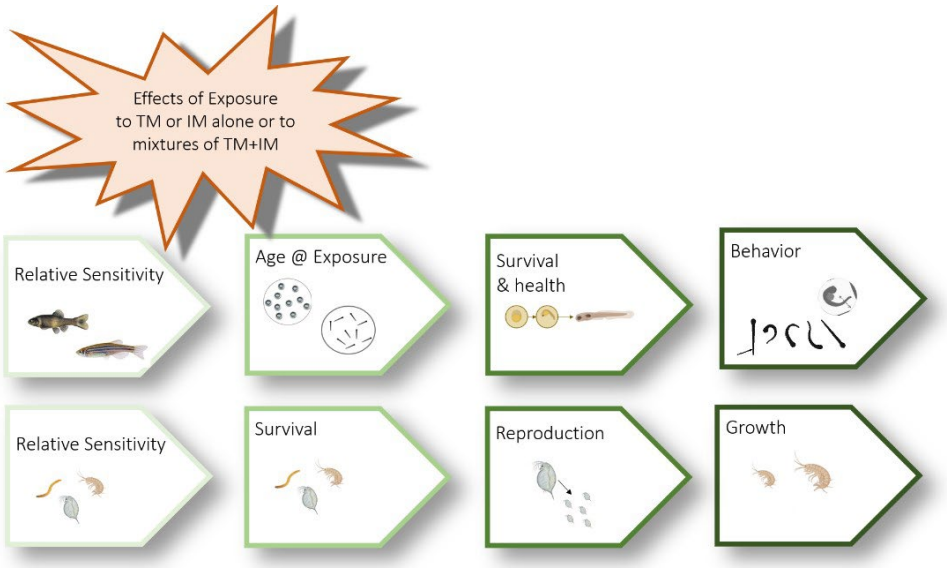
FINAL REPORT

PROJECT TITLE:
Sublethal Effects of Chronic Exposure to Neonicotinoid Pesticides on Aquatic Organisms

PROJECT ID:
**DATCP2020-2**




INVESTIGATORS:

Tisha King-Heiden, University of Wisconsin- La Crosse (Principal Investigator)
Elisabeth Harrahy, University of Wisconsin- Whitewater (Co-Principal Investigator)

December 2022

# Table of Contents



## LIST OF FIGURES



## LIST OF TABLES

## PROJECT SUMMARY

**Title:** Sublethal Effects of Chronic Exposure to Neonicotinoid Pesticides on Aquatic Organisms

**Project ID:** DATCP2020-2

**Investigators:** Tisha King-Heiden, Professor, Department of Biology, UW-La Crosse
Elisabeth Harrahy, Associate Professor, Department of Biology, UW-Whitewater

**Period of Contract:** June 2019 – December 2022

**Background/Need:** Neonicotinoid insecticides were designed to specifically target the nervous system of invertebrate pests (specifically the nicotinic Acetylcholine receptor, nAChR) and deemed not overtly toxic to vertebrates. Initially hailed as an exceptional replacement for older insecticides, their reputation was quickly tarnished by their association with honeybee declines and impacts on other non-target terrestrial insects. Following decades of widespread use, neonicotinoids are now major contaminants of concern in aquatic ecosystems, and their impacts on the aquatic community are not fully understood. Thiamethoxam (TM) and imidacloprid (IM) are found above ecological threshold levels in groundwater and surface waters in Wisconsin, particularly within the Central Sands Region. This poses a potential risk to aquatic invertebrates, and emerging evidence suggests that fish may be susceptible to adverse effects following chronic exposure to these insecticides.

**Objectives:** The overall objectives of our work were to better understand the chronic toxicity of TM and IM to key aquatic species within the Central Sands Region of Wisconsin, as well as an additional fish species that is a model organism in aquatic toxicology. Since neonicotinoids are commonly found in mixtures that are dominated by TM and IM, we also evaluated the toxicity of these compounds in mixtures.

**Methods:** We used standard laboratory assays to determine the acute toxicity of TM and IM singly and as a mixture to select aquatic invertebrates (*Ceriodaphnia dubia* water fleas, *Chironomus dilutus* midges, and *Gammarus pseudolimnaeus* amphipods), and the chronic toxicity of sublethal exposure of TM and IM singly and as a mixture to the water flea and amphipod. We also evaluated the toxicity of TM in the native fish fathead minnow (*Pimephales promelas*) embryos and larvae and zebrafish (*Danio rerio*) embryos, commonly used in toxicology studies. Static renewal toxicity tests were conducted in accordance with U.S. EPA and OECD guidelines. Mixture tests conducted with invertebrates utilized a toxic unit approach based on acute toxicity of each chemical alone, and mixture tests conducted with fish used specific ratios based on environmentally realistic concentrations. Endpoints measured included survival, growth, reproduction, and ecologically relevant behaviors (e.g., avoidance and foraging efficiency for fish). Finally, we used *in silico* approaches to estimate the binding affinity of TM and IM to the vertebrate nAChR in comparison to nicotine to better understand sublethal responses in fish.

**Results and Discussion:** We did not observe standard concentration-dependent mortality for invertebrates, but exposure to high concentrations of TM and IM increased mortality. Calculated LC/EC50 (lethal concentration/effective concentration for 50% of the individuals) values were well above concentrations of TM and IM typically found in streams and lakes. Amphipods were most sensitive to TM, while water fleas were most sensitive to IM. Reproduction of water fleas generally declined with increasing concentrations of TM and IM. Exposure to <6 μg/L of TM did not impact total length of amphipods, but length of amphipods exposed to 25 μg/L IM was significantly lower and growth was impaired over the course of the test. Mixture tests suggest that TM and IM act additively for invertebrates.

We observed no significant differences in survival for water fleas or amphipods. However, when we let the amphipod test continue beyond the standard duration (six days in clean water), survival was decreased in those exposed to the highest concentration of IM. We observed no significant effects of a mixture of TM and IM on reproduction of water fleas or growth of amphipods.

Chronic exposure to environmentally relevant concentrations of both TM and IM caused increased mortality by ~20% in fish larvae and caused subtle alterations in behaviors of survivors that could potentially impact their ability to reach adulthood. While computer modeling suggests that these insecticides can bind to the vertebrate nAChR, non-linear dose responses observed in our studies supports emerging findings that these compounds may cause toxicity in vertebrates through a different mode of action other than the nervous system - they may disrupt hormones like estrogen or thyroid hormones. Exposure to binary mixtures of TM and IM did not reveal a clear relationship with respect to their potential to act in an additive or synergistic fashion in fish, but we did observe slightly different toxic responses to mixtures compared to exposure to TM or IM singly.

**Conclusions/Implications/Recommendations:** Our laboratory data indicate that long-term impacts of neonicotinoid pesticides on aquatic communities warrant additional research. Exploration of potential modes of action for observed toxicity in vertebrates should be evaluated. We recommend increasing the observation period following exposure for invertebrates. In the case of *G. pseudolimnaeus* amphipods, this would require determining the best food for this species first. In the environment, neonicotinoid insecticides often enter streams and lakes in pulses. While we may not be able to capture short-term inputs of these chemicals, such short-term exposure may be enough to exert longer-term effects on survival, reproduction, growth and behavior of fish and aquatic invertebrates, and community-related impacts should be evaluated.

**Current Publications and Awards:**

S Victoria†, M Hein*, E Harrahy and TC King-Heiden. 2022. Potency matters: Impacts of embryonic exposure to nAChR agonists thiamethoxam and nicotine on hatching success, growth, and neurobehavior in larval zebrafish. J Toxicol Env Health, A. 85(18):767-782.

S Victoria†, S Duffy*, E Harrahy and TC King-Heiden. 2022. Embryonic exposure to thiamethoxam reduces survival and alters neurobehavior of fathead minnows. Env. Tox. and Chem. 41(5):1276-1285.

AJ Jeninga (MS student, UW-La Crosse) awarded Best Presentation Awards at (1) the Mississippi River Research Consortium Annual Meeting and (2) the Midwest Society of Environmental Toxicology and Chemistry annual meetings in 2022. S Victoria (MS, UW-La Crosse) won the best 3-min grad presentation (2021).

**Key Words:** Neonicotinoid, thiamethoxam, imidacloprid, fish, fathead minnow, zebrafish, aquatic invertebrate, water flea, chironomid, amphipod

**Funding:** This project was funded through the Wisconsin Department of Agriculture, Trade and Consumer Protection (DATCP2020-2). Supplemental funding was also provided by the Freshwater Collaborative of WI, UW-La Crosse College of Science and Health, UWL Graduate Studies Program RSEL grants to S Victoria and AJ Jeninga, and UW-Whitewater Summer Undergraduate Research Fellowship Program grants to AJ Jeninga and Jacob Lacki.

**Final Report:** A final report containing detailed information on this project is included in the Groundwater Project Repository of the Wisconsin Groundwater Research and Monitoring Program (WGRMP).

## INTRODUCTION

Over the past two decades, neonicotinoid insecticides have become the most widely used insecticides in agriculture around the world (Simon-Delso et al., 2015; Casida and Durkin, 2013). At least 600 products registered for use on Wisconsin crops contain one or more neonicotinoid as active ingredients (DATCP, 2018; Kelly Products, Inc., 2017). Neonicotinoids function as neuroactive insecticides, over-stimulating nicotinic acetylcholine receptors (nAChR), leading to paralysis and death in target species. Since they have a relatively low affinity for mammalian nAChRs, they are considered a safer alternative to organophosphate, carbamate, and organochlorine pesticides (Simon-Delso et al., 2015; Tomizawa and Casida, 2005). Not surprisingly, non-target insects have been shown to be sensitive to exposure to low concentrations of these pesticides, and their unintended impacts on non-target species such as honeybees and other pollinators have garnered considerable attention (Goulson, 2013; Lundin et al., 2015). There is widespread concern regarding the potential large-scale risks they pose to ecosystem structure and function by impacting non-target species directly or indirectly by altering food webs (Chagnon et al., 2015; Gibbons et al., 2015). And while much is known about the toxicity of these insecticides to non-target terrestrial invertebrates (Goulson, 2013; Lundin et al., 2015), less is known about the toxicity of these pesticides to aquatic species (Anderson et al., 2015; Chagnon et al., 2015; Goulson, 2013).

Newly implemented monitoring programs suggest that aquatic organisms are at risk from exposure to neonicotinoid pesticides because their chemical properties make them susceptible to transport into surface waters via runoff and/or groundwater movement, and regular use enhances their persistence within the environment (Anderson et al., 2015; Armbrust and Peeler, 2002; Sánchez-Bayo et al., 2016; CCME, 2007; EFSA, 2008; Tisler et al., 2009). A study conducted by the Wisconsin Department of Agriculture, Trade and Consumer Protection (DATCP) recently detected neonicotinoids in surface waters and groundwater across the state, which were particularly high within the Central Sands Region (DATCP, 2018). Thiamethoxam, for which there are limited toxicity data available, was frequently detected, ranging in concentration from ~ 0.05 – 0.30 µg/L and concentrations of imidacloprid ranged from ~ 0.05 – 0.09 µg/L (DATCP, 2018). These concentrations exceed established U.S. EPA benchmarks for chronic exposure of invertebrates to imidacloprid, but not thiamethoxam (0.01 µg/L and 0.74 µg/L (US EPA, 2017), although there is considerably less information about the toxicity of thiamethoxam to support these target concentrations (Anderson et al., 2015). Concentrations in this area also exceeded the ecological thresholds recommended by Morrissey et al. (2015) for chronic exposure (0.035 µg/L) and acute exposure (0.2 µg/L) of aquatic invertebrates to neonicotinoids.

Evaluating the potential ecological risks of these neonicotinoids within the Central Sands Region ecosystem will require careful examination of the effects of chronic exposure to sublethal concentrations on non-target aquatic species. Prolonged exposure to sublethal concentrations of neonicotinoids is of particular concern for invertebrates that have long aquatic life stages (Morrissey et al., 2015), and while less sensitive than invertebrates, there is evidence that these compounds can have both direct and indirect impacts on fish as well (Gibbons et al., 2015; DeCant and Barrett, 2010; Cox, 2001). Because more than one neonicotinoid may be found together in groundwater and in surface waters, it is also important to examine the potential for these insecticides to have additive or even synergistic effects when organisms are exposed to mixtures.

Given the limited amount of empirical data on sublethal effects of chronic exposure to thiamethoxam, as well as the impact of co-exposure to more than one neonicotinoid, this project examined the effects of chronic exposure to sublethal concentrations of neonicotinoid insecticides (thiamethoxam or imidacloprid, as well as mixtures of thiamethoxam + imidacloprid) to two aquatic invertebrates (*Ceriodaphnia dubia* and *Gammarus pseudolimnaeus)* and to a fish species (*Pimephales promelas*) found in aquatic ecosystems of the Wisconsin Central Sands Region and Wisconsin. Given the lack of toxicity

data for thiamethoxam, we also ran toxicity tests using another model fish (zebrafish, *Danio rerio*) for comparison. Acute toxicity tests were conducted with *Chironomus dilutus*, *Ceriodaphnia dubia*, and *Gammarus pseudolimnaeus* to determine LC50 (lethal concentration for 50% of the population) or EC50[1] (effective concentration for 50% of the population) values and to better determine concentrations to be used in the chronic toxicity tests conducted with these invertebrates. Acute exposure studies were not performed for fish since LC50s are predicted/known to be at an order of magnitude greater than measured environmental concentrations. Endpoints for chronic studies focused on sublethal responses that could contribute to population declines.

## PROCEDURES AND METHODS

**Preparation and Confirmation of Test Solutions:** Thiamethoxam (TM) and imidacloprid (IM) were purchased from Sigma-Aldrich (>98% purity) and test solutions were made in the appropriate dilution water for each species (e.g., moderately hard reconstituted water for the invertebrates). Samples of test solutions were stored in amber glass bottles with Teflon lids and stored frozen until analysis. Concentrations were confirmed by high-performance liquid chromatography with tandem mass spectrometry (HPLC/MS/MS) performed by the Lumigen Instrument Center at Wayne State University. In all experiments, test solutions were 100% renewed each day, for the duration of exposure.

