

FINAL REPORT

Phytoplankton Responses to Lake Nitrogen Enrichment in Mount Rainier, North Cascades, and Olympic National Parks

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Prepared For:

National Park Service Air Resources Division

Prepared By:

Jason Williams and Marc Beutel

Washington State University Department of Civil & Environmental Engineering

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Rainbow Lake, NOCA



Hidden Lake, MORA



Hoh Lake, OLYM

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Acronyms

ANOVA	analysis of variance
Chl <i>a</i>	chlorophyll <i>a</i>
CV	coefficient of variation
DIN	dissolved inorganic nitrogen (NO ₃ -N + NH ₄ -N)
K _s	Monod growth half-saturation constant
LDPE	low density polyethylene
MORA	Mount Rainier National Park
NADP	National Atmospheric Deposition Program
NO ₃ -N	nitrate nitrogen
NH ₄ -N	ammonium nitrogen
NMDS	non-metric multi-dimensional scaling
NOCA	North Cascades National Park
NPS	National Park Service
Nr	reactive nitrogen
NTN	National Trends Network
OLYM	Olympic National Park
PERMANOVA	permutation analysis of variance
PRISM	Parameter-elevation Relationships on Independent Slopes Model
RR _{biomass}	biomass relative response (treatment mean Chl <i>a</i> / control mean Chl <i>a</i>)
RR _{taxa}	taxa relative response (1 + treatment mean cell density / 1 + control mean cell density)
TP	total phosphorus
μ	specific growth rate (d ⁻¹)
μ _m	maximum specific growth rate (d ⁻¹)

Executive Summary

The National Park Service (NPS) faces mandates from the Organic Act, Wilderness Act, and 1977 Clean Air Act amendments to protect national parks from adverse effects of air pollution. In the Pacific Northwest, NPS has identified a need for information about the effects of atmospheric nitrogen deposition on park resources (Cummings et al. 2014). Nitrogen emitted through fossil fuel combustion and agricultural activities can be transported through the atmosphere and deposited on parks as wet or dry nitrogen deposition. Elevated nitrogen deposition can reduce biodiversity, alter species composition, alter biogeochemical cycling, and contribute to acidification in both aquatic and terrestrial ecosystems.

In many mountain lakes throughout the northern hemisphere, nitrogen deposition has increased lake nitrate concentrations, and thereby stimulated changes in phytoplankton abundance and community composition. It is not yet clear if nitrogen deposition has caused widespread changes in Pacific Northwest mountain lakes. Previous investigations (Saros 2009, Sheibley et al. 2014) found evidence for deposition-induced phytoplankton changes in only one lake (Hoh Lake at Olympic National Park), and highlighted a need to better understand factors affecting lake sensitivity to nitrogen deposition. At least three conditions must be met for nitrogen deposition to induce phytoplankton changes in mountain lakes. Nitrogen deposition inputs must increase lake nitrogen concentrations, phytoplankton growth must be limited by nitrogen, and lake nitrogen concentration increases must be sufficient to stimulate phytoplankton growth.

In this study, we tested all three of these conditions for mountain lakes at Mount Rainier (MORA), North Cascades (NOCA), and Olympic (OLYM) national parks. We conducted nutrient enrichment experiments in three lakes at each park to determine if phytoplankton growth is nitrogen limited and quantify lake nitrogen concentration thresholds necessary to stimulate phytoplankton changes. Results indicate phytoplankton species and biomass growth was nitrogen-limited or co-limited by nitrogen and phosphorus. Dissolved inorganic nitrogen ($\text{DIN} = \text{NO}_3\text{-N} + \text{NH}_4\text{-N}$) concentrations of $13\text{-}25\ \mu\text{g N L}^{-1}$ were necessary to stimulate an increase in lake phytoplankton biomass, and DIN concentrations $0.02\text{-}50\ \mu\text{g N L}^{-1}$ can stimulate growth of nitrogen-sensitive species, depending on the species. We compared the minimum concentration necessary to increase phytoplankton biomass ($13\ \mu\text{g N L}^{-1}$) to concentrations observed in previous lake surveys at the parks. This threshold is currently exceeded in at least 27% of sampled lakes at NOCA, 26% of sampled lakes at MORA, and 0 out of 9 sampled lakes at OLYM.

Phytoplankton in mountain lakes appear highly sensitive to future increases in lake nitrogen. However, it is not clear if nitrogen deposition strongly affects lake nitrogen concentrations. We tested for a correlation between nitrogen deposition rates and lake nitrate across MORA, NOCA, and OLYM using lake chemistry data from previous lake surveys. We observed a weak positive correlation between deposition and lake nitrate across the three parks. However, many lakes receiving similar deposition rates had nitrate concentrations that differed by orders of magnitude. This result implies that watershed characteristics such as land cover type, slope, and geology strongly mediate the relationship between nitrogen deposition and lake nitrate. Additional research is needed to establish if future increases or decreases in nitrogen deposition will increase lake nitrogen concentrations, and to identify lakes where deposition-induced concentration increases are most likely.

1.0 INTRODUCTION

Atmospheric nitrogen (N) deposition rates have more than doubled since the nineteenth century due to fossil fuel combustion, agricultural and industrial nitrogen fixation, and other human activities that convert inert molecular N₂ to reactive N compounds (Nr) (Galloway et al. 2008). Once created, Nr can be transported regionally or globally through the atmosphere and subsequently deposited onto terrestrial and aquatic ecosystems (Fowler et al. 2013).

Anthropogenic N deposition has thereby altered the chemistry and ecology of lakes throughout the northern hemisphere (Elser et al. 2009a, Baron et al. 2011). It has increased lake nitrate, simulated phytoplankton biomass growth (Bergström et al. 2008, Elser et al. 2009b), shifted phytoplankton nutrient limitation from N to P (Elser et al. 2009a), and altered phytoplankton community composition (Hobbs et al. 2010).

Remote subalpine and alpine (hereafter ‘mountain’) lakes are typically most sensitive to these effects. Mountain lake watersheds often have landcover, geologic, and geomorphic characteristics that facilitate efficient flux of deposited N in to lakes (Clow et al. 2010, Nanus et al. 2012), where phytoplankton growth is often N-limited (Elser et al. 2009a, Baron et al. 2011). Thus, relatively small increases in N deposition can increase lake N and trigger phytoplankton changes. Threshold or ‘critical load’ deposition rates for diatom community changes in mountain lakes are 1.0-1.5 kg wet inorganic N ha⁻¹ yr⁻¹ (Baron 2006, Saros et al. 2010, Sheibley et al. 2014), which is lower than those for other ecological receptors (Pardo et al. 2011). Diatom critical loads therefore have been used as an indicator of early stage eutrophication in aquatic ecosystems, and to develop target deposition rates in regional air quality agreements (Porter and Johnson 2007).

In the Rocky Mountain region of the Western United States, substantial evidence indicates N deposition has induced chemical and phytoplankton changes in mountain lakes. Mountain lake nitrate concentrations are positively correlated with N deposition rates (Nanus et al. 2012). Lakes in high deposition regions of the Rockies ($> 6 \text{ kg wet inorganic N ha}^{-1} \text{ yr}^{-1}$) have higher nitrate concentrations, higher algal biomass, and are more likely to have P-limited algal growth compared to lakes in low ($< 2 \text{ kg wet inorganic N ha}^{-1} \text{ yr}^{-1}$) deposition regions (Elser et al. 2009b). Lake sediment cores indicate diatoms *Asterionella formosa* and *Fragilaria crotonensis* have increased in relative abundance as N deposition rates increased along with regional human population growth and industrialization (Saros et al. 2003, 2005, Wolfe et al. 2003, Baron 2006). The current distribution and abundance of these diatoms in the Rockies is also positively related to lake nitrate concentrations (Saros et al. 2005). Nutrient enrichment experiments indicated that *A. formosa* and *F. crotonensis* are N-limited in some Rocky Mountain lakes, and have N and P resource requirements that allow them to grow rapidly in response to small lake N increases (Saros et al. 2005, Michel et al. 2006, Arnett et al. 2012, Nanus et al. 2012).

In contrast, in the Pacific Northwest, it is not clear if N deposition has increased mountain lake N and induced widespread phytoplankton responses. Annual N deposition rates measured at northwest National Atmospheric Deposition Program (NADP) National Trends Network (NTN) monitoring sites range from $0.38\text{--}3.24 \text{ kg wet inorganic N ha}^{-1} \text{ yr}^{-1}$ (<http://nadp.sws.uiuc.edu/data/ntn/>), and nitrate concentrations in mountain lakes are frequently at or near detection limits ($1 \text{ } \mu\text{g N L}^{-1}$) (Larson et al. 1999, Clow and Campbell 2008). However, the regional relationship between N deposition and lake N has not been examined to our knowledge. Previous studies have yielded mixed evidence for effects of N deposition on

mountain lake phytoplankton in the northwest. N deposition rates at northwest NTN sites are frequently near or above the 1-1.5 kg wet inorganic N ha⁻¹ yr⁻¹ threshold at which historic diatom community changes have been observed in sediment cores collected in the Rocky Mountains and Sierra Nevada (Baron 2006, Saros et al. 2010). Consistent with patterns in other regions, Sheibley et al. (2014) documented increases in the relative abundance of *A. formosa* and *Fragilaria tenera* as wet inorganic N deposition rates increased above 1-1.2 kg N ha⁻¹ yr⁻¹ in the 1969-1975 timeframe at Hoh Lake in Olympic National Park. Larson et al. (1998) also observed that lake nitrate was the primary chemical variable influencing phytoplankton community composition in mountain lakes at North Cascades National Park. However, Saros (2009) conducted nutrient enrichment experiments in two Washington State mountain lakes and found that phytoplankton biomass and diatom growth was P limited. In addition, multiple sediment cores collected in Cascade Mountains lakes indicate no changes in phytoplankton community structure, relatively few occurrences of indicator species *F. crotonensis* and *A. formosa* and diverse phytoplankton communities that differ substantially among lakes (Saros 2009, Sheibley et al. 2014). These patterns could indicate that critical loads are not yet widely exceeded, mountain lakes are predominantly P-limited, or that diatom taxa responsive to N deposition in the Pacific Northwest are different from those in other regions.

To help address these uncertainties, we tested for a relationship between N deposition and lake nitrate in regional mountain lakes and conducted nutrient enrichment experiments to characterize phytoplankton responses associated with lake N increases. We used data from previous lake surveys at Mount Rainier (MORA), North Cascades (NOCA), and Olympic (OLYM) national parks to test for a relationship between N deposition and lake nitrate. We also conducted *in situ* nutrient enrichment experiments in three mountain lakes at each park.

