Nutrients and warming alter mountain lake benthic algal structure and function

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Abstract: In recent years, benthic algae have been increasing in abundance in the littoral zones of oligotrophic lakes, but causality has been hard to assign. We used field and laboratory experiments to explore the implications of increasing water temperature and nutrient availability for benthic algal assemblages and ecosystem processes in a Colorado alpine lake. We tested the effect of nutrient enrichment on the relative abundance of algal taxonomic groups in situ using nutrient diffusing substrata. We manipulated temperature and nutrient concentrations in laboratory assays to assess their interactive effects on ecosystem function of chlorophyte-dominated benthic assemblages. Nutrient enrichment with both N and P favored Chlorophyta (green algae) in field experiments and produced the highest overall algal biomass. In the absence of nutrient enrichment, the relative abundance of Bacillariophyta (diatoms) was substantially greater than that of Chlorophyta and cyanobacteria. In laboratory assays, N uptake increased but net ecosystem production decreased with warming temperatures, resulting in reduced N-use efficiency. Even though dissolved organic C (DOC) substantially increased in solution after all laboratory incubations, lower DOC concentrations in the assays with added P and warmer temperatures suggest nutrients and warming stimulated heterotrophic microorganisms as well as primary producers. Our results demonstrate that nutrient availability stimulates Chlorophyta in benthic algal assemblages and that the increase in chlorophytes may alter ecosystem processes with ongoing, rapid environmental change, including N cycling and metabolic functions in oligotrophic lake littoral habitats.

Key words: periphyton, filamentous green algae, Bacillariophyta, algal function, Chlorophyta, temperature–nutrient interactions

Two of the most influential drivers of global environmental change are climate warming and increases in nutrient availability (Steffen et al. 2015). For freshwater aquatic ecosystems, interactions between these drivers have the potential to significantly alter the structure and function of primary producer assemblages (Cross et al. 2015). Warming increases algal growth rates and favors taxa with high temperature growth optima (Kalf and Watson 1986, Lürling et al. 2013, Lepori et al. 2018). Temperature is also a factor in the seasonal succession of algae in freshwaters (Reynolds 1984, Litchman and Klausmeier 2008). Excessive loading of N, P, or both (N + P) are major causes of eutrophication. While P has long been the principal nutrient assumed to cause eutrophication in freshwater ecosystems, N or N + P limitation of algal growth is increasingly recognized (Cooper et al. 2016, Lewis et al. 2020).

Research on the causes of increasing lake productivity in the late 20th and early 21st centuries has largely focused on phytoplankton with comparatively little attention paid to benthic algal assemblages, in spite of many papers and textbooks acknowledging the dominant role of the benthos in total lake ecosystem productivity (Wetzel 1996, Moss et al. 2003, Winder et al. 2009, Petchey and Belgrano 2010, Yvon-Durocher et al. 2010). However, there are increasing

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DOI: 10.1086/713068. Received 23 August 2019; Accepted 3 September 2020; Published online 12 February 2021; Associate Editor, Michael Vanni. Freshwater Science. 2021. 40(1):88–102. © 2021 by The Society for Freshwater Science. All rights reserved. This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0), which permits non-commercial reuse of the work with attribution. For commercial use, contact journalpermissions@press.uchicago.edu.
reports of benthic algal blooms in historically oligotrophic lakes around the world (Vadeboncoeur et al. 2020). Despite their importance, these benthic assemblages are globally understudied, making it difficult to disentangle the mechanisms underpinning the assemblage shifts occurring in some lake ecosystems (Vadeboncoeur and Steinman 2002, Vadeboncoeur et al. 2002).

Alpine Sky Pond in the Loch Vale watershed, Rocky Mountain National Park, Colorado, USA, is among those freshwater systems where benthic algal mats have proliferated. Complex changes in autotrophic structure in Loch Vale lakes have occurred over the past 50 to 100 y. Whole-lake primary producer assemblages, historically dominated by benthic Bacillariophyta (diatoms), have shifted in recent decades toward benthic Chlorophyta (green algae) and planktonic Bacillariophyta (Oleksy et al. 2020). In addition to N inputs to Loch Vale from atmospheric deposition and warming trends in air and water temperatures (Wolfe et al. 2003, Mast et al. 2014). P from either deposition or weathering has been implicated in the shifts toward chlorophyte-dominated algal assemblages over the last several decades (Oleksy et al. 2020). While increases in benthic algal biomass and restructuring of algal assemblages can be indicative of local-scale disturbance, such as shoreline development and point-source pollution (Rosenberger et al. 2008, Hampton et al. 2011, Schneider et al. 2014), the changes in Loch Vale lakes are indicative of regional and global changes because these headwater lakes are protected from direct human impact (Stoddard et al. 2016, Moser et al. 2019). In addition to effects on benthic algae, changes in temperature and nutrient loads may further influence ecosystem function by altering pathways of energy flow through the microbial loop via changing dissolved organic C (DOC) quality (Wyatt et al. 2014).

Elevated nutrients in alpine lakes can come from a variety of sources in addition to atmospheric deposition, including increased aeolian dust inputs, glacier and rock glacier meltwaters, and thaw of discontinuous permafrost (Clow et al. 2003, Barnes et al. 2014, Leopold et al. 2015). The types of nutrients that are delivered to alpine lakes can differ depending on the source. Atmospheric deposition is a large source for reactive N (Elser et al. 2009), and dust deposition can include significant P minerals (Stoddard et al. 2016). In the Arctic, thawing permafrost, and the resulting mobilization of previously locked-up P, is a major driver of ecological change in aquatic systems (Frey and McClelland 2009, Lougheed et al. 2015, Kendrick et al. 2018). Thawing glaciers and rock glaciers above alpine lakes subsidize headwater aquatic ecosystems with weathering products including P, silica, and base cations (Barnes et al. 2014), and legacy nutrients released from thawing ice can also contribute N, P, and labile C (Saros et al. 2010, Brighenti et al. 2019, Fegel et al. 2019, Ren et al. 2019).

