SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 9999, No. 9999, pp. 1–9, 2016

Printed in the USA

Environmental Toxicology

COMBINED EFFECTS OF ROAD SALT AND AN INSECTICIDE ON WETLAND COMMUNITIES

Aaron B. Stoler,* Brent M. Walker, William D. Hintz, Devin K. Jones, Lovisa Lind, Brian M. Mattes, Matthew S. Schuler, and Rick A. Relyea

Department of Biological Sciences, Rensselaer Polytechnic Institute, Troy, New York, USA

(Submitted 6 June 2016; Returned for Revision 12 September 2016; Accepted 2 October 2016)

Abstract: As the numbers of chemical contaminants in freshwater ecosystems increase, it is important to understand whether contaminants interact in ecologically important ways. The present study investigated the independent and interactive effects of 2 contaminants that frequently co-occur in freshwater environments among higher latitudes, including a commonly applied insecticide (carbaryl) and road salt (NaCl). The hypothesis was that the addition of either contaminant would result in a decline in zooplankton, an algal bloom, and the subsequent decline of both periphyton and periphyton consumers. Another hypothesis was that combining the contaminants would result in synergistic effects on community responses. Outdoor mesocosms were used with communities that included phytoplankton, periphyton, zooplankton, amphipods, clams, snails, and tadpoles. Communities were exposed to 4 environmentally relevant concentrations of salt (27 mg $Cl^- L^{-1}$, 77 mg $Cl^- L^{-1}$, 277 mg $Cl^- L^{-1}$, and 727 mg $Cl^- L^{-1}$) fully crossed with 4 carbaryl treatments (ethanol, 0 $\mu g L^{-1}$, 5 $\mu g L^{-1}$, and 50 $\mu g L^{-1}$) over 57 d. Contaminants induced declines in rotifer and cladoceran zooplankton, but only carbaryl induced an algal bloom. Consumers exhibited both positive and negative responses to contaminants, which were likely the result of both indirect community interactions and direct toxicity. In contrast to the hypothesis, no synergistic effects were found, although copepod densities declined when high concentrations of both chemicals were combined. The results suggest that low concentrations of salt and carbaryl are likely to have mostly independent effects on aquatic communities. *Environ Toxicol Chem* 2016;9999:1–9. © 2016 SETAC

Keywords: Aquatic invertebrates Deicer Ecotoxicology *Lithobates clamitans* Mixtures

INTRODUCTION

Chemical contamination can trigger changes in ecological communities that alter species composition and ecosystem stability [1]. To understand and mitigate the impacts of chemical contamination, traditional approaches rely on examining the effects of individual chemicals on test organisms during short-duration laboratory studies (e.g., median lethal concentration [LC50] tests; [2]). However, the effects of individual chemicals can strongly depend on community interactions [3]. For example, predation and competition generate additional stressors that can decrease the tolerance of biota to chemical contaminants [4]. In addition, the effects of individual chemical stressors are often mediated by interactions with other chemical contaminants that can induce synergistic effects on exposed organisms [3]. Realistic management strategies must be informed by studies that explore the interactions between chemical contaminants in varying community contexts.

Among ecosystems that commonly experience chemical contamination, freshwater environments are particularly vulnerable because they are often in close proximity to areas where chemicals are released [3]. For example, more than 450 million kg of pesticides are used in the United States annually [5], and many of these chemicals find their way to freshwater systems through aerial drift, overspray, and runoff [6]. Surveys reveal that up to 95% of freshwater streams and many associated groundwater resources are contaminated with at least 1 pesticide [7]. The ecological effect of pesticides depends on community structure and the range of sensitivities among community members [3,8]. For example, several species of zooplankton are far more sensitive to low concentrations of insecticides than are larger, benthic grazers (e.g., tadpoles [9]). However, zooplankton mortality can also lead to dramatic algal blooms that shade the benthos, resulting in the decline of benthic algal resources (i.e., periphyton) and grazer survival [10].

Such community interactions are equally important for understanding the effects of contaminants that frequently co-occur with pesticides in aquatic systems. For example, the application of salt as a deicing agent has dramatically increased in developed and developing areas over the past several decades, particularly in northern latitudes. More than 22 million metric tons of salt are currently applied on roadways annually, most commonly in the form of sodium chloride (NaCl) [11]. Spring snowmelt and storm water runoff can lead to an accumulation of chloride in ponds and wetlands throughout the growing season [12]. Indeed, past studies have observed chloride concentrations up to 500 mg Cl^- by the end of the growing season [13,14], and temporary chloride concentrations up to 5000 mg L^{-1} following severe runoff events [12]. Short-term (1–7 d) laboratory tests suggest that aquatic taxa greatly vary in their tolerance to chloride contamination but are generally tolerant to concentrations below 1000 mg $Cl^- L^{-1}$, with the exception of some sensitive zooplankton species [12,15,16]. Such variations can have important, long-term effects in ecological communities. For example, both Van Meter et al. [17] and Dananay et al. [18] demonstrated that increasing chloride concentrations up to 900 mg L^{-1} leads to the mortality of salt-sensitive zooplankton species and to an increase in phytoplankton density.

Because the interaction between chemical contaminants can result in synergistic effects [3], we examined the independent and interactive effects of NaCl and a common insecticide, carbaryl, on wetland communities. We hypothesized that the addition of either contaminant would result in a decline of

* Address correspondence to abstoler@gmail.com

Published online 24 October 2016 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/etc.3639

zooplankton abundance, a subsequent increase in phytoplankton density, and a decrease in periphtyon biomass as a result of phytoplankton shading. We predicted that such a trophic cascade would also result in a decline in benthic grazer growth and survival because of a reduction in food resources. We further hypothesized that synergistic effects between salt and carbaryl would occur whereby the combination of the 2 contaminants would lead to negative synergistic effects on zooplankton survival and grazer growth and survival. We tested our hypotheses in an outdoor mesocosm experiment with a full-factorial combination of 3 carbaryl concentrations and 4 chloride concentrations.

METHODS

Pesticide background

Carbaryl (CAS no. 63-25-2; Sigma-Aldrich) is a broad-spectrum insecticide that works by inhibiting acetylcholinesterase [19]. This mode of action is similar to many other carbamate insecticides, and past studies have found that the effects of carbaryl are generalizable to many other pesticides [20]. It is estimated that 1.8 million to 2.7 million kilograms of carbaryl are applied annually in the United States to control a variety of agricultural pests [5]. Although laboratory studies indicate that carbaryl has a relatively short half-life (12 d) in a neutral aquatic environment [19], several studies have indicated that carbaryl can be highly toxic to nontarget organisms [19]. Moreover, sublethal effects might occur through indirect interactions such as trophic cascades [21].

