Effects of nutrient loading, temperature regime and grazing pressure on nutrient limitation of periphyton in experimental ponds

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SUMMARY

1. We studied nutrient limitation of periphytic algae (henceforth periphyton) in 24 mesocosms simulating shallow lakes with two nutrient levels, enriched (with added nitrogen, N, and phosphorus, P) and unenriched (control), and three temperature scenarios, ambient, A2 from the Intergovernmental Panel on Climate Change (IPCC) and A2 + 50%. Periphyton growth (measured as chlorophyll *a*) was investigated four times *in situ* using nutrient-diffusing substrata. The effect of grazing was also manipulated using exclusion cages.

2. We found that periphyton responded differently to nutrient addition bioassays (N and P) depending on the background nutrient concentration and warming scenario. Our results indicate that single-nutrient limitation prevailed for periphyton in our experimental temperate shallow lakes. The responses were season sensitive.

3. Periphyton in the unenriched mesocosms were P-limited in early summer in the ambient and A2 scenarios, N-limited in late summer in these two climate scenarios, not nutrient-limited in autumn and P-limited in spring in all climate scenarios. Periphyton in the A2 + 50% scenario showed a positive response to N and P added together in early summer.

4. In contrast, periphyton in the enriched mesocosms showed no clear nutrient limitation, except for short-term periods of P limitation in the warmer systems. Grazers did not affect the quantitative response of periphyton to nutrient addition, and the concentrations of P and N as well as mean monthly temperature were the main environmental factors driving P or N limitation.

5. We conclude that warming in low-productivity lakes affects the seasonality of N limitation and changes the single-nutrient limitation of periphyton into NP co-limitation. This last observation suggests that warming reduces the sensitivity of temperate shallow lakes to bottom-up perturbations.

Keywords: grazers, nitrogen, phosphorus, primary producers, temperate lakes

Introduction

The role of nitrogen (N) and phosphorus (P) in regulating the structure and functioning of aquatic and terrestrial ecosystems has long been the subject of biogeochemical and ecological studies (Elser *et al.*, 2007; Harpole *et al.*, 2011; Lewis, Wurtsbaugh & Paerl, 2011). In aquatic environments, such investigations are motivated by the long-standing recognition that increased nutrient loading results in significant shifts in the ecological structure and functioning of ecosystems (Smith, Tilman & Nekola, 1999). Predicting and mitigating the effects of altered nutrient loading require an understanding of where, and to what extent, these key nutrients limit primary production.

Phosphorus is generally considered the most important nutrient regulating community composition and growth

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of aquatic primary producers in lakes (Vollenweider, 1976; Schindler, 1977; Schindler *et al.*, 2008). With increasing P loading, succession occurs among aquatic primary producers in shallow lakes, involving a shift in dominance by submerged macrophytes and periphyton towards complete dominance by phytoplankton (Sand-Jensen & Borum, 1991). However, N may also be the primary or co-limiting nutrient for phytoplankton (Elser *et al.*, 2007; Conley *et al.*, 2009; Allgeier, Rosemond & Layman, 2011; Harpole *et al.*, 2011). Indeed, many observations suggest that in most lakes, N and P occur in relatively balanced proportions relative to the demands of phytoplankton (Sterner, 2008; Moss *et al.*, 2012). In turn, this implies that relatively subtle changes in the supplies of N and P might shift phytoplankton between N- and P-limited growth. Consequently, the growth of lake periphyton, which is an important contributor to lake productivity in the littoral zone (Vadeboncoeur, Lodge & Carpenter, 2001), is also often limited by the availability of N and P (Borchardt, 1996; Hillebrand & Kahlert, 2001; Maberly *et al.*, 2002). However, phytoplankton and periphyton differ in their uptake of nutrients. Phytoplankton is suspended in the water column and can more easily access available nutrients than periphyton, which is constrained by the shape and boundary layer effects of the biofilm (Riber & Wetzel, 1987). Furthermore, periphyton and phytoplankton from the same lake may differ in the extent to which they are influenced by light and grazing (Hill, Boston & Steinman, 1992; Steinman, 1996; Elser & Urabe, 1999; Hillebrand & Kahlert, 2001; Hillebrand, 2005). It is now well established that nutrient (N, P) limitation of primary producers leads to elevated carbon (C) / nutrient ratios in primary producer biomass. This may result in decreased energetic (or C) growth efficiency of herbivores due to limitation of their growth by low N or P in the diet (Sterner & Elser, 2002), unless grazers take up these nutrients from the sediment or other nutrient hotspots in the water (Schatz & McCauley, 2007; Cazzanelli *et al.*, 2012). Moreover, herbivores regulate the responses of benthic algae to nutrients by suppressing algal accumulation but increasing productivity through nutrient recycling (Rober, Wyatt & Stevenson, 2011).

Another factor that might affect the *in situ* nutrient status of lake primary producers is temperature, currently increasing due to climate warming. Climate warming is expected to affect nutrient cycles in various ways (Jeppesen *et al.*, 2009, 2011; Veraart, de Klein & Scheffer, 2011). In particular, higher temperatures increase the release of P and N from bottom sediments (Jensen & Andersen, 1992; Feuchtmayr *et al.*, 2009), increase denitrification (Veraart *et al.*, 2011) and stimu-

late the production of dissolved organic carbon (Evans, Monteith & Cooper, 2005). Phosphorus availability may directly contribute to increased primary productivity (Elser *et al.*, 2007), while enhanced denitrification and prolongation of NO_3-N-depleted conditions might result in increasing occurrence of N-fixing cyanobacteria (Hyenstrand, Blomqvist & Pettersson, 1998; Ferber *et al.*, 2004; Weyhenmeyer *et al.*, 2007; Rober *et al.*, 2011). Burgmer & Hillebrand (2011) observed that elevated temperature resulted in lower phytoplankton variability in a long-term microcosm experiment. In a mesocosm experiment, Kratina *et al.* (2012) also showed that warming made phytoplankton communities more susceptible to the cascading effects of predators but reduced their sensitivity to nutrient enrichment (e.g. less mean and temporal variation in primary producer biomass). Finally, Jeppesen *et al.* (2009, 2011) and Kosten *et al.* (2012) evidenced a positive relationship between warming and the percentage contribution of cyanobacteria to the total phytoplankton biomass of shallow lakes and synergistic effects with nutrient enrichment.

