CONSEQUENCES OF INCREASING NITROGEN:PHOSPHORUS RATIOS FOR PELAGIC LAKE FOOD WEBS

Dissertation

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Every action has consequences radiating in every direction. You cannot dam a small river in the highlands without having some effect on the river estuary perhaps hundreds of kilometers away. You cannot farm the land and expect a downstream lake to stay the same as it was before. You cannot change the climate and expect everything to go on as usual. The land and the freshwaters together have to be seen and discussed as a whole. Freshwater science is a microcosm of the whole of environmental science. There is something of everything in it!

Brian Moss 2010

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Summary

Lake ecosystems are faced with increasing depositions of nitrogen (N), deriving from anthropogenic activities such as the use of fertilizers and fossil fuels. The consequences are, however, still far from being understood. Since the great majority of pre-alpine lakes are limited in phosphorus (P), one would not immediately expect that an increasing N input would have serious consequences for these lake ecosystems. However, there is evidence that the consequences of rising N depositions, especially for the trophic systems of these lakes, are more far-reaching than would be initially assumed. To investigate the influence of increasing N loading in pre-alpine lakes, I conducted various mesocosm and microcosm experiments and large-scale monitoring of 11 pre-alpine lakes.

The mesocosm experiments were performed in three pre-alpine lakes in southern Germany. The natural phyto- and zooplankton communities and juvenile whitefish (Coregonus spec.) were exposed to different N concentrations or to gradients of dissolved inorganic nitrogen to total phosphorus (DIN:TP) ratios. These experiments were carried out in the spring, immediately after ice-break (March–May), for periods between 63 and 76 days.

The aim of the microcosm experiments was to estimate the toxic thresholds of ammonium or ammonia (NH_4^+ or NH_3) for the survival of a *Daphnia hyalina* complex. In these microcosm experiments, *Daphnia* were exposed to a NH_4^+ gradient with NH_4^+ concentrations between 0–87.8 mg L^{-1}. In contrast, the large-scale lake-monitoring included 11 pre-alpine lakes in southern Germany. These lakes were located between Lake Königssee in the east and Lake Constance in the west of southern Germany. Over a period of three years, the lakes were sampled twice a year for nutrient concentrations, and different parameters concerning phytoplankton-, zooplankton- and whitefish communities (including whitefish of all age classes).

I found that increasing N concentrations had significant effects on organisms from different trophic levels in the pelagic food-webs of pre-alpine lakes. Phytoplankton communities responded with measurable qualitative effects to increasing N inputs (changes in community composition, in biomass stoichiometry and in biochemical composition). In contrast, zooplankton communities and whitefish were both, qualitatively but also quantitatively affected. Observed effects on zooplankton levels were changes in community composition and decreasing *Daphnia* abundances. With increasing N, whitefish showed a decrease in growth and a lowered condition factor. These findings indicate a transfer of N-derived effects from the primary producers at the base of pelagic food-webs through all trophic levels up to top

consumers such as planktivorous whitefish. However, rising N concentrations had no toxic effects on *Daphnia* survival, neither in the mesocosm experiments nor in natural lakes.

For the survival of the *Daphnia* clone used in the microcosm experiments, I found a toxic threshold concentration of ammonium of NH_4^+ = 4.22 mg L^{-1}. This is more than two times higher than the artificially established ammonium concentrations in the mesocosms ($NH_4^+{}_{max}$ = 1.88 mg L^{-1}) and over 13 times higher than the natural ammonium concentrations found in the monitored lakes ($NH_4^+{}_{max}$ = 0.32 mg L^{-1}).

In conclusion, my results emphasize the importance of increasing N loading for organisms in pre-alpine lake ecosystems. This is especially true as the observed N-derived effects in pre-alpine lakes were clearly measurable, although the productivity of those lakes is known to be predominantly P-limited. Furthermore, the paradigm that P concentrations alone determine the functioning and transfer efficiency of lake food-webs comes into question. I suggest that for future lake-management programs not only the P concentrations but also N enrichment and consequent N:P ratios should be considered.

1. Introduction

Recently, the nitrogen (N) cycle on earth has started to change drastically. The reasons for this are anthropogenic activities that had already started at the time of the industrial revolution. Since then, processes such as the rising intensification of fertilizer use in agriculture and the continuously increasing combustion of fossil energy sources have slowly started to change earth's N cycle. However, some profound knowledge does not yet exist, let alone any reliable forecasts concerning the emerging consequences for ecosystems and organisms on our planet. Moreover, some leading scientists in this field have recently announced major concerns about thresholds for earth system processes that have already been crossed; beyond these thresholds irregularities may be irreversible, as seems to be true for the global N cycle (Rockström et al. 2009, Steffen et al. 2012).

1.1 The Global Nitrogen Cycle

The cycling of N on earth is one of the major planetary systems and delineates the circulation of N atoms between abiotic and biotic environments on earth. All N on earth circulates in a reversible biogeochemical pathway from the atmosphere into terrestrial, marine and freshwater ecosystems. Once in the ecosystems, biotic N compounds pass from organism to organism (Alberts et al. 2017) until they are transformed back into abiotic N, via microbial denitrification, and released to the atmosphere again (Fowler et al. 2013) (Fig. 1). About 50% of N on earth exists as atmospheric dinitrogen (N_2), where it constitutes 78% of the chemical composition of the earth's atmosphere (Holleman et al. 1985). This unreactive atmospheric N_2 exists only in its gaseous form as molecular N_2 at standard pressure and standard temperature. Dinitrogen has a very strong triple bond (N≡N), which is the second strongest chemical bond (after the C≡O bond in carbon monoxide) in diatomic molecules (Huheey and Cottrell 1958). It is the strong triple bond that makes the process of atmospheric N fixation very energy-consuming. Therefore, the great majority of organisms cannot metabolize N_2, only lighting events and a few organisms (i.e., leguminous plants and cyanobacteria) are able to break this bond and fix N in nature.

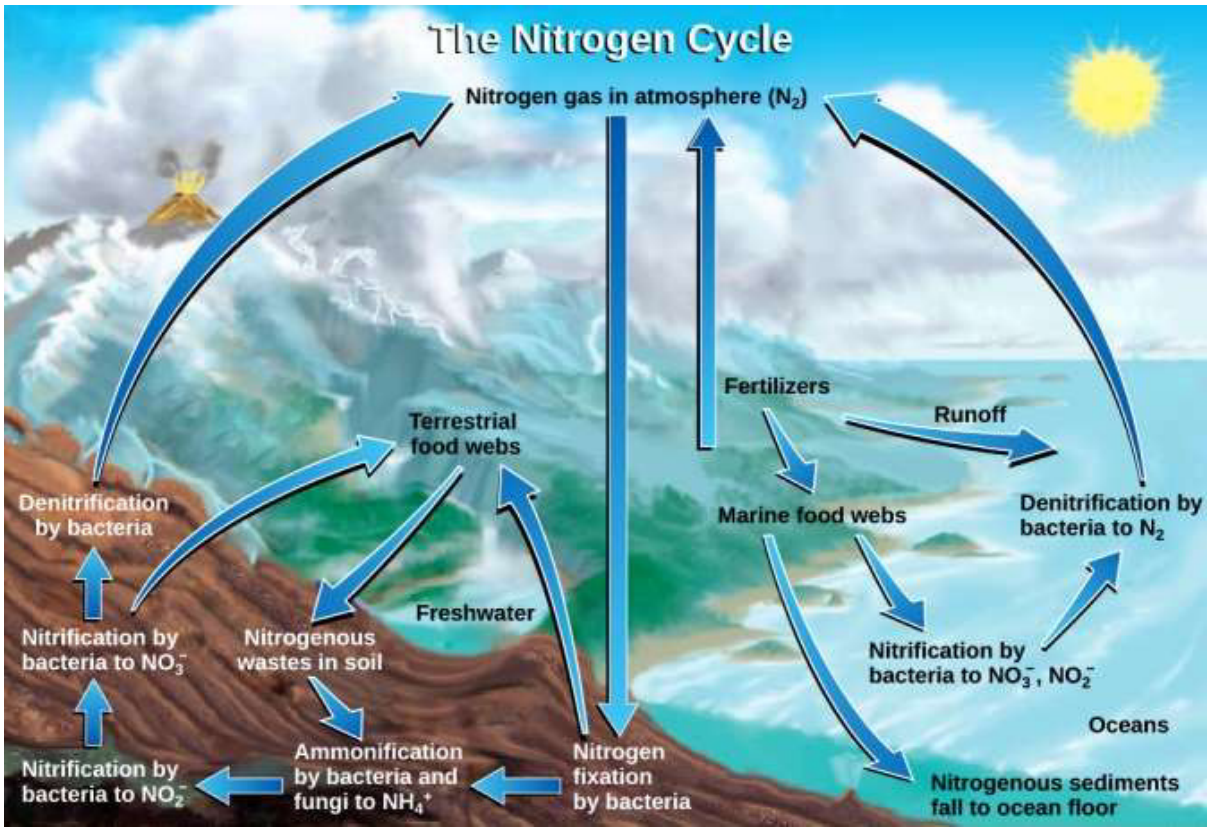

Fig. 1: Schematic illustration of the natural N cycle on earth. Blue arrows display the path of N compounds from the atmosphere through ecosystems, food webs and organisms and finally back to the atmosphere (Fowler et al. 2013).

Less than 1% of all N on earth occurs in a reactive or "fixed" form and is usable for organisms (BMUB 2017). Reactive N (Nr) comprises N compounds with oxygen (O), such as nitrogen oxides NO_x (i.e., nitrogen oxide NO, nitrite NO_2 or nitrate NO_3) and nitrous oxide (N_2O), and N forms with hydrogen (H) in the form of NH_x (i.e., ammonia NH_3, ammonium NH_4^+). These molecules are essential for all living organisms because they are needed for the biosynthesis of many organic molecules, such as proteins, DNA (deoxyribonuclein acid), RNA (ribonuclein acid) and others.

With the invention of the "Haber Bosch Process" at the beginning of the twentieth century, in which unreactive N_2 is converted into reactive NH_3, humans have also started to fix atmospheric N (Erisman et al. 2008). From that date, the anthropogenic fixation of N has increased from zero tons per year before 1910, to over 100 million tons per year in 2000. More than 85% of the anthropogenic Nr was used as fertilizer for food production (Galloway et al. 2003) to meet the rising food demand of the increasing world population (Zhang et al. 2015). Together with the human-induced biological N fixation caused by the cultivation of legumes and the combustion of fossil fuels, human Nr production has already greatly exceeded natural

production (Ciais et al. 2013) (Fig. 2). Total anthropogenic N fixation is predicted to rise a further 50% by 2050 (Sutton and Bleeker 2013).

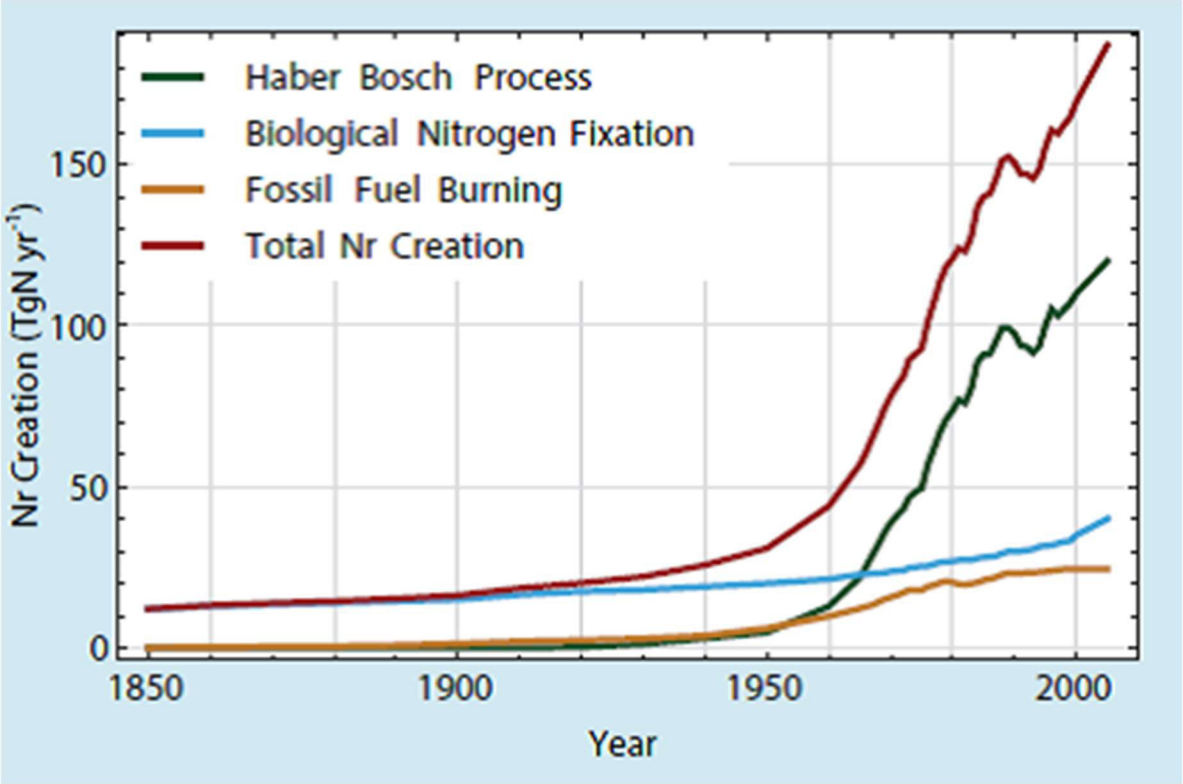

Fig. 2: Creation rates for the three main sources of anthropogenic reactive N (Nr) production (TgN yr^{-1}), shown from 1850–2005: the Haber Bosch process (green line), biological N fixation induced by the cultivation of crops such as legumes (blue line), burning of fossil fuels (orange line) and total anthropogenic Nr (red line) (Galloway et al. 2003, Galloway et al. 2008, Ciais et al. 2013).

However, a remarkable feature of human-fixed N is that it is directly released to the atmosphere, distributed around the world and deposited in extensive amounts into terrestrial and aquatic ecosystems (Schlesinger 2009). Liu and colleagues (2013) found that, for China, the patterns of atmospheric NO_x and NH_3 depositions over the last three decades were correlated with rising anthropogenic NO_x and NH_3 emissions. Hence, for ecosystems on earth, the direct consequence of increasing anthropogenic Nr production is rising atmospheric Nr deposition, even for remote areas (Fig. 3) (Galloway et al. 1995, Dentener et al. 2006).

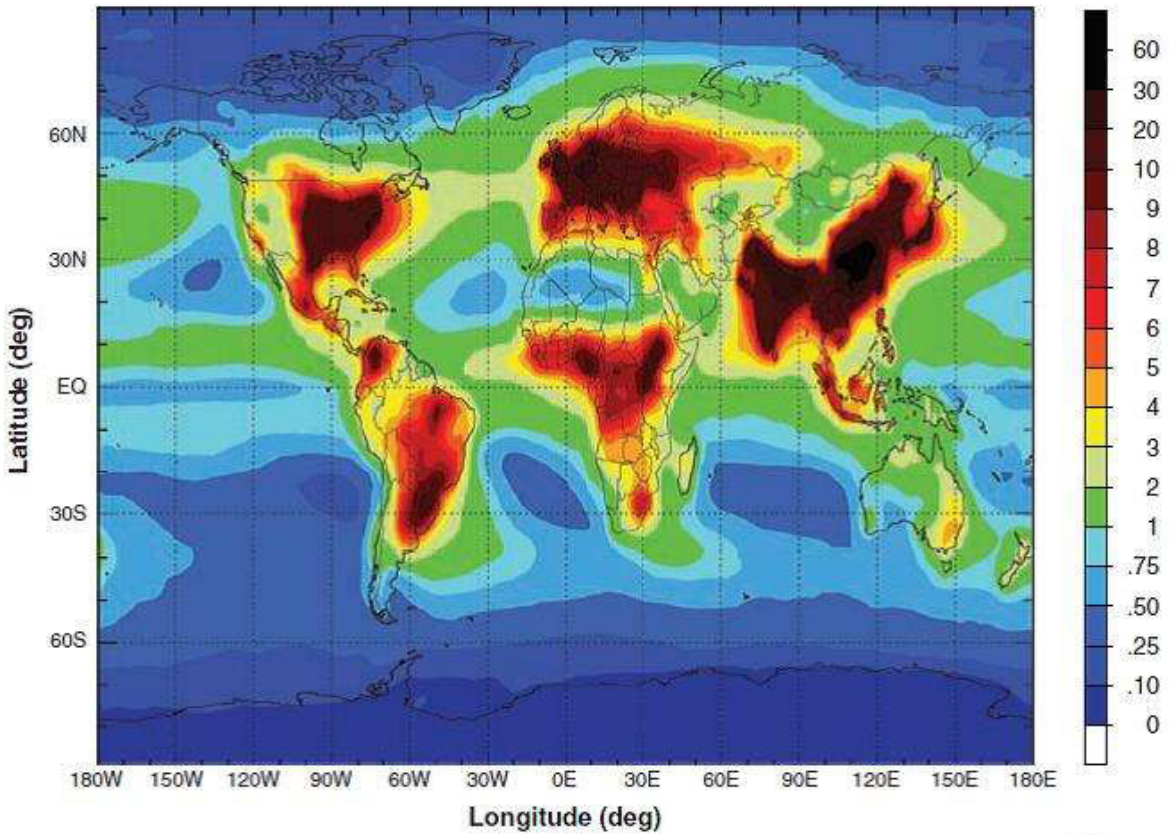

Fig. 3: Estimates of total global deposition of Nr (kg ha^{-1} yr^{-1}) into ecosystems for the year 2000 (Dentener et al. 2006, Galloway et al. 2008).

Atmospheric Nr distribution is the main N transportation process on earth (Galloway et al. 2008) and besides has significant impacts on the receiving ecosystems (Achermann and Bobbink 2003, Galloway et al. 2008). The orders of magnitudes of N depositions further highlight the importance of the process. The natural N deposition rate without any anthropogenic influence are ~0.5 kg N ha^{-1} yr^{-1} (Dentener et al. 2006); currently, a large part of the world's ecosystems experience average N loading rates of 10 kg N ha^{-1} yr^{-1} or more (Galloway et al. 2008). By the year 2050, these N loading rates are expected to double. Furthermore, for some regions, N depositions are expected to rise to an estimated rate of 50 kg N ha^{-1} yr^{-1} (Galloway et al. 2004). These future perspectives are consistent with the assumptions of other scientists and support the suggestion that we are now in the middle of a massive conversion of the worldwide N cycle (Steffen et al. 2015) (Fig. 4).

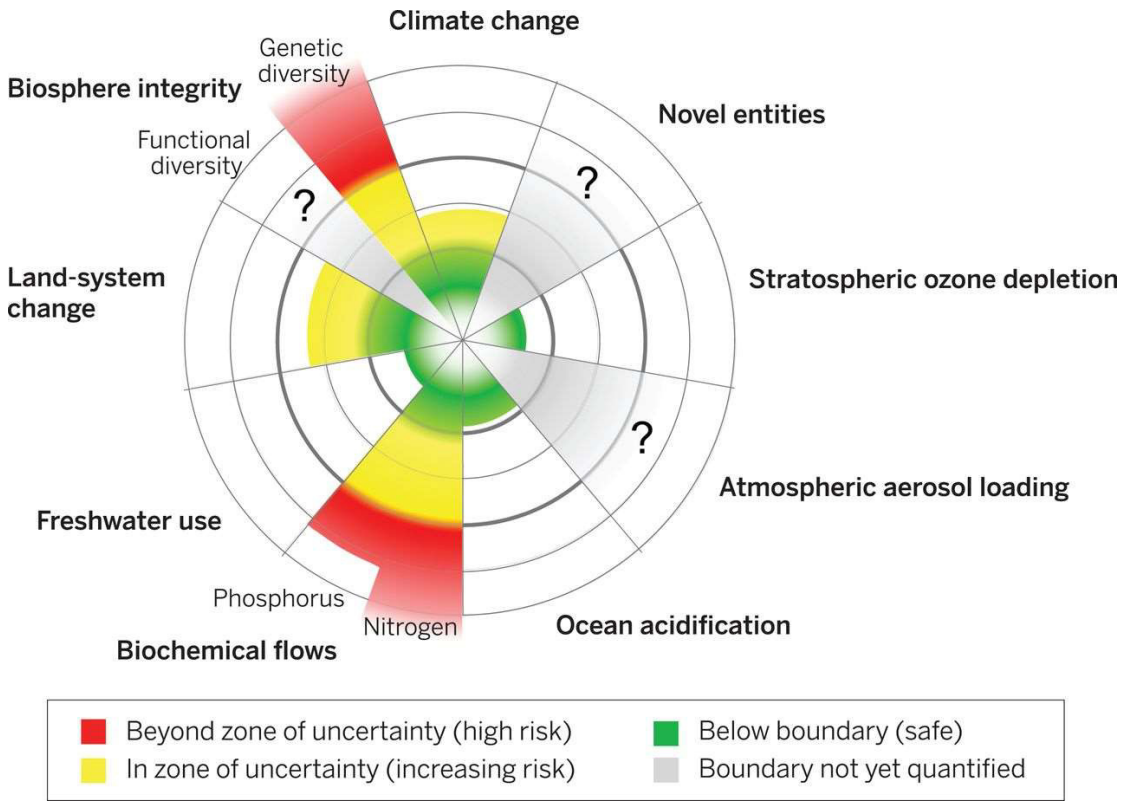

Fig. 4: Estimates of the current status of control variables for seven planetary boundaries (year 2015). Green area: safe operating space; yellow area: zone of uncertainty (increasing risk); red area: high-risk zone. The planetary boundary is located at the intersection of the green and yellow areas. Earth system processes for which global-level boundaries cannot yet be quantified are displayed by gray wedges and question marks; these are atmospheric aerosol loading, novel entities and the functional role of biosphere integrity (Steffen et al. 2015).

1.2 Nitrogen in Ecosystems

The availability of nutrients is an essential factor that determines the productivity of ecosystems. Nitrogen is one of the key nutrients in ecosystems and often the limiting factor for primary production. This is true especially for primary producers in terrestrial (LeBauer and Treseder 2008) and marine (Dugdale and Goering 1967, Glibert 1998) ecosystems. Hence, increased N loading in terrestrial and marine ecosystems increase their primary production (Smith 1998, Smith et al. 1999). Freshwater lakes have different nutritional background conditions compared to terrestrial and marine ecosystems: although N-limitation is also known in lakes (Elser et al. 2007, Lewis and Wurtsbaugh 2008), the great majority of lakes are P-limited (Schindler 1977) and N is available in excess. Therefore, one would at first not expect that additional N loading in lakes could have any consequences for primary production in lake ecosystems.

1.3 Freshwater Lake Ecosystems

Freshwater lakes provide delineated habitats for an incredible diversity of species, ranging in size from a virus a few nanometers in diameter to some fish species with a body length of two meters or more (Brönmark and Hansson 2005). Although two-thirds of the earth's surface is covered with water, almost all of it is saltwater, held by the oceans. Freshwater is scarce and most of the earth's freshwater is located in polar caps, soils and rocks (Moss 2010). Only less than on ten-thousandth of all water on earth can be found in rivers and lakes (Jackson et al. 2001), which makes these ecosystems precious assets and worth preserving. Apart from this, the importance of lakes first becomes fully apparent when we directly consider their measurable and tangible value for human daily life. Humans rely upon the ecosystem services provided by lakes (O´Reilly et al. 2003) and the species inhabiting them. Numerous people are dependent on the goods provided by freshwater lakes, and the demand for these goods will further increase during the coming century.

Lakes are highly complex systems, and myriad factors can influence the functioning of a lake system. The so-called "abiotic factors," the lake's specific physical and chemical characteristics, determine the suitability of a lake to provide a habitat for various groups of aquatic organisms. The most important physical factors in this context are light, temperature, the morphometric structure of the lake basin (depth, constitution of littoral zone and pelagic zone, thermal stratification, etc.) and the presence or absence of lake inlets and drains. The chemical composition of the lake water is also of great significance and must fit the respective requirements of inhabiting organisms. The concentrations of numerous chemicals must meet the individual tolerance ranges of all organisms living in the lake. In this regard, the determining key parameters are pH; the concentrations of mineral nutrients, such as N, phosphorus (P) and silicate (Si); the concentrations of dissolved gases, such as oxygen (O_2) and carbon dioxide (CO_2); and the concentrations of pollutants (Lampert and Sommer 2007). The combination of these physical and chemical factors builds a lake's specific, abiotic frame that determines the overall living conditions for all inhabiting organisms (Brönmark and Hansson 2005).

In lakes, the open water, or "pelagic zone," provides a habitat for two large organism groups: the plankton and the nekton. Planktonic organisms, such as microscopic algae (phytoplankton) and small animals (zooplankton), mainly drift passively through the water column. The nekton, in contrast, including the great majority of fish, is able to swim actively (Lampert and Sommer 2007). Lake organisms are strongly interconnected and "organized" in a complex network, a

so-called “food web”. All organisms within a lake’s food web hold their specific trophic position. According to this trophic position, they are generally classified into two groups: primary producers and consumers. The group of primary producers forms the trophic basis of an aquatic food web and includes photoautotroph and mixotroph organisms. The consumers represent the next trophic levels in food webs and can be categorized into various subclasses. The primary consumers, represented by herbivores such as zooplankton, feed directly on phytoplankton and also act as food prey for higher consumer levels. Insect larvae, jellyfish and planktivorous fish are the main taxa forming the group of secondary consumers. Depending on the biodiversity of an aquatic ecosystem, there can be variations in the number of consumer levels, but the highest trophic position in a lake food web is occupied by the top consumers, mainly piscivorous fish. All members of the different trophic groups are directly or indirectly interconnected with each other and with their abiotic environment. However, every single connection is highly susceptible to external stressors. Therefore, any disturbance can be a severe threat to the functioning of the food webs and for their internal trophic interactions.

Due to these highly complex structures, lake systems are extremely sensitive and often respond directly and rapidly to external interference, such as environmental changes. Some scientists have even proposed lakes as valid sentinels for climate change (Adrian et al. 2009). They may be good indicators to reveal incipient changes in external environmental factors. This is also true for gradual changes in nutrient regimes, which can often be unnoticed for a long time. This capacity further underlines the suitability of freshwater lakes to directly indicate the effects of increasing N input. Furthermore, it highlights the ability of lakes to provide reliable estimations for the future consequences of the ongoing, rising N loading into ecosystems.

1.3.1 Phytoplankton

Phytoplankton, as the major primary producer, represents the link between the nonliving world and living organisms. As it creates biomass using nothing more than elements, inorganic nutrients, biochemical molecules and energy provided by the sun, phytoplankton is of crucial importance for aquatic ecosystems and lake food webs (Sterner and Elser 2002). It is the production of energy-rich organic matter via photosynthesis that gives phytoplankton its extraordinary position. In photosynthesis, CO_2 and water (H_2O) are converted, using sunlight, into energy-rich glucose ($C_6H_{12}O_6$) and oxygen O_2 (Eq. 1):

$$6\ CO_2 + 6\ H_2O \rightarrow C_6H_{12}O_6 + 6\ O_2 \quad (1)$$

For the majority of organisms, glucose is an important energy supplier and essential to cellular respiration, which is the biochemical generation of energy in eukaryotic cells. Thus, autotrophic phytoplankton represents the origin for the transfer of energy and matter through all trophic levels.

Phytoplankton communities in lakes can be very diverse and include different taxa of prokaryotic and eukaryotic algae (Schwoerbel and Brendelberger 2005). Besides the great taxonomic diversity of phytoplankton communities, the different groups of algae also often show great variation in size, body shape, life-history pattern, mode of nutrition, chemical composition or biochemical composition. In aquatic food webs, these algal traits are first and foremost important for herbivorous zooplankton, as they determine the food quality of phytoplankton communities. This phytoplankton–zooplankton interface forms a critical threshold for the efficiency of energy transference in aquatic food webs (Hastings and Conrad 1979, Brett 1993, Sterner and Hessen 1994, Brett and Müller-Navarra 1997). The variability of the flow of energy, and also elements and biochemical compounds, across this interface is massive (Cahoon 1981, Ambler 1986, Müller-Navarra 1995, Brett and Müller-Navarra 1997).

1.3.2 Zooplankton

In pelagic food webs, zooplankton acts as phytoplankton grazer, nutrient recycler (Andersen and Hessen 1991) and food prey (Brooks 1968, Brooks and Dodson 1965) and thereby represents the link between primary producers and zooplanktivorous consumers. The zooplankton communities in freshwater lakes consist of different species that also vary in physiological properties and ecological functions. In terms of density, biomass, production, grazing and nutrient-recycling, copepods, rotifers and cladocerans are the most important taxa (Frey and Hutchinson 1967, Haney 1973, Hrbácek 1977, Porter 1977, Makarewicz and Likens 1979, Pace and Orcutt 1981). However, there is one group of cladocerans that is of outstanding importance for pelagic food webs because it occupies a central position within these systems: the water flea, *Daphnia* spec. (Lampert 2006). Besides their high biomass, it is primarily their crucial role in the trophic interactions between different food web levels that gives *Daphnia* the position of a keystone species (Gaedke and Straile 1998). Due to their high abundance and their extraordinary grazing efficiency, *Daphnia* can rapidly reduce the phytoplankton communities in lakes and thereby significantly decrease food for other herbivores, but also increase the recycling of nutrients (Polis et al. 1996, Lampert 2006, Moss 2010). *Daphnia* also play an important role as prey organisms for fish, since almost all fish feed on zooplankton, at least during their juvenile states (Welker et al. 1994, Lampert and Sommer 2007) (Fig. 5).

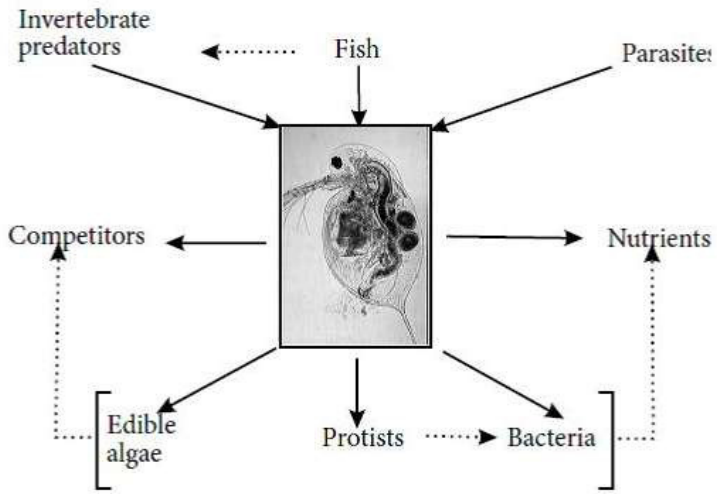

Fig. 5: *Daphnia* as keystone species. The central position of *Daphnia* in lake food webs and its interactions with numerous other components of aquatic food webs are shown (Lampert 2006).

1.3.3 Phytoplankton–Zooplankton Interface

Zooplankton growth and development is directly dependent on the transfer efficiency of matter and energy from phytoplankton. This makes zooplankton the first trophic level affected if the transfer fails. The great majority of zooplankton species are phytoplankton grazers, hence the food quality and quantity of phytoplankton are directly connected to zooplankton growth and reproduction (Hessen 1992). Whereas algal food quantity is more or less linearly correlated with phytoplankton biomass (Peters 1984), the food quality of algae is determined by multiple factors. The main factors are the taxonomical composition of the phytoplankton communities, the phytoplankton stoichiometry (the elemental composition of the phytoplankton biomass) and the presence or absence of essential fatty acids in the phytoplankton biomass.

In terms of the taxonomical composition of phytoplankton communities, it is mainly the edibility and resistance to digestion of individual algal taxa that determine the suitability of algae as food for zooplankton (Sterner and Schulz 1998). For example, large phytoplankton colonies are more difficult to capture for filter feeders such as *Daphnia* than small, single cells (Vanni and Lampert 1992). In addition, gelatinous algae are seen as poor-quality food because they often pass undigested through guts and are excreted relatively unharmed (Porter 1973, 1977, Vanni and Lampert 1992).

Increasing N input in lakes can alter the stoichiometric composition of phytoplankton towards higher N:P ratios in the algal biomass. In general, the N:P ratios in phytoplankton biomass are 16:1, which is also known as the *Redfield ratio* (Redfield 1958, Klausmeier et al. 2004). However, phytoplankton is very flexible in their biomass N:P ratios and can adjust N and P according to the concentrations of the surrounding water (Hessen 1990, Sterner and Elser 2002, Sommer et al. 2012). In contrast, zooplankton biomass stoichiometry is highly homeostatic, so

large deviations from the demanded N and P amounts in food can cause severe mismatches for zooplankton. Moreover, individual taxa have specific food requirements; *Daphnia,* for example, depend on P-rich food to ensure growth and reproduction, whereas most copepod species continue to grow and reproduce well even when supplied with P-deficient food (Sterner et al. 1992, Sterner und Elser 2002). Accordingly, high N:P ratios in lakes would favor high N:P zooplankton groups, such as copepods, and disfavor cladocerans, such as the *Daphnia* species.

Phytoplankton food quality for zooplankton is also determined by the amounts of fatty acids in the biomass (Müller-Navarra 1995, Müller-Navarra et al. 2000). As with the composition of nutrients in phytoplankton, the composition of essential fatty acids can also be a limiting factor for the growth and reproduction of zooplankton. This is because, apart from phytoplankton, no aquatic organisms are able to synthesize some essential fatty acids on their own. In particular, the concentrations and compositions of polyunsaturated fatty acids (PUFA) are the factors that determine the quality of phytoplankton as food for zooplankters. For zooplankton, the supply of the PUFAs alpha-linoleic acid, C18:3ω3 (ALA), eicosapentaenoic acid, C20:5ω3 (EPA) and linoleic acid, C18:2ω6 (LA) are important (Strandberg et al. 2015).

1.3.4 Fish

Natural fish communities in lakes include three different functional groups in terms of feeding: the omnivorous fish (feeding on invertebrates and plants), the planktivorous fish (feeding on phytoplankton or zooplankton or both) and the piscivorous fish (feeding on other fish) (Moss 2010). Independent of species or trophic group, the preferred habitats of fish often change during their life cycle. Fish larvae mainly use the littoral zone as habitat during the first months after hatching. The littoral offers a rich structure, due to the presence of macrophytes, and thereby provides shelter from predation pressure. As fish grow, most species start moving and use the benthic or pelagic zones as habitat for the rest of their lives (Lampert and Sommer 2007, Moss 2010). As with habitat preferences, the feeding behavior of fish species can also change with age. After a short phase directly after hatching when fish live off their yolk sack, they start using algae and small zooplankton (e.g., rotifers) as their diet. Once juvenile fish are no longer gape-limited, they start feeding on larger, pelagic zooplankton taxa, such as cladocerans (Lampert and Sommer 2007, Moss 2010). This is true for the majority of fish taxa in temperate lakes. Almost all juvenile fish go through a period of mainly feeding on zooplankton, regardless of their mode of nutrition as adults (Welker et al. 1994, Lampert and Sommer 2007). However, in pre-alpine lakes there is one important group of fishes that

remains zooplanktivorous for their entire life: the whitefish (*Coregonus* spec.) (Bergstrand 1982, Kahilainen et al. 2004, Lampert and Sommer 2007, Eckmann 2013). Whitefish usually inhabit the pelagic zones of deep alpine and pre-alpine lakes in regions with a temperate climate (Pothoven and Nalepa 2006). The great majority of whitefish taxa prefer the upper pelagic zone as habitat, where they feed on zooplankton, especially during their main feeding period from May until September (Mookerji et al. 1998, Skurdal et al. 1985, Becker and Eckmann 1992). Taxonomically, the group *Coregonus* is located within the family of Salmonidae and comprises about 78 species (Froese and Pauly 2017). However, the genetic classification of some whitefish species is unclear, and various hybrid forms may exist (Gum et al. 2014). Whitefish are a significant economic factor for fisheries located at alpine and pre-alpine lakes. For example, in Southern Bavaria, Germany, whitefish are traditionally called the "Brotfisch" by professional fishermen, reflecting the enormous economic importance of whitefish for the local fishermen and the accompanying gastronomic and touristic businesses of these regions (Mayr 2001, Gerdeaux 2004).

2. Central Research Questions

My intention in this PhD thesis is to study the consequences of rising N:P ratios for organisms of different trophic levels in pre-alpine lakes. Furthermore, I want to reveal the possible impacts of changing N:P regimes on their trophic interactions and the dynamics of lake food webs. By performing microcosm experiments in the laboratory, large-scale mesocosm experiments in different lakes and a three-year lake-monitoring exercise involving 11 ecologically and economically important pre-alpine lakes, I attempt to evaluate the effects of increasing N:P ratios on: 1. The phytoplankton community composition, phytoplankton biomass stoichiometry and phytoplankton biochemical composition; 2. The zooplankton community composition and zooplankton abundance; and 3. The growth as shown by the condition of planktivorous whitefish in those lakes (Fig. 6).

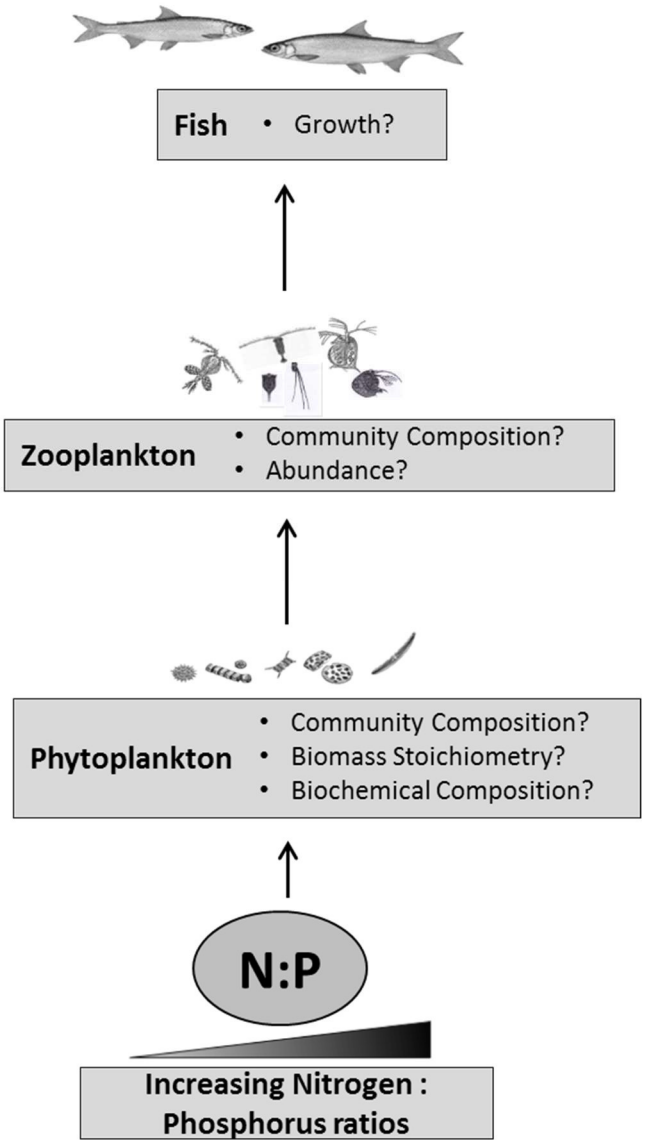

Fig. 6: Schematic, simplified illustration of pelagic food webs in pre-alpine lakes. Displayed are (from bottom to top) the most important nutrients (nitrogen and phosphorus) as the N:P ratio and the different food web levels from phytoplankton as primary producers, to herbivorous zooplankton as primary consumers, to planktivorous fish as secondary consumers. The terms in grey boxes show parameters which are expected to change with increasing N:P ratios in pre-alpine lakes. Arrows indicate possible paths for an effect to transfer from the base to the top consumers in a lake food web.

My central research questions for this thesis are as follows:

2.1 Do increasing N:P ratios influence phytoplankton

2.1.1 via changes in community composition? (Publication I)

2.1.2 via changes in biomass stoichiometry? (Publications I–III)

2.1.3 via changes in biochemical composition? (Publication IV, Manuscript I)

2.2 Do increasing N:P ratios influence zooplankton

2.2.1 via phytoplankton community composition? (Publication I)

2.2.2 via phytoplankton stoichiometry? (Publications II and III)

2.2.3 via phytoplankton biochemical composition? (Publication IV, Manuscript I)

2.2.4 via toxic effects? (Manuscript II)

2.3 Do increasing N:P ratios influence pelagic whitefish

2.3.1 growth patterns? (Publication I)

2.3.2 fitness, expressed by their condition factors? (Publications II and III, Manuscript I)

3. Publications and Manuscripts

PUBLICATION I

The effect of increased nitrogen load on phytoplankton in a phosphorus limited lake

Monika Poxleitner, Gabriele Trommer, Patrick Lorenz and Herwig Stibor

Freshwater Biology 61:1966-1980

2016

Freshwater Biology

Freshwater Biology (2016) doi:10.1111/fwb.12829

The effect of increased nitrogen load on phytoplankton in a phosphorus-limited lake

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SUMMARY

1. Widespread use of artificial fertilisers and the burning of fossil fuels and/or biomass release a large amount of reactive nitrogen into the atmosphere. So far, the effects of increasing nitrogen deposition from the atmosphere have mainly been studied in nitrogen-limited limnic and marine systems. Interestingly, in phosphorus-limited lakes, additional nitrogen input might not affect phytoplankton biomass, but rather increase mainly the degree of phosphorus limitation. The resulting effects on plankton communities are difficult to predict and quantify.
2. To estimate the effects of increasing nitrogen load on a spring phytoplankton community in a primarily phosphorus-limited system, a mesocosm experiment was performed in an oligotrophic lake, in which a gradient of six increasing nitrogen enrichment levels was applied.
3. During the initial phytoplankton growth phase (spring bloom), molar, seston nitrogen:phosphorus ratios increased from 43 to 72 and carbon:phosphorus ratios from 328 to 542 with increasing nitrogen enrichment, indicating increased phosphorus limitation. Three commonly used phytoplankton biomass proxies (phytoplankton biovolume, chlorophyll *a* and particulate organic carbon) showed only minor responses to nitrogen enrichment. Different groups and species of phytoplankton varied in their responses to the nitrogen enrichment in both the growth phase (spring bloom) and the descending phase (clear water phase).
4. Overall, we detected an effect of nitrogen enrichment on phytoplankton stoichiometry and community composition. The observed changes in the phytoplankton community combined with changes in abundances of heterotrophic nanoflagellates and ciliates indicate bottom-up driven alterations of the basal food web due to increased nitrogen loads.

Keywords: biomass, community composition, mixotrophy, nitrogen deposition, phytoplankton

Introduction

The increasing release of reactive nitrogen into the atmosphere is of global concern (Vitousek *et al*., 1997; Ciais *et al*., 2013). Industry, agriculture, as well as the burning of fossil fuels and biomass release a variety of nitrogen-containing gases (Galloway & Cowling, 2002). The contribution of anthropogenic atmospheric reactive nitrogen is four times greater than natural contribution from sources such as atmospheric losses from soil and creation through lightning (Ciais *et al*., 2013). Reactive nitrogen in the atmosphere can easily be distributed through precipitation and accumulates in this way in ecosystems (Lamarque *et al*., 2005; Elser *et al*., 2009a). This results in nutrient-related alteration (eutrophication) of even remote ecosystems that are not directly influenced by human activities (Elser *et al*., 2009b).

To date, the influence of increased nitrogen loads has mainly been studied in nitrogen-limited ecosystems (Bergström & Jansson, 2006; Duce *et al*., 2008). In such systems, the effects of increased nitrogen input can be severe. Studies showed, for example, that increased nitrogen deposition can be linked to an increase of phytoplankton biomass (Bergström & Jansson, 2006; Duce *et al*., 2008). However, increased nitrogen load can also lead to acidification (Dillon & Molot, 1990; Galloway, 2001), loss of biodiversity (Galloway *et al*., 2003; Bobbink *et al*., 2010) or higher susceptibility to stress factors

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(Pardo *et al.*, 2011) in communities. Therefore, increasing nitrogen supply can potentially also influence non-nitrogen-limited ecosystems, in which a biomass increase due to effects of higher nitrogen loads is primarily not expected.

Temperate lake ecosystems are often phosphorus-limited or nitrogen–phosphorus co-limited, rather than being solely nitrogen-limited (Elser *et al.*, 2007). Nonetheless, an increased nitrogen supply might affect phytoplankton dynamics of temperate lakes through the different nutrient demands (Quigg *et al.*, 2003) and usage by individual phytoplankton species (Domingues *et al.*, 2011). Additionally, nitrogen input also affects the relationship between different dissolved nutrients in aquatic ecosystems such as nitrogen:phosphorus (N:P) and nitrogen:silicon (N:Si) ratios (Elser *et al.*, 2009a). It is well established that such changes can influence phytoplankton species composition (Sommer, 1994; Roberts, Davidson & Gilpin, 2003). In both laboratory and mesocosm experiments, changes in species abundance of diatoms were shown along a Si:N gradient (Sommer, 1994), with higher diatom abundances and lower flagellate abundances resulting from increasing Si:N ratios (Sommer, 1994; Roberts *et al.*, 2003). Long-term dynamic shifts in nutrient ratios might even lead to a loss of species in phytoplankton (via competitive exclusion) due to a nutrient ratio-related decrease in competitive abilities. Such shifts in community composition might result in changes in stoichiometry, biochemical composition and edibility of phytoplankton, and thus change the quality of food available for secondary consumers.

