Phytoplankton nitrogen and phosphorus limitation and the N_2-fixation potential of Nostocales at varying nitrogen supplies and light intensities in lakes

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Abstract

Anthropogenic eutrophication is one of the biggest threats to freshwater ecosystems. A main consequence of eutrophication is the massive increase in phytoplankton biomass. There is an extended debate in the literature over whether nitrogen (N) or phosphorus (P) is mainly limiting the phytoplankton. The variety of the results suggests that the limiting nutrient may vary with lake type and season. In order to lower the phytoplankton biomass it is theoretically most effective to reduce the nutrient that is actually limiting. However, it is widely assumed that, due to their competitive advantage when inorganic N sources are scarce, the abundance and N_2-fixation rate of N_2-fixing cyanobacteria (Nostocales) would increase in response to reduced N loading, and thereby render efforts to improve water quality by N reduction ineffective. Nostocales N_2-fixation has a huge energy demand and consequently the light intensity may affect the response of Nostocales biovolume and N_2-fixation to varying N additions. This led to the following aims of this thesis:

(i) Determination of the seasonal dynamic of N- and P-limitation for four lakes of differing lake types in the German lowlands and testing the power of four N:P ratios (TN:TP, DIN:TP, DIN:SRP and TN:SRP) to predict the limiting nutrient.

(ii) Determination of the response of Nostocales cyanobacteria biovolume and N_2-fixation to varying N additions.

(iii) Determination of the effect of the light intensity on the response of Nostocales cyanobacteria biovolume and N_2-fixation to varying N additions.

Three different sets of nutrient enrichment experiments were conducted in order to accomplish these aims:

(i) To identify the seasonal pattern of N- and P-limitation of phytoplankton in three shallow and one deep lake, biweekly experiments (bioassays) were conducted during the vegetation period 2011. Lake water samples were enriched with N, P or both nutrients and incubated under two different light intensities. Chlorophyll a (Chla) fluorescence was measured and a model selection procedure was used to assign bioassay outcomes to different limitation categories. N and P were both limiting at some time in each lake. For the shallow lakes there was a trend from P limitation in spring to N or light limitation in summer and autumn, while the deep lake remained predominantly P limited. To determine the ability of in-lake N:P ratios to predict the relative strength of N vs. P limitation, four separate regression models were fitted. In these models the log-transformed ratio of Chla in the P and N treatments (Response ratio = RR) was used as the response variable and those of ambient total phosphorus:total nitrogen (TN:TP), dissolved inorganic nitrogen:soluble reactive phosphorus (DIN:SRP), TN:SRP and DIN:TP mass ratios as predictors. All four N:P ratios had significant positive relationships with RR, such that high N:P ratios were associated with P limitation, and low N:P ratios with N limitation. The TN:TP and DIN:TP ratios performed better than the DIN:SRP and

TN:SRP in terms of misclassification rate and the DIN:TP ratio had the highest R^2 value. Nitrogen limitation was predictable, frequent and persistent, suggesting that nitrogen reduction could play a role in water quality management.

(ii) To assess the response of Nostocales cyanobacteria biovolume and N_2-fixation to varying N additions, a microcosm experiment was performed, using water from an N limited lake (Langer See). In this six day experiment phosphorus additions were held constant, while a gradient of N addition simulated a reduction in N loading. Nostocales biovolume increased over time in all microcosms, regardless of the N addition rate, so that no difference in Nostocales biovolume developed between high and low N microcosms. In contrast, the biovolumes of other taxa were lower at low N addition rates. N_2-fixation increased in low N microcosms. To quantify the extent to which Nostocales cyanobacteria compensated for the varying N addition rate, we calculated a compensation rate (CR), defined here as the proportion of omitted N addition that would be replaced by N_2-fixation. By the end of the experiment a compensation rate of 36 % was reached. However, this compensation rate was achieved at Nostocales biovolumes far higher than those typical in the studied lake. At biovolumes typical for summer the compensation rate would be much lower. Therefore, in shallow polymictic lakes like Langer See, reduced N loading may lower both in-lake N concentrations and biovolumes of non-fixing phytoplankton without significantly impacting Nostocales biovolume.

(iii) To assess the effect of the light intensity on the response of Nostocales cyanobacteria biovolume and N_2-fixation to varying N additions, another microcosm experiment with water from Langer See was performed. In this experiment six treatments along a light gradient were used, where each was divided into two nutrient treatments. In both nutrient treatments phosphorus additions were held constant but N was only added to one of them to again simulate a reduction in N loading. Generally, the biovolume of all taxa increased with increasing light intensity and over time. While at low and intermediate light intensities the Nostocales biovolume was the same or even lower in the treatment without N addition compared to the treatment with N and P addition, the reduction of N addition led to an increase in Nostocales biovolume at high light intensities. The N_2-fixation per Nostocales biovolume increased with increasing light intensity and at reduced N addition at all light intensities. This positive response to a reduction in N addition increased with light intensity at low and intermediate light intensities, but was decreased again at high light intensities. This experiment showed that Nostocales cyanobacteria may take advantage of being able to fix N_2 mainly at high light intensities and therefore it is important to consider the light intensity when assessing their potential to compensate for reduced N loading.

In conclusion this study showed that N-limitation is mainly occurring in shallow polymictic lakes in summer and autumn. Under the here tested conditions Nostocales cyanobacteria responded to reduced N loading by increasing their N_2-fixation rate per biovolume without increasing the biovolume itself. They are able to partly compensate for the reduction. Finally this study showed that the response of Nostocales to varying N additions is affected by the light intensity. At high light intensities related to lower trophic conditions the compensation rate determined in (ii) may be underestimated.

Zusammenfassung

Anthropogene Eutrophierung ist eine der größten Gefahren für Gewässerökosysteme. Eine Hauptfolge der Eutrophierung ist das massive Wachstum der Phytoplanktonbiomasse. In der Literatur herrscht eine langwierige Debatte ob Stickstoff (N) oder Phosphor (P) hauptsächlich das Phytoplankton limitieren. Die Vielfalt der Ergebnisse deuten an, dass der limitierende Nährstoff saisonal und zwischen Seetypen variiert. Zur Verringerung der Phytoplanktonbiomasse ist es theoretisch am effektivsten den tatsächlich limitierenden Nährstoff zu reduzieren. Es wird allerdings häufig angenommen, dass die Abundanz und die N_2-Fixierungsrate von N_2-fixierenden Cyanobakterien (Nostocales) in Folge einer Verringerung der N-Einträge zunehmen und dadurch die Bemühungen die Wasserqualität zu verbessern ineffektiv machen. Die N_2-Fixierung durch Nostocales hat einen großen Energiebedarf und dementsprechend ist es möglich, dass die Lichtintensität einen Einfluss auf die Reaktion des Nostocalesbiovolumen und der N_2-Fixierung auf variierende N-Zugaben hat. Dies führte zu den folgenden Zielen dieser Arbeit:

(i) Bestimmung der saisonalen Dynamik der N- und P-Limitation in vier Seen mit unterschiedlichen Seetypen in der Deutschen Tiefebene und Prüfung der Vorhersagbarkeit des limitierenden Nährstoffes durch vier N:P-Verhätlnisse (TN:TP, DIN:TP, DIN:SRP und TN:SRP).

(ii) Bestimmung der Reaktion des Nostocalen Biovolumens und der N_2-Fixierung auf variierende N-Zugaben.

(iii) Bestimmung des Einflusses der Lichtintensität auf die Reaktion des Nostocalen Biovolumens und der N_2-Fixierung auf variierende N-Zugaben.

Zur Erreichung dieser Ziele wurden drei Reihen von Nährstoffzugabeexperimenten durchgeführt:

(i) Zur Identifizierung des saisonalen Musters der Stickstoff- und Phosphorlimitation des Phytoplanktons in drei flachen und einem tiefen See, wurden während der Vegetationsperiode alle zwei Wochen Experimente (Bioassays) durchgeführt. Dabei wurden Seewasserproben mit Stickstoff (N), Phosphor (P) oder beiden Nährstoffen angereichert und bei zwei verschiedenen Lichtintensitäten inkubiert. Die Fluoreszenz des Chlorophyll a (Chla) wurde gemessen und die Ergebnisse der Bioassays wurden anschließend mit Hilfe eines Modelauswahlverfahrens in verschiedene Limitationskategorien eingeteilt. Sowohl N- als auch P-Limitation wurden nachgewiesen. In den polymiktischen Flachseen wurde ein Wechsel von P-Limitation im Frühling zu N-Limitation später im Jahr beobachtet, während der tiefe geschichtete See vorwiegend durch P limitiert war. Die Möglichkeit die relative Stärke der N und P-Limitation durch N:P-Verhältnisse in den Seen vorherzusagen sollte getestet werden. Dafür wurden vier separate Regressionsmodelle gefittet. Das log-transformierte Verhältnis vom Chla des P- und des N-Ansatzes (Reaktionsverhältnis = RR) diente dabei als Zielgröße und die

Massenverhältnisse im See von gesamt N zu gesamt P (TN:TP), gelöster anorganischer N zu gelöstem reaktiven P (DIN:SRP), TN:SRP und DIN:TP dienten als Einflussgröße. Alle vier N:P Verhältnisse zeigten einen signifikanten positiven Zusammenhang mit dem RR, so dass hohe N:P Verhältnisse mit P-Limitation und niedrige N:P Verhältnisse mit N-Limitation verbunden sind. Die Verhältnisse von TN:TP und DIN:TP schnitten am besten in Bezug auf die Misklassifikationsrate ab, wobei das DIN:TP-Verhältnis den höchsten R^2-Wert hatte. Stickstofflimitation konnte häufig und beständig nachgewiesen werden. Daher könnte die Reduktion von Stickstoff eine Rolle in der Bewirtschaftung von Gewässern spielen.

(ii) Um die Reaktion des Nostocalen Biovolumens und der N_2-Fixierung auf variierende N-Zugaben zu bestimmen wurde ein mit Wasser aus dem überwiegend N-limitierten Langen See (LAN) ein Mikrokosmosexperiment durchgeführt. In diesem sechs Tage Experiment wurde die P-Zugabe konstant gehalten, während ein Gradient der N-Zugabe eine Reduktion des N-Eintrages simulieren sollte. Das Biovolumen der Nostocales nahm über die Zeit in allen Mikrokosmen unabhängig von der N-Zugabe zu, so dass sich kein Unterschied zwischen hoher und niedriger N-Zugabe entwickelte. Im Gegensatz dazu war das Biovolumen der anderen Taxa bei niedriger N-Zugabe geringer. Die N_2-Fixierung nahm bei niedriger N-Zugabe zu. Zur Quantifizierung der Kompensation durch Nostocales wurde eine Kompensationsrate berechnet, die hier als der Anteil am verringerten N-Eintrag, der durch N_2-Fixierung ersetzt wird, definiert ist. Bis zum Ende des Experimentes wurde eine Kompensationsrate von 36 % erreicht. Diese Kompensationsrate wurde allerdings bei einem Nostocales Biovolumen erreicht, das deutlich über den üblich im LAN beobachteten Biovolumina lag. Bei für den Sommer typischen Biovolumina wäre die Kompensationsrate wesentlich geringer. Daher könnte in polymiktischen Flachseen, wie dem Langen See, eine Verringerung der N-Einträge, sowohl die N-Konzentration, als auch das Biovolumen des nichtfixierenden Phytoplanktons verringern, ohne das Biovolumen der Nostocales zu beeinflussen.

(iii) Um den Einfluss der Lichtintensität auf die Reaktion des Nostocalen Biovolumens und der N_2-Fixierung auf variierende N-Zugaben zu bestimmen wurde ein weiteres Mikrokosmosexperiment mit Wasser aus dem Langen See durchgeführt. Dieses Experiment bestand aus sechs Lichtansätzen entlang eines natürlichen Gradienten, wobei jeder Lichtansatz auf zwei Nährstoffansätze aufgeteilt wurde. In beiden Nährstoffansätzen wurde die P-Zugabe konstant gehalten, aber N wurde nur zu einem hinzugegeben, um wieder eine Verringerung des N-Eintrages zu simulieren. Allgemein nahm das Biovolumen aller Taxa mit zunehmender Lichtintensität und über die Zeit zu. Während bei niedrigen und mittleren Lichtintensitäten kein Unterschied beim Biovolumen der Nostocales dem Nährstoffansatz mit N- und P-Zugabe und dem ohne N-Zugabe beobachtet wurde, führte die Verringerung der N-Zugabe bei hohen Lichtintensitäten zu einem Anstieg des Biovolumens der Nostocales. Die N_2-Fixierung nahm allgemein mit zunehmender Lichtintensität zu und auch eine Reduktion der N-Zugabe führte zu einem Anstieg. Die positive Reaktion auf die Verringerung der N-Zugabe nahm bei niedrigen und mittleren Lichtintensitäten mit steigender Lichtintensität zu, verringerte sich bei hohen Lichtintensitäten aber wieder. Dieses Experiment zeigte, dass Nostocale Cyanobakterien von ihrem Vorteil der N_2-Fixierung hauptsächlich bei hohen Lichtintensitäten profitieren. Daher ist es wichtig, die

Lichtintensität bei der Bestimmung des Kompensationspotentials zu berücksichtigen.

Schlussfolgerung: Diese Studie zeigte, dass N-Limitation hauptsächlich in polymiktischen Flachseen im Sommer und Herbst auftritt. Unter den hier getesteten Bedingungen reagierten Nostocales auf eine Reduzierung der N-Einträge mit einem Anstieg der N_2-Fixierung pro Biovolumen, nicht aber mit einer Zunahme des Biovolumens selbst. Sie sind in der Lage eine Reduktion der N-Einträge teilweise auszugleichen. Schließlich zeigte diese Studie, dass die Reaktion der Nostocales auf variierende N-Zugaben von Lichtintensität beeinflusst wird. Bei hohen Lichtintensitäten könnte die unter (ii) bestimmte Kompensationsrate unterschätzt worden sein.

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List of abbreviations

α	Initial slope of a light response curve
Bv_{max}	Maximum biovolume in a light response curve
C:	Carbon
Chla:	Chlorophyll a
CR:	Compensation rate
Ctrl:	Control treatment
DIN:	Dissolved inorganic nitrogen
ELA:	Canadian experimental lake area
F_{max}	Maximum fixation rate in a light response curve
I_{mix}:	mean photosynthetically active radiation in the mixed upper part of the water column
ISL:	In situ light conditions
LAN:	Langer See
MR:	Misclassification rate
MUEG:	Müggelsee
N:	Nitrogen
P:	Phosphorus
PC:	Polycarbonate
PE:	Polyethylene
R^2:	Coefficient of determination
RR:	Response ratio
SCH:	Scharmützelsee
SL:	Standard light intensity
SRP:	Soluble reactive phosphorus
TN:	Total nitrogen
TP:	Total phosphorus
UH:	Untere Havel
z_{SD}:	Secchi depth

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1 General introduction

Freshwater lakes form the basis for several human activities such as drinking water production, transportation, fishing or recreation and are the vital habitat for a huge variety of organisms. One of the biggest threats to lakes worldwide is still eutrophication caused by anthropogenic pollution with nutrients, mainly nitrogen (N) and phosphorus (P) (Carpenter et al. 1998; Smith 2003). Among others, the main consequences of increasing availability of nutrients are enhanced growth and biomass of the phytoplankton. The increased phytoplankton biomass leads to decreases in water column transparency, and the decomposition of high biomasses can result in deep water oxygen depletion and fish kills as a consequence (Dodds and Whiles 2010). Another effect of eutrophication may be a change in phytoplankton species composition (Proulx et al. 1996), where the community often shifts to bloom forming cyanobacteria (Steinberg and Hartmann 1988). These cyanobacteria can form surface scums, bad taste and odours (Wnorowski 1992) and many of them can produce several toxins (Carmichael et al. 1985; Lawton and Codd 1991), which may lead to illness or death of aquatic organisms (Penaloza et al. 1990) and wild or domestic animals (Carmichael et al. 1985). In particular, drinking water production suffers from taste, smell, toxins and filtration problems (Wnorowski 1992). In general, most of these effects cause a decrease in the aesthetic benefits of the lake.

Human activities have heavily impacted the biogeochemical cycles of N and P. The application of P fertilizers has increased the amount of P cycling through the ecosystems by 20 to 30 % (Chapin et al. 2002) and the use of N fertilizers, N_2-fixing crops and fossil fuel combustion has at least doubled the amount of N (Vitousek et al. 1997).

An assessment in course of the European Water Framework Directive (Solheim et al. 2012) showed that 44 % of 19 000 reported lakes failed to reach “good” ecological status and presented nutrient enrichment from diffuse sources as one of the main reasons. Water quality impairment due to eutrophication can cause immense negative economic effects. Dodds et al. (2009) estimated the value loss as a result of eutrophication in U.S. freshwaters to be approximately $2.2 billion annually. However, the reduction of nutrient emissions to aquatic ecosystems is also cost intensive (Butt and Brown 2000; Rabotyagov et al. 2009); therefore it may be more cost and time effective to match reduction measures for specific nutrients to regions or phases when the nutrient in question is limiting and thus may have an immediate effect on water quality.

1.1 Overview of methods to determine nutrient limitation

For our understanding of lake ecology and for water management to control eutrophication it is central to know which nutrient is limiting the phytoplankton in a specific lake at a specific point in time. Different methods have been used in the

past to determine the limiting nutrient of phytoplankton growth or biovolume, which will be reviewed in the following.

The most used approach is the nutrient enrichment bioassay (Elser et al. 1990), in which different nutrients are added to separate water samples and the response of the phytoplankton is measured after a set incubation time. The nutrient that provokes a reaction of a response parameter, compared to a control treatment without nutrient addition, is defined as the limiting nutrient. Two types of limitation, which are determined by different response parameters, can be distinguished: Limitation of the final yield of a plant crop (here phytoplankton), the so called Liebig limitation (Liebig 1842) and limitation of rate processes, such as photosynthesis, the so called Blackman limitation (Blackman 1905).

A wide range of different response parameters have been used in the literature: Phytoplankton biomass equivalent parameters like Chlorophyll a (e. g. White and Payne 1977; Morris and Lewis 1988; Tamminen and Andersen 2007) or biovolume (e. g. Burger et al. 2007), growth rate and carbon assimilation (e. g. Sakamoto 1971; Beardall et al. 1982; Tamminen and Andersen 2007). In a single water sample different response parameters may indicate different nutrients as being limiting. For example while chlorophyll a is biased to indicating N-limitation carbon assimilation is biased to indicating P-limitation (Tamminen and Andersen 2007).

Nutrient enrichment bioassays may vary in scales of size and running time between microcosm experiments in bottles running for days (e. g. Sommer 1989; Weithoff and Walz 1999), mesocosm experiments in lakes running for weeks (e.g. Levine and Schindler 1999; Vrede et al. 2009) and whole lake experiments running for multiple years (Holmgren 1984; Schindler et al. 2008). Hecky and Kilham (1988) nicely described the advantages and disadvantages of different experiment scales. The larger the scale of the experiments the more processes are included and the naturalness increases. However, it is difficult to have replicates in large experiments and the addition of nutrients to a whole lake may have a huge negative impact on the water quality of the lake. Therefore, whole lake experiments can only be used to test general rules of nutrient limitation in lakes and not to identify the limiting nutrients of individual lakes. Small experiments have the advantage of tight control over experimental conditions (e.g. temperature or light intensity), better replication and statistical power, however the containment of the natural phytoplankton communities in enclosures may lead to a change in species composition (Venrick et al. 1977).

The concept of nutrient enrichment experiments in general also has some drawbacks. A single experiment can only indicate the limiting nutrient at a single point in time for a specific lake and repeated experiments over longer time periods or for many lakes are cost intensive. Furthermore, the addition of one nutrient can induce limitation of other nutrients, which may lead to a false interpretation that the phytoplankton was co-limited by more than one nutrient. In experiments in the laboratory it is important not to incubate at light intensities or temperatures higher than the in situ conditions, without testing for limitation of these factors.

Otherwise some kind of nutrient limitation may be observed when in fact the phytoplankton was actually limited by light or temperature.

There are several alternative experimental methods and parameters to define nutrient limitation, like biochemical and molecular approaches, interactions between nutrient status and algal metabolism, specific enzyme markers for nutrient limitation or variable Chlorophyll a fluorescence (for review see Beardall et al. 2001). In general, most types of experiments are time and/or resource consuming. A cost effective alternative is to draw conclusions on the limiting nutrient from cross-lake statistical relationships between nutrient concentrations and phytoplankton biomass that are already measured in most monitoring programs (e.g. Sakamoto 1971; OECD 1982; Smith 1982; Dolman and Wiedner 2015). However, this method only works with large data sets, usually from multiple lakes, and is not suitable for determining the limiting nutrient for a specific lake.

Other approaches to determining the limiting nutrient from in-lake nutrient concentrations or ratios for single lakes are based on the seminal work of Redfield (1958). In his study he observed that N and P are assimilated by phytoplankton on average in the molar ratio of 16:1 (mass ratio of about 7), the so called Redfield ratio. Accordingly a higher N:P ratio in the water would indicate P limitation while a lower ratio would indicate N limitation of phytoplankton growth. The Redfield ratio is very generalised and the optimal N:P ratio for phytoplankton growth may vary locally, seasonally (Sterner et al. 2008), with species (Sterner and Hessen 1994) and is also affected by availability of other nutrients like carbon or other limiting factors like light (see Beardall et al. 2001 for a review). In addition, it is not trivial to determine the availability of the different nutrient fraction (e. g. for nitrogen total nitrogen (TN), dissolved inorganic nitrogen (DIN) or dissolved organic nitrogen) for phytoplankton growth (Beardall et al. 2001). Therefore several indices have been tested in Colorado mountain lakes (Morris and Lewis 1988), boreal and alpine lakes (Bergström 2010) and the Baltic Sea (Ptacnik et al. 2010). In all of these studies the DIN:TP ratio has been found to be the best predictor for the limiting nutrient. However, this has not yet been tested for German lakes.

Of course, these ratios can only indicate the relative deficiency of one nutrient to the other, while the absolute concentrations of nutrients decide whether they may be limiting. In other words: even if the N:P ratio indicates that for example N is limiting, at very high nutrient concentrations it is unlikely that any nutrient is limiting and instead factors like light or temperature may limit the phytoplankton. Maberly et al. (2002) observed that P and N limitation was possible at SRP and DIN concentrations < 10 µg L^{-1} and < 100 µg L^{-1} respectively. However, these thresholds could, like the N:P ratio, vary and have not been tested yet for German lakes.

1.2 History of nutrient limitation paradigms in lakes

For approximately 50 years there has been an intensive discussion about which nutrient controls the productivity of phytoplankton in lakes. Phytoplankton blooms were first linked with increased nutrient loadings (mainly N and P) from anthropogenic activities in the 1960's (Lund 1967). Although the studies of Vollenweider (1968) and Edmondson (1970) provided strong evidence that eutrophication may be controlled by the reduction of P effluents, there was resistance against this hypothesis. In order to keep on selling phosphorus-based products the soap and detergent manufacturers especially emphasized results from (flawed) bioassay experiments, which suggested that phytoplankton biovolume was limited by carbon (C) rather than P (see review by Schindler 2006). The Canadian experimental lake area (ELA) with 46 lakes was founded in 1967 in north-western Ontario to push forward eutrophication research (Johnson and Vallentyne 1971). In the first whole lake experiment at ELA, oligotrophic Lake 227, despite low content of dissolved inorganic carbon, quickly became eutrophic after adding only N and P. By showing that depleted dissolved inorganic C contents will be replenished with atmospheric CO_2, the hypothesis of carbon control on eutrophication was rejected (Schindler et al. 1971; Schindler et al. 1972; Schindler et al. 1973).

Further experiments at the ELA in the 1970s tried to examine the effects of N vs. P on the phytoplankton biomass. Lake 226 got divided by a curtain into two almost identical basins: Only the basin receiving also P as well as C and N developed a bloom of N_2-fixing cyanobacteria (Schindler 1974). In Lake 227 the total phytoplankton biomass remained high despite lowered N additions in 1975, which, however resulted in a shift of the species composition to dominance of N_2-fixing cyanobacteria. Schindler (1977) concluded that, as for C, a deficiency of N will be balanced by atmospheric N sources, while this is not possible for P. The results of these experiments led, together with strong relationships between total phosphorus (TP) and standing crop of phytoplankton in a wide variety of lakes (Vollenweider 1968; Sakamoto 1971; Dillon and Rigler 1974), to a general agreement that focus should be on controlling P in order to fight eutrophication (Schindler 1977). The P limitation paradigm was restated in most textbooks. Interestingly, the certainty with which authors presented it, seemed to increase between 1994 and 2005, although there was no primary research paper justifying the increasing confidence (Sterner 2008).

On the contrary there is an increasing number of nutrient addition bioassays indicating algal limitation by N (see meta-analyses by Elser et al. 1990; Elser et al. 2007). It is often stated that N limitation is only observed in small scale bioassays and that these cannot reflect the natural conditions in a lake. However, it was shown that the results of small-scale experiments can be applied to larger systems (Spivak et al. 2011) and in fact N-limitation was also observed in mesocosm and whole lake experiments (Lewis et al. 2011). There are several factors and processes influencing the absolute and relative supplies of N and P, like N_2-fixation (Howarth et al. 1988), denitrification (Downing and McCauley 1992; Seitzinger et al. 2006), nutrient recycling in the sediments (Welch and Cooke 1995; Grüneberg et al. 2011; Holmroos et al. 2012), atmospheric deposition (Bergström et al. 2005;

Elser et al. 2009), and land use in the catchment area (Downing and McCauley 1992; Vanni et al. 2011). Consequently the ratio of N to P in lakes varies widely so that many also have a deficit of N relative to P (Downing and McCauley 1992). Together with the variety in observations of the limiting nutrient this suggests that rather than a paradigm of a single nutrient that determines productivity in lakes the limiting nutrient may vary with lake type/morphology (Chaffin et al. 2013; Dolman et al. 2016), trophic status (Downing and McCauley 1992), region (Downing et al. 1999; Abell et al. 2010) and season (Morris and Lewis 1988; Chaffin et al. 2013; Dolman et al. 2016).