Experimental design and setup

We conducted our experiment during summer 2015 at the Rensselaer Aquatic Lab (Troy, NY, USA). Our experiment consisted of a full-factorial design with 4 salt treatments (0 mg $Cl^- L^{-1}$, 50 mg $Cl^- L^{-1}$, 250 mg $Cl^- L^{-1}$, and 700 mg $Cl^- L^{-1}$) crossed with 4 insecticide treatments (0 μg carbaryl L^{-1}, 5 μg carbaryl L^{-1}, and 50 μg carbaryl L^{-1}, and an ethanol control). We added salt to the ambient chloride concentration in our water supply, which was 27 mg $Cl^- L^{-1}$, to achieve nominal concentrations of 27 mg $Cl^- L^{-1}$, 77 mg $Cl^- L^{-1}$, 277 mg $Cl^- L^{-1}$, and 727 mg $Cl^- L^{-1}$. We selected salt and pesticide concentrations that are representative of concentrations measured in freshwater ecosystems [12,22,23]. We replicated each of the 16 treatment combinations 4 times for a total of 64 experimental units. Our experimental units consisted of 100-L plastic wading pools.

We filled mesocosms with aged tap water on 8 July. We placed a 60% shade cloth cover on each pool to simulate moderate canopy cover and to prevent oviposition of insects and frogs. On 13 July, we added 5 g of rabbit chow (Blue Bunny 16; Kent Nutrition Group) to each mesocosm to provide an initial source of organic nutrients that would normally be present in a natural wetland [24]. On the same day, we added 100 g of air-dried black oak (*Quercus velutina*) litter collected in the spring. This amount of litter mimics natural densities of litter inputs and is common in mesocosm studies [25]. To generate microbial, algal, and zooplankton communities, we added 0.34 L of homogenized water collected from 2 local wetlands. To provide a substrate to sample macroinvertebrate grazers, we added 2 coarse-mesh bags (10-mm opening) containing 5 g of litter to each pool. The mesh size of the bag allowed entry by all consumers except for late-stage tadpoles. To measure periphyton biomass, we placed a single ceramic tile (surface area = 112.5 cm^2) on the east-facing side of each pool.

After allowing microbes, algae, and zooplankton communities to develop for 2 wk, we established communities of macroconsumers found in temperate wetlands [26]. We collected all organisms from nearby wetlands immediately adjacent to roadways (<2 m). To avoid the potential introduction of parasites typically associated with freshwater snails, we added 20 pouch snails (*Physa acuta*) as hatchlings collected from a culture of 100 adults maintained in aged tap water for 2 mo. We also added 15 amphipods (*Hyallela azteca*) and 8 fingernail freshwater clams (*Musculium transversum*) of similar size. Finally, we added 10 green frog tadpoles (*Lithobates clamitans*) to each mesocosm. We collected green frogs as 3 newly laid egg masses from a single wetland and reared them under common garden conditions in outdoor culture pools filled with aged tap water. We fed tadpoles rabbit chow ad libitum and added them to mesocosms once they reached a safe handling stage (Gosner stage 25 [27]; 0.017 ± 0.001 g). We set aside 20 tadpoles to assess 24-h survival as a result of handling stress, which was 100%.

Following the addition of all organisms to our mesocosms, we applied our carbaryl and salt treatments on 22 and 23 July, respectively. We first dissolved technical grade carbaryl in ethanol to create a working solution, dosed the appropriate amounts of this working solution into 500 mL of water from each mesocosm, and then distributed the solution across the surface of the respective mesocosms. For salt additions, we used 100% NaCl rock salt (Ice-a-Away Rock Salt Ice Melter®; Compass Minerals) that was first dissolved in 1 L of water taken from each mesocosm and then redistributed across the surface of the mesocosm. After the addition of salt and pesticides, we used a plastic cup to gently push water around the mesocosm and homogenize the concentration of solute. For mesocosms that did not receive any salt or carbaryl additions, we removed and poured 1 L of water across the surface of the mesocosm and also pushed water around the tank to equalize disturbance between treatments. The day of salt addition is termed day 0 of the experiment.

To ensure that our chemical additions achieved nominal concentrations, we measured both carbaryl and salt concentrations. Immediately after carbaryl additions on day 0, we took 60 mL of water from each carbaryl treatment, pooled and homogenized all samples, preserved 500 mL with 2 mL methylene chloride, and tested them for actual pesticide concentration via gas chromatography-mass spectrometry (Center for Environmental Sciences and Engineering, University of Connecticut, Storrs, CT, USA). Actual carbaryl concentrations were 0 μg L^{-1}, 6.3 μg L^{-1}, and 56.3 μg L^{-1} in our nominal treatments of 0 μg L^{-1}, 5 μg L^{-1}, and 50 μg L^{-1} of carbaryl, respectively. We did not detect carbaryl in our ethanol vehicle treatment. On day 29, we measured chloride concentrations with a hand-held meter (YSI). Actual chloride concentrations were 27 μg $Cl^- L^{-1}$, 84 μg $Cl^- L^{-1}$, 279 μg $Cl^- L^{-1}$, and 746 mg $Cl^- L^{-1}$ in the 27 mg $Cl^- L^{-1}$, 77 mg $Cl^- L^{-1}$, 277 mg $Cl^- L^{-1}$, and 727 mg $Cl^- L^{-1}$ treatments, respectively. Because actual chloride and carbaryl concentrations were within 10% of nominal concentrations, we report all results using nominal values.

Abiotic measurements

On day 29, we measured the pH, dissolved oxygen, and temperature in each mesocosm. We conducted these measurements with a hand-held multimeter (YSI).

Biotic measurements

Throughout the study, we collected measurements on algal and microbial resources, as well as on consumer responses. On

days 11 and 27, we measured phytoplankton as the concentration of chlorophyll *a* in the water column using the fluorometric method of Arar and Collins [28] without acid correction. On day 20, we measured periphyton as the oven-dried biomass of attached material brushed from the clay tile. On days 4 and 19, we collected and preserved zooplankton samples to enumerate the densities of rotifers, copepods, and cladocerans. In ecotoxicological studies, zooplankton are frequently grouped by these larger taxonomic groups because species within each group respond similarly to insecticides [29]. To measure amphipod and snail abundance, we preserved and enumerated all organisms that were rinsed from leaf packs into a 250-μm sieve on days 28 and 56. At the conclusion of our study (day 57), we removed all remaining leaf litter (i.e., litter not placed into bags) and collected clams and tadpoles using a 0.5-mm mesh aquarium net. Because some clams might attach to leaf litter, we visually inspected all leaves for attached organisms before removing them. After preserving all individuals in 70% ethanol, we assessed the abundance and oven-dried biomass of clams. We also assessed the survival and average individual blot-dry mass of tadpoles in each mesocosm. We did not measure amphipod or snail biomass because oven-dried mass values were often very close to or below the detection limits of our balance. All methods were performed in concordance with the Rensselaer Polytechnic Institute Institutional Review Board and with Institutional Animal Care and Use Committee protocol REL-001-15.

