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Nutrient availability and phytoplankton nutrient limitation across a gradient of atmospheric nitrogen deposition

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Abstract. Atmospheric nitrogen (N) deposition to lakes and watersheds has been increasing steadily due to various anthropogenic activities. Because such anthropogenic N is widely distributed, even lakes relatively removed from direct human disturbance are potentially impacted. However, the effects of increased atmospheric N deposition on lakes are not well documented. We examined phytoplankton biomass, the absolute and relative abundance of limiting nutrients (N and phosphorus [P]), and phytoplankton nutrient limitation in alpine lakes of the Rocky Mountains of Colorado (USA) receiving elevated (>6 kg $N \cdot ha^{-1} \cdot yr^{-1}$) or low (<2 kg $N \cdot ha^{-1} \cdot yr^{-1}$) levels of atmospheric N deposition. High-deposition lakes had higher NO_3-N and total N concentrations and higher total N : total P ratios. Concentrations of chlorophyll and seston carbon (C) were 2–2.5 times higher in high-deposition relative to low-deposition lakes, while high-deposition lakes also had higher seston C:N and C:P (but not N:P) ratios. Short-term enrichment bioassays indicated a qualitative shift in the nature of phytoplankton nutrient limitation due to N deposition, as high-deposition lakes had an increased frequency of primary P limitation and a decreased frequency and magnitude of response to N and to combined N and P enrichment. Thus elevated atmospheric N deposition appears to have shifted nutrient supply from a relatively balanced but predominantly N-deficient regime to a more consistently P-limited regime in Colorado alpine lakes. This adds to accumulating evidence that sustained N deposition may have important effects on lake phytoplankton communities and plankton-based food webs by shifting the quantitative and qualitative nature of nutrient limitation.

Key words: alpine lakes; nitrogen; nitrogen deposition; nutrient limitation; phosphorus; phytoplankton; Rocky Mountains, Colorado (USA).

Introduction

The role of limiting nutrient elements, such as nitrogen (N) and phosphorus (P), in regulating the structure and function of aquatic and terrestrial ecosystems has long been the subject of biogeochemical and ecological study (Schlesinger 1997). In the aquatic sciences this work has been motivated by the long-standing recognition that increased nutrient loading results in major shifts in ecological structure and function structure and deterioration of ecosystem conditions, such as reduced water transparency and bottom water oxygenation ("cultural eutrophication"; Hutchinson 1973, Harper 1992). Thus identifying the role of various nutrients in driving lake eutrophication has been a major focus in limnology for several decades (Likens 1972, Smith et al. 1999). Work in the 1960s and 1970s highlighted the critical role of P in lake eutrophication. Prominent among these studies was the classic work at the Experimental Lakes Area (Ontario, Canada), which clearly demonstrated that large increases in phytoplankton biomass only occurred when P was enriched (Schindler et al. 1971, 1973). Based on this work, Schindler (1977) proposed a paradigm in which P limitation was the natural condition of most lakes, arguing that ecosystems deficient in N would respond with increased levels of N fixation and thus alleviate N limitation.

However, because there are a variety of ecological and environmental limitations that can inhibit the success of nitrogen fixers (Vitousek et al. 2002), in many situations lakes may be unable to compensate for N limitation as proposed in the P-limitation paradigm, and thus many lakes may often harbor N-limited phytoplankton (Lewis and Wurtsbaugh 2008). This possibility is supported by meta-analyses of lake phytoplankton N- and P-limitation bioassays (Elser et al. 1990, 2007) and of whole-lake nutrient enrichment experiments (Elser et al. 1990) showing that instantaneous N limitation and P limitation are approximately equal in frequency and severity and that strong increases of phytoplankton biomass are usually observed only when N and P are added together.

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These observations suggest that in most lakes, N and P are provided in relatively balanced proportions relative to the demands of phytoplankton (Sterner 2008). In turn, this implies that relatively subtle changes in the supplies of N and P might shift phytoplankton between N- and P-limited growth. For example, when total N:total P (TN:TP) ratios are relatively balanced, differential consumer-driven nutrient recycling of N and P by stoichiometrically contrasting zooplankton species can shift lake phytoplankton between N and P limitation (Sterner et al. 1992, Elser et al. 1995).

Another relatively subtle factor that might affect in situ nutrient status of lake phytoplankton is input of anthropogenic N from the atmosphere. Rates of atmospheric N deposition have increased dramatically in various parts of the world in response to urbanization, industrialization, and agricultural intensification (Galloway et al. 2008). Since alterations of atmospheric inputs primarily involve N, these shifts potentially increase the N:P ratio of lake nutrient supplies and could shift phytoplankton from N limitation to P limitation. Indeed, such effects have already been proposed for temperate European and North American lakes (Bergström et al. 2005, Bergström and Jansson 2006) and for Lake Tahoe (California and Nevada, USA; Goldman et al. 1993).

Here we test whether atmospheric N deposition to lakes can affect nutrient availability and phytoplankton nutrient status by performing a regional survey of high-elevation Colorado lakes receiving background (<2 $kg \cdot ha^{-1} \cdot yr^{-1}$) or elevated (>6 $kg \cdot ha^{-1} \cdot yr^{-1}$) levels of atmospheric N deposition. Our results show increased N availability and increased frequency of P limitation along with decreased N limitation in high-deposition lakes. These data indicate that anthropogenic influences can alter important ecological parameters in lakes seemingly far from direct human disturbance.

Study Sites and Methods

Study sites

Our study involved field sampling of lakes in regions receiving different levels of N deposition in the central Rocky Mountains of the western USA (Fig. 1; Appendix A). On the eastern slopes of the Rocky Mountains near the Niwot Ridge Long Term Ecological Research (LTER) site (NWT; 40°3′ N latitude, 105°35′24″ W longitude) and the Loch Vale Watershed Research Site (LVWS), atmospheric N deposition rates have been steadily increasing over the past 20 years due to transport of pollutants from fossil fuel combustion and agricultural practices (Nanus et al. 2003, Burns 2004, Musselman and Slauson 2004) and have reached levels of 6–8 kg/ha annual deposition of inorganic nitrogen (Fig. 1) (National Atmospheric Deposition Program; data *available online*).[5] In contrast, lakes in central or western Colorado near the Rocky Mountain Biological Laboratory (RMBL, Crested Butte; 38°57′36″ N latitude, 106°58′48″ W longitude) or the Mountain Studies Institute (MSI, Silverton; 37°48′36″ N latitude, 107°39′47″ W longitude) receive low atmospheric N deposition (<1.5 kg/ha and <2 kg/ha annually for RMBL and MSI, respectively), similar to values at NWT 20 years ago (Fig. 1). The lakes occupy a geologically diverse set of substrata (Kent and Porter 1980). In the Front Range in the vicinity of Rocky Mountain National Park, the underlying geology is dominated by Precambrian-age igneous and metamorphic rocks, including granite, gneiss, and schist. Geological substrata in the vicinity of RMBL are complex; geologic parent materials include rhyolitic Tertiary intrusives and Cretaceous/Jurassic sedimentary rocks along with Cretaceous/Paleozoic metamorphics and intrusives. The San Juan mountains surrounding the MSI in southwestern Colorado involve extensive volcanic deposits (including andesitic lavas and tuffs) but also include felsic gneisses and granites of the Uncompahgre formation.