However, phytoplankton species composition is only one parameter describing a phytoplankton community that might be affected by increasing nitrogen loads. Phytoplankton biovolume, chlorophyll *a* content (representing the amount of the main photosynthetic pigment) and carbon content (an indicator for the food quantity of higher trophic levels) are other important parameters characterising a phytoplankton community. All of these factors are commonly used as phytoplankton biomass proxies (Bergström & Jansson, 2006; Reynolds, 2006; Berger *et al.*, 2010). Each proxy represents a different aspect of the phytoplankton influencing pelagic food-web dynamics. The dynamics of phytoplankton biomass proxies have been shown in field studies to be both spatially and temporally variable (Felip & Catalan, 2000) and it has been shown in experimental studies that the biomass response to changes in nutrient limitation varied (Gilpin, Davidson & Roberts, 2004). The aim of this study was to investigate whether increased nitrogen input in multiple atmospheric amounts is able to influence phytoplankton community biomass, composition and stoichiometry in a phosphorus-deficient lake system.

We expected that nitrogen enrichment (within recent and future atmospheric wet deposition range) does not have a large effect on phytoplankton biomass in an otherwise phosphorus-deficient system, but rather on phytoplankton species composition and stoichiometry.

We performed a mesocosm experiment in an oligotrophic lake with a usual spring dissolved inorganic nitrogen (DIN):total phosphorus (TP) molar ratio of >1000:1 (H. Stibor, unpubl data from regular lake monitoring). A gradient of six nitrogen enrichments from 0 to 32 times the natural nitrogen wet deposition was applied, and the resulting effects on water chemistry, phytoplankton biomass, seston stoichiometry, amount of chlorophyll *a* and community composition were estimated.

Methods

Study site and experimental design

The mesocosm experiment was performed in the oligotrophic lake Brunnensee in Upper Bavaria, Germany (47°59′ N, 12°26′ E). The lake is located close to the Limnological Research Station of the LMU Munich, where all water and plankton analyses were conducted. Lake Brunnensee is a small (5.8 ha) kettle lake with a maximum depth of 19 m. It is fed by silica- and nitrate-rich ground water and has high dissolved nitrogen concentrations (~4 mg L^{-1}) and a low TP content (<10 μg L^{-1}) leading to a lake water DIN:TP stoichiometric ratio of >1000:1 after winter mixing.

In early spring 2013 (27.03.13–31.05.13), 12 enclosures (cylindrical bags made of white PE foil, with 150 μm thickness; dimensions: 4 m deep, 0.95 m diameter; Biofol Film GmbH, Unseburg) were filled with lake water by uplifting them from a depth of ~8 m to the water surface, thereby trapping a well-mixed sub-sample of the phytoplankton and zooplankton community of the lake. Enclosures were attached to anchored rafts in the lake and were open to the atmosphere. The experimental setup was covered with a transparent foil roof to avoid external wet deposition of nutrients, but still allowing gas exchange and penetration of the full light spectrum. This way, the experimental setup excludes input of nitrogen through wet deposition as well as inputs from the surrounding water due to the impermeable plastic foil.

For nitrogen fertilisation, calculations of natural input were based on 75 mg m^{-2} nitrate (NO_3^-) and

25 mg m^{-2} ammonium (NH_4^+) per week (data provided by the Bavarian Environment Agency) and a weekly precipitation of approximately 25 L m^{-2} (German Meteorological Survey). This resulted in a weekly nitrogen input of 60 mg NO_3^- and 20 mg NH_4^+ per enclosure surface as a simulated natural input. Treatments followed a replicated gradient design (Cottingham, Lennon & Brown, 2005). Six increasing nitrogen enrichment treatments (replicated twice) were established, including sodium nitrate and ammonium chloride as a nitrogen source. The treatments consisted of 0, 1, 2, 8, 16 and 32 times the natural regional nitrogen wet deposition. The 0 treatment therefore did not receive any nitrogen input except ambient atmospheric dry deposition. From a stock solution with a concentration of 30 g L^{-1} NO_3^- and 10 mg L^{-1} NH_4^+, 0, 1, 2, 8, 16 and 32 mL were added to distilled water to a final volume of 1 L. Nitrogen enrichment was performed twice a week over a period of 9 weeks. In order to ensure sufficient distribution of the nitrogen in the enclosures, they were mixed with a Secchi disc.

Sampling programme and measurements

Nutrients: Water samples were taken once a week (2 days after a nitrogen addition) using an integrated tubular water sampler with a volume of 2 L (KC DenmarkA/S Research Equipment, Silkeborg) from a depth of 1–3 m from each enclosure. The water was pre-filtered through a 250-μm gauze to exclude mesozooplankton. Nitrate and nitrite content was measured in an ion chromatograph system (Dionex ICS-1100 Basic Integrated IC System; Thermo Scientific, Germering) after 0.45 μm filtration (CS 400 Syringe Filters Cellulose Acetate 0.45 m; Nalgene, Rochester). Ammonium was measured fluorometrically (Trilogy Laboratory Fluorometer Module CDOM/NH_4; Turner Designs, San Jose) using a working reagent including orthophtalate, sodium sulphite and borate buffer (after Holmes *et al.*, 1999). TP was measured spectrophotometrically (Shimadzu UV-1700; Shimadzu Corporation, Duisburg) using the molybdenum blue method (Wetzel & Likens, 1991).

Phytoplankton and heterotrophic protists: Water samples were taken twice a week as described above. Chlorophyll *a* concentration, based on multispectral fluorescence analyses, was measured *in vivo* with an AlgaeLabAnalyser (bbe Moldaenke GmbH, Schwentinental). Samples were dark adapted (20 min) prior to measurement. Once a week, phytoplankton samples were fixed in Lugol's iodine and later counted using an inverted microscope, following established methods (Utermöhl, 1958). Sedimentation chambers were filled with 25–50 mL of the Lugol fixed sample and the composition and abundances of phytoplankton, ciliates and heterotrophic nanoflagellates (HNF) were counted. Phytoplankton and HNF biovolume was then calculated from abundance data by multiplying species cell counts by individual phytoplankton cell volume. For all species, besides diatoms, cell volumes were obtained from existing data of our own monitoring programmes and measurements of phytoplankton of the lake Brunnensee (shape estimates of Hillebrand *et al.*, 1999). For the highly variable diatoms, cell volumes were calculated for each sampling day using our own measurements based on geometric shape estimates of Hillebrand *et al.* (1999). Relevant dimensions of at least 100 individuals per diatom species were measured using analySIS software (Pro 2.11.006; Soft-Imaging Software GmbH, München).

Zooplankton: Once a week, zooplankton was sampled by net hauls (105 μm) through the 4-m water column of each enclosure. Zooplankton sampling started on day 22 as we did not want to disturb zooplankton dynamics at the initially very low densities. Zooplankton samples were fixed with 70% ethanol and counted at 25× magnification using a Wild M3Z stereomicroscope.

Seston stoichiometry: For weekly measurements of particulate organic carbon (POC), nitrogen (PN) and phosphate (PP) 150–400 mL water was filtered on glass fibre filters (GF/F). Filters were frozen at −20 °C until later analysis. POC and PN were measured in an elemental analyzer (vario MicroCube, Elementar, Hanau) and PP was measured spectrofluorometrically after molybdate reaction following sulphuric acid digestion (Wetzel & Likens, 1991). Seston stoichiometric ratios of C:N:P were calculated accordingly.

Statistical analysis

Statistical analyses were performed using Sigma Plot 11.0 (Systat Software, 2008, Erkrath). Linear (lr) and quadratic (qr) regression models were applied with log (base 10) transformed data for biovolume, nitrogen and POC data to meet statistical assumptions (Sokal & Rohlf, 1995). Averaged values are given as mean ± SE. To determine possible chronological shifts with increasing nitrogen enrichment, the peak day for each of the three biomass proxies, chlorophyll *a*, POC and biovolume were identified for each enclosure separately. For statistical analyses of the phytoplankton succession, the experiment was divided into two important phases: an initial growth phase (spring bloom) up to a peak and a descending phase after the peak resulting in low

chlorophyll *a* levels (clear water phase). This classification is based on chlorophyll *a* data, due to the higher temporal resolution of the chlorophyll *a* measurements and was applied for all total biomass proxies, biovolume on phytoplankton group and species level, and seston stoichiometry data. The following parameters in each phase were analysed separately by calculating averages per enclosure: POC, chlorophyll *a*, total biovolume, biovolume on phytoplankton group and species level and stoichiometry data (C, N, P). Finally, the following ratios were calculated: (i) chlorophyll *a*:algal biovolume, (ii) chlorophyll *a*:POC and (iii) algal biovolume:POC, to estimate possible nitrogen-dependent variations of the algal chlorophyll *a* content (i) and the contribution of algal pigments and algal biovolume to POC > 0.7 μm (ii, iii). For all ratio calculations (biomass proxies and stoichiometry), the ratios were first calculated for each time point and then averaged over time for each of the two phases. Additionally, initial exponential growth rates (based on biovolumes) were calculated from the start of the experiment up to the total biovolume peak of each enclosure. This was done for total biovolume and the biovolume of major phytoplankton groups. We refer to significance levels between 0.05 and 0.1 as trends.

Results

Chemical analyses

Dissolved NH_4^+ concentrations in the enclosures showed a steady increase over time following the continuous nitrogen enrichment in the nitrogen enrichment treatments (Figure S1). Dissolved NO_3^- concentrations fluctuated over time showing a slow increase and interim peaks on days 21 and 49 (Fig. 1). NO_3^- and NH_4^+ concentrations were 16.9 and 0.058 mg L^{-1} at the beginning of the experiment and rose up to 25.3 and 2.1 mg L^{-1} by the end of the experiment in the highest nitrogen treatment. Final concentrations of dissolved nitrogen compounds (NH_4^+ and NO_3^-) correlated significantly with nitrogen enrichment treatments (NH_4^+: $P < 0.01$, $R^2 = 0.98$; NO_3^-: $P < 0.01$, $R^2 = 0.89$). Dissolved nitrite was rarely detectable and concentrations were on average below 0.04 mg L^{-1} throughout the experiment. TP declined from 6.7 ± 0.18 to 3.0 ± 0.20 μg L^{-1} over time and was unaffected by nitrogen enrichment.

Seston stoichiometry. PP declined from 3.6 ± 0.08 μg L^{-1} at the beginning of the experiment to 2.8 ± 0.13 μg L^{-1} at the end, following the general TP trend (data not shown). At the beginning of the experiment, POC concentrations were on average 324.92 ± 12.18 μg L^{-1} (Fig. 1a). An increase of POC was observed for all enclosures followed by a decline (starting from day 35) to an average of 287.29 ± 14.85 μg L^{-1} at the end of the experiment (Fig. 1a). PN showed large fluctuations with a general declining trend; starting on average with values around 60.92 ± 1.65 μg L^{-1} and end concentrations of 37.92 ± 1.94 μg L^{-1}. Seston stoichiometric ratios were in the range of 477.36 ± 1.98 and 63.5 ± 0.24 for C:P and N:P, indicative of typical phosphorus-limitation signatures. Seston C:N ratios increased from on average 6.21 ± 0.33 to 8.87 ± 0.52 at the end of the experiment.

Statistical analyses of seston stoichiometry data revealed a significant effect of nitrogen enrichment for seston C:P ratios (Fig. 2b; lr: $P = 0.01$, $R^2 = 0.47$) and N:P ratios (Fig. 2c; lr: $P < 0.01$, $R^2 = 0.57$) for the growth phase. However, no trend was observed for C:N ratios during this phase (Fig. 2a; lr: $P = 0.28$, $R^2 = 0.12$). On the

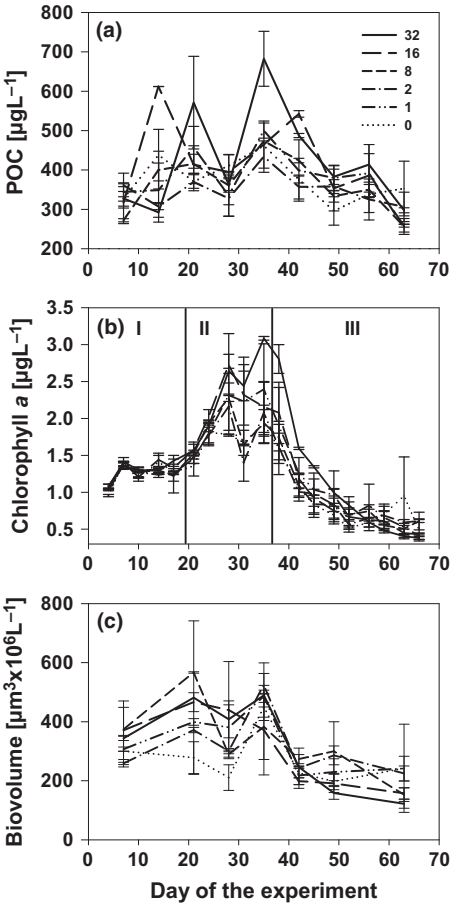


Fig. 1 Time course of the three phytoplankton biomass proxies: (a) particulate organic carbon (μg L^{-1}), (b) *In vivo* chlorophyll *a* (μg L^{-1}) [lines divide the time course in: (I) initialising, (II) growth phase and (III) descending phase], (c) Biovolume (μm^3 × 10^6 L^{-1}). Lines plot mean values from the two respective enclosures per treatment and whiskers show standard errors.

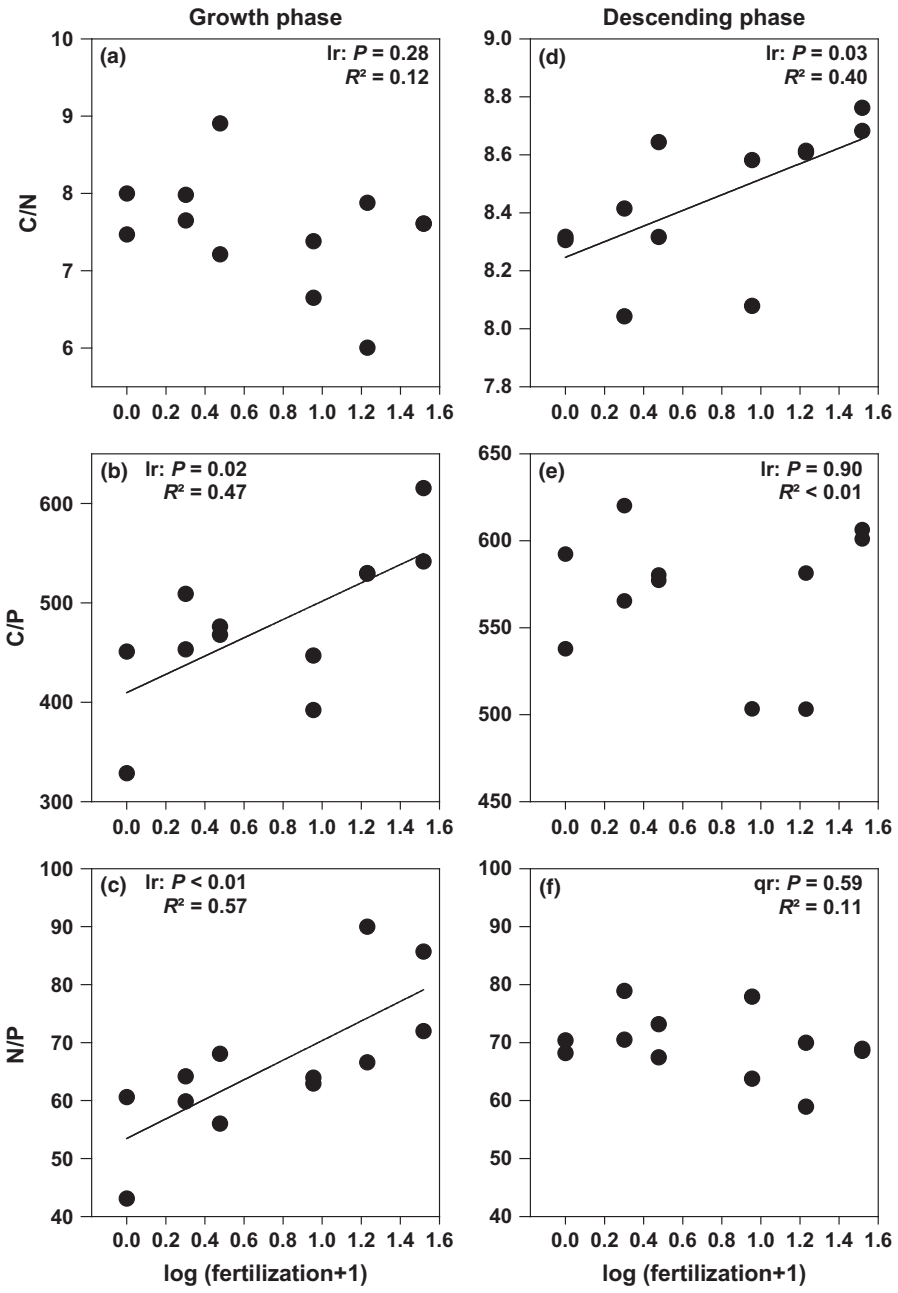


Fig. 2 Responses of stoichiometry ratios to nitrogen enrichment. The left column presents data from the growth phase and the right column presents data from the descending phase: (a, d) C:N, (b, e) C:P, (c, f) N:P. Regression lines show significant responses with $P < 0.05$.

contrary, during the descending phase, only seston C:N ratio showed a positive response with increasing nitrogen enrichment (Fig. 2d; lr: $P = 0.03$, $R^2 = 0.40$) and no patterns were observed in seston C:P and N:P ratios (Fig. 2e–f).

Phytoplankton biomass and community composition

Chlorophyll a and POC. In the phytoplankton development of all treatments, a peak was observed, representing the typical seasonal spring development for oligotrophic temperate lakes (Sommer *et al.*, 1986). Chlorophyll *a* increased from on average 1.03 ± 0.01 to 2.27 ± 0.20 μg L^{-1} on day 28, when six of the enclosures reached their chlorophyll *a* maximum and an average of 2.61 ± 0.22 μg L^{-1} on day 35 for the remaining six enclosures (Fig. 1b). A continuous and rapid decrease in chlorophyll *a* concentrations of all enclosures was observed after day 35 (Fig. 1b). From the mean chlorophyll *a* values, we defined that the overall growth phase began on day 17 (excluding the initial lack phase when no growth was observed) and ended on day 35. The descending phase began on day 38 and ended on day 63. The seasonal development of the chlorophyll *a*

concentration did not differ greatly between the treatments, and the timing of the day on which the chlorophyll *a* peak occurred in each enclosure was not influenced by nitrogen enrichment (lr: $P = 0.72$, $R^2 = 0.01$).

Chlorophyll *a* showed a trend to higher concentrations with increasing nitrogen enrichment for the growth phase (Fig. 3b; lr: $P = 0.06$, $R^2 = 0.31$). For the descending phase, no effect of nitrogen enrichments on chlorophyll *a* could be observed (Fig. 3e; lr: $P = 0.28$, $R^2 = 0.12$).

Particulate organic carbon showed a quadratic response to increasing nitrogen enrichment during the growth phase (Fig. 3a; qr: $P = 0.02$, $R^2 = 0.59$) with the lowest values found in the twofold nitrogen increase treatment. During the descending phase, there was a trend of increasing POC with increasing nitrogen enrichment (Fig. 3d; lr: $P = 0.08$, $R^2 = 0.27$). The timing of the day on which the POC peak occurred was not influenced by nitrogen enrichment (lr: $P = 0.90$, $R^2 < 0.00$).

Microscopic analyses of phytoplankton biovolume. Throughout the whole experiment, the phytoplankton community was dominated by bacillariophyceae, dinoflagellates, chrysophyceae and cryptophyceae. Only a few individuals of chlorophyceae and cyanophyceae

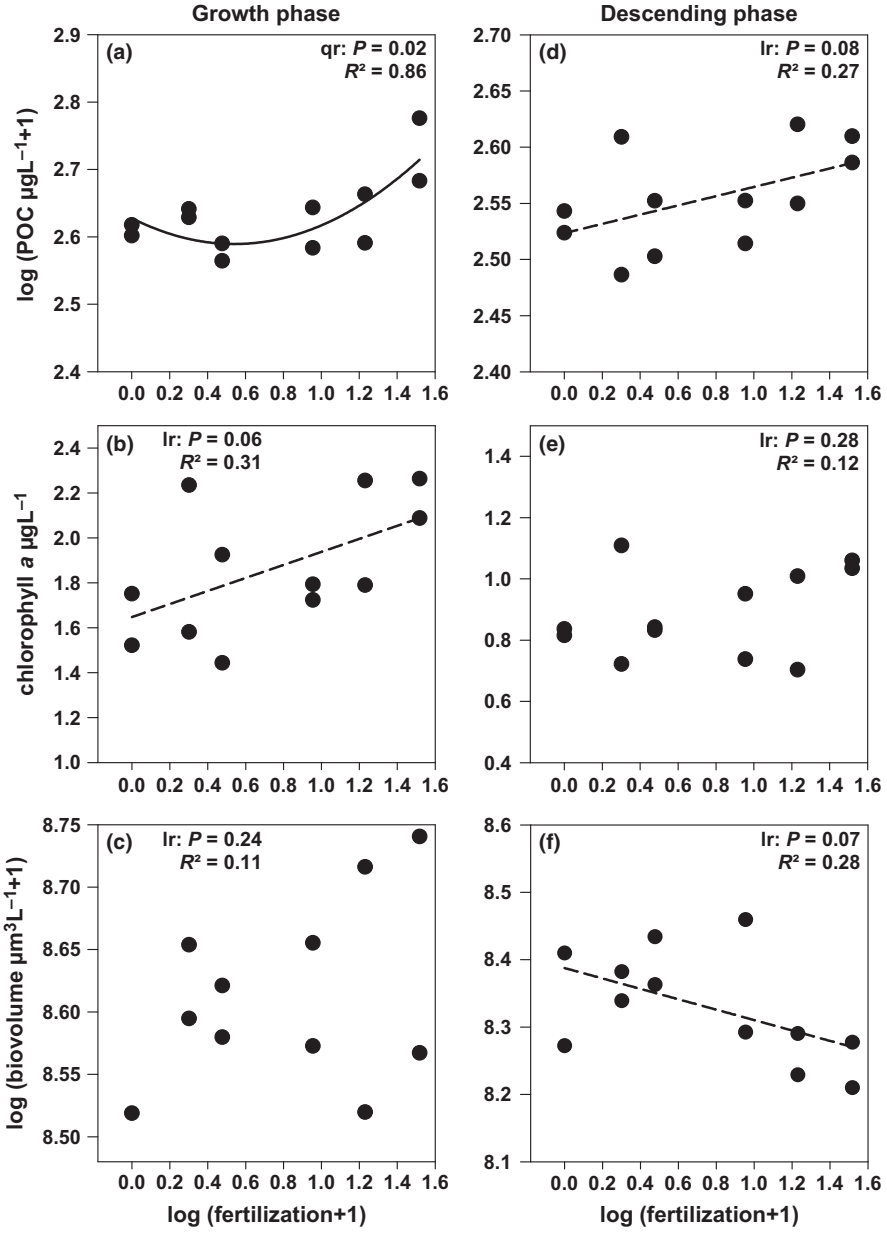


Fig. 3 Responses of the three phytoplankton biomass proxies to nitrogen enrichment. In the left column for the growth phase and in the right column for the descending phase: (a, d) particulate organic carbon, (b, e) chlorophyll *a*, (c, f) biovolume. Regression lines are solid for significant responses with $P < 0.05$ and dashed for trends with $0.05 < P < 0.1$.

(mainly *Anabaena* sp.) were present. The most abundant species within the bacillariophyceae were *Asterionella formosa*, *Cyclotella* sp. and *Fragilaria crotonensis*. Other highly abundant species were the chrysophyceae species *Dinobryon divergens* and the dinoflagellate *Ceratium hirundinella*.

Peak phytoplankton biovolume was reached in the enclosures between day 21 (highest average biovolume of $451.6*10^6 \pm 11.7*10^6$ µm³ L^{-1}) and day 35 (Fig. 1c) and occurred earlier with increasing nitrogen enrichment (Fig. 4a; lr: $y = 36.2–9.5*x$; $P < 0.01$, $R^2 = 0.64$). Total phytoplankton growth rates (based on biovolumes) estimated up to the phytoplankton peak in each enclosure, did show a significant response to increasing nitrogen enrichment (Fig. 4b; lr: $P = 0.02$, $R^2 = 0.46$). However, growth rates of individual phytoplankton groups showed different responses to the N enrichment. Although initial growth rates (up to the total phytoplankton peak) of mixotrophic chrysophyceae, especially *Dinobryon* sp., and dinoflagellates showed a clear increase with increasing N (chrysophyceae: Fig. 4c; lr: $y = 0.12 + 0.05*x$; $P < 0.01$, $R^2 = 0.77$; dinoflagellates: Fig. 4d; lr: $y = 0.04 + 0.04*x$; $P < 0.01$, $R^2 = 0.51$) growth rates of more autotrophic groups, such as chlorophyceae or bacillariophyceae, did not respond in a significant directed way to nitrogen addition (Fig. 4e–f).

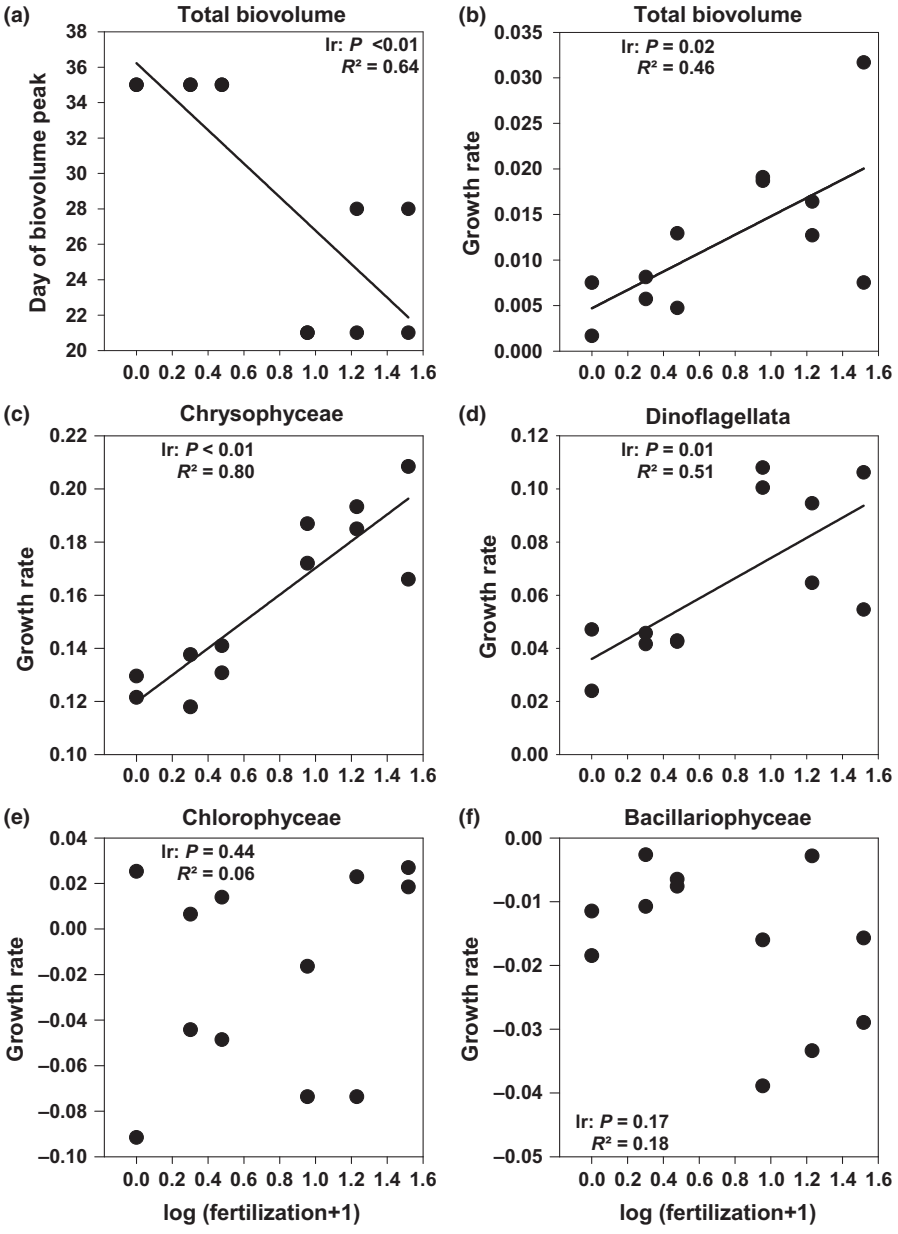


Fig. 4 Response of the timing of the biovolume peak (a) and growth rates of major phytoplankton groups to nitrogen enrichment. (b) Total biovolume, (c) chrysophyceae, (d) dinoflagellata, (e) chlorophyceae, (f) bacillariophyceae. Regression lines show a significant responses with $P < 0.05$

Within the phytoplankton groups, a clear peak in biomass development could be observed for chlorophyceae (peak on day 21, $34.0*10^6 \pm 8.6*10^6$ µm³ L^{-1}), dinoflagellates (day 21, $142.6*10^6 \pm 37.2*10^6$ µm³ L^{-1}) and chrysophyceae (day 35, $71.4*10^6 \pm 7.0*10^6$ µm³ L^{-1}) (Fig. 5a, b,d). Within the dinoflagellates, *C. hirundinella* reached peak densities on day 28 ($21.0*10^6 \pm 2.0*10^6$ µm³ L^{-1}); *D. divergens*, representing the majority of chrysophyceae found, reached its peak on day 35 ($20.1*10^6 \pm 1.6*10^6$ µm³ L^{-1}) (Fig. 5f,i). A fluctuation over time with no obvious biovolume peak development was observed in cryptophyceae ($72.8*10^6 \pm 6.6*10^6$ µm³ L^{-1}) and bacillariophyceae ($103.3*10^6 \pm 4.1*10^6$ µm³ L^{-1}) (Fig. 5c,e). However, within the bacillariophyceae, different species showed succession patterns with varying time courses. At the beginning of the experiment, bacillariophyceae were mainly represented by *A. formosa* ($119.9*10^6 \pm 5.06*10^6$ µm³ L^{-1}), which subsequently decreased over time and were almost absent after day 49 (Fig. 5g). *Cyclotella* sp. showed the reverse pattern, having a classical peak development, with an increase until day 42 ($85.4*10^6 \pm 7.0*10^6$ µm³ L^{-1}) and a decrease afterwards (Fig. 5j). Finally, *F. crotonenis* started to increase after day 42 and continued to increase until the end of the experiment ($21.1*10^6 \pm 5.3*10^6$ µm³ L^{-1}) (Fig. 5h). Even though *F. crotonensis* increased over time, the maximum abundance (biovolume) at the end of the experiment was low compared to the abundances (biovolumes) of the other bacillariophyceae species.

Total algal biovolume did not show a response to increased nitrogen enrichment in the growth phase (Fig. 3c; lr: $P = 0.24$, $R^2 = 0.11$). On group level, only chrysophyceae biovolume showed an increase with increasing nitrogen enrichment (Fig. 6b; lr: $P = 0.05$, $R^2 = 0.33$) when comparing treatments during the growth phase. In other algal groups, no significant effects of nitrogen on biovolume could be observed when comparing treatments during growth phase (Fig. 6 a–f, column 1). When considering the dynamics of individual species (Fig. 6n–r, column 3), we found that the chrysophyceae *D. divergens* biovolume showed a trend to increase with increasing nitrogen enrichment (Fig. 6o; lr: $P = 0.09$, $R^2 = 0.27$). Within diatoms, *A. formosa* declined in all treatments over time (Fig. 5g) but showed a trend to increase with increasing nitrogen enrichment when comparing treatments during growth phase (Fig. 6p; lr: $P = 0.06$, $R^2 = 0.32$), *Cyclotella* sp. showed a trend towards a quadratic relationship, with nitrogen

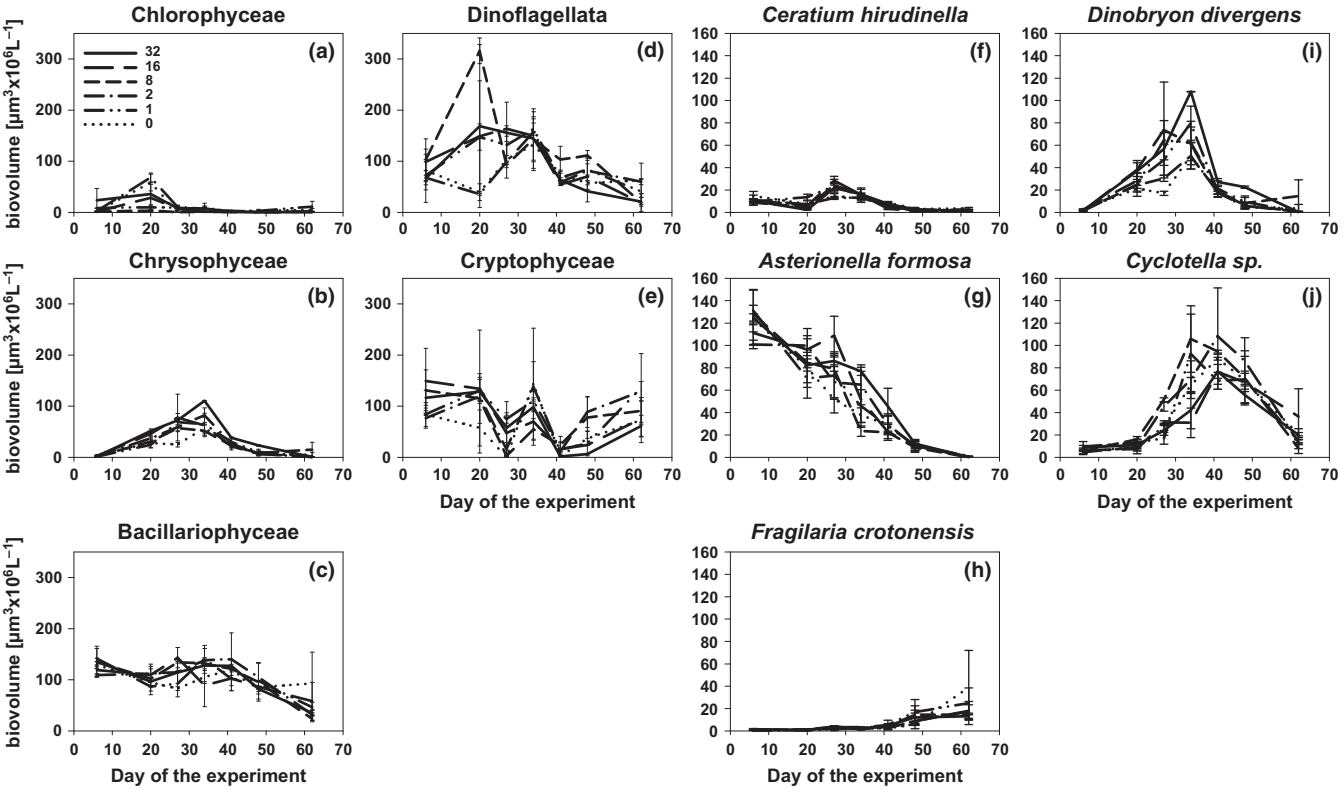


Fig. 5 Biovolume developments over time for the most abundant algae groups and species. The left two columns show group data and the right two columns show species data.

peaking just after the fourfold natural nitrogen input (Fig. 6q; qr: $P = 0.10$, $R^2 = 0.40$).

When comparing treatments in the descending phase, total algal biovolumes showed a trend to decrease with increasing nitrogen enrichment (Fig. 3f; lr: $P = 0.07$, $R^2 = 0.28$). A significant decrease with nitrogen enrichment, when comparing treatments in the descending phase, was found for the chlorophyceae (Fig. 6g; lr: $P = 0.03$, $R^2 = 0.38$). Additionally, a quadratic relationship (trend) was found for the dinoflagellates, with a peak between the two- and eightfold nitrogen enrichment (Fig. 6k; lr: $P = 0.07$, $R^2 = 0.44$). Cryptophyceae showed a similar pattern with increasing nitrogen enrichment (Fig. 6l; qr: $P = 0.01$, $R^2 = 0.61$). In the other groups and species, no significant responses could be observed (Fig. 6g–m and s–w, columns 2 and 4).

Comparing relationships between the different biomass proxies (chlorophyll *a*:biovolume, chlorophyll *a*:POC, biovolume:POC ratios) revealed a quadratic response of biovolume:POC ratio to nitrogen enrichment during growth phase (Fig. 7c; $P = 0.05$, $R^2 = 0.48$) and no response for the other two proxies (Fig. 7a,b). However, a significant influence of increasing nitrogen enrichment could be observed for the chlorophyll *a*:biovolume ratio (Fig. 7d; qr: $p < 0.01$, R^2=0.74) and biovolume:POC ratio (Fig. 7f; qr: $P < 0.01$, $R^2 = 0.69$) in the descending phase. While chlorophyll *a*:biovolume ratio was lowest around the twofold nitrogen enrichment treatment, biovolume:POC ratio was highest at that treatment. For the chlorophyll *a*:POC ratio in the descending phase, a declining trend with increasing nitrogen enrichment could be observed (Fig. 7e; lr: $P = 0.09$, $R^2 = 0.26$).

Protist and zooplankton development

Nanoflagellates showed a peak development with highest biovolumes of $6.5*10^7 \pm 3.2*10^7$ µm³ L^{-1} on day 35 (Fig. 8a). In the phytoplankton growth phase, they slightly increased with increasing nitrogen enrichment (lr: $P = 0.08$, $R^2 = 0.28$) and in the descending phase, no response was observed (lr: $P = 0.12$, $R^2 = 0.23$).

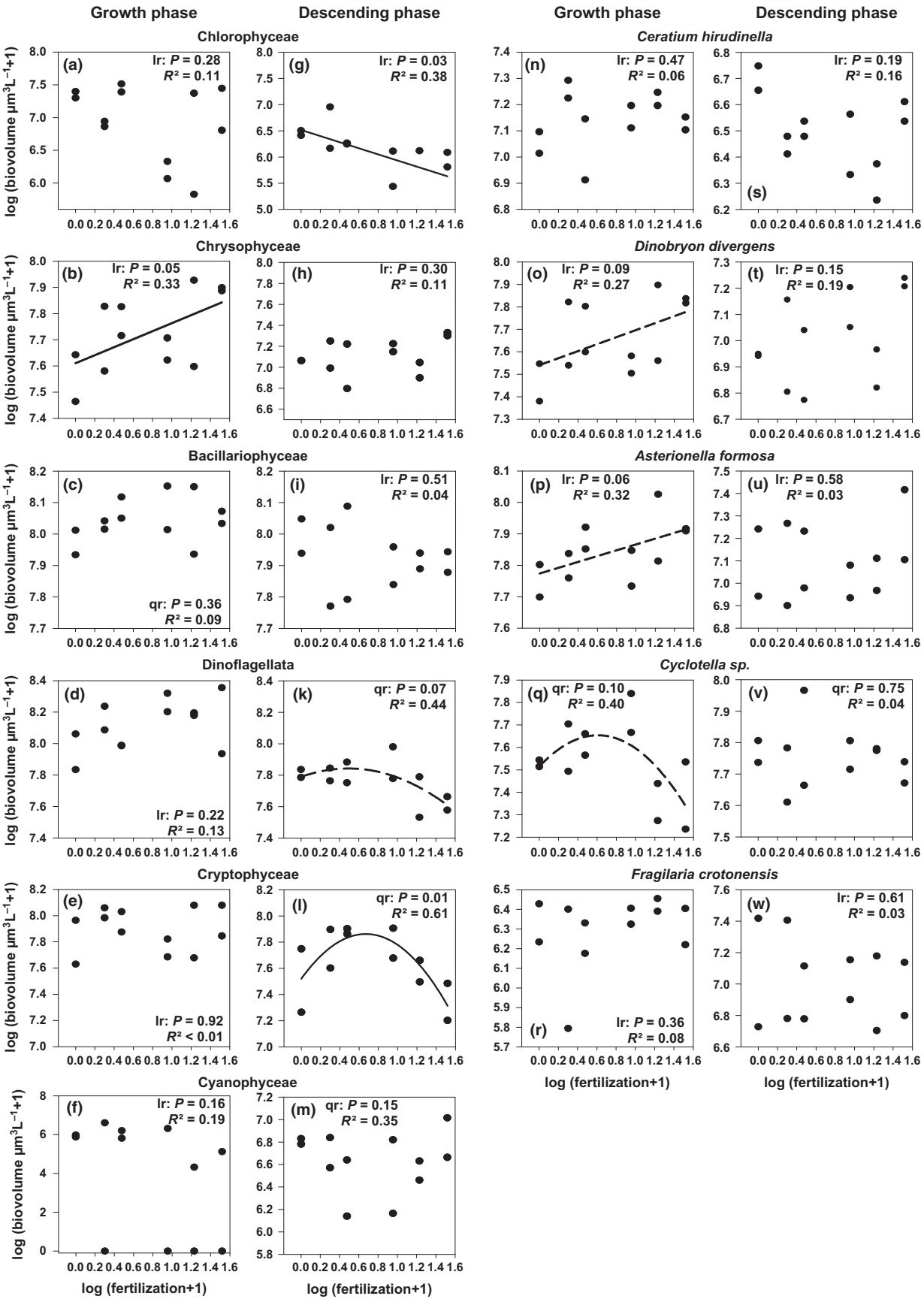
Ciliates decreased during the course of the experiment from $14.71*10^3 \pm 2.04*10^3$ to $2.12*10^3 \pm 0.30*10^3$ individuals L^{-1} (Fig. 8b). During the initial phytoplankton growth phase, ciliate abundances were positively correlated with nitrogen enrichment (lr: $P = 0.04$, $R^2 = 0.36$). During the descending phase, no effect of nitrogen enrichment on ciliate abundances could be observed (lr: $P = 0.52$, $R^2 = 0.04$).

Crustacean mesozooplankton (Cladocera and copepods) increased over time from 2.98 ± 0.22 to 10.72 ± 0.98 individuals L^{-1} and very rapidly between days 29 and 36 (Fig. 8c). Neither during the phytoplankton growth phase nor during their descending phase, a response of mesozooplankton to the nitrogen enrichment could be observed (phytoplankton growth phase: qr: $P = 0.16$, $R^2 = 0.33$; phytoplankton descending phase: qr: $P = 0.20$, $R^2 = 0.30$).

Discussion

We investigated the effects of increased nitrogen deposition on phytoplankton biomass, community composition and seston stoichiometry in an oligotrophic lake. The lake is characterised by high DIN:TP ratios, suggesting severe P limitation. Several studies have shown that high N : P ratios are indicators of P limitation in plankton communities, with DIN:TP values being one of the best predictors for nutrient limitation (Morris & Lewis, 1988; Bergström, 2010). DIN:TP molar ratios of higher than 1.5 (as calculated from Bergström, 2010) are considered to be indicators of P limitation. Since we found an average molar ratio of DIN:TP of >1000:1 in our study lake, we expected the phytoplankton to be heavily phosphorus-limited. This is indicated by seston C:P and N:P molar ratios, which had average values of 232 ± 26 C:P and 37 ± 2 N:P at the beginning of the experiment.

