PLOS ONE

Phytoplankton-Specific Response to Enrichment of Phosphorus-Rich Surface Waters with Ammonium, Nitrate, and Urea

Derek B. Donald[1], Matthew J. Bogard[1¤], Kerri Finlay[2], Lynda Bunting[1], Peter R. Leavitt[1]*

1 Limnology Laboratory, Department of Biology, University of Regina, Regina, Saskatchewan, Canada, 2 Department of Biology, University of Regina, Regina, Saskatchewan, Canada

Abstract

Supply of anthropogenic nitrogen (N) to the biosphere has tripled since 1960; however, little is known of how *in situ* response to N fertilisation differs among phytoplankton, whether species response varies with the chemical form of N, or how interpretation of N effects is influenced by the method of analysis (microscopy, pigment biomarkers). To address these issues, we conducted two 21-day *in situ* mesocosm (3140 L) experiments to quantify the species- and genus-specific responses of phytoplankton to fertilisation of P-rich lake waters with ammonium (NH_4^+), nitrate (NO_3^-), and urea ($[NH_2]_2CO$). Phytoplankton abundance was estimated using both microscopic enumeration of cell densities and high performance liquid chromatographic (HPLC) analysis of algal pigments. We found that total algal biomass increased 200% and 350% following fertilisation with NO_3^- and chemically-reduced N (NH_4^+, urea), respectively, although 144 individual taxa exhibited distinctive responses to N, including compound-specific stimulation (*Planktothrix agardhii* and NH_4^+), increased biomass with chemically-reduced N alone (*Scenedesmus* spp., *Coelastrum astroideum*) and no response (*Aphanizomenon flos-aquae*, *Ceratium hirundinella*). Principle components analyses (PCA) captured 53.2–69.9% of variation in experimental assemblages irrespective of the degree of taxonomic resolution of analysis. PCA of species-level data revealed that congeneric taxa exhibited common responses to fertilisation regimes (e.g., *Microcystis aeruginosa*, *M. flos-aquae*, *M. botrys*), whereas genera within the same division had widely divergent responses to added N (e.g., *Anabaena*, *Planktothrix*, *Microcystis*). Least-squares regression analysis demonstrated that changes in phytoplankton biomass determined by microscopy were correlated significantly ($p<0.005$) with variations in HPLC-derived concentrations of biomarker pigments ($r^2 = 0.13–0.64$) from all major algal groups, although HPLC tended to underestimate the relative abundance of cyanobacteria. Together, these findings show that while fertilisation of P-rich lakes with N can increase algal biomass, there is substantial variation in responses of genera and divisions to specific chemical forms of added N.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: Peter.Leavitt@uregina.ca

¤ Current address: Département des sciences biologiques, Pavillon des sciences biologiques, Université du Québec à Montréal, Montréal, Québec, Canada

Introduction

Human activities such as farming and industrial fixation of atmospheric nitrogen (N) have tripled the supply of N to the biosphere since 1960 and are expected to double present levels of N influx by 2050 to meet future demands for food production [1,2]. In particular, application of N fertilisers will exceed 275 Tg N year^{-1} and will be concentrated in regions where centuries of farming may have saturated soils with phosphorus (P) [3,4], increased P export to lakes [5], and overloaded surface waters with P [6]. In these regions, lakes already exhibit poor predictive relationships between P influx and algal abundance [7], continuously high concentrations of dissolved P despite abundant phytoplankton [3,8], insufficient biological fixation of N to support primary production [9,10], and strong positive correlations between N influx and total algal or cyanobacterial abundance [6,11]. Taken together, these findings suggest that persistent

fertiliser application has weakened the regulatory role of P [12,13], and that pollution with N may further degrade water quality in P-rich lakes [11].

At present, most evidence shows that freshwater eutrophication ultimately arises from persistent increases in P influx from urban and other anthropogenic sources [14–16]. However, synthesis of laboratory studies [17,18], *in situ* mesocosm experiments [19,20], whole-ecosystem manipulations [21] (but see) [22], catchment-scale mass balances [11,23], regional surveys [24] and palaeo-limnological reconstructions [6,11] also demonstrates that rises in N influx can independently increase algal biomass, alter the proportion of diazotrophic cyanobacteria, and increase toxicity of some algae, particularly in lakes with total P (TP) concentrations over 100 μg P L^{-1} and N : P mass ratios below ~20: 1 [25]. Lack of reconciliation between these two robust data sets has resulted in vigorous and occasionally acrimonious debate over the unique and
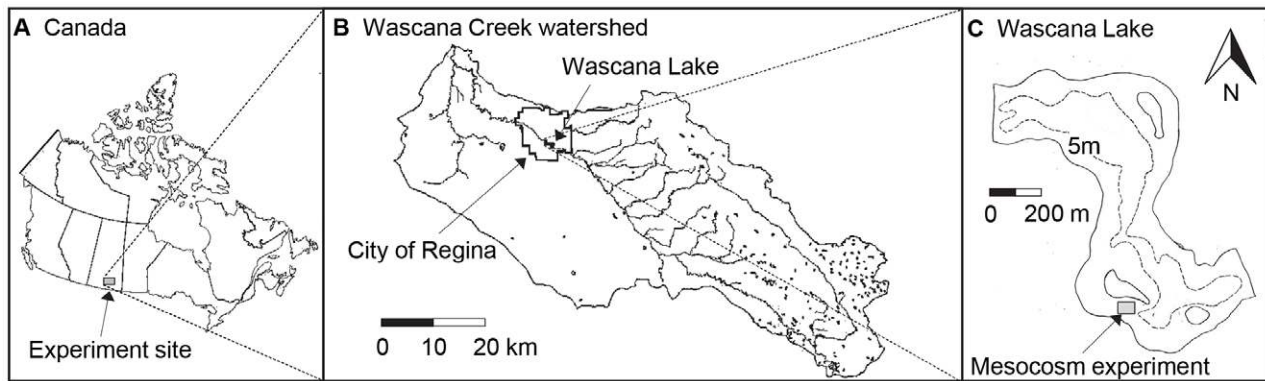
Figure 1. Map of Wascana Lake, Saskatchewan, Canada. Map shows a) the continental location, b) the gross drainage area (1400 km^2) and c) depth contour map with the location of the mesocosm experiment (shaded box).
doi:10.1371/journal.pone.0053277.g001

interactive roles of N in regulating baseline lake productivity [26,27] and cultural eutrophication [13,28,29].

Continuing uncertainty over ecosystem consequences of N pollution may arise in part because of comparatively limited understanding of how effects of N may vary with phytoplankton identity, nutritional capability and status, and the chemical form of added N [18,30,31]. At a coarse taxonomic level, preliminary evidence suggests that fertilisation of eutrophic waters with ammonium (NH_4^+) or urea ([NH_2]$_2$CO) increases the *in situ* abundance of non-N-fixing cyanobacteria [20,25] which exhibit efficient light use [32] and superior NH_4^+ uptake kinetics [33], as well as chlorophytes which can sustain rapid growth using diverse N sources if sufficient light is present [34,35]. In contrast, the competitive advantage of diazotrophic cyanobacteria can be lost following N fertilisation because N uptake suppresses formation of heterocysts and nitrogenase enzyme complexes needed for biological fixation of N_2 [20,36]. Finally, addition of nitrate (NO_3^-) to P-rich waters can favour production of diatoms if silica (Si) is available [37,38], possibly due to non-saturating uptake kinetics for this compound [39]. However, despite these broad generalizations, substantial uncertainty surrounds the *in situ* response of individual species or genera of phytoplankton to fertilisation with N [40–42] due to low taxonomic resolution of prior N studies (division-level), substantial overlap among algae in *in vitro* nutrient-uptake capabilities (N half-saturation constant, K_s, = 1–14 μg N L^{-1}) and maximum growth rates (N-sufficient V_{max} = 0.2–8.0 ln units day^{-1}) [30,31,43], and the high degree of environmental simplification in laboratory and microcosm studies [44].

In this study, we conducted 21-day long mesocosm (3140 L) experiments in summer and autumn to quantify the *in situ* response of over 140 individual phytoplankton taxa to enrichment of P-rich freshwaters with NH_4^+, NO_3^- and urea. Effects of N addition on total algal abundance and gross community composition in this experiment have been analysed previously using high performance liquid chromatography (HPLC) and reported in [25]. Instead the unique objectives of the present study are four-fold: 1) to use microscopic analysis to quantify interspecific variation among algae in the response to N amendments; 2) to determine how phytoplankton-specific responses vary with the chemical form of added N; 3) to evaluate the influence of the taxonomic resolution of microscopic analysis (division, genus, species) on interpretation of N effects on phytoplankton, and; 4) to compare changes in phytoplankton assemblages derived from microscopic enumeration of cell densities and chromatographic analysis of algal pigments. As suggested elsewhere, biomarker-based analyses might be biased by limited taxonomic resolution, phylogenetic variation in cellular pigment content, or changes in ambient environmental conditions (light, temperature, nutrient availability) which uniquely influence the cellular quota of pigments [45–47]. Improved understanding of the nature of taxon-specific responses to N influx may help protect aquatic ecosystems against future pollution with agricultural N [2], optimise wastewater treatment procedures [28], and resolve on-going debate concerning the respective roles of N and P in regulating cultural eutrophication [48].

Methods

Study Site

The experiments were conducted in Wascana Lake (Fig. 1), an unstratified, 0.5 km^2, 7-m deep basin located within the central urban park of the City of Regina, Saskatchewan, Canada (50°26.17′N, 104°36.91′W). Regional evaporation (~60 cm year^{-1}) exceeds precipitation by two-fold, the climate is classified as sub-humid continental, and mean monthly air temperatures vary by up to 35°C (19°C in July, −16°C in January) [49]. Wascana Lake is fed by Wascana Creek, a permanent stream which drains a 1400 km^2 agricultural basin [49]. Snow melt during March–April typically accounts for 80% of annual runoff in the region [50] resulting in seasonally variable, but generally low, water residence in the lake (decadal mean ~0.07 yr) [49].

