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Cascading effects of insecticides and road salt on wetland communities[☆]

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ABSTRACT

Novel stressors introduced by human activities increasingly threaten freshwater ecosystems. The annual application of more than 2.3 billion kg of pesticide active ingredient and 22 billion kg of road salt has led to the contamination of temperate waterways. While pesticides and road salt are known to cause direct and indirect effects in aquatic communities, their possible interactive effects remain widely unknown. Using outdoor mesocosms, we created wetland communities consisting of zooplankton, phytoplankton, periphyton, and leopard frog (*Rana pipiens*) tadpoles. We evaluated the toxic effects of six broad-spectrum insecticides from three families (neonicotinoids: thiamethoxam, imidacloprid; organophosphates: chlorpyrifos, malathion; pyrethroids: cypermethrin, permethrin), as well as the potentially interactive effects of four of these insecticides with three concentrations of road salt (NaCl; 44, 160, 1600 Cl^- mg/L). Organophosphate exposure decreased zooplankton abundance, elevated phytoplankton biomass, and reduced tadpole mass whereas exposure to neonicotinoids and pyrethroids decreased zooplankton abundance but had no significant effect on phytoplankton abundance or tadpole mass. While organophosphates decreased zooplankton abundance at all salt concentrations, effects on phytoplankton abundance and tadpole mass were dependent upon salt concentration. In contrast, while pyrethroids had no effects in the absence of salt, they decreased zooplankton and phytoplankton density under increased salt concentrations. Our results highlight the importance of multiple-stressor research under natural conditions. As human activities continue to imperil freshwater systems, it is vital to move beyond single-stressor experiments that exclude potentially interactive effects of chemical contaminants.

1. Introduction

The use of synthetic chemicals has introduced novel stressors to freshwater ecosystems worldwide (Dugan et al., 2017; Stehle and Schulz, 2015). Among these stressors, pesticides, heavy metals, and deicers are known to cause direct lethal and sublethal effects on non-target species within aquatic environments (Gonçalves et al., 2007; Jackson et al., 2016; Schuler and Relyea, 2018; Van Wijngaarden et al., 2005). While single-species laboratory toxicological tests are vital to investigate individual- and population-level effects of single chemical exposures, such studies lack the natural complexities found within aquatic communities. For instance, single-species toxicity tests are unable to examine the effects of contaminants on trophic interactions, and thus, food webs (Clements and Rohr, 2009; Relyea and Hoverman, 2006). Moreover, aquatic ecosystems are regularly exposed to multiple chemical stressors simultaneously (Gilliom, 2007; Schuler and Relyea, 2018). Understanding the effects of multiple-chemical exposure on aquatic communities is of the utmost importance as the use and production of synthetic chemicals outpaces other agents of global change (Bernhardt et al., 2017).

Pesticides are commonly found contaminating freshwater ecosystems (Berrill et al., 1993; Malaj et al., 2014; Stone et al., 2014). They have become widespread in the environment due to the annual use of more than 2.3 billion kg of active ingredient globally in agricultural, residential, and industrial settings (Antle, 2006; Atwood and Paisley-Jones, 2017). Pesticide application occurs in agricultural areas that are highly integrated with aquatic ecosystems as they function as both water sources and drainage facilities

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(Van Wijngaarden et al., 2005). This integration and close proximity inevitably results in some pesticide contamination of aquatic ecosystems through spray drift, runoff, and leaching (Ganzelmeier et al., 1995; Marrs et al., 1989; Pimentel, 1995; Preftakes et al., 2019).

Once in aquatic environments, pesticides can cause direct lethal effects on sensitive non-target species as well indirect effects that cascade through freshwater communities (Berrill et al., 1993; Fleeger et al., 2003; Relyea, 2009; Rohr and Crumrine, 2005). Insecticides, in particular, are highly toxic to non-target species, and can cause significant mortality. Among the reported effects of insecticide contamination are the decline of total species richness (Duchet et al., 2018; Relyea, 2005), reduction of zooplankton abundance by decreasing cladocerans (Relyea, 2005, 2009), and loss of predatory insect diversity and biomass (Relyea, 2005). Furthermore, the direct, toxic effects of insecticide exposure can cause trophic cascades as documented in outdoor mesocosm experiments (Bendis and Relyea, 2016; Relyea and Diecks, 2008). Given the temporal and spatial variation in pesticide application, it is important to investigate how such agrochemicals interact with other freshwater contaminants (Main et al., 2016).

In temperate latitudes, deicing salts (primarily sodium chloride (NaCl)) are used to improve road conditions with around 22 billion kg applied annually (Findlay and Kelly, 2011). However, snow melt and precipitation events carry salt ions into adjacent aquatic environments (Forman and Alexander, 1998; Herbert et al., 2015). Although commonly thought of as a winter problem, road salt concentrations in freshwater stay high throughout the spring and summer months. In many areas, freshwater salinization is becoming a year-round phenomenon due to long-term storage and transport of dissociated road salt ions (e.g., chloride, sodium, magnesium) in groundwater and soils (Kaushal et al., 2005, 2019) with concentrations ranging from 5 to 5000 Cl^- mg/L (Environment Canada and Health Canada, 2001).

Although clearing roads of snow and ice is important to human safety, the accumulation of salt in adjacent aquatic habitats can negatively affect ecosystem health, biodiversity, and cause trophic cascades (Cañedo-Argüelles et al., 2013; Herbert et al., 2015; Hintz et al., 2017; Karraker et al., 2008; Van Meter et al., 2011a, 2011b). For example, exposure to 645 mg Cl^-/L NaCl reduces zooplankton abundance causing increases in phytoplankton and periphyton biomass (Van Meter et al., 2011). As freshwater salinization continues, it will be important to understand how increased salinity influences species-, population-, and community-level responses to increasing agrochemical contamination.

In water bodies from colder regions of the world, freshwater communities are invariably exposed to insecticides and road deicing salt simultaneously, yet we know little about their potential to interact. Some studies suggest there may be an antagonistic effect as juvenile rainbow trout (*Oncorhynchus mykiss*) have reduced mortality to chlorpyrifos exposure after transitioning and acclimating to hypersaline conditions (Amiri et al., 2018; reviewed by Velasco et al., 2019). Conversely, in Australian agricultural areas with salinization due to rising saline water tables, pesticide and salt concentration had no interactive effects on the community structure of macroinvertebrates (Szöcs et al., 2012). As for road salt, we are aware of only one study that has examined the interactive effects with an insecticide (carbaryl). Stoler et al. (2016) found an interactive effect that caused a reduction in copepod abundance. Clearly, much remains unknown about the possible interactive effects of pesticides and road salts, especially across different families that have unique modes of action. For example, pyrethroids cause prolonged sodium channel openings in invertebrate animals (Brander et al., 2012; Burr and Ray, 2004; Werner and Moran, 2008) whereas organophosphates bind to acetylcholinesterase (Giddings et al., 2014; Widder and Bidwell, 2006).