Total algal biomass can be stimulated by single nutrient additions of N or P, but because algal taxa differ in their nutrient acquisition strategies and requirements, resulting in limitation by different nutrients, the proportions of N and P derived from wet or dust deposition, ice meltwaters, and permafrost can influence the mix of algal species (Elser et al. 2007, Mette et al. 2011). Chlorophytes, for instance, are often stimulated by the additions of both N and P in freshwaters, including in oligotrophic lakes (Reynolds 1984, Rhew et al. 1999, Nydick et al. 2004, Eichel et al. 2014). Collectively, different sources of nutrients to alpine lakes could select for algal species with different life histories, nutrient acquisition kinetics, and growth forms than historical algal populations.

We postulated that warming and nutrients are shifting benthic algal assemblages toward chlorophyte dominance because genera such as Spirogyra, Zygnema, Cladophora, and Mougeotia tend to grow well in high N:P environments and warm temperatures (Hawes 1988, Frossard et al. 2014, Middleton and Frost 2014). The 1st goal of our study was to understand how nutrient availability influences the development of periphyton, with a focus on total algal biomass and the relative abundance of different algal taxonomic groups within the overall assemblage. We expected additions of both N and P to increase total benthic algal biomass, which is a common response in nutrient diffusing substrate (NDS) experiments in oligotrophic environments (Beck et al. 2017). The 2nd goal of the study was to understand the ecosystem response of benthic chlorophyte-dominated assemblages to different temperature and nutrient conditions. We asked how nutrient amendments and temperature individually and interactively affect metabolic functions, such as gross primary production (GPP) and ecosystem respiration (ER), of Chlorophyta-dominated periphyton assemblages. We also investigated the effects of nutrients and temperature on benthic chlorophyte N uptake, which we expected to be high given the affinity of green algae for nutrient-enriched conditions (Eichel et al. 2014). Lastly, we quantified net DOC exudation because DOC factors strongly in freshwater ecosystems as a C subsidy for bacterial respiration (Wyatt et al. 2014).

METHODS

We conducted field and laboratory experiments to understand the effects of warming and nutrient inputs on benthic algal assemblages and their ecosystem functions in Sky Pond. We measured responses of benthic algal assemblages during an NDS field experiment. To assess benthic algal ecosystem functions, we conducted short-term laboratory experiments that included in-situ NDS incubations and laboratory metabolism assays under warming conditions.

Study area

Sky Pond (40.27814 N, –105.66837 W; 3322 m a.s.l.) is an alpine lake in the Loch Vale Watershed, Rocky Mountain National Park, USA. The surface area of Sky Pond is 3 ha,
and the lake has an approximate volume of 1.2 × 105 m³, maximum depth of 7.3 m, and average depth of 4.5 m (Baron 1992). Multiple inlets supply water to Sky Pond. These inlets generally flow through talus fields below a rock glacier and also include unchanneled flow through boulder fields (Baron 1992). The landscape surrounding Sky Pond is characterized by sparse vegetation (Arthur et al. 1992) and permafrost. In the late 1990s, as much as 11% of the basin was estimated to be underlain by permafrost, in addition to cryic features like rock glaciers and glaciers (Clow et al. 2003). At a neighboring research station at similar elevations, permafrost was recorded in the 1970s (Ives and Fahey 1971), but its extent and thickness has diminished significantly in recent decades (Leopold et al. 2015).

NDS field experiment
Preparation and deployment of NDS treatments We built and deployed NDS using methods outlined by Tank et al. (2017). We filled forty-eight 30-mL plastic vials with 4% agar solution, with 12 vials/treatment. In addition to agar-only controls, our treatments included amending vials with 0.5 M NaNO₃ (N treatment), 0.031 M KH₂PO₄ (P treatment), or 0.5 M NaNO₃ plus 0.031M KH₂PO₄ (molar ratio ~11.5; N + P treatment). We placed a fritted 5.7-cm² glass disk on top of each agar-filled plastic vial to serve as substrate for algae colonization (Tank et al. 2017). We labeled, capped, and randomized the vials and attached them to 6 angle iron bars for a total of 48 experimental units. Each vial had a ~3-cm buffer around it. We anchored all 6 bars in the littoral zone of Sky Pond where they would not be disturbed by national park visitors. We deployed the experiment for 16 d during August 2017. At the end of the experiment, we wrapped each glass frit individually in aluminum foil and immediately placed them in a −40°C freezer until further analysis.

We selected NaNO₃ as the inorganic N carrier because NO₃⁻ is the primary inorganic N source in Sky Pond, and we chose KH₂PO₄ because it is the most commonly used phosphate chemical in NDS experiments (Beck et al. 2017). In selecting our starting P concentration, we aimed to simulate P hotspots that may exist at the sediment–water interface, although our P additions were smaller than in many other NDS manipulations. Diffusion tests by Lepori and Robin (2014) at similar starting concentrations confirmed that nutrients diffused at the end of the experiment at a rate of ~13 ± 5 µg P/h. Control treatment agar did not have nutrients added, causing periphyton to rely on ambient water quality (Table 1).

