






Salty fertile lakes: how salinization and eutrophication alter the structure of freshwater communities

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Abstract. The quality of freshwater ecosystems is decreasing worldwide because of anthropogenic activities. For example, nutrient over-enrichment associated with agricultural, urban, and industrial development has led to an acceleration of primary production, or eutrophication. Additionally, in northern areas, deicing salts that are an evolutionary novel stressor to freshwater ecosystems have caused chloride levels of many freshwaters to exceed thresholds established for environmental protection. Even if excess nutrients and road deicing salts often contaminate freshwaters at the same time, the combined effects of eutrophication and salinization on freshwater communities are unknown. Thus by using outdoor mesocosms, we investigated the potentially interactive effects of nutrient additions and road salt (NaCl) on experimental lake communities containing phytoplankton, periphyton, filamentous algae, zooplankton, two snail species (*Physa acuta* and *Viviparus georgianus*), and macrophytes (*Nitella* spp.). We exposed communities to a factorial combination of environmentally relevant concentrations of road salt (15, 250, and 1000 mg Cl⁻/L), nutrient additions (oligotrophic, eutrophic), and sunlight (low, medium, and high) for 80 d. We manipulated light intensity to parse out the direct effects of road salts or nutrients from the indirect effects via algal blooms that reduce light levels. We observed numerous direct and indirect effects of salt, nutrients, and light as well as interactive effects. Added nutrients caused increases in most producers and consumers. Increased salt (1000 mg Cl⁻/L) initially caused a decline in cladoceran and copepod abundance, leading to an increase in phytoplankton. Increased salt also reduced the biomass and chl *a* content of *Nitella* and reduced the abundance of filamentous algae. Added salt had no effect on the abundance of pond snails, but it caused a decline in banded mystery snails, which led to an increase in periphyton. Low light negatively affected all taxa (except *Nitella*) and light levels exhibited multiple interactions with road salt, but the combined effects of nutrients and salt were always additive. Collectively, our results indicate that eutrophication and salinization both have major effects on aquatic ecosystems and their combined effects (through different mechanisms) are expected to promote large blooms of phytoplankton and periphyton while causing declines in many species of invertebrates and macrophytes.

Key words: algae; eutrophication; food web; freshwater ecosystems; macrophyte; salinization; zooplankton.

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INTRODUCTION

Lake ecosystems offer multiple ecosystem services, such as the provisioning of drinking water, water for industry and agriculture, recreation, and fisheries (Malmqvist and Rundle 2002, Keeler et al. 2012). Humans have exploited and contaminated lake resources causing extensive degradation and loss of biodiversity (Naiman et al. 2002). The contaminants can dramatically alter the structure and function of freshwater lake ecosystems, triggering a loss of ecosystem services (Hintz et al. 2017). Eutrophication and salinization are two major threats to lake ecosystems (Carpenter et al. 1985, Jackson et al. 2016, Dugan et al. 2017) and, while they co-occur throughout much of the world, their additive and synergistic effects are not well understood. Thus, it is important to understand whether these co-occurring disturbances interact to affect lakes ecosystems and food webs, and what the implications might be for lake ecosystem services and future mitigation efforts.

Over the last two centuries, human activities have enriched freshwater ecosystems with nutrients that have altered the trophic state of systems around the world (Conley et al. 2009). Nutrient pollution frequently occurs in human-dominated systems, causing eutrophication, harmful algal blooms, hypoxia, and changes in aquatic food webs (Conley et al. 2009, Paerl and Paul 2012). Moreover, algal blooms can reduce light availability, negatively affecting primary producers that are unable to migrate, drift, or extend toward the water surface (e.g., vascular plants and benthic algae; Cronin and Lodge 2003, Havens et al. 2003). Therefore, increased pelagic primary productivity might limit the persistence and growth of benthic primary producers (Scheffer et al. 1993).

Human activities in higher latitudes have also contaminated freshwater ecosystems by the usage of deicing salts, for the purpose of increasing driving safety during winter (Novotny et al. 2008, Corsi et al. 2010, Cañedo-Argüelles et al. 2016). The most common deicer is rock salt that consists mainly of sodium chloride (NaCl; Thunqvist 2004, Novotny et al. 2008, Rogora et al. 2015), and in 2013, the annual rock salt use on roads was 20.4 million metric tonnes in the United States, 5 million tonnes in Canada, and 0.2–0.3 million

tonnes in Sweden (Thunqvist 2004, Howard and Maier 2007, Bolen 2013). As snow and ice melt, the dissolved salt runs off into streams, rivers, and lakes or infiltrates soil and groundwater (Thunqvist 2004). During runoff events, chloride levels can reach 4300 mg/L in streams and 5000 mg/L in ponds and wetlands (Environment Canada 2001). These levels far exceed the current chronic (230 mg Cl⁻/L) and acute (860 mg Cl⁻/L) thresholds that were established for the protection of freshwater biota by the U.S. Environmental Protection Agency (EPA 1988).

Organisms in freshwater ecosystems vary in their tolerance to NaCl, and both chronic and acute chloride concentrations (from NaCl) negatively affect macroinvertebrate species richness in streams (Horrigan et al. 2005, Kefford et al. 2006). Chronic and acute chloride concentrations also negatively affect the abundance of lake and pond zooplankton (Sarma et al. 2006, Van Meter et al. 2011, Hintz et al. 2017, Stoler et al. 2017). Increased chloride concentrations have also been linked to algal blooms, which can reduce light availability and alter food-web structure in freshwater ecosystems (Dananay et al. 2015, Cañedo-Argüelles et al. 2016). Consequently, increased salinization can alter the freshwater community structure (Petranka and Doyle 2010, Cañedo-Argüelles et al. 2016). Although studies have been conducted on the salinity tolerance of freshwater macrophytes, these studies often focus on biogeochemical and molecular mechanisms on a cellular level, and not on food-web implications or changes in macrophyte abundance (Haller et al. 1974, Rout et al. 1997, Rout and Shaw 2001, Parida and Das 2005).

It is particularly valuable to understand how macrophytes respond to changes in their environment (e.g., light conditions), since macrophytes play an essential role in freshwater ecosystems (Carpenter and Lodge 1986, Scheffer et al. 1993), and since conditions in waterbodies have been altered by different types of pollution and invasion of species (Anderson et al. 2002, Kovalenko et al. 2010). Most aquatic plants cannot tolerate salt concentrations greater than 10 g/L, but tolerance varies among species, life stage, type of salt, and the duration and intensity of the exposure (Deegan et al. 2005, Lacoul and Freedman 2006). Furthermore, environmentally relevant chloride concentrations have been shown

to favor salt-tolerant species and alter the biomass of primary producers (Petranka and Doyle 2010, Van Meter et al. 2011, Hintz et al. 2017).

Our objective was to examine the combined effects of salt contamination and nutrient pollution on freshwater communities. We expected both contaminants to cause an algal bloom, but through different mechanisms. Road salts would indirectly cause an algal bloom by reducing the abundance of zooplankton (i.e., top-down), whereas nutrients would directly increase the growth rate of algae (i.e., bottom-up). Because algal blooms can have cascading effects on food webs and ecosystems by reducing light transmission through the water column, we also manipulated light levels to parse out the direct and indirect effects of an algal bloom caused by nutrients or salt. We employed outdoor mesocosms, which are commonly used to test the effects of anthropogenic impacts on aquatic systems, because they provide a venue to manipulate and replicate conditions in a controlled manner (e.g., Rowe and Dunson 1994, Downing and Leibold 2002, Hua and Relyea 2014).

We hypothesized that the combination of stressors would cause additive and interactive effects on primary and secondary biomass and abundance. Specifically, we predicted that (1) higher chloride concentrations will cause a decrease in zooplankton and other animals and a subsequent bloom in phytoplankton, (2) higher nutrient concentrations will cause an increase in productivity of all primary producers, (3) increased phytoplankton production due to elevated nutrient or salt levels will have an indirect negative effect on the benthic macroalgae (*Nitella* spp.) and grazers, due to reduced light availability, (4) low light levels will mimic the effects of reduced light transparency caused by algal blooms produced by increased salt and nutrients, and (5) increased salt, increased nutrients, and reduced light will have multiple interactive effects.