For the three common phytoplankton biomass proxies, algal biovolume represents the most direct estimate for *in situ* biomass, while chlorophyll *a* represents the main photosynthetic pigment within algae, and POC also includes other non-planktonic suspended particles <250 µm. In our study, the chlorophyll *a* measurements showed a clear peak development, while POC and phytoplankton biovolume levels showed less pronounced patterns. Slight differences in the timing of the algal biomass peaks were found, depending on which biomass proxy was used (biovolume: between days 21 and 35, chlorophyll *a*: day 28 and 35, and POC: between days 14 and 35, Fig. 1). Temporal differences between seasonal dynamics of biovolume and chlorophyll *a* have been shown before in an oligotrophic lake over a 14-month monitoring project (Felip & Catalan, 2000). In our study, temporal differences between the peaks of different proxies were independent of the applied nitrogen enrichment treatments. Differences between the seasonal dynamics of chlorophyll *a* and POC among enclosures were also independent of nitrogen enrichment. However, differences in the timing of the phytoplankton biovolume peak were nitrogen dependent, with higher


Growth phase
Descending phase
Growth phase
Descending phase
Chlorophyceae
Ceratium hirudinella
Chrysophyceae
Dinobryon divergens
Bacillariophyceae
Asterionella formosa
Dinoflagellata
Cyclotella sp.
Cryptophyceae
Fragilaria crotonensis
Cyanophyceae
(a) lr: P = 0.28 R² = 0.11
(g) lr: P = 0.03 R² = 0.38
(n) lr: P = 0.47 R² = 0.06
(s) lr: P = 0.19 R² = 0.16
(b) lr: P = 0.05 R² = 0.33
(h) lr: P = 0.30 R² = 0.11
(o) lr: P = 0.09 R² = 0.27
(t) lr: P = 0.15 R² = 0.19
(c) qr: P = 0.36 R² = 0.09
(i) lr: P = 0.51 R² = 0.04
(p) lr: P = 0.06 R² = 0.32
(u) lr: P = 0.58 R² = 0.03
(d) lr: P = 0.22 R² = 0.13
(k) qr: P = 0.07 R² = 0.44
(q) qr: P = 0.10 R² = 0.40
(v) qr: P = 0.75 R² = 0.04
(e) lr: P = 0.92 R² < 0.01
(l) qr: P = 0.01 R² = 0.61
(r) lr: P = 0.36 R² = 0.08
(w) lr: P = 0.61 R² = 0.03
(f) lr: P = 0.16 R² = 0.19
(m) qr: P = 0.15 R² = 0.35
log (biovolume µm³L⁻¹+1)
log (fertilization+1)

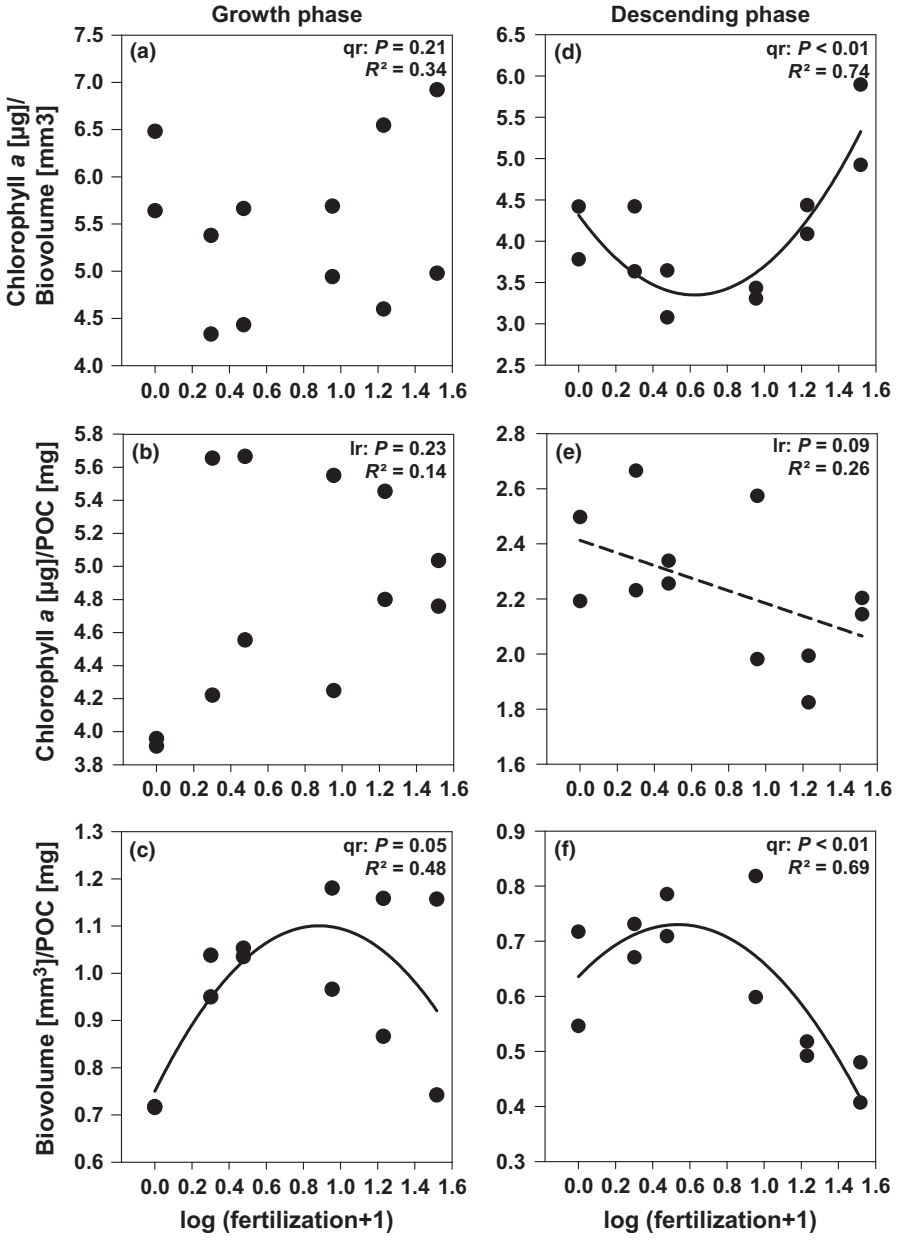


Fig. 7 Ratios between biomass proxies versus nitrogen enrichment. The left column shows the growth phase and the right column shows the descending phase: (a, d) chlorophyll *a*:biovolume, (b, e) chlorophyll *a*:particulate organic carbon (POC), (c, f) biovolume:POC. Regression lines show significant responses with $P < 0.05$, dotted lines show trends with $0.05 < P < 0.1$.

nitrogen enrichment resulting in earlier phytoplankton biovolume peaks. It is likely that this was due to bottom-up effects, as phytoplankton growth rates were positively linked to nitrogen fertilisation. This was mainly due to the positive response of primarily mixotroph chrysophytes and dinoflagellates growth rates to nitrogen enrichment. It is possible that N enrichment resulted in an increase of bacterial prey promoting the growth of mixotrophs. In support of this, algal species not combining auto- and phagotrophy, such as chlorophytes and diatoms, did not show a positive response in their initial growth rates to N enrichment.

Additionally, there was no direct relationship between the timing of the biovolume peak and mesozooplankton densities which would otherwise explain the observed time shift by grazing. Nevertheless, ciliate abundances showed – similar to chrysophytes – a positive relationship with increasing nitrogen enrichment during the phytoplankton growth phase. This was probably also due to higher availability of small prey (Berninger,

Fig. 6 Biovolume versus nitrogen enrichment for algae groups (left two columns) and most abundant species (right two columns): (a–f) algae groups in the growth phase (days 21 till 35), (g–m) algae groups in the descending phase (days 42 till 63), (n–r) species in the growth phase, (s–w) species in the descending phase. Regression lines are solid for significant responses with $P < 0.05$ and dashed for trends with $0.05 < P < 0.1$.

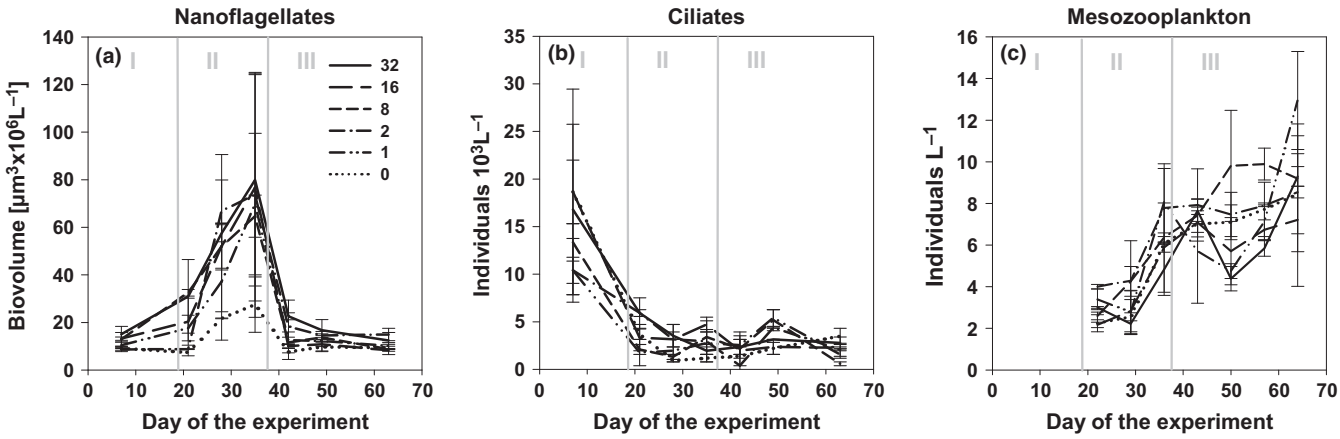


Fig. 8 Development of nanoflagellates, ciliates and mesozooplankton over time. Lines plot mean values from the two respective enclosures per treatment and whiskers show standard errors. Grey lines divide the time course in phytoplankton growth phases: (I) initial phase, (II) phytoplankton growth phase, (III) phytoplankton descending phase.

Wickham & Finlay, 1993) such as bacteria promoted by nitrogen enrichment (Roberts *et al*., 2003).

While the timing of phytoplankton peaks in terms of biovolume showed a clear response to nitrogen enrichment, probably due to enhanced initial mixotroph growth rates, total algal biovolume during the growth phase (days 17–35) did not differ between treatments. This indicates that the planktonic community was indeed not nitrogen-limited as the additional nitrogen could not support higher biovolumes. However, the effects of nitrogen enrichment on algal biomass dynamics were detectable after the peak phase (days 38–66), when zooplankton started to grow to larger densities. The observed decrease of total algal biovolume with increasing nitrogen supply in the descending phase (see Fig. 3f) suggests that interactions with the zooplankton might have been affected, and that indirect effects of variable grazing patterns along the nitrogen enrichment gradient lead to changes in phytoplankton biovolume. Although we could not detect direct responses of mesozooplankton grazers on phytoplankton biovolume during the descending phase, top–down effects are still possible as interactions are complex and not only defined by abundances.

Using the phytoplankton proxies POC and chlorophyll *a* instead of algal biovolume for the analysis, phytoplankton dynamics showed different patterns. We observed the highest values of chlorophyll *a* (growth phase) and of POC (both phases) at the highest nitrogen enrichment treatment (see Fig. 3). The increasing POC values must not necessarily imply higher phytoplankton contribution. Besides photoautotrophic organisms, our POC measurements included all other seston particles in the range of approximately 0.7–250 µm, including heterotrophic organisms. The increase of POC with increasing nitrogen enrichment could therefore also be caused by increasing abundances of heterotrophic organisms, which is supported by higher ciliate and nanoflagellate abundances with increasing nitrogen enrichment during the initial growth phase. A mesocosm experiment comparing two different ratios of N:Si showed a shift to higher abundances of heterotrophic species (including dinoflagellates, microflagellates and ciliates) with N enrichment (high N:Si ratios) (Roberts *et al*., 2003). Our N enrichment experiment also resulted in N:Si ratio shifts as Si concentrations were the same in all treatments. The observed increase in POC with increasing nitrogen enrichment may therefore partly result from increasing abundances of heterotrophic organisms, additionally indicating higher microbial food-web activities.

Collecting data on the responses of the three biomass proxies of phytoplankton to the nitrogen enrichment also allowed us to examine their relationships along the nitrogen gradient. Nitrogen enrichment had a significant effect on the chlorophyll *a*:biovolume ratio, the chlorophyll *a*:POC ratio, as well as on the biovolume:POC ratio within algal communities in the descending phase. This could either result from different algal communities developing along the nitrogen enrichment gradient, or from direct effects of nitrogen on chlorophyll *a* synthesis and carbon fixation. Obtaining lower phytoplankton biovolume:POC and chlorophyll *a*:POC ratios with increasing nitrogen enrichment supports our findings of a larger contribution of heterotrophic organisms to seston POC <250 µm. For chlorophyll *a*:biovolume ratios, the lowest values were observed for intermediate nitrogen enrichment and highest values for high nitrogen

enrichment. In the descending phase, cryptomonad and dinoflagellate biovolumes were highest at intermediate nitrogen enrichment (see Fig. 6k,l). These two groups, representing 12–29 and 18–33% of the algae community in terms of biovolume, might have been the drivers of the response in the chlorophyll *a*:biovolume ratio. The relative chlorophyll *a* content of dinoflagellates is low compared to other algae groups indicated, for example, by decreasing chlorophyll *a*:biovolume ratios with increasing dinoflagellate abundances (Felip & Catalan, 2000). The co-occurrence of the chlorophyll *a* peak and that of *D. divergens* (together with the absence of a total biovolume peak on that day) additionally stresses the importance of community structure on chlorophyll *a*:biovolume ratios. A temporal overlap of chlorophyll *a* with high abundances of *D. divergens* has been observed before (Fee, 1976) and is probably due to comparable high chlorophyll *a* contents of *D. divergens*. It can be concluded that changes in chlorophyll *a*:biovolume ratios, as well as in biovolume:POC ratios, appear to be driven by changes in the community structure rather than by direct effects of nitrogen on the chlorophyll *a* synthesis or the photosynthetic carbon fixation.

Instead of large phytoplankton biomass changes, we rather expected to find changes in phytoplankton community composition and/or seston stoichiometry. Stoichiometric differences in terms of POC:nutrient ratios were detected during the phytoplankton growth phase (see Fig. 2). Both, seston C:P and N:P ratios, went up with increasing nitrogen enrichment and drift further away from the classical Redfield ratio of 106:1 (C:P) and 16:1 (N:P). Within the growth phase, ratios increased from around 400 (C:P) and 50 (N:P) in the lowest nitrogen enrichment treatment, up to values around 600 (C:P) and 80 (N:P) in highest nitrogen enrichment treatments (see Fig. 2b,c). However, we did not observe any decrease in seston C:N ratios with nitrogen enrichment within the growth phase. The increase in C:P ratios indicates an increase of phosphorus limitation of the phytoplankton with increasing nitrogen enrichment. The observed shifts in C:P ratios with nitrogen enrichment could have further effects on zooplankton growth. P limitation of zooplankton growth can already be expected in the low nitrogen treatments, since seston C:P ratios above 300 have been frequently reported to result in a decrease in the growth of daphniids (Urabe & Watanabe, 1992; DeMott, Gulati & Donk, 2001).

During the initial growth phase, algal communities shifted towards a higher contribution of chrysophyceae at higher nitrogen enrichment levels. A large number of species of that group are known to be mixotrophs that are able to take up nutrients from particulate sources such as bacteria (Holen & Boraas, 1995). In our experiment, the main species within that group was *D. divergens*, which is known to be strongly mixotrophic (Jones, 2000 and citations therein). Similar to Roberts *et al.* (2003), nitrogen enrichment could additionally have promoted bacterial growth in our experiment, as indicated by both increasing POC and increasing POC:biovolume ratios with increasing nitrogen enrichment. Therefore, increased food availability for mixotrophic species could have been a potential driver of the observed increasing chrysophyceae abundances. A higher proportion of mixotrophic species within phytoplankton would have far reaching consequences for food-web dynamics. Mixotroph algal species direct carbon and nutrient flows from bacteria towards phytoplankton, and as a consequence, the total carbon budgets of pelagic systems would become more heterotrophic. Accordingly, the fuelling of pelagic carbon flows with allochthonous dissolved organic carbon would become more important. Additionally, chrysophytes are well known to be a lower quality food source for herbivores, thereby reducing food-web transfer efficiencies (Katechakis *et al.*, 2005; Taipale *et al.*, 2013). Altogether, with higher abundances of mixotrophic chrysophyceae, ciliates and heterotrophic nanoflagellates, within the initial growth phase, we observed strong signs of increasing nitrogen enrichment boosting the microbial loop within a phosphorus-limited system. Microbial food webs represent crucial components of aquatic food webs and play an important role in the carbon and nutrient turnover in aquatic systems (Sherr & Sherr, 2002; Löder *et al.*, 2011). Therefore, changes in the microbial food web, resulting from nitrogen enrichment may, on a long-term perspective, have far reaching consequences for food-web dynamics, even in strongly P-limited systems.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Nitrate (left) and ammonium (right) content over time. The lines show mean values from the two respective enclosures per treatment and the whiskers show standard errors.

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PUBLICATION II

Altered food web dynamics under increased nitrogen load in phosphorus deficient lakes

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RESEARCH ARTICLE

Altered food-web dynamics under increased nitrogen load in phosphorus deficient lakes

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Abstract Atmospheric nitrogen deposition predominantly influences ecosystems by shifting their available nutrient budgets towards excess nitrogen conditions. In temperate lakes nitrogen is often naturally in excess and phosphorus is deficient, when compared with the optimal Redfield ratio of 16:1. To investigate effects of future increasing nitrogen conditions on lake plankton communities, we performed mesocosm experiments in three different nitrogen rich lakes, all characterised by high nitrogen to phosphorus ratios. In order to determine functional responses to increased nitrogen loading, we conducted six nitrogen fertilization treatments. Nitrogen fertilization was based upon existing nitrate and ammonium concentrations in natural wet deposition and multiple loadings of these concentrations. Despite the initial conditions of excess nitrogen, removal of additional nitrogen by the plankton community was observed in all of the lakes. In one lake, an increasing phosphorus limitation became visible in seston stoichiometry. Over all of the lakes and within each lake's experimental nitrogen gradient, we found evidence for decreased mesozooplankton due to nitrogen enrichment. The negative responses of mesozooplankton to N enrichment were mainly restricted to cladocerans and nauplii. The results indicate that nitrogen enrichment within the magnitudes of projected future atmospheric nitrogen depositions may lead to a long-term reduction of mesozooplankton in phosphorus deficient lakes. The transfer of nitrogen enrichment effects on lower food-web dynamics could have consequences for higher trophic levels, such as fish.

Keywords Nitrogen · Phosphorus · Phytoplankton · Zooplankton · Lakes · Food-web

Introduction

As a result of continuously rising global economic activity, together with the energy and the food demands of the human population (Galloway et al. 2008), the nitrogen (N) cycle is considered to be the most anthropogenically altered biogeochemical nutrient cycle. Nitrogen excess is one of the critical risks for a sustainable human life on earth (Rockström et al. 2009; Steffen et al. 2015). Over the last century, a three- to fivefold increase in the reactive forms of nitrogen emissions (nitrate and ammonium) has been observed (Denman et al. 2007; Ciais et al. 2013). Due to their widespread atmospheric distribution, the subsequent locally uncontrollable deposition leads to rising concentrations of reactive N in ecosystems, even in remote regions (Bergström et al. 2005; Crowley et al. 2012; Kim et al. 2014). It has been shown that an increased N deposition is able to alter the nutrient status of ecosystems (Vitousek et al. 1997; Aber et al. 2003) due to the increment of nitrification rates (Aber et al. 2003), as well as to an increase of N leaching from forests and soils (Gundersen et al. 1998; Reay et al. 2008; Lovett et al. 2013).

An increasing N supply is of biological importance since it leads to changes in the available nutrient ratios, and eventually, to excess N conditions and a phosphorus (P) deficiency in (terrestrial and aquatic) ecosystems

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(Bergström and Jansson 2006; Elser et al. 2009a, b; Crowley et al. 2012). An increment of N is the first and foremost concern for ecosystems in which the primary producers are limited by N, potentially increasing biomass and primary production (Tyrrell 1999; Reich et al. 2001; Elser et al. 2009b; Hessen 2013). In addition, autotrophs, such as phytoplankton, have the flexibility to alter their elemental (stoichiometric) composition according to the surrounding resource conditions (Sterner et al. 1997; Klausmeier et al. 2004). It has recently been shown that for predominantly N-limited lakes, in a gradient of atmospheric N deposition, the availability of higher dissolved N:P ratios can result in higher seston biomass N:P ratios (Elser et al. 2009b; Hessen 2013).

Seston stoichiometry is an indicator of food quality for aquatic herbivores (Andersen and Hessen 1991; Hessen 1992), whose performance is related to both the producer food quantity and quality (Hessen et al. 2002). Food quality needs to meet specific nutrient requirements for each herbivore, which include seston stoichiometry in general (Andersen and Hessen 1991; Hessen 1992; Elser et al. 2000), fatty acid composition (Müller-Navarra 1995; Müller-Navarra et al. 2000), as well as the edibility of algae (Sommer et al. 1986). In terms of mineral nutrient limitation, a copepod's growth is rather limited by N-deficient food through the higher organismic demands of N (Hessen 1992; Sterner and Hessen 1994). In contrast, the growth and the reproduction of the abundant freshwater cladoceran *Daphnia* are commonly limited by the amount of available P (Sommer 1992; Sterner and Hessen 1994; Urabe et al. 1997). There is evidence from natural lake systems that an algal P limitation, expressed in high seston C:P ratios, is transferred to the trophic level of secondary producers and affects the growth of *Daphnia* (Elser et al. 2001; Berger et al. 2006). Besides seston stoichiometry, the fatty acid content of phytoplankton is essential for herbivore growth and may be reduced under an increased P limitation (Müller-Navarra 1995; Müller-Navarra et al. 2000). Hence, effects of increased N load in traditionally P deficient temperate lakes (Schindler 1977) would not be expected to be of great magnitude. It is conceivable that the growth and the reproduction of P-demanding herbivores should be derogated to a greater extent, and thus might lead to lower herbivore abundance under increasing N:P conditions.

In order to estimate the degree to which effects of an increased N load in P deficient lakes are transferred to the zooplankton trophic level, we experimentally investigated the consequences of N enrichment on epilimnion plankton communities in mesocosm field studies. To be able to detect overarching effects of an increased N load in P deficient systems, we performed experiments in three lakes with different trophic conditions. Experiments were performed synchronously and covered a broad N:P nutrient supply range. We hypothesised that the zooplankton groups with high P requirements would be the first to be negatively affected by an additional N enrichment. We did not expect that increased N loads would primarily lead to changes in the phytoplankton biomass, but rather to shifts in the seston stoichiometry.

Materials and methods

Study sites and experimental design

The mesocosm field experiments were performed during the spring of 2013 in three lakes with different trophic statuses (Lake Brunnsee 27.03.13–31.05.13, Lake Klostersee and Lake Thalersee 27.03.13–28.05.13) in Bavaria, Germany. The lakes were chosen for their different nutrient backgrounds (Table 1), in which the dissolved N:P ratios (nitrate and ammonium compared to total phosphorus concentrations) varied from >50:1 N:P (Lake Klostersee), >400:1 N:P (Lake Thalersee), to >1000:1 N:P (Lake Brunnsee). This was far higher than the classical Redfield ratio of 16:1 N:P. All of the lakes are hard water lakes with bedrocks of rubble and lie in a nature reserve. The total N supply of the lakes is continuously affected by atmospheric deposition and leaching, but also, the groundwater discharges and the surface runoffs contribute to the total N in the lakes. Lake Brunnsee (18.6 m max. depth, 5.9 ha, 502×10^3 m^3) is mainly groundwater fed, whereas Lake Klostersee (16 m max. depth, 47 ha, 2762×10^3 m^3) and Lake Thalersee (7 m max. depth, 3.8 ha, 166×10^3 m^3) have small streams running through them. Lake Brunnsee and Lake Klostersee are typically dimictic, with a stable stratification from spring to autumn, and Lake Thalersee is a shallow polymictic lake.

In the mesocosm experiments at each lake, we simulated a continuous N supply on a stratified pelagic water column. The natural phytoplankton and zooplankton communities were enclosed in 12 mesocosm bags (4 m deep, 0.95 m in diameter, made of white polyethylene foil, 150 µm, Biofol Film GmbH, Germany), which were sealed at the bottom and open to the atmosphere. Those

Table 1 Background nutrient data (TP µg l^{-1}, NO_3 mg l^{-1}, NH_4 µg l^{-1} and dissolved reactive N:P ratio: $N–NO_3 + N–NH_4$:TP) of the lakes Brunnsee, Klostersee and Thalersee

	Brunnsee	Klostersee	Thalersee
TP µg l^{-1}	6.6	12	13
NO_3 mg l^{-1}	17	1.3	12
NH_4 µg l^{-1}	56	304	29
Dissolved N:P ratio	>1000	>50	>400

mesocosms were filled by lowering the bags into the water column (6–8 m, if possible) and then lifting them back to the surface. The mesocosm bags were fixed to anchored rafts and were equipped with a transparent covering to control for the N wet deposition, while ensuring that there were natural light conditions, with regard to light quantity and spectrum. The N supply was manipulated in a gradient of six N treatments (two replicates per treatment), starting from a zero N deposition, to 1, 2, 8, 16, and 32-fold the natural regional N wet deposition. Since we could not foresee the range of N deposition amounts, where responses in the plankton community could be expected, we chose a log-linear experimental design to cover a reasonably wide magnitude of N deposition. The natural N wet deposition was based on average amounts, which are effectively supplied to the lake surfaces in about 25 l m^{-2} of weekly precipitation in the region (German Meteorological Survey) with 3 mg l^{-1} nitrate (NO_3), and 1 mg l^{-1} ammonium (NH_4) (~1:1 mol:mol). Thus, this ambient wet deposition contained an average supply of 75 mg m^{-2} NO_3 and 25 mg m^{-2} NH_4 per week (Bavaria regional state office), with yearly peak values of 490 mg m^{-2} NO_3 and 245 mg m^{-2} NH_4 per week. Only a doubling of the maximum N deposition, combined with a doubling in the weekly precipitation (Denman et al. 2007), would result in conceivable future N depositions in the range of 32 times the average wet deposition.

The N fertilization of the mesocosms was carried out twice a week by the addition of 1 l of an appropriate N solution per mesocosm and by a thorough mixing using a Secchi disc. The respective N solutions were freshly prepared from a stock solution prior to fertilization (41.1 mg ml^{-1} $NaNO_3$, 29.7 mg ml^{-1} NH_4Cl). These were calculated according to the natural N deposition in relation to the enclosure surface. For each treatment, 1, 2, 8, 16, and 32 ml of stock solution was placed into a 1-l PE bottle, which was then filled up with distilled water. The control treatment (0) received 1 l of distilled water only. An initial fertilization, equivalent to 4 weeks of a fertilization amount, was given on 28 March 2013, the first day of the experiment. This was in order to mimic the high availability of dissolved nutrient levels after the winter/spring circulation, and to ensure the immediate treatment differences at the start of the algal bloom phase in early spring. The sampling was performed once a week for water chemistry and zooplankton analyses, and twice a week for the phytoplankton analyses. Temperature was monitored in 20 randomly chosen mesocosms during the experiment by a sensor below the water surface and connected to a data logger (SE-309; Conrad Electronic, Germany).

Laboratory and data analyses

The samples for water chemistry and phytoplankton analyses were taken with an integrated tubular water sampler (2 m; KC Denmark A/S research equipment) from a water depth of between 1 and 3 m and then were pre-filtered over a 250 µm mesh to exclude the mesozooplankton. The weekly water chemistry analyses started on 30 March 2013 and included measurements for NO_3 by ion chromatography (Dionex ICS-1100, Thermo Scientific, USA), and measurements of NH_4 by fluorometry (Trilogy Laboratory Fluorometre Module CDOM/NH4; Turner Designs, USA), using the orthophthalate method (Holmes et al. 1999). This was done in addition to the measurements of total phosphorus levels (TP) by spectrophotometry (Shimadzu UV-1700, Shimadzu Cooperation, Germany), using the molybdenum blue method (Wetzel and Likens 1991). For particulate C, N, and P analyses, between 100 and 350 ml of enclosure water was filtered in duplicates onto a pre-combusted acid-washed GF/F filter (Whatman) and then frozen (−20 °C) until further analyses. The measurements for particulate organic carbon (POC) and particulate N were accomplished with an elemental analyzer (vario Micro cube, Elementar, Germany)—and for the particulate P (PP) with a spectrophotometer applying the molybdenum blue method. In weeks 4, 6, 8, and 10, biogenic silicate (Si) was analyzed by filtering between 50 and 200 ml of enclosure water onto cellulose-acetate filters (0.6 µm pore size, Satorius) and then frozen (−20 °C) until further analyses. The filters were subsequently extracted in a water bath (95 °C, for 4 h, in 0.2 mol NaOH) (Ragueneau and Tréguer 1994) and measured spectrophotometrically using the molybdenum blue method. From the particulate measurements, atomic seston C:N, C:P, N:P, and N:Si ratios, were calculated.

The phytoplankton groups were optically analyzed twice a week in vivo with an AlgaeLabAnalyser (bbe Moldaenke, Germany), which measures the total of chlorophyll *a*, and additionally separates the excitation spectra of four pigment groups into spectrally characterised algae groups. These spectrally characterised algae groups are named "green algae" (chlorophyta), "chromophytes" (with heterokontophyta, haptophyta, and also dinophyta), "blue-green algae" (cyanobacteria) and "cryptophytes" (Beutler et al. 2002).

The zooplankton sampling started on 17 April 2014 in order to allow for an initially undisturbed population growth. From then on, a 105 µm net (12 cm diameter) was hauled through the 4 m water column once a week. The zooplankton were immediately fixed to a 70% ethanol final concentration. The zooplankton communities were subsequently analyzed under a stereo microscope by counting splits of the fixed samples that contained at least 400 specimens and by determining those individuals to a species level if possible. For biomass calculation, the

length of up to 30 individuals of the most abundant zooplankton taxa of cladocerans and copepods were measured if available and applied to established length-weight regression equations from the literature (Watkins et al. 2011; Bottrell et al. 1976). Rotifer biomass was estimated with given dry weights from the literature (Pauli 1989). As a direct and counting independent second biomass proxy, carbon measurements were performed weekly on the mesozooplankton (>250 µm) from the water samples that were filtered through a 250 µm gauze. The samples were taken with an integrated tubular water sampler from a water depth of between 1 and 3 m. The mesozooplankton were first counted alive (copepods and cladocerans) and then it was brought onto a pre-combusted GF/F filter for POC measurements using an elemental analyzer (vario Micro cube, Elementar, Germany).

Following the logarithmic fertilization design, the statistical analyses were performed with regression models (linear or unimodal) using SigmaPlot 11.0 (Systat Software 2008) against the log-transformed N fertilization loadings or, against the observed dissolved reactive N or dissolved N:P ratios. In the case of the phytoplankton development, the maximum chlorophyll *a* values were defined as being the highest values after the growth phase. The "peak phase" was defined according to the maximum of total chlorophyll *a* values in Lake Brunnsee and Lake Thalersee (chromophytes in the case of Lake Klostersee), including two measurements before and after the maximum chlorophyll *a* values. Before and after the peak phase, the exponential growth phase and the subsiding phase were determined. We statistically analyzed the total chlorophyll *a* levels and all of the spectral algal classes for their maximum and average chlorophyll *a* levels. For zooplankton from microscopic counting, the abundances and biomass of copepods, cladocerans, and rotifers, together with the total zooplankton species, were analyzed. Calculations were performed for the average, and the maximum ("peak") during the experimental period. Zooplankton biomass parameters of all lakes (mesozooplankton carbon measurements, copepod and cladoceran carbon estimated by their relative biomass abundances and calculated total zooplankton biomass from microscopic countings) were analysed as averages for the second half of the experiment (after day 43), when responses of mesozooplankton communities to bottom up driven effects of N fertilization would be expected to be visible. Multivariate analyses were performed as canonical correspondence analyses (CCA, Legendre and Legendre 1998) using PAST 2.17 Software (Hammer et al. 2001). Explanatory variables included the TP levels, the dissolved reactive N levels, and the dissolved N:P ratios. Log-transformed abundances of calanoid copepods, nauplii, cladocerans, and rotifers, were used as species variables.

Results

Nitrogen enrichment

In all of the three lakes, the required nitrogen gradient was established in the mesocosms (linear regression over all of the lakes versus N fertilization by the end of the experiment: NO_3: $R^2=0.69$, $F=316$, $P<0.001$; NH_4: $R^2=0.25$, $F=23.7$, $P<0.001$). It varied between 3.8 and 5.7 mg l^{-1} NO_3–N (0.01–1.7 mg l^{-1} NH_4–N) in Lake Brunnsee, 0.3–2 mg l^{-1} NO_3–N (0.14–.8 mg l^{-1} NH_4–N) in Lake Klosterse, and 2.8–4.9 mg l^{-1} NO_3–N (0.004–1.7 mg l^{-1} NH_4–N) in Lake Thalersee. In contrast, the TP concentrations in the mesocosms were not correlated with N fertilization ($R^2=0.01$, $F=1.99$, $P=0.16$) and ranged between 3 and 8 µg l^{-1} in Lake Brunnsee, 6–15 µg l^{-1} in Lake Klostersee, and 6–17 µg l^{-1} in Lake Thalersee. Over the experimental duration, the temperatures in the mesocosms were on average about 15.2 ± 0.1 °C in Lake Brunnsee, 15.3 ± 0.1 °C in Lake Klostersee and 15.5 ± 0.1 °C in Lake Thalersee.

When comparing the fertilization treatments at the end of the experiments and comparing the observed versus the predicted concentrations of NH_4, the final NH_4 concentrations were consistently below the predicted concentrations in all of the N treatments (negative intercepts of linear regressions, Supplementary Table S1). The lower than predicted NH_4 concentrations indicated a significant NH_4 conversion into biomass or other oxidized molecular forms in all of the lakes. For Lake Thalersee, the observed NO_3 concentrations for the higher N treatments were higher than what would be expected from the fertilization alone (slope >1, Supplementary Table S1), whereas in Lake Klostersee, an increasing NO_3 removal could be observed in the applied 8–32-fold N treatments (slope <1, Supplementary Table S1). In Lake Brunnsee, the NO_3 removal occurred only in the lower N treatments (0–2-fold) but this was reduced in the 8–32-fold N treatments (slope >1, Supplementary Table S1).

Phytoplankton

In terms of the phytoplankton growth, the mesocosms in oligotrophic Lake Brunnsee reached the highest chlorophyll *a* values between the days 28 and 35 (Fig. 1a). The mesocosms in Lake Thalersee had already reached the highest chlorophyll *a* values between days 7 and 10 (Fig. 1b) and it lacked an exponential growth phase. Over the entire experimental duration, the phytoplankton communities in Lake

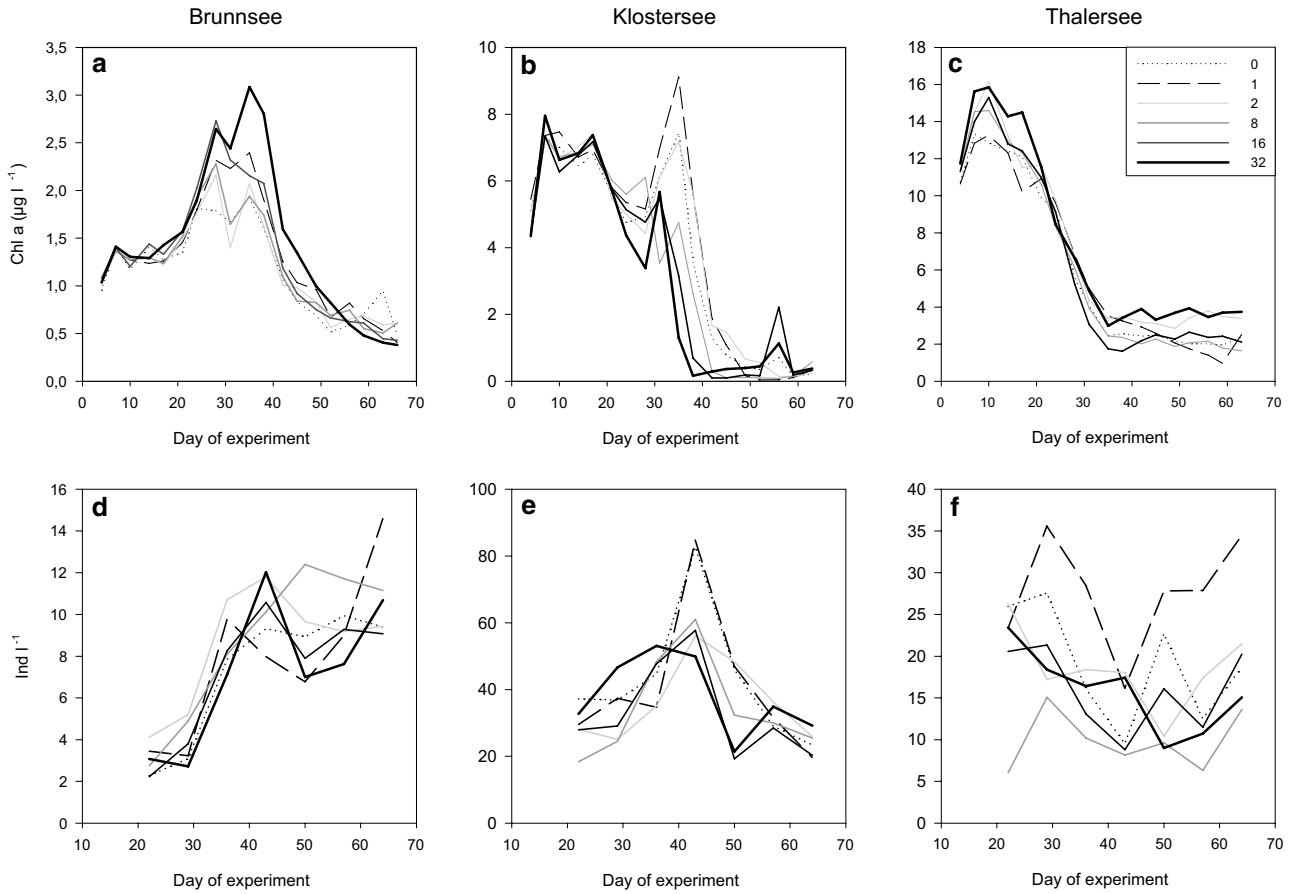

Fig. 1 Development of the chlorophyll *a* concentration (µg l^{-1}) (**a**–**c**) and the zooplankton densities (Ind l^{-1}) (**d**–**f**) over the experimental duration. Displayed are the average concentrations (N = 2) of each of the six N fertilization treatments for lakes Brunnsee (*left*), Klostersee (*middle*) and Thalersee (*right*). The legend in **c** applies to all *panels*

Thalersee consisted equally of green algae and chromophytes and in Lake Brunnsee, it was mainly chromophytes (Supplementary Fig. S2). The experiment in Lake Klostersee was dominated by high green algae concentrations during the first 3 weeks, after which the adjoining "peak phase" of the spring bloom between the days 31 and 35 (Fig. 1c) was primarily due to chromophytes. In Lake Klostersee, the highest chlorophyll *a* concentration levels after the growth phase of the chromophytes were lower than at the start of the experiment in some of the mesocosms.

In terms of the N fertilization, different responses of the chlorophyll *a* concentration levels could be observed between the lakes. In two of the lakes (Brunnsee and Thalersee), the chlorophyll *a* levels increased with the increment of N fertilization during the peak phase (Table 2). In Lake Brunnsee, we observed a positive relationship between the maximum chlorophyll *a* concentrations and the N fertilization (Table 2), as well as with the average chlorophyll *a* concentrations of the peak phase (Table 2; Fig. 2a). This trend was mainly driven by the chromophytes, which made up >95% of the total chlorophyll *a* concentration levels, and therefore, this group also showed a positive relationship of the chlorophyll *a* peaks with N fertilization ($R^2 = 0.41$, $F = 6.82$, $P < 0.05$). In Lake Thalersee, the

Table 2 Linear regression results (slope, R^2, *P* value) for the maximum total chlorophyll *a* values and the average chlorophyll *a* values of the peak phase versus N fertilization (log-transformed data) of the lakes Brunnsee, Klostersee and Thalersee

	Brunnsee	Klostersee	Thalersee
Maximum total chl *a*	0.61, 0.42, <**0.05**	−0.55, 0.14, 0,23	1.5, 0.29, 0.07
Average chl *a* peak phase	0.44, 0.38, <**0.05**	−1.72, 0.45, <**0.05**	1.09, 0.43, <**0.05**

Bold values are statistically significant ($P < 0.05$)

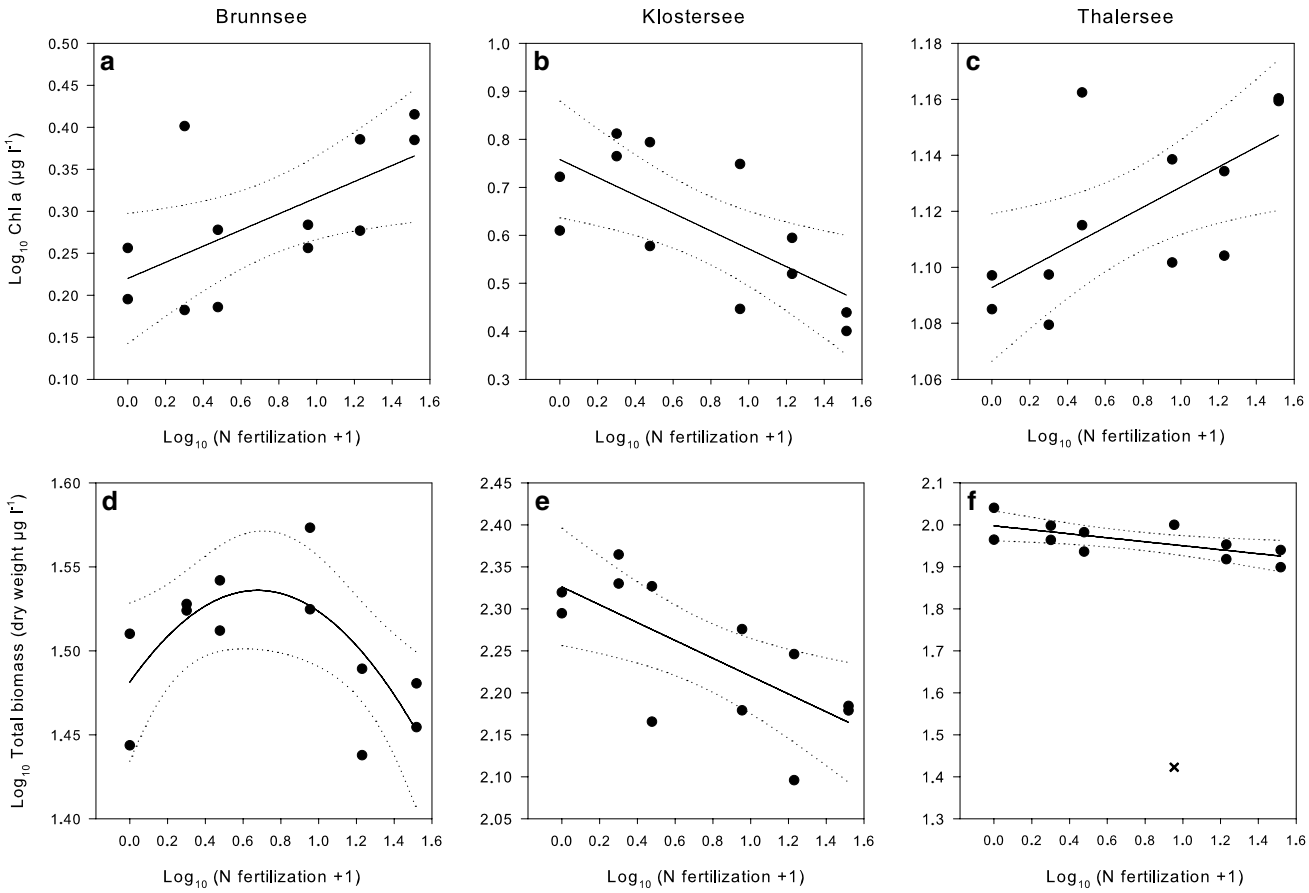

Fig. 2 Observed relationships due to the experimental nitrogen fertilization in lakes Brunnsee (*left*), Klostersee (*middle*) and Thalersee (*right*): the average chlorophyll *a* concentrations (µg l^{-1}) of the peak phase (**a**–**c**) and the total zooplankton biomass (µg l^{-1}) (**d** average biomass, **e, f** peak biomass). Displayed are the significant regression curves (*solid* $P<0.05$; equations in Table 2 for chlorophyll *a* and Table 6 for zooplankton) and the 95% confidence bands (*dotted*); in **f** without the outlier (*cross*)

average chlorophyll *a* concentrations increased with the increment of N fertilization during the peak phase (Table 2; Fig. 2c). In contrast, Lake Klostersee showed a decrease of average chlorophyll *a* concentrations during the peak phase with increasing N fertilization. The regression analyses revealed a significant negative relationship of maximum and average chlorophyll *a* levels with the increment of the N fertilization (Table 2; Fig. 2b).

