

Nitrate, ammonium, and phosphorus drive seasonal nutrient limitation of chlorophytes, cyanobacteria, and diatoms in a hyper-eutrophic reservoir

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Abstract

Nitrogen (N) and phosphorus (P) inputs influence algal community structure and function. The rates and ratios of N and P supply, and different N forms (e.g., NO₃ and NH₄), from external loading and internal cycling can be highly seasonal. However, the interaction between seasonality in nutrient supply and algal nutrient limitation remains poorly understood. We examined seasonal variation in nutrient limitation and response to N form in a hyper-eutrophic reservoir that experiences elevated, but seasonal, nutrient inputs and ratios. External N and P loading is high in spring and declines in summer, when internal loading becomes more important, reducing loading N:P ratios. Watershed NO₃ dominates spring N supply, but internal NH₄ supply becomes important during summer. We quantified how phytoplankton groups (diatoms, chlorophytes, and cyanobacteria) are limited by N or P, and their N form preference (NH₄ vs. NO₃), with weekly experiments (May–October). Phytoplankton were P-limited in spring, transitioned to N limitation or colimitation (primary N) in summer, and returned to P limitation following fall turnover. Under N limitation (or colimitation), chlorophytes and cyanobacteria were more strongly stimulated by NH₄ whereas diatoms were often equally, or more strongly, stimulated by NO₃ addition. Cyanobacteria heterocyte development followed the onset of N-limiting conditions, with a several week lag time, but heterocyte production did not fully alleviate N-limitation. We show that phytoplankton groups vary seasonally in limiting nutrient and N form preference, suggesting that dual nutrient management strategies incorporating both N and P, and N form are needed to manage eutrophication.

The flow of nitrogen (N) and phosphorus (P) into aquatic ecosystems greatly impacts algal biomass and community structure, and excess inputs of these nutrients can cause harmful algal blooms (HABs; Schindler 1971; Brooks et al. 2016; Paerl et al. 2018; Wurtsbaugh et al. 2019). Ecologists and resource managers have had long-standing interest regarding what elements, or combination of elements, limits productivity and growth (Liebig 1855; Elser et al. 2007; Harpole et al. 2011; Marañón et al. 2018). Understanding phytoplankton nutrient limitation status provides insight on what watershed management strategies can be implemented to reduce or prevent eutrophication. Therefore, considerable effort has been devoted to assessing the factors governing nutrient limitation. These studies have produced a rich body of work spanning spatial and temporal scales from bottle or flask incubations, mesocosm studies, and whole lake manipulations (Carpenter et al. 1995; Spivak et al. 2010; Harpole

et al. 2011; Paerl et al. 2016). Some studies present evidence that phytoplankton growth is primarily P-limited, while others show primary N limitation or N and P colimitation (Sterner 2008; Harpole et al. 2011; Paerl et al. 2016). This variation in limitation status has given rise to a lively conversation on how best to mitigate eutrophication by controlling limiting nutrients (e.g., Schindler et al. 2008; Conley et al. 2009; Schelske 2009; Schindler and Hecky 2009). Studies also suggest that the form of nitrogen supplied, particularly NH₄ vs. NO₃, can affect algal communities, including the biomass and species composition of potentially harmful cyanobacteria (Gobler et al. 2016). Though it is well established that phytoplankton are influenced by the timing, quantity, quality, and relative availability of nutrients (Tilman et al. 1982; Berg et al. 2003; Donald et al. 2011), we know comparatively little about how seasonal variation mediates phytoplankton N vs. P limitation and response to different N forms.

The quantity, forms, and stoichiometric ratios of external and internal nutrient loading to aquatic ecosystems can vary seasonally, and this can drive temporal changes in nutrient limitation. The Redfield ratio (N:P = 16 mol:mol) is often thought of as the transition between N and P limitation, with values below 16 indicating N limitation and values above 16 indicating P limitation

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Additional Supporting Information may be found in the online version of this article.

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(Redfield 1958). However, experimental studies have found that the transition between N and P limitation can occur at higher N:P ratios (Maberly et al. 2002; Elser et al. 2009; Ptacnik et al. 2010). In many areas, stream discharge (and thus nutrient loading) reflects seasonal trends in snowmelt, precipitation, and evapotranspiration by watershed vegetation (Mulholland and Hill 1997; Frost et al. 2009). High springtime and low summertime discharge can alter the extent to which algae in downstream lakes and reservoirs are limited by N or P (Bukaveckas and Crain 2002; Royer et al. 2006; Vanni et al. 2006a). In temperate regions, high baseline stream discharge in spring often results in elevated nutrient loading to aquatic ecosystems and is associated with high N:P loading ratios that can promote P limitation, especially in agriculturally impacted systems (Mulholland and Hill 1997; Williamson et al. 2018). In contrast, lower stream discharge during the summer reduces nutrient loading and N:P ratios, promoting N limitation (Williamson et al. 2018). However, storm driven high discharge events, which occur more in spring than in summer, may reduce N:P for short periods of time because stream P increases disproportionately compared to N during storm events (Williamson et al. 2018; Kelly et al. 2019).

Discharge can also influence the relative abundance of different N forms. The dominant NO_3 pathway to streams is often via groundwater, and large precipitation events with substantial overland flow can shift the balance of N loading from NO_3 to other forms such as NH_4 or urea (Mulholland and Hill 1997). Additionally, patterns of uptake and denitrification within the watershed landscape may further reduce the relative abundance of NO_3 in streamflow, especially during the growing season when temperatures are warm, nutrient demand by terrestrial plants is high, and streamflow is low (Jacobs and Gilliam 1985; Chinnasamy and Hubbart 2015).

Internal nutrient loading often supplies a substantial fraction of nutrient demand, and also varies seasonally in both magnitude and stoichiometric ratio (Orihel et al. 2015; Bormans et al. 2016; Wu et al. 2017). Remineralization by animals and microbes can be an important source of limiting nutrients, especially during relatively warm and dry periods with low watershed discharge (Williamson et al. 2018). During these periods, consumer biomass and metabolic rates are often high, and internal nutrient loading from consumers can exceed external nutrient sources (Shostell and Bukaveckas 2004; Williamson et al. 2018). Though variable, consumers often supply limiting nutrients at relatively low N:P ratios when compared to discharge driven externally derived nutrient sources (Downing and McCauley 1992; Vanni and McIntyre 2016; Williamson et al. 2018). Other biogeochemical processes such as P recycling from anoxic sediments and N removal via denitrification may further serve to reinforce seasonally low N:P stoichiometry in mid-summer (Seitzinger et al. 2006; Bormans et al. 2016; Maranger et al. 2018). Internal recycling by consumers and denitrification also lower the $\text{NO}_3:\text{NH}_4$ ratio as NH_4 is the primary N form excreted by animals and microbes, and denitrification removes NO_3 . The

interaction between external nutrient loading and internal lake processes (e.g., denitrification, immobilization, recycling, burial in sediments, etc.) likely modulates phytoplankton assemblage through variation in nutrient concentrations, N:P ratios, and the relative abundance of different N forms (Tilman et al. 1982; Sommer 1984; Heil et al. 2007; Swarbrick et al. 2019).

Whether N or P is the limiting nutrient strongly affects the structure and function of algal communities. Nutrient limitation status (N vs. P limitation) can influence phytoplankton assemblage, the nutritional quality of algae for consumers, the concentration of algal toxins, and N-fixation rates (Sterner and Hessen 1994; Frangópulos et al. 2003; Vrede et al. 2009; Scott et al. 2013). Under P-limiting conditions, chlorophytes and diatoms generally occupy more of the phytoplankton assemblage in both marine and freshwater systems (Tilman et al. 1982; Heil et al. 2007; Swarbrick et al. 2019). Cyanobacteria often become the dominant algal group under N limiting conditions; this is partly because some species can fix atmospheric N_2 gas (Howarth et al. 1988b), though some non-N-fixing cyanobacteria taxa also become abundant at low N:P (Gobler et al. 2016). N fixation in conjunction with potentially higher temperatures for optimum growth and the ability to vertically migrate in stratified waters may provide a competitive advantage for cyanobacteria under the N-limiting and stratified conditions that often occur in summer (Paerl and Huisman 2009; Lürting et al. 2013, 2018). However, N fixation is energetically expensive, and therefore occurs only during periods when reactive N is scarce and N:P ratios are low (Howarth et al. 1988a,b). Additionally, many cyanobacteria (e.g., *Anabaena*, *Microcystis*, *Cylindrospermopsis* spp.) can produce toxins that are responsible for HABs (Carmichael 1992; Anderson et al. 2002). Therefore, increased cyanobacteria abundance could result in more toxin producing species and higher HAB frequency and severity (Paerl et al. 2018).

