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Co-limitation by N and P Characterizes Phytoplankton Communities Across Nutrient Availability and Land Use

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ABSTRACT

Historically, freshwater lakes have been widely assumed to be singly limited by phosphorus (P) because the dominant paradigm assumes that nitrogen fixation (N_2 fixation) will compensate for any nitrogen (N) deficits. However, a growing body of evidence demonstrates that primary producer response to nutrient manipulation most frequently indicates co-limitation by N and P. Differences in N and P supply ratio have been shown to influence the identity and severity of nutrient limitation, but whether N and P concentration and the ratio of N to P concentrations can explain the frequency of co-limitation in aquatic primary producer assemblages remains unclear, especially in ecosystems subject to human perturbation that strongly increase nutrient availability. We determined how resource availability influences nutrient limitation by N and P of phytoplankton primary production across 12 lakes in Minnesota that vary in watershed land use and lake nutrient levels. We measured epilimnetic lake metabolism and indicators of N_2 fixation to evaluate their influence on nutrient limitation status of planktonic algal assemblages. Despite large differences in land use (agricultural, urban, and suburban) and water column N and P availability, planktonic algal response to nutrient manipulation was consistently characterized by co-limitation by N and P across years and months. Neither P availability (as concentrations of total and inorganic forms) nor N_2-flux rate predicted responses to nutrient additions. N availability significantly influenced responses of phytoplankton to nutrient additions across years, but this effect was small. The ratio of total N to total P significantly influenced the response to single additions of N and P (these effects were negative and positive, respectively) in summer 2013. Importantly, higher lake primary production and heterocyte count (number of nitrogen fixing cells) were also associated with a stronger, positive response to N + P addition. Overall, these data suggest that planktonic algal assemblages are predominantly characterized by co-limitation by N and P despite large and diverse human impacts on nutrient inputs. Additionally, higher rates of primary production increase the likelihood of co-limitation. Together, these results further support the paradigm shift toward dual management of N and P in aquatic ecosystems.

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INTRODUCTION

Nitrogen (N) and phosphorus (P) are the most ubiquitous water pollutants (United States Environmental Protection Agency 2015) and together threaten the health of ecosystems at both local and global scales via downstream transport. N and P availability often limits primary production and in excess can fuel large algal blooms and subsequent decomposition and depletion of dissolved oxygen (Dodds and others 2009). Nutrient impairment of freshwater and estuarine ecosystems has led to widespread problems such as the dead zone in the Gulf of Mexico (Rabalais and Wiseman 2002; Turner and Rabalais 2013), harmful algal blooms (Paerl and others 2015), and eutrophication of local streams in urban areas (Walsh and others 2005). Reduction of both N and P in aquatic systems is an important goal for management at multiple scales and across land use types (Cotner 2016; Schindler and others 2016, 2017). Management practices have historically focused on mitigation of P since the prevailing paradigm assumes that the availability of P limits the growth of algae (Schindler and others 2016).

The current 'phosphorus paradigm' (sensu Sterner and others 2008) asserts that nitrogen fixation (N_2 fixation) will compensate for any N deficits, ultimately resulting in P limitation (Schindler 1977; Sterner 2008; Schindler and others 2016). Thus, lakes and streams are frequently assumed to be P-limited (Schindler 2006). N has also been shown to contribute to freshwater eutrophication (Vitousek and others 1997; Bergström and Jansson 2006; Elser and others 2007; Abell and others 2010) and is also often cited as the main culprit of coastal eutrophication (Howarth and Marino 2006; Paerl and others 2014). Meta-analyses have demonstrated that algal communities are most frequently limited by the availability of *both* N and P (Francoeur and others 1999; Tank and Dodds 2003; Elser and others 2007; Harpole and others 2011; Donnell and others 2017). Despite this important finding, there is still much debate about whether eutrophication should be managed by controlling for P alone or both N and P (United States Environmental Protection Agency 2015; Cotner 2016; Schindler and others 2016, 2017; Paerl and others 2018).

The majority of the studies that have documented nutrient co-limitation in aquatic primary producer assemblages have occurred in ecosystems that experience very little human use. For example, the whole-lake manipulations by Schindler and others took place in lakes in the Canadian Shield (Schindler 1977, 2012) and have influenced the management of freshwaters across the USA despite the fact that these northern lakes experience very little human use. Additionally, meta-analyses that have demonstrated the frequency of co-limitation across aquatic primary producer assemblages are primarily composed of field studies in landscapes that also are not subject to human use (Francoeur and others 1999; Tank and Dodds 2003; Elser and others 2007). Subsequent studies of co-limitation have primarily focused on data from pristine ecosystems such as the oligotrophic open ocean (Saito and others 2008; Harpole and others 2011). It is unclear how broadly these results can apply to freshwaters that are subject to urban and agricultural land, yet this information is critical to the successful management of eutrophication.

Human activity has dramatically altered nutrient availability with important consequences for growth of primary producers. The global input of reactive N to landscapes has doubled (Vitousek and others 1997), and availability of P has increased fourfold (Cordell and others 2009), mostly due to conventional agricultural practices. Land use changes associated with urban development can also play a dominant role in the high N and P inputs to aquatic ecosystems that lead to eutrophication, especially at local scales due to high population densities (Van Drecht and others 2009; Lin and others 2014; Zhang and others 2015). These changes are mostly due to drainage intensification (that is, storm drains) and sewage, but other sources of nutrients may be important, such as pet waste and leaf litter (Bratt and others 2017; Hobbie and others 2017; Janke and others 2017). The relative and absolute amounts of these N and P inputs can also vary dramatically across different land use. Agricultural runoff often has high amounts of N and P and a high ratio of N to P, whereas urban runoff often carries high amounts of P as well (due to storm water) but with a lower ratio of N to P (Hobbie and others 2017). Differences in N and P ratio of supply have been shown to influence the identity and severity of nutrient limitation (Vanni and others 2011), but whether N and P concentration and the ratio of N to P concentrations can explain the frequency of co-limitation in aquatic primary producer assemblages remains unclear, especially in ecosystems subject to

human perturbation that strongly increases nutrient availability.

This research aimed to address the following questions: (1) In ecosystems heavily influenced by human land are epilimnetic phytoplankton use co-limited by N and P? (2) Does resource availability predict nutrient limitation by N and P in these ecosystems? Based on the results of Elser and others (2007) and Harpole and others (2011) that have demonstrated that primary producer response to nutrient manipulation most frequently indicates co-limitation by N and P, we hypothesize that co-limitation is likely also common across urban and agricultural landscapes. Additionally, we hypothesize that resource availability will influence nutrient limitation in these ecosystems subject to urban and agricultural land use that strongly increase nutrient availability.

