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Effects of N : P loading ratios on phytoplankton community composition, primary production and N fixation in a eutrophic lake

TOBIAS VREDE*, ASHLEY BALLANTYNE†, CECILIA MILLE-LINDBLOM*, GRETE ALGESTEN*, CRISTIAN GUDASZ‡, SANDRA LINDAHL* AND ANNA KRISTINA BRUNBERG*

**Department of Ecology and Evolution/Limnology, Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden*
†Department of Civil and Environmental Engineering, University of Washington, Seattle, WA, U.S.A.
‡Department of Taxonomy and Ecology, Babes-Bolyai University, Cluj-Napoca, Romania

SUMMARY

1. The aim of this study was to assess the effects of different nitrogen (N) to phosphorus (P) loading ratios on phytoplankton community composition and primary production in a naturally eutrophic lake. Furthermore, the sources of N fuelling primary production were estimated using ^{15}N stable isotope tracers.
2. A mesocosm experiment was performed with the same amount of P added to all mesocosms (similar to internal loading rates) but with a range of N additions (0–86 μM N), resulting in a gradient of N : P supply ratios.
3. Low N : P supply ratios resulted in a significant shift in the phytoplankton assemblage to a community dominated by N-fixing cyanobacteria and a supply of atmospheric N_2 estimated to be up to 60% of total supply.
4. The N : P loading ratio had no significant effect on primary production, total nitrogen (TN) concentration or particulate N concentration.
5. Our results imply that a reduced N : P ratio of the nutrient load does not necessarily result in a lower TN concentration and downstream N export due to compensation by N-fixing cyanobacteria.

Keywords: ^{15}N stable isotopes, eutrophication, nitrogen, phosphorus, stoichiometry

Introduction

Human activities have greatly altered the biogeochemical cycles of nitrogen (N) and phosphorus (P). In the case of N, artificial fertilizers, fossil fuel combustion, N-fixing crops and N-mobilization have increased the amount of N available in the biosphere. For example, it has been estimated that the amount of atmospheric N_2 fixed has doubled due to industrial fixation (Vitousek *et al.*, 1997). Furthermore, atmospheric N deposition has increased threefold, mainly as a result of fossil fuel combustion (Galloway & Cowling, 2002). This anthropogenically caused increase in N availability is reflected by a positive correlation between riverine N discharge and human

Correspondence: Tobias Vrede, Department of Ecology and Environmental Science, Umeå University, SE-90187 Umeå, Sweden. E-mail: tobias.vrede@emg.umu.se

Present address: Ashley Ballantyne, Department of Geological Sciences, University of Colorado, Boulder, CO, U.S.A.

Present address: Grete Algesten, The County Administrative Board in Värmland, Karlstad, Sweden.

Present address: Cristian Gudasz, Department of Ecology and Evolution/Limnology, Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden.

population density within catchments (Howarth *et al.*, 1996). One of the main environmental effects of the increased load of non-point source P and N is that many lakes and coastal areas have become enriched with nutrients (Carpenter *et al.*, 1998). Abundant research has been conducted on eutrophication, nutrient availability and productivity in freshwater ecosystems, and P has often been considered the ultimate limiting nutrient (Schindler, 1977; Vollenweider & Kerekes, 1982). However, several studies also indicate that primary production can be N-limited or co-limited by N and P (Elser, Marzolf & Goldman, 1990; Jansson *et al.*, 1996; Vrede *et al.*, 1999), and recent studies indicate that increased atmospheric N deposition may result in a shift towards P limitation of freshwater ecosystems (Bergström, Blomqvist & Jansson, 2005; Bergström & Jansson, 2006). Since the export of nutrients, and in particular N, from terrestrial and freshwater ecosystems to marine areas has also contributed to eutrophication of coastal areas (Howarth & Marino, 2006), reducing the N load has been considered as a way to ameliorate coastal eutrophication. However, since lakes are often the primary recipients of both point and non-point sources, we must consider what the effects of such a strategy would be on freshwater ecosystems.

A reduction of the N load without a proportional reduction of the P load will result in lower N : P ratios, ultimately leading to N-limitation of primary producers. The N : P ratio is a good predictor of the phytoplankton community structure in freshwater ecosystems. Lakes with N : P molar ratios below approximately 60 are frequently dominated by N-fixing cyanobacteria that are able to access the abundant supply of atmospheric N_2 (Smith, 1983; Brettum, 1989), although other phytoplankton taxa can also dominate in lakes with low N : P ratios, both in oligotrophic (Diaz *et al.*, 2007) and hypertrophic (Jeppesen *et al.*, 2005) systems. Cyanobacterial taxa are often associated with eutrophication (Edmondson & Anderson, 1956), release of toxins and an overall decline in water quality (Welch & Lindell, 1992). Because freshwater phytoplankton assemblages are sensitive to alterations of the N : P supply ratio, reductions of N loading intended to ameliorate eutrophic conditions will likely induce a shift in phytoplankton community composition. Since changes in the phytoplankton community may have strong effects on higher trophic levels as well as on nutrient fluxes and system metabolism, elucidating phytoplankton community response to altered nutrient supply ratios or availability is critical for our understanding and management of aquatic ecosystems.

The objective of this study was to test experimentally the effects of different N : P supply ratios on (i) primary production; (ii) phytoplankton community composition; (iii) inorganic and organic N concentrations and (iv) the contribution of fixed atmospheric N_2 to particulate organic N, which was estimated using a stable N isotope tracer technique. The study was performed in mesocosms within a eutrophic lake. Our working hypothesis was that a reduction in the loading of N relative to P would result in dominance of cyanobacteria capable of fixing atmospheric N_2. Thus, a reduction of N load would actually result in lower water quality due to increasing dominance of N-fixing cyanobacteria, but no decrease in total nitrogen (TN) or particulate N concentrations.

Methods

Study site and field sampling

Field experiments were conducted in temperate, eutrophic Lake Limmaren, Sweden (59°43′N, 18°43′E). Lake Limmaren has a surface area of 5.9 km^2, a maximum depth of 7.8 m, a mean depth of 4.7 m and a mean water residence time of 5.8 years (Brunberg & Blomqvist, 2000). The lake is normally ice-covered during winter and, because of its size and shallow depth, the lake is not thermally stratified during summer. Although relatively shallow, the lake morphometry prevents extensive colonization of macrophytes as only 13% of the lake area is less than 2 m deep (Pettersson & Lindqvist, 1993).