The relative abundance of different N forms (e.g., NH_4 , NO_3) under N-limiting conditions likely influences the competitive efficiency of different algal groups, and thus influences the phytoplankton assemblage. It is generally acknowledged that phytoplankton prefer NH_4 relative to NO_3 , due to higher NH_4 absorption affinity, faster NH_4 absorption rates, and the high energetic cost of reducing NO_3 to NH_4 (Blomqvist et al. 1994; Glibert et al. 2016). However, diatom preference for NO_3 has been demonstrated in marine and estuarine systems (Lomas and Glibert 2000; Glibert et al. 2016). For instance, Berg et al. (2003) found that in estuaries with excess available NH_4 , cyanobacteria and chlorophytes dominated the phytoplankton assemblage, whereas excess NO_3 promoted diatom dominance. However, less is known about algal preference for NO_3 vs. NH_4 under N-limiting conditions in freshwater systems (Donald et al. 2011, 2013).

In this study, we explored the seasonality of nutrient limitation patterns and N form preference of phytoplankton groups in a hyper-eutrophic midwestern U.S.A. reservoir. Specifically, we

hypothesized that (1) we would observe a seasonal transition from P to N limitation for all phytoplankton groups, in response to lower N:P supply ratios in summer; (2) that cyanobacteria would dominate the phytoplankton assemblage during periods of N limitation, as some species within this group are capable of N-fixation, (3) that heterocyte abundance would increase during periods of N limitation, and (4) cyanobacteria and chlorophytes would show a stronger relative preference for NH_4 while diatoms would show a stronger preference for NO_3 .

Methods

Study site

We tracked the seasonal dynamics of phytoplankton nutrient limitation status in Acton Lake, a hypertrophic reservoir in southwestern OH, U.S.A. (39.582 N, -84.757 W). Acton has a surface area of 2.4 km² and a mean depth of 3.9 m (Vanni et al. 2006b). The lake has a relatively large watershed of 257 km² (watershed area: lake area = 111), 70–80% of which is in row crop agriculture, primarily corn and soybeans (Vanni et al. 2001, 2006b; Renwick et al. 2018). Therefore, the lake receives large nutrient inputs from the terrestrial landscape (Williamson et al. 2018; Kelly et al. 2019). This promotes high phytoplankton biomass, with an average summer (July–September) chlorophyll, total phosphorous, and total nitrogen concentrations of 63 $\mu\text{g L}^{-1}$, 94.6 $\mu\text{g P L}^{-1}$, and 3.22 mg N L⁻¹, respectively, from 1994 to 2014 (Kelly et al. 2018). Phytoplankton consist of a mixed assemblage of chlorophytes, cyanobacteria, diatoms, and cryptophytes with frequent dominance by cyanobacteria in summer (Dickman et al. 2006; Hayes et al. 2015). In years when P limits phytoplankton in summer, the cyanobacteria assemblage is dominated by taxa that do not fix N, but in years with N limitation, heterocystous N fixers are common (Hayes et al. 2015). In general, external loading to Acton has a high N:P ratio; however, over the past decade, N concentrations and loads, as well as the load N:P, from tributary streams have been declining (Renwick et al. 2018; Kelly et al. 2019). Changes in agricultural practices over the past two decades, especially a pronounced shift to conservation tillage, may be the cause for this decline in N (Renwick et al. 2018; Kelly et al. 2019). As N:P ratios have declined, phytoplankton communities have become increasingly N-limited in recent years (Vanni et al. unpubl.), whereas historically they were usually P-limited except in summers of drought years when N limitation occurred (Hayes et al. 2015). We measured the limitation status, and N form preferences, of phytoplankton across one growing season in relation to seasonal changes in nutrient availability.

Lake physical and chemical parameters

Lake sampling

Lake sampling began on 21 May 2018 and continued weekly until 08 October 2018 for a total of 21 weeks. We sampled two locations: the Inflow site, which is shallow, thermally unstratified,

and located near the entrance points of most tributaries; and the Outflow site, which is 8 m deep, near the dam, and thermally stratified during summer. At each site, two replicate vertically integrated water samples were collected from the euphotic zone with an integrated tube sampler. Euphotic zone depth was calculated as depth stratum with $\geq 1\%$ of surface photosynthetically available radiation (PAR). For the Outflow site, vertical light profiles were measured using a LI-COR LI-1400 PAR sensor (LI-COR Biosciences, Lincoln, NE). We sampled the entirety of the water column at the Inflow site (≤ 1.5 m) irrespective of light conditions, as this shallow area remains unstratified. Temperature (°C) in the euphotic zone was also determined at each site using a YSI Pro-ODO probe (Yellow Springs Instruments, Yellow Springs, OH). Water samples were held in a translucent bottle in a dark cooler at approximately lake surface temperature until sample processing began later that day.

Lake discharge and nutrient loading

We estimated watershed tributary discharge and watershed nutrient loading to Acton Lake during the study period. A full treatment of these methods can be found in Vanni et al. (2001), Renwick et al. (2018), and Williamson et al. (2018). We do not yet have stream nutrient data for 2018; however, we estimated loading rates of NO_3 , NH_4 , and soluble reactive phosphorus (SRP) using regressions of daily stream discharge vs. daily loads obtained for the years 2015–2017 (using only May–October dates) and 2018 daily stream discharge (see Supporting Information for details). We further estimated N and P excretion by gizzard shad (*Dorosoma cepedianum*) a detritivorous fish that comprises > 90% of fish biomass in Acton Lake. See Williamson et al. (2018) for a full description of the methods used to estimate fish biomass and N and P excretion rates, and Supporting Information for a summary of our approach.

Nutrient analysis

Dissolved nutrients ($\text{NO}_3\text{-N}$, $\text{NH}_4\text{-N}$, and SRP) were quantified on integrated samples after they were filtered (nominal pore size, 1.0 μm ; Type A/E glass fiber filter, Pall Corp., New York, NY), acidified to pH < 2 with H_2SO_4 , and stored at 4°C until analysis. Dissolved nutrient concentrations were quantified with a Lachat QC 8000 autoanalyzer (Lachat Instruments, Loveland, CO).

Phytoplankton biomass, composition, and heterocyte production

To measure phytoplankton biomass, chlorophyll *a* (Chl *a*) concentration was determined by filtering 25 mL of the integrated sample water onto 1.0 μm glass filters (Type A/E glass, Pall Corp., New York, NY); Chl *a* was then extracted with 95% ethanol for 24 h, and analyzed with a Turner TD-500 fluorometer (Turner Designs, San Jose, CA).

Concentrations ($\mu\text{g chlorophyll L}^{-1}$) of four phytoplankton groups (chlorophytes, cyanobacteria, diatoms, and cryptophytes) from the integrated samples were determined using a spectrofluoroprobe (FluoroProbe, bbe moldaenke, Schwentinental, Germany; Beutler et al. 2002, Gregor et al. 2005). Each algal group has varying

photosystem II accessory pigments that fluoresce differently allowing the fluoroprobe to accurately detect the concentrations of each algal group (Gregor et al. 2005). Concentrations for two replicates per sample were averaged.

To characterize the abundance of potential N-fixing cyanobacteria, we quantified heterocyte abundance weekly at both sampling sites. Integrated water samples were preserved in

Lugol's solution and stored in the dark (Lund et al. 1958). We settled 2 mL of sample for 48 h in Utermoehl chambers. Heterocytes were counted at $\times 400$ magnification with an inverted microscope across five equally spaced transects amounting to 1% of chamber area with an inverted microscope (Lund et al. 1958). When heterocytes were observed, we also identified the taxa carrying the heterocytes.