We chose a set of suitable lakes in the vicinity of each of these three main research sites. Lakes in the Colorado Front Range (NWT, LVWS, and vicinity) were designated as "Eastern" and placed in the "high-deposition" category. Lakes in the vicinity of Crested Butte were designated as "Central" and those near Silverton as "Western," and both of these sets were placed in the "low-deposition" category. Lakes were selected if they were within ~8 km of a trailhead, relatively small (<20 ha), and deep (generally >5 m maximum depth). To minimize confounding effects of vegetation type or land use, we limited sampling to alpine and high subalpine lakes having small, mostly or entirely unforested watersheds. As a result, lakes encompassed a relatively narrow elevation range (2973–3935 m above sea level). Detailed information about the lakes included in the sampling is given in Appendix A.

Sampling and sample preparation

Most lakes were visited on a single day during summer 2006 (three Eastern lakes were visited twice, separated by one month); dates of sampling for each lake are given in Appendix A. Sampling in the three regions was staggered so that sampling of lakes in high- and low-deposition regions was not strongly skewed by date. High-deposition (Eastern) lakes were sampled in early July and early August, while low-deposition lakes were sampled in late July (Central) and late August/early September (Western). Sampling generally took place in the morning to avoid afternoon thunderstorms.

Lakes were sampled from an inflatable raft. The deepest part of the lake was found using a portable echo sounder after which a vertical profile of temperature and dissolved oxygen was made at ~1-m intervals using a YSI model 85 temperature–oxygen probe (YSI, Yellow Springs, Ohio, USA). Water samples (usually four

[5] ⟨http://nadp.sws.uiuc.edu/⟩←

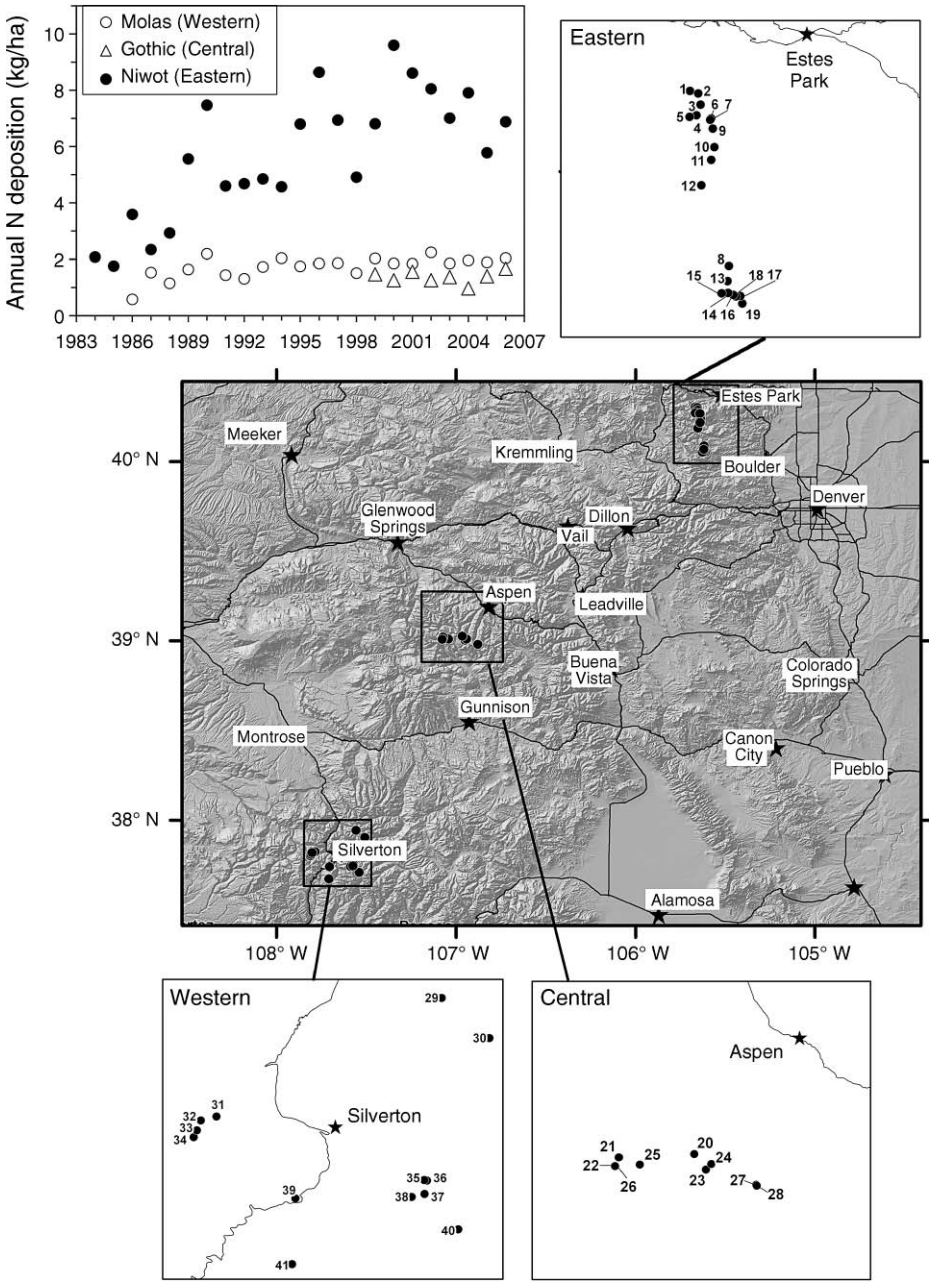

FIG. 1. Map of west-central Colorado (USA) showing the main study areas. Lakes in the Eastern area receive high atmospheric N deposition, while lakes in the Central and Western areas receive low deposition. The graph at the top left shows the dynamics of annual deposition (wet) of inorganic N at monitoring stations close to each of the three sampling regions. A key to the individual lakes (numbered in the insets) is given in Appendix A.

independent replicates) were taken using a battery-powered submersible pump fitted with tubing to take in water at 1–1.5 m depth to fill 4-L acid-washed and lakewater-rinsed cubitainers. Water was pumped through 80-μm mesh Nitex (Wildlife Supply, Buffalo New York, USA) to remove ambient macrozooplankton. Samples were returned immediately to the laboratory. Processing of water samples generally occurred with two to four hours of sampling, although on some occasions the delay was closer to six hours due to travel distances.