MATERIALS AND METHODS

Experimental design

We conducted the experiment at Rensselaer Polytechnic Institute's Aquatic Research Laboratory in Troy, New York, USA, during the summer of 2015. We used a completely randomized design that employed a full factorial combination of three

salt concentrations (15, 250, and 1000 mg Cl⁻/L), two nutrient levels (high eutrophic and ambient oligotrophic), and three light levels (low [10%], medium [35%], and high [70%] of ambient sunlight). As noted earlier, the three sunlight manipulations acted as a proxy for the shading effect of an algal bloom that is independent of the other impacts that an algae bloom can have on a food web. Four replicates of each of the 18 treatment combinations resulted in 72 experimental units.

Our experimental units were 1200-L plastic mesocosms (i.e., cattle tanks). On 15 June, we filled the mesocosms with 850 L of water from Lake George (Warren County, New York, USA) due to its low chloride concentration (15 mg Cl⁻/L) and oligotrophic state. Two days later, we added 140 L (5 cm deep) of sand substrate to the mesocosms and allowed the water to sit undisturbed for 10 d, until the soil particles settled. When the water was clear, we placed two unglazed clay tiles (10 × 15 cm) vertically on the north side of each mesocosm to serve as periphyton samplers during the experiment.

We established highly similar ecological communities in each mesocosm. We initiated a zooplankton community on 27 June by collecting zooplankton from Lake George using a zooplankton net (64 μm) and adding 600 mL of the concentrated zooplankton slurry to each of the mesocosms. In addition to zooplankton, the collected water also introduced microbial and algal assemblages to each mesocosm. On the same day, we collected banded mystery snails (*Viviparus georgianus*) from a local lake and added six individuals (two large and four small) to each mesocosm. On 1 July, we collected a mixture of the *Nitella* species (*N. flexilis*, *N. opaca*, and *N. tenuissima*) from Lake George and placed 200 g (wet weight) on the bottom of each mesocosm after rinsing and removing undesirable species (e.g., macrophytes and snails) that were visible. *Nitella* spp. are macroalgae (Characeae) that live in monoculture meadows in deep water (7–12 m; Boylen et al. 2014), and since its growth form resembles an aquatic plant, it is categorized as a macrophyte (Cushing and Allan 2001). The collected *Nitella* also contained attached pond snails (*Physa acuta*), so we quantified the number of attached pond snails and estimated that an average of four individuals (3–6) were introduced to each mesocosm.

On 7 July (defined as day 1 of the experiment), we applied the light treatments by covering the mesocosms with mesh lids with three different light transmittance percentages (10, 35, and 70% sunlight). Prior to this date, all mesocosms had identical mesh lids that allowed 35% light transmittance. These lids also prevented organisms from colonizing or leaving the mesocosms (Howeth and Leibold 2010).

On 10 July, we added road salt to the mesocosms in the form of NaCl (Solar Salt; Morton Salt, Chicago, Illinois, USA; 99.8% pure NaCl; 60.7% chloride, free of additives). Given that the ambient chloride concentration of the lake water was 15 mg Cl⁻/L, we added salt to reach medium and high concentrations (250 and 1000 mg Cl⁻/L, respectively). We chose these three concentrations because the U.S. EPA maximum acceptable level for drinking water is 250 mg Cl⁻/L (EPA, 2016), and 1000 mg Cl⁻/L exceeds the standards for acute events but is representative of North American lakes with the highest road salt concentrations (Novotny et al. 2008). The highest concentration observed in North American ponds and wetlands is approximately 4300 mg Cl⁻/L (Environmental Canada 2001). We added the sodium chloride to each mesocosm by extracting 5 L of water and mixing the salt with the water until it was dissolved. We added the chloride to each mesocosm assigned to a salt treatment in a slow, circular movement to ensure that the mixture was evenly dispersed. On the next day, we measured the chloride concentrations to ensure that we reached our goals for each mesocosm.

On the same day as salt additions, we applied our nutrient treatments. In the low-nutrient treatment, no nutrients were added to represent an oligotrophic lake (Lake George, TP mean of 4.36 µg/L over 30 yr; Boylen et al. 2014). For the high-nutrient treatment, we added 0.185 g of potassium phosphate and 4.2 g of sodium nitrate (16N:1P) on 10 July. We set the target eutrophic conditions at 100 µg/L of P and 1600 µg/L of N (see, e.g., Schuler et al. 2017a). We dosed the mesocosms assigned to the high-nutrient treatment a second time on 4 August to maintain higher nutrient levels since there is a 5% day⁻¹ loss of nutrients to the bottom substrate (Howeth and Leibold 2010). To control for disturbance, we gently agitated the surface water of all mesocosms not receiving nutrients or salt.

Response variables

We quantified phytoplankton abundance on days 8, 16, 20, 30, 42, and 78, with an average of 13 days between sampling occasions (Table 1). Phytoplankton were sampled to reflect when we observed the most dramatic changes. We sampled phytoplankton by collecting 450 mL of water from the middle of each mesocosm and vacuum-filtered all samples through GF/C glass fiber filters (Whatman, Inc., Massachusetts, USA). Each filter was wrapped in aluminum foil and frozen (-20°C) to prevent chlorophyll breakdown. We later measured the concentration of chlorophyll *a* in each filter using a fluorometer (Model ED-700; Turner Designs) following Arar and Collins (1997).

We measured periphyton biomass on days 32 and 77 (Table 1), by removing one tile each time from each mesocosm and scrubbing the tile with a brush. We rinsed the tile and brush, and the resulting slurry was filtered through pre-dried (60°C for 48 h) and pre-weighed 1.2-µm glass fiber filters (Whatman GF/C). After drying the filters at 60°C for 48 h, we re-weighed them to determine dry periphyton biomass.

We also sampled the living *Nitella* from each mesocosm on day 80 (Table 1) to assess final biomass and chlorophyll *a* content. We rinsed the samples to remove attached filamentous algae and snails and then dried the *Nitella* samples at 60°C for 48 h. After drying, we weighed each sample. From each *Nitella* sample, we also clipped a 4-cm piece to analyze it for chlorophyll *a* content. The *Nitella* pieces were wrapped in aluminum foil and frozen (-20°C) to prevent chlorophyll breakdown. We later measured the concentration of chlorophyll *a* in each sample by shaking the bottle for 1 min until the fragile plant tissue had become suspended in acetone solution (Gitelson et al. 2003). We then used the fluorometer to quantify

Table 1. Schematic overview of sampling intensity of each response variable or group of response variables.

Response variables	Day					
	7-8	14-16	19-21	30-32	42-43	77-80
Phytoplankton	X	X	X	X	X	X
Periphyton	—	—	—	X	—	X
Macroalgae	—	—	—	—	—	X
Snails	—	—	—	—	—	X
Zooplankton	—	—	X	—	—	X
Abiotic	X	X	X	X	X	X

chlorophyll *a* (Model ED-700; Turner Designs) following Arar and Collins (1997).

On day 80, we also estimated the abundance of the filamentous algae (floating and submerged). Three observers each made a single estimate of the amount of filamentous algal cover in each mesocosm by ranking it from 1 to 5 (1 = 1–5%, 2 = 5–10%, 3 = 10–25%, 4 = 25–65%, 5 = 65–100%), and these rankings were then averaged.

We quantified the abundance of juvenile snails on day 80 (Table 1) by collecting a benthic sample of snails. The benthic sample was collected by sweeping an aquarium net (width = 10 cm, mesh size = 250 μm) against the bottom, from the center of the mesocosm advancing to the side wall in all four cardinal directions. We preserved all snails in 70% ethanol and later enumerated the number of banded mystery snails and pond snails (juveniles were <0.5 cm). We only enumerated the number of juvenile snails because adult snails were very rare in all samples.

To quantify zooplankton abundance when they were diverging among treatments, we sampled the mesocosms two times during the experiment (Table 1). On days 19 and 78, we sampled 450 mL of water from six locations in a given mesocosm and then pooled the six samples. We poured the pooled sample through a 64- μm net and preserved the collected zooplankton in 30% ethanol to ensure that the zooplankton stayed intact. We later enumerated the zooplankton in three taxonomic categories: copepods, cladocerans, and rotifers.