Statistics

Abiotic responses. We used analysis of variance (ANOVA) to assess the effects of carbaryl and salt on abiotic responses. We first assessed abiotic responses (pH, dissolved oxygen, temperature, and decay rate) with a multivariate ANOVA (MANOVA). After finding a significant multivariate effect, we conducted univariate ANOVAs. We used Tukey's honest significant difference (HSD) post hoc comparison tests to determine significant treatment differences.

Biotic responses. We also employed ANOVA to assess the effects of salt and pesticides on all community responses. Because we measured phytoplankton concentrations on 2 sample dates, we employed repeated-measures ANOVA. In a similar manner, we assessed the effects of time and treatments on snail and amphipod abundances. If we found a significant interaction between treatment and time, we conducted univariate analyses within sample date and employed Tukey's HSD post hoc comparison tests to determine significant treatment differences.

To analyze zooplankton responses, we first conducted a repeated-measures MANOVA (rm-MANOVA) on copepod, cladoceran, and rotifer densities. After finding a significant multivariate effect, we conducted univariate rm-ANOVAs on individual zooplankton groups, and conducted univariate analyses within sample date if there was an interaction between treatment and time. We employed Tukey's HSD post hoc comparison tests to determine significant treatment differences.

We quantified the remaining community responses only once (periphyton biomass, clam biomass, and average individual tadpole mass), so we analyzed these responses with univariate ANOVAs. We employed Tukey's HSD post hoc comparison tests to determine significant treatment differences.

When necessary, we log- or square root-transformed the data to fit parametric assumptions of ANOVA. We rank-transformed amphibian survival data. We verified normality of individual responses by assessing the linearity of residual Q-Q plots. When required by the analysis, we verified multivariate normality of response sets by examining the scatterplot of χ^2-values with squared Mahalanobis distances, and assuming normality if the line was reasonably straight. We removed a single replicate (27 mg $Cl^- L^{-1}$/5 μg carbaryl L^{-1}) from all analyses because of a leak in the mesocosm. We also removed 4 replicates of periphyton biomass (2 replicates of 27 mg $Cl^- L^{-1}$/50 μg carbaryl L^{-1}, 1 replicate of 277 mg $Cl^- L^{-1}$/ethanol, and 1 replicate of 277 mg $Cl^- L^{-1}$/50 μg carbaryl L^{-1}), and a single replicate of zooplankton (277 mg $Cl^- L^{-1}$/5 μg carbaryl L^{-1}) because of mistakes in the processing of samples. We accounted for the loss of replicates in our statistics. We analyzed all data in R (version 3.1.2) using the packages *MVN*, *vegan*, *car*, and *agricolae* [30–33].

RESULTS

Throughout the following discussion of the results, we omit mention of treatment comparisons with the ethanol controls because the results of these treatments never differed from treatments with 0 μg carbaryl L^{-1}. However, the values for all statistical tests account for the inclusion of the ethanol control.

Abiotic measurements

We found an effect of salt on pH but found no other abiotic response (i.e., dissolved oxygen, temperature, and leaf litter decay rate) and no effect of carbaryl or a salt × carbaryl interaction (measured on day 6; Table 1). Univariate analyses revealed a strong effect of salt on pH ($F_{3,47} = 8.2$, $p < 0.001$); pH decreased from 7.9 to 7.6 between chloride

Table 1. Analysis of variance results for abiotic responses, phytoplankton concentration, and periphyton biomass

	Abiotic (pH, DO, temperature, and decay rate)[a]		Phytoplankton concentration[b]		Periphyton biomass	
Factor	F[c]	*p*	F[c]	*p*	F[c]	*p*
Salt	$3.4_{12,114}$	<0.001	$4.4_{3,47}$	0.008	$0.1_{3,43}$	0.942
Carbaryl	$0.9_{12,114}$	0.543	$9.7_{3,47}$	<0.001	$2.2_{3,43}$	0.100
Salt × carbaryl	$1.2_{36,163}$	0.201	$0.7_{9,47}$	0.672	$0.6_{9,43}$	0.749
Time			$161.6_{1,47}$	<0.001		
Time × salt			$1.2_{3,47}$	0.315		
Time × carbaryl			$4.4_{3,47}$	0.008		
Time × salt × carbaryl			$1.0_{9,47}$	0.466		

[a]Values represent statistics for a multivariate analysis of variance on pH, dissolved oxygen (DO), temperature, and decay rate.
[b]Values represent statistics for a repeated-measures analysis of variance on measurements over 2 sample dates.
[c]Subscripts of F values represent treatment and error degrees of freedom.

concentrations of 27 mg L^{-1} and 727 mg L^{-1}. We did not find any univariate effects on dissolved oxygen, temperature, or litter decay rate ($p \geq 0.092$).

Biotic measurements

Phytoplankton and periphyton. For phytoplankton density, we detected effects of salt, carbaryl, and a carbaryl × time interaction (Figure 1 and Table 1). Phytoplankton density was 54% higher, with 727 mg Cl^- L^{-1} relative to 277 mg Cl^- L^{-1} ($p = 0.006$), but not significantly higher than 77 mg Cl^- L^{-1} or 27 mg Cl^- L^{-1} treatments ($p \geq 0.245$). We detected effects of carbaryl on day 11 ($F_{3,47} = 4.9$, $p = 0.004$) and day 27 ($F_{3,47} = 7.9$, $p < 0.001$). On day 11, phytoplankton density was 55% higher, with 50 µg carbaryl L^{-1} relative to 0 µg L^{-1} ($p = 0.002$). On day 27, phytoplankton density was 61% to 182% higher, with 50 µg carbaryl L^{-1} relative to all other treatments ($p \leq 0.006$). We did not detect any significant effects of salt or carbaryl on periphyton biomass (Table 1).

Zooplankton. We detected effects of salt, carbaryl, and time on zooplankton densities, as well as several interactions (Figure 2 and Table 2). For cladocerans, we detected univariate effects of salt (Table 2), which was generated by 38% to 51% lower cladoceran densities with 727 mg Cl^- L^{-1} relative to all other salt concentrations (averaged across both sample dates; $p \leq 0.039$). We also found effects of carbaryl and a carbaryl × time interaction (Table 2). On the first sample date (day 4; $F_{3,46} = 47.3$, $p < 0.001$), cladoceran densities were 93% to 99% lower in the 0 µg L^{-1} carbaryl treatments relative to the 5 mg L^{-1} and 50 µg L^{-1} carbaryl treatments, respectively ($p < 0.001$). On the second sample date (day 19; $F_{3,46} = 24.5$, $p < 0.001$), we found 98% to 99% lower densities in 50 µg L^{-1} carbaryl treatments relative to all other treatments ($p < 0.001$).

