; LC_{50}=6.06 ppm		Wang et al. (2012b)
Eisenia fetida	Thiacloprid	China	Contact toxicity survival; soil toxicity survival	–; ––	LC_{50}=0.45 μg cm^{-2}; LC_{50}=10.96 ppm		Wang et al. (2012a)
Eisenia fetida	Thiacloprid	UK	Cocoon production; weight change	–; ––	EC_{50}=0.968; EC_{50}=19.0 ppm	0.291; 1.91 ppm	Gomez-Eyles et al. (2009)
Eisenia fetida	Acetamiprid	China	Contact toxicity survival; soil toxicity survival	–; ––	LC_{50}=0.0088 μg cm^{-2}; LC_{50}=1.52 ppm		Wang et al. (2012a)
Eisenia fetida	Nitenpyram	China	Contact toxicity survival; soil toxicity survival	–; ––	LC_{50}=0.22 μg cm^{-2}; LC_{50}=3.91 ppm		Wang et al. (2012a)
Lumbricus terrestris	Imidacloprid	France	Survival; biochemical (hsp70); avoidance	0; +; 0		4 ppm	Dittbrenner et al. (2012)
Lumbricus terrestris	Imidacloprid	France	Survival; body mass	0; –		2 ppm	Dittbrenner et al. (2011b)
Lumbricus terrestris	Imidacloprid	USA	Feeding activity; abundance	–; –		43 mg m^{-2}	Tu et al. (2011)
Lumbricus terrestris	Imidacloprid	France	Burrowing	–		2 ppm	Dittbrenner et al. (2011b)
Lumbricus terrestris	Imidacloprid	France	Body mass change; cast production	–; ––	NA; EC_{50}=0.84 ppm	0.66; 0.66 ppm	Dittbrenner et al. (2010)
Lumbricus terrestris	Imidacloprid	France	Cast production; body mass change	–; –	LC_{50}=10.7 ppm	1.89; 0.189 ppm	Capowiez et al. (2010)
Lumbricus rubellus	Imidacloprid and thiacloprid mixture	UK	Survival; weight change; cocoon production; metabolism	0; –; ––; 0	EC_{50} imidacloprid=1.46 and EC_{50} thiacloprid=1.28 ppm		Baylay et al. (2012)
Aporrectodea caliginosa	Imidacloprid	France	Survival; biochemical (hsp70); avoidance	0; –; ++		2; 2 ppm	Dittbrenner et al. (2012)
Aporrectodea caliginosa	Imidacloprid	France	Survival; body mass	–; ––		2; 0.66 ppm	Dittbrenner et al. (2011a)
Aporrectodea caliginosa	Imidacloprid	France	Burrowing	–		0.2 ppm	Dittbrenner et al. (2011b)
Aporrectodea caliginosa	Imidacloprid	France	Body mass change; cast production	–; ––	NA; EC_{50}=0.76 ppm	0.66; 0.66 ppm	Dittbrenner et al. (2010)
Aporrectodea nocturna	Imidacloprid	France	Weight loss; avoidance; burrowing	–; +; –		0.5; 0.1; 0.05 ppm	Capowiez and Berard (2006)
Aporrectodea nocturna	Imidacloprid	France	Burrowing	–		0.1 ppm	Capowiez et al. (2006)
Aporrectodea nocturna	Imidacloprid	France	Survival, weight loss	–; –	LC_{50}=3.74 ppm	0.1 ppm	Capowiez et al. (2005)
Aporrectodea nocturna	Imidacloprid	France	Burrowing	–		0.01 ppm	Capowiez et al. (2003)
Allolobophora icterica	Imidacloprid	France	Weight loss; avoidance; burrowing	–; +; ––		0.5; 0.01; 0.05 ppm	Capowiez and Berard (2006)
Allolobophora icterica	Imidacloprid	France	Burrowing	–		0.1 ppm	Capowiez et al. (2006)
Allolobophora icterica	Imidacloprid	France	Survival, weight loss	–; ––	LC_{50}=2.81 ppm	0.1 ppm	Capowiez et al. (2005)
Allolobophora icterica	Imidacloprid	France	Burrowing	–		0.01 ppm	Capowiez et al. (2003)
Dendrobaena octaedra	Imidacloprid	Canada	Survival; leaf decomposition	0; –		31 ppm	Kreutzweiser et al. (2009)
Dendrobaena octaedra	Imidacloprid	Canada	Survival; weight loss; reproduction; leaf decomposition	–; ––; –; –	LC_{50}=5.7 ppm	3; 3; 7; 7 ppm	Kreutzweiser et al. (2008b)

Table 6 (continued)

Taxa	Insecticides	Location	Measurement endpoint	Impact	LC/EC_{50}	Lowest effective concentration	Reference
Dendrobaena octaedra	Imidacloprid	Canada	Survival; weight loss; reproduction; leaf decomposition	0; −; 0; −		11; 3.2 ppm	Kreutzweiser et al. (2008a)
Eisenia andrei	Imidacloprid	Brazil	Survival; reproduction; avoidance	−; —; ++	LC_{50}=25.53; EC_{50}=4.07; EC_{50}=0.11 mg/kg	25; 0.75; 0.13 ppm	Alves et al. (2013)
Pheretima group	Imidacloprid	South Africa	Survival	–	LC_{50}=3.0 ppm		Mostert et al. (2002)
Pheretima group	Fipronil	South Africa	Survival	0		>300 ppm	Mostert et al. (2002)
Apporectodea spp.	Clothianidin	USA	Abundance; biomass; cast production	−; −; −	NA, field applications		Larson et al. (2012)

adverse effects at 0.7 ppm. Kreutzweiser et al. (2008b) tested the effects of imidacloprid in forest litter on the litter-dwelling earthworm *Dendrobaena octaedra* and reported significant reductions in cocoon production among surviving earthworms at 7 ppm.

Effects on behaviour

A number of studies focused on behavioural endpoints under the premise that effects on behaviour are often ultimately linked to population or community effects (Little 1990; Dittbrenner et al. 2012). The behavioural attributes considered here are avoidance behaviour, burrowing, cast production and weight change (as an indicator of feeding behaviour). Among the 31 reported values for behavioural effects, weight change was the most common, followed by burrowing, avoidance behaviour and cast production (Table 6). Only a few studies gave median effective concentrations (EC_{50}), and they ranged from 0.1 (avoidance) to 19 (weight change) ppm, with a mean EC_{50} of 3.7 and median of 1.3 ppm. These behavioural EC_{50}s were about 1.5 to 2.8 times lower than the mean and median lethal concentrations of 5.8 and 3.7 ppm.

However, many more studies reported lowest concentrations at which behavioural effects were detected, and those ranged from 0.01 to 14 ppm with a mean of 1.2 and median of 0.5 ppm. Thus, measurable behavioural effects were more sensitive endpoints than measurable survival effects. Measurable behavioural effects occurred at concentrations of about two to four times lower than the mean and median lowest effective concentrations on survival of 4.7 and 1.0 ppm. Burrowing (smaller, shorter, more narrow burrows) was the most sensitive behavioural endpoint with effects detected at mean and median concentrations of 0.3 and 0.07 ppm (range 0.01 to 2, n=8). Avoidance behaviour was the next most sensitive endpoint with effects detected at mean and median concentrations of 0.5 and 0.13 ppm (n=5), followed by cast production (mean 1.1, median 0.7 ppm, n=3) and weight change (mean 2.1, median 0.7 ppm, n=13). All of these indicate that measurable adverse effects on earthworm behaviour would be expected at neonicotinoid concentrations below 1 ppm in soil.

Risks to earthworms

The actual risk of harmful effects on earthworm populations posed by neonicotinoid insecticides will depend on exposure concentration, exposure duration, route of exposure, rate of uptake and inherent species sensitivity. From the toxicity studies reviewed here, it appears that individual earthworms across all common species are at risk of mortality if they consume soil or organic particles with neonicotinoid insecticide concentrations of about 1 ppm or higher for several days. Higher numbers (up to 50 %) of earthworms would be

expected to be at risk of mortality when concentrations reach about 3 ppm and higher. Although it was difficult to compare the exposure concentrations to standard bioassays, it appears that the risk of mortality from surface contact exposure can be ten times or more higher than the risk of mortality from consumption of contaminated soils (Wang et al. 2012a). On the other hand, the route of exposure can affect the likelihood of lethal effects on earthworms. When earthworms were exposed to foliar residues in leaf litter from imidacloprid-injected trees, a significant feeding inhibition effect was detected that reduced leaf consumption but did not cause earthworm mortality, even at concentrations of about 10 ppm (Kreutzweiser et al. 2008a).

The risk of sublethal effects on some important behavioural attributes is higher than the risk of mortality to individuals. Insecticide effects on burrowing and avoidance behaviours would be expected at concentrations of about 0.1 to 0.5 ppm and higher. Whilst alterations in burrowing behaviour, especially reductions in burrowing depths, have implications for the transfer properties of soils (Capowiez et al. 2006; Dittbrenner et al. 2011b), the consequences in real-world field conditions are not clear. Fewer, smaller and shorter burrows could reduce air, water and solute transport through soils affecting overall soil ecology, but none of the studies we found actually tested these implications in experimental or field settings.

The concentrations that pose risk of mortality (assuming high toxicity by contact exposure) and sublethal effects on earthworms fall within the range of reported field concentrations, albeit at the upper end of that range of concentrations. Dittbrenner et al. (2011b) indicate that predicted environmental concentrations for imidacloprid in agricultural soils would be about 0.3 to 0.7 ppm, suggesting risks of at least sublethal effects on earthworms could be quite high. Bonmatin et al. (2005) reported that imidacloprid in soils can reach several hundred parts per billion shortly after sowing of treated seeds. Soil samples from a tea plantation treated with clothianidin had average concentrations of up to 0.45 ppm shortly after application (Chowdhury et al. 2012). Donnarumma et al. (2011) found concentrations of imidacloprid in soils at about 0.6 to 0.8 ppm by 2 weeks after application of treated seeds. Ramasubramanian (2013) reported clothianidin concentrations in soils of 0.27 to 0.44 ppm up to 3 days after single applications and 0.51 to 0.88 ppm by 3 days after double applications of water-soluble granules. Collectively, these studies show that operational applications of neonicotinoids can result in soil concentrations that are likely to pose a high risk of sublethal effects and potential risk of lethal effects (especially by contact toxicity) to earthworms.

At least two issues related to the assessment of risk to earthworms from exposure to neonicotinoids have not been adequately addressed in the published literature. The first is the length of exposure periods in toxicity testing compared to the length of exposure to persistent concentrations in natural soils. Most toxicity tests are short term, in the order of days to weeks. On the other hand, neonicotinoid residues can persist in soils for months to years (Bonmatin et al. 2014, this issue). For most pesticides, lethal or effective concentrations become lower as exposure periods increase, and this is likely the case for neonicotinoids (Tennekes 2010; Tennekes and Sánchez-Bayo 2012, 2013; Rondeau et al. 2014). It is plausible that long-term low-level concentrations of neonicotinoids in soils may pose higher risk to earthworms than what can be inferred from the published toxicity tests. The second issue pertains to the heterogeneous distribution of neonicotinoid residues in natural soils. When residues enter the soil at the surface from spray or granule deposition or from litter fall, concentrations in soils are likely to be higher on or near the surface than in deeper soils. Residues entering soils from planted seed or from contaminated water are likely to be higher at or near the source of contamination than elsewhere. Both situations would result in concentration "hot spots" near the points of entry. Conversely, most toxicity tests prepare test concentrations as parts per million (or equivalent) and assume complete mixing. Therefore, levels of exposure to earthworms at or near those hot spots in natural soils will consequently be higher than would be predicted from residue analyses of bulk samples from laboratory or field test systems.

Mortality or behavioural effects on individual earthworms do not necessarily translate to population effects with ecological consequences. Populations of organisms with short generation times (e.g. several generations per year as is the case for most earthworm species) and/or high dispersal capacity have a higher likelihood of recovery from pesticide-induced population declines than those with longer regeneration periods and limited dispersal capacity (Kreutzweiser and Sibley 2013). However, the tendency for neonicotinoids to persist in organic soils reduces the likelihood of this recovery pathway because subsequent generations may be exposed to concentrations similar to those to which the parent generation was exposed. Life history strategies and their influences on community responses and recovery from pesticide effects have been demonstrated by population modelling of other non-target organisms (Wang and Grimm 2010), and similar principles may apply to assessing risks to overall earthworm populations and communities. Population models that account for differential demographics and population growth rates within communities have been shown to provide more accurate assessments of potential pesticide impacts on populations and communities than conventional lethal concentration estimates can provide (Stark and Banks 2003). The use of ecological models to incorporate a suite of factors including seasonal variations, community assemblage mechanisms and lethal and sublethal insecticide effects and their influences on the risks to organisms, populations or communities can provide useful insights into receptor/pesticide interactions and

can thereby improve risk assessments (Bartlett et al. 2010). Ecological and population modelling combined with pesticide exposure modelling and case-based reasoning (drawing on past experience or information from similar chemical exposures) can provide further refinements and improve risk assessment for earthworm communities and their ecological function (van den Brink et al. 2002). Empirical field studies of earthworm population responses to realistic field concentrations of neonicotinoids are lacking and would greatly improve risk assessment efforts.

Aquatic invertebrates

Freshwater invertebrates

Aquatic invertebrates are extremely important components of aquatic ecosystems. They play roles as decomposers, grazers, sediment feeders, parasites and predators. They also provide much of the food that vertebrates associated with these systems feed upon. Pesticides, including neonicotinoids, reach surface waters through various routes, but in particular through atmospheric deposition (drift) after application by various sprayers, by surface runoff and by seepage of contaminated groundwater. Aquatic invertebrates are particularly susceptible to pesticides. Unlike terrestrial organisms, aquatic organisms generally cannot avoid exposure easily by moving to uncontaminated areas, particularly when pesticides are water soluble. Uptake of pesticides in aquatic invertebrates occurs through respiration (gills and trachea), feeding and through the epidermis, be it cuticle or skin.

Neonicotinoids have been used for a comparatively shorter period of time than other insecticides. However, they are found in freshwater systems more and more frequently. For example, surface water monitoring for pesticides in California has revealed that imidacloprid has frequently exceeded water quality guidelines of 1 ppb (Starner and Goh 2012). In the Washington State, USA, the State Department of Ecology and the State Department of Agriculture have been monitoring salmon-bearing rivers and streams for pesticides, including imidacloprid for a number of years and this insecticide is frequently found (http://agr.wa.gov/PestFert/natresources/SWM/).

However, even though imidacloprid and other neonicotinoids are present in freshwater systems, the question remains to what extent such concentrations affect aquatic organisms in the field. Here we discuss a number of studies dealing with neonicotinoid toxicity to aquatic invertebrates and make some observations about their potential impact on aquatic ecosystems.

Laboratory studies

Crustacea and Amphipoda Several laboratory studies have been published on the toxicity of the neonicotinoid imidacloprid on a range of aquatic invertebrates (Table 7). Stark and Banks (2003) developed acute toxicity data and population-level toxicity data for the water flea *Daphnia pulex* exposed to thiamethoxam (Actara). Thiamethoxam was the least toxic insecticide evaluated in this study of seven insecticides, and its LC_{50} of 41 ppm was well above any anticipated concentration expected to be found in surface water systems.

Chen et al. (2010) estimated the acute toxicity of imidacloprid to the water flea, *Ceriodaphnia dubia* (LC_{50}= 2.1 ppb), and the chronic toxicity to *C. dubia* populations. The effects of the adjuvant, R-11 alone and in combination with imidacloprid were also assessed. In the population study, exposure of *C. dubia* to imidacloprid concentrations of 0.3 ppb reduced population size to 19 % of the control population. This concentration is well below the U.S. EPA's expected environmental concentration of 17.4 ppb, indicating that imidacloprid may cause damage to aquatic invertebrates in the field.

The acute and chronic effects of imidacloprid on the amphipod *Gammarus pulex* were studied by Nyman et al. (2013). Feeding by *G. pulex* and body lipid content were significantly reduced after exposure to a constant imidacloprid concentration of 15 ppb. Furthermore, *G. pulex* individuals were unable to move and feed after 14 days of constant exposure resulting in a high level of mortality.

Interestingly, the standard test organism *Daphnia magna* is especially insensitive to neonicotinoids (Beketov and Liess 2008). An acute LC_{50} of around 7,000 ppb is several orders of magnitude above effective concentrations found for several other invertebrates. This implies that *D. magna* cannot be used as a sensitive test organism protective for many species.

Insecta Acute toxicity estimates of neonicotinoids on aquatic insects have also been published. LC_{50} estimates for aquatic insects range from 3 to 13 ppb. Imidacloprid LC_{50} estimates for the mayfly *Baetis rhodani*, the black fly *Simulium latigonium* (Beketov and Liess 2008) and the mosquito *Aedes taeniorhynchus* (Song et al. 1997) are 8.5, 3.7 and 13 ppb, respectively. LC_{50} estimates for *B. rhodani* and *S. latigonium* exposed to thiacloprid were 4.6 and 3.7 ppb, respectively (Beketov and Liess 2008). A chronic LC_{50} of 0.91 ppb was reported for the midge *Chironomus tentans* for imidacloprid (Stoughton et al. 2008). A study on the effects of imidacloprid as a mixture with the organophosphate insecticides dimethoate and chlorpyrifos on the midge *Chironomus dilutus* found that imidacloprid acted synergistically with chlorpyrifos and antagonistically with dimethoate (LeBlanc et al. 2012).

Table 7 Selection of studies on the effects of imidacloprid on freshwater macrophauna

	Compound	Experimental design	Effect	LC_{50}/EC_{50}	LOAEL	Reference
Aquatic taxa						
Oligochaeta	Imidacloprid	10 day exposure to contaminated sediment	Survival, growth, behaviour, avoidance		<0.05 mg/kg	Sardo and Soares (2010)
Chironomus tentans and *Hyalella azteca*	Imidacloprid	Standard toxicity test	Survival	0.91 μg/l (28 days)		Stoughton et al. (2008)
Mesocosm communities	Neonics and other insecticides		Drift response			Berghahn et al. (2012)
Daphnia, *Gammarus pulex*	Imidacloprid		Survival			Ashauer et al. (2011)
Mayflies	Imidacloprid		Nymph abundance emergence patterns and adult body size			Alexander et al. (2008)
Ceriodaphnia dubia	Imidacloprid	Lab toxicity tests	Mortality Population growth rate	2.1 ppb		Chen et al. (2010)
D. magna	Imidacloprid	Lab toxicity tests	Mortality	10.4 mg/l		Song et al. (1997)
Aedes aegypti	Imidacloprid	Lab toxicity tests	Mortality	44 ppb		Song et al. (1997)
Aedes taeniorhynchus		Lab toxicity tests	Mortality	13 ppb		Song et al. (1997)
Mayflies, Oligochaetes	Imidacloprid		Feeding inhibition			Alexander et al. (2008)
Odonata, Libellulidae	Imidacloprid, fipronil	Field	Larval and adult survival, emergence			Jinguji et al. (2013)
Macro-invertebrate community	Imidacloprid	Stream mesocosm	Community diversity, leaf litter breakdown			Pestana et al. (2009)
Crustacean: *Asellus aquaticus*, *Gammarus fossarum*	Imidacloprid and atrazine	Standard toxicity test	Survival, respiration, electron transport system			Lukancic et al. (2010)
Caddisfly: *Seriocostoma*, *Chironomis ripartus*	Imidacloprid	Standard toxicity test	Burrowing behaviour; antipredator behaviour			Pestana et al. (2009)
Ostracoda, *Daphnia magna*	Imidacloprid	Lab toxicity test	Survival			Sánchez-Bayo (2006)
Chironomus diutus	Imidacloprid+mixtures (chlorpyrifos, dimethoate)	Lab toxicity test	Survival			LeBlanc et al. (2012)
Terrestrial taxa						
Aphidius ervi	Imidacloprid+cadmium	Lab toxicity tests	Population growth rate			Kramarz and Stark (2003)

Oligochaetes Sardo and Soares (2010) investigated the effects of imidacloprid on the aquatic oligochaete *Lumbriculus variegatus*. They exposed this worm species to imidacloprid concentrations ranging from 0.05 to 5.0 mg/kg sediment. Mortality was fairly low (35 % in the highest concentration), but *L. variegatus* avoided imidacloprid-contaminated sediment. Furthermore, individual growth (biomass) was inhibited at all concentrations tested compared to the control.

Mesocosm studies Alexander et al. (2008) examined the effect of imidacloprid as a 12 day pulse or 20 day continuous exposure on the mayflies *Epeorus* spp. and *Baetis* spp. Nymph densities were reduced after both types of exposures. Sublethal effects were observed as well. Adults were smaller and had smaller head and thorax size after exposure to imidacloprid concentrations as low as 0.1 ppb. However, these effects were only found in males.

Within community test systems, neonicotinoids had strong effects especially on insects (Hayasaka et al. 2012). However, to our knowledge, all experiments investigating a dose–response relationship observed effects at the lowest concentrations evaluated. Hence, it is difficult to establish a NOEC. Within outdoor mesocosm studies, a LOEC of 1.63 ppb was estimated for imidacloprid. Adverse effects on benthic communities with 5 % reductions in the abundance of invertebrates were observed by Pestana et al. (2009). For thiacloprid, strong effects on sensitive long living insects were observed at pulsed exposure to 0.1 ppb (Liess and Beketov 2011), the lowest effective concentration observed so far in communities.

Berghahn et al. (2012) conducted stream mesocosm studies whereby 12 h pulses of imidacloprid (12 ppb) were introduced three times at weekly intervals. Results showed that drift of insects and the amphipod *Gammarus roeseli* increased after exposure to pulses of imidacloprid. These results indicated that imidacloprid was having a negative effect on *G. roeseli*.

In another stream mesocosm study, Böttger et al. (2013) evaluated pulses of imidacloprid on *G. roeseli*. The number of brood carrying females was reduced in the imidacloprid treatments compared to the control groups in the last 3 weeks of the study.

The populations of an aquatic invertebrate, the common mosquito *Culex pipiens*, exposed over several generations to repeated pulses of low concentrations of the neonicotinoid thiacloprid, continuously declined and did not recover in the presence of a less sensitive competing species, the water flea *D. magna*. By contrast, in the absence of a competitor, insecticide effects on the more sensitive species were only observed at concentrations one order of magnitude higher, and the species recovered more rapidly after a contamination event. The authors conclude that repeated toxicant pulse of populations that are challenged with interspecific competition may result in a multigenerational culmination of low-dose effects (Liess et al. 2013).

Risk to aquatic ecosystems A species sensitivity distribution (SSD) of acute toxicity data for a wider range of species, including ostracods, cladocerans and other aquatic organisms, predicts a hazardous concentration for 5 % of aquatic species (HC5) for imidacloprid in water in the range 1.04–2.54 ppb (Sanchez-Bayo and Kouchi 2012).

Van Dijk et al. (2013) developed a regression analysis for abundance of aquatic macro-invertebrate species and nearby imidacloprid concentrations in Dutch surface waters. Data from 8 years of nationwide monitoring covering 7,380 different locations of macro-invertebrate samples and 801 different locations of imidacloprid samples were pooled. Next, the biological samples (macro-invertebrate abundance counts) were combined with nearby (in space and time) chemical samples (imidacloprid concentrations), and next, a statistical analysis was done on the complete pooled combined dataset. They found that macro-invertebrate abundance consistently declines along the gradient of increasing median nearby imidacloprid concentration in the pooled dataset. This pattern turned out to be robust: it is independent of year and location. Overall, a significant negative relationship ($P<0.001$) was found between abundance of all macro-invertebrate species pooled and nearby imidacloprid concentration. A significant negative relationship was also found for abundance of each of the pooled orders Amphipoda, Basommatophora, Diptera, Ephemeroptera and Isopoda, and for several species separately. The order Odonata had a negative relationship very close to the significance threshold of 0.05 ($P=0.051$). In accordance with previous research, a positive relationship between abundance and nearby imidacloprid pollution was found for the order Actinedida. However, other pesticides were not included into the analyses by Van Dijk et al. (2013). Therefore, possible co-linearity or synergisms between neonicotinoids and other pollutants still need to be further explored (Vijver and Van den Brink 2014).

Pesticide exposure was identified to strongly reduce the amount and abundance of vulnerable invertebrate species in streams using the SPEAR approach (Liess and von der Ohe 2005). The approach was extended from German streams to Australian, Danish, French and Finnish streams revealing the same effects of pesticide exposure on vulnerable invertebrate species (Rasmussen et al. 2013; Liess et al. 2008; Schäfer et al. 2012). Beketov et al. (2013) analysed the effect of pesticide presence on invertebrate species richness in European (Germany and France) and Australian streams. They found an overall reduction of 42 % for Europe and 27 % for Australia in species richness between uncontaminated and heavily contaminated streams. The limitation of these studies in the context of assessment of neonicotinoid impact is that toxicity was mainly due to insecticides, other than neonicotinoids, as general usage of the latter only increased recently.

The results of laboratory and semi-field (mesocosm) studies indicate that aquatic invertebrates are very sensitive to the neonicotinoid insecticides. However, most of the studies we found in the literature were conducted with imidacloprid. For pesticide risk assessment, the published results to date indicate that it may be difficult to predict community-level effects using the tiered aquatic effect assessment scheme and acute and chronic toxicity data. When extrapolating from acute and chronic single species test systems, the assessment factors identified by the uniform principle of the relevant EU legislation (1107/2009) do not predict safe concentrations in multi-species outdoor mesocosms. For example, acute laboratory effects of thiacloprid on sensitive insect species show that effects occur after exposure to the range of 3–13 ppb. Accordingly, an assessment factor of 100 would indicate a safe concentration of 0.03 to 0.13 ppb for thiacloprid. However, outdoor mesocosm results employing a pulsed exposure show a LOEC below 0.1 ppb for thiacloprid (Liess and Beketov 2011). Lower concentrations were not investigated. Obviously, an assessment factor higher than 100 is needed to identify safe concentrations on the basis of acute test results. For the HC5 calculated on acute lethal concentrations, an assessment factor of larger than 10 is necessary (Liess and Beketov 2012). Additionally, in a laboratory study, chronic effects of sensitive insect species were exhibited after exposure to 0.91 ppb imidacloprid. Employing an assessment factor of 10 would indicate a safe concentration of approximately 0.1 ppb imidacloprid. However, this concentration is not safe according to the results obtained in complex community investigations. Unfortunately, to the best of our knowledge, no community-level investigation with imidacloprid evaluating a range of concentrations below 0.1 ppb has been published. This type of study would help with determining a NOEC for imidacloprid. Overall, the results of the published literature indicate that certain neonicotinoids have the potential to cause significant damage to aquatic ecosystems by causing negative effects in individuals and populations of aquatic invertebrates at very low concentrations. Protective concentrations for these products in aquatic systems still need to be determined.

Marine and coastal invertebrates

There is very limited information regarding the assessment of the environmental toxicology and contamination of neonicotinoids in marine ecosystems. Standardised environmental toxicological characterization focuses on only a few species models and rarely examines species that represent keystone organisms in marine or coastal ecosystems (CCME 2007). Monitoring and surveillance of neonicotinoid pollution in marine coastal habitats are non-existent.

Toxicology The earliest published marine ecotoxicological studies of neonicotinoids were with opossum shrimps (*Mysidopsis bahia*) which are distributed in marine coastal waters (Ward 1990, 1991; Lintott 1992). Median LC_{50} (96 h) for the technical grade of imidacloprid was 34.1 ppb with a mortality-NOEC of 13.3 ppb (Ward 1990). Exposure to a commercial formulation (ADMIRE) of imidacloprid resulted in a 96 h mortality-NOEC of 21 ppb. Maximum acceptable toxicant concentrations for *M. bahia* to imidacloprid were 23 parts per trillion (ppt) for growth effects and 643 ppt for reproductive effects (Ward 1991).

Toxicology for other marine arthropods includes *Artemia* spp. and a brackish water mosquito (*Aedes taeniohynchus*). The 48 h LC_{50} for *Artemia* was 361 ppm, whilst *Aedes* exhibited a 72 h LC_{50} of 21 ppb, and a 48 h LC_{50} of 13 ppb for an early instar stage of development (Song et al. 1997; Song and Brown 1998). Osterberg et al. (2012) demonstrated that in the blue crab (*Callinectes sapidus*), megalopae were an order of magnitude more sensitive than juveniles to lethal effects of imidacloprid (24 h-LC_{50}=10 ppb for megalopae vs 24 h-LC_{50}=1,1 ppb for juveniles).

There are no known published OECD/EPA parameter-based studies on non-arthropod marine invertebrates. For the marine mussel, *Mytilus galloprovincialis*, a transcriptomic and proteomic survey was conducted as a response to imidacloprid and thiacloprid exposures (Dondero et al. 2010). This study concluded that the two neonicotinoids induced distinct toxicodynamic responses and that caution should be heeded when conducting ecological risk assessments for chemical mixtures that target the same receptor. Rodrick (2008) demonstrated that imidacloprid had an effect on oyster hemocyte immunocompetence and that there was an additive effect when oysters were exposed to a compound stress of salinity and exposure to imidacloprid. Tomizawa et al. (2008) used the gastropod *Aplysia californica* as a model to characterize imidacloprid and thiacloprid as agonists of the acetylcholine-binding protein, indicating that neonicotinoids could also affect marine gastropods.

Environmental pollution There are no published works regarding the marine environmental contamination of neonicotinoids. Until recently, there has been little public concern of neonicotinoid non-point source pollution of marine environments from land runoff. At least within the USA, this attitude is beginning to change. In the State of Washington 2013, the Willapa-Grays Harbor Oyster Growers Association received a conditional registration from the U.S. Environmental Protection Agency to use imidacloprid to control native burrowing shrimp in Willapa Bay, Washington that may threaten commercial shellfish beds (EPA Reg. no. 88867–1). In Hawaii, there have been public protests and scrutiny over the use of neonicotinoid pesticides in their industrial agricultural practices and their likely negative impacts on coral reefs and sea grass beds (Sergio 2013). For both Hawaii and the U.S. Virgin Islands, there is concern that the use of

neonicotinoids as a method for termite control may be polluting and impacting coastal resources.

Conclusion

At field-realistic levels of pollution, neonicotinoids and fipronil generally have negative effects on physiology and survival for a wide range of non-target invertebrates in terrestrial, aquatic, marine and benthic habitats. Effects are most often found by in vitro testing, using a limited number of test species. This basically means that there is a deficit of information for the grand majority of other invertebrates. In vitro testing to establish safe environmental concentration thresholds is hindered by the fact that most test protocols are based on older methodology, validated for pesticides with very different chemical and toxicological characteristics. New and improved methodologies are needed to specifically address the unique toxicology of these neurotoxic chemicals, including their non-lethal effects and synergistic effects for a variety of terrestrial, aquatic and marine organisms.

The amount of published in vivo field tests is small and experimental setups often suffer from inability to control for variation in (semi)natural circumstances or have insufficient statistical power due to the high financial costs of large robust field experiments. Given the clear body of evidence presented in this paper showing that existing levels of pollution with neonicotinoids and fipronil resulting from presently authorized uses frequently exceed lowest observed adverse effect concentrations and are thus likely to have large-scale and wide ranging negative biological and ecological impacts, the authors strongly suggest that regulatory agencies apply more precautionary principles and tighten regulations on neonicotinoids and fipronil.

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Reciprocal effects of pesticides and pathogens on amphibian hosts: The importance of exposure order and timing☆

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ABSTRACT

Ecological communities are increasingly exposed to natural and anthropogenic stressors. While the effects of individual stressors have been broadly investigated, there is growing evidence that multiple stressors are frequently encountered underscoring the need to examine interactive effects. Pesticides and infectious diseases are two common stressors that regularly occur together in nature. Given the documented lethal and sublethal effects of each stressor on individuals, there is the potential for interactive effects that alter disease outcomes and pesticide toxicity. Using larval wood frogs (*Lithobates sylvaticus*), we examined the reciprocal interaction between insecticides (carbaryl and thiamethoxam) and the viral pathogen ranavirus by testing whether: (1) prior ranavirus infection influences pesticide toxicity and (2) sublethal pesticide exposure increases susceptibility to and transmission of ranavirus. We found that prior infection with ranavirus increased pesticide toxicity; median lethal concentration (LC50) estimates were reduced by 72 and 55% for carbaryl and thiamethoxam, respectively. Importantly, LC50 estimates were reduced to concentrations found in natural systems. This is the first demonstration that an infection can alter pesticide toxicity. We also found that prior pesticide exposure exacerbated disease-induced mortality by increasing mortality rates, but effects on infection prevalence and transmission of the pathogen were minimal. Collectively, our results underscore the importance of incorporating complexity (i.e. order and timing of exposures) into research examining the interactions between natural and anthropogenic stressors. Given the environmental heterogeneity present in nature, such research will provide a more comprehensive understanding of how stressors affect wildlife.

1. Introduction

Pesticides are a ubiquitous environmental stressor, with thousands of registered chemicals used worldwide and millions of kilograms of active ingredient applied annually (Grube et al., 2011). These chemicals often enter natural systems, where they influence non-target organisms and disrupt natural processes (Köhler and Triebskorn, 2013; Relyea and Hoverman, 2006). In non-target organisms, pesticides have been linked to endocrine disruption, developmental abnormalities, altered immune function, behavioral changes, and mortality (Brühl et al., 2013; Di Prisco et al., 2013; Egea-Serrano et al., 2012; Gill et al., 2012; Hayes et al., 2010; Mason et al., 2013; McKinlay et al., 2008). Moreover, changes that affect reproduction, survival, and species interactions have been implicated in trophic cascades in terrestrial and aquatic systems (Beketov et al., 2013; Cahill et al., 2008; Chiron et al., 2014; Hallmann et al., 2014; Relyea et al., 2005; Rohr et al., 2008b; Whitehorn et al., 2012). While our understanding of how pesticides influence ecological systems has increased, non-target organisms experience a multitude of stressors, both anthropogenic and natural, which may interact with one another to alter individual physiology, population dynamics, and community structure (Blaustein et al., 2011; Goulson et al., 2015; Koprivnikar, 2010; O'Gorman et al., 2012). A comprehensive understanding of pesticide contamination in ecological systems must therefore incorporate the interactive effects of pesticides and additional stressors.

One stressor in particular that may interact with pesticides is infectious disease. Infectious disease is a fundamental component of ecological communities (Wood and Johnson, 2015). Indeed, wildlife populations encounter a diversity of pathogenic organisms (e.g., viruses, fungi, nematodes) that can influence host morbidity and mortality, population dynamics, and community interactions

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(De Castro and Bolker, 2004; Johnson et al., 2015; Smith et al., 2006). These disease agents often comprise a substantial proportion of biomass in natural systems, perform important functions in food webs, and regulate host population sizes (Kuris et al., 2008; Lafferty et al., 2006; Scott and Dobson, 1989). While infectious diseases are a natural component of communities, there is concern that environmental stressors may exacerbate disease outcomes (Smith et al., 2009, 2006). Anthropogenic stressors such as climate change, habitat alteration, and agrochemical contamination have been implicated in the disruption of infectious disease dynamics by altering the availability of competent hosts, changing optimal environmental conditions for pathogens, and influencing host susceptibility to infection (Bradley and Altizer, 2007; Rohr and Raffel, 2010).

Pesticide contamination has been singled out as a particularly influential stressor because it can influence disease dynamics in a variety of ways (Marcogliese and Pietrock, 2011; Mason et al., 2013). Pesticides can disrupt mechanisms of resistance and tolerance in hosts, often turning relatively benign parasites into pathogenic threats (Marcogliese et al., 2010). Pesticide-induced immunosuppression, namely the reduction of leukocyte counts and down-regulation of immunoregulatory proteins, has been linked to increased disease risk in amphibians, pollinators, and fish (Christin et al., 2003; Di Prisco et al., 2013; Marcogliese et al., 2010). These physiological changes can lead to increased morbidity and mortality in host species (Coors et al., 2008; Rohr et al., 2013). These effects can also cascade through communities by changing host and parasite abundance, as demonstrated with the increase in trematode abundance in wetland communities due to pesticide-mediated increases in intermediate host abundance (Rohr et al., 2008b). While the existing literature provides strong evidence that pesticide contamination can alter disease dynamics in natural systems, there are several gaps in the literature. Previous research has largely focused on susceptibility to infection, yet few studies have addressed the influence of pesticides on parasite transmission between hosts, an important component of disease dynamics (Rohr et al., 2008). Additionally, most studies examine how pesticides alter disease dynamics while few have addressed whether pathogens alter the toxicity of pesticides (Budischak et al., 2009). Given that exposure to pathogens may occur prior to pesticide exposure, infection may damage tissues or modify resource allocation and ultimately alter mechanisms of pesticide tolerance. Infections that damage the liver in particular (e.g., malaria, leishmaniasis) have been shown to reduce xenobiotic metabolizing cytochrome P450s and glutathione s-transferases in rodents, hindering their ability to tolerate chemicals (Ahmad and Srivastava, 2007; Samanta et al., 2003; Tekwanl et al., 1988). Research on coinfecting disease agents has highlighted the importance of priority effects in determining disease outcomes (Hoverman et al., 2013). However, a similar emphasis on order of exposure in pesticide-disease research is needed. In particular, the incorporation of environmental stressors into traditional toxicity tests (e.g., median lethal concentration (LC50) estimates) may provide a more comprehensive understanding of pesticide toxicity in variable environments (Budischak et al., 2009).

Amphibians provide a prime model system for studying pesticide-disease interactions because of the pervasiveness of pesticide contamination in wetland environments and the suite of disease agents implicated in their global population declines (Daszak et al., 2003; Relyea and Hoverman, 2006). Due to the immunosuppressive effects of pesticide exposure, pesticides can increase parasite loads and parasite-induced mortality in larval amphibians (Christin et al., 2003; Koprivnikar, 2010; Rohr et al., 2013, 2008a). Pesticides can also increase exposure to parasites by facilitating the population size of intermediate hosts (e.g., freshwater snails; Rohr et al., 2008b). Consequently, pesticide concentrations in wetlands have been found to be the primary driver of parasite abundance in amphibian populations (Rohr et al., 2008b). Collectively, this research demonstrates that pesticides can alter disease dynamics in amphibians, yet most of this research has focused on trematodes and the fungal pathogen *Batrachochytrium dendrobatidis*. The influence of pesticides on ranavirus, a widespread amphibian disease agent, has been largely understudied.

Ranaviruses are viral pathogens of amphibians that infect the liver, kidney, and spleen and cause edema, lesions, and hemorrhaging, often leading to death (Bollinger et al., 1999; Docherty et al., 2003; Jancovich et al., 1997). Moreover, they have been implicated in worldwide mass mortality events (Ariel et al., 2009; Fox et al., 2006; Green et al., 2002; Une et al., 2009). While pesticides have been implicated as drivers of disease emergence, few studies have experimentally tested the interaction between ranavirus and pesticides. Interestingly, studies that have examined this interaction have found conflicting results. For example, pesticides were shown to increase ranavirus susceptibility in tiger salamanders (*Ambystoma tigrinum*; Forson and Storfer, 2006a; Kerby and Storfer, 2009) but decreased susceptibility in long-toed salamanders (*Ambystoma macrodactylum*; Forson and Storfer, 2006b). Pesticide-induced immunosuppression was argued to be the leading driver of increased ranavirus susceptibility (Forson and Storfer, 2006a), while pesticide-induced immunostimulation and a potential reduction in viral efficacy were proposed as explanations for decreased susceptibility (Forson and Storfer, 2006b). These conflicting results could be due to the experimental designs; individuals were exposed to pesticides and ranavirus simultaneously. With a simultaneous exposure, it becomes difficult to differentiate between the effects of the stressors on the host and the stressors on each other. By controlling the timing and sequence of exposure, we can more directly assess the reciprocal effects of pesticides and ranavirus on amphibians.

The objectives of our study were to determine whether: (1) ranavirus infection affects pesticide toxicity estimates, (2) sublethal pesticide exposure affects ranavirus disease outcomes (e.g., mortality rates, viral load), and (3) sublethal pesticide exposure affects ranavirus transmission. We expected that ranavirus infection would damage host liver and kidney tissues, reducing the ability to metabolize and excrete pesticides, leading to increased pesticide toxicity estimates (lower LC50 values) in infected individuals. If pesticide exposure impairs immune function, we expected an increase in susceptibility to ranavirus indicated by increased mortality rates and viral loads. If increased viral loads resulting from pesticide exposure are observed, we expected this to correlate with an increase in viral shedding rate and transmission to conspecifics.

2. Materials and methods

2.1. Species collection and husbandry

All experiments were carried out using wood frogs, *Lithobates sylvaticus*, collected as 10 partial egg masses from a woodland pond in Nashville, IN on 28 March 2015. Egg masses were reared outdoors in 100-L pools filled with ~70 L of well water and covered with 70% shade cloth. After hatching, tadpoles were fed rabbit chow *ad libitum* until the start of the experiments. Tadpoles were brought inside and acclimated to laboratory conditions (23 °C, 12:12 h day:night photoperiod) for 24 h prior to the start of each experiment. Unless noted otherwise, water changes were conducted every 4 d and tadpoles were fed Tetramin *ad libitum* every 2 d during all experiments.

Ranavirus was isolated from an infected larval green frog, *Lithobates clamitans*, collected from the Purdue Wildlife Area (PWA) in

West Lafayette, IN. The virus was cultured using a protocol adapted from Hoverman et al. (2010) wherein virus was passaged through fathead minnow cells incubated at 28° C without CO_2 and fed with Eagle's minimum essential medium (MEM) with Hank's salts and 5% fetal bovine serum. The virus was on the second passage since original isolation and was stored at −80 °C until used in the experiments.

2.2. Pesticide application

We selected two insecticides with different modes of action for the study: (1) the carbamate carbaryl, an acetylcholinesterase inhibitor and (2) the neonicotinoid thiamethoxam, a nicotinic acetylcholine receptor agonist. Both insecticides are widely used, with approximately 100,000 to 500,000 kg applied annually in the contiguous U.S. (Baker and Stone, 2015). Because carbaryl is capable of targeting both vertebrate and invertebrate nervous systems, it has been widely studied for its non-target effects on aquatic systems (Story and Cox, 2001). Thiamethoxam represents a newer class of insecticides lauded for its invertebrate specificity (Maienfisch et al., 2001). However, few studies have examined its effects on aquatic systems (Morrissey et al., 2015).

For each experiment, we used commercial grade carbaryl (22.5% Sevin) and thiamethoxam (21.6% Optigard Flex). Lethal concentrations of each pesticide were determined using pilot studies prior to the start of the experiments. We created working solutions by adding 1 mL of pesticide to 9 mL of filtered, UV-irradiated water to achieve 23,600 mg L^{-1} of carbaryl and 24,400 mg L^{-1} of thiamethoxam; experimental concentrations were made by adding working solutions to filtered, UV-irradiated well water. Nominal pesticide concentrations were verified at the Bindley Bioscience Center Metabolite Profiling Facility at Purdue University (Table 1).

2.3. Experiment 1 — effects of ranavirus exposure on LC50 values

We performed LC50 tests to determine the effects of ranavirus exposure on pesticide toxicity estimates. Our experiment was a randomized factorial design consisting of seven pesticide treatments and two virus treatments. The pesticide treatments consisted of a control (no pesticide) and three concentrations (0.3, 3, and 30 mg L^{-1}) of each pesticide. These concentrations allowed us to measure both the expected LC50 values for healthy individuals and the theoretically reduced values for infected individuals, while providing the minimum number of concentrations needed to produce statistically sound results. The ranavirus treatments consisted of a no-virus control and exposure to ranavirus at a concentration of 10^3 PFU mL^{-1}. Experimental units were 2-L plastic tubs filled with 1 L of filtered, UV-irradiated aged well water. We randomly assigned 10 tadpoles at Gosner stage 28 (Gosner, 1960) to each unit. We replicated the 14 treatments four times for a total of 56 experimental units.

We began the experiment by adding 1.43 mL of the virus (original titer 7×10^5 PFU mL^{-1}) to each virus treatment to achieve a final concentration of 10^3 PFU mL^{-1}. Previous studies have demonstrated that this dosage is sufficient for initiating infection in wood frogs and other ranids (Hoverman et al., 2011, 2010). For instance, 95% infection prevalence was documented using identical exposure conditions (Hoverman et al., 2011). We added 1.43 mL of MEM to the experimental units not assigned to the virus treatment to serve as a control. After 24 h, tadpoles were moved to new containers containing fresh water for 3 d before conducting the LC50 test. We chose to begin the LC50 test on day 4 of ranavirus exposure because we wanted to examine pesticide toxicity after virus infection, but before individuals experienced disease-induced mortality. Previous work has demonstrated that mortality due to ranavirus increases sharply on day 7 following exposure (Hoverman et al., 2011). Given the 48 h window of the LC50 test, the 4 d ranavirus exposure period allowed us to detect treatment differences before the day 7 mortality spike would occur.

The LC50 tests were initiated on day 4 by randomly assigning experimental units from each virus treatment to the pesticide treatments. We applied the pesticide concentrations to the experimental units and tadpoles were subsequently monitored for mortality every 8 h for 48 h. Dead individuals were removed and preserved in 70% ethanol. At the end of the experiment, all individuals were euthanized using a 2 g L^{-1} solution of MS-222 and preserved in 70% ethanol. A randomly selected subset of 4 tadpoles from each treatment was tested to ensure infection in ranavirus-exposed tadpoles and no infection in control tadpoles.

2.4. Experiment 2 — effects of pesticides on ranavirus susceptibility

To determine the effect of pesticide exposure on susceptibility to ranavirus, we conducted a randomized factorial experiment consisting of three pesticide treatments and three ranavirus treatments. The pesticide treatments consisted of a control (no pesticide) and exposure to carbaryl (1 mg L^{-1}) or thiamethoxam (1 mg L^{-1}). These concentrations were sublethal to tadpoles in our pilot studies and are both representative of concentrations measured in natural surface waters (Main et al., 2014; Norris et al., 1983). Ranavirus treatments consisted of a no-virus control, immediate exposure to ranavirus at a concentration of 10^3 PFU mL^{-1} following pesticide exposure, and ranavirus exposure (10^3 PFU mL^{-1}) 14 days following pesticide exposure. The two exposures were chosen to determine if ranavirus susceptibility changes with time since pesticide exposure, with 14 days chosen to avoid allowing tadpoles to metamorphose. The experimental units were 2-L plastic tubs filled with 1 L of filtered, UV-irradiated aged well water. We randomly assigned 10 tadpoles at Gosner stage 29 (Gosner, 1960) to each unit. We replicated each treatment four times for a total of 36 experimental units.

We exposed tadpoles to their respective pesticide treatments for 7 d, which has been shown to be sufficient in altering susceptibility to infection (Rohr et al., 2008a), and pesticide solutions were

Table 1
Nominal and actual concentrations of carbaryl and thiamethoxam.

Insecticide (common name; % active ingredient)	Nominal Concentration	Actual Concentration
Carbaryl (Sevin; 22.5%)	0.3 mg L^{-1}	0.2 mg L^{-1}
	1.0 mg L^{-1}	0.8 mg L^{-1}
	3.0 mg L^{-1}	1.7 mg L^{-1}
	30.0 mg L^{-1}	14.3 mg L^{-1}
Thiamethoxam (Optigard Flex; 21.6%)	0.3 mg L^{-1}	0.2 mg L^{-1}
	1.0 mg L^{-1}	0.7 mg L^{-1}
	3.0 mg L^{-1}	2.3 mg L^{-1}
	30.0 mg L^{-1}	25.2 mg L^{-1}

renewed with each water change. Given the estimated half life of each pesticide, concentrations were expected to remain fairly stable between water changes (carbaryl, 10 d at pH = 7; thiamethoxam, 200 d at pH = 7; Maienfisch et al., 2001). After 7 d, tadpoles were moved to fresh water and exposed to their respective virus treatments. Tadpoles in the immediate virus exposure treatment were exposed to virus immediately after pesticide exposure on day 8. We added 1.43 mL of the virus (original titer 7×10^5 PFU mL^{-1}) to achieve a final concentration of 10^3 PFU mL^{-1}. Tadpoles in the delayed virus exposure treatment remained in fresh water for 2 wk before being exposed to virus on day 22 (10^3 PFU mL^{-1}). We added 1.43 mL of MEM to the experimental units not assigned to the virus treatment to serve as a control. After 24 h of virus exposure, the tadpoles were moved to fresh water for the remainder of the experiment. Tadpoles in the virus treatments were monitored for mortality every 12 h until 100% mortality was observed. Dead individuals were immediately removed and preserved in 70% ethanol for ranavirus testing. At the end of the experiment, surviving individuals were euthanized with MS-222 and preserved in 70% ethanol.

Each individual was weighed, measured for snout-vent length (SVL) and total length, and staged. Then, the individual was necropsied and sections of the liver and kidney were pooled into one 1.5 mL microcentrifuge tube for ranavirus testing. From each sample, we extracted DNA using a DNeasy Blood and Tissue Kit (Qiagen) and stored at −80°C until qPCR analysis. To prevent cross contamination during necropsies, we scrubbed and soaked all tools and surfaces in 10% bleach for 10 min and changed gloves between samples.

2.5. Experiment 3 — effects of pesticides on ranavirus transmission

To determine the effect of pesticide exposure on the transmission of ranavirus, we conducted an experiment analyzing two components of ranavirus transmission from a focal host to a naïve host: (1) viral shedding rate of the focal host and (2) infection in naïve hosts. The experiment was a completely randomized 3 × 2 factorial design manipulating pesticide and ranavirus exposure on the focal tadpoles. The pesticide treatments consisted of a control (no pesticide) and sublethal exposure to carbaryl or thiamethoxam (1.0 mg L^{-1}). The ranavirus treatments consisted of a no-virus control and exposure to ranavirus at a concentration of 10^3 PFU mL^{-1}. We replicated each treatment 10 times for a total of 60 experimental units. The experimental units were 2-L plastic tubs filled with 1 L of filtered, UV-irradiated well water aged for 24 h prior to use. We randomly assigned one focal tadpole to each experimental unit.

We exposed focal tadpoles to their respective pesticide treatments for 7 d followed by virus exposure for 24 h. After exposure to ranavirus for 24 h, tadpoles were rinsed with fresh water and moved to new containers with fresh water to ensure no virions from the initial exposure remained in the tubs. Every 24 h for 3 d, 40 mL water samples were taken from each experimental unit and frozen at −80°C to test for ranavirus. We stirred the water in each unit before sampling to ensure homogeneity, and changed water after each sampling. After 3 d, focal tadpoles were euthanized using MS-222 and stored in 70% ethanol for ranavirus testing. Water from the experimental units was kept unchanged for the next portion of the experiment. To each experimental unit, we added 5 naïve tadpoles, which had never been exposed to pesticides or virus. Naïve tadpoles were maintained in the contaminated water for 3 d before being euthanized in MS-222 and stored in 70% ethanol for ranavirus testing. Tadpoles were processed as described above.

To extract ranavirus from the water samples, we used a protocol adapted from Kirshtein et al. (2007). In brief, the thawed 40 mL water samples were filtered through 0.2 μm PVDF syringe filters. The filters were incubated using DNA extraction reagents (Qiagen). Extracted DNA was transferred to 1.5 mL microcentrifuge tubes and frozen at −80° C until qPCR analysis. All tools and surfaces were soaked in 10% bleach, and gloves and syringes were changed between samples.

2.6. Ranavirus testing

We used quantitative polymerase chain reaction (qPCR) to determine the viral load of each sample using the methods of Forson and Storfer (2006a,b). The PCR reaction mixture included 6.25 μL of TaqMan® Universal PCR Master Mix (Applied Biosystems), 2.75 μL of DNA grade water, 1.0 μL of a mixture of each primer at 10 pmol μL^{-1} (rtMCP-F [5′-ACA CCA CCG CCC AAA AGT AC-3’] and rtMCP-R [5′-CCG TTC ATG ATG CGG ATA ATG-3’]) and a fluorescent probe rtMCP-probe (5′- CCT CAT CGT TCT GGC CAT CAA CCA-3′). Each well included 2.5 μL of its respective template DNA or DNA grade water for a final volume of 12.25 μL. We ran qPCR reactions using a Bio-Rad real-time PCR system. Each qPCR run included a standard curve and a negative control. The DNA standard was a synthetic double-stranded 250bp fragment of the highly conserved *Ranavirus* major capsid protein (MCP) gene (gBlocks Gene Fragments; Integrated DNA Technologies). A standard curve was created using a log-based dilution series of 4.014×10^9 viral copies μL^{-1} to 4.014×10^6 viral copies μL^{-1}. All samples, including standard curves, negative controls, and unknowns, were run in duplicate. For each sample, the concentration of genomic DNA (ng of DNA μL^{-1}) was measured using a NanoDrop 2000c (Thermo Scientific). Using these measurements, we calculated viral load as viral copies ng^{-1} of DNA.

2.7. Statistical analyses

To compare LC50 values in experiment 1, we followed the methods of Budischak et al. (2009). Experimental units from each virus treatment were randomly assigned to cohorts such that each cohort contained the full range of pesticide concentrations (0, 0.3, 3, and 30 mg L^{-1}). We calculated LC50 values for each cohort individually using probit analysis, which produced four replicate LC50 values for each virus treatment. We used individual one-way analyses of variance (ANOVAs) to compare LC50 values between virus and no-virus treatments for each pesticide separately. LC50 estimates were adjusted according to the actual verified pesticide concentrations. For all analyses in experiment 2, we used general linear mixed models with experimental unit as a random factor. Of our size measurements, SVL was found to have the strongest positive correlation with time to death and viral load ($p < 0.02$) and was therefore included as a covariate in our analyses for experiment 2. Separately for each virus exposure regime, we compared time to death among pesticide treatments. Additionally, we examined the relationship between time to death and viral load in our treatments using general linear mixed models with experimental unit as a random factor. This was done to determine whether we could assess the effects of pesticide exposure on tolerance (Read et al., 2008). Overall, there was no relationship between time to death and viral load in our treatments ($P > 0.08$). The one exception was for individuals exposed to thiamethoxam immediately before ranavirus exposure. In this treatment, there was a positive relationship between viral load and time to death ($F_{1,35} = 7.76$, $p = 0.01$). We expect that this is a result of *in vivo* viral replication over time, where individuals that survived longer had higher viral loads. Given the general lack of a relationship between viral load and time to death, we did not explore additional analyses of tolerance but focus instead on the effects of pesticide exposure

on time to death. For individuals across both virus exposure regimes, we determined if there was an interactive effect of pesticide and the timing of virus exposure on time to death. For this test, time to death was inverse transformed to meet the assumption of homoscedasticity. We also compared viral load among pesticide treatments. For experiment 3, we assessed the effects of pesticide treatment on the mean viral load of focal and naïve tadpoles with one-way ANOVAs. The no-virus treatment was excluded from the analysis because no individuals were infected. Based on Pearson's correlations, none of our size variables were correlated with viral load ($p > 0.06$) and were therefore excluded from the analyses for this experiment. In analyzing viral loads of the naïve tadpoles, we calculated the mean viral load for all tadpoles housed within each experimental unit. Because viral concentrations in the water samples were too low to be detected, no statistical analyses were conducted. All analyses were performed using SPSS 23.0 (SPSS Inc., Chicago, IL, USA) at $\alpha = 0.05$.

3. Results

3.1. Experiment 1 – effects of ranavirus exposure on LC50 values

Virus exposure significantly increased the toxicity of carbaryl ($F_{1,6} = 23.06$, $p = 0.003$) and thiamethoxam ($F_{1,6} = 11.65$, $p = 0.01$; Fig. 1, Fig. A1). LC50 estimates were 72% and 55% lower in the virus treatment for carbaryl and thiamethoxam, respectively, compared to the no-virus treatments. We observed 100% infection in the ranavirus treatment and 0% infection in the no-virus control based on a randomly selected subset of tadpoles from each treatment. Within this subsample, there was no effect of pesticide treatment on viral load ($F_{2,30} = 1.27$, $p = 0.30$).

3.2. Experiment 2 – effects of pesticides on ranavirus susceptibility

Time to death decreased (i.e. tadpoles died faster) when tadpoles were exposed to pesticides prior to ranavirus infection ($F_{2,9} = 3.75$, $p = 0.07$; Fig. 2a). However, the effect was dependent on the pesticide. Based on post-hoc comparisons, carbaryl significantly decreased time to death compared to control ($p = 0.02$) but thiamethoxam did not ($p = 0.16$). When ranavirus exposure was delayed 2 wk following pesticide exposure, there was no effect of the pesticide treatments on time to death ($F_{2,8} = 2.97$, $p = 0.11$; Fig. 2b). Moreover, time to death was significantly higher in the delayed exposure ($F_{1,53} = 105.94$, $p < 0.001$), and there was an interactive effect of pesticide and the timing of virus exposure on time to death ($F_{2,24} = 6.26$, $p = 0.01$). However, in both the immediate and delayed exposures, pesticide exposure did not influence infection prevalence (100% of tadpoles were infected in the ranavirus treatment) or viral load at time of death (Immediate, $F_{2,9} = 0.15$, $p = 0.87$; Delayed, $F_{2,9} = 3.24$, $p = 0.09$; Fig. 3). Furthermore, there was no difference in viral load between the immediate and delayed exposure regimes ($F_{1,39} = 0.75$, $p = 0.39$).

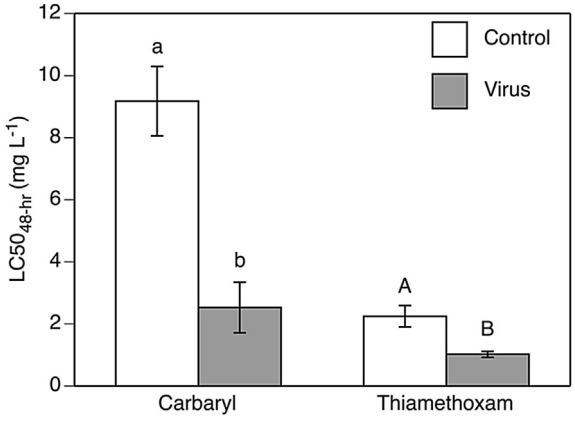

Fig. 1. $LC50_{48\text{-hr}}$ values for carbaryl and thiamethoxam for ranavirus-exposed and unexposed larval wood frogs. Treatments sharing uppercase or lowercase letters are not statistically different ($p > 0.05$). Data are means ± 1 SE.

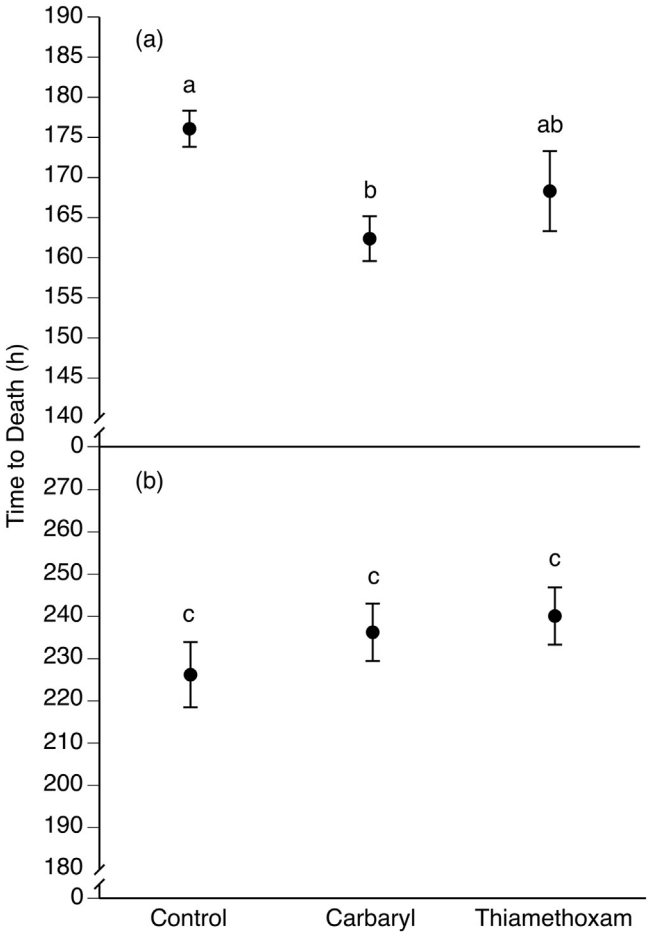

Fig. 2. Time to death of ranavirus-exposed larval wood frogs across pesticide treatments. (a) Individuals were exposed to ranavirus immediately after pesticide exposure. (b) Individuals were exposed to ranavirus 2 wk after pesticide exposure. Treatments sharing lowercase letters are not statistically different ($p > 0.05$). Data are means ± 1 SE.

3.3. Experiment 3 – effects of pesticides on ranavirus transmission

Sublethal pesticide exposure had no effect on the viral load of the focal tadpoles ($F_{2,27} = 4.01$, $p = 0.14$; Fig. 4). All focal hosts exposed to ranavirus were infected with an average viral load of 75,892 viral copies ng DNA^{-1}. While we were unable to detect shed virions in the water of the focal tadpoles, there was evidence of transmission to the naïve tadpoles because 100% of naïve tadpoles were infected with ranavirus. Additionally, the viral load of naïve tadpoles differed among pesticide treatments ($F_{2,27} = 5.44$ $p = 0.01$; Fig. 4). Compared to the control, viral load was lower in the carbaryl treatment ($p = 0.006$). There was no difference between the control and thiamethoxam treatments ($p = 0.79$). Finally, mean viral load was 65% lower in naïve tadpoles compared to focal tadpoles.

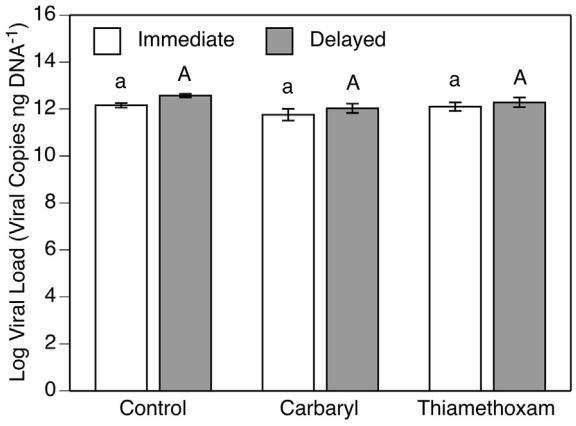

Fig. 3. Viral load (viral copies ng DNA^{-1}) at time of death for ranavirus-exposed larval wood frogs that were previously exposed to no pesticides (control), carbaryl (1 mg L^{-1}) or thiamethoxam (1 mg L^{-1}). Individuals were either exposed to ranavirus immediately ("Immediate") after pesticide exposure or 2 wk after pesticide exposure ("Delayed"). Treatments sharing uppercase and lowercase letters are not statistically different ($p > 0.05$). Data are means ± 1 SE.

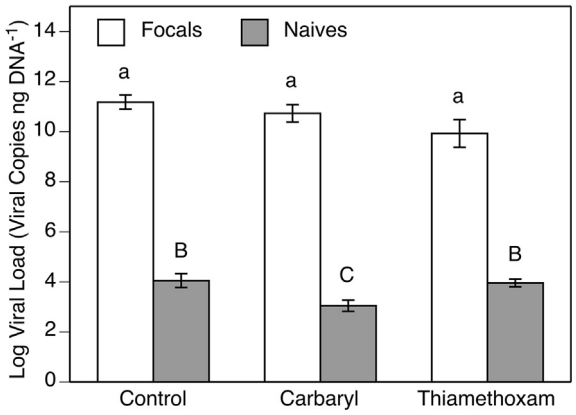

Fig. 4. Viral load (viral copies ng DNA^{-1}) at time of death for ranavirus-infected focal and naïve larval wood frogs. Focal larvae were previously exposed to one of three insecticide treatments (a control, carbaryl at 1 mg L^{-1}, or thiamethoxam at 1 mg L^{-1}) before virus addition. Naïve larvae were not previously exposed to insecticides or ranavirus before addition to containers with water from focals. Treatments sharing uppercase or lowercase letters are not statistically different ($p > 0.05$). Data are means ± 1 SE.

4. Discussion

There is a growing interest in addressing the interactive effects of pesticide exposure and disease on hosts. While there is evidence for altered disease dynamics as a result of pesticide exposure across host taxa, considerable research is needed for many understudied disease systems (Coors et al., 2008; Di Prisco et al., 2013; Marcogliese et al., 2010; Rohr et al., 2013). Moreover, research that addresses the effects of prior infection on estimates of pesticide toxicity is needed. We examined these interactions in the amphibian-ranavirus system, focusing both on the effects of pesticides on ranavirus dynamics and the effects of ranavirus infection on pesticide toxicity. We found that prior ranavirus infection can increase pesticide toxicity, and that pesticide exposure can alter disease outcomes.

We found that prior ranavirus infection increased the toxicity of the insecticides carbaryl and thiamethoxam to larval wood frogs by 72% and 55%, respectively. Notably, infection shifted LC50 values to concentrations measured in surface waters for thiamethoxam (~2.0 mg L^{-1}; J. Hoverman, M. Sepúlveda, and C. Krupke, unpublished data) and carbaryl (4.8 mg L^{-1}; Norris et al., 1983). Given the widespread prevalence of ranavirus infection and the ubiquity of pesticide contamination, this interaction could have considerable impacts on amphibian populations (Ariel et al., 2009; Fox et al., 2006; Green et al., 2002; Une et al., 2009). Because many pesticides have immunosuppressive effects on non-target organisms, research on pesticide-disease interactions has primarily focused on pesticide-mediated effects on disease outcomes (Christin et al., 2003; Di Prisco et al., 2013; Mason et al., 2013). While these effects are important, they assume that hosts are exposed to pesticides prior to disease agents. However, wild populations are likely to experience temporally varied exposure to pesticides and disease agents. Our results underscore the importance of considering scenarios in which pesticide exposure occurs following infection. Additionally, our results highlight the value in incorporating natural stressors into measurements of toxicity. Traditional toxicity tests, such as LC50 determinations, generally exclude the effects of natural stressors. However, by considering these effects, we can gain a better understanding of contaminant toxicity in natural environments. Similar effects on pesticide-induced mortality have been found for other stressors, such as predator cues (Relyea and Mills, 2001), but the effect of disease has rarely been addressed (Budischak et al., 2009). Given the ubiquity of parasites in natural systems, there is a need for further investigation involving other species and disease systems.

