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Chronic toxicities of neonicotinoids to mayfly nymphs

# Chronic Toxicities of Neonicotinoids to Nymphs of the Common New Zealand Mayfly *Deleatidium* spp.

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## Acknowledgement

We thank William Doucette, Joan McLean and Joseph Stewart (Utah Water Laboratory) for use and assistance with the LC/MS/MS analysis in their laboratory, and Nicola McHugh for developing the initial HPLC methods. We acknowledge financial support for this research to Sam Macaulay from the University of Otago and thank Paul Klerks and three anonymous reviewers for their constructive comments which improved the quality of this manuscript.

## Data Accessibility

Data are deposited in figshare at DOI:

## Abstract

Neonicotinoid insecticides have been shown to have high chronic relative to acute toxicity, therefore short-term toxicity tests of ≤ 96 hours in duration may underestimate their environmental risks. Among non-target aquatic invertebrates, insects of the orders Diptera and Ephemeroptera have been found to be the most sensitive to neonicotinoids. To undertake more accurate assessment of the risks posed by neonicotinoids to freshwater ecosystems, more data are needed from long-term

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/etc.4556.

tests using the most sensitive taxa. Using nymphs of the common New Zealand mayfly genus *Deleatidium* spp., we performed 28-day static-renewal exposures with the widely used neonicotinoids imidacloprid, clothianidin and thiamethoxam. We monitored survival, immobility, impairment, and mayfly moulting propensity at varying time points throughout the experiment. Imidacloprid and clothianidin exerted strong chronic toxicity to *Deleatidium* nymphs, with respective 28-day LC50s of 0.28 and 1.36 µg/L, while thiamethoxam was the least toxic, with a 28-day LC50 > 4 µg/L (highest concentration tested). Mayfly moulting propensity was also negatively affected by clothianidin (during 3 of 4 weeks), imidacloprid (2 of 4) and thiamethoxam (1 of 4). Comparisons with published neonicotinoid chronic toxicity data for other mayfly taxa and larvae of the midge genus *Chironomus* showed similar sensitivities for mayflies and midges, suggesting experiments using these taxa provide reliable assessments of the threats of neonicotinoids to the most vulnerable freshwater species.

## Keywords

Imidacloprid, clothianidin, thiamethoxam, freshwater toxicology, aquatic invertebrates, pesticides

## Introduction

The rapid rise in global use of neonicotinoids over the last few decades has resulted in their widespread contamination of surface waters where they can pose a considerable threat to non-target aquatic organisms, especially insects. Previous research to investigate the effects of neonicotinoids on non-target insects has consistently shown high toxicity to the insect taxa tested (Wood and Goulson 2017). A comprehensive review of neonicotinoid effects in aquatic ecosystems by Morrissey et al. (2015) demonstrated that there is considerable variation in toxicity among invertebrate taxa (more than six orders of magnitude in difference within aquatic arthropods) and that aquatic insect taxa are the most sensitive. This is not surprising, given that neonicotinoids were designed to target nicotinic receptors of terrestrial pest insects (Matsuda et al. 2001) and, among aquatic invertebrates, the physiology and nicotinic-receptor binding sites of aquatic insects are most similar to those of terrestrial insects. Consequently, when conducting ecotoxicological studies with neonicotinoids, it is important to consider species belonging to these more sensitive taxa, which exhibit lethal and sublethal effects (e.g. impaired mobility, feeding, reproduction, growth, emergence) at concentrations where crustacean test species are not affected (Anderson et al. 2015).

Morrissey et al. (2015) reviewed data from toxicity studies conducted with 49 arthropod species, including 178 acute and 36 chronic tests, and concluded that mayflies (Ephemeroptera) were the most sensitive taxonomic group. Using 24–96 h median lethal concentrations (LC50s; 137 tests) and median effective concentrations evaluated with sublethal endpoints (EC50s; 77 tests), they calculated an acute geometric mean L[E]C50 of 3.9 µg/L for Ephemeroptera. Cladocerans were the least sensitive group, including the widely used test species *Daphnia magna* which had an

acute geometric mean L[E]C50 of 43,926.5 µg/L. This finding is concerning given *D. magna* has traditionally been considered the global industry standard invertebrate test species, having been used for 82% of chemicals tested (Sánchez-Bayo 2006).

Mayflies are used worldwide as biological indicators of stream health because of their high sensitivity to pollutants and their critical role in freshwater food webs (Sánchez-Bayo et al. 2016). For these reasons and because they are among the most sensitive aquatic taxa to neonicotinoids, they are prime study organisms for evaluating environmental risks of neonicotinoids in fluvial ecosystems. In New Zealand, the mayfly genus *Deleatidium* spp. (Leptophlebiidae) is distributed ubiquitously in running waters across the country. *Deleatidium* nymphs are periphyton grazers that feed on biofilm growing on rock surfaces and are an important food source for fish, other aquatic insects and birds (Scrimgeour 1991; Winterbourn 1974), with a seasonal and temperature-dependent larval cycle resulting in several overlapping generations per year (Huryn 1996; Winterbourn 1974). Nymphs that hatch in spring or early summer can achieve their maximum size in 3 months, but those hatching in late summer can have a larval stage of up to 12 months (Scrimgeour 1991). These long aquatic larval stages are likely to render them vulnerable to exposure to neonicotinoids in streams draining agricultural land.

A consistent pattern observed in the literature concerning the aquatic ecotoxicity effects of neonicotinoids is a high acute-chronic ratio (ACR) indicating considerably lower chronic-effect concentrations than those required to cause acute toxicity (Sánchez-Bayo et al. 2016). In the review by Morrissey et al. (2015), sublethal end-points in chronic studies were frequently an order of magnitude or more below those for acute tests. It has been proposed that irreversible binding of neonicotinoids to the insect nicotinic acetylcholine receptor, causing continual firing of electrical impulses and eventually neuronal death (Tennekes 2010), is the reason for their adverse effects to accumulate with time (Tennekes and Sánchez-Bayo 2013). Consequently, initial toxicity assessments based on acute tests with standard test species underestimated the risks posed by neonicotinoids to aquatic ecosystems (Sánchez-Bayo et al. 2016). However, there is also some evidence of the potential for reversible binding, with freshwater invertebrate recovery from an impaired state observed following short-term pulses of imidacloprid (Raby et al. 2018a).

To account for ACRs and other uncertainties in environmental risk assessment, ‘uncertainty factors’ are applied to acute LC50s or EC50s determined from highly standardised, short-term laboratory tests (Heugens et al. 2001) run in the first tier of tiered risk assessment schemes (e.g. EFSA 2013). However, when initial acute tests are run with a highly tolerant test organism (e.g. *D. magna* in the case of neonicotinoids) and when combined with high ACRs, the risks may still be underestimated even after the cautionary uncertainty factors have been applied (Tennekes and Sánchez-Bayo 2011; van Wijngaarden et al. 2015). Due to the higher expense of performing experiments longer than 96 hours (Smith et al. 1991) and the difficulty of maintaining sufficient control survivorship for the exposure duration for some taxa, acute tests still dominate the toxicological literature (83% of those reviewed by Morrissey et al. 2015 spanned ≤ 96 h) and there is a lack of chronic

neonicotinoid toxicity data, especially for sensitive test species (Anderson et al. 2015). Yet chronic laboratory studies lasting 28 days or longer and field or mesocosm studies are recommended as the primary guides for determining regulatory acceptable concentrations for neonicotinoids, because these studies give more accurate and realistic assessments of the environmental risks (Morrissey et al. 2015).

Currently only three publications have investigated chronic toxicities of neonicotinoids to mayfly larvae (Roessink et al. 2013; Van den Brink et al. 2016; Raby et al. 2018b). The first of these determined acute and chronic toxicities of imidacloprid to the European mayflies *Cloeon dipterum* and *Caenis horaria*, three other aquatic insects and two crustaceans. Large ACRs of at least 10 were found for all species tested (the largest ratio being 336 for *C. dipterum*), leading the authors to conclude that acute toxicity data are inappropriate for assessing effects of long-term exposure to imidacloprid. Several studies have also reported on chronic effects of neonicotinoids on larvae of the freshwater dipterans *Chironomus riparius* (Finnegan et al. 2017; Langer-Jaesrich et al. 2010; Saraiva et al. 2017), *Chironomus dilutus* (Cavallaro et al. 2017; Maloney et al. 2018a; Raby et al. 2018b), *Chironomus tentans* (Stoughton et al. 2008) and *Chaoborus* sp. (Finnegan et al. 2017; Roessink et al. 2013). However, only four studies have reported chronic laboratory effects of clothianidin on aquatic insects: the mayfly *Neocloeon triangulifer* was investigated by Raby et al. (2018b), *C. dilutus* by Cavallaro et al. (2017), Maloney et al. (2018a) and Raby et al. (2018b), and *C. riparius* by Drottar et al. (2000).

The aim of the present study was to expand the limited knowledge about the effects of chronic exposure of pollution-sensitive freshwater mayflies to neonicotinoids (imidacloprid, clothianidin and thiamethoxam). We used nymphs of the ubiquitous endemic New Zealand mayfly, *Deleatidium* spp., as our test organism, making our study just the second to provide chronic toxicity data for clothianidin to mayfly nymphs. Neonicotinoids have been registered for use to control the pests of a variety of pasture and forage crops throughout New Zealand since 1991 (Chapman 2010), but their presence in surface waters is currently not monitored. Based on the related studies on European mayfly species mentioned above, we predicted all three neonicotinoids would elicit chronic toxicity to *Deleatidium* nymphs with 28-day LC50s < 1 µg/L.