We evaluated the effects of six broad-spectrum insecticides (neonicotinoids: thiamethoxam, imidacloprid; organophosphates: chlorpyrifos, malathion; pyrethroids: cypermethrin, permethrin), as well as the possible interactive effects of four of the insecticides (two organophosphates and two pyrethroids) with three concentrations of road salt (NaCl) on simulated wetland communities containing zooplankton, phytoplankton, periphyton, and tadpoles. We made the following predictions: 1) insecticide exposure will reduce zooplankton abundance, tadpole mass, and tadpole activity; 2) insecticides with same mode of action (i.e. the same insecticide class) will produce similar effects on the communities; 3) road salt exposure alone will decrease zooplankton abundance and tadpole mass; and 4) given the potential interactive effects of road salt (NaCl) and pyrethroid insecticides (opening of Na—K channels), exposure to pyrethroid insecticides and road salt will be more detrimental compared to when organophosphate insecticides are combined with salt. We do not expect either stressor to have a direct impact on phytoplankton, however, if zooplankton abundance is negatively affected by pesticide, road salt, or their interaction we expect a cascade effect subsequently causing phytoplankton abundance to increase due to a lack of grazing.

2. Materials and methods

2.1. Experimental set-up

Experiment 1. —To evaluate the effects of six pesticides (imidacloprid, thiamethoxam, malathion, chlorpyrifos, cypermethrin, and permethrin) representing three insecticide families (neonicotinoids, organophosphates, and pyrethroids) on wetland communities, we conducted an outdoor mesocosm experiment at Rensselaer's Technology Park (Troy, New York, USA). The experiment used a completely randomized design and employed a factorial treatment combination that exposed communities to low and high concentrations of each insecticide (Table S1). We included a vehicle control (ethanol) and no-pesticide control (water) resulting in a total of 14 treatments. Each treatment was replicated four times for a total of 56 experimental units.

Experiment 2. —To evaluate the potentially interactive effects of pesticides and road salt, we used a factorial combination of four insecticides (malathion, chlorpyrifos, cypermethrin, permethrin) at two concentrations (low, high) to create eight pesticide treatments (Table S1). We then added a vehicle control (ethanol) and no-pesticide control (water) to the experimental treatments. These 10 treatments were crossed with three nominal road salt (NaCl) concentrations: low salt (44 mg Cl^-/L), medium salt (160 mg Cl^-/L), and high salt (1693 mg Cl^-/L). The 30 treatment combinations were replicated four times for a total of 120 experimental units.

Since the chloride concentration in the water used to fill the outdoor mesocosms (44 mg Cl^-/L) represented the low-salt treatment, we were able to share the no-pesticide control, vehicle control, malathion, chlorpyrifos, cypermethrin, and permethrin treatments in Experiment 1 with the low-salt treatment combinations in Experiment 2. This not only reduced the number of organisms needed but decreased the experimental size to 136 combined experimental units. All experiments were run simultaneously.

Our experimental units were 100-L plastic wading pools that we filled with 82 L of chlorinated tap water on 11—13 May 2015. We covered each pool with 65% shade-cloth lid to reduce heating effects from sunlight and to prevent insects from ovipositing. We then waited several days for the chlorine to off-gas from the tap water. On 15 May, we added 100 g of dried oak leaf litter (*Quercus* spp.) to each mesocosm to provide structure and a slow-release nutrient source, 5 g of rabbit chow (Blue Seal Bunny 16; Londonderry, NY, USA) as an initial nutrient source, and an aliquot of pond water containing zooplankton, periphyton, and phytoplankton to establish biotic communities. Pond water was collected from four local fishless ponds (Rennselaer County, New York, USA), visually screened to remove any invertebrate predators, homogenized, and then distributed in 177-mL aliquots to each mesocosm.

We collected five freshly oviposited leopard frog (*Rana pipiens*) egg masses on April 30, 2015 from a local wetland (Albany County, New York, USA). Eggs were held in 300-L plastic culture pools containing 250 L aged tap water. Once the eggs hatched, the tadpoles were fed rabbit chow *ad libitum*. On 28 May, we haphazardly collected tadpoles (body mass: 78.15 ± 13.6 mg) from all egg masses and selected 10 individuals to add to each mesocosm.

2.2. *Insecticide and salt exposure*

We used two nominal concentrations for each insecticide that fell within or near the range of estimated environmental concentrations in aquatic systems (Table S1). Concentrations were also selected to avoid the median lethal concentration values for invertebrates. Within each insecticide family, we used the same concentration for each pair of insecticides. For the organophosphates and neonicotinoids, we set the nominal high concentrations at 20 μg/L and then set the low concentrations at one-fourth of the high concentration (i.e., 5 μg/L); for the much more lethal pyrethroids, we set the high nominal concentration at 0.5 μg/L and the low concentrations at one-fourth of the high concentration (i.e., 0.125 μg/L). To achieve these nominal concentrations, we created 5 mg active ingredient (a.i.)/L stock solutions for the neonicotinoid and organophosphate insecticides, and 2 mg (a.i.)/L stock solutions for the pyrethroid insecticides by dissolving each insecticide's active ingredient in ethanol. We applied the twelve pesticide treatments on May 29, 2015 (day 0 of the experiment) as a singular pulse stressor as pesticides break down in water in a about a week. The no-pesticide and vehicle controls received an equivalent volume of water and ethanol, respectively, that matched the highest volume of insecticide stock solution added matching that which was applied to the pesticide treatments.

Following pesticide application, we collected water samples from all replicates within each treatment to analyze the actual insecticide concentrations (Table S1). Within replicates of each insecticide treatment, we collected a 200-mL water sample from the center of each mesocosm, homogenized the four samples, and extracted a final 500-mL sample in pre-cleaned, glass amber jars (VWR # 89126-604). Water samples were preserved using the addition of 2 mL methylene chloride (CAS # 75-09-2; Fisher Scientific, Pittsburgh, PA, USA) and immediately refrigerated (1–2 °C). The samples were then packed on ice and transported to the University of Connecticut's Center for Environmental Sciences and Engineering (Storrs, Connecticut, USA). Water samples from the vehicle control and no-pesticide control had no detectable pesticide concentrations (analysis detection limit = 0.1 ppb).

To create the three salt concentrations, we added NaCl to mesocosms assigned to road salt treatments. The salt concentrations used reflect the range of concentrations detected in natural ponds. In this environment, chloride concentrations have been reported covering a wide range of concentrations ranging from 5 to 5000 Cl^- mg/L (Environment Canada and Health Canada, 2001). As the Cl^- concentration of the tap water was 44 mg Cl^-/L (156 μS/cm), it was defined as our low salt concentration. On 30 May (day 1 of the experiment), we added 22 g NaCl and 230 g NaCl to obtain the nominal concentrations of 160 Cl^- mg/L (actual: 558 μS/cm; ~168 Cl^- mg/L) and 1600 Cl^- mg/L (actual: 3616 μS/cm; ~1033 Cl^- mg/L) for the medium and high concentrations, respectively. Since salt does not break down, the salt concentrations stayed consistent throughout the study, acting as a press stressor on the communities.

