Received: 3 December 2020 | Revised: 15 September 2021 | Accepted: 23 September 2021

DOI: 10.1111/fwb.13836

ORIGINAL ARTICLE

Freshwater Biology WILEY

Factors regulating lake periphyton biomass and nutrient limitation status across a large trophic gradient

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Abstract

1. Because of the historical focus of limnology on pelagic processes, the factors controlling lake periphyton growth and nutrient limitation are understudied compared to the phytoplankton.
2. We deployed nutrient-diffusing substrata at 28 sites spanning a wide trophic status gradient in Lakes Superior and Michigan to assess periphyton biomass accrual on control substrata and the response of periphyton to single and combined phosphorus (P) and nitrogen (N) additions.
3. Periphyton growth was unimodally related to a composite metric of site trophic status, with highest biomass at mesotrophic sites and lower growth at oligotrophic and highly eutrophic sites. Contrary to expectations, P limitation was rare. Instead, several lines of evidence pointed to primary N or N + P co-limitation of periphyton. Limitation extent was negatively related to site trophic status, with stronger nutrient limitation at oligotrophic sites.
4. Our results support the hypothesis that phytoplankton and periphyton biomass respond differently to nutrient enrichment and suggest that different nutrients may limit pelagic and benthic primary production, even in the same system.
5. Our findings also support the use of periphyton as an early warning indicator of nutrient pollution and help explain why large, oligotrophic lakes may be especially susceptible to localised benthic algal blooms.

KEYWORDS
benthic algae, eutrophication, Laurentian Great Lakes, nitrogen, phosphorus

1 | INTRODUCTION

Most illuminated surfaces in lakes are colonised by periphyton—a mixture of autotrophic and heterotrophic micro- and macroorganisms, extracellular exudates, and detritus. Periphyton can account for a large fraction of total ecosystem primary production and is an important energy source for lake food webs (Hecky & Hesslein, 1995; Sierszen et al., 2014; Vander Zanden & Vadeboncoeur, 2002). Periphyton can also be responsible for significant water quality degradation. Benthic algal blooms are source of concern and the target of costly management programmes in many lakes. Benthic algal blooms can produce toxins, harbour pathogens, and have negative impacts on littoral biodiversity and food web structure (Belykh et al., 2016; Chun et al., 2013; Dodds & Gudder, 1992; Gladyshev & Gubelit, 2019). Detached benthic algae can clog water intakes and fishing nets and accumulate on shorelines, interfering with recreation and reducing shoreline property values (Dodds & Gudder, 1992; Higgins et al., 2005). There is concern that benthic algal blooms are becoming more common, even in lakes that are considered oligotrophic based on pelagic indicators of trophic status (Gladyshev & Gubelit, 2019; Timoshkin et al., 2016; Vadeboncoeur et al., 2021).

The productivity of aquatic autotrophs is constrained mainly by the availability of light and essential nutrients. Phosphorus (P) and nitrogen (N) are the primary limiting nutrients in most marine and freshwater ecosystems (Elser et al., 2007; Harpole et al., 2011). Thousands of studies have examined the factors controlling the abundance of lake phytoplankton, the relationship between nutrient supply and phytoplankton productivity, and the relative importance of P and N in limiting pelagic primary production. Studies of phytoplankton consistently show a positive monotonic relationship between nutrient concentrations and phytoplankton biomass, with higher phytoplankton densities with increasing lake trophy (Quinlan et al., 2020; Schindler, 1978). Nutrient limitation studies show that P is the nutrient ultimately limiting phytoplankton biomass in most lakes (Guildford et al., 2000; Schindler et al., 2016). However, N limitation and N + P co-limitation (when additions of both elements are needed to stimulate production) can occur in some lakes or be important at certain periods of the year (Elser et al., 2007; Guildford et al., 2000; North et al., 2007; Paerl et al., 2016).

Fewer studies have examined controls on the biomass and nutrient limitation status of lake periphyton. Early studies failed to identify clear nutrient–biomass relationships for lake periphyton (Cattaneo, 1987). Over the last 3 decades, several authors have suggested that periphyton biomass is unimodally related to lake trophic status, peaking at intermediate nutrient concentrations due to competition with phytoplankton over light and nutrients (Fork et al., 2020; Hansson, 1992; Liboriussen & Jeppesen, 2006; Vadeboncoeur et al., 2002). While this hypothesis has been supported by several studies, the ubiquity of the unimodal periphyton biomass–trophic status relationship remains less well established than the monotonic relationship between nutrient availability and phytoplankton biomass. In addition, relatively few studies examined the role of N and P in limiting lake periphyton biomass, often reaching contrasting conclusions, even from the same data (Elser et al., 2007; Maberly et al., 2002). Thus, it is presently not well known whether periphyton in specific lakes is limited by the same nutrients as phytoplankton and how periphyton nutrient limitation varies with trophic status and other environmental conditions.

Improved knowledge of factors controlling the growth of lake periphyton is important for managing benthic algal blooms and understanding the role of periphyton in lake food webs. The goal of this study was to investigate patterns of summer (July–August) periphyton biomass and nutrient limitation across a large trophic status gradient. We deployed nutrient-diffusing substrata (NDS) at 28 sites in the upper Laurentian Great Lakes to address three specific objectives: (1) identify environmental controls on periphyton biomass; (2) determine the form and extent of periphyton nutrient limitation at multiple study sites; (3) investigate the role of abiotic environmental factors (nutrient concentrations and light) in determining periphyton limitation status. We hypothesised that: (1) periphyton biomass will have a unimodal relationship with trophic status and will be highest at mesotrophic sites where both light and nutrient availability are relatively high; (2) phosphorus will be the primary limiting nutrient for periphyton, due to the importance of P in limiting Great Lakes phytoplankton and the high water column N:P ratios at many of our study locations; and (3) nutrient limitation extent will be negatively related to trophic status, and will be lowest at eutrophic sites, where nutrient supply is high and light levels low.

2 | METHODS

2.1 | Nutrient-diffusing substrata construction and deployment

Nutrient-diffusing substrata design (Figure S1) was similar to Ozersky et al. (2018). Four rows of aluminium netting (gutter guards) were attached to concrete blocks (50 × 50 cm) with screws and washers. Individual NDS cups were attached to the metal netting using zip ties and electrical tape. To construct individual NDS cups, 30-ml polypropylene jars were filled with 2% (by weight) microbiology-grade agar (Millipore Sigma). Control NDS cups contained just 2% agar, while N-, P-, and N + P-enriched NDS cups contained agar with 0.5 M NH_4Cl, KH_2PO_4, or both, respectively (Tank et al., 2006). NDS cups were capped with a flat, *c.* 1.6-mm (1/16″) thick, 38-mm diameter, 10-μm pore-size porous, polyethylene disc (GenPore). Prior to use, discs were soaked in 10% HCl overnight, and then rinsed thoroughly with ultrapure water. The rigid and porous polyethylene discs allowed for diffusion of nutrients out of the agar and provided a surface for periphyton colonisation. A 35-mm diameter hole was cut into each of the jar lids and the discs were secured underneath. Treatments were done in replicates of five, for a total of 20 individual NDS cups at each site.