Wascana Lake has been monitored biweekly (May–Aug) since 1996 and exhibits elevated but annually-variable mean (±SD) summer concentrations of Chl *a* (39±48 μg L^{-1}), soluble reactive P (SRP) (192±161 μg P L^{-1}), total dissolved P (TDP) (299±208 μg P L^{-1}), NO_3^- (119±217 μg N L^{-1}), NH_4^+ (94±203 μg N L^{-1}), total dissolved N (TDN) (1327±726 μg N L^{-1}), and dissolved organic carbon (DOC) (16.2±4.0 mg C L^{-1}). Consequently, mean mass ratios of TDN : SRP are low (6.9±6.6). Typical plankton phenology includes a progression from a spring community composed of diatoms, cryptophytes, and copepods (*Diacyclops thomasi, Leptodiaptomus siciloides*), through a pronounced June clearwater phase with high densities of large-bodied Cladocera (*D. magna, D. pulicaria, D. galeata mendotae*) [51], to a summer and autumn community composed of small-bodied zooplankton (*Eubosmina coregoni, Bosmina longirostris, Diaphanosoma birgei, Daphnia retrocurva, Ceriodaphnia, Chydorus,* rotifers) and abundant cyanobacteria [8,49]. Overall, cryptophytes, chrysophytes and chlorophytes comprise <20% of summer algal biomass. Instead, N-fixing *Aphanizomenon flos-aquae* and *Anabaena* spp. are
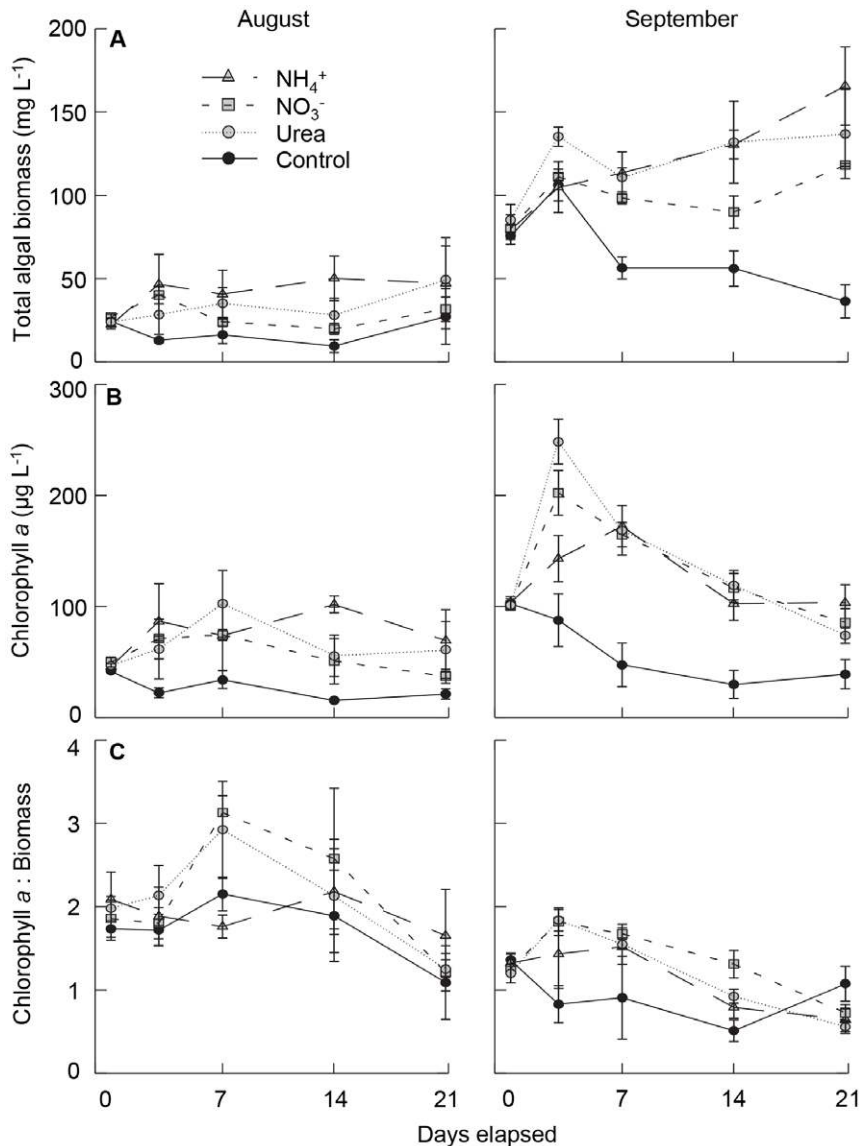
Figure 2. Biomass responses of total algal response to fertilisation with nitrogen in mesocosms conducted in August and September. Time series include; a) total phytoplankton biomass (mg wet mass L^{-1}), b) Chl a (μg L^{-1}) and c) the ratio of Chl a : total phytoplankton biomass. Symbols represent mean and standard error ($\pm$ SE, $n = 3$) for each nitrogen treatment, including amendments with NH_4^+ (shaded triangle, coarse dashed line), NO_3^- (shaded square, medium dashed line) and urea (shaded circle, fine dashed line), as well as unamended (control) mesocosms (solid circle, solid line).

doi:10.1371/journal.pone.0053277.g002

abundant immediately after the clearwater phase, *Microcystis* spp. are common in the warm ($>25^{\circ}$C) surface waters during August, *Planktothrix agardhii* is dominant during late-August through September, and *Phormidium* (and *Cyclotella*) spp. increase thereafter. Consequently, late-summer concentrations of the cyanobacterial toxin microcystin (MC) can be 10-fold greater than the upper limits recommended by the World Health Organization for drinking water (1 μg MC L^{-1}).

Mesocosm Experiments

Three-week long mesocosm experiments were conducted during both August and September of 2008, as described in [25]. Briefly, 2-m wide by 1-m deep, cylindrical, opaque white poly-weave enclosures (3140 L) were open to the atmosphere, closed to the sediments, and located in a sheltered embayment

(Fig. 1). Enclosures were filled passively (drawn up from depth), attached to anchored floating frames, and assigned treatments at random. No attempt was made to circulate these well-mixed mesocosms or to modify zooplankton densities, although minnow traps were added to each enclosure and checked routinely to remove planktivorous fish. Advantages and limitations of this experimental design for eutrophication studies are detailed in [20] who studied division-level effects of urea on algal communities and [25] who also contrasted urea with nitrate and ammonium effects, but did not analyse species- and genus-level responses.

Each experimental treatment consisted of three replicates to which N was added on days 0, 7, and 14 as sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl) or urea ((NH_2)$_2$CO). All trials received 6 mg N L^{-1} per week, whereas unamended enclosures served as controls. Nitrogen additions were intended

Table 1. Repeated-measures analysis of variance of total phytoplankton biomass, chlorophyll *a*, chlorophyll *a* : biomass ratio, and biomass of major algal groups.

Response variable	August		September	
	p	*Post hoc*	*p*	*Post hoc*
Total phytoplankton biomass				
Treatment	**0.041**	NH, U, NO>U, NO, C	**0.000**	U, NH, NO>C
Interaction	**0.042**		**0.000**	
Chlorophyll *a*				
Treatment	**0.043**	NH, U, NO>U, NO, C	**0.000**	U, NO, NH>C
Interaction	**0.016**		**0.000**	
Chlorophyll *a* : biomass ratio				
Treatment	0.335	–	0.077	–
Interaction	0.457		**0.000**	
Cyanobacteria				
Treatment	0.110	–	**0.000**	NH, U, NO>C
Interaction	**0.035**		**0.000**	
Chlorophytes				
Treatment	**0.048**	NH, U, NO>U, NO, C	**0.001**	NH>U, NO, C
Interaction	0.053		**0.000**	
Diatoms				
Treatment	**0.004**	NO, U, C>C, NH	**0.009**	NO, U, C>U, C, NH
Interaction	**0.000**		**0.000**	
Chrysophytes				
Treatment	0.092	–	**0.005**	NH, U>U, C, NO
Interaction	**0.032**		**0.004**	
Cryptophytes				
Treatment	**0.025**	NO, U, C>U, C, NH	**0.020**	U, NO, C>NO, C, NH
Interaction	**0.007**		**0.002**	
Dinoflagelates				
Treatment	**0.009**	C, U, NO>NO, NH	0.345	–
Interaction	**0.000**		0.847	

Probability (*p*) values were calculated for treatment and treatment-time interaction effects. Tukey's HSD *post hoc* results represent mean treatment values ordered from largest to smallest and significant differences (>) at $\alpha = 0.05$, for urea (U), nitrate (NO), ammonium (NH), and the control (C). If a treatment falls on both sides of a ">" this indicates no significant difference from the treatments on either side. All phytoplankton biomass (mg L^{-1}) data, but not chlorophyll *a* concentrations (µg L^{-1}), were $\log_{10}(x+1)$ transformed prior to analysis to meet assumptions of normality. Probabilities were not corrected for number of comparisons. See Methods for additional information.

doi:10.1371/journal.pone.0053277.t001

to increase soluble N : P to >22: 1 by mass to suppress N-fixing cyanobacteria [52], were based on decadal mean concentrations of SRP and TDN, and were within the range of N content observed in regional lakes [50].

Sampling took place on days 0, 3, 7, 14 and 21 between 10 00 h and 14 00 h, immediately after N addition on day 0, and before N additions on days 7 and 14. Water was collected from mesocosms using a 6-L Van Dorn bottle deployed centrally at 0.5-m depth. All water samples were screened (247-µm mesh) in the field to remove invertebrates, filtered through 0.45-µm pore membrane filters within 2 hr, and stored in darkness at 4°C until analysed for chemical solutes. Samples of 100 mL were collected from Van Dorn bottles and preserved with Lugol's iodine solution for microscopic analysis of phytoplankton species composition and abundance. In addition, particulate organic matter (POM) was concentrated on GF/C glass fibre filters (nominal pore 1.2-µm), and stored frozen (−10°C) in darkness until analysis of biomarker pigments using HPLC. Physical (Secchi disk transparency,

temperature) and chemical parameters (conductivity, dissolved O_2, pH) were also recorded in the field following standard protocols [11,49] and are reported in [25].