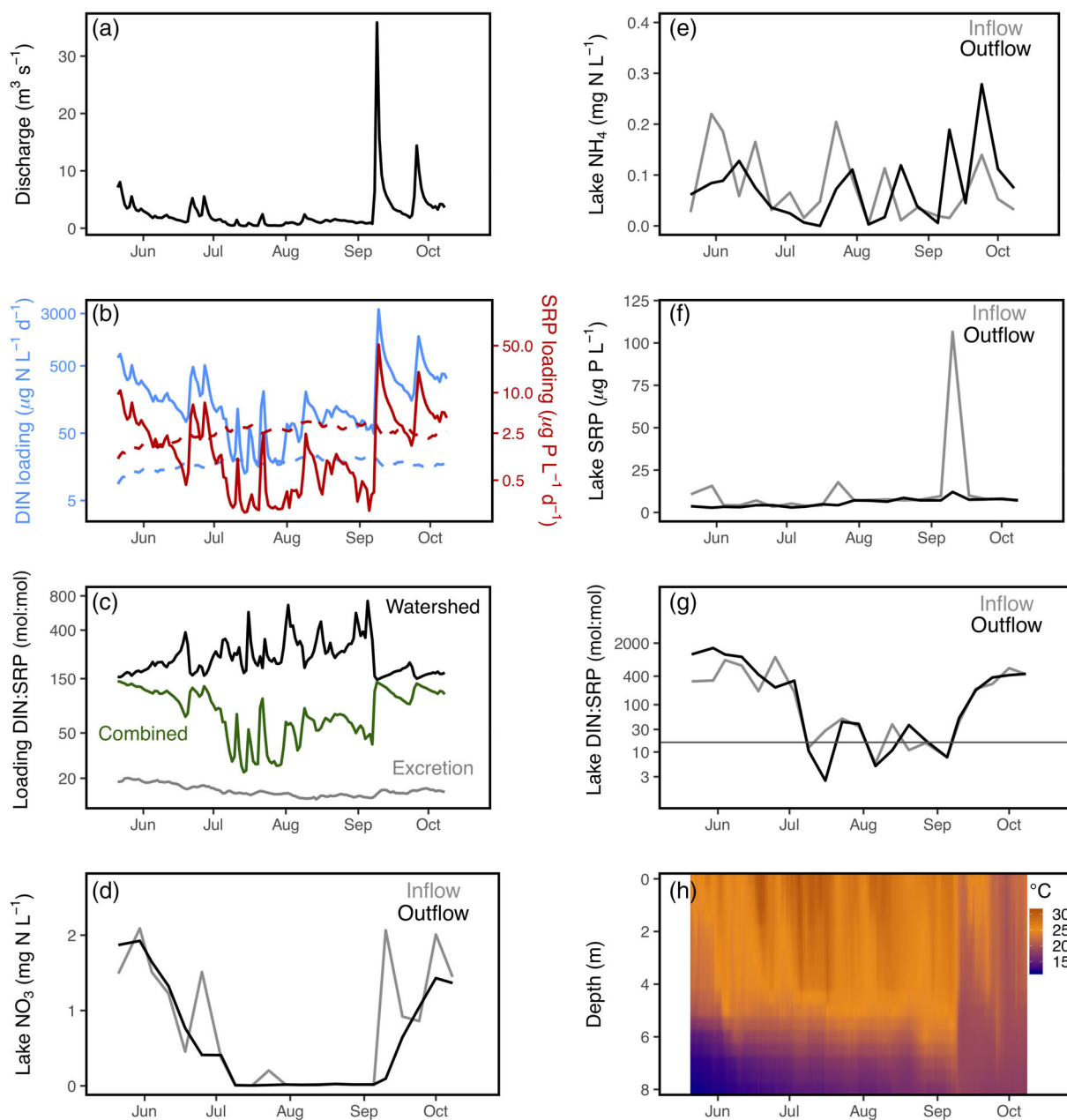


Fig. 1. Watershed discharge from tributary streams (a). DIN (summed $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$) loading from watershed streams (solid line) and gizzard shad (*D. cepedianum*) excretion (dashed line; b). SRP from watershed streams (solid line) shad excretion (dashed line; b). Loading DIN:SRP ratios (mol:mol) for watershed loading, shad excretion, and combined watershed loading and shad excretion (c). Lake $\text{NO}_3\text{-N}$ concentrations (d), $\text{NH}_4\text{-N}$ (e), and SRP concentrations (f) in the Inflow and Outflow sampling sites in Acton Lake during the study period. The lake DIN:SRP ratio (mol:mol) during the study period with the horizontal line at the Redfield ratio of 16:1 N:P (note the log scale; g). Heatmap of lake temperature at the Outflow sampling location only (h). The Inflow is too shallow (~ 1.5 m) to thermally stratify.

Nutrient limitation and N form preference

Phytoplankton nutrient limitation status was determined with standard nutrient addition bioassay experiments (Harpole et al. 2011; Hayes et al. 2015). Water samples from both sites were screened through a 63 μm filter to remove macrozooplankton (Vanni et al. 2006a). We transferred 175 mL of screened sample water into 250 mL Erlenmeyer flasks and exposed phytoplankton to one of four treatments (in duplicate): no nutrients added (control), +N (700 $\mu\text{g N L}^{-1}$ as NH_4NO_3), +P (80 $\mu\text{g P L}^{-1}$ as NaH_2PO_4), and both +N and +P together. To quantify N form preference (NH_4 vs. NO_3) of phytoplankton groups as well as the total phytoplankton assemblage, we added an additional four nutrient treatments beginning on 09 July, after N limitation had been detected: NO_3 (700 $\mu\text{g N L}^{-1}$ as NaNO_3), NH_4 (700 $\mu\text{g N L}^{-1}$ as NH_4Cl), $\text{NO}_3 + \text{P}$, and $\text{NH}_4 + \text{P}$ (as in the +P treatments above, 80 $\mu\text{g P L}^{-1}$ was added as NaH_2PO_4). Flasks were incubated for 48 h at the average euphotic zone temperature of the lake (range 11–31; mean 25°C) and a light intensity of $\sim 200 \mu\text{mol PAR m}^{-2} \text{ s}^{-1}$ (Hayes et al. 2015). Initial and postincubation phytoplankton biomass (Chl *a*) and phytoplankton assemblage were analyzed with spectrofluorometry, as described above.

Data analysis

Severity of nutrient limitation and N form response

We quantified phytoplankton nutrient limitation and response to different N forms as the growth response (ΔR) during the 48 h incubation period. Growth response was calculated for each taxonomic group as:

$$\Delta R = \log_{10}(\text{avg chl treatment} / \text{avg chl control}) / t$$

where avg chl is the mean chlorophyll concentration for that group, treatment is N, P, N and P, NO_3 , NH_4 , NO_3 and P, or NH_4 and P, and t is the incubation period (2 d). Cryptophytes were generally too scarce to evaluate due to both low initial concentrations in lake water, and a propensity to decline over the incubation period. Consequently, we did not quantify their nutrient limitation status.

Statistical analysis

For each experiment, we assessed whether phytoplankton growth responded to nutrient additions using a one-way ANOVA on the mean Chl *a* concentrations at the end of the incubation period. A Tukey HSD post hoc test was subsequently performed to assess the relative significance of the individual nutrient treatments and assign limitation status (N, P, colimited, or no limitation detected) and NO_3 vs. NH_4 preference. The ANOVA models were fit with the “aov” function, and the Tukey HSD test was fit with the “TukeyHSD” function in the R package “stats” (R Core Team 2018). We examined the significance of apparent threshold responses in heterocyte production as a function of nutrient concentrations and ratios, using segmented regression with the “segmented” function in the R package “segmented” (Vito and Muggeo 2003, 2008; R Core Team 2018). We used linear

regression to assess the relationship between heterocyte abundance and the relative strength of N vs. P limitation. Regressions were fit with the “lm” function in the R package “stats” (R Core Team 2018). Heterocyte abundance was \log_e transformed when appropriate, to meet model assumptions.

Results

Lake physical, biological, and chemical parameters

Stream discharge into the lake was low for much of the study period, with the exception of two large storm events in September (Fig. 1a). Mean daily discharge (SD) for the study period was $3.73 \pm 3.70 \text{ m}^3 \text{ s}^{-1}$, increasing to maximum daily mean discharge of 35.8 and 14.4 $\text{m}^3 \text{ s}^{-1}$ during the two fall storm events. During these two events, maximum daily mean discharge was in the 99th

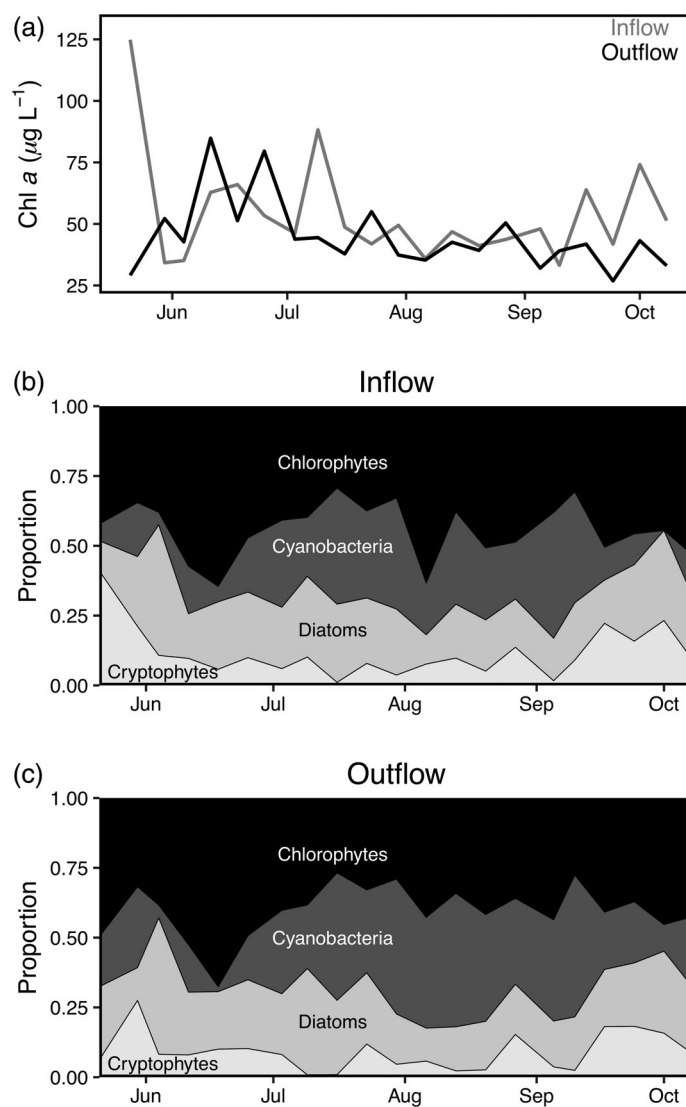


Fig. 2. Chl *a* concentrations in the Inflow and Outflow sampling sites in Acton Lake during the study period (a). Phytoplankton assemblage in the Inflow (b) and Outflow (c) sampling sites during the study period.