2.3. *Response variables*

We surveyed zooplankton abundance twice during the experiment (on days 7 and 21). At day 7 we were looking for direct effects of both stressors, while at day 21 we were looking for longer-term effects of initial insecticide exposure and continued exposure to road salt. Sampling was done by plunging a 0.2-L tube sampler into each mesocosm at four locations (three equidistant (120°) positions and once in the center). The four samples were then filtered through a 62-μm Nitex screen and captured zooplankton were preserved in 70% ethanol. We used a high-powered dissection microscope (Olympus SZX16) to enumerate and identify preserved individuals, categorizing zooplankton into four groups: cladocerans, cyclopoid copepods, calanoid copepods, and rotifers.

Phytoplankton abundance was assessed once during the experiment (on day 24). We collected a 400-mL water sample from the middle of each mesocosm by inverting a plastic container, and then vacuum filtered the sample through a Whatman GF/C filter (Whatman Inc., Florham Park, NJ, USA). Filters were then sealed individually in aluminum foil and frozen (−20 °C) prior to analysis. Phytoplankton was measured as chlorophyll *a*, and quantified following the methods of Arar and Collins (1997), including the acidification protocol and using a calibrated fluorometer (TD-700; Turner Instruments, Sunnyvale, California, USA).

We sampled abiotic conditions (i.e. pH, dissolved oxygen (DO), temperature, and conductivity) on days 7, 14, and 21 of the experiment. Abiotic water quality variables were surveyed using a calibrated, multiparameter water meter (YSI, Yellow Springs, Ohio, USA).

To investigate the effects of the pesticide and road salt exposure on leopard frog tadpoles, we quantified activity, survival, and mass. On day 12, we assessed leopard frog activity. Thirty minutes prior to observations, we removed the shade-cloth lids from each mesocosm and allowed tadpoles to acclimate to the new conditions. Three observers then quietly approached the uncovered mesocosms to count the number of tadpoles they could see and the number of seen tadpoles that were active (e.g., actively swimming or moving in the water column). Each observer recorded activity in all tanks three times, such that we had nine observations of each mesocosm. For each mesocosm, we quantified the proportion of active tadpoles by dividing the number moving individuals by the total number of tadpoles seen. The overall mean activity of the nine observations was used as our behavioral response variable for each mesocosm.

We terminated the experiment on day 25 (23 June) prior to tadpole metamorphosis. All surviving tadpoles were euthanized using an MS-222 overdose, weighed (g), and then preserved in 10% buffered formalin.

2.4. *Statistical analysis*

To evaluate the effect of pesticide treatment, road salt concentration, and their possible interaction on abiotic conditions and wetland community responses, we employed analyses of variance (ANOVA). Repeated-measures analyses of variance (rm-ANOVA) were used to analyze data that were sampled multiple times during the experiment, such as abiotic data and zooplankton abundance. If a significant time-by-treatment interaction was detected, we conducted subsequent Dunnett's tests for *post hoc* analyses using the emmeans package (R Core Team, 2019) comparing treatments to the no-pesticide control mean for each sampling date and within a salt concentration.

ANOVAs were used to analyze responses measured only once during the experiment, such as phytoplankton abundance and amphibian activity and mass. We did not analyze tadpole survival because survival was high across all treatments (range = 98–100%). When main effects were significant, we conducted subsequent Dunnett's tests as *post hoc* tests using the emmeans package (R Core Team, 2019) to compare the experimental treatment values to that of the no-pesticide control treatment at each salt concentration. For each analysis, if the errors were heterogeneous or assumptions of normality were violated, we log-transformed the data. Statistical analyses were all conducted in R version 3.6.1 (R Core Team, 2019).

3. Results

3.1. Experiment 1 —insecticide exposure

Zooplankton abundance. —The zooplankton were sampled early and late in the experiment (days 7 and 21). For cladocerans, the rm-ANOVA indicated effects of pesticides, time, and their interaction (Table 1). The subsequent Dunnett's test within each sampling date indicated organophosphates at both concentrations significantly decreased cladoceran abundance on both dates compared to the no-pesticide control (Fig. 1). In contrast, the neonicotinoids and pyrethroids had no effect on day 7. However, cladoceran abundance on day 2L was reduced in the low concentration of imidacloprid and the high concentrations of thiamethoxam and permethrin.

While calanoid copepods exhibited no significant main effects or interaction, the abundance of cyclopoid copepods was significantly influenced by pesticides, time, and the pesticide-by-time interaction (Table 1). The *post hoc* tests revealed significant decreases in cyclopoid copepod abundance on day 7 following exposure to high concentrations of malathion, and on day 21 following exposure to high concentrations of chlorpyrifos (Fig. 1).

The rm-ANOVA investigating rotifer abundance identified significant effects of time and pesticide-by-time interaction (Table 2). However, further *post hoc* tests within each sample date showed no significant differences in rotifer abundance between any pesticide treatment and the no-pesticide control.

Phytoplankton abundance. —We sampled phytoplankton late in the experiment (day 24). The ANOVA identified an effect of pesticide (Table 1). In treatments with low concentrations of malathion and low and high concentrations of chlorpyrifos a significant or marginally significant increases in Chl *a* was observed (Fig. 2).

Abiotic conditions. —We assessed abiotic conditions early, middle, and late in the experiment (days 7, 14, and 21). Neither pH nor conductivity significantly changed. Mesocosm pH ranged from 7.34 to 7.44 and conductivity ranged from 149 to 161 μS/cm across all days. In contrast, pesticide exposure corresponded with elevated temperatures on day 7 and 14 compared to the control (Fig. S1). DO

Table 1
Experiment 1, results of ANOVAs and repeated-measure ANOVAs that examined how the pesticide treatments affected a number of biotic response variables. *F*-values for ANOVAs and chi square values for rm-ANOVAs are given, while *p*-values are in parentheses. Significant *p*-values are in bold font.