Since changes in periphyton growth can have strong effects on higher trophic levels, as well as on nutrient fluxes and system metabolism, investigation of the response of periphyton to altered nutrient supply ratios or availability is critical for our understanding of aquatic ecosystems in a future warmer world. In this study, we elucidated how the combined effects of increased nutrient loading and climate warming modulate nutrient limitation of periphyton in shallow lakes. For this purpose, experiments were performed using nutrient diffusion substrata (NDS, diffusion substrata releasing N, P and combined N and P) in a unique advanced flow-through mesocosm system consisting of 24 experimental ponds with two contrasting nutrient levels and three temperature scenarios. We hypothesised that (i) warming will shift the timing of nutrient limitation for periphyton growth as the growth season would start earlier in the heated mesocosms, (ii) warming will make periphyton less sensitive to nutrient enrichment (lowering its mean and temporal variation) and also makes periphyton less susceptible to the effects of grazers related to, for instance, cyanobacteria blooms, (iii) nutrient enrichment will stimulate periphyton accumulation, but grazers should regulate the response of algae to nutrients by suppressing their accumulation and increasing productivity via nutrient recycling. Thus, grazers may alter periphyton nutrient limitation patterns. To our knowledge, this is the first field-scale study of changes in seasonal variations in N and P limitation of periphyton run under contrasting climate scenarios.

Methods

The experimental set-up (Fig. 1) consisted of outdoor mesocosms (cylindrical stainless steel tanks 1.9 m in diameter and 1.5 m in total depth, filled with 2.8 m^3 of water) established in August 2003 in a lowland valley in Central Jutland, Denmark (56°14′N, 9°31′E). The mesocosms were equipped with a flow-through system where a timer-controlled magnetic valve (Danfoss Group, EV 220B) automatically adds ground water to the mesocosms every sixth hour, while an overflow pipe (diameter = 2.6 cm) drains off excess surface water. This flow system ensures a constant water level of 1.0 m. The system was maintained at two nutrient levels: unenriched and enriched ground water (weekly loading rates equivalent to a concentration increase in the mesocosms of 19 µg L^{-1} of P, 768 µg L^{-1} of N, and N / P (atomic) of 88 crossed with three temperature treatments: ambient (¨reference¨) and heated according to the IPCC (Houghton *et al.*, 2001) scenarios, A2 (predicted temperature in the period 2071–2100 downscaled to local 25 × 25 km grid cells) and A2 + 50%. Each treatment combination had four replicates, resulting in a total of 24 mesocosms.
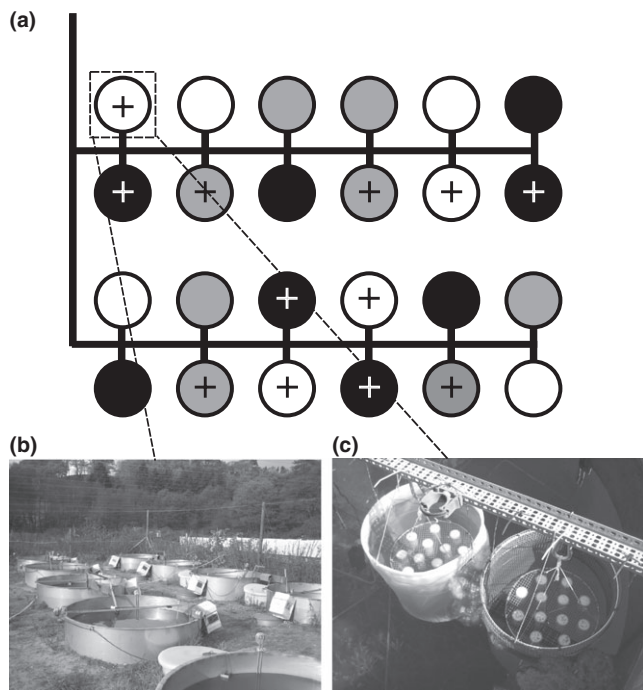

Fig. 1 Experimental design showing the nutrient diffusion substrata (NDS) in place: (a) mesocosm set-up with two nutrient loadings, unenriched (no symbol) and enriched (+ symbol), and three temperatures, ambient (white circles), A2 (grey circles) and A2 + 50% (black circles); (b) mesocosms; (c) NDS placed inside the cages.

The water retention time of the mesocosms averaged 2.5 months. Electrical elements heated the water, and paddles provided continuous stirring. The highest temperature difference between the ambient and heated mesocosms occurred from August to January (max. 4.4 and 6.6 °C in September for A2 and A2 + 50%, respectively) compared to the rest of the year (minimal 2.5 and 3.7 °C in June, respectively). The sediment and mixture of active plankton communities inoculated in the mesocosms came from nearby lakes and ponds. The mesocosms also contained three-spined sticklebacks (*Gasterosteus acuelatus*) close to natural densities in Danish lakes according to the nutrient treatment: one male in each unenriched mesocosm, while 12 fish (males and females) were stocked in each enriched mesocosm, thus allowing reproduction. This strategy was chosen as the mesocosms were too small to have piscivores, which usually exert strong control of plankti-benthivorous fish at low nutrient level, but not at high nutrient level in shallow temperate lakes (Jeppesen *et al.*, 2000). For further details, see Liboriussen *et al.* (2005).

Nutrient enrichment bioassays

We used NDS to determine the effect of added inorganic N and P on the development of periphyton biofilm in the 24 mesocosms mimicking natural ponds with contrasting nutrient states and different temperatures.