Water chemistry To assess the environmental characteristics at Sky Pond, we measured water temperature and conductivity in situ and collected samples for chemical analyses. We used an Orion™ 3-Star hand-held probe (Thermo Fisher Scientific, Waltham, Massachusetts) to measure water temperature and conductivity at the deployment site (littoral zone) as well as in the epilimnion. For chemical analyses, we collected 4 grab samples from a depth of 0.5 m below the surface during the experiment. We collected 2 of these samples (littoral zone and epilimnion) at the beginning and 2 samples (littoral and epilimnion) at the end of the experiment near the NDS deployment site.

| Parameter     | Sky Pond environmental conditions during NDS experiment | Laboratory incubation experiment initial conditions |
|---------------|------------------------------------------------------|--------------------------------------------------|
|               | Littoral | Lake | Control | N     | P     | N + P   |
| Conductivity (µS/cm) | 13.2    | 12.3 | –       | –     | –     | –       |
| DOC (mg/L)     | 0.65 ± 0.05 | 0.52 ± 0.05 | 0.66 ± 0.05 | 0.66 ± 0.05 | 0.66 ± 0.05 | –       |
| TN (µM)        | 22.5 ± 1.0 | 14.9 ± 1.0 | 18.1 ± 1.1 | 66.6 ± 0.4 | 17.8 ± 0.7 | 66.6 ± 1.0 |
| Chlorophyll a (µg/L) | –    | 9 ± 0.5 | –       | –     | –     | –       |
| TP (µg/L)      | 10 ± 1    | 10 ± 1 | –       | –     | –     | –       |
| PO₄ (µM)       | 0.8       | 0.8   | 0.8 ± 0.3 | 0.8 ± 0.3 | 8.7 ± 0.3 | 8.3 ± 0.3 |
| DIN (mg/L)     | 0.23 ± 0.01 | 0.18 ± 0.01 | 0.18 ± 0.01 | 0.84 ± 0.03 | 0.18 ± 0.01 | 0.8 ± 0.03 |
| DIN:TP (molar) | 51.0      | 41.3  | –       | –     | –     | –       |
| DIN:PO₄ (molar) | 20.5     | 22.4  | 22.4    | 82.5  | 2.1   | 7.6     |

Table 1. Water chemical properties for Sky Pond nutrient diffusing substrate (NDS) and incubation experimental initial conditions. Littoral chemical parameters are reported as the mean of 2 samples taken where the NDS was deployed, 1 at the start and 1 end of the experiment. Lake chemical parameters are reported as means ±SD of 2 pelagic water samples collected 0.5 m below the surface at the deepest part of the lake. In the incubation experiment, the treatments received ~4× ambient lake dissolved inorganic N (DIN) concentrations (N, N + P treatments) and ~10× ambient lake PO₄⁻ concentrations (P, N + P treatments). DOC = dissolved organic carbon. TDN = total dissolved nitrogen. TP = total phosphorus.
At each of these samplings, we collected water samples for analysis of major ions in 500-mL translocan, acid-washed high-density polyethylene (HDPE) bottles (Nalgene®, Rochester, New York); samples for phosphate (PO$_4^{3-}$) and total P analyses in 60-mL translocan, acid-washed HDPE bottles; and samples for DOC and total dissolved nitrogen (TDN) analyses in 480-mL baked silicate bottles. We returned all samples to the laboratory within 5 h of collection, and they were either refrigerated (4°C; major ions, DOC/TDN) or frozen (−18°C; total P) until analysis.

We conducted chemical analyses according to standard procedures for the Loch Vale program (https://www2.nrel.colostate.edu/projects/lvws/data.html). Inorganic N (NO$_3^-$ and NH$_4^+$) and phosphate (PO$_4^{3-}$) were measured by spectrophotometry using a flow injection analyzer (Lachat Instruments, Milwaukee, Wisconsin) at the Rocky Mountain Research Station Biogeochemistry Laboratory, Fort Collins, Colorado. We also used Rocky Mountain Research Station facilities to quantify DOC and total N on a Shimadzu TOC-V Combustion Analyzer (Kyoto, Japan) (United States Environmental Protection Agency [EPA] 415.1 and ASTM D5176 methods, respectively). Total P (TP) was analyzed using persulfate digestion at High Sierra Water Laboratory (Tahoe City, California; EPA method 365.1). We extracted planktonic chlorophyll a (Chl a) with 90% acetone and analyzed Chl a concentrations with a Trilogy® benchtop fluorometer (Turner Designs, San Jose, California; EPA method 445.0).

**Biomass and assemblage structure** We quantified total benthic algal biomass as total Chl a, and we quantified the biomass of pigments specific to certain algal groups to assess abundance of different taxa within the overall assemblage. Fucoxanthin served as an indicator for Bacillariophyta (diatoms), myxoxanthophyll for cyanobacteria, and total chlorophyll b (Chl b) for Chlorophyta (green algae; Leavitt and Hodgson 2001, Steinman et al. 2017). To extract pigments, we placed each glass frit individually in an 85°C methanol : water solution in glass dram vials for 24 h in a dark cycle. We refreshed the dram vials for 7 d) in Sky Pond in early July 2017. Each 5 × 5-inch tile (n = 3) had 25 to 30 small (5.7 cm$^2$), rough, porous fritted glass disks, as used for the NDS experiment, attached with non-toxic aquarium sealant (Aqueon, Franklin, Wisconsin). The tiles were incubated in a shallow bay where Spirogyra have grown previously in mats (IAO, personal observation). After 2 w, we gently wrapped the Chlorophyta-covered tiles and fritted disks in aluminum foil and transported them on ice in an insulated, soft-sided cooler back to the laboratory with minimal disturbance. We confirmed dominance of Spirogyra by using an Olympus Vanox microscope (Tokyo, Japan) equipped with differential interference contrast optics and a 1.3 NA objective. We then incubated tiles in an 8°C water bath containing 10 L of filtered lake water. The water bath was kept inside a growth chamber where the light/dark cycle was 16:8 h (∼100 µmol photons m$^{-2}$ s$^{-1}$ irradiance). We refreshed the water bath daily with filtered lake water to offset evaporative losses. Algae continued to grow on the fritted glass disks in the growth chambers for 7 d prior to the initiation of the experiment.

