

Nitrogen limitation of the phytobenthos in Alpine lakes: results from nutrient-diffusing substrata

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SUMMARY

1. In the Alps, as in other mountainous regions across the world, nitrogen (N) enrichment of ecosystems via atmospheric deposition is a major environmental concern. Nitrogen enrichment can cause acidification in poorly buffered lakes and streams, but here we assessed the less well-known effects of eutrophication on the phytobenthos of lakes and its consequences for grazing.
2. To simulate the effects of N deposition, we conducted experimental nutrient additions (N and N+P) in two lakes in the French Alps, in summer 2012. In each lake, we deployed nutrient-diffusing substrata for 22 days. The substrata were placed within cages that either allowed or prevented access by macrograzers. We tested the hypotheses that N enrichment: (i) increases phytobenthic biomass, (ii) alters phytobenthic taxonomic composition and (iii) reduces benthic grazing.
3. Supporting the first hypothesis, at the end of the experiments N-enriched substrata had a greater phytobenthic biomass than unenriched substrata, indicating N limitation. Supporting the second hypothesis, the taxonomic composition of the phytobenthos differed between N-enriched and control substrata. Green algae, including filamentous forms, were favoured by N enrichment to a greater extent than diatoms and cyanobacteria. By contrast, the third hypothesis was not supported. Grazing had the same effect on the biomass of the phytobenthos regardless of nutrient treatment.
4. Our results highlight the sensitivity of Alpine lakes to deposition of atmospheric N. Its ecological effects are not limited to acidification, but also include increased biomass (eutrophication) and altered taxonomic composition of the phytobenthos. Because filamentous green algae favoured by N enrichment are usually unpalatable to macrograzers, inputs of atmospheric N could reduce the efficiency of transfer of energy from the phytobenthos up through the food web.

Keywords: atmospheric deposition, eutrophication, food web, global change, nutrient limitation

Introduction

Remote fresh waters are increasingly affected by global pressures. During the last century, deposition of atmospheric nitrogen (N) has grown to influence fresh waters as remote as Arctic lakes (Wolfe, Cooke & Hobbs, 2006; Holtgrieve *et al.*, 2011). However, unproductive lakes and streams in mountains near heavily populated regions, such as the Alps, the Rocky Mountains, the Californian Sierra Nevada and the Adirondacks are most affected (e.g. Baron *et al.*, 2000; Wolfe, Baron & Cornett, 2001; Aber *et al.*, 2003; Fenn *et al.*, 2003; Lepori, Barbieri & Ormerod, 2003a). In these ecosystems, N deposition

can lead to both acidification and eutrophication. However, whereas acidifying effects are now well-documented (e.g. Barmuta *et al.*, 1990; Baker *et al.*, 1996; Lepori, Barbieri & Ormerod, 2003b; Lafrancois *et al.*, 2004; Passy, 2006), evidence of eutrophication effects remains fragmentary (Bergström & Jansson, 2006; Elser *et al.*, 2009a).

Research on eutrophication of fresh waters induced by N deposition has focussed so far on the pelagic (planktonic) system of lakes. Enriching effects of deposited N on pelagic systems might at first seem unlikely. Extensive research on the causes of eutrophication in the 1960s and 1970s culminated in a

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hypothesis of single-nutrient limitation by phosphorus (Schindler, 1977), and this hypothesis established a deep-rooted tenet of modern limnology (Lewis & Wurtsbaugh, 2008; Sterner, 2008). Nitrogen, by comparison, has been considered non-limiting under most circumstances owing to the ability of some cyanobacteria to fix N from the atmosphere, an inexhaustible source (Schindler, 1977). Nonetheless, subsequent research has questioned these generalisations (Elser *et al.*, 2007), particularly with regard to unproductive, undisturbed lakes in mountain or boreal environments. In these lakes, N limitation of phytoplankton, as determined by nutrient bioassays, is common (Morris & Lewis, 1988; Maberly *et al.*, 2002; Lafrancois *et al.*, 2004; Nydick *et al.*, 2004; Elser *et al.*, 2009a,b). Moreover, consistent with evidence of N limitation, large-scale surveys of oligotrophic lakes across Sweden and the Northern Hemisphere have indicated that increasing atmospheric deposition of N is associated with increasing phytoplankton biomass (Bergstrom, Blomqvist & Jansson, 2005; Bergström & Jansson, 2006). These lines of evidence strongly suggest that the pelagic system of unproductive lakes is sensitive to eutrophication through atmospheric N inputs.

In addition to increasing production and biomass, N enrichment can also influence the taxonomic composition of the phytoplankton. Inputs of N to lakes cause shifts in the N:P ratio of the water column (e.g. Elser *et al.*, 2009a). Given that planktonic algae and cyanobacteria have species-specific optimal N:P supply ratios (Tilman, Kilham & Kilham, 1982), such shifts will benefit some species and be deleterious for others (Schindler, 1977; Smith, 1983; Interlandi, Kilham & Theriot, 1999). The ensuing compositional changes can have far reaching consequences for pelagic food webs. Phytoplanktonic species favoured by high N:P supply ratios may be less palatable or of poorer nutritional quality to grazers (Lafrancois *et al.*, 2004). As a result, N enrichments may indirectly reduce pelagic grazing and the efficient transfer up the food web of energy and nutrients from primary producers (Elser *et al.*, 2009b).

In comparison with the effects on phytoplankton, the effects of N enrichment on the biomass and composition of the phytobenthos (benthic algae and cyanobacteria) of unproductive lakes are poorly investigated (Maberly *et al.*, 2002; Nydick *et al.*, 2004). Moreover, we are not aware of any studies examining the effects on benthic grazing. This lack of research is surprising, for three reasons. First, in unproductive lakes, phytobenthic production can account for most of the primary production of the whole lake, including the pelagic and benthic zones

and the sediments (Hecky & Hesslein, 1995; Vadeboncoeur, Vander Zanden & Lodge, 2002). Second, phytobenthic production can determine the production and biomass of the primary and secondary consumers, that is, benthic invertebrates and fish (Karlsson *et al.*, 2009). Third, benthic and pelagic systems can show different responses to nutrient additions (Nydick *et al.*, 2004). Therefore, the response of the benthic system to N enrichment could strongly influence how energy flows through the whole-lake food web.