**Aquatic Invertebrate Studies:** Water fleas (*Ceriodaphnia dubia*) and midges (*Chironomus dilutus*) were cultured in the laboratory and amphipods (*Gammarus pseudolimnaeus*) were collected from Bluff Creek in Whitewater, WI. Culture and toxicity test methods followed standard guidelines (US EPA, 2002a; US EPA, 2002b; US EPA, 2002c; US EPA, 2000; Buikema and Cairns, 1980). All tests were conducted in environmental chambers with a 16 h light/8 h dark cycle and a temperature of 25°C (water fleas and chironomids) or 17°C (amphipods). Water quality parameters were measured daily in every test and included temperature, dissolved oxygen, conductivity and pH. Hardness and alkalinity were measured in the moderately hard reconstituted dilution water.

In the acute toxicity tests, organisms were exposed to high concentrations for short durations to determine the effects of TM or IM singly, or as a mixture, on survival. In the single insecticide toxicity tests, organisms were exposed to a control and seven or eight concentrations of thiamethoxam or imidacloprid using a 60% dilution series for 48 h (water fleas and chironomids) or 96 h (amphipods). Nominal concentrations of test solutions ranged from 56 to 2000 or from 28 to 1000 µg/L. In the mixture toxicity tests, organisms were exposed to a control, a TM alone treatment, an IM alone treatment, and four mixture treatments, using a toxic unit approach, where one toxic unit was equal to the nominal LC/EC50 value for TM or IM for the test species, based on results of the single insecticide acute toxicity tests. Three of the mixture treatments were ratios of the two insecticides that should have added up to a total of one toxic unit (e.g., 0.5 LC50 for TM plus 0.5 LC50 for IM), and one of the mixture treatments was equal to two toxic units (1.0 LC50 for TM plus 1.0 LC50 for IM). The TM alone and IM alone treatments were equal to the LC/EC50 value for that chemical, for the test species. The toxic unit study design can allow us to determine if two chemicals act in an additive, synergistic, or antagonistic manner (Hepditch et al., 2020). For example, if the combined effects of the two insecticides in a given treatment result in greater than 50% mortality (i.e., > one toxic unit), then the interaction may be synergistic (greater than additive). For all experiments, there were five water fleas (<24 h old) in each of four replicates, ten chironomids (2nd instar) in each of four replicates, or one amphipod (3-6 mm) in each of 20 replicates per treatment. Mortality was used to calculate LC/EC50 values using the trimmed Spearman-Karber method (Hamilton

[1]EC50 values are used for very small organisms like water fleas and midges where it might be difficult to determine death. Immobilization is used as an endpoint instead. We did examine all organisms under a microscope in these tests to confirm mortality, but follow standard reporting of an EC50.

et al. 1977), and analysis of variance (ANOVA) and Tukey's multiple comparison test were used to determine if there were significant differences in survival among treatments.

In the chronic toxicity tests, organisms were exposed as for acute studies to lower concentrations for longer durations (seven days for water fleas or ten days for amphipods) to determine the effects of TM or IM singly, or as a mixture, on reproduction of water fleas and growth of amphipods. In the water flea tests, there was one water flea in each of ten replicate glass beakers to better follow reproduction for each water flea. Nominal concentrations in the single insecticide tests ranged from 5.4 to 200 (water fleas) and 0.93 to 20 (60% dilution; amphipods) or 0.02 to 20 (10% dilution; amphipods), based on results of the acute toxicity tests. In the chronic mixture tests, a toxic unit was equal to 0.5 LC/EC50 instead of 1.0 LC/EC50 (based on nominal concentrations). Water fleas were fed YTC (yeast, cerophyl, trout chow) and algae (*Selenastrum capricornutum*), and amphipods were fed stream-conditioned leaves. Water flea reproduction was measured as number of neonates produced. All surviving amphipods were collected, preserved in 70% ethanol and photographed under a microscope. Image J software was used to measure total length as a measure of growth. ANOVA and Tukey's multiple comparison tests were used to determine if there were significant differences in survival, reproduction, or growth among treatments.

**Fish Studies:** Exposure concentrations for assessing the toxicity of TM or IM alone encompassed environmental concentrations found in the Central Sands Region of Wisconsin: 0.02, 0.02, 0.2, or 2 µg/L, as well as higher concentrations, 20 or 200 µg/L. For mixtures studies, we used 1:1 ratios of IM:TM to assess potential additive responses or environmentally relevant ratios of 1:3, 1:4 or 1:5 IM:TM. Zebrafish embryos were exposed to TM beginning just after fertilization through larval development (5 days), while fathead minnows were exposed to TM for 8 days as embryos (beginning just after fertilization through larval development) or as larvae (beginning at post-hatch). Fathead minnow embryos were exposed to IM or mixtures of TM and IM for 8 days. In addition to monitoring survival and general health (hatching success and signs of malformations in developing fish), we assessed behaviors that rely on proper activation of the nAChR such as embryonic motor activity (spontaneous movement of the tail before the fish hatches from its chorion), the "C-start response" (an innate swimming response used to avoid predators), and/or foraging efficiency (timed ability to capture prey items). Finally, we used computer modeling software (Maestro, Schrödinger, LLC) to predict the strength/ability of both TM and IM to bind to a vertebrate nAChR in comparison to nicotine to better interpret our findings in fish. For all studies, we used Kaplain-Meier survival analysis to evaluate treatment-related effects on survival, and ANOVA and Tukey's post hoc or ANOVA on Ranks with Dunn's post hoc to evaluate treatment related impacts on growth and behavior. Chi Square analyses and linear regression analyses were also performed to gain additional insight into observed effects.

## RESULTS AND DISCUSSION

**Aquatic Invertebrate Studies:** Results of the single insecticide acute toxicity tests are summarized in Table 1. Our results can be compared to those published in the literature and presented in Table 1 in Appendix B. We were able to calculate LC/EC50 values for both TM and IM, with the exception of *C. dilutus* midges for which we were unable to calculate an EC50 for IM. In that test, survival was significantly lower than the control, starting at a concentration of 390 µg/L IM. In some of the acute toxicity tests, we observed non-monotonic dose responses, in which survival was higher for individuals exposed to higher concentrations than for those exposed to moderate concentrations. Raby et al. (2018c) and Raby et al. (2018a) also observed "poor concentration-response relationships" in examining the effects of imidacloprid and thiamethoxam on select aquatic invertebrates including *C. dilutus*. In some tests, there was high variability in response among individuals within a treatment, which led to broader 95% confidence intervals around the LC/EC50 values. And where tests were repeated, there was some

variability in LC/EC50 values calculated for the same chemical for the same species, even when actual (versus nominal) concentrations were taken into account.

**Table 1. Impacts of acute exposure to thiamethoxam or imidacloprid on survival of aquatic invertebrates.** LC50 (lethal concentration for 50% of the population; amphipods) or EC50 (effective concentration for 50% of the population; midges and water fleas) values and 95% confidence intervals were calculated using a trimmed Spearman-Karber method following 48 hours (midges and water fleas) or 96 hours (amphipods) exposure.

| Chemical | Species | LC50/EC50 µg/L | 95% CI µg/L |
|---|---|---|---|
| Thiamethoxam | *Chironomus dilutus* | 134 | 107 - 168 |
| | | 373 | 304 - 458 |
| | *Ceriodaphnia dubia* | 146 | 1.36 - >1,000 |
| | | 223 | 155 - 321 |
| | *Gammarus pseudolimnaeus* | 48.8 | 25.3 - 94.2 |
| | | 127 | 74.8 - 215 |
| Imidacloprid | *Chironomus dilutus* | EC50 not calculable. Survival sig < control at 390 | -- |
| | *Ceriodaphnia dubia* | 31.7 | 8.77 - 114 |
| | | EC50 not calculable. Survival sig < control at 98.8 | -- |
| | *Gammarus pseudolimnaeus* | 189 | 120 - 298 |
| | | 145 | 112 - 188 |

Our 48 h EC50 values for *C. dubia* water fleas exposed to IM (31.7 µg/L) and TM (146 and 223 µg/L) were significantly lower than those found in the literature for IM, while our 48 h EC50 values for *C. dilutus* exposed to TM (134 and 373 µg/L) were significantly higher (less sensitive) than those reported in the literature (see Table 1, Appendix B). We were unable to calculate a 48 h EC50 value for *C. dilutus* exposed to IM because survival remained at 60% or higher, even at the highest concentration tested. However, survival was significantly reduced, compared to the control, at a concentration of 390 µg/L. Many of the chironomids exposed to IM and TM in our test were immobilized (barely moving under the microscope), or impaired (exhibited muscle spasms). In the wild, immobilized or impaired individuals may be more likely to die through inability to forage or eat, increased drift and increased predation. Relative sensitivity among the species differed by chemical. *G. pseudolimnaeus* amphipods were most sensitive to TM, while *C. dubia* water fleas were most sensitive to IM. This is in contrast to studies in the literature that have shown water fleas to be less sensitive than amphipods to IM (Raby et al., 2018a; Finnegan et al., 2017). However, to the best of our knowledge, we are the first to examine the effects of neonicotinoid insecticides on *G. pseudolimnaeus*, and less is known about the sensitivity of this species of amphipod.

LC/EC50 values were all well above concentrations typically found in surface waters (Metcalfe et al., 2019; Hladik and Kolpin, 2016) and groundwater (Bradford et al., 2018). But having the acute toxicity data was useful in determining the best concentrations to use in the single chemical chronic tests and in the acute and chronic mixture tests. These data can also be useful in the case of a spill, and in calculating water quality criteria and secondary values.

Results of the single insecticide chronic toxicity tests are summarized in Figure 1. The number of neonates produced by the water fleas generally declined with increasing concentrations of IM and TM. Reproduction was significantly different from the control at 175 µg/L IM and 82 µg/L TM. These are much lower effect concentrations than those determined by others (Table 1, Appendix B). The decrease in reproduction was not significant until the concentration of IM was higher than the EC50 value for lethality (31.7 µg/L), but the lack of significance at lower concentrations may be due to variability within a treatment. There seems to be a good deal of intraspecific variability in sensitivity. In addition, the dose response curve in the acute test for water fleas exposed to IM was atypical, with higher survival at higher concentrations than at moderate concentrations. There was no significant difference in total length among amphipods exposed to seven concentrations of TM. However, while the highest nominal concentration was 20 µg/L, it turned out the measured concentration for that treatment was only 6.0 µg/L, and this may not have been high enough to elicit an effect. Total length of amphipods exposed to 25 µg/L IM (nominal of 20 µg/L) was significantly lower than length of amphipods in the control, and amphipods in this treatment did not significantly grow over the course of the 10-d test compared to an initial sample. There was no significant difference in length of amphipods exposed to concentrations as high as 2.5 µg/L IM.

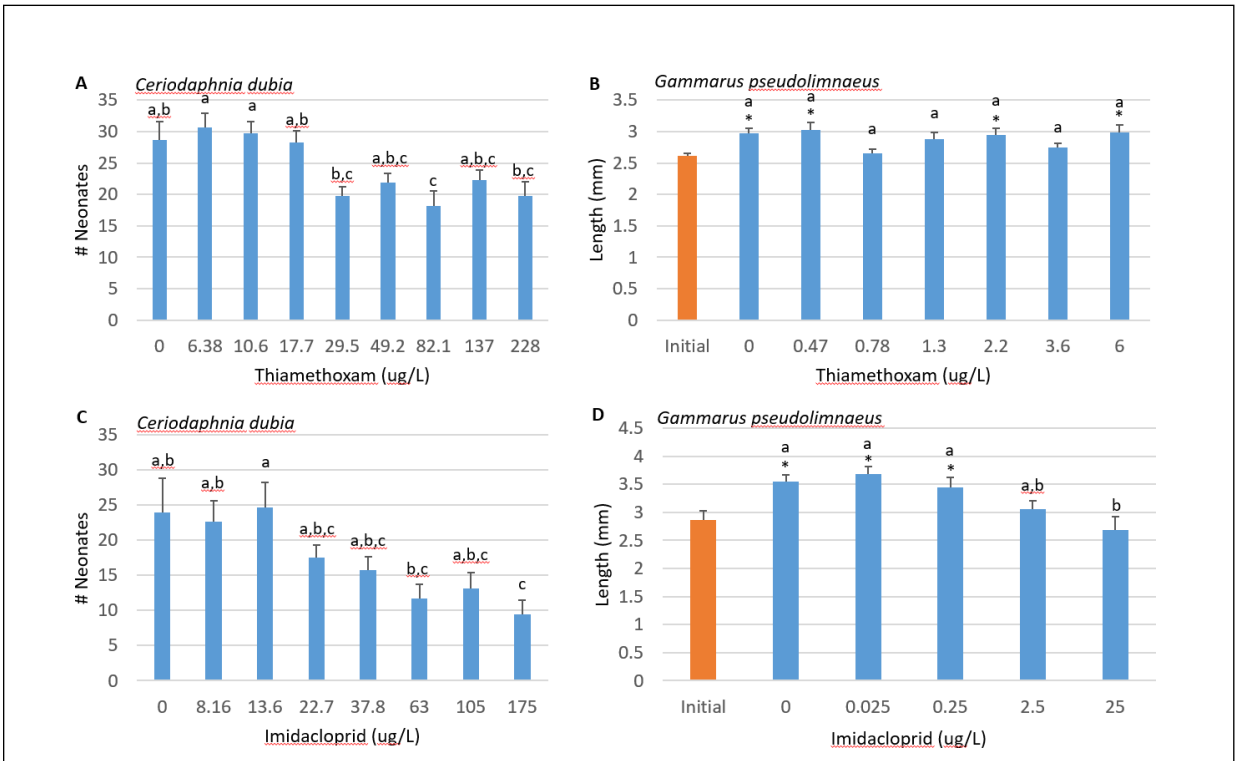



**Figure 1. Effects of chronic exposure to thiamethoxam or imidacloprid on reproduction and growth of aquatic invertebrates.** Effects of the neonicotinoid insecticides thiamethoxam or imidacloprid on reproduction (as number of neonates) of *Ceriodaphnia dubia* water fleas (**A** and **C**, respectively) and growth (as length) of *Gammarus pseudolimnaeus* amphipods (**B** and **D**, respectively). Duration of exposure was 7 days (water fleas) or 10 days (amphipods). Endpoints were compared among treatments using analysis of variance and Tukey's multiple comparison procedure. Treatments with the same letter are not significantly different from each other. Amphipods in treatments with an "*" had an average length that was significantly greaterthan those in the initial sample. Error bars are +1 standard error.