The NDS experiments were deployed at approximately 1.5-m depths at 33 study sites along the shorelines of Lake Superior and Lake Michigan (Figure 1). Sites were chosen to span a large geographic range and trophic status gradient and based on ease of shoreline access. The natural substrate at all sites was either rocky (bedrock, boulder, cobble, or pebble) or sandy. Macrophytes and invasive dreissenid mussels (which can stimulate periphyton growth through nutrient excretion; Ozersky et al., 2013) were absent at all but one site (GB6) due to the high energy environment at the shallow deployment depths of our experiments. Experiments were deployed between 11 and 31 July 2017 and retrieved between 8 and 28 August 2017. All experiments were in the lake for between 28 and 29 days. Of the 33 deployed experiments, 28 were recovered, with five experiments lost (presumably to vandalism).

Several environmental variables were measured at each site during the deployment and retrieval of the NDS experiments. Temperature was recorded at approximately 0.75-m depth using an EXO2 multiparameter sonde (YSI). Samples for nutrient analysis (total phosphorus, TP; total nitrogen, TN; nitrate, NO_3^-) were taken just below the surface. Water for NO_3^- analysis was syringe-filtered in the field through a 25-mm diameter, 0.2-μm pore-size cellulose nitrate filter. Duplicates for TP were taken from one bottle on the deployment trip, while duplicates were taken from two separate bottles on the retrieval trip. Only one sample for TN and NO_3^- was taken on the deployment trip. All water samples were frozen until

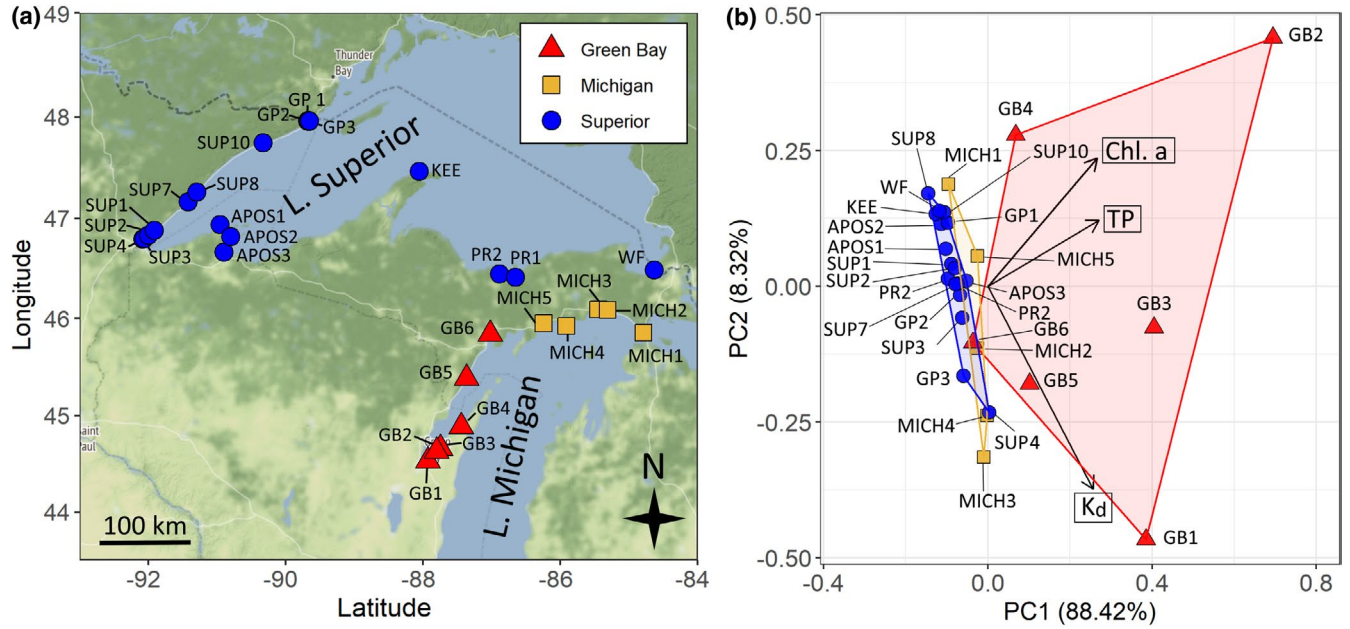

FIGURE 1 Map of study sites (a) and principal component (PC) analysis on standardised site environmental characteristics (b). Map was created using the *ggmap* package for R (Kahle & Wickham, 2013). Symbol shapes and colours are the same for both panels

analysis. Duplicate samples of phytoplankton biomass (measured as chlorophyll *a* [Chl-*a*]) were obtained only during the experiment retrieval by filtering 30–60 ml of water (depending on the turbidity of the sample) through a 25-mm diameter, 0.2-μm pore-size cellulose nitrate filter. The filter was frozen until analysis. The light environment was characterised at experiment deployment and retrieval using a photosynthetically active radiation LI-192 cosine sensor (LI-COR Biosciences). Photosynthetically active radiation was measured at the surface, 0.5, and 1.0 m depths. Light extinction (K_d) was calculated for 0.5 and 1.0 m depths using the equation: $K_d = (\ln(I_o) - \ln(I_d)) \times d^{-1}$, where I_o = Light intensity at surface, I_d = Light intensity at depth, d = measurement depth in m. The K_d from 0.5 and 1.0 m depths was then averaged. The average K_d from the deployment and retrieval measurements was used in data analysis.

2.2 | Sample analysis

Periphyton biomass on NDS was estimated as Chl-*a* and as ash-free dry weight (AFDW). Upon retrieval of NDS experiments, plastic discs were carefully removed from cups and cut into two equal pieces along the centre of each disc. One half was used for periphyton Chl-*a* analysis and the other for AFDW determination. Discs were wrapped in aluminium foil and frozen until analysis. To determine Chl-*a* amounts on NDS, half discs were first freeze-dried for 24 hr in the dark (Hagerthey et al., 2006) and then extracted in 10 ml of 90% acetone for 24 hr in the dark. Chl-*a* was then measured using a UV-1800 Shimadzu spectrophotometer (Shimadzu) and a 10-mm quartz cuvette following the protocol of Steinman et al. (2006). Phaeophytin-corrected Chl-*a* concentrations were expressed as μg Chl-*a*/cm^2. AFDW was determined by first scraping the periphyton on the surface of NDS discs into pre-weighed aluminium cups using a razor blade. All visible macroinvertebrates were removed from the sample to avoid confounding effects on periphyton biomass estimates. The scraped samples were dried at 60°C for 24 hr and weighed. They were then combusted at 450°C for 4 hr and weighed again. AFDW (mg/cm^2) was calculated by subtracting the initial dry weight from the combusted dry weight then dividing by the area of the disc substrate. In a small number of samples (<2%), sample AFDW was measured as 0 following combustion; we replaced those values with 0.01 mg/cm^2 (corresponding to approximately half of the detection limit of our balance) to avoid zeros in statistical analyses.