**Experiment 1: Estimates of biomass, nutrient uptake, and algal exudates** We conducted laboratory incubations to better understand how the combination of warming and nutrients affected algal nutrient uptake and exudation and
overall heterotrophic activity. For our experimental treatments, we manipulated temperature (8, 12, and 16°C) and nutrients (control, N, P, N + P) for 8 d in a 3 × 4 factorial experiment. Each treatment took place in a separate 250-mL glass beaker, with a total of 72 separate beakers so that each treatment combination had 6 replicates. Each beaker contained 1 colonized fritted glass disk in 175 mL of filtered lake water, to which we added nutrients. Nutrient treatments included a control (filtered lake water only), elevated N (N treatment; 4× higher than ambient lake water), elevated P (P treatment; 10× higher than ambient lake water), and elevated N and P (N + P treatment; 4 and 10× higher than ambient lake water concentrations, respectively; Table 1). Because of atmospheric N deposition, ambient lake water (control) had an average NO3-N concentration of 0.19 μg/L, NO2-N and an N:P molar ratio of ~120 (Elser et al. 2009). Our coldest temperature treatment (8°C) was equivalent to the base temperature in the growth chamber where we incubated the beakers. For the other temperature treatments, we placed beakers in water baths warmed with Tetra® HT submersible aquarium heaters (Blacksburg, Virginia) to raise the temperatures to 12 and 16°C. The 8°C treatment represented 2015 to 2017 mid- to late-summer averages for the littoral zone of Sky Pond. The 12°C temperature treatment represented 2015 to 2017 peak maximum temperature in the littoral zone. The warmest temperature treatment (16°C) represented a warming scenario comparable to littoral conditions in a downstream subalpine lake, The Loch. We conducted the experiment under a 16:8 h light:dark cycle, and all beakers were exposed to the same intensity of light (~100 μmol photons m−2 s−1 irradiance). We monitored the experiment daily, and we corrected for evaporative loss by supplementing with deionized water. To measure TDN and DOC, we collected 30 mL from each water (control) had an average NO3-N concentration of 0.19 μg/L, NO2-N and an N:P molar ratio of ~120 (Elser et al. 2009). Our coldest temperature treatment (8°C) was equivalent to the base temperature in the growth chamber where we incubated the beakers. For the other temperature treatments, we placed beakers in water baths warmed with Tetra® HT submersible aquarium heaters (Blacksburg, Virginia) to raise the temperatures to 12 and 16°C. The 8°C treatment represented 2015 to 2017 mid- to late-summer averages for the littoral zone of Sky Pond. The 12°C temperature treatment represented 2015 to 2017 peak maximum temperature in the littoral zone. The warmest temperature treatment (16°C) represented a warming scenario comparable to littoral conditions in a downstream subalpine lake, The Loch. We conducted the experiment under a 16:8 h light:dark cycle, and all beakers were exposed to the same intensity of light (~100 μmol photons m−2 s−1 irradiance). We monitored the experiment daily, and we corrected for evaporative loss by supplementing with deionized water. To measure TDN and DOC, we collected 30 mL from each beaker at the beginning and end of the experiment and conducted chemical analyses using methods described above. We controlled for changes in nutrients of the filtered lake water by incubating beakers with blank glass frits in triplicate for the duration of the experiment, but we detected no changes in water chemistry.

We used a BenthoTorch™ fluorescence probe (bbe Moldaenke, Schwentinental, Germany) to measure relative changes in total algal biomass (as Chl a) at two different times: before and after the incubation. While we are aware of concerns related to quantitative uses of the BenthoTorch, in a trial experiment we found high correlation between Chl a (as a proxy for chlorophyte biomass) and BenthoTorch-quantified green algal biomass indices (as Chl a equivalent; r2 = 0.71; Fig. S2).

At the conclusion of the 8-d experiment, we poured all overlying water from the beakers into acid-washed HDPE plastic bottles (Nalgene) for chemical analyses and estimates of nutrient uptake. We filtered water (GF/F; Whatman, Maidstone, United Kingdom) within 6 h of the end of the experiment. We calculated areal TDN uptake rates as the difference between initial dissolved N and final dissolved N concentrations divided by incubation length and surface area of algal substrate (Williamson et al. 2015). We calculated %N uptake as the change in TDN multiplied by 100, assuming zero loss of N to the atmosphere. We calculated N-use efficiency (NUE) as mean net ecosystem production (NEP; described below) per unit N taken up (μmol N cm⁻² h⁻¹; Pastor and Bridgham 1999, Williamson et al. 2015). We calculated final DOC, which was assumed to be almost entirely composed of algal exudates, as the final minus initial concentrations of DOC.