In terms of the seston stoichiometry, we observed significant changes in the meocosms of Lake Brunnsee and Lake Thalersee due to the N fertilization, but none in Lake Klostersee (Table 3). For Lake Brunnsee, we observed an increase in the particulate C ($R^2=0.04$, $F=4.79$, $P<0.05$) and in the particulate N ($R^2=0.04$, $F=3.97$, $P<0.05$) with the N fertilization, as well as an increase in the seston N:Si ratio (Table 3). A higher seston stoichiometric P limitation with an increasing N fertilization was observed at the time of the chlorophyll *a* peak, where the seston C:P ratios (day 28: $R^2=0.42$, $F=7.38$, $P<0.05$; day 35: $R^2=0.49$, $F=7.58$, $P<0.05$), as well as the seston N:P ratios (day 35: $R^2=0.42$, $F=5.81$, $P<0.05$), significantly increased with the N fertilization. In Lake Brunnsee, the seston C:P ratios over the entire experimental duration correlated significantly with the dissolved N:P ratios ($R^2=0.05$, $F=5.37$, $P<0.05$). In Lake Thalersee, we observed decreasing seston C:N ratios with the N fertilization (Table 3), which might indicate a higher N uptake in the higher fertilized treatments—but there were no changes in the seston C:P, in the N:P, or in the N:Si ratios, when increasing the N load. Over all of the lakes including all of the experimental treatments, a general response of the seston stoichiometry was observed for average seston C:P and N:P ratios, which increased with the dissolved reactive N and the dissolved N:P ratios (Table 4; Fig. 3a), as well as for seston C:N ratios, which decreased respectively (Table 4).

Zooplankton

The community composition in all of the lakes included copepods, cladocerans, and rotifers. In the mesocosms of

Table 3 Seston stoichiometry data of the lakes Brunnsee, Klostersee and Thalersee. Given are the total sample size (N), the average values ± standard deviation and the P values of linear regression results of the mesocosm averages (N = 12) versus the log-transformed N fertilization (significant results bold and with leading sign of the slope)

	Brunnsee			Klostersee			Thalersee		
	N	Av ± Stdev	*P*	N	Av ± Stdev	*P*	N	Av ± Stdev	*P*
C:N	105	7.5 ± 1.5	0.78	84	8.0 ± 1.2	0.58	108	8.2 ± 1.0	**−<0.01**
C:P	107	498 ± 253	0.95	108	348 ± 102	0.55	108	526 ± 154	0.94
N:P	106	63 ± 26	0.13	84	44 ± 16	0.36	108	65 ± 21	0.24
N:Si	46	2.5 ± 0.9	**+<0.01**	48	2.3 ± 1.9	0.37	48	0.6 ± 0.3	0.62

Table 4 Linear regression results of the average seston stoichiometry data (log-transformed) over all the lakes (Brunnsee, Klostersee and Thalersee) versus the dissolved inorganic N (DIN mg L^{-1}) and the log-transformed dissolved N:P ratios (N = 36)

All lakes	slope	R^2	*P*
C:P versus N	+	0.54	**<0.001**
C:P versus N:P	+	0.49	**<0.001**
C:N versus N	−	0.13	**<0.01**
C:N versus N:P	−	0.19	**<0.05**
N:P versus N	+	0.60	**<0.001**
N:P versus N:P	+	0.53	**<0.001**

Given are the leading signs of the slope, R^2 and the *P* values (bold: significant)

Lake Brunnsee, the copepods were on average the most abundant taxonomical group and were followed by the cladocerans (Table 5). On the contrary, in Lake Klostersee and Lake Thalersee, the cladocerans were the most abundant group, followed by the copepods. In all of the lakes, the rotifers were the least abundant taxonomical group. In the lakes Brunnsee and Klostersee, the zooplankton populations increased in all of the mesocosm treatments over the course of the experiment and they reached peak densities after the chlorophyll *a* maxima between the days 35 and 45 (Fig. 1d, e). In Lake Brunnsee, the absolute zooplankton densities remained low compared to the other two lakes, but the cladoceran densities increased until the end of the experiment. In Lake Thalersee, no clear growth phase was observed and the zooplankton numbers showed a high variability and fluctuated between the treatments over the experimental duration (Fig. 1f). The CCA analysis of the data revealed that 80.2% of the variance in the entire data set could be explained by the first axis ($P < 0.01$), which was first correlated with the TP (R = 0.54) and then with the dissolved N:P ratios (R = −0.16) (Fig. 4). The remaining 19.2% of the variance in the data set was explained by the second axis, which was correlated with the highest of the dissolved reactive N (r = −0.25), followed by the dissolved N:P ratios (R = −0.19) and the TP (R = 0.14). The analysis supports that samples from TP-rich and low N:P environments, as in Lake Thalersee, were generally characterised by the presence of cladocerans, whereas the copepods were associated with the higher dissolved N:P ratios, as found in Lake Brunnsee (Fig. 4). The nauplii held an intermediate position and since the Thalersee nauplii started to grow at the end of the experiment under the highest treatment N concentrations, they plot together with the high dissolved reactive N (Fig. 4).

We found decreasing zooplankton densities and their biomasses with a higher N fertilization in all the lakes (mainly negative slopes in Table 6, Supplementary Table S3). However, significant zooplankton parameters

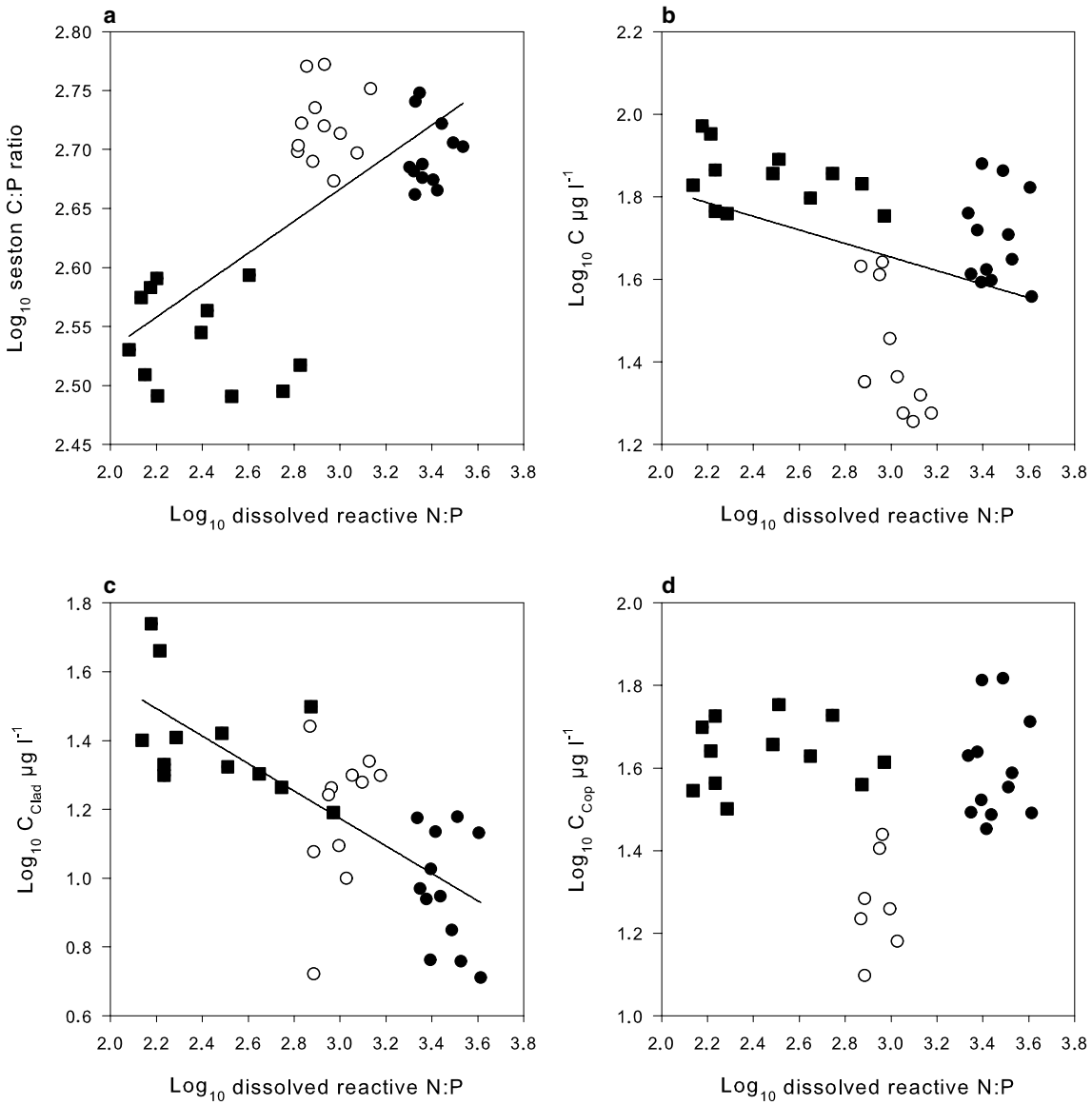

Fig. 3 Regression analyses of **a** the seston C:P ratios, **b** the total zooplankton biomass (C µg l^{-1}), **c** the cladoceran biomass (C µg l^{-1}), and the copepod biomass (C µg l^{-1}) versus the dissolved reactive N:P ratios in the mesocosms (n = 36). Linear regressions: **a** R^2 = 0.49, $P < 0.001$ (Table 4); **b** R^2= 0.13, $P < 0.05$ (Table 7); **c** R^2= 0.53, $P < 0.001$ (Table 7). *Black squares* Lake Klostersee, *circles* Lake Thalersee, *dots* Lake Brunnsee

differed between the lakes. In Lake Klostersee and Lake Thalersee, the peak zooplankton was linearly decreasing over the N fertilization gradient (Table 6, Supplementary Table S3; Fig. 2e, f). In Lake Brunnsee, declining average zooplankton was observed in the N treatments higher than 8 times the natural wet deposition due to a unimodal relationship (Fig. 2d; Table 6). For Lake Brunnsee, this unimodal trend was also found in the nauplii abundances (Supplementary Table S3). In Lake Klostersee, the nauplii (Table 6, Supplementary Table S3) and the cladocerans (Table 6, Supplementary Table S3 and the carbon biomass average: R^2 = 0.38, $F = 6.14$, $P < 0.05$, and peak: R^2= 0.59, $F = 14.46$, $P < 0.01$) were negatively affected and showed a significant decrease with the N fertilization in the average, and in case of cladocerans the peak parameter. In Lake Thalersee, only the cladocerans were negatively affected by the N fertilization and declined (Table 6, Supplementary Table S3). Interestingly, in Lake Brunnsee and in Lake Klostersee, the rotifer biomass showed some positive relationship with the increased N fertilization (Table 6, Supplementary Table S3). The average zooplankton biomass after day 43 (carbon biomass data from elemental analyses as well as the calculated zooplankton biomass from microscopic countings) showed a declining relationship

Table 5 Composition of the zooplankton community of the lakes Brunnsee, Klostersee and Thalersee

	Brunnsee (%)	Klostersee (%)	Thalersee (%)
Calanoid copepods	30.3	16.1	3.7
Cyclopoid copepods	11.7	6	14.7
Nauplii	16	8.4	29.6
Daphnia sp	14.6	14	6.3
Bosmina longirostris	3.8	36.4	40.9
Ceriodaphnia sp	3.9	3.4	2
Pseudochydorus sp	–	1.2	0.6
Rotifers	19.7	14.5	2.2

Given are percentages of most abundant species and taxonomical groups. Rotifers comprised mainly of the species *Kellicottia sp*., *Filinia longiseta, Keratella sp*., and *Asplanchna sp*.

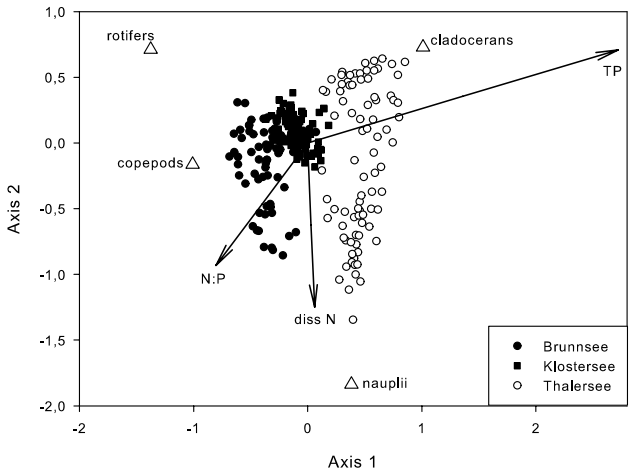

Fig. 4 Canonical correspondence analysis of the log transformed absolute zooplankton abundances (Ind l^{-1}) (calanoid copepods, cladocerans, rotifers, nauplii) and the explanatory variables (TP, dissolved N:TP ratios, dissolved reactive N) over the entire experimental duration (n = 252, $P < 0.01$). *Black squares* Lake Klostersee, *circles* Lake Thalersee, *dots* Lake Brunnsee

over all of the lakes across a wide range of dissolved N:P ratios (wider than experimentally achieved within each lake) (Table 7; Fig. 3b, dry weight µg l^{-1} versus dissolved N:P: $R^2 = 0.72$, $P < 0.001$). This overarching pattern was observed in the total carbon biomass of cladocerans (Fig. 3c) but not in the total carbon biomass of copepods (Fig. 3d).

Discussion

Our study indicates that an increased N load influences the food-web dynamics in pelagic ecosystems that are P deficient. All mesocosm experiments showed measurable effects of N enrichment, although they were conducted in three lakes with differences in nutrient concentrations, dissolved N:P ratios ranging from >50:1–1000:1, and differences in other lake parameters such as depth, water exchange and plankton community assemblages. An in situ bioassay study (Ilic 2014) characterized the lakes Brunnsee and Klostersee as primary P limited (classification of Andersen et al. 2007) with synergistic N + P co-limitation effects. This characterization is in accordance with findings in most freshwater systems (Elser et al. 1990, 2007). The experiments showed effects on zooplankton and chlorophyll *a*, although they received much lower N fertilization amounts in contrast to previous eutrophication studies (0.33 mg l^{-1} N per week in the highest 32 N treatment compared to 6 mg l^{-1} N per week in Donald et al. 2013). The resulting N concentrations lie within the naturally observed total N concentrations in Scandinavian lake systems under an increased N deposition (Elser et al. 2009a).

It was not necessarily expected that under the already high environmental N:P conditions effects of N enrichment on the zooplankton would be measurable. During spring succession each individual lake experiences a diversity of interacting mechanisms, which are affecting the lake's biology over all trophic levels (Sommer et al. 2012). Nevertheless, we observed declines of zooplankton at high fertilization levels in all lake experiments. The qualitative relationships of N load and zooplankton were markedly lake specific (two were linear, one was unimodal) and restricted to distinct zooplankton parameters (2-times peak, once average biomass). Thereby, Lake Brunnsee, with highest dissolved N:P ratios, showed weakest responses of zooplankton to N fertilization, and Lake Klostersee, with lowest dissolved N:P ratios, showed strongest responses compared to the other lakes (Table 6). Additionally, a negative correlation of the total zooplankton biomass with dissolved N:P ratios was seen over the entire data set including all treatments from the three lakes (Table 7; Fig. 3b). Despite this experimental variability, the observed relationship of N load and zooplankton also seems to occur across all three investigated lakes. This general relationship supports the idea that trophic transfer of increasing N load to the zooplankton community follows an overarching trend but does so via lake-specific mechanisms.

A negative effect of the N enrichment was primarily expected for the cladocerans, which have typically higher P requirements, but to a lesser degree for the copepods with typically higher N requirements (Sterner and Hessen 1994). Indeed, a negative relationship between N load (and dissolved N:P ratios) and cladocerans existed over all of the lakes (Table 7; Fig. 3c) but was expressed in different magnitudes in two lakes (Table 6). Strongest effects of N enrichment on cladoceran dry weights were observed in the peak biomass of the mesotrophic Lake Klostersee (Table 6). The generally supposed constraints of the N and P requirements

Table 6 Zooplankton statistics of the taxonomic groups: regression results of the biomass (µg l^{-1}) versus N fertilization (log-transformed) of average and peak densities (N = 12)

Zooplankton	Brunnsee				Klostersee				Thalersee			
Data	Model	Slope	R^2	P	Model	Slope	R^2	P	Model	Slope	R^2	P
Average												
Total	2		0.50	*	1	–	0.17	0.19	1[a]	–	0.17	0.21
Copepoda cal	1	–	0.03	0.61	1	–	0.01	0.80	1	–	0.11	0.33
Nauplii	1	–	0.15	0.21	1	–	0.43	*	1	–	0.19	0.18
Cladocera	1	–	0.07	0.42	1	–	0.42	*	1 [a]	–	0.18	0.19
Rotifera	1	+	0.25	0.1	1	+	0.58	**	1	+	0.01	0.73
Peak												
Total	1	+	0.04	0.53	1	–	0.51	**	1[a]	–	0.45	*
Copepoda cal	1	–	0.09	0.36	1	–	0.14	0.23	1	–	0.30	+
Nauplii	1		0.11	0.29	1	–	0.07	0.42	1	–	0.19	0.19
Cladocera	1	–	0.00	0.80	1	–	0.77	***	1[a]	–	0.45	*
Rotifera	1	+	0.41	*	1	+	0.80	***	1	+	0.03	0.62

Regression models are either linear regression (1) or Gauss fit (2). Gauss fit was used when Levene's test failed for the linear regression assumption. Leading signs of linear regression slopes are indicated as positive (+) or negative (−). The coefficient of determination (R^2) as well as the P-value (+: <0.1, *<0.05, **<0.01, ***<0.001) for the regression fit is given

[a]Outlier (enclosure 8) removed

of cladocerans compared to copepods are supported by the multivariate analysis. In the data set over all three lakes, the cladocerans clearly correlate to a low N:P ratio and the copepods to higher N:P conditions (Fig. 4). Subsequently, with increasing N:P ratios, the cladocerans are expected to be the first zooplankton group to be negatively affected. This is indeed true for Lake Thalersee and Lake Klostersee (Table 6, adjoining lakes in Fig. 4), which were richer in cladocerans (Table 5). In case of Lake Brunnsee and Lake Klostersee (adjoining lakes in Fig. 4) nauplii were sensitive to increased N load (Table 6, Supplementary Table S3). There is evidence for commonalities between the cladocerans and the nauplii, which both have low N:P body stoichiometry (<12:1), indicating high P demands (Andersen and Hessen 1991; Carrillo et al. 2001; Sterner and Elser 2002; Meunier et al. 2015), and similar optimal food size spectra (Hansen et al. 1994). The seston N:P ratios in this study were >44:1 (Table 3) indicating non-favourable food conditions for filter feeders with low biomass N:P ratios. Therefore, by increasing the P deficiency due to N enrichment, it is conceivable that this directly affects these two zooplankton groups, although it remains to be verified if the copepod egg production and/or the hatching success indirectly affect the nauplii abundances.

In stoichiometric theory, a trophic transfer effect of an N enrichment to the zooplankton can be related to a decrement of food quality in terms of seston stoichiometry (Hessen et al. 2013). This negatively affected the zooplankton in Lake Brunnsee, since in this lake increasing seston C:P ratios were observed. However, previous investigations of other lakes also revealed that higher dissolved N:P resource supply ratios, together with an increased P deficiency, were not necessarily reflected in a significantly higher seston N:P stoichiometry (Elser et al. 2009a). Similarly, it has been shown that zooplankton nutrient recycling can diminish the seston stoichiometric signatures despite having very different initial nutrient supply ratios (Trommer et al. 2012). Therefore, the absence of clear responses of seston stoichiometry to an N enrichment within the lakes Klostersee and Thalersee, does not necessarily mean that trophic food quality effects were not present. The role of seston stoichiometry as an underlying ecological mechanism for the observed negative zooplankton response in our study was supported by the increasing C:P (and N:P) ratios with dissolved N:P ratios over all of the lakes (Table 4; Fig. 3a). The negative effects of seston C:P ratios >300:1 (Urabe et al. 1997) and across a range of similar seston C: P ratios of natural lakes (Brett et al. 2000) have already been observed for the growth of the daphnid species.

Table 7 Linear regression results of the mesozooplankton biomass data over all the lakes (Brunnsee, Klostersee and Thalersee) (N = 36) versus the dissolved inorganic N (DIN mmol l^{-1}) and the dissolved N:P ratios (log-transformed)

C µg/l	N		N:P	
	R^2	P	R^2	P
All Mesozoo	0.21	**<0.01**	0.13	**<0.05**
All Copepoda	0.01	0.12	0.02	0.47
All Cladocera	0.40	**<0.001**	0.53	**<0.001**

Given are R^2 and the P values (bold: significant)

Other than the seston stoichiometry, the food quality transfer to the higher trophic levels can also be related to other energetic pathways. It is for example known that the fatty acid composition of algae plays an essential role for food quality and can significantly influence the growth of *Daphnia sp*. (Müller-Navarra et al. 2000; Wacker and Elert 2001). There are indications that the abundance of fatty acids correlates with the relative P availability for algae to some extent, since some species grown in P-limited media produce less essential fatty acids than in P saturated media (Müller-Navarra 1995). Additionally, *Daphnia* growth is seasonally stronger correlated to specific fatty acid concentrations than seston C:P ratios (Wacker and Elert 2001), which might also be related to physiological adaptations to high energy and low nutrient environments (Mulder and Bowden 2007). Higher metabolic costs from higher feeding activity (Plath and Boersma 2001), higher respiration rates (Darchambeau et al. 2003) or higher alkaline phosphatase activity (Elser et al. 2010; McCarthy et al. 2010) could also have contributed to an energetic mismatch for cladocerans in the lakes Klostersee and Thalersee through an increasing P deficiency.

The immediate causes for unfavourable growth conditions for the zooplankton under a high N fertilization might also be related to the toxic effects of high N concentrations. However, the highest NO_3 concentrations (5.7 mg l^{-1} NO_3–N in the Lake Brunnsee) did not reach critical values for the cladocerans (>14 mg l^{-1} NO_3–N, Camargo et al. 2005). In terms of an NH_4 toxicity, concentrations >0.6 mg l^{-1} ammonia nitrogen (NH_3–N) can result in chronic toxicity for *Daphnia magna* (Gersich and Hopkins 1986). These are higher than the NH_3–N concentrations in our experiments (<0.2 mg l^{-1} NH_3–N at 1.7 mg l^{-1} NH_4–N, pH 8, <20 °C).

In terms of effects of food quantity for zooplankton, the phytoplankton biomass was hypothesised not to change with higher N fertilization, since the dissolved N:P ratios were clearly above Redfield ratios in all of the lakes. Nevertheless, we found positive relationships of chlorophyll a with N fertilization in Lake Brunnsee and Lake Thalersee. However, the chlorophyll *a* content per cell can be variable (Paasche 1971; Levasseur et al. 1993; Poxleitner et al. 2016), and chlorophyll *a* per phytoplankton biomass does vary with the community composition (Felip and Catalan 2000; Schindler et al. 2008). Besides the indirect responses of phytoplankton to herbivore grazing pressure (Sommer et al. 1986), the addition of NH_4 in our fertilization solution might have promoted the growth of certain algal taxa (Donald et al. 2013; Glibert et al. 2016) or altered the chlorophyll *a* content per cell (Collos and Harrison 2014). It is known that the utilisation of NH_4 and NO_3 is highly species specific. It can range from a preference of NH_4 to the inhibition of the NO_3 uptake (Dortch 1990), and thus, may change the community depending on the original phytoplankton composition. Therefore, detailed investigations on the phytoplankton community composition shifts that are caused by different NO_3:NH_4 supply ratios are required.

This study suggests that N amounts in the range of an increased atmospheric deposition can lead to changes of food-web dynamics in P deficient ecosystems. Effects of increased N load are well known with focus on phytoplankton; mainly in studies with regard to eutrophication (e.g. Paerl 2009; Rabalais et al. 2009; Donald et al. 2013) with fewer studies in unproductive systems (Bergström et al. 2005; Poxleitner et al. 2016). Here, we observed a decline in the mesozooplankton with N enrichment across all of the three lakes and within the experimental manipulations. The data indicate that increasing dissolved N:P ratios due to N enrichment are able to negatively affect zooplankton, most notably, the cladocerans. The observed lake-specific responses of the different taxa would imply that N enrichment could result in community composition changes, towards a more copepod and rotifer dominated community, containing fewer cladocerans. Since the cladocerans typically represent the most convenient food source for the planktivorous fish (Brooks 1968; Vanni et al. 1987), the predicted progressing P deficiency in ecosystems (Vitousek et al. 2010; Goll et al. 2012) may cause deteriorating food conditions for fish populations.

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PUBLICATION III

Impacts of increasing nitrogen:phosphorus ratios on zooplankton community composition and whitefish (Coregonus macrophthalmus) growth in a pre-alpine lake

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Impacts of increasing nitrogen:phosphorus ratios on zooplankton community composition and whitefish (*Coregonus macrophthalmus*) growth in a pre-alpine lake

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Abstract

1. The combination of increasing atmospheric depositions of reactive nitrogen (N) and the highly effective diminishing of external phosphorus (P) loadings can change key nutrient ratios in lake ecosystems. Consequently, ratios of dissolved inorganic N (DIN) to dissolved P (DP) in lakes are increasing. However, potential consequences for aquatic organisms are as yet far from understood.
2. We formulated three hypotheses on the potential effects of rising DIN:DP ratios on a lake food web: (1) increasing DIN:DP ratios intensify the P limitation of phytoplankton communities and lower their food quality; (2) densities of P rich zooplankters (e.g. cladocerans) will be negatively affected by P-limited food algae; (3) as result, planktivorous fish will experience a reduction of their main prey (especially *Daphnia* species) and respond with lowered growth.
3. These hypotheses were tested in a mesocosm experiment conducted in a pre-alpine lake in southern Germany, Lake Brunnensee. For 76 days, the natural phytoplankton and zooplankton communities were exposed to a wide gradient of DIN:DP ratios. At the end of the experiment, juvenile planktivorous whitefish (*Coregonus macrophthalmus*) were released into the mesocosms and allowed to feed on zooplankton communities for 72 hr.
4. Along the gradient of DIN:DP ratios, we found evidence for a rising P limitation of autotroph growth, which was indicated by increasing ratios of N:P (15:1–157:1) and C:P (91:1–797:1) in seston biomass. The rising P limitation in algae reduced the nutritional food quality for the majority of herbivorous zooplankton. Both the total zooplankton biomass and the *Daphnia* biomass declined substantially with increasing DIN:DP ratios. In contrast, increasing DIN:DP ratios favoured rotifer species, showing strong positive correlation with rotifer biomass. Whitefish weights decreased with increasing rotifer biomass and increased with rising *Daphnia* biomass in zooplankton communities.
5. In summary, our results provide an experimental demonstration that increasing DIN:DP ratios can cause stoichiometric shifts in the biomass of primary producers towards higher N:P and C:P ratios. Effects on zooplankton were changes in the taxonomical community composition towards lower cladoceran biomass (mainly *Daphnia* spp.). The reduction in *Daphnia* biomass in turn caused significantly

reduced growth rates of whitefish in our experiment. Our experimental results therefore support the assumption that stoichiometric effects can travel up the food chain. General consequences of such multi-trophic effects induced by altered nutrient ratios could be potentially visible during re-oligotrophication of water bodies, often resulting in high N:P ratios. Further empirical studies could look for signatures of these effects on the yield of economically important species.

KEYWORDS
nitrogen, phosphorus, pre-alpine lake, whitefish growth, zooplankton

1 | INTRODUCTION

Over the decades, anthropogenic activities such as fertiliser production, the cultivation of legumes and the use of combustion engines have substantially exceeded the natural production of reactive nitrogen (N) compounds (Ciais et al., 2013). Transported through the atmosphere, N is easily distributed and deposited in extensive amounts in terrestrial and aquatic ecosystems (Schlesinger, 2009), even in remote areas (Galloway, Schlesinger, Levy, Michaels, & Schnoor, 1995). The ongoing atmospheric N deposition implies changes in biogeochemical cycles, for which lakes are important sentinels (Adrian et al., 2009). Key nutrients of lake ecosystems that are indispensable for primary production are phosphorus (P) and N (Hecky & Kilham, 1988). However, the limiting nutrient for phytoplankton growth in lakes remains primarily P (Schindler, 1977). Therefore, an additional N load would not necessarily be considered to have observable and evident effects on a P-limited lake food web. In fact, the total concentration of a single limiting nutrient is not the only determinant for autotrophic growth. Phytoplankton growth is, apart from physical (light, temperature, others) and chemical factors (e.g. carbon, macro- and micronutrients; Lampert & Sommer, 2007), also dependent on the ratio in which nutrients are supplied (Rhee, 1978). Recently, N loadings into lakes by groundwater fluxes or airborne wet deposition are increasing on a global scale (Galloway et al., 1995; Hampton et al., 2018; Vitousek, Mooney, Lubchenco, & Melillo, 1997). However, in European pre-alpine lakes at the same time P depositions are decreasing as a result of effective re-oligotrophication programs (Gerdeaux, Anneville, & Hefti, 2006; Jeppesen et al., 2005). Thus, the concentrations of key nutrients in lake ecosystems are shifting, with increasing ratios of dissolved inorganic N (DIN) to dissolved P (DP) as result.

Organisms and ecosystems are closely linked through their specific elemental compositions (stoichiometry; Sterner, 1995), and any changes in stoichiometry would be expected to affect organisms and their trophic interactions. In general, ideal stoichiometric conditions for the growth of primary producers in aquatic ecosystems are given at an ambient nutrient ratio of N:P = 16:1 (by atoms), which is commonly known as the *Redfield ratio* (Redfield, 1958). However, high deviations from this ratio can be found in the biomass composition of various phytoplankton taxa. Most phytoplankton species are extremely flexible and able to adjust their stoichiometric N:P ratios to ambient nutrient conditions (Sommer et al., 2012). Thus, high concentrations of dissolved N in ambient water can cause higher N:P ratios in phytoplankton biomass. These variations in phytoplankton N and P contents are known to have strong effects on the food quality for herbivorous zooplankton (Andersen & Hessen, 1991; Hessen, 1992; Sterner, 1990). In contrast to phytoplankton, herbivorous zooplankton taxa require a relatively constant N:P ratio in diet (Sterner & Elser, 2002), and the range of adequate N:P ratios for sustainable growth appears to be narrow. In addition, different zooplankton taxa have different optimal biomass N:P ratios (Sterner & Elser, 2002). For example, cladocerans have N:P ratios between 12 and 18, whereas calanoid copepods have much higher N:P ratios of 30 and above (Andersen & Hessen, 1991; Carillo, Villar-Argaiz, & Medina-Sánchez, 2001; Hessen & Lyche, 1991). This means that both the increasing DIN:DP ratios in lake water as well as the accompanying rising N:P ratios in algal food can induce stoichiometric constraints, first and foremost for *Daphnia* (Trommer et al., 2017) and other cladocerans. Rotifers also have a preference for low N:P ratios in food (Rothhaupt, 1995), implying comparable stoichiometric mismatches for rotifers if DIN:DP ratios increase. However, it is known that, in lakes with high abundances of cladocerans such as *Daphnia*, rotifers are suppressed through competition (Gilbert, 1988a and references therein). In contrast, if large cladocerans are removed, for example by visually and size-selectively feeding planktivorous fish, rotifers can immediately increase in abundance (Brooks & Dodson, 1965; Gilbert, 1985; Hall, Threlked, Burns, & Crowley, 1976). This in turn means that, if increasing N:P ratios in phytoplankton biomass cause decreasing cladoceran densities, rotifers would derive benefit from competitive advantage. It is, however, not clear whether high N:P ratios in food algae could offset the advantage for rotifers and if rotifers should actually increase in abundance.

In general, zooplankton act as both phytoplankton grazers and prey for planktivores (Brooks, 1968; Brooks & Dodson, 1965). Therefore, taxonomic shifts in zooplankton community composition can also impact organisms at higher trophic levels. For size selective planktivores, such as numerous fish species, shifts in zooplankton communities from cladoceran dominance towards dominance by

small rotifer species implies a reduction in prey densities. In temperate lakes, the majority of fish species could be affected by those shifts, since they feed mainly on zooplankton, at least as juveniles (Lampert & Sommer, 2007).

Coregonus species (whitefish) are the dominant fish species in the majority of Bavarian pre-alpine lakes and one of the most important species for commercial fisheries (Mayr, 2001). Most whitefish use zooplankton and in particular cladocerans as a principal food source at all stages of their life cycle (Bergstrand, 1982; Eckmann, 2013; Kahilainen, Malinen, Tuomaala, & Lehtonen, 2004). Variations in nutrient concentrations are known to impact resident whitefish (Eckmann, 2013). It has been demonstrated in various studies that decreasing P concentrations lead to lower fish production and lower yields for fisheries (Eckmann & Rösch, 1998; Müller & Bia, 1998). The recently reported slowdown in whitefish growth (Schubert & Härth, 2017) together with the dropping yields of fisheries in pre-alpine lakes (Koops, 2016) further underline the relevance of this topic. However, despite the large number of existing studies about dynamics of whitefish, there still remain many open questions. Above all, any potential impacts on whitefish resulting from shifts in the concentrations of key nutrients are still far from understood (Eckmann, 2013).

In this study, we investigated the effects of increasing DIN:DP ratios on different trophic levels of the food web using three hypotheses. (1) Increased N input in primarily P-limited lakes intensifies phytoplankton P limitation and subsequently decreases food quality for P rich zooplankton such as cladocerans. (2) Decreased stoichiometric food quality changes the taxonomic composition of zooplankton communities towards increasing abundances of species with low P demands (copepods) and decreasing abundances of zooplankters with high P demands (cladocerans). (3) The effects of rising DIN:DP ratios on lake plankton are passed on to planktivorous whitefish.

To test these hypotheses, we conducted a mesocosm experiment in an oligotrophic, primarily P-limited lake in southern Germany. An artificial gradient of DIN:DP ratios was established by fertilising mesocosms with different amounts of N (nitrate NO_3^- and ammonium NH_4^+), reflecting multiples of the present atmospheric N deposition. The effects of increasing DIN:DP ratios on lake food webs were investigated at various trophic levels, including phytoplankton, zooplankton, and planktivorous fish.

2 | METHODS

2.1 | Study site and experimental design

The mesocosm experiment was performed in Lake Brunnensee, which is located in Upper Bavaria, Germany (47°59′03″N, 12°26′09″E). It has an area of 5.8 ha, a maximum depth of 19 m and an average depth of 8.5 m. During the experimental period, from 11 March (Julian day [JD] 70) to 26 May (JD 146) 2014 (duration: 76 days), the lake water had average concentrations of total P (TP) = 8.7 ± 1 (±1 *SD*) µg/L, NO_3^- = 19.6 ± 0.4 (±1 *SD*) mg/L and NH_4^+ = 71.1 ± 132.2 (±1 *SD*) µg/L.

The cylindrical enclosures (diameter 0.95 m, depth 4 m, volume 2,835 L) were made out of a white silage foil (thickness 150 µm; Afolen-Standard; Biofol Film GmbH, Germany). They were closed at the bottom and open to the atmosphere. Enclosures were filled with lake water by lifting them from 4 m depth to the surface. In order to establish a wide range of DIN:DP ratios, the experiment was based on a gradient fertilisation design with increasing DIN supply. Four fertilisation treatments were applied, with three replicates per treatment (enclosures: n = 12) and two fertilisation procedures per week. The quantity of N fertilisation was calculated as a multiple (0 × N, 1 × N, 4 × N, 16 × N; Figure 1) of the N amount deposited into the lake by natural precipitation (25 L/m^2 of precipitation with a concentration of 3 mg/L NO_3^- and 1 mg/L NH_4^+). These data were based on NO_3^- and NH_4^+ measurements in our own local rain samples of the years 2012 and 2013, which were verified by regional data from the German Meteorological Service and the Bavarian State Office for the Environment.

As fertiliser, a stock solution containing NO_3^- and NH_4^+ in form of sodium nitrate ($NaNO_3$) and ammonium chloride (NH_4Cl) was applied in a molar ratio of 1:1. To compensate for natural nutrient loss through sedimentation, low background fertilisation was given twice a week. The amount of nutrient input as background fertilisation was equivalent to a TP loss of 0.06 µg L^{-1} day^{-1}. This was measured in a previous mesocosm experiment conducted in the same lake, during the same season and with an identical mesocosm size (Poxleitner, Trommer, Lorenz, & Stibor, 2016). The background TP fertilisation was supplemented by N (NO_3^-:NH_4^+ = 1:1 [mol:mol]) and Si input in the molar Redfield Ratio (P:N:Si = 1:16:16; Redfield, 1958). For fertilisation, the appropriate amounts of stock solution were transferred into 1-L PE bottles, filled up with distilled water and poured into the enclosures. To guarantee an equal nutrient distribution within the water column, all enclosures were vertically mixed with a Secchi disk immediately after fertilisation. In order to promote the establishment of different DIN concentrations right from the beginning of the experiment, a nutrient pulse, equivalent to 2 weeks of the DIN fertilisation amounts for respective treatments, was given on the first fertilisation event on 13 March (JD 72).

2.2 | Sampling procedures and analyses

Samplings for nutrients, zooplankton, chlorophyll *a*, total seston biovolume, and total seston cell densities were carried out once per week. Water samples for the analyses of nutrients and phytoplankton parameters were taken with a 2-m long integrated water sampler (Integrated tubular water sampler; KC Denmark A/S Research Equipment, Denmark). Integrated water samples were taken from the centre of each enclosure at 1–3-m water depth. Water was pre-filtered (250 µm) to exclude large mesozooplankton and transported in darkness to the laboratory for further analyses. Ammonium was measured using a fluorometric method modified after Holmes (Holmes, Aminot, Kérouel, Hooker, & Peterson, 1999). An aliquot of 2.5 ml enclosure water was mixed with 10 ml

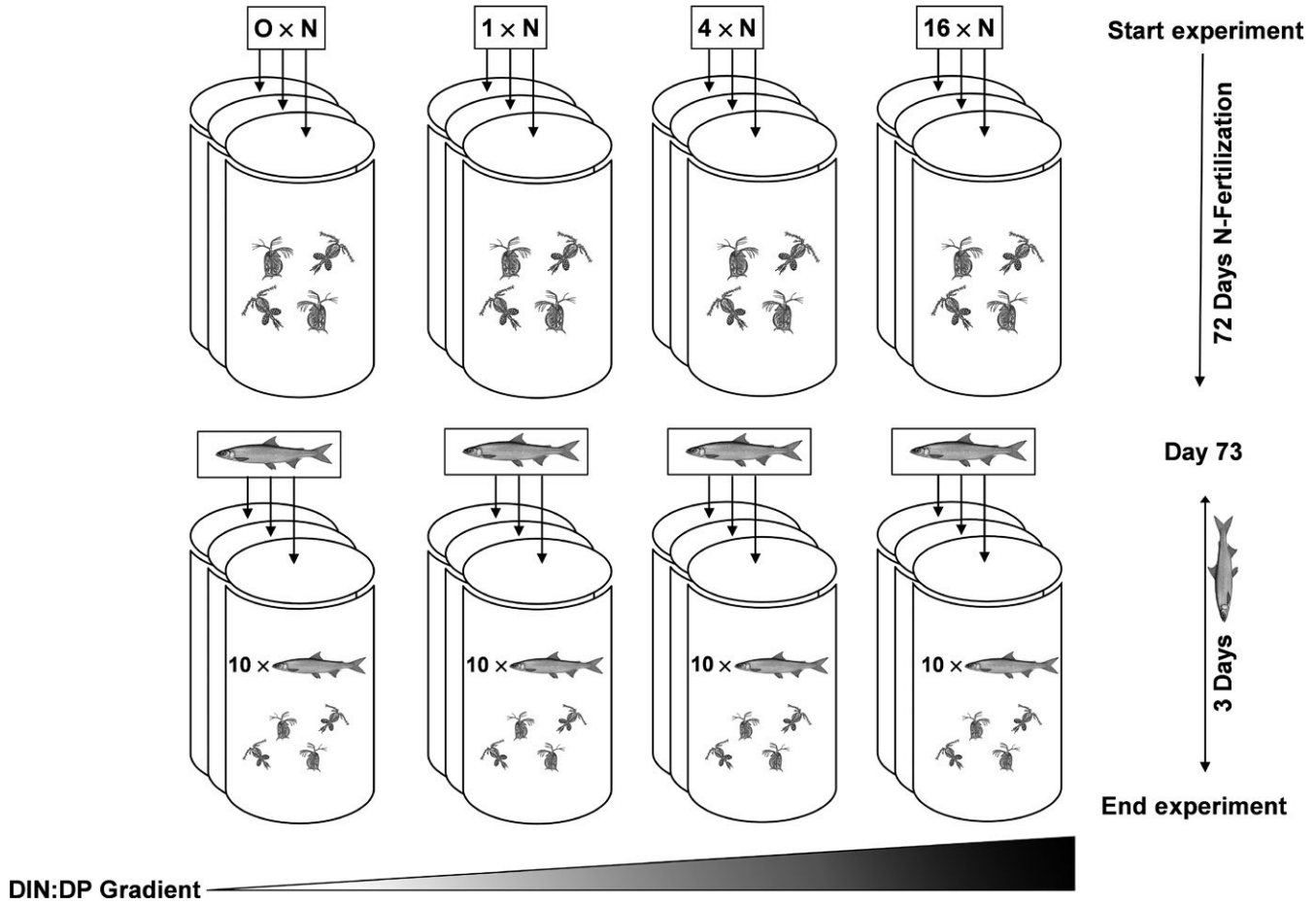

FIGURE 1 Schematic illustration of the experimental design applied in this mesocosm experiment. We used four N addition treatments with three replicates per treatment. The mesocosms (V = 2,835 L) received different N amounts (calculated as a multiple of natural N deposition: 0 × N, 1 × N, 4 × N, 16 × N) two times per week. After 72 days (*c*. 10 weeks) of N fertilisation, a large DIN:DP gradient could be established along the four treatments. From day 73 until day 76 (end of experiment), 10 juvenile whitefish (*Coregonus macrophthalmus*) were kept in every enclosure. Whitefish were allowed to feed on zooplankton communities grown in different DIN:DP regimes for 72 hr. On day 76, all whitefish were recaptured and brought to the laboratory for further analyses

of working reagent in scintillation vials. The working reagent was composed of borate buffer (40 g/L), sodium sulfite (40 mg/L), and orthophthaldialdehyde in ethanol (EtOH; 50 ml/L). The samples were incubated for 2 hr in darkness and afterwards measured fluorometrically (Trilogy Laboratory Fluorometer Module CDOM/NH4; Turner Designs, USA). To determine nitrite (NO_2^-) and NO_3^- concentrations, an aliquot of enclosure water was pre-filtered with a 0.45-µm cellulose acetate membrane filter (CS 400 Syringe Filters Cellulose Acetate 0.45 µm; Nalgene, USA) and measured with an ion-chromatograph (Dionex ICS-1100 Basic Integrated IC System; Thermo Scientific, USA). Total phosphorus was measured with a spectrophotometer (Shimadzu UV-1700; Shimadzu Corporation, Germany) using the molybdenum blue method (Wetzel & Likens, 1991). The N compounds for DIN were calculated as the sum of the concentrations (mol/L) of NH_4^+-N, NO_3^--N and NO_2^--N in the enclosure water. For DP, the difference in concentrations (mol/L) from TP to particulate P (PP) was used (TP – PP = DP). Thus, DP includes all dissolved organic and inorganic P compounds of the enclosure water.

2.3 | Phytoplankton

Measurements of the chlorophyll *a* concentration and taxonomic composition of phytoplankton communities were determined fluorometrically in vivo by using an AlgaeLabAnalyser (bbe Moldaenke GmbH, Germany). To examine the composition of phytoplankton communities, the taxa were, according to their excitation fluorescence spectra, grouped into five pigment groups, green algae, cyanobacteria, diatoms, cryptophytes (Beutler et al., 2002). Water subsamples were fixed in Lugol's iodine for further analyses of total seston cell densities and total seston biovolumes. Total seston cell densities and total seston biovolumes were measured in fixed water samples by using a CASY cell counter with a 150 µm capillary (CASY1 Modell TTC; Schärfe System, Germany); hence, seston cell counts and seston cell volumes were used as a proxy for phytoplankton cell densities and biovolumes.

To quantify seston C (particulate organic C) and N (particulate N), 100–300 ml of enclosure water was filtered onto pre-combusted (4 hr, 450°C), acid washed (10% HCl) glass microfiber filters with a

pore size of 0.7 μm (GF/F; Whatman, USA). To evaporate the inorganic C on filters as CO_2, filters were treated for 30 s with 5 ml of 0.5 molar dihydrogenphosphate acid (H_2PO_4). After filtration, filters were wrapped in aluminium foil and stored frozen (−18°C) until further analyses. For the measurements, all filters were defrosted, dried (60°C, 24 hr), compacted in tin foil and burned in a CN analyser (vario MICRO cube; Elementar Analysensysteme GmbH, Germany). For the determination of seston P (particulate P) concentrations, the water samples were filtered on pre-combusted (4 hr, 450°C), acid washed (10% HCl) glass microfiber filters with a pore size of 0.7 μm (GF/F; Whatman, USA). The filters were wrapped in aluminium foil and stored frozen (−18°C) until the PP amount was quantified with a spectrophotometer using the molybdenum blue method (Wetzel & Likens, 1991). Subsequently, the seston C:N:P ratios were calculated and used as a proxy for phytoplankton stoichiometry to evaluate the nutritional food quality of phytoplankton for herbivorous grazers.