Water column Chl-*a* was measured using a fluorometer. Filters were extracted in 10 ml of 90% acetone for 24 hr in the dark. Non-pheophytin corrected Chl-*a* (μg/L) was then determined using a Turner Designs 10-AU fluorometer with an excitation wavelength of 436 nm and emission of 680 nm. TP and NO_3^- analyses were performed on an AQ 400 nutrient auto analyser (Seal Analytical) using standard EPA methods 365.1 and 353.2, respectively. TN was measured using a Shimadzu TOC-VSH auto analyser (Shimadzu) using ASTM method D8083. The averages of NDS deployment and retrieval values for TP, NO_3^-, TN, and Chl-*a* were used in subsequent analyses.

2.3 | Statistical analysis

Relationships between site environmental variables (water temperature, K_d, TP, TN, TN:TP, NO_3^-, water column Chl-*a*) were examined using a scatterplot matrix and Spearman non-parametric correlation tests. Because of strong correlations between most

environmental variables, we used principal component analysis (PCA) to summarise three key indictors of trophic status: TP, K_d, and water column Chl-*a* concentrations. Variables were standardised (centred and scaled to calculate *z*-scores) prior to PCA and site PC1 scores were used as a summary indicator of site trophic status. The variables included in the PCA correspond to the variables that comprise Carlson's Trophic State Index (Carlson, 1977), linking our PC1 scores to a definition of trophic state familiar to most limnologists.

Spearman correlation tests were used to determine the relationship between periphyton biomass measured as Chl-*a* and as AFDW across all treatments as well as for individual treatments (control, P, N, and N + P). Further analyses of the relationships between site trophic state, periphyton biomass and limitation status were carried out separately for Chl-*a*- and AFDW-based measurements of biomass.

The relationship between site trophic status and periphyton biomass on control substrata was assessed using linear regression analysis. Biomass was regressed against standardised PC1 scores (as a summary of site trophic status) as well as against indicators of site chemical and physical conditions. Biomass values from the five control replicates were averaged for each site prior to analysis and regressions were performed on these averages. Each relationship was modelled as either a simple linear fit or as a second order polynomial relationship. We then used model comparison based on analysis of variance and Akaike information criterion (Crawley, 2013) to determine whether simple linear or second order polynomial relationships were most appropriate in each case. Normality and equal variance were assessed using quantile–quantile and residual plots and transformation of the response and predictor variables were used to satisfy assumptions.

We used log response ratios (LRRs) to assess periphyton nutrient limitation status and response to nutrient enrichment. LRRs were calculated for Chl-*a* and for AFDW as the natural log of the ratio between Chl-*a* (or AFDW) on a nutrient enriched treatment and average Chl-*a* (or AFDW) on control substrata. A response ratio of zero indicates no biomass response to the addition of nutrients relative to controls, a negative value indicates a decrease in biomass on nutrient enriched substrata, and a positive value indicates an increase. An LRR = 1 represents approximately a tripling of biomass relative to control whereas LRR = −1 corresponds to an approximately 3-fold decrease. We used 95% confidence interval (CI) overlaps of site-averaged LRRs to determine the nutrient limitation status across all study sites for periphyton Chl-*a* and AFDW. Overlaps of 95% CI for treatment LRRs with 0 were interpreted as no response to enrichment relative to control and 95% CI overlaps between pairs of treatments were interpreted as lack of pairwise differences between the treatments. We assessed whether Chl-*a* and AFDW LRRs for different nutrient amendments were spatially autocorrelated using Moran's I (R package *ape*; Paradis & Schliep, 2019). The Chl-*a* LRRs on P-enriched (Moran's $I = 0.20$, $p = 0.02$) and N + P-enriched (Moran's $I = 0.22$, $p = 0.01$) substrata showed significant spatial autocorrelation, suggesting comparisons of 95% CIs for Chl-*a* LRRs may be somewhat biased due to violation of independence.

We also determined the nutrient limitation status at each study site using 95% CI overlaps of Chl-*a* and AFDW LRRs. Interpretations of LRR results to determine limitation status followed Harpole et al. (2011):

1. Simultaneous colimitation: LRR of N or P treatments alone not greater than 0, but LRR on N + P treatments greater than 0.
2. Independent colimitation: LRR of both N and P treatments greater than 0. LRR of N + P treatment greater than 0 and than N and P treatments.
3. Serial colimitation: LRR of either the N or P treatment greater than 0. LRR of the N + P treatment greater than 0 and than LRRs of treatments with the primary limiting nutrient.
4. Strict primary limitation: LRR of the N or P treatment greater than 0. LRR of the N + P treatment greater than 0, but not different from LRR for the primary limiting nutrient treatment.
5. Negative response to enrichment: LRRs of N, P, or N + P treatments below 0.
6. No limitation: LRRs of N, P, or N + P treatments not different from 0.

Log response ratios were also used to assess the magnitude of nutrient limitation at each site in relation to environmental conditions. Chl-*a* and AFDW LRRs for each nutrient treatment were regressed against the PC1 summary trophic status indicator and individual indicators of site chemical and physical conditions. As with periphyton biomass on control substrata, we used model comparison to determine whether simple linear or second order polynomial regressions were most appropriate for describing the relationships between limitation extent and environmental variables.

All statistical analyses and data visualisation were carried out using the R statistical computing environment (R Core Team, 2014) with packages *ape* (Paradis & Schliep, 2019) and *ggmap* (Kahle & Wickham, 2013).

3 | RESULTS

3.1 | Site characteristics

Study sites spanned large spatial and trophic status gradients (Figure 1, Table 1). Most Lake Superior sites were characterised by low TP and water column Chl-*a* concentrations, low temperatures, and high water clarity (low light attenuation coefficients, K_d). Green Bay sites had high TP and phytoplankton concentrations, relatively high temperatures, and low water clarity (high K_d). Lake Michigan sites were intermediate along these parameters. Across all sites, TP ranged 2.1–76.5 μg/L, water column Chl-*a* ranged 0.1–11.9 μg/L, TN ranged 291–708 μg/L, NO_3^- ranged 0.7–338 μg/L, molar TN:TP ratios ranged 20.5–407, and temperatures ranged

TABLE 1 Site location and mean water column chlorophyll *a* (Chl-*a*), light extinction (K_d), NO_3^-, total phosphorous (TP), and total nitrogen (TN) concentrations (averaged from nutrient-diffusing substratum deployment and retrieval sampling)