To characterize the lake in terms of nutrient status, water chemistry and phytoplankton community composition, samples were collected from June 1998 to August 2000. The sampling frequency was once every second week from May to September and once per month from October to April (a few winter samples were not taken due to thin ice). Water for a depth-integrated composite sample was collected with a 2 m plexiglass tube at five sampling stations (two to three tubes per station), and pooled in a large bucket. Subsamples for phytoplankton biomass, chlorophyll-*a*, N, P and water colour analyses were then drawn from the composite sample. The net accumulation rate

of P in the pelagic zone was calculated as the difference between summer maximum and minimum total phosphorus (TP) concentration divided by the time between these sampling occasions. Since the anthropogenic sources of P are very small, the lake has a long water renewal time, the discharge is low during summer and surface sediments are frequently resuspended in this relatively shallow lake, we consider this net accumulation to be an estimate of internal loading from the sediments.

Mesocosm experiment

A mesocosm experiment was conducted in Lake Limmaren from 21 June to 10 July 2000. The enclosures were polyethylene bags (Noax, Stockholm, Sweden) which were closed to the sediment but open to the atmosphere. The cross-section area of the bags was 1.0 m^2 and the total length 3.0 m, of which 0.5 m was above the water level. The enclosures were filled with 2.5 m^3 surface water and attached to wooden frames anchored to polystyrene foam pontoons. The treatments were assigned in random order to the enclosures. To manipulate the N and P concentrations, and to achieve different N : P ratios, different doses of N were applied to the mesocosms whereas the same amount of P was added to all bags. The P dose was dimensioned to mimic ambient P loading rates in the lake.

The experimental design consisted of three treatments performed in triplicate: no N addition, addition of totally 43 μM N and addition of totally 86 μM N. N was added as $Ca(NO_3)_2$ with a $\delta^{15}N$ isotopic signature of 33.9 ± 1.7‰ (mean ± SD), which is easily distinguishable from the reference standard atmospheric N_2 ($\delta^{15}N$ = 0 ‰). To all bags, totally 1.9 μM P was added as KH_2PO_4, corresponding to an average P loading rate of 100 nM P day^{-1}. The nutrient additions thus had N : P molar ratios of 0, 23 and 45. The nutrients were added in four equally sized batches (on the 1st day of the experiment, and then every 5th day). Each nutrient addition thus consisted of 0.48 μM P (to all nine mesocosms) and 0, 11 or 21 μM N (to three mesocosms each). Because planktivorous fish are important components of the pelagic food-web, both as predators and nutrient regenerators (Vanni & Layne, 1997), roach (*Rutilus rutilus* L.) were added to the mesocosms to make the system response more realistic. Roach were caught in the littoral zone with a fine-mesh hand-held trawl, and three individuals of similar size (48 ± 5 mm average fork length ± SD) were added to each enclosure. Samples for chemical and biological analyses were taken on five occasions, immediately before the nutrient addition, except for the initial samples for TN, particulate C and N, and $\delta^{15}N$ analyses, which were taken immediately after nutrient addition. The mesocosms were thoroughly mixed before water was sampled with a 2 m plexiglass tube sampler.

Analyses

Dissolved and TN and TP concentrations were analysed on a flow injection analyser with a spectrophotometric detector (FIAstar Foss Teacator, Hillerød, Denmark). Ammonium was analysed with the hypochlorite method (Strickland & Parsons, 1972). Nitrate plus nitrite (NO_3 + NO_2) was determined with the sulphanilamide method after reduction in a cadmium column (Wood, Armstrong & Richards, 1967). Soluble reactive phosphorus (SRP) was analysed with the molybdate-blue method (Murphy & Riley, 1962). For measurements of TP and TN, samples were oxidized with 5% potassium persulphate for 60 min in an autoclave (Menzel & Corwin, 1965) and P and N were then measured by the molybdate-blue and sulphanilamide methods respectively. Water colour was measured on filtered water (GF/C glass fiber filters; Whatman, Springfield Mill, U.K.) using a colour comparator (Lovibond; Tintometer GmbH, Dortmund, Germany) calibrated against standard solutions containing K_2PtCl_6 and $CoCl_2{\cdot}6H_2O$. Samples for chlorophyll-*a* determinations were collected on GF/C glass fibre filters, which were stored in a freezer. Chlorophyll-*a* was extracted in 95% ethanol and measured spectrophotometrically (Strickland & Parsons, 1972). For analysis of phytoplankton biomass, 100 mL of water was preserved with Lugol's solution. A 1.1 mL sample was sedimented and phytoplankton were counted with an inverted phase contrast microscope (Olrik *et al.*, 1998). Phytoplankton biovolume was calculated from species-specific geometric formulas, and converted into carbon biomass according to Olrik *et al.* (1998).

Primary production was measured with the ^{14}C-method with acidification and bubbling (Schindler, Schmidt & Reich, 1972; Bell & Kuparinen, 1984). Duplicate 60 mL light bottles and one 60 mL dark bottle were filled with water from each mesocosm, spiked with 1 μCi $NaH^{14}CO_3$ (Amersham

CFA3) and incubated at 0.5 m depth for 3 h in the early afternoon each sampling day. Adding formaldehyde to a final concentration of 0.4% stopped the incubations. Two 1.5 mL subsamples from each incubation bottle were acidified with 50 μL 0.5 M HCl, and the vials were shaken for 1 h before 15 mL scintillation cocktail (Pharmacia Wallać Optiphase Hisafe 2, Perkin Elmer, Waltham, MA, U.S.A.) was added. The ^{14}C activity was measured in a scintillation counter (Rackbeta 1217; LKB Wallac, Turku, Finland) at least 24 h after the addition of scintillation cocktail. To measure the amount of ^{14}C added, another 1.5 mL subsample was taken from each bottle, and 50 μL NaOH and 15 mL scintillation cocktail was added. The ^{14}C activity was measured as above. Internal standards (0.05 μCi; Wallac) were used to estimate counting efficiency.