METHODS

Study Sites

All study sites were located within Minnesota (Figure 1). We sampled 6 lakes during summer (June–August) 2013 and 9 lakes during summer 2014 (Figure 1, Table 1). Each lake was visited three times each summer (June, July, and August), yielding 51 unique lake–date combinations. Lakes were chosen to span a wide range of nitrogen and phosphorus availability and land use (for example, urban, agricultural, and suburban, Table 1). Lake area and maximum depth varied across lakes from 27.7 to 320.4 hectares and 3.4–20.7 m, respectively (Table 1). The lakes we chose are typical of glacial lakes that are used recreationally across the state of Minnesota, in terms of area and maximum depth (Minnesota Conservation Department 1968).
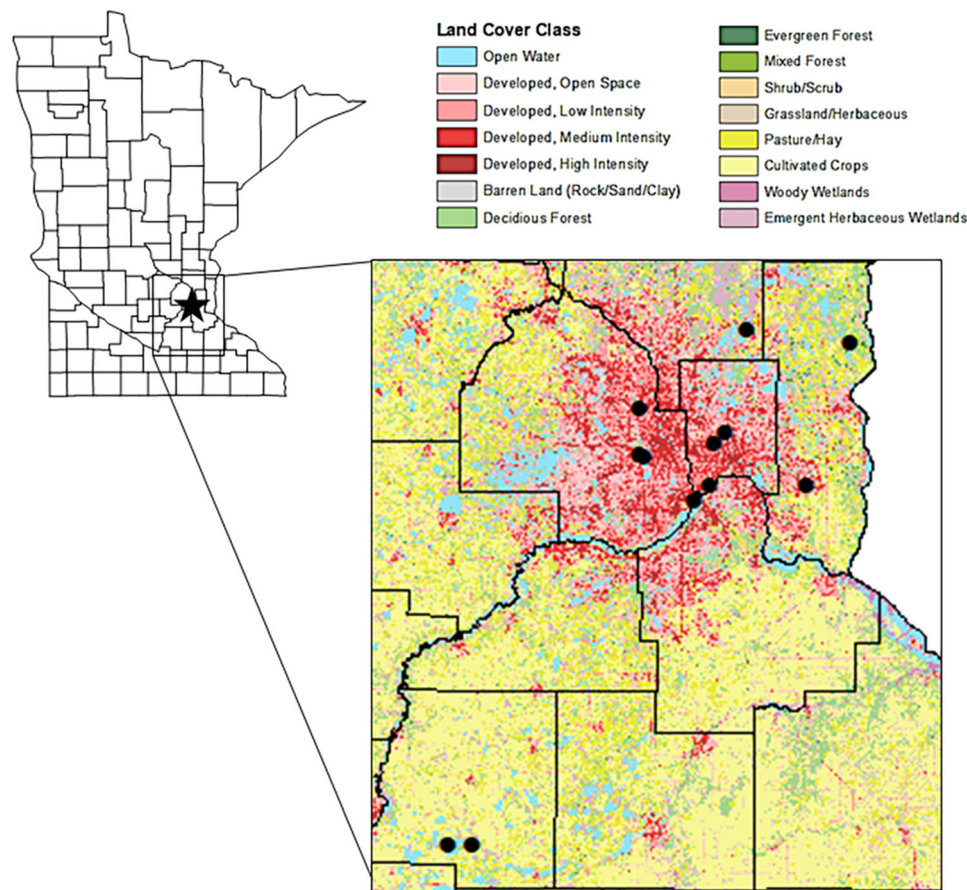
Figure 1. Map of sampling sites across Minnesota. Black dots represent lakes. Lines outline counties. Land cover data are from National Land Cover Dataset (2011). Star represents location of Twin Cities metro area.

Table 1. Characteristics of study lakes

Lake	Years sampled	Land use	Management	Location (lat, long)	Area (ha)	Maximum depth (m)	Total impervious cover (%)
Lake of the Isles	2013	Urban residential[α]	Milfoil harvest, alum	44° 57′ 17.3″ N 93° 18′ 32.8″ W	44.1	9	37.5
Cedar	2013	Urban residential[α]	Alum	44° 57′ 35.7″ N 93° 19′ 16.3″ W	68.8	15.5	30.7
Como	2013, 2014	Urban residential, open spaced[β]	Alum	44° 58′ 45.8″ N 93° 08′ 27.1″ W	27.7	4.7	31.8
Snelling	2013	Commercial*	Fish stock	44° 52′ 45.6″ N 93° 11′ 12.1″ W	41.7	10	41.2
McCarrons	2013, 2014	Urban residential[β]	Alum	44° 59′ 54.4″ N 93° 06′ 47.4″ W	29.5	17.3	24.7
Ryan	2013, 2014	Urban residential[Φ]	Fish stock	45° 02′ 27.0″ N 93° 19′ 19.1″ W	7.3	11	34.4
Crosby	2013, 2014	Urban residential, commercial[Ψ]		44° 54′ 16.4″ N 93° 08′ 58.4″ W	19.4	6	17.2
Square	2013, 2014	Grassland, woodland[κ]	Fish stock	45° 09′ 18.0″ N 92° 48′ 03.2″ W	78.1	20.7	2.50
Peltier	2014	Pasture and open[δ]	Fish stock, aeration	45° 10′ 35.1″ N 93° 03′ 33.6″ W	195. 5	5.5	3.10
Colby	2014	Suburban residential[λ]	Fish stock, aeration	44° 54′ 22.6″ N 92° 54′ 37.1″ W	23.8	3.4	20.0
West Jefferson	2014	Agriculture[η]	Fish stock	44° 16′ 28.9″ N 93° 47′ 09.6″ W	177. 6	7.3	4.30
German	2014	Agriculture[η]	Fish stock	44° 16′ 27.3″ N 93° 43′ 28.8″ W	320. 4	15.5	2.25

[α]*Minneapolis Park and Recreation Board (2012)*, [β]*Capitol Region Watershed District (2013)*, *Metropolitan Airport Commission (1996)*, [Φ]*Minnesota Pollution Control Agency (2007)*, [Ψ]*Capitol Region Watershed District (2010)*, [κ]*Carnelian-Marine St. Croix Watershed District (2012)*, [δ]*Minnesota Pollution Control (Agency 2009)*, [λ]*South Washington Watershed District (2011)*, [η]*Pallardy and others (2013)*.

Water Samples

Bulk water samples were collected at 0.5-m (m) depths from the center of the lake. Water samples were stored in Nalgene carboys and transported in coolers to the University of Minnesota (UMN) within 8 h of collection. Carboys were rinsed once with lake water before filling. Water for chemical analysis was filtered the same day as collection and filtered for nutrient bioassays within 24 h. A portable meter (YSI model 556) was used to record dissolved oxygen and temperature in 0.25-m intervals from the surface of each lake to the bottom.