Objectives of nutrient enrichment experiments were to i) assess nutrient limitation status of phytoplankton biomass and specific taxa, ii) characterize patterns of biomass and taxon responses to lake N enrichment, and iii) estimate N concentration thresholds at which species and biomass responses occur. Once quantified, concentration thresholds can be used to identify the number of lakes in a region above or below thresholds, to calculate a critical load N deposition rate at which phytoplankton responses occur (Nanus et al. 2012), or to help explain diatom community changes observed in sediment cores (Saros et al. 2005, Michel et al. 2006, Arnett et al. 2012).

2.0 METHODS

2.1 Study Area

Lakes in this study are located in three national parks within the Cascade and Olympic mountain ranges of western WA (Figure 1). Annual precipitation rates range from 100-500 cm across the parks and are strongly influenced by regional terrain, elevation gradients, and weather systems that move eastward from the Pacific Ocean. Most mountain lakes within the parks range from 1,000 – 2,000 m elevation, and are cirque, tarn, or moraine-dammed lakes formed by past glacial activity, and are typically ice-free from mid-July through October. Park mountain lakes are oligotrophic, and frequently have N and P concentrations below or near detection limits (Clow and Campbell 2008, Glesne et al. 2012). Nitrogen deposition rates measured at NTN sites within and near the three parks range from 0.38-3.24 kg wet inorganic N ha⁻¹ yr⁻¹ (<http://nadp.sws.uiuc.edu/data/ntn/>). No significant trend in annual N deposition rates is present at the parks from the 1980's to the present (Du et al. 2014). N deposition rates at high elevations appear to be greater than those measured at NTN sites (< 500 m elevation), but data at elevation

is limited (Sheibley et al. 2014). Among the three parks, N deposition rates are consistently highest at NOCA, and lowest at OLYM.

2.2 Nitrogen Deposition and Lake Nitrate

N deposition is positively correlated with mountain lake nitrate in the Rocky Mountains (Nanus et al. 2012), so we tested for the same pattern in the Pacific Northwest. A lake nitrate database was assembled for the three parks using data from previous lake surveys (Larson et al. 1994, 1999, Sheibley et al. 2014) and unpublished National Park Service (NPS) data. The assembled database included 675 nitrate observations from 125 lakes sampled between 1988-2014 at MORA, 82 lakes sampled between 1989-2013 at NOCA, and 9 lakes sampled between 2005-2013 at OLYM. Some lakes were sampled in multiple years, and some lakes were sampled only once. A mean nitrate concentration was calculated for each lake across years. Lakes below 1200 m elevation were excluded from the database to avoid lakes where nitrate concentrations may be influenced by N-fixing red alders. Detection limits were $1 \mu\text{g NO}_3\text{-N L}^{-1}$ across datasets used to construct the database, with 70% of data points below detection.

N deposition rates at each lake in the database were calculated as the mean of 1988-2013 annual wet inorganic N deposition. Annual deposition rates at each lake were calculated using a three-step process. First, lake annual precipitation was estimated using the Parameter-elevation Relationships on Independent Slopes Model (PRISM) (Daly et al. 2008). Second, annual precipitation was multiplied by the annual inorganic N concentration in precipitation at the relevant park NTN site to calculate annual deposition ($\text{mg N L}^{-1} \times \text{cm yr}^{-1} \times 0.1 = \text{kg N ha}^{-1} \text{ yr}^{-1}$). Third, mean 1988-2013 deposition was calculated at each lake, using data only from years where NTN performance criteria were met for NTN measurements. This approach has previously been

used to calculate diatom critical loads (Sheibley et al. 2014, Saros 2010), and more accurately predicts wet inorganic N deposition at high elevation than other modeling approaches at MORA (Williams et al., in prep).

We tested for a positive rank correlation between lake mean deposition rates and lake mean $\text{NO}_3\text{-N}$ using a Kendall tau rank correlation test ($p < 0.05$). In addition, we used logistic regression to evaluate the relationship between lake mean deposition rates and the probability that lake mean nitrate exceeds the detection limit. Although ammonium data were also available for lakes within the database, we only evaluated the relationship between deposition and nitrate. Ammonium detection limits were not constant across data sets and in some cases relatively high ($10 \mu\text{g N L}^{-1}$) which complicates analysis and interpretation or correlations.

2.3 Nutrient Enrichment Bioassays

Three lakes within each park were selected as bioassay study sites (Table 1). We selected lakes above 1200 m where available chemistry data indicated phytoplankton N-limitation was likely (Bergstrom et al. 2010), or where known N-sensitive diatom species such as *Asterionella formosa*, *Fragilaria crotonensis*, *Fragilaria tenera*, or *Discostella stelligera* were present (Wolfe et al. 2003, Saros et al. 2005, Arnett et al. 2012, Sheibley et al. 2014). We targeted lakes above 1200 m to avoid the potential influence of N-fixing red alder (*Alnus* sp.) on lake N. We deliberately chose lakes where responses to N addition were likely because our goal was to characterize phytoplankton responses to N enrichment in deposition-sensitive lakes. Selected lakes were remote, accessible only by foot, required 3 –11 miles of hiking one-way to access, and in several cases lake access and sampling required a multi-day expedition.

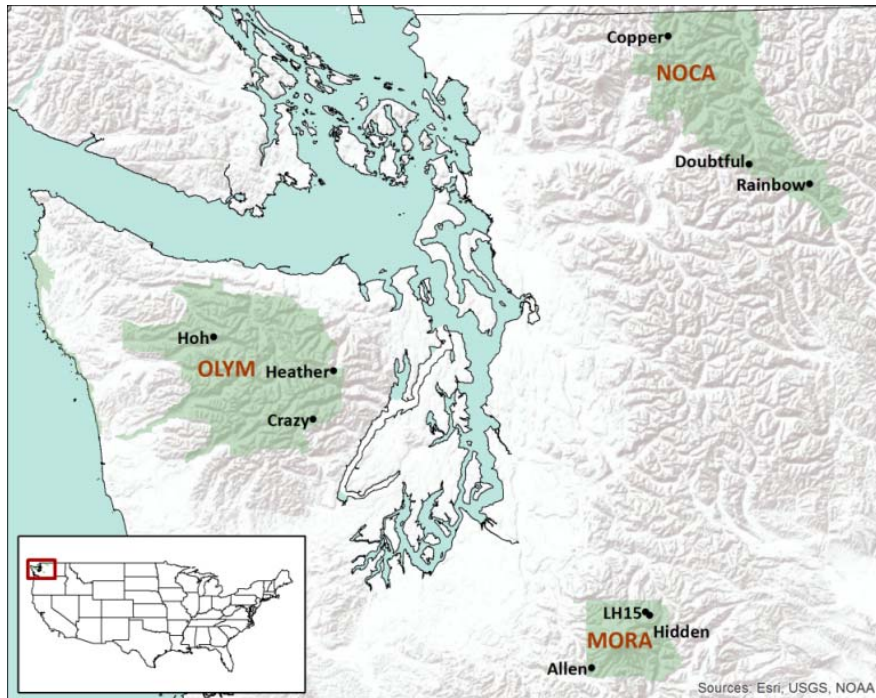


Figure 1. Locations of Mount Rainier (MORA), North Cascades (NOCA), and Olympic National Park (OLYM) and bioassay lakes.

Table 1. Physical characteristics, incubation depth, and experiment dates for study lakes.

Park	Lake	Elevation (m)	Area (ha)	Max Depth (m)	Secchi Depth (m)	Incubation Depth (m)	Incubation Length (days)	Incubation Temperature (C)
North Cascades (NOCA)	Rainbow	1,717	6.3	10.4	10.4	5.5	7	17.3
	Doubtful	1,642	12.2	17.7	9.5	4.75	7	13.8
	Copper	1,601	5.2	22.6	19*	9	7	7.6
Mount Rainier (MORA)	LH15	1,659	3.1	10	10	5	7	16.8
	Hidden	1,806	2.2	7	7	3.5	7	16.5
	Allen	1,397	1.9	7.2	7	3.5	7	16.3
Olympic (OLYM)	Crazy	1,457	1	7.7	7.7	3.5	11	14
	Heather	1,589	0.4	6.6	6.6	3	8	7.9
	Hoh	1,383	7.4	14.9	11	5.5	8	14.5

*Secchi disk depth recorded by NPS in 2011. A Secchi disk depth of 13 m was recorded on Aug 29, 2013

during cloudy skies and heavy rain.

Bioassays were conducted during late summer (August or September) in 2013 (NOCA) and 2014 (MORA and OLYM). Secchi disc depth was measured at each lake from an inflatable packraft at the deepest point on the lake. One half of the Secchi disc depth was used as water collection and experimental incubation depth. This depth typically has sufficient light intensity for phytoplankton growth, but does not cause algal photoinhibition (Morris and Lewis 1988). In lakes where the Secchi disk could be seen on the lake bottom, experimental containers were incubated at lake mid-depth. At each lake, 30-40 L of water was collected into 4 L LDPE cubitainers using a battery-powered submersible sampling pump and Tygon tubing. The pump and Tygon tubing were flushed with lake water from the selected depth for 2-3 minutes prior to collecting water used in experiments. A piece of 100 μm Nitex mesh was placed on the pump intake to screen out large-bodied zooplankton. Collected water was subsampled to establish the initial Chl *a*, nutrient chemistry, and phytoplankton community composition data for experiments. Triplicate Chl *a* samples were collected by filtering a known volume of water through 0.7- μm Whatman GF/F filters. Filters were placed in petri dishes, which were then wrapped in aluminum foil. Triplicate 60 mL water subsamples were collected and filtered through a pre-rinsed 0.4- μm polycarbonate filter into a HCl-washed sample bottle for analysis of dissolved N and P species. Three additional 60 mL subsamples were filtered for analysis of dissolved silica (Si). Three 120 mL unfiltered subsamples were collected into HCl-washed sample bottles for analysis of total N (TN) and total phosphorus (TP). Chl *a* filters and N and P water samples were stored in a cooler bag packed with snow in the field and immediately frozen upon return. Water samples collected for Si were cooled in the field and refrigerated upon return. Six 60 mL subsamples were preserved with Lugol's iodine solution and used to determine initial

phytoplankton community composition and species densities. Phytoplankton samples collected for identification and enumeration were refrigerated after returning from the field.