Throughout the 80 d of the experiment, we quantified temperature ($^{\circ}\text{C}$), pH, dissolved oxygen ($\text{mg O}_2/\text{L}$), and chloride concentrations ($\text{mg Cl}^-/\text{L}$) on days 7, 14, 21, 32, 43, and 78 (Table 1) using a calibrated digital water meter (YSI Professional Plus, Yellow Springs, Ohio, USA). We measured all abiotic parameters at approximately half the water depth. The nominal salt concentration of 15 and 1000 $\text{mg Cl}^-/\text{L}$ differed from actual salt concentrations of 11 and 1072 $\text{mg Cl}^-/\text{L}$ by 26%, and 7%. On average there was no difference between actual and nominal concentration for 250 $\text{mg Cl}^-/\text{L}$.

Statistical analysis

This study includes a large number of response variables, some of which were measured multiple times. Therefore, we used several different

analyses to examine the effects of our treatments. We used univariate repeated-measures analysis of variance (ANOVA) for phytoplankton (sampled at six time points) and periphyton (sampled at two time points). We conducted two additional multivariate analyses of variance (MANOVAs): one on macroalgae (*Nitella* dry biomass, *Nitella* chlorophyll *a* content, and filamentous algae rank abundance) and one on juvenile snail abundance (pond snails and banded mystery snails). For *Nitella* biomass, we analyzed the dry mass of *Nitella* because wet and dry mass were highly correlated (Pearson correlation = 0.98). We conducted repeated-measures MANOVAs (rm-MANOVA) on zooplankton (cladocerans, copepods, and rotifers) which were sampled at two time points and on the abiotic water quality variables which were measured at six time points (temperature, pH, and DO). When we found significant multivariate effects, we conducted subsequent univariate repeated-measures ANOVAs on each response variable. When we detected time-by-treatment interactions, we used ANOVAs to analyze the treatment effects on each sample date. For all significant ANOVAs, we conducted Tukey's HSD post hoc test because the number of possible mean comparisons was low. Data were log-transformed when needed to fit parametric assumptions of normality and homogeneity of variance. All analyses were performed in R version 3.3.1, using packages *vegan* and *car*.

RESULTS

Phytoplankton

We found effects of salt, light, nutrients, and salt-by-light and nutrient-by-light interactions with time on phytoplankton chlorophyll *a* concentration; the other interactions were not significant. (Appendix S1: Table S1). Given the time-by-treatment interactions, we analyzed phytoplankton for each sample date, and detailed descriptions are available in Appendix S1.

On the first sample date, there was no main effect of salt, but there was a main effect of light, nutrients, and a light-by-nutrient interaction (Table 2, Fig. 1a). Added nutrients caused an increase of phytoplankton, and the magnitude of the increase was greatest under high light. On the second sample date (day 16), there were main effects of salt, light, and nutrients, but no

Table 2. Results of an ANOVA for phytoplankton chl *a* concentration at each sample day based on significant interactions from rm-ANOVA.

Day	Salt (df = 2,54)		Light (df = 2,54)		Nutrient (df = 1,54)		Salt × Light (df = 4,54)		Light × Nutrient (df = 2,54)	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
8	2.6	0.083	14.4	<0.001	37.3	<0.001	1.54	0.201	15.83	<0.001
16	8.0	<0.001	3.5	0.038	6.3	0.015	1.07	0.380	1.0	0.363
20	0.1	0.922	4.9	0.011	6.3	0.015	0.29	0.887	3.4	0.039
30	5.0	0.010	1.4	0.266	4.1	0.047	1.17	0.334	0.4	0.648
42	5.5	0.006	0.6	0.553	0.8	0.376	1.18	0.330	1.4	0.260
78	4.1	0.022	0.7	0.524	0.1	0.756	1.01	0.409	1.2	0.310

Notes: Bold numbers indicate statistical significance at the alpha level of 0.05. Additional interactions were not significant in the rm-ANOVA (Appendix S1: Table S1).

interactions among treatments (Table 2, Fig. 1b). Once again, the combination of added nutrients and high light caused an increase in phytoplankton. In addition, the high chloride concentration caused an increase in phytoplankton. On the third sample date (day 20), there was no effect of salt, but there were effects of light, nutrients, and a light-by-nutrient interaction (Table 2, Fig. 1c). The increase in phytoplankton with added nutrients and high light was once again apparent. On the fourth sample date (day 30), there was an effect of salt and nutrients on phytoplankton, but not light (Table 2, Fig. 1d). The added nutrients and high salt concentration both caused an increase in phytoplankton. On the fifth and sixth sampling dates (days 42 and 78), phytoplankton was only affected by salt (Table 2, Fig. 1e–f). In both cases, the high concentration of salt caused an increase in phytoplankton.

Periphyton

The repeated-measures ANOVAs on periphyton revealed effects of salt and nutrients, but no effects of light or any interactions (Appendix S1: Table S1). However, there was a nearly significant effect of time, because it was only at the second sampling that elevated nutrients caused a 68% increase in periphyton biomass (day 32: $F_{1,54} = 2.1$, $P = 0.149$; day 78: $F_{1,54} = 6.3$, $P = 0.015$; Fig. 2). In 1000 mg Cl⁻/L, periphyton biomass was 110% and 78% higher, respectively, compared to 15 mg Cl⁻/L (day 32: $F_{2,54} = 3.9$, $P = 0.024$; day 78: $F_{2,54} = 3.1$, $P = 0.051$).

Macroalgae

The MANOVA on *Nitella* dry biomass, *Nitella* chl *a*, and filamentous algae rank abundance

indicated a multivariate effect of salt (Wilks' λ , $F_{2,54} = 11.5$, $P < 0.001$) and a nearly significant effect of light (Wilks' λ , $F_{2,54} = 2.0$, $P = 0.072$), but no effect of nutrients (Wilks' λ , $F_{1,54} = 1.2$, $P = 0.314$) or any treatment interactions. We then conducted separate ANOVAs for each response variable.

In the analysis of *Nitella* dry biomass, we found main effects of salt, light, and a salt-by-light interaction (Table 3, Fig. 3a). The post hoc comparison revealed that the *Nitella* biomass in low light declined by about half with 250 mg Cl⁻/L ($P = 0.013$) and by 98% with 1000 mg Cl⁻/L ($P < 0.001$), compared to 15 mg Cl⁻/L. In medium sunlight, *Nitella* biomass was 77% higher in 15 mg Cl⁻/L than in 1000 mg Cl⁻/L ($P = 0.003$). In high sunlight, *Nitella* biomass was much lower and there were no effects of salt treatments ($P > 0.7$). When we examined the effect of sunlight within each salt treatment, we found that *Nitella* biomass declined by 65% between high and low light in 15 mg Cl⁻/L ($P = 0.004$). However, there were no effects of light within the 250 and 1000 mg Cl⁻/L salt treatments (all: $P > 0.7$).

The analysis of chlorophyll *a* in *Nitella* revealed an effect of salt (Table 3, Fig. 3b) and a nearly significant negative effect of light, but no salt-by-light interaction. Post hoc comparisons showed that there was 53–54% higher chl *a* concentration in *Nitella* exposed to 15 or 250 mg Cl⁻/L compared to *Nitella* exposed to 1000 mg Cl⁻/L (all: $P < 0.001$). There was also 29% lower chl *a* concentration in *Nitella* with high light compared to low light ($P = 0.049$; Fig. 3b).

