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CUMULATIVE TOXICITY OF NEONICOTINOID INSECTICIDE MIXTURES TO *CHIRONOMUS DILUTUS* UNDER ACUTE EXPOSURE SCENARIOS

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Abstract: Extensive agricultural use of neonicotinoid insecticide products has resulted in the presence of neonicotinoid mixtures in surface waters worldwide. Although many aquatic insect species are known to be sensitive to neonicotinoids, the impact of neonicotinoid mixtures is poorly understood. In the present study, the cumulative toxicities of binary and ternary mixtures of select neonicotinoids (imidacloprid, clothianidin, and thiamethoxam) were characterized under acute (96-h) exposure scenarios using the larval midge *Chironomus dilutus* as a representative aquatic insect species. Using the MIXTOX approach, predictive parametric models were fitted and statistically compared with observed toxicity in subsequent mixture tests. Single-compound toxicity tests yielded median lethal concentration (LC50) values of 4.63, 5.93, and 55.34 μg/L for imidacloprid, clothianidin, and thiamethoxam, respectively. Because of the similar modes of action of neonicotinoids, concentration-additive cumulative mixture toxicity was the predicted model. However, we found that imidacloprid–clothianidin mixtures demonstrated response-additive dose-level–dependent synergism, clothianidin–thiamethoxam mixtures demonstrated concentration-additive synergism, and imidacloprid–thiamethoxam mixtures demonstrated response-additive dose-ratio–dependent synergism, with toxicity shifting from antagonism to synergism as the relative concentration of thiamethoxam increased. Imidacloprid–clothianidin–thiamethoxam ternary mixtures demonstrated response-additive synergism. These results indicate that, under acute exposure scenarios, the toxicity of neonicotinoid mixtures to *C. dilutus* cannot be predicted using the common assumption of additive joint activity. Indeed, the overarching trend of synergistic deviation emphasizes the need for further research into the ecotoxicological effects of neonicotinoid insecticide mixtures in field settings, the development of better toxicity models for neonicotinoid mixture exposures, and the consideration of mixture effects when setting water quality guidelines for this class of pesticides. *Environ Toxicol Chem* 2017;36:3091–3101. © 2017 SETAC

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INTRODUCTION

Neonicotinoid insecticides are the fastest-growing and largest-selling group of insecticides worldwide. Used in a multitude of agricultural products, neonicotinoids are commonly applied as seed treatments, soil drenches, or foliar sprays to protect young crops from biting–sucking pests. Because of their versatility, broad-spectrum insecticidal action, and low toxicity to vertebrates, neonicotinoids have recently come to dominate the agrochemical market, representing more than 24% of agrochemicals and 80% of seed treatments sold worldwide [1]. Of the 7 commercially available neonicotinoid compounds, the most commonly applied are the second-generation seed treatments thiamethoxam and clothianidin and the first-generation seed treatment imidacloprid [2]. Extensive application has raised concerns about the environmental impacts of these compounds, particularly in aquatic environments surrounding areas of intensive use where multiple neonicotinoids may be found.

These 3 compounds display many physiochemical characteristics that facilitate their movement into and persistence in aquatic environments [3]. Following application, a large portion of active ingredient (up to 90%) moves from the treated seed directly into the soil and soil water [4]. These compounds can then easily move into nearby surface water and groundwater systems via leaching, drainage, run-off, or snowmelt processes [5]. Once in aquatic and terrestrial environments, neonicotinoids can exhibit extended persistence (e.g., thiamethoxam max aquatic and terrestrial half lives = 43 and 6931 d, respectively) [4,6]. This has resulted in the widespread and frequent detection of clothianidin, thiamethoxam, and imidacloprid residues in diverse water bodies in Canada [5,7], Australia [8,9], the United States [10–12], Europe [13,14], and Asia [15].

One environmental concern is the impact of these neonicotinoid residues on nontarget aquatic organisms. Many aquatic macroinvertebrates are relatively sensitive to these compounds [3], and thus may be adversely affected by neonicotinoid exposure. Of the aquatic macroinvertebrate taxa, insects that have an aquatic larval stage and emerge as adults (e.g., Ephemeroptera, Trichoptera, Diptera) are particularly sensitive to neonicotinoids [3,16]. Neonicotinoids elicit neurotoxicity in insects by interfering with neural transmission. These compounds bind to and activate postsynaptic nicotinic acetylcholine receptors (nAChR), continuously exciting cholinergic neurons, resulting in muscle tremors and cell energy exhaustion. This can be followed by neural desensitization to acetylcholine (ACh), which blocks neural transmission and results in paralysis or lethality [3]. The relative sensitivity of

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these insect species is of concern, as aquatic insects play important roles in both aquatic and terrestrial ecosystems.

Rather than being present as single compounds, neonicotinoid residues are often found as mixtures in aquatic environments. In a recent survey of the Canadian Prairie Pothole Region, binary or ternary neonicotinoid mixtures were detected in 11 to 63% of wetlands sampled, with cumulative concentrations ranging from 0.004 to 1.66 μg/L [17]. Because of the similar mechanism of action among neonicotinoids, mixtures of these insecticides are expected to display some form of cumulative toxicity (e.g., concentration-addition). Indeed, concentration-additive cumulative toxic effects have been reported for binary mixtures of imidacloprid and thiacloprid in some invertebrate species [18,19]. However, deviation from this assumption of directly additive cumulative toxicity has also been reported [18–21], with the cumulative toxicity of neonicotinoids deviating based on test species, toxicological endpoint of interest, and mixture constituents. In Canada, the United States, and the European Union, neonicotinoids are currently regulated as single compounds [3,22–25], with regulations primarily based on toxicity data for imidacloprid only [22]. This is of concern for 2 reasons. First, the use of a single-compound toxicity value to protect aquatic organisms does not account for the potential cumulative effects of neonicotinoid mixtures in aquatic environments. Second, thiamethoxam and clothianidin account for most neonicotinoid use across Canada, especially in densely agricultural regions like the Canadian Prairies [17]. Furthermore, the mixture effects of the neonicotinoid compounds most frequently detected in aquatic environments (clothianidin, thiamethoxam, and imidacloprid) have yet to be formally tested in mixtures with an ecologically relevant test species. Therefore, it is essential to characterize the cumulative toxicity of these neonicotinoid mixtures to understand the impacts of mixture exposures on sensitive aquatic insects, and to determine whether using a single-compound water quality guideline value would be adequately protective of sensitive aquatic life.