For rotifers, we found significant effects of salt, time, salt × time, and carbaryl × time (Table 2). On day 4, we detected an effect of salt on rotifer density ($F_{3,46} = 9.0$, $p < 0.001$). Specifically, 727 mg Cl^- L^{-1} reduced rotifer densities by 44% and 49% relative to the 77 mg Cl^- L^{-1} and 27 mg Cl^- L^{-1} treatments, respectively. On day 19, we detected a nearly significant effect of salt ($F_{3,46} = 2.7$, $p = 0.055$) but no significant post hoc treatment comparisons. We did not detect a significant effect of carbaryl on either sample date ($p \geq 0.200$).

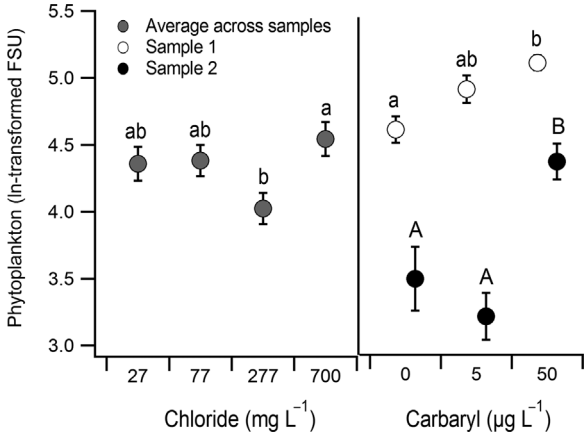

Figure 1. Effects of salt and carbaryl on phytoplankton densities among chloride and carbaryl treatments. Values are log-transformed relative fluorescence units (FSUs). Because there was no interaction between time and salt treatment, averaged responses across sample dates are presented. Letters above points denote significant differences within treatment type and within sample date. Bars are ± 1 standard error.

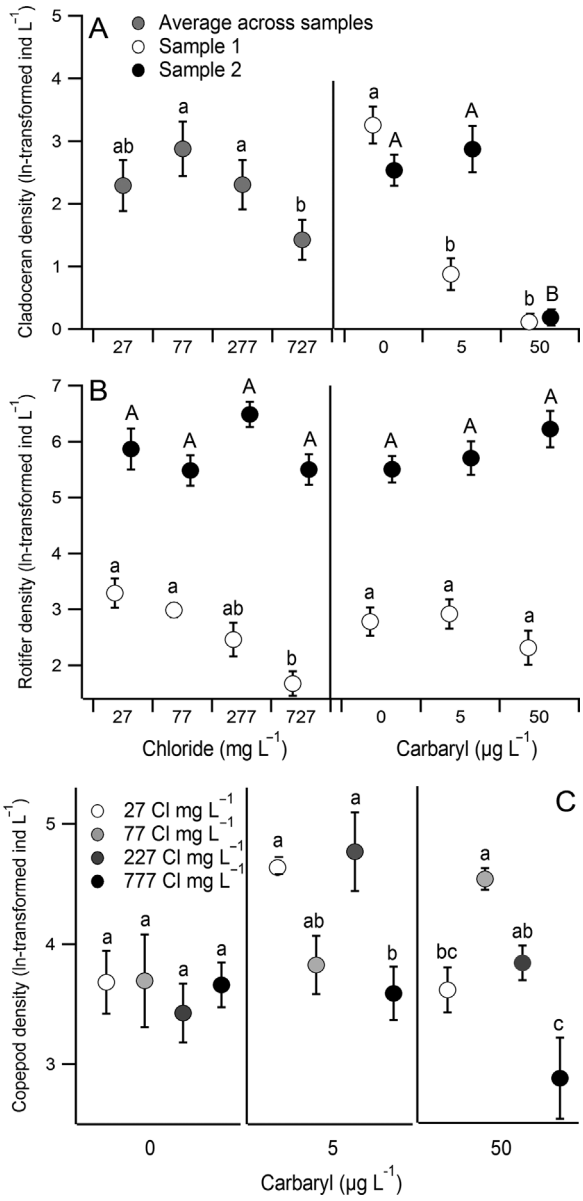

Figure 2. Effects of salt and carbaryl on densities of cladocerans (**A**) and rotifers (**B**) with values that represent averaged responses across sample dates in the absence of a treatment × time interaction or average treatment means within sample date when there was an interaction. For copepod densities (**C**), we present only data for the second sample date. Because of the presence of a significant salt × carbaryl interaction, we present values for salt within carbaryl treatments. Letters above points denote significant differences within treatment type and within sample date (when provided). For copepod densities (**C**), letters indicate significant differences within carbaryl treatment. Bars are ± 1 standard error.

For copepods, we detected effects of salt, time, and a 3-way interaction with salt, carbaryl, and time (Table 2). Although no terms were significant on day 4 ($p \geq 0.420$), all 3 terms were significant on day 19 (salt: $F_{3,46} = 2.9$, $p = 0.013$; carbaryl: $F_{3,46} = 3.3$, $p = 0.02$; salt × carbaryl: $F_{9,46} = 3.0$, $p = 0.007$). On this date, we detected differences in copepod densities among salt treatments, but only when carbaryl was present. At 5 µg carbaryl L^{-1}, 727 mg Cl^- L^{-1} induced 63% and 70% lower densities relative to 27 mg Cl^- L^{-1} and 277 mg Cl^- L^{-1} treatments, respectively ($p \leq 0.045$). Similarly, 50 µg carbaryl L^{-1} combined with 727 mg Cl^- L^{-1} induced 77% and 55% lower densities relative to the 77 mg and 277 Cl^- L^{-1} treatments,

Table 2. Multivariate and univariate results for analyses on zooplankton densities

	MANOVA		Cladocerans		Rotifers		Copepods	
Factor	F[a]	*p*	F[a]	*p*	F[a]	*p*	F[a]	*p*
Salt	$22.9_{3,46}$	<0.001	$9.0_{3,46}$	$3.1_{3,46}$	0.038	<0.001	$4.9_{3,46}$	0.005
Carbaryl	$16.6_{3,46}$	<0.001	$52.0_{3,46}$	$2.1_{3,46}$	0.118	<0.001	$0.3_{3,46}$	0.832
Salt × carbaryl	$1.1_{9,46}$	0.412	$1.6_{9,46}$	$1.8_{9,46}$	0.104	0.148	$1.1_{9,46}$	0.391
Time	$395.4_{1,46}$	<0.001	$<0.1_{1,46}$	$4.9_{1,46}$	0.032	0.859	$388.7_{1,46}$	<0.001
Time × salt	$2.5_{3,46}$	0.068	$0.2_{3,46}$	$2.3_{3,46}$	0.094	0.862	$6.2_{3,46}$	0.001*
Time × carbaryl	$8.3_{3,46}$	<0.001	$15.2_{3,46}$	$2.3_{3,46}$	0.093	<0.001	$3.0_{3,46}$	0.042
Time × salt × carbaryl	$2.1_{9,46}$	0.047	$1.3_{9,46}$	$2.3_{9,46}$	0.029	0.273	$1.0_{9,46}$	0.429

[a]Subscripts of F values represent treatment and error degrees of freedom.
*Not significant at the multivariate level.
MANOVA = multivariate analysis of variance.

respectively ($p \leq 0.033$), and 27 mg $Cl^- L^{-1}$ induced 58% lower densities relative to the 77 mg $Cl^- L^{-1}$ treatment ($p = 0.040$).