## Materials and Methods

### Artificial stream water (ASW)

The base water for all laboratory experiments was prepared according to the American Society for Testing Materials artificial soft water (ASW) recipe and consisted of deionised water (Gemini-MB Ultra High Purity Water System; Aries FilterWorks, West Berlin, NJ, U.S.A.) containing (in mM/L): 0.57 $NaHCO_3$, 0.17 $CaSO_4 \cdot 2H_2O$, 0.25 $MgSO_4 \cdot 7H_2O$, and 0.03 KCl. ASW was stored in 25-L carboys at climate room temperature and aerated overnight prior to use.

## Experimental design

Three separate 28-day, static-renewal laboratory experiments were performed with clothianidin (May−June 2017), thiamethoxam (June−July 2017) and imidacloprid (May−June 2018). In each experiment, *Deleatidium* nymphs were exposed to ten neonicotinoid concentrations ranging from 0 to 4 µg/L. This range was selected because prior experiments with *Deleatidium* nymphs exposed to imidacloprid had shown strong effects on mayfly survival (partial-$\eta^2$ effect size 0.67) and impairment (partial-$\eta^2$ effect size 0.76) at concentrations within this range (0.9 and 2.1 µg/L) after 9 days of exposure (Hunn 2016). Therefore, we expected this range to cover a full concentration-response profile across 28 days for the three neonicotinoids tested. Treatment concentrations were randomly allocated to 50 × 1.16 L aerated glass chambers (19.9 × 14.4 × 6.3 cm, see Figure S1), with five replicates per concentration and at least 15 *Deleatidium* nymphs per replicate (see below). Exposure solutions were renewed every 7 days.

## Insecticide application, sampling and quantification

Working stock solutions of 10 mg/L imidacloprid, clothianidin and thiamethoxam were prepared using 10 mg/mL each insecticide (PESTANAL® analytical standard grade, Sigma-Aldrich, Castle Hill, NSW, Australia). The ten exposure concentrations (0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 2.4, 3.2 and 4.0 µg/L) were prepared by dosing 2.5 L of ASW with the required amount of working stock. From the glass chambers, 2-mL water samples were collected for analysis from each of three replicates of the 0.05, 0.4 and 4.0 µg/L concentrations. These samples were collected at the beginning and end of each test week and stored in 4-mL glass vials with Teflon caps in the dark at –20 °C until shipping to the analytical laboratory with ice packs, where they were again stored at –20 °C until analysis.

All neonicotinoid standards used for neonicotinoid quantitation (including Clothianidin-d3, see below) were PESTANAL® standard analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Neonicotinoids were quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS). Analyte separation was achieved using an Agilent 1290 Infinity Binary Pump connected to an Agilent Poroshell 120 EC-C18 column (4.6 mm x 100 mm x 2.7 µm) and a gradient elution method (see Supporting Information, Table S1). The column was held at 30.0 °C, the total flow rate was 0.600 mL/min and sample injection volumes were 100 µL. Quantification was achieved with an Agilent 6490 Triple Quadrupole Mass Spectrometer in positive electrospray ionization mode, using multiple reaction monitoring. The source temperature was 200 °C, the gas flow rate was 14 L/min and the nebulizer pressure was 20 psi. The sheath gas temperature was 325 °C, sheath gas flow rate was 11 L/min and the capillary voltage was 3500 V. Internal calibration curves were prepared from the ratio of the peak area of the target analyte to the internal standard (Clothianidin-d3), with target analyte concentrations ranging from 0.05–20 µg/L. The sample-specific estimated detection limits, calculated using United States Environmental Protection Agency (2007) Method

8280b, were 0.003 µg/L for clothianidin and 0.002 µg/L for imidacloprid and thiamethoxam. Additional analyte quantification details are provided in Table S2.

## Mayfly food supply

*Deleatidium* were fed periphyton grown on 10 × 10 × 1 cm ceramic tiles *in situ* in Lindsay Creek, located in North East Valley, Dunedin (45.8420°S, 170.5408°E). This site was chosen for its ease of accessibility, concealment from public view, presence of *Deleatidium*, and because the small stream drained a nature reserve clad in native forest that was unlikely to flood (causing bed disturbance) during rainfall. A period of two weeks was deemed sufficient for periphyton growth which was assumed to be consistent in quantity and composition. One randomly chosen periphyton tile was placed in each chamber. This tile also acted as a substratum for the nymphs and was exchanged with a fresh tile from Lindsay Creek every 7 days, at the same time as renewal of the exposure solutions.

## Test specimen collection and acclimation conditions

*Deleatidium* specimens were collected on three separate occasions, once prior to each experiment, from Silver Stream, a fourth-order stream located in an unpolluted native forest catchment about 15 km from Dunedin, New Zealand (45.8096°S, 170.4211°E). On each occasion, specimens were collected from the same stream reach (ca. 75 m) using a pulsed DC backpack electro-shocker (Kainga EFM300; NIWA, Christchurch, New Zealand). Electric fishing strongly increases invertebrate drift rates and is a fast, efficient method for collecting stream invertebrates including mayflies (Taylor et al. 2001). The technique has been used successfully in New Zealand and North America for ecological experiments where large numbers of live invertebrate specimens were needed (e.g. McIntosh and Townsend 1994; Peckarsky and McIntosh 1998). Specimens drifting downstream were caught in a pole-net (0.9 × 0.7 m, mesh size 3 mm) and transferred to large holding bins for sorting on-site. Small to medium-sized nymphs (head-and-body length 5-15 mm) were preferentially targeted because in previous experiments, late instars (especially those with visible wing-pads) had been less tolerant to laboratory conditions and more likely to emerge during experiments than was the case for earlier instars.

To maximise specimen survival during transport from the collection site to the climate-controlled room, battery-operated air pumps (Aqua One Battery Air 250; Aqua One, Ingleburn, NSW, Australia) and crushed ice were used to maintain high dissolved oxygen levels. Stream temperatures during collections ranged from 3.7–7.5 °C. Nymphs were left in aerated buckets with stream water overnight to allow acclimation to the temperature of the climate-controlled room (12 °C). The following morning, all specimens were transported to holding containers (40 L) with aerated ASW that was already at climate-room temperature and contained 15 ceramic tiles pre-colonised with periphyton (see above) for a further 48 hours of acclimation. During all acclimation and test periods, the climate-controlled room was maintained at 12 ± 1°C with a 16:8 hour light:dark regime (with a 1-h ramp from light−dark and vice versa). A random sample of at least 15 (maximum 17, mean 15.5), healthy

nymphs with all limbs and cerci intact, representative of the range of sizes and instars collected, were distributed to each treatment replicate at the start of each exposure period.

## Recording invertebrate responses

At the time of each tile exchange and renewal of exposure solutions, mayfly survival and two sublethal responses indicating mayfly nymph motility were recorded. Nymphs were gently inverted using forceps; if they were unable to right themselves by performing a normal swimming movement, but were still able to walk, they were classified as "impaired". Nymphs that were unable to move either by swimming or walking and that could only twitch their appendages were classified as "immobile". These sublethal responses for motility follow the continuum of observed toxicity symptoms described by Camp and Buchwalter (2016) for nymphs of the mayfly *Isonychia bicolor* exposed to imidacloprid. According to their classification, "righting inability" occurs during the "impairment" phase of responses and "immobility" occurs following onset of muscle spasms and unresponsiveness, immediately preceding mortality. This continuum of toxicity symptoms means, for the purpose of calculating chronic toxicities from our concentration-response experiments, all dead nymphs were assumed to have previously been impaired and immobilised, and all immobile nymphs were assumed to have been impaired prior to immobilisation (e.g. immobile = dead + immobile; impaired = dead + immobile + impaired). Tests were considered valid when control immobility was < 10% (as per Roessink et al. 2013). If control immobility exceeded 10%, the results were considered to be indicative.

Survival, impairment and immobility were also recorded four days after each tile exchange, i.e. on Days 11, 18 and 25. Further, "moulting propensity" was calculated as the number of moults occurring during each experimental week (1–4) out of the number of *Deleatidium* nymphs that had been alive on the start of each week; this was recorded because acute exposure of imidacloprid to *Deleatidium* had significantly affected moulting frequency in previous experiments (Macaulay et al. 2019). Dead specimens and shed exuviae were removed daily from the chambers and used to calculate survivorship and moulting propensity. Emerged mayflies were also removed and their final moults recorded as part of the moulting response. Emergence was too infrequent to be considered as a response variable on its own.