In the present study, cumulative toxicities of binary and ternary mixtures of imidacloprid, clothianidin, and thiamethoxam were investigated under acute exposure scenarios to gain a preliminary understanding (proof of principle) of whether neonicotinoid mixture toxicity can be adequately predicted using single-compound toxicity values. A regression-based, dose–response computational method developed by Jonker et al. [26], MIXTOX, was used to analyze toxicological deviations of the neonicotinoid mixtures from direct additivity (i.e., synergism/antagonism, dose-level–dependent deviation, dose-ratio–dependent deviation) using a sensitive aquatic insect, *Chironomus dilutus,* as a representative test species. The objectives were: 1) to assess the relative acute toxicity of the individual neonicotinoid insecticides, imidacloprid, clothianidin, and thiamethoxam; and 2) to characterize the joint acute toxicity of these binary and ternary mixtures to *C. dilutus* larvae. Because of their common mechanism of action, we hypothesized that these neonicotinoid insecticides would display a cumulative toxicity that may be adequately described by the assumptions of a concentration-addition mixture model.

MATERIALS AND METHODS

Test organisms and culture conditions

Chironomus dilutus were obtained from a laboratory culture maintained at the Toxicology Centre, University of Saskatchewan (Saskatoon, SK, Canada). Organisms were cultured in a controlled environmental chamber with a temperature of 23 ± 1 °C, a 16:8-h light:dark photoperiod, and an illumination intensity of 500 to 1000 lux. Cultures were sustained in 20-L aquaria, and maintenance was based on the protocol outlined by Environment Canada [27]. Culture water consisted of carbon-filtered, biofiltered, Saskatoon municipal water, aerated in 50-L Nalgene® carboys prior to use. Culture tanks were fed with 15 mL of Nutrafin® (Rolf C. Hagen) fish food slurry (100 g/L) 3 times a week. Water quality was monitored monthly, with parameters as follows (mean ± standard deviation [SD]): dissolved oxygen (DO), 7.54 ± 0.55 mg/L; unionized ammonia (NH_3), 0.63 ± 1.34 mg/L; pH, 8.13 ± 0.19; conductivity, 510 ± 20 μS/cm; total hardness, 174 ± 11 mg/L as $CaCO_3$; and alkalinity, 127 ± 16 mg/L as $CaCO_3$.

Chironomus dilutus larvae were obtained for experimentation by isolating and breeding adults from the laboratory culture [28]. Adult *C. dilutus* were collected into a 300-mL Erlenmeyer flask via aspiration, and then transferred into a 1-L glass breeding jar containing 200 mL of culture water, a floating Parafilm® platform, 2 rectangular plastic pieces of mesh (serving as mating platforms), and a screened lid. Breeding jars were placed in enclosed cardboard containers to deter visual disturbances, and left in environmental chambers until egg mass production occurred (up to 48 h). Egg masses were transferred to fresh 20-L glass aquaria containing aerated culture water and a 1-cm layer of washed silica sand (250–425 μm). Nutrafin® slurry (5 mL @ 100 g/L) was introduced to tanks at the time of hatch (48–96 h post transfer), and subsequently every 2 d until time of experimentation. After 6 or 7 d, larvae were transferred to a glass tray, and organisms were selected for experimental use.

Experimental compounds

Three technical-grade neonicotinoids were used as experimental compounds: imidacloprid (98.8% pure; *N*-[1-[(6-chloropyridin-3-yl)methyl]-4,5-dihydroimidazol-2-yl]nitramide), clothianidin (99.6% pure; 1-[(2-chloro-1,3-thiazol-5-yl)methyl]-2-methyl-3-nitroguanidine), and thiamethoxam (98.8% pure; (*NE*)-*N*-[3-[(2-chloro-1,3-thiazol-5-yl)methyl]-5-methyl-1,3,5-oxadiazinan-4-ylidene]nitramide). Imidacloprid and clothianidin were acquired from Bayer CropScience, and thiamethoxam was acquired from Syngenta Crop Protection. Stock solutions were prepared by dissolving the technical product in purified, reverse-osmosis water (Barnstead® Diamond™ NANOpure, 18 MΩ/cm; Barnstead International) and stored in amber glass bottles at 4 °C in the dark until experimental use. To avoid degradation and contamination, fresh stock solutions were prepared monthly, and stock solutions were chemically analyzed prior to every experiment.

Experimental procedures

Toxicity tests were performed in a controlled environmental chamber at the Toxicology Centre, University of Saskatchewan. Experimental conditions remained consistent with those used to culture test animals. Acute (96-h) static toxicity tests were conducted using 300-mL glass beakers containing 50 g of washed, dried 250- to 425-μm silica sand and 200 mL of test solution. Experimental solutions were prepared by spiking 1 L of culture water with concentrated stock solutions to achieve desired test concentrations. Beakers were gently aerated to maintain adequate DO concentrations (>6 mg/L), and covered with borosilicate glass to prevent neonicotinoid photodegradation. Ten early-instar (approximately 6–7 d old) *C. dilutus* larvae were placed in each beaker and exposed to test solutions

for 96 h. To feed test organisms, 60 μL of a 10-g/L Nutrafin® slurry was introduced to each beaker daily. Following the exposure period, live organisms were retrieved and counted to assess survival. Mortality of test organisms in control solutions never exceeded 10%, thus meeting experimental validity requirements.

Single-compound toxicity tests

Acute toxicity (median lethal toxicity [LC50]) was assessed for each neonicotinoid compound in single-compound toxicity tests. *Chironomus dilutus* larvae were exposed to anywhere from 6 to 10 concentrations of insecticide (imidacloprid, clothianidin, or thiamethoxam), along with untreated controls. Each treatment was replicated 4 times ($n = 40$ organisms/treatment). Nominal concentrations of imidacloprid (0.4–20.61 μg/L), clothianidin (0.4–20.61 μg/L), and thiamethoxam (0.4–482.9 μg/L) were based on range-finding tests and previous studies [28,29].

Binary mixture tests

Mixture tests were designed based on the toxic unit (TU) concept, where a TU is defined as the actual concentration of a chemical (c) divided by its toxicity threshold (in this case the LC50; Equation 1)

$$\mathrm{TU} = \frac{c}{\mathrm{LC50}} \quad (1)$$

Exposure scenarios were based on a fixed-ray experimental design. Compounds were tested at 5 TU dose ratios (1:0, 3:1, 1:1, 1:3, 0:1), and 6 dose levels ($\Sigma\mathrm{TU} = 0.25, 0.5, 1.0, 1.5, 2.0, 3.0$), yielding 18 different binary mixtures and 12 single-compound exposures (Figure 1A–D). Nominal exposure concentration ranges were as follows: imidacloprid, 0.29 to 13.89 μg/L; clothianidin, 0.37 to 17.79 μg/L; and thiamethoxam, 3.46 to 166.02 μg/L. The fixed-ray design necessitates reduced replicates (2 per treatment) to allow for an increased number of exposure combinations. As the analysis of the mixture toxicity data is regression based, the statistical strength is maintained via adequate coverage of the toxicological response surface [26].