This study assessed the effects of N enrichment on the benthic food web of two small and unproductive lakes in the Alps (Rhône-Alpes, France). To simulate the effects of N deposition, we deployed substrata diffusing N (and a mixture of N and P) in the littoral zone for 22 days. Using a factorial experiment, we tested the effects of N enrichment on the biomass and taxonomic composition of the phytobenthos and its consumption by grazing macroinvertebrates. Our main hypotheses were that N enrichment would (i) increase the biomass of phytobenthos, (ii) alter the taxonomic composition, and (iii) reduce grazing compared to control substrata with no enrichment (hypotheses i–iii) or enrichment with a mixture of N and P (hypotheses ii–iii).

Methods

Study sites

We chose two small alpine lakes located south of Grenoble, France: Lac Canard (latitude, +45°3'41"; longitude, +5°55'54"; altitude, 2060 m; surface area, 1.13 ha) and a nameless lake, which we dubbed Lac Crécerelle for this study (latitude, +45°3'05"; longitude, +5°55'49"; altitude, 2060 m; surface area, 0.24 ha; Fig. 1). Even though they were only 1 km apart, the lakes differed markedly in several respects. Lac Canard had emergent vegetation of *Sparganium* sp. and *Carex* sp. along the shores and slightly brown (humic) water. Lac Crécerelle was barren and strikingly clear. Thus, the study lakes represent endpoints of a spectrum of water colour, from clear to slightly brown, found locally and across the Alps, not including wetland ponds. The geology of the catchments consisted of a crystalline basement (made of gneiss, schists and amphibolite) and isolated outcrops of dolomite. The landscape was a mix of alpine grass meadows, rock debris and, around Lac Canard, bog. Land use was limited to light grazing by sheep. In 2010, total atmospheric deposition of N was *c.* 7 kg N ha⁻¹ y⁻¹ (European Monitoring & Evaluation Programme (EMEP), 2012).

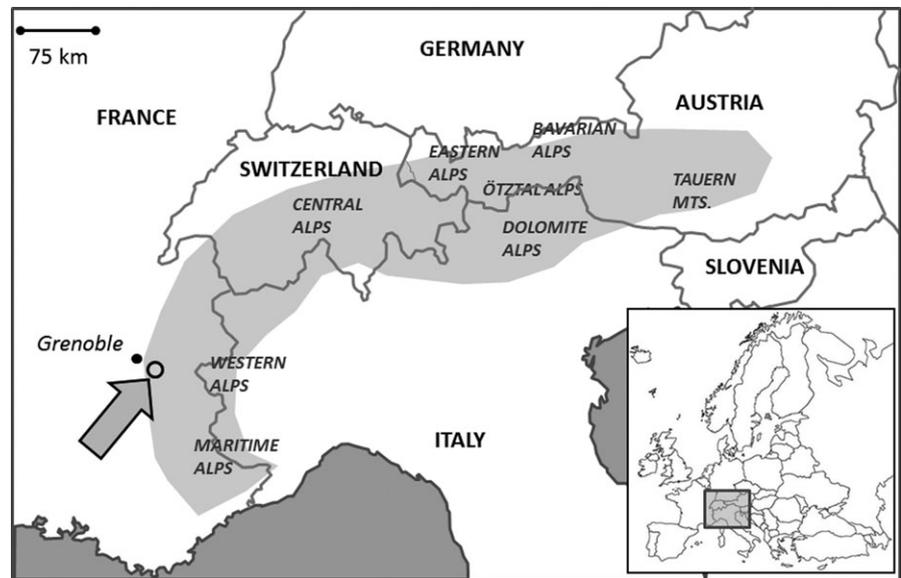


Fig. 1 Location of the study lakes.

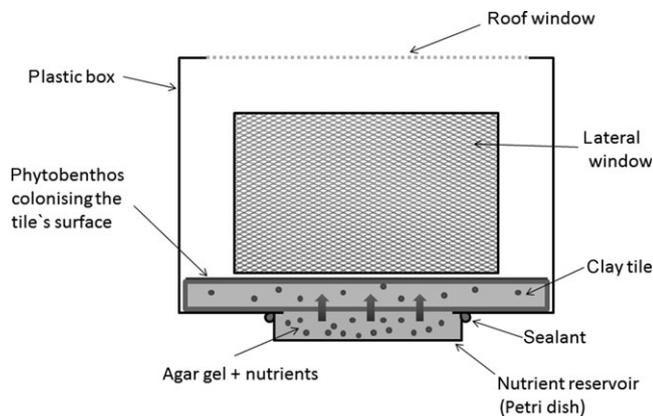


Fig. 2 Schematic drawing of the section of an experimental unit, consisting of a nutrient-diffusing substratum enclosed in a cage. Drawing not to scale.

Experimental design and set-up

We used a crossed factorial design with two factors: grazing (two levels: yes or no) and nutrients (three levels: unenriched, enriched with N and enriched with N and P). In addition, we used a reference (described below) to assess cage effects, that is, the effects of our experimental units on colonisation by phytobenthos and macrograzers.

The experimental units consisted of diffusing substrata set within cages (Fairchild, Lowe & Richardson, 1985), which allowed us to manipulate a) nutrient supply and b) access to substrata by macrograzers (Fig. 2). The cages were made of 1 L plastic boxes (17.5 × 15.9 × 6.5 cm), from which we cut out a roof window (9 × 9 cm), two lateral windows (9 × 3.5 cm)

and a circular opening (diameter: 8 cm) through the bottom. Each substratum consisted of a 10.3 × 10.2 × 1.4 cm porous clay tile (intended for flooring; manufactured by Céramiques du Beaujolais, Villefranche sur Saône, France) placed on the bottom of the cage. The tile provided a surface colonisable by phytobenthos and macrograzers. Beneath the box, we hot-glued a Petri dish (diameter: 8.8 cm, depth: 1.8 cm; volume = 110 mL), which served as a nutrient reservoir. Previous assays in Alpine streams (F. Lepori, *unpublished*) suggested that 110 mL reservoirs would have been ample to ensure nutrient diffusion throughout the assays, and we checked this assumption using diffusion tests (see below). The added nutrients reached the colonisable surface by diffusing from the Petri dish through the tile.