## Data analysis

Separate log-logistic (binomial) generalized linear regressions were performed in R (version 3.5.1. R Core Team) to determine the individual effects of imidacloprid, clothianidin and thiamethoxam on the responses of *Deleatidium* survivorship, impairment, immobility and moulting propensity on Days 7, 11, 14, 18, 21, 25 and 28. The significance level α for all regressions was set conservatively at $p = 0.01$ so that any effects significant at this α would be clearly visible in the data. All regressions were highly significant ($p < 0.001$) unless stated otherwise. Median lethal concentrations (LC50s), median immobilising concentrations (IC50s) and effective concentrations causing impairment (EC50s) were calculated with 95% confidence

intervals (CI) for all significant regressions using the dose.p function in R package MASS (Venables and Ripley 2002). McFadden's pseudo-$R^2$ values ($\rho^2$) and dispersion parameters were calculated for each model. McFadden's $\rho^2$ (McFadden 1974) tend to be considerably lower than those of the linear $R^2$ measure and are therefore to be interpreted differently to linear regression standards of model fit (Domencich 1975). According to McFadden (1977), $\rho^2$ of 0.2–0.4 represents an excellent model fit, therefore we consider $\rho^2 > 0.4$ a strong effect and $\rho^2 < 0.2$ a weak effect. Log-logistic regression summaries for survivorship, immobility and impairment regressions are presented in Table 1. Summaries for mayfly moulting propensity are presented in Table 2.

## Results

### Neonicotinoid exposures

The verified concentrations for imidacloprid, clothianidin and thiamethoxam deviated on average by -19%, -38% and 66% from nominal, respectively (see Table S3 for all verified concentrations). However, having verified concentrations for only three of the ten treatments meant it was more feasible to use the nominal concentrations in our analyses. Implications of the considerably higher achieved concentrations for thiamethoxam are discussed below. Mean initial and final concentrations for the lowest, middle and highest treatments (0.05, 0.4 and 4 µg/L) across the three experiments were 0.04 and 0.04 µg/L, 0.48 and 0.48 µg/L, and 4.38 and 4.24 µg/L, respectively. Given the high consistency in mean initial and final concentrations (taken at the beginning and end of each week), we were satisfied that there was minimal pesticide degradation over the week-long static-renewal periods.

### Time-to-effect observations and comparative toxicities

Overall, the toxicity of each neonicotinoid on survivorship and mayfly mobility (immobility and impairment) increased over time, as indicated by decreasing median lethal, immobilising and effective concentrations (Table 1; Figures 1–3). When comparing the three insecticides, imidacloprid was the most toxic, clothianidin intermediate, and thiamethoxam the least toxic. For example, after 18 days of exposure imidacloprid caused 50% mortality at 0.46 µg/L, whereas clothianidin had a corresponding LC50 of 2.75 µg/L. Exposure to thiamethoxam caused some significant reductions in *Deleatidium* survival, but this did not drop below 50% over the entire 28-day exposure period and all median effect concentrations were > 4 µg/L (Table 1). Control mortality (and immobility) in the imidacloprid experiment exceeded 10%, therefore these results should be considered indicative. However, survivorship remained > 75% at the 0.4 µg/L imidacloprid treatment throughout the entire exposure period. Above this concentration, survival dropped sharply due to imidacloprid exposure. This can be verified by the lack of an immobilising or impairing effect on the specimens still alive in all imidacloprid treatments below 0.4 µg/L (see Figures S2 and S3).

### Week 1

Only imidacloprid had significantly reduced *Deleatidium* survivorship after the first 7 days of exposure (Figure 1a). Considering the sublethal response of immobility (in addition to mortality) resulted in a stronger effect of imidacloprid and a significant, albeit weak effect of clothianidin (Figure 2a-b). Including the sublethal response of impairment as well showed an even stronger effect of imidacloprid, with almost 100% of *Deleatidium* nymphs impaired at the highest concentration (4 µg/L) after just 7 days (Figure 3a). Therefore, a 7-day EC50 of 1.21 µg/L (the concentration at which 50% of *Deleatidium* nymphs were adversely affected, in this case, impaired) could be calculated for imidacloprid. No significant effects of thiamethoxam were observed in the first week.

### Week 2

After 14 days, survivorship had been reduced below 50% at 0.8 µg/L imidacloprid (Figure 1a; LC50 = 0.86 µg/L). Both imidacloprid and clothianidin had strongly increased *Deleatidium* immobility (Figure 2a and b) and impairment (Figure 3a and b), with respective 14-day EC50s of 0.4 µg/L and 2.46 µg/L. While a significant effect of thiamethoxam did occur for *Deleatidium* survival ($p$ = 0.008; Figure 1c) and impairment (Figure 3c), these effects were not strong enough to be considered biologically relevant.

### Week 3

By Day 21, imidacloprid and clothianidin had both strongly reduced *Deleatidium* survivorship ($\rho^2 > 0.4$). Their respective 21-day LC50s were 0.38 and 2.12 µg/L. The effects of imidacloprid and clothianidin were stronger when considering the sublethal mayfly responses, especially impairment, compared to mortality just alone, with lower median effect concentrations of 0.30 and 1.47 µg/L. As in the second week, the effects of thiamethoxam were still too weak to consider biologically relevant.

### Week 4: final 28-day toxicities

The respective 28-day LC50s of imidacloprid, clothianidin and thiamethoxam were 0.28, 1.36 and > 4 µg/L, corresponding to strong effects of imidacloprid and clothianidin and a weak effect of thiamethoxam on *Deleatidium* survivorship (Figure 1). As observed in the first three weeks, including the sublethal mayfly responses marginally increased the strengths of the effects of each neonicotinoid, which was most evident when evaluating the impairment responses (Figure 3). The corresponding 28-day EC50s for imidacloprid, clothianidin and thiamethoxam were 0.19, 1.02 and > 4 µg/L, respectively.

## Effects on moulting propensity

Overall, *Deleatidium* moulting propensity was negatively affected by neonicotinoid exposure (Table 2). During the first week of exposure, there were no observable effects of the neonicotinoids on moulting propensity (Table 2; Figure 4). During the

second week, moulting propensity decreased significantly with increasing concentrations of imidacloprid and clothianidin while thiamethoxam had no effect. These effects mirrored the strong increases in immobility and impairment with exposure to imidacloprid and clothianidin (and lack of effect for thiamethoxam) that had also occurred by this stage (Figures 2 and 3). Similar patterns were observed in the third week when reductions in moulting propensity occurred with increased concentrations of imidacloprid and clothianidin (Figure 4a-b), but now a weak reduction in moulting propensity with increasing concentration of thiamethoxam was also observed (Figure 4c). In the fourth week, a significant reduction in moulting propensity was observed for clothianidin only (Figure 4b) whereas neither thiamethoxam nor imidacloprid had an effect.

## Discussion

This study assessed the chronic effects of three neonicotinoids (imidacloprid, clothianidin and thiamethoxam) on the winter nymphs of the ubiquitous New Zealand mayfly *Deleatidium* spp. Over the 28-day exposure period, all three neonicotinoids elicited some degree of chronic toxicity, but this occurred to different degrees. Imidacloprid, the most toxic, was the only neonicotinoid to cause effects consistent with those predicted, resulting in a 28-day LC50 below 1 µg/L (0.28 µg/L; 0.21–0.36). Clothianidin was slightly less toxic than imidacloprid, with a 28-day LC50 of 1.36 µg/L (1.22–1.5). Compared to imidacloprid and clothianidin, thiamethoxam was considerably less toxic to *Deleatidium* nymphs; there were no cases in which it led to more than 50% mortality, immobility or impairment over the entire exposure period, resulting in a 28-day LC50 which was outside the range of concentrations tested (> 4 µg/L). Although the achieved concentrations for thiamethoxam were somewhat higher than the nominal concentrations, this only strengthens our findings that thiamethoxam was less toxic.

## Comparison of chronic toxicities for mayflies and midges

Table 3 summarizes all published results of neonicotinoid chronic toxicities to mayflies and midges. To our knowledge, only three previous studies reported chronic toxicities of imidacloprid to mayfly nymphs. Our findings for *Deleatidium* were closely matched by the observations of Roessink et al. (2013) for chronic toxicities of imidacloprid to two European mayflies, *Cloeon dipterum* and *Caenis horaria*, with 28-day LC50s of 0.20 and 0.32 µg/L, respectively. Van den Brink et al. (2016) observed a higher tolerance to imidacloprid in the winter generation of *C. dipterum* they tested compared to the summer generation tested by Roessink et al. (2013), calculating a 28-day LC50 four times higher (0.85 µg/L). Given we used an overwintering population of *Deleatidium* nymphs this difference suggests imidacloprid, and potentially other neonicotinoids, could be even more toxic if tested on a summer generation of *Deleatidium* nymphs. When testing the chronic toxicities of six neonicotinoids to the mayfly *Neocloeon triangulifer*, Raby et al. (2018b) calculated an LC50 for imidacloprid higher than those already mentioned (1.75 µg/L). Contrary to our findings, their LC50 for clothianidin was lower than for imidacloprid (0.91 µg/L).

Raby et al. (2018b) is the only other study to test the chronic toxicity of clothianidin to mayfly larvae. They also determined chronic neonicotinoid toxicities to larvae of the freshwater midge, *Chironomus dilutus* (see below). Three further studies have assessed the chronic toxicity of clothianidin to *Chironomus* spp. The first of these, Drottar et al. (2000), is no longer available but was referenced in Morrissey et al. (2015, Supplemental Data Table A.2), with a 28-day EC50 of 1 µg/L for *Chironomus riparius* (using successful emergence), which is equal to the 28-day EC50 for clothianidin we calculated using the endpoint of impairment for *Deleatidium* nymphs. Cavallaro et al. (2017), Maloney et al. (2018a) and Raby et al. (2018b) assessed chronic toxicities of imidacloprid, clothianidin and thiamethoxam to *C. dilutus* and, measuring emergence rates, calculated 40-day, 28-day and 56-day EC50s. Across the three studies, these were between 0.24–0.5 µg/L for imidacloprid, 0.28–0.71 µg/L for clothianidin and 4.13–12.95 µg/L for thiamethoxam, results which are remarkably consistent with the chronic median lethal and effective concentrations we calculated for *Deleatidium* nymphs (Table 3).