Ternary mixture test

The ternary mixture test also followed a fixed-ray experimental design based on the toxic unit concept (Figure 1E and F). Mixtures were tested at 10 dose ratios (1:0:0, 0:1:0, 0:0:1, 1:1:1, 2:1:1, 1:2:1, 1:1:2, 2:2:1, 2:1:2, 2:2:1), and 6 dose levels ($\Sigma\mathrm{TU} = 0.25, 0.5, 1.0, 1.5, 2.0, 3.0$), yielding 42 different ternary mixtures and 18 single-compound exposures. Nominal exposure concentration ranges were as follows: imidacloprid, 0.23 to 13.89 μg/L; clothianidin, 0.46 to 17.79 μg/L; and thiamethoxam, 3.46 to 166.02 μg/L.

Water quality

Water quality was assessed at the beginning (day 0) and end (day 4) of each test. A 20-mL sample of water was removed from test beakers and analyzed for pH, conductivity, total hardness, and alkalinity. The pH was measured with an ORION® PerpHect LogR meter, model 370 (ORION Research), conductivity with an ORION® Conductivity meter (model 170), and hardness and alkalinity using a Hach Digital Titrator (model 16900). In addition, temperature, DO, and ammonia (NH_3) concentrations in test beakers were evaluated every 2 d to ensure that adequate experimental conditions were maintained. The DO and temperature were measured with a Thermo ORION® dissolved oxygen meter, model 835, and ammonia was measured with a VWR™ SB301 sympHony ISE ammonia meter paired with a Thermo ORION® 95-12 ammonia electrode.

Chemical analysis

Test solutions were sampled at the start (day 0) and end (day 4) of each test and analyzed to determine actual concentrations of neonicotinoid exposure. For each treatment, 50 mL of test solution was collected from each replicate beaker, pooled, and stored in a 250-mL amber glass bottle at 4 °C until time of analysis. In the single-compound tests, both new (day 0) and old (day 4) water samples were analyzed for each treatment to ensure minimal degradation and constant exposure concentrations. Because of the complexity of the experimental designs for the mixture tests, only a subset of samples (TU = 1.0 at each dose ratio) was analyzed at both day 0 and day 4. The remaining samples were pooled across sample times (day 0 + day 4) prior to analysis.

Samples were analyzed at the National Hydrology Research Centre, Environment and Climate Change Canada (Saskatoon, SK, Canada) using methods described in Main et al. [5]. Briefly, analytical standards of imidacloprid, clothianidin, and thiamethoxam were obtained from Chem Service. The internal standards (d_4-imidacloprid and d3-thiamethoxam) were obtained from CDN Isotopes. Neonicotinoid concentrations were quantified via solid-phase extraction (SPE) followed by high-performance liquid chromatography (HPLC) paired with tandem mass spectrometry (LC–MS/MS). The SPE was performed by loading samples onto OASIS® HLB cartridges (Waters), rinsing with deionized water to remove any salts, and eluting the retained solutes with methanol. The eluted samples were then dried via evaporation, reconstituted in deionized water, and spiked with the internal standards. The LC-MS/MS was performed using a Waters 2695 Alliance HPLC system equipped with a Waters XTerra MS-C8 column (3.5 μm diameter particle size; 2.1 × 100 mm), paired with a Micromass Quattro Premier triple quadrupole mass spectrometer (Waters) equipped with an electrospray ionization interface (positive ion mode). The mobile phase consisted of an 80/20 mix of solvent A (99.9% water, 0.1% formic acid) and solvent B (90% acetonitrile, 9.9% water, 0.1% formic acid). The injection volume was 20 μL, the flow rate was 200 μL/min, and the average runtime was 10 min. Calibration curves were run, allowing for quantification of neonicotinoids to the following mean (± standard error [SE]) limits of quantification: imidacloprid 0.008 (± 0.002) μg/L, clothianidin 0.009 (± 0.003) μg/L, and thiamethoxam 0.015 (± 0.002) μg/L. Mean recoveries from Milli-Q water spiked with neonicotinoid concentrations of 0.125 μg/L were as follows: imidacloprid 88.8 (± 1.5) %, clothianidin 85.2 (± 1.6) %, and thiamethoxam 88.7 (± 4.7) %. All measured neonicotinoid concentrations reported were recovery corrected prior to use in statistical analysis and MIXTOX modeling.

Statistical analysis and MIXTOX modeling

Single-compound toxicity was evaluated by fitting survival data to a 3-parameter logistic dose–response curve (Equation 2) using SigmaPlot statistical software, Ver 11.0 (Systat Software). The toxicological response (Y_i) is a function of maximum response (Y_{max}), concentration of exposure (c_i), LC50, and the slope of the response curve (B_i)

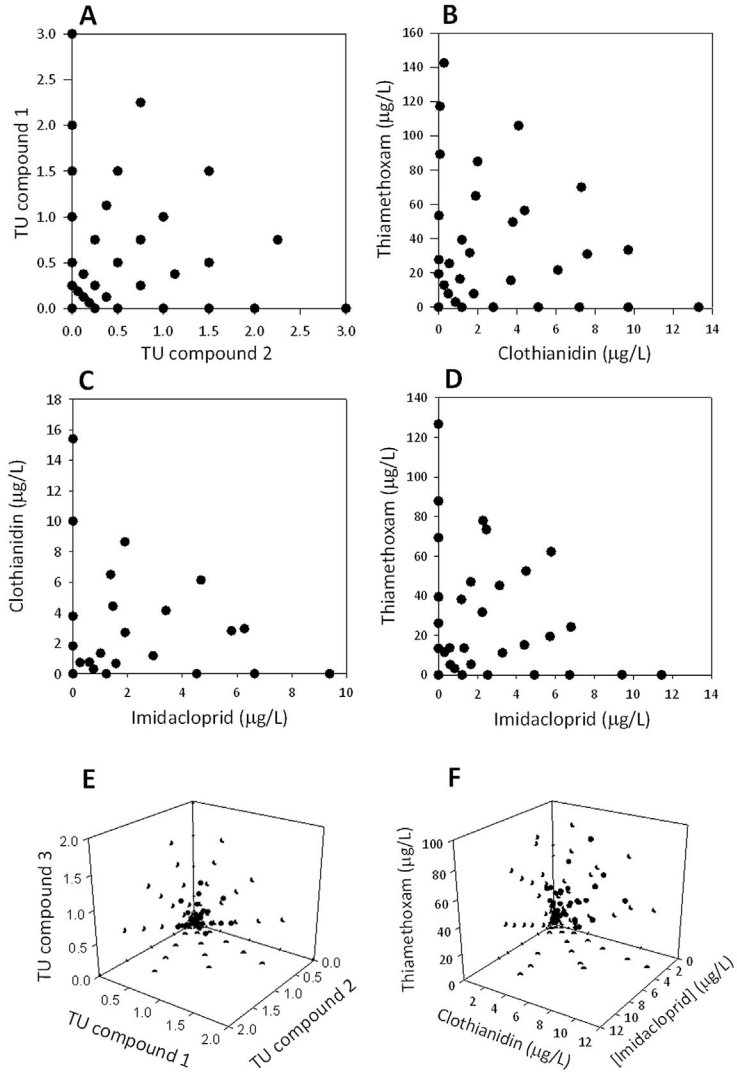

Figure 1. Fixed-ray experimental design applied in binary (**A**) and ternary (**E**) mixture toxicity tests, compared with actual concentrations of exposure in clothianidin-thiamethoxam (**B**), imidacloprid-clothianidin (**C**), imidacloprid–thiamethoxam (**D**), and imidacloprid–clothianidin–thiamethoxam (**F**) mixture studies. TU = toxic unit.

$$Y_i = \frac{Y_{\max}}{1 + (C_i/\mathrm{LC50})B_i} \qquad (2)$$

The LC50 values were estimated using the trimmed Spearman–Kärber method [30,31] and compared with those derived through fitting the dose–response curve (Equation 2), to assess the reliability of parameter estimates.