Water Chemistry Analyses

Samples were analyzed for dissolved and particulate N and P in laboratories at UMN per Janke and others (Janke and others 2014). Water samples were filtered through pre-ashed 0.7-µm Whatman GF/F filters and analyzed for total dissolved N (TDN), total dissolved phosphorus (TDP), and soluble reactive phosphorus (SRP). TDN samples were analyzed with a Shimadzu TOC Vcpn analyzer (Shimadzu Scientific Instruments, Columbia, MD). SRP was analyzed by molybdate colorimetry, and TDP was analyzed using the same method after persulfate digestion. Particulates were collected on pre-ashed 0.7-µm Whatman GF/F filters. Particulate P (PP) was analyzed using molybdate colorimetry, similar to TDP, and particulate N (PN) was determined using near-infrared spectroscopy (NIRS). Subsets of PN samples were analyzed using standard analytical methods on a PerkinElmer CHN analyzer (PerkinElmer, Waltham, MA) for calibration of spectroscopic method ($R^2 = 0.93$ and slope = 1.07 for a fit of the two methods). Total N (TN) was calculated as TDN + PN, and TP was calculated as TDP + PP. Cholorophyll-a (chl-a) was collected on 0.2-µm cellulose nitrate filters, extracted in 90% acetone, and measured using a narrow-band fluorometer (Welschmeyer 1994).

Nutrient Bioassays

Nutrient limitation was assayed using laboratory incubations. Incubations were established within 24 h of sample collection. Incubations were performed using a 90:10 filtered/unfiltered water dilution, and particles were removed with an acid-cleaned all-polypropylene 0.45-µm capsule filter. Chl-a content of the diluted water was measured to establish the initial chl-a concentration for incubations. One hundred milliliters of the water was added to 250-ml polycarbonate flasks which were treated with 70 µM NH_4NO_3 (N) and 5 µM PO_4 (P) nutrient spikes to alleviate both N and P limitation. We applied these treatments in a complete factorial design with three replicates. Flasks were incubated for 72 h at the water temperature of the lake, under a 12-h light–dark cycle. Initial chl-a and final chl-a were collected and determined as above with duplicate samples from each flask. Growth rates were calculated as the difference between initial and final chl-a concentrations normalized by incubation time. We also calculated response ratios as follows for the ease of data visualization and analysis:

$$RR = \ln(\text{treatment growth rate/control growth rate}). \tag{1}$$

Response ratios are absolute differentials of growth between specified treatments, in this case N, P, and N + P versus control treatments.

Epilimnetic Lake Metabolism

To determine the effect of trophic status on nutrient limitation of planktonic algal assemblages, we estimated lake metabolism based on gas flux rates across lakes during summer 2014. Epilimnetic lake metabolism was measured for all lakes in both July and August using methods outlined by Staehr and others (2010). Gross primary production (GPP), ecosystem respiration (R), and net ecosystem production (NEP) were estimated from diel, 'free-water' changes in dissolved oxygen (DO) using high-frequency sonde data. A sonde (miniDO$_2$T Logger or Hobo Dissolved Oxygen Logger) was deployed to record continuous DO and water temperature at 15-min intervals for 3 days at each lake. Sondes were located 0.5 m beneath the surface over the deepest part of the lake. Salinity influence was negligible (conductivity ranged from 200 to 600 µS/cm/cm). We acquired daily barometric pressure and wind speed data required to estimate GPP, R, and NEP from the National Oceanic and Atmospheric Administration (NOAA) National Climatic Data Center for the St. Paul Minneapolis international airport station. Areal estimates of daily GPP, R, and NEP values (g O_2/m^2 day) were modeled from raw data using the mathematical equations outlined in Table 2 of Staehr and others (2010).

Dinitrogen Gas Flux

To determine the effect of biological N$_2$ inputs on nutrient limitation of planktonic algal assemblages, we measured rates of N$_2$-flux as a proxy for biological N$_2$ fixation for all lakes during June, July,

and August of 2014. We incubated 125 ml of unfiltered epilimnetic water collected from 0.5 m depth in eight replicate gas-tight, glass 125-ml serum bottles with rubber septa. Bottles were incubated in situ at 0.5 m depth for 2 h. N_2-flux incubations were conducted only on sunny, clear days to minimize variation across sites due to differences in light availability. Water was sampled from bottles at both the beginning (T_0) and end of the incubation (T_2) and stored in 20-ml gas-tight, crimp top glass vials. Samples were immediately preserved with 1 μL 1% ZnCl, crimped and stored on ice in the dark. Vials were kept in the refrigerator (4°C) until analyzing for dissolved N_2 gas using a membrane inlet mass spectrometer (MIMS, Bay Instruments, detector by Pfeiffer, Prisma QMS 200 quadrupole). We assume that differences in concentration of N_2 gas between the T_2 bottles and the T_0 bottles (N_2-flux) were due to biological N_2 fixation over a 2-h period. We acknowledge that denitrification could have been co-occurring in these bottles (thereby increasing dissolved N_2), but dissolved oxygen measurements at the end of the 2-h incubation showed that water column conditions remained oxic over this period.

Community Composition

Phytoplankton community composition was determined using the FlowCam dynamic imaging particle analysis system (Fluid Imaging Technologies, Inc.). Automated imaging technologies are an increasingly common tool in the field of plankton identification because they are less labor intensive than microscope identification of preserved samples, though software has limitations in the accuracy of identification (Benfield and others 2007).

Images were generated using FlowCam Visual Spreadsheet software (version 3.2). A minimum of 10,000 images for each lake–date combination were captured; however, individual particles (much of which can be debris) can be imaged multiple times by this software, and therefore, this did not necessarily represent 10,000 individual plankton. Phytoplankton were visually identified and enumerated manually. Image resolution limited identification of phytoplankton to the level of broad taxonomic groups, including chlorophytes, cyanobacteria, diatoms, cryptomonads, and dinoflagellates. We used principal component analysis (PCA) and non-metric multidimensional scaling (NMDS) on the abundances of each taxonomic group for each lake–date combination to determine whether the community composition was different between lakes or land uses. Hetero-

cytes were also counted and distinguished by identification of one or more clear heterocytes with homogenous cell contents by eye within the subset of images of cyanobacteria.

Data Analysis

To determine nutrient limitation status, response ratios (RR_N, RR_P, and RR_{NP}) of phytoplankton were analyzed with factorial analysis of variance (ANOVA) analysis of covariance on all nutrient addition experiments grouped together by year. We grouped data by year because there were different responses to treatment across years. We aimed to target lakes across more land uses in 2014, so the results are not directly comparable (see Table 2). We also measured more variables (GPP and N_2-flux).

We used factorial analysis of covariance (ANCOVA) to assess the effects of adding P and N on growth rates (as determined by final chl-a concentration) across gradients of water column N and P availability, N_2-flux (as a proxy of N_2 fixation), and epilimnetic lake metabolism. The ANCOVA model contained the factors P and N (experimental treatments), the covariates TP, TN, TP/TN (lake water concentrations from the same date), N2-flux (2014 only), and epilimnetic lake metabolism (2014 only), and all second-degree interactions between factors. Again, we used separate models for 2013 and 2014. We followed ANCOVA methods based on Zuur and others (2009).

We further investigated the individual effects of significant covariates (as determined by above model) on response ratios (RR_x) using repeated-measures ANOVA. Finally, we used ANCOVA to assess the effects of land use (as estimated by % impervious cover and categorical land use outlined in Table 1) and depth on absolute and relative nutrient availability. All statistical analyses were conducted using R (R Core Team 2018). ANCOVA analyses were conducted using the lme4 package in R (Bates and others 2015). p values less than 0.05 were considered significant.