Chronic toxicity of thiamethoxam to mayfly nymphs was also assessed by Van den Brink et al. (2016), for *C. dipterum*. In this case, the over-wintering generation had the same 28-day EC50 as for imidacloprid (0.68 µg/L). Finnegan et al. (2017) summarised the acute and chronic effects of thiamethoxam to a wide range of aquatic organisms, including three freshwater insect larvae, *Chaoborus* sp., *Chironomus riparius* and *Chironomus dilutus*. They observed that *C. riparius* was the most sensitive, with a 30-day EC50 (emergence) of 11.4 µg/L. Saraiva et al. (2017) also evaluated chronic effects of thiamethoxam exposure to larvae of *C. riparius*. After 28 days they observed a significantly lower emergence rate in treatments above 6.5 µg/L compared to controls, with successful emergence being only 12.5% at 10.5 µg/L compared to a 77.5% control emergence success. This led to their generation of a No Observable Effect Concentration (NOEC) of 6.5 µg/L and a Lowest Observable Effect Concentration (LOEC) of 10.5 µg/L. Median effect concentrations (EC50 using emergence) were not presented.

Based on these studies with *C. riparius* and *C. dilutus*, it appears that imidacloprid, clothianidin and thiamethoxam have comparable sublethal toxicities to midge larvae (assessed using emergence rates) as to *Deleatidium* nymphs (assessed using mortality, impairment and immobility) and perhaps to those of other mayfly nymphs, although more data are needed to test this possibility. Regardless, the common finding that aquatic insect taxa are most sensitive to neonicotinoids among aquatic invertebrates is supported, with chronic effect concentrations for imidacloprid and clothianidin being frequently < 1 µg/L and ~10 µg/L (an order of magnitude higher) for thiamethoxam. These findings are encouraging for risk assessment procedures employing *Chironomus* spp. as test organisms. Given mayflies have been found to be the most sensitive taxa to neonicotinoids (Morrissey et al. 2015), it would seem that neonicotinoid risk assessments using *Chironomus* spp. may also be protective for the most vulnerable freshwater taxa.

## Effects on moulting propensity

*Deleatidium* moulting propensity decreased with increasing exposure to imidacloprid and clothianidin in the second and third weeks of exposure, which was likely related to increased impairment and immobility of the mayflies. In week 4, mortality in the imidacloprid treatments with concentrations above 0.4 µg/L was probably so high that moulting was too rare to allow detecting a significant imidacloprid impact. Because moulting is controlled by the neuroendocrine system, neonicotinoid exposure could have directly disrupted moulting processes, though only a handful of studies have investigated this phenomenon and mechanistic explanations remain unstudied. For example, Song et al. (1997) observed a reduction in successful moulting and increased moult-related mortality in Yellow-fever mosquito larvae, *Aedes aegypti*, with increasing concentration of imidacloprid over a 48-h exposure. They hypothesised that neurological disruption induced by imidacloprid may interrupt the moulting process, causing death during moulting. Further, in the abovementioned chronic experiments with *C. dilutus*, Cavallaro et al. (2017) found that imidacloprid, clothianidin and thiamethoxam all caused moult-related mortality during emergence. Similarly, Stoughton et al. (2008), who tested the effects of imidacloprid on the life-cycle of another midge, *Chironomus tentans*, observed a 55% reduction in emergence (final moult) under chronic exposure to 1.14 µg/L imidacloprid compared to controls after 28 days. These findings suggest there may indeed be the potential for neonicotinoid-induced moulting disruption, although the specific mechanisms behind this remain unclear.

During our experiments, *Deleatidium* nymph mortality associated with incomplete moulting was irregularly observed but was not recorded. Regardless, the trend toward reduced moulting frequency with exposure to increasing concentration of imidacloprid and clothianidin matches a pattern observed in 96-h exposures to imidacloprid with *Deleatidium* nymphs (Macaulay et al. 2019). In this experiment, moulting was significantly reduced compared to controls with acute exposure to 8 µg/L imidacloprid and further reduced at 40 µg/L. Whether there is more to these patterns than a simple reflection of the overall effect of impaired mayfly mobility caused by these insecticides requires further investigation.

## Relative toxicities of neonicotinoids

The relative toxicities of imidacloprid, clothianidin and thiamethoxam to non-target aquatic insect taxa discussed above contrast with the findings of Van den Brink et al. (2016), who found thiamethoxam to be just as toxic to *C. dipterum* nymphs as imidacloprid. Their findings were supported by one comparison in Morrissey et al.'s (2015) review, where the latter authors calculated the geometric mean of all available acute L[E]C50s for aquatic insects and found no difference between imidacloprid and thiamethoxam. However, Morrissey et al. (2015) also ranked the available acute toxicity data for *C. riparius* by comparing the acute LC50 for imidacloprid (20 µg/L; 0.08 µmol/L) to the acute EC50s for clothianidin (22 µg/L; 0.09 µmol/L) and thiamethoxam (35 µg/L; 0.12 µmol/L), a ranking which supports the trends observed for larvae of *C. dilutus* and mayfly nymphs in the present experiment and several

other recent studies (Bartlett et al. 2018; Cavallaro et al. 2017; Maloney et al. 2018a; Raby et al. 2018b; Raby et al. 2018c). In all these studies, imidacloprid and clothianidin were more toxic than thiamethoxam, with imidacloprid also regularly displaying a higher toxicity than clothianidin. Hazardous concentrations for 5% of species (HC5s) were determined using toxicity data for crustaceans by Whiteside et al. (2008). Although their assessment was not based on neonicotinoid toxicities to aquatic insects, they also concluded imidacloprid presented the greatest risk to crustaceans with an HC5 of 0.70 µg/L, followed by clothianidin (HC5 = 39 µg/L) and thiamethoxam was the least toxic with an HC5 of 430 µg/L.

## Conclusions and further research

More toxicity data are needed to elucidate the relative toxicities of different neonicotinoids to non-target invertebrates, and Morrissey et al. (2015) especially highlighted a lack of toxicity data for clothianidin (particularly for insect taxa). While the observed differences in toxicity among neonicotinoids are relatively minor compared to the differences in toxicity among taxonomic groups, when we consider the environmentally relevant concentrations and the most sensitive aquatic insect taxa, they are still relevant. For instance, the geometric mean for average global surface water concentrations of 0.73 µg/L for imidacloprid calculated by Sánchez-Bayo et al. (2016) is higher than the 28-day LC50 and EC50 for imidacloprid we calculated for *Deleatidium* (0.28 and 0.19 µg/L, respectively). In contrast, the 28-day LC50s we calculated for clothianidin and thiamethoxam (1.36 and > 4 µg/L, respectively) are well above this concentration (Sánchez-Bayo et al. 2016 do not report the specific geometric means for these compounds in their review). In New Zealand, neonicotinoids are registered for use to control pests of a variety of pasture and forage crops (Chapman 2010), however their usage and concentrations in surface waters are currently unmonitored. Very little is therefore known about the detection frequencies, concentrations and potential ecological impacts of neonicotinoids and other pesticides in New Zealand streams.

Full-factorial mixture experiments would allow more accurate evaluation of the comparative toxicities between the three neonicotinoids. Such experiments would also elucidate the interactive effects of multiple neonicotinoids acting simultaneously—a scenario which has been shown to occur in surveys of surface waters in many locations (Hladik and Kolpin 2016; Main et al. 2014; Sánchez-Bayo and Hyne 2014). These interactions were predicted to be additive rather than synergistic because of their shared modes of action, which suggests a concentration-addition model of combined toxicity (Rodney et al. 2013;Morrissey et al. 2015). However, several studies have found that this prediction does not always hold (Loureiro et al. 2010; Pavlaki et al. 2011;Maloney et al. 2017; Maloney et al. 2018a) with varying antagonistic and synergistic effects observed, though these rarely met accepted thresholds of model deviations, and mainly additive effects have been observed in field trials (Maloney et al. 2018b; Rico et al. 2018). More tests with sensitive freshwater species such as *Deleatidium* spp. mayflies in environmentally realistic experiments would be beneficial to improve our understanding of these multiple-stressor scenarios.

## Supplemental Data

The supporting information are available in the online version of this manuscript

## Data Accessibility

Data are deposited in figshare at DOI:

## References

Anderson JC, Dubetz C, Palace VP. 2015. Neonicotinoids in the Canadian aquatic environment: A literature review on current use products with a focus on fate, exposure, and biological effects. Sci Total Environ. 505:409-422.

Bartlett AJ, Hedges AM, Intini KD, Brown LR, Maisonneuve FJ, Robinson SA, Gillis PL, de Solla SR. 2018. Lethal and sublethal toxicity of neonicotinoid and butenolide insecticides to the mayfly, *Hexagenia* spp. Environ Pollut. 238:63-75.

Camp AA, Buchwalter DB. 2016. Can't take the heat: Temperature-enhanced toxicity in the mayfly *Isonychia bicolor* exposed to the neonicotinoid insecticide imidacloprid. Aquat Toxicol. 178:49-57.

