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Environmental Toxicology and Chemistry, Vol. 33, No. 3, pp. 648–653, 2014

Printed in the USA

IMIDACLOPRID PERTURBS FEEDING OF *GAMMARUS PULEX* AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS

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(Submitted 17 September 2013; Returned for Revision 7 October 2013; Accepted 21 November 2013)

Abstract: Changes in food uptake by detritivorous macroinvertebrates could disrupt the ecosystem service of leaf litter breakdown, necessitating the study of shredding under anthropogenic influences. The impact of the neonicotinoid insecticide imidacloprid on the feeding rate of individual *Gammarus pulex* was measured at a daily resolution both during and after a 4-d exposure period. The authors found that imidacloprid inhibits feeding of *G. pulex* during exposure at concentrations $\geq$30 μg/L and that there was no recovery in feeding on transfer into clean media for 3 d. Exposure to imidacloprid at concentrations $\geq$0.81 μg/L and $\leq$9.0 μg/L resulted in increased feeding after exposure even though there was no significant effect on feeding during the exposure itself. Comparison with the literature shows that concentrations found to influence feeding lie within the range of estimated and measured environmental concentrations. Additionally, effects on feeding rate were observed at concentrations 2 orders of magnitude lower than those causing mortality. The lethal concentration for 50% of test organisms after 4 d of exposure (270 μg/L, literature data) and the effect concentration for a reduction in feeding by 50% (5.34 μg/L) were used for this comparison. The present study discusses the potential that effects on feeding may evoke effects at the population level or disturb leaf litter breakdown in the environment. *Environ Toxicol Chem* 2014;33:648–653. © 2013 SETAC

Keywords: Aquatic invertebrate Leaf litter breakdown Sublethal effect Behavioral toxicology Pesticide

INTRODUCTION

Gammarus pulex is an aquatic detritivorous macroinvertebrate that has a key role in litter breakdown in aquatic environments through fragmentation of leaf material [1,2]. Therefore, changes in food uptake by detritivorous macroinvertebrates could disrupt this ecosystem service, necessitating the study of shredding behavior (e.g., by measuring feeding rates) under anthropogenic influences. Studies on food uptake and the energy budgets of detritivores show that food uptake is affected by xenobiotics at much lower concentrations than those causing mortality [3–7]. Food uptake of detritivorous organisms has been measured in situ and ex situ for several decades [3,7,8], and it was demonstrated that laboratory feeding assays are representative of leaf decomposition in the field [9]. Therefore, effects on feeding can be an indicator of effects at the ecosystem level, for example, with respect to leaf litter breakdown and related elemental cycling.

Nevertheless, studies observing impacts of xenobiotics on food uptake of gammarids need to be improved. Concentration and exposure patterns of xenobiotics in aquatic environments fluctuate and can change rather quickly, especially in flowing waters [10–13]. Both exposure and effects may vary over short timescales. Thus, detection of impacts from realistic exposure patterns requires an appropriate temporal resolution of measurement. Furthermore, the observation of recovery to normal feeding and/or the potential increase of feeding as a compensation for a decrease during exposure should be investigated for a more realistic assessment of effects on decomposition of leaf litter in the field.

For the present study, we worked with *G. pulex* to improve the ex situ method of feeding assays with this species by increasing its temporal resolution and facilitating measurements for individual organisms. Generally, ecotoxicological studies have measured the composite feeding rate over periods from 4 d to 7 d [8] and recovery potential has not been included (for an exception, see Nyman et al. [14]). The only studies we are aware of where feeding of gammarids was measured at a resolution of 1 d [15,16] or 2 d [17,18] were carried out in ecological studies without chemical stressors.

Imidacloprid is a neonicotinoid insecticide that is generating concern regarding potential impacts on ecosystems [19]. The compound has the potential to reach surface waters as a result of its chemical and physical properties; it has been estimated to potentially reach such waters in concentrations up to 36 μg/L and has been detected in surface waters at concentrations up to 14 μg/L [20]. For daphnids, the most commonly used aquatic invertebrate test species, such concentrations were not relevant for any observed effects, as concentrations causing effects were in the range of several milligrams per liter. Nevertheless, effects from short-term exposure were shown [21] and led to increased vulnerability for populations of daphnids to subsequent stress [22]. Gammarids are known to be more sensitive to imidacloprid than daphnids with respect to mortality [23]. Because feeding behavior is a particularly sensitive end point and prolonged starvation contributes to mortality caused by imidacloprid in gammarids [14], effects are more likely to occur at field-relevant concentrations of imidacloprid. The present study investigated whether imidacloprid affects feeding of *G. pulex* at environmentally relevant concentrations and whether a feeding assay at the individual level is sufficiently sensitive to allow testing at a daily resolution. Furthermore, we explored the

All Supplemental Data may be found in the online version of this article.
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Published online 4 December 2013 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.2480

benefits of extending the feeding assay to include a recovery phase.

MATERIALS AND METHODS

Gammarus pulex were collected in August from a small stream in Bishop Wilton, United Kingdom (grid reference SE7963, latitude 53.985, longitude −0.787) and fed prior to experimentation with horse chestnut leaves (*Aesculus hippocastanum* [L.]) that had been stored in tap water for at least 3 mo. These leaves were conditioned with *Cladosporium* sp. at room temperature. The organisms were maintained prior to and within experimentation under continuous ventilation at 13 ± 1 °C and with a 12:12-h light:dark photoperiod at 750 lux to 900 lux in artificial pond water [6]. Organisms were left to acclimatize to those conditions for 3 d before the start of the experiments.

Food source for the experiment

Leaf discs with a diameter of 1.6 cm were prepared for the experiment using horse chestnut leaves collected in November. Leaves were stored after drying in the dark and at room temperature (20 ± 2 °C) until further preparation. Leaf discs were conditioned with *Cladosporium* sp. for 2 wk following the description of Naylor et al. [6] and subsequently stored as leaves previously collected. All leaf discs were rewetted in artificial pond water for 2 d prior to use.

Experimental design

Gammarids with a dry body mass between 3.8 mg and 15.0 mg and without visible infection with acanthocephalan parasites were kept individually in 90 mL artificial pond water, and each individual was provided with 3 leaf discs at all times. All food was exchanged every 24 h, and the artificial pond water was replaced every 48 h. Oxygen content and pH in the old and new media were measured, and mortality and moulting status were recorded daily. The feeding rate of organisms that moulted during the experiment was discounted from analysis because the exact impact of moulting on the feeding rate is unreported; previous observations show that organisms might stop eating during the period before changing the carapax [18]. Body mass of the organisms was measured after the experiment by drying the organisms for 48 h at 65 °C and weighing to a precision of 0.01 mg.

The actual experiment was divided into 2 phases, which were a 4-d exposure phase (2 × 2 d) and a 3-d recovery phase. Prior to the experiment and after the acclimatization to the laboratory conditions (3 d), organisms were further acclimatized to test conditions for 2 d (i.e., food source and separation). Five test concentrations of imidacloprid (0.81 μg/L, 2.7 μg/L, 9.0 μg/L, 30 μg/L, and 100 μg/L) were selected to range between approximately 0.2% and 20% of the lethal concentration for 50% of the test organisms (LC50) determined after 96 h of exposure [24,25]. The largest concentration tested was similar to the lethal concentration for 10% of the test organisms (LC10) for *G. pulex* after 96 h (99.5 μg/L [25]). No formulations or solvents were used (analytical standard 99.0% purity, PESTANAL®; Sigma-Aldrich). Samples of the stock solution and the media were taken at the beginning and end of both parts of the exposure phase and frozen at −22 °C until preparation and chemical analysis using high-performance liquid chromatography (HPLC).

A total of 10 individuals were used for each test concentration and the control. Additionally, the experiments included a leaf disc control (3 replicates) on each day. These controls detect differences in the weight associated with the drying and weighing procedure and furthermore prevent an overestimation of the feeding rate associated with weight loss of the leaf discs caused by leaching and/or decomposition during the experiment. All row data are available in the Supplemental Data, Table S1. All measurements of weights refer to dry weight.

Measurement of feeding rate

The individual feeding rate, *FR* (mg [food] / (mg [gammarid] × d)), was calculated at a daily resolution (t = exactly 24 h) by dividing the amount of food eaten within the observed period, $FE[t]$ (mg/d, $FE[t] = F[t-1] / F[t]$), by the body mass of the individual, G (mg); $FR = FE[t] / G$). The measured food at the end of the period, $F[t]$, was corrected with the leaching decomposition factor, ld ($FE[t] = F[t-1] - F[t] / ld$). The leaching decomposition factor was obtained by dividing the weight of the control leaves at the end of the measuring period by the initial weight. The initial weight of the leaf discs was determined by weighing the leaf discs for each replicate prior to the 2-d rewetting phase in artificial pond water.

Chemical analysis

The 3 smallest test concentrations of imidacloprid (0.81 μg/L, 2.7 μg/L, and 9 μg/L) and 4 standards (range 0.35–17.65 μg/L) were preconcentrated on C_{18} cartridges (Strata; 8B-S001-EBJ; C18-E; 55 μm, 70A) prior to chemical analysis. The cartridges had a bed mass of 100 mg and a column volume of 3 mL. Cartridges were activated with 3 mL methanol, loaded with 10 mL sample at 1 mL/min, and then eluted with 3 mL methanol. The eluted sample was evaporated to dryness under nitrogen and then redissolved in 0.25 mL methanol and water (50:50 v/v).

Analysis of imidacloprid was by injection of a 75-μL sample onto HPLC (Agilent 1100 Series; Agilent Technologies) equipped with an ultraviolet detector (254 nm) and a Discovery® C_{18} column (15 cm × 4.6 mm, 5 μm; Supelco) maintained at 25 °C. The mobile phase was methanol and water (45:55, v/v) with a flow rate of 0.5 mL/min.

The limit of detection for imidacloprid (retention time 5.6 min) was ≤14 μg/L (equivalent to ≤0.35 μg/L in the original samples subjected to preconcentration), and the recovery through the preconcentration step was 109 ± 9%.

Statistics

A one-way analysis of variance (ANOVA) was performed with the control data over time. The Shapiro-Wilk test for normal distribution and the Levene-Mediane test for equal variance were performed prior to ANOVA. The feeding rate of each replicate was used as input for this test. Similarly, but with a two-way ANOVA, the relative feeding rate of treatments was tested against the control. A modified probit analysis was performed to generate the median effect concentration (EC50) values. Statistical analysis of feeding rates was undertaken with SigmaPlot 11. The EC50 values were determined using ToxRat Professional 2.10 (ToxRat Solutions).

RESULTS AND DISCUSSION

Environmental conditions

The pH ranged between 7.4 and 7.9, the oxygen content was always higher than 75% saturation, and the temperature ranged between 12.2 °C and 14.0 °C. The measured pH lies within the optimum (7.2–7.8) for the organism given by Schellenberg [26]. Oxygen content and temperature of the test medium fulfilled the conditions preferred by *G. pulex* [27].

The maximal difference between measured and nominal concentrations of imidacloprid was 11%, whereas the difference for samples that were not preconcentrated was <5%. A decrease in imidacloprid concentrations by $5.7 \pm 0.4\%$ during exposure was detected. For further analysis, imidacloprid was assumed to be constantly present during the exposure phase at the nominal concentrations tested.

Feeding rate over time

A two-way ANOVA on relative feeding rates during the whole experiment revealed an overall significant effect of treatment ($p = 0.025$). In addition, feeding rate in the control and the 3 smallest concentrations of imidacloprid differed from the highest concentration (100 μg/L) throughout ($p < 0.032$). The relative feeding rates during and after exposure to imidacloprid as a function of time are presented in Figure 1. There was a clear trend of concentration-dependent influences on the feeding rate in both exposure and recovery phases. Feeding inhibition in the exposure phase increased with increasing concentration (Figure 2) and, at least for the 2 highest concentrations tested, with exposure time (Figure 1). In both cases the impact on feeding was significant within 1 or more feeding periods at concentrations ≥30 μg/L. Those concentrations were similar to or higher than those reported to cause drift [24,28] and immobility of *G. pulex* [25]. It could be argued that reduced feeding was caused by the inability of organisms to reach food as a result of loss of movement. However, the food that was provided in the present study covered the whole base of the test vessel, meaning that active movement to reach food was unnecessary. Furthermore, it was observed that organisms were always holding a leaf disc at the time when food was exchanged. Hence, the effects observed were the result of reduced feeding and not driven by loss of ability to reach the food. Lost ability to coordinate the feeding apparatus could be the driving factor for reduced feeding.

Another pattern was observed in the recovery phase. It seems that organisms not showing any (0.81 μg/L) or showing nonsignificant (2.7 μg/L and 9.0 μg/L) effects on feeding during exposure were nevertheless affected by imidacloprid (Figures 1 and 2, recovery phase). When the compound was removed from the test vessel, those organisms ate significantly more food than in the exposure phase and more than the control organisms (Figure 2). However, this recovery was restricted to those concentrations that did not alter feeding significantly during exposure (Figure 2). Furthermore, recovery was not directly related to exposure concentration in terms of the feeding rate itself but rather in terms of the time when the feeding rate reached (recovery) or exceeded (compensational feeding) that of the control (Figure 1). At the smallest concentration tested (0.81 μg/L), organisms recovered to the control level even before exposure ended (day 4) and feeding exceeded that of the control in the following days (Figure 1), leading to a significantly increased overall feeding in the recovery phase compared to the exposure phase (Figure 2). Organisms at the next highest concentration (2.7 μg/L) recovered 1 d later (within 1 d after exposure) and exceeded the feeding rate of the control in the following days more intensively compared to organisms exposed to 0.81 μg/L (Figure 1). The highest test concentration did not allow the organisms to recover (e.g., reaching or exceeding the feeding rate of the control on the same day) within 3 d after exposure.

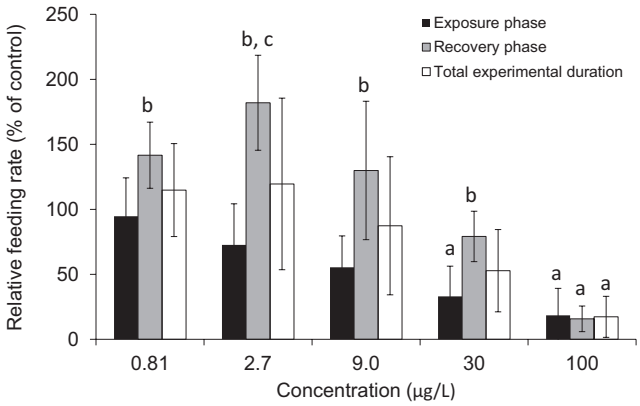

Figure 2. Relative individual feeding rate of *Gammarus pulex* as a function of nominal imidacloprid concentrations. Average ± standard deviation ($n = 10$) calculated for different phases of the experiment. a = significantly reduced compared with control; b = significantly increased compared with the exposure phase; c = significantly increased compared with the control (all at $p < 0.05$).

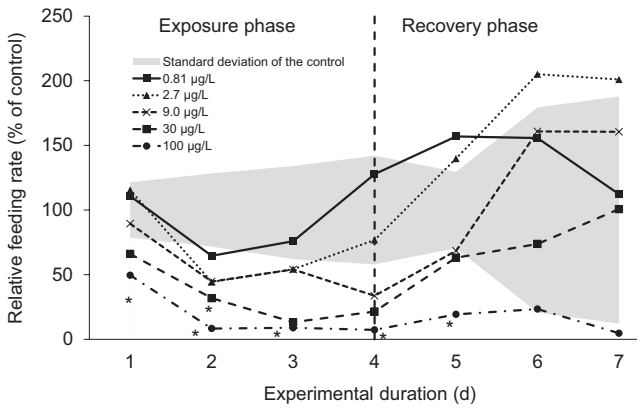

Figure 1. Relative individual feeding rate of *Gammarus pulex* as a function of time within and after exposure to 5 nominal imidacloprid concentrations. Values are the average of treatments ($n = 10$) and the standard deviation of the control ($n = 10$) where the control is set at 100%. *Significant difference from control ($p < 0.05$).

We cannot distinguish whether the compensational feeding after exposure is a direct consequence of impacts on feeding or acts as compensation for other influences requiring energy, such as detoxification or increased energy demand of any other kind. Nevertheless, we show that low concentrations which do not cause significant effects on feeding (present study), drift [24,28], immobility [25], or survival [23–25] during exposure can alter feeding subsequent to exposure; this may indicate potential vulnerability to additional and subsequent stress. Roessink et al. [25] reported a 10% effect concentration (EC10) for immobilization of *G. pulex* after exposure to imidacloprid for 96 h of 3.6 μg/L. This concentration is 4 times larger than the smallest concentration tested in the present study for which increased feeding after exposure was observed.

Concluding the test with a recovery phase not only revealed influences at very low concentrations but also led to better insights about the effects. *Gammarus pulex* did not recover within 3 d from a 4-d exposure to imidacloprid at concentrations ≥30 μg/L (Figure 2). This result conforms with observations that imidacloprid blocks the postsynaptic nicotinic acetylcholine receptors virtually irreversibly (e.g., in insects [29]). Our results also show that recovery at lower concentrations is possible, suggesting that some degradation of the compound or regeneration of nicotinoid receptors is possible. In fact, it was

shown that imidacloprid is eliminated by 95% after 11.2 d in clean water [30] and that this elimination does not involve formation of metabolites at detectable concentrations [31].

Effect concentrations

Effect concentrations causing 10% and 50% reduction in feeding rate after 1, 2, 3, and 4 d of exposure are presented in Table 1. Effects on feeding rate were observed at concentrations 2 orders of magnitude lower than those causing mortality [23–25] (when comparing the average 96-h LC50 of 270 μg/L [23] and the 96-h EC50 of 5.3 μg/L [Table 1]), emphasizing previous findings that feeding rate is a sensitive end point for *G. pulex* [4–6]. A reduction in feeding rate by 50% was observed at a concentration that is within the range of measured and estimated environmental concentrations (Table 2). Hence, our results from the exposure phase indicate that effects on the feeding rate of *G. pulex* caused by imidacloprid might already occur in the environment. Impacts of imidacloprid at concentrations of approximately 10 μg/L to 30 μg/L have already been reported. Nyman et al. [14] observed that gammarids were able to recover feeding between pulses of exposure to 15 μg/L and to survive repeated pulsed exposures. The present study demonstrates such recovery of feeding. *Gammarus pulex* exposed to 30 μg/L were observed to show extra, pesticide-induced, drifting activity on top of their natural drifting activity [24]; and it was recently demonstrated that organisms drifted downstream after exposure to 12 μg/L [28]. We show that strong impacts on feeding (EC50) occur at slightly lower concentrations. A critical issue would be to determine whether patterns of exposure in the field match those in experiments as it is frequently found that exposure concentrations vary markedly over fairly short periods of time.

Limitations of the test design

Measurement for individual organisms showed that the feeding rate is not stable over 1 wk. There was a significant difference in the control feeding rate between the first day and the last 3 d ($p < 0.008$, data not shown). The feeding rate decreased from 0.17 ± 0.04 mg [food] / (mg [gammarid] × d) to 0.05 ± 0.04 mg [food] / (mg [gammarid] × d), accompanied by an increase in variability (average divided by standard deviation) as the standard deviation remained stable. Thus, improvement of the test design is needed for longer experiments. Such experiments would be desirable for testing multiple pulses and to determine effects on growth and reproduction, end points important for extrapolating effects to the population level. Furthermore, there was a high variability in the feeding rate measured at a daily resolution, which is most likely driven by a range of natural factors including age or size, food source, and water quality [32]. Hence, measurement at a daily resolution for individuals requires a large number of replicates to determine impacts at low effect intensity. A statistical power analysis for the data generated on day 1 of the experiment revealed that 20 replicates per treatment would be needed to detect a reduction in feeding rates by 20% at a significance level of 95%. However, such a study would not be guaranteed to succeed because data obtained from moulting individuals should be removed from the analysis and because feeding rate decreases over time.

Mortality

No organisms died in the control or the 3 smallest test concentrations. After 4 d of exposure to imidacloprid, 1 organism died in the largest test concentration of 100 μg/L. On the last day of the experiment, 2 organisms died in the second largest concentration of 30 μg/L. Lethal concentrations for half of the test organisms (LC50) of imidacloprid to *G. pulex* were investigated in 3 studies where similar LC50 values after 96 h of exposure were found. Beketov and Liess [24] reported an LC50 of 270 μg/L (95% confidence interval 170–450 μg/L). Ashauer et al. [23] and later Roessink et al. [25] found an almost identical LC50 3 yr later and 5 yr later, respectively. Having 1 individual out of 10 die after 4 d of exposure to 100 μg/L matches the LC10 of 99.5 μg/L (32.2–307 μg/L) reported by Roessink et al. [25]. Nevertheless, it seems that another mechanism also occurred, causing the mortality observed at the end of the experiment when individuals were exposed to 30 μg/L. The literature gives evidence that might explain the delayed mortality, which certainly complicates the extrapolation of effects measured in acute toxicity tests to the field. Hervant et al. [33] reported death of *Gammarus fossarum* after 7 d of starvation; Agatz et al. [21] demonstrated that feeding inhibition resulting from imidacloprid exposure was the only cause of effects on growth, maturation, reproduction, and survival of another crustacean, *Daphnia magna*. A further study showed that imidacloprid has the potential to indirectly cause lethality as a result of interference with feeding [14]. Their multiple stress model explained the mortality by a combination of direct chemical stress and starvation. Apparently, mortality can occur from even lower concentrations when feeding behavior is affected for longer durations [14]. Tennekes and Sánchez-Bayo [34] reviewed the temporal aspects of imidacloprid exposure assessment. The incorporation of time as a dose factor is especially important for neonicotinoids and other xenobiotic groups that can bind irreversibly to receptors because repeated exposure at low doses has clear potential to cause adverse effects. Additionally, additive effects of different compounds with the same mode of action are possible.

GENERAL DISCUSSION

We showed a temporary increased feeding rate following exposure to low test concentrations, which may have an impact on overall leaf litter breakdown in the field and, thus, could lead to changes at the ecosystem level. Various studies show that food uptake of detritivores is directly related to the critical ecosystem-level process of leaf litter breakdown [1–5,7]. Furthermore, changed feeding could lead to effects at the population level. Naylor et al. [6] showed that influences on the scope for growth (i.e., food uptake as the most sensitive part of the scope-for-

Table 1. Concentrations giving 10% and 50% inhibition (EC10 and EC50, respectively; plus 95% confidence intervals) of individual feeding rate mg [food]/(mg [gammarid] × d) measured in dry weight for different time points from the start of exposure of Gammarus pulex to imidacloprid

Exposure time (h)	EC10 (μg/L)	95% Confidence interval	EC50 (μg/L)	95% Confidence interval
24	9.05	5.15–12.10	18.96	14.93–23.05
48	3.28	0.005–8.81	20.59	6.48–72.01
72	2.03	NA	10.50	NA
96	2.05	NA	5.34	NA

Table 2. Measured and estimated imidacloprid concentrations in surface and groundwater

Location	Type of determination	Concentration (μg/L)	Reference
Sea Wilapa Bay, USA	m	1.6	[20][a]
Surface water, Florida, USA	m	1.0	[20][a]
Lake Wales Ridge, USA	m	14	[20][a]
Groundwater, New York, USA	m	6.7	[20][a]
Surface water, USA	m	3.29	[48]
Surface water (river), USA	m	0.56	[48]
Surface water, USA	e (acute exposure)	36.0	[20][a]
Surface water, USA	e (chronic exposure)	17.2	[20][a]
Groundwater, USA	e (acute exposure)	2.09	[20][a]
Groundwater, USA	e (chronic exposure)	2.09	[20][a]
EU[b]	e (acute exposure)	<6.1[c]	[49]

[a]Original reference given in this reference.
[b]End point identified by the European Union (EU) as relevant for member states when applying the Uniform Principles.
[c]Depending on the treated monoculture.
m = measured; e = estimated.

growth concept) can be related to reproduction of *G. pulex* [35]. Alterations in population abundance and population structure caused by short-term feeding inhibition as a result of imidacloprid exposure and its consequences for population vulnerability to subsequent stress have recently been shown for *D. magna* [22]. It is unlikely that a single short-term inhibition of feeding leads to similar influences at the population level for gammarids because the life cycles of daphnids and gammarids are very different in terms of timescale. Nevertheless, instability in population development cannot be ruled out. Multiple pulses of the same compound or of compounds acting in the same manner likely result from the long life span of *G. pulex*. The combination of multiple exposure and slow elimination of imidacloprid from *G. pulex* [30] increases the likelihood of additive adverse effects. Compounds that have been shown to affect feeding of aquatic invertebrates include, but are not restricted to, the following pesticides or pesticide metabolites: fenvalerate [36,37], endosulfan [38], diazinon [38], pentachlorophenol [39], clorpyrifos [39], naphthol [39], tebuconazole [40], molinate [41], carbendazim [42], and propanil [43]. Hence, there is a potential risk to aquatic nontarget organisms from the possibility of additive effects on feeding following temporal and spatial co-occurrence of substances influencing feeding in surface waters of agricultural areas.

Whether short-term feeding depression of gammarids, as shown in the present study, is a matter of concern at the population and ecosystem levels could be investigated in different ways. A possible technique would be the extrapolation of temporal and spatially explicit measures of reduced feeding to the ecosystem process leaf litter breakdown and the assessment of impacts on the nutrient cycle via changes in shredding activity. Including the extrapolation of individual feeding to shredding activity at the population scale might be a potential or even vital addition for understanding ecosystem-level effects of pesticide exposure. Generally, exposure models [44] and ecological models [45,46] have been developed, and their combination could address those questions. The incorporation of compensational feeding would be vital for assessing effects of environmentally realistic exposure, but the actual mechanism of compensational feeding has yet to be determined. To understand the physiological basis of compensational feeding, we need to understand how impaired feeding affects an organism's energy requirements and energy budgets. Individual-based, time-resolved feeding rate measurements following different starvation intervals are needed to achieve such understanding. However, the measurement of feeding of individuals needs to be improved in order to conduct the necessary experiments.

When considering acute toxicity, *G. pulex* are 2 orders of magnitude more sensitive to neonicotinoids than the commonly used standard test species, *D. magna* [23]. However, insects are even more sensitive than *G. pulex* by about a factor of 35 [47], which suggests that feeding rates of insects may also be affected by imidacloprid at much lower concentrations than those used in the present study.

CONCLUSION

A toxicity study determining sublethal effects with an observation period beyond exposure is time-intensive. However, the extension yielded information on recovery potential and indicated that a low concentration of a stressor can have subsequent effects even though no effects were observable during exposure. Imidacloprid reduced feeding at low concentrations (30 μg/L) that are at the upper end of those likely to occur in the environment. Feeding rates were increased after exposure at even lower concentrations (0.81 μg/L) to compensate for earlier impacts, but recovery did not occur at the higher concentrations because of slow elimination of imidacloprid. To what extent the effects on feeding have the potential to evoke effects at the population level or disturb leaf litter breakdown in the environment needs further investigation. There is a need to improve methods for laboratory experiments with gammarids, to enhance culturing of these organisms, and to develop models that can help to quantify the propagation of effects along the various levels of organization from the individual (e.g., feeding, growth, reproduction) to the ecosystem (e.g., leaf litter decomposition, food web).

SUPPLEMENTAL DATA

Table S1. (74 KB XLS).

Acknowledgment—This research was financially supported by the European Union under the 7th Framework Programme (project CREAM, contract PITN-GA-2009-238148).

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SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 26, No. 8, pp. 1726–1732, 2007

Printed in the USA
0730-7268/07 $12.00 + .00

EFFECTS OF INSECTICIDE EXPOSURE ON FEEDING INHIBITION IN MAYFLIES AND OLIGOCHAETES

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(*Received* 8 *January* 2007; *Accepted* 9 *March* 2007)

Abstract—The present study examined the effects of pulse exposures of the insecticide imidacloprid on the mayfly, *Epeorus longimanus* Eaton (Family Heptageniidae), and on an aquatic oligochaete, *Lumbriculus variegatus* Müller (Family Lumbriculidae). Pulse exposures of imidacloprid are particularly relevant for examination, because this insecticide is relatively soluble (510 mg/L) and is most likely to be at effect concentrations during runoff events. Experiments examined the recovery of organisms after a 24-h pulse exposure to imidacloprid over an environmentally realistic range of concentrations (0, 0.1, 0.5, 1, 5, and 10 μg/L). Effects on feeding were measured by quantifying the algal biomass consumed by mayflies or foodstuffs egested by oligochaetes. Imidacloprid was highly toxic, with low 24-h median lethal concentrations (LC50s) in early mayfly instars (24-h LC50, 2.1 ± 0.8 μg/L) and larger, later mayfly instars (24-h LC50, 2.1 ± 0.5 μg/L; 96-h LC50, 0.65 ± 0.15 μg/L). Short (24-h) pulses of imidacloprid in excess of 1 μg/L caused feeding inhibition, whereas recovery (4 d) varied, depending on the number of days after contaminant exposure. In contrast to mayflies, oligochaetes were relatively insensitive to imidacloprid during the short (24-h) pulse; however, immobility of oligochaetes was observed during a 4-d, continuous-exposure experiment, with 96-h median effective concentrations of 6.2 ± 1.4 μg/L. Overall, imidacloprid reduced the survivorship, feeding, and egestion of mayflies and oligochaetes at concentrations greater than 0.5 but less than 10 μg/L. Inhibited feeding and egestion indicate physiological and behavioral responses to this insecticide.

Keywords—Sublethal effects Imidacloprid Insecticide Feeding rate

INTRODUCTION

The inhibition of invertebrate feeding in response to contaminant exposure can indicate the potential for a stressor to produce sublethal population-level responses [1]. Feeding inhibition is a particularly relevant endpoint for measuring the effects of modern chemicals, such as insecticides, that are applied in low doses and that produce exposure regimes of short duration and low magnitude. One example of this new generation of chemicals is imidacloprid, a soluble (510 mg/L) insecticide commonly applied in North America [2]. Imidacloprid is a chemically stable mimic of nicotine that has well-documented toxicity to a variety of pest species, including fleas, thrips, and the Colorado potato beetle [3]. Because imidacloprid is relatively water soluble (510 mg/L), mobile in soil [4,5], and persistent in organic sediments (>400 d [6]), this compound has the potential to enter streams in concentrated pulses after rain events. In New Brunswick (Canada) and Prince Edward Island (Canada), agricultural runoff of imidacloprid has been measured over a range (mean ± standard error) of 0.25 ± 0.07 to 15.88 ± 0.99 μg/L [7]. Also, because imidacloprid attacks the nervous system by binding to the nicotinic acetylcholine receptor (nAcChR) [8], a receptor common in most invertebrate taxa, it is hypothesized to cause effects in nontarget aquatic invertebrates.

Imidacloprid has been found in streams and rivers and is likely to be bioavailable to aquatic organisms. Because few studies have examined the toxicity of imidacloprid to relevant lotic species, the present study investigated the impact of environmentally relevant concentrations of imidacloprid on the feeding and egestion of two common aquatic species, the larval mayfly, *Epeorus longimanus* Eaton (Family Heptageniidae), and an aquatic oligochaete, *Lumbriculus variegatus* Müller (Family Lumbriculidae). Effects were measured using a combination of traditional toxicological (median effective concentration [EC50] and median lethal concentration [LC50]) and sublethal (feeding and egestion) endpoints to determine the impact of low-magnitude (μg/L) and short-duration (24-h) pulses of imidacloprid. We hypothesized that because imidacloprid induces tremors and lethargy in insects, the reduced activity associated with imidacloprid exposure also would reduce feeding and egestion. Furthermore, imidacloprid is not permanently bound to the nAcChR [9]; therefore, we also examined the latency of effects by measuring feeding rate over a 4-d recovery period.

MATERIALS AND METHODS

Organism collection and culturing

Mayfly larvae (*E. longimanus*) were collected in the Nashwaak River, a tributary of the larger Saint John River near Stanley (NB, Canada; 46°17.18′N, 66°44.14′W) and immediately transported to the laboratory. *Epeorus longimanus* are grazing mayflies that reside on the cobble substrate found in fast-flowing rivers; they were chosen for this study because mayflies are abundant in streams [10] and sensitive to pesticides [11]. Larvae were equilibrated to 20 ± 1°C in Percival®

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(Percival Scientific, Boone, IA, USA) growth chambers over a 24-h period with a 50% water exchange to dechlorinated (model 20-36 dechlorinator; Culligan, NB, Canada) groundwater (pH, 8.1 ± 0.1; conductivity, 261 ± 5 μS). The groundwater supplied to our animal care facility at the University of New Brunswick (Fredericton, NB, Canada) is provincially monitored, and monthly reports indicated insignificant changes in water quality for the duration of our experiments. Feeding rate and lethality experiments were conducted using early instar mayflies collected throughout the spring of 2004 (body length, 3.01 ± 0.26 mm; $n_{measured} = 120$, $n_{total} = 600$), and later-instar larvae were collected in July of 2005 (body length, 7.5 ± 0.34 mm; $n_{measured} = 72$, $n_{total} = 200$). Only the later-instar mayflies were used to examine feeding recovery over time.

Oligochaetes (*L. variegatus*, strain 285120; Aquatic Ecosystems, Apopka, FL, USA) were cultured in the laboratory using the methods described by Williams [12]. Oligochaetes were maintained in 10-L aquaria with dechlorinated (model 20-36 dechlorinator; Culligan) groundwater flowing through the aquaria at a rate of 200 ml/min. Oligochaetes were fed a 1:1 mixture of ground Tetramin® (Tetra, Blacksburg, VA, USA) and *Spirulina* spp. sinking-pellets (SP1; Aquatic Ecosystems), with 5 to 25 g provided to the oligochaete aquaria on alternate days. A 1-cm layer of shredded paper towel served as substrate. Oligochaetes chosen for lethality and egestion experiments had similar mass and length to minimize any potential confounding effects of body size [12]. Oligochaetes selected for experiments were approximately 2.5 cm in length and had a dry mass of 1.17 ± 0.02 mg ($n_{measured} = 42$, $n_{total} = 150$).

Lethality and immobility tests

In tandem with feeding tests (described below), 24- and 96-h lethality and immobility tests were conducted over a range of imidacloprid concentrations (0, 0.1, 0.5, 1, 5, 10, 100, and 240 μg/L) by employing standard acute-toxicity test techniques [13]. Mayflies and oligochaetes were exposed to aqueous solutions of imidacloprid in glass beakers (diameter, 12 cm; volume, 300 ml). Either five mayflies or 25 oligochaetes were exposed in each treatment, and each test vessel was replicated three times. Early mayfly tests were repeated several times to confirm the low LC50 result. Three 24-h LC50 tests were conducted with early instar mayflies, whereas LC50 was examined once with respect to both the 24- and 96-h exposures in later-instar mayflies. For oligochaetes, immobility was used as the endpoint to estimate the 96-h EC50 (i.e., only conducted once). Immobility was evaluated as the percentage of oligochaetes moving after gentle agitation with a transfer pipette. No immobility was detected in oligochaetes during the 24-h pulse exposure.

Mayfly feeding tests

Mayfly foodstuffs were cultured in the laboratory on ceramic tiles. *Nitzschia* spp. diatoms (strain F110; University of Toronto, Toronto, ON, Canada) were maintained in the exponential growth phase in S-diatom media [14,15] before being subcultured to ceramic tiles (2.5 × 2.5 cm) to form single-species diatom mats within 7 to 10 d. All cultures were raised at 20 ± 1°C under a 16:8-h light:dark photoperiod. Diatom tiles were provided ad libitum to the mayflies at a rate of four tiles/replicate stream/d.

All sublethal experiments were conducted using a 24-h pulse scenario. Experiments with early instar mayflies included five replicates of each imidacloprid concentration (0, 0.1, 0.5, 1, 5, and 10 μg/L) prepared in dechlorinated groundwater, whereas late-instar treatments were replicated 10 times to ensure that at least five replicate treatments were available for feeding rate studies. The concentrations that we chose to examine in the sublethal mayfly studies overlapped the LC50s, which were determined in tandem with our feeding studies. In the mayfly feeding experiments, each acid-washed glass beaker (diameter, 12 cm) contained four tiles covered with *Nitzschia* spp. and five larval mayflies collected from the field as described above. A stir bar was used to generate water velocity in the beakers to increase water flow over the external gills, therefore eliminating the need for aeration. Flow rates were maintained at 20 ml/min to prevent damage to mayflies drifting between tiles. Following 24-h exposure to the contaminant, mayflies were transferred to 300-ml, flow-through, artificial streams [16] that were void of imidacloprid and had four diatom-covered tiles each. Artificial streams were placed in growth chambers (Percival Scientific) and supplied with dechlorinated groundwater that recirculated through the streams in a closed system for 4 d postexposure.

Daily diatom consumption by mayflies in the postexposure streams was measured by scraping the remaining diatomaceous material from selected tiles, then washing this material onto a 47-mm, glass-fiber filter (GF-C; Whatman C grade filters; pore size, 1.2 μm; Fisher Scientific, Fair Lawn, NJ, USA). Each stream received fresh tiles daily. Preweighed filter papers were dried for 4 h at 350°C, then reweighed and the mass of diatoms remaining on the tiles after mayfly feeding determined by the difference between the initial and measured final filter mass. Feeding rate was quantified as the per-capita mass of diatoms removed from the tiles per mayfly per day (μg/mayfly/d). Because the unit of measure is 1 d, the rate is expressed as μg/mayfly throughout. Six tiles from each *Nitzschia* culture batch were retained, and batches were found not to be significantly different ($p > 0.05$). Nonconsumptive losses were measured by chlorophyll *a* analysis of the aqueous treatment solutions by fluorometry (lower limit, 0.05 μg/L; model 10 series; Turner Design, Sunnyvale, CA, USA). For each treatment replicate, a 10-ml aliquot of treatment water was filtered (GF-C grade, as above), and chlorophyll *a* was extracted from the filter with 90% ethanol for 5 min in an 80°C water bath. The resulting readings (μg/L) were converted (with respect to dilution) and the resultant biomass measures subtracted from the feeding rate totals to account for nonconsumptive losses, such as careless feeding and/or sloughing of diatomaceous material because of mayfly activity within the beakers and artificial streams. In the absence of mayflies, ambient diatom sloughing was less than 0.2 ± 0.0009 μg per 300-ml test vessel.

Oligochaete egestion tests

All oligochaete egestion experiments were conducted using 24-h pulses of imidacloprid (0, 0.1, 0.5, 1, 5, and 10 μg/L) prepared in dechlorinated groundwater. Exposures were performed in glass beakers (diameter, 5 cm; volume, 80 ml), with five replicates per treatment and with each replicate containing five oligochaetes. Each beaker contained 4 g of lake sediment (organic matter, 16% ± 0.1%) capped with a fine layer of inorganic sand (Ottawa Sand Standard; 20-30 mesh; Fisher Scientific). The sand was necessary to separate the sediment and aqueous layer for subsequent fecal pipetting. Each treatment beaker also contained 60 ml of dechlorinated groundwater.

Before exposure, the oligochaetes were held in dechlorinated groundwater overnight to purge their guts of any pre-

viously ingested material prior to the initiation of the test. During the holding period, the sediment slurries were prepared by mixing dry (preweighed) Little Magaguadavic Lake (45°47.62′N, 67°13.48′W; near Fredericton, NB, Canada) sediment with dechlorinated groundwater that contained prepared aliquots of imidacloprid. Likewise, during the exposure period, sediment slurries devoid of imidacloprid were made ready for the subsequent transfer of oligochaetes from the treatment vessels (volume, 80 ml; as above). These postexposure egestion rates were used to estimate the reversibility of sublethal feeding effects. Lake Magaguadavic sediment was chosen because it had been used in previous *L. variegatus* studies and is known to be free of pesticides and other contaminants [12].

The *L. variegatus* egestion rate was measured by collecting surface-deposited fecal pellets generated by the consumption of sediment particles. Fecal pellets were deposited by oligochaetes on the sand layer separating the aqueous and sediment layers. Fecal pellets were then easily removed from the sediment surface with a glass transfer pipette at 1-d intervals. Four beakers containing sediment but devoid of worms were used to correct for pipetting error, whereby the overlying sand was transfer pipetted as though fecal pellets were being collected and the average value was subtracted from the daily measurements. Fecal material was filtered onto preweighed, 47-mm, glass-fiber filters (GF-C grade; Fisher Scientific), then dried at 110°C and reweighed to determine the dry mass of material egested per day (mg/oligochaete/d). Because the unit of measure is 1 d, the rate is expressed as μg/oligochaete throughout.

Chemical analyses

The present study examined imidacloprid at concentrations much less than the median detection limit of most commercial laboratories (~2 μg/L). The various imidacloprid concentrations were created by diluting 1-ml aliquots of 240 g/L of Admire® (Bayer CropScience, Toronto, ON, Canada), then performing a standard serial dilution to achieve the desired treatment concentrations in dechlorinated groundwater. Chemical analyses of the imidacloprid samples were conducted at the National Water Research Institute (Environment Canada, Saskatoon, SK) on a Micromass Quattro Ultima liquid chromatography–mass spectrometer equipped with a stainless-steel column (MS Xterra C-8; 100 × 2.2 mm; Waters, Milford, MA, USA). Given imidacloprid's relatively high solubility (with respect to other pesticides; ~510 mg/L), precautions were taken to account for matrix effects of the dechlorinated groundwater used in the creation of the treatment solutions by supplying the Saskatoon laboratory with monthly water-quality monitoring data for the New Brunswick water source. Samples for imidacloprid analyses were taken from three replicate exposure beakers (both mayflies and oligochaetes) per treatment concentration per experiment. These samples were collected in 40-ml, amber-glass vials (U.S. Environmental Protection Agency vials; Fisher Scientific) and stored at 4°C until shipment to the laboratory (within 10 d). The imidacloprid samples were injected directly into the liquid chromatography–mass spectrometry system. The mobile phase contained 40% aqueous acetonitrile and 0.2% formic acid (v/v). The flow rate was 200 μl/min, and injection volumes were 10 to 20 μl. Calibration of the instrument was performed on the stock solutions used for creating the test solutions. Results from the laboratory yielded a correlation between nominal and actual values of $r^2 = 0.999$ for mayflies and oligochaetes, respectively. For mayflies, the correlation of actual to nominal was $y_{(actual)} = 1.0019x_{(nominal)} + 0.3098$; for oligochaetes, the correlation of actual to nominal was $y = 0.9979x - 0.2682$. Actual values for both the mayfly and oligochaete experiments can be found in Table 1. Because of this high laboratory performance, concentrations are presented in nominal rather than actual concentrations throughout.

Table 1. Comparison of the actual values (mean ± standard error [SE]) of imidacloprid as determined by liquid chromatography-mass spectrometry with respect to nominal

Mayfly			Oligochaete		
Nominal (μg/L)	Actual (μg/L)	SE	Nominal (μg/L)	Actual (μg/L)	SE
0.10	0.09	±0.01	0.10	ND[a]	—
0.50	0.60	±0.05	0.50	0.19	±0.01
1.00	1.12	±0.04	1.00	0.69	±0.05
5.00	4.75	±0.05	5.00	4.68	±0.01
10.00	9.79	±0.34	10.00	9.67	±0.34
100.00	107.23	±5.69	100.00	99.50	±5.47
240.00	238.57	±1.93	240.00	239.24	±2.48

[a] No imidacloprid was detected in the 0.1 μg/L oligochaete treatment.

Statistical analyses

Standard toxicity tests were assessed using traditional LC50 and EC50 probit analysis (STATA Ver 8.02; SAS, Cary, NC, USA). Analysis of variance (ANOVA) was performed on feeding and egestion data using both the SAS (Ver 8.02) and Statistica (Ver 6; Statsoft, Tulsa, OK, USA) software packages. During the 24-h pulse (day-1) exposure, one-way ANOVAs were performed on the feeding and egestion rate data. The effect of imidacloprid treatment with respect to feeding and egestion rate over the 4-d recovery period was assessed repeatedly (days 2, 3, 4, and 5) from each replicate vessel using repeated-measures ANOVA. Repeated measures was chosen as an appropriate analysis because this model does not assume that the measurements were independent and could compare the rate changes to imidacloprid exposure, day, and number of surviving nymphs (or oligochaetes) per replicate treatment. Because imidacloprid induced mayfly mortality at the environmentally relevant concentrations that were previously thought to be sublethal, the feeding rate tests incorporated nymph number as a covariate in the statistical analyses. Assumptions of ANOVA, including normality (Shapiro-Wilk *W* test) and homogeneity of variance (Cochran's *C* test), were tested, and when these assumptions were not met, data were transformed and the residuals checked to ensure that test assumptions were met. Analyses were performed on both raw and transformed data. When significant effects were detected, the Bonferroni sequential post-hoc test was used [17,18]. The Bonferroni test incorporates the Bonferroni adjustment, which divides α by the number of tests conducted during post-hoc testing. All post-hoc testing was one-tailed and only evaluated whether the response variables are lower than the control because of treatment.

RESULTS

Mayfly test results indicated that imidacloprid is toxic to these larvae in the low-μg/L range. Early and late-instar mayflies had 24-h LC50s of 2.1 ± 0.8 and 2.1 ± 0.5 μg/L, respectively. The 96-h LC50 of late instars was 0.65 ± 0.15 μg/L. Oligochaetes were an order of magnitude less sensitive

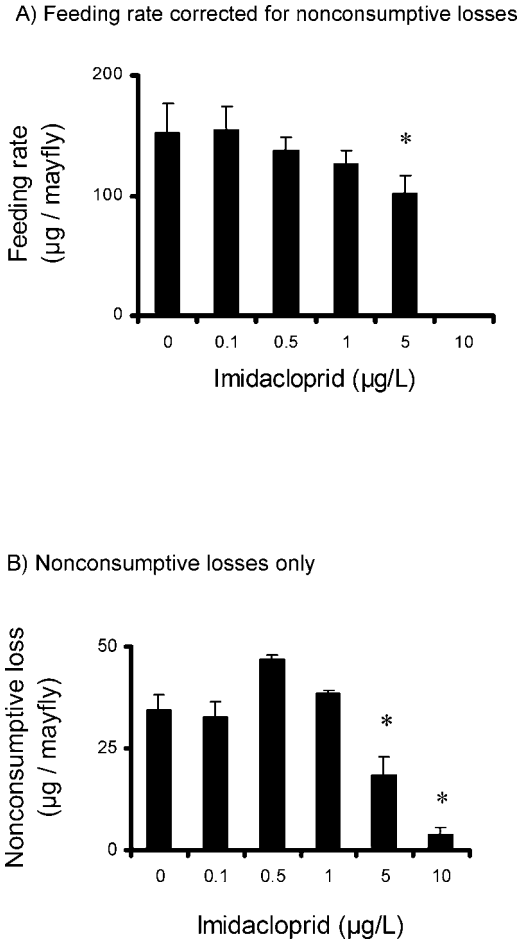

Fig. 1. (**A**) Feeding rate (µg/mayfly, mean ± standard error) of early instar mayflies during the 24-h pulse (day 1 only) at 0, 0.1, 0.5, 1, 5, and 10 µg/L concentrations of imidacloprid. No feeding was measured in the 10 µg/L treatment. (**B**) Nonconsumptive losses (µg chlorophyll *a*/mayfly, mean ± standard error) for early instar mayflies during the 24-h pulse (day 1 only) at µg/L concentrations of imidacloprid (as above). In both graphs, an asterisk denotes a significant reduction (Bonferroni, $p < 0.01$) in comparison to the control.

to imidacloprid, with a 96-h EC50 (immobility) of 6.2 ± 1.4 µg/L.

Mayfly feeding tests

Exposure to imidacloprid concentrations of 5 µg/L or greater caused significant reductions in early instar mayfly feeding during the 24-h pulse exposure ($F_{5,23} = 4.70$, $p < 0.013$) (Fig. 1A). Concentrations of imidacloprid as low as 0.5 µg/L also showed a trend toward reduction; however, these differences (0.5 and 1 µg/L) were not significant. No feeding was observed at concentrations of 10 µg/L because of mortality of all mayflies in the five replicate streams. Latent recovery to normal feeding levels could not be examined in these experiments because mortality (24-h LC50, 2.1 ± 0.8) and insufficient replication precluded comparing imidacloprid treatments over time. Treatments of 5 µg/L or greater had a significant decrease in nonconsumptive losses compared to the control level ($F_{5,23} = 3.835$, $p < 0.05$), suggesting a significant reduction in insect activity and feeding. In contrast, larvae in the 0.5 and 1 µg/L treatments exhibited higher nonconsumptive chlorophyll readings, which although nonsignificant may indicate less efficient scraping and feeding at these sublethal exposures (Fig. 1B). In replicate treatments that contained 10 µg/L of imidacloprid, only nonconsumptive losses were observed, likely because all the mayflies had died.

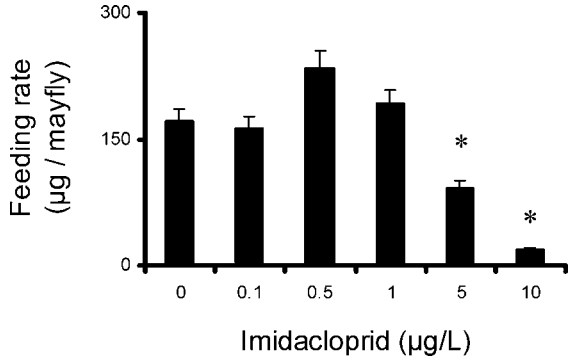

Fig. 2. Comparison of the feeding rate (µg/mayfly, mean ± standard error) of late-instar mayflies during the 24-h pulse (day 1 only) at 0, 0.1, 0.5, 1, 5, and 10 µg/L of imidacloprid. The feeding rate has been adjusted with respect to nonconsumptive losses. An asterisk indicates a significant reduction in feeding rate (Bonferroni, $p < 0.01$) compared to the control.

During the imidacloprid exposure, later-instar mayflies had reduced feeding rates in the 5 and 10 µg/L exposures, a trend consistent with the early instar results (Fig. 2). Even after correction for nonconsumptive losses, however, the later-instar mayflies appear to have increased feeding rates at some treatment levels (0.5 and 1 µg/L). This may suggest that increased feeding activity might be an adaptive response to low-dose imidacloprid exposure. This is consistent with the nonconsumptive losses observed in the early instar mayflies (Fig. 1B): Early instars were unable to incorporate the material, whereas later instars could. Whether the consumed *Nitzschia* sp. was suitably digested, however, was not evaluated in the present study.

After imidacloprid exposure in the day-1 treatment vessel, mayflies were transferred to flow-through artificial streams of the same dimensions as the test beaker. Postexposure, recovery feeding was significantly affected by imidacloprid treatment ($F_{5,60} = 2.47$, $p = 0.042$) and the number of days following exposure ($F_{3,60} = 9.45$; $p < 0.001$) (Fig. 3). Because all treatments (including control) had depressed feeding rates during the recovery (days 2–5) experiment as a result of handling, comparisons are only made within the 4-d recovery period

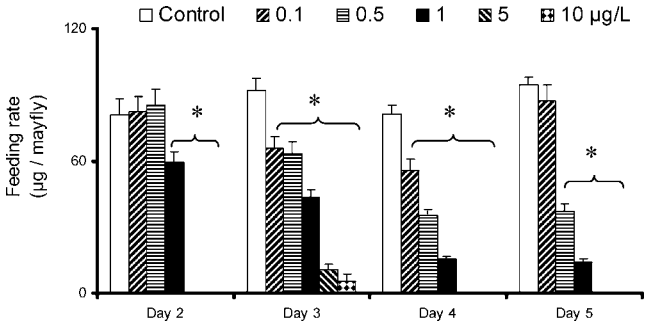

Fig. 3. Comparison of the feeding rate (µg/mayfly, mean ± standard error) of late-instar mayflies on days 2 to 5 following imidacloprid exposure. Feeding rate was reduced within days 2 through 5 because of imidacloprid. Recovery to control feeding rates may occur in some imidacloprid treatments (0.1 µg/L) but not in treatments of greater than 0.5 µg/L in the 4 d of recovery examined. The lack of bars in some treatments indicates that no feeding was detected. An asterisk indicates a significant reduction in feeding rate compared to control values within the day of recovery (Bonferroni, $p < 0.01$).

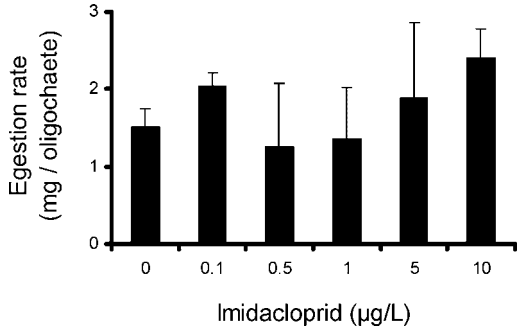

Fig. 4. Comparison of the egestion rate (mg/worm, mean ± standard error) for oligochaetes during the 24-h pulse (day 1 only) at 0, 0.1, 0.5, 1, 5, and 10 µg/L of imidacloprid. No reduction in egestion rate was detected across imidacloprid treatments.

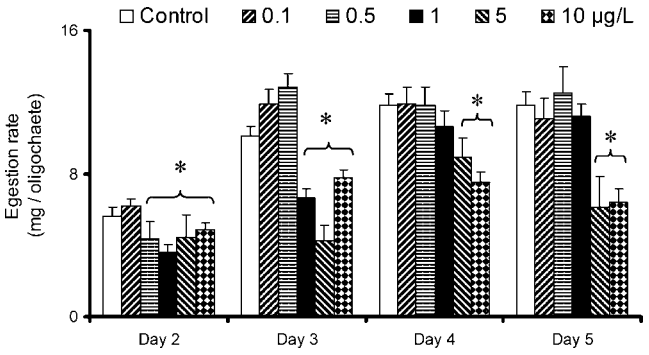

Fig. 5. Comparison of the egestion rate (mg/worm, mean ± standard error) on days 2 to 5 following imidacloprid exposure. An asterisk indicates reduced egestion rate compared to control feeding rate within each day of recovery (Bonferroni, $p < 0.01$).

(Fig. 3). Although transferring the mayflies in the laboratory between the exposure and test vessels did result in reduced feeding in the control group, the intense reduction in feeding rate during the recovery period demonstrates the feeding inhibition of mayflies exposed to imidacloprid.

Feeding rate of mayfly larvae was affected by time since imidacloprid pulse and exposure concentration (Fig. 3). Only larvae in the lowest-dose exposure (0.1 µg/L) recovered to control (day-5) feeding rates, although feeding depression was observed in this treatment on days 3 and 4. Mean feeding rates for larvae in treatments with an imidacloprid concentration equal to or greater than 0.5 µg/L decreased during the 4-d recovery period. Thus, significant sublethal effects were observed for up to 4 d after exposure and at an order of magnitude lower than what was observed following the initial 24-h imidacloprid challenge.

Oligochaete egestion tests

Oligochaetes were rendered immobile by 96-h (continuous) exposures to imidacloprid when concentrations exceeded 5 µg/L (96-h EC50, 6.2 ± 1.4 µg/L). Shorter (24-h) exposures of 10 µg/L or less, however, did not affect egestion or mobility ($F_{5,42} = 1.49$, $p = 0.21$) (Fig. 4). Following 24-h exposure to imidacloprid, animals in treatments with less than 1 µg/L increased egestion rate over the 4-d recovery period. As a result of handling, egestion rates initially were lower after each vessel transfer. Because oligochaetes were transferred twice, initially into exposure containers and later into recovery vessels, egestion was reduced at the beginning of the test. Treatments that had a sufficient dose of imidacloprid (>5 µg/L), however, maintained a depressed egestion rate for the duration of the test ($F_{3,108} = 46.65$, $p < 0.001$) (Fig. 5). The delayed recovery in the 0.5 µg/L (after day 2) and 1 µg/L (after day 4) treatments may suggest an extended period of anesthesia in annelids. Oligochaete recovery occurred at pulse concentrations an order of magnitude higher than those for mayflies (~1 µg/L for oligochaetes vs ~0.1 µg/L for mayflies).

DISCUSSION

Imidacloprid was designed to target invertebrate pests and is highly effective at selectively binding the insect nAcChR while being virtually nontoxic to higher vertebrates [8,9,19,20]. The nAcChR is common across insect taxa, however, because the origin of this receptor can be found deep in the evolution of neurochemical signaling [21]. Therefore, this compound can affect both aquatic and terrestrial invertebrates. Mayflies and oligochaetes were chosen for the evaluation of this insecticide because they are important contributors to the functioning of lotic food webs [22,23] and can be exposed to imidacloprid either through rain-event pulses or contaminated groundwater. Imidacloprid exposure produced significant nontarget effects in mayflies by causing mortality and by inhibiting feeding rate at low concentrations. In oligochaetes, imidacloprid induced immobility and reduced sediment egestion. Also, the effect of imidacloprid can be latent, with feeding and egestion inhibited upward of 4 d postexposure in some treatments.

This experiment demonstrated that mayflies exposed to 24-h pulses of imidacloprid greater than 0.5 µg/L did not recover to control feeding rates within 96 h (4 d) postexposure. Imidacloprid also caused mayfly mortality at environmentally relevant concentrations (24-h LC50, 2.1 µg/L) in both early and late-instar organisms. The use of the two mayfly life stages originally was conceived to account for potential tolerance differences in the different instar groups. Both groups had similar LC50s, however, which may reflect insignificant differences in the ability to metabolize imidacloprid between the two age classes. Because the upper concentrations in the recovery experiment incorporated the 24-h LC50, it was only with increased replication that sufficient later instars survived to compare the mayfly feeding inhibition over time. Therefore, the recovery results are the estimated feeding rate and recovery of less than 50% of the experimental *E. longimanus* population. These results are in keeping with population studies on the effects of imidacloprid, in which a 12-h pulse exposure of 10 µg/L or greater caused the loss of all male *Epeorus* from the adult cohort (A.C. Alexander, MSc thesis, University of New Brunswick, Fredericton, NB, Canada). Thus, the feeding rate at concentrations greater than the LC50 may reflect the continued feeding of the female mayflies only. Given the potential impact of imidacloprid on sex ratios and population viability, further study is warranted.

Because mayflies only feed as larvae, reduced consumption of foodstuffs can result in hampered larval development, reduced emergence, and smaller adult imagoes [24–26]. Nonconsumptive losses result from grazing disturbance [16], and our nonconsumptive results suggest that mayflies were less active in moderate to high imidacloprid exposures (5 and 10 µg/L) and, alternatively, exhibited increased activity in response to low imidacloprid exposure (0.5 and 1 µg/L). Feeding was reduced for at least 4 d following exposure to insecticide concentrations as low as 0.5 µg/L. This may be caused by the tremor and/or anesthetic action of imidacloprid [3], which would interfere with foraging activity. We hypothesize that

low doses of imidacloprid (0.5 and 1 μg/L) induce mild tremors that increase nonconsumptive losses and reduce foraging efficiency, whereas higher concentrations produce more severe tremors, which limit foraging activity. Ultimately, larval mayflies were unable to recover to preexposure feeding rates (~160 μg/mayfly [14]) during the 4-d recovery period, and the observed response could be either immediate (>5 μg/L) or latent (>0.5 μg/L). Feeding rates also decreased because of handling between the exposure and recovery periods. Mayflies in low-dose exposures, however, resumed feeding, similar to the control organisms, whereas mayflies in higher-dose exposures did not. Altered activity patterns are important, because other species may be affected, for example, changes in grazer-dislodged material (nonconsumptive losses) may be an important source of energy for downstream consumers [27–29].

Oligochaetes were more tolerant than mayflies of imidacloprid (oligochaete 96-h EC50 was 10-fold higher than the mayfly 96-h EC50) and were not affected during the 24-h pulse exposure in the egestion experiment. The 4-d postexposure experiment validated the 96-h EC50 (immobility) value of ~6 μg/L (aqueous exposure), with no recovery to normal egestion rates (~12 mg/worm [12]) observed for concentrations of 5 μg/L or greater. Oligochaete egestion rate was reduced at the onset of the tests because of handling. As above, however, oligochaetes in low-dose exposures recovered to baseline egestion rates similar to the control organisms, whereas oligochaetes in higher-dose exposures did not. The response of oligochaetes to imidacloprid 96 h following exposure was primarily that of prolonged reduced egestion, lethargy, and immobility, whereas mayflies exhibited tremors, paralysis, and death [3]. This taxonomic difference in response may be caused, in part, by functional differences in the acetylcholine receptors of insects versus annelids.

Previous research identified imidacloprid as an insecticide of concern in agricultural watersheds [7]. Studies seeking to measure imidacloprid in surface waters, however, have encountered difficulty acquiring water samples that contain the compound. Because of the relative solubility of imidacloprid, it may pass through river systems at concentrations greater than analytical detection thresholds primarily during storm discharge events. Likewise, imidacloprid is difficult to detect with the chromatographic columns currently in use for the detection of more common nonpolar pesticides. Even when the detection of imidacloprid is possible using modified techniques, the detection limit employed in many commercial laboratories (~2 μg/L) is well above the concentrations at which we detected effects on aquatic biota (~0.5 μg/L). Clearly, improved monitoring and detection limits for imidacloprid are needed to track concentrations in the field. A similar problem arises with the detection of imidacloprid metabolites. Imidacloprid degrades into several nonpolar and highly persistent derivatives from either photodegradation [30,31] or decomposition (>400 d) [6,31]. To our knowledge, these derivatives are not currently being examined by regulatory agencies. Considering the toxic potential of these metabolites, future research should examine whether these metabolites accumulate in lotic food webs.

The present study was intended to evaluate the occurrence of sublethal responses caused by environmentally relevant concentrations of imidacloprid. Subsequently, we have determined not only that are reductions in feeding rate occurring but also that adult abundance and body size is reduced when larval mayflies are exposed to low-dose, pulse exposures of imidacloprid [32]. Presently, these endpoints can be explained best by sublethal effects of imidacloprid exposure on feeding. Combined with the larval mortality associated with imidacloprid exposure, it seems likely that this insecticide could have impacts on the abundance and success of mayflies in streams. Consequences of reduced mayfly activity potentially include differential grazing in streams [33], whereas consequences of reduced mayfly abundance, especially of dominant taxa such as heptageniid mayflies [10], could include reduced foodstuff availability for fish.

Because delayed responses appear to be common for oligochaetes with respect to imidacloprid treatment, an even longer-term experiment may be warranted. This may be particularly salient, because soluble imidacloprid is a potential groundwater contaminant. Therefore, long-term, low-dose imidacloprid exposure may be common in sediment-dwelling organisms. Also, laboratory studies have identified a number of nonpolar metabolites of imidacloprid that may contribute to depressed egestion rates because of residual toxicity [30,31]. Given the sublethal effectiveness of imidacloprid, further studies may yield indications regarding the impact of this insecticide on oligochaete success in streams. For example, the reduction in oligochaete movement could increase predation risk by limiting the ability to avoid capture [34], whereas a reduction in egestion rates has the potential to alter organic matter processing and bioturbation in streams [35].

CONCLUSIONS

Imidacloprid exposure reduced the survivorship, feeding, and egestion of mayflies and oligochaetes at concentrations from 0.5 to 10 μg/L. Concentrations of imidacloprid greater than 5 μg/L limited oligochaete movement (24-h EC50, 6.2 ± 1.4 μg/L) and reduced egestion rate, with no recovery occurring in 4-d postexposure (>5 μg/L) experiments. Imidacloprid exposures at or exceeding 0.5 μg/L over a 24-h period caused significant long-term reductions in the feeding rate of late-instar mayflies that did not recover to control levels even 4 d after exposure. Of concern is that all these effects, including the mayfly 24-h LC50 of approximately 2 μg/L, were measured at exposure concentrations that overlap with observed environmental concentrations (0.25 ± 0.07 to 15.88 ± 0.99 μg/L). In Atlantic Canada, imidacloprid is an important insecticide used in the protection of potato crops [7]. Because Canada has adopted a seed-coat application technique for runoff protection of stream resources, spring rain events are thought to be the primary threat for large influxes of imidacloprid to streams in potato-farming regions. Because imidacloprid continues to be detected in agricultural stream surface waters (3.6% of samples; C. Murphy, 2006; Environment Canada, Charlottetown, PE), continued monitoring that employs lower analytical detection limits is warranted along with future examination of the persistence and toxicity of imidacloprid metabolites.

Acknowledgement—We would especially like to acknowledge the technical advice and assistance of J. Bailey, D. Halliwell, C. Casey, E. Irving, P. Williams, K. Heard, E. Luiker, and D. Hryn. Earlier drafts of the manuscript benefited from critical review by D. Baird, G. Benoy, K. Kidd, and three anonymous reviewers. Support for this research was provided by financial assistance to A.C. Alexander from the University of New Brunswick and the Science Horizons internship program; an Environment Canada Pesticide Science Fund grant to J. Culp, K. Liber, and A. Cessna; and a Natural Sciences and Engineering Research Council of Canada Discovery grant to J.M. Culp.

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Chapter 3

Predicting the Effects of Insecticide Mixtures on Non-Target Aquatic Communities

Alexa C. Alexander and Joseph M. Culp

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53356

1. Introduction

In this study two questions will be posed: firstly, how can single-species, single-compound toxicity test data on non-target aquatic insects predict patterns in stream communities exposed to the same compounds individually and jointly? Secondly, can mixtures of two or three insecticides be treated additively using a concentration addition, Toxic Unit (TU) approach in an aquatic community context? To evaluate these questions, the following studies examined the responses of field-collected benthic (bottom-dwelling) invertebrates exposed to mixtures of organophosphorus insecticides (chlorpyrifos and dimethoate) in detail as well as a preliminary investigation of the effects of adding a third insecticide to the mixture, the neo-nicotinoid (imidacloprid).

Non- target aquatic organisms are routinely exposed to pesticides because these compounds are widely used and are regularly detected during stream biomonitoring [1]. Mixtures of insecticides are particularly worrisome because these compounds can directly alter the abundance and diversity of aquatic insects; consequently, these effects can reshape aquatic food webs. Organophosphorus insecticides are particularly relevant for consideration because they are extensively used in agriculture worldwide and, for example, constitute ~ 40% of the insecticides applied in the United States [2]. In this study, two organophosphorus insecticides were selected, chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) and dimethoate (O,O-dimethyl S-[2-(methylamino)-2-oxoethyl] phosphorodithioate) to examine in detail because both are among the most commonly used in North America. Both are also routinely applied jointly or sequentially for the protection of more than 40 crops globally [2,3].

Chlorpyrifos and dimethoate are also highly toxic to non-target, aquatic species. According to van Wijngaarden *et al.* [4], the 48-h LC_{50} (median lethal concentration to affect 50% of the

population) for chlorpyrifos on the non-target mayfly, *Cloeon dipterum* is approximately 1 μg/L and similarly, Baekken and Aanes [5], report that the 96-hr LC_{50} for the mayfly, *Baetis rhodani*, is in the range of 7 μg/L for dimethoate. The third insecticide, imidacloprid (1-((6-Chloro-3-pyridinyl)methyl)-N-nitro-2-imidazolidinimine), is also highly toxic to non-target aquatic species (e.g., the mayfly, *Epeorus longimanus* 24-h LC50 = 2.1 ± 0.5 μg/L, see [6]). Unlike chlorpyrifos and dimethoate however, the primary mode of action of imidacloprid is semi-permanent binding to the acetylcholine receptor rather than the ACh enzyme [7]. This difference may increase toxicity of the ternary mixture because all three insecticides bind the same enzyme and receptor system.

Organophosphorus insecticides are thought to primarily target the acetylcholinesterase (AChE) enzyme, preventing the removal of acetylcholine (ACh) by the enzyme from the post-synaptic gap [8]. Therefore, excessive acetylcholine is bound and continuous nerve signals are sent to cholinergic receptors, which can result in trembling, respiratory duress and ultimately death [8]. Notably, in order for most organophosphorus compounds to become toxic they must first be transformed into their active form, an oxon [9,10]. However, insecticides such as chlorpyrifos and dimethoate are chemically diverse and are able to interact with multiple metabolic pathways and targets. Therefore, indirect biochemical or ecological effects of these compounds may be responsible for observed differences in their toxicity [8,9,10].

In this study, two organophosphorous insecticides (chlorpyrifos and dimethoate) with the same primary mode of action were tested individually and jointly on a natural, macroinvertebrate assemblage using a toxic unit approach. The primary question asked was whether the joint-action of these two insecticides can be reasonably evaluated at a community level using additive assumptions of toxicity. This question was evaluated by determining the appropriate concentrations in toxic units of chlorpyrifos and dimethoate by compiling single-species toxicity test data for orders of insects commonly thought to be sensitive indicators in aquatic biomonitoring of streams and rivers namely, Ephemeroptera, Plecoptera and Trichoptera, or E.P.T. taxa. A 20 day artificial stream experiment was conducted where field-collected benthic (bottom-dwelling) macroinvertebrate assemblages were exposed to four toxic unit (TU) doses of either chlorpyrifos or dimethoate individually (control, 0.2, 0.4 and 0.8 TU) and two, 1:1 mixture doses (0.2 + 0.2 TU and 0.4 + 0.4 TU) of both insecticides applied jointly. Subsequently, responses in the benthos in a community were examined using Principle Components Analysis (PCA). Macroinvertebrate abundance, richness and guild structure was assessed using a factorial ANOVA and a chi-square (χ^2) approach to compare observed responses to control values as well as to predicted responses to treatment across a toxic unit gradient.

2. Methods

This 20-d study was conducted from 12 July to 2 August, 2007 at the Environment Canada mesocosm facility 10-km southeast of Fredericton (New Brunswick, Canada). Aquatic inver-

tebrates were collected in the Nashwaak River (sampling location: 46º14294´N, 66º36722´W). The Nashwaak River is a relatively pristine tributary of the larger Saint John River and runs more than 100 km through forested and rural communities of less than 500 inhabitants in central New Brunswick.

Subsampled invertebrate assemblages were inoculated into 88 outdoor, artificial streams (Figure 1, see also [11,12]). Each partial flow-through stream was circular and had a planar area of 0.065 m^2 and a 10-L volume. Three treatments of organophosphorus insecticides ($n_{replicates\ per\ treatment}$ = 8) were examined in detail: chlorpyrifos (control, 0.2, 0.4 and 0.8 TU), dimethoate (control, 0.2, 0.4 and 0.8 TU) and a 1:1 mixture of both insecticides (0.1 + 0.1, 0.2 + 0.2 and 0.4 + 0.4 TU). An additional ternary 1:1:1 mixture of all three insecticides was also examined as a pilot study and included imidacloprid as well as chlorpyrifos and dimethoate (0.1 + 0.1 + 0.1 TU). Treatment solutions were housed in polyethylene reservoirs and manifolds were used to distribute the treatment solutions at uniform flow rates to each replicate stream. Groundwater from the extensive Saint John River aquifer was used to provide water to the artificial streams. Wastewater from each stream was passed through carbon filters (Culligan Inc.; activated carbon filter cylinder, Moncton, NB, CAN) to remove all contaminants before any water was discharged to the environment.

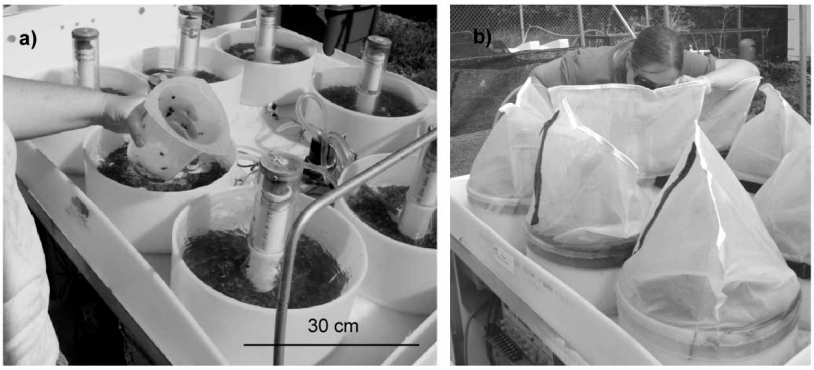

Figure 1. Cylindrical artificial streams. We inoculated 88 outdoor, artificial streams with a field-collected benthic invertebrate assemblage. Each flow-through stream was circular with a planar area of 0.065 m2 and a 10-L volume. In Fig. 1a, 8 streams were inoculated with gravel (coarse and fine) as well as 5 cobbles per stream. Protruding from the centre of each replicate stream is a motorized, rotating paddle that regulated the velocity of water in each stream. In Fig. 1b, streams post inoculation where each stream is covered with mesh to facilitate the collection of adult emergent insects.

2.1. Establishment of the aquatic community

2.1.1. Mimicking in-stream habitats

Prior to initiating the experiment, benthic substrates were introduced into each replicate stream. A realistic benthic substrate was created by inoculating each stream with a mix-

ture of 25% fine gravel (2 - 4 mm) and 75% gravel (4 - 30 mm) that was obtained from gravel beds adjacent to the invertebrate sampling site on the Nashwaak River (Figure 1a). Cobblestones (7-10 cm) were also collected from this site with five stones randomly assigned to each replicate stream. Cobble and gravel were gently washed to remove any attached invertebrates while maintaining the periphyton community. This procedure established a lotic substrate consisting of a 2-3 cm layer of gravel-cobble plus surface stones that were covered with periphyton and was similar to the original habitat of the benthic community examined (Figure 1a).

2.1.2. Field collection

Benthic invertebrates were collected in a single riffle upstream of the gravel collection site on the Nashwaak River with U-nets (area = 0.06 m^2). The subsampling procedure consisted of the collection of twenty-five (25) U-nets collected 8 times by 5 samplers working systematically upstream within the riffle. Twenty-five U-nets were selected to slightly increase (~10%) the ambient density of aquatic invertebrates in the artificial streams, thus offsetting any mortality due to transport from the river to the test site. Each set of 25 U-nets were divided into 16 community subsamples with 5 reference subsamples from each set retained to determine the initial composition of the aquatic community. Streams were systematically inoculated with a subsample from each of the 8 sets of the 25 U-nets collected. Such that each of the 11 treatments levels (low, medium, high or chlorpyrifos, dimethoate, binary mixture, as well as a single comparison of a low ternary mixture and the control) received a portion of the same stream assemblages collected in the field (Figure 2).

2.2. Establishment of treatments

The 96-h LC50s (as 95% C.I.) were estimated for chlorpyrifos (4.68 – 5.69 μg/L) and dimethoate (23.96 – 26.57 μg/L) by curve-fitting single-species, single-compound toxicity test data compiled from public databases (U.S. Environmental Protection Agency Ecotox database [13], Figure 3). Appropriateness of doses was also assessed using tandem laboratory testing of chlorpyrifos and dimethoate on laboratory-reared *Chironomus tentans* and field-collected Heptageniidae mayflies from the Nashwaak River [14]. For imidacloprid (96-h LC50 0.8 – 3.1 μg/L 95% C.I.), where less data was available, appropriate doses were determined in comparison to previous artificial stream studies in our region [15]. Only genera of the orders Ephemeroptera, Plecoptera and Trichoptera (E.P.T. taxa) were included in the estimated riverine community 96-h LC50 (the median lethal concentration that will affect 50% of E.P.T. taxa) because the abundance of these insects is generally thought to be indicative of healthy streams and is widely used in stream biomonitoring [16].

Insecticide solutions were mixed in agricultural grade stock tanks, a 2000-L stock tank for chlorpyrifos, a 520-L stock tank for dimethoate and a 200–L stock tank of each component of the ternary mixture. All solutions were mixed using groundwater from the extensive Saint John River aquifer. Stock solutions of chlorpyrifos (70 μg/L) were made by serial dilution of Lorsban -4E© (NAF-163, Dow AgroSciences, Indianapolis, IN, USA). Stock solutions of dimethoate (200 μg/L) were made by serial dilution of Lagon 480E © (9382, United Agri Prod-

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ucts Canada Inc., Dorchester, ON, Canada) and finally, imidacloprid (240 μg/L) by dilution of Admire 240® (Bayer CropScience, Calgary, AB, CAN). The insecticide-treated groundwater was delivered to one of eleven treatment reservoirs by positive displacement pumps (Viking Pumps, Pulsefeeder 25-H duplex pump, Cedar Falls, IA, USA). Secondary pumps then delivered the treatment solutions from each reservoir through a manifold to generate uniform flow rates into the base of each partial flow-through, replicate stream.

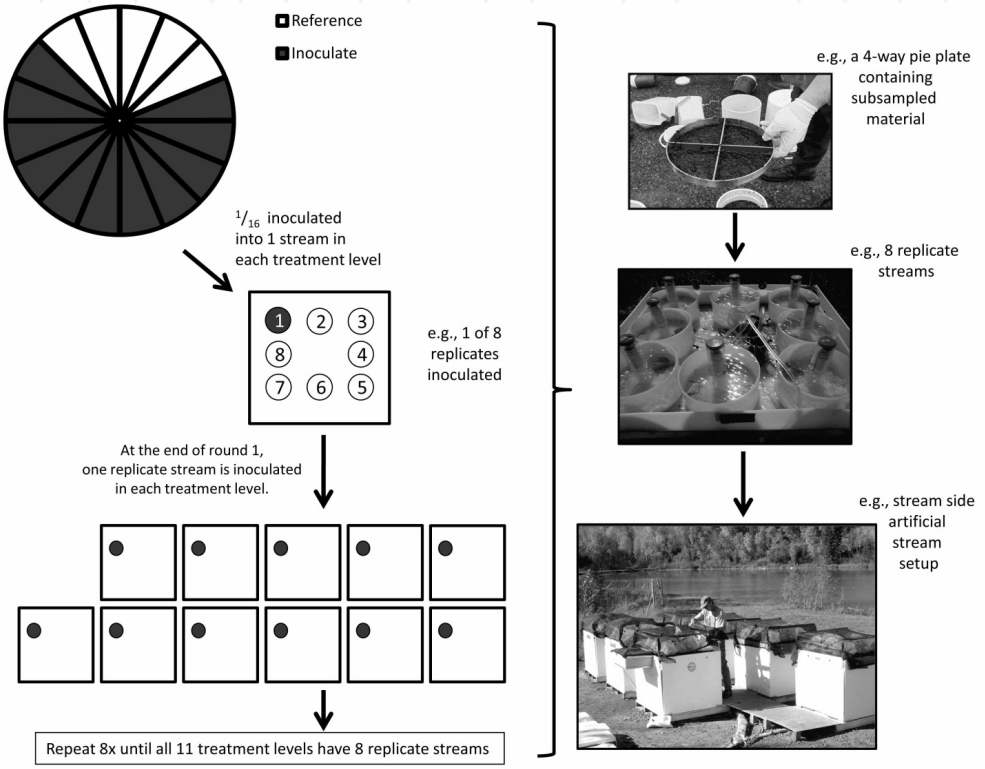

Figure 2. Benthic community subsampling and inoculation procedure for 88 replicate streams (11 treatments each containing 8 replicates). Sets of 25 U-nets (5 samplers collecting 5 U-nets each) were subsampled into 16 equal parts using a pie-plate made from 44 μm mesh. One sixteenth (1/16) of every 20 U-nets collected was inoculated into one replicate stream in every treatment level. This procedure was repeated eight times with each additional set of 25 U-nets systematically inoculated into adjacent replicate streams (one per treatment level). Thus, if the initial stream community had been significantly different in composition differences would have been allocated between treatments. Differences in community composition were not detected between subsamples (*Wilks-L* > 0.86; *P* > 0.99, in both cases).

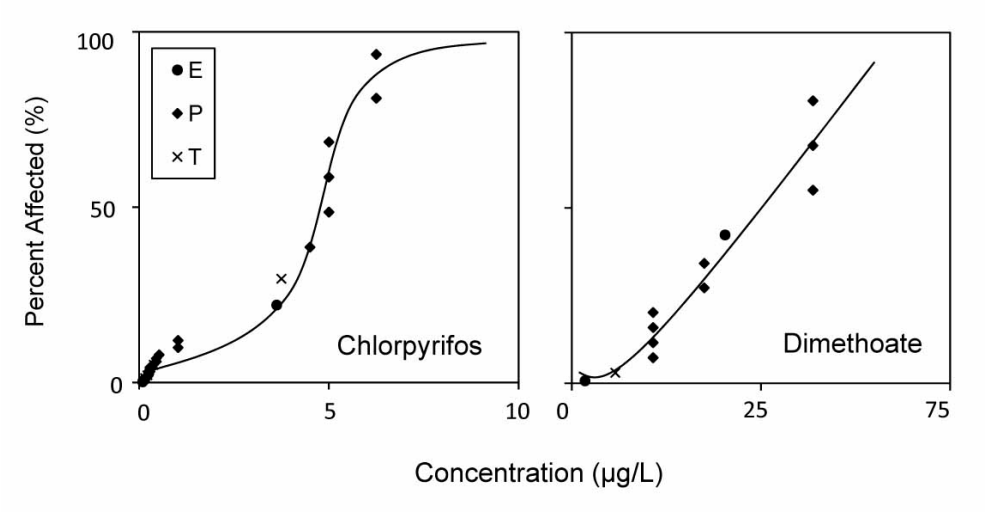

Figure 3. Percent Affected (96-h) of E.P.T. taxa as reported in the literature for the insecticides chlorpyrifos and dimethoate. For imidacloprid (96-h LC50 0.8 – 3.1 µg/L 95% C.I.), where less data was available, appropriate doses were determined in comparison to previous studies in our region [6,15]. Additional, tandem laboratory testing of chlorpyrifos and dimethoate on laboratory-reared *Chironomus tentans* and field-collected Heptageniidae mayflies from the Nashwaak River further corroborated dose selection [14]. Only genera of the E.P.T. Orders (Ephemeroptera, Plecoptera and Trichoptera) were used because the abundance of these insects is thought to be indicative of healthy stream conditions.

Chemical analysis determined the actual concentrations (Table 1) of the three insecticides individually and in mixture. Analyses were conducted at the National Water Research Institute (Environment Canada) in Saskatoon (SK, Canada) using a Waters 2695 Alliance HPLC System interfaced to a Micromass Quattro Ultima triple quadrupole mass spectrometer (LC-MS-MS) equipped with an electrospray ionization interface set to positive ion mode. For chlorpyrifos and dimethoate, chromatography was achieved using a Waters Xtera MS C_{18} (100 mm x 2.1 mm i.d., 3.5-µm particle size, Milford, MA, USA) analytical column and an aqueous acetonitrile mobile phase containing 0.1% formic acid (v/v). For imidacloprid, the mobile phase contained 40% aqueous acetonitrile and 0.2% formic acid (v/v). Water samples were collected in each treatment level on three occasions (July 13, 14, 17 in 2007) during the 96-h insecticide exposure period which began at noon on 13 July. Samples were collected in 500-mL amber vials (EPA vials, Fisher scientific, Fair Lawn, NJ, USA) and stored at 4ºC until shipment to Saskatoon for analysis. The samples were subjected to solid-phase (dimethoate) or liquid-phase (chlorpyrifos) extraction, the extracts taken to dryness, and the extract residue dissolved in deionized water (1.0 mL) prior to analysis by LC-MS-MS. All of the actual concentrations overlapped the target concentrations (Table 1) with an even distribution of under- and over- dosing for each target. Therefore, concentrations were comparable to those determined by laboratory bioassays in the published literature.

Treatment in Toxic Units (TU)	0.2 TU	0.4 TU	0.8 TU	
Target [chlorpyrifos]	0.94 – 1.14	1.87 – 2.28	3.74 – 4.55	
Actual [chlorpyrifos]	0.47 – 1.31	1.64 – 2.70	2.41 – 6.89	
Target [dimethoate]	3.79 – 5.31	9.58 – 10.63	19.17 – 21.26	
Actual [dimethoate]	1.04 – 4.80	9.32 – 12.07	19.93 – 22.96	
Target [imidacloprid]	N/A	N/A	N/A	
Actual [imidacloprid]	N/A	N/A	N/A	
Mixtures in Toxic Units (TU x n)	**0.1 TU x 2**	**0.2 TU x 2**	**0.4 TU x 2**	**0.1 TU x 3**
Target [chlorpyrifos]	0.24 - 0.57	0.94 – 1.14	1.87 – 2.28	0.24 - 0.57
Actual [chlorpyrifos]	0.19 - 0.86	0.78 – 1.61	1.39 – 4.02	0.12 - 0.38
Target [dimethoate]	2.40 - 2.66	4.79 – 5.31	9.58 – 10.63	2.40 - 2.66
Actual [dimethoate]	2.13 – 3.54	2.36 – 5.88	8.18 – 16.43	2.18 - 2.80
Target [imidacloprid]				0.24 - 0.57
Actual [imidacloprid]				0.47 - 0.69

Table 1. Comparison of treatments in toxic units (TU) with respect to the 95% confidence interval (95% CI) of the estimated range of targeted doses and the actual concentrations for chlorpyrifos, dimethoate and the 1:1 binary (x2) mixtures of chlorpyrifos and dimethoate compared to 1:1:1 ternary (x3) insecticide mixtures of chlorpyrifos, dimethoate and imidacloprid. All concentrations are in µg/L. Target concentrations for each insecticide are presented as ranges to reflect the uncertainty in the LC50 estimate.

2.3. Final data collection

At the end of the 20-d experiment, the streams were dismantled and the contents collected. Water samples, periphyton samples and invertebrates were collected from each replicate stream. Benthic macroinvertebrates were collected from each stream and preserved (10% formalin, transferred to 70% ethanol after 1 week) for subsequent laboratory sorting and identification using dissecting microscopes (Leica© Microsystems Ltd., Cambridge, UK). Aquatic specimens were sorted and identified to genus at the end of the experiment according to Environment Canada protocols, with a minimum of 20% of the collected material checked by a certified taxonomist to achieve 95% confidence in the identifications [17]. Some taxa were only identified to Order given time constraints and available expertise (e.g., Oligochaeta, Nematoda, Gastropoda, Collembola and 1st instar Plecoptera). Guilds were inferred from the literature in order to infer the habits of organisms [16,18]. Adult insects were also collected over the course of the 20-d experiment in 2-d intervals and in some cases were used to corroborate the presence of cryptic genera.

2.4. Statistical approaches

Community responses were examined in the factorial portion of the experiment (chlorpyrifos x dimethoate) using Principal Components Analysis (PCA) because the data were continuous with respect to both of the treatment level factors of interest (e.g., actual

concentrations of insecticides) as well as the density of in-stream macroinvertebrates [19]. A correlation matrix was used to prevent the different variances in the variables to influence the analysis. Responses in different taxa and guilds were also examined using factorial ANOVA (for chlorpyrifos and dimethoate only) and chi-square (χ^2) approaches. In this study, factorial ANOVA approaches examined response variables with respect to explicit treatment categories: a gradient of toxic units (TU, throughout); different insecticide treatments (I) and the interaction between the dose and the insecticide treatments (TU x I). Post-hoc testing, where applicable, was conducted using 1-tailed Dunnett's tests [20] and compared specific treatments to control levels (ANOVA approach, marked 'a' in corresponding figures). Where necessary (e.g., total and scraper abundance), data were transformed to satisfy assumptions (ln transformation, [21]). Whether the treatments initiated predictable reductions in abundance (of taxa, groups or guilds) was examined by comparing observed differences to those expected (or predicted) using chi-square (χ^2) tests. Expected values were determined by calculating the predicted reduction compared to control values for each invertebrate metric, in abundance from the toxic unit treatment range. Predicted values with respect to control appear throughout and significant deviations from predicted values by the χ^2 approach are marked 'c' in the corresponding figures. Preliminary comparisons of differences between the low binary (0.1 TU x 2) and low ternary (0.1 TU x 3) mixtures (1-way ANOVA) are also made for the six response variables of interest with respect to control, predicted, binary and ternary mixture treatment levels. To simplify, although differences in density per cm^2 were tested for significance, the responses are shown as the percent reduction in response between the ternary and the binary mixtures at 0.1 TU.

3. Results

3.1. Responses to treatment with chlorpyrifos and dimethoate

Principal Components Analysis (PCA) of the 38 genera and 5 orders of benthic macroinvertebrates identified in this experiment were highly responsive to increasing TU treatment and responded differently to treatment with either chlorpyrifos or dimethoate (Figure 4). Factor 1, (Eigenvalue 7.08, 44.3% of variance) was composed of the combined loadings of treatment in toxic units (TU, Pearson's $r = 0.34$) as well as the action of chlorpyrifos (Pearson's $r = 0.58$) or dimethoate (Pearson's $r = -0.15$). Increased insecticide treatment in Toxic Units (TU) reduced the breadth of taxa present in the community assemblage, as indicated by the decreased variation in the distribution of taxa and guilds from left to right along the horizontal axis (Factor 1 in Figure 4). Interestingly, community responses to treatment with either chlorpyrifos or dimethoate were in opposing directions, although both insecticides were important contributors to the distribution of taxa, guilds and treatments in Factor 2 (Eigenvalue 2.36, 14.7%; TU, Pearson's $r = 0.01$; chlorpyrifos, Pearson's $r = -0.31$; dimethoate, Pearson's $r = 0.29$). In particular, chlorpyrifos was an important contributor to the removal of taxa with streams treated with 0.8 TU of chlorpyrifos (C0.8TU) occurring in the PCA quadrant with the fewest taxa (bottom right, Figure 4). By contrast, responses to treatment with dimethoate occurred in the opposite quadrant suggesting firstly, that different members of the benthic

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macroinvertebrate assemblage were responding to chlorpyrifos versus dimethoate, and that treatment with dimethoate did not decrease density and diversity of taxa as forcefully as treatment with chlorpyrifos (top left, Figure 4). Interestingly, medium dose mixture treatments (M0.4TU) are located in the same quadrant as the equivalent dimethoate treatments (e.g., D0.4TU and D0.8TU) whereas high dose mixtures (M0.8TU) were more closely associated with predictions of additive toxicity in toxic units (Factor 1).