Grazing treatments were created by either closing the two lateral windows with nylon mesh (250 µm) to exclude macrograzers (benthic insects and tadpoles) or leaving the windows open to allow their access. Hereafter, we refer to these treatments as CLOSED and OPEN, respectively. The roof window was closed with similar nylon mesh (250 µm) in both treatments. Some micrograzers (including herbivore zooplankton) could pass through the mesh and might have consumed phytobenthos in CLOSED units. However, we assumed that this consumption was minor compared to consumption by macrograzers and also similar across treatments. We also assumed that the nylon mesh did not affect colonisation by phytobenthos.

Nutrient treatments were created as follows: for the unenriched treatment, we filled the Petri dishes (i.e. the nutrient reservoirs) with 2% agar gel; for the N-enriched

treatment, we filled the reservoirs with agar containing 0.5 M of sodium nitrate, NaNO_3 , and for the N- and P-enriched treatment, we filled the reservoirs with agar containing 0.5 M NaNO_3 and 0.031 M KH_2PO_4 . This gives a nominal atomic N:P ratio of 16:1, which is probably optimal for the growth of algae (Redfield, 1958). Hereafter, we refer to these treatments as UNENRICHED, N and N+P, respectively. The diffusion tests confirmed that the substrata were still diffusing nutrients at the end of the bioassays. N was diffusing at $113 \pm 65 \mu\text{g N h}^{-1}$ (N treatment) or $181 \pm 82 \mu\text{g N h}^{-1}$ (N+P treatment) and P at $13 \pm 5 \mu\text{g P h}^{-1}$ (all data are mean \pm SE; $n = 4$). Therefore, the actual N:P supply ratio of the N+P treatment, in atoms, was c. 30:1. The diffusion rate of N was not significantly different between N and N+P treatments (t -value = -0.59 ; $P = 0.578$). The reference was created by deploying bare tiles (without the cage) directly on the lake bottom.

All tiles used in the bioassays were placed out in shallow water (20–70 cm) in the study lakes for three (Lac Crécerelle) or five (Lac Canard) weeks before the bioassays. The purpose was to permit some colonisation by phytobenthos before the start of the bioassays and purge any salts that might have been present on the new tiles. The tiles used in Lac Crécerelle were actually placed in a similar neighbouring lake, in which we intended to perform the experiment. We transferred the tiles to Lac Crécerelle for the experiment because we realised that the lake we had originally chosen was frequented by walkers, so we feared disturbance.

The bioassays started on 8 August (Lac Canard) or 14 August 2012 (Lac Crécerelle) and lasted 22 days. At the beginning of each bioassay, we retrieved the tiles from the lake and, having carefully removed the invertebrates present, fixed them at the bottom of the experimental cages using silicone sealant (except for the references, which consisted of bare tiles). In each lake, 42 cages (six treatments \times seven replicates) and seven replicate references were deployed on the bottom near the shore, arranged along a square grid (c. 5×5 m) made of seven rows (parallel to the shore) and seven columns (perpendicular to the shore). Each row included one replicate of each treatment and a reference, randomly distributed. The grids were laid out within areas that were homogeneous in terms of emergent vegetation and sediment composition. Depths within these experimental areas ranged from 59 to 80 cm at Lac Canard (mean, 68 cm) and from 18 to 61 cm at Lac Crécerelle (mean, 50 cm).

At the end of the experiment, we collected the cages (treatments) and bare tiles (references) by sliding a

large aquarium net (27×20 cm, mesh size: 0.5 mm) underneath them, lifting the net out of the water and placing the cages in a tray lying on the bank. We then opened the cages and collected all the macroinvertebrates within. We also collected the invertebrates that escaped from the OPEN cages upon retrieval and were trapped in the aquarium net. The invertebrates were stored in alcohol-filled vials. Next, we detached the tiles, wrapped them in aluminium foil and placed them in a cooler box. References (bare tiles) were processed in the same way. All tiles were transported to the laboratory on the same day that they were retrieved from the lakes, where they were frozen until further analysis.

Ambient conditions in the lakes

To characterise the ambient physicochemical conditions during the experiments, we measured water temperature and collected water samples from each lake at the beginning and at the end. The samples were analysed for conductivity, pH, alkalinity, N (i.e. NO_3 , NO_2 , NH_4 and total N, TN), P (i.e. soluble reactive P, SRP and total P, TP), soluble reactive silica (SRSi) and total organic carbon (TOC). All analyses were carried out at the laboratory of the Centre Alpin de Recherche sur les Réseaux Trophiques et Ecosystèmes Limniques (Thonon-Bains, France) according to national standards. Dissolved inorganic N (DIN) was calculated as $\text{NO}_3 + \text{NO}_2 + \text{NH}_4$. Dissolved inorganic carbon (DIC) was calculated from alkalinity, pH and temperature following McCutcheon, Martin & Barnwell (1993; online calculator at www.microcosmofscience.com).

Laboratory analyses

Chlorophyll-a. Phytobenthic biomass was estimated as the mass per unit surface area (mg cm^{-2}) of chlorophyll-*a*. Chlorophyll-*a* was extracted by immersing each tile, whole, in 80 mL of 96% ethanol. Its phaeophytin-corrected mass was measured spectrophotometrically (Steinman & Lamberti, 1996; Montana Department of Environmental Quality (MDEQ), 2008). In calculating the chlorophyll-*a* mg cm^{-2} , a small tile area from which the phytobenthos had been removed for taxonomic analysis (see below, *Phytobenthos composition*) was taken into account. The use of chlorophyll-*a* as a proxy for phytobenthic biomass should be carried out with caution (Bergström & Jansson 2006), although in this study chlorophyll-*a* correlated well with cell counts ($r = 0.75$; $P < 0.001$), which supports its use.