Consequently many authors urged to introduce a dual nutrient control of both N and P instead of the often applied control of P only (e.g. Elser et al. 2007; Sterner et al. 2008; Lewis and Wurtsbaugh 2008; Paerl et al. 2016). As a result, policy directives have been established demanding the reduction of N and P to counter eutrophication in the European Union (European Commission and Directorate-General for the Environment 2009) and the USA (USEPA 2015). In New Zealand, there is discussion over whether dual nutrient control or even N only control should be established (Abell et al. 2010).

1.3 Nostocales cyanobacteria, N_2-fixation and controlling factors

The main argument of proponents of the P-only control to counter eutrophication is that cyanobacteria capable of fixing atmospheric N_2 (mostly Nostocales cyanobacteria) will rise in biovolume, due to their advantage at low N conditions, and render N reduction measures effortless (e.g. Schindler et al. 2008; Welch 2009; Schindler 2012). However, despite N limiting conditions Nostocales have been absent in many lakes (Lewis et al. 2008) and it has been shown that N_2-fixation is not always able to balance load reductions (Scott and McCarthy 2010). The contribution of N_2-fixation to the total N input varies greatly between lakes (Howarth et al. 1988). Next to deficiency of N there are many biogeochemical (Howarth, Marino, and Cole 1988) and physical (Carr and Whitton 1982; Paerl 1988) factors that may control the rates of N_2-fixation and therefore the abundance of Nostocales in lakes.

Biological N_2-fixation is restricted to prokaryotic microorganisms possessing an enzyme complex called nitrogenase, which catalyses the reduction of molecular N_2 to ammonium (Stal 2013):

$$N_2 + 8[H] + 16ATP \rightarrow 2NH_4^+ + H_2 + 16ADP + 16P_i \quad (1.1)$$

The most important group of organisms, capable of fixing atmospheric N_2 in lakes are Nostocales cyanobacteria (Howarth et al. 1988). In these species the nitrogenase is located in specialised, thick walled cells, called heterocytes (Wolk and Wojciuch 1971a; Wolk and Wojciuch 1971b), to protect it from inactivation by oxygen (Fay 1992).

A main control factor for N_2-fixation is the availability of dissolved inorganic N (DIN), especially NH_4 and NO_3 (Horne and Commins 1987). Deficiency in DIN may promote N_2-fixation (Vanderhoef et al. 1974) and is the main initialization factor of heterocyst development (Ogawa and Carr 1969). The amount of energy needed to assimilate N for phytoplankton growth varies between different N sources in the following order: $NH_4 < NO_3 < N_2$ (de Marsac and Houmard 1993). Consequently Nostocales growth rates are significantly lower when relying on N_2-fixation compared to NO_3 or NH_4 (Rhee and Lederman 1983; De Nobel et al. 1997). To prevent a waste of energy the presence of NH_4 or NO_3 can suppress the nitrogenase synthesis (Horne and Goldman 1972; Magasanik 1977) or activity (Ohmori and Hattori 1974; Reich and Böger 1989) and the heterocyst formation (Horne et al. 1979). Although Nostocales cyanobacteria should have an advantage over other phytoplankton species incapable of N_2-fixation under conditions of low DIN (Tilman 1982), they have been absent in many N-limited lakes (Lewis et al. 2008). In these cases N_2-fixation was likely limited by other factors.

The reaction of N_2-fixation has a great energy demand (Howard and Rees 1994). Therefore, the availability of P and a sufficient light intensity are essential factors for N_2-fixation. To reduce one molecule of N_2 a minimum of 16 ATP (containing P) are hydrolysed (equation 1.1). The energy needed to synthesize the ATP is supplied by the cyclic photophosphorylation via photosystem I (Cox and Fay 1969; Fay 1970; Kohl et al. 1982). Consequently, the limitation of N_2-fixation by P (Stewart and Alexander 1971; Liao 1977; Tõnno and Nõges 2003) and light (Lewis and Levine 1984; Stal and Walsby 1998) has been observed in several studies.

Other factors that may control rates of N_2-fixation and Nostoclaes abundance are trace metals like iron (Wurtsbaugh and Horne 1983) or molybdenum (Howarth and Cole 1985), which are needed for nitrogenase synthesis (Mortenson and Thorneley 1979), pH (Fernandez Valiente and Leganes 1990), availability of external CO_2 (Leganés and Fernández Valiente 1991), or temperature (McQueen and Lean 1987; Brauer et al. 2013).

1.4 Scope of the thesis

The work for this thesis was integrated into the BMBF-FONA project: “Nitrogen limitation in freshwaters – Is nitrogen reduction ecologically meaningful and economically feasible?” (NITROLIMIT). The overall question of the project was whether the reduction of N in freshwaters may result in a better ecological status and whether that is economically feasible.

It was foreseeable that the majority of German freshwaters would not achieve the “good” ecological status required by European Water Framework Directive by 2015. While to date the main effort to reduce nutrient loading into freshwaters was targeted at P, recent research indicated that the importance of N in many freshwaters may have been underestimated and a reduction of N loading could be considered. However, the reduction of N loadings is expensive and it is still uncertain whether the water quality will improve sufficiently to justify the

additional costs. Therefore, using a comprehensive multidisciplinary approach NITROLIMIT aimed at assessing the impact of N on the ecological state in freshwaters, estimating N input and turnover for lakes and rivers, analyzing the costs and benefits of measures to reduce N-input and compiling recommendations for sustainable freshwater management.

It is important to know which nutrient is limiting in which lake and when, in order to plan appropriate nutrient reduction measures. The limitation status of the phytoplankton may vary between different lakes (lake types) and seasons. Nutrient enrichment bioassays for determination of the limiting status are time and cost intensive and cannot be repeated for large numbers of sites or over long periods. Therefore, it would be useful if the results of these experiments can be predicted by in lake nutrient concentrations, which are included in most water monitoring programs. This led to the following aims:

Determination of the seasonal dynamic of N- and P-limitation for four lakes of differing lake types using nutrient enrichment bioassays. Testing the power of four N:P ratios (TN:TP, DIN:TP, DIN:SRP and TN:SRP) to predict the limiting nutrient (Chapter 2).

In N-limited lakes the reduction of N-loadings could be ecologically meaningful to lower the trophic status and improve the water quality. However, it is still not resolved whether Nostocales cyanobacteria would compensate for the reduction in N-loading by N_2-fixation. To compensate Nostocales would need to increase in biovolume, N_2-fixation rate per biovolume or both. This led to the following aim:

Determination of the response of Nostocales cyanobacteria biovolume and N_2-fixation to varying N additions (Chapter 3).

N_2-fixation is a very energy demanding process and Nostocales cyanobacteria are dependent on sufficient light intensities in order to be able to compensate a for a reduction in N-loading. The quality of the light climate in lakes is subject to diurnal and seasonal changes. This led to the following aim:

Determination of the effect of the light intensity on the response of Nostocales cyanobacteria biovolume and N_2-fixation to varying N additions (Chapter 4).

1.5 Publications and author contributions

This thesis is based on manuscripts that were published in scientific journals or prepared for submission. The manuscripts and the relative contributions of the authors are listed in Table 1.1.

Table 1.1: Publications & manuscripts used for this thesis and contributions of authors. Order of authors is descending with proportion of contribution. The field and laboratory work was strongly supported by technical and laboratory staff and students (see acknowledgement). AMD = Andrew M. Dolman, AK = Antje Köhler, JK = Jan Köhler, SK = Sebastian Kolzau, JR = Jacqueline Rücker, MV = Maren Voss, CW = Claudia Wiedner.

Chapter	Study design	Field and laboratory work	Data preparation and statistics	Writing
2	*Seasonal Patterns of Nitrogen and Phosphorus Limitation in Four German Lakes and the Predictability of Limitation Status from Ambient Nutrient Concentrations* Kolzau, S., Dolman, A. M., Wiedner, C., Rücker, J., Köhler, J., & Köhler, A. PLOS ONE, published April 22, 2014, doi:10.1371/journal.pone.0096065			
	SK, CW, AMD, JR, JK & AK	SK	SK, AMD, JR & JK	SK with contributions of AMD, CW & JR
3	*The response of nitrogen fixing cyanobacteria to a simulated reduction in nitrogen loading* Kolzau, S., Dolman, A. M., Wiedner C. & Voss, M. International Review of Hydrobiology, submitted November 23, 2016			
	SK, CW & AMD	SK	SK & AMD	SK with contributions of AMD & CW
4	*Effect of light intensity on the response of nitrogen fixing cyanobacteria to varying N additions* Kolzau, S., Dolman A. M., Wiedner C.			
	SK, CW & AMD	SK	SK & AMD	SK with contributions of AMD

2 Seasonal patterns of nitrogen and phosphorus limitation in four German lakes and the predictability of limitation status from ambient nutrient concentrations

2.1 Introduction

Anthropogenic eutrophication is one of the biggest threats to freshwater ecosystems. Its consequences include changes in phytoplankton species composition and increases in biovolume that are accompanied by unpleasant odors, oxygen depletion, decreases in water transparency and a loss of biodiversity (Carpenter et al. 1998; Smith 2003). There has been an extended debate over whether N or P is the nutrient that ultimately determines productivity in lakes (Schindler et al. 2008; Lewis and Wurtsbaugh 2008; Sterner 2008). Early work emphasized P as the main nutrient controlling phytoplankton biovolume in most lakes based on inferences from the stoichiometry of N and P in phytoplankton and the relative availability of these elements in nature (Lewis and Wurtsbaugh 2008). This view was further reinforced by observation of the close statistical relationship between chlorophyll a and P concentration (Dillon and Rigler 1974) and the results from early lake manipulation experiments (Schindler 1977). However, subsequent nutrient addition experiments have found N to be just as often limiting as P (Elser et al. 1990; Elser et al. 2007) and it is now clear that the ratio of N to P in lakes varies widely so that many have a deficit of N relative to P (Downing and McCauley 1992). Some authors stated that N limitation can only be observed in short-term, small-scale experiments that may not be relevant to dynamic lake systems, and argued that P is the ultimate limiting nutrient over time due to N_2-fixation by cyanobacteria (Schindler et al. 2008; Schindler 2012). However Spivak et al (2011) showed that the results from small-scale experiments can be applied to larger more natural systems and in fact there are cases where N limitation was observed in mesocosms and whole lake experiments (Lewis et al. 2011). Scott and McCarthy (2010) even interpreted the results of Schindler et al. (2008) as proof that N_2-fixing cyanobacteria cannot fully compensate for nitrogen limitation, as the total N concentration and chlorophyll a concentration decreased after the N fertilization was stopped. Paterson et al. (2011) however responded in a comment with 4 more years of data for the studied lake showing that the N_2-fixation increased and the chlorophyll a concentration remained at a high level without N fertilization.

The variety of results suggests that rather than a single nutrient determining lake productivity, the limiting nutrient may vary with lake type, trophic status and season. For example, Downing and McCauley (1992) showed that average N:P ratios decline with trophy, so that N limitation is more likely to occur in eutrophic lakes while most oligotrophic lakes are likely limited by P. Reynolds (2006) indicated that in deeper lakes at higher altitudes, P sets the upper limit of

phytoplankton biovolume, but that this is less likely to apply to smaller, shallower lakes at all altitudes. Morris and Lewis (1988) found 5 of 8 lakes in the Colorado mountains where the limiting nutrient changed during the year. However, studies that investigated limitation in multiple lakes differing in mixing type, and over whole growing seasons, are still rare.

Reducing nutrient inputs from sewage plants or agriculture is expensive (Butt and Brown 2000; Rabotyagov et al. 2009); therefore it may be more cost and time effective to match reduction measures for specific nutrients to regions or phases when the nutrient in question is limiting and thus may have an immediate effect on water quality. The most commonly used method to identify the limiting nutrient is the enrichment bioassay, (e.g. Levine and Schindler 1999), in which different nutrients are added to separate water samples and the response of the phytoplankton is monitored. These experiments can be conducted on different temporal and spatial scales, ranging from bioassays in small bottles with duration of hours to a few days, (e.g. Sommer 1989), to whole lake manipulations that can run indefinitely, (e.g. Schindler 1977; Holmgren 1984). Small scale experiments offer tight control over experimental conditions, like temperature and light intensity, but may exclude important processes operating in natural systems. With increasing size and duration, experiments more closely replicate natural systems and include processes such as nutrient fluxes at the water-sediment interface; however, this comes at a cost of reduced experimental control, smaller sample sizes (Hecky and Kilham 1988) and a lack of replication. Furthermore, due to their ecological impact, whole lake experiments usually cannot be used to determine limiting nutrients in lakes. There are some downsides to identifying the limiting nutrient by experiments. Even small scale nutrient enrichment bioassays are time and cost intensive and cannot be repeated for large numbers of sites or over long periods. Therefore, it would be useful to be able to predict the outcome of these experiments from in-lake nutrient concentrations, which are part of most monitoring sampling programs. Theories of predicting the limiting nutrient with the elemental composition of the phytoplankton or the composition of the water bear on the work of Redfield (1958) who observed that on average phytoplankton assimilate C, N and P in the molar ratio of 106:16:1 (mass N:P ratio of about 7). This very generalized ratio has to be used carefully because it may vary with ecosystem and scale of analysis (Sterner et al. 2008). Morris and Lewis (1988) tested nine indices to predict the limiting nutrient in Colorado mountain lakes and found the DIN:TP ratio to be the best predictor. Subsequent studies have similarly found DIN:TP to predict the limiting nutrient the best in boreal and alpine lakes (Bergström 2010) and the Baltic Sea (Ptacnik et al. 2010).

The aim of this study was to compare the seasonal patterns of N and P limitation in four German lowland lakes, differing in mixing type, and to test which N:P ratio best predicted the limiting nutrient. Biweekly bioassays were conducted between the end of March and September 2011, in one deep-stratified, two shallow-polymictic and one riverine lake in the Berlin/Brandenburg lowlands. Each bioassay experiment then was classified into limitation categories by model selection. The seasonal pattern of limitation was compared with the seasonal dynamics of nutrients, available light and phytoplankton biovolume to identify drivers of the limiting factors. As quantitative measure of nutrient limitation a

response ratio was calculated. With linear regression this response ratio was then used to test the predictive power of the DIN:SRP, TN:TP, TN:SRP and DIN:TP ratio. We found that the seasonal patterns of limitation differed between lakes of different mixing type and that the limiting nutrient could be predicted by DIN:TP and TN:TP ratio.

2.2 Material and methods

2.2.1 Study sites, nutrient concentrations, phytoplankton biovolume and light availability

The nutrient and light limitation status of four lakes in the German states of Berlin and Brandenburg were studied from the end of March to September 2011: a deep stratified lake (Scharmützelsee = SCH), a very shallow polymictic lake (Langer See = LAN), a shallow temporarily stratified lake (Müggelsee = MUEG) and a shallow riverine lake (Untere Havel = UH). The main characteristics of the lakes are shown in Table 2.1; for more detailed information, see Grüneberg et al. (2011), Nixdorf and Deneke (1997), Köhler et al. (2005) and Knösche (2006).

Table 2.1: Morphometric data, geographic coordinates, mean concentration of TN, TP and chlorophyll *a* for the lakes from the end of March to September 2011.

Lake	Mixis	A (km^2)	Geographic coordinates	z_{max} (m)	z_{mean} (m)	TN ($\mu g\ L^{-1}$)	TP ($\mu g\ L^{-1}$)	Chla ($\mu g\ L^{-1}$)
SCH	di	12.1	52.216°N, 14.024°E	29.5	8.9	594	26	15
LAN	poly	1.6	52.243°N, 13.786°E	3.8	2.1	836	61	61
MUEG	poly	7.5	52.438°N, 13.645°E	8.9	4.8	1278	84	31
UH	poly	11.7	52.449°N, 13.157°E	10.7	5.6	1491	103	24

A = lake area; z_{max} = maximum depth; TP = total phosphorus; Chla = chlorophyll *a*.

Water sampling was performed biweekly at the deepest point of SCH, in the southern main basin of UH, in the middle of LAN, and weekly at five stations spread across MUEG. For SCH and MUEG, subsamples were taken from the mixed part of the water column (i.e. the epilimnion during thermal stratification or the whole water column during mixing periods) at 1 m depth intervals with the volume taken at each depth proportional to the lake volume at that depth. For UH and LAN, equal volume subsamples were taken at 0.5 m depth intervals. Subsamples were mixed together and used for the following experiments and analyses. Concentrations of SRP, TP, nitrate plus nitrite (NO_x-N), ammonia (NH_4-N) TN were measured according to standard methods (DEV 1981). Herein we refer to the sum of NO_x-N and NH_4-N as DIN. Phytoplankton biovolume and species composition were estimated according to Utermöhl (1958) using an inverted microscope. Secchi depth (z_{SD}) and depth profiles of water temperature were measured. The mean photosynthetically active radiation in the mixed upper part of

the water column (I_{mix}) was assumed to approximate the in situ light conditions for phytoplankton and was calculated according to Riley (1957) modified by Behrendt and Nixdorf (1993):

$$I_{mix} = 0.45 \cdot I_0 \left(\frac{1 - e^{-K_d \cdot z_{mix}}}{K_d \cdot z_{mix}} \right) \quad (2.1)$$

where z_{mix} is the mixing depth, K_d is the vertical attenuation coefficient and I_0 is the mean global radiation for that specific calendar week. Global radiation data from the meteorological observatory in Lindenberg were used. For LAN and UH the mean depth, and for SCH and MUEG the depth of the epilimnion (from the surface to the point where the change in water temperature was greater than 1°C per meter), was used as mixing depth. In cases where the epilimnion was deeper than the mean depth of the lake, the mean depth was used as mixing depth. The mean depth was determined with bathymetric maps drawn with sonar and GPS data. For SCH, LAN and UH the vertical attenuation coefficients were calculated from Secchi depth using an equation derived from long-term data of regional turbid lakes of different trophic states as given in Hilt et al. (2010):

$$K_d = 1.3611 \cdot {z_{SD}}^{-0.7105} \quad (2.2)$$

For MUEG the vertical attenuation coefficients were calculated according to Kirk (2011):

$$K_d = \frac{\ln I_1 - \ln I_2}{z_1 - z_2} \quad (2.3)$$

where I_1 and I_2 are the PAR at depth z_1 and z_2 respectively.

2.2.2 Bioassays

Nutrient addition experiments (bioassays) were conducted every two weeks between the end of March and September 2011. The full experimental design (Fig. 2.1) consisted of six treatments, four incubated under standard light conditions and two under in situ light conditions. In all cases, a control with no nutrient addition (Ctrl); an addition of 500 µg L^{-1} nitrogen in the form of 250 µg N L^{-1} $(NH_4)_2SO_4$ and $NaNO_3$ (+N); an addition of 200 µg L^{-1} phosphorus in the form KH_2PO_4 (+P) and the addition of both nutrients like in the single nutrient additions (+NP) were incubated under a standard light intensity (SL) of 100 µmol photons m^{-2} s^{-1}. 100 µmol photons m^{-2} s^{-1} were chosen because at this light intensity we did not expect light to be limiting nor inhibiting. Before each experiment, and for each lake, the in situ I_{mix} was estimated (see above). When the in situ light conditions were expected to be below 75 µmol photons m^{-2} s^{-1}, separate replicates of the control and +NP treatment were additionally incubated under the estimated in situ light conditions (ISL). Between 75 and 100 µmol photons m^{-2} s^{-1} we expected the effect on growth of the difference between SL and ISL treatments to be too small to be reliably detected and therefore decided to conduct ISL treatments only when I_{mix} was below 75 µmol photons m^{-2} s^{-1}. Therefore, SL and ISL treatments were

both performed in 20 experiments and just the SL treatments were performed in 29 experiments. Osram Lumilux cool daylight fluorescent tubes were used as the light source.

All bioassays were started on the same day as sampling, with water from the same sample as that for the nutrient analyses. Larger zooplankton were removed from the water by prefiltering through a 200 µm gauze. For all treatments three replicates of 150 ml lake water were incubated, gently shaken in glass Erlenmeyer flasks in a growing chamber (KBW 400, Binder), for three days at the measured water temperature of the epilimnion (± 2°C) under a 12 h: 12 h light: dark regime. The bottles were closed with cotton plugs to maintain air supply and bottle positions were switched daily to adjust for a light gradient in the growing chamber. The response of the phytoplankton was determined by measuring chlorophyll a concentration after three days with a fluorescence probe (FluoroProbe, bbe-Moldaenke).

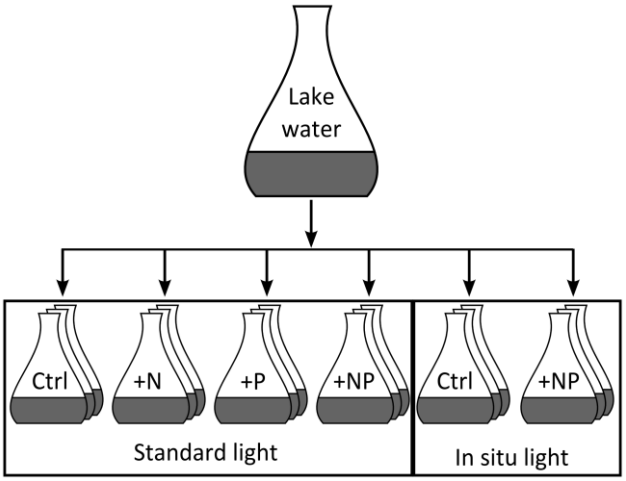

Figure 2.1: Experimental design of the bioassays. Treatments: Ctrl = control (no nutrient addition); +N = 250 µg N L^{-1} each of $NaNO_3$ and $(NH_4)_2SO_4$; +P = 200 µg P L^{-1} of KH_2PO_4; +NP = combined N+P addition; Standard light = 100 µmol photons m^{-2} s^{-1}; In situ light = predicted I_{mix}

2.2.3 Limitation categories

The outcomes of the bioassays were classified according to the 8 nutrient limitation categories defined by Harpole et al. (2011) and illustrated in Fig. 2.2:

– Single limitation (N or P): response to only one of the single nutrient treatments (+N or +P) and the response to the combined treatment (+NP) is no different (Fig. 2.2 a).

– Serial limitation (N or P): response to only one of the single nutrient treatments (+N or +P) but a larger response to the +NP treatment (Fig. 2.2 b).

– Independent co-limitation (primary N or P): response to both single nutrient treatments and a larger response to the +NP treatment; the single

treatment with the larger response indicates the primary limiting nutrient (Fig. 2.2 c).

– Simultaneous co-limitation: response only to the +NP treatment (Fig. 2.2 d).

– No nutrient limitation: no response to any nutrient treatment (not shown).

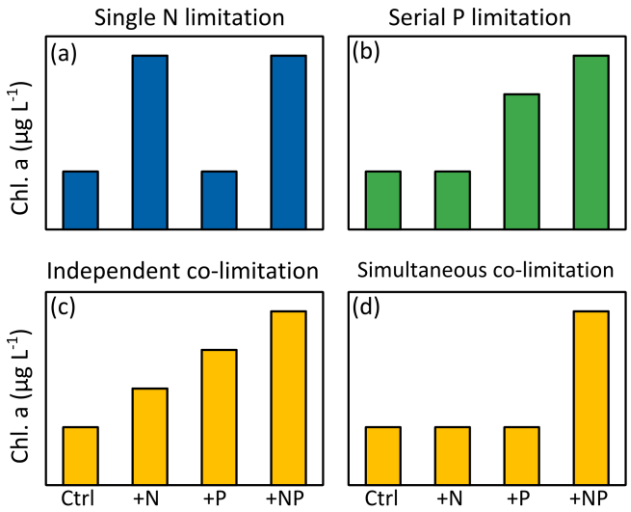

Figure 2.2: Example chlorophyll a response patterns. These patterns correspond to a subset of the nutrient limitation categories defined by Harpole et al. (2011). a) Single N limitation: a response to only one of the single treatments, in this example +N and the response to the +NP treatment is no different. b) Serial P limitation: a response to only one of the single nutrient treatments, in this example +P and a larger response to the +NP treatment. c) Independent co-limitation (primary P): a response to both single nutrient treatments with a larger response to +P and an even larger response to the +NP treatment; d) Simultaneous co limitation: a response only to the +NP treatment.

In addition, three light limitation categories were distinguished:

– Light limitation: a lower response to the Ctrl and +NP treatments when incubated under in situ light conditions compared to the response when incubated under standard light intensity and no difference between the Ctrl and +NP treatment when incubated with in situ light (Fig. 2.3 a).

– Co-light-nutrient limitation: the +NP treatment response is greater than the Ctrl for both standard and in situ light, but responses to Ctrl and +NP treatments are lower under in situ light than standard light (Fig. 2.3 b)

– No light limitation: no difference between the in situ and standard light incubation for either the Ctrl or +NP treatment (Fig. 2.3 c).