RESULTS

Epilimnetic Water Chemistry

Lakes varied dramatically in terms of both absolute and relative nutrient availability (Table 1). Absolute availability of water column TN ranged from 0.43 mg/L in urban Lake Snelling to 2.62 in agriculturally impacted Lake West Jefferson. TP ranged from 16.3 to 159 ug/L suburban oligotrophic Square Lake and suburban Lake Peltier, respectively. N/P ratio ranged from 16.5 molar TN/TP in

Table 2. Water chemistry and algal productivity of study lakes

Lake	Mean DIN (ug/L)	Mean TN (mg/L)	Mean SRP (ug/L)	Mean TP (ug/L)	TN/TP (molar)	Algal biomass (g chl-a/L)	N_2-flux (mg N fixed/L hr)	GPP (g O_2/m^3 h)	Summer stratification
Lake of the Isles	0.01 (0.02)	1.24 (0.20)	5.72 (2.90)	26.3 (12.8)	121 (50.8)	118 (84.7)	–	–	June, July, Aug
Cedar	0.01 (0.02)	0.83 (0.09)	4.94 (2.43)	21.6 (13.4)	107 (58.2)	46.1 (17.8)	–	–	June, Aug
Como	0.05 (0.09); 35.7 (30.5)	1.30 (0.30); 0.74 (0.05)	67.5 (18.5); 51.7 (28.5)	153 (39.5); 143 (54.3)	19.9 (8.35)	69.7 (57.0); 13.1 (6.27)	10.0 (10.5); 14.7 (8.85)	4.67 (0.99)	June July, Aug
Snelling	0.01 (0.02)	0.43 (0.08)	2.78 (0.26)	23.4 (10.4)	48.2 (24.1)	7.46 (3.19)	–	–	June July, Aug
McCarrons	0.01 (0.02); 62.6 (60.3)	0.52 (0.21); 0.59 (0.21)	5.96 (1.12); 5.65 (1.11)	19.7 (0.50); 47.7 (12.1)	59.2 (25.6)	14.0 (9.59); 27.0 (3.51)	10.1 (5.55)	0.98 (0.26)	June, July, Aug
Ryan	0.01 (0.01); 142 (114)	0.70 (0.25); 0.73 (0.36)	10.4 (8.13); 18.4 (19.1)	35.5 (17.0); 45.3 (10.0)	46.2 (15.3)	22.7 (15.6); 34.8 (13.7)	12.6 (5.67)	5.68 (3.00)	July, Aug (2013); June, July, Aug (2014)
Crosby	0.02 (0.03); 447 (743)	0.53 (0.48); 2.38 (2.12)	37.2 (11.8); 93.3 (10.9)	103 (43.8); 232 (83.9)	9.26 (8.35)	58.9 (40.7); 31.9 (39.4)	2.50 (2.43)	2.18 (3.12)	June, Aug
Square	0.01 (0.01); 18.2 (13.0)	0.36 (0.08); 0.76 (0.30)	2.85 (1.00); 7.89 (5.16)	13.3 (2.43); 18.3 (6.52)	59.5 (5.41)	5.63 (1.41); 103 (67.1)	1.66 (2.00)	0.52 (0.20)	June July, Aug
Peltier	134 (47.6)	1.63 (0.43)	57.9 (37.9)	159 (14.9)	22.6 (5.54)	17.1 (17.5)	2.60 (3.60)	2.45 (1.88)	July
Colby	77.0 (106)	1.46 (0.70)	18.2 (8.25)	155 (43.7)	24.0 (17.9)	46.1 (37.7)	4.77 (8.27)	4.01 (5.06)	June
West Jefferson	132 (152)	2.62 (1.33)	7.92 (0.11)	58.1 (18.0)	120 (104)	21.3 (9.72)	7.89 (7.72)	5.60 (1.19)	June
German	52.3 (65.2)	2.49 (1.79)	7.32 (0.04)	73.1 (8.03)	72.6 (45.8)	41.7 (9.09)	3.20 (3.55)	3.73 (0.54)	June, August

Mean (SD in parentheses) dissolved inorganic nitrogen (DIN), total nitrogen (TN), soluble reactive phosphorus (SRP), total phosphorus (TP), TN/TP, algal biomass [estimated by chlorophyll-a (chl-a)], N_2-flux rate, and gross primary production (GPP) are mean values from all sample dates across all seasons (n = 3). Lakes that were studied across summers 2013 and 2014 have two mean values and SD, with 2013 at left and 2014 at right. Summer stratification indicates which months of the summer lakes were stratified based on temperature and O_2 vertical profiles taken from the middle of each lake.

Lake Como to 121 molar TN/TP in Lake of the Isles, respectively (both urban and alum-treated lakes). Urban Lake Crosby also had low TN/P (18.2), and agriculturally impacted Lake West Jefferson also had high TN/TP (120). Nutrient availability varied dramatically, both across and within land use (Tables 1, 2). However, there was no significant effect of land use (using the categorical variables and percent impervious cover outlined in Table 1) or depth on within-lake nutrient concentrations (both absolute and relative) in these lakes. Nutrient availability also varied seasonally, but this varied from lake to lake and across years (sometimes increasing, decreasing, or peaking in mid-summer).

Nutrient Limitation of Phytoplankton Communities

Planktonic algal assemblages in the lakes we sampled responded most strongly to N + P additions and were characterized as co-limited across land use and temporal variation (seasonal and yearly, Figures 2, 3). When all 51 lake–dates were pooled together for each summer, the response ratio (RR) was significantly higher for the N + P addition than the N or P additions (Figure 3, ANOVA, $p < 0.01$). Of the 51 lake–date combinations, 28 showed the strongest response to the N + P treatment (Figures S1–S2). However, there were exceptions to this result.

Some lakes were consistently characterized by single-nutrient limitation. Of the 51 lake–date combinations, 12 responded most strongly to N additions and 8 to P additions (Figures S1–S2). These responses did not show evidence of a seasonal pattern. Urban Lakes Crosby and Como were characterized by N limitation for all lake–date combinations. Lake Peltier also showed N limitation but only in June 2014. Interestingly, oligotrophic, suburban Square Lake was characterized by P limitation throughout summer 2014, but was co-limited throughout summer 2013. Agriculturally impacted Lake West Jefferson was P-limited in June and August 2014, but co-limited in July. Urban Lake McCarrons was characterized by P limitation in August 2013 and June 2014 but was otherwise co-limited by N and P. Finally, suburban Colby Lake was P-limited in August 2014, but co-limited in June and July.

Some planktonic algal assemblages did not respond or responded negatively to nutrient additions. In August 2013, suburban Square Lake did not response to nutrient additions and the same response occurred in urban lakes Como and Ryan in June 2014. Phytoplankton frequently responded negatively to P additions and, at times, to N addi-

tions (Figures 2, 3, S1–S2). This phenomenon primarily occurred in summer 2013 (Figure 3) and was driven by responses in urban lakes (Cedar, Como and Lake of the Isles, Figure S1). In late summer 2013, suburban Square Lake responded negatively to all nutrient additions and responded negatively to N addition in July and August 2014 (Figure S1).