For analysis of the N stable isotope signature and particulate C and N of seston, 80–400 mL samples were filtered onto pre-combusted (550 °C, 2 h) GF/C glass fibre filters which were air-dried and packed into tin capsules. On all sampling dates, the water was pre-filtered through a nylon net with 40 μm mesh size to remove zooplankton (which are likely to have a higher $\delta^{15}N$ signature than phytoplankton due to trophic enrichment of ^{15}N). On the last two sampling dates, additional samples with unfiltered water were also taken. The samples were analysed on a Thermo Finnegan Delta Plus continuous-flow isotope ratio mass spectrometer in line with a Carlo Erba elemental analyser (Thermo Fisher Scientific, Waltham, MA, U.S.A.). All data of stable N isotope ratios are reported relative to an atmospheric ^{15}N standard according to convention as $\delta^{15}N$ (‰):

$$\delta^{15}N = \frac{{}^{15}N_{sample}/{}^{14}N_{sample} - {}^{15}N_{standard}/{}^{14}N_{standard}}{{}^{15}N_{standard}/{}^{14}N_{standard}} \times 1000 \quad (1)$$

Calculations and statistical analyses

Average values of seston $\delta^{15}N$ measured on each sampling date were used for calculating the fractional contribution of various sources of N. In the treatment with no addition of nitrate, the contribution of fixed atmospheric N_2 to seston N was calculated with a conventional two end member isotopic mixing model (Robinson, 2001):

$$f_{N2} = \frac{\delta^{15}N_{sample} - \delta^{15}N_{initial}}{\delta^{15}N_{N2} - \delta^{15}N_{initial}} \quad (2)$$

where f_{N2} is the fractional contribution of fixed N_2 in the sample, $\delta^{15}N_{sample}$ is the isotopic signature of the sample, $\delta^{15}N_{initial}$ is the initial isotopic signature of seston and $\delta^{15}N_{N2}$ is the isotopic signature of fixed N_2 ($\delta^{15}N_{N2} = 0‰$). In the treatments with nitrate addition, the sources contributing to N in the sample are three: seston present initially, fixed atmospheric N_2 and N from added nitrate ($\delta^{15}N = 33.9‰$). Because the sources are one too many to allow a unique solution to a mixing model, we used the approach of Phillips & Gregg (2003) to calculate possible solutions of the proportional contribution of these sources. The calculations were made with ISOSOURCE version 1.1 (US EPA, Western Ecology Division, Corvallis, OR, U.S.A.). A source increment of 1% and a mass balance tolerance of 1.5‰ (equal to the average within treatment SD) were used (Phillips & Gregg, 2003). In many cases, the solutions had very broad frequency distributions for the three N sources. Therefore, the solutions were further constrained by using particulate N data to select solutions that met the following mass balance criterion:

$$\frac{N_{sample}}{N_{initial}} = \frac{f_{initial} + f_{N2} + f_{NO3}}{f_{initial}} \quad (3)$$

where N_{sample} is the N concentration in seston, $N_{initial}$ is the initial seston N concentration and f is the fractional contribution of initial seston N, fixed N_2, and added nitrate to the particulate N in the sample. For each treatment and date, a 95% confidence interval for this ratio was estimated: 1000 random values of N_{sample} and $N_{initial}$ (normal distribution, average and SD obtained from the particulate N measurements) were generated with JMP version 5.0.1 (SAS Institute, Cary, NC, U.S.A.). The ratio $N_{sample} \times N_{initial}^{-1}$ was calculated, and the 97.5% and 2.5% percentiles of the distribution of the calculated ratios were used as upper and lower limits for selecting allowable combinations of $(f_{initial} + f_{N2} + f_{NO3}) \times f_{initial}^{-1}$ among the possible solutions. It should be noted that the estimates of fractional contribution of different sources above assume no isotopic fractionation. This assumption is not strictly valid, and therefore the estimates should be regarded as approximations.

Correlations between parameters were tested with the Spearman's rank correlation coefficient, ρ (Conover, 1980). The responses of TN and particulate N and of phytoplankton biomass and

production in the mesocosm experiment were analysed with time-series ANOVA (Quinn & Keough, 2002). The statistical analyses were performed using JMP version 5.0.1.

Results

Study site

Lake Limmaren is a eutrophic and weakly coloured lake with a relatively low TN : TP ratio and very high chlorophyll concentration (Table 1, Fig. 1). TP and TN concentrations exhibit a high seasonal variation, with the highest concentrations occurring during late summer (Fig. 1a). TN, TP and chlorophyll concentrations were all strongly correlated (ρ = 0.84–0.86, $P < 0.0001$). The net accumulation of P in the pelagic system was 23 nM P day^{-1} in 1998, 71 nM P day^{-1} in 1999 and 39 nM P day^{-1} in 2000. The TN : TP ratio varied approximately one order of magnitude with maximum values occurring during winter and summer values of approximately 40 (Fig. 1b). The dissolved inorganic N (DIN) to TP ratio showed a similar pattern with high ratios during winter, and very low ratios during summer when DIN concentrations are very low (Fig. 1b). The summer phytoplankton biomass was very high, and the community was dominated by N-fixing cyanobacteria [Cyanophyceae, mainly *Aphanizomenon flos-aquae* (L.) and *Anabaena* spp.], non-N-fixing cyanobacteria (Cyanophyceae, mainly *Microcystis* spp.) and diatoms (Bacillariophyceae, mainly *Stephanodiscus* spp.) (Fig. 1c). There are conspicuous differences in the relative abundance of different phytoplankton taxa in the different years that appear to be connected to the net accumulation of P in the pelagic; the higher the TP increase in the water column, the more N-fixing cyanobacteria appear.

Table 1 Summer (June–August) average concentrations of total nitrogen (TN), total phosphorus (TP), TN : TP ratio, dissolved inorganic nitrogen (DIN) to TP ratio, chlorophyll-*a* concentration and water colour in Lake Limmaren, 1998–2000

		Average ± SD	*n*
TP	μM	2.5 ± 1.5	19
TN	μM	85 ± 23	19
TN : TP	mole : mole	39 ± 9	19
DIN : TP	mole : mole	0.9 ± 1.3	19
Chlorophyll-*a*	μg L^{-1}	32 ± 20	19
Water colour	mg Pt L^{-1}	20 ± 7	18

SD, standard deviation; *n*, number of samples.