Macrograzer biomass. At the end of the experiments, macrograzers included four invertebrate taxa. Of these, *Cloeon simile* (Ephemeroptera: Baetidae) feeds mostly on algae, whereas *Paraleptophlebia submarginata* (Ephemeroptera: Leptophlebiidae), *Agrypnia obsoleta* (Trichoptera: Phryganeidae) and Chironomidae non-Tanyptodinae (Diptera) consume algae as part of a broader diet (Tachet *et al.*, 2010). At the end of the experiments, all individuals of these macrograzers found associated with the substrata were collected, identified, counted and measured (± 0.1 mm), so that their biomass could be estimated from published length–biomass regression models (Benke *et al.*, 1999). Tadpoles of the common frog, *Rana temporaria*, may be important grazers in the lakes and were present at the beginning. However, they had metamorphosed into froglets and left the lake before the end of the experiments (F. Lepori, pers. obs.). Therefore, the biomass of macrograzers measured at the end of the bioassays probably underestimated the biomass present at the beginning.

Composition of the phytobenthos. A subsample of phytobenthos was removed from each substratum before the extraction of chlorophyll-*a*, to be examined for taxonomic composition. The subsample was obtained by scraping off the biofilm from a small area (8 cm²) of each tile with a sharp scalpel. The scrapings were stored in Transeau's solution (six parts of water, three parts of 95% ethanol and one part of formalin) until identification. The composition of the phytobenthic assemblage was determined after Uthermühl (AFNOR, 2006). Briefly, the whole sample was placed in a sedimentation chamber, and, once settled, all algae and cyanobacteria within selected fields of view were identified to genus and counted under an inverted microscope. We counted a minimum of 400 cells per genus. The abundance of algae and cyanobacteria was expressed as density (number of cells cm⁻²).

Statistical analyses

Differences in chlorophyll-*a* and macrograzer biomass among treatments were tested using ANOVA. For each response variable and each lake, we ran two analyses. First, we compared the OPEN-UNENRICHED treatment with the reference (bare tiles) to test for any cage effects. Second, we tested the effects of the main treatments and their interaction using overall ANOVA tests and Tukey's pairwise comparisons. Phytobenthic composition was assessed at two levels of taxonomic resolution: major divisions (diatoms, green algae and cyanobacteria)

and genus. Treatment effects on taxonomic composition at the division level were tested using multivariate ANOVA (MANOVA). In addition, to interpret N effects on the composition of the major phytobenthic divisions (see hypothesis ii), we tested the effects of N enrichment separately for each level of the grazing factor, using one-way ANOVA. Effect sizes were quantified using Glass' Δ , the difference of the group means divided by the standard deviation of the control group (here, the UNENRICHED treatment). Simple (rather than main) effects were computed because the meaning of main effects was often obscured by significant interactions. The effects on genus-level composition were tested using ANOVA, with the analysis restricted to common genera (defined as genera totalling $\geq 5\%$ of the total number of the cells counted). Data were log (chlorophyll-*a* mass) or log($x + 1$)-transformed (grazer biomass and cell density) to meet the parametric assumptions of the tests.

Results

Ambient conditions

Thermal and chemical conditions were similar between lakes, although Lac Canard had a lower concentration of dissolved inorganic N (DIN) and a higher concentration of total organic C (TOC) than Lac Crécerelle (Table 1). Ambient nutrient concentrations were modest at both lakes and varied little over the course of the experiments. The atomic N:P ratios of the two lakes, measured as DIN:TP, lay on either side of the Redfield ratio, averaging 3:1 at Lac Canard and 27:1 at Lac Crécerelle.

Table 1 Physicochemical conditions of the lakes at the beginning (B) and end (E) of the experiments

Variable	Unit	Lac Canard		Lac Crécerelle	
		B	E	B	E
Temperature	°C	17.8	15.9	13.2	11.2
pH		7.15	7.08	7.94	7.47
Alkalinity	meq L ⁻¹	0.11	0.18	0.42	0.48
DIC	mmol C L ⁻¹	0.13	0.22	0.43	0.53
DIN	µg N L ⁻¹	30	20	80	130
SRP	µg P L ⁻¹	3	10	4	6
TP	µg P L ⁻¹	16	23	10	8
DIC:TP	(in atoms)	251	295	1336	2036
DIN:TP	(in atoms)	4	2	19	35
SRSi	mg Si L ⁻¹	0.62	0.62	1.76	2.2
TOC	mg C L ⁻¹	6.16	7.43	0.37	0.35

Macrograzer biomass

In Lac Canard, four macrograzer taxa (all benthic insects) colonised the substrata (Fig. 3). Macrograzer biomass was similar between Reference and OPEN-UNENRICHED substrata (mean \pm SE, 40 ± 14 versus 70 ± 14 mg m⁻²), indicating no cage effect on colonisation (Table 2). Grazing had a significant effect, reflecting the fact that CLOSED cages successfully excluded nearly all macrograzers (biomass was 1 ± 0 mg m⁻² on CLOSED substrata versus 85 ± 10 mg m⁻² on OPEN substrata). In contrast, the nutrient factor and the nutrient \times grazing interaction had no effects. Macrograzer biomass was 35 ± 12 mg m⁻² on UNENRICHED substrata, 51 ± 16 mg m⁻² on N-enriched substrata and 43 ± 16 mg m⁻² on N+P-enriched substrata.

In Lac Cr  cerelle, macrograzers were limited to Chironomidae and their biomass was low overall (Fig. 3). Again, macrograzer biomass was similar between Reference and OPEN-UNENRICHED substrata (3 ± 2 versus 7 ± 5 mg m⁻²), indicating no cage effect (Table 2). As in Lac Canard, grazing had a significant effect (macrograzer biomass on CLOSED substrata was 0 ± 0 mg m⁻² versus 8 ± 3 mg m⁻² on OPEN substrata), whereas the nutrient factor and the nutrient \times grazing interaction had no effects. The biomass of Chironomidae was 4 ± 3 mg m⁻² on UNENRICHED substrata, 2 ± 1 mg m⁻² on N-enriched substrata and 7 ± 3 mg m⁻² on N+P-enriched substrata.