We also found that prior exposure to pesticides can influence disease outcomes in wood frogs. However, these effects were dependent on the pesticide and timing of ranavirus exposure following pesticide exposure. Time to death for tadpoles exposed to carbaryl was 8% shorter compared to control tadpoles. However, we did not observe this effect with thiamethoxam. Moreover, when the ranavirus exposure occurred two weeks post pesticide exposure, neither pesticide influenced time to death. These results suggest that pesticide exposure can influence disease-induced mortality, but the effects can be eliminated if individuals are given the opportunity to metabolize pesticides. Importantly, these results were not influenced by differences in susceptibility to infection; all individuals exposed to ranavirus become infected. Conversely, Forson and Storfer (2006a, 2006b) found that simultaneous exposure to the herbicide atrazine altered susceptibility to ranavirus infection in ambystomatid salamanders. Additionally, Rohr et al. (2013) determined that early-life exposure to atrazine increased *Bd*-induced mortality in later developmental stages of Cuban treefrogs, indicating that pesticide metabolism did not ameliorate mortality effects. However, differences in species, disease agents, pesticide modes of action, and order of exposure may all contribute to variation in susceptibility and mortality effects. In comparing viral load among pesticide treatments, we found no differences in both the immediate and delayed exposure regimes. Given that all measurements were taken at time to death, this indicates that individuals may experience mortality at similar viral loads. Additionally, wood frogs are highly susceptible to ranavirus infection with case mortality rates >95% (Hoverman et al., 2011), which may explain why there were no detectable differences in viral load. Because there is considerable variability in ranavirus dynamics among species (Hoverman et al., 2011), there is a need for research on other amphibian species to assess generality. For example, Forson and Storfer (2006a) also found that pesticide exposure did not affect viral load in ranavirus-infected tiger salamanders, suggesting that this may be a general trend for the amphibian-ranavirus system. Conversely, in other systems, pesticides have been shown to increase viral load, as seen with honey bees infected with deformed wing virus (Di Prisco et al., 2013). Infecting individuals with lower viral concentrations may also aid in detecting

subtle changes in viral load by preventing individuals from reaching the high viral load threshold where they appear to experience mortality. Future studies that generate variability in mortality and viral load will be necessary to determine how pesticide exposure affects the relationship between host fitness and parasite burden (i.e. tolerance of infection; Read et al., 2008). Collectively, our results suggest that pesticide exposure can increase disease-induced mortality rates, but this effect may be ameliorated if there is sufficient time to metabolize pesticides before pathogen exposure.

In addition to susceptibility, we examined the effects of pesticide exposure on ranavirus transmission. We found no effect of pesticide exposure on the viral load in focal hosts, suggesting that any differences in transmission were not due to pesticide-mediated effects on ranavirus infection. We did not recover ranavirus from the water samples and could not determine if ranavirus shedding rates differed among pesticide treatments. However, it was clear that transmission occurred because all naïve hosts were infected following exposure to water from the focal hosts. There were no differences in infection success among the naïve hosts, but viral loads were lower for naïve hosts in the carbaryl treatment. Therefore, pesticide exposure may affect transmission dynamics, either by affecting shedding rate or by affecting the virulence of shed particles. Viral shedding rates may be fairly low because we were unable to detect virus concentrations in the water. Additionally, viral loads for the naïve hosts were considerably lower than for the directly infected focal hosts. To our knowledge, there are no previous studies investigating ranavirus shedding rates. Therefore, considerable work is needed to understand this route of exposure and the influence of pesticide contamination.

5. Conclusions

Across taxa, species experience a variety of natural and anthropogenic stressors that may co-occur and interact, often with variable outcomes. For example, predator stress can magnify the effects of pesticides, ameliorate these effects, or influence how future generations respond to pesticide exposure (Gergs et al., 2013; Relyea, 2012; Trekels et al., 2013). Given the highly context-dependent nature of multiple stressor interactions, there is a need for research that addresses the details of these interactions to fully understand how they might influence species. We found that pesticide exposure and ranavirus infection have interactive effects on an amphibian host, and importantly, these effects are sensitive to the order and timing of exposure, providing further evidence that stressors can interact in context-dependent ways. When pesticide exposure preceded ranavirus infection, disease-induced mortality rates increased. Moreover, when we reversed the order of exposure, prior ranavirus infection increased the toxicity of pesticides and lowered LC50 values to environmentally relevant concentrations. In disease systems, we see similar priority effects when host organisms are coinfected with multiple pathogens in different orders (Hoverman et al., 2013), but rarely is a connection drawn to pesticide-disease interactions. These results emphasize the value of addressing these priority effects in studies of pesticides and disease dynamics by utilizing study designs that manipulate the order and timing of exposure. Additionally, they highlight the importance of incorporating natural stressors into traditional toxicity tests, which generally do not account for environmentally relevant scenarios. Given the multitude of natural and anthropogenic stressors that commonly co-occur and the context-dependency of their interactions, it is imperative that we form a comprehensive understanding of how stressors interact in varied systems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.11.086.

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Short Communication

THE NEONICOTINOID IMIDACLOPRID SHOWS HIGH CHRONIC TOXICITY TO MAYFLY NYMPHS

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Abstract—The present study evaluated the acute and chronic toxicity of imidacloprid to a range of freshwater arthropods. Mayfly and caddisfly species were most sensitive to short-term imidacloprid exposures (10 tests), whereas the mayflies showed by far the most sensitive response to long-term exposure of all seven arthropod species tested (28-d EC10 values of approximately 0.03 μg/L). The results indicated a high aquatic risk of chronic exposure of imidacloprid to mayflies. Environ. Toxicol. Chem. 2013;32:1096–1100.
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Keywords—Pesticides Imidacloprid Aquatic macroinvertebrates Chronic toxicity Laboratory toxicity tests Ecological risk assessment

INTRODUCTION

The systemic insecticide imidacloprid is a chloronicotinoid insecticide that has been used for insect pest control since the early 1990s. The compound is taken up by insects via contact and ingestion and binds to the nicotinic acetylcholine (nAcChR) receptor, thereby disrupting nerve impulses. It is selective for insect nAcChR receptors and consequently has much less pronounced effects in mammals [1–3]. Commercial crop protection uses include seed treatment and spraying to protect against sucking insects, soil insects, and chewing insects. Domestic uses include termite control and flea control in companion animals ([1]; http://npic.orst.edu/ingred/imid.html).

Within the agricultural context, non-target aquatic organisms potentially could be exposed to imidacloprid via input from spray drift, leaching, or runoff. After imidacloprid enters a body of water, its dissipation is dominated mainly by photolysis. Furthermore, the rate of dissipation has been shown to vary in different aquatic systems [4]. Thuyet et al. [5] reported a dissipation time 50% for the water phase ($DT50_{water}$) of 1 d in a rice paddy system (mainly due to photodegratation). In a pond microcosm system in Germany, a $DT50_{water}$ of 8.2 d was recorded [6]. The European Food Safety Authority (EFSA) reported $DT50_{water}$ values of 30 to 150 d for three water-sediment studies, performed at 22°C in the laboratory in the dark [4]. Based on these and other data, EFSA experts have agreed that, for the EU level assessment, it was appropriate to use a $DT50_{water}$ value of 90 d [4], possibly leading to long-term exposure of aquatic ecosystems.

Although long-term exposure may occur, the chronic toxicity data for imidacloprid is sparse. In a review, Posthuma-Doodeman [6] found few chronic values for macroinvertebrates, with the lowest 28-d no-observed-effect concentration (NOEC) of 1.14 μg/L for *Chironomus tentans* (see Supplemental Data, Table S1). To address this knowledge gap, the present study generated acute and chronic toxicity data for a range of non-standard, freshwater macroinvertebrate species. The present study was undertaken to determine the chronic toxicity of imidacloprid and to identify species of concern and the acute-to-chronic toxicity ratios for several species.

MATERIALS AND METHODS

Test animals and conditions

For the toxicity tests, taxa belonging to the orders Ephemeroptera (*Cloeon dipterum* and *Caenis horaria*), Diptera (*Chaoborus obscuripes*), Hemiptera (*Plea minutissima*, *Micronecta* spp., and *Notonecta* spp.), Trichoptera (Limnephilidae), Megaloptera (*Sialis lutaria*), Isopoda (*Asellus aquaticus*), and Amphipoda (*Gammarus pulex*) were used (Table 1). Test organisms were chosen based on their availability in the field and our ability to obtain a diverse selection of taxa within the arthropods.

The test animals were collected from uncontaminated aquatic ecosystems at the outdoor research site De Sinderhoeve, Renkum, The Netherlands (www.sinderhoeve.org). Macrocrustacean juveniles and early larval insect instars were used for the studies, except for *P. minutissima*, which was tested using adults. After collection, the animals were kept in aerated tanks in the laboratory—first in a mixture of field and test water, then in only in test water—for at least 3 d to acclimate them to laboratory conditions. Animals that tend to exhibit cannibalistic behavior (*Notonecta* spp. and *S. lutaria*) were kept individually in 500-ml glass jars. During the acclimation period, the test animals were fed *ad libitum* with the food items described in Table 1. During the acclimation and testing period, all jars containing the test animals were placed in a water bath maintained at a temperature of 18 ± 1°C with a light regime of 12:12 hours light:dark.

Acute tests

The test system for *Notonecta* spp. and *S. lutaria* consisted of 0.5-L jars filled with 300 ml of copper-free water, whereas for the other test species, 1.5 L jars were filled with 1 L copper-free water. In addition, stainless steel meshes were introduced into

All Supplemental Data may be found in the online version of this article.
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Table 1. Test species used in the acute and chronic toxicity studies and foodstuff used during acclimatization and chronic toxicity studies

Species	Order	Foodstuffs	Amount provided to each animal per day
Macrocrustaceans			
Asellus aquaticus	Isopoda	Conditioned *Populus* leaf	3.7 ± 0.2 mg/d
Gammarus pulex	Amphipoda	Conditioned *Populus* leaf	3.7 ± 0.2 mg/d
Insects			
Chaoborus obscuripes	Diptera	Copepod and *Daphnia* (zooplankton)	1.5 ml/d
Sialis lutaria	Megaloptera	Annelids and chironomidae larvae	2–3 pieces/d
Plea minutissima	Hemiptera	Copepod and *Daphnia* (zooplankton)	1.5 ml/d
Notonecta spp.	Hemiptera	Copepod and *Daphnia* (zooplankton)	1.5 ml/d
Micronecta spp.	Hemiptera	Copepod and *Daphnia* (zooplankton)	1.5 ml/d
Limnephilidae	Trichoptera	Organic matter and biofilm (*Populus* leaf)	2.5 ± 0.2 mg/d
Cloeon dipterum	Ephemeroptera	Biofilm, organic matter and periphitic algae	Pre-cultured in test vessels
Caenis horaria	Ephemeroptera	Biofilm, organic matter and periphitic algae	Pre-cultured in test vessels

the test system to serve as substrate. For each test, six treatments (including a control) were used. Each treatment had two or three replicates except for *Notonecta* spp. and *S. lutaria*, which were housed individually to avoid cannibalism (see Table 2 for experimental details).

Immediately after the animals were transferred into the test jars containing test water, an appropriate volume of imidacloprid stock solution was spiked using a capilettor (see the *Chronic tests* section for further analytical procedures). After 4 d, individuals were scored as immobile when no movement of any kind was observed for a period of 20 s and were scored as dead when no response of any kind was observed during 3 to 5 s of gentle stimulation using a Pasteur's capillary pipette. Dead organisms were removed daily from the test vessels. The test was considered valid when control immobilization did not exceed 10% [7]. If control immobility exceeded 10%, the results were considered to be indicative. The physicochemical variables of pH, dissolved oxygen, electrical conductivity, and temperature were measured at the start and end of the test in the control and highest treatment level only.

Chronic tests

Chronic tests were performed for the same species as the acute tests, with the exception of *Notonecta* spp., *Micronecta* spp., and Limnephilidae. For all species, the same test systems were used as in the acute tests, except for *C. horaria*, for which we used 0.5-L glass jars rather than 1.5-L glass jars (see Table 2 for experimental details). The experimental conditions and spiking procedure of imidacloprid were the same as in the acute tests. For non-air breathing animals, gentle aeration was provided. The animals were fed with appropriate species-specific food (Table 1). Every week, the jars were completely refreshed with new test medium, and the living test animals were transferred with care to the new test system. Immobility and mortality effect endpoints were monitored at the end of the test period, and the physicochemical parameters were measured weekly. Emerged individuals were removed weekly and were counted as missing in the statistical analysis because after emergence, it is no longer possible to determine whether the individual would have been affected. The test was considered valid when control immobilization did not exceed 10% [8].

Imidacloprid application, sampling, and analysis

Dosing solutions were prepared using a soluble concentrate (SL) formulation containing 200 g imidacloprid/L and made up in 2.5- or 7.5-ml dosing aliquots for application using a 2.5-ml capilettor. Samples were taken from the dosing solutions to confirm imidacloprid concentrations.

Exposure concentrations at the start of the acute and chronic tests were characterized using the measured concentration in the dosing solution, the amount of dosing solution applied, and the amount of receiving test volume. During the chronic tests, water samples from the control and highest treatments were collected for residue analysis at the end of each test week. Samples of approximately 3 ml were collected with a glass Pasteur's pipette and transferred into 4 ml glass vials containing 1 ml of

Table 2. Test concentrations used in the acute and chronic tests, which included a control, and the time-weighted average measured concentrations in the chronic tests as a percentage of the nominal concentrations[a]

	Acute tests		Chronic tests		
Species	Test concentration (μg/L)	No. of replicates (No. of individuals per replicate)	Test concentration (μg/L)	No. of replicates (No. of individuals per replicate)	Measured concentration as percentage of nominal ± SD
Macrocrustaceans					
Asellus aquaticus	10, 30, 100, 300, 1000	3 (10)	1, 3, 10, 30, 100	3 (10)	95.3 ± 1.1
Gammarus pulex	10, 30, 100, 300, 1000	3 (10)	1, 3, 10, 30, 100	3 (10)	97.0 ± 2.7
Insects					
Chaoborus obscuripes	1, 10, 30, 100, 300	3 (10)	0.3, 1, 3, 10, 30	3 (10)	91.7 ± 4.2
Sialis lutaria	1, 10, 30, 100, 300	15 (1)	0.3, 1, 3, 10, 30	15 (1)	95.3 ± 1.5
Plea minutissima	1, 10, 30, 100, 300	3 (10)	0.3, 1, 3, 10, 30	3 (10)	92.4 ± 5.5
Notonecta spp.	1, 10, 30, 100, 300	15 (1)	NP		NP
Micronecta spp.	1, 10, 30, 100, 300	3 (10)	NP		NP
Limnephilidae	1, 10, 30, 100, 300	2 (10)	NP		NP
Cloeon dipterum	1, 10, 30, 100, 300	3 (10)	0.03, 0.1, 0.3, 1, 3	3 (10)	86.4 ± 3.7
Caenis horaria	1, 3, 10, 30, 100	3 (10)	0.01, 0.03, 0.1, 0.3, 1	3 (10)	84.9 ± 4.5

[a]Time-weighted averages were determined for four weeks and three replicates ($n = 12$).
SD = standard deviation; NP = test not performed.

acetonitrile, shaken thoroughly by hand, and subsequently stored in a freezer at –20°C prior to analysis. The samples were analysed by liquid chromatography–tandem mass spectrometry with an Agilent Zorbax Eclipse XDB C18 column (4.6 × 150 mm, 5 μm). Eluents were isocratic Milli-Q water +0.1% v/v formic acid and methanol +0.1% v/v formic acid, with a flow of 0.7 ml/min. The fragmentator voltage was 88 V and 209 *m/z* and 175.1 *m/z* for the respective quantifier and qualifier ions. The collision energy used was 18 and 14 V respectively. Dr. Ehrenstorffer imidacloprid was used as an external standard.

Data analysis

The chronic, time-weighted average exposure concentrations were calculated using the following formula

$$TWA_t = \frac{c_0}{k \times t_{TWA}} \left(1 - e^{(-k \times t_{TWA})}\right)$$

where TWA_t is the average concentration (μg/L) over a period of t days; c_0 is the start concentration (μg/L); k is the dissipation rate coefficient (1/d); and t_{TWA} is the period over which the *TWA* was calculated (d). The dissipation rate constant, k, was calculated using

$$k = \frac{-\ln\left(\frac{c_t}{c_0}\right)}{\Delta t}$$

where c_t is the concentration (μg/L) at the end of the period Δt, and Δt is the length of the period (d).

Dose–response relationships were fitted using the nominal exposure concentrations for both endpoints with both the acute and chronic data using the following equation

$$y(conc) = \frac{1 - c}{1 + e^{-b \times (\ln conc - a)}}$$

where y is the fraction of dead or affected test animals (–), *conc* is the nominal concentration (μg/L), a is ln(median effective concentration [EC50]) or ln(median lethal concentration [LC50]) (μg/L), b is the slope (L/μg), and c is the fraction of control mortality or immobilisation (–).

The log-logistic regression was performed using GenStat 15th edition (Laws Agricultural Trust; VSN International). Species sensitivity distributions were constructed for both acute and chronic EC50 and effective concentration for 10% (EC10) values values using the ETX2.0 program [9], and the median 5th percentile hazardous concentrations (HC5) were calculated. The Anderson–Darling test (incorporated into the ETX2.0 program) was used to check whether the data fit the log-normal distribution ($p = 0.05$).

Acute-to-chronic toxicity ratios (ACR) were calculated as the mean of the ratio based on lethal concentration and effective concentration data

$$ACR = \frac{\left(\frac{96h,\ \mathrm{LC50}}{28d,\ \mathrm{LC10}}\right) + \left(\frac{96h,\ \mathrm{EC50}}{28d,\ \mathrm{EC10}}\right)}{2}$$

RESULTS AND DISCUSSION

Imidacloprid exposure

Concentrations of imidacloprid measured in the dosing solutions used were, on average, 97.5% (±7.1, $n = 10$) and 95.5% (±4.3, $n = 7$) of the intended concentration for the acute and chronic tests, respectively. Hence, we concluded that, based on the amount of dosing solution applied and the amount of receiving test volume, all tests received their appropriate dosage.

During the four-week test period of the chronic tests, the time-weighted average imidacloprid concentrations were 91.9% (±4.6, $n = 7$) of the intended test concentrations (Table 2). Based on these results, the chronic effects were expressed as nominal test concentrations following the recommendation by the European Commission [10]. Because the same application procedure was used in the acute tests as in the chronic tests, and the application procedure has been validated in the chronic tests by the analytical measurements, we also expressed the acute effects in terms of nominal concentrations.

Physicochemical parameters

In both the acute and chronic tests, physicochemical variables did not show a significant increase or decrease over the experimental period except in the acute test with *G. pulex*. The dissolved oxygen (DO) level decreased drastically during the acute test performed with *G. pulex*. The 96-h DO values observed for the control and highest treatment were 3.95 ± 0.30 mg/L and 5.29 ± 0.45 mg/L, respectively. A pesticide concentration dependent increase was observed in all tests for pH and electrical conductivity parameters. In general (excluding the values for the acute test with *G. pulex*), the observed ranges of the variables during the experimental period were pH (7.36–8.30), DO (7.06–9.59 mg/L), effective concentration (179–208 μS/cm), and temperature (17.7–19.7°C).

Toxicity

The acute data showed that mayflies (*C. dipterum* and *C. horaria*) and caddisflies (Limnephilidae) are the most sensitive species tested (Table 3). Although all insects possess the nicotinic acetylcholine receptor, freshwater insects such as *C. obscuripes*, *Notonecta* spp., and *S. lutaria* were relatively tolerant to short-term imidacloprid exposure, as were the macrocrustacean species (*A. aquaticus* and *G. pulex*). The acute tests with *G. pulex* and *Micronecta* spp. were the only tests that had control immobilization above 10%; therefore, their results should be considered to be indicative. The control mortality of *G. pulex* can be explained by the observed decrease in dissolved oxygen levels. The 96-h LC50 value of *G. pulex* was very similar to that reported by Beketov and Liess [11] of 270 μg/L, whereas the 96-h EC50 was very similar to the concentration of 14.2 μg/L, which Böttger et al. [12] reported for *Gammarus roeseli*. Note that *Notonecta* spp. and *S. lutaria* did not show any treatment-related mortality in the acute experiments (Table 3). Based on the 96-hour EC50 values, the calculated hazardous concentration 5% ($HC5_{96h\ EC50}$) was 0.630 (0.065–2.27) μg/L, and the HC5 based on 96-h EC10 values ($HC5_{96h\ EC10}$) was 0.084 (0.005–0.422) μg/L. The $HC5_{96h\ EC50}$ corresponds very well with the microcosm NOEC of 0.6 μg/L, which was derived from a microcosm experiment performed in Germany that evaluated two applications of imidacloprid with a 21-d interval [6]. The EFSA [4] concluded from this microcosm study that Ephemeroptera are very sensitive due to their long larval development, but it was not possible to draw a clear conclusion on the effects and recovery of sensitive mayfly species, because abundances were too low to allow reliable statistical evaluation. The acute data listed by Posthuma-Doodeman [6] for macrocrustaceans and insects are generally higher than those reported here, but they consist almost exclusively of LC50 values, and the duration of

Table 3. Results of acute toxicity studies performed with imidacloprid expressed as 96-h LC50 and EC50 and 96-h LC10 and EC10 values (μg/L), the slope parameter of the dose–response function and observed control mortality and immobilization

	Mortality						Immobilization					
Species	96-h LC50	(95% CI)	96-h LC10	(95% CI)	Slope (b)	Control mortality (%)	96-h EC50	(95% CI)	96-h EC10	(95% CI)	Slope (b)	Control immobilisation (%)
Macrocrustaceans												
Asellus aquaticus	316	(216–461)	61.6	(34.1–111)	1.34	0	119	NC	24.7	NC	1.40	0
Gammarus pulex	263	(155–446)	99.5	(32.2–307)	2.26	33[a]	18.3	(8.84–37.8)	3.63	(0.916 – 14.4)	1.36	33[a]
Insects												
Chaoborus obscuripes	294	(247–350)	178	(66.1–481)	4.39	3	284	NC	223	NC	9.06	3
Sialis lutaria	> 10000	(0 – > 10000)	> 10000	(0 – > 300000)	NC	0	50.6	(30.9–82.8)	15.7	(6.95–35.4)	1.88	0
Plea minutissima	37.5	NC	32.3	NC	14.8	7	35.9	(31.1–41.5)	30.4	(26.1–35.4)	13.1	7
Notonecta spp.	> 10000	(0 – > 10000)	> 10000	(0 – > 300000)	NC	0	18.2	(9.24–35.7)	3.00	(0.779–11.5)	1.22	0
Micronecta spp.	28.2	(17.6–45.2)	8.87	(3.43–22.9)	1.90	20[a]	10.8	(9.72–12.0)	9.41	(8.34–10.6)	15.9	20[a]
Limnephilidae	25.7	(18.1–36.5)	9.86	(5.09–19.1)	2.29	5	1.79	(0.993–3.22)	0.532	(0.220–1.29)	1.81	5
Caenis horaria	6.68	(4.19–10.6)	2.55	(0.952–6.85)	2.29	10	1.77	(1.05–2.99)	0.325	(0.105–1.00)	1.29	10
Cloeon dipterum	26.3	(17.7–39.1)	6.16	(2.69–14.1)	1.52	0	1.02	(0.460–2.28)	0.100	(0.018–0.554)	0.944	0

[a]Because the control immobilization in these tests exceeded 10%, their results should be considered to be indicative.
LC50 = median lethal concentration; CI = confidence interval; LC10 = lethal concentration for 10%; EC50 = median effective concentration; EC10 = effective concentration for 10%; NC = confidence interval or slope could not be calculated.

most of those studies was also shorter (24 or 48 h) than those reported in the present study.

For chronic exposures, the insect *C. dipterum* was the most sensitive to imidacloprid exposure, followed by *C. horaria* (Table 4). These species showed much higher sensitivity than the other species tested. Based on chronic EC50 values, these species were approximately 50 times more sensitive to imidacloprid than *P. minutissima* (Table 4). The Anderson–Darling test did not indicate log-normality at $p = 0.05$; therefore, no HC5 were reported. As in the acute test, *S. lutaria* showed the largest difference in response to the long-term exposure between the two assessment endpoints (mortality and immobility). Based on chronic EC50 values, the macrocrustaceans (*G. pulex* and *A. aquaticus*) and *C. obscuripes* were relatively tolerant. The chronic threshold value for *G. pulex* (28-d EC10 = 2.95 μg/L) is, however, much lower than the 28-d NOEC of 64 μg/L reported for swimming behavior for the same species (see Supplemental Data). The 28-d NOEC values reported for the two *Chironomus* species (1–3 μg/L; see Supplemental Data) are in the same range as observed with some of the chronic insect endpoints in the present study (Table 4).

All ACRs were greater than 10 (Table 4). *Plea minutissima* showed the lowest ratio and *C. dipterum* showed the highest ratio. Because our experiments showed large ACRs for some arthropod species, this implies that acute data are not appropriate to assess the effects of long-term exposure to imidacloprid.

Ensminger et al. [13] detected imidacloprid in 50% of water samples taken in Sacramento County and Orange County, California, USA, with median concentrations of 0.05 μg/L and maxima of almost 0.70 μg/L. Phillips and Bode [14] detected imidacloprid in 40% of the samples taken from the Kisco River in south-eastern New York state, USA, in 2000 and 2001, with a maximum concentration of 0.13 μg/L. Jemec et al. [2] reported a maximum concentration of 14 μg/L for Lake Wales Ridge, Florida, USA. If such measured concentrations occur over chronic time periods, there may be effects on sensitive insect species.

Table 4. Results of chronic toxicity studies performed with imidacloprid expressed as 28-d LC50 and EC50 and 28-d LC10 and EC10 values (μg/L), the slope parameter of the dose–response function (–), and the observed control mortality and immobilisation (%)

	Mortality						Immobilization						
Species	28-d LC50	(95% CI)	28-d LC10	(95% CI)	Slope (b)	Control mortality (%)	28-d EC50	(95% CI)	28-d EC10	(95% CI)	Slope (b)	Control immobilisation (%)	ACR
Macrocrustaceans													
Asellus aquaticus	20.3	(8.61–47.9)	1.35	(0.164–11.0)	0.810	20	11.9	(5.94–23.7)	1.71	(0.386–7.55)	1.13	20	152
Gammarus pulex	33.8	(20.9–54.6)	5.77	(1.92–17.3)	1.24	7	15.4	(9.80–24.1)	2.95	(1.15–7.59)	1.33	7	26
Insects													
Chaoborus obscuripes	12.6	(7.33–21.6)	1.99	(0.523–7.60)	1.19	12	11.8	(8.17–17.1)	4.57	(2.05–10.2)	2.31	12	105
Sialis lutaria	32.5	NC	25.1	NC	8.55	7	3.46	(1.86–6.44)	1.28	(0.382–4.31)	2.22	7	40[a]
Plea minutissima	9.80	(7.61–12.6)	4.35	(2.66–7.11)	2.71	0	6.45	(4.81–8.64)	2.03	(1.26–3.28)	1.91	0	13
Cloeon dipterum	0.195	(0.113–0.338)	0.041	(0.013–0.124)	1.40	8	0.123	(0.075–0.201)	0.033	(0.012–0.090)	1.67	8	336
Caenis horaria	0.316	NC	0.235	NC	7.43	13	0.126	(0.070–0.228)	0.024	(0.006–0.091)	1.32	17	51

[a]Based only on effective concentration values.
LC50 = median lethal concentration; CI = confidence interval; LC10 = lethal concentration for 10%; EC50 = median effective concentration; EC10 = effective concentration for 10%; ACR = acute-to-chronic toxicity ratio; NC = confidence interval could not be calculated.

SUPPLEMENTAL DATA

Table S1. Published data on chronic toxicity (test duration $>$ 20 d) of imidacloprid to freshwater invertebrates. (41 KB DOC).

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Assessment of the Effects of the Pesticide Imidacloprid on the Behaviour of the Aquatic Oligochaete *Lumbriculus variegatus*

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Abstract Contaminants, such as pesticides, can cause direct toxic effects when released into aquatic environments. Suitably sensitive species can help us understand and predict the impacts of such pollutants. Automated sediment toxicity testing and biomonitoring has grown rapidly, and biomonitoring instruments have proven appropriate for studying the effects of pollutants. A new approach in online biomonitoring, using the multispecies freshwater biomonitor was developed in the present study, using whole-sediment toxicity tests and behavioural responses of the freshwater oligochaete *Lumbriculus variegatus*. Endpoints, such as mortality and growth, were used to study the effects of the pesticide imidacloprid and to achieve a gradient of responses; exposures to contaminated sediments were performed over 10 days' duration (short-term tests). High mortality was observed in the three highest concentrations of imidacloprid, and inhibition of behaviour was monitored along a gradient of pesticide concentration. Exposure to imidacloprid-contaminated sediments affected growth, behaviour, and avoidance in *L. variegatus*.

Mortality, bioaccumulation, growth, and reproduction have been the most common endpoints used in the majority of studies in environmental toxicology (Leppänen and Kukkonen 1998). Data are lacking on sublethal toxicologic endpoints, such as effects on morphology or behaviour. Without this kind of information, complex biologic actions cannot be fully understood, and reliable predictions of ecologic impacts of environmental toxicants cannot be made (Rogge and Drewes 1993). Since the 1980s, there has been increasing interest in investigating sublethal endpoints (Aisemberg et al. 2005). Behaviour may be an important endpoint to elucidate mechanisms of toxicity (Macedo-Sousa et al. 2007). Once quantified, a behaviour has the potential to be used as a biomarker in the assessment of stress (Beitinger 1990). Biomonitoring offers a useful tool for the assessment of metal pollution in aquatic ecosystems (Zhou et al. 2008), and should rely on sublethal endpoint rather than on mortality alone (Macedo-Sousa et al. 2007).

Construction and functioning of the multispecies freshwater biomonitor (MFB) has been described elsewhere. Briefly, it measures online the different behaviours of aquatic species and is based on the registration of changes in a high-frequency alternating current caused by movements of organisms in their test chambers (Macedo-Sousa et al. 2008). The individual test organism is placed in a test chamber with two pairs of stainless steel-plate electrodes. Different types of behaviour (movements) generate characteristic electrical signals (Macedo-Sousa et al. 2008) that can be characterized by their amplitude and frequency. For *L. variegatus*, two different movements can be measured: peristaltic movements (0.5–1 Hz) and locomotion (1–3 Hz). The electrical signals are processed by a discrete fast Fourier transformation and generate a histogram of the occurrence of all signal frequencies in percentages (summarized in intervals of 0.5 Hz from 0 to 10 Hz), yielding a "fingerprint" of the behavioural pattern of the organism. This transformation gives the percentage of occurrence of each single frequency during a period of 4 min. The unit for measurement is the test chamber, which can have different sizes, forms, materials, and arrangements of electrodes. This method has been shown to be a valuable

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biomonitoring and toxicity testing tool using epibenthic crustaceans, insects, and planktonic and pelagic species of fish and tadpoles (Gerhardt 2000).

Aquatic species are important contributors to the functioning of lotic foodwebs (Benke and Jacobi 1994; Thorp and Delong 2002), which is why they were chosen as test species for the present study. Oligochaete worms are key macroinvertebrate constituents of terrestrial and freshwater ecosystems (Edwards and Lofty 1977; Brinkhurst and Gelder 1991). The locomotion and other behavioural activities of these organisms are significant determinants of the physical, chemical, and biologic properties of soils and sediments. In addition, locomotor functions are the cornerstone of such vital functions as foraging, sexual reproduction, predator avoidance, dispersal, and general orientation to environmental cues (Drewes 1997). Aquatic oligochaetes have an extremely long history of use in pollution assessment (Chapman 2001). *Lumbriculus variegatus* (Müller 1774) is recommended for use in toxicity tests with sediments based on its ease of culture and handling, known chemical exposure history, adequate tissue mass for chemical analysis, tolerance to a wide range of sediments' physicochemical characteristics, low sensitivity to contaminants associated with sediment, and amenability to long-term exposure without feeding (Ingersoll et al. 2003). Judging by the number of internationally published articles, the most common oligochaete species used in evaluations of freshwater toxicity has been *L. variegatus* (Leppänen 1999; Aisemberg et al. 2005). This species was proposed by the American Society of Testing and Materials (ASTM 1995) as a standard organism for tests of sediment bioaccumulation and is listed by the Organization for Economic Co-operation and Development (1992) as a good organism for bioaccumulation studies. *L. variegatus* is a freshwater oligochaete known to have remarkable powers of segmental regeneration (Hyman 1916). Reproduction under laboratory conditions is always by asexual fragmentation, during which a worm spontaneously divides into two or more body fragments. Each surviving fragment then undergoes rapid regeneration of body segments to form a new head, tail, or both (Lesiuk and Drewes 1999).

All pesticides on the market have been evaluated by sets of standardized protocols (the so-called a priori evaluation). One of the aims of standardized tests is to evaluate the negative effects of pesticides on terrestrial and aquatic ecosystems. Because the direct impact on ecosystems is difficult to study, the tests are based on the detrimental effects observed in a set of model organisms that play key roles in ecosystem structure and function. However, although the European Commission (EC) encouraged the development of tests to determine sublethal effects on model organisms, most of these protocols focused on mortality (Capowiez et al. 2005). The EC recognized the importance of sublethal tests, for earthworms in particular, when the active substance is potentially persistent or applied more than once (EC 2003).

Imidacloprid [1-(6-chloro-3-pyridylmethyl)-2nitroimino-imidazolidine] is a relatively new systemic insecticide (product names Admire, Confidor, Gaucho, and Provado, manufactured by Bayer Cropscience). It was the first member of a new family, the neonicotinoids, and is chemically related to the nicotinic acetylcholine receptor (nAChR) agonists nicotine and epibatidine (Matsuda et al. 2001). It acts as an agonist of acetylcholine (Bai et al. 1991) and is therefore effective against many insects currently resistant to carbamates, organophosphates, and pyrethroids. It was first introduced to the United Kingdom in 1998 and is now marketed in >120 countries to protect >140 crops (Simms et al. 2006). It is widely used in agriculture for controlling sucking insects, as a seed dressing, for soil treatment, and as a foliar treatment for a variety of crops, including orchards. It is also used for controlling cockroaches and termites and is found in many products used for domestic pets and in gardens (Cox 2001). Some studies have shown that imidacloprid can induce behaviour modifications in parasitoid hymenoptera (Stapel et al. 2000) and termites (Thorne and Breisch 2001) (foraging and burrowing activities, respectively). The effects of imidacloprid on earthworms have been studied to a certain extent. Luo et al. (1999) and Zang et al. (2000) found sperm deformities in *Eisenia fetida* at imidacloprid concentrations as low as 0.5 mg/kg in dry soil. More recently, Mostert et al. (2000, 2002) showed that the LC_{50} for worms of the Pheretima group was 3 mg/kg in dry soil and that no effect was observed on earthworm weight at 0.66 mg/kg in dry soil. Finally, Lal et al. (2001) observed a decrease in the production of earthworm casts during a period of 120 days in field conditions. Capowiez et al. (2003) found that the behaviour of earthworms was significantly altered, noting decreases in burrow length, rate of burrow reuse, and distance covered, at concentrations of imidacloprid between 0.5 and 1 mg/kg in dry soil.

Imidacloprid has been found in streams and rivers and is likely to be bioavailable to aquatic organisms. Because few studies on the toxicity of imidacloprid have been relevant to lotic species (Alexander et al. 2007), we investigated the impact of imidacloprid on the behaviour of the aquatic oligochaete *L. variegatus*. In this study, a new automated sediment toxicity test using *L. variegatus* was developed to assess the effects of short-term exposures to different concentrations of the pesticide. In addition, this study was an attempt to investigate the use of the behaviour of *L. variegatus* as a tool to assess the sublethal effects of a toxic substance. Our hypothesis was that the exposure to the pesticide would cause behavioural early warning responses, particularly locomotion and peristaltic movements.

Materials and Methods

Culture

Laboratory cultures of *L. variegatus*, used throughout these tests, originated from the University of Joensuu, Finland. Animals were reared in polyethylene aquariums (8.5 × 17.5 × 12 cm), covered with lids, that contained ASTM (1980) medium (pH 7.6 ± 0.3; 20°C) in a temperature-controlled room (16:8-h light-to-dark cycle and 50% humidity). A commercially available sand-pebble mixture (grain sizes 0–8 mm) was acid washed (pH 2), ashified (for 4 h at 450°C), and used as sediment. The aquaria contained a 2-cm layer of sediment with continuous and moderated aeration. The worms were fed with Tetraphyll, applied two or three times a week (approximately 5 mg/30 worms).

Spiking

Whole sediment (sediments and associated pore water that have had minimal manipulation (United States Environmental Protection Agency [USEPA] 2000) used in the experiments had the following characteristics: 4.9% sand, 74.4% clay, and 20.7% silt; pH 6.77; ammonia 3.04 mg/kg; and total carbon content 0.54%. Solutions were prepared by dissolving the appropriate amount of imidacloprid ($C_9H_{10}ClN_5O_2$; supplied by Sigma-Aldrich) in distilled water. These solutions were immediately added to the sediment, which was then capped and rapidly shaken for 1 min. The spiked sediments were equilibrated in the dark (due to the light sensitivity of the pesticide) for a minimum of 48 h to allow the pesticide to adsorb to the sediment particles. During this time, the sediments were shaken every day for approximately 2 min. The contaminated water was replaced by ASTM water before adding the worms. The nominal imidacloprid concentrations in the sediment were 0 (uncontaminated sediment control), 0.05, 0.5, 1.0, 2.5, and 5.0 mg/kg. The sediment was sampled for imidacloprid analysis at the start and end of each test, and the overlying medium (ASTM) was sampled only at the end of the tests.

Exposure Design

Short-term (10-day) tests were performed using contaminated sediment and clean water. The exposures were conducted at 20°C in a temperature-controlled room (16:8-h light-to-dark cycle and 50% humidity) in 100-ml plastic beakers containing 35 g whole sediment and 20 ml ASTM medium under static conditions. Seven replicates per concentration were used, each with six young worms (approximately 1.5 cm; totalling 42 organisms per concentration) that were carefully introduced into the beakers with the help of a plastic Pasteur pipette. Mortality, growth (size class 1 = worm <2 cm; size class 2 = worm >2 cm but <2.5 cm; size class 3 = worm >2.5 cm), colour, and presence in sediment or water were monitored every 48 h. For monitoring, the worms were removed from the test beakers and carefully observed and measured. The sediments were replaced by newly spiked ones at day 5, and no food was added during the test. The surviving worms were collected, dried at 40°C for approximately 24 h, and then weighed. Before drying, the worms were rinsed rapidly in distilled water and gently dried with filter article. There was no attempt to remove the sediment from the intestines of *L. variegatus* by allowing a depuration period (putting the worms in water for 24 h) because an increase in worm water content could decrease or erase the negative effects on weight (Dalby et al. 1996; Capowiez et al. 2005). Imidacloprid levels in water, sediment, and whole-body samples were analysed after extraction with acetonitrile by ultraviolet high pressure liquid chromatography at 270 nm (limit of quantitation [LOQ] 0.1 µg/l for water, 0.001 mg/kg for sediment, and 0.01 mg/kg for whole body). Before analysis, the sediment, water, and whole-body samples were kept in the dark. Biomass, i.e., the dry weight of surviving organisms divided by the initial number of organisms (United States Environmental Protection Agency [USEPA]) 2000), was also calculated. Bioaccumulation factors (BAFs) were calculated according to the following formula (Barron 1995; Eq. 1):

$$\mathrm{BAF} = \text{contaminant concentration (mg/kg dry wt) in tissue/contaminant concentration (mg/kg dry wt) in sediment.} \quad (1)$$

Behavioural Responses

Behaviour was measured using the MFB. Seven worms per concentration were used, and behaviour was record for 2 h (plus a 30-min acclimation period). The lower half of each test chamber was filled with sediment; the upper half contained ASTM medium (ASTM 1980). One worm was added to each test chamber, and three chambers without worms were used as controls.

Statistical Analysis

Regression analyses were carried out using Excel software (Microsoft). For each tested concentration, selected behavioural signal frequencies (ranges 0–1 and 1–3 Hz) were plotted over time. Normality and homoscedasticity were tested using SigmaStat for Windows (version 3.5) software. Original behavioural MFB data were arcsin

transformed, and the overall effect of imidacloprid concentrations on population behaviour was investigated using Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks ($p < 0.001$) followed by *posthoc* Dunn's test ($p < 0.05$) to test for significant differences (Zar 1996).

Results

The normal reddish-brown colour of the worms did not change after exposure to imidacloprid. Avoidance was measured by counting the number of worms that were not in the sediment. As seen in Fig. 1, *L. variegatus* clearly avoided contaminated sediments, and avoidance was greater (100%) for sediments contaminated with higher concentrations of the pesticide. Furthermore, the controls never avoided the sediment and were not found in the water. avoidance increased during the test period for worms exposed to 0.05 and 0.5 mg/kg.

Mortality (Fig. 2) in the controls was low (2%), demonstrating that the holding facilities and handling techniques were acceptable for conducting such tests, as required in the standard protocol, in which mean survival for controls should be 90% (ASTM 1990). After 10 days of exposure, high mortalities were observed in worms exposed to 1.0, 2.0, and 5.0 mg/kg. There was a clear positive relation between mortality and imidacloprid concentrations: Higher mortalities were observed in worms exposed to higher pesticide concentrations. A power trendline ($R^2 = 0.84$) showed that mortality increased at a specific rate.

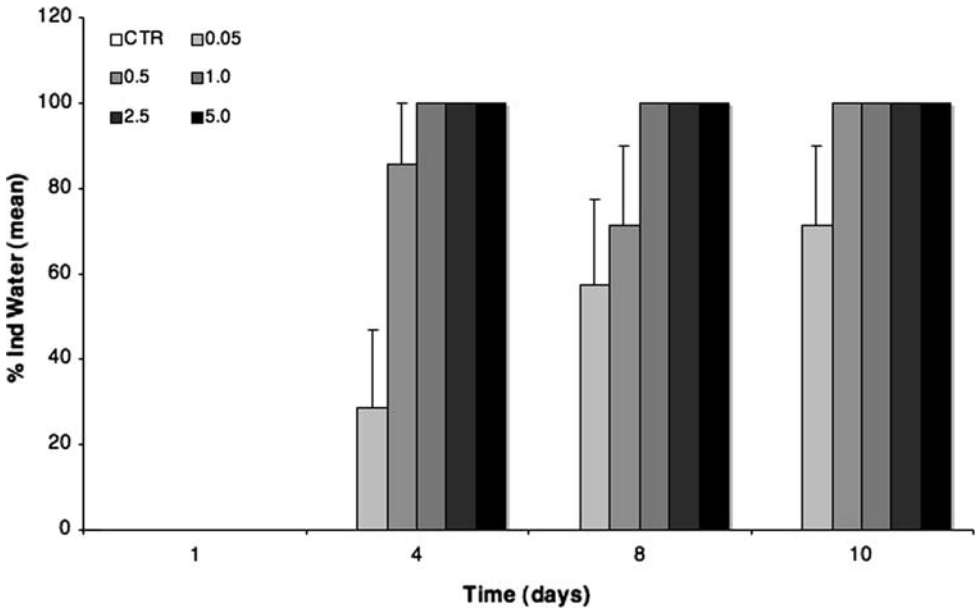

Fig. 1 Percentage of individuals in water (mean ± STDEV) throughout the test

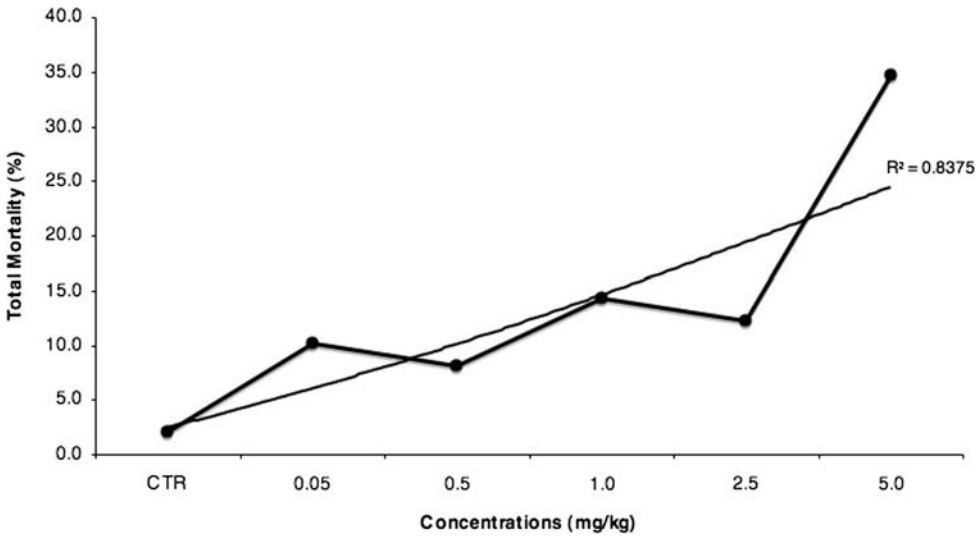

Fig. 2 Total mortality (%) of *L. variegatus* at the end of the short-term (10-day) test

After the short-term test, it was clear that the growth of the worms was inhibited by their exposure to sediments contaminated with imidacloprid (Fig. 3). All tested concentrations induced a growth inhibition; in fact, worms exposed to higher concentrations (2.5 and 5.0 mg/kg) did not grow at all. Biomass, according to the USEPA (2000), is the dry weight of surviving organisms divided by the initial number of organisms. Biomass data (Table 1) corroborated the results of mortality and growth. As expected, biomass decreased with increasing imidacloprid concentrations because mortality was higher and growth was lower. Biomass values (Fig. 3) decreased in a concentration-dependent fashion.

Behavioural tests showed that exposure to imidacloprid strongly inhibited both locomotion and peristaltic movements (Fig. 4). The average frequency of peristaltic movements on day 1 (Fig. 4a) was high for all worms, with no significant differences between sediments ($p > 0.05$). However, after 10 days of exposure to collected sediments (Fig. 4b), significant differences ($p < 0.05$) were found among the worms. Activity decreased with increased pesticide concentrations. The variances were not homogeneously distributed, but the results of one-way ANOVA on ranks indicated a significant concentration effect ($p < 0.05$) for both locomotion and peristaltic movement.

Sediment, Water, and Whole-Body Analysis

Concentrations of pesticide present in the initial and final sediment samples were as expected (Table 2), thus confirming the adequacy of the protocol designed for spiking the sediments with imidacloprid. Differences were found between the initial and final values of imidacloprid in the sediment: after 10 days, despite frequent sediment change, concentrations of the pesticide were much lower. Water samples collected at the end of the test showed some imidacloprid present due to pesticide degradation. As expected, whole-body tissues presented a higher level of imidacloprid when exposed to higher concentrations of the pesticide (Table 1). Worms exposed to higher levels of imidacloprid (2.5 and 5.0 mg/kg) had higher concentrations of the pesticide in whole-body tissues, which confirms a great ability to absorb and store this chemical.

Table 1 Bioaccumulation and biomass data[a]

Concentration (mg/kg)	Bioaccumulation (mg/kg)	Biomass (mg)
CTR	<LOQ	502.7
0.05	4.06	456.9
0.5	4.02	452.3
1.0	6.16	443.3
2.5	9.73	308.0
5.0	27.8	219.0

[a] LOQ 0.001 mg/kg

Discussion

Growth of *L. variegatus* was particularly affected by exposure to contaminated sediments. Even the lowest concentration of imidacloprid (0.05 mg/kg) was observed to inhibit growth. Hence, growth was inhibited by exposure to imidacloprid-contaminated sediments. Acute (short-term) tests are useful for identifying highly toxic chemicals, but they do not test key life events, during which

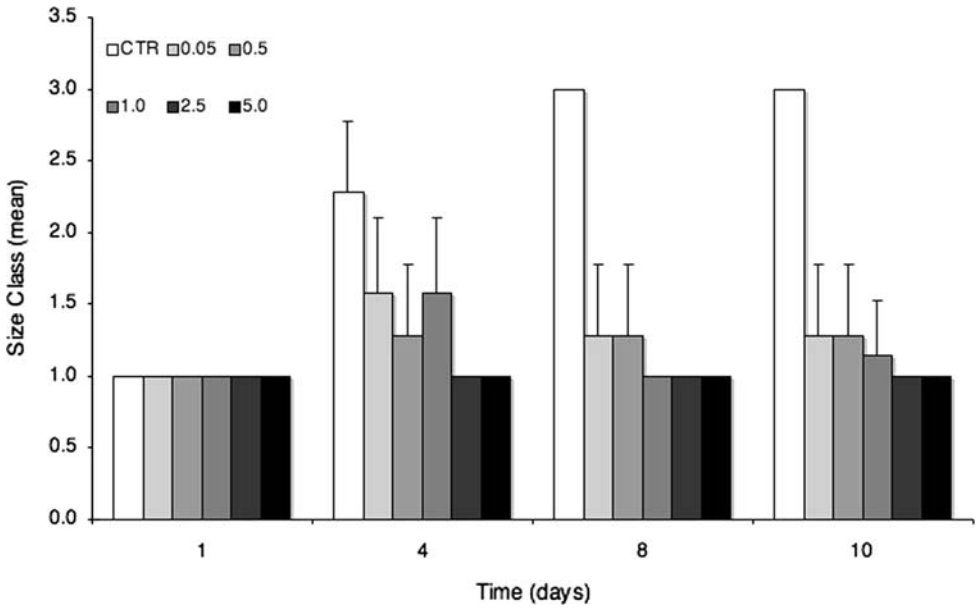

Fig. 3 Growth (size class) of worms (mean ± STDEV) throughout the test

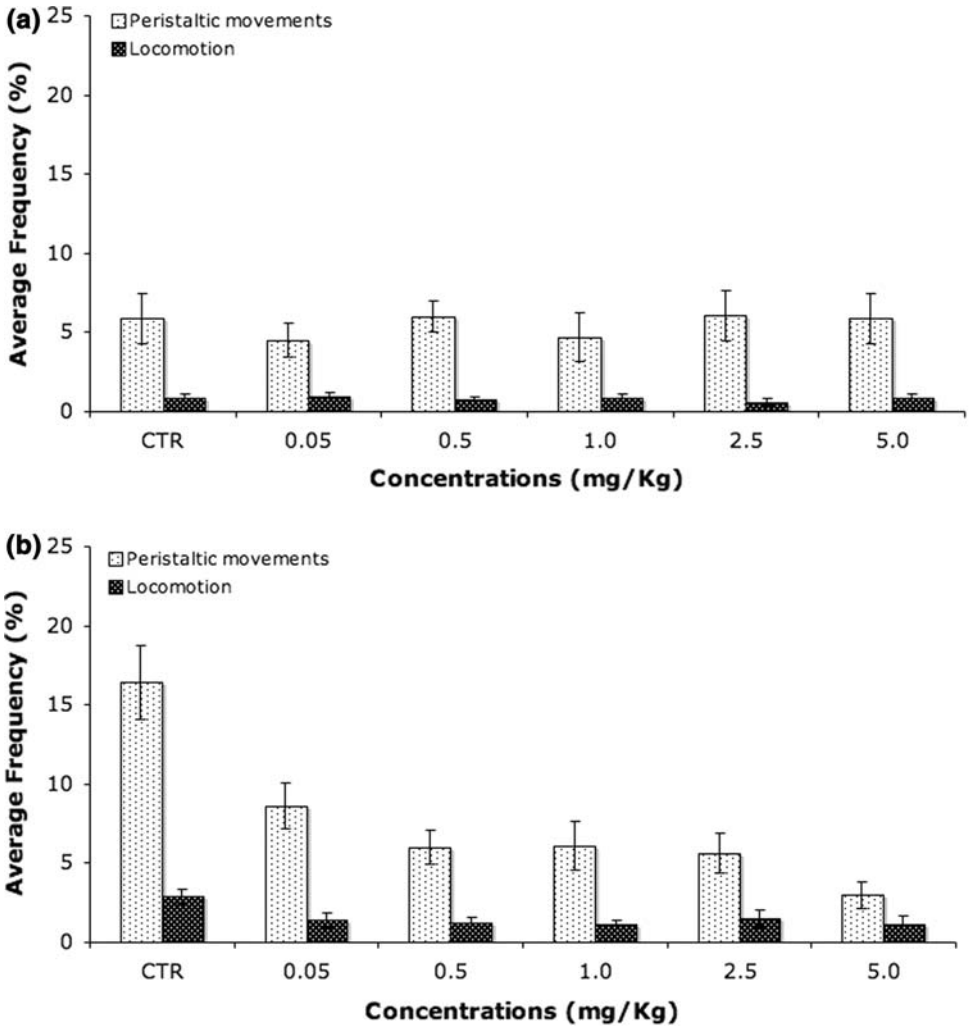

Fig. 4 Average frequency (%) (±SE) of locomotion and peristaltic movements throughout the test (A = day 1; B = day 10)

Table 2 Imidacloprid concentration in sediment and water samples (mean ± STDEV)[a]

Concentration (mg/kg)	Sediment in initial samples (mg/kg)	Sediment in final samples (mg/kg)	Water in final samples (mg/L)
CTR	<LOQ	<LOQ	<LOQ
0.05	0.05 ± 0.032	0.05 ± 0.051	0.39 ± 0.0004
0.5	0.58 ± 0.030	0.14 ± 0.009	0.55 ± 0.0006
1.0	0.80 ± 0.211	0.20 ± 0.021	0.86 ± 0.0009
2.5	2.27 ± 0.030	0.30 ± 0.016	1.38 ± 0.0014
5.0	4.58 ± 0.428	0.64 ± 0.103	2.99 ± 0.0030

[a] LOQ 0.001 mg/kg

sensitivity to toxicants may be increased (Scarlett et al. 2007). Because *L. variegatus* does not have a chitinous exoskeleton, it can only accumulate toxicants in soft tissues, simplifying the interpretation of the relation between survival and body concentrations of toxic materials (Meyer et al. 2002). Considerable quantities of imidacloprid were found in the worms' whole-body tissues. The chloragogen cells of lumbricid worms (such as *L. variegatus*), which surround the gut and the large blood vessels, contain numerous granules and chloragosomes, which are capable of binding toxic cations and organic xenobiotcs, thus enabling the worms to survive mild poisoning (Fischer 1977). Contaminants may accumulate from ingested sediment particles by desorption followed by absorption across the gut wall in the presence of digestive fluids (Weston et al. 2000), which can explain the high values of imidacloprid present in the whole-body tissues.

As hypothesised, it was possible to detect early warning signals in exposed worms, proving that behavioural parameters may be included in risk-assessment protocols.

These experiments showed that imidacloprid significantly changed the behaviour of *L. variegatus* in terms of both locomotion and peristaltic movements, confirming the findings of Alexander et al. (2007). The decreased oligochaete movement could increase predation risk by limiting the ability to avoid capture (Drewes 1997). Previous experiments using earthworms showed that imidacloprid can also change burrowing behaviour at concentrations between 0.5 and 1 mg/kg (Capowiez et al. 2003). In another experiment, Capowiez and Bérard (2006) observed that several aspects of earthworm behaviour (e.g., distance travelled, oscillations) or of the resulting burrow systems (e.g., area, topology, sinuosity, and depth) were affected by imidacloprid concentrations. Imidacloprid is a potential contaminant of surface and ground waters because of its persistence in soil (half-life of 48–190 days), high solubility (514 mg/L at 20°C), and low octanol water partition coefficient (log kow = 0.57) (Fossen 2006). It is not easy to spike sediments homogeneously with a contaminant, but, bearing this in mind, the effectiveness of our spiking protocol appears satisfactory.

Decrease in biomass seems to be a sensitive endpoint because it was possible to detect decreases in biomass even at low concentrations of imidacloprid. Capowiez et al. (2005) observed that weight loss in earthworms was a sensitive biomarker for exposure to imidacloprid even at low concentrations (0.5 mg/kg dry soil). To explain weight decrease, these investigators proposed different but not exclusive factors: (1) inactivity, as a direct response to the insecticide or as a mechanism of avoidance or (2) physiologic causes, such as less efficient assimilation or development of a costly mechanism of detoxification. We believe that these two factors may also be responsible for the biomass decrease observed in the present study.

An interesting observation was that all surviving worms exposed to concentrations >0.05 mg/kg were found together in a single aggregate. This also seems to be a result of exposure to imidacloprid and raises many questions. Is it a defense mechanism? Does it help the worms survive longer? Further investigation is needed to address these questions.

Oligochaetes feed on subsurface sediments and egest onto the sediment's surface, hence recycling deposited material. At high worm densities, reworking can considerably modify the structure of sediments (Krezoski and Robbins 1985; McCall and Fisher 1980). Thus, when the presence of imidacloprid affects the survival, growth, and behaviour of *L. variegatus*, it also affects the balance of the ecosystem. Because it is likely that behaviour inhibition could have a severe effect on oligochaete performance in the environment, this endpoint is important to evaluate the impacts of pollutants and pesticides on sediment ecosystems. Although behaviour cannot replace standard toxicity endpoints, we suggest that it should be introduced as an additional parameter. It is a rapid approach (faster than mortality and growth) and thus important in early warning systems. Behaviour integrates many cellular processes and is essential to the viability of the organism, its population, and its community. Using behaviour as a parameter, results can also be obtained at ecologically relevant concentrations (lower than lethal concentrations), which does not always happen with mortality and growth. Therefore, observations of behaviour provide a unique toxicologic perspective, linking the biochemical and ecologic consequences of environmental contamination (Little and Finger 1990). Because behaviour is important in activities such as predator avoidance, sexual interactions, and feeding, an impact on individual behaviour leads to an impact on population dynamics.

Conclusion

The pesticide imidacloprid proved to affect the population of *L. variegates* by decreasing survival, inhibiting behaviour, interfering with the growth process, and shortening life span. Growth and avoidance proved to be sensitive sublethal endpoints for imidacloprid contamination. Behaviour tests, in addition to classical sediment tests, should be conducted.

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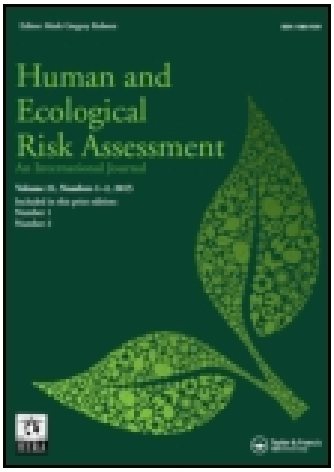

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Ecotoxicity of Imidacloprid to Aquatic Organisms: Derivation of Water Quality Standards for Peak and Long-Term Exposure

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ABSTRACT

The neonicotinoid insecticide imidacloprid is among the pesticides that most frequently exceed current water quality standards in Dutch surface waters. Recent research shows that effects of imidacloprid on water organisms occur at concentrations below these standards. Mayflies appear to be particularly sensitive with chronic No Observed Effect Concentrations in the nanogram per liter range. The aim of this study was to derive updated water quality standards in accordance with the methodology of the European Water Framework Directive by evaluating the available recent literature on acute and chronic ecotoxicity of imidacloprid to aquatic organisms in laboratory and semi-field experiments. It is concluded that the standard for long-term exposure should be lowered to 8.3 nanograms per liter; the standard for short-term concentration peaks can be maintained at the current value of 0.2 micrograms per liter. The European Commission set restrictions to the use of imidacloprid-based products to reduce the risks for bees and the Dutch national authorities issued emission reduction measures to protect aquatic life. Future monitoring data will ultimately reveal if these measures are sufficient to meet the newly proposed standards.

Key Words: imidacloprid, water quality standards, aquatic toxicity.

INTRODUCTION

The neonicotinoid insecticide imidacloprid is among the pesticides that most frequently exceed current water quality standards in Dutch surface waters (De Snoo and Vijver 2012). Products based on imidacloprid are used for a variety of crops, including maize, beets, and various greenhouse crops. The compound is systemic,

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meaning that after uptake it is distributed throughout the whole plant, and exerts its toxicity to sucking and biting insects via sap or leaves consumption. The products can be applied in various ways (*e.g.*, via seed and bulb treatment, by addition to nutrient solution or compost, by dripping or pouring, and via spray application). Authorized uses also include household biocide applications for ant and fly control and veterinary use in flea collars.

In 2013, the European Commission restricted the use of imidacloprid and two other neonicotinoid pesticides because the European Food Safety Authority (EFSA) identified potential high risks for bees due to exposure to dust from treated seeds, and from residues in pollen, nectar, or guttation fluid (EFSA 2013a,b,c). However, the European restrictions do not apply to imidacloprid use in greenhouses and full-field applications after flowering, and will not affect potential emissions to surface water from these sources, nor from the biocidal and veterinary applications, although the latter are likely to consist of lower tonnages.

In 2007, a literature review was carried out to update the then indicative Dutch environmental risk limit for imidacloprid and to derive water quality standards according to the European Water Framework Directive (WFD) (Posthuma-Doodeman 2008). A water quality standard in this context means the concentration of a chemical in surface water below which no unacceptable effects are expected to occur. The WFD distinguishes two types of water quality standards. One is a long-term Environmental Quality Standard (EQS), expressed as an Annual Average concentration (AA-EQS) and normally based on chronic ecotoxicity data. This standard aims to protect the ecosystem against adverse effects resulting from long-term exposure. The other is a standard that aims to protect the ecosystem from short-term concentration peaks, referred to as a Maximum Acceptable Concentration EQS (MAC-EQS) and based on acute ecotoxicity data (EC 2011a). The AA-EQS should not only protect aquatic organisms, but should also provide protection for indirect exposure of humans and predatory birds or mammals via consumption of fish or shellfish. However, following WFD methodology, these routes are not relevant for imidacloprid in view of the absence of bioaccumulation potential (log K_{ow} 0.57; EC 2006). The current Dutch AA-EQS is 0.067 μg/L, based on the lowest No Observed Effect Concentration (NOEC) of 0.67 μg/L from a semi-chronic test with the midge *Chironomus tentans* (Anatra-Cordone and Durkin 2005) and applying an assessment factor of 10. The MAC-EQS is 0.2 μg/L, based on the NOEC from a mesocosm experiment (EC 2006) with an assessment factor of 3 (Posthuma-Doodeman 2008). The study with *C. tentans* was the only valid non-acute toxicity test with imidacloprid on insects that was available at that time.

During the past years, a large number of studies on aquatic ecotoxicity of imidacloprid have been published, probably because of the debate on the presumed relationship between the use of neonicotinoids and worldwide bee health decline. Among these studies are chronic laboratory tests with sensitive aquatic arthropod species such as *Hyalella azteca*, *C. tentans* (Stoughton *et al*. 2008), and *C. riparius* (Pestana *et al*. 2009a). The NOEC values published for these species are in the range of the NOEC used for standard setting. However, Alexander *et al*. (2007) showed that mayflies (Ephemeroptera) might be much more sensitive than the taxa tested so far. The acute LC50 of 0.65 μg/L obtained for *Epeorus longimanus* is similar to the chronic NOEC for *C. tentans*, suggesting that much lower endpoints could be

expected for mayflies when tested chronically. This was confirmed by Roessink *et al*. (2013), who found EC10-values of 24 and 33 ng/L for *Caenis horaria* and *Cloeon dipterum* after 28 days of exposure. Based on this information, the Dutch Ministry of Infrastructure and the Environment decided to update the water quality standards again and commissioned the National Institute for Public Health and the Environment (RIVM) to (re-)evaluate the available literature, including micro- and mesocosm studies, and, if necessary, to propose new values. This article describes the process of data collection, evaluation, and standard derivation, and discusses the implications for water quality assessment for The Netherlands and other countries where imidacloprid is used.

METHODS AND MATERIALS

The methodology for deriving water quality standards is described in the *Technical Guidance for Deriving Environmental Quality Standards under the Water Framework Directive* (EC 2011a). The WFD-guidance builds on the guidance developed by the European Chemicals Agency (ECHA 2008) within the context of the European regulation for Registration, Evaluation and Authorisation of Chemicals (REACH). Additional national guidance was used for those aspects that were not (fully) addressed in the WFD-guidance (Brock *et al*. 2011; Smit *et al*. 2013; Van Vlaardingen and Verbruggen 2007). Basically, the derivation consists of a four-step approach: collection of literature, evaluation of the scientific reliability, selection of relevant endpoints, and derivation of the EQSs. Depending on the available data, the AA-EQS and MAC-EQS can be derived in three ways: by applying an Assessment Factor (AF) to the lowest endpoint (AF-approach), by statistical extrapolation using Species Sensitivity Distributions (SSD-approach), and on the basis of micro or mesocosm studies (model ecosystem approach). When enough data are available, all three methods have to be performed and the selection of the final value should be made based on expert judgment, taking into consideration the remaining uncertainty associated with, *e.g.*, the number of data available and the extrapolation of laboratory data to the field situation. Preference is given to the results from the SSD-approach or from model ecosystem-studies, since these entail a more robust approach towards assessing ecosystem effects (EC 2011a). In the present study, all three methods have been considered.

The starting point for collection of data was the 2008 report that includes data from the Draft Assessment Report prepared within the context of the former European pesticides directive 91/414/EEC (EC 2006) and public scientific literature until 2007. Additional literature published from 2007–August 2013 was collected using SCOPUS (http://www.scopus.com/). The Competent Authority Report (CAR) prepared for the evaluation of imidacloprid under the former European biocides directive 98/8/EC was also consulted (EC 2011b) as well as a Swiss report on water quality standards (Oekotoxzentrum 2013). The registration holder in The Netherlands for products based on imidacloprid (Bayer CropScience) provided an additional study (Roessink and Hartgers 2013).

All references were checked for relevant endpoints related to population health (*e.g.*, mortality, growth, reproduction) or ecosystem effects and evaluated with

respect to scientific validity. For this, studies were rated with a Reliability index (Ri) of 1 to 4, following Klimisch *et al.* (1997). Ri 1 (reliable without restrictions) generally applies to studies according to international test guidelines, preferably performed according to Good Laboratory Practice (GLP) with full documentation of data. Ri 2 (reliable with restrictions) relates to studies or data (mostly not performed according to GLP) in which the test parameters do not totally comply with the specific testing guideline or for which no guideline is available, but that are nevertheless well documented and scientifically acceptable. Ri 3 (not reliable) concerns studies with inadequate methodology and/or reporting, while Ri 4 is used for studies that do not give sufficient experimental detail (*e.g.*, data listed in summaries or reviews without further information). Laboratory studies were summarized in tables with explanatory notes regarding the reliability assessment (see Supporting Information 1 in the supplementary information).

Micro- and mesocosm studies were summarized and evaluated according to De Jong *et al.* (2008), who present a detailed checklist for summarizing and evaluating this type of studies. Key items in the evaluation are the representativeness of the aquatic community with respect to trophic levels, taxa richness, and abundance of potentially sensitive species, the experimental set-up, the exposure regime and the statistical and ecological evaluation of the observed effects in relation to the mode of action of the compound (see Supporting Information 2).

Because imidacloprid is susceptible to photolysis (EC 2006), special attention was paid to maintenance of exposure concentrations. The available laboratory data are inconclusive on the occurrence of photolysis under laboratory conditions. In some cases lower toxicity was found under light conditions as compared to darkness (*e.g.*, Sánchez-Bayo and Goka 2006a), probably caused by a decrease in concentrations of imidacloprid as a result of photodegradation. Therefore, endpoints from studies performed under light were only accepted if analytical verification of test concentrations was included. Endpoints were based on actual concentrations if these deviated more than 20% from nominal.

For the AF- and SSD-approach, a single endpoint per species should be used as input, representing the most sensitive relevant parameter reported (EC 2011a). For any species, whenever multiple reliable values were available for the same endpoint obtained in similar tests with species from comparable life stages, the geometric mean of these values was taken as single endpoint per species. For any species, whenever reliable endpoints were available from tests with different durations, the most relevant duration was selected based on existing guidelines (Smit *et al.* 2013). For the purpose of quality standard derivation, tests with active substances are preferred. The reason for this is that potential side effects of formulations are assumed to be limited to edge-of-field surface waters immediately after emission, and may thus be less relevant for larger water bodies. To decide whether or not similar results from ecotoxicity studies with formulated products and active substance could be pooled into a geometric mean, an arbitrary cut-off criterion was used. If the difference between (no) effect values was a factor of 3 or less, the data were pooled. Otherwise the value for the active substance was taken forward, even when this was higher than that obtained for the product. However, if the most critical test result for a species was obtained in a test with a formulated product, and no value was available for a comparable endpoint from a test with the active substance, the result

obtained with the formulation was used. Detailed information on data treatment can be found in EC (2011a), Brock *et al.* (2011), Smit *et al.* (2013), and Van Vlaardingen and Verbruggen (2007).