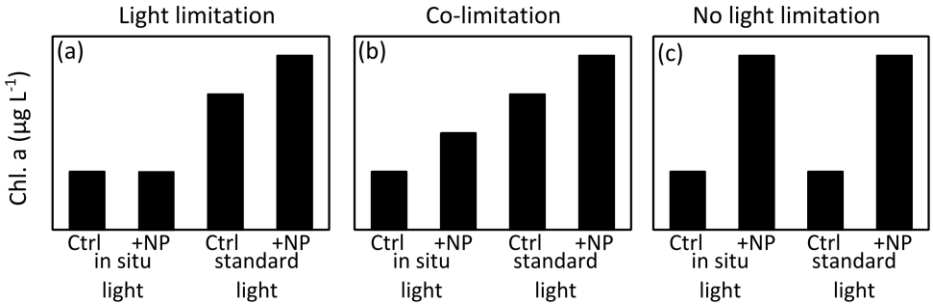

Figure 2.3: Possible light limitation patterns. a) Light limitation: a lower response to the Ctrl and +NP treatments when incubated under in situ light conditions and no difference between the Ctrl and +NP treatment when incubated with in situ light. b) Co light-nutrient limitation: the +NP treatment response is greater than the Ctrl for both standard and in situ light, but responses to Ctrl and +NP treatments are lower under in situ light than standard light. c) No light limitation: no difference between in the in situ and standard light incubation for either the Ctrl or +NP treatment.

A model selection procedure was used to assign bioassay outcomes to one of the above categories in a similar manner to Andersen et al (2007). Nutrient and light limitation categorization were performed separately. For treatments incubated under standard light, a set of linear models were fit to each bioassay outcome where each model represents one of the nutrient limitation categories outlined above. The simplest model corresponds to a no-response classification and has a single parameter b_0 representing the mean chlorophyll a (Chla) for all treatments:

$$\text{Chla} = b_0 + \epsilon \tag{2.4}$$

The model representing single N limitation has two parameters: one representing the mean chlorophyll a for all treatments where N was added $b_{(+N,+NP)}$ and one for all other treatments $b_{(Ctrl,+P)}$:

$$\text{Chla} = b_{(+N,+NP)} + b_{(Ctrl,+P)} + \epsilon \tag{2.5}$$

The most complex model has a separate parameter for all treatments:

$$\text{Chla} = b_{Ctrl} + b_{+N} + b_{+P} + b_{+NP} + \epsilon \tag{2.6}$$

Akaike's Information Criterion, corrected for small sample size (AIC_c), and Akaike weights (AIC_w), were used to assess the relative fit of the models (Burnham and Anderson 2002) and the model with the highest AIC_w was taken as the indicated limitation type. To avoid the situation where a model was selected because certain treatments inhibited chlorophyll a development, i.e. chlorophyll a was lower than the control in those treatments, bioassay outcomes were first screened for inhibitory effects and those models and treatments removed from the candidate list, outcomes were labeled as showing +N, or +P inhibition.

Model selection was similarly used separately on the Ctrl and +NP treatments under both standard and in situ light to further classify outcomes as indicating light, co light-nutrient, or non-light limitation (Fig. 2.3).

2.2.4 Relative N vs. P limitation and in-lake N:P ratios

To measure the relative strength of N versus P limitation a log response ratio RR was calculated as follows:

$$RR = \ln\left(\frac{Chla_P}{Chla_N}\right) \quad (2.7)$$

where $Chla_P$ and $Chla_N$ are the mean chlorophyll a concentrations of the three replicates at the end of the incubation in the +P and +N treatments respectively. Negative values indicate N, and positive values indicate P, as the primary limiting nutrient. RR was only calculated for experiments in which nutrient limitation was identified by the model selection (see above).

To determine the ability of in-lake N:P ratios to predict N vs. P limitation, for each N:P ratio a separate linear regression model was fit with RR as the response variable and logged in-lake TN:TP, DIN:SRP, TN:SRP and DIN:TP mass ratios as predictors. From each fitted model, the N:P ratio at which RR is predicted to be zero was used as an estimate of the ratio at which lake phytoplankton switch from being P to N limited.

Additionally, the sign of the predicted RR was used to predict outcomes as being N or P limited and a misclassification rate (MR) was calculated according to

$$MR = \frac{n_F}{n_T} \quad (2.8)$$

with n_F being the number of false predictions and n_T being the total number of experiments. A prediction was defined as false when from the N:P ratio the limiting nutrient was predicted to be N but RR was positive or to be P but RR was negative. Only experiments that showed nutrient limitation were used for this analysis.

All analyses were performed using R vers. 2.15.3 (R Core Team 2013).

2.3 Results

2.3.1 Bioassays

Nitrogen, phosphorus, and light were all at some point indicated to be the primary factor limiting phytoplankton biomass. There was a general trend from P limitation in spring to N limitation later in the year but also differences between lakes in the relative frequency of N and P limitation (Fig. 2.4; Table 2.2).

In the deep stratified lake, SCH, the phytoplankton were predominantly limited by P (Fig. 2.4 a). From April until the end of July, P was identified as the primary limiting nutrient in 6 of 7 experiments. In late summer the limiting nutrient was more variable and switched repeatedly between P and N. Co-limitation by

nutrients and light and by N and P was observed in SCH one and three times, respectively. The largest absolute value of RR, indicating the strongest limitation, was observed in September during a phase when limitation in SCH was categorized as being single P (Fig. 2.4 e).

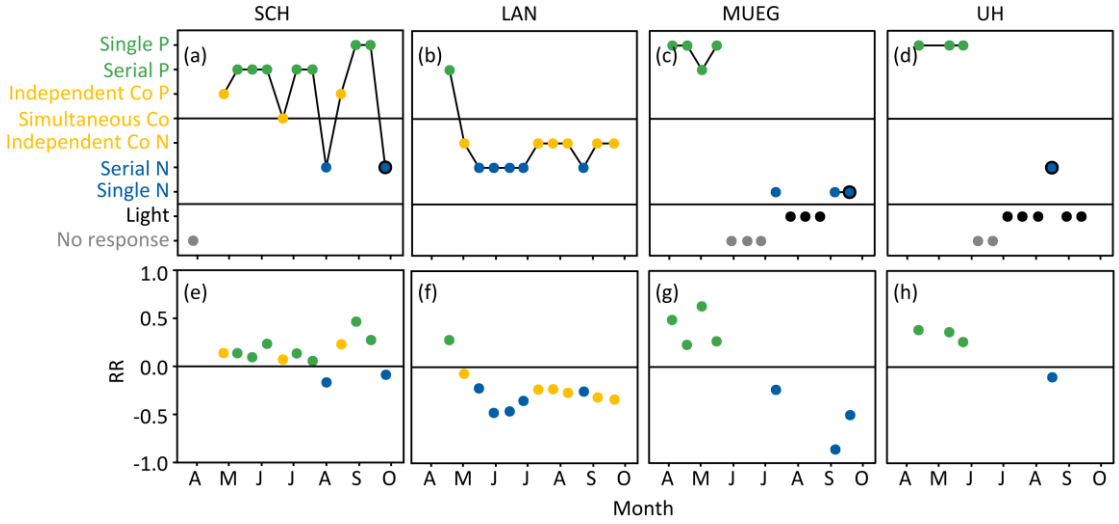

Figure 2.4: Seasonal variation of limitation types and response ratio determined by a series of bioassays (2011). a-d) Single and serial P limitation (green), independent (primary P or N) and simultaneous co-limitation (yellow), serial and single N limitation (blue), light limitation (black), co-limitation between light and nutrients (black circle around the colored point) and no response (grey). e-h) Response ratio (RR) indicating the relative strength of N versus P limitation. Negative values indicate N and positive values indicate P as the primary limiting nutrient. The colors are the same as those in a-d. RR for experiments showing no response or pure light limitation are not shown.

Table 2.2: Number of observations of the different limitation types in the four lakes.

	Number of Observations								
Lake	N	P	Co	Primary N	Primary P	Light	Co light/Nut.	No Response	n
SCH	2	7	3	2	9	0 (n=4)	1 (n=4)	1	13
LAN	5	1	6	11	1	0 (n=0)	0 (n=0)	0	12
MUEG	3	4	0	3	4	3 (n=7)	1 (n=7)	3	13
UH	1	3	0	1	3	5 (n=9)	1 (n=9)	2	11
Total	11	15	9	17	17	8 (n=20)	3 (n=20)	6	49

N: serial + single N limitation; P: serial + single P limitation; Co: independent + simultaneous co-limitation; Primary N: N + independent co-limitation (primary N); Primary P: P + independent co-limitation (primary P); Light: number of experiments showing light limitation; Co light/Nut.: number of co-limitation between light and Nutrients, n: number of experiments. In the columns Light and Co light/Nut. n gives the number of experiments where light limitation was tested.

In the shallow lake, LAN, phytoplankton were limited by P in early spring, but after a shift in early May they remained either N limited, or independent co-limited with

N as the primary limiting nutrient, for the rest of the studied period (Fig. 2.4 b). The highest absolute values of RR were found in June during a period of serial N limitation (Fig. 2.4 f). In LAN only serial limitation (N and P) and no single limitation was observed.

In the temporarily stratified shallow lake, MUEG, the phytoplankton also showed a shift from P to N limitation (Fig. 2.4 c) but the period of P limitation in spring lasted longer and was followed by a period in Jun-July in which there was no response to any nutrient addition treatment. One experiment in late July indicated N limitation and then during August the phytoplankton were limited by light before returning to N limitation in September.

In the riverine lake, UH, the phytoplankton were P limited from April to the end of May, but in June they did not respond to nutrient or light addition and from July to October they were light limited with just one co-limitation between light and N (Fig. 2.4 d).

2.3.2 Nutrient concentrations, light and phytoplankton biovolume

The four studied lakes showed differences in their trophic status (Table 2.1) and in the seasonal dynamics of nutrient concentrations (Fig. 2.5 and 2.6). Overall the highest nutrient concentrations were observed in UH (Fig. 2.6 b and d) and lowest in SCH (Fig. 2.5 a and c). DIN concentration in UH and MUEG was much higher than in SCH and LAN. DIN concentration in all lakes decreased in spring (Fig. 2.5 a and b and 2.6a and b). Both UH and MUEG showed a rapid increase of SRP and TP concentration in early summer (Fig. 2.6 c and d).

SRP was very low during phases of P limitation (predominantly below 10 µg P L^{-1}) and DIN was very low during phases of N limitation (predominantly below 100 µg N L^{-1}) in all lakes (Fig. 2.7). Although in SCH and LAN the dissolved forms of both nutrients were very low during the entire studied period, TP was much higher in LAN than in SCH, and SCH was predominantly P limited while LAN was N limited. The seasonal changes from P to N limitation in LAN and MUEG and from P to light limitation in UH were accompanied by a decrease of DIN in MUEG and LAN and an increase of SRP and TP in MUEG and UH. The change happened in LAN in spring and in MUEG in summer. LAN started out with lower N:P ratios than MUEG and DIN in LAN already decreased in early spring (data not shown).

The seasonal dynamic of I_{mix} is shown in Fig. 2.5 e and f and 2.6 e and f. The highest values of I_{mix} were observed in early summer in all lakes. The threshold of I_{mix} (75 µmol photons m^{-2} s^{-1}) below which an extra treatment was conducted in the nutrient enrichment bioassays was reached in SCH and MUEG in spring and late summer. In UH the measured I_{mix} was below 75 µmol photons m^{-2} s^{-1} on almost all sampling days. Light limitation was only observed when both DIN and SRP were close to or above 100 and 10 µg L^{-1} respectively (Fig. 2.7).

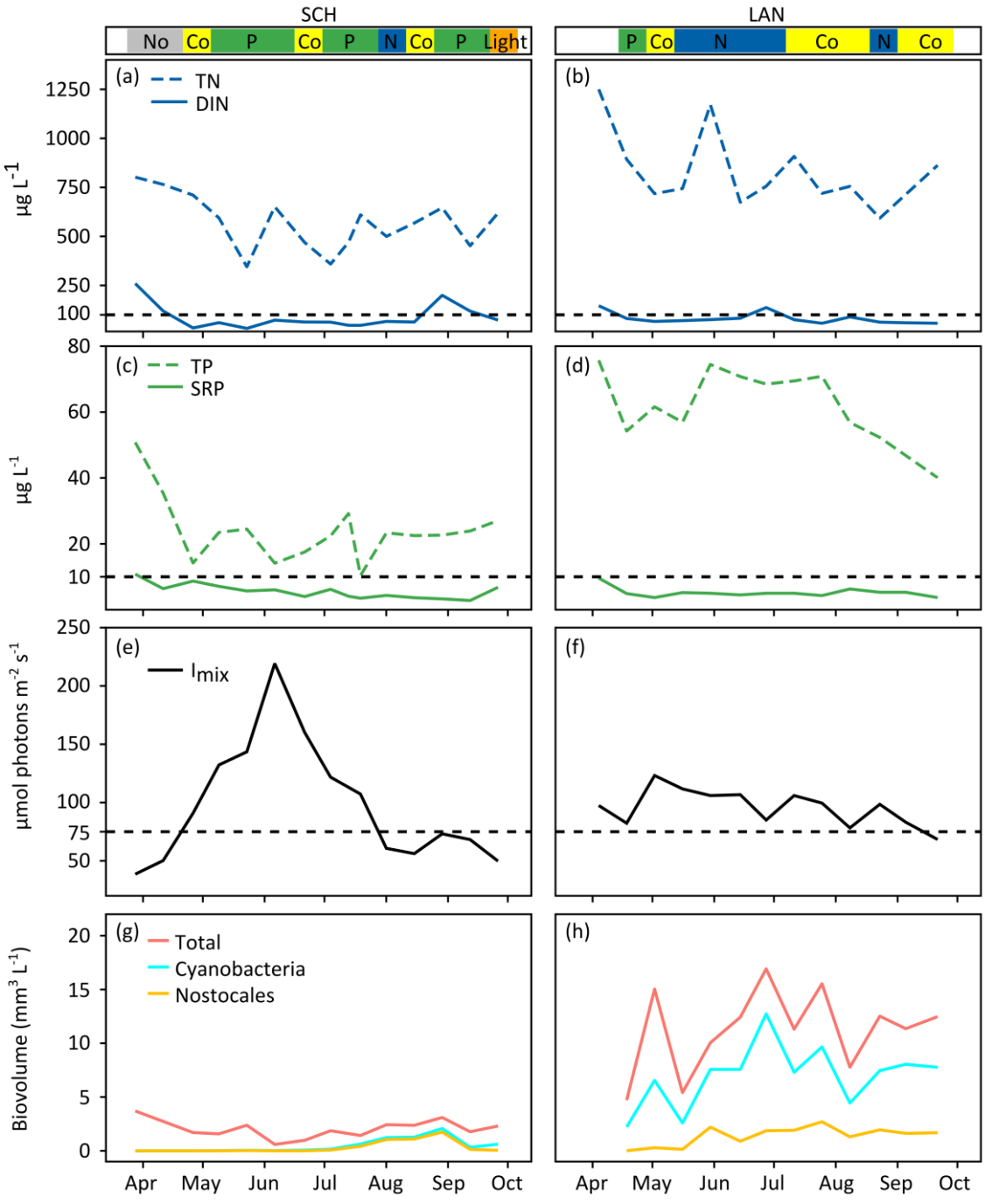

Figure 2.5: Seasonal pattern of nutrient concentration, I_{mix} and phytoplankton biovolume measured in SCH and LAN (2011). The colored bands above the graphs indicate the limitation type identified by the bioassays: where No is no limitation; Co is simultaneous or independent co-limitation; P is serial or single P limitation; N is serial or single N limitation; Light is light or co-limitation between light and nutrients. a-d) TN, DIN, TP and SRP; the horizontal lines mark the DIN and SRP concentrations below which N or P limitation are possible according to Maberly et al. (2002). e and f) I_{mix}, the horizontal line marks the light intensity below which in situ light treatments were conducted in the bioassays. g and h) Phytoplankton biovolume estimated according to Utermöhl (1958).

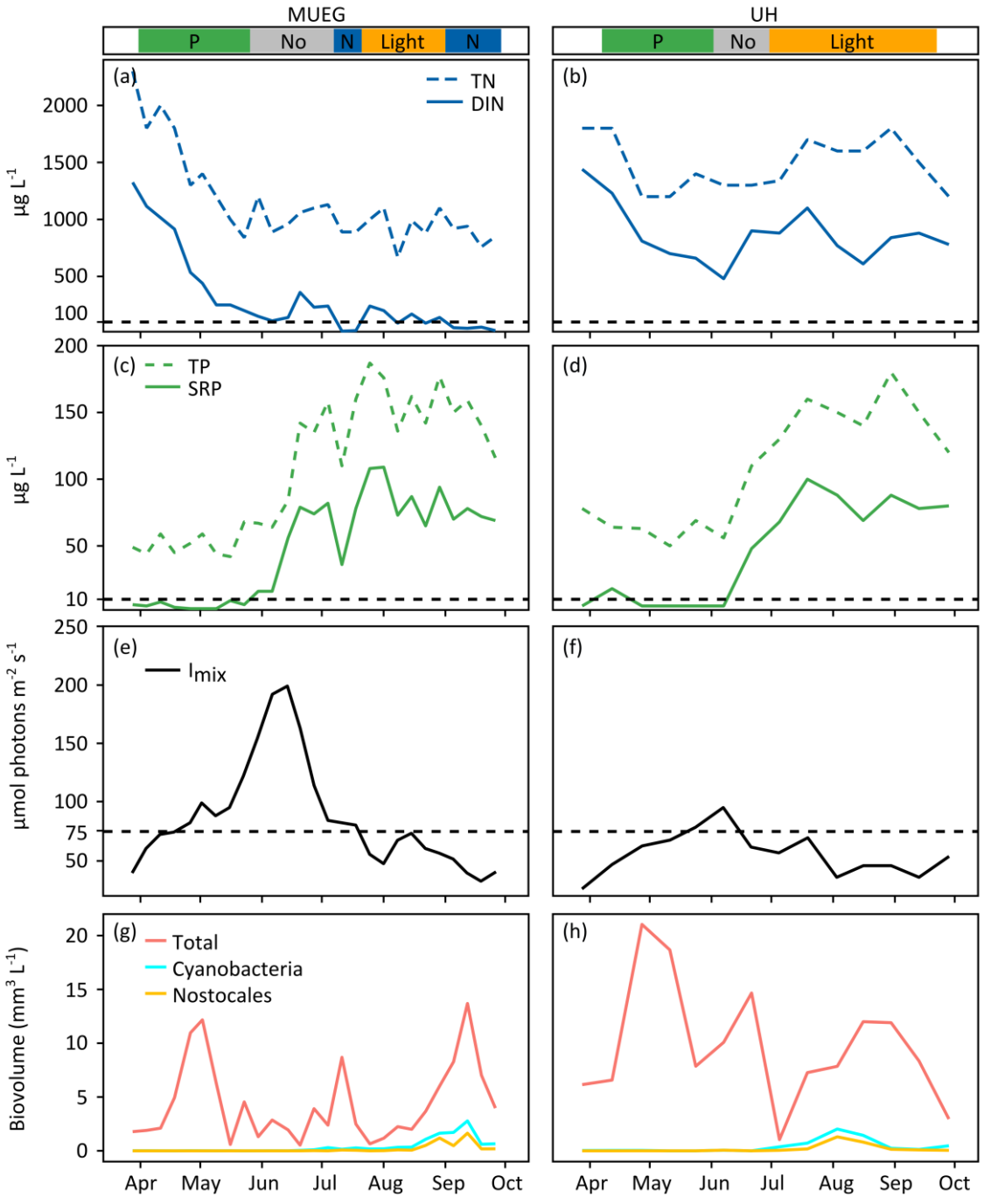

Figure 2.6: Seasonal pattern of nutrient concentration, I_{mix} and phytoplankton biovolume measured in MUEG and UH (2011). The colored bands above the graphs indicate the limitation type identified by the bioassays: where No is no limitation; Co is simultaneous or independent co-limitation; P is serial or single P limitation; N is serial or single N limitation; Light is light or co-limitation between light and nutrients. a-d) TN, DIN, TP and SRP; the horizontal lines mark the DIN and SRP concentrations below which N or P limitation are possible according to Maberly et al. (2002). e and f) I_{mix}, the horizontal line marks the light intensity below which in situ light treatments were conducted in the bioassays. g and h) Phytoplankton biovolume estimated according to Utermöhl (1958).

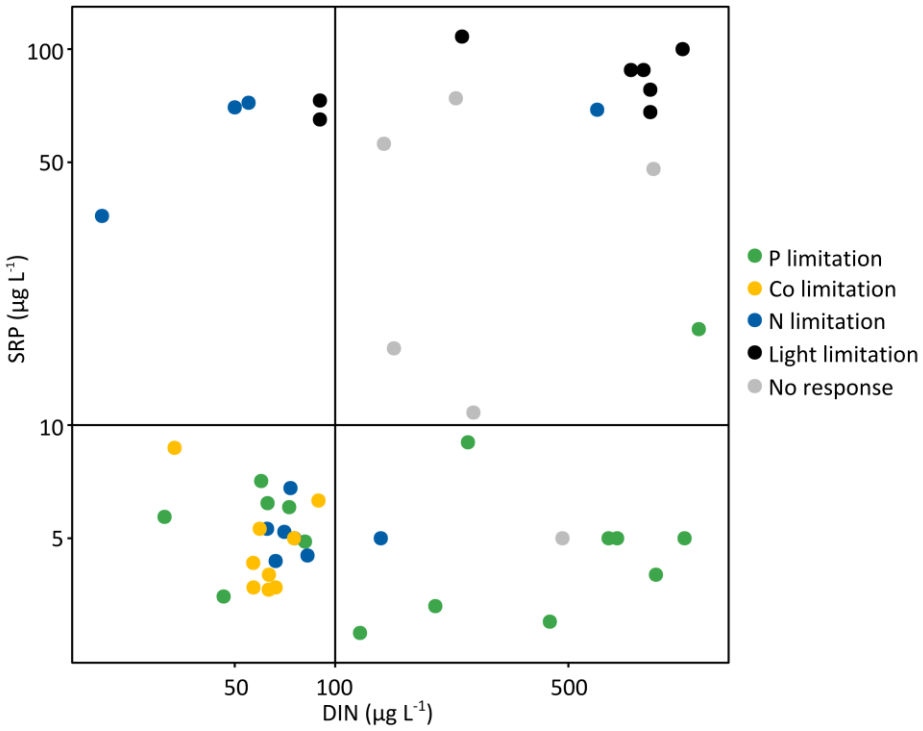

Figure 2.7: Relationships between the ambient DIN and SRP concentrations and the limitation categories. The vertical line marks the DIN concentration and the horizontal line marks the SRP concentration below which N or P limitation are possible according to Maberly et al. (2002). This plot shows that the results of the bioassays agree with the values given by Maberly et al. as SRP was predominantly below 10 µg L^{-1} when P limitation was observed and DIN was predominantly below 100 µg L^{-1} when N limitation was observed. Both dissolved nutrients were usually above these thresholds when light limitation or no response was observed.

The seasonal dynamic of total phytoplankton, cyanobacteria and nostocalean cyanobacteria biovolume is shown in Fig. 2.5 g and h and 2.6 g and h. In all lakes Nostocales occurred predominantly during phases of N limitation. In SCH, MUEG and UH Nostocales, and cyanobacteria in general, occurred only in late summer, while they were observed in LAN during the whole studied period. The highest absolute biovolumes of Nostocales were observed in LAN, but the highest relative biovolume was observed in SCH in late summer.

2.3.3 Prediction of the limiting nutrient by N:P ratios

The relationships between the in-lake DIN:SRP, TN:TP, TN:SRP and DIN:TP ratios and the P vs. N response ratio RR are shown in Fig. 2.8. All four ratios have significant positive relationships with RR, such that high N:P ratios were associated with P, and low N:P ratios with N limitation, but R^2 values for the DIN:TP ratio were higher than that for the TN:TP, DIN:SRP and TN:SRP ratios (Fig. 2.8).

Negative and positive values of RR indicate N and P respectively as the primary limiting nutrient. For each ratio, the position where the linear fit crosses the horizontal line at RR = 0 indicates the value of that ratio at which the

phytoplankton are predicted to switch between N and P limitation. These points were 17, 18.5, 120 and 2.6 for the DIN:SRP, TN:TP, TN:SRP and DIN:TP ratios respectively and are indicated by vertical black lines (Fig. 2.8). The predicted limiting nutrient for experiments in the top left and bottom right quadrants would therefore be wrong if those values were used as criteria. The number of incorrect predictions were much higher for the DIN:SRP and TN:SRP than the TN:TP or DIN:TP ratios, which is reflected in their higher misclassification rates.