Each replicate sample was then processed for analysis of various chemical parameters and nutrient concentrations. An unfiltered subsample from each sample was refrigerated and then brought or shipped within one week to the Kiowa Chemical Analysis Laboratory at the Mountain Research Station for determination of pH (using an Accumet AR10 pH meter [Fisher Scientific,

Pittsburgh, Pennsylvania, USA]) and acid neutralizing capacity (by Gran titration with hydrochloric acid). Subsamples were filtered through Pall Type A/E glass fiber filters (Pall, East Hills, New York, USA) and then held refrigerated until being brought or shipped to the Kiowa lab for analysis of dissolved nutrient species. Ammonia/ammonium (NH_4-N) was analyzed using the indophenol colorimetric method (APHA 2005) by segmented flow analysis on an OI Analytical Flow Solution IV Instrument (OI Analytical, College Station, Texas, USA). Nitrate + nitrite (hereafter: NO_3-N) was determined by ion chromatography using a Metrohm 761 Compact Ion Chromatography instrument fitted with a Metrosep A Supp 5 anion column (Metrohm USA, Riverview, Florida, USA). Soluble reactive phosphorus (SRP) was quantified with the ammonium molybdate colorimetric method (APHA 2005) using a Lachat QuikChem 8000 Flow Injection Automated Ion Analyzer (Hach, Loveland, Colorado, USA). Additional unfiltered subsamples were frozen for later analysis of total nitrogen (TN) and total phosphorus (TP). TN was analyzed by the in-line persulfate/UV oxidation method using a Lachat QC8000 Flow Injection Analyzer. Concentrations of TP in the study lakes were generally very low. To reduce the detection limit and obtain reliable data, oxidized TP samples were concentrated fivefold using the method of Solorzano and Sharp (1980) after which P concentrations were analyzed using the ammonium molybdate method (APHA 2005).

Three subsamples from each replicate water sample were also filtered onto a Whatman GF/C filter (Whatman, Piscataway, New Jersey, USA) for chlorophyll analysis or onto two precombusted Whatman GF/F filters, one of which was analyzed for C and N and the other for P. Chlorophyll samples were frozen and then extracted by soaking for 24 h in 100% methanol. Extracts were analyzed on a Turner BioSystems Model TD-700 fluorometer (Turner Biosystems, Sunnyvale, California, USA) with internal phaeophytin correction. Seston samples were dried and held on desiccant until analysis. Seston C and N samples were analyzed using a Perkin Elmer Model 2400 elemental analyzer (Perkin Elmer, Waltham, Massachusetts, USA). Seston P samples were analyzed by colorimetric analysis after persulfate oxidation (APHA 2005).

Bioassays

Nutrient enrichment bioassay experiments involved assessment of phytoplankton growth responses to factorial manipulations of N and P availability over three days. For each experiment, ~10 L of epilimnetic lake water was pumped through 80-μm mesh Nitex to remove zooplankton and then returned to the laboratory. In the lab, 16 250-mL clear polycarbonate bottles were filled with lake water; four received an enrichment of 7.5 μmol/L N (as NH_4NO_3), four received 0.5 μmol/L P (as KH_2PO_4), four received both N and P at the same concentration, and four were unamended as controls. Triplicate subsamples from the initial lake water sample were filtered onto GF/C filters and frozen. Bottles were incubated in a water bath held at approximately the epilimnetic temperature of the lake with illumination provided from fluorescent tubes producing a light intensity of ~70 μmol quanta·m^2·sec. Bottles were shaken twice daily. After four days, a subsample from each bottle was filtered onto a GF/C filter and then frozen. Samples were processed for chlorophyll concentration as described for the lake sampling (see *Study sites and methods: Sampling and sample preparation*).

Data analysis

Averages of all measured values in replicate epilimnetic samples ($n = 3$ or 4) for each lake were calculated. High- and low-deposition lakes were then compared using a simple t test. To evaluate effects on nutrient ratios in total nutrient pools (TN:TP) and in seston, analysis of covariance (ANCOVA) was also used.

Phytoplankton bioassay responses were analyzed by individual two-way analysis of variance (ANOVA; generally, df = 3, 12) to evaluate main effects of N and P and their interaction for each lake's experiment. Each lake's phytoplankton response to enrichment was then classified into several categories of nutrient limitation depending on the ANOVA results and, in particular, the presence and nature of N × P interaction effects using the following nomenclature. In this approach, if there were no main effects, the response was classified as "no limitation." If there was a single main effect but no significant N × P interaction, then the response was classified as "single nutrient limitation (X)," where X is N or P, or as "additive dual nutrient limitation" if there were two significant main effects. The remaining categories involve experiments that produced a significant N × P interaction term. For these cases we first assessed whether the interactive effect was superadditive or subadditive by calculating a parameter $R = (Chl_{NP} - Chl_C)/((Chl_N - Chl_C) + (Chl_P - Chl_C))$, where Chl_X is the final chlorophyll concentration in the N- and P-enriched (Chl_{NP}), the N-enriched (Chl_N), the P-enriched (Chl_P), or the unenriched (Chl_C) bottles. When $R > 1$, the N × P interaction effect was classified as superadditive but subadditive when $R < 1$. Superadditive effects were further classified by performing sequential contrasts between the single-enrichment treatment means and the control. If there were no significant contrasts, the experiment was classified as "strict co-limitation" (in which a difference from the control is only observed when *both* N and P are added). If there was only one significant single-enrichment contrast with the control, then the experiment was classified as "sequential co-limitation (X)," where X is the nutrient (N or P) that produced the significant pairwise contrast with the control; e.g., an experiment in which $Chl_{NP} > Chl_N > Chl_C = Chl_P$ would be classified as "sequential co-limitation (N)." If there were two significant pairwise contrasts with the control, then the response was

classified as "synergistic co-limitation." Finally, cases with $R < 1$ were further broken down into response categories by looking at all pairwise comparisons of the four treatments. If the contrasts between enriched treatments and controls were positive, then the response was classified as "constrained," indicating that growth was limited by one or both of the nutrients, but the response was less than expected when N and P were enriched simultaneously. If the contrasts were negative, the response was classified as "antagonistic."