After subsampling, collected water was divided among clean 1 L LDPE cubitainers that were pre-rinsed three times with lake water. Water in each cubitainer was amended with N (as NaNO_3), P (as NaH_2PO_4) and Si (as Na_2SiO_3) as required to create 10 treatments, each with 3 replicates. Four treatments (control, $112 \mu\text{g N L}^{-1}$, $31 \mu\text{g P L}^{-1}$, $112 \mu\text{g N L}^{-1} + 31 \mu\text{g P L}^{-1}$) were designed to discriminate between N, P, or co-limitation using concentrations similar to those in other bioassays designed to assess nutrient limitation status (Elser et al. 2009b, Slemmons and Saros 2012, Bergström et al. 2013). Six additional treatments were designed to create an N concentration gradient and quantify diatom Monod growth curves (section 2.4). Treatment N additions were 0.14, 0.7, 1.4, 7, 14, and $56 \mu\text{g N L}^{-1}$. $31 \mu\text{g P L}^{-1}$ and 2.8 mg L^{-1} Si were also added to these treatments to prevent these nutrients from limiting growth and isolate responses to N additions. The $56 \mu\text{g N L}^{-1}$ treatment was used at MORA and OLYM, but not at NOCA; it was added based on results observed in NOCA lakes. Concentrations for these treatments were selected to be comparable to those used in similar experiments in the Rocky Mountains (Nanus et al. 2012). Throughout this document, these treatments where N, P and Si were all added are referred to as the ‘N gradient treatments’. Lake water nutrient concentrations were not known in the field when treatments were created. Treatment nutrient concentrations are the sum of nutrient additions and initial concentrations.

Cubitainers were suspended in each lake at the incubation depth for 7-11 d, depending on access constraints (Table 1). Three replicate buoys, each with one cubitainer from each treatment, were anchored to the lake bottom. At the end of the incubation period, buoys were removed from the lake and each cubitainer was subsampled to collect one Chl *a* and

phytoplankton taxonomy sample as described above. Water temperatures were recorded at the lake incubation depth either continuously during the experiment by a NPS temperature logger or using a submersible digital thermometer at the beginning or end of the experiment.

2.4 Laboratory Analyses

Chl *a* filters were extracted in 10 ml of 90% acetone within three weeks of collection. The extract was then clarified by centrifugation and Chl *a* was analyzed using a Shimadzu RF-501 spectrofluorometer (emission at 670 nm, excitation at 430 nm light). Chl *a* samples were not corrected for pheophytin interference. Detection limits, defined as 3 times the mean of filter blanks, were 0.04 $\mu\text{g L}^{-1}$ or less in each analytical run, and all results were above detection limits. Water samples were analyzed for nutrients using standard methods (APHA 2011). N and P analyses were performed using a Lachat Autoanalyzer according to standard methods. Detection limits were 1-2 $\mu\text{g N L}^{-1}$ for nitrate plus nitrite, 4 $\mu\text{g N L}^{-1}$ for ammonium, 2 $\mu\text{g P L}^{-1}$ for ortho-phosphate and TP, and 2 $\mu\text{g N L}^{-1}$ for TN. Si analyses were performed at the Oregon State University Cooperative Chemical Analytical Laboratory using a Technicon Auto-Analyzer II using standard method 4500-SiO₂ E. Phytoplankton samples were settled in an Utermöhl -style chamber and identified to the lowest practical level (typically species or genus) under 400 or 600x magnification on a Nikon TS100 inverted microscope.

2.5 Nutrient Limitation

Nutrient limitation of biomass growth in each lake was classified using an approach similar to Slemmons and Saros (2012). A one-way ANOVA and Tukey HSD means separation test were applied to Chl *a* data from the control, N-only, P-only and N+P limitation treatments. Data were log-transformed, ANOVA homogeneity of variance and normality assumptions were

tested with Levene's and Shapiro-Wilks tests, and significant differences between means were assessed at $p < 0.05$. Statistical analyses were performed using the R statistical software (version 3.1.1) (www.r-project.org). If only the N-only or P-only treatment was significantly greater than the control, growth was classified as 'N-limited' or 'P-limited'. If a single-element treatment was greater than the control, and $N+P > \text{single element} > \text{control}$, growth was classified as 'serial N limitation' or 'serial P limitation'. Growth was classified as 'co-limited' if only $N+P > \text{control}$, or if both $N > \text{control}$ and $P > \text{control}$. If only significant decreases relative to the control were observed, growth was classified as 'constrained'. Growth was classified as 'not limited' if there were no significant differences between treatments and the control. At the taxon level, nutrient limitation was classified by applying the procedure described above for biomass to cell densities. Taxa growth responses were also described by calculating cell density relative responses ($RR_{\text{taxa}} = (1 + \text{treatment mean cell density}) / (1 + \text{control mean cell density})$). Cell density RR_{taxa} was calculated for N-only, P-only, and N+P treatment to quantify the magnitude of responses relative to the control.

2.6 Taxa Monod Growth Curves

Monod growth curves were constructed for taxa demonstrating a significant difference in cell densities between the N-only or N+P treatments and the control. Results from the control, N gradient treatments (0.14, 0.7, 1.4, 7, 14, and $56 \mu\text{g N L}^{-1}$, plus $31 \mu\text{g P L}^{-1}$ and $2.8 \text{ mg L}^{-1} \text{ Si}$), and $112 \mu\text{g N} + 31 \mu\text{g P}$ treatments were used to create growth curves. The $112 \mu\text{g N} + 31 \mu\text{g P}$ treatment was used for growth curve calculations despite not including Si because initial lake Si concentrations and observed growth responses indicated Si did not limit growth (see results). Growth rates (μ, d^{-1}) were calculated as:

$$\mu = \frac{\ln(F) - \ln(I)}{T}$$

where F is the cell concentration at the end of the incubation (cells mL⁻¹), I is the mean cell concentration (cells mL⁻¹) from triplicate initial lake water samples, and T is the incubation time (d) (Table 1). Growth curves were created by fitting the Monod equation to experiment data using nonlinear least squares regression with the ‘nls’ function in R:

$$\mu = \mu_m \left(\frac{S}{K_s + S} \right)$$

where μ_m is the maximum specific growth rate (d⁻¹), S is the dissolved inorganic nitrogen (DIN = NO₃-N + NH₃-N) concentration (µg N L⁻¹) calculated as the sum of initial and added DIN, and K_s is the half-saturation coefficient for growth (S when $\mu = \mu_m/2$). Growth affinities ($\mu_m \cdot k_u$) were calculated to characterize the relative competitive ability of each taxon in each lake; higher values indicate a greater initial growth curve slope and thus theoretically a greater competitive ability. Taxa were categorized as responsive to N if they exhibited N-limitation, serial N limitation, or were co-limited and showed a strong Monod response to increasing N concentration in gradient treatments in at least one lake.

2.7 Phytoplankton Community Changes

Phytoplankton community changes in response to N and P enrichment were visualized with nonmetric multidimensional scaling (NMDS) plots and tested for statistical significance using permutation multivariate analysis of variance (PERMANOVA) (Anderson 2001a, 2001b). NMDS and PERMANOVA analyses were applied to the control, N-only, P-only, and N+P treatments. The metaMDS function from the Vegan package in R was used to construct a similarity matrix of pairwise Bray-Curtis distances after log₁₀(x+1) transformation of taxa cell

densities. Data were then visualized using 2-dimensional NMS. NMDS plots were constructed for each lake, and again after combining data across lakes. Significance of phytoplankton community changes were tested statistically using PERMANOVA (Anderson 2001a, 2001b). Within each lake, a two-way PERMANOVA was used to test for main effects of N and P, and NxP interactions. A nested two-way PERMANOVA was also applied to limitation treatment data from all lakes with lake as the nesting factor to test for significant effects of N and P addition across lakes. PERMANOVA analyses used 999 permutations and were performed on Bray-Curtis similarity matrices constructed after $\log_{10}(x+1)$ transformation of cell densities, were applied at the taxa level (the level at which taxa were identified and enumerated), and were performed using Adonis from the vegan package (version 2.2-1) in R (Oksanen et al. 2015).

2.6 Chlorophyll a Response Thresholds

Chl *a* results from the N gradient treatments were used to estimate the threshold lake DIN concentration where biomass increases occur. Biomass relative response (RR_{biomass}) was calculated for each treatment as the ratio of treatment mean Chl *a* to control mean Chl *a*. Simple linear regression was used to estimate two biomass response thresholds: the DIN concentration for any increase in biomass relative to the control ($RR_{\text{biomass}} > 1$), and the DIN concentration required to increase biomass above typical lake inter-annual variability. We defined typical inter-annual variability as the average of Chl *a* coefficients of variation (CV) observed in 21 mountain lakes sampled annually by the National Park Service (NPS) in late summer 2009-2013. A CV value was calculated for each lake and then used to calculate an average Chl *a* CV ($CV_{\text{mean}} = 0.73$, range = 0.26-1.6). Data for calculating CV_{mean} were provided by NPS (NPS, unpublished data). DIN (initial DIN + added N) was used as the predictor variable and lake mean RR as the response variable in regressions, using data from the N gradient and 112 $\mu\text{g N} + 31 \mu\text{g P}$

treatments. Regressions were performed for 1) individual lakes, 2) using data from all lakes, and 3) using data from only N-limited and sequential co-limitation (N) lakes (Table 4) for comparison. Best-fit regression equations were solved for DIN where $RR_{\text{biomass}}=1$ or where $RR_{\text{biomass}}=(1 + CV)$ to estimate thresholds for each lake and across lakes.

We then used biomass thresholds calculated from bioassays to estimate the number of mountain lakes at each park where DIN ($\text{NO}_3\text{-N} + \text{NH}_3\text{-N}$) exceeds thresholds. Using the lake database described in section 2.1, we calculated lake mean lower bound and upper bound mean DIN by substituting either $0 \mu\text{g N L}^{-1}$ for or the detection limit for values that were below detection, respectively. We then compared lower and upper bound lake mean DIN to thresholds, in order to estimate lower and upper bound estimates for the number of lakes where DIN exceeds thresholds.

3.0 RESULTS

3.1 N Deposition and Lake Nitrate

The relationship between lake mean N deposition and lake mean nitrate across parks is plotted in Figure 2. There is not a clear positive relationship between N deposition and nitrate; lakes with similar deposition rates had nitrate concentrations that differed by orders of magnitude (Figure 2). However, there was a statistically significant positive correlation between lake mean wet inorganic N deposition and lake mean nitrate across parks (Kendall tau, $p < 0.001$, $\tau = 0.16$, $z = 3.34$). A logistic regression model with lake mean wet inorganic N deposition as the predictor and the binary response of nitrate above or below the detection limit was marginally better than a null model with only an intercept (deviance = 4.1, $df = 1$, $p = 0.04$). In the logistic model, deposition was marginally positively related to the occurrence of nitrate values above the

detection limit ($b = 0.48$, $b_{SE} = 0.24$, $p = 0.05$, $df_{model} = 214$). The odds ratio for N deposition (e^b) indicates a one $\text{kg N ha}^{-1} \text{yr}^{-1}$ increase in lake mean N deposition increases the probability that lake mean nitrate will exceed the detection limit by a factor of 1.6.