Amphipods. We found a significant effect of carbaryl on amphipod abundance, but no effect of salt, time, or any interaction (Figure 3A and Table 3). Amphipod abundance was 73% lower in 50 μg carbaryl L^{-1} treatments relative to 5 μg L^{-1} treatments (averaged across sample dates; $p \leq 0.049$), but not relative to the 0 μg L^{-1} control ($p = 0.118$).

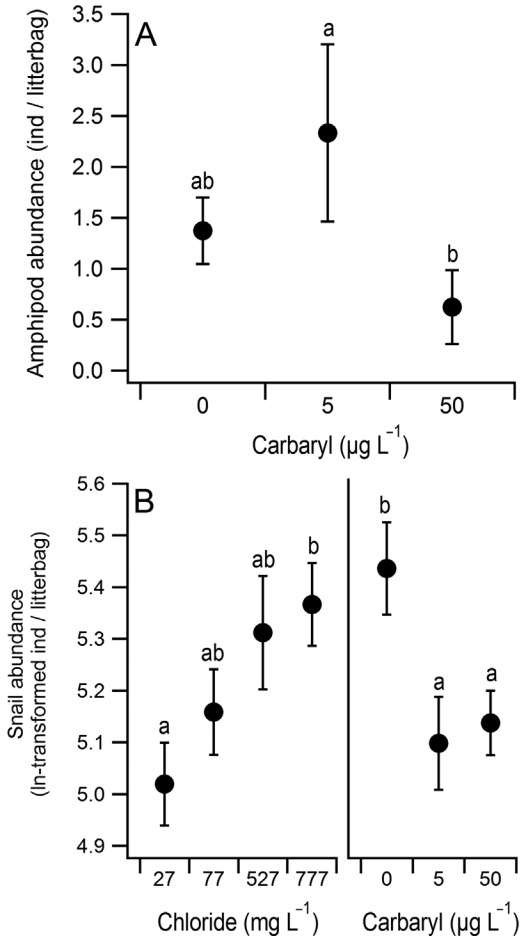

Figure 3. Effects of salt and carbaryl on the abundance of amphipods (**A**) and snails (**B**) in litter bags. There was no effect of chloride concentrations on amphipods, so these values are not shown. Values represent averaged responses across sample dates. Letters above points denote significant differences within treatment type. Bars are ± 1 standard error.

Snails. We found significant effects of salt, carbaryl, and time on snail abundance, but no interactions (Figure 3B and Table 3). Snail abundance was 42% higher with 727 mg $Cl^- L^{-1}$ relative to 27 mg $Cl^- L^{-1}$ (averaged across sample dates; $p = 0.022$) and 40% higher with 0 μg carbaryl L^{-1} relative to 5 μg carbaryl L^{-1} ($p = 0.022$). We also found 38% higher snail abundance with 50 μg carbaryl L^{-1} relative to 0 μg carbaryl L^{-1} ($p = 0.060$). Between sample dates, we recorded a 22% increase in snail abundance across all treatments.

Clams. We found significant effects of salt and carbaryl on clam responses, but no effect of their interaction (Figure 4 and Table 3; measured on day 57). For both abundance and biomass, we found univariate effects of salt (abundance: $F_{3,47} = 7.4$, $p < 0.001$; biomass: $F_{3,47} = 4.1$, $p = 0.012$) and carbaryl (abundance: $F_{3,47} = 10.3$, $p < 0.001$; biomass: $F_{3,47} = 7.6$, $p < 0.001$). We found 61% to 68% lower abundance in 700 mg $Cl^- L^{-1}$ treatments relative to all other chloride treatments ($p \leq 0.016$), as well as 59% and 71% lower biomass in 700 mg $Cl^- L^{-1}$ treatments relative to 50 mg $Cl^- L^{-1}$ and 250 mg $Cl^- L^{-1}$ treatments, respectively ($p \leq 0.017$), but not relative to 0 mg Cl L^{-1} ($p = 0.353$). We also detected 103% greater clam biomass in 250 mg Cl L^{-1} treatments relative to 0 mg $Cl^- L^{-1}$ treatments ($p = 0.003$). Regarding the effects of carbaryl, we found 44% lower abundance in 50 μg L^{-1} treatments relative to 5 μg L^{-1} treatments ($p = 0.026$), but not relative to 0 μg L^{-1} controls ($p = 0.784$). We also found 97% and 174% higher biomass in 5 μg L^{-1} treatments relative to 0 and 50 μg L^{-1} treatments, respectively ($p \leq 0.050$).

Amphibians. We found a significant effect of carbaryl on green frog tadpole responses, but no effect of salt or their interaction (Figure 5 and Table 3). For average individual mass, we found an effect of carbaryl ($F_{3,47} = 3.2$, $p = 0.032$); pairwise comparisons revealed 37% greater individual mass in 50 μg carbaryl L^{-1} treatments relative to 5 μg L^{-1} treatments ($p = 0.024$), but not relative to 0 μg L^{-1} controls ($p = 0.363$). We found no effect of carbaryl on tadpole survival ($F_{3,47} = 1.8$, $p = 0.165$), which averaged 99% across all treatments.

DISCUSSION

The present study is the first to examine the combined effects of environmentally relevant concentrations of carbaryl and NaCl on wetland communities. Moreover, our study adds to the growing body of evidence regarding interactions among chemical contaminants [34]. As expected, we found that carbaryl and road salt induced declines in some groups of zooplankton, although this resulted in an algal bloom only in the presence of carbaryl. We also found significant positive effects

Table 3. Analysis of variance results for amphipod abundance, snail abundance, clams, and tadpole individual mass

	Amphipod abundance		Snail abundance		Clams (biomass and abundance)[a]		Tadpoles (survival and individual mass)[b]	
Factor	F[c]	*p*	F[c]	*p*	F[c]	*p*	F[c]	*p*
Salt	$0.3_{3,47}$	0.792	$3.0_{3,47}$	0.041	$6.9_{6,92}$	<0.001	$0.8_{6,92}$	0.606
Carbaryl	$4.0_{3,47}$	0.014	$3.1_{3,47}$	0.034	$4.3_{6,92}$	0.001	$2.5_{6,92}$	0.026
Salt × carbaryl	$1.3_{9,47}$	0.268	$1.6_{9,47}$	0.155	$1.6_{18,92}$	0.086	$1.0_{18,92}$	0.490
Time	$0.5_{1,47}$	0.466	$5.5_{1,47}$	0.023				
Time × salt	$1.7_{3,47}$	0.179	$0.6_{3,47}$	0.592				
Time × carbaryl	$0.6_{3,47}$	0.590	$1.4_{3,47}$	0.264				
Time × salt × carbaryl	$0.9_{9,47}$	0.529	$0.6_{9,47}$	0.754				

[a]Values represent statistics for a multivariate analysis of variance on biomass and abundance.
[b]Values represent statistics for a multivariate analysis of variance on survival and individual mass.
[c]Subscripts of F values represent treatment and error degrees of freedom.

of road salt on snails and clams, as well as a positive effect of carbaryl on tadpoles. In contrast to our hypothesis of synergistic effects, we found that the effects of both contaminants were largely independent for measured responses, although we did find an interactive effect of salt and carbaryl on copepod densities.