While *G. pseudolimnaeus* is an ecologically and locally important species, it proved difficult to culture in the laboratory. Survival of our wild caught amphipods did not allow for longer tests (survival of the control amphipods declined below 80% between ten and 14 days, likely because they were not getting enough nutrition). It is possible we would have seen greater differences in length among treatments, and in overall growth compared to initial size had we run the tests for a longer duration, but such studies would require improved culturing techniques or use of other species that are easier to maintain in the lab (e.g., *Hyalella azteca;* Bartlett et al., 2019). Feeding studies also proved difficult for this species. While most amphipods are considered to be shredders, and other researchers have been able to conduct long-term studies that examined the effects of neonicotinoid insecticides on feeding of leaves (e.g., Nyman et al., 2013), we observed a high level of intraspecific variability in feeding in our *G. pseudolimnaeus*. Some individuals consumed the leaves we provided them, and other individuals consumed only portions or none of the leaves. In fact, we ran a test to compare their preference for four different species of leaves collected from the same stream from which we obtained the amphipods: cut-leaf water parsnip (*Berula erecta*), watercress (*Nasturium officinale*), curly-leaf pondweed (*Potamogeton crispus*) and Canadian waterweed (*Canadian waterweed*). While each leaf species was eaten by some individuals, the cut-leaf water parsnip, seemed to be favored (*unpublished data*). This was not surprising, given we find more of the amphipods living on and around this plant species in the stream. We also tried feeding the amphipods sugar maple leaves that had been stream-conditioned in Bluff Creek for 30 days in leaf pack bags. Regardless, no one leaf species was regularly consumed by all individuals. This limited our ability to conduct studies designed to determine the effects of TM and IM on feeding. An initial feeding study conducted with amphipods exposed to IM in their water while being fed stream-conditioned maple leaves indicated a potential dose response in feeding rate (those exposed to higher concentrations ate fewer leaves), but the difference was not significant due to high variability among individuals within a treatment. *Gammarus* spp. may also eat the biofilm growing on the leaves before (Bottger et al., 2013), or instead of, shredding the leaves, which can be harder to quantify. Taken together, our work suggests studies to develop standardized rearing and feeding of this ecologically important species may prove worthwhile.

Results of the acute toxicity tests conducted with a mixture of TM and IM are presented in Figure 2. Since TM and IM target the same receptor and have the same predicted mode of toxicity, we would predict that mixtures of these two neonicotinoids would cause additive toxicity (the effects of each sum together, like 2+2=4) versus greater than additive toxicity (synergism; the sum of the effects is greater than when only one is present, like 2+2=6) or less than additive toxicity (antagonism; the sum of the effects is less than when only one is present, like 2+2=1). When acute tests were run for the standard duration (two days for the water fleas and four days for the amphipods), we observed no significant differences in survival among treatments (control, single insecticides and four different mixtures) for water fleas or amphipods, although again, there was significant variability in survival within treatments that may have affected these results. For example, survival was 100% for water fleas in the control, and only 55% for water fleas in one of the mixture treatments, but these were not significantly different (ANOVA, Tukeys, $p<0.05$). However, when we let the amphipod mixture test continue beyond the standard duration of four days to ten days (four days exposure followed by six days in clean water), we observed significant decreases in survival among some of the treatments, while the control survival remained at 100%. Delayed effects have been observed in other studies (e.g., Bottger et al., 2013). In the present study, survival was lowest where concentrations of IM were highest, so IM may, at first, appear to account for a higher proportion of the observed mortality than TM. However, actual concentrations of IM were closer to nominal, while concentrations of TM were only about half of nominal. So, using the toxic unit approach, we would expect the treatments with higher proportions of IM to be more toxic in this case.

In the chronic toxicity tests conducted with a mixture of TM and IM, we observed no significant effects on reproduction of water fleas or growth of amphipods. It is possible that effects on growth of amphipods

may have been observed if the test had been conducted for a longer duration of exposure, or incorporated a post-exposure observation period as observed by Cavallaro et al. (2017) where the longer the duration of exposure, the lower the effective concentration.

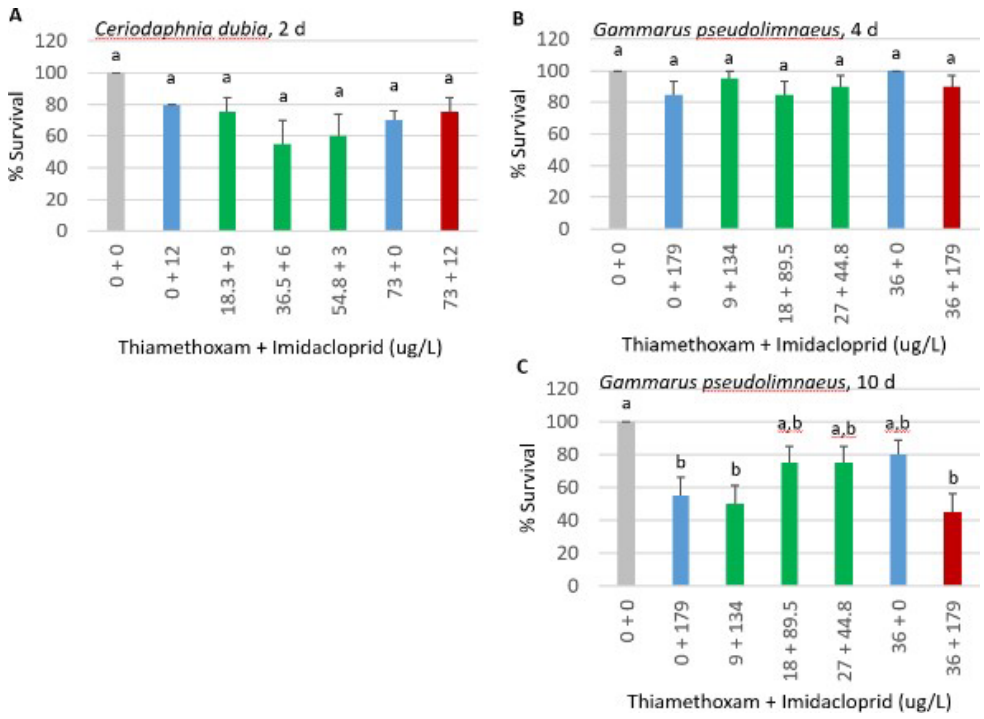



**Figure 2. Effects of acute exposure to mixture of the neonicotinoid insecticides thiamethoxam and imidacloprid on survival of aquatic invertebrates**. **(A)** *Ceriodaphnia dubia* water fleas after 2 d exposure, and **(B)** *Gammarus pseudolimnaeus* amphipods after 4 d exposure or **(C)** 10 d exposure. A toxic unit approach was used such that each combination of the two insecticides was expected to kill about 50% of individuals (green bars) or 100% of individuals (red bars), but this was based on nominal concentrations used in the single insecticide toxicity tests. Actual concentrations are presented here. The EC50 and LC50 values calculated based on actual concentrations can be found in Table 1. Survival was compared among treatments using analysis of variance and Tukey's multiple comparison procedure. Treatments with the same letter are not significantly different from each other. Error bars are +1 standard error.

Overall, our findings suggest that mixtures of TM and IM cause additive toxicity in the aquatic invertebrates we tested, at the concentrations we tested. We did not observe greater-than-additive action of IM and TM mixtures in our acute or chronic mixture tests. Few studies have been conducted that have examined the effects of mixtures of two or more neonicotinoid insecticides on aquatic invertebrates. Those that have been conducted have indicated that mixtures of neonicotinoid insecticides do not always act in an additive manner. Maloney et al. studied the acute (2017) and chronic (2018b) toxicity of binary and ternary mixtures of clothianidin, IM, and TM in *C. dilutus*, and used a MIXTOX modeling approach to test the assumption of concentration additive cumulative toxicity. In their acute tests, they determined that mixtures of IM and TM exhibited a "response-additive dose-ratio-dependent synergism," with toxicity shifting from antagonism to synergism as the relative concentration of TM increased. In their chronic tests, they also determined that a mixture of IM and TM displayed a "dose-ratio dependent

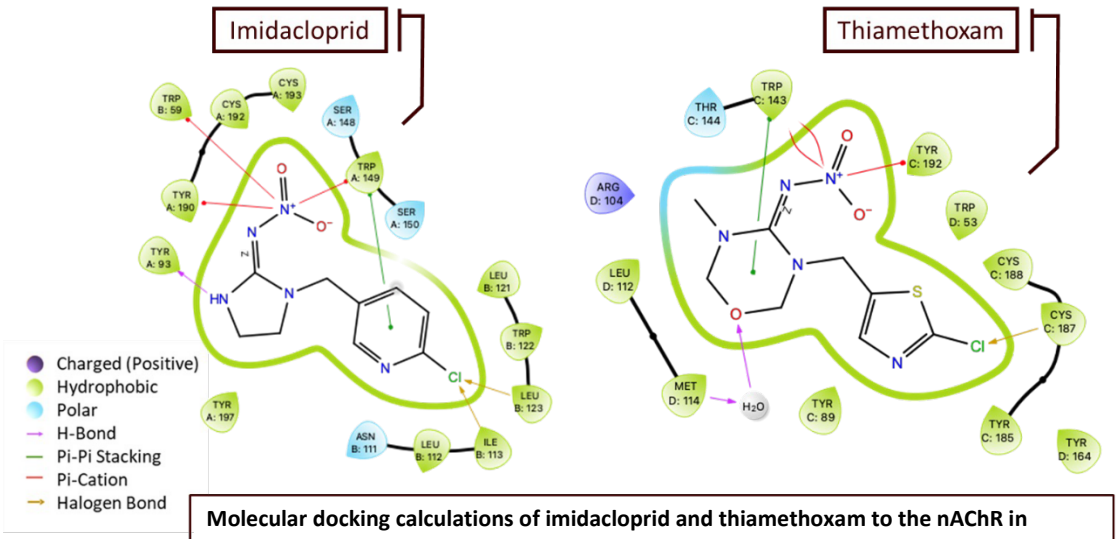
synergism," in which emergence of *C. dilutus* was reduced to a higher degree than would be predicted by concentration addition alone, but only when mixtures contained higher concentrations of TM than IM. However, in a semi-controlled field setting using limnocorrals, Maloney et al. (2018a) found that emergence and biomass responses of *C. dilutus* to a mixture of IM and TM were strictly additive.

**Fish Studies:** Summative information regarding the toxicity of thiamethoxam and imidacloprid singly and in combination are summarized in Table 2.

**Table 2: Summary of toxicity observed in fish following chronic exposure to thiamethoxam and imidacloprid.**

| Species & age at exposure | Mortality | Impaired Hatching | Embryonic Motor Activity | Predator Escape | Foraging Efficiency |
|---|---|---|---|---|---|
| **Thiamethoxam** | | | | | |
| Fathead minnow embryo | ≥ 1.6 µg/L ~20% increase | no effect obs; >155 µg/L | - reduced 155 µg/L -25% of fish exposed to ≥ 1.6 µg/L | - delayed larvae exposed to ≥ 0.16 µg/L - ~25-58% of fish decreased burst speed | -Slight reduction in 40-50% of all exposed fish |
| Fathead minnow larvae | no effect obs; >155 µg/L | no effect obs; >155 µg/L | no effect obs; >155 µg/L | no effect obs; >155µg/L | no effect obs; >155µg/L |
| Zebrafish embryo | 163 µg/L | 0.21; 163 µg/L | no effect obs; >163 µg/L | -0.21 µg/L delayed response and burst speed | no effect obs; >163 µg/L |
| **Imidacloprid** | | | | | |
| Fathead minnow embryo | ≥ 1.6 µg/L 10-40% increase | ≥ 1.6 µg/L | Reduced ≥ 1.6 µg/L | ~42% exposed to ≥ 0.02 µg/L had delayed response -174 µg/L decreased burst speed | Not assessed |
| **Mixtures of thiamethoxam and imidacloprid** | | | | | |
| Fathead minnow embryo 1:1 IM:TM | ≥ 0.2 µg/L 1:1 10-30% increase | ≥ 20 µg/L 1:1 Increase in hatching | Reduced ≥ 0.2 µg/L 1:1 | No observed effect | Not assessed |
| Fathead minnow embryo 1:3, 1:4, or 1:5 IM:TM | No observed effect | No observed effect | No observed effect | 1:5 ratio delayed | Not assessed |

Computer modeling predicts that, as expected, neither thiamethoxam nor imidacloprid are capable of binding the nAChR with sufficient affinity to cause overt toxicity (Figure 3), which is supported by our experimental findings as described below.




**Molecular docking calculations of imidacloprid and thiamethoxam to the nAChR in comparison to nicotine**

| Compound | Ionization Penalty (kcal/mol) | State Penalty (kcal/mol) | Docking Score | Glide Score |
|---|---|---|---|---|
| Imidacloprid | <0.01 | <0.01 | -6.7 | -6.7 |
| Thiamethoxam | <0.01 | <0.01 | -4.5 | -4.5 |
| Nicotine | 0.03 | 0.03 | -9.1 | -9.1 |

**Figure 3. Estimated binding affinity of imidacloprid and thiamethoxam compared to nicotine for the vertebrate nAChR.** Computer models indicate that both imidacloprid and thiamethoxam are capable of binding to the vertebrate nAChR; however, based upon docking and glide scores, the affinity is much lower as predicted (the more negative these scores, the stronger the molecule binds the receptor). Imidacloprid may have a slightly higher binding affinity for the nAChR than thiamethoxam.