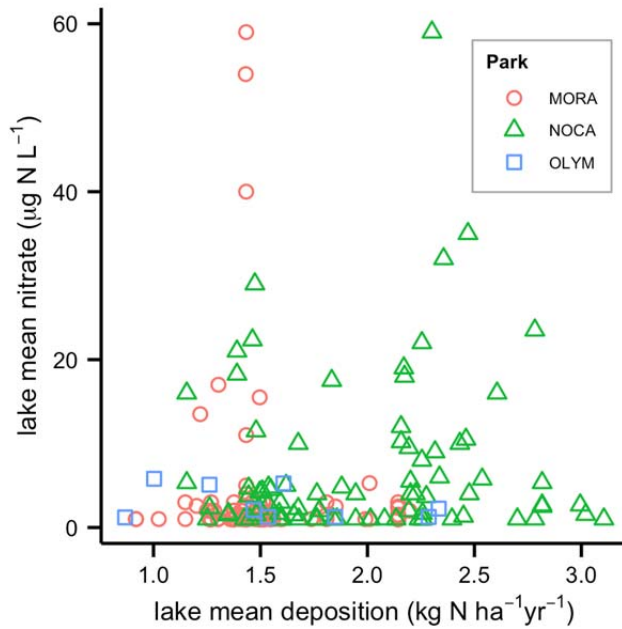


Figure 2. Relationship between lake mean annual wet inorganic N deposition and lake mean nitrate across parks.

3.2 Bioassay Initial Conditions and Enclosure Effects

Initial lake mean Chl *a* concentrations were $0.05\text{--}0.65 \mu\text{g L}^{-1}$ in lake water prior to nutrient additions and ammonium, nitrate plus nitrite, and orthophosphate concentrations were near or below detection limits in lake water prior to nutrient additions (Table 2). Initial TP concentrations were $4\text{--}8 \mu\text{g P L}^{-1}$ across lakes. The mass ratio of dissolved inorganic nitrogen to total phosphorus (DIN:TP) was less than 1.5 and therefore predicted biomass N-limitation in 4 lakes (Rainbow, Hidden, Allen, and Heather). according to the metric developed by Bergstrom et

al. (2012) Initial Si concentrations ranged from 0.7 – 3.3 mg L⁻¹, and thus were above concentrations typically associated with Si-limited diatom growth (< 0.5 mg Si L⁻¹) (Reynolds 2006).

Comparison of mean Chl *a* concentrations in initial lake water (Table 2, Table 3) and in control treatments after incubation indicated enclosure effects were small relative to treatment effects in all lakes except Hoh Lake. Mean Chl *a* concentrations in control treatments were generally similar to, but slightly lower than, Chl *a* in initial lake water. Within each lake differences in initial and control treatment Chl *a* were 3-40 times smaller than treatment responses observed for significant treatments. One exception was Hoh Lake, where initial mean Chl *a* (0.59 µg L⁻¹) was substantially higher than mean Chl *a* in the control treatment after incubation (0.11 µg L⁻¹). The difference between the initial and control treatment was larger than that between the N-only treatment and the control, but less than that between the N+P treatment and the control (Table 3, Table 4). Enclosure effects are more difficult to assess at the taxon level due to higher within-treatment variability in cell densities. However, in the relatively few cases where substantial negative enclosure effects were observed (control mean / initial mean < 0.5), positive treatment effects on cell density were orders of magnitude larger than enclosure effects. Higher control mean cell densities compared to the initial mean were more common, but in these cases large significant effects of nutrient enrichment were also observed. No Chl *a* or taxon results were excluded from analyses due to enclosure effects.

Table 2. Nutrient and Chl *a* concentrations prior to nutrient additions. Values are averages where N=3.

Park	Lake	N	Total P ($\mu\text{g P L}^{-1}$)	Ammonium ($\mu\text{g N L}^{-1}$)	Ortho- phosphate ($\mu\text{g P L}^{-1}$)	Nitrate plus Nitrite ($\mu\text{g N L}^{-1}$)	Si (mg L^{-1})	DIN:TP ^a	Chl <i>a</i> ^b ($\mu\text{g L}^{-1}$)
NOCA	Rainbow	3	7	5	2	< 1	0.90	0.9	0.65
	Doubtful	3	5	9	2	7	0.65	4.2	0.33
	Copper	3	4	6	2	5	0.63	2.6	0.28
MORA	Hidden	1	6	< 4	< 2	< 2	2.66	1.00	0.63
	LH15	1	6	5	3	15	1.98	3.33	0.25
	Allen	1	8	< 4	< 2	< 2	2.34	0.75	0.12
OLYM	Heather	1	7	< 4	< 2	3	1.23	1.00	0.05
	Crazy	1	4	< 4	< 2	3	0.71	1.75	0.2
	Hoh	1	6	< 4	< 2	6	1.22	1.67	0.59

^aDIN:TP calculated as [(nitrate+nitrite) + (ammonium)]/ TP; detection limits were used for calculations when results were below detection

^bChl *a* values are means of triplicate samples

Table 3. Mean +/- standard error chlorophyll *a* (µg/L) concentrations from control, N-only, P-only, and N+P treatments and inferred limitation type. * denotes treatments significantly different from the control (p<0.05, Tukey HSD), and ^ denotes cases where N + P > N > control (p<0.05, Tukey HSD).

Park	Lake	Control	31 µg P L ⁻¹	112 µg N L ⁻¹	112 µg N L ⁻¹ + 31 µg P L ⁻¹	Limitation Type
	Copper	0.18 ± 0.02	0.21 ± 0.03	0.23 ± 0.04	0.53 ± 0.01 *	Co-limitation
NOCA	Doubtful	0.30 ± 0.03	0.27 ± 0.04	0.97 ± 0.05 *	3.21 ± 0.36 *^	Serial N limitation
	Rainbow	0.53 ± 0.14	0.47 ± 0.07	1.07 ± 0.16	5.31 ± 0.49 *	Co-limitation
	Hidden	0.27 ± 0.12	0.12 ± 0.02	0.47 ± 0.06	2.2 ± 0.74 *	Co-limitation
MORA	Allen	0.08 ± 0.01	0.1 ± 0.01	0.20 ± 0.03 *	0.64 ± 0.12 *^	Serial N limitation
	LH15	0.13 ± 0.01	0.05 ± 0.01	0.12 ± 0.03	1.13 ± 0.43 *	Co-limitation
	Crazy	0.12 ± 0.05	0.09 ± 0.01 *	0.27 ± 0.04	1.3 ± 0.05 *	Co-limitation
OLYM	Heather	0.04 ± 0.01	0.04 ± 0.00	0.1 ± 0.02 *	0.12 ± 0.04 *	N limitation
	Hoh	0.11 ± 0.01	0.12 ± 0.02	0.22 ± 0.03 *	0.86 ± 0.04 *^	Serial N limitation

3.2 Nutrient Limitation

Phytoplankton biomass (measured as Chl *a*) in each lake increased in response to N or N+ P addition, but not to addition of P alone. Biomass growth was classified as N-limited (strict N limitation or serial N limitation) in four lakes, and as co-limited by N and P in five lakes (Table 3). In N-limited lakes, addition of N alone increased biomass 2-3 times relative to the control (Table 3). Addition of P alone did not increase biomass relative to the control in any of the study lakes (Table 3). Addition of N and P together significantly increased biomass relative to the control in all 9 lakes ($p < 0.05$). Addition of N+P together increased Chl *a* concentrations 3-10 times relative to the control, with the largest increases in co-limited lakes (Table 3).

In each lake, a total of 27 to 50 taxa were observed. Diatom (bacilliarophyta), chrysophyte (chrysophyta), chlorophyte (chlorophyta), and cyanophyte (cyanophyta) taxa were numerically most abundant based on cell densities (Figure 3). Most taxa that responded positively to nutrient addition responded to N and/or N+P addition, but not to addition of P alone. Among abundant taxa, there were 11 instances of N limitation, 13 instances of serial N limitation, 21 instances of co-limitation, 1 instance of P limitation, 1 instance of serial P limitation, and 5 instances of constrained growth, and 21 instances of no responses to enrichment. Strong Monod growth responses were observed for multiple N-limited, serial N limited, and co-limited taxa.

We identified 21 distinct taxa that responded to N enrichment (Table 4), meaning that in at least one lake they were N-limited, serial N limited, or were co-limited and showed a strong Monod response. Of these N-responsive taxa, 8 were diatoms, 6 were chlorophytes, 3 were cyanophytes, 2 were chrysophytes, and 2 were not identified to the phylum level. Many diatom

species responded to N addition in multiple lakes, including *Aulacoseira pusilla*, *Cyclotella distinguenda*, *Discostella glomerata*, *Fragilaria crotonensis*, and *Fragilaria tenera* (Table 4). Two chlorophyte taxa (coccoid green algae and *Tetraspora* sp.), one chrysophyte taxon (small golden w/o flagella), and one cyanophyte taxon (*Synechocystis* type coccoid cyanobacteria) also responded to N addition in multiple lakes (Table 4). Among N-responsive taxa, addition of N alone increased cell densities 1.6 to 29 times relative to the control, and addition of N+P increased cell densities 1.8 to 326 times relative to the control (Table 4).

3.3 Taxa Monod Growth Curves

Monod growth curves were constructed for 18 of 21 N-responsive taxa (those demonstrating a significant difference in cell densities between the N-only or N+P treatments and the control) (Table 4). Monod growth curves could not be constructed for all responsive taxa due to cell density variability for some taxa. Monod parameters and RR_{taxa} values for all abundant taxa in each lake are presented in Appendix A. Monod half-saturation constants ranged from 0.02 to 77 $\mu\text{g DIN L}^{-1}$ across most N-responsive taxa. Monod half saturation constants exceeded 100 $\mu\text{g DIN L}^{-1}$ for three cases where responses appeared more linear than Monod-like (*Discostella glomerata* in LH15, *Tetraspora* in Hoh, *Microsystis* sp. colony in Crazy). Standard errors for K_s estimates in many instances were larger than K_s values, which is common for K_s measurements due to limited data points at very low concentrations (Grover et al. 1999, Michel et al. 2006). Values of μ_{max} ranged from 0.1 to 0.6 for most taxa, but were higher for the three taxa mentioned above which showed a linear response (Table 4). Growth affinities ($\mu_{\text{max}}: K_s$) ranged from 0.01 to 11, with all but 4 values falling between 0.01 and 0.05 (Table 4).