RESULTS AND DISCUSSION

Laboratory Toxicity Data

A total of 215 acute and 106 chronic ecotoxicity results were collected, the vast majority from studies with freshwater organisms (for full dataset, see Supporting Information 1). Valid acute data are presented in Table 1, including L(E)C50 values for 31 species of freshwater bacteria, algae, crustaceans, insects, fish and annelids, and for a marine crustacean, mollusk (unbound value), and fish. A total of 19 valid chronic NOEC or L(E)C10 values were obtained for algae, crustaceans, insects, a marine mollusk (unbound value), and fish (Table 2). Toxicity data for freshwater and marine species were pooled since there are no indications of a difference in sensitivity between freshwater and marine organisms of the relevant taxonomic groups (EC 2011a).

Acute toxicity data are presented in Figure 1, where bound L(E)C50-values for different taxonomic groups are plotted on a log-scale. From the data in Table 1 and Figure 1 it is clear that there is a large variation in sensitivity among the species tested, between taxa as well as within taxa. Within a taxon, even closely related species show large differences in sensitivity towards imidacloprid, despite similar life-forms and feeding strategies (see, *e.g.*, *Daphnia magna* and *Ceriodaphnia dubia*, *Gammarus pulex*, and *G. roeseli*). Crustaceans and insects are overall most sensitive. Based on the single value for *Lumbriculus variegatus*, annelids may also belong to the sensitive taxa. Within the group of aquatic insects, Ephemeroptera (represented by the mayflies *Caenis horaria*, *Cloeon dipterum*, and *Epeorus longimanus*) and Diptera (represented

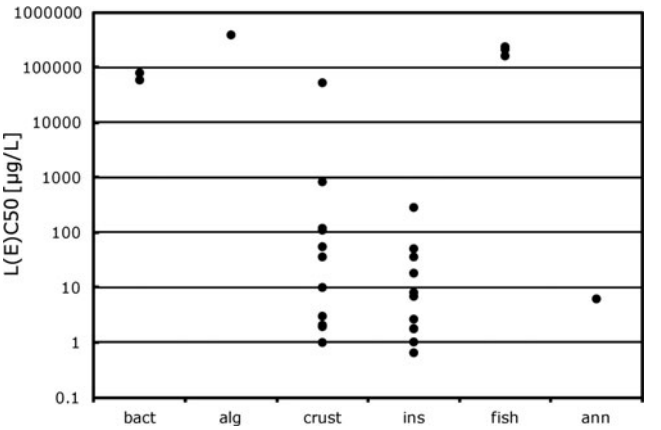

Figure 1. Representation of acute toxicity of imidacloprid to water organisms. Acute L(E)C50-values for bacteria, algae, crustaceans, insects, fish, and annelids are plotted on the Y-axis. Note that the Y-axis is presented on a log-scale.

Table 1. Selected aquatic ecotoxicity data for imidacloprid from acute toxicity studies with freshwater and marine species (indicated with sw). L(E)C50 in μg imidacloprid/L.

Taxon/species	L(E)C50 [μg/L]	Reference
Bacteria		
Vibrio fischerii	58,876[a]	Tišler *et al.* (2009)
V. qinghaiensis sp.	79,255	Zhou *et al.* (2010)
Algae		
Desmodesmus subspicatus	389,000[b]	Tišler *et al.* (2009)
Pseudokirchneriella subcapitata	>100,000[c]	EC (2006)
Crustaceans		
Americamysis bahia (sw)	35.9[d]	Anatra-Cordone and Durkin (2005), EC (2006)
Asellus aquaticus	119[e]	Roessink *et al.* (2013)
Ceriodaphnia dubia	2.07	Chen *et al.* (2010)
Chydorus sphaericus	832	Sánchez-Bayo and Goka (2006a)
Cypretta seuratti	1	Sánchez-Bayo and Goka (2006a)
Cypridopsis vidua	10[e]	Sánchez-Bayo and Goka (2006a)
Daphnia magna	52,455[f]	EC (2006), Tišler *et al.* (2009)
Gammarus pulex	110[e]	Ashauer *et al.* (2011)
Gammarus roesseli	1.94[g]	Böttger *et al.* (2012)
Hyallella azteca	55	Stoughton *et al.* (2008)
Ilyocypris dentifera	3[e]	Sánchez-Bayo and Goka (2006a)
Insects		
Caenis horaria	1.77[e]	Roessink *et al.* (2013)
Chaoborus obscuripes	284[e]	Roessink *et al.* (2013)
Chironomus dilutus	2.65	LeBlanc *et al.* (2012)
Chironomus tentans	6.9[h]	Stoughton *et al.* (2008)
Cloeon dipterum	1.02[e]	Roessink *et al.* (2013)
Epeorus longimanus	0.65[i]	Alexander *et al.* (2007)
Limnephilidae	1.79[e]	Roessink *et al.* (2013)
Notonecta spp.	18.2[e]	Roessink *et al.* (2013)
Plea minutissima	35.9[e]	Roessink *et al.* (2013)
Sialis lutaria	50.6[e]	Roessink *et al.* (2013)
Simulium vittatum	8.1[j]	Overmyer *et al.* (2005)
Fish		
Danio rerio	227,099[k]	Tišler *et al.* (2009)
Leuciscus idus melanotus	237,000	EC (2006)
Oncorhynchus mykiss	211,000	EC (2006)
Cyprinodon variegatus (sw)	161,000	Anatra-Cordone and Durkin (2005), EC (2006)
Molluscs		
Crassostrea virginica (sw)	>145,000[c,l]	Anatra-Cordone and Durkin (2005), EC (2006)
Annelids		
Lumbriculus variegatus	6.2	Alexander *et al.* (2007)

[a]Geometric mean of 61,900 and 56,000 μg/L for tests with active and formulation; marine species tested in freshwater; [b]test with active, endpoint for formulation >3 times lower; [c]unbound values are not used for EQS-derivation, value included to show that species has been tested; [d]geometric mean of 37.7, 34.1, and 36 μg/L from tests with active and formulation; [e]lowest relevant endpoint, immobility; [f]geometric mean of 30,000, 85,000, and 56,600 μg/L, 48 h tests with formulation and active, endpoint immobility; [g]most sensitive life-stage: spring collected early adults; [h]geometric mean of 10.5 and 5.75 μg/L, lowest relevant endpoint from tests with active; [i]endpoint from most relevant test duration; [j]geometric mean of 6.75, 8.25, and 9.54 μg/L; [k]geometric mean of 241,000 and 214,000 μg/L, tests with active and formulation; [l]highest concentration without 50% effect. For details on individual tests, see Supporting Information 1 in the online supplementary information.

Table 2. Selected aquatic ecotoxicity data for imidacloprid from chronic toxicity studies with freshwater and marine species (indicated with sw). NOEC or L(E)C10 in μg imidacloprid/L.

Taxon/species	NOEC/L(E)10 [μg/L]	Reference
Algae		
Desmodesmus subspicatus	106,000[a]	Tišler *et al.* (2009)
Pseudokirchneriella subcapitata	<100,000[b]	EC (2006)
Crustaceans		
Asellus aquaticus	1.35[c]	Roessink *et al.* (2013)
Daphnia magna	1768[d]	Jemec *et al.* (2007)
Gammarus pulex	2.95[c]	Roessink *et al.* (2013)
Hyallella azteca	0.47[e,f]	Stoughton *et al.* (2008)
Insects		
Caenis horaria	0.024[c]	Roessink *et al.* (2013)
Chaoborus obscuripes	1.99[f]	Roessink *et al.* (2013)
Chironomus riparius	<0.4[b,g]	Pestana *et al.* (2009a)
Chironomus tentans	0.42[f]	Stoughton *et al.* (2008)
Cloeon dipterum	0.033[c]	Roessink *et al.* (2013)
Plea minutissima	2.03[c]	Roessink *et al.* (2013)
Pteronarcys dorsata	14.5[h,i]	Kreutzweiser *et al.* (2007, 2008)
Sericostoma vittatum	≥5.0[f,i]	Pestana *et al.* (2009a)
Sialis lutaria	1.28[c]	Roessink *et al.* (2013)
Tipula sp.	34[f,i]	Kreutzweiser *et al.* (2008)
Fish		
Danio rerio	300,000	Tišler *et al.* (2009)
Oncorhynchus mykiss	1200[j]	Anatra-Cordone and Durkin (2005)
Molluscs		
Crassostrea virginica (sw)	≥23,300[b,k]	Anatra-Cordone and Durkin (2005) EC (2006)

[a]Test with active, endpoint for formulation >10 times lower; [b]unbound values are not used for EQS-derivation, value included to show that species has been tested; [c]lowest relevant endpoint, immobility; [d]lowest relevant endpoint, number of neonates; geometric mean of 1250 and 2500; [e]endpoint from most relevant test duration; [f]lowest relevant endpoint, mortality; [g]lowest relevant endpoint, development rate; [h]geometric mean of 15.8 and 13.3, 14-d LC10; [i]test duration semi-chronic; [j]lowest relevant endpoint, growth; [k]lowest concentration without effects. For details on individual tests, see Supporting Information 1 in the supplementary information.

by the midges *Chironomus dilutus* and *C. tentans*, and the blackfly *Simulium vittatum*) are most sensitive. The midge *Chaoborus obscuripes* seems to be an exception with a rather high acute EC50 in comparison to the other midges, but the chronic toxicity result for this species is low (Table 2).

The selected bound chronic results per species are presented in Figure 2. The previously used semi-chronic 10-days NOEC for *C. tentans* of 0.67 μg/L could be replaced by a NOEC of 0.42 μg/L from a test with a longer duration (28 days). The LC10-values of 14.5 μg/L for *Pteronarcys dorsata* and 34 μg/L for *Tipula* sp. originate from a 14-days test, which is shorter than the minimum test duration for chronic

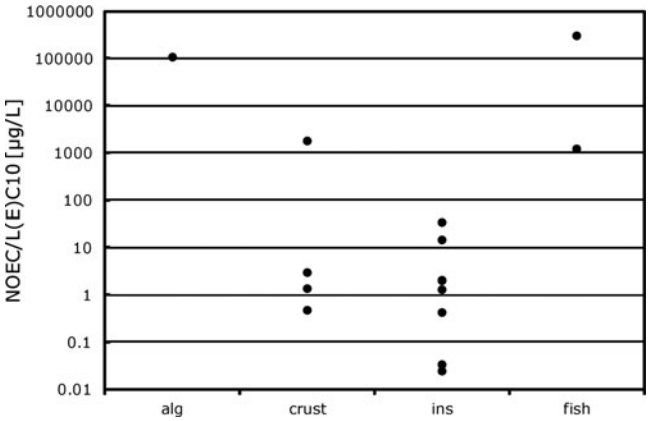

Figure 2. Representation of chronic toxicity of imidacloprid to water organisms. Chronic NOEC or L(E)C10-values for algae, crustaceans, insects, and fish are plotted on the Y-axis. Note that the Y-axis is presented on a log-scale.

tests with arthropods. However, because larvae were tested it was considered justified to include the data in the chronic dataset. The NOEC of ≥5.0 μg/L for *Sericostoma vittatum* was also obtained with larvae, but this test lasted only 6 days. Since the result is a "≥-value," the result was not used directly in the calculation of the AA-EQS but is included in the table to show that valid data for this particular species are present.

The chronic data show a similar, high variation in sensitivity among species as observed in the acute dataset. Again, crustaceans and insects represent the sensitive species groups, but the ranking of individual species as regards their relative sensitivity differs between the acute and chronic dataset. Based on acute and chronic data, *D. magna* is least sensitive while *C. dipterum*, *C. horaria*, and *C. tentans* are the most sensitive. In between, species switch positions when comparing the acute and chronic data. This emphasizes the importance of testing a range of species within a taxon. In addition, the comparison of acute and chronic effect concentrations points at the high Acute-to-Chronic Ratio (ACR) for imidacloprid. For those species for which both an acute L(E)C50 and a chronic NOEC or L(E)C10-value are available, the ratio between the two values ranges from 16 for *C. tentans*, to 143 for *C. obscuripes*. This indicates that the factor of 10, which is usually assumed to cover the difference between acute L(E)C50-values and chronic NOECs, underestimates the effects of prolonged exposure to imidacloprid. The time-cumulative effect of imidacloprid was pointed out by Tennekes (2010) and Tennekes and Sánchez-Bayo (2013) and a high ACR was also demonstrated for other species (Charpentier *et al*. 2014). Although these studies mostly refer to lethal effects, they underpin the conclusion of Roessink *et al*. (2013), that acute studies are not appropriate to assess the effects of long-term exposure to imidacloprid. It also indicates that semi-field studies should be critically evaluated with respect to exposure time, because effects may be underestimated if exposure duration has been too short. In general, chronic studies are considered indispensable for derivation of any AA-EQS and consideration

should be given to critical ecological traits of the test species compared to relevant field species.

Microcosms, Mesocosms, and Other Studies

A total of 15 bioassay experiments and micro/mesocosm studies were collected. Some of them were indoor, single, or multiple species tests under more realistic conditions (Böttger *et al*. 2013; Kreutzweiser *et al*. 2007, 2008), but did not examine the effects on whole aquatic communities. If valid, results of such tests were added to the laboratory dataset. Other (semi–)field studies were not included because they were performed in rice paddy test systems with application types that are not relevant to the Dutch situation; for example, by using nursery boxes or lysimeters with treated seedlings (Hayasaka *et al*. 2012a,b; Jinguji *et al*. 2013; Sánchez Bayo and Goka 2005, 2006b). It is noted, however, that these studies confirm the outcome of the other valid and relevant micro/mesocosm studies. These latter are summarized in Table 3 and briefly discussed below (for full summaries, see Supporting Information 2).

Study 1. This outdoor pond study with two applications of Confidor 200 SL at a 21-days interval was included in the European authorization of imidacloprid (EC 2006; Brock 2005; Ratte and Memmert 2003). Effects were found on community parameters such as taxa richness, diversity, similarity and principal response of the community, with Chironomidae and Baetidae being the most sensitive. The NOEC was established as 0.6 μg a.s./L based on initial concentrations. Decline of concentrations was moderately fast, the DT50 for dissipation from the water phase ranged from 5.8 to 13.0 days (average DT50 8.2 days) and 12–20% of the nominal concentrations was present in the water phase just before the second application. According to criteria given by Brock *et al*. (2011) and EFSA (2013d), this study may be used to derive acute and chronic risk limits, because exposure was characterized by peak exposure (relevant for the MAC-EQS), while concentrations of imidacloprid in between applications were sufficiently maintained (relevant for the AA-EQS). However, according to the European assessment, the variability in insect species sensitivity to imidacloprid was not fully covered in this study, and the most sensitive taxon of the current laboratory dataset, Ephemeroptera, was not adequately represented. EFSA (2008) advised to use a safety factor of 1–3 when deciding on authorization of products based on imidacloprid.

Study 2. Colombo *et al*. (2013) treated outdoor pond enclosures with three applications of technical imidacloprid at 0.6 to 40 μg/L at a 7-days interval. Clear effects on abundance and emergence of several macroinvertebrate taxa were observed at the two highest initial concentrations of 17.3 and 40 μg/L. Ephemeroptera were most sensitive and showed effects on emergence at 3.2 μg/L, no significant effects were present at 1.4 μg/L. Imidacloprid disappeared rapidly from the water phase with a DT50 of 28 h, consequently the study was only considered for derivation of the MAC-EQS.

Study 3. Alexander *et al*. (2008) exposed benthic communities in outdoor artificial streams to a single 12-h pulse of Admire 240 g/L at 0.1 to 10 μg a.s./L or to a 20-days continuous treatment with 0.1 to 1 μg a.s./L. The 12-h NOEC for the pulse treatment was established as 3.9 μg a.s./L (actual measured) based on effects on emergence and abundance of the mayfly species *Epeorus* spp. (Ephemeroptera:

Table 3. Summary of results from mesocosm and related tests. NOEC represents treatment level without significant effects, expressed in μg imidacloprid/L.

Study type	Treatment	NOEC [μg/L]	Critical effect	Reference
1. Outdoor pond	2 × 0.6–23.5 μg a.s./L, 21-days interval	0.6[a]	community effects, mainly Ephemeroptera (Baetidae), Diptera (Chironomidae)	EC (2006), Brock (2005), Ratte and Memmert (2003)
2. Outdoor pond enclosure	2 × 0.6–40 μg a.s./L, 7-days interval	1.4[a]	abundance of Chironomidae, Ephemeroptera	Colombo *et al.* (2013)
3. Outdoor stream	12-h pulse 0.1–10 μg a.s./L	3.9[b]	emergence of *Epeorus* spp. (Ephemeroptera)	Alexander *et al.* (2008)
		<0.1[b]	adult male thorax length of *Baetis* and *Epeorus* spp.	
	20-h continuous 0.1–10 μg a.s./L	0.1[b]	emergence of *Epeorus* spp. (Ephemeroptera)	
		<0.1 / 0.1[b]	adult male thorax length of *Baetis* and *Epeorus* spp.	
4. Outdoor stream	3 × 24-h pulse 2 or 20 μg a.s./L, 7-days interval	1.63[b]	effects on Ephemeroptera, Plecoptera and Tricoptera, Oligochaetes at next dose (20 μg a.s./L)	Pestana *et al.* (2009b)
5. Indoor stream	3 × 12-h pulse of 12 μg a.s./L, 7-days interval; treatment repeated after ca. 50 d	<12[b]	abundance and emergence of Ephemeroptera (affected after single pulse), Trichoptera (id.), Chironomidae and Gammaridae	Berghahn *et al.* (2012), Mohr *et al.* (2012)
6. Outdoor enclosure	2 × 0.097–3.8 μg a.s./L, 21-days interval	1.52[a]	abundance of *Cloeon dipterum* larvae	Roessink and Hartgers (2013)

a.s. = active substance. [a]Actual initial concentration, [b]average actual during treatment. For details of individual tests, see Supporting Information 2 in the online supplementary information.

Heptageniidae). For *Baetis* spp. (Ephemeroptera: Baetidae), the NOEC was ≥ 9.1 μg a.s./L (actual measured). For the continuous treatment, the 20-days NOEC for emergence of *Epeorus* spp. was 0.1 μg a.s./L, the NOEC for *Baetis* spp. was 0.3 μg a.s./L, based on measured concentrations. In both treatments, significant effects on adult thorax and/or head length were observed at the lowest concentration of 0.1 μg a.s./L. Although the ecological implications of reduced head or thorax length are not clear, the authors point at a potential impact on, for example, mating success. The exposure duration of 12 h for pulse treatment and 20 days for continuous treatment is shorter than the duration of the laboratory tests used for derivation of the MAC- and AA-EQS, respectively. Moreover, species and community interactions were not reported. Consequently, the study could only be used as additional information.

Study 4. Pestana *et al*. (2009b) exposed benthic macroinvertebrates and periphyton in outdoor artificial stream mesocosms to three 24-h pulses of Admire 240 g/L at 2 and 20 μg a.s./L at an interval of 7 days. Observations were made after the last pulse. The high dose caused a significant reduction (69%) in combined Ephemeroptera, Plecoptera, and Tricoptera taxa, Oligochaetes were sensitive as well. Coleoptera were less affected (ca. 29% reduction). No effects were seen on Chironomidae. The NOEC was set to the average measured concentration of imidacloprid over the 24-hours exposure time at the low dose, which was 1.63 μg/L. This treatment level was considered for derivation of the MAC-EQS, taking account of the fact that exposure duration was shorter than in the laboratory studies used for MAC-derivation.

Study 5. Berghahn *et al*. (2012) and Mohr *et al*. (2012) incubated straw litterbags in reference streams. After colonization the collected invertebrates were exposed to two series of three weekly 12-h pulses of imidacloprid (99.9% pure) at 12 μg/L in indoor stream mesocosms. They observed significant effects on several insect taxa, with Ephemeroptera (affected after single pulse), Trichoptera (id.), Chironomidae and Gammaridae being most sensitive. Consequently the NOEC of this study was set to <12 μg/L. The systems were re-stocked with aquatic organisms before the second pulse series. This is a kind of recolonization that under natural conditions is only possible when an undisturbed community is present upstream. This makes the study less relevant for EQS-derivation. Again, the exposure duration was shorter than in the laboratory studies used for MAC-derivation.

Study 6. Roessink and Hartgers (2013) treated outdoor enclosures that were additionally stocked with *C. dipterum*-larvae with two applications of imidacloprid SL 200 at 0.097 to 3.8 μg/L at a 21-days interval. Abundance was followed until 37 days after application. The timing of application (October) did not allow for assessment of reproduction and emergence. About 40% of the initial concentration was present in the water phase just before the second application, exposure can therefore be considered chronic. A decrease in abundance was observed in one of the replicates of the 3.8 μg a.s./L treatment. Although this decrease was not significant and not consistent with the other replicates, the authors considered it as a treatment-related effect and set the NOEC to 1.52 μg a.s./L nominal. This is much higher than the 28-days laboratory EC10 for immobility of 0.033 μg/L reported for the same species by Roessink *et al*. (2013) (Table 2). A possible explanation could be that the summer generation that was used in the laboratory test is more sensitive than animals preparing for overwintering. A comparison between spring and autumn collected animals was made in an acute study with *G. roeseli* (Böttger *et al*. 2012), but

no conclusions could be drawn from this experiment because test water and feeding were varied as well. It was concluded that the NOEC of outdoor study 5 should not be used to replace the lower endpoints observed for mayflies in the other laboratory and outdoor experiments.

Derivation of the MAC-EQS for Peak Exposure

AF-approach

According to the WFD-guidance, the MAC-EQS may initially be derived by applying an assessment factor of 100 to the lowest acute L(E)C50-value; this factor can be lowered to 10 if the compound has a known mode of toxic action and representative species for the most sensitive taxonomic group are included in the dataset (EC 2011a). This is the case for imidacloprid and using the lowest EC50 of 0.65 μg/L for *E. longimanus*, this results in a MAC-EQS$_{AF}$ of 0.065 μg/L (65 ng/L).

SSD-approach

For using SSDs, the WFD- and REACH-guidance require that the database contains preferably more than 15, but at least 10 datapoints, from different species covering at least eight specified taxonomic groups (EC 2011a; ECHA 2008). The acute dataset does not fully cover the specified taxa, since data on aquatic macrophytes are missing. However, because imidacloprid is an insecticide with a specific mode of action, and other primary producers are clearly not sensitive, it was considered justified to use the SSD-approach without macrophytes. Shown in Figure 3 is the acute SSD constructed with the program *ETX* 2.0 (Van Vlaardingen *et al*. 2004) fitting all available acute data to a log-normal distribution. It is apparent that there

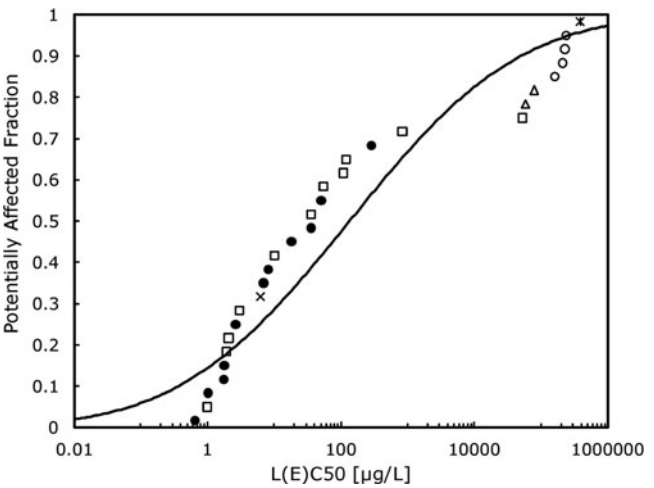

Figure 3. Species Sensitivity Distribution for imidacloprid based on acute toxicity data for all available aquatic species. The X-axis represents the L(E)C50-values in μg/L for algae (*), annelids (x), bacteria (△), crustaceans (□), insects (●), and fish (○), the Y-axis represents the fraction of species potentially affected.

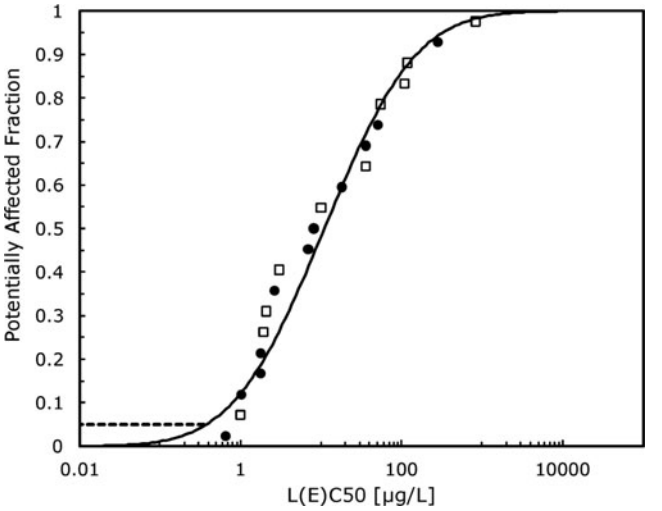

Figure 4. Species Sensitivity Distribution for imidacloprid based on acute toxicity data for aquatic arthropods combined, endpoint for *Daphnia magna* omitted. The X-axis represents L(E)C50-values for crustaceans (□) and insects (•) μg/L, the Y-axis represents the fraction of species potentially affected. The dashed line represents the Hazardous Concentration for 5% of the species (HC5 = 0.36 μg/L).

is a distinction between bacteria, algae and fish at the upper right hand side of the distribution, and crustaceans and insects at the lower left hand side. The overall fit is poor and the assumption of a normal distribution is rejected by the tests included in the E_TX-package (Anderson-Darling, Kolmogorov-Smirnov, Cramer von Mises).

If a clear distinction in sensitivity exists, the WFD-guidance offers the option to construct an SSD for the taxa that are most sensitive in line with the mode of action. Because the data for insects and crustaceans overlap, both groups were included in such a specific SSD. An exception was made for *D. magna*. According to EFSA (2013d), when differences in sensitivity are 1 or 2 orders of magnitude (factor 10–100), care should be taken for a bias in the effect assessment due to insensitive species. The endpoint for *D. magna* was left out because the EC50 is more than 3000 times higher than the geometric mean of all arthropods (including *D. magna*). The resulting SSD is shown in Figure 4. The median estimate of the HC5 is 0.36 μg/L (95% confidence interval 0.09 and 0.97 μg/L). This is almost a factor of 2 lower than the lowest available endpoint (0.65 μg/L for *E. longimanus*). The WFD-guidance recommends to apply a default assessment factor of 10 to the HC5 when L(E)50 data are used in a generic SSD; this factor should account for the extrapolation from a 50% effect level to the no-effect level associated with the MAC-EQS, and cover remaining uncertainty regarding the extrapolation from a laboratory-based SSD to the field situation.

No guidance is given, however, as to which assessment factor should be used in case a specific SSD is constructed for the potentially most sensitive species group(s). A lower assessment factor may be sufficient because including particularly sensitive

species reduces uncertainty, but the factor should still correct for the extrapolation from 50% effect to no effect, and for the extrapolation from lab to field. Taking this into account, Brock *et al.* (2011) proposed an assessment factor of 6 for this situation. Using this value a MAC-EQS_{SSD} of 0.06 μg/L was derived, which is slightly lower than the value obtained by the AF-approach. Given the position of the two lowest data points on the right hand side of the SSD-curve (Figure 4), the HC5 is probably worst case.

Considering the fact that at the level of the MAC-EQS no effects should occur after short-term exposure, using acute L(E)C10-values instead of L(E)C50-values would be most appropriate for derivation of this standard. For the 10 aquatic arthropods tested by Roessink *et al.* (2013), the acute LC10 ranges from 2.55 to >10,000 μg/L, while the acute EC10 ranges from 0.1 to 223 μg/L. The HC5 based on acute EC10-values was reported as 0.084 μg/L by the authors. Leaving the EC10 for *Gammarus pulex* and *Micronecta* sp. out of consideration because of high control mortality, the remaining eight EC10-values would lead to an HC5 of 0.05 μg/L. This value is very similar to the above derived MAC-EQS_{SSD} of 0.06 μg/L based on acute L(E)C50-values with an assessment factor of 6.

Mesocosm-approach

The available micro- and mesocosm studies were summarized above. Five studies were considered reliable and potentially useful for derivation of the MAC-EQS: outdoor pond study 1 (two applications, moderately fast decline of imidacloprid concentrations between applications), outdoor pond enclosure study 2 (three applications, fast dissipation from the water phase), outdoor stream study 3 (single 12-hours pulse application), outdoor stream study 4 (repeated 24-hours pulse application), and indoor stream study 5 (repeated 12-hours pulse application). Below, the use of these studies for derivation of the MAC-EQS is discussed in the context of exposure duration and ecological reality.

When using mesocosm data for derivation of water quality standards it should first be decided how to express the NOEC from such a study. When concentrations decline during the experiment, using the initial concentration may underestimate the risk since in reality the organisms have been exposed to lower concentrations. EFSA (2013d) advises to use the time window of the critical laboratory tests for calculation of the time weighted average (TWA) concentration after the highest peak in the NOEC-treatment. Similarly, based on the duration of acute ecotoxicity tests, Brock *et al.* (2011) proposed to use the 48-h TWA concentration in the NOEC-treatment for derivation of the MAC-EQS_{MESO}. For the outdoor pond study (study 1), the NOEC was set to 0.6 μg/L (initial), which is equivalent to a 48-h TWA of 0.51 μg/L. Expressed as a 48-h TWA concentration, the NOEC of the outdoor pond enclosure (study 2) equals 0.82 μg/L. The NOECs from stream mesocosms with single or repeated 12–24 h pulse applications were 3.9, (study 3), 1.63 (study 4), and <12 μg/L (study 5), respectively, based on concentrations during the pulses. The WFD-guidance proposes to put an assessment factor of 5 on the lowest NOEC of a single valid mesocosm. Based on a comparison of multiple studies, Brock *et al.* (2011) argued that lower factors are sufficient and suggested an assessment factor of 2–3 in case of a single application design, and a factor of 1–2 when multiple

applications are used. The lower factors of these ranges (2 for single applications, 1 for multiple applications) may be applied when more studies are available, as is the case here. These factors are in line with recommendations of EFSA (2013d).

According to the EU risk assessment (EC 2006), pond study 1 did not fully address the variability in insect species sensitivity, and Ephemeroptera were not adequately represented. This taxon was, however, included in the other studies, but the exposure duration in the stream studies (studies 3–5) was shorter than the minimum standard test duration for arthropods of 48 hours. Together, this would be a reason not to use the lowest assessment factor. Both pond study 1 and pond enclosure study 2 involved multiple applications, which would be a reason for a lower assessment factor. In pond study 1, however, the application interval was large and effects were already present after the first application. This was also the case in the indoor stream study that delivered the NOEC of <12 μg/L (study 5). The NOEC of 1.63 μg/L (stream study 4) was obtained after multiple applications, but it cannot be judged if a single pulse would have resulted in a higher NOEC.

In addition, the NOEC for effects on thorax and/or head length of *Baetis* ssp. and *Epeorus* ssp. was <0.1 μg/L. Although the ecological consequences are not clear, this is a reason for concern. Based on these arguments, it was decided to use the lowest NOEC of 0.51 μg/L with the higher assessment factor of 3 proposed by Brock *et al.* (2011) and set the MAC-EQS$_{MESO}$ to 0.17 μg/L. This is still higher than the NOEC for thorax/head length, and also higher than the 96-hours laboratory EC10 for *C. dipterum* of 0.1 μg/L reported by Roessink *et al.* (2013). However, the other 96-hours EC10-values reported by Roessink *et al.* (2013) are a factor of 2 or more higher, and the lowest 96-hours LC10 of 2.55 μg/L for *C. horaria* is a factor of 15 higher than this MAC-EQS$_{MESO}$.

Selection of the MAC-EQS

The MAC-EQS$_{AF}$ is 0.065 μg/L, the MAC-EQS$_{SSD}$ is 0.06 μg/L, and the MAC-EQS$_{MESO}$ is 0.17 μg/L. The difference between lowest and highest value is a factor of 2.8. The SSD-based MAC-EQS is similar to the value obtained with the AF-approach. As indicated above, the MAC-EQS should preferably be based on the SSD- or mesocosm-approach. The MAC-EQS$_{SSD}$ of 0.06 μg/L is similar to the HC5 based on acute EC10-data, but it is lower than the lowest acute EC10 of 0.1 μg/L reported by Roessink *et al.* (2013) and more than a factor of 5 lower than the other acute EC10-values. Considering the acute LC10-values, the difference is more than a factor of 40. As shown above, the MAC-EQS$_{MESO}$ of 0.17 μg/L is protective for almost all species when considering the acute EC10-values of Roessink *et al.* (2013) and 15 times lower than the lowest acute LC10. Since the mesocosms represent the most ecologically relevant way of exposure and effects testing, preference was given to the mesocosm-based MAC-EQS, and it is concluded that the current Dutch MAC-EQS of 0.2 μg/L can be maintained. This value is twice as high as the Swiss proposal for the MAC-EQS of 0.1 μg/L (Oekotoxzentrum 2013), based on the acute EC50 for *Cypretta seuratti* (Table 1) with an assessment factor of 10. The Swiss assessment did not include SSDs as an option, the mesocosm studies were not considered because they were performed with formulated products rather than with the active substance.

Derivation of the AA-EQS for Long-Term Exposure

AF-approach

According to the WFD- and REACH-guidance (EC 2011a; ECHA 2008), an assessment factor of 10 can be applied to the lowest EC10 of 0.024 μg/L for the mayfly *C. horaria* because chronic NOEC or L(E)C10-values are available for algae, *Daphnia*, and fish, and the acutely most sensitive taxon is included in the chronic dataset. This results in an AA-EQS$_{AF}$ of 0.0024 μg/L (2.4 ng/L).

SSD-approach

The taxa represented in the chronic dataset (Table 2) do not meet the criteria of the WFD-guidance for constructing a generic SSD. However, based on the same considerations as presented above for the derivation of the MAC-EQS, constructing a specific SSD was considered for derivation of the AA-EQS. Insects and crustaceans were combined into one dataset for arthropods, and *D. magna* was left out since the NOEC for this species is over 900 times higher than the geometric mean of all arthropods. The SSD is shown in Figure 5. The median estimate of the HC5 is 0.025 μg/L (95% confidence interval 0.002–0.1 μg/L), which is similar to the lowest NOEC (0.024 μg/L for *C. horaria*). The WFD- and REACH guidance recommend to apply a default assessment factor of 5–1 to the HC5 when chronic NOEC/L(E)10 data are used in a generic SSD (EC 2011a; ECHA 2008). However, a lower assessment factor may be appropriate in case a specific SSD is constructed for the potentially

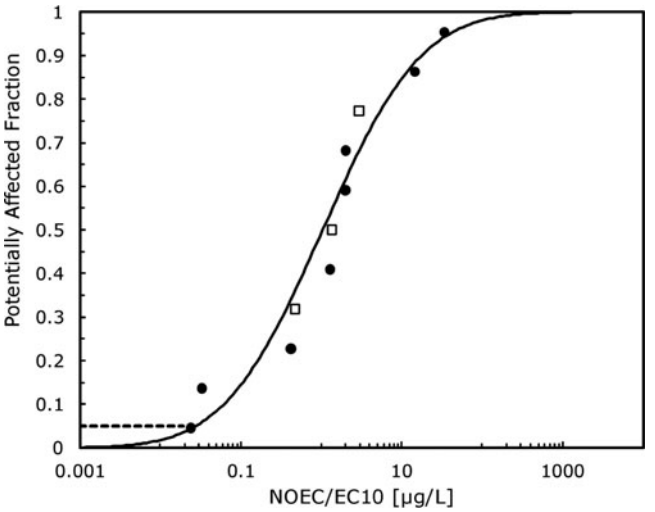

Figure 5. Species Sensitivity Distribution for imidacloprid based on chronic toxicity data for aquatic arthropods combined, endpoint for *Daphnia magna* omitted. The X-axis represents NOEC/L(E)C10-values for crustaceans (□) and insects (●) in μg/L, the Y-axis represents the fraction of species potentially affected. The dashed line represents the Hazardous Concentration for 5% of the species (HC5 = 0.025 μg/L).

most sensitive species groups. For this, a default assessment factor of 3 was proposed by Brock *et al.* (2011), which is consistent with EFSA (2013d). The dataset is limited and does not meet the requirements of a generic SSD and the number of data points for sensitive taxa is only just above the minimum of 10. Although the data cover the species groups that have consistently been shown to be sensitive, the high ACR is an indication that if other acutely sensitive species would have been tested chronically, a number of relatively low endpoints might be added to the chronic dataset. This would potentially lead to a lower HC5 and favors the use of a higher assessment factor. On the other hand, the results of the mesocosm- and related studies, although not considered adequate as a direct basis for AA-EQS (see below), indicate that the assessment factor of 3 as proposed by Brock *et al.* (2011) might be sufficiently protective for the sensitive aquatic taxa. Using this factor, the AA-EQS_{SSD} is 0.0083 μg/L (8.3 ng/L).

Mesocosm-approach

Two studies were available in which chronic exposure was sufficiently maintained: outdoor pond study 1 and outdoor stream study 3 (Table 3). Following EFSA (2013d), the NOEC of the pond study was expressed as the 28-days TWA-concentration, being 0.23 μg/L, based on the duration of the critical laboratory test with *C. horaria*. Mayflies were not adequately represented in this study, and a lower NOEC of 0.1 μg/L was derived for the Ephemeroptera *Epeorus* spp. and *Baetis* spp. in the stream study. Species or community interactions were not included in this study and the duration of exposure was 20 days, which is shorter than in the critical laboratory studies (28 days). Given the high ACR of imidacloprid for insects, longer exposure may have led to increased effects. In addition, the NOEC for effects on thorax and/or head length of *Baetis* sp. and *Epeorus* sp. was <0.1 μg/L. In view of the available information, it was not considered justified to use the mesocosm studies directly for derivation of the AA-EQS.

Selection of the AA-EQS

The AA-EQS_{AF} is 0.0024 μg/L (2.4 ng/L), the AA-EQS_{SSD} is 0.0083 μg/L (8.3 ng/L). The difference is a factor of 3.5. The WFD-guidance gives preference to an SSD-based AA-EQS since this is a more robust approach towards ecosystem effects; it was therefore decided to set the AA-EQS to 0.0083 μg/L (8.3 ng/L). This is a factor of 8 lower than the current Dutch AA-EQS (0.067 μg/L). Being a factor of 3 below the lowest laboratory NOEC for mayflies and a factor of 12 lower than the NOEC that was observed for the same taxon in the stream mesocosm, the new AA-EQS is considered protective for effects on the most sensitive taxa in the current dataset. The value is in line with the Swiss proposed EQS of 0.013 μg/L (13 ng/L) (Oekotoxzentrum 2013). The U.S. Environmental Protection Agency (USEPA) uses a chronic toxicity benchmark of 1.05 μg/L (OPP 2014), which seems rather high given the fact that the acute LC50 for some species is below this value (Table 1). Canada uses a value of 0.23 μg/L, based on a 28-day EC15 for *C. riparius* with a safety factor of 10 (CCME 2007). The LOEC used to derive the Canadian standard is based on initial concentrations in the water phase of a

water/sediment study (EC 2006), and these are likely to overestimate the actual exposure concentrations in the water phase during the test. When based on actual concentrations in the water phase, the LOEC would probably be much lower. All cited standards have been derived before the mayfly data of Roessink *et al.* (2013) became available.

Implications of the New Standards

Monitoring data for imidacloprid in The Netherlands are presented in the Dutch Pesticide Atlas (CML and RWS-WVL 2014). Concentrations at individual sampling locations frequently exceed current water quality standards. In 2012, the MAC-EQS of 0.2 μg/L was exceeded at 45 out of 451 locations (10%), the AA-EQS of 0.067 μg/L was exceeded at 54 out of 451 monitoring locations (12%). Exceedance is detected the whole year round, but less in winter (CML and RWS-WVL 2014). Kreuger *et al.* (2010) measured pesticide residues in samples from six water courses in a greenhouse horticulture area in Sweden and detected imidacloprid in 39% of the samples, the highest concentration being 15 μg/L. Concentrations of 39 and 89 μg/L were found in drainage water from greenhouses. Widespread occurrence of imidacloprid is also confirmed for regions outside Europe. In a survey of rivers around Sydney, Sánchez-Bayo and Hyne (2014) detected imidacloprid in 93% of the samples, with concentrations up to 4.6 μg/L in the vicinity of a turf farm. Starner and Goh (2012) analyzed 75 surface water samples from agricultural areas in California in 2010–2011, and detected imidacloprid in 89% of the samples. Maximum concentrations were between 1.38 and 3.29 μg/L and the authors report that 19% of the samples exceeded the U.S. toxicity benchmark of 1.05 μg/L, while 73% and 88% of the samples exceeded the current Dutch AA-EQS of 0.067 μg/L and MAC-EQS of 0.2 μg/L, respectively (Starner and Goh 2012). Comparing concentrations in single samples with the AA-EQS is not fully justified, since this should be done on the basis of the annual average concentration per location. However, at one sampling location Starner and Goh (2012) found concentrations between 0.162 and 0.488 μg/L in monthly samples taken from May to August, suggesting that exposure was above the critical level for a longer period of time. Similarly, Lamers *et al.* (2011) detected imidacloprid on six consecutive sampling dates between April and June when monitoring river water in a rice cultivation area in Northern Vietnam. Imidacloprid concentrations of about 0.5 μg/L were reported shortly after pesticide application, and mean measured concentrations were around 0.2 μg/L, which is well above the proposed AA-EQS.

The available monitoring data indicate that the proposed water quality standards for imidacloprid are likely to be exceeded unless measures are taken to reduce emissions. Based on some of the recently published studies on aquatic arthropods that are also included in this article, the Dutch board for the authorization of plant protection products and biocides (Ctgb) lowered the Regulatory Acceptable Concentration (RAC) and restricted the use of several imidacloprid-based products (Ctgb 2014a,b). Treatment of discharge water from greenhouses and further drift reduction measures for field applications were made compulsory. If applied correctly, these measures may lead to reduced emissions to surface water. However, due to differences in methodology and dataset, the RAC was set to a chronic HC5

of 0.027 μg/L without an assessment factor, and is thus a factor of 3 higher than the revised AA-EQS proposed in this study. Moreover, simultaneous or consecutive use of different products with the same active substance on different crops is not accounted for in the authorization procedure. This means that if a safe use is identified according to the provisions for authorization, this is no guarantee that the new WFD-water quality standards will be met in the field. The overall impact of the newly proposed standard on the assessment of Dutch surface water quality thus remains unclear until new monitoring data are available.

Van Dijk *et al.* (2013) linked the observed decline in abundance of some aquatic invertebrate taxa in The Netherlands to contamination of surface water by imidacloprid, and used these ecological observations to motivate that a lower water quality standard be needed. In a recent response, Vijver and Van den Brink (2014) concluded that the status of aquatic ecosystems in the highly managed landscape of The Netherlands is the result of a complex suite of stressors, of which pesticides are one factor. Imidacloprid, although important in terms of ecological risks, is one of many pesticides being applied. They argue that water quality standards should not be solely based on field observations but should largely rely on the results of controlled experiments, in order to separate stress from a single pesticide from other stressors (Vijver and Van den Brink 2014). The present study confirms, based on the analysis of such experiments, that the current water quality standard for imidacloprid should indeed be lowered.

It is noted that both pesticide authorization and water quality assessment according to the WFD are performed on a substance-by-substance basis, and do not take into account the presence of other pesticides. In case of neonicotinoids, this is of particular importance because different active substances share a common mode of action. An initial assessment of the impact of combined exposure may be made by adding up the risk ratios of different pesticides found at a single location when comparing monitoring data with quality standards (Syberg *et al.* 2009; Teuschler and Herzberg 1995). If such an analysis points at a potential risk caused by a combination of multiple pesticides, risk mitigation should be focused on the package of compounds, rather than on single substances. For greenhouse applications, the treatment of discharge water issued for imidacloprid-based products will probably also lead to reduced emissions of other substances and potentially lower the combined exposure to pesticides.

CONCLUSIONS

Based on an up-to-date evaluation of acute and chronic laboratory studies and semi-field experiments, it is concluded that the water quality standard for long-term exposure to imidacloprid should be set to 8.3 ng/L. The standard for short-term peak exposure of 0.2 μg/L can be maintained. Based on these values, it is expected that imidacloprid will remain a problematic substance for Dutch water quality. Future monitoring data will ultimately reveal if the measures that were taken to reduce emissions are sufficient to meet the newly proposed standards. Since imidacloprid is only one of the large number of pesticides used, the presence of other pesticides should be taken into account when assessing water quality.

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SUPPLEMENTAL MATERIAL

Supplemental data for this article can be accessed on the publisher's website.

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Agricultural insecticides threaten surface waters at the global scale

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Compared with nutrient levels and habitat degradation, the importance of agricultural pesticides in surface water may have been underestimated due to a lack of comprehensive quantitative analysis. Increasing pesticide contamination results in decreasing regional aquatic biodiversity, i.e., macroinvertebrate family richness is reduced by ∼30% at pesticide concentrations equaling the legally accepted regulatory threshold levels (RTLs). This study provides a comprehensive metaanalysis of 838 peer-reviewed studies (>2,500 sites in 73 countries) that evaluates, for the first time to our knowledge on a global scale, the exposure of surface waters to particularly toxic agricultural insecticides. We tested whether measured insecticide concentrations (MICs; i.e., quantified insecticide concentrations) exceed their RTLs and how risks depend on insecticide development over time and stringency of environmental regulation. Our analysis reveals that MICs occur rarely (i.e., an estimated 97.4% of analyses conducted found no MICs) and there is a complete lack of scientific monitoring data for ∼90% of global cropland. Most importantly, of the 11,300 MICs, 52.4% (5,915 cases; 68.5% of the sites) exceeded the RTL for either surface water (RTL_{SW}) or sediments. Thus, the biological integrity of global water resources is at a substantial risk. RTL_{SW} exceedances depend on the catchment size, sampling regime, and sampling date; are significantly higher for newer-generation insecticides (i.e., pyrethroids); and are high even in countries with stringent environmental regulations. These results suggest the need for worldwide improvements to current pesticide regulations and agricultural pesticide application practices and for intensified research efforts on the presence and effects of pesticides under real-world conditions.

global surface waters | insecticide contamination | agriculture | regulatory risk assessment | biodiversity

At present, 15.3×10^6 km^2 of available croplands (Fig. 1) are cultivated worldwide; thus, agriculture (croplands and pasture) constitutes the world's largest terrestrial biome (1). Agricultural expansion and intensification led to a >750% increase in pesticide production between 1955 and 2000 (2). Moreover, pesticides represent a US$50 billion market worldwide (3). However, agricultural pesticide use leads to the exposure of nontarget ecosystems such as surface waters (4, 5). In this study, we focused on insecticides because they exhibit a high potential toxicity to aquatic organisms (6) that are crucial for ecosystem functions (7), and we analyzed exposure data obtained for surface waters because these waters are likely to be exposed to agricultural insecticide inputs (4, 5, 8) while providing essential environmental and human health-related ecosystem services (9).

Although the importance of nutrient levels and habitat degradation for surface water impairment is well understood (9), the same cannot be said for insecticides or pesticides in general (5, 9) (Fig. 1). A recent study (10) showed that in Europe, organic chemicals and pesticides specifically threaten freshwater integrity. Based on model predictions, another study (8) identified river fragmentation and nutrient loading as greater threats to aquatic biodiversity than pesticides; however, this study did not consider differences in pesticide toxicities. In response to the inherent toxicity of pesticides and their intentional release into the environment, elaborate environmental risk assessment procedures (*SI Appendix*, *SI Discussion*) (11, 12) defining a legally accepted regulatory threshold level (RTL) for each compound (see *SI Appendix*, Table S1 for the RTLs of the 28 insecticides considered here) have been developed; thus, pesticides are among the most intensively tested and regulated chemicals (13) (*SI Appendix*, Table S2), possibly contributing to the general perception of their environmental safety.

A recent study (14) using field data obtained from Germany, France, and Australia showed that elevated pesticide levels affect regional freshwater invertebrate biodiversity. This analysis ruled out confounding factors and used exposure data based on methods reflecting short-term pesticide concentrations. Transferring the standard toxicity values used in this study into RTLs clearly illustrates that species richness is reduced at the taxonomic family level by ∼30% at the RTL and by ∼12% at a factor of 10 below the RTL (Fig. 2*A*). Field studies (15, 16) reporting measured insecticide concentrations (MICs) up to 250 times RTL detected decreases in family richness of up to 63%. Any exceedance of the RTL thus indicates a risk of incurring clearly unacceptable effects on aquatic biodiversity. The overarching question now is how widespread and common this risk is, i.e., do MICs exceed their RTLs in the surface waters globally?

The few large-scale studies of insecticide exposure in surface waters have either examined sites in spatially restricted areas (10, 17, 18); lacked a quantitative data analysis (4); or followed other, rather specific objectives (18, 19) (*SI Appendix*, *SI Discussion*). However, the results obtained in these studies suggest that exceedances of threshold values occur, particularly for insecticides. These studies also showed that insecticides are only present for very short periods

Significance

Agricultural systems are drivers of global environmental degradation. Insecticides, in particular, are highly biologically active substances that can threaten the ecological integrity of aquatic and terrestrial ecosystems. Despite widespread insecticide application to croplands worldwide, no comprehensive field data-based evaluation of their risk to global surface waters exists. Our data show, for the first time to our knowledge at the global scale, that more than 50% of detected insecticide concentrations (n = 11,300) exceed regulatory threshold levels. This finding indicates that surface water pollution resulting from current agricultural insecticide use constitutes an excessive threat to aquatic biodiversity. Overall, our analysis suggests that fundamental revisions of current regulatory procedures and pesticide application practices are needed to reverse the global environmental impacts of agrochemical-based high-intensity agriculture.

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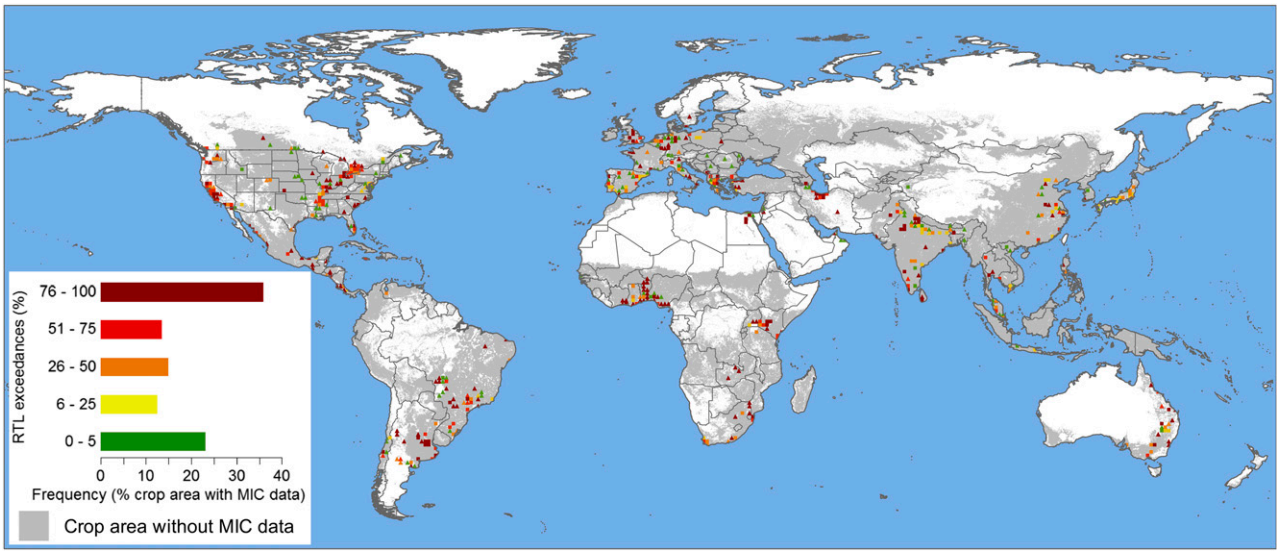

Fig. 1. Global crop area and the distribution of regulatory threshold level (RTL) exceedance rates for reported measured insecticide concentrations (MICs, $n = 10{,}659$) aggregated in 1° grid cells. Information on insecticide surface water exposure was available for only 1.62 million km^2 (10.6%) of the 15.3 million km^2 of global croplands (1). Rectangles ($n = 307$) represent subclassified cropped areas with five or more MICs, and triangles ($n = 290$) display grid cells with fewer than 5 MICs. Please note that 641 MICs could not be allocated to a specific grid cell due to the provision of imprecise location information in the studies. The horizontal bars in the legend illustrate the relative distributions of the respective insecticide RTL exceedance classes among the global cropped area with information on insecticide exposure.

(i.e., less than 1% of the year) in agriculturally influenced surface waters. It follows that most traditionally operated, fixed interval-based sampling campaigns inherently miss insecticide exposure (20). To avoid bias resulting from an excessive number of samples without quantifiable insecticide levels, exposure assessments of insecticides using monitoring data must be based solely on quantifiable concentrations in aquatic environments, i.e., those above the limit of quantification (LOQ) (*SI Appendix*, *SI Discussion*) (20).

Based on prior investigations (4, 10, 17, 18), we hypothesized that MICs in surface waters exceed their RTLs at dimensions underestimated by regulators and the general public. We tested this first hypothesis using a metaanalysis of global insecticide monitoring data from international peer-reviewed publications (*Methods*).

The majority of the 28 insecticides included in our analysis (*SI Appendix*, Table S1) are currently approved in the United States and the European Union. They represent all major insecticide classes and those compounds that are important for global agriculture in terms of annual application rates (*SI Appendix*, *SI Methods*). A total of 11,300 MICs [representing an estimated 2.6% of the population of analyses conducted (*SI Appendix*, *SI Discussion*)] caused by agricultural nonpoint source pollution from 838 studies published between 1962 and 2012 were compared with their respective RTLs for surface water (RTL_{SW}; $n = 8{,}166$) or sediment (RTL_{SED}; $n = 3{,}134$). Specifically, we used the RTL_{SW} derived from the official US Environmental Protection Agency's regulatory risk assessment for the evaluation of MIC_{SW} detected

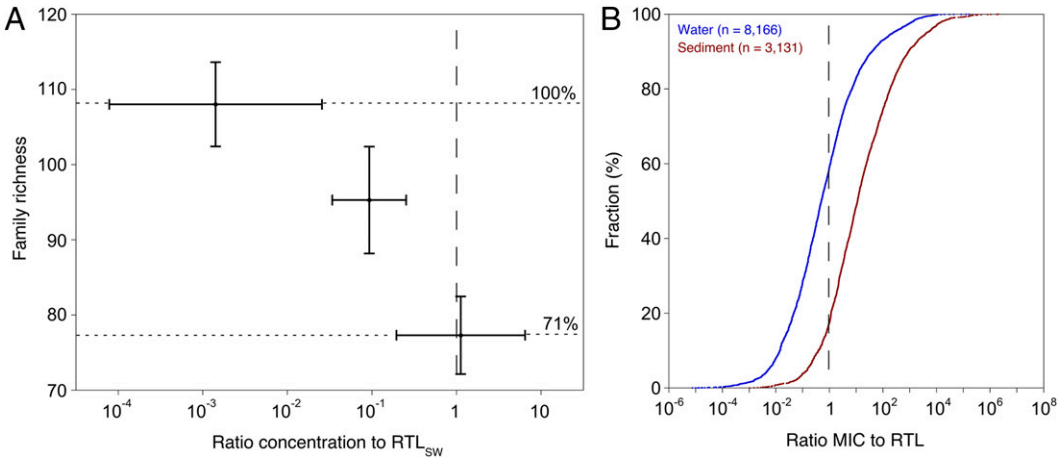

Fig. 2. Observed ecological effects of pesticide exposure on regional surface water biodiversity and distribution curves for global reported measured insecticide concentrations (MICs) in water and sediment relative to regulatory threshold levels (RTLs). (*A*) Dependency of mean macroinvertebrate family richness at 60 agricultural stream sites on mean aqueous pesticide concentration to RTL_{SW} ratios. Data on family richness, pesticide exposure levels, and categories were taken from ref. 14. The vertical dashed line indicates the RTL_{SW}, and the error bars denote 95% confidence intervals. (*B*) Blue represents the concentrations in water relative to the substance-specific RTL_{SW} ($n = 8{,}166$), and brown represents the concentrations in sediment relative to the substance-specific RTL_{SED} ($n = 3{,}131$). The vertical dashed line indicates the RTL.

in the United States and Canada, the official European RTL_{SW} for the evaluation of MIC_{SW} detected in European Union member states, and the average of the two values for the evaluation of MIC_{SW} detected in other parts of the world (*SI Appendix*, Table S1 and *Methods*). Notably, the United States' and European Union's RTL_{SW} values do not differ consistently, i.e., some individual RTL_{SW} values are higher in the United States or the European Union. Our analysis is based on more than 2,500 surface water sites located in 73 countries worldwide (Fig. 1 and *SI Appendix*, Fig. S1 and *SI Discussion*) and includes freshwater (n = 9,910 concentrations) and estuarine (n = 1,390 concentrations) systems with catchment sizes between 0.002 and 3,400,000 km^2 (*SI Appendix*, Table S3).

Results and Discussion

Our global analysis shows that no scientific investigations of insecticide surface water exposure exist for large portions (i.e., ∼90%) of high-intensity agricultural areas (Fig. 1). For example, no MICs were reported for Russia or several other post-Soviet states or from large parts of Africa or northwestern South America, although croplands dominate large areas in these regions. The most important outcome of our study is that among the 11,300 insecticide concentrations detected, 52.4% exceeded their specific threshold levels. Approximately 40.8% of the MIC_{SW} values (which are considered directly bioavailable due to their presence in the water phase) (21, 22) were above their respective RTL_{SW} values (Fig. 2*B*). Thus, our results demonstrate that in at least 3,331 cases distributed globally (Fig. 1), the regional biodiversity of surface waters is at risk for impairment due to insecticide contamination (Fig. 2 *A* and *B*) (14). Importantly, these risks were defined only for individual compounds, without considering the potential effects of mixture toxicity (see below on this topic). The application of only the United States (54% RTL_{SW} exceedances) or European Union (35.1% RTL_{SW} exceedances) RTL_{SW} to global MIC_{SW} did not alter the overall findings of our metaanalysis. When the dataset was rigorously restricted based on land use and entry routes to only those exposure incidents that were definitely linked to agricultural nonpoint entries (*SI Appendix*, *SI Discussion*), the results were even more striking (49.7% RTL_{SW} exceedance; *SI Appendix*, Table S4).

The 82.5% RTL_{SED} exceedances (2,584 cases) reported herein (Fig. 2*B*) also signify remarkable environmental risks. Sediment samples reflect exposure conditions over longer time spans compared with those of water samples, and the high exceedance levels (*i*) support the data reported for water, (*ii*) are likely due to the high hydrophobicity of many insecticides, (*iii*) imply long-term (chronic) risks to sediment-dwelling organisms (23), and (*iv*) indicate that both major aquatic ecosystem components are at risk.

Overall, the data regarding insecticide exposure (Fig. 2*B*) and their attributable ecological effects (Fig. 2*A*) reveal for the first time to our knowledge at the global scale that, in concert with nutrients and habitat degradation, agricultural insecticide use is likely a driver for biodiversity loss in agriculturally impacted aquatic ecosystems (8, 9, 24). This synthesis responds to a request to quantify the "concentrations of [. . .] pollutants in the global environment" (25), made with regard to pollution as one of the two planetary boundaries that have not yet been quantified. Our approach is based on an extended version of the approach used in ref. 8 as it analyzes empirical monitoring data and employs for the first time to our knowledge a global risk-based evaluation that considers the fact that individual insecticide toxicities span several orders of magnitude. Applying the available insecticide monitoring results to areas that currently lack information on insecticide exposure (i.e., ∼90% of global cropland) reveals that the surface waters located in ∼65% of global cultivated areas are at risk for exposure to insecticide RTL exceedance rates of more than 25% (Fig. 1). However, future studies are needed to quantify the uncertainty related to extending the present risk predictions to all global cropland.

Please note that there are a number of aspects that require further consideration in the assessment of insecticide risks. First, the published insecticide monitoring results to which we refer in our analysis most likely underestimate the actual exposure levels because it is extremely difficult to capture transient insecticide peak concentrations; ∼84.4% of the reported water-phase concentrations were measured using sampling strategies likely to miss the short-term insecticide peaks (20). Highly transient exposures are, according to ref. 20, typical for insecticides in agricultural surface waters. Even considerably contaminated sites regularly exhibit detectable insecticide concentrations for only a few (i.e., 3–4) hours during ∼4–6 d/y coinciding with typical application patterns (e.g., in the spring/summer). Organisms present at such sites receive their entire annual insecticide exposure dose during these short time periods during which short-term peak exposure incidents occur, and these incidents may cause long-term ecological perturbations (4, 14) due to the high intrinsic toxicity of insecticides (6, 26). Therefore, environmental science is faced with the challenge of being able to detect very low absolute levels of insecticides occurring stochastically in time and space that lead to negative ecological impacts. It is thus likely that insecticides are regularly underestimated in their importance as a driver of aquatic biodiversity decline. Second, an in-depth evaluation of the field studies underlying this metaanalysis showed that the majority of sites received either repeated contamination peaks over short periods or concurrent exposure to a number of different pesticides. For example, 81.3% of the samples that were analyzed for the presence of additional compounds (n = 4,198) contained up to 31 additional pesticides; this finding indicates that although disregarded in the regulatory risk assessment (11, 27), overall pesticide effects in the field are driven by repetitive exposure peaks and mixture toxicity (the simultaneous exposure of organisms to a multitude of different compounds). Third, unacceptable ecological effects on aquatic organisms are likely to occur in the field at concentrations well below the RTL (Fig. 2*A*) (7, 14). Applied to the data compiled here, this consideration means that in virtually all cases where an insecticide had been detected (ratio MIC to RTL $\geq 10^{-3}$; Fig. 2*B*), the consequence is a negative impact on regional biodiversity (Fig. 2*A*).

Based on these three considerations, both the actual insecticide contamination of surface waters and the resulting ecological risks are, in reality, even greater than indicated in this study based on the assessed literature and current regulatory procedures for insecticide risk assessment. In this context, the comparison of MIC_{SW} to other established threshold levels such as science-based environmental quality standards (EQSs) [which, in contrast to RTLs, do not tolerate (transient) clear effects on aquatic organisms], leads to an even higher threshold level exceedance rate of 70.1% (n = 7,821; *SI Appendix*, *SI Methods*). However, a concentration exceeding the RTL measured at a given site does not necessarily indicate that large stretches of the associated surface water are exposed and therefore harbor risks to aquatic fauna. For example, aquatic vegetation can reduce the negative impacts of pesticides (26). Nonetheless, the fact that RTL exceedances are so widespread and lead to detectable biodiversity reductions clearly highlights the global problem we are facing as a result of insecticide use in agriculture.

In addition to improving the efficiency of insecticides and reducing insect/pest resistance, the research and development (R&D) of insecticide compounds have focused on being more environmentally friendly, with the intention of reducing risks to surface waters as nontarget ecosystems (28, 29). However, a recent study (18) showed that the FOCUS model, used for the regulatory exposure assessment in the European Union, underpredicts field concentrations of newer, increasingly used insecticides such as hydrophobic pyrethroids. Specifically, the ratio of the predicted insecticide surface water concentrations to the MIC_{SW} was significantly lower for pyrethroids than for organochlorines and organophosphorus insecticides. The authors partially attributed these

results to the inadequacies of the runoff model termed "pesticide root zone model" (PRZM), which is also used for the authorization of pesticide compounds in other countries such as the United States (30). Therefore, our second hypothesis was that newer, more recently developed and registered insecticide classes (*SI Appendix*, Table S5) show higher RTL exceedances.

Contemporary insecticides, such as pyrethroids, showed a significantly higher percentage of RTL_{SW} exceedance (65.8%) compared with both organophosphates (43.7%; $P < 0.001$) and organochlorines (24.4%; $P < 0.001$), and the latter two also differed significantly ($P < 0.001$; Fig. 3*A* and *SI Appendix*, Table S6). Although first introduced to the global crop protection market in 1973 (*SI Appendix*, Table S5), pyrethroids have gained prominence in part due to concerns over organophosphates and human health. In our comparison of insecticide classes, we specifically considered differences in bioavailability and the ratios between the RTL_{SW} and the LOQ in additional linear model analyses; neither aspect altered the general picture of significant differences among the compound classes. In particular, considering only the freely dissolved [and therefore directly bioavailable (31)] fraction analyzed in water samples of the highly hydrophobic [organic carbon/water partitioning coefficients (K_{OC}) of 10^5–10^7 (32)] pyrethroids did not reduce their concentration to RTL_{SW} ratios (*SI Appendix*, Table S7 and *SI Discussion*). This finding indicates that the significantly higher RTL_{SW} exceedance frequency for highly sorptive pyrethroids is not biased by potential bioavailability limitations. In addition, considering the lower RTL_{SW} of pyrethroids associated with their comparably higher toxicity to aquatic organisms, and thus lower distances between RTL_{SW} and LOQs (*SI Appendix*, Table S8), did not disprove our findings; however, the discrepancies among insecticide classes were reduced (*SI Appendix*, Table S9 and *SI Discussion*).

Overall, we conclude that the environmental risk is even higher for newer-generation insecticides, such as pyrethroids, compared with older-generation insecticides. Further, these increased risks indicate a failure of R&D efforts to develop more environmentally friendly insecticides to improve surface water protection. Current risk management obligations and application practices for pyrethroids in agriculture obviously do not result in surface water exposure levels that adhere to the strict RTLs triggered by their extremely high invertebrate toxicities (6). However, in contrast to pyrethroids, a valid conclusion for neonicotinoid MIC_{SW} (RTL_{SW} exceedances: 6.1%; $n = 131$) is hindered due to insufficient data. Nonetheless, recent studies (19, 33) on agricultural neonicotinoid use reveal environmental concerns for both aquatic and terrestrial ecosystems.