Site	Lake	Latitude (N)	Longitude (W)	Chl-*a* (μg/L)	K_d (m^{-1})	NO_3^- (μg/L)	TP (μg/L)	TN (μg/L)	Periphyton C:N
GB1	Green Bay	44.6377	87.8037	3.47	2.96	5.15	48.2	526	8.9
GB2	Green Bay	44.5371	87.9278	11.9	2.72	0.67	76.4	708	9.2
GB3	Green Bay	44.6685	87.7473	7.81	2.66	1.46	34.3	403	10.2
GB4	Green Bay	44.8914	87.4300	4.18	1.24	69.4	14.1	418	10.1
GB5	Green Bay	45.3885	87.3637	3.74	1.95	126	6.09	433	11.8
GB6	Green Bay	44.6377	87.8037	0.58	1.38	126	7.38	474	11.9
MICH1	Michigan	45.8542	84.7836	0.77	0.84	148	6.50	325	10.8
MICH2	Michigan	46.0808	85.3092	1.05	1.44	145	5.53	297	11.2
MICH3	Michigan	46.086	85.4446	0.36	1.73	138	5.92	321	11.6
MICH4	Michigan	45.9208	85.9100	0.98	1.67	178	5.73	352	11.6
MICH5	Michigan	45.9478	86.2406	1.58	1.22	203	8.15	384	15.6
APOS1	Superior	46.9399	90.9582	0.28	0.97	338	4.53	345	12
APOS2	Superior	46.8188	90.8055	0.32	0.88	320	3.94	324	12.9
APOS3	Superior	46.6641	90.9053	0.66	1.19	248	8.42	291	26.8
GP1	Superior	47.9629	89.6523	0.68	0.93	274	4.28	364	12.4
GP2	Superior	47.9627	89.6823	0.48	1.18	279	5.98	366	15.8
GP3	Superior	47.9545	89.6636	0.29	1.40	311	3.23	352	12.4
KEE	Superior	47.4689	88.0577	0.26	0.83	318	2.61	390	14.2
PR1	Superior	46.4126	86.6500	0.39	1.13	335	6.29	374	15.9
PR2	Superior	46.4468	86.8854	0.21	1.06	318	3.84	326	14.8
SUP1	Superior	46.8819	91.9176	0.32	1.04	309	5.41	349	12.5
SUP2	Superior	46.8371	92.0028	0.40	1.08	283	6.09	369	14.1
SUP3	Superior	46.8022	92.0681	0.42	1.25	304	5.90	390	14.3
SUP4	Superior	46.7958	92.0826	1.08	1.68	308	6.09	408	16
SUP7	Superior	47.1653	91.4244	0.39	1.12	325	5.39	362	10.7
SUP8	Superior	47.2606	91.2934	0.07	0.72	329	2.06	378	19.9
SUP10	Superior	47.7457	90.3321	0.52	0.88	313	4.56	408	11.4
WF	Superior	46.4849	84.6307	0.50	0.85	327	2.74	394	14.8

Note: Also included are C:N ratios of periphyton from natural substrata at the study sites (from Camilleri & Ozersky, 2019).

10.6–25.8°C. K_d ranged 0.72–2.95, corresponding to between 49% and 1.2% of surface light reaching the NDS colonisation surfaces at 1.5 m depth.

Many site environmental parameters were strongly correlated with each other (Figure S2). For example, sites with high TP also had high water column Chl-*a* concentration ($r_s = 0.78$), high temperature ($r_s = 0.73$), low water clarity ($r_s = 0.66$), and low TN:TP ($r_s = -0.91$). TN had a relatively weak association with other trophic status parameters and was high at many Lake Superior locations, owing to high NO_3^- concentrations in Lake Superior, which dominate the TN pool there (Table 1). A PCA on trophic status indicators (K_d, TP, water column Chl-*a*) efficiently summarised the variation among sites (Figure 1), with PC1 explaining 88.4% of the variation and PC2 an additional 8.3%. TP, Chl-*a*, and K_d all loaded positively on PC1, meaning that the higher PC1 scores correspond to more eutrophic conditions (Figure 1b).

3.2 | Spatial variation and controls on periphyton biomass

Periphyton AFDW and Chl-*a* were significantly correlated across all nutrient treatments (Figure S3). However, there was considerable spread in the relationship and the degree of correlation varied among different nutrient treatments, being strongest in control treatments ($r_s = 0.65$) and weakest in P-enriched treatments ($r_s = 0.43$). Periphyton biomass on control substrata varied among the study sites and was, on average, lowest at Lake Superior locations and highest at Green Bay locations (Figure 2). Periphyton biomass measured as either AFDW or Chl-*a* was significantly explained by site PC1 axis scores (Figure 2), with second-order polynomial regressions providing a better fit than simple linear regressions for both AFDW and Chl-*a*. Both metrics of periphyton biomass showed a unimodal relationship with site PC1 axis scores.