Effects of carbaryl

As expected from previous studies with carbaryl and other insecticides [10,21,34–37], the presence of carbaryl induced a decline in large-bodied cladocerans and a subsequent increase in phytoplankton. Large-bodied cladocerans are sensitive to carbaryl at concentrations $<10\ \mu g\ L^{-1}$ (median effective concentrations for *Daphnia pulex* and *D. magna* range from $5.6\ \mu g\ L^{-1}$ to $10.1\ \mu g\ L^{-1}$[19]), and their decline typically results in an increase of phytoplankton resources [38]. Although a decline in cladocerans can lead to an increase in rotifers, which are considered to be poor competitors with cladocerans [39], we did not observe any change in rotifer densities among carbaryl treatments. It is possible that sublethal effects of carbaryl induced lower rotifer reproduction. Although rotifers generally exhibit the highest tolerance to insecticides of all zooplankton groups [39], other studies have found reduced reproductive and population growth rates of rotifers at increasing insecticide concentrations [40].

Similar to the effects of carbaryl on cladocerans and phytoplankton, the effects of carbaryl on macroconsumers in our study were likely the result of both community interactions and toxic effects. For example, we found that filter-feeding clams increased in abundance at a low concentration of carbaryl but decreased at a high concentration. This pattern suggests that the clams benefitted from the increase in phytoplankton density at a low carbaryl concentration, but suffered from direct toxicity at a higher concentration. To our knowledge, there are no studies that document the sensitivity of *M. transversum* to insecticides, and future work should verify whether the observed decline was an effect of direct toxicity.

As phytoplankton blooms, we expected to observe a decline in periphyton resources and a parallel decline in periphyton grazers. In contrast, we did not observe any variation in periphyton standing crop. Although we did find a decline in

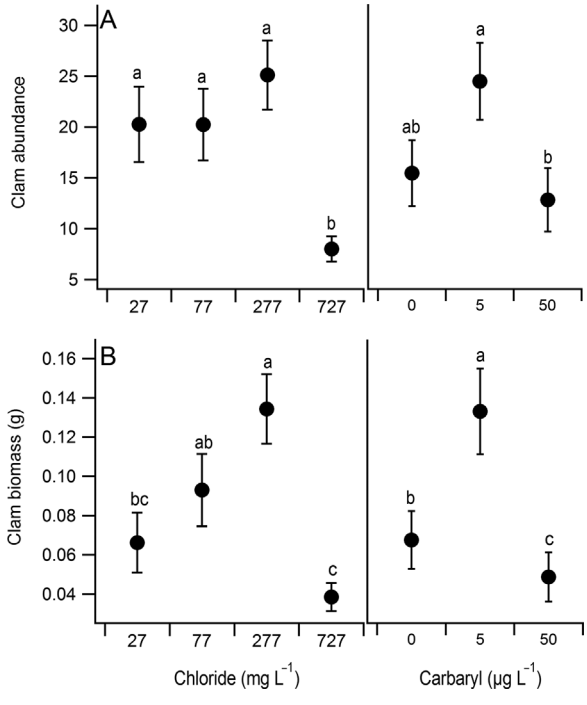

Figure 4. Effects of salt and carbaryl on the abundance (**A**) and biomass (**B**) of fingernail clams collected at the end of the experiment. Letters above points denote significant differences within treatment type. Bars are ± 1 standard error.

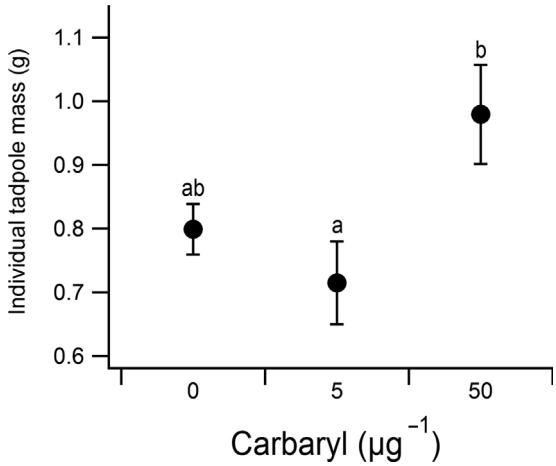

Figure 5. Effect of carbaryl on average individual mass of tadpoles at the end of the experiment. Letters above points denote significant differences within treatment type. Bars are ± 1 standard error.

amphipod abundance at 50 μg carbaryl L^{-1}, this was not likely a consequence of declining periphyton productivity but rather because amphipods cannot tolerate carbaryl levels greater than 15 μg L^{-1} [19]. Moreover, we found that 5 μg carbaryl L^{-1} increased amphipod abundance. A possible explanation for this is that there were changes in the nutritional value of periphyton. In combination with endosulfan insecticide, cyanobacteria exhibit reduced growth, photosynthetic pigments, and carbohydrates [41]. This reasoning might also explain why we observed a reduction in snail abundance, even though *P. acuta* is known to tolerate carbaryl concentrations up to 27 000 μg L^{-1} [19]. Although a reduction in periphyton resources should also have an impact on tadpole grazers, we actually observed an increase in tadpole mass. However, evidence suggests that green frog tadpoles can filter-feed large-bodied phytoplankton in addition to grazing periphyton [42]. Future studies should explicitly examine the effects of carbaryl on the composition and stoichiometry of algal and microbial resources, and their interaction with the breadth of consumer diets.