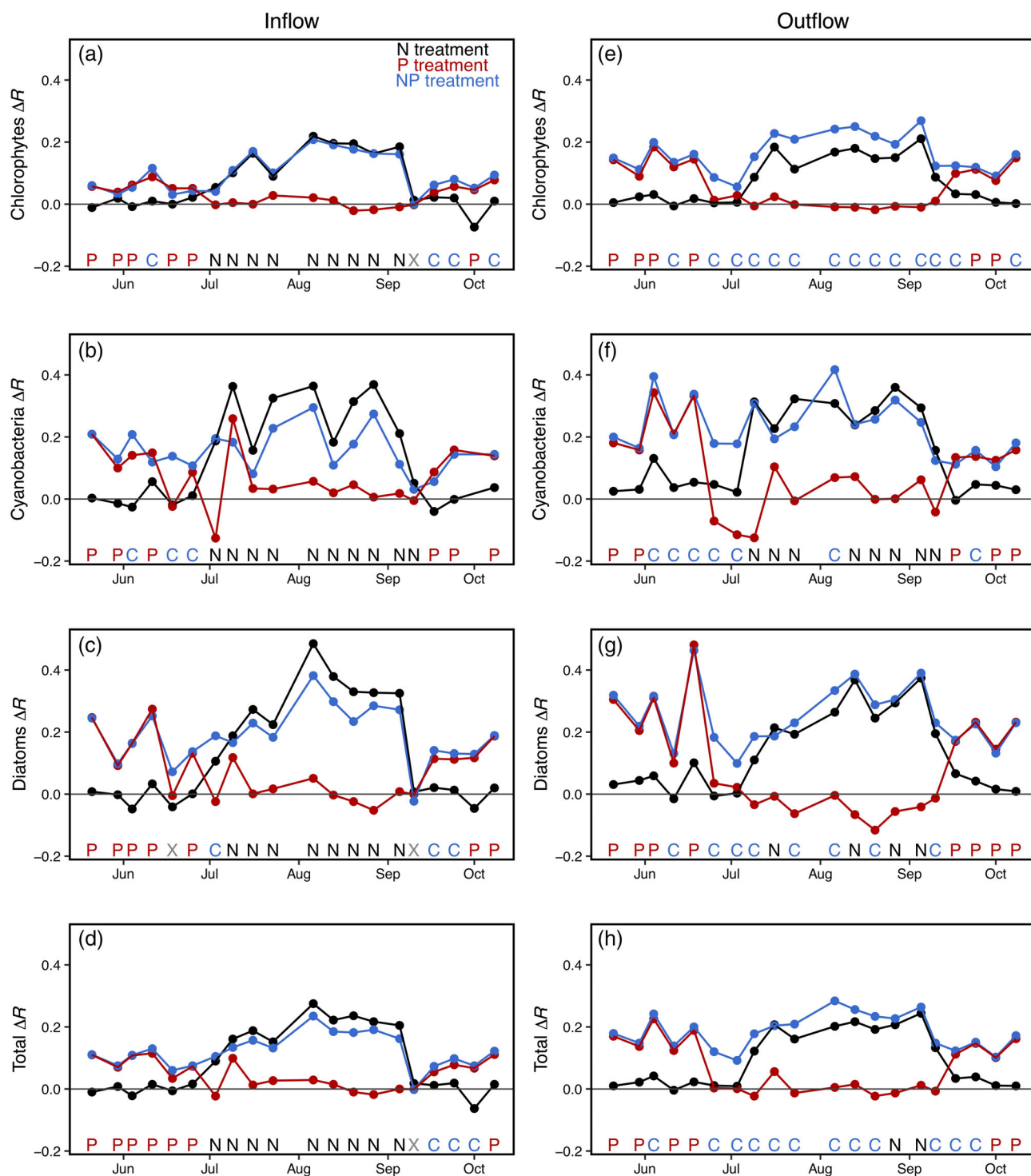


Fig. 3. Nitrogen (N; as NH_4NO_3), phosphorus (P; as NaH_2PO_4) limitation, and N and P colimitation for the total phytoplankton assemblage, chlorophytes, cyanobacteria, and diatoms in the Inflow sampling site (a–d) and the Outflow sampling site (e–h). Limitation values are expressed as ΔR , the \log_{10} ratio of the treatment growth relative to the control per day. Values at or below zero (solid horizontal line) indicate no or negative growth in response to nutrient additions. Colored letters indicate significant limitation response for each date (one-way ANOVA; $\alpha = 0.05$) with C indicating significant colimitation and X indicating no significant growth response to nutrient additions.

and 96th percentile for all days within the period 1994–2018 (Williamson et al. 2018). Watershed dissolved inorganic nitrogen (DIN) and SRP loading over the study period largely mirrored

trends in discharge (Fig. 1b). Mean DIN loading was $236 \pm 358 \mu\text{g N L}^{-1} \text{d}^{-1}$ and mean SRP loading was $3.0 \pm 5.4 \mu\text{g P L}^{-1} \text{d}^{-1}$ (Fig. 1b). Excretion by gizzard shad was relatively constant over

the study period, with a mean DIN excretion of $17 \pm 2.8 \mu\text{g N L}^{-1} \text{ d}^{-1}$, and a mean SRP excretion of $2.5 \pm 0.6 \mu\text{g P L}^{-1} \text{ d}^{-1}$ (Fig. 1b). Watershed loading DIN:SRP remained high and variable for the study period ($242 \pm 101 \text{ mol}$), whereas the DIN:SRP of shad excretion was low and relatively less variable ($15.6 \pm 1.8 \text{ mol}$; Fig. 1c). However, the DIN:SRP ratio of combined watershed loading and nutrient limitation was high in spring, declined during the summer, and returned high again following the fall storm events (Fig. 1c).

NO_3 concentrations at the Inflow site were relatively high ($\sim 2 \text{ mg N L}^{-1}$) in June and steadily declined to $< 0.2 \text{ mg N L}^{-1}$ during summer, likely due to a combination of reduced loading from the watershed, phytoplankton uptake, and denitrification; concentrations returned to fairly high levels following the large storm events in September (Fig. 1d). NO_3 dynamics were similar at the Outflow, except that concentration response to storm discharge was more muted, likely because of uptake, denitrification, and dilutive mixing (Fig. 1d). NH_4 concentrations remained consistently low relative to NO_3 and variable (range $0\text{--}0.29$, mean 0.07 mg N L^{-1}) throughout the study period without a clear seasonal trend (Fig. 1e), likely due to high uptake demand and because concentrations in tributary streams are much lower than NO_3 concentrations (Renwick et al. 2018). Similarly, SRP concentrations were low (near the detection limit; mean concentration $10.5 \mu\text{g SRP L}^{-1}$) much of the study period, except immediately following the first large September storm event (Fig. 1f). During this large storm event, we observed a sharp increase in SRP at the Inflow ($106.5 \mu\text{g SRP L}^{-1}$) but not at the Outflow ($12.1 \mu\text{g SRP L}^{-1}$), likely due to high phytoplankton uptake demand (Fig. 1f).

Trends in the DIN:SRP ratio in the lake were largely reflective of changing DIN:SRP loading ratios from combined watershed nutrient loading and shad excretion over the study period (Fig. 1g). Lake DIN:SRP was high at the beginning and end of the sampling period, but during July and August it was low, frequently below Redfield (Fig. 1g). Thermal stratification at the Outflow site further influenced nutrient availability, as the Outflow site remained stratified for much of the study period. The September storm events completely mixed the water column at the Outflow site, bringing hypolimnetic NH_4 into the epilimnion (Fig. 1h). The Outflow site restratified briefly the following week before completely mixing, and remained mixed for the rest of the study period due to fall turnover in the lake (Fig. 1h). The two September spikes in NH_4 concentration at the Outflow site correspond to these mixing events (Fig. 1e,h). Although hypolimnetic SRP concentrations were higher than those in the epilimnion, we do not see a spike in epilimnetic SRP, probably because of rapid uptake by phytoplankton.

Phytoplankton biomass and assemblage

Total Chl *a* concentrations and trends in phytoplankton composition were similar at the Inflow and Outflow (Fig. 2a–c). Chlorophytes were the dominant phytoplankton group for

much of the study period, representing $44\% \pm 9\%$ and $40\% \pm 9\%$ of total biomass at the Inflow and Outflow sites, respectively (Fig. 2b,c). Cyanobacteria represented $22\% \pm 7\%$ and $22\% \pm 8\%$, and diatoms represented $21\% \pm 13\%$ and $27\% \pm 14\%$, of biomass at the Inflow and Outflow sites, respectively (Fig. 2b,c). At the beginning of the study, cyanobacteria accounted for a relatively small fraction of the assemblage, which increased substantially in late June–early July (Fig. 2b,c), corresponding with the onset of N limitation (see below). Cryptophytes were the least common group, representing only $11\% \pm 9\%$ and $8\% \pm 6\%$ of phytoplankton biomass at the Inflow and Outflow sites, respectively (Fig. 2b,c).