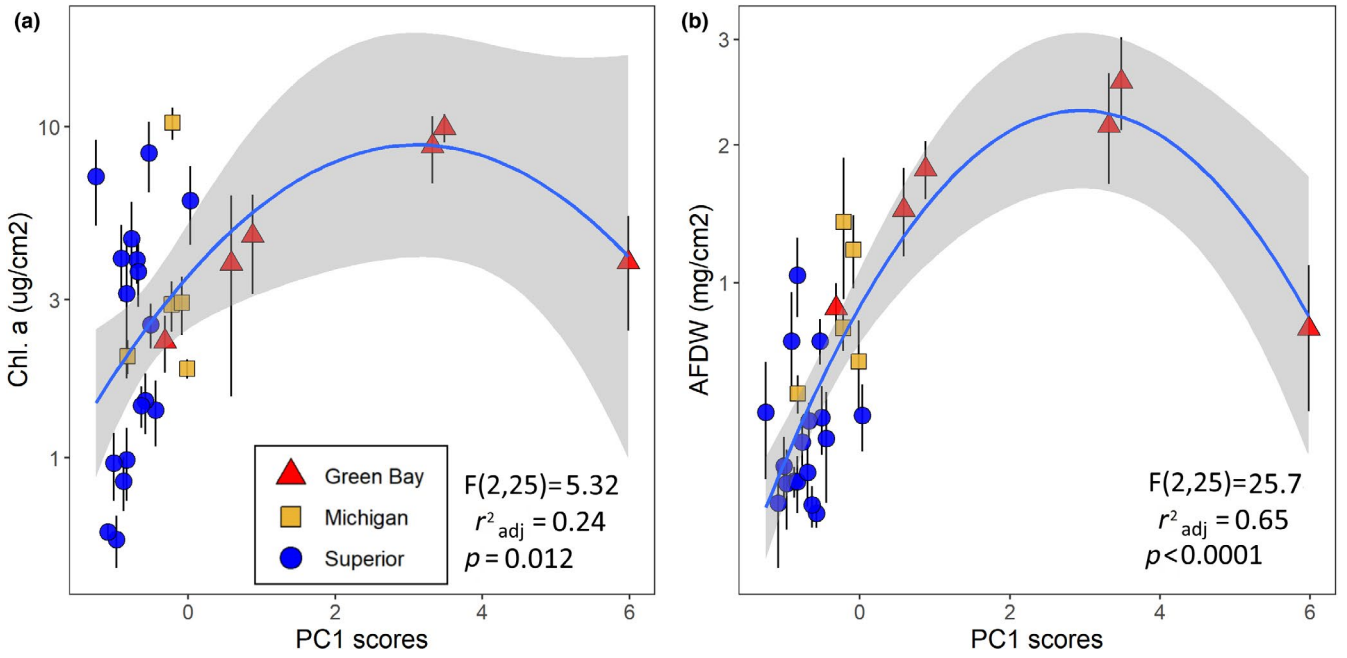

FIGURE 2 Relationship between average site periphyton biomass as chlorophyll *a* (Chl-a; a) and as ash-free dry weight (AFDW; b) and site PC1 axis scores. Statistical results are from linear regression analysis on site-averages biomass. Error bars represent one standard deviation of the mean for each sampling location; grey areas are 95% confidence intervals. Symbol shapes and colours are the same for both panels

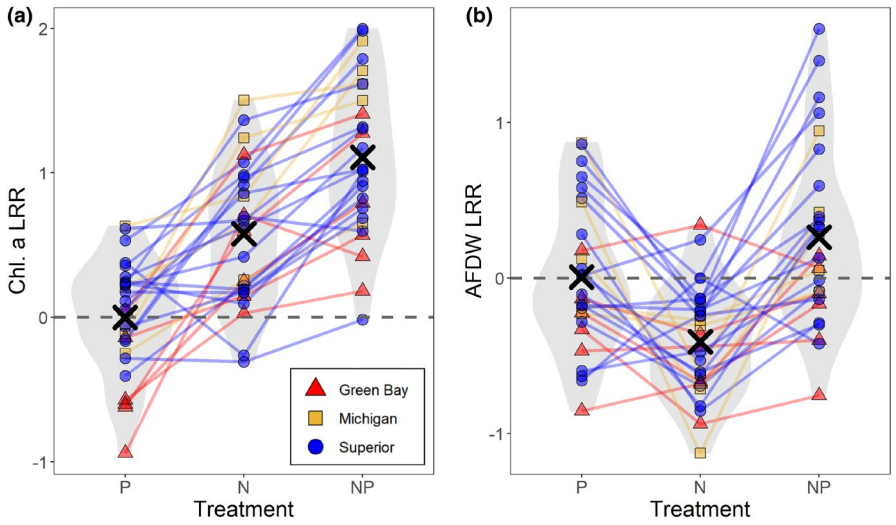

FIGURE 3 Site average periphyton chlorophyll a (Chl-a; a) and ash-free dry weight (AFDW; b) log response ratios (LRRs) on P-, N-, and N + P-enriched nutrient-diffusing substrata. Crosses represent treatment means. Grey areas represent kernel density distributions of values. The dashed line corresponds to no response relative to control. LRR values of 1 and −1 represent an approximately three-fold increase or decrease (respectively) of biomass relative to control

Periphyton biomass was low at low PC1 scores (corresponding to low TP and Chl-*a* concentrations and high light), increased with PC1 scores and then decreased at the highest PC1 scores (eutrophic Green Bay sites). The relationship between periphyton biomass and PC1 axis scores was stronger for AFDW than Chl-*a* (Figure 2).

Examined individually, site environmental variables displayed a variety of relationships with periphyton biomass on control substrata (Figure S4). Both periphyton Chl-*a* and AFDW were positively correlated with K_d (higher periphyton biomass at turbid sites) and $\log_{10}$(TP). Neither metric of periphyton biomass showed a significant correlation with TN and both metrics had a negative simple linear relationship with NO_3^-. The strongest relationship observed was that between periphyton AFDW and the non-NO_3^- portion of TN, which represents reduced dissolved N along with particulate N. Both Chl-*a* and AFDW showed a negative, curvilinear relationship with water column TN:TP ratios. Periphyton AFDW, but not Chl-*a*, showed a positive relationship with water temperature.

3.3 | Form and extent of periphyton nutrient limitation

Across all sites, periphyton Chl-*a* LRRs on P-, N-, and N + P-enriched substrata had respective means and 95% CIs of 0 (95% CI −0.15 to 0.15), 0.58 (95% CI 0.39–0.76), and 1.1, (95% CI 0.90–1.32). Thus, across all sites, there was no response of periphyton Chl-*a* to P enrichment, positive responses to N and N + P enrichment, and significant differences among all pairs of treatments (Figure 3a). These results show that, across all study sites, periphyton Chl-*a* exhibited primary N limitation and secondary P limitation (i.e., serial colimitation *sensu* Harpole et al., 2011). At the individual site level, colimitation of Chl-*a* by N and P was observed at 14 of the 28 sites. Of those, seven sites had simultaneous colimitation, five sites had serial colimitation (four sites with primary P and secondary N limitation and one site had primary N and secondary P limitation), and two sites showed independent colimitation (Table S1). Strict N limitation occurred at 11 sites and three sites showed no limitation. Chl-*a* showed a significant negative response to P enrichment alone at four sites.

Across all sites, periphyton AFDW LRRs on P-, N-, and N + P-enriched substrata had respective means and 95% CIs of 0 (95% CI −0.18 to 0.19), −0.41 (95% CI −0.52 to −0.30), and 0.26, (95% CI 0–0.49). Thus, across all study sites, there was no response of periphyton AFDW to P enrichment, a negative response to N enrichment and a weak positive response to N + P enrichment, with significant differences of means among P- and N-enriched substrata, N- and N + P- enriched substrata, but not P- and N + P-enriched substrata (Figure 3b). This indicates that, across all study sites, periphyton AFDW biomass did not show individual N or P limitation, instead exhibiting simultaneous N + P colimitation and a negative response to N enrichment. At the individual site level, 19 sites had no N or P limitation of AFDW, two sites displayed primary P limitation, and seven displayed simultaneous colimitation by N and P (Table S2). AFDW showed a significant negative response to N, P, and N + P enrichment alone at 12, six, and one sites, respectively.