For fish, we found that TM was not toxic to fathead minnow larvae if exposure began as larvae (post-hatch), but did cause adverse health and behavioral outcomes if exposure began as embryos (just after fertilization) (Figure 4). Further, fathead minnows are more sensitive to TM compared to zebrafish larvae. We were surprised to see increased mortality for both fathead minnow and zebrafish following chronic exposure to concentrations of TM that are an order of magnitude lower than reported LC50s for other fish species. Chronic exposure to TM induced subtle alterations in behaviors (embryonic motor activities and predator escape responses) in both fathead minnows and zebrafish, indicative that TM may bind the nAChR in vertebrates with a low affinity; this is supported by our computer modeling. Further studies would be needed to clarify NOEC or LOEC values for thiamethoxam, but using a risk quotient, calculated using concentrations detected in WI, and our NOEC of 14.6 µg TM/L for behavioral alterations in fathead minnows, chronic exposure to surface waters with concentrations below 1.5 µg TM/L would pose low risk, concentrations between 1.5-15 µg TM/L would pose medium risk and concentrations >15

µg TM/L would pose high risk with respect to altering behaviors that are essential for survival. Our findings also support the need to further test the hypothesis that TM may be able to interfere with thyroid hormone or estrogen hormone regulation of early development (Zhu et al. 2019).

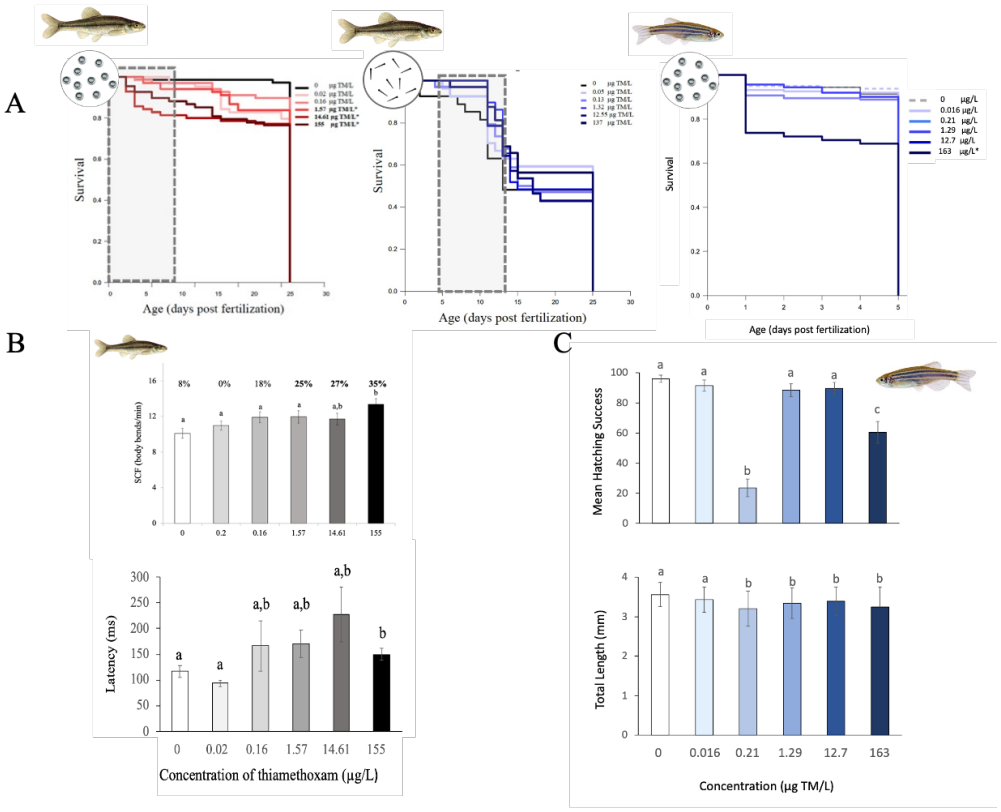



**Figure 4. Effects of chronic exposure to thiamethoxam in fish.** TM reduces survival by approximately 20% in both fathead minnows and zebrafish when exposure begins at early development, but TM is not toxic to fathead minnows if exposed post-hatch (A). Embryonic exposure to TM has a larger impact on behavior in fathead minnows (B) but has more general adverse health outcomes in zebrafish (C). Taken together, chronic exposure to TM poses low to medium risk in fish. Letters denote significant differences, and proportions (%) in bold are significantly different from control groups.

Imidacloprid appears to be slightly more toxic to fathead minnow larvae than thiamethoxam, which may be due to its potential to bind the nAChR at a greater affinity. Chronic exposure to IM beginning just after fertilization reduced survival by 20-40%, impaired growth by 7-24%, and altered embryonic motor activities (Figure 5). Interestingly, IM did not impair the predator escape response other than slowing the overall swimming speed following exposure to very high concentrations (174 µg IM/L). Further studies would be needed to clarify NOEC or LOEC values for imidacloprid, using our NOEC of 0.16 µg IM/L for mortality and reduced growth in fathead minnows to calculate a risk quotient, chronic exposure to surface waters with concentrations below 0.016 µg TM/L would pose low risk, concentrations between 0.016-0.16 µg IM/L would pose medium risk and concentrations 0.16 > µg IM/L would pose high risk to wild fish.

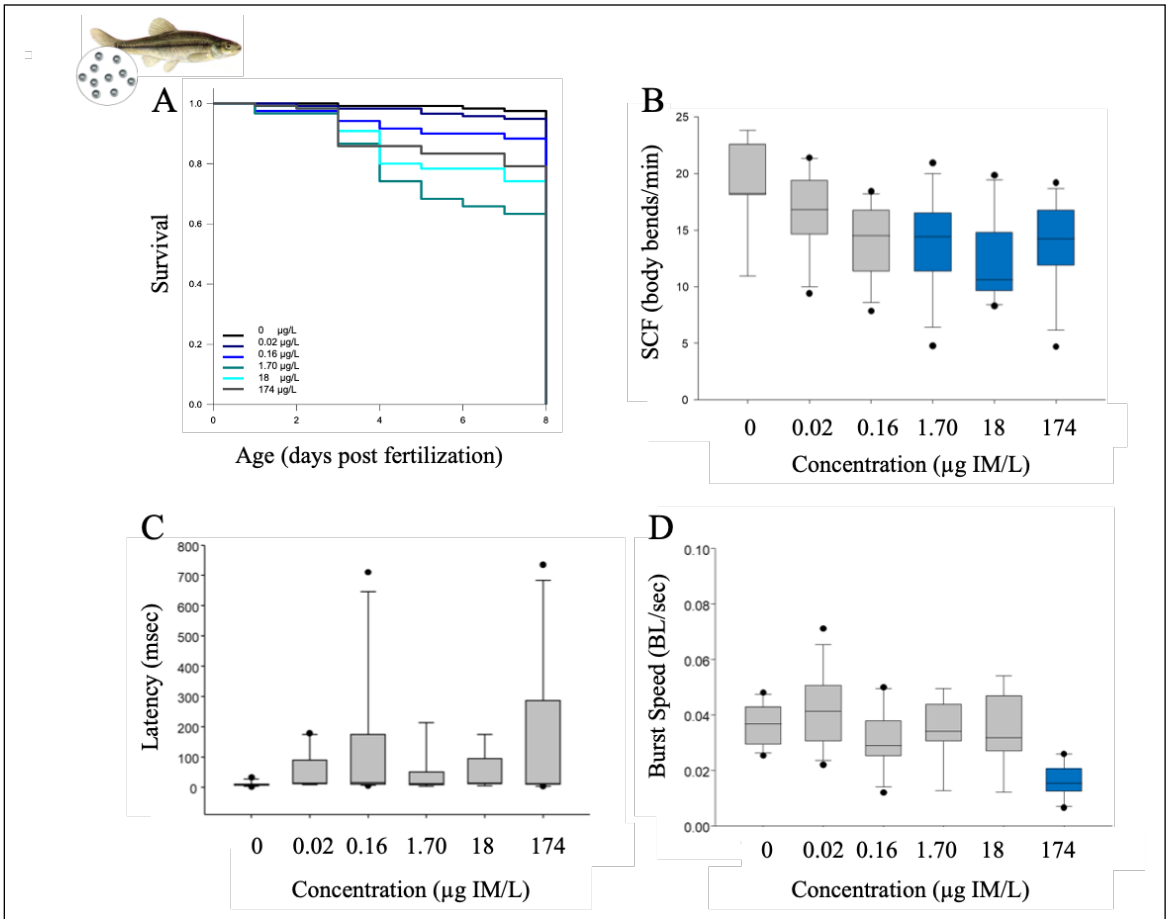



**Figure 5. Effects of chronic exposure to imidacloprid in fish.** IM reduces survival by approximately 20-40% in fathead minnows, but not in a dose-response manner (A). Embryonic exposure to ≥ 1.7 µg IM/L resulted in increased embryonic motor activity (B), but the predatory response was only altered following exposure to very high concentrations of IM (C,D). Blue bars indicate data that was significantly different from control. Taken together, chronic exposure to IM poses low to medium risk in fish.

When fish were exposed to mixtures of imidacloprid and thiamethoxam, imidacloprid appeared to drive the overall toxicity. Imidacloprid and thiamethoxam do not appear to cause additive toxicity in fish (Figure 6); however, there were some subtle differences in toxic response of IM when TM was present at equivalent nominal concentrations (confirmation of exposure concentrations are in progress). For example, when exposed to IM alone, significant mortality was observed following chronic exposure to concentrations ≥ 0.16 µg IM/L, but when IM and TM were present in equivalent concentrations, significant mortality was only seen following exposure to approximately 1:1 mixtures of 0.2 or 2 µg/L of IM and TM. Further, decreased embryonic motor activity was observed following exposure to a lower concentration of IM when TM was present at an equivalent nominal concentration. Our computer modeling suggests that IM and TM may interact with slightly different residues on the nAChR, indicating that observed toxicities following exposure to mixtures of IM and TM could be explained by potential competitive interactions between the two molecules and the nAChR. Further, since it is possible that TM may cause its toxicity in fish through a different mode of action all together, that could also support the lack of any observed additive responses observed in these studies. Taken together, our data indicate that mixtures of TM and IM do not pose an

increased direct risk to wild fish populations compared to each compound together, especially at the environmentally relevant mixtures observed in the Central Sands Region of WI.

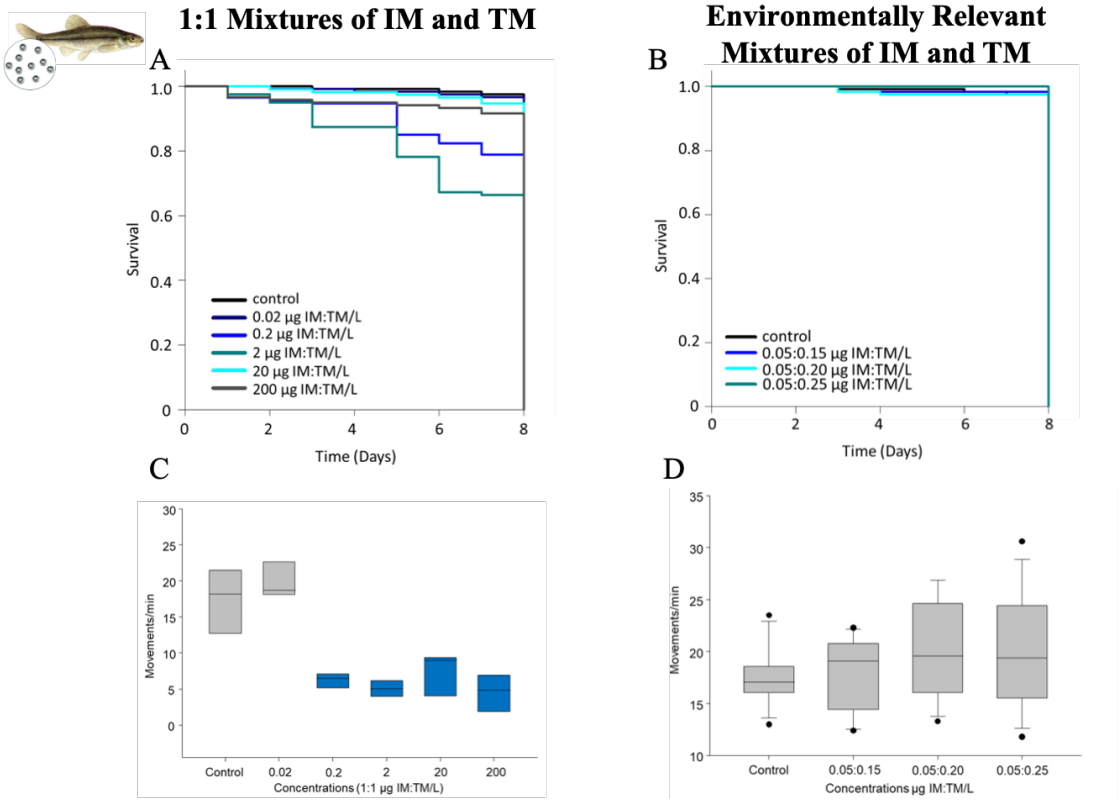



**Figure 6. Effects of chronic exposure to mixtures of thiamethoxam and imidacloprid in fish.** Chronic exposure to mixtures of IM and TM reduces survival by approximately 20-40% in fathead minnows, but not in a dose-response manner (A, B). Embryonic exposure to approximate mixtures of ≥ 0.2 µg IM/L and 0.2 µg TM/L resulted in increased embryonic motor activity (C, D). Blue bars indicate data that was significantly different from control. The predator escape response was not altered.

## CONCLUSIONS AND RECOMMENDATIONS

We examined the effects of two neonicotinoid insecticides (IM and TM) on three species of aquatic invertebrates and two species of fish: *Ceriodaphnia dubia* (water fleas), *Chironomus dilutus* (midges; single chemical acute tests only), *Pimephales promelas* (fathead minnow) and *Danio rerio* (zebrafish); we are the first to report acute and chronic effects of neonicotinoids on *G. pseudolimnaeus,* important in the structure (as prey) and function (organic matter processing) of local aquatic ecosystems. In some cases, acute and chronic exposure to IM and TM individually, and in combination, resulted in decreased survival, reproduction, and growth, as well as changes in ecologically-relevant behaviors in both aquatic invertebrates and fish. In addition, we used *in silico* computer modeling approaches to estimate the binding affinity of TM and IM to the vertebrate nAChR (nicotinic acetylcholine receptor) to better

understand sublethal responses in fish. We saw no evidence in any of the mixture studies that TM and IM together acted in any way other than additively for invertebrates. Mixtures likely present higher risk for aquatic invertebrates compared to fish.