Laboratory Analysis

Preserved phytoplankton were identified, enumerated and measured using a Leica model DM IRB inverted light microscope (Leica Microsystems, Concord, Canada) and the Utermöhl sedimentation technique [53]. Aliquots of 2-mL were settled for 24 h prior to enumeration and analysis. Counts of cells, filaments and colonies were conducted at 100×, 400× or 1000× magnification, depending on the size and abundance of algal units, and were identified following the taxonomic conventions of [54–56]. Enumeration at 100× was made on every second transect within 50% of the depositional area of slides, those at 400× on one horizontal and one vertical transect, and counts at 1000× on 30 random fields of view, such that over 300 algal units were recorded for each sample. Cell measurements were conducted for abundant
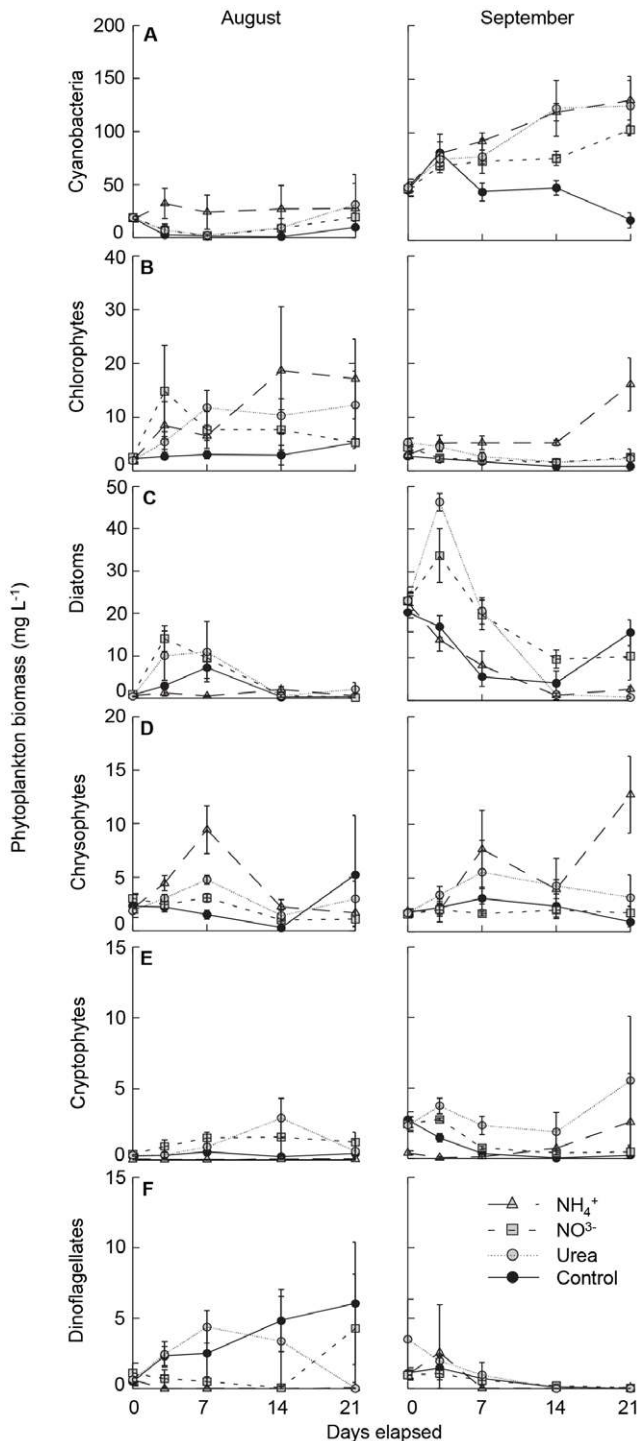
Figure 3. Biomass responses of major phytoplankton groups to fertilisation with nitrogen in mesocosms conducted in August and September. Algal groups (mg wet mass L^{-1}) include; a) cyanobacteria, b) chlorophytes, c) diatoms, d) chrysophytes, e) cryptophytes and f) dinoflagellates. Symbols represent mean and standard errors ($\pm$ SE, $n = 3$) for each of the nitrogen treatments, included amendments with NH_4^+ (shaded triangle, coarse dashed line), NO_3^- (shaded square, medium dashed line) and urea (shaded circle, fine dashed line), as well as unamended (control) mesocosms (solid circle, solid line).
doi:10.1371/journal.pone.0053277.g003

taxa at $1000\times$ magnification on 30 randomly-selected individuals from a composite sample representing all enclosures and sampling events. Biovolume calculations followed [57] and were converted to cellular biomass by assuming that 1 mm^3 of volume was equivalent to 1 mg of wet-weight biomass.

Phytoplankton pigments were extracted from POM using a mixture of acetone : methanol (80: 15, by volume) and were quantified using standard methods including spectrophotometry [58] and HPLC analyses [59]. HPLC used an Agilent model 1100 system (Agilent Technologies Inc, Mississagua, Canada) fitted with photodiode-array and fluorescence detectors and was calibrated using authentic pigment standards. Chlorophyll a (Chl a) concentrations were used to estimate total algal abundance (μg L^{-1}), while other taxonomically-diagnostic pigments (nmol L^{-1}) were used to quantify changes in siliceous algae (mainly diatoms and chrysophytes) (fucoxanthin), cryptophytes (alloxanthin), dino-flagellates (peridinin), chlorophytes (Chl b) and colonial cyanobac-teria (myxoxanthophyll). Pigments from N-fixing cyanobacteria (canthaxanthin, aphanizophyll) were near detection limits during the 2008 experiments and were not included in further analyses.

Concentrations of dissolved nutrients were conducted at the University of Alberta Water Chemistry Laboratory following [60], are reported in [25], and included TDP, SRP, TDN, $NH_4^+ + NH_3$ (as NH_4^+ hereafter), and $NO_3^- + NO_2^-$ (as NO_3^- hereafter). Concentrations of urea [61] and dissolved organic carbon (DOC) [62] were determined at University of Regina Environmental Quality Analysis Laboratory using standard protocols reported in [25].

Numerical Analysis

Repeated measures analysis of variance (RM-ANOVA) was used to estimate the statistical significance of differences in phytoplankton abundance among treatments, as well as interac-tions between time and treatment effects [20,25]. Briefly, data were transformed ($\log_{10}$ [x+1]) as necessary prior to analysis and appropriate critical F-statistics selected for experiments with four treatment levels, three replicates and five sampling events (i.e. $F_{treatment} = 4.07$, $F_{time} = 2.78$, $F_{treatment \times time} = 2.18$). Statistically-significant differences among treatments were tested using Tukey's Honestly Significant Difference (HSD) post hoc test. No correction was made for the number of comparisons as one of our intentions was to quantify the general patterns of phytoplankton response to N amendments, although we recognize that this approach may inflate the number of apparently-significant algal responses. Least-squares regression analysis was used to quantify the linear relationship between microscopic- and pigment-based estimates of phytoplankton abundance. RM-ANOVA and linear regressions were conducted using software from SPSS version 11 (IBM, Armonk, NY, USA) and SYSTAT version 10 (SYSTAT Software Inc., Chicago, IL, USA), respectively.

Principal component analysis (PCA) was used to summarise the main patterns of phytoplankton community response to N fertilisation, and to evaluate how patterns of response varied with the taxonomic resolution of microscopic analysis, including division or class (division hereafter), genus and species. These levels of classification were selected because they are commonly used in limnological studies, but differ considerably in the total effort required for microscopic analysis [56]. An individual genus or species was included in PCA only if their mean biomass for the experiment was >1% of the total algal abundance in at least one of the twelve experimental enclosures. Estimates of algal abun-dance were $\log_{10}$ (x+1)-transformed prior to analysis using CANOCO version 4.5 software (Microcomputer Power, Ithaca, NY, USA). As our intention was to evaluate algal response to N
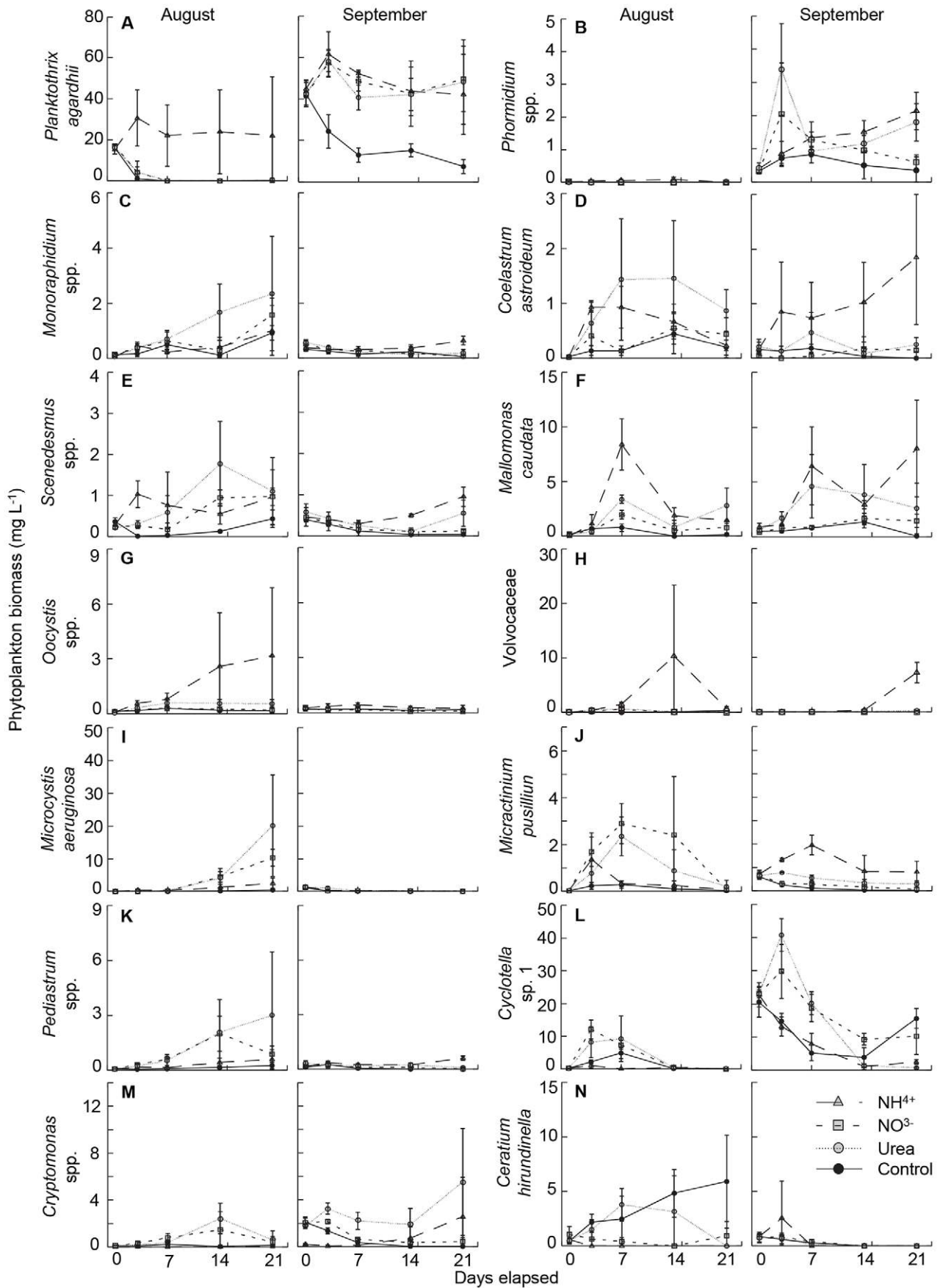
fertilisation, categorical N treatments (e.g.,+or − urea) were included as passive variables in each PCA, whereas other environmental variables were not included in the ordinations.

