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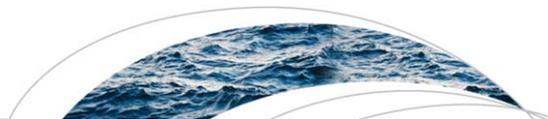
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RESEARCH ARTICLE

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Special Section:

Responses to Environmental Change in Aquatic Mountain Ecosystems

Key Points:

- Montane lakes can be used as early indicators of the impacts of environmental stressors on aquatic communities at lower elevations
- Zooplankton from a fishless and fish-present lake were subjected to acidification and nutrient enrichment from nitrogen deposition
- Zooplankton responded differently to nitrogen by lake type and the combination of acid and nitrogen resulted in ecological surprises

Supporting Information:

- Supporting Information S1

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The Interactive Effect of Multiple Stressors on Crustacean Zooplankton Communities in Montane Lakes

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Abstract Nonnative fish introductions have altered thousands of naturally fishless montane lakes, resulting in cascading food web repercussions. Nitrogen deposition has been recognized as an anthropogenic contributor to acidification and eutrophication of freshwater ecosystems, which may affect the abundance and composition of planktonic communities. This study identified responses of zooplankton communities from two lakes (fish present versus absent) in Mount Rainier National Park to manipulations simulating an episodic disturbance of acidification and eutrophication via nitrogen addition in mesocosms. Zooplankton communities from lakes with different food web structure (i.e., fish present or absent) responded differently to the singular effects of acid and nitrogen addition. For instance, zooplankton biomass decreased in the acid treatment of the fishless lake experiment, but increased in response to acid in the fish-present experiment. In contrast, the combination of acid and nitrogen often resulted in weak responses for both lake types, resulting in nonadditive effects, i.e., the net effect of the stressors was in the opposite direction than predicted, which is known as a reversal or “ecological surprise.” This experiment demonstrates the difficulty in predicting the interactive effects of multiple stressors on aquatic communities, which may pose significant challenges for habitat restoration through fish removal.

1. Introduction

The remote montane lakes found in high elevation regions have been referred to as sentinels of change for their use in monitoring shifting climate regimes and levels of anthropogenic influence across the landscape (Battarbee et al., 2009; Williamson et al., 2008). Although these high elevation lakes are often regarded by the public as pristine ecosystems, many have experienced extensive disturbance, such as nonnative species introductions (Eby et al., 2006), atmospheric pollution (Baron et al., 2000), climate change (Battarbee et al., 2002), and an increased susceptibility to UV radiation (Schindler et al., 1996).

The stocking of nonnative fishes into mountain lakes is a common practice globally (Pister, 2001; Schindler & Parker, 2002). Nonnative fishes can significantly alter food web structure by assuming the position of top predator (Vander Zanden et al., 1999). For instance, trout stocking has resulted in declines of amphibians, macroinvertebrates, and plankton communities in lakes that were historically fishless (Knapp et al., 2001; Pilliod & Peterson, 2001; Schilling et al., 2009). One of the most significant effects of introducing planktivorous fishes is size-selective predation of large-bodied zooplankton, like *Daphnia* (Brooks & Dodson, 1965). In lakes where fish populations are abundant, less efficient small-bodied grazers will dominate the zooplankton community as a result of size-selective predation (Hall et al., 1976) and primary productivity is expected to increase. In lakes where fish are absent, a higher abundance of large-bodied zooplankton is expected (Liss et al., 2002).

An additional stressor in mountain lakes is the deposition of atmospheric nitrogen (N), which has been linked to elevated lake nitrate concentrations and shifts in phytoplankton communities (Baron et al., 2000). Nitrogen deposition can impact aquatic systems by increasing the amount of reactive nitrogen, which could result in both acidification and eutrophication (Galloway et al., 2003). Mountain lakes are particularly susceptible to the effects of acidification because of poor buffering capacity (Armstrong & Schindler, 1971; Brakke & Loranger, 1987; Clow et al., 2002). Acidification can alter zooplankton communities through reductions in abundance, biomass, diversity, and a loss of acid-sensitive species (i.e., herbivores, large-bodied species) (Confer et al., 1983; Havens et al., 1993; Keller & Yan, 1998; McCullough & Horwitz, 2010). Phytoplankton biomass may increase in acidified lakes as a result of increases in large inedible phytoplankton that zooplankton cannot readily consume (Schindler, 1990).

In temperate lakes, nutrient additions can lead to enhanced growth rates of phytoplankton production, as well as nuisance and harmful algal blooms (Anderson et al., 2002). However, in ultraoligotrophic lakes it is possible that slight nitrogen increases may not lead to degradation, but rather enrich the system by providing a limiting nutrient, after which further perturbation pushes the system beyond its normal operating range, leading to stress effects (Odum et al., 1979). Williams et al. (2016) observed increased phytoplankton biomass in response to elevated nitrogen concentrations in bioassays from oligotrophic mountain lakes. However, the effects of increased nutrients on zooplankton are equivocal. In oligotrophic systems, bottom-up control is most important, where phytoplankton are limited by nutrient availability, which limits zooplankton, and can result in positive correlations between zooplankton and phytoplankton (McQueen et al., 1986). However, top-down regulation of phytoplankton by zooplankton may also result in a negative relationship, particularly if *Daphnia* (a keystone species) is present (McQueen et al., 1986). Mesocosm experiments suggest that nutrients can have a positive effect on zooplankton biomass in shallow eutrophic lakes with fish removed (Vakkilainen et al., 2004) and in fishless oligotrophic lakes (Neill, 1984), but this effect could be mediated by the presence of planktivorous fish.

Multiple stressors are common in natural systems, yet it is difficult to predict whether their interaction will exacerbate or ameliorate the effects of individual stressors on communities (Folt et al., 1999). A recent review of multiple stressors in freshwater ecosystems showed that the combined effects of stressors most often elicited an antagonistic response, indicating that the combined effects of stressors were less than the sum of the stressors independent effects (Jackson et al., 2016). In mountain lakes, the preexisting condition of the ecosystem largely determines whether acidification or eutrophication will occur first; however both stressors can result in a loss of phytoplankton diversity (Baron et al., 2011) and shifts in phytoplankton species composition (Lafrancois et al., 2004). However, no studies have explored how these stressors impact plankton communities based on differences in food web structure that result from historical fish stocking.

Our objective was to identify the response of zooplankton communities in montane lakes of differing fish stocking histories to acidification and eutrophication manipulations. In the western United States, 95% of >16,000 historically fishless lakes were stocked with fish (Bahls, 1992). Research on the ecological effects of nitrogen deposition has occurred in the U.S. Rocky Mountains and Sierra Nevada; however, little is known about the effects of N deposition in other regions (Fenn et al., 2003; Nanus & Clow, 2004). The Cascade Mountains of Oregon and Washington contain a relatively high percentage of lakes believed to be N-limited, thus making them susceptible to chemical and biological changes from nitrogen deposition (Baron et al., 2011; Elser et al., 2009).