Response variable	Pesticide	Time	Pesticide × Time
Cladoceran abundance	**272.1 (<0.001)**	**4.1 (0.042)**	**99.8 (<0.001)**
Calanoid copepod abundance	10.3 (0.669)	1.0 (0.308)	4.5 (0.985)
Cyclopoid copepod abundance	**38.4 (<0.001)**	**16.6 (<0.001)**	**36.0 (<0.001)**
Rotifer abundance	9.2 (0.755)	**4.5 (0.035)**	**25.4 (0.021)**
Phytoplankton abundance	**7.7 (<0.001)**		
Tadpole activity	1.4 (0.222)		
Tadpole mass	**6.4 (<0.001)**		

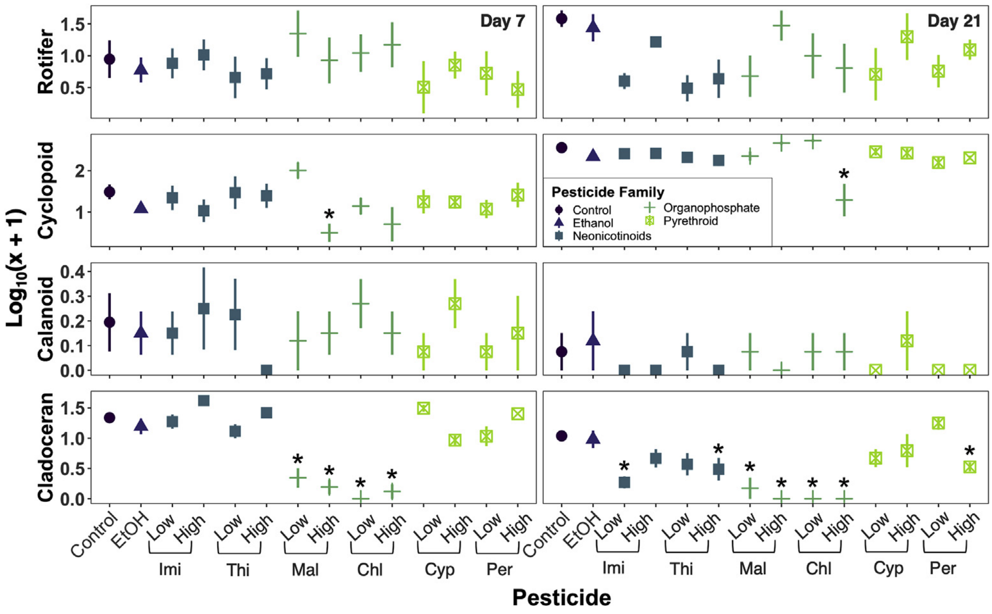

Fig. 1. Zooplankton abundance ($\log_{10} + 1$) for four zooplankton groups in response to 12 pesticide treatments (Imi = imidacloprid, Thi = thiamethoxam, Mal = malathion, Chl = chlorpyrifos, Cyp = cypermethrin, and Per = permethrin) and two controls (Experiment 1). Data are means (±1 SE). Asterisks indicate significant differences between each pesticide treatment and the water control for each zooplankton group on each sampling day ($p < 0.05$).

Table 2
Experiment 2, results of ANOVAs and repeated-measure ANOVAs that examined how the pesticide and salt treatments affected biotic response variables. *F*-values are given, while *p*-values are in parentheses. Significant *p*-values are in bold font.

Response variable	Pesticide	Salt	Time	Pesticide × Salt	Pesticide × Time	Salt × Time	Pesticide × Salt × Time
Cladoceran abundance	**23.6 (<0.001)**	0.9 (0.398)	3.1 (0.080)	**2.9 (<0.001)**	**4.0 (<0.001)**	2.0 (0.140)	1.0 (0.502)
Calanoid copepod abundance	6.3 (0.714)	0.2 (0.908)	1.2 (0.281)	21.6 (0.249)	2.1 (0.989)	3.4 (0.180)	23.6 (0.168)
Cyclopoid copepod abundance	**4.4 (<0.001)**	**9.4 (<0.001)**	**16.4 (<0.001)**	**2.1 (0.009)**	**3.6 (<0.001)**	1.2 (0.320)	0.9 (0.586)
Rotifer abundance	10.2 (0.332)	4.3 (0.118)	**4.2 (0.041)**	12.5 (0.818)	**19.9 (0.019)**	1.2 (0.546)	**56.7 (<0.001)**
Phytoplankton abundance	**6.7 (<0.001)**	**11.6 (<0.001)**		**6.9 (<0.001)**			
Tadpole activity	1.4 (0.220)	2.8 (0.067)		1.1 (0.333)			
Tadpole mass	**6.3 (<0.001)**	**3.3 (0.040)**		**2.8 (<0.001)**			

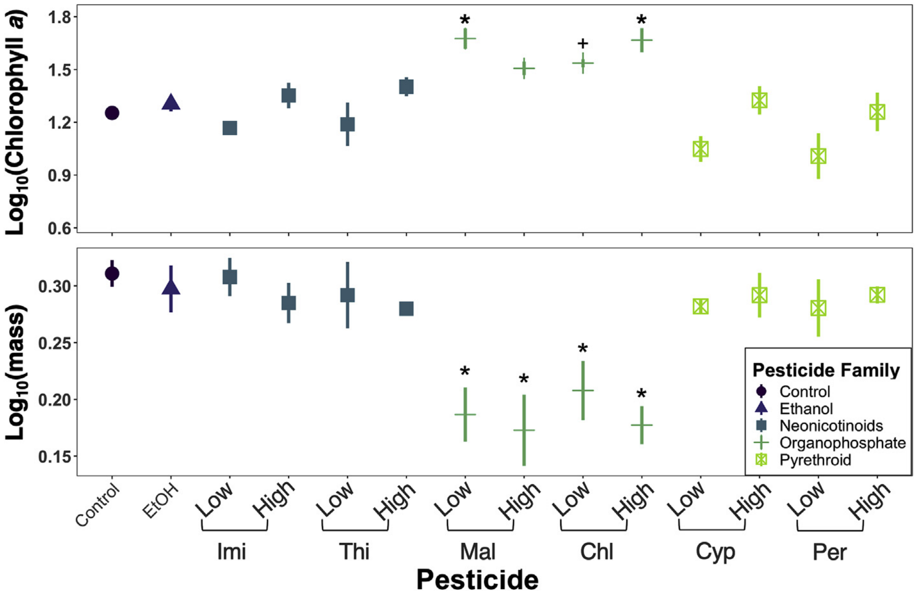

Fig. 2. Phytoplankton concentration at day 24 (i.e. $\log_{10}$ values of chlorophyll *a*) and Leopard frog tadpole mass ($\log_{10}$) on day 25 in response to 12 pesticide treatments (Imi = imidacloprid, Thi = thiamethoxam, Mal = malathion, Chl = chlorpyrifos, Cyp = cypermethrin, and Per = permethrin) and two controls (Experiment 1). Data are means (±1 SE). Asterisks indicate significant differences between each pesticide treatment and the water control ($p < 0.05$); crosses (+) indicate marginally significant differences ($0.05 < p < 0.1$).

did not have an effect with time or an interaction but increased with organophosphate exposure (Table S2; Fig. S2). Although significant, these temperature differences are mostly an artifact of the order in which the treatments were sampled as the day got warmer, with the exception of the organophosphates on day 7. See supplementary document for further information.

Tadpoles. —Effects on tadpoles were assessed looking at activity mid experiment (day 12) and mass at the end of the experiment (day 25). We found no effect of the pesticides on tadpole activity (day 12), but there was an effect on tadpole mass (day 25; Table 1). Exposure to organophosphates, regardless of concentration, reduced tadpole body mass (Fig. 2). In contrast, exposure to neonicotinoids or pyrethroids had no effect on tadpole mass.