Cavallaro MC, Morrissey CA, Headley JV, Peru KM, Liber K. 2017. Comparative chronic toxicity of imidacloprid, clothianidin, and thiamethoxam to *Chironomus dilutus* and estimation of toxic equivalency factors. Environ Toxicol Chem. 36(2):372-382.

Chapman RB. 2010. A review of insecticide use on pastures and forage crops in New Zealand. Christchurch, NZ: Insect Science Ltd.

Domencich TA. 1975. Urban travel demand: a behavioral analysis: a Charles River Associates research study. Amsterdam: New York: North-Holland Pub. Co. American Elsevier.

EFSA. 2013. Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. EFSA Journal. 11(7):268.

Finnegan MC, Baxter LR, Maul JD, Hanson ML, Hoekstra PF. 2017. Comprehensive characterization of the acute and chronic toxicity of the neonicotinoid insecticide thiamethoxam to a suite of aquatic primary producers, invertebrates, and fish. Environ Toxicol Chem. 36(10):2838-2848.

Heugens EHW, Hendriks AJ, Dekker T, Straalen NMv, Admiraal W. 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Crit Rev Toxicol. 31(3):247-284.

Hladik ML, Kolpin DW. 2016. First national-scale reconnaissance of neonicotinoid insecticides in streams across the USA. Environmental Chemistry. 13(1):12-20.

Hunn JG. 2016. Imidacloprid effects on New Zealand mayfly nymphs: acute and chronic exposure and interactions with natural stressors. Bsc Honours Thesis. University of Otago.

Huryn AD. 1996. Temperature-dependent growth and life cycle of *Deleatidium* (Ephemeroptera: Leptophlebiidae) in two high country streams in New Zealand. Freshwat Biol. 36(2):351-361.

Langer-Jaesrich M, Köhler H-R, Gerhardt A. 2010. Assessing toxicity of the insecticide thiacloprid on *Chironomus riparius* (Insecta: Diptera) using multiple end points. Arch Environ Contam Toxicol. 58(4):963-972.

Loureiro S, Svendsen C, Ferreira ALG, Pinheiro C, Ribeiro F, Soares AMVM. 2010. Toxicity of three binary mixtures to *Daphnia magna*: Comparing chemical modes of action and deviations from conceptual models. Environ Toxicol Chem. 29(8):1716-1726.

Macaulay SJ, Buchwalter DB, Matthaei CD. 2019. Water temperature interacts with the insecticide imidacloprid to alter acute lethal and sublethal toxicity to mayfly larvae. N Z J Mar Freshwat Res.1-16.

Main AR, Headley JV, Peru KM, Michel NL, Cessna AJ, Morrissey CA. 2014. Widespread use and frequent detection of neonicotinoid insecticides in wetlands of Canada's Prairie Pothole Region. Plos One. 9(3):e92821.

Maloney EM, Morrissey CA, Headley JV, Peru KM, Liber K. 2017. Cumulative toxicity of neonicotinoid insecticide mixtures to *Chironomus dilutus* under acute exposure scenarios. Environ Toxicol Chem. 36(11):3091-3101.

Maloney EM, Morrissey CA, Headley JV, Peru KM, Liber K. 2018a. Can chronic exposure to imidacloprid, clothianidin, and thiamethoxam mixtures exert greater than additive toxicity in *Chironomus dilutus*? Ecotoxicol Environ Saf. 156:354-365.

Maloney EM, Liber K, Headley JV, Peru KM, Morrissey CA. 2018b. Neonicotinoid insecticide mixtures: Evaluation of laboratory-based toxicity predictions under semi-controlled field conditions. Environ Pollut. 243:1727-1739.

Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M, Sattelle DB. 2001. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. Trends Pharmacol Sci. 22(11):573-580.

McFadden D. 1974. Conditional logit analysis of qualitative choice behavior. In: Zarembka P, editor. Frontiers in Econometrics. Berkeley, California: Academic Press. p. 105-142.

McFadden D. 1977. Quantitative methods for analyzing travel behaviour of individuals: Some recent developments. CT, USA: Cowles Foundation for Research in Economics, Yale University.

McIntosh AR, Townsend CR. 1994. Interpopulation variation in mayfly antipredator tactics: differential effects of contrasting predatory fish. Ecology. (7):2078-2090.

Morrissey CA, Mineau P, Devries JH, Sánchez-Bayo F, Liess M, Cavallaro MC, Liber K. 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: a review. Environ Int. 74:291-303.

Pavlaki MD, Pereira R, Loureiro S, Soares AMVM. 2011. Effects of binary mixtures on the life traits of *Daphnia magna*. Ecotoxicol Environ Saf. 74(1):99-110.

Peckarsky BL, McIntosh AR. 1998. Fitness and community consequences of avoiding multiple predators. Oecologia. 113(4):565-576.

R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Raby M, Zhao XM, Hao CY, Poirier DG, Sibley PK. 2018a. Chronic effects of an environmentally-relevant, short-term neonicotinoid insecticide pulse on four aquatic invertebrates. Sci Total Environ. 639:1543-1552.

Raby M, Zhao X, Hao C, Poirier DG, Sibley PK. 2018b. Chronic toxicity of 6 neonicotinoid insecticides to *Chironomus dilutus* and *Neocloeon triangulifer*. Environ Toxicol Chem. 37(10):2727-2739.

Raby M, Nowierski M, Perlov D, Zhao XM, Hao CY, Poirier DG, Sibley PK. 2018c. Acute toxicity of 6 neonicotinoid insecticides to freshwater invertebrates. Environ Toxicol Chem. 37(5):1430-1445.

Rodney SI, Teed RS, Moore DRJ. 2013. Estimating the toxicity of pesticide mixtures to aquatic organisms: A review. Hum Ecol Risk Assess. 19(6):1557-1575.

Roessink I, Merga LB, Zweers HJ, Van den Brink PJ. 2013. The neonicotinoid imidacloprid shows high chronic toxicity to mayfly nymphs. Environ Toxicol Chem. 32(5):1096-1100.

Rico A, Arenas-Sánchez A, Pasqualini J, García-Astillero A, Cherta L, Nozal L, Vighi M. 2018. Effects of imidacloprid and a neonicotinoid mixture on aquatic invertebrate communities under Mediterranean conditions. Aquat Toxicol. 204:130-143.

Sánchez-Bayo F. 2006. Comparative acute toxicity of organic pollutants and reference values for crustaceans. I. Branchiopoda, Copepoda and Ostracoda. Environ Pollut. 139(3):385-420.

Sánchez-Bayo F, Goka K, Hayasaka D. 2016. Contamination of the aquatic environment with neonicotinoids and its implication for ecosystems. Frontiers in Environmental Science. 4:1-14.

Sánchez-Bayo F, Hyne RV. 2014. Detection and analysis of neonicotinoids in river waters - Development of a passive sampler for three commonly used insecticides. Chemosphere. 99:143-151.

Saraiva AS, Sarmento RA, Rodrigues ACM, Campos D, Fedorova G, Zlabek V, Gravato C, Pestana JLT, Soares A. 2017. Assessment of thiamethoxam toxicity to *Chironomus riparius*. Ecotoxicol Environ Saf. 137:240-246.

Scrimgeour GJ. 1991. Life history and production of *Deleatidium* (Ephemeroptera: Leptophlebiidae) in an unstable New Zealand river. N Z J Mar Freshwat Res. 25(1):93-99.

Smith AD, Bharath A, Mallard C, Orr D, Smith K, Sutton JA, Vukmanich J, McCarty LS, Ozburn GW. 1991. The acute and chronic toxicity of ten chlorinated organic compounds to the American flagfish (*Jordanella floridae*). Arch Environ Contam Toxicol. 20(1):94-102.

Song MY, Stark JD, Brown JJ. 1997. Comparative toxicity of four insecticides, including imidacloprid and tebufenozide, to four aquatic arthropods. Environ Toxicol Chem. 16(12):2494-2500.

Stoughton SJ, Liber K, Culp J, Cessna A. 2008. Acute and chronic toxicity of imidacloprid to the aquatic invertebrates *Chironomus tentans* and *Hyalella azteca* under constant-and pulse-exposure conditions. Arch Environ Contam Toxicol. 54(4):662-673.

Taylor BW, McIntosh AR, Peckarsky BL. 2001. Sampling stream invertebrates using electroshocking techniques: implications for basic and applied research. Can J Fish Aquat Sci. 58(3):437-445.

Tennekes HA. 2010. The significance of the Druckrey-Küpfmüller equation for risk assessment--the toxicity of neonicotinoid insecticides to arthropods is reinforced by exposure time. Toxicology. 276(1):1-4.

Tennekes HA, Sánchez-Bayo F. 2011. Time-dependent toxicity of neonicotinoids and other toxicants: Implications for a new approach to risk assessment. Journal of Environmental & Analytical Toxicology. 01(S4).

Tennekes HA, Sánchez-Bayo F. 2013. The molecular basis of simple relationships between exposure concentration and toxic effects with time. Toxicology. 309:39-51.