We conducted two simultaneous mesocosm experiments to assess the singular and interactive effects of acid and nitrogen, one using plankton communities from a fish-present lake and another using plankton from a fishless lake (Figure 1). We tested three main hypotheses. First, we expected that acidification would decrease zooplankton abundance, with greater effects on communities from fishless lakes. These communities may be less resilient due to a greater abundance of acid-sensitive taxa, i.e., large-bodied crustaceans (Havens et al., 1993). Second, we predicted that the nitrogen treatment would increase phytoplankton (e.g., Williams et al., 2016), but the presence of larger-bodied zooplankton in the fishless community would more effectively control phytoplankton growth (Sarnelle & Knapp, 2005) compared to the less efficient grazers in the fish-present zooplankton community. This should lead to an increase in zooplankton abundance in the fishless community. Third, we hypothesized that the positive effects of nitrogen would buffer the negative effects of acid, such that there would be minor or no change in zooplankton communities in the nitrogen + acid treatment. Using systems with these legacies can help to identify the interactive effects of multiple stressors, allowing for projections of how lakes in more temperate climates could respond in the future.

2. Methods

2.1. Study Site and Experimental Setup

The experiment was conducted in Mount Rainier National Park at the park headquarters in Longmire, WA, USA. Minimal disturbance to native flora and fauna was achieved by placing the main tank array outside of the designated wilderness area, and using a common garden experimental design to source water from a single site, Mowich Lake, which has similar physical and chemical characteristics to other study lakes, discussed below (Table 1). A common garden design is ideal for this type of study because both communities

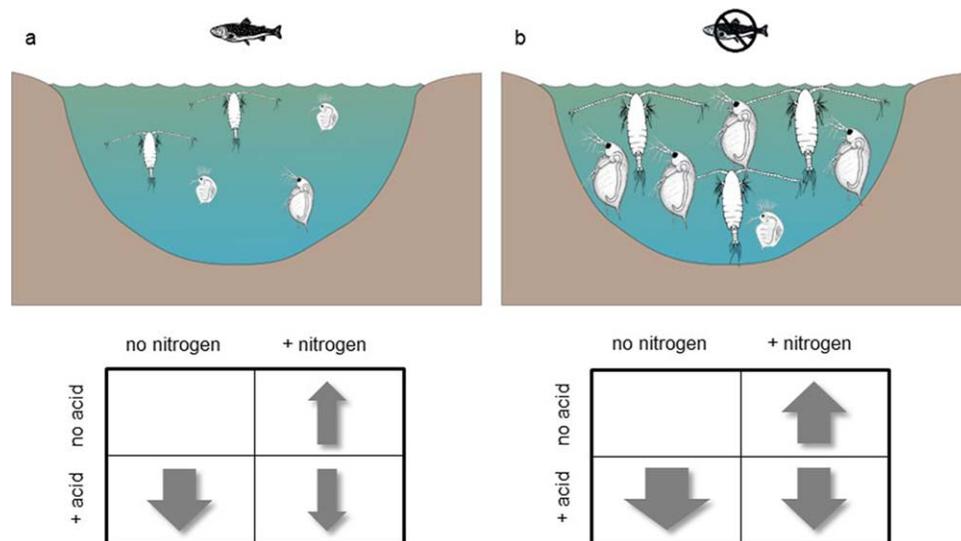


Figure 1. Conceptual diagram of experimental design, where the (a) fish-present lake is characterized by smaller zooplankton in low abundances, in contrast to the (b) fishless lake, which is characterized by larger zooplankton in greater abundances. Directional arrows indicate predicted changes to zooplankton abundance resulting from acidification and nitrogen addition based on fish presence or absence, while width indicates magnitude of change. We hypothesized that the acid treatment would have the most negative effects on zooplankton, but that the positive effects of the nitrogen treatment would moderate the effects of the acid in the nitrogen + acid treatment combination. We propose that the magnitude of effects will be greatest in the fishless lake because of the dominance of acid-sensitive large-bodied taxa.

were subjected to the same conditions, allowing for changes to be attributed to the treatments. Mowich Lake is the deepest lake in the park, and park officials determined water withdrawal from Mowich posed the smallest disturbance compared to the smaller study lakes. Like many lakes in the Cascades, these sites are oligotrophic and remain frozen over for up to 8 months of the year.

A 2 × 2 factorial design was used to allow two treatments (acid and nitrogen) to be studied at two levels (with and without addition). Each treatment combination was replicated four times. The experiment was replicated using plankton from a fishless lake (Clover Lake) and from a fish-present lake (Snow Lake), such that the two factorial experiments were run simultaneously. Zooplankton were collected from Snow Lake and Clover Lake, which were both historically fishless waterbodies, but were subjected to a legacy of fish stocking. The park began recording stocking data for both lakes in 1926, which included introductions of steelhead and rainbow trout (*Oncorhynchus mykiss*), cutthroat trout (*Oncorhynchus clarkii*), and brook trout (*Salvelinus fontinalis*) until 1962 (Clover Lake) and 1970 (Snow Lake). Snow Lake continues to support

Table 1
Physicochemical Conditions of Study Lakes

Variable	Mowich Lake (water source)	Snow Lake (fish-present experiment)	Clover Lake (fishless experiment)
Latitude (N)	46°56'16.08"	46°45'27.85"	46°55'44.75"
Longitude (W)	121°51'44.75"	121°41'52.96"	121°35'36.41"
Elevation (m)	1,500	1,424	1,743
Maximum depth (m)	60.0	10.9	14.0
Area (ha)	45.0	2.6	2.5
pH	7.02 ^a	6.90 ^a	6.90 ^a
Specific conductance ($\mu\text{S}\cdot\text{cm}^{-1}$)	12.5 ^a	10.8 ^a	14.0 ^a
Secchi depth (m)	19.0 ^a	7.0 ^a	12.0 ^a
Total phosphorus ($\mu\text{g}\cdot\text{L}^{-1}$)	3.3 ^b	4.8 ^b	4.1 ^b
Total nitrogen ($\mu\text{g}\cdot\text{L}^{-1}$)	35.0 ^b	20.0 ^b	53.8 ^b

^aValues averaged across study period, and collected from surface water where applicable. ^bUnpublished seasonally averaged surface water data from Robert Hoffman (U.S. Geological Survey, Corvallis, Oregon).

populations of fish, while the absence of fish in Clover Lake was confirmed by snorkel surveys in 2013. Though these fish species are not typically known as planktivores, there are multiple lines of evidence that they readily consume zooplankton in these resource-limited mountain systems (Eby et al., 2006 and references therein). For instance, a study from Utah found that both brook trout and cutthroat trout consume diatomid copepods, *Daphnia*, and other crustacean zooplankton in mountain lakes (Carlisle & Hawkins, 1998). Recent surveys (1999–2015) have indicated that the fish present in Snow Lake are small: brook trout averaged 210 mm (range 179–239 mm), while cutthroat trout averaged 217 mm (range 193–241 mm), which suggests that zooplanktivory is likely (e.g., Tremblay & Magnan, 1991).