We made the NDS by amending 2% agar with NH$_4$NO$_3$ (N treatment) and NaH$_2$PO$_4$ (P treatment; modified from Gibeau & Miller, 1989). We used four treatments of NDS: control (agar only), N addition (0.05 M NH$_4$NO$_3$), P addition (0.025 M NaH$_2$PO$_4$) and N + P combined (0.05 M NH$_4$NO$_3$ + 0.025 M NaH$_2$PO$_4$). We poured the amended agar solutions into 30-mL polystyrene vials. After the agar mixture had solidified, we placed GF/F glass fibre filters (0.7 µm retention; Whatman, Kent, U.K.) on the top of the containers to cover the agar completely. We held the filter (the growth surface) in place with a tight fitting snap-on cap, exposing a 2-cm-diameter circle on the top of the substratum for periphyton colonisation.

We selected the concentrations of N and P and the N / P nutrient ratios used in the NDS experiments based on published data and a pilot experiment. We chose 0.025 and 0.05 M (for P and N, respectively) to ensure concentrations that would not affect the nutrient dynamics of the 24 mesocosms (taking into account total water volume, retention time and N and P concentrations in the mesocosms). We assumed that these nutrient levels would saturate algal growth rates because they

exceeded concentrations reported to be limiting for benthic algae in studies reviewed by Borchardt (1996). Besides, Capps *et al.* (2011) found that the N / P ratios (e.g. 1 : 1 or 16 : 1) in the combined treatments do not affect limitation patterns on plastic-cup NDS in environments with low ambient nutrient levels. Finally, our pilot experiment results indicated that release rates of NDS using the selected concentrations of P and N decreased log-linearly over 21 days (the time period for the NDS experiments). After 21 days, we calculated a mean release rate of around 8% of the initial rate for N (both NH$_4$-N and NO$_3$-N) at 0.05 M (20 µg h^{-1}) and 11% of P at 0.025 M (8.4 µg h^{-1}). These release rates are high compared with the nutrient concentrations in our study mesocosms and therefore represented a nutrient source for periphyton growth.

We added NDS to the 24 mesocosms on four occasions: July–August 2008 (late summer), October–November 2008 (autumn), April 2009 (spring, directly after ice break) and June 2009 (early summer). Experimental NDS sets consisted of a plastic circular rack, each containing 12 randomly distributed NDS (three replicates of each treatment) of which two were placed in each mesocosm, one inside a cage (cylindrical containers with a diameter of 30 and 35 cm long) made of 0.5-mm mesh (without grazers, WOG) and the other in a cage made of 5-mm mesh with grazers (WG; Fig. 1). In the WG treatment, we also cut larger holes in the base of the cages to allow colonisation by meso- and macrograzers (see Ventura *et al.*, 2008 for detailed information on species composition). All the cages were open at the top to ensure natural light availability and were placed 5 cm above the water surface to avoid grazer entrance from the top. We secured the racks containing the NDS inside the cages with nylon strings. The filters for periphyton colonisation hung 15 cm below the water surface. We collected the filters from each substratum after 21 days for chlorophyll *a* (Chl-*a*) analyses.

Physical, chemical and biological parameters

We recorded water temperature continuously via temperature sensors (sensor type: Pt 100; maximum error ±0.15 °C at 0 °C, temperature transmitter type: TT-5333; PR electronics products, Rønde, Denmark) placed centrally in each mesocosm. We measured pH (light-duty submersion type connected to a Manta pH measurement system; OxyGuard, International, Birkerød, Denmark) in 12 of the mesocosms at the same time and rotated among the mesocosms every fourth week. Calibration of the probes occurred weekly, and we recorded water

temperature and pH every 30 min. In addition, we calculated the light attenuation coefficient (K_d PAR) in the mesocosms at 15 cm using a PAR irradiance sensor (2100 Series; Biospherical Instruments, San Diego, CA, USA) as K_d (m^{-1}) = [log (irradiance at 0 cm) − log (irradiance at 15 cm)]/0.15.

We measured concentrations of phosphate (PO$_4$-P) (Murphy & Riley, 1962), total phosphorus (TP), nitrate (NO$_3$-N) (Grasshoff, Ehrhardt & Kremling, 1983), ammonium (NH$_4$-N), total N (TN) (Solórzano & Sharp, 1980) and total iron (Rebsdorf, Søndergaard & Thyssen, 1989). We determined phytoplankton Chl-*a* and nutrient concentrations monthly from a pooled integrated water sample collected with a core sampler in open water at three random sites. We measured Chl-*a* using ethanol extraction of filter residues [Whatman glass microfibre filters (GF/C, 47 mm)] according to Jespersen & Christoffersen (1987). For the NDS experiments, we also measured periphyton Chl-*a* concentrations (µg cm^{-2}) (Jespersen & Christoffersen, 1987) and corrected for phaeophytin (Standard Methods, 1998).

We estimated coverage of macrophytes and filamentous green algae once every month in the upper (0–0.5 m depth) and lower (0.5–1.0 m depth) parts of each mesocosm from August 2008 to June 2009. We assigned macrophytes and filamentous green algae coverage to the following categories: 0, >0–5%, >5–25%, >25–50%, >50–75% and >75–100%. In addition, we measured upper and lower heights/lengths of the macrophytes and filamentous green algae, and we calculated total plant volume inhabited (PVI, sensu Canfield *et al.*, 1984) for each mesocosm as %PVI = % coverage × plant height/water depth.

Data analyses

We performed repeated-measures (RM) ANOVAs with autoregressive variance structure to analyse for differences in each biological, chemical and physical parameter across seasons. Also, we calculated averages of all measured values in replicate NDS treatments (n = 3) for each mesocosm on each sampling occasion. Then, we evaluated the results from each experiment using log-transformed ratio effect-size criteria. This was carried out by quantifying the response by the periphyton of each mesocosm to N, P or combined N and P enrichment by normalising the final periphyton Chl-*a* concentration in a given nutrient enrichment treatment to the final periphyton Chl-*a* concentration in the control (C)/ relative response (RR-X) = log (Chl$_X$/Chl$_C$), where X is N, P or NP (combined N and P enrichment). The

response was then evaluated for the effects of nutrient status (unenriched, enriched), temperature (ambient, A2, A2 + 50%) and season (late summer, autumn, spring, early summer; WOG, WG) as a nested factor (RM ANOVAs with autoregressive covariance structure and plotted also for each treatment combination).