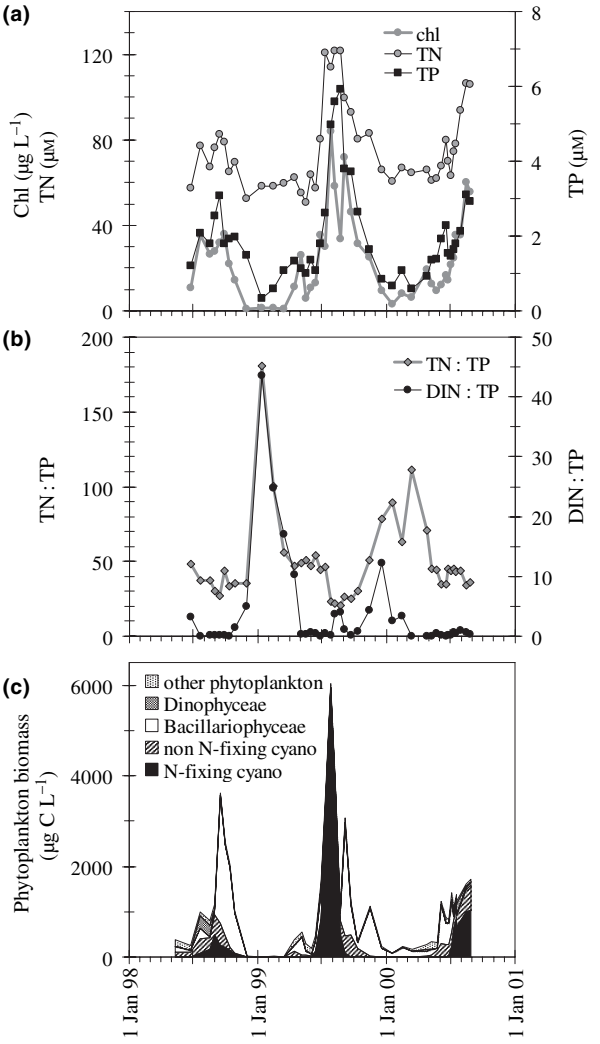

Fig. 1 Nutrients and phytoplankton in Lake Limmaren from June 1998 to August 2000. (a) Concentrations of chlorophyll-*a* (chl), total phosphorus (TP) and total nitrogen (TN); (b) TN : TP molar ratio, dissolved inorganic nitrogen to TP molar ratio; (c) phytoplankton biomass.

Mesocosm experiment

There was a clear indication of N fixation in response to low N : P ratios in the mesocosm experiment. The TN concentration approximately doubled during the experiment from 70 to 140 μM N in all mesocosms regardless of treatment (Fig. 2a). The effect of time was highly significant, whereas the treatment effect was non-significant, and the treatment × time interaction effect was barely significant (Table 2). The particulate N concentrations (<40 μm) showed a similar pattern, with a highly significant three- to fourfold increase during the

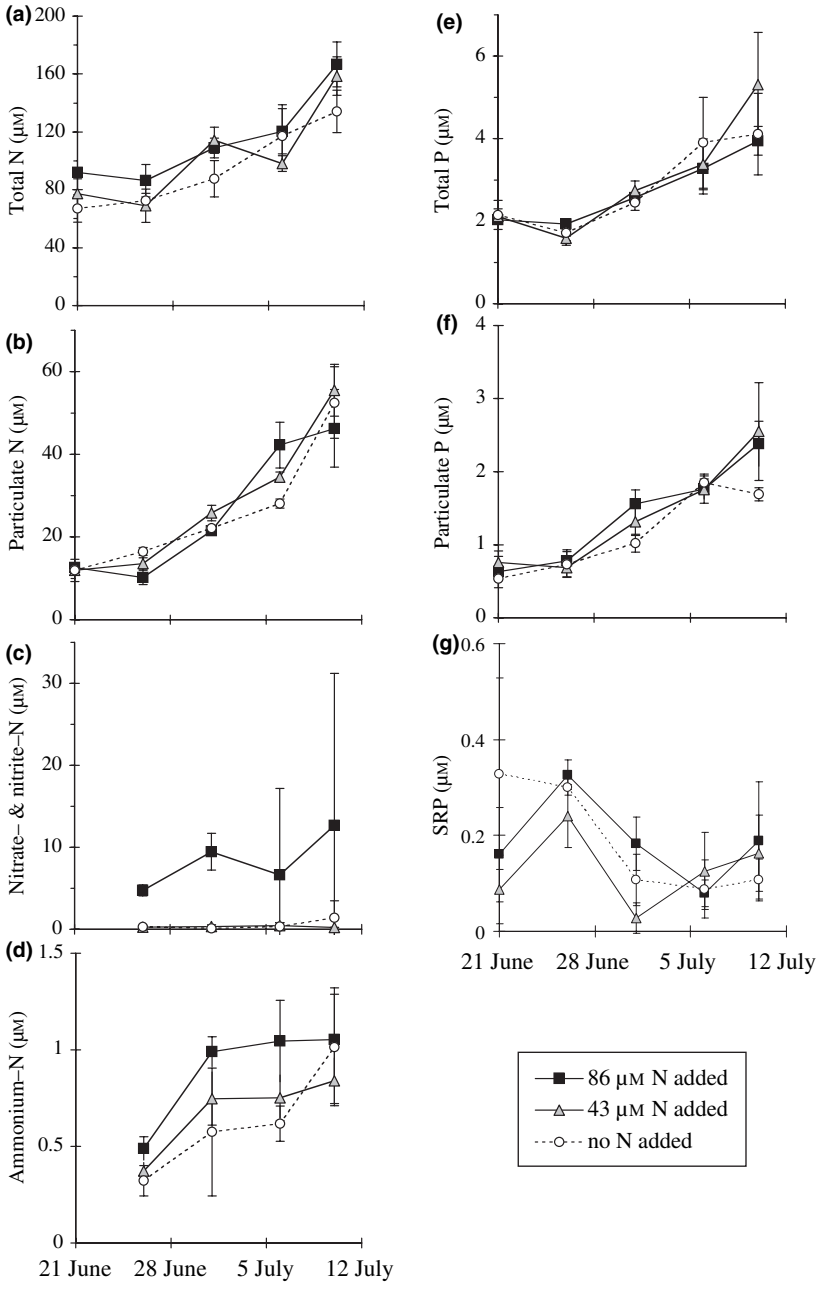

Fig. 2 Nitrogen (N) and phosphorus (P) concentrations over time in the mesocosm experiment. (a) total N; (b) particulate N, <40 μm fraction; (c) nitrate plus nitrite; (d) ammonium; (e) total P; (f) particulate P, <40 μm fraction; (g) soluble reactive P. Note different scales on *Y*-axes. Each data point represents the mean of three mesocosms and error bars show SD.

experiment, and non-significant treatment and treatment × time effects (Fig. 2b, Table 2). The N-fixation rate in treatments without N addition was 8.4 mmole N m^{-2} day^{-1} over the entire experimental period. Initial nitrate plus nitrite and ammonium concentrations were not measured in the mesocosms, but lake samples from 20 June had a nitrate plus nitrite concentration of 0.14 μM N and an ammonium concentration below 0.36 μM N. Nitrate plus nitrite concentrations remained very low throughout the experiment except in the treatment with the highest nitrate dose (Fig. 2c). Ammonium concentrations increased in all treatments during the experiment (Fig. 2d). As a result of the additions of P, both TP (Fig. 2e) and particulate P (Fig. 2f) increased significantly throughout the experiment (Table 2) In contrast, SRP did not increase over time (Fig. 2g, Table 2). TP, particulate P and SRP concentrations did not differ among treatments (Table 2).