The body size, composition, and density of zooplankton in our study lakes was largely reflective of other fishless and fish-present mountain lakes in the region and beyond (e.g., Donald et al., 2001). Most strikingly, zooplankton densities in our fishless lake were more than 2 orders of magnitude larger than the fish-present lake, with densities averaging $\sim 7,605$ individuals·m⁻³ compared to ~ 73 individuals·m⁻³, respectively. At the beginning of the experiment, the fish-present lake was dominated by two copepods: *Hesperodiaptomus franciscanus* (70%) and *Eucyclops agilis* (29%), with no *Daphnia* or *Holopedium* present (supporting information S1, Figure S1-1). This trend continued throughout the summer, with cladocerans comprising, on average, $\sim 2\%$ of the community. Copepods are generally thought to be better than cladocerans at avoiding predation by fish (Hambright & Hall, 1992). By contrast, the fishless lake was comprised of *Hesperodiaptomus franciscanus* (69%), *Holopedium gibberum* (18%), and *Daphnia pulicaria* (13%) at the start of the experiment, but switched to codominance by *Daphnia* later in the summer (supporting information S1, Figure S1-1). In addition, the average body size of zooplankton in the fishless lake was ~ 200 μm larger than zooplankton in the fish-present lake at the start of the experiment (fishless: 948 μm ; fish-present: 768 μm). It is possible that differences in zooplankton abundance or biomass, body size, and community composition could be the result of factors other than fish, such as water chemistry, lake morphometry, productivity, and water temperature (e.g., Messner et al., 2013; Strecker et al., 2008). Water chemistry, including pH and specific conductance, and lake morphometry variables were similar across study lakes (Table 1). Conversely, total phosphorus and Secchi depth suggest that Snow Lake (fish) is likely more productive than fishless Clover Lake (Table 1), which should lead to larger and more abundant zooplankton; however, this is the opposite of what we observed (supporting information S1, Figure S1-1). Water temperature is another factor that can affect zooplankton: fishless Clover Lake is warmer than fish-present Snow Lake (supporting information S1, Figure S1-2), which could have affected zooplankton abundance. However, survey data from mountain lakes indicate that warmer fishless lakes have lower zooplankton biomass than colder fish-present lakes (Messner et al., 2013). Additionally, warmer water temperatures have experimentally been shown to decrease zooplankton biomass in alpine lakes (Strecker et al., 2004). Thus, our data do not support these alternative hypotheses and the presence of fish seems to be the most parsimonious explanation for differences in zooplankton communities.

Thirty-two 140 L gray Rubbermaid polyethylene tanks were used to simulate ambient lake conditions. Tanks were filled with water from the epilimnion of Mowich Lake, which was filtered through 80 μm mesh to remove Mowich Lake zooplankton. Zooplankton were collected mid-afternoon at the deepest spot of the fish-present and fishless lakes, from 2 m above the lake bottom to the surface (accounting for the length of the net) using a 30 cm diameter plankton net with 80 μm mesh. Zooplankton were stored in 19 L carboy containers, and transported to the experimental tanks, where they were added to tanks at 1.5X ambient density from each source lake. Aquatic predators, such as mosquito larvae, were removed by hand. Tanks were inoculated with zooplankton from the fish-present lake (Snow) and the fishless lake (Clover) on 14 August and 15 August 2013, respectively. Tanks were located in partial shade and covered with mesh to prevent aerial colonization.

In order to ensure that the source water did not have an undue influence on zooplankton communities in the experiment, we established reference mesocosms using water from either the fishless lake or fish-present lake (i.e., Clover zooplankton in Clover water, Snow zooplankton in Snow water). These reference mesocosms were situated at the lakes in order to obtain water for the experiments, in contrast to the main experimental mesocosms, which were located at the park headquarters. Despite some early initial differences, zooplankton abundance was not significantly greater in reference tanks (using Clover or Snow Lake water) compared to control tanks (using Mowich Lake water) over the course of the experiment (supporting

information S1, Figure S1-2, Table S1-1), indicating that source water had no significant influence on the experiment. These early differences are likely due to zooplankton acclimation and stress from transport.

Treatments of nitrogen and acid (as KNO_3 and HCl , respectively) were added on 17 August 2013 to randomly assigned tanks, simulating a single episodic pulse characteristic of a spring melt event. Tanks with nitrogen addition received $69.3 \mu\text{g}\cdot\text{L}^{-1}$ of nitrogen as KNO_3 to mimic a 30–40% increase above ambient levels, representing potential nitrogen concentrations resulting from spring glacial melt as observed in other mountainous areas of the West (Baron et al., 2011; Clow & Campbell, 2008; Nanus et al., 2012). Though this nitrogen addition is modest, it is within the range of concentrations observed to promote increases in phytoplankton in mountain lakes (Williams et al., 2016). Tanks with acid addition were titrated from a pH ~ 7 to an endpoint of pH 5.5 to mimic moderate acidification, which has been used in similar studies to identify impacts related to atmospheric deposition (Lafrancois et al., 2004). Large-bodied adult crustaceans begin to decline at pH < 6.0 (Havens et al., 1993), thus our treatment is reasonable.

2.2. Sampling and Sample Processing

Sampling of experimental tanks took place weekly, from 16 August 2013 (week 1, pretreatment baselines) to 20 September 2013 (week 6). The lakes were also sampled each week to compare differences in environmental conditions and plankton composition to the tanks. Water temperature, dissolved oxygen, and specific conductance were measured from mid-depth in the tanks and in the lakes at surface and 1 m intervals using a YSI ProPlus (Yellow Springs, OH) at midday. Water temperature was consistent between treatments over time; on average, temperature varied less than 0.30°C between all tanks (supporting information S1, Table S2-1, Figure S2-1). Water temperature of the mesocosms was usually warmer than the lake surface temperature for the fish-present lake (mesocosm average = $14.8^\circ\text{C} \pm 1.9$ SD; lake average = $12.1^\circ\text{C} \pm 1.3$ SD), but roughly the same in the fishless lake (mesocosm average = $14.8^\circ\text{C} \pm 1.9$ SD; lake average = $16.1^\circ\text{C} \pm 1.1$ SD). Dissolved oxygen ($\text{mg}\cdot\text{L}^{-1}$) was not significantly different between tanks throughout the experiment for either community (supporting information S1, Table S2-1, Figure S2-1), though the mesocosms had lower dissolved oxygen (% saturation) than the lakes, likely due to reduced mixing (fish-present averages: mesocosms = $71.1\% \pm 5.9$ SD; lake = $93.6\% \pm 8.2$ SD; fishless averages: mesocosms = $72.2\% \pm 4.8$ SD; lake = $87.0\% \pm 6.7$ SD). The pH of surface water was measured in situ using an Orion 290A pH meter (Thermo Fisher Scientific, Waltham, MA). Specific conductance and pH of the mesocosms was similar to the lake, though specific conductance in the fish-present lake was lower than the mesocosms, reflecting the different source water origin (Table 1; mesocosm average = $17.0 \mu\text{S}\cdot\text{cm}^{-1} \pm 2.6$ SD; lake average = $10.7 \mu\text{S}\cdot\text{cm}^{-1} \pm 0.4$ SD).