Multiple Monod curves were constructed for *Fragilaria crotonensis*, *Fragilaria tenera*, coccoid green alga sp., *Tetraspora* sp., and coccoid cyanobacteria, and for some taxa Monod parameters varied substantially between lakes (Table 4). Monod growth curves were not constructed for any taxa from NOCA lakes (Copper, Doubtful, Rainbow) due to taxon response patterns and treatments applied at NOCA. Taxon-level responses were most prominent in N+P treatments in NOCA lakes, with limited responses in gradient treatments. The 56 $\mu\text{g N L}^{-1}$ treatment was not used at NOCA lakes, which likely also affected our ability to construct Monod curves in these lakes. *Discostella stelligera* in Doubtful Lake was the only abundant taxon in NOCA lakes that responded to N alone. Other abundant taxa responded either only to the N+P treatment or did not respond to enrichment at all (Appendix A).

3.4 Phytoplankton Community Structure

NMDS plots developed using control, N-only, P-only, and N+P treatments (Figure 4) qualitatively indicated changes in phytoplankton community structure in all lakes except Copper Lake (NOCA). Plots indicate community composition changes due to N+P addition, and distinct effects of N and N+P addition (Figure 4). NMDS plots suggest community structure was generally similar in control and P-only treatments, which is consistent with patterns in Figure 2. Qualitative patterns in NMDS plots were consistent with PERMANOVA results. In two-way PERMANOVA analyses applied to each lake, there were significant (< 0.05) N and P main effects and NxP interactions in all lakes except Copper (NOCA) and Heather (OLYM). In Copper Lake, there were no significant main effects or interactions. In Heather Lake, there was a significant main effect of N, but no significant effect of P or NxP interaction. There was also a significant main effects and interaction in a nested two-way PERMANOVA applied to data from all lakes with lake as the nesting factor. Clustering of lakes in NMDS plots indicated

phytoplankton community differences between lakes were generally larger than differences in phytoplankton community composition within lakes across treatments (Figure 4).

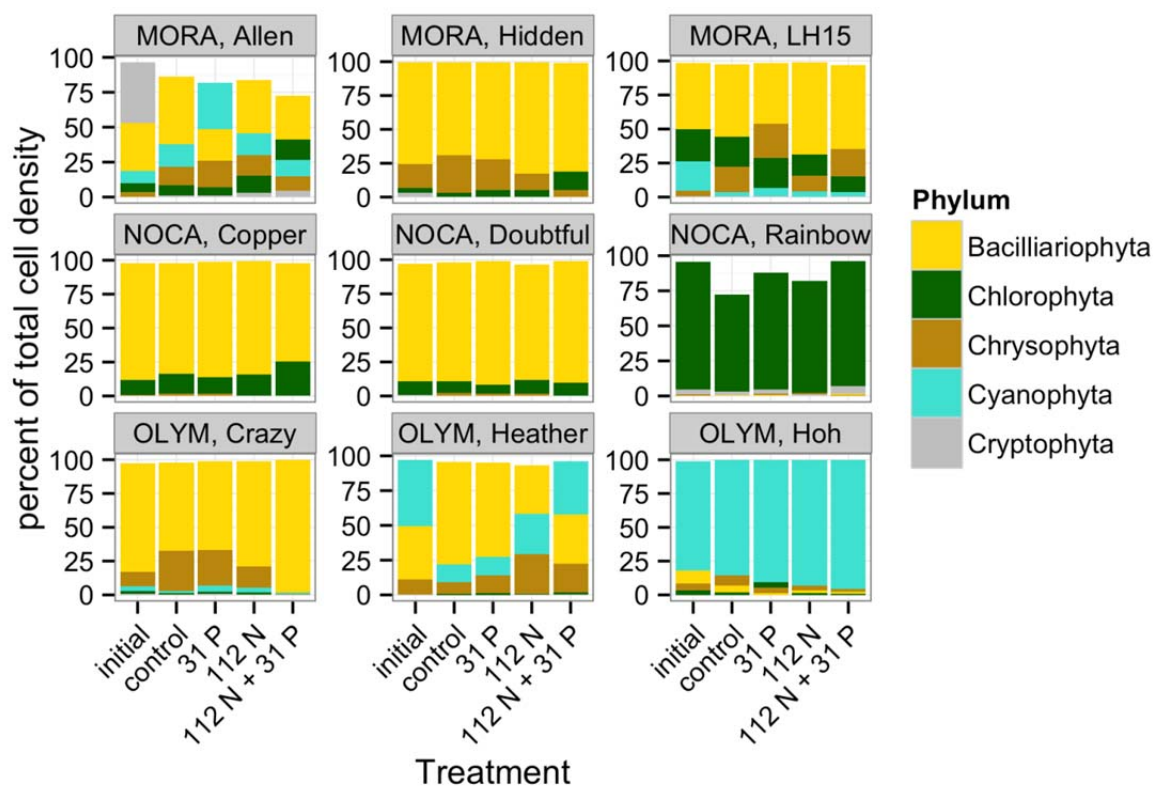


Figure 3. Relative proportions of common phytoplankton groups in each lake.

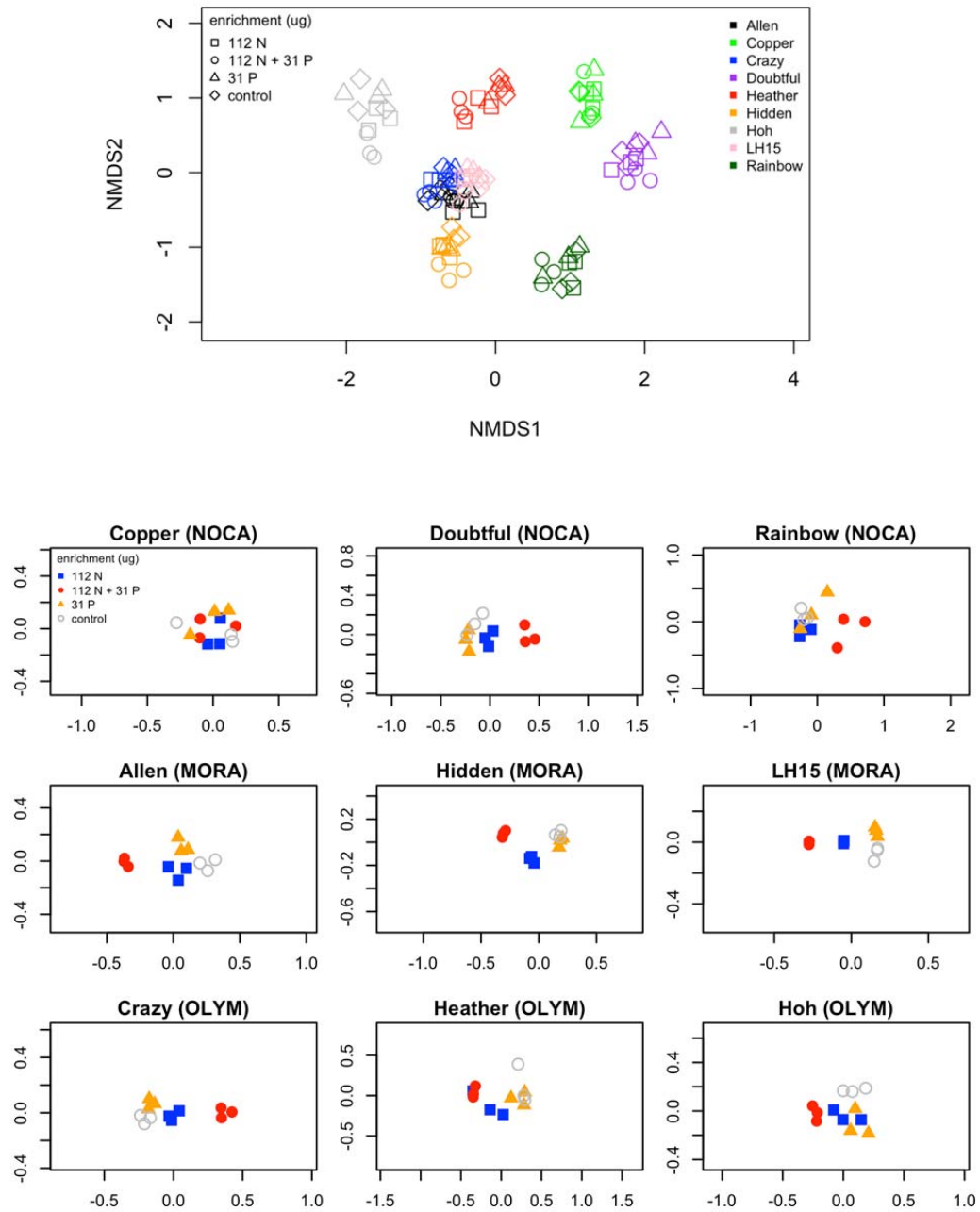


Figure 4. Nonmetric multidimensional scaling (NMDS) of phytoplankton community responses in control, N only, P only, and N+P treatments when for lakes plotted together (top) and for individual lakes (bottom). Distances between shapes reflect community similarity, with greater distances indicating larger differences.

Table 4. Cases where abundant taxa (> 1% total cell density) were N limited, serial N limited, or were co-limited and showed a strong Monod growth response. $\mu_{\max}$ is the maximum growth rate, K_s is the Monod half-saturation constant, and $\mu_{\max}:K_s$ is the taxon growth affinity. * indicates statistically significant Monod parameters. -- indicates a Monod curve could not be constructed. BAC = Bacilliarophyta, CHL = Chlorophyta, CHR = Chrysophyta, CYA = Cyanophyta, UND = Undetermined.