In our analysis of the filamentous algae rank abundance, we found an effect of salt, but no effects of light or nutrients (Table 3). Post hoc

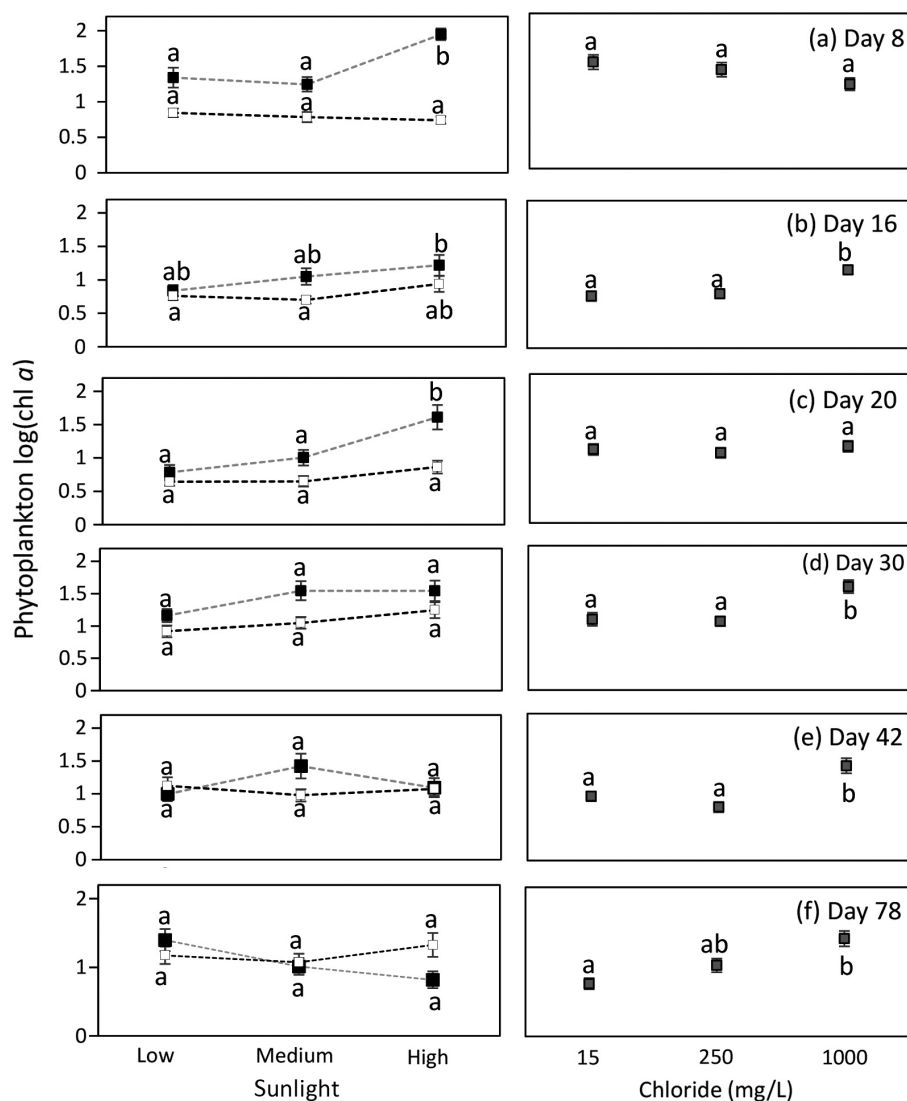


Fig. 1. Effects of nutrients (black boxes indicate high and white boxes indicate low nutrient concentration), sunlight (low 10%, medium 35%, and high 70%), and salt (15, 250, and 1000 mg Cl⁻/L) on phytoplankton (chl *a*) concentration on six sampling dates (a–f). Letters indicate significant difference between and within treatments. Data are logged means \pm 1 SE.

comparisons showed that there was no difference between 15 and 250 mg Cl⁻/L ($P = 0.822$). However, filamentous algae did not decline from 15 to 250 mg Cl⁻/L, but it did decline from 15 and 1000 mg Cl⁻/L ($P < 0.001$) and 250 to 1000 mg Cl⁻/L ($P < 0.001$).

Snails

The MANOVA on juvenile snail abundance revealed a multivariate effect of salt, light,

nutrients, and a salt-by-light interaction (Appendix S1: Table S2). In the subsequent univariate analysis on pond snails, we found main effects of light and nutrients, with a 70% increased abundance from low to high light ($F_{1,54} = 14.9$, $P < 0.001$) and a 60% increased abundance in mesocosms with added nutrients ($F_{2,54} = 4.5$, $P = 0.019$; Fig. 4a). There was no effect of salt ($F_{2,54} = 1.9$, $P = 0.158$) or a salt-by-light interaction ($F_{4,54} = 0.9$, $P = 0.496$).

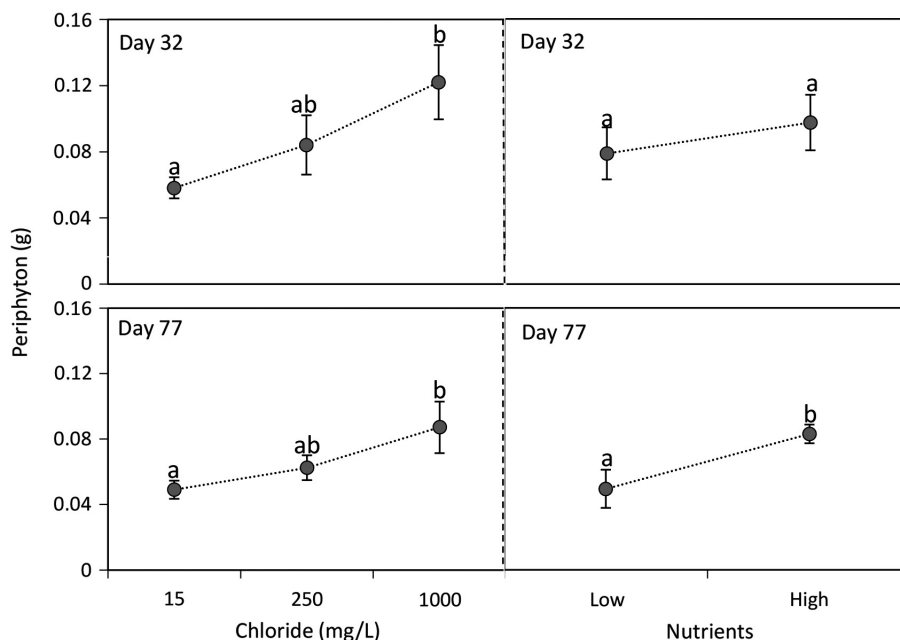


Fig. 2. Effects of nutrients (low and high) and salt (15, 250, and 1000 mg Cl⁻/L) on total periphyton biomass (g/15 cm²) on two sampling dates. Data are means ± 1 SE.

Table 3. ANOVA table for *Nitella* biomass, *Nitella* chlorophyll, and filamentous algae rank abundance.

Source of variation	<i>Nitella</i> biomass		<i>Nitella</i> chl <i>a</i>		Filamentous algae rank	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Salt	24.9 _{2,54}	<0.001	19.6 _{2,54}	<0.001	29.8 _{2,54}	<0.001
Light	5.5 _{2,54}	0.006	2.9 _{2,54}	0.060	2.1 _{2,54}	0.134
Salt × Light	2.9 _{4,54}	0.029	2.0 _{4,54}	0.101	0.2 _{4,54}	0.961

Notes: Bold numbers indicate statistical significance at the alpha level of 0.05. Subscripted numbers indicate degrees of freedom. The preceding MANOVA indicated no effects of nutrients or its interactions.

In the univariate analysis on banded mystery snails, there were no effects of nutrients, but we found effects of salt ($F_{2,54} = 18.8$, $P < 0.001$), light ($F_{2,54} = 13.1$, $P < 0.001$), and a salt-by-light interaction ($F_{4,54} = 5.0$, $P = 0.002$; Fig. 4b). Under low-light conditions, snail abundance was low and there were no differences among the salt treatments. Under medium-light conditions, banded mystery snail abundance was 83% higher with 250 mg Cl⁻/L than with 1000 mg Cl⁻/L ($P = 0.001$), but there was no difference between 15 and 250 mg Cl⁻/L ($P = 0.131$) or between 15 and 1000 mg Cl⁻/L ($P = 0.777$). Under high-light conditions, snail abundance declined sharply by 92% and 94% with 1000 mg Cl⁻/L compared to 15 and 250 mg Cl⁻/L, respectively (all: $P < 0.001$).

Zooplankton

The MANOVA on cladocerans, rotifers, and copepods revealed multivariate effects of light, nutrient, time, and a salt-by-time interaction; no other interactions were significant (Appendix S1: Table S3). The subsequent univariate analyses also showed interactions between salt and time, and we therefore analyzed all groups of zooplankton within sampling dates for salt treatments (Tables 4 and 5).