United States Environmental Protection Agency. 2007. Method 8280B, Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans by high resolution gas chromatography/low resolution mass spectrometry (HRGC/LRMS). Available from: https://www.epa.gov/sites/production/files/2015-12/documents/8280b.pdf

Van den Brink PJ, Van Smeden JM, Bekele RS, Dierick W, De Gelder DM, Noteboom M, Roessink I. 2016. Acute and chronic toxicity of neonicotinoids to nymphs of a mayfly species and some notes on seasonal differences. Environ Toxicol Chem. 35(1):128-133.

van Wijngaarden RP, Maltby L, Brock TC. 2015. Acute tier-1 and tier-2 effect assessment approaches in the EFSA Aquatic Guidance Document: are they sufficiently protective for insecticides? Pest Manage Sci. 71(8):1059-1067

Venables WN, Ripley BD. 2002. Generalized linear models. In Chambers J, Eddy W, Härdle W, Sheather S, Tierney L, eds, *Modern Applied Statistics with S,* 4th ed–Statistics and Computing. Springer, New York, NY, U.S.A., pp 183–210.

Whiteside M, Mineau P, Morrison C, Knopper LD. 2008. Comparison of a score-based approach with risk-based ranking of in-use agricultural pesticides in Canada to aquatic receptors. Integr Environ Assess Manage. 4(2):215-236.

Winterbourn M. 1974. The life histories, trophic relations and production of *Stenoperla prasina* (Plecoptera) and *Deleatidium* spp. (Ephemeroptera) in a New Zealand river. Freshwat Biol. 4(6):507-524.

Wood TJ, Goulson D. 2017. The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. Environmental Science and Pollution Research International. 24(21):17285-17325.

Figures

**Figure 1.** Mean mayfly survivorship over 28 days of exposure to a) imidacloprid b) clothianidin, and c) thiamethoxam. Error bars are +/- standard error ($n$ = 5). Solid red lines represent slopes of the log-logistic regressions; dotted blue lines are 95% CIs.

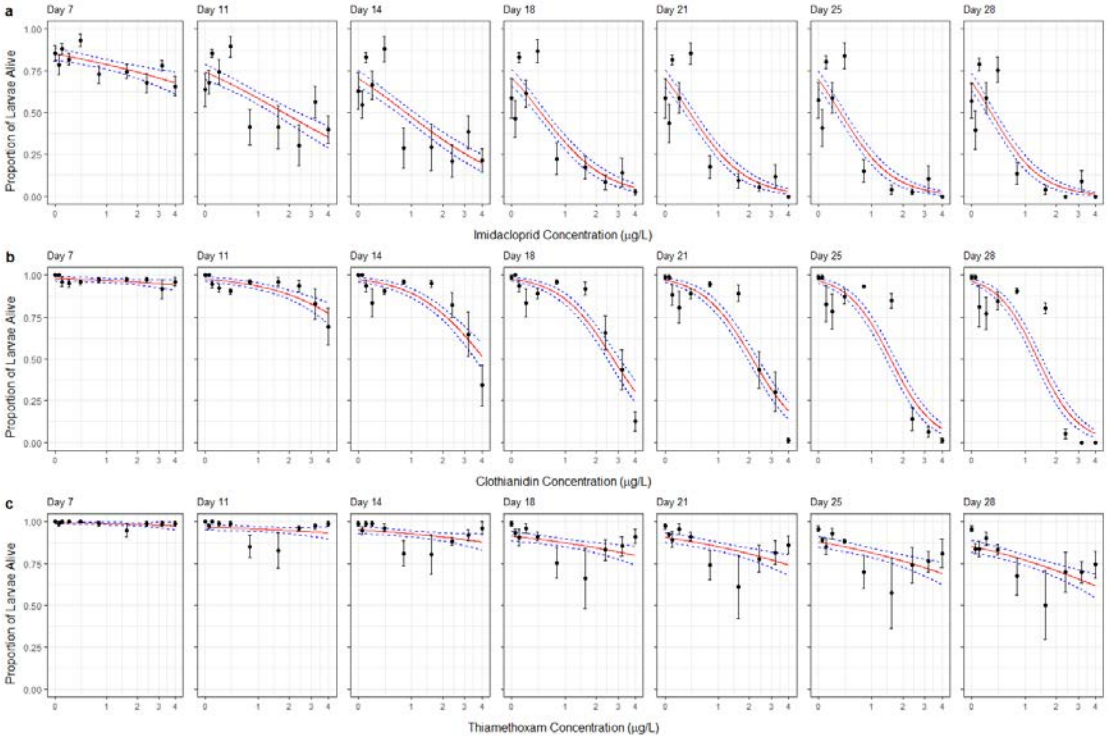

**Figure 2.** Mean mayfly immobility (including mortality) over 28 days of exposure to a) imidacloprid b) clothianidin, and c) thiamethoxam. Error bars are +/- standard error ($n$ = 5). Solid red lines represent slopes of the log-logistic regressions; dotted blue lines are 95% CIs.

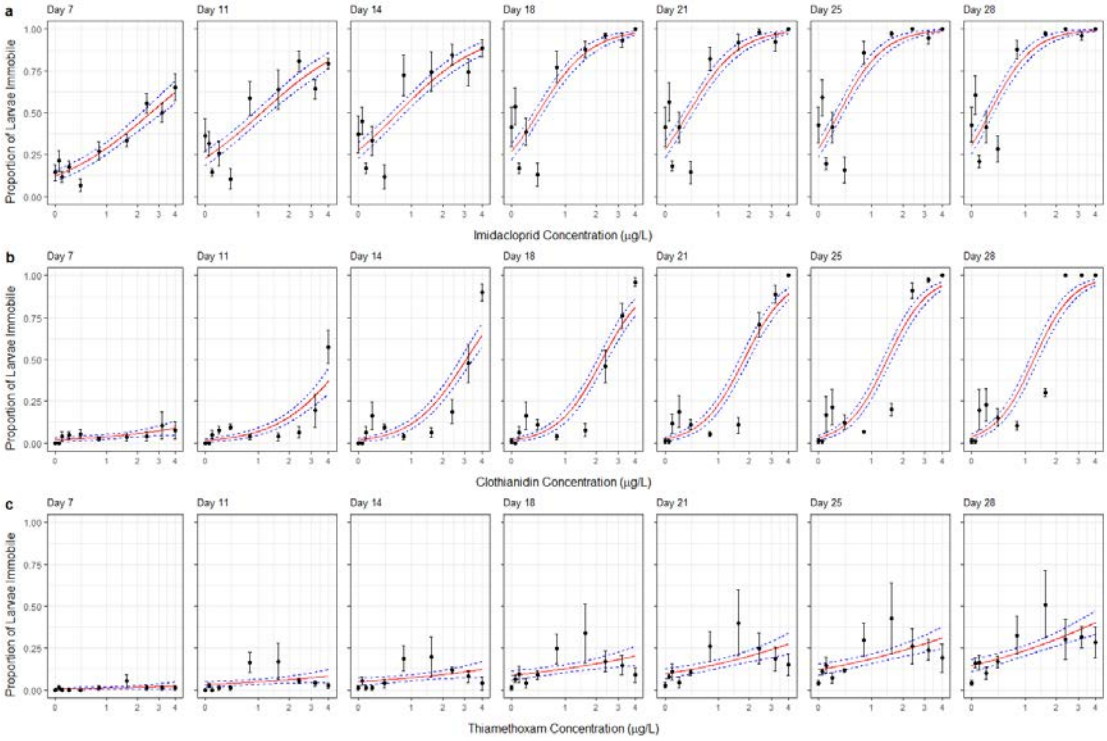

**Figure 3.** Mean mayfly impairment (including immobility and mortality) over 28 days of exposure to a) imidacloprid b) clothianidin, and c) thiamethoxam. Error bars are +/- standard error ($n$ = 5). Solid red lines represent slopes of the log-logistic regressions; dotted blue lines are 95% CIs.

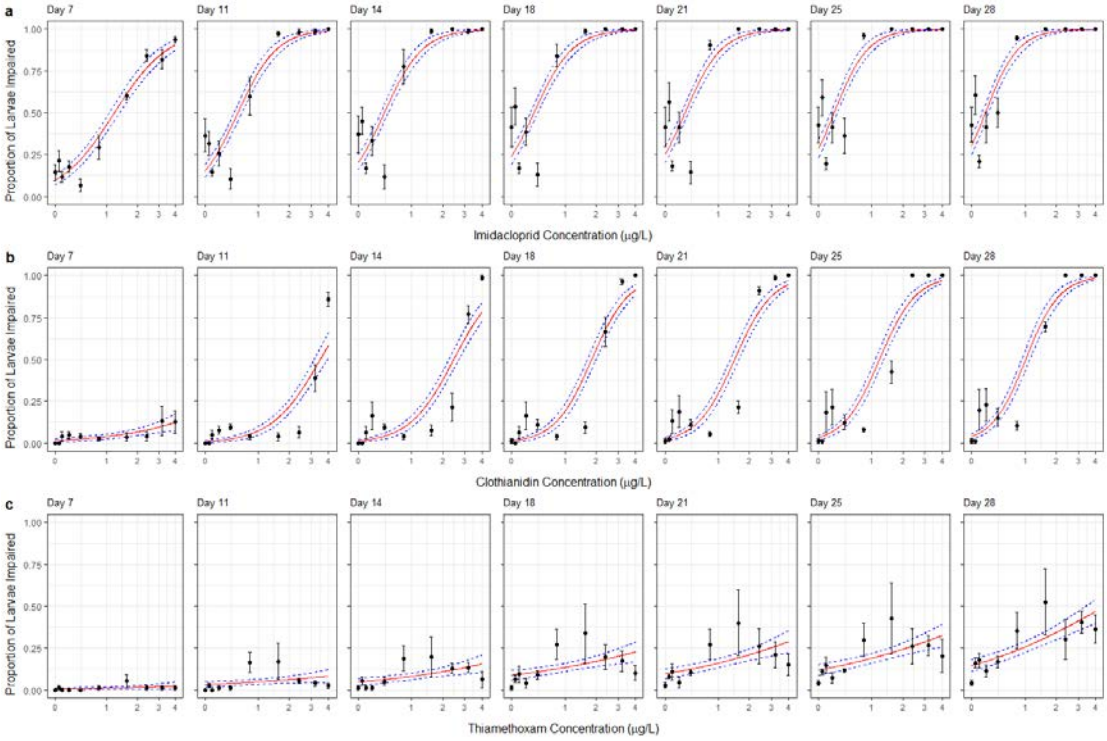

**Figure 4.** Mean mayfly moulting propensity (proportion of moulted *Deleatidium* nymphs that were alive at the start of each week) over 28 days of exposure to a) imidacloprid b) clothianidin, and c) thiamethoxam. Error bars are +/- standard error ($n$ = 5). Solid red lines represent slopes of the log-logistic regressions; dotted blue lines are 95% CIs.