3.2. Experiment 2. —salt and insecticide exposure

Zooplankton abundance. —Zooplankton were sampled early and late in the experiment (days 7 and 21). For cladocerans, we found effects of pesticide, marginal effects of time, and two interactions (Table 2). Exposure to organophosphates decreased cladoceran abundance on both dates across all salt concentrations (Fig. 3). In contrast, the pyrethroid effects changed under different salt concentrations. Under low salt concentrations, all pyrethroid concentrations had no effect on both dates. Under medium salt concentrations, however, low concentrations of cypermethrin increased cladoceran abundance early in the experiment, but not later in the experiment. Under high salt concentrations, two pyrethroid treatments reduced cladoceran abundance on day 7 and all four pyrethroid treatments reduced cladoceran abundance by day 21 (Fig. 3).

While we observed no effects of the treatments on calanoid copepods, cyclopoid copepods were affected by pesticide, salt, time, and several interactions (Table 2). The identity and concentration of the organophosphate insecticide treatments influenced the reduction in cyclopoid abundance on both days but varied among salt concentrations (Fig. 4). Under low salt concentrations, cyclopoid abundance decreased in high concentrations of malathion and chlorpyrifos on day 7, but reduced abundance remained only in high concentrations of chlorpyrifos by day 21. Under medium salt concentrations, the abundance of cyclopoid copepod decreased in high chlorpyrifos treatments on days 7 and 21. Under high salt concentrations, organophosphate exposure had no effect on cyclopoid copepod abundance. In contrast, regardless of salt concentration, exposure to pyrethroids did not influence cyclopoid abundance on either sampling date (Table 2).

For rotifers, we found significant effects of time and two interactions (Table 2). Early in the experiment, none of the pesticide treatments affected rotifer abundance within any of the salt treatments. Later in the experiment, there were no effects of the pesticides under low or high salt concentrations. Under medium salt

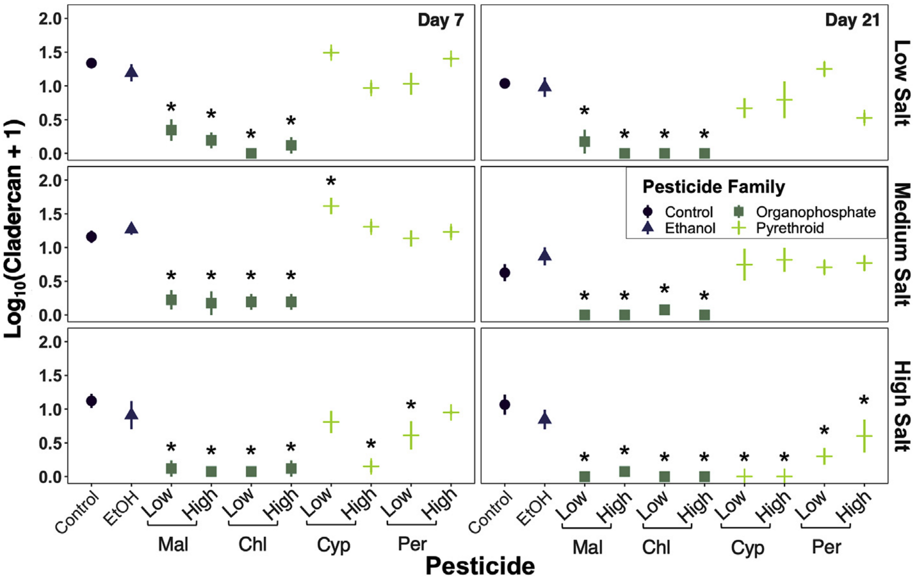

Fig. 3. Cladoceran abundance ($\log_{10} + 1$) in response to eight pesticide treatments (Mal = malathion, Chl = chlorpyrifos, Cyp = cypermethrin, and Per = permethrin) and two controls, crossed with three salt concentrations (low, medium, and high) on days 7 and 21 (Experiment 2). Data are means (±1 SE). Asterisks indicate significant differences between each pesticide treatment and the water control for each salt concentration on each day ($p < 0.05$).

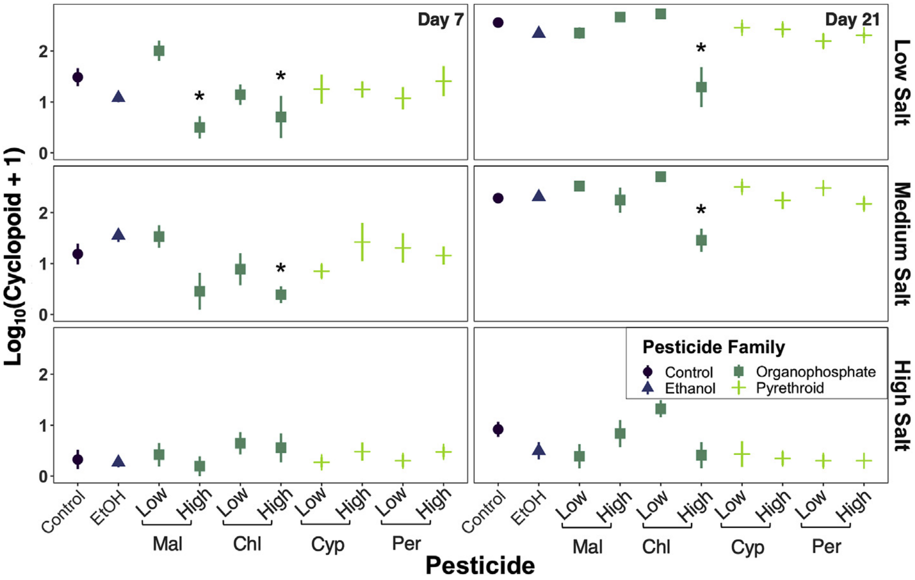

Fig. 4. Cyclopoid copepod abundance ($\log_{10} + 1$) in response to eight pesticide treatments (Mal = malathion, Chl = chlorpyrifos, Cyp = cypermethrin, and Per = permethrin) and two controls, crossed with three salt concentrations (low, medium, and high) on days 7 and 21 (Experiment 2). Data are means (±1 SE). Asterisks indicate significant differences between each pesticide treatment and the water control for each salt concentration on each day ($p < 0.05$).

concentrations, however, rotifer abundance increased with both concentrations of chlorpyrifos (Fig. 5).

Phytoplankton abundance. —We sampled phytoplankton late in the experiment (day 24). We found effects of pesticide, salt, and their interaction (Table 2; Fig. 6). Under low salt concentrations, two of the organophosphate treatments increased Chl *a*. Under medium salt concentrations, all four organophosphate and three of the pyrethroid treatments increased Chl *a*. Under high salt concentrations, both concentrations of malathion and three of the four pyrethroid treatments decreased Chl *a*.