Nutrient (N vs. P) limitation

Phytoplankton at both Inflow and Outflow sites were primarily P-limited in spring, but by summer had transitioned to either N limitation, or colimitation with primary N limitation. At both sites, phytoplankton transitioned back to P limitation (or co-limitation with primary P limitation) following the fall storm events and lake turnover. Within this general seasonal

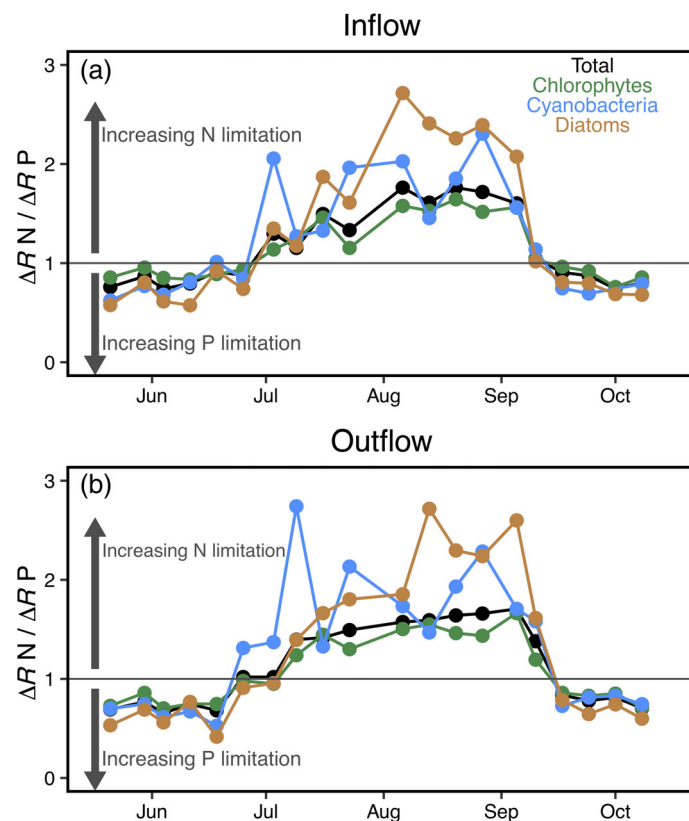


Fig. 4. The ratio of the nitrogen (N) and phosphorus (P) ΔR limitation response (\log_{10} ratio of nutrient treatment growth relative to the control per day) for the total phytoplankton assemblage, chlorophytes, cyanobacteria, and diatoms in the Inflow (a) and Outflow (b) sampling sites in Acton Lake. Values above 1 (solid horizontal line) indicate relatively a stronger response to N additions, and values below 1 indicate a relatively stronger response to P additions.

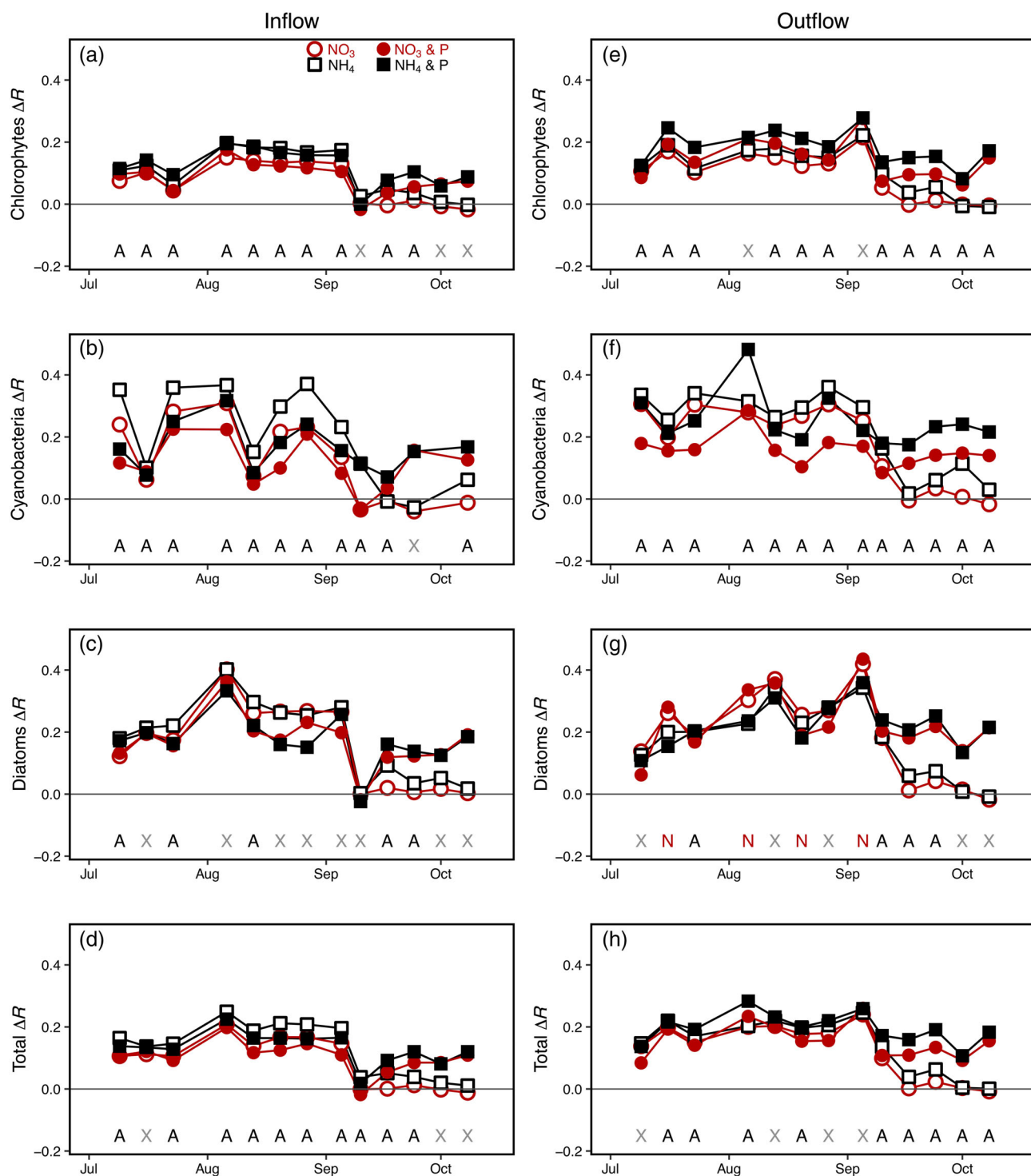


Fig. 5. Nitrate (NO_3 ; as NaNO_3), ammonium (NH_4 ; as NH_4Cl), NO_3 and phosphorus (P; as NaH_2PO_4), limitation for the total phytoplankton assemblage, chlorophytes, cyanobacteria, and diatoms in the inflow sampling site (a–d) and the outflow sampling site (e–h). Limitation values are expressed at ΔR , the log₁₀ ratio of the treatment growth relative to the control per day. Values at or below 0 (solid horizontal line) indicate no or negative growth in response to nutrient additions. Letters indicate significant preference for N form in the presence and absence of P (one-way ANOVA; $\alpha = 0.05$); N indicates NO_3 preference, A indicates NH_4 preference, and X indicates no significant N form preference.

trend, we found site-specific differences in the timing of transitions and the relative strength of colimitation vs. single element limitation among the different phytoplankton taxonomic groups. At the Inflow site, chlorophytes were P-limited from late May through June and transitioned to N limitation in early July (Fig. 3a). This transition occurred abruptly over 1 week, from P limitation in the last week of June to N limitation in the first week of July (Fig. 3a). Chlorophytes at the Inflow site returned to P limitation in mid-September following nutrient pulses delivered by the September storm events and fall turnover. Inflow cyanobacteria and diatoms generally followed a similar temporal transition in limitation status (Fig. 3b,c). However, cyanobacteria experienced more periods of colimitation during the springtime transition from P to N limitation. Interestingly, during the period of N limitation (July and August), cyanobacteria and diatom growth response in +N treatment was consistently higher than in the +N and P treatment (Fig. 3b,c). Limitation patterns for the total phytoplankton assemblage in the Inflow site were largely reflective of those for chlorophytes (Fig. 3d). This is not surprising

because chlorophytes largely dominated the phytoplankton assemblage the majority of time.

Much like the Inflow site, the Outflow phytoplankton assemblage was dominated by chlorophytes for most of this study; therefore, trends in the total assemblage and chlorophyte responses to nutrient treatments were similar. During July and August, Outflow chlorophytes experienced persistent colimitation with N as the primary limiting nutrient; growth in the +N and P treatment was much higher than in the +N treatment (Fig. 3e). In contrast, Outflow cyanobacteria and diatoms experienced a few weeks of colimitation during the transition from P to N limitation, then were mostly N-limited with intermittent colimitation (Fig. 3f,g). However, the colimitation response (growth in the +N vs. +N and P treatments) was weaker than that of chlorophytes (Fig. 3f,g). We also observed seasonal transitions in limitation status in the total assemblage at the Outflow; however, the shifts in nutrient limitation occurred a week later than the Inflow (Fig. 3h). Furthermore, the total phytoplankton assemblage (total Chl *a*) remained colimited at the Outflow for much of the summer (Fig. 3h).