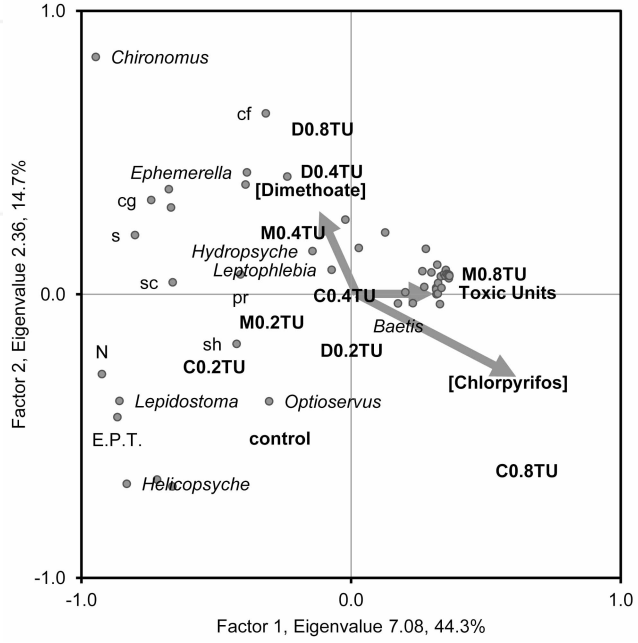

Figure 4. Principal Components Analysis (PCA) of differences in responses of 38 genera and 5 orders of benthic macroinvertebrates (each indicated, •) associated with chlorpyrifos or dimethoate insecticide treatment in Toxic Units (as vectors, above). Each treatment level is indicated (e.g., C0.2 TU, Chlorpyrifos at 0.2 TU). Factor 1 explained 44.3 % of the variance in the assemblages and was primarily driven by increased insecticide treatment in Toxic Units and secondarily by chlorpyrifos treatment. Dimethoate treatment was associated with different assemblages predominantly contributing to pattern in Factor 2 which explained an additional 14.7 % of the variance. Additional notes: guilds are indicated by codes cf = collector-filterers; cg = collector-gatherers; sc = scrapers; sh = shredders; pr = predators; total abundance per cm^2 = N; total richness per cm^2 = s; E.P.T. = sum density of Ephemeroptera, Plecoptera and Trichoptera orders. Remaining labels indicate genera of aquatic insect taxa (e.g., *Chironomus* spp.).

Significant change in measures of average total density per cm^2 and average taxa richness per cm^2 (Figure 5) were only found at the highest dose of chlorpyrifos tested (0.8 TU, abundance or richness, $P < 0.01$). The highly significant interactions (total density, TU x I, $F_{5, 69} = 68.23$, $P < 0.01$; or richness, TU x I, $F_{5, 69} = 709.03$, $P < 0.01$) were the result of total density and richness being decreased as predicted under exposure to chlorpyrifos, while dimethoate had no such effect. Throughout this study, dimethoate was non-toxic with respect to total density and richness and no negative effects of insecticides were detected irrespective of dose.

Additionally, mixture treatments were not different than control levels for either total density or richness (e.g., total density in M0.8TU, $P = 0.97$; richness in M0.8TU, $P = 0.75$). Stream communities were significantly more dense than predicted in high dose treatments containing dimethoate including the high mixture (M0.8TU, $\chi^2_{7} = 20.24$, $P < 0.01$) and the high dimethoate treatment (D0.8TU, $\chi^2_{7} = 16.90$, $P < 0.01$). In contrast, taxa richness was not found to be significantly different than predicted.

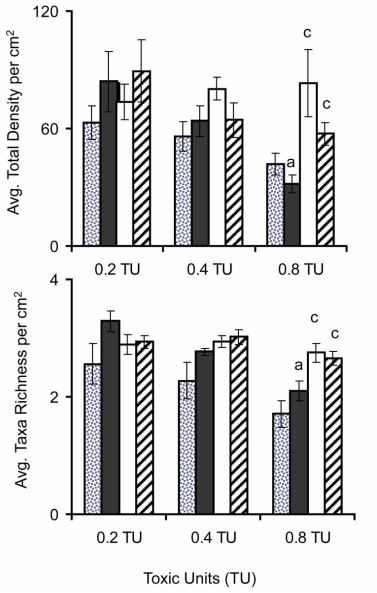

Figure 5. Total abundance and richness per cm^2 (± 1 SE, n = 8) of aquatic macroinvertebrates compared to treatment with the insecticides chlorpyrifos (black bars), dimethoate (white bars) or a 1:1 mixture of both insecticides (patterned bars). Letters indicate: 'a' significant differences compared to control (ANOVA approach), and 'c' differences in specific treatments (χ2 approach).

Responses in the average density of E.P.T. taxa and *Chironomus* spp. per cm^2 (Figure 6) were only found to significantly differ from control values in the highest chlorpyrifos treatment level (0.8 TU, E.P.T. or *Chironomus*, $P < 0.01$). Highly significant interactions were evident (E.P.T., TU x I, $F_{5, 69} = 53.91$, $P < 0.01$; or *Chironomus*, TU x I, $F_{5, 69} = 50.02$, $P < 0.01$) because density of E.P.T. and *Chironomus* decreased due to chlorpyrifos but not due to dimethoate. However, *Chironomus* midges were highly negatively affected by 0.8 TU of chlorpyrifos and the mean density of larvae in this treatment level was reduced 96% compared to controls (predicted decrease at 0.8 TU = 40%; C0.8TU, $\chi^2_{7} = 31.45$, $P < 0.01$). E.P.T. taxa were highly sensitive to high dose treatment with chlorpyrifos (C0.8TU, $\chi^2_{7} = 12.75$, $P < 0.01$), however, treatments containing dimethoate (e.g., dimethoate and mixture) were much less toxic than predicted (e.g., mean E.P.T. density in 0.8TU mixture, 37 % greater than predicted).

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Scraper density was not different than the control, although predators were highly responsive to all high dose insecticide treatments ($P < 0.01$, Figure 6). Once again, significant interactions were found for both guilds (scrapers, TU x I, $F_{5, 69} = 12.46$, $P < 0.01$; predators, TU x I, $F_{5, 69} = 26.35$, $P < 0.01$). However, the extent of significant interactions in scraper genera appeared to be largely due to the high variation in the density of the guild in the low dose, chlorpyrifos treatment (0.2 TU). Doses of 0.2 to 0.4 TU of chlorpyrifos and 0.2 TU of dimethoate all contained more scrapers than predicted (e.g., 74 % greater than predicted scraper density in chlorpyrifos 0.2 TU, $\chi^2_{7} = 50.03$, $P < 0.01$). In contrast, responses in predators were unique in that they responded to high insecticide doses (0.8 TU) by significantly decreasing abundance in these treatments, irrespective of the insecticide applied (e.g., 0.8TU mixture, 46 % less than predicted, $\chi^2_{7} = 28.38$, $P < 0.01$). Finally, the bell-shaped abundance pattern in predators with increased dimethoate treatment, compared with the linear decrease in abundance of the chlorpyrifos treatment, suggests that responses in predators were more complex than in other groups, potentially as a result of indirect effects due to reduced prey density.

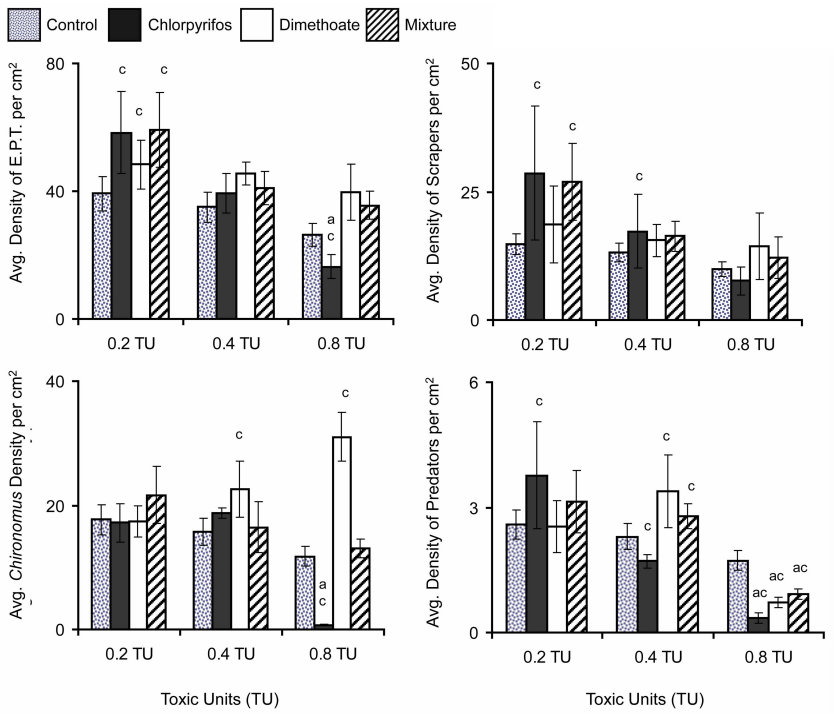

Figure 6. Density of E.P.T., Chironomus spp., scrapers and predators per cm² (± 1 SE, n = 8) compared to treatment with the insecticides chlorpyrifos (black bars), dimethoate (white bars) or a 1:1 mixture of both insecticides (patterned bars). Letters indicate: 'a' significant differences compared to control (ANOVA approach), and 'c' differences in specific treatments (χ2 approach).

3.2. Preliminary findings comparing binary and ternary mixtures

Statistical comparisons of the differences in density between binary (0.1 TU x 2) and ternary (0.1 TU x 3) mixtures of insecticides determined that the average total density ($P = 0.02$), taxa richness ($P < 0.01$) and *Chironomus* spp. ($P < 0.01$) were all significantly reduced due to the addition of imidacloprid to the mixture (Figure 7). In contrast, the average density of E.P.T. genera, scrapers and predators were not found to be significantly reduced in the presence of imidacloprid ($P > 0.06$, all cases). On average, the addition of a third insecticide resulted in a 62.9 ± 13.0 % reduction in average density. Density was more greatly reduced in some groups than others with scrapers the most affected (-111.6 ± 16.9 %) and taxa richness the least affected (-18.2 ± 16.5 %).

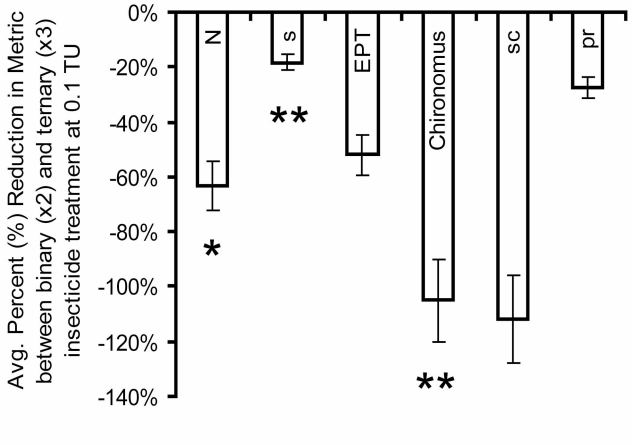

Figure 7. Comparison of % reduction in metrics due to treatment with the ternary mixture of 0.1 TU versus the binary mixture with the same doses. Each 0.1 TU dose should reduce the density of sensitive taxa by 5% because 1 TU = LC50. Therefore, reductions greater than 5% in the density of aquatic taxa is of biological interest even if differences in the density of organisms were not found to be statistically significant.

4. Discussion

4.1. Responses to chlorpyrifos and dimethoate

All of the metrics of benthic invertebrate responses measured also had significant interaction terms (TU x I, $P < 0.1$) suggesting that not all taxa, groups or guilds were equally sensitive to insecticide treatment. Differential toxicity within the organophosphorus insecticides has been reported previously and is predominantly due to the complexity of the biochemical pathway to reach what is considered the primary target, acetylcholinesterase [8,9,10]. Specifically, the toxic potency of organophosphorus insecticides depends on the creation of an

oxygen analogue (oxon) via metabolic bioactivation, creating an excretable endproduct which is also potentially toxic [9]. It is the oxon that binds acetylcholinesterase (AChE) and prevents the capture and removal of acetylcholine in the synapses, creating a positive feedback loop whereby uncontrolled neural signalling is initiated. Therefore, increased or decreased toxicity, even from the standpoint of a single mode of action (AChE), is due to the interaction of at least five factors: firstly, in/efficient creation of the oxygen analogue (oxon), i.e., differences in basal metabolism; secondly, insufficient binding of the target esterase(s) and/or binding to alternative targets; thirdly, insufficient accumulation of acetylcholine in the synaptic gap, due to inherent neurochemical differences or deficiencies, e.g., Myasthenia gravis; fourthly, other forms of tolerance and/or resistance, e.g., species, strain or regional differences (e.g., as reported in [22]), and finally, excretion and/or uptake efficiency of the parent toxicant or its metabolites. Furthermore, organophosphates also bind other receptors (e.g., muscarinic and nicotinic receptors), which in themselves can up or down regulate the effectiveness of the insecticide dose [23].

Despite the equivalent toxic unit doses employed in this study, treatment with dimethoate was associated with increased abundance of different taxa and guilds with the exception of predators, which were found to be substantially negatively impacted by all high dose treatments. In mixture treatments, the density of taxa often fell between that of either of the two insecticides individually, or, resembled the relatively non-toxic dimethoate at 0.4 and 0.8 TU. The highly significant declines in abundance of different taxa and guilds due to chlorpyrifos treatment, and the lack of similar findings due to dimethoate treatment are troubling because this study determined the appropriate doses from standard bioassays of the same genera from public databases of the published literature. For instance, according to a Norwegian study by Baekken and Aanes [5], the 96-hr LC_{50} for *Baetis rhodani* exposed to dimethoate was ~ 7 μg/L. In this study *Baetis* not only survived but emerged as adults (37 females and 26 males, not shown) in the 0.8 TU treatment where the dimethoate concentration was in the range of 19.93 – 22.96 μg/L. Disparities such as these invite speculation. If regional differences in sensitivity are as pronounced as the above finding suggests, then modeling may be restricted to more local scales. Alternatively, regional variation in data quality also invites speculation.

This study generally found that the mixture pattern at high doses had intermediate toxicity. Specifically, invertebrate responses to the binary mixtures were between that of dimethoate or chlorpyrifos individually. LeBlanc *et al.* [14] also found mixtures of chlorpyrifos and dimethoate to exhibit dose-level dependency in concurrent laboratory studies using chlorpyrifos and dimethoate in both binary mixtures (i.e., low dose antagonism to high dose synergy). Although high dose exposures are likely less common than sublethal effects (as described in [24]), high dose synergy is a concern because isolated high-dose events (e.g., a rain event) could significantly alter the composition of aquatic communities. Additionally, in more complex mixtures where multiple modes of action may be the norm, the concentration that initiates a synergistic effect may be lower than implied from bioassay results using single-species and single compounds.

4.2. Preliminary findings for responses in binary versus ternary mixtures

In this study, the addition of a third insecticide at 0.1 TU resulted in an average reduction in invertebrate density of approximately 60% (-62.9 ± 13.0%). However, the addition of 0.1TU of imidacloprid should, in theory, only result in a reduction of 5% in the abundance of organisms because 0.1 TU equals the 5% median lethal concentration or the LC5. Therefore, average density was reduced 50% more with the addition of one more insecticide to the mixture despite the addition occurring at what would otherwise be considered a very low dose. The implication of these findings is that the presence of imidacloprid in a mixture, an insecticide with a similar mode of action to chlorpyrifos and dimethoate, may cause significantly greater than additive reductions in invertebrate density in naturally occuring assemblages such as those tested in this study. These findings are similar to those of Leblanc *et al.* [14] where the combined action of imidacloprid resulted in greater than additive toxicity of mixtures of the same insecticides used in this study.

Although we did not detect significant differences when comparing the density of predators in low dose binary versus ternary mixtures, responses in groups such as predators continue to be of interest because of the importance of certain feeding groups in food webs (e.g., see [25]). For predators, the average percent reduction in density was -27.4 ± 9.9% at a dose that in theory will cause a 15% reduction in density (0.3 TU = LC15). However, if the addition of one insecticide can cause (at best) a 30% reduction in density, then what effects are likely for more complex mixtures acting on highly interconnected aquatic communities? Gilliom has previously reported that mixtures of up to 5 insecticides are routinely found in the environment [1]. If the patterns found in this study are true of more complex mixtures, then 5 insecticides at 0.1 TU could remove more than half the invertebrate population (> LC50) at individual doses that are thought to cause a mere 5% reduction in density. Clearly, further study of the effects of mixtures on keystone species, such as predators, will be important for untangling community responses to multiple stressors.

4.3. Implications to additive models: a biological argument

It is questionable whether additive predictions of responses can be made for these insecticides despite having the same (or similar) primary modes of action. Clearly, chlorpyrifos and dimethoate were not sufficiently similar in their actions on organisms in the community assemblage studied here to warrant additive treatment, even though their effects may be similar *in vitro*. In this study, dose-level dependency and genus or guild specific differences were the norm. Therefore, although the use of additivity to predict effects of insecticide mixtures has the appeal of simplicity, pest managers and regulators may be better informed by focused study of common mixtures of multiple compounds on relevant assemblages of organisms. Differences in sensitivity and tolerance may be region or system specific due to the predisposition of different populations to up or down-regulate the production of alternative substrates to which these insecticides can bind [9,26,27].

Thus, arbitrary grouping of two similar insecticides based on their primary mode of action, is inappropriate, particularly in an ecological context. Although grouping organophosphorus insecticides to model responses additively has been demonstrated to be appropriate

chemically (as in [28,29]), there appears to be little empirical evidence to support the uniform toxicity, or activity of organophosphorus compounds in biota (see [9]). Rather, non-additive responses appear to be the norm in real systems, perhaps because effects in real systems are mediated by biotic filters such as trait-mediated indirect effects [30,31]. We suggest that grouping these compounds into potency subclasses, as first suggested by Mileson *et al.* [23] will aid modelling efforts to overcome dose dependent effects of similar mixtures with variable potency. This is particularly warranted because dose-dependency appears to be a common mixture pattern [32]. Although concentration addition is widely thought to be a conservative approach to modelling impacts in streams (as in [33]), regional differences in sensitivity, or alternatively data quality, will reduce the usefulness of additive models. Finally, current toxicological models such as concentration addition and independent action, do not consider biological interactions between species. Interactions between species in a community can increase or mask organismal responses to stress and may be more important than isolated laboratory responses for the prediction of community level patterns.

5. Conclusions

In this study, when chlorpyrifos and dimethoate were both applied these mixtures were often intermediately toxic to aquatic invertebrates with the exception of predators that were severely impacted by all elevated insecticide treatments. In contrast, ternary mixtures were generally more toxic than expected and predators were highly affected even at the very low doses tested. Although only an additional 0.1 TU (= LC5) was added of a third insecticide, imidacloprid, responses in the density of different benthic macroinvertebrate metrics were reduced on average by more than 20%. From a community standpoint, it is apparent that different taxa and guilds within the macroinvertebrate community tested were not equally sensitive to treatment with different insecticides despite the use of equivalent toxic unit doses drawn from published bioassays on the same genera of aquatic insects as those examined in this study. As such, additive assumptions of toxicity in a community context are questionable. This is particularly true given that the interactions between species are rarely measured in ecotoxicology and thus, significant biological effects are likely ignored. Pest managers and regulators concerned with the impact of complex mixtures on naturally occurring communities may be better informed by focused study of common mixtures of multiple compounds on locally and regionally relevant assemblages of organisms than predictions derived from laboratory based mode of action models.

Acknowledgements

Many thanks to Kristie Heard (Environment Canada, Fredericton) for her assistance with the taxonomy and subsampling procedure, to Dave Hryn (Environment Canada, Fredericton) for his technical expertise and assistance in conducting the artificial stream experiment, and to Jon Bailey (Environment Canada, Saskatoon) who conducted the chemical analyses.

This work was made possible by the support of Environment Canada and a grant from Health Canada's Pesticide Science Fund. Further support was provided by a National Sciences and Engineering Research Council (NSERC) Discovery Grant to JMC and a NSERC (PGS-D3) to A.C. Alexander.

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Journal of Freshwater Ecology

ISSN: 0270-5060 (Print) 2156-6941 (Online) Journal homepage: http://www.tandfonline.com/loi/tjfe20

Sensitivity of freshwater molluscs to hydrilla-targeting herbicides: providing context for invasive aquatic weed control in diverse ecosystems

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To cite this article: Jennifer M. Archambault, Christine M. Bergeron, W. Gregory Cope, Robert J. Richardson, Mark A. Heilman, J. Edward Corey III, Michael D. Netherland & Ryan J. Heise (2015) Sensitivity of freshwater molluscs to hydrilla-targeting herbicides: providing context for invasive aquatic weed control in diverse ecosystems, Journal of Freshwater Ecology, 30:3, 335-348, DOI: 10.1080/02705060.2014.945104

To link to this article: http://dx.doi.org/10.1080/02705060.2014.945104

Journal of Freshwater Ecology, 2015
Vol. 30, No. 3, 335–348, http://dx.doi.org/10.1080/02705060.2014.945104

Sensitivity of freshwater molluscs to hydrilla-targeting herbicides: providing context for invasive aquatic weed control in diverse ecosystems

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(*Received 11 April 2014; accepted 6 June 2014*)

Hydrilla (*Hydrilla verticillata*) is an invasive aquatic weed that has spread rapidly throughout the USA, especially in the southeast. A common control method is the application of aquatic herbicides, such as fluridone and endothall. However, there is limited documentation on the effects of herbicides commonly used to control hydrilla and other aquatic weeds on many non-target freshwater species and no published information exists on the toxicity of these herbicides to freshwater molluscs. We exposed juveniles (96 h) and glochidia (48 h) of the unionid mussel *Lampsilis siliquoidea* and adults (28 d) of *Lampsilis fullerkati* to a formulation of fluridone (Sonar – PR®) in laboratory toxicity tests. The early life stages of *L. siliquoidea* were also exposed to a formulation of the dipotassium salt of endothall (Aquathol – K®) in separate tests. Juveniles of the freshwater gastropod snail, *Somatogyrus viriginicus* (Lithoglyphidae), were exposed (96 h) to the Sonar – Genesis® fluridone formulation. Endpoints were survival (all species and life stages) as well as siphoning behavior and foot protrusion (adult mussels). Median lethal fluridone concentrations (LC50s) were 865 μg/L (95% CI, 729–1,026 μg/L) for glochidia (24 h), 511 μg/L (309–843 μg/L) for juvenile *L. siliquoidea* (96 h), and 500 μg/L (452–553 μg/L) for juvenile *S. viriginicus* (96 h). No mortality occurred in the 28-d exposure of adult *L. fullerkati* and we found no statistically significant effect of fluridone concentration on foot protrusion ($p = 0.06$) or siphoning behavior ($p = 0.08$). The 24-h LC50 for glochidia exposed to the dipotassium salt of endothall was 31.2 mg/L (30.3–32.2 mg/L) and the 96-h LC50 for juvenile mussels was 34.4 mg/L (29.3–40.5 mg/L). Freshwater molluscs were more sensitive to fluridone and endothall than most other species previously tested. Fluridone and endothall concentrations typically recommended for hydrilla treatment (5–15 μg/L and 1–5 mg/L, respectively) were not acutely toxic to the molluscs we tested and a 28-d exposure to fluridone was not lethal to adult mussels even at the highest concentration (300 μg/L), indicating minimal risk of short-term exposure effects.

Keywords: fluridone (Sonar); endothall (Aquathol); unionid mussels; snails; LC50; toxicity; invasive species

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Introduction

Freshwater systems are subject to many stressors, including point and non-point source pollution, extreme climatic events, habitat modification (e.g., dams), and invasive species (commonly anthropogenically introduced). Hydrilla (*Hydrilla verticillata*, Hydrocharitaceae) is a non-native aquatic invasive weed that was introduced into the United States in Florida in the early 1950s and has spread rapidly throughout the country, especially in the southeast (Gordon & Thomas 1997). Included on the Federal Noxious Weed List (USDA APHIS 2012), hydrilla can form vast monocultures, shade out native vegetation (FWC 2013), alter water quality parameters including dissolved oxygen (Pesacreta 1988), and can serve as a vector for a neurotoxic cyanobacteria that has been linked to avian vacuolar myelinopathy in several water birds and their predators (e.g., bald eagle *Haliaeetus leucocephalus* and great horned owl *Bubo virginianus*; Wiley et al. 2008; Williams et al. 2009). Hydrilla produces numerous vegetative propagules (e.g., tubers, turions, and shoot fragments), and is frequently dispersed by humans via boat motors, trailers, and angling gear. Given the longevity of tubers in bottom sediments, eradication and/or long-term maintenance control is difficult (Langeland 1996). The most common control methods include application of aquatic herbicides, introduction of non-native grass carp (*Ctenopharyngodon idella*), and mechanical removal (Langeland 1996). Fluridone (Sonar®), a carotenoid synthesis inhibitor herbicide, is among the most commonly used aquatic herbicides for hydrilla management, and is typically prescribed for one to four months depending on the management objective and plant maturity. The dipotassium salt of endothall (Aquathol®) is also among the most commonly used aquatic herbicides for control of hydrilla and is typically prescribed two to three times during the growing season, each for a period of days. The impetus for this study was the recent introduction and persistence of hydrilla in two North Carolina, USA, ecosystems (Lake Waccamaw and the Eno River) with high biodiversity, high rates of endemism, and the presence of threatened and endangered species (Stager & Cahoon 1987; Smith et al. 2002; NCWRC 2005; LeGrand et al. 2013; NatureServe 2013). Here, the targeted use of herbicides has been recommended as the most effective hydrilla control method that is least likely to negatively affect native vegetation. However, increased information is needed on the potential effects of these herbicides on other non-target organisms.

Lake Waccamaw is a unique Carolina Bay Lake located in the southeastern coastal plain of North Carolina, USA, because it has a neutral pH, unlike other bay lakes and blackwater systems, which enable it to support high biodiversity (Stager & Cahoon 1987). It has been called a 'notable center of endemism in the southeast' (Smith et al. 2002), supporting several endemic and other rare species, including two endemic unionid mussels (state-listed threatened Waccamaw fatmucket *Lampsilis fullerkati* and state-listed endangered Waccamaw spike *Elliptio waccamawensis*) and two endemic freshwater snails (Waccamaw snail *Amnicola sp. 1* and Waccamaw siltsnail *Cincinnatia sp. 1*; NCWRC 2005; LeGrand et al. 2013). The Eno River is located in the Piedmont region of North Carolina (USA), and supports a variety of rare species, including the Carolina madtom (*Noturus furiosus*, state-listed threatened), one state-threatened (*Lampsilis radiata*) and three state-endangered (*Fusconaia masoni*, *Lampsilis cariosa*, *Lasmigona subviridis*) freshwater mussels, and the only confirmed population of panhandle pebblesnail (*Somatogyrus viriginicus*) in the state (LeGrand et al. 2013).

Though toxicity data exist for some freshwater invertebrates and fishes (Crosby & Tucker 1966; Hamelink et al. 1986; Paul et al. 1994; Yi et al. 2011), to our knowledge, no information has been published on the toxicity of fluridone or endothall to freshwater

molluscs. Understanding the potential risks to this non-target faunal group is especially important because both freshwater mussels and snails are simultaneously highly imperilled and critically important to the functional ecology of freshwater systems (Lydeard et al. 2004; Downing et al. 2010; Allen et al. 2012; Johnson et al. 2013). The southeastern USA, where hydrilla is most prevalent, has the highest unionid mussel biodiversity and endemism compared to any other region on the planet and >71% of North America's unionid species are endangered, threatened, or of special concern (Williams et al. 1993). Similarly, of the 703 freshwater gastropod species in USA and Canada, 278 (40%) are federally listed as endangered and >74% are considered imperilled (Johnson et al. 2013). Moreover, non-pulmonate snails and the early life stages of freshwater mussels are among the most sensitive aquatic organisms to several contaminants (e.g., atrazine, carbaryl (Conners & Black 2004); copper, ammonia (Besser et al. 2009)), and glyphosate-based chemicals which are among the most widely used herbicides (Bringolf et al. 2007). Potential risks of specific aquatic herbicides to freshwater molluscs should be assessed and balanced appropriately against the significant biological threat posed by invasive aquatic weeds like hydrilla. Further endangerment to these organisms may push some species to extinction and reduce common species to rare status.

Fluridone (market formulations tested: granular Sonar – PR® and liquid Sonar – Genesis®) and the dipotassium salt of endothall (hereafter, simply 'endothall'; market formulation Aquathol – K®) applications are commonly prescribed for management of hydrilla and both were considered for management of hydrilla in Lake Waccamaw (NC DENR 2013) and the Eno River. Unlike many aquatic systems in the southeastern USA that hydrilla has invaded and which have relatively low biodiversity (e.g., reservoirs, canals, and ponds), the two aforementioned ecologically unique systems in North Carolina, as well as others requiring similar conservation management, dictate a more thorough assessment of potential hazards to non-target biota from herbicide treatment. Therefore, we chose species of direct relevance to these systems for toxicity testing in this study. The purpose of this study was to determine the sensitivity of freshwater mussels and snails to herbicides commonly used in control and management of hydrilla and other aquatic weeds and to consider those results in the context of typically proposed hydrilla treatments (e.g., Lake Waccamaw) and potential future treatment of other sensitive ecosystems (e.g., Eno River).

Methods

Test organisms

Freshwater mussels are especially important non-target organisms for toxicity testing. They have been demonstrated as particularly susceptible to toxicants and other environmental stressors, in part because their larval life stage, glochidia, is an obligate parasite that requires encystment on a host fish to transform into the juvenile life stage (Cope et al. 2008). Therefore, juveniles and glochidia of the unionid mussel *Lampsilis siliquoidea* (fatmucket) were used in fluridone (Sonar – PR®) and endothall acute toxicity tests; *L. siliquoidea* is routinely used in toxicity testing due to its wide availability and ease of laboratory culture. *Lampsilis siliquoidea* is a congener of the Lake Waccamaw endemic and state-listed as threatened Waccamaw fatmucket (*Lampsilis fullerkati*). *Lampsilis siliquoidea* were supplied by the mussel culture laboratory at Missouri State University (Springfield, Missouri, USA). All glochidia were harvested from females <24 h before initiation of each test. All juveniles were propagated via host-fish infection, using

standard propagation and culture methods (Barnhart 2006), and ranged in age from 1 to 3 d, with an average shell length of 0.25 mm (± 0.14 mm, SD).

Adult *L. fullerkati* mussels were used in a 28-d chronic experiment. They were 33 months old at the time of the experiment, with an average shell length of 46.6 mm (± 3.3 mm; range 37.5−53.9 mm) and mean weight of 9.9 g (± 2.0 g; range 6.3−14.8 g). *Lampsilis fullerkati* were propagated at the Aquatic Epidemiology Conservation Laboratory, North Carolina State University College of Veterinary Medicine (Raleigh, North Carolina, USA), using a standard *in vitro* propagation protocol (Owen 2009).

The freshwater snail *Somatogyrus virginicus* was used in acute toxicity tests with the Sonar − Genesis® fluridone formulation; *S. viriginicus* (Lithoglyphidae) is a rare, non-pulmonate snail with patchy distribution in Atlantic Slope streams of Virginia, North Carolina, and South Carolina (USA; NatureServe 2013). *Somatogyrus viriginicus* is an annual species, in which most adults die soon after reproducing (Johnson et al. 2013). Juveniles were collected on 6 August 2013 from a viable population in the Eno River (near Hillsborough, North Carolina, USA) and were immediately transported to our laboratory at North Carolina State University for testing. Average shell length, as measured from the top down, perpendicular to the spiral, was 1.84 mm (± 0.37 mm). Based on earlier sampling in the Eno River on 2 May 2013, in which only adults and eggs were found, the juveniles tested were <3 months old.

Experimental conditions

We selected herbicide treatment concentrations based on recommended application rates for treatment of hydrilla, maximum application rates reported on the product label, and acute toxicity data reported for other taxa in peer-reviewed literature (Crosby & Tucker 1966; Sanders 1969; Hamelink et al. 1986; Paul et al. 1994; Yi et al. 2011) and on Material Data Safety Sheets (SePRO Corporation 2009, 2010, 2011, 2012; UPI 2011, 2012). An analytically verified 1304 μg/L (parts per billion) stock solution of Sonar − PR® (fluridone) formulation was prepared and provided by the SePRO Research and Technology Campus (Whitakers, North Carolina, USA). An analytically verified stock solution of Sonar − Genesis® formulation was prepared at 1383 μg/L. The fluridone formulations were shipped via overnight courier to our laboratory at North Carolina State University and immediately refrigerated until use in toxicity tests. Acute test concentrations of fluridone formulations ranged from 2.5 to 200 μg/L with an additional treatment at the stock solution concentrations (1304 μg/L for PR® and 1383 μg/L for Genesis®). Concentrations of Sonar − PR® in the chronic (28-d) experiment ranged from 5 to 300 μg/L. A concentrated formulation of endothall (Aquathol-K®), labeled as 4.23 lb/gal (~506,866 mg/L), was hand delivered by collaborating personnel in the Department of Crop Science, North Carolina State University, and subsequently diluted to a working stock of 1000 mg/L (parts per million). Test concentrations of endothall ranged from 0.5 to 1000 mg/L. Composite water samples (10 mL from each of three replicates, 30 mL total volume) were collected for herbicide concentration verification prior to placing organisms into the chambers, and again at 48 h; samples were stored at 4 °C until they were shipped to SePRO analytical laboratory (fluridone quantified via HPLC) or the US Army Engineer Research and Development Center's Environmental Laboratory (endothall quantified via immunoassay; Gainesville, Florida, USA).

All experiments were static-renewal tests conducted in reconstituted soft water (ASTM 2007), with 90%−100% water renewal at 48 h in the 96-h acute non-aerated tests, and at 72-h intervals in the 28-d aerated experiment. Soft water was selected because it most

closely approximated the water quality parameters in most of the test organisms' native ranges (e.g., Lake Waccamaw, Eno River). Quality assurance and control were ensured by conducting all tests according to the *Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels* (ASTM 2006). No formalized guidelines exist for conducting experiments with freshwater snails or adult mussels, so the mussel guideline was used (ASTM 2006), as per protocol in other studies (Besser et al. 2009; Archambault et al. 2013). Organisms were acclimated from their culture water to the test water by placing them in a 50:50 solution of culture/reconstituted water for 2 h, then further diluting the culture water to a 25:75 ratio with reconstituted water, and held for an additional 2 h before being placed in 100% reconstituted water (ASTM 2006, 2007). Tests were conducted in light and temperature-controlled environmental chambers (Precision Model 818, Thermo Fisher Scientific, Marietta, Ohio, USA), held at 20 °C and LD 16:8. In the 24-h tests, ~150 glochidia were placed in each of three replicates per treatment. In the 96-h experiments, seven mussels or snails were placed in each of three replicates per treatment, with 10 organisms per replicate in controls (0 μg/L). Snails were transferred to untreated ASTM water at the conclusion of the test and held for 48 h for a post-exposure survival assessment to identify potential latent mortality effects (per J. Besser, 2013, e-mail to WGC; unreferenced). Mean water quality conditions among acute experiments were 27.8 mg $CaCO_3$/L alkalinity, 39.0 mg $CaCO_3$/L hardness, 220 μS/cm conductivity, 7.50 pH, and 8.47 mg/L dissolved oxygen ($n = 4$ for alkalinity and hardness, $n = 36$ for all other variables). In the 28-d experiment, five adult mussels were placed in each of three replicates per treatment. Mussels were fed a mixture of 1-mL Instant Algae® Shellfish Diet and 0.5-mL *Nannochloropsis* (Nanno 3600) concentrate diluted in 500 mL deionized water. Approximately, 6.25 mL of food mixture was added to each replicate (administered concentrations of 50,000 and 850,000 cells/mL solution, respectively; Reed Mariculture, Campbell, California, USA) every 72 h at least 2 h before each solution renewal (Mosher et al. 2012; Leonard 2013). Mean water quality conditions in the chronic experiment were 26.7 mg $CaCO_3$/L alkalinity, 44.9 mg $CaCO_3$/L hardness, 168 μS/cm conductivity, 7.49 pH, and 8.54 mg/L dissolved oxygen ($n = 9$ for alkalinity and hardness, $n = 54$ for pH, $n = 60$ for all other variables).

Data collection and statistical analysis

Viability was assessed at 24 h for a subsample of approximately 50 glochidia in each replicate. We assessed viability by exposing glochidia to a saturated NaCl solution and viewing them under a stereomicroscope; glochidia that exhibited a shell-closure response to salt were considered viable (ASTM 2006). At the end of each 96-h exposure, survival of juvenile mussels was assessed by viewing them under a stereomicroscope; juveniles that exhibited foot movement outside of the shell, foot movement inside the shell, or a detectable heartbeat within a five-minute observation period were considered alive (ASTM 2006). Snail survival was assessed similarly, by observing for righting or movement within five minutes. In the chronic experiment, survival of adult mussels was assessed visually every 72 h by observing for foot retraction or valve closure in response to dewatering during renewal in mussels with open shells. Because the shell of *L. fullerkati* is thin and fragile, we made no attempt to check for resistance to opening, and mussels with tightly closed shells were assumed to be alive.

The effects of herbicide concentration on the survival of mussels and snails were analyzed by using survival data to generate median lethal concentrations (LC50s) and 95% confidence intervals (CIs) via the Trimmed Spearman-Karber method (Comprehensive

Environmental Toxicity Information Software (CETIS)™, v1.8.0.12, Tidepool Scientific, LLC, McKinleyville, California, USA). The LC50 was defined as the concentration that caused mortality in 50% of the individuals in the exposed sample, and the LC05 was defined as the concentration that caused mortality in 5% of the sample. LC values were considered significantly different when their 95% CIs did not overlap (i.e., $\alpha = 0.05$).

In the 28-d experiment with adults, we made observations every 72 h of siphoning behavior and foot protrusion; mussels were given a binary designation of siphoning or not siphoning and assigned a binary score of foot protrusion or no foot protrusion (Leonard 2013). The effect of herbicide concentration on siphoning behavior and foot protrusion was analyzed using a repeated measures analysis of variance (PROC MIXED; SAS version 9.3; SAS Institute, Inc., Cary, North Carolina, USA). Significant effects ($\alpha = 0.05$) of fluridone concentration were further analyzed using a Dunnett's *post hoc* test.

Results

Herbicide concentration analysis

Exposure accuracy (i.e., measured herbicide concentration compared to target concentration) was calculated as: exposure accuracy $= (P_m)/(P_t) \bullet 100$, where P_m is the measured herbicide concentration and P_t is the target concentration. The measured concentration of the fluridone stock solution used in tests with mussels (Sonar − PR®) was 108.3% of the reported prepared concentration of 1304 μg/L, and the mean exposure accuracy in experiments was 119.9% (range 102%−176%) of target treatment concentrations. The verified concentration of the Sonar − Genesis® formulation at the time of testing with juvenile snails was 87.0% of the initial reported concentration of 1383 μg/L, and had a mean exposure accuracy of 85.2% (range 80%−102%) in treatments prepared from the stock. The mean exposure accuracy in endothall (Aquathol-K®) experiments was 109.0% (range 100%−114%) of target treatment concentrations.

Mussel toxicity

Control viability at 24 and 48 h in glochidia tests was >90% of the initial viability that was assessed on arrival to the laboratory for all experiments, in accordance with testing guidelines (ASTM 2006). Control survival in experiments with juveniles was >90%, except in the endothall experiment at the 96-h time point. Even though control survival (73.3%) at 96 h in the endothall experiment was slightly below the 80% recommended in the standard guideline for toxicity tests with juvenile mussels (ASTM 2006), results are reported herein because survival was >90% in three of the low concentration treatments (1, 5, and 10 mg/L).

The 24-h LC50 for *L. siliquoidea* glochidia exposed to fluridone (Sonar − PR®) was 865 μg/L (95% CI, 729−1026 μg/L) and the 48-h LC50 was 978 μg/L (787−1214 μg/L). The experiment with juveniles yielded a 48-h LC50 of 1197 μg/L (569−2522 μg/L) and a 96-h LC50 of 511 μg/L (309−843 μg/L; (Table 1)). The 24-h LC05 for glochidia was 290 μg/L (0−598 μg/L); LC05s in the juvenile tests and at the 48-h time point of the glochidia test could not be determined due to the lack of two or more partial mortality responses among treatments. A chronic LC50 could not be determined for *L. fullerkati* at any time point during the 28-d test because no mortality occurred. Adult mussels exhibited only minor foot protrusion behavior during the experiment. Moreover, the degree of foot extension observed was minimal. Initially, foot extension was recorded using four

Table 1. Median lethal concentrations (LC50s) for mussels and snails (with 95% CI) in acute and chronic exposures to herbicides commonly used to treat *Hydrilla verticillata*. ND = value could not be determined. 48-h post = post-exposure assessment.

Species	Life stage	Time point	Fluridone (μg/L)	Endothall (mg/L)
Lampsilis siliquoidea	Glochidia	24 h	865 (729−1026)	31.2 (30.3−32.2)
		48 h	978 (787−1214)	27.6 (25.5−29.9)
	Juvenile	48 h	1197 (569−2252)	214 (134−342)
		96 h	511 (309−843)	34.4 (29.3−40.5)
Somatogyrus virginicus	Juvenile	48 h	ND	–
		96 h	500 (452−553)	–
		48-h post	409 (329−509)	–
Lampsilis fullerkati	Adult	28 d	ND − no mortality	–

categories: (1) shell closed and/or foot not visible; (2) foot visible, but not extended beyond mantle margin; (3) foot extended beyond mantle; and (4) foot extended and swollen. Because observations ($n = 1050$) were recorded as category 1 (67.5% of observations) or 2 (32.4%) in all but one case, foot protrusion data were analyzed as a binary function (i.e., foot extended or not) like the siphoning data. A category 3 observation was recorded only once, and category 4 was never observed. We found no statistically significant effect of fluridone concentration on foot protrusion ($p = 0.06$) or siphoning behavior ($p = 0.08$).

In endothall exposures, the glochidia 24-h LC50 was 31.2 mg/L (30.3−32.2 mg/L), and the 48-h LC50 was 27.6 mg/L (25.5−29.9 mg/L). The experiment with juvenile *L. siliquoidea* yielded a 48-h LC50 of 214 mg/L (134−342 mg/L) and a 96-h LC50 of 34.4 mg/L (29.3−40.5 mg/L; (Table 1)). The 48-h LC05 for juveniles was 34.6 mg/L (3.90−80.0 mg/L); other LC05s were not determined due to the lack of two or more partial mortality responses among treatments or poor fit.

Snail toxicity

The 96-h LC50 for *S. virginicus* exposed to fluridone (Sonar − Genesis®) was 500 μg/L (452−553 μg/L) and the LC50 at 48-h post exposure was 409 μg/L (329−509 μg/L; (Table 1)). The overlapping CIs between the two assessment time points indicate that there was no significant latent mortality in the exposed snails ($\alpha = 0.05$). An LC50 at the 48-h time point and LC05s could not be determined due to the lack of two or more partial mortality responses among treatments.

Discussion

Our results indicate that the early life stages of *L. siliquoidea* are more acutely sensitive to fluridone than most other aquatic organisms that have been tested. In a multi-laboratory study evaluating the effects technical grade fluridone (i.e., active ingredient only) and a commercial formulation of Sonar® on freshwater and marine invertebrates and fishes, Hamelink et al. (1986) reported a mean LC50 of 4.3 mg/L for invertebrates ($n = 15$ tests among six species) and a mean LC50 of 10.4 mg/L for fishes ($n = 28$ tests among five species) (Table 2). By comparison, at 24 h, *L. siliquoidea* glochidia were approximately five times more sensitive than invertebrates they tested and 12 times more sensitive than

Table 2. Comparative acute toxicities of freshwater species exposed to technical or commercial formulations of fluridone and dipotassium salt of endothall.

Species	Common name	Chemical grade	Time point	LC50 (mg/L)	Source
		FLURIDONE			
Invertebrates					
Arrenurus sp.	A water mite	Formulation	96 h	0.010	Yi et al. (2011)
		Technical	48 h	0.891	Yi et al. (2011)
		Technical	96 h	0.631	Yi et al. (2011)
Chironomus plumosus	A midge	Formulation	48 h	1.3	Hamelink et al. (1986)
		Technical	48 h	1.3	Hamelink et al. 1986
Gammarus pseudolimnaeus	An amphipod	Technical	96 h	2.1–4.1	Hamelink et al. 1986
		Formulation	96 h	>32	Hamelink et al. (1986)
Daphnia magna	A water flea	Formulation	48 h	3.6–3.9	Hamelink et al. (1986)
		Technical	48 h	3.9–6.3	Hamelink et al. (1986)
Fishes					
Sander vitreus	Walleye	Formulation	96 h	1.8	Paul et al. (1994)
Oncorhyncus mykiss	Rainbow trout	Technical	96 h	4.2–11.7	Hamelink et al. (1986)
		Formulation	96 h	7.1–8.1	Hamelink et al. (1986)
Ictalurus punctatus	Channel catfish	Technical	96 h	8.2–15.0	Hamelink et al. (1986)
		Formulation	96 h	13.2	Hamelink et al. (1986)
Micropterus dolomieu	Smallmouth bass	Formulation	96 h	7.6	Paul et al. (1994)
Lepomis macrochirus	Bluegill sunfish	Formulation	96 h	12.0	Hamelink et al. (1986)
		Technical	96 h	12.1–13.0	Hamelink et al. (1986)
Micropterus salmoides	Largemouth bass	Formulation	96 h	13	Paul et al. (1994)
Pimephales promelas	Fathead minnow	Technical	96 h	22	Hamelink et al. (1986)

(*continued*)

Table 2. (*Continued*)

Species	Common name	Chemical grade	Time point	LC50 (mg/L)	Source
		ENDOTHALL			
Invertebrates					
Daphnia magna	A water flea	Technical	26 h (IC50)	46	Crosby and Tucker (1966)
Gammarus lacustris	An amphipod	Formulation	24 h	>100	Sanders (1969)
Fishes					
Sander vitreus	Walleye (8−10 d)	Formulation	96 h	16	Paul et al. (1994)
	Walleye (41−43 d)	Formulation	96 h	54	Paul et al. (1994)
Micropterus dolomieu	Smallmouth bass (<1 d)	Formulation	96 h	47	Paul et al. (1994)
Micropterus salmoides	Largemouth bass (9−13 d)	Formulation	96 h	130	Paul et al. (1994)

fishes, and juveniles at 96 h were approximately eight times more sensitive than other invertebrates and 20 times more sensitive than fishes. The closest relative to *L. siliquoidea* in their study was the eastern oyster (*Crassostrea virginica*); oyster embryos had a 48-h LC50 of 6.8 mg/L. *Lampsilis siliquoidea* was approximately 8 (24-h glochidia LC50) to 13 (96-h juvenile LC50) times more sensitive than oyster embryos. Another study determined the 96-h LC50s of fluridone for the early life stages of walleye (*Sander vitreus*), smallmouth bass (*Micropterus dolomieu*), and largemouth bass (*M. salmoides*) (Paul et al. 1994; Table 2), which were all more tolerant than the mussels tested here (Table 1). In a recent investigation of the toxicity of fluridone on male water mites (*Arrenurus* sp.), Yi et al. (2011) reported toxicities to technical grade fluridone similar to our Sonar − PR® commercial formulation results; however, they found water mites were 60 times more sensitive in tests with another commercial formulation (Sonar − AS®; Table 2).

In the context of typical treatment prescriptions for hydrilla, all of the mussel toxicity data generated in tests with Sonar® PR, including those generally reported for regulatory purposes (24 h for glochidia, 96 h for juveniles), are two or more orders of magnitude greater than the water column treatment maximum target concentration for Lake Waccamaw (5 μg/L), and are more than three times higher than the maximum label application rate of 150 μg/L (SePRO Corporation 2012).

As with fluridone, freshwater mussels were also more acutely sensitive to endothall than most other tested organisms. Median effective concentrations (EC50s) and LC50s for 11 species range from >100 to 1071 mg/L; channel catfish (*Ictalurus punctatus*) and coho salmon (*Oncorhynchus kisutch*) were the most sensitive in the group, and the bluegill sunfish (*Lepomis macrochirus*) was the most tolerant (UPI 2012). The nearest relative to unionid mussels included in the ecotoxicity data was the eastern oyster, which had a 96-h sublethal EC50 (shell deposition) of 335 mg/L (UPI 2012), approximately 10 times greater than our 24-h glochidia and 96-h juvenile LC50s for *L. siliquoidea*. Acute values reported for some species in other studies showed sensitivities more similar to those of unionid mussels, including early life stage smallmouth bass (aged <1 d) and walleye fry (aged 41−43 d; Paul et al. 1994), and *Daphnia magna* (26-h median immobilization concentration (IC50); Crosby & Tucker 1966) (Table 2). Walleye 8−10 days old (96-h LC50 = 16 mg/L; Paul et al. 1994) were approximately twice as sensitive as the *L. siliquoidea* tested here (Tables 1 and 2). There was good agreement in our data among the LC50s for glochidia and the 96-h juvenile LC50, suggesting a defined threshold of tolerance; most mussels survived at concentrations ≤10 mg/L and experienced complete mortality at concentrations ≥100 mg/L. Despite being among the most sensitive species tested to date, the toxicity data for *L. siliquoidea* are 6−34 times higher than the recommended application rate of endothall for treatment of hydrilla (1−5 mg/L; UPI 2011). The 24-h glochidia and 96-h juvenile LC50s are approximately one order of magnitude greater than the application rate, indicating a smaller margin of error in applying endothall compared to fluridone. It should be stressed that an LC50 is not protective of a population (i.e., only 50% are expected to survive at the LC50 concentration).

We did not find any significant effects of fluridone on lethal or sublethal endpoints in tests with *L. fullerkati*, suggesting that adult mussels were tolerant to the range of concentrations used over 28 d, and may be tolerant to seasonal exposures at 5 μg/L during treatment of hydrilla infestations. However, many other endpoints could be explored, and some may provide more insight into effects from chronic exposure. Relevant toxicological endpoints in sublethal studies of freshwater mussel sensitivity to other contaminants that may be applied in future fluridone and endothall studies include growth (in juveniles, Bringolf et al. 2007; Wang et al. 2007, 2011, 2013), glochidial metamorphosis success

(Hazelton et al. 2013), female mantle lure display (Bringolf et al. 2010; Hazelton et al. 2013; Leonard 2013), hemolymph and tissue analysis (Archambault et al. 2013; Leonard 2013), movement and burrowing (Flynn & Spellman 2009; Archambault et al. 2013; Hazelton et al. 2014), and metabolomics (Leonard 2013). We attempted to evaluate female mantle lure display in our experiment, but we had few females per replicate, thus there was insufficient statistical power to make sound inferences. We did note, however, that mussels in all treatments except for the highest concentration (300 μg/L) were periodically observed displaying mantle lures (stage 3 or higher, as per Bringolf et al. 2010). Our observations suggest that fluridone applied at a typically prescribed rate of 5 μg/L may not affect unionid mantle lure display. However, more statistically robust experimentation is needed to confirm a lack of effect, and to elucidate any other potential reproductive effects of fluridone.

The freshwater snail, *S. viriginicus*, was equally sensitive to the fluridone formulation Sonar – Genesis® as juvenile mussels were to the Sonar – PR® formulation (Table 1), and much more sensitive than other animals previously tested in commercial formulations of Sonar® (Hamelink et al. 1986; Paul et al. 1994), except for water mites (Yi et al. 2011) (Table 2). The reported acute values for Sonar – Genesis® were 1.8 mg/L (96-h LC50) for walleye and 3.6 mg/L (48-h EC50) for *Daphnia* (SePRO Corporation 2011), which are values 3.6 to 7.2-fold higher than the snail 96-h LC50. In experiments with *S. viriginicus*, both the 96 and 48-h post exposure LC50s were approximately two orders of magnitude higher than typical treatment concentrations recommended for hydrilla, and more than three times higher than the maximum label rate of application (SePRO Corporation 2010). Moreover, adult snails suffered no mortality in previous tests in our laboratory that had a maximum treatment concentration of 500 μg/L ((96-h LC50 > 500 μg/L), Archambault, Bergeron, and Cope, unpublished data). However, caution should be used in interpreting acute duration data, because slow-release or slow-acting herbicides like fluridone typically require extended exposure when treating hydrilla. Further, whole life cycle studies are especially important for *S. viriginicus* and other species that have an annual reproductive ecology, where the timing of hydrilla and other weed growth – and therefore herbicide treatment – coincides with egg laying, juvenile hatching and growth, and adult senescence. Because *S. viriginicus* adults die after reproduction (Johnson et al. 2013), negative effects to one cohort could result in further species decline.

In summary, we found that the fluridone and endothall concentrations typically recommended for hydrilla treatment were not acutely toxic to the freshwater molluscs tested in this study, and a 28-d exposure to fluridone was not lethal to adult mussels even at the highest concentration, indicating minimal risk of short-term effects to non-target species, including several protected and rare species. We also found that freshwater molluscs were more sensitive to fluridone and endothall than most other species previously tested. The mussels and snails studied here represent hundreds of highly imperilled freshwater gastropods and unionids, and our findings may signal their greater sensitivity to herbicides than other species commonly studied in aquatic toxicity testing (e.g., *Daphnia* spp., *Hyalella* spp., fathead minnows (*Pimephales promelas*)). Furthermore, chemicals like fluridone and endothall are sometimes used in combination to increase effectiveness against aquatic weeds, and are rarely the only chemicals present in surface waters (i.e., aquatic contaminants). They also are typically applied over a longer duration than our test exposures. Though fluridone and endothall have been used for aquatic weed management for decades, more research is needed to elucidate any potential risk to less-studied non-target taxa, including molluscs, especially given hydrilla's encroachment into

more systems across the country with high native biodiversity and endemism (e.g., Lake Waccamaw, Eno River). By providing a more thorough picture of the potential ecological risk associated with applying such herbicides for control of invasive aquatic weeds, resource managers can more confidently evaluate them as an option among other management choices (e.g., no treatment, grass carp control, and mechanical removal) and their associated risks. Topics warranting future study include acute exposures of endothall to snails; chronic exposures of juvenile mussels and snails to fluridone and endothall; evaluation of short- and long-term sublethal effects to juvenile and adult molluscs (e.g., reproduction, transformation success, growth, and biomarkers); indirect effects (e.g., effects on diet/food availability); whole life cycle exposures; and multi-stressor studies.

Acknowledgements

The authors received no directed funds to conduct this research, other than the support of their respective agencies and institutions. We thank Tom Fox and Jay Levine (Aquatic Epidemiology and Conservation Laboratory, North Carolina State University) for donating *Lampsilis fullerkati*, Haywood Perry (SePRO) for analyzing herbicide concentrations in test samples, Andrew Wilcox (Department of Statistics, North Carolina State University) for assistance with statistical analyses, and Cody Hale, Steve Hoyle, Justin Nawrocki, and Angela White for field assistance. Chris Barnhart (Mussel Culture Laboratory, Missouri State University) provided *Lampsilis siliquoidea*. North Carolina Division of Parks and Recreation permitted access to the Eno River for collection of *Somatogyrus virginicus*. The Lake Waccamaw Technical Advisory Committee provided context and impetus for this research.

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Pesticide Biochemistry and Physiology 121 (2015) 78–87

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Review

The global status of insect resistance to neonicotinoid insecticides

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ARTICLE INFO

Article history:
Received 2 February 2015
Accepted 9 April 2015
Available online 28 April 2015

Keywords:
Neonicotinoids
Imidacloprid
Nicotinic acetylcholine receptor
Resistance management
Resistance mechanisms
Sucking pests

ABSTRACT

The first neonicotinoid insecticide, imidacloprid, was launched in 1991. Today this class of insecticides comprises at least seven major compounds with a market share of more than 25% of total global insecticide sales. Neonicotinoid insecticides are highly selective agonists of insect nicotinic acetylcholine receptors and provide farmers with invaluable, highly effective tools against some of the world's most destructive crop pests. These include sucking pests such as aphids, whiteflies, and planthoppers, and also some coleopteran, dipteran and lepidopteran species. Although many insect species are still successfully controlled by neonicotinoids, their popularity has imposed a mounting selection pressure for resistance, and in several species resistance has now reached levels that compromise the efficacy of these insecticides. Research to understand the molecular basis of neonicotinoid resistance has revealed both target-site and metabolic mechanisms conferring resistance. For target-site resistance, field-evolved mutations have only been characterized in two aphid species. Metabolic resistance appears much more common, with the enhanced expression of one or more cytochrome P450s frequently reported in resistant strains. Despite the current scale of resistance, neonicotinoids remain a major component of many pest control programmes, and resistance management strategies, based on mode of action rotation, are of crucial importance in preventing resistance becoming more widespread. In this review we summarize the current status of neonicotinoid resistance, the biochemical and molecular mechanisms involved, and the implications for resistance management.

1. Neonicotinoid insecticides

Neonicotinoids are one of the most important chemical classes of insecticides globally due to their high efficacy against a range of important insect pests and their versatility of use [1,2]. They are registered in more than 120 countries worldwide [2] and are particularly active against numerous sucking pests, and also several coleopteran, dipteran, and lepidopteran pest species by foliar, soil and seed treatment applications [3]. Neonicotinoids are selective agonists of the insect nicotinic acetylcholine receptor (nAChR), a pentameric cys-loop ligand-gated ion channel located in the central nervous system of insects [1]. The mode of action classification scheme of the Insecticide Resistance Action Committee (IRAC) lists seven commercial neonicotinoids in Group 4A (nAChR agonists) (Sparks and Nauen, in this issue). The first neonicotinoid launched was imidacloprid in 1991, followed by nitenpyram and acetamiprid in 1995, and others such as thiamethoxam in 1998 (Fig. 1). Based on total global insecticide sales the market share of neonicotinoids was greater than 25% in 2014, with thiamethoxam, imidacloprid and clothianidin accounting for almost 85% of the total neonicotinoid sales in crop protection in 2012 (Fig. 2). The main regions of neonicotinoid use are Latin America, Asia and North America (75%), with Europe accounting for 11% of total global sales (Fig. 2). Increases in use have inevitably led to a mounting selection pressure for resistance to neonicotinoids. This review summarizes the global status of neonicotinoid resistance in a range of important insect pests with a particular focus on the biochemical and molecular mechanisms underlying resistance, and on information reported since the last comprehensive review of this subject published ten years ago [4].

2. Neonicotinoid resistance: from mechanisms to field failure

The first report of neonicotinoid resistance was published in 1996, describing low efficacy of imidacloprid against Spanish greenhouse populations of cotton whitefly, *Bemisia tabaci* [5]. Since then more than 500 peer-reviewed papers have been published on neonicotinoid resistance issues (SciFinder® 2014, American Chemical Society) in different pest insects (Fig. 3). A substantial proportion

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http://dx.doi.org/10.1016/j.pestbp.2015.04.004
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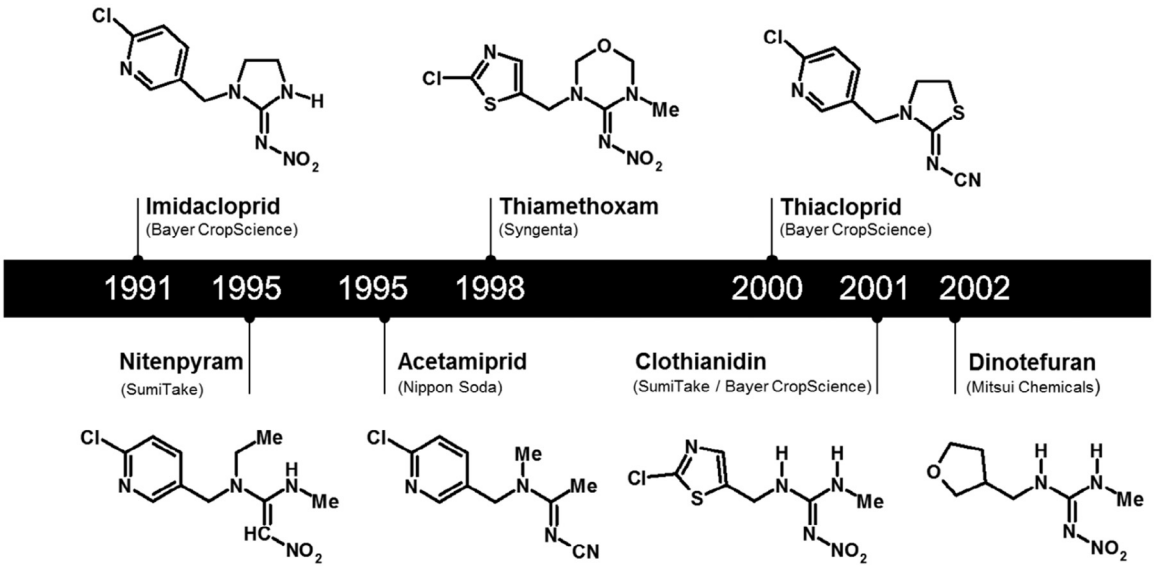

Fig. 1. Important neonicotinoid insecticides (manufacturers) and year of market introduction.

of these refer specifically to imidacloprid resistance. The Arthropod Pesticide Resistance Database (APRD) [6] lists more than 330 cases of imidacloprid resistance, followed by ca. 130 and 50 cases of thiamethoxam and acetamiprid resistance, respectively. Unsurprisingly, the number of arthropod species with resistance to neonicotinoids has increased with time (Fig. 4). However, most cases of neonicotinoid resistance (all compounds combined) concern *B. tabaci* followed by the green peach aphid, *Myzus persicae*, the cotton aphid, *Aphis gossypii*, and the rice brown planthopper, *Nilaparvata lugens*. Other pests targeted by neonicotinoid insecticides with at least 10 assigned cases of resistance in the APRD are houseflies, *Musca domestica*, Colorado potato beetle, *Leptinotarsa decemlineata* and glasshouse whitefly, *Trialeurodes vaporariorum* (Fig. 5). In the sections below we treat each of these seven species separately, but then combine others with fewer than 10 cases reported.

2.1. Bemisia tabaci

The cotton whitefly, *B. tabaci* (Gennadius) is a highly destructive and invasive sucking pest, damaging plants by direct feeding, honeydew excretion (as a nutritional source for sooty mold) and transmission of numerous plant viruses [7]. At least 24 cryptic and morphologically indistinguishable *B. tabaci* biotypes have been identified by recent phylogenetic comparisons based on DNA sequencing [8,9]. However, two widespread biotypes, the Middle East–Asia Minor 1 biotype (MEAM1, also referred to as biotype B) and the Mediterranean biotype (MED, also referred to as biotype Q), are of particular importance as crop pests [10]. Both biotypes have developed resistance to multiple classes of insecticide [11,12] including neonicotinoids [4]. Neonicotinoid resistance has been widely reported in both B and Q type *B. tabaci* from several geographic regions [4,12–19] particularly against imidacloprid. Resistance ratios for neonicotinoids in *B. tabaci* often exceed 1000-fold and lead to serious control failures [4].

Neonicotinoid resistance in *B. tabaci* is mainly conferred by enhanced detoxification by microsomal monooxygenases [17,20], and recently a single, constitutively overexpressed, cytochrome P450, CYP6CM1, was shown to be highly correlated with imidacloprid resistance in B- and Q-type whiteflies [21]. Functional expression of CYP6CM1 revealed its capacity to detoxify imidacloprid by hydroxylation of position 5 of the imidacloprid imidazolidine ring system [22], but also its inability to metabolize other neonicotinoids such as acetamiprid [23]. Resistance to imidacloprid in cotton whiteflies was shown to be age-specific [24] and correlated with the expression of CYP6CM1 in different life stages [25]. Recently it was shown that CYP6CM1 also detoxifies pymetrozine by hydroxylation, an insecticide with a different mode of action and chemically very different from neonicotinoids [26]. These results provided the molecular basis for the observed cross-resistance between neonicotinoids and pymetrozine in *B. tabaci* [27]. Transgenic lines of *Drosophila melanogaster* expressing CYP6CM1 were shown to be

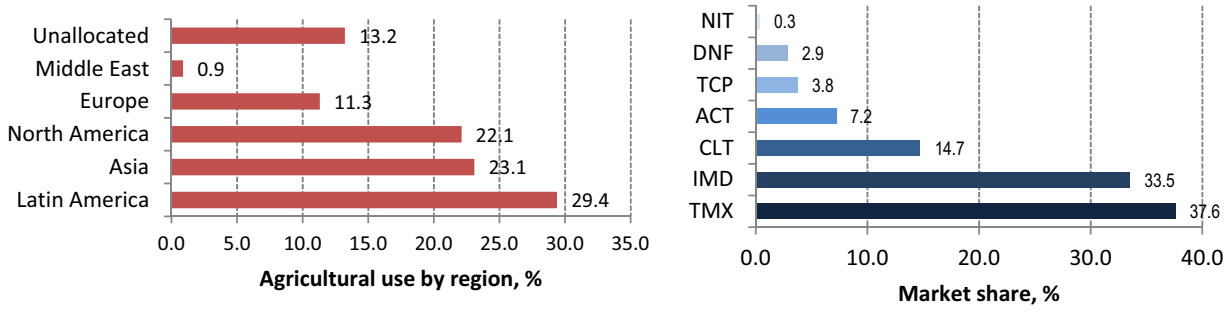

Fig. 2. Agricultural use by region and market share of individual neonicotinoids in percent (total market share 2012: 3.192 bn US$; Source: Wood Mackenzie). Abbreviations: TMX (thiamethoxam), IMD (imidacloprid), CLT (clothianidin), ACT (acetamiprid), TCP (thiacloprid), DNF (dinotefuran), NIT (nitenpyram).

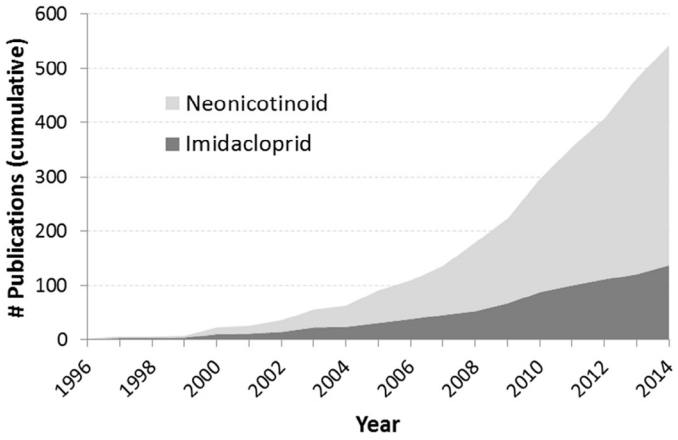

Fig. 3. Cumulative number of published peer-reviewed papers on resistance to neonicotinoids generally and to imidacloprid specifically.

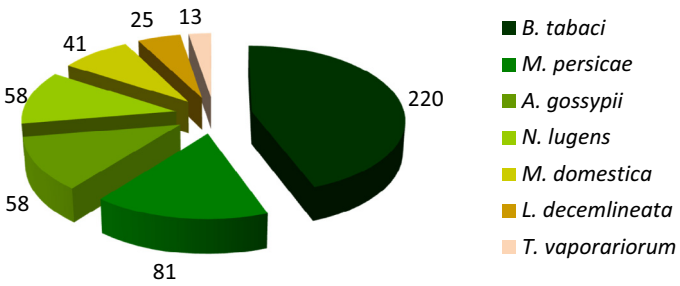

Fig. 5. Number of reported cases of neonicotinoid resistance up to 2014 (Arthropod Pesticide Resistance Database, Michigan State University). Only those pests with >10 reported cases are shown.

less susceptible to imidacloprid, providing further functional evidence of its role in imidacloprid resistance in *B. tabaci* [28]. Next generation sequencing (RNAseq) has provided further insights into the diversity of detoxification genes over-expressed in a *B. tabaci* strain resistant to neonicotinoid insecticides such as thiamethoxam [29]. Another study on thiamethoxam resistance in *B. tabaci* also revealed stage-specific expression of CYP6CM1, but also other detoxification enzymes such as glutathione S-transferases [30]. Even though other cytochrome P450s such as CYP4C64 have been reported to be over-expressed in neonicotinoid-resistant *B. tabaci*, the main P450 gene consistently over-expressed is *CYP6CM1* [31]. To date, no target-site mutations in *B. tabaci* nAChR subunits have been described.

2.2. Myzus persicae

The green peach aphid, *M. persicae* (Sulzer), is the most economically important aphid crop pest worldwide. Unlike other species in which differences in response to neonicotinoids emerged several years after first exposure to these compounds, low but statistically-significant variation in susceptibility to imidacloprid in *M. persicae* was reported in tandem with the first commercial releases of this insecticide [32,33]. Suspicions that such variation was a by-product of tolerance to nicotine, selected during the adaption of some populations of *M. persicae* (so-called *M. persicae* subsp. *nicotianae*) to feeding on tobacco, have been reinforced by research attributing resistance to over-production of a single P450 (CYP6CY3) [34,35]. Survival following exposure to discriminating concentrations of nicotine (and neonicotinoids) for a range of aphid clones from the UK, Greece, southern Africa and Japan was closely and positively correlated with levels of CYP6CY3 mRNA expression [34,35]. Expression of recombinant CYP6CY3 enzyme in Sf9 insect cells showed it to be highly efficient at metabolizing nicotine and two neonicotinoids – imidacloprid and clothianidin – to less toxic metabolites [34]. Overexpression appears attributable both to a modification of the promoter region and to structural amplification of the CYP6CY3 gene, with some clones possessing up to 100 copies. Thus, in contrast to the usual case of resistance traits being selected *de novo* by chemicals used for aphid control, this appears to be a rare example of pre-selection resulting from host–plant adaptation and an expansion in host range [34]. At present it is unclear to what extent CYP6CY3-mediated resistance occurs in or has spread to non-tobacco-adapted *M. persicae* as a consequence of gene flow between races, or as a result of subsequent selection by neonicotinoids themselves.

The microarray study that initially implicated CYP6CY3 in resistance also showed a number of ESTs encoding cuticular proteins to be up-regulated in a resistant clone, suggesting that modified penetration through the cuticle might be operating in concert with enhanced detoxification to determine the resistance phenotype [35]. Further evidence for an additional mechanism in clones overexpressing CYP6CY3 came from incomplete suppression of resistance by enzyme inhibitors [36], the differential expression of resistance in feeding and contact bioassays [35], and *in vivo* penetration assays with radiolabelled imidacloprid [35]. However,

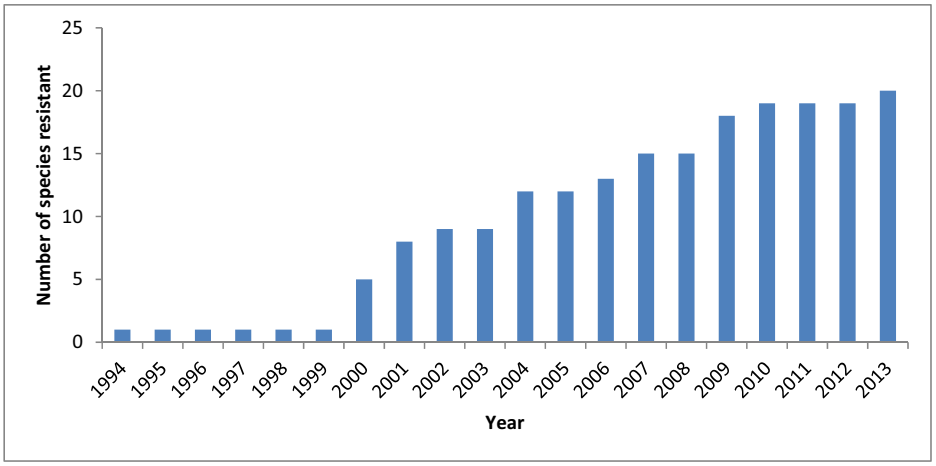

Fig. 4. Cumulative number of arthropod species with neonicotinoid resistance (Arthropod Pesticide Resistance Database, Michigan State University).

without an unambiguous marker for a mechanism based on reduced penetration it has not been possible to quantify its importance and contribution to resistance, singly or alongside different levels of overexpression of CYP6CY3.

Receptor radioligand binding studies and nucleotide sequencing of nAChR subunit genes have also been undertaken to explore the possible occurrence of target-site resistance to neonicotinoids in *M. persicae*. These yielded negative results until a clone (termed FRC) was collected in 2009 from peach at a site experiencing a marked loss of control efficacy with neonicotinoids [37]. Resistance in FRC was markedly more resistant than any clone studied previously. In topical application bioassays with imidacloprid and thiamethoxam, resistance was impossible to quantify due to survival at the highest doses it was feasible to apply [37]. CYP6CY3 was overexpressed in FRC at levels similar to those in resistant clones studied previously, but in addition, sequencing of nAChR subunit genes identified a point mutation in the loop D region of the β1 subunit that causes an arginine to threonine substitution (R81T). Loop D of β1 has a known role in binding of the natural ligand acetylcholine and of synthetic neonicotinoids [38] and the R81 residue specifically has been shown through homology modelling to modulate neonicotinoid binding [39]. Indeed, the presence of threonine at this residue in most vertebrate receptors compared to the ubiquity of arginine in insects is considered a primary determinant of the selective toxicity of neonicotinoids. Hence it seems unequivocal that R81T is directly implicated in conferring a level of neonicotinoid resistance unrecorded previously in *M. persicae*. Its discovery represented the first proven case of a target-site modification leading to control failure with neonicotinoids under field conditions.

Using a PCR-based diagnostics the current distribution of the R81T mutation has been shown to extend in a band from southern Spain, through southern France to northern and Central Italy [40,41]. This distribution remains closely coincident with the cultivation of peach and closely-related crops. Extensive monitoring has failed to detect its presence further north in Europe despite continuing and extensive reliance on neonicotinoids for aphid control in countries such as the UK (S. Foster, pers. comm. 2014). It seems likely that the transition from holocycly in the south of Europe to obligate anholocycly in the north is constraining the ability of the mutation to spread from its point of origin and/or establish in new localities. This is being investigated further.

2.3. Aphis gossypii

Like *M. persicae*, the cotton-melon aphid, *A. gossypii* (Glover) is highly polyphagous with a long history of resistance to insecticides. Its host plants, which include cucurbits, cotton and solanaceous crops, are often intensively treated with neonicotinoids and resistance to these products, although only confirmed relatively recently, now appears to be geographically widespread. Systematic monitoring of aphids on cotton in Australia and the USA has documented a temporal decline in sensitivity related to increased reliance on neonicotinoids as seed treatments and foliar sprays [42,43]. Discriminating concentration assays complemented by full dose–response testing of insects from Australian cotton showed a gradual change from 2006–7 to 2008–9, with resistance factors in the latter season peaking at 6.4-fold for acetamiprid, 22-fold for thiamethoxam and 6-fold for clothianidin, respectively [43]. This trend continued in 2009–2010 when 96% of samples contained resistant individuals [43]. To combat this trend there are recommendations to avoid foliar sprays of neonicotinoids against *A. gossypii* but these are compromised by the continuing importance of neonicotinoids for controlling other pests including whiteflies and mirids [43].

Monitoring of *A. gossypii* between 2008 and 2011 from cotton-growing regions of the southern USA that were reporting diminished efficacy of neonicotinoids showed a 48-fold range of LC_{50} values for thiamethoxam across the four years, with resistance tending to be higher for fields that had received at least one foliar application of a neonicotinoid insecticide [42]. Interestingly, resistance factors were much higher after 48 h exposure in a leaf-dip bioassay than after 72 h, although the broad association between resistance and field treatment history was evident at both endpoints.

The mechanism(s) underpinning resistance in Australia and the USA remain to be elucidated, whereas in eastern Asia there is mounting evidence for the same target-site R81T amino acid substitution as found in *M. persicae*. Samples of *A. gossypii* collected from six sites in South Korea in 2012 gave maximum resistance of 1500-fold to imidacloprid, 2600-fold to acetamiprid and 14,000-fold to clothianidin [44]. Even more remarkably, laboratory selection with imidacloprid of a strain (IMI-R) collected in 2011 led to resistance factors of 36,000 to imidacloprid, 69,000 to acetamiprid, and 285,000 to thiacloprid [44]. Bioassays using synergists and enzyme assays yielded no evidence of enhanced detoxification in IMI-R compared to a susceptible strain, whereas full length cloning showed R81T to be present in the β1 nAChR subunit of IMI-R and five of the field samples collected in 2012. Sixty generations of laboratory selection with imidacloprid of an originally susceptible strain collected in Shandong province in China in 2009 resulted in 66-fold resistance to this compound [45]. Cloning of six α and the β1 subunits again showed R81T to be present in the latter.

One notable discrepancy between these two studies suggesting R81T to be the primary sole cause of neonicotinoid resistance is in the magnitude of resistance factors: up to 36,000-fold for imidacloprid in Korea but only 66-fold in the selected strain from China. One explanation might be the different bioassay methods utilized: dipping of leaves and apterous aphids in test solutions by Shi et al. [45], and placing untreated aphids on previously dipped and dried leaves by Koo et al. [44]. Side-by-side testing using both methods would be valuable for disclosing the importance of the route of exposure in influencing the phenotypic expression of resistance traits, as already documented when comparing systemic and topical application methods for *M. persicae* [46]. The parallel appearance of R81T in *M. persicae* and *A. gossypii* is of evolutionary significance, highlighting again the limited scope for target-site mutations that confer appreciable resistance while retaining normal receptor function.

2.4. Nilaparvata lugens

The brown planthopper, *N. lugens* (Stål), is the most economically significant pest of rice (*Oryza sativa* L.) throughout Asia, causing damage through direct feeding and the transmission of rice viruses [47]. The control of *N. lugens* has relied heavily on the use of synthetic insecticides with resistance developing to all of the older compounds used for control [48]. The first neonicotinoid, imidacloprid, was introduced against *N. lugens* in the early 1990s and because of its excellent efficacy and the fact that it was largely unaffected by resistance that had evolved to older compounds rapidly became a mainstay for control. After a decade of use populations of *N. lugens* were reported with reduced efficacy/resistance to imidacloprid, and resistance is now widespread in populations collected from across Asia with resistance factors of 600–800-fold recently described [48–52].

The first mechanism of resistance to neonicotinoids reported for *N. lugens* involved a target-site modification [53] with a strain of *N. lugens* selected with imidacloprid for 35 generations exhibiting over 250-fold resistance compared to a lab susceptible strain in insecticide bioassays. Radioligand binding experiments to whole body membrane preparations revealed a significant lower level of [^{3}H]imidacloprid-specific binding to preparations of the resistant strain suggesting a target-site resistance mechanism [53]. Sequenc-

ing of nAChR subunit genes identified a single point mutation at a conserved position (Y151S) in two nAChR subunits, Nlα1 and Nlα3, with confirmation of the causative effect of these mutations coming from expression of hybrid nAChRs containing *N. lugens* α and rat β2 subunits, with the presence of Y151S associated with a substantial reduction in specific [^{3}H]imidacloprid binding [53]. Surprisingly, since these findings were reported, this mechanism has never been identified in any field-collected population. Rather, several studies have provided both indirect and direct evidence that enhanced P450 activity contributes to the neonicotinoid resistance of field collected populations of *N. lugens* throughout Asia [4,54,55]. Use of the metabolic enzyme inhibitor piperonyl butoxide (PBO) and the model substrate 7-ethoxycoumarin were initially used to implicate P450-mediated detoxification in resistance [54,56]. However, more recently, molecular studies have identified the overexpression of two possible P450 enzymes with imidacloprid resistance in lab and field populations. The first of these, CYP6ER1, was identified as the only member of 32 tentative unique P450s annotated from two recent sequencing projects as highly overexpressed (up to 40-fold) by quantitative RT-PCR in a range of resistant strains, with the level of expression observed in the different strains significantly correlated with the resistance phenotype [57]. The second P450, CYP6AY1, was one of six genes identified by quantitative RT-PCR as significantly overexpressed (~18-fold) in a laboratory strain selected with imidacloprid for 40 generations [58]. This P450 was also overexpressed in four field strains (4–9-fold) compared to a susceptible strain [58]. This finding was surprising as CYP6AY1 was down-regulated (or neutrally expressed) in the resistant strains compared to the susceptible strain examined in the study by Bass et al. [57]. Nevertheless, functional expression of CYP6AY1 and RNAi experiments provided evidence that CYP6AY1 has the capacity to metabolize imidacloprid to 4/5-hydroxy-imidacloprid and confer resistance [58]. More recently polymorphisms in the promoter of CYP6AY1 were identified between a resistant field-collected and lab susceptible strain that were shown to enhance promoter activity in reporter gene assays and may be acting as cis-acting factors to enhance the expression of CYP6AY1 [59]. Further work is required to elucidate the relative contribution of CYP6ER1 and CYP6AY1 in the imidacloprid resistance of *N. lugens* populations across Asia.

2.5. Musca domestica

The housefly, *M. domestica* L., is a passive vector for a range of debilitating human and animal diseases and is consequently an important pest on animal farms across the world. Like the other pest species highlighted in this review, effective control is often reliant on the use of pesticides and houseflies have similarly proved highly adept at developing resistance, with reports of over 60 different compounds now listed in the APRD [6]. Neonicotinoids, primarily imidacloprid and thiamethoxam, are effective against a range of public hygiene pests and have been used as feeding baits and in spray applications to control houseflies in animal facilities for a number of years [60]. Early studies showed good efficacy of imidacloprid against laboratory strains carrying resistance to other insecticide classes [61] and initial monitoring of field populations prior to the introduction of neonicotinoids for housefly control confirmed only limited variation in their response [62,63]. Recent studies have, however, revealed more significant resistance in field collected populations from several parts of the world, including the U.S. [64], Europe [65,66], Pakistan [67] and China [68], with further laboratory selection of these strains resulting in resistance factors for imidacloprid ranging from 100-fold [66] to over 2000-fold [69].

Attempts to investigate the underlying mechanisms of resistance in these strains have implicated possible roles for both metabolic enzymes and target site modification, but have yet to unambiguously assign the metabolic activity to a specific enzyme or identify the exact target alteration(s) responsible. For example, both imidacloprid and thiamethoxam resistance in field-collected strains from Denmark was partly synergized by treatment with the cytochrome P450 inhibitor, PBO [66] and this was correlated with increased expression of several P450 genes (*CYP6A1*, *CYP6D1*, *CYP6D3*, *CYP6G4*) after neonicotinoid exposure [66,70]. However, as yet none of these genes have been functionally expressed and shown conclusively to metabolize these compounds. The metabolic resistance was accompanied by an apparent 60% reduction in the expression level of the α2 nicotinic acetylcholine receptor subunit (Mdα2) in the same resistant strains and was suggested as a possible additional mechanism that contributes to their reduced sensitivity [71], although it should be pointed out that no other nicotinic subunits were investigated for either altered expression or target site modification in this study.

Interestingly, the high level of imidacloprid resistance (2300-fold) selected from a Florida field strain was not synergizable by PBO [69], suggesting a possible target site alteration similar to that described in aphids. This resistance was mapped to autosomes 3 and 4, both of which carry nicotinic acetylcholine receptor subunit genes, and would therefore seem to be a fruitful area for further investigation. The publication of a full genome sequence for *M. domestica* [72] offers new opportunities for a more detailed characterization of nAChR genes in this and other resistant strains, and should facilitate a clearer understanding of the molecular basis of resistance in this species.

2.6. Leptinotarsa decemlineata

The Colorado potato beetle, *L. decemlineata* (Say), is a serious pest of potatoes and other solanaceous crops, particularly in North America and Europe. This species has gained notoriety for rapidly developing resistance to almost all of the insecticides used for its control [6]. The neonicotinoid imidacloprid was first introduced for *L. decemlineata* control in Northern America in 1995. Widespread monitoring of imidacloprid susceptibility in populations from North America and Europe collected over 1995–1998 revealed up to 29-fold variation in response [73]. Much of this variation was not a result of selection from imidacloprid use per se, as most of the populations assayed were never exposed to this compound, but was likely a consequence of cross-resistance from chemicals used earlier. The least sensitive strains described in this study came from Long Island, New York, an area with a history of intensive insecticide use against *L. decemlineata* [73]. In support of this finding a report published in the same year described 100-fold levels of resistance to imidacloprid in adults of an *L. decemlineata* population collected as early as 1997 from an imidacloprid-treated commercial potato field [74]. Subsequent monitoring of samples from Long Island has reported further increases in resistance to imidacloprid (309-fold) with lower levels of cross-resistance also observed to dinotefuran, clothianidin, acetamiprid, thiacloprid, thiamethoxam, and nitenpyram, despite these never having been used in the field up to this point [75].

The precise mechanism(s) underlying neonicotinoid resistance in *L. decemlineata* have not been fully characterized; however, several studies have advanced our understanding of the possible mechanisms involved. Two studies of resistant strains from Long Island using insecticide synergists have suggested that P450-mediated detoxification plays a significant role in resistance, with esterases possibly also involved; however, the fact that enzyme inhibitors did not completely eliminate resistance in resistant strains suggests additional mechanisms may be involved [74,75]. In contrast to these findings pharmacokinetic experiments with other strains of *L. decemlineata* showed no significant difference in *in vivo* metabolism of radiolabelled imidacloprid [76]. The potential role of target-site modification in the neonicotinoid resistance of *L. decemlineata*

has also been explored using binding assays with tritiated imidacloprid. Initial results failed to reveal differences in imidacloprid affinity to nAChRs from head membrane preparations of neonicotinoid-resistant and susceptible beetles (Nauen et al., unpublished). Further work has compared the neural activity of imidacloprid on the spontaneous activity of a motor nerve leaving the isolated central nervous system of susceptible and resistant beetles [77]. Although no differences were seen in the sensitivity of the central nervous system of resistant and susceptible beetles to excitation by imidacloprid, significant reductions in the sensitivity of CNS preparations of the resistant strain to inhibition by imidacloprid were observed, suggestive of a possible change in the sensitivity of at least one subgroup of nAChRs [77]. Although the origin of the decreased sensitivity to block neural activity by imidacloprid in the resistant beetles requires further characterization, it is likely that it relates to the observed resistance to imidacloprid.

2.7. Trialeurodes vaporariorum

The glasshouse whitefly, *T. vaporariorum* (Westwood), is an economically important pest of protected vegetable and ornamental crops in most temperate regions of the world. As for many of the other pests detailed in this review resistance of this species to a range of older insecticide classes, such as the pyrethroids and organophosphates [78], led to the increasing reliance on neonicotinoid insecticides for control after their introduction. The first cases of neonicotinoid resistance were reported in *T. vaporariorum* strains collected in 2004/2005 from the United Kingdom, The Netherlands and the U.S. [79,80]. More recent work has described neonicotinoid resistance in *T. vaporariorum* strains from the UK, Turkey, Spain, China, Germany [81] and Greece [82] with reduced susceptibility to imidacloprid also reported in strains from Finland [83]. Taken together these results suggest resistance to neonicotinoids in *T. vaporariorum* may now be widespread in global populations.

Interestingly, neonicotinoid resistance in *T. vaporariorum* shows several parallels with that of the tobacco whitefly *B. tabaci*. Cross-resistance bioassays and selection experiments revealed a clear correlation in the observed responses of *T. vaporariorum* to neonicotinoids and pymetrozine, strongly suggestive of cross-resistance between the two classes [81]. Furthermore, resistance to the neonicotinoid imidacloprid and pymetrozine was shown to be age-specific, with resistance in nymphs failing to compromise recommended application rates [81]. Taken together these results suggest a similar mechanism may underlie resistance in *B. tabaci* and *T. vaporariorum*. As detailed above, resistance to both imidacloprid and pymetrozine in *B. tabaci* results from enhanced expression of the P450 CYP6CM1. Recent sequencing of the transcriptome of *T. vaporariorum* has allowed the identification of several P450 genes (*CYP6CM2*, *CYP6CM3*, *CYP6CM4*) that share significant homology with *B. tabaci CYP6CM1* and therefore represents candidates for a potential role in resistance in *T. vaporariorum* [84].

2.8. Other pests

Neonicotinoid resistance has also been reported in several other insect pest species in addition to those listed above and it is beyond the scope of this review to provide an exhaustive list, nevertheless, in some cases multiple reports of resistance have suggested a growing resistance problem for certain species and these are summarized below.

The white-backed planthopper, *Sogatella furcifera* (Horvath), and small brown planthopper, *Laodelphax striatellus* (Fallén), are two important pests of rice in Asia. Screening for imidacloprid resistance in *S. furcifera* populations collected in 2006 from East and South-East Asia revealed that, in contrast to *N. lugens*, most populations displayed full sensitivity to this compound [85]. However, in the same study the first evidence of field resistance was detected in a single population from Japan. More recent monitoring of field populations of *S. furcifera* in China has suggested resistance has since become more widespread with ~30% of populations collected from 2010 to 2013 showing moderate resistance (<15-fold) to imidacloprid [86,87]. Despite these findings all populations tested remained susceptible to thiamethoxam [86,87]. Initial monitoring of the sensitivity of *L. striatellus* populations in China found high levels of resistance to imidacloprid in strains collected from Jiangsu province suggestive of a local hotspot of resistance [88]. However, more recent monitoring of populations in China (including from Jiangsu province) found that all populations collected from 2011 to 2013 were susceptible to both imidacloprid and thiamethoxam [87].

The Asian citrus psyllid, *Diaphorina citri* (Kuwayama), is one of the most economically important pests of citrus worldwide, primarily due to its status as a vector of citrus greening disease. Monitoring of populations of this pest in Florida collected in 2009/2010, where it is a significant problem to citrus growers, revealed reduced sensitivity in certain populations to imidacloprid and thiamethoxam, with 35- and 13-fold resistance to the two compounds respectively observed in the most resistant strain [89]. These findings suggested neonicotinoid/insecticide resistance may be becoming an emerging problem in this species in Florida; however, more recent monitoring has revealed, in contrast to other insecticide classes, a slight decrease in resistance to neonicotinoids [90]. Beyond Florida monitoring of *D. citri* populations collected from lime orchards in Central West Mexico has recently revealed widespread, mostly moderate, resistance (<25-fold) to both imidacloprid and thiamethoxam [91]. However, a strain collected from one site (Apatzingan, Michoacan) displayed extremely high resistance to imidacloprid (>4000-fold) suggesting the emergence of more potent resistance in this area [91].

The codling moth, *Cydia pomonella* L., is a major pest of pome fruit worldwide. The N-cyano-imino neonicotinoids thiacloprid and acetamiprid are relatively effective for codling moth control and have been widely adopted since their introduction. Resistance to both compounds has been reported in *C. pomonella* populations from Europe [92,93], the U.S. [94] and Argentina [95], with low level resistance to thiacloprid also reported in populations from Canada [96]. Surprisingly, resistance to thiacloprid in Europe has been observed in countries/regions prior to their use by growers and this is associated with cross-resistance with older compounds. A similar phenomenon has also been reported for acetamiprid with resistance to this compound correlated with levels of azinphos-methyl resistance in populations from the U.S. [94]. Both of these cases are suggestive of an underlying metabolic resistance mechanism that confers broad cross-resistance to a range of compounds. In relation to this several studies have also reported enhanced activity of detoxification enzymes, including P450s, glutathione-S-transferases and esterases, to be correlated with resistance in biochemical assays [92,93,97]. However, to date, the precise enzymes involved in neonicotinoid resistance have not been characterized.