Phytobenthic (chlorophyll-*a*) biomass

In Lac Canard, cages had no effect on phytobenthic biomass, indicated by similar chlorophyll-*a* mass on OPEN-UNENRICHED substrata and References (0.56 ± 0.05 versus 0.42 ± 0.05 μ g cm⁻²; Table 2). Both grazing and nutrients had significant main effects (Table 2; Fig. 4). With regard to grazing, CLOSED substrata had more chlorophyll-*a* than OPEN substrata (1.52 ± 0.18 versus 1.17 ± 0.14 μ g cm⁻²). With regard to nutrients, substrata enriched with N and N+P (2.03 ± 0.15 and 1.37 ± 0.16 μ g cm⁻², respectively) had more chlorophyll-*a* than UNENRICHED substrata (0.64 ± 0.04 μ g cm⁻²; Tukey tests; *t*-values = 9.30 and 5.62, respectively; *P* < 0.001). Unexpectedly, substrata enriched with N+P had less chlorophyll-*a* than substrata enriched with N only (*t*-value = -3.68; *P* = 0.002). Grazing and nutrients had no interactive effect.

In Lac Cr  cerelle, cages had a negative effect on chlorophyll-*a*, as indicated by the greater chlorophyll-*a* mass on Reference than on OPEN-UNENRICHED substrata (1.12 ± 0.21 versus 0.57 ± 0.05 μ g cm⁻²; Table 2). As in Lac Canard, grazing and nutrients had significant main effects (Table 2; Fig. 4). Again, CLOSED substrata had more chlorophyll-*a* than OPEN substrata (2.28 ± 0.29 versus 1.20 ± 0.12 μ g cm⁻²), and substrata enriched with N and N+P (2.03 ± 0.19 and 2.52 ± 0.35 μ g cm⁻², respectively) had more chlorophyll-*a* than UNENRICHED substrata (0.69 ± 0.06 μ g cm⁻²; Tukey tests; *t*-values = 10.47 and 11.88, respectively; *P* < 0.001). In

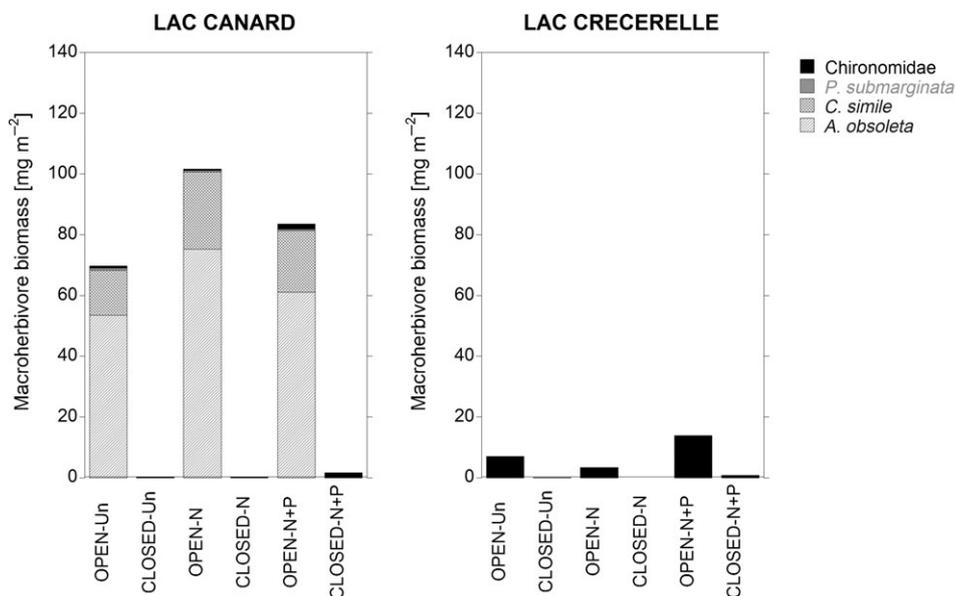
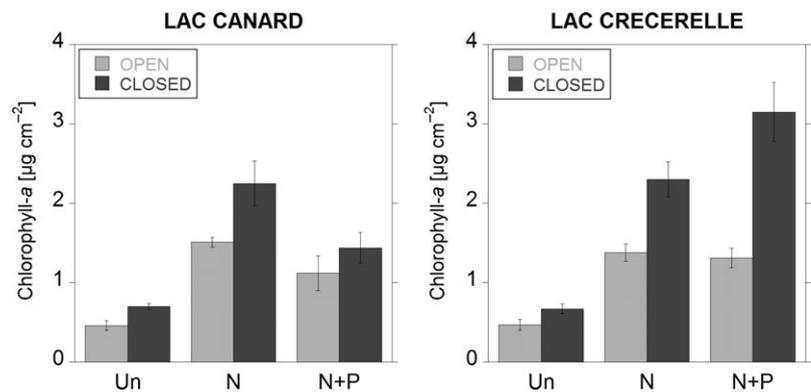


Fig. 3 Biomass of macrograzers on the substrata at the end of the experiments, by grazing and nutrient treatment.