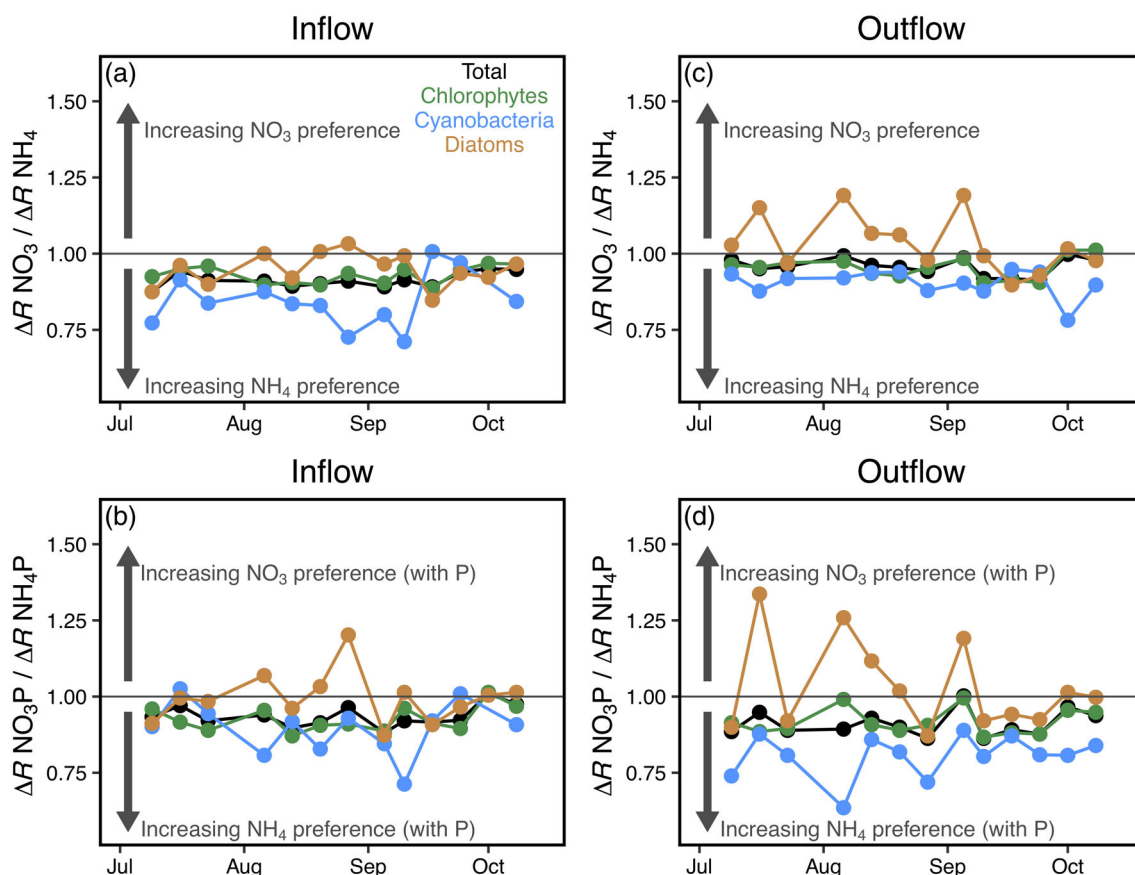


Fig. 6. The ratio of the nitrate (NO₃) to ammonium (NH₄) ΔR limitation response (\log_{10} ratio of nutrient treatment growth relative to the control per day) with and without phosphorus (P) for the total phytoplankton assemblage, chlorophytes, cyanobacteria, and diatoms in the Inflow (a, b) and Outflow (c, d) sampling sites in Acton Lake. Values above 1 (solid horizontal line) indicate relatively a stronger preference for NO₃ additions, and values below 1 indicate a relatively stronger preference for NH₄ additions.

During the transition from P to N limitation, we observed periods when P addition inhibited cyanobacterial growth (two experiments at the Inflow site and three at the Outflow site; Fig. 3b,f). This generally occurred during the period before heterocytes increased, but this inhibitory effect largely disappeared afterwards (see below). At both Inflow and Outflow sites, we found that chlorophytes generally had the lowest growth response to either N or P addition under both P- and N-limiting conditions (Fig. 4a,b). Diatoms were generally the most severely limited group in terms of both P (June) and N (July) limitation. Cyanobacteria were usually intermediate between diatoms and chlorophytes in terms of the severity of nutrient limitation, except during the transition to N limitation, when they were the most strongly N-limited group, especially at the Outflow (Fig. 4a,b).

NO₃ vs. NH₄ preference

During the N limitation period, we added NO₃ and NH₄ separately, with and without P to assess taxonomic group preference for these two N forms. As with overall N and P limitation, we found site-specific differences in the timing and relative preference for N form among the different taxonomic groups. During July and August at the Inflow, chlorophytes

and cyanobacteria responded more strongly to NH₄ than to NO₃ (Fig. 5a,b). Following the early fall transition back to P limitation, we found that the chlorophytes and cyanobacteria responded most strongly to NH₄ and P addition; however, over the last 2 weeks of the study, N addition did not affect the growth response, regardless of N form (Fig. 5a,b). In contrast, during the N limitation period (July and August), diatoms at the Inflow did not show consistent preference for NH₄; rather, most weeks they showed no, or equal, preference for either N form (Fig. 5c). Additionally, following the transition back to P limitation, Inflow diatom growth responded similarly in the +NH₄ and P and +NO₃ and P treatments (Fig. 5c). As to be expected, the response of the total phytoplankton assemblage to N form was similar to that of chlorophytes (Fig. 5d).

At the Outflow during July and August, chlorophytes generally showed a preference for NH₄, with a greater response when P was added, from July to October (Fig. 5e). Outflow cyanobacteria showed a preference for NH₄ in every experiment; after the switch to P limitation during the fall storm events and lake turnover, this preference was manifested primarily when P was also added (Fig. 5f). In contrast, diatoms at the Outflow preferred NH₄ on four dates, NO₃ on four dates,

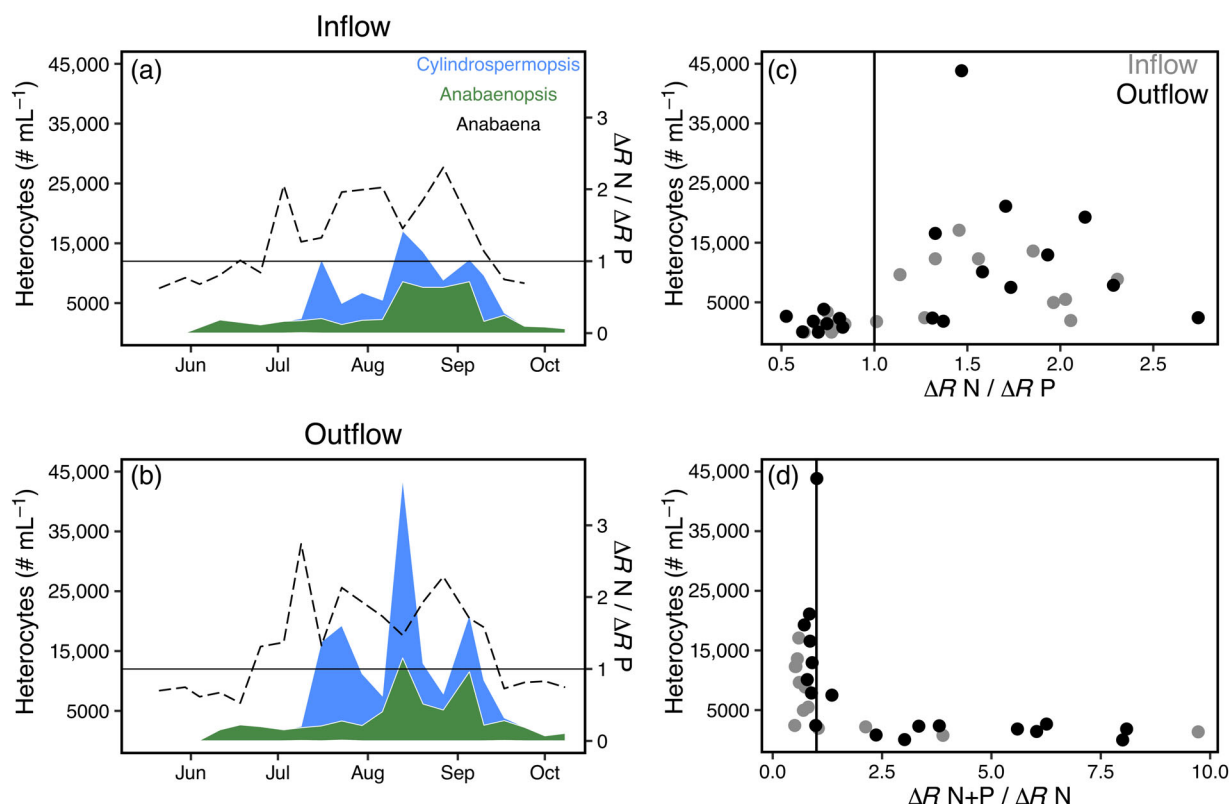


Fig. 7. Heterocyte abundance by genera and the ratio of nitrogen (N) to phosphorus (P) ΔR limitation response (dashed lines; log₁₀ ratio of nutrient treatment growth relative to the control per day) of cyanobacteria in the Inflow (a) and Outflow (b) sampling sites. Anabaena heterocytes, while present, are too scarce to show on these figures. The relationship between the $\Delta R N / \Delta R P$ limitation response of cyanobacteria and heterocyte abundance (c). The relationship between the ratio of N and P ΔR limitation response to the N ΔR limitation response and heterocyte abundance (d).