For cladoceran abundance, there was a main effect of light and nutrients, and there was also an interaction between salt and time (Table 4, Fig. 5). Cladocerans also showed a 43% increase from low to medium light ($P = 0.043$) and a 53% increase from low to high light ($P = 0.009$). They

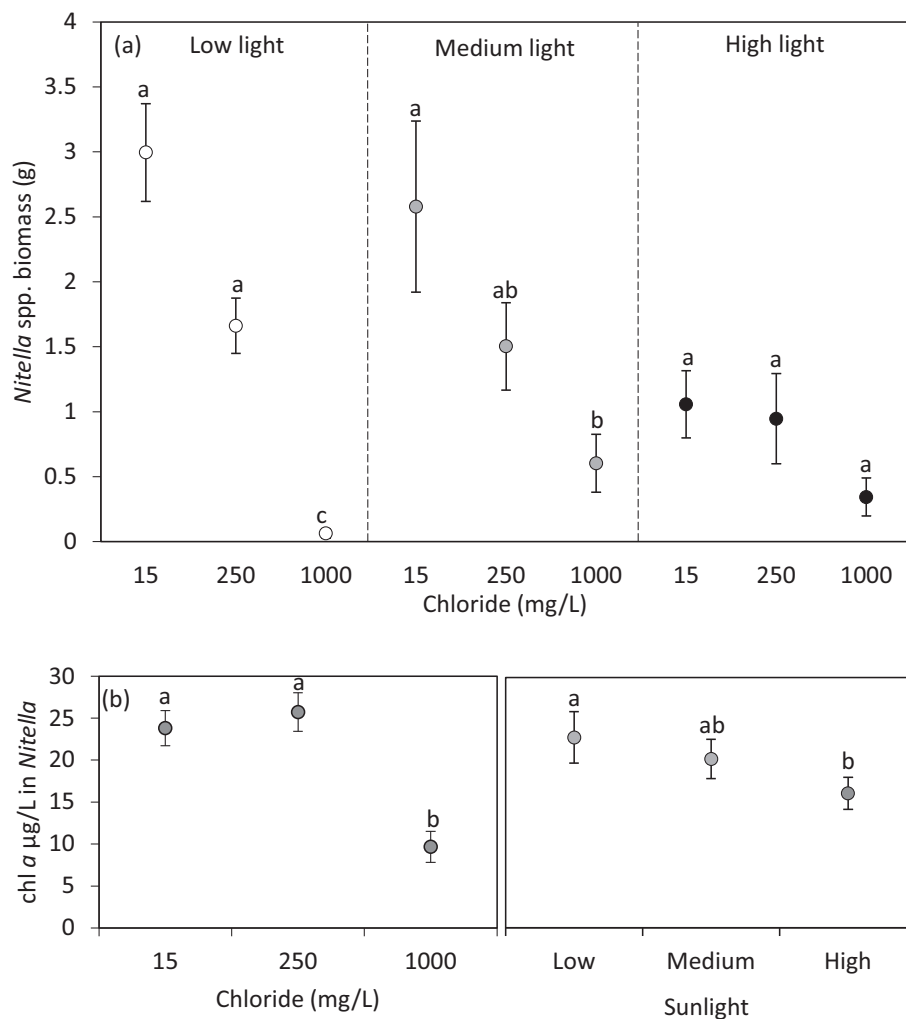


Fig. 3. (a) Effects of sunlight (low 10%, medium 35%, and high 70%) and salt (15, 250, and 1000 mg Cl⁻/L) on *Nitella* biomass. (b) Effects of salt (15, 250, and 1000 mg Cl⁻/L) and sunlight (low 10%, medium 35%, and high 70%) on the chlorophyll *a* content in *Nitella*. Data are means \pm 1 SE.

experienced a 41% increase from low to high nutrients ($P = 0.004$). On the first sample date, cladocerans exhibited a 38–40% lower abundance with 1000 mg Cl⁻/L compared to the 250 and 15 mg Cl⁻/L (all: $P \leq 0.04$). On the second sample date, there was no effect of salt.

For rotifer abundance, there was a main effect of nutrients and light and a marginal effect of salt (Table 4, Fig. 5). Post hoc comparisons showed that rotifers were also 57–70% less abundant in low light compared to medium and high light ($P \leq 0.05$). They also experienced a 50% increase with added nutrients ($P = 0.031$). On the second

sample date, there was a 53–63% higher abundance with 1000 mg Cl⁻/L compared to 15 and 250 mg Cl⁻/L ($P \leq 0.049$). On the first sample date, there were no effects of salt.

Copepod abundance was affected by salt, nutrients, and a salt-by-time interaction (Table 4, Fig. 5). Copepods experienced a 64% increase with increased nutrients (Table 5). On the first sampling, copepods were 76% more abundant with 15 mg Cl⁻/L than with 1000 mg Cl⁻/L ($P = 0.006$). On the second sample date, copepods were 86% more abundant with 250 mg Cl⁻/L than with 1000 mg Cl⁻/L treatments ($P < 0.001$).

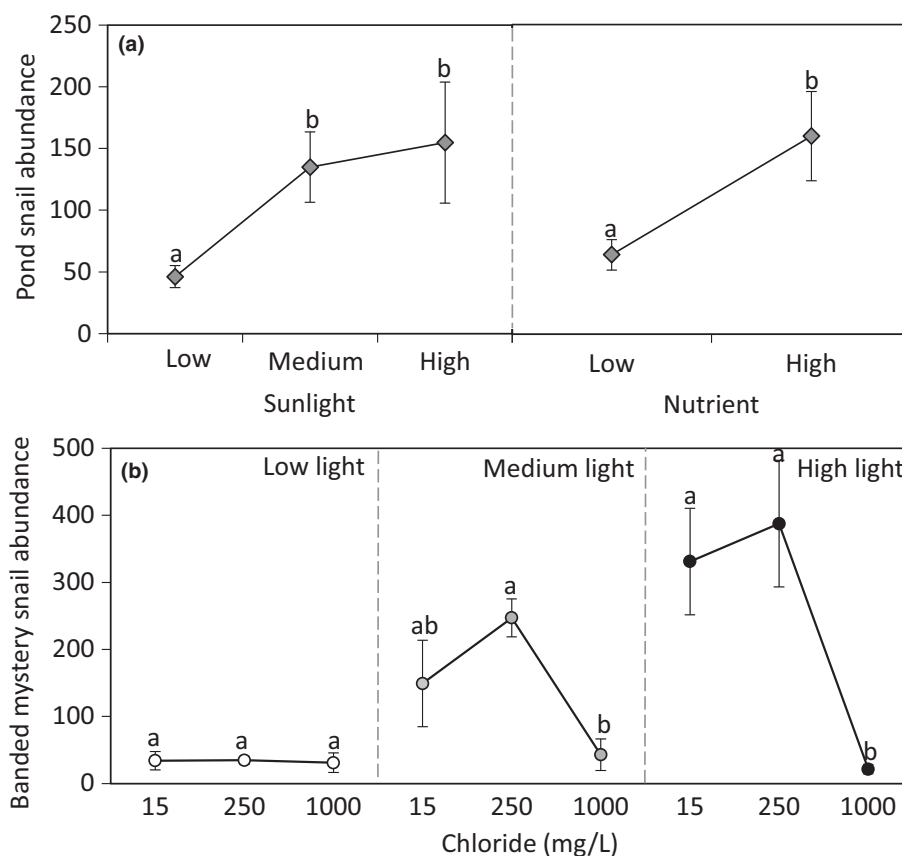


Fig. 4. Effects of sunlight (low 10%, medium 35%, and high 70%) and salt (15, 250, and 1000 mg Cl⁻/L) on (a) juvenile pond snails and (b) juvenile banded mystery snails. The white, gray, and black dots represent different sunlight levels (10, 35, and 70%). Data are means \pm 1 SE.

Abiotic conditions

The analysis of abiotic measurements detected multivariate effects of salt, light, nutrients, time, and their interactions (Appendix S1: Table S4). We

Table 4. Univariate repeated-measures ANOVA on copepods, cladocerans, and rotifers for those factors found to be significant in the rm-MANOVA (see Appendix S1: Table S3).