Community Composition

Although the phytoplankton communities trended toward being distinct, there was much variability and these differences were not statistically significant (see Figures S-4 and S-5). We explored these communities using PCA and NMDS on the abundances of each taxonomic group for each lake–date combination to determine whether the community composition was different between lakes or land uses, but points did not separate out in a meaningful way. At this course resolution of taxonomic diversity, we could not distinguish differences between communities.

## Covariate Effects on Response to Nutrient Additions

Our analysis of covariance (ANCOVA) further demonstrated that phytoplankton response to nutrient manipulation most frequently supported co-limitation. N addition increased growth rates across both years (Tables 2, 3). P addition actually decreased growth rates overall in 2013 but increased them in 2014. There was a significant effect of the factorial nutrient addition (N * P, beyond the effect of adding these nutrients separately) in 2014. This effect was not significant in 2013, likely due to the strong negative effect of P addition that year.

Multiple covariates influenced the phytoplankton response to nutrient additions. There was a significant, but small effect of TN on phytoplankton response to nutrient additions across both years. This effect was negative in 2013 and positive in 2014 (Tables 3, 4). TN/TP ratio significantly affected responses to nutrient additions in summer 2013 (Table 3, Figure 4). N/P ratio had the largest effect on responses in 2013; overall, this factor
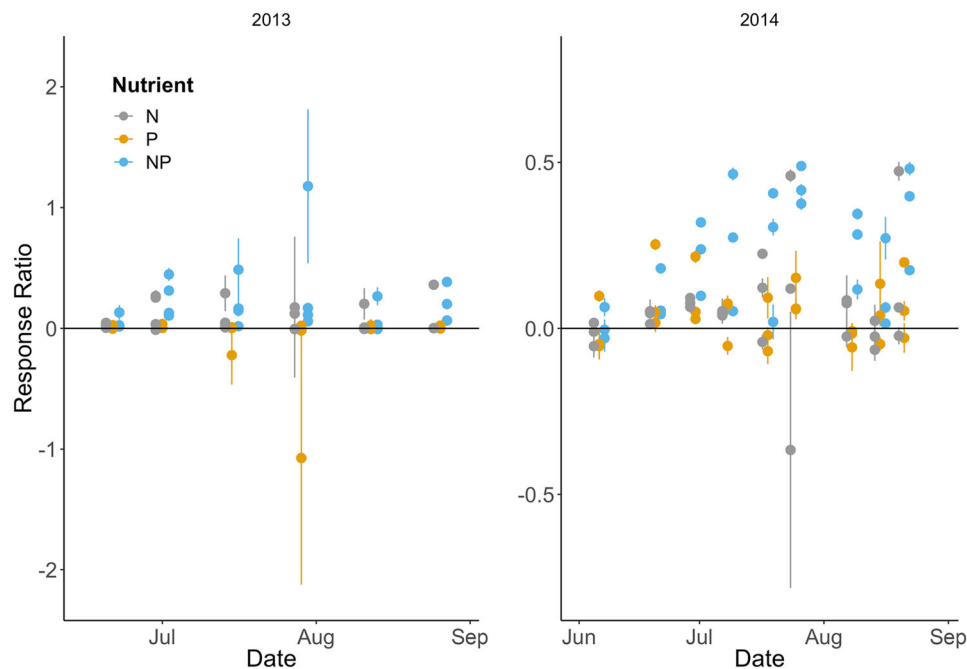
Figure 2. Seasonal variation in response ratio across lakes determined by bioassays in planktonic algal assemblages during summers 2013 and 2014. Response ratio was measured from chlorophyll-a determined growth rates in response to nutrient enrichment and indicates the relative strength of nitrogen (N), phosphorus (P), and NP limitation compared to control treatments. Points represent means of three bottles per treatment combination. Vertical lines represent ± 1 standard error. Horizontal line indicates where the response ratio equals 0—above this value there was a positive response to the nutrient enrichment compared to control treatments and a negative response for values below 0. Note that the y-axes differ.
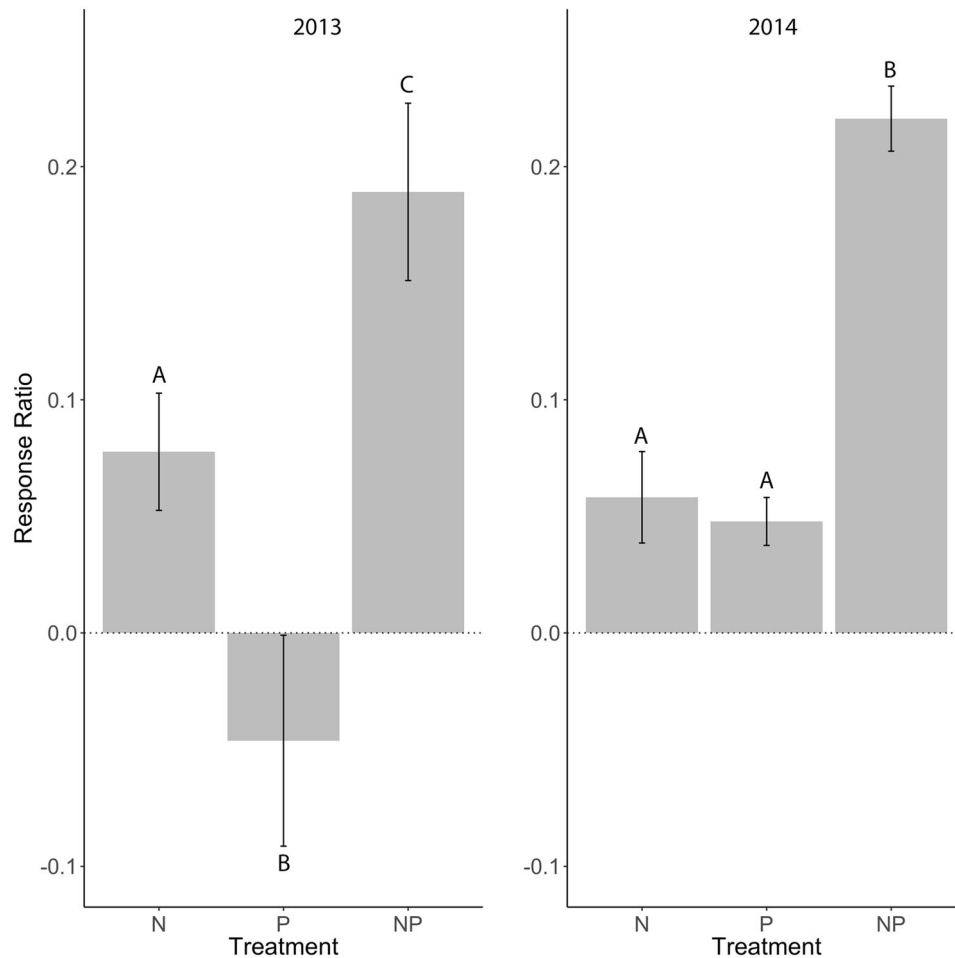
Figure 3. Differences in response to nutrient amendments in planktonic algal assemblages for all lake–date combinations during summers 2013 and 2014. Response ratio was measured from chlorophyll-a determined growth rates and indicates the relative strength of N, P, and NP limitation compared to control treatments. Bars represent means of all lake–date treatments ($N = 24$ or 27 in 2013 and 2014, respectively). Error bars represent $\pm$ 1 standard error. Letters within each panel indicate significant differences between means ($p < 0.01$).