In terms of survival, invertebrates (as expected) were more sensitive than fish. Invertebrate sensitivity varied with insecticide, but little is understood about the underlying reasons for species sensitivities towards neonicotinoids. Potential mechanisms could include differences in binding affinities for the nACh receptor, differences in ventilation, uptake and bioaccumulation, oxidative stress, and enzymes used in detoxification and elimination (Maloney et al., 2021; Wei et al., 2020; Chandran et al., 2018; Azevedo-Peeira et al., 2011).

In general, effect concentrations were higher than concentrations typically found in the environment. However, fish and crustaceans such as water fleas and amphipods tend to be less sensitive. Recent studies have shown mayflies are among the most sensitive (to neonicotinoids) species, and we recommend including them in future studies. Most mayflies cannot be cultured in the laboratory, but *Neocloeon triangulifer* is parthenogenetic and has been shown to be a useful model species (Chou et al., 2017).

While the present study and other studies have shown water fleas to be less sensitive to neonicotinoid insecticides than other aquatic invertebrates such as mayflies, they remain important test organisms given their role in calculation of water quality criteria and secondary values. In Wisconsin, a secondary value may be calculated for the protection of aquatic life from a toxic chemical when database requirements necessary to calculate a water quality criterion have not been fulfilled. A secondary value cannot be calculated without acute data for one of three genera of water fleas, including *Ceriodaphnia* sp. (Wis. Admin. Code Ch. NR 105). A longer-term goal might be to amend data requirements for calculation of secondary values to include more sensitive species such as mayflies. This could ultimately lead to better protection of fish and aquatic life.

Future studies designed to examine the toxicity of neonicotinoids should be run for a longer duration than required by standard protocols to allow delayed effects to be observed. Delayed toxicity is not taken into account by standard methods used for regulatory purposes, which means toxicity and risk may be underestimated (Li et al., 2021).

Future studies should also consider pulse exposures. In our study, organisms were subjected to constant exposures. In the environment, neonicotinoid insecticides often enter streams and lakes in pulses. While we may not be able to capture short-term inputs of these chemicals, such short-term, repeated exposures may be enough to exert longer-term effects on survival and health of longer-lived aquatic invertebrates and fish, especially if there is carry-over toxicity (Li et al., 2021; Raby et al., 2018b).

Our studies were all conducted in the laboratory (some with field-caught organisms). And while this allows better control of variables, it lacks environmental realism in terms of presence of other species, other chemicals, sediment, etc. Future studies should include a field biomonitoring component to determine if lab test results are predictive of effects in the field. And while measurements of insecticide concentrations in surface water and groundwater offer a snapshot in time, bioaccumulation and various aquatic invertebrate biomonitoring endpoints can integrate effects over the long-term. For example, *Gammarus pulex* amphipods have been shown to bioaccumulate neonicotinoid insecticides, including IM and TM in agricultural streams (Shahid et al., 2018). And while neonicotinoid pesticides were designed to target the nAChR, growing evidence suggests that they may have a different mode of action in fish (e.g., endocrine disruption) which warrant further study. Finally, impacts on survival, growth, reproduction, and behavior of aquatic invertebrates and fish could have cascading effects on the structure and function of aquatic ecosystem.

## REFERENCES


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

Armbrust, K.L., H.B. Peeler. 2002. Effects of formulation on the run-off of imidacloprid from turf. Pest Manag. Sci. 58:702–706.

Azevedo-Pereira, M.F.L. Lemos, A.M.V.M. Soares. 2011. Effects of imidacloprid exposure on *Chironomus riparius* Meigen larvae: Linking acetylcholinesterase activity to behaviour. Ecotoxicol. Environ. Saf. 74:1210-1215.

Bartlett, A.J., A.M. Hedges, K.D. Intini, L.R. Brown, F.J. Maisonneuve, S.A. Robinson, P.L. Gillis, S.R. de Solla. 2019. Acute and chronic toxicity of neonicotinoid and butanolide insecticides to the freshwater amphipod, *Hyalella azteca*. Ecotoxicol. Environ. Saf. 175:215-223.

Bottger, R. M. Feibicke, J. Schaller, G. Dudell. 2013. Effects of low-dosed imidacloprid pulses on the functional role of the caged amphipod *Gammarus roeseli* in stream mesocosms. Ecotoxicol. Environ. Saf. 93:93-100.

Bradford, B.Z., A.S. Huseth, R.L. Groves. 2018. Widespread detections of neonicotinoid contaminants in central Wisconsin groundwater. PLoS ONE 13:e0201753.

Buikema A, J. Cairns. 1980. Aquatic Invertebrate Bioassays. ASTM STP 715. ASTM International: ASTM Special Technical Publication.

Casida, J.E., K.A. Durkin. 2013. Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. Annu. Rev. Entomol. 58:99–117. https://doi.org/10.1146/annurev-ento-120811-153645.

Cavallaro, M.C., C.A. Morrissey, J.V. Headley, K.M. Peru, K. Liber. 2017. Comparative chronic toxicity of imidacloprid, clothianidin, and thiamethoxam to *Chironomus dilutus* and estimation of toxic equivalency factors. Environ. Toxicol. Chem. 36:372-382.

CCME. 2007. Canadian Water Quality Guidelines: Imidacloprid. Scientific Supporting Document. Canadian Council of Ministers of the Environment, Winnipeg.

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

Chagnon, M., D. Kreutzweiser, E.A.D. Mitchell, C.A. Morrissey, D.A. Noome, J.P. Van der Sluijs. 2015. Risks of large-scale use of systemic insecticides to ecosystem functioning and services. Environ. Sci. Pollut. Res. 22:119–134.

Chou, H., W. Pathmasiri, J. Deese-Spruill, S. Sumner, D.B. Buchwalter. 2017. Metabolomics reveal physiological changes in mayfly larvae *Neocleon triangulifer* at ecological upper thermal limits. J. Insect Physiol. 101:107-112.

Cox, C., 2001. Insecticide factsheet: imidacloprid. J Pestic Reform 21:15–21.

DATCP. 2018. Neonicotinoid pesticides in Wisconsin groundwater and surface water. Final Draft. Department of Agriculture, Trade and Consumer Protection.

DeCant, J., M. Barrett. 2010. Environmental fate and ecological risk assessment for the registration of clothianidin for use as a seed treatment on mustard seed (oilseed and condiment) and cotton. U.S. Environmental Protection Agency, Washington.
EFSA. 2008. Conclusion regarding the peer review of the pesticide risk assessment of the active substance imidacloprid. European Food Safety Authority Scientific Report. European Food Safety Authority.

Finnegan, M.C., L.R. Baxter, J.D. Maul, M.L. Hanson, P.F. Hoekstra. 2017. Comprehensive characterization of the acute and chronic toxicity of the neonicotinoid insecticide thiamethoxam to a suite of aquatic primary producers, invertebrates, and fish. Environ. Toxicol. Chem. 36:2838-2848.

Gibbons, D., C. Morrissey, P. Mineau. 2015. A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. Environ. Sci. Pollut. Res. 22:103–118. https://doi.org/10.1007/s11356-014-3180-5

Goulson, D. 2013. Review: An overview of the environmental risks posed by neonicotinoid insecticides. J. Appl. Ecol. 50:977–987.

Hamilton, M.S. R.C. Russo, R. Thurston. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. US EPA. EPA/600/J-77/178 (NTIS PB81191918).

Hepditch, S.L.J., O. Birceanu, M.P. Wilkie. 2021. A toxic unit and additive index approach to understanding the interactions of 2 piscicides, 3-trifluoromethyl-4-nitrophenol and niclosamide in rainbow trout. Environ. Toxicol. Chem. 40:1419-1430.

Hladik, M.L., D.W. Kolpin. 2016. First national-scale reconnaissance of neonicotinoid insecticides in streams across the USA. Environ. Chem. 13:12-20.

Kelly Products, Inc. 2017. Kelly Registration Systems portal for State Department of Agriculture Registrants and Licensees. Retrieved from http://www.kellysolutions.com/wi/pesticideindex.asp.

Li, H., Q. Zhang, H. Su, J. You, W-X. Wang. 2021. High tolerance and delayed responses of *Daphnia magna* to neonicotinoid insecticide imidacloprid: Toxicokinetic and toxicodynamic modeling. Environ. Sci. Technol. 55:458-467.

Lundin, O., M. Rundlöf, H.G. Smith, I. Fries, R. Bommarco. 2015. Neonicotinoid insecticides and their impacts on bees: A systematic review of research approaches and identification of knowledge gaps. PLoS one 10:e0136928. https://doi.org/10.1371/journal.pone.0136928

Maloney, E.M., K. Liber, J.V. Headley, K.M. Peru, C.A. Morrissey. 2018a. Neonicotinoid insecticide mixtures: Evaluation of laboratory-based toxicity predictions under semi-controlled field conditions. Environ. Pollut. 243:1727-1739.

Maloney, E.M., C.A. Morrissey, J.V. Headley, K.M. Peru, K. Liber. 2018b. Can chronic exposure to imidacloprid, clothianidin, and thiamethoxam mixtures exert greater than additive toxicity in *Chironomus dilutus*? Ecotoxicol. Environ. Saf. 156:354-365.

Maloney, E.M., C. Morrissey, J.V. Headley, K.M. Peru, K. Liber. 2017. Cumulative toxicity of neonicotinoid insecticide mixtures to *Chironomus dilutus* under acute exposure scenarios. Environ. Toxicol. Chem. 36:3091-3101.

Maloney, E.M., E. Taillebois, N. Gilles, C.A. Morrissey, K. Liber, D. Servant, S.H. Thany. 2021. Binding properties to nicotinic acetylcholine receptors can explain differential toxicity of neonicotinoid insecticides in Chironomidae. Aquat. Toxicol. 230:105701.

Metcalfe, C.D., P. Helm, G. Paterson, G. Kaltenecker, C. Murray, M. Nowierski, T. Sultana. 2019. Pesticides related to land use and watersheds of the Great Lakes basin. Sci. Total Environ. 648:681-692.

Morrissey, C.A., P. Mineau, J.H. Devries, F. Sanchez-Bayo, M. Liess, M.C. Cavallaro, K. Liber. 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: A review. Environ. Int. 74:291–303. https://doi.org/10.1016/j.envint.2014.10.024

Nyman, A-M., A. Hintermeister, K. Schirmer, R. Ashauer. 2013. The insecticide imidacloprid causes mortality of the freshwater amphipod *Gammarus pulex* by interfering with feeding behavior. PLoS one 8:1-13.

Raby, M. M. Nowierski, D. Perlov, X. Zhao, C. Hao, D.G. Poirier, P.K. Sibley. 2018a. Acute toxicity of 6 neonicotinoid insecticides to freshwater invertebrates. Environ. Toxicol. Chem. 37:1430-1445.

Raby, M. X. Zhao, C. Hao, D.G. Poirier, P.K. Sibley. 2018b. Chronic effects of an environmentally-relevant, short-term neonicotinoid insecticide pulse on four aquatic invertebrates. Sci. Total Environ. 639:1543-1552.

Raby, M., X. Zhao, C. Hao, D.G. Poirier, P.K. Sibley. 2018c. Chronic toxicity of 6 neonicotinoid insecticides to *Chironomus dilutus* and *Neocloeon triangulifer*. Environ. Toxicol. Chem. 37:2727-2739.

Sánchez-Bayo, F., D. Goulson, F. Pennacchio, F. Nazzi, K. Goka, N. Desneux. 2016. Are bee diseases linked to pesticides? A brief review. Environ. Int. 89–90:7–11. https://doi.org/10.1016/j.envint.2016.01.009

Shahid, M., J.M. Becker, M. Krauss, W. Brack, M. Liess. 2018. Pesticide body burden of the crustacean *Gammarus pulex* as a measure of toxic pressure in agricultural streams. Environ. Sci. Technol. 52:7823-7832.

Simon-Delso, N., V. Amaral-Rogers, L.P. Belzunces, J.M. Bonmatin, M. Chagnon, C. Downs, L. Furlan, D.W. Gibbons, C. Giorio, V. Girolami, D. Goulson, D.P. Kreutzweiser, C.H. Krupke, M. Liess, E. Long, M. McField, P. Mineau, E.A.D. Mitchell, C.A. Morrissey, D.A. Noome, L. Pisa, J. Settele, J.D. Stark, A. Tapparo, H. Van Dyck, J. Van Praagh, J.P. Van der Sluijs, P.R. Whitehorn, M. Wiemers. 2015. Systemic insecticides (neonicotinoids and fipronil): Trends, uses, mode of action and metabolites. Environ. Sci. Pollut. Res. Int. 22:5–34. https://doi.org/10.1007/s11356-014-3470-y

Tisler, T., A. Jemec, B. Mozetic, P. Trebse. 2009. Hazard identification of imidacloprid to aquatic environment. Chemosphere 76:907–914. https://doi.org/10.1016/j.chemosphere.2009.05.002

Tomizawa, M., J.E. Casida. 2005. Neonicotinoid insecticide toxicology: Mechanisms of selective action. Annu. Rev. Pharmacol. Toxicol. 45:247–268. https://doi.org/10.1146/annurev.pharmtox.45.120403.095930

U.S. EPA. 2017. Aquatic life benchmarks and ecological risk assessments for registered pesticides. U.S. EPA Office of Pesticide Programs. Accessed 10/30/18. https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/aquatic-life-benchmarks-and-ecological-risk

U.S. EPA. 2002a. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Fifth edition. Office of Water. EPA-821-R-02-012.

U.S. EPA. 2002b. Method 1000.0: Fathead minnow, *Pimephales promelas*, larval survival and growth: Chronic toxicity. Office of Research and Development and Office of Water. EPA/660/3-75009.