Division	Taxa	Lake	Limitation	$\mu_{\max} (d^{-1}) \pm SE$	$K_s (\mu g\ N\ L^{-1}) \pm SE$	$\mu_{\max}:K_s$
BAC	<i>Aulacoseira alpigena</i>	LH15	N	$0.49 \pm 0.05^*$	$27.17 \pm 6.64^*$	0.02
BAC	<i>Aulacoseira pusilla</i> 6 μm	Allen	co-limitation	$0.12 \pm 0.02^*$	3.90 ± 3.31	0.03
BAC	<i>Aulacoseira pusilla</i> 6 μm	Crazy	serial N	--	--	
BAC	<i>Aulacoseira pusilla</i> 6 μm	LH15	serial N	--	--	
BAC	<i>Cyclotella < 7 μm distinguenda</i>	LH15	serial N	--	--	
BAC	<i>Cyclotella < 7 μm distinguenda</i>	Crazy	serial N	--	--	
BAC	<i>Discostella glomerata</i>	Heather	N	--	--	
BAC	<i>Discostella glomerata</i> 7-8 μm	Crazy	N	$0.06 \pm 0.03^*$	9.46 ± 5.76	0.01
BAC	<i>Discostella glomerata</i> 7-8 μm	LH15	co-limitation	3.73 ± 3.22	782 ± 761	0.00
BAC	<i>Discostella stelligera</i>	Doubtful	serial N	--	--	
BAC	<i>Fragilaria capucina</i>	Crazy	co-limitation	$0.51 \pm 0.01^*$	$1.50 \pm 0.21^*$	0.15
BAC	<i>Fragilaria crotonensis</i>	Allen	co-limitation	$0.63 \pm 0.10^*$	$32.12 \pm 13.52^*$	0.02
BAC	<i>Fragilaria crotonensis</i>	Crazy	serial N	$0.30 \pm 0.01^*$	$6.25 \pm 0.83^*$	2.14
BAC	<i>Fragilaria tenera</i>	Crazy	serial N	$0.34 \pm 0.02^*$	$24.67 \pm 4.76^*$	0.01
BAC	<i>Fragilaria tenera</i>	Hidden	serial N	--	--	
BAC	<i>Fragilaria tenera</i>	Hoh	co-limitation	$0.42 \pm 0.04^*$	$14.52 \pm 3.61^*$	0.02
CHL	<i>Ankistrodesmas</i> sp.	Allen	co-limitation	0.64 ± 0.54	77.60 ± 128	0.01
CHL	Cocoid green alga sp.	Allen	N	$0.51 \pm 0.04^*$	0.05 ± 0.05	10.65
CHL	Cocoid green alga < 5 μm sp.	LH15	N	$0.28 \pm 0.04^*$	9.95 ± 5.87	0.03
CHL	Gonium-like individuals	Hidden	serial N	$0.45 \pm 0.15^*$	10.20 ± 12.43	0.04
CHL	<i>Nonmotile green algae</i>	Heather	co-limitation	$0.21 \pm 0.04^*$	13.49 ± 6.62	0.01
CHL	<i>Schroederia</i> sp.	LH15	N	0.40 ± 0.06	57.0 ± 17.4	0.01
CHL	<i>Tetraspora</i> sp.	LH15	co-limitation	$0.02 \pm 0.07^*$	0.57 ± 16.51	0.04
CHR	<i>Chrysolepidomonas</i> sp.	Allen	co-limitation	$0.25 \pm 0.02^*$	0.02 ± 0.04	11.14
CHR	<i>Chrysolepidomonas</i> sp.	LH15	co-limitation	$0.82 \pm 0.12^*$	$27.3 \pm 9.95^*$	0.03
CHR	Small golden w/o flagella	Heather	N	--	--	
CHR	Small golden w/o flagella	LH15	N	$0.15 \pm 0.04^*$	36.70 ± 24.0	0.00
CYA	Cocoid cyanobacteria sp.	Allen	co-limitation	0.26 ± 0.10	18.26 ± 22.68	0.01
CYA	Synechocystis type cocoid cyanobacteria sp.	Hoh	serial N	$0.38 \pm 0.03^*$	14.13 ± 2.51	0.02
CYA	Microcystis sp. colony	Crazy	N	0.84 ± 1.62	391 ± 935	0.00
CYA	Synechocystis type cocoid cyanobacteria sp.	Heather	serial N	--	--	
UND	Large yellow balls w/o flagella	Allen	serial N	$0.40 \pm 0.03^*$	0.06 ± 0.07	6.50

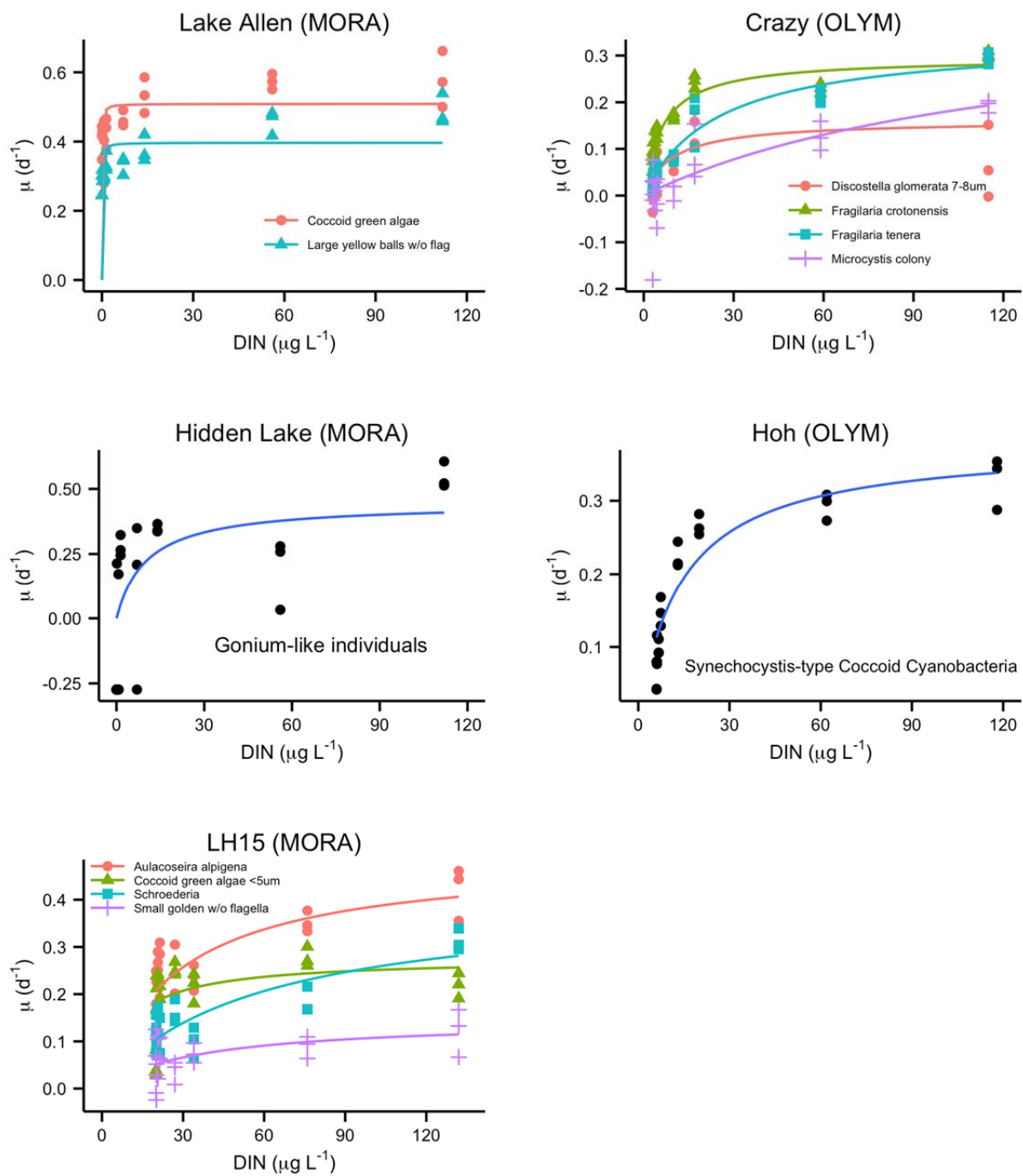


Figure 5. Monod growth curves for N limited and serial N limited taxa.

3.3 Chlorophyll a Response Thresholds

There was a statistically significant positive relationship between DIN and RR_{biomass} in all 9 bioassay lakes (Figure 6, Table 5). The relationship was linear in all lakes except Hoh Lake (OLYM), where it was logarithmic (Table 5). Regression models explained $> 65\%$ of variation in RR_{biomass} data in all lakes except Heather Lake (OLYM) ($r^2 = 0.48$) (Table 5). DIN thresholds for any increase in biomass ($RR > 1$) and for an increase beyond typical inter-annual variation ($RR_{\text{biomass}} > 1.73$) ranged from 7 to 33 $\mu\text{g DIN L}^{-1}$ and 13 to 67 $\mu\text{g DIN L}^{-1}$ across lakes, respectively. The type of lakes included in the regression (N-limited versus co-limited biomass growth) did not strongly affect calculated threshold values (Table 5). Among lakes where water chemistry data were available, mean nitrate concentrations exceeded the 13 $\mu\text{g N L}^{-1}$ threshold for any increase in biomass in 6 lakes (5%) at MORA, 14 lakes (17%) at NOCA and 0 out of 9 lakes at OLYM. Lake mean nitrate concentrations were above the threshold for exceeding typical inter-annual variation in Chl *a* (25 $\mu\text{g N L}^{-1}$) in 3 lakes at MORA (2%), 4 lakes (5%) at NOCA, and 0 out of 9 lakes at OLYM. Using upper and lower bound lake mean DIN, 33-45 lakes (26-36%) at MORA, 22-28 lakes at NOCA (27-34%), and 0 lakes at OLYM exceeded the 13 $\mu\text{g N L}^{-1}$ threshold for any increase in biomass. Lake mean DIN exceeded the 25 $\mu\text{g N L}^{-1}$ threshold for exceeding typical biomass inter-annual variation in 17-19 lakes at MORA (13-15%), 6-10 lakes at NOCA (7-12 %), and 0 lakes at OLYM.

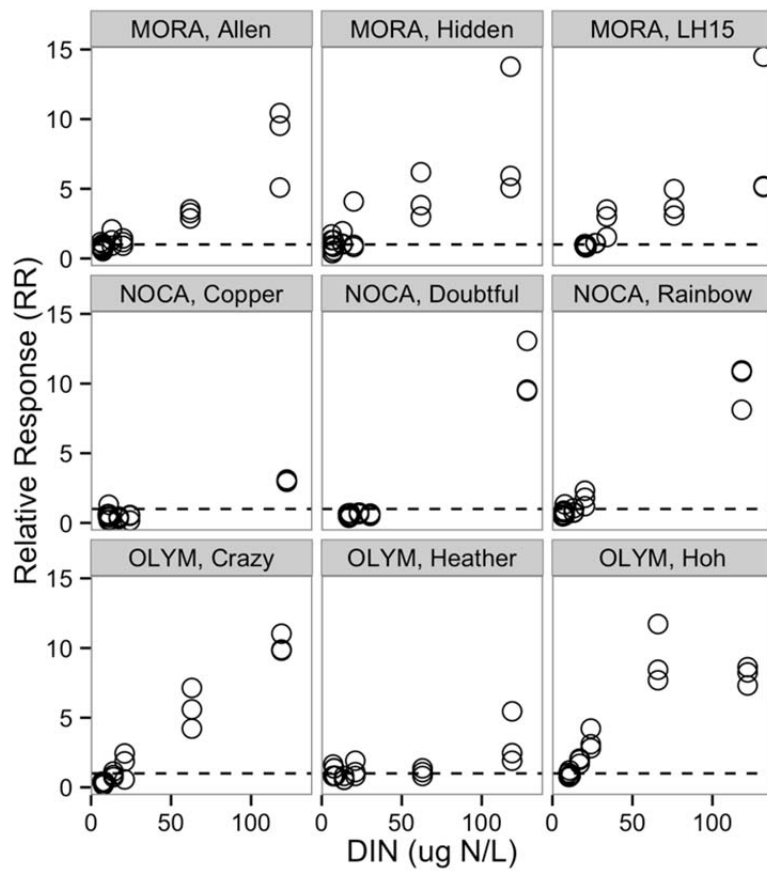


Figure 6. Relationship between biomass relative response (RR_{biomass}) and DIN across gradient treatments. RR_{biomass} = treatment replicate Chl *a* divided by control mean Chl *a*. The dashed horizontal line indicates $RR_{\text{biomass}} = 1$.