Results from each experiment were further analyzed by quantifying the response of each lake's phytoplankton community to N, P, or combined N and P enrichment by normalizing the final chlorophyll concentration in a given nutrient enrichment treatment to the final chlorophyll concentration in the control: RR-X (relative response) = Chl_X/Chl_C where X is N, P, or NP (combined N and P enrichment). These responses were then evaluated for an effect of N deposition (by t test comparing high- vs. low-deposition sets of lakes) and also plotted against each other or against various limnological parameters for each lake. The effect of N deposition on the qualitative nature of nutrient limitation (e.g., nutrient response categories) was tested via chi-square analysis. Statistical analyses were performed using the software JMP, version 5.0.1.2 (SAS Institute, Cary, North Carolina, USA).

Results

Water chemistry and seston

Study lakes varied considerably in acid neutralizing capacity (ANC), with values ranging from 30 to 2196 μEq/L (Appendix B). Lakes receiving high levels of atmospheric N deposition had significantly lower ANC (75 vs. 508 μEq/L) as well as lower pH (6.5 vs. 7.1) than low-deposition lakes (t test: $P < 0.0001$, df = 41 for both ANC and pH). The study lakes were generally highly oligotrophic, with a maximum total phosphorus (TP) concentration of 0.28 μmol/L and generally undetectable levels of soluble reactive phosphorus (SRP; Appendix B). Importantly, high- and low-deposition lakes did not differ in SRP or TP concentrations (Fig. 2A for TP; $P > 0.65$, df = 41), indicating that edaphic and watershed conditions that might have affected overall P loading did not differ substantially between the study regions, despite the range of geologic substrata encompassed in the three study areas.

Concentrations of NH_4-N were generally low (<0.50 μmol/L; Appendix B) but somewhat variable (e.g., >3 μmol/L in two lakes in the Western region) and did not differ between low- and high-deposition lakes ($P > 0.35$, df = 41). In contrast to P concentrations and to NH_4-N, concentrations of NO_3-N were nearly three times higher in high-deposition lakes relative to low-deposition lakes (Fig. 2B; $P < 0.0001$, df = 41). Reflecting these high NO_3-N concentrations, high-deposition lakes had significantly higher TN concentrations (Fig. 2C; $P < 0.001$, df = 39) and indeed, lake TN was highly positively correlated with NO_3-N ($r^2 = 0.65$, $P < 0.0001$). Lake TP concentration was uncorrelated with NO_3-N ($P > 0.18$). High-deposition lakes had significantly higher TN:TP ratios than low-deposition lakes (Fig. 2D; $P < 0.001$, df = 39), a result directly attributable to elevated NO_3-N levels, given the strong correlation of TN and NO_3-N. In an ANCOVA for TN, with TP as a covariate, deposition level (low vs. high) was highly significant ($P < 0.0001$, df = 1, 38), indicating increased overall N levels for a given level of P in Colorado lakes under high N deposition. Thus nutrient chemistry analyses are consistent with an impact of atmospheric N deposition on overall availabilities of N relative to P.

Consistent with the oligotrophic nature of the study lakes, chlorophyll concentrations were low, ranging from ~0.3–14 μg/L (Appendix C). On average, chlorophyll concentrations were ~2.5 times higher in lakes receiving high N deposition relative to low N deposition lakes (Fig. 2E; t test: $P < 0.002$, df = 40), consistent with a eutrophying impact of atmospheric N deposition. As for chlorophyll, seston C concentrations were also significantly higher under high N deposition ($P < 0.01$, df = 40) by about a factor of two (Fig. 2F). However, neither seston N nor seston P concentrations differed significantly between the two regions, although the significance level for seston P was marginal (Appendix C). Considering the overall averages by each deposition level, seston N comprised a substantial proportion (57%) of total N in the low-deposition lakes (average seston N of 4.2 μmol/L vs. TN of 7.4 μmol /L) but only contributed 31% (5.1 μmol/L seston N, 16 μmol/L TN) in high-deposition lakes, a result that suggests weaker N limitation in high-deposition lakes. Seston contributed ~100% of TP in both high and low N deposition lakes.

Seston C:N and C:P ratios were both higher in high N deposition lakes relative to low deposition (Fig. 2G), but this difference was significant ($P < 0.006$, df = 40) only for seston C:N ratio. Seston N:P showed no difference (Fig. 2H). Results of analysis of covariance were consistent with these observations. In an ANCOVA for seston C with seston N as a covariate, deposition level (low vs. high) was highly significant ($P < 0.0004$, df = 1, 39), indicating increased seston C levels for a given level of seston N in lakes under high N deposition. The ANCOVA for seston C with seston P as a covariate yielded similar results, although the result was only marginally significant ($P < 0.06$, df = 1, 39). No significant effect of deposition was identified for the ANCOVA considering seston N and P.

Phytoplankton nutrient limitation and relation to nutrient concentrations

A total of 36 factorial bioassay experiments were performed, 20 in lakes receiving low N deposition and 16 in lakes receiving high N deposition (in 13 lakes). To check for seasonal changes, experiments were performed on two dates for three of the lakes. Experiments showed