Binary mixture toxicity data were analyzed using the MIXTOX approach [26]. A descriptive approach to modeling cumulative toxicity of complex mixtures, MIXTOX compares observed data with fitted parametric models of mixture effects, calculated from single-compound toxicity data, thus enabling quantification of the deviation of observed data from reference models of concentration addition (CA), which assumes concentration-additive cumulative toxicity, or independent action (IA), which assumes response-additive cumulative toxicity, (Figure 2; concentration addition shown under CA). Deviation from the reference models could take the form of synergism or antagonism, dose-ratio–dependent deviation, or dose-level–dependent deviation, and was assessed via a stepwise addition of extra parameters, a and b. The first parameter a describes a synergistic (greater than expected toxicological effect) or antagonistic (lower than expected toxicological effect) deviation from CA or IA (Figure 2; synergistic deviation shown under SYN). The models were then further extended with b. Two forms of the b parameter exist, b_i and b_{DL}, where b_i describes a dose-ratio–dependent deviation from the reference model, indicating a shift between synergism and antagonism dependent on the ratio of mixture constituents (Figure 2; dose-ratio–dependent deviation shown under D-R), and b_{DL} describes a dose-level–dependent deviation from the reference model, indicating a shift between synergism and antagonism dependent on the cumulative magnitude of TUs (Figure 2; dose-level–dependent deviation shown under D-L). Interpretation of numerical values derived in the MIXTOX analysis can be found in the Supplemental Data (Table S1).

Ternary mixture toxicity was analyzed using an extension of the MIXTOX approach, called the Ternary-Plus model [32]. In this analysis, data from all 3 binary mixture studies were analyzed alongside the empirical ternary mixture data, to account for the toxicological effects of binary mixtures when predicting ternary-mixture response. A ternary deviation parameter, $a_{1,2,3}$, was introduced, describing the deviation of

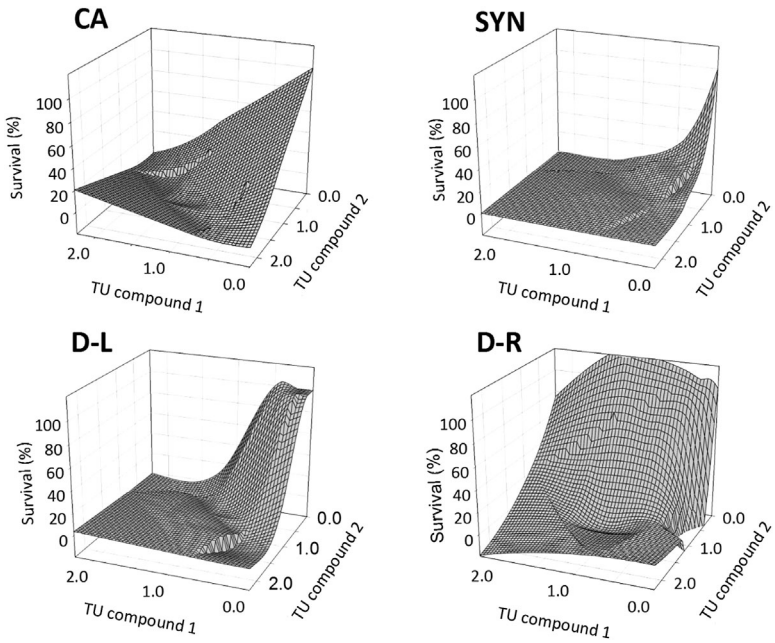

Figure 2. Three-dimensional binary mixture dose–response surfaces depicting concentration addition (CA) and 3 deviation patterns from this reference model: synergistic deviation (SYN), dose-level–dependent deviation (D-L), and dose-ratio–dependent deviation (D-R). (Adapted from Jonker et al. [26]). TU = toxic unit.

the measured ternary response surface from the response surface predicted by a combination of binary deviation functions. This parameter describes a synergistic or antagonistic deviation from IA or CA. Because the Ternary-Plus model is still in development, this equation could not be further extended to model dose-level and dose-ratio–dependent deviations from the synergism/antagonism model. An interpretation of the numerical values derived in the Ternary-Plus model can be found in the Supplemental Data (Table S1).

Mean measured (not nominal) neonicotinoid concentrations were used in the MIXTOX analysis to more accurately characterize the cumulative effect of these neonicotinoid mixtures. Adequate coverage of the toxicological response surface was evaluated through scatterplots of measured concentrations tested in mixture toxicity tests (Figure 1). Experimental data were fit to parametric models using maximum likelihood estimation. First, measured data were fit to reference models. Because of their similar mechanism of action at the nAChR, mixtures of neonicotinoids were hypothesized to have a cumulative effect that is best described by the CA reference model. However, to comprehensively assess the cumulative toxicity of the neonicotinoid mixtures evaluated in the present study, both reference models (CA and IA) were initially fit to mixture datasets. Models were then further extended with parameters indicating deviation from direct additivity (i.e., synergism/antagonism, dose-level deviation, and dose-ratio deviation). As the sequential addition of parameters resulted in the formation of a series of nested models, the fit of parametric models could be directly assessed through pairwise model comparison and significance testing. Following extension of reference models with additional parameters, improved fit was confirmed by a reduction in the residual sum of squares (RSS) and the statistical significance of this improvement determined via Chi-squared tests (χ^2) with degrees of freedom equal to the difference in number of parameters in the 2 models. Further information regarding the derivation and statistical interpretation of parametric MIXTOX models can be found in Jonker et al. [26].

RESULTS

Water quality and chemical analysis

Because of their consistency, routine water quality variables were averaged across all single-compound and mixture toxicity tests. Mean values ($\pm$ SE) were as follows: DO 7.8 ($\pm$ 0.1) mg/L; temperature, 23.0 ($\pm$ 0.4) °C; pH, 8.03 ($\pm$ 0.04); conductivity, 328 ($\pm$ 41) μS/cm^3; total hardness, 111 ($\pm$ 3) mg/L as $CaCO_3$; and alkalinity, 117 ($\pm$ 6) mg/L as $CaCO_3$. Ammonia concentrations increased over the duration of each test, but remained well below the total ammonia 96-h LC50 for *C. dilutus* (82 mg N/L) [33], with a mean value ($\pm$ SE) of 0.69 ($\pm$ 0.2) mg N/L.

