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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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Abstract: Nontarget aquatic insects are susceptible to chronic neonicotinoid insecticide exposure during the early stages of development from repeated runoff events and prolonged persistence of these chemicals. Investigations on the chronic toxicity of neonicotinoids to aquatic invertebrates have been limited to a few species and under different laboratory conditions that often preclude direct comparisons of the relative toxicity of different compounds. In the present study, full life-cycle toxicity tests using *Chironomus dilutus* were performed to compare the toxicity of 3 commonly used neonicotinoids: imidacloprid, clothianidin, and thiamethoxam. Test conditions followed a static-renewal exposure protocol in which lethal and sublethal 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-d median lethal concentrations for imidacloprid, clothianidin, and thiamethoxam were 1.52 μg/L, 2.41 μg/L, and 23.60 μg/L, respectively. The 40-d median effect concentrations (emergence) 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 3-point response average of equivalencies calculated at 20%, 50%, and 90% lethal and effect concentrations. Relative to imidacloprid (toxic equivalency factor [TEF] = 1.0), chronic (lethality) 14-d TEFs for clothianidin and thiamethoxam were 1.05 and 0.14, respectively, and chronic (emergence inhibition) 40-d 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, the authors caution that under field conditions, thiamethoxam readily degrades to clothianidin, thereby likely enhancing toxicity. *Environ Toxicol Chem* 2017;36:372–382. © 2016 SETAC

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

INTRODUCTION

Chemical input into aquatic environments from agricultural runoff remains a challenging global threat 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 sublethal exposure to insecticides. Systemic insecticides, which typically feature a low octanol–water partition coefficient [4], are particularly susceptible to leaching and runoff into aquatic environments. Growing concern over the loss of biodiversity from the intensification of agricultural operations necessitates further assessment of the threats that 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 applied broadly on a suite of crop types worldwide and over a variety of landscapes where environmental conditions, active ingredients, 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] reported 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, which is not surprising because 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 the present study, because 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 postsynaptic nAChRs, functionally interfering with normal

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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 postsynaptic membrane. Unlike acetylcholine, the activity of neonicotinoids is not limited by acetylcholinesterase; indeed, 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, as a result of the conserved nature of insect neurophysiology, both pest and nontarget 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 neonicotinoids' 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 sublethal effects [15]. Furthermore, some studies have found that repeated, short-term exposure to neonicotinoids may have a delayed lethal and sublethal effect on freshwater invertebrates [20,25].

Chironomidae (nonbiting 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 2 separate studies. Toxicity tests with clothianidin and thiamethoxam have been conducted using different study species, durations, and methodologies, which makes it difficult to compare the toxic potency of individual compounds [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 several toxicants, including the legacy insecticide lindane [28].

Worldwide, regulatory aquatic life benchmarks have only been established for imidacloprid, which is only 1 of 7 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 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, whereas 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].

Although 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. Whereas, exposure to sublethal contamination throughout their immature life stages is more common and of greater environmental relevance. The present 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 3 different neonicotinoids. Data generated from the present study allowed for the calculation of toxic equivalency factors (TEFs) for these 3 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 an environmental chamber at the Toxicology Centre, University of Saskatchewan, at $23.0 \pm 1.0\ ^{\circ}C$ with a 16:8-h light:dark photoperiod following the protocols 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 (5 cm × 5 cm) piece of Parafilm®, 2 plastic screens (5 cm × 12 cm) for mating surfaces, and approximately 150 mL of water. Adults were given 3 d to produce egg masses; otherwise, they were 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 ± standard deviation) were as follows: pH, 8.2 ± 0.3; conductivity, 475 ± 63 μS/cm; total hardness, 137 ± 7 mg/L as $CaCO_3$; and alkalinity, 85 ± 9 mg/L as $CaCO_3$. Rearing tanks were fed with 5-mL of macerated fish food (Tetramin®) every other day. After 7 d, 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; 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. Stock solutions were prepared in 1-L volumetric flasks with reverse osmosis water (Barnstead® Diamond™ NANOpure, 18.2 MΩ/cm) and then diluted to the desired test concentrations using culture water.