Finally, we generated general linear models (GLMs) with the various indices of nutrient limitation from the bioassays (log RR-Chl$_X$) as response variables and nutrient availability measurements (TN, TP and total iron), biological measurements (phytoplankton Chl-*a*, filamentous green algae %PVI and macrophyte %PVI) and physical measurements (temperature, pH and K_d) as quantitative covariates, and we also included the factor season in the models. We ran the models with autoregressive covariance structure and reduced the initial model in a stepwise manner, using the log-likelihood test to compare the models. We log-transformed or square-root arcsine-transformed the data, when needed, to fulfil the requirements of homoscedasticity and normal distribution of residuals.

Results

Physical, chemical and biological parameters

The temperature in the mesocosms differed significantly under the three climate scenarios (Fig. 2a, Table 1). The enriched mesocosms had higher concentrations of TN, NH$_4$-N, NO$_3$-N, TP and PO$_4$-P than the unenriched
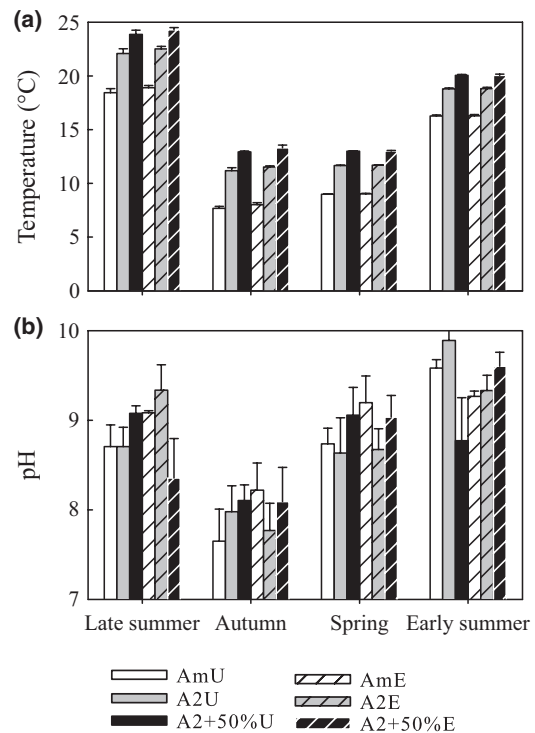

Fig. 2 (a) Temperature and (b) pH data (mean ± 1 SE) for the six mesocosm treatment combinations. AmU, ambient unenriched; A2U, A2 unenriched; A2 + 50%U, A2 + 50% unenriched; AmE, ambient enriched; A2E, A2 enriched; A2 + 50%E, A2 + 50% enriched.

mesocosms (Fig. 3a,c,d,e, Table 1). NO$_3$-N concentrations were lower in the A2 + 50% scenario compared to the ambient scenario (Table 1). Also, phytoplankton Chl-*a*

Table 1 Results of repeated-measures ANOVAs of physical, chemical and biological parameters using the effects of nutrients (two levels, unenriched and enriched) and temperature (three levels, ambient, A2 and A2 + 50%)

Treatments	Nutrient	Temperature	Nutrient × temperature	Tukey's test $P < 0.05$
	F-values			
Temperature	0.116 N.S	22.951***	0.002 N.S	Ambient < A2 < A2 + 50%
pH	0.251 N.S	0.037 N.S	0.261 N.S	
K_d	0.098 N.S	0.644 N.S	0.250 N.S	
TN	234.581***	0.089 N.S	1.995 N.S	Unenriched < enriched
NH$_4$-N	25.705***	0.721 N.S	0.344 N.S	Unenriched < enriched
NO$_3$-N	34.525***	3.209*	2.118 N.S	Unenriched < enriched Ambient > A2 + 50%
TP	24.051***	0.521 N.S	0.121 N.S	Unenriched < enriched
PO$_4$-N	37.455***	0.341 N.S	1.226 N.S	Unenriched < enriched
Dissolved inorganic nitrogen/TP	0.122 N.S	1.578 N.S	1.080 N.S	
Total iron	0.093 N.S	0.125 N.S	0.140 N.S	
Chl-a	26.268***	0.333 N.S	0.880 N.S	Unenriched < enriched
Macrophytes %PVI	20.951***	3.181 N.S	2.155 N.S	Unenriched > enriched
Filamentous green algae %PVI	1.159 N.S	1.921 N.S	0.214 N.S	