3.4 | Environmental factors and periphyton limitation status

The response of periphyton to nutrient enrichment on NDS was related to site environmental characteristics. Chl-*a* LRRs showed a significant negative relationship with site PC1 scores for P- and N + P-enrichment, but not N enrichment (Figure 4). When examined against individual site environmental variables (Figure S5), several patterns were observed. The Chl-*a* LRR for P enrichment was significantly and negatively related to K_d, $\log_{10}$(TP), TN, and (TN-NO_3^-) concentrations. It was positively related to NO_3^- concentration and showed an overall positive, concave unimodal relationship with water column TN:TP ratios and an overall negative, concave unimodal relationship with water temperature. The Chl-*a* LRRs for N displayed only two significant relationships with environmental variables: a convex unimodal relationship with TN and a concave unimodal relationship with water temperature. The LRRs for N + P enrichment showed a negative, convex unimodal relationship with TN, and a concave relationship with water temperature. AFDW response ratios showed a significant negative relationship with site PC1 scores for N + P-enrichment but not P- or N-enrichment alone. Examined against individual site environmental variables (Figure S6), only the AFDW LRRs for N + P enrichment were significantly related to environmental parameters, with significant negative relationships with K_d, TN and the non-NO_3^- portion of TN (TN-NO_3^-), a positive relationship with NO_3^- and a concave, unimodal relationship with $\log_{10}$(TP).

4 | DISCUSSION

4.1 | Spatial variation and controls on periphyton biomass

The positive relationship between nutrients and lake phytoplankton biomass is well established (Quinlan et al., 2020; Schindler, 1978). The nature of the relationship between nutrients and lake periphyton was, until relatively recently, less clear. Over the past 3 decades, several studies have found a unimodal, concave relationship between trophic status and periphyton biomass (Fork et al., 2020; Hansson, 1992; Liboriussen & Jeppesen, 2006; Vadeboncoeur et al., 2002). This pattern is explained by changes in relative availability of light and nutrients along the trophic status continuum. In oligotrophic systems, light availability is high, but nutrients are limiting, resulting in low periphyton biomass. In highly eutrophic systems, nutrients are plentiful but shading by abundant phytoplankton reduces light penetration to the benthos, causing light limitation of periphyton and suppressing its growth. Peak periphyton biomass is therefore predicted at intermediate nutrient levels, where severe nutrient limitation of periphyton is alleviated, but light is still relatively plentiful (Hansson, 1992; Vadeboncoeur et al., 2008).

Our results also showed a unimodal relationship between periphyton biomass and a composite metric of site trophic status (PC1 axis scores from PCA of water column TP, Chl-*a*, and water clarity). Periphyton biomass, measured both as Chl-*a* and as AFDW, peaked at mesotrophic and meso-eutrophic sites in Lake Michigan and in Green Bay, and was lower at oligotrophic Lake Superior sites and the most eutrophic Green Bay locations. Overall, periphyton AFDW showed stronger relationships with trophic status indicators than periphyton Chl-*a*. This is probably because cellular chlorophyll concentrations of algae can change in response to changes in environmental conditions (e.g., light, temperature) without a corresponding change in biomass, complicating the use of Chl-*a* to compare periphyton biomass across sites spanning large environmental gradients (Baulch et al., 2009).

When periphyton biomass was examined against individual indicators of site chemical and physical conditions (rather than

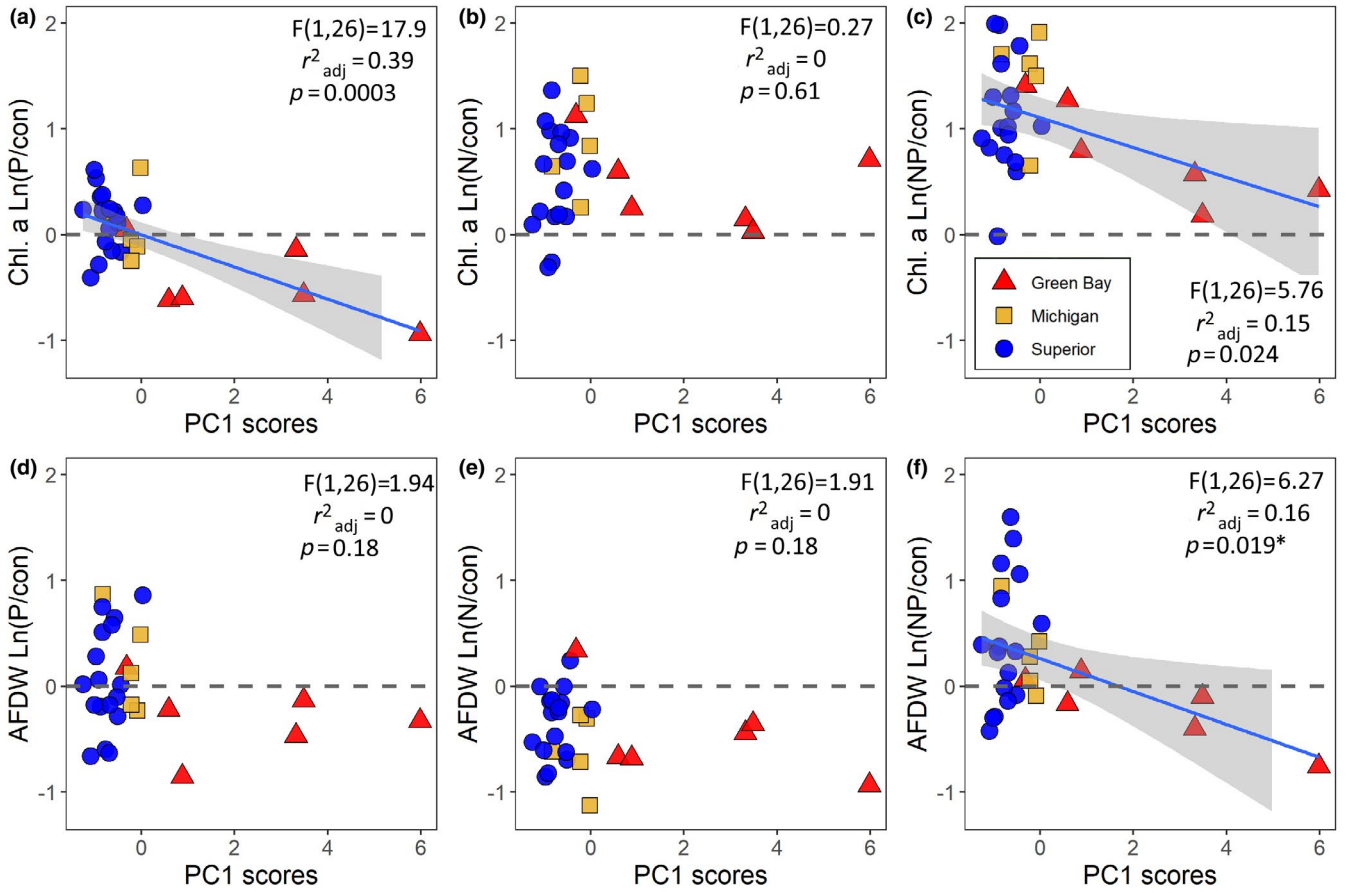

FIGURE 4 Relationships between site-averaged log response ratios of periphyton biomass as chlorophyll *a* (Chl-a) on P, N and N + P enriched nutrient-diffusing substrata (panels a, b, c, respectively) and site environmental characteristic PC1 scores. Panels d, e and f are as above, but for periphyton ash-free dry weight (AFDW). Log response ratio values of 1 and −1 represent an approximately three-fold increase or decrease (respectively) of biomass relative to control. Statistical results are from linear regression analysis on site-averages biomass. Grey areas are 95% confidence intervals. Asterisks by *p*-values represent cases where the homogeneity of variance assumption could not be satisfied. Symbol shapes and colours are the same for all panels