Chronic, static-renewal toxicity tests were 40 d in duration and used 8 replicate glass beakers, each containing 10 second-instar *C. dilutus* larvae (6–7 d old), 50 mL of washed silica sand, and 200 mL of treatment water. Beakers were continuously aerated

gently to maintain adequate oxygen saturation ($\geq 80\%$), and roughly 150 mL of water from each beaker was changed every third day. Larvae were fed daily by adding 60 μL of macerated fish food (50 g dry weight 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, 4 replicates from each treatment were removed, organisms were counted to assess survival, and surviving larvae were weighed. The remaining 4 replicates were maintained for an additional 26 d to allow larvae to emerge as adults. Emerging adults were collected daily and weighed, and their sex was determined. 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 2-d span. Adult chironomids were considered to have emerged successfully 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 4 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 decaying food and feces. Temperature and dissolved oxygen (DO) were measured with an ORION® dissolved oxygen meter (model 835). Water hardness and alkalinity were quantified with a Hach Digital Titrator (model 16900), pH was measured with an ORION PerpHect LogR meter (model 370), and ammonia levels were assessed with a YSI Photometer (model 9300).

Neonicotinoid analysis

Water (60 mL) was sampled from 4 randomly selected replicate beakers per treatment, pooled into a 250-mL amber bottle, and stored at 4 °C until analyzed. Both old and new water samples (every third day) were collected, and a subset of samples was analyzed to determine insecticide exposure and determine whether any degradation had occurred. Water samples were analyzed at the National Hydrology Research Centre, Environment Canada, using analytical methods previously described by Main et al. [2]. Analytical standards of imidacloprid, clothianidin, and thiamethoxam were acquired from Chem Service and the internal standard from CDN Isotopes. Water samples were solid phase extracted using Oasis HLB cartridges (Waters). 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 mm × 2.2 mm; Waters MS Xterra C-8). Water samples were injected into the liquid chromatography–tandem mass spectrometry 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 in water samples were as follows: imidacloprid, 0.004 ± 0.002 μg/L; clothianidin, 0.004 ± 0.001 μg/L; and thiamethoxam, 0.011 ± 0.001 μg/L. Mean recoveries from Milli-Q water spiked at 500 μg/L, 100 μg/L, and 0.005 μg/L were as follows (mean ± standard deviation): imidacloprid, $91.3 \pm 6.7\%$; clothianidin, $79.0 \pm 4.0\%$; and thiamethoxam, $86.3 \pm 4.2\%$. 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-d (mortality) median lethal concentrations (LC50) values and 40-d (emergence) median effect concentrations (EC50) using the trimmed Spearman–Kärber method [33,34]. Dry weights of larvae (day 14) and number and dry weight of successfully emerged adults (day 40) were used to estimate 20%, 50%, and 90% effect concentrations (EC20, EC50, and EC90, respectively) using the US Environmental Protection Agency (USEPA) inhibition concentration (ICp) program [35].

All other statistical analyses were performed using SigmaPlot™ Ver 13.0 (Systat Software) 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 (ANOVA; *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 ANOVA followed by a Dunnett's post hoc test was performed on the mean cumulative proportion emerged on the day when 50% of the controls had successfully emerged. If data did not fit a normal distribution, a nonparametric 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 of the more controversial no-observed-effect concentration and lowest-observed-effect concentration estimates [36,37].

Toxic equivalency factors were estimated through a 3-point response average of equivalencies calculated at 20%, 50%, and 90% lethal and effect concentrations (LC/EC[20,50,90]) for both day 14 (lethality) and day 40 (emergence inhibition) endpoints. The relative potency of both clothianidin and thiamethoxam were compared with imidacloprid (TEF = 1). Acute and chronic endpoints were graphed on a Probit scale to visually verify assumptions of parallelism of slopes for the 3 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. In addition, at no time during the thiamethoxam tests was clothianidin detected in any water sample, indicating that no degradation had occurred,