As a consequence of the increase in both particulate N and particulate P in all treatments, the C: N : P stoichi-

Table 2 Results from repeated measures ANOVA of the response of nitrogen, phosphorus, phytoplankton and δ^{15}N isotope ratio in the mesocosm experiment

Response variable	Source of variation	*F*	Wilk's λ	d.f.$_{num}$	d.f.$_{den}$	*P*-value
TN	Treatment	0.78		2	6	0.18
	Time	168		4	3	0.001
	Time × treatment		0.017	8	6	0.032
PartN	Treatment	1.55		2	6	0.06
	Time	153		4	3	0.001
	Time × treatment		0.026	8	6	0.06
TP	Treatment	0.08		2	6	0.92
	Time	48		4	3	0.005
	Time × treatment		0.022	8	6	0.05
PartP	Treatment	4.8		2	6	0.06
	Time	142		4	3	0.0009
	Time × treatment		0.069	8	6	0.19
SRP	Treatment	2.91		2	6	0.13
	Time	18.8		4	3	0.02
	Time × treatment		0.058	8	6	0.16
Total phyto	Treatment	0.569		2	6	0.26
	Time	37.4		2	5	0.0001
	Time × treatment		0.538	4	10	0.49
N-fix cyano	Treatment	4.72		2	6	0.005
	Time	119		2	5	<0.0001
	Time × treatment		0.075	4	10	0.007
non-N-fix cyano	Treatment	1.35		2	6	0.077
	Time	12.5		2	5	0.0015
	Time × treatment		0.176	4	10	0.051
Bacillario	Treatment	0.242		2	6	0.52
	Time	0.840		2	5	0.22
	Time × treatment		0.726	4	10	0.78
Other phyto	Treatment	2.05		2	6	0.035
	Time	5.61		2	5	0.0089
	Time × treatment		0.346	4	10	0.22
Prim prod	Treatment	0.861		2	6	0.16
	Time	30.1		4	3	0.01
	Time × treatment		0.233	8	6	0.62
δ^{15}N	Treatment	15.7		2	6	0.0002
	Time	68.9		4	3	0.004
	Time × treatment		0.0004	8	6	0.0002

The treatment (none, medium or high nitrate addition) is the fixed factor.
The response variables: TN, total N concentration; PartN, particulate N < 40 μm; TP, total P concentration; PartP, particulate *P* < 40 μm; SRP, soluble reactive phosphorus; Total phyto, total phytoplankton biomass; N-fix cyano, biomass of N-fixing Cyanophyceae; non-N-fix cyano, non-N-fixing Cyanophyceae; Bacillario, Bacillariophyceae; Other phyto, other phytoplankton, mainly consisting of green algae; Prim prod, primary production; δ^{15}N, δ^{15}N in seston <40 μm.

ometry of seston changed in a similar fashion in all treatments (Fig. 3). The particulate C : N ratio in the <40 μm fraction was initially 10.2 ± 0.5 (average molar ratio of all enclosures ± SD). At the conclusion of the experiment, particulate C : N ratios had decreased in all treatments and were 6.7 ± 0.3 in the treatment without N addition, 8.0 ± 0.7 in the treatment receiving 43 μM N, and 7.9 ± 0.8 in the treatment receiving 86 μM N (Fig. 3a). This general decrease over time was statistically significant (repeated measures ANOVA, $P = 0.02$), but neither treatment nor treatment × time were significant (repeated measures ANOVA, $P = 0.23$ and 0.37, respectively). The particulate C : P ratio was on average 208 ± 65 initially and 183 ± 53 at the end of the experiment, and there were no statistically significant effects (repeated measures ANOVA, $P = 0.32$) (Fig. 3b). The particulate N : P ratio was initially 20.4 ± 6.2, decreased slightly to 18.4 ± 4.0 on 1 July, and then increased to a final ratio of 24.6 ± 7.4, but there was no statistically significant treatment effect on the N : P ratio (repeated

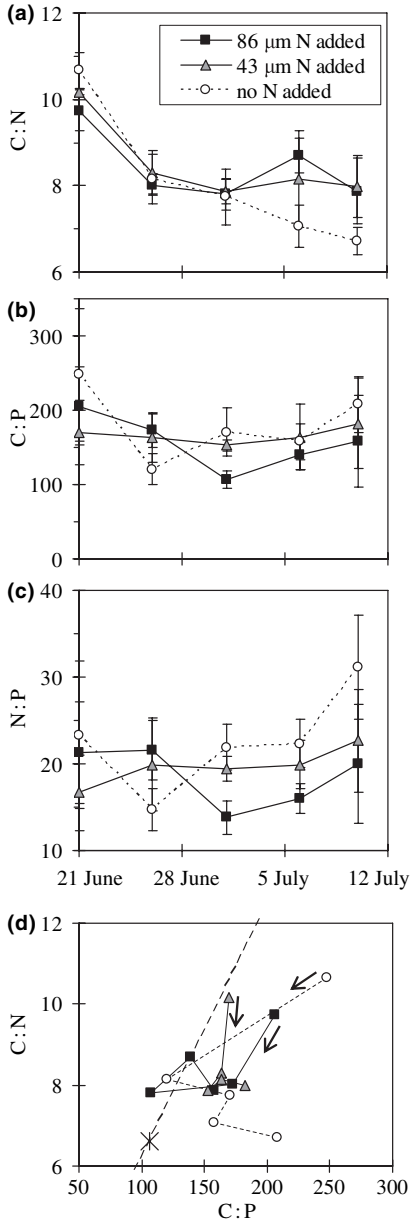

Fig. 3 C : N : P stoichiometry of particulate organic matter <40 μm in the mesocosm experiment. (a) molar C : N ratios over time; (b) molar C : P ratios over time; (c) molar N : P ratios over time; (d) scatter plot of C : N over C : P with the broken line indicating the Redfield N : P ratio (16 : 1) and the cross the Redfield C : N : P ratio (106 : 16 : 1). Arrows indicate direction of time sequence from start of the experiment. Each data point represents the mean of three mesocosms and error bars show SD.

measures ANOVA, $P = 0.07$) (Fig. 3c). When C : N and C : P were plotted against each other, it is evident that the seston stoichiometry tended to approach the Redfield ratio, especially in the N addition treatments. The no N addition treatment, however, deviated in having a lower final C : N and higher final C : P ratio than the other treatments (Fig. 3d).