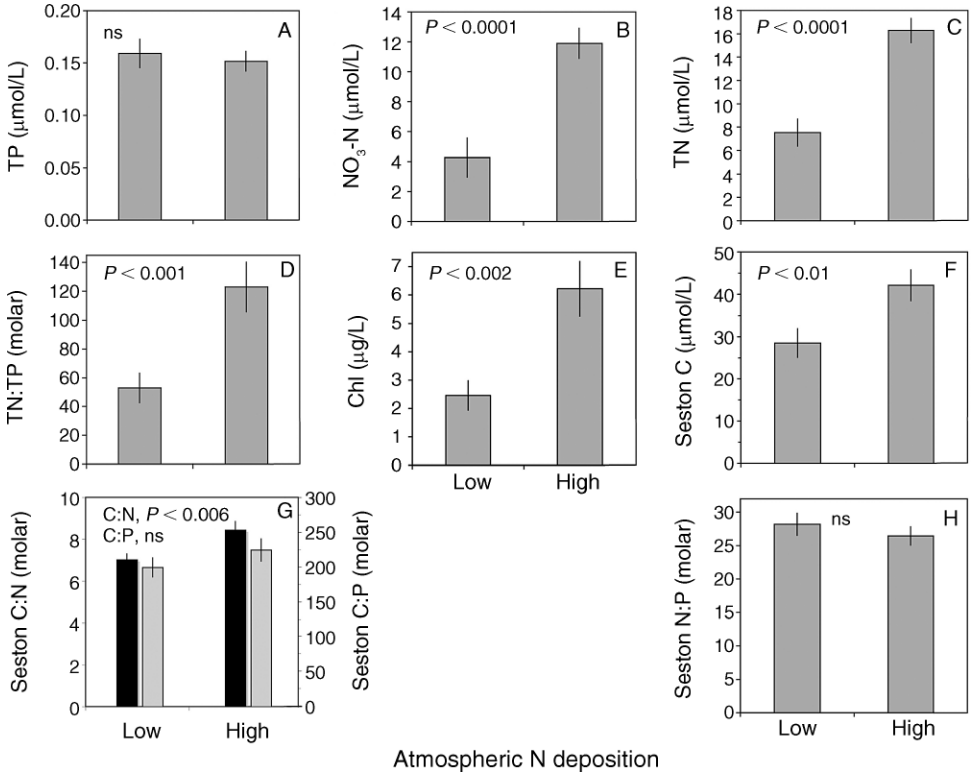

FIG. 2. Nutrient and seston concentrations and ratios for lakes receiving low or high atmospheric N deposition: (A) total phosphorus, TP; (B) NO_3-N; (C) total nitrogen, TN; (D) TN:TP ratio; (E) chlorophyll; (F) seston C; (G) seston C:N and C:P ratios; and (H) seston N:P ratio. Error bars indicate ±SE. The result of a *t* test comparing low- and high-deposition means for each parameter is given in each panel.

a variety of responses according to the nomenclature described in the Methods (Table 1; Appendices D and E), including no limitation (four experiments), single nutrient limitation (15 experiments), co-limitation (eight experiments), dual limitation (three experiments), and constrained or antagonistic (six experiments).

N deposition appeared to increase the importance of P and decrease the importance of N as a limiting nutrient (Table 1). Lakes receiving high deposition displayed a high frequency of primary P limitation (single limitation by P or sequential co-limitation with P as the primary limiting nutrient; nine of 16 lakes in these categories) with limited evidence of N limitation (only one of 16 experiments showed direct N limitation). In contrast, lakes under low N deposition displayed a lower frequency of P response (four of 20 experiments showing single limitation by P) and a higher frequency of N response (five of 20 with single limitation by N or sequential limitation with N as the first limiting nutrient). Phytoplankton in six of 20 lakes under low deposition showed strict co-limitation or dual limitation, while phytoplankton in only one of 16 experiments showed such a response under high N deposition.

To evaluate if high- and low-deposition lakes differed significantly in the frequency of nutrient limitation categories, we classified each experiment as "N-limited" (single N or sequential co-limitation [N]), "P-limited" (single P or sequential co-limitation [P]), "NP-limited" (strict co-limitation, dual limitation), or "other" (antagonistic, constrained, no limitation). Including all the categories together, chi-square analysis indicated that experiments from low- vs. high-deposition areas differed significantly in the relative frequency of these response categories ($P < 0.04$, df = 3, 30), although some response categories had very low frequencies, and thus chi-square results should be interpreted with caution. We also split the data into three bivariate categories: P (single P and sequential co-limitation [P]) vs. non-P (all other categories); N (single N and sequential co-limitation [N]) vs. non-N (all others); and NP-limited (co-limitation or dual limitation) vs. non-NP (all others). Experiments in high-deposition lakes had a significantly higher frequency ($P < 0.02$, df = 1, 34) of P limitation (nine of 16) than in low-deposition lakes (four of 20), but the frequency of N limitation was, surprisingly, nonsignificant ($P = 0.11$, df = 1, 34) for high- (one of 16) vs. low- (five of 20) deposition areas. The frequency of NP-limited responses was higher in low- (six of 20) than in high- (one of 16) deposition experiments, although this difference was only marginally significant ($P = 0.06$, df = 1, 34).

The quantitative nature of phytoplankton nutrient limitation was examined by considering RR-*X* values

TABLE 1. Summary of phytoplankton responses to N, P, and combined N and P enrichment in the study lakes in 2006 in the Rocky Mountains of Colorado, USA.

Deposition level, area, and lake	2006 date	RR-P	RR-N	RR-NP	RR-N/RR-P	*P* value N	*P* value P	*P* value N × P	Type
Low deposition, Central									
Copper (lower)	29 Jul	1.30	0.95	1.43	0.73	ns	<0.01	ns	single (P)
Copper (upper)	28 Jul	0.44	1.69	5.00	3.84	<0.05	ns	(<0.10)	single (N)
Emerald	20 Jul	1.67	0.7	1.97	0.42	ns	<0.01	ns	single (P)
Galena	23 Jul	1.42	1.98	4.65	1.39	<0.0001	<0.0001	<0.01	strict co-limitation
Monkey	1 Aug	1.64	1.01	1.92	0.62	<0.05	<0.0001	<0.10	additive dual limitation
Twin (large)	2 Aug	1.46	1.92	1.06	1.32	ns	ns	<0.01	constrained
Twin (small)	2 Aug	0.63	0.49	1.33	0.78	ns	ns	<0.01	antagonistic
Yule (large)	30 Jul	1.86	1.01	1.69	0.54	ns	<0.0001	ns	single (P)
Yule (small)	30 Jul	1.58	0.94	1.38	0.59	ns	<0.001	ns	single (P)
Low deposition, Western									
Clear	4 Sep	0.85	1.65	2.14	1.94	<0.001	ns	ns	single (N)
Crater	2 Sep	0.83	1.51	2.76	1.82	<0.0001	<0.0001	<0.0001	sequential co-limitation (N)
Eldorado	6 Sep	1.06	2.06	9.08	1.94	<0.0001	<0.0001	<0.0001	strict co-limitation
Fuller	8 Sep	1.26	1.37	2.53	1.09	<0.0001	<0.0001	<0.0001	sequential co-limitation (N)
Highland Mary (large)	2 Sep	0.72	1.09	1.99	1.51	<0.05	ns	<0.05	antagonistic
Highland Mary (small)	11 Sep	1.14	2.28	4.84	2.00	<0.0001	<0.0001	<0.0001	sequential co-limitation (N)
Horseshoe	7 Sep	2.53	1.13	2.88	0.45	<0.01	<0.0001	ns	additive dual limitation
Ice	2 Sep	0.63	0.85	0.83	1.35	ns	ns	ns	no nutrient limitation
Island	1 Sep	1.80	1.16	2.19	0.64	<0.001	<0.0001	(<0.10)	additive dual limitation
Little Molas	4 Sep	3.00	0.99	2.36	0.33	<0.05	<0.0001	<0.05	constrained
Verde	8 Sep	1.08	1.59	2.69	1.47	<0.0001	<0.01	<0.01	strict co-limitation
Mean, all low-deposition lakes		1.35	1.32	2.74	1.24				
SE, all low deposition lakes		0.15	0.11	0.44	0.19				
High deposition, Eastern									
Albion	6 Aug	1.20	0.80	1.36	0.67	ns	(<0.10)	ns	single (P)
Bluebird	11 Jul	0.98	1.14	2.16	1.16	<0.0001	<0.0001	<0.0001	strict co-limitation
Green 1	6 Aug	1.27	1.24	1.69	0.98	(<0.10)	<0.05	ns	single (P)
Green 1	30 Jun	1.51	1.04	1.5	0.69	ns	<0.0001	ns	single (P)
Green 2	22 Aug	2.15	0.99	3.39	0.46	<0.01	<0.0001	<0.01	sequential co-limitation (P)
Green 3	2 Jul	2.09	1.04	2.13	0.50	ns	<0.0001	ns	single (P)
Green 3	14 Aug	1.82	0.83	2.33	0.46	ns	<0.05	ns	single (P)
Green 4	6 Jul	2.23	1.01	2.4	0.45	ns	<0.001	ns	single (P)
Green 4	18 Aug	1.06	1.51	1.67	1.42	<0.0001	ns	ns	single (N)
Green 5	6 Aug	1.10	0.90	1.27	0.82	ns	ns	ns	no nutrient limitation
Lake of Glass	23 Aug	0.99	0.58	1.11	0.59	ns	ns	ns	no nutrient limitation
Haiyaha	26 Aug	2.13	0.87	1.98	0.41	<0.05	<0.0001	ns	single (P)
Isabelle	11 Aug	0.86	0.9	1.03	1.05	ns	ns	ns	no nutrient limitation
The Loch	23 Aug	2.85	0.94	2.63	0.33	ns	<0.0001	ns	single (P)
Sky	23 Aug	1.00	1.42	0.92	1.42	ns	<0.05	<0.05	constrained
Thunder	25 Aug	1.31	0.36	0.55	0.27	<0.01	ns	ns	antagonistic
Mean, all high-deposition lakes		1.53	0.97	1.76	0.73				
SE, all high-deposition lakes		0.15	0.07	0.19	0.10				