U.S. EPA. 2002c. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Fourth edition. Office of Water. EPA-821-R-02-013.

U.S. EPA. 2000. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates, Second Edition. Office of Research and Development and Office of Water. EPA/600/R-99/064.

Wei, F., D. Wong, H. Li, P. Xia, Y. Ran, J. You. 2020. Toxicogenomics provides insights to toxicity pathways of neonicotinoids to aquatic insect, *Chironomus dilutus*. Environ. Pollut. 260:114011.

Wis. Admin. Code Ch. NR 105. Surface water quality criteria and secondary values for toxic substances. Register July 2022 No. 799.

Zhu, L. W. Li, J. Zha, N. Li, and Z. Wang. 2019. Chronic thiamethoxam exposure impairs the HPG and HPT axes in adult Chinese rare minnow (*Gobiocypris rarus*): Docking study, hormone levels, histology and transcriptional responses. Ecotox. Environ. Safety. 185:109683.

## APPENDIX A

**PUBLICATIONS** (*undergraduate student; †Master of Science graduate student)

S Victoria†, M Hein*, E Harrahy and TC King-Heiden. 2022. Potency matters: Impacts of embryonic exposure to nAChR agonists thiamethoxam and nicotine on hatching success, growth, and neurobehavior in larval zebrafish. J Toxicol Env Health, A. 85(18):767-782. https://pubmed.ncbi.nlm.nih.gov/35650526/

S Victoria†, S Duffy*, E Harrahy and TC King-Heiden. 2022. Embryonic exposure to thiamethoxam reduces survival and alters neurobehavior of fathead minnows. Env. Tox. and Chem. 41(5):1276-1285. https://setac.onlinelibrary.wiley.com/doi/abs/10.1002/etc.5301

**PRESENTATIONS AND AWARDS** (Presenting author underlined; *undergraduate student; †Master of Science graduate student)

### International/Global Research Conferences

A Jeninga†, S Victoria†, S Duffy*, Z Wallace*, E Harrahy, and TC King-Heiden. 2022. You act differently around your friends: The developmental and neurobehavioral toxicities of imidacloprid and thiamethoxam change during binary exposures. SETAC-NA[2] Annual meeting, Pittsburg, PA ~2000 attendees

TC King-Heiden, S Victoria†, S Duffy*, M Hein*, and E Harrahy. 2022. Incorporating ecologically relevant behaviors, age at exposure, and two species in assessing the toxicity of thiamethoxam in fish. SETAC-NA Annual meeting, Pittsburg, PA ~2000 attendees

S (Michel) Victoria†, M. Hein*, E Harrahy, and TC King-Heiden. 2021. Effects of chronic thiamethoxam exposure on fathead minnow larvae. SETAC- NA virtual meeting. ~2000 attendees

### Midwest Regional Research Conferences

A Jeninga†, S Duffy*, E Harrahy, and TC King-Heiden. 2022. Toxicity of single and binary exposures of the neonicotinoids thiamethoxam and imidacloprid on *Pimephales promelas*. Mississippi River Research Consortium Annual Meeting ~300 attendees ***Awarded Best Student Poster Presentation***

A Jeninga†, S Duffy*, E Harrahy, and TC King-Heiden. 2022. Do the neonicotinoids thiamethoxam and imidacloprid exhibit additive toxicity in *Pimephales promelas* larvae? MRC-SETAC[3] annual meeting ~100 attendees. ***Awarded Best Student Poster Presentation***

S (Michel) Victoria†, M Hein*, A Helgeson*, S Duffy*, E Harrahy, and TC King-Heiden. 2021. Adverse effects of chronic exposure to thiamethoxam in zebrafish and fathead minnow embryos. Joint meeting between MRC-SETAC and Northland Society of Toxicology. ~100 attendees

[2] SETAC-NA: Society of Environmental Toxicology and Chemistry-North America
[3] MRC-SETAC: Midwest Regional Chapter of SETAC

J Lacki*, A Draper*, A Jeninga*, TC King-Heiden and E Harrahy. 2021. Acute and chronic toxicity of the neonicotinoid insecticides thiamethoxam and imidacloprid to select aquatic invertebrates. Platform presentation. MRC-SETAC annual meeting with Northland Society of Toxicology, April 29. Virtual. ~100 attendees

S (Michel) Victoria†, S Duffy*, T deDianous*, E Harrahy, and TC King-Heiden. 2021. Local Fish Species exposed to the pesticide, thiamethoxam, exhibits changes to survival, embryonic motor activity, and predator escape in early life stages. Mississippi River Research Consortium. Virtual ~300 attendees

T King-Heiden. 2021. It's a matter of time: Delayed toxic responses in fathead minnow larvae following chronic exposure to environmentally relevant concentrations of thiamethoxam. Puget Sound Toxics Workshop, WA. Virtual ~50 attendees

E Harrahy, A Draper*, J Lacki*, A Jeninga*, TC King-Heiden. 2021. Acute and chronic toxicity of the neonicotinoid insecticides thiamethoxam and imidacloprid to select aquatic invertebrates. Platform presentation. Wisconsin Chapter of the American Water Resources Association Annual Meeting, March 3-4. Virtual. ~100 attendees

### Presentations to Community Members/Outreach Presentations

***Note:*** Due to the COVID-19 pandemic, several planned outreach presentations were cancelled

S Duffy*, S Victoria†, E Harrahy, and TC King-Heiden. 2022. Secret life of fish: bee-harming pesticide makes fish larvae susceptible to predators. Research on the Rotunda, WI State Capitol, Madison, March 9. Poster presentation to various UW system administrators, state legislators, and the public. ~200 attendees

F Fisher*, A Draper*, J. Lacki*, A Herrmann*, A. Jeninga*, TC King-Heiden, and E Harrahy. 2022. Effects of a mixture of two neonicotinoid insecticides on reproduction of water fleas and growth of amphipods. Research in the Rotunda, WI State Capitol, Madison, March 9. Poster presentation to various UW system administrators, state legislators, and the public. ~200 attendees

### Presentations at Undergraduate Research Conferences

F Fisher*, J Lacki*, A Herrmann*, TC King-Heiden, and E Harrahy. 2022. Effects of a mixture of two neonicotinoid insecticides on reproduction of water fleas and growth of amphipods. Poster presentation. UW System Symposium for Undergraduate Research, UW-Whitewater, April 22. ~400 attendees

J Lacki*, A Draper*, A Jeninga*, TC King-Heiden, and E Harrahy. 2021. Acute and chronic toxicity of the neonicotinoid insecticides thiamethoxam and imidacloprid to select aquatic invertebrates. Poster presentation. UW System Symposium for Undergraduate Research, UW- Whitewater, April 28. Virtual. ~400 attendees

A Draper*, J Lacki*, TC King-Heiden, and E Harrahy. 2020. Effects of two neonicotinoid insecticides on survival and growth of *Gammarus pseudolimnaeus* amphipods. Poster presentation. Fall Undergraduate Research Day, UW-Whitewater, November 11. Virtual. ~200 attendees

J Lacki*, A Draper*, TC King-Heiden, and E Harrahy. 2020. Acute and chronic toxicity of neonicotinoid insecticides thiamethoxam and imidacloprid to the water flea, *Ceriodaphnia dubia*. Poster presentation. Fall Undergraduate Research Day, UW-Whitewater, November 11. Virtual. ~200 attendees

A Jeninga*, A Draper*, TC King-Heiden, and E Harrahy. 2020. Acute and chronic toxicity of the insecticide thiamethoxam to select aquatic macroinvertebrates. Poster presentation. Spring Undergraduate Research Day, UW-Whitewater, May 1. Virtual. ~200 attendees

A Jeninga*, A Draper*, TC King-Heiden, and E Harrahy. 2019. Acute and chronic toxicity of the insecticide thiamethoxam to select aquatic macroinvertebrates. Poster presentation. Fall Undergraduate Research Day, UW- Whitewater, November 13. ~200 attendees

## STUDENTS FUNDED OR SUPPORTED BY GRANT AND CURRENT AFFILIATIONS

Undergraduate students

- Megan Hein (UWL), meganhein98@gmail.com, graduated May 2021; Marquette PA program
- Sarah Duffy (UWL), duffy8630@uwlax.edu, expected graduation (BS) May 2023
- Taylor deDianous (UWL), dedianous8885@uwlax.edu, expected graduation (BS) May 2023
- Zion Wallace (UWL), wallace1025@uwlax.edu, expected graduation (BS) May 2024
- Anya Jeninga (UWW), jeninga@wisc.edu, MS program UWL, graduated 2022
- Austin Draper (UWW), austin.draper@usm.edu, M.S. student at University of Southern Mississippi
- Jacob Lacki (UWW), jl466s@MissouriState.edu, M.S. student at Missouri State University
- Faune Fisher (UWW), faune@epic.com, Quality Manager at Epic Systems, Verona, WI
- Lina Han (UWW), HanLH28@uww.edu, expected graduation (BS) May 2023
- Connor Hodgson (UWW), HodgsonCG10@uww.edu, expected graduation (BS) May 2024
- Ciara Hynes (UWW), HynesCE30@uww.edu, expected graduation (BS) May 2024

Graduate students (Masters of Science)

- Shayla Victoria (UWL), srvictor@go.olemiss.edu, PhD program University of Mississippi
- Anya Jeninga (UWL), jeninga@wisc.edu, WI Division of Public Health, Department of Health Services

## Impact of the Work

Insecticides, both natural and synthetic, have become an integral component of our agricultural industry allowing food growers to increase crop yields while keeping the costs of our food down. It is important that we balance the need for using insecticides with their potential risks to wildlife and human health. Neonicotinoid insecticides are uniquely designed to reduce risks to some non-target species by specifically targeting the nicotinic acetylcholine receptors (nAChR) in insects, which has been shown to reduce their toxicity to vertebrate species such as fish. In addition, they are frequently applied as a seed coating instead of as a spray to reduce environmental contamination. Unfortunately, their widespread use has resulted in their presence in aquatic ecosystems in Wisconsin, posing a potential risk for aquatic invertebrates and fish living in our streams, rivers, and lakes, especially in the Central Sands Region. For this reason, we sought to better understand their effects on aquatic invertebrates and fish. Our work shows that the two most prevalent neonicotinoids found in Wisconsin's waters, thiamethoxam and imidacloprid,

do cause toxicity in aquatic invertebrates and fish, impacting survival, growth, and various ecologically relevant behaviors. Further, these insecticides are typically found as mixtures in the environment, and our work indicates that for the invertebrate species we tested, their combined toxicity is additive, while for the fish species we tested, we saw no evidence for additive toxicity. Our data can be used to help develop use restrictions where necessary to maintain water quality. Future avenues of research, including determining longer-term effects of cumulative short-term exposures to important, local species will allow a better understanding of potential cascading effects of neonicotinoids on Wisconsin's aquatic ecosystems.

### Potential use for management:

- Since the toxicity of a mixture of thiamethoxam and imidacloprid appears to be additive for invertebrates, it may be important to consider total concentrations of neonicotinoid pesticides in deriving criteria, much the same as has been done for atrazine and its metabolites in groundwater (Chapter NR 140, Wisconsin Administrative Code).
- Relative sensitivities of exposure to thiamethoxam and imidacloprid varied among species. In general, water flea species are less sensitive to neonicotinoids than other aquatic invertebrates such as mayflies (based on data from the literature). However, in Wisconsin, a secondary value cannot be calculated for the protection of aquatic life without acute data for one of three genera of water fleas, including *Ceriodaphnia* sp. (Wis. Admin. Code Ch. NR 105). A longer-term goal might be to amend data requirements for calculation of secondary values to include more sensitive species such as mayflies. This could ultimately lead to better protection of fish and aquatic life.
- While concentrations shown to be lethal to invertebrates in this study were orders of magnitude higher than concentrations detected in surface waters in WI, it should be noted that these were shorter-term exposures. Future studies and management decisions should take into account potential longer-term cumulative effects of repeated pulse exposures.
- Incorporating earlier age-at exposure (beginning exposure pre-hatch) for toxicity tests in standardized fish toxicity assays may better predict the potential risks that neonicotinoids pose to wild fish populations.
- Incorporating behavioral endpoints that are essential for survival and recruitment of wild fish populations could also enhance our understanding of the risks that environmental contaminants pose to wild fish populations, particularly if the target mode of action for a contaminant is part of the nervous system.