Table 1. Calculated (mean ± standard error) neonicotinoid exposure concentrations (μg/L) measured in water over 3-d 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 (μg/L)								
Days 1–14[a]	<LOQ	0.11 ± 0.01	0.25 ± 0.02	0.78 ± 0.08	2.62 ± 0.45	7.82 ± 0.93	NA	NA
Days 1–40[b]	<LOQ	0.10 ± 0.01	0.26 ± 0.01	0.79 ± 0.06	2.82 ± 0.30	8.24 ± 0.80	NA	NA
Clothianidin concentrations (μg/L)								
Days 1–14[a]	<LOQ	0.04 ± 0.01	0.21 ± 0.06	0.42 ± 0.05	1.86 ± 0.12	4.67 ± 0.40	NA	NA
Days 1–40[b]	<LOQ	0.05 ± 0.01	0.20 ± 0.04	0.48 ± 0.06	1.87 ± 0.10	4.97 ± 0.46	NA	NA
Thiamethoxam concentrations (μg/L)								
Days 1–14[a]	<LOQ	0.07 ± 0.01	0.27 ± 0.01	0.74 ± 0.03	2.23 ± 0.12	5.90 ± 0.45	11.99 ± 2.00[c]	33.76 ± 4.60[c]
Days 1–40[b]	<LOQ	0.07 ± 0.01	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 d of exposure.
[b]Mean neonicotinoid concentration calculated for the entire 40-d study.
[c]Thiamethoxam treatments >10.0 μg/L were required to calculate median lethal and effect concentration values.
LOQ = limit of quantification; NA = not applicable.

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 (± standard error) for the 3 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 with 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 d of exposure, imidacloprid was the most toxic and thiamethoxam the least toxic of the 3 compounds to *C. dilutus* larvae (Table 2). The 14-d LC50 values for imidacloprid, clothianidin, and thiamethoxam were 1.52 μg/L, 2.41 μg/L, and 23.60 μg/L, respectively. A significant decrease in survival relative to the controls was observed at mean concentrations greater than 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 the clothianidin test ($F = 3.04$, $df = 4$, $n = 20$, $p = 0.051$). Larval biomass was reduced by 50% at mean concentrations of 2.23 μg/L, 1.83 μg/L, 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 μg/L, 0.1 μg/L, 0.3 μg/L, 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 μg/L, 0.28 μg/L, 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

Table 2. Calculated lethal (LC) and sublethal (EC) toxicity values (in μg/L; 95% confidence interval in parenthese) for *Chironomus dilutus* larvae exposed to technical grade imidacloprid, clothianidin, or thiamethoxam over periods of 14 d and 40 d

	Day 14 LC (larvae survival)			Day 40 EC (emergence)		
LC/EC*x*	Imidacloprid	Clothianidin	Thiamethoxam	Imidacloprid	Clothianidin	Thiamethoxam
20[a]	0.47 (0.29–0.98)	0.34 (0.12–1.27)	1.94 (1.58–7.42)	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)	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]	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 (α = 0.05) using the US Environmental Protection Agency (USEPA) inhibition concentration (ICp) program [35].
[b]Median lethal (or effect) concentration calculated with the trimmed Spearman–Kärber method by Hamilton et al. [33].
[c]Concentrations estimated to produce a 90% effect ± confidence intervals (α = 0.05) using the USEPA ICp method [35].
[d]Calculation extrapolated due to <90% effect observed at highest treatment; used extrapolated EC90 for calculation of toxic equivalency factor.

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

	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 ratio (day 40).
[c]No confidence intervals could be calculated with the provided data.
EC20 = 20% effect concentration; EC50 = 50% effect concentration; CI = confidence interval.

apparent in some surviving individuals in nominal treatments greater than 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 counted as mortalities.

Emergence timing and cumulative emergence were consistent among control treatments in the 3 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 emerged successfully (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 at which adults emerged in the clothianidin and thiamethoxam treatments, relative to the cumulative total, increased with exposure concentration (Figure 2D 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 and 27 (Figure 2B). Adult dry weight was significantly reduced in the 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-d test. Sex ratios were skewed in favor of a male dominant population at concentrations greater than or equal to 0.17 μg/L, 0.46 μg/L, and 3.60 μg/L for imidacloprid, clothianidin, and thiamethoxam, respectively (Table 3). The 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 (USEPA ICp [35]; $\alpha = 0.05$), they may be ecologically important.