2.4 | Zooplankton

For zooplankton sampling, a plankton net with a mesh size of 105 μm was hauled up from the bottom of the enclosure to the surface. Samples were immediately filled into 100-ml PE bottles, which were prepared with a fixative containing sugar, glycerine, and EtOH (EtOH final concentration 70%). Samples were stored in the refrigerator (4°C) until microscopic analyses were performed. Species were determined, individuals counted, and lengths measured by using a light stereomicroscope (WILD M3Z; WILD Heerbrugg, Switzerland). To measure lengths, an ocular micrometer with a 0.1 mm division scale was used. Dependent on zooplankton densities, some samples were split one or two times using a zooplankton splitter built after Motoda (1959). At least 400 specimens were counted per sample. To evaluate *Daphnia* egg production, the number of eggs and embryos of 20 randomly chosen individuals (if sufficiently present in the sample) were counted by using a light stereomicroscope (WILD M3Z; WILD Heerbrugg, Switzerland).

To assess the zooplankton biomass, length–weight relationships were used as a function of mean measured taxa lengths or taxa dry weights given in the literature. The biomass of crustaceans was estimated by applying Equation (1) for length–weight regressions:

$$\mathrm{Ln}\,(w) = \mathrm{Ln}\,(\alpha) + \beta\,\mathrm{Ln}\,(L), \tag{1}$$

where w = dry weight in μg, L = length in mm, β = regression slope, and Ln (α) = intercept. Coefficients for the terms Ln (α) and β were chosen from Bottrell et al. (1976), Dumont, Van de Velde, and Dumont (1975), and Michaloudi (2005). The biomass of rotifers was estimated by using the mean values of given dry weights by Bottrell et al. (1976) and Pauli (1989).

2.5 | Fish-feeding experiment

The juvenile whitefish (*Coregonus macrophthalmus*) were approximately 3 months old, reared in net enclosures in the nearby Lake Chiemsee, and raised with natural lake zooplankton. Prior to their introduction into the mesocosms, all fish were kept in net enclosures in Lake Brunnensee for 3 days (72 hr) to allow adaption. On day 73 (JD 143) after 10 weeks of N fertilisation, 10 randomly chosen fish were introduced in every enclosure (Figure 1). On the same day (day 73, JD 143), 10 additional fish were brought alive to the laboratory, where fork lengths (FL) were recorded in order to determine the starting conditions of the fish lengths and the fish weights. To avoid effects of food limitation on fish growth, the fish were removed from the enclosures after 72 hr. The amount of enclosed zooplankton in mesocosms with a water volume of 2,835 L is limited. Thus, long feeding periods of the fish would certainly cause starvation effects, due to a lack of prey in the mesocosms. In contrast, too short feeding periods would cause weak growth responses. In general, fish have the highest growth rates during the larval and early juvenile phase (Eckmann, 2013). Juvenile whitefish have specific growth rates of up to 33%/day at water temperatures around 16°C (Troschel & Roesch, 1991). Therefore, we considered a feeding period of 72 hr to be long enough to receive distinct growth responses.

On day 76 (JD 146), the fish were removed with landing nets, transferred into buckets with enclosure water and brought alive to the laboratory. From the total number of 120 fish, all except for three fish (enclosure 1: 0 × N, enclosure 4: 1 × N, enclosure 6: 1 × N) could be recaptured and used for analyses. In the laboratory, pictures of all fish were taken and fork lengths were measured to the nearest 0.001 mm by using the open source software *ImageJ* (Schneider, Rasband, & Eliceiri, 2012). Fish wet weights were estimated by employing Equation (2) for the length–weight relationship:

$$W = a \times L^b, \tag{2}$$

where W = wet weight in g and L = total length (TL) in cm (Froese & Pauly, 2017). Coefficients for a = 0.0066 and b = 3.08 were chosen for *C. macrophthalmus* (Klein, 1992). Total lengths of the fish were obtained by applying a length–length relationship with Equation (3), as suggested by Binohlan, Froese, Pauly, and Reyes (2011):

$$\mathrm{TL} = \frac{\mathrm{FL}}{0.861}, \tag{3}$$

where TL = total length in cm and FL = fork length in cm.

To evaluate if whitefish could generally use the available zooplankton size classes as prey, we estimated fish mouth gape sizes. These analyses were carried out on the 10 randomly chosen fish which had also been used to determine the starting conditions for fish lengths and fish weights (see above). To estimate mouth gape sizes, the lengths of upper maxillae were measured to the nearest 0.001 mm by using a light stereomicroscope (WILD M3Z; WILD Heerbrugg, Switzerland) and the open source software *ImageJ* (Schneider et al., 2012). Mouth gape sizes were calculated by applying Equation (4), as suggested by Shirota (1970):

$$D = \sqrt{2AB}, \tag{4}$$

where *D* is mouth gape size in mm and *AB* is the length of upper maxilla in mm (*AB* was measured with open mouth; Araújo, Silva-Falcão, & Severi, 2011). Calculations of the specific growth rates (sG) for the whitefish were performed after Equation (5), suggested by Brett, Shelbourn, and Shoop (1969):

$$sG = 100\frac{(\ln W_2 - \ln W_1)}{t}, \quad (5)$$

where sG = specific growth rate in %/day, W_2 = mean fish wet weight in g at the end of the experiment, W_1 = mean fish wet weight in g at the beginning of the experiment and t = duration of the experiment in days.

2.6 | Statistical analyses

All statistical analyses were carried out with the software SigmaPlot (SigmaPlot 11.0; Systat Software 2008, USA).

Based on our experimental design, the data were analysed by using regression models. We used either a linear function ($f = y0 + a^*x$), a peak function ($f = a^*\exp(-0.5^*((x - x0)/b)^2)$) or a hyperbolic saturation function ($f = a^*x/(b + x)$) for analyses. Regression models for respective analyses were chosen after evaluating the adjusted R^2 values and the Akaike information criterion (Akaike, 1973). Data were log(x) or log(x + 1) transformed if necessary. For all analyses, we used mean values over ecologically meaningful time scales as independent variables and response variables. Mean values of chlorophyll *a*, seston N:P, and C:P and C:N ratios were calculated for the entire sampling period (days 15–71, JD 85–141) and for the clear water period (days 50–71, JD 120–141). Only herbivorous zooplankton was included in the statistical analyses. For analyses of *Daphnia* egg production, no distinction between eggs and embryos was made during counting. To calculate egg production per adult *Daphnia*, the total number of eggs was divided by the total number of egg-carrying *Daphnia* in respective samples. Regressions of zooplankton biomass, *Daphnia* egg production and fish wet weights were conducted with the mean values of the last two samplings (days 64–71, JD 134–141).

3 | RESULTS

The applied N fertilisation was effective, and we could successfully establish a broad gradient of DIN:DP ratios in our mesocosms. Over the experimental period of 76 days, phytoplankton and zooplankton of different treatments were exposed to DIN:DP ratios ranging from 16:1 to 250,000:1 (mol:mol). Among the treatments, the mean DIN concentrations ranged from 323–405 µmol/L (NO_3^-: 305–386 µmol/L, NO_2^-: 0–3 µmol/L, NH_4^+: 0.05–89 µmol/L), the mean DP concentrations ranged from 0–3 µmol/L and the mean TP concentrations were within the range of 0.1–24 µmol/L (Supporting Information Figure S1).

3.1 | Phytoplankton

We observed a typical phytoplankton spring succession from 11 March (JD 70) until 26 May (JD 146) 2014. Total seston biovolume and total chlorophyll *a* showed peaks around day 50 (JD 120) and subsequent minima around day 64 (JD 134; Figures 2 and 3a). Total chlorophyll *a* concentrations increased from 0.8 µg/L at the beginning of the experiment to 4.1 µg/L at peak phase around day 50 (JD 120); subsequently, they dropped to 1 µg/L at day 64 (JD 134; Figure 3a). The phytoplankton communities in all treatments were in general dominated by diatoms (60.5%) and cryptophytes (30.6%); less abundant were taxa from cyanobacteria (4.9%) and green algae (4%). Phytoplankton communities at the chlorophyll *a* peak (day 50,

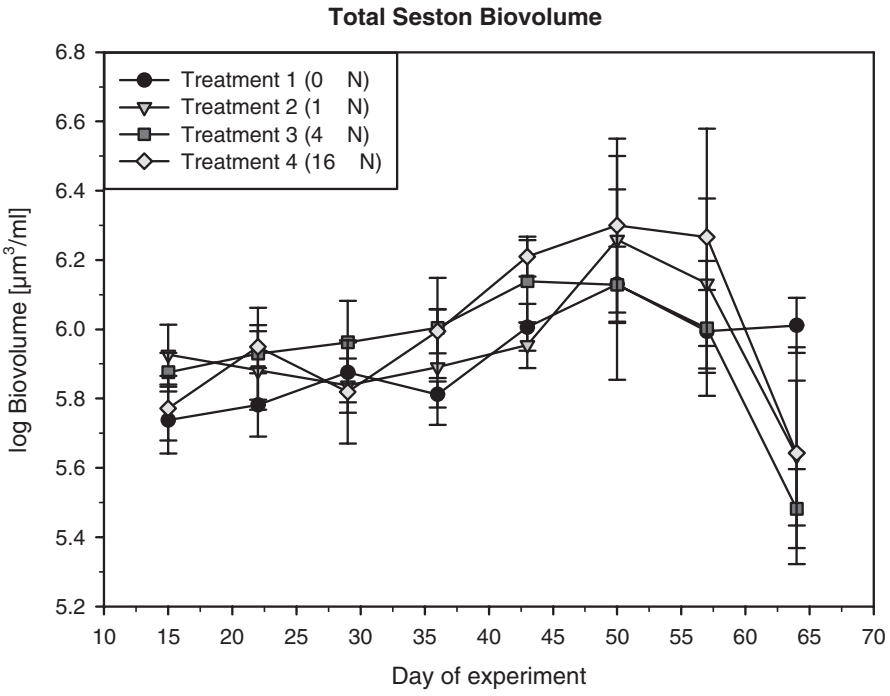

FIGURE 2 For treatments 1–4, the temporal dynamics of total seston biovolume (µm³/ml) shown for the experimental period from day 15 to day 64 (Julian days 85–134). Error bars display ± 1 standard deviation

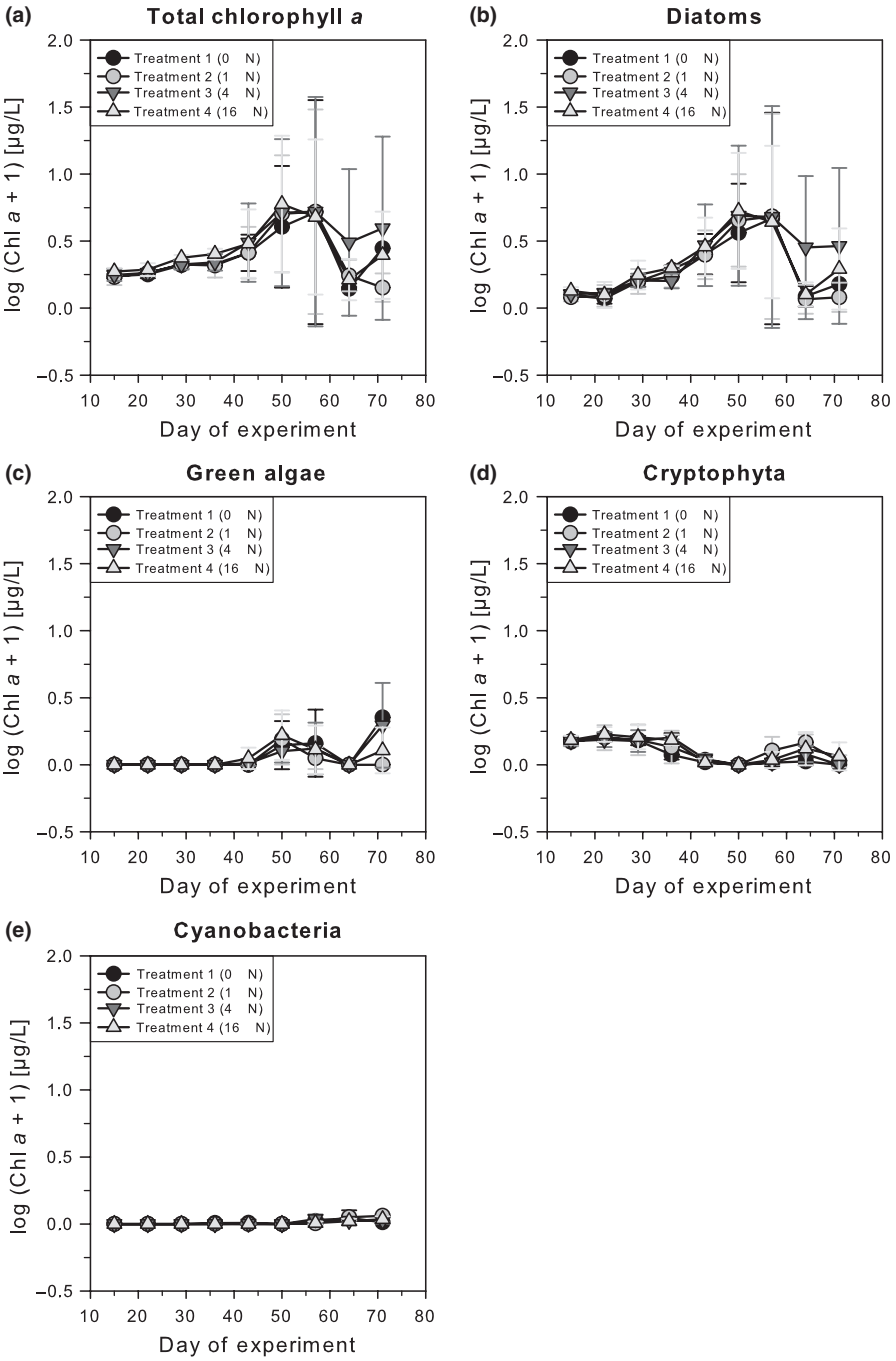

FIGURE 3 Temporal dynamics over the entire experimental period (days 15–71, Julian days 85–41) of (a) total chlorophyll *a*, (b) diatom chlorophyll *a*, (c) green algae chlorophyll *a*, (d) cryptophyta chlorophyll *a*, and (e) cyanobacteria chlorophyll *a* (µg/L). Different symbols display different treatments 1–4, and error bars display ± 1 standard deviation

TABLE 1 Regression results (adjusted R^2 and *p* values) with respective regression models for the analyses of DIN:DP ratios against total seston biovolume (µm^3/ml), total seston cell densities (cells/ml), concentrations of total chlorophyll *a* (µg/L), seston biomass N:P (mol:mol), seston biomass C:P (mol:mol), and seston biomass C:N (mol:mol), for the clear water period (days 50–71, Julian days 120–141)

	Adjusted R^2	*p*	Regression model
Total seston biovolume (µm^3/ml)	0.00	0.98	Linear
Total seston cell densities (cells/ml)	0.00	0.72	Linear
Total chlorophyll *a* (µg/L)	0.48	**<0.05**	Peak
Seston N:P (mol:mol)	0.69	**<0.001**	Linear
Seston C:P (mol:mol)	0.75	**<0.001**	Linear
Seston C:N (mol:mol)	0.00	0.33	Linear

Note. All data were log(*x*) or log(*x* + 1) transformed prior to analyses. Values in bold are statistically significant.

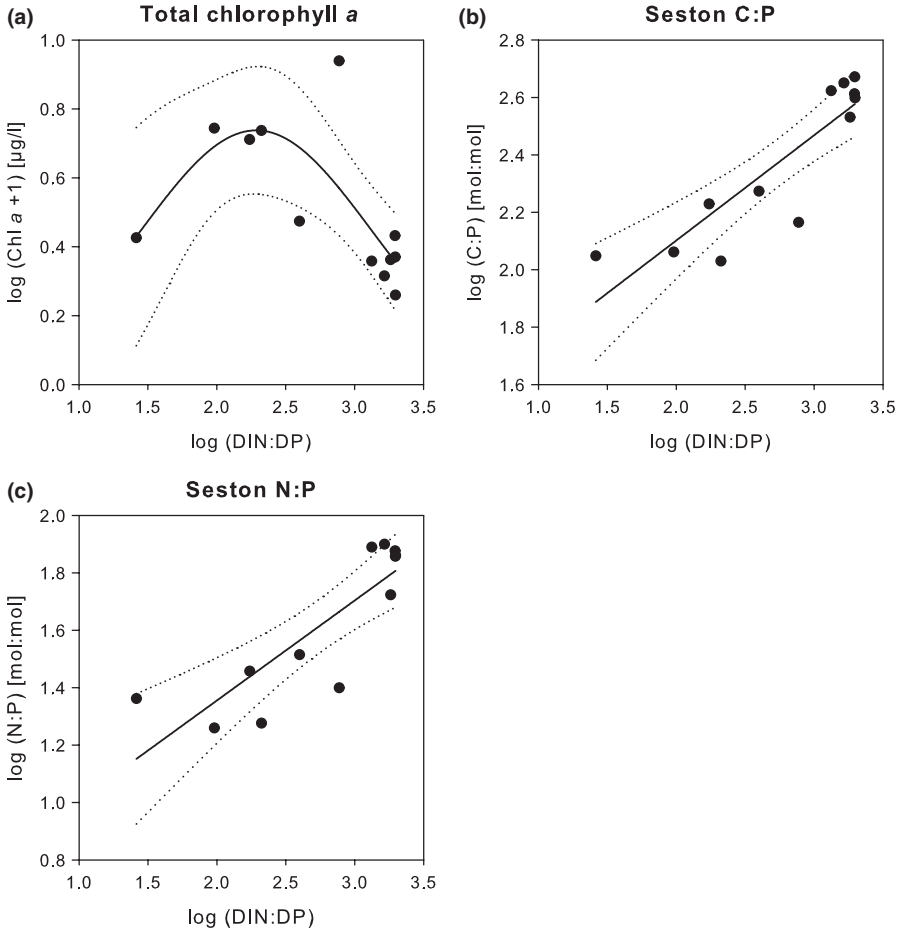

FIGURE 4 Relationships of DIN:DP ratios with (a) total chlorophyll *a* (µg/L), (b) seston C:P ratios (mol:mol) and (c) seston N:P ratios (mol:mol), during the clear water period (day 50–71, Julian days 120–141). All data were log(*x*) or log(*x* + 1) transformed prior to analyses. Regression lines indicate significance at a level of $p < 0.001$, and dotted lines display 95% confidence intervals. Statistical parameters for regressions are shown in Table 1

JD 120) were highly dominated by diatoms (90%; Figure 3b). The second most abundant group during phytoplankton peak was green algae (>9%; Figure 3c). Cryptophytes (Figure 3d) and cyanobacteria (Figure 3e) did not contribute to the composition of the phytoplankton community during the peak phase. For the clear water period (day 50–71, JD 120–141), total chlorophyll *a* showed a significant unimodal relationship with increasing DIN:DP ratios (Table 1, Figure 4a). In contrast, total seston biovolume and total seston cell densities did not show any significant relationship with increasing DIN:DP ratios during the clear water period (day 50–71, JD 120–141; Table 1). For the entire experimental period (day 15–71, JD 85–141), chlorophyll *a* concentrations of the different phytoplankton groups showed also no significant correlation to increasing DIN:DP (Table 2). Whereas, total seston cell densities (Table 2, Figure 5a) and total seston biovolume (Table 2, Figure 5b) were significantly positively correlated to increasing DIN:DP ratios.

TABLE 2 Linear regression results (adjusted R^2 and *p* values) for the analyses of DIN:DP ratios against total chlorophyll *a* (µg/L), diatom chlorophyll *a* (µg/L), green algae chlorophyll *a* (µg/L), cryptophyta chlorophyll *a* (µg/L), cyanobacteria chlorophyll *a* (µg/L), total seston cell densities (cells/ml), and total seston biovolume (μm^3/ml), for the entire experimental period (days 15–71, Julian days 85–141)

	Adjusted R^2	*p*
Total chlorophyll *a* (µg/L)	0.02	0.30
Diatom chlorophyll *a* (µg/L)	0.04	0.25
Green algae chlorophyll *a* (µg/L)	0.00	0.68
Cryptophyta chlorophyll *a* (µg/L)	0.00	0.71
Cyanobacteria chlorophyll *a* (µg/L)	0.00	0.87
Total seston cell densities (cells/ml)	0.22	**0.07**
Total seston biovolume (μm^3/ml)	0.23	**0.06**

Note. All data were log(*x*) or log(*x* + 1) transformed prior to regression analyses. Values in bold are statistically significant.

3.2 | Seston stoichiometry

The stoichiometric composition of seston biomass showed great variability along the DIN:DP gradient. With rising ambient ratios of DIN:DP, significantly increasing N:P (Table 1, Figure 4c) and C:P ratios (Table 1, Figure 4b) in seston biomass indicated an intensification of P limitation for autotroph growth. Observed N:P ratios ranged from N:P = 15:1 (mol:mol) at DIN:DP = 20 to N:P = 157:1 (mol:mol) at DIN:DP = 8,642. The C:P ratios ranged from C:P = 91:1 (mol:mol) at DIN:DP = 25 to C:P = 797:1 (mol:mol) at DIN:DP = 1,374. Seston C:N ratios remained relatively constant (C:N = 6:1) and showed no significant relationship to increasing DIN:DP ratios (Table 1).

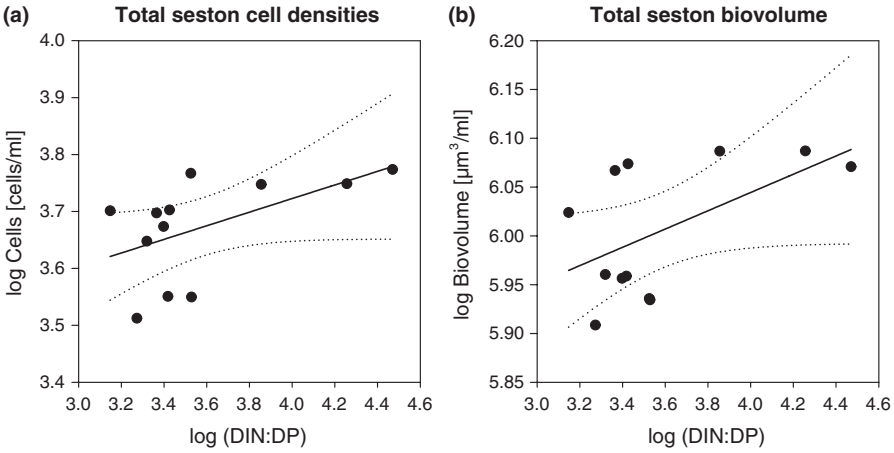

FIGURE 5 Relationships of DIN:DP ratios with (a) total seston cell densities (cells/ml) and (b) total seston biovolume (μm^3/ml) during the entire experimental period (days 15–71, Julian days 85–141). All data were log(x) or log(x + 1) transformed prior to regression analyses. Detailed statistical parameters for regressions are shown in Table 2

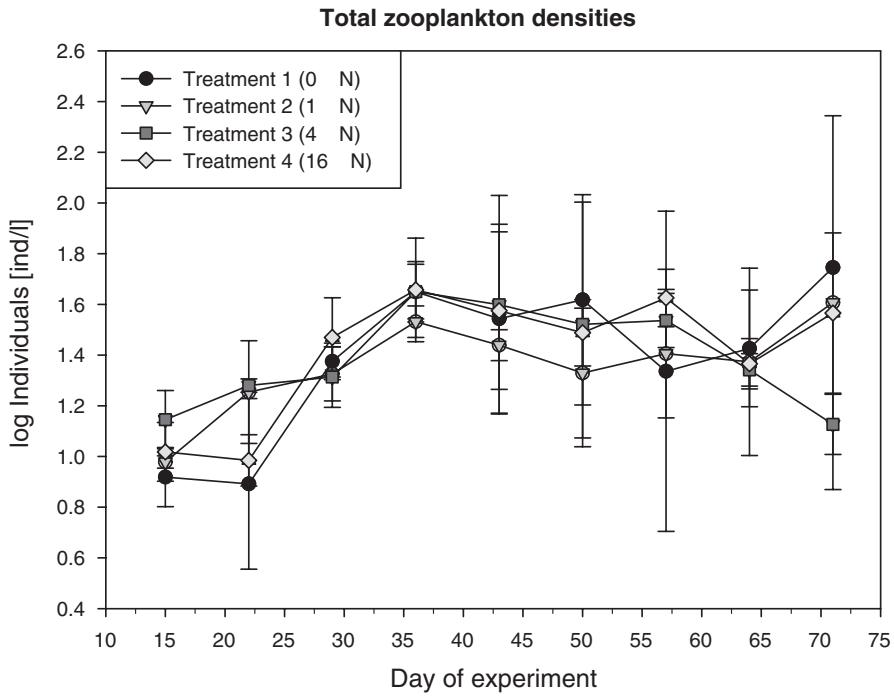

FIGURE 6 For treatments 1–4, the temporal dynamics of total zooplankton densities (individuals/L) shown for the entire experimental period (days 15–71, Julian days 85–141). Error bars display ± 1 standard deviation

3.3 | Zooplankton

Densities of zooplankton increased during the experiment, from an average of 10 individuals per litre (ind/L) on day 15 (JD 85) to average peak densities around day 43 (JD 113) of 42 ind/L. After a slight decrease until day 64 (JD 134), total zooplankton densities in treatments 1 (0 × N), 2 (1 × N), and 4 (16 × N) started to increase again, with a maximum on day 71 (JD 141) of 55 ind/L in treatment 1 (Figure 6). The zooplankton community consisted of copepods, cladocerans and rotifers. The most abundant species of copepods were *Eudiaptomus gracilis* and *Diaptomus castor*. The most abundant species of cladocerans were *Daphnia hyalina*, *Bosmina longirostris*, and *Ceriodaphnia quadrangular*. *Filinia longiseta*, *Keratella quadrata*, and *Keratella cochlearis* dominated rotifers.

From the start of the experiment, the biomass of cladocerans (Figure 7a) and of *Daphnia* (Figure 7b) increased continuously and reached highest values around day 71 (JD 141). In contrast, the temporal dynamics of copepod biomass and rotifer biomasses displayed unimodal patterns during the experimental period. Copepod biomass showed peak values around day 30 (JD 100), followed by a decrease until day 71 (JD 141; Figure 7c). Rotifer biomass was highest between days 36 (JD 106) and 57 (JD 127) and decreased afterwards till the end of the experiment (day 71, JD 141; Figure 7d).

Regarding the responses of zooplankton biomass to N fertilisation, total zooplankton biomass (Table 3, Figure 8a), cladoceran biomass (Table 3, Figure 8b), and *Daphnia* biomass (Table 3) were significantly negatively correlated to increasing DIN:DP ratios, and declined linearly along the DIN:DP gradient. Copepod biomass showed no significant correlation to rising DIN:DP ratios (Table 3). In contrast, the biomass of rotifers increased significantly with rising DIN:DP (Table 3, Figure 8c). As a result of rising rotifer biomass in combination with decreasing cladoceran

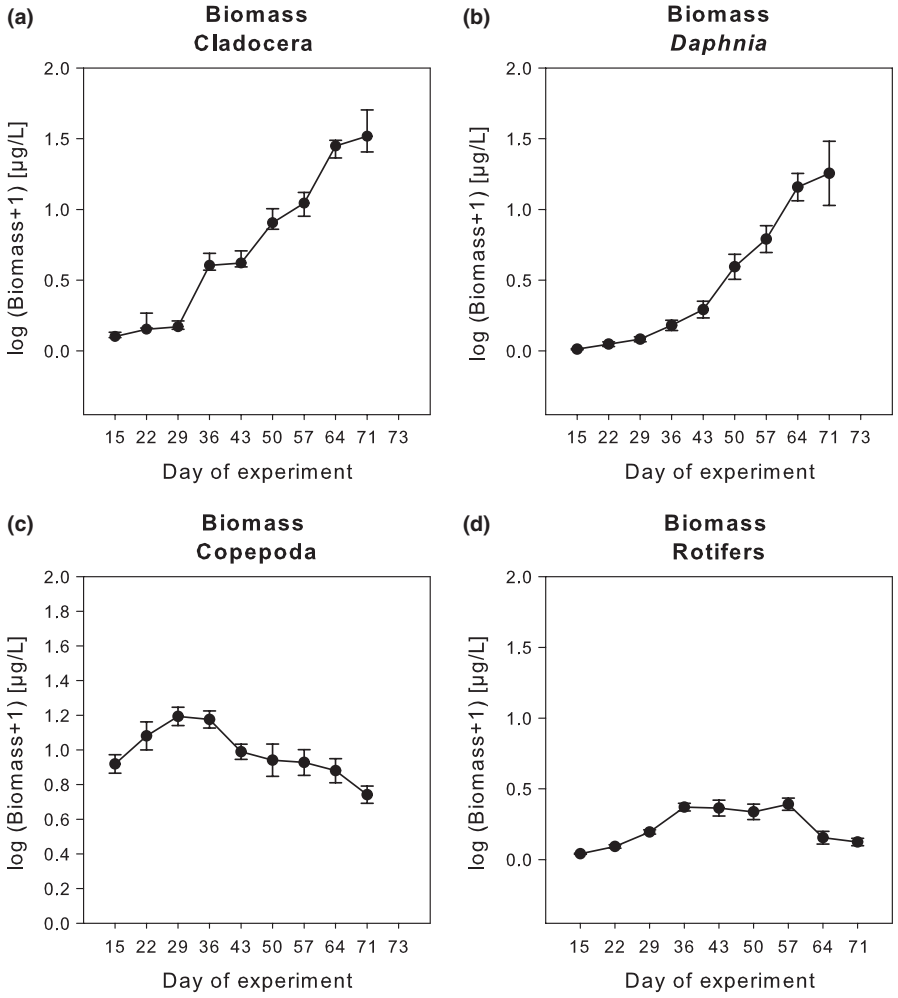

FIGURE 7 Temporal dynamics over the entire experimental period (days 15–71, Julian days 85–141) of (a) cladoceran biomass (µg/L), (b) *Daphnia* biomass (µg/L), (c) copepod biomass (µg/L), and (d) rotifer biomass (µg/L). All data were log(*x*) or log(*x* + 1) transformed prior to analyses. Error bars display ± 1 standard error

TABLE 3 Linear regression results (adjusted R^2 and *p* values) for the analyses of DIN:DP ratios against the biomass (µg/L) of total zooplankton, cladocerans, *Daphnia*, *Ceriodaphnia*, rotifers, copepods, nauplii, and the ratio of rotifer:cladocera (biomass:biomass), at the end of the experiment (days 64–71, Julian days 134–141)

Biomass (µg/L)	Adjusted R^2	*p*
Total zooplankton	0.34	**<0.05**
Cladocera	0.31	**<0.05**
Daphnia	0.29	**<0.05**
Ceriodaphnia	0.00	0.81
Rotifera	0.38	**<0.05**
Copepoda	0.16	0.11
Nauplii	0.00	0.82
Rotifera:Cladocera	0.31	**<0.05**

Note. All data were log(*x*) or log(*x* + 1) transformed prior to regression analyses. Values in bold are statistically significant.

biomass, we found significantly increasing ratios of rotifera:cladocera (biomass:biomass) along the gradient of DIN:DP (Table 3), indicating changes in the composition of zooplankton communities. Egg production of *Daphnia* was negatively correlated to increasing DIN:DP ratios. We found a significant unimodal relationship of increasing DIN:DP ratios with the number of eggs per *Daphnia* (regression: peak; adjusted $R^2 = 0.63$, $p < 0.01$; Figure 9a). The number of egg-carrying *Daphnia* decreased significantly along the DIN:DP gradient (regression peak; adjusted $R^2 = 0.8$, $p < 0.0001$; Figure 9b).

3.4 | Fish-feeding experiment

Fish were introduced on day 73 (JD 143); at this time, the highest cladoceran (Figure 7a) and *Daphnia* (Figure 7b) biomass was available in the enclosures. Since cladocerans and especially *Daphnia* are supposed to be the preferred prey of planktivorous fish (Brooks, 1968), best possible food conditions for young-of-the-year fish could be provided on this day.

During the fish-feeding experiment, the mean temperature in the enclosure water column was 16.9°C. The wet weight of 10 randomly chosen fish at the start (day 73, JD 143) was 0.357 ± 0.08 g (mean ± 1 *SD*), fork length was 3.13 ± 0.22 cm (mean ± 1 *SD*) and the total length was 3.63 ± 0.26 cm (mean ± 1 *SD*). After an exposure time of 72 hr, the fish wet weight was 0.459 ± 0.082 g (mean ± 1 *SD*), fork length was 3.4 ± 0.2 cm (mean ± 1 *SD*) and the total length was 3.95 ± 0.24 cm (mean ± 1

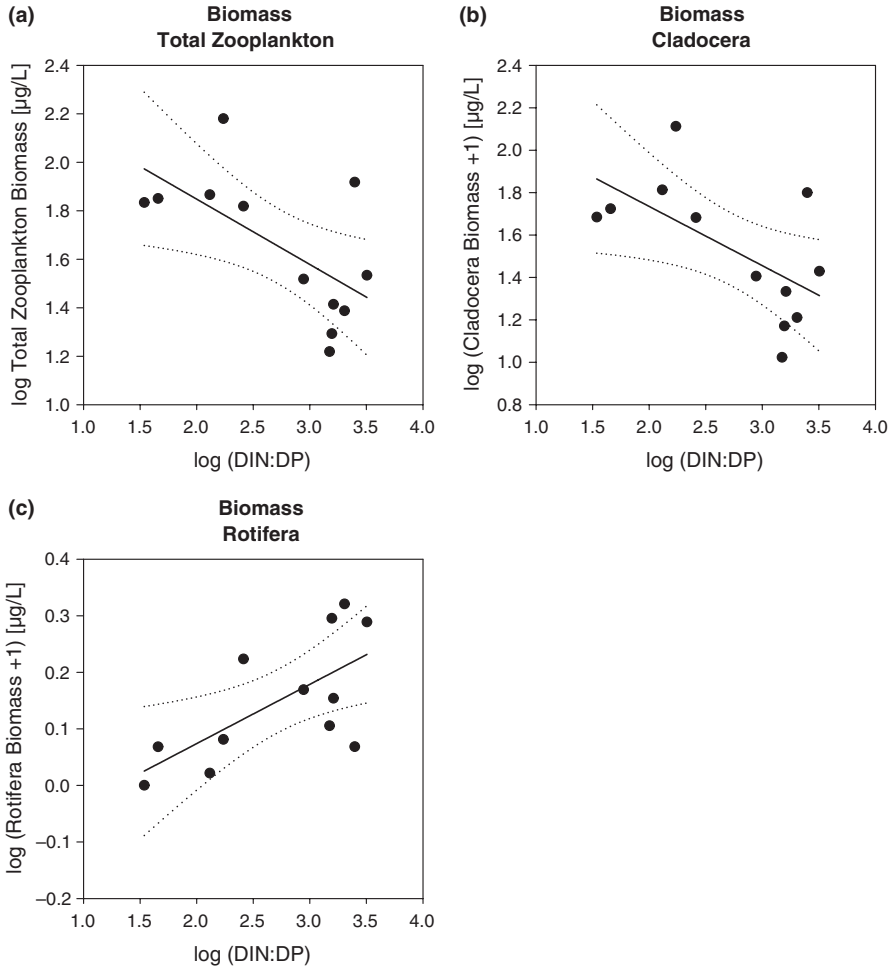

FIGURE 8 Relationships of DIN:DP ratios with (a) total zooplankton biomass (µg/L), (b) cladoceran biomass (µg/L) and (c) rotifer biomass (µg/L) during the end of the experiment (days 64–71, Julian days 134–141). All data were log(x) or log(x + 1) transformed prior to analyses. Regression lines indicate significance at a level of $p < 0.05$, and dotted lines display 95% confidence intervals. Detailed statistical parameters for regressions are shown in Table 3

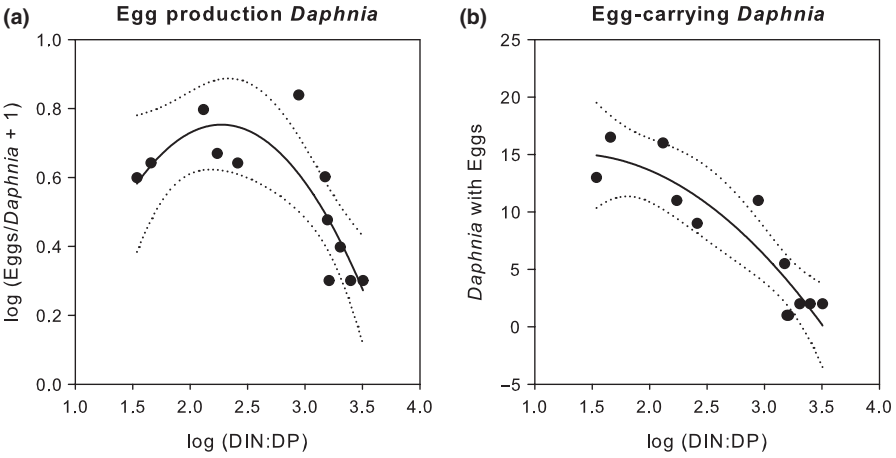

FIGURE 9 Relationships of DIN:DP ratios with (a) number of eggs per *Daphnia* and (b) number of egg-carrying *Daphnia*. All data were log(x) or log(x + 1) transformed prior to analyses. Regression lines indicate significance for (a) at a level of $p < 0.01$ (adjusted $R^2 = 0.63$) and for (b) at a level of $p < 0.001$ (adjusted $R^2 = 0.8$). Dotted lines display 95% confidence intervals

SD). Hence, within 3 days, the fish gained on average 0.102 g of wet weight (0.034 g/day), 0.27 cm of fork length (0.09 cm/day) and 0.32 cm of total length (0.17 cm/day). Based on fish wet weights, we found a specific growth rate (sG) [%/day] of sG = 3.4% for our fish. To evaluate if whitefish could use available *Daphnia* as food, the mean mouth gape size of fish was estimated. We found a mouth gape size of 1.65 ± 0.14 mm (mean ± 1 *SD*). This indicates that the mouth gapes of our whitefish were large enough to use *Daphnia* (body length *Daphnia* = 0.84 ± 0.11 mm [mean ± 1 *SD*]) as prey. We found a positive response of fish weights to increasing *Daphnia* biomass (Table 4, Figure 10a). In contrast, fish weights were significantly negatively correlated to increasing rotifer biomass (Table 4, Figure 10b). No significant correlation could be found in regressions of fish weights against copepod biomass (Table 4) or fish weights against total zooplankton biomass (Table 4).

4 | DISCUSSION

This mesocosm experiment was performed in order to investigate the impacts of increasing N deposition on different trophic levels of an oligotrophic lake food web. The first hypothesis tested was that increasing N loads in a P-limited lake can intensify the P limitation of phytoplankton growth. As a consequence, the food quality of algae for herbivorous zooplankton decreases, in particular for zooplankton taxa with high P demands, such as cladocerans. Our results support this hypothesis, and the significantly increasing seston N:P and C:P ratios found along the DIN:DP gradient indicate an amplification of P limitation for phytoplankton. This was to be expected, since phytoplankton are known to be very flexible in their stoichiometric composition (Sommer, 1989; Sterner & Elser, 2002). It is also known that if both elements N and P are supplied in excess, seston biomass displays a typical N:P ratio of 16:1 (by atoms; Redfield, 1958). However, if the supply ratio of the two nutrients changes, most phytoplankton species are able to adjust their elemental composition accordingly. Due to the high flexibility of phytoplankton biomass large shifts in seston C:P or N:P ratios can result (Schindler, 1977). In contrast, for herbivorous zooplankton, any shifts in phytoplankton stoichiometry could imply changes in food quality causing more or less serious effects on growth and reproduction (Elser, Hayakawa, & Urabe, 2001; Hessen, 1992).

TABLE 4 Regression results (adjusted R^2 and p values) with respective regression models for the analyses of whitefish weights (g) against total zooplankton biomass (µg/L) and the biomass (µg/L) of the zooplankton taxa, *Daphnia*, rotifers, and copepods

Biomass (µg/L)	Adjusted R^2	p	Regression model
Total zooplankton	0.07	0.21	Linear
Daphnia	0.17	**0.10**	Hyperbolic saturation
Rotifera	0.31	**0.04**	Linear
Copepoda	0.02	0.29	Hyperbolic saturation

Note. All data were log(x) or log(x + 1) transformed prior to analyses. Values in bold are statistically significant.

Our second hypothesis included the assumption that impacts on zooplankton level, induced by the decreasing food quality of algae would cause a shift in zooplankton community composition. Due to their different stoichiometric constraints, we suggested that copepods would be favoured and cladocerans would be negatively affected by high DIN:DP conditions (Trommer et al., 2017). However, our results cannot completely confirm this hypothesis. Even though we found declining cladoceran biomass along the DIN:DP gradient, copepods did not show a significant response to increasing DIN:DP ratios. However, our data indicate that the rising P deficiency of food algae was at least one reason that cladoceran biomass declined along the gradient of DIN:DP ratios. Numerous studies have already shown that P-limited food significantly lowers the growth and reproduction success of zooplankton, even if food is supplied in excess (DeMott, Gulati, & Siewertsen, 1998; Elser et al., 2001; Sterner & Hessen, 1994; Sterner & Schulz, 1998). This is, above all, true for *Daphnia* species, which accounted for over 62% of cladoceran biomass in the mesocosms. Ideally, *Daphnia* require food with an atomic C:P ratio of <300:1 (Olsen et al., 1986; Sterner, 1997; Urabe, Clasen, & Sterner, 1997; Urabe & Watanabe, 1992). Food algae with C:P > 300:1 are classified as low-quality food and known to generate P limitation for cladoceran net production (Brett, Müller-Navarra, & Sang-Kyu, 2000; Sterner, 1997, 1998; Urabe et al., 1997). We found a range of seston biomass C:P ratios from 90:1 to 800:1 along the DIN:DP gradient. This implies that *Daphnia* in high DIN:DP enclosures were mainly supplied with highly P-limited food. As a consequence, somatic growth and reproduction success of these *Daphnia* were significantly reduced. Support is given to this by the observed decrease in the reproductive output of *Daphnia* when exposed to increasing DIN:DP ratios. Therefore, it is apparent that seston C:P ratios exceeding C:P = 300:1 must have contributed to the low *Daphnia* biomass in high DIN:DP treatments.

Unexpectedly, copepod species were not affected by our experimental manipulations. Since copepods grow and reproduce more slowly than cladocerans, their numerical responses have a certain temporal delay (Main, Dobberfuhl, & Elser, 1997). Furthermore, as copepods usually have only one or two reproduction events per season (Allan, 1976; Larsson, 1978) the experimental duration of

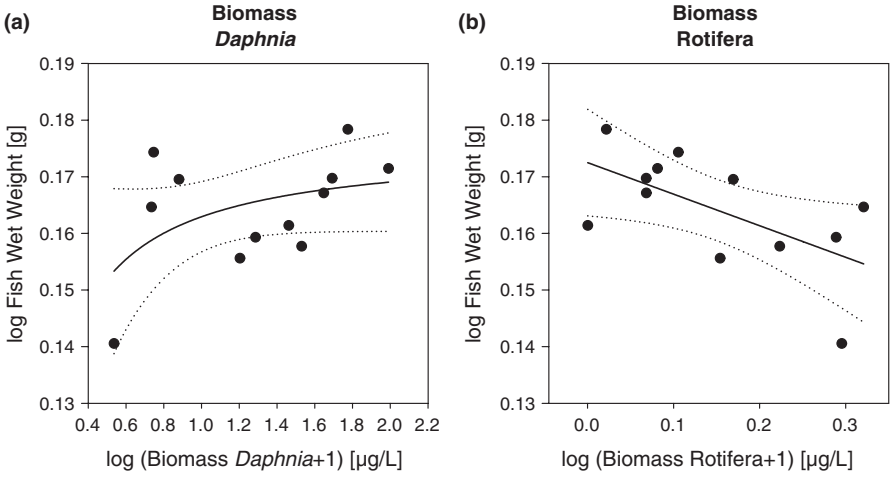

FIGURE 10 Relationships of fish wet weights (g) with available biomass (µg/L) of (a) *Daphnia* species and (b) rotifer species. All data were log(x) or log(x + 1) transformed prior to analyses. Solid regression lines indicate statistical significance at a level of p < 0.05–0.1. Dotted lines display 95% confidence intervals. Detailed statistical parameters for respective regressions are shown in Table 4

10 weeks could have been too short for copepods to fully respond to the N fertilisation. There are also other on specific traits that could explain the differences in copepod and *Daphnia* responses to increasing DIN:DP ratios. Carillo et al. (2001) described a biomass P content for calanoid copepods of about 0.5% (percentage of dry weight), which is two- to five-fold less than in cladocerans. *Daphnia*, for example, have a P content of about 1.2% of dry weight. This disparity between the P contents of copepods and cladocerans can partly be attributed to the different growth rates of the two taxa (Elser, Dobberfuhl, Mackay, & Schampel, 1996). Copepods have lower growth rates compared to cladocerans, resulting in a reduced need of P rich ribosomal RNA (Main et al., 1997) to maintain somatic growth. The resultant lower P demand enables copepods to cope better with P poor food. Furthermore, copepods are, in contrast to cladocerans, able to feed selectively (Sommer & Stibor, 2002 and references therein; Meunier, Boersma, Wiltshire, & Malzahn, 2015). This provides copepods with the opportunity to select food particles prior to uptake and avoid ingestion if necessary (Flynn, Davidson, & Cunningham, 1996; Sommer & Sommer, 2006). Selection criteria can be size, motility (Tiselius & Jonsson, 1990), or chemical quality (DeMott, 1988) of food particles. Thus, copepods in high DIN:DP mesocosms could have refused the uptake of P deficient food, thereby avoiding any possible implications for growth and reproduction.