Western flower thrips, *Frankliniella occidentalis* (Pergande), is a major insect pest of several vegetable, fruit and ornamental crops. The first report of resistance of this species to neonicotinoids was in a laboratory strain originating from the United States which displayed moderate resistance to imidacloprid (RR 14-fold) [98]. Interestingly imidacloprid had not been used against this species at this time and therefore the observed resistance was almost certainly a result of cross-resistance from older insecticides [98]. More recent work has reported resistance to both imidacloprid and acetamiprid in strains of *F. occidentalis* originating from Japan and China [99]. Synergism bioassays using the metabolic enzyme inhibitor piperonyl butoxide (PBO) suggested that metabolism by P450s may be involved in acetamiprid resistance in these strains, and cloning

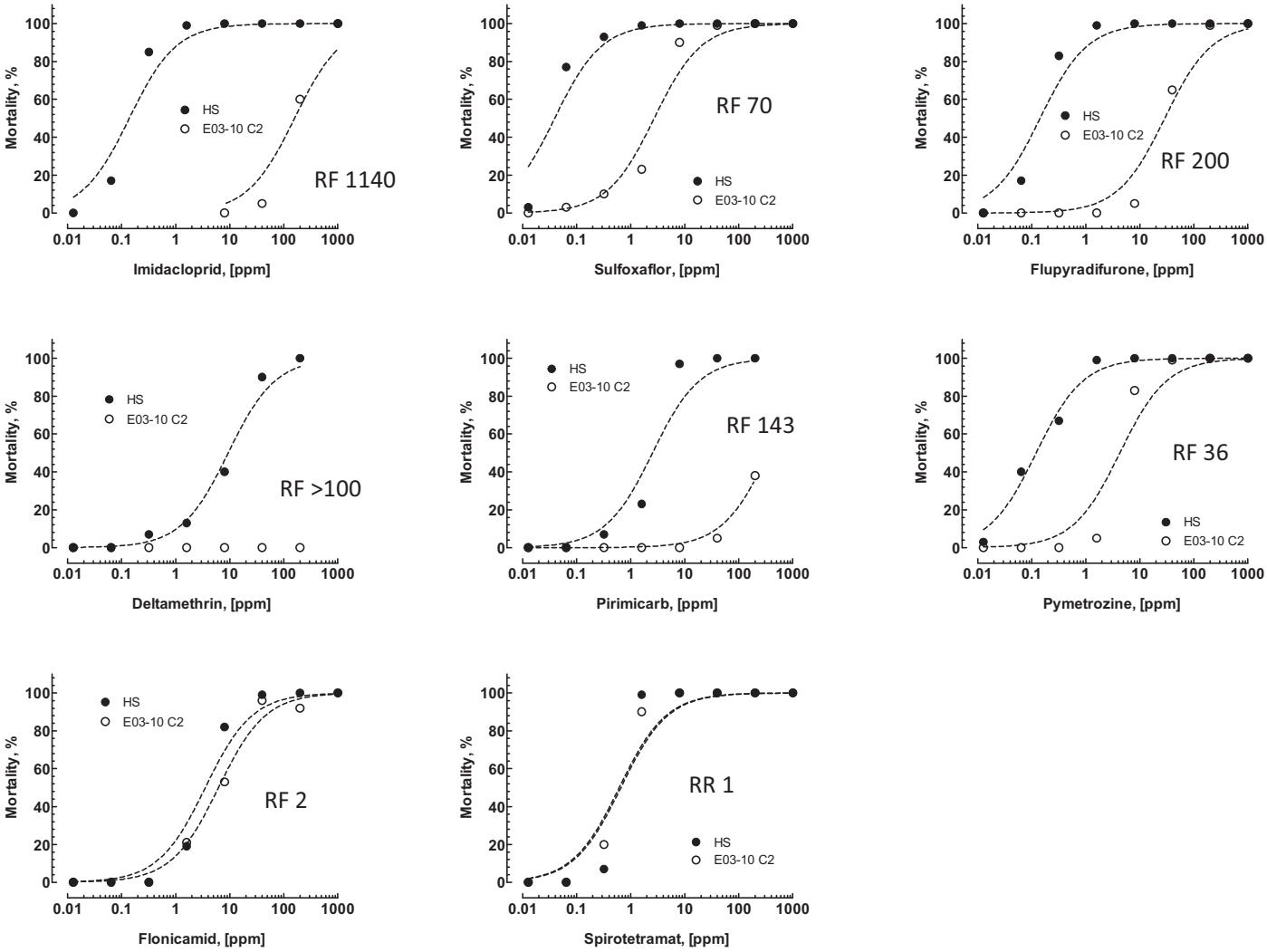

Fig. 6. Dose–response curves for different insecticides against 3rd instar nymphs of *Myzus persicae* in leaf-dip bioassays (72 h). Strain HS is susceptible to insecticides, whereas clone E03-10 C2 is derived from a field strain collected in Spain in 2010 and homozygous for the R81T mutation in the ß1-subunit of the nAChR, conferring cross-resistance to neonicotinoids, sulfoxaflor and flupyradifurone. This clone also carries mutations in AChE (MACE) and voltage-gated sodium channel (kdr/skdr). RF refers to the resistance factor (EC_{50}-value obtained for clone E03-10 C2 divided by the EC_{50}-value of strain HS).

and sequencing of nicotinic acetylcholine receptor (nAChR) subunits provided no evidence of a target-site mechanism [99]. Finally, modest levels of resistance to thiamethoxam (15-fold) were also recently reported in a strain of *F. occidentalis* selected in the laboratory with this compound for 55 generations [100]. Interestingly this strain showed high levels of cross-resistance to the neonicotinoid imidaclothiz (392.1-fold) but no or very low cross-resistance to the neonicotinoids imidacloprid, acetamiprid, dinotefuran and nitenpyram. This finding might be explained by a metabolic resistance mechanism that exhibits substrate preference for 2-chloro-1,3-thiazol-5-ylmethyl neonicotinoids such as thiamethoxam and imidaclothiz. In this regard thiamethoxam efficacy against the resistant strains was synergized by PBO and triphenyl phosphate (TPP), and biochemical assays showed modest increases in monooxygenase and carboxylesterase activity, suggesting a possible involvement of these enzyme systems in resistance [100].

3. Implications and conclusions

It is no coincidence that most species exhibiting economically-significant resistance to neonicotinoids are ones that have gained notoriety for resistance to a broad range of other insecticide groups. The same agronomic and biological traits that have predisposed them to resist older products must also underpin the evolution of resistance to neonicotinoids. This propensity for accumulating multiple resistance greatly constrains the implementation of approaches recommended for combating resistance in general [101] and to neonicotinoids specifically [5,102]. The most widely advocated tactic for managing resistance, other than the obvious one of minimizing reliance on chemicals per se, is the alternation of groups with different modes of action to avoid continuous selection for the same resistance mechanism(s). In the above cases, a lack of effective alternatives combined with the unprecedented versatility of neonicotinoids has led to intensive use of these compounds and enhanced the risk of resistance developing [4,103]. Bioassay results for several insecticides tested against a multi-resistant Spanish strain of the aphid *M. persicae* (Fig. 6) exemplify well how the accumulation of resistance mechanisms can deplete the supply of compounds available for alternation schemes. The appearance of strong resistance to imidacloprid caused by the R81T target-site mutation (see above) in a genetic background already containing mechanisms conferring target-site insensitivity to the carbamate pirimicarb and synthetic pyrethroids [104] results in only two of the tested products (flonicamid and spirotetramat) retaining high

levels of activity against this strain. Interestingly this field-collected strain also shows moderate resistance to pymetrozine (IRAC subgroup 9B), but not flonicamid (subgroup 9C). Both insecticides are known to act as modulators of chordotonal organs (IRAC main group 9), but are chemically different.

One of the major limitations to resistance management is the occurrence of cross-resistance. Insect pests very rarely resist just one compound; resistance mechanisms commonly encompass most or all chemicals within a particular mode-of-action group and can, much less predictably, affect other groups as well. The literature reviewed above contains numerous cases of resistance initially reported to one neonicotinoid being found through bioassays to extend to other compounds in this class. The magnitude of resistance factors to different molecules may vary considerably, presumably as a consequence of differences in the substrate specificity of detoxifying enzymes. However, based on the collective results of work so far it is impossible to identify consistent and exploitable patterns of cross-resistance across commercially-available neonicotinoids. Recommendations advanced previously [102,103], reinforced by a common IRAC mode of action classification (Group 4A) (Sparks and Nauen, in this issue), to treat the seven commercial neonicotinoids as a single group for resistance management purposes unquestionably remain appropriate when designing insecticide alternation strategies.

Interesting questions about cross-resistance arise with the introduction of new molecules targeting the same site as ones developed previously, but considered to display unique properties that distinguish them from predecessors. The sulfoximine, sulfoxaflor [105], and the butenolide, flupyradifurone [106], are unquestionably nAChR agonists but structurally distinct from neonicotinoids and thus have been placed in new subgroups (4C and 4D, respectively) in the IRAC classification scheme. This distinction is supported by data showing that aphids and whiteflies with metabolic resistance to imidacloprid and other conventional neonicotinoids remain almost fully susceptible to sulfoxaflor and flupyradifurone [105–107]. However, a strain of *M. persicae* with the still geographically-restricted R81T mutation showed appreciable resistance to both of these new compounds (Fig. 6). Thus, anticipating risks of cross-resistance involving novel members of a broad mode-of-action group requires caution as these risks can be mechanism-specific.

The predominance (so far) of enhanced metabolism, as opposed to target-site modification, as a cause of resistance to neonicotinoids increases the possibility of resistance extending to compounds with contrasting modes of action. The best documented example to date is cross-resistance between neonicotinoids and the azomethine pymetrozine in the whiteflies *B. tabaci* [27] and *T. vaporariorum* [81]. Examples of species showing variation in response to neonicotinoids at the time of their introduction can raise suspicions of resistance pre-selected by earlier used groups [73], although the exact nature of such cross-resistance remains to be investigated.

Since the last comprehensive review of this subject [4], there have been additional pest species acquiring neonicotinoid resistance, and changes in the extent and severity of cases of resistance already documented ten years ago. Most notably, there has been significant progress with characterizing the genetic and molecular basis of resistance mechanisms, providing exciting evolutionary insights and also techniques for rapid diagnosis and monitoring of resistance genotypes. These achievements can contribute not only to tracking and helping to contain known cases of resistance but also to anticipating the emergence and nature of new resistance outbreaks.

Acknowledgments

We thank past and present scientists who have worked on neonicotinoid resistance and apologize that, due to space constraints, we have not been able to cite all the research on this important topic. Rothamsted Research receives grant aided support from the Biotechnology and Biological Sciences Research Council of the UK.

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Pesticides reduce regional biodiversity of stream invertebrates

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Edited by David Pimentel, Cornell University, Ithaca, NY, and accepted by the Editorial Board May 13, 2013 (received for review March 25, 2013)

The biodiversity crisis is one of the greatest challenges facing humanity, but our understanding of the drivers remains limited. Thus, after decades of studies and regulation efforts, it remains unknown whether to what degree and at what concentrations modern agricultural pesticides cause regional-scale species losses. We analyzed the effects of pesticides on the regional taxa richness of stream invertebrates in Europe (Germany and France) and Australia (southern Victoria). Pesticides caused statistically significant effects on both the species and family richness in both regions, with losses in taxa up to 42% of the recorded taxonomic pools. Furthermore, the effects in Europe were detected at concentrations that current legislation considers environmentally protective. Thus, the current ecological risk assessment of pesticides falls short of protecting biodiversity, and new approaches linking ecology and ecotoxicology are needed.

environmental impacts | environmental risk assessment | plant protection products | macroinvertebrates | spatial scale

The losses of biodiversity caused by anthropogenic activities during the past 50 y are unprecedented in human history (1). Despite general concern and several international initiatives (2–4), the current rate of biodiversity loss appears to be accelerating rather than slowing (5, 6). The future consequences of this crisis may be dramatic, as the latest analyses show that a planetary-scale ecosystem shift to an unknown and irreversible state may occur (7).

To date, no unequivocal link has been established between the measured exposure (i.e., the concentration of toxicants in the environment) and quantitative measures of regional biodiversity (i.e., the regional taxonomic richness pool). The only exceptions are two studies that addressed effects of salinity (8, 9). Hence, although chemical contaminants are well known as an important driver for biodiversity loss (1, 10–28), there is scarce empirical evidence to support such opinion for the large-scale taxonomic pools.

This problem holds true even for agricultural pesticides, which are among the best ecotoxicologically characterized and regulated groups of contaminants. Essentially, it remains unknown whether, to what degree, and at what concentrations pesticides cause the species losses at the regional scale. However, there are many investigations showing the effects on the local biodiversity-related parameters in both freshwater (16–23) and terrestrial systems (14, 15, 24–27). Thus, the previous studies with freshwater invertebrates reliably measured the aquatic pesticide concentrations and identified local (site-scale) changes in the abundance of the taxa specifically vulnerable to pesticides and structural community alterations, e.g., using the species-at-risk $(SPEAR)_{pesticides}$ indicator (16–21), or the abundance of separate species (22) (for different taxonomic groups, see ref. 23). Similarly, numerous investigations in the agroecosystems revealed various effects of pesticides on the terrestrial arthropod communities and their local biodiversity metrics (e.g., site- or farm-scale taxonomic richness; 14, 15, 24–27). Most of these impacts detected in both freshwaters and agroecosystems have a clear potential to propagate to alterations of the large-scale taxonomic pools, i.e., regional biodiversity (14, 15, 21), but such effects on the regional scale remained to be proven and quantified empirically.

A fundamental measure of biodiversity is taxa richness, i.e., the number of taxa inhabiting a certain region or a set of sites. Despite its simplicity, taxa richness is an elusive quantity, as it is strictly dependent on the sampling effort and abundance: as more individuals and samples are collected, more species will be recorded (29, 30). Therefore, taxonomic richness can only be reliably measured using taxa accumulation or rarefaction curves. Such curves represent a relationship between the number of samples or individuals and the number of taxa recorded (29) (Fig. 1).

Recently, the term "contaminant category richness" was introduced by Kefford et al. (9) to describe the taxa richness of stream invertebrates peculiar to different water salinity levels and quantified by rarefaction curves (8, 9). This richness is conceptually similar to richness in latitudinal, altitudinal, or marine barometric zones (31) and reflects the taxonomic pool of a large set of sites having a certain contamination level (9). The contaminant category richness is a measure of the regional richness constrained by the contamination level, and essentially, it represents the split of the regional taxonomic pool characterized by a certain contamination level. This approach differs fundamentally from the point richness or site-specific richness (i.e., the number of taxa per sample or site) that is commonly used, as the latter type of richness only reflects a small fraction of the taxonomic pool and, therefore, was suggested to be defined as taxon density (29).

In the present study, we applied the contaminant category richness to investigate the effects of pesticides on stream invertebrates using the data from Europe [Germany (16) and France (17)] and Australia [southern Victoria (18)]. These data were chosen as they included (*i*) exposure assessment using methods designed to capture episodic pesticide exposure, (*ii*) records of stream invertebrates, and (*iii*) data on the principal environmental factors that may confound the effects of pesticides. The taxonomic richness was compared across site groups characterized by different levels of pesticide contamination (i.e., contamination category). The contamination categories were as follows: 1, reference—sites with log-transformed toxic units (TUs) < -4; 2, slightly contaminated—sites with $-4 < TU < -2$; and 3, highly contaminated—sites with $TU > -2$ (following ref. 32). These three contamination categories correspond to <1/10,000, 1/10,000–1/100, and >1/100, respectively, of the median acute effect concentration (EC_{50}) values for the reference species *Daphnia magna* (for details, see *Materials and Methods*).

Author contributions: M.A.B. and B.J.K. designed research; M.A.B., R.B.S., and M.L. performed research; M.A.B. and B.J.K. analyzed data; and M.A.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. D.P. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1305618110/-/DCSupplemental.

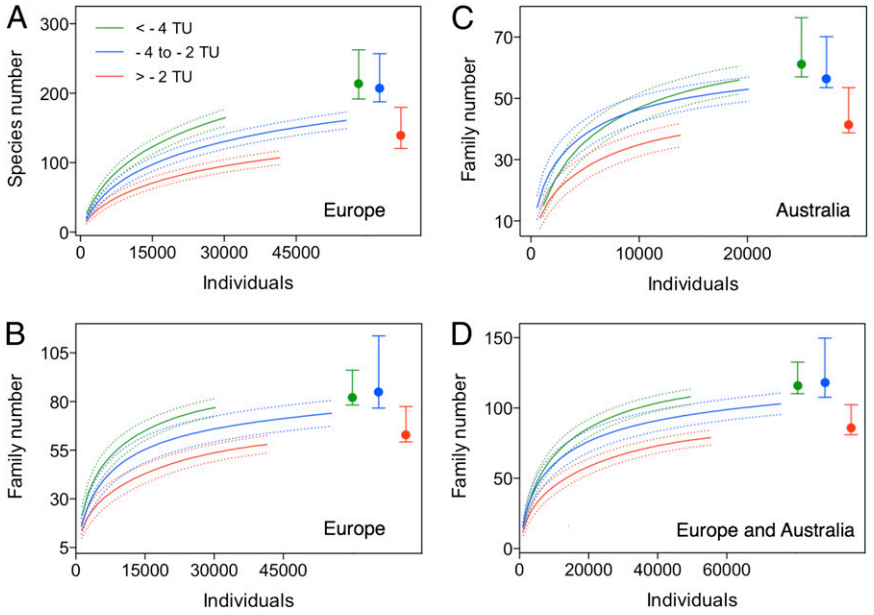

Fig. 1. Taxonomic richness of stream macroinvertebrates in the site groups characterized by different levels of pesticide contamination. Data from Europe (*A*, species level, and *B*, family level), Australia (*C*, family level only), and the combined dataset (*D*). The richness is expressed as taxa rarefaction curves (left side of each graph), showing the dependence of the richness on the sampling efforts, and the richness estimator Chao 2 (right side of each graph), showing the richness predicted for an infinite number of samples. The site groups are reference (TU < –4), slightly contaminated (–4 < TU < –2), and highly contaminated (TU > –2).

Results and Discussion

The rarefaction analysis revealed significant differences in taxonomic richness among all three of the contamination categories for both the species- and family-level data from Europe, as indicated by the nonoverlapping 95% confidence intervals (Fig. 1 *A* and *B*). For Australia, the rarefaction analysis for family-level data only showed a difference between the highly contaminated category and both the reference and slightly contaminated categories (Fig. 1*C*). The curves based on the combined dataset were similar to those found for the European family-level data (Fig. 1*D*). The percentage decrease in taxonomic richness between the uncontaminated and highly contaminated categories ranged from 42% for the European species-level data to 27% for the Australian family-level data, as calculated for the highest

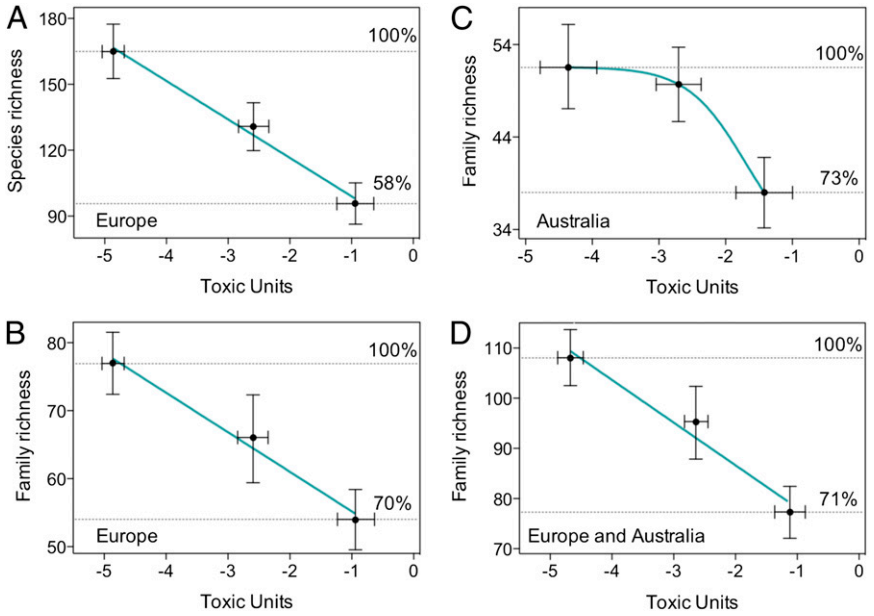

Fig. 2. Concentration–response dependence between the mean pesticide concentration and mean overall taxa richness in the three site groups characterized by different levels of pesticide contamination. Data from Europe (*A*, species level, and *B*, family level), Australia (*C*, family level only), and the combined dataset (*D*). The taxa richness values are derived from the rarefaction curves (Fig. 1) for the highest number of samples available for all three groups for each case. The regression lines are derived by linear (*A*, *B*, and *D*) and log logistic (*C*) regression models to illustrate the trends. The dashed horizontal lines indicate the maximum and minimum mean richness and are marked with the percentages of maximum richness. The error bars indicate 95% confidence intervals.

numbers of samples available for all three of the site groups (Fig. 2).

The richness predicted for an infinite number of samples by the estimator Chao 2 differed significantly between the highly contaminated sites and both the reference and slightly contaminated sites for all datasets examined (Fig. 1). The differences between the empirical (i.e., the rarefaction curves, Fig. 1) and predicted richness (i.e., Chao 2 in Fig. 1, right upper corners) indicate that pesticides may cause severe declines in the abundance and/or localization of certain taxa rather than their full absence from contaminated sites. Such taxa can only be found through excessive sampling effort and are unlikely to sustain viable populations [i.e., sink populations (33)]. Therefore, the relatively weaker pesticide effects detected by the Chao 2 estimator should be interpreted with caution.

To discriminate the possible confounding factors from the effects of pesticides, we used two lines of analyses. First, we checked whether the observed declines in taxa richness are based on the losses of taxa that are particularly vulnerable to pesticides due to their high physiological sensitivity and combination of eco/biological traits following the classification of the highly pesticide-specific SPEAR approach (16–21, 32). We found that pesticide contamination was, indeed, associated with a decrease in the shares of pesticide-vulnerable taxa (Fig. 3). Thus, the observed losses in taxonomic diversity were, to a large degree, determined by the loss of those taxa specifically vulnerable to pesticides (for details, see Fig. S1 and Tables S1 and S2).

Second, we analyzed whether any other available water quality and habitat variable would explain the differences between the three contamination categories (Tables S3 and S4), revealing only a significant difference in the electrical conductivity (a measure of salinity) of the water between the slightly and heavily contaminated sites in Australia. However, there was no consistent linear trend in water conductivity, with only the slightly contaminated sites having disproportionately low conductivity values (Fig. S2). Hence, water conductivity is very unlikely to be a major determinant of the observed diversity patterns.

Our results demonstrate that pesticides do produce pronounced negative effects on the regional biodiversity of stream invertebrates in both Europe and Australia. Furthermore, the effects on the taxa richness in Europe were identified in the contaminant category with TUs ranging from 1/10,000 to 1/100 of the model species *D. magna* EC_{50} (–4 < TU < –2; Figs. 1 and 2), i.e., at a concentration level that is considered to be protective by the current European regulation for agricultural pesticides (34, 35),

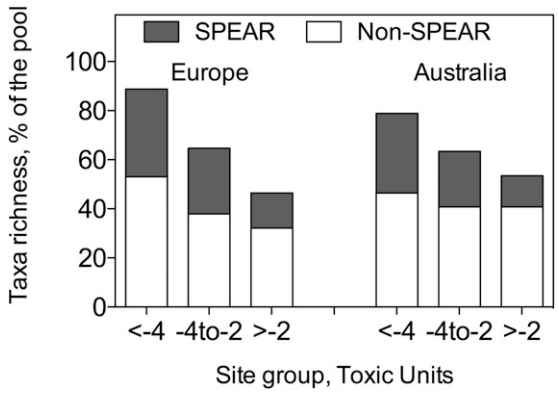

Fig. 3. Taxa richness expressed as a percentage of the entire species pool and shares of the pesticide-vulnerable SPEAR taxa and not vulnerable Non-SPEAR taxa. The values are given for the site groups in Europe and Australia characterized by different level of pesticide contamination: reference (TU < –4), slightly contaminated (–4 < TU < –2), and highly contaminated (TU > –2).

which states that no effects should occur below 1/100 of the EC_{50} of *Daphnia* spp. or fish (for discussion, see ref. 21).

Thus, the current risk assessment standards and/or their implementation in agricultural practice are not protective for regional biodiversity of the stream invertebrates. These findings are in accordance with previous studies on the site-scale effects on the abundance of taxa specifically vulnerable to pesticides, as shown in a metaanalysis of eight studies (21). Importantly, the present analyses show that the effects previously identified on the site scale for pesticide-sensitive organisms are actually translated into the alteration of the entire taxonomic pools in the contamination categories.

The present outcomes indicate that the aim to reduce the rate of biodiversity loss and to meet the 2020 targets set by the Convention on Biological Diversity (3, 5, 36) is jeopardized for freshwater ecosystems. Our analysis shows that the pesticides most of which are currently in use in Europe and Australia may cause declines of up to 42% of the stream invertebrates' species pools (Fig. 2). Such an extensive decline is comparable to the effects of other drivers and, as already demonstrated (17, 21), can be translated into functional impairments (1). Pesticide use has not decreased in the last decade (e.g., Eurostat Database; http://epp.eurostat.ec.europa.eu) and is predicted to increase in the next decades due to climate change (37) and thus may be a more important driver of biodiversity loss in the future.

The current prioritization of the biodiversity loss drivers may be misleading if pesticides are not considered. The measurement of the environmental concentrations of pesticides is difficult and expensive due to their episodic and low-level exposure and the multitude of substances (12, 38). Therefore, the actual effects of pesticides can easily be misattributed to other more "traditional" drivers (e.g., N and P levels and habitat degradation), which are better understood and can be more easily investigated.

If the aims of slowing the biodiversity loss rate (3, 4) and minimizing the effects of contaminants on biodiversity (34, 35) are to be achieved, the existing pesticide registration, methods of application to fields, and mitigation practices (e.g., nonsprayed buffer zones near waterways) should be developed toward more protective standards.

More generally, ecotoxicology as any applied ecological discipline should be matched to scales relevant for management practices. So with pesticides applied at the field scale and generally regulated at the national or supernational scale, ecotoxicology investigations should cover these scales. There is a clear need to better incorporate ecological theory and new large-scale-oriented approaches (e.g., 9, 16) to estimate and predict effects of contaminants across various spatial and temporal scales including the regional scale (12).

Materials and Methods

Datasets. To investigate the effects of agricultural pesticides on the taxa richness of stream invertebrates, we used datasets for the effects of pesticides on macroinvertebrates in small streams in two different biogeographical regions of Europe, including a central plains region in Germany (16) and a western plains region in France (17), and in southern Victoria, Australia (18). The datasets include the results of extensive pesticide analyses based on methods that reflect short-term peak pesticide exposure (see below). The datasets also include information on macroinvertebrate communities (abundance of taxa), and basic water quality parameters (Tables S3 and S4). In all, the datasets comprise information on 48 and 24 sampling sites in Europe and Australia, respectively. The general characteristics of the streams investigated were as follows: current velocity ranging between 0.1 and 0.5 m/s, maximum stream depth of 0.8 m, no drying up in summer, no dredging in the present or past year, and presence of adjacent fields (except for several reference sites in Australia) with grape vines, orchards, berries, vegetables, corn, sugar beet, or oil-seed crops. The sites were evaluated with field surveys and maps to ensure that they had no wastewater treatment plants, industrial facilities, or mining drainage upstream. Thus, pollution other than from agricultural sources was unlikely (for details, see refs. 16–18).

Pesticide Sampling and Analyses. The pesticide monitoring was designed to capture episodic runoff events, as this is a major input path for pesticides in small streams (16–21). The substances for the analyses were selected based on regulatory monitoring programs, pesticide use information from local agricultural advisory boards, and all other available information. Additionally, to select the most toxic compounds for the monitoring, the compounds were ranked according to their toxicity, as indicated by the 48-h acute median lethal concentration (LC50) for *Daphnia magna* taken from ref. 39 or the FOOTPRINT Pesticide Properties Database (http://sitem.herts.ac.uk/aeru/footprint/). The lists of measured pesticides differed between the study regions due to differences in the crops, pests, and pesticide authorization. The numbers of compounds analyzed were 21, 10, and 97 for Germany, France, and Australia, respectively (Table S5).

In Germany, two event-controlled runoff sampling systems were used: (*i*) an automated active sampler triggered by a conductivity decrease and water level increase and (*ii*) runoff-triggered 1-L bottle samplers passively triggered by a water level increase and retrieved after heavy rain events. The latter sampling system was also used in the study in France. In Australia, three methods were used: grab water sampling with a 1-L bottle, passive sampling using low-density polyethylene (LDPE) bags filled with 2,2,4-trimethylpentane (TRIMPS), and sediment samples. The TRIMPS passive samplers consisted of prefabricated LDPE membrane bags that were prerinsed overnight in 2,2,4-trimethylpentane and subsequently deployed for ∼28 d. The sediments were sampled using a dip net, wet-sieved on site to 64 μm, and decanted into a 1-L solvent-rinsed jar after a 15-min settling period (for details, see refs. 16–18). Although the sampling methods differed between the regions, the outcomes are comparable, as very similar relationships between the estimated pesticide toxicity in terms of the TUs and biotic endpoints were obtained (compared in ref. 21).

Expression of Pesticide-Related Water Toxicity. To compare the toxicity associated with the pesticide concentrations measured in the sampling sites, the TUs were computed from the maximum peak water concentrations measured at each site (16):

$$TU_{(D.\ magna)} = \max_{i=1}^{n}(\log(C_i/LC50_i)),$$

where $TU_{(D.\ magna)}$ is the maximum toxic unit value of the n pesticides detected at the site considered, C_i is the concentration (in micrograms per liter) of pesticide i, and $LC50_i$ is the 48-h LC50 of pesticide i for *D. magna* (in micrograms per liter), as given in ref. 34 or Footprint database (for the extended discussion on applicability of this approach, see ref. 21).

Macroinvertebrate and Environmental Variables. In Europe, macroinvertebrates were collected with a Surber sampler (area of 0.062 m^2, four replicate samples collected randomly over a stream length of 50 m per site/date). The macroinvertebrates were sorted, counted, and identified to the lowest possible taxonomic level, which was the species/genus for most of the taxa (16, 17). In Australia, the macroinvertebrate sampling was conducted according to the rapid bioassessment method of the Environment Protection Authority Victoria (40) and involved taking a sample from the edge/pool habitat with a kick net and, where riffles were present, a kick net (41). The taxa were identified to the family level due to the lack of taxonomic information on lower levels for many taxonomic groups (18). The measured environmental variables are summarized in Tables S3 and S4. The measurements of the water physicochemical parameters and assessment of the habitat and landscape parameters were performed on site.

Species Richness Calculations and Data Analyses. Taxonomic richness was quantified using the sample-based rarefaction curves calculated without replacement (Fig. 1) (in the terminology in ref. 24). The curves were calculated with 95% confidence intervals according to the analytical equations (42). Sample-based rarefaction was chosen to account for the natural levels of sample heterogeneity (patchiness) in the data. Following ref. 29, the sample-based rarefaction curves were plotted as a function of the cumulative number of individuals, not the cumulative number of samples, to avoid possible biases based on systematic differences in the mean number of individuals per sample. We used the classic richness estimator Chao 2 to predict the taxonomic richness for an infinite number of samples (43). This estimator generates asymmetrical confidence intervals that are based on the assumption that $\log(S_{estimated} - S_{observed})$ is normally distributed (where S is taxa richness). This assumption is reasonable in that the lower confidence bound cannot be less than the observed number of species (38, 39). The species richness calculations were performed using EstimateS 8.2 software (University of Connecticut, Storrs, CT; http://viceroy.eeb.uconn.edu/EstimateS/) (44).

To illustrate the concentration–response dependence between the estimated pesticide toxicity and regional taxa richness, we calculated a linear regression model between the mean TU per contamination-category site group and mean overall taxa richness in this site group (i.e., the three site groups characterized by different levels of pesticide contamination; Fig. 2). A nonlinear log logistic regression model was only fitted for the Australian data because it showed an obvious nonlinear relationship (Fig. 2*C*).

To determine whether the observed declines in the taxa richness are based on the losses of taxa that are particularly vulnerable to pesticides, we applied the SPEAR approach, which is known to have a high specificity with regard to pesticides (16–21, 27). The SPEAR approach divides the stream invertebrate taxa according to a binary classification including "species at risk" and "species not at risk" (where the "species" can be any taxonomic category, e.g., species, genus, family) according to the following biological traits: physiological sensitivity to organic toxicants, generation time, presence of aquatic stages in water during the maximum pesticide use period, and migration abilities. We calculated the fractions of the SPEAR taxa in the taxonomic pools of the three site groups characterized by different levels of pesticide contamination (i.e., contamination categories) to ascertain whether the declines in the taxa richness are based on the losses of the SPEAR taxa (Fig. 3).

To determine whether factors other than pesticides can explain the observed taxa richness patterns, we compared the three groups of the sites with different pesticide contamination levels with respect to the available physicochemical water characteristics and habitat and landscape parameters (Tables S3 and S4). The comparisons were performed with a nonparametric multiple comparison test of the Behrens–Fischer type, followed by a Holm–Bonferroni correction. A nonparametric test was selected due to violations of normality identified by the Kolmogorov–Smirnov test. In addition, a Levene test was performed to check for differences in the variance between the categories. A high variation in habitat/water quality variables may lead to a greater number of species as a result of a wider niche. However, this latter test revealed no statistically significant differences between the categories.

The statistical computations were performed using the open-source software package R, version 2.7 for Mac OS X (www.r-project.org) and Prism 5.0b for Mac OS X (GraphPad Software).

ACKNOWLEDGMENTS. We are grateful to everyone who helped with the fieldwork and the associated chemical analyses. We thank Graham Pyke for valuable comments on the manuscript. This work was supported by the Helmholtz Association of German Research Centers (Project ECOLINK, HRJRG-025). A visit to Germany by B.J.K. was funded by the University of Technology Sydney's International Researcher Development Scheme. Melbourne Water funded the collection of the pesticide data in the Australian study.

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Forest Ecology and Management 360 (2016) 152–158

Contents lists available at ScienceDirect

Forest Ecology and Management

journal homepage: www.elsevier.com/locate/foreco

Consequences of imidacloprid treatments for hemlock woolly adelgid on stream water quality in the southern Appalachians

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ARTICLE INFO

Article history:
Received 17 July 2015
Received in revised form 15 October 2015
Accepted 16 October 2015
Available online 26 October 2015

Keywords:
Imidacloprid
Streams
Water quality
Hemlock woolly adelgid
Resource management

ABSTRACT

Imidacloprid, a neonicotinoid pesticide, is commonly used in hemlock woolly adelgid, *Adelges tsugae* (Annand) (HWA) (Hemiptera: Adelgidae), pest management programs to preserve hemlock resources. Great Smoky Mountains National Park (GRSM) has an extensive HWA integrated pest management program, with more than 200,000 individual hemlocks in the Park having received imidacloprid soil treatments. A retrospective study was conducted in cooperation with GRSM to assess imidacloprid and two of its insecticidal metabolites (5-hydroxy and olefin) are present in surface waters (i.e., streams) associated with HWA imidacloprid treatment areas.

Thirty stream locations were sampled in GRSM to assess the presence and concentration of imidacloprid, 5-hydroxy, and olefin. Water samples were collected from 10 streams downstream from riparian areas where hemlocks received imidacloprid soil treatments and immediately upstream from hemlock treatment areas in each of the selected 10 streams. In addition, water samples were collected from 10 control streams each in close proximity to one of the 10 streams flowing through treatment areas. The concentrations of imidacloprid, 5-hydroxy, and olefin in parts per trillion (ppt) were determined by liquid chromatography mass spectroscopy (LC/MS). Data analysis included historical treatment data from GRSM. Data were analyzed using a Kruskal–Wallis test ($P < 0.05$), least significant difference (LSD), and a multiple regression ($P < 0.05$).

Imidacloprid, in concentrations ranging from 28.5 to 379 ng L^{-1}, was detected in 7 of the 10 downstream sampling locations. Upstream or adjacent stream locations did not have detectable concentrations of imidacloprid. Five-hydroxy and olefin were not detected in any streams. A positive relationship between the total amount of imidacloprid applied to a hemlock treatment area and the concentration of detectable imidacloprid in the associated stream was observed. However, while imidacloprid was detected in streams associated with hemlock treatment areas, the concentrations are below USEPA chronic and acute aquatic life benchmarks for fish (1200 and 41,500 µg L^{-1}, respectively) and aquatic macroinvertebrates (1.05 and 34.5 µg L^{-1}, respectively). Since the amount of imidacloprid applied in a treatment area has an influence on the concentration of imidacloprid in streams, resource managers must carefully consider the frequency and extent of imidacloprid applications to meet management goals while providing minimal environmental impact.

1. Introduction

Hemlock woolly adelgid, *Adelges tsugae* (Annand) (Hemiptera: Adelgidae) (HWA), an invasive insect from southern Japan (Havill et al., 2006), was unintentionally introduced to the eastern United States in the 1950s (Stoetzel, 2002). HWA feeds on eastern hemlock, *Tsuga canadensis* (L.) Carriére, a slow-growing species that inhabits a distinctive ecological niche and is an important component of many forest types (Orwig and Foster, 1998; Ward et al., 2004). As the dominant shade-tolerant conifer in its habitat, eastern hemlock plays a vital ecological role in southern Appalachian forests, and that role cannot be filled by any other native evergreen tree species (Orwig and Foster, 1998; Ward et al., 2004). Many species depend on eastern hemlock and will be negatively impacted by its decline (Wallace and Hain, 2000; Hakeem, 2008; Dilling

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http://dx.doi.org/10.1016/j.foreco.2015.10.028
0378-1127/

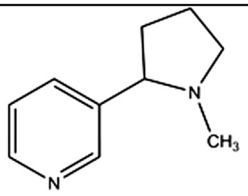
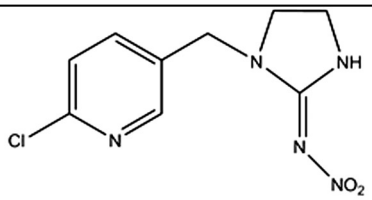
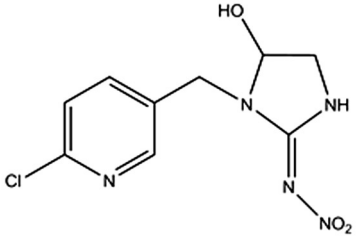
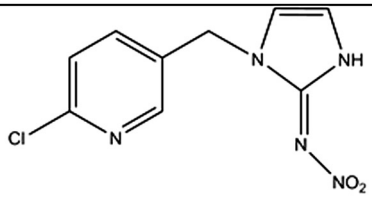
et al., 2007, 2009; Coots et al., 2012). Unfortunately, as eastern hemlock has exhibited no visible resistance against the adelgid (McClure, 1995) and no native predators are capable of suppressing adelgid populations (McClure, 1987), excessive mortality and decline have occurred throughout most of the natural range of this native tree species in the eastern United States (Lambdin et al., 2006).

Great Smoky Mountains National Park (GRSM) launched an aggressive integrated pest management (IPM) program against HWA to reduce damage to its hemlock resources once HWA was documented in the Park in 2002. Horticultural oil sprays, biological control (i.e., predatory beetles), and systemic imidacloprid applications have been employed to suppress HWA populations. Imidacloprid, a neonicotinoid pesticide, is the primary management tactic used in this program in the Park, where it is applied in GRSM as soil injections within 30 cm of the hemlock trunk, basal drench (i.e., imidacloprid solution is poured on the soil within 30 cm of the hemlock trunk), stem injections, or as a dissolvable pellet (Core Tect). Over 200,000 trees, many in riparian areas, have received imidacloprid soil treatments.

Imidacloprid has been used for pest control since the early 1990s (Diehr et al., 1991) and is applied in agricultural, forestry, and urban settings to suppress a variety of pest species (Jeschke et al., 2011; Goulson, 2013). The chemical structure of imidacloprid is similar to nicotine (Fig. 1) (Matsuda et al., 2001), and it functions similarly by acting on nicotinic acetylcholine receptors in the central nervous system of insects (Nauen and Bretschneider, 2002). Neonicotinoids are commonly used because they are selective for treating arthropod pests, have low fish and mammalian toxicity, and can be applied by various methods (Sánchez-Bayo and Hyne, 2014). However, concerns about the potential negative impacts of imidacloprid to surface water quality, aquatic macroinvertebrates, pollinators, and other non-target organisms have been expressed (USEPA, 2008b; Dilling et al., 2009; Pestana et al., 2009; Goulson, 2013).

Because imidacloprid can be toxic to aquatic macroinvertebrates if the dosage is high enough (Alexander et al., 2007; Pestana et al., 2009), its ability to leach into surface water and persistence in aquatic systems are important. Movement of imidacloprid through the soil is a route of potential impact to surface water quality (USEPA, 2008b). Similar to other pesticides, once in the environment, imidacloprid begins to degrade by biotic, abiotic, and photolytic degradation (Wamhoff and Schneider, 1999), and some degradation products of imidacloprid, such as olefin, 5-hydroxy, 4-hydroxy, and dihydroxy, have insecticidal properties (Nauen et al., 1998, 1999). The persistence of imidacloprid and its metabolites in the environment will influence their potential to cause negative non-target impacts.

The persistence of imidacloprid in the soil, determined by its ability to bind to soil and its degradation in the soil column (Cox et al., 2004), can affect which compounds enter surface waters. The sorption of imidacloprid into soil is dependent on the concentration of imidacloprid and the organic matter content in the soil, as imidacloprid binds to organic matter (Mullins and Christie,

Common Name	IUPAC Name	Structure
Nicotine	3-(1-methyl-2-pyrrolidinyl)pyridine	
Imidacloprid	1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine	
5-hydroxy	1-(6-chloro-3-pyridylmethyl)-2-(nitroimino)imidazolidin-5-ol	
Olefin	1-(6-chloro-3-pyridylmethyl)-*N*-nitro-1,3-dihydro-imidazol-2-ylideneamine	

[1]IUPAC = International Union of Pure and Applied Chemistry

Fig. 1. The IUPAC[1] names and chemical structures of nicotine, imidacloprid, and two insecticidal imidacloprid metabolites (5-hydroxy and olefin).

1995; Cox et al., 1998). In soils with high organic matter content, such as those in GRSM, less leaching is expected (Cox et al., 1998).

Once imidacloprid enters surface water its ability to persist may be limited because imidacloprid photodegrades in water (Moza et al., 1998; Wamhoff and Schneider, 1999). The half-life of imidacloprid in water has been recorded from one hour to three days (Agüera et al., 1998; Moza et al., 1998; Wamhoff and Schneider, 1999), and half-life can vary by season, ranging from estimates of 8.6–52.8 h, with slower photodegradation occurring in the winter (Lu et al., 2015). In the absence of light, imidacloprid is stable in water for more than 12 h. However, when exposed to light complete degradation has been documented in less than five hours (Agüera et al., 1998).

Possible non-target effects of imidacloprid in eastern hemlock systems in both terrestrial and aquatic habitats have been investigated by numerous researchers (Hakeem, 2008; Dilling et al., 2009; Churchel et al., 2011). Imidacloprid applied to hemlocks by soil injection can move laterally and horizontally through the soil (Knoepp et al., 2012). In numerous studies imidacloprid has been documented in surface waters associated with soil applications of imidacloprid in agricultural areas (Starner and Goh, 2012; Hladik et al., 2014; Main et al., 2014). Imidacloprid and its metabolites may move into the water column through leaf degradation, since imidacloprid, olefin and 5-hydroxy have been detected in hemlock foliage tissue (Dilling et al., 2010; Coots et al., 2013). A similar scenario has been documented in the laboratory using ash leaves, where imidacloprid was found to enter the water column as leaves from treated ash trees degraded (Kreutzweiser et al., 2007). Given the presence of imidacloprid in surface waters via various routes, imidacloprid treatments for hemlock conservation may pose potential risks to surface water quality. The purpose of this study is to assess the potential risks of imidacloprid in surface waters in GRSM by determining the presence and concentration of imidacloprid, 5-hydroxy, and olefin in surface waters and if any treatment area and timing factors contribute to observed concentrations of the insecticidal chemicals in water.

2. Materials and methods

Ten streams flowing through hemlock-dominant or co-dominant forest types in treatment areas were selected for this study (Table 1). Ten locations, one in each stream, were selected 10–100 m downstream from a treatment area, hereafter referred to as downstream. As a control, ten locations, one in each stream, were selected 10–100 m upstream from the treatment areas, hereafter referred to as upstream. In addition, ten streams were selected in hardwood-dominant forest types, in the same watersheds as the streams in treatment areas, and are henceforth referred to as adjacent streams. No imidacloprid treatments were applied upstream from the adjacent stream locations; thus, these locations serve as an additional control. Water samples were collected from 30 stream locations (10 upstream, 10 downstream, and 10 adjacent stream) (Fig. 2) in GRSM to assess the presence and concentration of imidacloprid and two of its metabolites (5-hydroxy and olefin) (Fig. 1).

Treatment areas contained between 100 and 1000 hemlocks that received imidacloprid treatments. Hemlocks in the riparian corridors of treatment areas were treated one to eight years before sampling and received between one and three treatment cycles, depending on the site (Table 1). A treatment cycle may refer to a time when most trees in a treatment area were treated or when the hectarage of a treatment area was expanded. Due to hemlock health in selected treatment areas and the expansion or contraction of the size of treatment areas, the number of trees per treatment area was not consistent among treatment cycles. For example, a larger treatment area may have had many treated hemlocks initially, but with hemlock mortality due to HWA in that area, fewer trees would have been treated during the next cycle. A few trees near campsites also may have had an initial treatment and later the larger area around the campsite was treated.

Imidacloprid was applied as a basal drench, which involves pouring a wettable powder solution of imidacloprid around the base of hemlock trees approximately 30 cm from the trunk. Trees smaller than 25 cm diameter at breast height (dbh) were treated with 0.7 g.a.i. (grams of active ingredient) per 2.5 cm dbh, and trees 25 cm dbh and larger were treated with 1.4 g.a.i. per 2.5 cm. Imidacloprid rates per hectare did not exceed the maximum allowable rate of treatment (0.4 kg per hectare per year) (Bayer, 2006).

Samples were collected from each selected location (either upstream, downstream, or adjacent stream) during a single sampling event. During a sampling event three replicate water samples (1 L) were collected mid-channel and mid-depth from each stream sampling location using amber glass bottles (1 L) with Teflon lined lids. Glass bottles were placed into the water column, lid down. The bottle was then turned with the opening facing upstream to allow the bottle to fill with stream water. Containers were transported to and from the field in cooler bags (25 × 15 × 15 cm). Sampling locations were often in remote areas, so the cooler bags

Table 1
Imidacloprid treatment histories for streams in treatment areas where imidacloprid was used for the management of hemlock woolly adelgid, Great Smoky Mountains National Park.

Stream	First treatment	Last treatment	Sampling date	Treated hectares	Total kg.a.i.[a,b] applied	kg.a.i. 1 yr prior to sampling[c]	Treated stream length (m)	Treatment cycles
Alum Creek	9/2004	8/2011	6/2012	19.0	14.8	0.2	4008	5
Camel Hump Creek	N/A[d]	N/A	5/2012	N/A	N/A	1.2	353	N/A
Cane Creek	2/2005	10/2010	2/2013	14.5	6.3	0	4178	3
Chasteen Creek	1/2005	6/2009	12/2012	42.6	16.8	0	8766	4
Dunn Creek	4/2005	9/2010	6/2012	47.1	114.0	0	1046	6
Indian Camp Creek	5/2005	9/2010	6/2012	N/A	N/A	0	9899	N/A
Indian Creek	9/2005	6/2011	8/2012	47.2	38.3	0	5046	5
Kingfisher Creek	5/2004	10/2012	10/2012	29.4	20.9	9.7	1773	4
Panther Creek	8/2011	4/2012	8/2012	26.6	1.8	1.8	3811	1
Shop Creek	4/2011	6/2011	10/2012	23.3	7.6	0	2249	1

[a] Kilograms active ingredient.
[b] Total kg.a.i. applied in the treatment area.
[c] kg.a.i. applied in the treatment area one year before water samples were collected.
[d] All data were not available for Camel Hump Creek and Indian Camp Creek.

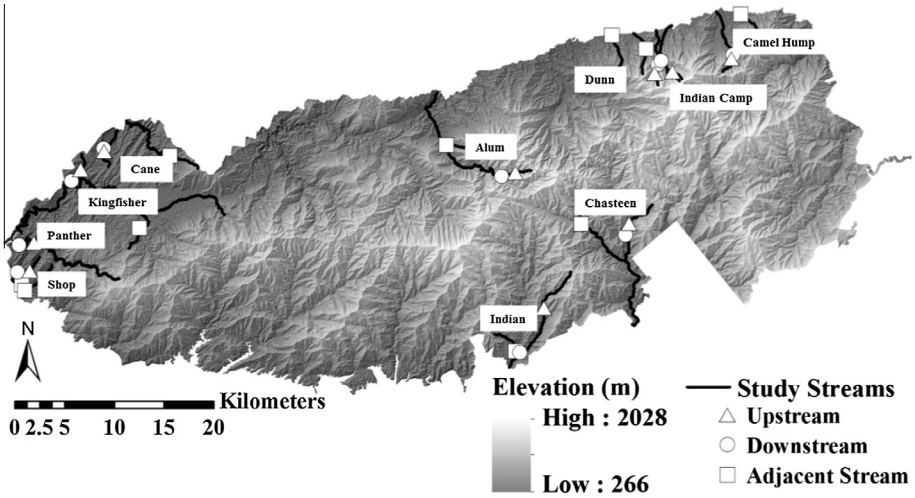


Fig. 2. Stream sampling locations in the Great Smoky Mountains National Park.

were placed in large backpacks for transport to the laboratory, where samples were stored in a walk-in cooler at 4 °C until processed. Samples were processed within three weeks of the collection date. All samples were collected between May 2012 and January 2013.

The amount of sample collected was sufficient to allow for concentration detection in parts per trillion (ng L^{-1}). All methods were optimized for greater sensitivity to determine low levels of imidacloprid in the environmental samples using liquid chromatography mass spectroscopy (LC/MS). Sample preparation prior to analysis utilized an Empore aqueous extraction system (Mueller et al., 2000; Mersie et al., 2002; Senseman et al., 2003). This procedure passes the water sample through a 17 mm C 18 embedded filter allowing the matrix to pass through unimpeded and capturing the analytes of interest, imidacloprid, 5-hydroxy, and olefin. Preliminary studies examined the recovery of fortified imidacloprid samples using our methodology, and indicated that recovery was 49.0–52.5% (data not shown). Repeated attempts to increase recovery trying a range of different solvents and operating parameters were not successful. While recovery in the import system of 49.0–52.5% was not ideal, the consistency and relative goodness of that 50% across several validation runs encouraged the use of the described procedures. In addition, the Empore solid phase extraction platform is widely recognized as an appropriate sample processing and concentrating procedure. Thus, the determination of concentration recovery in our samples was 50%.

The entire water sample (1 L) was passed through an Empore disk (3 M) on an Empore 6 station extraction manifold and processed using standard laboratory procedures to prepare a given water sample for LC/MS analysis. First, the Empore disk was conditioned using methanol. Once the disk was conditioned, the water sample was added to a reservoir, which holds the water above the disk. Water was then drawn through the disk using a vacuum pump (GAST model P104 oil-less pump) operated at 0–7 bar of negative pressure. Residual water was removed from the disk using the vacuum pump to dry the disk. The sample was eluted using 10 mL of methanol and collected in a 12 mL vial. This sample preparation resulted in a highly concentrated sample that was prepared for LC/MS. Processed samples were stored at 4 °C until LC/MS analysis.

Chromatographic conditions included use of the C 18 column (Phenomenex, Inc.) and isocratic mobile phase of 30% acetonitrile and 70% water (both with 0.1% formic acid to maintain constant ionic strength). Mass spectroscopy conditions included drying gas flow of 5.0 L, nebulizer pressure at 4.14 bar, drying gas temperature of 300 °C, vapor temperature 250 °C, capillary voltage 2000 V, Corona current was set at 1.0, charging voltage was set at 2000, and the fragmentor setting was 70. The ionization hardware used was mixed mode-ESI–APCI. Apparent molecular mass units using the select ion monitoring mode determined the imidacloprid, 5-hydroxy, and olefin simultaneously. Approximate retention times for imidacloprid, 5-hydroxy, and olefin were 8.50, 5.98, and 5.26 min, respectively.

They were analyzed as a group and each run included individual standards for the parent and metabolites, with an external standard technique used. The conservative limit of detection (LOD) was 20 ng L^{-1}. Given the difficulty of collecting and storing samples, due to remote site location, the decision was made not to collect blank water samples or fortify deionized water samples in the field. Method development strongly indicated that procedures used in this study were robust and highly precise for the detection and quantification of the target compounds.

Rainfall data were obtained from the National Oceanic and Atmospheric Administration (NOAA) climate data website (NOAA, 2015). NOAA weather stations closest to the watersheds of interest were used. Data three days prior to sample collection were used to determine how much rainfall had recently fallen in the sampled watersheds. Data were not used in the analyses because rainfall records were not available for Camel Hump Creek and incomplete for Cane Creek and Chasteen Creek.

All data were stored using an Excel file (Microsoft, Redmond, WA). The three replicate samples collected at each sampling location were averaged, to obtain one concentration for each sampling location for use in data analyses. A Kruskal–Wallis Test was used to determine significant differences, if any, among ranks of concentration of imidacloprid found in upstream, downstream, and adjacent stream sampling locations ($P < 0.05$). The mean ranks were separated using least significant difference (LSD). A multiple regression analysis was used to determine if a relationship existed between treatment area information and time variables and the concentration of imidacloprid documented in streams in GRSM ($P < 0.05$). A backward elimination selection method was used to select the model that best explained the data. All data analyses were conducted using SAS (SAS Institute, 2008). The Camel Hump Creek treatment area was never isolated as a separate site from a larger treatment area in regards to data entry, so accurate numbers on treatment time and site variables specifically to that smaller watershed are not available. Indian Camp Creek flows through numerous treatment areas, but does not have a distinct treatment

drainage area for treatment time and site variables. Because all site data are not available for Camel Hump Creek and Indian Camp Creek they were not included in the multiple regression analysis.

3. Results

Imidacloprid was detected in streams associated with imidacloprid treatments for the control of HWA in this study (Table 2). All stream locations where imidacloprid was detected were downstream from imidacloprid treatment areas. Imidacloprid was detected in seven out of ten downstream locations, and imidacloprid concentrations ranged from 28.5 to 379.1 ng L^{-1}. In six of the streams where imidacloprid was detected the concentration of imidacloprid was below 100 ng L^{-1}. Dunn Creek, with a documented imidacloprid concentration of 379.1 ng L^{-1}, was the only stream where the concentration of imidacloprid was in excess of 100 ng L^{-1}. Three downstream locations, Camel Hump Creek, Cane Creek, and Panther Creek, had no samples that exceeded the LOD for imidacloprid. Samples from all upstream and adjacent stream locations did not exceed the LOD for imidacloprid (data not shown). All samples were below the LOD for 5-hydroxy and olefin (data not shown).

Rainfall amounts and imidacloprid concentrations detected in streams do not have a clear pattern, which may be, in part, due to the variety of treatment area conditions in the study. The two highest concentrations recorded, 379.1 and 78.0 ng L^{-1}, were detected in Dunn Creek and Indian Creek, respectively. Nearly 1 cm of rainfall occurred three days prior to sample collection, which may have influenced the observed concentrations. However, rainfall events in excess of 2 and 3 cm occurred before samples were collected at Alum Creek and Indian Creek, respectively. While imidacloprid was detected in those streams, concentrations were only 28.5 and 31.2 ng L^{-1}, respectively. Little to no rain occurred prior to sampling at Panther Creek, Chasteen Creek, and Kingfisher Creek. Imidacloprid was detected at both Kingfisher Creek (33.6 ng L^{-1}) and Chasteen Creek (36.8 ng L^{-1}). Given the diversity of hectarage and imidacloprid usage in treatment areas, it would be difficult to perceive overall trends in imidacloprid concentrations in stream water based on rainfall.

A significant difference among upstream, downstream, and adjacent stream categories was detected ($X^2 = 52.92$, df = 2, $P < 0.001$; Kruskall–Wallis). Downstream locations have a significantly higher mean rank for imidacloprid concentrations than upstream and adjacent stream locations ($P < 0.05$; LSD test). Locations downstream from imidacloprid treatment areas had significantly higher concentrations of imidacloprid than upstream and adjacent stream locations, both of which did not have detectable concentrations of imidacloprid.

The selected multiple regression model, which includes months since the first and last imidacloprid treatments, the number of treated hectares, and the total amount of imidacloprid applied to treatment areas, explains 97% of the variation in the data. The model overall was significant ($P = 0.009$), and all variables could explain at least 48% of the variation adjusted for the other variables. Given the adjustments made for the other variables in the model, the concentration of imidacloprid found in streams is positively related to the total amount of imidacloprid applied to treatment areas (Partial $R^2 = 0.96$, $P = 0.002$) (Fig. 3 and Table 3). Cane Creek and Panther Creek, two sites where imidacloprid was not detected, had the smallest amounts of imidacloprid applied to their treatment areas, 6.3 and 1.8 kg.a.i., respectively. Dunn Creek, which had an imidacloprid concentration of 379.1 μg L^{-1}, also received the greatest amount of imidacloprid applied to the treatment area (114.0 kg.a.i.). The concentration of imidacloprid detected at Dunn Creek is largely responsible for the slope of the relationship between the concentration of imidacloprid and the amount of imidacloprid applied to treatment areas. No significant relationship was detected between imidacloprid concentrations and treatment area variables when Dunn Creek was removed from the analysis and only lower concentration data points were considered (data not shown). However, data collected from Dunn Creek are valid and explain much of the relationship between imidacloprid concentration in streams and the amount of imidacloprid applied in treatment areas (Table 3).

4. Discussion

The potential risk of imidacloprid from hemlock treatments to leach through soil, enter surface water, and cause associated negative impacts on water quality and aquatic biota is an issue that scientists, regulators, and land managers must consider. According to the USEPA, the Chronic and Acute Aquatic Life Benchmarks of imidacloprid for fish is 1200 and 41,500 μg L^{-1}, respectively. Aquatic invertebrates have much lower Chronic and Acute Aquatic Life Benchmarks of 1.05 and 34.5 μg L^{-1}, respectively (USEPA, 2008a). The LC_{50} (the concentration at which 50% of individuals of a taxa are killed) of imidacloprid for aquatic macroinvertebrates in 96 h exposure studies ranges from 0.65–12.94 μg L^{-1} (Alexander et al., 2007; Stoughton et al., 2008; Pestana et al., 2009). Sublethal effects on aquatic macroinvertebrates have been documented at

Table 2
Concentration in ng/L (parts per trillion) of imidacloprid at downstream locations and rainfall totals three days prior to sample collection, Great Smoky Mountains National Park.

Stream name	Imidacloprid concentration[a]	Rainfall (cm)
Alum Creek	28.5 ± 3.8	2.44
Camel Hump Creek	<LOD[b]	na[c]
Cane Creek	<LOD	0.53[d]
Chasteen Creek	36.8 ± 3.4	0[d]
Dunn Creek	379.1 ± 7.9	0.97
Indian Camp Creek	78.0 ± 8.0	0.97
Indian Creek	31.2 ± 1.5	3.35
Kingfisher Creek	33.6 ± 6.6	0
Panther Creek	<LOD	0.20
Shop Creek	82.2 ± 25.8	0.71

[a] Means (± standard deviation) are an average of the concentrations of the three samples collected at each sample location.
[b] Imidacloprid concentration was below the limit of detection (LOD) (20 ng/L).
[c] Rainfall data for Camel Hump Creek were not available.
[d] Complete rainfall data were not available during the three day time period prior to sampling.

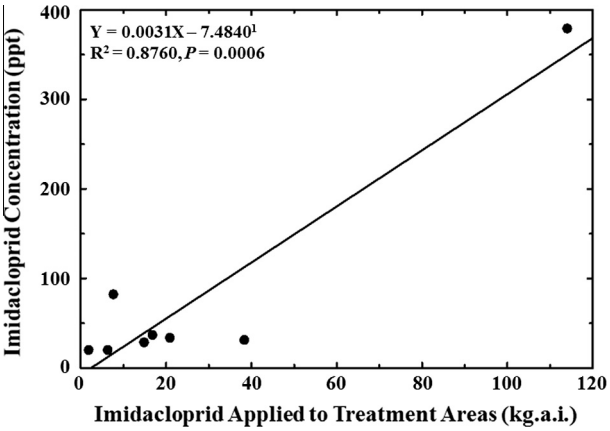


Fig. 3. Relationship between the amount of imidacloprid applied to treatment areas and concentration of imidacloprid observed in streams. [1]No adjustments are made for other variables.

Table 3
Multiple regression associating imidacloprid concentration in streams with treatment area information and time variables.

Variable	DF	Parameter estimate	Standard error	*t* Value	Pr > |t|	Partial R^2
Intercept	1	99.3703	36.8975	2.69	0.074	–
Mo. Since First Treatment	1	−0.9091	0.3260	−2.79	0.069	0.7216
Mo. Since Last Treatment	1	1.4638	0.8674	1.69	0.190	0.4870
Hectares	1	−2.8644	1.1769	−2.43	0.093	0.6638
Total kg.a.i. Applied[a]	1	0.00402	0.0004	9.72	0.002	0.9694

[a] Total kg.a.i. applied in the treatment area.

concentrations of 0.10–3.00 μg L^{-1} in 96 h exposure trials (Azevedo-Pereira et al., 2011; Roessink et al., 2013). Sublethal effects of imidacloprid were observed in a mesocosm experiment using 12 μg L^{-1} imidacloprid pulses simulating rainfall event frequency and duration (Mohr et al., 2012).

Short-term exposure data are currently used to set water quality standards. Because the cumulative effect of exposure to low imidacloprid concentrations may have sublethal impacts on aquatic macroinvertebrates, concern has been raised regarding the use of these methods. In addition, the USEPA Aquatic Life Benchmark concentrations are higher than standards set by Canada, Europe, and the Netherlands (Morrissey et al., 2015). While negative effects of imidacloprid exposure on aquatic macroinvertebrates have been documented, most concentrations observed in this study are below concentrations documented to have negative acute and chronic effects. Six streams had documented imidacloprid concentrations that were less than 0.10 μg L^{-1}, which is one tenth of the USEPA Chronic Aquatic Life Benchmark. Dunn Creek was the only sampling location where imidacloprid concentration was in excess of 100 ng L^{-1}, and the observed concentration (379.1 ng L^{-1}) is below the above-mentioned USEPA Chronic and Acute Aquatic Life Benchmarks of imidacloprid for both aquatic macroinvertebrates and fish. In addition, preliminary results from a complementary study assessing aquatic macroinvertebrates in the upstream and downstream locations indicate similar abundance and taxa richness of environmentally sensitive aquatic macroinvertebrate taxa (unpublished data). Examination of the aquatic community composition among sites will be addressed in a separate publication.

Imidacloprid has been previously documented in a stream associated with imidacloprid treatments for suppression of HWA. In that study, four streams were sampled for approximately 2 yr after imidacloprid soil applications, and only one sample, collected over 700 d after treatment, tested positive for imidacloprid. The concentration of imidacloprid in the only positive sample was <1 μg L^{-1}. However the LOD for their study was 0.6 μg L^{-1} (Churchel et al., 2011), which is 30 times higher than the LOD in the current study. All documented concentrations of imidacloprid in our study were lower than the 0.6 μg L^{-1} LOD in Churchel et al. (2011). If methods used in that study had allowed for a lower LOD, then more positive samples may have been detected in streams associated with imidacloprid treatments for HWA. In addition to low documented presence of imidacloprid in streams, no negative effects on aquatic macroinvertebrates were observed in the stream where imidacloprid was documented (Churchel et al., 2011).

The absence of olefin and 5-hydroxy in stream samples was not unexpected. Olefin and 5-hydroxy are not the main metabolites of imidacloprid produced via photodegradation in water (Agüera et al., 1998; Moza et al., 1998; Redlich et al., 2007). However, since both metabolites are highly toxic insecticidal metabolites produced in numerous plant systems, including hemlock, it is important to establish the absence of olefin and 5-hydroxy in streams flowing through HWA treatment areas (Nauen et al., 1998, 1999; Coots et al., 2013).

Eastern hemlock is an important component of southern Appalachian riparian ecosystems with many aquatic and terrestrial species depending on its presence. With hemlocks in eastern forests declining, land managers must make difficult decisions involving positive and negative trade-offs of treatments for the protection of hemlock resources. Assessment of the presence and concentration of imidacloprid in streams as a result of imidacloprid treatments to hemlocks is an initial step to determine what negative consequences to surface water quality must be considered when making management decisions. Because the amount of imidacloprid applied in a treatment area has a significant effect on the concentration of imidacloprid observed in streams in this study, the frequency and extent of imidacloprid applications must be carefully considered. Land managers must decide if the risk of imidacloprid exposure to aquatic macroinvertebrates adjacent to areas of treated hemlock outweighs the benefits of preserving hemlock, which is a key species in many systems.

5. Conclusions

Imidacloprid was present downstream from imidacloprid treatment areas in seven of ten streams, and the presence of imidacloprid was not observed in upstream and adjacent stream samples. The highest concentration observed, 379.1 ng L^{-1}, was below USEPA Aquatic Life Benchmarks for chronic toxicity of imidacloprid to aquatic invertebrates. Six of the seven streams where imidacloprid was documented had concentrations below 100 ng L^{-1}, less than one tenth of the USEPA Chronic Aquatic Life Benchmark. No obvious trends existed between the amount of rainfall prior to sampling and the observed concentration of imidacloprid in streams. A positive relationship between the total amount of imidacloprid that was applied in treatment areas and the imidacloprid concentration in streams was documented. Months since the first and last imidacloprid treatments as well as hectares treated explained at least 48% of the observed variation in imidacloprid concentration data. The insecticidal metabolites olefin and 5-hydroxy were not documented in any of the sampled streams. Knowledge about the presence and concentration of imidacloprid in multiple streams associated with HWA treatment areas can help land managers make calculated assessments of the risks and benefits of treating hemlocks with imidacloprid for the suppression of HWA. Based on these results, imidacloprid does appear in streams associated with HWA treatment areas. Concentrations detected are below USEPA Chronic and Acute Aquatic Life Benchmarks.

While chronic, sublethal effects are possible (Morrissey et al., 2015), according to guidelines currently set by the USEPA, detected imidacloprid concentrations are not expected to impact the aquatic community.

Acknowledgements

The authors thank personnel at Great Smoky Mountains National Park for assistance with site selection and field work. This work was partially funded by the National Park Service (Agreement No. J547110059) and the United States Forest Service (Agreement No. 11-DG-11083150-021).

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Environmental impacts of an imidacloprid-containing formulation: from soils to waters

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Impactos ambientales de una formulación que contiene imidacloprid: de los suelos a las aguas

Impactes ambientals d'una formulació que conté imidacloprid: dels sòls a les aigües

Recibido: 26 de junio de 2015; aceptado: 1 de julio de 2015

RESUMEN

El pesticida neonicotinoide imidacloprid se encuentra entre los agroquímicos más vendidos en todo el mundo. Debido a su amplio uso en mezclas con diferentes disolventes y co-adyuvantes, estudiar el impacto ambiental de las formulaciones comerciales derivadas se ha convertido en obligatorio. En este estudio se utilizaron ensayos ecotoxicológicos de laboratorio para cuantificar el impacto del Confidor® 20SL (formulación que contiene imidacloprid) en los compartimentos terrestre y acuático. Los efectos letales y subletales de las dosis recomendadas de aplicación del producto fueron evaluadas en los invertebrados terrestres *Eisenia foetida* y *Folsomia candida* mientras que la toxicidad de los lixiviados de los suelos contaminados se evaluó en los organismos acuáticos modelo *Daphnia magn*a y *Raphidocelis subcapitata* (anteriormente *Selenastrum capricornutum*). La exposición a concentraciones ambientalmente relevantes de imidacloprid no causó mortalidad en las lombrices de tierra (CL_{50} de 4.23 mg de imidacloprid por kg de suelo seco) pero alteró los patrones de comportamiento y reproducción (valores de CE_{50} de 0.43 y 1.40 mg de imidacloprid por kg de suelo seco en los ensayos de alejamiento y reproducción respectivamente). Los efectos en los colémbolos *F. candida* fueron despreciables. El imidacloprid presentó una lixiviabilidad moderada, con tasas de recuperación en los extractos acuosos que fueron del 25.4 al 50.4% de la cantidad presente en los suelos y concentraciones de 13.05 a 71.8 µg por litro. Las pruebas estándar de ecotoxicidad acuática no fueron capaces de detectar toxicidad aguda o crónica en los organismos de ensayo. Sin embargo, las concentraciones de insecticida en los extractos fueron lo suficientemente grandes como para representar una amenaza letal para otros organismos acuáticos no estándar.

Palabras clave: Imidacloprid; ecotoxicidad; extractos acuosos; lombrices de tierra.

RESUM

El pesticida neonicotinoide imidacloprid es troba entre els agroquímics més venuts a tot el món. Degut al seu ampli ús en mescles amb diferents dissolvents i co-adjuvants, estudiar l'impacte ambiental de les formulacions comercials que en deriven ha esdevingut obligatori. En aquest estudi es van utilitzar assajos ecotoxicològics de laboratori per a quantificar l'impacte del Confidor® 20SL (formulació que conté imidacloprid) en els compartiments terrestre i aquàtic. Els efectes letals i subletals de les dosis recomanades d'aplicació del producte van ser avaluades en els invertebrats terrestres *Eisenia foetida* i *Folsomia candida* mentre que la toxicitat dels lixiviats dels sòls contaminats es va avaluar en els organismes aquàtics model *Daphnia magna* i *Raphidocelis subcapitata* (anteriorment *Selenastrum capricornutum*). L'exposició a concentracions ambientalment rellevants d'imidacloprid no va causar mortalitat en els cucs de terra (CL_{50} de 4.23 mg d'imidacloprid per kg de sòl sec) però en va alterar els patrons de comportament i reproducció (valors de CE_{50} de 0.43 i 1.40 mg d'imidacloprid per kg de sòl sec en els assajos d'allunyament i reproducció respectivament). Els efectes en els col·lèmbols *F. candida* van ser menyspreables. L'imidacloprid va presentar una lixiviabilitat moderada, amb taxes de recuperació en els extractes aquosos que van anar del 25.4 al 50.4% de la quantitat present en el sòls i concentracions de 13.05 a 71.8 µg per litre. Les proves estàndard d'ecotoxicitat aquàtica no van ser capaces de detectar toxicitat aguda o crònica ens els organismes d'assaig. No obstant això, les concentracions d'insecticida en els extractes van ser prou grans com per a representar una amenaça letal per a altres organismes aquàtics no estàndard.

Paraules clau: Imidacloprid; ecotoxicitat; extractes aquosos; cucs de terra

SUMMARY

The neonicotinoid pesticide imidacloprid is among the top sold agrochemicals worldwide. Due to its widespread use in mixtures with different solvents and co-adjuvants, studying the environmental impact of its derived commercial formulations has become mandatory. In this study we used laboratory ecotoxicological tests to quantify the impact of the imidacloprid-containing formulation Confidor®

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20SL on the terrestrial and aquatic compartments. Lethal and sublethal effects of recommended application doses of the product were assessed on standard terrestrial invertebrates *Eisenia fetida* and *Folsomia candida* whereas the toxicity of leachates from contaminated soils was evaluated in the aquatic model organisms *Daphnia magna* and *Raphidocelis subcapitata*. The exposure to environmentally relevant concentrations of imidacloprid caused no mortality to earthworms (LC_{50} of 4.23 mg imidacloprid kg^{-1} dry soil) but altered their behavior and reproduction patterns (EC_{50} values for avoidance and reproduction tests of 0.43 and 1.40 mg imidacloprid kg^{-1} dry soil, respectively). Effects on collembolans *F. candida* were negligible. Imidacloprid presented moderate leachability, with recovery rates that ranged from 25.4 to 50.4% of the amount present in soils and concentrations in water extracts from 13.05 to 71.8 µg L^{-1}. Standard aquatic ecotoxicity tests were not able detect chronic or acute toxicity in standard test organisms. Nonetheless, concentrations of the insecticide in water extracts were high enough to pose a lethal threat to several other non-standard aquatic organisms.

Keywords: *Imidacloprid, ecotoxicity, water-extracts, earthworms*

1. INTRODUCTION

Despite the potential harmful effects of pesticides, the massive application of plant protection products is necessary in order to provide enough food to satisfy the demands of an increasing human population. Neonicotinoids are a relatively new group of systemic insecticides developed in the 1980s and first commercially available in the form of imidacloprid since early 1990s (Kollmeyer et al. 1999). They bind to the post-synaptic nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects, thereby disrupting their nerve impulses. Due to their systemic activity, high toxicity to insects, low toxicity to vertebrates and versatile application, neonicotinoids are among the largest selling and most used pesticides worldwide (Elbert et al. 2008; Jeschke et al. 2011; Main et al. 2014). Within this group of insecticides, imidacloprid-containing formulations account for up to 41% of the neonicotinoids market, becoming the second most used agrochemical worldwide (Jeschke et al. 2011; Pollack 2011).

The prophylactic use of imidacloprid during the last decades has led to serious environmental concerns because of its chemical properties. Regardless of the application route of imidacloprid-containing formulations, the bulk of the active ingredient ends up in soil, where it is subjected to various transformation and transportation processes. Due to its high persistence because of a generally long half-live in soils, non-target soil organisms and terrestrial pollinators are usually exposed to fluctuating concentrations of the insecticide. During the last decades, detrimental effects after exposure to imidacloprid have been documented in terrestrial snails (Radwan and Mohamed. 2013), beetles (Russell et al. 2010), earthworms (Luo et al. 1999; Capowiez et al. 2003; Dittbrenner et al. 2010; Dittbrenner et al. 2011), collembolans (Idinger 2002; Alves et al. 2014) and bees (Decourtye et al. 2004; Dively et al. 2015) among others. Furthermore, its high water solubility, high partitioning and low soil sorption enhance the movement of the neonicotinoid from the terrestrial to the aquatic compartment by spray drift, leaching or surface runoff (Roessink et al. 2013). Concentrations of imidacloprid have been measured in surface and ground waters worldwide (Lamers et al. 2011; Starner and Goh 2013) and toxic effects have been documented in many aquatic non-target organisms (Tisler et al. 2009; LeBlanc et al. 2012, Roessink et al. 2013; Pérez-Iglesias et al. 2014 among others).

In the European Union, ecotoxicological laboratory tests are used as a preliminary step in the assessment of the environmental impacts of pesticides and are required prior to the sale of plant protection products (EC 2009). Most laboratory tests follow standardized guidelines to study the toxic effects that pesticides cause to a set of non target model organisms that play key roles in ecosystem structure and function. Among the invertebrate species mostly recommended for terrestrial ecotoxicological assays, acute and chronic effects of imidacloprid have been reported in *Eisenia fetida* (Dittbrenner et al. 2011; Alves et al. 2013) and *Folsomia candida* (Idinger 2002; Alves et al. 2014). Similarly, aquatic ecotoxicology have been traditionally applied for the toxicity determination of aquatic pollutants (Lopez-Roldan et al. 2012), industrial effluents (Riva et al. 1993; Riva and Valles 1994; Riva et al. 2007) or elutriates of sediments (Pereira-Miranda et al. 2011) among others. Effects of imidacloprid on the aquatic environment have been mostly studied through standard aquatic toxicity tests with the model organisms *Daphnia magna* (Crustacea) and *Raphidocelis subcapitata* (Chlorophyta) (Pavlic et al. 2005; Jemec et al. 2007; Tisler et al. 2009; Malev et al. 2012). Unfortunately, the application of ecotoxicity tests for the regulation of pesticides have traditionally focused on parental compounds, passing over the fact that are commercial formulations instead of pure active ingredients the ones applied in the environment. This approach neglects the effects of some co-formulants and solvents present in commercial formulations that can be more important than the active substances to non-target organisms (Anderson and Roberts 1983; Neves et al. 2001) due to its own toxicity or through the modification of the toxicity and bioavailability of the pesticide (Malev et al. 2012). Furthermore, it is known that the leaching potential of pesticides is affected by the type of formulation, surfactants and adjuvants (Camazano et al. 1995; Hall et al. 1998).

Despite the amount of available data regarding the impacts of imidacloprid to non-target organisms, data on the toxicity of imidacloprid-containing formulations is scarcer. Data on such commercial products is required since some studies revealed a higher toxicity and leaching potential of the commercial formulation in comparison with the active ingredient (Gupta et al. 2002; Jemec et al. 2007; Malev et al 2012). In order to widen the available information on this formulation, we studied the environmental impacts associated to the field application rates of Confidor® 20SL.

Table 1. *Physical-chemical parameters of the test soil. C/N: carbon-nitrogen ratio; CEC: cation exchange capacity*

Moisture (%)	pH	Organic carbon (%)	Organic matter (%)	Total nitrogen (%)	C/N	$N\text{-}NO_3$ (mg/kg)	CEC meq/100g	Textural class
3.0	7.2	6.2	10.7	0.4	16.9	15	22.8	Loamy

Effects on the terrestrial compartment were assessed through standard ecotoxicity tests that evaluated the mortality, inhibition of reproduction and avoidance behavior of earthworms *E. fetida* and avoidance of collembolans *F. candida* after exposure to treated soils. Impacts on the aquatic compartment were assessed through the leaching of treated soils and the evaluation of the acute effects of the water extracts to the non-target aquatic invertebrate *D. magna* and the microalgae *R. subcapitata*. Following this methodology, the main objective of this study was to characterize via lower-tier standard ecotoxicological tests the risk that the application of the recommended field rates of the commercial formulation Confidor® 20SL poses to the aquatic and terrestrial compartments.

2. MATERIALS AND METHODS

A soil from a known natural uncontaminated area near the laboratory was selected for the performance of the tests. Samples were collected from the topsoil (0-20 cm depth), air-dried and sieved through a 2 mm mesh. Several soil parameters were analyzed: moisture, pH, organic carbon, organic matter, total nitrogen, C/N ratio, N-NO_3, cation exchange capacity and texture (Table 1).

The insecticide Confidor® 20SL (soluble concentrate, 20% imidacloprid (w/v)) was purchased from Bayer (Germany). Toxicity tests were performed in a range of concentrations that included the lowest and highest application rates recommended by the manufacturer (0.5 and 4 L Confidor ha^{-1}, respectively), two intermediate concentrations (1 and 2 L Confidor ha^{-1}) and a concentration of 8 L Confidor ha^{-1} to cover the worst case scenario of an excessive application of the insecticide. Assuming a depth of incorporation in the soil profile of 0-5 cm and a density of 1.5 g/cm^3, the application rates of Confidor amounted to 0.78-1.56-3.1-6.20-12.4 mg per kg of soil dry weight (dw) and corresponded to 0.13-0.26-0.5-1-2 mg of imidacloprid kg^{-1} dry soil respectively. The application of the formulation into the soil consisted in preparing a stock solution of 1000 mg Confidor L^{-1} in deionized water. Different spiking solutions were applied to the soil in order to provide the desired concentrations of test substance and a moisture content of 60% of the WHC. Soils were carefully mixed to ensure an evenly distribution of the pesticide and left overnight for equilibration. Only deionized water was added to the controls.

Water-extracts were obtained from each soil following the British Standard EN 12457-2 (2002). Soil samples were incorporated to 2-L glass vessels at a ratio of 1 kg/10 L, corresponding to 0.1 kg of soil per liter of deionized water. Vessels were placed at a rotating apparatus and mixed during 24 hours at a temperature of 20±2ºC. After a settling period of 15 minutes, samples were centrifuged (2000g, 10 minutes) and filtered. The supernatant was kept refrigerated until use. The concentration of imidacloprid in the leachates was analyzed by SAILab (Cerdanyola del Vallès, Barcelona, Spain) by High Performance Liquid Chromatography/MS (Agilent 1200 LC/ Applied Biosystems 3200 LMS).

Synchronized cultures of earthworms *E. fetida* and collembolans *F. candida* were obtained from the Centre for Research and Innovation in Toxicology of the Technical University of Catalonia (UPC) in Terrassa (Spain). Earthworms were bred in a cow manure–peat mix (1:1, w/w) at a temperature of 20±2 ºC and under a 16:8 light:dark photoperiod and were fed once a week with moistened bread. Forty-eight hours prior to starting the tests, adult clitellate animals were acclimated to the untreated soil. Only individuals weighting between 300 and 600 mg were selected. Collembolans were cultured in vessels filled with a substrate of plaster of Paris and charcoal (8:1 w/w) at 20±2ºC. Individuals were fed twice a week with granulated dry yeast added in small amounts to avoid spoilage by fungi. Organisms between 10 and 20 days old were selected for avoidance tests. Terrestrial bioassays were performed in a climate-controlled room at 20±2ºC and under a 16:8 light-dark photoperiod except for the acute toxicity test with earthworms that was carried out under constant illumination (400-800 lux).

Lethal effects to *E. fetida* were assessed following the recommendations by the OECD guideline 207 (OECD 1984). Ten individuals were placed in plastic containers containing 500 g of spiked soil (dw). Four replicates were prepared per test concentration. The percentage of mortality and pathological symptoms were monitored after 7 and 14 days of exposure. As no mortality was expected at field application rates of the pesticide, higher concentrations of Confidor were included in order to estimate the LC_{50}.

Effects on the reproduction of earthworms were studied by means of the OECD 222 (2004) guideline. Ten earthworms were placed in 1-L plastic containers filled with 500 grams of dry soil. Four replicates per test concentration and 6 replicates for the control were prepared. Animals were fed weekly with 2 grams of moistened bread during 4 weeks. After 28 days of exposure, surviving earthworms were sorted by hand and the mortality and changes in biomass were recorded. Juvenile worms and cocoons remained in the test vessels for another 28 days. The number of juveniles was recorded after 56 days by heating the soils in a warm bath at 60ºC for 20-25 minutes and waiting for the juveniles to emerge.

Avoidance tests with *E. foetida* and *F. candida* were carried out according to the ISO 17512 (2008) and ISO 17512 (2011) standards respectively. Tests were performed in plastic containers divided into two equal sections by a vertically introduced plastic card. In the test with earthworms, each side of the vessel (control and test) was filled with 350g (dw) of the corresponding soil and the divider was removed. Ten adult earthworms were placed in the line separating both soils. In the test with collembolans, 25 g (dw) of soil were filled into the corresponding section and twenty springtails were carefully placed on top of the soils. In both cases tests ran with five replicates per concentration. At the end of the test period the plastic card was reinserted and the number of individuals at each section counted. In tests with collembolans, the soil from each section was carefully emptied into two different vessels and flooded with water. After gentle stirring the animals floating on the water surface were counted. Missing animals were considered as dead organisms and discarded for the later calculations. Dual-control tests were carried out with both methodologies (5 replicates each) to guarantee the homogeneous distribution of the organisms in the absence of the test substance.

Toxicity in the aquatic compartment was tested in two model species, the cladocera *D. magna* and the microalgae *R. subcapitata*. Cultures of 15 daphnids were maintained in 2.5 L ASTM hard synthetic water kept at 20±2ºC in a 16:8h light:dark cycle. Culture media were changed

three times per week and an organic extract and a concentrate of *Chlorella vulgaris* were added as food. Neonates were collected daily and only those less than 24 hours old were used in tests. Cultures of the algae *R. subcapitata* were kept under a constant illumination of 4000-5000 lux at 20±2ºC. Only populations in the exponential phase were used for the assays. The acute toxicity test with *D. magna* was carried out according to the OECD Guideline 202 (1984). Four replicates were prepared per leachate. Each replicate consisted in a glass tube with 10 mL of the corresponding leachate and 5 daphnids. The test was performed in an incubator at 21ºC and in the dark. Immobilization was visually recorded after 24 and 48 hours of exposure. Chronic toxicity to *D. magna* was evaluated following the OECD Guideline 211 (1998) for a semi static exposure system. Ten replicates per leachate were prepared, each consisting of a 250 mL glass vessel filled with 75 mL of test solution and one daphnid. During the assay, test solutions were replaced and enriched with seaweed extract three times per week. Animals were fed with a concentrate of *Chlorella vulgaris* (0.1-0.2 mg per day). The assay was carried out in a controlled room for 21 days at a temperature of 20±2ºC and a light:dark cycle of 16:8 hours. The growth inhibition test with *R. subcapitata* was carried out following the recommendations of the OECD Guideline 201 (1984). The test ran with 3 replicates for each water extract from contaminated soils plus the leachate from the control soil and an additional control with algae culture medium. Each replicate consisted in 9 mL of test solution and 1 mL of algal inoculum of known concentration. In order to avoid interferences in the spectrometric measure of the leachates at the end of the test, one extra tube was prepared with 9 mL of leachate, 1 mL of culture medium and no algae. The tubes were placed in a controlled room at 20±2 ºC under constant light (4000-5000 lux) and agitation. After 72 hours of incubation, the absorbance of each replicate was measured at 665 nm with a CECIL CE9200 spectrophotometer in order to determine the final algal concentration.

Results of toxicity tests were calculated as percentages. Differences between treatment means (i.e., different concentrations of Confidor) were tested through Analysis of Variance (ANOVA)(P<0.05). When significant differences were detected, the Dunnet post-hoc test was applied to compare treatment means with the control using SPSS 19.0 (NY, USA) software. NOEC (No observed effect concentration) and LOEC (Lowest observed effect concentration) values were established through this procedure. The percentage of avoidance was calculated following the equation presented in the ISO standards 17512 (2008) and 17512 (2011):

$$x = \left(\frac{n_c - n_t}{N}\right) \times 100$$

where x is avoidance, expressed as a percentage; n_c is the number of individuals in the control soil; n_t is the number of individuals in the test soil and N is the total number of individuals. The significance of the avoidance responses were analyzed using the Fisher Exact test (Zar 1998). A two-tailed test were used in the analysis of the dual-control test and a one-tailed test was used for the polluted soils. The null hypothesis assumed an even distribution of individuals between both soil sections and was rejected for a probability equal or lower than 0.05. Median lethal concentration (LC_{50}) values and effective median concentration values (EC_{50}) were estimated by the Probit method following logistic regressions with Statistica software version 8.0 (OK, USA) and Minitab 13.20 software (PA, USA) respectively.

3. RESULTS AND DISCUSSION

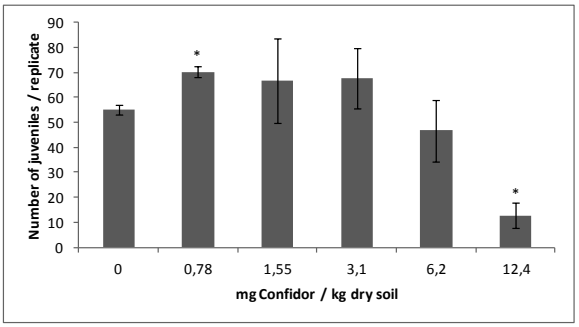
The exposure of soil invertebrates to field doses of Confidor in standard ecotoxicity tests showed marked differences in sensitivity between endpoints and test species. Mortality of earthworms occurred at concentrations higher than 19.77 mg Confidor kg^{-1} (soil dw) (LOEC) (Table 2) and the LC_{50} was estimated at 24.71 mg kg^{-1} dry soil (corresponding to 4.23 mg imidacoprid kg^{-1} dry soil), indicating that the recommended doses of the formulation did not represent a lethal threat to *E. fetida.* Similar toxicity values were reported by Luo et al. (1999) and Gomez-Eyles et al. (2009) using pure imidacloprid as test substance (LC_{50} values of 2.30 mg kg^{-1} soil dw and 2.36 mg kg^{-1} soil dw respectively). On the other hand, studies by Kreutzweiser et al. (2008) and Alves et al. (2013) reported LC_{50} values 10 times higher (25 and 25.53 mg imidacloprid kg^{-1} soil dw respectively) after applying the commercial imidacloprid-containing formulations Merit Solupak® and Gaucho®. Differences in LC_{50} values between studies were partly explained by variations in experimental parameters like soil organic matter, texture or time of exposure (Kula and Larink 1997) although the influence of certain components from commercial formulations to the overall toxicity of the product was not discarded.

Table 2: *EC_{50} (effect concentration 50%), LC_{50} (lethal concentration 50%), confidence intervals (95%), LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) values of Confidor / imidacloprid estimated for earthworm mortality, reproduction and avoidance tests. Values presented in [mg Confidor /kg soil dw] / [mg Imidacloprid /kg soil dw]*

Test	EC_{50}(LC_{50})	Lower limit (95%)	Upper limit (95%)	LOEC	NOEC
Mortality	24.71/4.23	23.30/3.99	26.20/4.48	19.77/3.38	15.21/2.6
Reproduction	8.41/1.40	5.38/0.90	12.87/2.15	12.40/2	6.20/1
Avoidance	2.57/0.43	1.86/0.31	3.21/0.54	0.78/0.13	<0.78/<0.13

The reproduction test gave varying results depending on the concentration of pesticide in soil. *E fetida* produced a significantly higher number of juveniles (Dunnet's test, P < 0.05) in soils treated with the lowest application rate of imidacloprid than in untreated soils (Fig. 1). On the other hand, significant detrimental effects on the reproductive output occurred at twice the highest recommended dose (12.4 mg Confidor kg^{-1} soil dw)(LOEC). The EC_{50} for the reproduction test was estimated at 8.41 mg Confidor kg^{-1} soil dw (corresponding to 1.40 mg imidacloprid kg^{-1} soil dw) (Table 2), a concentration that could be easily reached if the formulation is not properly employed in terms of applied concentrations or time between applications. A similar EC_{50} value (1.41 mg kg^{-1} soil dw) was reported by Gomez-Eyles et al. (2009) using pure imida-

cloprid as test substance whereas a study by Alves et al. (2013) observed a significantly lower toxicity (EC_{50} value of 4.07 mg imidacloprid kg^{-1} soil dw) of a imidacloprid-containing formulation. Luo et al. (1999) and Capowiez and Berard (2006) linked the decrease in the reproductive output to the damage exerted by imidacloprid to spermatozoa of earthworms. It was not concluded whether differences in toxicity between studies were due to the experimental conditions or to the nature of the test substance (active ingredient or commercial formulation). Additionally, it is noteworthy the hormetic response that Confidor triggered in the reproductive output of exposed earthworms. An enhanced reproduction rate was previously documented by Senapati et al. (1992) and Suthar (2014) after exposing earthworms to low concentrations of the pesticides malathion and methyl parathion respectively although the biochemical mechanism of this response is not clear yet. Similar results have not been reported for other neonicotinoids or neonicotionid-based formulations. Regarding the reduction of body weight, it followed the same pattern than juvenile production, with an average weight loss lower than controls at low application rates and significantly higher at high test concentrations (Fig. 1).