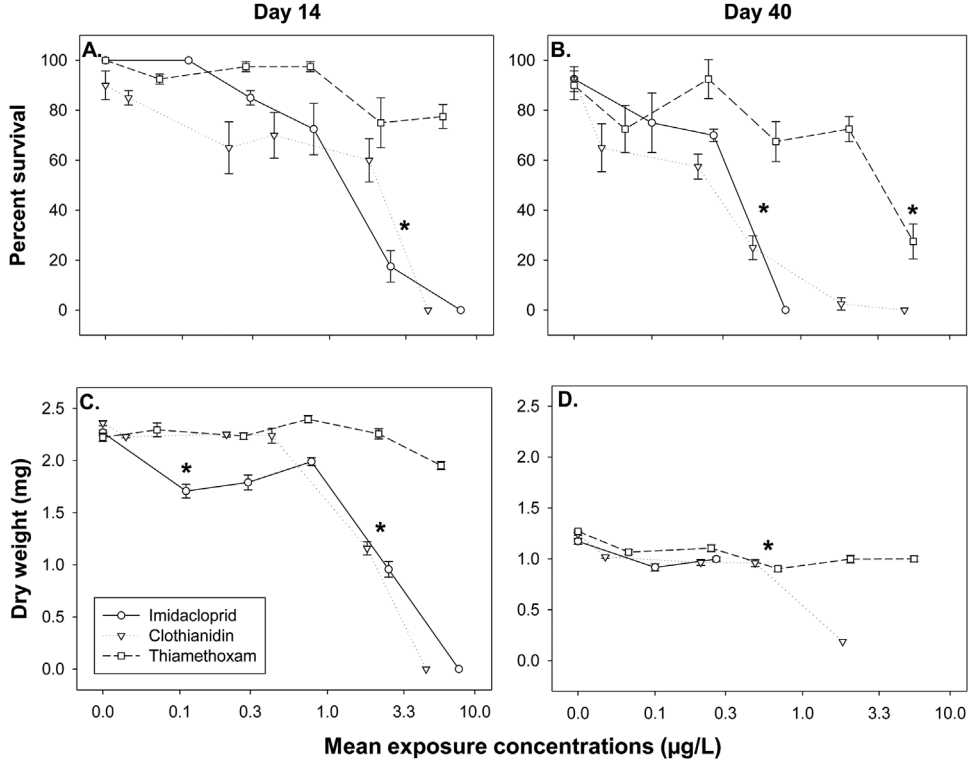

Figure 1. Percent survival or emergence (mean ± standard deviation) and dry weight (mean ± standard deviation) of *Chironomus dilutus* larvae on day 14 (**A**, **C**) and adults on day 40 (**B**, **D**) plotted against mean measured neonicotinoid concentration. (See Table 1 for mean exposure concentrations for each compound tested.) Asterisk (*) indicates significant difference from the control as determined by a Dunnett's post-hoc test ($p \leq 0.05$).