This study obtained all necessary permits and approvals required by Environment Canada, Saskatchewan Environment, Transport Canada, and the Wascana Centre Authority (City of Regina) and adhered to all ethical and environmental regulations of the University of Regina and the Natural Sciences and Engineering Research Council of Canada.

Results

Lake and Mesocosm Conditions

As presented in [25], nutrient concentrations in Wascana Lake were elevated (~125–175 µg P L-1, ~1.2–1.6 mg N L-1), TDN : TDP mass ratios were low (~10–15), and bottle bioassays revealed that phytoplankton growth exhibited instantaneous limitation by N supply during both August and September. As expected, mesocosm fertilisation elevated TDN to 15–20 mg N L-1, but had few measured effects on mesocosm water chemistry (pH, conductivity, oxygen, etc.) other than a rapid decline in SRP from ~100 µg P L-1 to <5 µg P L-1, and a 50% decline in TDP, by day 14 in all N-amended enclosures. Further details and interpretations of water chemistry change are presented in [25].

Community Response to N fertilisation

Total algal biomass measured microscopically or using ubiquitous Chl a increased ~200% and ~350% when fertilised with NO_3^- and chemically-reduced N (urea, NH_4^+), respectively (Fig. 2). Biomass response to NH_4^+ fertilisation during August and all N amendments in September was statistically significant ($p_{treatment}$ <0.05) relative to control enclosures (Table 1), although there was no significant effect of treatment on the ratio of Chl a : biomass ($p_{treatment}$ >0.05).

The biomass response of algal divisions varied substantially with both phylogenetic group and chemical form of added N (Fig. 3, Table 1). In particular, abundance of cyanobacteria (Fig. 3a), chlorophytes (Fig. 3b) and chrysophytes (Fig. 3d) increased 400–800% following fertilisation with NH_4^+ and secondarily urea, changes which were significant during September ($p_{treatment}$ <0.005), but only marginally so during August (0.05<$p_{treatment}$ <0.11) due to high variability among NH_4^+ enclosures. In contrast, biomass of diatoms (Fig. 3c) and cryptophytes (Fig. 3e) increased ~300% and ~600% in treatments receiving NO_3^- and urea, respectively, but were suppressed by addition of NH_4^+ ($p_{treatment}$ <0.025) (Table 1). Finally, dinoflagellates were ~500% more abundant in control mesocosms and those receiving urea during August relative to enclosures fertilised with NH_4^+ or NO_3^- ($p_{treatment}$ = 0.009), but showed little response to N additions during the September experiment (Fig. 3f).

Species and Genus Response to N fertilisation

Phytoplankton assemblages in mesocosms consisted of 144 phytoplankton taxa, of which 51 responded significantly to N treatments (Table S1), 45 exhibited an increase in biomass following N fertilisation, and 14 had a wet-weight biomass of more than 1 mg L^{-1} on at least one sampling date (Fig. 4, Table 2). Overall, the biomass-dominant species exhibited distinctive

responses to specific N compounds. For example, fertilisation with NH_4^+ consistently increased growth of *Planktothrix agardhii* (Fig. 4a), a species which accounted for up to 60% of total biomass, some members of the family Volvocaceae (*Pandorina morum*, *Pledorina illinoisensis*, *Gonium pectorale*) (Fig. 4h) (up to 50% of biomass) and *Oocystis* spp. (Fig. 4g) (~10% of biomass). Similarly some algae were stimulated by both forms of chemically-reduced N, including *Phormidium* spp., *Monoraphidium* spp., *Scenedesmus* spp., *Coelastrum astroideum* and *Mallomonas caudata* (Fig. 4b–f). In contrast, species from the genera *Cyclotella* (Fig. 4l) and *Cryptomonas* (Fig. 4m) (10–40% of biomass) were stimulated more by NO_3^- and urea than by NH_4^+, while the dinoflagellate *Ceratium hirundinella* (Fig. 4n) (25–50% of biomass) and rare diazotrophic cyanobacteria (*Aphanizomenon flos-aquae*, *Anabaena viguieri*, *Anabaena* spp.) grew poorly in fertilised enclosures. Finally, some abundant algae (*Microcystis aeruginosa*, *Micractinium pusillum*, *Pediastrum* spp.) (Figs 4i, j, k) (10–40% of biomass) showed inconsistent responses to fertilisation, with highest biomass recorded following amendment with NO_3^- or urea in August, and with NH_4^+ during September.

Influence of Taxonomic Resolution on Interpretation of N effects

PCA captured 53.2–69.9% of variation in phytoplankton community composition on the first two ordination axes when phytoplankton was resolved to level of division, genus or species (Fig. 5). Total explained variation decreased only slightly with increased taxonomic resolution. In general, axis 1 explained more variance in the September experiment (40.9–55.4%) than during August trials (32.4–43.6%). During August, axis 1 was associated positively with NH_4^+ at all taxonomic levels and negatively with NO_3^- and urea for PCAs with genus or species resolution, while axis 2 was associated most strongly with control mesocosms at all taxonomic resolutions. During September, axis 1 was correlated positively with NH_4^+ treatments and negatively with NO_3^- treatments at the division and genus levels, but was not related linearly to any N treatment in the PCA of algal species. In contrast, axis 2 was associated positively with the urea, control, and NO_3^- treatments at the division, genus and species levels, respectively, and negatively with the control, urea, and NH_4^+ trials at the division, genus and species levels, respectively.

PCA of algal divisions confirmed that total cyanobacterial biomass was associated positively with NH_4^+ treatments in both experiments, while that of chrysophytes and euglenoids responded positively to NH_4^+ in September alone (Figs. 5a, b). In contrast, chlorophyte, cryptophyte and diatom abundances were elevated in mesocosms treated with urea and NO_3^- during August, but NO_3^- (diatoms) or urea alone (chlorophytes and cryptophytes) during September. Finally, dinoflagellate abundance was associated mainly with control enclosures in August, but not during September.

Multivariate analysis at a finer taxonomic resolution demonstrated that genera within algal divisions often exhibited individualistic responses to N treatments (Fig. 5c, d). Among cyanobacteria, *Planktothrix* was strongly associated with NH_4^+ treatments in both experiments, whereas *Microcystis* was abundant in enclosures amended with urea and NO_3^- only during August, and *Phormidium* was abundant in urea treatments during September. Similarly, the chlorophytes *Closterium* and *Pleodorina* were associated positively

Table 2. Repeated-measures analysis of variance for the response of selected phytoplankton taxa to added nitrogen.

Response variable	August		September	
	p	Post hoc	p	Post hoc
Planktothrix agardhii				
Treatment	**0.003**	NH>U, NO, C	**0.000**	NH, NO, U>C
Interaction	**0.015**		**0.001**	
Phormidium spp.				
Treatment	0.103	–	**0.014**	U, NH, NO>NO, C
Interaction	0.101		**0.010**	
Monoraphidium spp.				
Treatment	0.174	–	**0.041**	NH, U, NO>U, NO, C
Interaction	0.524		**0.002**	
Coelastrum astroideum				
Treatment	0.056	–	0.117	–
Interaction	0.364		0.222	
Scenedesmus spp.				
Treatment	**0.013**	U, NH, NO>NO, C	**0.011**	NH, U>U, NO, C
Interaction	0.144		**0.002**	
Mallomonas caudata				
Treatment	**0.001**	NH, U>U, NO>NO, C	**0.001**	NH,U>U, NO>NO, C
Interaction	**0.006**		**0.046**	
Oocystis spp.				
Treatment	0.244	–	**0.010**	NH, U>U, C, NO
Interaction	0.523		0.746	
Volvocaceae				
Treatment	0.059	–	**0.000**	NH>U, C, NO
Interaction	0.247		**0.000**	
Microcystis aeruginosa				
Treatment	0.122	–	0.092	–
Interaction	**0.003**		**0.046**	
Micractinium pusillum				
Treatment	**0.047**	NO, U, NH>U, NH, C	**0.000**	NH>U>NO, C
Interaction	**0.024**		0.063	
Pediastrum spp.				
Treatment	0.249	–	**0.004**	NH, U>U, NO>NO, C
Interaction	0.262		**0.000**	
Cyclotella spp.				
Treatment	**0.013**	NO, U, C>C, NH	**0.012**	NO, U, C>U, C, NH
Interaction	**0.002**		**0.000**	
Cryptomonas spp.				
Treatment	0.084	–	**0.023**	U, NO>NO, C, NH
Interaction	**0.018**		**0.002**	
Ceratium hirundinella				
Treatment	**0.003**	C, U>U, NO>NO, NH	0.906	–
Interaction	**0.006**		0.995	

Probability (*p*) values were calculated for treatment and treatment-time interaction effects. Tukey's HSD *post hoc* results represent mean treatment values ordered from largest to smallest and significant differences (>) at α = 0.05, for urea (U), nitrate (NO), ammonium (NH), and the control (C). If a

Table 2. Cont.

treatment falls on both sides of a ">" this indicates no significant difference from the treatments on either side. All phytoplankton biomass (mg L^{-1}) data were log$_{10}$(x+1??transformed prior to analysis to meet assumptions of normality. Probabilities were not corrected for number of comparisons.
doi:10.1371/journal.pone.0053277.t002

with NH_4^+ treatments during August; however, *Micractinium* and *Coelastrum* were common in urea amendments during September, while *Monoraphidium* and *Pediastrum* were associated with urea treatments in both experiments and with NO_3^- in during August. Divergent responses were also recorded for cryptophytes genera, with *Cryptomonas* being associated positively with NO_3^- fertilisation during August and urea treatments in both months, and *Komma* exhibiting elevated abundance within control enclosures during August. Finally, the chrysophyte *Mallomonas* exhibited the fundamentally different responses to those of *Chromulina* and *Ochromonas*.