PC1 scores), departures from predicted unimodal patterns were observed. For example, the relationships with TP and light availability were best explained as, respectively, simple positive and negative relationships. One possible explanation for this discrepancy is that individual metrics of site trophic status (which are based on two samples—at NDS deployment and retrieval) are more affected by high temporal variability in nearshore conditions (Reisinger et al., 2019) than the composite metric provided by PC1 and therefore do not adequately capture the average conditions at our study sites. The sparseness of observations at the upper end of the trophic spectrum—and their consequent high statistical leverage—provides another possible explanation for the discrepancy in the patterns observed between individual indicators of water quality and PC1 scores. Interestingly, of the individual metrics of water quality, non-NO_3^- TN (corresponding to reduced forms of dissolved N, plus particulate N), performed best to predict periphyton AFDW biomass. This finding adds weight to the importance of N in limiting Great Lakes periphyton biomass, a finding that was also supported by results from NDS experiments (see next section).

4.2 | Extent and form of periphyton nutrient limitation

The phytoplankton of the Great Lakes are believed to be primarily P-limited (Guildford et al., 2000; Lin & Schelske, 1981; Millard et al., 1996; North et al., 2007; Sterner et al., 2004; Stoermer et al., 1978). This, along with high water column TN:TP ratios at many of our sites (molar average 154, range 20–407), led us to expect widespread P limitation of the periphyton. While NDS experiments showed that nutrient limitation of benthic Chl-*a* was common, primary P limitation was never observed. Instead, some form of N limitation or N and P co-limitation occurred at 25 of our 28 sites. Some support for N limitation is also provided by C:N ratios of natural periphyton communities from our study sites (Camilleri & Ozersky, 2019). Healey (1975) identified cellular C:N ratios >8.3 and >14.6 as the respective thresholds for moderate and severe N-limitation of phytoplankton, and Hillebrand and Sommer (1999) showed that periphyton cellular C:N ratios >10 may indicate N limitation, especially when periphyton N:P ratios are below 13. In Camilleri and Ozersky (2019), we found that C:N ratios of periphyton from natural substrata at the

same sites as the NDS experiments averaged 13.3, with 26 sites having periphyton C:N ratios >10 and eight having C:N ratios >14.6. C:N ratios of natural periphyton from our sites showed positive correlations with LRRs of Chl-*a* and AFDW on P-, N-, and N + P-enriched substrata, although the relationship was only significant for the LRR of periphyton AFDW on N + P-enriched substrata (Spearman correlation, $p = 0.014$).

Several other researchers have studied lentic periphyton nutrient limitation. While a meta-analysis of several studies by Elser et al. (2007) found that primary P limitation was common for lake periphyton, a study of 30 lakes in the U.K. (Maberly et al., 2002), several locations in oligotrophic Lake Baikal (Ozersky et al., 2018), and 10 lakes in northern Sweden (Fork et al., 2020) rarely observed primary P limitation, finding that, instead, N or N + P co-limitation were most common. In the Great Lakes, Francoeur et al. (2015), showed primary P limitation at a mesotrophic site in Lake Huron's Saginaw Bay. In contrast, Carrick and Lowe (2007), working at two locations in Lake Michigan, showed N and Si co-limitation of benthic algae. Cooper et al. (2016) studied periphyton nutrient limitation in 54 coastal wetlands of Lake Huron and Michigan; they never observed primary P limitation, reporting either primary N or N + P co-limitation at 43% and 18% of their locations, respectively. Together, these findings suggest that N limitation of lentic benthic algae may be widespread, even in systems where phytoplankton are P-limited. The efficient retention and recycling of P within the periphyton matrix (Mulholland et al., 1994; Noe et al., 2003), possibly along with removal of bioavailable nitrogen through denitrification (Ishida et al., 2008; Triska & Oremland, 1981), may help explain why periphyton are less likely to exhibit P limitation and more likely to exhibit N limitation than phytoplankton.

Many lake and stream studies of periphyton nutrient limitation use the photosynthetic pigment chl-*a* as a metric of periphyton biomass. Periphyton, however, is a complex mixture of diverse photosynthetic organisms, fungi, bacteria, micro- and macroscopic animals, extracellular exudates, and organic and inorganic detritus (Young, 1945). It has been shown that different components of periphyton may be limited by different factors (Bechtold et al., 2012; Cattaneo, 1987; Ferragut & de Campos Bicudo, 2010; Ozersky et al., 2018; Sanches et al., 2011). This may explain the discrepancy in response to enrichment that we observed when measuring periphyton biomass as Chl-*a* and as AFDW. When using AFDW as a biomass metric, nutrient limitation of any kind was observed only at nine of the 28 sites (compared to 25 sites based on Chl-*a*). Other researchers have found divergent responses of Chl-*a* and AFDW to enrichment (Bechtold et al., 2012; Sanches et al., 2011; Scott et al., 2009; Vizza et al., 2018). These studies, along with our findings, indicate that N enrichment can increase the Chl-*a* to organic carbon ratio of periphyton. One interpretation of this pattern is that autotrophs are often limited by N but the heterotrophic components of periphyton are not, thereby causing N additions to increase the proportion of autotrophs in the periphytic matrix (Bechtold et al., 2012). More research is needed to understand how the various components of periphyton respond to nutrient additions and how these responses affect the role of periphyton in nutrient cycling, food web dynamics, and formation of nuisance blooms (Bechtold et al., 2012; Ribot et al., 2015).

In addition to indicating different prevalence of nutrient limitation across our study sites, Chl-*a* and AFDW also showed differences in their negative (inhibitory) responses to nutrient enrichment. Periphyton Chl-*a* was significantly inhibited by P additions at four of our sites but was never inhibited by N or N + P additions. In contrast, significant negative responses of AFDW to N additions were common (12 sites). Inhibitory effects of both N and P additions are sometimes reported in nutrient enrichment experiments (Bernhardt & Likens, 2004; Francoeur, 2001; Harpole et al., 2011; Ribot et al., 2015). Several explanations for inhibitory effects of single nutrient additions have been offered, including selective grazing by invertebrates on periphyton growing on enriched substrata, changes in community composition of periphyton in response to enrichment, or toxicity due to overly high concentration of nutrients (Bernhardt & Likens, 2004). Harpole et al. (2011) suggest that stoichiometric imbalance in nutrient supply, rather than strict toxicity, can also lead to suppressive responses to single nutrient additions. While we can not distinguish among these four possibilities, our results are consistent with the stoichiometric imbalance explanation (Harpole et al., 2011), since sites that showed negative responses to single nutrient additions typically showed either positive or no response to combined N and P additions.