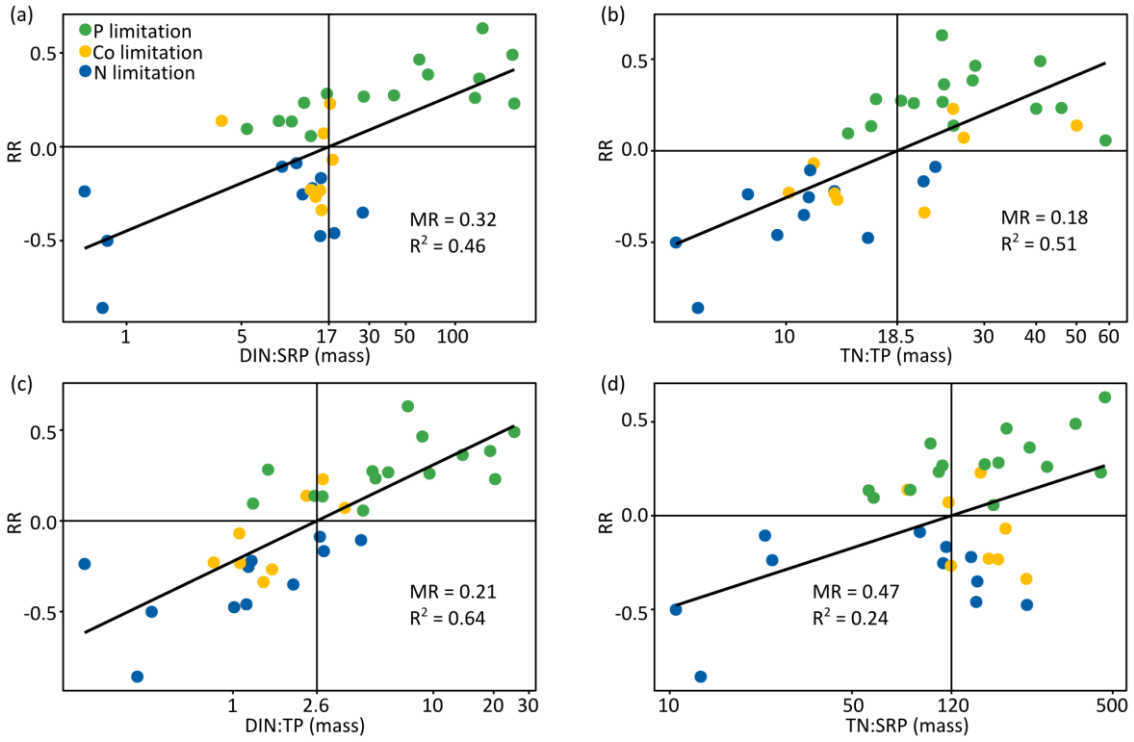

Figure 2.8: Relationships between the ambient N:P ratios and the response ratio observed in the bioassays. a) DIN:SRP, b) TN:TP, c) DIN:TP and d) TN:SRP. A positive response ratio (RR) indicates P limitation and a negative N limitation. The point at which the fitted line crosses RR = 0 identifies the ratio at which phytoplankton switch from being N to P limited. MR: Misclassification rate, R^2: Coefficient of determination of the linear regression. Experiments showing no nutrient limitation were excluded.

2.3.4 N:P ratios

The three N:P ratios TN:TP, DIN:TP and DIN:SRP were all more variable in SCH than in the other three lakes (Fig. 2.9 a, c and e). On the two occasions in SCH when N limitation was observed all three ratios were low, although they were sometimes even lower during P limitation. In LAN the N:P ratios showed little variability (Fig. 2.9 b, d and f); they were low during the entire studied period and were not appreciably higher on the one occasion when P limitation was observed. In MUEG and UH all three ratios showed a similar trend with very high values in spring and a sharp decrease to an extended period of low values in summer (Fig. 2.10) during which N limitation, and co-limitation between N and light, were observed. The more eutrophic lakes LAN, MUEG and UH showed, at least in summer (MUEG and

UH), lower N:P ratios and higher numbers of observed N limitation (light limitation in UH) than SCH.

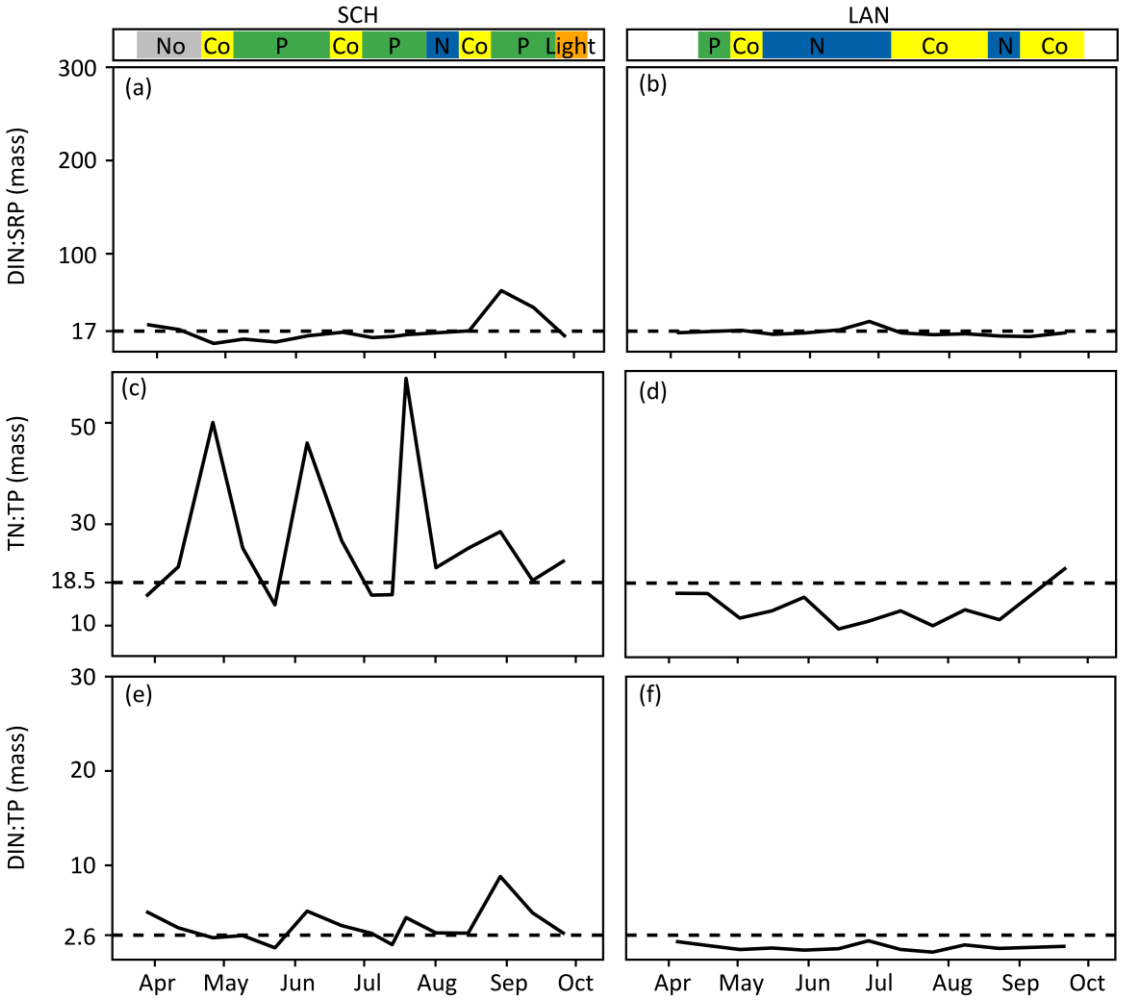

Figure 2.9: Seasonal pattern of the N:P mass ratios measured in SCH and LAN (2011). a and b) DIN:SRP, c and d) TN:TP, e and f) DIN:TP mass ratios. The colored bands above the graphs indicate the limitation type identified by the bioassays; where No is no limitation; Co is simultaneous or independent co-limitation; P is serial or single P limitation; N is serial or single N limitation; Light is light or co-limitation between light and nutrients. The horizontal lines mark the N:P ratio at which phytoplankton switched from being N to P limited based on an analysis of all four lakes combined (see Fig. 8).

2.4 Discussion

The aim of this study was to compare the seasonal patterns of N and P limitation in four lakes of different mixing types and to test whether the limiting nutrient could be predicted from ambient nutrient concentrations and ratios.

Nutrient addition bioassays showed that the seasonal pattern of N and P limitation differed between the lakes. The deep stratified lake was predominantly limited by P, while the three shallow polymictic lakes showed a seasonal shift, with P limitation in spring and N or light limitation later in the year. These patterns of

limitation matched the seasonal dynamics of nutrients and light availability, with high N:P ratios in spring and early summer and low N:P ratios and low light availability later in the year.

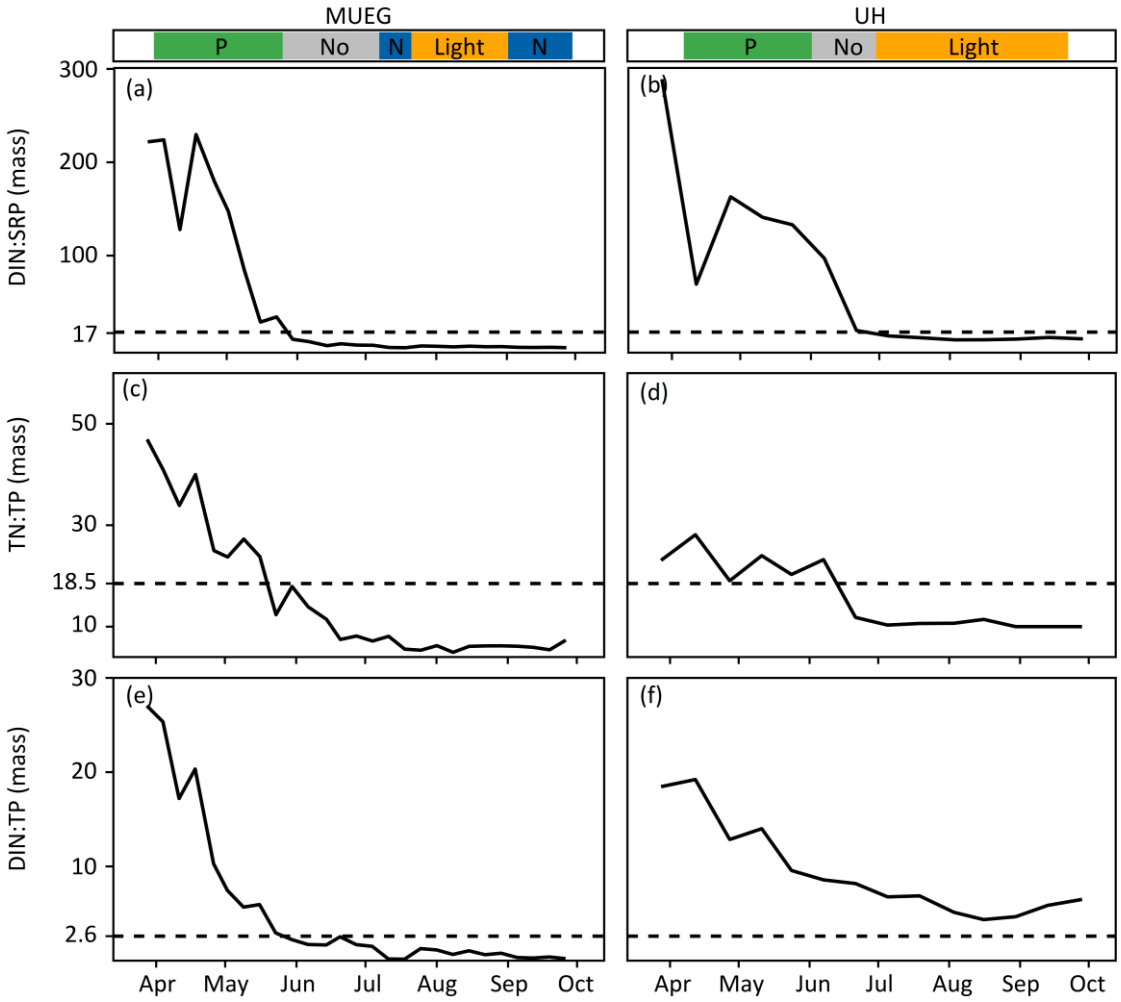

Figure 2.10: Seasonal pattern of the N:P mass ratios measured in MUEG and UH (2011). The colored bands above the graphs indicate the limitation type identified by the bioassays, where No is no limitation; Co is simultaneous or independent co-limitation; P is serial or single P limitation; N is serial or single N limitation Light is light or co-limitation between light and nutrients. The horizontal lines mark the N:P ratio at which phytoplankton switched from being N to P limited based on an analysis of all four lakes combined (see Fig. 8).

Ratios can only indicate the deficiency of one nutrient relative to the other; it is the absolute concentrations that determine whether nutrient limitation actually occurs. Here P limitation was observed only when SRP < 10 µg L^{-1}, and N limitation when DIN < 100 µg L^{-1}, confirming the observations of Maberly et al. (2002). However, they contrast with those of Reynolds (2006), who doubted that P limitation is possible at SRP > 3 µg L^{-1} and N limitation at DIN > 30 µg L^{-1}, as in our study N and P limitation were observed at concentrations well above these values. Nutrient affinities differ between phytoplankton species (Gotham and Rhee 1981a; Gotham and Rhee 1981b), so differences in the phytoplankton community may explain the different findings.

When some form of nutrient limitation occurred, the primary limiting nutrient could be well predicted from ambient N:P ratios. Predictions from the DIN:TP and TN:TP ratios were more or less equally accurate and better than those from the DIN:SRP and TN:SPR ratios. This is partly in contrast to the findings of Bergström (2010) and Morris and Lewis (1988), where DIN:TP performed best and much better than TN:TP, but the identified values of the DIN:TP and the TN:TP mass ratios at which the phytoplankton switched from being P to N limited (2.6 and 18.5 respectively) were in good agreement with the values found in a wide range of lakes and ocean sites (see Table 2.3). The threshold for the TN:TP ratio we found here was higher than the Redfield ratio of 7 (Redfield 1958). This is in agreement with Klausmeier et al. (2004) who predict a low optimal N:P ratio of 3.7 for phytoplankton under exponential growth, but higher ratios of 16, 17 and 20 when phytoplankton are light, N, or P limited as they mostly were here. They conclude that the Redfield N:P ratio is not a universal biochemical optimum, but instead represents an average of species-specific N:P ratios.

Table 2.3: Thresholds from this study and from the literature of TN:TP and DIN:TP mass ratios that separate N and P limitation.

	TN:TP		DIN:TP			
System	N	P	N	P	*Notes	Reference
German lowland lakes	<18.5	>18.5	<2.6	>2.6		This study
American mountain lakes	<15	>25	<0.5	>4	a	(Morris and Lewis 1988)
Several lake and ocean sites	<9	>22.6	-	-	b	(Guildford and Hecky 2000)
American + Swedish mountain lakes	<28	>28	<2.2	>2.2		(Bergström 2010)
Baltic sea	<45	>55	<2	>5	c	(Ptacnik et al. 2010)

*a) ratios were taken from Fig. 2 of (Morris and Lewis 1988). b) mass ratios were calculated from the molar ratios given by Guildford and Hecky (Guildford and Hecky 2000) c) ratios were taken from Fig. 5 of (Ptacnik et al. 2010).

A reduction in N:P ratios, accompanied by a shift from P to N limitation, was observed in the three studied shallow lakes. As described by Moss et al (2012) this may be a general feature of lakes and is likely due to seasonal changes in the rates of denitrification, a major sink of N in lakes (Lijklema 1994), and P release from the sediment, which can be an important internal P source (Hupfer and Lewandowski 2008). Decreasing oxygen concentrations at the sediment-water interface (Wetzel 2001) and increasing temperatures in spring and summer promote both denitrification and the release of P (Jensen and Andersen 1992; Veraart et al. 2011). Large increases in both TP and SRP concentration were observed in MUEG and UH during June and were likely due to release from the sediment, which has been documented previously in MUEG (Köhler et al. 2005) and in other parts of the UH river system (Schauser and Chorus 2009). There was no obvious increase in

phytoplankton biovolume following these summer P increases. However, although their N:P ratios declined into the range where N limitation might be expected, absolute DIN concentrations remained high, particularly in UH. MUEG showed only occasional N limitation, while UH was predominantly limited by light, and therefore N limitation cannot be wholly credited for the lack of a response in biovolume.

The fact that MUEG and UH are deeper compared to LAN leads to a lower average light availability in the completely mixed water and there was frequent light limitation in MUEG and UH. Nevertheless, the bioassays for these lakes conducted under standard light intensity showed a bigger response to +N treatment then to +P. So with more light available they would have been limited by N. Furthermore, in the studied polymictic lakes the phytoplankton could not profit from P release from the sediment as it was limited by light or nitrogen at that time.

In the deep stratified lake SCH no clear seasonal shift in limitation was observed and this is likely explained by the isolating effect of stratification. While in shallow lakes P released from the sediment is mixed into the entire water column, during stratification of a deep lake the released P is trapped in the hypolimnion and is largely unavailable to the phytoplankton. Similarly, denitrification at the sediment-water interface is isolated from the epilimnion during stratification. Denitrification rates may also be higher in shallow lakes due to overall higher temperatures and a larger relative surface area of sediment compared with deep lakes (Scheffer 2004).

N limited LAN and P limited SCH both had low SRP concentrations; only TP was higher in LAN than in SCH. “Luxury uptake” may explain why LAN was not P limited despite its low SRP concentrations. Many phytoplankton species are able to take up P faster than it is deployed and with this intracellular storage they are able to sustain up to four cell doublings without new P input (Reynolds 2006).

A potential weakness of this study is that light limitation was tested only when I_{mix} was below 75 µmol photons m^{-2} s^{-1} with extra treatments under an in situ (I_{mix}) in addition to the standard light intensity of 100 µmol photons m^{-2} s^{-1} (20 experiments). In other cases experiments were performed only under the standard light intensity of 100 µmol photons m^{-2} s^{-1} (29 experiments).

In 11 of the 29 experiments where light limitation was not tested, the phytoplankton were incubated at a higher light intensity than in situ (I_{mix}). In these cases the phytoplankton were classified as being nutrient limited (because there was a response to at least one of the nutrient treatments) but in fact may have been either co limited by light, or indeed exclusively limited by light. However, exclusive light limitation on these occasions seems unlikely as the ambient dissolved nutrient concentrations were very low (DIN < 100 µg L^{-1} and/or SRP < 10 µg L^{-1}).

In a further 18 of the 29 experiments where light limitation was not tested, the standard light intensity was either equal to, but in most cases lower than in situ I_{mix}. In all but 3 of these phytoplankton were classified as nutrient limited when they may more correctly have been classified as co limited by light and nutrients.

As they already showed a response to nutrients under 100 µmol photons m^{-2} s^{-1} a classification of exclusive light limitation would not occur even if they were to show a reaction to a higher light intensity.

In the remaining 3 experiments, where light limitation was not tested and in situ I_{mix} was higher than the standard incubation intensity, phytoplankton were classified as limited by neither light nor nutrients. Under higher light intensities the phytoplankton may have shown a response to nutrients but the high ambient concentrations of dissolved nutrients do not support this idea (DIN > 100 µg L^{-1} and SRP > 10 µg L^{-1}). In summary, while the frequency of co-limitation by light may have been underestimated, the relative frequency of N vs. P limitation should be correct, and a greater frequency of exclusive light limitation is unlikely given the ambient nutrient concentrations and light intensities.

In the studied lakes, nostocalean cyanobacteria reached their highest biovolume in the predominantly N limited LAN, where they may have an advantage due to their ability to fix atmospheric N_2 (Smith and Bennet 1999). Unexpectedly, the highest relative abundance of nostocalean cyanobacteria was found in SCH in late summer. This might have been triggered by the short periods of N and co limitation that were observed in SCH but still it shows that nostocalean cyanobacteria can reach high relative abundances in lakes predominantly limited by P (Dolman et al. 2012).

2.5 Conclusion

In order for water managers to best allocate resources it may be useful to know which nutrient limits phytoplankton in which lake and when. This study has shown that the frequency of nitrogen and phosphorus limitation varies between lakes and with the season and that the limiting nutrient is predictable. This study has shown that nitrogen limitation is frequent and persistent especially in shallow lakes. However it will be vital to determine whether phytoplankton biovolume can indeed be controlled by limiting the N supply and that nostocalean cyanobacteria cannot compensate by fixing N_2 when P is plentiful as this is still controversially discussed in literature (Scott and McCarthy 2010; Paterson et al. 2011; Schindler 2012).

3 The response of nitrogen fixing cyanobacteria to varying nitrogen additions

3.1 Introduction

Many lakes worldwide are suffering from eutrophication due to anthropogenic nutrient pollution (Carpenter et al. 1998). P was at one time thought to be the main nutrient limiting phytoplankton biomass in the majority of lakes (Schindler 1977); however, since the 1970s, empirical and experimental studies have demonstrated that N is also important in many lakes (see Lewis and Wurtsbaugh 2008; Sterner 2008 for review). In cross-lake analyses, phytoplankton biovolume is more closely related to N when N:P ratios are low (Dolman and Wiedner 2015) and conditions indicative of N limitation seem to predominate in shallow polymictic and riverine lakes (Dolman et al. 2016). In lakes that are identified as N limited, N loading reduction could be an ecologically meaningful way to improve water quality; however, concerns exist that compensatory N_2-fixation by cyanobacteria (Nostocales) could render N load reduction measures ineffective (Schindler 1977; Schindler et al. 2008; Schindler 2012). These concerns are based on two assumptions about the response of Nostocales to reduced N loading: (i) that Nostocales become more abundant, and (ii) that N_2-fixation per biovolume of Nostocales increases.

Evidence for a causal link between reduced N loading and Nostocales abundance is mixed. Many cross-lake studies have shown an association between Nostocales abundance, or less specifically cyanobacteria, and low N:P ratios. However, because N:P ratios generally decline with increasing trophic state (Downing and McCauley 1992) it is difficult to separate the effect of low N:P ratios from the larger influence of higher absolute nutrient concentrations (Pick 2016) and after controlling for the association between N:P ratio and overall trophy, Nostocales taxa show a variety of responses to relative N and P enrichment (Dolman et al. 2012). Additionally, many studies reported an increase in Nostocales as a percentage of total phytoplankton biovolume (e.g. Smith 1983; Smith et al. 1995; Hellström 1996; Havens et al. 2003), which might also be explained by a decrease in other, non N_2-fixing taxa, in response to lower N availability.

An increase in both Nostocales biovolume and N_2-fixation, in response to reduced N loading, has been found in some mesocosm (Levine and Schindler 1992; Levine and Schindler 1999; Vrede et al. 2009) and whole lake experiments (Schindler 1977; Flett et al. 1980; Findlay et al. 1994; Schindler et al. 2008). However, in a reanalysis of data presented by Schindler et al (2008), Scott and McCarthy (2010) showed that TN concentrations and N:P ratios in lake 227 had decreased following N loading reduction and took this as indication that increasing N_2-fixation was not sufficient to offset the decrease in external N inputs. There have also been experimental studies in the Neuse River Estuary and in lake Taihu in which reduction of N loading did not promote, or only marginally promoted, an increase

in Nostocales abundance (Piehler et al. 2002; Paerl et al. 2014). In a dynamic, mechanistic, molecular-level model of N_2-fixing cyanobacteria in a hypothetical lake, reduction of N loading by 50 % was only partly compensated for by fixation and led to a 33 % reduction in chlorophyll a (Hellweger et al. 2016).

There are several reasons why Nostocales may not be able to compensate for a reduction in N loading. Fixation of atmospheric N_2 is energy demanding (Paerl 1990) and therefore often limited by light intensity (Lewis and Levine 1984; Stal and Walsby 1998). It can also be limited by P concentration (Paerl 1990) and by the availability of micronutrients like iron, which is essential for the synthesis of the nitrogenase enzyme (Wurtsbaugh 1988). Nostocales gain a competitive advantage over other phytoplankton taxa when N is scarce, but their growth rates are significantly lower when relying on N_2-fixation compared to nitrate or ammonium as their N source (Rhee and Lederman 1983; De Nobel et al. 1997). Furthermore, Nostocales lose their competitive advantage over non-fixing taxa when N_2-fixation is restricted by low light (Wiedner et al. 2002; Lewis et al. 2008), P (Stockner and Shortreed 1988) or iron (McQueen and Lean 1987), and their biovolume can remain low, or even absent, despite N limited conditions.

Evidence is increasing for the importance of N limitation in regulating phytoplankton biovolume and therefore the ability of N reduction to improve the ecological status of lakes, but the extent to which Nostocales may compensate for N load reduction across multiple timescales is still unknown. The aim of this study was to determine this compensation potential over short timescales relevant to population growth. To achieve this we carried out a microcosm experiment in which P loading was kept constant, while a gradient of N-addition from 200 to 0 µg l^{-1} d^{-1} simulated a reduction in nitrogen loading. The biovolume of Nostocales, other cyanobacteria and eukaryotic phytoplankton as well as the N_2-fixation rate, was measured over 6 days and the potential of Nostocales to compensate for N reduction was calculated.

3.2 Material and methods

3.2.1 Study area “Langer See”

For the microcosm experiments we used water from LAN, a shallow (mean depth 2.1 m) polymictic and eutrophic lake in northeast Germany (52.243°N, 13.786°E), previously shown via nutrient enrichment bioassays to be nitrogen limited in summer (see chapter 2 and Kolzau et al. 2014). LAN in 2012 was routinely sampled fortnightly at the deepest point of the lake like described in chapter 2.2.1.

3.2.2 Microcosm experiment

The experimental setup consisted of 10 treatments (Fig. 3.1). In nine treatments we added a daily constant dose of P (20 µg L^{-1} d^{-1}) to simulate an unchanged P loading, but nine different doses of N (a gradient ranging from 200 to 0 µg L^{-1} d^{-1};

see Fig. 3.1) to simulate a reduction in N loading. One treatment served as a control, without any P or N addition, and was performed in triplicate. With the high P addition, we aimed to assure N limitation of the phytoplankton. P release from the sediment resulting in a high availability of P is a common process in many shallow polymictic lakes in summer (e.g. Köhler et al. 2005; Isles et al. 2015). However, a side effect of the nutrient additions is an increase in the trophic status.