Source of variation	df	Copepods		Cladocerans		Rotifers	
		F	P	F	P	F	P
Salt	2,54	8.5	<0.001	1.8	0.166	2.8	0.065
Light	1,54	2.0	0.134	6.6	0.002	5.8	0.004
Nutrient	2,54	21.8	<0.001	8.6	0.004	4.7	0.032
Salt \times Time	2,54	3.0	0.053	4.7	0.011	1.0	0.387

Notes: Bold numbers indicate statistical significance at the alpha level of 0.05. Subscripted numbers indicate degrees of freedom.

therefore conducted rm-ANOVAs on each response variable to understand which response variables were driving the multivariate effects and then used subsequent ANOVA for each response variable at

Table 5. Univariate tests on copepods, cladocerans, and rotifers within each sample time for effect of salt.

Zooplankton	Salt	
	F	P
Day 19		
Copepods	5.1 _{2,54}	0.009
Cladocerans	4.2 _{2,54}	0.020
Rotifers	0.4 _{2,54}	0.670
Day 78		
Copepods	8.1 _{2,54}	0.001
Cladocerans	2.5 _{2,54}	0.086
Rotifers	4.3 _{2,54}	0.018

Notes: Bold numbers indicate statistical significance at the alpha level of 0.05. Subscripted numbers indicate degrees of freedom.

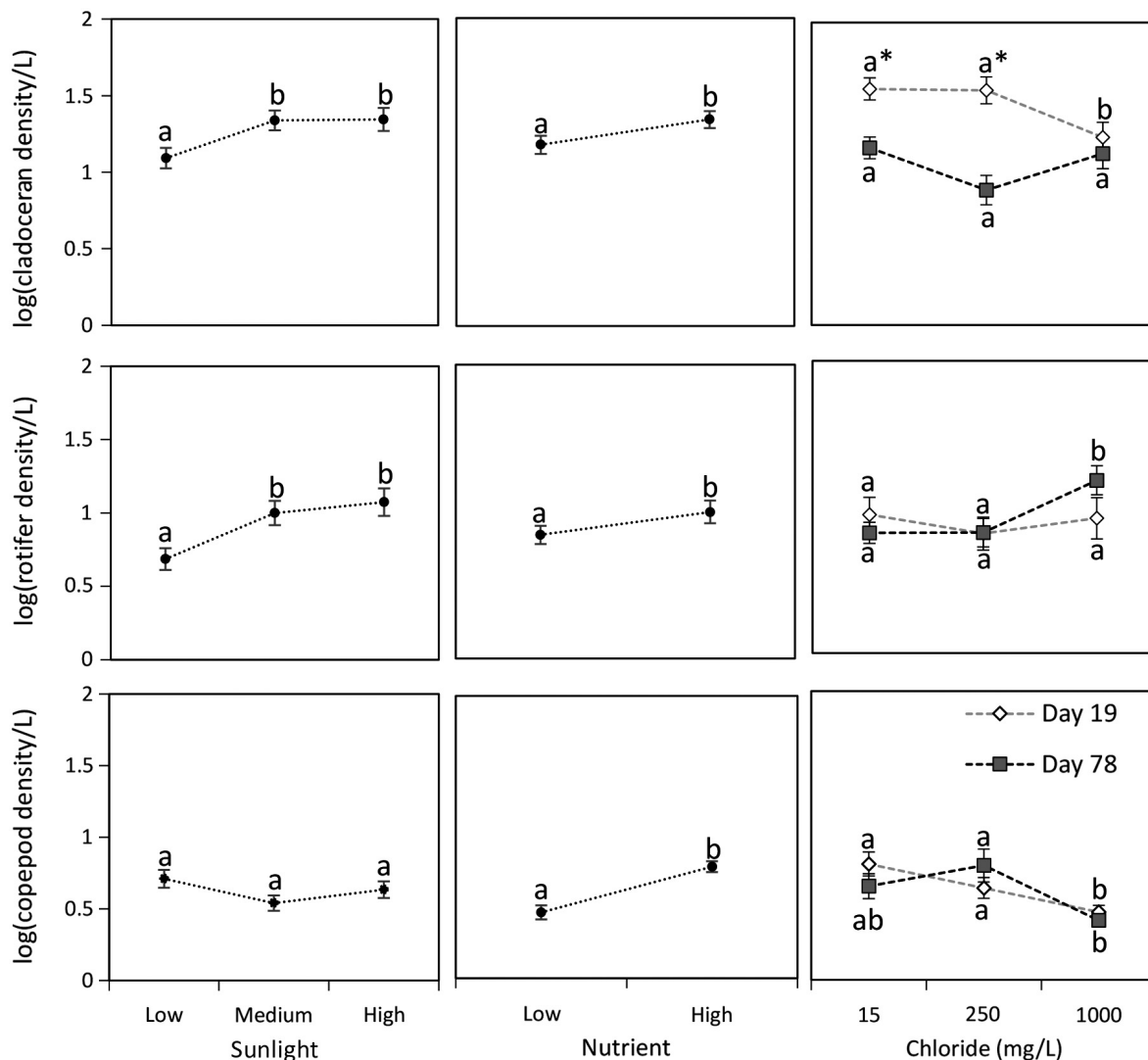


Fig. 5. Effects of nutrients (low and high), sunlight (low 10%, medium 35%, and high 70%), and salt (15, 250, and 1000 mg Cl^-/L) on cladocerans, rotifers, and copepods on two sampling dates (days 19 and 78). Letters indicate significant differences between treatments, and asterisks (*) indicate significant differences between sampling occasions. Data are logged means \pm 1 SE.

each sample date if treatments interacted with time (Fig. 6, Appendix S1: Tables S5–S6). Detailed supplementary results for each sampling date for DO, temperature, and pH are available in Appendix S1. During the experiment, the chloride concentrations remained the same; that is, they did not have an interaction with time (all: $P > 0.1$).

The univariate analysis of DO revealed effects of light, salt, nutrients, time, and light-by-nutrient, salt-by-time, and nutrient-by-time interactions (Appendix S1: Table S5). Averaged over time, DO

levels were higher with high light and added nutrients (Fig. 6; Appendix S1: Table S6).

For temperature, the analysis revealed effects of light, time, and light-by-time interaction (Fig. 6; Appendix S1: Tables S5–S6). During the first part of summer, temperatures were highest in high sunlight treatment, whereas in the later parts of summer, temperatures were highest in the low sunlight treatment.

Analysis of pH showed effects of salt, light, nutrient, time, and salt-by-time and nutrient-by-time