Water for nutrient analysis was collected from the epilimnion using a 10 m long, 2.54 cm diameter tygon tube, and from the tanks with grab samples once they had been mixed. Water was transferred into 125 mL HDPE bottles and kept cool until frozen in the laboratory. Total phosphorus (TP) samples were digested with a persulfate solution and heat, and analyzed on a Shimadzu UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan) using the molybdenum blue colorimetric method (method detection limit: $0.002 \text{ mg}\cdot\text{L}^{-1}$; precision: $\pm 0.004 \text{ mg}\cdot\text{L}^{-1}$) (Cooperative Chemical Analytical Laboratory, 2010). In order to test whether the nitrogen addition was effective, a subset of tanks that received nitrogen ($n = 8$) were randomly chosen and sampled after the treatment application on week 2, in addition to analyses of all tanks on week 6. Total nitrogen samples (TN) were digested with a persulfate solution and analyzed on a Technicon Auto-Analyzer II (Seal Analytical, Mequon, Wisconsin) with a colorimetric determination of nitrate and nitrite (method detection limit: $0.01 \text{ mg}\cdot\text{L}^{-1}$; precision: $\pm 0.01 \text{ mg}\cdot\text{L}^{-1}$) (Cooperative Chemical Analytical Laboratory, 2013).

Water for chlorophyll *a* analysis was collected from the epilimnion using the aforementioned tygon tube, and from the tanks with grab samples once they had been mixed. Water for chlorophyll analysis was divided into two fractions, one passing through a $35 \mu\text{m}$ filter to represent the highly edible algal fraction (Cyr & Curtis, 1999), and one unfiltered to represent total algae, using chlorophyll *a* as a proxy of algal biomass. Though zooplankton can consume larger size fractions of phytoplankton, we believe that $35 \mu\text{m}$ represents a realistic benchmark of highly edible taxa. Each fraction was filtered onto a $1.2 \mu\text{m}$ pore size glass fiber filter and frozen. Periphyton was collected on two porcelain tiles, which were placed in the tanks on week 1, individually scraped at two intervals (weeks 3 and 6), and filtered through a glass fiber filter. Chlorophyll *a* was extracted with acetone for 20 h in a dark refrigerator, and concentrations were measured on a

TD-7200 fluorometer (Turner Designs, Sunnyvale, CA), using the modified fluorometric technique (Arar & Collins, 1997).

Zooplankton were sampled from each tank by mixing the water and collecting from the bottom to the top of the tank using a 2.5 L bucket, and poured through an 80 μm mesh filter. Six replicate hauls, representing a total filtered volume of 10% of the tank volume, were collected. Weekly samples were taken from each lake using identical methods to those described above for the experimental setup. Zooplankton were preserved in a 70% ethanol solution. Taxonomic guides were used to identify adult crustacean taxa (Haney, 2013; Thorp & Covich, 2009) to species level when possible using a Leica M165C microscope and IC80HD camera (Leica Microsystems Inc., Buffalo Grove, IL), following EPA protocol (Environmental Protection Agency, 2003). Zooplankton abundance was calculated by counting the entire sample, which ranged from 0 to 459 individuals (mean = 71.2 ± 86.6 SD). Biomass was estimated by measuring the length of a subset of 10 animals per taxa in each sample, which were averaged to calculate biomass with length-weight regressions (Culver et al., 1985; Lawrence et al., 1987; McCauley, 1984). Zooplankton body size was calculated based on length measurements, using abundance-weighted averages in each treatment.

2.3. Statistical Analyses

Two-factor analysis of variance (ANOVA), two-factor repeated measures ANOVA (RM-ANOVA), and a permutational multivariate analysis of variance (MANOVA) were used to test the singular and interactive impacts of eutrophication and acidification using R version 3.0.1 (R Core Team, 2013) with the *EZ* and *vegan* libraries (Lawrence, 2013). Environmental conditions (temperature, dissolved oxygen, specific conductance, pH, and total phosphorus), total chlorophyll *a*, and edible chlorophyll *a* were compared between treatments using a RM-ANOVA performed separately on the two lake types (fish-present and fishless lakes) for weeks 2–6 (excluding week prior to treatments being added). A two-way ANOVA was used on total nitrogen for week 6. Periphyton growth as measured in chlorophyll *a* was tested with a two-factor RM-ANOVA on samples for weeks 4 and 6. Variables with statistically significant interactions were visually inspected using interaction plots. The response of zooplankton community composition to treatments for weeks 2–6 was tested using a permutational MANOVA ($n = 9,999$ permutations) (Anderson, 2001). Species abundances were Hellinger transformed prior to testing (Legendre & Gallagher, 2001).

The assumptions of normality, homogeneity, and sphericity were tested using the Shapiro-Wilk, Levene's, and Mauchly's tests, respectively. Violations of sphericity used Greenhouse-Geisser corrections. Environmental conditions and zooplankton community data between treatments were compared on week 1 (prior to treatment additions) with a two-factor ANOVA and determined that there were no significant differences prior to treatment application, with the exception of body size in the nitrogen treatment of the fishless experiment (supporting information S1, Table S2-2). However, this difference is likely not biologically relevant, as the difference in body size was $<100 \mu\text{m}$ (control: $1162 \mu\text{m} \pm 230$ SD; nitrogen: $1248 \mu\text{m} \pm 92$ SD). Last, in order to evaluate the combined effect of stressors, we calculated the predicted additive effect (X_p) of zooplankton community metrics following Jackson et al. (2016):

$$X_p = (X_n - X_u) + (X_a - X_u) + X_u \quad (1)$$

where X_u is the control, X_n is the nitrogen treatment, and X_a is the acid treatment.

3. Results

3.1. Environmental Conditions

The treatment applications in the tanks created the desired conditions and suggest that these simulations were responsible for impacting the plankton communities rather than the minor differences that existed in other environmental variables (e.g., temperature). As expected, pH was significantly reduced in the acid treatment for both sets of experiments: pH decreased by 17% (average 6.0 in acid treatments versus 6.6 in nonacid treatments) and 16% (average 5.9 in acid treatments versus 6.6 in nonacid treatments) in the fish-present and fishless experiment, respectively (supporting information S1, Table S2-1, Figure S2-1). Total nitrogen was significantly higher (33%) in the tanks with nitrogen addition (average $280 \mu\text{g}\cdot\text{L}^{-1}$) compared to tanks without nitrogen addition on week 2 (average $210 \mu\text{g}\cdot\text{L}^{-1}$), indicating that the nitrogen addition was successful (supporting information S1, Table S2-3, Figure S2-2). Total nitrogen was not observed to be significantly different between treatments on week 6 (supporting information S1, Figure S2-2), which was

Table 2
Statistical Summary of RM-ANOVA on Total and Edible Chlorophyll *a* Fractions for Weeks 2–6, While Periphyton Statistics Are for Weeks 4 & 6

Variable	Treatment	Fish-present experiment			Fishless experiment		
		F-ratio	p-value	ES	F-ratio	p-value	ES
Total chlorophyll <i>a</i>	Nitrogen [1,12]	6.496	0.026**	↑126%	1.492	0.245	
	Acid [1,12]	0.001	0.971		2.587	0.134	
	Nitrogen × Acid [1,12]	0.144	0.711		3.483	0.087*	↑48%
	Time [4,48]	3.098	0.024**		0.778	0.545	
	Time × Nitrogen [4,48]	0.332	0.855		0.417	0.795	
	Time × Acid [4,48]	0.442	0.777		0.222	0.925	
Edible chlorophyll <i>a</i>	Nitrogen × Nitrogen × Acid [4,48]	0.247	0.910		0.814	0.522	
	Nitrogen [1,12]	9.790	0.009**	↑135%	3.489	0.086*	↓52%
	Acid [1,12]	2.870	0.116		1.156	0.303	
	Nitrogen × Acid [1,12]	5.485	0.037**	↑203%	4.64	0.052*	↓46%
	Time [4,48]	4.828	0.002**		1.069	0.382	
	Time × Nitrogen [4,48]	0.796	0.534		0.779	0.544	
Periphyton	Time × Acid [4,48]	0.654	0.627		0.916	0.463	
	Time × Nitrogen × Acid [4,48]	3.322	0.018**	↑203%	1.14	0.345	
	Nitrogen [1,12]	2.484	0.141		3.040	0.106	
	Acid [1,12]	4.411	0.058*	↓68%	0.225	0.644	
	Nitrogen × Acid [1,12]	2.201	0.163		0.821	0.383	
	Time [2,23]	3.708	0.078*		11.137	0.006**	
	Time × Nitrogen [2,23]	1.680	0.219		2.927	0.113	
	Time × Acid [2,23]	2.071	0.176		0.205	0.658	
	Time × Nitrogen × Acid [2,23]	2.099	0.173		4.199	0.063*	↑83%