We performed a 2-way analysis of covariance (ANCOVA) to examine the effects of nutrient treatment and temperature (both categorical variables) on continuous response variables (N uptake rates, %N uptake, DOC exudation) after controlling for a continuous covariate, changes in BenthoTorch-estimated benthic algal biomass. Prior to running models, we ran an initial model including interactions between the covariate and grouping variables (temperature and nutrient treatments), and we determined that there were no such interactions (based on p > 0.05) in this initial model, confirming homogeneity of regression slopes. We assessed normality of residuals with the Shapiro–Wilk test and homogeneity of variances with Levene’s test. If there was evidence for a substantial interaction between temperature and nutrient treatments, we used the emmeans_test function in the rstatix package (Kassambara 2020) to test for a simple main effect followed by pairwise comparisons (with Bonferroni adjustments) among temperature or nutrient treatments.

**Experiment 2: Estimates of metabolism** We conducted short-term metabolism assays on each of the fritted glass disks used in the previous incubation experiment to estimate GPP and ER following methods developed by Reisinger et al. (2016). To conduct the metabolism assays, we placed each fritted disk into a 50-mL acid-washed Falcon tube (n = 12 for each nutrient treatment). We filled the tubes with nutrient-amended filtered Sky Pond water from 3.75-L buckets (1 bucket prepared for each nutrient treatment) and capped the tubes underwater to avoid air bubbles. We measured the temperature and dissolved oxygen (DO) of the bucket water, which served as the experimental starting conditions, with a multimeter probe (Hach, Loveland, Colorado). We inverted the Falcon tubes and exposed them to the same temperature conditions as in the 8-d experiment. All tubes were initially exposed to ~100 μmol
photon m⁻² s⁻¹ irradiance for 3 to 4 h. After the light incubation, we measured the DO concentrations of each tube. We refilled the falcon tubes with fresh filtered lake water, incubated them in a dark environmental chamber for 3 to 4 h, and measured them for changes in DO. Three to 4 h was long enough to observe substantial changes in DO concentrations. We controlled for changes in DO of the filtered lake water by incubating (in triplicate) blank disks in falcon tubes containing only filtered lake water.

We used the DO measurements from the incubations to estimate NEP and ER, and subsequently calculate GPP, for each treatment combination. We estimated NEP (μg O₂ cm⁻² h⁻¹) as the increase in DO in the light incubation and ER (μg O₂ cm⁻² h⁻¹) as the decrease in DO during the dark incubation, with each glass disk representing the ecosystem. We calculated GPP (μg O₂ cm⁻² h⁻¹) as the sum of NEP and ER. We conducted 2-way ANCOVAs with BenthoTorch-estimated Chl a as a covariate, as described above for the previous experiment, to examine the effects of nutrient treatment and temperature on GPP and ER.

RESULTS

NDS field experiment

Water chemistry Sky Pond waters were highly dilute, with low conductivity (13.2 μS/cm), low DOC concentrations (mean ± SD; 0.52 ± 0.05 mg/L), and summer phytoplankton biomass averaging 9 ± 0.5 μg/L Chl a in 2017. DIN concentrations were 0.18 ± 0.01 mg/L, resulting in high DIN:TP ratios (51.0 and 41.3 for littoral and epilimnion samples, respectively). TDN, DOC, and DIN were slightly higher in the littoral habitat than in the epilimnion (Table 1).

Biomass and assemblage structure Nutrient additions in the field experiment had a substantial effect on the total amount of algal biomass as Chl a (F₁,₃₀ = 19.059, p < 0.001; Table 2). Total biomass was highest with the N + P treatment, which had substantially more biomass compared to all other treatments (Fig. 1A). The effect of N-only additions was no different than the control. When P was added alone, there was a slight decrease in algal biomass compared to the control.

Chlorophyta dominated in the treatments enriched with N (N and N + P treatments), whereas Bacillariophyta had the highest relative pigment composition in the control and P treatments (Fig. 2). Nutrient enrichment had a strong positive effect on Chlorophyta biomass (as Chl b), which was substantially greater in the N and N + P treatments than either the control or P treatments (χ²3,₆₀ = 23.199, p < 0.001; Fig. 1B). The opposite was observed for Bacillariophyta biomass (as fucoxanthin), which had the highest values in the control and P treatments (Fig. 1C). A Tukey’s test following 1-way ANOVA revealed that the control had the highest mass of fucoxanthin compared to all nutrient treatments (F₁,₃₀ = 31.459; p < 0.001), and the relative pigment composition of Bacillariophyta was highest in the control and P treatments (Fig. 2). Cyanobacteria biomass (as myxoxanthophyll) was low in the control, N, and P treatments and was substantially higher in the N + P treatment than in the control (χ²3 = 7.337, p < 0.001; Fig. 1D). Ultimately, cyanobacteria had the lowest relative pigment composition of the 3 taxonomic groups examined (Fig. 2).

Laboratory incubation experiments

Biomass estimates The small observed changes in BenthoTorch-quantified benthic algal biomass were included as a covariate in our ANCOVA models, but they showed very small effect sizes with limited explanatory power. Chl a concentrations did not change over the course of the incubations (beginning mean ± SD value: 6.92 ± 1.05 μg/cm², ending value: 6.94 ± 1.62 μg/cm²; Fig. S1).