Rotifer taxa responded completely different to DIN:DIP manipulations and showed significantly increasing biomass with rising DIN:DP ratios. However, since rotifers are also known to require P rich food (Allan, 1976; Rothhaupt, 1995) this response was most likely not an effect of N fertilisation but rather an effect of interspecific competition. Rotifers are known to be suppressed by cladocerans, through exploitative and mechanical interference (Dodson, 1974; Gilbert, 1988a, 1988b). In our high DIN:DP enclosures with low numbers of *Daphnia*, the suppression of rotifers by daphnids was, however, considerably reduced. This was especially pronounced at the end of the experiment (days 64–71, JD 134–144) when *Daphnia* abundances were negatively affected by DIN:DP manipulations. The significantly increasing ratios of rotifer:cladoceran biomass along the DIN:DP gradient at the end of the experiment indicate a certain advantage for rotifers. In contrast, at the beginning of the experiment (days 15–22, JD 85–92) when *Daphnia* abundances in high DIN:DP treatments had not yet been affected, rotifers did not show any biomass increase along the DIN:DP gradient. Summarising, these findings imply that the observed dynamic in rotifer biomass derives rather from a competitive advantage than from the stoichiometric implications described above.

The third hypothesis was that the effects of increasing DIN:DP ratios can be transferred via phyto- and zooplankton to planktivorous fish. We found evidence in our data for the existence of such an effect transfer. Fish growth depends directly on the availability of food (Eckmann, 2013). However, the growth of zooplanktivorous fish also depends on the taxonomic composition of zooplankton communities, which determines the suitability of zooplankton taxa as diet (Müller, Breitenstein, Bia, Rellstab, & Kirchhofer, 2007). Many studies report that cladocerans, especially *Daphnia* species, are the preferred prey zooplankters of subadult and adult whitefish, at least during the main growing season (Becker & Eckmann, 1992; Brooks, 1968; Mookerji, Heller, Meng, Bürgi, & Müller, 1998; Müller et al., 2007; Rufli, 1979). Nonetheless, whitefish do not feed exclusively on cladocerans; juvenile whitefish can also use rotifers as prey but usually only for a few days after hatching (Brooks, 1968; Northcote & Hammar, 2006; Ponton & Müller, 1990). About 1 month after hatching, coregonid larvae can physically feed on cladocerans larger than 1 mm (Karjalainen, 1991). This is in accordance with our data, as we showed that whitefish were not gape limited and thus were morphologically able to feed on prey items in the size class of *Daphnia*. Furthermore, we found a positive correlation between *Daphnia* biomass and fish wet weight. These results provide compelling evidence that whitefish in our mesocosms must have used *Daphnia*, as prey, if available.

In conclusion, the data presented here support our assumptions that the growth of planktivorous whitefish can be negatively affected by increasing DIN:DP ratios. Bottom-up mechanisms in lake food webs can provide a pathway for the effect transfer through a tri-trophic lake food web up to planktivorous whitefish. The taxonomic composition of zooplankton communities can hereby play a crucial role. A reduction in abundance of essential prey taxa, such as *Daphnia*, could have severe effects on the growth of zooplanktivorous fish. Above all, this conclusion is consistent with those of numerous other studies that showed that for higher trophic levels, the food quality of zooplankton is determined by zooplankton community composition, fatty acid composition (Dabrowski, 1984; Smyntek, Teece, Schulz, & Storch, 2008) and stoichiometric composition (Boersma et al., 2008; Hendrixson, Sterner, & Kay, 2007; Vanni, 2002). Hence, we empirically support the assumption of Boersma et al. (2008) that "nutritional limitation can travel up the food chain."

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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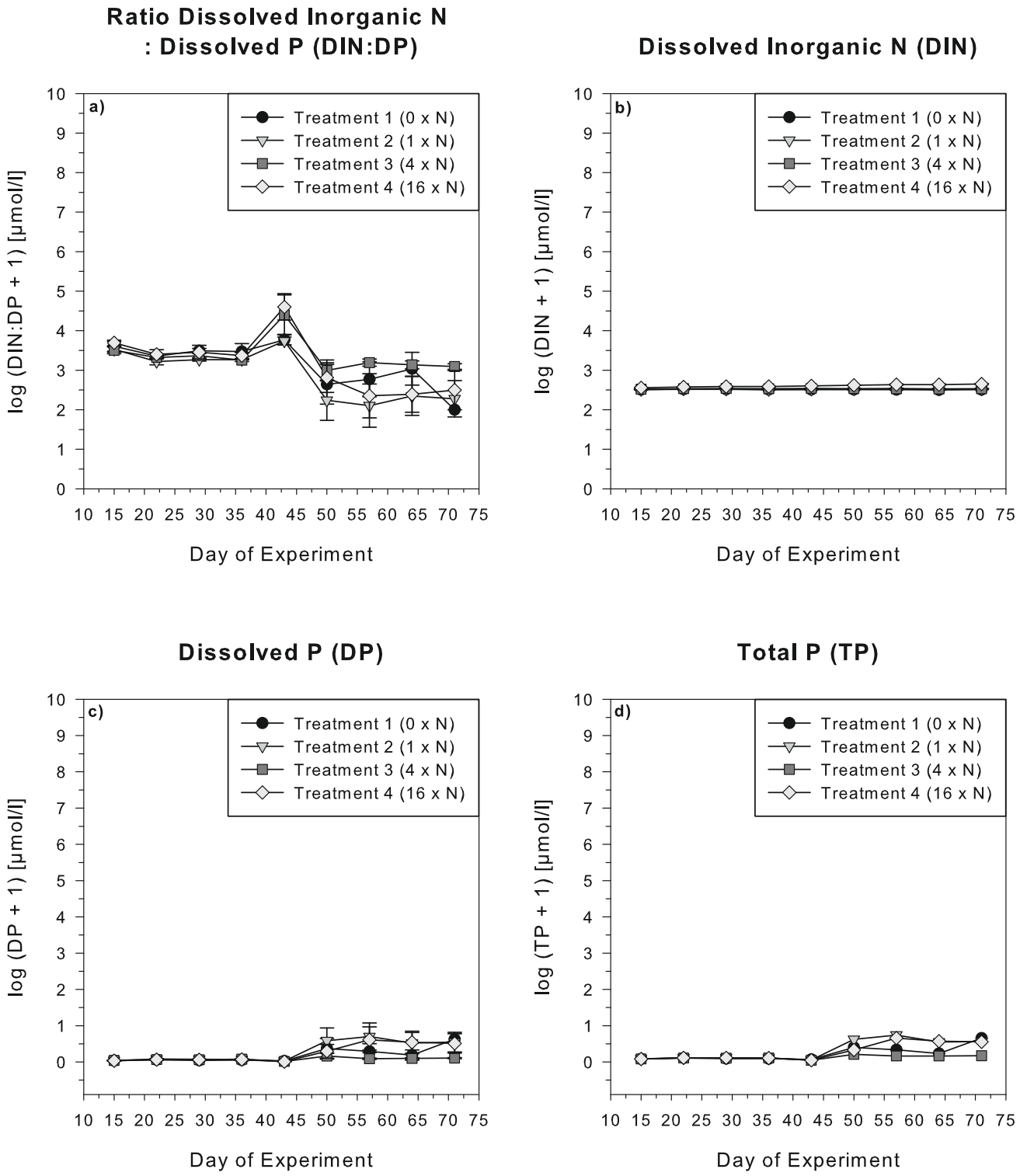

Supplementary Figure S1: For treatments 1-4, the temporal dynamics over the entire experimental period (days 15-71, JD 85-141) of a) DIN:DP ratios, b) DIN (µmol/l), c) DP (µmol/l) and d) TP (µmol/l). All data were log(x) or log(x+1) transformed prior to analyses.

PUBLICATION IV

Nitrogen enrichment leads to changing fatty acid composition of phytoplankton and negatively affects zooplankton in a natural lake community

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Nitrogen enrichment leads to changing fatty acid composition of phytoplankton and negatively affects zooplankton in a natural lake community

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Secondary production in freshwater zooplankton is frequently limited by the food quality of phytoplankton. One important parameter of phytoplankton food quality are essential polyunsaturated fatty acids (PUFAs). Since the fatty acid composition of phytoplankton is variable and depends on the algae's nutrient supply status, inorganic nutrient supply may affect the algal PUFA composition. Therefore, an indirect transfer of the effects of nutrient availability on zooplankton by changes in algal PUFA composition is conceivable. While the phosphorus (P) supply in lakes is largely decreasing, nitrogen (N) inputs continue to increase. This paper presents data from a mesocosm field experiment in which we exposed phytoplankton communities to increasing N enrichment. As a consequence, the PUFA composition of the phytoplankton community changed. With increasing nitrogen fertilisation, we observed lower quantities of essential PUFAs, together with a decrease in the abundances of the dominant herbivorous zooplankton *Daphnia sp*. Their biomass was significantly correlated with phytoplankton PUFA content (C18:3 ω3, C20:5 ω3, C18:2 ω6). Our data therefore indicate that changes in nitrogen supply, together with the resultant changes in phytoplankton food quality, can negatively affect the secondary production of herbivorous zooplankton by reducing the availability of essential polyunsaturated fatty acids.

Global anthropogenic activities drastically alter nutrient cycles by increasing energy consumption and biomass production[1] thereby strongly affecting global ecosystem services[2,3]. Since the key nutrients nitrogen (N) and phosphorus (P) are essential components of the biomass of organisms' and often limit primary production, changes in their biogeochemical flows can have drastic consequences on ecosystem dynamics. The biogeochemical pathways of both elements are to a large degree influenced by anthropogenic activities resulting in increasing amounts of N and P entering the ecosystems by means of waste water, excessive fertiliser application and soil erosion soils[1,4,5]. There has been an increasing effort for several decades to reduce nutrient loads, especially in freshwater systems where P often limits the primary production[6]. Replacing the P compounds in detergents and/or providing purification plants with highly efficient P elimination techniques has resulted in successful P reduction and the reoligotrophication of water bodies[7,8]. However, N loads have continued to increase, since the diffuse N inputs are difficult to control by means of targeted measures. N is much more mobile than P, and its high dispersal potential means that it can easily enter groundwater and atmospheric pools[9].

In lakes, P enrichment is often clearly visible by an apparent increase in primary production[6]. Furthermore, P enrichment can potentially result in eutrophication characterised by blooms of toxic or undesired algal species, the oxygen reduction of deep waters, and other undesirable consequences[10]. By contrast, N enrichment of fresh

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waters is often much more inconspicuous. Only in lakes where N availability limits primary production, eutrophication signatures are visible and observed[11–13]. However, in addition to the quantitative effects of increasing primary production, more subtle qualitative effects can occur in terms of community and biochemical composition. For example, N enrichment can favor certain algal groups, such as mixotrophic algae[14] via an alteration of the bacterioplankton composition[15]. Such changes in algal community composition can result in changes in food quality for herbivorous zooplankton, as not all algal groups are equally well-suited as zooplankton food. Consequently, recent mesocosm experiments with natural plankton communities suggest that N enrichment is accompanied by a lower trophic transfer efficiency[16]. While zooplankton growth decreases with an increasing N supply, the decline is most pronounced in the case of cladoceran zooplankton[16], which are a particularly important food source for fish. Cladoceran zooplankton, especially *Daphnia* sp., have been thoroughly investigated and several food quality related factors that influence their fitness have been identified[17,18]. These factors are algal size, gelatinous sheaths, and toxicity, but also nutrient stoichiometry and biochemical composition[17–19]. The fatty acid composition of phytoplankton, especially the contribution of polyunsaturated fatty acids (PUFAs), is a biochemical factor that is already well known from laboratory studies for its consequences for *Daphnia* growth[20–22]. There is evidence that the PUFA composition of phytoplankton appears to exert a higher influence on the somatic growth rates and reproduction of *Daphnia sp*. than do stoichiometric effects such as the C:P or N:P ratio of the phytoplankton biomass[23,24].

There is a general mismatch in nutrient management strategies that recent reoligotrophication continues to reduce the amount of P in a large number of water bodies, whereas the N supply continues to increase. This unbalanced reoligotrophication can have various undesirable effects. Although large quantitative effects from N supply to P limited systems are not expected, qualitative changes on the biomass stoichiometry[14] or biochemical composition cannot be excluded. We therefore investigated the qualitative effects of increasing N enrichment on biochemical phytoplankton PUFA composition in an already P deficient system. We exposed a natural spring plankton community to a gradient of N enrichment in an enclosure experiment. The experimental N enrichment was conducted over an ecologically meaningful time scale of ten weeks[25,26] in order to include not only the short-term direct enrichment effects on phytoplankton, but also the subsequent bottom up effects on higher trophic levels.

Results

Water chemistry. Prior to fertilisation, the nutrient concentrations in Lake Brunnensee were 8.4 mg L^{-1} for NO_3, 121 µg L^{-1} for NH_4 and 6.4 µg L^{-1} for TP. Nitrite (NO_2) was on average 0.09 ± 0.01 (st.dev.) mg L^{-1} over the experimental period. During the experiment, the NO_3 concentrations ranged from on average 8.4 ± 0.1 mg L^{-1} in the control treatments up to a maximum of 12.5 ± 0.2 mg L^{-1} in the 32 x N treatments. The NH_4 concentrations ranged from on average 61.9 ± 4.3 µg L^{-1} in the control treatments up to 1169 ± 16.3 µg L^{-1} in the 32 x N treatments. Over the experimental period, the control treatments lost dissolved inorganic N in the form of NH_4 and NO_3, whereas in the N supply treatments, NH_4 and NO_3 remained at the same level or increased with time in the higher fertilisation levels (Fig. 1A,B). The TP concentration was on average 8.7 ± 4.2 µg L^{-1} over all the treatments and declined on day 56 to 2.7 ± 2.7 µg L^{-1} (Fig. 1C), possibly due to sedimentation loss and microscopic wall growth (periphyton) in the last two weeks. The PO_4 concentrations were most of the time below detection limit. The silicate concentrations were on average 7.1 ± 0.4 mg L^{-1} in all the treatments and declined at the end of the experiment to ~6.7 ± 0.4 mg L^{-1}. According to the experimental design, the average dissolved N:P (based on NH_4 and NO_3 to TP) ratio increased significantly with N fertilisation (Fig. 1D).

Phytoplankton. The total chlorophyll *a* concentration in all the experimental treatments showed a parallel increase from 0.84 µg L^{-1} at the beginning of the experiment to on average 2.2 ± 0.4 µg L^{-1} until day 28 (Fig. 2A). In the mesocosms, maximum chlorophyll *a* concentrations were reached from days 21 to 38. In the control treatment, chlorophyll *a* decreased after day 28, whereas in the higher N treatments chlorophyll *a* started to decrease later after day 40. Higher chlorophyll *a* concentrations with N fertilisation were observed only on day 42 ($p = 0.04$, $r^2 = 0.24$), which is related to the fertilisation design.

Microscopic analyses of the phytoplankton community revealed a flagellate dominated community, comprising chlorophytes and other unclassified small flagellates (which were not further identified). The major taxa from days 21 to 49 were *Chlamydomonas sp*. (on average 10 ± 11%, up to 35%) and unclassified pigmented flagellates of approximately the same size (on average 66 ± 19%, up to 98% on day 21) (Fig. 2B). Colonial chlorophytes (*Botryococcus sp*., *Dictyosphaerium sp*., *Coelastrum sp*., *Gloeocystis sp*., and *Coenochloris sp*.) contributed on average to 10.2 ± 9.3% of the phytoplankton biomass. Cryptophytes were represented by *Cryptomonas sp*. and *Rhodomonas sp*. and comprised on average 1.9 ± 1.8% of the total phytoplankton biomass. Chrysophytes (mainly *Dinobryon sp*.) contributed on average to 4.9 ± 11.9% (up to 16% on day 49) of the phytoplankton biomass. Diatoms contributed on average to only 1.5 ± 1.7% of the phytoplankton community and were represented by the genera *Achnanthes sp*., *Asterionella sp*., *Cyclotella sp*., *Cymbella sp*., *Fragilaria sp*., *Navicula sp*., and *Synedra sp*. Coccoid chlorophytes (2.3 ± 3.3%), dinophytes (0.6 ± 0.7%), and cyanobacteria (2.6 ± 6.0%) represented on average only a minor proportion of the phytoplankton community, with slightly higher abundances on day 49 (Fig. 2B). The statistical analyses revealed a significant relationship between total phytoplankton biovolume and N fertilisation on days 21 and 28, which was due to the large proportion of unclassified small flagellates present (Table 1). On day 49, the biomass of the chlorophyte colonies increased with N fertilisation (Table 1), which was driven by one single mesocosm (No. 18). Relative abundances of chlorophyte colonies did not show any correlation to N fertilisation, only the 2 x N treatment was lower than the control and 32 x N treatment (see Table 1, ANOVA results). No differences between treatments were observed in the other phytoplankton groups on that day (chrysophytes: $p = 0.80$, $r^2 = 0.00$; coccoid chlorophytes: $p = 0.16$, $r^2 = 0.08$; cyanophytes: $p = 0.30$, $r^2 = 0.07$).

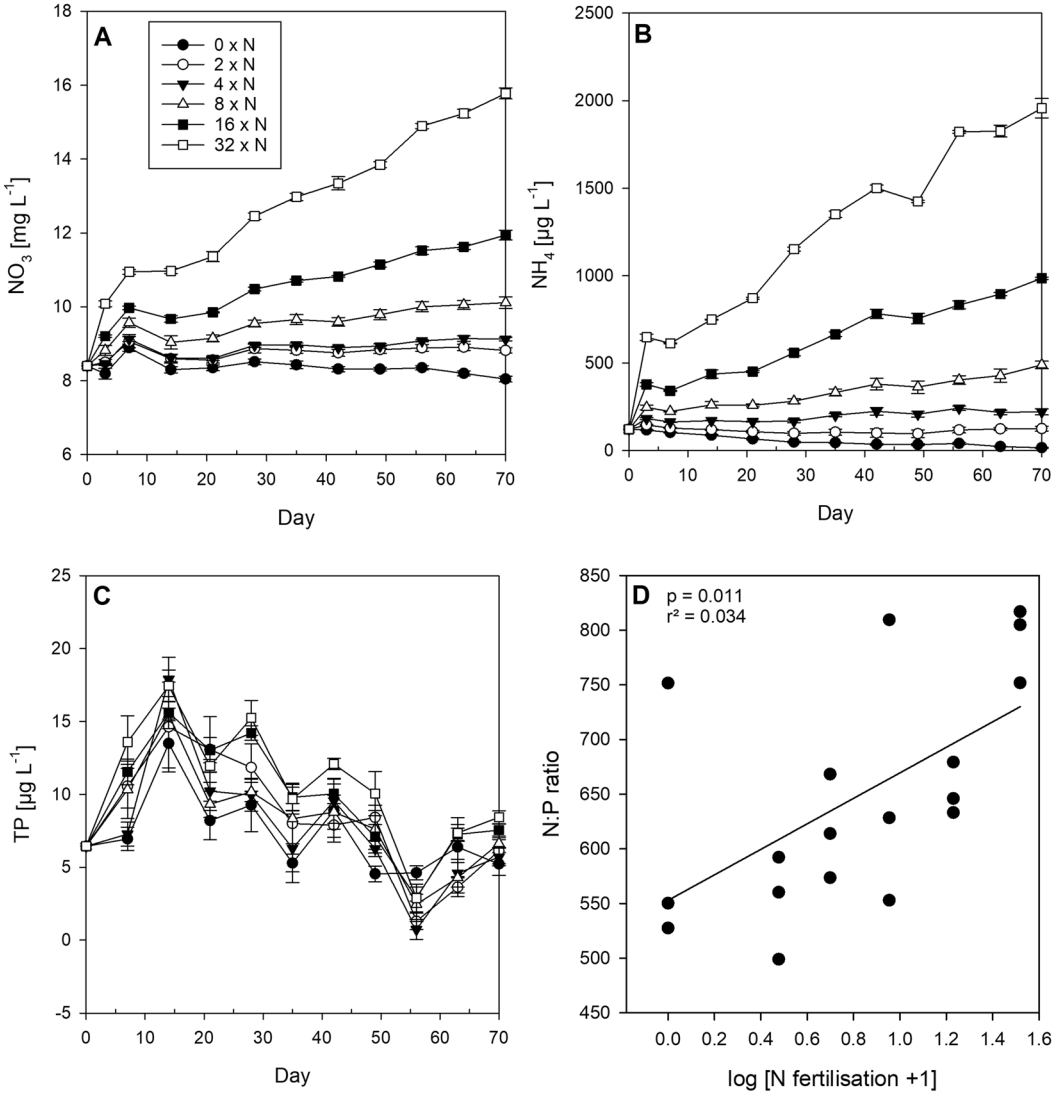

Figure 1. Nutrient development in all six N fertilisation treatments. (**A**) NO_3 (mg L^{-1}), (**B**) NH_4 (µg L^{-1}) and (**C**) TP concentrations (µg L^{-1}, mean ± 1 SE of n = 3 replicates) in all six N fertilisation treatments over time. (**D**) Average N:P ratios over time (mean ± 1 SE of n = 3 replicates) against N fertilisation treatment. Significant linear regression line in solid ($p < 0.05$).

Seston stoichiometry. From days 21 to 63, concentrations of particulate organic carbon were on average 0.71 ± 0.28 mg L^{-1}, particulate nitrogen 0.17 ± 0.06 mg L^{-1}, and particulate P 5.0 ± 2.2 µg L^{-1}. The resulting seston C:P ratios fluctuated over the course of the experiment, from a minimum of 173 to a maximum of 710, and were on average 387 ± 97 (see Supplementary Fig. S1). The seston N:P ratios fluctuated from a minimum of 41 to a maximum of 172 and were on average 80 ± 24; the seston C:N ratios ranged from a minimum 2.3 to a maximum of 7.2 (5.0 ± 1 on average, see Supplementary Fig. S1). However, N fertilisation did not significantly affect the biomass stoichiometric ratios, neither as averages over the investigated period (C:P ratio: $p = 0.44$, $r^2 = 0.04$; N:P ratio: $p = 0.09$, $r^2 = 0.17$; C:N ratio: $p = 0.24$, $r^2 = 0.09$) nor on day 49 (C:P ratio: $p = 0.39$, $r^2 = 0.05$; N:P ratio: $p = 0.13$, $r^2 = 0.13$; C:N ratio: $p = 0.29$, $r^2 = 0.07$).

Phytoplankton fatty acids. A total of 32 fatty acids were identified (see Supplementary Table 1). The total fatty acid content indicated no response to increasing N enrichment ($p = 0.47$, $r^2 = 0.03$). However, the fatty acid composition of phytoplankton changed slightly along the N fertilisation gradient (see Supplementary Fig. S2). The total omega-3 PUFAs tended to decrease ($p = 0.07$, $r^2 = 0.2$), while ALA (alpha-linoleic acid, C18:3 ω3) declined substantially with increasing N enrichment (Fig. 3A). Most importantly, the essential PUFA fatty acid EPA (eicosapentaenoic acid, C20:5 ω3) showed a significant negative relationship to increasing N enrichment (Fig. 3B). The total amounts of omega-6 PUFAs and omega-9 monounsaturated fatty acids (MUFAs) did not show a significant relationship to increasing N fertilisation ($p = 0.20$, $r^2 = 0.10$ and $p = 0.22$, $r^2 = 0.09$). Only the relative contribution of omega-9 MUFAs on total fatty acids increased significantly with increasing N fertilisation from 19.1 ± 0.1% in the control treatment to 25.2 ± 0.3% in the 32 x N treatment ($p = 0.01$, $r^2 = 0.35$). On the contrary,

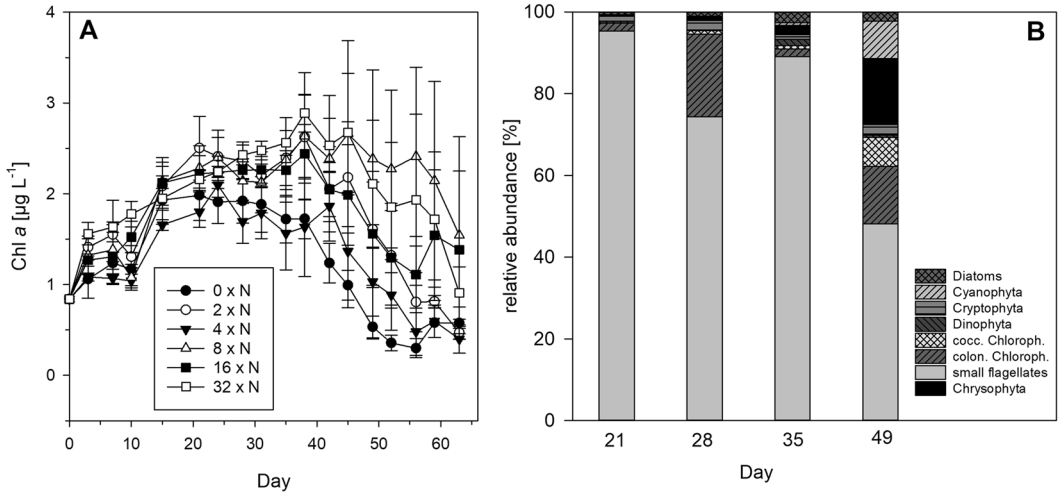

Figure 2. Phytoplankton data in all six N fertilisation treatments. (**A**) Chlorophyll *a* concentrations (µg L^{-1}, mean ± 1 SE of n = 3 replicates) in all six N fertilisation treatments over time. (**B**) Relative abundances of the phytoplankton groups in the six treatments as averages per sampling day (differences between the treatments see Table 1).

Day, No. observations (n), Degrees of freedom (df)	model		Total biovolume	Chlorophyta colonies	Other flagellates
21 n = 18 df = 17	Lr ANOVA	p r^2 p F	**0.02** −0.31	0.39[a] 0.04 0.35[b] 5.61	**0.01** −0.38 0.08[b] 9.73
28 n = 18 df = 17	Lr ANOVA	p r^2 p F	**0.01** −0.35	0.07 0.20 0.18 1.84	**0.01** −0.37 0.10 2.44
35 n = 13 df = 7	Lr ANOVA	p r^2 p F	0.84 0.00	0.41[a] 0.06 0.44[b] 4.81	0.86 0.00 0.47 1.02
49 n = 18 df = 17	Lr ANOVA	p r^2 p F	0.32 0.06	**0.02** +0.29 **0.01** 5.31	0.05 0.22 0.80 0.46

Table 1. Statistical results of the main phytoplankton groups (>5% abundance of total phytoplankton biomass). Linear regression (lr) of the phytoplankton biovolume and One-Way-ANOVA (ANOVA) of the relative abundances against N fertilisation treatments. Normality and equal variance tests passed if not indicated. Significant results are shown in bold. The leading sign of slope for significant linear regression is indicated (+, −). [a]Normality test failed. [b]H-value (not normally distributed: Kruskal-Wallis ANOVA on Ranks).

the relative contribution the omega-6 PUFAs showed a decreasing trend with increasing N fertilisation ($p = 0.06$, $r^2 = 0.21$).

Zooplankton. Total zooplankton biomass, including cladocerans, copepods, and rotifers, increased during the experiment from a mean dry mass of 2.9 ± 1.2 µg L^{-1} on day 22 to a mean dry mass of 13.3 ± 8.4 µg L^{-1} on day 63 (see Supplementary Fig. S3). *Daphnia* biomass started to increase on day 35 (see Supplementary Fig. S3). In the control treatment without N fertilisation, *Daphnia* showed the fastest biomass increase and reached the highest biomass (12.9 µg L^{-1}, resembling approximately 5 Ind L^{-1}) on day 55. In all the other treatments *Daphnia* biomass continued to increase until the end of the experiment on day 63.

Daphnia biomass showed a decreasing trend with increasing N concentrations (day 50: $p = 0.10$, $r^2 = 0.16$), which equals an average decline of 66 ± 38% of *Daphnia* biomass over the experimental N gradient. No relationship between the *Daphnia* biomass and seston stoichiometry could be observed (C:P ratio: $p = 0.73$, $r^2 = 0.01$; N:P ratio: $p = 0.23$, $r^2 = 0.09$; C:N ratio: $p = 0.53$, $r^2 = 0.03$). However, *Daphnia* biomass was strongly positively correlated to the PUFA content in the seston (Fig. 4A). In particular, we found a significant increase of *Daphnia* biomass with ALA, EPA, and LA (linoleic acid, Fig. 4B–D).

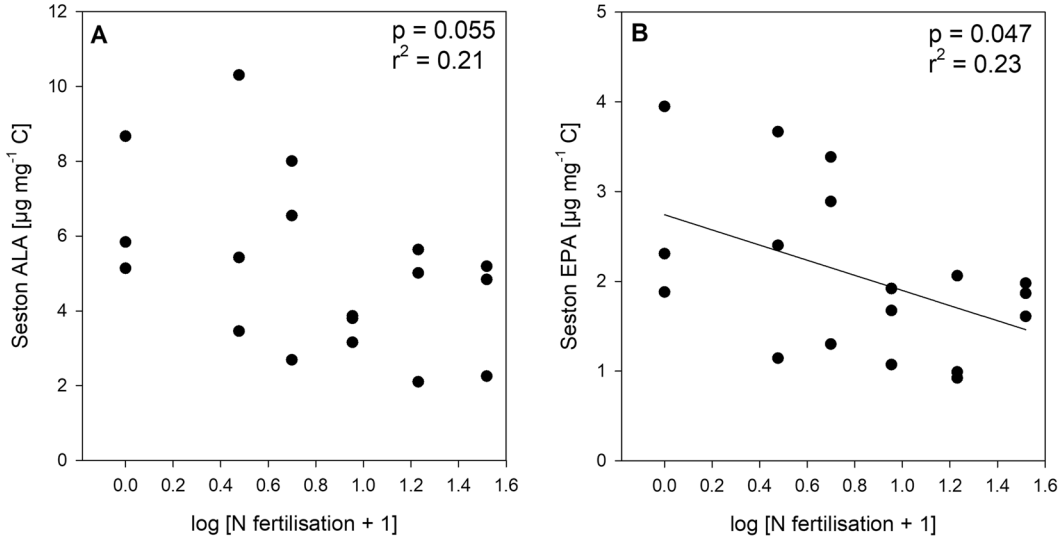

Figure 3. Relationship of fatty acids with N fertilisation treatment. Concentration of (**A**) alpha-linoleic acid (ALA, C18:3 ω3) and (**B**) eicosapentaenoic acid (EPA, 20:5 ω3) against the N fertilisation treatment. Significant linear regression line in solid ($p < 0.05$).

Discussion

Our data indicate that an increase in N supply into the studied lake resulted in a decline of PUFA contents in natural phytoplankton communities. Such a reduction of PUFA contents in natural phytoplankton communities can lead to a lower *Daphnia* biomass. The significant shifts in phytoplankton fatty acid composition caused by increased N enrichment became visible in lower quantities of EPA and ALA, which are known to be key PUFAs in freshwater food webs[20–22]. It had already been demonstrated that the PUFA yield in natural phytoplankton communities is negatively correlated with the P concentration and trophic status across lakes[27], and is additionally influenced by multiple other environmental stressors such as temperature, light or brownification[28–31]. In this study, we demonstrate that N load can also affect the PUFA composition of natural lake phytoplankton communities. It is well known that algal taxonomic groups differ in their PUFA composition[32]. EPA for example is a major FA of diatoms[33,34] and ALA for chlorophytes[33]. However, the negative relationship of EPA and ALA with increasing N fertilisation in our study can not be sufficiently explained by changes in the phytoplankton communities. With increasing N fertilisation, we found a weak positive relationship with chlorophyte colony biomass (but not relative abundances) and no relationship with diatoms (both biomass and relative abundances), which would allow this conclusion. Biomass and relative abundances of other unidentified flagellates were also not related to N fertilisation during the period of the *Daphnia* biomass increase, although we cannot fully exclude taxonomic differences within this group. Therefore, we assume that the shifts in PUFA composition in our study were independent of larger taxonomic changes in the phytoplankton community. We find no evidence in our experiment that the reduction in PUFAs with N enrichment was due to a succession of algal groups with different PUFA contents, but was rather dependent on a shift in the PUFA content of the phytoplankton cells *per se*.

Laboratory studies[35–38] have demonstrated that N deprivation can trigger higher lipid production in green algae, which were also an abundant component of the natural communities in our enclosures. Biochemically, the expression of PUFA biosynthesis genes is upregulated by N depletion[37]. Nitrogen depletion usually increases algal lipid synthesis and the abundance of lipid-related transcripts[37]. In our experiment under conditions of semi-continuous N enrichment, the lipid synthesis appears to be repressed to some extent, leading to lower phytoplankton PUFA contents. In particular, the omega-3 and -6 PUFAs decreased, while the omega-9 MUFAs gained relative importance with N enrichment. Our results provide first evidence that N enrichment not only affects fatty acid dynamics in laboratory strains of cultured microalgae, but also in natural phytoplankton communities. While our study focused on phytoplankton responses, one could potentially also expect such effects on functionally similar microalgal-periphyton. Given the importance of omega-3 and -6 PUFAs in the dietary quality of algae for higher trophic levels, this has potentially far reaching consequences for entire lake food webs.

In our field experiment, the *Daphnia* biomass correlated strongly with the PUFA content of the phytoplankton community and not with seston stoichiometry. This relationship indicates a clear transfer of N enrichment effects through the food web via N-enrichment dependent changes in phytoplankton PUFAs. Despite the natural variation associated with the phytoplankton and zooplankton communities, the relationship between N, *Daphnia* population growth, and the ALA and EPA content of phytoplankton became clearly visible. This is even more remarkable considering the relatively low N enrichment in our study, in comparison to previously described N enrichment field experiments (0.33 mg L^{-1} N per week in the highest 32 x N treatment compared to e.g. 6 mg L^{-1} N per week[39]). The phytoplankton-zooplankton-dynamics indicate that *Daphnia* increased earlier (after day 35) in the control treatment, and that this was followed by an earlier chlorophyll *a* decrease due to grazing, than was the case in the higher N treatments. These dynamics indicate that higher N enrichment and presumably lower PUFA concentrations may lead to worse growth conditions for *Daphnia* in spring, and subsequently could result

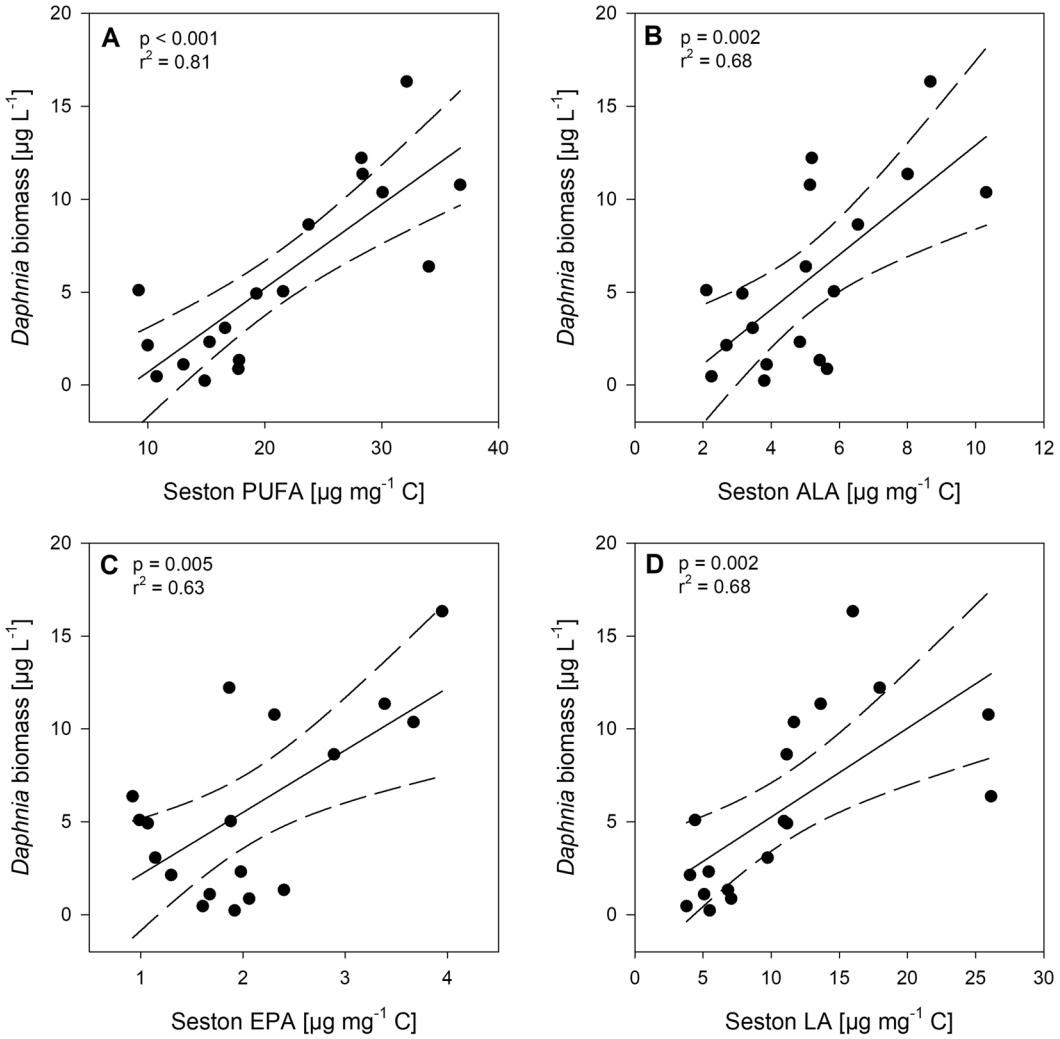

Figure 4. Relationship of *Daphnia* biomass with fatty acid concentrations. *Daphnia* biomass against the concentrations of (**A**) PUFAs, (**B**) alpha-linoleic acid (ALA, C18:3 ω3), (**C**) eicosapentaenoic acid (EPA, 20:5 ω3) and (**D**) linoleic acid (LA, C18:2 ω6). Significant linear regression lines in solid ($p < 0.05$), 95% confidence interval in dashed lines.

in a delayed clear-water-phase (after day 50 in treatments 8–32 x N). Poor growth and recruitment of zooplankton under low PUFA conditions was already suggested to have a negative feedback on the control of phytoplankton biomass[40]. To our knowledge our experiments are the first supporting this idea with natural plankton communities. Nitrogen enrichment affected phytoplankton food quality by bottom-up effects but also phytoplankton population dynamics by negative feedback effects of zooplankton grazing on phytoplankton with low food quality. This emphasizes the general importance of PUFAs for pelagic food web dynamics including bottom-up and top-down processes.

Our results suggest that continuous N enrichment and accumulation leads to lower PUFA (especially ALA and EPA) contents, and therefore also to lower food quality of lake phytoplankton. Subsequently, zooplankton and particularly the *Daphnia* biomass will decrease, even if the P concentrations remain unaltered. We can exclude large phytoplankton community composition changes as the reason for the lower *Daphnia* population growth observed, together with the presence of toxic algae or detectable amounts of algae with indigestible gelatinous sheaths. Community shifts towards a lower abundance of EPA (and ALA) rich taxa, such as diatoms and cryptophytes[41], could in theory cause similar effects to those observed here, which would cause a reduced phytoplankton PUFA content, with subsequent negative consequences for *Daphnia* sp.

Beside an N enrichment, an increase in P limitation may also change the seston stoichiometric composition or the PUFA composition in phytoplankton. Mineral limitation by P in *Daphnia* sp. is predicted at seston C:P ratios >300[42], as it was the case in our experiment. While previous studies have demonstrated increasing P limitation with N enrichment[14,43], we were unable to detect signs of increasing P limitation with increasing N enrichment, such as increasing seston N:P ratios. Additionally we did not observe any correlation of seston stoichiometry with *Daphnia* biomass, which does not exclude the possibility that this mechanism could operate supplementary. However, our data suggest an N dependent change in the lipid syntheses of phytoplankton *per se*.

Fertilisation amounts of N treatments	0 x N	2 x N	4 x N	8 x N	16 x N	32 x N
NO_3: mL mg	0 0	2 60	4 120	8 240	16 480	32 960
NH_4: mL mg	0 0	2 20	4 40	8 80	16 160	32 320

Table 2. Experimental design of the applied N fertilisation in each N treatment with given volumes (mL) and respective amounts (mg) of NO_3 and NH_4. The amounts were given twice a week and in a 1:1 molar ratio.

N enrichment has recently been shown to be negatively linked to zooplankton and *Daphnia* biomass across three lakes with different trophic status[16]. However, no mechanism has yet been identified that can explain the relationship observed. The effect of N enrichment on phytoplankton PUFA composition found in this study represents a mechanistic and ecologically important link to how N enrichment can affect higher trophic levels, even in lakes where primary production is P limited and the quantitative food web effects of N enrichment are not typically expected. Based on spring phytoplankton communities that are the nutritional base for zooplankton growth early in the season[25,26], our experiment demonstrates that "non-limiting" nutrients can also have strong trophic effects on food web dynamics and production. By increasing the non-limiting nutrient, N is able to reduce the secondary production of zooplankton in a similar way as reducing the limiting nutrient P would do.

Our experimental system included a typical plankton community (phyto- and zooplankton) that is characteristic of the majority of P deficient temperate lakes, including the full spring succession dynamics of temperate lakes (PEG model[25,26]). The causal relationship described between N enrichment and reduced biochemical food quality might also be relevant to other lake systems, although the qualitative effects of N enrichment could be masked by the quantitative effects of phytoplankton growth in N limited lake systems[39] and upscaling from mesocosms to lake systems may have its limitations[44]. However, an N related reduction in secondary production could even affect higher trophic levels, such as fish[43]. Cladoceran zooplankton are known to represent the primary food source for planktivorous fish in temperate lakes[45], and cladoceran production is positively correlated to fish biomass in lakes e.g.[46,47]. Therefore, the extent to which increasing N enrichment, including organic N forms, contributes to poor food conditions for planktivorous fish has to be further evaluated. Given than non-limiting nutrients can also affect food web dynamics and the production of higher trophic levels, they should be considered in management strategies for freshwater lakes in order to understand the potential ecosystem consequences.

Material and Methods

Study site and experimental design. The mesocosm field experiment was performed in Lake Brunnensee in southern Germany during the spring of 2015 (March 17th to May 19th), and started directly after the ice melting. Lake Brunnensee is an oligotrophic lake with total phosphorus (TP) concentrations of less than 10 µg L^{-1} and nitrate (NO_3) concentrations of ~8 mg L^{-1}. Accordingly, the N:P ratios were >600:1 at the beginning of the experiment, indicating highly P limiting conditions for the primary producers. The zooplankton community of Lake Brunnensee consists predominantly of calanoid copepods (*Eudiaptomus* sp.) and cladocera (*Daphnia cf. longispina*).

The mesocosms were made of transparent polyethylene foil (4 m deep, 0.95 m in diameter, ~2.84 m^3 in volume). They were closed at the bottom and open at the top, where they were attached to a raft anchored in the centre of the lake. Natural phytoplankton and zooplankton communities were enclosed by lowering the mesocosms into the water column and lifting them back to the surface. A total of 18 mesocosms were filled, and transparent coverings were installed above them in order to minimize the influence of natural precipitation, while ensuring natural light penetration.

The increasing N treatments were based on multiple amounts of natural N fertilisation by nitrate and ammonium (0-, 2-, 4-, 8-, 16- and 32-times the concentration in atmospheric wet deposition). The natural atmospheric wet deposition of the region contains an average supply of 75 mg m^{-2} NO_3 and 25 mg m^{-2} NH_4 per week (Bavaria regional state office), with on average 25 L m^{-2} of weekly precipitation (German Meteorological Survey). The control treatment (0) received no N fertilisation, the treatments with 2-times the N concentration in atmospheric wet deposition received an equivalent to 150 mg m^{-2} NO_3 and 50 mg m^{-2} NH_4 per week, and so forth for the higher fertilisation treatments.