PCA of common algal species (>1% of biomass) revealed low variability among congeneric taxa in response to N treatments (Figs. 5e, f). For example, *Microcystis aeruginosa*, *M. botrys* and *M. flos-aquae* were all associated weakly with urea and NO_3^- treatments during August. Similarly *Cryptomonas ovata* and *C. erosa*, as well as *Pediastrum duplex* and *P. boryanum*, responded positively to NO_3^- and urea treatments, whereas *Synedra acus* and *S. ulna* were common in NH_4^+ treatments. Congruent ordination of congeneric species was also evident for members of the genera *Cyclotella* and *Mallomonas*.

Comparison of Microscopy and HPLC

Changes in phytoplankton biomass determined by microscopy were correlated significantly ($p_{treatment} < 0.005$) with variations in concentrations of taxonomically-diagnostic pigments for all major algal groups (Table 3). The strongest correlations were observed for Chl *b*-chlorophytes ($r^2 = 0.42-0.63$), myxoxanthophyll-colonial cyanobacteria ($r^2 = 0.31-0.52$), and fucoxanthin-siliceous algae ($r^2 = 0.29-0.46$), although significant linear relationships were recorded for alloxanthin-cryptophytes ($r^2 = 0.18-0.28$) and peridinin-dinoflagellates ($r^2 = 0.13-0.36$).

Patterns of temporal change in gross community composition (division level) were also very similar when analysed by microscopy or HPLC (Fig. 6). Overall, HPLC analysis tended to underestimate cyanobacterial contributions to the phytoplankton community, whereas chlorophytes and cryptophytes composed a greater fraction of total abundance when based on biomarker pigments. Similarly, siliceous algae (diatoms+chrysophytes) were slightly overrepresented by pigment analysis in the September experiment, but not during trials conducted in August (Fig. 5e, f).

Discussion

Three-fold expansion of agricultural fertilisation [1], exponential growth of cities [2], high infrastructure costs to eliminate waste N [63], and scientific debate concerning the role of N in eutrophication [13,28] have combined to prolong N pollution and degrade some freshwater ecosystems [11,64]. In part, uncertainty over best management practices may arise because we do not often distinguish clearly how algal response to N pollution differs among taxonomic groups, whether the response of individual taxa varies with the chemical form of N, or how differences in analytical approach (microscopy, pigment biomarkers) affect the interpretation of N effects on water quality. Analysis of experiments herein demonstrates that growth of >30% of phytoplankton species in eutrophic lake waters was stimulated
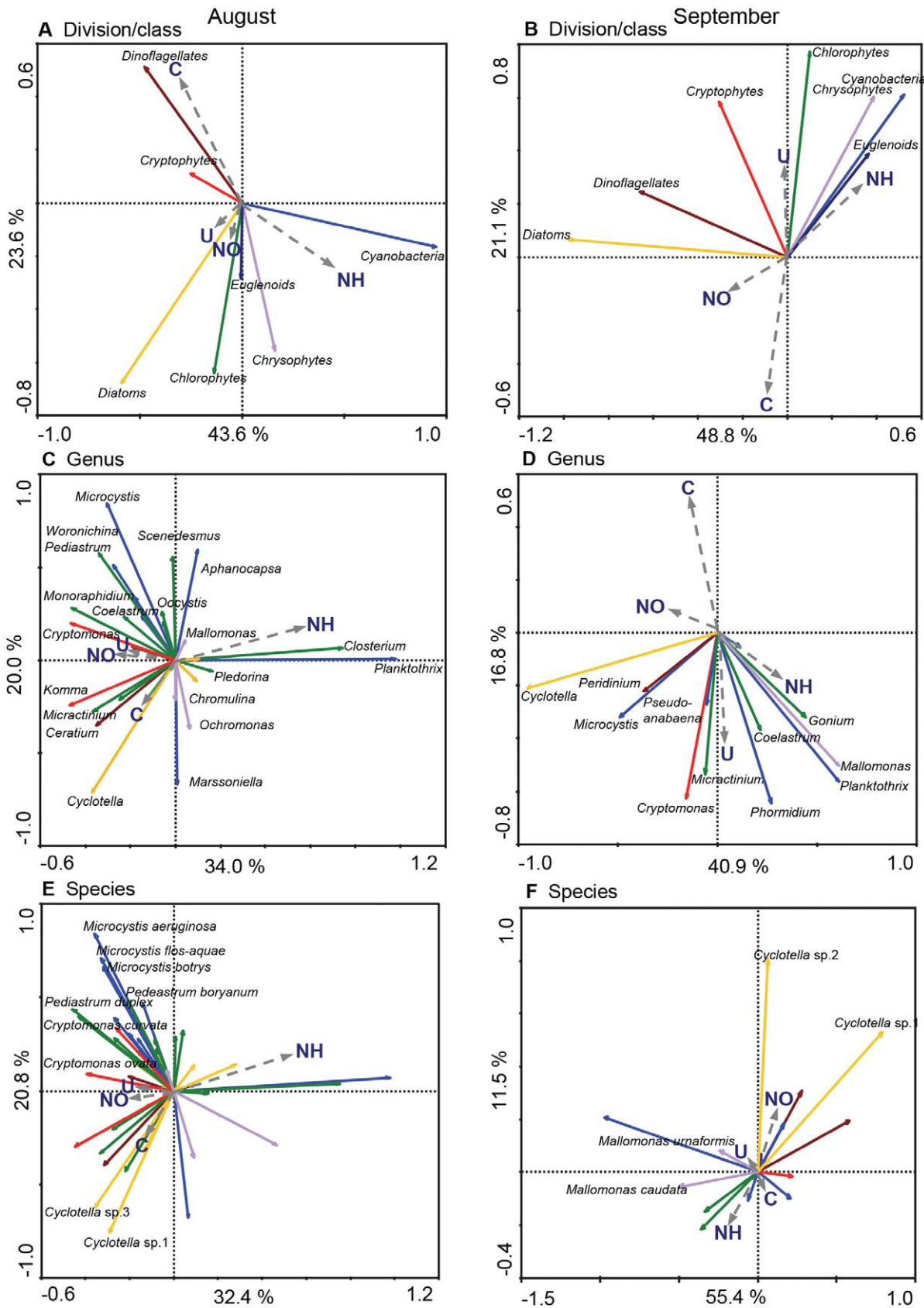
Figure 5. Principal component analysis of experimental phytoplankton assemblages at the a) division, b) genus, and c) species level of taxonomic resolution. Genera and species were selected if their cumulative biomass over the course of each experiment was more than 1% of the total for any of the 12 enclosures. Algal densities were $\log_{10}(x +1)$-transformed as needed, and categorical nitrogen treatments (e.g.,+or –

urea) were included as passive variables. All samples were included in each PCA; however, to simplify presentation, sample ordination points are not presented and only select taxa are identified. Coloured arrows indicate cyanobacteria (blue), chlorophytes (green), cryptophytes (red), diatoms (yellow), dinoflagellates (brown), and chrysophytes (purple). Proportion of total variation explained by first (x) and second (y) principle axes are presented.

significantly by N fertilisation (Table S1), and that the differential response of algal divisions to each chemical form of N (Fig. 3) resulted from distinct responses of algal genera rather than from unique responses of congeneric species (Fig. 5) consistent with [65]. Specifically, fertilisation with NH_4^+ increased total algal abundance $\sim$350% (Fig. 2) and cyanobacterial biomass over 500% (Fig. 3a) because *Planktothrix agardhii* accounted for up to 60% of phytoplankton biomass and its growth increased nearly six-fold following addition of chemically-reduced N (Fig. 4a). In contrast, *Microcystis aeruginosa* responded mainly to NO_3^- addition, particularly when waters were warm [20,66]. These experiments also documented significant correlations between microscopic- and HPLC-based estimates of algal abundance (Table 3) consistent with previous calibration exercises [45,46,67]. As well, the highly similar patterns of temporal change in assemblage composition (Fig. 6) infer that pigment-based investigations accurately represent how N can degrade lake ecosystems [6,11,20,25]. When considered in the context of previous laboratory [17,18], mesocosm [19,20], whole-lake [21,68], catchment [11,23,24] and palaeolimnological studies [6,11], the present analysis confirms that pollution with diverse forms of N can degrade P-rich lakes by promoting toxic cyanobacteria such as *Microcystis* and *Planktothrix*, but shows that there is no unique response of 'cyanobacteria' as has been suggested in management studies [28,48].

Response of Predominant Phytoplankton to N fertilisation

Addition of NH_4^+ to P-rich lake water favoured a community composed predominantly of nondiazotrophic *Planktothrix agardhii*, a taxon known to produce high levels of microcystin [69,70]. Elevated growth of *P. agardhii* likely reflects its lower Km for reduced N [33], higher maximum uptake rates [71] relative to other taxa [38], and the general preference of cyanobacteria for

chemically-reduced forms of N [17,36]. In addition, low energetic requirements for NH_4^+ assimilation would have allowed *P. agardhii* to maintain a high biomass and shade out competitors, thereby perpetuating a competitive advantage for this low-light-adapted taxon [72,73]. Continued dominance of *P. agardhii* into mid-September was also consistent with algal phenologies seen in other unstratified lakes [6] and with this species' higher tolerance to low temperatures relative to that of *Microcystis* [66,74]. Such selective stimulation of *Planktothrix* growth by N can also result in five- to 10-fold increases in water-column concentrations of microcystin [20,25], as transcription of toxin synthesis genes is also up-regulated by N assimilation [75].

Nitrate amendment favoured initial growth of large centric diatoms such as *Cyclotella* spp. (Figs. 3c, 4l), before giving way to colonial *Microcystis* spp. during August (Fig. 4i). As shown elsewhere, members of the genus *Cyclotella* are often abundant in NO_3^- rich eutrophic waters [37,38,76], possibly because they exhibit higher affinity for NO_3^- [77] and non-saturating uptake kinetics for that compound [39]. However, under conditions in which their growth becomes limited by the supply of P, Si or light [37,78], diatoms can be replaced by dense blooms of slower-growing *Microcystis* spp. [43]. Members of this latter genus can exhibit relatively low Km [33] and high V_{max} [79] for NO_3^- under *in vitro* conditions, are capable of substantial storage of P [10], and use vertical migration to optimize energy receipt [80]. However, we recommend caution when interpreting the mechanisms underlying these rapid ($<$7 day) changes in algal composition both because our experimental design may favour limitation of diatom growth by Si [20], and because *Microcystis* abundance is also suppressed by water temperatures less than 20°C [66,81]. Consequently, although our mesocosms are suitable for evaluation of many pelagic processes [20], we suggest that further *in situ* experimentation be conducted to evaluate controls of diatom abundance, including use of mesocosms which include benthic habitats.