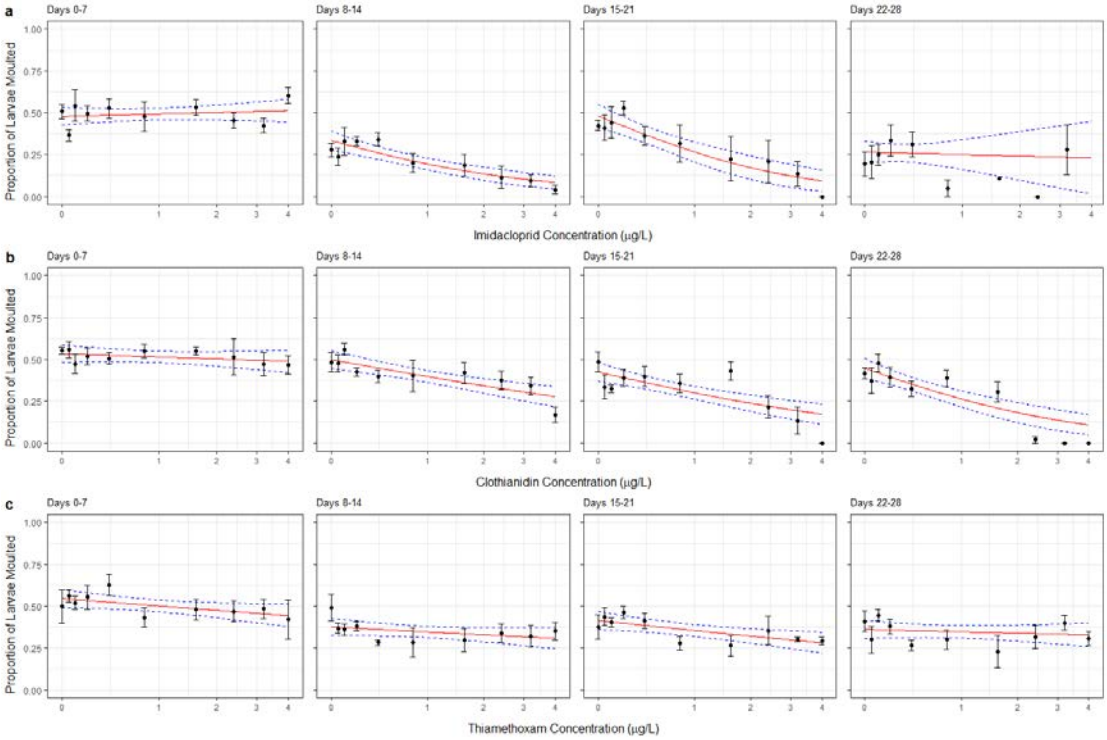


**Table 1. Results summary of log-logistic generalized linear models for *Deleatidium* survivorship, immobility (including mortality) and impairment (including immobility and mortality) responses from 28-day chronic exposures to imidacloprid, clothianidin and thiamethoxam (*p*-values for all regressions are < 0.001 unless stated otherwise)**

| | | Survivorship | | | | | Immobility | | | | | Impairment | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Experiment | Day | LC50 (µg/L) | (95% CI) | Slope[a] | $\rho^2$ | Control mortality (%) | IC50 (µg/L) | (95% CI) | Slope[a] | $\rho^2$ | Control immobility (%) | EC50 (µg/L) | (95% CI) | Slope[a] | $\rho^2$ |
| Imidacloprid[b] | 7 | >4 | NC | -0.6 | 0.21 | 14.5 | 2.60 | (2.13 −3.13) | 1.5 | 0.62 | 14.5 | 1.21 | (1.06 −1.36) | 2.8 | 0.82 |
| | 1 | 1.7 | (1.36 –2.29 | -1. | 0.2 | 36.3 | 1.0 | (0.89 −1.30 | 1. | 0.4 | 36.3 | 0.5 | (0.46 −0.61 | 4. | 0. |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 8 | ) | 0 | 5 | | 9 | ) | 6 | 9 | | 3 | ) | 0 | 74 |
| | 14 | 0.86 | (0.66−1.07) | -1.4 | 0.32 | 37.0 | 0.68 | (0.54−0.84) | 1.8 | 0.44 | 37.0 | 0.40 | (0.34−0.47) | 4.0 | 0.68 |
| | 18 | 0.46 | (0.36−0.57) | -2.4 | 0.52 | 41.4 | 0.41 | (0.33−0.5) | 2.9 | 0.59 | 41.4 | 0.34 | (0.27−0.4) | 4.0 | 0.66 |
| | 21 | 0.38 | (0.29−0.47) | -2.7 | 0.55 | 41.4 | 0.36 | (0.28−0.44) | 3.0 | 0.59 | 41.4 | 0.30 | (0.24−0.36) | 4.2 | 0.66 |
| | 25 | 0.33 | (0.25−0.41) | -2.9 | 0.66 | 42.8 | 0.31 | (0.24−0.38) | 3.4 | 0.61 | 42.8 | 0.22 | (0.17−0.28) | 4.6 | 0.7 |
| | 28 | 0.28 | (0.21−0.36) | -3.1 | 0.59 | 42.9 | 0.26 | (0.2−0.33) | 3.5 | 0.66 | 42.9 | 0.19 | (0.14−0.25) | 4.7 | 0.74 |
| Clothianidin | 7 | >4[c] | NC | -0.7 | 0.07 | 0.0 | >4[d] | NC | 1.0 | 0.13 | 0.0 | >4 | NC | 1.4 | 0.23 |
| | 11 | >4 | NC | -1.6 | 0.35 | 0.0 | >4 | NC | 2.2 | 0.49 | 0.0 | 3.48 | (3.11−3.89) | 3.1 | 0.63 |
| | 14 | >4 | NC | -2.2 | 0.49 | 0.0 | 3.07 | (2.74−3.44) | 2.9 | 0.59 | 0.0 | 2.46 | (2.24−2.69) | 3.5 | 0.67 |
| | 18 | 2.75 | (2.46−3.06) | -2.9 | 0.64 | 1.3 | 2.25 | (2.05−2.47) | 3.4 | 0.71 | 1.3 | 1.83 | (1.68−2.00) | 4.2 | 0.76 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 21 | 2.12 | (1.91−2.34) | -3.1 | 0.66 | 1.3 | 1.80 | (1.63−1.97) | 3.6 | 0.73 | 1.3 | 1.47 | (1.33−1.62) | 4.1 | 0.77 |
| | 25 | 1.56 | (1.41−1.72) | -3.6 | 0.67 | 1.3 | 1.45 | (1.31−1.60) | 3.8 | 0.72 | 1.3 | 1.21 | (1.09−1.34) | 4.2 | 0.76 |
| | 28 | 1.36 | (1.22−1.50) | -3.8 | 0.71 | 1.3 | 1.24 | (1.12−1.38) | 4.0 | 0.73 | 1.3 | 1.02 | (0.91−1.13) | 4.6 | 0.8 |
| Thiamethoxam[e] | 14 | >4[f] | NC | -0.6 | 0.06 | 1.3 | >4[f] | NC | 0.6 | 0.06 | 1.3 | >4 | NC | 0.8 | 0.11 |
| | 18 | >4 | NC | -0.6 | 0.07 | 1.3 | >4 | NC | 0.6 | 0.07 | 1.3 | >4 | NC | 0.7 | 0.08 |
| | 21 | >4 | NC | -0.8 | 0.1 | 2.8 | >4 | NC | 0.8 | 0.11 | 2.8 | >4 | NC | 0.8 | 0.12 |
| | 25 | >4 | NC | -0.7 | 0.11 | 4.2 | >4 | NC | 0.7 | 0.11 | 4.2 | >4 | NC | 0.8 | 0.12 |
| | 28 | >4 | NC | -0.8 | 0.14 | 4.2 | >4 | NC | 0.9 | 0.16 | 4.2 | >4 | NC | 1 | 0.23 |

[a]Slope estimates of the log-logistic regression curves.

[b]Because the control immobility in the imidacloprid test exceeded 10%, these results should be considered indicative.