Effects of salt

The magnitude of negative effects from chloride was less than the effects of carbaryl, and effects were actually positive for some consumers. Among zooplankton, we found that densities of both rotifers and cladocerans declined with increasing chloride concentration, which is similar to the findings of Van Meter et al. [17]. Although a decrease in zooplankton densities is likely to result in a phytoplankton bloom, we found no evidence for an increase in phytoplankton but actually detected a decrease in phytoplankton densities at 277 mg Cl^- L^{-1}. This contrasts with the study of Van Meter et al. [17], which notes an increase in phytoplankton between 0 mg Cl^- L^{-1} and 645 mg Cl^- L^{-1}. However, their study did not include any filter feeders other than zooplankton, whereas the present study included freshwater clams. Indeed, we found a substantial increase in clam biomass at 277 mg Cl^- L^{-1}, which would account for the slight decrease in phytoplankton. However, we found a decline in clam abundance and biomass at the highest salt concentration. This decline is somewhat surprising given that *M. transversum* has a reported 96-h LC50 value of 1930 mg Cl^- L^- [43]. However, studies have also reported population die-offs of *M. transversum* along the Mississippi River (USA) and suggest that the species is intolerant to many contaminants [44]. In contrast to clams, we observed a consistent increase in the abundance of *P. acuta* up to 700 mg Cl^- L^{-1}, which supports prior evidence that this species has a relatively high tolerance to salt pollution [45]. Similarly, we found no difference in the survival or mass of green frog tadpoles among chloride treatments. Karraker [46] also found no effect of chloride concentrations on green frog tadpoles up to 465 mg Cl^- L^{-1}, but did note a slight decrease in survival at 945 mg Cl^- L^{-1}. In comparative studies among several species of North American anurans, green frogs exhibit intermediate tolerances to elevated chloride levels, with LC50 values at approximately 3000 mg Cl^- L^{-1} [16]. Such interspecific variation in salt tolerance likely exists for most families of freshwater organisms, so mitigation strategies must be tailored to the tolerance of species comprising local communities.

Interactive effects of salt and carbaryl

Among all measured responses, the only part of our mesocosm communities that exhibited an interaction between salt and carbaryl inputs was copepod density. We found no difference in densities among salt concentrations with control and ethanol treatments. However, we found that densities tended to increase with lower concentrations of both carbaryl and chloride. This might be because of an increase in rotifer prey following the decline of cladocerans. In contrast, we detected substantial declines in copepod densities when the highest carbaryl and chloride concentrations were combined. These results suggest that moderate levels of both chloride and carbaryl additions might increase copepod reproduction until a threshold of contamination is reached. Previous studies have found that copepods are tolerant to the highest level of carbaryl in the present study [47]. Salt tolerance varies substantially among copepod species [15], but previous research shows a decline in density at concentrations of 645 mg Cl^- L^{-1} [17]. The lack of copepod declines among salt treatments within control and low carbaryl treatments suggests that our species (primarily *Skistodiaptomus* spp. and *Microcyclops* spp.) might be more tolerant than those present in Van Meter et al. [17]. However, the decline of copepod densities in the high contamination treatment indicates that individuals were nearing their tolerance threshold, and that higher concentrations of either contaminant might generate further adverse effects.

Higher concentrations of chloride might also alter the effects of insecticide contamination as a result of changing abiotic conditions. We found that chloride reduced pH levels by 0.3 units between the control and highest concentration. This is likely because of the formation of hydrochloric acid following the dissolution of NaCl in the water column. Although the range of pH is not very large, the breakdown rate of carbaryl in aquatic systems is greatly reduced at pH levels below 7 [19]. For this reason, it is possible that further elevated chloride concentrations might extend the life span of carbaryl and result in more substantial effects. However, it is worth noting that Karraker et al. [47] found higher pH in roadside ponds contaminated with 91 mg Cl^- L^{-1} to 250 mg Cl^- L^{-1} salt relative to forest ponds without chloride contamination. Because their study was a survey of natural ponds, it is unclear whether this change is because of the effects of salt inputs or other factors (e.g., external subsidies). Hence, it is possible that road salt contamination might alleviate or exacerbate the effect of pesticides at higher chloride concentrations, and the effects of salt on pH warrant further research.

CONCLUSIONS

Although we did find an interactive effect of carbaryl and salt on copepod density, most other components of the community exhibited independent responses to either contaminant. Given that this effect only occurred by combining the highest concentrations of salt and carbaryl, our results suggest that additional interactive effects might occur at even higher concentrations of road salt and carbaryl. It is important to note that the phenomenon observed in our mesocosm communities might be substantially altered by the effects of other natural stressors such as competition and predation. Such stressors are known to exacerbate the negative effects of chemical contaminants, even at sublethal concentrations [48]. Hence, it will be crucial for future research to consider the breadth of other factors that structure natural aquatic systems to fully elucidate when and where synergistic interactions among chemical contaminants might occur. In addition, it is uncertain whether one can generalize our results to the effects

of other pesticides and salt alternatives. There is a substantial body of literature examining the comparability of community effects from exposure to pesticides of similar and differing modes of action [49], yet comparatively little is known regarding the comparability of salts such as magnesium chloride and calcium chloride. Such salt alternatives are increasing in use as potentially safer and more effective solutions, and further work is needed to understand their effects on aquatic communities.

Acknowledgment—We thank K. Sudol, M. Kaloustian, G. Agostini, J. Narendran, J.J. Hu, C. Van Der Laarschott, and A. Brooks for field assistance. Research funding was provided by an RPI SURP grant awarded to B. Walker and The Jefferson Project at Lake George, which is a collaboration of Rensselaer Polytechnic Institute, IBM, and The FUND for Lake George.

Conflict of Interest—The authors declare no conflicts of interest.

Data Availability—Data are available upon request from the authors.