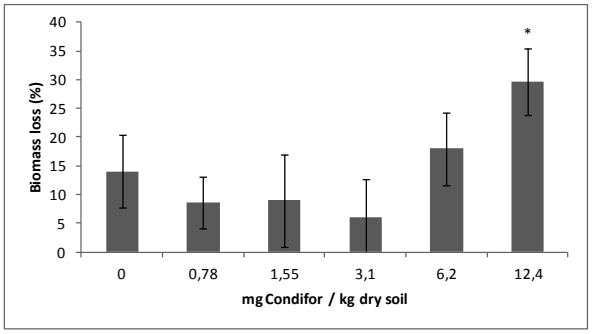


Figure 1: *Effects of varying concentrations of Confidor on the reproductive output and weight loss of E. Fetida in reproduction tests. Data presented as treatment means ± SD(N=4). Asterisks indicate significant differences with controls (Dunnet's test, P < 0.05).*

Earthworms exhibited a significant avoidance behavior in response to the presence of all test concentrations of the formulation (Figure 2). The LOEC value was established at the lowest tested concentration, corresponding to the minimum application rate recommended by the manufacturer (Table 2). Furthermore, the EC_{50} value was estimated at 2.57 mg Confidor kg^{-1} soil dw, within the range of recommended doses. According to Hund-Rinke and Wiechering (2001), soils contaminated with concentrations of Confidor higher than 1.56 mg kg^{-1} soil dw presented a reduced habitat function and should be considered as toxic to earthworms since they presented avoidance responses higher than 60% (i.e more than 80% of individuals remained at the control section of the test chamber). Our results were in accordance with those from Alves et al. (2013) who estimated an EC_{50} value of 0.11 mg kg^{-1} in *Eisenia andrei* for a commercial formulation of imidacloprid. In contrast, Capowiez and Bérard (2006) reported no avoidance response of earthworm species *Aporrectodea nocturna* and *Allolobophora icterica* after exposure to 0.5 and 1 mg kg^{-1} (soil dw) of Confidor® 200 SL despite previous studies documented behavioral alterations on burrow length, overall distance travelled and rate of burrow reuse under the same experimental conditions (Capowiez et al. 2003). Similarly, earthworms exposed to the pesticide in our study presented an altered locomotion pattern. After the increase in the avoidance response observed at 0.78 and 1.56 mg Confidor kg^{-1} soil dw, the behavioral response turned stable while increasing test concentrations. A study by Pereira et al. (2010) reported that the exposure of *E. Andrei* to the carbamate insecticide methomyl induced a inhibition of the Acetylcholine esterase activity that led to hyperactivity in the test organisms and in consequence to the adoption of an irregular avoidance behavior. Similar conclusions were postulated by Martínez Morcillo et al. (2013) after exposing earthworms from the species *Lumbricus terrestris* to chlorpyrifos, another insecticide known to affect the nervous system of soil invertebrates. Based on behavioral alterations reported by Capowiez et al. (2003) and the mechanism of action of imidacloprid, we hypothesized that the exceeding of certain toxicity threshold somehow altered the locomotive ability of the test organisms and led to an erratic movement pattern, thus causing the stabilization of the avoidance response. In the case of collembolans, an avoidance behavior in response to the application of Confidor recommended doses was not detected at any test concentration. Furthermore, a significant preference for the contaminated soil (Fisher exact test, $P < 0.05$) was observed at concentrations of 3.1 and 12.4 mg Confidor/kg dw (data not shown).

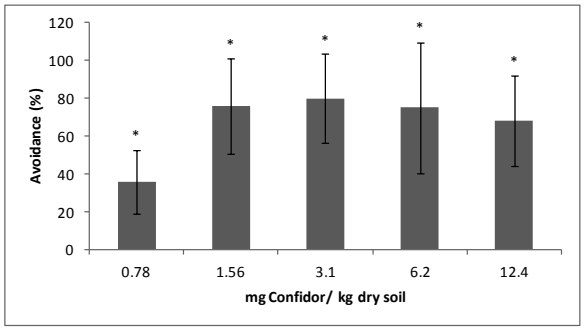


Figure 2: *Avoidance response (%) of E. fetida (mean ± SD)(N=5) to varying concentrations of Confidor in avoidance tests. Asterisks indicate significant differences with the control (Fisher's test, P < 0.05).*

To determine the leaching potential of imidacloprid and its risk for aquatic organisms, concentrations of imidacloprid were determined in water extracts from contaminated soils (Table 3). The concentrations of active ingredient in leachates ranged from 13.05 µg L^{-1} (corresponding to the soil treated with 0.26 mg imidacloprid kg^{-1} dw) to 71.8 µg L^{-1} (2 mg imidacloprid kg^{-1} soil dw) and were positively correlated with concentrations in test soils ($r = 0.910$, $P < 0.05$,

Spearman). The concentrations of imidacloprid in water extracts were within the range estimated by Fossen (2006) for chronic and acute surface water exposures (17.24 and 36.04 µg L^{-1} respectively) or after accidental direct spray in a pond or stream (22 µg L^{-1})(SERA 2005). The recovery of the pesticide ranged from 25.4% to 50.4% of the total amount previously spiked in soil. Recovery rates were in accordance with the relatively high water solubility (0.5 to 0.6 g L^{-1}) and low octanol-water partitioning coefficient (Log (Pow)=0.57) of imidacloprid reported by other authors (Gupta et al. 2002; Kurdwadkar et al. 2014) but were higher than expected according to the high organic carbon content of our soil, a parameter positively correlated with imidacoprid sorption in soils (Cox et al. 1998).

Table 3: *Concentration of imidacloprid in water extracts from contaminated soils. Means ± Standard deviations (N=3).*

mg Confidor / kg soil (dw)	mg imidacloprid / kg soil (dw)	Water extract (µg/L leachate)	Recovery rates (%)
0.78	0.13	< QL	-
1.56	0.26	13.05±3.04	50.35±11.95
3.1	0.5	16.35±4.60	32.70±9.19
6.2	1	25.4±8.21	25.4±8.21
12.4	2	71.8±0	35.9±0

QL (quantification limit): 1 µg/L

Although the highest concentration of imidacloprid determined in water extracts was almost 10^3 times lower than LC_{50} values found in bibliography for *D. magna* (85 mg L^{-1}) (Fossen 2006), mortality tests were performed since previous studies reported the higher toxicity of imidacloprid-containing commercial formulations to *D. magna* due to the presence of toxic adjuvants (Jemec et al. 2007). The exposure to the leachates caused no mortality after 48 hours of exposure in the acute toxicity test and 21 days in the reproduction test. Similarly, differences with the control in the number of neonates per adult, brood size, day of first brood and number of broods per adult in the chronic test were not detected (LOEC value in chronic tests estimated between 2.5 and 10 mg L^{-1} (Jemec et al. 2007)). Regarding the effects on the microalgae *R. subcapitata,* algal growth rates in water extracts from all soils (including the untreated soil) were significantly lower than in algal culture medium (data not shown). However, no significant differences in growth inhibition were found between soil leachates. Consequently, algal growth inhibition was related to the fact that water parameters deviated from the standard test medium and not to the presence of the insecticide in soil leachates. Results with this model organism were expected based on the insecticidal type of action of imidacloprid and its estimated EC_{50} values (> 600 mg L^{-1})(Daam et al. 2013) although previous studies reported the high toxicity to algae of some Confidor® 200 SL co-formulants (Malev et al. 2012). We hypothesized that the lower toxicity detected in our study was related to the fact that in previous studies the commercial formulation was directly spiked into water while we used leachates from contaminated soils. Since the purpose of adjuvants is associated to the fixation of the pesticide in soil, we expected a lower leachability of potentially toxic co-adjuvants.

Despite the low toxicity of leachate concentrations of imidacloprid to the standard organisms *D. magna* and *R. subcapitata*, the presence of the active ingredient in the water extracts was high enough to represent a lethal or sublethal threat to several other non-standard, freshwater macroinvertebrate species. Based on the available bibliography, Daam et al. (2013) reported that a concentration of 52 µg of imidacloprid L^{-1} (value that could be easily reached in soils if Confidor is improperly applied) was expected to produce 50% affection to 25% and 79% of the crustacean and insect taxa respectively. Furthermore, Roessink et al. (2013) documented LC_{50} and EC_{50} values for the non-standard insect species *Notonecta* spp., *Micronecta* spp*., Limnephilidae*, *Caenis horaria* and *Cloeon dipterum* and the macrocustacean *Gammarus pulex* close or below 25 µg imidacloprid L^{-1} , a concentration of active ingredient reached in our leachates.

4. CONCLUSION

Our study pointed out that the application of recommended field doses of the imidacloprid-containing formulation Confidor® 20SL represents a potential threat for the environment. Although mortality was not reported, the exposure to the pesticide caused sublethal effects to *E. fetida* earthworms. The influence of some co-adjuvant and solvents to the overall toxicity of pesticide formulations was observed after comparing results from terrestrial ecotoxicity tests with imidacloprid with those from commercial products. Confidor presented toxicity levels in terrestrial standard ecotoxicity tests closer to those from the active ingredient than to other commercial formulations. Additionally, reproduction and avoidance tests with earthworms showed responses that had not been previously reported, highlighting the need to keep studying the impacts of massively-applied pesticides.

The application of Confidor® 20SL to agricultural soils posed a risk to the aquatic compartment due to the high leachability of imidacloprid. Despite the low response of aquatic standard ecotoxicity tests to the presence of the pesticide or to other components of the formulation, final concentrations of the insecticide in the aquatic compartment were high enough to represent a lethal threat to many other non-standard, non-target aquatic organisms, thus emphasizing the need for testing organisms from different taxonomical groups when assessing the environmental risks posed by pesticides.

ACKNOWLEDGMENTS

The authors thank Dr. Juan Ribó for his support in the performance of the study. This research was funded by Universitat Politècnica de Catalunya (UPC) and R&D Gestió i Serveis Ambientals S.L. (Spain) through a doctoral grant to Jaume Bori (Beca UPC Recerca 2012-2016) and by the Spanish Ministry of Economy and Competitiveness through the project SOILBIOMONITOR (CTM2010-18167).

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Food and Energy Security
Open Access
REVIEW

Pesticides, environment, and food safety

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Keywords
agrochemicals, environmental health, food production, pesticides, residues

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Received: 20 February 2017; Revised: 28 April 2017; Accepted: 1 May 2017

Food and Energy Security **2017; 6(2): 48–60**

doi: 10.1002/fes3.108

Abstract

Agrochemicals have enabled to more than duplicate food production during the last century, and the current need to increase food production to feed a rapid growing human population maintains pressure on the intensive use of pesticides and fertilizers. However, worldwide surveys have documented the contamination and impact of agrochemical residues in soils, and terrestrial and aquatic ecosystems including coastal marine systems, and their toxic effects on humans and nonhuman biota. Although persistent organic chemicals have been phased out and replaced by more biodegradable chemicals, contamination by legacy residues and recent residues still impacts on the quality of human food, water, and environment. Current and future increase in food production must go along with production of food with better quality and with less toxic contaminants. Alternative paths to the intensive use of crop protection chemicals are open, such as genetically engineered organisms, organic farming, change of dietary habits, and development of food technologies. Agro industries need to further develop advanced practices to protect public health, which requires more cautious use of agrochemicals through prior testing, careful risk assessment, and licensing, but also through education of farmers and users in general, measures for better protection of ecosystems, and good practices for sustainable development of agriculture, fisheries, and aquaculture. Enhanced scientific research for new developments in food production and food safety, as well as for environmental protection, is a necessary part of this endeavor. Furthermore, worldwide agreement on good agriculture practices, including development of genetically modified organisms (GMOs) and their release for international agriculture, may be urgent to ensure the success of safe food production.

Introduction

Pesticides and agrochemicals, in general, became an important component of worldwide agriculture systems during the last century, allowing for a noticeable increase in crop yields and food production (Alexandratos and Bruinsma 2012). Notwithstanding, the exponentially growing human population further stresses the need for enhancing food production. This need is aggravated by conflicts that paralyze food production and dislocate millions of refugees and, together with the effects of climate changes on agriculture, worsen scarcity of food in many regions and call for renewed efforts in food production (UN 2015).

At the same time, during the last decades we realized that agrochemical residues did spread in the environment, causing significant contamination of terrestrial ecosystems and poisoning human foods (Carson 1962; EEA 2013). In addition, contamination of aquatic systems by pesticide residues around the world – illustrated herein with case studies in tropical coastal ecosystems – repeatedly compromised also aquatic food resources, fisheries, and aquaculture.

Paths, alternative to the intensive use of crop protection chemicals, are open to trial and assessment. However,

the selection of future paths for enhanced food production shall be made through wise and science-based decision-making processes. Scientific research for developing food production and enhancing food safety, as well as environmental protection, is thus a necessary part of this process.

This article reviews the main issues related to pesticide residues, their environmental fate, and effects and discusses pathways for enhanced food safety.

The Role of Fertilizers and Pesticides in Agriculture

Agricultural production markedly increased since the beginning of the 20th century to cope with demographic growth. In about one century, population numbers exploded from 1.5 billion in 1900 to about 6.1 billion in 2000, which corresponds to an increase in world population three times greater than during the entire history of humanity. The world has added one more billion people since 2003, and at the current growth rates, it is estimated that world population will be of about 9.4–10 billion by 2050 (UN 2015).

The increase of world population in the 20th century would not have been possible without a parallel growth in food production, and this was achieved due to fertilizers. Organic fertilizers ("guano") were incipiently used by the end of the 19th century, but the introduction of mineral phosphate fertilizers took over in the beginning of the 20th century and continuously increased up to our days (Gilland 2015). The use of phosphates, together with development of improved crop varieties with higher yields, allowed for an unprecedented increase in agriculture productivity, the "green revolution," and the production of cereals more than duplicated per unit surface area of agriculture land (Brown 1995; Carvalho 2006). For example, in the USA from 1950 to 1990, the cereal production grew at 2.2% per year, although it has slowed down afterward (Brown 1995 2011). The growth of human population and the world production of phosphates for use as fertilizers were significantly and positively correlated over the last century (Roser and Ortiz-Ospina 2017), with a R^2 = 0.97 for the period 1900–1988 (Hendrix 2011) (Fig. 1).

From the 1940s onwards, further increase in food production was allowed by the introduction of synthetic crop protection chemicals. Worldwide pesticide production increased at a rate of about 11% per year, from 0.2 million tons in 1950s to more than 5 million tons by 2000 (FAO 2017; Fig. 2). Pesticides, or crop protection chemicals, include several groups of compounds, namely organochlorine, organophosphate, carbamate, pyrethroids, growth regulators, neonicotinoids, and now biopesticides, which have been developed one after the other. Pesticide sales

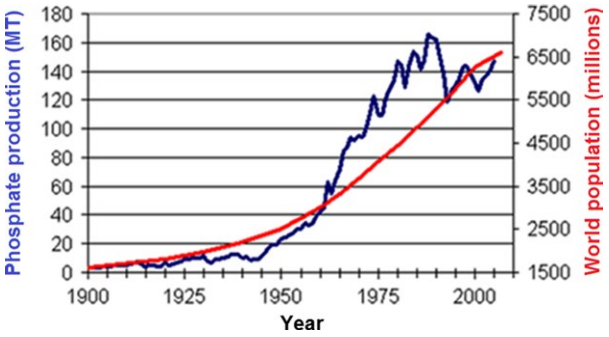

Figure 1. Increase of world population and phosphate rock production during last century (Modified from Roser and Ortiz-Ospina 2017).

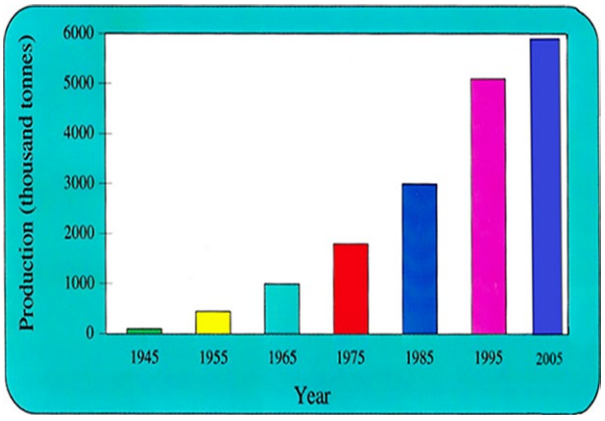

Figure 2. World production of formulated pesticides (based on FAO Statistics).

have increased for all types of pesticides, but herbicides were the group that expanded the most followed by insecticides and fungicides (Fig. 3).

The use of pesticides has not been the same across the world due to the cost of the chemicals (most of them patented), but also due to the cost of man power and the specific pests of each climatic/geographic region. Average application rates of pesticides per hectare of arable land have been computed by FAO and the highest average values, attaining 6.5–60 kg/ha, occurred in Asia and in some countries of South America (Fig. 4). While in North America and West Europe, the use of herbicides intensively applied in agriculture and in urban areas boomed in the last decades; in Asia, the use of herbicides remained low and contrasting with the use of insecticides that was very high (Fig. 4).

Early synthetic pesticides developed to control agriculture pests, such as DDT, were intensively used also for control of cattle ticks and human parasites in North America, Europe, and elsewhere (Fig. 5) and, although banned today, still are popular food preservatives of sun dry fish

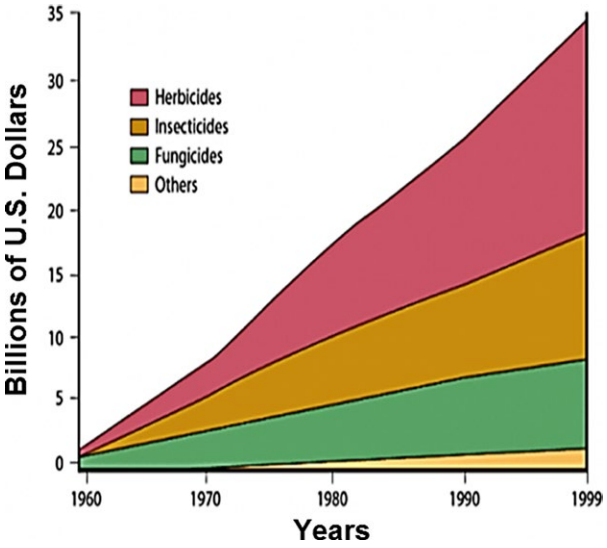

Figure 3. Estimated worldwide annual sales of pesticides (herbicides, insecticides, fungicides, and others in billion dollars; modified from Roser and Ortiz-Ospina 2017).

in South Asia and remain in use, sometimes illegally, to control malaria vectors and household pests in urban areas in the tropics (Taylor et al. 2003).

Environmental Fate and Effects of Pesticide Residues

Application of pesticides in agriculture has been made with the help of several techniques, from the manual spraying by workers on foot to truck- and airplane-based spraying techniques. At different times in different regions, some or all these techniques have been used.

Many cases of intoxication of farmers, rural workers, and their families did occur during pesticide applications and were documented in reports on poisoning and effects of synthetic chemicals on human health. It was reported that unintentional poisonings kill an estimated 355,000 people globally each year, and such poisonings are strongly associated with excessive exposure and inappropriate use of toxic chemicals (WHO 1990, 2012, Alavanja 2009; Alavanja and Bonner 2012).

Dispersion of pesticide residues in the environment and mass killings of nonhuman biota, such as bees, birds, amphibians, fish, and small mammals, were also reported (Köhler and Triebskorn 2013; Paoli et al. 2015; WHO 2017). Early reports and structured incident reporting systems certainly helped to develop regulations for pesticide applications, including dosage of chemicals and best periods of application (Hester and Harrison 2017). Over the years, a considerable research effort was developed also to understand the behavior of these chemicals in the environment, including their cycling and fate as well as their toxicity to biota.

Soon after the start of synthetic chemicals use, it was realized that the application of crop protection pesticides was causing contamination not only at local scale but also at global scale (Carson 1962; Fig. 6).

At local scale, chemicals applied on crops, as for example toxaphene applied in cotton crops in Nicaragua, remained in soils year after year and were carried by surface runoff to watersheds and coastal lagoons where residues contaminated aquatic biota (Carvalho et al. 1992, 2003). DDT applied to crops was often reported also to be transported to the aquatic environment where it is rapidly metabolized to DDE and bio-accumulated in aquatic food chains being returned eventually to humans (Kale et al. 1999). Endosulfan

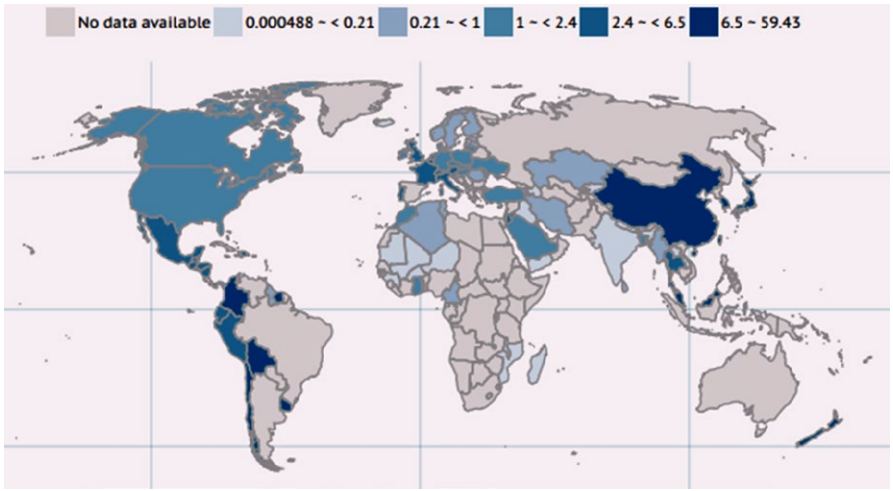

Figure 4. Use of pesticides per hectare of arable land, kg/ha, in the years 2005–2009 (FAO 2013).

Figure 5. DDT application on humans and cattle, around the 1940s (photos from Internet).

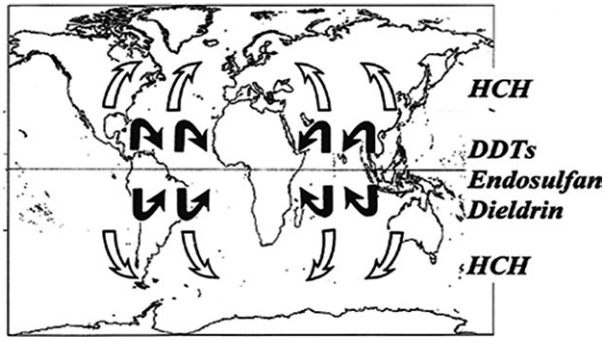

Figure 6. Volatilization and atmospheric transport of pesticides from tropical regions toward the poles.

was found to be metabolized by bacteria into endosulfan sulfate and could persist in soils and in aquatic sediments as a toxic chemical (Carvalho et al. 2002a,b). In general, these chemical compounds could undergo several chemical transformations and be transferred among environmental compartments, reaching other ecosystems outside the area of application and exerting toxic effects on nontarget species (Taylor et al. 2003).

At global scale, compounds such as hexacyclohexanes (HCH), chlordane, and toxaphene applied in fields in the south of USA were volatilized, transported by atmospheric processes, condensed in cooler climates, and deposited from the atmosphere onto the Great Lakes at Canada (Li and Jin 2013). The same did occur with HCH applied on rice fields in South Asia and transported to higher latitudes (Iwata et al. 1993; Simonich and Hites 1995). The most volatile compounds were more rapidly transported by atmospheric processes, reaching regions far away from the application areas (Fig. 6). This evaporation-condensation process was first observed with organochlorine compounds (OCs), but later was reported also for organophosphates (OPs), such as chlorpyrifos, that volatilized from application on banana plantations in the inter tropical region of Central America and reached the ice pack in the Artic (Garbarino et al. 2002). This global scale dispersion process could have been predicted based on Henri's Law, which relates the volatility (fugacity) of compounds from liquid media to the air as inversely related to water solubility, and on van 't Hoff equation that parameterizes the effect of temperature on volatility of compounds (Rand 1995).

The organochlorine (OC) pesticides of first generation were soon reported as environmentally persistent, remaining long time in soils and sediments and accumulating in nonhuman organisms with devastating toxic effects at population level (Köhler and Triebskorn 2013). Organochlorine residues are generally transferred also in the food chains with impact on human health (discussed further in section Human Exposures to Residues and Public Health Concerns, below). Development of resistance by pests to these OC chemicals urged to replace them by new and less persistent chemicals, such as organophosphate (OP), carbamate, and pyrethroid compounds, supposedly more specific in the fight to pests too (The Agrochemicals Handbook 1991).

Research on all these chemicals, in particular using carbon-14 (^{14}C)-labeled compounds, shed light on the degradation rates in soils and in aquatic environments, and in accumulation by nontarget biota (e.g. Carvalho et al. 1992, 1997). Organochlorine compounds, such as DDT, HCH, heptachlor, toxaphene, and lindane, are in general, much more persistent and their residues may remain in soils and sediments over days, weeks, and even years (Fig. 7; Carvalho et al. 2002a,b, 2003). In the aquatic environment, OPs were expected to degrade rapidly, but experimental research has shown that they persist days/weeks and are accumulated by crustacean and fish (Carvalho et al. 1992). Moreover, once released into the aquatic systems, these compounds are bio-accumulated in a few minutes and undergo also partitioning between water and particulate matter/sediment, with partitioning coefficients

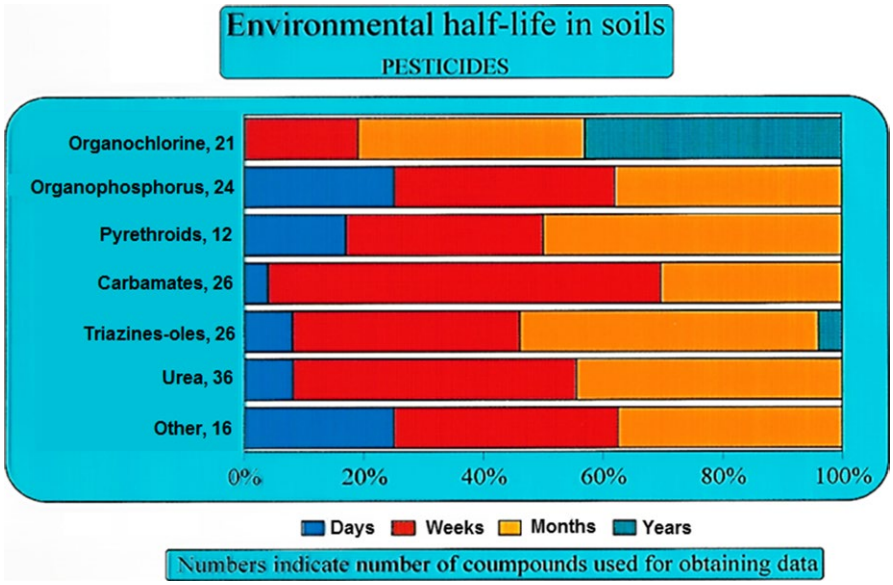

Figure 7. Environmental persistence (half-lives) of pesticides in soils by chemicals group. Numbers indicate number of compounds for which data are available (modified from Carvalho et al. 1997).

(K_p) that are positively correlated with the octanol-water partitioning coefficients (k_{ow}) of the compounds. Experimental studies in mesocosmos have shown that compounds, such as endosulfan, could persist long time as well and were accumulated up to the point to represent a toxicological risk to aquatic biota (Carvalho et al. 1999, 2002a,b; Nhan et al. 2002). Taking into account chemical properties and persistence in the environment, chemicals applied in agriculture fields may be transported and reach other ecosystems (Fig. 8). As predicted from results of experimental studies using ^{14}C-labeled compounds, the endosulfan applied in coffee and leguminous plantations and at the time seen as a nonpersistent compound, through field investigations was consistently found in aquatic systems near agriculture regions in central and North America countries (Carvalho et al. 2002b, 2009a,b). Later, it was verified that endosulfan residues are widespread in the environment and it is considered nowadays a "global pollutant" (Weber et al. 2010).

Compounds of different chemical groups have different toxic mechanisms and act on pest organisms in different ways. Organochlorine compounds (insecticides, e.g., aldrin, DDT, HCH, heptachlor, chlordane, endosulfan) are in general very effective contact insecticides, and they are structurally related to steroid hormones and act on the respective hormone receptor (Tebourbi et al. 2011). Organophosphates (mostly insecticides, e.g., parathion, malathion, chlorpyrifos, diazinon, dichlorvos) and carbamates (mostly herbicides and fungicides, e.g., aldicarb, carbofuran, ethienocarb, fenobucarb, methomyl) act as acetylcholinesterase (AchE) inhibitors causing disruption of nervous impulse transmission at synaptic level. Pyrethroids (insecticides, e.g., cypermethrin, deltamethrin, esfenvalerate, fenvalerate) act on the voltage gated-sodium channels in cell membranes disrupting the Na^+ ion flux. The neonicotinoids (insecticides, e.g., acetamiprid, clothianidin, dinotefuran, imidacloprid) act as agonists at the nicotinic acetylcholine receptors (nAChRs), are neurotoxic, and act on the insect's nervous system, resulting in paralysis and death (Tomizawa and Casida 2005).

The mechanism for toxic action is not restricted to target pests, and toxicity is exerted also on nontarget similar organisms causing damage to biodiversity and ecosystems health. OCs impacted heavily the top predators in terrestrial food chains, as birds of prey, and accumulate in adipose tissues of animals and humans, being transferred to newborns with the milk fat, and act as endocrine disruptor (EEA 2013). Organophosphates were reported as highly toxic to arthropods in general, which includes insects but also shrimp, crabs and other crustacean, and also to vertebrates. Pyrethroids have also impact on insects and vertebrates. Many other compounds used, as herbicides have shown effects also on central nervous system and excretory system of mammals (Casida 2009; Singh et al. 2016).

Due to reports on contamination of the environment and toxic effects on biota, considerable efforts have been made to design new chemicals, improve pesticide formulations, application devices, and chemical delivery mechanisms such as the use of degradable nanoparticles as a vehicle to pesticides in an attempt to reduce exposure of biota and environmental contamination (De et al. 2014).

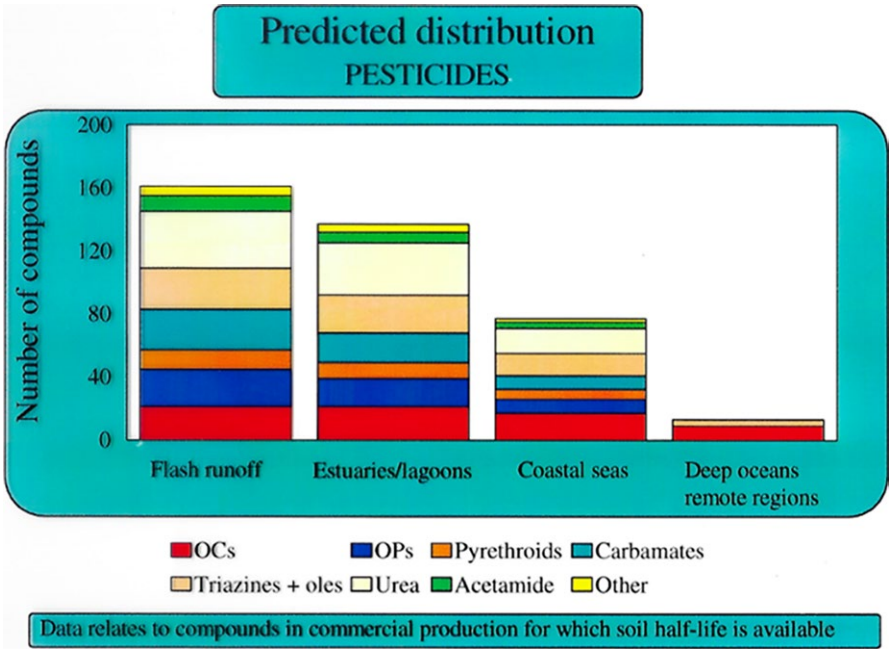

Figure 8. Potential for transport and dispersion of pesticides in the environment with ecosystems that they may reach. Data relate to compounds in commercial production for which soil half-life is available (modified from Carvalho et al. 1997).

Nevertheless, this has not resolved the collateral effects of pesticides and recurrent episodes with new chemicals, including neonicotinoids, have been reported (Bouwman et al. 2013; Hallmann et al. 2014; Park et al. 2015).

Residues in Soils and in Aquatic Environments

Persistent and bio-accumulative chemical compounds, such as DDT, HCH, toxaphene, aldrin, and dieldrin, were banned by the Stockholm Convention, approved in 2002, and have been replaced by environmentally friendly and less bio-accumulative chemicals. This has been the trend over the last decades, and it was driven by the toxicity of chemical residues present in food to humans as well as to chemicals' persistence in the environment and toxicity to nonhuman biota. However, from the massive application of OCs in the past, they are still present in soils, in sediments, and in the biosphere and are toxic. For example, toxaphene in cotton fields is not used anymore in Nicaragua but many years after cessation of applications, the deposit in agriculture soils was still a source of contaminants transported by surface runoff to aquatic environment and a threat to shrimp farming in coastal lagoons (Carvalho et al. 2002a,b, 2003).

Indeed, the ban of persistent OC compounds in agriculture abated application of OC pesticides in many regions but was not the end of concerns about toxic effects of these compounds. Today, we still find these OC compounds in environmental compartments as a legacy of past applications. Soils are the main reservoir of persistent OCs, and soil erosion, surface runoff, and river discharges carry and cycle significant amounts of persistent OCs in the environment. For example, results from the annual surveys of USA pollution trends reported pesticide residues in coastal sediments and biota (mussels and oysters) originated in river catchments. Many years after the ban of these compounds (e.g. DDT, chlordane), they were still present in the coastal environment where they degraded very slowly, as reflected by decreasing concentrations in biota over the years (Fig. 9). Similarly slow decrease of residue concentrations was also recorded in coastal environments of Mediterranean Sea in Europe (Villeneuve et al. 1999).

From a vast study carried out in tropical coastal ecosystems worldwide, it was concluded that pesticide residues were everywhere and were concentrated by marine fauna (Taylor et al. 2003). Other case studies showed similar conclusions, such as in the Manila Bay, Philippines, Mekong River Delta in Vietnam, coastal lagoons of NW Mexico, Laguna de Terminus, Caribbean Sea, Mexico, Todos-os-Santos Bay in Salvador, Brazil, coastal areas of Florida in USA, and North Sea and Baltic Sea in Europe (Carvalho et al. 1997, 1999, 2008, 2009a,b,c). In all these coastal areas, the residues of a large collection of crop protection chemicals, such as DDTs, HCHs, lindane, aldrin, toxaphene, and endosulfan, were determined (Carvalho et al. 2002a,b, 2003; Kimbrough et al. 2008;

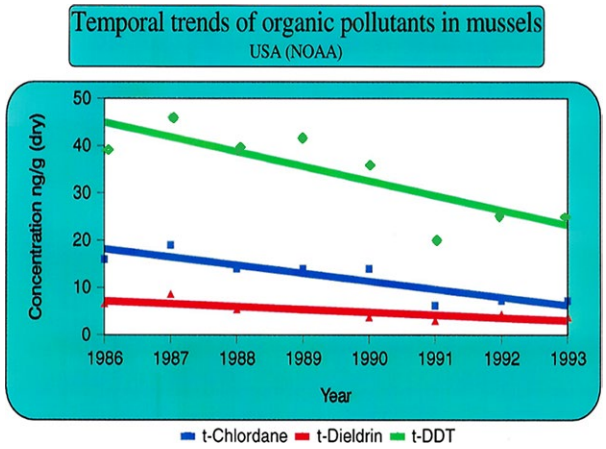

Figure 9. Temporal trends of organochlorine pesticides in coastal mussels. Average concentrations based on data from USA-NOAA Status and Trends Reports. Environmental half-lives in mussels are from 6 to 12 years depending on compound.

Moreno-Gonzalez and Leon 2017). More modern pesticides and other chemicals used in industry, such as PCBs, tributyltin, and pharmaceutical drugs, have been detected also in river waters and coastal areas and often originate in urban wastewater discharges (Barceló and Petrovic 2008).

Pesticide residues carried to the sea are also a threat to large marine ecosystems such as coral reefs. Agrochemical residues are currently monitored in sea water by the Great Barrier coral reef, Australia, where recent studies demonstrated the widespread contamination by pesticides, particularly herbicides which may impinge on symbiotic algae and destroy the coral reef (Lewis et al. 2009; Smith et al. 2012). Similarly, residues of persistent organic contaminants were found in biota in the deep sea, by many still seen as a remote and pristine environment (Jamieson et al. 2017).

Contamination of aquatic bodies by residues from periodic application of pesticides in crops was, thus, found in many environments, and this called for preventive measures. Not much was done at global scale, but near golf greens, where the use of herbicides and fertilizers is intensive, contamination of watercourses and groundwater by residues was of concern, and improved area management was advised and locally introduced through using constructed wetlands (Klaine et al. 1988).

Persistent OCs are not anymore used in Europe also, but HCH, DDT, and lindane still are present in rivers in Europe and are bioaccumulative (McKnight et al. 2015; Rasmussen et al. 2015). Organochlorine compounds residues are almost always present in environmental samples although in decreasing concentrations with the years, as reported, for example, in Denmark. There, the presence of OC residues in river waters is from leaching of legacy applications persisting in soils, but their potential for toxic effects on aquatic fauna remains current (Rasmussen et al. 2015).

More recently, introduced and more degradable pesticides, such as chlorpyrifos, parathion, isoproturon, and mecroprop, are often detected also in river waters (Barceló and Petrovic 2008; Moreno-Gonzalez and Leon 2017). Residues of these new chemicals show the opposite concentration trend in surface waters, with concentrations often increasing over the years, such as for glyphosate (McKnight et al. 2015; Portier et al. 2016). This is very worrisome because this widespread presence of chemical residues compromises natural resources such as water for human consumption including groundwater and water for aquaculture activities.

Water Quality and Biodiversity in Freshwater and Coastal Ecosystems

The monitoring of water quality has been subject to stricter control with the EU Water Directive Framework (Directive 2000/60/EC of the European Parliament and of the Council) that required each contaminant concentration to be below 1 pg/L. Water quality for aquaculture is, however, more difficult to ensure because aquaculture itself makes use of chemicals. For example, salmon production, which in EU provided about 1/3 of fish for human consumption, uses antifouling agents, antibiotics, and chemicals for protection against lice, the main parasite of farmed salmon (SEP 2015). Residues from these chemicals, plus natural toxins from harmful algal blooms, dioxins, and PCBs, put the quality of coastal waters and the aquaculture production under pressure of contamination (SEP 2015). Although there has been a decrease of farmed salmon contamination by organic chemicals over the years, still the consumption of fish is a matter of concern and advice on intake limitation has been given to consumers (Nøstbakken et al. 2015; Ruzzina et al. 2015).

Discharges of industrial waste water and urban sewage into water lines and coastal zones have been a common procedure in most countries. The adoption of urban sewage treatment and their success has always been introduced, or attained late. One may recall the impact of pesticide residues on corals, fisheries, and shrimp aquaculture to understand that contamination has been always followed by ecological disasters and public health impacts, before regulations and mitigation measures were adopted. Increasing awareness of contamination multiplied the monitoring efforts that are continuously developing now. For example, the EU project Ocean of Tomorrow included a several initiatives to control chemical residues in sea food and development of a real-time monitoring system

to respond to these challenges (e.g. Research Project Sea-on-a-Chip; http://www.sea-on-a-chip.eu). Removal of emerging contaminants from industrial waters and treatment of urban waters are also progressing (Barceló and Petrovic 2008).

The importance of controlling contamination of aquatic systems goes beyond the immediate need for water with quality for human consumption. Toxic residues in aquatic systems may eliminate aquatic species, reduce biodiversity, and compromise the functioning of ecosystems. A large research effort has been made in aquatic toxicology to understand bioaccumulation mechanisms and define toxicity levels to species selected as representative (plants, crustacean, fish) and elaborate guidelines for pollution control within tolerated limits (Rand 1995). However, toxic substances even in very low concentrations always bioconcentrate and may act on sensitive species or larval stages of biota impairing the ecosystem healthy functioning and compromising their services (Chagnon et al. 2015; Gilbert 2016). Dramatic examples are the reduction of pollinating insects, elevated concentrations of PCBs, and pesticides in farmed salmon, and the dying of the Great Barrier Coral Reef which may compromise entire ecosystems (Smith et al. 2012; Nøstbakken et al. 2015; Park et al. 2015). Eventually, instead refining toxicity testing and determination of LD_{50}, we should move the efforts to develop processes to remove contaminants from soils and effluents and prevent them to attain aquatic systems and bioaccumulate in food chains.

Human Exposures to Residues and Public Health Concerns

Worldwide, about 25 million agricultural workers experience unintentional pesticide poisonings each year, and it is estimated that approximately 1.8 billion people engage in agriculture and most use pesticides to protect food and commercial products that they produce. A few more are occupationally exposed during the use pesticides in sanitary campaigns and for lawn and garden applications (Alavanja 2009).

To reduce further exposure of population from widespread environmental contamination by these chemicals, it is not surprising that residues from both legacy applications and current agricultural, industrial, and household applications need to be controlled tightly in the environment and in the foods (EFSA 2016).

Currently, pesticide residues in North America and in EU are thoroughly monitored. In general, market foods are compliant with maximum permissible concentrations (MPC) and percentages of samples with detected residues exceeding MPCs fortunately are in small number. For example in the EU, among more than 83,000 food samples from 28 Member States analyzed in 2014, 97% of samples analyzed were within legal limits; of these, 53.6% were free of quantifiable residues, and 43.4% contained residues that were within permitted concentrations (EFSA, 2016). Notwithstanding, in plant products, 154 different substances were found in measurable concentrations including recent and old crop protection chemicals and, although the food authority EFSA assessed the risk to consumers as low, recommendations were deemed necessary to further improve food safety and abate consumers exposure through diet (EFSA 2016).

Exposure to pesticides and synthetic chemicals were related to cancer, obesity, endocrine disruption, and other diseases in humans (Gorell et al. 1998; Bassil et al. 2007; George and Shukla 2011; Mrema et al. 2013; Araújo et al. 2016; WHO 2017). Phasing out persistent chemicals, as agreed in the Stockholm convention, contributed to reduce human exposure to toxic chemicals. Indeed, over the last decades, studies carried out in several countries have shown a consistent decrease of DDTs in human adipose tissues and milk (EEA 2013). Notwithstanding, exposure to chemical residues via water and food ingestion remains for the members of the public (i.e. without occupational exposure), a subject of concern and a burden to public health. Recent reviews of exposure and health impact of pesticides on human health have underlined the burden on human health and re-evaluated the current toxicity of legacy pesticide residues (Mrema et al. 2013). The WHO and IARC, among other organizations, keep under close scrutiny and revision the advisories on toxicity of new and old chemicals. Many agrochemicals were related to prostate cancer and other types of cancer and are increasingly regulated (Singh et al. 2016; ECA 2017).

At present, there is a widespread concern about effects of herbicides on human health, such as glyphosate that is of common use in agriculture and in cities to control weeds, and is a main carcinogenic agent (Araújo et al. 2016; Benbrook 2016). Glyphosate is the most widely applied pesticide worldwide, and in the USA, in 2014 farmers applied glyphosate at a rate of about 1 kg/ha in croplands (Benbrook 2016). The EU set the daily chronic reference dose for glyphosate to 0.5 mg/kg body weight per day, while the US EPA has set glyphosate daily chronic reference dose at 1.75 mg/kg body weight per day. However, recent compilation of toxicological data on glyphosate supports the need for reducing further the daily chronic reference dose to 0.1 mg/kg body weight per day (Antoniou et al. 2012).

In general, the maximum tolerated limits of residues in foods have been decreasing over the years, although exposure has not decreased sufficiently still due to legacy compounds in the environment and new chemicals

introduced. Furthermore, it was recognized that most of the work done in the toxicity field has been reactive to problems and with marginal efficiency in anticipating and preventing the collateral toxic effects (EEA 2013).

Current Trends in Chemicals Control

The data base CAS Registry (www.cas.org) provided by the American Chemical Society includes more than 129 million unique organic and inorganic chemical substances and more than 67 million nucleotide sequences (by April 2017). More than 4000 new substances are added each day. The number of chemicals increased exponentially over the years with an average annual growth rate of about 15% in the last decades (Binetti et al. 2008). In this universe of chemicals, a small fraction is pesticides. In the data base of the US Pesticide Action Network (PAN), 6,400 pesticide active ingredients and their transformation products, as well as adjuvants and solvents used in pesticide products, were listed (www.pesticideinfo.org/). In the EU pesticide database, there are 1359 entries, not all approved for use, and about 700 registered chemicals are in use as pesticides (Eurostat, 2017). However, toxicological information about these chemicals is very poor for most of them.

In the USA, an EPA report of 1998 indicated that no information on toxicity was available for 43% of high production volume chemicals and a full set of toxicity data was available for 7% of them only (USEPA, 1998). A similar situation occurred also in the EU, and a study carried out in Denmark for 100,000 substances listed in the European Inventory of Existing Commercial Chemical Substances (EINECS) concluded that for 90% of them few toxicological data were available (Niemelä 1992).

The EU adopted in 2007 the new policy to control industrial chemicals called REACH (Registration, Evaluation, and Assessment of CHemicals), intended to create a central database on chemicals and entrusting the industry with the responsibility to evaluate and manage the risks of chemicals. In spite of large progress made in improving the knowledge about toxicity and environmental impact of chemicals, control of risks is far from being grasped and controlled (EUROSTAT 2012; EEA 2013). In a recent report, it was appreciated that, in the decade 2004–2013 in the EU, the production of environmentally harmful chemicals averaged about 150 million tons per year, representing about 40% of the total production of industrial chemicals (EUROSTAT, 2014). It was registered also a shift in production from more harmful to less harmful chemicals (based on aquatic toxicity and persistence), but still far from the objectives of sustainable development (EUROSTAT 2014).

EU objectives for 2020 foresee further action to implement REACH and achieve improvements in human life quality and environmental management regarding chemicals (7th EU Environment Action Plan). However, as pointed out before, experiments on hazards and risks cannot follow the same increasing trends for chemicals produced, because this would require very large amounts of expertise and very large amounts of human and laboratory resources to carry out complex tests (Binetti et al. 2008). Thus, timely risk assessment may be delayed.

Can we do better?

The need for producing more food to feed the growing human population is likely to increase (UN, 2015). To meet this goal, several options are open. One option might be to continue the path of intensive use of agrochemicals, including pesticides, with subsidiary research to produce more selective pesticides and improved application techniques. Other alternative options have been proposed and include the use of genetically modified organisms for better yield crops and crops resistant to pests, organic farming, development of new cultivars and recuperation of old cultivars, increased use of bio-pesticides and pheromone traps to control pests, and change of dietary habits of human populations.

The current pathway of applying synthetic crop protection chemicals has been walked through on a circular approach consisting of identification of a pest, development of a chemical, observation of collateral effects and rise of new problems, development of new chemicals, etc. We could consider this as an approach based on the trial and error method. There has been results temporarily achieved, certainly, but they always have come with an associated cost. Today, food and environment contamination with toxic chemicals impinging on public health over several human generations is considered unaffordable. We need to learn the lessons from the past and, desirably, this circle of trial and error should come to an end.

Probably, agriculture and intensive food production may not dispense the use of current agrochemicals in the next few years. Several measures could be introduced to better mitigate their collateral effects in the meantime. For example, introduction of precision application of agrochemicals (as well as precision irrigation) could reduce the amount of chemicals (and water) applied over the fields. Some other simple measures could be also immediately applied everywhere, such as: a) recovery and treatment of contaminated agriculture runoff with installation of wetland stripes suitable to clean up runoff and water drainage; b) reinforce education of farmers and the public in general about chemical hazards; and c) thorough toxicity testing

and proper registration of chemicals and formulations. These measures may help to gain some extra time.

Meanwhile, we should look beyond the present time for sustainable solutions. There is a consensus that intensified research on better food production and production of food with better quality is needed. Furthermore, it is recognized that productive soil is a finite resource (as water) and, in order to ensure continued production of food, the agriculture must go side by side with soil and ecosystems preservation, restoration, and agronomic research on better yield cultivars. Therefore, it is urgent to achieve a generalized agreement on pesticide application and adoption of good agriculture practices, with consideration to Integrated Pest Management (IPM) techniques.

Consumers and the public in general have rejected already the environmental and health costs of hazardous chemicals, and awareness of chemical residues in foods created the demand for clean foods. More food and safer food is, therefore, required, but the human population and natural ecosystems may not survive longer to poor planning and poor agriculture practices. A systematic application of the precautionary principle in the introduction and application of all chemicals, including pesticides, is needed (EEA 2013). This requires thorough risk assessment of chemicals toxicity to environment and humans.

Emerging alternative paths in food production, such as development of GMO varieties and their release for international agriculture without application of the precautionary principle and satisfactory risk assessment, must be avoided. This issue deserves urgent international discussion. An agreement should be reached based on science and on ethical principles for ensuring food security and food safety. Moreover, alternative paths for food production should not repeat the mistakes of pesticide applications and must succeed in ensuring food safety and food security.

Conflict of Interest

None declared.

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COMPARATIVE CHRONIC TOXICITY OF IMIDACLOPRID, CLOTHIANIDIN, AND THIAMETHOXAM TO *CHIRONOMUS DILUTUS* AND ESTIMATION OF TOXIC EQUIVALENCY FACTORS

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Environ Toxicol Chem., **Accepted Article** • DOI: 10.1002/etc.3536

Environmental Toxicology **Environmental Toxicology and Chemistry**
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Running title: Chronic Neonicotinoid Toxicity and Toxic Equivalency Factors

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Submitted 22 February 2016; Returned for Revision 10 April 2016; Accepted 21 June 2016

Abstract: Non-target aquatic insects are susceptible to chronic neonicotinoid insecticide exposure during the early stages of development from repeated run-off events and prolonged persistence of these chemicals. Investigations on the chronic toxicity of neonicotinoids to aquatic invertebrates have been limited to a few species, under different laboratory conditions that often preclude direct comparisons of the relative toxicity of different compounds. Here, full life-cycle toxicity tests using *Chironomus dilutus* were performed to compare the toxicity of three commonly used neonicotinoids: imidacloprid, clothianidin, and thiamethoxam. Test conditions followed a static-renewal exposure protocol where lethal and sub-lethal endpoints were assessed on days 14 and 40. Reduced emergence success, advanced emergence timing, and male-biased sex ratios were sensitive responses to low-level neonicotinoid exposure. The 14-day LC50 values for imidacloprid, clothianidin, and thiamethoxam were 1.52 µg/L, 2.41 µg/L, and 23.60 µg/L, respectively. The 40-day EC50 (emergence) values for imidacloprid, clothianidin, and thiamethoxam were 0.39 µg/L, 0.28 µg/L, and 4.13 µg/L, respectively. Toxic equivalence, relative to imidacloprid, was estimated through a three-point response average at L(E)C(20, 50, 90) and plotted concentration-response curves. Relative to imidacloprid (TEF=1.0), chronic (lethality) 14-day TEFs for clothianidin and thiamethoxam were 1.05 and 0.14, respectively, and chronic (emergence inhibition) 40-day TEFs were 1.62 and 0.11, respectively. These population-relevant endpoints and TEFs suggest that imidacloprid and clothianidin exert comparable chronic toxicity to *C. dilutus,* whereas thiamethoxam induced comparable effects only at concentrations an order of magnitude higher. However, we caution that under field conditions thiamethoxam readily degrades to clothianidin, thereby likely enhancing toxicity.

Keywords: Chronic toxicity, Neonicotinoid insecticides, Macroinvertebrate, Static-renewal test, Toxic equivalency factor

INTRODUCTION

Chemical input into aquatic environments from agricultural run-off remains one of the most challenging global threats to the quality of freshwater resources [1], and extensive contamination of both lotic and lentic systems is well-documented [2, 3]. Aquatic arthropods inhabiting watersheds dominated by conventional agriculture operations can be at risk from both lethal and sub-lethal exposure to insecticides. In particular, systemic insecticides, which typically feature a low octanol-water partition coefficient [4], are particularly susceptible to leaching and run-off into aquatic environments. Growing concern over the loss of biodiversity from the intensification of agricultural operations necessitates further assessment of the threats systemic insecticides pose to aquatic invertebrate populations and associated ecosystem structure and function [5, 6].

Neuroactive, systemic insecticides are currently the most abundant form of arthropod pest-control globally [7]. Among the principal classes of insecticides used in crop protection are the neonicotinoids. Neonicotinoids are broadly applied on a suite of crop types worldwide and over a variety of landscapes where environmental conditions, active ingredient, and application rates can differ substantially. One of the most recent controversies regarding neonicotinoids concerns the broad use of seed treatment application [8]. This is a prophylactic application method that protects seedlings and mature plants from phytophagous invertebrate pests by translocating and incorporating the insecticide throughout the plant during its development [9]. Jeschke and Nauen [10] report that nearly 80% of all seed treatments (e.g., canola, corn, lentils, cereals) are coated with a neonicotinoid-based insecticide. In North America, imidacloprid, clothianidin, and thiamethoxam have frequently been detected in surface water bodies; not surprisingly since they represent some of the most water-soluble insecticides ever used on a large scale [2, 11, 12]. Once in water, neonicotinoid compounds break down at multiple molecular sites into various metabolites; a characteristic important in this study as thiamethoxam can be transformed into clothianidin by ring methylene hydroxylation. Furthermore,

clothianidin undergoes *N*-demethylation to form subsequent metabolites which retain insecticidal properties [13, 14].

Neonicotinoids are agonists of the nicotinic acetylcholine receptors (nAChRs) in insect nerve synapses [13]. They disrupt neural activity in invertebrates by binding to the post-synaptic nAChRs, functionally interfering with normal neural activity [13]. When the neonicotinoid compound reaches the nAChR, subsequent activation causes an increase in sodium ion conductance, followed by a depolarization of the post-synaptic membrane. Unlike acetylcholine, the activity of neonicotinoids is not limited by acetylcholinesterase; neonicotinoids consequentially produce prolonged neuronal activation, which leads to hyper-excitation of the insect nervous system, followed by convulsions, paralysis, and ultimately death [13]. The binding of neonicotinoids to the nAChRs is believed to be largely irreversible and to some extent cumulative over time [15]. Even low doses over time can promote adverse effects in invertebrates, such as inhibited growth and development [16], altered behavior [17], limited mobility [18], decreased adult emergence [19, 20], and reduced feeding [21, 22]. Moreover, due to the conserved nature of insect neurophysiology, both pest and non-target species are affected by neonicotinoids [23], albeit to varying degrees among species.

To date, the majority of the aquatic invertebrate toxicity studies conducted with neonicotinoids have focused on imidacloprid using single-species under acute exposure scenarios. Of the 214 single-species aquatic invertebrate laboratory studies on neonicotinoids reviewed by Morrissey et al. [24], 178 (83%) were acute whereas only 36 (17%) were chronic. Given their environmental persistence and high water solubility, chronic studies on sensitive aquatic taxa are still needed. Several studies have shown a direct relationship between more persistent neonicotinoid exposure and increased mortality or other sub-lethal effects [15]. Furthermore, some studies have found that repeated, short-term exposure to neonicotinoids may have a delayed lethal and sub-lethal effect on freshwater invertebrates [20, 25].

Chironomidae (non-biting midges) are ideal model organisms for freshwater toxicity tests, especially for insecticides. However, acute and chronic neonicotinoid toxicity tests conducted with

Chironomus dilutus (previously *C. tentans*; taxonomic description found in Shobanov et al. [26]) only exist for imidacloprid and clothianidin in two separate studies. Toxicity tests with clothianidin and thiamethoxam have been conducted using different study species, durations, and inter-laboratory methodologies, which creates comparing the toxic potency of individual compounds difficult [27]. Other model *Chironomus spp.* used in neonicotinoid research include *C. riparius* and *C. tepperi*. Previous toxicity studies comparing *C. dilutus* and *C. riparius* have shown that *C. dilutus* is typically more sensitive to a number of toxicants, including the legacy insecticide lindane [28].

World-wide, regulatory aquatic life benchmarks have only been established for imidacloprid, which is only one of seven common neonicotinoid active ingredients. Toxicity of the different neonicotinoids has been assumed equivalent for the different compounds [24], however this assumption has not been formally tested. The maximum allowed levels of imidacloprid in freshwater ecosystems for the protection of aquatic life range from 0.0083 μg/L in the Netherlands [29] to 1.05 μg/L in the United States [4]. The Canadian Council of Ministers of the Environment (CCME) lists the interim Canadian Water Quality Guidelines for the Protection of Aquatic Life for imidacloprid as 0.23 μg/L [30]. Morrissey et al. [24] documented that 66% of all the neonicotinoid laboratory toxicity tests reviewed tested imidacloprid, while clothianidin and thiamethoxam accounted for only 3.7% and 4.2% of published studies, respectively. Given the prevalence of clothianidin and thiamethoxam in aquatic environments, it remains unclear whether these benchmarks for imidacloprid are appropriate for all neonicotinoid compounds [24].

While the evaluation of lethality during an aquatic insect larval stage is important for evaluating toxicity, these organisms rarely experience the level of insecticide exposure in the field necessary to invoke a lethal response restrictive to a portion of their life span. However, exposure to sub-lethal contamination throughout their immature life stages is more common and of greater environmental relevance. This research aimed to compare the chronic toxicity of technical grade imidacloprid, clothianidin, and thiamethoxam to the model benthic macroinvertebrate species, *C. dilutus*. The chronic

toxicity was evaluated under field-relevant exposure durations and identical laboratory test conditions for three different neonicotinoids. Data generated from this study allowed for the calculation of toxic equivalency factors for these three common neonicotinoids thus helping improve the cumulative risk assessment of neonicotinoid insecticides and inform protective aquatic life benchmarks for clothianidin and thiamethoxam.

MATERIALS AND METHODS

Experimental animals

A population of *C. dilutus* was cultured in environmental chambers at the Toxicology Centre, University of Saskatchewan, Saskatoon, SK at 23.0 ± 1.0º C with a 16:8 (L:D) photoperiod following the modified protocol outlined by Environment Canada [31] and Benoit et al. [32]. Briefly, adult *C. dilutus* were collected with an aspirator into a 500-mL Erlenmeyer flask. Adults were transferred to 1-L glass mason jars each containing a small (5x5 cm) piece of Parafilm®, two plastic screens (5x12 cm) for mating surfaces, and approximately 150-mL of water; adults were given three days to produce egg masses or discarded. To avoid disturbance, breeding jars were isolated in cardboard boxes and checked for egg masses daily. New egg masses ≤ 24 h old were transferred to new 18.9-L tanks with culture water and 2.5 cm of washed silica sand. The culture water used in all experiments was carbon-filtered, biofiltered City of Saskatoon municipal water. Water quality parameters (mean ± SD) were as follows: pH 8.2 ± 0.3, conductivity 475 ± 63 µS/cm, total hardness 137 ± 7 as mg/L $CaCO_3$, and alkalinity 85 ± 9 as mg/L $CaCO_3$. Rearing tanks were fed with 5-mL of macerated fish food (Tetramin®) every other day. After 7 days, larvae were removed from the rearing tank and placed into test beakers with their cases to reduce transfer stress.

Chronic tests

All toxicity tests were performed at the Toxicology Centre, University of Saskatchewan, under conditions similar to those used for culturing the test animals. Technical grade imidacloprid (98.8% pure; 1-[(6-Chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine) and clothianidin (99.6% pure;

[*C(E)*]-*N*-[(2-Chloro-5-thiazolyl)methyl]-*N*'-methyl-*N''*-nitroguanidine) were obtained from Bayer CropScience (Mississauga, ON, Canada); technical grade thiamethoxam (98.9% pure; 3-(2-chloro-thiazol-5-ylmethyl)-5-methyl-[1,3,5]oxadiazinan-4-ylidene-*N*-nitro-amine) was acquired from Syngenta Crop Protection, LLC (Guelph, ON, Canada). Stock solutions were prepared in 1-L volumetric flasks with reverse osmosis water (Barnstead® Diamond™ NANOpure, 18.2 megohm/cm; Barnstead International, Dubuque, IA, USA) and then diluted to the desired test concentrations using culture water.

Chronic, static-renewal toxicity tests were 40 days in duration and used eight replicate beakers, each containing 10 second-instar *C. dilutus* larvae (6-7 days old), 50-mL of washed silica sand, and 200-mL of treatment water. Beakers were continuously aerated gently to maintain adequate oxygen saturation (≥ 80%) and roughly 150-mL of water from each beaker were changed every third day. Larvae were fed daily by adding 60-µL of macerated fish food (50 g d.w. Tetramin®/500-mL Barnstead® water) into each beaker. To prevent photo-degradation of test compounds, borosilicate glass was place on top of the beakers. Larvae were exposed to nominal concentrations of 0 µg/L (control), 0.1 µg/L, 0.3 µg/L, 1.0 µg/L, 3.3 µg/L, and 10.0 µg/L of each insecticide. On day 14, four replicates from each treatment were removed, organisms counted to assess survival, and surviving larvae weighed. The remaining four replicates were maintained for an additional 26 days to allow larvae to emerge as adults. Emerging adults were collected daily, their sex determined, and weighed. Larvae and adults were dried at 60° C to a constant weight. A cumulative total of emerged males and females for each beaker ensured that all individuals were accounted for and served to determine emergence synchrony across treatments. Emergence synchrony was defined as an emergence event representing the greatest proportion of the cumulative total of adults emerging within a two-day span. Adult chironomids were considered to have successfully emerged when the adult completely dissociated from its pupal exuvia and exited the water [32].

Water quality

A water sample (10 mL per beaker) was removed from four beakers in each treatment and pooled for water quality analysis before and after each partial water change. Water changes were conducted every third day to maintain static test concentrations and prevent significant ammonia buildup related to feeding. Temperature and dissolved oxygen (DO) were measured with an ORION® dissolved oxygen meter (model 835; ORION Research, Beverly, MA, USA). Water hardness and alkalinity were calculated with a Hach Digital Titrator (model 16900; Hach Company, Loveland, CO, USA), pH was measured with an ORION® PerpHect LogR meter (model 370; ORION Research, Beverly, MA, USA), and ammonia levels were assessed with a YSI Photometer (model 9300; YSI, Inc, Yellow Springs, OH, USA).

Neonicotinoid analysis

Water (60 mL) was sampled from four randomly selected replicate beakers per treatment, pooled into a 250-mL amber bottle, and stored at 4º C until analyzed. Both old and new water samples (every third day) were collected, and a subset of samples were analyzed to determine insecticide exposure and determine whether any degradation had occurred. Water samples were analyzed at the National Hydrology Research Centre, Environment Canada, Saskatoon, SK using analytical methods previously described by Main et al. [2]. Analytical standards of imidacloprid, thiamethoxam, and clothianidin were acquired from Chem Service (West Chester, PA, USA) and the internal standard from CDN Isotopes (Pointe-Claire, QC, Canada). Water samples were solid phase extracted using Oasis HLB cartridges (Waters, Mississauga, ON, Canada). Neonicotinoid analytes within the sorbent bed were reconstituted in de-ionized water with the addition of the internal standard. Neonicotinoid residues were quantified using a Waters® Model 2695, high performance liquid chromatograph interfaced with a Micromass Quattro Premier mass-spectrometer with a stainless steel column (100 x 2.2 mm; Waters® MS Xterra C-8) (Waters Corp., Milford, MA, USA). Water samples were injected into the LC/MS/MS system; the average flow through run time was 10 min, with an injection volume

of 20 µL (16 µL of 99.9% water and 0.1% formic acid, and 4 µL of 90% acetonitrile, 9.9% water, and 0.1% formic acid). Limits of quantification (LOQ) in water samples were as follows: imidacloprid, 0.0038 ± 0.002 µg/L; clothianidin, 0.004 ± 0.001 µg/L; and thiamethoxam, 0.011 ± 0.001 µg/L. Mean recoveries from MilliQ and river water spiked at 500, 100, and 0.005 µg/L were as follows: imidacloprid, 91.3 ± 6.7%; clothianidin, 78.97 ± 4.0%; and thiamethoxam, 86.3 ± 4.2%; (mean ± SD). Controls and laboratory blanks were all below the limits of detection, and all water concentration data were recovery corrected to allow for comparison among runs. Data and calculated endpoint values are reported on measured concentrations.

Data analysis

Survival data were used to calculate 14-day (mortality) LC50 values and 40-day (emergence) EC50 values (median lethal effective concentrations) using the trimmed Spearman–Kärber method [33, 34]. Dry weights of larvae (day 14) and successfully emerged adults (day 40) were used to estimate EC20, EC50, and EC90 values (observed 20%, 50%, and 90% effect) using the U.S. EPA ICp program [35].

All other statistical analyses were performed using SigmaPlot™ Version 13.0 (Systat Software, Inc., San Jose, CA, USA) with a 95% ($\alpha = 0.05$) level of confidence. Significant differences among treatments within an individual test (compound) for day 14 and day 40 survival and biomass endpoints were assessed using one-way analysis of variance (*F* statistic) followed by a Tukey post-hoc test for all multiple pairwise comparisons. To determine significant differences in adult emergence relative to the controls, a one-way analysis of variance (ANOVA) followed by a Dunnett's post-hoc test was performed on the mean cumulative proportion emerged on the day where 50% of the controls had successfully emerged. If data did not fit a normal distribution, a non-parametric Kruskal-Wallis test (*H* statistic) was used to determine significance. Degrees of freedom (df) varied between tests and among compounds due to mortality in some treatments. For the purposes of comparing similar endpoints that

include dose-response relationships, independent of dose choice, an EC20 was calculated as an appropriate derivation in favor over the more controversial NOEC and LOEC estimates [36, 37].

Toxic equivalency factors (TEFs) were estimated through a three-point response average at L(E)C(20, 50, 90) for both day 14 (lethality) and day 40 (emergence inhibition) endpoints. The relative potency of both clothianidin and thiamethoxam were compared to imidacloprid (TEF=1). Acute and chronic endpoints were graphed on a Probit scale to visually verify assumptions of parallelism of slopes for the three compounds. This was followed by a one-way analysis of covariance (ANCOVA) to statistically validate the assumption of equal slopes for both the day 14 and day 40 endpoints.

RESULTS

Water chemistry

Active ingredient concentrations in old and new water confirmed that neonicotinoid exposures remained constant throughout the experimental period of each test. Mean measured concentrations for imidacloprid, clothianidin, and thiamethoxam were 83.1%, 51.4%, and 59.7% of the target nominal doses (Table 1). All calculated toxicity endpoints were based on analyzed neonicotinoid water concentrations. Additionally, at no time during the thiamethoxam tests was clothianidin detected in any water sample, indicating that no degradation had occurred, an observation corroborated by Nauen et al. [38]. Therefore, each test evaluated the toxic effects of a single active ingredient.

Routine water quality parameters were measured during each experiment and all mean values represent an average across treatment means. There was no difference in the water quality means among treatments. Mean values (± SE) for the three chronic tests were as follows: DO 7.2 ± 0.09 mg/L, temperature 23.0 ± 0.1° C, pH 8.2 ± 0.02, conductivity 459 ± 14 μS/cm, total hardness 136 ± 1.2 as mg/L $CaCO_3$, and alkalinity 86 ± 3.5 as mg/L $CaCO_3$. Dissolved oxygen remained >7.0 mg/L throughout each test. Ammonia concentration, food consumption, and waste production increased over time as larval growth increased. On average, we observed lower ammonia concentrations in new water

samples (0.42 ± 0.07 mg/L) when compared to old water samples (1.4 ± 0.2 mg/L), but mean and peak concentrations were well below the reported ammonia LC50 value of 121.9 mg/L for *C. dilutus* [39].

Larval chronic toxicity endpoints

After 14 days of exposure, imidacloprid was the most toxic and thiamethoxam the least toxic of the three compounds to *C. dilutus* larvae (Table 2). Fourteen-day LC50 values for imidacloprid, clothianidin, and thiamethoxam were 1.52, 2.41, and 23.60 µg/L, respectively. A significant decrease in survival relative to the controls was observed at mean concentrations >2.62 µg/L (H=17.799, df=4, *p*=0.001) for imidacloprid. At the same nominal dose group, a nearly significant decrease in survival was observed in clothianidin test (F=3.04, df=4, n=20, *p*=0.051). Larval biomass was reduced by 50% at mean concentrations of 2.23, 1.83, and 21.39 µg/L for imidacloprid, clothianidin, and thiamethoxam, respectively (Table 3). Larval dry weight was consistent in the thiamethoxam and clothianidin treatments at nominal test concentrations of 0, 0.1, 0.3, and 1.0 µg/L. Both imidacloprid and clothianidin caused a statistically significant reduction in average larval dry weight at the mean exposure concentrations of 2.62 µg/L (H=17.00, df=4, n=20, *p*=0.002) and 1.86 µg/L (F=117.7, df=4, n=20, p=<0.001), respectively. Interestingly, only imidacloprid treatments caused significant decreases in survival at concentrations greater than 2.82 ± 0.30 µg/L in addition to decreases in mean dry weight. Larvae exposed to thiamethoxam displayed significant reduction in mean dry weight at 5.69 µg/L (F=10.87, df=5, n=24, *p*=<0.05).

Adult chronic toxicity endpoints

The EC50 (emergence) values for imidacloprid, clothianidin, and thiamethoxam were 0.39, 0.28, and 4.13 µg/L, respectively (Table 2). Both imidacloprid and clothianidin displayed significant decreases in percent survival at mean exposure concentrations of 0.80 µg/L (H=16.94, df=4, n=20, *p*=0.002) and 0.48 µg/L (H=12.31, df=3, n=16, *p*=0.006), respectively (Figure 1). Thiamethoxam emergence success was significantly higher than for imidacloprid and clothianidin (H=10.05, df=2, n=72, *p*=0.007), which was consistent with the other measured endpoints. A significant decrease in

emergence in the thiamethoxam test was only observed at a mean concentration of 5.75 µg/L. Developmental complications were apparent in some surviving individuals in nominal treatments > 3.3 µg/L across all active ingredients tested. For example, individuals attempting to complete metamorphosis would occasionally become fixed to the resulting pupal exuvia. This led to adults drowning, unable to breach the surface of the water and were counted as mortalities.

Emergence

Emergence timing and cumulative emergence were consistent among control treatments in the three tests, with the greatest production occurring between days 21 and 22 (Figure 2). For each test, we compared the mean proportion of adults emerged on the day where 50% of the controls had successfully emerged (day 22 for imidacloprid and thiamethoxam and day 21 for clothianidin). Emergence timing of adults was similar in the clothianidin and thiamethoxam tests; however, the 0.53 µg/L clothianidin treatment was significantly earlier than the controls (H=15.74, df=5, n=24, p=0.008; Figure 2D). The rate with which adults emerged in the clothianidin and thiamethoxam treatments, relative to the cumulative total, increased with exposure concentration (Figure 2C and 2F) although the thiamethoxam test did not yield a statistically significant response (H=6.27, df=5, n=24, p=0.28). Although not statistically significant, the most pronounced delay in emergence relative to the control was observed in the imidacloprid test (H=4.20, df=2, n=12, p=0.145), where a comparable proportion of adults (50%) emerged on days 26-27 (Figure 2B). Adult dry weight was significantly reduced in clothianidin and thiamethoxam treatments >0.20 µg/L (H=10.19, df=3, n=16, p=0.017) and >0.68 µg/L (H=18.14, df=5, n=24, p=0.003), respectively; adults emerging from the 0.10 µg/L imidacloprid treatment were also significantly lower in weight than adults emerging from the controls (H=8.80, df=2, n=12, p=0.001).

Sex ratios

Adult *C. dilutus* are sexually dimorphic and exhibit protrandry, where males emerge before females [40]. Sex ratios were evaluated as a proportion average among each replicate after each 40-day

test. Sex ratios were skewed in favor of a male dominant population at mean concentrations of 0.17, 0.46, and 3.60 µg/L for imidacloprid, clothianidin, and thiamethoxam, respectively (Table 3). EC50 (sex ratio) values for imidacloprid and thiamethoxam were lower than their respective EC50 (emergence) values, suggesting that skewed sex ratio may be an even more sensitive population endpoint than survivorship to emergence. Although differences between EC50 (sex ratio) values and EC50 (emergence) values were not statistically significant (US EPA ICp; $\alpha = 0.05$), but may be ecologically important.

Toxic equivalency factors

Toxic equivalency factors, relative to imidacloprid, were estimated through a three-point response average at LC/EC(20, 50, 90) and plotted as concentration-response curves (Figure 3). Relative to imidacloprid (TEF=1.0), acute (lethality) 14-day TEFs for clothianidin and thiamethoxam were 1.05 and 0.14, respectively. Chronic (emergence) 40-day TEFs were 1.62 and 0.11, respectively. Slopes for both the 14-day lethality (F=0.26, df=2, n=9, p=0.785) and 40-day emergence (F=0.35, df=2, n=9, p=0.731) endpoints passed the equality of slopes ANCOVA test, thus meeting the assumption of parallelism.

DISCUSSION

This is the first study we are aware of that has compared the chronic toxicity of three different neonicotinoid active ingredients, including the well-studied imidacloprid against the second generation compounds, clothianidin and thiamethoxam, under identical laboratory conditions. Compared to imidacloprid, both clothianidin and thiamethoxam have been largely overlooked in the peer-reviewed literature and in setting regulatory guidelines. Due to the widespread use of imidacloprid and the associated rise in pest insect tolerance, second generation neonicotinoids were developed to improve crop protection [41]. Together, clothianidin and thiamethoxam are now the most heavily applied neonicotinoid active ingredients in both North America and the United Kingdom, but concerns remain around their prolonged environmental persistence in soil and their high water solubility which may lead

to adverse affects on aquatic biota [41, 42, 43]. Furthermore, recent studies have identified ecological and abiotic variables, such as the presence/absence of specific plant species and communities [44], or runoff of cold spring snow meltwater [45], as factors that may extend neonicotinoid exposure to aquatic organisms.

Previous literature reviews of neonicotinoid toxicity data have identified imidacloprid as the most toxic neonicotinoid active ingredient, or as equally toxic as some other neonicotinoid compounds to aquatic invertebrates, followed by clothianidin and thiamethoxam [24, 46]. Whiteside et al. [46] conducted a rank-based risk assessment focusing on the adverse effects of agrochemicals on aquatic communities, including algae, invertebrates, fish, and other aquatic organisms. Of the 206 compounds evaluated, imidacloprid ranked the highest of the three neonicotinoids at number 51, followed by clothianidin at 143 and thiamethoxam at 190. A similar pattern surfaced in a review by Morrissey et al. [24], with imidacloprid, clothianidin, and thiamethoxam displaying similar acute toxicity geometric means (LC50 data from 24 h to 96 h tests), but the lack of clothianidin and thiamethoxam toxicity data in the primary literature preclude accurate comparison across compounds. From the identical test concentrations and endpoints evaluated in this study, we confirmed the order of toxicity to be similar to previous reports, with imidacloprid having the lowest L(E)C50 values for every endpoint except day 40 (emergence) value, where clothianidin was marginally lower (0.28 vs. 0.39 µg/L). In general, however, imidacloprid and clothianidin displayed similar toxicity to *C. dilutus* larvae, while thiamethoxam was approximately one order of magnitude less toxic. In addition, the toxicity thresholds for imidacloprid were within the range of values reported from other studies. Stoughton et al. [16] described both the acute and chronic toxicity of imidacloprid to *C. dilutus*, and reported chronic L(E)C50 values of 3.17 µg/L at day 10 and 0.91 µg/L at day 28. Similar chronic studies investigating the sensitivity of *C. riparius* to second generation neonicotinoids have reported 1.0 µg/L (EC50) for clothianidin and 10.0 µg/L (NOEC) for thiamethoxam after a 28-day exposure period [47]. However, details on the methodology for both chronic tests were not disclosed in the original documents. Additionally, the

endpoint values appear to be based on nominal exposures with no analytical validation. Acute (96 h LC50) values of 2.32 μg/L and 35 μg/L, for clothianidin and thiamethoxam, respectively, were reported for *C. dilutus* and *C. riparius* exposed to technical grade active ingredients (>98% pure) [48, 49]. Recent comparative neurophysiological studies offer further insight to the mechanism of action for imidacloprid, clothianidin, and thiamethoxam; Vehovsky et al. [50] documented the excitatory post-synaptic potential (EPSP) inhibition of the VD4-RPeD1 synaptic connection (acetylcholine-evoked membrane) in the central nervous system of the pond snail, *Lymnaea stagnalis*, for five neonicotinoid active ingredients. Imidacloprid and clothianidin were found to significantly inhibit the EPSP amplitudes at identical dose ranges (10.0-100.0 mg/L), whereas thiamethoxam exhibited negligible effects. Acetylcholine in *L. stagnalis* controls both excitatory and inhibitory neurotransmission, suggesting a similar neural response between imidacloprid and clothianidin.

The family Chironomidae is one of the most sensitive taxa to neonicotinoids and our results corroborate this; only species of Ephemeroptera and Trichoptera appear to be more sensitive [18, 24, 27]. The ephemeropteran species *Cloeon dipterum* and *Caenis horaria* have estimated 28-day imidacloprid LC50 values of 0.195 μg/L and 0.316 μg/L, respectively [18]. A recent study by van den Brink et al. [51] comparing the toxicity of imidacloprid, thiacloprid, and thiamethoxam to the mayfly, *Cloeon dipterum*, found comparable 28-day LC50 values for imidacloprid (0.85 μg/L) and thiamethoxam (0.94 μg/L). Similar LC50 values for imidacloprid and thiamethoxam indicate a parallel conclusion that these neonicotinoids, albeit possibly acting on different nAChR receptor subunits [52], exert similar toxic effects on this species. Differences in LC50 or EC50 values between species are likely attributed to the metabolic biotransformation rate of thiamethoxam to clothianidin [38]. Given that van den Brink et al. [51] conducted less intensive but adequate water changes, some thiamethoxam may have degraded into clothianidin in the aqueous solution or *in vivo* which, in the present study, displayed similar toxicity to imidacloprid. Limnephilidae (caddisflies) are also sensitive to imidacloprid

with a 96 h (immobilization) EC50 value of 1.79 μg/L [18]. Phylogenetically related taxa to the Chironomidae include members from the genera *Chaoborus* and *Culex*. *Chaoborus obscuripes* has an estimated 28-day imidacloprid LC50 value of 12.6 μg/L [18] and *Culex pipiens* a 14-day thiacloprid LC50 value of 6.04 μg/L [25].

In the present study, there is a direct relationship between duration of exposure and the concentration required to induce an adverse effect, emphasizing the importance of chronic neonicotinoid toxicity tests with aquatic insects. Furthermore, day 14 LC50 and day 40 EC50 values both represent a measure of lethality (i.e., LC50 larval mortality and EC50 adult emergence inhibition). Our data demonstrate a relationship well established in the literature where toxicity increases with exposure duration (i.e., incipient LC50s were not reached in 14 days). Day 14 LC50 values were 3.9, 8.6, and 5.7 times higher than day 40 EC50 values for imidacloprid, clothianidin, and thiamethoxam, respectively. This trend was similarly observed by Roessink et al. [18] and van den Brink [51]. Compared to previous chronic studies, we evaluated toxicity at day 14, not day 10 [16]. This difference allowed for exposure to occur for >80% of the larvae's life-cycle, to within a day from pupation in some instances.

Differences in sensitivity among taxa, especially at low exposure concentrations, can, at least partly, be broadly explained by environmental conditions, physiological status, and life history traits [53, 54], and by inherent differences in sensitivity among different taxa. Life history traits that are associated with increased sensitivity include generations per year, mobility in the aquatic environment, and reactions to biotic or abiotic stress [53]. A recent study by Rico and van den Brink [27] calculated the mode-specific sensitivity of synthetic insecticide classes to aquatic invertebrates using traits such as potential maximum size, mode of respiration, lifecycle length, temperature preference, and exoskeleton sclerotization. Members from the family Chironomidae were among the best represented taxa in the analysis due to their popularity as test species. The relative sensitivity of chironomids to all five

insecticide classes from most toxic to least toxic were as follows: organophosphates, carbamates, organochlorines, neonicotinoids, and pyrethroids. Available neonicotinoid toxicity data for the Chironomidae are summarized in Table 5.

Insect metabolism, and associated growth and development, is largely governed by environmental conditions and available resources. Alexander et al. [21] found a decrease in feeding rates of the mayfly, *Epeorus longimanus,* when exposed to 0.5 µg/L of imidacloprid for 24 h. A reduction in feeding rate was also observed at a mean 24 h exposure concentration of 1.0 µg/L, well below the LC50 of 2.1 µg/L [21]. In our study, fourth-instar chironomid larvae were observed to be excessively active on the substrate surface at nominal concentrations below 1.0 µg/L, but larval dry weights decreased only at concentrations >1.0 µg/L; EC50 (biomass) estimates were 1.83 to 2.23 µg/L for clothianidin and imidacloprid, respectively.

All toxicity tests in the present study started with second-instar larvae. Numerous studies have shown that earlier instars are more sensitive to contaminants than prepupal larvae [55]. The most important immature stages for growth and development are instars second to fourth [40], which completely overlaps with the exposure scenario used in the present study. In addition to exhibiting excessive locomotive activity, surviving fourth-instar larvae in nominal treatments >3.3 µg/L of clothianidin and thiamethoxam were periodically observed to build elongated silk cases, in some instances measuring 6-7 cm. This extra case building activity may have negatively influenced emergence success in higher concentrations, promoting vulnerability during pupal development. Unnecessarily utilizing resources to build an uncharacteristically long case may have both energetic and physical consequences during this critical stage in development.

By altering feeding habits, body size, and emergence timing, pesticide exposure can dictate the success and speed of metamorphosis. High treatments of imidacloprid, clothianidin, and thiamethoxam caused lower emergence success of *C. dilutus*. In some cases, the leg sheaths of individuals attempting to emerge would become tangled in the pupal exuvia, causing the pharate adult to sink and drown.

Song et al. [56] found similar molt related mortality with the IGH inhibitor tebufenozide and imidacloprid. Premolt-related mortality was displayed in the mosquito species, *Aedes aegypti,* after exposure to imidacloprid in a 48 h acute test with molting difficulties increasing with concentration [56]. In the present study, all three of the active ingredients tested caused molt-related mortality of *C. dilutus* during emergence. Previous studies have shown neonicotinoids to influence aquatic insect emergence across several taxonomic groups. Although we recognize the substantial variation that can occur among species sensitivities within similar taxonomic groups, the taxon shown to be most affected by a single pulse exposure of neonicotinoids is Trichoptera [20], whereas Diptera and Ephemeroptera were most affected by repeated exposure [20]. Similar to the present study, imidacloprid was found to reduce successful emergence of *C. dilutus* by 55% at 1.14 µg/L (EC50) during a 28-day full life-cycle toxicity test [16]. Full life-cycle tests therefore contribute more robust data to population-level risk assessments than short-term tests, particularly for holometabolous insect species.

In swarming dipertan species, changes in sex ratio can influence swarming success and subsequent egg mass fertility. Large chironomid swarms with even sex ratios are documented as having more fertile egg masses [40]. We found that relative to the other neonicotinoids tested, imidacloprid exerted the greatest effects on adult *C. dilutus* sex ratios (Table 3). Imidacloprid, clothianidin, and thiamethoxam shifted sex ratios in favor of male-dominant populations with increasing exposure concentrations. This observation is consistent with results from full life-cycle toxicity tests using dichlorodiphenyltrichloroethane (DDT) [57]. Female adults require more time to develop than males, and most adult females carry fully-formed ovary follicles and other egg mass constituents [40]. Longer developmental times prolong exposure to aquatic contaminants, which may help explain their increased sensitivity. Another hypothesis includes the greater physiological demand on females during transition from pupae to adult. Since the sex of *C. dilutus* is genetically predetermined, female sensitivity appears to be attributed to complications during development. Compared to the control treatment, the greater proportional loss of female adults at higher insecticide concentrations may have contributed to the

lower EC50 values and significant decreases in adult dry weight. Adult female *Chironomus sp.* can weigh up to 57.3% more than males [58]. This proportional loss of female adults could compromise wild chironomid populations and should be further explored. Studies focused on the intergenerational effects of chronic neonicotinoid exposure may shed further light on long-term population viability.

While individual current-use pesticides continue to receive the most research attention, in the environment these pesticides are often found as mixtures of similar or different active ingredients. The toxicity of neonicotinoid mixtures to aquatic life is still largely speculative with studies estimating cumulative environmental exposure by summing total neonicotinoid concentrations [2], or standardizing among compounds by molecular weight [24]. The data presented here provide a first opportunity to better describe the relative toxicity of three common neonicotinoids, an essential step towards calculating appropriate toxic equivalency factors (TEFs) of multiple neonicotinoids (Table 4). When plotting the concentration-response curves for the three insecticides used to estimate LC_{XX} and EC_{XX} values, the slopes of the lethality and the sub-lethal effect lines are reasonably parallel. From these lines, we were able to calculate the TEF for clothianidin and thiamethoxam relative to imidacloprid. For each compound, the LC_{XX} and EC_{XX} estimates were plotted to create a three point-estimate curve (Figure 3). Acknowledging the limited data used to calculate these TEFs, and the slight deviation of the curves from being truly parallel, these TEFs do provide a first attempt at appropriately standardizing and summing the estimated toxicity from mixtures of imidacloprid, clothianidin, and thiamethoxam. Based on the relative potencies described here, mixture toxicity of these three neonicotinoids can be approximated by equations (1) and (2):

(1) 14-day neonicotinoid exposure (lethality)

[Imidacloprid toxic equivalence] = [IMI conc.] + 1.05[CLO conc.] + 0.14[THX conc.]

(2) 40-day neonicotinoid exposure (emergence inhibition)

[Imidacloprid toxic equivalence] = [IMI conc.] + 1.62[CLO conc.] + 0.11[THX conc.]

These imidacloprid toxic equivalence values could subsequently be used where multiple neonicotinoids are found in water in order to compare summed equivalence to existing regulatory water quality benchmarks for imidacloprid, such as the Canadian water quality guideline for the protection of aquatic life of 0.23 μg/L [30]. Future research could aim to strengthen the dataset on which these TEFs are calculated, but in the interim the TEFs presented here could be used to provide a reasonable, or at least improved, estimate of the hazard of imidacloprid, clothianidin, and thiamethoxam mixtures to non-target insect in aquatic ecosystems.

Acknowledgment—We acknowledge funding to C. Morrissey and K. Liber from the Natural Sciences and Engineering Research Council of Canada and the Department of Fisheries and Oceans Canada. We thank C. Carter, S. Crawford, K. Raes, and S. Schiffer for their guidance and assistance in maintaining laboratory *Chironomus dilutus* cultures, and M. Hauck and J. Fehr for the neonicotinoid analysis. Pure technical grade imidacloprid and clothianidin were supplied by Bayer CropScience and thiamethoxam from Syngenta Crop Protection, LLC. No external parties influenced the experimental design, objectives, or results of this study.

Data Availability—Data are available upon request. Please contact M.C. Cavallaro (cavallaromc15@gmail.com) or K. Liber (karsten.liber@usask.ca) for all data inquires.

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Figure 1. Percent survival or emergence (mean ± SD) and dry weight (mean ± SD) of *Chironomus dilutus* larvae on day 14 (A, C) and adults on day 40 (B, D) plotted against nominal treatments (See Table 1 for mean exposure concentrations for each compound tested).

* Significantly different from the control as determined by a Dunnett's post-hoc test ($p \leq 0.05$).

Figure 2. Total emergence (A, C, E) and proportion of surviving individuals that emerged (B, D, F) of *Chironomus dilutus* adults from days 15 to 40 exposed to aqueous solutions of thiamethoxam, clothianidin or imidacloprid at one of four nominal test concentrations (0, 0.1, 1.0, 10.0 µg/L). Results for nominal treatments of 0.3 and 3.3 µg/L left out for clarity.

Figure 3. Concentration-response curves for imidacloprid, clothianidin, and thiamethoxam to *Chironomus dilutus* based on (A) 14-day lethality and (B) 40-day emergence inhibition. The y-axis response plots the individual LC/EC 20/50/90 estimates for the three compounds on a probit scale.

Table 1. Calculated (mean ± SE) neonicotinoid exposure concentrations (µg/L) measured in water over 3-day intervals during separate full *Chironomus dilutus* larval static-renewal lifecycle tests with imidacloprid, clothianidin and thiamethoxam.