A second line of evidence for substantial N-fixation was the change in phytoplankton community composition. The total phytoplankton biomass was initially 1270 ± 420 μg C L^{-1} (mean ± SD, $n = 9$), and the community was dominated by diatoms, which constituted 75 ± 6% of the total biomass (Fig. 4). The total biomass increased strongly during the course of the experiment, but neither the treatment effect nor the treatment × time effect were significant (Fig. 4a, Table 2). The phytoplankton community composition changed over time. This was mainly an effect of the increase of N-fixing and non N-fixing cyanobacteria, and other phytoplankton (dominated by green algae) in the N addition treatments, as well as an increase of N-fixing cyanobacteria in the treatment with no N addition (Fig. 4, Table 2). At the conclusion of the experiment, N-fixing cyanobacteria constituted 50 ± 12% of the phytoplankton biomass in treatments without N addition, whereas N-fixing cyanobacteria constituted 32 ± 4% and 25 ± 1% of the biomass in treatments receiving N additions. The primary production varied between approximately 100 and 800 μg C L^{-1} h^{-1} during the experiment with a general increase during the experiment, but there was no significant treatment or treatment × time effect (Fig. 4f, Table 2).

Stable isotope data clearly show that N-fixation rates were higher in mesocosms with low N : P supply ratios. During the course of the experiment, phytoplankton was reliant on N sources with distinctly different $\delta^{15}N$ signatures. This is reflected by the temporal development of the $\delta^{15}N$ in seston (Fig. 5). Initially, the average $\delta^{15}N$ signature was between 9.9 ± 0.8‰ and 13.2 ± 1.3‰ in seston <40 μm (Fig. 5a). In the treatment without N addition, the $\delta^{15}N$ decreased to 5.6 ± 0.5‰ on 1 July, indicating substantial fixation of atmospheric N_2. The $\delta^{15}N$ then remained low throughout the experiment. In the treatments with nitrate addition, $\delta^{15}N$ first increased, indicating uptake of the ^{15}N enriched nitrate. During later phases of the experiment, the increase in $\delta^{15}N$ levelled off at 20‰ in the treatment with the highest nitrate addition, and decreased to 12‰ in the treatment with the lower nitrate addition. The effects of treatment, time and treatment × time on $\delta^{15}N$ were all significant (Table 2). Isotopic measurements of hand-picked colonies of *Anabaena* spp. from the treatment

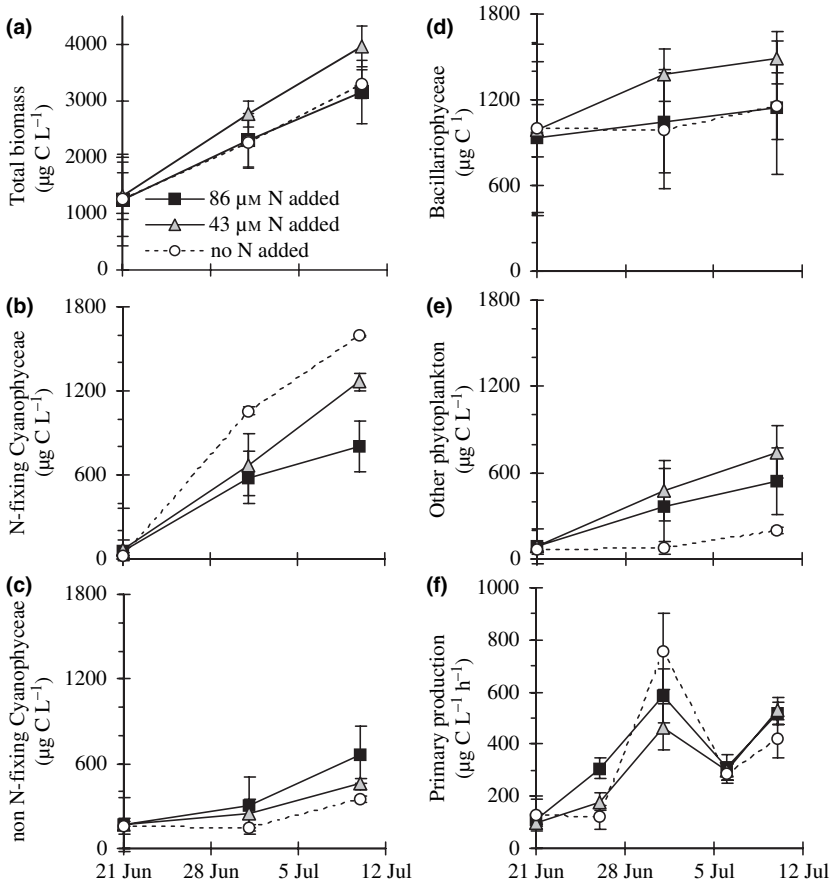

Fig. 4 Phytoplankton biomass and primary production in the mesocosm experiment. (a) total phytoplankton biomass; (b) biomass of N-fixing cyanobacteria; (c) biomass of non N-fixing cyanobacteria; (d) diatom biomass; (e) biomass of other phytoplankton; (f) primary production. Each data point represents the mean of three mesocosms and error bars show SD. Note different scales on *Y*-axes.

with no N addition yielded $\delta^{15}N$ values of 1.9 ± 3.4‰ towards the end of the experiment, reflecting the atmospheric ^{15}N signature. The $\delta^{15}N$ signature of total seston was similar to the $\delta^{15}N$ signature in the <40 μm fraction on the two sampling dates it was measured (Fig. 5b). The estimated fractional contribution of fixed N_2 to seston N increased in the treatment without N addition. It reached a maximum of 60% on 1 July, and then decreased slightly (Fig. 6). In the treatment with 43 μM N added, the fractional contribution of fixed N_2 increased throughout the experiment, eventually becoming the major source, constituting 38–55% of the particulate N. In contrast, fixed N_2 was the least important N source in the treatment with 86 μM N added, constituting 6–34% of the particulate N at the end of the experiment, and much less on previous sampling dates.