## APPENDIX B

Table 1. Acute and chronic toxicity estimates for imidacloprid and thiamethoxam, for water fleas, amphipods, chironomids and mayflies. Note: This table is not meant to be exhaustive.

| Neonic | Species | Endpoint | LC/EC50/ NOEC/NOEC/ Duration | Estimate µg/L unless noted | Citation |
|---|---|---|---|---|---|
| **IMIDACLOPRID: ACUTE** | | | | | |
| **Water Fleas** | | | | | |
| IMD | *Ceriodaphnia dubia* | Survival | LC50 6 d | 8,420 (5360-11480) | Raby et al. 2018c |
| IMD | *Ceriodaphnia dubia* | Survival | EC50 48 h | 571.62 (289.63-841.19) | Hayasaka et al. 2013 |
| IMD | *Ceriodaphnia dubia* | Survival | LC50 48 h | 72,124.9 (51,000.0, 102,000.0) | Raby et al. 2018a |
| IMD | *Daphnia magna* | Survival | LC50 21 d | 35,440 (22,780-48,090) | Raby et al. 2018c |
| IMD | *Daphnia magna* | Survival | LC50 96 h | 8,470 (6,070-11,800) | Li et al. 2021 |
| IMD | *Daphnia magna* | Survival | LC50 48 h | 85,000 | *In* Jamec et al. 2007 |
| IMD | *Daphnia magna* | Survival | LC50 48 h | 10,400 | *In* Jamec et al. 2007 |
| IMD | *Daphnia magna* | Survival | EC50 48 h | 56,600 | *In* Jamec et al. 2007 |
| IMD | *Daphnia magna* | Survival | LC50 48 h | >102,000 | Raby et al. 2018a |
| **Amphipods** | | | | | |
| IMD | *Hyalella azteca* | Survival | LC50 96 h juveniles | 526 | *In* Jamec et al. 2007 |
| IMD | *Hyalella azteca* | Survival | LC50 96 h 14-21 d old | 51,800 | *In* Jamec et al. 2007 |
| IMD | *Hyalella azteca* | Survival | LC50 96 h 7-21 d old | 94,800 | *In* Jamec et al. 2007 |
| IMD | *Hyalella azteca* | Survival | LOLC 96 h 14-21 d old | 43,800 | *In* Jamec et al. 2007 |
| IMD | *Hyalella azteca* | Survival | LC50 7 d | 230 | Bartlett et al. 2019 |
| IMD | *Hyalella azteca* | Survival | LC50 28 d | 90 | Bartlett et al. |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | 2019 |
| IMD | *Hyalella azteca* | Survival | LC50<br>96 h | 65.43<br>(39.78, 107.62) | Stoughton et al. 2008 |
| IMD | *Hyalella azteca* | Survival | LC50<br>96 h | 363.2<br>(301.3, 425.1) | Raby et al. 2018a |
| IMD | *Gammarus pulex* | Survival | EC50<br>96 h | 18<br>(8.8, 38) | Van Den Brink et al. 2016 |
| IMD | *Gammarus pulex* | Survival | EC50<br>96 h | 49<br>(29, 81) | Van Den Brink et al. 2016 |
| IMD | *Gammarus pulex* | Survival | LC50<br>96 h | 263<br>(155-446) | Roessink et al. 2013 |
| IMD | *Gammarus pulex* | Survival | LC50<br>28 d | 33.8<br>(20.9, 54.6) | Roessink et al. 2013 |
| IMD | *Gammarus roeseli* | Survival | EC50<br>96 h | 14.2 | Bottger et al. 2012 |
| **Chironomids** | | | | | |
| IMD | *Chironomus dilutus* | Survival | LC50<br>14 d | >1.43 | Raby et al. 2018b |
| IMD | *Chironomus dilutus* | Survival | LC50<br>96 h | 11.8<br>(8.3, 15.4) | Raby et al. 2018a |
| IMD | *Chironomus dilutus* | Survival | LC50<br>96 h | 4.63 | Maloney et al. 2017 |
| IMD | *Chironomus dilutus* | Survival | LC50<br>14 d | 1.52<br>(0.99, 1.82) | Cavallaro et al. 2017 |
| IMD | *Chironomus riparius* | Survival | LC50<br>24 h | 31.5<br>(15.1, 75.9) | Chandran et al. 2018 |
| IMD | *Chironomus riparius* | Survival | LC50<br>10 d | 2.33<br>(1.30, 4.41) | Chandran et al. 2018 |
| IMD | *Chironomus tentans* | Survival | LC50<br>96 h<br>2nd instar | 10.5 | *In* Jamec et al. 2007 |
| IMD | *Chironomus tentans* | Survival | LOLC<br>96 h<br>2nd instar | 3.39 | *In* Jamec et al. 2007 |
| IMD | *Chironomus tentans* | Survival | LC50<br>96 h | 5.75<br>(4.10, 8.08) | Stoughton et al. 2008 |
| **Mayflies** | | | | | |
| IMD | *Cloeon sp.* | Survival | LC50<br>96 h | 1,152.0<br>(5,13.1, 1,790.8) | Raby et al. 2018a |
| IMD | *Cloeon dipterum* | Survival | LC50<br>96 h | 26.3<br>(17.7, 39.1) | Roessink et al. 2013 |
| IMD | *Cloeon dipterum* | Survival | LC50<br>28 d | 0.195<br>(0.113, 0.338) | Roessink et al. 2013 |
| IMD | *Neocloeon triangulifer* | Survival | LC50<br>96 h | 5.2<br>(4.2, 6.2) | Raby et al. 2018a |

| | | | | | |
|---|---|---|---|---|---|
| IMD | *Isonychia bicolor* | Survival | LC50<br>96 h (@15oC) | 18.77 | Camp and Buchwalter 2016 |
| IMD | *Deleatidium spp.* | Survival | LC50<br>28 d | 0.28<br>(0.21, 0.36) | Macaulay et al. 2019 |
| IMD | *Hexagenia* sp. | Survival | LC50<br>96 h | 900<br>(290, 2800)<br>NOMINAL | Bartlett et al. 2018 |
| **IMIDACLOPRID: CHRONIC** | | | | | |
| **Water Fleas** | | | | | |
| IMD | *Ceriodaphnia dubia* | Reproduction | EC50<br>6 d | 2,980<br>(2,590-3,370) | Raby et al. 2018c |
| IMD | *Daphnia magna* | Reproduction | EC50<br>21 d | 4,590<br>(4,230-5,050) | Raby et al. 2018c |
| IMD | *Daphnia magna* | Reproduction | LOEC<br>21 d | 7,300 | *In* Jamec et al. 2007 |
| IMD | *Daphnia magna* | Reproduction | LOEC<br>21 d | 2,500 | *In* Jamec et al. 2007 |
| IMD | *Daphnia magna* | Body residue | 96 h<br>LR50<br>Internal exposure threshold (body residue) | 10,200<br>(7,300-14,200) | Li et al. 2021 |
| **Amphipods** | | | | | |
| IMD | *Hyalella azteca* | Immobility | LOEC<br>96 h<br>juveniles | 0.97 | *In* Jamec et al. 2007 |
| IMD | *Hyalella azteca* | Immobility | NOEC<br>96 h<br>7-21 d old | 94,800 | *In* Jamec et al. 2007 |
| IMD | *Hyalella azteca* | Growth | EC50<br>28 d | 4.3 | Bartlett et al.<br>2019 |
| IMD | *Gammarus pulex* | Immobility | EC50<br>96 h | 18.3<br>(8.84, 37.8) | Roessink et al. 2013 |
| IMD | *Gammarus pulex* | Immobility | EC50<br>28 d | 15.4<br>(9.80, 24.1) | Roessink et al. 2013 |
| IMD | *Gammarus pulex* | Feeding rate (inhibition) | EC50<br>96 h | 5.34 | Agatz et al. 2013 |
| **Chironomids** | | | | | |
| IMD | *Chironomids (mult. spp; community)* | Emergence | Sig. diff.<br>3 weeks? | ≥2.0 | Williams and Sweetman 2019 |
| IMD | *Chironomus dilutus* | Emergence | EC50<br>40 d | 0.39<br>(0.31, 0.42) | Cavallaro et al.<br>2017 |
| IMD | *Chironomus dilutus* | Emergence | EC50<br>28 d | 0.5<br>0.37, 0.59) | Maloney et al. 2017 |

| | | | | | |
|---|---|---|---|---|---|
| IMD | *Chironomus dilutus* | Emergence | EC50<br>14 d | 0.24<br>(0.22, 0.27) | Raby et al. 2018b |
| IMD | *Chironomus dilutus* | Sex ratios | 40 d | No sig diff | Cavallaro et al. 2017 |
| IMD | *Chironomus riparius* | Ventilation | Sig. diff.<br>96 h<br>48 h post exposure | <br>0.55<br>0.3 | Azevedo-Pereira et al. 2011 |
| IMD | *Chironomus riparius* | Locomotion | Sig. Diff.<br>96 h<br>48 h post exposure | <br>1.2<br>1.2 | Azevedo-Pereira et al. 2011 |
| IMD | *Chironomus riparius* | AChE decline | Sig. diff.<br>96 h<br>48 h post exposure | <br>1.2<br>0.3 | Azevedo-Pereira et al. 2011 |
| IMD | *Chironomus riparius* | Growth | EC50<br><br>NOEC<br>LOEC<br>10 d | 5.03<br>(4.23, 6.00)<br>0.625<br>1.25 | Chandran et al. 2018 |
| **Mayflies** | | | | | |
| IMD | *Cloeon dipterum* | Immobility | EC50<br>96 h | 1.02<br>(0.460, 2.28) | Roessink et al. 2013 |
| IMD | *Cloeon dipterum* | Immobility | EC50<br>28 d | 0.123<br>(0.075, 0.201) | Roessink et al. 2013 |
| IMD | *Neocloeon triangulifer* | Imago Emergence | EC50<br>14 d | 1.75<br>(1.42, 2.09) | Raby et al. 2018b |
| IMD | *Deleatidium spp.* | Immobility | IC50<br>28 d | 0.26<br>(0.2, 0.33) | Macaulay et al. 2019 |
| IMD | *Deleatidium spp.* | Impairment | EC50<br>28 d | 0.19<br>(0.14, 0.25) | Macaulay et al. 2019 |
| IMD | *Isonychia bicolor* | Immobility | EC50<br>96 h | 5.88 | Camp and Buchwalter 2016 |
| IMD | *Hexagenia* sp. | Behavior (remaining in burrows) | EC50<br>96 h | 10<br>(2.5, 42)<br>NOMINAL | Bartlett et al. 2018 |
| IMD | *Hexagenia* sp. | Behavior (remaining in burrows) | NOEC<br>LOEC<br>96 h | 1<br>10<br>NOMINAL | Bartlett et al. 2018 |
| **THIAMETHOXAM: ACUTE** | | | | | |
| **Water Fleas** | | | | | |
| TMX | *Ceriodaphnia dubia* | Survival | LC50<br>6 d | Not calculable<br>> 80,000 | Raby et al. 2018c |
| TMX | *Ceriodaphnia dubia* | Survival | LC50<br>48 h | >80,000 | Raby et al. 2018a |
| TMX | *Daphnia magna* | Survival | LC50<br>48 h | >80,000 | Raby et al. 2018a |

| | | | | | |
|---|---|---|---|---|---|
| TMX | *Daphnia magna* | Survival | EC50<br>24 h<br>static | >100,000 | Finnegan et al. 2017 (Syngenta) |
| TMX | *Daphnia magna* | Survival | EC50<br>48 h<br>static | >100,000 | Finnegan et al. 2017 (Syngenta) |
| TMX | *Daphnia pulex* | Survival | EC50<br>24 h<br>static | >100,000 | Finnegan et al. 2017 (Syngenta) |
| **Amphipods** | | | | | |
| TMX | *Hyalella azteca* | Survival | LC50<br>7d | 290 | Bartlett et al.<br>2019 |
| TMX | *Hyalella azteca* | Survival | LC50<br>28 d | 220 | Bartlett et al.<br>2019 |
| TMX | *Hyalella azteca* | Survival | LC50<br>96 h | 801.0<br>(518.7, 1,083.3) | Raby et al. 2018a |
| TMX | *Gammarus kischineffensis* | Survival | LC50<br>96 h | 3,751 | Ugurlu et al. 2015 |
| TMX | *Gammarus sp.* | Survival | EC50<br>24 h<br>static | 15,000<br>(10,000-23,000) | Finnegan et al. 2017 (Syngenta) |
| TMX | *Gammarus sp.* | Survival | EC50<br>48 h<br>static | 2,800<br>(1,700-4,100) | Finnegan et al. 2017 (Syngenta) |
| **Chironomids** | | | | | |
| TMX | *Chironomus dilutus* | Survival | LC50<br>96 h | 54.3<br>(49.1, 60.1) | Phillips et al. 2021 |
| TMX | *Chironomus dilutus* | Survival | LC50<br>96 h | 58.5<br>(49.3, 69.4) | Phillips et al. 2021 |
| TMX | *Chironomus dilutus* | Survival | LC50<br>10 d | 31.8<br>(28.2, 35.9) | Phillips et al. 2021 |
| TMX | *Chironomus dilutus* | Survival | LC50<br>10 d | 30.3<br>(29.1, 31.7) | Phillips et al. 2021 |
| TMX | *Chironomus dilutus* | Survival | LC50<br>96 h | 61.9<br>(45.4, 78.4) | Raby et al. 2018a |
| TMX | *Chironomus dilutus* | Survival | LC50<br>14 d | 45.88 | Raby et al. 2018b |
| TMX | *Chironomus dilutus* | Survival | LC50<br>96 h | 55.34 | Maloney et al. 2017 |
| TMX | *Chironomus dilutus* | Survival | LC50<br>14 d | 23.60<br>(20.36, 26.89) | Cavallaro et al. 2017 |
| TMX | *Chironomus dilutus* | Survival | NOEC<br>LOEC<br>10 d<br>Static renewal<br>Sediment application | 1,300 µg/g<br>2,600 µg/g<br>Sediment dry wt | Finnegan et al. 2017 (Syngenta) |

| | | | | | |
|---|---|---|---|---|---|
| TMX | *Chironomus riparius* | Survival | LC50<br>48 h | 86.41<br>(74.35, 100.04) | Saraiva et al. 2017 |
| TMX | *Chironomus riparius* | Survival | EC50<br>24 h<br>static | 61<br>(50, 75) | Finnegan et al. 2017 (Syngenta) |
| TMX | *Chironomus riparius* | Survival | EC50<br>48 h<br>static | 35<br>(30, 41) | Finnegan et al. 2017 (Syngenta) |
| TMX | *Chironomus riparius* | Survival | EC50<br>48 h<br>static | 45<br>(not calculable) | Finnegan et al. 2017 (Syngenta) |
| TMX | *Chironomus riparius* | Survival | EC50<br>48 h<br>static | 71<br>(34, 194) | Finnegan et al. 2017 (Syngenta) |
| TMX | *Chironomus riparius* | Survival | LC50<br>48 h<br>static | 260<br>(130, 520) | Finnegan et al. 2017 (Syngenta) |
| **Mayflies** | | | | | |
| TMX | *Cloeon sp.* | Survival | LC50<br>96 h | 4,633.6<br>(1,835.8, 7,431.3) | Raby et al. 2018a |
| TMX | *Neocloeon triangulifer* | Survival | LC50<br>96 h | 5.5<br>(3.9, 7.8) | Raby et al. 2018a |
| TMX | *Cloeon dipterum* | Survival | EC50<br>48 h<br>static | 21<br>(not calculable) | Finnegan et al. 2017 (Syngenta) |
| TMX | *Cloeon dipterum* | Survival | LC50<br>48 h<br>static | 53<br>(38, 73) | Finnegan et al. 2017 (Syngenta) |
| TMX | *Cloeon dipterum* | Survival | EC50<br>48 h<br>static | 44<br>(42, 45) | Finnegan et al. 2017 (Syngenta) |
| TMX | *Cloeon sp.* | Survival | EC50<br>24 h<br>static | 19<br>(16, 23) | Finnegan et al. 2017 (Syngenta) |
| TMX | *Cloeon sp.* | Survival | EC50<br>48 h<br>static | 14<br>(11, 17) | Finnegan et al. 2017 (Syngenta) |
| TMX | *Deleatidium spp.* | Survival | LC50<br>28 d | >4 | Macaulay et al. 2019 |
| TMX | *Hexagenia* sp. | Survival | LC50<br>96 h | >10,000<br>NOMINAL | Bartlett et al. 2018 |
| **THIAMETHOXAM: CHRONIC** | | | | | |
| **Water Fleas** | | | | | |
| TMX | *Ceriodaphnia dubia* | Reproduction | EC50<br>6 d | Not calculable<br>>80,000 | Raby et al. 2018c |
| TMX | *Daphnia magna* | Reproduction | NOEC<br>LOEC<br>14 d | 100,000<br>>100,000 | Finnegan et al. 2017 (Syngenta) |