Our third hypothesis is that countries with a high environmental regulatory quality (HERQ) should exhibit markedly less frequent RTL exceedances than those with a low environmental regulatory quality (LERQ) (*SI Appendix*, Table S10). RTL_{SW} exceedances were indeed significantly more frequent in the LERQ countries ($P < 0.001$; *SI Appendix*, Table S6). This pattern also holds true when accounting for differences in RTL/LOQ ratios (*SI Appendix*, Table S9). Although not unexpected, this finding is alarming considering that recent and anticipated future agricultural expansion and intensification have occurred and will occur in biodiversity-rich tropical LERQ countries (1). In these countries, pesticide regulations are insufficiently enforced (5, 34) (*SI Appendix*, *SI Discussion*) and surface waters are already exposed to numerous other stressors (9). The absolute percentage of the detected RTL_{SW} exceedance (39.9%) in the HERQ countries (such as the United States, Canada, Germany, Japan, and Australia), is only slightly lower than that in the LERQ countries (42.2%; Figs. 1 and 3*B*). Therefore, our data show that the actual extent to which surface waters are contaminated with insecticides is not controlled effectively by increasingly stringent environmental regulations at present. However, in the LERQ countries, substantially larger surface water systems and longer sampling intervals were considered in the monitoring campaigns (*SI Appendix*, Table S11), decreasing the likelihood of determining insecticide peak exposure incidences (*SI Appendix*, Table S6) (20). The application of more targeted insecticide sampling strategies (20) is needed in the future to adequately reflect the risks to the surface waters of LERQ countries.

Overall, RTL exceedances depend on multiple factors, including insecticide classes, environmental regulatory standards, catchment size, sampling regime, and sampling date (*SI Appendix*, Table S6). We identified a significant interaction among insecticide class, the quality of countries' regulatory standards, and sampling date (*SI Appendix*, Tables S6, S12, and S13, Fig. S2, and *SI Discussion*).

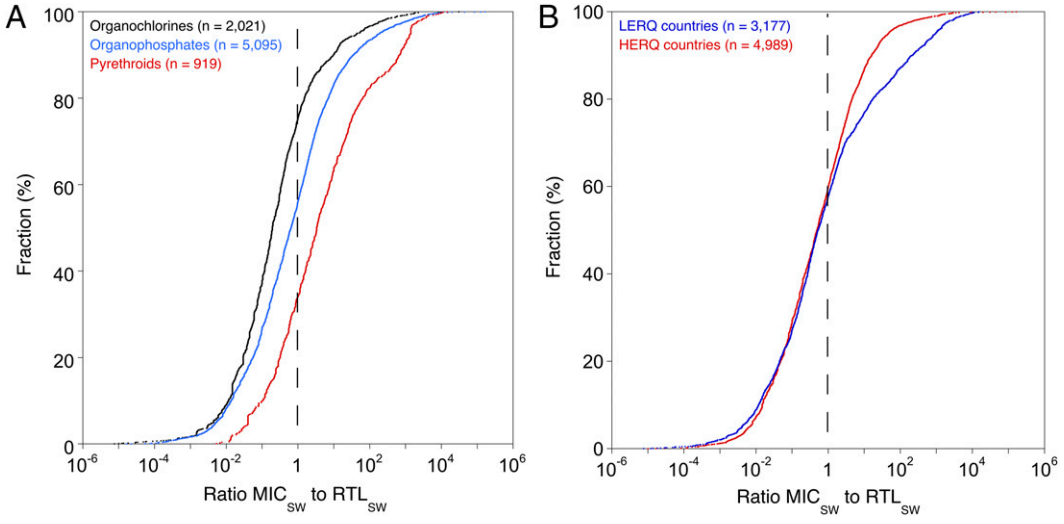

Fig. 3. Effect of insecticide class and country environmental regulations on the distribution curves for reported measured insecticide concentrations in the water phase (MIC_{SW}) relative to substance-specific regulatory threshold levels (RTL_{SW}). (*A*) Black represents data obtained for organochlorine insecticides (n = 2,021), blue represents data obtained for organophosphate insecticides (n = 5,095), and red represents data obtained for pyrethroid insecticides (n = 919); 6.1% of the MIC_{SW} of neonicotinoids (n = 131) exceeded the RTL_{SW} (not displayed). (*B*) Distribution curves for MIC_{SW} relative to substance-specific RTL_{SW}. Blue represents concentrations measured in countries with low environmental regulatory quality (LERQ; n = 3,177), and red represents data measured in countries with high environmental regulatory quality (HERQ; n = 4,989). The vertical dashed lines indicate the RTL_{SW}.

Unlike in HERQ countries, the risks of organochlorine and organophosphorus insecticide exposure in LERQ countries have increased over the last three decades due to increased insecticide use and simultaneously weak or even nonexistent pesticide regulation schemes.

Taken together, our results seriously challenge the protectiveness of the current regulatory insecticide risk assessments and management procedures at the global scale. Although, for example, major EU and US pesticide legislations were already enforced at the beginning of the 1990s (*SI Appendix*, Table S2), 54.2% (n = 4,686; and 49.5%, n = 2,681 when considering HERQ countries only) of the MICs reported since 2000 have exceeded their respective RTLs (*SI Appendix*, Fig. S3 *A* and *B*). Targeted postregistration monitoring schemes and regulatory actions are needed, considering that 18 and 24 of the 28 insecticide compounds included in our metaanalysis are currently approved in EU countries and in the United States, respectively. The high numbers of threshold exceedances worldwide are caused by failures of either regulatory exposure assessment (18) or farmers' adherence to prescribed risk management obligations (35).

Edge-of-field runoff was an important route of entry for insecticides in our dataset, comprising 72.4% of cases for which an entry route was specified (*SI Appendix*, Table S3). In addition to application patterns and geographical and meteorological conditions, the physicochemical properties of an insecticide (such as its hydrophobicity) are crucial components of its potential to enter a surface water via runoff (36, 37). Empirical studies (38, 39) suggest that lower runoff losses to surface waters occur for strongly sorbed compounds. This potential provides opportunities for the more efficient use of insecticides based on modeling of their runoff potential. However, the potential risks of insecticide surface water impairments are driven not only by the respective entry pathways and probabilities of exposure but also by the intrinsic toxicity, which varies considerably among different classes of insecticides (40). Thus, any risk mitigation attempt must consider both entry probability and toxicity.

To date, agriculture occupies ∼40% of the world's land surface and agricultural production is forecast to undergo substantial intensification (1, 2). This situation leads to the projection that future agricultural activities may rival climate change in their environmental impacts (2). Reforming conventional agricultural systems and adopting promising approaches from organic farming (41), including the elimination of pesticides wherever applicable, in concert with the closing of yield gaps on underperforming lands (1, 42) and precision agricultural techniques (43), are possible ways to meet the twin challenges of providing sufficient food for a growing human population and reversing the global environmental impacts of agrochemical-based high-intensity agriculture.

Methods

We conducted a comprehensive literature search of multiple databases to identify scientific studies in eight different languages reporting on agricultural insecticide concentrations in global surface waters. We evaluated more than 200,000 database entries and examined ∼20,000 articles in greater detail. The studies had to meet the following selection criteria to be included in our meta-analysis: (*i*) only peer-reviewed studies were considered to ensure that minimum scientific standards were met; (*ii*) the studies had to be written in one of the following eight languages: Chinese, English, French, German, Japanese, Russian, Spanish, and Portuguese; and (*iii*) the MICs reported resulted from agricultural nonpoint source pollution (excluding urban, industrial, and public health activities; aquaculture; atmospheric deposition; forest application; sheep dipping; golf course applications; accidental spills; intentional water contamination; and in-crop use) and were detected in perennial freshwater or estuarine surface water bodies (*SI Appendix, SI Methods*).

Regulatory threshold levels were applied as follows to assess the ecological importance of reported insecticide exposure data (*SI Appendix, SI Methods*, and Table S1): aqueous concentrations measured in the United States, Canada, or the European Union were compared with the respective regulatory threshold levels (RTL_{SW}), which are defined as part of the US (differentiated further into freshwater and estuarine RTL_{SW}) or EU pesticide legal registration procedures; and aqueous concentrations measured in other parts of the world were compared with the average values of the US and EU RTL_{SW} (*SI Appendix*, Table S1), as both regulatory risk assessments are considered highly elaborated and science based. Sediment or suspended-particle exposure was evaluated using the respective RTL_{SED}. The concentration of each insecticide was compared with its respective RTL, irrespective of how many compounds were measured in a given sample. To focus on the potential ecological risks of the highly relevant short-term exposure peaks of insecticides in surface waters, and considering that insecticide exposure occurs less than 1% of the time per year, we used only insecticide concentrations above the LOQ, as suggested by ref. 20 (see also *SI Appendix, SI Discussion* for further details). The aggregate exceedance frequencies for all studies considered were computed across multiple sites and plotted as distribution curves.

In addition to information on insecticide concentrations, we collected information on several covariates (i.e., sampling location, catchment size, sampling interval, and sampling date) that might influence insecticide exposure and used these data in a linear model analysis (*SI Appendix, SI Methods*) with the logarithm of the MIC_{SW} to RTL_{SW} ratio as the dependent variable to test for differences among specific insecticide classes (organochlorines, organophosphates/carbamates, and pyrethroids) and between countries' environmental regulatory standards (HERQ vs. LERQ countries, classified based on environmental, regulatory, and economic indices) (*SI Appendix, SI Methods*). We also evaluated the effects of the organic carbon/water partitioning coefficient (K_{OC}), the bioavailability of highly sorptive pyrethroids, and the differences in the RTL_{SW}/LOQ ratios on the concentration to RTL_{SW} ratios using two additional linear model analyses (*SI Appendix, SI Discussion*).

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Acute and Chronic Toxicity of Imidacloprid to the Aquatic Invertebrates *Chironomus tentans* and *Hyalella azteca* under Constant- and Pulse-Exposure Conditions

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Abstract The toxicity of imidacloprid, a nicotinic mimic insecticide, to the aquatic invertebrates *Chironomus tentans* and *Hyalella azteca*, was first evaluated in static 96-hour tests using both technical material (99.2% pure) and Admire®, a commercially available formulated product (240 g a.i. L^{-1}). The 96-h lethal concentration (LC)50 values for technical imidacloprid and Admire® were 65.43 and 17.44 μg/L, respectively, for *H. azteca*, and 5.75 and 5.40 μg/L, respectively, for *C. tentans*. Admire® was subsequently used in 28-day chronic tests with both species. Exposure scenarios consisted of a constant- and a pulse-exposure regime. The pulse exposure lasted for four days, after which time the animals were transferred to clean water for the remaining 24 days of the study. Assessments were made on both day 10 and day 28. In the *C. tentans* under constant exposure, larval growth on day 10 was significantly reduced at 3.57 μg/L imidacloprid, the lowest-observed-effect concentration (LOEC). The no-observed-effect concentration (NOEC) and LOEC for the 28-day exposure duration (adult survival and emergence) were 1.14 and greater than 1.14 μg/L, respectively; the associated LC50 and LC25 were 0.91 and 0.59 μg/L, respectively. The LOEC for the pulse treatment was greater than 3.47 μg/L, but the day 10 LC25 was 3.03 μg/L. In the *H. azteca* tests, the day 10 and 28 constant exposure, as well as the day 28 pulse exposure, LOEC (survival) values were similar at 11.95, 11.46, and 11.93 μg/L, respectively. The day 10 and 28 constant exposure effective concentration (EC)25s (dry weight) were also similar, at 6.22 and 8.72 μg/L, respectively, but were higher than the pulse-exposure day 10 LOEC and EC25 (dry weight) values of 3.53 and 2.22 μg/L, respectively. Overall, *C. tentans* was more sensitive to acute and chronic imidacloprid exposure, but less sensitive to a single pulse, than *H. azteca*. Chronic, low-level exposure to imidacloprid may therefore reduce invertebrate survival and growth, but organisms are able to recover from short-term pulse exposure to similar imidacloprid concentrations if the stressor is removed after four days.

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Imidacloprid, 1-((6-chloro-3-pyridinyl)methyl)-*N*-nitro-2-imidazolidinimine ($C_9H_{10}ClN_5O_2$), is a nicotine mimic (nicotinoid insecticide) that belongs to a group of insecticides referred to as the chloronicotinyl group (Kidd and James 1991; Cox et al. 1997; Tomlin 1997). It produces toxicity by binding to and overstimulating nicotinic acetylcholine (Ach) receptors on the postsynaptic membranes of neurons (Kidd and James 1991; Song et al. 1997; Tomlin 1997). Imidacloprid has both stomach (systemic) and contact action, and is effective against a wide variety of insect pests, including the Colorado potato beetle, *Leptinotarsa decemlineata*. In Canada, this pesticide is used on potato, tomato, lettuce, canola, and other vegetable crops, as well as for control of fleas on domestic cats and dogs (Cox 2001). In crop production, imidacloprid can be

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Factors influencing the occurrence and distribution of neonicotinoid insecticides in surface waters of southern Ontario, Canada

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HIGHLIGHTS

- Conducted a survey of neonicotinoids used in full range of agricultural activities in surface waters of Ontario.
- Statistical correlation of individual compounds with land use was investigated.
- Relationship between neonicotinoid occurrence and hydrology of water courses was assessed.
- Imidacloprid, clothianidin, and thiamethoxam detection frequency over 90% at over half the sites sampled.
- At 2 sites, the Canadian freshwater guideline value for imidacloprid (230 ng/L) was exceeded in 75% of samples.

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ABSTRACT

The widespread use of neonicotinoid insecticides and recent increased regulatory scrutiny requires the generation of monitoring data with sufficient scope and resolution to provide decision makers with a better understanding of occurrence and distribution in the environment. This study presents a wide-scale investigation of neonicotinoid insecticides used across the range of agricultural activities from fifteen surface water sites in southern Ontario. Using statistical analysis, the correlation of individual compounds with land use was investigated, and the relationship between neonicotinoid occurrence and hydrologic parameters in calibrated water courses was also assessed. Of the five neonicotinoids studied, imidacloprid, clothianidin and thiamethoxam exhibited detection rates above 90% at over half the sites sampled over a three year period (2012–2014). At two sites in southwestern Ontario, the Canadian Federal freshwater guideline value for imidacloprid (230 ng/L) was exceeded in roughly 75% of the samples collected. For some watersheds, there were correlations between the occurrence of neonicotinoids and precipitation and/or stream discharge. Some watersheds exhibited seasonal maxima in concentrations of neonicotinoids in spring and fall, particularly for those areas where row crop agriculture is predominant; these seasonal patterns were absent in some areas characterized by a broad range of agricultural activities.

1. Introduction

In the past decade, the use of organophosphorous insecticides has been superseded by neonicotinoid insecticides (Hladik et al., 2014; Hladik and Kolpin, 2015; Morrissey et al., 2015; Anderson et al., 2015). Neonicotinoids are active against a wide range of insects, are effective at low concentrations, are systemic, and can be applied using a variety of methods (Anderson et al., 2015). Registered uses of neonicotinoids in Canada include control of insects on field and greenhouse crops, orchards and nurseries, woodlots, flea control on household pets, and control of turf pests in urban areas, sod farms and golf courses. Neonicotinoids are regulated nationally by Health Canada's Pest Management Regulatory Agency (PMRA) with additional provincial restriction under the Province of Ontario's 2009 ban on cosmetic use of pesticides on lawns and gardens under the Ontario Pesticides Act (Ontario, 2016). Neonicotinoid formulations are also used for seed treatment of row crops such as corn, soybeans and canola, which has led to widespread use in Ontario (McGee et al., 2010; Farm and Food Care

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Ontario, 2016).

There has been growing concern about use of neonicotinoid pesticides and possible ecological and ecotoxicological effects on pollinators and invertebrates, and possible indirect effects on songbirds and waterfowl (Anderson et al., 2015). Anderson et al. (2015) recently reviewed fate, exposure and biological effects of neonicotinoids in the Canadian aquatic environment, while Morrissey et al. (2015) reviewed neonicotinoid contamination in surface waters globally and potential risk to aquatic invertebrates. The United States Geological Survey (USGS) has conducted both national- (Hladik and Kolpin, 2015) and regional-scale (Midwestern United States, Hladik et al., 2014) reconnaissance studies of neonicotinoids in streams in the United States. Imidacloprid, clothianidin and thiamethoxam were the most frequently detected; in the U.S. national study, clothianidin and thiamethoxam were positively correlated with percentage of land use in cultivated crop production, while imidacloprid was positively correlated with percentage of urban area (Hladik and Kolpin, 2015).

To make informed decisions with respect to use, registration, and effects guidelines, there is a requirement for knowledge of occurrence and distribution of neonicotinoid insecticides across jurisdictions. The purpose of this study was to assess occurrence and distribution of neonicotinoids in surface waters in different agricultural and urban areas of southern Ontario as part of a comprehensive pesticide monitoring program. The neonicotinoids analyzed were thiamethoxam, clothianidin, imidacloprid, thiacloprid and acetamiprid; registration for the first three compounds is currently being re-evaluated by the PMRA. Morrissey et al. (2015) identified a scarcity of neonicotinoid insecticide data globally that enables inferences regarding the fate of these compounds in relation to water body features and land use. Using statistical analysis, the correlation of individual compounds with land use was investigated and the relationship between neonicotinoid occurrence and hydrologic parameters in calibrated water courses. This study presents the first wide-scale investigation of neonicotinoid insecticides in surface waters across the range of agricultural activities in southern Ontario.

2. Methods

Fifteen sites in southern Ontario consisting of nine streams near agricultural areas (drainage area <100 km^2), and six larger streams/rivers (drainage area >100 km^2) were sampled (Fig. S1). These stream sites reflected a range of agricultural activities including row crops, fruits and vegetables, orchards and grapes, greenhouses, ornamental nurseries, and turf. The sites also included an urban stream (Indian Creek) and a reference stream (Spring Creek) located adjacent to a national park removed from agricultural activities. All neonicotinoid insecticide concentrations in samples from Spring Creek were below the method detection limits (Table S1). Precipitation was sampled at one additional site (Bear Creek).

2.1. Sampling methods

Whole water samples were collected by submersing sample bottles (1L amber glass with Teflon® lids) at mid-stream to a depth of 10–20 cm, and stored in coolers with ice packs for transport. Samples were collected bi-weekly through the growing season (May–September) with monthly sampling in April, October, November and December. Duplicate field samples and field blanks were collected for QA/QC purposes. General water quality characteristics including temperature, pH, conductivity and dissolved oxygen were also measured during each sampling event using a YSI® sonde.

2.2. Sample preparation

Surface water and precipitation samples (800 mL stored at 4 °C) were extracted at 5 mL/min using a Waters OASIS HLB (0.5 g) solid phase extraction (SPE) cartridge. The cartridge was rinsed with 5 mL of 5% methanol in water (v/v) and then dried on-line with nitrogen for 1 min. The cartridge was eluted with 10 mL of methanol at a flow rate of 2 mL/min. The final extract was concentrated to ~0.9 mL and 50 μL of internal standard (acetamiprid-d_3 at 0.97 μg/mL, imidacloprid-d_4 at 1.3 μg/mL and thiamethoxam-d_3 at 1.0 μg/mL) was added and the extract volume-adjusted with water to a 1.5 mL final volume.

2.3. Analysis

The five neonicotinoids analyzed were acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam. An Agilent 1100 series HPLC system equipped with a Phenomenex Synergi Hydro-RP analytical column (3 × 100 mm i.d., 2.5 μm particle size) was used at a column temperature of 40° C and mobile phase flow rate of 250 μL/min. The mobile phase solvents were water (A) and 90% methanol (v/v) in water (B), each containing 5 mM ammonium formate used in a gradient elution program; initial composition 90% A:10% B; 90% A:10% B at 0.1 min; 5% A:95% B at 5.0 min and then held for duration of the 12 min run. The column was equilibrated for 5 min between 5 μL sample injections.

Neonicotinoid compounds were analyzed using an Applied Biosystems/Sciex API 2000 tandem mass spectrometer (MS) using an electrospray ionization (ESI) source in positive ion mode. The optimized positive ESI-MS conditions were; curtain gas (CUR) 35 psi, collision gas (CAD) 4 psi, TurboIon Spray source voltage (IS) 3000 v, heated nebulizer temperature 500° C, nebulizing gas (GS1) at 80 psi and auxiliary/heater gas (GS2) at 80 psi. The dwell time for each ion-pair was 50 ms. Resolution was set to achieve unit mass resolution for quadrupoles 1 and 3.

2.4. Statistical analysis

Summary statistics were estimated using the Kaplan-Meier method to account for values below detection limits using the NADA package in R (Helsel, 2012; R Core Team, 2016). Principal components analysis (PCA) was used to identify relationships between land-use, crop type, and neonicotinoid concentrations (Helsel and Hirsch, 2002). Prior to analysis by PCA all data were transformed to standard scores (z-score) as variables included have different units of measure. Association of individual neonicotinoids and association with precipitation and stream discharge were assessed using the Kendal rank correlation coefficient (Kendall's tau, τ). The PCA and correlation analyses were performed using JMP® Version 10 (SAS Institute Inc., Cary, NC).

3. Results and discussion

3.1. Occurrence and distribution of neonicotinoid insecticides in southern Ontario surface waters

As observed in other North American studies of neonicotinoids, imidacloprid, thiamethoxam and clothianidin were the most ubiquitous; occurrence and distribution data including surface water concentrations and frequency of detection for southern Ontario surface waters are shown in Table S1 and Fig. S2. Table S1 also includes the number of samples that exceeded the Canadian Council of Ministers of the Environment (CCME) interim freshwater guideline for protection of aquatic life value for imidacloprid (230 ng/L, CCME, 2007); this guideline is currently the only

Canadian federal freshwater guideline available for any neonicotinoids registered for use in Canada, and was used as a benchmark to compare with observed concentrations in this study.

Registered uses of imidacloprid in Canada include control of insects on field and greenhouse crops, orchards and nurseries, and household and turf applications (PMRA, 2001, 2016). In Ontario, imidacloprid replaced diazinon for lawn care use and turf applications (Struger and Fletcher, 2002), prior to the Province of Ontario's 2009 ban on cosmetic use of pesticides on lawns and gardens. Imidacloprid is applied to control insects across the entire range of agricultural activities using a variety of application methods including soil application, foliage spray treatment, and seed treatment (PMRA, 2001, 2016). Typical application rates to foliage or soil range are determined by crop, but typically range from 42 to 480 g a.i./ha (PMRA, 2016). However, the application rate for imidacloprid on fruiting vegetables for control of the Colorado potato beetle and aphids can be as high as 560 g a.i./ha (PMRA, 2016). The range of applications for imidacloprid result in potential for entry into aquatic systems through a variety of vectors, including spray drift, atmospheric deposition, soil erosion and runoff (CCME, 2007).

Imidacloprid was detected in all samples at 8 sites, which was the highest level of occurrence for all compounds (Table S1). In general, very high occurrences of detection were observed across the entire study area, presumably due to the broad range of applications of imidacloprid. There was roughly a 6000-fold range in measured concentrations from low ng/L to 10,400 ng/L (Table S1). Imidacloprid was particularly prevalent in southwestern Ontario along the Lake Erie shoreline (Lebo Drain and Sturgeon Creek, Fig. 1) and at Two Mile Creek in the Niagara Peninsula (Fig. 1). At Lebo Drain and Sturgeon Creek, roughly 75% of the samples contained imidacloprid at concentrations exceeding the CCME guideline value (230 ng/L). The watersheds of these water courses are characterized by high percentages of row crop agriculture; roughly 20% corn and 40% soybean for Lebo Drain and 26% corn and 33% soybean for Sturgeon Creek, Table S2). This area of southern Ontario is also home to the largest concentration of commercial greenhouses (representing 9% of the watershed, Table S2) in North America with roughly 1800 acres in vegetable production in 2012 (Ontario Greenhouse Vegetable Growers, 2012); cucumbers, peppers and tomatoes represent the majority of greenhouse crops in this area. In addition, 4.6% of the Sturgeon Creek watershed is dedicated to field tomato production. Two Mile Creek in the Niagara Region was the only other sampling station where concentrations of imidacloprid exceeded the CCME guideline (maximum value of 816 ng/L, Table S1). In contrast to watersheds in southwestern Ontario dominated by row crops, the Two Mile Creek watershed is represented by over 50% vineyards and orchards (Table S2). These observations indicated imidacloprid is preferred as an insecticide for a broad range of agricultural activities. These results contrasted with previous Canadian studies from 2000 to 2005 where imidacloprid was rarely detected.

Acetamiprid was one of the less ubiquitous compounds with only 6 sites exhibiting detection rates greater than 50% (Table S1, Fig. S3). As with imidacloprid, the highest concentrations and frequencies of detection were associated with sites in southwestern Ontario (Sturgeon Creek, Lebo Drain) and the Niagara Peninsula (Two Mile Creek, Prudhomme Creek and Four Mile Creek). In four samples acetamiprid was detected at concentrations greater than the imidacloprid CCME guideline value of 230 ng/L; these samples were associated with Two Mile Creek (1 sample), Lebo Drain (2 samples) and Sturgeon Creek (1 sample). Crops on which acetamiprid is routinely applied include pome fruits, leafy vegetables and ornamental plants and flowers; these crops are widely grown in both regions. In addition, acetamiprid is applied to grapes; the Niagara Region represents the majority of the 17,000 acres of grapes in production in Ontario with lesser production in the southwestern and eastern parts of the province (Grape Growers of Ontario, (2016)). Grapes comprise 35% of the value of Ontario commercial fruit crops. The Two Mile Creek and Four Mile Creek watersheds are characterized by roughly 26% orchard/33% vineyard and 15% orchard/28% vineyard, respectively (Table S2). Prudhomme Creek has watershed characteristics similar to those of Two Mile Creek and Four Mile Creek.

Thiacloprid exhibited the lowest levels of occurrence, distribution, and rate of detection (Table S1 and Fig. S4); Prudhomme Creek was the only site where thiacloprid was detected in greater than 50% of samples. The concentration of thiacloprid exceeded the imidacloprid CCME guideline value (230 ng/L) in only three samples; all of these occurrences were in Prudhomme Creek in the Niagara Region. The occurrence and distribution of thiacloprid is limited by its relatively narrow range of applications; in the case of its detection at sites in southwestern Ontario (Sturgeon Creek and Lebo Drain) and the Niagara Peninsula (Prudhomme Creek, Two Mile Creek and Four Mile Creek), application on pome fruits represents the most likely source, as registration in Canada is restricted to use on these crops. Although designated as the urban control site, thiacloprid was detected at 17% of samples collected at Indian Creek (Table S1, Fig. S4). Thiacloprid is not registered for domestic use in Canada; these detections may be the result of some limited agricultural activity in the watershed that includes orchards (Table S2).

Although clothianidin is registered for a fairly broad range of applications in Canada, it is commonly used as a seed treatment for canola and corn, and on grains and soybean. Of the twelve clothianidin commercial products registered for use in Canada, nine are for field crops; the remaining three are for turf grass, orchards and vegetables. The widespread prevalence of row crop agriculture in southern Ontario has resulted in clothianidin being ubiquitous as evidenced by its detection in over 80% of samples at 10 of the 15 sites in the study (Table S1, Table S2, Fig. S5). In addition to its frequent detection at sites in southwestern Ontario and the Niagara Region, clothianidin was prevalent in central and northern parts of southern Ontario where row crop agriculture is predominant (Fig. S5). For example, the Nissouri Creek (100% detection rate) watershed is roughly 40% corn production (Table S2). The concentration in only one sample in Two Mile Creek in the Niagara Peninsula exceeded the imidacloprid CCME guideline value of 230 ng/L; in general mean clothianidin concentrations were in the tens of ng/L (Table S1). Clothianidin is also the primary metabolite of thiamethoxam, although the current study provided no insights into the relationship of these compounds from a parent – breakdown product perspective.

Thiamethoxam is similar to clothianidin in that many registered commercial products are specifically for seed treatment; as a result there are similarities in the occurrence and distribution for both compounds (Figs. S5 and S6). However, thiamethoxam is registered for a broader range of applications compared to clothianidin, including control of house flies, ornamentals, greenhouse, and fruits and vegetables. As with clothianidin, typical mean concentrations for thiamethoxam are in the tens of ng/L range. Mean concentrations for thiamethoxam were highest at Twenty Mile Creek, Lebo Drain and the Sydenham River (Table S1); these mean concentrations also exceeded the 230 ng/L CCME guideline value.

The measured concentrations in the current study can be compared with those reported in other areas of North America and globally. Geometric means for average and maximum concentrations of all neonicotinoids in surface waters based on 29 studies carried out in 9 countries world-wide were 130 ng/L and 630 ng/L, respectively (Morrissey et al., 2015). For imidacloprid in the current

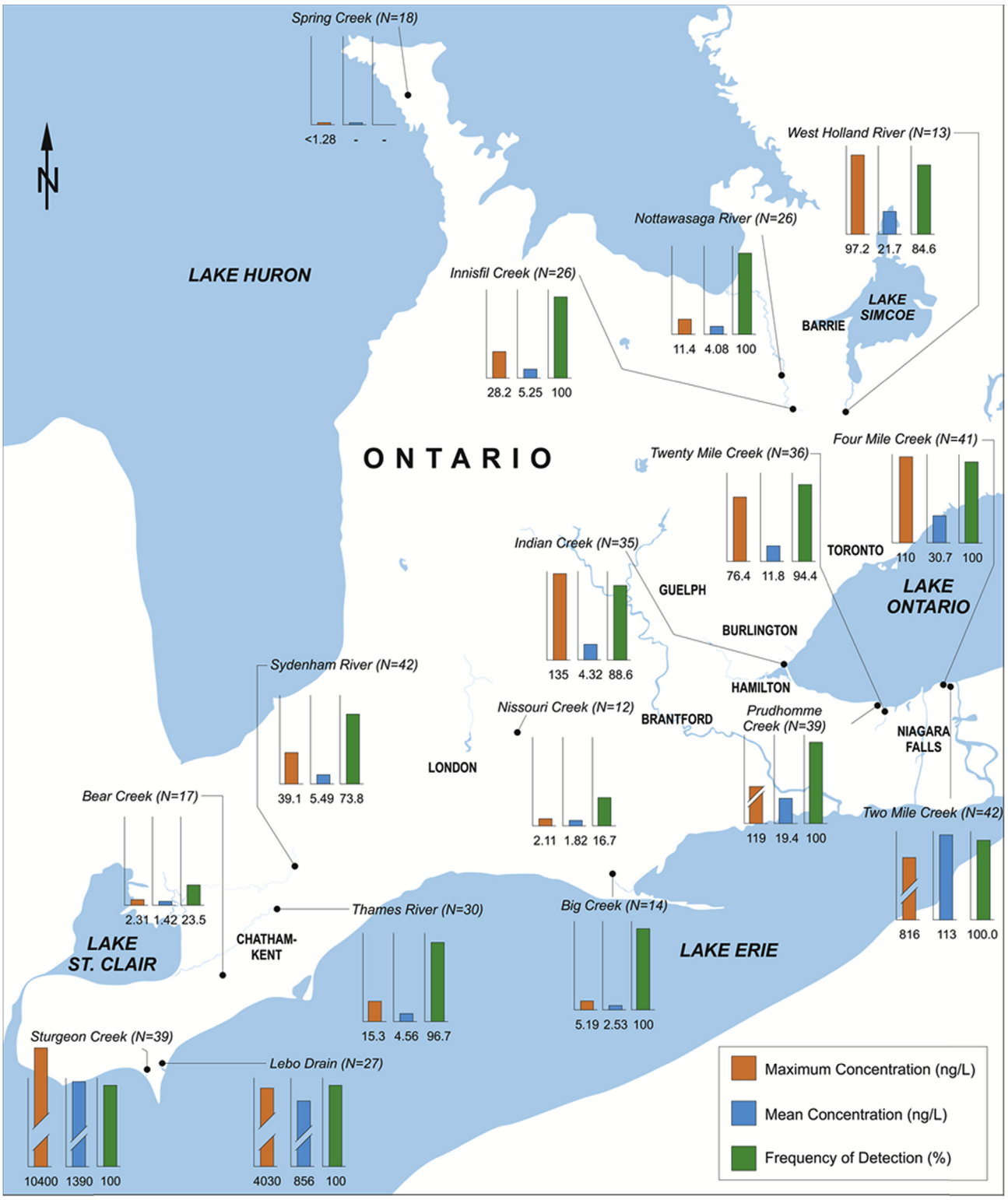

Fig. 1. Occurrence and distribution of imidacloprid in southwestern Ontario surface waters. Maximum and mean concentrations are expressed in ng/L while frequency of detection represents the percentages of samples in which imidacloprid was detected.

study, the mean average and maximum concentrations at the thirteen sites where the rate of detection was greater than 70% were 190 ng/L and 1210 ng/L, respectively. Morrissey et al. (2015) also presented a summary of ecological quality reference values for neonicotinoid insecticides, including the Canadian benchmark of 230 ng/L. The USEPA average (1050 ng/L) and maximum (35,000 ng/L) reference values are much higher than the Canadian CCME guideline value; however, the most recent benchmark for an average concentration adopted by the Netherlands (8.3 ng/L) is roughly 30-fold lower than the Canadian guideline. A study of

neonicotinoids in the Province of Quebec (Canada) determined that 3.2%–48% of surface water samples collected from watercourses where corn and soybean were the predominant agricultural crops exceeded the Netherlands reference value of 8.3 ng/L (Quebec, 2014). Morrissey et al. (2015) proposed reference values for average and maximum concentrations of 35 ng/L and 200 ng/L, respectively; these values were developed in consideration of weighting and standardizing all neonicotinoid insecticides to imidacloprid.

3.2. Correlation of land use with occurrence and distribution of neonicotinoid insecticides in southern Ontario

The occurrence and distribution of neonicotinoid insecticides in southern Ontario surface waters were presumably primarily influenced by agricultural activities. To assess the importance of land use, we performed a Principal Components Analysis (PCA) to identify correlations between land use and neonicotinoids (Fig. 2). In general, results of the PCA corroborated our previous interpretations of the data; as expected thiamethoxam and clothianidin were positively correlated with row crops, particularly soybeans and corn, while imidacloprid and acetamiprid were strongly correlated with greenhouse activity, vegetables and other agriculture including vineyards and orchards. Thiacloprid was more associated with fruit production, as was expected given this

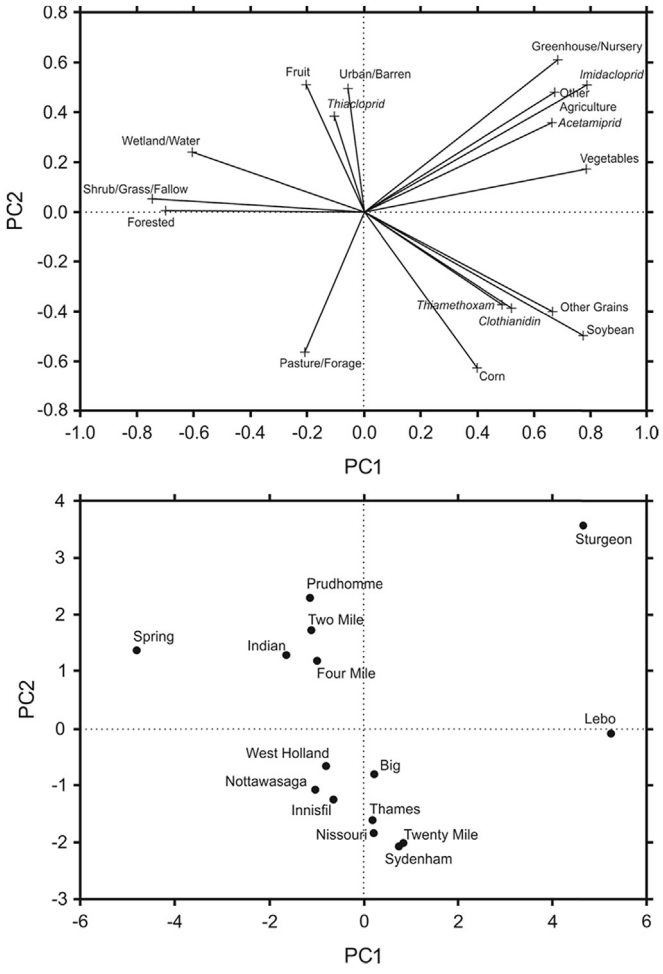

Fig. 2. Principal component analysis (PCA) of land use, crop type, and measured concentrations of neonicotinoid insecticides. The top panel shows the loadings of each factor and the bottom panel shows the distribution of stations in the ordination.

insecticide is commercially registrated in Canada for use on pome fruits (apples and pears). The most recent survey of pesticide use in Ontario (Farm and Food Care Ontario, 2016) reported that both corn and soybean acreage increased by over 20%, while associated pesticide usage increased by 38% for corn and 32% for soybeans; as a result, use of neonicotinoid insecticides for these two crops can be expected to continue to be robust. In their national-scale reconnaissance of neonicotinoids in the USA, Hladik and Kolpin (2015) found a positive statistical relationship between row crops and both clothianidin and thiamethoxam, while imidacloprid exhibited a positive relation to urban land-use. In terms of the co-occurrence of clothiainidin and thiamethoxam, Hladik and Kolpin (2015) also identified transformation of thiamethoxam to clothianidin as a potential factor.

Sampling sites in the PCA were generally grouped according to geography, and correspondingly, land use (Fig. 2). One grouping contained sites in the Niagara Peninsula (Prudhomme Creek, Two Mile Creek, Four Mile Creek, Fig. 2) and the urban control site (Indian Creek), while the other contained the sites in southwestern and southcentral Ontario. Twenty Mile Creek was also in the latter grouping as a result of this watershed representing primarily row crop agricultural activities, in contrast to the other sites in this area that exhibit a diversity of agricultural activities that include orchards and vineyards (Table S2). Interestingly, the Lebo Drain and Sturgeon Creek sites are significantly separated in the PCA; these two watercourses are adjacent to each other and routinely monitored for water quality. For both Lebo Drain and Sturgeon Creek, intensive horticultural activities, not exclusively limited to greenhouses, have contributed to elevated nutrient levels at the mouths of both watercourses (OMOE, 2012). The relatively greater influence of greenhouse activity in the Sturgeon Creek watershed (roughly 3-fold greater on a percentage basis, Table S2), and differences in maximum and mean neonicotinoid concentrations due to differences in the physical characteristics of these watersheds, is apparent.

3.3. Seasonal trends in occurrence and distribution of neonicotinoid insecticides in southern Ontario

Multi-year monitoring of neonicotinoid insecticides during field season (April to December) allows for assessment of seasonal trends in occurrence and distribution. In general, we observed two types of distributions dependent on insecticide and/or crop type. We selected Four Mile Creek and Prudhomme Creek, and Lebo Drain and Sturgeon Creek as representative examples of these different seasonal distributions. In all cases, we observed high rates of detection and high concentrations of neonicotinoid insecticides in spring in concert with snow melt, spring rains and subsequent crop planting; this “spring flush” phenomenon has been observed in other studies (e.g., Hladik et al., 2014).

In the case of Four Mile Creek and Prudhomme Creek, there was no apparent seasonal trend in the occurrence of imidacloprid, with high rates of detection throughout the spring/summer/fall time periods (Fig. 3). We attribute the lack of a seasonal trend at the Niagara sites to a wide range of agricultural activities potentially requiring multiple applications across a broader period of time and throughout the growing season. For example, imidacloprid can be used for preventative purposes in mid-summer; in addition, this compound is applied using a variety of techniques including soil treatment and foliar spray application that in turn increases the number of potential vectors for entry into watercourses. A similar temporal distribution was observed at the Four Mile Creek and Prudhomme Creek sites for acetamiprid (data not shown). In contrast, the occurrence of imidacloprid at Sturgeon Creek and Lebo Drain exhibited a bimodal distribution with maxima in late

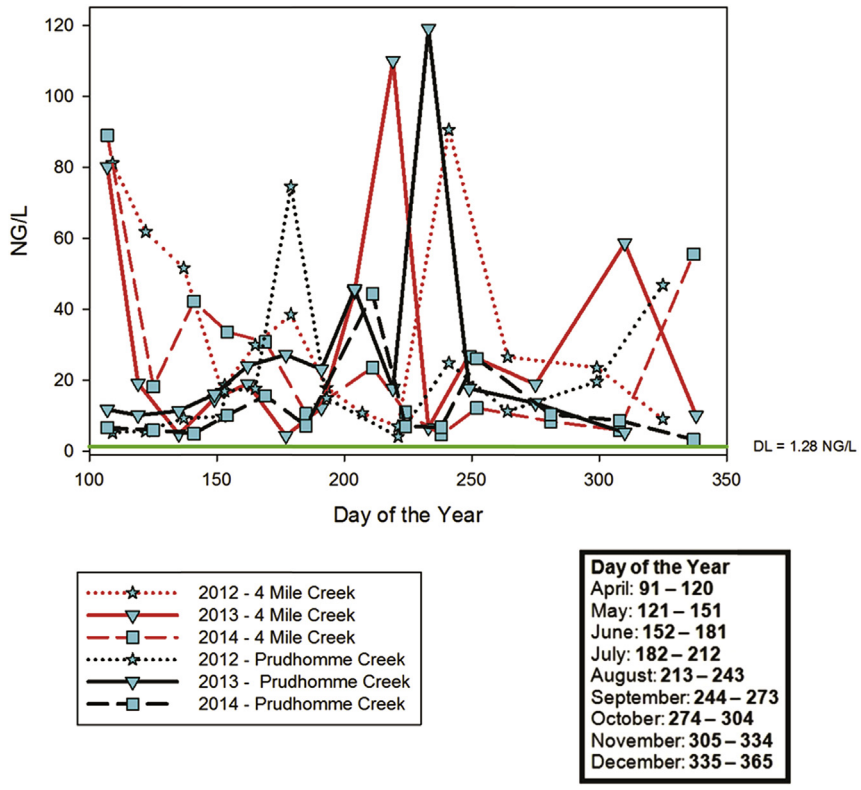

Fig. 3. Occurrence of imidacloprid at Four Mile Creek and Prudhomme Creek in the Niagara Region of southern Ontario resulting from sampling conducted from April to December 2012–2014.

spring and late summer/early fall (Fig. S7). We attribute this observation to greenhouse and/or vegetable applications; these watercourses have the highest level of greenhouse activity of all watersheds surveyed (Table S2, Fig. 2). Typical application periods for insecticides on row crops include spring and fall; however, it has also been reported that imidacloprid, clothianidin and thiamethoxam can be detected at significant concentrations (>100 ng/L) five-to-six months after use as seed treatments (Mineau and Palmer, 2013). Therefore, the bimodal distributions we observed at some sites could be the result of seasonal application, release of residues several months after application, or a combination of both circumstances.

Seasonal patterns for detection of neonicotinoid insecticides applied primarily for protection of row crops were more definitive; the occurrence of thiamethoxam at Four Mile Creek and Prudhomme Creek in the Niagara Region is shown in Fig. S8. The distribution of occurrence was bimodal and exhibited maximum concentrations in the late spring and fall time periods. We observed similar distributions for both thiamethoxam and clothianidin at other sampling sites, including Lebo Drain and Sturgeon Creek (data not shown). However, in the case of clothianidin at Four Mile Creek and Prudhomme Creek, we observed a unimodal distribution corresponding to maximum concentrations primarily in the spring (Fig. S9). We are unsure of the reasons for the lack of detections of clothianidin at the Niagara Region sites later in the year, compared to other areas of southern Ontario, but presume that preventative and/or curative applications in summer and fall were generally not required using this compound in these areas over the period of the study. Over the course of the three-year sampling program, we observed a broader time period when soybeans were being planted (e.g., into late-June), compared to corn which is not planted after May in Ontario.

3.4. Correlation of precipitation with occurrence and distribution of neonicotinoid insecticides in southern Ontario

The high water solubility of some neonicotinoid insecticides, including imidacloprid, was a primary impetus for investigating the relationship between precipitation and occurrence, as runoff is an important vector for entry of neonicotinoid insecticides into the aquatic environment. In their national-scale reconnaissance of neonicotinoids in the USA, Hladik and Kolpin (2015) identified precipitation as an important driver of neonicotinoid runoff to watercourses. Sampling in conjunction with rain events has been identified as a crucial factor in interpreting both peak and mean concentrations of neonicotinoids in surface waters, and the associated exposure of aquatic species (Morrissey et al., 2015). The occurrence and fate in surface waters are influenced by light, pH, temperature, formulation and microbial processes (Anderson et al., 2015). Three watercourses (Sydenham River, Four Mile Creek, Twenty Mile Creek, Fig. S1) were selected for assessment of the relationship among occurrence and rainfall events and water flow(stream discharge); supplemental monitoring of these sites resulted in availability of precipitation data of sufficient frequency and resolution to enable statistical analysis. However, it should be noted that precipitation measurements were not conducted at the exact location of surface water sampling.

A statistical analysis of the correlations among neonicotinoid insecticides and stream discharge, precipitation on the day preceding sampling, and stream discharge on the day of sampling was performed (Table 1). In addition, the correlations between the individual compounds were calculated (Table S3). There were no correlations between neonicotinoids and precipitation on the day of sampling; all significant correlations were associated with stream discharge and/or precipitation on the day preceding

Table 1
Non-parametric correlation coefficients (Kendall's τ) of concentrations of selected neonicotinoid insecticides with precipitation on day of sampling, precipitation on the day preceding sampling, and stream discharge on day of sampling measured at three locations (2012–14). Correlation coefficients in *italics* designate p-values <0.10 while **bold** designates p-values <0.05.

	Stream discharge (m^3/s)	Precipitation - sampling day (mm)	Precipitation - day preceding sampling (mm)
Imidacloprid			
Sydenham River	−0.085	−0.045	0.177
Four Mile Creek	0.136	−0.048	*0.197*
Twenty Mile Creek	**0.346**	0.080	0.149
Clothianidin			
Sydenham River	**0.345**	−0.003	0.002
Four Mile Creek	0.105	0.120	*0.217*
Twenty Mile Creek	**0.255**	**0.251**	0.143
Thiamethoxam			
Sydenham River	0.022	−0.063	0.042
Four Mile Creek	**0.219**	0.077	**0.341**
Twenty Mile Creek	**0.264**	**0.280**	0.195
Acetamiprid			
Sydenham River	−0.025	0.145	0.133
Four Mile Creek	0.150	0.121	0.167
Twenty Mile Creek	0.150	0.045	0.220
Thiacloprid			
Sydenham River	0.168	−0.201	0.083
Four Mile Creek	0.096	0.086	0.167
Twenty Mile Creek	0.079	−0.108	**0.337**

sampling (Table S3). In terms of correlations between individual neonicotinoids, thiamethoxam and clothianidin were correlated in all three watercourses due to similarities in crop types to which they are applied (Table S3).

For the Sydenham River, two significant positive correlations were observed; precipitation on the day preceding sampling for imidacloprid ($p < 0.10$) and stream discharge for clothianidin ($p < 0.10$). As shown by the hydrograph for the Sydenham River (Fig. S10), we anticipated difficulty in attributing occurrence of neonicotinoids with precipitation events due to the fact this watercourse is a major tributary characterized by high flow volumes and discharge; as a result any runoff from the watershed could be rapidly diluted. For the Sydenham River, there was no correlation between precipitation and stream discharge.

In the case of Four Mile Creek, precipitation on the day of sampling and stream discharge were significantly correlated; as a result precipitation has a significant impact on stream discharge throughout the year (Fig. S11). Clothianidin was positively correlated ($p < 0.10$) with precipitation the day preceding sampling while thiamethoxam was more strongly correlated ($p < 0.05$) for both precipitation the day preceding sampling and stream discharge, indicating that precipitation events and the associated runoff are significant contributors of loadings of neonicotinoids primarily associated with row crop agriculture to watercourses (Table 1). For Twenty Mile Creek, precipitation preceding the day of sampling and stream discharge were significantly correlated (Fig. 4). Compared to Four Mile Creek, Twenty Mile Creek exhibited high stream discharges in the late winter – early spring time period. Imidacloprid ($p < 0.05$) and clothianidin ($p < 0.10$) were both correlated with stream discharge for this watercourse, while thiamethoxam ($p < 0.05$) was correlated with precipitation the day preceding sampling (Table 1). The observations of neonicotinoid occurrence in

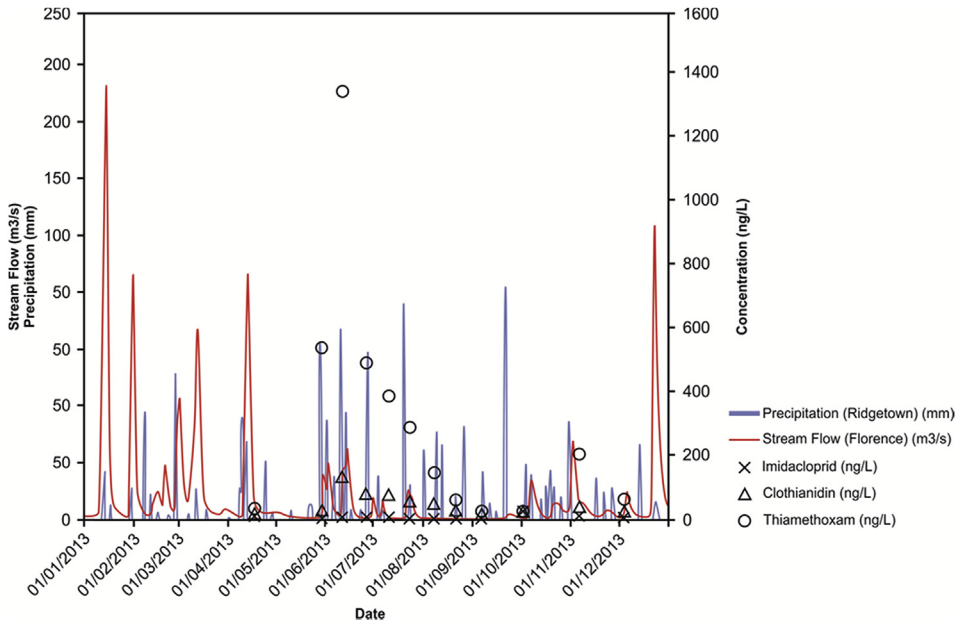

Fig. 4. Hydrograph showing stream flow (m^3/s), precipitation (mm) and neonicotinoid insecticide concentrations (ng/L) for sampling in 2013 in Twenty Mile Creek.

the Niagara Region show that in cases where precipitation and stream discharge are correlated, sampling associated with precipitation events is important for assessing the influence of agricultural activities on smaller watercourses in that runoff can exhibit relatively high concentrations and represent significant loadings, compared to base flow conditions. In the case of thiamethoxam in Twenty Mile Creek, the hydrograph also showed the influence of precipitation events in the late spring and summer months that can result in release of neonicotinoids to watercourses (Fig. 4).

Neonicotinoids were rarely detected in precipitation at Bear Creek in 2013; most detections were during the period of 14–31 May 2013. While detections of imidacloprid, thiacloprid and acetamiprid were within a factor of two of the method detection limit, concentrations in precipitation of thiamethoxam and clothianidin on May 14th, 2013 were 114 ng/L and 120 ng/L, respectively. We speculate these detections may have been the result of drift of dust generated during application on row crops, or planting of treated seeds during the spring planting period, given that the Bear Creek site is in proximity to the Lebo Drain and Sturgeon Creek stations, both of which are characterized by greater than 60% row crop agriculture.

4. Conclusions

The most widely used of the neonicotinoid insecticides; imidacloprid, thiamethoxam and clothianidin, were detected in over 90% of samples from over half of the sites surveyed during the three years of the study (2012–2014). Based on usage information for the Province of Ontario, the ubiquity of these compounds was not unexpected, and our data corroborate findings of studies conducted elsewhere in Canada and the United States. In the case of imidacloprid, the broad range of registered uses combined with multiple methods of application resulted in a high frequency of detections in surface waters across southern Ontario; however, there was a roughly 6000-fold range in concentrations detected. In addition to high frequencies of detection of neonicotinoids in spring samples, at some sites in the Niagara Peninsula area of southern Ontario imidacloprid was detected over the breadth of the spring – summer – fall timeframe, which indicated multiple applications during the planting and growing seasons. In one area of southwestern Ontario, three quarters of the samples exceeded the Canadian guideline value for imidacloprid (230 ng/L) indicating this compound is environmentally relevant and should continue to be the focus of further research and monitoring activities.

As expected, the occurrence and distribution of thiamethoxam and clothianidin were also correlated with row crop agriculture resulting from their wide use as seed treatments for canola, corn, grains and soybean. Seasonal patterns of detection for these two compounds were bimodal in nature, with maximum concentrations observed in late spring and fall. Recent information from the Province of Ontario indicates continued increases in acreage devoted to row crop agriculture which could significantly influence use of neonicotinoids. The results of the current study in southern Ontario also emphasize the importance of greenhouse activity in influencing the occurrence and distribution of neonicotinoids in surface waters. In terms of the impact of precipitation and watercourse characteristics on the occurrence of neonicotinoid insecticides, the results of our study were more definitive in cases where precipitation and discharge were linked, i.e., for smaller watercourses; in these cases precipitation events and subsequent runoff and increased discharge resulted in higher concentrations and loadings.

The results of our study emphasize the need for targeted event-based sampling to determine maximum concentrations and their duration in surface waters, and the requirement for ecotoxicological studies to investigate potential acute and chronic effects on a range of aquatic biota.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.11.036.

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Hazard identification of imidacloprid to aquatic environment

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ABSTRACT

The use of a very effective insecticide against sucking pests, neonicotinoid imidacloprid, has been increasing extensively. For this reason elevated concentrations are expected in aquatic environment. Despite this fact, there is still a lack of data available on its possible risk for the environment. In this study, the potential hazards of imidacloprid and its commercial product Confidor SL 200 to aquatic environment were identified by the acute and chronic toxicity assessment using bacteria *Vibrio fischeri*, algae *Desmodesmus subspicatus*, crustacean *Daphnia magna*, fish *Danio rerio* and the ready biodegradability determination. We found out, that imidacloprid was not highly toxic to tested organisms in comparison to some other environmental pollutants tested in the same experimental set-up. Among the organisms tested, water flea *D. magna* proved to be the most sensitive species after a short-term (48 h EC50 = 56.6 mg L^{-1}) and long-term exposure (21 d NOEC = 1.25 mg L^{-1}). On the contrary, the intensified toxicity of Confidor SL 200 in comparison to analytical grade imidacloprid was observed in the case of algae and slight increase of its toxicity was detected testing daphnids and fish. The activities of cholinesterase, catalase and glutathione S-transferase of daphnids were not early biomarkers of exposure to imidacloprid and its commercial product. Imidacloprid was found persistent in water samples and not readily biodegradable in aquatic environment. Due to increased future predicted use of commercial products containing imidacloprid and the findings of this work, we recommend additional toxicity and biodegradability studies of other commercial products with imidacloprid as an active constituent.

1. Introduction

Worldwide production and application of pesticides have increased progressively during the last two decades. It is important to know that only a small portion of applied pesticide in the field reaches the final biological target. A great part of applied pesticide is released into the environment, where it can provoke problems, such as toxicity to non-target organisms and accumulation. Polluted soil, surface and ground waters involve risk to the environment and also to human health due to possible direct or indirect exposures. For this reason there is a need to monitor and assess possible adverse effects of applied pesticides on ecosystems (Tomlin, 1997; Wamhoff and Schneider, 1999; Nemeth-Konda et al., 2002).

Imidacloprid [1-(6-chloro-3-pyridylmethyl)-*N*-nitro-imidazolidin-2-ylideneamine], a new promising insecticide, has been commercially introduced to the market in 1991 by Bayer AG and Nihon Tokushu Noyaku Seizo KK and has been increasingly used ever since. It is a worldwide used insecticide, used mainly to control sucking insects on crops, (e.g. aphids, leafhoppers, thrips, whiteflies, termites) (Tomlin, 1997; Tomizawa and Casida, 2005) and parasites (e.g. fleas) of dogs and cats (Dryden et al., 2000). It is a systemic insecticide used for seed treatment, soil and foliar applications. Imidacloprid belongs to the group of nicotine-related insecticides referred to as neonicotinoids, which act as agonists of the postsynaptic nicotinic acetylcholine receptors (nAChRs) (Matsuda et al., 2001) resulting in the impairment of normal nerve function. It is now considered a possible replacement for the insecticides, which are in the process of phased revocation (US EPA, 2004).

Data on the environmental fate of imidacloprid are rather inconsistent. Some authors consider imidacloprid as relatively immobile in soil and do not expect its leaching to groundwater (Mullins, 1993; Tomlin, 1997; Krohn and Hellpointner, 2002), while some studies indicate the opposite (Felsot et al., 1998; Gonzales-Pradas et al., 1999; Armbrust and Peeler, 2002; Gupta et al., 2002). Literature data reported that in aqueous samples imidacloprid is quite stable to hydrolysis at environmentally relevant pH values (Yoshida, 1989) but it undergoes photolytic degradation rapidly (Hellpointner, 1989; Krohn and Hellpointner, 2002).

Although imidacloprid is not intended for use in water, it may pass into water bodies by spray drift or by run-off after application. In comparison to other widely used insecticides, only few toxicity studies have been performed on the effects of imidacloprid on aquatic organisms despite its increasing use (Jemec et al., 2007).

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It is therefore important to assess the concentrations at which these chemicals are toxic to aquatic organisms. There is also a lack of data on the environmental fate of imidacloprid in the aquatic ecosystems, e.g. biodegradation, bioaccumulation. Furthermore, no attention was paid to the effects of commercial formulations of imidacloprid, e.g. Confidor SL 200, Gaucho, Admire, Provado, which usually contain other toxic ingredients, such as solvents. Namely, possible interactions between the pesticide and solvents could alter the toxicity of commercial preparation.

The aim of the study was to identify the potential hazard of imidacloprid and its commercial formulation Confidor SL 200 to aquatic environment by the assessment of their toxicity using a battery of test organisms, stability and ready biodegradability. We also assessed whether the toxicity of Confidor SL 200 is mainly on the account of solvent mixture or active ingredient present in this commercial formulation. A base set of test species from different taxonomic groups, which are most frequently used for toxicity identification of chemicals and biocides, was selected. These include: bacteria *Vibrio fischeri*, algae *Desmodesmus subspicatus,* crustacean water flea *Daphnia magna* and fish *Danio rerio*. In the case of daphnids, sublethal changes, such as the activities of enzymes: cholinesterase (ChE; involved in nerve signal transmission); catalase (CAT; enables the degradation of hydrogen peroxide formed during oxidative stress) and glutathione S-transferase (GST; involved in the biotransformation of xenobiotics) were also evaluated.

2. Materials and methods

2.1. Chemicals

Imidacloprid and Confidor SL 200 were provided by Bayer CropScience AG, Monheim, Germany. A standard stock solution of imidacloprid was prepared in distilled water with no addition of solvents. A commercially available product Confidor SL 200 contains 200 g L^{-1} of active ingredient and some solvents, such as dimethylsulfoxide (38.4%; v/v) and 1-methyl-2-pyrrolidone (37.5%; v/v). Dibasic and monobasic potassium phosphate, 1-chloro-2,4-dinitrobenzene, L-glutathione (reduced form), 5,5′ dithiobis-2-nitrobenzoic acid, sodium hydrogen carbonate, acetylthiocholine chloride, sodium sulphate and ethylenediaminetetraacetic acid were obtained from Sigma (Germany) and HPLC grade acetonitrile from J.T. Baker. BCA Protein Assay Reagent A and BCA Protein Assay Reagent B were purchased from Pierce (USA). All chemicals were of the highest commercially available grade, typically 99% or higher.

2.2. Stability of imidacloprid in distilled water and stream water

To ensure reliable toxicity data, we checked the stability of imidacloprid in distilled and stream water under the same conditions and concentrations as in the toxicity tests (controlled room temperature 21 ± 1 °C, room light illumination). For the purposes of storage, we also checked if the solution of imidacloprid in distilled water is stable in the dark at fridge temperature 3 ± 2 °C.

Imidacloprid solutions were prepared in distilled water in the following concentrations: 0; 8.75; 17.5; 35; 70; 105 and 140 mg L^{-1}. Each solution was aliquoted in five flasks (100 mL), two of them were kept in the dark at fridge temperature (3 ± 2 °C) and the rest three on light at controlled room temperature (21 ± 1 °C). The solid phase extraction (SPE) of imidacloprid from distilled water solutions was performed immediately after the experiment set up (0 d), and 1, 2, 3, 7, 10, 14, 17 and 22 d from the experiment outset. The SPE extraction with methanol used for the stability studies yielded the extraction recoveries of (95 ± 10) % for imidacloprid. For quantification purposes a calibration curve in the concentration range from 5 ppm to 150 mg L^{-1} was prepared. The *r*-square values for regression line was $r^2 = 0.998$. All determinations were performed in six (for the calibration curve and the experiments in the sunlight) and four (for the experiments in the dark) with relative errors of 5–15%.

Imidacloprid solutions were prepared also in local stream water (pH 8.4, total hardness 140 mg CaO/L, alkalinity 131 mg CaO/L), which was used for fish acute toxicity tests. The stability of 215, 230, 245, 260 and 280 mg L^{-1} of imidacloprid was checked right after the experiment set up (0 d) and at the end of it (after 4 d). Imidacloprid water samples (1 mL) were taken in duplicates.

2.2.1. Sample preparation and HPLC-DAD analysis

Imidacloprid extraction was performed on Strata C18-E columns (100 mg) according to Baskaran et al. (1997). The columns were initially preconditioned with 5 mL of methanol followed by 5 mL of distilled water. Imidacloprid water sample (1 mL) was loaded on the column and the retained imidacloprid was eluted with 2 mL of methanol. In the next step methanol was removed by rotary (Büchi–Rotavapor R-124, Flawil, Switzerland) evaporation in vacuum (T = 30 °C) (Büchi–Waterbath B-480; Germany, Flawil, Switzerland) and dried leftover was rediluted in 1 mL of acetonitrile–water (20:80 v/v) solution (HPLC solvent mixture). Prepared samples were stored at 4 °C until subjected to HP 1000 Series liquid chromatograph (HPLC) equipped with diode array detection (DAD) as described previously (Baskaran et al., 1997). All HPLC-DAD analyses were performed in duplicates on Zorbax C8 (4.6 × 250 mm, 5 μm particle size) column at 25 °C using an isocratic separation with mobile phase of acetonitrile–water (20:80 v/v) at a flow rate 1.25 mL min^{-1}. The stability of imidacloprid was followed from the imidacloprid peak areas at 270 nm, which was identified on the basis of retention time comparison with authentic standard.

2.3. Toxicity tests

At least one preliminary and two definitive trials for each test species were conducted. In each definitive toxicity experiment five concentrations and a control in two replicates were tested. In the case of Confidor SL 200, the solvents listed on the data sheet provided by the supplier (38.4%; dimethylsulphoxide, and 37.5%; v/v 1-methyl-2-pyrrolidone) (further referred to as solvent mixture) at the concentrations used in each toxicity test were tested to investigate the possible toxic effects of the solvents.

2.3.1. Toxicity to bacteria

Luminescence of *V. fischeri* NRRL-B-11,177 was measured using a LUMIStox 300 luminometer (Dr. Lange GmbH, Düsseldorf, Germany). Reactivated liquid-dried bacteria were exposed to 0.78; 1.56; 3.13; 6.25; 12.5; 25; 50; and 100 mg L^{-1} of imidacloprid; 0.016%; 0.031%; 0.063%; 0.13%; 0.25%; and 0.5% (v/v) of Confidor SL 200, and 0.0313%; 0.0625%; 0.125%; 0.25% and 0.5% (v/v) of solvent mixture for 30 min at 15 ± 0.2 °C on a temperature-controlled block (ISO 11348-2, 1998). The percentage of luminescence inhibition was calculated for each concentration relative to the control.

2.3.2. Toxicity to algae

The green, unicellular algae *D. subspicatus* Chodat 1926 (CCAP 276/22; Culture Collection of Algae and Protozoa, Cumbria, United Kingdom) were cultured according to Jaworski (Thompson et al., 1988) on an orbital shaker at 150 rpm (alternately 15 min agitation and resting) at a constant room temperature of 21 ± 1 °C, and fluorescent illumination (4000 lux). In the toxicity tests, the flasks were agitated permanently at 150 rpm and 7000 lux. The algal density and growth rate were determined after 72 h by counting the algal cells in a Bürker counting cell. The tested concentrations

of imidacloprid were 100; 144; 207; 299; and 430 mg L^{-1} and 0.001%; 0.005%; 0.01%; 0.05%; and 0.1% (v/v) of Confidor SL 200, and 0.001%; 0.005%; 0.01%; 0.05% and 0.1% (v/v) of solvent mixture. The inhibition of specific growth rates for each concentration was calculated in comparison to the control (ISO 8692, 2004).

2.3.3. Toxicity to daphnids

Water fleas *D. magna* Straus 1820 were obtained from the Institut für Wasser, Boden und Lufthygiene, des Umweltbundesamtes (Berlin). They were cultured in 2.5 L of modified M4 media (Kühn et al., 1984) at 21 ± 1 °C and 16:8 h light/dark regime (1800 lux) with a diet of the algae *D. subspicatus* Chodat 1926 corresponding to 0.13 mg carbon/daphnia per day.

2.3.3.1. Acute toxicity to daphnids. In the acute toxicity tests, neonates less than 24 h old, derived from the second to fifth brood, were exposed to 10, 40, 70, 100, 130 mg L^{-1} of imidacloprid and 0.0025%; 0.005%; 0.01%; 0.02%; and 0.04% (v/v) of Confidor SL 200, and 0.05%; 0.1%; 0.25%; 0.5% and 1% (v/v) of solvent mixture. After a 24 h and 48 h exposure period the immobile daphnids were counted (ISO 6341, 1996). On the basis of the 48-h EC_{10} and EC_{50} values determined in these range finding tests, the concentrations for further toxicity tests followed by enzyme analyses were selected.

2.3.3.2. Sublethal effects on daphnids after acute exposure. After the acute (48 h) exposure of water fleas sublethal effect of imidacloprid and Confidor SL 200 were studied by measuring their effects on the activities of ChE, GST and CAT. Namely, five test containers containing 20 daphnids/50 mL of test solution were prepared for each concentration of imidacloprid (10, 20, 30 and 40 mg L^{-1}). After a 48-h exposure period, the immobile daphnids were counted, removed, and all mobile animals (70–100) were combined into one sample. Each acute toxicity test was repeated three times.

The animals were homogenized for 3 min in 0.7 mL of homogenization buffer (50 mM phosphate buffer pH 7.0), using a glass–glass Elvehjem–Potter homogenizer. The excess imidacloprid was removed from the homogenizer and the surface of the animals by rinsing three times with 2 mL of the homogenization buffer combined with 5 mM EDTA. The homogenate was centrifuged for 15 min at 15000*g* and 4 °C (Jemec et al., 2007).

ChE activity was determined according to Ellman et al. (1961), and Jemec et al. (2007) using microtiter plates (Bio-Tek® Instruments, USA; PowerWave™ XS). The reaction mixture was prepared in 100 mM potassium phosphate buffer pH 7.3 containing acetylthiocholine chloride and 5,5′ dithiobis-2-nitrobenzoic acid in the final concentrations of 1 mM and 0.5 mM, respectively. Protein supernatant (100 μL) was added to start the reaction, which was followed spectrophotometrically at 412 nm and 25 °C for 15 min.

GST activity was determined using the method described by Habig et al. (1974) and Jemec et al. (2007), using microtiter plates (Bio-Tek® Instruments, USA; PowerWave™ XS) and 1-chloro-2,4-dinitrobenzene as a substrate. The final reaction mixture contained 1 mM of 1-chloro-2,4-dinitrobenzene and 1 mM of reduced glutathione. 50 μL of protein supernatant were added to start the reaction. The reaction was followed spectrophotometrically at 340 nm and 25 °C for 3 min.