Addition of urea to eutrophic environments stimulated the growth of many phytoplankton taxa (Table S1; Figs 4, 5), similar to findings from earlier laboratory [18,30,82] and field studies [20,83]. Preference for urea as a N source may be widespread among algae because this compound enters cells by passive diffusion or light-independent transporters [84], transports two NH_4^+ for every molecule acquired [85], is assimilated into organic matter without intracellular chemical reduction [36], and releases CO_2 following assimilation, partly reducing the need for active uptake of HCO_3^- at high pH [86]. In fact, energetic costs for assimilation of chemically-reduced N species are less than half that associated with atmospheric N or dissolved NO_3^- [17], consistent with our observation that total algal biomass was $\sim$1.5-fold higher when phytoplankton received NH_4^+ or urea than when NO_3^- was added (Fig. 2). However, despite expected energetic benefits of urea and NH_4^+, the wide variety of phytoplankton species response to different chemical forms of N (Fig. 3, 4) demonstrates that factors other than simple energetic costs of assimilation must also influence algal response to N, including temperature [20,25], nutrient co-limitation [31,37], or cellular stoichiometry [77,87].

Interestingly, all forms of N amendment increased growth of chlorophyte algae (Figs 3b, 5e), including 26 of 69 taxa (Table S1). Chlorophytes are sometimes associated with N-enriched eutrophic environments [19], particularly those of shallow lakes where light may penetrate to benthic substrates [21,34]. In general, green algae are

Table 3. Least-squares regression analysis of the linear relationship between microscopic and chromatographic estimates of phytoplankton abundance.

Model		August		September	
Pigment	Algal group	r^2	p	r^2	p
Chlorophyll *a*	Total biomass	0.641	0.000	0.418	0.000
Myxoxanthophyll	Colonial cyanobacteria	0.313	0.000	0.523	0.000
Chlorophyll *b*	Chlorophytes	0.630	0.000	0.416	0.000
Fucoxanthin	Chrysophytes and diatoms	0.292	0.000	0.458	0.000
Alloxanthin	Cryptophytes	0.280	0.000	0.181	0.000
Peridinin	Dinoflagellates	0.355	0.000	0.129	0.005

Phytoplankton biomass was measured by microscopy, while concentrations of taxonomically-diagnostic biomarker pigments were analysed by spectrophotometry (chlorophyll *a*) and high performance liquid chromatography (all other pigments). Data were log$_{10}$(x+1) transformed prior to analysis (*df* = 58). Algal biomass was summed according to distribution of indicator pigments prior to statistical analysis.
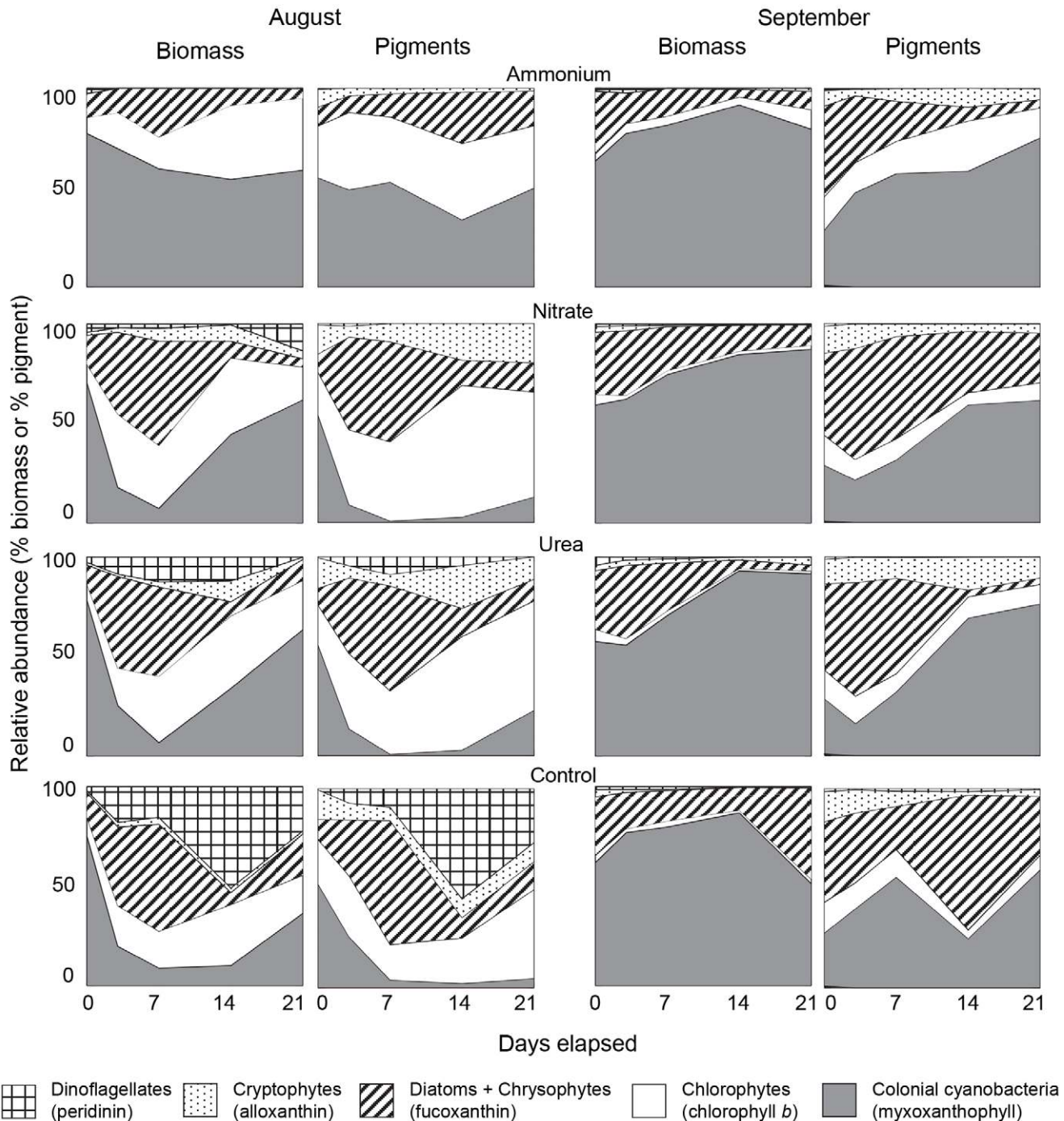doi:10.1371/journal.pone.0053277.t003

Figure 6. Mean relative abundance of the major phytoplankton groups in the mesocosms subject to addition of nitrogen. Treatment include addition of ammonium, nitrate, urea and no nitrogen (control) (n = 3). Phytoplankton abundance was determined by microscopic enumeration of biomass and by high performance liquid chromatography of algal pigments in experiments conducted during August and September 2008. Algal groups (and pigments) include dinoflagellates (peridinin), cryptophytes (alloxanthin), diatoms and chrysophytes (fuoxanthin), chlorophytes (chlorophyll *b*) and colonial cyanobacteria (myxoxanthophyll).
doi:10.1371/journal.pone.0053277.g006

thought to have high light requirements [32] and, if sufficiently illuminated, are competitive with other phytoplankton due to high rates of cell division and diverse mechanisms of N assimilation [88]. However, the lack of consistent response among green algal genera to individual forms of N (Fig. 4) suggests that pronouncements of division-level response of chlorophytes to pollution with N are premature [48], and that further research is needed to evaluate the complex

relationships between lake depth, irradiance regime, and N influx as factors regulating growth of chlorophyte algae [34].

Effects of Taxonomic Resolution on Interpretation of N effects

Comparison among PCAs (Fig. 5) revealed that generalisations concerning algal response to N addition depended on the taxonomic resolution of the microscopic analysis. Within the algal divisions, genera exhibited high variability in response to added N (Fig. 5c, d), likely reflecting substantial differences in morphology, growth capabilities and nutrient uptake kinetics (see above). Given the wide range in Km and V$_{max}$ observed in studies of isolated algae and natural communities [41,89,90], it has been inferred that variations among genera mainly reflect substantial differences in colony size (μm-mm) and cell volume [91] for many divisions [42,43]. In contrast, similar responses of closely-related species to N amendments (Fig. 5e, f) may occur because the fundamental niche of individual taxa has not yet diverged from shared lineages [65,90,92], recent speciation has conserved important morphological and physiological traits [93] which control of nutrient use [91], or local ecological interactions have already selected for congeneric species with similar ecological attributes. However, irrespective of the precise mechanism, the highly similar responses of congenetic species to added N (e.g., *Microcystis aeruginosa*, *M. flos-aquae*, *M. botrys*) combined with the low congruence of genera within a given division (e.g., *Anabaena*, *Planktothrix*, *Microcystis*, *Aphanizomenon*) (Table S1), suggests that important ecological insights on the role of N in lake eutrophication may be obtained without laborious identification of all phytoplankton to species identity [92], assuming patterns observed herein generalise well to other lakes.

Effects of Analytical Method on Interpretation of N effects

Empirical (Fig. 6) and statistical (Table 3) comparison of phytoplankton community composition derived from phytoplankton analysis by microscopy and HPLC demonstrates that changes in concentrations of biomarker pigments were influenced mainly by variation in abundance of the predominant algae rather than by the precise species composition of phytoplankton, irradiance regime or nutrient availability [46,59,67]. Although cellular pigment content (e.g., Chl a cell^{-1}) increases directly with N availability and inversely with irradiance levels in the laboratory [47], cellular quotas did not appear to be the main factor regulating HPLC-based inference of algal abundance and pigment-biomass correlations were of a similar magnitude to those quantified in earlier studies [46,59,67]. That said, we recognize that the uneven distribution of some indicator pigments among genera within some functional groups (e.g., aphanizophyll among N-fixing cyanobacteria) [59] may complicate pigment-based interpretations of algal community change unless augment-ed by microscopic enumeration of critical samples [6]. Fortunately, the generally high correspondence between microscopic and chromatographic analyses (Fig. 6), combined with the widespread use of similar HPLC and microscopic protocols [59,94], suggests that previous investigations based solely on biomarker pigments have provided robust and reliable information about N effects on aquatic ecosystems.