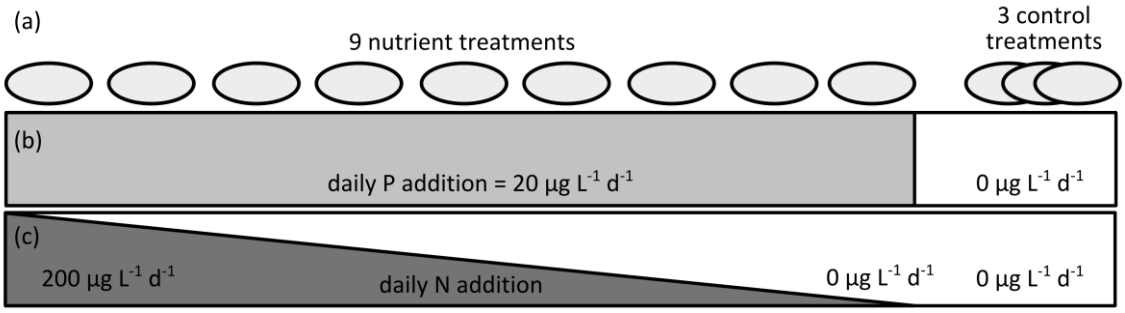

Figure 3.1: The Experimental design consisted of nine nutrient treatments and a triplicate control treatment without nutrient additions (a). All nine nutrient treatments received a constant daily P dose of 20 µg L^{-1} d^{-1} (b) while the N addition was varied along a gradient between 200 and 0 µg L^{-1} d^{-1} (c) to simulate a reduction in N loading.

The experiment was carried out using a mixed sample of the water column from LAN taken on 17.08.2012. Larger zooplankton were removed from the sample by filtering the water through a 200 µm gauze and a sample was taken to determine the phytoplankton biovolume at the start of the experiment. Thereafter the sample was split into twelve aliquots of 5 L. Nine aliquots were used for the P and N addition treatments and three for the control. All aliquots were filled into polyethylene (PE) bags and P was added in the form of KH_2PO_4 while N was added in the form of $(NH_4)_2SO_4$ and $NaNO_3$ in equal molar N measure. The PE bags were then incubated in an experimental pond at a depth with an average light intensity of 100 µmol photons m^{-2} s^{-1} for a period of six days from 17.08. to 23.08.2012. Each afternoon during the experiment nutrients were added and the water in the bags was aerated with a membrane pump until oxygen saturation was lowered back to approximately 100%, measured with a Clark electrode. Subsamples to measure N_2-fixation were taken after the nutrient addition on the starting day and after the aeration and nutrient addition on days 3 and 6. Further samples to measure phytoplankton biovolume were taken before the aeration and nutrient addition on days 3 and 6.

N_2-fixation was measured using the $^{15}N_2$ stable isotope method (Montoya et al. 1996). Polycarbonate (PC) bottles (~ 630 ml) were filled with water from the PE bags with no headspace, and 0.6 ml of $^{15}N_2$ (98 atom %, Aldrich chemistry) were added through a Teflon-covered butyl rubber septum with a gas-tight syringe. Each bottle was gently shaken for 5 minutes to achieve equilibrium of $^{15}N_2$ and dissolved $^{14}N_2$. The bottles were then incubated in the experimental pond for approximately 24 hours at the same depth as the PE bags. After incubation the bottles were recovered and particulate organic matter was filtered onto precombusted GF filters (MN 85/90 BF, Macherey-Nagel) and dried for at least 12

h at 60°C. The filters were wrapped in tin cups and analysed with a Delta V Advantage Isotope mass spectrometer, with Conflo IV and Flash Elemental Analyzer (Thermo Scientific), at the Leibniz Institute for Baltic Sea Research Warnemünde, Germany. The amount of fixed N_2 was calculated according to Montoya et al. (1996). N_2-fixation per Nostocales biovolume was calculated using the biovolume at the start of the incubation. We are aware of the discussion around the delayed isotopic equilibrium when labelled N_2 gas (bubble) instead of dissolved N_2 is added to a sample (see Wilson et al. 2012). Wilson observed lower N_2-fixation rates when the incubation time was only a few hours long, but as our incubation period was a full 24 hours the effect of delayed equilibration should be minimal. A potential underestimate of the rates may be as low as 1.4 according to measurements in the Atlantic Ocean (Mulholland et al. 2012).

Table 3.1: Mean light intensity (I_z) and temperature (T) during the experiment, and days when N_2-fixation was measured.

Day	I_z (µmol photons m^{-2} s^{-1})	T (°C)	N_2-fix. incubation
0-1	121	23	x
1-2	127	23	-
2-3	105	23	-
3-4	110	23	x
4-5	98	23	-
5-6	111	23	-
6-7	56	23	x

The water temperature and the average light intensity for the phytoplankton in the PE bags and PC bottles (I_z) during the incubations can be found in Table 3.1. I_z was calculated according to the Lambert-Beer law as:

$$I_z = I_0 \cdot e^{K_d \cdot z} \tag{3.1}$$

where I_0 is the global radiation, K_d is the attenuation coefficient and z is the incubation depth. The target for the incubation light intensity was the median of I_{mix} observed in summer (May - September) from 2009 to 2014 in LAN (84 µmol photons m^{-2} s^{-1}). During the time of the experiment the global radiation was higher than expected, resulting in an incubation light intensity of 104 µmol photons m^{-2} s^{-1} (Fig 3.2).

3.2.3 Statistics

The response of the biovolume, percentage of Nostocales, heterocyst abundance and N_2-fixation to the manipulated daily N and P additions were tested using the following linear regression model:

$$y = b_0 + b_{Nadd} \cdot Nadd + b_{Padd} \cdot Padd + \epsilon \quad (3.2)$$

where y is the dependent variable (e.g. biovolume). N_{add} is the amount of N added per day as a continuous variable in µg L^{-1} d^{-1}, and P_{add} is a binary variable indicating whether P was added or not. For all regressions, the intercept (b_0), slope (b_{Nadd}) and the change in the intercept when P was added (b_{Padd}) are given in Table 3.2, together with their 95 % confidence intervals. ε is a normally distributed error term. The analyses were performed using R vers. 3.2.2 (R Core Team 2015).

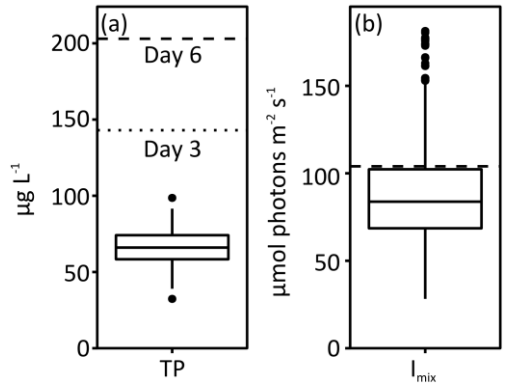

Figure 3.2: Boxplots of the TP concentrations (a) and the mean photosynthetically active radiation in the whole water column (b) observed in summer (May - September) from 2009 to 2014 in Langer See. The horizontal lines show the TP concentrations (start value + addition) on day 3 and 6 and the mean I_{mix} of the microcosm experiment.

3.3 Results

3.3.1 Nutrient concentrations and phytoplankton composition in LAN 2012

During the summer of 2012 (May - September), the DIN and SRP concentrations in LAN were consistently below thresholds of 100 µg N L^{-1} and 10 µg P L^{-1} (Fig. 3.3), indicating both N and P limitation of the phytoplankton (Maberly et al. 2002; Kolzau et al. 2014). However, TN:TP and DIN:TP mass ratios below 18.5 and 2.6 respectively are indicative of N limitation in this region (Kolzau et al. 2014) and by those criteria N was more likely than P limitation in LAN in summer 2012.

There was a mixed phytoplankton community in 2012, comprising eukaryotes and both non-fixing and Nostocales cyanobacteria (Fig. 3.3), with non-fixing cyanobacteria being the largest group from June until the end of the year. Nostocales reached a relative biovolume of 7 - 27 % (0.5 - 4.3 mm^3 L^{-1}) of the total phytoplankton biovolume, which ranged from 7.8 to 16.7 mm^3 L^{-1} (Fig. 3.3).

In August, when the microcosm experiment was performed, Nostocales cyanobacteria accounted for 14 % (2.9 mm^3 L^{-1}) of the total phytoplankton biovolume (22.8 mm^3 L^{-1}). *Aphanizomenon spp* was the most abundant Nostocales species and accounted for 71 % (2.1 mm^3 L^{-1}) of Nostocales biovolume, followed by *Dolichospermum spp* with 15 % (0.4 mm^3 L^{-1}). These biovolumes were typical for

LAN. Over the period 2009 -2014, Nostocales biovolumes observed between May and September were below 3.0 mm^3 L^{-1} on 75 % of occasions, with a maximum observed biovolume of 10.5 mm^3 L^{-1} (Fig. 3.5 c).

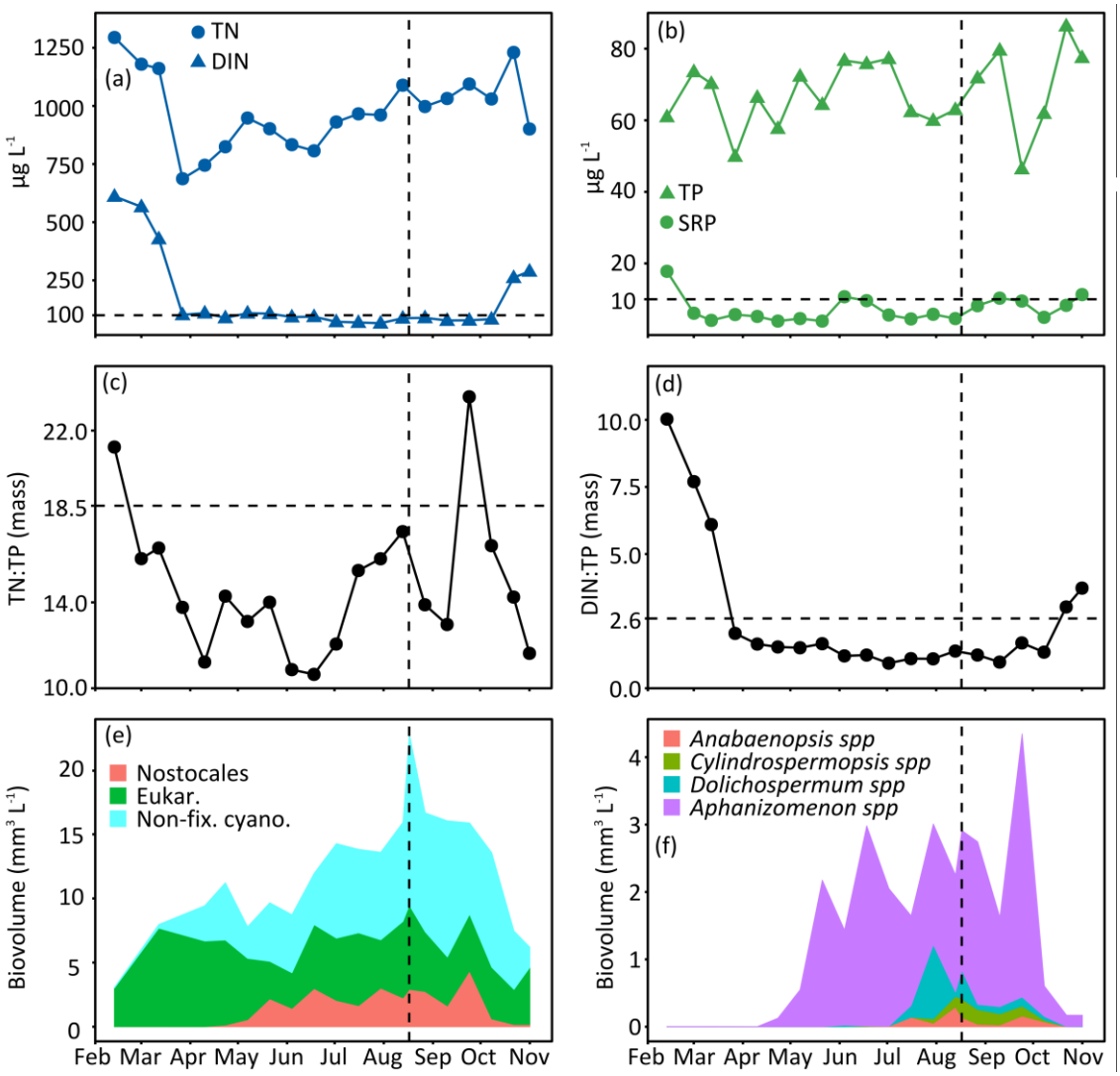

Figure 3.3: Seasonal pattern of nutrient concentrations (a and b), N:P mass ratios (c and d) and phytoplankton biovolume (e and f) measured in Langer See (2012). The vertical lines mark the day when the microcosm experiment was started. TN, DIN, TP and SRP; the horizontal lines mark the DIN and SRP concentrations below which N or P limitation are possible (a and b) and the TN:TP and DIN:TP ratios at which phytoplankton is predicted to switch from being N to P limited (c and d) according to the findings in chapter 2.

3.3.2 Microcosm experiment

Nostocales biovolume in the microcosms increased more than 5-fold during the experiment (Fig. 3.4 a). However, it increased at approximately the same rate in all treatments along the N addition gradient, so that there was no difference in absolute Nostocales biovolume between low and high N loading rate treatments. In contrast, the biovolume of non-fixing cyanobacteria and eukaryotic phytoplankton was significantly lower at low N addition rates (Fig. 3.4 and c and Table 3.2) and as

a consequence, relative Nostocales biovolume (Nostocales biovolume as a percentage of the total) increased with declining N addition rates (Fig. 3.4 e and Table 3.2). The Nostocales species composition changed slightly during the experiment. The percentage on the total Nostocales biovolume *of Aphanizomenon spp* increased from 71 % at the beginning of the experiment to 82 – 87 % at the end (data not shown).

Table 3.2: Estimated parameters with 95% confidence intervals for linear regression models between various response variables and the N and P addition.

Response variable	Day	b_0	b_{Nadd}	b_{Padd}
Nostocales biovolume	3	4.78 (3.78, 5.78)	0.001 (-0.009, 0.010)	1.30 (-0.11, 2.71)
	6	5.88 (3.01, 8.75)	-0.010 (-0.037, 0.017)	11.24 (7.19, 15.28)
Non-fixing cyanobacteria	6	17.45 (11.70, 23.19)	0.096 (0.042, 0.149)	-3.78 (-11.89, 4.32)
Eukaryotes biovolume	6	5.20 (2.94, 7.45)	0.022 (0.001, 0.043)	1.58 (-1.59, 4.76)
Total biovolume	6	28.53 (20.90, 36.16)	0.107 (0.036, 0.178)	9.04 (-1.72, 19.80)
Percentage Nostocales	6	20.70 (16.50, 24.91)	-0.102 (-0.141, -0.062)	23.80 (17.88, 29.73)
Heterocyst abundance	3	7.99 (5.93, 10.05)	-0.008 (-0.028, 0.011)	3.13 (0.23, 6.04)
	6	9.78 (5.37, 14.19)	-0.053 (-0.094, -0.012)	22.09 (15.87, 28.31)
N_2-fixation per litre	0	14.21 (12.30, 16.12)	-0.003 (-0.021, 0.015)	3.00 (0.30, 5.69)
	3	28.72 (16.55, 40.88)	-0.149 (-0.263, -0.036)	43.85 (26.70, 61.01)
	6	27.76 (16.84, 38.67)	-0.350 (-0.452, -0.248)	76.26 (60.87, 91.65)
N_2-fix. per 10^6 hetero.	0	2.91 (2.52, 3.31)	-0.001 (-0.004, 0.003)	0.61 (0.06, 1.17)
	3	3.68 (1.94, 5.41)	-0.009 (-0.025, 0.007)	2.96 (0.52, 5.41)
	6	2.89 (2.35, 3.42)	-0.009 (-0.014, -0.004)	0.52 (-0.24, 1.27)
N_2-fix. per Nosto. biovol.	0	4.88 (4.22, 5.53)	-0.001 (-0.007, 0.005)	1.03 (0.10, 1.95)
	3	6.16 (3.80, 8.51)	-0.024 (-0.046, -0.002)	5.78 (2.46, 9.10)
	6	4.76 (3.75, 5.77)	-0.020 (-0.030, -0.011)	1.56 (0.14, 2.98)

b_0 is the intercept indicating the value for the response variable when no N or P was added. b_{Nadd} is the slope indicating by how much the response variable changed when the N addition was increased by 1 µg L^{-1} d^{-1}; b_{Padd} is the change in the intercept indicating how much the value of the response variable changed when P was added compared to the control. Bold text indicates significant correlations ($p < 0.05$) between the response variable and the N and P additions. The unit for biovolumes is mm^3 L^{-1}, for N_2-fixation per litre it is µg N L^{-1} d^{-1}, for the N_2-fixation rate per Nostocales biovolume it is µg N mm^{-3} d^{-1} and for the N_2-fixation rate per heterocyst it is µg N (10^6 heterocysts)$^{-1}$ d^{-1}.

N_2-fixation per litre was significantly higher at low rates of N addition after three and six days (Fig. 3.5 and Table 3.2). We also observed significantly higher heterocyst abundance, and higher N_2-fixation per heterocyst and per Nostocales biovolume, at low N addition rates (Fig. 3.4 f and 3.6 and Table 3.2). N_2-fixation rates per Nostocales biovolume also varied between the three sampling dates, with higher values on day 0 and 3 (4.0 – 13.3 µg N mm^{-3} d^{-1}) than on day 6 (1.8 – 7.5 µg N mm^{-3} d^{-1}). Light intensity was higher during the days with higher fixation rates (Table 3.1), indicating light limitation of N_2-fixation. The slope of the relationship between N_2-fixation per Nostocales biovolume and N addition did not differ significantly between day 3 and day 6 (Fig. 3.6 b); therefore, the mean slope was used for further analysis.

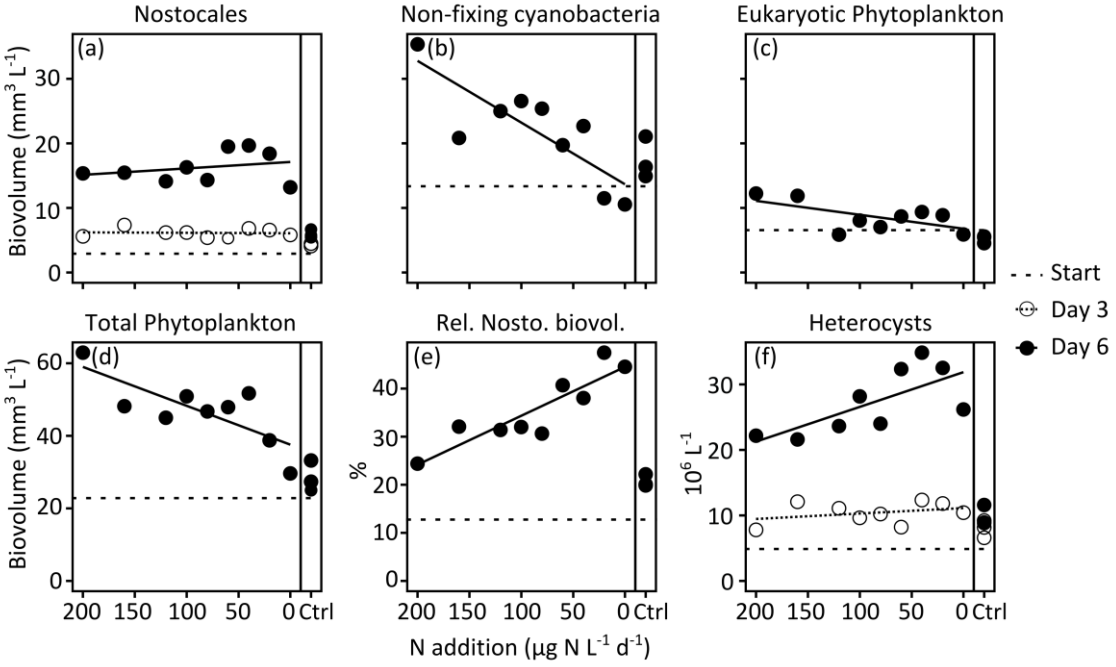

Figure 3.4: Components of phytoplankton biovolume along the daily N addition gradient: Nostocales (a), non-fixing (b), eukaryotic (c) and total (d) phytoplankton biovolume, Nostocales cyanobacteria as a percentage of total phytoplankton biovolume (e) and total heterocyst abundance (f). Note that the x-axis is reversed so that N input declines from left to right and that the y-axis scale differs between the upper and lower rows. The treatments with no nutrient addition (Ctrl) are shown to the right of the vertical line. The biovolume of the non-fixing cyanobacteria and eukaryotic phytoplankton was determined only at the start (dashed line) and on day 6.

The high P addition in our microcosms produced a strong increase in Nostocales biovolume, heterocyst abundance and N_2-fixtion per liter and per Nostocales biovolume (compared with the control; Fig. 3.4, 3.5 and 3.6 and Table 3.2), indicating P limitation of Nostocales growth and N_2-fixation. In contrast, total phytoplankton biovolume was unaffected by addition of P alone, only responding to joint addition of N and P (Fig. 3.4 d and Table 3.2), indicating N limitation of the total phytoplankton biovolume.

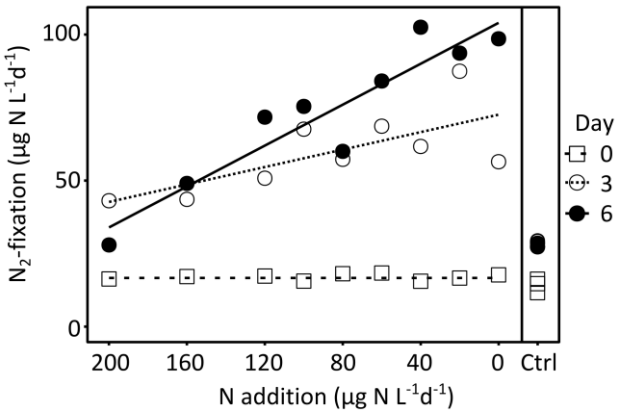

Figure 3.5: Correlation of the N_2-fixation rate per litre and day with the daily N addition. Notice that the x-axis is reversed so that N input declines from left to right. The same amount of P was added to each treatment. The treatments with no nutrient addition (Ctrl) are shown to the right of the vertical line.

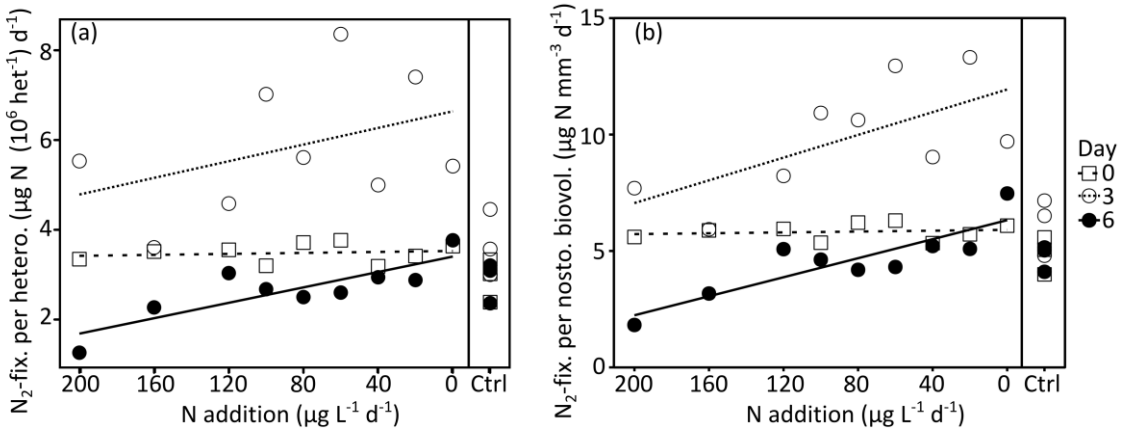

Figure 3.6: Correlation of the N_2-fixation per 10^6 heterocysts (a) and per Nostocales biovolume (b) with the daily N addition. Notice that the x-axis is reversed so that N input declines from left to right. The same amount of P was added to each treatment. The treatments with no nutrient addition (Ctrl) are shown to the right of the vertical line.

3.3.3 Compensation rate

Finally, to quantify the extent to which Nostocales cyanobacteria compensated for the varying N addition rate, we calculated a compensation rate (CR), defined here as the proportion of omitted N addition that would be replaced by N_2-fixation and was calculated as:

$$CR = BV_{Nost.} \cdot b_{Nfix} \cdot 100 \quad (3.3)$$

with b_{Nfix} being the slope of the relationship between N_2-fixation per Nostocales biovolume and N addition (mean of the slopes on day 3 and 6 in Fig. 3.6 b) and $BV_{Nost.}$ being the Nostocales biovolume. An assumption for this calculation was that the N_2-fixation per Nostocales biovolume varied with the N addition rate (Fig. 3.6 b), but the biovolume itself was unchanged (Fig 3.4 a).