Abiotic conditions. —We sampled abiotic conditions on days 7, 14, and 21. As in experiment 1, pH did not significantly change and ranged between 7.18 and 7.44 throughout the duration of the experiment. For temperature, we found significant effects of pesticide treatment, time, and several interactions (Table S3). Under all salt concentrations, organophosphate and pyrethroid exposure increased temperature on day 7 and 14 (Fig. S3). While on day 21 only pyrethroid treatments increased temperature. Similarly, to experiment 1, these temperature differences likely are an artifact of the order in which the treatments were sampled as the day got warmer. See supplementary document for further information.

For DO, we found a marginally significant effect of pesticide and two significant interactions (Table S3). Both pyrethroid and organophosphate exposure effected DO across the salt gradient. When there was an effect of pesticide treatment, DO tended to

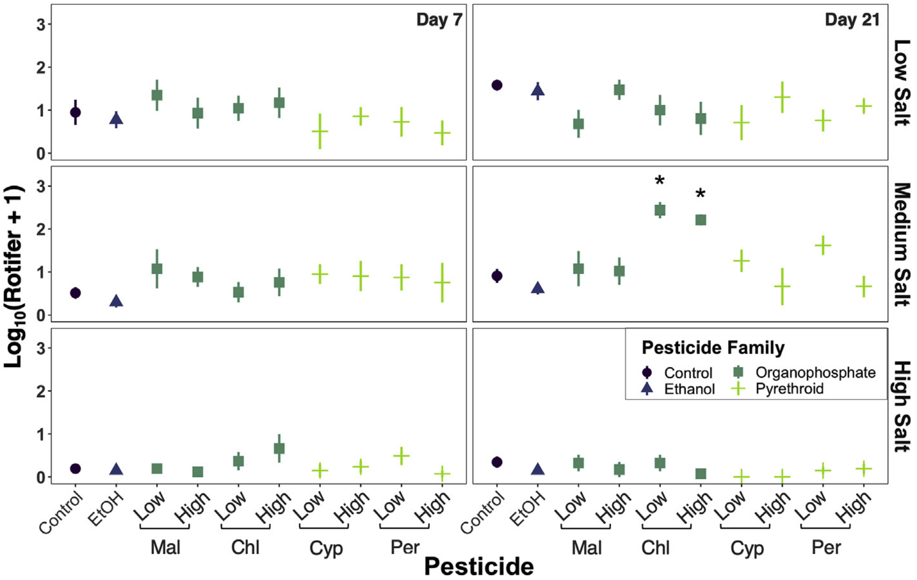

Fig. 5. Rotifer abundance ($\log_{10} + 1$) in response to eight pesticide treatments (Mal = malathion, Chl = chlorpyrifos, Cyp = cypermethrin, and Per = permethrin) and two controls, crossed with three salt concentrations (low, medium, and high) on days 7 and 21 (Experiment 2). Data are means (±1 SE). Asterisks indicate significant differences between each pesticide treatment and the water control for each salt concentration on each day ($p < 0.05$).

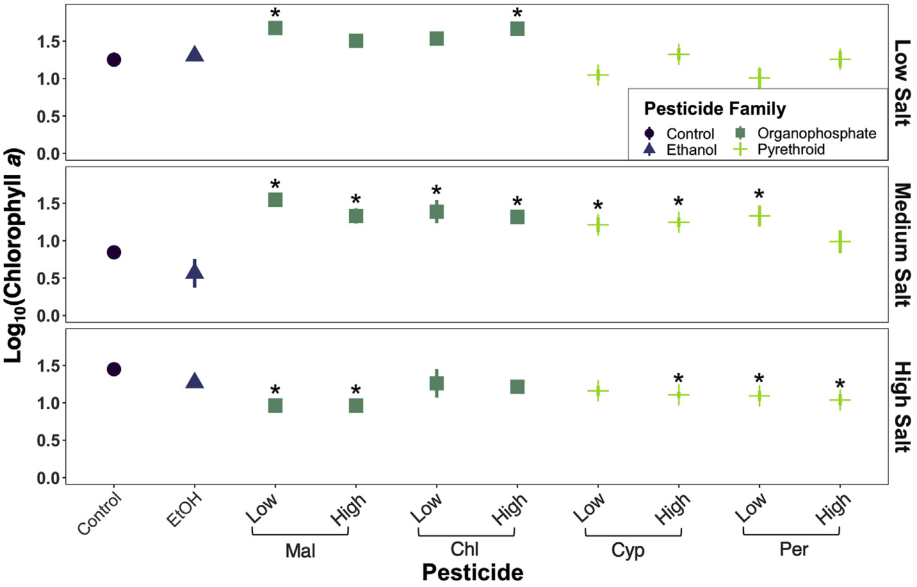

Fig. 6. The concentration of phytoplankton at day 24 ($\log_{10}$ values of chlorophyll *a*) in response to response to eight pesticide treatments (L = low concentration, H = high concentration, Mal = malathion, Chl = chlorpyrifos, Cyp = cypermethrin, and Per = permethrin) and two controls (C = water control, E = ethanol control), crossed with three salt concentrations (low, medium, and high) (Experiment 2). Data are means (±1 SE). Asterisks indicate significant differences between each pesticide treatment and the water control for each salt concentration ($p < 0.05$).

increase, however, pyrethroid exposure under high salt on day 7 DO decreased (Fig. S4). By day 21, there were no differences in DO among pesticide treatments at any salt concentration. See supplementary document for further information.

Tadpoles. —Effects on tadpoles were assessed by quantifying activity mid experiment (day 12) and mass at the end of the experiment (day 25). Tadpole activity showed no effect of pesticide, a marginal effect of salt, and no interaction (Table 2). Subsequent *post hoc* tests of the salt effect, however, showed no effect of medium or high salt concentrations compared to the control. For tadpole mass, we found a significant effect of pesticide, salt, and their interaction (Table 2). Under low salt concentrations, all four organophosphates caused a decrease in mass (Fig. 7). Under medium salt concentrations, the low concentration of permethrin caused increased mass. Under high salt concentrations, there was no effect of pesticide treatments.