and neither N form on five dates; all instances of NO_3 preference occurred during the period of N limitation (Fig. 5g). The Outflow total phytoplankton assemblage shifted between NH_4 preference and no preference for N form; in some weeks, the response to N was stronger when P was also added (Fig. 5h). During the P limitation period following the fall storm events, the total phytoplankton assemblage at the Outflow showed a strong preference for NH_4 over NO_3 and this preference was greater with P addition (Fig. 5e).

Overall, the three phytoplankton groups showed distinct N form preferences (Fig. 6). Cyanobacteria showed the strongest preference for NH_4 among the groups, and this preference was enhanced by P addition, especially at the Outflow site. In contrast, diatoms often showed a preference for NO_3 over NH_4 , especially at the Outflow site. Chlorophytes consistently preferred NH_4 , although their preference for this form over NO_3 was not nearly as strong as that of cyanobacteria. Trends in N form preference for the total phytoplankton assemblage followed those of chlorophytes (Fig. 6).

Heterocyte dynamics

We found three genera of heterocyte-forming cyanobacteria at both sites: *Cylindrospermopsis*, *Anabaenopsis*, and *Anabaena* (Fig. 7a,b). During springtime P limitation (May

through late June), heterocytes were scarce at both sites, ranging from 0 to 2662 heterocytes mL^{-1} (mean 1224 ± 1097 ; Fig. 7a,b). Heterocyte abundance increased greatly in mid-July, 2–3 weeks after cyanobacteria became N-limited (Fig. 7a,b). Following this, heterocyte abundance was generally lower and less variable at the Inflow (mean 8577 ± 5091) than at the Outflow (mean $13,153 \pm 12,376$) during the period of strong N limitation (03 July through 05 September; Fig. 7a,b). Interestingly, we observed out-of-phase oscillations in the relative severity of N and P limitation in cyanobacteria and heterocyte abundance. Peaks in heterocyte abundance occurred at 3–4 week intervals and generally coincided with declines in the ratio of relative N vs. P limitation ($\Delta\text{RN}/\Delta\text{RP}$; Fig. 7a,b). Heterocyte abundance was positively and significantly correlated with relative N vs. P limitation ($R^2 = 0.18$; $p = 0.015$; Fig. 7c). We found that heterocyte abundance was highest under strict N-limiting conditions, and declined markedly under N and P colimitation (Fig. 7d).

Predicting nutrient limitation with nutrient concentrations and ratios

We found significant threshold responses in the relationship between N and P limitation vs. the DIN:SRP ratio, although we found no difference in threshold response among

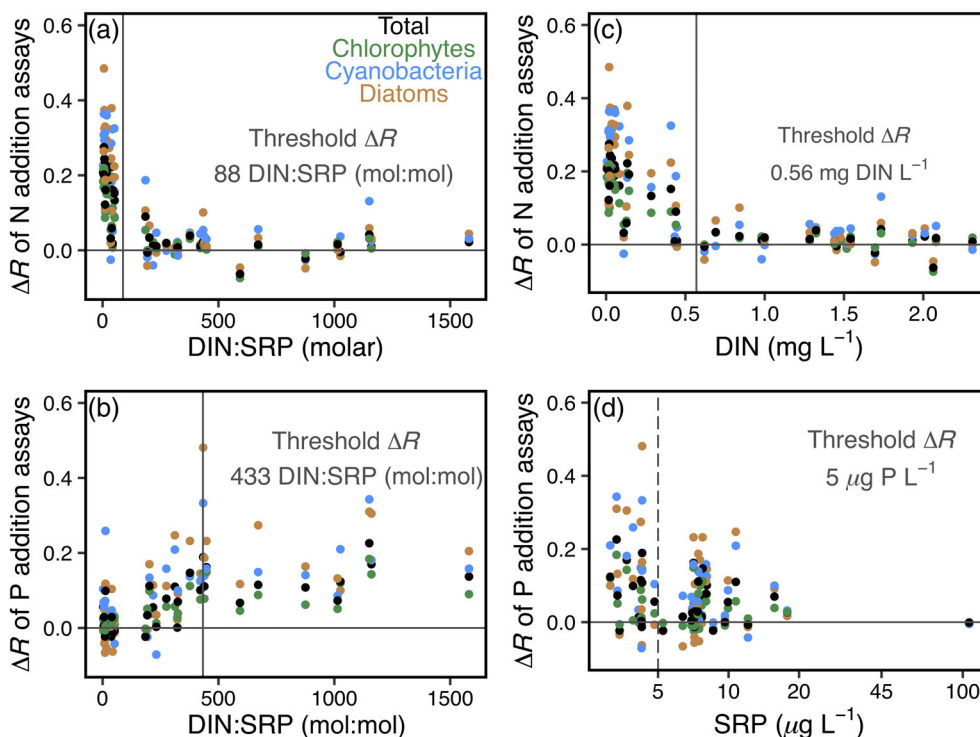


Fig. 8. Threshold relationship between DIN (the sum of $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$) and the nitrogen (N) addition ΔR limitation response (\log_{10} ratio of nutrient treatment growth relative to the control per day) for the total phytoplankton assemblage, chlorophytes, cyanobacteria, and diatoms (a). Threshold relationship between the DIN to SRP ratio and the nitrogen (N) addition ΔR limitation response (b). Threshold relationship between SRP concentration and the P addition ΔR limitation response (c), and the threshold relationship between the DIN:SRP ratio and the P addition ΔR limitation response (d). Note: Solid vertical lines indicate statistical significance ($\alpha < 0.05$), and dashed vertical lines indicate marginal significance ($\alpha > 0.05$ and < 0.10).

the phytoplankton groups (Fig. 8a,b). The threshold ΔR in the N and P addition assays occurred at 88 and 433 DIN:SRP (mol: mol), respectively (Fig. 8a,b). We found a significant threshold relationship between N limitation and DIN concentrations ($0.56 \text{ mg DIN L}^{-1}$; Fig. 8c). We found a marginally significant relationship between SRP concentration and the severity of P limitation ($5 \text{ } \mu\text{g P L}^{-1}$; $p = 0.065$; Fig. 8d).

Discussion

We demonstrate pronounced seasonality in N vs. P limitation and N form preference of phytoplankton in a hypertrophic reservoir. Consistent with our hypotheses, we observed seasonal patterns in limitation status that were broadly reflective of nutrient concentrations and ratios in the lake, as well as the N:P of nutrient supply from the watershed and excretion by fish. However, there were important site- and taxon-specific differences in limitation patterns. In support of our hypotheses, we found that cyanobacteria represented a proportionally larger fraction of the phytoplankton assemblage under N-limiting conditions. Heterocyte abundance increased under N-limiting conditions, suggesting that upregulation of N fixation compensated partially, but not fully, for N deficiency. We found that chlorophytes and cyanobacteria had a higher growth response to NH_4 relative to NO_3 . In contrast, diatoms preference for NO_3 vs. NH_4 was mediated by site and the primary limiting nutrient (N vs. P). This is consistent with our hypothesis that diatoms would be the only group to potentially prefer NO_3 relative to NH_4 .

Phytoplankton response to nutrient limitation

Nutrient limitation status often governs phytoplankton assemblage (Berg et al. 2003; Vrede et al. 2009; Cunha et al. 2017). Indeed, we observed seasonal trends in phytoplankton assemblage that are at least partially due to changing concentrations and ratios of limiting nutrients. We found a strong threshold in N limitation response to ambient dissolved N:P ratios that was relatively high but close to the range reported by others (Maberly et al. 2002; Elser et al. 2009; Ptacnik et al. 2010; Kolzau et al. 2014). We also found a threshold response in N limitation to DIN concentration consistent with Chaffin et al. (2014). We found a weak, but suggestive, threshold response in P limitation to SRP concentration and a significant threshold response to N:P ratios. Furthermore, the thresholds were generally quite similar among phytoplankton groups. Chlorophytes and diatoms occupied more of the assemblage under P-limiting conditions, as has been found in both marine and freshwater systems (Tilman et al. 1982; Heil et al. 2007; Swarbrick et al. 2019). Interestingly, there were site-specific differences in limitation patterns among these two groups. Inflow chlorophytes transitioned between P and N limitation while Outflow chlorophytes transitioned from P to colimitation for much of the study period. Inflow diatoms similarly transitioned from P to strict N limitation with only

brief periods of colimitation, while Outflow diatoms alternated between N and colimitation during the summer. Inflow diatoms under N limitation experienced a relatively lower growth response in treatments that received conutrient (N and P) additions, which suggests an inhibitory effect of P addition that was mediated by site.