Note. Numbers in brackets indicate degrees of freedom for RM-ANOVA. Effect size (ES) is calculated for significant treatments by comparing marginal means. ES for significant interaction terms compares the nitrogen + acid treatment to the control. * $p < 0.10$; ** $p < 0.05$.

likely due to biological uptake (e.g., periphyton) or sedimentation of N, which was not reapplied after the episodic simulation on week 2.

Total phosphorus increased significantly in all tanks during the experiment and was also significantly greater in the acid treatment over time in both the stocked and fishless lake experiments, with 20 and 25% increases, respectively, compared to control tanks (supporting information S1, Table S2-1, Figure S2-1). A follow-up experiment indicated that the most likely source of the increased phosphorus was a leaching effect from the tanks themselves (supporting information S1, Text S3-1, Figure S3-1). There is no indication that these increases in TP had any noticeable effects on the experimental outcomes. For instance, we would expect an increase in TP to stimulate phytoplankton, particularly in acid treatments, which had the largest increases in TP (supporting information S1, Table S2-1, Figure S2-1). This, however, was not the case, as there were no significant effects of acid on chlorophyll *a* concentrations (with the exception of one significant interaction), discussed below. Both TN and TP were an order of magnitude higher in the mesocosms and lake samples at the beginning of the experiment (supporting information S1, Figure S2-1, Figure S2-2) compared to historical data (Table 1), likely due to the capture of nutrient run-off following ice-off in July.

Total chlorophyll *a* concentrations were significantly greater in the nitrogen treatment in the fish-present experiment (Table 2, Figure 2), exhibiting, on average, a 126% increase following treatment application compared to treatments without nitrogen. There was a significant nitrogen × acid interaction in the fish-present experiment, whereby nitrogen had a positive effect on edible chlorophyll *a* concentration in the presence of acid, exhibiting a threefold increase over controls (Table 2, Figure 2). There were some treatment effects that approached significance in the fishless lake mesocosms, including a 48% increase in total chlorophyll *a* in the nitrogen + acid treatment compared to the control and a 46% decrease in edible chlorophyll *a* concentration in the nitrogen + acid treatment (Table 2, Figure 2). Although there were no differences between treatments in periphyton growth, there were trends that approached significance: a 68% decline in the acid treatment in the fish-present experiment and an 83% increase in the nitrogen + acid treatment, both compared to the control (Table 2, Figure 2). Overall, chlorophyll concentrations in the

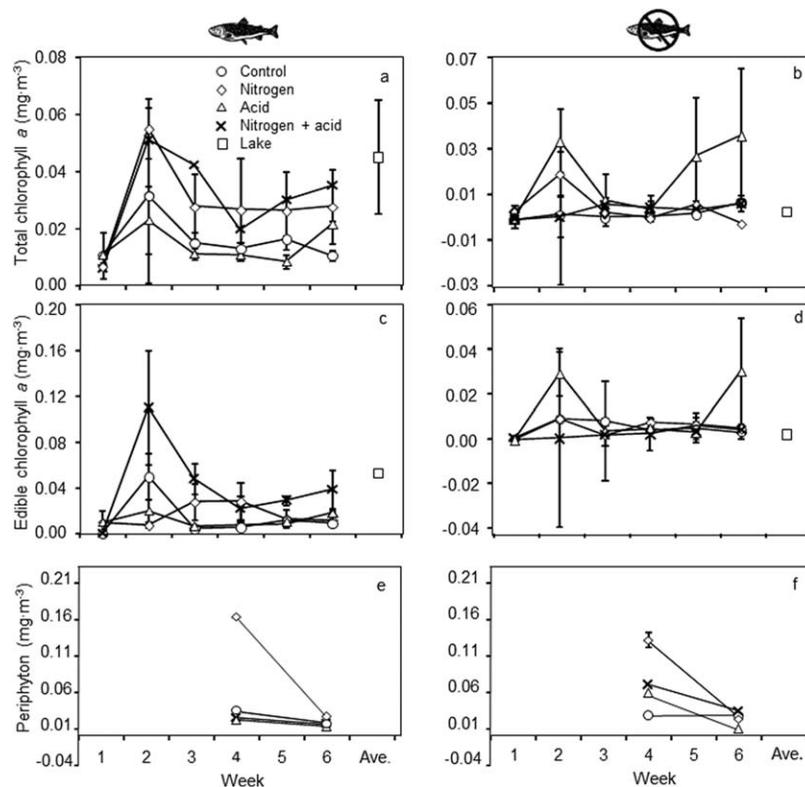


Figure 2. Average chlorophyll *a* concentration ($\mu\text{g}\cdot\text{L}^{-1}$) for both experiments for weeks 1–6 ($\pm\text{SE}$). (a and b) Total chlorophyll, (c and d) edible chlorophyll, (e and f) periphyton, in the fish-present community (a, c, and e), and the fishless lake community (b, d, and f). The week 2 data point for the nitrogen \times acid treatment in Figure 2c is skewed by two tanks with unusually high values, which gives the appearance of the edible fraction being larger than total. The symbol *Lake* represents epilimnetic chlorophyll *a* values from the respective fish-present and fishless lake, averaged over the experiment.

treatment tanks were lower than previously reported and observed in regional lakes, which may have been the result of photoinhibition (Powles, 1984).

3.2. Zooplankton Community Responses

Zooplankton abundance in the fish-present experiment increased slightly across all treatments; however there were no significant treatment effects (Table 3, Figures 3a and 4a). Zooplankton biomass spiked on week 2 in the acid treatment, and then declined rapidly on week 3 until the end of the experiment, resulting in a significant difference over time (Table 3, Figures 3c and 4c). There was a significant interaction of nitrogen \times acid in relation to body size in the fish-present experiment, whereby zooplankton were larger in the presence of both nitrogen and acid compared to treatments with just nitrogen or just acid (Table 3, Figures 3e and 4e). The combined effect of stressors was always greater than the predicted additive effect for zooplankton community metrics (Figure 4), suggesting that these stressors have nonadditive effects. In fact, the net effect of the stressors was in the opposite direction (i.e., more positive) than predicted, which is referred to as a reversal or “ecological surprise” (Jackson et al., 2016). Zooplankton abundance and biomass in the treatment tanks was typically greater than values measured in the lake, likely reflecting a release from predation pressure (Figures 3a and 3c). This may also result from differences in sampling protocols between mesocosms and lakes, where zooplankton could have been missed in lake sampling as a result of diel vertical migration away from predators and/or UV (Williamson et al., 2011).