N.S., Not significant; PVI, plant volume inhabited; TN, total nitrogen; TP, total phosphorus.
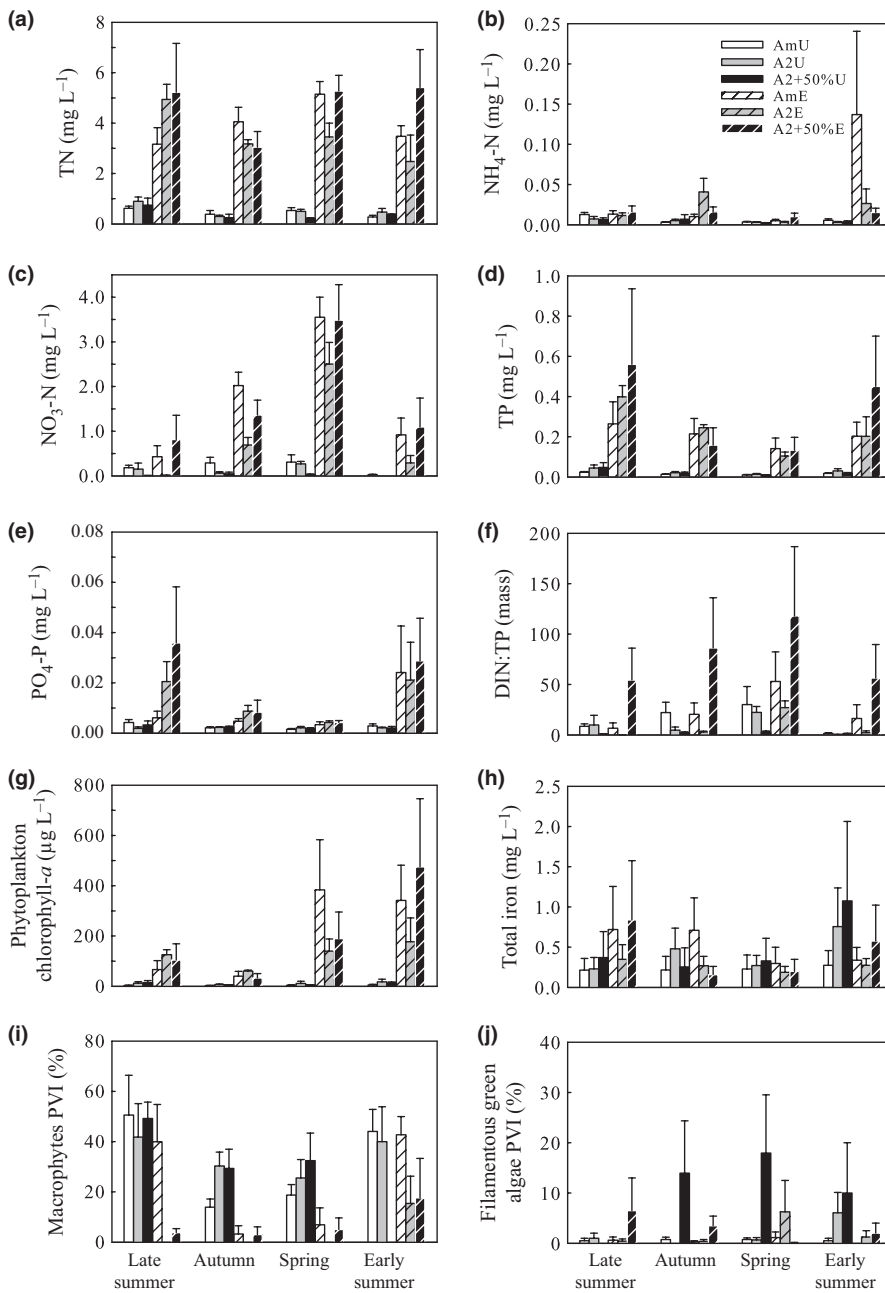Significance levels: *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$.

Fig. 3 Nutrient concentrations and biological parameters for the six mesocosm treatment combinations: (a) total nitrogen, TN; (b) ammonium, NH_4-N; (c) nitrate, NO_3-N; (d) total phosphorus, TP; (e) phosphate, PO_4-P; (f) dissolved inorganic nitrogen (DIN)/TP mass ratio; (g) phytoplankton chlorophyll *a*; (h) total iron; (i) macrophytes; (j) filamentous green algae. Data show mean ± 1 SE. For abbreviations, see Fig. 2.

concentrations were significantly higher in the enriched than in the unenriched mesocosms (Fig. 3g, Table 1). In the unenriched mesocosms, phytoplankton chlorophyll concentrations were relatively low (Am: 1.1–13.8 µg L^{-1}; A2: 1.2–47.4 µg L^{-1} and A2 + 50%: 2–39 µg L^{-1}). On average, phytoplankton Chl-*a* concentrations were 21 times higher in the enriched mesocosms (Am: 4.8–738 µg L^{-1}; A2: 15.5–412 µg L^{-1} and A2 + 50%: 2.8–1004 µg L^{-1}; Fig. 3g). Finally, macrophyte %PVI was significantly higher in the unenriched mesocosms (Fig. 3i, Table 1). The treatments did not differ in pH (Fig. 2b; e.g. mean: 8.7 and 8.8 for the unenriched and enriched

mesocosms, respectively), K_d (data not shown), dissolved inorganic nitrogen (DIN) (NO_3-N + NH_4-N)/TP mass ratio, total iron or filamentous green algae %PVI (Fig. 3f, h,j, Table 1).

Analyses of the quantitative response of periphyton growth

The quantitative nature of periphyton nutrient limitation was examined by considering the log-transformed RR-X values for all treatments as a function of time (nested ANOVAs factors: season, nutrient status, temperature WG as a nested factor; Figs 4 & 5, Table 2). None of the
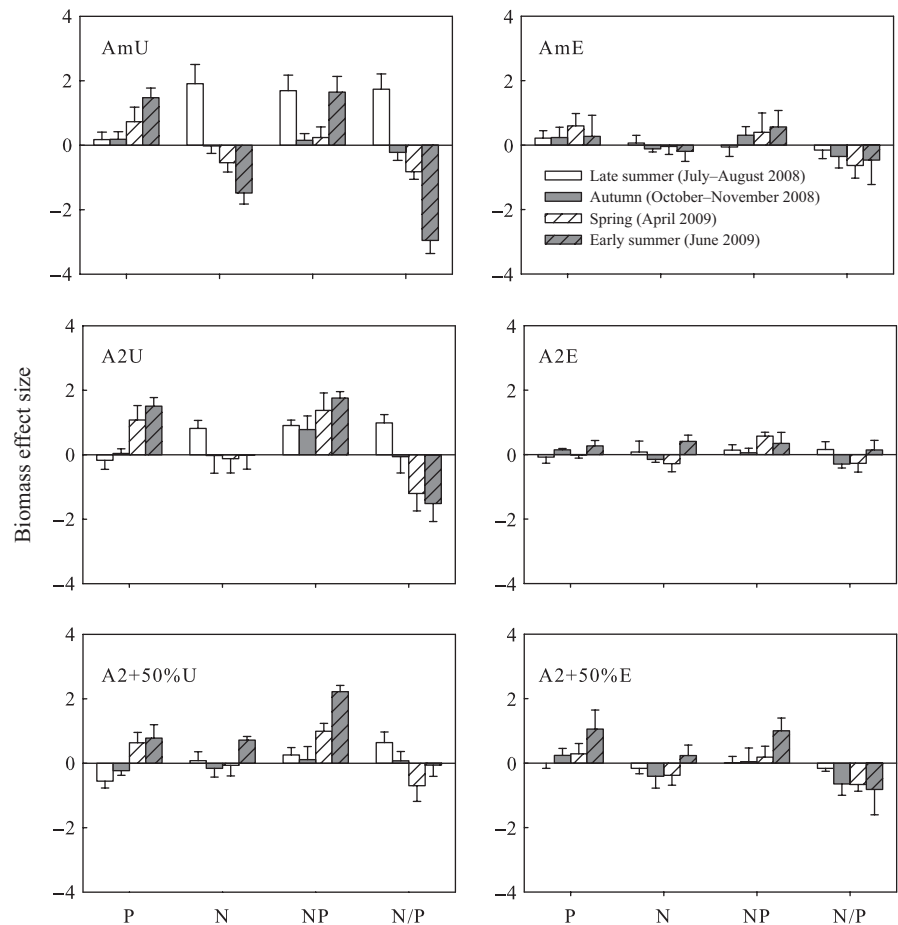
Fig. 4 Periphyton responses to N and/or P addition for mesocosms with two nutrient loadings (unenriched, enriched) and three temperatures (ambient, A2, A2 + 50%) given as the ratio of final chlorophyll concentration in the +P, +N or +N+ P relative to the chlorophyll concentration in the control in the without-grazer treatment (WOG). The response ratio, log RR, has no units. Relative response to P addition alone (P); relative response to N addition alone (N); relative response to combined N and P addition (NP); relative response to N versus P (N/P, equivalent to final Chl_N/final Chl_P). Data show mean ± 1 SE.