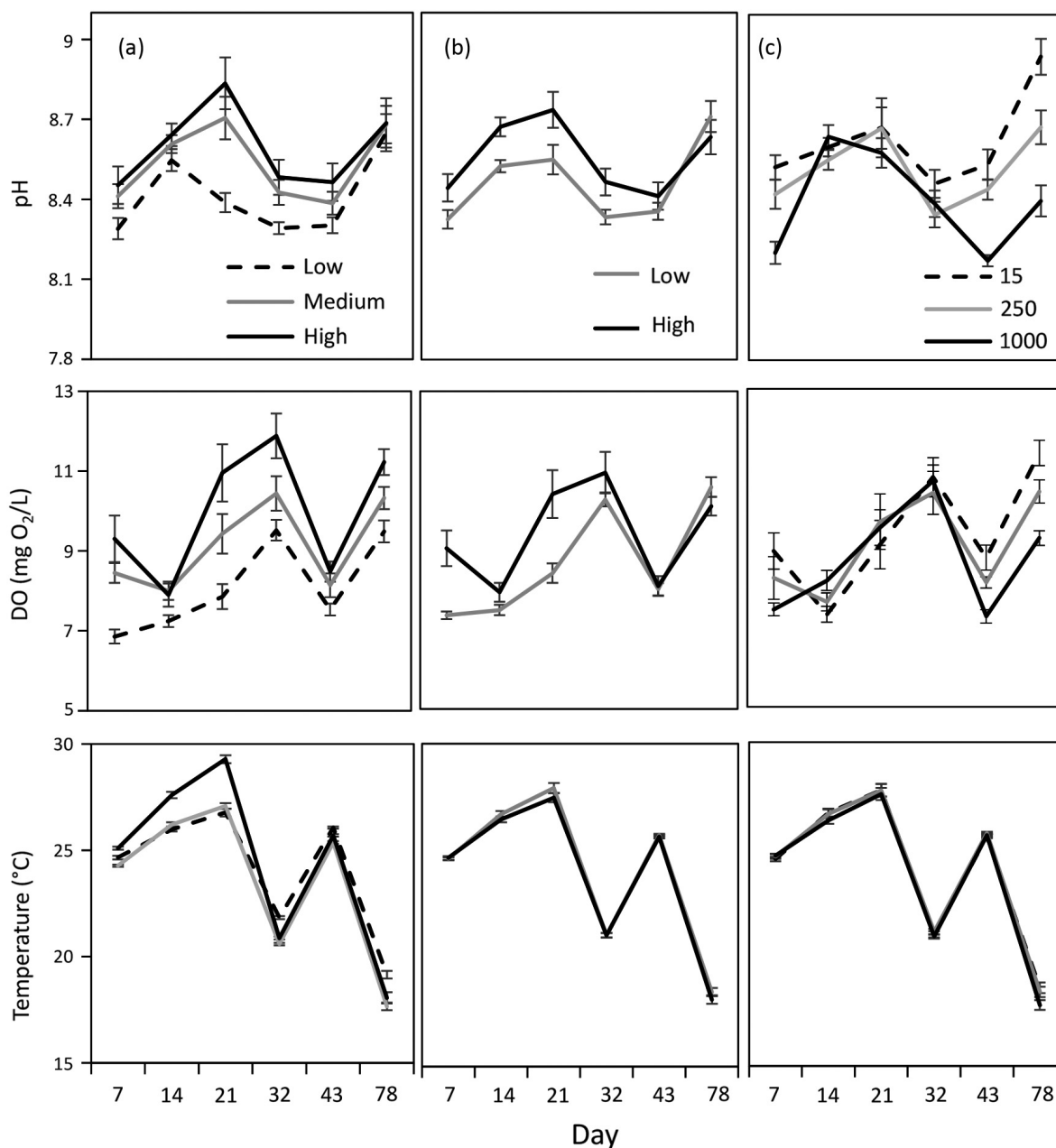


Fig. 6. pH, DO (dissolved oxygen; mg O₂/L), and temperature (°C) for each sampling day and each treatment: (a) sunlight (low 10%, medium 35%, and high 70%), (b) nutrients (low and high), and (c) salt (15, 250, and 1000 mg Cl⁻/L).

interactions (Fig. 6; Appendix S1: Tables S5–S6). The pH levels were generally increasing with increasing light and nutrients levels while decreasing with increasing salt concentrations. The pH ranged between 7.46 and 9.95 with an average of 0.2 pH units lower in 1000 vs. 15 mg Cl⁻/L.

DISCUSSION

Salinization and eutrophication in freshwater ecosystems is a serious environmental problem (Van Meter et al. 2011, Paerl and Paul 2012, Cañedo-Argüelles et al. 2016, Hintz et al. 2017).

We discovered that altered nutrients, sunlight, and salt concentrations altered the structure of ecological communities by causing direct effects as well as top-down and bottom-up indirect effects that altered the abundance of primary producers and consumers. Adding nutrients caused our community to experience increased productivity, including the increased growth of phytoplankton and periphyton, one of the two snail species, and all three zooplankton groups. Adding salt caused declines in two of the three zooplankton groups, an increase in phytoplankton and periphyton, sharp declines in the *Nitella* macroalgae, and sharp declines in the abundance of banded mystery snails. In short, we found that the combination of increased chloride and nutrients creates a highly eutrophied ecosystem with decreasing macrophyte coverage, higher pelagic primary production, and altered abundances of consumers for higher trophic levels. Reduced sunlight caused a decline in the abundance of pond snails and banded mystery snails (although the latter depended on salt concentration), declines in phytoplankton (although only under high-nutrient conditions), and declines in cladocerans and rotifers. However, there was no evidence that declines in sunlight caused by salt- and nutrient-induced increases in phytoplankton or macroalgae caused any indirect effects on the food web. Below, we elaborate on these findings and interpretations.

Phytoplankton and periphyton

Phytoplankton and periphyton increased in our experiment when exposed to high nutrients or elevated salt concentrations (Figs. 1 and 2). The increase in phytoplankton under high-salt conditions was likely caused by a decline in the copepods and cladocerans that consume phytoplankton. This outcome has also been observed in past lake and wetland ecosystems (Van Meter et al. 2011, Hintz et al. 2017) and is similar to that seen for other contaminants that are lethal to zooplankton, including insecticides (Hua and Relyea 2014, Bendis and Relyea 2016).

While it was not surprising that phytoplankton became more abundant when nutrients were added (Conley et al. 2009, Paerl and Paul 2012), it was interesting that nutrients and sunlight had interactive effects; reduced sunlight had no effect under low-nutrient conditions but caused a large

decline in phytoplankton under high-nutrient conditions. This suggests that while phytoplankton abundance is commonly nutrient-limited, it becomes light-limited when nutrients are abundant (e.g., Karlsson et al. 2009).

We hypothesized that the increases in phytoplankton caused by added nutrients or salt would shade the deeper periphyton and thereby reduce the biomass of periphyton. When we added nutrients, periphyton initially showed no change in biomass, but after 77 d, it showed an increase. The increase exhibited no interaction with light levels, suggesting that while the periphyton was nutrient-limited, it was not light-limited (unlike phytoplankton). In the case of added salt, we also observed an increase in periphyton, which was in contrast to our shading hypothesis: that the increase of phytoplankton caused by nutrient and salt additions would indirectly cause a decline in periphyton due to a shading effect. The most likely explanation for our observation of increased periphyton with salt addition is that the salt was toxic to one of the major periphyton grazers (e.g., banded mystery snails); as a result, the lower grazing pressure by snails in the high-salt treatments allowed for an increase in periphyton. Consistent with this result are other recent studies that have found periphyton increases with elevated salt (e.g., Van Meter et al. 2011, Dananay et al. 2015). However, the novel takeaway message is that increases in nutrients and salts appear to affect phytoplankton and periphyton abundance additively and not synergistically.

Macroalgae

We also found several surprising responses to our manipulations on the *Nitella* macroalgae. First, *Nitella* showed no increase in biomass when we added nutrients (Fig. 3). This suggests not only that this macroalga is not limited by nutrients, but also that it is not limited by the reduced sunlight availability that occurred as the added nutrients initiated a phytoplankton bloom. This resiliency of *Nitella* under different nutrient conditions may reflect its ability to extract and store considerable amount of nutrients from the water (Kufel and Kufel 2002).

Clear evidence that *Nitella* is not harmed by reduced sunlight availability comes from the results of our light manipulations (Fig. 3). In the absence of added salt (e.g., 15 mg Cl⁻/L), large

reductions in sunlight resulted in substantial higher *Nitella* biomass. Previous studies have shown that charophytes, such as *Nitella*, can use low light intensities effectively and can therefore survive in deep water (Blindow 1992, Kufel and Kufel 2002). The fact that *Nitella* did not respond favorably to the shading effect of the phytoplankton bloom that occurred with added nutrients suggests that the reduced light availability from the phytoplankton bloom was weaker than the reduced light availability in our light manipulations, or might have caused some type of resource competition that we did not measure.

We also found, for the first time, that *Nitella* is highly sensitive to increased salt. This is surprising given that *Nitella* has been found in salinities up to 5000 mg/L (James et al. 2003). When growing well under low-light conditions, increases in salt severely reduced *Nitella* biomass in our treatments. Moreover, photosynthetic pigment concentration can indicate the physiological status of a plant (Peñuelas et al. 1995) and the lower chl *a* concentration in *Nitella* in high-salt treatments also indicates that the macrophyte was experiencing physiological stress caused by elevated salt concentration. While the impact of salt disappeared under high-light conditions, this was simply because *Nitella* grew so poorly under high-light conditions that there was very little remaining scope for a response to salt.

This high sensitivity to increased salt concentrations is particularly relevant given that many salt-polluted lakes can achieve salt concentrations of 250 to 1,000 mg Cl⁻/L (Novotny et al. 2008). An additional concern arises if tributaries carry high salt concentrations into lakes and then this water sinks to lake bottoms (due to the higher density of the salty water). Under this scenario, concentrated salt water would descend to the deeper waters where *Nitella* lives and this would cause a major decline in *Nitella* abundance, with potential cascading effects on the animals that depend on the *Nitella* meadows for habitat. In summary, our results suggest that *Nitella* meadows are very susceptible to increased salt, but do not respond to increases in nutrients.