CAT activity was determined according to Aebi (1984). We added 50 μL of protein supernatant to 750 μL of H_2O_2 solution (10.8 mM) prepared in 50 mM potassium phosphate buffer pH 7.0. The reaction was followed spectrophotometrically at 240 nm and 25 °C for 5 min on a Shimadzu UV-2101PC spectrophotometer (Japan). The concentrations of substrates used for all enzymes were saturating and ensured the linear changes of absorbance with time and the concentration of proteins.

One enzyme unit (EU) was determined as the amount of ChE that hydrolyses 1 nmole of acetylthiocholine/min (ε_{412} = 13,600 M^{-1} cm^{-1}), the amount of CAT that degrades 100 μmoles of hydrogen peroxide/min (ε_{240} = 43.6 M^{-1} cm^{-1}), and the amount of GST that conjugates 100 nmoles of reduced glutathione/min (ε_{340} = 9600 M^{-1} cm^{-1}). These enzyme units were chosen to facilitate comparison of all enzyme activities for each chemical.

Protein concentration was determined using a BCA™ Protein Assay Kit, a modification of the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

2.3.4. Toxicity to fish

2.3.4.1. Zebrafish survival. Specimens of zebrafish *Danio rerio* Hamilton Buchanan, obtained from a commercial supplier, were initially acclimated to the test conditions in water obtained from an unpolluted stream (pH 8.4, total hardness 140 mg CaO/L, alkalinity 131 mg CaO/L) 7 d prior to the experiment. They were fed daily with commercial fish food and illuminated with fluorescent bulbs for 12 h per day.

During the toxicity tests, the animals were placed in 2.5 L of slightly aerated test solution at 21 ± 1 °C (ISO 7346-1, 1996). Dead fish were counted and removed from the tanks daily during a 96 h exposure period. The concentration of oxygen in the test solutions was measured at the beginning and end of the experiment using an oxygen electrode (WTW Oximeter, OXI 96). The percentage of mortality for each tested concentration of Confidor SL 200 (0.075%; 0.1%; 0.11%; and 0.13%; v/v) and 200; 215; 260; 280; and 300 mg L^{-1} of imidacloprid, and 0.075%; 0.1%; 0.11%; and 0.13% (v/v) of solvent mixture was calculated after 24, 48, 72, and 96 h of exposure.

2.3.4.2. Zebrafish embryo test. A detailed description of zebrafish breeding to obtain eggs was published by Kammann et al. (2004). Briefly, adult zebrafish were bred in a temperature-controlled room in aquarium (60 × 30 × 30 cm) containing 45 L of tap water with constant temperature (26 °C) and photoperiod (12 h light:12 h dark). Filtration was provided by internal bioactive filter device. Fish were fed three times daily with commercially available dried fish food (Nutrafin, Tetramin). A day before breeding a plastic spawning box covered with stainless steel mesh was placed in the breeding tank. On the following day, one hour after the light cycle started, the spawning plastic box was removed from the tank and eggs were collected and rinsed with synthetic medium prepared according to ISO 15088 (2007).

The toxicity test was performed according to the same ISO standard. Fertilized eggs in the four to eight cell stages were placed in 24-well plates; each well contained 1 mL of synthetic ISO medium with different concentrations of imidacloprid (10, 40, 60, 80, 160 and 320 mg L^{-1}); Confidor SL 200 (0.1%, 0.2%, 0.4%, 0.6% and 0.8%; v/v), and 0.3%; 0.4%; 0.5% and 0.6% (v/v) of solvent mixture. For each experiment a control containing only synthetic ISO medium was prepared. After 24 h and 48 h of exposure at 26 °C lethal malformations, i.e. egg coagulation, missing heartbeat, missing somites, missing tail detachment from the yolk sac, and non-lethal malformations, i.e. no eye and body pigmentation, missing blood flow, spine deformation, yolk sac edema, incomplete eye and ear development were observed. The percentages of each malformation were calculated for the exposed concentrations of imidacloprid and Confidor SL 200. The reference chemical 3,4-dichloroaniline (2, 2.5 and 3.7 mg L^{-1}) was used as a positive control. After 48 h of exposure 2 mg L^{-1} of 3,4-dichloroaniline caused the changes of the majority of endpoints in 10% of specimens, at 2.5 mg L^{-1} in 30% of specimens, while at 3.7 mg L^{-1} of the reference chemical, from 30% to 100% of the specimens were affected when different end-points were evaluated. Based on this, the tests fulfilled the validity criteria prescribed by the standard (ISO 15088,

2007), which states, that at least one effect at 3.7 mg L^{-1} of 3,4-dichloroaniline should be observed in more than 10% of specimens. We consider the later validity criteria very broad, and recommend that either concentration 2 mg L^{-1} or 2.5 mg L^{-1} be rather used as a reference concentration.

2.4. Biodegradability

Prior to imidacloprid biodegradation test, its toxicity to a mixed bacterial community was assessed. The activated sludge microorganisms (the final concentration was 150 mg L^{-1} of suspended solids) from the aeration tank of the municipal laboratory waste water treatment plant were exposed to increasing concentrations of imidacloprid according to ISO 8192 (1986). Oxygen consumption was measured with an oxygen electrode (WTW Oximeter, OXI 96) following biochemical degradation of meat extract, peptone, and urea every 30 min during 3 h. The inhibition of oxygen consumption rate compared to the control was determined for imidacloprid (100, 150, 200, 300 and 400 mg L^{-1}). Based on these preliminary results, the biodegrability of Confidor SL 200 was not tested due to extensive consumption of oxygen as a result of solvents degradation.

The aerobic biodegradability of imidacloprid was studied in a closed respirometer (Baromat, WTW, BSB-Messgerät, Model 1200). The same source of activated sludge was used as in a toxicity test with mixed bacterial community; concentration 30 mg L^{-1} of suspended solids was used. The oxygen consumption was measured during 28 d or until the plateau was reached (ISO 9408, 1991) in the samples containing 250 and 450 mg L^{-1} of imidacloprid.

2.5. Statistical analyses

2.5.1. Bacteria

The 30 min IC_{20}, IC_{50} with 95% confidence limits and IC_{80} values for luminescence bacteria were calculated using a linear regression analysis supported by computer software (Dr. Bruno Lange, 2000). The IC_{20} was considered a toxicity threshold. In a case of mixed bacterial community the percentages of inhibition of oxygen consumption were plotted against corresponding concentrations of imidacloprid on semi-logarithmic paper and the IC_{20}, IC_{50}, and IC_{80} values were determined using linear regression analysis. The IC_{20}, IC_{50}, IC_{80} stand for inhibition concentration that causes 20%, 50% and 80% inhibition of luminescence or oxygen consumption compared to the control.

2.5.2. Algae

The percentages of inhibition of specific growth rates were plotted against concentration on semi-logarithmic paper and the 72 h IC_{10}, IC_{50}, and IC_{90} values (inhibition concentrations that cause 10%, 50% and 90% inhibition of algal growth in comparison to the control, respectively) were estimated using linear regression analysis.

2.5.3. Daphnids and fish

The percentages of immobile daphnids, fish lethal and sublethal end-points were analysed with probit analysis to determine the effective (EC_{10}, EC_{50}, EC_{90}) and lethal (LC_{10}, LC_{50}, LC_{90}) concentrations that cause 10%, 50% and 90% of daphnids immobility, fish dead or sublethal effects, respectively. The 95% confidence limits are provided for the EC_{50} (LC_{50}) values (US EPA, 1994).

2.5.4. Enzyme analyses

The effects of the imidacloprid on enzymes were compared by Kruskal–Wallis analysis and non-parametric Mann–Whitney *U* test ($P < 0.05$), using Statgraphics software (Statgraphics Plus for Windows 4.0, Statistical Graphics Corporation). Homogeneity of variance was tested using Levene's test. The percentages given in the results represent the change in medians of ChE, GST and CAT activity in exposed animals compared to control.

2.5.5. Biodegradability

Biodegradation curves were plotted as the percentages of biodegradation for each sample of imidacloprid versus time. A final level of biodegradation, a lag phase and a degradation time were the parameters used for biodegradability assessment.

3. Results and discussion

3.1. Stability of imidacloprid in distilled and stream water

The results of HPLC-DAD measurements have shown the same levels of imidacloprid at any of the tested concentrations when stored in the dark at fridge temperature for 22 d (Fig. 1a).

The stability of imidacloprid solution stored at room light and 21 ± 1 °C depended on the concentration of imidacloprid. For example, the concentrations of imidacloprid up to 70 mg L^{-1} did not change during 22 d, while the highest tested concentrations 105 mg L^{-1} and 140 mg L^{-1} of imidacloprid in the same period decreased by 16% and 24% in comparison to their initial concentrations (Fig. 1b). This could be explained by the presence of sunlight. Slight variations of imidacloprid levels were noticed at higher concentrations (70, 105 and 140 mg L^{-1}) until day three. This variability is probably the result of an experimental error.

The concentrations of imidacloprid measured in the stream water from the fish toxicity tests at the beginning of the experiment were slightly lower (up to 5%) as initial values. Instead of 215, 230, 245, 260 and 280 mg L^{-1} of imidacloprid, the following levels were measured: 216, 216, 232, 250 and 270, respectively.

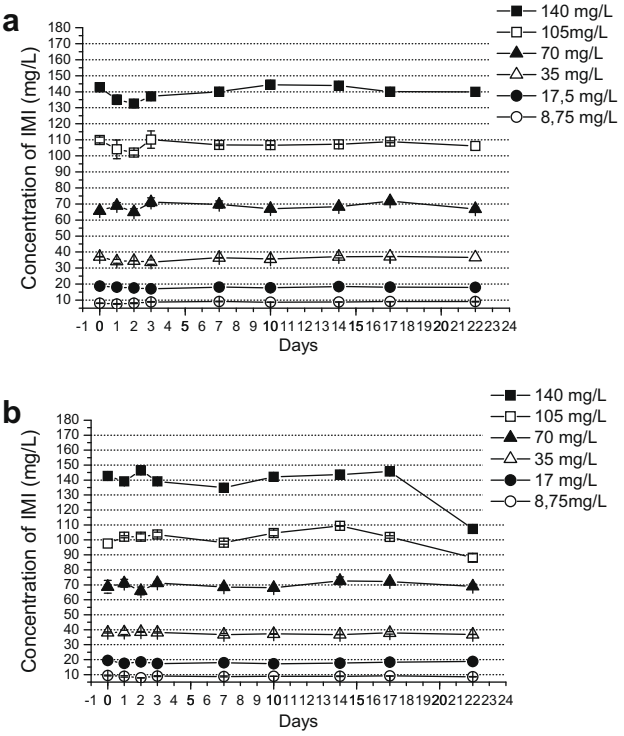

Fig. 1. The effect of storage conditions: (a) dark and fridge temperature (2–5 °C); (b) light and room temperature (21 ± 1 °C) on the stability of imidacloprid in distilled water (mean of six (Fig. 1a) and four replicates (Fig. 1b) ± standard error of mean).

The concentrations of imidacloprid were stable during the experiment (up to 4 d).

Different literature data are available on the stability of imidacloprid in aqueous medium. Similarly as in our study, Overmyer et al. (2005) reported that imidacloprid was stable during 48 h of toxicity tests using aquatic insects *Simulium vittatum* (20 °C, 16:8-h light:dark period). Several studies reported the stability of imidacloprid under simulated environmentally relevant conditions. Namely, Kagabu and Medej (1995) determined a short half live of imidacloprid (1–3 h) when exposed to simulated sunlight (250 W at 30 °C). On the contrary, Sarkar et al. (1999) reported longer half lives (31–43 d) of commercial preparation Confidor SL 200 depending on the temperature and pH.

3.2. Toxicity tests

The toxicity values for analytical grade imidacloprid, Confidor SL 200, the amount of imidacloprid in Confidor SL 200 and solvent mixture in this formulation are provided in Fig. 2 and Tables 1–3. We compare the toxicity of analytical grade imidacloprid to its commercial formulation Confidor SL 200 for each species and assess whether the toxicity of Confidor SL 200 is mainly on the account of solvent mixture or imidacloprid present in Confidor SL 200.

3.2.1. Acute toxicity to bacteria, daphnids and zebrafish

Analytical grade imidacloprid was similarly toxic to *V. fischeri* as imidacloprid formulated as Confidor SL 200. Also, the solvent mixture alone was significantly less toxic than Confidor SL 200. This indicates, that the toxicity of Confidor SL 200 to *V. fischeri* is mainly due to imidacloprid action, and not because of solvents (Fig. 2a, Table 1) There are no other reported data concerning the toxicity of imidacloprid to aquatic bacteria (SERA, 2005).

When imidacloprid was formulated as Confidor SL 200, it was more toxic to daphnids than analytical grade imidacloprid. Also, Confidor SL 200 was significantly more toxic than the solvents alone (48 h EC_{50} of Confidor SL 200 was 20 times lower). Namely, when the amount of solvent mixture, contained in the highest tested concentrations of Confidor SL 200 was tested, no toxicity to daphnids was observed. This implies, that the toxicity to daphnids cannot be attributed either to solvents or imidacloprid alone, but a combination of both increases the toxicity of this commercial formulation in comparison to analytical grade imidacloprid (Fig. 2b, Table 1).

The 48 h EC50 obtained for *D. magna* in our research was 56.6 mg L^{-1} of imidacloprid, which is in the range of the literature data reported: 48 h LC50 and the 48 h EC50 values obtained for *D. magna* were 17.36 mg L^{-1} (Song et al., 1997) and 85 mg L^{-1} (Young and Blakemore, 1990; SERA, 2005), respectively. Imidacloprid impairs the nerves function and consequently the normal mobility

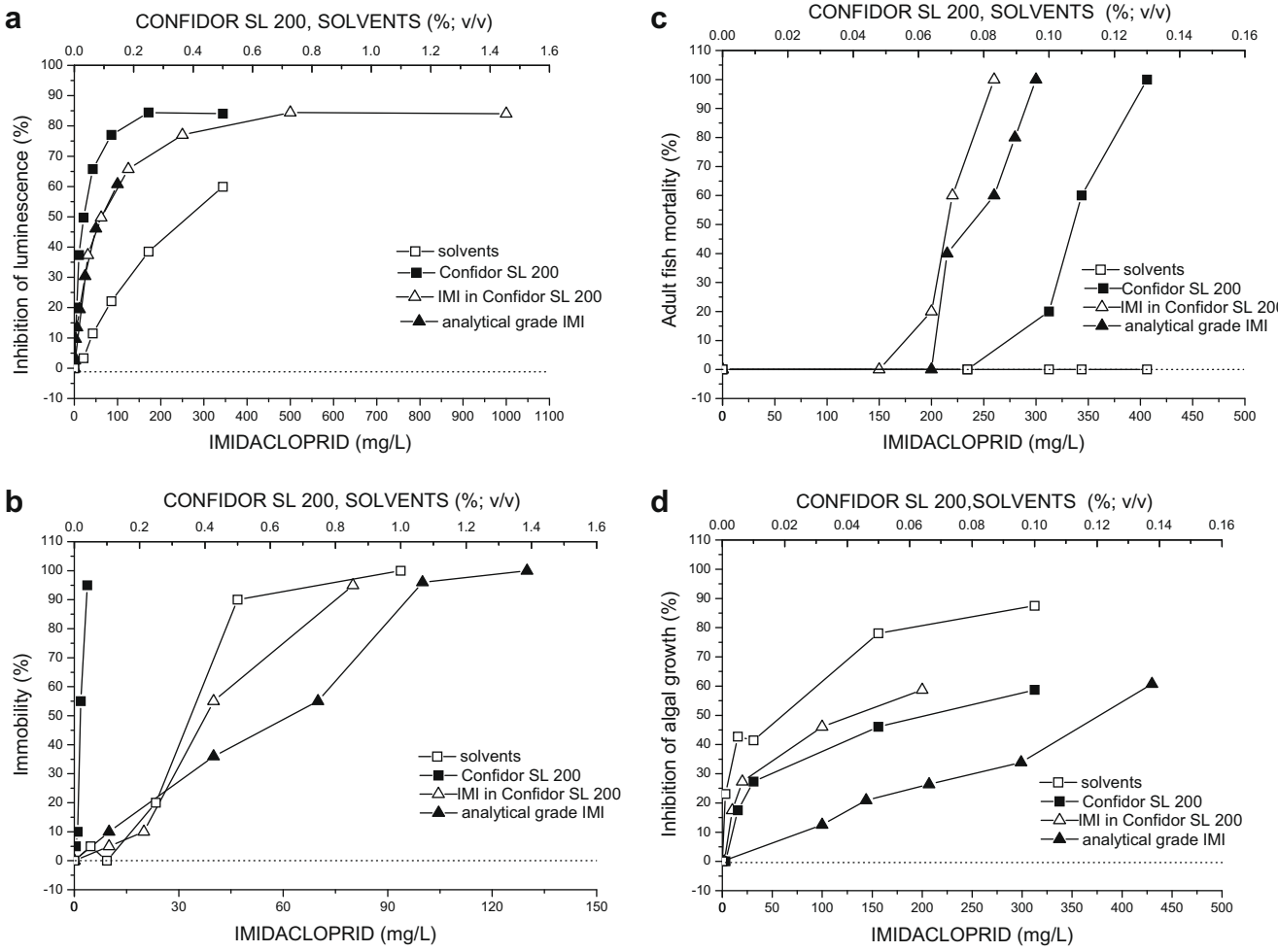

Fig. 2. Toxicity of imidacloprid and Confidor SL 200 to (a) *Vibrio fischeri*, (b) *Daphnia magna*, (c) adult *Danio rerio* and (d) algae *Desmodesmus subspicatus*. The lower *x*-axis (in mg L^{-1}) stands for analytical grade imidacloprid and the concentration of imidacloprid in Confidor SL 200. The upper *x*-axis (in %) stands for solvents and Confidor SL 200. The concentrations of imidacloprid in Confidor SL 200 applied on lower *x*-axis do not correspond to concentrations of Confidor SL 200 on upper *x*-axis.

Table 1
ECx/ICx/LCx values (effective, inhibition and lethal concentrations) of imidacloprid and Confidor SL 200 to *Daphnia magna*, *Vibrio fischeri* and adult *Danio rerio*.

Species	IMI[a] (mg L^{-1})		Confidor SL 200 (%; v/v)		IMI[b] (mg L^{-1})	
D. magna	24 h	48 h	24 h	48 h	24 h	48 h
EC_{10}	36.8	22.5	0.011	0.008	22	12
EC_{50}	97.9	56.6	0.019	0.018	38	30
(95% CL)	(81.4–127.7)	(34.4–77.2)	(0.016–0.024)	(0.014–0.022)	(32–48)	(28–44)
EC_{90}	260	142	0.035	0.038	70	70
V. fischeri	30 min		30 min		30 min	
IC_{20}	11.9		0.0056		11.2	
IC_{50}	61.9		0.028		56	
(95% CL)	(61.9–62.0)		(0.015–0.041)		(30–82)	
IC_{80}	320		0.140		280	
D. rerio	96 h		96 h		96 h	
LC_{10}	201		0.097		194	
LC_{50}	241		0.107		214	
(95% CL)	(224–257)		(0.101–0.115)		(202–230)	
LC_{90}	290		0.118		236	

[a] Analytical grade imidacloprid.
[b] Concentration of IMI in corresponding %, v/v Confidor SL 200 solution, CL – corresponding 95% confidence limits.

Table 2
LCx/ECx (lethal and effective concentrations) of imidacloprid, Confidor SL 200 and solvent mixture used in Confidor SL 200 based on the development of zebrafish embryos after 48 h.

	Danio rerio – development of embryos (48 h) Confidor SL 200 (%; v/v)								
	Egg coagulation[b]			Missing heartbeat[b]			Missing tail detachment[b]		
	Confidor SL 200 (%; v/v)	IMI[a] (mg L^{-1})	Solvents[d] (%; v/v)	Confidor SL 200 (%; v/v)	IMI[a] (mg L^{-1})	Solvents (%; v/v)	Confidor SL 200 (%; v/v)	IMI[a] (mg L^{-1})	Solvents (%; v/v)
LC_{10}	0.442	884	0.228	0.150	300	0.237	0.406	812	0.254
LC_{50}	0.580	1160	0.452	0.251	502	0.350	0.575	1150	0.400
(95% CL)	(0.500–0.658)	(1000–1316)	(0.314–0.758)	(0.194–0.315)	(388–630)	(0.261–0.404)	(0.486–0.668)	(972–1336)	(0.311–0.472)
LC_{90}	0.762	1524	0.896	0.418	836	0.517	0.814	1628	0.631
	Missing somites[b]			Missing eye pigmentation[c]			Missing body pigmentation[c]		
LC/EC_{10}	0.172	344	0.287	0.174	348	0.196	0.160	320	0.166
LC/EC_{50}	0.413	826	0.445	0.366	732	0.419	0.313	626	0.368
(95% CL)	(0.307–0.553)	(614–1106)	(0.222–0.560)	(0.275–0.466)	(550–932)	(0.142–0.672)	(0.236–0.394)	(472–788)	(0–0.487)
LC/EC_{90}	0.993	1986	0.689	0.767	1534	0.894	0.613	1226	0.812
	Missing blood flow[c]			Incomplete eye development[c]			Incomplete ear development[c]		
EC_{10}	0.111	222	0.237	0.181	362	0.248	0.168	336	0.150
EC_{50}	0.204	408	0.350	0.380	760	0.423	0.313	626	0.284
(95% CL)	(0.154–0.262)	(308–524)	(0.261–0.404)	(0.287–0.485)	(574–970)	(0.320–0.523)	(0.238–0.391)	(476–782)	(0.009–0.363)
EC_{90}	0.373	746	0.517	0.799	1598	0.717	0.585	1170	0.537

[a] Concentration of IMI in corresponding % (v/v) of Confidor SL 200 solution.
[b] Lethal endpoints.
[c] Sublethal endpoints, CL – corresponding 95% confidence limits.
[d] Solvents refer to solvent mixture used in Confidor SL 200 solution.

Table 3
Chronic toxicity of imidacloprid and Confidor SL 200 to *Desmodesmus subspicatus* and *Daphnia magna* (Jemec et al., 2007).

Test species	IMI[b] (mg L^{-1})	Confidor SL 200 (%; v/v)	IMI[c] (mg L^{-1})
D. subspicatus			
72 h IC_{10}	106	2.8×10^{-3}	5.6
72 h IC_{50}	389	5.8×10^{-2}	116
72 h IC_{90}	1425	1.18	2351
Daphnia magna[a]			
21 d LOEC	2.50	2.5×10^{-3}	5.0
21 d NOEC	1.25	1.25×10^{-4}	2.5

[a] Jemec et al. (2007).
[b] Analytical grade imidacloprid.
[c] Concentration of IMI in corresponding % (v/v) of Confidor SL 200 solution.

of organisms, which is the most frequent observed endpoint of the acute toxicity test with water fleas. In comparison to some other pesticides, e.g. diazinon, imidacloprid is not highly toxic to daphnids (Jemec et al., 2007). On the contrary, some invertebrate species revealed high sensitivity to imidacloprid; the highest toxicity was observed for *Hyalella azteca* and *Chironomus tentans* with the corresponding 96 h LC50 values 0.526 mg L^{-1} and 0.0105 mg L^{-1}, respectively (SERA, 2005).

When imidacloprid was formulated as Confidor SL 200, it was slightly more toxic to adult zebrafish than analytical grade imidacloprid. When the amount of solvent mixture, contained in the highest tested concentrations of Confidor SL 200 (0.13%; v/v) was tested, no toxicity to adult fish was observed. Again, as in the case of daphnids, the combination of active ingredient imidacloprid and solvents increase the toxicity of commercial formulation (Fig. 2c, Table 1)

No toxicity of analytical grade imidacloprid to development of zebrafish embryos was observed even at 320 mg L^{-1}. However Confidor SL 200 revealed high toxicity to all observed endpoints; the most sensitive was found to be blood circulation and heartbeat comparing the obtained LC50/EC50 values. The toxic effects of

solvent mixture used in Confidor SL 200 on embryos were similar to Confidor SL 200 (Table 2). This indicates that probably the toxicity of this commercial preparation to zebrafish embryos is mainly on the account of solvents.

The survival of adult zebrafish exposed to Confidor SL 200 was more affected than the embryos development comparing the LC50/EC50 values (Tables 1 and 2). Literature review indicated that the sensitivity of adult and embryos of zebrafish depends on tested chemical and its mode of toxic action (Lange et al., 1995; Roex et al., 2002; Kammann et al., 2006). No previous data on the toxicity of imidacloprid to zebrafish are available. Our result is similar to those reported to golden ide *Leuciscus idus melanotus* as the 96 h LC50 was obtained at 237 mg L^{-1} (Pfeuffer and Matson, 2001). The reported 96 h LC50s for rainbow trout *Oncorhynchus mykiss* and common carp *Cyprinus carpio* were 211 mg L^{-1} and 280 mg L^{-1}, respectively (SERA, 2005; Fossen, 2006).

The comparison of acute toxicity values (Tables 1 and 2) for different species showed, that imidacloprid and Confidor SL 200 were found to be the most acutely toxic to daphnids, followed by bacteria *V. fischeri* and zebrafish adults and embryos.

3.2.1.1. Effects on enzyme activities. The activities of ChE, GST and CAT did not change during acute exposure of daphnids to imidacloprid or Confidor SL 200. The values of ChE, CAT and GST activities in control animals were 3.48 ± 0.13; 1.29 ± 0.049 and 1.42 ± 0.036 EU/mg protein, respectively in the case of imidacloprid and 3.03 ± 0.38, 1.15 ± 0.09 and 1.33 ± 0.047 EU/mg protein in the case of Confidor SL 200. This suggests that these enzyme activities are not an early, sensitive biomarker of exposure to imidacloprid or Confidor SL 200. Similarly was shown in our previous work (Jemec et al., 2007), where the activities of the same enzymes were decreased in daphnids chronically exposed up to 40 mg L^{-1} of imidacloprid and 0.02% Confidor SL 200, but these changes were shown to be due to generally impaired physiological state of an organism and not specific action of imidacloprid and Confidor SL 200. Only one study was previously published on the acute effects of imidacloprid on ChE and GST activities, where no changes of the latter were found in earthworms exposed up to 1 mg L^{-1} of imidacloprid (Capowiez et al., 2003).

3.2.2. Chronic toxicity to algae and daphnids

The results of chronic toxicity tests with algae and daphnids are given in Fig. 2d and Table 3. The 72 h IC50 value obtained for *D. subspicatus* was 389 mg L^{-1} indicating the lowest toxicity of imidacloprid observed among the selected tested organisms. It was found that the imidacloprid in Confidor SL 200 was much more toxic than active ingredient alone. Solvents contributed a major part to toxicity for algae, because the tested solvents alone inhibited the algal growth already at 0.005 v/v%. Literature data showed that the highest tested concentrations in toxicity tests (10 mg L^{-1} and 119 mg L^{-1} of analytical grade imidacloprid) did not cause adverse effects on *D. subspicatus* and *Selenastrum capricornutum* (SERA, 2005). Diatom *Navicula pelliculosa* was found to be the most sensitive algal species as the 4 d NOAEC and the LOAEC were 6.69 mg L^{-1} and 9.88 mg L^{-1} of imidacloprid, respectively (SERA, 2005).

In our laboratory, the highest toxicity of imidacloprid among the species tested in the present study was previously reported on the reproduction of daphnids: the 21 d NOEC was 1.25 mg L^{-1} of imidacloprid (Jemec et al., 2007). Contrary to the acute toxicity observations with daphnids, bacteria, and zebrafish, the toxicity of imidacloprid to the reproduction of daphnids did not increase when testing the Confidor SL 200. The obtained 21 d NOEC was even higher as those obtained for pure chemical. Similar result was reported by Young and Blakemore (1990) as they determined the 21 d NOAEC at 1.8 mg L^{-1} of imidacloprid using the immobility as endpoint. Also data for other aquatic crustaceans show high toxicity of imidacloprid, i.e. the NOAEC for *Mysidopsis bahia* was found at 0.000163 mg L^{-1} after the chronic exposure (SERA, 2005).

At the moment, imidacloprid is not regularly monitored in aquatic environments. Very few data are available and they indicate low environmental levels of imidacloprid; the lowest and the highest measured environmental concentrations were 1 μg L^{-1} and 14 μg L^{-1} of imidacloprid (Pfeuffer and Matson, 2001; US Geological Survey, 2003). These concentrations are lower than chronic levels observed for daphnids. However, some local point-source contamination which can occur as a consequence of an accidental spill could pose a potential chronic risk to *D. magna* according to the results obtained in our study. Moreover, the acute risk for more sensitive crustacean species than daphnids, e.g. *Hyalella azteca* and *Chironomus tentans* exists (SERA, 2005).

3.3. Ready biodegradability

Initially, acute toxicity of imidacloprid was determined using activated sludge to eliminate possible inhibition of biodegradation due to potential toxicity of imidacloprid to microorganisms. Imidacloprid was non-toxic to mixed bacterial community of activated sludge as the inhibition of oxygen consumption at the highest concentration tested (400 mg L^{-1}) was 6% compared to the control. In the case of Confidor SL 200 toxicity to activated sludge could not be evaluated due to intensive degradation of the solvents present in the Confidor SL 200.

The samples with 250 and 450 mg L^{-1} of imidacloprid were tested for biodegradability. Tested samples were non-toxic to microorganisms and biodegradation started immediately without a lag phase. The final levels of biodegradation were between 9% and 12%. The samples containing 250 and 450 mg L^{-1} of imidacloprid were not readily biodegradable according to the recommendations for the ready biodegradability classification of pure chemicals as the "pass level" of biodegradation in the O_2 test was not achieved (Struijs and van den Berg, 1995). The obtained persistence is in agreement with the statement that imidacloprid is likely to remain in water column in aquatic systems (Overmyer et al., 2005). The degradation and elimination of imidacloprid was investigated in water–sediment systems (Spiteller, 1993; Krohn and Hellpointner, 2002). It was found that radioactively labelled imidacloprid disappeared quickly from the water phase to the sediment phase. At the same time formation of CO_2 by microbes due to mineralization was observed throughout the experiments although its proportion was quite low (0.7–2.0%) and the process was slow. The calculated DT50 values (time after which half of the initial concentration of imidacloprid was disappeared) were 30 d for elimination from the water phase and between 130 and 160 d for different types of sediments. Henneböle (1998) demonstrated that the DT50 was reduced to some days under the influence of sunlight using a water–sediment system. It was also reported that the elimination of imidacloprid was lower in the oligotrophic system (Bayer, 2000) contrary to the fast disappearence in eutrophic conditions. In our experiment, oligotrophic system with low concentration of microorganisms and nutrients was used and consequently the low biodegradability of imidacloprid was determined.

4. Conclusions

The results of this study show that imidacloprid is not highly toxic to tested aquatic organisms in comparison to some other environmental pollutants tested in the same experimental set-up (Tišler and Zagorc-Končan, 2002; Tišler et al., 2004; Tišler and Kožuh Eržen, 2006). Water fleas *D. magna* were the most sensitive

species after a short-term (48 h EC50 = 56.6 mg L^{-1}) and long-term exposure (21 d NOEC = 1.25 mg L^{-1}) followed by *V. fischeri*, zebrafish and algae. The activities of enzymes ChE, GST and CAT of daphnids were not early, sensitive biomarkers of exposure to imidacloprid and its commercial product. Imidacloprid was found persistent in water samples and not readily biodegradable in aquatic environment. The toxicity of commercial formulation Confidor SL 200 was intensified in comparison to the analytical grade imidacloprid to daphnids, zebrafish and especially in a case of algae the solvents highly elevated the adverse effects. Therefore, due to the increased predicted use of commercial products containing imidacloprid in the future and the obtained findings of this study we recommend additional toxicity and biodegradability studies of other commercial products containing imidacloprid as an active ingredient in the aquatic environment. Only, these studies will provide the final answer, whether imidacloprid is an appropriate substitution for other more toxic pesticides.

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Metabolomics to Explore Imidacloprid-Induced Toxicity in the Central Nervous System of the Freshwater Snail *Lymnaea stagnalis*

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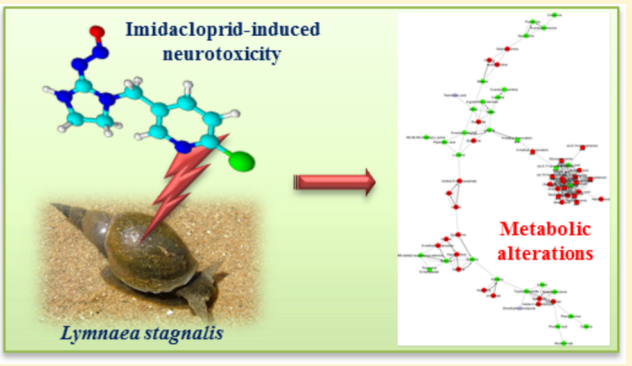
Supporting Information

ABSTRACT: Modern toxicology is seeking new testing methods to better understand toxicological effects. One of the most concerning chemicals is the neonicotinoid pesticide imidacloprid. Although imidacloprid is designed to target insects, recent studies have shown adverse effects on nontarget species. Metabolomics was applied to investigate imidacloprid-induced sublethal toxicity in the central nervous system of the freshwater snail *Lymnaea stagnalis*. The snails (n = 10 snails) were exposed for 10 days to increasing imidacloprid concentrations (0.1, 1, 10, and 100 μg/L). The comparison between control and exposure groups highlighted the involvement and perturbation of many biological pathways. The levels of several metabolites belonging to different metabolite classes were significantly changed by imidacloprid exposure. A change in the amino acids and nucleotide metabolites like tryptophan, proline, phenylalanine, uridine, and guanosine was found. Many fatty acids were down-regulated, and the levels of the polyamines, spermidine and putrescine, were found to be increased which is an indication of neuron cell injury. A turnover increase between choline and acetylcholine led us to hypothesize an increase in cholinergic gene expression to overcome imidacloprid binding to the nicotinic acetylcholine receptors. Metabolomics revealed imidacloprid induced metabolic changes at low and environmentally relevant concentration in a nontarget species and generated a novel mechanistic hypothesis.



INTRODUCTION

Over the past decades, the presence of manufactured chemicals in the environment has raised concerns because of their potentially lethal and sublethal effects on organisms, resulting in ecosystem functionality damages.[1] The environmental risk assessment guidelines (e.g., Water Framework Directive, 2000) are currently based on phenotypical end point effects, measured by acute and chronic lethal concentrations and with experiments focusing on effects such as mobility, ventilation, and reproduction (www.OECD.org). These types of toxicity testing are incapable of mimicking a realistic environmental exposure scenario and are failing to predict effects in anything other than the target species.[2]

To test the possible sublethal toxic effects of environmental pollutants in the past decades, several biochemical biomarkers have been developed.[3] Among these, enzymatic assays indicate toxic effects because the activity of these biomarker enzymes has been linked to oxidative stress.[4] One of these enzymatic tools is the Ellman's cholinesterase assay, which provides a simple colorimetric determination of acetylcholinesterase (AChE) activity. One commom method used to investigate toxic effects induced by pesticides is to apply the AChE bioassay; this is especially effective in determining the toxicity of organophosphate and carbamate pesticides because these compounds directly block AChE.[5]

Nowadays, after the introduction on the market of the neonicotinoid pesticide imidacloprid in 1991 by Bayer CropScience, the global insecticides market is dominated by this new class of pesticides.[6,7] Due to its extensive application and combined with the high persistency and leaching potential,[8,9] imidacloprid concentrations in water bodies have been found to exceed the regulatory norms in several countries.[10−13] Recently, this compound gained attention due to its significant ecotoxicological effects.[14] The pesticide is considered to be insect-specific, as it acts mainly as an agonist of the nicotinic acetylcholine receptors (nAChRs) on the postsynaptic membrane of neuronal cells of insects.[15,16] However, recent reports indicate a decline in nontarget species in surface waters contaminated with imidacloprid, demonstrating serious cascading effects of imidacloprid on aquatic and terrestrial ecosystem functionality.[10,17,18]

Due to the different mode of action of imidacloprid, an alternative strategy to the AChE bioassay should be found to warrant the investigation of imidacloprid-induced toxicity in nontarget species. To this extent, promising alternatives to

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traditional toxicity testing are found in the "omics" field.[19] Transcriptomics, proteomics, and metabolomics can measure changes in intracellular functioning upon exposure to toxicants at lower concentrations than traditional toxicity testing methods and can focus on numerous end points (genes, proteins, and metabolites) simultaneously.[20] The combination of these "omics" techniques in a system biology approach will enable a more accurate determination of the mechanism of action of toxicants, which may improve environmental risk assessment.[21,22] Compared to the other omics, metabolomics has the advantage that metabolites are more conserved across species and therefore this approach is more suited to determine conserved end points.[23] Metabolomics is adding to the base of knowledge on the ecotoxicological effects of compounds that are of immediate concern to environmental health.[22−24]

To further improve the base of evidence of imidacloprid toxicity, the effect of imidacloprid on nontarget species should be more thoroughly investigated. A promising species to study imidacloprid-induced sublethal effects is the freshwater snail *Lymnaea stagnalis*. This species is a globally distributed inhabitant of freshwater ecosystems and a model organism in environmental toxicology and neurobiology.[25,26] *L. stagnalis* has been applied as a model organism in the omics field, as shown by the increasing number of recent publications.[25,27−30]

In this study, a 10-day exposure to imidacloprid at environmentally relevant concentrations (0.1 and 1.0 μg/L) and higher concentrations (10 and 100 μg/L) was carried with *L. stagnalis*. Each exposure group comprised 10 snails, and along with the exposure experiment, a control group with 10 snails not exposed to imidacloprid was used. Effects on reproduction and on the activity of acetylcholine esterase (AChE) of the snails' central nervous systems (CNSs) were examined. To assess the toxicity of imidacloprid at the molecular level, multiple metabolomics approaches were applied and compared to traditional toxicity assessment methods. A metabolomics-targeted approach based on hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry (MS) was performed to profile neurotransmitters in the CNSs of the exposed *L. stagnalis*. A nontargeted metabolomics strategy based on liquid chromatography (LC) and gas chromatography (GC) coupled to high-accuracy MS was used to investigate changes in hydrophilic and hydrophobic metabolites after imidacloprid exposure. Multivariate data analysis (MVDA) and multiple *t* test with false discovery rate (FDR) correction was employed to determine the metabolites contributing to the differences between the control group and exposed groups and potential biomarkers of exposure were identified. Biochemical networks were created to provide mechanistic insights into the metabolic pathways associated with imidacloprid toxicity.

MATERIALS AND METHODS

Reagents and Materials. Milli-Q water was obtained from a Millipore purification system (Waters-Millipore Corporation, Milford, MA). HPLC-grade acetonitrile (ACN) and methanol (MeOH) were from JT Baker Chemical (Phillipsburg, NJ). MS-grade formic acid (98% purity) and sodium formate salt (purity ≥99%) were obtained from Fluka (Steinheim, Germany). Chloroform, hexane, and isooctane were obtained from Sigma-Aldrich (Schnelldorf, Germany).

Hydrophilic standards (amino acids, sugars, organic acids, neurotransmitters, and nucleotides) and the hydrophobic standard mixture, consisting of 37 fatty acid methyl esters, were purchased from Sigma-Aldrich. The hydrophilic metabolites were mixed in ACN/H_2O 90:10 v/v at a concentration of 1 mg/L, and the fatty acid methyl esters mixture was diluted to 1 mg/L in isooctane. These standards were used as quality control (QC) for the LC-MS and GC-MS analysis, respectively. The MS metabolite library of standards (MSMLS) was obtained from IROA Technologies (Ann Arbor, Michigan), and the mixtures of metabolites were prepared as described by the manufacturer. A list of all the analytical standards used can be found in Table S1 (Supporting Information). The stable isotope-labeled internal standards of 3-MT-d_4, acetylcholine-d_4, serotonin-d_4, 5-HIAA-d_5, L-tryptophan-d_3, and GABA-d_6 were from CND Isotopes (Quebec, Canada). DOPA-d_3, dopamine-d_4, L-tyrosine-d_4, epinephrine-$^{13}C_2$ ^{15}N, choline-d^{13} and glutamate-d_5 were obtained from Cambridge Isotope Laboratories (Andover, MA). Glutamine-^{13}C ^{15}N, norepinephrine-d_6 and 5-hydroxy-L-tryptophan-d_4 were bought from Toronto Research Chemicals (Toronto, Ontario, Canada). Imidacloprid analytical standard (99.9%) and imidacloprid-d_4 were purchased from Sigma-Aldrich.

***L. stagnalis* Selection and Exposure.** *L. stagnalis* snails used in our study were 16 weeks old, with an average shell length of 26.14 ± 0.69 mm, and from a synchronized population cultured at the VU University Amsterdam, The Netherlands. In the breeding facility, the snails were kept in a circulation system of copper-free freshwater (average water characteristics: hardness 1.48 mmol/L, pH 8.12, total organic carbon 1.9 mg/L) at 20 ± 1 °C in a 12 h light/12 h dark cycle and fed on lettuce leaves ad libitum.

The snails were individually exposed for 10 days to different concentrations of imidacloprid (control, 0.1, 1.0, 10, and 100 μg/L). The control and exposure groups were composed of 10 snails each. Two days prior to the exposure, the snails were acclimatized in glass beakers with copper-free water. Afterward, the glass beakers were filled with 150 mL of copper-free water spiked with different concentrations of imidacloprid, previously dissolved in copper-free water. The beakers were placed in a climate room at 20 °C in an 8:16-h light−dark cycle. A suspension of 250 μL TetraPhyll fish feed in copper-free water (133 g/L) was added daily.

Effects on reproduction were assessed by measuring the number of laid eggs and the dry weight of the egg clutches. Every other day, egg clutches were collected, and the eggs were counted using the cell counter plug-in of the image analysis software, ImageJ. The egg clutches were dried at 50 °C for 12 h and weighed (Supporting Information).

Sample Preparation. After the exposure experiment, the snails were sacrificed by snap freezing in liquid nitrogen. The CNSs were dissected, and the sample preparation was carried out following a two-step extraction with the Precellys24 Dual device (Bertin Technologies, France) operating at 6500 rpm for 2 cycles of 10 s with a 15 s break between cycles. The first extraction step was performed with Milli-Q water. From the aqueous homogenate, 10 and 15 μL were withdrawn from the homogenate to perform the Bradford and Ellman assays, respectively. Chloroform and a mixture of neurotransmitters stable isotope-labeled internal standards in MeOH was added to the homogenate and in order to reach the final solvent composition of 1:1:1 v/v/v H_2O/MeOH/$CHCl_3$ in the final volume of 500 μL. The homogenates were kept in ice for 10 min to allow the metabolite partitioning in the biphasic mixture. The samples were centrifuged in a precooled centrifuge (Heraeus Biofuge Stratos, Heraeus Instruments, Germany) at

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

4 °C for 10 min at 17 000 rpm, and the hydrophilic fractions were dried in a Centrivap Concentrator (Labconco Co., Kansas City, MO) for 240 min at 20 °C. The residues were reconstituted in 100 μL of mobile phase, vortexed, and centrifuged again. The clear solutions were transferred to autosampler vials for analysis. The chloroform fractions containing the lipophilic metabolites were dried under a gentle flow of N_2 and then derivatized with 500 μL of BF_3 methanolic solution kept for 30 min at the temperature of 80 °C. After cooling, a liquid−liquid extraction was performed three times with 500 μL of hexane. The hexane fractions were reconstituted in an autosampler vial and evaporated until dryness with nitrogen, and finally, 200 μL of isooctane was added.

Acetylcholinesterase Activity Assay. The experimental setup of the AChE bioassay is described in the Supporting Information.

Imidaclopid Exposure Concentrations. The exposure media solutions were refreshed every second day. Samples of the exposure solutions were collected and analyzed with LC triple quadrupole (QqQ) mass spectrometer (QqQ) to determine actual exposure concentrations. The internal standard was added to the water samples in a final concentration of 5 ng/mL. Analyses were carried out with a Agilent (Palo Alto, CA) 1260 infinity binary liquid chromatography system coupled to an Agilent (Palo Alto, CA) QqQ 6410 series. A pentafluorophenyl column (100 × 2.1 mm 3.5 μm particle size) from Phenomenex was used at a flow rate of 0.2 mL/min. The mobile phase composition was H_2O 0,1% formic acid and MeOH and the elution was achieved with a gradient from 20 to 90% of MeOH in 6 min. The electrospray source (ESI) was operated in positive mode and the following parameters were set: gas temperature, 350 °C; gas flow, 6 L/min; nebulizer pressure, 40 PSI; and ESI capillary voltage, 3000 V. The MS data acquisition was carried out in multiple reaction monitoring (MRM) mode. The calibration line ranged from 0.05 μg/L (LOQ) to 1000 μg/L and was linear with a correlation coefficient (R^2) of >0.98. The MRM transitions monitored for imidacloprid were 256.1 → 175.1 (identification) and 256.1 → 209.1 (quantification) and for imidacloprid-d_4 were 260.1 → 213.1 (identification) and 260.1 → 179.2 (quantification). The fragmentor was set to 90 V, the collision energy was set to 30 eV, and the dwell time was set at 50 ms for all transitions. Data acquisition and analysis using the QqQ was performed with a MassHunter Workstation by Agilent. The actual concentrations of the 0.1 and 1 μg/L) groups were in the range of the nominal concentrations (Supporting Information, Figure S3). The actual concentrations of the 10 and 100 μg/L groups were about 3 times lower than the nominal concentrations. The stability of imidacloprid concentrations in the exposure media were assessed before carrying out the exposure experiment (Supporting Information).

Metabolomics. A targeted metabolomics analysis of neurotransmitters, precursors and metabolites was performed with MS/MS based on the method by Tufi et al.[31] A cross-platform nontargeted metabolomics approach, based on HILIC high resolution Time of Flight (ToF) and GC-HRToF coupled to atmospheric pressure chemical ionization (APCI), was used to profile the hydrophilic metabolites and the chloroform fraction respectively according to Tufi et al.[28]

Data Analysis. The data were normalized for the CNS protein content and outliers were removed using the Dixon's Q test. The analysis of variance (ANOVA) with post hoc Tukey's honestly significant difference (HSD) was performed with the software SPSS (IBM).

The high-resolution (HR) time-of-flight (ToF) chromatograms were analyzed with Compass DataAnalysis software (Bruker Daltonik, Bremen, Germany) where a mass accuracy below 5 ppm was assured by calibrating chromatograms using sodium nitrate calibration curves. The chromatograms were analyzed with the software DataAnalysis 4.0 (Bruker Daltonik, Bremen, Germany). The first data treatment step consisted of a internal calibration of the spectra using the enhanced quadratic mode. The peak intensities of the detected metabolites in all HILIC-ToF and GC-ToF data were normalized for the CNS protein content.

On the basis of the MSMLS, we created a target list, was performed a batch targeted analysis with the software PathwayScreener (Bruker Daltonik, Bremen, Germany). The acquired LC and GC HR-MS chromatograms were then automatically screened for the accurate masses of metabolites in the target list. The results were exported to ProfileAnalysis 2.1 (Bruker Daltonik) that performs exclusion of outliers based on the interquartile ranges and multiple comparison *t*-test with *p*-value adjustment based on false discovery rate (FDR) for the comparison between control and exposed groups. Fold changes were calculated by dividing the average of metabolites in exposed groups by the average of metabolites in the control group. Pathway over-representation analysis based on the web tool IMPaLA was performed to investigate which pathways were involved in imidacloprid exposure.[32] Biochemical network maps were generated using Metamapp[33] and visualized in the open source software platform Cytoscape.[34]

RESULTS AND DISCUSSION

Neuronal Metabolism Disruption. On the basis of imidacloprid mode of action, we carried out a biologically driven targeted metabolomics analysis. Because imidacloprid binds to the ACh receptor on the postsynaptic membrane of neuronal cells, the neuronal metabolism was investigated by quantifying the levels of the main neurotransmitters, their metabolites and precursors. Of the 12 quantified neurotransmitters, metabolites and precursors, the levels of 8 metabolites were significantly (p-value < 0.05, t test) changed by imidacloprid (Figure S4). These metabolites are choline, acetylcholine, glutamate, glutamine, serotonin, tryptophan, phenylalanine and histidine. Four of these metabolites were significantly different at more than two exposure concentrations: choline, acetylcholine, phenylalanine, and histidine.

Most of the changes appear indeed to be implicated with the cholinergic system in accordance with the mode of action of imidacloprid. Acetylcholine decreases and the observed increase of the choline/acetylcholine ratio (Figure 1) might indicate a possible increase in the cholinergic gene expression.

This mechanism might take place in the synaptic cleft to enhance the clearance of acetylcholine, which accumulates because of the binding of imidacloprid to the nACh receptors. This compensatory strategy would then lead to a feedback increase of acetylcholine esterase (AChE) that has been previously observed in association with acute stress and AChE inhibitors.[35] The CNS extracts of the exposed snails were tested in the AChE activity assay. A slight increase in the AChE activity related to increased exposure concentrations was observed (Figure S5). The group exposed to the highest imidacloprid concentration showed an average AChE activity of 156.2 ± 33.6% compared to the control group (unpaired t test,

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

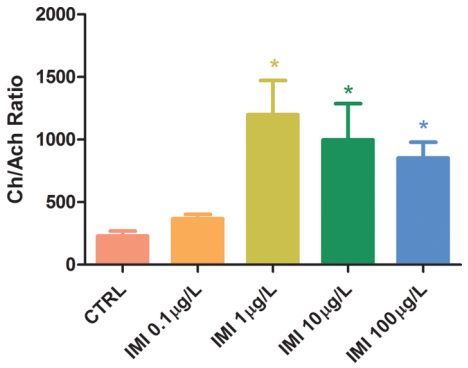

Figure 1. Choline (Ch)/ acetylcholine (ACh) ratio in *L. stagnalis* CNS exposed to increasing concentration of imidacloprid (IMI). Statistical significance (n = 10; error bars = SD; * = p-value <0.05, t test).

confidence interval 90%). Despite the large number of biological replicates, the coefficients of variation (CV%) in the exposed groups were above 30%. The incubation period of 10 days may have been too short to show any significant effects on AChE activity. However, a significant reduction of AChE activity after only 7 days of exposure at 25 μg/snail was previously reported in the land snail *Helix aspera*.[36] A significant inhibition of AChE activity was observed at 0.1 and 1.0 mg/L in exposed mussels gills.[37] In blood and brain of rats exposed to imidacloprid an enzyme inhibition was shown as well.[38,39] Phenotypical end points like locomotion and ventilation of *Chironomus riparius* Meigen larvae showed to be more sensitive to imidacloprid exposure than AChE activity.[40] On the contrary, an increase in AChE activity was observed in caged bees in response to imidacloprid exposure.[41] Even though AChE has been suggested as potential biomarker of imidacloprid exposure[38,41] the effect of imidacloprid on AChE activity appears to be species-specific or not significant. On the basis of these results, AChE cannot be used to assess imidacloprid-induced sublethal effects and is not suitable as biomarker for imidacloprid exposure.

Metabolome Perturbation. To further explore what metabolite levels were altered due to exposure of imidacloprid, the HILIC and GC chromatograms were screened for a larger number of metabolites. This approach allowed increasing the number of detected metabolites, and in combination with statistics, it enhanced the chances to discover exposure biomarkers. Many metabolites in our standard library (Table S1) were accurately identified on the basis of three quality parameters (mass accuracy, retention time, and isotopic pattern) and were found to be statistically significant (p-value < 0.05, t test FDR). For the exposure at environmental concentrations of 0.1 μg/L and 1 μg/L we have identified 22 and 25 significant metabolites, respectively. At 10 and 100 μg/L, 27 and 30 identified metabolites, respectively were significantly different (Table 1). The fold changes and p-values of all the identified metabolites in the different exposure groups are given in Table S2.

The fold changes for metabolites which were significantly changed in at least two exposure concentrations are shown in Figure 2. Decreased levels were found for tryptophan, linolelaidic acid, linoleic acid, cis-10-heptadecenoic acid, 3-methoxy-4-hydroxymandalate, uridine, stearic acid, oleic acid, inosine, heptadecanoic acid, guanosine, γ-linolenic acid, elaidic acid, acetylcholine, and 3-methyl-2-oxovaleric acid. An increase in the level of spermidine, proline, leucine, histidine, betaine, 5-methylthioadenosine, putrescine, 4-methyl-2-oxovaleric acid, valine, creatinine, 4-guanidino-butanoate, phenylalanine, choline, and carnitine was observed.

Table 1. Number of Metabolites Identified in the Different Exposure Groups, Number of Significantly Different Metabolites based on p-Values Corrected by False Discovery Rate, and Percentage of Significantly Different Metabolites of the Identified Metabolites

exposure group	no. of identified metabolites	no. of significant metabolites	significant metabolites of identified metabolites (%)
control vs 0.1 μg/L	71	22	31
control vs 1.0 μg/L	56	25	45
control vs 10 μg/L	61	27	44
control vs 100 μg/L	68	30	44

The significant biomarkers can be used as biomarkers of exposure. Several biomarkers for imidacloprid exposure have been determined, and their accuracy has been assessed by the receiver-operating characteristic (ROC) curve analysis. Of the 29 metabolites that showed a statistically significant change at least at two exposure concentrations (shown in Figure 2), 12 showed an area under the curve (AUC) above 0.8 (Table S3 and Figure S8). These metabolites are carnitine, elaidic acid, γ-linolenic acid, linolelaidic acid, stearic acid, 3-methyl-2-oxovaleric acid, acetylcholine, creatinine, guanosine, inosine, phenylalanine and tryptophan. Among these metabolites, acetylcholine is directly related to mode of action of imidacloprid. The profile of these metabolites can be used as a biomarker of imidacloprid exposure.

Biochemical networks were built to provide information on the mechanism of toxicity and the metabolic pathways affected. The networks were based on p-values and fold changes between the control group and exposed groups (Figure 3). Using biochemical network maps, the biological interpretation is facilitated since it allows the visualization of consistent changes among the exposure concentrations. Pathway over-represented analysis was performed with the web-tool IMPaLA on the list of significantly changed metabolites. This tool analyzes whether these metabolites are significantly associated with a particular pathway or set of pathways. The metabolic pathways in which significantly changed metabolites are involved are reported in Table 2. In this table ,the pathway name, the database source, the pathway size, the number of metabolites overlapping to the metabolic pathway, and the percentage of the pathway coverage are provided. In addition, the p-value and q-value corrected by FDR for each pathway are reported.

With an increase in the exposure concentration, a down-regulation was found for the fatty acids biosynthesis and the cholinergic system, whereas an increase was observed for many amino acids for which several amino acid biosynthesis pathways were involved.

The decrease in the levels of many fatty acids indicates a down-regulation of fatty acid biosynthesis and up-regulation of fatty acids degradation through the mechanism of β-oxidation. In this metabolic breakdown of long-chain fatty acids, carnitine and acylcarnitines play the key role of carriers that assist the transportation across the inner mitochondrial membrane. The acetyl-CoA generated in the β-oxidation enters the TCA cycle, where it is further oxidized to CO_2, producing more reduced energy carriers, NADH and $FADH_2$. Another destination of acetyl-CoA is the production of ketone bodies by the liver that

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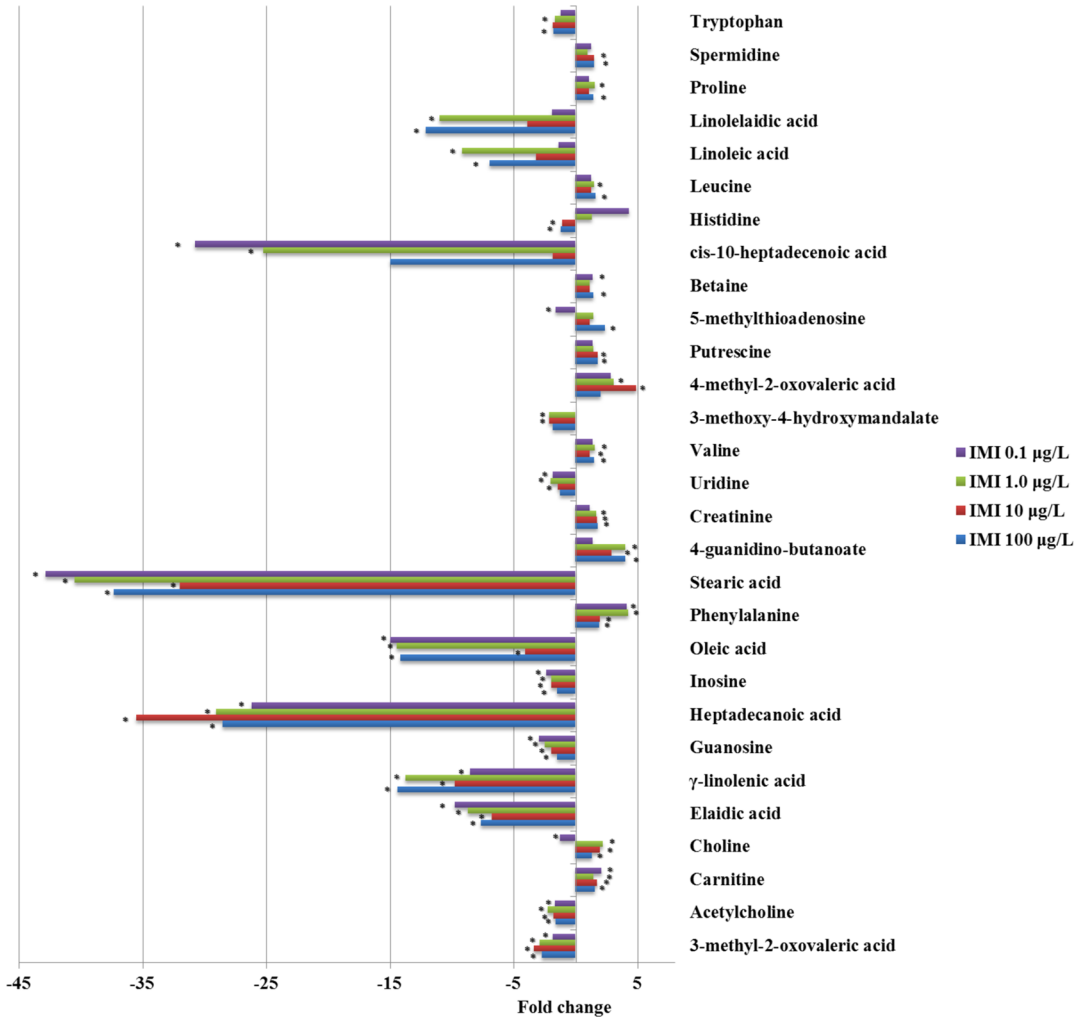

Figure 2. Fold changes of the significantly different metabolites (* p-value <0.05, t test FDR) for two or more imidacloprid (IMI) exposure concentrations.

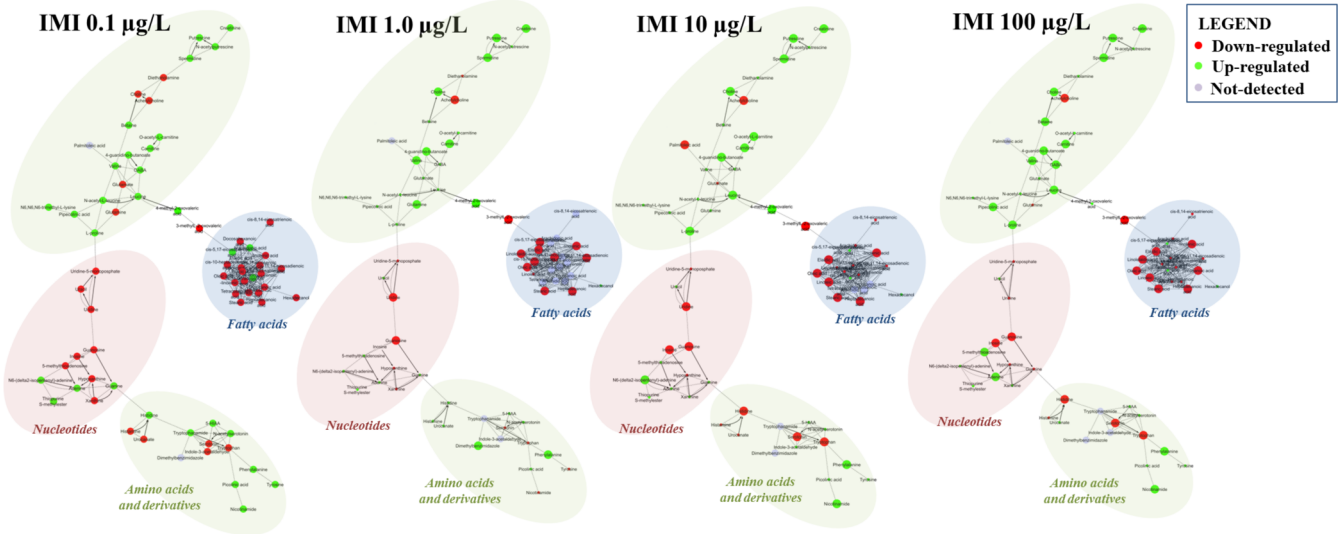

Figure 3. Biochemical network mapping for the comparison between control and exposed groups to increasing concentrations of imidacloprid (IMI). In the networks, the size of the nodes (metabolites) depends on the p-values. Statistically significant metabolites (p-value <0.05, t test FDR) are shown by bigger nodes, whereas not significant metabolites are represented by smaller nodes. The color of the node represents the fold change: (red) down-regulated, (green) up-regulated, and (gray) not detected metabolites. The clusters of the fatty acids metabolite class is shown in blue, the nucleotides are clustered within the red circle and in green the cluster comprising amino acids and derivatives is shown.

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Table 2. Pathway Over-Represented Analysis of the Significantly Changed Metabolites at More than Two Concentration Exposure of Imidacloprid and P and Q Values (FDR) for the Pathways

pathway name	pathway source	no. of overlapping metabolites	no. of all pathway metabolites	pathway coverage (%)	P value	Q value (FDR)
metabolism of amino acids and derivatives	Reactome	12	181	6.6	6.70×10^{-11}	5.90×10^{-08}
metabolic disorders of biological oxidation enzymes	Reactome	11	305	3.6	3.40×10^{-07}	7.20×10^{-05}
biological oxidations	Reactome	7	220	3.2	1.90×10^{-04}	6.80×10^{-03}
immune system	Reactome	6	87	6.9	7.70×10^{-06}	3.70×10^{-04}
urea cycle and metabolism of arginine, proline, glutamate, aspartate and asparagine	EHMN	6	125	4.8	6.20×10^{-05}	2.50×10^{-03}
Adaptive Immune System	Reactome	5	48	10.4	6.50×10^{-06}	3.30×10^{-04}
antigen processing-cross presentation	Reactome	5	29	17.2	4.80×10^{-07}	9.10×10^{-05}
arginine and proline metabolism	KEGG	5	91	5.5	1.50×10^{-04}	5.50×10^{-03}
glutathione conjugation	Reactome	5	38	13.2	2.00×10^{-06}	2.20×10^{-04}
glutathione synthesis and recycling	Reactome	5	30	16.7	5.80×10^{-07}	1.00×10^{-04}
leukotriene biosynthesis	HumanCyc	5	29	17.2	4.80×10^{-07}	9.10×10^{-05}
methionine metabolism	SMPDB	5	41	12.2	2.90×10^{-06}	2.20×10^{-04}
biosynthesis of unsaturated fatty acids	KEGG	4	54	7.4	2.40×10^{-04}	8.30×10^{-03}
valine, leucine, and isoleucine biosynthesis	KEGG	4	23	17.4	7.50×10^{-06}	3.70×10^{-04}
valine, leucine, and isoleucine degradation	KEGG	4	40	10	7.20×10^{-05}	2.80×10^{-03}
β-alanine metabolism	KEGG	3	31	9.7	7.20×10^{-04}	2.10×10^{-02}
biogenic amine synthesis	Wikipathways	3	17	17.6	1.10×10^{-04}	4.30×10^{-03}
branched-chain amino acid catabolism	Reactome	3	36	8.3	1.10×10^{-03}	3.20×10^{-02}
metabolism of polyamines	Reactome	3	30	10	6.50×10^{-04}	2.00×10^{-02}
nucleotide metabolism	Wikipathways	3	17	17.6	1.10×10^{-04}	4.30×10^{-03}
spermidine and spermine biosynthesis	SMPDB	3	17	17.6	1.10×10^{-04}	4.30×10^{-03}

are transported to tissues such as heart and brain tissue for energy. The observed increase in carnitine and acetyl-carnitine associated with the decrease in fatty acid levels suggest a possible alteration in mitochondrial metabolism, energy production, and acute oxidative stress.[42]

Pathway analysis revealed the involvement of metabolic pathways associated with biological oxidation, immune system and inflammation process. Glutathione metabolism is usually correlated to oxidative stress[43] and leukotriene biosynthesis is related to the occurrence of an inflammatory reaction in tissue injuries caused by xenobiotic.[44] A significant difference was found in the biogenic amine synthesis in which the main neurotransmitters are synthesized starting from their amino acid precursors, confirming the involvement of the neuronal metabolism.

An alteration in many amino acids was observed, such as arginine and proline metabolism, methionine metabolism, and β-alanine metabolism. Branched chained amino acids like valine, leucine, and isoleucine metabolism were also affected by imidacloprid exposure. Pathways of valine, leucine and isoleucine biosynthesis and degradation and branched-chain amino acid catabolism appear to be involved. The levels of the 3-methyl-2-oxovaleric acid, isoleucine alpha-keto acid and precursor were decreased whereas the levels of valine and leucine were enhanced, suggesting an increasing turnover of these amino acids biosynthesis. The levels of other amino acids like phenylalanine and proline were found to be increased whereas a decrease in tryptophan was found.

Nucleotides were involved as well, showing a decrease in the levels of inosine, uridine and guanosine.

Polyamine levels were also significantly changed and, as a consequence, the polyamine metabolism, spermidine levels, and spermine biosynthesis were dysregulated by imidacloprid exposure. Putrescine and spermidine had increased, showing an up-regulation in polyamine metabolism. The enzymatic reaction of spermidine synthetase catalyzes the production of spermidine from putrescine that is involved in the amino acid pathways such as arginine and proline metabolism, β-alanine metabolism, cysteine and methionine metabolism, and glutathione metabolism. Increases in putrescine levels have been related to cell injuries in the CNS associated with pathological conditions and neurotoxin exposure.[45,46]

To our knowledge, this is the first time a metabolomics study has been applied to investigate the metabolic alterations in the CNSs of the freshwater snail *L. stagnalis*. With this approach, the molecular mechanism of imidacloprid toxicity in a nontarget species was investigated. This quantitative and biologically driven approach was effective to single out metabolites whose levels were affected by the exposure of the snails to different levels of imidacloprid, showing the importance of the followed strategy. Because metabolomics focuses simultaneously on multiple endpoints our study found indications that, besides the binding of imidaclorpid to the AChE, this neonicotinoid can probably cause inflammation and neuron cell injury. This should be further investigated. Metabolomics was more sensitive than tradition toxicity testing because it enabled to determine metabolic alterations at low and environmentally relevant concentrations. The combination of metabolomics with statistical and visualization tools, such as biochemical networks and pathway analysis, facilitated the biological interpretation of the results and a better understanding of the undergoing toxicity mechanism. However, the proposed hypothesis of an increase in the cholinergic gene expression should be further studied by applying gene expression techniques and future research should investigate the validity of the exposure biomarkers also in other species.