	Nominal concentrations (µg/L)							
Test period	Control	0.1	0.3	1	3.3	10	20	40
				Imidacloprid concentrations				
Days 1-14[a]	<LOQ[c]	0.108 ± 0.010	0.25 ± 0.02	0.78 ± 0.08	2.62 ± 0.45	7.82 ± 0.93	NA[e]	NA
Days 1-40[b]	<LOQ	0.100 ± 0.010	0.26 ± 0.01	0.79 ± 0.06	2.82 ± 0.30	8.24 ± 0.80	NA	NA
				Clothianidin concentrations				
Days 1-14	<LOQ	0.043 ± 0.010	0.21 ± 0.06	0.42 ± 0.05	1.86 ± 0.12	4.67 ± 0.40	NA	NA
Days 1-40	<LOQ	0.046 ± 0.010	0.20 ± 0.04	0.48 ± 0.06	1.87 ± 0.10	4.97 ± 0.46	NA	NA
				Thiamethoxam concentrations				
Days 1-14	<LOQ	0.061 ± 0.004	0.27 ± 0.01	0.74 ± 0.03	2.23 ± 0.12	5.90 ± 0.45	11.99 ± 2.00[d]	33.76 ± 4.60[d]
Days 1-40	<LOQ	0.066 ± 0.004	0.24 ± 0.01	0.68 ± 0.04	2.11 ± 0.12	5.69 ± 0.36	NA	NA

[a]Mean neonicotinoid concentration calculated for the initial 14 days of exposure
[b]Mean neonicotinoid concentration calculated for the entire 40-day study
[c]LOQ = limit of quantification
[d]Thiamethoxam treatments >10.0 µg/L were required to calculate L(E)C50 values
[e]NA = not applicable

Table 2. Calculated lethal (LC) and sub-lethal (EC) toxicity values (µg/L (95% CI)) for *Chironomus dilutus* larvae exposed to technical grade imidacloprid, clothianidin, or thiamethoxam over periods of 14 and 40 days.

Day 14 (larvae survival)				Day 40 (emergence)			
LC_{XX}	Imidacloprid	Clothianidin	Thiamethoxam	EC_{XX}	Imidacloprid	Clothianidin	Thiamethoxam
20[a]	0.47 (0.29-0.98)	0.34 (0.12-1.27)	1.94 (1.58-7.42)	20	0.06 (0.05-0.17)	0.02 (0.019-0.036)	0.48 (0.05-2.76)
50[b]	1.52 (0.99-1.82)	2.41 (1.73-2.83)	23.60 (20.36-26.89)	50	0.39 (0.31-0.42)	0.28 (0.20-0.33)	4.13 (3.53-4.76)
90[c]	4.83 (2.48-7.03)	4.21 (4.76-5.07)	>33.76 (± 4.6)[d]	90	0.71 (0.81-0.83)	1.48 (1.32-1.74)	>5.69 (± 0.36)[d]

[a]Concentrations estimated to produce a 20% effect ± confidence intervals ($\alpha = 0.05$) using the ICp method U.S. EPA [35]
[b]Median lethal (or effect) concentration calculated with the trimmed Spearman-Kärber method Hamilton et al. [34]
[c]Concentrations estimated to produce a 90% effect ± confidence intervals ($\alpha = 0.05$) using the ICp method U.S. EPA [35]
[d]Calculation extrapolated due to <90% effect observed at highest treatment; used extrapolated EC90 for calculation of TEF

Table 3. Calculated sub-lethal toxicity endpoints of biomass and sex ratio (EC20 and EC50 µg/L; 95% CI) for *Chironomus dilutus* larvae exposed to technical grade imidacloprid, clothianidin, or thiamethoxam over periods of 14 and 40 days.

	Imidacloprid	Clothianidin	Thiamethoxam
Biomass[a]			
EC20	0.81 (0.10-0.94)	0.89 (0.74-0.98)	10.17 (7.38-14.58)
EC50	2.23 (2.09-2.54)	1.83 (1.74-2.08)	21.39 (17.38-28.65)
Sex Ratio[b]			
EC20	0.11 (0.02-0.14)	0.15[c]	0.31 (0.12-0.75)
EC50	0.17 (0.05-0.19)	0.46 (0.29-1.17)	3.60[c]

[a]Dry weight of larvae (Day 14)
[b]Adult males to females M:F (Day 40)
[c]No confidence intervals could be calculated with the provided data

Table 4. Toxic equivalency factors (TEFs) for clothianidin and thiamethoxam relative to imidacloprid.

	Imidacloprid	Clothianidin	Thiamethoxam
Day 14[a]			
20	1.0	1.38	0.24
50	1.0	0.63	0.06
90	1.0	1.15	0.12
Mean ± SD	-	1.05 ± 0.38	0.14 ± 0.09
Day 40[b]			
20	1.0	3.0	0.13
50	1.0	1.39	0.09
90	1.0	0.48	0.11
Mean ± SD	-	1.62 ± 1.28	0.11 ± 0.02

[a]Larvae survival; LC_{XX}: lethal concentration data for day 14
[b]Adult emergence: EC_{XX}: effective concentration data for day 40

Table 5. Available neonicotinoid toxicity data for Chironomidae species.

Species	Active Ingredient	Study Duration	Study Type	Endpoint	Toxicity (µg/L)	Reference
Chironomus riparius	Acetamiprid	28 d	Chronic	NOEC[a]	5	[47]
	Clothianidin	48 h	Acute	EC50[b]	22	[30]
	Clothianidin	48 h	Acute	EC50	29	[47]
	Clothianidin	28 d	Chronic	EC50[e]	1	[47]
	Clothianidin	28 d	Chronic	EC15[e]	0.72	[60]
	Imidacloprid	24 h	Acute	LC50[c]	55.2	[59]
	Imidacloprid	48 h	Acute	LC50	19.9	[17]
	Imidacloprid	96 h	Acute	EC50[d]	12.94	[61]
	Imidacloprid	28 d	Chronic	EC50[e]	3.11	[59]
	Imidacloprid	28 d	Chronic	EC50[e]	3.6	[59]
	Thiacloprid	28 d	Chronic	EC50	1.8	[47]
	Thiamethoxam	N.A.	Acute	EC50	35	[60]
	Thiamethoxam	30 d	Chronic	NOEC	10	[47]
Chironomus dilutus	Clothianidin	96 h	Acute	LC50	2.32	[48]
	Imidacloprid	48 h	Acute	EC50	69	[30]
	Imidacloprid	96 h	Acute	LOEC[a]	3.39	[30]
	Imidacloprid	96 h	Acute	LC50	10.5	[30]
	Imidacloprid	96 h	Acute	LC50	10.5	[16]
	Imidacloprid	96 h	Acute	LC50	5.4	[16]
	Imidacloprid	96 h	Acute	LC50	5.75	[16]
	Imidacloprid	96 h	Acute	LC50	2.65	[62]
	Imidacloprid	10 d	Chronic	LC50	3.17	[16]
	Imidacloprid	28 d	Chronic	EC50[e]	0.91	[16]
Chironomus tepperi	Thiacloprid	24 h	Acute	LC50	1.58	[25]

[a]N(L)OEC = no (low) observed effect concentration
[b]EC50 = effective concentration to 50% of test population
[c]LC50 = lethal concentration to 50% of test population
[d]Immobilization
[e]Emergence

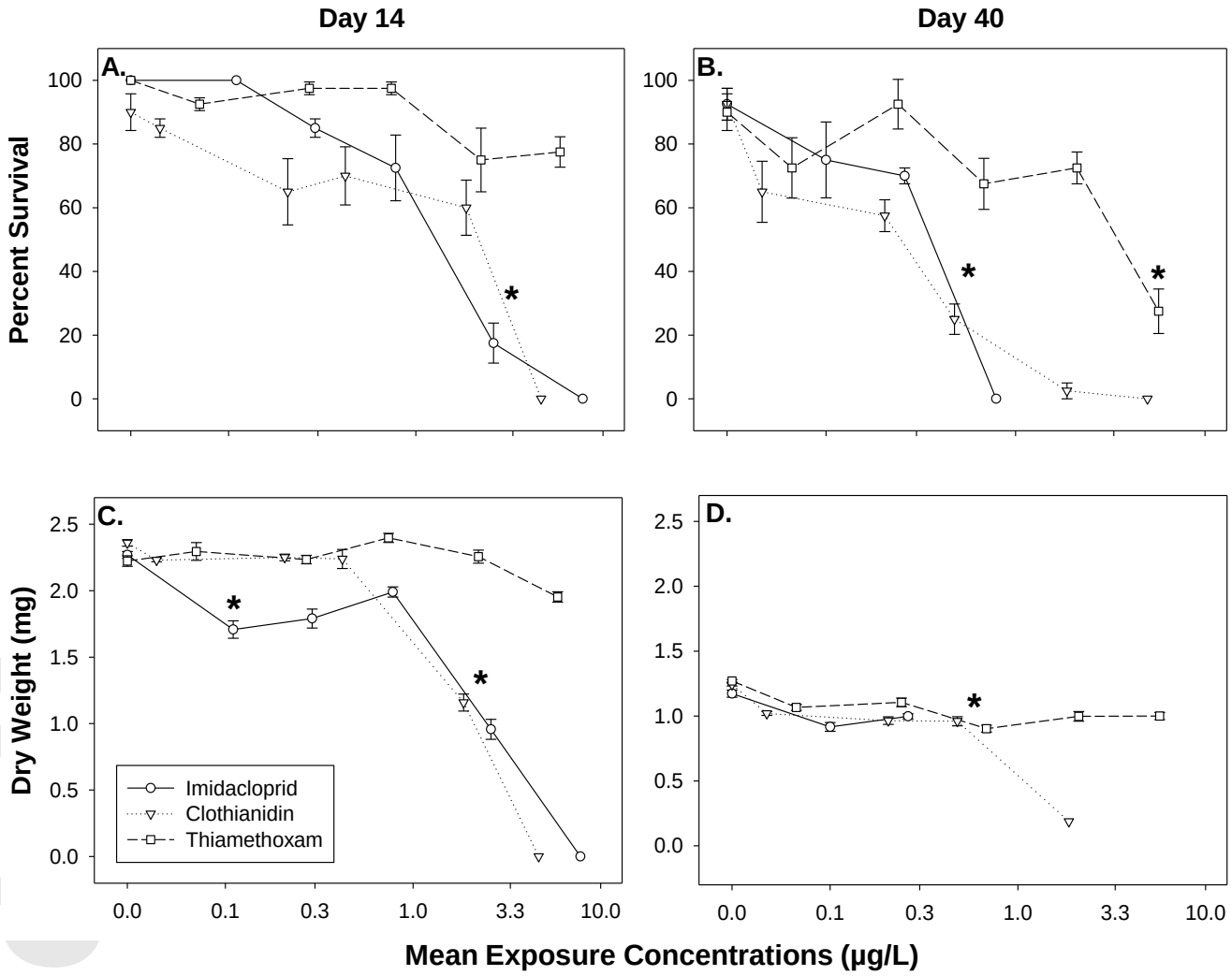

Figure 1

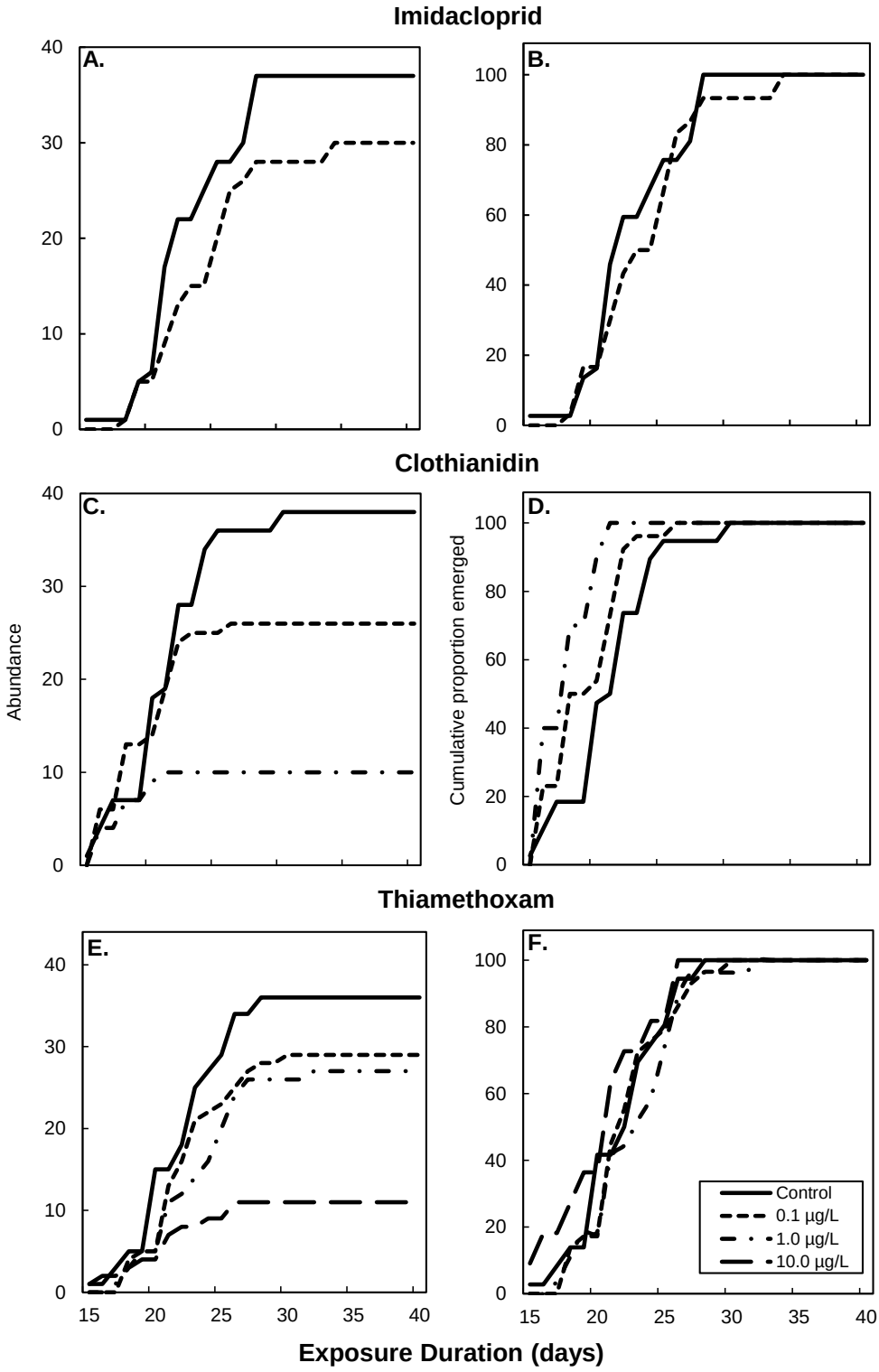

Figure 2

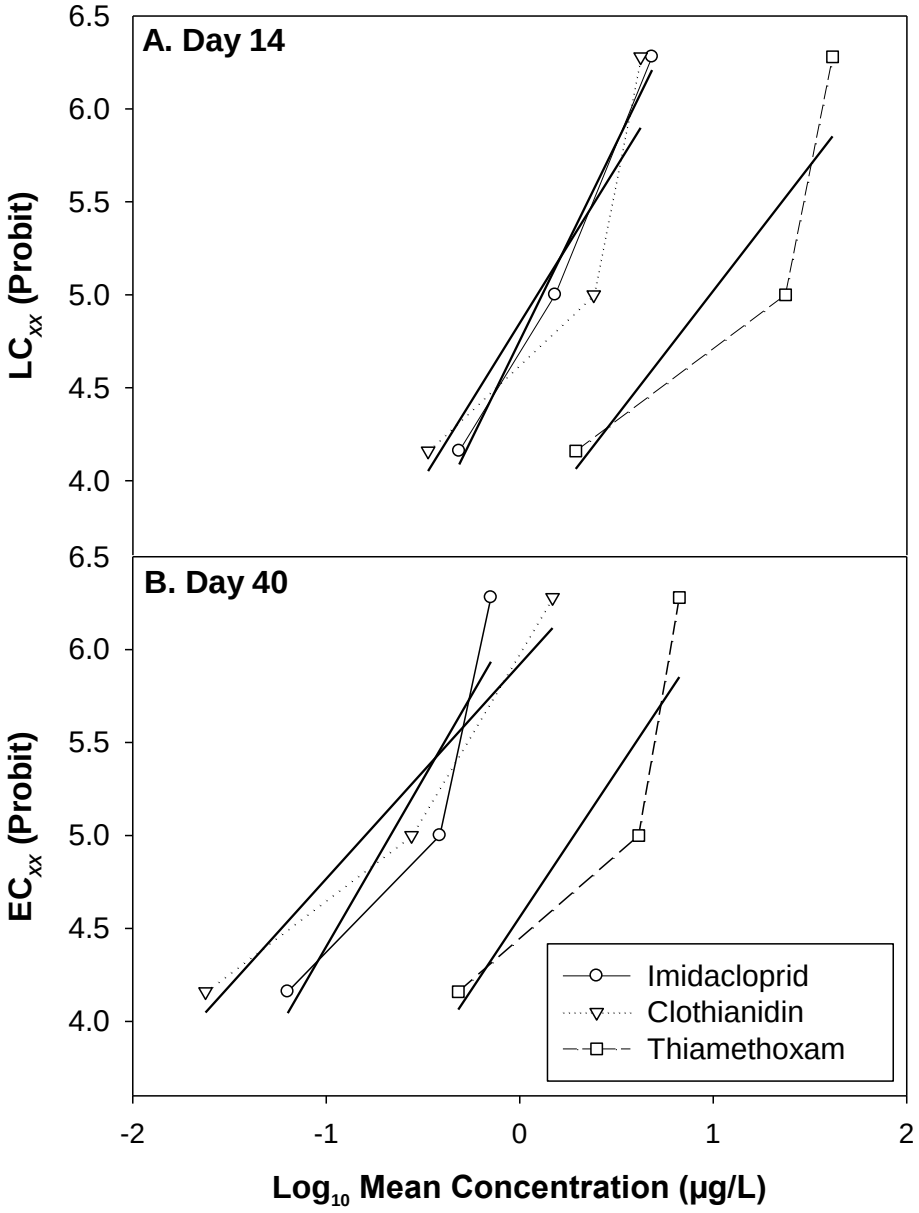

Figure 3

Structural Changes in a Macrozoobenthos Assemblage After Imidacloprid Pulses in Aquatic Field-Based Microcosms

Archives of Environmental Contamination and Toxicology

November 2013, Volume 65, Issue 4, pp 683–692 | Cite as

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Article
First Online: 01 August 2013
Received: 09 December 2012
Accepted: 05 July 2013

Abstract

A field-based microcosm experiment was performed to investigate the effects of repeated pulses of the neonicotinoid insecticide imidacloprid on a lentic benthos assemblage. This specific microcosm method was chosen because it allows for both testing of a wide range of organisms under natural conditions and as well as gaining insight into intraspecific and interspecific interactions. The macrozoobenthos that colonised the microcosms was exposed to three pulses each 1 week apart at nominal concentrations ranging from 0.6 to 40 µg/L. Imidacloprid underwent fast aqueous photolysis due to optimal sunlight conditions during the test phase (half-life = 28 ± 8 h [monitored for 21 days]). Nonetheless, decreased abundance and emergence of Ephemeroptera and decreased survival of chironomid species of the subfamilies Tanypodinae and Orthocladiinae were observed at time-weighted average concentrations of 2.3 µg/L. In contrast, the gastropod *Radix* sp. became dominant at high imidacloprid concentrations, probably due to decreased competition for food with sensitive species. The results of this study show that repeated short-term contamination of imidacloprid at low concentration levels may affect aquatic ecosystems even under optimal conditions for photodegradation. The microcosm approach, with its simple and field-relevant design, proved to be a useful tool for assessing the effects of imidacloprid contamination.

Imidacloprid is the active component of many commercial pesticides. Compared with other insecticides, its irrefutable advantages are its high insecticidal toxicity at low concentrations and low toxicity to mammals (Tomlin 2000). Imidacloprid belongs to the neonicotinoids, a class of neuroactive insecticides, and it binds to the nicotinic acetylcholine receptors (Buckingham et al. 1997; Goedkoop and Markensten 1998; Matsuda et al. 2001). Such receptors are present in all animals; however, they are made of different subunits: those of arthropods are very susceptible to imidacloprid, whereas those in vertebrates are not; this confers to imidacloprid selectivity on specific taxa (Tomizawa et al. 2000). This also indicates that imidacloprid is effective probably against virtually every insect. These properties have made it a successful replacement for many formerly used insecticides in numerous types of applications (Liu and Casida 1993).

Imidacloprid was designed to be an insecticide with low impact on the environment; it is considered harmless to the nontarget aquatic ecosystem (Bayer CropScience AG 2002) because of its rapid and complete degradation accelerated by sunlight. For example, Moza (1998), under laboratory conditions, found a half-life (DT_{50}) of 4 h at irradiation of 290 nm. A number of other physicochemical properties, however, raised the concern that imidacloprid has a high potential to contaminate surface waters. For example, its DT_{50} in soil ranges from 104 to 228 days (IUPAC Footprint Database [http://sitem.herts.ac.uk/aeru/iupac/397.htm (http://sitem.herts.ac.uk/aeru/iupac/397.htm)]); this property, in combination with its moderate water solubility, may result in high contamination potential during runoff (Armbrust and Peeler 2002). Finally, imidacloprid is also considered harmless because of its low toxicity to *Daphnia magna* (LC_{50} = 85 mg/L).

Single-species laboratory tests have shown that aquatic organisms—e.g., the mayfly *Epeorus longimanus* (LC_{50} at 96 h = 0.65 μg/L [Alexander et al. 2007]) and the nonbiting midge *Chironomus tentans* (LC_{50} at 96 h = 5.75 and 5.40 μg/L, respectively, for the technical and the commercial formulation of Admire [Stoughton et al. 2008])—are affected by imidacloprid even at low concentrations. The strengths of laboratory, single-species tests are as follows: standardisation, which implies reproducibility, and reliability, which helps to understand the mode of action of a chemical and hence the building of a cause–effect relationship. These tests are often performed under artificial conditions using laboratory-reared organisms to restrain abiotic and biotic factors that may influence the bioavailability of chemicals or the fitness of the organisms and hence the outcome of the test. The flaw inherent in this practice is that nature's complexity becomes simplified. The ecological relevance of the laboratory tests is strongly reduced (Forbes and Forbes 1993), and the risk of erroneous conclusions is increased. Most of the laboratory tests performed with imidacloprid have focused on exposure scenarios in which the concentration of the insecticide has been kept constant during the experiment. However, recent investigations have focused on the simulation of realistic exposure scenarios. Repeated pulse exposures best reflect pollution events caused by the existence of many pollution sources (e.g., spray drift, stormwater runoff in urban areas, nurseries, orchards, etc.), and this scenario is more relevant for describing the fate of imidacloprid in the environment (Canadian Council 2007; Pestana et al. 2009; Mohr et al. 2012). Several imidacloprid pulses could have the potential to cause cumulative effects as has

been indicated amongst others by Tennekes (2010), Tennekes and Sanchez-Bayo (2011), and Berghahn et al. (2012). However, this hypothesis needs further validation. In addition, Pestana et al. (2009) and Mohr et al. (2012) tested the effects of imidacloprid pulses on entire aquatic communities in stream mesocosms and observed that insects' larvae were negatively affected.

So far, there is no published study dealing with the effects of repeated imidacloprid pulses on lentic aquatic macroinvertebrate assemblages. Exposure in lentic and lotic ecosystems are different: In a lotic system, a stream section is exposed to a pulse for a short duration due to the flow velocity; in a lentic system the pulse will be subject to degradation processes rather than dilution processes due to water flow and turbulence. The use of field-based microcosms (Pettigrove and Hoffmann 2005) provides an ecologically relevant method to evaluate the effects of pesticides on lentic aquatic organisms. Unlike mesocosms, the small size and low costs of these microcosms allow for a high number of replicates and treatments and also for randomised distribution in the field. The microcosms are set up in water bodies or at a shore and are allowed to be colonised by indigenous, egg-laying insect species. The assemblage of macroinvertebrates of the study site in the season under investigation is partially represented in the microcosms. Furthermore, a large number of species can be investigated.

In this study, the effects of repeated imidacloprid pulses on an indigenous assemblage of lentic macrozoobenthos organisms was investigated. It was hypothesized that imidacloprid would rapidly dissipate from the water phase of the shallow microcosms due to its fast aqueous photolysis. The aim was to investigate whether imidacloprid would still cause lethal or sublethal effects on aquatic organisms under these conditions. Finally, it was intended to compare the results of this lentic microcosm study with those of the lotic mesocosms study by Mohr et al. (2012). Abundance, number of species, and emergence (as total number of adults) were chosen as end points to detect significant structural changes in the macroinvertebrate community that developed in the microcosms.

Materials and Methods

Field-Based Microcosm Design

Each microcosm consisted of a 20-L polypropylene container (45.5 × 30 × 21 cm, KIS System Box 8605; ABM Italy Spa). A total of 56 microcosms were used.

The microcosms were exposed in the reservoir pond at the field station of the German Federal Environment Agency (UBA) in Berlin, Germany. This location was chosen because it fulfilled the prerequisites for this microcosm method. It was unpolluted and closed to the public, which excluded possible sources of disturbance during the experiment. The setup followed the method described by Pettigrove and Hoffmann (2005) with some modifications. Each microcosm contained 750 mL of fine, homogenized sediment (silt and clay loam with ~3 % organic matter). The sediment was

taken from an uncontaminated lake on the island Ruegen (Germany) and treated as described in Mohr et al. (2007). Approximately 15 L of filtered water from the reservoir pond of the field station were added into each microcosm.

The microcosms were fixed in pipe-framed rafts and left to float on the reservoir pond to achieve a homogeneous colonisation. Each raft consisted of eight microcosms distributed in a stratified random design: two microcosms for the controls and one for each of the six treatments. The microcosms were covered with a coarse net (mesh size ~2 cm) to prevent colonisation by large predatory macroinvertebrates, such as dragonflies, whereas small flying insects were still able to lay eggs inside the microcosms. To decrease the risk of heat accumulation, the microcosms were two-thirds submerged in the water. The colonisation phase lasted 3 weeks (late May to June). During this time, imidacloprid was added in three pulses, each 1 week apart, to simulate imidacloprid pulses caused by different pollution events. The third pulse represented the end of the colonization phase, and all of the microcosms were covered with stockings (XL to XXL champagne colour; Ja) that acted as a fine nylon mesh. This mesh was fine enough to retain emerging insects and to prevent new ones from colonizing the microcosms; however, it was sufficiently coarse to allow for aeration inside the microcosms.

The collection of the emerged insects started shortly after the third pulse, lasted 7 weeks, and was concluded when the emergence rate strongly decreased. Adults were removed weekly, however, at irregular intervals, due to logistic constraints using a mechanical, modified battery-powered aspirator (Hausherr's Machine Works U.S.). At the end of the experiment, the content of each microcosm was filtered through a 500-μm sieve to collect the remaining insect larvae. The larvae were stored in 80 % ethanol and identified using a stereoscope (Stemi 2000; Zeiss).

In situ measurements of water temperature, turbidity, conductivity, dissolved oxygen, and pH were recorded once a week during the experiment (sensor = Cond 340i; Multi 340i; turbidity = TURB 555IR). The water temperature of the reservoir pond, air temperature, and ultraviolet (UV) radiation (UBA meteorology station) were also recorded.

Imidacloprid Exposure and Measurements

Macroinvertebrates were exposed to six nominal concentrations of imidacloprid (0.6, 1.4, 3.2, 7.5, 17.3, and 40 μg/L), which were applied 3 times as pulse with each pulse applied 1 week apart. These concentrations are within the average range measured in contaminated water bodies (Lamers et al. 2011; Starner and Goh 2012).

Each test concentration had 7 replicates apart from the control, which had 14 replicates. Exposure to imidacloprid was monitored by analyzing overlying water samples from all of the treatments. 1 L of water was collected at 6 h, 1 and 6 weeks after each pulse and at the end of the experiment in all treatments. Sacrificial tanks for one treatment (17.3 μg/L nominal concentration) were set up. Water was additionally collected here at 1, 2, 3, and 7 days after each pulse. The whole sediment of these sacrificial microcosms was collected

for chemical analysis. Imidacloprid was extracted using a solid-phase extraction column of modified polystyrene–divinylbenzene resin (ENV + 200 mg/6 mL of IST Biotage). Derivatization of imidacloprid for analysis by gas chromatography–mass spectrometry (GC–MS) was performed with pentafluorobenzoyl chloride (CAS no. 2251-50-5) instead of hepta-fluorobutyric anhydride and pyridine. For water, the detection limit was 4–5 ng/m, and the determination limit was 12–15 ng/mL (= 500 mL). For the water sample, the detection limit was 0.01 µg/L, and the determination limit was 0.03 µg/L. For sediment, the detection limit was extraction of 75 ng/mL. For 20 g of frozen sample, 3.8 ng/g TM = 3.8 µg/kg TM. All chemical analyses were performed on a gas GC–MS system (gas-phase chromatograph HP 6890/MSD HP 5973; capillary column 50 cm, CP Sil 8, carrier gas helium; Hewlett Packard [MacDonald and Meyer 1998]).

Imidacloprid-Dissipation Analysis

For calculating degradation rates, the decrease of imidacloprid was displayed as a function of time (Fig. 1). Under the simplified assumption that the amount of degraded imidacloprid remains constant and independent of its concentration, the reaction rate decreases proportionally in function of time (single first-order kinetic) and follows an exponential decay according to the following equation (Eq. 1):

$$A(t) = A_0 \mathrm{e}^{-kt} \quad (1)$$

where $A(t)$ denotes the residual concentration at time t; A_0 represents the initial imidacloprid concentration; and k, the reaction rate, is the rate constant. The rate constant k is measured as the slope of the plot of the logarithm of concentration *versus* the reaction time and was determined using the maximum likelihood estimation in Origin 8. The rate constant was also used to determine the half-life (DT_{50}), according to the following equation (Eq. 2):

$$t_{1/2} = \frac{\ln(2)}{k} \quad (2)$$

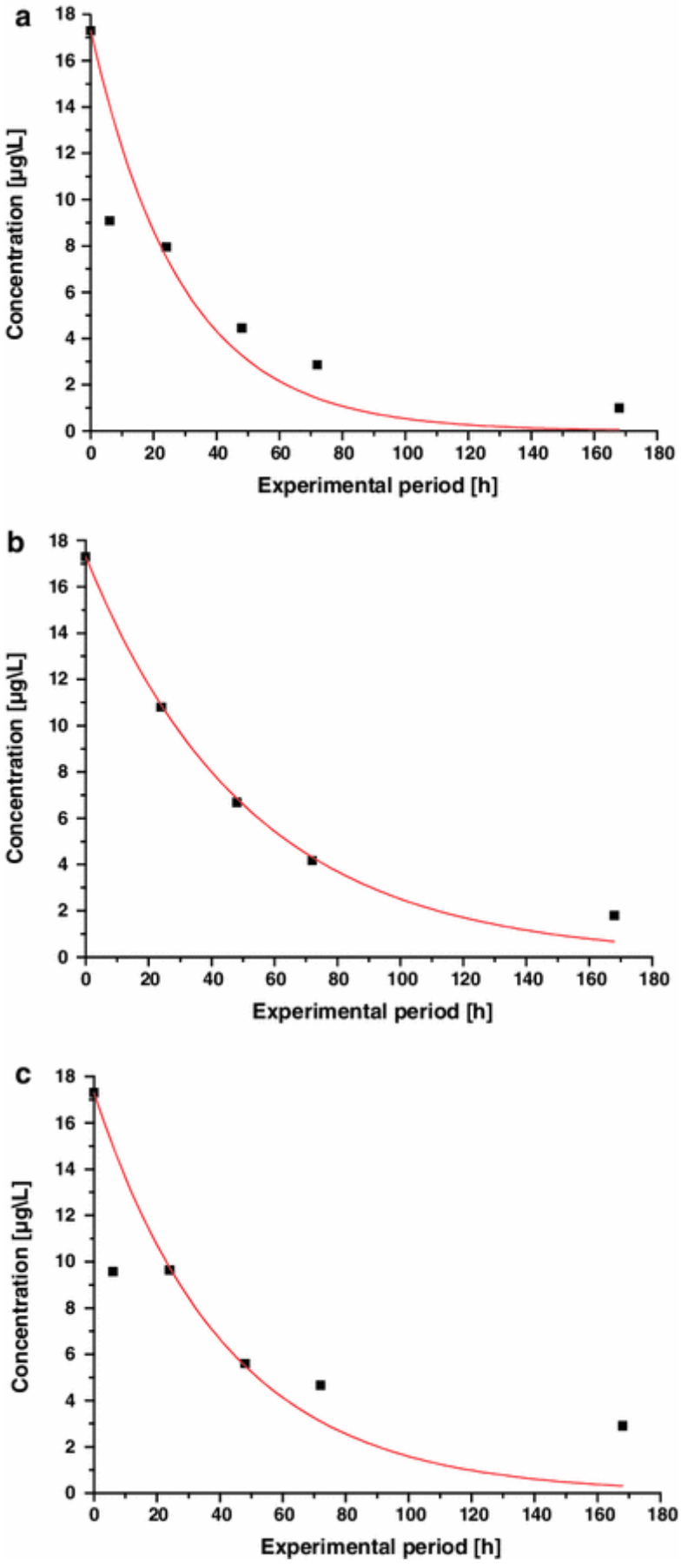
a
b
c
Concentration [µg\L]
Experimental period [h]
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
120
140
160
180

Fig. 1

Degradation of imidacloprid in water after each pulse in the 17.3 μg/L (nominal concentration) treatment. **a** Pulse 1: correlation R^2 = 0.83; K = 0.035; DT_{50} = 20 h; TWA = 7.01 μg/L; mean UVB = 2.35 μW/cm². **b** Pulse 2: correlation R^2 = 0.99; K = 0.02; DT_{50} = 36 h; TWA = 5.32 μg/L; mean UVB = 2.78 μW/cm². (**c**) Pulse 3: correlation R^2 = 0.71; K = 0.023; DT_{50} = 29 h; TWA = 3.23 μg/L; mean UVB = 2.75 μW/cm². More details are in "Materials and Methods" section

where $t_{1/2}$ denotes the time after which half of the chemical has been chemically transformed.

Data Analysis

Total abundance (larvae plus emerged adults), number of species, and emergence (number of adults) of common taxa were monitored as end points of the experiment. Statistical analyses and graphs were performed using the statistical program R (R Development Core Team 2008) and SPSS version 19. Because the data were nonnormally distributed (Kolmogorov–Smirnov test), nonparametric tests were chosen. The treatments are graphically displayed using parallel boxplots. Median and interquartile ranges (IQR [25th and 75th quartiles]) are used to give an idea of centrality and spread. For comparison among treatments of the total abundance and abundance of common species Kruskal–Wallis tests were performed. When a difference among treatments was detected, two-tailed Mann–Whitney *U* tests were used for identifying which pairs of treatments were different. Jonckheere–Terpstra trend test was used to test whether the diversity, abundance, and emergence of common taxa would gradually decrease with increasing imidacloprid concentrations (Jonckheere 1954). Differences between treatments were tested for significance at the 5 % level. Power analysis (*G* test) was run where "no statistical significance" was found to assure that the statistical tests had enough power to detect an effect.

Results

Dissipation of Imidacloprid in the Water Phase

All three pulse applications took place on sunny days with air temperature average ranging from a minimum of 10 °C during the night and 24 °C during the day and relatively high levels of ultraviolet radiation (UV-B between 6 and 11 μW/cm²). The water of all microcosms was clear during the whole experiment and became temporarily turbid only during rain events (≤53 nephelometric turbidity unit) due to dispersed sediment.

The physicochemical parameters of the water in the microcosms were similar within the treatments and also similar to those of the reservoir pond. The pH ranged between 8 and 9 and was similar to the pond's pH of 8. The water temperature ranged from 16 to 22 °C (pond temperature 17 to 21 °C). The conductivity decreased over time from 835 μS/cm at the start to 615 μS/cm at the end of the experimental period. The conductivity of the pond water was ~950 μS/cm.

Detailed chemical analysis of the water performed for the 17.3 μg/L nominal concentration treatment showed that the DT_{50} value for each of the three pulses was 20, 36, and 29 h (monitored for 21 days). After each pulse, a rapid initial decrease in insecticide concentration was observed within 6 h after application suggesting that aqueous photolysis was the main breakdown pathway (Fig. 1). At the end of the experiment, 0.13 μg/Kg and 1.72 μg/L, respectively, were the highest imidacloprid concentrations found in the sediment and in the water of the treatment with the highest contamination (see Table 1).

Table 1

Imidacloprid concentrations (μg/L [water] and μg/Kg [sediment])

Treatments	Control	0.6 (μg/L)	1.4 (μg/L)	3.2 (μg/L)	7.5 (μg/L)	17.3 (μg/L)	40 (μg/L)
Water	0.0	0.0	0.06	0.13	0.37	0.99	1.72
Sediment	0.0	0.0	0.0	0.0	0.02	0.04	0.13

Water and sediment samples collected at the end of the experiment (7 weeks after pulse 3)

The rapid dissipation rates of imidacloprid in the microcosms indicated that when assessing of the level of toxic response, the use of nominal concentrations would be misleading, whereas the use of time-weighted average (TWA) concentrations would be more appropriate (see Discussion for more details). The TWA concentrations of the different treatments ranged from 0.2 to 12 μg/L (Table 2). In the following text, both the nominal (nc) and TWA concentrations will be displayed in the figures, whereas the text will exclusively refer to the TWA concentrations.

Table 2

Imidacloprid concentrations: Nominal and corresponding TWA concentrations (μg/L) after each pulse

Nominal concentrations (µg/L)	TWA for pulse 1	TWA for pulse 2	TWA for pulse 3	Mean TWA (µg/L)
0.6	0.24	0.18	0.11	0.2
1.4	0.57	0.43	0.26	0.4
3.2	1.3	0.98	0.6	1.0
7.5	3.04	2.31	1.4	2.3
17.3	7.01	5.32	3.23	5.2
40	16.22	12.3	7.46	12

Colonization Success of Macroinvertebrate Taxa in Control Microcosms

An average number of 680 individuals/microcosm, ranging from 347 to 1010 (interquartile [IQR] spread) was collected during the entire experiment. The macroinvertebrate assemblage was dominated by Chironomidae (Diptera) (65 %) from the subfamilies Chironominae, Tanypodinae, and Orthocladiinae. The second most abundant and frequent family was Gastropoda (~18 %), represented by the pulmonate snail *Radix* sp., which probably entered the microcosms at the planktonic stage with the water. Other relatively abundant insect families were Ephemeroptera (*Caenis* sp. and *Cloeon* sp.), whereas Ceratopogonidae. Chaoboridae, Culicidae, other Diptera, and Nematoda were present in only a small number of microcosms.

Total Number of Species and Common Taxa in Treatments

A significant decrease in total number of species and abundance of Chironomidae was observed in the two highest treatments of 5.2 and 12 µg/L TWA. Of the 6 (2 to 8 IQR) most common genera found in the controls, only 4 (1 to 4 IQR) were found at the highest concentration (Kruskal–Wallis $p = 0.002$; Mann–Whitney $Up = 0.025$ for control vs. 5.2 µg/L TWA and $p = 0.001$ for control vs. highest concentration). The average number of chironomids found in the control was 275 (105.7–357 IQR), which decreased to 148 (130–212 IQR) and further to 51 (0–109 IQR) at 5.2 and 12 µg/L TWA, respectively (Kruskal–Wallis test $p \leq 0.05$; Mann–Whitney U test $p = 0.003$ for control *vs*. highest concentration; Jonckheere–Terpstra $p \leq 0.05$; Fig. 2). *Paratanytarsus grimmii* was

excluded from the statistical analysis because it reproduces through parthenogenesis and is able to breed in the microcosms.

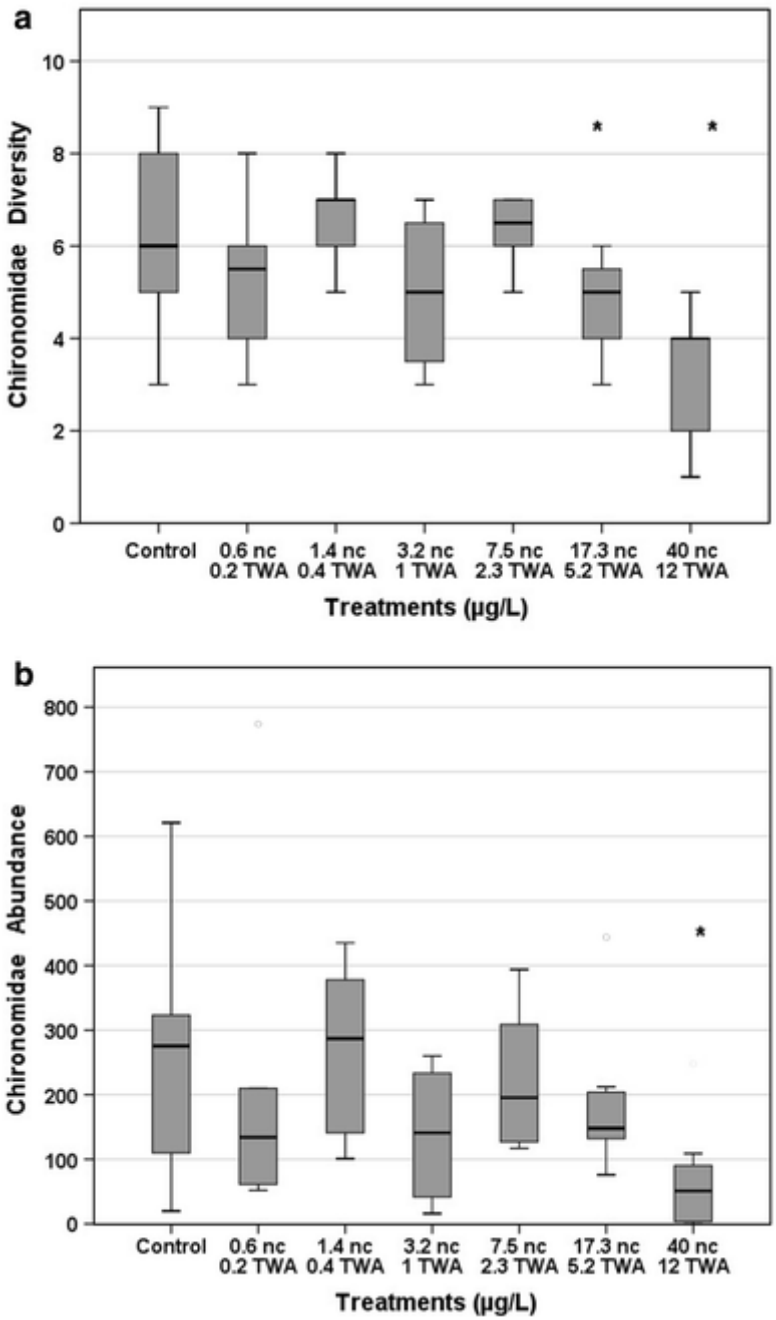

Fig. 2

Comparison of chironomid diversity and abundance across imidacloprid concentrations. * Treatments significantly different from control ($p < 0.05$). *Numbers* and *signs* without *asterisks* indicate outliers

Three species belonging to the subfamily Orthocladiinae were particularly sensitive to imidacloprid (Fig. 3). Of the four species that colonised the microcosms, three (*Corynoneura* sp., *Cricotopus* sp., and *Eukiefferiella* sp.) were strongly affected because none of them was present at the highest concentration, thus contributing strongly to the

general decrease of chironomids species (Kruskal–Wallis $p \leq 0.05$; Mann–Whitney $Up = 0.001$ for control *vs*. highest concentration; Jonckheere–Terpstra $p \leq 0.05$). *Psectrocladius* sp. was the only Orthocladiinae species still present at 12 µg/L TWA, although its abundance was also reduced. The total abundance of Orthocladiinae was significantly lower at the highest concentration (Kruskal–Wallis $p \leq 0.05$). Among the Tanypodinae, the abundance of *Ablabesmyia* sp. decreased starting from 2.3 µg/L TWA concentration (Kruskal–Wallis $p = 0.007$; Jonckheere–Terpstra $p = 0.002$) and was significantly affected at the highest concentration (Mann–Whitney $Up < 0.001$, Fig. 4).

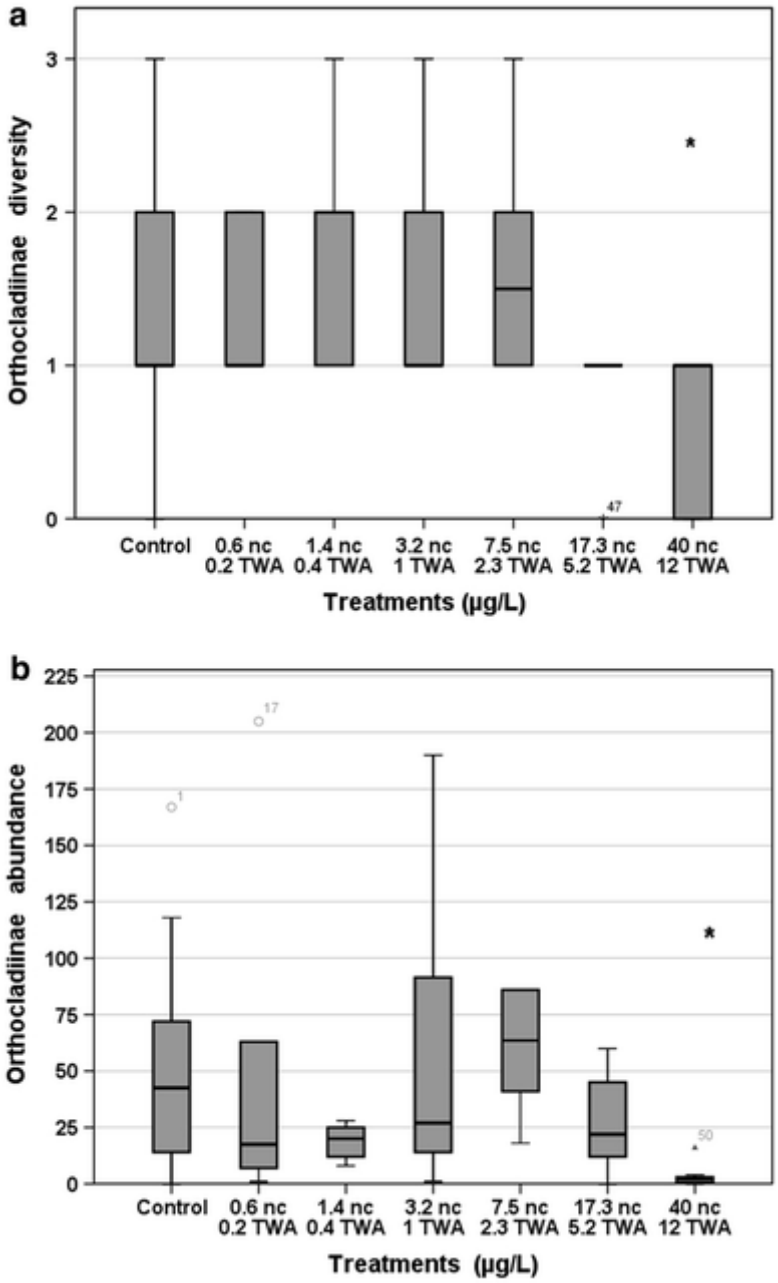

Fig. 3

Decrease of Orthocladiinae diversity and total abundance across imidacloprid concentrations. * Treatments significantly different from control ($p < 0.05$). *Numbers* and *signs* without *asterisks* indicate outliers

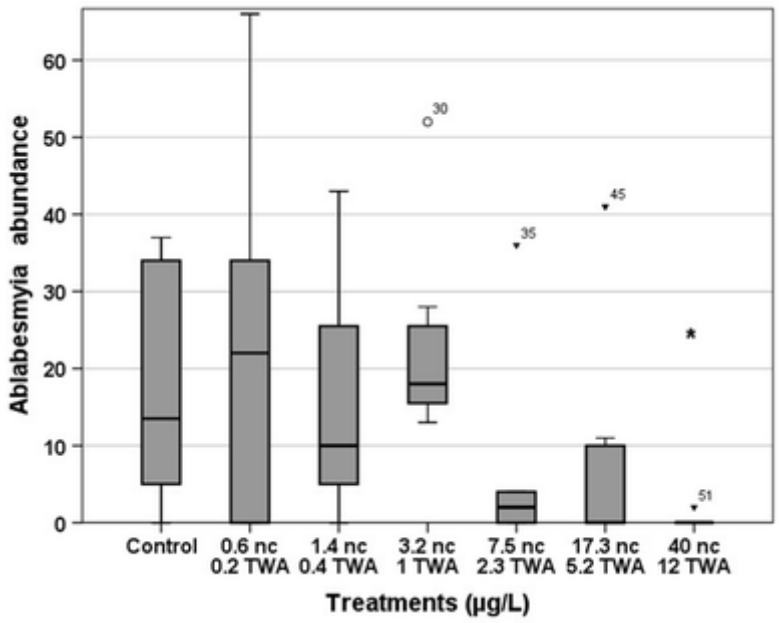

Fig. 4

Total abundance of *Ablabesmyia* sp. (Tanypodinae) across imidacloprid concentrations. * Treatments significantly different from control ($p < 0.05$). *Numbers* and *signs* without *asterisks* indicate outliers

The number of *Radix* sp. (Gastropoda) increased significantly at the highest imidacloprid concentration. An average of 55 (19.5–241.5 IQR) snails was found in the control compared with 399 (222 to 615 IQR) snails in the treatment with the highest imidacloprid concentration (Kruskal–Wallis $p \leq 0.05$; Mann–Whitney $Up = 0.007$; Fig. 5).

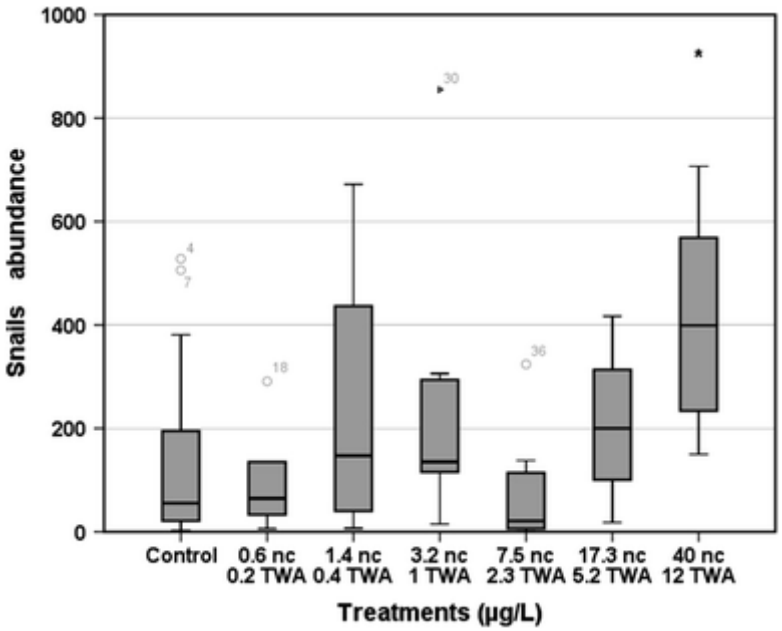

Fig. 5

Increased abundance of *Radix* sp. (Gastropoda) across imidacloprid concentrations. * Treatments significantly different from control ($p < 0.05$). *Numbers* and *signs* without *asterisks* indicate outliers

Finally, the abundance of Ephemeroptera, represented by only two taxa (*Caenis* sp. and *Cloeon* sp.) decreased at the highest imidacloprid concentration; however, this was not significant (Kruskal–Wallis $p = 0.2$). At the highest imidacloprid concentration, *Caenis* sp. was present in only two of the seven microcosms (Fig. 6b), whereas Baetidae species *Cloeon* sp. was completely absent. Unfortunately, *Cloeon* sp. colonised only a few control microcosms and was therefore too infrequent to run a powerful statistic test (*G* test $p < 0.05$).

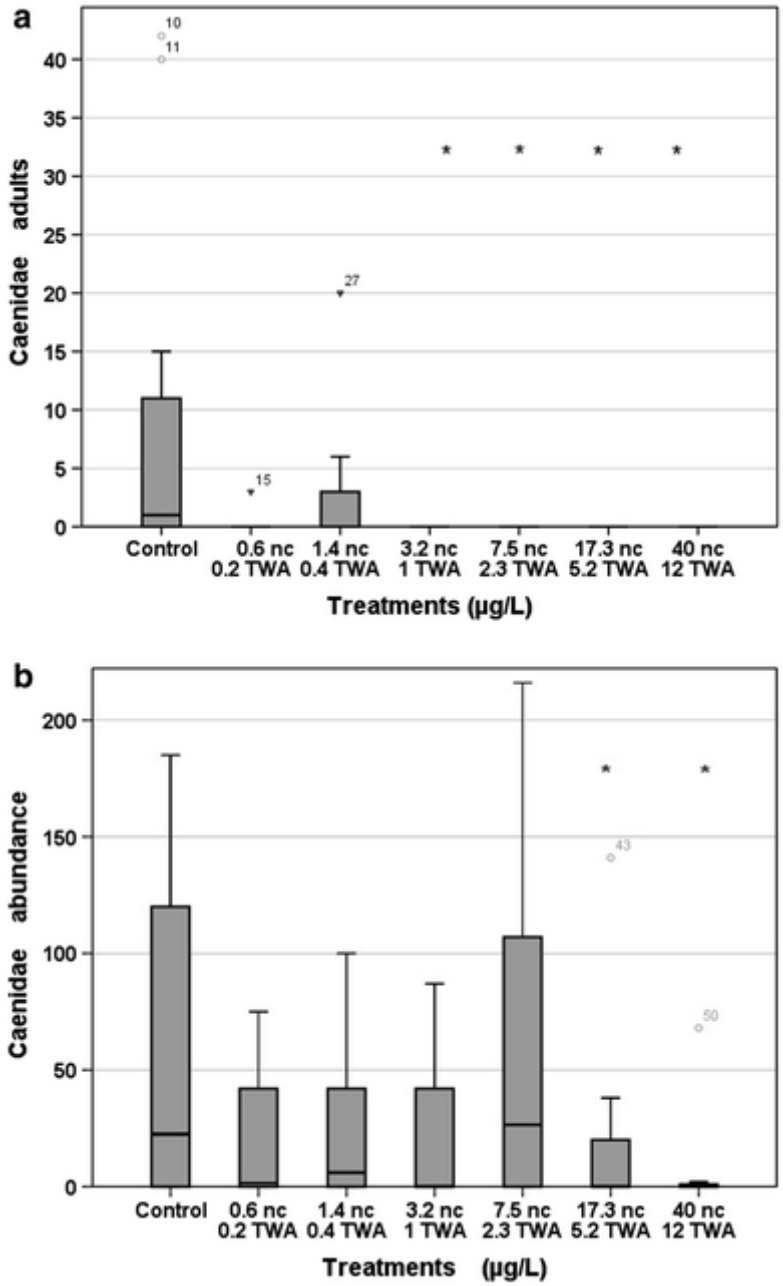

Fig. 6

Emergence of adult *Caenis* sp. (Caenidae, Ephemeroptera) and its total abundance (larvae and adults) across imidacloprid concentrations. * Treatments significantly different from control ($p < 0.05$). *Numbers* and *signs* without *asterisks* indicate outliers

Effects of Imidacloprid on Emergence (as Number of Adults)

The total number of adult Chironomidae decreased with increasing imidacloprid concentrations; however, this was not significant. The number of *Ablabesmyia* sp. adults decreased significantly (Kruskal–Wallis $p = 0.002$; Jonckheere–Terpstra $p = 0.001$); however, this decrease, similar to the general decrease of Chironomidae adults, reflected decreased survival (hence decreased abundance) rather than delayed emergence.

The number of adult Ephemeroptera appeared to be sensitive to increased amounts of imidacloprid (Jonckheere–Terpstra $p = 0.037$). In particular, *Caenis* sp. adults were absent from microcosms with imidacloprid concentrations >0.4 μg/L TWA (Kruskal–Wallis $p = 0.016$; Jonckheere–Terpstra $p < 0.001$; Fig. 6a).

Discussion

Fate of Imidacloprid in Microcosms

In this study, imidacloprid rapidly dissipated from the water column of the microcosms after each pulse with a mean DT_{50} of 28 ± 8 h monitored for 21 days in the field-based microcosms. This result is similar to those reported by Moring et al. (cited in CCME 2007), Moza et al. (1998), and Wamhoff and Schneider (1999), who determined a half-life of 30 h in outdoor microcosms. In them study by Moring et al. imidacloprid was added in four pulses each spaced 2 weeks apart. In that study as well as the current study, chemical analyses of sediment and porewater did not indicate major residues, which supports the hypothesis of photolysis as main breakdown pathway. During the present experiment, the UV radiation levels were high, and the transmission of light in the water column was almost unhindered due to both the absence of turbidity and color substances. The water in the microcosms was also moderately alkaline (pH 8–9), which may also have contributed to the rapid breakdown of imidacloprid (Zheng and Liu 1999). The clear water in the microcosms simulated lentic ecosystems exposed to spray drift events rather than to storm-water runoff.

Once imidacloprid enters aquatic ecosystems, the sediment could also become a source of pollution. In this study, the absence of high concentrations of imidacloprid in the sediment may be due to the rapid breakdown in its degradation products in the water column. Some hydrolytic metabolites of imidacloprid (e.g., 5-hydroxy imidacloprid and olefin) are equally toxic to insects (Suchail et al. 2001) and may contribute to overall toxicity.

Regarding the high concentrations found in many surface waters it may also be argued that due to factors such as turbidity, water colour, and pH, the half-life of imidacloprid in aquatic environment and in sediment may be considerably higher than expected. In any case, the concentrations in many surface waters are much greater than in those in the present study, which rather simulated a best-case scenario.

Some studies have suggested that imidacloprid and pesticides in general enter water bodies more frequently than what has been estimated so far (Liess et al. 1999; Neumann et al. 2002; Phillips and Bode 2004; Knabel et al. 2012). Analyses of sediments and survey techniques employing passive samplers showed that the actual amount of imidacloprid in water bodies is often underestimated. In two creeks in Germany, the mean concentration of imidacloprid in water detected by chemical analysis was 0.1 μg/L, whereas the sediment analyses indicated concentration of 344 μg/Kg (Höcker 2001).

On the Use of Nominal Concentrations: Are They Representative?

This study can be considered a chronic study in which the concentration of the toxicant was not held constant and the entire life cycle of a number of taxa and species was investigated. Due to the rapid photolysis of imidacloprid (Fig. 1), the use of nominal concentrations would overestimate imidacloprid concentrations during the experiment. In general, the use of TWA concentrations is recommended for substances that show relevant decay during the study such that appropriate reference concentrations for describing hazardous effects can be determined (Organisation for Economic Co-operation and Development 2008). In the case of imidacloprid, the use of TWA concentrations for describing the effects in this study is better because of the rapid photolysis and the low potential for bioaccumulation due to its K_{ow} (International Union of Pure and Applied Chemistry (CCME 2007).

In this experiment, Ephemeroptera started being affected at a nominal concentration of 3.2 μg/L, which corresponds to a three times lower TWA concentration of 1 μg/L, whereas the rest of the aquatic community was affected at the nominal concentration of 7.5 μg/L, which corresponds to a TWA concentration of 2.3 μg/L. These TWA values are in agreement with concentrations found in other community studies (Sánchez-Bayo and Goka 2006; Hayasaka et al. 2012).

Imidacloprid Effects on Macroinvertebrate Assemblage

The macroinvertebrate community that colonized the microcosms was affected by the repeated imidacloprid pulses although imidacloprid rapidly disappeared from the water phase. The assemblage was remarkably affected in the two highest treatment levels of 5.2 and 12 μg/L TWA, respectively. Some adverse effects were also visible at lower

concentrations: at 1 μg/L TWA, a concentration that seems to have an effect on Ephemeroptera emergence and at 2.3 μg/L TWA where there was a significant decrease in the abundance of *Ablabesmyia* sp. (Tanypodinae).

Chironomidae are pioneer species (Armitage et al. 1995) and hence, not surprisingly, were the most common insect found in the microcosms. Different species are known to have different ranges of sensitivity toward pollutants in general (Pettigrove and Hoffmann 2005), which was also evident in this study.

The decrease in the survival of Tanypodinae and the decreased emergence of *Caenis* sp. Were also observed in a stream mesocosms study, in which the organisms were exposed to three pulses of 12 μg/L of imidacloprid (corresponding to a 0.85 μg/L TWA concentration (Mohr et al. 2012). These similar results, although found in two different systems (lentic and lotic), may indicate that low repeated imidacloprid pulses are likely to have effects on aquatic benthic communities.

The findings of reduced adult numbers (emergence success) were also supported by Handy (1994), who postulated that sporadic pollution pulses, if not immediately lethal, may still have long-term effects (or sublethal effects) and may become lethal when ulterior stressors are added. In tests where the imidacloprid pulses did not reach lethal concentrations, often sublethal effects, such as feeding inhibitions or a reduction of body size, occurred (Alexander et al. 2007).

In addition, Tennekes and Sanchez-Bayo (2011) showed that one short-term exposure to a low imidacloprid concentration, which is expected to cause only sublethal effects, had the same or even stronger effect as one single exposure to a high concentration. They inferred that imidacloprid has a cumulative effect. The effects observed in our study were probably cumulated effects caused by repeated pulses. However, colonization of the microcosms was still possible until the third pulse. This means that some invertebrates were potentially exposed to only one pulse instead of three. If outcomes caused by the cumulative effect were present at the low concentrations, they may have been masked by the organisms exposed to only one pulse. Nevertheless, our results, based on total survival and emergence (as number of adults), are comparable with those obtained in a stream mesocosms study with a similar exposure (Mohr et al. 2012) despite differences in size, system (lentic/lotic), and complexity.

The findings of this study are particularly relevant for univoltine organisms that may be frequently exposed to imidacloprid pulses during their life cycle and are therefore likely to be more strongly affected by short-term, sublethal contaminations than short-living groups (e.g., chironomids). It should also be considered that under natural conditions, organisms are exposed to a plethora of stressors: mixture of chemicals, predators, changing abiotic and environmental conditions, etc. As a result, cumulative or synergistic effects can occur, or the time available for detoxification mechanisms and recovery may not be sufficient (Ashauer et al. 2012).

This study also made clear that the identification of organisms only to the family level could become a confounding variable: Often different species have different sensitivities, and therefore the identification to at least the genus level is recommended. For example, in this study, *Procladius* sp. and *Ablabesmyia* sp., although belonging to the same family, the Tanypodinae, showed a completely different response to imidacloprid. *Ablabesmyia* sp. was very sensitive, whereas *Procladius* sp. seemed to be tolerant. Interspecific differences in sensitivity to imidacloprid were found also in mayflies (Roessink et al. 2013).

Implication for the Ecosystem

The microcosm method used in this experiment has the same advantage as mesocosms studies because it allows for insight in potential intraspecific and interspecific interactions (indirect effects). A typical indirect effect is the increase in abundance of tolerant species. In this study, an increase in the number of the pulmonate gastropod *Radix* sp. was observed with increasing imidacloprid concentrations. Because an increase in population density of Gastropoda has been also observed elsewhere as an indirect effect in insecticide-stressed aquatic ecosystems and community-level studies (CCME 2007) we could hypothesise that this could be an example of an indirect effect caused by imidacloprid. This increase could be explained by (1) molluscs in general having moderate tolerance to imidacloprid due to its mode of action and (2) the reduction of competition for food and space with other species that were more sensitive. In the microcosms, the snails took over the functional role of the more sensitive grazers, such as chironomids and mayflies. This phenomenon is referred to as “functional redundancy” (Tilman et al. 1997). In this respect, functional end points may be useful in gaining complementary information; however, structural end points seem to be more adequate to detect a toxicant’s effects as also shown by Pestana et al. (2009).

The survival of only the tolerant species has clear consequences for any ecosystem exposed to pollutants. Cucker (1983) observed that the increased density of *Lymnaea* sp. caused a reduction of biomass in all food competitors (free-living scrapers and grazers and tube-dwelling scrapers) and even in mobile predators. According to Cucker (1983), snails themselves became a stressor: For example, they affected the chironomids by direct contact, thus causing larval displacement and a temporary disruption of foraging activity. Even for the tube-dwellers, the chironomids’ frequent snail encounters would result in damaged tubes, increased maintenance costs, and simultaneously increased exposure to predators. In this experiment, some of the sensitive species already affected by imidacloprid may have become further stressed by interaction and competition with a greater number of snails and other tolerant species. Indirect effects may have even more far-reaching consequences if tolerant species are vectors for diseases or hosts of parasites, which is often the case in snails.

Indirect effects observed in multispecies tests may not reflect what may occur in a natural environment in detail; however, they are an inevitable and unpredictable consequence of the direct effects and as such are worthwhile to be considered. Depending

on the end points chosen, an increase in abundance of tolerant species may mask the effects of the toxicant, e.g., a decrease of abundance or emergence of sensitive species.

Conclusion

This study showed that repeated short-term pulses of imidacloprid at low concentration levels affected aquatic ecosystems even under favourable conditions for photolysis of imidacloprid in water. As a consequence, an ecosystem may experience direct and indirect effects, imbalance, and cascading effects on many trophic levels and not those restricted to the aquatic food web. The implications of this are not easily foreseen. Considering the increased use of imidacloprid and of pesticides in agriculture and gardening in general, episodic contaminations (pulses) will not be rare events and thus represent a realistic and recurrent risk. The proposed microcosms approach, with its field-relevant and simple design, proved to be a useful tool for assessing the effects of imidacloprid contaminations.

Notes

Acknowledgments

The authors are grateful to the entire team of the UBA unit IV 2.5 especially to S. Loth and S. Meinecke and the analytical section for their kind support. A. Günther, J. Schott, and K. Grohmann are acknowledged for assistance during field work and sampling. We also thank C. Knowles and two anonymous reviewers for the valuable comments.

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Cite this article as:
Colombo, V., Mohr, S., Berghahn, R. et al. Arch Environ Contam Toxicol (2013) 65: 683. https://doi.org/10.1007/s00244-013-9940-2

- DOI (Digital Object Identifier) https://doi.org/10.1007/s00244-013-9940-2
- Publisher Name Springer US
- Print ISSN 0090-4341
- Online ISSN 1432-0703

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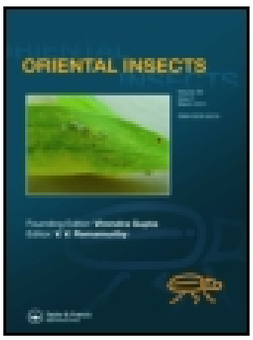

Oriental Insects

ISSN: 0030-5316 (Print) 2157-8745 (Online) Journal homepage: http://www.tandfonline.com/loi/toin20

Cross-resistance among common insecticides and its possible mechanism in Laodelphax striatellus Fallén (Hemiptera: Delphacidae)

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To cite this article: Mohammed Esmail Abdalla Elzaki, Jian Pu, Yuxuan Zhu, Wanfang Zhang, Haina Sun, Min Wu & Zhaojun Han (2017): Cross-resistance among common insecticides and its possible mechanism in Laodelphax striatellus Fallén (Hemiptera: Delphacidae), Oriental Insects, DOI: 10.1080/00305316.2017.1316784

To link to this article: http://dx.doi.org/10.1080/00305316.2017.1316784

ORIENTAL INSECTS, 2017
https://doi.org/10.1080/00305316.2017.1316784

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Cross-resistance among common insecticides and its possible mechanism in *Laodelphax striatellus* Fallén (Hemiptera: Delphacidae)

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ABSTRACT

Laodelphax striatellus Fallén, is a serious pest of rice, has developed resistance to various chemical insecticides. Thus, clear documentation of resistance and cross-resistance is required for good resistance management. This study examined cross-resistance among common insecticides acting on different targets and its mechanism. First, the *L. striatellus* strains selected with chlorpyrifos, deltamethrin and imidacloprid were tested for cross-resistance to common insecticides. Then, these three resistant strains underwent mixed breeding for two generations, as an original population and reselected for seven generations by corresponding insecticides, resulting in three new resistant strains. These strains were tested for confirmation of the cross-resistance and the mechanism was analysed by comparing the expression levels of related detoxification enzyme genes. The results demonstrated that cross-resistance existed among chlorpyrifos, deltamethrin and imidacloprid. The detoxification enzyme gene *CYP6AY3v2*, *CYP306A2v2* and *CYP353D1v2* were found to be up-regulated in the chlorpyrifos-selected strain; *CYP6AY3v2*, *CYP6FU1*, *CYP353D1v2*, and *CYP439A1v3* in the Deltamethrin-selected strain; and *CYP4C72*, *CYP6AY3v2* and *CYP353D1v2* in the Imidacloprid-selected strain. Furthermore, overexpression of *CYP6AY3v2* and *CYP353D1v2* was in concert with cross-resistance in selected strains. These results suggest that *CYP6AY3v2* and *CYP353D1v2* might be associated with the cross-resistance among chlorpyrifos, deltamethrin and imidacloprid in *L. striatellus*.

ARTICLE HISTORY
Received 19 May 2016
Accepted 4 April 2017

KEYWORDS
Chlorpyrifos; cross-resistance; Cytochrome P450; deltamethrin; imidacloprid; *Laodelphax striatellus*

1. Introduction

The small brown planthopper *Laodelphax striatellus* Fallén (Homoptera: Delphacidae) found widely throughout China and Southeast Asia is one of the

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Supplemental data for this article can be accessed https://doi.org/10.1080/00305316.2017.1316784

most serious pests of rice, maize and wheat. This insect causes major yield reductions by sucking plant sap and transmitting plant viruses (Duan et al. 2010), and was usually controlled by chemical insecticides. However, overuse of insecticides was driving a force of insects to develop a serious resistance to various insecticides, such as organophosphate, carbamate (Nagata et al. 1979; Nagata & Ohira 1986; Endo et al. 2002; Ban et al. 2012), neonicotinoid (Gao et al. 2008; Otuka et al. 2010; Sanada-Morimura et al. 2010), phenylpyrazole (Elzaki et al. 2015), pyrethroid and insect growth regulator insecticides (Yanhua et al. 2010, Xu et al. 2014).

In order to understand and manage this resistance, the resistance mechanisms of deltamethrin, chlorpyrifos and imidacloprid have been studied (Xu et al. 2013, 2014; Elzaki et al. 2016). However, little is known about the cross-resistance among insecticides; especially between the insecticides have a different mode of action. Cross-resistance avoidance is the key for pesticide selection in pest control. Traditionally, the insecticides with the same targets were thought to possess cross-resistance. For example, the cross-resistance among pyrethroids or organophosphates caused by insensitively alternated voltage-gated sodium channel or acetylcholinesterase, respectively (Bisset et al. 1997; Rodríguez et al. 2002). Additionally, cross-resistance has also been found between insecticides with a different mode of action (Gorman et al. 2010), which usually results from enhanced detoxification in the pest. However, the molecular mechanism is unclear, and no occurrence regularity had been found, because detoxification enzymes are encoded by supergene families.

The previous work in our laboratory revealed that some cytochrome P450 oxidase (P450) and esterase genes were involved and over-expressed in the small brown planthopper resistant to different kinds of insecticides. Thus, with resistant strains selected with different insecticides, this study tries to declare the cross-resistance among common insecticides and its mechanism in *L. striatellus*.

2. Materials and methods

2.1. Insects and resistance selection

The susceptible (Sus) and the three resistant strains (chlorpyrifos (Chlor-R), deltamethrin (Delt-R) and imidacloprid (Imida-R)) were laboratory-selected strains (selected for more than 30 generations), originally collected from Jianhu in 2009. The Mix strain was established by mixing the three resistant strains with similar portions and breeding together for two generations (200 m and 200 f for each strain). Then, as the original the mixed population was selected for seven generations with corresponding insecticides for developing Chlorpyrifos-selected (Chlor-selected), deltamethrin-selected (Delt-selected) and imidacloprid-selected (Imida-selected) strains.

Resistance selection was carried out by spraying the insect colonies every generation with the LC_{50} of their parents. One or two days after the insecticide

application, the surviving insects (mainly adults and older nymphs) were transferred to new fresh rice seedlings without insecticides and were reared routinely for breeding and further selection.

In this study, all insects were reared on rice seedlings planted in plastic boxes tissue-laid (soil-less) at 26 (±2) °C under a photoperiod 12:12 h light: dark regime at 70–80% relative humidity.

2.2. Insecticides and chemicals

The insecticides used were technical grade purchased from Invitrogen Biotechnology Co., Ltd, Shanghai, China (avermectin 92%, chlorpyrifos 96.5%, deltamethrin 98%, ethiprole 97.4% and imidacloprid 97%) and acetone was obtained from Shanghai Ling Feng Chemical Reagent Co., Ltd, Shanghai, China.

2.3. Toxicity bioassay

Topical application (Immaraju et al. 1990) was used for testing the LD_{50} of avermectins, chlorpyrifos, deltamethrin, ethiprole and imidacloprid with female adults (3–5 days old) as the test animal. Technical grade insecticides were dissolved in acetone and serially diluted into five concentrations as treatments. A group of 30 female adults were anesthetized with CO_2 for 30 s, and then treated individually with a droplet (0.05 µL) of the insecticide solutions topically applied on the pronotum with a Microapplicator (Burkard Manufacturing Co Ltd, Rickmansworth, UK). The control was treated with acetone alone. The treated insects were reared in plastic cups containing fresh rice seedlings covered with a cloth net and were kept under the same rearing conditions mentioned above. For each concentration, 25–30 insects were used in three replicates. The results were checked after three days for all tested insecticides. The resistance ratio (RR) was calculated by dividing the LD_{50} of tested strain by the LD_{50} of the susceptible strain.

2.4. RNA extractions and cDNA synthesis

Total RNA was isolated from 20 adults of the Sus, Chlor-selected, Delt-selected and Imida-selected strains. RNA was extracted with TRIzol Reagent (Invitrogen). Three different samples were extracted for each strain. The quality and quantity of the RNA were measured using 1% gel electrophoresis and Nano-Drop spectrophotometer (NanoDrop Technologies). Ten micrograms of total RNA from each of the three biological replicates were used as templates for cDNA synthesis using the protocol (TaKaRa, Dalian, Liaoning, China) according to the manufacturer's suggestion. The quantity of cDNA was measured using Nano-Drop spectrophotometer.

2.5. Detoxification genes and quantitative real-time PCR

Eight P450s and two esterase (EST) genes were checked, *CYP4C71v2*, *CYP4C72*, *CYP6AY3v2*, *CYP6FU1*, *CYP306A2v2*, *CYP314A1v2*, *CYP353D1v2*, *CYP439Av3*, *LSCE12* and *LSCE35*, which were insecticide resistance-related genes in *L. striatellus* (Xu et al. 2014; Elzaki et al. 2016). Genes sequence were got from NCBI and the primers were designed by Beacon Designer 7.0 (Premier Biosoft Inter National, Palo Alto, CA, USA) (Supplementary Table 1). The amplification efficiency of the primer pairs was measured using the equation $E = 10^{-1/slope}$, where the slope was determined from the standard curve based on *Ct* values vs. fivefold dilutions of the cDNA templates. The relative expression levels of the mentioned genes were checked in Mix strain and three-selected strains and compared with susceptible strain as control.

The quantitative real-time PCR system was done with SYBR Premix Ex TaqTM (TaKaRa, Dalian, Liaoning, China) and the Applied Biosystems 7500 Real Time PCR system. Each reaction contained a gene specific primer pair with 20 μL final volume consisting of 1.0 μL cDNA, 10 μL SYBR Premix Ex Taq™, 0.4 μL of each primer (10 μM), 0.4 μL Rox Reference Dye II (50×) and 7.8 μL ddH_2O. PCR reactions were performed at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. Three biological samples with three technical replicates were tested for each gene. Beta-actin was used as the internal control (Yanhua et al. 2010). The $2^{-\Delta\Delta Ct}$ method (Zhang et al. 2010) was used for calculating the relative level of expression using the ABI 7500 analysis software.

2.6. Statistical analysis

The Polo Plus® program (LeOraSoftware 2002) was used for a probit analysis of the dose-response data. The statistical significance differences in *Ct* between two samples were calculated with a *t*-test using SPSS software. A *P*-value ≤0.05 was considered significantly different or ≤0.01 level was considered significantly different.

3. Results

3.1. Resistances of different insecticides-selected strains

The toxicity of avermectin, chlorpyrifos, deltamethrin, ethiprole and imidacloprid was tested against Sus, Chlor-R, Delt-R and Imida-R strains (Table 1). The result demonstrated that the Delt-R strain exhibited a high level of resistance to deltamethrin (264-fold), chlorpyrifos (104-fold), imidacloprid (119-fold) and ethiprole (45-fold), whereas, low level of resistance to avermectin (9-fold). The Chlor-R strain showed high resistance to chlorpyrifos (104-fold) as well as deltamethrin (49-fold), moderate resistance to imidacloprid (20-fold), however no obvious resistance to avermectins and ethiprole (four and threefold, respectively). The

Table 1. Toxicity of common insecticides on different strains of *Laodelphax striatellus*.

	Susceptible	Delt-R		Chlor-R		Imid-R	
Insecticides	LD_{50} μg/insect (95% CL)	LD_{50} μg/insect (95% CL)	RR	LD_{50} μg/insect (95% CL)	RR	LD_{50} μg/insect (95% CL)	RR
Deltamethrin	0.0003 (0.0001–0.0007)	0.0793 (0.0501–0.1256)	264	0.0147 (0.0047–0.0463)	49	0.0073 (0.0054–0.0098)	24
Chlorpyrifos	0.0002 (0.0002–0.0003)	0.0207 (0.0147–0.0292)	104	0.0207 (0.0157–0.0274)	104	0.0125 (0.0089–0.0175)	63
Imidacloprid	0.0004 (0.0002–0.0008)	0.0476 (0.0270–0.0839)	119	0.0081 (0.0062–0.0106)	20	0.0134 (0.0067–0.0270)	34
Ethiprole	0.0001 (0.0001–0.0002)	0.0045 (0.0029–0.0070)	45	0.0003 (0.0002–0.0006)	3	0.0002 (0.0001–0.0004)	2
Avermectins	0.0004 (0.0002–0.0007)	0.0034 (0.0024–0.0048)	9	0.0017 (0.0009–0.0032)	4	0.0086 (0.0048–0.0154)	22

Note: RR (resistance ratio) = LD_{50} of tested strain/LD_{50} of susceptible strain.

Imida-R strain displayed moderate to high level of resistance to imidacloprid, chlorpyrifos, avermectin and deltamethrin (34-, 63-, 22- and 24-fold, respectively), whereas, no resistance to ethiprole (twofold).

For the susceptible and the three resistant strains were developed from the same field population by breeding without contacting any insecticides or continuously selected with the corresponding insecticide, the resistance showed for each resistant strain might result from the selection of corresponding insecticide as well as the heredity of the original field population, which had exposed to different insecticide spraying. Thus, it could be interfered that the resistance shown to the insecticides other than the selected one may be cross-resistance or multiple resistances. In general, cross-resistance should increase in step during the selection, whereas the multiple resistances would not. Therefore, the cross-resistance was confirmed by checking resistance increase during reselection with deltamethrin, chlorpyrifos and imidacloprid as shown below.

3.2. *The resistance level of mixed and reselected strains of* L striatellus

To confirm the observed cross-resistance, the three resistant strains were mixed and reselected again with deltamethrin, chlorpyrifos and imidacloprid. Then, the resistance of the three reselected strains was tested and compared to the original Mix strain. Figure 1 shows that the original Mix strain had only moderate resistance to deltamethrin, chlorpyrifos and imidacloprid (52, 32 and 18-fold, respectively). After selection with three insecticides, the resistance to deltamethrin, chlorpyrifos and imidacloprid increased dramatically in the first generation (to 99.0, 152.0 and 71.5-fold, respectively). In the third generation, the increase of resistance was very slow as compared to the first generation; it was 125.7, 174.5 and 92.8-fold, respectively.

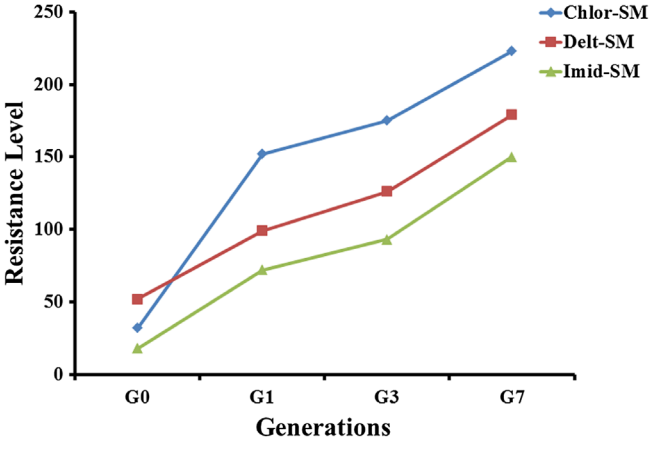

Figure 1. The dymanics of selection of Mix strain (G0) against three different insecticides.
Notes: chlorpyrifos for chlor-SM strain, deltamethrin for Delt-SM and imidacloprid for Imida-SM.

Table 2. Toxicity of deltmethrin, chlorpyrifos and imidacloprid in Mix and three selected strains of *Laodelphax striatellus*.

Insecticides	Strains	LD_{50} μg/insect	(95% CL)	RR
Deltamethrin	Suceptible	0.0003	(0.0001–0.0007)	1
	Mixed	0.0155	(0.0105–0.0230)	52
	Delt-selected	0.0537	(0.0260–0.1107)	179
	Chlor-selected	0.0104	(0.0051–0.0212)	35
	Imida-selected	0.0106	(0.0043–0.0260)	35
Chlorpyrifos	Suceptible	0.0002	(0.0002–0.0003)	1
	Mixed	0.0063	(0.0043–0.0090)	32
	Delt-selected	0.0236	(0.0181–0.0306)	118
	Chlor-selected	0.0446	(0.0313–0.0634)	223
	Imida-selected	0.0138	(0.0095–0.0201)	69
Imidacloprid	Suceptible	0.0004	(0.0002–0.0008)	1
	Mixed	0.0071	(0.0046–0.0110)	18
	Delt-selected	0.0277	(0.0142–0.0540)	69
	Chlor-selected	0.0118	(0.0079–0.0177)	30
	Imida-selected	0.0601	(0.0257–0.1407)	150

Note: RR (resistance ratio) = LD_{50} of tested strain/LD_{50} of susceptible strain.

The cross-resistance in the selected strains was tested in the seventh generations as showed in Table 2. The Deltamethrin-selected strain shows increase in the resistance to deltamethrin from 52-fold to 179-fold. Meanwhile, resistance to chlorpyrifos and imidacolprid increased from 32- to 118-fold and 18- to 69-fold, respectively. These result showed clear cross-resistance among deltamethrin, chlorpyrifos and imidacloprid.

The resistance of Chlorpyrifos-selected strain to chlorpyrifos increased from 32-fold to 223-fold. Meanwhile, the resistance to imidacloprid increased from 18- to 30-fold, while the resistance to deltamethrin decreased from 52- to 35-fold. These data showed clear cross-resistance between chlorpyrifos and imidacloprid. Conversely, there was no cross-resistance to deltamethrin.

The imidacloprid resistance in resulting Imidacloprid-selected strain increased from 18-fold to 150-fold. Meanwhile, the resistance to chlorpyrifos increased from 32- to 69-fold, whereas, the resistance to deltamethrin decreased from 52- to 35-fold. These data showed clear cross-resistance between chlorpyrifos and imidacloprid, however no cross-resistance to deltamethrin.

It is clear that cross-resistance existed among the three tested insecticides. However, deltamethrin selection could increase obviously the resistance to chlorpyrifos and imidacloprid, but neither chlorpyrifos nor imidacloprid selections could increase the resistance to deltamethrin. Thus, the cross-resistance among deltamethrin, chlorpyrifos and imidacloprid was not reciprocal.

3.3. Expression levels of related detoxification enzyme genes in different strains of L. striatellus

Ten detoxification enzyme genes have been found involved in insecticide resistance of *L. striatellus* (Xu et al. 2013, 2014; Elzaki et al. 2016). Thus, the relative expression levels of these genes were checked in the Mix, Chlor-selected,

Delt-selected and Imida-selected strains compared to Sus strain. The Sus strain was reared in parallel with the selected strains, which was sampled as the control, and tested at the same time for either Mix or the selected strains.

Figure 2 shows the relative expression levels of eight cytochrome P450s and two EST genes in the Mix strain. *CYP4C71v2*, *CYP4C72*, *CYP6AY3v2*, *CYP6FU1*, *CYP306A2v2*, *CYP314A1v2*, *CYP353D1v2*, *CYP439A1v3*, *LSCE12* and *LSCE35* were 0.68, 4.8, 1.8, 2.2, 1.9, 1.9, 6.6, 4.4, 2 and 0.5 fold higher than those in the susceptible strain, respectively. Eight of the ten genes were significantly up-regulated.

Figure 3 displayed the relative expression levels of detoxification genes in the Chlor-selected strain, where only three genes were found to be overexpressed,

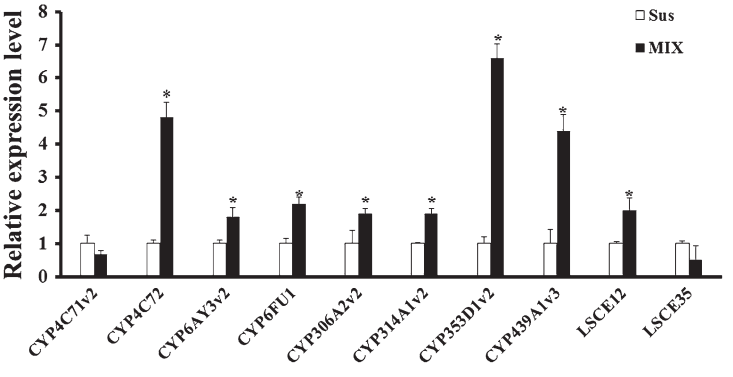

Figure 2. Expression levels of detoxification genes between Mix strain and Susceptible strain (Sus).

Notes: Each bar indicated the mean of three biological samples, each implemented in replicates. Error bars represented the standard deviation from the mean. Data were normalised to the expression of β-actin. The significant differences between strains and population were compared with Susceptible strain, were marked by stars. *significantly different at 0.05 level and **significantly different at 0.01 level.

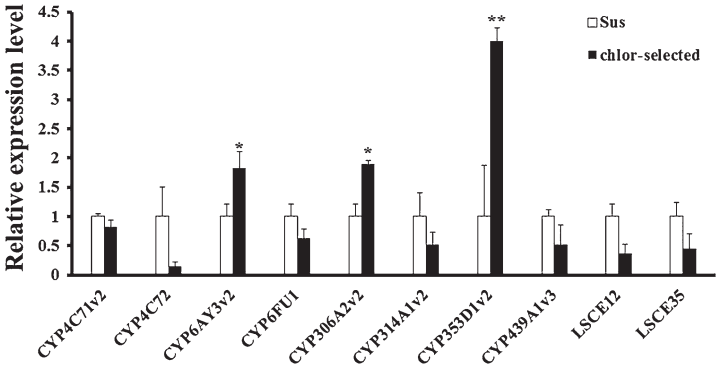

Figure 3. Expression levels of detoxification genes between Chlorpyrifos-selected strains (chlor-SM) and Susceptible strain(Sus).

Notes: Each bar indicated the mean of three biological samples, each implemented in replicates. Error bars represented the standard deviation from the mean. Data were normalised to the expression of β-actin. The significant differences between strains and population were compared with Susceptible strain, were marked by stars. *significantly different at 0.05 level and **significantly different at 0.01 level.

CYP6AY3v2 (1.83-fold), *CYP306A2v2* (1.9-fold) and *CYP353D1v2* (fourfold); and significantly different as compared to the susceptible strain.

Figure 4 exhibits the relative expression levels of detoxification genes in the Delt-selected strain, and four genes were found up-regulated, *CYP6AY3v2* (1.8-fold), *CYP6FU1* (twofold), *CYP353D1v2* (1.8-fold) and *CYP439A1v3* (4.5-Fold) and were significantly different as compared to the susceptible strain.

Figure 5 presents the relative expression levels of detoxification genes in the Imida-selected strain, three genes were found overexpressed and significantly different as compared to the susceptible strain, *CYP4C72* (1.55-fold), *CYP6AY3v2* (1.95-fold) and *CYP353D1v2* (1.97-fold).

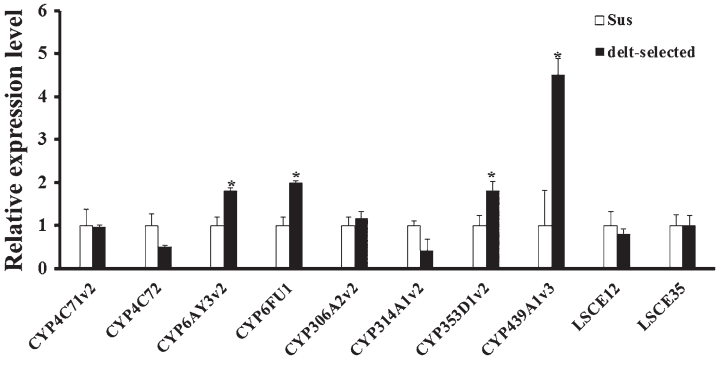

Figure 4. Expression levels of detoxification genes between Deltamethrin-selected strains (Delt-SM) and Susceptible strain(Sus).

Notes: Each bar indicated the mean of three biological samples, each implemented in replicates. Error bars represented the standard deviation from the mean. Data were normalised to the expression of β-actin. The significant differences between strains and population were compared with Susceptible strain, were marked by stars. *significantly different at 0.05 level and **significantly different at 0.01 level.

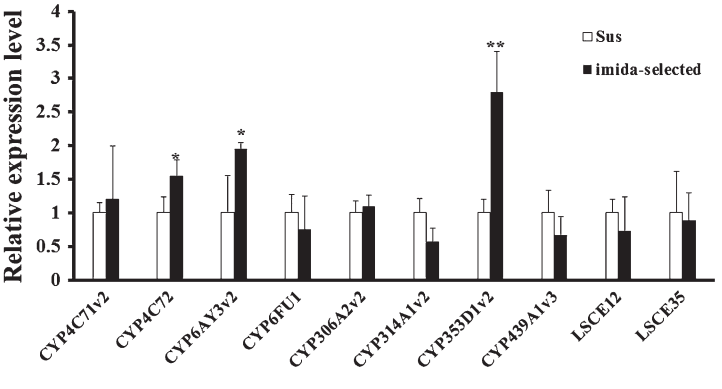

Figure 5. Expression levels of detoxification genes between Imidacloprid-selected strains (Imida-SM) and Susceptible strain(Sus).