Discussion

Like many other lakes in lowland areas of the Baltic Sea catchment, Lake Limmaren is geologically young and is a naturally eutrophic lake, mainly due to its morphometry. Although a relatively shallow lake, the basin is sufficiently deep to allow accumulation of nutrient rich sediments in areas that are too dark to allow benthic primary production (Brunberg, Blomqvist & Rydin, 2002). The substantial internal P loading, indicated by our calculations as well as from earlier investigations (Pettersson & Lindqvist, 1993), might be explained by several different processes occurring simultaneously or alternating with the local environmental conditions (reviewed by Søndergaard, Jensen & Jeppesen, 2003). Frequent mixing of this polymictic lake prevents persistent oxygen depletion and low redox conditions in the bottom waters. However, whenever these conditions do occur the iron-rich sediments would easily release P from the redox-sensitive P fractions that constitute a significant part of the sediment P (Pettersson, 1986; Pettersson & Lindqvist, 1993). Increasing temperature during summer, even in deeper parts of the lake due to the mixed water column, will also enhance microbially-mediated P release from sediments (Boström *et al.*, 1988;

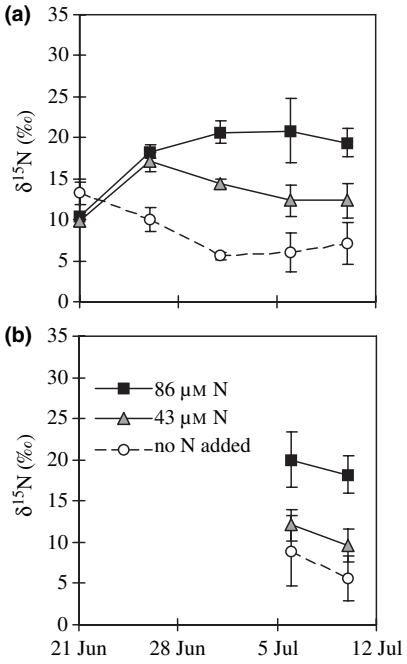

Fig. 5 $\delta^{15}N$ values of seston in the mesocosm experiment. (a) seston <40 μm; (b) total seston. Each data point represents the average of three mesocosms. Error bars show SD. The variability in $\delta^{15}N$ measurements is constrained by the nitrate tracer ($\delta^{15}N$ = 33.9‰) and atmospheric N_2 ($\delta^{15}N$ = 0.0‰).

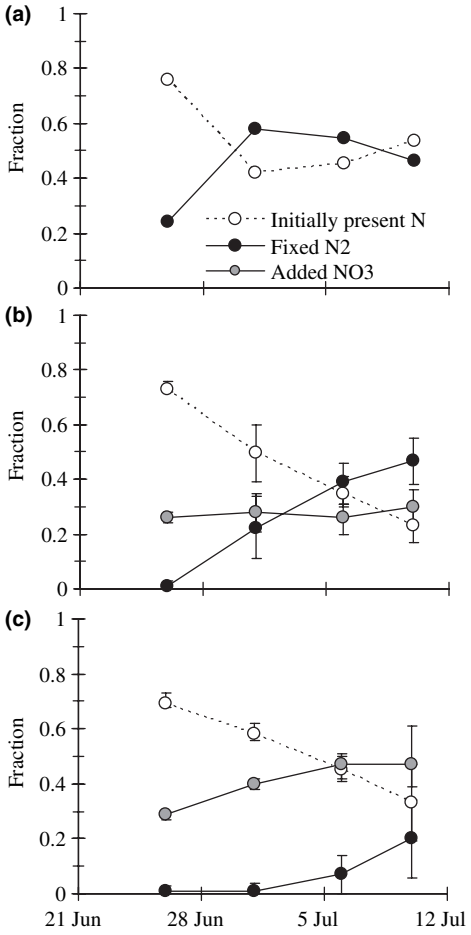

Fig. 6 Sources of origin of N in seston <40 μm in the mesocosm experiment, given as the fractional contribution to seston N of initially present N, fixed N_2 and added nitrate in the three treatments. (a) no N addition; (b) 43 μM NO_3–N added; (c) 86 μM NO_3–N added. In the treatment with no nitrate addition, there is a unique solution for each sampling date, whereas in the two treatments with nitrate addition, the fractional contribution is given as the mean and range of the feasible solutions constrained by a mass balance criterion (see Methods for a more complete description).

Gächter & Meyer, 1993). In addition, high pH of the water during summer algal blooms may promote additional release of P. Laboratory experiments with sediments from Lake Limmaren (Pettersson, 1986) revealed that high pH levels substantially increased the P release to the lake water. Although these kinds of experiments, where NaOH is used to enhance pH, can be criticised for non-natural artefacts, the natural pH increase in Lake Limmaren caused by photosynthesising algal blooms is high enough (≥9 pH) to induce significant enhance of P release also under natural conditions (cf. Boers, 1991). The internal loading of P can be observed as a strong increase in TP during the summer. Since there are no major point sources of P to this lake, the only alternative explanation for this threefold increase in TP would be that P is imported from the catchment. However, a strong negative correlation between lake TP concentration and discharge, as well as a long water residence time, suggest that the high TP concentrations in late summer were due to internal loading, and not the result of P import from the catchment. Thus, in this lake with high internal P loading, there is often a deficiency in N, which favours dominance by N-fixing cyanobacteria, as in many eutrophicated lakes. As a consequence of this massive internal P loading, the biomass of N-fixing cyanobacteria increases and TN concentration increases as a result of N-fixation, efficiently balancing the N and P concentrations, as evidenced by the temporal covariance of TN and TP. The magnitude of the internal P loading differs between years, which may at least partly be an effect of inter-annual climatic variation, and this is reflected in differences in both the magnitude of the phytoplankton biomass and the phytoplankton community composition. However, the dominance by N-fixing

cyanobacteria during periods with high net accumulation of P in the pelagic appears not to be a universal phenomenon. When considering the full range of lake TP concentrations it is evident that other factors besides N : P ratio are important determinants of phytoplankton community structure. For example, in hypertrophic lakes with high external or internal nutrient loading rapidly growing green algae tend to become dominant despite low N : P ratios (Jensen *et al*., 1994; Jeppesen *et al*., 2005).