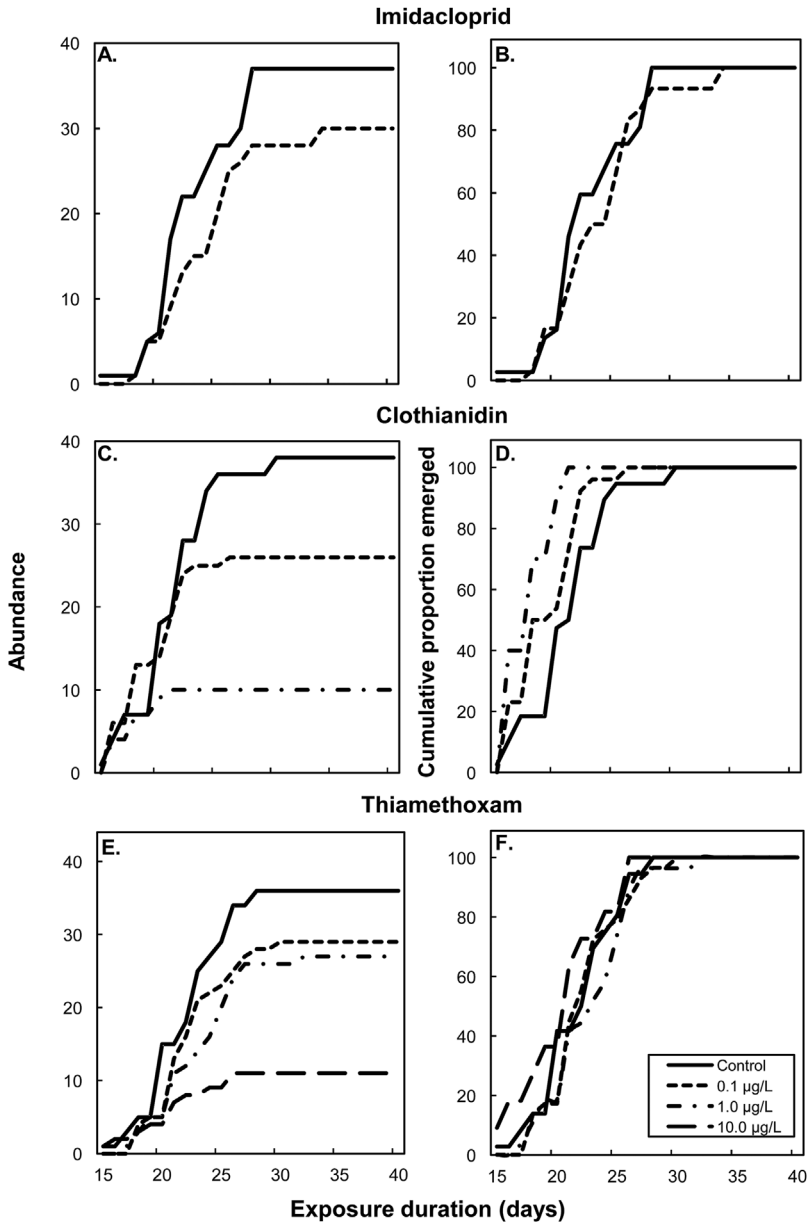

Figure 2. Total emergence (**A**, **C**, **E**) and proportion of surviving individuals that emerged (**B**, **D**, **F**) of *Chironomus dilutus* adults from days 15 to 40 exposed to aqueous solutions of thiamethoxam, clothianidin, or imidacloprid at 1 of 4 nominal test concentrations (0 μg/L, 0.1 μg/L, 1.0 μg/L, and 10.0 μg/L). Results for nominal treatments of 0.3 μg/L and 3.3 μg/L left out for clarity. Where no line is shown for 1.0 μg/L or 10.0 μg/L no emergence occurred.

Toxic equivalency factors

Toxic equivalency factors, relative to imidacloprid, were estimated through a 3-point response average at LC/EC-(20,50,90; Table 5). Relative to imidacloprid (TEF = 1.0), acute (lethality) 14-d TEFs for clothianidin and thiamethoxam were 1.05 and 0.14, respectively. Chronic (emergence) 40-d TEFs were 1.62 and 0.11, respectively. Slopes for both the 14-d lethality ($F = 0.26$, $df = 2$, $n = 9$, $p = 0.785$) and 40-d 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 (Figure 3).

DISCUSSION

To our knowledge, the present study is the first that has compared the chronic toxicity of 3 different neonicotinoid active ingredients, including the well-studied imidacloprid against the second-generation compounds clothianidin and thiamethoxam, under identical laboratory conditions. Compared with imidacloprid, both clothianidin and thiamethoxam have been largely overlooked in the peer-reviewed literature and in setting regulatory guidelines. As a result of the widespread use of imidacloprid and the associated increase 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 regarding 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