We fertilised the 18 mesocosms using six N treatments (3 replicates, randomly scattered over the experimental rafts) over a period of 10 weeks, with two fertilisations per week to simulate a semi-continuous N supply. The fertilisation solutions comprised nitrate and ammonium in a 1:1 molar ratio (stock solution: 41.1 mg mL^{-1} $NaNO_3$, 29.7 mg mL^{-1} NH_4Cl). A basic P and Si solution (247.2 mg L^{-1} KH_2PO_4, 6164.49 mg L^{-1} $Na_2SiO_3 \times 5$ H_2O) was prepared in order to counteract nutrient loss by sedimentation (based on 0.056 µg L^{-1} day^{-1} total P in previous years). The respective amounts of the fertilisation solutions (0, 2, 4, 8, 16, and 32 mL of stock solution) were transferred into labelled 1 L polyethylene bottles for each treatment in the laboratory (Table 2). 10 mL of basic P and Si solution was added to all the bottles, which were then filled with distilled water. The control treatment (0) received only 10 mL of the basic P and Si solution. The nutrient solution was given to each mesocosm out on the lake, and a Secchi disk was lowered twice to ensure the mixing of the added nutrients. In order to ensure different starting conditions for the individual treatments, the first N fertilisation was given on day 2, with four times the common fertilisation amount.

Sampling and laboratory analyses. Sampling for chlorophyll *a* occured twice a week, and sampling for water chemistry, phytoplankton, and zooplankton occurred once a week. All water samples for chlorophyll *a*,

water chemistry, and phytoplankton, were taken with an integrated water sampler (KC DenmarkA/S research equipment) of between 1 and 3 metres. The water was filtered through a 250 µm gauze to exclude mesozoplankton.

The water chemistry analyses included NO_3 and NO_2 measurements, which were performed by ion chromatography (Dionex ICS-1100, Thermo Scientific, USA) after 0.45 µm filtration of enclosure water (CS 400 cellulose acetate syringe filters; Nalgene, USA). The NH_4 was measured by fluorometry (Trilogy Laboratory Fluorometre Module CDOM/NH4; Turner Designs, USA) using the orthophthalate method[48]. Prior to the measurement, 2.5 mL of mesocosm water was mixed with 10 mL of a working reagent, which included orthophtalate, sodium sulphite, and borate buffer, and was incubated for two hours in darkness. Dissolved inorganic phosphorus (PO_4) was measured by ion chromatography (Dionex ICS-1100, Thermo Scientific, USA) after 0.45 µm filtration of enclosure water (CS 400 cellulose acetate syringe filters; Nalgene, USA). The total phosphorus (TP) was measured by means of spectrophotometry (Shimadzu UV-1700, Shimadzu Cooperation, Germany) on 12 mL of mesocosm water, using the molybdenum blue method[49]. The silicate concentrations were analysed on April 7th (day 21), April 21th (day 35), May 5th (day 49) and May 19th (day 63). Silicate was analysed by filtering 100 to 200 mL of enclosure water onto cellulose-acetate filters (0.6 µm pore size, Satorius). The filters were subsequently extracted in a water bath (95 °C, for 4 h, in 0.2 mol NaOH)[50] and were measured spectrophotometrically using the molybdenum blue method.

For the analyses of particulate organic carbon (POC), particulate nitrogen (PN), and particulate phosphorus (PP), 100 mL to 250 mL of enclosure water was filtered onto pre-combusted (4 h, 450 °C), acid washed (10% HCl) glass fibre filters (GF/F; Whatman, USA), which were subsequently frozen (−20 °C). The samples from the biomass maximum to the end of the experiment (April 7th to May 19th 2015) were analysed as being representative of food quality for zooplankton. The measurements for POC and PN were conducted using an elemental analyser (vario Micro cube, Elementar, Germany), after thawing and drying the filters, and compacting them into small tin caps. The PP was measured after sulfuric acid digestion of the filters with a spectrophotometer (Shimadzu UV-1700, Shimadzu Cooperation, Germany) by applying the molybdenum blue method. Afterwards, the stoichiometric ratios of biomass C:N, N:P and C:P were calculated.

In order to follow the phytoplankton development, chlorophyll *a* measurements were performed twice a week *in vivo* using an Algae lab Analyser (bbe Moldaenke, Germany). This device measures the total chlorophyll *a*, and additionally separates the excitation spectra of four pigment groups into the blue, green, brown, and mixed spectral group[51].

The microscopic counting of the phytoplankton community was conducted on Lugol fixed samples for the biomass maximum (April 7th, 14th, and 21st 2015) and the biomass decline (May 5th 2015, day 49) (n = 18, except for April 21st 2015 where only 13 samples could be counted). Applying the Utermöhl method[52], we placed 25 to 50 mL of each sample into sedimentation chambers. The algae were settling down for at least 24 hours and then they were counted under an inverted microscope (Leica, Germany). In the case of large taxa (e.g. *Ceratium hirudinella*), the whole area of the sedimentation chamber was counted using a magnification of 40. Large diatoms, other ciliates and green algae colonies were counted using a magnification of 200, by counting at least two stripes or 100 individuals of the most abundant taxa. Small green algae, small diatoms, flagellates, and heterotrophic nanoflagellates were counted using a magnification of 400, by counting at least two stripes or 100 individuals of the most abundant taxa. The phytoplankton biomass was estimated after measuring the size of 10 individuals of the most abundant taxa, or by using measurements from earlier studies in this lake[53].

In order to draw conclusions about the fatty acid quality of the zooplankton's available food, the phytoplankton fatty acid composition was analysed before the increase in zooplankton densities that occurred during phytoplankton biomass decline (on day 50). To this end, 1 to 1.5 L of 250 µm pre-filtered water was filtered onto glass fibre filters (GF/F; Whatman, USA) and submerged in glass vials, together with 5 mL of a 2:1 (vol:vol) mixture of dichloromethane and methanol (chromatography grade).

The phytoplankton lipids were extracted twice with 5 mL of dichloromethane and methanol (2:1), after the addition of 20 µg methyl heptadecanoate and methyl tricosanoate as internal standards for each sample. The resulting extracts were pooled and evaporated to dryness at 40 °C under a stream of N_2 gas. The lipid residue was then re-dissolved in 5 mL of 3 N methanolic hydrochloric acid (SUPELCO) and the lipid-bound and free fatty acids were transesterified to fatty acid methyl esters (FAMEs) for 20 min at 70 °C. Subsequently, the FAMEs were extracted from the hydrochloric acid using 3 × 2 mL iso-hexane. The hexane supernatants were pooled and evaporated to dryness under a stream of N_2 gas at 40 °C. The residue was taken up in 100 µL iso-hexane of which 1 µL was injected (splitless) into the inlet (200 °C, 1.5 mL min^{-1} He as carrier gas) of an Agilent 6890 N GC system equipped with a J&W DB-225 fused silica capillary column (30 m, 0.25 mm, 0.25 µm) at 60 °C. The initial oven temperature of 60 °C was held for one minute, followed by a 20 °C min^{-1} temperature ramp to 150 °C, then 7 °C min^{-1} to 220 °C followed by a final 14 minutes at 220 °C as described elsewhere[54]. The FAMEs were quantified via the internal standards and response factors determined for each FAME relative to the standard from mixtures of known composition for details see[54,55].

The zooplankton sampling was performed once a week from day 22 (April 8th 2015). The samples were taken by slowly hauling up a 105 µm plankton net through a four metre water column in the centre of each mesocosm. Each sample was immediately poured into a 100 mL polyethylene bottle and immediately fixed to a 70% ethanol end concentration. The samples were stored in the fridge (4 °C) until the zooplankton communities were analysed using a stereo microscope (Wild M3Z; Wild Heerbrugg, Switzerland).The specimens were determined to species level if possible. Length measurements were performed by applying an ocular micrometre with a 0.1 mm division scale. The *Daphnia* biomass (consisting of *D. longispina*) was calculated by using the length-weight relationship of Eq. (1):

$$\mathrm{Ln}(w) = \mathrm{Ln}(\alpha) + \beta\,\mathrm{Ln}(L) \quad (1)$$

where w = dry weight in µg, L = length in mm, β = regression slope and Ln(α) = intercept. The coefficients Ln(α) = 1.073 and β = 2.89 were chosen for *Daphnia longispina*[56].

Statistical analyses. On the basis of our exponential experimental fertilisation design, linear regression models against logarithmic N fertilisation were applied to the response variables (df = 17 unless indicated otherwise). The applied treatment design was tested using linear regression for the average dissolved N:P ratios against logarithmic N fertilisation. In order to analyse the phytoplankton responses, the weekly data of chlorophyll *a* were tested using linear regression models against logarithmic N fertilisation, while the seston stoichiometric ratios (Seston C:P, seston N:P and seston C:N) were tested as an average over the experimental period and on day 49. The responses of microscopic estimated biovolumes of phytoplankton to N treatments were analysed for the most abundant phytoplankton groups (>5% abundance on total phytoplankton) for each day (21 to 49) against logarithmic N fertilisation. In addition, the relative abundances of the individual algal groups (>5% abundance on total phytoplankton) were tested using One-Way ANOVAs for differences between the treatments (with N treatment as the fixed factor). The microscopically estimated total biovolume of phytoplankton was tested using linear regression against logarithmic N fertilisation. The phytoplankton fatty acid composition was tested during chlorophyll *a* decline (indicating strong grazing through *Daphnia*, day 50) as the absolute amounts (PUFA, omega-3 PUFA, omega-6 PUFA, omega-9 MUFA, EPA, ALA, LA) and the relative contribution with linear regression against the N fertilisation treatments. In order to analyse the effects of N enrichment, seston stoichiometry, and biochemical food composition on *Daphnia*, the *Daphnia* biomass on day 50 was analysed using linear regression against the N fertilisation treatments, seston stoichiometry (day 49) and phytoplankton fatty acid composition. The statistical analyses were performed with Systat Software (Sigma Plot 11.0).

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Author contributions

G.T. was responsible for the conception, acquisition, analyses and interpretation of the data. P.L., A.L. and P.F. contributed to the analyses and interpretation of the data. H.S. contributed to the conception and interpretation of the data. All authors contributed to the text and have read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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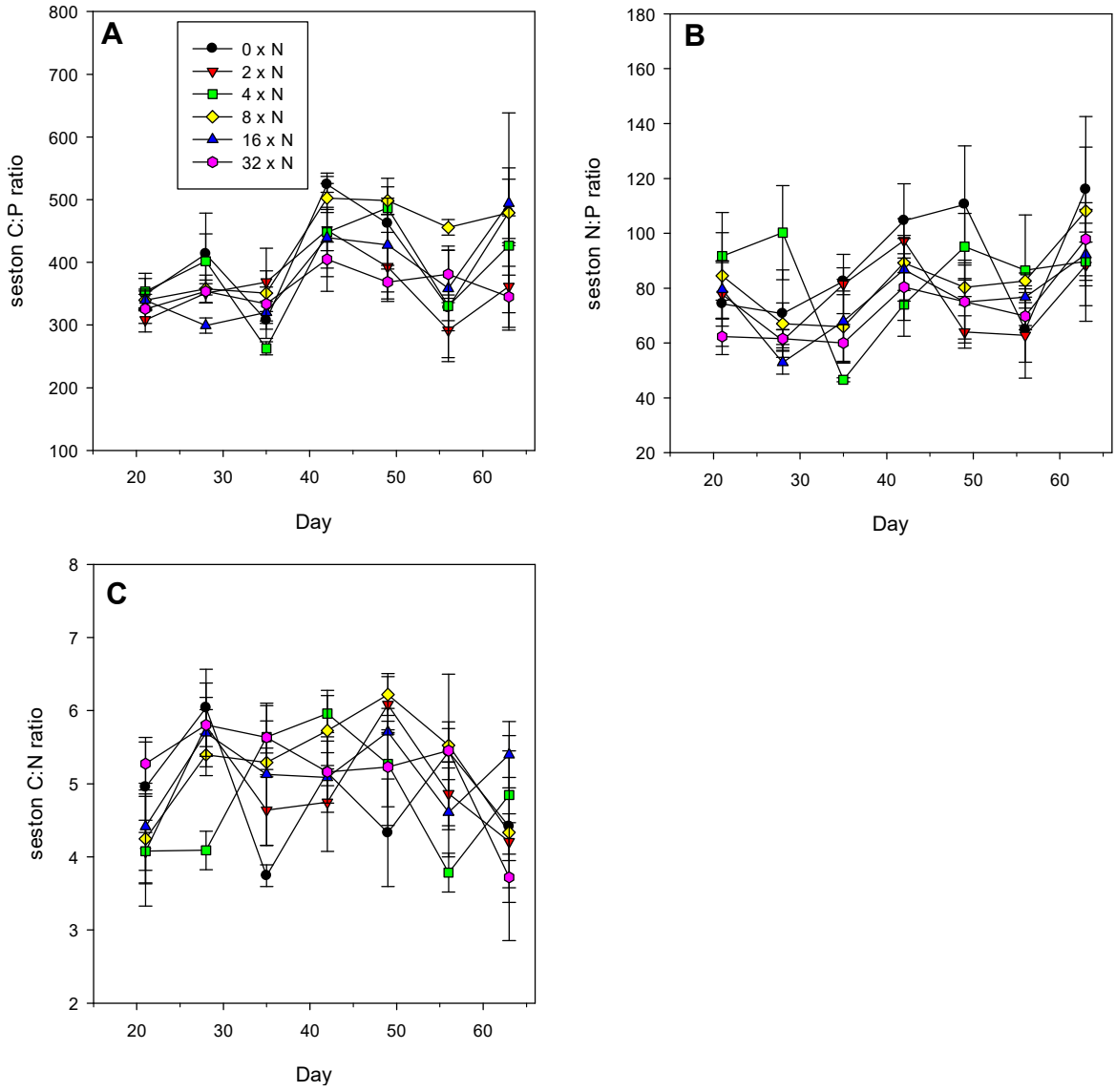
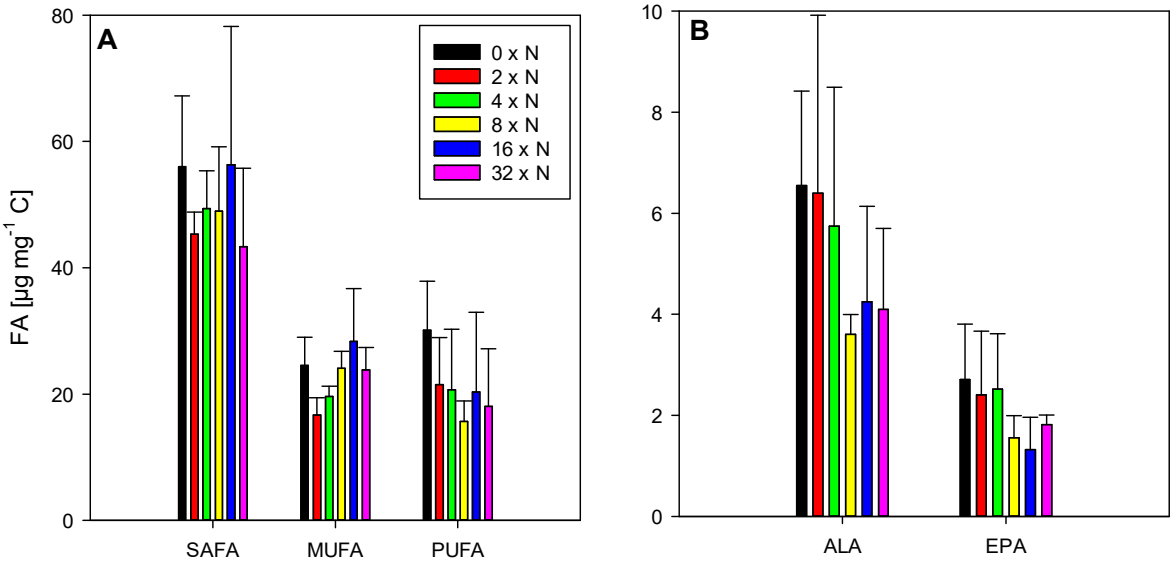
Nitrogen enrichment leads to changing fatty acid composition of phytoplankton and negatively affects zooplankton in a natural lake community

Gabriele Trommer, Patrick Lorenz, Ameli Lentz, Patrick Fink, Herwig Stibor

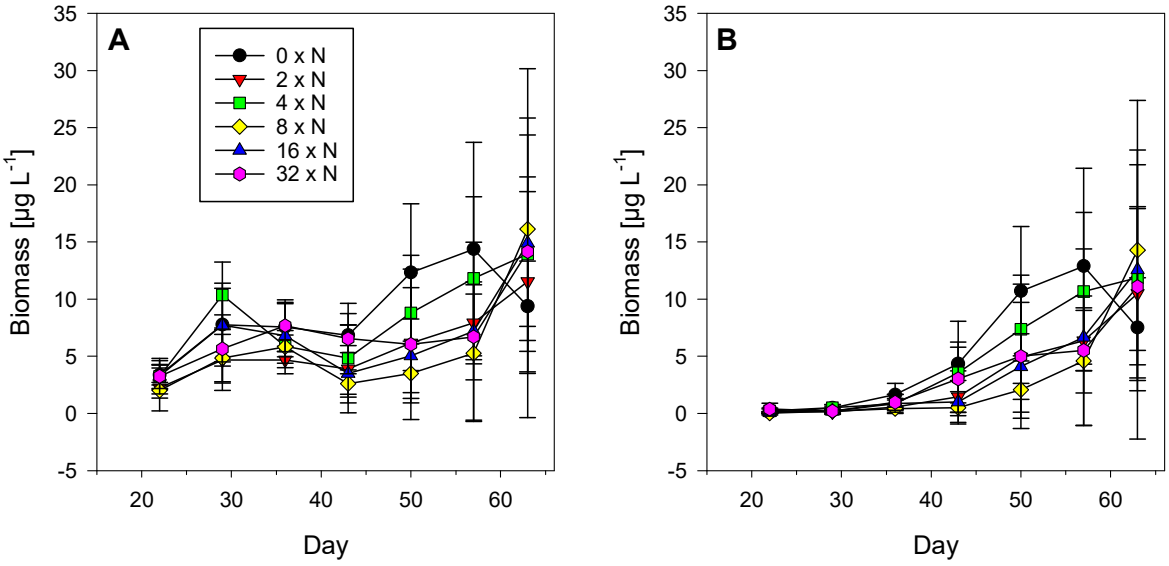
Supplementary Information



Supplementary Figure S1. Seston stoichiometry in all six N fertilisation treatments. (A) seston C:P ratios, (B) seston N:P ratios and (C) seston C:N ratios (mean ± 1 SE of n = 3 replicates) over time.



Supplementary Figure S2. Fatty acid composition in all six N fertilisation treatments. (A) Saturated fatty acids (SAFAs), mono unsaturated fatty acids (MUFAs), PUFAs and (B) ALA and EPA (mean ± 1 SE of n = 3 replicates).



Supplementary Figure S3. Time course of zooplankton biomass in all six N fertilisation treatments. (A) Total zooplankton biomass and (B) *Daphnia sp*. biomass (µg L^{-1}, mean ± 1 SE of n = 3 replicates) in all six N treatments over time.

Supplementary Table 1: Seston fatty acid composition (ng/mg C) of the mesocosms (day 49).

Mesocosm No.	Date	Treatment No.	Treatment N fertilization	Total FA	C10:0	C11:0	C12:0	C13:0	C14:0	C14:1 ω9	C15:0	C15:1 ω9	C16:0	C16:1 ω9	C17:0	C17:1 ω9	C18:0	C18:1 ω9 t	C18:1 ω9 c	C18:1 ω7	C18:2 ω6 t	C18:2 ω6 c	C18:3 ω6	C18:3 ω3	C18:4 ω3
01	06.05.15	1	0	115034,5	0,0	0,0	619,3	292,8	804,6	902,2	823,8	0,0	17424,7	3953,0	903,2	0,0	6180,2	0,0	15474,5	3535,3	0,0	15981,3	0,0	8669,0	691,5
02	06.05.15	1	0	85617,6	0,0	0,0	567,4	270,0	585,3	579,7	696,4	0,0	12767,3	4160,9	592,7	0,0	5526,1	0,0	12129,3	2563,7	0,0	10937,0	317,7	5839,9	463,6
03	06.05.15	1	0	131541,7	0,0	0,0	775,9	0,0	4248,3	707,3	256,1	0,0	23158,6	1111,8	327,6	0,0	7796,3	0,0	23428,6	2832,9	0,0	25933,2	840,8	5139,2	1294,2
04	06.05.15	2	2	76758,8	0,0	0,0	389,6	0,0	422,2	546,8	548,7	0,0	12284,5	1815,5	347,2	0,0	4522,9	0,0	10438,2	1961,3	0,0	9736,8	386,6	3454,6	1042,3
05	06.05.15	2	2	92520,2	0,0	0,0	386,8	0,0	1051,0	940,4	0,0	0,0	14235,7	1392,9	859,6	0,0	5292,1	0,0	12683,2	4093,5	0,0	11670,3	0,0	10305,5	541,2
06	06.05.15	2	2	81308,5	0,0	0,0	0,0	209,4	9087,4	976,6	0,0	514,0	14360,8	1897,1	621,4	0,0	4103,8	0,0	7429,9	2983,8	0,0	6834,6	0,0	5424,8	694,5
07	06.05.15	3	4	92157,1	0,0	0,0	0,0	272,1	704,0	853,3	0,0	513,3	14456,1	1515,5	739,8	0,0	5324,6	0,0	11510,0	3287,8	0,0	11135,7	421,6	6545,6	579,2
08	06.05.15	3	4	102702,8	0,0	0,0	0,0	0,0	731,4	822,5	0,0	487,7	15231,9	1225,3	798,3	0,0	5891,2	0,0	13408,6	2901,0	0,0	13623,5	0,0	8003,1	676,8
09	06.05.15	3	4	74465,4	0,0	0,0	0,0	0,0	7738,3	743,7	206,0	316,8	10169,7	12977,2	359,1	0,0	2421,7	0,0	4833,4	2136,7	0,0	4045,4	275,2	2687,0	668,6
10	06.05.15	4	8	103222,8	0,0	0,0	742,0	424,3	2689,6	491,5	642,6	0,0	11487,6	3248,7	344,9	0,0	5937,5	0,0	13543,8	2080,5	0,0	11159,4	469,2	3155,3	1794,4
11	06.05.15	4	8	88112,9	0,0	0,0	398,2	204,5	8558,2	1310,4	608,5	0,0	12622,4	15462,9	372,5	0,0	3179,6	0,0	6985,4	2752,7	0,0	5487,0	266,5	3797,9	660,0
12	06.05.15	4	8	75109,0	0,0	0,0	356,4	0,0	7676,7	1144,8	615,4	0,0	11224,9	12498,3	336,0	0,0	3055,9	0,0	5843,0	2204,7	0,0	5082,5	247,0	3867,4	588,3
13	06.05.15	5	16	87547,7	0,0	0,0	0,0	0,0	6004,7	1222,2	740,1	0,0	12419,0	10542,7	399,3	0,0	3587,2	0,0	8398,3	2971,2	0,0	7064,2	399,6	5637,2	669,1
14	06.05.15	5	16	74080,8	0,0	0,0	0,0	0,0	8535,7	1334,8	206,2	0,0	11385,1	14219,8	291,5	169,0	3083,9	0,0	5672,9	2190,2	0,0	4415,2	245,2	2100,8	632,5
15	06.05.15	5	16	153407,5	0,0	0,0	726,9	308,7	4579,9	1003,1	1273,3	0,0	24858,1	6699,5	360,9	0,0	10106,2	0,0	25745,7	3452,0	0,0	26124,3	659,8	5014,7	1310,2
16	06.05.15	6	32	113606,1	0,0	0,0	557,8	0,0	550,8	643,5	985,7	0,0	18487,3	5627,7	382,9	0,0	7905,4	0,0	17878,1	3007,0	0,0	17951,8	684,7	5192,5	1136,1
17	06.05.15	6	32	74871,6	0,0	165,2	449,9	194,3	626,9	1152,3	171,8	0,0	11864,8	11984,4	477,1	0,0	3094,0	0,0	7057,8	2840,9	0,0	5419,8	292,5	4840,8	726,9
18	06.05.15	6	32	67359,8	0,0	130,8	0,0	138,1	7852,6	655,1	0,0	256,3	10354,7	12474,0	297,4	0,0	2364,1	0,0	4721,8	2131,3	0,0	3793,4	275,2	2250,4	979,8

Mesocosm No.	Date	Treatment No.	Treatment N fertilization	C19:0	C20:0	C20:1 ω9	C20:1 ω7	C20:2 ω6	C20:3 ω6	C20:4 ω6	C21:0	C20:3 ω3	C20:5 ω3	C22:0	C22:1 ω9	C22:2 ω6	C23:0	C22:6 ω3	C24:0	C24:1 ω9
01	06.05.15	1	0	15533,4	0,0	0,0	0,0	567,1	0,0	1240,1	0,0	1036,3	3947,9	523,6	397,3	0,0	15533,4	0,0	0,0	0,0
02	06.05.15	1	0	10686,4	0,0	0,0	891,2	430,2	0,0	615,7	961,3	321,2	1880,3	393,6	0,0	754,3	10686,4	0,0	0,0	0,0
03	06.05.15	1	0	14076,5	0,0	0,0	498,1	0,0	0,0	880,1	0,0	316,9	2307,4	947,2	588,0	0,0	14076,5	0,0	0,0	0,0
04	06.05.15	2	2	12474,8	0,0	0,0	421,5	0,0	0,0	487,8	0,0	198,9	1142,2	382,3	1129,2	150,3	12474,8	0,0	0,0	0,0
05	06.05.15	2	2	10319,5	0,0	0,0	0,0	0,0	0,0	1462,0	0,0	2423,4	3667,0	341,9	534,8	0,0	10319,5	0,0	0,0	0,0
06	06.05.15	2	2	10343,6	0,0	0,0	0,0	0,0	0,0	1272,3	0,0	1163,1	2401,3	277,2	369,4	0,0	10343,6	0,0	0,0	0,0
07	06.05.15	3	4	14079,4	0,0	0,0	111,9	365,3	0,0	1014,1	0,0	797,0	2889,8	446,5	515,1	0,0	14079,4	0,0	0,0	0,0
08	06.05.15	3	4	15939,6	0,0	0,0	0,0	522,3	0,0	1077,4	0,0	1077,8	3385,7	475,3	483,8	0,0	15939,6	0,0	0,0	0,0
09	06.05.15	3	4	11014,0	0,0	0,0	106,0	0,0	0,0	742,9	0,0	254,7	1298,8	176,5	279,9	0,0	11014,0	0,0	0,0	0,0
10	06.05.15	4	8	18827,8	0,0	0,0	4315,5	0,0	0,0	0,0	0,0	370,4	1070,1	341,8	0,0	1258,3	18827,8	0,0	0,0	0,0
11	06.05.15	4	8	9870,4	379,4	0,0	479,6	0,0	0,0	1457,5	0,0	812,8	1919,0	200,7	0,0	456,4	9870,4	0,0	0,0	0,0
12	06.05.15	4	8	8449,1	0,0	0,0	0,0	0,0	0,0	1010,7	0,0	564,7	1675,3	218,8	0,0	0,0	8449,1	0,0	0,0	0,0
13	06.05.15	5	16	11265,0	565,1	145,8	0,0	290,5	0,0	880,5	0,0	734,2	2061,6	285,2	0,0	0,0	11265,0	0,0	0,0	0,0
14	06.05.15	5	16	8615,4	0,0	0,0	125,0	0,0	0,0	601,3	0,0	220,4	988,8	236,3	195,6	0,0	8615,4	0,0	0,0	0,0
15	06.05.15	5	16	19126,3	0,0	0,0	478,6	0,0	0,0	0,0	0,0	0,0	922,0	934,6	596,5	0,0	19126,3	0,0	0,0	0,0
16	06.05.15	6	32	14044,0	0,0	283,8	230,8	345,3	0,0	714,3	0,0	368,5	1866,7	717,4	0,0	0,0	14044,0	0,0	0,0	0,0
17	06.05.15	6	32	9543,8	0,0	0,0	0,0	311,4	0,0	949,1	0,0	747,3	1979,7	254,5	182,5	0,0	9543,8	0,0	0,0	0,0
18	06.05.15	6	32	7343,4	0,0	0,0	103,5	0,0	0,0	809,1	0,0	224,6	1607,7	218,6	237,1	93,2	7343,4	704,5	0,0	0,0

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Increasing N:P ratios change seston fatty acids, zooplankton growth and whitefish growth conditions in pre-alpine lakes

Patrick Lorenz, Gertrud Spoerl, Gabriele Trommer, Bernhard Gum, Michael Schubert, Thomas Hansen and Herwig Stibor

In preparation

Increasing N:P ratios change seston fatty acids, zooplankton growth and whitefish growth conditions in pre-alpine lakes

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***Running title*:** Increasing N:P ratios change seston FA, zooplankton and whitefish

***Keywords*:** nitrogen, phosphorus, fatty acids, zooplankton, whitefish, pre-alpine lakes

Summary

Anthropogenic activities result in changes in nutrient loadings and increasing nitrogen (N) to phosphorus (P) ratios in freshwater lakes. It is known that imbalances in N and P concentrations can induce changes in seston stoichiometry and in the composition of seston polyunsaturated fatty acids (PUFAs). These shifts can negatively affect food web transfer efficiency from phytoplankton to zooplankton and to planktivorous fish. However, most studies dealing with this subject have been performed under controlled, and hence non-natural, conditions in laboratory and field mesocosm experiments. Here we investigate if the food web effects of N enrichment can also be seen in natural pelagic communities that show the full complexity of pre-alpine lake systems.

The questions to be answered in this paper are: 1. Does increasing N loading alter seston biochemical food quality (PUFA composition)? 2. Are *Daphnia* abundances negatively affected by potentially decreasing seston food quality? 3. To what extent are potential trophic effects further transferred to planktivorous whitefish (*Coregonus* spec.)?

For this purpose a large-scale lake-monitoring program involving 11 pre-alpine lakes in southern Germany (Ammersee, Chiemsee, Lake Constance, Klostersee, Königssee, Riegsee, Simssee, Staffelsee, Starnberger See, Tegernsee, Walchensee) was conducted, with two samplings per year (spring and autumn) for a period of three years (2016–2018).

We found significantly decreasing seston carbon to N (C:N) ratios with rising nitrate-N (NO_3^--N) concentrations in the lakes. Furthermore, we observed significantly decreasing proportions of total PUFAs, ω3-PUFAs, eicosapentaenoic acid C20:5ω3 (EPA), docosahexaenoic acid 22:6ω3 (DHA) and linoleic acid 18:2ω6C (LA-C) in the total fatty acids in the seston biomass with increasing N:P ratios. *Daphnia* biovolume and whitefish condition factor K were also negatively correlated to increasing N:P ratios. Whereas, *Daphnia* biovolume was significantly positively correlated to seston DHA and the whitefish condition factor K was significantly positive correlated to seston C-18 PUFAs.

Our results indicate that the effects of increasing N concentrations are also evident in the natural and non-experimentally manipulated pelagic communities of pre-alpine lakes. Moreover, as demonstrated by experimental studies that manipulated N:P ratios, the natural differences in N:P ratios in lakes seem to be linked to quantitatively and qualitatively similar changes in the elemental and biochemical composition of the phytoplankton. This lake

comparison shows that decreasing *Daphnia* abundances and a reduced growth of planktivorous whitefish are accompanying the observed changes in phytoplankton along the natural N:P gradient.

1. Introduction

Nutrient regimes in lakes are affected by anthropogenic activities and are changing rapidly (Galloway et al. 2003, Geist 2015). The emerging consequences may be of crucial significance for aquatic organisms. This is true primarily for the concentrations of the key nutrients in lakes: phosphorus (P) and nitrogen (N). There are mainly two underlying reasons for the ongoing changes in nutrient concentrations. On the one hand, there is the highly effective P detraction from wastewater that significantly decreases P loading into lakes (Gerdaux et al. 2006, Jeppesen et al. 2005). For example, the P concentrations in Lake Constance have been decreased by 90% since the 1970s (Geist 2015). On the other hand, rising anthropogenic N emissions are responsible for a continuous increase in N depositions (Schlesinger 2009, Steffen et al. 2015).

The outcome of these two processes results in steadily increasing N:P ratios in lake waters and a continuously increasing P limitation for phytoplankton in P-limited lakes (Bergström and Jansson 2006, Elser et al. 2009a, Elser et al. 2009b, Hessen 2013). Even if algae prefer the macronutrients N and P in a certain amount for growth (Hecky and Kilham 1988, Klausmeier et al. 2004), the majority of phytoplankton taxa are flexible in their stoichiometry (biomass composition of carbon, N and P) (Sterner and Elser 2002). If supplied with a surplus of N, phytoplankton can also accumulate more N, resulting in increasing biomass N:P ratios. Increasing N:P ratios in food algae can, however, negatively influence the growth and reproduction of some zooplankton groups (Sterner et al. 1993). In particular, zooplankters with high P demands, such as cladocerans, are known to experience a nutritional mismatch when supplied with P-deficient food (Sterner 1993).

Another biochemical factor that defines the food quality of algae for zooplankton is the content of fatty acids in their diet. In particular, the amount of essential polyunsaturated fatty acids (PUFAs) in the phytoplankton biomass determines the suitability of algae as food for planktivorous zooplankton (Ahlgren et al. 1990, Stottrup and Jensen 1990, Müller-Navarra 1995a). Herbivorous zooplankton (and other aquatic animals) cannot synthesize PUFAs themselves and are thus dependent on the supply of essential fatty acids from their natural, algal diet (Müller-Navarra et al. 2000, Von Elert 2002). Although phytoplankton is known to

increase the production of fatty acids under N limitation (Roessler 1990, Müller-Navarra 1995b, Ahlgren and Hyenstrand 2003, Bi et al. 2014) this increase is mainly due to non-essential, saturated fatty acids (Shifrin and Chisholm 1981, Parrish and Wangersky 1990). In contrast, phytoplankton can even reduce the synthesis of unsaturated fatty acids when grown under P limitation (Müller-Navarra 2004). Currently, the proximate cause for the dominance of either a mineral or a biochemical depletion of algal food is not completely understood. Studies suggest that biochemical limitation is to some extent a function of mineral limitation and hence correlates with nutrient stress among phytoplankton (Andersen and Hessen 1991, Harrison et al. 1990, Müller-Navarra 1995b, Bi et al. 2012, Bi et al. 2014).

A recent mesocosm experiment, conducted in 2015 in a P-limited pre-alpine lake (Brunnsee) in southern Germany, first provided evidence for decreasing concentrations of essential PUFAs in phytoplankton when exposed to increasing N loading. Furthermore, *Daphnia* biomass in this experiment was significantly correlated with seston fatty acids and decreased with decreasing alpha-linoleic acid C18:3ω3 (ALA), eicosapentaenoic acid C20:5ω3 (EPA) and linoleic acid C18:2ω6 (LA) concentrations in the phytoplankton biomass (Trommer et al. 2019). However, it is not easy to estimate whether the observed responses to an artificial enrichment with N were unique patterns of lake Brunnsee or if they are transferable to other lakes. Since *Daphnia* are known to be a key link in the trophic transfer of aquatic food webs (Gaedke and Straile 1998 and references therein) and they are of particular importance as prey for planktivorous fish (Lampert and Sommer 2007), any decreases in *Daphnia* biomass may also negatively influence planktivorous fish communities.

The purposes of this study were: (1) To estimate to what extent the results of the above-mentioned mesocosm experiment showing that N affects phytoplankton quality and zooplankton growth can also be seen in observational data originating from natural lake communities, and 2) To determine whether available fish data from lakes support the idea that the observed effects of N on plankton can influence organisms in higher trophic levels, such as fish. To do this we monitored 11 pre-alpine lakes, which are located along the east–west axis of southern Germany, for a period of three years (2016–2018). The studied lakes differ in various factors, such as size, depth, temperature and nutrients, in particular N and P concentrations. The lakes were sampled twice each year. The parameters investigated were nutrient concentrations, seston fatty acid composition, *Daphnia* biomass and the nutritional conditions of whitefish (*Coregonus* spec.) populations.

2. Material and Methods

2.1 *The lakes studied*

The 11 pre-alpine lakes studied are located along an east–west axis on the northern edge of the European Alps in Bavaria, in southern Germany: Lake Ammersee, Lake Chiemsee, Upper Lake Constance (subsequently referred to as *Lake Constance*), Lake Klostersee, Lake Königssee, Lake Riegsee, Lake Simssee, Lake Staffelsee, Lake Starnberger See, Lake Tegernsee and Lake Walchensee (Fig. 1). All the lakes have economically meaningful stocks of whitefish (*Coregonus* spec.) and differ considerably in size, volume, maximum and mean depth, size of catchment area and trophic state (Tab. 1).

2.2 *Sampling procedures*

The 11 lakes were sampled twice each year from 2016 to 2018. To account for seasonal differences, one sampling was performed in spring (including the months March to July) and one sampling in autumn (August to September). Whitefish samples were taken during the night between 6:00 pm and 7:00 am. *Daphnia* samples and water samples for nutrients, FA and seston C:N ratios (particulate organic carbon:particulate N ratios) were taken close to the fishing nets in the pelagic zone of the lakes during the first half of the day, between 7:00 am and noon. The water samples for nutrients, seston FA and seston C:N ratios were taken either from the surface (early in the year, when the lakes had no thermal stratification) or as integrated water samples from the epilimnion between 0–16 m (when the lakes were thermally stratified). All water samples were taken by using a two-meter-long, integrated water sampler (Integrated Tubular Water sampler, KC Denmark A/S Research Equipment, Denmark). The water was immediately pre-filtered (mesh size: 250 µm) to remove large zooplankton and kept in darkness for further analyses.

2.3 *Nutrients*

Water samples for nutrient analyses were transported to the laboratory, where concentrations of NH_4^+, NO_3^- and TP were measured. Ammonium was measured fluorometrically by applying a method modified after Holmes (Holmes et al. 1999). A volume of 2.5 ml lake water was mixed with 10 ml of working reagent (borate buffer 40 g/l, sodium sulfite 40 mg/l and orthophthaldialdehyde in ethanol (EtOH) 50 ml/l) and incubated for two hours in darkness. The samples were then measured with a fluorometer (Trilogy Laboratory Fluorometer Module

CDOM/NH4, Turner Designs, USA). The NO_3^- concentrations in lake water were quantified after pre-filtering an aliquot of sample water with a 0.45 µm cellulose acetate membrane filter (CS 400 Syringe Filters Cellulose Acetate 0.45 µm, Nalgene, USA) by using an ion chromatograph (Dionex ICS-1100 Basic Integrated IC System, Thermo Scientific, USA). For quantifying TP concentrations, the molybdenum blue method (Wetzel and Likens, 1991) was applied and measurements were performed by using a spectrophotometer (Shimadzu UV-1700, Shimadzu Corporation, Germany).

2.4 *Seston fatty acids*

For measurements of seston FA concentrations, a method modified after Christie (1989) was used, in which FAs were analyzed with a gas chromatograph after they were transferred into fatty acid methyl esters (FAMEs). Prior to analysis, water samples were filtered with pre-combusted (450°C, 24 h), acid-washed (10% HCl), glass, microfiber filters with a pore size of 0.7 µm (GF/F, Whatman, USA). After the addition of 100 µl nonadecanoic acid (FA C19:0) and 100 µl heneicosanoic acid (FA C21:0) as internal standards, the filters were incubated (−20°C, 12 h) in chloroform:dichlormethane:methanol (1:1:1; v:v:v) for FA extraction (Arndt and Sommer 2014).

Prior to esterification, a purification step with potassium chloride (KCl) (1 M) was performed. Subsequently, the FAMEs were produced by heating up the FA extract (50°C, 12 h) with methylbenzene and sulfuric acid (H_2SO_4, 1%) in methanol. After a second purification step using sodium chloride (NaCl, 5%), the FAMEs were extracted with 100 µl n-hexane and analyzed with a gas chromatograph (Trace GC Ultra AS 3000, Thermo Scientific, Germany). For final quantification of FAMEs, the software Chrome-Card (Thermo Scientific, Germany) was used and the FAME peaks obtained were compared against respective standards (Supelco 37 Component FAME Mix, Sigma-Aldrich, USA).

2.5 *Seston C:N ratios*

Seston C:N ratios were determined by measuring the amounts of C (POC, particulate organic C) and N (PN, particulate N) in seston biomass. For this purpose, 100–400 ml of lake water was filtered using acid-washed (10% HCl), pre-combusted (450°C, 4 h) GF/F microfiber filters with a pore size of 0.7 µm (GF/F, Whatman, USA). The inorganic C on the filters was then evaporated by treating them for 30 s with 5 ml of 0.5 molar dihydrogenphosphate acid (H_2PO_4). The filters where subsequently stored in freezers (−20°C) until further analysis. To perform POC and PN measurements, the filters were defrosted, dried (60°C, 24 h), compacted

in tin foil and analyzed in an elemental CN analyzer (vario MICRO cube, Elementar Analysensysteme GmbH, Germany). The seston C:N ratios were then calculated and used as a proxy for the stoichiometric C:N composition of the phytoplankton communities in the lakes.

2.6 *Daphnia*

Daphnia samplings were conducted with a plankton net (mesh size 250 µm, diameter 26 cm) that was sunk (to the same depth from which the respective water samples were taken) and slowly hauled up to the surface. The caught zooplankton was immediately flushed (using filtered lake water) into 100 ml PE bottles containing either a fixative with sugar, glycerin and EtOH (EtOH final concentration 70%) or a fixative with a 4% sugar-formalin solution. *Daphnia* samples were stored in a refrigerator at 4°C until counting of the samples. The counting was performed by using a light stereomicroscope (WILD M3Z, WILD Heerbrugg, Switzerland). Only *Daphnia* species were counted and taxonomic determinations of *Daphnia* were performed only to genus level. If possible, at least 400 specimens were counted per sample. If zooplankton densities were very high, samples were split once or twice using a zooplankton splitter built after Motoda (1959). To estimate *Daphnia* biovolumes, mean values for *Daphnia* biovolumes [mm^3/l] were calculated for respective lakes by using biovolume data from existing monitoring programs by the water-management administration of Bavaria. The mean values obtained for the *Daphnia* biovolumes of each lake were then multiplied with the respective *Daphnia* densities found in the 11 lakes:

$$Daphnia\ bv_{(lakeX)} = mean\ Daphnia\ bv_{(lake\ X)} * Daphnia\ dens_{(lakeX)} \quad (1)$$

where *Daphnia bv* = *Daphnia* biovolume in mm^3/l and *Daphnia dens* = *Daphnia* density in individuals/l.

2.7 *Whitefish*

Whitefish samplings were conducted either by a professional fisherman of the district administration of upper Bavaria in cooperation with local fishermen, or by the Institute of Fisheries of the Bavarian State Ministry for Nutrition, Agriculture and Forestry (StMELF). Fish were caught using floating, pelagic, multi-mesh gill nets (height: 5–7 m, length: 300–700 m) set at different locations in the lake (based on the experiences of local fishermen) at depths between 7–16 m (lower edge). To catch fish of all relevant age classes, the gill nets were separated into six or seven sections (length/section: 50–100 m) of different mesh sizes in the

range of 20–45 mm (for lake specific details, see Tab. 2). After catching, the whitefish were killed, stored on ice and brought to the laboratory for further analysis. In the laboratory, fish fork lengths were measured to the nearest 0.1 cm and wet weights were measured to the nearest 0.1 mg. To estimate the current nutritional conditions of the whitefish, the length–weight relationships of all captured individuals were estimated by using equation (2) of Fulton's condition factor (K) (Ricker 1975):

$$K = 100 \frac{W}{L^3} \quad (2)$$

where W = wet weight in grams, L = length in cm and the factor 100 is used to bring K close to one.

2.8 *Statistical analyses*

For statistical analyses, the software SigmaPlot14 (SigmaPlot 14.0, Systat Software 2008, USA) was used. Data were analyzed using either linear regression models (f = y0 +a*x) or regressions from the type of exponential decay (f = a*exp (−b*x)). Regression analyses of seston FAs were performed using the relative amounts of respective FAs [%] as the dependent variable. If necessary, data were log(x) or log(x+1) transformed prior to analysis. Seston C:N ratios were calculated as mmol/l:mmol/l and NO_3^--N:P ratios as mol/l:mol/l.

In pre-alpine lakes, annual fluctuations of growth conditions for primary producers (e.g., shifts in nutrient concentrations, mixing depth, light intensity) can have significant impacts on plankton communities and, hence, also on the nutritional situation of coregonids. Regular seasonal patterns of growth dynamics in lakes, such as plankton peaks and clear-water phases in spring, are well summarized within a conceptual framework, the so-called PEG model (Sommer et al. 1986, Sommer et al. 2012). Hence, to take the transient dynamics of seasonal growth into account, we analyzed spring and autumn data for each lake separately.