Table 2 Effects of cages and experimental treatments on macrograzer biomass, chlorophyll-*a* mass and composition of the phytoplankton at the end of the experiments. Effects tested by ANOVA (biomass) or MANOVA (composition)

Lake	Comparison/treatment	Macrograzer biomass			Chlorophyll- <i>a</i> mass			Phytoplankton composition		
		<i>F</i>	d.f.	<i>P</i>	<i>F</i>	d.f.	<i>P</i>	Wilks's λ	<i>F</i>	<i>P</i>
Lac Canard	Reference versus OPEN-UNENRICHED	2.04	1,12	0.178	4.26	1,12	0.061	12.12	2,11	0.002
	Grazing (G)	418.78	1,36	0.001	6.81	1,36	0.013	127.24	3,34	<0.001
	Nutrients (N)	1.26	2,36	0.269	43.88	2,36	<0.001	47.18	6,68	<0.001
	Interaction (G×N)	2.05	2,36	0.144	0.01	2,36	0.992	42.11	6,68	<0.001
Lac Crécerelle	Reference versus OPEN-UNENRICHED	0.08	1,12	0.777	13.48	1,12	0.003	0.16	2,11	0.853
	Grazing (G)	16.89	1,36	<0.001	44.77	1,36	<0.001	42.56	2,35	<0.001
	Nutrients (N)	1.98	2,36	0.153	84.21	2,36	<0.001	19.70	4,70	<0.001
	Interaction (G×N)	0.21	2,36	0.810	3.14	2,36	0.055	10.80	4,70	<0.001

**Fig. 4** Mass of chlorophyll-*a* on the substrata at the end of the experiments, by grazing and nutrient treatment.

this lake, however, enrichment with N or N+P did not produce different results (t -value = 1.40; P = 0.349). The nutrient \times grazing interaction was not significant.

Phytoplankton composition

In Lac Canard, cages influenced the composition of phytoplankton at the major division level, due to the presence of green algae on some OPEN-UNENRICHED substrata, which were absent from the References (Table 2). In Lac Crécerelle, cages had no effect. In both lakes, the division-level composition of the phytoplankton displayed significant main effects of grazing and nutrients, as well as a significant grazing \times nutrients interaction (Table 2). Inspection of plotted data and univariate analyses indicated that N enrichment nearly always increased cell density (Table 3; Fig. 5). However, the magnitude of the effects depended on phytoplanktonic division (Table 3). Green algae displayed the largest effects, whereas cyanobacteria (present only in Lac Canard) showed effects comparable to those of diatoms.

The responses of common phytoplanktonic genera to the treatments varied among taxa and between lakes (Table 4). The responses of diatoms appeared complex, although in several cases, enrichment with N had negative effects. Common genera of green algae were positively affected by N enrichment in Lac Canard, where they were represented by *Dictyosphaerium* and *Mougeotia*, and by either N or N+P enrichment in Lac Crécerelle, where they were represented by *Crucigenia*. In Lac Canard, the cyanobacteria *Anabaena* and *Isocystis* were absent from the OPEN-UNENRICHED treatment (and the References, results not shown), but were relatively abundant in all other treatments. Overall, these generic-level responses indicate that each treatment, in each lake, was associated with a relatively distinct phytoplanktonic assemblage.

Discussion

Effect of N on phytoplanktonic biomass

Supporting our first hypothesis, phytoplanktonic biomass was N-limited in the study lakes. This accords with the

Table 3 Effect size and significance (tested by ANOVA) of N enrichment on the density (cells cm⁻²) of major taxonomic divisions in the phytobenthos, by grazing treatment (OPEN and CLOSED) and lake

	Average density Unenriched	Average density N-enriched	Effect size (Glass' Δ)	Significance
Lac Canard				
<i>Diatoms</i>				
OPEN	1382	2197	2.3	**
CLOSED	1455	1193	-0.7	NS
<i>Green algae</i>				
OPEN	66	1896	17.8	***
CLOSED	189	1826	15.0	**
<i>Cyanobacteria</i>				
OPEN	0	1299	1.7	***
CLOSED	1687	3304	2.3	*
Lac Crécerelle				
<i>Diatoms</i>				
OPEN	3039	3855	1.2	*
CLOSED	3865	4995	1.8	*
<i>Green algae</i>				
OPEN	23	142	3.0	†
CLOSED	53	308	2.4	*

NS, non-significant.

†*P* < 0.1; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

findings of other studies that have investigated the effect of N enrichment on phytoplankton biomass in mountain or upland lakes. In a late-summer experiment in a high-altitude lake in Wyoming, U.S.A., N amendments increased phytoplankton biomass by a factor of *c.* 3.5 (Nydick *et al.*, 2004). Nutrient-diffusing bioassays in 30 upland lakes in the U.K. indicated that the phytoplankton was N-limited in 20% of them (Maberly *et al.*, 2002). In comparison, a nutrient assay in a Swiss Alpine lake found no evidence of N limitation (Niederhauser & Schanz, 1993). In this case, the phytoplankton was limited by P and C. However, this lake received high N deposition (the mean wet deposition at a nearby station was 15 kg N ha⁻¹ year⁻¹ in 1996–2007; Steingruber & Colombo, 2010), whereas N limitation may be expected only where N deposition is at most moderate (Bergström & Jansson, 2006; Elser *et al.*, 2009b).

Nitrogen limitation of the phytoplankton is perhaps not surprising. Whereas speculations about phytoplankton limitation have gravitated towards a paradigm of single-nutrient limitation by P (Sterner, 2008), it is generally agreed that the factors limiting the phytoplankton are varied and include N alongside other nutrients, light and space (Lowe, 1996). Nonetheless, N limitation of the phytoplankton seems at variance with the notion that freshwater primary producers circumvent N deficiencies thanks to the ability of certain cyanobacteria to fix

atmospheric N (Schindler, 1977). However, such internal compensation may develop only in relatively productive lakes. In unproductive lakes, P supply may be insufficient to support the development of N fixers, either planktonic or benthic (Suttle & Harrison, 1988; Downing, Watson & McCauley, 2001; Maberly *et al.*, 2002). Therefore, in mountain lakes and other infertile fresh waters, N deficiency may not be completely offset.

As suggested by Maberly *et al.* (2002), difficulties in compensating for N deficiency may also explain N limitation of the phytoplankton at high ambient N:P ratios. At Lac Crécerelle, the N:P ratio was 27:1, which is considerably higher than the Redfield ratio of 16N:1P (Redfield, 1958), commonly used to set the boundary between N limitation and P limitation in aquatic ecosystems. On the other hand, at Lac Canard, the low 3N:1P ratio suggests considerable N deficiency (presumably due to denitrification in peripheral bogs, absent around Lac Crécerelle; Fisher & Acreman, 1999). The different ambient N:P ratio of the study lakes might have been involved in causing the different response to the N+P treatment (lower than the response to N alone in Lac Canard, similar in Lac Crécerelle), but at this time, we cannot propose a mechanism to account for this unexpected result.