| | | | | | |
|---|---|---|---|---|---|
| | | | semi-static | | |
| TMX | *Daphnia magna* | Reproduction | NOEC<br>LOEC<br>21 d<br>semi-static | 100,000<br>>100,000 | Finnegan et al. 2017 (Syngenta) |
| **Amphipods** | | | | | |
| TMX | *Hyalella azteca* | Growth | EC50<br>28 d | 200 | Bartlett et al. 2019 |
| **Chironomids** | | | | | |
| TMX | *Chironomus dilutus* | Sex ratios | 40 d | No sig diff | Cavallaro et al. 2017 |
| TMX | *Chironomus dilutus* | Emergence | EC50<br>40 d | 4.13<br>(3.53, 4.76) | Cavallaro et al. 2017 |
| TMX | *Chironomus dilutus* | Emergence | EC50<br>28 d | 8.91<br>(5.79, 12.37) | Maloney et al. 2017 |
| TMX | *Chironomus dilutus* | Emergence | EC50<br>14 d | 12.95<br>(8.54, 17.35) | Raby et al. 2018b |
| TMX | *Chironomus dilutus* | Growth | IC25<br>10 d | 17.8<br>(12.2, 20.1) | Phillips et al. 2021 |
| TMX | *Chironomus dilutus* | Growth | IC25<br>10 d | 17.3<br>(4.2, 29.6) | Phillips et al. 2021 |
| TMX | *Chironomus dilutus* | Growth | NOEC<br>LOEC<br>MATC<br>10 d | 21<br>42<br>29.7 | Phillips et al. 2021 |
| TMX | *Chironomus dilutus* | Growth | NOEC<br>LOEC<br>MATC<br>10 d | 16<br>32<br>22.6 | Phillips et al. 2021 |
| TMX | *Chironomus dilutus* | Growth ash-free dw | NOEC<br>LOEC<br>10 d<br>Static renewal<br>Sediment application | 600 µg/g<br>1,300 µg/g<br>Sediment dry wt | Finnegan et al. 2017 (Syngenta) |
| TMX | *Chironomus riparius* | Growth | NOEC<br>LOEC<br>10 d | 10.5<br>18 | Saraiva et al. 2017 |
| TMX | *Chironomus riparius* | Emergence | NOEC<br>LOEC<br>10 d | 6.5<br>10.5 | Saraiva et al. 2017 |
| TMX | *Chironomus riparius* | Emergence | NOEC<br>LOEC<br>30 d<br>Static<br>Sediment application | 20 µg/g<br>100 µg/g<br>Sediment dry wt | Finnegan et al. 2017 (Syngenta) |

| | | | | | |
|---|---|---|---|---|---|
| TMX | *Chironomus riparius* | Development rate | NOEC<br>LOEC<br>30 d<br>Static<br>Sediment application | >10 µg/g<br>>10 µg/g<br>Sediment dry wt | Finnegan et al. 2017 (Syngenta) |
| **Mayflies** | | | | | |
| TMX | *Cloeon dipterum* | Larval abundance | NOEC<br>34 d | 3.0 | Pickford et al. 2018 (Syngenta) |
| TMX | *Cloeon dipterum* | Larval abundance | LOEC<br>34 d | 10 | Pickford et al. 2018 (Syngenta) |
| TMX | *Cloeon dipterum* | Emergence | NOEC<br>35 d | 0.3 | Pickford et al. 2018 (Syngenta) |
| TMX | *Cloeon dipterum* | Emergence | LOEC<br>35 d | 1.0 | Pickford et al. 2018 (Syngenta) |
| TMX | *Deleatidium spp.* | Immobility | IC50<br>28 d | >4 | Macaulay et al. 2019 |
| TMX | *Deleatidium spp.* | Impairment | EC50<br>28 d | >4 | Macaulay et al. 2019 |
| TMX | *Neocloeon triangulifer* | Imago Emergence | EC50<br>14 d | 2.18<br>(1.60, 3.20) | Raby et al. 2018b |
| TMX | Heptageniidae (*Stenacron, Stenonema, Maccaffertium*) | Survival | EC50<br>96 h<br>Trials with diff pops of each genus | Ranged from 19.8 to 86.5 µg/L | Rackliffe and Hoverman 2020 |
| TMX | *Hexagenia* sp. | Behavior (remaining in burrows) | EC50<br>96 h | 630<br>(140, 2900)<br>NOMINAL | Bartlett et al. 2018 |
| TMX | *Hexagenia* sp. | Behavior (remaining in burrows) | NOEC<br>LOEC<br>96 h | 100<br>1,000<br>NOMINAL | Bartlett et al. 2018 |

NOTES:
LC50 = median lethal concentration (50%)
EC50 = median effective concentration (50%)
LOLC = lowest observed lethal level
NOEC = no observed effect concentration = highest concentration tested that was not significantly different from the control
LOEC = lowest observed effect concentration = lowest concentration tested that was significantly different from the control
MATC = maximum allowable toxicant concentration = geometric mean of the NOEC and LOEC concentrations

LITERATURE CITED IN TABLE 1, APPENDIX B

Agatz, A., R. Ashauer, and C.D. Brown. 2014. Imidacloprid pertubs feeding of Gammarus pulex at environmentally relevant concentrations. Environmental Toxicology and Chemistry 33(3):648-653.

Azevedo-Pereira, M.F.L. Lemos, and A.M.V.M. Soares. 2011. Effects of imidacloprid exposure on *Chironomus riparius* Meigen larvae: Linking acetylcholinesterase activity to behaviour. Ecotoxicology and Environmental Safety 74:1210-1215.

Bartlett, A.J., A.M. Hedges, K.D. Intini, L.R. Brown, F.J. Maisonneuve, S.A. Robinson, P.L. Gillis, and S.R. de Solla. 2019. Acute and chronic toxicity of neonicotinoid and butanolide insecticides to the freshwater amphipod, *Hyalella azteca*. Ecotoxicology and Environmental Safety 175:215-223.

Bartlett, A.J., A.M. Hedges, K.D. Intini, L.R. Brown, F.J. Maisonneuve, S.A. Robinson, P.L. Gillis, and S.R. de Solla. 2018. Lethal and sublethal toxicity of neonicotinoid and butanolide insecticides to the mayfly, *Hexagenia* spp. Environmental Pollution 238:63-75.

Bottger, R., J. Schaller and S. Mohr. 2012. Closer to reality—the influence of toxicity test modifications on the sensitivity of *Gammarus roeseli* to the insecticide imidacloprid. Ecotoxicology and Environmental Safety 81:49-54.

Camp, A.A. and D.B. Buchwalter. 2016. Can't take the heat: Temperature-enhanced toxicity in the mayfly *Isonychia bicolor* exposed to the neonicotinoid insecticide imidacloprid. Aquatic Toxicology 178:49-57.

Cavallaro, M.C., C.A. Morrissey, J.V. Headley, K.M. Peru, K. Liber. 2017. Comparative chronic toxicity of imidacloprid, clothianidin, and thiamethoxam to *Chironomus dilutus* and estimation of toxic equivalency factors. Environmental Toxicology and Chemistry 36:372-382.

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

Finnegan, M.C., L.R. Baxter, J.D. Maul, M.L. Hanson, and P.F. Hoekstra. 2017. Comprehensive characterization of the acute and chronic toxicity of the neonicotinoid insecticide thiamethoxam to a suite of aquatic primary producers, invertebrates and fish. Environmental Toxicology and Chemistry 36:2838-2848.

Hayasaka, D., K. Suzuki, T. Nomura, M. Nishiyama, T. Nagai, F. Sanchez-Bayo, and K. Goka. 2013. Comparison of acute toxicity of two neonicotinoid insecticides, imidacloprid and clothianidin, to five clodoceran species. J. Pestic. Sci. 38(1):44-47.

Jemec, A., T. Tisler, D. Drobne, K. Sepcic, D. Fournier, P. Trebse. 2007. Comparative toxicity of imdacloprid, of its commercial liquid formulation and of diazinon to a non-target arthropod, the microcrustacean *Daphnia magna*. Chemosphere 68:1408-1418.

Li, H., Q. Zhang, H. Su, J. You, and W-X. Wang. 2021. High tolerance and delayed responses of *Daphnia magna* to neonicotinoid insecticide imidacloprid: Toxicokinetic and toxicodynamic modeling. Environmental Science & Technology 55:458-467.

Macaulay, S.J., K.J. Hageman, R.E. Amumbaugh, S.M. Lyons, J.J. Piggott, and C.D. Matthaei. 2019. Chronic toxicities of neonicotinoids to nymphs of the common New Zealand mayfly Deleatidium spp. Environmental Toxicology and Chemistry 38:2459-2471.

Maloney, E.M., C. Morrissey, J.V. Headley, K.M. Peru, and K. Liber. 2017. Cumulative toxicity of neonicotinoid insecticide mixtures to *Chironomus dilutus* under acute exposure scenarios. Environmental Toxicology and Chemistry 36:3091-3101.

Phillips, B.M., J.P. Voorhees, K. Siegler, L. McCalla, P. Meertens, J. Bower, and R.S. Tjeerdema. 2021. Acute and chronic effects of clothianidin, thiamethoxam and methomyl on *Chironomus dilutus*. Bulletin of Environmental Contamination and Toxicology 108:884-889.

Pickford, D.B. M.C. Finnegan, L.R. Baxter, W. Bohmer, M.L. Hanson, P. Stegger, U. Hommen, P.F. Hoekstra, M. Hamer. 2018. Response of the mayfly (*Cloeon dipterum*) to chronic exposure to thiamethoxam in outdoor mesocosms. Environmental Toxicology and Chemistry 37:1040-1050.

Raby, M. M. Nowierski, D. Perlov, X. Zhao, C. Hao, D.G. Poirier, and P.K. Sibley. 2018a. Acute toxicity of 6 neonicotinoid insecticides to freshwater invertebrates. Environmental Toxicology and Chemistry 37:1430-1445.

Raby, M., X. Zhao, C. Hao, D.G. Poirier and P.K. Sibley. 2018b. Chronic toxicity of 6 neonicotinoid insecticides to *Chironomus dilutus* and *Neocloeon triangulifer*. Environmental Toxicology and Chemistry 37:2727-2739.

Raby, M. X. Zhao, C. Hao, D.G. Poirier and P.K. Sibley. 2018c. Relative chronic sensitivity of neonicotinoid insecticides to *Ceriodaphnia dubia* and *Daphnia magna*. Ecotoxicology and Environmental Safety 163:238-244.

Rackliffe, D.R., J.T. Hoverman. 2020. Population-level variation in neonicotinoid tolerance in nymphs of Heptageniidae. Environmental Pollution 265:114803.

Roessink, I., L.B. Merga, H.J. Zweers, P.J. Van den Brink. 2013. The neonicotinoid imidacloprid shows high chronic toxicity to mayfly nymphs. Environmental Toxicology and Chemistry 32:1096-1100.

Saraiva, A.S., R.A. Sarmento, A.C.M. Rodrigues, D. Campos, G. Fedorova, V. Zlabek, C. Gravato, J.L.T. Pestana, and A.M.V.M. Soares. 2017. Assessment of thiamethoxam toxicity to *Chironomus riparius*. Ecotoxicology and Environmental Safety 137:240-246.

Stoughton, S.J., K. Liber, J. Culp and A. Cessna. 2008. Acute and chronic toxicity of imidacloprid to the aquatic invertebrates *Chironomus tentans* and *Hyalella azteca* under constant and pulse exposure conditions.

Ugurlu, P., E. Unlu, and E.I. Satar. 2015. The toxicological effects of thiamethoxam on *Gammarus kischineffensis* (Schellenberg 1937) (Crustacea: Amphipoda). Environmental Toxicology and Pharmacology 39:720-726.

Van Den Brink, P.J., J.M. Van Smeden, R.S. BekeleW. Dierick, D.M. De Gelder, M. Noteboom and I. Roessink. 2016. Acute and chronic toxicity of neonicotinoids to nymphs of mayfly species and some notes on seasonal differences. Environmental Toxicology and Chemistry 35:128-133.

Williams, N. and J. Sweetman. 2020. Effects of neonicotinoids on the emergence and composition of chironomids in the Prairie Pothole Region. Environmental Science and Pollution Research 26:3862-3868.

## PUBLICATIONS CURRENTLY IN PREPARATION:

*undergraduate student; †Masters of Science graduate student

A Jeninga†, Z Wallace*, S Victoria†, E Harrahy, and TC King-Heiden. Chronic exposure to environmentally relevant concentrations of imidacloprid impact survival and ecologically relevant behaviors of fathead minnow larvae.

E Harrahy, A Jeninga, and TC King-Heiden. Acute and chronic toxicity of imidacloprid and thiamethoxam to select aquatic invertebrates.

E Harrahy, A Jeninga†, S Duffy*, and TC King-Heiden. Exposure to binary mixtures of thiamethoxam and imidacloprid causes additive toxicity in aquatic invertebrates, but not fish.