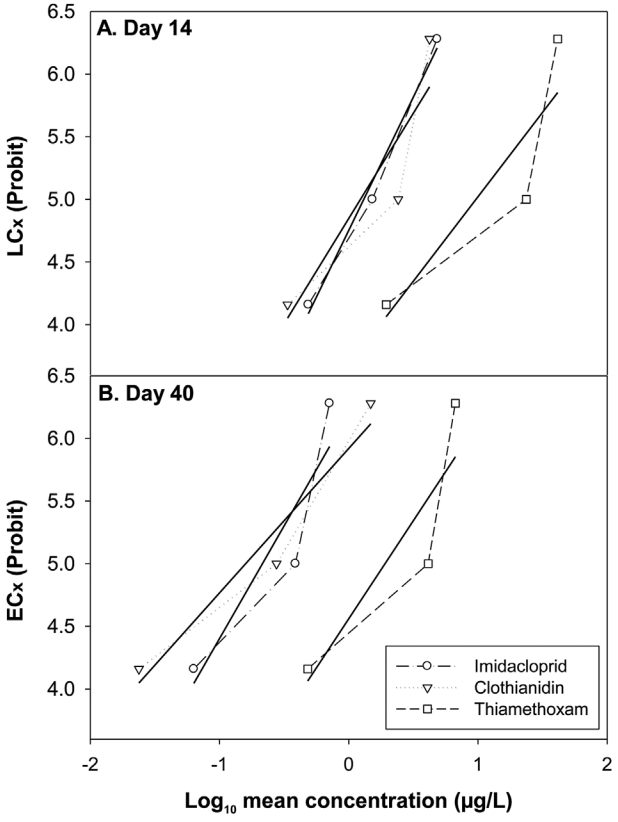

Figure 3. Concentration–response curves for imidacloprid, clothianidin, and thiamethoxam to *Chironomus dilutus* based on (**A**) 14-d lethality (LC*x*) and (**B**) 40-d emergence inhibition (EC*x*). The *y*-axis response plots the individual 20%, 50%, and 90% lethal and effect concentration estimates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

species and communities [44], or runoff of spring snow melt water [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 3 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 the present study, we confirmed the order of toxicity to be similar to previous reports, with imidacloprid having the lowest LC/EC50 values for every endpoint except the day 40 (emergence) value, where clothianidin was marginally lower (0.28 μg/L clothianidin vs 0.39 μg/L imidacloprid). In general, however, imidacloprid and clothianidin displayed similar toxicity to *C. dilutus* larvae, whereas thiamethoxam was approximately 1 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 LC/EC50 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 (no-observed-effect concentration) for thiamethoxam after a 28-d exposure period [47]. However, details on the methodology for both chronic tests were not disclosed in the original documents. In addition, 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 postsynaptic potential inhibition of the VD4-RPeD1 synaptic connection (acetylcholine-evoked membrane) in the central nervous system of the pond snail, *Lymnaea stagnalis*, for 5 neonicotinoid active ingredients. Imidacloprid and clothianidin were found to significantly inhibit the excitatory postsynaptic potential 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-d 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-d LC50 values for imidacloprid (0.85 μg/L) and thiamethoxam (0.94 μg/L). Similar LC50 values for imidacloprid and thiamethoxam indicate 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 different metabolic biotransformation rates 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-d imidacloprid LC50 value of 12.6 μg/L [18] and *Culex pipiens* a 14-d thiacloprid LC50 value of 6.04 μg/L [25].

In the present study, there was 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). The present study's data demonstrate a relationship well established in the literature, where toxicity increases with exposure duration (i.e., incipient LC50s were not

reached in 14 d). Day 14 LC50 values were 3.9 times, 8.6 times, 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 with 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 be broadly explained, at least partly, by environmental conditions, physiological status, and life history traits [53,54], as well as by inherent differences in sensitivity among different taxa. Life history traits 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 a test species. The relative sensitivity of chironomids to all 5 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 4.

Insect metabolism, and associated growth and development, is governed largely by environmental conditions and available resources. Alexander et al. [21] found a decrease in the feeding rate 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 the present 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 μg/L 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 cm to 7 cm. This extra case-building activity may have negatively influenced emergence success in higher concentrations, promoting vulnerability during pupal development. Unnecessarily using 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 imidacloprid and the insect growth hormone inhibitor tebufenozide. Premolt-related mortality was displayed in the mosquito species, *Aedes aegypti,* after exposure