Notes: RR-P, RR-N, and RR-NP indicate the response ratio, i.e., the chlorophyll concentration in a given treatment (+P, +N, or +N + P, respectively) relative to its value in the unenriched control. An index of the relative responses to N or P is given as the ratio of RR-N to RR-P (note that this is equivalent to final Chl_N/final Chl_P). The results of the factorial ANOVA indicating the statistical significance of main effects of N and P and of the N × P interaction are given. The type of phytoplankton response (see *Study sites and methods: Data analysis*) is indicated. The actual results for each individual experiment are shown in Appendices D and E. Results that were not significant are denoted "ns."

for different nutrient treatments (Table 1). Average phytoplankton response to N (RR-N) was significantly higher (*t* test: $P < 0.02$, df = 35) in low-deposition experiments (1.32) than in high N deposition experiments (0.97) (Fig. 3B). Similarly, RR-NP was higher in low-deposition experiments (2.74 vs. 1.76), although this difference was only marginally significant ($P = 0.06$, df = 35; Fig. 3C). RR-P was somewhat higher in high N deposition experiments (1.49 vs. 1.36), but this difference was not statistically significant ($P = 0.28$, df = 35; Fig. 3A). To further examine the relative importance of N vs. P limitation in the study lakes, for each experiment an N vs. P index was calculated by dividing its RR-N value by its RR-P value (Table 1). Note that this ratio is equivalent to the ratio of the final chlorophyll concentrations in the N and P enrichment treatments, respectively). This N vs. P index was significantly ($P < 0.03$, df = 35; Fig. 3D) higher in low-deposition experiments (1.23, indicating N response larger than P response) than in high-deposition experiments (0.73, indicating P response larger than N response).

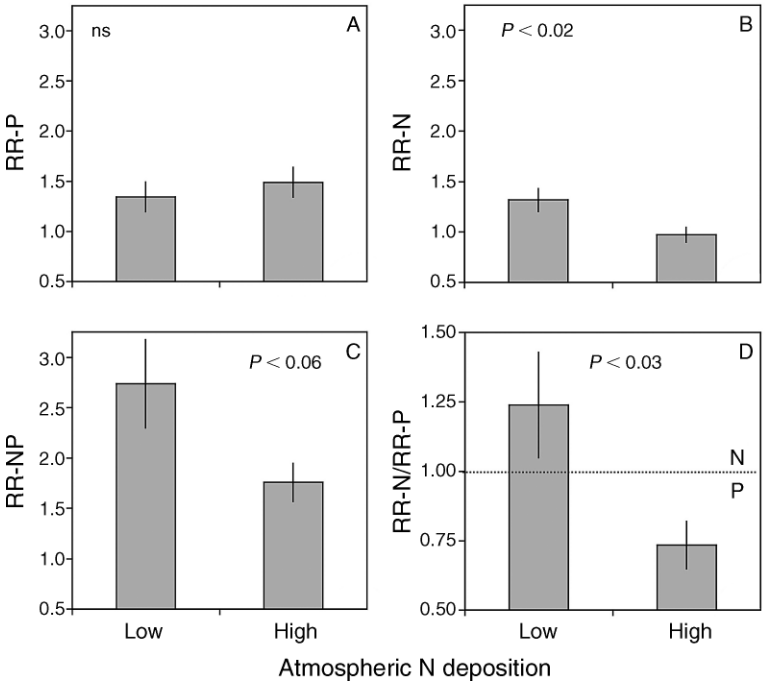

FIG. 3. Phytoplankton responses to N and/or P enrichment for lakes receiving low or high atmospheric N deposition given as the ratio of final chlorophyll concentration in the enriched treatment (+P, +N, or +N + P) to the chlorophyll concentration in the unenriched control. The response ratio, RR, has no units. (A) Response to P enrichment alone (RR-P); (B) response to N enrichment alone (RR-N); (C) response to combined N and P enrichment (RR-NP); (D) relative response to N vs. P (RR-N/RR-P, equivalent to final Chl_N/final Chl_P). For RR-N/RR-P, a value >1 indicates stronger N limitation while a value <1 indicates stronger P limitation. Error bars indicate ±SE. The result of *t* tests comparing low- and high-deposition means for each parameter are given in each panel.