Effect of N on phytoplankton composition

Supporting our second hypothesis, N enrichment influenced the taxonomic composition of the phytoplankton, causing a greater relative abundance of green algae. Effects of N enrichment on phytoplankton composition have been studied in a variety of freshwater habitats (e.g. Fairchild *et al.*, 1985; Flecker *et al.*, 2002; Wyatt, Stevenson & Turetsky, 2010). Across studies, compositional shifts have been common, although the taxa affected positively or negatively have varied, defying generalisation. However, N-induced shifts towards a greater abundance of green algae, both planktonic and benthic, have been observed in other experiments conducted in unproductive mountain lakes (Lafrancois *et al.*, 2004; Nydick *et al.*, 2004). Changes in composition of the phytoplankton may be expected under the premise that different N:P supply ratios favour different taxa (Stelzer & Lamberti, 2001). Accordingly, increases in green algae following N enrichment might suggest that species within this division have high N:P optima. However, this was probably not the case in our study, where green algae were favoured by N alone and by a balanced mixture of N+P. Therefore, they appeared to respond to absolute increases in N supply rather than shifts towards higher N:P supply ratios.

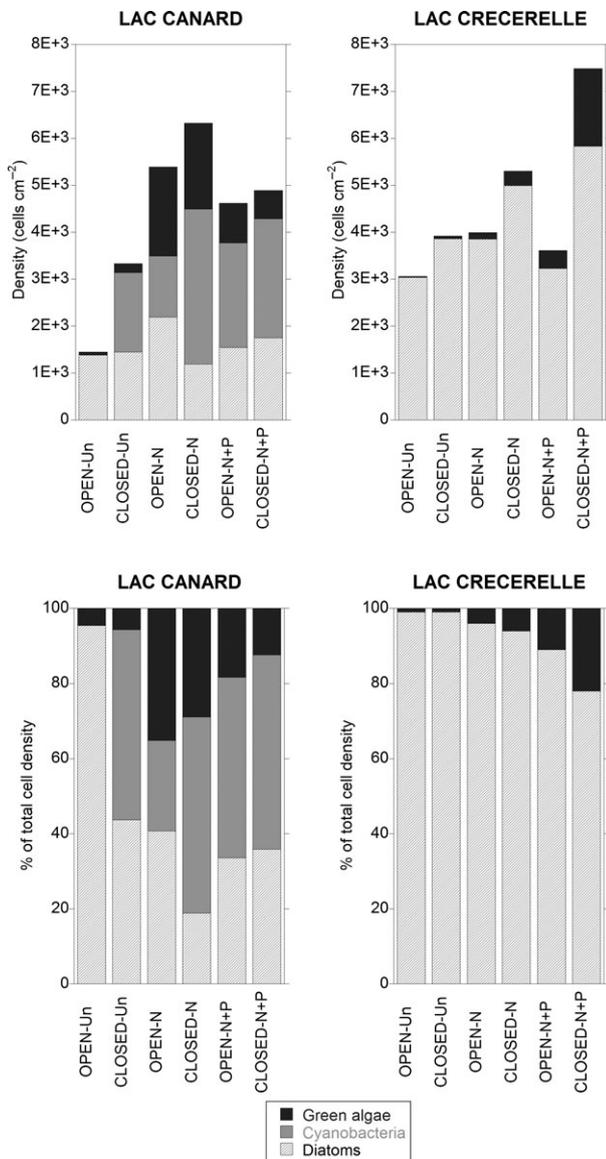


Fig. 5 Taxonomic composition of the phytoplankton on the substrata at the end of the experiments, by grazing and nutrient treatment. The graphs at the top represent abundance as absolute density (cells cm^{-2}), those at the bottom as percentage of total cell numbers.

Changes in taxonomic composition induced by N can have ramifications for food-web processes, because they could alter the palatability or the nutritional quality of the phytoplankton to grazers. As a rough generalisation, diatoms are consumed by most benthic grazers, whereas filamentous green algae and cyanobacteria are unpalatable or difficult to harvest (Allan & Castillo, 2007). Therefore, at least in Lac Canard, where compositional changes were led by a large increase in the filamentous green alga *Mougeotia*, N enrichment probably caused a greater relative abundance of unpalatable phytoplankton. Additionally, N enrichment might increase the cellular C:P ratio of algae,

as shown for phytoplankton (K. Nydick, unpubl. data cited in Nydick *et al.*, 2004). Ecological stoichiometry suggests that this shift would reduce the nutritional quality of this resource for grazers with high dietary requirements for P (Sterner & Elser, 2002).

Effect of N on benthic grazing

Despite changes in phytoplankton composition, and contrary to our third hypothesis, grazing was not reduced by N enrichment. In most freshwater systems, grazers have strong effects on the phytoplankton (Steinman, 1996). Albeit with a *caveat* (explained below), our results suggest that grazing also controlled phytoplankton biomass in our study lakes. However, the effect of grazing was similar, regardless of nutrient treatment, suggesting two possibilities: either N enrichment did not reduce the availability of palatable taxa (notwithstanding a possible increase in unpalatable forms) or macrograzers were not food-limited in our lakes.

The interpretation of the effect of the grazing treatment is complicated by the fact that, in Lac Cr cerelle, some silt settled on substrata in OPEN cages during the experiment and shaded the phytoplankton (F. Lepori, pers. obs.). In this lake, where benthic invertebrates were scarce, we suggest that this shading, rather than grazing, was the most plausible cause of the reduced phytoplankton biomass on OPEN substrata. Substrata in Lac Canard were not affected by deposition, owing to more stable sediments.