These inferences drawn from our lake observations were supported by our mesocosm experiment, in which the phytoplankton community composition shifted towards a larger dominance of N-fixing cyanobacteria when the nutrient load to the mesocosms had a low N : P ratio. In the treatment with no N addition, the N-fixing cyanobacteria comprised as much as 50% of the final phytoplankton biomass. Similar shifts in phytoplankton community composition at low N : P supply ratios have previously been reported from several whole-lake, mesocosm and laboratory studies (e.g. Schindler, 1977; Smith, 1983; Bulgakov & Levich, 1999; Levine & Schindler, 1999; Smith & Bennett, 1999). There was also an increase in N-fixing cyanobacterial biomass in the treatments with N addition, although the biomass decreased with increasing N addition. The presence of N-fixing cyanobacteria in the high N treatment is consistent with previously reported TN : TP thresholds for dominance of N-fixing cyanobacteria (Smith & Bennett, 1999; and references therein) since the TN : TP ratio was as low as approximately 40 at the end of the experiment. It was only in the treatment with the highest N : P ratio that a pool of nitrate remained present. In this treatment, the final biomass of N-fixing cyanobacteria was only half of that observed in the treatment without N addition, and eukaryotic phytoplankton contributed 53% of the total biomass. It is known that cyanobacteria have a low competitive ability to use nitrate as their N source, and consequently eukaryotic phytoplankton should become dominant when there is nitrate available (Hyenstrand, Blomqvist & Pettersson, 1998).

In contrast to the effect of N : P supply ratio on the phytoplankton community composition, there was no or only a very weak treatment effect on TN concentration, particulate N concentration and primary production. The reason for this large increase in TN is that the total production of the community was set by the availability of resources other than N (e.g. P, inorganic C and/or light), and most of all, that N fixation could compensate for the low N availability. The relative importance of the P load in this context has been demonstrated in a mesocosm experiment in the Baltic Sea, where large increases in particulate N were observed in mesocosms receiving P, but not in mesocosms without P addition (Rydin *et al*., 2002). Likewise, Hellström (1996) showed in a survey of 38 lakes that N fixation was positively related to TP concentration.

The stable isotope data provide further evidence that atmospheric N contributes to the TN pool and allow us to estimate this contribution. Fixation of atmospheric N_2 was the dominant source of N in the treatments without N addition. Atmospheric N_2 was a smaller, yet significant, source for treatments receiving N additions, which implies that the N demand was larger than the nitrate supply even in the N addition treatments. The observed N fixation rates in mesocosms without N addition (*c.* 8.6 mmole N m^{-2} day^{-1} over the entire experimental period) are comparable to or even higher than previously published values from eutrophic systems: Rydin *et al.* (2002) found a N-fixation rate of 9.3 mmole N m^{-2} day^{-1} in a study of a brackish-water phytoplankton community, and Howarth *et al.* (1988b) report N fixation rates ranging between 14 and 660 mmole N m^{-2} $year^{-1}$, which corresponds to 0.2–10 mmole N m^{-2} day^{-1} (assuming 65 days of N-fixation per year). In contrast to these high N-fixation rates in eutrophic lakes, N-fixation rates are usually lower in estuaries, possibly due to iron limitation (Howarth, Marino & Cole, 1988a). Iron is required as a co-enzyme in the nitrogenase complex, and is therefore essential for N-fixation (Stryer, 1981). Iron concentrations are often sufficiently high in lakes to meet the demands of N-fixing cyanobacteria. Thus, in lakes with molar N : P ratios lower than 16 : 1 and total inorganic N concentrations <3.6–7.1 μM (Horne & Commins, 1987), N-fixation can take place, given that other environmental factors such as pH, temperature and mixing conditions are favourable. In our experiment, N fixation was apparently regulated by the availability of N relative to P, and resulted in very similar TN concentrations in all treatments throughout the experiment. Our results thus contrast with those of Ferber *et al.* (2004) who reported that N fixation contributed only a minor fraction to the N uptake by a cyanobacteria-dominated phytoplankton community in a

eutrophic lake. We acknowledge that other mechanisms, including light limitation of primary production and uptake of benthic sources of ammonium, may be key explanatory factors for cyanobacterial dominance, but argue that our results strongly suggest that inorganic N deficiency can be of importance.

It has been suggested that the phytoplankton C : N ratio may provide a proxy for the degree of N-limitation (Weithoff & Waltz, 1999), with higher C : N ratios indicative of greater N limitation. In the mesocosm experiment, the C : N ratios of seston reveal some interesting patterns. First, the highest measured C : N ratios of the <40 μm seston fraction were measured at the inception of the experiment, suggesting that phytoplankton were N deficient at the beginning of the experiment. At the conclusion of the experiment, all treatments had experienced a substantial decline in C : N ratio. However, the treatment receiving no N addition had the lowest C : N ratio, whereas the treatments receiving N addition had higher C : N ratios. We therefore conclude that treatments subjected to lower relative amounts of N were more than able to compensate for nutrient deficiencies by fixing atmospheric N.

From an applied point of view, our results at least suggest that caution should be exercised when proposing to decrease the N load currently being delivered to lakes without a corresponding decrease of the P load. Although the intent is eventually to decrease nutrient loading to coastal marine ecosystems, thereby ameliorating eutrophic conditions in those ecosystems, reducing N loading may have detrimental effects on freshwater ecosystems. The present study adds to a growing body of evidence that reduced N loading creates conditions that greatly benefit N-fixing cyanobacteria, which are able to outcompete other phytoplankton by importing considerable amounts of N from the atmosphere (e.g. Bulgakov & Levich, 1999; Smith & Bennett, 1999). Thus, without a commensurate decrease in other nutrients, especially P, programmes that seek to improve water quality in coastal marine ecosystems by N loading reduction may actually reduce water quality in freshwater ecosystems while ultimately failing to reduce N loading to marine ecosystems.

In conclusion, our experiment showed that N fixation contributes a substantial amount of N to the summer phytoplankton community of Lake Limmaren, and that the N fixation rate depends on the N : P loading ratio. As a consequence, decreasing the N load (without a proportional reduction of the P load) to this eutrophic lake during the summer did not result in lower TN concentrations or lower primary productivity. The presence of N-fixing cyanobacteria during that period acted as a conduit between the abundance of N in the atmosphere and the N-deprived biosphere, eventually relieving the phytoplankton community growth from N limitation. Although reductions in the N : P ratio had little effect on the amount of available N or total primary production in this aquatic ecosystem, reduced N : P ratios had a significant impact on the phytoplankton assemblage. While not all lakes will necessarily respond like Lake Limmaren, our results indicate that reducing N load to lakes without a corresponding reduction in P load can prove an inappropriate action.

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