We also considered the correlations among various indices of nutrient limitation from the bioassays and nutrient availability measurements. None of the nutrient limitation indicators were significantly correlated with lake TP concentration ($P > 0.21$). However, as one would expect if nutrient limitation patterns were being driven by changing N availability, RR-P was positively correlated with NO_3-N ($P < 0.001$, $r^2 = 0.16$), TN ($P < 0.02$, $r^2 = 0.18$), and TN:TP ($P < 0.001$, $r^2 = 0.18$), while RR-N was negatively correlated with NO_3-N ($P < 0.02$, $r^2 = 0.16$) and (marginally) TN ($P < 0.09$) and TN:TP ($P < 0.10$). Following these trends, the index of N vs. P limitation (RR-N/RR-P) was negatively correlated with NO_3-N ($P < 0.006$, $r^2 = 0.21$), TN ($P < 0.02$, $r^2 = 0.19$), and TN:TP ($P < 0.02$, $r^2 = 0.16$). RR-NP showed no correlations with any of these nutrient parameters.

DISCUSSION

Our data indicate that increased inputs of N from the atmosphere appear to have overridden local watershed- and lake-specific factors governing relative N and P supplies and shifted phytoplankton in these lakes into a more purely P-limited regime in which N plays a limited role. In this way, our data provide further support for the contention, based on time-series and paleolimnological studies in the Colorado Front Range, that even the relatively low levels of N deposition in the Front Range have produced fundamental ecological changes in these ecosystems (Baron et al. 2000, Wolfe et al. 2001, 2003). They are also consistent with the results of a mid-1980s survey of nutrient limitation in Colorado mountain lakes (Morris and Lewis 1988) that indicated widespread predominance of primary N limitation of phytoplankton growth throughout Colorado, including lakes in the Rocky Mountain Front Range (our Eastern high-deposition lakes). Our study identifies potential effects of N deposition on ecological parameters by directly comparing a set of lakes sampled in a region of high deposition (Eastern) with a set of lakes sampled in regions of low deposition (Central, Western), an approach that involves several limitations. Before discussing their implications any further, first we consider several caveats and assumptions that potentially bear on our results and their interpretation.

Interpretation of our nutrient limitation bioassays and study approach

First, our bioassay data rely on chlorophyll changes to infer phytoplankton response. It is possible that nutrient-limited phytoplankton may alter their cell-specific chlorophyll quotas in response to nutrient enrichment prior to, or perhaps instead of, an overall biomass response. We did not obtain overall biomass responses by cell enumeration or other means, and thus

we cannot assess the extent to which such physiological responses impinge on our data.

Second, while we staggered our sampling of high- and low-deposition regions to try to avoid confounding seasonal effects with possible effects of N deposition, it remains possible that seasonal shifts may have impacted our data. However, we believe that this can be discounted. While the data are limited, results of the bioassays performed on two dates for three lakes (Green Lakes 1, 3, and 4) suggested that seasonal changes did not influence our results in a major way. For two of the lakes (Green Lake 1, Green Lake 3), both bioassays indicated single limitation by P. However, for Green Lake 4, the experiment in July demonstrated single limitation by P while the August experiment indicated single limitation by N. A relative lack of seasonal impacts on our data is also supported by correlation analyses that indicate no significant associations of bioassay responses (RR-X) or limnological parameters (concentrations of dissolved nutrients, seston, or chlorophyll) with sampling date (data not shown).

Third, not only N but also P can enter lakes via atmospheric vectors (Lewis et al. 1985, Sickman et al. 2003), and this may also be affected by human activities. Indeed, Neff et al. (2008) concluded that lakes of western Colorado have received elevated inputs of aeolian dust, including P, during the past century, and aeolian dust has been documented in soils and sediments of the eastern Front Range (Muhs and Benedict 2006). We considered this possibility and examined existing data from the National Atmospheric Deposition Program/National Trends Network (NADP/NTN; *available online*; see footnote 5). Unfortunately, analytical methods for P in precipitation have changed during the monitoring record, complicating the results. Nevertheless, a consideration of the available NADP/NTN data indicates that atmospheric P inputs to lakes are higher in the Eastern (high N deposition) region than in the Central and Western (low N deposition) regions (average annual rates from 2000–2007 of 0.078 kg PO_4/ha vs. 0.019 kg PO_4/ha and 0.027 kg PO_4/ha, respectively). If, indeed P deposition is greater for the high N (Eastern) vs. low N deposition lakes as the NADP/NTN data suggest, this would drive the high N deposition lakes away from P limitation toward N limitation, which is the opposite of our results.

Fourth, while lakes were chosen to be relatively similar in elevation, size, depth, and watershed conditions, they differed in parent material. Lakes in the Eastern (high-deposition) region are found in granitic rock, while lakes in the Central and Western regions encompassed a larger diversity of volcanic, metamorphic, and sedimentary parent materials (Blair 1996). All the regions experienced post-Pleistocene glacial retreat between 12 000–15 000 yr BP, and alpine soils of Colorado are classified as Inceptisols (Bowman and Seastedt 2001). Sedimentary rocks of the Central and Western regions may contain N-bearing minerals (Inyan and Williams 2001), in which case these lakes should have had higher, not lower, NO_3 concentrations, which is the opposite of our results. Since both regional and hemispheric lake surveys have shown a strong relation between atmospheric N deposition, lake NO_3 concentrations, and lake N:P ratios regardless of bedrock composition (Fenn et al. 2003, Bergstrom and Jansson 2006), we concluded that variations on geologic substrates were unimportant in the determination of lake chemistry and phytoplankton stoichiometry.

Implications for mountain lakes

Bearing these caveats in mind, our study provides several lines of evidence consistent with a conclusion that atmospheric N deposition can shift the relative availabilities of nitrogen and phosphorus and alter the nutrient limitation status of lake phytoplankton. First, our data show that Colorado alpine lakes with high N deposition have considerably elevated growing season concentrations of NO_3-N, higher total N concentrations, and higher TN:TP ratios (Fig. 2B–D). Second, these differences are accompanied by increased phytoplankton biomass (as indexed by chlorophyll concentration) and seston C concentration, as well as by increased C:nutrient ratios in high N deposition lakes (Fig. 2E–G). Finally, lakes in districts receiving low levels of N deposition appear to show a considerable variety of responses to nutrient enrichments (Table 1), including not only a greater tendency for direct N limitation but also an increased frequency and magnitude of interactive, synergistic responses to combined N and P enrichment. This diversity suggests that, while there does appear to be a primary overall importance of N as a limiting nutrient in unimpacted Colorado lakes (Table 1, Fig. 3), these lakes have relatively balanced supplies of N and P, and the relative importance of N vs. P for any given lake is likely set by watershed-specific (vegetation characteristics, wetland denitrification) or lake-specific (depth, extent of sediment nutrient processing, internal food-web structure) factors.