Nutrient uptake After controlling for changes in benthic algal Chl a, there was a temperature by nutrient treatment interaction on TDN uptake rates (F₆,₅₈ = 44.1, p < 0.001). In the N and N + P treatments, TDN uptake increased as a function of increasing temperature (p < 0.001), but TDN uptake rates did not respond to temperature in the control and P treatments. Averaged across all temperature treatments, TDN uptake rates were 4× lower in the control and P treatments compared to the N treatment, and TDN uptake rates in the N + P treatment were 2× higher than in the N treatment (Fig. 3A). The increase in TDN uptake rates with rising temperatures was strongest in the N-only treatments, where TDN uptake rates increased from 4.6 μg N cm⁻² d⁻¹ at 8°C to 7.2 μg N cm⁻² d⁻¹ at 12°C and peaked near 13.0 μg N cm⁻² d⁻¹ at 16°C. TDN uptake rates were highest in the N + P treatment and varied somewhat with temperature: mean uptake rates increased from 15 to 18 μg N cm⁻² d⁻¹ between the 8°C and 12°C treatments but then decreased slightly in the 16°C treatment to 17 μg N cm⁻² d⁻¹.

Given that N was supplied in different quantities across treatments, investigating the percentage of initial N that was taken up provided further context to describe N-cycling dynamics. As with N uptake rates, there was also a strong interaction between temperature and nutrient amendments for %N uptake (F₆,₅₈ = 19.9, p < 0.001). Warming increased the percentage of N taken up in all N and N + P treatments (p < 0.001), but there was no effect of temperature on the percentage of N taken up in the control or P treatments. Only in the control and N treatments, when P was in low supply relative to N, did we see large differences in the %N uptake among temperature treatments (Fig. 3B). In all temperature treatments, the %N uptake was highest in the N + P treatment.
N-use efficiency was also affected by an interaction between nutrient and temperature treatments after controlling for changes in benthic algal Chl \( a \) (F\(_{6,54} = 12.6, p < 0.001; \) Fig. 3C), but the relationship was in the opposite direction compared to the N-uptake results (Fig. 3A). Regardless of temperature, NUE was highest in the control and P treatments (mean \( \pm \) SD of 103 \( \pm \) 27.5 and 76.7 \( \pm \) 37 \( \mu \)mol O\(_2\)/\( \mu \)mol N, respectively), and regardless of nutrient treatment, NUE decreased with increasing temperature, though the largest decreases were observed in the control. Thus, whereas N-uptake rates were highest in warm N-enriched treatments, NUE was lowest in those treatments. In spite of N concentrations approaching 4\( \times \) higher than background in the N and N \( + \) P treatments, the benthic assemblages still took up to 88\% of available inorganic N. At 8 and 12\( ^\circ \)C, the percentage of N taken up decreased as a function of increasing N:P (\( p < 0.001 \)), but there was no relationship between the percentage of N uptake and N:P for treatments incubated

| Experiment | Response | Statistical model | Term                      | df | Test statistic* | p-value |
|------------|----------|-------------------|---------------------------|----|----------------|---------|
| NDS        | Chl \( a \) | ANOVA             | Nutrient trt              | 3  | 19.1           | <0.001  |
|            | Fucoxanthin | ANOVA             | Nutrient trt              | 3  | 31.5           | <0.001  |
|            | Chl \( b \) | Kruskal–Wallis    | Nutrient trt              | 3  | 23.2           | <0.001  |
|            | Myxoxanthophyll | Kruskal–Wallis | Nutrient trt              | 3  | 7.3            | <0.001  |
| Incubation | TDN uptake | ANCOVA            | Temperature trt           | 2  | 57.3           | <0.001  |
|            |           |                   | Nutrient trt              | 3  | 1070.4         | <0.001  |
|            |           |                   | Interaction               | 6  | 44.1           | <0.001  |
|            |           |                   | Biomass change            | 1  | 0.01           | 0.92    |
| Incubation | % TDN taken up | ANCOVA       | Temperature trt           | 2  | 26.6           | <0.001  |
|            |           |                   | Nutrient trt              | 3  | 132.7          | <0.001  |
|            |           |                   | Interaction               | 6  | 19.9           | <0.001  |
|            |           |                   | Biomass change            | 1  | 0.3            | 0.56    |
| Incubation | N-use efficiency | ANCOVA | Temperature trt           | 2  | 98.2           | <0.001  |
|            |           |                   | Nutrient trt              | 3  | 229.8          | <0.001  |
|            |           |                   | Interaction               | 6  | 12.6           | <0.001  |
|            |           |                   | Biomass change            | 1  | 0.22           | 0.22    |
| Incubation | DOC       | ANCOVA            | Temperature trt           | 2  | 35.4           | <0.001  |
|            |           |                   | Nutrient trt              | 3  | 16.3           | <0.001  |
|            |           |                   | Interaction               | 6  | 4.2            | <0.001  |
|            |           |                   | Biomass change            | 1  | 1.9            | 0.16    |
| Incubation | logGPP    | ANCOVA            | Temperature trt           | 2  | 28.3           | <0.001  |
|            |           |                   | Nutrient trt              | 3  | 2.1            | 0.11    |
|            |           |                   | Interaction               | 6  | 1.6            | 0.16    |
|            |           |                   | Algal biomass             | 1  | 0.8            | 0.36    |
| Incubation | logER     | ANCOVA            | Temperature trt           | 2  | 32.0           | <0.001  |
|            |           |                   | Nutrient trt              | 3  | 1.5            | 0.23    |
|            |           |                   | Interaction               | 6  | 2.8            | 0.02    |
|            |           |                   | Algal biomass             | 1  | 0.54           | 0.46    |

* For ANOVA and ANCOVA models, this is the F-statistic. For Kruskal–Wallis, this is the chi-squared value.
Slopes for 8 and 12°C were not statistically different ($p = 0.8427$).