negatively influenced N effect sizes (Figure 4A) and was positive for P (Figure 4B). Epilimnetic lake metabolism had a significant and relatively large effect on phytoplankton response to nutrient additions in 2014 (Table 4, Figure 5, note that we did not measure GPP in 2013). Additionally, heterocyte count had a significant but small effect on phytoplankton response to nutrient additions in 2013 (Table 3) and was overall negatively related to RR_N and RR_P (Figure S6). We found no effect of N_2-flux rate and responses to nutrient enrichment (Tables 3, 4). Although we collected data on the phytoplankton communities in these lakes, there was too much variation at this course scale of diversity (functional) to determine any community effects on the response to nutrient addition (Figures S3–S4).

DISCUSSION

Meta-analyses have shown that algal assemblages are most frequently limited by the availability of both N and P (Francoeur and others 1999; Tank and Dodds 2003; Elser and others 2007; Harpole and others 2011). Most of the study systems included in these meta-analyses were not directly impacted by anthropogenic activity or at least were not specifically chosen because of these impacts, which strongly increases nutrient availability. Here, we show that across 12 urban, suburban, and agriculturally impacted lakes in Minnesota, phytoplankton were most frequently limited by the availability of N and P together, despite large variation in the TN/TP ratio of availability (that is, the ratio that is available to phytoplankton from the water column) in these lakes.

Table 3. ANCOVA of final growth rates in the nutrient addition experiments

Effect/source	Parameter estimate	Chi sqr	p
Treatments			
N	0.56	16.2	< 0.0001***
P	− 0.05	2.38	< 0.0001***
N * P[1]	0.86	0.08	0.12
Covariates			
TN (ug/L)	− 0.002	19.02	< 0.0001***
TP (ug/L)	− 0.0005	0.49	0.49
TN/TP	− 0.010	7.49	< 0.001**
Heterocyte count	0.007	7.65	< 0.001**
Error	1.20		

Mixed effects ANCOVA of chlorophyll-a-based growth rates of all nutrient addition experiments during summer 2013. Lake was the random effect (SD = 0.25). Conditional R^2 = 0.51.
[1]*This represents effect of the factorial addition of N and P.*

Table 4. Mixed effects ANCOVA of chlorophyll-a-based growth rates of all nutrient addition experiments during summer 2014

Effect/source	Parameter estimate	Chi sqr	p
Treatments			
N	0.08	20.7	< 0.0001***
P	0.04	12.4	< 0.001**
N * P[1]	0.09	5.32	0.02*
Covariates			
TN (ug/L)	0.00008	23.5	< 0.0001***
TP (ug/L)	0.0001	0.09	0.77
TN/TP	− 0.007	3.26	0.07
N_2-flux (mg N_2 fixed L^{-1} h^{-1})	− 0.004	1.72	0.19
GPP (g O_2 m^{-3} h^{-1})	− 0.01	5.50	0.02*
Heterocyte Count	− 0.0005	3.11	0.08
Error	0.09757		

*Lake was the random effect (SD = 0.09). Conditional R^2 = 0.66.*
[1]*This represents effect of the factorial addition of N and P.*

Nutrient Limitation Across Land Use

Overwhelmingly, the response of planktonic algal assemblages from the lakes we studied indicates that phytoplankton were co-limited by N and P. This aligns with previous findings across meta-analyses of nutrient addition experiments (Francoeur and others 1999; Elser and others 2007; Harpole and others 2011) and recent arguments made by Kaspari and Powers (2016). This is surprising considering the large variation in both absolute and relative availabilities of N and P in these lakes. These results somewhat align with recent work across lakes subject to similar agricultural land use in Ohio (Hayes and others 2015). Hayes and others found evidence of both N and P

limitation in lake phytoplankton communities of agriculturally impacted lakes (although they did not add N and P together), but the identity of the limiting nutrient depended on drought status (N inputs are primarily delivered by storm events and are thus reduced during drought conditions). However, our study lakes did not experience drought during the study period and so our results cannot explain changes in precipitation.

The phytoplankton responses we captured varied little with season or across years, but phytoplankton did respond more strongly to nutrient addition with time during summer 2014 (Figures 2, S2). Additionally, there was an overall greater response to nutrient additions in 2013 compared to 2014 (see y-axes in Figure 2). This is likely due to an
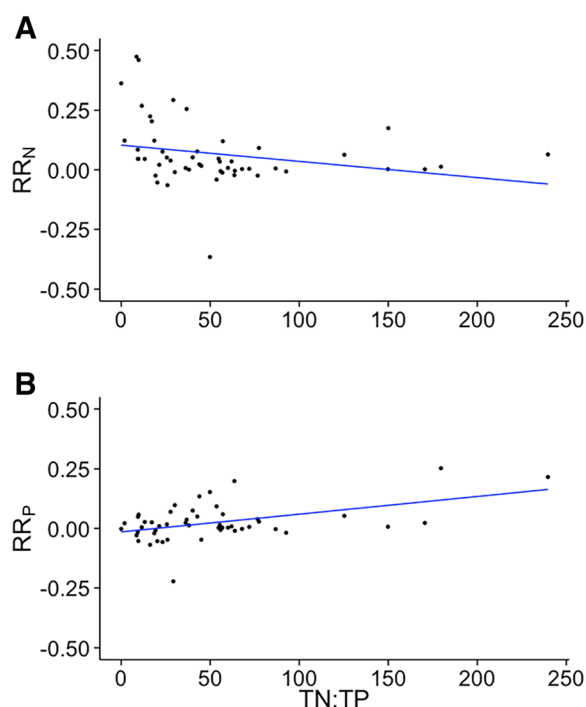
Figure 4. Relationship between molar ratio of total nitrogen (TN) to total phosphorus (TP) and response to **A** nitrogen addition (RR_N) and **B** phosphorus additions (RR_P) in planktonic algal assemblages for all lake–date combinations during summers 2013 and 2014. Points represent single lake–date sampling events (each lake was sampled three times). Lines represent trendline for significant effects of TN/TP on N and P response ratios (repeated-measures ANOVA, $p < 0.001$).
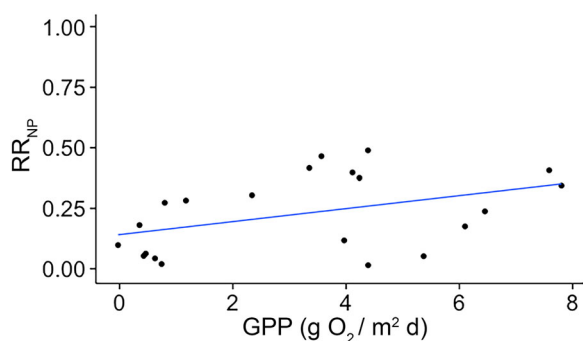
Figure 5. Relationship between gross primary production (GPP) and response to nitrogen and phosphorus additions in planktonic algal assemblages (RR_{NP}) for all lake–date combinations during summer 2014. Points represent single lake–date sampling events. Lines represent trendline for significant positive effects of GPP on N * P effect size (repeated-measures ANOVA, $p < 0.001$).

earlier spring in 2013 (January–March in 2014 were much colder than the same months in 2013) causing an earlier warm season and higher chl-a concentrations in 2013 (see Table 2). These differences in antecedent winter conditions could underlie the overall higher growth response in 2013 compared to 2014 and the stronger response with time in 2014 (assuming logistic growth conditions). Despite this overall trend, our analysis captured important exceptions to the dominant co-limitation response between lakes as well.