The compensation rate is illustrated in Fig. 3.7a as a function of Nostocales biovolume. At the biovolumes observed in the microcosms, the compensation rate was 14 % on day 3 and 36 % on day 6 (Fig. 3.7 a and b). However, these biovolumes were much higher than those typically observed under natural summer conditions in LAN (Fig. 3.7 c), and much higher than they were in either the controls or at the start of the experiment. At the maximum Nostocales biovolume observed in LAN over the period 2009 – 2014, and with the increase in N_2-fixation per Nostocales biovolume observed here, a compensation rate of 23 % would be possible. However, at more typical Nostocales biovolumes of 0.8 - 3 mm^3 l^{-1} (the 25th and the 75th percentiles) the compensation rate would be only 2 - 7 % (Fig. 3.7 a and c).

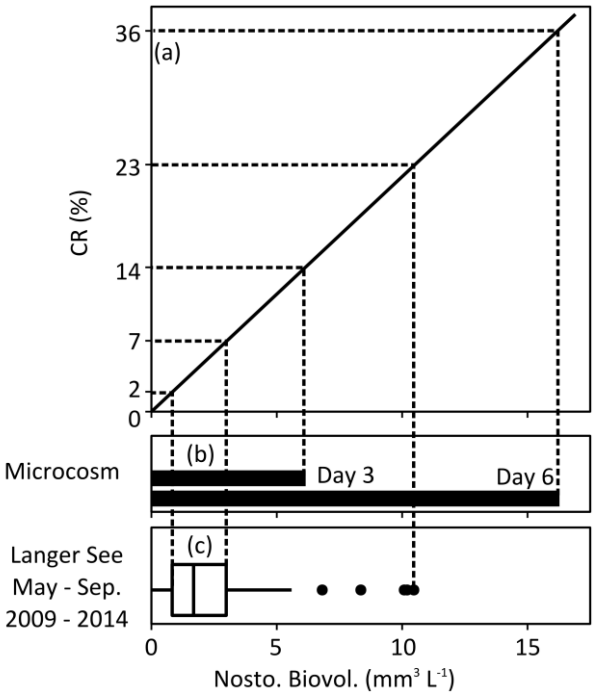

Figure 3.7: The nitrogen compensation rate (CR) as a function of Nostocales biovolume (a). CR was calculated according equation (5), using the slope of the relationship between N_2-fixation per Nostocales biovolume and daily N addition (mean of days 3 and 6 in Fig. 4 b). This assumes that only N_2-fixation per Nostocales biovolume changes with decreasing N addition, while the Nostocales biovolume itself remains constant. Mean Nostocales biovolume in the microcosms on day 3 and 6 (excluding the controls; b). Boxplot of the Nostocales biovolume observed in summer (May - September) from 2009 to 2014 in LAN (c).

3.4 Discussion

To compensate for reduced N loading, Nostocales biovolume, N_2-fixation rate per biovolume, or both, would need to increase. In our experiment, relative Nostocales biovolume increased with decreasing N addition, matching the pattern found in many field studies that show higher percentages of Nostocales at low N:P ratios (e.g. Smith 1983; Smith et al. 1995; Hellström 1996; Havens et al. 2003). However, this relative increase in Nostocales was driven exclusively by decreases in non-fixing cyanobacteria and eukaryotic algae, while absolute Nostocales biovolume

showed no response. Similar results have been found elsewhere (e.g. Paerl et al. 2014), and this highlights the importance of differentiating between relative and absolute biovolume, as only an increase in absolute biovolume is relevant for compensatory N_2-fixation.

In contrast to biovolume itself, N_2-fixation per Nostocales biovolume increased significantly in response to reduced N addition. This supports the hypothesis that Nostocales react to reduced N loading by enhancing N_2-fixation, which is in line with many previous experimental studies (Lean et al. 1978; Levine and Schindler 1992; Findlay et al. 1994; Piehler et al. 2002; Schindler et al. 2008; Vrede et al. 2009). Increased N_2-fixation was a consequence of both increased heterocyst abundance and increased N_2-fixation per heterocyst at lower N addition rates. Nitrogen stress is the main factor controlling initiation of heterocyst development (Ogawa and Carr 1969) and N_2-fixation is regulated by DIN with low DIN concentrations promoting; and high DIN concentrations inhibiting N_2-fixation (Horne and Goldman 1972; Ohmori and Hattori 1974; Vanderhoef et al. 1974).

In addition to the N addition effect, we also found evidence for P and light limitation of N_2-fixation, both of which have been observed in numerous experimental studies (Stewart and Alexander 1971; Liao 1977; Lewis and Levine 1984; Stal and Walsby 1998; Tõnno and Nõges 2003; Bradburn et al. 2012). Both the P concentrations and light intensities in our microcosms were higher than those typical during summer in LAN (Fig. 3.2), and in addition to explaining the higher biovolumes and N_2-fixation rates in the microcosms, they may also have improved the Nostocales ability to adapt to the N addition gradient. Nevertheless, the rates measured in our manipulated microcosms (1.8 to 13.3 µg N mm^{-3} d^{-1}) were of the same magnitude as those observed under natural conditions (0.06 to 18.4 µg N mm^{-3} d^{-1}) in the studies of Gu et al. (1997) and Horváth et al. (2012). We converted published per litre rates to rates per Nostocales biovolume using Nostocales biovolumes extracted from Fig. 4 (Gu et al. 1997) and from Fig. 2 and Table 2 (Horváth et al. 2012), assuming 12 hours of sufficient light for fixation per day.

The ability of increased N_2-fixation to cancel out N reduction measures depends on the proportion of the omitted N that would be compensated for. We defined that here as the compensation rate CR. In our microcosms the CR was up to 36 % (Fig. 3.7); however, this relatively high compensation rate was achieved by Nostocales biovolumes of up to 16 mm^3 l^{-1}, which, due to the high P additions, far exceeded the 0.8 to 3 mm^3 l^{-1} (25^{th} and the 75^{th} percentile) normally observed during summer in LAN. At these more typical biovolumes, the compensation rate would be just 2-7 % and therefore rather negligible. Furthermore, compensatory N_2-fixation will be limited to the summer months because significant Nostocales biovolumes occur only in summer in LAN (Fig. 3.3 e), as do substantial N_2-fixation rates in temperate lakes in general (Scott et al. 2008; Marcarelli and Wurtsbaugh 2009).

In contrast to our findings, other mesocosm studies (Levine and Schindler 1992; Levine and Schindler 1999; Vrede et al. 2009) and whole lake experiments (Schindler et al. 2008) have shown an increase not only in N_2-fixation but also in absolute Nostocales biovolume in response to a reduced N additions. One possible

explanation for these findings could be a difference in Nostocales species composition, as there are differences between Nostocales species in growth (Robarts and Zohary 1987; Mehnert et al. 2010) and N_2-fixation rates (Stal and Walsby 2000; Bradburn et al. 2012). Another explanation could be the high addition of P in our experiment: while under natural conditions a reduction in N may lessen the P limitation of Nostocales and consequently lead to an increase in biovolume, in our experiment the P limitation was possibly relieved in all treatments along the N gradient, leading to an increase in all treatments.

The larger size and longer time scales of other studies may also explain some of the differences. While large scale experiments better reproduce the real conditions and functions of lake ecosystems, small scale experiments like ours have the advantage of tight control over experimental conditions (Hecky and Kilham 1988) and their results often do scale to larger more natural systems (Spivak et al. 2011). However, both our and the study of Spivak et al (2011) only address short-term dynamics of phytoplankton populations, and because these are highly variable (Carpenter and Kitchell 1988), our study may yield contradictory results to whole ecosystem studies (Carpenter 1999). Several factors and processes in a natural lake may influence the absolute and relative supplies of N and P, such as N_2-fixation (Howarth et al. 1988), denitrification (Downing and McCauley 1992; Seitzinger et al. 2006), nutrient recycling in the sediments (Welch and Cooke 1995; Grüneberg et al. 2011; Holmroos et al. 2012), atmospheric deposition (Bergström et al. 2005; Elser et al. 2009), and land use in the catchment area (Downing and McCauley 1992; Vanni et al. 2011). Therefore, it is possible that the short-term limitations observed here may be overcome by larger-scale processes (Sterner et al. 2008).

Consequently, Schindler (2012) argues that even if N_2-fixation contributes only a small proportion of algal N requirements in a single year, over many years, fixation, together with recycling of sedimented N, would satisfy long-term algal demand. However, we think this is unlikely in shallow polymictic lakes like LAN, because of their rapid sedimentation-resuspension cycle (Scheffer 2004) and their hydraulic residence times much shorter than a year (Mischke and Nixdorf 2003). In shallow lakes, the majority of fixed N can be flushed out before it is recycled and therefore cannot meet phytoplankton demand in the next season. Scott and Grantz (2013) and Barica (1990) came to the same conclusion for other lakes that were unable to accumulate sufficient fixed N for internal loading to alleviate N limitation during the growing season.

3.5 Conclusion

In conclusion, our study suggests that Nostocales cyanobacteria would not render N reduction efforts in LAN ineffective because despite sustained in-lake N limiting conditions Nostocales biovolume is usually low and did not increase in response to reduced N loading. Furthermore, the observed increase in N_2-fixation per Nostocales biovolume would not be enough to reach high compensation rates. Therefore, in N limited shallow polymictic lakes like LAN, a reduction in N loading

could be ecologically meaningful and lead to a reduction in trophic status. However, further investigation is needed into the response of Nostocales to reduced N loadings over longer timescales.

4 Effect of light intensity on the response of nitrogen fixing cyanobacteria to varying nitrogen additions

4.1 Introduction

It is widely assumed, that due to their competitive advantage in low N conditions, the abundance and N_2-fixation rate of Nostocales would increase in response to reduced N loading, and thereby render efforts to improve water quality by N reduction ineffective (e.g. Schindler 2012). However, there is growing evidence against this hypothesis. In chapter 3 a reduction in N-additions admittedly led to increased N_2-fixation, but still the total phytoplankton biovolume declined and the Nostocales biovolume did not increase. These findings are in line with other studies suggesting that reduced N-loadings will promote no or only a small increase in Nostocales biovolume (Piehler et al. 2002; Paerl et al. 2014) and an increase in N_2-fixation often not big enough to fully offset a decrease in external N inputs (Scott and McCarthy 2010; Hellweger et al. 2016).

Nostocales N_2-fixation has a huge demand for energy, which is supplied in the form of ATP primarily generated in the heterocysts by the cyclic photophosphorylation via photosystem I (Cox and Fay 1969; Fay 1970; Kohl et al. 1982). Consecutive N_2-fixation is correlated to the light intensity and the shape of light-response curves of N_2-fixation is similar to that of photosynthesis (Lewis and Levine 1984). The quality of the light climate for phytoplankton in lakes is dependent on (mixing) depth and on absorption and scattering by particulate materials (e.g. phytoplankton and detritus) and humic substances (Kirk 2011). Further, the light climate is subject to daily and seasonal changes. Therefore, in order to estimate the potential of Nostocales to compensate for a reduction in N loading it is important to understand how light intensity controls the input of fixed N_2 into a lake.

When phytoplankton species compete for nutrients or light, competition theory predicts that under nutrient limited conditions the species with the lowest critical (minimum) nutrient requirements (Tilman 1982), and under light limited conditions the species with the lowest critical light requirements (Huisman and Weissing 1995), will succeed (Passarge et al. 2006). N_2-fixing cyanobacteria are not reliant on DIN as a source of N. Therefore, they have an advantage at N-limiting conditions, however, as N_2-fixation is energetically expensive they usually have higher light requirements compared to many non-fixing phytoplankton taxa (Agawin et al. 2007). *Aphanizomenon* for example could not achieve positive growth rates at low light intensities (Ward and Wetzel 1980). Accordingly, only at sufficient light intensities Nostocales would be able to increase significantly in biovolume in response to reduced N-loading. This hypothesis was approved in a multispecies culture study (de Tezanos Pinto and Litchman 2010), but from our knowledge there are no studies with natural phytoplankton communities estimating the interactive effects of varying light intensities and N-loading on the development of Nostocales and N_2-fixation.

The aim of this study was to determine the influence of light intensity on the response of Nostocales biovolume and N_2-fixation to varying N additions. To achieve this we carried out a microcosm experiment along a light gradient (0 - 200 µmol photons m^{-2} s^{-1}) in which two nutrient treatments with the same P-additions but different N-additions (70 and 0 µg L^{-1} d^{-1}) simulated a reduction in nitrogen loading. The biovolume of Nostocales and of the other phytoplankton (non-fixing cyanobacteria and eukaryotes) as well as the N_2-fixation were measured over seven days.

4.2 Material and methods

4.2.1 Microcosm experiment

For the microcosm experiments we used water from LAN (see chapter 2.2.1 and 3.2.1 for description of the study site). The experimental setup consisted of six treatments along a light gradient, where each was divided into two nutrient treatments (Fig 4.1). In both nutrient treatments we added a daily constant dose of P (7 µg L^{-1} d^{-1}) but only to one of them we added N (70 µg L^{-1} d^{-1}). The treatment with both nutrients (NP treatment) simulated N and P loading into a lake, while the treatment with only the P addition (P treatment) simulated a lake with reduced N loading. With the high P addition, we aimed to assure N limitation of the phytoplankton. P release from the sediment resulting in a high availability of P is a common process in many shallow polymictic lakes in summer (e.g. Köhler et al. 2005; Isles et al. 2015). However, a side effect of the nutrient additions is an increase in the trophic status.

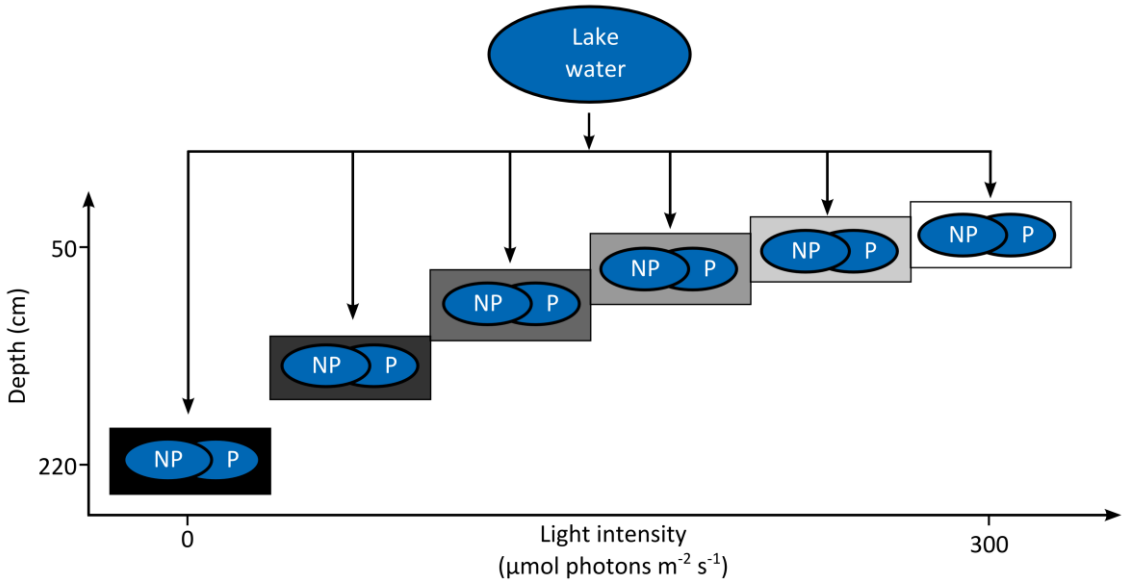

Figure 4.1: The experimental design consisted of two nutrient treatments incubated along a natural light gradient. Both nutrient treatments received a constant daily P dose of 7 µg L^{-1} d^{-1} while only the NP treatment received 70 µg N L^{-1} d^{-1} to simulate a reduction in N loading from the NP to the P treatment.

The experiment was carried out with a mixed sample of the water column from LAN taken on 06.07.2015. To lower the phytoplankton biovolume at the start the 62 L water sample was diluted with a 12.4 L subsample filtered through a 0.45 µm membrane filter. Larger zooplankton were removed from the sample by filtering the water through a 200 µm gauze and a sample was taken to determine the phytoplankton biovolume at the start of the experiment. Thereafter the sample was split into twelve aliquots of 5.5 L. All aliquots were filled into polyethylene (PE) bags and P was added in the form of KH_2PO_4 while N was added in the form of $(NH_4)_2SO_4$ and $NaNO_3$, in equal measure. Afterwards the PE bags were incubated in an experimental pond at 6 different depths (Table 4.1) to create a gradient of light intensity for a period of 7 days from 06.07. – 13.07.2015. Each afternoon nutrients were added and the water in the bags was aerated with a membrane pump until oxygen saturation was approximately 100 %, measured with an optode. Subsamples to measure N_2-fixation were taken after the nutrient addition on the starting day and after the aeration and nutrient addition on day 2, 4 and 6. Further samples to measure phytoplankton biovolume nutrient concentrations were taken before the aeration and nutrient addition. Every day between 0.5 and 0.9 L samples were taken out of the bags. Before the last sampling 1.5 L of water remained in each bag. The nutrient additions have been adjusted accordingly.

The N_2-fixation rate was determined as described in chapter 3.2.2, except that the Samples were analysed at the Stable Isotope Facility of the University of California Davis, USA using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

Table 4.1: Incubation depth and average water temperature.

	Day						
	0-1	1-2	2-3	3-4	4-5	5-6	6-7
Depth (m)	Temperature (°C)						
0.5	24.9	25.2	23.9	22.2	21.0	21.2	21.1
0.8	24.6	24.5	23.8	22.2	20.9	20.9	20.8
1.0	24.1	24.0	23.8	22.2	20.9	20.7	20.8
1.3	22.5	22.8	23.1	22.0	20.8	20.5	20.5
1.6	20.5	21.2	21.6	21.7	20.7	20.2	20.1
2.2	18.3	18.7	19.0	19.6	19.8	19.5	19.4

The average water temperature in the PE bags and PC bottles during the incubations can be found in Table 4.1. Temperature and the light intensity were measured with HOBO temperature/light data loggers (UA-002-64, Onset), attached to the frames that held the PE bags at the several depths. Because this type of light logger is most useful for determining relative changes, rather than absolute values of intensity, several depth profiles were measured during the experiment with a spherical light sensor (LI-193SA, LI-COR). Afterwards a linear model (R^2 = 0.94) was fitted to the parallel measurements to calibrate the HOBO light logger.

While the N_2-fixation rate is primarily affected by the current light intensity, the biovolume determined at a specific time point t is the result of the growth during the period before t and therefore also affected by the light intensity during that period. In the plots showing the N_2-fixation along the light gradient, the light intensity is therefore given as an average over the incubation time of the N_2-fixation measurement (I_{da}), while in the plots for the biovolume, it is given as the moving average between the beginning of the experiment and the specific sampling date. The moving average I_{ma} for the nth day of the experiment was calculated as:

$$I_{ma} = \frac{1}{n}\sum_{i=0}^{n-1} I_{da} - i \tag{4.1}$$

4.2.2 Statistics

A reparameterized model, which was originally developed by Jassby and Platt (1976) to describe the relationship between light and photosynthesis, was used to describe the relationship between light and N_2-fixation (F) and biovolume (bv), respectively:

$$F = F_{max} \cdot \tanh(\alpha \cdot I) \tag{4.2}$$

$$Bv = Bv_{max} \cdot \tanh(\alpha \cdot I) + b \tag{4.3}$$

where F_{max} and Bv_{max} are the maximum N_2-fixation rate and biovolume, respectively, reached at light saturation, α is the initial slope of the curve at zero light intensity, I is the light intensity and b is the intercept on the y axis.

Models were fit using RStan version 2.12.1 (Stan Development Team 2015) with 3 chains, each with 5000 iterations, of which 2500 were used for the burn-in phase and discarded. Chains were examined visually to check convergence, convergence was good for all models, with all Gelman-Rubin convergence statistics <= 1.02 (Gelman and Rubin 1992). Weakly informative priors were used for all fitted parameters.

The final 2500 iterations from each chain were pooled and inferences made from these 7500 posterior samples. The means and 0.025 and 0.975 quantiles of parameter draws were used as point estimates and 95% confidence intervals.

The analyses were performed using R vers. 3.3.2 (R Core Team 2016).

4.3 Results

4.3.1 Light intensity, nutrient concentrations and phytoplankton composition in LAN 2015

The mean photosynthetically active radiation in the mixed water column (I_{mix}) in LAN 2015 showed a typical seasonal pattern with the highest light intensities in summer, intermediate light intensities in spring and autumn and lowest light intensities in winter (Fig. 4.2). Two weeks before the start of the experiment I_{mix} reached a maximum of 144 µmol photons m^{-2} s^{-1} but decreased rapidly to 70 µmol photons m^{-2} s^{-1} by the start of the experiment due to a decrease in global radiation.

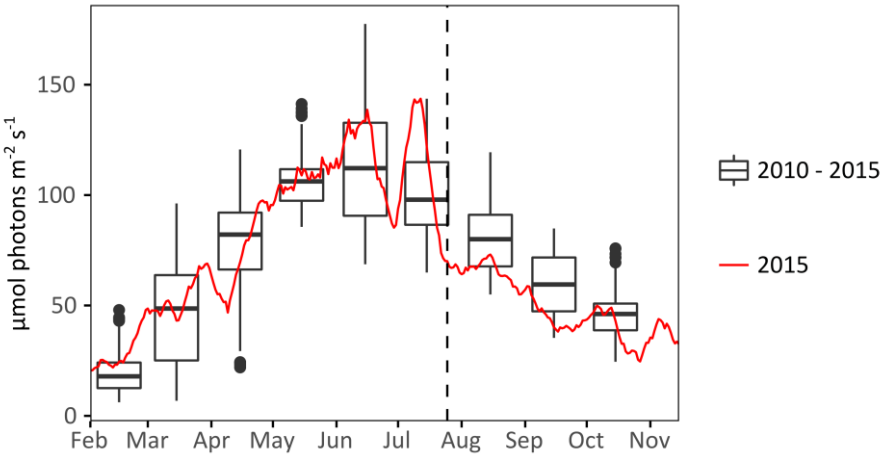

Figure 4.2: Seasonal pattern of I_{mix} measured in LAN 2015 (red line) and between 2010 and 2015 (boxplots), the dotted line mark the day when the microcosm experiment was started.

The DIN and SRP concentrations and the TN:TP and DIN:TP ratios in LAN in summer (May – September) 2015 were low (Fig. 4.3 a - d), indicating, like in summer 2012 (see chapter 3), that the phytoplankton was probably limited by nitrogen.

The mixed phytoplankton community in LAN in 2015 comprised eukaryotes and both non-fixing and Nostocales cyanobacteria (Fig 4.3 e). In spring eukaryotes formed the largest group while in late summer it was formed by non-fixing cyanobacteria. Nostocales species were almost absent until July, but between July and September they reached a relative biovolume of 9 - 58 % (2.8 – 10.2 mm^3 L^{-1}) of the total phytoplankton biovolume, which during that time ranged from 8.7 to 32.5 mm^3 L^{-1}. In the first week of July the biovolume of Noctocales cyanobacteria started to increase quickly and by the start of the microcosm experiment it accounted for 34 % (3 mm^3 L^{-1}) of the total phytoplankton biovolume (8.7 mm^3 L^{-1}). *Aphanizomenon spp* was the dominant Nostocales species and represented 82 % (2.4 mm^3 L^{-1}) of Nostocales biovolume, followed by *Dolichospermum spp* with 18 % (0.5 mm^3 L^{-1}) (Fig. 4.3 f). One week after the experiment was started Nostocales in LAN reached their highest relative biovolume in 2015 with 58 % (10.2 mm^3 L^{-1}).