Notes: Each bar indicated the mean of three biological samples, each implemented in replicates. Error bars represented the standard deviation from the mean. Data were normalised to the expression of β-actin. The significant differences between strains and population were compared with Susceptible strain, were marked by stars. *significantly different at 0.05 level and **significantly different at 0.01 level.

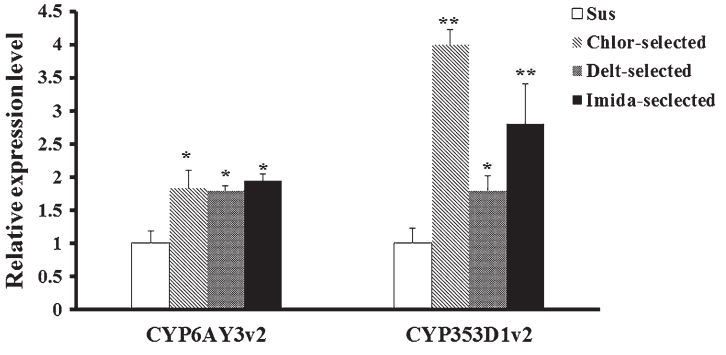

Figure 6. Expression levels of *CYP6AY3v2* and *CYP353D1v2* in Chlorpyrifos-selected, Deltmethrin-selected and Imidacloprid-selected strains.

Notes: Each bar indicated the mean of three biological samples, each implemented in replicates. Error bars represented the standard deviation from the mean. Data were normalised to the expression of β-actin. The significant differences between strains and population were compared with Susceptible strain, were marked by stars. *significantly different at 0.05 level and **significantly different at 0.01 level.

Furthermore, both *CYP6AY3v2* and *CYP353D1v2* genes were found overexpressed in all these three resistant strains. Figure 6 showed the expression levels of *CYP6AY3v2* and *CYP353D1v2* in the Chlor-selected, the Delt-selected and the Imida-selected strains were (1.83, 1.80 and 1.95-fold) and (4.00, 1.80 and 1.97-fold), respectively.

4. Discussion

Cross-resistance arises when resistance to one selection compound also confers protection against others. However, accurate evaluation depends on proper test methods. In the past, cross-resistance was assessed by checking the selection strain with ready baseline data as standards. This method is scarcely used now, because of depending on the baseline data used. Nowadays, the original strain is used as the control to see if the resistance to other insecticides increases after selection (Xu et al. 2014; Yorulmaz-Salman & Ay 2014). When the resistant strains (or field resistant populations) are used as original, this method may underestimate cross-resistance because further resistance increase is difficult in this case. In addition, the resistant original strain divided into two groups. One group is used for the resistance selection and the other is reared parallel for recovery to the relatively susceptible strain. Then, the parallel reared susceptible strain is used as the control to see if the resistance to other insecticides is selected (Faheem et al. 2013; Xu et al. 2014). However, this method usually overstates cross-resistance because selection could slow down the susceptibility recovery to some insecticides in the selected strain, which makes the selected strain more resistant than the parallel reared susceptible strain (Gao 2008). In our work, different methods were used and all results indicated that cross-resistance existed among chlorpyrifos, deltamethrin and imidacloprid, except the un-reciprocal one between deltamethrin and

the other two insecticides when the Mix strain original was considered as control during resistance ratio calculations.

Cross-resistance is most common among members of the same chemical classes because of the target insensitive alternation. For example, cross-resistance among pyrethroids in *Anopheles gambiae* (Chandre et al. 1999) and among organophosphates in Codling Moth, *Lucilia cuprina* and *L. striatellus* (Campbell et al. 1998; Dunley & Welter 2000; Wang et al. 2010). This study has reported cross-resistance among chlorpyrifos, deltamethrin and imidacloprid in the resistant strains of *L. striatellus*. Similarly, cross-resistance among different insecticides was also reported in different kinds of insect pests such as: *Spodoptera exigua* (Ishtiaq et al. 2012), *Bemisia tabaci* (Gorman et al. 2010), *Choristoneura rosaceana*, *Pandemis pyrusana* (Dunley et al. 2006), and *Cydia pomonella* (Sauphanor et al. 1998).

Insecticide resistance is raised mainly by insensitive targets and enhanced detoxification. Thus, cross-resistance among different kinds of insecticides could be due to target site mutation only when the cross couples share the same target, such as cross-resistance between pyrethroid and DDT in *Aedes aegypti* (L.) correlated with novel mutation in voltage-gated sodium channel gene (Brengues et al. 2003). For those insecticides with different action targets, cross-resistance might result from enhanced detoxification, as well as reduced penetration rate or even increased excretion, which acts on both insecticides of the cross couples. In addition, detoxification enzymes that could metabolise different xenobiotic compounds might drive to cross-resistance within and among various insecticides.

Our results showed that most identified detoxification genes were overexpressed in the Mix strain, which was developed from multiple resistant strains. When the Mix strain was selected with chlorpyrifos, deltmethrin and imidacloprid, only few genes kept overexpressed, such as *CYP6AY3v2*, *CYP306A2v2* and *CYP353D1v2* in Chlor-selected strain; *CYP6AY3v2*, *CYP6FU1*, *CYP353D1v2*, and *CYP439A1v3* in Delt-selected strain and *CYP4C72*, *CYP6AY3v2* and *CYP353D1v2* in Imida-selected strain. In general, insecticide resistance-unrelated genes should keep constant expression levels. The expression of most genes might decrease due to insecticide treatment or the systematic error for testing the Mix and the selected strains in different time. Taking into consideration the genes kept up-regulated in the selected strains, they should have higher expression level than those in the original Mix strain.

The overexpressed CYP genes *CYP6AY3v2*, *CYP306A2v2* and *CYP353D1v2* in Chlorpyrifos-selected strain had previously been proved to be associated with chlorpyrifos resistance in *L. striatellus* (Xu et al. 2014). Likewise, *CYP6AY3v2*, *CYP6FU1*, *CYP353D1v2*, and *CYP439A1v3* genes were also identified as conferring resistance to deltamethrin in *L. striatellus* (Xu et al. 2013). *CYP6AY3v2*, *CYP4C71v2*, *CYP4C72* and *CYP353D1v2* were found to be associated with imidacloprid resistance in *L. striatellus* (Elzaki et al. 2016). Thus, our results indicate that the overexpressed CYP genes identified in different re-selected strains are associated with resistance to chlorpyrifos, deltamethrin and imidacloprid.

Furthermore, *CYP6AY3v2* and *CYP353D1v2* were over-expressed in all three selected strains against chlorpyrifos, deltamethrin and imidacloprid in concert with high resistance to the mentioned insecticides, these results suggest that *CYP6AY3v2* and *CYP353D1v2* might be associated with cross-resistance among chlorpyrifos, deltamethrin and imidacloprid. Cytochrome P450s have a wide range of substrates, and overexpression of some CYP genes had been reported associated with cross-resistance in different insects. For example, *CYP6M2* was associated with the resistance of two classes of insecticides in *A.gambiae* (Mitchell et al. 2012). *CYP6CM1* conferred cross-resistance to imidacloprid, other neonicotinoid insecticides and pymetrozine in *B. tabaci* (Nauen et al. 2013). *CYP6g1* was associated with resistance to neonicotinoids, organophosphate and organochlorines in *Drosophila melanogaster* (Daborn et al. 2002; Le Goff et al. 2003; Joußen et al. 2008, 2010).

In conclusions, our study revealed that overexpression of *CYP6AY3v2*, *CYP306A2v2* and *CYP353D1v2* were associated with chlorpyrifos resistance, *CYP6AY3v2*, *CYP6FU1*, *CYP353D1v2*, *CYP439A1v3* were related with deltamethrin resistance, and *CYP4C72*, *CYP6AY3v2*, *CYP353D1v2* were related to imidacloprid resistance. In addition, high to moderate level of cross-resistance among chlorpyrifos, deltamethrin and imidacloprid were revealed in concert with overexpression of *CYP6AY3v2* and *CYP353D1v2*. These results suggest that *CYP6AY3v2* and *CYP353D1v2* might play an important role in cross-resistance among chlorpyrifos, deltamethrin and imidacloprid. Therefore, functional studies about the *CYP6AY3v2* and *CYP353D1v2* should be conducted.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by the Projects of National Natural Science Foundation of China [grant number 31130045]; Development Plan of the State Key Fundamental Research [grant number 2010CB126204]; and the Special Fund for Agro-scientific Research in the Public Interest of China [grant number 201303017].

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SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 36, No. 10, pp. 2838–2848, 2017
Published 2017 SETAC
Printed in the USA

COMPREHENSIVE CHARACTERIZATION OF THE ACUTE AND CHRONIC TOXICITY OF THE NEONICOTINOID INSECTICIDE THIAMETHOXAM TO A SUITE OF AQUATIC PRIMARY PRODUCERS, INVERTEBRATES, AND FISH

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(Submitted 5 February 2017; Returned for Revision 13 March 2017; Accepted 1 May 2017)

Abstract: Thiamethoxam is a neonicotinoid insecticide used widely in agriculture to control a broad spectrum of chewing and sucking insect pests. Recent detection of thiamethoxam in surface waters has raised interest in characterizing the potential impacts of this insecticide to aquatic organisms. We report the results of toxicity testing (acute and chronic) conducted under good laboratory practices for more than 30 freshwater species (insects, molluscs, crustaceans, algae, macrophytes, and fish) and 4 marine species (an alga, a mollusc, a crustacean, and a fish). As would be anticipated for a neonicotinoid, aquatic primary producers and fish were the least sensitive organisms tested, with acute median lethal and effect concentrations (LC50/EC50) observed to be $\geq$80 mg/L in all cases, which far exceeds surface water exposure concentrations. Tested molluscs, worms, and rotifers were similarly insensitive (EC50 $\geq$ 100 mg/L), except for *Lumbriculus* sp., with an EC50 of 7.7 mg/L. In general, insects were the most sensitive group in the study, with most acute EC50 values $<$ 1 mg/L. However, the crustaceans *Asellus aquaticus* and Ostracoda exhibited a sensitivity similar to that of insects (acute EC50 $<$ 1 mg/L), and the midge larvae *Chaoborus* sp. were relatively insensitive compared with other insects (EC50 $>$ 5.5 mg/L). The most sensitive chronic response was for *Chironomus riparius*, with a 30-d no-observed-effect concentration (NOEC; emergence) of 0.01 mg/L. Observed toxicity to the tested marine organisms was comparable to that of freshwater species. We used the reported data to construct species sensitivity distributions for thiamethoxam, to calculate 5% hazard concentrations (HC5s) for acute data (freshwater invertebrates), and compared these with measured concentrations from relevant North American surface waters. Overall, based on acute toxicity endpoints, the potential acute risk to freshwater organisms was found to be minimal (likelihood of exceeding HC5s $<$ 1%). *Environ Toxicol Chem* 2017;36:2838–2848. © 2017 The Authors. Environmental Toxicology and Chemistry published by Wiley Periodicals, Inc. on behalf of SETAC.

Keywords: Insecticide Aquatic toxicology Species sensitivity distributions Neonicotinoid Thiamethoxam

INTRODUCTION

The insecticide thiamethoxam was first approved for use in agriculture by the US Environmental Protection Agency (USEPA) in 1999. It is now used around the world to control a wide range of insect pests on major agricultural crops. Approved uses in the United States include seed treatments, foliar and soil applications. As a neonicotinoid insecticide, thiamethoxam acts selectively on insects by interfering with nicotinic acetylcholine receptors [1]. Globally, neonicotinoid insecticides comprise the most widely used insecticide class in agriculture [2] and have largely replaced a variety of older chemistries (e.g., organophosphates, carbamates, and organochlorine pesticides). This is because of their broad-spectrum control of numerous insect pests (e.g., aphids, whiteflies, flea beetles, thrips, and wireworms), versatile use pattern (e.g., crops such as maize, canola, sugar beet, and cotton), reduced risk profile to human health and the environment, and resulting agronomic benefit [1].

With their increased use, neonicotinoid insecticides have increasingly been included in surface water monitoring programs, with a number of publications citing detections of these compounds. Morrissey et al. [3] recently reported that 29 studies from 9 countries have published detectable concentrations of neonicotinoid insecticides in puddles, streams, rivers, wetlands, and irrigation channels. Government monitoring appears to be expanding in recent years, with surveillance programs now reporting findings from Canada and the United States [4–7]. While not systematically monitored, the presence of neonicotinoid insecticides in marine environments has also been reported [8]. These detections have led to suggestions that aquatic ecosystems may be impacted by neonicotinoid insecticides [9,10].

To quantify this potential impact, there is a need to first understand the potential for direct impacts on aquatic organisms. As highlighted by Anderson et al. [9] and Pisa et al. [11], there are relatively few published reports of the effects of neonicotinoid insecticides on freshwater and marine species. Furthermore, most publications have focused on the neonicotinoid imidacloprid [3], with fewer than 10 published, peer-reviewed experimental studies of freshwater organisms identified to date for thiamethoxam [12,13], and none for marine species.

Although not published as traditional peer-reviewed literature, a significant amount of aquatic toxicity data for neonicotinoid insecticides has been generated as part of regulatory packages for registration of these pesticides. Regulatory risk assessment follows a tiered approach, which

This article includes online-only Supplemental Data.

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Published online 11 May 2017 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.3846

applies high levels of conservatism at its base, and increasing realism at higher tiers. At the lowest tier, assessment factors are applied to toxicity estimates (e.g., median lethal concentrations [LC50s]) in the risk characterization to give conservative threshold concentrations, which, if not exceeded, are considered to present an acceptable risk. If preliminary estimates of exposure indicate that the threshold concentrations may be exceeded, subsequent testing and analysis may be employed to refine the understanding of risk, including the creation of species sensitivity distributions (SSDs). Initial studies early in the process typically focus on single-species acute and chronic laboratory toxicity assays with indicator species. Subsequent higher-tier studies may include testing additional species and/or testing under more realistic conditions, such as community-level studies in aquatic mesocosms.

Because these tests are required for regulatory purposes to support the registration of an active ingredient, they are typically generated using standardized procedures (e.g., following guidelines of the Organisation for Economic Co-operation [OECD], the USEPA, and ASTM International), by laboratories accredited under the OECD's Good Laboratory Practice (GLP) program [14], resulting in a substantial body of high-quality data. It should be noted that much of the ecotoxicology data published in the peer-reviewed literature is generated from studies that do not follow the rigors and quality control associated with GLP procedures. With the current level of interest in understanding neonicotinoid insecticide toxicity to aquatic organisms, the aim of the present study was to compile previously unpublished data from a suite of laboratory toxicity assays conducted with the neonicotinoid insecticide thiamethoxam for freshwater and marine primary producers, invertebrates, and fish. We did not address in detail the aquatic risk assessment for thiamethoxam, as this is clearly dependent on locale and duration-defined exposure profiles and specific protection goals that are highly context-dependent. We did create SSDs to better understand the general risk to aquatic species under currently reported concentrations of thiamethoxam in freshwater ecosystems. Overall, the extensive collection of high-quality data we report will assist those seeking to conduct formal ecological risk assessments for thiamethoxam and those aiming to gain a greater understanding of the potential impact of detected concentrations of thiamethoxam in surface waters.

METHODS

In total, 30 separate laboratory toxicity studies (24 freshwater, 6 marine) were conducted, with up to 12 single-species tests per study (Table 1). The total freshwater dataset includes 5 tests of aquatic primary producers, 34 tests with aquatic invertebrates (29 acute and 5 chronic), and 6 tests of fish (4 acute and 2 chronic). Experiments with marine species included 1 algal test, 1 mollusc test, 2 tests with a marine crustacean (1 acute and 1 life-cycle), and 2 tests with fish (1 acute and 1 chronic early life stage). All tests were conducted by GLP-accredited laboratories and, with the exception of 2 studies conducted with nonstandard invertebrate species (studies 11 and 12), all tests followed published standard guidelines, with few deviations (as noted in Supplemental Data, Tables S1–S7). We have summarized all relevant test information (e.g., replication, exposure concentrations, and assay conditions) in tabular form, as noted in the following sections, for each test. Species were selected based on availability of widely accepted protocols, organism accessibility, and a desire to better understand the range of possible responses to a variety of nontarget organisms. The names of some species are different in the report titles from those referred to in this paper. This is due to changes in taxonomic identification since the original studies were performed.

All tests were conducted with technical-grade thiamethoxam provided by Syngenta Crop Protection, with purity greater than 98% (Supplemental Data, Tables S1–S7). No organic solvents were used in the preparation of test solutions, except for the chronic test with *Chironomus riparius* (study 17), where acetone was used as a vehicle for sediment treatment. A solvent control was employed in this test, and no significant differences from the negative control were found for any endpoint (Supplemental Data, Table S4). All studies included analytical confirmation of thiamethoxam concentrations at multiple time-points by high-performance liquid chromatography, except for study 18, where analysis was by liquid chromatography–tandem mass spectrometry. Measurements of general water quality included pH, dissolved oxygen, temperature, light level, and in certain studies (as indicated), ammonia, salinity, and total organic carbon in sediment. Details of each study methodology are provided (Supplemental Data, Tables S1–S7). Concerns about thiamethoxam degrading via photolysis to clothianidin (a neonicotinoid), and confounding toxicity have been raised. When the formation of this metabolite has been observed, it is not a major metabolite (typically $<1\%$); and for some studies under field conditions, is not observed at all [15]. However, to address this issue, many of the tests conducted were flow-through or static renewal.

Tests with freshwater primary producers

Assessment of effects of thiamethoxam on primary producers included 2 tests with the green alga *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum* and then *Pseudokirchneriella subcapitata*), 1 test with the filamentous cyanobacteria *Anabaena flos-aquae*, 1 test with the diatom *Navicula pelliculosa*, and 1 test with the macrophyte *Lemna gibba*. All tests assessed sublethal effects (i.e., exposure durations of 72 or 96 h for algae, 7 d for *L. gibba*). Endpoints were growth rate and biomass based on cell density at the start and end of the study (algae), or based on frond count and frond dry weight (*L. gibba*). For each study, no-observed-effect concentration (NOEC) and lowest-observed-effect concentration (LOEC) values were calculated by Dunnett's multiple comparison test, and effective concentration (EC*x*) values were calculated by logit analysis or linear interpolation of the response (the inhibition concentration [ICp] method of Norberg-King [16]). Details of each test, including duration, source of organisms, test concentrations, replication, test conditions, statistics, and validity of the test are described in Supplemental Data, Table S1.

Tests with freshwater invertebrates

Twenty-nine acute toxicity tests were conducted with freshwater aquatic invertebrates for 21 different organisms (insects, molluscs, and aquatic worms). All acute tests were 24- or 48-h, water-only, static tests, with endpoints of immobilization and/or mortality. The NOEC values were typically determined empirically. The EC50 values (concentration causing an increase in immobilization by 50% compared with controls) and LC50 values (concentration causing an increase in mortality by 50% compared with controls) were calculated according to the maximum likelihood method using the logit or probit model. It should be noted that the organism's specific life stage (i.e., instar) was not determined for some tests, which may increase uncertainty around the resulting estimates of toxicity. Details of each test, including duration, source of organisms, test

Table 1. Reference study ID, authors, year, species tested, and original report title as submitted to regulatory agencies and referenced in the present study

Study ID	Authors, year	Species	Title
Freshwater			
1	R. Grade, 1996	*Raphidocelis subcapitata*	Growth Inhibition Test of CGA 293343 tech. to Green Algae (*Selenastrum capricornutum*) in a Static System
2	R. Grade, 1998	*Raphidocelis subcapitata*	Growth Inhibition Test of CGA 293343 tech. to Green Algae (*Selenastrum capricornutum*) Under Static Conditions
3	M. Staggs, 2014	*Anabaena flos-aquae*	Thiamethoxam—96-Hour Toxicity Test with the Cyanobacterium, *Anabaena flos-aquae*
4	M. Staggs, 2014	*Navicula pelliculosa*	Thiamethoxam—96-Hour Toxicity Test with the Freshwater Diatom, *Navicula pelliculosa*
5	R. Grade, 1998	*Lemna gibba*	Acute Toxicity Test of CGA 293343 tech. to the Duckweed *Lemna gibba* G3 under Semi-Static Conditions
6	L. Sayers, 2008	*Procambarus clarkii*	Acute Toxicity to Red Swamp Crayfish (*Procambarus clarkii*), Under Static-Renewal Conditions
7	C. Neumann, 1996	*Daphnia magna*	Acute Toxicity Test of CGA 293343 to the Cladoceran *Daphnia magna* Straus Under Static Conditions
8	K. Knauer, 2000	*Gammarus* sp.	Acute Toxicity Test of CGA 293343 tech. to *Gammarus* sp. Under Static Conditions
9	K. Knauer, 2000	*Daphnia pulex* Leydig, *Thamnocephalus platyurus*, *Brachionus calyciflorus*	Acute Toxicity Test (24 h) of CGA 293343 tech. to Three Invertebrate Species *Daphnia pulex* Leydig, *Thamnocephalus platyurus*, and *Brachionus calyciflorus* Under Static Conditions
10	K. Knauer, 2000	Ostracoda, *Chaoborus* sp., *Lymnea stagnalis*, *Radix peregra*	Acute Toxicity Test of CGA 293343 tech. to Individual Invertebrate Species and Molluscs from a Natural Pond Assemblage Under Static Conditions
11	J. Ashwell and R. Dark, 2002	*Asellus aquaticus*, Copepoda, *Cloeon dipterum*, *Chaoborus crystallinus*, *Chironomus riparius*, Dytiscidae, *Crangonyx pseudogracilis*, *Coenagrionidae*, *Lymnea stagnalis*, Erpobdellidae, *Lumbriculus sp.*, Planariidae	Acute Toxicity to Aquatic Insects
12	J. Pickervance, R. Dark, and J. Ashwell, 2003	*Asellus aquaticus*, *Cloeon dipterum*, *Chironomus riparius*, Dytiscidae, *Crangonyx pseudogracilis*	CGA293343 (Thiamethoxam technical) and CGA322704 (Thiamethoxam metabolite) Acute Toxicity to a Range of Aquatic Invertebrates
13	K. Knauer, 2000	*Cloeon* sp.	Acute Toxicity Test of CGA 293343 tech. to the Ephemeroptera *Cloeon* sp. Under Static Conditions
14	M. Mank and H. Kruegar, 1998	*Chironomus riparius*	CGA 293343 Technical: A 48-hour Static Acute Toxicity Test with the Midge (*Chironomus riparius*)
15	C. Neumann, 1997	*Daphnia magna*	*Daphnia magna* Reproduction Test: Effects of CGA 293343 on the Reproduction of the Cladoceran *Daphnia magna* Straus in a Semi-Static Laboratory Test
16	R. Grade, 2002	*Chaoborus* sp.	Toxicity Test of CGA 293343 tech. on *Chaoborus* sp. (Invertebrate, Insect) Under Static Conditions in a Sediment-Water-Test System
17	R. Grade, 1998	*Chironomus riparius*	Toxicity Test of CGA 293343 tech. on Sediment-Dwelling *Chironomus riparius* (syn. *Chironomus thummi*) Under Static Conditions
18	M. Bradley, 2015	*Chironomus dilutus*	10-day Toxicity Test Exposing Midge (*Chironomus dilutus*) to Thiamethoxam Applied to Sediment Under Static-Renewal Conditions
19	H. Rufli, 1996	*Oncorhynchus mykiss*	Acute Toxicity Test of CGA 293343 tech. to Rainbow Trout (*Oncorhynchus mykiss*) in the Flow-Through System
20	H. Rufli, 1997	*Oncorhynchus mykiss*	Acute Toxicity Test of CGA 293343 tech. to Rainbow Trout (*Oncorhynchus mykiss*) Under Flow-Through Conditions
21	K. Drottar and J. Swigert, 1996	*Lepomis macrochirus*	A 96-hour Flow-Through Acute Toxicity Test with the Bluegill (*Lepomis macrochirus*)
22	S. Maynard, 2003	*Cyprinus carpio*	Thiamethoxam (CGA 293343 technical): Acute Toxicity to Mirror Carp (*Cyprinus carpio*)
23	H. Rufli, 1997	*Oncorhynchus mykiss*	Prolonged Toxicity Test of CGA 293343 tech. to Rainbow Trout (*Oncorhynchus mykiss*) in the Flow-Through System
24	K. Drottar, W. Graves, and J. Swigert, 1997	*Oncorhynchus mykiss*	An Early Life-Stage Toxicity Test with the Rainbow Trout (*Oncorhynchus mykiss*)
Marine			
25	M. Staggs, 2014	*Skeletonema costatum*	Thiamethoxam—96-hour Toxicity Test with the Marine Diatom, *Skeletonema costatum*
26	K. Drottar and J. Swigert, 1997	*Crassostrea virginica*	CGA-293343: A 96-Hour Shell Deposition Test with the Eastern Oyster (*Crassostrea virginica*)
27	L. Sayers, 2015	*Americamysis bahia*	Thiamethoxam—Life-Cycle Toxicity Test with Mysids (*Americamysis bahia*)
28	K. Drottar and J. Swigert, 1997	*Americamysis bahia*	CGA-293343: A 96-Hour Flow-Through Acute Toxicity Test with the Saltwater Mysid (*Mysidopsis bahia*)
29	K. Drottar and J. Swigert, 1997	*Cyprinodon variegatus*	CGA-293343: A 96-Hour Flow-Through Acute Toxicity Test with the Sheepshead Minnow (*Cyprinodon variegatus*)
30	L. Sayers, 2015	*Cyprinodon variegatus*	Thiamethoxam—Early Life-Stage Toxicity Test with Sheepshead Minnow, *Cyprinodon variegatus*

concentrations, replication, test conditions, statistics, and validity of the test, are described in Supplemental Data, Tables S2–S5.

Assessments of chronic toxicity were conducted for 4 aquatic invertebrates—the crustacean *Daphnia magna*, and the insect larvae *Chaoborus* sp., *Chironomus riparius*, and *Chironomus dilutus*. The larval tests included a sediment phase, and toxicity to *C. riparius* was assessed for both sediment- and water-phase applications of thiamethoxam. Endpoints for *D. magna* were mortality and immobilization of adults and number of young produced, endpoints for *Chaoborus* sp. and *C. riparius* were based on emergence (number, rate), and endpoints for *C. dilutus* were survival and growth of the larvae. Details of each test, including duration, source of organisms, test concentrations, replication, test conditions, statistics, and validity of the test, are described in Supplemental Data, Table S5.

Tests with freshwater fish

Four tests for assessment of acute toxicity (96 h) were conducted with freshwater fish, including 2 flow-through tests with the rainbow trout *Oncorhynchus mykiss*, 1 flow-through test with the bluegill sunfish *Lepomis macrochirus*, and 1 static test with the common carp, *Cyprinus carpio*. For all acute tests, the endpoint was mortality, and LC50s were estimated by visual interpretation. In addition, an assessment of chronic toxicity to juvenile *O. mykiss* was conducted (28-d exposure), along with an assessment of early life stage toxicity (88-d exposure from embryo stage). Chronic test endpoints included a variety of sublethal effects. Details of each test including duration, source of organisms, test concentrations, replication, test conditions, endpoints, statistics, and validity of the test are described in the Supplemental Data, Table S6.

Tests with marine species

Marine species tested include a diatom (*Skeletonema costatum*), the Eastern oyster (*Crassostrea virginica*), the opossum shrimp (*Americamysis bahia*), and the sheepshead minnow (*Cyprinodon variegatus*). Acute (96-h) tests were conducted for all species, along with a life-cycle test (28-d) with *A. bahia*, and an early life stage toxicity test (33 d) with *C. variegatus*. Acute test endpoints were based on measurements of cell density (*S. costatum*), shell growth (*C. virginica*), or mortality (*A. bahia, C. variegatus*). Chronic test endpoints included survival, reproduction, and growth-based metrics (organism length, weight). Details of each test are provided in the Supplemental Data, Table S7.

Preliminary assessment of the acute risk from thiamethoxam

To begin placing the data reported in the present study in a broader ecological context, we performed a preliminary risk assessment focusing on acute toxicity data for freshwater invertebrates, as these are the most sensitive organisms and have the greatest amount of data. We did not examine risk via chronic exposure using SSDs for 2 primary reasons. First, it can be reasonable to assume that a point measurement of exposure can be reflective of acute exposure. This is not necessarily the case with chronic exposure, which requires more continuous monitoring and is highly site specific. Second, we lack data on a sufficient number of organisms to construct a robust SSD for chronic responses at this time.

For the effects assessment, SSDs were created as described by Solomon et al. [17] and 5% hazard concentrations (HC5s) were calculated from the resulting distributions. These HC5s describe the concentration estimated to produce an adverse impact according to the assessed endpoint in 5% of species. Because a minimum of 6 reported responses is recommended for HC calculations, the datasets for 24- to 48-h EC50 (immobility) and 48-h LC50 estimates were chosen as the basis for the constructed SSDs. No distinctions were made as to the relative quality of individual responses; rather, the lowest reported value for each species was utilized to be conservative. Data were plotted as a cumulative frequency distribution using a

Table 2. Regression coefficients, intercepts, and concentrations estimated to cause effects in 5% of species (HC5) for thiamethoxam acute toxicity species sensitivity distributions as calculated using the Weibull equation[a]

	$y = ax + b$[b]						% of species impaired at median	% of species impaired at 75th percentile	% of species impaired at 90th percentile	% of species impaired at 95th percentile	% of species impaired at 99th percentile
Distribution	*a*	*b*	r^2	No.[c]	HC5 (μg/L)	Likelihood of exceeding HC5 (%)	0.0125 (μg/L)	0.0250 (μg/L)	0.0320 (μg/L)	0.0538 (μg/L)	0.4000 (μg/L)
All invertebrate acute 24–48-h EC50s	0.550	–2.035	0.95	22	5.1	0.15	0.103	0.178	0.214	0.314	1.211
Insect acute 48-h EC50s	0.727	–1.732	0.94	8	1.3	0.51	0.092	0.189	0.241	0.397	2.161
Invertebrate (excluding insect) acute 24–48-h EC50s	0.471	–2.314	0.92	14	26.3	0.01	0.066	0.108	0.127	0.180	0.619
All invertebrate acute 48-h LC50s	0.604	–2.466	0.97	13	22.9	0.01	0.015	0.030	0.038	0.061	0.340
Insect acute 48-h LC50s	0.758	–2.303	0.96	6	7.4	0.12	0.009	0.022	0.029	0.055	0.460
Invertebrate (excluding insect) acute 48-h LC50s	0.684	–3.473	0.96	7	470.2	<0.001	0.001	0.0002	0.0003	0.0007	0.0090

[a]The percentage of species impaired by exposure to thimethoxam based on an exposure distribution at specific percentiles are also provided. Where multiple responses for the same species and time point were available, the lower concentration was used.
[b]These values are transformed into units of log and probit for the purposes of regression and back-transforms were used to calculate the intercepts. The distribution units were in μg/L.
[c]Number of data points used in the ranking.

probability scale as a function of the log concentration. Plotting positions were expressed as percentages and calculated using the Weibull formula with a total of 6 SSDs created (Table 2). Three distributions were constructed each for LC50s and EC50s and consisted of all invertebrates, insects only, and invertebrates excluding insects. Separate distributions were created to be as conservative as possible in the identification of sensitive groups of organisms. In addition, we calculated HC5s using the USEPA CADDIS Species Sensitivity Distribution Generator Ver 1 software for the same acute insect EC50 and LC50 data in order to test results using a different model [18].

For the exposure assessment, a review of the published literature was conducted to compile a broad dataset of measured environmental concentrations. Peer-reviewed literature, published reports, and online databases were searched for thiamethoxam analyses of freshwater habitats (or potential habitats) within the United States and Canada as of November 2016. If not published, raw data were requested from the authors. Duplicated values were identified and removed using a search formula that matched latitude, longitude, sampling date, and sampling time. In cases in which multiple samples were obtained within a 24-h period at a site, the greatest value was retained in the dataset as a conservative method. Only studies or databases that reported limits of detection or quantitation were retained. Samples with nondetectable concentrations were assigned a value of one-half of that sample's respective limit of detection (LOD) or limit of quantitation (LOQ), whichever was greater. The LOD/LOQs ranged from 1 to 50 ng/L across all data sources. The peer-reviewed sources consulted are given in the *References* section [4,5,19–22]. A list of the online or unpublished data sources consulted is provided in the Supplemental Data, Table S8 and the raw data used in the distribution are provided as a separate Excel file. In total, 6906 data points were assembled, of which 1322 (19.1%) reported thiamethoxam concentrations at or above the LOD.

Once the HC5s were calculated, the likelihood of observing a concentration that would impair at least 5% of species was calculated. In addition, the percentages of species that could be impaired at the median, 75th, 90th, 95th, and 99th percentiles of the exposure distribution were determined for each constructed SSD.

RESULTS

Test concentrations

Results of analytical confirmations of test concentrations, along with LOQ/LOD values, are provided in Supplemental Data, Tables S1–S7. Except for final concentrations in chronic static tests, measured concentrations were typically 80 to 120% of nominal, which fall within guideline recommendations for use in statistical analyses. All measured concentrations in control units were below the test LOD/LOQ, except where noted (study 7: *D. magna*). Statistical analyses are based on nominal or mean measured concentrations, as described in Supplemental Data, Tables S1–S7.

Effects on freshwater primary producers

All measured responses (raw data) for primary producers are provided in Supplemental Data, Tables S9–S13. In the 96-h test with *R. subcapitata*, exponential growth occurred in controls, and conditions for validity of the test were met according to the primary guideline method [23]. Similarly, conditions for validity were met in the test with *L. gibba* (10-fold increase in biomass in 7 d) [24]. For tests with *A. flos-aquae* and *N. peliculosa*, 2 of the 5 OECD [25] conditions for validity of tests with freshwater algae and cyanobacteria were not met (coefficients of variation for mean yield and section-by-section growth rate exceeded requirements). However, results were considered typical of those species, because growth is more variable than for the unicellular algae used to define the criteria [26]. Conditions for validity of the tests are described in the Supplemental Data, Table S1.

Estimated NOEC, LOEC, and EC50 values for primary producers are presented in Table 3. First-tier algal and plant studies demonstrate that, as expected for an insecticide, thiamethoxam has low toxicity to algae and aquatic macrophyte species. Estimated 72-h and 96-h EC50 values were all greater

Table 3. No-observed-effect concentrations (NOECs), lowest-observed-effect concentrations (LOECs), and median effect/lethal concentration (EC50/LC50) estimates for freshwater primary producers exposed to thiamethoxam[a]

Organism	Test type	Study ID	Endpoint	NOEC (mg/L)	LOEC (mg/L)	ECx_x (mg/L)
Raphidocelis subcapitata	72 h (static)	1	Biomass ($_b$) and growth rate ($_r$)	$NOEC_r/NOEC_b$: 81.8[b]	>81.8	$EC50_{r/b}$: >81.8
	96 h (static)	2	Biomass ($_b$) and growth rate ($_r$)	$NOEC_r/NOEC_b$: 100[b]	>100	$EC50_{r/b}$: > 100
Anabaena flos-aquae	96 h (static)	3	Avg. specific growth rate ($_r$); biomass (area under growth curve) ($_b$); yield ($_y$)	72-h $NOEC_{r,b,y}$: 97[b] 96-h $NOEC_{r,b,y}$: 47	72-h $LOEC_{r,b,y}$: >97 96-h $LOEC_{r,b,y}$: 97	72-h $EC10_b$: 51 (ne – 61) 72-h $EC20_b$: 62 (ne – 74) 72-h $EC10_y$: 53 (ne – 64) 72-h $EC20_y$: 64 (ne – 87) 72-h $EC10_{yr}$: 88 (32 – ne) 72-h $EC20_{yr}$: >97 72- and 96-h EC50 $_{r/b/y}$: > 97
Navicula pelliculosa	96 h (static)	4	Avg. specific growth rate ($_r$); biomass (area under growth curve) ($_b$); yield ($_y$)	72-h $NOEC_{r,b,y}$ 98[b] 96-h $NOEC_{r,b,y}$: 98[b]	72-h $LOEC_{r,b,y}$: >98 96-h $LOEC_{r,b,y}$: >98	72-h $EC10_b$: 66 (16 – ne) 72-h $EC20,50_b$: >98 72-h $EC10,20,50_{r/y}$: >98 96-h $EC50_{r/b/y}$: >98
Lemna gibba	7 d (semistatic)	5	Inhibition of frond number (growth rate ($_r$) and biomass ($_b$) as area under the growth curve); frond dry weight	Frond number $NOEC_{r,b,y}$: 90.2[b] Weight NOEC: 90.2[b]	>90.2	Frond number $EC50_{r/b}$: >90.2

[a]Species, test durations, and endpoints as indicated.
[b]Greatest tested concentration.
ne = not estimable.

than the greatest concentrations tested (≥81.8 mg thiamethoxam/L). In all but 1 case, NOEC values were equivalent to the highest concentration tested, and the minimum reported NOEC was 47 mg thiamethoxam/L (96-h growth rate, biomass, and yield for *A. flos-aquae*).

Effects on freshwater invertebrates

All measured responses (raw data) are provided in the Supplemental Data, Tables S14–S33. In all acute tests with aquatic invertebrates, <10% response (immobility/mortality) was observed in control units, indicating that criteria for validity were met according to standard guidelines [27]. Similarly, all criteria for validity were met in chronic tests (Supplemental Data, Table S5).

Among tested crustaceans, a wide range of sensitivities were observed (Table 4). Thiamethoxam had low toxicity to daphnids (*D. magna* and *Daphnia pulex*) in both acute and chronic tests and to Copepoda (acute tests only were conducted), with estimated EC50 values all exceeding 100 mg thiamethoxam/L. For other species tested (*Asellus aquaticus, Gammarus* sp., Ostracoda, *Thamnocephalus platyurus, Procambarus clarkii*), 48-h EC50 estimates ranged from 0.084 mg thiamethoxam/L (*A. aquaticus*) to 3.0 mg thiamethoxam/L (*P. clarkii*).

With the exception of the phantom midge larvae *Chaoborus* sp. (48-h EC50s of 5.5 and 7.3 mg thiamethoxam/L), aquatic insects were the most sensitive species tested, with 48-h EC50 estimates for each species reported below 1 mg thiamethoxam/L (Table 5). Values ranged from 0.014 mg thiamethoxam/L (*Cloeon* sp.) to 0.98 mg thiamethoxam/L (Coengrionidae). Among insects tested under chronic conditions, *C. riparius* was the most sensitive, with a 30-d NOEC (emergence) of 0.01 mg/L.

Other aquatic invertebrates tested include 2 snails, a rotifer, and 3 worms. With the exception of the blackworm, *Lumbriculus* sp. (48-h EC50 = 7.7 mg/L), all EC50 estimates were >100 mg/L (Table 6).

Effects on freshwater fish

In all tests with fish (acute and chronic), conditions for validity of the test were met (Supplemental Data, Table S6). Acute tests demonstrated that all tested species (*O. mykiss*, *L. macrochirus*, *C. carpio*) were relatively insensitive to thiamethoxam (Table 7). All NOEC values in the 96-h tests were equivalent to the maximum tested concentration (≥100 mg thiamethoxam/L). Endpoints included mortality for all species, as well as sublethal symptoms for *O. mykiss* (swimming behavior, loss of equilibrium, respiratory function, exophthalmos, pigmentation). Chronic-duration tests were conducted with *O. mykiss*, including a 28-d prolonged toxicity test and an early life stage test (28-d hatch time, 60-d post hatch time). The NOEC values in these tests were also equivalent to the greatest concentration tested (100 mg thiamethoxam/L and 20 mg thiamethoxam/L, respectively). Endpoints in the 28-d test were mortality, growth rate (length and weight), food conversion efficiency, and sublethal effects (feeding activity, swimming behavior, respiratory movement, pigmentation,

Table 4. No-observed-effect concentrations (NOECs), lowest-observed-effect concentrations (LOECs), and median effect/lethal concentration (EC50/LC50) estimates for freshwater crustaceans exposed to thiamethoxam[a]

Organism	Common name	Study ID	Test type	NOEC/LOEC (mg/L)	EC50 (mg/L; 95% CL)	LC50 (mg/L; 95% CL)
Acute studies						
Asellus aquaticus	Water louse	11	48 h (static)	—	0.084 (0.044–0.16)	2.3 (0.82–7.32)
		12		—	>0.32 (ne)	—
Copepoda	N/A	11	48 h (static)	—	>100 (ne)	>100 (ne)
Daphnia magna	Water flea	7	24 h (static)	—	>100	
			48 h (static)	NOEC = 32	>100	—
Daphnia pulex	Water flea	9	24 h (static)	—	>100 (ne)	—
Gammarus sp.	N/A	8	24 h (static)	—	15 (10– 23)	—
			48 h (static)	—	2.8 (1.7–4.1)	—
Ostracoda	Seed shrimp	10	24 h (static)	—	0.24 (0.20–0.29)	—
			48 h (static)	—	0.18 (0.15–0.22)	—
Thamnocephalus platyurus	Fairy shrimp	9	24 h (static)	—	>100 (ne)	—
Procambarus clarkii	Crayfish	6	24 h (static renewal)	—	3.7 (2.7–5.1)	110 (13–ne)[c]
			48 h (static renewal)	—	3.0 (2.1–4.6)	17 (6.0–14 000)[c]
			72 h (static renewal)	—	2.8 (2.0–3.9)	12 (4.9–580)[c]
			96 h (static renewal)	NOEC = 0.65	2.3 (1.6–3.2)	10 (4.5–360)[c]
Chronic studies						
Daphnia magna (parent and juvenile)	Water flea	15	14 d (semi-static)	Reproduction (no. of young/female): NOEC = 100[b]; LOEC = >100	—	—
			21 d (semi-static)	Reproduction (no. of young/female); adult length; time to first brood: NOEC = 100[b]; LOEC = >100	Reproduction (no. of young/female); immobilization: >100[b]	—

[a]The endpoint for all NOEC and EC50 values is immobilization, except for study 6 (NOEC for immobility and mortality combined); study 12 (immobilization and mortality combined), and study 15 (as indicated). The LOEC endpoints are as indicated.
[b]Greatest tested concentration.
[c]LC50 calculated by probit analysis, but response considered insufficient (max. 50% mortality) to produce adequate effect estimate.
ne = not estimable.

Table 5. No-observed-effect concentrations (NOECs), lowest-observed-effect concentrations (LOECs), and median effect/lethal concentration (EC50/LC50) estimates for freshwater aquatic insects[a]

Organism	Common name	Study ID	Test type	NOEC/LOEC (mg/L)	EC50 (mg/L; 95% CL)	LC50 (mg/L; 95% CL)
Acute studies						
Chaoborus cristallinus	Glassworm (phantom midge larvae)	11	48 h (static)	—	7.3 (5.4–10)	11 (7.9–17)
Chaoborus sp.	Glassworm (phantom midge larvae)	10	24 h (static)	—	6.9 (5.7–8.3)	—
			48 h (static)	—	5.5 (4.4–6.6)	—
Chironomus riparius	Bloodworm (harlequin fly larvae)	14	24 h (static)	—	0.061 (0.050–0.075)	—
			48 h (static)	NOEC = 0.013	0.035 (0.030–0.041)	—
		11	48 h (static)	—	0.045 (ne)	0.26 (0.13–0.52)
		12	48 h (static)	—	0.071 (0.034–0.194)	—
Cloeon dipterum	Species of mayfly (larvae)	11	48 h (static)	—	0.021 (ne)	0.053 (0.038–0.073)
		12	48 h (static)	—	0.044 (0.042–0.045)	—
Cloeon sp.	Species of mayfly (larvae)	13	24 h (static)	—	0.019 (0.016–0.023)	—
			48 h (static	—	0.014 (0.011–0.017)	—
Coengrionidae	Species of damselfly (larvae)	11	48 h (static)	—	0.98 (ne)	1.6 (0.82–2.9)
Crangonyx pseudogracilis	None	11	48 h (static)	—	0.42 (0.20–0.87)	20 (7.28–96)
		12	48 h (static)	—	1.491 (1.029–2.403)	—
Dytiscidae	Predacious diving beetle	11	48 h (static)	—	0.069 (ne)	0.34 (0.17–0.62)
		12		—	0.047 (0.022–0.094)	—
Chronic studies						
Chaoborus sp.	Glassworm (phantom midge larvae)	16	34 d (static; with sediment; water application)	NOEC: total emergence: 0.64; development rate: 1.28; LOEC: total emergence: 1.28; development rate: >1.28	Total emergence: 0.48 (ne) Development rate: ne	—
Chironomus dilutus	Species of nonbiting midge	18	10 d (static renewal; (sediment application)	NOEC: survival: 1.3; growth (ash-free dry wt): 0.60 mg/kg sediment dry wt LOEC: survival: 2.6; growth: 1.3 mg/kg sediment dry wt	Growth: >2.6 (ne) mg/kg sediment dry wt	2.0 (1.9–2.1) mg/kg sediment dry wt
Chironomus riparius	Bloodworm (harlequin fly larvae)	17	30 d (static) (sediment application)	NOEC: emergence and development rate: 0.10 mg/kg sediment dry wt LOEC: emergence: 0.020 development rate: >0.010[b]	Emergence: 0.11 Development rate: >0.10 mg/kg sediment dry wt[b]	-
			30 d (static) (water application)	NOEC: emergence and development rate: 0.010 LOEC: emergence: 0.020 development rate: >0.010[b]	Emergence: 0.0114 Development rate: >0.010[b,c]	-

[a]Endpoint for all NOEC and EC50 values is immobilization, except for studies 12 and 14 (immobilization and mortality), and chronic studies (as indicated). LOEC endpoints as indicated.
[b]Greatest concentration where emergence occurred.
[c]Because of a 100% effect on emergence at 0.02 and above, the effect on development rate could not be calculated.
ne = not estimable.

Table 6. No-observed-effect concentrations (NOECs) and median effect/lethal concentration (EC50/LC50) estimates for other freshwater aquatic invertebrates[a]

Organism	Common name	Study ID	Test type	NOEC (mg/L)	EC50 (mg/L; 95% CL)	LC50 (mg/L; 95% CL)
Lymnaea stagnalis	Great pond snail	10	48 h (static)	100[b,c]	>100 (ne)	—
		11	48 h (static)	—	>100 (ne)	>100 (ne)
Radix peregra	None (pond snail)	10	48 h (static)	100[c]	>100 (ne)	—
Brachionus calyciflorus	None (planktonic rotifer)	9	24 h (static)	100[c]	>100 (ne)	—
Erpobdellidae	None (leech)	11	48 h (static)	—	100[c] (ne)	>100 (ne)
Lumbriculus sp.	Blackworm	11	48 h (static)	—	7.7 (ne)	>32 (ne)
Planariidae	Flatworm	11	48 h (static)	—	>100 (ne)	>100 (ne)

[a]The NOEC/EC50 endpoint was immobilization. LOEC values were not estimated.
[b]Greatest concentration with 10% or less immobilization because this amount is considered acceptable for control performance.
[c]Greatest tested concentration.
ne = not estimable.

Table 7. No-observed-effect concentrations (NOECs), lowest-observed-effect concentrations (LOECs), and median lethal concentration estimates (LC50) for freshwater fish

Organism	Common name	Study ID	Test type	Endpoint	NOEC (mg/L)	LOEC (mg/L)	LC50 (mg/L)
Acute studies							
Oncorhynchus mykiss	Rainbow trout	19	96 h (flow-through)	Mortality; sublethal symptoms (swimming behavior, loss of equilibrium, respiratory function, exophthalmos, pigmentation)	125[a]	—	24, 48, 72, 96 h: >125
		20		Mortality; sublethal symptoms (swimming behavior, loss of equilibrium, respiratory function, exophthalmos, pigmentation)	100[a]	—	24, 48, 72, 96 h: >100
Lepomis macrochirus	Bluegill sunfish	21	96 h (flow-through)	Mortality	114[a]	—	24, 48, 72, 96 h: >114
Cyprinus carpio	Common carp	22	96 h (static)	Mortality	120[a]	—	3, 24, 48, 72, 96 h: >120
Chronic studies							
Oncorhynchus mykiss	Rainbow trout	23	Prolonged toxicity 28 d (flow-through)	Mortality; growth rate (length and weight); food conversion efficiency; sublethal effects: feeding activity, swimming behavior, respiratory movement, pigmentation, exophthalamus, loss of equilibrium, reaction to external stimulus	100[a]	>100	—
		24	Early life stage 88 d (flow-through)	Hatching success and time to hatch, time to swim up, larvae and fry survival, growth (d 31 length, d 31 and d 60 length and weight)	20[a]	>20	—

[a]Greatest tested concentration.

Table 8. No-observed-effect concentrations (NOECs), lowest-observed-effect concentrations (LOECs), and median effect/lethal concentration estimates (EC50/LC50) for marine organisms

Organism	Common name	Study ID	Test type	Endpoints	NOEC (mg/L)	LOEC (mg/L)	EC_x (mg/L; 95% CL)	LC50 (mg/L; 95% CL)
Skeletonema costatum	None	25	96 h (static)	Avg. specific growth rate ($_r$); biomass (area under growth curve) ($_b$); yield ($_y$)	72- and 96-h $NOEC_{r,y}$: 99[b]72- and 96-h $NOEC_b$: 48	72- and 96-h $NOEC_{r,y}$: >99 72- and 96-h $NOEC_b$: 99[b]	72-h $EC10_b$: 8.7 (4.0–35) 72-h $EC20_b$: 28 (ne–87) 72-h $EC10_y$: 8.8 (ne–75) 72-h $EC20_y$: 30 (ne) 72-h EC10, 20_r: >99 72-h and 96-h $EC50_{r/b/y}$ > 99	N/A
Crassostrea virginica	Eastern oyster	26	96 h (flow-through)	Inhibition of shell growth	119[b]	—	96-h EC50: >119	—
Americamysis bahia	Opossum shrimp	27	28 d (flow-through)	Postpairing survival ($F_{0f,m}$); overall 28-d survival (F_{0all}); F1 96-h survival (F_1); no. of offspring; male and female length ($L_{m,f}$) and weight ($W_{m,f}$)	Survival: $F_{0f,m}$: 1.1 F_{0f}: 3.9[b] F_{0all}: 0.56 F_1: 2.0[b] No. of offspring: 2.0 $L_{m,f}$ and $W_{m,f}$: 3.9[b]	Survival: $F_{0f,m}$: 2.0 F_{0f}: >3.9 F_{0all}: 1.1 F_1: >2.0 No. of offspring: 3.9 $L_{m,f}$ and $W_{m,f:}$ >3.9	—	—
Americamysis bahia	Opossum shrimp	28	96 h (flow-through)	Mortality	—	—	—	24 h: >16 48 h: >14 (11–25)[a] 72 h: 9.3 (7.6–12) 96 h: 6.9 (5.8–8.4)
Cyprinodon variegatus	Sheepshead minnow	29	96 h (flow-through)	Mortality	—	—	—	24, 48, 72, 96 h: >111
Cyprinodon variegatus	Sheepshead minnow	30	33 d (flow through)	Hatching success (Hs); Live, normal hatch (Hn); Larval survival (S_L); Length (L); Wet and dry wt ($W_{w,d}$)	Hs,n, S_L, $W_{w,d}$: 9.9[b]L: 1.7	Hs,n, S_L, $W_{w,d}$: >9.9 L: 4.1	Hs,n, S_L, $W_{w,d}$,L: >9.9	Hs,n, S_L, $W_{w,d}$,L: >9.9

[a]Concentration–response relationship not demonstrated over a reasonable range (<37 to >63% dead).
[b]Greatest tested concentration.
ne = not estimable; N/A = not available.

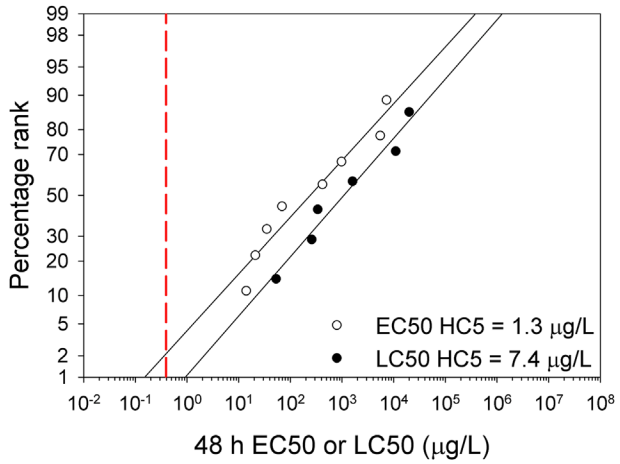

Figure 1. The species sensitivity distributions for insect 48-h median effective concentrations (EC50s) and median lethal concentrations (LC50s). The red line represents the 99th centile of exposure to thiamethoxam (derived from Figure 3). HC5 = 5% hazard concentration.

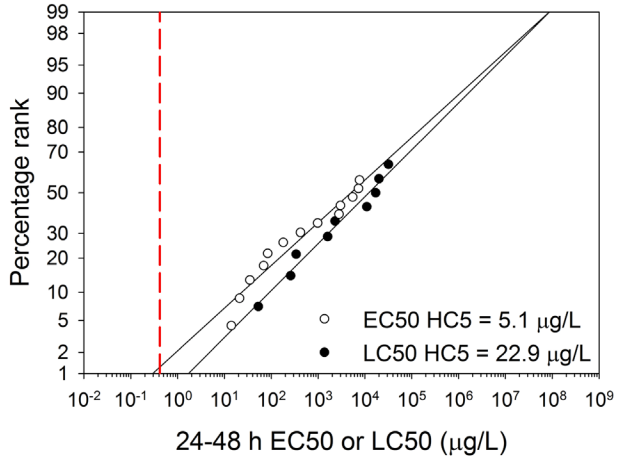

Figure 2. The species sensitivity distributions for invertebrates, including insect, 24- and 48-h median effective concentrations (EC50s) and 48-h median lethal concentrations (LC50s). The red line represents the 99th centile of exposure to thiamethoxam (derived from Figure 3). HC5 = 5% hazard concentration.

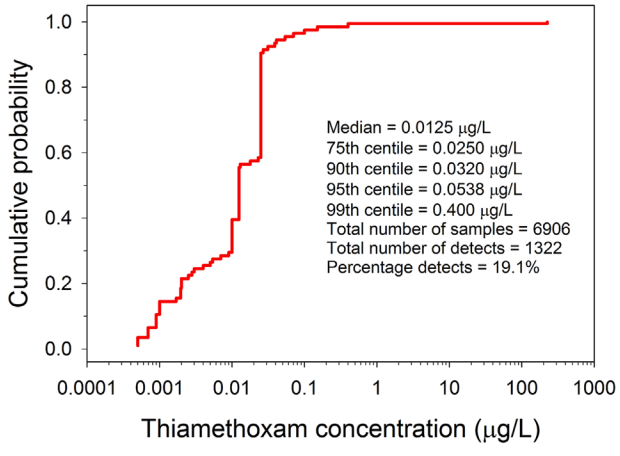

Figure 3. Cumulative probability of all surface water monitoring data for thiamethoxam. The cumulative probability distribution includes all data ($n = 6906$), including detects and nondetects. Nondetects were treated as one-half of the respective limit of detection or limit of quantitation. Inset table contains statistics that characterize the dataset.

exophthalmos, loss of equilibrium, reaction to external stimulus). Endpoints in the early life stage test were hatching success, time to swim up, larvae and fry survival, and growth (31-d length, 31-d and 60-d length and weight).

Effects on marine organisms

All measured responses (raw data) are provided in the Supplemental Data, Tables S38–S46. When provided in the test guideline, all criteria for validity of the marine tests were met (Supplemental Data, Table S7). As with freshwater species, the marine diatom, mollusc, and fish were insensitive to acute exposure to thiamethoxam (96-h EC/LC50 values exceeded 99 mg/L for all species tested; Table 8). Estimated EC50 values in the early life stage test with *C. variegatus* also exceeded the greatest concentration tested (9.9 mg/L) for all measured endpoints (hatching success, normal appearance at hatch, larval survival 28-d posthatch, and 28-d larval length and weight). The most sensitive marine organism was the opossum shrimp (*A. bahia*), with a 96-h LC50 of 6.9 (95% confidence interval [CI], 5.8–8.4) mg/L. In the life cycle test, males and females were paired after 13 d of exposure (following brood appearance), and the following endpoints were assessed: postpairing survival, overall 28-d survival, F1 96-h survival, number of offspring/female, and 28-d male/female length and weight. The LOEC values ranged from 1.1 mg/L (overall 28-d survival) to >3.9 mg/L (female postpairing survival, male/female length and weight).

Assessment of the acute risk from thiamethoxam

The resulting SSDs, HC5s, and likelihood of exceeding specific centiles of exposure can be found in Table 2 and the representative SSDs in Figures 1 and 2. The resulting exposure distribution from surface water samples collected from North America can be found in Figure 3. It is unlikely (<~0.5%) that current concentrations of thiamethoxam found in these aquatic environments will exceed the HC5 for any of the 6 distributions.

Using the USEPA CADDIS SSD software [18], the HC5 (with 95% CIs) derived for 48-h acute EC50 insect data was 3.27 (0.51, 21.14) µg/L thiamethoxam, with an r^2 of 0.924. The HC5 (with 95% CIs) derived for 48-h acute LC50 insect data was 21.06 (4.4, 99.97) µg/L thiamethoxam, with an r^2 of 0.956. These HC5 estimates are greater than those reported in Table 2, with likelihoods of exceedance for our North American exposure distribution of <0.2% and <0.02%, respectively.

DISCUSSION

As would be anticipated for a neonicotinoid insecticide, aquatic primary producers and fish were the least sensitive organisms tested, with acute median effect concentrations exceeding the greatest tested exposure concentrations (≥80 mg/L) in all cases. The tested molluscs, worms, and single rotifer species were similarly insensitive; among these, only *Lumbriculus* sp. exhibited a median effect concentration lower than 100 mg/L. In general, insects were the most sensitive species, with the majority of EC50 values < 1 mg/L. However, the freshwater crustaceans *A. aquaticus* and Ostracoda exhibited a similar sensitivity, while the midge larvae *Chaoborus* sp. were relatively insensitive compared with other insects (EC50 > 5.5 mg/L).

To date, relatively few assessments of the toxicity of thiamethoxam to aquatic species have been published in the peer-reviewed literature. Here we compare the thiamethoxam dataset generated by Syngenta for product registration (as described in the present study) with values reported in the

literature to date, and find that there is general agreement in the observed responses.

In our search, and to the best of our knowledge, no peer-reviewed studies examining thiamethoxam toxicity to any marine species, freshwater primary producers, rotifers, worms, or fish have been published. Two published studies have examined effects of thiamethoxam on crustaceans. As in Syngenta study 6, Barbee and Stout [28] assessed 96-h toxicity of thiamethoxam to crayfish (*P. clarkii*). Both tests followed ASTM standard E729-96, "Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians" (a static-renewal method) [29]. Barbee and Stout [28] reported 96-h LC50s of 0.967 (95% CI = 0.879–1.045) mg thiamethoxam/L. Although mortality response in study 6 was not sufficient to produce statistically robust LC50 values (maximum 50% mortality observed; Table 3), the EC50 value based on immobility was estimated as 2.3 mg (95% CI, 1.6–3.2) thiamethoxam/L. Acute toxicity to the amphipod *Gammarus kischineffensis* was tested by Ugurlu et al. [30], with reported LC50s for 24-h and 48-h exposures of 75.6 and 23.5 mg thiamethoxam/L, respectively. That study used the pesticide formulation, rather than the technical grade product, and 50% water changes were completed daily to renew exposure solutions. In study 8, mortality was not recorded separately from immobility. Estimates of 24-h and 48-h EC50s (immobility) were 15 (95% CI, 10–23) mg/L, and 2.8 (95% CI, 1.7–4.1) mg/L, respectively, for *Gammarus* sp. tested under static conditions, with technical-grade thiamethoxam.

Three published studies reporting 24-h or 48-h acute effects to insects were identified. Riaz et al. [31] examined toxicity to larvae of the mosquito *Aedes aegypti* and reported a 24-h LC50 of 0.183 (95% CI, 0.162–0.205) mg thiamethoxam/L. Similarly, Stevens et al. [32] determined a 24-h LC50 for *Chironomus tepperi* larvae of 0.121 (95% CI, 0.108–0.136) mg thiamethoxam/L. Finally, van den Brink et al. [33] assessed toxicity of thiamethoxam to *Cloeon dipterum* nymphs over 96 h and reported a 24-h EC50 (immobility) of 0.092 (95% CI 0.085–0.099) mg/L. Among our studies, 24-h toxicity of thiamethoxam to insect larvae was determined for the midge larvae *Chaoborus* sp. (study 10) and *C. riparius* (study 14), and the mayfly larva *Cloeon* sp. (study 13). The LC50 estimates were not separately determined, but 24-h EC50 values based on immobility were estimated at 6.9 (95% CI, 5.7–8.3), 0.061 (95% CI, 0.050–0.075), and 0.019 (95% CI, 0.016–0.023) mg thiamethoxam/L, respectively. Our assessments of 48-h toxicity to *Cloeon* sp. (LC50 of 0.053 mg/L [study 11] and EC50 [immobility] of 0.044 mg/L [study 12]) were also similar to those reported in van den Brink et al. [33], with an EC50 (immobility) of 0.049 (95% CI, 0.038–0.064) mg/L. These data demonstrate an overall agreement in acute toxicity estimates within insect species groups.

Two chronic duration tests with insects were found in the peer-reviewed literature. Van den Brink et al. [33] assessed the toxicity of thiamethoxam to *C. dipterum* nymphs after a 28-d exposure. The test was conducted under a static-renewal method with analytical confirmation of test concentrations. The reported EC50 for immobility after 28 d was 0.00068 mg/L (95% CI, 0.00038–0.0012 mg/L). Cavallaro et al. [13] conducted a full life cycle assessment using the midge *C. dilutus*, under a static-renewal method with analytical confirmation of test concentrations. That study reported an EC50 value for emergence after 40 d of 0.00413 (95% CI, 0.00353–0.00476) mg thiamethoxam/L. Among the reports in our study, a 30-d life cycle test was conducted with *C. riparius* larvae, with an EC50 for emergence of 0.0114 mg/L (95% CI not estimated), based on nominal concentrations. However, this test was conducted under static conditions (study 17), and by day 30, all concentrations were lower than the LOD (Supplemental Data, Table S31). A 34-d life cycle test was also conducted with the midge larvae *Chaoborus* sp. under static conditions, with an EC50 for emergence of 0.48 mg/L, based on nominal concentrations. Again, however, concentrations of thiamethoxam in the water phase declined substantially over the test, and were <38% of nominal concentrations by test termination.

No readily comparable toxicity estimates for molluscs were found to be available. However, Prosser et al. [12] assessed toxicity of thiamethoxam to the snail *Planorbella pilsbryi* after 7-d exposures, as well as to a freshwater mussel, *Lampsilis fasciola,* after 48-h exposure. For *P. pilsbryi*, the 7-d LC50 value was estimated at 6.195 (95% CI, 2.9078–9.4822) mg thiamethoxam/L, and 48-h EC50 estimates based on viability for *L. fasciola* exceeded the highest concentration tested (0.691 mg/L). Our studies included only 48-h tests with 2 different snail species (*Lymnaea stagnalis* and *Radix peregra*), and EC50 (immobility) values were estimated to exceed the highest test concentration (100 mg/L).

Our preliminary risk assessment reveals little likelihood of acute toxicity at current environmental concentrations for freshwater invertebrates, even using conservative models and including the most sensitive responses. Although our methods did not include an assessment of quality in the exposure concentration dataset, this approach should be included in HC5 calculations for more formal purposes [34]. Based on results of our studies and those reported previously in the literature, primary producers and fish (acute and chronic) are not sensitive to thiamethoxam, and current environmental concentrations pose no risk to these organisms. Invertebrates, and specifically insects, are significantly more sensitive, but current environmental concentrations are unlikely to exceed our calculated HC5s. Although the sizeable dataset assembled in the present study allows for the calculation of freshwater invertebrate HC5s based on acute endpoints (immobility and mortality), additional assessments of toxicity for sensitive insects (and potentially some crustacean species) should be generated to develop more complete SSDs, especially for chronic exposures. These resulting acute and chronic HC5 estimates should then be further confirmed through mesocosm studies.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3846.

Acknowledgment—The present study was funded by Syngenta. L.R. Baxter and M.L. Hanson were compensated for their assistance in assembling this manuscript. We thank 3 anonymous reviewers for their valuable feedback and comments.

Conflict of Interest—The toxicity tests were funded by the registrant of thiamethoxam (currently Syngenta). Baxter and Hanson were compensated for assisting with the writing of the manuscript and had full access to all data, as well as editorial control.

Data availability—The raw data behind the toxicity values are provided in the Supplemental Data. Full GLP reports are available upon request from the contact author.

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Journal of Applied Ecology 2013, **50,** 977–987 doi: 10.1111/1365-2664.12111

REVIEW

An overview of the environmental risks posed by neonicotinoid insecticides

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Summary

1. Neonicotinoids are now the most widely used insecticides in the world. They act systemically, travelling through plant tissues and protecting all parts of the crop, and are widely applied as seed dressings. As neurotoxins with high toxicity to most arthropods, they provide effective pest control and have numerous uses in arable farming and horticulture.

2. However, the prophylactic use of broad-spectrum pesticides goes against the long-established principles of integrated pest management (IPM), leading to environmental concerns.

3. It has recently emerged that neonicotinoids can persist and accumulate in soils. They are water soluble and prone to leaching into waterways. Being systemic, they are found in nectar and pollen of treated crops. Reported levels in soils, waterways, field margin plants and floral resources overlap substantially with concentrations that are sufficient to control pests in crops, and commonly exceed the LC_{50} (the concentration which kills 50% of individuals) for beneficial organisms. Concentrations in nectar and pollen in crops are sufficient to impact substantially on colony reproduction in bumblebees.

4. Although vertebrates are less susceptible than arthropods, consumption of small numbers of dressed seeds offers a route to direct mortality in birds and mammals.

5. *Synthesis and applications*. Major knowledge gaps remain, but current use of neonicotinoids is likely to be impacting on a broad range of non-target taxa including pollinators and soil and aquatic invertebrates and hence threatens a range of ecosystem services.

Key-words: bee, clothianidin, environmental fate, half-life, imidacloprid, non-target wildlife, soil water, systemic insecticide

An introduction to neonicotinoids

Neonicotinoids were developed in the 1980s, and the first commercially available compound, imidacloprid, has been in use since the early 1990s (Kollmeyer *et al*. 1999). They are nicotinic acetylcholine receptor agonists; they bind strongly to nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects, causing nervous stimulation at low concentrations, but receptor blockage, paralysis and death at higher concentrations. Neonicotinoids bind more strongly to insect nAChRs than to those of vertebrates, so they are selectively more toxic to insects (Tomizawa & Casida 2005). They can be classified into one of three chemical groups, the *N*-nitroguanidines (imidacloprid, thiamethoxam, clothianidin and dinotefuran), nitromethylenes (nitenpyram) and *N*-cyanoamidines (acetamiprid and thiacloprid; Jeschke *et al*. 2011). They are generally toxic to insects in minute quantities; for example, the LD_{50} (dose that kills 50% of individuals) for ingestion of imidacloprid and clothianidin in honeybees is 5 and 4 ng per insect, respectively, which for comparison is approximately 1/10 000th of the LD_{50} for dichlorodiphenyltrichloroethane (DDT; Suchail, Guez & Belzunces 2000). Neonicotinoids are water soluble and are readily absorbed by plants via either their roots or leaves and then are transported throughout the tissues of the plant. This provides many advantages in pest control, for they protect all parts of the plant; for example, they are effective against boring insects and root-feeding insects, both of which cannot easily be controlled using foliar sprays of non-systemic compounds. Concentrations in plant tissues and sap between 5 and 10 ppb (parts per billion) are generally regarded as sufficient to provide protection against pest insects (Castle *et al*. 2005; Byrne & Toscano 2006). For example, in citrus trees treated with imidacloprid via irrigation water, 5 ppb in xylem fluids was sufficient to

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control the sap-sucking insect *Homalodisca coagulata* (Castle *et al.* 2005).

In developed countries, neonicotinoids are predominantly used as seed dressings for a broad variety of crops such as oilseed rape, sunflower, cereals, beets and potatoes (primarily imidacloprid, clothianidin and thiamethoxam). For example, in the UK, use as a seed dressing accounted for 91% of all neonicotinoid use in farming in 2011 (Defra 2012a; note that this does not include garden or amenity use). Globally, 60% of neonicotinoids are used in this way (Jeschke *et al.* 2011). One attraction of seed dressings is that they require no action from the farmer, prophylactically protecting all parts of the crop for several months following sowing, and they are also regarded as providing better targeting of the crop than spray applications (Jeschke *et al.* 2011). However, the widespread adoption of neonicotinoids is partly down to their flexibility of use, for they can be applied in many other ways (Jeschke *et al.* 2011); they are commonly used as foliar sprays on horticultural crops such as soft fruits and on some arable crops such as soya, and they are sold for garden use as a spray on flowers and vegetables. They are used in bait formulations for domestic use against cockroaches and ants and also as granular formulations for the treatment of pasture and amenity grasslands against soil-dwelling insect pests. They can be applied as a soil drench or in irrigation water to defend perennial crops such as vines, and they can be injected into timber to combat termites or into trees to protect them against herbivores, where a single application can provide protection for several years (e.g. Oliver *et al.* 2010). Finally, they are commonly used in topical applications on pets such as dogs and cats to control external parasites.

Their advantages of low toxicity to vertebrates, high toxicity to insects, flexible use and systemic activity led to neonicotinoids swiftly becoming among the most widely used pesticides globally; they are now used more than any other class of insecticides and comprise approximately one quarter of all insecticides used. They are licensed for use in more than 120 countries and have a global market value of ~$2·6 billion, with imidacloprid alone comprising 41% of this market and being the second most widely used agrochemical in the world (Jeschke *et al.* 2011; Pollack 2011). Detailed data on use by country are generally not available, but figures for the UK illustrate the rapid adoption of neonicotinoids in the last 20 years, with UK use rising from three tonnes in 1994 to nearly 80 tonnes in 2011 (Fig. 1a).

The widespread adoption of neonicotinoids as seed dressings has led to a move away from integrated pest management (IPM), a philosophy of pest management predicated on minimizing use of chemical pesticides via monitoring of pest populations, making maximum use of biological and cultural controls, applying chemical pesticides only when needed and avoiding broad-spectrum, persistent compounds (Metcalf & Luckmann 1994).

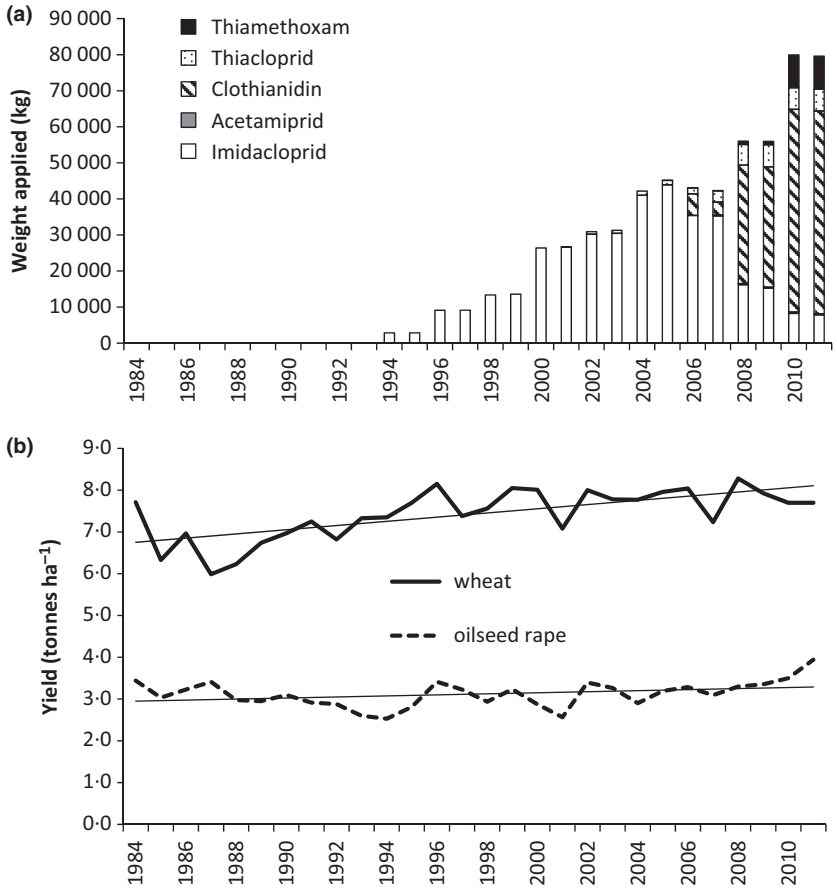

Fig. 1. (a) Annual usage (kg) of neonicotinoids in agriculture and horticulture in the UK, one of few countries from which detailed records are available (Defra 2012a). Note that these figures do not include garden or amenity use, or use for treatment of pets. In 2011, the area of land treated was approximately 1·3 million ha. (b) UK yields of two crops that are now widely treated with neonicotinoids as a seed dressing (Defra 2012b). There has been no significant rise in oilseed rape yield since its introduction, while winter wheat yields have risen slightly (linear regressions, $F_{1,26} = 4{\cdot}01$, ns and $F_{1,26} = 21{\cdot}1$, $P < 0{\cdot}001$, respectively).

Of necessity, seed dressing has to be applied prophylactically to crops before any information is available on likely pest problems in the coming year.

Economic benefits of neonicotinoids

There is abundant evidence that neonicotinoids can provide effective control of a broad range of insect pests (reviewed in Jeschke *et al.* 2011). It is less clear to what extent the widespread adoption of neonicotinoids has contributed to yield increases in farming or whether neonicotinoids offer economic benefits compared to alternatives. Yields per hectare of almost all arable crops have increased markedly over the last 60 years as a result of many changes, including improved crop varieties, widespread use of artificial fertilizers, new agronomic techniques and the development of successive generations of pesticides. However, the pace of yield increases has slowed, and yield increases in the last 20 years in developed countries have been modest, with some crops such as oilseed rape showing no increase coincident with the introduction of neonicotinoids; for example, in the UK, yields of oilseed rape were the same pre-1994 (when no neonicotinoids were available) as they are today, when close to 100% of crops are treated (Parry & Hawkesford 2010; Defra 2012a,b; Fig. 1b). Where yield increases have occurred in recent years, it is hard to disentangle the contribution of neonicotinoids from the effects of other changes in agronomic practices.

Given their widespread use, it is surprising that few studies have attempted to compare the effectiveness of neonicotinoids with alternative means of pest control. Bueno *et al.* (2011) compared managing soya pests in Brazil using either an IPM approach or prophylactic use of insecticides (the latter primarily based on imidacloprid). Crop yields were indistinguishable in the two treatments, but pesticide use and costs were much lower in the IPM treatment, demonstrating that this remains the best alternative in this system. In North America, Seagraves & Lundgren (2012) compared yield of either imidacloprid or thiamethoxam seed dressings on soya with untreated controls and found no difference in yield in either of the 2 years of their study, but populations of beneficial natural enemies were depressed in treated plots. In this system, the evidence would suggest that the cost of seed treatment (~\$30 ha^{-1}) is not being recouped by the farmer. This is in accordance with a several similar studies of soya which found either no yield benefits (McCornack & Ragsdale 2006; Cox, Shields & Cherney 2008; Ohnesorg, Johnson & O'Neal 2009) or yield benefits below those which could be achieved more economically using foliar insecticides applied only when pests exceeded a threshold (McCornack & Ragsdale 2006; Johnson *et al.* 2009). Similarly, studies of the efficacy of imidacloprid dressing of winter wheat in North America suggest that yield benefits are small (compared to unprotected, control crops) and often exceeded by the cost of the pesticide (Royer *et al.* 2005). In contrast, in Western Australia, McKirdy, Jones & Nutter (2002) demonstrated that application of an imidacloprid seed dressing to spring wheat is cost-effective compared to using no pest control, but that using foliar applications of alpha-cypermethrin (which is much cheaper) provided a significantly higher economic return.

There is clearly a need for further studies of other crops and geographical regions to establish in which instances use of neonicotinoids is cost-effective and whether alternatives such as pyrethroid sprays or IPM systems offer a more cost-effective approach. Such studies would need to incorporate the additional labour and application costs associated with crop monitoring and responsive spray applications.

Persistence of neonicotinoids in soils

Studies of the uptake of neonicotinoid seed dressings into the target crop suggest that between 1·6 and 20% of the active ingredient is absorbed by the crop (Sur & Stork 2003). Thus, although seed dressings are often stated to provide accurate targeting of the crop (e.g. Jeschke *et al.* 2011), they result in a considerably smaller proportion of the active ingredient ending up in or on the crop than do traditional spray applications to foliage, which commonly exceed 50% efficiency (Graham-Bryce 1977).

Of the 80–98% of the active ingredient in seed dressings, which is not absorbed by the crop, a small proportion (<2%) is lost as dust during sowing (Tapparo *et al.* 2012). This aerial dust can be sufficient to cause direct mortality in honeybees flying nearby (Marzaro *et al.* 2011; Tapparo *et al.* 2012) and is deposited on field margin vegetation at concentrations ranging from 1 to 9 ppb (Krupke *et al.* 2012). Release of active ingredient in dust is exacerbated when talcum powder or graphite is added to the seeds to lubricate their flow, as is common practice in North America (Krupke *et al.* 2012). Deflectors can be fitted to drilling equipment which direct this dust at the soil surface and reduce the amount of powder drifting in the air by 50–95%, although of course the active ingredient is then on the soil surface (Biocca *et al.* 2011).

By far the bulk of the active ingredient, typically more than 90%, enters the soil. Neonicotinoids are water soluble and have a half-life in soil, which varies greatly among compounds, soil type and across studies. No systematic attempt has been made to understand what factors affect their persistence or why published values are so variable. The primary sources of data are commonly not available for inspection since they are studies commissioned by industry to comply with regulatory requirements. For the most commonly used seed treatments, reported half-lives in soil typically range from 200 to in excess of 1000 days (range 28–1250 days for imidacloprid; 7–353 days for thiamethoxam [correction added on 28 June 2013 after first online publication: range changed from 7–3001 to 7–353 days; see footnote to Table 1]; 148–6931 days for clothianidin; Table 1). Half-lives appear to be shorter for

Table 1. Estimated dissipation times (DT_{50}) for neonicotinoids in soil

Compound	DT_{50} (days)	Laboratory or field study	Soil type	Location	Reference
Acetamiprid	450	Laboratory	Silty clay loam	NA	Reported in Anon (2004)
Acetamiprid	388	Laboratory	Clay loam	NA	Reported in Anon (2004)
Acetamiprid	Mean 31	Field	Various	Europe	Reported in Anon (2004)
Dinotefuran	82	Laboratory	NA	NA	PPDB (2013)
Dinotefuran	75	Field	NA	NA	PPDB (2013)
Imidacloprid	990–1230	Laboratory	Sandy loam	Australia	Baskaran, Kookana & Naidu (1999)
Imidacloprid	455–518	Laboratory	Sandy loam	Spain	Fernández-Bayo, Nogales & Romero (2009)
Imidacloprid	233–366	Laboratory	Silty clay loam	Spain	Fernández-Bayo, Nogales & Romero (2009)
Imidacloprid	34–45	Laboratory	Alluvial	India	Sarkar *et al.* (2001)
Imidacloprid	28–44	Laboratory	Lateritic	India	Sarkar *et al.* (2001)
Imidacloprid	36–46	Laboratory	Coastal alkaline	India	Sarkar *et al.* (2001)
Imidacloprid	1250	Field	Loam	UK	Calculated from data in Anon (2006)
Clothianidin	6931	Laboratory	Fuquay loamy sand	USA	Rexrode *et al.* (2003)
Clothianidin	1386	Field	Clay loam	North Dakota	Reported in De Cant & Barrett (2010)
Clothianidin	1155	Laboratory	Elder loam	USA	Rexrode *et al.* (2003)
Clothianidin	990	Laboratory	Howe sandy loam	USA	Rexrode *et al.* (2003)
Clothianidin	693	Laboratory	Susan silt loam	USA	Rexrode *et al.* (2003)
Clothianidin	578	Laboratory	Crosby silt loam	USA	Rexrode *et al.* (2003)
Clothianidin	533	Laboratory	Sparta sand	USA	Rexrode *et al.* (2003)
Clothianidin	533	Laboratory	Quincy loamy sand	USA	Rexrode *et al.* (2003)
Clothianidin	495	Laboratory	Loamy sand	Germany	Rexrode *et al.* (2003)
Clothianidin	365	Field	Silt loam	Ontario	Reported in De Cant & Barrett (2010)
Clothianidin	315	Field	Silt loam	Ohio	Reported in De Cant & Barrett (2010)
Clothianidin	277	Field	Sandy soil	Wisconsin	Reported in De Cant & Barrett (2010)
Clothianidin	239	Laboratory	Laacher Hof AII silt loam	Germany	Rexrode *et al.* (2003)
Clothianidin	148	Laboratory	Hofchen silt	Germany	Rexrode *et al.* (2003)
Clothianidin	Negligible dissipation in 25 months	Field	Silty clay loam	Saskatchewan	Reported in De Cant & Barrett (2010)
Nitenpyram	8	Laboratory	NA	NA	PPDB (2013)
Thiacloprid	>1000	Laboratory	NA	NA	Reported in Anon (2009b)
Thiacloprid	74	Laboratory	Sandy loam	Australia	Reported in Anon (2001b)
Thiacloprid	3·4–27	Field	NA	Australia	Reported in Anon (2001b)
Thiamethoxam	294–353	Laboratory	Sandy loam	USA	Reported in Anon (2001c)
Thiamethoxam	34–233	Laboratory	Silty loam	NA	Reported in Anon (2001c)
Thiamethoxam	7–109	Field	NA	NA	Reported in Anon (2001c)
Thiamethoxam	46–301*	Laboratory	NA	NA	Gupta, Gajbhiye & Gupta (2008)

*Correction added on 28 June 2013 after first online publication: Range listed from the study by Gupta *et al.* (2008) changed from 46-3001 to 46-301 days. This was only one of 12 studies reporting numerous dissipation times for neonicotinoids, and does not substantially alter the main conclusions of the article. Although it could suggest that thiamethoxam may have a shorter dissipation time than the other N-nitroguanidines, and thus be more similar to the N-cyanoamidines, it is important to note that the range of dissipation times are extremely variable across studies and we lack sufficient data to be able to make generalizations of this sort.

the *N*-cyanoamidines (thiacloprid and acetamiprid, ranges 3–74 and 31–450 days, respectively) [but see footnote to Table 1].

Given these estimates, we would expect repeated applications of neonicotinoids in successive years to result in accumulating concentrations in soils, but data here are sparse. The only studies available, from spray applications of imidacloprid to orchard soil in Germany and when used as a seed treatment on winter wheat in the UK, do show significant accumulation (Fig. 2, Anon 2006). For example, in the UK study, concentrations ranging from 6 to 18 ppb remained in the soil 1 year after sowing. After 6 years of repeated applications, soil concentrations 1 year after the final application ranged from 18 to 60 ppb, depending on the application rate. Concentrations may have continued to rise, but the experiment was terminated (Fig. 2).

Given their long life and potential for accumulation in soil, we would expect most arable soils to contain detectable, variable quantities of neonicotinoids, depending on cropping history, rainfall and soil properties. Bonmatin *et al.* (2005) randomly sampled 74 farmland soils in France and screened them for imidacloprid. Seven soils from organic farms contained no imidacloprid. Of the remaining 67 samples, 62 contained detectable imidacloprid (>0·1 ppb) and 65% of samples contained >1 ppb. Some of these positive samples had not been treated with imidacloprid in the previous 2 years, and only ten of the positive samples were from fields treated in the current year. Nine samples contained between 10 and 100 ppb,

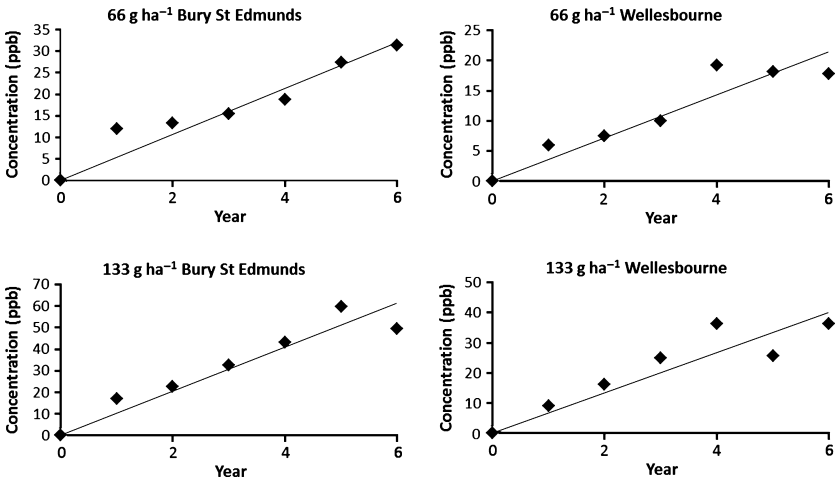

Fig. 2. Levels of imidacloprid detected in soil into which treated winter wheat seeds were sown each autumn (1991–1996). Both study sites are in the east of England. Treatment rates were 66 and 133 g a.i. ha^{-1} except in the first year, when it was 56 and 112 g, respectively. Data from Placke, FJ, reported in Anon (2006).

and three exceeded 100 ppb. They did not screen for other neonicotinoids, but given their widespread use and similar persistence, we would expect broadly similar levels of clothianidin and thiamethoxam. Since Bonmatin *et al.*'s study, neonicotinoid use has increased greatly – in the UK, it has approximately doubled – so current levels in arable soils are likely to be higher. It seems likely that most soil-dwelling organisms in conventional arable farmland are chronically exposed to fluctuating concentrations and mixtures of neonicotinoids in the range from 1 to >100 ppb.

Contamination of other environments

Loss of neonicotinoids from agricultural soils is presumably via degradation or leaching in soil water, but the relative importance of these routes cannot be clearly established from existing data. The pattern of loss is commonly biphasic, with an initial rapid phase followed by a much slower second phase, probably reflecting sorption of a proportion of the active ingredient onto soil particles which then slows dissipation (Gupta, Gajbhiye & Gupta 2008). This biphasic pattern will lead to an underestimation of persistence if dissipation studies are performed over short periods. Leaching is lower and sorption is higher in soils with high organic matter content (Cox, Koskinen & Yen 1998; Selim, Jeong & Elbana 2010). Before they become bound to soil, neonicotinoids readily leach so that significant levels might be predicted in groundwater and run-off immediately after application, particularly if there is heavy rainfall at this time, where the soil organic content is low, and on steep slopes (Scorza *et al.* 2004; Anhalt, Moorman & Koskinen 2008; Selim, Jeong & Elbana 2010; Thuyet *et al.* 2012). For example, Gupta, Gajbhiye & Gupta (2008) leached 79% of applied thiamethoxam from soil by simulating 65 cm of rainfall in the laboratory. Dissolved organic carbon appears to compete with neonicotinoids for soil sorption sites, increasing leaching (Flores-Céspedes *et al.* 2002). Accordingly, neonicotinoids have been detected in groundwater, streams, storm-water ponds and tidal creeks (Anon 2007; Lamers *et al.* 2011; DeLorenzo *et al.* 2012). For example, Starner & Goh (2012) detected imidacloprid in 89% of water samples taken from rivers, creeks and drains in California, with 19% of samples exceeding the US Environmental Protection Agency guideline concentration of 1·05 ppb. In the Netherlands, concentrations of up to 200 ppb in groundwater, streams and ditches have been reported (van Dijk 2010). However, neonicotinoids are absent from many groundwater and run-off samples collected in areas where they are deployed (e.g. Anon 2007). This may be because they are only present for a short period after application and so are likely to be missed by most sampling regimes and also because imidacloprid, clothianidin and thiamethoxam (but not thiacloprid and acetamiprid) rapidly degrade through photolysis in clear water (Anon 2007; Pena, Rodriguez-Liebana & Mingorance 2011). Many water-monitoring programmes do not screen for the metabolites of neonicotinoids such as imidacloprid olefin, but these can be as toxic as the parent compound (Anon 2007). Notably, no neonicotinoids feature in the EU Water Framework Directive's list of priority substances for aquatic pollution monitoring (Anon 2001a), and so they are not specifically targeted, and screening methods may not be well suited to their detection.

One aspect of the environmental fate of neonicotinoids for which few data are available is with regard to their uptake from soil and soil water by non-target plants. Given their persistence and accumulation in soils, we might predict hedgerow plants and trees, field margin vegetation and naturally regenerating fallows to take up neonicotinoids. Data on persistence of neonicotinoids once taken up by plants are sparse. However, vines treated in spring via irrigation maintain levels of imidacloprid sufficient to control pests through the growing season (Byrne & Toscano 2006), and levels of imidacloprid and thiamethoxam in citrus trees remain sufficient to suppress pests for 5 months following a single application (Castle *et al.* 2005). Similarly, a single application of imidacloprid to maple trees protected them against insect pests for 4 years (Oliver *et al.* 2010). Hence, there is the potential for non-target vegetation growing near arable crops to be

contaminated for much or all of the year via uptake from roots, supplemented annually by neonicotinoid dust deposition during sowing. This could deliver chronic exposure to herbivorous insects. However, other than the isolated study of Krupke *et al.* (2012) (which describes concentrations up to 9 ppb in dandelions in field margins), such vegetation does not appear to have been screened for neonicotinoids, so it is not possible to evaluate exposure of non-target organisms via this route.

Patterns of toxicity across taxa

Given the scale of use of neonicotinoids, their persistence in soils, leaching into waterways and their systemic nature within plants, there is no doubt that most organisms inhabiting arable environments will be exposed to them. The key question is whether typical levels of exposure are likely to lead to significant individual- or population-level impacts.