Some phytoplankton responses to nutrient manipulation were consistently characterized by single limitation by N or P across years and months. Crosby Lake and Como Lakes were characterized by N limitation for all lake–date combinations, which can be explained by their characteristic low TN/TP ratio of availability (mean 18.2 and 16.2 from all lake–dates, respectively) driven by high P availability. Phytoplankton from Square Lake were characterized by co-limitation in 2013, but indicated P limitation for all lake–date combinations in summer 2014. This was likely due to the doubling of TN availability in 2014 (mean 0.4 mg/L TN in 2013 vs. 0.8 in 2014). West Jefferson was characterized by P limitation in June and August 2014, but was co-limited in July. This could be due to the higher availability of DIN in June and August (304 and 70 ug/L DIN, respectively) compared to July (21 ug/L DIN). McCarrons Lake was P-limited in August 2013 and June 2014, as was Colby Lake in August 2014. These strong responses to P additions could be driven by the slightly lower availability of SRP we observed during those months compared to other months (~ 4 ug/L in McCarrons and 9 in Colby, Bratt, unpublished data).

TN/TP ratio could not have predicted these nuanced, single-limitation responses. For example, both Square Lake and West Jefferson frequently demonstrated P limitation and had a high TN/TP ratio of availability (mean 82.9 and 120 from all lake–dates, respectively); however, other lakes with similar, high TN/TP ratio of availability responded more to N + P enrichment than P enrichment alone. For example, Cedar Lake and Lake of the Isles both had very high TN/TP ratios (mean 107 and 121, from all lake–dates, respectively), but responded much more strongly to the N + P nutrient addition than to P addition. Overall, these results demonstrate that at most commonly occurring N/P ratios (that is, a wide range that is

captured by these lakes), co-limitation is the most relevant characterization of nutrient limitation in planktonic algal assemblages. Further, we acknowledge that while water chemistry can explain some of our responses to nutrient additions, there is much variation that could not be explained.

We found a small, but significant effect of TN across both years and TN/TP ratio on phytoplankton response to nutrient additions in summer 2013, suggesting that the availability of N and its relationship to P availability can influence nutrient limitation of these phytoplankton assemblages. N/P ratio negatively influenced N effect sizes and had a positive effect for P. However, overall TN/TP ratios were a weak predictor of nutrient limitation, which is surprising considering the wide range of TN/TP ratios included in this dataset. These findings are consistent with other recent studies that show a lack of support for using TN/TP ratios to predict nutrient limitation status of phytoplankton. For example, Keck and Lepori (2012) found that TN/TP ratio was the best predictor of N limitation of benthic primary production in streams; however, these predictions were uncertain except at extreme TN/TP ratios (< 1:1 and > 100:1, Keck and Lepori 2012). Additionally, Ptacnik and others (2010) evaluated chlorophyll-response-based threshold ratios for determining nutrient limitation and found it to be far below Redfield, with N limitation below 2:1 and P limitation above 5:1 (Ptacnik and others 2010). Taken together, these studies in combination with our results indicate co-limitation at moderate N/P, with increasing importance of P as N/P increases and N as it decreases toward extreme values that are not commonly observed.

Phytoplankton consistently responded most strongly to nutrient additions of N and P together, despite large variation in water sources across lakes. For example, Lake McCarrons receives most of its water from street surface runoff; some of it routed through engineered storm water retention ponds. Many of the lakes are in high density, residential watersheds and receive storm water. Crosby Lake is located in the floodplains of the Mississippi and Minnesota Rivers and is subject to seasonal inundation. Square Lake has no inlet or outlet and is groundwater fed and located in a primarily forested watershed. German and West Jefferson Lakes are located in a chain of lakes that are subject to overland flow from the surrounding cropland and inputs from groundwater. Our results are surprising considering this wide variation in water source, which is also often connected to the variation in N and P availabilities.

Surprisingly, we did not find a statistical effect of land use on N and P availability, which has been connected to the variation in surface water N and P in other studies (Easton and Petrovic 2008; Fraterrigo and Downing 2008; Taranu and others 2009; Hall and others 2016; Collins and others 2017; Halbach 2017; Read and others 2018). This could be due to the relatively diverse land use across our study lakes, compared to other studies that often focus solely on agriculture or urban land cover metrics. Additionally, this diversity of land use makes statistical analyses challenging since there is no metric that encompasses both urban and agriculture land use (note, we used the categorical variables and percent impervious cover outlined in Table 1). It is worth considering how the diversity of human land uses can be collapsed into one variable, considering they all have implications for water quality (as our dataset clearly demonstrates). This problem is beyond the purview of this study, but future work on land use effects on water quality should consider this limitation.

Taken together, our dataset characterizing the nutrient limitation status of phytoplankton across lakes subject to urban and agricultural land use supports the emerging conception of nutrient limitation that embraces the likelihood of co-limitation (see Kaspari and Powers 2016). Our dataset did show evidence of single-nutrient limitation at certain times and places. These responses can at least partially be explained by shifts in nutrient availability (for example, Colby and McCarrons Lakes likely responded to decreased availability of SRP). However, other lakes included in this study experienced similar shifts in nutrient availability and still responded most strongly to simultaneous additions of N and P. This suggests that these phytoplankton are able to somehow overcome the nutrient status that should be dictated by their environment. Thus, our data show at least partial support for the multiple limitation hypothesis (MLH, coined in Gleeson and Tilman 1992; Rastetter and Shaver 1992) which predicts co-limitation in primary producer assemblages. The MLH is founded on the idea that communities are not static. Species differ in their competitive abilities for different resources, and there is plasticity in an autotroph's ability to shift allocation of resources to acquire limiting elements. Additionally, community composition can shift in response to resource availability. The MLH asserts that over time, individuals and communities should adjust to fully exploit their resource supply, thus locally depleting multiple resources to a limiting level (Chapin 1980; Bloom 1985; Interlandi and Kilham 2001). This

framework considers that primary producer communities not only respond to nutrient supply, but can also mediate this supply. We assert that our results support this conception of nutrient limitation.