relative responses showed an effect of grazers (Table 2). The average log periphyton Chl-*a* relative response to N (log RR-N) differed between warmed and ambient mesocosms (being negative in the latter) in early summer. Moreover, the periphyton response to N addition was significantly higher in late summer in the unenriched ambient and A2 mesocosms compared to the A2 + 50% mesocosms (Table 2 and Tukey's test $P < 0.05$). The log relative response to P addition (RR-P) was significantly higher in the unenriched compared to the enriched mesocosms in spring and early summer (Table 2 and Tukey's test $P < 0.05$). Following the trends for single-nutrient additions, log RR-NP was higher in the unenriched mesocosms in spring and in early and late summer (Table 2 and Tukey's test $P < 0.05$). Moreover, log RR-NP was significantly higher in early summer in the A2 + 50% compared to the ambient mesocosms, and the opposite occurred in late summer (Table 2 and Tukey's test $P < 0.05$). To further examine the relative importance of N versus P limitation in the study mesocosms, for each experiment, an N versus P index was calculated using the log of the ratio RR-N to RR-P. This ratio is equivalent to the ratio of the final

periphyton chlorophyll concentrations in the +N and +P treatments, respectively (i.e. if the average ratio of the response to N is equal to the response to P, the log of that ratio is 0). This log N to P index (RR-N/RR-P) was significantly positive in late summer in the unenriched mesocosms (indicating N limitation), while stronger P limitation was observed in the unenriched mesocosms in spring for all climate scenarios and in the ambient and A2 scenarios in early summer (Table 2 and Tukey's test $P < 0.05$).

Relationships of relative responses to physical, chemical and biological parameters

We also considered the GLMs among various indices of nutrient limitation from the bioassays and nutrient availability measurements and biological and physical parameters. None of the models of nutrient limitation indicators included total iron concentrations, filamentous green algae %PVI or K_d in the mesocosms (Table 3). The final regression model obtained for RR-P included TN, TP, macrophyte %PVI and pH interacting with time (season) as significant covariates (Table 3). TN and RR-P
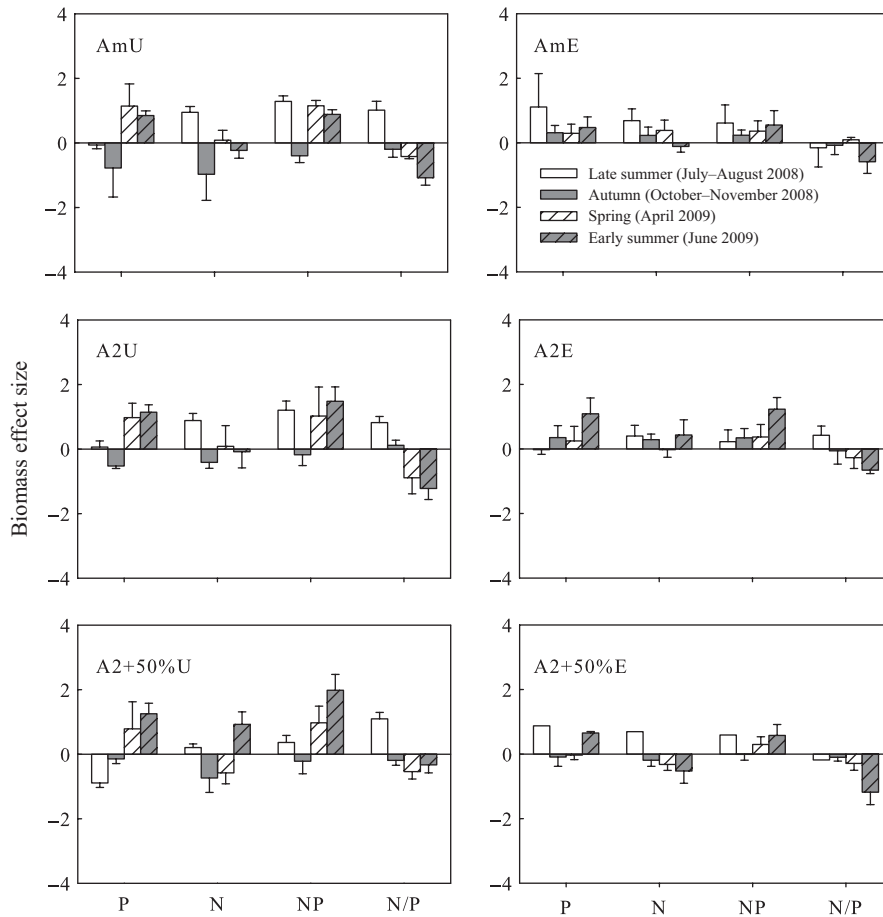
Fig. 5 Periphyton responses to N and/or P addition for mesocosms with two nutrient loadings (unenriched, enriched) and three temperatures (ambient, A2, A2 + 50%) given as the ratio of final chlorophyll concentration in the +P, +N or +N+ P to the chlorophyll concentration in the control in the with-grazer treatment (WG). For further details, see Fig. 4. Data show mean ± 1 SE.