**Estimates of metabolism**  
GPP was insensitive to changes in nutrients but was slightly higher on average in the 16°C treatment ($F_{2,58} = 28.3, p < 0.001$; Table 2, Fig. 5A). ER rates were influenced by an interaction between nutrients and temperature ($F_{6,58} = 2.8, p = 0.02$; Fig. 5B). However, the only substantial difference in ER between nutrient
DISCUSSION

In mountain ecosystems, global environmental change is leading to both warming temperatures and changes in nutrient supplies (Catalan et al. 2006). These changes can, in turn, aid the proliferation of benthic green algae in heretofore oligotrophic lakes (Vadeboncoeur et al. 2020). Our field and laboratory experiments were conducted to see if different combinations of nutrients and temperature could lend explanatory power to the recent observed increase in benthic chlorophytes in alpine Sky Pond in Rocky Mountain National Park, Colorado (Oleksy et al. 2020). N additions above extant concentrations stimulated benthic chlorophytes, and warming further altered ecosystem properties: N-uptake rates were substantially greater in warm, nutrient-rich, low N:P green algal mats, and NEP declined with warming, whereas ER increased with no net change in GPP. Although our experiments were small in scale, our results suggest some possible N- and temperature-related mechanisms for this unprecedented change in algal assemblages in Sky Pond.

Nutrient effects on algal assemblages and ecosystem processes

The changes in algal assemblage structure that we observed in our field experiment are consistent with the findings of other studies. An increase in lake nitrate concentrations, ca. 1950 in Sky Pond and elsewhere, from the rise in atmospheric N deposition was responsible for an initial shift in Bacillariophytes, at that time, from benthic-dominated to planktonic-dominated species (Wolfe et al. 2003, Saros et al. 2005). Paleolimnological reconstructions using algal pigments showed benthic chlorophytes also began to increase mid-20th century, potentially due to a competitive advantage of chlorophytes over diatoms under conditions of increased N availability or colonization of a vacated niche (Oleksy et al. 2020). Since the mid-20th century, benthic diatoms have declined while green algae have continued to increase in biomass. Even in the relatively high N environment of Sky Pond, Chlorophyta responded...
strongly to N additions in our field NDS experiment. This finding is consistent with other studies in oligotrophic lakes that found filamentous green algae to increase in biomass with N or N + P enrichments (Nydick et al. 2004, Hogan et al. 2014, Lepori and Robin 2014). In contrast, and in keeping with the pattern observed in Sky Pond lake sediments, benthic Bacillariophytes dominated when N concentrations were low. These results suggest Bacillariophyta outcompeted Chlorophyta as a result of differing nutrient-dependent growth and uptake optima in relatively low-N conditions, as found by others (Litchman et al. 2007, Wen et al. 2012, Thomas et al. 2017). Warming, too, could shift benthic algal structure to favor taxa with flexible stoichiometry, such as filamentous green algae (Middleton and Frost 2014). Indeed, paleolimnological records in the Arctic show that Chlorophyta dominated over Bacillariophyta during warmer periods (e.g., Holocene Thermal Maxima), when summer temperatures were elevated and growing seasons were long (Florian et al. 2015).

In our field experiment, we expected to see increased benthic algal biomass with P additions because the surface waters of Sky Pond are high in inorganic NO₃⁻, which has previously been shown to cause P limitations in lake phytoplankton (Nydick et al. 2003, Elser et al. 2009). Instead, total biomass was highest with N + P additions; P alone had no effect or a negative effect on benthic algal biomass. One potential explanation for the aforementioned result is that bacterial growth outpaced algal growth in the P-only treatment (Bernhardt and Likens 2004, Beck et al. 2017), but we did not measure heterotrophic responses in the NDS experiment.

Several mechanisms may explain how Chlorophyta biogeochemical functions changed as a result of nutrient availability and temperature in our laboratory incubations. Nitrate uptake in algal cultures increases as a function of temperature (Reay et al. 1999), and we found this pattern for %N uptake and N-uptake rates, particularly when N was in high supply (N and N + P treatments). This result is consistent with findings by Thrane et al. (2017), who found that the optimal N:P ratio of planktonic chlorophytes

Figure 4. A.—Final dissolved organic C (DOC) concentration averaged across nutrient enrichments for each temperature treatment (blue = 8°C, green = 12°C, and red = 16°C). B.—Final DOC concentration across each temperature (panel) by nutrient enrichment (shading: no-nutrient control [Ctrl], N, P, and N + P) combination. The boxplots show 25 to 75% quantiles with whiskers extending to 1.5× the interquartile range, and the thick black lines are the treatment medians. If the treatment was different from the control (p < 0.05), it is noted with an asterisk (*).

Figure 5. Metabolism assay results. A.—Gross primary production (GPP). B.—Ecosystem respiration (ER). C.—ER across each temperature (panel; 8, 12, and 16°C) and nutrient enrichment (shading: no-nutrient control [Ctrl], N, P, and N + P) combination. The boxplots show 25 to 75% quantiles with whiskers extending to 1.5× the interquartile range, and the thick black lines are the treatment medians. If the treatment was different from the control (p < 0.05), it is noted with an asterisk (*).
changed with temperature such that N demand was higher at higher temperatures owing to greater ribosomal content. Chlorophytes may be able to use additional N to acquire P through the stimulation of additional P scavenging pathways in low-P environments (e.g., synthesis of extracellular phosphatase enzymes; Bracken et al. 2015). While relatively low N:P resulted in higher NUE, we also found lower NUE at warmer temperatures. This result is in opposition to theory laid out by Cross et al. (2015), who postulated nutrient-use efficiency should increase with warmer temperatures. Respiration outpaced productivity in our metabolism assays, leading to decreases in NEP and, thus, lower NUE (calculated as NEP divided by N uptake) with warmer temperatures. It is possible that heterotrophs growing in association with the chlorophytes in our experimental treatments may have driven this pattern, as discussed further below.