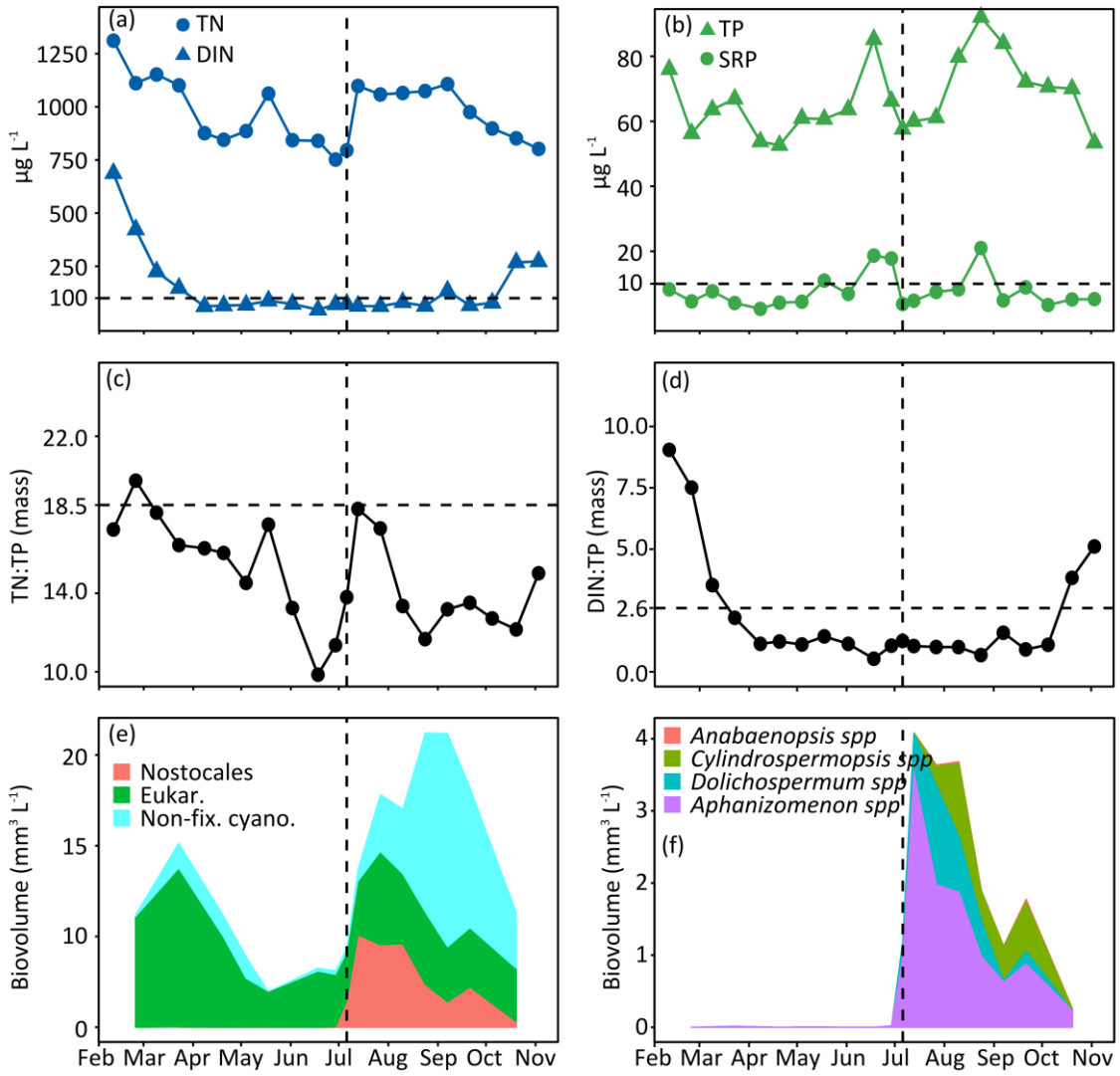

Figure 4.3: Seasonal pattern of nutrient concentration, N:P mass ratios and phytoplankton biovolume measured in LAN (2015). The vertical lines mark the day when the microcosm experiment was started. A and b) TN, DIN, TP and SRP; the horizontal lines mark the DIN and SRP concentrations below which N or P limitation are possible according to the findings in chapter 2 (c and d) TN:TP and DIN:TP ratios, the horizontal lines mark the N:P ratio at which phytoplankton is predicted to switch from being N to P limited according to the findings in chapter 2 (e and f) Phytoplankton biovolume estimated according to (Utermöhl 1958).

4.3.2 Microcosm experiment

The biovolume of Nostocales and the other phytoplankton taxa (non-fixing cyanobacteria and eukaryotic algae) along the light gradient are shown in Fig. 4.4. Generally, the biovolume of all taxa increased with increasing light intensity. In all light treatments, apart from the lowest light intensity, the biovolume of all taxa also increased over time. Although the biovolume of Nostocales was lower at the start than that of the other phytoplankton taxa, by day 3 both groups reached the same biovolume along the whole light gradient in both nutrient treatments.

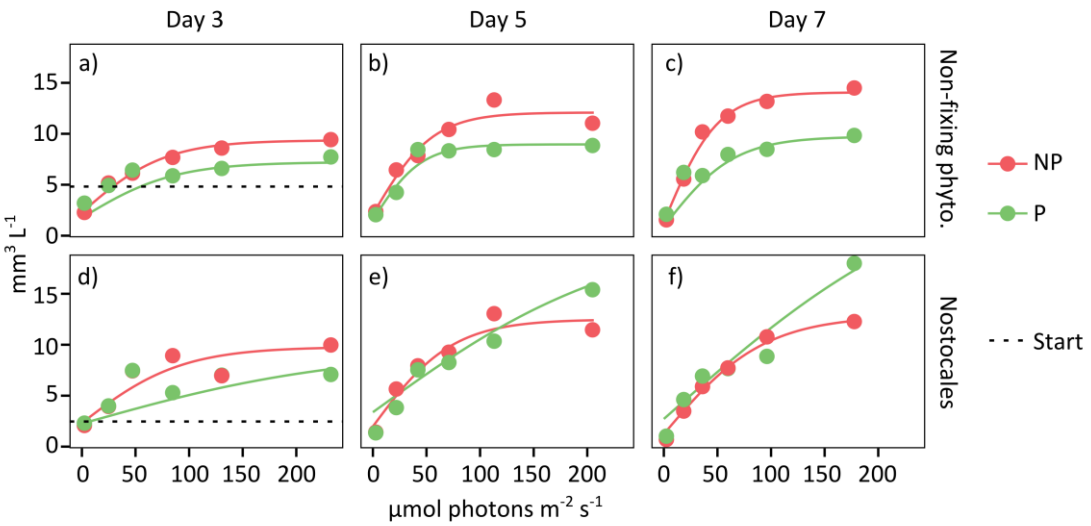

Figure 4.4: Biovolume of non-fixing phytoplankton (a-c) and Nostocales (d–f) along the light gradient

The model parameters of the fitted light-biovolume curves for the non-fixing phytoplankton are presented in Table 4.2 and for the Nostocales in table 4.3. The non-fixing phytoplankton reached a significantly higher maximum biovolume (bv_{max}) in the treatment with N and P addition compared to the treatment with no N addition, while the initial slope of the curve at zero light intensity (α) for both treatments was similar (Fig 4.4 a - c). The Nostocales showed an unusual pattern along the light gradient (Fig 4.4 d - f). At low and intermediate light intensities the biovolumes for both treatments are similar and show the typical shape with a steep initial increase at low light intensities, which starts flattening out at intermediate light intensities. However, while the Nostocales biovolume in the treatment with N and P addition reached a maximum at high light intensities, the biovolume in the treatment without N addition showed a second increase at day 5 and 7. This pattern could not be described by the model by Jassby and Platt (Equation 4.3). Instead the initial almost linear increase of the model was fit to the whole range of the light intensity gradient.

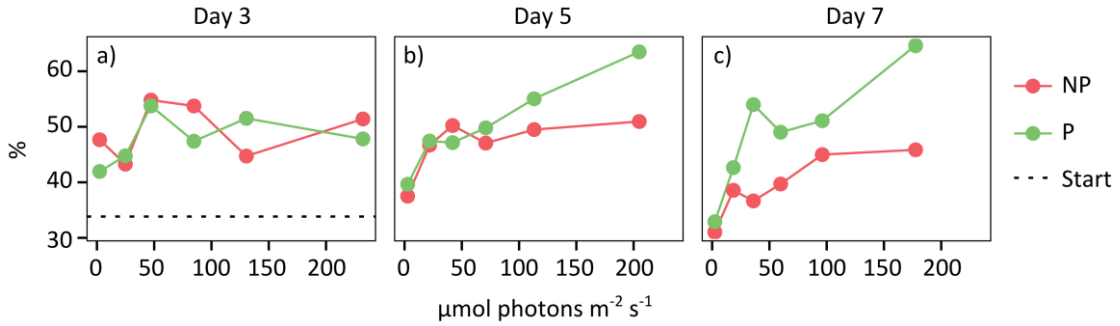

Figure 4.5: Nostocales cyanobacteria as a percentage of total phytoplankton biovolume along the light gradient

The relative biovolume of Nostocales increased with increasing light intensity and over time (Fig. 4.5). On day 3 there was no difference between the nutrient treatments but by days 5 and 7 Nostocales reached a higher relative biovolume in the treatment without N addition compared to the treatment with N and P addition.

Table 4.2: Parameters for the model fit of the light-biovolume curves of the non-fixing phytoplankton. α is the initial slope of the curve at zero light intensity and bv_{max} is the maximum biovolume reached at light saturation for the NP and P treatments and for the difference between the treatments. Bold text indicates where the difference of a parameter between the P and the NP treatment is statistically significant. The 95 % credible interval of the posterior distribution for the parameters is given in brackets. Bold text indicates where the difference of a parameter between the P and the NP treatment is statistically significant.

Day	Treatment	α	bv_{max}
3	NP	0.012 (0.005, 0.02)	9.362 (7.964, 11.715)
3	P	0.016 (0.003, 0.045)	7.215 (5.861, 9.532)
3	Diff.	0.003 (-0.011, 0.031)	-2.148 (-4.243, 0.259)
5	NP	0.016 (0.011, 0.022)	12.085 (10.843, 13.491)
5	P	0.02 (0.011, 0.032)	8.958 (7.865, 10.18)
5	Diff.	0.005 (-0.007, 0.018)	**-3.127 (-4.881, -1.414)**
7	NP	0.017 (0.012, 0.023)	14.063 (12.696, 15.55)
7	P	0.016 (0.005, 0.033)	9.724 (8.057, 12.352)
7	Diff.	-0.001 (-0.013, 0.016)	**-4.34 (-6.575, -1.55)**

To visualize the influence of light intensity on the response of the biovolume to a reduction in N-addition we plotted the difference of the biovolume between the P and the NP treatment (Fig. 4.6). The biovolume of the non-fixing phytoplankton decreased with decreasing N-addition along most of the light gradient and the response increased with increasing light intensity. The Nostocales biovolume predominantly showed no response or even a small decrease. However, the Nostocales biovolume increased in response to the reduced N-additions on days 5 and 7 at high light intensities.

The N_2-fixation per Nostocales generally increased with increasing light intensity but decreased over time (Fig 4.7 a - d). In the treatment without N addition α was significantly higher compared to the treatment with N and P addition (Table 4.4). The difference of maximum N_2-fixation rate (F_{max}) between the nutrient treatments was not statistically significant. However, F_{max} was always higher in the treatment without N addition and therefore it is possible that a higher nutrient addition may have a statistically significant effect on the F_{max} parameter.

The ratio of added P to total phytoplankton biovolume as a measure for the available P per biomass decreased over time and with increasing light intensity, while there was no difference between the two nutrient treatments (Fig. 4.7 e - h).

The influence of light intensity on the response of the N_2-fixation per Nostocales biovolume to a reduction in N addition is, like the response of the biovolume, shown as the difference between the P and the NP treatment (Fig. 4.7 i - l). At low light intensities the difference between the nutrient treatments increased with increasing light intensity, but at higher light intensities it decreased again.

Table 4.3: Parameters for the model fit of the light-biovolume curves for the Nostocales. α is the initial slope of the curve at zero light intensity and bv_{max} is the maximum biovolume reached at light saturation for the NP and P treatments and for the difference between the treatments. The 95 % credible interval of the posterior distribution for the parameters is given in brackets. Bold text indicates where the difference of a parameter between the P and the NP treatment is statistically significant.

Day	Treatment	α	bv_{max}
3	NP	0.011 (0.003, 0.024)	9.788 (7.555, 13.57)
3	P	0.008 (0.001, 0.032)	9.503 (5.733, 17.989)
3	Diff.	-0.004 (-0.019, 0.019)	-0.285 (-5.012, 7.928)
5	NP	0.012 (0.006, 0.021)	12.486 (10.426, 15.027)
5	P	0.004 (0.002, 0.008)	21.309 (13.597, 37.747)
5	Diff.	**-0.008 (-0.017, -0.001)**	**8.823 (0.804, 25.033)**
7	NP	0.01 (0.005, 0.016)	12.857 (10.217, 17.502)
7	P	0.004 (0.002, 0.007)	28.304 (16.337, 50.84)
7	Diff.	**-0.006 (-0.013, -0.001)**	**15.447 (3.247, 38.052)**

4.4 Discussion

The aim of this study was to determine the influence of light intensity on the response of Nostocales biovolume and N_2-fixation to varying N additions. Overall this study shows that increasing light intensity leads to an increase in Nostocales absolute and relative biovolume and N_2-fixation. This is consistent with the high energy demand of N_2-fixation (Howard and Rees 1994) and similar light response curves for N_2-fixation have been observed in other studies before (Lewis and Levine 1984). In contrast there are also studies suggesting that cyanobacteria in general are correlated with low light intensities (Smith 1986) and have lower light requirements than eukaryotic algae (Richardson et al. 1983). However, the cyanobacteria communities in these studies consisted mainly, or even only, of non-fixing cyanobacteria, whereas other studies observed that an increase of Nostocales biovolume preceded low light intensity rather than tracking it, suggesting that the Nostocales were the cause and not the consequence of rare light (Levine and Schindler 1999) and blooms of Nostocales were regularly replaced by non-fixing cyanobacteria when the light availability decreases in late summer (Wiedner et al. 2002). Additionally, Nostocales species *Aphanizomenon spp* could only reach positive growth rates when relying on N_2-fixation if light

intensities were high (Ward and Wetzel 1980). Consequently recent competition models assumed that N_2-fixing cyanobacteria usually have higher light requirements compared to many non-fixing phytoplankton taxa (e.g. Agawin et al. 2007).

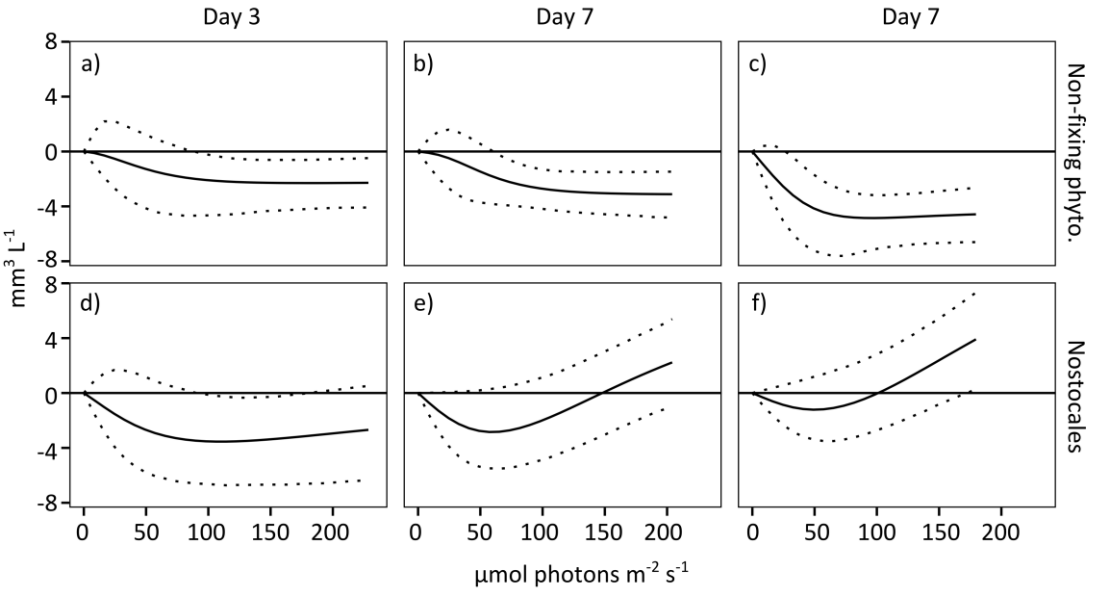

Figure 4.6: Difference of the biovolume of non-fixing phytoplankton (a-c) and Nostocales (d-f) between the P and the NP treatment along the light gradient. The dotted lines mark the 95 % credible interval of the posterior distribution.

Interestingly, the N_2-fixation rates per Nostocales biovolume decreased overall between the start and the end of our experiment. A possible explanation is the increasing phytoplankton biovolume during the course of the experiment, while the addition of P was kept constant. Therefore, later in the experiment there was less P available per biomass, which is reflected by a decreasing rate of added P per biovolume (Fig. 4.7 e - h). Consequently, the N_2-fixation may have been increasingly limited by P, as P is a frequently limiting factor for N_2-fixation studies (see Chapter 3 and Stewart and Alexander 1971; Liao 1977; Tõnno and Nõges 2003).

The main result of this study is that next to the influence of light intensity on the growth rates and N_2-fixation of Nostocales cyanobacteria, light intensity also influences the response of these two parameters to a reduction in N addition. The biovolume of Nostocales at low light intensities remained constant or even decreased in response to a reduction in N addition, while at high light intensities it increased. In contrast the biovolume of the non-fixing phytoplankton along the whole light gradient showed a decrease in biovolume in the treatment without N addition. Accordingly, Nostocales can make use of their advantage at N limiting conditions primarily at high light intensities, although they also show an advantage at low light intensities as they decrease less in response to reduced N additions compared to non-fixing phytoplankton species. This is in agreement with competition theory (Tilman 1982; Huisman and Weissing 1995; Passarge et al. 2006): Under N limiting conditions the species with the lowest critical N

requirements will succeed. Nostocales usually have a low critical N requirement, due to their ability to fix atmospheric N_2. As their ability to fix N_2 increases with light intensity (Fig 4.7 a - d), their critical N requirement decreases with increasing light intensity, which is reflected by the increasing positive response in N_2-fixation to a reduction in N addition (Fig 4.7 i - l). Similar observations have been made before in a culture study (de Tezanos Pinto and Litchman 2010), but our study showed this first for a natural phytoplankton community. In the equation 3.3 in chapter 3 for calculation of the compensation rate it was assumed that the Nostocales biovolume does not change with reduction of N addition. In the present chapter we showed that this is only true under low and intermediate light intensities. Accordingly, equation 3.3 cannot be valid for high light intensities.

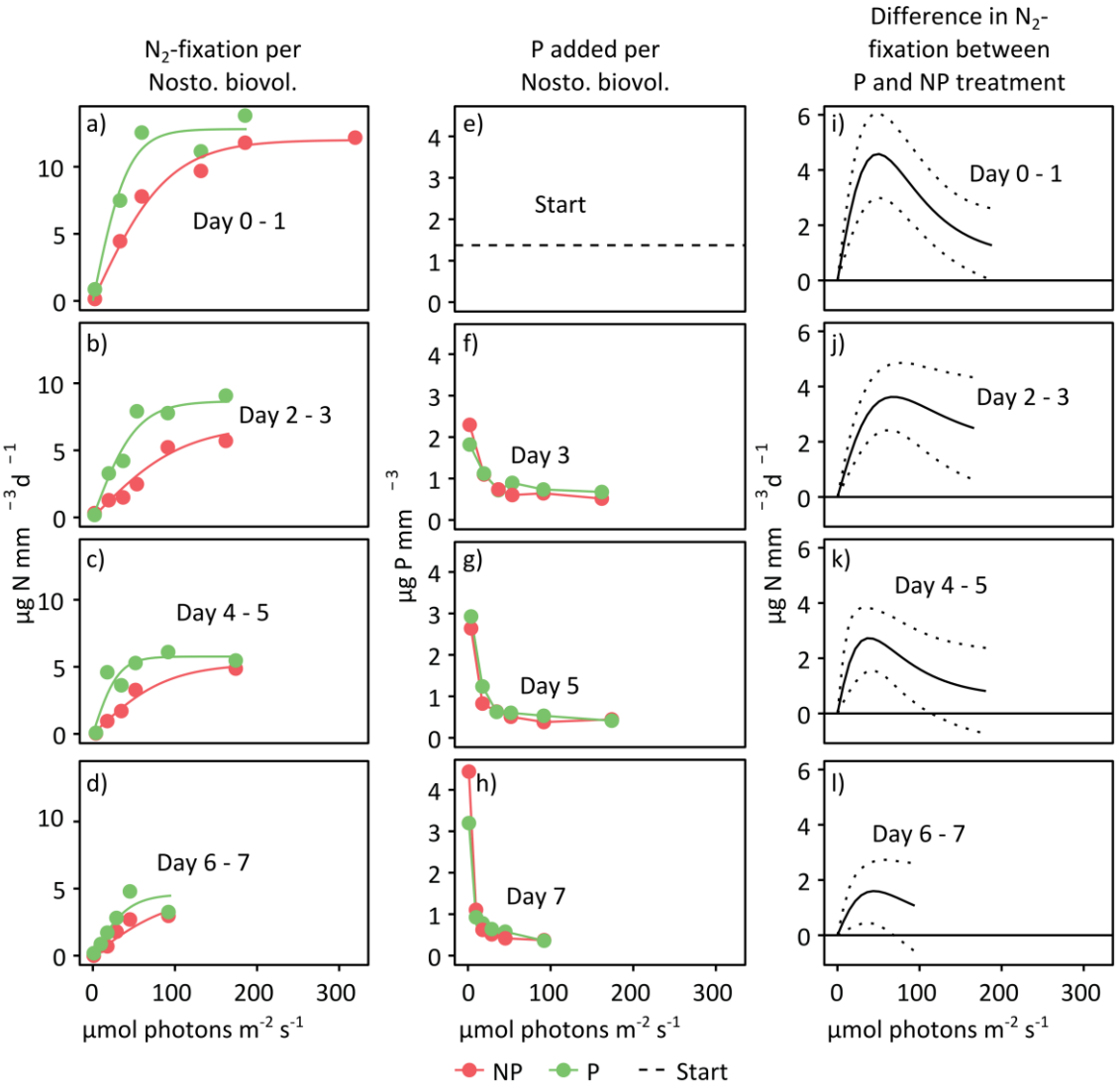

Figure 4.7: N_2-fixation per Nostocales biovolume (a-d), P added per phytoplankton biovolume (e-h) and the difference of N_2-fixation per Nostocales biovolume between the P and NP treatment (i-l) along the light gradient. The dotted lines mark the 95 % credible interval of the posterior distribution.

The influence of the light intensity on the response of N_2-fixation per Nostocales biovolume to a reduction in N-addition shows an interesting pattern in this study: The positive response initially increases and reaches the maximum at intermediate light intensities, but then decreases again with further increasing light intensity. This suggests that the biggest response of N_2-fixation to a reduction in N addition would occur at intermediate light intensities. As N_2-fixation has a huge energy demand, this observation is initially surprising and we would have expected the biggest response at high light intensities.

Table 4.4: Parameters for the model fit of the light-N_2-fixation per Nostocales biovolume curves. α is the initial slope of the curve at zero light intensity and F_{max} is the maximum rate reached at light saturation for the NP and P treatments and for the difference between the treatments. The 95 % credible interval of the posterior distribution for the parameters is given in brackets. Bold text indicates where the difference of a parameter between the P and the NP treatment is statistically significant.

Day	Treatment	α	F_{max}
0 - 1	NP	0.009 (0.007, 0.012)	12.00 (10.84, 13.23)
	P	0.020 (0.015, 0.026)	12.81 (11.79, 13.86)
	Diff.	**0.011 (0.005, 0.017)**	0.81 (-0.71, 2.30)
2 - 3	NP	0.008 (0.005, 0.011)	6.89 (5.13, 9.30)
	P	0.016 (0.011, 0.023)	8.66 (7.51, 10.18)
	Diff.	**0.008 (0.003, 0.015)**	1.78 (-0.35, 4.01)
4 - 5	NP	0.010 (0.006, 0.015)	5.23 (3.81, 6.97)
	P	0.031 (0.015, 0.062)	5.77 (4.78, 6.92)
	Diff.	**0.021 (0.006, 0.052)**	0.54 (-1.31, 2.21)
6 - 7	NP	0.010 (0.005, 0.017)	4.47 (2.58, 7.21)
	P	0.023 (0.009, 0.043)	4.58 (3.20, 6.79)
	Diff.	**0.013 (0.001, 0.033)**	0.11 (-2.34, 2.14)

However, this study also shows that the N addition has an influence on the parameters of the light response curve for N_2-fixation (Table 4.4) This is a possible explanation for the observed unexpected maximum response of N_2-fixation at intermediate light intensities, which will be shown in this paragraph: Let us approach this result visually, with a set of theoretical light response curves of N_2-fixation, where the parameters F_{max} and α vary in the treatment without N addition compared to the treatment with N addition (Fig. 4.8): When the parameters F_{max} and α are the same in both treatments there is no difference in N_2-fixation between the two treatments (top left plot). When a reduction in N addition causes only an increase in F_{max} while α remains constant, the difference in N_2-fixation between the treatments increases with light intensity until a maximum is reached (left column of plots). When a reduction in N addition causes only an increase in α while F_{max} remains constant, the difference in N_2-fixation between the treatments initially increases with light intensity but eventually decreases back to zero (top row of

plots). However, when a reduction in N addition causes both an increase in α and an increase in F_{max}, the difference in N_2-fixation between the treatments initially increases with light intensity but eventually decreases again until it remains constant at a value equal to the difference in F_{max} between the two treatments, like it was observed in our study. An increase in F_{max} and α in response to decreasing DIN availability was also observed in a field study (Bradburn et al. 2012) suggesting that this may be a general pattern.