In contrast, lakes receiving high N deposition appear to be predominantly P-limited (Table 1, Fig. 3), based both on the predominance of experiments showing single limitation by P and by the relative lack of positive synergistic responses of N and P added together. This pattern is more obvious if you contrast the similarity of average RR-NP and average single-nutrient RR-X values in the high-deposition lakes (1.76 vs. 1.49 [P] and 0.97 [N]) with the strong difference for low-deposition lakes (2.74 vs. 1.35 [P] and 1.32 [N]; Table 1, Fig. 3). This relative lack of synergistic response to combined N and P enrichment in high-deposition lakes is not surprising, given that many of the high-deposition lakes had undetectable soluble reactive phosphorus (SRP) concentrations but substantial levels of NO_3-N (12 μmol/L on average) well into the summer growing season (Fig. 2B; Appendix B). In contrast, in low-deposition lakes both NO_3-N and SRP levels were

generally low and frequently below the detection limit (Fig. 2B; Appendix B).

Our findings suggest that phytoplankton growth in Colorado alpine lakes is predominantly N-limited when lakes are unimpacted by atmospheric N deposition, a result consistent with a variety of previous studies showing frequent phytoplankton N limitation in oligotrophic western North American lakes that have been relatively free of direct anthropogenic disturbance. These studies include early years in Lake Tahoe (California and Nevada, USA; Goldman et al. 1993); Colorado (USA) mountain lakes (Morris and Lewis 1988); Wyoming (USA) mountain lakes (Nydick et al. 2004); Pyramid Lake (Nevada, USA; Reuter et al. 1993); Mono Lake (California USA; Jellison and Melack 2001); and Alaskan (USA) lakes (Levine and Whalen 2001). While these results are in contrast with the paradigm that P limitation predominates in lake ecosystems because of a natural tendency for lakes to compensate for N deficiency via increased N fixation (Schindler 1977, Schindler et al. 2008), they are consistent with a view that phytoplankton N limitation might be common in oligotrophic lakes due to insufficient P supplies to support proliferation of N-fixing cyanobacteria (Lewis and Wurtsbaugh 2008). Widespread N limitation is also understandable in low N deposition, high-elevation lakes due to low terrestrial N fixation in the alpine watersheds, along with a lack of vegetation that could sequester P derived either from weathering or aeolian inputs.

The widespread alterations of nutrient loading conditions and amplification of phytoplankton P limitation due to atmospheric N deposition indicated by our data have important ecological implications in at least three areas. First, long-distance transport of atmospheric N to oligotrophic alpine lakes is likely to induce eutrophication (at least until P becomes strongly limiting), even in lakes protected by nature preserves and parks and seemingly far from human influence. While atmospheric N loading is unlikely to induce the intense eutrophication impacts that accompany other anthropogenic nutrient perturbations, such as fertilizer runoff or sewage inputs (Harper 1992), it may be sufficient to alter desirable properties of sensitive mountain lakes, such as their high water clarity (e.g., Lake Tahoe).

Second, beyond the widely recognized effects of eutrophication on lake ecosystems, N deposition may impact ecological diversity and ecosystem services in lakes via indirect means, such as effects on resource ratios (Grover 1997, Interlandi and Kilham 2001). By distorting relative N and P supplies from relatively balanced values to high N:P ratios, atmospheric N deposition may result in lower species diversity in the phytoplankton by favoring those few taxa best suited for uptake and sequestration of P and able to sustain growth with low cellular P quotas. Indeed, paleolimnological reconstructions of diatom assemblages in lakes in the western USA that have received increased N deposition indicate a switch from evenly distributed types of diatoms to dominance by a few species (Wolfe et al. 2001, 2003, Saros et al. 2003, 2005), suggestive of a loss of biodiversity in response to N deposition. Such effects of N deposition on biodiversity have already been noted in terrestrial ecosystems (Phoenix et al. 2006). These authors also note that projected future increases in atmospheric N deposition for various regions of the world are largest in regions of high terrestrial biodiversity. Studies considering how future patterns of N deposition to lakes correspond to patterns of lake biodiversity have not yet been performed.

Third, insights from ecological stoichiometry (Sterner and Elser 2002) suggest that amplification of phytoplankton P limitation may be especially deleterious for the functioning of planktonic food webs. P-limited phytoplankton are poor food for higher trophic levels, especially for the keystone herbivore *Daphnia*, which has a high dietary P requirement. Indeed, a mesocosm NO_3 enrichment in a low N deposition lake in Wyoming resulted in decreased zooplankton biomass, consistent with inducement of poor stoichiometric food quality (Lafrancois et al. 2004). While our data indicate that N deposition to Colorado lakes has produced only modest changes in seston C:P and N:P ratios, it should be noted that N deposition in the Colorado Front Range ($\sim$8 $kg \cdot ha^{-1} \cdot yr^{-1}$) is relatively low compared to other regions of the world (current N deposition levels in central Europe and parts of China and southern Asia can exceed 20 $kg \cdot ha^{-1} \cdot yr^{-1}$; Galloway et al. 2004) and to future projections of N deposition (estimated to reach and exceed 50 $kg \cdot ha^{-1} \cdot yr^{-1}$ in several regions by the year 2050; Galloway et al. 2004). Lakes experiencing higher levels of N deposition than those in the Colorado Front Range may have higher seston C:P and N:P ratios than we observed. We are not aware of published data along these lines nor have any studies comprehensively examined the nutritional status (P limitation) of zooplankton in lakes as a function of atmospheric N deposition.

In sum, our data indicate that increased atmospheric inputs of N to Colorado alpine lakes have differentially altered the supplies of N and P available to phytoplankton and shifted phytoplankton nutrient limitation from predominant N and joint N and P limitation to predominant P limitation. These changes may alter planktonic community structure and trophic interactions and suggest that further increases in atmospheric N inputs such as those projected for many areas of the world (Galloway et al. 2004) may have major ecological ramifications for lake ecosystem structure and function, even in protected lakes far from direct human disturbance.

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APPENDIX A

Study lakes according to N deposition level and geographic region (*Ecological Archives* E090-220-A1).

APPENDIX B

Average values for various chemical parameters for epilimnetic samples for the study lakes (*Ecological Archives* E090-220-A2).

APPENDIX C

Average values for chlorophyll and seston (C, N, P) concentrations and C:N, C:P, and N:P ratios (molar) for the study lakes (*Ecological Archives* E090-220-A3).

APPENDIX D

Final chlorophyll concentrations in the four bioassay treatments for lakes receiving low atmospheric N deposition (Central and Western regions) (*Ecological Archives* E090-220-A4).

APPENDIX E

Final chlorophyll concentrations in the four bioassay treatments for lakes receiving high atmospheric N deposition (Eastern region) (*Ecological Archives* E090-220-A5).