Table 4. 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	5	[47]
	Clothianidin	48 h	Acute	EC50	22	[30]
	Clothianidin	48 h	Acute	EC50	29	[47]
	Clothianidin	28 d	Chronic	EC50[b]	1	[47]
	Clothianidin	28 d	Chronic	EC15[b]	0.72	[59]
	Imidacloprid	24 h	Acute	LC50	55.2	[60]
	Imidacloprid	48 h	Acute	LC50	19.9	[17]
	Imidacloprid	96 h	Acute	EC50[a]	12.94	[61]
	Imidacloprid	28 d	Chronic	EC50[b]	3.11	[60]
	Imidacloprid	28 d	Chronic	EC50[b]	3.6	[60]
	Thiacloprid	28 d	Chronic	EC50	1.8	[47]
	Thiamethoxam	NA	Acute	EC50	35	[59]
	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	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[b]	0.91	[16]
Chironomus tepperi						
	Thiacloprid	24 h	Acute	LC50	1.58	[25]

[a]Immobilization.
[b]Emergence.
NOEC = no-observed-effect concentration; LOEC = lowest-observed-effect concentration; EC50 = effective concentration to 50% of test population; LC50 = lethal concentration to 50% of test population.

to imidacloprid in a 48-h acute test with molting difficulties increasing with imidacloprid concentration [56]. In the present study, all 3 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-d 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 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. Because the sex of *C. dilutus* is genetically predetermined, female sensitivity appears to be attributed to complications during development. Compared with 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 explored further. Studies focused on the intergenerational effects of chronic neonicotinoid exposure may shed further light on long-term population viability.

Although 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 in the present study provide a first opportunity to better describe the relative toxicity of 3 common neonicotinoids, an essential step toward calculating appropriate toxic equivalency factors of multiple neonicotinoids (Table 5). When plotting the concentration–response curves used to estimate LC*x* and EC*x* values for the 3 insecticides, the slopes of the lethality and the sublethal effect lines are reasonably parallel. From these lines, we were able to calculate the TEFs for clothianidin and thiamethoxam relative to imidacloprid. For each compound, the LC*x* and EC*x* estimates were plotted to create a 3-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 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 in the present study, mixture toxicity of these 3 neonicotinoids can be approximated by Equations 1 and 2

Table 5. Toxic equivalency factors for clothianidin and thiamethoxam relative to imidacloprid

	Imidacloprid	Clothianidin	Thiamethoxam
Day 14[a]			
LC20	1.0	1.38	0.24
LC50	1.0	0.63	0.06
LC90	1.0	1.15	0.12
Mean ± SD	—	1.05 ± 0.38	0.14 ± 0.09
Day 40[b]			
EC20	1.0	3.00	0.13
EC50	1.0	1.39	0.09
EC90	1.0	0.48	0.11
Mean ± SD	—	1.62 ± 1.28	0.11 ± 0.02

[a]Larvae survival: Lethal concentration to 20%, 50%, or 90% (LC20, LC50, LC90, respectively) of test population on day 14.
[b]Adult emergence: Concentration affecting 20%, 50%, or 90% (EC20, EC50, EC90, respectively) of test population on day 40.
SD = standard deviation.

$$\begin{aligned}&\text{14-d neonicotinoid exposure (lethality)}\\&[\text{IMI toxic equivalence}] = [\text{IMI conc.}]\\&+1.05[\text{CLO conc.}] + 0.14[\text{THX conc.}]\end{aligned} \quad (1)$$

$$\begin{aligned}&\text{40-d neonicotinoid exposure (emergence inhibition)}\\&[\text{IMI toxic equivalence}] = [\text{IMI conc.}]\\&+1.62[\text{CLO conc.}] + 0.11[\text{THX conc.}]\end{aligned} \quad (2)$$

where IMI is imidacloprid, CLO is clothianidin, and THX is thiamethoxam.

These imidacloprid toxic equivalence values could be used where multiple neonicotinoids are found in water to compare summed equivalence to existing regulatory water quality benchmarks for imidacloprid, such as the interim Canadian Water Quality Guidelines 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; in the interim, however, the TEFs presented in the present study could be used to provide a reasonable, or at least improved, estimate of the hazard of imidacloprid, clothianidin, and thiamethoxam mixtures to nontarget insects in aquatic ecosystems.

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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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