For filamentous algae, we did not find any effects of increased nutrients or light. However, we found filamentous algae to decrease with elevated

salt indicating that filamentous algae have a similar salinity tolerance as *Nitella*. Hintz et al. (2017) also found that the biomass of filamentous algae decreased substantially with elevated salt levels. Thus, this may turn out to be a common observation in salt-impacted freshwater habitats.

Snails

While neither snail species performed well under low-light conditions, they had unique responses to increased salt and nutrients. The negative response to low-light conditions is likely a response to low periphyton productivity. While we measured periphyton standing crop, which did not respond to light, it appears that the productivity of periphyton growth was quite limiting to the growth of pond snails. Further support for this conclusion can be found in the pond snails, which experienced a higher abundance when nutrients were added, which increased periphyton standing crop. In contrast, banded mystery snails did not respond to the nutrient addition (Fig. 4).

Banded mystery snails are more commonly found in mesotrophic and eutrophic lakes and ponds (Browne 1978, Lee et al. 2002), although they also can be abundant in some oligotrophic lakes (e.g., Lake George, New York, USA). We therefore expected an increase in banded mystery snails with nutrient addition. The difference in sensitivity to nutrients may reflect differences in their feeding habits. Banded mystery snails are primarily detritivores, whereas pond snails are primarily periphyton grazers (Lee et al. 2002, Evans-White and Lamberti 2009). As a result, increased nutrients that cause increased periphyton productivity should favor an increased production of pond snails but have weaker effects on banded mystery snails.

A major difference between the two snail species was in their response to increased salt. Pond snails exhibited no harmful effects of increased salt, whereas banded mystery snails were nearly exterminated by high salt concentrations. However, the harmful impact of salt on banded mystery snails could only be observed under medium- and high-light conditions, since low-light conditions caused very few banded mystery snails to survive. Collectively, this suggests that the two snail species have dramatically different tolerances to salt. Moreover, as detritivores,

banded mystery snails assimilate contaminants from the sediments (which can have higher salt concentrations since saltier water has a higher density), while pond snails are grazers and would be less likely to directly assimilate contaminants from the sediments (Lee et al. 2002, Evans-White and Lamberti 2009). As a result, even if banded mystery snails and pond snail have a similar tolerance to salt, banded mystery snails may be exposed to higher levels of salt because of their habit of feeding on the detritus of sediments. Previous studies have shown pond snails to have a high tolerance to salinity (Kefford and Nugegoda 2005, Hintz et al. 2017) and that the tolerance increases with life stage (Kefford et al. 2004, 2007), while no studies on the salt tolerance of banded mystery snails were found. We clearly need much more information on the variation in salt tolerance among gastropods to better understand how salt will alter species assemblages in freshwater habitats. However, the data from our study indicate that the impacts of salt and nutrient inputs on snails are additive rather than synergistic.

Zooplankton

The zooplankton responded positively to increased sunlight and nutrients (Fig. 5). The positive response to increased sunlight and nutrients is not particularly surprising, since both of these factors combined produce a larger standing crop of phytoplankton. Increases in phytoplankton driving increases in zooplankton populations are a common observation in freshwater ecosystems (Canfield and Jones 1995, Ger et al. 2014). When more than 50% of total phytoplankton biomass are cyanobacteria, negative effects from eutrophication start to occur (Ger et al. 2014). However, zooplankton abundance did not suffer any negative effects under high-nutrient conditions; on the contrary, zooplankton populations in our mesocosms tracked the increase in phytoplankton following nutrient addition.

The more novel finding was the decline in zooplankton with increased salt and that this dynamic changed over time. We found that cladocerans and copepods experienced declines in abundance as we increased salt from 250 to 1000 mg Cl⁻/L (Fig. 5). These declines are consistent with past studies of zooplankton sensitivity

to salt (e.g., Petranka and Doyle 2010, Van Meter and Swan 2014, Hintz et al. 2017, Stoler et al. 2017). We also found rotifers to be less sensitive to increased salt, which is also consistent with previous studies (Sarma et al. 2006, Hintz et al. 2017). Collectively, these studies suggest that the decline in copepods that we observed in our mesocosm study was the result of direct toxicity to the added salt.

Given the direct toxicity of the high-salt treatment and given the fact that sodium and chloride do not break down or leave the system, it is quite interesting that the negative impact of salt diminished over time. A similar observation was made recently by Hintz et al. (2017) who tracked zooplankton abundance over time, and a follow-up study provided the underlying explanation. In the case of cladocerans, Cold-snow et al. (2017) found that large populations that experience high concentrations of salt are initially greatly reduced in abundance but not completely eliminated. The few that persist possess salt tolerance and, over time, these salt-tolerant cladocerans reproduce and ultimately rise to an abundance that is similar to the abundance of cladocerans that were never exposed to salt. Given this discovery, it may be the case that the copepods also evolve increased tolerance during the experiment. Many copepods experience different feeding modes during their development with some copepods changing from feeding on phytoplankton to becoming predatory (Brandl 2005). Therefore, the increase in cladocerans and rotifers in the second sampling occasion might be an indirect effect of released predation pressure as higher amount of copepods might still be in earlier developmental stages. However, even if there is always a chance of missing some patterns throughout the sampling period, our samples were taken in response to when communities were diverging among the treatments. Temperature could also influence zooplankton reproduction; however, we did not see any general drastic drop in the zooplankton community in the second sampling occasion that would indicate such a pattern (Figs. 5 and 6). In terms of our focus on the combined effects of added nutrients and road salts, our results suggest that the two anthropogenic factors have additive effects and not synergistic or antagonistic effects.

CONCLUSIONS

Eutrophication and salinization are two ecosystem stressors that are being experienced in aquatic ecosystems around the world. While eutrophication has been studied for decades, the ecological effects of salinization are only recently receiving attention and the combined effects of the two stressors have received no attention. Our study has found that the combined effects of the two stressors—across the range of values examined—are entirely additive for all of the taxa we examined including phytoplankton, periphyton, macroalgae, snails, and zooplankton. While the impacts of anthropogenic additions of nutrients and salt are not synergistic, their combined effects on aquatic ecosystems are still of tremendous concern since they both contribute to major changes including phytoplankton and periphyton blooms (via bottom-up and top-down mechanisms, respectively). Equally important are the impacts of salinization alone, including causing a major decline in numerous taxa including zooplankton, snails, and macroalgae. One would reasonably predict that such declines would have further cascading effects on consumers that rely on the salt-sensitive prey and on species that rely on the expansive *Nitella* meadows (and perhaps other salt-sensitive macrophyte species) in freshwater lakes for habitats. Overall, the combined effects of salinization and eutrophication might fast-forward the process of lakes becoming hypertrophic, and this could potentially result in devastating algal blooms and poor water quality.

As the first study to examine the combined effects of salt and nutrients, there is clearly much more work to be done. For example, the striking negative effects of NaCl road salt on macroalgae suggest that many other macroalgae species, and perhaps many aquatic plant species that are adapted to low salinities, may be highly susceptible to road salt pollution in freshwater ecosystems. The rebounding of both cladocerans and copepods after initial declines following salt exposure suggests evolved tolerance, but our current insights into this possibility are limited to only one species of cladoceran (Coldsnow et al. 2017). There has also been growing interest in using other road salts (or mixtures of salts) for deicing roads including MgCl₂ and CaCl₂. Little research has examined

the ecological impacts of these alternative salts and organic salt additives (but see Schuler et al. 2017b, Schuler and Relyea 2018). As we move forward on these frontiers, we will have a much more holistic idea of how anthropogenic impacts are altering aquatic ecosystems and develop management strategies for their mitigation.

Through these direct and indirect temperature effects, in combination with reduced wind speed and reduced cloudiness, summer heatwaves boost the development of harmful cyanobacterial blooms. These findings warn that climate change is likely to yield an increased threat of harmful cyanobacteria in eutrophic freshwater ecosystems.

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