Neonicotinoid concentrations did not change significantly throughout the duration of the test (analysis of new and old water) with concentrations of imidacloprid, clothianidin, and thiamethoxam on day 4 remaining within $101.8 \pm 10.5\%$, $107.2 \pm 15.0\%$, and $99.8 \pm 16.3\%$ of original (day 0) concentrations, respectively. Measured neonicotinoid concentrations were close to nominal concentrations, with measured imidacloprid, clothianidin, and thiamethoxam concentrations (mean $\pm$ SE) being within $98.6 \pm 4.7\%$, $102.9 \pm 5.5\%$, and $94.6 \pm 10.8\%$ of nominal concentrations, respectively. Neonicotinoid concentrations in control treatments remained lower than the limit of quantitation. Measured neonicotinoid concentrations for single-compound and mixture toxicity tests are presented in the Supplemental Data (Tables S2 and S3).

Single-compound toxicity

Dose–response curves generated from the single-compound toxicity tests are shown in Figure 3. *Chironomus dilutus* demonstrated the greatest sensitivity to imidacloprid, with a 96-

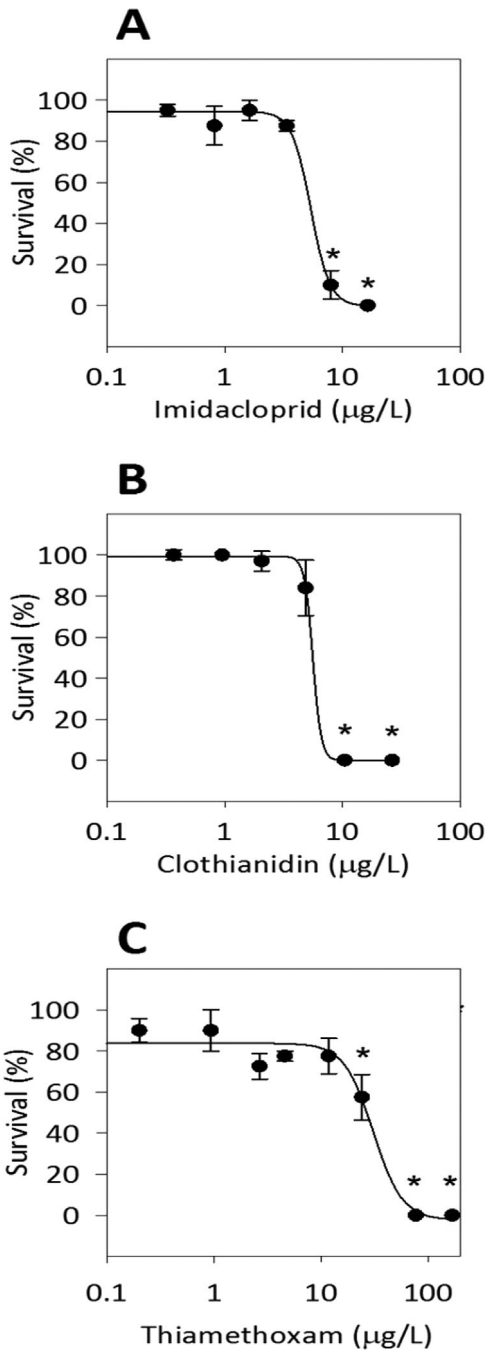

Figure 3. Survival (mean ± standard deviation) of *Chironomus dilutus* larvae after exposure to imidacloprid (**A**), clothianidin (**B**), and thiamethoxam (**C**) for 96 h ($n = 4$, 10 organisms/replicate, 10 organisms/replicate). Asterisk (*) indicates significant difference from the untreated control, calculated via one-way analysis of variance with the Tukey post hoc test ($p \leq 0.05$).

h LC50 of 4.63 (3.96–5.41) µg/L. Clothianidin displayed similar toxicity, with a 96-h LC50 of 5.93 (5.29–6.63) µg/L. Thiamethoxam was the least toxic compound to *C. dilutus*, with a 96-h LC50 approximately 10 times lower, at 55.34 (43.98–69.64) µg/L.

Single-compound 96-h LC50 values were also determined for each mixture constituent in the binary and ternary mixture toxicity tests (Table 1). To evaluate the accuracy and replicability of the laboratory tests, the LC50 values for imidacloprid, clothianidin, and thiamethoxam were compared across both single-compound and mixture toxicity tests. For imidacloprid and clothianidin, single-compound toxicity remained consistent across all tests, with LC50 values ranging from 2.79 to 6.83 µg/L and 5.08 to 6.15 µg/L respectively. For thiamethoxam, single-compound toxicity was consistently lower in mixture tests, with LC50 values ranging from 35.4 to 39.4 µg/L. This variation is likely because of either the difference in concentration ranges applied with the narrower concentration ranges in mixture tests resulting in a more accurate prediction of single-compound toxicity, or the difference in statistical method used to calculate LC50 estimates, with single-compound tests relying on the trimmed Spearman–Kärber method [30,31], and mixture tests fitting survival data to a 3-parameter logistic dose–response curve (Equation 2).

Mixture toxicity

Binary mixture: Imidacloprid–clothianidin. Imidacloprid and clothianidin mixtures were best described by IA (IA RSS = 1695; CA RSS = 88 176). Extension of the IA model with the synergism/antagonism (S/A) parameter (a) significantly improved model fit (RSS = 527.2, $\chi^2 = 50.7$, $p < 0.0001$), with $a = -10.8$ indicating a synergistic effect. The IA-S model was further extended with a dose-ratio (D-R) parameter (b_{DR}), which failed to significantly improve model fit (D-R: RSS = 516.94, $\chi^2 = 50.7$, $p = 0.48$). However, extension of the model with the dose-level (D-L) parameter (b_{DL}) significantly improved model fit (D-L: RSS = 325.5, $\chi^2 = 78.9$, $p = <0.0001$). Parameters $a = -10.9$ and $b_{DL} = 0.89$ indicate synergism across all concentration levels, with the magnitude of synergism being effect level dependent (i.e., up to 56% decrease in survival). Therefore, the model of best fit was IA-DL, with the imidacloprid–clothianidin mixture demonstrating dose-level–dependent synergistic cumulative toxicity (Figure 4A and Supplemental Data, Table S4). The IA-DL model was found to explain 99.6% of the variability in the imidacloprid–clothianidin mixture data (Figure 4B).