4. Discussion

We sought to examine the potentially interactive effects of pesticides and road salt given the widespread contamination of freshwater systems in an effort to better predict the direct and indirect multi-stressor effects on aquatic communities. We discovered that insecticide exposure not only causes cascading

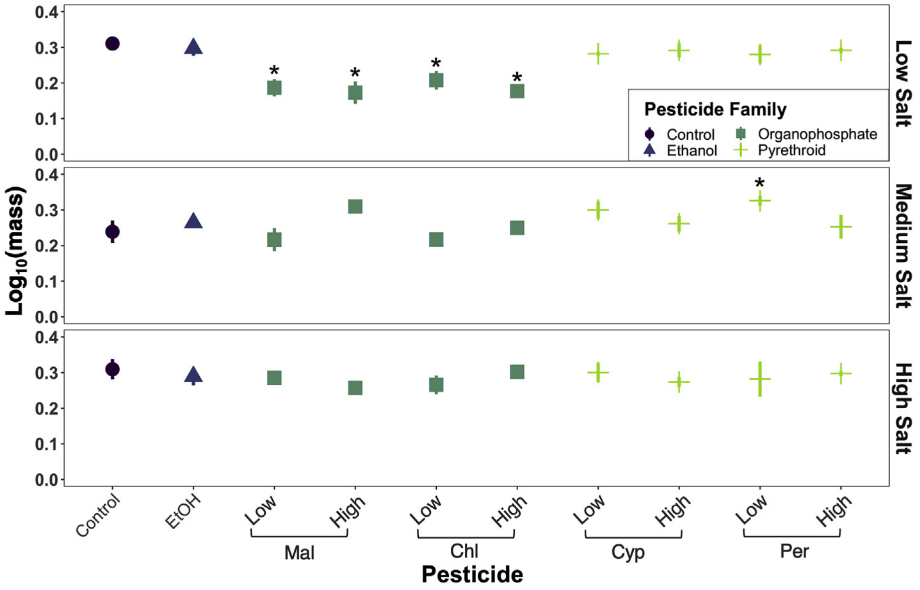

Fig. 7. Leopard frog tadpole mass ($\log_{10}$) in response to eight pesticide treatments (L = low concentration, H = high concentration, Mal = malathion, Chl = chlorpyrifos, Cyp = cypermethrin, and Per = permethrin) and two controls (C = water control, E = ethanol control), crossed with three salt concentrations (low, medium, and high) on day 25 (Experiment 2). Data are means (±1 SE). Asterisks indicate significant differences between each pesticide treatment and the water control for salt concentration ($p < 0.05$).

effects in aquatic communities but can also interact with environmentally relevant concentrations of road salt to alter the abundance and body condition of sensitive, non-target organisms. As expected, the impacts on communities differed among insecticide families, either due to their toxicity or their differences in concentration ranges used. Organophosphate exposure significantly reduced zooplankton abundance, increased phytoplankton biomass, and reduced tadpole mass. Neonicotinoid and pyrethroid exposure also decreased zooplankton abundance, albeit to a lesser extent than organophosphates, and had no significant effect on phytoplankton or tadpoles. The effects of insecticide exposure on aquatic communities were dependent upon the degree of salinization. For instance, only in high salt environments did pyrethroid exposure reduce zooplankton abundance and phytoplankton biomass. Lastly, while organophosphates significantly decreased zooplankton at all salt concentrations, their impacts on phytoplankton abundance and tadpole mass varied with salt concentrations.

4.1. Individual effects of insecticides

Insecticide exposure caused cascading effects in the experimental communities. The abundance of phytoplankton increased following direct lethal effects on sensitive zooplankton. While zooplankton were highly sensitive to our concentrations of chlorpyrifos and malathion (organophosphates) exposure, they were less sensitive to pyrethroid and neonicotinoid exposure. The reduction in cladoceran and cyclopoid copepod abundance following organophosphate exposure was sustained throughout the duration of the experiment. This is consistent with other studies showing reduced zooplankton abundance when exposed to malathion (20–110 μg/L; Odenkirchen and Wente, 2007; Cothran et al., 2011) and chlorpyrifos (0.035–1.47 μg/L; López-Mancisidor et al., 2008; Giddings et al., 2014). Moreover, tolerance of zooplankton varies with family; whereas cladocerans are sensitive to organophosphate exposure (5.8–1000 μg malathion/L and 3.2 μg chlorpyrifos/L; Relyea, 2005; 2009; Smith et al., 2018), copepods appear to be tolerant of organophosphates, and in some cases, increase in abundance after the competitive release following cladocerans mortality (35–320 μg malathion/L; Relyea, 2005; Groner and Relyea, 2011; Hua and Relyea, 2012). In contrast, we found cyclopoid copepod abundance decreased in response to high concentrations (9.57–19.48 μg/L) of organophosphates. Cyclopoid copepod abundance declined at 7 d after malathion exposure and by 21 d after chlorpyrifos exposure. Our results are consistent with Smith et al. (2018), who observed that very high concentrations of malathion (250–1000 μg/L) decreased cyclopoid copepod abundance.

While we observed reduced cladoceran abundance following exposure to the high permethrin concentration on day 21, we did not observe any other direct toxic effects of pyrethroid insecticides on zooplankton abundance. Although pyrethroid insecticides are known to be extremely highly toxic to aquatic invertebrates (Anderson, 1989; Antwi and Reddy, 2015), exposure to 0.130–0.324 μg/L of cypermethrin and permethrin caused minimal changes in our zooplankton communities. Previous studies have documented exposure to cypermethrin concentrations as low as 0.13 μg/L over 11 days caused a rapid decrease in cladoceran and copepod zooplankton in small in-situ enclosures in an eutrophic lake (Friberg-Jensen et al., 2003). Under laboratory conditions, pyrethroid concentrations of 1 μg/L or greater significantly reduced cladoceran populations, while reduction in copepod and rotifer abundance occurred at higher concentrations (>10 μg/L; Day, 1989). Thus, our pyrethroid concentrations may have been too low to cause a discernible effect in our aquatic communities.

Similar to the temporal effect of pyrethroid exposure, zooplankton abundance was reduced later in the experiment following initial exposure to imidacloprid and thiamethoxam. Specifically, cladocerans abundance was significantly lower following exposure to low imidacloprid and high thiamethoxam concentrations. However, the median LC_{50} values for most cladoceran species (not including *Ceriodaphnia dubia* — LC_{50} = 2.07 μg/L imidacloprid) exposed to neonicotinoids are much higher than the concentrations used in our experiment (>572 μg/L; reviewed by Morrissey et al., 2015), suggesting our concentrations were too low to cause an immediate decline in abundance. This is consistent with our results at 7 d. Moreover, the decreased abundance among cladocerans on day 21 suggests lasting, toxic effects from neonicotinoid exposures on sensitive organisms not found using short-term, laboratory studies. In fact, the application of neonicotinoids in watersheds surrounding Lake Shinji, Japan resulted in low lake surface water concentrations (<0.01–0.07 μg/L), which coincided with an 83% decrease in zooplankton biomass (Yamamuro et al., 2019). It should be noted, however, that a similar mesocosm study indicated no effect of thiamethoxam (1, 3, 10, 30, and 100 μg/L) on zooplankton abundance across all 80 d of the experiment (Finnegan et al., 2018).