Despite evidence that growth of 45 phytoplankton species (31.5% of taxa) was stimulated by addition of dissolved N to P-rich lake waters (Table S1, Fig. 5), further research is required to evaluate the reasons for limited response of the remaining 99 taxa, including 6 species whose growth was suppressed by N amendments (Fig. 4n). For example, bottle bioassays of the nutritional status of algae in Wascana and other regional lakes [20,25] suggests that addition of NH$_4^+$ during May can suppress primary production when phytoplankton are composed mainly of diatoms, chrysophytes and cryptophytes [49] and that additional seasonal analysis of N effects is warranted. Further, improved understanding of the effects of N influx on the relative proportion of chlorophytes and non-N-fixing cyanobacteria is also needed [34], and would benefit from analysis of gross community changes along a gradient of N addition, as well as from comparisons of chlorophyte response in lakes of differing depths or water clarity (see above). Taken together, such refinement of our understanding of the phytoplankton-specific responses to N pollution may provide the best means of averting future damage to aquatic ecosystems arising from doubled N influx by 2050.

Supporting Information

Table S1 Phytoplankton taxa and response to nitrogen fertilisation in August and September experiments.
(DOC)

Author Contributions

Standardized taxomony: LB. Conducted microscopic and HPLC analyses: DBD. Conceived and designed the experiments: DBD KF LB PRL. Performed the experiments: DBD MJB. Analyzed the data: DBD MJB KF PRL. Contributed reagents/materials/analysis tools: PRL KF. Wrote the paper: DBD MJB KF LB PRL.

References

1. Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, et al. (1997) Human alteration of the global nitrogen cycle: Sources and consequences. Ecological Applications 7: 737–750.
2. Millennium Ecosystem Assessment (2005) Ecosystems and human well-being, vol. 1. Current state and trends: Findings of the condition and trends working group (Millennium Assessment Series). Washiington, DC: Island Press.
3. Foy RH, Smith RV, Jordan C, Lennox SD (1995) Upward trend in soluble phosphorus loadings to Lough Neagh despite phosphorus reduction at sewage treatment works. Water Research 29: 1051–1063.
4. Carpenter SR (2005) Eutrophication of aquatic ecosystems: Bistability and soil phosphorus. Proceedings of the National Academy of Sciences of the USA 102: 10002–10005.
5. MacDonald GK, Bennett EM, Potter PA, Ramankutty N (2011) Agronomic phosphorus imbalances across the world's croplands. Proceedings of the National Academy of Sciences of the USA 108: 3086–3091.
6. Bunting L, Leavitt PR, Gibson CE, McGee EJ, Hall VA (2007) Degradation of water quality in Lough Neagh, Northern Ireland, by diffuse nitrogen flux from a phosphorus-rich catchment. Limnology and Oceanography 52: 354–369.
7. Allan RJ (1980) The inadequacy of existing chlorophyll-a-phosphorus concentration correlations for assessing remedial measures for hypertrophic lakes. Environmental Pollution Series B 1: 217–231.
8. Patoine A, Graham MD, Leavitt PR (2006) Spatial variation of nitrogen fixation in lakes of the northern Great Plains. Limnology and Oceanography 51: 1665–1677.
9. Patoine A, Leavitt PR (2008) Landscape analysis of the role of N$_2$ fixation in satisfying algal demand for N in eutrophic lakes. Verhandlungen Internationale Vereinigung Limnologie 30: 366–370.
10. Paerl HW, Scott JT (2010) Throwing fuel on the fire: Synergistic effects of excessive nitrogen inputs and global warming on harmful algal blooms. Environmental Science and Technology 44: 7756–7758.

11. Leavitt PR, Brock CS, Ebel C, Patoine A (2006) Landscape-scale effects of urban nitrogen on a chain of freshwater lakes in central North America. Limnology and Oceanography 51: 2262–2277.

12. Lewis WM Jr, Wurtsbaugh WW (2008) Control of lacustrine phytoplankton by nutrients – Its causes and aquatic consequences. International Review of Hydrobiology 93: 446–465.

13. Lewis WM Jr, Wurtsbaugh WW, Paerl HW (2011) Rationale for control of anthropogenic nitrogen and phosphorus to reduce eutrophication of inland waters. Environmental Science and Technology 45: 10300–10305.

14. Carpenter SR, Caraco NF, Correll DL, Howarth RW, Sharpley AN, et al. (1998) Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecological Applications 8: 559–568.

15. Schindler DW (2006) Recent advances in the understanding and management of eutrophication. Limnology and Oceanography 51: 356–363.

16. Smith VH, Schindler DW (2008) Eutrophication science: Where do we go from here? Trends in Ecology and Evolution 24: 201–207.

17. Turpin DH, Layzell DB, Elrifi IR (1985) Modeling the C-economy of *Anabaena flos-aquae* - Estimates of establishment, maintenance, and active costs associated with growth on NH_3, NO_3^-, and N_2. Plant Physiology 78: 746–752.

18. Berman T, Chava S (1999) Algal growth on organic compounds as nitrogen sources. Journal of Plankton Research 21: 1423–1437.

19. Levine SN, Schindler DW (1999) Influence of nitrogen to phosphorus supply ratios and physicochemical conditions on cyanobacteria and phytoplankton species composition in the Experimental Lakes Area, Canada. Canadian Journal of Fisheries and Aquatic Sciences 56: 451–466.

20. Finlay K, Patoine A, Donald DB, Bogard MJ, Leavitt PR (2010) Experimental evidence that pollution with urea can degrade water quality in phosphorus-rich lakes of the Northern Great Plains. Limnology and Oceanography 55: 1213–1230.

21. Barica J, Kling H, Gibson J (1980) Experimental manipulation of algal bloom composition by nitrogen addition. Canadian Journal of Fisheries and Aquatic Sciences 37: 1175–1183.

22. Lathrop RC (1988) Evaluation of whole-lake nitrogen-fertilization for controlling blue-green-algal blooms in a hypereutrophic lake. Canadian Journal of Fisheries and Aquatic Sciences 45: 2061–2075.

23. James C, Fisher J, Moss B (2003) Nitrogen driven lakes: The Shropshire and Cheshire Meres? Archiv für Hydrobiologie 158: 249–266.

24. Giani A, Bird DF, Prairie YT, Lawrence JF (2005) Empirical study of cyanobacterial toxicity along a trophic gradient of lakes. Canadian Journal of Fisheries and Aquatic Sciences 62: 2100–2109.

25. Donald DB, Bogard MJ, Finlay K, Leavitt PR (2011) Comparative effects of urea, ammonium, and nitrate on phytoplankton abundance, community composition, and toxicity in hypereutrophic freshwaters. Limnology and Oceanography 56: 2161–2175.

26. Elser JJ, Bracken MES, Cleland EE, Gruner DS, Harpole WS, et al. (2007) Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. Ecology Letters 12: 1135–1142.

27. Sterner RW (2008) On the phosphorus limitation paradigm of lakes. International Review of Hydrobiology 93: 433–445.

28. Schindler DW, Hecky RE, Findlay DL, Stainton MP, Parker BR, et al. (2008) Eutrophication of lakes cannot be controlled by reducing nitrogen input: Results of a 37-year whole-ecosystem experiment. Proceedings of the National Academy of Sciences of the USA 105: 11254–11258.

29. Conley DJ, Paerl HW, Howarth RW, Boesch DF, Seitzinger SP, et al. (2009) Controlling eutrophication: Nitrogen and phosphorus. Science 323: 1014–1015.

30. Solomon CM, Collier JL, Berg GM, Glibert PM (2010) Role of urea in microbial metabolism in aquatic systems: A biochemical and molecular review. Aquatic Microbial Ecology 59: 67–88.

31. Edwards KF, Klausmeier CA, Litchman E (2011) Evidence for a three-way trade-off between nitrogen and phosphorus competitive abilities and cell size in phytoplankton. Ecology 92: 2085–2095.

32. Schwaderer AS, Yoshiyama K, Pinto PT, Swenson NG, Klausmeier CA, et al. (2011) Eco-evolutionary differences in light utilization traits and distributions of freshwater phytoplankton. Limnology and Oceanography 56: 589–598.

33. Blomqvist P, Pettersson A, Hyenstrand P (1994) Ammonium-nitrogen - A key regulatory factor causing dominance of non-nitrogen-fixing cyanobacteria in aquatic systems. Archiv für Hydrobiologie 132: 141–164.

34. Jensen JP, Jeppesen E, Olrik K, Kristensen P (1994) Impact of nutrients and physical factors on the shift from cyanobacterial to chlorophyte dominance in shallow Danish lakes. Canadian Journal of Fisheries and Aquatic Sciences 51: 1692–1699.

35. Hyenstrand P, Burkert U, Pettersson A, Blomqvist P (2000) Competition between the green alga *Scenedesmus* and the cyanobacterium *Synechococcus* under different modes of inorganic nitrogen supply. Hydrobiologia 435: 91–98.

36. Flores E, Herrero A (2005) Nitrogen assimilation and nitrogen control in cyanobacteria. Biochemical Society Transactions 33: 164–167.

37. Tilman D, Kiesling R, Sterner RW, Kilham SS, Johnson FA (1986) Green, bluegreen and diatom algae - Taxonomic differences in competitive ability for phosphorus, silicon and nitrogen. Archiv für Hydrobiologie 106: 473–485.

38. McCarthy MJ, James RT, Chen Y, East TL, Gardner WS (2009) Nutrient ratios and phytoplankton community structure in the large, shallow, eutrophic, subtropical Lakes Okeechobee (Florida, USA) and Taihu (China). Limnology 10: 215–227.

39. Lomas MW, Glibert PM (1999) Temperature regulation of nitrate uptake: A novel hypothesis about nitrate uptake and reduction in cool-water diatoms. Limnology and Oceanography 44: 556–572.

40. Reynolds CS, Huszar V, Kruk C, Naselli-Flores L, Melo S (2002) Towards a functional classification of the freshwater phytoplankton. Journal of Plankton Research 24: 417–428.

41. Burger DF, Hamilton DP, Hall JA, Ryan EF (2007) Phytoplankton nutrient limitation in a polymictic eutrophic lake: Community versus species-specific responses. Fundamental and Applied Limnology 169: 57–68.

42. Kruk C, Peeters ETHM, Van Nes EH, Huszar VLM, Costa LS, et al. (2011) Phytoplankton community composition can be predicted best in terms of morphological groups. Limnology and Oceanography 56: 110–118.