Binary mixture: Clothianidin–thiamethoxam. Clothianidin and thiamethoxam mixtures were best described by the CA reference model (RSS CA = 1401; RSS IA = 10 408). Extension of the CA model with the S/A parameter significantly improved model fit (RSS = 1039, $\chi^2 = 9.26$, $p = 2.0 \times 10^{-3}$), with $a = -0.05$ indicating a weakly synergistic effect across all concentration levels (i.e., up to 12% decrease in survival). The CA-S model was further extended with D-R and D-L parameters; however, neither parameter significantly improved model fit (D-R: RSS = 1039, $\chi^2 = 0$, $p = 1$; D-L: RSS = 3462, $\chi^2 = -37.3$, $p =$ n/a). Therefore, the model of best fit was CA-S, with the clothianidin–thiamethoxam mixture demonstrating a synergistic cumulative toxicity (Figure 4C). The CA-S model was found to explain 99.6% of the variability in the clothianidin–thiamethoxam mixture data (Figure 4D and Supplemental Data, Table S5).

Binary mixture: Imidacloprid–thiamethoxam. Imidacloprid and thiamethoxam mixtures were best described by the IA reference model (IA RSS = 6326; CA RSS = 25 076). Extension of the model with the S/A parameter significantly improved model fit (RSS = 4,520, $\chi^2 = 12.33$, $p < 0.0001$), with $a = -2.32$ indicating a synergistic effect. The IA-S model was further extended with a D-L parameter, which failed to significantly improve model fit (RSS = 4071, $\chi^2 = 2.93$, $p = 0.09$). However, extension of the IA-S model with the D-R parameter significantly improved the model fit further (RSS = 3294, $\chi^2 = 7.44$, $p = 0.0006$), with parameters $a = 57.17$ and $b_{DR} = -86.89$ indicating synergism at dose ratios with higher imidacloprid (i.e., up to a 28% decrease in survival), and antagonism at dose ratios with higher thiamethoxam (i.e., up to a 35% increase in survival). Therefore, the

Table 1. Median lethal concentrations (96-h LC50; μg/L) and slopes (β) for *Chironomus dilutus* larvae exposed to single-compound positive controls in the mixture toxicity tests[a]

	Imidacloprid (IMI)		Clothianidin (CLO)		Thiamethoxam (TMX)	
	LC50	β	LC50	β	LC50	β
IMI–CLO	6.83	8.31	5.76	19.69	—	—
CLO–TMX	—	—	6.15	8.12	35.40	11.16
IMI–TMX	4.96	12.57	—	—	39.40	171.0
IMI–CLO–TMX	2.79	3.53	5.08	13.50	36.48	19.73

[a]Calculated via maximum-likelihood estimation through MIXTOX analysis [26].

model of best fit was IA-D-R, with imidacloprid–thiamethoxam demonstrating dose-ratio–dependent cumulative toxicity (Figure 4E and Supplemental Data, Table S6). This model was found to explain 94.3% of the variability in the imidacloprid–thiamethoxam mixture data (Figure 4F).

Ternary mixture: Imidacloprid–clothianidin–thiamethoxam. The ternary mixture of imidacloprid, clothianidin, and thiamethoxam demonstrated a cumulative effect that was best described by the IA reference model (IA RSS = 62 924; CA RSS = 74 560). Extension of this model with the S/A

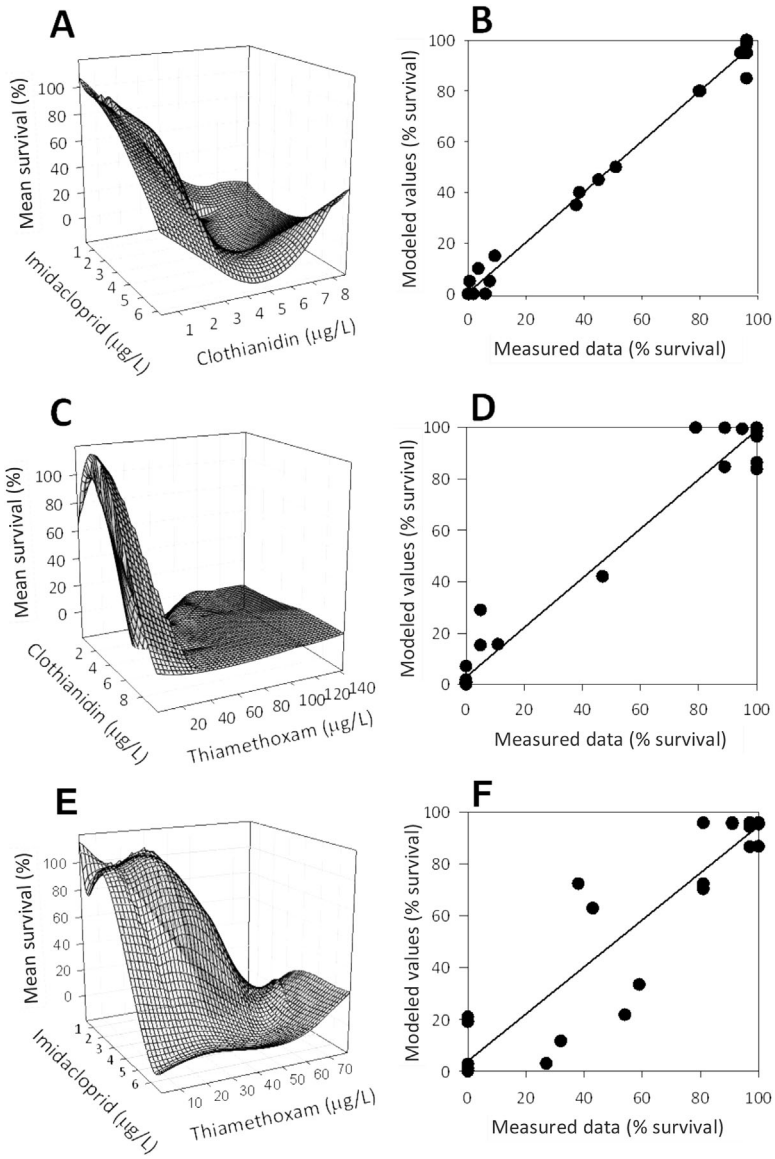

Figure 4. Survival (mean) of *Chironomus dilutus* larvae following 96-h exposures to imidacloprid–clothianidin (**A**), clothianidin–thiamethoxam (**C**), and imidacloprid–thiamethoxam (**E**) mixtures. Relationships between measured survival data and modeled values the most statistically significant parsimonious deviation model are shown for imidacloprid–clothianidin (**B**), clothianidin–thiamethoxam (**D**), and imidacloprid–thiamethoxam (**F**) mixtures. A diagonal line (1:1 relationship) indicates ideal model description.