Many studies have examined the toxicity of neonicotinoids to both target and non-target organisms, including mammals, birds, fish, insects, crustacean, molluscs and annelids (Table S1 in Supporting Information). Insects are consistently among the most sensitive taxa, whether exposed via contact or ingestion. Typical LD_{50} values vary from 0·82 to 88 ng per insect, with much of the variation between species due to the size of the insect (Table S1, Supporting Information). For example, the most sensitive species, the brown planthopper, *Nilaparvata lugens*, weighs approximately 1 mg, while the least sensitive, the Colorado potato beetle, *Leptinotarsa decemlineata,* weighs approximately 130 mg, so that the LD_{50} values expressed as ng mg body per weight are similar (0·82 and 0·67, respectively). LC_{50} values (the concentration which kills 50% of individuals) for aquatic insects vary from 0·65 to 44 ppb (Table S1, Supporting Information). Here, the variation between studies is partly explained by differences in the duration of exposure. For example, the LC_{50} for the mayfly *Epeorus longimanus* falls from 2·1 ppb at 24 h to 0·65 ppb at 96 h (Alexander *et al.* 2007). Most studies assess only mortality and are carried out over short periods, but it is clear that important sublethal effects (such as reduced feeding, movement and reproduction) can be elicited by much lower doses. For example, feeding of *E. longimanus* nymphs was reduced for 4 days following exposure to water containing 0·1 ppb of imidacloprid for 24 h (Alexander *et al.* 2007).

The widespread prophylactic use of neonicotinoids has led to some insect pests developing resistance (e.g. Horowitz, Kontsedalov & Ishaaya 2004; Szendrei *et al.* 2012). For example, Szendrei *et al.* (2012) describe Colorado potato beetle populations with a 26-fold increase in resistance to thiamethoxam and a 100-fold increase in resistance to imidacloprid. The first strains with increased resistance to imidacloprid were detected in 1998, just 3 years after the chemical was first used against this pest. Given the increasing ubiquity of neonicotinoids and their persistence, insect populations in arable ecosystems are likely to be chronically exposed to them, a situation which will inevitably lead to increasing resistance in pest species (which tend to have large populations and short generation times).

Studies of toxicity to crustaceans are few, but they appear to be highly variable in their susceptibility to neonicotinoids, with LC_{50} values ranging from 7·1 ppb (over 28 days) in the amphipod *Hyalella azteca* to 361 000 ppb (over 48 h) in the brine shrimp *Artemia* sp. (Table S1, Supporting Information). Most crustaceans are considerably less susceptible than insects. Studies of annelids are also scarce, but suggest lower susceptibility than insects (Table S1, Supporting Information).

Toxicity to vertebrates is also low compared to insects, but varies greatly among neonicotinoids; for example, the LD_{50} value in rats varies from 140 mg $kgbw^{-1}$ (mg of active ingredient per kilogram body weight) for acetamiprid up to 5000 mg $kgbw^{-1}$ for clothianidin (Table S1, Supporting Information). Birds appear to be generally more susceptible than rats, with LD_{50} values ranging from 14 mg $kgbw^{-1}$ for imidacloprid in grey partridge up to 1333 mg $kgbw^{-1}$ for clothianidin in mallard ducks. Fish are markedly less susceptible than aquatic insects, with LC_{50} values ranging from 16 to 177 ppm (parts per million; Table S1, Supporting Information).

Risks to granivorous vertebrates

Although neonicotinoids do show relatively low toxicity to vertebrates, we might expect seed-eating vertebrates to be exposed to lethal doses if they consume treated seeds spilled during sowing. Typically, maize seeds are treated with ~1 mg of active ingredient per seed, beet seeds with 0·9 mg and the much smaller oilseed rape seeds with 0·17 mg (Rexrode *et al.* 2003; Anon 2012; Krupke *et al.* 2012). A grey partridge, typically weighing approximately 390 g, therefore needs to eat ~5 maize seeds, six beet seeds or 32 oilseed rape seeds to receive an LD_{50}. A grey partridge typically consumes ~25 g of seeds per day (Liukkonen-Anttila, Putaala & Hissa 1999), equivalent to ~600 maize seeds, so clearly there is the potential for birds to swiftly consume a lethal dose. By a similar calculation, three maize seeds treated with imidacloprid would deliver more than the LD_{50} to a mouse. The US Environmental Protection Agency estimated that ~1% of drilled seeds remain accessible to granivorous vertebrates (i.e. they are not buried during drilling), and this does not include spillages which may occur, for example, when transporting grain or loading hoppers. With typical sowing rates of ~50 000 seeds ha^{-1} for maize and 800 000 seeds ha^{-1} for oilseed rape, we might expect sufficient seed to be available on the soil surface to deliver an LD_{50} to 100 partridge or 167 mice for every hectare sown.

Lopez-Antia *et al.* (2013) fed imidacloprid-dressed wheat seed to red-legged partridge *Alectoris rufa* for 10 days and obtained 58% mortality, with the survivors

exhibiting a range of sublethal effects. This mortality rate, although considerable, is less than we might expect from the calculations above. Lopez-Antia *et al.* report anecdotally that partridge did not avoid dressed seed when offered both dressed and undressed, but speculate that treated birds ate less than control birds and so received a lower dose than expected. This requires further investigation, in this and other species, to determine how much treated seeds vertebrates actually consume in the field. De Snoo, Scheidegger & de Jong (1999) describe incidents of poisoning of wild partridge, pigeon and duck by seed dressed with imidacloprid, reported by members of the public in France in 1994–1995 (a time when neonicotinoid use was very low), but other evidence for effects in the field is lacking, and it is unclear whether public reporting is an efficient means of detecting such incidents.

There are other knowledge gaps. Susceptibility of most granivorous vertebrates that occur in farmland, which includes various rodents and a large number of bird species, has not been evaluated. Sublethal effects on invertebrates are poorly understood, although in birds they are known to include hyporeactivity, ataxia, wing drop, diarrhoea, opisthotonos (rigidity and severe arching of the back), immobility, intoxication, eggshell thinning, reduced egg hatching rate and low weight in chicks; and in mammals, they include reduced reproduction, premature deliveries and deformities in foetuses (Rexrode *et al.* 2003; Anon 2007; Lopez-Antia *et al.* 2013). Bal *et al.* (2012a) report reduced sperm production in rats exposed to imidacloprid at 2 mg $kgbw^{-1}$ day^{-1}, a dose representing ~1/250th of the LD_{50} per day, equivalent to a rat eating one treated maize seed (see also Bal *et al.* 2012b for a related study on clothianidin). Thus, one might expect doses considerably lower than the LD_{50} (which is derived from short-term laboratory tests) to have significant impacts on the long-term survival or reproductive success of vertebrates living in natural environments where they are exposed to other stressors. For example, many treated crops are sown in October; birds or mammals that consume seeds at this time will shortly have to survive the winter, and any factors that reduce their fitness at this time are likely to result in substantially reduced overwintering survival.

Impacts on pollinators

Much of the controversy over the use of neonicotinoids has focussed on their effects on bees. Neonicotinoids are routinely used to dress seeds of oilseed rape, sunflower and maize, and these crops are major forage sources for both managed honeybees and wild pollinators in arable landscapes. Being systemic, small concentrations of neonicotinoids are found in both pollen and nectar of seed-treated crops. Neonicotinoids are also routinely applied as foliar sprays to fruit crops such as raspberries (mainly thiacloprid), which are visited by both managed and wild pollinators (Lye *et al.* 2011; Defra 2012a). Widespread but unquantified use of neonicotinoids as foliar sprays in gardens, where they are recommended for use on both vegetables and flowers, provides a further route of exposure for pollinators.

Limited information is available on the actual concentrations of neonicotinoids typically found in pollen and nectar of treated crops (reviewed in EFSA 2012 and USEPA 2012; see also Stoner & Eitzer 2012). Concentrations in nectar are generally lower than those in pollen. When applied as seed dressings, concentrations in nectar range from <1 to 8·6 ppb (mean maximum level ±SE from 20 studies = 1·9 ± 0·5 ppb, EFSA 2012), with concentrations in pollen ranging from <1 to 51 ppb (mean maximum level ±SE from 20 studies = 6·1 ± 2·0 ppb). Generally higher concentrations are found when neonicotinoids are applied directly to the soil (e.g. in irrigation water), ranging from 1 to 23 ppb in nectar and 9 to 66 ppb in pollen (USEPA 2012). The highest concentrations recorded in nectar and pollen appear to result from foliar applications; Dively & Kamel (2012) report concentrations in pollen of 36 to 147 ppb for dinotefuran and 61 to 127 ppb for thiamethoxam when sprayed on pumpkin, plus significant concentrations of toxic metabolites. Concentrations in nectar were approximately 10-fold lower, ranging from 5 to 11 ppb for dinotefuran and 6 to 9 ppb for thiamethoxam.

Given the oral LC_{50} value for imidacloprid in honeybees of 5 ng bee^{-1} (Suchail, Guez & Belzunces 2000), and taking the mean values for seed-treated crops calculated here, a bee would need to consume nearly 1 g of pollen or 2·6 ml of nectar to obtain an LC_{50} dose. This seems unlikely in the short term for a honeybee, which weighs ~0·1 g, but could easily be accumulated over a number of days or weeks, so the actual effect of field exposure on mortality is likely to depend on the rate at which neonicotinoids are metabolized or excreted. A recent meta-analysis based on 13 studies of the impacts of imidacloprid on honeybees found that field-realistic doses (for seed-treated crops) under laboratory and semi-field conditions had no significant lethal effects (Cresswell 2011). Overall, the balance of evidence at present suggests that field-realistic exposure of bees to neonicotinoids in nectar and pollen of seed-treated crops is unlikely to cause substantial direct mortality (although exposure to dust released during drilling can cause direct mortality, Marzaro *et al.* 2011; Tapparo *et al.* 2012). However, only honeybees and bumblebees have been investigated; no information is available of susceptibility of other pollinating taxa such as hoverflies or butterflies. Also, if pollinators forage on crops treated with neonicotinoids via irrigation water or as a foliar application, direct mortality is likely; this has not yet been investigated, with attention largely focussed on exposure of bees to seed-treated crops.

Although there is little convincing evidence for direct mortality in bees, there is strong evidence for important sublethal effects. Exposure to sublethal doses of neonicotinoids is known to reduce learning, foraging ability and

homing ability in both honeybees and bumblebees (Yang *et al.* 2008; Han *et al.* 2010; Mommaerts *et al.* 2010; Henry *et al.* 2012). Such effects will not be revealed in standard safety-testing protocols that typically involve laboratory or cage trials with *ad lib* food, but would be much more marked under natural conditions when colonies rely on their workers to locate patches of flowers across the landscape. However, very few studies have been carried out in which bees that have been exposed to pesticides have to navigate across realistic distances.

In one such study, Henry *et al.* (2012) showed that honeybees, after being fed with sublethal doses of the neonicotinoid thiamethoxam, had a lower chance of finding their home colony than control bees. Importantly, the effect was much stronger when foragers had to return from an unfamiliar location at 1 km from their hive, compared to familiar locations or when closer to the hive. However, the dose given was higher than that bees might commonly be expected to receive in a single feed. Recently Gill, Ramos-Rodriguez & Raine (2012) found that bumblebee *Bombus terrestris* workers from colonies exposed to field-realistic concentrations of imidacloprid in nectar suffered from impaired foraging ability when gathering food in a natural setting, particularly when collecting pollen. As a result, treated colonies grew more slowly.

In the only well-replicated field study that has looked at the impacts of neonicotinoids on bee colony reproduction, Whitehorn *et al.* (2012) first simulated exposure of bumblebee colonies to a crop of treated flowering oilseed rape in the laboratory using realistic concentrations (6 ppb in pollen and 0·7 ppb in nectar). Colonies were then allowed to develop naturally in the field, gathering food for themselves. They recorded reduced nest growth and an 85% drop in queen production resulting from exposure to imidacloprid compared to control colonies. This study and Gill, Ramos-Rodriguez & Raine (2012) provide complementary evidence that reduced foraging efficiency following exposure to realistic levels of imidacloprid can result in a strong colony-level effect, which is likely to impact upon bumblebee populations in the long term. However, both studies placed treated food in the nests (and in the case of Whitehorn *et al.,* no other food was provided during the exposure phase), so we cannot be certain that the concentrations to which bees were exposed are representative of what happens under field conditions. For example, if bees detect and avoid neonicotinoid-treated crops, they may be exposed to less that we would otherwise expect. Easton & Goulson (2013) demonstrate that pollinating flies and beetles avoid pan traps containing imidacloprid at as low as 0·01 ppb, but whether bees avoid contaminated crops is unknown. If they do, this could have consequences for crop pollination.

Studies to date have focussed almost exclusively on exposure of adult bees. However, Yang *et al.* (2012) recently showed that learning of adult bees was impaired if they had been treated with 0·04 ng larva^{-1} of imidacloprid in the larval stage (<1/100th of the LC_{50} for adult bees). It seems highly likely that bee larvae are routinely exposed to such very low concentrations, but we have no data on whether this has long-term repercussions for colony fitness. This also raises the interesting question as to whether the exposure of other insects to low levels of neonicotinoids during development has effects on adult behaviour, an area which has not been investigated.

In summary, there is clear evidence that exposure of bees to field-realistic levels of neonicotinoids has significant sublethal impacts and that in the case of bumblebees, this has been demonstrated to have major impacts on colony success. To understand how widespread these effects are, further studies are needed to determine the range of concentrations of neonicotinoids to which wild bumblebee colonies and managed honeybee colonies are actually exposed in different environments (especially in urban areas for which we have no data). We also have a poor understanding of how the effects of neonicotinoids interact with other stressors, such as other pesticides, diseases and food stress, all of which undoubtedly influence bee health (Goulson, Lye & Darvill 2008; Moritz *et al.* 2010). At present, we have no data on impacts on pollinators other than bees. The major knowledge gaps concerning possible impacts of neonicotinoids on pollinators are usefully summarized in recent reviews of this issue conducted by the European Food Standards Agency (EFSA 2013a,b,c).

CONCLUSIONS

The adoption of prophylactic use of neonicotinoids as seed dressing has led to the abandonment of the long-established principles of IPM, an approach which uses monitoring of pest populations to indicate when treatment is necessary, avoids broad-spectrum pesticides wherever possible and avoids use of pesticides that persist in the environment (Metcalf & Luckmann 1994). This minimizes pesticide use, reduces the likelihood of the development of resistance in pests and minimizes impacts on non-target organisms.

At the Convention on Biological Diversity in 2002, world leaders committed to achieving a significant reduction in the rate of loss of biodiversity. By almost all indices, we have failed to reach this target (Butchart *et al.* 2010). In many developing countries, the reasons for this are clear: ongoing loss and degradation of species-rich habitat. Continuing declines of biodiversity in the European Union are more surprising, particularly given the real-term increase in spend on conservation, notably through a range of agri-environment schemes intended to boost biodiversity on farmland. For example, in England alone in 2009, 58 000 farmers were paid a total of £400 million per year to farm in a more environmentally sensitive manner (Anon 2009a). Despite this, UK indices for bees, butterflies, moths, carabid beetles and birds (the

Table 2. Knowledge gaps and suggestions for further research

Gap	Required Research
Acute toxicity to most taxa has not been investigated: for example, almost all pollinators apart from honeybees; many soil arthropods; non-target herbivores such as butterfly larvae; most farmland vertebrates	Further LD/LC_{50} studies conducted over long time-scales, for example 28 days
Sublethal impacts on learning, behaviour and fecundity unstudied for almost all taxa	Studies including behavioural and fecundity assays under realistic scenarios
Impacts of chronic exposure during development on neuronal development and adult behaviour are largely unknown	Assessment of adult fitness of insects following exposure as a larvae
Possible synergies between neonicotinoids and other stressors such as disease are largely unknown	Trials exposing insects to multiple stressors
Consumption of treated seeds by vertebrates has not been quantified	Trials to establish whether treated seeds are consumed, and if so what mortality this causes
Very few data are available on actual levels of neonicotinoids in arable soils and on whether accumulation with repeated application is common	More sampling of soils, long-term accumulation studies
No data from most countries on levels of neonicotinoids found in waterways	Sampling of waterways, particularly in the period following sowing of treated seed
No data are available on the extent to which field margin vegetation and hedgerow plants draw up neonicotinoids from arable soils	Screening of non-target vegetation, manipulative studies
No data are available on the extent of use of neonicotinoids in gardens	Collection of data via gardening outlets or random sampling of gardens
Few data are available on the agronomic or economic benefits of neonicotinoids	More field trials to compare the efficacy of alternative control strategies

LD_{50}, dose that kills 50% of individuals; LC_{50}, concentration that kills 50% of individuals.

groups for which good data are available) all show significant overall declines in recent years, particularly in farmland (Biesmeijer *et al*. 2006; Fox *et al*. 2006; Wilson, Evans & Grice 2010; Brereton *et al*. 2011; Brooks *et al*. 2012). Although data are sparse for many taxa, similar ongoing declines are evident across Europe (e.g. De Heer, Kapos & Ten Brink 2005; Gregory *et al*. 2005; Van Dyck *et al*. 2009). The reasons for these declines remain unclear and are the subject of ongoing debate.

The evidence presented here suggests that the annually increasing use of neonicotinoids may be playing a role in driving these declines. The concentrations accumulating in soil (1 to >100 ppb), waterways (often in excess of 1 ppb, sometimes up to 200 ppb), field margin plants (1–9 ppb) and nectar and pollen of flowering crops (1–50 ppb) exceed levels in crop tissues needed to control pest insects (5–10 ppb) and overlap with LC_{50} values for a range of non-target insects. They would appear to be sufficient to cause both direct mortality in the more sensitive non-target species and chronic sublethal effects in many more. The groups most at risk are likely to include soil-dwelling insects, benthic aquatic insects, granivorous vertebrates and pollinators. Herbivorous insects feeding on field margin and hedgerow plants may also be exposed.

Of course all pesticides are harmful to non-target organisms to some degree. Reconciling conserving biodiversity with food production requires a balance to be found. If it is not, then biodiversity loss will threaten vital ecosystem services upon which food production depends. Use of neonicotinoids appears to pose a particular threat to pollination services and also to soil health which depends on soil invertebrates that play major roles in nutrient cycling and maintaining soil structure. However, there are major knowledge gaps at present, so it is not possible to fully evaluate these threats (Table 2). Overall, there is an urgent need to re-evaluate whether current patterns of usage of neonicotinoids provide the optimum balance between meeting the demands of food production and farming profitability in the short term, vs. the need to sustainably manage global biodiversity to ensure the long-term health of ecosystems (including farmland) upon which all life depends.

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Received 19 February 2013; accepted 19 April 2013
Handling Editor: David Kleijn

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Additional data and references on toxicity of neonicotinoids to various taxa.

Integrated Environmental Assessment and Management — Volume 7, Number 3—pp. 426–436

Using Field Data to Assess the Effects of Pesticides on Crustacea in Freshwater Aquatic Ecosystems and Verifying the Level of Protection Provided by Water Quality Guidelines

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(Submitted 29 April 2010; Returned for Revision 28 June 2010; Accepted 6 October 2010)

ABSTRACT

The purpose of this study was to investigate how well single-species laboratory data predict real-world pesticide toxicity effects on Crustacea. Data from field pesticide exposures from experimental mesocosm and small pond studies were converted into toxicity units (TUs) by dividing measured pesticide concentrations by the L(E)C50 for *Daphnia* or acute 5% hazard concentration for Crustacea (HC5-C). The proportion of crustacean taxa significantly affected by the pesticide treatment, called the count ratio of effect, was used in logistic regression models. Of 200 possible logistic model combinations of the TUs, fate, physicochemical variables, and structural variables versus the count ratio of effect for the mesocosm data, the best model was found to incorporate log(TU HC5-C). This model was used to convert pesticide water quality guidelines from around the world into estimates of the proportion of crustacean taxa predicted to be impacted by exposure to a pesticide at the water quality guideline concentration. This analysis suggests 64% of long-term water quality guidelines and 88% of short-term pesticide water quality guidelines are not protective of the aquatic life they are designed to protect. We conclude that empirically derived data from mesocosm studies should be incorporated into water quality guideline derivation for pesticides where available. Also, interspecific differences in susceptibility should be accounted for more accurately to ensure water quality guidelines are adequately protective against the adverse effects of pesticide exposure. Integr Environ Assess Manag 2011;7:426–436.

Keywords: Pesticides Crustacea Water quality guidelines Mesocosms Risk assessment

INTRODUCTION

Around the world, water quality guidelines, or standards, are developed to protect aquatic life from the adverse effects of pesticide exposure (CCME 1999; ANZECC 2000; EU 2006; UKTAG WFD 2008; USEPA 2009). The approach to establishing what is a safe concentration varies among jurisdictions; however, water quality guidelines are, in general, derived from single-species toxicity laboratory experiments on a series of organisms designed to represent taxa from different trophic levels in an aquatic ecosystem. All water quality guidelines are intended to minimize the impact on nontarget organisms of pesticide addition to a water body. For most jurisdictions, water quality guidelines are intended to protect at least 95% of aquatic species; Australia and New Zealand produce trigger values to protect 99% of aquatic taxa (ANZECC 2000). While single-species studies provide important information about how a pesticide may affect aquatic organisms, laboratory studies cannot provide information about how abiotic and ecological variation in the environment affect how toxicants function in and on natural systems (Ravera 1989; Joern and Hoagland 1996; Pratt and Cairns 1996; Shaw and Kennedy 1996; Selck et al. 2002). The challenge for risk managers is to measure how many organisms are impacted by incidental pesticide addition to an aquatic ecosystem.

Model ecosystems, such as microcosms and mesocosms, are an important tool in determining actual ecosystem impacts of nontarget pesticide exposure from runoff, spray drift, and leaching, because they contain diverse communities of microorganisms, plants, zooplankton, and benthic invertebrates in natural lake or river water under local climatic conditions. Although physicochemical and fate characteristics of a pesticide are used during toxicity assessment to estimate exposure levels, these characteristics typically are not factored in during the derivation of water quality guidelines for pesticides, because water quality guidelines are intended to be as generic as possible, so they can be applied to the widest possible variety of water bodies. Not including physicochemical and fate characteristics in water quality guidelines may result in values that under-or overestimate the toxicity of the pesticide in the ecosystem. Dosing pesticides into model ecosystems can help to evaluate toxicological endpoints at population or community levels, such as predation and competition and can also allow observation of indirect impacts of chemical application, such as the loss of a food source (Ravera 1989; Pratt and Cairns 1996; Boxall et al. 2002; Selck et al. 2002). Dosing pesticides into model ecosystems also can allow the assessment of population and

All Supplemental Data may be found in the online version of this article.

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Published online 16 November 2010 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/ieam.143

community recovery and latency of effects. Further, model ecosystems can be used to integrate physicochemical and fate processes with exposure and consequently to measure the effects of transformation products. Model ecosystem studies can provide more realistic assessments, but results can be difficult to interpret and extrapolation to other systems is problematic and depends on the design of the mesocosm study (Maund et al. 1999; Boxall et al. 2002).

This study investigates how well single-species laboratory toxicity data can predict real-world toxicity effects on Crustacea in a sample of mesocosms and small pond studies. The toxicity of pesticides to Crustacea in freshwater systems is documented extensively, and if water quality guidelines are not protective enough of Crustacea, they will be even more ''hit and miss'' for other taxa. Field exposures are converted into toxicity units (TUs) by dividing measured pesticide concentrations by a standard metric of the toxic effect on a species, such as the L(E)C50 in *Daphnia* or 5% hazard concentration (HC5) values derived from species sensitivity distributions (Brock, Lahr et al. 2000; Brock, van Wijngaarden et al. 2000; Maltby et al. 2005; Van den Brink et al. 2006; Malby et al. 2009). The HC5 is an increasingly accepted measure incorporating interspecies susceptible to toxicants (Posthuma et al. 2002; CCME 2007; Whiteside et al. 2008). Exposure standardization through the calculation of TUs enables comparison among pesticide studies. Logistic models are developed using these derived TUs to predict the proportion of taxa significantly affected by pesticide exposure. Finally, these models are used to convert pesticide water quality guidelines from around the world into estimates of the proportion of crustacean taxa impacted by exposure to a pesticide at the water quality guideline concentrations. This analysis allows an estimation of how well existing water quality guidelines for pesticides protect Crustacea found in natural lentic systems.

METHODS AND MATERIALS

Data acquisition

Mesocosm studies. Data on the acute toxic effects of pesticides on crustacea were collected from mesocosm and pond studies published in the primary literature. Studies were chosen for inclusion based on the following criteria:

1) Studies were performed in a freshwater, lentic aquatic ecosystem larger than 80 L, containing a natural plankton assemblage and no fish.
2) The experimental design was clear, included a pretreatment or untreated control system, and the statistical significance of the results was reported.
3) Effect data were available for a single direct pesticide application, and the pesticide water concentration was measured.
4) Systems did not include any other pesticides.

For each study, the trade name and formulation of the pesticide, along with the solvent into which it was dissolved, application method, regime, rate, and date were recorded. Mesocosm or pond dimensions (length, diameter, width, or depth) and water volume were recorded and used to calculate surface area to volume ratios where possible. Peak pesticide concentration in the water column, the number and names of taxonomic groups monitored, and the number of taxa declining significantly within a week of pesticide application were also recorded. Results for replicate enclosures were recorded as averages. A list of the studies used in our analysis is provided in the Supplementary Material (available online).

Laboratory data. For those pesticides with suitable model ecosystem studies, the log K_{OW}, K_{OC}, aerobic aquatic biotransformation half-life (AAB) and water photolysis half-life (WPHL) were collected from several sources, including Tomlin (2003), Whiteside et al. (2008), the Canadian Pest Management Regulatory Agency (PMRA, unpublished data), European Commission Pesticide Review reports, the Extension Toxicology Network (EXTOXNET), Pesticide Action Network (PAN), USEPA pesticide fate database, and InChem (Table 1).

When more than 1 value for a property for the same pesticide was found, the geometric mean of the values was calculated. Where possible, half lives were matched to the average pH of the system; when not possible, the hydrolysis half-life for a neutral pH was used. Missing AAB values were filled in using an equation based on a correlation analysis between aerobic soil biotransformation half-life and AAB values using 117 pesticides in a database containing the Canadian Pest Management Regulatory Agency's unpublished regulatory data ($R^2 = 0.41$, $p < 0.001$).

Fate properties for persistent pesticides were given a value greater than the highest half-life for the pesticides in the database. Carbendazim and hexazinone were assigned an arbitrary value of 1095 d for WPHL. Carbendazim, hexazinone, linuron, metribuzin, and permethrin were assigned a hydrolysis half-life of 1825 d (Table 1).

Single-species acute toxicity values, L(E)C50 for *Daphnia* spp., and acute 5% hazard concentration for crustacea (HC5-C), were obtained from Whiteside et al. (2008). Where more than 1 L(E)C50 for *Daphnia* spp. were reported for a pesticide of interest, the geometric mean of all available values was calculated.

Water quality guidelines. Pesticide water quality guidelines or standards from around the world were obtained from the Internet (Table 2). Canada has Canadian Water Quality Guidelines (CWQG) for the protection of aquatic life for 5 pesticides: Carbaryl, chlorpyrifos, deltamethrin, lindane, and permethrin (CCME 2009). Recently, long- and short-term pesticide ideal performance standards (IPS) for 20 priority pesticides in water were developed under the National Agri-Environmental Standards Initiative (Demers and Jiapiazian 2009). While the IPS were developed to be voluntary water quality objectives for use by the agricultural community, they were derived following the CWQG derivation protocol (CCME 2007) and many are in the process of being adopted as CWQG. The US Environmental Protection Agency (USEPA) Office of Pesticides Programs has chronic and acute aquatic life benchmarks to protect freshwater species from 127 pesticides. These benchmarks were developed using toxicity values obtained during the decision-making process for USEPA's pesticide registration (USEPA 2009). The Australia and New Zealand Environment and Conservation Council established chronic trigger values for 24 pesticides (ANZECC 2000). These trigger values were derived using laboratory-derived NOEC data for freshwater biota. In 2008, the UK Technical Advisory Group on the Water Framework

Table 1. Laboratory data for pesticides used in mesocosm studies[a]

CAS number[b]	Common name	log K_{OW}	K_{OC}	AAB (d)	WPHL (d)	HHL (d)	*Daphnia* L(E)C50[a]	HC5-C[a]
86-50-0	Azinphos-methyl	2.96	789.7	3.61	3.2	37	1.11	0.14
22781-23-3	Bendiocarb	1.72	385	7.5	14.3	3	29.2	0.47
10605-21-7	Carbendazim	1.38	297.5	61	1095	1825	711.64	12.58
2921-88-2	Chlorpyrifos	4.7	4875	51	30	24.6	0.64	0.05
52315-07-8	Cypermethrin	6.6	42 367	59.7	1.9	1	5.00	0.0018
52918-63-5	Deltamethrin	4.5	116 033.5	87.5	48	20	0.68	0.0015
35367-38-5	Diflubenzuron	3.89	7350	11.3	28	63.7	4.28	0.312
122-14-5	Fenitrothion	3.43	1127	12.7	3.7	186	12.50	0.137
55-38-9	Fenthion	4.84	2346	5.7	0.01	56.8	—	0.067
77182-82-2	Glufosinate-ammonium	4.81	69.4	3	6.8	365	693 465.5	4511.6
51235-04-2	Hexazinone	1.2	26.5	60	1095	1825	197 901.6	14 561
58-89-9	Lindane	3.5	3380	202.7	1095	172.8	1127.2	0.4582
330-55-2	Linuron	3	246.5	42.1	49	1825	25 000	170.8
298-00-0	Methyl parathion	3	522.9	4.1	10	21	0.89	0.205
21087-64-9	Metribuzin	1.6	17	100	0.18	1825	13 839.4	2240.92
74223-64-6	Metsulfuron- methyl	0.018	6.5	43.6	1095	1825	150 000	9294.98
52645-53-1	Permethrin	6.1	40 867.5	32.5	0.13	1825	2.04	0.014
298-02-2	Phorate	3.92	454.5	0.46	1.1	3	3.42	0.007
112410-23-8	Tebufenozide	4.25	478.5	100	326.3	1034	974.06	22.86
3383-96-8	Temephos	4.91	100 000	17.2	15	460	0.011	0.019

[a]L(E)C50 are toxicity values for *Daphnia* for pesticides used in mesocosm studies; HC5-C = acute 5% hazard concentration for crustacea.
[b]AAB = aerobic aquatic biotransformation half-life; CAS = Chemical Abstract Service; HHL = hydrolysis half-life; WPHL = water photolysis half-life.

Directive (UKTAG WFD) published updated water quality standards for 9 pesticides considered as ''Specific Pollutants on List II of the Dangerous Substances Directive of the Water Framework Directive'' (UKTAG WFD 2008). These standards are for pesticides for which enough data exist to calculate a reasonably solid predicted no effect concentration. The European Union (EU) directive COM(2006)397 final contains maximum acceptable concentrations (short-term) and annual average concentration (long-term) standards for 10 pesticides, among the 41 substance standards in the directive (EU 2006).

Data modeling and analysis

Once assembled, the mesocosm effect data were standardized to common units. Results of each measured taxon were coded binomially: 1 = significant effect and 0 = no significant effect. Despite variation in experimental design and power, responses to pesticide treatment were considered significant according to the original author's analysis, after the numerical response associated with each effect reported as ''significant'' was verified as the result of direct toxicity of the pesticide under investigation. Significant declines in taxa abundance ranged from 37% to 100%, indicating reported effects were numerically and biologically important.

To differentiate between primary effects of the pesticide addition and the secondary effects of ecosystem disruption, such as predator release or loss of a food source, only significant decreases occurring within 7 d of application were included in the analysis. Choosing this sampling window also allowed the inclusion of studies that dosed the mesocosms again after a week. It is possible that by choosing a short exposure period, we may have missed delayed, direct toxic effects on Crustacea. Significant increases were assessed on a case-by-case basis to evaluate whether they occurred as a direct result of pesticide additions through competitor or predator release, or simply reflected natural population growth or recovery of affected organisms. Significant increases generally occurred among the Copepoda because they recovered fastest once free from cladoceran competition.

We derived a simple measure of the proportion of crustacean taxa significantly affected by the pesticide treatment, which we called the count ratio of effect. The count ratio of effect was calculated for all Crustacea taxa enumerated in a study by dividing the number of affected taxa by

Table 2. A selection of freshwater quality guidelines, standards, and benchmarks to protect aquatic life from pesticides and their associated log(water photolysis half-life) and log(K_{OW}) values[a]

CAS number[b]	Pesticide	CWQG long-term (μg/L)	CWQG short-term (μg/L)	USEPA acute invert (μg/L)	USEPA chronic invert (μg/L)	ANZECC 99% chronic[c] (μg/L)	UK long-term[d] (μg/L)	UK short-term[d] (μg/L)	EU AA (μg/L)	EU MAC (μg/L)
94-75-7	2,4-D	13	4860	—	—	140	0.3	1.3	—	—
94-82-6	2,4-DB	—	—	7500	—	—	—	—	—	—
30560-19-1	Acephate	—	—	550	150	—	—	—	—	—
62476-59-9	Acifluorfen	—	—	14 050	—	—	—	—	—	—
15972-60-8	Alachlor	—	—	1600	110	—	—	—	0.3	0.7
1912-24-9	Atrazine	12.53	82.12	360	60	0.7	—	—	0.6	2
86-50-0	Azinphos-methyl	—	—	0.08	0.036	0.01	—	—	—	—
131860-33-8	Azoxystrobin	—	—	130	44	—	—	—	—	—
741-58-2	Bensulide	—	—	290	—	—	—	—	—	—
25057-89-0	Bentazon	—	—	> 50 000	—	—	—	—	—	—
133-06-2	Captan	—	—	4200	560	—	—	—	—	—
63-25-2	Carbaryl	0.20	3.30	0.85	0.5	—	—	—	—	—
1563-66-2	Carbofuran	0.11	8.58	1.115	0.75	0.06	—	—	—	—
1897-45-6	Chlorothalonil	—	—	1.8	0.6	—	—	—	—	—
2921-88-2	Chlorpyrifos	0.002	0.02	0.05	0.04	0.00004	—	—	0.03	0.1
1702-17-6	Clopyralid	—	—	56 500	—	—	—	—	—	—
52315-07-8	Cypermethrin	0.0002	0.041	0.21	0.069	—	0.0001	0.0004	—	—
52918-63-5	Deltamethrin	0.0004	—	—	—	—	0.01	0.02	—	—
333-41-5	Diazinon	0.0016	0.24	0.105	0.17	0.00003	—	—	—	—
1918-00-9	Dicamba	—	—	17 300	—	—	—	—	—	—
1194-65-6	Diclobenil	—	—	1850	560	—	—	—	—	—
87674-68-8	Dimethenamid	—	—	6000	1020	—	—	—	—	—
60-51-5	Dimethoate	—	—	21.5	0.5	0.1	0.48	1	—	—
2764-72-9	Diquat	0.15	13.97	—	—	0.01	—	—	—	—
330-54-1	Diuron	—	—	80	160	—	—	—	0.2	1.8
115-29-7	Endosulfan	0.003	0.06	2.9	0.07	0.03	—	—	0.005	0.01
759-94-4	Ethyl dipropyl-thiocarbamate	—	—	3245	810	—	—	—	—	—
66230-04-4	Esfenvalerate	—	—	0.025	0.017	0.001[b]	—	—	—	—
55283-68-6	Ethalfluralin	—	—	30	24	—	—	—	—	—
122-14-5	Fenitrothion	—	—	1.15	0.087	0.1	0.01	—	—	—
55-38-9	Fenthion	—	—	2.6	0.013	—	—	—	—	—
142459-58-3	Flufenacet	0.1	0.2	—	—	—	—	—	—	—
81406-37-3	Fluroxypyr	10.9	—	—	—	—	—	—	—	—
1071-83-6	Glyphosate	730	27 000	26 600	49 900	—	—	—	—	—

(*Continued*)

Table 2. (*Continued*)

CAS number[b]	Pesticide	CWQG long-term (μg/L)	CWQG short-term (μg/L)	USEPA acute invert (μg/L)	USEPA chronic invert (μg/L)	ANZECC 99% chronic[c] (μg/L)	UK long-term[d] (μg/L)	UK short-term[d] (μg/L)	EU AA (μg/L)	EU MAC (μg/L)
51235-04-2	Hexazinone	—	—	75 800	20 000	—	—	—	—	—
81334-34-1	Imazapyr	—	—	50 000	97 100	—	—	—	—	—
138261-41-3	Imidacloprid	—	—	35	1.05	—	—	—	—	—
36734-19-7	Iprodione	—	—	120	170	—	—	—	—	—
141112-29-0	Isoxaflutole	—	—	>750	—	—	—	—	—	—
58-89-9	Lindane	0.01	—	0.5	54	0.07	—	—	0.02	0.04
330-55-2	Linuron	—	—	60	0.09	—	0.5	0.9	—	—
121-75-5	Malathion	0.01	0.48	0.005	0.000026	0.002	0.01	—	—	—
8018-01-7	Mancozeb	—	—	290	—	—	—	—	—	—
12427-38-2	Maneb	—	—	60	—	—	—	—	—	—
2039-46-5	MCPA DMAS	—	—	41 000	11 000	—	—	—	—	—
94-81-5	MCPB	—	—	25 000	—	—	—	—	—	—
57837-19-1	Metalaxyl	—	—	6250	1200	—	—	—	—	—
10265-92-6	Methamidophos	0.23	4.13	13	4.5	—	—	—	—	—
16752-77-5	Methomyl	0.080	11.4	2.5	0.7	0.5	—	—	—	—
298-00-0	Methyl parathion	—	—	0.485	0.25	—	—	—	—	—
51218-45-2	Metolachlor	—	—	550	1	—	—	—	—	—
21087-64-9	Metribuzin	—	—	2100	1.29	—	—	—	—	—
300-76-5	Naled	—	—	-	0.045	—	—	—	—	—
15299-99-7	Napropamide	—	—	7150	1100	—	—	—	—	—
23135-22-0	Oxamyl	—	—	90	180	—	—	—	—	—
42874-03-3	Oxyfluorfen	—	—	40	13	—	—	—	—	—
40487-42-1	Pendimethalin	0.5	0.5	140	14.5	—	—	—	—	—
52645-53-1	Permethrin	0.004	—	0.0106	0.0014	—	0.01	—	—	—
298-02-2	Phorate	—	—	0.3	0.21	—	—	—	—	—
732-11-6	Phosmet	—	—	1	0.8	—	—	—	—	—
1918-02-1	Picloram	—	—	34 150	11 800	—	—	—	—	—
7287-19-6	Prometryn	—	—	9295	1000	—	—	—	—	—
60207-90-1	Propiconazole	—	—	2400	—	—	—	—	—	—
23950-58-5	Propyzamide	—	—	>2800	600	—	—	—	—	—
82-68-8	Quintozene	3	10	—	—	—	—	—	—	—
122-34-9	Simazine	—	—	500	2000	—	—	—	1	4
141776-32-1	Sulfosulfuron	—	—	> 48 000	102 000	—	—	—	—	—
79538-32-2	Tefluthrin	0.002	0.006	—	—	—	—	—	—	—
3383-96-8	Temephos	—	—	5	—	—	—	—	—	—

Table 2. (*Continued*)

CAS number[b]	Pesticide	CWQG long-term (μg/L)	CWQG short-term (μg/L)	USEPA acute invert (μg/L)	USEPA chronic invert (μg/L)	ANZECC 99% chronic[c] (μg/L)	UK long-term[d] (μg/L)	UK short-term[d] (μg/L)	EU AA (μg/L)	EU MAC (μg/L)
5902-51-2	Terbacil	—	—	32 500	640	—	—	—	—	—
13071-79-9	Terbufos	—	—	0.1	0.03	—	—	—	—	—
79277-27-3	Thifensulfuron-methyl	0.16	1.59	—	—	—	—	—	—	—
137-26-8	Thiram	0.056	2.47	105	170.6	0.01	—	—	—	—
87820-88-0	Tralkoxydim	—	—	> 87 000	2100	—	—	—	—	—
2303-17-5	Triallate	—	—	45.5	13	—	—	—	—	—
82097-50-5	Triasulfuron	—	—	> 50 000	105 000	—	—	—	—	—
52-68-6	Trichlorfon	0.009	1.09	—	—	—	—	—	—	—
55335-06-3	Triclopyr	—	—	850	80 700	—	—	—	—	—
1582-09-8	Trifluralin	—	—	280	2.4	—	—	—	0.03	—
137-30-4	Ziram	—	—	24	39	—	—	—	—	—

[a]Long- and short-term Canadian water quality guidelines (CWQG) contain a mix of approved water quality guidelines (CCME 1999) and ideal performance standards (Demers and Jiapizian 2009). United States Environmental Protection Agency (USEPA) values are benchmarks designed to protect aquatic invertebrates from the adverse effects of pesticides (USEPA 2009). The Australia and New Zealand Environment and Conservation Council (ANZECC) values are trigger values for pesticides designed to protect 99% of aquatic species (ANZECC 2000). The European Union (EU) annual averages (AA; long-term) and maximum acceptable concentration (MAC; short-term) standards are for pesticides listed in the European Union's directive COM(2006) 397 (EU 2006).
[b]CAS = Chemical Abstract Service.
[c]The ANZECC trigger value for esfenvalerate is the 95% protection value because no 99% protection trigger value was available (ANZECC 2000).
[d]The United Kingdom long- and short-term standards are proposed values (UKTAG WFD 2008).

the total number of crustacean taxa studied in a study. For example, in the Jahr et al. (2000) mesocosm study of the effects of fenitrothion, diflubenzuron, deltamethrin, and bendiocarb, the authors reported results for 8 crustacean taxa (Cladocera: *Ceriodaphnia quadrangula*, *Moina micrura*, *Diaphanosoma senegal*; Copepoda: *Mesocyclops kieferi*, *Thermocyclops deipiens*, *Paradiaptomus rex*; Ostracoda: *Heterocypris symmetrica*; and Anostraca: *Streptocephalus* spp.). In the 0.62 μg/L deltamethrin treatment, the abundance of *C. quadrangula* and *Streptocephalus* spp. significantly declined. The count ratio of effect for the treatment was thus calculated as $2/8 = 0.25$. Laboratory data were standardized to the number of TUs by dividing the peak pesticide water column concentration in the mesocosms by the geometric mean of the L(E)C50 for *Daphnia* spp. or the acute HC5-C reported in Whiteside et al. (2008). Thus, following the example of Jahr et al. (2000), the HC5-C for deltamethrin is 0.001467, according to Whiteside et al. (2008). The TU for this treatment was $0.62/0.001467 = 436.3$.

STATISTICA 6.0 was used for all modeling and statistical analysis. Prior to modeling, the normality of the fate, physicochemical and structural properties of the system, TUs and quantified changes was tested by visual examination of normal probability plots. All variables were log10-transformed as required to meet requirements of homogeneity of variance and normality.

To predict the probability of nontarget taxa being impacted by pesticides in an aquatic ecosystem, and because the count ratio of effect is bounded by 0 (no effect) and 1 (all taxa affected), simple logistic regression was used to investigate the relationship between the count ratio of effect and each TU.

Multiple logistic regression was used to investigate whether the fate and physicochemical and structural properties of the study systems (Table 1) could improve the relationships between each TU and the count ratio of effect. Prior to modeling, correlations were tested among the independent variables. Highly correlated variables ($r \geq 0.6$) were not included in modeling runs together. Thus, results containing both TU HC5-C and TU L(E)C50, for and/or K_{OW} and K_{OC}, were not considered. Models with 2 or more chemical fate parameters also were not considered for inclusion.

The best model was chosen using Akaike's information criterion corrected for small sample sizes (AICc) (Burnham and Anderson 2002). The AICc is derived from maximum likelihood estimation and penalizes modeling results based on the number of parameters in a model, thereby favoring parsimony. Akaike's weights ratio (w_i) was also calculated because it indicates the probability that the model is the best among the complete set of candidate models. For example, a w_i of 0.75 indicates that the model has a 75% chance of being the best model among the candidate models. Once a list of candidate models was selected, the parameters were assessed using backward- and forward-stepping multiple logistic regression to ensure that the models were statistically robust. Relative model performance was measured by assessing the proportion of data classified correctly.

The US, Australia and New Zealand Environment and Conservation Council, EU, United Kingdom, and Canadian water quality guidelines for pesticides were converted to TUs by dividing the guideline concentration by the appropriate L(E)C50 for *Daphnia* or the HC5-C values reported in

Whiteside et al. (2008). TU values were converted to count ratios of effect using the best chosen logistic regression equation.

RESULTS

Sixty-nine independent water bodies contributed to the models. The data points were extracted from 25 experiments in 20 publications, examining 21 pesticides: 1 fungicide, 5 herbicides, and 15 insecticides. The Cladocera, Calanoida, and Cyclopoida were the most studied crustaceans, with pesticide effect data for these zooplankton orders found in 89% of studies. The effects of pesticides on other Crustacea, such as Amphipoda and Ostracoda, were found in 30% of studies.

Studies on herbicides and fungicides contributed data for the lower TU values in the plots of log(TU L(E)C50) and log(TU HC5-C) against the count ratio of effect; they are typically less toxic to *Daphnia* in particular and Crustacea in general (Figure 1). Using the full dataset, the logistic regression of log(TU L(E)C50) against the count ratio of effect correctly predicted an adverse effect 62.2% of the time and predicted no effect in 74.0% of cases, for an overall correct prediction rate of 68.6% (Figure 1a). Modeling using log(TU HC5-C) alone produced a logistic model predicting adverse impacts correctly 76.4% of the time and predicted no effect 73.7% of the time, for an overall correct prediction rate of 75.0% (Figure 1b).

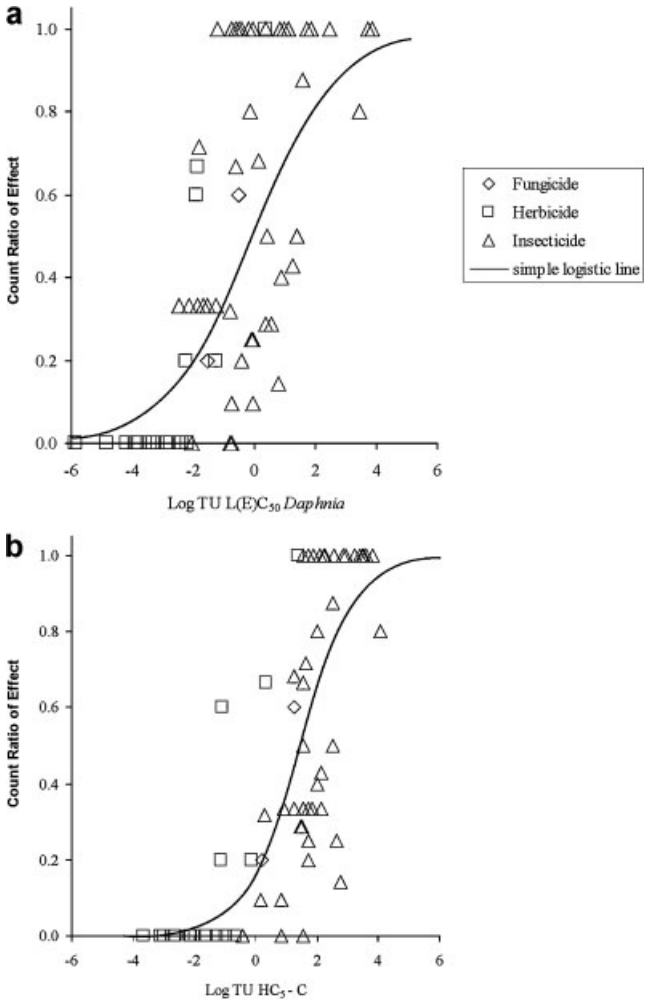

Figure 1. The log(TU L(E)C50) values for *Daphnia* (**a**) and log(TUHC5-C) (**b**) versus the count ratio of effect, or the proportion of species significantly affected by pesticide treatment, for 18 pesticides from 20 studies grouped by functional group.

Of 200 possible model combinations of the TUs and the fate, physicochemical, and structural variables, 48 were considered to be good approximating models once equations were culled following the rules outlined in the *Methods and Materials* section. The log(TU HC_5-C) was the highest ranked model, with a single variable (AICc = 248.86) and it was the only toxicity measure included in the top 15 equations (Table 3). The best approximating model includes log(TU HC_5-C) and log WPHL (AICc = 244.99). The next 2 models have very similar AICc scores and include log K_{OC} with these 2 variables (AICc = 245.63) or log K_{OW}(AICc = 245.75). Burnham and Anderson (2002) suggest that models within a $\Delta AICc \leq 2$ are essentially equivalent. The other 2 models within $\Delta AICc = 2$ incorporate the surface area to volume ratio. Forward and backward stepping multiple regression confirmed the best model incorporated log(TU HC_5-C) and log(WPHL). This model had an overall correct probability of classification of 73.2%. Curiously, log(WPHL) was negatively correlated to the count ratio of effect, suggesting that chemicals undergoing quick photolysis are more toxic than persistent pesticides. Because this result was counterintuitive, we examined the data and discovered that our modeling database included several organothiophosphate pesticides. These pesticides are designed to degrade quickly into organophosphates, so we speculated that they may be driving the regression equations. However, removing the organothiophosphates from the analysis did not change the relationship, although the probability of correct classification increased to 78.3%. Further investigation also suggested a relationship between log K_{OW} and WPHL that we could not explain. Concerned that the results perhaps were affected by the specific pesticides in the modeling data set, we chose the simplest model, with log(TU HC_5-C) alone, as the best model with which to explore the adequacy of water quality guidelines for pesticides.

$$Count\ Ratio\ of\ Effect = \frac{1}{(1 + e^{\,1.6696 - 1.12678 \times \log TUHC5})} \quad (1)$$

Based on the training set, this model was balanced, correctly predicting an adverse effect 76.4% of the time and no effect 73.7% of the time.

Long-term (acute) and short-term (chronic) water quality guidelines for 80 pesticides were converted to TUs using acute HC5-C values reported in Whiteside et al. (2008). Applying Equation 1 to the TU values allowed us to estimate a count ratio of effect corresponding to the water quality guideline concentration. The analysis was conducted on 69 pesticides with 115 short-term (acute) water quality guidelines and on 77 pesticides with 103 long-term (chronic) water quality guidelines. Typically, chronic water quality guidelines are derived using chronic data from toxicological studies conducted on a limited number of species. In general, these guidelines should have lower threshold values because they

Table 3. A selection of the best models ranked by increasing AICc[a] for predicting the count ratio of effect for crustacean species from TU HC5-C[b], pesticide fate properties, and structural properties of the mesocosms

Variable 1	Variable 2	Variable 3	Variable 4	K[d]	AICc	w_i ratio	L ratio χ^2	p
L TU HC5-C	L WPHL	—	—	4	244.99	0.145	199.37	0.000
L TU HC5-C	log K_{OC}	L WPHL	—	5	245.63	0.199	201.05	0.000
L TU HC5-C	log K_{OW}	L WPHL	—	5	245.75	0.212	200.92	0.000
L TU HC5-C	L TSA/V	L WPHL	—	5	246.52	0.310	200.16	0.000
L TU HC5-C	L TSA/V	log K_{OW}	L WPHL	6	247.08	0.412	201.98	0.000
L TU HC5-C	L TSA/V	log K_{OC}	L WPHL	6	247.10	0.415	201.97	0.000
L TU HC5-C	LAAB	—	—	4	247.48	0.503	196.88	0.000
L TU HC5-C	L TSA/V	LAAB	—	5	248.84	0.991	197.84	0.000
L TU HC5-C[c]	—	—	—	**3**	**248.86**	**1.000**	**193.26**	**0.000**
L TU HC5-C	log K_{OW}	LAAB	—	5	249.60	1.453	197.07	0.000
L TU HC5-C	log K_{OC}	LAAB	—	5	249.72	1.539	196.96	0.000
L TU HC5-C	L HHL	—	—	4	250.17	1.926	194.19	0.000
L TU HC5-C	L TSA/V	log K_{OW}	LAAB	6	251.00	2.917	198.07	0.000
L TU HC5-C	L TSA/V	log K_{OC}	LAAB	6	251.18	3.195	197.89	0.000
L TU HC5-C	L TSA/V	—	—	4	250.73	2.555	193.63	0.000

[a]AICc = Akaike's information criterion corrected for small sample sizes.
[b]HC5-C = acute 5% hazard concentration for crustacea; HHL = hydrolysis half-life; L = log; TU = toxicity units; WPHL = water photolysis half-life; K = the number of estimated parameters included in the logistic model; w_i = Akaike's weight ratio; TSA/V = total surface area/volume of the experimental mesocosm; AAB = aerobic Aquatic biotransformation.
[c]The proposed model appears in boldface in the body of the table. It is not the most parsimonious model but it has better balanced error.

integrate subtle behavioral and developmental changes. Where the TUs based on acute data are lower than chronic water quality guidelines, the results suggest chronic guidelines generated from a limited number of species are not sufficient; we expect a higher proportion of guidelines to fail to protect 95% of species from chronic effects.

Of the long-term (chronic) values, 41 of 115 are at or below the 0.05 acute effect level, the level the majority of water-quality guidelines are designed to protect (Figure 2a).

The protective water quality guidelines include the Canadian water quality guidelines for thifensulfuron-methyl, flufenacet, diquat, methomyl, atrazine, diazinon, pendimethalin, lindane, endosulfan, malathion, cypermethrin, chlorpyrifos, trichlorfon, and 2,4-D; USEPA benchmarks for malathion, linuron, metribuzin, metolachlor, atrazine, methomyl, and oxyfluorfen; Australia and New Zealand Environment and Conservation Council trigger values for diquat, diazinon, atrazine, chlorpyrifos, malathion, thiram, methomyl, and azinphos-methyl; the EU annual average water quality standards for atrazine, alachlor, simazine, diuron, trifluralin, endosulfan, and hexachlorocyclohexane (lindane); and the United Kingdom standards for 2,4-D, linuron, malathion, diazinon, cypermethrin, and fenitrothion. Of the 8 Australia and New Zealand Environment and Conservation Council trigger values designed to protect 99% of species, only the points for diquat, diazinon, atrazine, chlorpyrifos, and malathion are at or below the 0.01 effect level.

For the acute (short-term) values, only 12 of 103 are close to or below the 0.05 effect level, the level they are designed to protect (Figure 2b). The short-term Canadian water quality guidelines for thifensulfuron-methyl, flufenacet, pendimethalin and atrazine; the EU maximum acceptable concentrations for alachlor, atrazine, simazine, diuron, and endosulfan; the United Kingdom standard for 2,4-D and linuron, and the US benchmark for malathion were the only standards to correctly approximate the effects of these pesticides on the crustacean community.

The pesticide water quality guideline chronic concentrations predicted to affect greater than 50% of taxa based on our field impact model include the Australia and New Zealand Environment and Conservation Council and United Kingdom standards for dimethoate and the US pesticide benchmarks for oxamyl, phorate, triasulfuron, cypermethrin, dimethoate, lindane, methamidophos, thiram, and picloram. For acute guidelines, water concentrations at the Canadian water quality guidelines for carbofuran and methamidophos; at the US pesticide benchmarks for fenthion, 2,4-DB, phorate, imidacloprid, captan, mancozeb, cypermethrin, prometryn, thiram, temephos, methamidophos, picloram, and dimethoate; and at the United Kingdom water quality standard for dimethoate are all predicted to affect more than 50% of taxa in natural lentic ecosystems.

DISCUSSION

The goal of ecological risk assessment is to determine the probability of an adverse impact of a chemical on an ecological system (Joern and Hoagland 1996; Shaw and

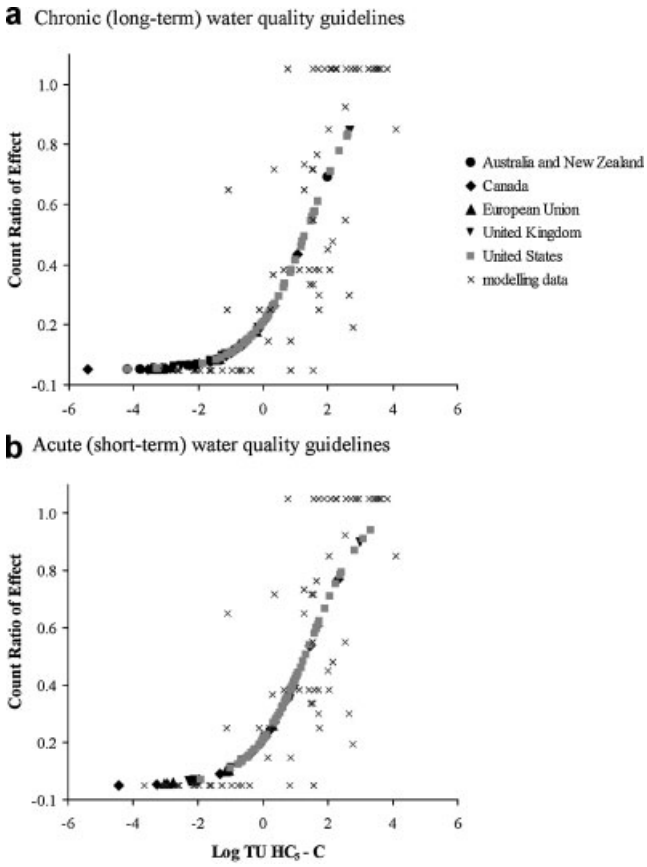

Figure 2. The log(TU acute 5% hazard concentration for crustacea) (HC_5-C) values versus the count ratio of effect for chronic, or long-term, water quality guidelines (**a**) and acute, or short-term, water quality guidelines for 80 pesticides from 5 jurisdictions (**b**) (CCME 1999; ANZECC 2000; EU 2006; UKTAG WFD 2008; USEPA 2009). The count ratio of effect is a measure of the proportion of crustacean species significantly affected by pesticide treatment.

Kennedy 1996). While water quality guideline derivation approaches vary from jurisdiction to jurisdiction, the goal of all water quality standards is to protect as many, if not all, nontarget species in a water body as possible. Most jurisdictions aim to establish water quality guidelines protecting at least 95% of nontarget species (CCME 1999; ANZECC 2000; EU 2006; UKTAG WFD 2008; USEPA 2009). Australia and New Zealand are unique in setting standards to protect either 95% or 99% of taxa in an aquatic ecosystem (ANZECC 2000).

In this study, we developed empirically based models relating TUs, derived either from *Daphnia* L(E)C50 data or HC5 for crustacea, and pesticide fate and physicochemical properties to predict real-world effects as estimated using data from mesocosm studies. TU HC5-C proved to be the single variable that best predicted the proportion of crustacean taxa significantly affected by the pesticide treatment. It is not surprising the TU HC5-C is a better predictor than the TU L(E)C50 from *Daphnia*, because its calculation incorporates a broader array of crustacean species, which should more closely reflect the range of toxicity sensitivities present in a water body. In essence, the HC5-C produces a laboratory-based toxicity measure more reflective of the broader crustacean community.

Among the structural and pesticide fate and physicochemical property variables in our modeling training data set, the count ratio of effect was positively correlated to the surface area to volume ratio (TSAV) and log K_{OW} and negatively correlated to the WPHL. At first glance, this finding is surprising, because it suggests that hydrophobic pesticides and those that undergo rapid photolysis are more toxic to Cladocera than persistent pesticides. Further investigation of the data suggested that both the WPHL and log K_{OW} variables are integrating aspects of water body shape. TSAV is positively correlated to the count ratio of effect, suggesting that a greater proportion of Cladocera are impacted by pesticide exposure in shallow water bodies with large surface areas. Photolysis can be accelerated or inhibited depending on how much and how deeply sunlight can penetrate the water. Also, a shallow pond would have more surfaces, such as macrophytes and sediment, onto which lipophilic compounds can bind. These results illustrate how water body shape can greatly influence the toxic effects seen. Adding the total surface area to volume variable to the TU HC5-C WPHL model increased the overall correct classification of results from 73% to 83%. Our training dataset did not allow us to explore these relationships any further and inclusion of TSAV would have necessitated specifying a specific water body type for predictive purposes; thus we decided not to include the structural and physical parameters in our predictive model. These interactions may be central to understanding how pesticides react in the natural environment, and they warrant further investigation.

Perhaps the most surprising result of our study was the discovery that, in many cases, the level of protection projected from water quality guidelines fell far short of expectation. Many did not adequately protect against acute effects in Crustacea. Clearly, protection afforded to other important aquatic groups (e.g., Insecta), or protection from indirect or more subtle or delayed toxicological effects, is probably illusory as well. It is not clear exactly why this situation exists. On the surface, water quality guidelines for pesticides from the 5 jurisdictions vary widely. For example, the short-term (acute) water quality guidelines for 2,4-D range from 4860 μg/L in Canada to 1.3 μg/L in the United Kingdom. Similarly, long-term (chronic) values for 2,4-D range from 140 μg/L in Australia and New Zealand to 0.3 μg/L in the United Kingdom, with Canada intermediate, at 12.53 μg/L (Table 2). Because the protection goals are similar, this variation reflects differences in how water quality guidelines are derived around the world. The Canadian long-term water quality guidelines for 2,4-D have recently been updated using species sensitivity distribution curves composed predominantly of no-effect thresholds for the long-term value and 96-h LC50 values for the short-term values (CCME 2007). A short-term, chronic water quality guideline is intended to be a maximum exposure value to protect against pulsed exposures such as a spill, whereas a long-term value is designed to protect aquatic life from adverse effects of lifelong pesticide exposure. The USEPA criteria are freshwater life benchmarks for the impacts of pesticides on invertebrates that are derived from ecological risk assessments for pesticide registration decisions and rely on data for the most sensitive effects concentration for a given taxon. In contrast, the USEPA ambient water quality criteria are based

on data from at least 8 families. In Europe, pesticide registration criteria are derived according to the Uniform Principles (EU 1997) by applying a safety factor of 0.01 to the lowest acute L(E)C50 for fish or *Daphnia* and 0.1 to the lowest EC50 for algae. The average exposure concentrations may not be higher than 0.1 times the 21-d NOEC for *Daphnia* and the 28-d NOEC for fish.

In their assessment of European herbicide and insecticide water quality criteria compared to NOECs derived from freshwater model ecosystems, Brock, Lahr et al. (2000) and Brock, van Wijngaarden et al. (2000) concluded the criteria set by using the Uniform Principles to protect freshwater life against most herbicides and pesticides depending on the exposure regime. Water quality guidelines for auxin-stimulating herbicides tend to underestimate the effects of pesticides by a factor as high as 100. Maltby et al. (2005) found the lower 95% confidence interval value of HC5, which is roughly equivalent to 0.1 times TU HC5-C, to be protective of aquatic ecosystems. Our study agrees with this benchmark and predicts that only 6% of Crustacea may be affected by pesticide exposure at concentrations of 0.1 times TU HC5-C. When water quality guidelines are converted into a count effect ratio, our results also suggest that 63% (73 out of 115) of chronic and 88% (91 out of 103) of acute water quality guidelines are insufficiently protective and would lead to adverse impacts in greater than 5% of exposed species. This finding suggests pesticide water quality guidelines derived from laboratory data underestimate the impacts of pesticides in real-world freshwater bodies, in part because they do not account for interactions between the pesticide and the abiotic properties of a water body. More importantly, the interspecies variation in sensitivity may have been underestimated, a problem also seen in regulatory evaluations of pesticides (Luttik et al., in press).

CONCLUSIONS

In conclusion, a model based on empirical field data using a species sensitivity distribution-derived measure of TUs appears to be reasonably predictive of the number of crustacean taxa affected by exposure to pesticides. One caveat to this finding is that our model did not consider postapplication recovery of affected taxa. It is very likely that, were recovery to have been considered, product persistence would have played a much more important role.

The majority of water quality guidelines, as currently derived in several jurisdictions, do not appear to protect nontarget organisms sufficiently. This situation may arise because the water quality guideline derivation protocols do not incorporate environmental conditions and other important real-world, abiotic factors. Also, the variation in interspecies sensitivity to pesticides may be underestimated during the water quality guideline derivation process.

We recommend that an empirical field-based approach such as the one derived in this paper should be considered by jurisdictions when formulating concentration-based water quality guidelines for pesticides. A very large number of provisional water quality guidelines could be generated in minutes and based on our analysis, might be more accurate than water quality guidelines for many pesticides now in circulation.

Acknowledgment—This research was conducted with funds from Agriculture and Agri-Food Canada through the National Agri-Environmental Standards Initiative to P Mineau. L Singh was supported by a partial scholarship from the Organisation of American States (OAS) administered through the Latin American Scholarship Program of American Universities (LASPAU) and the Public Service Ministry, Government of Guyana. We thank Clare Morrison, Melanie Whiteside, and Katie Harding for help compiling data for this project, and Peter Delorme, Scott Findlay, and Mark Forbes for their participation in L Singh's supervisory committee and their comments on the graduate thesis that originated this article. We also thank 2 anonymous reviewers for their insightful comments that helped improve the manuscript.

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Differences in susceptibility of five cladoceran species to two systemic insecticides, imidacloprid and fipronil

Article *in* Ecotoxicology · March 2012

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Differences in susceptibility of five cladoceran species to two systemic insecticides, imidacloprid and fipronil

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Accepted: 23 September 2011

Abstract Differences in susceptibility of five cladocerans to the neonicotinoid imidacloprid and the phenyl-pyrazole fipronil, which have been dominantly used in rice fields of Japan in recent years, were examined based on short-term (48-h), semi-static acute immobilization exposure tests. Additionally, we compared the species sensitivity distribution (SSD) patterns of both insecticides between two sets of species: the five tested cladocerans and all other aquatic organisms tested so far, using data from the ECOTOX database of U.S. Environmental Protection Agency (USEPA). The sensitivity of the test species to either imidacloprid or fipronil was consistent, spanning similar orders of magnitude (100 times). At the genus level, sensitivities to both insecticides were in the following descending order: *Ceriodaphnia* > *Moina* > *Daphnia*. A positive relationship was found between body lengths of each species and the acute toxicity (EC_{50}) of the insecticides, in particular fipronil. Differences in SSD patterns of imidacloprid were found between the species groups compared, indicating that test cladocerans are much less susceptible than other aquatic species including amphibians, crustaceans, fish, insects, mollusks and worms. However, the SSD patterns for fipronil indicate no difference in sensitivity between cladocerans tested and other aquatic organisms despite the greater exposure, which overestimates the results, of our semi-static tests. From these results, *Ceriodaphnia* sp. should be considered as more sensitive bioindicators (instead of the standard *Daphnia magna*) for ecotoxicological assessments of aquatic ecosystems. In addition, we propose that ecotoxicity data associated with differences in susceptibility among species should be investigated whenever pesticides have different physicochemical properties and mode of action.

Keywords Acute toxicity · *Ceriodaphnia* · *Daphnia* · Moina · Pesticide · Species sensitivity distribution · Zooplankton

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Introduction

Pesticides are developed to protect crops against pests, and are indispensable to assure agricultural quality and productivity. However, pesticides can have adverse impacts on some non-target organisms in the aquatic ecosystem. Especially, rural areas including paddies play an important role as habitats for many species (Bambaradeniya and Amerasinghe 2003). Even if complex experimental systems such as micro- and mesocosms (e.g., Chang et al. 2005; Sánchez-Bayo and Goka 2006a; Beketov et al. 2008) are essential for effective higher-tier ecological risk assessment to pesticides (Campbell et al. 1999), acute ecotoxicity data still play an important role in first-tier risk assessments for regulatory purposes.

In Japan, as in most developed countries, the ecotoxicity of pesticides to aquatic organisms is estimated using only laboratory single-species tests based on the OECD guidelines (1982). These guidelines recommend using three test species: a zooplankton crustacean (typically *Daphnia*

Published online: 05 October 2011

magna), a small fish (e.g. *Oryzias latipes*) and an aquatic algae (e.g. *Pseudokirchneriella subcapitata*). In particular, since zooplankton are prey to fish and aquatic insects while being consumers of phytoplankton, zooplankton organisms are important links in the aquatic food chain and the function of freshwater ecosystems (Chang et al. 2005; Steiner et al. 2005). It is also important to take species sensitivities into consideration for a proper evaluation of laboratory acute toxicity tests. Different species can vary significantly in their sensitivity to toxic contaminants (Wogram and Liess 2001; Posthuma et al. 2002). However, information on the susceptibility among zooplankton species to many modern pesticides such as neonicotinoid and phenyl-pyrazole is deficient, and most of our knowledge is based on carbamate insecticides and metallic compounds (e.g. Sakamoto et al. 2005; Vesela and Vijverberg 2007; Mano et al. 2010), whereas most ecotoxicity data refers to *Daphnia magna* (Sánchez-Bayo 2006).

In this study, we examined the relative sensitivities of five cladoceran species to two new systemic insecticides imidacloprid and fipronil, which belong to the neonicotinoid and phenyl-pyrazole chemical classes, respectively, and have different chemical properties. Our comparison is based on the 48-h acute toxicity test, taking their body size into account (Gliwicz 1990; Mano et al. 2010). In addition, we compared the sensitivity of the five test cladocerans and that of other species of aquatic vertebrates and invertebrates, using species sensitivity distribution (SSD) curves for the respective insecticides. The concept of SSD is to statistically predict the safe environmental concentration of a toxicant that is protective of most species (usually above 95% in a community) (Posthuma et al. 2001).

Table 1 Physicochemical properties and acute toxicity of imidacloprid and fipronil

	Imidacloprid	Fipronil
Physicochemical properties		
Water solubility at 20°C (mg/l)	610[a]	3.78[c]
Octanol: water partition coefficient at 20°C (logPow)	0.57[c]	4[c]
Hydrolysis half-life at 25°C (days)	>30[b]	>100[d]
Aqueous photolysis half-life at 25°C (days)	0.0398[b]	0.33[d]
Sorption in soil (Koc)	132–310[b]	542–1176[e]
Acute toxicity		
Crustaceans (48-h LC_{50}: µg/l)		
Daphnia magna	10440–64873[f]	>100[f]
Fish (96-h LC_{50}: µg/l)		
Lepomis macrochirus (bluegill)	>105000[f]	25–83[f]
Oncorhynchus mykiss (rainbow trout)	83000–229100[f]	39–246[f]

[a] Data from Tomlin (2001–2002)

[b] Data from Kollman and Segawa (1995)

[c] Data from Japan Plant Protection Association (2005)

[d] Data from Gunasekara et al. (Gunasekara et al. 2007)

[e] Data from Ying and Kookana (2001)

[f] Data from ECOTOX database (http://cfpub.epa.gov/ecotox/)

Materials and methods

Physicochemical properties and acute toxicity of target insecticides

Physicochemical data of imidacloprid and fipronil are given in Table 1. Imidacloprid has high water solubility, and though the active ingredient disappears quickly from surface waters (Kollman and Segawa 1995), it is more persistent in underground water environments (Felsot et al. 1998; Nemeth-Konda et al. 2002). By contrast, fipronil has low water solubility, is more stable and it is adsorbed more strongly onto soil (USEPA 1996; Ying and Kookana 2001; US Geological Survey 2006; Gunasekara et al. 2007) than imidacloprid. The penetration rates of imidacloprid and fipronil, which are dominantly used in rice fields of Japan, are 18.9 and 24.8%, respectively (Ministry of Agriculture, Forestry and Fisheries 2005). Since these insecticides can be absorbed by rice seedlings and stored in their tissues, they are usually applied to nursery boxes in granular formulation before planting, to protect crops against pests. From the acute toxicity data, it appears that fipronil is 100–1000 times more toxic than imidacloprid to *Daphnia magna* and two species of fish (Table 1).

Test species

All test cladocerans in this study (*Ceriodaphnia dubia*, *Ceriodaphnia reticulata*, *Daphnia magna*, *Daphnia pulex* and *Moina macrocopa*) were obtained from the National Institute for Environmental Studies, Tsukuba, Japan. Except for non-indigenous species such as *D. magna* and *C. dubia*, all others occur commonly in freshwater environments in Japan, including rice fields (Hanazato 1998). These stock cultures have been maintained for 30 years at the institute. Stock cultures were kept at a constant temperature of 22 ± 1°C with a light:dark cycle of 16:8-h. The five cladocerans were separately cultured in 1 l glass beakers filled with dechlorinated tap water and fed daily, using green alga *Chlorella vulgaris* as their exclusive diet. Parameters of the tap water used are follows: pH 7.8; turbidity, <0.1; water hardness, 76 mg/l; and total organic carbon (TOC), 0.9 mg/l.

Toxicity bioassays (immobilization test, 48-h EC_{50})

In this test, we used wettable powders of imidacloprid and fipronil to make the insecticidal solutions. Commercial imidacloprid [Admire® Flowable, imidacloprid/water and surfactant (20:80, v/v)] was obtained from BASF Japan Ltd. and fipronil [Prince® Flowable, fipronil/water and surfactant (5:95, v/v)] from Kumiai Chemical Industry Co., Ltd., both from Tokyo, Japan.

The bioassays were performed following OECD guideline no. 202 (1984, 2004) for acute immobilization tests and good laboratory practice. Female neonates (<24-h old) from the second or later broods were used in all tests. The nominal concentrations of imidacloprid and fipronil, and number of tests for each species/treatment are shown in Table 2. The concentration ratio between successive solutions in all the tests was 2.0. Nominal chemical concentrations were prepared by serial dilution with dechlorinated tap water of stock solutions in distilled water. For each concentration, four replicates were used, each replicate beaker containing five neonates of the same species, which were placed in 50 ml of the test solutions. Each species was tested separately. Controls were prepared in the same way but using only dechlorinated tap water. No food was provided during the test period. Because of the fast aqueous photolysis of both insecticides (Table 1), the acute immobilization test in this study was semi-static, with chemical solutions being renewed daily according to the test guideline for longer exposure tests (OECD 1984). This means our results may be slightly overestimated when compared to those from static 48-h tests. The test beakers were kept at 21 ± 1°C with a light:dark cycle of 16:8-h for 48-h. The endpoint used for all bioassays was immobility, i.e., the inability to swim within 15 s after gentle agitation of the test container. Test organisms were checked after 48-h from the beginning of the tests.

Abiotic factors such as pH and dissolved oxygen (DO) were measured at the beginning and end of the tests in the controls and beakers with the highest test substance concentrations. Water pH and DO were measured by a portable multi-meter (DM-32P; TOA DKK-TOA Corporation, Tokyo, Japan).

Initial values of pH were 7.92 ± 0.07, and though they decreased slightly after 48-h (7.84 ± 0.06), the change was not significant. The values of DO at the start and the end were 8.30 ± 0.20 and 8.04 ± 0.15, respectively.

Body length of neonates of the five test cladocerans

To clarify the relationship between the EC_{50} values of imidacloprid and fipronil and the body sizes of the test species, we measured body lengths of their neonates (Table 3). Prior to the bioassays, 30–40 female neonates, randomly selected from the stock culture of each organism, were preserved in formalin (4%). Body lengths, from the crown of the head to the base of the tail spine (Mano et al. 2010), were measured using graphic software (IE-500, Leica Microsystems AG, Switzerland) under a dissecting microscope (Leica DFC490, Leica, Wetzlar, Germany).

Data analysis

All observations were recorded at 48-h exposures to determine the corresponding acute EC_{50} (immobilization), which was calculated by the Probit method (Finney 1971) using the program EcoTox-Statics ver. 2.5 (http://www.intio.or.jp/jset/ecotox.htm). The relationship between estimated EC_{50} values of imidacloprid and fipronil among each test species and their body lengths was analyzed by Pearson's correlation coefficient.

To examine differences in the patterns of other aquatic organisms (i.e., except cladocerans) to imidacloprid and fipronil, we compared our results with the acute toxicity data (LC_{50} and EC_{50}) from the ECOTOX database (http://cfpub.epa.gov/ecotox/), using all data available for these insecticides to amphibians, crustaceans, fish, insects, mollusks and worms. Species sensitivity distributions (SSDs) of each insecticide were used to this purpose. Based on laboratory single-species acute toxicity tests, SSDs are constructed by fitting a cumulative density function to a plot of species toxicity data against rank-assigned percentiles (Aldenberg and Jaworska 2000). From the distribution of such data the 5% hazardous concentration (HC5) of each insecticide was calculated, which would indicate the

Table 2 Nominal concentrations of imidacloprid and fipronil used in the acute tests

Species	Imidacloprid		Fipronil	
	Range (μg/l)	Number of treatments	Range (μg/l)	Number of treatments
Ceriodaphnia dubia (C.dub)	390.63–6250	5	0.39–12.5	6
Ceriodaphnia reticulata (C.ret)	781.25–50000	7	0.39–100	9
Daphnia magna (D.mag)	12500–400000	6	9.77–625	7
Daphnia pulex (D. pul)	6250–200000	6	9.77–625	7
Moina macrocopa (M. mac)	6250–20000	6	6.25–200	6

Table 3 Acute toxicity (immobilization), of imidacloprid and fipronil to five cladocerans

Species	Sample size (*n*)	Mean body length (mm)	Imidacloprid 48-h EC_{50} (μg/l)	Fipronil 48-h EC_{50} (μg/l)
Ceriodaphnia dubia	34	0.34 ± 0.06	571.62 ± 289.6–841.2	0.99 ± 0.62–1.42
Ceriodaphnia reticulata	30	0.37 ± 0.07	5552.9 ± 4213.3–7387.8	8.83 ± 5.52–17.57
Daphnia magna	25	1.11 ± 0.06	43265 ± 34302–53592	88.30 ± 64.20–141.12
Daphnia pulex	32	0.73 ± 0.10	36872 ± 28399–48106	40.392 ± 30.04–53.50
Moina macrocopa	41	0.56 ± 0.07	45271 ± 34378–62218	29.57 ± 16.83–9.E + 7

Body length is indicated by the mean and standard deviation

concentration that has a negligible effect on natural biocenosis. Differences in SSD patterns between the five test cladocerans and other aquatic organisms to the two insecticides were analyzed by paired *t*-test. The statistical analysis was conducted using SPSS ver 11.5 J (SPSS Japan, Tokyo, Japan).

Results

Calculated acute toxicities values (48-h EC_{50}) of the test organisms to imidacloprid and fipronil are given in Table 3. In this study, the values of *D. magna* for imidacloprid and fipronil were 43,265 and 88.3 μg/l, respectively. These values are in the same range as reported for this species on the ECOTOX database (6,029–85,200 μg/l for imidacloprid, and 29–190 μg/l for fipronil).

Clear differences in susceptibility among the cladocerans tested were found for both imidacloprid and fipronil. The degree of sensitivity of the species to the two insecticides spanned similar orders of magnitude: about 100 times from the most sensitive (*Ceriodaphnia dubia*) to the least. Toxicity of imidacloprid was in the following decreasing order: *C. dubia* > *C. reticulata* > *D. pulex* > *D. magna* > *M. macrocopa*. For fipronil: *C. dubia* > *C. reticulata* > *M. macrocopa* > *D. pulex* > *D. magna*. *Ceriodaphnia dubia* and *C. reticulata* showed the highest sensitivities to the two insecticides, and *D. magna* exhibited the lowest (Table 3). At the genus level, *Ceriodaphnia* spp. are more sensitive, whereas *Daphnia* spp. and Moina sp. are less susceptible to either imidacloprid or fipronil (Table 3).

The relationship between mean body lengths and EC_{50} values of the species tested to the two insecticides are shown in Fig. 1. Although a weak relationship between the two factors was found in the case of imidacloprid ($r^2 = 0.585$, $P = 0.132$), the acute toxicity of fipronil was significantly correlated with body length ($r^2 = 0.989$, $P < 0.001$). On the other hand, there were no clear differences in susceptibility to the insecticides between indigenous (*C. reticulata*, *D. pulex* and *M. macrocopa*) versus non-indigenous species (*C. dubia* and *D. magna*) (Fig. 1).

Comparative results of SSD patterns between the test five species of cladocerans tested in this study and other aquatic organisms are shown in Fig. 2. For imidacloprid, the 5% hazardous concentration (HC5) values calculated from the ECOTOX database (all aquatic organisms except cladocerans) and our data (five cladoceran species) to imidacloprid were 0.67 and 513.68 μg/l, respectively. Those of fipronil were 0.10 and 0.88 μg/l, respectively. In the case of imidacloprid, a significant difference in SSD patterns was found between the cladocerans and other aquatic organisms ($t = -3.112$, $P < 0.01$), with cladocerans being less sensitive than other species. However, similar SSD patterns were found between the two species groups compared in the case of fipronil ($t = 1.239$, $P = 0.231$).

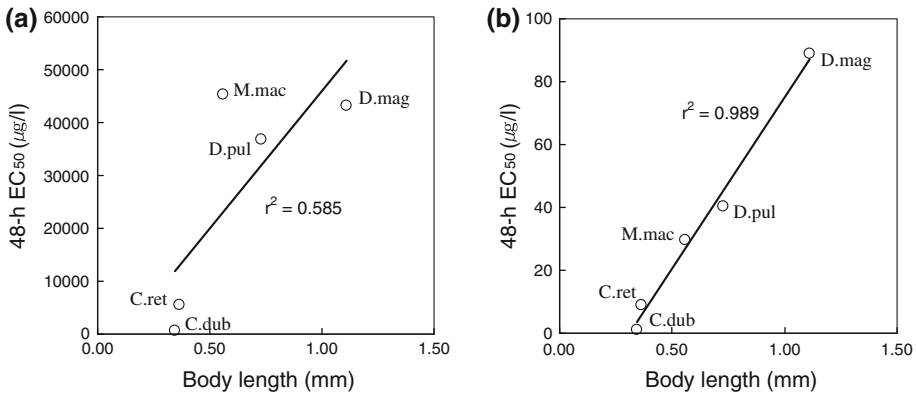

Fig. 1 Relationships between the acute toxicity of insecticides (48-h EC_{50} in μg/l) and body size of five cladoceran species: **a** imidacloprid, **b** fipronil. Abbreviations of the test species: *C.dub Ceriodaphnia dubia*; *C.ret Ceriodaphnia reticulata*; *D.mag Daphnia magna*; *D.pul Daphnia pulex* and *M.mac Moina macrocopa*. *Solid line* indicates a regression line of the relationship between the two factors

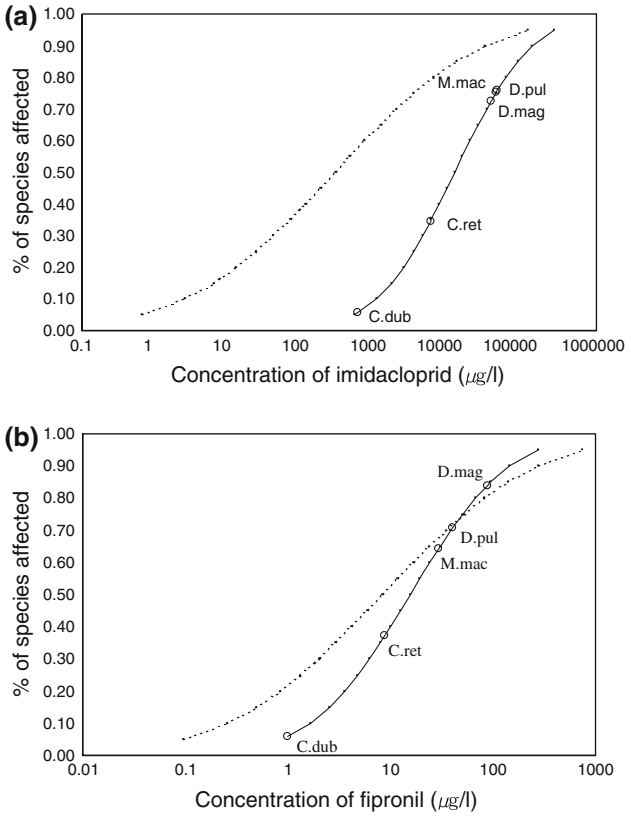

Fig. 2 Species sensitivity distribution (SSD) of five cladoceran species in this study (*solid line*) and other aquatic organisms (*dotted line*) using data from ECOTOX database for **a** imidacloprid and **b** fipronil. Abbreviations of the test species as in Fig. 1. Other aquatic organisms include amphibians, crustaceans, fish, insects, mollusks and worms

Discussion

Sensitivities of the five cladoceran species tested here to imidacloprid and fipronil varied depending on the body size of species and taxonomic (genus) level rather than species status (indigenous and non-indigenous) (Fig. 1; Table 3). Our results showed the wide interspecific variation in the susceptibility of test cladocerans to both insecticides. Similar finding is reported by Hose and Van den Brink (2004) that the sensitivity of organisms to toxicants is independent of their geographic origin. Body size was positively related to the capacity of these organisms to withstand the stress caused by the two insecticides (Fig. 1), since smaller species tend to be more sensitive to toxic stress than the larger ones (Wong et al. 2009). Similar findings have been reported by other researchers when testing for metals and cholinesterase-inhibitor insecticides (e.g. Sakamoto et al. 2005; Vesela and Vijverberg 2007), whereas a review by Hanazato (1998) indicates that larger cladoceran species are more sensitive to insecticides than smaller zooplankton species such as rotifers. A comprehensive comparative study by Sánchez-Bayo (2006) found that there is no significant effect of size on the sensitivities of zooplankton crustaceans to most toxic chemicals. In fact, positive correlations with size appear to be the exception (16% of toxicants) rather than the norm, and are found with preference among chemicals with specific mode of action such as insecticides, which are usually the most toxic (Vaal et al. 1997). In our study, fipronil showed a clear correlation with the size of the five species tested ($r^2 = 0.989$), but that of imidacloprid was not as strong ($r^2 = 0.585$). Thus, differences in the insecticide impacts on biocenosis may depend on the mode of action of the chemicals as well as the cladoceran species composition (Mano et al. 2010). Previous studies reported the high tolerance capability of *M. macrocopa* (Hatakeyama and Sugaya 1989; Mano et al. 2010) and by contrast the high sensitivity of *Ceriodaphnia* sp. (Hatakeyama et al. 2010; Mano et al. 2010) to carbamate pesticides. The bioassay tests results shown here, which consider different chemical classes of insecticides (neonicotinoid and phenyl-pyrazole) also showed a similar tendency (Table 3). *Ceriodaphnia reticulata* is known to consume micro-organisms such as bacteria more efficiently than other cladocerans, perhaps because of its small size (Geller and Müller 1981).

From these results, because of their high sensitivity to these two insecticides, *Ceriodaphnia* spp. may be more suitable bioindicators of ecological disturbance by imidacloprid and fipronil in aquatic ecosystems than the current OECD surrogate species, *Daphnia magna*. Low sensitivity of *D. magna* to neonicotinoid thiacloprid was also found by Beketov and Liess (2008). Mano et al. (2010) indicate that a decrease in the abundance of *Ceriodaphnia* spp., in particular *C. reticulata* by carbamate insecticides such as carbaryl and methomyl may reduce the energy flow through the microbial loop, since heterotrophic micro-organisms such as bacteria are consumed by zooplanktons.

Species sensitivity distribution (SSD) of ecotoxicological data is one of the most effective approaches for ecological risk assessment to pesticides because it aims at protecting biodiversity (Posthuma et al. 2001; Nagai et al. 2011). Clear differences in SSD patterns of the two insecticides tested here, in particular imidacloprid, were found between the five cladocerans used in this study and other aquatic organisms (Fig. 2). Among the zooplanktons, cladocerans are more sensitive than rotifers and copepods to a large range of pollutants (Hanazato and Yasuno 1990; Sierzen and Lozano 1998; Wong et al. 2009), and have been attractive test organisms also due to their short generation cycle and ease of culture and maintenance in laboratories (Benfield and Buikema 1980). In addition, Dodson et al. (1995) reported that prey zooplankton such as cladocerans are more sensitive to toxicants than their

predators, and therefore are preferred as sentinel bioindicators of the ecosystem (Sakamoto et al. 2005). The significant differences in HC5 values for imidacloprid between the two groups compared (Fig. 2a) suggests, however, that imidacloprid residues in water can have larger adverse effects on aquatic organisms other than cladocerans: indeed, most aquatic taxa are about 500 times more sensitive to imidacloprid than cladocerans. In particular, ostracods are two to three orders of magnitude more susceptible to imidacloprid than cladocerans (Sánchez-Bayo and Goka 2006b). By constant, the sensitivity of cladocerans to fipronil is no different from that of other aquatic taxa (Fig. 2b). The SSD patterns shown here are in agreement with the finding reported by Vaal et al. (1997), who documented that reactive and specific mode of action chemicals such as insecticides usually have the largest intraspecific variation, as shown by the less steep slope of a SSD curve of toxicity data from aquatic species.

On the other hand, Hose and van den Brink (2004) indicate that arthropod taxa in mesocosm were less sensitive than in laboratory tests, which suggests that laboratory single-species data used on SSDs may be overprotective of field populations. However, Hayasaka et al. (2011) report that imidacloprid in paddy mesocosms can have adverse effects on zooplankton, neuston, nekton and benthic communities at concentrations well below the HC5 protective value of the test cladocerans, whereas small impacts of fipronil on the same aquatic organisms were found. This discrepancy may not be surprising because many researchers have shown similar tendencies with other insecticides (e.g., Liess and von der Ohe 2005; Schäfer et al. 2007). However, the results from the semi-static tests in this study may be regarded as slightly overestimated due to the greater exposure.

As mentioned above, ecotoxicological assessment protocols for aquatic organisms are standardized by the OECD guidelines. Harmon et al. (2003) and Wu et al. (2007) indicate that although the regulating authorities accept the test organisms and protocols, they do not always reflect local taxa or site-specific conditions. For instance, Wu et al. (2007) have suggested that *Daphnia carinata* is a more suitable test species for tropical and subtropical regions, where *D. magna* is not found, while other authors have criticized the use of *D. magna* on size considerations (Koivisto 1995).

The strong differences in susceptibility among cladocerans to the pesticides imidacloprid and fipronil were clarified in this study, and our findings agree well with interspecific differences shown by many other authors using insecticides. Therefore, we conclude that toxicological data associated with differences in susceptibility among species should be investigated whenever pesticides have different physicochemical properties and mode of actions. Such information may help define uncertainty factors to extrapolate from laboratory acute toxicity tests based on OECD test guidelines (i.e. *Daphnia magna*) to other species with similar ecological function in the ecosystems.

Acknowledgments We thank Masako Ikejima for her kind assistance with the acute toxicity tests. We are indebted to Fusae Oyama for providing the culture organisms and their food. The authors wish to thank Dr. Hiroyuki Mano and Dr. Takashi Nagai for valuable technical advices. The article benefited from the constructive comments of two anonymous reviewers.