Table 5. Threshold DIN concentrations required for $RR_{\text{biomass}} = 1$ and $RR_{\text{biomass}} = 1 + CV_{\text{chla}}$.

Model	Regression Equation	r^2	p	RR = 1 (μg DIN/L)	RR = 1+ CV_{chla} (μg DIN/L)
All lakes	$RR = 0.159 + 0.063 \cdot \text{DIN}$	0.69	<0.001	13	25
N-limited lakes only	$RR = 0.226 + 0.062 \cdot \text{DIN}$	0.66	<0.001	13	24
Allen (MORA)	$RR = 0.306 + 0.064 \cdot \text{DIN}$	0.86	<0.001	11	22
Hidden (MORA)	$RR = 0.525 + 0.065 \cdot \text{DIN}$	0.70	<0.001	7	19
LH15 (MORA)	$RR = -0.333 + 0.063 \cdot \text{DIN}$	0.67	<0.001	21	33
Copper (NOCA)	$RR = 0.268 + 0.022 \cdot \text{DIN}$	0.85	<0.001	33	67
Doubtful (NOCA)	$RR = -1.18 + 0.092 \cdot \text{DIN}$	0.95	<0.001	24	32
Rainbow (NOCA)	$RR = 0.137 + 0.083 \cdot \text{DIN}$	0.97	<0.001	10	19
Crazy (OLYM)	$RR = -0.176 + 0.088 \cdot \text{DIN}$	0.97	<0.001	13	22
Heather (OLYM)	$RR = 0.739 + 0.018 \cdot \text{DIN}$	0.48	<0.001	15	55
Hoh (OLYM)	$RR = -7.25 + 3.46 \cdot \ln(\text{DIN})$	0.88	<0.001	11	13

1+ $CV_{\text{chla}} = 1.73$; CV_{chla} is the average of coefficients of variation (CV) observed in 21 mountain lakes NPS staff have sampled at least once annually in late summer 2009-2013.

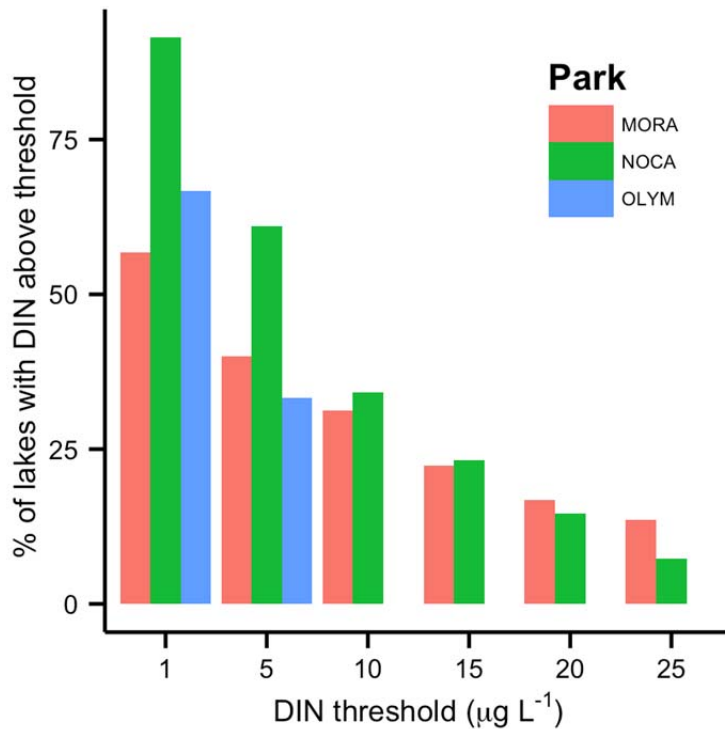


Figure 7. Minimum percent of sampled lakes at each park where DIN thresholds are exceeded for DIN threshold values ranging from 1 to 25 µg N L⁻¹

4.0 DISCUSSION

At least three conditions must be met for N deposition to induce phytoplankton changes in mountain lakes. N deposition inputs must increase lake N concentrations, N-limited phytoplankton must be present, and lake N increases must be sufficient to stimulate growth of N-limited phytoplankton. Our results indicate phytoplankton in park mountain lakes are highly sensitive to future increases in lake N. Phytoplankton biomass and taxon growth was primarily limited by N or co-limited by N and P in our study lakes (Table 3, Table 4, Appendix A). We identified 21 taxa that responded to N additions (Table 4), and only two instances of taxon P-limitation were observed. The DIN concentration thresholds we quantified for biomass (13-25 µg N L⁻¹) and species responses (0.02-50 µg N L⁻¹ for most N-limited taxa) are low and suggest

relatively small increases in lake DIN could stimulate growth of sensitive taxa or an increase in biomass. We estimate that at least 26% of sampled lakes at MORA and NOCA have DIN concentrations that exceed $13 \mu\text{g N L}^{-1}$, and thus also exceed response thresholds for biomass increases (Table 5) many taxa (Table 4). Biomass and taxon thresholds developed in this study should be protective of all mountain lakes in the three parks because they assume growth is N-limited.

However, it still not clear if N deposition is the primary cause of elevated lake N concentrations. Although we observed a positive relationship between deposition and lake nitrate and a ‘dog leg’ pattern in Figure 2 consistent with that in other regions of the Western U.S. (Baron et al. 2011), lakes that receive similar deposition rates have mean nitrate concentrations that differ by orders of magnitude. Watershed characteristics thus likely strongly control lake nitrate responses to deposition. In other Western U.S. regions, lake nitrate is positively correlated with watershed characteristics that decrease watershed biological N uptake and increase hydrologic flow rates. For example, the percent of watershed area that is unvegetated, has steep slopes, or is covered by talus is positively correlated with nitrate in Yosemite National Park (Clow et al. 2010) and in the Rocky Mountains (Nanus et al. 2012). Similar patterns likely occur for MORA, NOCA, and OLYM lakes. At NOCA, lake nitrate is positively correlated with watershed percent barren area (Williams, in prep). In other regions, N deposition is not always among the variables that best explains regional patterns of mountain lake nitrate (Clow et al. 2010, Nanus et al. 2012). Therefore, multivariate analyses are needed to determine if N deposition is a primary cause of phytoplankton threshold exceedances, and to estimate critical N deposition rates that cause lake N to exceed DIN thresholds quantified in this study.

4.1 Phytoplankton Biomass Responses

We estimated that a DIN concentration of $13 \mu\text{g N L}^{-1}$ are required for an increase in biomass, and a concentration of $25 \mu\text{g N L}^{-1}$ is necessary to increase biomass beyond typical lake inter-annual variation. These thresholds are similar to those estimated by Heard (2013) based on experiments in Sierra Nevada mountain lakes. Heard (2013) enriched lake water with a range of N concentrations and used Michaelis-Menten and Monod growth models to estimate the 10%, 50%, and 90% ‘effective doses’, or N concentrations that resulted in 10%, 50%, and 90% of saturated biomass growth rates ($\mu\text{g day}^{-1}$). In contrast to Heard’s (2013) experiments, this study used a smaller N concentration gradient ($0\text{-}112 \mu\text{g N L}^{-1}$ versus $0\text{-}700 \mu\text{g N L}^{-1}$), did not observe biomass growth saturation except perhaps in Hoh Lake (Figure 5), and modeled biomass responses using linear regression and RR values. Despite these methodological differences, our DIN thresholds overlap with those estimated by Heard (2013). Heard’s estimated 10%, 50%, and 90% effective doses to be $4.6\text{-}12.5 \mu\text{g N/L}$, $14\text{-}56 \mu\text{g N/L}$, and $43.4\text{-}252 \mu\text{g N/L}$, respectively. The 10% and 50% effective doses of Heard (2013) overlap with our thresholds (Table 5).

Relatively large increases in lake DIN may be necessary to stimulate a biomass increase beyond typical inter-annual variation in our study lakes. DIN concentrations in our study lakes and in many other park lakes are at or near detection limits ($5\text{-}10 \mu\text{g N L}^{-1}$) (Clow and Campbell 2008, Glesne et al. 2012), meaning DIN concentrations would need to more than double in many lakes to exceed the $25 \mu\text{g N L}^{-1}$ threshold. Inter-annual variation in late summer Chl *a* is relatively high ($\text{CV}_{\text{mean}}=0.73$, range = $0.26\text{-}1.6$) because Chl *a* is affected by a wide variety of other factors, such as timing of ice-out, stratification, light availability, zooplankton grazing, and concentrations of other nutrients (DOC, micronutrients). Our biomass thresholds are also likely underestimates for some lakes because they assume P concentrations of at least $31 \mu\text{g P L}^{-1}$. We

added 31 $\mu\text{g P}$ along with N in treatments used for biomass regressions, and thus presumably alleviated P-limitation for many taxa. However, P availability likely constrains the magnitude of biomass responses in some N-limited or co-limited lakes. For example, in co-limited and serial N-limited lakes examined here, addition of N alone resulted in much smaller biomass relative response than would be predicted by our regression models (Table 3, Table 5). Our regression models and DIN thresholds should be protective of all lakes because they assume only N is limiting. However, they likely overestimate the magnitude of biomass responses in lakes with serial N limitation, co-limitation, or P limitation.