Table 2 Results of repeated-measures ANOVA tests on various indices of nutrient limitation (log RR-X) using the effects of nutrients (two levels, unenriched and enriched), temperature (three levels, ambient, A2 and A2 + 50%) and season (four levels: late summer, autumn, spring and early summer) with grazers (without grazers, WOG versus with grazers, WG) as a nested factor

Treatments/RR-X	d.f.	F-values RR-P	F-values RR-N	F-values RR-NP	F-values RR-N/RR-P
Season	3	16.313***	11.604***	19.773***	32.471***
Nutrient	1	2.119 N.S.	1.461 N.S.	22.376***	2.131 N.S.
Temperature	2	0.723 N.S.	2.455 N.S.	0.672 N.S.	0.153 N.S.
Season × nutrient	3	5.537**	5.771**	6.119**	16.765***
Season × temperature	6	0.511 N.S.	5.362***	2.841*	1.135 N.S.
Nutrient × temperature	2	0.352 N.S.	1.034 N.S.	0.295 N.S.	1.551 N.S.
Season × Nutrient × Temperature	6	0.329 N.S.	3.010**	1.209 N.S.	2.485*
Season × Nutrient × Temperature (Grazers)	24	0.526 N.S.	1.041 N.S.	0.953 N.S.	0.997 N.S.

N.S., Not significant; RR-N, relative response to N; RR-P, relative response to P addition; RR-X, relative response.
Significance levels:*$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$.

showed a positive relationship, while RR-P was negatively related with macrophyte %PVI in spring and with TP and pH in early summer (Table 3). Mean monthly temperatures showed a positive relationship with RR-P in early summer. The final GLM for RR-N included time as a factor and the covariates TN and temperature interacting with season (Table 3). As is to be expected if nutrient limitation patterns were determined by changes in N availability, RR-N was negatively related with TN in late summer (Table 3). Also, RR-N and temperature were positively related in early summer and negatively related in late summer (Table 3). RR-NP was negatively related with TN in early and late summer, but was positively related with temperature in all seasons (Table 3). Following these trends, the index of N versus P limitation (RR-N/RR-P) and TN were positively related in

Table 3 Results of general linear models on the effects of TP, TN, total iron, phytoplankton Chl-*a*, macrophyte %PVI, filamentous green algae %PVI, pH, K_d and mean monthly temperature on log RR-Chl$_X$. The fixed factor season (time) was also included in the models

	d.f.	F-values	Partial coefficient	P-value
RR-P $R^2 = 0.930$				
Season	4	7.636	–	<0.0001
TN	1	9.256	0.171	0.004
TP × Season	4	14.450	–	<0.0001
Macrophyte %PVI × Season	4	3.935	–	0.006
pH × Season	4	8.117	–	<0.0001
TP : early summer	–	–	−0.467	<0.0001
Macrophyte %PVI : spring	–	–	0.647	<0.0001
pH : early summer	–	–	0.319	<0.0001
RR-N $R^2 = 0.818$				
Season	4	6.238	–	<0.0001
TN × Season	4	2.375	–	0.061
Mean monthly temperature × Season	4	5.745	–	<0.0001
TN : late summer	–	–	−0.248	<0.003
Mean monthly temperature : early summer	–	–	2.575	<0.005
Mean monthly temperature : late summer	–	–	−2.747	<0.0001
RR-NP $R^2 = 0.842$				
Mean monthly temperature	4	138.317	0.453	<0.0001
TN × Season	4	7.014	–	<0.0001
TN : early summer	–	–	−0.326	<0.0001
TN : late summer	–	–	−0.369	<0.0001
RR-N/P $R^2 = 0.804$				
TN × Season	4	7.168	–	<0.0001
Mean monthly temperature × Season	4	70.068	–	<0.0001
TN : late summer	–	–	−0.371	<0.0001
TN : early summer	–	–	0.137	0.027
Mean monthly temperature : early summer	–	–	0.174	<0.0001
Mean monthly temperature : late summer	–	–	0.405	<0.0001
Mean monthly temperature : autumn	–	–	0.251	<0.0001
Mean monthly temperature : spring	–	–	0.175	<0.0001

PVI, plant volume inhabited; RR-N, relative response to N; RR-P, relative response to P addition; TN, total nitrogen; TP, total phosphorus.

early summer and negatively related in late summer (Table 3), while during all seasons, RR-N/RR-P was positively related with temperature (Table 3).

Discussion

Our experiments clearly showed that periphyton responded differently to nutrient (N and P) addition depending on the nutrient status of the system and its warming scenario. Furthermore, these responses were time- (season-) sensitive. Not unexpectedly, increased nutrient loading seemed to shift the mesocosms into a regime where N and P supplies no longer limited the periphyton.

A meta-analysis of N and P limitation of primary producers in freshwater ecosystems by Elser *et al.* (2007) indicated that enrichment by either N or P can increase autotroph production, but that a simultaneous increase in both nutrients often leads to much higher levels of

production in nearly all situations, including benthic habitats. Harpole *et al.* (2011) provided further evidence for N as well as P limitation by showing that primary producers in freshwater systems are equally likely to be N- or P-limited and are most probably co-limited by both. Thus, the relative lack of a synergistic response to combined N and P enrichment in our study is somewhat surprising. We only observed a strict co-limitation (enhanced growth of periphyton only when N and P were added together, *sensu* Harpole *et al.*, 2011) in the warmest unenriched treatment in early summer.

N limitation of primary producers is more likely to occur if environmental factors limit nitrogen fixation (Howarth *et al.*, 1988; Vitousek & Howarth, 1991). In our investigation, N limitation occurred in the unenriched mesocosms under all temperature treatments, possibly due to the inability of N-fixing cyanobacteria to thrive in these systems where P concentrations were low. Indeed, Marcarelli & Wurtsbaugh (2006) found that the presence

of N fixers in oligotrophic streams was primarily limited by the supply of P and simultaneously controlled by water temperature. Furthermore, Jensen *et al.* (1994) analysed monitoring data from 94 systems, mainly shallow Danish lakes, and showed that dominance of N-fixing phytoplankton cyanobacteria was more driven by their high P affinity than by low concentrations of inorganic N. Another survey performed by Diaz *et al.* (2007) in Patagonian lakes with extremely low N / P ratios detected low abundance of phytoplankton with only occasional presence of N-fixing cyanobacteria and very low Chl-*a*/TP ratios, indicating absence of full compensation by N fixation.