Zooplankton abundance in the fishless lake experiment generally decreased over time (Figure 3b). There was a significant increase in abundance in the nitrogen treatment, where abundance increased by 31% over the treatment without nitrogen, likely driven by the peak on week 2 (Table 3, Figure 3b). There was a trend toward an increase in abundance and biomass in the presence of both nitrogen and acid as well in

Table 3
Statistical Summary of RM-ANOVA on Zooplankton Community Data for Weeks 2–6

Variable	Treatment	Fish-present experiment			Fishless experiment		
		F-ratio	p-value	ES	F-ratio	p-value	ES
Abundance	Nitrogen [1,12]	1.327	0.272		3.229	0.099*	↑31%
	Acid [1,12]	0.005	0.947		<0.001	0.992	
	Nitrogen × Acid [1,12]	0.423	0.527		3.216	0.098*	↑27%
	Time [4,48]	2.750	0.039**		19.115	<0.001**	
	Time × Nitrogen [4,48]	1.448	0.233		4.875	0.002**	↑31%
	Time × Acid [4,48]	0.687	0.604		1.550	0.203	
	Time × Nitrogen × Acid [4,48]	0.149	0.963		0.450	0.772	
Biomass	Nitrogen [1,12]	0.349	0.566		4.094	0.067*	↑48%
	Acid [1,12]	1.405	0.259		0.667	0.430	
	Nitrogen × Acid [1,12]	1.785	0.206		3.628	0.081*	↑59%
	Time [4,48]	2.143	0.090*		24.842	<0.001	
	Time × Nitrogen [4,48]	1.211	0.319		4.769	0.003**	↑48%
	Time × Acid [4,48]	3.228	0.020**	↑49%	3.043	0.026**	↓23%
	Time × Nitrogen × Acid [4,48]	0.214	0.930		1.779	0.149	
Body size	Nitrogen [1,12]	0.231	0.640		2.056	0.177	
	Acid [1,12]	0.122	0.733		0.071	0.795	
	Nitrogen × Acid [1,12]	6.928	0.022**	↑2%	0.085	0.775	
	Time [4,48]	1.821	0.140		2.913	0.031**	
	Time × Nitrogen [4,48]	2.019	0.107		0.545	0.703	
	Time × Acid [4,48]	1.434	0.237		1.395	0.250	
	Time × Nitrogen × Acid [4,48]	0.781	0.543		0.660	0.623	

Note. Numbers in brackets indicate degrees of freedom for RM-ANOVA. Effect size (ES) is calculated for significant treatments by comparing marginal means. ES for significant interaction terms compares the nitrogen + acid treatment to the control. * $p < 0.10$; ** $p < 0.05$.

the fishless lake experiment, though it was not significant (Figures 4b and 4d). Zooplankton biomass increased significantly over time in the fishless lake experiment nitrogen treatment compared to treatments without nitrogen (48%) (Table 3, Figures 3d and 4d). There was also significant effect of the acid treatment over time, where biomass decreased 23% compared to the treatment without acid (Table 3, Figures 3d and 4d). There was no effect of treatments on zooplankton body size in the fishless experiment, though body sizes declined across all treatments over the course of the study (Table 3, Figures 3f and 4f). As in the fish-present experiment, the combined effect of stressors was always more positive than the predicted additive effect (Figure 4). The mesocosms were similar to ambient lake densities and biomass throughout weeks 3–6 of the experiment (Figure 3b).

Species abundance data revealed that both communities are dominated by a small number of crustacean zooplankton taxa, with most inhabiting both lakes (Figure 5). Given these similarities in composition, their abundances within each community varied markedly and responded differently to treatment applications. A PERMANOVA was used to test if the treatments had an effect on community composition, and revealed a nearly significant trend with the acid treatment in the fishless community experiment ($F_{1,12} = 2.638$, $p = 0.063$). We observed a decline in the calanoid, *Hesperodiptomus franciscanus*, which dominated the composition in the first 2 weeks, and increases in the cladocerans *Daphnia rosea* and *Holopedium gibberum* through week 6 (Figure 5f). By contrast, the controls in the fishless experiment maintained consistent population sizes of *H. gibberum* and slower growth of *D. pulicaria* (Figure 5b).

4. Discussion

Nitrogen deposition can lead to acidification and eutrophication in high elevation lakes, many of which have been subjected to the introduction of top predator fishes. This combination of stressors may be particularly stressful for communities characterized by a few specialized species. Data from mesocosms at the beginning of the experiment indicate that zooplankton body size and biomass were greater in the fishless experiment compared to the fish-present experiment, supporting our hypothesis that fish predation is affecting zooplankton communities in these mountain lakes (Brooks & Dodson, 1965; Eby et al., 2006). We

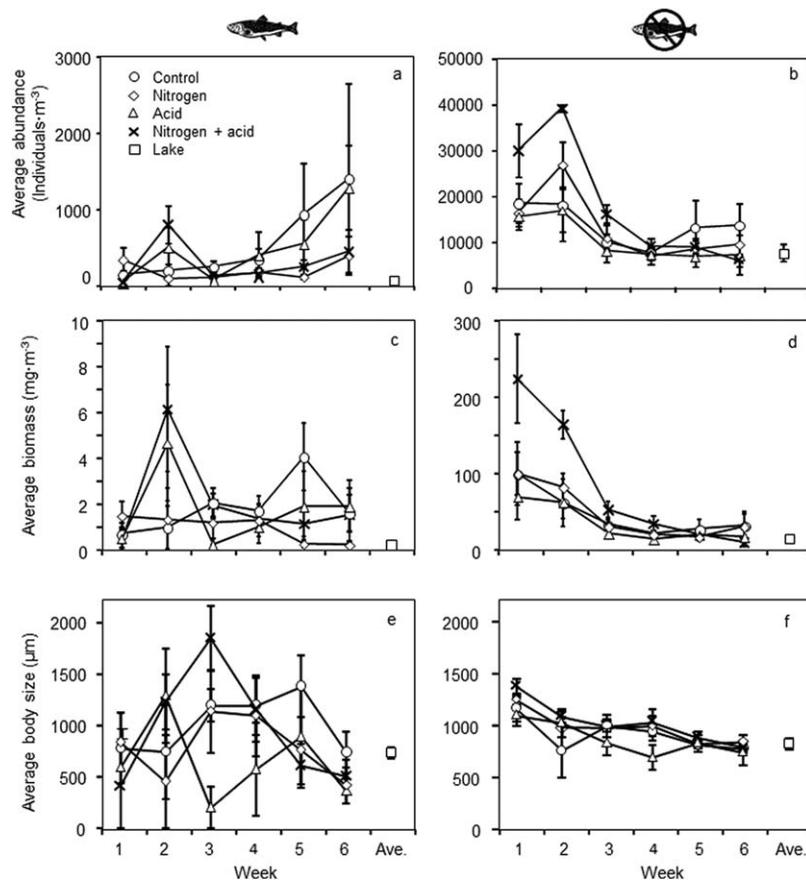


Figure 3. Average (a and b) zooplankton abundance ($\text{individuals}\cdot\text{m}^{-3}$), (c and d) biomass ($\text{mg}\cdot\text{m}^{-3}$), and (e and f) body size (μm) for weeks 1–6 for both experiments ($\pm\text{SE}$). Note differences in y axis scales between fish-present and fishless lake experiments. The symbol *Lake* represents zooplankton water column values from the respective fish-present and fishless lake, averaged over the experiment.

found that treatments simulating the effects of acidification and eutrophication sometimes had the opposite effect on chlorophyll concentrations and zooplankton communities from fishless versus fish-present communities. Additionally, we found that there were largely weak effects when acidification and eutrophication treatments were combined, frequently leading to reversals, where the combined effects were in the opposite direction of the predicted additive effect of individual stressors. These results suggest that nitrogen deposition may present complex challenges for managers attempting to protect and restore lakes following fish introductions.