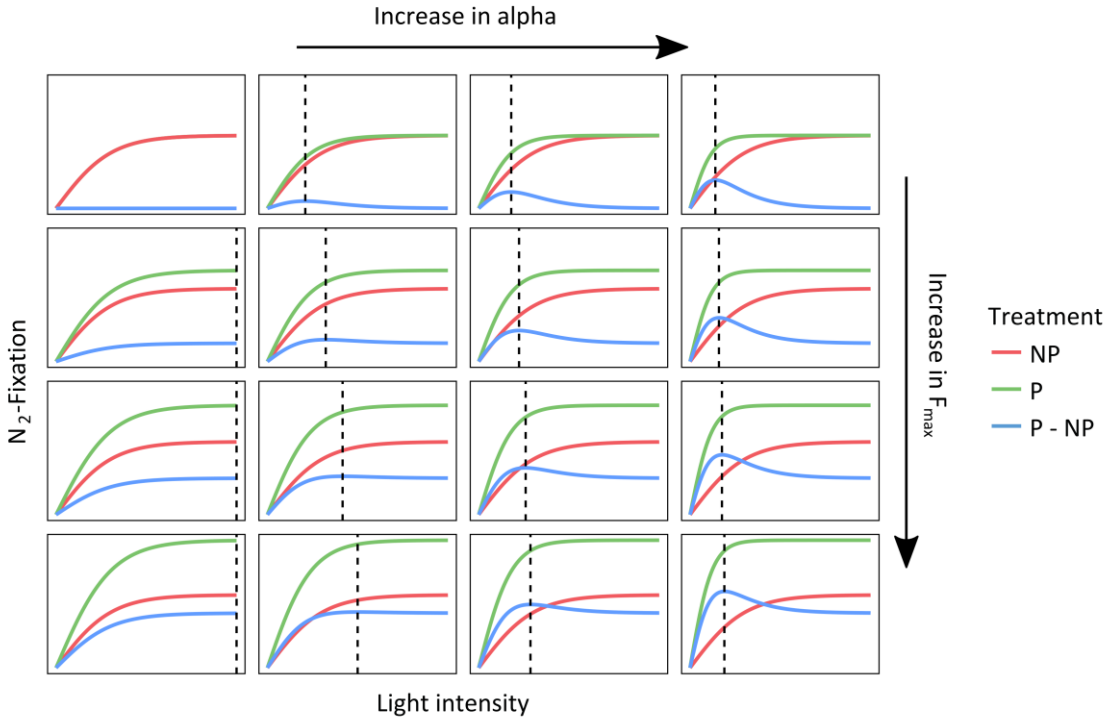

Figure 4.8: Theoretical light response curves of N_2-fixation and the difference in N_2-fixation between the treatments (response to the reduction in N addition; blue line) with varying parameters α and F_{max} in the P treatment. The parameters α and F_{max} for the NP treatment (red line) are the same in all plots, while they are varied for the P treatment (green line). In the top left plot the parameters for the P treatment are equal to the ones in the NP treatment. From left to right α in the P treatment increases and from top to bottom F_{max} in the P treatment increases. The dotted vertical lines indicate the maximum difference between the two treatments.

Although the biggest response in N_2-fixation to a reduction in N addition was observed at intermediate light intensities the biggest increase in Nostocales biovolume was observed at high light intensities (Fig. 4.6 and 4.7). Accordingly, next to the ability to fix N_2, Nostocales probably have another competitive advantage at high light intensities and low N availability. Further study is needed to investigate this.

The Nostocales cyanobacteria in our microcosm experiment performed better than non-fixing phytoplankton species, as their relative and absolute biovolume increased in both nutrient treatments at all and at all but the lowest light intensities, respectively. This could be due to the Nostocales community being healthier at the start of the experiment. In LAN the relative and absolute

Nostocales biovolume already started to increase steeply one week before the start and reached its 2015 maximum value one week after the start of the experiment. This could have affected the results of this study. However, another explanation could be that the Nostocales cyanobacteria benefited from the P additions in our experiment, which was also shown in chapter 3 and in other studies (Stewart and Alexander 1971; Liao 1977; Tõnno and Nõges 2003). The experiment in this study is addressing only short term processes under high trophic conditions. For a discussion of this see chapter 3.

4.5 Conclusion

In conclusion the results from this study suggest that light intensity is an important factor that needs to be considered when estimating the potential of Nostocales to compensate for a reduction in N-loading. Increasing light intensity may enhance the response of Nostocales biovolume and N_2-fixation to a reduction in N-loading. In cases where the reduction of N-loading into a lake leads to a short term reduction in phytoplankton biovolume, the resulting higher light intensity in the water may promote Nostocales and consequently the low compensation rates observed in chapter 3 may increase. However, further investigation is needed into the response of Nostocales to reduced N loadings under different light intensities over longer timescales and under natural trophic conditions.

5 Conclusions and outlook

5.1 Conclusions

To further conclude this thesis, the accomplishment of the aims set in the introduction will be commented upon:

Determination of the seasonal dynamic of N- and P-limitation for four lakes of differing lake types using nutrient enrichment bioassays. Testing the power of the four N:P ratios (TN:TP, DIN:TP, DIN:SRP and TN:SRP) to predict the limiting nutrient.

In order to effectively plan nutrient reduction measures it is important to know which nutrient is limiting in which lake and when. This study has shown that the occurrence of N- and P-limitation may vary between lake and seasons, and that we can predict it. While the deep stratified lake (SCH) in this study was predominantly limited by P, in the two shallow polymictic lakes (LAN and MUEG) the limiting nutrient switched seasonally from P-limitation in spring to N-limitation in summer, and the riverine lake (UH) was mainly not limited by nutrients but by light. These observations are supported by the seasonal dynamic of the nutrients and the light intensity in the four lakes. The predominance of P limitation in deep stratified lakes, the seasonal switch from P limitation in spring to N-limitation in summer/fall in shallow polymictic lakes and the predominance of other limiting factors apart from nutrients in riverine lakes seems to be a general pattern in temperate lakes as similar observations have been made in other studies (Søndergaard et al. 2005; Moss et al. 2012; Dolman et al. 2016).

Because the use of nutrient enrichment bioassays to identify the limiting nutrient is cost intensive it would be convenient to be able to predict their outcome by nutrient ratios and concentrations that are part of common monitoring routines. This study has shown that the results of nutrient enrichment bioassays can be predicted by the in-lake water chemistry. It was shown that N- and P-limitation are possible when the DIN and/or SRP concentrations in the water are below 100 and 10 µg L^{-1}, respectively. In these cases, the limiting nutrient can be predicted by in lake N:P ratios with the DIN:TP ratio (2.6) showing the best results.

Determination of the response of Nostocales cyanobacteria biovolume and N_2-fixation to varying N additions.

Many lakes have been shown to be limited by N indicating that a reduction in N loadings may lower the trophic status in these lakes. However, it is often assumed that Nostocales cyanobacteria will render these measures useless by fixing atmospheric N_2. This study has shown that in response to lowered N additions the N_2-fixation per Nostocales biovolume will increase. However, it has also shown that the absolute Nostocales biovolume will not increase. This study has presented a simple way to calculate the compensation rate under the assumption that Nostocales biovolume will not increase with decreasing N additions. As the

Nostocales biovolume in LAN is usually low, the increase of the N_2-fixation per Nostocales biovolume alone is not enough to fully compensate for a reduction in external N-loading. Consequently, such a reduction may be an appropriate measure to reduce the trophic status of LAN. However, these results were found in short term experiments under very high trophic conditions and need to be verified by experiments on a longer time scale at more natural trophic conditions with lower nutrient concentrations.

Determination of the effect of the light intensity on the response of Nostocales cyanobacteria biovolume and N_2-fixation to varying N additions.

Because Nostocales N_2-fixation has a huge energy demand the results observed in chapter 3 may be highly dependent on the light intensity. This study has shown that the biovolume of all phytoplankton taxa and the N_2-fixation rate will increase with light intensity. This study has also shown that the light intensity has an effect on the response of Nostocales biovolume and N_2-fixation to reduced N-additions. While at low and intermediate light intensities the reduction of N-addition has almost no effect on the Nostocales biovolume, at high light intensities a reduction of N addition leads to an increase of Nostocales biovolume. The increase in N_2-fixation in response to a reduction in N addition initially increases with increasing light intensity up to intermediate light intensities but decreases again at higher light intensities. Accordingly, this study has shown that it is important to consider light intensity when estimating the response of Nostocales to a reduction in N-loading.

5.2 Outlook

This study provided valuable information about the seasonal dynamic of N- and P-limitation in four lakes in the German lowlands. In agreement with the findings of other studies including small scale experiments, whole lake experiments and model approaches (Elser et al. 2007; Lewis et al. 2011; Dolman et al. 2016) it provided further evidence that N-limitation is common in many lakes.

Additionally, evidence was provided that Nostocales in LAN will not compensate for a reduction in external N-loadings. However, the results from one specific lake cannot always be transferred to other lakes, as the morphological conditions, the catchments and climate conditions influence the composition of the phytoplankton community. Therefore, similar experiments with water from other lakes would further improve our understanding of the effects of reduced N loadings on different lake systems/types with varying phytoplankton communities.

This study also showed as well as other studies (Stewart and Alexander 1971; Liao 1977; Tõnno and Nõges 2003) that P has a huge effect on Nostocales N_2-fixation. Studies with a gradient of the P addition would further improve our understanding of Nostocales potential to compensate for reduced N loading.

The small scale experiments used here cannot fully replicate the natural conditions and processes and a real (not just a simulated) reduction of N in these kinds of experiments is impossible. Therefore, there is the need for case studies, testing the effects of N reduction measures on the phytoplankton community in general and especially on the Nostocales biovolume and N_2-fixation at the whole ecosystem level and over longer time scales.

Finally, whole lake model approaches that include a detailed module for the N_2-fixation process, like in Hellweger et al. (2016) could further help answer the question whether in N limited lakes a reduction of N would be ecologically meaningful.

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Appendix

Raw data chapter 2

The raw data for chapter 2 can be found under:
journals.plos.org/plosone/article?id=10.1371/journal.pone.0096065#s5

Raw data chapter 3

Monitoring data Langer See 2012

Date	TN	DIN	TP	SRP	TN:TP	DIN:TP	Nosto.	Eukar	Non.fix.cyano	Anabaenop.	Cyli.	Ana.	Apha.
	(µg N L^{-1})	(µg N L^{-1})	(µg P L^{-1})	(µg P L^{-1})	(mass)	(mass)	(mm^3 L^{-1})	(mm^3 L^{-1})	(mm^3 L^{-1})	(mm^3 L^{-1})	(mm^3 L^{-1})	(mm^3 L^{-1})	(mm^3 L^{-1})
13.02.2012	1294.33	611.20	60.96	17.80	21.23	10.03	0.00	3.00	0.05	0.00	0.00	0.00	0.00
01.03.2012	1179.50	566.20	73.56	6.10	16.04	7.70	NA	NA	NA	NA	NA	NA	NA
12.03.2012	1161.00	428.40	70.24	4.10	16.53	6.10	0.00	7.68	0.29	0.00	0.00	0.00	0.00
27.03.2012	687.00	102.00	49.90	5.72	13.77	2.04	NA	NA	NA	NA	NA	NA	NA
10.04.2012	745.00	109.30	66.40	5.20	11.22	1.65	0.00	6.68	2.76	0.00	0.00	0.00	0.00
23.04.2012	824.50	88.80	57.70	3.90	14.29	1.54	0.13	6.63	4.47	0.00	0.00	0.00	0.13
07.05.2012	948.00	109.10	72.30	4.60	13.11	1.51	0.55	4.78	2.47	0.00	0.00	0.00	0.55
21.05.2012	902.00	106.90	64.40	3.90	14.01	1.66	2.18	2.92	4.57	0.00	0.00	0.00	2.18
04.06.2012	833.50	92.40	76.70	10.70	10.87	1.20	1.43	2.77	4.53	0.00	0.00	0.02	1.41
18.06.2012	807.00	93.70	75.80	9.60	10.65	1.24	2.98	4.97	4.01	0.00	0.01	0.00	2.97
02.07.2012	931.00	72.00	77.20	5.60	12.06	0.93	2.05	4.85	7.38	0.01	0.00	0.00	2.04
16.07.2012	966.00	68.80	62.39	4.50	15.48	1.10	1.65	5.67	6.51	0.14	0.00	0.17	1.33
30.07.2012	961.00	65.70	59.95	5.80	16.03	1.10	3.01	3.76	6.83	0.05	0.07	1.10	1.79
13.08.2012	1089.00	87.30	62.97	4.60	17.29	1.39	2.25	5.96	7.73	0.29	0.16	0.06	1.74
17.08.2012	NA	NA	NA	NA	NA	NA	2.91	6.56	13.36	0.14	0.27	0.43	2.08
27.08.2012	997.50	88.90	71.82	8.20	13.89	1.24	2.74	4.65	9.28	0.04	0.22	0.08	2.41
10.09.2012	1032.00	77.50	79.57	10.30	12.97	0.97	1.62	3.81	10.61	0.03	0.16	0.11	1.33
24.09.2012	1094.00	78.35	46.41	9.50	23.57	1.69	4.35	4.43	7.11	0.16	0.15	0.13	3.91
08.10.2012	1030.00	83.25	61.90	4.95	16.64	1.34	0.61	4.03	8.93	0.07	0.02	0.06	0.45
22.10.2012	1230.00	261.50	86.34	8.30	14.25	3.03	0.17	2.74	4.56	0.00	0.00	0.00	0.17
01.11.2012	901.00	289.10	77.50	11.30	11.63	3.73	0.17	4.46	1.57	0.00	0.00	0.00	0.17

Microcosm results biovolume

Day	Treatment	Replicate	Total ($mm^3 L^{-1}$)	rel_nosto (%)	hetero ($10^6 L^{-1}$)	nosto ($mm^3 L^{-1}$)	Non_fix ($mm^3 L^{-1}$)	Eukar ($mm^3 L^{-1}$)
Start	Ctrl	1	22.83	12.76	4.88	2.91	13.36	6.56
Start	Ctrl	2	22.83	12.76	4.88	2.91	13.36	6.56
Start	Ctrl	3	22.83	12.76	4.88	2.91	13.36	6.56
Start	0	1	22.83	12.76	4.88	2.91	13.36	6.56
Start	20	1	22.83	12.76	4.88	2.91	13.36	6.56
Start	40	1	22.83	12.76	4.88	2.91	13.36	6.56
Start	60	1	22.83	12.76	4.88	2.91	13.36	6.56
Start	80	1	22.83	12.76	4.88	2.91	13.36	6.56
Start	100	1	22.83	12.76	4.88	2.91	13.36	6.56
Start	120	1	22.83	12.76	4.88	2.91	13.36	6.56
Start	160	1	22.83	12.76	4.88	2.91	13.36	6.56
Start	200	1	22.83	12.76	4.88	2.91	13.36	6.56
3	Ctrl	1	NA	NA	6.59	4.10	NA	NA
3	Ctrl	2	NA	NA	9.21	5.76	NA	NA
3	Ctrl	3	NA	NA	8.17	4.48	NA	NA
3	0	1	NA	NA	10.42	5.82	NA	NA
3	20	1	NA	NA	11.81	6.57	NA	NA
3	40	1	NA	NA	12.35	6.83	NA	NA
3	60	1	NA	NA	8.21	5.30	NA	NA
3	80	1	NA	NA	10.21	5.39	NA	NA
3	100	1	NA	NA	9.63	6.19	NA	NA
3	120	1	NA	NA	11.09	6.18	NA	NA
3	160	1	NA	NA	12.09	7.35	NA	NA
3	200	1	NA	NA	7.79	5.60	NA	NA
5	Ctrl	1	25.01	22.17	8.92	5.55	14.93	4.54
5	Ctrl	2	27.36	19.86	8.84	5.43	16.35	5.58
5	Ctrl	3	33.21	20.08	11.59	6.67	21.06	5.47
5	0	1	29.63	44.54	26.17	13.20	10.54	5.89
5	20	1	38.76	47.47	32.52	18.40	11.49	8.87
5	40	1	51.71	38.03	34.84	19.67	22.68	9.37
5	60	1	47.90	40.71	32.34	19.50	19.72	8.68
5	80	1	46.72	30.64	24.00	14.32	25.37	7.03
5	100	1	50.89	32.01	28.17	16.29	26.55	8.05
5	120	1	44.98	31.40	23.64	14.12	24.99	5.86
5	160	1	48.16	32.09	21.60	15.45	20.84	11.87
5	200	1	62.92	24.39	22.17	15.35	35.34	12.23

Microcosm results N_2-fixation

Day	Treatment	Replicate	Nfix_per_nosto ($\mu g\ N\ mm^{-3}\ d^{-1}$)	Nfix_per_hetero ($\mu g\ N\ [10^6 het]^{-1}\ d^{-1}$)	Nfix_µg_d_l ($\mu g\ N\ L^{-1}\ d^{-1}$)
Start	200	1	5.60	3.35	16.31
Start	160	1	5.88	3.51	17.14
Start	120	1	5.95	3.55	17.32
Start	100	1	5.35	3.20	15.59
Start	80	1	6.22	3.71	18.11
Start	60	1	6.30	3.77	18.36
Start	40	1	5.34	3.19	15.54
Start	20	1	5.72	3.41	16.65
Start	0	1	6.09	3.64	17.73
Start	Ctrl	1	4.00	2.39	11.64
Start	Ctrl	2	5.06	3.02	14.72
Start	Ctrl	3	5.58	3.33	16.26
3	200	1	7.70	5.53	43.11
3	160	1	5.93	3.61	43.57
3	120	1	8.22	4.58	50.82
3	100	1	10.93	7.02	67.61
3	80	1	10.62	5.61	57.30
3	60	1	12.95	8.36	68.63
3	40	1	9.03	5.00	61.70
3	20	1	13.31	7.40	87.45
3	0	1	9.70	5.42	56.46
3	Ctrl	1	7.16	4.45	29.34
3	Ctrl	2	4.80	3.00	27.64
3	Ctrl	3	6.51	3.57	29.17
6	200	1	1.82	1.26	27.97
6	160	1	3.17	2.27	49.04
6	120	1	5.08	3.03	71.73
6	100	1	4.63	2.68	75.40
6	80	1	4.19	2.50	60.01
6	60	1	4.31	2.60	84.10
6	40	1	5.21	2.94	102.51
6	20	1	5.09	2.88	93.64
6	0	1	7.47	3.77	98.56
6	Ctrl	1	5.15	3.20	28.57
6	Ctrl	2	5.03	3.09	27.31
6	Ctrl	3	4.11	2.36	27.38

Raw data chapter 4

Monitoring data Langer See 2015

Date	TN ($\mu g\ N\ L^{-1}$)	DIN ($\mu g\ N\ L^{-1}$)	TP ($\mu g\ P\ L^{-1}$)	SRP ($\mu g\ P\ L^{-1}$)	TN:TP (mass)	DIN:TP (mass)	Nosto. ($mm^3\ L^{-1}$)	Eukar ($mm^3\ L^{-1}$)	Non.fix.cyano ($mm^3\ L^{-1}$)	Anabaenop. ($mm^3\ L^{-1}$)	Cyli. ($mm^3\ L^{-1}$)	Ana. ($mm^3\ L^{-1}$)	Apha. ($mm^3\ L^{-1}$)
10.02.2015	1311	687.3	75.99	8.3	17.25	9.04	NA	NA	NA	NA	NA	NA	NA
24.02.2015	1111	422.5	56.29	4.6	19.74	7.51	NA	12.16	0.26	0	0	0	0
09.03.2015	1152	224.7	63.61	7.7	18.11	3.53	NA	NA	NA	NA	NA	NA	NA
23.03.2015	1101	147.4	66.9	4.1	16.46	2.2	0.04	17.47	2.82	0	0	0	0.04
08.04.2015	876	61.4	53.76	2.3	16.3	1.14	NA	NA	NA	NA	NA	NA	NA
20.04.2015	845	65.1	52.63	4.2	16.06	1.24	0	9.9	2.41	0	0	0	0
04.05.2015	886	68	60.96	4.5	14.53	1.12	0.01	5.38	2.48	0	0	0	0.01
18.05.2015	1062	87.7	60.68	11	17.5	1.45	0.01	3.93	0.01	0	0	0	0.01
02.06.2015	843	72.6	63.62	6.9	13.25	1.14	NA	NA	NA	NA	NA	NA	NA
18.06.2015	840	45.6	85.21	18.7	9.86	0.54	0	6.16	0.38	0	0	0	0
29.06.2015	752	70.7	66.22	17.8	11.36	1.07	0.05	5.75	0.46	0	0	0	0.05
06.07.2015	795.5	72.6	57.62	3.8	13.81	1.26	2.96	5.29	0.5	0	0	0.52	2.43
13.07.2015	1098	63.1	60	4.8	18.3	1.05	10.21	5.84	1.41	0.02	0	1.34	8.86
27.07.2015	1058	61.9	61.13	7.5	17.31	1.01	9.09	10.25	6.34	0.04	0.67	3.43	4.94
10.08.2015	1065	80.5	79.75	8.3	13.35	1.01	9.21	7.67	7.2	0.07	2.52	1.93	4.69
24.08.2015	1074	62.9	92.03	21	11.67	0.68	4.77	7.92	19.76	0.04	1.02	1.23	2.47
07.09.2015	1107	133.8	83.94	4.9	13.19	1.59	2.81	6.01	23.58	0.01	1.19	0.05	1.56
21.09.2015	975	65.9	72.11	8.9	13.52	0.91	4.44	6.5	15.52	0.1	1.71	0.44	2.19
05.10.2015	898	77.85	70.59	3.5	12.72	1.1	NA	NA	NA	NA	NA	NA	NA
20.10.2015	852	268.3	70.04	5.2	12.16	3.83	0.64	5.81	6.23	0.01	0.05	0	0.58
03.11.2015	802	272.8	53.38	5.3	15.02	5.11	NA	NA	NA	NA	NA	NA	NA

Microcosm results biovolume

Day	depth (cm)	treatment	light_running_mean (µmol photons m^{-2} s^{-1})	Non.fix.phyto (mm^3 L^{-1})	Nosto. (mm^3 L^{-1})
Day 3	50	NP	232.21	9.43	9.97
Day 3	50	P	232.21	7.73	7.08
Day 3	80	NP	130.44	8.60	6.96
Day 3	80	P	130.44	6.60	7.01
Day 3	100	NP	84.68	7.68	8.93
Day 3	100	P	84.68	5.88	5.30
Day 3	130	NP	47.19	6.13	7.44
Day 3	130	P	47.19	6.43	7.47
Day 3	160	NP	24.82	5.17	3.94
Day 3	160	P	24.82	4.93	4.00
Day 3	220	NP	2.34	2.28	2.08
Day 3	220	P	2.34	3.19	2.31
Day 5	50	NP	204.89	11.03	11.46
Day 5	50	P	204.89	8.85	15.40
Day 5	80	NP	113.12	13.32	13.05
Day 5	80	P	113.12	8.45	10.35
Day 5	100	NP	70.89	10.42	9.27
Day 5	100	P	70.89	8.33	8.26
Day 5	130	NP	41.97	7.86	7.93
Day 5	130	P	41.97	8.45	7.54
Day 5	160	NP	21.87	6.45	5.65
Day 5	160	P	21.87	4.25	3.83
Day 5	220	NP	2.73	2.37	1.42
Day 5	220	P	2.73	2.06	1.35
Day 7	50	NP	177.64	14.49	12.27
Day 7	50	P	177.64	9.84	17.99
Day 7	80	NP	96.14	13.18	10.78
Day 7	80	P	96.14	8.47	8.85
Day 7	100	NP	59.81	11.74	7.72
Day 7	100	P	59.81	7.97	7.66
Day 7	130	NP	36.05	10.19	5.89
Day 7	130	P	36.05	5.90	6.92
Day 7	160	NP	18.56	5.56	3.49
Day 7	160	P	18.56	6.21	4.62
Day 7	220	NP	2.37	1.55	0.70
Day 7	220	P	2.37	2.10	1.03

Microcosm results N_2-fixation

Day	depth (cm)	Treatment	light_mean (µmol photons m^{-2} s^{-1})	nfix_biovol (µg N mm^{-3} d^{-1})
Day 0 - 1	50	NP	319.53	12.17
Day 0 - 1	80	NP	185.43	11.79
Day 0 - 1	80	P	185.43	13.82
Day 0 - 1	100	NP	131.63	9.69
Day 0 - 1	100	P	131.63	11.15
Day 0 - 1	130	NP	59.54	7.79
Day 0 - 1	130	P	59.54	12.55
Day 0 - 1	160	NP	33.07	4.45
Day 0 - 1	160	P	33.07	7.48
Day 0 - 1	220	NP	2.45	0.14
Day 0 - 1	220	P	2.45	0.86
Day 2 - 3	50	NP	161.95	5.70
Day 2 - 3	50	P	161.95	9.08
Day 2 - 3	80	NP	91.25	5.23
Day 2 - 3	80	P	91.25	7.78
Day 2 - 3	100	NP	53.59	2.48
Day 2 - 3	100	P	53.59	7.91
Day 2 - 3	130	NP	36.82	1.49
Day 2 - 3	130	P	36.82	4.21
Day 2 - 3	160	NP	19.37	1.28
Day 2 - 3	160	P	19.37	3.28
Day 2 - 3	220	NP	2.05	0.32
Day 2 - 3	220	P	2.05	0.18
Day 4 - 5	50	NP	174.02	4.87
Day 4 - 5	50	P	174.02	5.47
Day 4 - 5	80	P	91.69	6.10
Day 4 - 5	100	NP	51.93	3.27
Day 4 - 5	100	P	51.93	5.28
Day 4 - 5	130	NP	34.65	1.71
Day 4 - 5	130	P	34.65	3.62
Day 4 - 5	160	NP	17.54	0.96
Day 4 - 5	160	P	17.54	4.60
Day 4 - 5	220	NP	3.75	0.04
Day 4 - 5	220	P	3.75	0.08
Day 6 - 7	50	NP	92.12	2.97
Day 6 - 7	50	P	92.12	3.25
Day 6 - 7	80	NP	45.13	2.70
Day 6 - 7	80	P	45.13	4.79
Day 6 - 7	100	NP	28.77	1.79
Day 6 - 7	100	P	28.77	2.81
Day 6 - 7	130	NP	17.63	0.70
Day 6 - 7	130	P	17.63	1.72
Day 6 - 7	160	NP	9.71	0.72
Day 6 - 7	160	P	9.71	0.87
Day 6 - 7	220	NP	1.12	0.00
Day 6 - 7	220	P	1.12	0.18