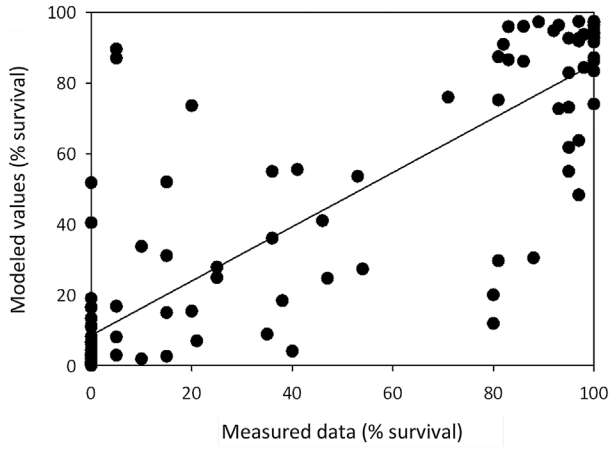

Figure 5. Relationship between measured data and modeled values for the most statistically significant parsimonious deviation model for the imidacloprid–clothianidin–thiamethoxam mixture. A diagonal line (1:1 relationship) indicates ideal model description.

parameters significantly improved model fit (RSS = 27 963, $\chi^2 = 103$, $p = <0.0001$), with the parameter $a_{imidacloprid,clothianidin,thiamethoxam} = -4.32$ indicating a synergistic effect across all concentration levels (i.e., up to a 6% decrease in survival). Interaction parameters of $a_{imidacloprid,clothianidin} = -1.16$ and $a_{imidacloprid,thiamethoxam} = -15.99$ indicated that the imidacloprid–clothianidin and imidacloprid–thiamethoxam binary mixtures had synergistic contributions to the cumulative toxicity of the ternary mixtures, whereas the interaction parameter of $a_{clothianidin,thiamethoxam} = 5.38$ indicated that the clothianidin–thiamethoxam binary mixture had an antagonistic contribution. Therefore, the model of best fit was IA-S, with a ternary mixture of imidacloprid–clothianidin–thiamethoxam demonstrating synergistic cumulative toxicity (Supplemental Data, Table S7). This model was found to explain 85.6% of the variation in the imidacloprid–clothianidin–thiamethoxam experimental data (Figure 5).

Model fit. In the present study, the strength of model fit is demonstrated in the statistical analysis (Supplemental Data, Tables S4–S7). Each model presented is the MIXTOX model of best fit, and each fit is statistically significant ($p < 0.05$). However, the strength of correlation between the modeled and measured data varies between mixture models. Although there is visibly strong correlation between modeled and measured data in the imidacloprid–clothiandin mixture (Figure 4A), the correlation between modeled and measured data in imidacloprid–thiamethoxam, clothianidin–thiamethoxam, and imidacloprid–clothianidin–thiamethoxam mixtures is less strong (Figures 4B,D and 5). This is potentially because of the complexity of interactions occurring in the insecticide mixtures. The MIXTOX method allows for extensive modeling of cumulative toxicity, but it presents a finite number of mixture models (i.e., CA/IA, S/A, D-R, and D-L for binary mixtures; CA/IA, S/A for ternary mixtures). Therefore, it is possible that goodness of fit of the clothianidin–thiamethoxam, imidacloprid–thiamethoxam, and imidacloprid–clothianidin–thiamethoxam models could be improved by fitting more complex models of mixture toxicity. However, further research is required to determine how the MIXTOX approach could be extended to increase the complexity of mixture models.

DISCUSSION

Acute toxicity of single neonicotinoid compounds

Although the toxicity of neonicotinoids has been extensively investigated for several nontarget invertebrate species, few published studies have compared the toxicity of different neonicotinoid compounds to sensitive insect species under consistent experimental conditions. Furthermore, to our knowledge, this toxicity comparison among multiple neonicotinoids has yet to be reported for *C. dilutus* under acute (96-h) exposure scenarios. In the present study, *C. dilutus* demonstrated the greatest sensitivity to imidacloprid (LC50 = 4.63 μg/L), followed closely by clothianidin (LC50 = 5.93 μg/L), and then thiamethoxam (LC50 = 55.34 μg/L), which was 10 times less toxic. These relative toxicities are in accordance with what has been previously reported for *C. dilutus* under longer exposure scenarios [29]. Under subchronic (14-d) exposure conditions, *C. dilutus* was found to display the highest sensitivity to imidacloprid (LC50= 1.52 μg/L), followed by clothianidin (LC50 = 2.41 μg/L), and thiamethoxam (LC50 = 23.6 μg/L) [29]. After 40 d of exposure, *C. dilutus* displayed slightly higher sensitivity to clothiandin (median effect concentration [EC50] = 0.28 μg/L) than imidacloprid (EC50 = 0.39 μg/L), followed by thiamethoxam (EC50 = 4.13 μg/L). Other studies with *C. dilutus* have reported similar acute toxicity values to those found in the present study: a 96-h LC50 for imidacloprid of 5.75 μg/L [28] and a slightly lower 96-h LC50 for clothianidin of 2.32 μg/L [34].

Under acute single-compound exposure scenarios, measured environmental concentrations above these would be expected to elicit lethal toxicity in *C. dilutus* and other sensitive aquatic arthropods (e.g., Ephemeroptera [16]). The single-compound toxicity values (96-h LC50) generated from the present study add to the growing body of literature used to evaluate current regulations and will be important for the development of new water quality guidelines, to ensure protection of sensitive aquatic insects from short-term neonicotinoid exposures.

Cumulative toxicity of neonicotinoid mixtures

Importantly, this study extends beyond the examination of single-compound neonicotinoid toxicity to characterize the cumulative toxicity of neonicotinoid mixtures. In the present study, acute exposures of *C. dilutus* to different neonicotinoid mixtures resulted in cumulative toxicity that deviated from that predicted based on the current mechanistic understanding of the toxicity (concentration addition) of neonicotinoid insecticides to insects. The mixture of imidacloprid and clothianidin displayed a response additive dose-level dependent synergistic deviation. The mixture of clothianidin and thiamethoxam displayed a concentration additive synergistic deviation, while the mixture of imidacloprid and thiamethoxam displayed a response-additive dose-ratio–dependent synergistic deviation. The ternary mixture (imidacloprid–clothianidin–thiamethoxam) displayed response-additive synergistic deviation.

To our knowledge, no other published studies to date have investigated the cumulative toxicity of neonicotinoid mixtures to any aquatic insect species. However, the deviation from direct additivity observed in the present study is consistent with what has been described for some invertebrate species in other neonicotinoid insecticide mixture studies. In *Caenorhabditis elegans,* a terrestrial roundworm, mixtures of imidacloprid and thiacloprid were found to have a dose-level–dependent synergistic effect on reproduction [19]. In the crustacean

Daphnia magna, mixtures of imidacloprid and thiacloprid had a dose-level–dependent synergistic effect on reproduction [18], a synergistic effect on lethality [20], and an antagonistic effect on feeding inhibition [20]. In addition, Bayer CropScience has patented synergistic activity of binary mixtures of imidacloprid, thiacloprid, and clothianidin for control of several invertebrate pest species [21]. Although the cumulative toxicities of these neonicotinoid insecticide mixtures vary when comparing across compounds, endpoints, and species, the present study, along with other neonicotinoid mixture studies, confirms a general trend of deviation from the hypothesized default CA model.