Acidification in freshwater systems has been shown to cause a variety of impacts to zooplankton, and those communities with low species richness may have limited abilities to adapt to increased acidity, potentially leading to a loss of ecosystem function (Yachi & Loreau, 1999). We predicted that both zooplankton communities would respond negatively to the acid treatment as a result of declines in the dominant acid-sensitive taxa such as *Daphnia pulicaria*, *Daphnia rosea*, and *Hesperodiaptomus franciscanus* (Havas & Hutchinson, 1982; Walton et al., 1982). Although there was a significant decline in zooplankton biomass in the acid treatment over the course of the fishless lake experiment, the biomass of the fish-present experiment increased over time (Figure 3). The acid treatment was dominated by small-bodied acid-tolerant crustaceans such as *Scapholeberis* and *Chydorus sphaericus* at the end of the fish-present experiment (Figure 5), in agreement with our hypothesis. However, these small-bodied taxa were largely absent from the fishless experiment, suggesting that zooplankton in fishless lakes may be more sensitive to the effects of acidification compared to lakes with fish. This result must be tempered by the nature of our experiment: in a natural lake setting with fish present, it is possible that predation would limit this response to acidification. However, *Scapholeberis* and *C. sphaericus* are littoral taxa (Walseng et al., 2003) and would likely be able to

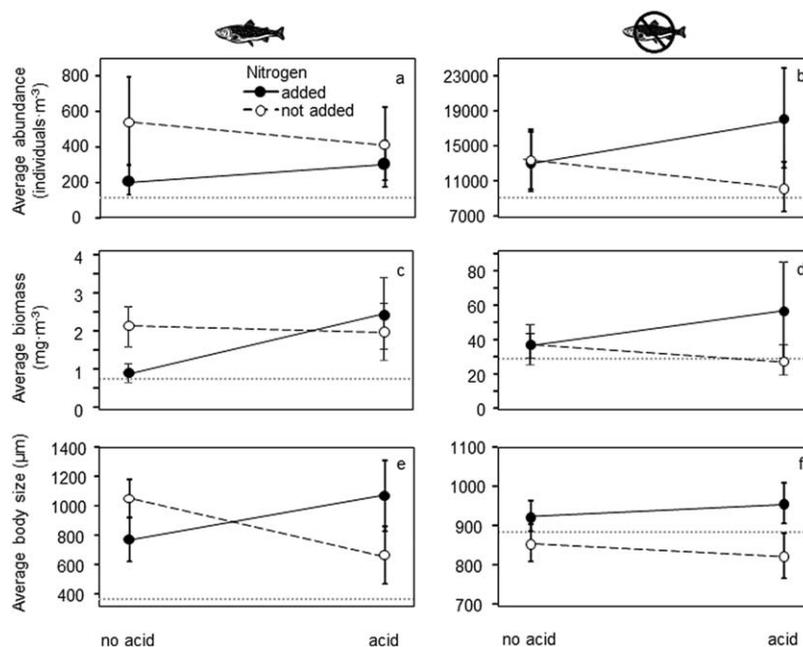


Figure 4. Interaction plots for average (a and b) zooplankton abundance ($\text{individuals}\cdot\text{m}^{-3}$), (c and d) biomass ($\text{mg}\cdot\text{m}^{-3}$), and (e and f) body size (μm) for weeks 2–6 for both experiments ($\pm\text{SE}$). Dashed gray line indicates the predicted additive effect of the nitrogen + acid treatment.

escape predation, suggesting that this result may persist in natural systems. Interestingly, *H. franciscanus* seemed to initially tolerate acid relatively well, dominating the community in the acid treatment in the first 2 weeks of the both experiments (Figure 5). However, this trend did not persist, with declines observed for the last 4 weeks of both experiments. Thus, the response of some zooplankton species and overall communities may be context-dependent, necessitating careful study of community responses to stressors.

The addition of limiting nutrients (nitrogen and phosphorus) has been shown to increase primary productivity and augment food availability for primary consumers (Elser et al., 2007). As predicted, the zooplankton community in the fishless lake experiment increased in abundance and biomass in response to the nitrogen treatment, driven by an early peak in abundance of *H. gibberum* and *H. franciscanus* (Figure 3). *Daphnia pulex* remained low in the fishless lake experiment until week 4 in all treatments, which is surprising considering that in other systems, *Daphnia* outcompete *H. gibberum* for food (Allan, 1973; Tessier, 1986). Given the equal additions of nitrogen in both the fishless and fish-present experiments, total chlorophyll *a* concentrations would have been expected to be more similar, yet concentrations were notably lower in the fishless lake experiment (average chlorophyll *a* with nitrogen addition: fishless = $0.007 \text{ mg}\cdot\text{m}^{-3} \pm 0.009 \text{ SD}$; fish-present = $0.031 \text{ mg}\cdot\text{m}^{-3} \pm 0.012 \text{ SD}$; Figure 2). The differences in chlorophyll concentrations between experiments could indicate that an early peak in available phytoplankton was consumed more aggressively by the herbivorous taxa in the fishless experiment in the first few days following nutrient addition. Thus, the lower abundance of zooplankton in the fish-present experiment may have resulted in less grazing and higher chlorophyll *a* concentrations.

Acidification and eutrophication can negatively affect aquatic biota; however this study sought to explore the interactions that could result from both stressors occurring at the same time. Previous work has observed declines in zooplankton biomass from fish-present lakes as a result of nitrogen and acid addition causing phytoplankton to become less palatable (Lafrancois et al., 2004). In our study, zooplankton abundance and biomass from the fish-present experiment were not affected by the combined treatment of acid and nitrogen, though average body size was affected (Figure 4). This interaction reflects the shift to smaller zooplankton taxa in the acid and nitrogen treatments, compared to larger taxa in the combined treatment of acid and nitrogen. The differences in our results from those of Lafrancois et al. (2004) could be due to our more subtle treatment additions, which failed to induce direct mortality. There were also no significant interactions between acid and nitrogen in the fishless lake experiment; however there were some moderate