The mechanisms responsible for differences in nutrient limitation between sites are not certain, but we can suggest some possibilities. Nutrient excretion by fish is an important flux in Acton Lake that supplies nutrients at a low N:P (Vanni et al. 2006a; Williamson et al. 2018), and excretion N:P (as well as N:P from both excretion plus the watershed) is correlated with patterns of N vs. P limitation (Fig. 1c). Excretion by fish is probably more important at the Inflow site, where gizzard shad, the dominant fish species can be more abundant (Supporting Information Fig. S1) and the water column is shallow. High nutrient excretion by fish, which occurs at a relatively low N:P, could promote N limitation at the Inflow site, compared to the Outflow site where excretion rates are likely to be lower and colimitation was more common. In addition, diffusive fluxes from the hypolimnion to the epilimnion at the Outflow are also likely to be supplied at relatively high N:P (Nowlin et al. 2005; Vanni et al. 2011), which may counter other internal cycling that provides nutrients at a lower N:P and promote colimitation. Differences in water depth between the two sites may also drive dissimilarity in limitation patterns. Water depth influences water residence time, P loss due to uptake or sedimentation, sediment P recycling under oxic/anoxic conditions, and N loss due to uptake and denitrification (Nowlin et al. 2005). Phytoplankton assemblages were similar at the two sites; we do not know if there were differences between sites at a finer taxonomic resolution (e.g., genus or species), but data from previous years suggest that species composition is similar at the two sites (M. J. Vanni unpubl. data). Thus, it seems unlikely that the different limitation patterns were caused by differences in composition.

Cyanobacteria generally performed well under N-limiting conditions, increasing after the onset of N limitation as diatoms decreased (Keating 1978; Tilman et al. 1982; Heil et al. 2007). This response in diatom abundance could be attributed to nutrient competition or potential allelopathic compounds and toxins released by cyanobacteria (Keating 1978; Leflaive and Ten-Hage 2007). Additionally, we found that heterocyte abundance mirrored (with a 1–2 week lag time) the onset of N limitation, and that heterocyte abundance increased markedly, but only under strict N-limited conditions. This suggests that N fixation is not upregulated during transitional or colimited periods. During the lag time between the onset of N limitation and heterocyte abundance, we observed occasional negative cyanobacteria growth responses in the +P treatments, suggesting that P addition inhibited cyanobacteria growth through a direct physiological effect or via competition from other phytoplankton groups or bacteria (Moisander et al. 2003). However, after heterocyte abundance increased, cyanobacteria

showed a weak positive growth response to P additions, suggesting that N fixation increased P demand. Interestingly, heterocytes disappeared rapidly without an apparent lag time following the fall transition back to P-limitation. The heterocyte counts we report are high, but within the range of literature reported values (e.g., Higgins et al. 2018).

During the N-limited period, heterocyte abundance oscillated at 3–4 week intervals, largely due to spikes in the number of *Cylindrospermopsis* heterocytes. These oscillations coincide with decreases in the severity of cyanobacteria N limitation. This suggests that cyanobacteria, in particular *Cylindrospermopsis*, increase N-fixation rates to partially compensate for N scarcity under N-limiting conditions, but this is not enough to completely alleviate N limitation of the cyanobacteria assemblage as a whole. Under N-limiting conditions, the addition of both N and P elicited a lower growth response than the N addition alone, although the effect was only observed at the Inflow. This is somewhat consistent with findings from marine systems which suggest that species within the genera *Aphanizomenon*, *Nodularia*, and *Anabaena* experience lower growth rates in N and P treatments relative to growth rates in N only treatments (Moisander et al. 2003); whether this can be attributed to direct P inhibition or via competition from other species remains unclear.

NO₃ vs. NH₄ preference

The relative supply of NO₃ and NH₄ may also regulate phytoplankton assemblage based on N form preference and relative handling efficiencies among different taxonomic groups (Donald et al. 2011; Glibert et al. 2016; Cunha et al. 2017). We found that cyanobacteria and chlorophytes showed a clear preference for NH₄ (Domingues et al. 2011; Gardner et al. 2017), whereas diatoms showed an equal preference for the two forms and occasionally a greater preference for NO₃ (Donald et al. 2011; Glibert et al. 2016); this NO₃ preference was more pronounced at the Outflow. What drives these site-specific variations remains unclear, however there are distinct environmental differences between the two sites. As previously mentioned, the Inflow is shallow, unstratified, and close to inlet tributaries, thus concentrations of suspended sediment are usually much higher here than at the Outflow, due to both sediment inputs from streams and resuspended sediment (Knoll et al. 2003). Some nutrients associated with suspended sediments may be available to phytoplankton, and it is possible that this would affect nutrient limitation status and response to N form (Franklin et al. 2018).

Irrespective of site level differences, diatoms clearly showed equal or higher growth with NO₃ addition relative to NH₄ addition. Although there is much evidence from marine ecosystems that diatoms prefer NO₃, there has been little previous evidence for freshwater diatoms (Donald et al. 2011, 2013). In general, phytoplankton should preferentially take up NH₄, due to the energetic cost of reducing NO₃ to NH₄ (Syrett and Lefty 1976; Glibert et al. 2016). However, it has been suggested that diatoms may have enhanced NO₃ handling and assimilatory efficiency

relative to other groups (Lomas and Glibert 1999). Diatoms may have dissimilatory pathways that directly use NO₃ as a reductant sink to rebalance cellular redox conditions, although evidence for these mechanisms in freshwater algae remain scarce (Lomas and Glibert 1999; McCarthy et al. 2009; Glibert et al. 2016). Furthermore, at a pH above 7 (euphotic zone pH in Acton is almost always well above 7 and often above 8), ammonium hydroxide may form, which can be toxic to diatoms and may promote preferential NO₃ uptake (Patrick 1977). As such, diatoms may maintain a higher cellular NO₃:NH₄ ratio, and may be favored in high pH environments with high NO₃:NH₄ ratios of available nutrients (McCarthy et al. 2009).

Management implications

Acton phytoplankton have historically been primarily P-limited from spring through fall (Vanni et al. 2006a,b), except in drought years when they became N-limited in summer (Hayes et al. 2015). However, in recent years, Acton phytoplankton have become more frequently N-limited, even in years with average precipitation and runoff (M. J. Vanni unpubl. data). These changes seem to be driven by a decrease in stream NO₃ loading and an increase in stream SRP loading over the past decade, perhaps due to long-term effects of increased conservation tillage (Renwick et al. 2018; Kelly et al. 2019), as well as an increase in gizzard shad, which excrete at low N:P (Williamson et al. 2018). Thus, seasonal variation in N vs. P supply, as well as the relative supply of the two N forms, from a combination of external and internal sources has become more pronounced. If N limiting conditions in lakes become more recurrent and prolonged, we may see negative effects expand to higher trophic levels. Cyanobacteria can be a poor-quality food resource for grazers due to low nutritional value or mucilaginous sheathing that poses high digestion resistance (DeMott and Tessier 2002; but see Perga et al. 2013). Thus, increased cyanobacterial dominance could decrease negatively impact grazers and ultimately zooplanktivorous fish (Hansson et al. 2007; Zi et al. 2018). Additionally, N-limiting conditions can favor the growth of toxin producing cyanobacteria which can lead to the development of HABs. High concentrations of algal toxins can reduce drinking water quality and negatively impact the recreational and commercial values of lakes (Paerl et al. 2016; Wolf et al. 2017).

Our findings suggest that a dual nutrient management strategy that focuses on reduction of both P and N will likely be required to successfully reduce eutrophication in many eutrophied lakes (Paerl et al. 2016; Wurtsbaugh et al. 2019). Management strategies that focus on single element reduction (N or P) will likely be ineffective given the seasonal patterns in limitation status that we observed (Sterner 2008; Paerl et al. 2018; Jankowiak et al. 2019). Currently, it is recognized that reducing P can successfully combat eutrophication, especially in low-P (or high N:P) lakes that remain consistently P-limited (Bormans et al. 2016; Schindler et al. 2016). However, much debate has surrounded the potential for eutrophication control through N reduction (Schindler et al. 2008; Conley et al. 2009; Schelske 2009; Schindler and Hecky

2009). The argument has been made that N limitation is a transitory phenomenon, and that long-term upregulation of N fixation will ultimately compensate for N deficiency (Schindler et al. 2008; Higgins et al. 2018; Wurtsbaugh et al. 2019). However, we found that N limitation persisted for a sustained period even during peak heterocyte abundance. This suggests that improved N control in lakes with relatively high P availability (or low N:P ratios) would be essential to reducing eutrophication.

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Conflict of Interest

None declared.

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