Mechanistic response to neonicotinoid mixtures

One potential explanation for this observed deviation from the CA model is the action of the mixture constituents at the nAChR. The nAChR is a pentameric receptor, with a wide variety of subunits that can be arranged into distinct nAChR subtypes [2]. At least 2 functionally distinct subtypes of nAChR exist: α-bungarotoxin (α-BGT)-sensitive (neonicotinoid-insensitive) nAChR, and α-BGT-insensitive (neonicotinoid-sensitive) nAChR [2]. These subtypes have been further categorized based on functional responses to specific neonicotinoid agonists. Within the α-BGT-insensitive nAChR group, subpopulations are defined by their sensitivity to imidacloprid: nAChR1 (imidacloprid-sensitive) and nAChR2 (imidacloprid-insensitive) [2]. Within the α-BGT-sensitive nAChR group, subpopulations are categorized based on their ability to desensitize the neuron following excitatory action [35]. Different neonicotinoids have been shown to differ in their ability to bind to and activate these receptor subpopulations. Clothianidin strongly activates both nAChR1 and nAChR2 subtypes and strongly desensitizes cockroach (*Periplaneta americana*) neurons following excitatory action [36,37]. Imidacloprid exclusively activates nAChR1 and desensitizes *P. americana* neurons, with the strength of neuronal desensitization dependent on length of exposure [38,39]. Thiamethoxam weakly interacts with nAChR receptors, with reversible (nondesensitizing) neuronal depolarization effects in *P. americana* [39]. Therefore, it is possible that the presence of multiple nAChR subtypes in larval *C. dilutus* could be influencing the observed cumulative toxicological effects of these neonicotinoid mixtures. However, to the best of our knowledge, the molecular composition and functional characterization of nAChR subtypes in *C. dilutus* have not been investigated. Thus, further research is required to make firmer conclusions regarding the molecular action of neonicotinoid mixtures at the receptor level in aquatic insects such as Chironomidae.

Implications for hazard assessment of neonicotinoid mixtures

The most common approach to hazard assessment of chemical mixtures in the environment involves an assumption of additive joint activity. When mixture constituents have similar sites and modes of action, the chemicals are thought to act as dilutions of each other, and the CA reference model is assumed to most accurately describe cumulative toxicity [40]. All neonicotinoid compounds act on the same general neuronal receptor, with the same general mechanisms of action; therefore CA was hypothesized to be the model of best fit for binary and ternary mixtures of imidacloprid, clothianidin, and thiamethoxam. However, in the present study, imidacloprid–clothianidin, imidacloprid–thiamethoxam, and imidacloprid–clothianidin–thiamethoxam mixtures displayed a cumulative toxicity that was much better described by the IA reference model. In addition, all neonicotinoid mixtures tested displayed cumulative toxicities that deviated from direct additivity. This statistically significant deviation from both CA and direct additivity was unexpected. Traditionally, the IA model is assumed to best describe the cumulative toxicity of mixtures of compounds that are strictly dissimilar in their sites and mechanisms of action [40]. In the IA model, cumulative toxicity is determined by summing toxicological responses of test organisms to each mixture constituent. Therefore, mixture effects must be predicted based on toxicological data rather than chemical concentration alone. In the present study, binary and ternary mixtures demonstrated similar deviation patterns when fit with both CA and IA reference models (Supplemental Data, Tables S4–S7). However, CA reference models consistently underpredicted the magnitude of deviation from direct additivity. In the imidacloprid–clothianidin mixture, for example, the CA model predicted a synergistic deviation from direct additivity ($a = -0.74$), whereas the IA model predicted a dose-level–dependent synergistic effect ($a = -10.8, b_{DL} = 0.89$; Supplemental Data, Table S4). Similarly, in the imidacloprid–thiamethoxam mixture the CA model predicted a slightly antagonistic effect ($a = 1.46$), whereas the IA model predicted dose-ratio–dependent deviation ($a = 57.17$, $b_{DR} = -86.89$) (Supplemental Data, Table S6). Finally, in the imidacloprid–clothianidin–thiamethoxam mixture, the CA model predicted direct additivity ($a = 0$), whereas the IA model predicted synergism ($a = -4.32$; Supplemental Data, Table S7).

Applications in field settings and risk assessment

Because MIXTOX is a descriptive, statistically based data analysis procedure, it cannot be used to directly identify the combination of mechanisms that lead to the cumulative toxicological effects observed in neonicotinoid mixtures [26]. Indeed, this analysis is typically carried out with an a priori assumption of mechanism of action of mixture constituents, (i.e., the reference model is selected prior to analysis). However, in the present study we found that cumulative toxicity of most neonicotinoid mixtures investigated deviated from the predicted reference model based on what is currently assumed about neonicotinoid mode of action. In the literature, there is no consensus on how similar the molecular sites or modes of action of mixture constituents must be to adequately employ either reference model [40]. Consequently, it is difficult to determine what model of mixture toxicity is best to apply in a risk assessment for acute exposures of neonicotinoid insecticide mixtures to aquatic insect species such as *C. dilutus*. We propose the application of a prediction window, incorporating both reference models into a probabilistic prediction of cumulative effects [40]. For risk assessment practices, this information should be further incorporated into a broad dataset of neonicotinoid mixture studies, including a range of neonicotinoid compounds, sensitive aquatic organisms, and exposure scenarios. This will aid in the further development of probabilistic mixture models, allowing for more accurate predictions of the ecotoxicological effects of neonicotinoid mixtures on aquatic insect communities.

Both the prevalence of neonicotinoid mixtures in aquatic environments [5] and the synergistic behavior of neonicotinoid mixtures observed in the present study indicate that neonicotinoids should not be regulated as single compounds. The use of neonicotinoid toxic equivalency factors [29] represents a reasonable approach for assessing cumulative toxicity; however, this may still underestimate risk. Furthermore, although exposure to acutely toxic concentrations of neonicotinoid mixtures could occur, nontarget aquatic organisms are more

likely to be chronically exposed to low concentrations of neonicotinoids in mixtures [3,17]. Therefore, caution should be taken when extrapolating the mixture responses observed in the present study to chronic exposure scenarios. However, until the effects of neonicotinoid mixtures on aquatic insects and arthropod communities under longer term, chronic exposure scenarios are further investigated, the cumulative synergism observed in the present study under acute exposure scenarios should be considered when setting water quality guidelines.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3878.

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Data Availability—Summarized Data, associated metadata, and calculation tools are available from the corresponding author (karsten.liber@usask.ca).

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