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Neonicotinoids in California's Surface Waters

A Preliminary Review of Potential Risk to Aquatic Invertebrates

Sarah Hoyle and Aimée Code

November 2016

Neonicotinoids, a relatively new class of insecticides, are the most widely used insecticides in the world. They are applied to a wide range of agricultural crops as well as in urban settings. Although neonicotinoids are less acutely toxic to mammals and other vertebrates than some older insecticides they have replaced, they are highly toxic to many beneficial invertebrates. Of the neonicotinoids, the nitroguanidine group (clothianidin, dinotefuran, imidacloprid, and thiamethoxam) are the most toxic and longest lived.

Recent reviews and reports have drawn more attention to the risks these insecticides pose to water quality and their potential effects on aquatic systems.[i] While there is still uncertainty, independent research and regulatory evaluations from other countries suggest that the US Environmental Protection Agency's (EPA) invertebrate aquatic life benchmarks may be substantially higher than levels of imidacloprid and other neonicotinoids in surface water that could cause harm to aquatic invertebrates and the systems they support.[ii] Aquatic invertebrates are essential to freshwater ecosystems and beyond. These invertebrates are preyed on by fish, birds, and other species; perform ecological services like shredding and nutrient retention; maintain biodiversity; and are important for human recreation, among other ecosystem functions.[iii] Effects on aquatic invertebrates could also indirectly cause harm to insectivorous fish and bird species, including protected species.

This white paper reviews current research on the effects of nitroguanidine neonicotinoids on aquatic invertebrates and compares the toxicological endpoints identified in those studies with California's surface water monitoring data. Since most aquatic toxicology and monitoring data is available for imidacloprid, our analysis focuses on this compound, but it also raises questions about the other nitroguanidine neonicotinoids. Sampling results show that imidacloprid contamination is

widespread and often detected at levels that can cause harms to foundational invertebrate species. From our initial review, it appears that the current aquatic life benchmarks for imidacloprid are under-protective. We are concerned that the levels of imidacloprid currently found in California's waters could harm aquatic species and potentially cause cascading effects up the food chain.

Xerces has brought this information to California's Department of Pesticide Regulation (CDPR) to request a timeline for a review of aquatic invertebrate toxicity data, potentially leading to the development of interim imidacloprid acute and chronic benchmarks to protect aquatic invertebrates. We also recommend that CDPR review the other nitroguanidine neonicotinoids to establish appropriate benchmarks that protect aquatic invertebrates. While the majority of available data is about imidacloprid, our findings raise questions about the effects of other nitroguanidine neonicotinoids as well.

Pesticide Sales and Use Reporting Data

The use of nitroguanidine neonicotinoids in California has climbed since their introduction, both in terms of number of applications and pounds applied. Pesticide use reports are collected by CDPR from agricultural and professional applicators across the state.[iv] This data does not include figures for seed coatings (used on California crops including cotton, corn, and wheat) or non-professional ornamental and urban applications, so it provides an underestimate of actual use.[v] The resulting data set can provide use trends, such as the rise in imidacloprid use over the last twenty years from 5,179 pounds in 1994 to 373,734 pounds in 2014 (Figure 1). The number of applications for clothianidin, thiamethoxam, and dinotefuran are all trending upward as well in recent years.

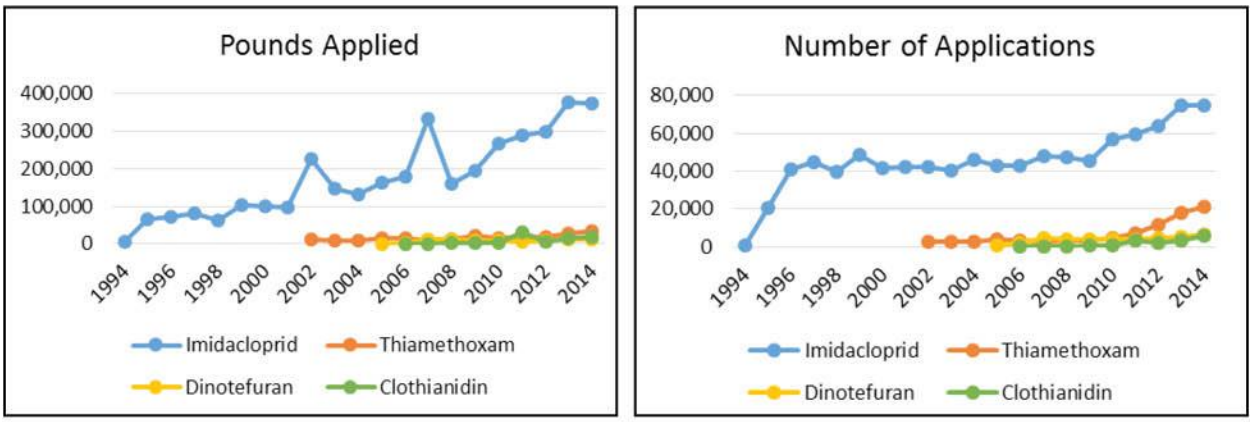

Figure 1: Pounds applied and number of applications of nitroguanidine neonicotinoids in California. This data does not include the planting of seed coated with neonicotinoids or non-professional ornamental and urban applications. The 2002 and 2007 outliers in imidacloprid pounds applied are likely data reporting errors.

California's use reporting data is currently only available up to 2014. Since then permitted uses of nitroguanidine neonicotinoids have expanded (for example, clothianidin has been approved for rice). To better understand possible increases in use since 2014, we reviewed California's pesticide sales data.[vi] Clothianidin sales jumped from 20,916 pounds in 2014 to 119,731 pounds in 2015, a 472% increase in a single year. Sales of the other nitroguanidines also increased notably between 2014 and 2015. Imidacloprid sales rose from 542,262 pounds in to 791,125 pounds (a 46% increase); thiamethoxam from 33,179 pounds to 53,381 pounds (a 61% increase); and dinotefuran from 13,170 pounds to 75,052 pounds (a 470% increase). The continued rise in neonicotinoid sales and use compels CDPR to address the impacts of imidacloprid on aquatic systems, and to review the effects of the other nitroguanidine neonicotinoids as their use increases.

California Surface Water Detections

California's water monitoring records provide valuable information on neonicotinoid water contamination. Imidacloprid monitoring data is available for 790 surface water samples taken at 132 sites from January 2010 to October 2015.[vii] Of those 132 sites throughout the state, 72 (55%) had at least one imidacloprid detection above the level of quantification (typically 0.05 µg/L).[viii] In the 790 samples, imidacloprid was detected 468 (59%) times, up to a maximum of 12.7 µg/L.[ix]

The EPA acute benchmark of 35 µg/L was not exceeded in any sample, but toxicological studies suggest that acute exposures could impact sensitive species well below this level, at concentrations detected in California surface water. Throughout this report detection frequencies and averages will exclude samples where imidacloprid was not detected. The average imidacloprid level among detections was 0.643 µg/L, which can cause sublethal effects in many aquatic invertebrates, especially sensitive groups of species like mayflies.[x] Imidacloprid was detected above the EPA chronic invertebrate benchmark of 1.05 µg/L in 65 (14%) instances.[xi] At or below this level, effects on aquatic species include death, downstream drift, reductions in larval emergence, reproductive impacts, and alterations in feeding behavior.

The prevalence of imidacloprid and other neonicotinoids in surface water samples throughout the state suggests that these compounds could be routinely entering aquatic ecosystems from a variety of sources. Detection levels are sufficient to raise concern for aquatic invertebrates and the ecosystems that depend on them.

Frequently-monitored areas signal risks

Imidacloprid detections are clustered throughout the state, and are particularly common in some agricultural areas like Santa Maria, the Salinas Valley, and the Imperial Valley that have been monitored more frequently (Figure 2). Of note, imidacloprid was detected in 91% (71 of 78) of

samples in the Santa Maria area; 82% (178 of 218) of samples in the Salinas Valley area; and 72% (31 of 43) of samples in the Imperial Valley area.[xii] The presence of clustered areas of imidacloprid detections suggests that discrete areas may be particularly at risk. Therefore, throughout this report, we present detections from the Santa Maria area to provide context for detection levels in an agricultural area that was well-studied and where imidacloprid was frequently present. Examining discrete areas separately from the entire state should provide a more representative understanding of surface water contamination in areas where imidacloprid use is high and monitoring data is available. Analyzing the data separately also reduces the potential that risk would be obscured by combining data from high-detection areas with data from locations with infrequent and/or low level detections.

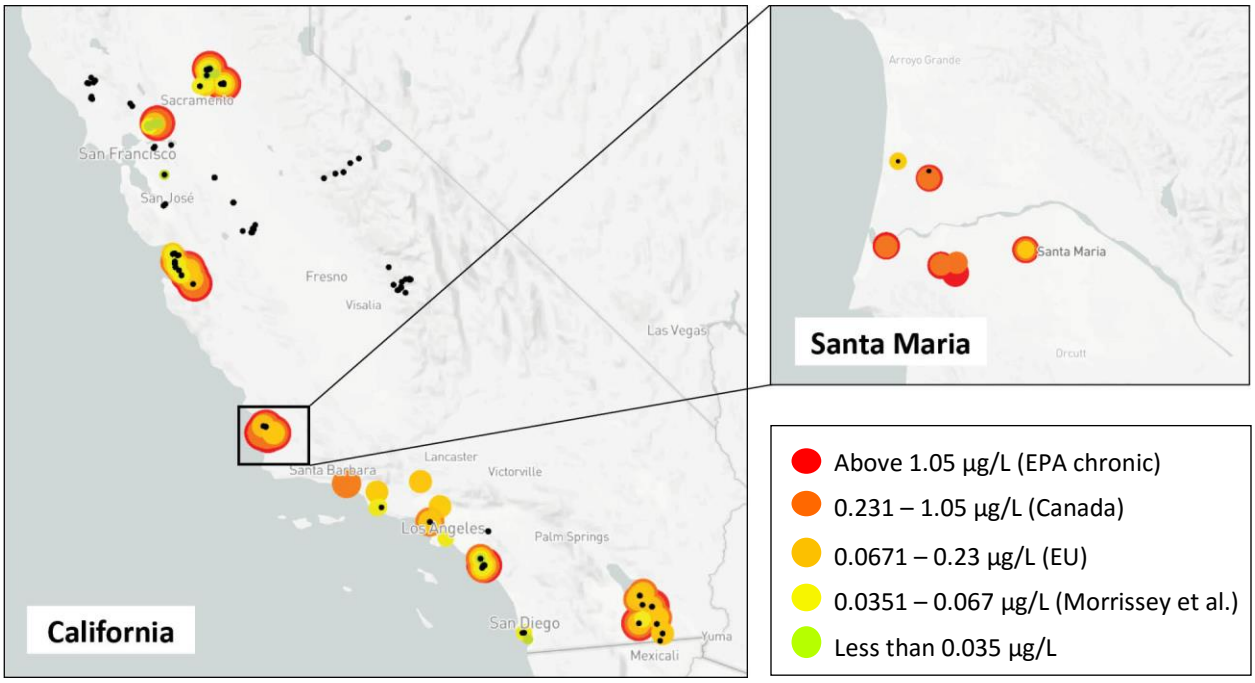

Figure 2: Imidacloprid detections from CDPR monitoring data. All California samples are mapped on the left, with a close-up of Santa Maria area samples on the right. Colors correspond to water quality guidelines for the US and other jurisdictions, black dots are samples where imidacloprid was not detected. No imidacloprid samples were taken north of the Sacramento region.

Imidacloprid in urban waters

Along with agricultural regions, imidacloprid has frequently been found in urban areas, particularly in the Santa Barbara, Los Angeles, and Sacramento regions (Figure 2). There are several potential sources of neonicotinoids in urban waterways, including landscaping, outdoor building products, and flea and tick control products used on pets.[xiii] Data on urban neonicotinoid

use is limited because there are no reporting requirements for independent non-professional applications.

Neonicotinoids used in urban areas can move into both storm and sanitary drains. Recent research has shown that neonicotinoids may not be removed during standard wastewater treatment, so they can be transferred to water bodies that receive effluent.[xiv] Urban sampling in the Sacramento area and Orange County from 2008–2011 found that imidacloprid was the second-most commonly detected insecticide, with a maximum of 0.67 µg/L.[xv] The city of Santa Barbara also conducted sampling for neonicotinoids and found imidacloprid in each wet-weather sample.[xvi] While the highest detection in Santa Barbara was 0.076 µg/L, the frequent presence of imidacloprid in urban waterways is concerning.[xvii]

Imidacloprid Toxicity to Aquatic Invertebrates

Imidacloprid toxicity studies have been conducted with a range of experimental designs, concentrations, and species. Experiments with both technical grade imidacloprid and formulated products containing imidacloprid have, in some cases, shown additional toxicity from formulations.[1,xviii] Furthermore, there is wide variation in the sensitivity of different invertebrates between and within taxa. The commonly-used test species for pesticide ecotoxicity studies, *Daphnia magna*, is orders of magnitude less sensitive to imidacloprid than many other invertebrates, particularly Ephemeroptera and Trichoptera species. The insensitivity of *D. magna* combined with the wide-ranging sensitivity of other species adds complexity to setting aquatic life benchmarks that are sufficiently protective. Independent testing completed since imidacloprid's registration has identified acute and chronic sensitivity in certain species at concentrations well below the aquatic life benchmarks. The range of concerning sublethal effects that have been identified could lead to mortality in individuals and population-level impacts. These effects include but are not limited to reproduction inhibition, impaired feeding, and downstream drift. Due to the nature of neonicotinoid binding, it is has been suggested that invertebrates are subject to cumulative and delayed effects from exposure.[xix] Both lethal and sublethal effects impact the structure and ecological functions of aquatic invertebrate communities, with far-reaching consequences for other species that depend on healthy freshwater ecosystems.[xx] Each experiment provides discrete information, but taken together they provide strong evidence that imidacloprid is toxic to freshwater aquatic invertebrates at levels below current EPA aquatic life benchmarks.

[1] Throughout this report, we note if a study used formulated products. Tisler et al. 2009.; *Daphnia magna* 21-day LOLC 40 mg/L for imidacloprid versus 10 mg/L for Confidor (Jemec et al. 2007); *Hyalella azteca* 96h LC_{50} 65.43 µg/L for imidacloprid versus 17.44 µg/L for Admire (Stoughton et al. 2008).

Acute Risks

Our literature review of independent imidacloprid toxicity studies revealed wide-ranging sensitivity among invertebrates (see Appendix A for additional detail on each study). Researchers have defined toxicological endpoints for a range of species, some of which are displayed in Table 1. The commonly used pesticide test species *Daphnia magna* is significantly less sensitive to imidacloprid (48-hour EC_{50} for immobility of 56,500 µg/L[xxi]) than many other species (for example the 48-hour LC_{50} for the mayfly *Baetis rhodani* is 8.49 µg/L[xxii]). In particular, species from the key groups Ephemeroptera and Trichoptera are particularly at risk.

The EPA acute freshwater invertebrate benchmark is set at 35 µg/L, a level that was not seen in Californian monitoring. However, LC_{50}s for certain sensitive species range from 0.65 to 8.49 µg/L (Table 1), suggesting the acute limit may be under-protective. California surface water samples have detected imidacloprid in or above this range in 124 (26%) of 468 detections from 2010 to 2015.[xxiii] Aquatic life benchmarks should be reconsidered given the sensitivity of certain species.

Table 1: Imidacloprid Toxicity for Selected Sensitive and Test Species (µg/L)

	Endpoint	Value (µg/L)	Citation
Lethal Endpoint			
Baetis rhodani (mayfly)	48h LC_{50}	8.49	Beketov and Liess 2008
Chironomus dilutus (midge)	14d LC_{50}	1.52	Cavallaro et al. 2016
Chironomus tentans (midge)	96h LC_{50}	5.75	Stoughton et al. 2008
Epeorus longimanus (mayfly)	24h LC_{50}	2.1*	Alexander et al. 2007
Epeorus longimanus (mayfly)	96h LC_{50}	0.65*	Alexander et al. 2008
Sublethal Endpoints			
Baetis rhodani (mayfly)	Downstream drift (48h)	1	Beketov and Liess 2008
Chironomus dilutus (midge)	40d EC_{50} (emergence)	0.39	Cavallaro et al. 2016
Daphnia magna (daphnid)	48h EC_{50} (immobility)	56,500	Tisler et al. 2009
Daphnia magna (daphnid)	21d NOEC (immobility)	1,250	Tisler et al. 2009

*Testing done with formulated product, Admire (imidacloprid).

Community structure impacts

Lethality from imidacloprid contamination can impact the community structure in aquatic systems by triggering declines in sensitive species while leaving more tolerant species unaffected. In an experiment designed to simulate the effects of spray drift on lentic communities, researchers applied imidacloprid on sunny days when photolysis was expected to play a role in degradation. When the time-weighted average imidacloprid level was 1 µg/L from three weekly pulses, Ephemeroptera declined and certain species were absent.[xxiv] Surface water samples in California equaled or exceeded 1 µg/L in 75 (16%) detections and 30 (42%) Santa Maria detections.[xxv]

Chironomidae species declined significantly in trials with a time-weighted average of 5.2 µg/L of imidacloprid, a level exceeded in 8 (2%) California detections and 4 (6%) Santa Maria detections.[xxvi]

In a separate experiment, imidacloprid applied to stream mesocosms as formulated Admire caused reductions in the total benthic insect population from three weekly 24-hour pulses of 17.60 µg/L (the time-weighted average concentration was not reported, but it would have been significantly lower that the level applied).[xxvii] The researchers saw a 69% decline in Ephemeroptera, Plecoptera, and Trichoptera (EPT) species pooled together and a 75% decline in Oligochaete density.[xxviii] EPT species abundance is commonly used to indicate water quality. Decomposition of leaf matter in coarse bags in the mesocosm also declined significantly, signaling a reduction in ecological functions.[xxix] Because this study reported only the concentration of the pulse dose, it cannot be directly compared to California surface water detections. Shifts in community structure as more sensitive species decline can affect freshwater aquatic ecosystems, altering trophic relationships and functional roles.

Chronic Sublethal Risks

Beyond the lethal effects of imidacloprid on many species, there are various sublethal effects that can impact aquatic invertebrates. The sublethal effects that have been observed include changes in feeding rates, change in individual size, downstream drift, impeded emergence, and declines in reproductive success. Each of these effects has consequences for individual fitness, and thus the resiliency of the individual and how well it can fulfill its ecological role. Shifts in individual health can manifest as changes at the community level that potentially leave more sensitive species behind as tolerant species outcompete them or survive the exposures. Adding uncertainty to assessing chronic risks, research suggests that neonicotinoids can bind irreversibly to receptors, so repeated low doses have the potential to cause harm and some effects can persist in individuals even after the contamination has ceased.[xxx] This preliminary analysis could not determine the potential scope of chronic exposure from available California water monitoring data. Yet, the frequency of detections in the dataset demonstrates a need to further explore chronic risks in order to avoid unreasonable harm.

<u>Reproductive impacts and larval survival</u>

Neonicotinoids can reduce the reproductive fitness of aquatic invertebrates and thus impact the success of their populations. The number of brood-carrying females declined in a long-term chronic study of *Gammarus roeseli*, indicating the potential for delayed reproductive effects from pulsed exposure.[2,xxxi] Adult emergence can also be impacted in certain species. A stream mesocosm study identified *Neureclipsis* spp. caddisflies as the most sensitive to three 12-hour

[2] Brood-carrying females declined in the last 3 weeks of a 70-day course of exposure to 12 µg/L weekly 12-hour pulses of imidacloprid.

pulses of 12 µg/L of imidacloprid, and also saw significant reductions in emergence among mayflies.[xxxii] Dipteran and ephemerid larvae declined more after the second and third imidacloprid pulses, indicating that they were unable to detoxify the compound in the seven days between pulses.[xxxiii] Each of these studies used 12-hour weekly pulses of 12 µg/L of imidacloprid that was then flushed from the system.[xxxiv] These results cannot be directly compared to Californian surface water monitoring because the time-weighted average was not reported (which would be lower and within the realm of California detections), but the maximum detection in the state was 12.7 µg/L, suggesting that while uncommon, these levels could be present in the environment.

Other reproductive effects can include impacts on emergence success and sex ratios. An experiment with chronic exposures to Admire (imidacloprid) found reduced *Epeorus* spp. and *Baetis* spp. mayfly nymph density (20 days of 0.8 µg/L) and *Epeorus* spp. male emergence (no male emergence in 0.25 and 0.8 µg/L), as well as reductions in male thorax lengths for emerged *Epeorus* from all treatment groups.[xxxv] California surface water exceeded 0.25 µg/L in 239 (51%) detections [65 (92%) in Santa Maria], and 0.8 µg/L in 98 (21%) detections [38 (54%) in Santa Maria] (Figure 3).[xxxvi] Over time, reductions in mating success and emergence of aquatic invertebrates could negatively impact their populations, as maintaining reproductive fitness is crucial to healthy populations.

Alterations in feeding behavior

Imidacloprid can also directly impact individual behavior in sublethal doses, with lasting effects that are not captured in short-term acute tests. Individual *Gammarus pulex* feeding rates that were not affected during a four-day constant exposure to imidacloprid (0.81, 2.7, and 9.0 µg/L) increased after the exposure ended, suggesting that compensational feeding could be a response to sublethal contamination.[xxxvii] Imidacloprid exceeded 0.81 µg/L in 98 (21%) California detections and in 38 (54%) Santa Maria detections. In experiments with *Epeorus longimanus* mayflies using the formulated product Admire (imidacloprid), researchers followed the treatment groups for four days after the 24-hour exposure, and noted that only the 0.1 µg/L group fully recovered to control feeding levels.[3,xxxviii] This suggests there may be ongoing sublethal effects after exposures that can be detected but are routinely missed in testing. Many toxicological studies do not follow sublethal effects after the exposure period ends, so researchers and regulators may not have crucial information about an individual's ability to recover. Furthermore, alterations in feeding behavior can cause broader ecosystem effects such as changing the rates of leaf litter breakdown that are crucial to aquatic ecology.

Incidence of downstream drift

Downstream drift of aquatic invertebrates is a common response to disturbance. While drift can be protective at an organism level, at a community level it can disrupt population structure and

[3] The other groups that did not recover to normal rates were 0.5, 1, 5, and 10 µg/L (all the mayflies in the 10 µg/L treatments died).

ecological functions. Experiments with mayflies, amphipods, and blackflies showed that imidacloprid, thiacloprid, and acetamiprid all triggered downstream drift within two hours of exposure.[4,xxxix] The short time frame after exposure suggests that pulses of contaminants in the field may be triggering drift. Imidacloprid triggered drift of *Baetis rhodani* mayflies at 1 µg/L, a level equaled or exceeded in 75 (16%) California detections and 30 (42%) Santa Maria detections (Figure 3).[xl] Another mesocosm experiment saw passive drift in Ephemeroptera and Orthocladiinae from three 12-hour pulses of 12 µg/L of imidacloprid that in some cases lasted after the imidacloprid was flushed out of the system.[xli] These studies suggest that drift has the potential to interrupt functional communities of invertebrates even after imidacloprid concentrations have declined.

Enhanced toxicity from other stressors

Environmental stressors such as food quality and temperature can impact the toxicity of compounds. Researchers provided *Daphnia magna* with algae of varying phosphorous content to assess the effect of lower food quality, finding that individuals consuming the lowest quality food also were affected by the lowest concentrations of imidacloprid.[xlii] While the doses were high and less field-relevant (mortality EC_{10} of 60 µg/L after 7 days of exposure), these results are worth noting here because they show that variable resource conditions in the natural world can affect the toxicity of compounds, and particularly that resource-stressed individuals may be more susceptible to pesticides. A study with *Isonychia bicolor* mayflies examined the effects of temperature on imidacloprid toxicity and found that increasing water temperature decreased the amount of time until impairment occurred.[xliii] For exposures to the EC_{50} (5.75 µg/L) at 15°C, impairment was evident at 60 hours and immobility at 76 hours, while at 24°C impairment occurred at 6 hours and immobility at 26 hours.[xliv] The authors noted that immobility occurred after other forms of impairment, suggesting that more sensitive endpoints would be more appropriate to quantify harm.[xlv] While detections have occurred above 5.75 µg/L in California, this experiment documents a trend at a higher level than commonly found in California's water samples.[xlvi]

Taken together, the lethal, sublethal, and indirect effects described in the literature show that even small concentrations of imidacloprid can trigger harmful effects. Concentrations of imidacloprid that can cause sublethal effects occur commonly in California (Figure 3). Although sublethal endpoints can be difficult to assess, their effects can still negatively affect functional community structures. Reductions in individual fitness can cascade into trophic disruptions and alterations in ecosystem services.

[4] Imidacloprid was tested on mayflies and amphipods, and significantly impacted both; thiacloprid significantly affected blackflies only; and acetamiprid significantly affected mayflies only.

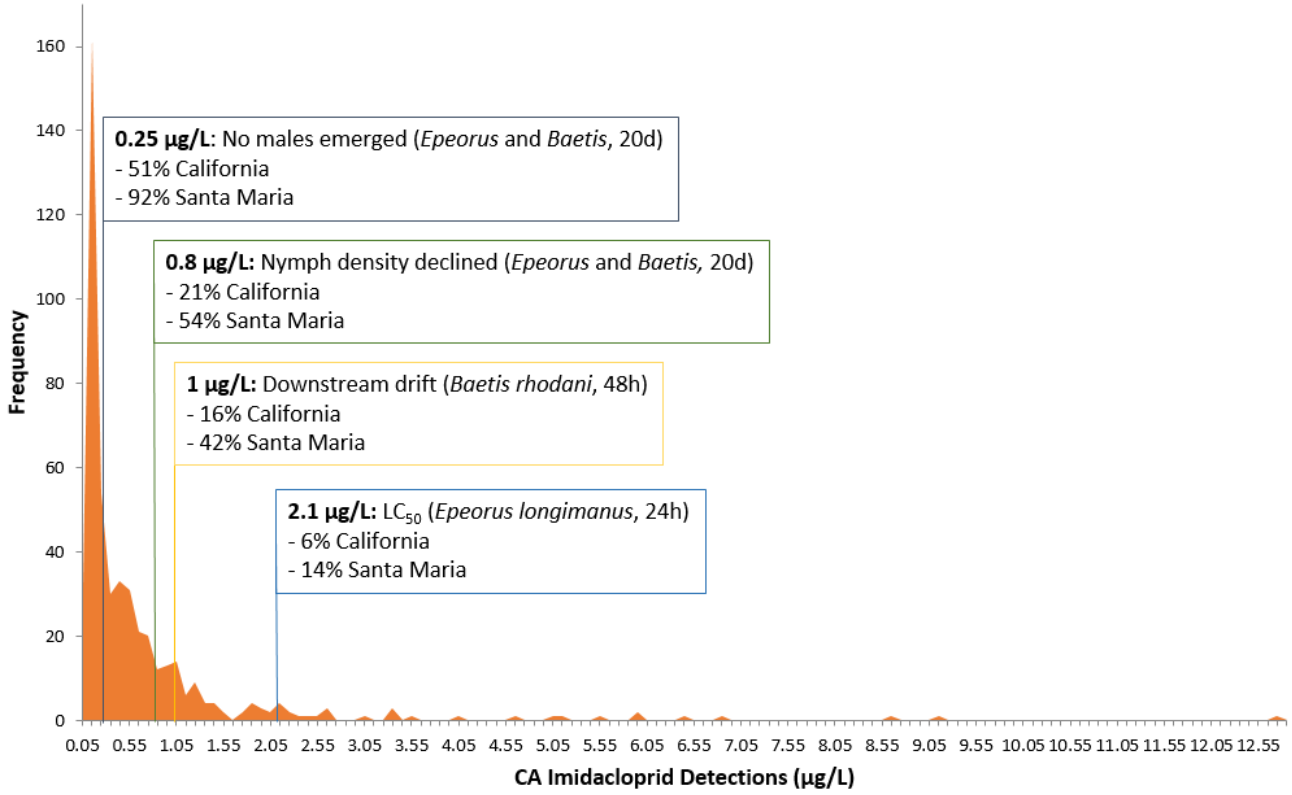

Figure 3: Histogram of imidacloprid surface water detections in California with the percentage of detections in California and Santa Maria that exceed levels shown to cause harm in mayfly species. No male *Epeorus* spp. or *Baetis* spp. emerged at 0.25 µg/L (20-day exposure to formulated Admire, Alexander et al. 2008), at 0.8 µg/L *Epeorus* spp. and *Baetis* spp. nymph density was reduced (20-day exposure to formulated Admire, Alexander et al. 2008), at 1 µg/L downstream drift of *Baetis rhodani* was initiated (Beketov & Liess 2008), and the 24h LC_{50} of *Epeorus longimanus* is 2.1 µg/L (Alexander et al. 2007).

Relative Toxicity of Other Nitroguanidine Neonicotinoids

There has been little research done to identify the relative toxicity of various neonicotinoids or to assess the potential for synergistic effects in aquatic invertebrates. One recent study sought to fill this data gap by comparing the chronic toxicity of imidacloprid, clothianidin, and thiamethoxam to *Chironomus dilutus* (Table 2).[xlvii] They calculated toxic equivalency factors based on 14-day LC_{50} values for clothianidin and thiamethoxam of 1.05 and 0.14, respectively (relative to imidacloprid values).[xlviii] Their results show that imidacloprid and clothianidin have similar toxicities, while thiamethoxam was less toxic—although thiamethoxam degrades into clothianidin. Another pair of studies evaluated the response of *Daphia magna* to Admire (imidacloprid) and Dantotsu (clothianidin).[xlix] In comparing the toxicity of the two chemicals, the studies noted wide variability in responses to the formulated products containing imidacloprid and clothianidin.[l]

Table 2. Chronic toxicity in *Chironomus dilutus* (µg/L)

	Chronic invertebrate aquatic benchmark	**14 day LC_{50}**	**40 day EC_{50} (emergence)**	**Shifts in sex ratio (40 day)**
Imidacloprid	1.05	1.52	0.39	0.17
Clothianidin	1.1	2.41	0.28	0.46
Thiamethoxam	none	23.60	4.13	3.60

Loss of Ecological Services

The toxicological tests outlined above show the wide variation in sensitivity among aquatic species. The most sensitive tend to be species in the orders Ephemeroptera and Trichoptera (mayflies and caddisflies). Both of these are extremely important to freshwater ecosystems. Mayflies are a commonly used water quality indicator because of their sensitivity to disturbance. Immature mayflies feed on detritus, diatoms, and algae, making them a valuable decomposer in aquatic systems.[li] Caddisflies are also good water quality indicators, partially because of their specific habitat requirements.[lii] They are crucial to aquatic food chains because they eat both plant and animal material, providing shredding services and making finer particulate organic matter available to other invertebrates.[liii]

Both mayflies and caddisflies are components of many fish, bird, bat, reptile, and amphibian diets, so any population-level disturbances can impact food resources for these species. Other species that feed on the predators of aquatic invertebrates can also be affected by changes in their abundance. Studies and reports have linked insectivorous bird declines to neonicotinoid use, as bird reproductive success may be affected by food availability.[liv] Populations of aquatic insects can be affected by neonicotinoid water contamination. Herbivorous insects that are a key food source for birds can be exposed to neonicotinoids through their presence in leaves and other parts of plants.[lv] Both of these exposure routes, terrestrial and aquatic, can reduce invertebrate abundance and limit food resources for birds and other insectivorous wildlife.

Water Quality Reference Values

EPA and other jurisdictions have established aquatic life benchmarks for imidacloprid and other neonicotinoids. Currently, the EPA imidacloprid acute aquatic invertebrate benchmark is 35 µg/L and the chronic benchmark is 1.05 µg/L. Canada, which collaborates with the United States on some pesticide risk assessments, has set their water quality guideline at a single value of 0.23 µg/L.[lvi] For reference to Californian detections, the Canadian guideline was exceeded in 246 (53%) detections, and 67 (94%) Santa Maria detections. The European Union, which relies more heavily on the precautionary principle while designing risk assessments, established a chronic guideline

of 0.067 µg/L, a level exceeded in 416 (89%) Californian detections and every Santa Maria detection.[lvii] The Netherlands set its chronic reference value even more conservatively at 0.0083 µg/L based on a wider analysis of toxicological information from a species sensitivity distribution approach.[lviii]

EPA benchmarks fail to protect sensitive species

In the case of imidacloprid, there is strong evidence that the EPA aquatic life benchmarks are under-protective of invertebrates. The EPA neonicotinoid risk assessments rely heavily on data for water fleas and midges, which do not represent the greater sensitivity of species like mayflies and caddisflies. Relying on these few less-sensitive test species does not ensure sufficient protection of aquatic invertebrates in instances where a compound's toxicity varies greatly between species, as it does for imidacloprid. A study that sought to quantify the proportion of crustacean species that would be adversely affected by pesticide contamination at water quality guidelines found that more than half of crustaceans could be impacted by imidacloprid at EPA benchmark levels.[lix]

Acute testing does not adequately simulate chronic risks

Water quality benchmarks that are based primarily on acute data may not provide adequate protection from chronic exposures. In a comparison of acute and chronic toxicity for several species, a study found that mayflies and caddisflies were the most acutely sensitive, while mayflies were the most sensitive to chronic exposures.[lx] The acute to chronic ratios the authors derived were all greater than ten.[lxi] Discrepancies between the acute and chronic sensitivity of species can lead to water quality benchmarks that are under-protective, especially for low-level chronic exposures. The recent Dutch review also identified wide variation in sensitivity both between taxa and species, as well as high acute-to-chronic ratios which implied that the typical Dutch 10x safety factor would not be protective for translating acute results into chronic values.[lxii] The discrepancies between acute testing and chronic effects for imidacloprid and other nitroguanidine neonicotinoids mean that there is no straightforward way to predict what percentage of a species' LC_{50} will cause chronic effects. In designing and reviewing risk assessment protocols, regulators must ensure that chronic testing is adequate to identify lasting effects after the exposure and that gaps between acute and chronic tests are considered.

Recommendations

This preliminary review suggests that the current aquatic life benchmarks for imidacloprid may be under-protective of sensitive species, especially those in the orders Ephemeroptera and Trichoptera. As such, current contamination of California's surface water could be causing unreasonable adverse effects to aquatic invertebrate populations. Effects of repeated, chronic exposures to neonicotinoids are a major area of uncertainty in risk assessments. Imidacloprid's large acute-to-chronic ratio introduces additional uncertainty into risk assessments that are based primarily on acute data. Given the critical ecological roles of mayflies and caddisflies, some of the most sensitive aquatic insects, imidacloprid water quality benchmarks must be reviewed and updated to ensure they are protective of sensitive species.

Additional research is needed to quantify and further investigate the impacts of imidacloprid and other nitroguanidine neonicotinoids on California's aquatic life. As the use of these compounds is continuing to rise, now is the time to take action to review potential risks, update aquatic life benchmarks, and identify and implement risk mitigation strategies. Xerces recommends CDPR take the following actions:

1. **Develop an action plan and timeline for reviewing nitroguanidine neonicotinoid aquatic toxicity.** We recommend that CDPR work to develop a plan and timeline for reviewing the aquatic impacts of the nitroguanidine neonicotinoids. A data synthesis and analysis (similar to the one prepared for fipronil[lxiii]) may help CDPR quantify the risks and define regulatory objectives.

2. **Create interim aquatic life benchmarks.** While there are uncertainties in quantifying the exposures that aquatic ecosystems face and the prevalence of acute versus chronic effects, our overall conclusion is that the current aquatic life benchmarks for imidacloprid are out of date. CDPR should create interim aquatic life benchmarks for all the nitroguanidine neonicotinoids if their preliminary review confirms our initial conclusions that the EPA benchmarks are under-protective.

3. **Require risk mitigation strategies.** Mitigation measures, including buffer strips and reductions in use or application rate, should be required to reduce surface water loading and protect sensitive aquatic ecosystems.

4. **Gather more data on surface water contamination.** California should bolster its surface water sampling efforts for neonicotinoid pesticides, especially the nitroguanidine group. Monitoring should particularly target storm events, irrigation returns, and urban areas, including municipal wastewater treatment plants. Including passive monitors could provide valuable additional information along with current snapshot monitoring methods.

5. **Strengthen pesticide use reporting requirements.** California's pesticide use reporting system is among the most robust in the country. Still, gaps in the system, such as the lack of reporting on use of insecticide-coated seeds or insecticide-impregnated outdoor building materials make it difficult to confidently assess pesticide sources and to identify the most effective mitigation measures. Requiring reporting of these unregistered uses would improve accuracy of California's pesticide use reporting system.

6. **Fund additional research on aquatic invertebrate toxicology.** Aquatic life benchmarks are limited in part by their reliance on a few key species selected by registrants to meet EPA's relatively limited aquatic toxicity testing requirements. The toxicological literature on imidacloprid alone demonstrates the wide range of sensitivity among aquatic invertebrates, even within the same taxa. Further toxicological information is lacking for the other nitroguanidines. Additional research would inform regulation and fill critical data gaps to ensure sufficient protection for aquatic species. Confounding factors including mixtures of pesticides and other stressors that invertebrates encounter in the real world should also be better represented in toxicity testing.

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SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 33, No. 3, pp. 621–631, 2014

Printed in the USA

IMPACT OF IMIDACLOPRID ON *DAPHNIA MAGNA* UNDER DIFFERENT FOOD QUALITY REGIMES

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(Submitted 12 July 2013; Returned for Revision 3 September 2013; Accepted 14 November 2013)

Abstract: Aquatic ecosystems are characterized by fluctuating conditions that have direct effects on aquatic communities but also indirect influences such as changing the toxicity of chemicals. Because the effect of food quality on pesticide toxicity has rarely been studied, in the present study *Daphnia magna* juveniles supplied with 4 different food quality levels were exposed to a range of imidacloprid concentrations for 21 d. Food quality was expressed as carbon:phosphorus ratios of algae *Pseudokirchneriella subcapitata* (C:P 35, C:P 240, C:P 400, and C:P 1300). Survival, growth rates, and reproduction of *D. magna* were monitored, and the combined effects of imidacloprid exposure and the phosphorus content of algae were analyzed. A stronger effect on survival was observed at the P-deficient diet (C:P 1300), confirmed by lower 10% effect concentration (EC10) values at days 7, 9, 15, and 21 compared with diets with higher phosphorus contents. Similarly, the growth rate was reduced when *D. magna* were supplied with algae of low phosphorus content at imidacloprid exposure conditions. The highest reproductive output was observed for *D. magna* fed the optimal phosphorus diet (C:P 240), both at control and exposed conditions. Poor food quality increased the sensitivity of nontarget species to pesticide exposure, potentially leading to an underestimation of adverse effects on aquatic communities in the field. *Environ Toxicol Chem* 2014;33:621–631.

Keywords: *Daphnia magna* Imidacloprid Algae Food quality Toxicity

INTRODUCTION

The toxicity of pesticides to aquatic invertebrate species is commonly assessed based on laboratory tests under controlled conditions, such as temperature, photoperiod, and standardized feeding regime [1]. Contrary to the laboratory setting, however, nature is characterized by fluctuating environmental conditions. Apart from physical conditions such as temperature, pH, and salinity, the ecological conditions for aquatic species, such as quantity and quality of food, also vary. The availability of phosphorus is an important factor controlling productivity of phytoplankton algae, which are primary producers in aquatic ecosystems. Aquatic algae in turn serve as a food source for primary consumers represented by zooplankton [2]. Aquatic invertebrates of the subphylum Cladocera constitute a dominant group of zooplankton mainly in freshwater ecosystems. The most well-known group is the daphnids, among which *Daphnia magna* Straus is a common species used in standard toxicity testing [1,3].

Literature mostly focuses on either the sensitivity of aquatic invertebrates to algal nutritional levels or on chemically induced effects. To date, the toxicity of only a few chemicals — including 3,4-dichloroaniline, fenoxycarb, and chlorpyrifos [4], endosulfan [5,6], and esfenvalerate [5]—to aquatic cladoceran species supplied with different algae cell concentrations (estimated as number of cells in 1 mL) has been studied. Organisms are sensitive not only to food quantity, however, but also to food quality. The elemental food composition (estimated as C:P ratio) is an important factor influencing the performance of cladocerans [7,8]. Sensitivity of daphnids to nutritional levels expressed as algae phosphorus content was described at the physiological level (growth rate [7,9–11], reproduction [9,11]) and at the biochemical level (calcium balance [12]). Yet the effect of the algal phosphorus concentration on the toxicity of chemicals to daphnids has been studied for only a few compounds: herbicide WeatherMAX Roundup (referred as concentration of glyphosate [13]) and antibiotic fluoxetine [14]. However, no study focused on the combined effects of nutritional quality of algae and neonicotinoid insecticides on *D. magna*. In the present study, we focus on the combined effects of insecticide imidacloprid and algae nutritional levels to *D. magna*.

Imidacloprid belongs to the group of neonicotinoid insecticides that block the nicotinergic neuronal pathway in invertebrates. This blockage of the nicotinic receptor in the neurons leads to the accumulation of the neurotransmitter acetylcholine [15], resulting in paralysis of the insect, and consequently death. The biochemical activity of imidacloprid in insects and other arthropods appears to be mainly agonistic [15]. Roessink et al. [16] reported a higher acute toxicity of imidacloprid to mayfly (Ephemeroptera) and caddisfly (Trichoptera) species compared with macrocrustaceans and insect species belonging to the orders Hemiptera, Megaloptera, and Diptera. The median effect concentration (EC50) of imidacloprid for microcrustacean *D. magna* is 85 mg/L (48-h test, immobility endpoint [17]), which is considerably higher than median lethal concentration values for mayfly species of 26.3 μg/L (*Cloeon dipterum*, 96-h test [16]). Despite its low acute toxicity to daphnids, in the semifield conditions imidacloprid caused significant reduction in the abundance of aquatic faunal assemblages [18,19]. This finding suggests a high potential for imidacloprid to cause adverse effects on nontarget species in the realistic environment.

All Supplemental Data may be found in the online version of this article.
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Published online 28 November 2013 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.2472

The aim of the present study was to quantify the effect of the insecticide imidacloprid at a range of nutritional levels (defined as algae C:P ratios) on *D. magna* (subphylum Crustacea, suborder Cladocera). In the present study, toxicological endpoints used were survival, growth, and reproduction, all relevant for population growth. To mimic the differences in food quality, 4 algal phosphorus levels were tested. We hypothesized that exposure to a range of imidacloprid concentrations at P-deficient conditions results in severe effects, such as reduced reproductive output, survival, and growth rate of *D. magna* compared with P-high conditions.

MATERIALS AND METHODS

Test species and culture conditions

Juveniles of *D. magna* were obtained from the laboratory culture of the National Institute of Public Health and the Environment (RIVM, Bilthoven, The Netherlands). Parent animals were cultured under standard laboratory conditions at 20 °C, and a 16:8-h light:dark photoperiod. Adult *D. magna* were raised in 1-L plastic jars in M4 medium described in Elendt [20]. The culture medium was renewed twice per week. Daphnids were fed with the algal cells *Pseudokirchneriella subcapitata*, which were cultured in 2-L bottles in a Woods-Hole medium. The culture medium was replaced once per week. Algae were centrifuged at 7500 RCF in 50-mL falcon tubes, suspended in M4 medium, and fed to *D. magna*.

Preparing different phosphorus levels

The 4 C:P levels of algae were selected for the experiment based on the analysis of literature reporting C:P levels limiting performance of daphnids [10,11,13,14,21]. To study the effect of the algal phosphorus content on *D. magna* responses, P-free Woods-Hole medium was prepared and divided between 4 2-L bottles. Four different concentrations of K_2HPO_4 and algae (*P. subcapitata*) were subsequently added to the different P levels. Phosphorus concentration in the P-optimal treatment (C:P 240) is the same as in the Woods-Hole medium used in the standard laboratory procedure for *P. subcapitata*.

The algae were adapted to these 4 different phosphorus conditions during 7 d to obtain algae cultures of different nutritional levels at the stationary growth phase. This procedure allowed for sufficient algal biomass to initiate the nutritional experiment. Algae cultures containing the 4 different phosphorus concentrations were kept in individual 2-L bottles with constant aeration and a 24-h light period. Measurement of carbon and phosphorus in algae cultures were made after 7 d adaptation. Table 1 depicts the nutritional levels tested during the experiment (C:P 35, C:P 240, C:P 400, C:P 1300).

For the determination of the organic carbon content, the algae culture was filtered through glass-fiber 45-μm pore size filters (Whatman GF/C). Dissolved organic carbon concentrations were determined using non-dispersive infrared analysis. Total organic carbon concentrations were quantified by high temperature combustion/direct injection. The concentration of dissolved and total phosphorus in the algae culture was determined according to the OMEGAM laboratory NEN 6663 (Amsterdam, the Netherlands). The concentration of particulate phosphorus was determined as the difference between total and dissolved phosphorus concentrations. Because the concentration of dissolved phosphorus was below the limit of detection at 4 treatments, half of the detection limit was used to calculate the concentration of particulate phosphorus.

Phosphate is taken up by algae quickly, leading to depletion in extracellular phosphorus concentration [22]. At the same time, algal cell density and internal phosphorus concentration increase [22]. For this reason, after 7 d, we found similar concentrations of external dissolved phosphorus in 4 algae cultures (<0.05 mg/L), even if the concentration of total phosphorus differed (Table 1). Total phosphorus in turn includes all forms of phosphorus: dissolved and particulate phosphorus. In the present study, particulate phosphorus means phosphorus bound to organic matter. Therefore, after 7 d, inorganic phosphorus was taken up by the algae and transformed to particulate phosphorus. Before being fed to daphnids, algae cultures were centrifuged at 7500 RCF and only the particulate fraction (algae cells dissolved in M4 medium) was used during the experiment.

Test setup

The *D. magna* neonates less than 24 h old were exposed for 21 d. The 6 different concentrations of imidacloprid and a blank at 4 algal phosphorus levels were prepared. Each experimental treatment consisted of 3 replicates with 5 neonates in each replicate chamber (this resulted in $7 \times 4 \times 3 = 84$ test chambers). The experiment was performed in 100-mL test chambers, with 50 mL media in each test chamber. The M4 media containing a range of imidacloprid concentrations were transferred to the test chambers. Algae containing 4 different P concentrations and *D. magna* neonates were subsequently added to the test chambers. All experiments were conducted in a 16:8-h light:dark photoperiod at 20 °C.

Test chambers were not aerated during the experiment. The M4 media containing imidacloprid were renewed every 3 d to ensure continuous exposure to imidacloprid and also to suppress bacteria and fungi growth. Feeding with algae cultured at 4 phosphorus levels was done on the same day as the medium renewal. Feeding with 4 different diets was normalized based on the amount of total organic carbon (0.05 mg C/Daphnia) for each of the 4 diets. Temperature, pH, oxygen saturation, and water hardness were recorded 3 times during the experiment at the time of medium renewal and in freshly prepared medium.

Preparing different imidacloprid concentrations

The concentration range was chosen based on reported acute and chronic toxicity data for imidacloprid: chronic 21-d

Table 1. Algae culture conditions and C:P levels used in the nutritional experiments with *Daphnia magna*

Reference	K_2HPO_4 addition (mg/L)	Dissolved P (mg/L)	Total P (mg/L)	Particulate P (mg/L)	DOC (mg/L)	TOC (mg/L)	Molar C:P ratio
P-high	16.80	<0.05	3.80	3.78	44.21	96.70	35
P-optimum	8.40	<0.05	1.00	0.98	32.79	87.50	240
P-low	2.80	<0.05	0.38	0.35	37.76	51.70	400
P-very low	0.28	<0.05	0.09	0.07	31.13	19.90	1300

Dissolved P = concentration of dissolved phosphorus; Total P = concentration of total phosphorus; Particulate P = concentration of particulate phosphorus (bound to algae); DOC = dissolved organic carbon concentration; TOC = total organic carbon concentration.

no-observed-effect concentration (NOEC) for *D. magna* with endpoint of reproduction, 1.8 mg/L; acute 48-h EC50 for *D. magna* with an endpoint of immobility, 85 mg/L; EC50 for *P. subcapitata* algae, >100 mg/L [17]. Nominal concentrations were 1.8 mg/L, 25 mg/L, 45 mg/L, 60 mg/L, 85 mg/L, and 130 mg/L. The 6 different imidacloprid concentrations were prepared by diluting an imidacloprid stock solution in M4 media. The concentration of the stock solution was 400 mg/L, which is lower than the water solubility limit of imidacloprid (610 mg/L), so no solvent was added [23]. The purity of the test substance as reported by the provider Sigma Aldrich Chemie BV was 99.7%.

Analytical measurements

Chemical analysis was performed for 3 imidacloprid concentrations (45 mg/L, 85 mg/L, and 130 mg/L; 1 replicate for each treatment) in freshly prepared medium and old medium (after 3 d exposure) in samples selected randomly in time. At least 2 measurements in fresh and old medium at 3 concentrations were made. Chemical analysis was performed using a 3200 Q Trap liquid chromatography–tandem mass spectrometer (LC/MS/MS; Applied Biosystems). External standard calibration was done using 6 calibration points (1 μg/L, 10 μg/L, 20 μg/L, 50 μg/L, 70 μg/L, and 120 μg/L) plus a blank. The limit of quantification was 0.01 μg/L. Samples were diluted before the analysis in the proportion 1:1000. Measured concentrations were 44.6 ± 3.1 mg/L; 94 ± 2.5 mg/L; and 158.0 ± 6.5 mg/L, respectively. Actual time-weighted mean concentrations of 2.0 mg/L, 27.6 mg/L, and 66.3 mg/L were estimated assuming similar deviation from the nominal concentrations (average 10.5%).

Because the concentration of imidacloprid was expected to decline slightly over the period of 3 d between medium renewals (half life time [DT50] in microcosm = 14.8 d [17]), the time-weighted mean concentration was calculated as follows:

$$TWConc = \frac{Conc\,0 - Conc\,1}{Ln(Conc\,0) - Ln(Conc\,1)} \times time \qquad (1)$$

where *TWConc* is the time-weighed concentration for the renewal period; *time* is the number of days in the renewal period; *Conc 0* is the measured concentration of imidacloprid at the start of the renewal period; and *Conc 1* is the measured concentration of imidacloprid at the end of the renewal period [1]. The average concentration per treatment was used in the statistical analysis [23].

Estimated endpoints

Survival and reproduction of parent animals was estimated daily during the 21-d experiment. Survival was calculated as the proportion of live animals. Animals were considered dead when no movement of antennae/appendages and no swimming behavior were observed. Offspring produced each day were counted daily and transferred to a new series of test chambers containing varying imidacloprid/phosphorus concentrations. Survival of juveniles was also recorded. The number of juveniles produced daily was divided by the number of live adults present. The net reproductive rate (R_0) was determined as the cumulative number of juveniles per adult produced in 21 d. Average reproduction per day was determined as average number of juveniles produced per adult per day. Average values for R_0 and average reproduction per day between the 3 replicates and standard deviation were calculated.

Body length of the parent animals was measured every 2 d under a microscope STEM SR Zeiss fitted with a micrometer eyepiece. At least 2 randomly selected live parent animals were measured from each test replicate (resulting in 6 size measurements per treatment, every 2 d). Live animals were placed in a petri dish, and the volume of water around the animals was reduced with a pipette to immobilize the animal, and then the animal was measured. *D. magna* body length was defined as the distance from the most posterior point on the head to the junction of the carapace with the tail spine [24].

Growth rate was estimated using 2 different methods. The somatic growth rate (SGR) provided information on body length increment per day. Additionally, the Von Bertalanffy growth model was fitted that is widely applied to study effects of various stressors on growth of animals.

The somatic growth rate (SGR) was calculated based on the formula

$$SGR = \frac{\ln(L2) - \ln(L1)}{time} \qquad (2)$$

where L_1 is the average measured length of neonates at the day of the initiation of the experiment, L_2 is the average measured length after 21 d, and time is the duration of the experiment (21 d). The average SGR per treatment and the standard error of the mean was used for statistical analysis. Additionally the Von Bertalanffy growth model was applied to estimate growth rates for *D. magna*, using mean length at time data

$$L_t = L_{\max}(1 + e^{-K(t-t0)}) \qquad (3)$$

where L_t is the body length of *D. magna* at time t; L_{max} is the length that can be reached at an infinite time, or a maximum potential length that can be reached at given conditions; K is the growth rate; t is the time (days); and t_0 is the theoretical age at $L_t = 0$. The parameters of the Von Bertalanffy growth model were obtained by constructing a Ford-Walford plot introduced by Ford [25] and Walford [26]. A Von Bertalanffy growth model was constructed for the control and the imidacloprid concentrations 2.0 mg/L and 27.6 mg/L, because animals at these treatments survived for 21 d, allowing comparison between food regimes. Mean length at time t (L_t) was then plotted versus L_t predicted by the Von Bertalanffy growth model, and the R^2 coefficient was estimated.

Data treatment

Two-way analysis of variance (ANOVA; 95% confidence interval) with replicates was performed to test the effect of 2 independent factors (imidacloprid and phosphorus concentrations) and the interaction between them on *D. magna* body length at days 3, 9, 15, and 21, as well as net reproductive rate (R_0). For the two-way ANOVA, analysis of body size measurements at days 3, 9, 15, and 21 at control conditions (C0), imidacloprid concentrations of 2.0 mg/L (C1), 27.6 mg/L (C2), and 44.6 ± 3.1 mg/L (C3) were used. Relationships between *D. magna* somatic growth rate and C:P ratio at different imidacloprid exposure conditions were analyzed with simple linear regression. A slope, intercept, and R^2 were derived for each imidacloprid concentration.

Dose–response relationships between *D. magna* survival and imidacloprid concentration were analyzed by plotting *D. magna* survival at days 5, 7, 9, 15, and 21 (for C:P 35, C:P 240, C:P 400, and C:P 1300) versus the corresponding imidacloprid

concentration (log transformed). GraphPad Software was used to obtain a logistic model following the equation

$$Y = \frac{(\max + \min)}{1 + \left(\frac{x}{EC50}\right)^{-H}} + \min \quad (4)$$

where *min* is the minimum response, *max* is the maximum response, *x* is the concentration of imidacloprid, and *EC50* is the concentration of imidacloprid that causes 50% of *D. magna* mortality, *H* is the Hill slope.

The EC10 values were calculated using the following equation

$$EC_F = \left(\frac{F}{100 - F}\right)^{1/H^*} EC50 \quad (5)$$

where EC_F is the EC10, H is the Hill Slope value, and F is 10.

The EC10 values were derived for 5, 7, 9, 15, and 21 d of exposure to compare effects of imidacloprid on *D. magna* fed with 4 diets at different ages. The EC50 values between 4 food regimes were compared using an extra sum-of-squares *F*-test.

Time-to-event analysis was applied to evaluate the median effective time that causes 50% mortality of *D. magna* (ET50) for 6 imidacloprid concentrations used in the experiment using the empirical model described in Sánchez-Bayo [27]. Calculations were made for each food quality regime. The ET50 (y) was calculated using the hyperbolic model

$$\mathrm{y} = a \times \mathrm{x}^{-b} \quad (6)$$

where *y* is the ET50 value, *x* is the concentration of imidacloprid.

To obtain coefficients *a* and *b*, time to 50% mortality of *D. magna* obtained in the experiment for days 5, 7, 9, 15, and 21 was plotted versus imidacloprid concentrations and fitted with linear regression [27]

$$\mathrm{Ln(ET50)} = \mathrm{a}' - \mathrm{b} \times \mathrm{ln(C)}, \mathrm{a}' = \mathrm{ln(a)} \quad (7)$$

Because reliable confidence intervals could not be derived for EC50 at C:P 1300 (days 15 and 21), it was excluded from the analysis. To validate the model, EC50 values were extrapolated using the hyperbolic model for days 5, 7, 9, 15, and 21. Estimated versus predicted EC50 values were analyzed with linear regression.

RESULTS

Effects of imidacloprid and phosphorus on the survival of Daphnia magna

Mortality increased with increasing imidacloprid concentrations in the water. Adverse effects on the survival of daphnids were shown to increase with decreasing food quality. Survival of *D. magna* fed with the low-phosphorus diet, C:P 1300, at an imidacloprid concentration of 44.6 ± 3.1 mg/L reached 0% at day 14, whereas at other diets it remained at 5% to 15% during the 21-d experiment (Figure 1).

Survival of *D. magna* at days 5, 7, 9, 15, and 21 can be found in the Supplemental Data, Tables S1 and S2, along with comparisons between all pairs of EC50 values at 4 food quality regimes. No trend was seen in EC50 values between food regimes derived for days 5, 7, and 9, whereas EC10 values were lower at C:P 1300 compared with other diets starting from day 7 (Table 2). Respective Hill slope values were also lower at C:P 1300 at days 7 through 21 than with other diets (Table 2). A more negative slope indicates a steeper curve and faster response to changing exposure conditions. At days 15 and 21, both EC50 and EC10 values were lower with a P-deficient diet, C:P 1300 (Table 2). However, a comparison between EC10 and EC50 between C:P 1300 and other diets for 15 and 21 d was not possible because the 95% confidence intervals for these parameters at C:P 1300 could not be fitted.

Highest absolute slope value (*b*) and intercept (*a*) between the time to 50% mortality and imidacloprid concentration was found for P-optimal conditions (C:P 240) and lowest for P-deficient conditions (C:P 400; Figure 2 and Table 3).

For an imidacloprid concentration of 2 mg/L, the highest predicted ET50 was found at C:P 240 (Figure 3; Supplemental Data, Table S3). At the imidacloprid concentrations 27.6 mg/L to 158 mg/L, the highest ET50 was derived at C:P 400 and the lowest at C:P 240 (Figure 3; Supplemental Data, Table S3). A relatively good fit was obtained between the estimated and predicted in the hyperbolic model EC50 values ($R^2 = 0.62$; Supplemental Data, Table S4 and Figure S1).

Effects on growth rate

The Von Bertalanffy growth model fitted with the experimental mean length at time data for *D. magna* showed that lowest values for maximum hypothetical length (L_{max}) were reached at P-deficient diet C:P 1300, at control and imidacloprid exposure conditions (Table 4). Body lengths of *D. magna* over the 21-d experiment at 4 diets can be found in the Supplementary Data, Figure S2. At control conditions, the highest *K* was observed at C:P 35; however, larger L_{max} was attained at C:P 240. At imidacloprid conditions, the highest L_{max} was observed at C:P 35 (Table 4 and Figure 4).

At all diets, imidacloprid induced a negative effect on the *D. magna* SGR (Figure 5). However, differences in SGR between the control and the lowest imidacloprid concentration of 2 mg/L were negligible. A negative regression slope between SGR and log C:P was found for the control and imidacloprid exposure conditions (Table 5). With increasing C:P level (lowering P content of algae), SGR decreased. The absolute slope value (*b*) was larger at higher imidacloprid concentrations of 27.6 mg/L to 44.6 mg/L (Table 5).

Results of the 2-way ANOVA showed significant effects of phosphorus, imidacloprid, and their interaction on the body length of *D. magna* at ages 3 d and 21 d (Table 6).

Effects on reproduction

Production of juveniles was observed at control exposure conditions and at imidacloprid concentrations of 2.0 mg/L. No reproduction was observed at the higher imidacloprid concentrations. Two-way ANOVA revealed a significant effect of phosphorus and imidacloprid on the reproductive output R_0 (Table 6). The effect of imidacloprid–phosphorus interaction was not significant ($p > 0.05$; Table 6). However, the mean net reproductive rate (R_0) was highest at C:P 240 (optimal conditions) compared with other diets at control and imidacloprid concentrations of 2 mg/L (Figure 6A, B). The lowest mean reproductive output, R_0, was observed for the P-deficient diet both at control and imidacloprid exposure conditions (C:P 1300) (Figure 6A, B). Average reproduction per day did not differ significantly for *D. magna* fed with different diets at

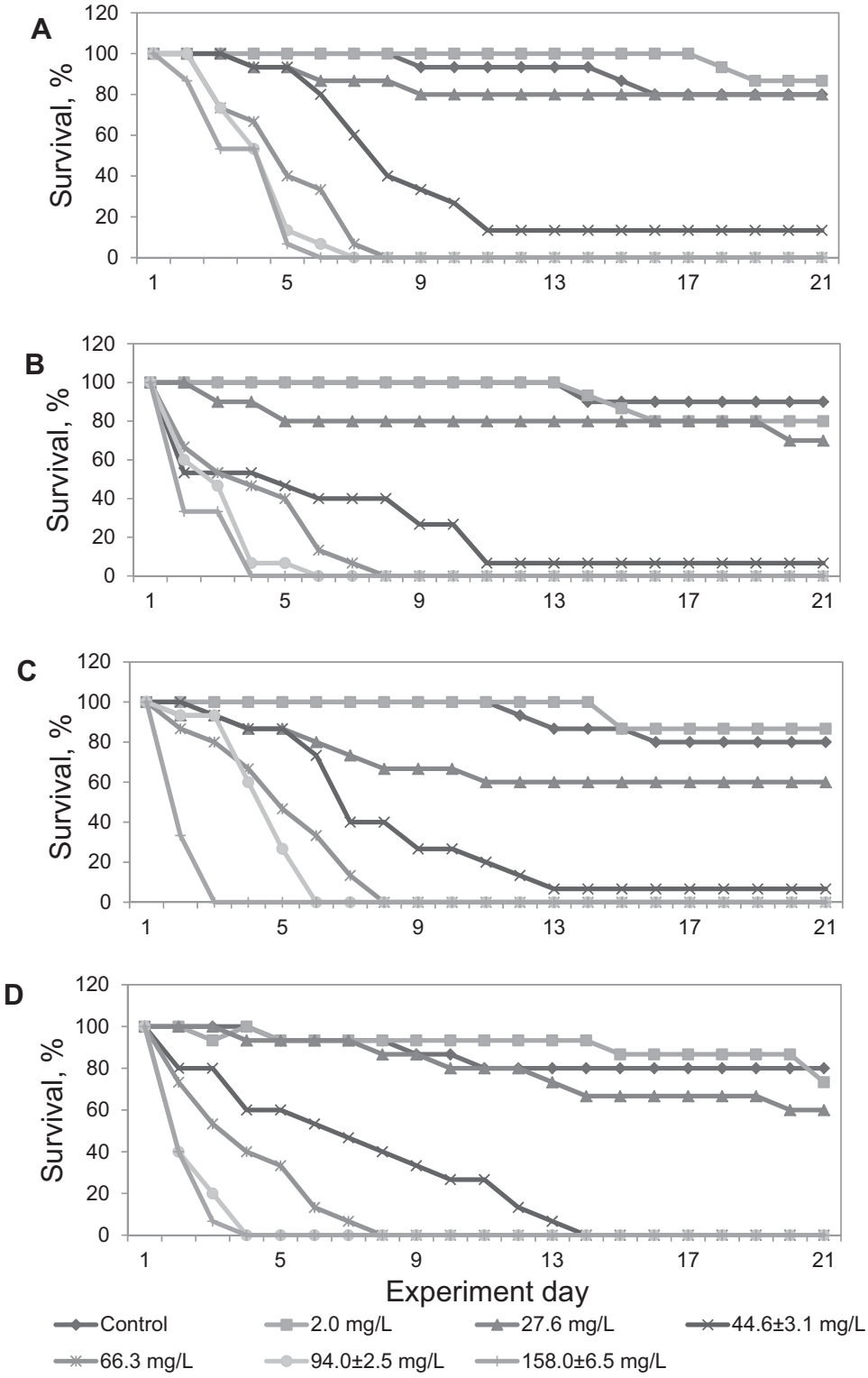

Figure 1. Effect of imidacloprid on the survival of *Daphnia magna* supplied with different food regimes (mean survival at C:P 35 [**A**], C:P 240 [**B**], C:P 400 [**C**], and C:P 1300 [**D**]).

control conditions and imidacloprid concentrations ($p > 0.05$) (Figure 6C, D).

DISCUSSION

Varying environmental conditions, including nutrient concentrations, are unavoidable characteristics of natural aquatic ecosystems. Within agricultural areas, concentrations of nutrients in surface waters vary significantly depending on local farming activities, fertilizer application, and the amount of precipitation. However, in ecological effect predictions the variable environmental conditions are hardly considered. Earlier research demonstrated that differences in toxicity between laboratory and field exposures range as a factor of 1.2 to 10 for the nutritional state [28]. When subjected to multiple stressors in a natural aquatic environment, organisms are more prone to diet change or

Table 2. Effective concentrations causing 50% and 10% mortality (EC50 and EC10, respectively) for 5 d, 7 d, 9 d, 15 d, and 21 d for *Daphnia magna* exposed to imidacloprid at 4 food regimes (endpoint survival)[a]

	C:P 35	C:P 240	C:P 400	C:P 1300
Day 5				
EC50	61.72 (56.05–67.96)	51.88 (37.63–71.53)	71.41 (54.14–94.19)	54.97 (44.43–68.01)
EC10	80.83 (73.03–88.63)	144.64 (97.38–191.89)	141.92 (102.12–181.72)	95.11 (74.71–115.51)
H	–8.15	–2.14	–3.20	–4.01
df	17	17	17	17
R^2	0.94	0.94	0.90	0.89
Day 7				
EC50	47.69 (44.74–50.84)	40.17 (35.00–46.11)	39.53 (34.10–45.81)	44.55 (40.13–49.46)
EC10	67.66 (63.33–71.99)	68.65 (59.16–78.14)	79.69 (67.89–91.49)	60.10 (53.81–66.40)
H	–6.28	–4.10	–3.13	–7.34
df	17	17	17	17
R^2	0.98	0.95	0.96	0.93
Day 9				
EC50	39.07 (35.61–44.77)	37.36 (32.70–42.70)	33.87 (29.88–38.40)	42 (36.71–48.04)
EC10	59.85 (52.98–66.71)	55.96 (48.47–63.45)	60.06 (52.50–67.61)	54.16 (46.86–61.47)
H	–5.43	–5.44	–3.84	–8.64
df	17	17	17	17
R^2	0.96	0.95	0.96	0.93
Day 15				
EC50	35.14 (31.26–39.51)	34.76 (28.78–41.98)	30.65 (26.67–35.22)	28.35 (no CI)
EC10	47.16 (52.69–41.62)	43.28 (35.06–51.50)	42.56 (36.62–48.50)	29.63 (no CI)
H	–7.47	–10.02	–6.69	–49.61
df	17	16	17	17
R^2	0.97	0.94	0.93	0.98
Day 21				
EC50	37.24 (31.83–43.58)	34.12 (29.26–39.78)	31.1 (26.89–35.98)	28.38 (no CI)
EC10	47.16 (39.72–54.60)	43.40 (36.71–50.09)	42.85 (36.59–49.11)	29.62 (no CI)
H	–9.30	–9.13	–6.86	–51.36
df	17	17	17	17
R^2	0.96	0.95	0.93	0.96

[a]95% Confidence intervals (CIs) shown in parentheses.
H = hillslope value; *df* = degrees of freedom; no CI = confidence intervals could not be fitted (very wide).

food deficiency [29]. Hence, extrapolation of results obtained in the laboratory to the field deals with high uncertainty [30].

Effects on survival

Lower EC10 values at days 7 through 21 were found at the P-deficient diet, C:P 1300, suggesting a greater effect of imidacloprid on *D. magna* survival at poor nutrient diet.

Results of time-to-event analysis indicated that *D. magna* supplied with P-optimal food had the highest absolute value of regression slope (*b*) between time to 50% mortality and imidacloprid concentration (Table 3 and Figure 3). Therefore, the gradient of response to imidacloprid at C:P 240 was larger compared with other diets (Table 3 and Figure 3). As a result, at C:P 240 *D. magna* ET50 estimated in a hyperbolic model (Equation 6) was lower compared with other food regimes. On the contrary, at lower phosphorus conditions of C:P 400, higher ET50 values were derived compared with other diets. This result was found for high imidacloprid concentrations of 27.6 to 158 mg/L. Reduced growth and reproduction at P-low conditions was possibly compensated for by larger time to mortality when exposed to high imidacloprid concentrations.

However, at the lowest imidacloprid concentration of 2 mg/L, the longest time-to-mortality was found for an optimal diet to be C:P 240. At the P-optimal treatment, the highest reproductive output was obtained at the control and the imidacloprid exposure of 2 mg/L (Figure 6). Therefore, when exposed to a low imidacloprid concentration, close to the NOEC (1.8 mg/L [17]), the optimal feeding regime C:P 240 was found to be the most favorable for reproduction and life duration of *D. magna*.

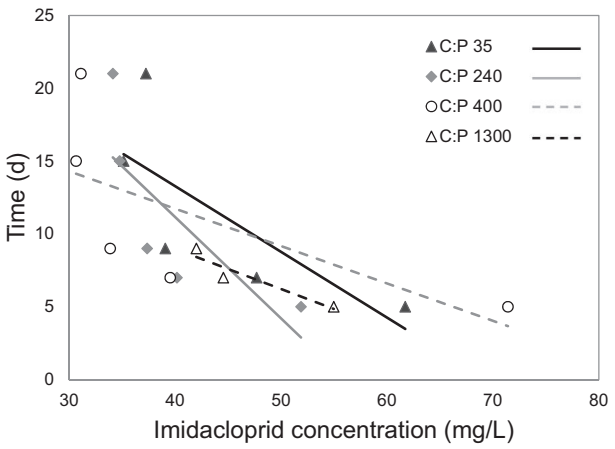

Figure 2. Time to 50% mortality of *Daphnia magna* plotted versus imidalcoprid concentration.

Effects on growth rate

Negative effect of low phosphorus content on the growth rate of *D. magna* was found at P-deficient conditions based on the

Table 3. Parameters of the regression equation, Equation 7, fitted to the data shown in Figure 2

C:P ratio	Intercept (*a*)	Slope (*b*)	R^2	*n*
35	31.316	–0.451	0.57	5
240	38.981	–0.696	0.59	5
400	21.998	–0.257	0.46	5
1300	19.958	–0.275	0.89	3

ET50 = median time to 50% effect.

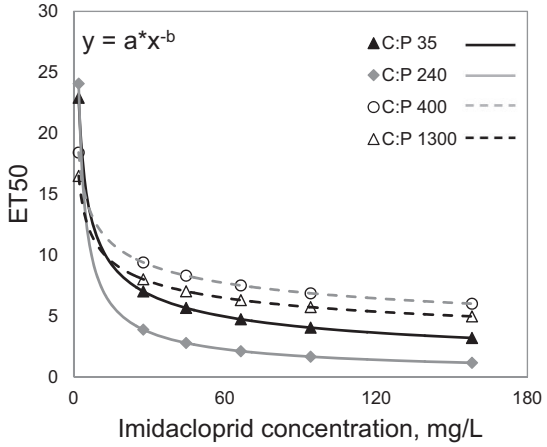

Figure 3. Relationship between median time to 50% effect (ET50) and imidacloprid concentration fitted with hyperbolic model.

results of the Von Bertalanffy growth model and somatic growth rate (Figures 4 and 5). Phosphorus is stored in algae cells as polyphosphate [31,32]. Addition of K_2HPO_4 to the phosphorus-sufficient algae results in the increase of total cellular phosphorus and polyphosphate [33]. On the contrary, at the conditions of starvation, the total cellular phosphorus content of algae decreases [34]. In the present study, the total phosphorus concentration of algae *Pseudokirchneriella subcapitata* was lowest at C:P 1300, which explains its poor nutritional quality for *D. magna* (Table 1). In previous studies, algae grown at conditions of P-deficiency increased the thickness of the cell wall, which resulted in lower digestion rates for *D. magna* and consequently reduced growth [11,35]. This was proposed to be a defensive mechanism of algae against grazing by *D. magna* at poor nutrient conditions [35]. DeMott and Van Donk [11] suggested that in the algae resistant to digestion, the cell wall remains undamaged when passing through the gut of daphnids. Therefore, in conditions of phosphorus deficiency, carbon and phosphorus of algae cannot be fully assimilated by daphnids [11]. Also, in the study by Frost et al [36], when the algae C:P ratio increased (meaning lowered P content), the percentage of P in the body mass of *D. magna* decreased at the control treatment. The growth rate of daphnids in turn depends on the amount of carbon assimilated [11]. Results of daphnids' growth rates, as determined in our experiment, especially at the high C:P levels, could therefore be a possible result of reduced carbon and phosphorus incorporation by *D. magna* fed with P-deficient algae. In poor nutrient conditions, values for both growth rate K and maximum hypothetical length L_{max} derived in the Von Bertalanffy model were lower compared with P-sufficient diets. At the same time, *D. magna* provided with algae of low P content could have higher filtering activity, which resulted in more energy spent for filtering and faster passage of algae through the gut [7]. As a result, higher energy costs for filtering activity may lead to a reduced growth rate and lower reproduction at a P-deficient diet. Therefore, the energy demand of *D. magna* supplied with algae of low phosphorus level (C:P 1300) may not be fulfilled. Similar results of the negative effects of low algal phosphorus content on the growth of daphnids were found in a number of previous studies [7,21,35]. Conversely, when supplied with P-sufficient algae, the feeding rate of *D. magna* is lower compared with P-deficient conditions [7]. Consequently, a lower amount of energy is allocated to filtering, that results in higher growth rates at P-sufficient conditions.

Table 4. Summary of parameters estimated in von Bertalanffy growth model for *Daphnia magna* supplied with 4 food regimes at control conditions (C0) and exposed to imidacloprid concentrations 2.0 mg/L (C1) and 27.6 mg/L (C2)

C:P ratio	Estimated parameters	C0 (0 mg/L)	C1 (2.0 mg/L)	C2 (27.6 mg/L)
C:P 35				
	K	0.43	0.39	0.30
	L_{MAX}	2400.9	2660.4	1987.8
	t_0	–1.76	–0.49	–0.74
	R^2	0.89	0.89	0.88
C:P 240				
	K	0.40	0.40	0.27
	L_{MAX}	2751.1	2639.6	1958.4
	t_0	–0.72	–0.44	–1.59
	R^2	0.90	0.86	0.87
C:P 400				
	K	0.38	0.33	0.28
	L_{MAX}	2546.4	2481.6	1707.0
	t_0	–0.99	–1.15	–1.21
	R^2	0.87	0.90	0.71
C:P 1300				
	K	0.36	0.29	0.30
	L_{MAX}	2209.4	2444.5	1622.5
	t_0	–0.37	–0.36	–2.53
	R^2	0.90	0.88	0.86

K = growth rate; L_{MAX} = hypothetical maximum length of *D. magna*; t_0 = constant at which an organism has a length $L_t = 0$; R^2 = correlation coefficient between observed and predicted in the model data.

According to the previous studies, the optimal effects of environmental conditions on *D. magna* growth rate were derived from the 21-d experiment. Differences in the modeled Von Bertalanffy growth estimates obtained in the 21-d and 41-d experiments were not significant in the study of Martínez-Jerónimo [37]. Similarly, in the present study, the increase in body size at 11 d to 21 d was generally smaller, likely because of the resource limitation (more energy allocated to reproduction and not to growth irrespectively of the diet). The experiment of 21 d was sufficient to estimate the effects of food limitation on the growth rate of *D. magna*.

Previous studies have suggested that the sorption of chemicals is positively related to their octanol–water partitioning coefficient (K_{OW}). In the study of Rose et al. [4] the hydrophobic fenoxycarb caused substantial toxicity to *D. magna* at the highest

Urabe et al. [21] confirmed that phosphorus determined food quality for *D. magna* and estimated the C:P ratio threshold for algae growth (C:P $\leq$ 300). *Daphnia magna* fed with algae of C:P lower than 300 are not limited by the phosphorus in food. This observation agrees with our results: lower growth and L_{max} were found at limited conditions of C:P 1300. Plath and Boersma observed reduced somatic growth rates at low C:P (approximately 30) [7]. These authors argued that this effect can be explained by a lower incorporation of carbon by *D. magna* as a result of the reduced feeding rate at P-rich conditions. This result could not be confirmed. However, the hypothetical body length L_{max} derived from the Von Bertalanffy model was higher at P-optimal conditions (C:P 240) than at P-rich (C:P 35 at control conditions). Additionally, in the study by Plath and Boersma, a significant reduction of somatic growth (approximately 3-fold) was observed at a P-deficient C:P level of approximately 640 [7]. The duration of their experiments (6 d) differed from the present study, and K_2HPO_4 was added to algae cultures 24 h before the start of the experiment [7]. In our study, algae were adapted to different nutritional levels during 7 d and likely changed their biochemical composition.

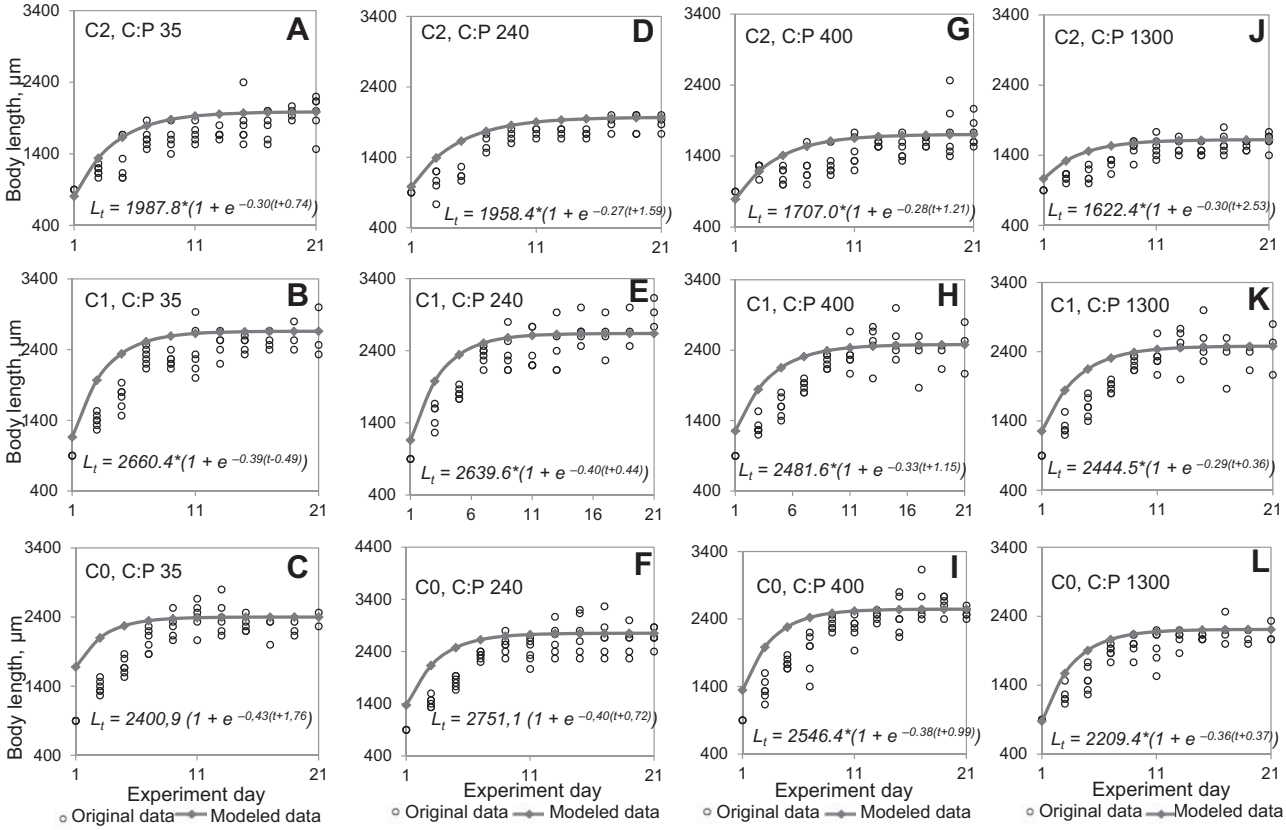

Figure 4. Body length of *Daphnia magna* supplied with diets C:P 35 (**A**–**C**), C:P 240 (**D**–**F**), C:P 400 (**G**–**I**), and C:P 1300 (**J**–**L**) at control conditions (C0; **C**,**F**,**I**,**L**) and exposed to imidacloprid concentrations 2.0 mg/L (C1; **B**,**E**,**H**,**K**) and 27.6 mg/L (C2; **A**,**D**,**G**,**J**) fitted with von Bertalanffy growth model. L_t = body length of *D. magna* at time t.

algae concentration used. This was likely because a larger amount of fenoxycarb was adsorbed to organic matter and harvested by animals supplied with a high food level [4]. A similar result of larger effect of herbicide glyphosate on *D. magna* growth supplied with P-rich food was found by Lessard and Frost [13]. This result was explained by lower incorporation of toxin by daphnids at P-deficient conditions [13]. Higher toxicity at a nutrient-rich diet was found for the pharmaceutical fluoxetine [14]. On the contrary, Barry et al. [5] proposed that the metabolic degradation of hydrophobic chemicals by algae can lead to lower effects on *D. magna* exposed at high food conditions [5]. However, this statement does not apply to the chemicals that also have toxic metabolite products.

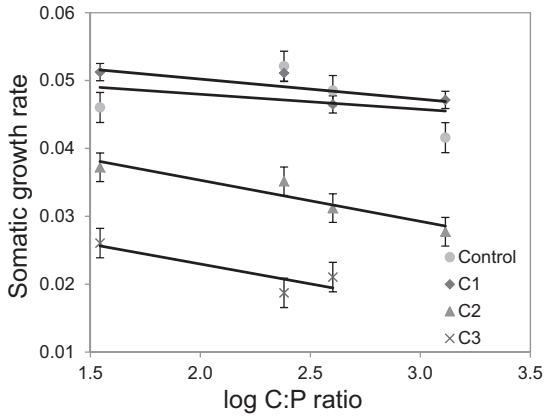

Figure 5. Somatic growth rate (µm/d) of *Daphnia magna* exposed to a range of imidacloprid concentrations plotted versus log C:P ratios (shown on the graph are mean somatic growth rate and standard error). C1 = 2.0 mg/L; C2 = 27.6 mg/L; C3 = 44.6 ± 3.1 mg/L.

Imidacloprid is a hydrophilic insecticide that has a lower tendency to bind to organic matter (water solubility = 610 mg/L, log $K_{OW} = 0.57$). Therefore, at the conditions of imidacloprid exposure, the quantity and quality of algae supplied to daphnids within the optimal feeding range does not affect toxic response. In our study, only at the conditions of phosphorus deficiency (C:P 1300) was the effect of imidacloprid on survival, growth, and reproduction more pronounced. Food limitation possibly acted as an additional stressor that led to higher toxicity when supplied with algae of low nutritional quality. Following the concept of Van Straalen [38], under sufficient food conditions invertebrates likely withstand easier additional stresses, and our results clearly show that at phosphorus-sufficient diets, high imidacloprid concentration was easier to battle.

Table 5. Parameters fitting regression equation $SGR = a + b \times \log(C:P)$, describing relationship between somatic growth rate (SGR) of *Daphnia magna* and C:P ratio at different imidacloprid exposure conditions and control

Imidacloprid concentration	Slope (b)	Intercept (a)	R^2	N
0 mg/L	−0.003	0.056	0.58	4
2.0 mg/L	−0.002	0.052	0.11	4
27.6 mg/L	−0.006	0.047	0.88	4
44.6 ± 3.1 mg/L	−0.006	0.035	0.76	3

Table 6. Summary statistics for the two-way analysis of variance explaining *Daphnia magna* body length at days 3, 9, 15 and 21 and net reproductive rate (R_0) at different exposure conditions

Parameter	Source of variation	*f stat*	*p* value	*f crit*
R_0				
	I	3.34	0.09**	4.49
	P	4.72	0.02*	3.24
	I × P	0.76	0.53	3.24
Body length day 3				
	I	25.31	*2.62E–12**	2.53
	P	3.71	*0.016**	2.76
	I × P	2.43	*0.012**	1.92
Body length day 9				
	I	193.50	*8.37E–27**	2.80
	P	2.09	*0.114*	2.80
	I × P	3.65	*0.002**	2.08
Body length day 15				
	I	76.11	*1.17E–13**	3.26
	P	10.29	*4.93E–05**	2.87
	I × P	1.51	*0.204*	2.36
Body length day 21				
	I	80.58	*5.09E–14**	3.26
	P	17.63	*3.29E–07**	2.87
	I × P	5.02	*0.0008**	2.36

f stat = F-statistic; *f crit* = F-critical; I = imidacloprid; P = phosphorus content of algae; I × P = interaction of imidacloprid and phosphorus.
*$p < 0.05$.
**$p < 0.1$

Effects on reproduction

The imidacloprid exposure concentration of 2.0 mg/L used in the experiment is close to the earlier reported NOEC for imidacloprid (1.8 mg/L in 21-d test, endpoint reproduction) [17]. Because a low imidacloprid concentration was used, average reproduction per day for exposed animals did not differ significantly from the control. At C:P 240 higher reproductive output was found at the exposed treatment (Figure 6A and B). The lowest value of R_0 (net reproductive rate) was observed at the P-deficient diet (C:P 1300) at the control conditions and at an imidacloprid concentration of 2.0 mg/L (Figure 6). As a result of lower growth rate at P-deficient conditions, smaller body size was reached. *D. magna* start reproducing when critical body size is achieved. Because of the reduced growth rate at P-deficient conditions, *D. magna* attained critical body length later than with the other diets. This has possibly led to delayed age at maturity and consequently lower reproduction at P-poor conditions. Under conditions of P-deficiency, *D. magna* is likely to allocate higher proportion of energy toward maintaining survival. Consequently, the proportion of energy available for reproduction is reduced [39]. The energy obtained by the organism is balanced between somatic maintenance (growth) and reproduction: when high growth is reached, less energy is available for reproduction [40]. This complies with the dynamic energy budget theory, which allows calculating costs that are made by organisms to deal with various natural and anthropogenic stressors [40]. Thus, in the present study we found a larger time to mortality (ET50) at P-poor conditions characterized by lower reproductive output.

Imidacloprid concentrations used in the experiment were significantly higher than usually found in Dutch surface waters (0.1–1.5 μg/L, Waterboard Rijnland, measurements of 2010 [41]). Selection of relatively high concentrations is also explained by the fact that cladoceran *D. magna* is more tolerant to imidacloprid compared with insect or other crustacean species [16]. This allowed detecting effects on *D. magna* survival and growth on a relatively short time scale of 21 d. In general, surface waters around intensively used arable fields contain phosphorus concentrations that are considerably higher compared with surface waters in areas with less intensive land

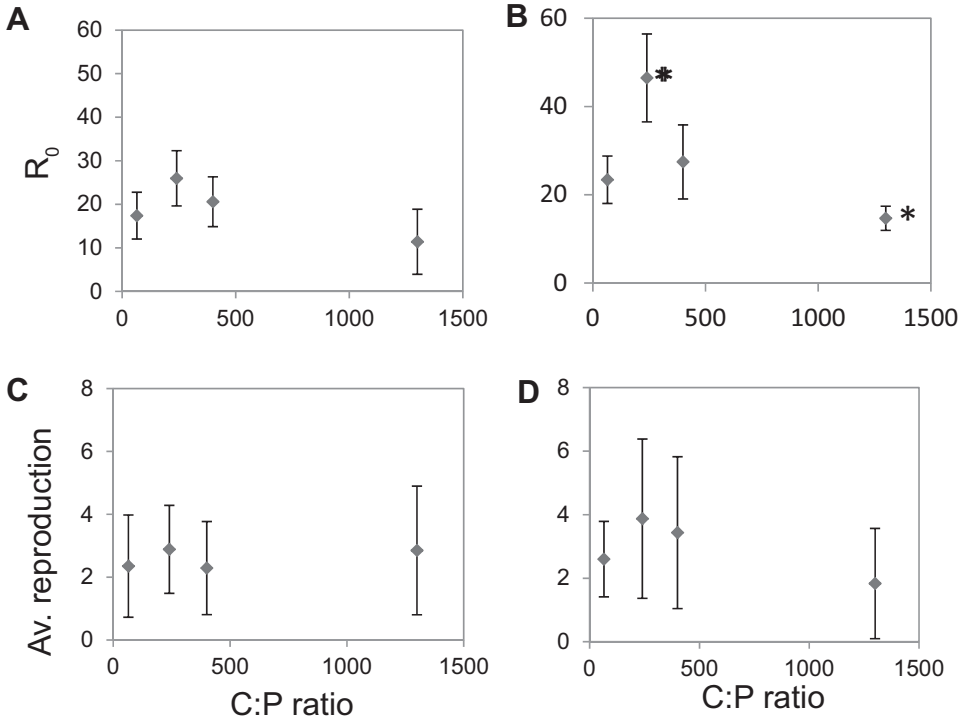

Figure 6. Net reproductive rate (R_0, juvenile/female), average reproduction per day (juvenile/female/d) of *Daphnia magna* at the control conditions (**A**, **C**) and imidacloprid concentration 2.0 mg/L (**B**, **D**). *Significantly different from other C:P levels at $p < 0.1$.

use and nature-protected areas (e.g., data waterboard Rijnland period 1993–2007 for the southern part of The Netherlands [42], or Gao et al. [43] period 2005–2006 for Southwestern China). Based on the results of the current study, we can conclude that under oligotrophic conditions (i.e., low P levels), imidacloprid pollution will result in more pronounced effects on crustaceans.

CONCLUSIONS

The interactive effect of imidacloprid exposure and the elemental composition of algae (C:P ratio) on the performance of *D. magna* was shown to be ambiguous. Higher impact on survival and growth of daphnids was observed at phosphorus-deficient conditions. Based on the experimental results, one can conclude that toxicity of imidacloprid increased at a P-deficient diet, as seen by the observed effects on survival, growth rate, and reproduction. This was confirmed by lower EC10 values, growth rates, and reproductive output of *D. magna* at the conditions of P-deficiency. Combined effects of toxicants and abiotic factors challenged the estimation of pesticide risks on daphnids populations in freshwater ecosystems. Results can be applied to predict limiting ratios of carbon:nutrients for daphnids at the conditions of toxic stress. In field situations, multiple abiotic factors are present, and, therefore, combined effects of chemicals and natural stressors can be expected. The interactive effects of resource limitation and toxic stress on organisms need to be considered in risk assessment of chemicals.

SUPPLEMENTAL DATA

Tables S1–S4.
Figures S1 and S2. (63 KB PDF).

Acknowledgment—O. Jeromina is supported by the Environmental Chemo-informatics (ECO) project, Marie Curie ITN-EU Framework 238701. The authors thank M. Wouterse for providing *Daphnia magna* culture animals and DOC/TOC measurements.

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