REFERENCES

1. Pimentel D, Edwards CA. 1982. Pesticides and ecosystems. *Issues Biol Educ* 32:595–600
2. Rand GM, 1995. *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment*, 2nd ed. Taylor & Francis, New York, NY, USA.
3. Relyea RA, Hoverman JH. 2006. Assessing the ecology in ecotoxicology: A review and synthesis in freshwater systems. *Ecol Lett* 9:1–15.
4. Jones DK, Hammond JI, Relyea RA. 2011. Competitive stress can make the herbicide Roundup more deadly to larval amphibians. *Environ Toxicol Chem* 30:446–454.
5. Grube A, Donaldson D, Kiely T, Wu L. 2011. Pesticides industry sales and usage: 2006 and 2007 market estimates. US Environmental Protection Agency, Washington, DC.
6. Clark JR, Lewis MA, Pait AS. 1993. Pesticide inputs and risks in coastal wetlands. *Environ Toxicol Chem* 12:2225–2233.
7. Gilliom RJ, Barbash JE, Crawford CG, Hamilton PA, Martin JD, Nakagaki N, Nowell LH, Scott JC, Stackelberg PE, Thelin GP, Wolock DM. 2007. The quality of our nation's waters: Pesticides in the nation's streams and groundwater, 1992–2001. US Department of the Interior & US Geological Survey, Washington, DC.
8. Fleeger JW, Carman KR, Nisbet RN. 2003. Indirect effects of contaminants in aquatic systems. *Sci Total Environ* 317:207–233.
9. Relyea RA. 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecol Appl* 15:618–627.
10. Relyea RA, Diecks N. 2008. An unforeseen chain of events: Lethal effects of pesticides on frogs at sublethal concentrations. *Ecol Appl* 18:1728–1742.
11. Findlay SEG, Kelly VR. 2011. Emerging indirect and long-term road salt effects on ecosystems. *Ann N Y Acad Sci* 1223:58–68.
12. Environment Canada. 2001. Canadian Environmental Protection Act, 1999: Priority substances list assessment report—Road salts. Minister of Public Works and Government Services, Toronto, Ontario, Canada.
13. Sriyaraj K, Shutes RBE. 2001. An assessment of the impact of motorway runoff on a pond, wetland and stream. *Environ Int* 26:433–439.
14. Sadowski E. 2002. The impacts of chloride concentrations on wetlands and amphibian distribution in the Toronto region. *Prairie Perspect* 5:144–162.
15. James KR, Cant B, Ryan T. 2003. Responses of freshwater biota to rising salinity levels and implications for saline water management: A review. *Aust J Bot* 51:703–713.
16. Collins SJ, Russell RW. 2009. Toxicity of road salt to Nova Scotia amphibians. *Environ Pollut* 157:320–324.
17. Van Meter RJ, Swan CM, Leips J, Snodgrass JW. 2011. Road salt stress induces novel food web structure and interactions. *Wetlands* 31:843–851.
18. Dananay KL, Krynak KL, Krynak TJ, Benard MF. 2015. Legacy of road salt: Apparent positive larval effects counteracted by negative postmetamorphic effects in wood frogs. *Environ Toxicol Chem* 34: 2417–2424.
19. US Environmental Protection Agency 2012. Aquatic life ambient water quality criteria for carbaryl—2012. EPA 820/R-12/007. Washington, DC.
20. Hua J, Cothran R, Stoler A, Relyea R. 2013. Cross-tolerance in amphibians: Wood frog mortality when exposed to three insecticides with a common mode of action. *Environ Toxicol Chem* 32:932–936.
21. Groner ML, Relyea RA. 2011. A tale of two pesticides: How common insecticides affect aquatic communities. *Freshw Biol* 56: 2391–2404.
22. Peterson HG, Boutin C, Martin PA, Freemark KE, Ruecker NJ, Moody MJ. 1994. Aquatic phyto-toxicity of 23 pesticides applied at expected environmental concentrations. *Aquat Toxicol* 28:275–292.
23. Relyea RA. 2003. Predator cues and pesticides: A double dose of danger for amphibians. *Ecol Appl* 13:1515–1521.
24. Stoler AB, Relyea RA. 2016. Leaf litter species identity alters the structure of pond communities. *Oikos* 125:179–191.
25. Stoler AB, Relyea RA. 2013. Bottom-up meets top-down: Leaf litter inputs influence predator-prey interactions in wetlands. *Oecologia* 173:249–257.
26. Stewart T., Downing JA. 2008. Macroinvertebrate communities and environmental conditions in recently constructed wetlands. *Wetlands* 28:141–150.
27. Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetelogica* 16:183–190.
28. Arar EJ, Collins GB. 1997. Method 445.0: *In vitro* determination of chlorophyll *a* and pheophytin *a* in marine and freshwater algae by fluorescence. US Environmental Protection Agency, Washington, DC.
29. Rubach MN, Baird DJ, Van der Brink PJ. 2010. A new method for ranking mode-specific sensitivity of freshwater arthropods to insecticides and its relationship to biological traits. *Environ Toxicol Chem* 29:476–487.
30. Fox J, Weisberg S. 2010. *An R Companion to Applied Regression*, 2nd ed. Sage, Thousand Oaks, CA, USA.
31. Korkmaz S, Goksuluk D, Zararsiz G. 2014. MVN: An R package for assessing multivariate normality. *The R Journal* 6:151–162.
32. de Mendiburu, F. 2014. agricolae: Statistical procedures for agricultural research. R Package Ver 1. 2–0. [cited 2016 September 30]. Available from: http://CRAN.R-project.org/package=agricolae
33. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H. 2015. vegan: Community Ecology Package. R Package Ver 2.2-1. [cited 2016 September 30]. Available from: http://CRAN.R-project.org/package=vegan
34. Côte IM, Darling ES, Brown CJ. 2016. Interactions among ecosystem stressors and their importance in conservation. *Proc R Soc B* 283: 20152592.
35. Havens KE. 1995. Insecticide (carbaryl, 1-napthyl-n-methylcarbamate) effects on a freshwater plankton community: Zooplankton size, biomass, and algal abundance. *Water Air Soil Pollut* 84:1–10.
36. Wijngaarden RPA Van, Brock TCM, Van den Brink P. 2005. Threshold levels for effects of insecticides in freshwater ecosystems: A review. *Ecotoxicology* 14:355–380.
37. Malby L, Blake N, Brock TCM, Van den Brink P. 2005. Insecticide species sensitivity distributions: Importance of test species selection and relevance to aquatic ecosystems. *Environ Toxicol Chem* 24: 379–388.
38. Hanazato T, Yasuno M. 1987. Effects of a carbamate insecticide, carbaryl, on the summer phyto- and zooplankton communities in ponds. *Environ Pollut* 48:145–159.
39. Hanazato T. 2001. Pesticide effects on freshwater zooplankton: An ecological perspective. *Environ Pollut* 112:1–10.
40. Xi Y, Chu Z, Xu X. 2007. Effect of four organochloride pesticides on the reproduction of freshwater rotifer *Brachionus calyciflorus* Pallas. *Environ Toxicol Chem* 26:1695–1699.
41. Kumar S, Habib K, Fatma T. 2008. Endosulfan induced biochemical changes in nitrogen-fixing cyanobacteria. *Sci Total Environ* 403: 130–138.
42. Seale DB, Beckvar N. 1980. The comparative ability of anuran larvae (genera: *Hyla*, *Bufo* and *Rana*) to ingest suspended blue-green algae. *Copeia* 3:495–503.
43. Canadian Council of Ministers of the Environment. 2011. Canadian water quality guidelines for the protection of aquatic life: Chloride. Winnipeg, Manitoba, Canada.
44. Wilson DM, Naimo TJ, Wiener JG, Anderson RV, Sandheinrich MB, Sparks RE. 1995. Declining populations of the fingernail clam

Musculium transversum in the upper Mississippi River. *Hydrobiologia* 304:209–220
45. Kefford BJ, Nugegoda D. 2005. No evidence for a critical salinity threshold for growth and reproduction in the freshwater snail *Physa acuta*. *Environ Pollut* 134:377–383.
46. Karraker NE. 2007. Are embryonic and larval green frogs (*Rana clamitans*) insensitive to road deicing salt? *Herpetol Conserv Biol* 2:35–41.
47. Karraker NE, Gibbs JP, Vonesh JR. 2008. Impacts of road deicing salt on the demography of vernal pool-breeding amphibians. *Ecol Appl* 18:724–734.
48. Rohr JR, Kerby JL, Sih A. 2006. Community ecology as a framework for predicting contaminant effects. *Trends Ecol Evol* 21:606–613.
49. Hua J, Relyea RA. 2014. Chemical cocktails in aquatic systems: Pesticide effects on the response and recovery of $>$ 20 animal taxa. *Environ Pollut* 189:18–26.