Assumptions and perspectives

An assumption in this study was that N limitation of phytoplankton was the natural regime of our lakes. We are not aware of human activities that could have driven the lakes away from this regime. The only apparent human pressure on the study area was atmospheric deposition. At the time of the study, N deposition (*c.* 7 kg N ha^{-1} year $^{-1}$; European Monitoring & Evaluation Programme (EMEP), 2012) was sufficient to influence the chemistry of lakes to some extent. In high-altitude catchments, sequestration of atmospheric N by terrestrial vegetation is limited; therefore, even modest deposition can cause leaching of N to surface waters. For example, in high-altitude catchments in the Rocky Mountains, atmospheric deposition as low as 1–2 kg N ha^{-1} year $^{-1}$ can result in increased DIN concentration in lakes (Baron *et al.*, 2011). However, N deposition should have driven our lakes towards P limitation, not N limitation.

In conclusion, our results indicate that atmospheric N deposition can cause eutrophication (increased

Table 4 Effects of the experimental treatments on the density (cells cm⁻²) of common phytoplanktonic genera, tested by ANOVA

Taxon	Relative abundance, %	Treatment and interaction effects	Comment
Lac Canard			
<i>Cyanobacteria</i>			
<i>Anabaena</i>	33	Grazing *** Nutrients *** Interaction ***	Absent in OPEN-Un, similarly abundant elsewhere.
<i>Isocystis</i>	7	Grazing ** Nutrients *** Interaction *	Absent in OPEN-Un, highest densities in N+P (both OPEN and CLOSED), similarly abundant elsewhere.
<i>Diatoms</i>			
<i>Gyrosigma</i>	5	Grazing NS Nutrients NS Interaction ***	Negatively affected by N in CLOSED cages, positively in OPEN CAGES.
<i>Meridion</i>	7	Grazing NS Nutrients NS Interaction ***	Negatively affected by N in CLOSED cages, positively in OPEN CAGES.
<i>Pinnularia</i>	11	Grazing NS Nutrients ** Interaction NS	N<Un, N+P
<i>Rhopalodia</i>	4	Grazing NS Nutrients ** Interaction NS	N<N+P
<i>Green algae</i>			
<i>Dictyosphaerium</i>	4	Grazing NS Nutrients *** Interaction NS	N>Un, N+P
<i>Mougeotia</i>	11	Grazing * Nutrients *** Interaction **	Positively affected by N (both OPEN and CLOSED) and by CLOSED-N+P
Lac Crécerelle			
<i>Diatoms</i>			
<i>Cyclotella</i>	8	Grazing NS Nutrients ** Interaction **	Negatively affected by N in CLOSED cages
<i>Cymbella</i>	4	Grazing NS Nutrients NS Interaction NS	
<i>Diatoma</i>	9	Grazing *** Nutrients *** Interaction ***	Positively affected by N and N+P in CLOSED cages, absent in OPEN cages.
<i>Gyrosigma</i>	17	Grazing *** Nutrients *** Interaction ***	Negatively affected by N and N+P in OPEN cages.
<i>Meridion</i>	26	Grazing NS Nutrients * Interaction NS	N<Un
<i>Nitzschia</i>	25	Grazing NS Nutrients NS Interaction NS	
<i>Green algae</i>			
<i>Crucigenia</i>	4	Grazing NS Nutrients *** Interaction NS	Un<N<N+P

NS, non-significant.

P* < 0.05; *P* < 0.01; ****P* < 0.001.

productivity) and shifts in taxonomic composition of the phytobenthos in high-altitude Alpine lakes. These effects probably accompany other perturbations, including eutrophication of pelagic systems (Bergström & Jansson, 2006; Elser *et al.*, 2009a) and acidification (e.g. Rogora, Marchetto & Mosello, 2001). At present, large regions of the Alps receive substantial atmospheric N deposition, in some cases exceeding $20 \text{ kg N ha}^{-1} \text{ year}^{-1}$ (Lepori *et al.*, 2003a). Therefore, the benthic and pelagic systems of high-altitude Alpine lakes have probably been modified by anthropogenic N.

Eutrophication of the benthic system can have important consequences for the structure and performance of Alpine lake ecosystems. For example, where increased productivity of the phytobenthos disproportionately involves unpalatable algae (this study; Nydick *et al.*, 2004), the transfer of energy between the phytobenthos and grazers could become inefficient. Given that in small, unproductive lakes animals feeding higher in the food web derive most energy and nutrients ultimately from the benthic habitat (Hecky & Hesslein, 1995; Vadeboncoeur *et al.*, 2002; Karlsson *et al.*, 2009), weakening this trophic link could diminish the efficiency of food chains. In addition, in pelagic systems, eutrophication reduces the diversity of phytoplankton by encouraging dominance by few mesotrophic species (Wolfe *et al.*, 2001; Wolfe, Van Gorp & Baron, 2003). Phytobenthic diversity may be liable to similar changes, although we are not aware of studies that have addressed these diversity effects.

In addition to causing effects on its own, N enrichment may influence mountain lake ecosystems through interactions with acidification, the other main effect of N deposition and the effects of other pressures, such as P deposition (Sickman, Melack & Clow, 2003; Morales-Baquero, Pulido-Villena & Reche, 2006a) and climate change (Beniston, 2005). For example, acidification often leads to the disappearance of key benthic grazers, including several mayflies, and this effect could further weaken the phytobenthos–grazer link (Winterbourn, Hildrew & Orton, 1992). In mountain fresh waters, production of phytoplankton (Lewis, 2011; Bergström *et al.*, 2013) and phytobenthos (F. Lepori, unpubl. data) is constrained not just by nutrients but also by water temperature. Therefore, N enrichment could amplify the response of lake ecosystems to warming (Bergström *et al.*, 2013). In a similar vein, high atmospheric N deposition drives lake ecosystems towards P limitation (Bergström & Jansson, 2006; Elser *et al.*, 2009b), rendering them more vulnerable to P inputs due to dust deposition (possibly linked to climate change) and biomass combustion (Morales-Baquero *et al.*, 2006b).

Thus, through individual and synergistic effects, N deposition is emerging as a key driver of ecological change in mountain lake ecosystems (Lepori & Keck, 2012).

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