43. Reynolds CS (1984) The ecology of freshwater phytoplankton. New York, NY: Cambridge University Press.

44. Carpenter SR (1996) Microcosm experiments have limited relevance for community and ecosystem ecology. Ecology 77: 677–680.

45. Foy RH (1987) A comparison of chlorophyll *a* and carotenoid concentrations as indicators of algal volume. Freshwater Biology 17: 237–250.

46. Millie DF, Paerl HW, Hurley JP (1993) Microalgal pigment assessments using high-performance liquid chromatography: A synopsis of organismal and ecological applications. Canadian Journal of Fisheries and Aquatic Sciences 50: 2513–2527.

47. Staehr PA, Henriksen P, Markager S (2002) Photo-acclimation of four marine phytoplankton species to irradiance and nutrient availability. Marine Ecology Progress Series 238: 47–59.

48. Schindler DW, Hecky RE (2009) Eutrophication: More nitrogen data needed. Science 324: 721–722.

49. McGowan S, Leavitt PR, Hall RI (2005) A whole-lake experiment to determine the effects of winter droughts on shallow lakes. Ecosystems 8: 694–708.

50. Pham SV, Leavitt PR, McGowan S, Wissel B, Wassenaar LI (2009) Spatial and temporal variability of prairie lake hydrology as revealed using stable isotopes of hydrogen and oxygen. Limnology and Oceanography 54: 101–118.

51. Dröscher I, Patoine A, Finlay K, Leavitt PR (2009) Climate control of spring clear-water phase through the transfer of energy and mass to lakes. Limnology and Oceanography 54: 2469–2480.

52. Smith VH, Bierman VJ Jr, Jones BL, Havens KE (1995) Historical trends in the Lake Okeechobee ecosystem: IV. Nitrogen : phosphorus ratios, cyanobacterial dominance, and nitrogen fixation potential. Archiv für Hydrobiologie Monographische Beiträge 107: 71–88.

53. Utermöhl H (1958) Zur Vervollkommnung der quantitativer Phytoplankton-methodik. Mittelungen International Vererin Limnologie 9: 1–38.

54. Prescott GW (1982) Algae of the Western Great Lakes Area, 2nd ed. Königstein : Otto Koeltz.

55. John DM, Whitton BA, Brook AJ (2002) The freshwater algae flora of the British Isles. New York, NY: Cambridge University Press.

56. Wehr JD, Sheath RG (2003) Freshwater algae of North America: Ecology and classification. NY, New York: Academic Press.

57. Hillebrand H, Durselen CD, Kirschtel D, Pollingher U, Zohary T (1999) Biovolume calculation for pelagic and benthic microalgae. Journal of Phycology 35: 403–424.

58. Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls *a*, *b*, *c*1 and *c*2 in higher-plants, algae and natural phytoplankton. Biochemical Physiology Pflanz 167: 191–194.

59. Leavitt PR, Hodgson DA (2001) Sedimentary pigments. In Smol JP, Birks HJB, Last WM, editors. Tracking environmental change using lake sediments, Volume 3: Terrestrial, algal and siliceous indicators, p. 295–325. Boston, MA: Kluwer.

60. Stainton MP, Capel MJ, Armstrong FAJ (1977) The chemical analysis of freshwater. 2nd ed. Canadian Fisheries Marine Service Miscellaneous Special Publication 25. Ottawa: Government of Canada.

61. Revilla M, Alexander J, Glibert PM (2005) Urea analysis in coastal waters: Comparison of enzymatic and direct methods. Limnology and Oceanography: Methods 3: 290–299.

62. Finlay K, Leavitt PR, Wissel B, Prairie YT (2009) Regulation of spatial and temporal variability of carbon flux in six hard-water lakes of the northern Great Plains. Limnology and Oceanography 54: 2553–2564.

63. United States Environmental Protection Agency (2007) Biological nutrient removal processes and costs. Report EPA-823-R-07–002. Washington, DC: United States Environmental Protection Agency, Office of Water. 13 p.

64. Waiser MJ, Tumber V, Holm J (2011) Effluent-dominated streams. Part 1: Presence and effects of excess nitrogen and phosphorus in Wascana Creek, Saskatchewan, Canada. Environmental Toxicology and Chemistry 30: 496–507.

65. Carneiro FM, Bini LM, Rodrigues LC (2010) Influence of taxonomic and numerical resolution on the analysis of temporal changes in phytoplankton communities. Ecological Indicators 10: 249–255.

66. Robarts RD, Zohary T (1987) Temperature effects on photosynthetic capacity, respiration, and growth rates of bloom-forming cyanobacteria. New Zealand Journal of Marine and Freshwater Research 21: 391–399.

67. Leavitt PR, Findlay DL (1994) Comparison of fossil pigments with 20 years of phytoplankton data from eutrophic Lake 227, Experimental Lakes Area, Ontario. Canadian Journal of Fisheries and Aquatic Sciences 51: 2286–2299.

68. Holmgren SK (1984) Experimental lake fertilisation in the Kuokkel Area, Northern Sweden. Phytoplankton biomass and algal composition in natural and

fertilised subarctic lakes. Internationale Revue der gesamten Hydrobiologie und Hydrographie 69: 781–817.

69. Dolman AM, Rücker J, Pick FR, Fastner J, Rohrlack T, et al. (2012) Cyanobacteria and cyanotoxin: The influence of nitrogen versus phosphorus. PLoS One 7(6): e38757. doi:10.1371/journal.pone.00038757.

70. Fastner J, Erhard M, Carmichael WW, Sun F, Rinehart KL, et al. (1999) Characterization and diversity of microcystins in natural blooms and strains of the genera *Microcystis* and *Planktothrix* from German freshwaters. Archiv für Hydrobiologie 145: 147–163.

71. Zevenboom W, Vaate AB, Mur LR (1982) Assessment of factors limiting growth rate of *Oscillatoria agardhii* in hypereutrophic Lake Wolderwijd, 1978, by use of physiological indicators. Limnology and Oceanography 27: 39–52.

72. Klemer AR (1976) The vertical distribution of *Oscillatoria agardhii* var. *isothrix*. Archiv für Hydrobiologie 78: 343–362.

73. Scheffer M, Rinaldi S, Gragnani A, Mur LR, Van Nes EH (1997) On the dominance of filamentous cyanobacteria in shallow, turbid lakes. Ecology 78: 272–282.

74. Fujimoto N, Sudo R, Sugiura N, Inamori Y (1997) Nutrient-limited growth of *Microcystis aeruginosa* and *Phormidium tenue* and competition under various N:P supply ratios and temperatures. Limnology and Oceanography 42: 250–256.

75. Ginn HP, Pearson LA, Neilan BA (2010) NtcA from *Microcystis aeruginosa* PCC 7806 is autoregulatory and binds to the microcystin promoter. Applied and Environmental Microbiology 76: 4362–4368.

76. Berg GM, Balode M, Purina I, Bekere S, Bechemin C, et al. (2003) Plankton community composition in relation to availability and uptake of oxidized and reduced nitrogen. Aquatic Microbial Ecology 30: 263–274.

77. Grover JP, Sterner RW, Robinson JL (1999) Algal growth in warm temperate reservoirs: Nutrient-dependent kinetics of individual taxa and seasonal patterns of dominance. Archiv für Hydrobiologie 145: 1–23.

78. Pérez-Martínez C, Cruz-Pizarro L (1995) Species-specific phytoplankton response to nutrients and zooplankton manipulations in enclosure experiments. Freshwater Biology 33: 193–203.

79. Takamura N, Iwakuma T, Yasuno M (1987) Uptake of C-13 and N-15 (ammonium, nitrate and urea) by *Microcystis* in Lake Kasumigaura. Journal of Plankton Research 9: 151–165.

80. Dokulil MT, Teubner K (2000) Cyanobacterial dominance in lakes. Hydrobiologia 438: 1–12.

81. Jöhnk KD, Huisman J, Sharples J, Sommeijer B, Visser PM, et al. (2008) Summer heatwaves promote blooms of harmful cyanobacteria. Global Change Biology 14: 495–512.

82. Sandgren CD, editor (1988) Growth and reproductive strategies of freshwater phytoplankton. New York, NY: Cambridge University Press.

83. Sanderson MP, Bronk DA, Nejstgaard JC, Verity PJ, Sazhin AF, et al. (2008) Phytoplankton and bacterial uptake of inorganic and organic nitrogen during an induced bloom of *Phaeocystis pouchetii*. Aquatic Microbial Ecology 51: 153–168.

84. Valladares A, Montesinos ML, Herrero A, Flores E (2002) An ABC-type, high-affinity urea permease identified in cyanobacteria. Molecular Microbiology 43: 703–715.

85. Herrero A, Muro-Pastor AM, Flores E (2001) Nitrogen control in cyanobacteria. Journal of Bacteriology 183: 411–425.

86. Badger MR, Price GD, Long BM, Woodger FJ (2006) The environmental plasticity and ecological genomics of the cyanobacterial CO_2 concentrating mechanism. Journal of Experimental Botany 57: 249–265.

87. Collier JL, Lovindeer R, Xi Y, Radway JC, Armstrong RA (2012) Differences in growth and physiology of marine *Synechococcus* (Cyanobacteria) on nitrate versus ammonium are not determined solely by nitrogen source redox state. Journal of Phycology 48: 106–116.

88. Fernandez E, Galvan A (2007) Inorganic nitrogen assimilation in *Chlamydomonas*. Journal of Experimental Botany 58: 2279–2287.

89. Lehman JT, Sandgren CD (1985) Species-specific rates of growth and grazing loss among freshwater algae. Limnology and Oceanography 30: 34–46.

90. Sommer U (1989) Nutrient status and nutrient competition of phytoplankton in a shallow, hypertrophic lake. Limnology and Oceanography 34: 1162–1173.

91. Edwards KF, Thomas MK, Klausmeier CA, Litchman E (2011) Allometric scaling and taxonomic variation in nutrient utilization traits and maximum growth rate of phytoplankton. Limnology and Oceanography 57: 554–566.

92. Litchman E, Klausmeier CA (2008) Trait-based community ecology of phytoplankton. Annual Review of Ecology and Systematics 39: 615–639.

93. Bruggeman J (2011) A phylogenetic approach to the estimation of phytoplankton traits. Journal of Phycology 47: 52–65.

94. Jeffrey SW, Mantoura RFC, Wright DW, editors (2003) Phytoplankton pigments in oceanography: Guidelines to modern methods. New York, NY: UNESCO.