We observed that the reduction and sustained suppression of zooplankton abundance led to elevated phytoplankton biomass measured as Chl *a*. Whereas organophosphate exposure indirectly increased phytoplankton biomass over the duration of the experiment with reduced zooplankton, exposure to neonicotinoids or pyrethroids did not influence phytoplankton abundance with no decline in zooplankton abundance at day 7. This is consistent with the current literature suggesting a cascade of effects such that severely decreased zooplankton abundance results in phytoplankton blooms due to reduced grazing (Bendis and Relyea, 2016; Brogan and Relyea, 2015; Downing et al., 2008; Hanazato and Yasuno, 1987; Hua and Relyea, 2014). Following this cascade, with increased phytoplankton, DO increased (Fig. S2) due to greater phytoplankton photosynthesis. Had we extended the duration of our experiment, we might have observed a lag-effect in phytoplankton abundance following the decrease in zooplankton abundance in communities exposed to low imidacloprid and high thiamethoxam concentrations, respectively, on day 21.

The direct toxic effects of insecticide exposure can also manifest as sublethal effects on sensitive organisms. We observed a significant reduction in leopard frog tadpole mass following organophosphate exposure, but no effects of insecticide exposure on survival. Tadpole mass decreased by 30% on average when exposed to organophosphate insecticides. These results are consistent with previous studies documenting the decrease in tadpole mass following exposure to chlorpyrifos (Widder and Bidwell, 2006, 2008). For instance, Widder and Bidwell (2008) reported a 20–35% reduction in tadpole mass of three species of anurans (Cope's gay tree frog (*Hyla chrysoscelis*), southern leopard frog (*R. sphenocephala*), and western narrow-mouthed toad (*Gastrophryne olivacea*)) after 4 d of exposure to 200 μg/L of chlorpyrifos. The high survival observed among pesticide treatments in the current study is consistent with other studies showing that leopard frog tadpoles are highly tolerant to imidacloprid (8.5 mg/L; Moe, 2017), thiamethoxam (250 μg/L; Robinson et al., 2019), malathion (5.8–320 μg/L; Relyea, 2005; 2009), chlorpyrifos (3.2 μg/L; Relyea, 2009), and permethrin (50 μg/L; Hua and Relyea, 2019).

We did not find an effect of insecticides on tadpole movement. In contrast, previous studies have reported reduced tadpole movement following insecticide exposure (Holtswarth et al., 2019; Materna et al., 1995; Relyea and Edwards, 2010; Wrubleswski et al., 2018). Our results may be due to the time frame in which we recorded activity. Studies indicate tadpoles responding within minutes to insecticide exposure (Relyea and Edwards, 2010) and that behavioral effects occur only within the first 24 h of exposure to organophosphate insecticides (Grue et al., 1997; Richards and Kendall, 2003). However, we measured activity 12 d after initial exposure. Studies that looked at the effect of organophosphates multiple days after exposure were consistent with our results and found no effect on activity (Widder and Bidwell, 2006, 2008).

4.2. Interactive effects of insecticides and road salt

Given contact with pyrethroid insecticides causes paralysis in exposed organisms following the inactivation of nerve cell sodium channels (Brander et al., 2012; Burr and Ray, 2004; Werner and Moran, 2008), we expected exposure to pyrethroid insecticides to have a more detrimental effect on aquatic biota when combined with increasing road salt concentrations. Our results supported our hypothesis; pyrethroid exposure only reduced zooplankton abundance in high salt environments, while organophosphates interacted less with heightened salt concentrations. This is not unexpected, as organophosphates bind to acetylcholinesterase which is unassociated salt. The reduction in zooplankton abundance following pyrethroid exposure in high salt environments was accompanied by the indirect increase of phytoplankton abundance. Surprisingly, we also observed a late increase in phytoplankton abundance after pyrethroid exposure in intermediate salt environments, void of any change in zooplankton abundance, suggesting the combined effects of pyrethroids and road salts at sublethal concentrations might influence sublethal physiological or behavioral traits of foraging zooplankton. Alternatively, road salt my affect metabolic rate and possible energy balance as suggested by Arnér and Koivisto (1993). An increase in respiration rate independent of any other metabolic challenges may increase uptake of pesticide through the gills.Whereas insecticide exposure caused indirect algal blooms under low salt, under high salt, phytoplankton declined below what was found in the control treatment. Since zooplankton abundance decreased in all organophosphate treatments regardless of salt concentration, we would expect, according to the reduced grazing hypothesis, to see Chl *a* increase in all organophosphate treatments. However, Chl *a* only increased in the organophosphate treatments under low and medium salt. Under high salt, phytoplankton decreased in all malathion treatments. Similarly, under high salt, pyrethroid treatments reduced zooplankton abundance, but the community did not have the corresponding increase in phytoplankton. Instead, we observed a reduction in phytoplankton. This suggests that the interaction between the insecticides and high road salt represses phytoplankton, such that the limited grazing from decreased zooplankton populations does not have a substantial positive effect on phytoplankton biomass. To our knowledge, this study is unique in its finding, as only one other study has examined possible interactive effects between insecticides (carbaryl) and road salt, and they did not find a decrease in phytoplankton (Stoler et al., 2016).

Leopard frog tadpole survival and activity were not affected by insecticide or salt exposure alone; however, salt had an antagonistic effect with organophosphate treatments diminishing their reductive effect on mass. Under low salt, tadpole mass decreased by an average of 30% when exposed to organophosphates, while under medium and high salt none of the insecticides caused a decrease in mass. Road salts have been reported to increase tadpole growth and mass (Dananay et al., 2015; Petranka and Francis, 2013; Van Meter and Swan, 2014) while our first experiment indicated that insecticides, specifically organophosphates, decrease tadpole growth and mass. Consequently, the combined effect of road salt and organophosphates, as indicated in this second experiment, negates both stressors' individual impacts with a net effect of neither an increase nor decrease in tadpole mass.

5. Conclusions

This study highlights the importance of examining simultaneous exposure to multiple stressors in order to understand the potential additive, antagonistic, or synergistic effects on natural communities. The current study, to our knowledge, is the first study to demonstrate that the effects of pesticides can be modified on a community level by a simultaneous exposure to road salt. This discovery is especially important given that insecticides and road salts are both commonly applied in northern temperate latitudes (Main et al., 2016). Moreover, salinization of freshwater habitats is common in dry climates with irrigated crops, resulting in a combination of both stressors. Future studies should examine whether other aquatic organisms are similarly affected by the interaction of road salt and pesticides. Furthermore, other pesticide families may interact with road salt. If so, then it is important to know the concentrations and type of road salt (e.g., NaCl, $CaCl_2$, KCl, $MgCl_2$) and pesticides that matter, the underlying mechanisms that cause the interaction, and the short- and long-term impacts of prolonged exposure.

CRediT author statement

Jacquelyn L. Lewis: Formal analysis, Writing - original draft, Writing - review & editing, Visualization. Gabriela Agostini: Conceptualization, Methodology, Investigation, Writing - review & editing. Devin K. Jones: Conceptualization, Methodology, Investigation, Writing - review & editing. Rick A. Relyea: Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.116006.

Main findings

Insecticide exposure not only causes cascading effects in aquatic communities but can also interact with environmentally relevant concentrations of road salt to alter the abundance and body condition of sensitive, non-target organisms.

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