■ ASSOCIATED CONTENT

Supporting Information

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Additional information as noted in the text. (PDF)

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Macro-Invertebrate Decline in Surface Water Polluted with Imidacloprid

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Abstract

Imidacloprid is one of the most widely used insecticides in the world. Its concentration in surface water exceeds the water quality norms in many parts of the Netherlands. Several studies have demonstrated harmful effects of this neonicotinoid to a wide range of non-target species. Therefore we expected that surface water pollution with imidacloprid would negatively impact aquatic ecosystems. Availability of extensive monitoring data on the abundance of aquatic macro-invertebrate species, and on imidacloprid concentrations in surface water in the Netherlands enabled us to test this hypothesis. Our regression analysis showed a significant negative relationship ($P<0.001$) between macro-invertebrate abundance and imidacloprid concentration for all species pooled. A significant negative relationship was also found for the orders Amphipoda, Basommatophora, Diptera, Ephemeroptera and Isopoda, and for several species separately. The order Odonata had a negative relationship very close to the significance threshold of 0.05 ($P = 0.051$). However, in accordance with previous research, a positive relationship was found for the order Actinedida. We used the monitoring field data to test whether the existing three water quality norms for imidacloprid in the Netherlands are protective in real conditions. Our data show that macrofauna abundance drops sharply between 13 and 67 ng l^{-1}. For aquatic ecosystem protection, two of the norms are not protective at all while the strictest norm of 13 ng l^{-1} (MTR) seems somewhat protective. In addition to the existing experimental evidence on the negative effects of imidacloprid on invertebrate life, our study, based on data from large-scale field monitoring during multiple years, shows that serious concern about the far-reaching consequences of the abundant use of imidacloprid for aquatic ecosystems is justified.

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Introduction

When neonicotinoids were introduced as new, systemic, insecticides in the 1990s, they were supposed to be much more efficient than the older generation of insecticides [1]. As a seed treatment they could be used in much lower quantities and they promised to be less polluting to the environment. Seed dressing makes spraying crops with insecticides unnecessary because the active substances are spread to all plant tissues when the plant grows. However, soon after the introduction of this new type of insecticides, concern rose that neonicotinoid residues in pollen and nectar might be harmful to honey bees [1,2], and several studies have provided supporting evidence for this [3].

Neonicotinoids are neuro-active insecticides which derive their toxicity to target species from acting mainly agonistically on nicotinic acetylcholine receptors (nAChRs) on the post-synaptic membrane [4–6]. This means that normal nerve impulses become impaired [7]. Some authors [8] have also indicated some antagonistic action. The binding sites in mammal nAChRs are different from those in insect nAChRs, and the neonicotinoid imidacloprid shows selective toxicity for insects over vertebrates. This partly attributable to a higher affinity of imidacloprid for insect nAChRs compared with their vertebrate counterparts [5].

In short-term (10-day) tests on the effects of imidacloprid [9] on the aquatic worm *Lumbriculus variegatus* a high mortality was observed at the highest concentrations of imidacloprid in the sediments (1 to 5 mg/kg). At lower concentrations (0.05 to 0.5 mg/kg) effects were observed on growth and behaviour of *L. variegatus*. In another test [10] the aquatic invertebrates *Chironomus tentans* and *Hyallella Azteca* were able to recover from a short-term pulse exposure, but a chronic low-level exposure (>1.14 µg l^{-1} for *C. tentans*) to imidacloprid reduced the species survival and growth. Different effects of imidacloprid exposure in an aquatic microcosm experiment were found for two species of stream insects [11]; while the survival of the stonefly, *Pteronarcys dorsata*, was significantly reduced at 48 and 96 mg l^{-1}, no significant mortality was found for the cranefly, *Tipula sp.*, although a change in behaviour was observed. In acute toxicity bioassays [12] of imidacloprid to zooplankton crustaceans, the imidacloprid 48-h LC_{50}-s for cladocerans (65–133 mg l^{-1}) were two orders of magnitude higher than for ostracods (301–715 µg l^{-1}). In an acute toxicity test on an amphibian [13] the 48-h LC_{50}-s for imidacloprid were found to be

165 mg l^{-1} for tadpoles of *Rana limnocharis* and 219 mg l^{-1} for tadpoles of *Rana nivalis*. The variation in susceptibility among different animal taxa indicates that certain biochemical traits particular to a group of organisms are responsible for a specific level of sensitivity [14].

Long-term alterations in aquatic invertebrate community structure occurred after single pulse contamination of a stream ecosystem with the neonicotinoid insecticide thiacloprid [15]. In other community studies, the caddisfly *Neureclipsis sp.* reacted very sensitively to a single pulse of imidacloprid, and Diptera and Ephemeroptera larvae were affected after repeated pulses [16]. In field mesocosms, zooplankton, benthic, nekton as well as neuston communities exposed to imidacloprid were significantly less abundant than non-treated controls [17].

At low concentrations of neonicotinoid insecticides sub-lethal effects can occur in invertebrates. Given the many limitations of acute toxicity as an indicator for impacts of agrochemicals on aquatic invertebrate communities, the sublethal effects must be considered for a complete and realistic assessment of the long term impact [18]. In a study [19] on the effect of imidacloprid exposure on the mayfly *Epeorus lingimanus* and the aquatic oligochaete, *Lumbriculus variegatus* a reduction of feeding and egestion was found. This indicates physiological and behavioural responses to this insecticide. In an extensive review Desneux et al. found that sub-lethal effects of neonicotinoids may occur on neurophysiology, larval development, molting, adult longevity, immunology, fecundity, sex ratio, mobility, orientation, feeding behaviour, oviposition behaviour, and learning [18]. All these effects have been reported for a wide range of invertebrates and all have a potential to produce population level and community level impacts on ecosystems. In bees an additional sub-lethal effect of imidacloprid was found namely an increased susceptibility to infections and parasites such as *Nosema ceranae* [20–22]. This effect seems not related to the immune system but to impairment of grooming and allogrooming, which leads to reduced hygiene in the individual and in the nest, and so gives the pathogens more chance to infect the insects.

Delayed and chronic toxicity to aquatic arthropods were found after exposure to very low concentrations of neonicotinoids in water [23]. Thiacloprid caused delayed lethal and sub-lethal effects after 4 to 12 days following exposure. In order to be able to predict the effects of toxicants and to determine safe levels of concentrations of neonicotinoids and other toxicants for organisms, exposure time should be taken into account [24]. As traditional approaches consider toxic effects at fixed exposure times, a new approach to risk assessment is needed in which the time-dependency of the toxicity is included, because lowering the concentrations only means an increase in the time to effect, which is only limited by the natural lifespan of the (unexposed) organism [24–26].

Large-scale use of neonicotinoid insecticides started around 2004, and has rapidly increased to make neonicotinoids the most widely used class of insecticides world-wide [27,28]. Imidacloprid now ranks second in the global top 10 of agrochemicals [29]. Only a small fraction of the pesticide doses used reaches its intended target. Sur and Stork [30] found that for systemic application via seed coating only 1.6 to 20% of the imidacloprid in the seed coating actually enters the crop to protect it. The remaining 80 to 98.4% of the applied amount ends up in the environment, and can accumulate in soil [31], especially because of its high persistence. There are various ways for imidacloprid to contaminate ground or surface water: by accidental spilling, leaching, overspray or spray-drift. Furthermore, imidacloprid used on grass, turf or hard surfaces such as lawns, golf courses or concrete may contaminate surface water through runoff and drainage [32,33].

Leaching of pesticides is one of the main mechanisms responsible for the contamination of groundwater and surface water. Felsot found that imidacloprid applied via drip chemigation leached significantly below the emitter depth [34]. The Groundwater Ubiquity Score (GUS) [35] of imidacloprid as calculated from the sorption coefficient (K_{oc}) and the soil halftime (DT_{50}) amounts to 3.76, indicating a high leaching potential [36]. However, the leaching process is highly variable across different soil types and pesticide formulations [37]. The presence of cracks or other macropores in the soil, or less structured soil can lead to preferential flows that bypass the most chemically and biologically reactive topsoil. Leaching from sandy soils is very high while imidacloprid is less mobile in, but still leaches substantially from, soil with a high organic matter content [38]. Estimated equilibrium partitioning over soil and water gives a soil to water ratio of 1 to 3 (log $P = 0.57$), indicating that most of the imidacloprid tends to end up in the water [39]. Note that this ration can vary with varying organic matter content of the soil [38].

Imidacloprid is generally persistent in water, and not easily biodegradable [31]. It is likely to remain in the water column in aquatic systems, and has an aerobic sediment and water half-life time of 30 to 162 days [36,40]. At pH values corresponding to environmental conditions, imidacloprid is stable to hydrolysis, but it can be rapidly degraded photolytically [31]. Some of the major metabolites of imidacloprid are equally neurotoxic, acting on the same receptors, and are also persistent [41].

Three environmental risk limits for surface water are currently in use in the Netherlands. These are technical-scientific advisory values for achieving environmental quality standards.

The MTR stands for Maximum Permissible Risk (Dutch: Maximaal Toelaatbaar Risico), and is the environmental concentration at which the species in an ecosystem are considered safe from effects caused by the substance, based on as many toxicity studies as possible. The MTR imidacloprid was 13 ng l^{-1} at the time the data used in this study were collected [42]. In the context of the European Water Framework Directive a Maximum Permissible Concentration (MPC) has been derived, which is the concentration at which aquatic ecosystems and humans should be protected from effects due to long-term exposure. The $MPC_{eco,water}$ for fresh water, based on ecotoxicological data for direct exposure, is set at 67 ng l^{-1} [43]. The Maximum Acceptable Concentration (MAC) is the concentration at which aquatic ecosystems should be protected from effects due to short-term exposure or concentration peaks. The $MAC_{eco,water}$ for fresh water, based on ecotoxicological data for direct exposure, is set at 200 ng l^{-1} [43].

As one of the most-used insecticides the Netherlands, imidacloprid came highest in a ranking of substances that exceeded the MTR in 2004 [44]. It has been in the top 3 of that list every year since 2004 and number 1 in most years. The MTR for imidacloprid has been exceeded in almost half of all 9037 water samples in our dataset; the highest exceedance, measured in 2005 near Noordwijkerhout, was 320 μg l^{-1} [42] – this is almost 25,000 times the MTR, and about 56 times the 96-h LC_{50} for *Chironomus tentans* of 5.75 μg l^{-1} [10]. It is also well within the acute toxicity (48-h EC_{50}) range (289–841.2 μg l^{-1}) of the cladoceran *Ceriodaphna dubia* [45]. Imidacloprid norm exceeding is not exclusive to the Netherlands. Almost one fifth of water samples taken in California, USA exceeded the United States Environmental Protection Agency's (EPA) Aquatic Life Benchmarks of 35 μg l^{-1} (acute) and 1.05 μg l^{-1} (chronic) for invertebrates and the concentrations

found there also often exceeded European and Canadian toxicity directives [46].

Much research has already been conducted on the influence of neonicotinoid insecticides on various species under controlled conditions in the lab and in mesocosms. Here, we combined eight years of Dutch monitoring data on imidacloprid in surface water with eight years of monitoring data on macrofauna abundance to look at this influence on a nationwide scale, something that had not been done before. We combined 680,147 species abundance measurements [*x, y, date, species, abundance*] at 7380 unique locations *[x,y]* with 9037 imidacloprid concentration measurements [*x, y, date, concentration*] at 801 locations. Locations and dates differed across both datasets. To combine the datasets we used $\leq$1 km distance and $\leq$160 days time difference as criteria for coupling the abundance data to the concentration data (see Methods section for details). This resulted in a combined dataset of 18,898 records [*concentration, abundance, species*] for the years 1998 and 2003–2009. We analysed this dataset to answer the question: is there a relationship between neonicotinoid residues in the surface water, and the number of observed individuals per non-target species, in the Netherlands? Note that our approach of statistical analysis of observational data implies that even if we find a correlation, this does not necessarily imply causality, because there could be other factors that could be the main driver of the observed patterns of abundance. In the discussion we will reflect on this issue in more detail.

Materials and Methods

Data Collection

Data on imidacloprid concentrations in surface water in the Netherlands were obtained from the Dutch pesticides atlas [42]. This is a database with nationwide results from routine monitoring of pesticide residues in Dutch surface water covering almost 700 pesticides and metabolites. The monitoring program is effectuated by the Dutch water boards, Leiden University and the Board for the Authorisation of Plant Protection Products and Biocides (Ctgb), and at the time we obtained the data, they were available for the years 1998, and 2003 to 2009.

For all samples in which no imidacloprid could be detected or quantified, the dataset reports the limit of reporting (LOR) instead. These numbers are flagged in the dataset to alert the user that they do not represent the measured concentration but the LOR. This is because the real imidacloprid concentration in samples that tested negative for imidacloprid can be anything within the range of 0 to the LOR of the particular measurement method used. The values of the reporting limits vary across water boards and across years; in the dataset, LORs ranged from 5 ng l^{-1} to 190 ng l^{-1}. Of those samples for which no true imidacloprid concentration was actually reported, we only included samples with LOR $\leq$7 ng l^{-1}, because we were interested in the effects of low imidacloprid concentrations. In these cases we used the reporting limit as the imidacloprid concentration for these samples.

Initially, data on the distribution and abundance of aquatic macro-invertebrate species in Dutch surface water were obtained from Limnodata Neerlandica (www.limnodata.nl), an online database developed and maintained by the Dutch Foundation for Applied Water Research (STOWA) and containing data provided by the water boards, the Provinces and Rijkswaterstaat. These data were used in an earlier study by Van Dijk [47]. However, Verdonschot and Van Oosten-Siedlecka [48] showed that the majority of the data in the Limnodata database were not copied properly from the original datasets, and therefore might not be reliable. Therefore, we requested the original macro-invertebrate datasets directly from the water boards, and received files from 23 of the total 26. We did not succeed in getting in contact with the very small water board Blija Buitendijks. The water boards Noorderzijlvest and Reest en Wieden did not supply data. We received data for various years, but could only use those for 1998 and 2003 to 2009 because of the limitations in the imidacloprid dataset; for the year 2009 we used the data from January to June.

The data files we received from the 23 water boards did not all have the same layout. We applied several operations (see text S1) to standardize the data and make them suitable for our analysis. The water boards collect these data by taking water samples at a fixed set of locations in the Netherlands, and from those samples the aquatic macro-invertebrate species and their abundance are determined. This means that all macro-invertebrate species found have at least one aquatic life stage. A standardized macro fauna net is used, with opening 0.30×0.20 m, depth 0.5 m and mesh size 0.5 mm. For each sample the standard net is moved through the water over a length of 5 m. Species in the samples are determined and individuals per species are counted. Only species present in the sample are reported, which implies that the minimum abundance of each species in each sample in the dataset is 1 and not 0. A detailed description of the sampling methods can be found in [49]. The definition of aquatic macro-invertebrate species is based on two criteria: the size of the representatives per taxonomic group (chiefly >0.5 mm), and the ease with which the taxonomic groups can be determined using common sampling methods.

Pairing Macro-invertebrate Data with Imidacloprid Data

The locations of the measurements of the imidacloprid concentrations and those of the samples of aquatic macro-invertebrates were mostly different. Chemical and biological samples were situated at various distances from each other. The same is true for the dates on which the measurements and samples were taken. To be able to investigate the relationship between imidacloprid concentration and species abundance, we paired the two datasets by making a selection based on a limited distance between the measurement location and sampling location and a small difference in dates between the measurement and sampling. For each macro-invertebrate sample we paired the data with the imidacloprid measurements located within a radius of 1 km, and no more than 160 days difference (one way). When more than one imidacloprid measurement was found that met these criteria, the median imidacloprid concentration of these measurements was used. The period of 160 days was based on the high end of the range of reported half-life times of imidacloprid in water [36,40]. Further, using the 160 days time window for our analysis allows for chronic and sublethal effects on population and reproduction to take effect, which would otherwise be overlooked in the analysis. In contrast to other pesticides, where recovery can occur after pulse exposure, aquatic invertebrate communities exposed to imidacloprid and other neonicotinoids take a long time to recover for two reasons: either the populations exposed die completely through chronic exposure, or they are unable to reproduce due to chronic weakness.

Statistical Analysis

All years and all places were pooled into one data set because we are mainly interested in the overall link between imidacloprid concentration and macro-fauna abundance and not in spatial temporal patterns. We tested the data for spatial autocorrelation using variograms exploring distances between samples up to 10 km. For the further analysis we used various ways of

aggregating the data for different species: all species pooled, species pooled per order (e.g. all Diptera pooled) and non-aggregated (analysis at species level). First, scatter plots were made to investigate the dependence of species abundance on imidacloprid concentration. Because of the skewed distribution of the data a $\log_{10}$-transformation was performed on the abundance data and imidacloprid concentration data. To enable easy comparison between species, a linear regression analysis was carried out on the log-transformed data. This is an over simplified metric for the strength of association but it enables an ordinal ranking of species according to strength of association. The significance of the regression coefficients was then tested with an Analysis of Variance (ANOVA).

Next, we reverted to an approach with a higher statistical power: a nonparametric test was performed to test the significance of the differences between the species abundance at imidacloprid concentrations above and below a water quality norm for imidacloprid (MTR, MPC, MAC). Because of the non-normal distribution of the non-transformed data on abundance, Mann-Whitney U tests were carried out to test the significance of differences in average abundance between the pooled samples above and the pooled samples below each water quality norm. Differences were considered significant at $P<0.05$. All datasets were analysed with the statistical package SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

Results

Relationship between Imidacloprid Concentration and Species Abundance

We did not find any spatial auto correlation in the abundance data. In the imidacloprid concentrations we found spatial autocorrelation for the short distances, but only between data points situated less than 3 km apart. Visual inspection of the scatter plots of abundance versus imidacloprid concentration (figures 1 and S1–S6) clearly show that at high imidacloprid concentrations, high abundance is rare while at low concentrations it is common. The simplified linear regression shows a significant negative relationship between species abundance and imidacloprid concentration for all species pooled, as well as for the separate orders Amphipoda (crustaceans), Diptera (true flies), Ephemeroptera (mayflies), Isopoda (custaceans) and Basommatophora (snails). For these orders the species abundance decreased significantly with increasing imidacloprid concentration (figure 1, table 1; figures S1–S6). The strongest decrease in species abundance was found for Amphipoda, with a slope of regression line $\beta=-0.180$ and $P<0.001$, and Ephemeroptera ($\beta=-0.157$, $P=0.001$). For each of the five orders mentioned above, one of the three most abundant species in the sampling data showed a significant negative relationship as well. Most of the other abundant species in these orders also showed a negative tendency, but those relationships were not significant at $P<0.05$. The negative relationship for the order Odonata (dragonflies and damselflies) was nearly significant.

For the order Actinedida (water mites), a reverse trend was observed. Here, a significant positive relationship was found, which means that species abundance for this order increases when the imidacloprid concentration in surface water increases. This was also found for the Actinedida species *Limnesia undulata*. *Polypedilum nubeculosum*, a species of Diptera, also showed a positive relationship ($\beta=0.187$, $P=0.008$), while *Glyptotendipes pallens*, the most abundant Diptera species in the water samples, had a significant negative relationship ($\beta=-0.434$, $P=0.001$). For the orders Neotaenioglossa (sea snails) and Trichoptera (caddisflies), one of the three most abundant species showed a significant negative relationship as well. The F ratio in table 1 indicates the ratio of the explained variance over the unexplained variance. The r^2 values in table 1 show that the oversimplified linear regression model leaves the major part of the variability unexplained. Note that we pooled all data irrespective of the time of the year of sampling, this means that the seasonal cycles in abundance may account for a substantial part of the variability for many species.

Water Quality Norms and Aquatic Macro-invertebrate Abundance

The three environmental risk limits used in the Netherlands to help achieve environmental quality are not met in many parts of the country [42]. This may influence species abundance in the surface water. Figure 2 shows the mean species abundance above and below the environmental risk limits for all species pooled. Clear and significant differences were found between species abundance below and above the limits of two water quality norms. The strictest norm, the MTR of 13 ng l^{-1} imidacloprid in surface water, showed the highest difference in average species abundance: a 3-fold difference (Mann-Whitney U test: $P<0.001$). The less strict MPC-norm, of 67 ng l^{-1} imidacloprid, also showed a significant difference in species abundance below and above the limit ($P<0.001$), but here the difference was smaller: a 2-fold difference. The MAC-norm of 200 ng l^{-1} imidacloprid in surface water, which is about 15 times less strict than the MTR-norm, showed a smaller difference in species abundance which was not significant ($P=0.065$).

Discussion

Visual inspection of the scatter plots convincingly shows that at high imidacloprid concentrations, high macro-fauna abundance is rare in comparison to high abundance at low imidacloprid concentrations. The simplified regression analysis showed a significant negative relationship between imidacloprid concentration and macro-invertebrate abundance. Such an association does not necessarily imply that imidacloprid is the main cause for lower species abundance, as there can be other factors and confounders that play a role in the observed patterns of abundance. In 1965 Sir Austin Bradford Hill [50] introduced nine criteria for distinguishing between a chance association and a true cause and effect: 1. strength of association, 2. consistency, 3. specificity, 4. temporality, 5. biological gradient, 6. biological plausibility, 7. biological coherence, 8. experimental evidence, and 9. analogy. These criteria are widely used by epidemiologists nowadays [51,52]. Their usefulness for the scientific inquiry on causal links, and for the justification of policy intervention based on the available evidence, has been widely recognized [53]. We will briefly discuss how the link between imidacloprid and reduced species abundance scores on these criteria.

Firstly, our statistical analysis shows a high strength of association with a high significance. The second criterion, consistency, also scores high; in our dataset we made a few random subsets of our data and found that the correlation (for all species pooled) is not sensitive to the years that we include in the analysis nor to the areas that we include: the pattern is consistent across time and space. Regarding the third criterion, specificity, the score is low because there are many potential factors that could reduce species abundance. However, the reason why we focussed our analysis on imidacloprid is that since 2004, it has been the insecticide with the highest number of samples that exceed the Dutch aquatic toxicity norm for surface water. On average, about half of all samples from the years 2004 to present in the nation-

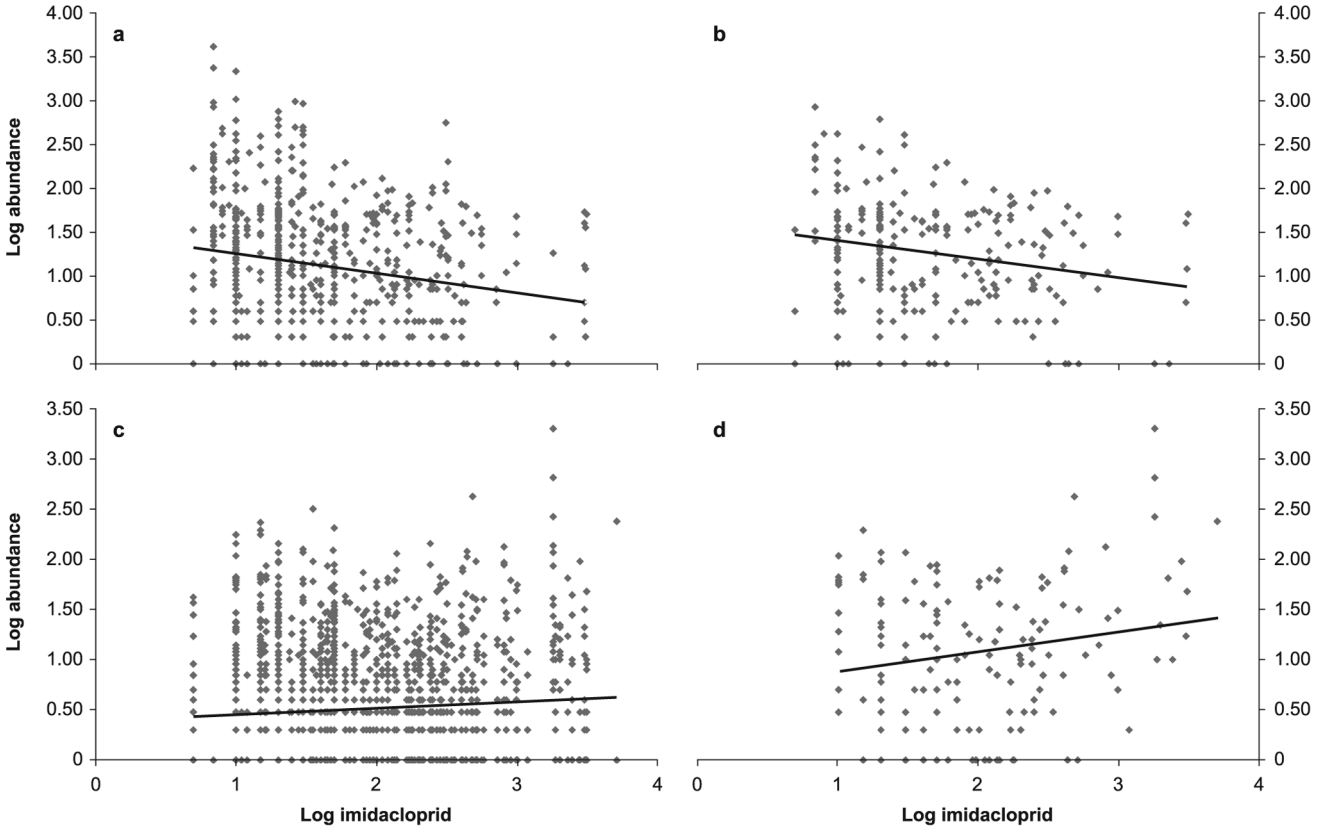

Figure 1. Relationship between log_{10} imidacloprid concentration and log_{10} Amphipoda and Actinedida abundance in surface water. a) Amphipoda ($P = <0.001$), b) its most abundant species *Gammarus tigrinus* ($P = 0.001$), c) Actinedida ($P = <0.001$), d) its most abundant species *Limnesia undulata* ($P = 0.022$).
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wide monitoring program violates this standard. Further, in these samples, the distance to the norm is extreme compared to other agrochemicals in the same surface waters. For that reason imidacloprid is a prime suspect compared to other pesticides. Also, we tested for spatial autocorrelation in the abundance data, both in the untransformed and in the log-transformed data, and did not find any autocorrelation (data not shown here). Consequently we have no reason to assume that landscape quality would be a major confounder in our case, but we cannot completely exclude it either. The fourth criterion scores high as in combining the datasets from biological and chemical sampling the date of the chemical sample is always before or at the date of the biological sample. We specifically used a range of 0 to 160 days for the (one way) time difference between the biological sampling and the chemical sampling, which is long enough for sublethal and chronic mechanisms to induce effects at population and community levels. For criterion 5, biological gradient, it is obvious from the data plots and the regression analysis that increased exposure to imidacloprid is associated with an increased effect. There clearly is a biological gradient, so this criterion also scores high. As regards criterion 6, the present day knowledge on sublethal effects of neonicotinoids on invertebrate reproduction adds to the biological plausibility that imidacloprid is indeed the main causal factor. On top of that, recent insights on the chronic toxicity profile of neonicotinoids, in particular the notion that the toxicity is reinforced by exposure time [24], implies that even the lowest concentrations, when sustained over a long period, will negatively impact invertebrates. Criterion 7, biological coherence, also scores high. Our study is consistent with a wide range of earlier studies as we will discuss further on in this section. The link between abundance and imidacloprid also scores high on criterion 8, experimental evidence. A large number of laboratory studies and mesocosm expriments discussed earlier in this paper all confirm the high toxicity of imidacloprid on invertebrates and clearly indicate community effects. Finally, the link also scores high on criterion 9, analogy, because for other neonicotinoids such as thiacloprid similar strong effects on community level have been observed in mesocosms (e.g. [54]).

While we still cannot exclude that our analysis overlooked confounders, the application of the causality criteria provides strong grounds to believe that the link between imidacloprid and abundance is indeed causal. Still, it remains advisable to further investigate whether a multivariate regression analysis, using a wider range of suspect chemicals still pinpoints imidacloprid as the main suspect, but the present data availability limits the statistical power of such a multivariate analysis, making extension of the systematic chemical and biological monitoring programs of surface water advisable as well.

Our findings are consistent with many other studies (see references in [47]) which reported a negative impact of neonicotinoid insecticides on a high number of non-target species. Flying insects appeared to be the most vulnerable to neonicotinoids in these studies [10,23,55–57]. In this study, the vulnerability to neonicotinoids of flying insects with an aquatic larval stage was also demonstrated: a significant negative relationship was found for the orders Diptera and Ephemeroptera, and a nearly

Table 1. Results of regression analysis on the relationship between imidacloprid concentration and species abundance for all macro-invertebrate orders together, for orders with a total species abundance n >300, and for the three most abundant species of each order.

Order	Species	F	β	n	P	r^2
All orders		71.863	−0.062	18898	<0.001 *	0.004
Amphipoda		21.733	−0.180	652	<0.001 *	0.032
	Gammarus duebeni	3.966	−0.364	28	0.057	0.132
	Gammarus tigrinus	10.984	−0.206	249	0.001 *	0.043
	Gammarus zaddachi	0.848	−0.257	14	0.375	0.060
Actinedida		12.206	0.075	2148	<0.001 *	0.006
	Arrenurus sinuator	0.516	0.062	134	0.474	0.004
	Limnesia undulata	5.373	0.185	153	0.022 *	0.034
	Unionicola crassipes	0.365	−0.058	112	0.547	0.003
Basommatophora		12.649	−0.086	1684	<0.001 *	0.007
	Gyraulus albus	5.410	−0.172	179	0.021 *	0.030
	Hippeutis complanatus	3.635	−0.181	109	0.059	0.033
	Physella acuta	2.523	−0.127	155	0.114	0.16
Coleoptera		0.435	0.018	1379	0.510	<0.001
	Haliplus fluviatilis	0.777	0.110	66	0.381	0.012
	Noterus clavicornis	0.145	0.041	86	0.705	0.002
	Noterus crassicornis	0.100	0.039	68	0.752	0.002
Diptera		25.799	−0.073	4757	<0.001 *	0.005
	Endochironomus albipennis	2.296	−0.101	227	0.131	0.010
	Glyptotendipes pallens	13.452	−0.434	60	0.001 *	0.188
	Polypedilum nubeculosum	7.122	0.187	198	0.008 *	0.035
Ephemeroptera		11.926	−0.157	471	0.001 *	0.025
	Caenis horaria	9.170	−0.352	67	0.004 *	0.124
	Caenis robusta	3.149	−0.174	103	0.079	0.030
	Cloeon dipterum	1.882	−0.098	197	0.172	0.010
Hemiptera		2.490	−0.040	1583	0.115	0.002
	Micronecta scholtzi	0.252	0.048	111	0.617	0.002
	Plea minutissima	0.448	−0.085	64	0.506	0.007
	Sigara striata	0.231	−0.031	246	0.631	0.001
Isopoda		5.127	−0.102	493	0.024 *	0.010
	Asellus aquaticus	0.011	−0.007	247	0.915	<0.001
	Proasellus coxalis	5.142	−0.210	114	0.025 *	0.044
	Sphaeroma hookeri	1.292	−0.252	21	0.270	0.064
Neotaenioglossa		0.260	−0.240	450	0.610	0.001
	Bithynia leachi	0.481	0.065	114	0.489	0.004
	Bithynia tentaculata	3.530	0.132	202	0.062	0.017
	Potamopyrgus antipodarum	7.155	−0.276	89	0.009 *	0.076
Odonata		3.817	−0.079	604	0.051 *	0.006
	Erythromma najas	0.480	−0.143	25	0.495	0.020
	Erythromma viridulum	0.594	−0.144	30	0.447	0.021
	Ischnura elegans	6.164	−0.175	197	0.014 *	0.031
Rhynchobdellae		0.006	−0.003	924	0.937	<0.001
	Alboglossiphonia heteroclita	0.169	−0.042	100	0.682	0.002
	Helobdella stagnalis	0.598	0.053	215	0.440	0.003
	Theromyzon tessulatum	0.455	−0.088	61	0.502	0.008
Trichoptera		0.157	−0.019	447	0.692	<0.001
	Mystacides longicornis	0.208	−0.071	43	0.651	0.005
	Oecetis lacustris	7.118	−0.397	40	0.011 *	0.158

Table 1. Cont.

Order	Species	*F*	*β*	*n*	*P*	r^2
	Triaenodes bicolor	0.461	0.127	30	0.503	0.016
Tubificidae		1.570	−0.035	1254	0.210	0.001
	Ophidonais serpentina	0.029	−0.018	89	0.865	<0.001
	Stylaria lacustris	0.873	−0.075	157	0.351	0.006
	Tubifex costatus	0.008	−0.032	10	0.930	0.001
Veneroida		0.081	−0.012	591	0.776	<0.001
	Dreissena polymorpha	0.014	−0.019	41	0.906	<0.001
	Pisidium nitidum	0.313	−0.068	69	0.578	0.005
	Sphaerium corneum	0.023	0.020	58	0.881	<0.001

*Indicates a significant relationship at $P<0.05$. *F* is the *F* ratio, β is the slope of the regression line. The data are log transformed so the numbers are dimensionless.
doi:10.1371/journal.pone.0062374.t001

significant relationship for Odonata (table 1). The caddisfly *Oecetis lacustris* of the order Trichoptera showed a strong negative relationship as well. Trichoptera are widely used in water quality assessments [58–60] and a high species richness of this order is generally assumed to indicate a good water quality. The strong decline we found for *Oecetis lacustris* at locations with higher imidacloprid concentrations can be seen as an indication that imidacloprid is an important factor reducing water quality.

With our approach we found effects at lower concentrations than known from mesocosm studies. A possible explanation is that mesocosm studies may underestimate the long term effects because the recovery observed in mesocosm studies is probably due to re-colonization by external individuals, not by recovery of the individuals affected by the exposure.

A reverse effect was found for the order Actinedida: our regression analysis showed a significant positive relationship between imidacloprid concentration and Actinedida abundance. This is consistent with the results of Szczepaniec et al. [61] who found spider mite outbreaks after the use of imidacloprid on trees. The outbreaks were probably caused by a positive effect of imidacloprid on mite reproduction by increasing the hatch rate [62]. However, positive relationships are exceptional in the case of imidacloprid (see table 1).

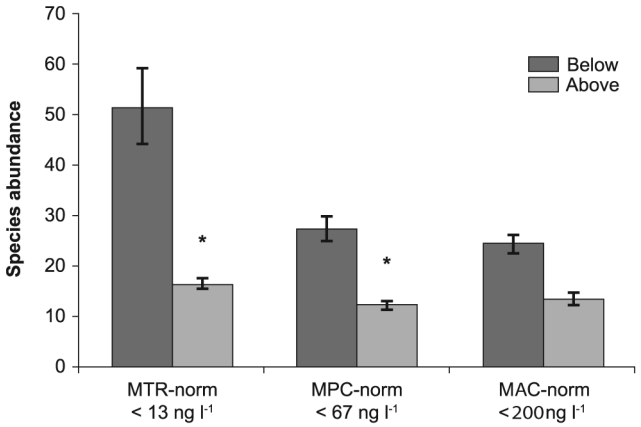

Figure 2. Macro-invertebrate abundance in surface water samples below and above Dutch imidacloprid norms for surface water. Mean and standard error of abundance is shown. We used median imidacloprid concentrations. Dependent variables were tested separately using the Mann-Whitney test. *Indicates significant differences at $P<0.05$. MTR = Maximum Permissible Risk imidacloprid, MPC = Maximum Permissible Concentration imidacloprid, MAC = Maximum Acceptable Concentration imidacloprid (see text).
doi:10.1371/journal.pone.0062374.g002

Besides the direct negative effects found on species living in the water, indirect effects of imidacloprid on the food chain can be expected as well. Experiments in imidacloprid-treated rice fields by Hayasaka et al. [63] showed direct negative effects on the species abundance of the zooplankton community, leading to the indirect effect of growth suppression in the fish feeding on the zooplankton species. Sanchez-Bayo and Goka [64] found indirect effects on algae growth in rice fields, after changes of the arthropod communities induced by imidacloprid. Indirect effects of the neoniconinoid thiacloprid on the food chain and ecosystem functions were also observed by Englert et al. [65] in a study on predator-prey interactions of gammarids and mayflies. Increased thiacloprid concentrations in surface water increased predation by *Gammarus fossarum* (Amphipoda) on *Baetis rhodani* (Ephemeroptera) nymphs, probably because of the impairment by thiacloprid of the predator avoidance behavior of *B. rhodani*. With the increased consumption of *B. rhodani* nymphs by *G. fossarum*, a reduction was observed in leaf consumption by *G. fossarum*, which can be explained by the preference of *G. fossarum* for food of high nutritional value. This reduced leaf consumption may translate into impairment of the ecosystem function of leaf litter breakdown. Other studies on aquatic decomposer organisms [11,66] also showed significant adverse effects (feeding inhibition) of imidacloprid on aquatic insects and high mortality. Antipredator responses to imidacloprid exposure were found by Pestana et al. [67] in the zooplanktonin grazer *Daphnia magna*.

Even at low levels of toxicants community-level effects can be found, as was shown in another study [54]. We suggest that not only organism-level effects should be considered for environmental risk assessment of insecticides, but community-level effects as well.

Leaf decomposition by leaf-shredding insects was found to be significantly reduced. Cumulative ecological impacts of insecticides were shown in experiments in rice fields with two successive annual treatments of imidacloprid and fipronil [63]. The abundance of aquatic organisms during both years was significantly lower in both insecticide-treated fields compared to the control, and large changes in aquatic community composition were observed. These results show that the impacts of insecticides cannot be accurately assessed during short-term monitoring studies. Like Wijngaarden et al. [68] suggested, we too recommend that the long-term ecological risks of their residues are

included in an assessment of insecticide effects at the community level.

Besides cumulative effects, imidacloprid is also known to act synergistically with other chemicals. For instance, eight days' exposure to a mixture of the nonylphenol polyethoxylate, R-11 and imidacloprid resulted in a population size which was three times smaller than with R-11 alone, and 13 times smaller than with imidacloprid alone in the crustacean *Ceriodaphnia dubia* [69]. The 96-h LC_{50} for imidacloprid in the presence of atrazine was significantly lower compared to imidacloprid alone for the daggerblade grass shrimp *Palaemonetes pugio* [70]. In the work of Loureiro et al. [71] on synergistic effects on *Daphnia magna*, synergism was observed for acute exposures of imidacloprid and thiacloprid mixtures (immobilization), and antagonism for feeding rates at sublethal concentrations. For imidacloprid and chlorpyrifos, antagonism was found in both exposures. In another study three widely used synergists were tested: piperonyl butoxide, triphenyl phosphate, and diethyl maleate. All tested synergists significantly amplified the toxic effect of imidacloprid on the wasp *Diaeretiella rapae*, piperonyl butoxide having the greatest impact [72]. Piperonyl butoxide, triflumizole and propiconazole increase the toxicity to honey bees of imidacloprid 1.70-, 1.85- and 1.52-fold respectively [73]. These substances are putative inhibitors of cytochrome P450s, a group of enzymes involved in the detoxification of xenobiotics such as pesticides, which explains their synergistic action.

Neonicotinoids have cumulative effects with exposure time [26], which become relevant for aquatic organisms which are constantly exposed to low levels of many contaminants. While most pesticides do not have toxic effects below a certain level (NOEC or NOEL), the cumulative effects of neonicotinoids imply that even the lowest concentrations have toxic effects if sustained over a long period, which is especially relevant for species with a long life span or a long aquatic stage [57].

Our results show that aquatic macro-invertebrates in Dutch surface water are less abundant at locations with higher imidacloprid concentrations. This provides reason for concern because the three water quality standards applied in the Netherlands to achieve ecological protection are not met in many parts of the country [42], and especially in agricultural areas with greenhouses and crops like bulbs, where concentrations up to hundreds of $\mu g\ l^{-1}$ imidacloprid are being found in the surface water.

Our results further show that – of the existing norms - the strictest norm, the MTR of 13 ng l^{-1} imidacloprid in surface water, makes the greatest difference for species abundance and is thus the only existing norm that could protect aquatic ecosystems. We cannot exclude that a norm lower than the best current Limit of Reporting of the measurement methods for imidacloprid concentration would even be more effective in protecting aquatic life. For the much less strict MAC-norm of 200 ng l^{-1}, there is no significant difference in average species abundance between the samples from locations where the norm is met and those where the norm is exceeded. It follows from the comparison of protectiveness of the various norms (figure 2) that a major drop in macro fauna abundance occurs when concentrations go up from exceeding 13 ng l^{-1} to exceeding 67 ng l^{-1}. Our findings imply that the MTR-norm of 13 ng l^{-1} seems more like a lowest effect concentration. If adequate protection of aquatic ecosystems is the goal, a stricter norm should be set. If we take a safety factor of 10, a standard of 1 ng l^{-1} is recommendable. Note that this is below the detection limit of the imidacloprid measurement methods currently in use by the Dutch Water Boards.

While a large amount of evidence exists from laboratory single-species and mesocosm experiments, our study is the first large-scale research based on multiple years of actual field monitoring data that shows that neonicotinoid insecticide pollution occurring in surface water has a strong negative effect on aquatic invertebrate life, with potentially far-reaching consequences for the food chain and ecosystem functions. The combination of nation-wide monitoring data on insecticide concentrations and aquatic macro-invertebrates creates a valuable instrument for the analysis of the impacts of different pesticides and the evaluation of environmental policy. Given the fact that the world-wide use of neonicotinoid insecticides is still growing, and given its high leaching potential and its high persistence in water and soil, it is important to sustain and extend chemical monitoring schemes of surface water, and further analysis of the major impacts this pollution has on biodiversity and ecosystem services.

Supporting Information

Figure S1 Relationship between log_{10} imidacloprid concentration and log_{10} Basommatophora and Diptera abundance in surface water. a) Basommatophora ($P<0.001$), b) its most abundant species *Gyraulus albus* ($P=0.021$), c) Diptera ($P<0.001$), d) its most abundant species *Endochironomus albipennis* ($P=0.131$). The first three relationships are significant at $P<0.05$.
(TIF)

Figure S2 Relationship between log_{10} imidacloprid concentration and log_{10} Ephemeroptera and Isopoda abundance in surface water. a) Ephemeroptera ($P=0.001$), b) its most abundant species *Cloeon dipterum* ($P=0.172$), c) Isopoda ($P=0.024$), d) its most abundant species *Asellus aquaticus* ($P=0.915$). The negative relationships for the orders are significant at $P<0.05$.
(TIF)

Figure S3 Relationship between log_{10} imidacloprid concentration and log_{10} Coleoptera and Hemiptera species abundance in surface water. a) Coleoptera ($P=0.510$), b) its most abundant species *Noterus clavicornis* ($P=0.705$), c) Hemiptera ($P=0.115$), d) its most abundant species *Sigara striata* ($P=0.617$).
(TIF)

Figure S4 Relationship between log_{10} imidacloprid concentration and log_{10} Neotaenioglossa and Odonata abundance in surface water. a) Neotaenioglossa ($P=0.610$), b) its most abundant species *Bithynia tentaculata* ($P=0.062$), c) Odonata ($P=0.051$), d) its most abundant species *Ischnura elegans* ($P=0.014$). The negative relationship for the order Odonata is nearly significant at $P<0.05$; the relationship for *Ischnura elegans* is significant.
(TIF)

Figure S5 Relationship between log_{10} imidacloprid concentration and log_{10} Rhynchobdellae and Trichoptera abundance in surface water. a) Rhynchobdellae ($P=0.937$), b) its most abundant species *Helobdella stagnalis* ($P=0.440$), c) Trichoptera ($P=0.692$), d) its most abundant species *Mystacides longicornis* ($P=0.651$).
(TIF)

Figure S6 Relationship between log_{10} imidacloprid concentration and log_{10} Tubificidae and Veneroida abundance in surface water. a) Tubificidae ($P=0.210$), b) its most abundant species *Stylaria lacustris* ($P=0.351$), c) Veneroida

($P = 0.776$), d) its most abundant species *Pisidium nitidum* ($P = 0.578$).
(TIF)

Text S1 The dataset and operations performed on the raw dataset of abundance data.
(DOC)

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Author Contributions

Conceived and designed the experiments: TVD MVS JVDS. Analyzed the data: TVD MVS JVDS. Contributed reagents/materials/analysis tools: TVD MVS JVDS. Wrote the paper: TVD MVS JVDS.

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Evolution of imidacloprid resistance in *Myzus persicae* in Greece and susceptibility data for spirotetramat

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Abstract

BACKGROUND: *Myzus persicae* s.l. is a major crop pest globally and has evolved resistance to a range of insecticide classes making it increasingly difficult to control in some areas. Here we compare bioassay monitoring data for two important compounds, imidacloprid and spirotetramat, on field samples/clones collected in Greece.

RESULTS: A total of 122 aphid samples/clones from central and northern Greece were examined in dose–response bioassays with imidacloprid. There was an overall increase in the level of resistance (resistance factor = 15–40) within tobacco-collected samples from 78.7% in 2007 to 86.7% in 2015. The corresponding frequencies for peach samples were 13.3% and 6.7%. These results were confounded however by the first identification of the R81T target mutation in Greece during 2015 (4.3% as heterozygotes in peach) and 2016 (21.3% as heterozygotes in peach). No resistance to spirotetramat was found at the 60 clones collected in 2015.

CONCLUSION: Resistance to imidacloprid is continuing to increase within Greek *M. persicae* s.l. populations and the situation is likely to deteriorate further with the recent identification of the R81T resistance mutation. Resistance to spirotetramat has not been found and is therefore a good alternative to neonicotinoids for resistance management.

Supporting information may be found in the online version of this article.

Keywords: neonicotinoids; spirotetramat; insecticide resistance; R81T; tobacco; peach

1 INTRODUCTION

Integrated pest management (IPM) in modern agriculture encompasses a range of approaches (e.g. biological, cultural, genetic, mechanical/physical and chemical control). However, in many cases chemical insecticides remain the primary tool for efficient pest control and with this comes the increasing problem of how to tackle the issues arising from insecticide resistance. Most people agree that insecticide resistance management (IRM) is the way to deal with the problem and is based largely on rotation approaches which use a 'window' or block strategy frequently defined by pest life cycle and the crop growth stages. These strategies involve alternation of insecticides with different mode of actions (MoAs). Furthermore, documentation and communication of the related data and information to scientists and growers are of primary importance (Insecticide Resistance Action Committee, IRAC, http://www.irac-online.org).[1,2] The challenge becomes more prominent as the number of available compounds is further reduced due to safety and regulatory restrictions and the costly and time-consuming development of new chemicals.[1,3] Therefore, the preservation of efficacy of current and new insecticides is an important aim for stakeholders involved in plant protection. Given that insecticide resistance is a dynamic phenomenon, studies on the long-term resistance status of the pest population in a given area/country provide valuable information for this aim to be achieved.

The green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), is a widespread pest species that has been recorded worldwide and is highly polyphagous, feeding on more than 400 plant species of 40 plant families, including many economically important crop plants. It causes damage by direct feeding and/or by virus transmission as it is a highly efficient plant-virus vector

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transmitting more than 100 plant viruses (see also Blackman RL, www.aphidsonworldsplants.info).[4,5] *M. persicae* has a typical aphid annual cycle (cyclical parthenogenesis) including a sexual generation on peach, *Prunus persica* (L.) (Rosaceae), in autumn, when the over-wintering eggs are laid, alternating with a number of asexual (all female; apomictic parthenogenesis) generations during spring on peach and on various herbaceous host-plants in summer and autumn. The sexual generation may be lost either totally (obligate parthenogenesis) or partially (functional parthenogenesis) when a few sexual forms are produced. Another trait of *M. persicae* is host-specialisation with the typical example of populations feeding on tobacco *Nicotiana tabacum* L. (Solanaceae) ('tobacco aphid') for which the sub-species name *Myzus persicae nicotianae* Blackman has been given.[5,6]

Owing to its economic importance, *M. persicae* s.l. is the target of extensive control programmes based largely on synthetic insecticides. As a response to the high selection pressure applied by the insecticides, the aphid has developed resistance to most chemical groups used for its control either through metabolic pathways or through mutations in the protein targets of insecticides.[7] *M. persicae* is among the 12 most resistant insect species and has developed resistance to 75 different compounds.[1] In Greece, both *M. persicae* s.s. and *M. persicae nicotianae* are economically important pests. Both taxa cause serious damage to peach orchards, while the latter also attack tobacco crops by direct feeding and transmission of non-persistent plant viruses.[8] The aphids are controlled mostly with chemical insecticides, with neonicotinoids proving to be most effective since their introduction in the 1990s.

The neonicotinoids are highly selective agonists of the insect nicotinic acetylcholine receptor (nAChR) (IRAC MoA group 4). They have been a valuable tool for pest control; however, as with other classes, their extensive and widespread use has led to cases of resistance which can compromise successful control.[9] In Greece, studies on populations mostly from peach and tobacco during the periods 1998–2007 and 2012–2013 revealed high resistance to organophosphates, carbamates and pyrethroids with several clones/field samples also exhibiting resistance to the neonicotinoid imidacloprid.[10–13] Elsewhere, neonicotinoid resistance in *M. persicae* has been associated with P450-mediated detoxification due to the amplification of the *CYP6CY3* P450 gene[13] and to the R81T mutation in the nAChR β sub-unit that makes the target-protein insensitive.[14] In our previous study, the R81T mutation was not found in 283 *M. persicae* s.l. clones collected in Greece during 2012–2013,[12] but P450-mediated detoxification was found to be involved in the neonicotinoid resistance of the samples examined in that study. The R81T mutation is, however, widespread in populations from peach in southern France, northern Spain and Italy (also in some samples from herbaceous hosts; see Bass *et al.*[7] and references therein).

Spirotetramat is a tetramic acid derivative, systemic insecticide (upward and downward moving through xylem and phloem), which has been developed for the control of sucking pests including aphids. It is an inhibitor of lipid biosynthesis (IRAC MoA group 23)[15,16] and is particularly effective on juvenile stages, while also reducing the fertility and fecundity of adult female aphids.[15–17] Field studies performed worldwide have shown that spirotetramat provided a very good level of control against various aphid species and other sucking pests and it has shown only low adverse effects on beneficial arthropods.[15,16] In Greece spirotetramat has been registered recently (2013) against *M. persicae* in various crops. The novel MoA, the high efficacy and the low adverse effects on beneficial arthropods, make spirotetramat a valuable tool for the control of populations that have developed resistance to other insecticides. There are no studies on the resistance status of *M. persicae* in Greece, although resistance has been reported in other species in various counties (see Discussion)[18–23] and therefore presents a potential risk in *M. persicae* especially in crops under intense chemical protection such as peach and tobacco in Greece.

Figure 1. Sampling sites in Greece. Samples tested for the R81T mutation were collected from all sites except Goules. Samples for the dose–response bioassays were collected from Meliki, Katerini, Velventos, Goules, Vathylakkos, Tyrnavos and Lehonia.

Within the framework of constant monitoring of *M. persicae* populations in Greece for insecticide resistance, the present study monitored with dose–response bioassays 122 field samples/clones (2007 and 2015 collections) for imidacloprid and 60 clones for spirotetramat (2015 collection). We further examined 925 aphid individuals from various regions and hosts in Greece during 2012–2016 for the presence of the R81T mutation.

2 MATERIALS AND METHODS

2.1 Aphid samples

Leaf samples infested with *M. persicae* s.l. were collected from peach orchards (2–3 ha) and tobacco fields (>5 ha) from various regions in mainland Greece in 2007 and 2015 (Fig. 1). Peach orchards were surveyed in late April to early June and one aphid sample was collected every four to five trees along the row. In tobacco fields, leaf samples were collected in June to August and one aphid sample was collected from infested plants every four to five rows and every 5 m along the row.

In 2007 the field samples were examined directly in dose–response bioassays using the FAO dip test.[24] One or two aphid samples (the collected leafs were pooled) from each orchard or field were examined. In each sample, only adult wingless females of one colour morph (red or green) were included, usually the most abundant morph. In 2015, aphid parthenogenetic lineages (clones) were established in the laboratory from one adult wingless female from each leaf sample. The clones were reared on Chinese cabbage *Brassica rapa pekinensis* Hanelt (Brassicaceae) leaves in Blackman boxes[25] at 20 °C, 16:8 h light:dark photoperiod and relative humidity (RH) 50–60% until dose–response bioassays were performed with a leaf dip test method.[16] One to two clones

Table 1. Number of field samples and clones of *Myzus persicae* s.l. examined in the bioassays with imidacloprid (in 2007 and 2015) and spirotetramat (in 2015)

		Field samples in 2007			Clones in 2015		
		Peach	Tobacco		Peach	Tobacco	
Region	Locality	Green	Green	Red	Green	Green	Red
Northern Greece	Meliki	9	10	10	10	6	4
	Katerini	–	9	10	–	7	3
	Velventos	–	–	–	10	–	–
	Goules[a]	–	4	4	–	–	–
	Vathylakkos[a]	–	–	–	–	6	4
						–	–
Central Greece	Tyrnavos	–	–	–	10	–	–
	Lehonia	6	–	–	–	–	–
Total		15	23	24	30	19	11

[a] Goules and Vathylakkos are near Velventos.

from each tobacco field or peach orchard were established and tested.

A total of 62 field samples (2007) and 60 (2015) clones of *M. persicae* s.l. were examined (Table 1 gives details of aphid collections). The aphids were collected mostly from tobacco-growing regions in northern Greece (56 field samples and 50 clones). Tobacco is not cultivated in the localities surveyed in central Greece. On the basis of previous findings, a reasonable working assumption is that the aphids from the tobacco-growing regions belong to the tobacco-adapted sub-species *M. persicae nicotianae*.[26–28] The insecticide-susceptible clones US1L and 4106A were used for comparison in the 2007 and 2015 bioassays, respectively. US1L was originally collected from sugar beet in England in 1974 and 4106A from potatoes in Scotland in 2000.[13,14,29]

2.2 FAO dip tests with imidacloprid (samples from 2007)

In the dip tests we used nine to 11 different concentrations (including only water as control) of water dispersions of imidacloprid (Confidor 200SL; Bayer CropScience Hellas, Marousi, Greece). Adult aphids from the field samples were dipped in the solutions for 10 s and then batches of 10 aphids were carefully brushed onto Chinese cabbage leaf discs placed on 1.1% agar (Applichem, Darmstadt, Germany) in plastic dishes (3.5 cm diameter). The lid had a hole, 2 cm in diameter, covered with fine muslin to allow ventilation. Usually 20 (19–23) adult wingless females were used per insecticide concentration (25–29 for the susceptible US1L). Treated aphids were kept at 23 ± 1 °C with a 16:8 h light:dark photoperiod and RH 50–60%, and their mortality was scored 48 h post-treatment. Dead and poorly co-ordinated aphids (irreversible symptoms) were classified together as affected.[30]

2.3 Leaf dip tests with imidacloprid and spirotetramat (clones from 2015)

Young (1–2 days old) adult wingless females were used in the bioassays with imidacloprid. Three- to four-day-old nymphs were assayed with spirotetramat because it is a lipid biosynthesis inhibitor and particularly effective against juvenile stages of sucking pests.[15,16] In the bioassays seven to nine different concentrations (including only water as control) of water dispersions of imidacloprid (Confidor 200SL; Bayer CropScience Hellas) and spirotetramat (Movento Gold 10SC; Bayer CropScience Hellas) were used. A total of 20 aphids per concentration were used for each clone (60 for the susceptible 4106A). Young leaves of Chinese cabbage were dipped for 10 s in the solutions and placed on 1.1% agar in plastic dishes (described above). In each plastic dish, 10 adults (for imidacloprid assays) or nymphs (for spirotetramat assays) were placed with a paint brush. Treated aphids were maintained at 21 ± 1 °C with a 16:8 h light:dark photoperiod and RH 50–60% and mortality was assessed after 72 h. Dead and poorly co-ordinated aphids (irreversible symptoms) were classified together as affected.[16]

2.4 Survey for the R81T mutation

In order to increase the probability of detection of the R81T mutation, we collected samples from different geographic areas in Greece. We also included samples of both sub-species, two colour forms and from both primary (peach) and secondary (pepper, tobacco, weeds) hosts.

A total of 925 aphid individuals were examined (Table 2). Aphids were collected following the sampling design described above and one individual per plant/tree was examined. Most of the aphids (75.7%) were collected from tobacco growing areas in northern Greece. The remaining aphids (24.3%) were from localities in central (Lehonia, Tyrnavos), south (Patras, Argos) and western (Ioannina) Greece and Crete where tobacco is not cultivated. The colour of the aphids was recorded (84.5% were green and the remaining were red) and then stored in absolute ethanol at −20 °C until analysis.

Genomic DNA was extracted from individual aphids using the sodium hydroxide method.[31] A new RFLP-based diagnostic[12] was used for the detection of the R81T mutation in 776 aphids collected in the years 2012–2015. In addition, 198 aphids from 2015–2016 were examined with a real-time TaqMan assay.[32] A subset (49) of the 2015 aphids were tested with both methods to validate the results.

2.5 Statistical analysis

LC_{50} values were calculated by probit analysis using Simply Probit 1.3 (Pisces Conservation Ltd, Lymington, UK). Simply Probit uses the maximum likelihood method of Finney[33] to undertake

Table 2. *Myzus persicae* s.l. individuals from peach (P), tobacco (T), pepper (Pep) and weeds (W) that were examined for the R81T mutation

		2012			2013			2014			2015			2016	
Region[a]	Locality	Pep	T	W	P	T	W	P	Pep	T	P[b]	Pep	T	P[b]	T
CG	Lehonia	–	–	–	10	–	–	–	–	–	–	–	–	–	–
	Tyrnavos	–	–	–	5	–	–	20	–	–	41	–	–	30 (36.7)	–
Crete	Ierapetra	–	–	–	–	–	–	–	10	–	–	–	–	–	–
	Lasithi	–	–	–	–	–	–	–	–	–	–	20	–	–	–
	Tymbaki	–	–	–	–	–	–	–	15	–	–	–	–	–	–
NG	Alexandria	–	–	–	1	–	–	–	–	–	–	–	–	–	–
	Katerini	–	31	–	1	15	–	–	–	30	–	–	44	–	–
	Meliki	21	3	1	85	134	10	20	–	30	48 (12.5)	–	46	10 (20.0)	–
	Vathylakkos	–	–	–	–	–	–	–	–	–	–	–	40	–	–
	Velventos	–	–	–	–	–	–	20	–	–	51	–	–	20 (5.0)	39
SG	Argos	–	–	–	14	–	–	–	–	–	–	–	–	20 (15.0)	–
WG	Patras	–	–	–	–	–	–	–	–	–	–	20	–	–	–
	Ioannina	–	–	–	–	–	–	–	–	–	–	20	–	–	–
Total		21	34	1	116	149	10	60	25	60	140	60	130	80	39

In brackets percentage (%) of heterozygous individuals for the resistant allele. All the other individuals were homozygous for the susceptible allele.
[a] NG, northern Greece; CG, central Greece; SG, southern Greece; WG, western Greece. Vathylakkos is near Velventos.
[b] The heterozygotes in the total sample from peach were 4.3% and 21.3% in 2015 and 2016, respectively.

the probit analysis. The mean LC_{50} values were compared between regions and crops using the non-parametric Kruskal–Wallis H test because the data deviated from normality (Shapiro–Wilk normality test). Pairwise post-hoc comparisons were made using the Tukey and Kramer (Nemenyi) test after Kruskal–Wallis H test. Frequencies of resistant samples were compared using the χ^2 test (with Yates' correction). In more than two frequencies when χ^2 returned a significant value, pairwise comparisons were performed using the Bonferroni correction. These analyses were conducted using R.[34]

3 RESULTS

The LC_{50} and the resistance factor (RF) values of the bioassays performed in 2007 and 2015 are summarised in Table 3 and Table 4, respectively, while the detailed data are given in Tables S1, S2 and S3 (supporting information). In all bioassays the response data fitted well the probit model (non-significant χ^2 values, $P > 0.05$).

In 2007 bioassays with imidacloprid, a field sample (07VelP02) showed a lower LC_{50} value compared to the laboratory susceptible US1L clone. The difference is statistically significant because the calculated 95% confidence intervals of lethal dose ratio (=RF) between 07VelP02 and US1L does not include 1 (i.e. 2.3, 1.3–4.3; ratio test described by Robertson *et al.*[35]). In addition, the likelihood ratio tests showed that the two probit regression lines are not equal ($\chi^2 = 10.26$, $df = 2$, $P = 0.006$; significant different intercepts), although the hypothesis of parallelism (i.e. equal slopes) is not rejected ($\chi^2 = 1.53$, $df = 1$, $P = 0.216$). Both ratio and likelihood ratio tests were performed with PoloPlus 2.0 (LeOra Software, Berkeley, CA, USA). Because we want to present the actual range of the RF values in the surveyed populations we used the 07VelP02 sample for their calculations (LC_{50} of a field sample/LC_{50} of 07VelP02). In the 2015 bioassays with spirotetramat, a field collected clone (15KatT01) had lower LC_{50} value compared to the laboratory susceptible clone (4106A), but the difference was not significant (95% confidence intervals of the lethal dose ratio included 1; hypothesis of equal lines – equal slopes and intercepts – was not rejected, $\chi^2 = 3.60$, $df = 2$, $P = 0.165$). However, for the aforementioned reason we used 15KatT01 for the RF values calculations. The RF values calculated using the susceptible laboratory clones are presented in Tables S1 and S3 (supporting information) for tentative comparisons. In 2015 bioassays with imidacloprid the laboratory susceptible clone 4106A showed the lowest LC_{50} value and it was used for RF calculations.

3.1 Bioassays with imidacloprid

The LC_{50} values in the aphid samples that were collected in 2007 and examined with the FAO dip test, ranged from 0.317 mg L^{-1} (active ingredient) in a sample from peach in Lehonia, central Greece to 11.860 mg L^{-1} in a sample from tobacco in Meliki, northern Greece (Table 3 and Table S1). Significant differences in the mean LC_{50} values were observed among regions/crops (Kruskal–Wallis H test, $\chi 2 = 30.58$, $df = 4$, $P = 0.001$). The mean values were lower in peach samples compared to those from tobacco and the significantly lowest mean value was observed in the peach samples from Lehonia. Furthermore, in the Meliki locality where tobacco fields are located near peach orchards, the mean LC_{50} value was significantly higher in the tobacco samples than those from peach (Table 3).

In the 2015 aphid clones, the LC_{50} values obtained by leaf dip tests ranged from 13.560 mg L^{-1} (active ingredient) in a clone from peach in central Greece (Tyrnavos locality) to 128.000 mg L^{-1} in a clone from tobacco in Meliki, northern Greece (Table 3 and Table S2). The LC_{50} values recorded in 2015 bioassays were ~10-fold higher than those in 2007. Presumably this is because of the different bioassay methods used. The differences among regions/crops followed the pattern observed in 2007. The mean LC_{50} values were lower in aphid samples from peach compared to those from tobacco, with the significant lowest mean values observed in the peach samples from Velventos, northern Greece and Tyrnavos, central Greece (Kruskal–Wallis H test, $\chi^2 = 42.49$, $df = 5$, $P < 0.001$). Significant differences were observed between peach and tobacco samples collected from the same (Meliki) or nearby (Velventos: peach, Vathyllakos: tobacco) localities in northern Greece (Table 3).

The frequencies of RF values showed similarities between the aphid samples collected in 2007 and 2015. The frequency of

Table 3. Summary statistics for the LC_{50} values (in mg L^{-1}) from bioassays with imidacloprid and spirotetramat according to region and host

Parameter	Host	Region[a]	Locality	N[b]	Mean ± SE[c]	Min	Max
Imidacloprid	Peach	CG	Lehonia	6	1.384 ± 0.281^{a}	0.317	2.172
2007 samples	Peach	NG	Meliki	9	3.696 ± 0.432^{ac}	1.100	5.416
FAO dip test	Tobacco	NG	Meliki	20	7.462 ± 0.560^{b}	3.415	11.860
	Tobacco	NG	Katerini	19	6.194 ± 0.601^{bc}	2.380	11.260
	Tobacco	NG	Goules	8	4.909 ± 0.461^{ab}	2.461	6.852
07VelP02	–	–	–	–	0.317	–	–
Imidacloprid	Peach	CG	Tyrnavos	10	24.515 ± 2.650^{a}	13.560	41.810
2015 samples	Peach	NG	Meliki	10	39.450 ± 5.167^{ab}	17.730	63.810
Leaf dip test	Peach	NG	Velventos	10	28.129 ± 3.805^{a}	13.970	49.930
	Tobacco	NG	Meliki	10	85.862 ± 7.568^{c}	50.300	128.000
	Tobacco	NG	Katerini	10	66.317 ± 4.962^{bc}	45.970	88.890
	Tobacco	NG	Vathylakkos	10	69.713 ± 5.457^{c}	46.500	108.700
4106A	–	–	–	–	3.442	–	–
Spirotetramat	Peach	CG	Tyrnavos	10	0.856 ± 0.024^{a}	0.724	0.976
2015 samples	Peach	NG	Meliki	10	0.842 ± 0.039^{a}	0.622	1.042
Leaf dip test	Peach	NG	Velventos	10	0.881 ± 0.034^{a}	0.716	1.037
	Tobacco	NG	Meliki	10	1.108 ± 0.092^{a}	0.625	1.588
	Tobacco	NG	Katerini	10	0.991 ± 0.091^{a}	0.495	1.399
	Tobacco	NG	Vathylakkos	10	0.928 ± 0.048^{a}	0.724	1.198
15KatT01	–	–	–	–	0.495	–	–

The values from the most susceptible clones/samples are given for comparison.
[a] NG, northern Greece; CG, central Greece. Goules and Vathylakkos are near Velventos.
[b] *N* = number of field samples (in 2007) and clones (in 2015) examined.
[c] Means followed by a different letter differ significantly at $P < 0.05$.

Table 4. Frequency of resistance factors (RFs) in bioassays with imidacloprid and spirotetramat

			RFs for imidacloprid						
Year	Crop	N[a]	1–5	5–10	10–15	15–20	20–30	30–40	45–73
2004–2006	Peach	39	74.36	12.82	10.26	2.56	0.00	0.00	0.00
2004–2005	Tobacco	49	10.20	24.49	30.61	18.37	4.08	4.08	8.16
2007	Peach	15	26.67	26.67	33.33	13.33	0.00	0.00	0.00
	Tobacco	47		6.38	14.89	40.43	19.15	19.15	
2015	Peach	30	16.67	50.00	26.67	6.67	0.00	0.00	0.00
	Tobacco	30	0.00	0.00	13.33	36.67	36.67	13.33	0.00
			RFs for spirotetramat						
			1–4	4–10	10–15	15–20	20–30	30–40	45–73
2015	Peach	30	100	0.00	0.00	0.00	0.00	0.00	0.00
	Tobacco	30	100	0.00	0.00	0.00	0.00	0.00	0.00

Data from pre-2007 surveys are from Margaritopoulos *et al.*[11]
[a] *N* = number of field samples (during 2004–2007) and clones (in 2015) examined.

tobacco samples/clones with RF values of 15–40, which is presumably an indication of resistance build-up, was 78.7% and 86.7%, respectively, for years 2007 (RF range >15: 15.2–37.4) and 2015 (RF range >15: 15.6–37.2) (comparison between years: $\chi^2 = 0.34$, $df = 1$, $P = 0.563$). The corresponding values for the samples/clones from peach were lower, 13.3% (RF range >15: 15.8–17.1) and 6.7% (RF range >15: 17.0–18.5) (comparison between years: $\chi^2 = 0.03$, $df = 1$, $P = 0.853$) (Table 4). Because we compared the RF values for imidacloprid found in our previous survey on Greek populations[11] with those in the present study (see Discussion), we chose to use the same statistical package (i.e. Simply Probit) for the calculations of the lethal doses. This excludes any minor differences due to the different statistical approach used by the packages. In addition, we calculated the RF values for the 2007 field samples using the value of the most susceptible field sample found in our previous survey.[11] The difference in the LC_{50} values between the two susceptible field samples was indiscernible (0.317 found here and 0.306 in our previous study) and not statistically different (95% confidence intervals of the lethal dose ratio included 1; hypothesis of equal lines – equal slopes and intercepts – was not rejected, $\chi^2 = 2.74$, $df = 1$, $P = 0.098$). Therefore, the frequency distribution of the RF values was not altered (results not shown).