4.3 | Environmental factors and periphyton limitation status

The third objective of this study was to examine spatial variation in, and identify controls of, the degree of periphyton nutrient limitation. Our results agree with other studies of freshwater periphyton (Cooper et al., 2016; Fork et al., 2020; Ren et al., 2019) and support our hypothesis that limitation strength is inversely proportional to site trophic status. Periphyton at oligotrophic sites showed a larger increase in biomass (both as Chl-*a* and AFDW) in response to combined N + P enrichment than periphyton at eutrophic sites. The negative relationship between trophic status and response to enrichment was also apparent for Chl-*a* on P-enriched substrata, but not for Chl-*a* on N-enriched substrata or for AFDW on either N-enriched or P-enriched NDS.

An interesting incongruity of the finding of primary N limitation emerges from the relationship between periphyton biomass, periphyton limitation extent, and NO_3^- concentrations. Periphyton Chl-*a* in Lake Superior, which has unusually high NO_3^- concentrations and TN:TP ratios (Sterner, 2011) was relatively low and showed consistently strong positive response to N and N + P addition. How can N be limiting given the very high NO_3^- concentrations at our study locations? A possible explanation for this unexpected finding may be Fe limitation. Synthesis of nitrate and nitrite reductase enzymes, required for effective assimilation of nitrate by algal cells, requires Fe. Fe limitation in the oceanic high-nitrate, low-chlorophyll zones is partly attributed to the inability of the phytoplankton there to

assimilate nitrate without additions of Fe (Marchetti et al., 2012). Studies have shown that in the Great Lakes, as in the ocean, nitrate uptake by phytoplankton may be Fe-limited (Havens et al., 2012; Ivanikova et al., 2007). Several studies have also shown that algae preferentially assimilate NH_4^+ over NO_3^- (Berg et al., 2003; Carpenter & Dunham, 1985; von Schiller et al., 2007). While NH_4^+ is present in only trace amounts in Lake Superior (Sterner, 2011), it constitutes the dominant fraction of the dissolved inorganic N pool in eutrophic Green Bay (Qualls et al., 2013). Thus, Fe-limitation of NO_3^- uptake, low NH_4^+ availability at many of our study sites, and our use of NH_4^+ (as NH_4Cl) in NDS substrata may help explain the widespread N limitation we saw, as well as stronger response to N enrichment at Lake Superior sites compared to the most eutrophic Green Bay sites. Studies of micronutrient limitation or the effects of different forms on N on lake periphyton are rare (but see Vizza et al., 2018) but could contribute to better understand nutrient limitation of lake periphyton and of the effects of nutrient pollution on lake ecosystems.

4.4 | Caveats, questions, and implications

Several caveats of this study should be mentioned. First, while NDS-based studies of periphyton avoid many of the problems inherent in use of bottle assays for determination of phytoplankton nutrient limitation (Schindler et al., 2016), they still suffer from methodological issues. For example, the purity of agar used, the material from which NDS are constructed, the length of deployment and the forms (e.g., P as mono- or dibasic potassium or sodium salt, N as NH_4^+ or NO_3^-), concentrations, and ratios of added nutrients have all been shown to affect study results (Beck & Hall, 2018; Capps et al., 2011; Carrick & Lowe, 1988; Vizza et al., 2018). Second, all our experiments were conducted in shallow water (1.5 m) and relatively high light levels; additional observations at lower light and higher nutrient levels would help further resolve the relationship between light and nutrient limitation of Great Lakes periphyton. Third, our conclusions are based on mid-summer observations and periphyton limitation status can vary seasonally (Bernhardt & Likens, 2004; Maberly et al., 2002; Trochine et al., 2014). Fourth, several factors that we did not explicitly consider here (such as water movement, micronutrient limitation, top-down effects of grazers) may have affected periphyton biomass and its response to enrichment (Carrick & Lowe, 2007; Cattaneo, 1990; Hillebrand & Kahlert, 2001). Finally, many potential predictor variables in our dataset were strongly correlated with each other (e.g., TP and temperature, NO_3 and TN:TP ratios), complicating interpretation of causal relationships.

Despite the potential limitations of this study, our findings have several implications for understanding lake periphyton ecology and managing nuisance benthic algal blooms. Several lines of evidence show widespread N limitation or N + P colimitation of Great Lakes periphyton and suggest that, at least in some lakes, phytoplankton and periphyton may be limited by different nutrients (see also Bonilla et al., 2005; Havens et al., 1996; Steinman et al., 2016). Thus, different nutrient management strategies may be needed to control pelagic and benthic algal blooms (Cooper et al., 2016), especially in large lakes where considerable gradients in nutrient ratios and availability may exist between nearshore and offshore environments. The strong nutrient limitation of periphyton we show at oligotrophic sites agrees with the idea that periphyton proliferation represents an early warning sign of eutrophication that responds to increasing terrestrial nutrient inputs before offshore nutrient concentrations or phytoplankton densities (Lambert et al., 2008; Rosenberger et al., 2008). Finally, our findings help explain why large, oligotrophic lakes may be particularly at risk of localised benthic algal blooms. Because of active horizontal mixing and a large volume of offshore waters, localised nutrient inputs from point and non-point sources into large oligotrophic lakes are unlikely to cause significant local stimulation of phytoplankton biomass and consequent shading of benthic substrates. However, these nutrients can be efficiently intercepted by benthic algae and cause their proliferation in these high-light environments. Given the important role of benthic algae in lake ecosystems, the ongoing increase in nuisance benthic algal blooms, and the many open questions that remain about lake periphyton ecology, we join others (DeNicola & Kelly, 2014; Lambert & Cattaneo, 2008; Vadeboncoeur et al., 2021) in calling for increased research on lake periphyton ecology and integration of periphyton into lake monitoring programmes.

ACKNOWLEDGEMENTS

We would like to thank Sandra Brovold, Julia Halbur, John Koets, Taylor Velander, and Kirill Shchapov for contributions to laboratory and fieldwork. Thanks are also due to Brenda Moraska Lafrancois and Brandon Seitz of the National Parks Service for coordinating access to sites, and to Margaret Watkins and the Grand Portage Reservation for boat transport. Comments by A. D. Steinman and three anonymous reviewers helped to substantially improve this manuscript. Funding for this project was provided to T.O. by the University of Minnesota Duluth.

DATA AVAILABILITY STATEMENT

The data for this manuscript have been deposited to the Data Repository for University of Minnesota and are freely accessible (Camilleri & Ozersky, 2021). Data include water column environmental parameters and periphyton biomass on NDS substrata as Chl-*a* and as AFDW.

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How to cite this article: Ozersky, T., & Camilleri, A. (2021). Factors regulating lake periphyton biomass and nutrient limitation status across a large trophic gradient. *Freshw Biol.*, 66, 2338–2350. https://doi.org/10.1111/fwb.13836