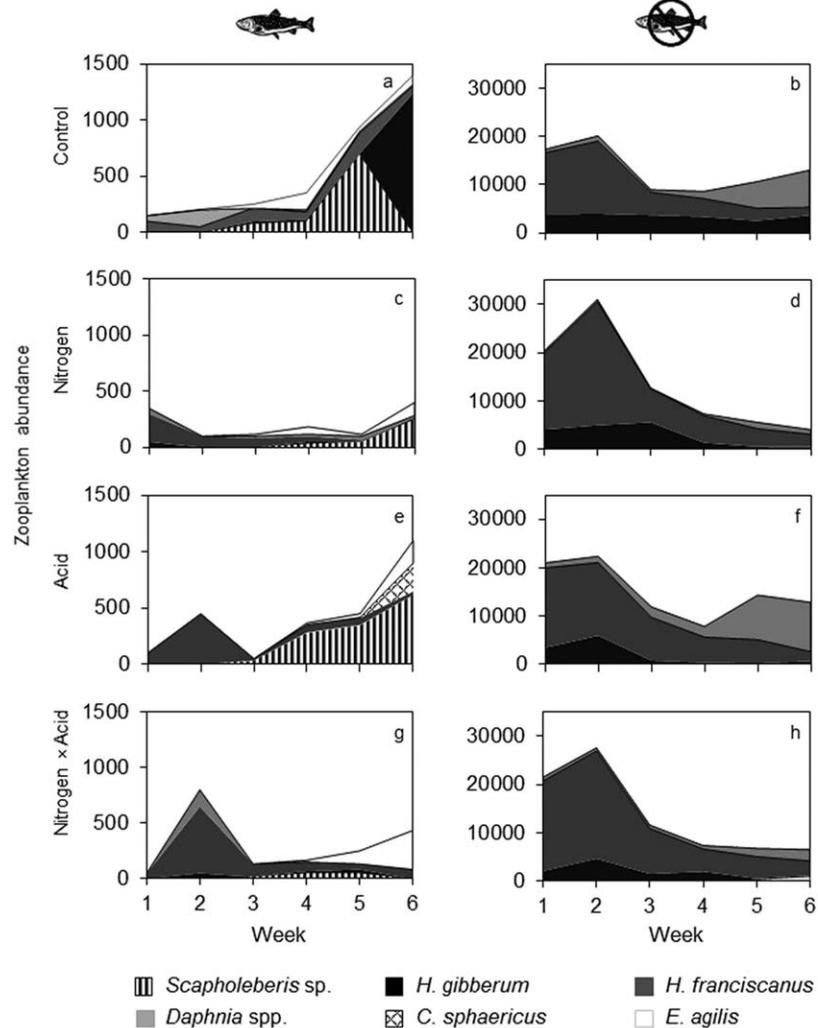


Figure 5. Species abundance (individuals·m⁻³) for weeks 1–6 for both experiments, averaged by treatment: (a and b) control, (c and d) nitrogen, (e and f) acid, (g and h) nitrogen + acid in the stocked lake experiment (a, c, e, and g) and the fishless lake experiment (b, d, f, and h).

trends of increased abundance and biomass with the addition of both nitrogen and acid (Figure 4). These interactions could be a result of the increase in some species before the acid treatment could cause chronic or delayed impacts (Figure 5). Delayed impacts of moderate pH on *Daphnia rosea* was also observed in the experimental acidification of a high elevation lake in the Sierra Nevada (Barmuta et al., 1990). Another possible explanation is that some taxa were released from competition as a result of loss of acid-sensitive taxa from the community, allowing other taxa to reproduce and grow, taking advantage of the higher nitrogen availability. The overall trend toward an increase or no change in abundance and biomass of the fishless and fish-present experiments, respectively, compared to controls was unexpected: instead of compounding negative individual effects of both variables, their sum was less harmful than predicted, resulting in reversals or ecological surprises (Jackson et al., 2016). It is noteworthy that reversals were the least common outcome of freshwater multiple stressor studies in the meta-analysis of Jackson et al. (2016). Our results support the hypothesis that reversals can occur when one of the stressors can act as a stimulant, such as nutrients (Boone & Bridges-Britton, 2006) or warmer temperatures (Jackson et al., 2016).

Our interpretations are based on the hypothesis that fish presence or absence had a significant role in structuring zooplankton communities in our study systems. As described in the Methods, the study lakes were chosen to be as similar as possible in terms of water chemistry, morphometry, temperature, and

productivity (Table 1). Although there were slight differences in productivity and temperature between fishless and fish-present lakes, if these factors were significant than we would have observed different patterns in the zooplankton communities (i.e., greater abundance, biomass, and body size in the fish-present lake). Alternately, if fish were not responsible for differences in initial community structure, another interpretation of our results is that the response of zooplankton communities to multiple stressors is dependent upon initial community composition. For instance, the acid treatment had an unexpected positive effect on zooplankton biomass in the fish-present lake, which we attribute to the presence of small-bodied acid-tolerant taxa, such as *Scapholeberis* and *Chydorus sphaericus*, which were either absent or in very low numbers in lake samples at the beginning of the experiment, respectively (supporting information S1, Figure S1-1). Thus, species traits may be a useful indicator of the potential response to multiple anthropogenic stressors.

In order to observe the most realistic responses, these experiments would have been conducted in the waterbodies themselves; however, due to park regulations, mesocosm tanks were used to recreate similar conditions (e.g., Strecker et al., 2004; Vakkilainen et al., 2004) posing little threat to the sensitive montane ecosystems. Small-scale experiments have been criticized for misrepresenting lake dynamics and attempting to extrapolate to the ecosystem level (Schindler, 1998). For instance, alterations to organisms that result from a mesocosm experiment (e.g., stress from collection and transportation, withdrawal, or addition of predators) can lead to bottle effects that could also be responsible for changes in response variables, in addition to the prescribed treatments. Additionally, our experiment lacked the trophic (e.g., fish, macroinvertebrates) and physical complexity of a lake environment. For example, high light levels encountered in our shallow mesocosms in a montane region may have reduced photosynthesis in algal cells via photoinhibition (Powles, 1984). However, the zooplankton and chlorophyll data reveal that values observed in the tanks were often within the range observed in the lakes (Figure 4), suggesting that our study was a realistic simulation. It is likely that the simplistic design used in this experiment did not account for the more complex pathways through which acidification and eutrophication occur in a natural setting, and the mediating effect of soils and vegetation in the watershed. Despite these drawbacks, this design is the most feasible way to decipher differences between zooplankton community responses to simulations of multiple stressors. Therefore, these experiments provide managers the most tangible estimates for how aquatic communities could respond to future changes, and assess the mechanisms that lead to changes in composition without whole-lake manipulations.

5. Conclusion

In low productivity systems, such as ultraoligotrophic montane lakes, additions of nitrogen could increase zooplankton abundance and biomass, offsetting the negative effects of acidification, and lead to an ecological surprise. However, the results from our experiment indicate that the addition of acid and nitrogen to a system may have differential impacts on zooplankton based on fish stocking history, and that not all stressors have negative effects. The removal of nonnative trout species is a current strategy for restoring native invertebrate populations in montane lakes. For example, the U.S. Forest Service has been restoring lakes to a fishless state since 2008 in the Lake Tahoe Basin Management Unit (Muskopf et al., 2011), while the National Park Service has been following an adaptive management plan that actively removes fish from lakes in most parks since 2009 (National Park Service, 2008). Little is known about how these systems will recover once returned to a fishless state and whether they will be more or less at risk to future perturbations. Future work should examine how other stressors, such as contaminants and novel invasive species, may affect mountain lake communities and whether ecological surprises or reversals are a widespread phenomenon in mountain ecosystems.

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