Negative Response to P Addition

Our data show negative responses to P additions for multiple lake–date combinations, especially during summer 2013. These negative response ratios (meaning the enriched phytoplankton grew less than the unenriched, control treatments) were surprisingly common, given that they are rarely discussed in the nutrient limitation literature. However, this response has been noted in a handful of studies. In a meta-analysis of factorial additions of N and P to primary producing communities by Harpole and others (2011), 15% of studies included showed some types of negative response. In over half of these cases, the reduction in biomass due to single addition of either N or P was reversed when N and P were added together, and these authors suggest that the reduction in biomass was perhaps due to stoichiometric constraints (Harpole and others 2011). This was often true for our data as well. However, it is unclear how stoichiometric constraints would lead to negative response ratios as opposed to a neutral response, and it is likely that other factors contributed to these negative response ratios.

Other biological mechanisms could explain the negative response to P addition we observed in our lakes. Schallenberg and Burns (2001) frequently observed a negative response to P additions in marine picoplankton communities. They hypothesized that this could be due to increased viral activity and subsequent lysing of algal cells with P additions (Schallenberg and Burns 2001). Fertilization can also increase herbivory rates by changing the amount and nutritional quality of vegetation; this has been documented in plant communities (see Gruner and others 2008) and algal communities (Sterner and others 2008). We filtered this water to remove macrozooplankton grazers that might mask phytoplankton response to nutrient addition, but microzooplankton cannot be removed this way and if present, these herbivores would have been included. Additionally, changes in the ratios of available nutrients could potentially drive changes in species composition and the production of the 'winning' species under fertilization, which may be very different from the original community. For example, fungi could respond strongly to the P addition, but this response would

not be detected by our chl-a response. The mechanisms that underlie the negative responses to P addition (and a few cases of N addition) in planktonic algal assemblages are unclear and warrant further investigation.

Predictors of Nutrient Limitation

We investigated potential factors that influenced the responses of the phytoplankton to our factorial additions of N and P. Mean TN/TP of the water column did significantly influence responses; mean TN/TP was positively correlated with response to P addition and negatively correlated with response to N addition, which makes sense considering that high TN/TP should promote P limitation (and thus a strong positive response to P additions) and vice versa. However, these effects, while significant, explained very little of the overall variation.

N_2-flux rates did not significantly influence responses to any of our nutrient additions (see mean rates for each lake in Table 2). We did find that heterocyte count was a weak predictor of responses to nutrient additions during summer 2013, but this effect was negative on RR_N and RR_P meaning that more heterocytes were associated with less of a response to these additions. Overall, the relationships between heterocyte count and response ratios were weak. These results were surprising since we expected that higher N_2-flux rates would increase P limitation and decrease N limitation. The ability of N_2-flux to moderate N demand to match P availability in aquatic systems is often invoked as an argument for management efforts to focus only on P (Schindler 1977, 2012; Schindler and others 2008, 2016). Our results indicate that N_2-flux rates did not contribute directly to nutrient limitation status in these heavily human-impacted lakes. This is not to say that N_2-flux does not contribute to nutrient availability, but that other factors influencing the availability of N in the water column are likely simultaneously at play, negating the direct effects of N_2-flux. We agree with others who have suggested that N_2 fixation cannot contribute enough N to make up for deficiencies and exert an effect on nutrient limitation (Scott and McCarthy 2010).

A key example of other factors that likely influence nutrient limitation status of phytoplankton is denitrification, which serves as an opposing influence on N availability. Denitrification reduces biologically reactive forms of N to dinitrogen gas, effectively reducing N availability both locally and also at the broader ecosystem scale. This removal of N has been argued to compensate for any fixation

of reactive N at the whole-lake level and thus motivates the argument for dual control of N and P to mitigate eutrophication (Conley and others 2009; United States Environmental Protection Agency 2015; Paerl and others 2018). Our results support this emerging paradigm of dual control of N and P in the ongoing debate on how to best manage eutrophication.

Epilimnetic lake metabolism was a significant predictor of phytoplankton response to N + P additions in 2014 (we did not measure metabolism in 2013). This suggests that among these lakes, eutrophic lakes were more likely to be co-limited. These results were unexpected given that co-limitation in oligotrophic systems has long been hypothesized to arise from the simultaneous scarcity of nutrients. For example, co-limitation has been well documented in the surface oceans (Saito and others 2008) and has also been observed in oligotrophic freshwater environments, such as the Great Lakes in North America (North and others 2007), Alaskan wetlands (Wyatt and others 2010), and subalpine streams (Marcarelli and Wurtsbaugh 2007). The co-limitation responses we documented in eutrophic systems could be due to increased diffusion rates in the same way that nutrient addition increases diffusion in oligotrophic systems. Perhaps in these hyper eutrophic systems, stoichiometric constraints limit the growth of algal cells until increases in the local availability of both N and P increase the diffusion rates of both of these elements into cells. Additionally, stoichiometric constraints could limit responses to single N and P additions in these systems meaning that the uptake and use of one resource (for example, P) is limited by the availability of another resource (for example, N). The biological mechanisms that underlie the co-limitation we documented in highly productive lakes are unclear and warrant further study.

Finally, we recognize that this approach to studying nutrient limitation (adding N and P over short time scales and monitoring responses) has many shortcomings. Our experiments assessed short-term nutrient limitation of phytoplankton and could not incorporate potentially important feedbacks that can occur at the lake scale such as establishment or increases in species like nitrogen fixers or denitrifying bacteria. This is a current topic of discussion in the literature (United States Environmental Protection Agency 2015; Cotner 2016; Schindler and others 2016, 2017), and we acknowledge there is much to be learned about how these small-scale experiments can be scaled up to inform management at the whole-lake scale.

However, our results do offer insight into how these phytoplankton currently function and how N availability and epilimnetic lake metabolism influence the nature of nutrient limitation. Interestingly, we found no relationship between TP availability and phytoplankton biomass (as determined by chl-a, Figure S-5A) across our bulk water sampling (n = 45) despite the large variation in TP concentrations. [We also found no relationship between TN and phytoplankton biomass (Figure S-5B)]. The prevalence of the positive relationship between chl-a and P is commonly cited for supporting the P paradigm (Schindler 1977; Lewis and Wurtsbaugh 2008; Sterner 2008). In combination with our nutrient limitation experiments, these results suggest that the P paradigm does not apply to these lakes that are subject to human land use. We argue these data are important to understanding how these communities function and will respond to reductions or increases in nutrient availability.

CONCLUSIONS

Overall, our results demonstrate that planktonic algal assemblages are most often co-limited by N and P. While we saw evidence for single-nutrient limitation in responses to additions of N and P (both in the few cases of N or P limitation and the decreased N response with higher TN/TP and vice versa for P), the significantly stronger response to the N + P additions across seasons and years suggests that these phytoplankton are much more likely to be co-limited than singly limited. This could be due to differences in nutrient limitation from species to species or across time, or due to shifts in allocation of resources within individual species such as using N to get P via enzymatic activity (sensu the multiple limitation hypothesis). Additionally, more productive phytoplankton assemblages are more likely to be co-limited. Taken together, our work provides evidence for the dual nutrient (control of both N and P) approach to mitigating eutrophication of freshwaters that are impacted by human land use.

Compliance with Ethical Standards

Conflict of interest   The authors declare that they have no conflict of interest.

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