[c]$p = 0.04$

[d]**$p$ = 0.001**

[e]**Regressions for thiamethoxam before day 14 were not significant to $p$ = 0.01 so are not presented.**

[f]**$p$ = 0.008**

**LC50 = median lethal concentration; CI = confidence interval; IC50 = median immobilizing concentration; EC50 = median effective concentration; NC = confidence interval could not be calculated.**

**Table 2. Results summary of log-logistic generalized linear models for *Deleatidium* mayfly moulting propensity from 28-day chronic exposures to imidacloprid, clothianidin and thiamethoxam ($p$-values are bolded where $p < 0.01$)**

| | | Moulting Propensity | | | |
|---|---|---|---|---|---|
| **Experiment** | **Day** | **df[a]** | ***p*-Value** | **Slope[b]** | **$\rho^2$** |
| **Imidacloprid** | **7** | **49** | **0.5** | **0.68** | **0.01** |
| | **14** | **49** | **<0.001** | **-1.05** | **0.35** |
| | **21** | **46** | **<0.001** | **-1.36** | **0.38** |
| | **28** | **37** | **0.8** | **-0.11** | **<0.01** |
| **Clothianidin** | **7** | **49** | **0.4** | **-0.11** | **0.02** |
| | **14** | **49** | **<0.001** | **-0.59** | **0.29** |
| | **21** | **49** | **<0.001** | **-0.79** | **0.22** |
| | **28** | **45** | **<0.001** | **-1.18** | **0.34** |
| **Thiamethoxam** | **7** | **49** | **0.04** | **-0.25** | **0.05** |
| | **14** | **49** | **0.2** | **-0.19** | **0.04** |
| | **21** | **49** | **0.007** | **-0.37** | **0.15** |

| | | | | |
|---|---|---|---|---|
| 28 | 49 | 0.5 | -0.09 | <0.01 |

[a]Degrees of freedom

[b]Slope estimates of the log-logistic regression curves

**Table 3. Published chronic (≥28 day) imidacloprid, clothianidin and thiamethoxam median lethal (LC50; when available) and effective (EC50) concentrations (µg/L) with 95% confidence intervals (CI; when available) for the mayfly (Ephemeroptera) and midge (Diptera) species tested.**

| Neonicotinoid | Order | Species/genus | Duration | LC50 | CI | EC50 | CI | Study |
|---|---|---|---|---|---|---|---|---|
| Imidacloprid | Ephemeroptera | Deleatidium spp. | 28 d | 0.28 | (0.21 - 0.36) | 0.19 | (0.14 - 0.25) | Present Study |
| | Ephemeroptera | Neocloeon triangulifer | 32 d | | | 1.75 | (1.42 - 2.09) | Raby et al. (2018) |
| | Ephemeroptera | Cloeon dipterum | 28 d | 0.85 | NA | 0.68 | (0.45 -1) | Van den Brink et al. (2016) |
| | Ephemeroptera | Cloeon dipterum | 28 d | 0.2 | (0.11 - 0.34) | 0.12 | (0.08 - 0.20) | Roessink et al. (2013) |
| | Ephemeroptera | Caenis horaria | 28 d | 0.32 | NA | 0.13 | (0.07 - 0.23) | Roessink et al. (2013) |
| | Diptera | Chironomus dilutus | 56 d | | | 0.24 | (0.22 - 0.27) | Raby et al. (2018) |
| | Diptera | Chironom | 28 d | | | 0.5 | (0.37 - | Maloney et al. (2018) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **a** | **us dilutus** | | | | | **0.59)** | |
| | **Diptera** | **Chironomus dilutus** | **40 d** | | | **0.39** | **(0.31 - 0.42)** | **Cavallaro et al. (2017)** |
| | **Diptera** | **Chironomus tentans** | **28 d** | | | **0.91** | **(0.73 - 1.12)** | **Stoughton et al. (2008)** |
| | **Diptera** | **Chironomus riparius** | **28 d** | | | **0.125, 0.625 (NOEC, LOEC†)** | **NA** | **Naveen et al. (2018)** |
| | **Diptera** | **Chaoborus obscuripes** | **28 d** | **12.6** | **(7.33 - 21.6)** | **11.8** | **(8.17 - 17.1)** | **Roessink et al. (2013)** |
| **Clothianidin** | **Ephemeroptera** | **Deleatidium spp.** | **28 d** | **1.36** | **(1.22 -1.5)** | **1.02** | **(0.91 - 1.13)** | **Present Study** |
| | **Ephemeroptera** | **Neocloeon triangulifer** | **32 d** | | | **0.91** | **(0.39 - 1.43)** | **Raby et al. (2018)** |
| | **Diptera** | **Chironomus dilutus** | **56 d** | | | **0.68** | **(0.60 - 0.77)** | **Raby et al. (2018)** |
| | **Diptera** | **Chironomus dilutus** | **28 d** | | | **0.71** | **(0.50 - 0.85)** | **Maloney et al. (2018)** |
| | **Diptera** | **Chironomus dilutus** | **40 d** | | | **0.28** | **(0.20 - 0.33)** | **Cavallaro et al. (2017)** |
| | **Diptera** | **Chironomus riparius** | **28 d** | | | **1** | **NA** | **Drottar et al. (2000) in Morrissey et al. (2015)** |
| **Thiamethoxa** | **Ephemeropte** | **Deleatidiu** | **28 d** | **>** | **NA** | **> 4** | **NA** | **Present Study** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **m** | **ra** | **m spp.** | | **4** | | | |
| | **Ephemeroptera** | **Neocloeon triangulifer** | **32 d** | | **2.18** | **(1.60 - 3.20)** | **Raby et al. (2018)** |
| | **Ephemeroptera** | **Cloeon dipterum** | **28 d** | | **0.68** | **(0.38 -1.2)** | **Van den Brink et al. (2016)** |
| | **Diptera** | **Chironomus dilutus** | **56 d** | | **12.95** | **(8.54 - 17.35)** | **Raby et al. (2018)** |
| | **Diptera** | **Chironomus dilutus** | **28 d** | | **8.91** | **(5.79 - 12.37)** | **Maloney et al. (2018)** |
| | **Diptera** | **Chironomus dilutus** | **40 d** | | **4.13** | **(3.53 - 4.76)** | **Cavallaro et al. (2017)** |
| | **Diptera** | **Chironomus riparius** | **30 d** | | **11.4** | **NA** | **Finnegan et al. (2017)** |
| | **Diptera** | **Chironomus riparius** | **28 d** | | **6.5, 10.5 (NOEC, LOEC†)** | **NA** | **Saraiva et al. (2017)** |
| | **Diptera** | **Chaoborus sp.** | **34 d** | | **480** | **NA** | **Finnegan et al. (2017)** |

**†No/lowest observable effect concentrations.**

## References

Cavallaro MC, Morrissey CA, Headley JV, Peru KM, Liber K. 2017. Comparative chronic toxicity of imidacloprid, clothianidin, and thiamethoxam to *Chironomus dilutus* and estimation of toxic equivalency factors. Environ Toxicol Chem. 36(2):372-382.

Finnegan MC, Baxter LR, Maul JD, Hanson ML, Hoekstra PF. 2017. Comprehensive characterization of the acute and chronic toxicity of the neonicotinoid insecticide thiamethoxam to a suite of aquatic primary producers, invertebrates, and fish. Environ Toxicol Chem. 36(10):2838-2848.

Maloney EM, Morrissey CA, Headley JV, Peru KM, Liber K. 2018. Can chronic exposure to imidacloprid, clothianidin, and thiamethoxam mixtures exert greater than additive toxicity in *Chironomus dilutus*? Ecotoxicol Environ Saf. 156:354-365.

Morrissey CA, Mineau P, Devries JH, Sánchez-Bayo F, Liess M, Cavallaro MC, Liber K. 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: a review. Environ Int. 74:291-303.

Naveen NC, Fojtova D, Blahova L, Rozmankova E, Blaha L. 2018. Acute and (sub)chronic toxicity of the neonicotinoid imidacloprid on *Chironomus riparius*. Chemosphere. 209:568-577.

Raby M, Zhao X, Hao C, Poirier DG, Sibley PK. 2018. Chronic toxicity of 6 neonicotinoid insecticides to *Chironomus dilutus* and *Neocloeon triangulifer*. Environ Toxicol Chem. 37(10):2727-2739.

Roessink I, Merga LB, Zweers HJ, Van den Brink PJ. 2013. The neonicotinoid imidacloprid shows high chronic toxicity to mayfly nymphs. Environ Toxicol Chem. 32(5):1096-1100.

Saraiva AS, Sarmento RA, Rodrigues ACM, Campos D, Fedorova G, Zlabek V, Gravato C, Pestana JLT, Soares A. 2017. Assessment of thiamethoxam toxicity to *Chironomus riparius*. Ecotoxicol Environ Saf. 137:240-246.

Stoughton SJ, Liber K, Culp J, Cessna A. 2008. Acute and chronic toxicity of imidacloprid to the aquatic invertebrates *Chironomus tentans* and *Hyalella azteca* under constant-and pulse-exposure conditions. Arch Environ Contam Toxicol. 54(4):662-673.

Van den Brink PJ, Van Smeden JM, Bekele RS, Dierick W, De Gelder DM, Noteboom M, Roessink I. 2016. Acute and chronic toxicity of neonicotinoids to nymphs of a mayfly species and some notes on seasonal differences. Environ Toxicol Chem. 35(1):128-133.