3.2 Bioassays with spirotetramat

The LC_{50} values in the clones collected in 2015 ranged from 0.495 mg L^{-1} (active ingredient) in a clone from tobacco in Katerini, northern Greece to 1.588 mg L^{-1} in a clone from tobacco in Meliki, northern Greece. The mean LC_{50} values did not differ significantly among regions/hosts (Kruskal–Wallis H test, $\chi 2 = 9.258$, $df = 5$, $P = 0.099$) and the RF values were very low 1.0–3.2 (Table 3, Table 4 and Table S3).

3.3 R81T mutation

The TaqMan and RFLP-based assays provided the same results for the aphids that were tested with both methods. The R81T mutation was not found in the 476 aphids collected from various hosts during the years 2012–2014. In 2015, the mutation was found only in 12.5% of the aphids from peach in Meliki, northern Greece. All the aphids with the mutation were heterozygous. The frequency of the heterozygous aphids in the total sample from peach and from all hosts were 4.3% (140 aphids; frequency of R allele: 2.1%) and 1.8% (330 aphids; frequency of R allele: 0.9%), respectively. The frequency increased in 2016, although the mutation was again detected only in samples from peach. Only heterozygous aphids were found in four localities, i.e. Argos (southern Greece), Tyrnavos (central Greece), Velventos and Meliki (both northern Greece) at frequencies of 15.0, 36.7, 5.0 and 20.0%, respectively. The frequency of heterozygous aphids in the total sample from peach and from all hosts were 21.3% (80 aphids; frequency of R allele: 10.6%) and 14.3% (119 aphids; frequency of R allele: 7.1%), respectively (Table 2). The χ^2 test showed that the increase in 2016 was statistically significant (peach samples: 4.3 vs. 21.3%; $\chi^2 = 13.89$, $df = 1$, $P < 0.001$).

4 DISCUSSION

Myzus persicae s.l. is one of the most resistant crop pests, exhibiting seven different resistant mechanisms and is therefore a difficult target for applied pest control programmes.[1,7,9]

Apart from the insecticide selection pressure other factors, both biotic (e.g. fitness–cost, life cycle, migration, reproduction rates and population genetics) and abiotic (e.g. crop rotation and distribution, winter severity) are also involved in the evolution of resistance and the spread of the resistance genes.[36,37] In temperate regions like Greece, the aphid populations are often holocyclic (alternating one sexual and many asexual generations) and alternate between peach and herbaceous hosts. These biological traits together with high migration rates, further favour the spread of resistance genes through sexual reproduction and novel resistant genotypes may be created, often carrying more than one resistance mechanism. It is possible for these traits to pass to long persistent asexual genotypes (functional parthenogens), because there is a degree of gene flow between sexually reproducing genotypes and functional parthenogens in aphids.[27,38] Resistance genes may be maintained in the parthenogenetic phase of the typical aphid's life cycle during the growing season or in long persistent asexual populations through many years. Indeed, populations/genotypes that exhibit all or most of the resistance mechanisms are not uncommon in Greece and their control with conventional chemical insecticides is becoming difficult.[11,12] Thus, regular monitoring of the aphid populations accompanied with historical data (see, for example, the evolution of carboxylesterase genes and associated chromosomal rearrangements in the *nicotianae* populations in Greece)[39] helps to advise for the potential of resistance development and provide information for efficient pest control and resistance management. For that reason we compared samples from different regions, crops and years and we included both *M. persicae* sub-species and populations with different frequencies of sexual/asexual genotypes (peach vs. tobacco).[40,41]

4.1 Imidacloprid

The data from the imidacloprid bioassays highlights two interesting points. There is variation in the response among the samples examined in both 2007 and 2015, which is mostly associated with the crop. The frequency of samples from tobacco that showed build-up of resistance (RF > 15) was 78.7% and 86.7% in 2007 and 2015, respectively, while these frequencies were lower in the aphids from peach (13.3% and 6.7%). This trend is also highlighted by the mean LC_{50} values among regions and crops where the differences were significant only between peach and tobacco. Such differences in resistance levels and frequencies of resistance mechanisms have been reported in previous studies focusing on peach and tobacco agro-ecosystems in Greece, and they have been attributed to reasons related to the aphid life cycle and insecticide selection pressure.[11,12] The aphid populations on peach consist of an enormous number of different genotypes as a result of sexual reproduction and often possess a range of resistance profiles (susceptible or resistant). The aphids migrate from peach to tobacco or other herbaceous crops in late May/June. During the whole season (April to early September) the aphid populations are under an intense selection pressure. Usually there are one to two sprays with neonicotinoids in peach orchards and an application in setting water in tobacco crops along with foliar sprays. Thus, in tobacco crops and other herbaceous crops under intense insecticide applications there is a selection of a few aphid resistant genotypes, which are proliferated through asexual reproduction leading to homogenised resistant populations. In addition, there is a movement of asexual genotypes (old clones) that over-winter on weeds to tobacco crops.[27,40,41] These genotypes may show neonicotinoid resistance as they are under selection from the chemical control programmes throughout the years/growing seasons. The data also showed differences, although not significant, in the mean LC_{50} values among regions (comparisons within crops), with the highest values observed in the Meliki locality. This might reflect differences in the intensity of chemical control applications among regions but other traits related to the bio-ecology and the genetic structure of these populations cannot be excluded.

It is also clear that resistance is increasing, especially in tobacco fields where the majority of aphid clones/samples showed moderate to high resistance and levels have increased since our previous monitoring during 2004–2005. The frequency of the RF > 15 was 34.7% in 2004–2005 (pooled data) and increased significantly in 2007 (78.7%) and 2015 (86.7%) ($\chi^2 = 29.19$, $df = 2$, $P < 0.001$; $P < 0.05$ for pairwise comparisons: 2004–2005 vs. 2007 and 2004–2005 vs. 2015). This increase was also observed in the peach samples, although the differences were not significant (2.6, 13.3 and 6.7%; $\chi^2 = 2.29$, $df = 2$, $P = 0.319$) (Table 4). Therefore, there are indications over the last decade that neonicotinoid effectiveness may be compromised in the future. In support of this, we have been informed of nenonicotinoid spray failures in peach orchards in Nausa, northern Greece in 2016 (Karatolos N, personal communication).

Neonicotinoid resistance has been attributed to enhanced detoxification by cytochrome P450s, due to over-expression of the *CYP6CY3* P450 gene[13] and to the R81T mutation in the nAChR.[7,14] Our recent study on Greek clones (2012 and 2013 collections)

concluded that the mechanism associated with imidacloprid resistance is the *CYP6CY3* over-expression (nine- to 36-fold). In that study, the R81T mutation was not detected in 283 clones that were collected from peach (central and northern Greece) and tobacco (northern Greece) in 2012 and 2013.[12] In the present study the sample size was substantially increased (925 aphids of *nicotianae and persicae* sub-species from peach and herbaceous hosts) and was more geographically diverse. The surveys of both studies suggest that the R81T mutation has been introduced into Greece very recently and was found only in peach samples from 2015 and 2016. Only heterozygous resistant genotypes were detected and their frequency was low in 2015 (4.3% in the total sample from peach), although this increased significantly in 2016 (21.3% in the total sample from peach). Because the mutation has been detected only in peach samples, it is probable that the resistant allele invaded Greece through sexual genotype(s) which spread the resistant allele through sexual reproduction events in peach. The increase observed in 2016 could be attributed to two factors: (1) selection of resistant genotypes through intense application of neonicotinoids and (2) spread of the resistant allele from the invader individuals to many genotypes through sexual reproduction on peach. The lack of genotypes carrying the mutation in the tobacco samples, even though the aphid migrates from peach to tobacco in northern Greece (sexual *nicotianae* genotypes),[26,27,40,41] cannot be adequately explained from the current data set. These genotypes maybe selected against in tobacco or have not reached this crop in adequate numbers to be detected in surveys. The R81T mutation has been found in southern France (2009–2010), northern Spain (2010) and Italy (2012).[42,43] These samples were mostly from peach orchards (in Italy some were from herbaceous crops), where control of *M. persicae* now relies on chemicals from different IRAC groups such as pymetrozine, spirotetramat and flonicamid.[7] As is discussed in our previous study, there are some differences in the samples tested from the different countries; e.g. the Greek samples included sexual and asexual genotypes, the *nicotianae* sub-species and many samples from herbaceous hosts.[12] The reasons why the R81T mutation has reached Greece recently and it has been found only in peach samples at low/medium frequencies (12.5% and 5.0–36.7% in peach samples in 2015 and 2016, respectively, were heterozygous) are not fully understood but may be related to minimum gene flow among Greek and western–southern European populations. In Greece there have only been heterozygous individuals detected so far, while in western–southern Europe homozygous R81T individuals have also been found. The reported frequencies were 6–100% in southern France, 17% in northern Spain and 10–100% in Italy. In most sampling sites in these countries, high frequencies of the resistant allele were found.[42,43] Therefore, monitoring should be kept up-to-date as it is likely that this resistance mechanism will spread further in Greece and its frequency will increase quickly. The fact that the R81T was found at low to medium frequencies in peach samples and the lack of this mutation in tobacco samples where the overall resistance to imidacloprid was higher than in those from peach, suggests that the main resistance mechanism to neonicotinoids in Greek *nicotianae/persicae* populations remains *CYP6CY3* over-expression as was suggested in our previous study on Greek populations.[12] More detailed surveys are clearly needed in order to validate the status of the *CYP6CY3* mechanism and this would clarify the relative importance of the two resistance mechanisms, especially in the peach–tobacco agroecosystems where between-crop differences have been observed. The lower resistance levels in peach samples, despite the presence of the R81T allele, could be also attributed to the fact that all genotypes found in this study were heterozygous. In a recent study all homozygous clones of *persicae* for the R81T mutation showed a higher level of resistance to both imidacloprid and thiacloprid than heterozygous and the wild homozygous clones. The authors reported also that the mutant allele is semi-recessive for both insecticides tested.[44]

4.2 Spirotetramat

In the present study no resistance was detected against spirotetramat. The RF values were low (1.0–3.2) and the LC_{50} values (0.495–1.588 mg L^{-1}) were comparable to those reported in a previous study using the same bioassay protocol. The reported LC_{50} values were 0.27–0.69 mg L^{-1} in three *M. persicae* clones, one was a susceptible reference clone while the other two showed resistance to organophosphates, carbamates and pyrethroids and they were collected from Japan and France.[45]

Spirotetramat has been shown to control a wide range of aphid species efficiently, in field trials on a range of crops in different parts of the world, and is also considered to be safe to most beneficial arthropods.[16] Given that *M. persicae* s.l. populations in Greece are susceptible to spirotetramat, it is a valuable tool to control the aphid in IPM schemes, especially in crops/regions where indications for resistance built-up have been noted for other products. It should also be considered an excellent rotation partner with existing aphidicides in IRM strategies.

However, there are published reports of resistance to tetronic acid compounds in other pests. In mite species (*Tetranychus* and *Panonychus*) certain populations/strains have developed resistance to spirodiclofen. The reported RF values for the resistant populations/strains were 12.0–90.8.[20–22,46] In another study on three *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae) populations from Germany, Turkey and UK the RF values for spiromesifen were 4.5–25.7.[23] A laboratory-induced spirotetramat resistant strain of *Aphis gossypii* Glover (Hemiptera: Aphididae) (579- and 15-fold resistance in adults and third instar nymphs, respectively) from China showed significantly increased transcriptional levels of *CYP6A2* P450 gene compared to the susceptible strain.[18] The authors suggested the involvement of the *CYP6A2* gene in the spirotetramat resistance and alpha-cypermethrin cross-resistance observed in this aphid strain.[18,19] *M. persicae* has the capacity to detoxify xenobiotic molecules, with the well documented example being the *CYP6CY3* gene that has been shown to metabolise nicotine and detoxify neonicotinoids.[7,13,47] Considering the traits of aphid biology (short generation times, high fecundity, high rates of population increase, combination of sexual and asexual reproduction) and that cases of rapid evolution events (i.e. changes over perceptible timescales) have already been reported in *M. persicae* (especially in the *nicotianae* sub-species),[37,39] the selection of a similar P450-based mechanism capable of detoxifying spirotetramat would seem a realistic possibility, especially when considering the current intense use of this compound. Thus, in order to ensure the sustainable use of spirotetramat, users should carefully follow the resistance management guidelines that have been designed for this product.[45]

5 CONCLUSIONS

M. persicae s.l. has been found to develop resistance to a number of important classes of insecticides resulting in increased dependence on remaining classes. Furthermore, resistance against neonicotinoids, which have been the most effective choice for over two

decades, is now increasing. Attempting to reverse insecticide resistance once it has developed is difficult[1] and this is also the case for *M. persicae* s.l. populations in Greece which are known to maintain resistance mechanisms long after the removal of selection pressure for certain aphidicides (see Voudouris *et al.*[12] for further discussion on this topic).

IRM strategies rely largely on rotation schemes where chemicals from different MoA groups (IRAC classification) are rotated regularly. However, the presence of metabolic resistance could be a potential threat for the rotation strategies under intense chemical control scenarios, given the broad substrate spectra of the monooxygenases.[48] Spirotetramat is an inhibitor of lipid biosynthesis with many characteristics that make it suitable for cyclical alternation to neonicotinoids within an IPM/IRM scheme. This way both insecticide groups may continue being effective for longer. In addition, pymetrozine, a pyridine azomethine, which causes irreversible cessation of feeding, could be used as another alternative to neonicotinoids as it has a completely different MoA (IRAC MoA group 9B) and there have been no reports of resistance in Greek and European populations of *M. persicae*.[49,50] In crops that suffer from aphid-transmitted plant-viruses pymetrozine could be a valuable alternative, as it significantly reduces virus transmission.[50]

Scientists and farmers likewise should be alert for the early detection of resistance to all currently used insecticides so that effective measures be taken quickly. Continuous monitoring is essential, both in the short and long term, especially in crops under intense chemical protection such as peach and tobacco in Greece.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Stormwater-related transport of the insecticides bifenthrin, fipronil, imidacloprid, and chlorpyrifos into a tidal wetland, San Francisco Bay, California

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HIGHLIGHTS

- Suisun Marsh, in California, provides habitat to several imperiled fish species.
- Pesticides were sampled in creek waters flowing to the marsh after a winter storm.
- Urban creeks were toxic to invertebrates due to bifenthrin and fipronil.
- No toxicity was seen in agriculture-affected creeks, at least during the winter.
- Fipronil was measurable in the marsh, but not toxic due in part to dilution.

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ABSTRACT

Suisun Marsh, in northern San Francisco Bay, is the largest brackish marsh in California, and provides critical habitat for many fish species. Storm runoff enters the marsh through many creeks that drain agricultural uplands and the urban areas of Fairfield and Suisun City. Five creeks were sampled throughout a major storm event in February 2014, and analyzed for representatives of several major insecticide classes. Concentrations were greatest in creeks with urban influence, though sampling was done outside of the primary season for agricultural pesticide use. Urban creek waters reached maximum concentrations of 9.9 ng/l bifenthrin, 27.4 ng/l fipronil, 11.9 ng/l fipronil sulfone, 1462 ng/l imidacloprid, and 4.0 ng/l chlorpyrifos. Water samples were tested for toxicity to *Hyalella azteca* and *Chironomus dilutus*, and while few samples caused mortality, 70% of the urban creek samples caused paralysis of either or both species. Toxic unit analysis indicated that bifenthrin was likely responsible for effects to *H. azteca*, and fipronil and its sulfone degradate were responsible for effects to *C. dilutus*. These results demonstrate the potential for co-occurrence of multiple insecticides in urban runoff, each with the potential for toxicity to particular species, and the value of toxicity monitoring using multiple species. In the channels of Suisun Marsh farther downstream, insecticide concentrations and toxicity diminished as creek waters mixed with brackish waters entering from San Francisco Bay. Only fipronil and its degradates remained measurable at 1–10 ng/l. These concentrations are not known to present a risk based on existing data, but toxicity data for estuarine and marine invertebrates, particularly for fipronil's degradates, are extremely limited.

1. Introduction

Insecticide residues entering aquatic habitats via runoff have been shown to have effects ranging from selection for pesticide-resistant genotypes (Weston et al., 2013), to mortality of indicator species (Bailey et al., 2009), to changes in community composition (Schulz and Liess, 1999). Organophosphate insecticides, such as diazinon and chlorpyrifos, have long been associated with aquatic toxicity following rain-related transport of residues into waterways (Bailey et al., 2009; Kuivila and Foe, 1995). Pyrethroids have been an increasingly important insecticide class for the past decade as organophosphate use has declined. They have been shown to enter creeks at toxic concentrations after rain events, even traveling downstream more than 20 km from their source while retaining their toxicity to aquatic life (Weston et al., 2014). In recent years, use of phenylpyrazole insecticides, especially fipronil, has become more common, and it is now commonly detected in urban creeks at concentrations acutely toxic to a variety of invertebrates (Weston and Lydy, 2014). Neonicotinoids, such as imidacloprid, are an

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emerging class of insecticides with potential for aquatic toxicity (Smit et al., 2015), though the research focus has largely been on their toxicity to pollinators (Cresswell, 2011).

The present study examined the potential for insecticide-related aquatic toxicity in agriculture and urban-influenced creeks flowing into Suisun Marsh, and in the sloughs of the marsh itself. Suisun Marsh is the largest brackish marsh in California, and is located near the confluence of the Sacramento and San Joaquin Rivers in northern San Francisco Bay. Freshwater enters the marsh through several upland creeks that flow through agricultural or urban lands before becoming sloughs as they enter the marsh, where they broaden and their flow becomes tidally influenced. Thus, there is the potential for these creeks to transport a variety of agricultural and/or urban contaminants into Suisun Marsh.

Intensive sampling was conducted during the largest storm event of the 2013/2014 winter rainy season. Sampling was done to quantify water concentrations of representatives from several insecticide classes (the organophosphate chlorpyrifos, the phenylpyrazole fipronil and its degradates, the neonicotinoid imidacloprid, and eight pyrethroids). The compounds were selected based on high use, prior linkage with aquatic toxicity in the region, or emerging use with little previous monitoring (imidacloprid). Since insecticide effects on fish within the marsh could be indirect through toxicity to their invertebrate prey, toxicity testing of water samples was conducted with the amphipod, *Hyalella azteca*, and the chironomid, *Chironomus dilutus*. While the study was focused on the Suisun Marsh watershed, findings should be internationally relevant, as many estuarine areas receive runoff from mixed-use urban and agricultural watersheds, and the insecticides investigated are used worldwide.

2. Material and methods

2.1. Description of study area

Suisun Marsh contains 470 km^2 of marsh, much of it diked and seasonally flooded to support waterfowl hunting. Among the diked wetlands is a network of tidal sloughs, with salinities temporally varying from 0 to 17 psu depending on the volume of river flow entering the Bay (Meng et al., 1994). Given that most of the wetlands surrounding San Francisco Bay have been lost to agriculture or urban development, the wetlands of Suisun Marsh are considered critical spawning and rearing habitat for a diverse assemblage of native and introduced fish species (Meng et al., 1994; Meng and Matern, 2001; O'Rear and Moyle, 2014). Of particular significance is the use of the marsh by several native species whose numbers have dramatically declined throughout the estuary in recent decades (Sommer et al., 2007), such as Sacramento splittail (*Pogonichthys macrolepidotus*), longfin smelt (*Spirinchus thaleichthys*), and delta smelt (*Hypomesus transpacificus*). The marsh and adjacent Suisun Bay provides summer and fall habitat for subadult and adult delta smelt (Sommer and Mejia, 2013), and there is evidence that it provides spawning habitat for delta smelt in winter and spring months as well (Bennett, 2005; Murphy and Hamilton, 2013).

2.2. Field sampling

Sampling addressed impacts of runoff following winter storms, as these events have been shown to result in both urban and agricultural pesticide inputs to estuarine waters in environments similar to the study site (Weston et al., 2014). We recognize that this focus does not address conditions during the summer growing season, when most agricultural pesticide application occurs. However, pesticide inputs at that time would be inherently unpredictable, as they depend on application and irrigation practices of individual growers, and the volume of pesticide-contaminated runoff from irrigation return flows is likely to be much smaller than the volume of runoff accompanying storm events. All sampling was conducted in response to a single major rain event, with light rain beginning 6 February 2014, and heavier rains from the night of 7 February until 9 February. Rainfall accumulations at Cordelia, California (gauge location = 38.172, −122.129) were 1.4 cm on the 6th, 2.7 cm on the 7th, 4.8 cm on the 8th, and 3.1 cm on the 9th. In this region of California, most rainfall occurs from November through March, but the 2013/2014 wet season had exceptionally little rainfall, and accumulation never exceeded 1 cm in any day of the entire wet season up until the February storm sampled for the present study. Thus, the sampled rain event can be considered a "first flush", the first major rain event of the season, often accompanied by high suspended sediment loads entering the San Francisco Bay estuary and with pesticides associated with those particles (Goodwin and Denton, 1991; Bergamaschi et al., 2001).

There were two types of sampling sites: creek samples and slough samples (Fig. 1). The former were collected from most of the major creeks that flow to Suisun Marsh, at the last vehicle-accessible location prior to their entry to the marsh. Creek sites were sampled in both morning and afternoon of 8 February, and in the morning of 9 February. Suisun Creek was the only sampled creek for which pesticide sources in the watershed were primarily agricultural (87% of developed land agricultural, 9% urban or residential; Fairfield-Suisun Sewer District, 2004). Laurel Creek and McCoy Creek watersheds were largely urban (3% agricultural, 95% urban/residential, and 14% agricultural, 68% urban/residential, respectively). Green Valley and Ledgewood Creek watersheds had mixed land uses (50% agricultural, 45% urban/residential, and 74% agricultural, 24% urban/residential, respectively). All the urban areas in the study area are served by storm drain systems that divert untreated runoff from the streets to nearby creeks. The region's municipal wastewater treatment plant discharges into Suisun Marsh, approximately midway between sites LLC and SSV, thus the discharge would not affect water quality at the creek sites, but could influence some of the slough sites, especially SSV and SSO.

Flow at the Laurel Creek sampling site (LLC) was weak compared to the other waterways because it only received runoff from urban storm drains in the immediate vicinity. The main flow from the upper reaches of Laurel Creek is diverted eastward, joining with flow from McCoy Creek, and their combined flow was characterized at site MCC.

As the creeks enter Suisun Marsh, velocities decrease, conductivity increases, and tidal action becomes significant. Five slough sites were sampled within the marsh daily from 8 to 10 February, and a sixth site (site SSO) was sampled 10 February near the outfall of the slough system into Grizzly Bay, an embayment of northern San Francisco Bay. In order to best represent freshwater flowing seaward from the sloughs, rather than tidally-driven brackish waters flowing into the marsh from Grizzly Bay, slough sites were sampled within the 3-h period preceding the lowest tide each day. Nightfall prevented sampling at low tide or shortly thereafter.

Water samples were collected just below the surface either from the bank or using a stainless steel bailer from bridges, depending on access at each site. The only exception was site SSO, which required boat access. Samples were collected in glassware certified clean for pesticide analysis (I-Chem 200 series, Fisher Scientific, Waltham, MA), using 4-l bottles for toxicity testing samples and 1-l bottles for chemistry samples. Hexane (10 ml) was added as a keeper solvent to samples intended for pyrethroid, chlorpyrifos, and fipronil analysis. Samples were kept at 4 °C, with toxicity testing done within 48 h and pesticide extractions done within 96 h. Total suspended solid (TSS) samples were collected in 250-ml glass bottles.

2.3. Analytical procedure

For those samples intended for analysis of pyrethroids, chlorpyrifos, fipronil, or fipronil degradates, the analytical surrogates 4,4′-dibromooctafluorobiphenyl (DBOFB) and decachlorobiphenyl (DCBP) (Supelco, Bellefonte, PA) were added to the samples, and approximately 850 ml of water was liquid:liquid extracted using U.S. Environmental Protection Agency Method 3510C (USEPA, 2013). Three sequential

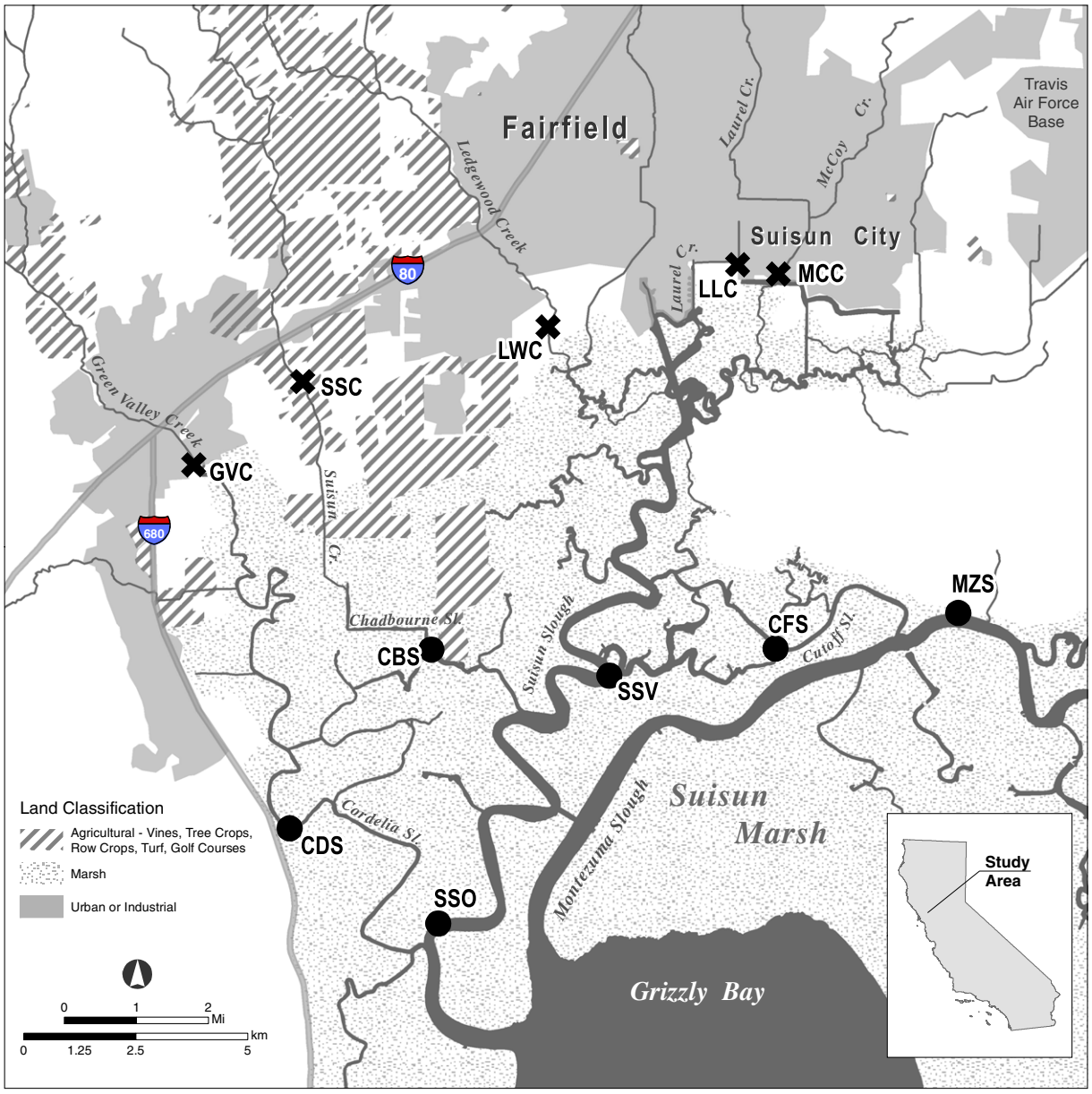

Fig. 1. Map of the study area, with regions of agricultural land use (cross-hatching) and urban land use (gray shading) designated. Creek sampling sites are indicated by "X" symbols. Slough sampling sites within Suisun Marsh are indicated by filled circles.

extractions were performed with 60 ml dichloromethane (all solvents from Fisher Scientific), with one aliquot also used to extract the empty sample bottle. All dichloromethane extracts were combined and reduced in volume to 5 to 10 ml under a stream of nitrogen for overnight shipment to the analytical laboratory at Southern Illinois University.

After arrival at the laboratory, extracts were solvent exchanged to hexane, concentrated to 1 ml, and eluted through a dual layer solid phase extraction cartridge (SPE) containing 300 mg of graphitized black carbon, 600 mg of primary/secondary amine and capped with anhydrous Na_2SO_4 (Wang et al., 2009). The SPE was primed with 3 ml of hexane prior to the introduction of the extract. The target pesticides were eluted with 10 ml of 1:1 hexane:acetone (v/v) solution, and solvent exchanged to 0.1% acetic acid in hexane with a final volume of 1 ml. The extracts were analyzed using an Agilent 6850 gas chromatograph 5975 XL mass spectrometer (GC–MS; Agilent Technologies, Palo Alto, CA) with negative-ion chemical ionization and selected-ion monitoring. Inlet, ion source, and quadrupole temperatures were 260, 150, and 150 °C, respectively. A HP-5 MS column (30 m × 0.25 mm × 0.25 μm film thickness) was used for separation of the analytes using helium as a carrier gas with the flow rate set at 1.8 ml/min. A 2 μl sample was injected into the gas chromatograph using pulsed splitless mode. The oven was set at 50 °C for 1 min, heated to 200 °C at 20 °C/min, then to 295 °C and held at 205 °C for 5 min. Quantification was performed using internal standard calibration. Calibration curves were based on area using concentrations of 2, 5, 10, 50, 100, 250, and 500 ng/ml of each pesticide and surrogate, while the concentrations of the internal standards were 20 ng/ml for each standard. Analytes included chlorpyrifos, bifenthrin, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, esfenvalerate, permethrin, tefluthrin, fipronil, fipronil desulfinyl, fipronil sulfide, and fipronil sufone. Data were reported down to a concentration of 1 ng/l. Quality assurance samples included a blank, lab control spike, matrix spike, matrix spike duplicate, and field duplicate, all run with every batch of 20 samples. Recovery in matrix spikes ranged from 36 to 110%, and averaged 77%.

For those samples intended for analysis of imidacloprid, the surrogates thiachloprid and acetamiprid (ChemService, West Chester, PA) were added to each sample of approximately 850 ml, as well as 35 g sodium chloride, and the samples were liquid:liquid extracted as detailed above. Cleanup methods were similar to those described above with the following exceptions: the SPE was primed with 3 ml of a 75:25 hexane:acetone (v/v) solution; unwanted interference was washed from the column with 7 ml of 90:10 hexane:dichloromethane (v/v) solution; and the target pesticide was eluted from the SPE with 3.5 ml of 1:1 acetonitrile:dichloromethane (v/v) solution. The eluent was evaporated to near dryness and reconstituted to 0.5 ml in 80:20 high performance liquid chromatography water:acetonitrile acidified by 0.1% of trifluoroacetic acid.

Quantification of imidacloprid was done using an Agilent 1260 HPLC interfaced with a 3200 Q Trap triple quadrupole/linear ion trap mass spectrometer (AB Sciex; Toronto, Canada). The HPLC system was equipped with a Waters Xterra® phenyl column (2.1 mm × 100 mm, 3.5 μm particle size) and the column temperature was maintained at 30 °C. The mobile phase consisted of water and methanol, both spiked with 0.1% formic acid (v/v). The mobile phase flow rate was 0.2 ml/min and the following gradient was employed: 10% methanol ramped to 70% methanol in 7 min (linear) and then ramped to 80% methanol in 6 min (linear), followed by a linear increase to 90% methanol in 2 min (held for 1 min) and then a change to 10% methanol in 1 min (held for 4 min). The mass spectrometry system was equipped with a Turbo Ion Spray® electrospray ionization probe operated in multiple reaction monitoring mode and in positive mode for quantitative determination. The ion pairs monitored were m/z 256.0 → 209.0 and 256.0 → 175.0. Quality control samples were as described above, and imidacloprid concentrations were reported down to 10 ng/l. Recovery in matrix spikes averaged 88%.

The TSS was quantified as the dried mass retained on a Whatman 934-AH filter (Whatman, Florham Park, NJ).

2.4. Toxicity testing of water samples

Samples were tested with *H. azteca*, using animals maintained in culture at the University of California Berkeley. Ten individuals, 7 to 10 d in age, were placed in 100-ml beakers containing 80 ml water, with five replicates per sample. A 1-cm^2 nylon screen was placed in each beaker to provide a substrate to which the animals cling, and its size was kept to a minimum to avoid adsorption of toxicants. Since pyrethroid toxicity is temperature dependent (Weston et al., 2009), tests were done at 13 °C to approximate the temperature at the sample sites, with the test animals acclimated to the test temperature by gradual decrease over three days. Testing was done under a 16-h light:8-h dark photoperiod. A yeast/cerophyll/trout food solution (1 ml per beaker) was provided on the second day, and after a 6-h feeding period, approximately 80% of the water was replaced with fresh sample. Renewal water was held in the dark at 4 °C after collection, but brought to test temperature prior to use. Conductivity, alkalinity, hardness and pH were measured at test initiation and termination; temperature and dissolved oxygen were measured at 0, 48 and 96 h. Tests were terminated at 96 h. Pyrethroids cause varying degrees of paralysis in *H. azteca*, ranging from animals that are motionless except for occasional twitching to others that attempt to swim but are unable to do so. Tests were scored for the number of dead amphipods and those that were alive but showing paralysis. All tests were accompanied by a control using moderately hard water (Smith et al., 1997), prepared by adding salts to Milli-Q purified deionized water. When testing slough samples, a high conductivity control (14,000 μS/cm) was also prepared by adding Instant Ocean (United Pet Group, Blacksburg, VA) to deionized water.

A single sample was also tested by addition of piperonyl butoxide (PBO), known to increase toxicity if due to pyrethroids (Amweg et al., 2006). Piperonyl butoxide at 50 μg/l was added to test waters in a methanol carrier, with methanol concentration at 12.5 μl/l. The PBO was renewed with the water change on the second day. A treatment control (laboratory water with PBO) was also included. The PBO test was performed at 17 °C, since nearly a week had elapsed by the time the sample was established as toxic by the initial testing, and we wished to minimize further delay that would have been necessary to temperature-acclimate additional *H. azteca* to the 13 °C of the previous tests.

Creek samples were also tested with *C. dilutus*, though the higher conductivity in the sloughs precluded their testing with this species. Test water (600 ml) was added to 1-l beakers, with five replicates per sample. A thin layer of washed sand (Fisher Scientific) was placed in each beaker to allow tube building. Ten 3rd-instar individuals were added to each beaker from cultures maintained at University of California Berkeley. Test temperature, light regime, feeding, and water change were as described above except that the second-day feeding consisted of 0.5 ml of a Tetrafin fish food slurry (United Pet Group). After 96 h, survivors were recorded, as well as those still alive but unable to perform typical thrashing movements when gently prodded (also referred to as unable to perform figure-8 movement; Pape-Lindstrom and Lydy, 1997).

Samples were compared to concurrent controls using CETIS software (Tidepool Scientific Software, McKinleyville, CA) by *t*-test if parametric assumptions were met, or by Wilcoxon Rank Sum if they were not.

3. Results and discussion

3.1. Relationship of sampling to rainfall patterns

The creek sites were repeatedly sampled as stormwater runoff entered and their flows increased. Water depth in Suisun Creek is monitored by the California Department of Water Resources, and was 0.4 m at the gauging station prior to the storm event. Samples were collected at stages of 0.5, 1.2 and 1.3 m on the rising limb of the hydrograph, with the stage peaking at 1.7 m. The other sampled creeks are not gauged, but stages at the times of sampling relative to peak stage would be comparable. Conductivity in the creeks ranged from 89 to 553 μS/cm, with temperatures of 11 to 13 °C (Table 1).

Within the sloughs of the marsh, conductivity rises and falls throughout each tidal cycle, depending on the relative proportions of freshwater from the creeks and brackish water from Grizzly Bay. In any given sample within the marsh sloughs, conductivity during the storm ranged from 854 to 10,302 μS/cm (Table 1). Prior to the rain event, conductivity at the mouth of Suisun Slough (near site SSO) fluctuated from 14,000 to 16,000 μS/cm throughout the tidal cycle, or approximately 8 to 9 psu. As runoff from the storm moved downstream into the marsh, the low conductivity excursions at low tides became more pronounced, eventually reaching a minimum of 5561 μS/cm at the mouth of Suisun Slough on 10 February (California Department of Water Resources, 2014). As sampling of slough sites occurred over three days from 8 to 10 February, we successfully captured the period of greatest runoff influence, and therefore our sampling should reflect the period of highest pesticide concentration during the runoff event.

3.2. Pesticide concentrations

Bifenthrin was detected in nearly every creek sample, and it was the only pyrethroid measurable in the creeks with the exception of two samples containing cyfluthrin just above the reporting limit (Table 2). Bifenthrin concentrations ranged from 3.2 to 9.9 ng/l in those creeks with the greatest urban influence, and the compound has consistently been the dominant pyrethroid in urban creeks throughout the U.S. (Weston et al., 2011; Kuivila et al., 2012). It was detected in only one of three samples in the creek draining only agricultural lands (1.5 ng/l; Suisun Creek), though most agricultural use would have occurred in the summer, about six months earlier. The magnitude of agricultural bifenthrin use is comparable to its non-agricultural use in Solano County,

Table 1
Sites sampled, including temperature and conductivity of surface waters.

Site	Waterway	Coordinates	Dates of sampling within Feb. 2014	Temp. range of samples (°C)	Conductivity range of samples (μS/cm)
Creek sites					
GVC	Green Valley Creek	38.2117, −122.1296	8, 8, 9	11.5–13.0	147–232
SSC	Suisun Creek	38.2248, −122.1077	8, 8, 9	11.5–12.4	150–553
LWC	Ledgewood Creek	38.2334, −122.0586	8, 8, 9	11.3–13.4	206–320
LLC	Laurel Creek	38.2432, −122.0206	8, 8, 9	11.8–13.4	89–121
MCC	McCoy Creek	38.2417, −122.0124	8, 8, 9	11.5–13.0	178–424
Suisun Marsh Sloughs					
CDS	Cordelia Slough	38.1545, −122.1103	8, 9, 10	12.0–14.0	854–1838
CBS	Chadbourne Slough	38.1826, −122.0819	8, 9, 10	12.2–14.6	1014–10,302
SSV	Suisun Slough	38.1785, −122.0462	8, 9, 10	11.1–13.1	3462–9678
CFS	Cutoff Slough	38.1828, −122.0130	8, 9, 10	11.1–13.3	6123–7778
MZS	Montezuma Slough	38.1884, −122.9763	8, 9, 10	11.4–12.4	8370–8464
SSO	Suisun Slough (outlet)	38.1394, −122.0805	10	13.2	8873

where the study area is located (590 kg/yr versus 523 kg/yr, respectively; 2012 data; California Department of Pesticide Regulation, 2015).

Fipronil and its environmental degradates were found in highest concentrations in those creeks with greatest urban influence (McCoy and Laurel Creeks), at lower concentrations in creeks with mixed urban and agricultural contributions, and were nearly always undetectable in samples from agricultural waterways. These results are consistent with fipronil use patterns, for while there is 227 kg/yr of fipronil used for non-agricultural purposes in Solano County, there are no approved agricultural uses of fipronil in California. In the predominantly urban waterways, fipronil concentrations were 14.5 to 27.4 ng/l, followed by fipronil sulfone at 8.3 to 11.9 ng/l, fipronil desulfinyl at 4.8 to 7.1 ng/l, and fipronil sulfide at 1.7 to 8.4 ng/l; values very similar to concentrations observed after rain in many urban waterbodies throughout northern California (Weston and Lydy, 2014).

Imidacloprid is primarily an agricultural-use insecticide with some urban applications (235 kg/yr versus 54 kg/yr, respectively, in Solano County). It was detected in particularly high concentrations (889 to 1462 ng/l) in Laurel Creek. The other predominantly urban waterway, McCoy Creek, contained much lower concentrations (26.5 to 33.2 ng/l). It is unclear why imidacloprid concentrations were dramatically higher in Laurel Creek, but at the point Laurel Creek was sampled, it only serves a very small watershed of residences and businesses within Suisun City, so it is possible that pesticide use at a very small number of homes could have led to dramatic differences in creek quality.

Chlorpyrifos was absent from the agricultural Suisun Creek, and found in urban-influenced creeks at <4 ng/l. Though nearly all urban-use products containing the compound were removed from retail stores over a decade ago, low concentrations such as those seen remain common in urban runoff in the region (Weston and Lydy, 2010a).

Within the sloughs of Suisun Marsh, bifenthrin was always undetectable, as was chlorpyrifos except for a single sample at the reporting limit. Fipronil or a degradate was found in almost every slough sample, though at low concentrations. Median fipronil concentration in the sloughs was 1.4 ng/l (versus 11.7 ng/l median in the creeks), and the maximum concentration was 5.1 ng/l. The low to immeasurable insecticide concentrations in the sloughs can be attributed in large part to dilution. Water entering the slough system from San Francisco Bay on rising tides had a conductivity of approximately 15,000 μS/cm, whereas freshwater entering via the creeks was typically near 300 μS/cm (range = 89 to 553 μS/cm). The conductivity of the slough samples ranged from 854 to 10,302 μS/cm, with a median of 6327 μS/cm. Thus, on the basis of salt content, it can be estimated that the slough samples were on average 60% freshwater from the creeks (range 30–95%). Dilution with San Francisco Bay water would have reduced insecticide concentrations by nearly one half (assuming no insecticides in Bay water), and hydrophobic pesticide concentrations would have been further reduced by processes such as particle deposition and adsorption of pesticides to plant material and other substrates (Moore et al., 2009). Concentrations of fipronil and its degradates remained measurable

Table 2
Pesticide concentrations (ng/l) in water from the creeks flowing to Suisun Marsh and in the sloughs of the marsh. Data are presented in the order samples were collected[a].

	Chlorpyrifos	Bifenthrin	Fipronil	Fip. desulfinyl	Fip. sulfide	Fip. sulfone	Imidacloprid
Urban creeks							
McCoy Creek	3.0, 2.8, 2.0	3.2, 7.7, 3.6	21.9, 14.5, 16.7	5.4, 5.4, 4.4	1.9, 2.0, 1.7	8.3, 9.0, 8.4	33.2, 26.5
Laurel Creek	4.0, 1.9, U	6.0, 9.9, 8.1[b]	22.2, 27.4, 23.4	4.9, 7.1, 4.8	8.4, 1.9, 1.7	9.5, 11.9, 11.1	1462, 889
Mixed urban and agricultural creeks							
Ledgewood Creek	1.7, 1.4, U	2.6, 4.4, 2.7	13.0, 11.7, 11.5	3.9, 4.2, 2.5	1.6, 1.9, 1.5	6.6, 7.3, 5.3	27.1, 69.2
Green Valley Creek	U, U, U	2.1, 1.3, 1.1	6.6, 3.5, 3.7	2.9, 1.3, 1.1	1.4, 1.2, 1.1	4.0, 2.0, 2.1	65.3, 13.5
Agricultural creek							
Suisun Creek	U, U, U	U, 1.5, U	U, U, 2.1	U, U, U	U, U, U	U, U, U	U, U
Suisun Marsh Sloughs							
Cordelia Slough	U, U, U	U, U, U	5.1, 2.7, 4.5	3.0, 1.3, U	1.3, 1.1, U	4.0, 2.0, 1.2	no data
Chadbourne Slough	U, U, U	U, U, U	U, 1.9, 1.4	U, U, U	1.0, 1.0, 1.3	U, U, U	no data
Suisun Slough	U, U, 1.0	U, U, U	1.4, U, 7.8	1.0, U, 3.1	1.1, U, 1.4	U, U, 4.0	no data
Cutoff Slough	U, U, U	U, U, U	U, 1.4, 1.6	U, 1.1, 1.1	1.2, 1.0, U	U, U, U	no data
Montezuma Slough	U, U, U	U, U, U	1.1, 1.2, U	U, U, U	U, 1.1, U	U, U, U	no data
Suisun Sl. (outlet)	U	U	1.7	1.3	1.1	1.1	no data

U indicates undetected at <1 ng/l for chlorpyrifos, bifenthrin, fipronil, and its degradates, and <10 ng/l for imidacloprid.

[a] Creek sites were sampled 8 Feb. morning, 8 Feb. afternoon, 9 Feb. morning, except imidacloprid was only analyzed in the two morning samples. Slough sites were sampled 8 Feb. afternoon, 9 Feb. afternoon and 10 Feb afternoon, except Suisun Slough Outlet was only sampled on 10 Feb. afternoon.

[b] Laurel Creek was the only site containing a pyrethroid other than bifenthrin. Cyfluthrin was found at U, 1.2, 1.7 ng/l.

throughout the sloughs both because of greater water solubility, and because initial concentration in the creeks were the highest of all analytes.

Total suspended solid concentrations ranged from 3 to 1476 mg/l in the creeks (median 79 mg/l) and 41–641 mg/l in the marsh (median 82 mg/l). It is recognized that the presence of suspended solids in the water can influence the toxicity of hydrophobic pesticides (particularly pyrethroids in the present study) with adsorption leading to less toxicity than expected based on pesticide concentration. However, in the six samples with *H. azteca* or *C. dilutus* toxicity (discussed in Section 3.3) and measurable bifenthrin concentrations, total suspended solid concentrations were relatively low at 12 to 59 mg/l, and would not have had an appreciable influence in most instances (Yang et al., 2006). Samples with measurable bifenthrin, but lacking toxicity, tended to have higher TSS concentrations (though only two samples, with 106 and 1476 mg/l).

3.3. Toxicity testing

Creek samples were tested in 96-h exposures using both *H. azteca* and *C. dilutus*. Only one sample caused *H. azteca* mortality significantly greater than that of the controls (a modest 14 ± 9% mortality in the 8 Feb. Laurel Creek sample), but impaired movement was often observed. *H. azteca* showed significant paralysis in 4 of 10 creek samples (Table 3), including both urban creeks (McCoy and Laurel Creeks) and Ledgewood Creek which carries both urban and agricultural runoff. Statistically significant mortality to *C. dilutus* was limited to a single sample (McCoy Creek, 9 Feb. = 64 ± 31% mortality), but inability to perform the typical thrashing motion when disturbed was observed in 6 of the 10 samples. The effects were greatest in the urban McCoy and Laurel Creeks, in which none or nearly none of the individuals were able to move normally. Lesser but still significant effects were seen in Ledgewood Creek.

To help identify the cause of toxicity, PBO was added to Laurel Creek water (a composite of two samples from the site, since sample volume was insufficient to test either sample alone). *H. azteca* were unaffected in the laboratory water control, either with or without PBO (only 6 and 0% dead or paralyzed, respectively). However, every individual exposed to Laurel Creek water in the presence of PBO was dead or paralyzed, a significant difference as compared to 84 ± 15% without PBO ($p < 0.05$; Kolmogorov–Smirnov two-sample test). This difference was consistent with pyrethroids as the cause, as it has been shown their toxicity is increased by PBO (Amweg et al., 2006).

Table 3
Results of toxicity tests using *H. azteca* or *C. dilutus* exposed to creek waters for 96 h. Asterisk indicates effect significantly greater than control. All sites were sampled twice (morning of 8 Feb. and morning of 9 Feb.; toxicity testing not done with afternoon 9 Feb. samples).

Sample site	% *H. azteca* affected[a] (±standard deviation)	% *C. dilutus* affected[a] (±standard deviation)
Laboratory controls	0 ± 0	8 ± 4
	8 ± 13	16 ± 15
Urban creeks		
McCoy Creek	26 ± 21*	94 ± 9*
	16 ± 15	100 ± 0*
Laurel Creek	94 ± 9*	98 ± 4*
	94 ± 5*	100 ± 0*
Mixed urban and agricultural creeks		
Ledgewood Creek	34 ± 18*	66 ± 13*
	8 ± 8	74 ± 17*
Green Valley Creek	7 ± 8	20 ± 19
	6 ± 5	30 ± 7
Agricultural creek		
Suisun Creek	2 ± 4	10 ± 17
	0 ± 0	42 ± 28

[a] Individuals not moving normally; either dead or showing paralysis.

Further evidence supporting bifenthrin as the cause of *H. azteca* toxicity is evident in the relationship between the proportion of individuals affected and the toxic units (TU) of bifenthrin present in the samples, where TU equals the measured concentration divided by the 96-h EC50 (3.3 ng/l; Weston and Jackson, 2009) (Fig. 2). Not only was there a significant correlation between effect and bifenthrin TU among the samples ($r = 0.920$; $p < 0.01$), but >50% effect would be expected in samples exceeding 1 TU if bifenthrin were the cause of toxicity, similar to the results seen.

Fipronil was unlikely to have contributed to the observed *H. azteca* toxicity. The species is extremely insensitive to fipronil and its degradates, with 96-h EC50s of 728, 458, and 213 ng/l for fipronil, the sulfone and the sulfide, respectively (Weston and Lydy, 2014). Even in the worst-case creek site (Laurel Creek, 8 Feb. morning sample) there was still <0.1 TU for *H. azteca* when summed for fipronil and its degradates.

The converse is true for *C. dilutus* with effect levels for bifenthrin at least 25 times greater than concentrations seen in the creeks (the maximum concentration observed of 9.9 ng/l compared to 96-h EC50 > 253 ng/l; Weston et al., 2015). However, *C. dilutus* toxicity would be expected at the observed concentrations of fipronil, fipronil sulfone and fipronil sulfide (96-h *C. dilutus* EC50 = 32.5, 7.7, and 9.9 ng/l, respectively; Weston and Lydy, 2014). A TU analysis suggests

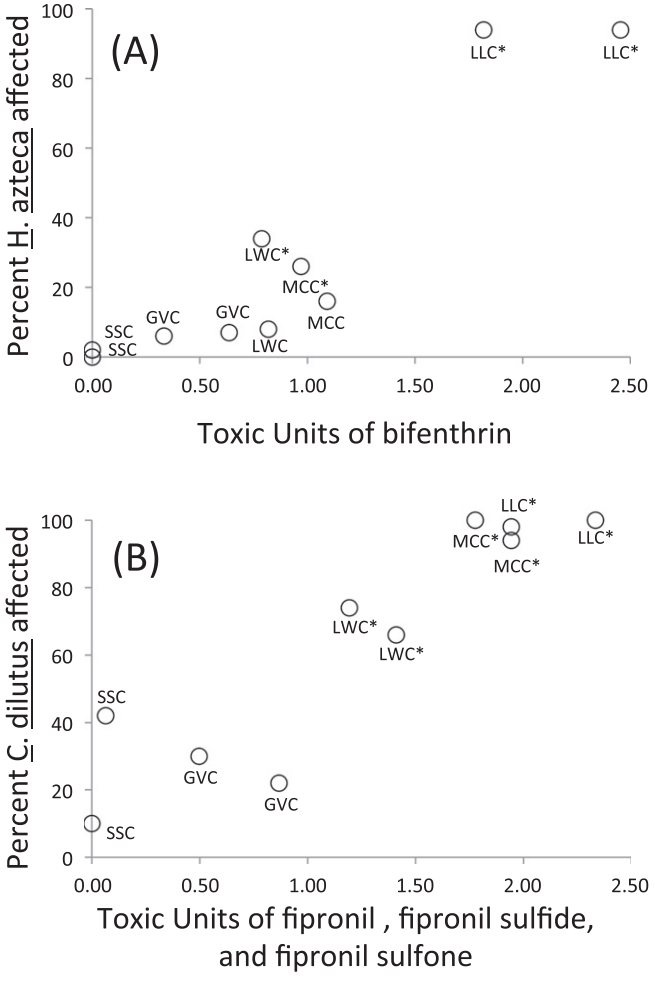

Fig. 2. Relationship between toxicity observed in creek samples and the toxic units (TU) of selected pesticides. (A) = Proportion of *H. azteca* paralyzed or dead as a function of bifenthrin TU in each sample. (B) = Proportion of *C. dilutus* unable to perform the characteristic thrashing motion as a function of sum TU of fipronil, fipronil sulfide and fipronil sulfone in each sample. Asterisks indicate effects significantly greater than control (LLC = Laurel Creek, MCC = McCoy Creek, LWC = Ledgewood Creek, GVC = Green Valley Creek, SSC = Suisun Creek).

fipronil and its degradates were responsible for the observed *C. dilutus* toxicity (Fig. 2). Not only did the proportion of individuals affected correlate with the sum TU of fipronil and its degradates ($r = 0.883$; $p < 0.01$), but >50% effects generally became evident at about 1 TU, precisely where they would be expected if these compounds were responsible.

Chlorpyrifos concentrations were below levels of concern with respect to both *H. azteca* and *C. dilutus* (96-h EC50s of 96 and 510 to 750 ng/l, respectively; Weston and Lydy, 2010a; Pape-Lindstrom and Lydy, 1997). Similarly, imidacloprid concentrations at creek sites (typically <70 ng/l; maximum 1462 ng/l) were below reported toxicity thresholds for the two species tested and were unlikely to have played a role in observed effects. *C. dilutus* is a relatively sensitive species, with an imidacloprid 96-h LC50 of 5750 ng/l, with concentrations about half of that reported to inhibit growth (Stoughton et al., 2008; though those tests were done with a specific commercial formulation, making comparisons to our data difficult). The reported *H. azteca* imidacloprid 96-h LC50s are 65,430 and 526,000 ng/l (Stoughton et al., 2008; England and Bucksath, 1991 as reported by Stoughton et al.), with a 96-h EC50 (immobilization) of 55,000 ng/l, and growth effects with as little as 2220 ng/l (Stoughton et al., 2008; using a specific commercial formulation).

While the urban creeks flowing to Suisun Marsh demonstrated acute toxicity when tested, and contained bifenthrin and the fipronil compounds at concentrations expected to cause toxicity, no toxicity was seen in the sloughs of Suisun Marsh. In tests of slough waters with *H. azteca*, the proportion of animals dead or paralyzed ranged from 0 to 12%, and were not significantly different from the control (6%) or the high conductivity control (7%). Given that bifenthrin appeared responsible for the *H. azteca* toxicity observed in the creeks, and the compound was undetectable in the slough samples, the lack of toxicity is not surprising.

We were not able to test the slough waters with *C. dilutus* because while *H. azteca* can be used for testing both fresh and estuarine waters, *C. dilutus* cannot (Munns et al., 2002). Mortality of *C. dilutus* would be expected simply due to the salt content of the slough samples (Sargent, 1978).

3.4. Risks to resident species

The bifenthrin concentrations observed in Laurel, McCoy, and Ledgewood Creeks were found to be toxic to *H. azteca*, as would be expected given the species EC50 (3.3 ng/l; Weston and Jackson, 2009), but they would also be on the threshold of toxicity to several other benthic invertebrates. Weston et al. (2015) determined bifenthrin 96-h EC50s for 12 benthic macroinvertebrates, most from northern California. Bifenthrin concentrations seen in the urban creeks flowing to Suisun Marsh were one-third to one-half the 48 to 96-h EC50 of two mayflies, a stonefly, and a caddisfly. Bifenthrin effects to fish within these creeks could occur through the food web, though sublethal effects on fish themselves tend to be at higher concentrations than those seen (30 to 140 ng/l for impairment of swimming, or 70 ng/l for gene transcription effects in fathead minnows; Beggel et al., 2010, 2011).

Laurel and McCoy Creeks are also a concern due to the presence of fipronil and its degradation products. Weston and Lydy (2014) determined EC50s of fipronil and its degradates for 14 macroinvertebrate species. The concentrations of fipronil and/or its sulfone degradate found in these two creeks were approximately half the EC50s for one-third of the species. As for bifenthrin, any fipronil effects on fish within the creeks are more likely to be manifested through the food web, as endocrine effects, gene transcription, and swimming performance in fathead minnows (Beggel et al., 2010, 2012; Bencic et al., 2013), as well as developmental defects in zebrafish (Stehr et al., 2006), have all been seen at concentrations at least three orders of magnitude higher than those we observed.

Though the creeks flowing into Suisun Marsh contain both bifenthrin and fipronil from urban runoff at concentrations representing a threat to a variety of benthic taxa, the threat is not unique to Fairfield and Suisun City, and is comparable to that seen in urban streams in many other northern California communities (Weston and Lydy, 2010b, 2012, 2014; Weston et al., 2014). Conditions elsewhere in the U.S. have not been well documented, though there are indications that they may be similar (Kuivila et al., 2012). It is noteworthy that regulatory monitoring of Fairfield-Suisun City urban creek waters, as done by the municipal stormwater utility, requires testing with *H. azteca*, *Selenastrum capricornutum*, *Ceriodaphnia dubia*, and *Pimephales promelas*. Excluding *H. azteca*, the utility's monitoring program is unlikely to show any insecticide toxicity to the other species, since their bifenthrin and fipronil LC50s far exceed the maximum concentrations observed (Konwick et al., 2005; Yang et al., 2006; USEPA, 2007; Werner and Moran, 2008; Baird et al., 2013). The utility's *H. azteca* monitoring only scores survival, an endpoint on which the present study found little effect, and is less than half as sensitive as the paralysis endpoint of the present study (Weston and Jackson, 2009). Their program also tests *H. azteca* at 23 °C, at which pyrethroids are one-third as toxic as at the in situ 13 °C used in the present study (Weston et al., 2009).

Excluding Laurel Creek (889–1462 ng/l), imidacloprid concentrations < 70 ng/l at the other sites are not known to represent a threat to resident macroinvertebrates. The most sensitive species known are mayflies, with 96-h LC50s of 650–1770 ng/l (Alexander, 2006; Roessink et al., 2013). For the protection of aquatic life in general, concentrations less than 8.3 and 200 ng/l have been recommended for chronic and acute exposure, respectively (Smit et al., 2015). The acute threshold is the more relevant to stormwater runoff events, and thus only Laurel Creek would exceed this benchmark.

Dilution and the other factors that reduce insecticide concentrations as creek waters move into Suisun Marsh substantially mitigate risks to estuarine species within the marsh habitat. Few data exist by which to compare fipronil concentrations found in the marsh to the tolerance of estuarine and marine species. A compilation of data by the U.S. Environmental Protection Agency (2007) suggests that no acute toxicity would be likely, though only five estuarine or marine species have been tested (most sensitive being *Americamysis bahia* with 96-h fipronil LC50 of 140 ng/l).

4. Conclusions

The present study documented considerable risk of insecticide toxicity to invertebrates within the urban creeks. Bifenthrin was likely responsible for toxicity to *H. azteca*, fipronil and its sulfone degradate likely responsible for toxicity to *C. dilutus*, and both compounds could pose a risk to multiple other macroinvertebrate species. The same compounds investigated in the present study are widely used in many other countries, and this work illustrates the co-occurrence and toxicity of multiple insecticides in urban runoff. Each compound has the potential for toxicity to a unique subset of species within receiving waters, thus toxicity testing with multiple species provides the best means to assess these risks. The co-occurrence of bifenthrin, fipronil, chlorpyrifos, and imidacloprid in the creeks, as well as many other urban runoff contaminants not measured in the present study, could also pose a risk due to additive or synergistic effects that are largely unknown given the current state of knowledge.

The present study failed to show risk once creek waters were diluted within the marsh, however it would be premature to entirely dismiss this potential. First, our study focused solely on winter rains as a transport mechanism for insecticide entry into the marsh. Agricultural sources are likely to be more significant during periods of peak pesticide use during the growing season, with irrigation runoff providing a mechanism for off-site movement of residues. Entry of agricultural insecticides into the marsh through this route is likely to be unpredictable and highly episodic, and therefore difficult to monitor.

Second, fipronil and its degradates were commonly found throughout the marsh following the rain event. While the concentrations measured are not known to be acutely lethal to the marine and estuarine invertebrates that have been tested, they are on the threshold of sublethal toxicity to the most sensitive freshwater invertebrate that has been tested (*C. dilutus*). Given the few estuarine/marine species tested with fipronil, and that estuarine/marine testing of fipronil degradates has been done with only a single species (*A. bahia*; USEPA, 2007), we cannot rule out possible adverse effects even at the <10 ng/l concentrations seen for fipronil and its degradates throughout the marsh.

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Environmental Toxicology and Chemistry

Hazard/Risk Assessment

ECOLOGICAL RISK ASSESSMENT FOR AQUATIC INVERTEBRATE COMMUNITIES EXPOSED TO IMIDACLOPRID DUE TO LABELED AGRICULTURAL AND NON-AGRICULTURAL USES IN THE UNITED STATES

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Abstract: A probabilistic ecological risk assessment (ERA) was conducted to determine the potential effects of acute and chronic exposure of aquatic invertebrate communities to imidacloprid arising from labeled agricultural and non-agricultural uses in the United States. Aquatic exposure estimates were derived using a higher tier refined modeling approach that accounts for realistic variability in environmental and agronomic factors. Toxicity was assessed using refined acute and chronic community-level effect metrics for aquatic invertebrates (i.e., species or taxon sensitivity distributions) developed using the best available data. Acute and chronic probabilistic risk estimates were derived by integrating the exposure distributions for different use patterns with the applicable species or taxon sensitivity distributions to generate risk curves, which plot cumulative probability of exceedance versus the magnitude of effect. Overall, the results of this assessment indicated that the aquatic invertebrate community is unlikely to be adversely affected by acute or chronic exposure to imidacloprid resulting from currently registered uses of imidacloprid in the United States.

Keywords: Probabilistic risk assessment, Neonicotinoids, Exposure modeling

1 INTRODUCTION

Imidacloprid is a first-generation neonicotinoid compound and one of the most widely used insecticides worldwide [1]. In 2009, imidacloprid accounted for over 40% of the neonicotinoid market in the United States [1]. Imidacloprid may be applied to either soil or foliage and is widely used in row crops (e.g., cotton, potatoes), greenhouse vegetables, forestry, vine crops, citrus, stone fruit and pome orchards, bush berries, and tree nuts to control a variety of pest insects, including aphids, Japanese beetles, lacebugs, leaf beetles, leafhoppers, leafminers, thrips, white flies, and others.

Although imidacloprid is not applied directly to water bodies, it may enter the aquatic environment through spray drift during application or storm water runoff shortly after application and has been detected in surface waters around the world [2]. In the aquatic environment, aquatic invertebrates have been shown to be more sensitive to imidacloprid than other aquatic taxa, due to the high specificity of imidacloprid and other neonicotinoids to the nicotinic acetylcholine receptors (nAChR) in the invertebrate central nervous system [3]. Therefore, the detection of imidacloprid in surface waters has raised concerns regarding potential direct negative impacts to aquatic invertebrate communities as well as indirect negative impacts to vertebrate species such as birds and fish that rely on aquatic invertebrates as prey [2-4].

The objective of this paper was to investigate the potential effects of acute and chronic exposure of aquatic invertebrate communities to imidacloprid arising from labeled agricultural and non-agricultural uses in the United States. This required a thorough review and evaluation of the available aquatic invertebrate toxicity data for imidacloprid, with a special focus on higher tier (mesocosm, semi-field and field) studies that considered more realistic exposure conditions. Additionally, exposure modeling was performed using a higher tier refined approach that accounted for variability in environmental and agronomic factors such as pesticide application date, weather, soils and slope of use sites, use site proximity to water bodies, and percent

cropped area of targeted watersheds to develop realistic yet conservative estimates of aquatic exposure concentrations. This refined exposure modeling relied on the best available national scale datasets for agricultural and non-agricultural labeled uses of imidacloprid. Finally, risk was characterized using a probabilistic approach that considers both the probability of exposure to a range of pesticide concentrations and the magnitude of effects (if any) predicted to occur at those concentrations [5, 6].

2 METHODS

2.1 Identification of the best available data

A review of the scientific literature, registrant-sponsored studies, EPA's EcoTox database, existing water quality guideline documents (e.g., [7, 8]), and grey literature (e.g., [9]) was carried out to identify the best available data for the hazard portion of this assessment. All studies were evaluated using a transparent and reproducible assessment scheme developed based on data quality assessment factors described by NAS [5], EPA [10], Breton et al. [11], Klimisch et al. [12] and Hall et al. [13]. This assessment scheme is discussed in Knopper et al. [14] and the specific scoring rubrics for laboratory-based and higher tier (mesocosm, semi-field, and field) studies are described in the Supporting Information (Sections S1 and S2, respectively). On the basis of this evaluation, endpoints from each study were rated as acceptable, supplemental, or unacceptable for use in an ecological risk assessment (Supporting Information, Section S3). A study rating of acceptable indicates that the endpoint is ecologically relevant for potential population level effects, all essential information was reported, and the study was performed according to a complete and transparent study protocol that follows acceptable laboratory practices. Acceptable data are relevant and robust and are suitable for quantitative use in an ecological risk assessment. Data ranked as supplemental are also ecologically relevant, but their reliability is uncertain because their study conditions or methods either deviated from recognized protocol or were not reported in sufficient detail to enable evaluation. Therefore, supplemental data are not preferred for quantitative use in risk assessment, but may be considered qualitatively

as part of a weight-of-evidence approach. Finally, data rated as unacceptable are not suitable for either quantitative or qualitative consideration in a risk assessment and should be excluded from further consideration.

2.2Effects assessment

Frequently, measures of effect used in risk assessments are selected by compiling the available effects data and then choosing the most sensitive effects metric from the most sensitive test species to represent effects to the receptors of concern (e.g., [15]). This conservative approach is appropriate for screening-level assessments or when few data are available. However, for this refined assessment, toxicity data that were determined to be acceptable for quantitative use in an ecological risk assessment were available for a diverse group of aquatic invertebrate taxa. In addition, the purpose of this assessment was to assess risk to the aquatic invertebrate community rather than to specific sensitive species. Given the "functional redundancy" in aquatic invertebrate communities in the temperate zone [16, 17], multiple species are generally present as potential food sources for higher trophic level species and to perform critical functions such as cycling nutrients and organic matter. Hence the impairment of a sensitive species is not expected to alter overall ecosystem function because of other functionally similar species [18-21]. Additionally, populations of sensitive invertebrate species often recover quickly following exposure because of their high reproductive fecundity [22-26] and rapid recolonization from nearby refugia [27]. Therefore, alternative methods for deriving effects metrics were pursued that make use of all of the best available data to determine endpoints protective of the aquatic invertebrate community rather than relying on single most sensitive endpoints.