Moreover, as hypothesised, we observed seasonal patterns in nutrient limitation with N limitation occurring earlier in the warmest climate scenario. We observed N limitation (actually N and P co-limitation) in the warmest scenario (A2 + 50%) in early summer in the unenriched mesocosms, while in late summer, N limitation occurred in ambient and A2 scenarios. We also found evidence of this trend in the positive relationship between RR-N and mean monthly temperature during early summer and an inverse relationship in late summer. A recent study by Veraart *et al.* (2011) demonstrated that a three-degree temperature rise may double denitrification rates due to a systematic decrease in oxygen concentrations with rising temperatures. Actually, we found that NO_3-N was lower in the A2 + 50% compared to the ambient scenario. Others (Kosten *et al.*, 2009; Özkan *et al.*, 2010), however, have observed accumulation of inorganic N in shallow lakes from warmer climates, indicating that the fate of N may differ among systems under the same climate warming scenario.

Our study showed P limitation for periphyton growth in spring (April) and early summer (June) in the unenriched mesocosms. The availability, in the water, of N (TN) appeared to be an important environmental factor controlling the extent of P limitation at any given time. The positive relationship in spring between the periphyton RR-P, macrophyte %PVI and pH, respectively, points to competition for P between the two primary producers, which makes sense as it is the period with strongest macrophyte biomass development (T.L. Lauridsen, unpublished data) and production (E. Jeppesen, unpublished data) in the mesocosms. The periphyton response to nutrient addition did not notably diminish under the warmer climate scenarios compared to the ambient scenario, which contradicts our hypotheses and differs as well from the findings of Kratina *et al.* (2012) on warming effects on eutrophication in experimental phytoplankton communities. Also, in agreement with previous work (e.g. Harpole *et al.*, 2011), we observed negative responses to addition of N and P. Unbalanced nutrient additions could have led to internal elemental imbalance [Liebscher's law of the optimum (Browne, 1942)], precipitating a negative effect such as growth inhibition. Our results showed statistically significant negative responses only in early summer for N addition in the ambient unenriched mesocosms (AmU); at this time, periphyton exhibited P limitation, and P and N+P supplies led to enhanced growth.

Periodically, the periphyton showed weak (not statistically significant) P limitation in the enriched mesocosms and no N limitation. This relative absence of nutrient limitation may be linked with increased fish-derived nutrient supply (Vanni, 2002; Vanni, Boros & McIntyre, 2013), as fish were abundant in this treatment, mimicking a condition found in shallow eutrophic lakes (Jeppesen *et al.*, 2000). It should be mentioned that in our study, the weekly nutrient loading may have also reduced nutrient limitation. In real temperate shallow lakes, external nutrient loading varies over the season, being lowest during summer. The TP concentration in summer is mainly driven by internal P loading (Boström, Jansson & Forsberg, 1982; Søndergaard *et al.*, 2002). By contrast, our results suggest that N limitation of periphyton may be of greater importance in summer in eutrophic lakes with low external N loading and perhaps stronger at higher temperatures due to enhanced denitrification. In support of this, in a study of northern shallow lakes, Weyhenmeyer *et al.* (2007) observed decreasing NO_3-N concentrations and ascribed these to a reduction in external N loading, including atmospheric deposition, and changes in climate (e.g. warmer temperatures, changes in the amount of water loading per unit of lake surface area). Thus, we conclude that our results are most representative for lakes with short retention times, and we speculate that P limitation in shallow eutrophic systems may occur in spring to early summer, while N limitation may happen in summer due to NO_3-depleted conditions (NO_3-N concentrations below 10 μg L^{-1}), particularly in a future warmer climate as suggested by Weyhenmeyer *et al.* (2007).

Periphyton productivity and nutrient limitation may be influenced by factors other than nutrients such as light and grazing (Steinman, 1996; Sterner *et al.*, 1997; Hillebrand & Kahlert, 2001; Hillebrand, 2005), and we therefore included both factors in our analyses. Light is the ultimate energy source for primary production and algal growth (Harris, 1980) and can modulate nutrient limitation (Sterner *et al.*, 1997), while grazing by various herbivorous species may dramatically reduce

periphyton biomass (Lamberti, Feminella & Resh, 1987; Jones & Sayer, 2003; Hillebrand, 2005) or alter the nutrient content or biomass-specific productivity of grazed compared to ungrazed periphyton (McCormick & Stevenson, 1991; Rosemond, Mulholland & Elwood, 1993). Interestingly and in opposition to our prediction, grazer presence did not affect the quantitative nutrient limitation results, which were consistent between grazer and grazer-free treatments. Also, in our study, none of the indices of nutrient limitation (RR-ChlX) related to light measurements (K_d). In addition, light showed no relationship with periphyton Chl-*a* during the experiments (data not shown). The absence of light effects, combined with the low ambient nutrient concentrations in the unenriched mesocosms, may explain the observed strong responses of periphyton to nutrient addition.

In recent years, the concept of resource limitation has shifted from an earlier paradigm of single-resource limitation towards concepts of co-limitation by multiple resources. However, synergistic interactions of N and P as limiting resources were uncommon for periphyton in our experimental temperate shallow lakes, indicating that co-limitation is not a constraint for these primary producers. Nonetheless, important changes in N and P loading to lakes can be expected with global warming, with consequent impacts on the ecological state and water quality of surface waters (Jeppesen *et al.*, 2009, 2011). Our results indicate that warming shifted the seasonality of N limitation and turned the single-nutrient limitation of periphyton into NP co-limitation in unproductive lakes. This last observation indicates that warming reduces the sensitivity of temperate shallow lakes to bottom-up perturbations.

Acknowledgments

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