Nutrient uptake kinetics are highly plastic in algae and increase with increasing nutrient availability and lower N:P (O’Brien et al. 2007, Bonachela et al. 2011), but increased nutrient uptake does not necessarily translate into higher GPP. In our laboratory study, N uptake increased in response to nutrient enrichment, but algal biomass and GPP did not. Our results mirror those from a whole-lake experiment in the Arctic, where enrichment with both N and P led to substantial uptake of nutrients by periphyton with no concomitant increase in benthic GPP and no impact on phytoplankton biomass (Gettel et al. 2013). Our finding could potentially be a result of luxury uptake, high-nutrient uptake capacity by benthic Chlorophyta, an artifact of the relatively short duration of the experiment, or a response to light stress as a consequence of relocation from the high-UV environment of an alpine watershed (Dodds and Gunder 1992, Middleton and Frost 2014, Gladyshev and Gubelit 2019). Additionally, separating respiration into autotrophic and heterotrophic components is difficult, and it is possible that nutrient additions (particularly NO₃⁻) fuel heterotrophic metabolism or denitrification, or both, in Arctic and alpine lakes (Daniels et al. 2015). Thus, GPP may appear not to increase because microbial decomposition of new organic matter might simultaneously increase.

Metabolic functional responses to temperature increase

Our results suggest that, at warmer temperatures, the initial N:P ratio is less of a factor in N uptake than it is at colder temperatures because of the effect temperature has on NUE (Fig. 3A–D). At 8 and 12°C, N uptake was more efficient when N:P was low (Fig. 3D), but these high rates of N uptake did not translate into higher NUE. Other researchers have found increased NEP leading to higher NUE with warming temperatures (Hood et al. 2018), but their experiments occurred over longer periods of time, which allowed time for assemblage shifts (e.g., more N-fixers) that were not a factor in our 8-d experiment. In our study, increases in heterotrophic respiration in the warmest waters may have contributed to decreased NEP, which is also supported by our finding, discussed below, of decreased DOC concentrations at higher temperatures (Fig. 4A).

We observed that ER rates increased more with warming than did GPP, similar to other studies (Martin et al. 2006, Yvon-Durocher et al. 2010, Rosa et al. 2013) because heterotrophic respiration is highly sensitive to temperature (Davidson et al. 2006). ER was highest in the warmest treatments where we also observed the lowest DOC concentrations. This finding suggests that heterotrophs in the periphyton matrix consumed algal-derived DOC, a major source of energy for heterotrophic bacteria in aquatic ecosystems (Haack and MCFeters 1982, Kaplan and Bott 1989, Baines and Pace 1991), but we did not measure heterotrophic microbes directly. At the highest temperatures, we measured substantially greater DOC concentrations in the control and N treatments than in the P and N + P treatments, a trend that may be explained by greater algal exudation of DOC under P limitation (Wyatt et al. 2014). Alternatively, P limitation may have prevented the use of labile organic C by heterotrophs despite higher ER rates at warmer temperatures. These results highlight the tight linkages between autotrophic and heterotrophic metabolism, which are difficult to disentangle in our study. Future investigations would benefit from examining additional heterotrophic functions of chlorophyte-dominated periphyton, such as extracellular enzyme activities, to better elucidate autotrophic–heterotrophic linkages and implications for whole-lake metabolism.

Broader implications

Shifts in algal assemblages have implications for biogeochemical function, particularly N uptake, C balance, and release of labile extracellular C. Chlorophyta like Zygnema and Spirogyra, which are now commonly observed in Sky Pond, may have higher temperature optima, high UV tolerance, and the ability to survive desiccation with lake-level changes, all of which are traits that may favor their dominance in late summer as climate and particularly hydrological conditions change in the future (Raven and Geider 1988, Holzinger et al. 2009, Holzinger and Karsten 2013). Our results provide experimental evidence suggesting that warming and nutrients together are changing the ecology of alpine lakes from historically clear oligotrophic systems to those whose benthos is dominated by productive green algae.

ACKNOWLEDGEMENTS

Author contributions: IAO conceived the study and performed the experiments and sample processing with some assistance from JSB. IAO, JSB, and WSB interpreted the data. The manuscript was written by IAO, with substantial contributions by JSB and WSB.

We acknowledge, with respect, that our field site, Sky Pond, is located on the traditional and ancestral homelands of the Ute, Arapaho, Cheyenne, and Comanche nations and peoples. This project is a product of the United States Geological Survey (USGS).
Western Mountain Initiative. IAO and WB were supported with National Science Foundation IGERT Grant No. DGE-0966346. 1-WATER: Integrated Water, Atmosphere, Ecosystems Education and Research Program. We gratefully acknowledge Daniel Bokker and Tim Weinmann for logistical support and Jacob Ritter and Mitch Ralson for assistance with building and deploying the field experiment. Laboratory experiments took place at the USGS Fort Collins Science Center, and we thank Alisha Shah for logistical assistance with the incubations. Claudia Boot helped develop the pigment analysis methods at the Central Instrument Facility at Colorado State University.

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