2.2.1 Acute effects

A species sensitivity distribution (SSD) was derived using the acute toxicity data that had an acceptable study rating (see Supporting Information Section S4). To derive the dataset for the SSD, only the most sensitive ecologically relevant endpoint for each species from each study

was used. If more than one study reported suitable endpoints for the same species, the geometric mean value was calculated using the lowest acceptable endpoint values from each study. The final condensed dataset for imidacloprid is shown in Table 1.

SSD models were fit to the data in Table 1 using SSD Master v3 [28], an Excel-based tool that fits four non-linear regression models in log space (normal, logistic, extreme value, and Gumbel) to determine the best-fitting cumulative distribution function (CDF). Initially, SSD models were generated that included the acute *Daphnia magna* endpoint of 131,000 µg a.i./L (Table 1). However, this endpoint is three orders of magnitude higher than the next highest endpoint and removing the *Daphnia magna* endpoint improved the fit of the SSD models. A similar observation was made by RIVM [29]. Therefore, the *Daphnia magna* endpoint was excluded from the final SSD analysis.

Of the SSDs generated by SSD Master v3 for acute laboratory-based toxicity data (Table 1) with *Daphnia magna* excluded, the best-fitting SSD was provided by the logistic model in log space based on the results of the Anderson-Darling (AD) goodness-of-fit test statistic (AD=0.26, $p>0.05$), mean square error (0.0034) and mean square error lower tail (0.0168). Visual inspection of the fitted CDFs also indicated that the logistic model closely followed the data (Figure 1). The logistic model is described by Equation 1.

$$f(x) = \frac{1}{1 + e^{-\frac{x-\mu}{s}}} \qquad \textbf{(1)}$$

where x is concentration (in µg a.i./L), and $f(x)$ is the proportion of taxa affected. The fitted location and scale parameters, μ and s, for this model were 1.44 and 0.409, respectively. The predicted 5% hazard concentration (HC_5) for this SSD was 1.73 µg a.i./L (95% CI from 1.01 to 2.97 µg a.i./L).

2.2.2 Chronic effects

The literature review and study evaluation process identified two distinct sets of chronic effects data for imidacloprid. The first dataset included the results of chronic toxicity tests conducted in laboratories according to standardized protocols that are well established, accepted, and reproducible. These laboratory-based studies generally tested single species exposed to constant concentrations of imidacloprid maintained by static renewal (e.g., Roessink et al., [30]) or flow-through test systems (e.g., Ward [31]). The second dataset included the results of chronic mesocosm, semi-field and field studies designed to more closely mimic natural conditions and exposure from registered used patterns. These studies considered factors such as varied exposure concentrations over time, the presence of sediment in the test system, the potential for natural recolonization and recovery, the effects of natural lighting and weather fluctuations, and/or the impacts of interactions between multiple species. Separate chronic effects metrics were developed using each of these two datasets.

Standard laboratory-based chronic effects

An SSD was derived using the chronic laboratory-based toxicity data that had an acceptable study rating (see Supporting Information section S5). The approach taken to derive the chronic SSD using laboratory-based data was the same as described above for the acute SSD. The final condensed chronic dataset is shown in Table 2.

SSD models were fit to the chronic effects metrics presented in Table 2 using SSD Master v3. As with the acute SSD, the *Daphnia magna* endpoint was excluded from chronic SSD analysis because the species is two orders of magnitude more tolerant than then the next most tolerant species.

Of the SSDs generated by SSD Master v3 for chronic laboratory based toxicity data (Table 2) with the *Daphnia magna* endpoint excluded, the best-fitting SSD was provided by the logistic model in log space. This model had the best fit based on the results of the Anderson-

Darling (AD) goodness- of-fit test statistic (AD=0.226, p>0.05), mean square error (0.0035) and mean square error lower tail (0.017). Visual inspection also indicated an excellent fit to the data (Figure 2). This model is described by Equation 1. The fitted location and scale parameters, μ and s, for this logistic model were 3.15 and 0.529, respectively. The HC5 was 0.039 µg a.i./L (95% CI from 0.02 to 0.075 µg a.i./L).

Chronic effects derived from chronic mesocosm, semi-field and field ('higher tier') studies

The higher tier toxicity data (i.e., NOECs for density, abundance, emergence, and mortality) that were judged to be acceptable are listed in full in the Supporting Information (Section S6). A comparison of these data with the chronic laboratory-based toxicity data reported in Table 2 reveals the degree to which the incorporation of more environmentally realistic conditions and species interactions within the higher tier studies can affect the apparent sensitivity of aquatic invertebrates to imidacloprid. For example, Roessink et al. [30] reported a chronic (28-day) LC10 of 0.041 µg a.i./L for *Cloeon dipterum* under standard laboratory conditions (Table 2), whereas chronic NOECs for this species reported from the higher-tier studies ranged from 0.243 to 9.4 µg a.i./L (Supporting Information Section S6, [25, 32, 33]). These results suggest that standard chronic laboratory test may significantly overestimate the sensitivity of aquatic invertebrates to imidacloprid under realistic field conditions. Therefore, significant consideration should be given to the higher tier study data when making risk conclusions.

In many of the higher tier studies, toxicity endpoints were reported for taxonomic groupings at a higher level of organization than species (e.g., at the genus, tribe, subfamily, or order level) (see Supporting Information Section S6). As such, we could not derive an SSD using these data. However, given the wealth of acceptable data available, we were able to derive a sensitivity distribution at a higher level of taxonomic characterization. Therefore, the chronic

higher-tier NOECs were grouped at the level of family, subfamily, or class to generate a Taxon Sensitivity Distribution (TSD, see data summary in Table 3).

To select the endpoints used to derive the TSD, the most sensitive acceptable NOEC for each taxonomic group from each study was compiled. Following the approach used for the acute and chronic laboratory-based SSDs, the geometric mean was used to summarize multiple endpoints reported for the same family, subfamily, or class from different studies (Table 3). In some cases, this approach excluded less sensitive NOECs from studies where recovery was taken into account. For example, in Moring et al. [22], the test system was observed for three months following cessation of treatment. During the initial exposure period, amphipods, copepods, and macroinvertebrates experienced declines in abundance, resulting in NOECs as low as 2 µg a.i./L (Table 3). However, full recovery of all species was observed within eight weeks of the final treatment. Therefore, Moring et al. [22] suggested that the next highest treatment concentration (6 µg a.i./L) should be adopted as a regulatory NOEC for these families. Likewise, Ratte and Memmert [25] reported NOECs as low as 0.6 µg a.i./L (Table 3) when recovery was not considered, but also reported "no observed ecologically adverse effect concentrations" (NOEAECs) ranging from 9.4 µg a.i./L to 23.5 µg a.i./L based on the complete recovery of emerging insects and zooplankton within eight weeks of the last application. Although these recovery-based endpoints were excluded from the TSD dataset (Table 3), they suggest that risk to aquatic invertebrates from chronic imidacloprid exposure may be overestimated when the potential for recovery is not accounted for.

Additionally, the NOECs reported in the higher tier studies were based on initial treatment concentrations following the first application (either nominal concentrations that were confirmed analytically or measured concentrations). However, the higher tier studies did not seek to maintain a constant concentration over the course of the study period. The pulse exposure regimes used in these studies varied with respect to number of applications (1 to 4 applications)

and retreatment interval (7, 14, or 21 d intervals) in order to mimic potential drift or runoff events. Following application, the imidacloprid concentration declined due to the natural degradation and dissipation of imidacloprid in aquatic systems. Therefore, to develop an effects metric that could be compared to a constant chronic exposure duration, time-weighted average concentration estimates were determined for the reported NOECs from the day of the first application to 21 days following the final application using the degradation half-life (DT50) of 11.6 days reported by Roessink et al. [32] and assuming first-order elimination kinetics per Equation 2. The 21 day interval was selected as this corresponded to the most common application interval in the higher tier studies with multiple applications. Additionally, a consistent cutoff was required to ensure that exposure estimates were not severely underestimated in studies that had very long durations.

$$\text{for } i = 1 \text{ to n}, \qquad C_i = \frac{C_{i-1}(1 - e^{-kt})}{kt} \tag{2}$$

Where n = The number of treatments applied in the study; C_i = The time-weighted average concentration (*µg a.i./L)* from application *i* to the next application or to 21 days following application *i* if there were no further applications. Note, C_0 represents the initial concentration immediately following the first treatment as reported in the study. t = the duration of time over which the time weighted average was calculated (i.e., the retreatment interval for *i* = 1 to n-1 and 21 days for *i=n.* k = ln(2)/DT50

For studies with only one treatment application, the time-weighted average concentration from the day of application to 21 days after application was calculated using Equation 2 directly. For studies with more than one treatment application, Equation 2 was applied to calculate time-weighted average concentrations between each application and from the final application to 21

days after that application and then an overall time-weighted average for the whole study was estimated using Equation 3.

$$\text{TWA} = \frac{(\sum_{i=1}^{n-1} C_i * t) + C_n * 21}{(n-1)t + 21} \quad (3)$$

Where

TWA = the overall study length time-weighted average concentration (µg a.i./L*)*

C_i = the time-weighted average concentration (µg a.i./L*)* from the *i*th application to the next application if there was a subsequent application or from the *i*th application to 21 days following that application if there were no further treatments (see Equation 2).

n = the number of treatments applied in the study

t = the length of the retreatment interval

The resulting time-weighted average NOEC estimates are reported in Table 3.

Although it is not possible to conclude that effects observed in the higher tier studies were the result of the longer term, lower exposure concentration represented by the TWA rather than due to an acute peak exposure or repeated peak exposures, using the TWA results in lower, more conservative NOEC values than those originally reported in the studies (see Table 3). Therefore, since the TWA approach is useful for standardizing results between different studies and exposure regimes while potentially increasing the conservatism of the assessment, it was considered appropriate and allowed for the use of all relevant studies in the assessment.

Taxa-specific sensitivity distributions (TSDs) were fit to the time-weighted average NOEC estimates in Table 3 using SSD Master v3. The best-fitting distribution was the Gumbel

model (in log space) based on the results of the Anderson-Darling (AD) goodness-of-fit test statistic (AD=0.612, p>0.05), mean square error (0.0061) and mean square error lower tail (0.055). The Gumbel model closely followed the data (Figure 3). This model is described by Equation 4:

$$f(x) = e^{-e^{\frac{(\mu - x)}{b}}} \quad \textbf{(4)}$$

where *x* is concentration (in µg a.i./L), and *f(x)* is the proportion of taxa affected. The fitted location and scale parameters, μ and *s*, for this model were 3.38 and 0.347, respectively. The predicted 5% hazard concentration (HC_5) for this TSD was 1.01 µg a.i./L (95% CI from 0.692 to 1.47 µg a.i./L).

2.3 Exposure assessment

The objective of the refined aquatic exposure modeling was to derive comprehensive and realistic distributions of aquatic estimated environmental concentrations (EECs) associated with representative imidacloprid use patterns. The approaches used to select use patterns and derive refined aquatic exposure estimates for agricultural and non-agricultural uses are described below.

2.3.1 Agricultural use patterns

An evaluation of all labeled agricultural uses of imidacloprid was not practical. Therefore, a subset of crop use patterns was selected to represent various crop types and geographic regions and to include higher vulnerability uses (i.e., uses predicted to have the highest EECs using standard Tier II aquatic exposure modeling, see Supporting Information Section S7). The selected crop use patterns (Table 4) included major crops (e.g., soybeans and potatoes) and minor crops (e.g., fruiting vegetables and leafy greens) as well as both row crops and orchard crops (citrus). Additionally, high vulnerability uses such as cucurbits were selected (see Supporting Information Section S7). Overall, the crop use patterns chosen for refinements represent a broad spectrum of the imidacloprid agricultural uses and are expected to provide a

comprehensive understanding of the likely exposure associated with registered agricultural uses of imidacloprid.

Exposure modeling for the agricultural use patterns incorporated EPA's standard aquatic exposure modeling tool, the Surface Water Concentration Calculator (SWCC) model version 1.106 [34], and the standard assumptions of a 10 hectare field draining into a 1 hectare, 2 meter deep farm pond. In addition, a specialized vegetative filter strip model (VFSMOD version 4.2.4) [35] was used to simulate the mitigating effects of the 10 foot vegetative filter strip (VFS) that is required on the imidacloprid agricultural label.

For all agricultural scenarios except for the two potato scenarios, Latin Hypercube sampling of the probability distributions of key characteristics (i.e., application date(s), weather stations, soil profile and land surface slope, pond-integrated spray drift fraction and percent cropped area) was used to develop 1,000 unique sets of the required model input parameters. This was done for each use scenario rather than relying on standard EPA screening level scenarios representative of high runoff and erosion potential to define model input values. Using these sets of parameters, 1,000 runs of 30-year simulations were modeled for each use pattern, resulting in a distribution of 30,000 annual maximum EECs for each exposure duration of interest (i.e., peak, 48-hour, 96-hour, 21-day, 60-day, and 90-day average periods).

For the potato scenarios, a different approach was applied that did not include the probabilistic characterization of the model inputs or accounting for a VFS using VFSMOD. Rather, refinements to the potato exposure modeling focused on parameterizing the application method to reflect in-furrow application at planting. This application method limits potential imidacloprid runoff to surface water exposure as the majority of the pesticide is applied at the planting depth and below the active runoff zone in the soil.

Further details regarding the models used as well as identification and development of the required model input values for the refined agricultural use exposure modeling are provided in Supporting Information, Section S8.

2.3.2 Non-agricultural use patterns

For the non-agricultural use patterns, the SWCC model (version 1.106, [34]) was also used to develop distributions of refined aquatic exposure estimates. Exposure refinements were achieved by simulating more realistic model inputs with respect to application timing, runoff vulnerability, off-target overspray, percent cropped area (PCA) (for golf course uses), and percent treated area (PTA). The specific model input refinements varied across the three types of non-agricultural use patterns assessed (i.e., golf course turf, residential, and nursery uses). These refinements are described in detail in Supporting Information, Section S9. Overall, these refinements produced distributions of annual maximum EECs with 30,000 values (i.e., 30 modeled years x 1,000 customized model input scenarios) for the golf course turf use patterns, 10,740 or 10,950 values for the residential use patterns (30 years modeled based on either 358 or 365 initial application dates), and between 19,890 and 24,750 values for the nursery use patterns (30 years modeled for each of 3 different runoff curve number assumptions for between 221 and 275 possible initial application dates).

2.4 Risk characterization

For the refined risk assessment, risk curves were generated by integrating the distributions of refined exposure estimates with the refined measures of effect. Risk curves plot the cumulative probability of exceedance versus the magnitude of effect (i.e., the probability of exceeding effects of differing magnitude). The use of such risk curves, also known as "joint probability curves" [6], in risk analysis has been recommended by both the NAS [5] and the EPA [36-38]. For each risk curve, the area under the curve (AUC) was calculated. Expressed as a percentage, AUC can range from 0% to 100%, with a lower AUC representing a lower overall probability and magnitude of risk. Although the AUC does not provide information about the

shape of the risk curve, it can be used to summarize and compare risk scenarios. For this assessment, the AUC of each risk curve was used to categorize risk qualitatively as *de minimis*, low, intermediate, or high for each use pattern as follows.

- If the AUC was less than the AUC associated with the curve produced by risk products (risk product = exceedance probability x magnitude of effect) of 0.25% (e.g., 5% exceedance probability of 5% or greater effect = 0.25%), then the risk was categorized as *de minimis*. The AUC for risk products of 0.25% is 1.75%;
- If the AUC was equal to or greater than 1.75%, but less than 9.82% (i.e., the AUC for a curve with a constant risk product of 2%), then the risk was categorized as low;
- If the AUC was equal to or greater than 9.82%, but less than 33% (i.e, the AUC for a curve with a constant risk product of 10%), then the risk was categorized as intermediate; and
- If the AUC was equal to or greater than 33%, then the risk was categorized as high.

The risk category boundaries, as described above, are shown in Figure 4. These risk categories are intended to be summary descriptors of the risks to aquatic invertebrates exposed to imidacloprid near treated areas. Similar risk categorization schemes have previously been applied to ecological risk assessments for other pesticides [39-41] and contaminated sites [42]. Overall, these risk boundaries are designed to be protective of the aquatic invertebrate community and therefore do not focus on effects to single species (or taxa). The high reproductive potential of most aquatic ecosystems enables them to rebound in a relatively short time after experiencing low to intermediate adverse effects [23, 24, 26, 43]. However, Liess and Schulz [44] showed that recovery may take longer (months to years) for aquatic invertebrates when local populations are extirpated. Thus, exposure scenarios in the high risk category would be of concern due to the long periods of time potentially required for population recovery.

RESULTS AND DISCUSSION

Exposure modeling results

Agricultural use patterns. A summary of the refined 90th percentile estimated environmental concentrations (EECs) at different exposure durations is provided in Table 5. Mississippi soybean had the highest 90th percentile concentrations, ranging from 0.30 µg a.i./L (annual maximum) to 0.03 µg a.i./L (annual average). The higher Mississippi soybean concentrations relative to the other crop scenarios were driven by higher spray drift potential resulting from the aerial application method and close proximity of treated areas to receiving water, as well as higher percent cropped areas (PCAs) around water bodies relative to some other crops. The California and Florida citrus scenarios followed soybean with 90th percentile concentrations ranging from 0.14 µg a.i./L (Florida, annual maximum) to 0.01 µg a.i./L (annual average). Concentration predictions for California and Florida citrus were impacted by the higher PCA distributions for those crop scenarios. Overall, the highest maximum concentrations for each scenario were driven by environments with high runoff potential, high drift and high PCA. The maximum concentrations for each scenario have a very low probability (1/30,000) of occurring and represent extreme worst-case conditions given that the corresponding 90th percentile concentrations are orders of magnitude lower than the maxima.

Scenarios with the soil chemigation/drench application method (no spray drift) resulted in lower concentrations than the scenarios with ground and aerial applications. Seed treatment application of imidacloprid has no potential for drift and represents much lower vulnerability use patterns than the use patterns evaluated in this refined assessment. Soil application results in lower surface water concentrations for all crops compared to foliar application of imidacloprid. Of the scenarios with soil application, California tomato had the highest refined 90th percentile annual maximum concentrations because it had some of the highest PCAs and the lowest median vegetative filter efficiency (41.7%, Supporting Information Section S8).

2.4.1 Non-agricultural use patterns

Summaries of the refined 90th percentile estimated environmental concentrations (EECs) at different exposure durations for golf course turf, residential, and nursery use patterns are provided in Table 6, Table 7, and Table 8, respectively. For each use pattern, EECs are shown both with and without the application of a percent treated area (PTA) refinement. As described in Supporting Information Section S9, the EECs derived for the 100% PTA condition assume that imidacloprid is applied to all possible use sites for the applicable use pattern. However, it was possible to refine the PTA estimates for each use pattern by combining various geographic datasets with imidacloprid sales data from 2013 and 2014 for golf course, residential, and nursery use products (see description in Supporting Information Section S9). Using these refined PTAs in the modeling produced EECs that reflect more realistic exposure conditions.

2.4.2 Comparison to surface water monitoring results

To provide context for the refined exposure modeling results, modeled concentrations were compared to available surface water monitoring data for imidacloprid from the EPA Storage and Retrieval (STORET) Data Warehouse [45], US Department of Agriculture (USDA) Pesticide Data Program (PDP) [46], US Geological Survey (USGS) National Water Quality Assessment (NAWQA) Program [47], and the USGS National Water Information System (NWIS) [48, 49]. More than a decade of water sample data were available from some monitoring stations in these federal monitoring programs. For example, in the USGS NAWQA program, water quality was monitored in 42 study units located across the US between 1991 and 2012. These study units were designed to "cover a variety of important hydrologic and ecological resources, critical sources of contaminants, including agriculture, urban and natural sources; and a high percentage of population served by municipal water supply and irrigated agriculture" [50]. Furthermore, the NAWQA study units are representative of a wide range of agricultural practices (e.g., crops, tillage, irrigation, drainage and chemical use) and landscapes (e.g., geology, soil type, topography, climate and hydrology) [51].

In these federal monitoring datasets, imidacloprid was detected in 4 to 18.5% of all samples, with detection limits ranging from 0.0015 to 0.5 µg a.i./L. Conservative estimates of 90th percentile imidacloprid concentrations in these datasets; calculated by setting all non-detect samples to the reported limit of detection, limit of quantitation, or reporting limit; ranged from 0.02 to 0.106 µg a.i./L (Supporting Information Section S10). Additionally, only 0.23% of these monitoring samples exceeded 1 µg a.i./L. In comparison, the refined exposure modeling in this assessment predicted 90th percentile annual maximum peak EECs ranging from 0.006 to 0.304 µg a.i./L for agricultural use patterns, from 0.18 µg a.i./L to 4.42 µg a.i./L for non-agricultural use patterns assuming 100% PTA, and from 0.0026 µg a.i./L to 0.061 µg a.i./L for non-agricultural use patterns with PTA refinements applied. Although a direct comparison between modeling data and monitoring data is not possible due to differences in the frequency and duration of data collection, the ranges of modeled EECs are similar to or exceed the ranges of values reported in the monitoring datasets, suggesting that the refined exposure assessment are generally realistic with a bias to being moderately conservative.

Risk characterization

Acute risk. Acute risk curves were generated by integrating the probabilistic distribution of the maximum peak EECs from each modeled year for each customized model input scenario with the acute lab-based SSD shown in Figure 1. Using the AUC statistic, risk to aquatic invertebrates from acute exposure to imidacloprid was categorized as *de minimis* for all agricultural use patterns (Table 9). For example, consider the risk curve for Mississippi Soybean (MS soybean), which had the highest AUC of the agricultural use patterns (0.342%, Table 9). This risk curve lies very close to the x- and y-axes, indicating very low probabilities of adverse effects (mortality) to the most sensitive species and negligible probabilities of effects to less sensitive species (Figure 5).

For the non-agricultural use patterns when 100% PTA was assumed, eight of 12 use patterns were categorized as posing *de minimis* risk (Table 10). The remaining four use patterns, which were categorized as posing a low risk, were nursery uses in Florida, Michigan, New Jersey, and Tennessee (Table 10). However, nursery use of imidacloprid is typically a spot treatment. The assumption of 100% PTA overestimates exposure and therefore risk. With PTA refinements (described in detail in Supporting Information Section S9), nursery uses in these states were categorized as being at *de minimis* risk (Table 10). For additional context, the acute risk curve for Golf Course Turf (Pennsylvania Turf, Refined PTA) is shown in Figure 6. This risk curve represents the worst case scenario (highest AUC) for acute risk due to non-agricultural uses for the refined PTA assumption. This worst-case risk curve lies very close to the x- and y-axes, indicating negligible probabilities of exceeding the effects metric used in the acute lab-based SSD (i.e., acute LC50 or EC50 (immobilization)) for any significant proportion of species (e.g., the probability of exceeding the effects metric for 5% of species is 0.00333%) (Figure 6). Overall, no unreasonable risk of adverse effects to aquatic invertebrates is expected from acute exposure to imidacloprid from agricultural or non-agricultural uses.

2.4.3 Chronic risk

Two sets of chronic risk curves were generated to characterize chronic risk. In the first set, risk curves were generated by integrating the distribution of the maximum 21-day average EECs from each modeled year with the chronic lab-based SSD. However, as noted in the chronic effects assessment, the chronic higher tier TSD is considered the best available data for chronic risk assessment as it is more directly relatable to community level effects and the studies used to generate the TSD were conducted under more realistic environmental conditions. Therefore, the second set of chronic risk curves integrated the same chronic exposure data with the chronic higher tier TSD (i.e., a taxon-specific sensitivity distribution composed of chronic higher-tier NOECs established at the family/sub-family taxonomic level).

For the agricultural use patterns with risk curves generated using the chronic lab-based SSD, seven out of 14 were categorized as 'low' risk, while the remaining were *de minimis* risk (Table 11). In contrast, chronic risk curves generated using the best available data (i.e., the chronic higher tier TSD) were categorized as *de minimis* for all 12 modeled agricultural use patterns (see Chronic II risk curves, Table 11). The worst-case (highest AUC) agricultural use risk curve generated using the chronic higher tier TSD was Florida Citrus (Florida Citrus, Figure 7), with an AUC of 0.0734% (Table 11). This risk curve shows an extremely low probability of exceeding the TSD effects metric for even a small percentage of taxa (Figure 7).

For the non-agricultural use patterns, the risk curves derived using the chronic lab-based SSD and EECs developed assuming 100% PTA indicated low risk for one use pattern, intermediate risk for eight use patterns and high risk for three use patterns (Table 12). However, when the PTA refinement was applied to the EECs for these non-agricultural use patterns, the risk curves generated using the chronic lab-based SSD indicated that risk was either *de minimis* (seven use patterns) or low (five use patterns), with the worst-case (highest AUC of 2.66%) risk curve for Golf Course Turf (Pennsylvania Turf) (Table 12). For risk curves derived using the chronic higher tier TSD, risk was categorized as *de minimis* for the majority of non-agricultural uses, regardless of whether the PTA refinement was applied (Table 12). Additionally, with the PTA refinement, all non-agricultural use patterns had *de minimis* chronic risk (Table 12). Of these, the worst-case scenario (highest AUC of 0.00160%, Table 12) was observed for Golf Course Turf (Florida Turf, Figure 8). This risk curve exhibits negligible probabilities of exceedance of the effects metric used in the chronic higher tier TSD (e.g., probability of exceeding the higher tier family/subfamily based NOECs for the 5% most sensitive taxa is 0.00667%, Figure 8). Given that the chronic higher tier TSD and the PTA-refined EECs represent the best available data, no unreasonable risk of adverse effects to aquatic invertebrates is expected from chronic exposure to imidacloprid from non-agricultural uses.

2.5 Assumptions and uncertainties

This assessment was developed with the best available data and information gained from long-term registered imidacloprid use in the United States. However, uncertainty is inherent in every assessment. The probabilistic exposure modeling approach accounted for several sources of uncertainty associated with imidacloprid use sites and receiving waters by sampling distributions of application timing, weather, soil characteristics, PCA, and drift fraction reflective of crop proximity relative to pond locations and pond surface area. This approach reduced the uncertainty in the model assumptions, inputs, and predicted imidacloprid concentrations by incorporating best available datasets to better characterize the range of conditions leading to potential imidacloprid exposure. In accounting for the uncertainty in the environmental and agronomic conditions assessed, the range of potential imidacloprid exposure concentrations increased, because a much broader set of conditions were considered.

Additionally, the exposure modeling maintained several conservative assumptions that may have overestimated imidacloprid EECs. For example, the EPA standard farm pond considered in the modeling does not account for potential outflow during large runoff events or for the addition of eroded sediment into the water body. Accounting for outflow would lead to lower EECs, as some chemical would be flushed downstream rather than being held in the static water body. A higher sediment concentration in the water body could lead to a larger portion of the pesticide in the water column binding to sediment, making it non-bioavailable. Other conservative assumptions included use of the maximum label application rate for non-agricultural scenarios although typical applications are often at lower rates, assuming that 100% of the crop area was treated for agricultural scenarios even though imidacloprid does not have 100% of the market share, ignoring agricultural practices that may reduce pesticide transport off-field (e.g., conservation tillage and avoiding pesticide application on rainy days), and assuming

that all pesticide on foliage at harvest time will be returned to the soil although harvested material may be removed from the field.

With respect to the measures of ecological effects applied in this assessment, a thorough literature review and study evaluation process was carried out to identify the best available data. Because only high quality data were considered, a high level of confidence can be placed on the data selected for use in this assessment. Additionally, acceptable toxicity data were identified for a much more diverse group of aquatic invertebrates than the core EPA pesticide registration data requirements (i.e., one acute freshwater invertebrate (EC50) study, one freshwater invertebrate life cycle (NOEC) study, and one acute estuarine invertebrate (LC50/EC50) per 40 CFR 40, §158.630 (2015)). The inclusion of data for a larger variety of aquatic invertebrates also helps to reduce uncertainty regarding the effects assessment. Finally, the effects assessment did not consider the potential for recovery of affected species through recolonization, high reproductive potential, or other means and was thus conservative.

CONCLUSIONS

Aquatic invertebrates are highly sensitive to imidacloprid exposure under certain conditions. For standard laboratory-based toxicity tests that received an acceptable rating in the study evaluation, acute LC50 values were as low as 2.07 µg a.i./L (Table 1) and chronic NOEC values were as low as 0.041 µg a.i./L (Table 2). The chronic NOECs observed in the higher-tier studies were considerably less sensitive, but still reflective of potentially environmentally relevant aquatic exposure concentrations. For example, the most sensitive acceptable chronic NOEC value used to develop the chronic higher-tier TSD was 0.243 µg a.i./L (Table 3) while the 90th percentile imidacloprid concentrations reported in federal monitoring datasets in recent years ranged from 0.02 to 0.106 µg a.i./L (Supporting Information Section S10). However, a direct comparison of monitoring data to effects data overlooks issues such as frequency and duration of exposure, geographic variability, and the potential for changes in use patterns or label instructions to alter exposure over time. Therefore, aquatic exposure modeling was used in this assessment to derive comprehensive and realistic exceedance probabilities of imidacloprid aquatic concentrations for various ecologically relevant durations. It was then possible to generate risk curves that plot cumulative probability of exceedance versus the magnitude of effect by integrating the modeled exposure distributions with relevant SSDs and TSDs derived using the best available data. The results of this probabilistic risk assessment found that based on the best available data (i.e., refined exposure modeling and higher-tier toxicity data), aquatic invertebrate communities are not likely to be at risk from acute or chronic exposure to imidacloprid from registered uses in the United States.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data Availability—For additional data, please see the supporting information file.

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Figure 1. Acute lab-based SSD constructed using acceptable laboratory-based acute toxicity endpoints for aquatic invertebrates (i.e., acute LC50 or EC50 (immobilization) values).

Figure 2. Chronic lab-based SSD constructed using acceptable laboratory-based chronic toxicity endpoints for aquatic invertebrates (NOEC, EC10, or LC10 values representative of ecologically relevant effects to growth, mortality, or reproduction).

Figure 3. Chronic higher-tier TSD constructed using estimated time weighted average NOECs from higher tier toxicity studies.

Figure 4. Risk curves defining the AUC boundaries for risk categorization.

Figure 5. Acute risk curve for Mississippi soybean.

Figure 6. Acute risk curve for Golf Course (Pennsylvania Turf) with percent treated area (PTA) refinement.

Figure 7. Chronic risk curve for Florida Citrus using chronic higher tier TSD.

Figure 8. Chronic risk curve for Golf Course (Florida Turf) with percent treated area (PTA) refinement using higher tier TSD.

Table 1. Acceptable laboratory-based acute toxicity endpoints (LC50 or EC50 (immobilization) values) for aquatic invertebrates used to derive acute SSD

Scientific Name (Duration)	***LC50 or EC50 (immobilization) (µg a.i./L)***	***Reference***
Ceriodaphnia dubia (48 h)	2.07	Chen et al. [52]
Caenis horaria (96 h)	6.68	Roessink et al. [30]
Simulium vittatum (48 h)	6.75	Overmyer et al. [53]
Chironomus tentans (96 h)	10.5	Gagliano [54]
Cloeon dipterum (96 h)	26.3	Roessink et al. [30]
Mysidopsis bahia (72 – 96 h)	34.8[a]	Lintott [55]; Ward [56]
Plea minutissima (96 h)	37.5	Roessink et al. [30]
Hyalella azteca (96 h)	55	England and Bucksath [57]
Chironomus riparius (24 h)	55.2	Dorgerloh and Sommer [58]
Chaoborus obscuripes (96 h)	294	Roessink et al. [30]
Asellus aquaticus (96 h)	316	Roessink et al. [30]
Daphnia magna (48 h)	131,000[b]	Riebschläger [59]

a. Geometric mean of 33.7 µg a.i./L [56] and 36 µg a.i./L [55].

[b] Not included in final SSD due to its relative insensitivity. Removing this endpoint improved overall fit of SSD.

Table 2 Acceptable laboratory-based chronic toxicity endpoints (NOEC, EC10, or LC10 values representative of ecologically relevant effects to growth, mortality, or reproduction) for aquatic invertebrates used to derive chronic laboratory-based SSD

Scientific Name	***Effect Concentration (µg a.i./L)***	***Reference***
Cloeon dipterum	0.041	Roessink et al. [30]
Mysidopsis bahia	0.163	Ward [31]
Caenis horaria	0.235	Roessink et al. [30]
Chironomus tentans	1.14	Stoughton et al. [60]
Asellus aquaticus	1.35	Roessink et al. [30]
Chironomus riparius	1.70[a]	Bruns [61, 62]; Dorgerloh [63-65]; Dorgerloh and Sommer [66-68]
Chaoborus obscuripes	1.99	Roessink et al. [30]
Plea minutissima	4.35	Roessink et al. [30]
Gammarus pulex	19.2[b]	Hendel [69], Roessink et al. [30]
Sialis lutaria	25.1	Roessink et al. [30]
Daphnia magna	1,236[c,d]	Heimbach [70]; Ieromina et al. [71]; Jemec et al. [72]; Pavlaki et al. [73]; Young and Blakemore [74]

a. Geometric mean of 0.56 µg a.i./L [62], 1.33 µg a.i./L [61], 1.8 µg a.i./L [63], 1.8 µg a.i./L [64], 1.87 µg a.i./L [68], 2.09 µg a.i./L [66], 2.28 µg a.i./L [67], 3.19 µg a.i./L [65]

b. Geometric mean of 5.77 µg a.i./L [30], 64 µg a.i./L [69]

c. Geometric mean of 320 µg a.i./L [70], 1250 µg a.i./L [72], 1,800 µg a.i./L [74], 2000 µg a.i./L [71], 2000 µg a.i./L [73]

[d]Not included in final SSD due to its relative insensitivity. Removing this endpoint improved overall fit of SSD

Table 3 Acceptable higher tier toxicity data for aquatic invertebrates (i.e., NOECs for density, abundance, emergence, and mortality) grouped by family, subfamily (Chironiminae, Orthocladinae, and Tanypodinae only), or class (Copepoda only) used to derive chronic higher-tier TSD

Scientific Name	***Effect Concentration Reported in Study (µg a.i./L)*** [a]	***Time-weighted Average NOEC Estimates (µg a.i./L)***	***Reference***
Baetidae	0.816[b]	0.581	Roessink et al. [32], Ratte and Memmert [25], Roessink and Hartgers [33], Moring et al. [22]
Chironominae	1.90[c]	1.48	Ratte and Memmert, [25], Moring et al., [22]
Hydrophilidae	2.00	1.87	Moring et al. [22]
Caenidae	2.00	1.87	Moring et al. [22]
Hydroptilidae	2.00	1.87	Moring et al. [22]
Naididae	3.80	2.47	Ratte and Memmert [25]
Chaoboridae	3.80	2.47	Ratte and Memmert [25]
Orthocladiinae	3.80	2.47	Ratte and Memmert, [25]
Copepoda	7.51[d]	5.85	Moring et al. [22], Ratte and Memmert [25]
Glossiphoniidae	9.40	6.12	Ratte and Memmert [25]
Daphniidae	9.40	6.12	Ratte and Memmert [25]
Planorbidae	9.40	6.12	Ratte and Memmert [25]
Tipulidae	12.0	6.84	Kreutzweiser et al. [75]
Tanypodinae	13.7[e]	10.7	Ratte and Memmert [25], Moring et al. [22]
Pteronarcyidae	24[f]	13.7	Kreutzweiser et al. [75], Kreutzweiser et al. [76]

a. Endpoints selected for this assessment represent the lowest endpoint for each taxa from a single study or a geometric mean of the lowest values from multiple studies. A conservative approach was taken for selecting the endpoints that did not account for recovery.
b. Geometric mean of 0.243 µg a.i./L [32], 0.6 µg a.i./L [25], 1.52 µg a.i./L [33], and 2 µg a.i./L [22].
c. Geometric mean of 0.6 µg a.i./L [25]and 6 µg a.i./L [22].
d. Geometric mean of 6 µg a.i./L [22] and 9.4 µg a.i./L [25].
e. Geometric mean of 9.4 µg a.i./L µg a.i./L [25]and 20 µg a.i./L [22].
[f]Geometric mean of 12 µg a.i./L [75] and 48 µg a.i./L [76]

Table 4 Imidacloprid crop use patterns for refined exposure assessment and the individual crop scenarios included in association with each use pattern

Crop Use Pattern	*US EPA Standard Crop Scenarios Assessed*
Cucurbit	FLcucumberSTD, MImelonSTD, NJmelonSTD, MOmelonSTD
Citrus	FLcitrusSTD, CAcitrus_WirrigSTD
Leafy greens	CAlettuceSTD
Fruiting vegetables	FLpeppersSTD, FLtomatoSTD_V2, PAtomatoSTD, CAtomato_WirrigSTD
Brassica	FLcabbageSTD
Potato	IDNpotato_WirrigSTD, MEpotatoSTD
Soybean	MSsoybeanSTD

Table 5 Summary of refined 90th percentile exposure predictions for agricultural use patterns (µg a.i./L)

Crop Scenario[a]	*Crop Use Pattern*	*Annual Maximum*	*48-hr Average*	*96-hr Average*	*21-day Average*	*60-day Average*	*90-day Average*	*Annual Average*
Florida citrus	Citrus, foliar airblast	0.14	0.14	0.13	0.10	0.06	0.05	0.01
California citrus	Citrus, foliar airblast	0.09	0.09	0.08	0.06	0.04	0.03	0.01
California lettuce	Leafy greens, foliar ground-boom	0.07	0.07	0.06	0.05	0.03	0.03	0.01
Florida cabbage	Brassica, foliar ground-boom	0.006	0.006	0.006	0.004	0.003	0.002	0.001
Mississippi soybean	Soybean, foliar aerial	0.30	0.29	0.28	0.21	0.13	0.10	0.03
Florida peppers	Fruiting vegetables, soil chemigation/drench	0.025	0.024	0.022	0.016	0.009	0.007	0.002
Florida tomato	Fruiting vegetables, soil chemigation/drench	0.013	0.013	0.012	0.009	0.005	0.004	0.001
Pennsylvania tomato	Fruiting vegetables, soil chemigation/drench	0.006	0.006	0.005	0.004	0.002	0.002	0.0005
California tomato	Fruiting vegetables, soil chemigation/drench	0.15	0.15	0.14	0.11	0.06	0.04	0.01
Florida cucumber	Cucurbits, soil chemigation/drench	0.021	0.020	0.019	0.014	0.010	0.009	0.001
Michigan melon	Cucurbits, soil chemigation/drench	0.018	0.017	0.016	0.012	0.007	0.005	0.002
New Jersey melon	Cucurbits, soil chemigation/drench	0.059	0.056	0.054	0.040	0.023	0.017	0.004
Idaho potato	Potato, in-furrow	0.03	NA[b]	0.025	0.019	0.012	0.008	0.002
Maine potato	Potato, in-furrow	0.21	NA[b]	0.20	0.16	0.12	0.09	0.03

a. For application rates (single and annual), application interval, and number of applications considered for each use pattern, see Supporting Information Section S7 (Table S7-3).
b. NA - 48 h average EECs are not a standard calculation in EPA's Tier II modeling.

Table 6 Summary of refined 90th percentile exposure predictions for golf course turf use (µg a.i./L) with and without percent treated area (PTA) refinement

Golf Course Scenario[a]	*Use Pattern*	*Peak*	*96-hr Average*	*21-day Average*	*60-day Average*	*90-day Average*	*Annual Average*
Florida Turf	Ground app., 100% PTA	0.38	0.34	0.24	0.13	0.09	0.02
	Ground app., 12.2% PTA	0.046	0.042	0.029	0.016	0.011	0.003
Pennsylvania Turf	Ground app., 100% PTA	0.66	0.61	0.46	0.31	0.25	0.07
	Ground app., 10.1% PTA	0.067	0.061	0.046	0.031	0.025	0.007

Notes:
a.

[a]For application rates (single and annual), application interval, and number of applications considered for each use pattern, see Supporting Information Section S9 (Table S9-1).

Table 7 Summary of refined 90th percentile exposure predictions for residential use patterns (California Residential/California Impervious Scenario) (µg a.i./L) with and without percent treated area (PTA) refinement

Use Pattern[a]	*Peak*	*96-hr Average*	*21-day Average*	*60-day Average*	*90-day Average*	*Annual Average*
Turf, 2 Apps., 100% PTA	0.60	0.55	0.41	0.25	0.19	0.05
Turf, 2 Apps, 2.08% PTA	0.012	0.011	0.008	0.005	0.004	0.001
Turf, 1 App, 100% PTA	0.53	0.48	0.36	0.22	0.17	0.04
Turf, 1 App., 2.08% PTA	0.011	0.010	0.007	0.005	0.003	0.001
Ornamentals., 100% PTA	0.33	0.30	0.23	0.14	0.11	0.03
Ornamentals, 2.08% PTA	0.007	0.006	0.005	0.003	0.002	0.001
Perimeter, 100% PTA	0.36	0.33	0.25	0.16	0.11	0.03
Perimeter, 2.08% PTA	0.008	0.007	0.005	0.003	0.002	0.001

[a]For application rates (single and annual), application interval, and number of applications considered for each use pattern, see Supporting Information Section S9 (Table S9-1).

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Table 8 Summary of refined 90th percentile exposure predictions for nursery use patterns (µg a.i./L) with and without percent treated area (PTA) refinement

Scenario[a]	*Peak*	*96-hr Average*	*21-day Average*	*60-day Average*	*90-day Average*	*Annual Average*
California Nursery, 100% PTA	0.22	0.21	0.14	0.08	0.06	0.02
California Nursery, 1.16% PTA	0.0026	0.0024	0.0017	0.0010	0.0007	0.0002
Florida Nursery, 100% PTA	4.42	4.05	2.97	1.68	1.24	0.29
Florida Nursery, 0.41% PTA	0.018	0.017	0.012	0.007	0.005	0.001
Michigan Nursery, 100% PTA	1.76	1.63	1.33	0.95	0.79	0.27
Michigan Nursery, 1.7% PTA	0.030	0.028	0.023	0.016	0.013	0.005
New Jersey Nursery, 100% PTA	2.58	2.38	1.82	1.16	0.88	0.23
New Jersey Nursery, 1.24% PTA	0.044	0.040	0.031	0.020	0.015	0.004
Oregon Nursery, 100% PTA	0.92	0.86	0.73	0.53	0.41	0.11
Oregon Nursery, 0.54% PTA	0.016	0.015	0.012	0.009	0.007	0.002
Tennessee Nursery, 100% PTA	2.69	2.47	1.85	1.11	0.89	0.21
Tennessee Nursery, 0.68% PTA	0.046	0.042	0.031	0.019	0.015	0.003

a. For application rates (single and annual), application interval, and number of applications considered for each use pattern, see Supporting Information Section S9 (Table S9-1).

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Table 9 Acute risk categories for agricultural use patterns

Scenario	*AUC (%)*	*Risk Category Based on AUC*
California citrus	0.143	*De minimis*
California lettuce	0.083	*De minimis*
California tomato	0.164	*De minimis*
Florida cabbage	0.0116	*De minimis*
Florida citrus	0.189	*De minimis*
Florida cucumber	0.0331	*De minimis*
Florida peppers	0.0373	*De minimis*
Florida tomato	0.0231	*De minimis*
Idaho potato	0.0253	*De minimis*
Maine potato	0.202	*De minimis*
Michigan melon	0.0229	*De minimis*
Mississippi soybean	0.342	*De minimis*
New Jersey melon	0.0638	*De minimis*
Pennsylvania tomato	0.0113	*De minimis*

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Table 10 Acute risk categories for non-agricultural use patterns with and without percent treated area (PTA) refinement

Scenario	*Percent Treated Area (PTA) Assumed*	*AUC (%)*	*Risk Category*
Golf Course (Florida Turf)	100% PTA	0.48	*De minimis*
	Refined PTA	0.0609	*De minimis*
Golf Course (Pennsylvania Turf)	100% PTA	0.855	*De minimis*
	Refined PTA	0.086	*De minimis*
Residential Turf, 2 Apps	100% PTA	0.745	*De minimis*
	Refined PTA	0.0271	*De minimis*
Residential Turf, 1 App	100% PTA	0.646	*De minimis*
	Refined PTA	0.0229	*De minimis*
Residential Ornamentals	100% PTA	0.413	*De minimis*
	Refined PTA	0.0162	*De minimis*
Residential Perimeter	100% PTA	0.451	*De minimis*
	Refined PTA	0.0182	*De minimis*
Nursery (California)	100% PTA	0.629	*De minimis*
	Refined PTA	0.007	*De minimis*
Nursery (Florida)	100% PTA	5.09	Low
	Refined PTA	0.0297	*De minimis*
Nursery (Michigan)	100% PTA	2.66	Low
	Refined PTA	0.0577	*De minimis*
Nursery (New Jersey)	100% PTA	3.34	Low
	Refined PTA	0.0617	*De minimis*
Nursery (Oregon)	100% PTA	1.38	*De minimis*
	Refined PTA	0.0326	*De minimis*
Nursery (Tennessee)	100% PTA	3.37	Low
	Refined PTA	0.062	*De minimis*

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Table 11 Chronic risk categories for agricultural use patterns generated with the chronic lab-based SSD (Chronic I) and the chronic higher tier TSD (Chronic II)

	Chronic I:		***Chronic II:***	
Scenario	***AUC (%)***	***Risk Category Based on AUC***	***AUC (%)***	***Risk Category Based on AUC***
California citrus	3.41	Low	0.0649	*De minimis*
California lettuce	2.54	Low	0.000628	*De minimis*
California tomato	3.25	Low	0.0632	*De minimis*
Florida cabbage	0.383	*De minimis*	0.000110	*De minimis*
Florida citrus	3.82	Low	0.0734	*De minimis*
Florida cucumber	0.897	*De minimis*	0.00306	*De minimis*
Florida peppers	1	*De minimis*	0.00238	*De minimis*
Florida tomato	0.633	*De minimis*	0.00128	*De minimis*
Idaho potato	1.13	*De minimis*	5.00E-08	*De minimis*
Maine potato	6.59	Low	5.00E-08	*De minimis*
Michigan melon	0.696	*De minimis*	2.93E-04	*De minimis*
Mississippi soybean	7.83	Low	0.031	*De minimis*
New Jersey melon	1.76	Low	0.00118	*De minimis*
Pennsylvania tomato	0.361	*De minimis*	8.81E-05	*De minimis*

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Table 12 Chronic risk categories for non-agricultural use patterns generated with the chronic lab-based SSD (Chronic I) and the chronic higher tier TSD (Chronic II) with and without percent treated area (PTA) refinement

Scenario	***Percent Treated Area (PTA) Assumed***	***Chronic I:***		***Chronic II:***	
		AUC (%)	***Risk Category Based on AUC***	***AUC (%)***	***Risk Category Based on AUC***
Golf Course (Florida Turf)	100% PTA	8.3	Low	0.317	*De minimis*
	Refined PTA	1.79	Low	0.0016	*De minimis*
Golf Course (Pennsylvania Turf)	100% PTA	13.5	Intermediate	0.721	*De minimis*
	Refined PTA	2.66	Low	0.00154	*De minimis*
Residential Turf, 2 Apps	100% PTA	14.6	Intermediate	0.0994	*De minimis*
	Refined PTA	0.758	*De minimis*	5E-08	*De minimis*
Residential Turf, 1 App	100% PTA	13	Intermediate	0.0868	*De minimis*
	Refined PTA	0.669	*De minimis*	5E-08	*De minimis*
Residential Ornamentals	100% PTA	10	Intermediate	0.00692	*De minimis*
	Refined PTA	0.482	*De minimis*	5E-08	*De minimis*
Residential Perimeter	100% PTA	10.8	Intermediate	0.00536	*De minimis*
	Refined PTA	0.527	*De minimis*	5E-08	*De minimis*
Nursery (California)	100% PTA	14	Intermediate	0.118	*De minimis*
	Refined PTA	0.388	*De minimis*	5E-08	*De minimis*
Nursery (Florida)	100% PTA	38.5	High	12.6	Intermediate
	Refined PTA	0.906	*De minimis*	5E-08	*De minimis*
Nursery (Michigan)	100% PTA	33.3	High	3.91	Low
	Refined PTA	1.92	Low	5E-08	*De minimis*
Nursery (New Jersey)	100% PTA	33.9	High	6.85	Low
	Refined PTA	2.13	Low	5.25E-08	*De minimis*
Nursery (Oregon)	100% PTA	23.1	Intermediate	0.978	*De minimis*
	Refined PTA	1.14	*De minimis*	5E-08	*De minimis*
Nursery (Tennessee)	100% PTA	32.8	Intermediate	6.72	Low
	Refined PTA	2.07	Low	5.37E-08	*De minimis*

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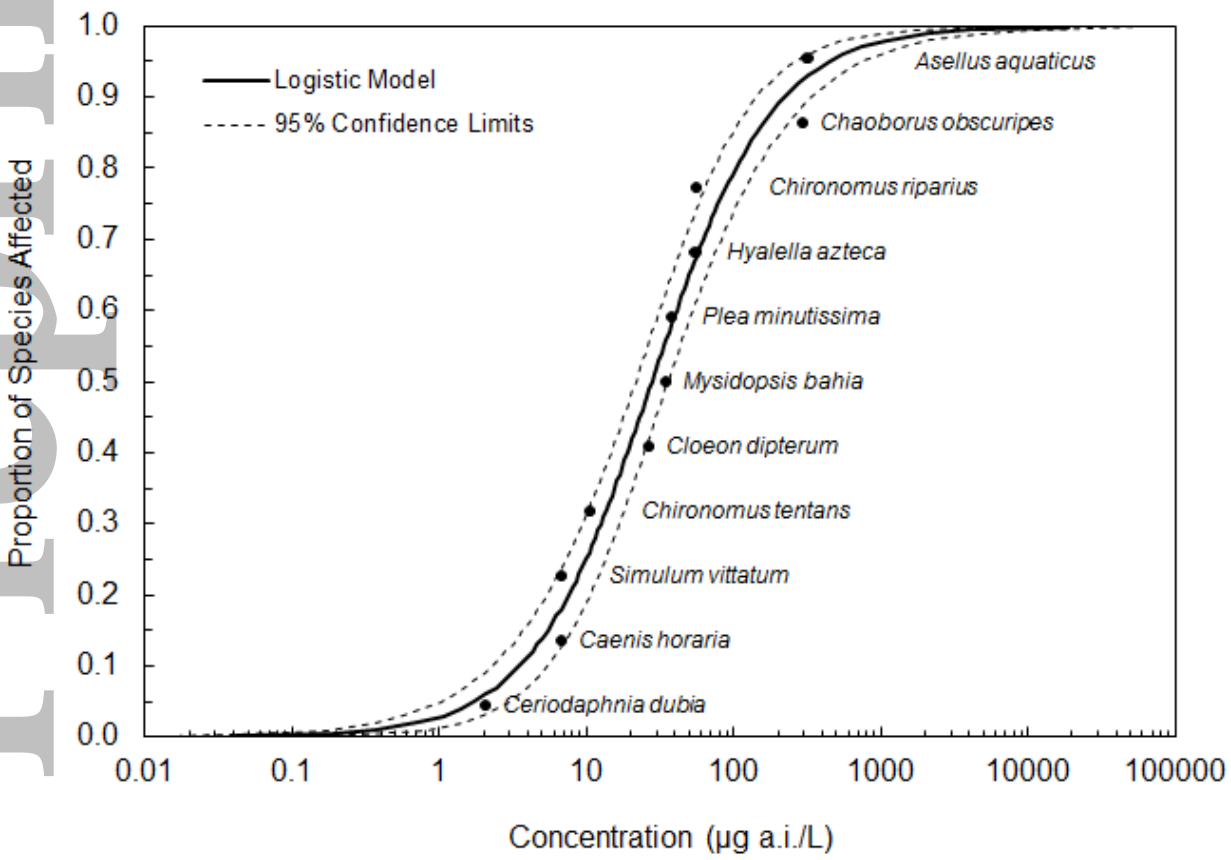

Figure 1

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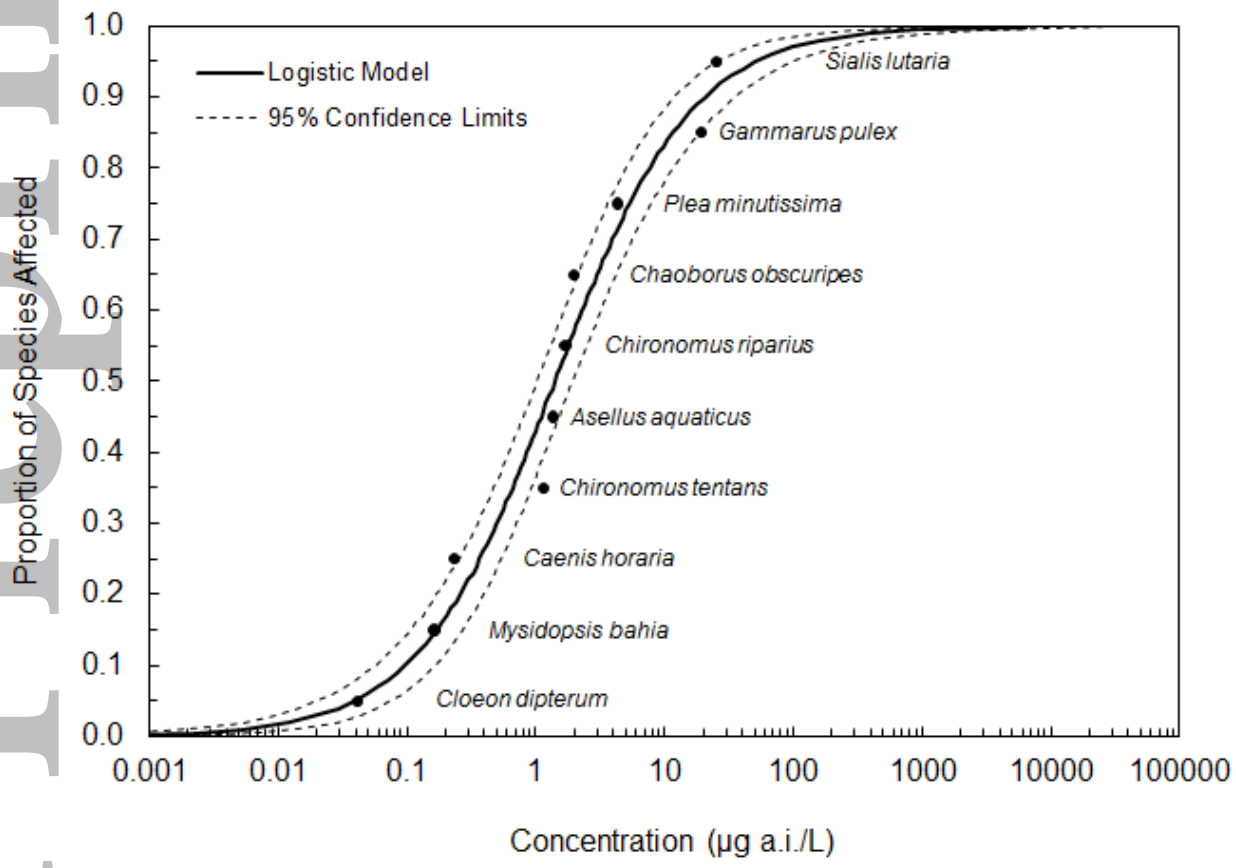

Figure 2

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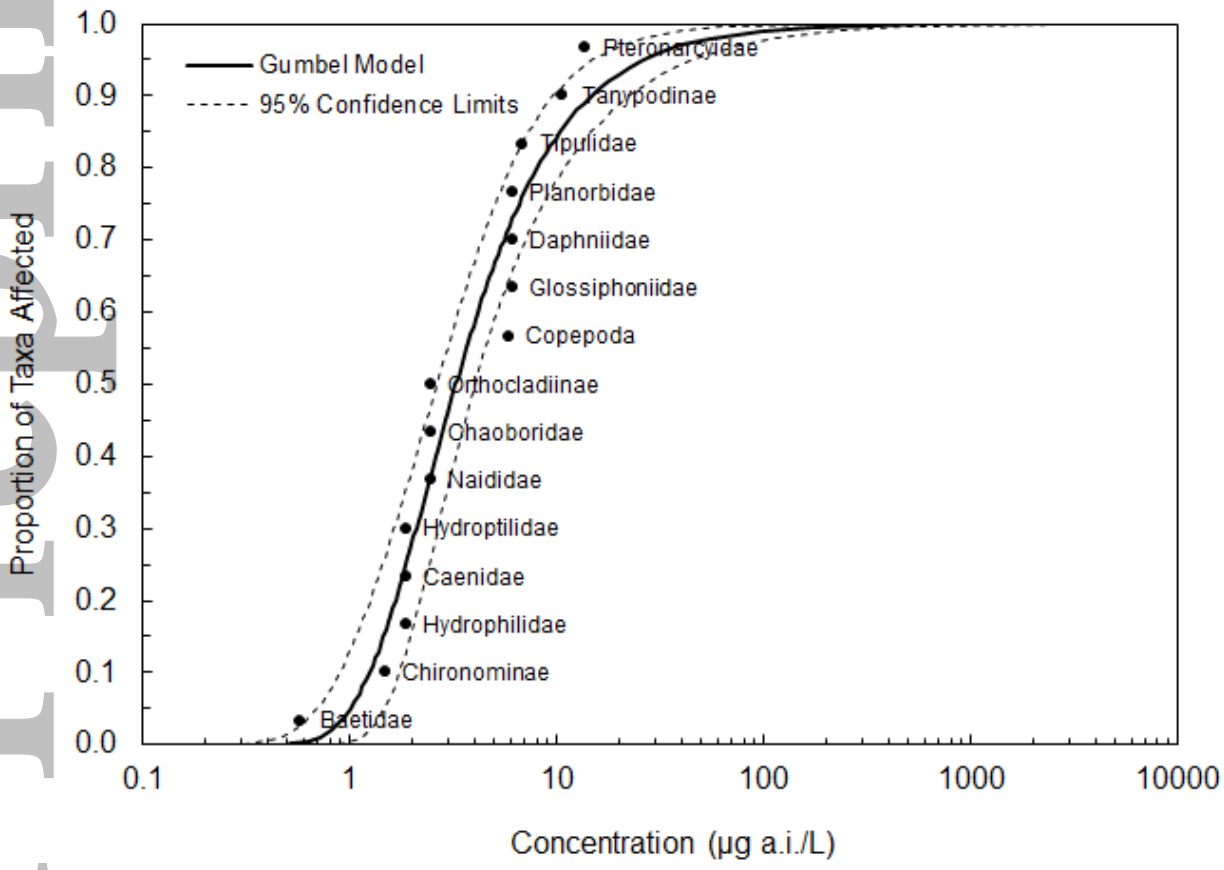

Figure 3

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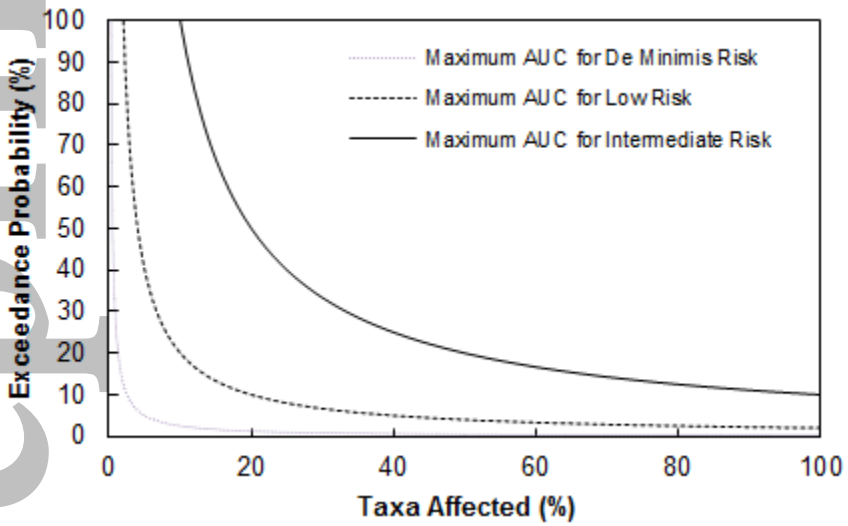

Figure 4

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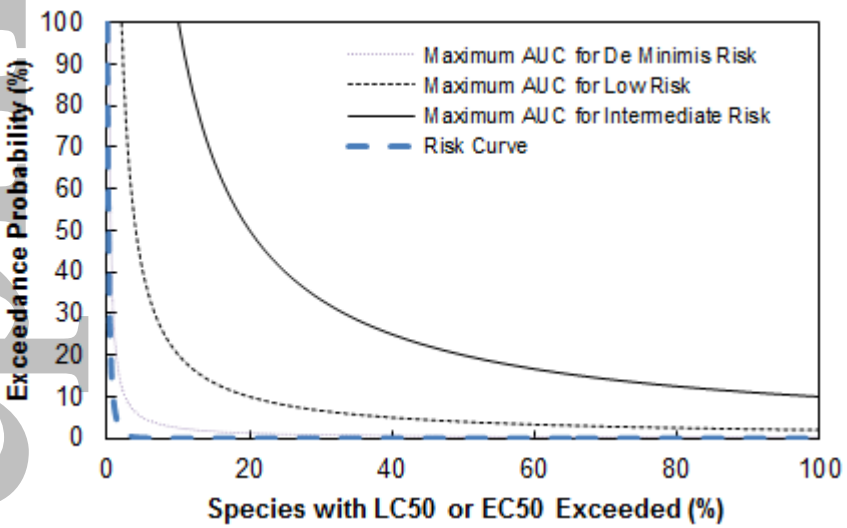

Species with LC50 or EC50 Exceeded (%)	Exceedance Probability (%)
5	0.11
10	<0.00333
25	<0.00333
50	<0.00333
75	<0.00333
90	<0.00333
95	<0.00333

Risk Summary	
AUC (%)	0.342
Risk Category	*De minimis*

Figure 5

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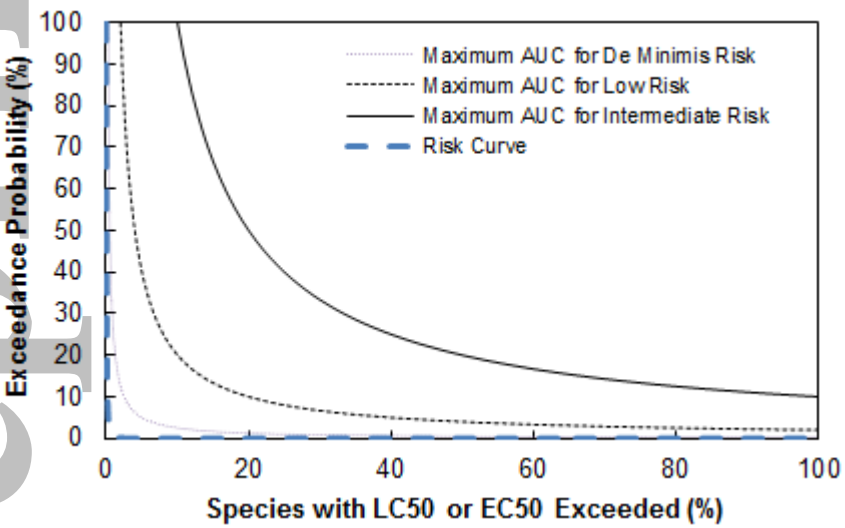

Figure 6

Species with LC50 or EC50 Exceeded (%)	Exceedance Probability (%)
5	0.00333
10	<0.0000333
25	<0.0000333
50	<0.0000333
75	<0.0000333
90	<0.0000333
95	<0.0000333

Risk Summary	
AUC (%)	0.0860
Risk Category	*De minimis*

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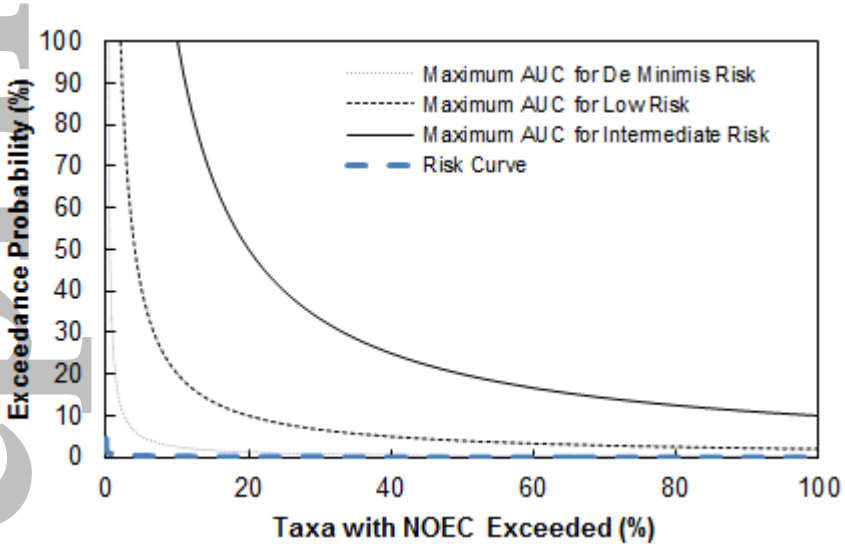

Figure 7

Taxa with NOEC Exceeded (%)	Exceedance Probability (%)
5	0.327
10	0.210
25	0.0767
50	0.0133
75	0.00333
90	<0.00333
95	<0.00333

Risk Summary	
AUC (%)	0.0734
Risk Category	*De minimis*

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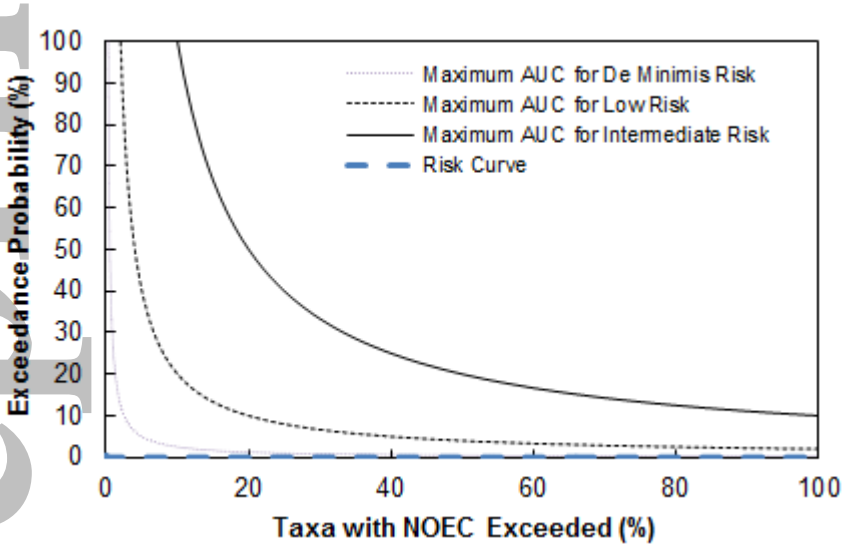

Figure 8

Taxa with NOEC Exceeded (%)	Exceedance Probability (%)
5	0.00667
10	0.00333
25	<0.0000333
50	<0.0000333
75	<0.0000333
90	<0.0000333
95	<0.0000333

Risk Summary	
AUC (%)	0.00160
Risk Category	*De minimis*

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