The mass ratio of DIN to total P (DIN:TP) is frequently used to predict algal nutrient limitation in mountain lakes with mixed results. Our observed nutrient limitation patterns were partially consistent with those predicted by DIN:TP thresholds developed by Morris and Lewis (1988) and Bergström (2010). Initial DIN:TP was less than the 3.4-4 thresholds for P limitation and thus accurately predicted the absence of exclusive P-limitation in eight of the nine examined lakes. Published DIN:TP thresholds were less successful at predicting the occurrence of N limitation. N limitation or serial N limitation was observed in four lakes; two of these lakes had initial DIN:TP consistent with Bergstrom's (2010) DIN:TP threshold for N limitation (<1.5), and no lakes had initial DIN:TP consistent with the <0.5 threshold from Morris and Lewis (1988). Co-limitation was also observed in several lakes that had DIN:TP below the threshold for N limitation developed by Bergström (2010). These DIN:TP thresholds have also been used to predict algal nutrient limitation in similar experiments with mixed success (Murphy et al. 2010, Daggett et al. 2015). The discrepancies between our findings and DIN:TP thresholds developed by Bergström (2010) and Morris and Lewis (1988) are not entirely surprising given that their thresholds were only designed to accurately discriminate between N and P limitation in 75% and

50% of cases, respectively. In addition, DIN:TP can be affected by how detection limits are handled and may be prone to misclassification if concentrations are near detection limits where analytical precision is often low relative to concentrations. Our results indicate one cannot assume biomass nutrient limitation patterns inferred based on DIN:TP or observed in nutrient enrichment experiments are representative of taxon-level limitation patterns. N enrichment stimulated taxon-level responses in both lakes where biomass growth was N-limited and co-limited.

4.2 Taxon Responses

Diatoms *Asterionella formosa*, *Fragilaria crotonensis*, and *Discostella stelligera* have been used as indicators of N enrichment in mountain lakes. In the Rocky Mountains, these species have increased in relative abundance in sediment cores at time periods corresponding to increases in anthropogenic N deposition (Saros et al. 2003, 2005, Wolfe et al. 2003), and phytoplankton surveys indicate the distributions of these species are positively correlated with lake N (Saros et al. 2005, Arnett et al. 2012). Nutrient enrichment experiments conducted in Rocky Mountain lakes also indicate that *A. formosa* and *F. crotonensis* are N-limited in lakes with low N concentrations (Saros et al. 2005). They have low P requirements and grow rapidly at low N concentrations, which enable their populations to increase rapidly as N deposition increases lake N in oligotrophic mountain lakes (Michel et al. 2006, Arnett et al. 2012, Nanus et al. 2012).

However, it was not previously clear if these taxa would also increase in response to lake N enrichment in the Pacific Northwest. Sheibley et al. (2014) collected sediment cores from 12 mountain lakes across NOCA, MORA, and OLYM, and looked for increases in *A. formosa* and

F. crotonensis consistent with those in the Rocky Mountains. Sheibley et al. (2014) observed *A. formosa* in only one lake, and did not observe *F. crotonensis* at all. In Hoh Lake at OLYM, *A. formosa* and *Fragilaria tenera* increased in relative abundance beginning in the 1969-1975 timeframe as N deposition rates began to exceed $1\text{--}1.2 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ (Sheibley et al. 2014). Sheibley et al. concluded these changes were likely caused by N deposition because they were consistent with patterns observed for *A. formosa* and *F. tenera* in the Rocky Mountains (Wolfe et al. 2003, Das et al. 2005, Saros et al. 2005). However, diatom community changes consistent with those observed in the Rocky Mountains were not observed in any other lakes examined by Sheibley et al. (2014). Sheibley et al. (2014) noted that the relative infrequency of *A. formosa* and *F. crotonensis* and deposition-induced diatom changes in park lakes could indicate that i) critical loads have not yet been exceeded for most of their study lakes, ii) their study lakes might not be N-limited, or iii) that N-responsive diatom taxa in the Pacific Northwest are different than those in the Rocky Mountains. Lake characteristics other than N and P concentrations can also influence the abundance and distribution of N-sensitive diatoms, including temperature (Arnett et al. 2012), conductivity (Saros et al. 2005, Arnett et al. 2012), and dissolved organic matter (Daggett et al. 2015).

Our study helps discriminate among these alternatives. As discussed above, taxa in our study lakes were primarily N-limited or co-limited by N+P. Our results suggest that N-responsive diatom species are similar in both regions. *A. formosa* was present at low densities in only a few treatment replicates in two lakes (Hoh, Copper), so we could not evaluate the N-sensitivity of this species. However, we conclude diatoms *F. crotonensis*, *D. stelligera*, and *F. tenera* could be used as N enrichment indicators in the Pacific Northwest because N responses observed in our experiments are consistent with patterns in sediment cores. Consistent with

sediment cores and nutrient enrichment experiments from the Rocky Mountains, both *F. crotonensis* and *D. stelligera* responded to N enrichment in at least one of our study lakes. In addition, *F. tenera* responded to N addition in three of our study lakes, including Hoh Lake, where *F. tenera* has increased in relative abundance starting in the 1969-1975 timeframe (Sheibley et al. 2014). Several other diatom species also responded to N addition in one or more lakes, including *Aulacoseira alpigena*, *Aulacoseira pusilla*, *Cyclotella distinguenda*, *Discostella glomerata*, and *Fragilaria capucina* (Table 4). These species also may be useful indicators of N enrichment, but there is less supporting evidence from sediment cores. *Aulacoseira* species were replaced by *A. formosa* and *F. tenera* in some Rocky Mountain lakes as N deposition increased (Wolfe et al. 2003, Hobbs et al. 2010), did not show substantial changes over time in Pacific Northwest Lakes examined by Sheibley et al. (2014), and in some cores *Aulacoseira* decreases have been attributed to warming (Catalan et al. 2013). The abundance of small centric diatoms such as *Cyclotella* and *Discostella* in sediment cores is strongly affected by complicated interactions among warming, light, and nutrients, which has made assigning causal factors to changes in sediment cores difficult (Saros et al. 2014, Saros and Anderson 2015). In addition, although *D. stelligera* responds to N in nutrient enrichment experiments (Table 4, Arnett et al. 2012), it often must compete with other N-sensitive species. In Rocky Mountain lakes, *A. formosa* and *D. stelligera* frequently co-occur, but the abundance of these two species is often inversely related (Arnett et al. 2012).

Some estimated Monod parameters quantified in our study lakes differ from those quantified for diatoms in other mountain lakes. The $\mu_{\max}$ values quantified in this study were similar to those quantified for diatoms in the Rocky Mountains, but our K_s values were higher (Michel et al. 2006, Arnett et al. 2012, Nanus et al. 2012). For *Fragilaria crotonensis*, we quantified K_s

values of $6.2 \mu\text{g N L}^{-1}$ in Crazy Lake and $32.1 \mu\text{g N L}^{-1}$ in Lake Allen, whereas Michel et al. (2006) quantified K_s values less than $1 \mu\text{g N L}^{-1}$ in Beartooth Mountains lakes. Values of K_s quantified for *Discostella stelligera* and *Asterionella formosa* in the Rocky Mountains range from 0.04 - $2.5 \mu\text{g N L}^{-1}$ (Michel et al. 2006, Arnett et al. 2012, Nanus et al. 2012). In contrast, diatom K_s values in this study ranged from 3.5 - $50 \mu\text{g N L}^{-1}$. The K_s values in this and other comparable studies have standard errors that in many cases exceed 100% of K_s (Grover et al. 1999, Michel et al. 2006). However, our diatom K_s values are higher even after accounting for this uncertainty. The higher K_s values in this study suggest lower growth rates at low N concentrations, which could result from sub-optimal light, temperature, or other conditions during our *in situ* incubations. Many of our study lakes had high transparency, and a Secchi disk was easily visible on the lake bottom. Photoinhibition of growth due to high light intensities is thus one potential explanation for lower growth rates at low N concentrations compared to previous studies. We did not measure lake light intensity because transporting a light meter to our remote study lakes was not feasible.

Our diatom K_s values were consistent with our nutrient limitation classifications based on results of the control N-only, P-only, and N+P treatments. For example, initial lake DIN concentrations were substantially below K_s values estimated for diatoms classified N-limited and serial N-limited diatoms in all lakes. However, our K_s values for *F. tenera* ($14.5 \mu\text{g N L}^{-1}$ in Hoh Lake, and $24.7 \mu\text{g N L}^{-1}$ in Crazy Lake) were higher than we expected. Considering Sheibley et al. (2014) documented an increase in *F. tenera* in Hoh Lake, we expected our *F. tenera* K_s to be near or below current Hoh Lake DIN concentrations, but DIN concentrations measured this study and by NPS in Hoh Lake are consistently below $10 \mu\text{g N L}^{-1}$. The reason for this discrepancy is currently not clear.

Several chlorophyte, chrysophyte, and cyanophyte taxa also responded strongly to N enrichment. Small coccoid chlorophytes and cyanophytes responded strongly to N enrichment and had several of the largest growth affinities we observed, suggesting they could respond to N enrichment more strongly than some diatom taxa. Chlorophyte, chrysophyte, and cyanophyte increases have also been observed in other nutrient enrichment experiments. Nydick et al. (2004) observed increases in cyanophyte and chlorophyte density in response to N and N + P enrichment in Snowy Range mountain lakes. Gardner et al. (2008) also documented strong increases in chlorophyte species *Chlamydomonas* sp. and *Scenedesmus* sp. in two Colorado mountain lakes.

4.3 Conclusions

Federal land management agencies in the U.S. face legal mandates for protecting national parks and other protected lands from adverse impacts of air pollution, including deposition-induced changes in mountain lakes (Porter and Johnson 2007, Cummings et al. 2014). We identified phytoplankton changes that may occur in sensitive Pacific Northwest mountain lakes if lake N concentrations increase due to N deposition, and estimated lake DIN thresholds associated with phytoplankton changes. Based on our results, we expect future lake N increases to increase phytoplankton biomass, and stimulate growth of diatom, chlorophyte, chrysophyte, and cyanophyte taxa. Diatom species that have increased in relative abundance throughout the Western U.S. along with increasing N deposition rates (*Fragilaria crotonensis*, *Fragilaria tenera*, *Discostella stelligera*) will also likely respond to lake N increases in the northwest. Growth stimulation of taxa can be expected in both lakes where biomass growth is N-limited and co-limited by N+P. Although we documented a positive relationship between N deposition and

lake nitrate, further investigation is required to determine if N deposition has increased lake N and induced phytoplankton changes in regional mountain lakes. The magnitude of deposition-induced lake N increase, type of phytoplankton change, or threshold exceedance that constitutes an 'adverse' impact which warrants action is ultimately a decision that must be made by NPS resource managers in the context of federal mandates for protecting NPS lands.

5.0 Works Cited

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