Some measurements, counts and analyses are still in progress; thus, at the time of manuscript preparation not all data were available for statistical analyses. However, as described below, a large data-set could be used for this publication, enabling the statistical evaluation of all relevant parameters. Currently, we have whitefish (condition factor K) and water chemistry (concentrations of NO_3^-, NH_4^+, TP) data for the entire experimental period (spring 2016–2018 and autumn 2016–2018). In terms of seston parameters, we have C:N ratios and FA concentrations from spring 2016, autumn 2016 and spring 2017. *Daphnia* biovolume data are available from spring 2016, autumn 2016 and spring 2017. For lakes Ammersee, Lake

Constance, Riegsee, Simssee, Starnberger See and Walchensee, 16 FA concentrations (1 × 18:3ω3 ALA, 4 × 22:6ω3 DHA, 4 × C20:5ω3 EPA, 7 × 18:2ω6C LA-C) were below the detection limit and hence, were omitted from analysis. The respective measurements are shown as "b.d.l." in supplementary table S1.

3. Results

3.1 *Water chemistry*

The 11 lakes examined in this study showed a broad range of nutrient concentrations. The NO_3^- concentrations ranged from 0.06 mg/l (Riegsee, spring 2016) to 3.92 mg/l (Ammersee, spring 2018). Lake Chiemsee (mean NO_3^- = 2.1 mg/l) and Lake Constance (mean NO_3^- = 2.53 mg/l) had the lowest mean NO_3^- concentrations, whereas lakes Klostersee (mean NO_3^- = 3.55 mg/l) and Riegsee (mean NO_3^- = 3.75 mg/l) showed the highest mean NO_3^- concentrations. Ammonium concentrations were between 0.82 µg/l (Starnberger See, spring 2018) and 316.34 µg/l (Klostersee, spring 2017). The highest mean NH_4^+ was found in lakes Klostersee (mean NH_4^+ = 187.07 µg/l]) and Riegsee (mean NH_4^+ = 60.17 [µg/l]). Königssee (mean NH_4^+ = 10.69 µg/l) and Lake Constance (mean NH_4^+ = 12.11 µg/l) had the lowest mean NH_4^+. Lake Tegernsee in March 2017 showed the lowest measured TP concentration, 1.88 µg/l, whereas the highest TP was measured in spring 2017 in Lake Simssee, with a TP of 16.52 µg/l. Over the experimental period the highest mean TP was measured in Klostersee (mean TP = 12.34 µg/L) and in Simssee (mean TP = 11.64 µg/L), the lowest mean TPs were in Walchensee (mean TP = 3.97 µg/l) and Tegernsee (mean TP = 4.33 µg/l). The resulting NO_3^--N:TP ratios were lowest in Lake Riegsee (NO_3^--N:TP = 3.7, spring 2016) and highest in Lake Tegernsee (NO_3^--N:TP = 535.08, spring 2017). The largest mean NO_3^--N:TP mass ratios were in Tegernsee (mean NO_3^--N:TP = 262.92) and Walchensee (mean NO_3^--N:TP = 237.81). Mean NO_3^--N:TP mass ratios were smallest in Klostersee (mean NO_3^--N:TP = 9.75) and Riegsee (mean NO_3^--N:TP = 12.73). The differences in NO_3^--N:P ratios were mainly driven by differences in NO_3^--N concentrations, as indicated by the relatively low magnitude of measured TP concentrations (1.88–16.52 µg/l) in comparison to the much larger variation in NO_3^--N concentrations (0.014–0.88 mg/l).

3.2 *Seston C:N ratios*

The seston C:N ratios ranged from C:N = 1.2 in Lake Constance (autumn 2018) to C:N = 9.2 in Walchensee (spring 2017). In terms of mean seston C:N ratios, we found the highest values in Riegsee (mean C:N = 6.9) and Simssee (mean C:N = 6.8). The lowest mean C:N was found in seston biomass of Chiemsee (mean C:N = 5.3) and Lake Constance (mean C:N = 5.4). Seston C:N ratios were significantly negatively correlated with NO_3^--N [mmol/l] concentrations (Fig. 2). We found no significant relationship between seston C:N ratios and NO_3^--N:TP ratios ($p = 0.96$, $R^2 = 9{,}744e^{-005}$).

3.3 *Seston PUFAs*

In total, 36 different FAs were identified in the seston biomass of the 11 study lakes (supplementary Tab. S1). For spring 2016, autumn 2016 and spring 2017, a change in seston FA composition along the NO_3^--N:TP gradient could be seen. We found significantly decreasing percentages (as percentages of total FAs) of total PUFAs (Fig. 3a), total ω3-PUFAs (Fig. 3b), EPA (Fig. 3c), DHA (Fig. 3d) and LA-C (Fig. 3e) along the NO_3^--N:TP gradient (for p and R^2 values see caption Fig. 3). The relative amounts of C-18 PUFAs showed no significant relationship to increasing NO_3^--N:TP ratios (Fig. 3f).

3.4 *Daphnia*

The *Daphnia* biovolume, as a measure of *Daphnia* abundance, ranged from 0.0002 mm^3/l (observed in Ammersee in autumn 2016) to 2.32 mm^3/l (Klostersee, spring 2017). Mean *Daphnia* biovolume in for the period of spring 2016, autumn 2016 and spring 2017 was highest in Klostersee (mean *Daphnia* biovolume = 2.32 mm^3/l) and Starnberger See (mean *Daphnia* biovolume = 0.6 mm^3/l). The lowest mean *Daphnia* biovolumes were observed in Ammersee (mean *Daphnia* biovolume = 0.006 mm^3/l) and Simssee (mean *Daphnia* biovolume = 0.03 mm^3/l). Concerning the relationship of *Daphnia* to N:P ratios, we found a significantly negative correlation of *Daphnia* biovolume with increasing NO_3^--N:TP ratios (Fig. 4). Significant correlations were also revealed for *Daphnia* with certain PUFAs. *Daphnia* biovolume in spring 2017 was significantly positively correlated to relative amounts (as a percentage of total FAs) of seston DHA (Fig. 5a) and seston ALA (Fig. 5b). Furthermore, in spring 2016 and spring 2017, *Daphnia* biovolume also showed a significantly positive relationship with the relative amount of DHA in seston biomass (Fig. 5c).

3.5 *Whitefish*

In the 11 sample lakes, the values for fish condition factor K were found to be within the range of K = 0.64 (Starnberger See, autumn 2016) and K = 0.95 (Riegsee, spring 2016). Over the experimental period, these two lakes also showed the highest mean K values (Riegsee: mean K = 0.939) and the lowest mean K values (Starnberger See: mean K = 0.731). Concerning the relationship of whitefish conditions to NO_3^--N:TP ratios, we found a significantly decreasing condition factor K with increasing NO_3^--N:TP ratios (Fig. 6a). In contrast, condition factor K was significantly positively correlated with the relative amounts of C-18 PUFAs (as a percentage of total fatty acids) in the seston biomass (Fig. 6b).

4. Discussion

This study was performed to evaluate the possible effects of increasing N concentrations on natural phytoplankton, zooplankton and whitefish (*Coregonus* spec.) communities in pre-alpine lakes. From a preceding mesocosm experiment performed in the oligotrophic, pre-alpine Lake Brunnsee (Trommer et al. 2019), it is known that seston PUFA content can decrease along an N gradient, resulting in significantly less *Daphnia* biomass under high N conditions. However, to our knowledge the negative influence of increasing N loading on seston PUFA composition and on *Daphnia* biomass has not be analyzed by comparing lakes differing in N. However, against the background of continuously rising N loading in lakes, (Vitousek et al. 1997, Galloway et al. 2003) it may be relevant to discover if the above-mentioned experimental mesocosm results can be transferred to other natural lake systems. Hence, to find potential evidence that the pattern between N loading and food web dynamics can also be observed in field data, we conducted a monitoring of 11 lakes, all inhabited by whitefish.

Our first objective was to determine whether increasing N, and thereby increasing N:P ratios, can significantly decrease the PUFA concentrations in the phytoplankton biomass of pre-alpine lakes. Data from the 11 observed lakes support the relationship found in a controlled mesocosm experiment, in which seston PUFA concentrations decreased with increasing N fertilization. One example is the negative correlation of EPA to increasing N:P ratios seen in the observational data from the 11 lakes. This empirically confirms our hypothesis and strongly suggests that rising N:P ratios originating from increasing N concentrations in pre-alpine lakes can cause decreases in phytoplankton PUFA concentrations.

In addition, the consistency between the results of both studies (the present study and that of Trommer et al. 2019) is remarkable. Due to the large differences between an enclosure

experiment in a single lake and an observational study of 11 natural lake systems, we did not expect to obtain such consistent results. Moreover, the range of NO_3^--N:TP ratios in the mesocosm experiment of Trommer and colleagues (2019) (NO_3^--N:TP = 475–1843, mean = 723, unpublished data) was relatively large in comparison to the spectrum of NO_3^--N:TP ratios found in the 11 lakes (NO_3^--N:TP = 4–535, mean = 146). Our data suggest that there is no threshold N:P ratio along the N:P gradient that triggers changes in phytoplankton PUFA composition. It seems that the response of phytoplankton to increasing N:P ratios is an exponential decrease in PUFA. However, the strength of these effects is still unclear. We therefore combined both datasets (data from the mesocosm experiment of Trommer and colleagues (2019) and data from the 11 lakes) and estimated the strength of potential PUFA decreases in phytoplankton for N:P ratios between 4–1843. Since, in both experiments, significantly decreasing amounts of 20:5ω3 EPA were observed, we used the essential PUFA EPA as a proxy (Fig. 7). Our data demonstrate an exponential decay, with the turning point at about N:P = 300–400. This means that increasing N:P ratios have the strongest influence on seston EPA concentrations below 300–400 (Fig. 7). Therefore, in the natural pre-alpine lake systems with measured N:P ratios between 4–535, seston EPA amounts may decrease much faster than was observed in the mesocosm experiment with measured N:P ratios of 475–1843. We want to point out that N:P ratios in the mesocosm experiment of Trommer et al. 2019 were the result of increasing N enrichment and not of P manipulations.

Hence, our monitoring data support the experimental evidence that increasing N input can affect phytoplankton food quality. Further increasing N:P ratios caused by rising N loads in the future may therefore cause continuously decreasing PUFA concentrations in phytoplankton communities, potentially affecting entire lake food webs.

The effect of N on phytoplankton food quality may be transferred to higher trophic levels via trophic interactions. Within a lake food web, herbivorous zooplankton in particular can be expected to experience severe consequences. Zooplankton (and most other animals) are not able to synthesize PUFAs; thus, herbivorous zooplankton is dependent on an adequate supply of essential PUFAs from its algal diet (Wacker and Von Elert 2001, Martin-Creuzburg and Von Elert 2008). Therefore, we also analyzed whether our observational data support a link between PUFA in phytoplankton and in herbivorous zooplankton taxa, especially *Daphnia* species.

We found significantly positive relationships between *Daphnia* biovolume and the relative amounts of the essential PUFAs DHA (for spring 2016 and spring 2017) and ALA (for spring

2017) in seston. The demand of *Daphnia* for essential PUFAs, such as DHA and ALA, is already known and has been shown in numerous studies (Ahlgren et al. 1990, Müller-Navarra 1995a, Jónasdóttir et al. 1995, Müller-Navarra et al. 2000, Ravet et al. 2003, Brett et al. 2009, others). Most of these studies were performed under relatively controlled conditions, in laboratories and mesocosm experiments. In these experiments, highly controlled environmental conditions enabled the detection of relatively weak effects because natural variances and overlaying interfering signals were reduced to a minimum in comparison to uncontrolled lake systems. Finding these patterns in large pre-alpine lakes under natural and uncontrolled conditions highlights the robustness of the effect and the importance of an adequate PUFA supply for *Daphnia*.

However, besides the insufficient supply of essential PUFAs there are also other parameters that are linked to N concentrations and can influence algal food quality, and thus zooplankton growth. Nitrogen-derived shifts in phytoplankton stoichiometry, phytoplankton community composition or both are also known to potentially decrease zooplankton abundance. This is especially true for zooplankton species with high P demands, such as cladocerans (Poxleitner et al. 2016, Lorenz et al. 2019 and references therein). The reduced growth of *Daphnia* is therefore probably not based on monocausal reasoning, and several effects may act simultaneously. Future experiments and studies are needed to disentangle under which environmental conditions which N-related changes in phytoplankton food quality prevail.

To obtain estimates of whether the effects of increasing N depositions and accompanying rising N:P ratios can affect higher trophic levels, we further examined the stocks of planktivorous whitefish (*Coregonus*. spec.) in the lakes. Our assumption was that whitefish would be affected by lower *Daphnia* abundance due to a loss of preferred prey. Although we observed significantly decreasing K values with increasing NO_3^--N:TP ratios, we did not find any significant relationship between fish condition factor K and *Daphnia* abundance in the 11 lakes. This indicates that other factors linked to the respective NO_3^--N:TP ratios in the studied lakes induced decreasing whitefish conditions along the observed NO_3^--N:TP gradient. However, whitefish condition factor K is not exclusively controlled by food quantity, and thus *Daphnia* densities, but also by the effects of food quality. It is known that for fish in general and for juvenile fish in particular, certain FAs are of special importance for growth, disease resistance and general well-being (Adams 1999, Olsen 1999, Sargent et al. 1999, Brett et al. 2009, Jardine et al. 2020). Moreover, there is evidence that to meet the physiological needs of adult fish, an adequate supply of PUFAs is essential. Especially during the main feeding

period, the supply of C-18 PUFAs, such as linoleic acid (18:2ω6) and α-linoleic acid (18:3ω3), is of high importance for numerous fish taxa (Strandberg et al. 2017, Taşbozan and Gökçe 2017, Jardine et al. 2020).

These facts, together with the observed positive correlation between fish condition and the proportions of C-18 PUFAs in the seston biomass in the 11 lakes, indicate that with increasing N:P ratios, whitefish probably experience a rising deficiency in PUFA supply, resulting in decreasing K values. This confirms the conclusions of Müller-Navarra and colleagues (2004) that in algal-based food webs the seston FA contents are conservatively transferred up the food web into fish.

However, the nutritional conditions of whitefish in pre-alpine lakes seem to represent an interplay between various factors. Our findings suggest that two of the main trophic factors potentially affecting the K value of whitefish are food quantity and food quality; both of these are in some way linked to the concentrations of N and P in pre-alpine lakes. Since primary production in these lakes is mainly P-limited, any changes in P concentrations would have direct effects on lake productivity. Hence, decreasing P concentrations would lower primary production and thereby decrease food quantity for planktivorous fish, with less phytoplankton and lower zooplankton densities. In contrast, shifts in N concentrations would have indirect effects, via food quality, on the zooplankton and whitefish communities of pre-alpine lakes. The data presented here suggest that the consequences for whitefish of rising N concentrations or decreasing P concentrations are very similar. The results in either case would be a reduction of growth, either because of a reduction in food quantity due to decreasing *Daphnia* abundances or by lowered food quality due to reduced proportions of essential PUFAs in the lower trophic levels.

We were able to show that increasing N concentrations and accompanying increasing N:P ratios decrease PUFA concentrations in the phytoplankton communities of natural pre-alpine lakes. For herbivorous zooplankton, this results in a decrease in the quality of food algae, especially reducing *Daphnia* growth in these lakes. The effects of a reduction in seston PUFA concentrations relative to total FAs in seston biomass are further transferred via zooplankton to planktivorous whitefish. Our study supports the conclusion that negative, N-derived effects on food-web efficiency, as seen in a previous mesocosm experiment manipulating N (Trommer et al. 2019), can probably also be seen in correlative data from lake observations. Further studies concerning the effects of N loading on food-web patterns and dynamics in P-limited lakes also are therefore urgently needed.

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Conflicts of Interest

None declared.

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Tables

Table 1: For all lakes, the area [km^2], volume [km^3], mean depth [m], maximum depth [m], size of catchment area [km^2] (UBA 2016) and trophic state according to the concentrations of total phosphorus are shown (Lampert and Sommer 2007).

Lake	**Area** [km²]	**Volume** [km³]	**Mean depth** [m]	**Max depth** [m]	**Catchment area** [km²]	**Trophic state**
Ammersee	46.6	1.75	37.6	81.1	993	oligotrophic
Lake Constance	571.5	48.52	85	254	11477	oligotrophic
Chiemsee	79.9	2.05	25.6	73.4	606.34	oligotrophic
Klostersee	0.5	0.003	5.9	16	4.5	mesotrophic
Königssee	5.22	511.79	98.1	180	136.5	oligotrophic
Riegsee	2	0.013	6.8	15.4	0.4	mesotrophic
Simssee	6.6	0.09	13.4	22.5	-	oligo-mesotrophic
Staffelsee	7.66	74.88	9.78	39.4	80.7	oligotrophic
Starnberger See	56.4	2.99	53.2	127.8	314.7	oligotrophic
Tegernsee	8.9	323.09	36.3	72.6	210.8	oligotrophic
Walchensee	16.11	1.3	80.8	189.5	783	oligotrophic

Table 2: Gill net lengths [m], net mesh sizes [mm] and gill net lengths per section [m] used for whitefish samplings in the studied lakes are shown. The last column displays the maximum number of whitefish per sampling and per mesh size used for the analyses.

Lake	**Gill net length** [m]	**Sections with mesh sizes** [mm]	**Gill net length/section** [m]	**Number of fish used for analyses/sampling**
Ammersee	600	20, 25, 30, 35, 40, 45	100	≤ 25
Lake Constance	700	20, 26, 32, 36, 38, 40, 44	100	≤ 50
Chiemsee	300	20, 25, 30, 35, 40, 45	50	≤ 25
Klostersee	300	20, 25, 30, 35, 40, 45	50	≤ 25
Königssee	300	20, 25, 30, 35, 40, 45	50	≤ 25
Riegsee	600	20, 25, 30, 35, 40, 44	100	≤ 50
Simssee	600	20, 25, 30, 35, 40, 45	100	≤ 25
Staffelsee	600	20, 25, 30, 35, 40, 45	100	≤ 25
Starnberger See	600	20, 25, 30, 35, 40, 44	100	≤ 50
Tegernsee	600	20, 25, 30, 35, 40, 45	100	≤ 25
Walchensee	600	20, 25, 30, 35, 40, 45	100	≤ 25

Figure Legends

Figure 1: Overview map displaying the geographical locations of the 11 studied pre-alpine lakes (Ammersee, Chiemsee, Lake Constance, Klostersee, Königssee, Riegsee, Simssee, Staffelsee, Starnberger See, Tegernsee and Walchensee) at the northern edge of the European Alps in Bavaria, southern Germany; provided by Google Maps (Google 2018).

Figure 2: Linear regression of log(x) transformed seston C:N ratios against NO_3^--N [mmol/l] ($p = 0.046$, $R^2 = 0.145$). Blue lines indicate the 95% confidence interval.

Figure 3: Linear regressions of the relative amounts [percentage of total FAs] of **a)** total PUFA ($p = 0.013$, $R^2 = 0.21$), **b)** total ω3 PUFA ($p = 0.037$, $R^2 = 0.16$), **c)** 20:5ω3 EPA ($p = 0.012$, $R^2 = 0.25$), **d)** 22:6ω3 DHA ($p = 0.015$, $R^2 = 0.24$), **e)** 18:2ω6c LA-C ($p = 0.0496$, $R^2 = 0.19$) and **f)** C-18 PUFAs ($p = 0.129$, $R^2 = 0.09$) against NO_3^--N:TP ratios. Blue lines indicate 95% confidence intervals.

Figure 4: Linear regression of *Daphnia* biovolume [mm^3/l] (log-scale for y-axis) against NO_3^--N:TP ratios ($y = -0.002x + 0.56$, $p < 0.05$, $R^2 = 0.18$). Blue lines indicate 95% confidence interval.

Figure 5: Linear regressions of *Daphnia* biovolume [mm^3/l] against **a)** (spring 2017) relative amounts [percentage of total FAs] of seston 22:6ω3 DHA ($p < 0.01$, $R^2 = 0.59$), **b)** (spring 2017) seston 18:3ω3 ALA ($p < 0.01$, $R^2 = 0.7$) and **c)** (spring 2016/17) relative amounts of seston 22:6ω3 DHA ($p < 0.01$, $R^2 = 0.4$) (log-scale for y-axis). Blue lines indicate 95% confidence intervals.

Figure 6: a) Linear regression of fish condition factor K against NO_3^--N:TP ratios for the entire experimental period ($p < 0.05$, $R^2 = 0.071$). **b)** Linear regression of fish condition factor K against the relative amount of C-18 PUFAs [percentage of total fatty acids] ($p = 0.05$, $R^2 = 0.21$). For **b)** the data for C-18 PUFAs are from spring 2016/17 and the data for fish condition factor K are from autumn 2016/17. Blue lines indicate 95% confidence intervals.

Figure 7: Regression (type: exponential decay) of the relative amount [percentage of total fatty acids] of seston 20:5ω3 EPA amounts ($p < 0.001$, $R^2 = 0.39$) against NO_3^--N:TP ratios. For this regression, data from the 11 lakes (triangles) and data from Trommer et al. 2019 (circles) were used. Blue lines indicate 95% confidence interval.

Figures

Figure 1

Figure 2

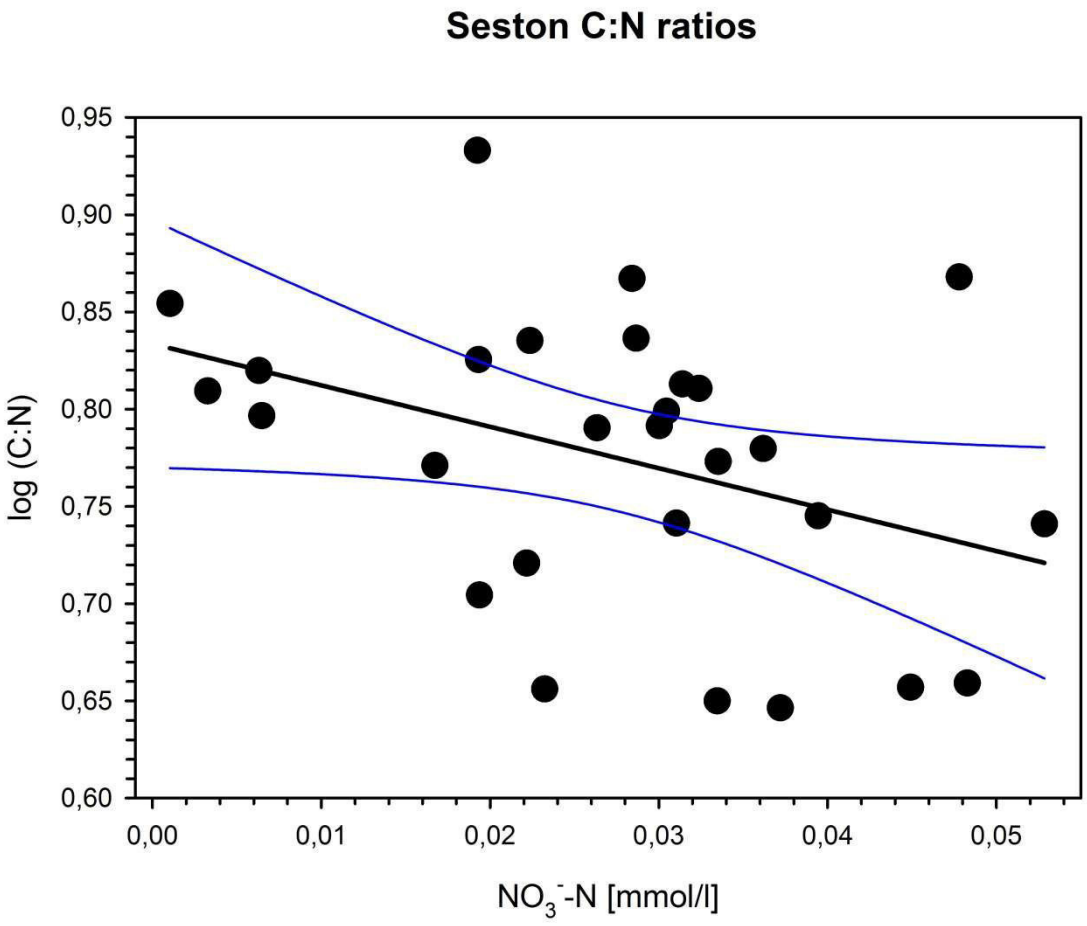

Seston C:N ratios
log (C:N)
0,95
0,90
0,85
0,80
0,75
0,70
0,65
0,60
0,00
0,01
0,02
0,03
0,04
0,05
NO3-N [mmol/l]

Figure 3

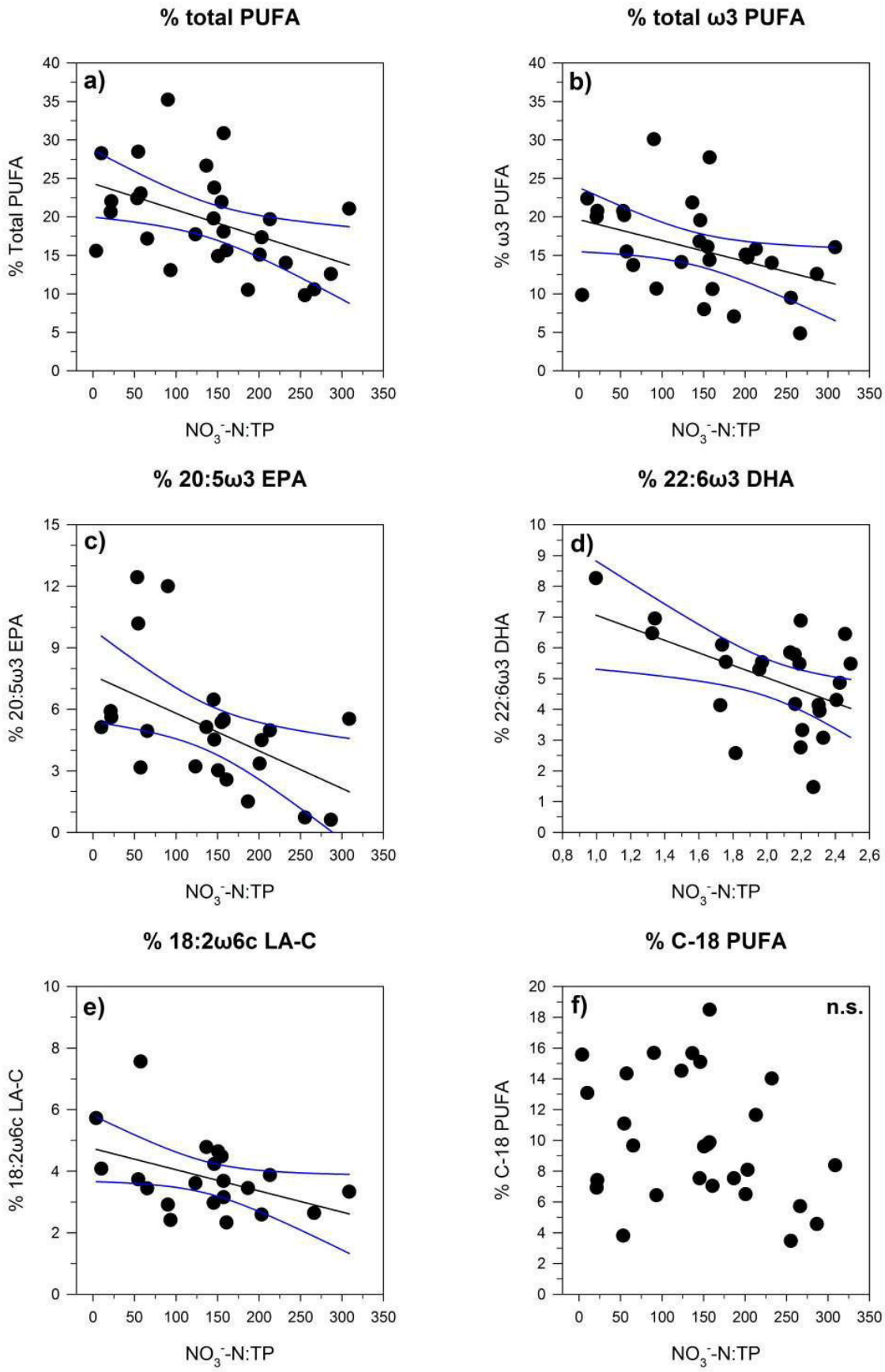
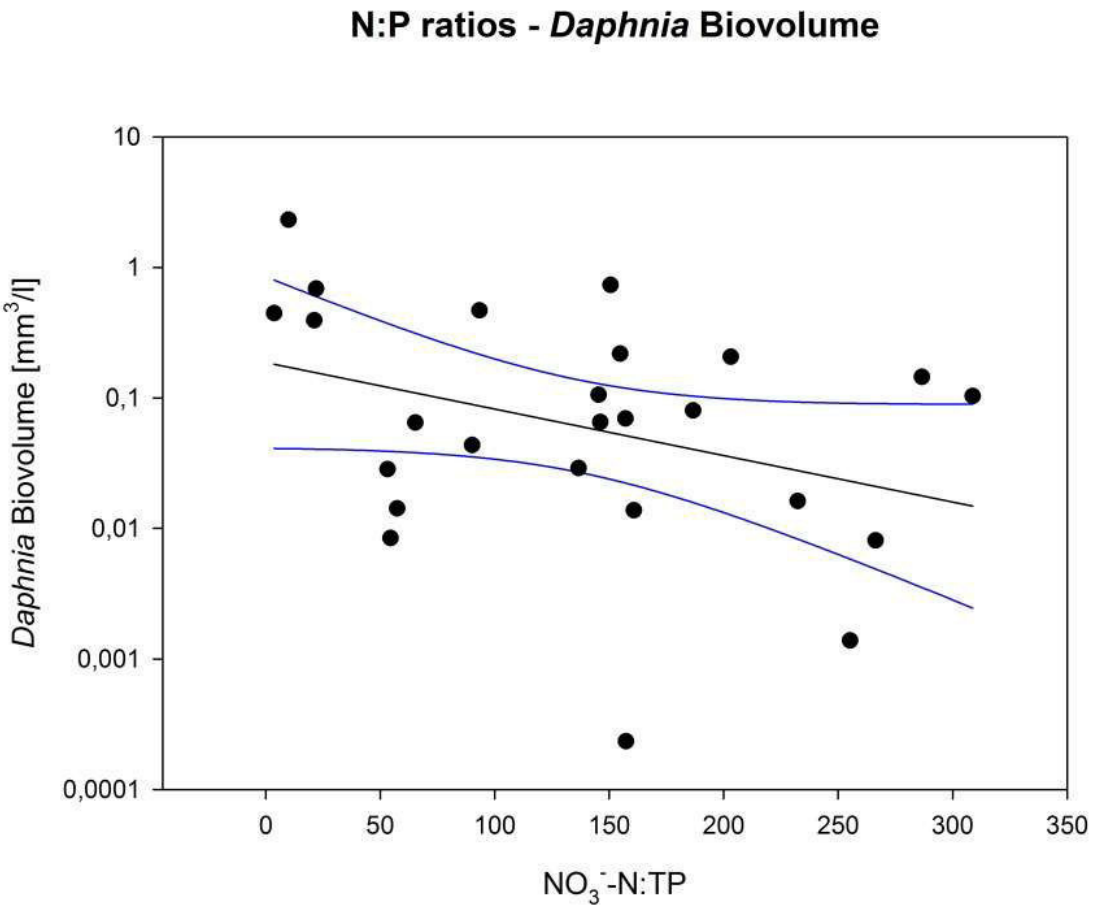

% total PUFA
a)
% Total PUFA
NO_3^--N:TP
% total ω3 PUFA
b)
% ω3 PUFA
NO_3^--N:TP
% 20:5ω3 EPA
c)
% 20:5ω3 EPA
NO_3^--N:TP
% 22:6ω3 DHA
d)
% 22:6ω3 DHA
NO_3^--N:TP
% 18:2ω6c LA-C
e)
% 18:2ω6c LA-C
NO_3^--N:TP
% C-18 PUFA
f)
n.s.
% C-18 PUFA
NO_3^--N:TP

Figure 4


N:P ratios - Daphnia Biovolume
Daphnia Biovolume [mm3/l]
10
1
0,1
0,01
0,001
0,0001
0
50
100
150
200
250
300
350
NO3--N:TP

.

Figure 5

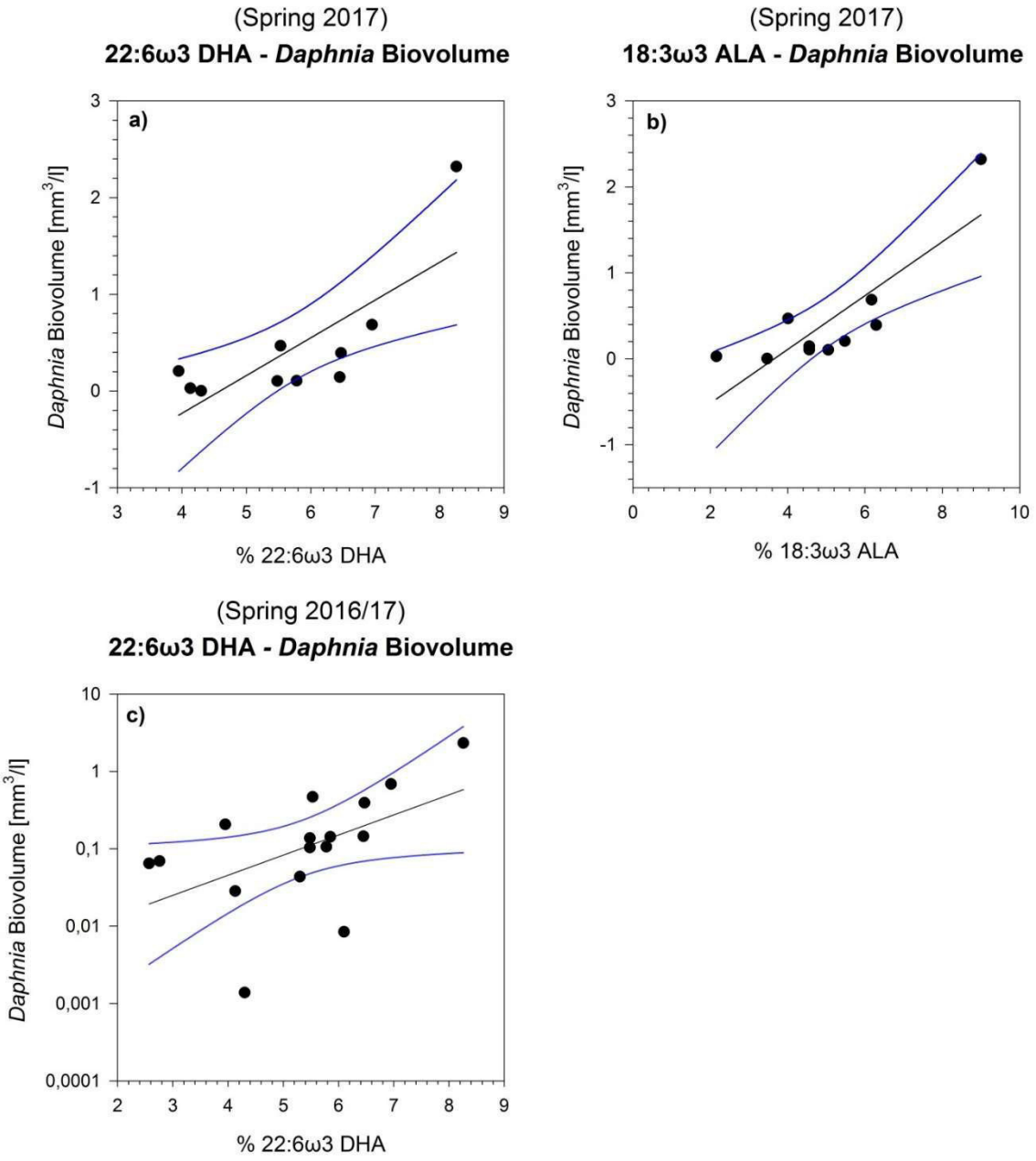

(Spring 2017)
22:6ω3 DHA - Daphnia Biovolume
a)
Daphnia Biovolume [mm3/l]
% 22:6ω3 DHA
(Spring 2017)
18:3ω3 ALA - Daphnia Biovolume
b)
Daphnia Biovolume [mm3/l]
% 18:3ω3 ALA
(Spring 2016/17)
22:6ω3 DHA - Daphnia Biovolume
c)
Daphnia Biovolume [mm3/l]
% 22:6ω3 DHA

Figure 6

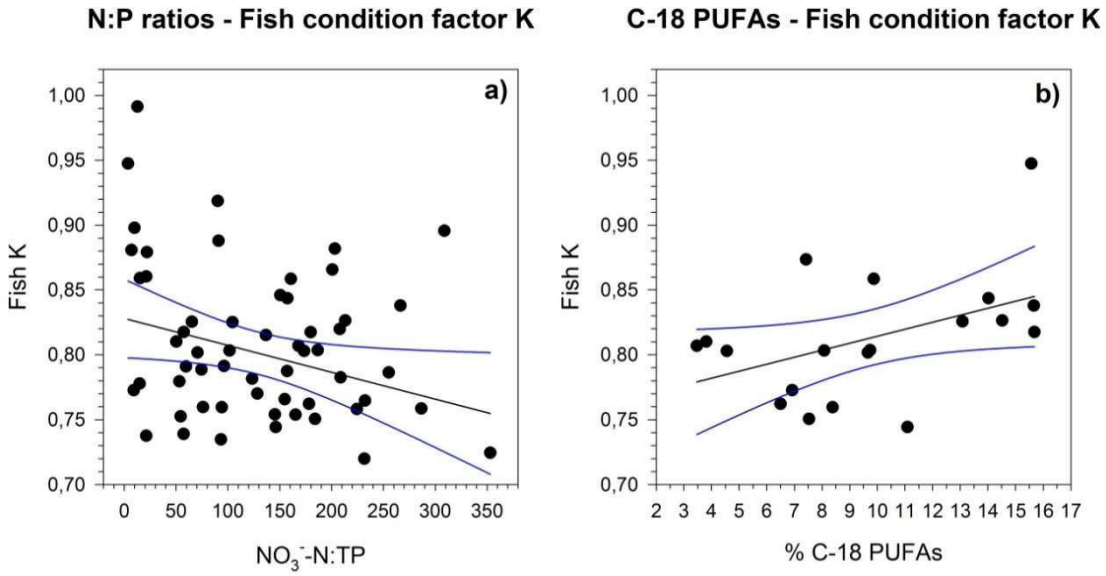
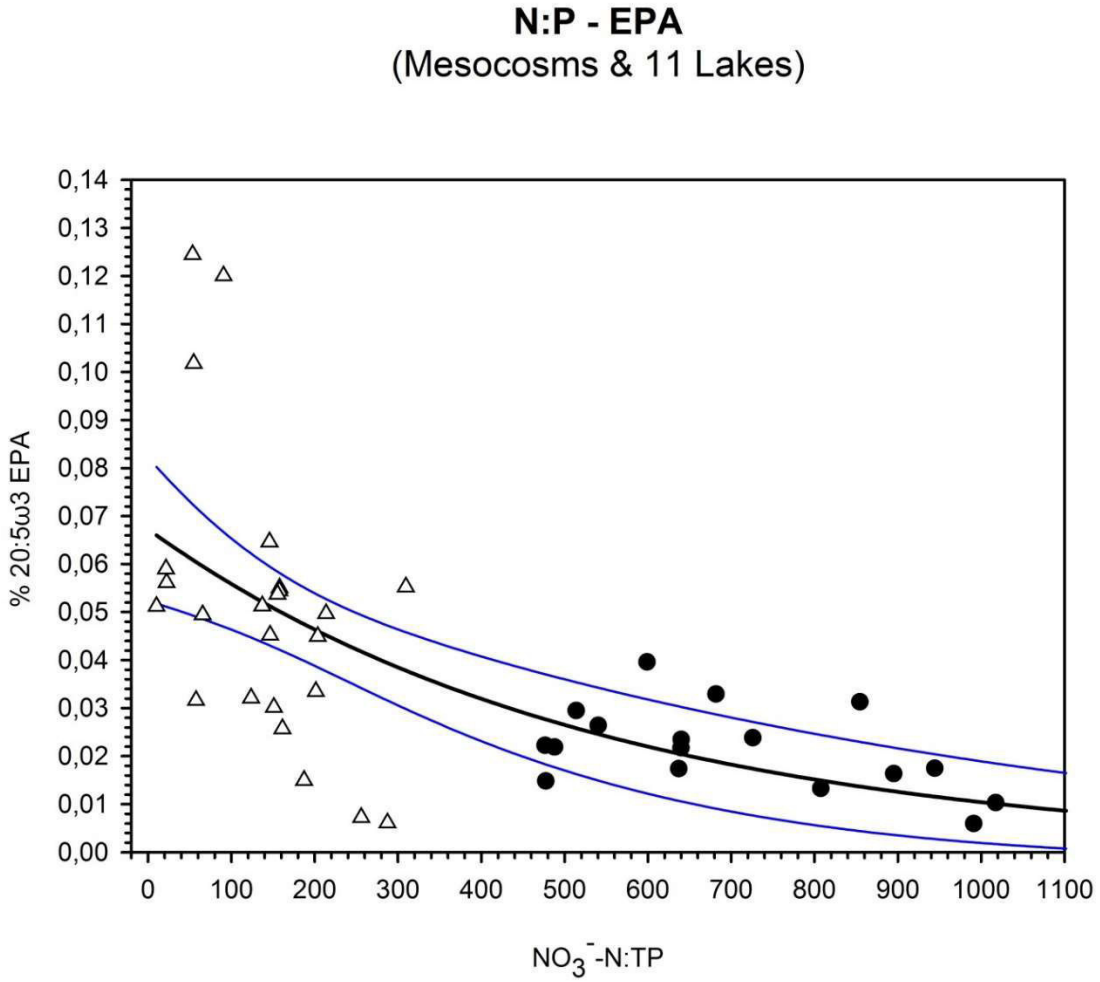

N:P ratios - Fish condition factor K
a)
Fish K
1,00
0,95
0,90
0,85
0,80
0,75
0,70
0 50 100 150 200 250 300 350
NO3-N:TP
C-18 PUFAs - Fish condition factor K
b)
Fish K
1,00
0,95
0,90
0,85
0,80
0,75
0,70
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
% C-18 PUFAs

Figure 7


N:P - EPA
(Mesocosms & 11 Lakes)
% 20:5ω3 EPA
0,14
0,13
0,12
0,11
0,10
0,09
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0,00
0
100
200
300
400
500
600
700
800
900
1000
1100
NO3-N:TP

Supplementary Table S1: Overview of all identified FAs in the seston biomass of the 11 study lakes. For each lake, any performed FA samplings are displayed with the corresponding sampling seasons, the respective concentrations of total identified FAs [ng/ml] (representing 100%) and the relative amounts of single FAs as a percentage. Fatty acid amounts that were below the detection limit are denoted with "b.d.l."

Lake	Season	FA total [ng/ml] (≙ 100%)	8:0	10:0	12:0	2-OH-C10:0	14:0	14:1	a-C15:0	2-OH-C12:0	C15	15:1	i-16:0	16:0	16:1	cis-9-10-C17:0	i-17:0	C17:0	17:1	2-OH-14:0	18:0
Ammersee	Spring16	3032,60	0,00	0,00	0,00	0,00	7,05	0,00	0,00	0,00	0,00	0,00	0,00	34,71	6,46	0,00	0,00	0,00	0,00	0,00	17,15
Ammersee	Spring17	15614,89	0,00	0,00	1,06	0,25	9,58	0,00	0,81	0,00	1,22	0,00	0,00	36,06	1,06	3,92	0,00	1,02	0,00	0,00	18,10
Ammersee	Autumn16	18122,78	0,00	0,00	0,00	0,00	8,19	0,00	0,00	0,00	0,00	0,00	0,00	32,52	6,80	0,00	0,00	0,00	0,00	0,00	13,17
Lake Constance	Spring16	2882,80	0,00	0,00	0,00	0,00	10,39	0,00	0,00	0,00	0,00	0,00	0,00	38,17	3,89	0,00	0,00	0,00	0,00	0,00	16,43
Lake Constance	Spring17	7865,00	0,00	0,00	0,90	0,00	18,52	0,00	1,31	0,00	0,75	0,00	0,00	27,61	1,03	0,00	0,00	0,00	0,00	0,73	5,95
Lake Constance	Autumn16	3620,05	0,00	0,00	0,00	0,00	9,84	0,00	0,00	0,00	0,00	0,00	0,00	35,56	9,70	0,00	0,00	0,00	0,00	0,00	19,06
Chiemsee	Spring16	8904,13	0,00	0,00	1,07	0,00	6,75	0,00	0,00	0,00	2,21	0,00	0,00	30,50	5,15	0,00	0,00	1,51	0,00	0,00	18,14
Chiemsee	Spring17	7196,53	0,00	0,00	0,79	0,00	16,24	1,01	1,38	0,71	1,39	0,00	0,00	32,42	0,95	0,00	0,00	2,12	0,00	0,00	17,10
Chiemsee	Autumn16	7110,72	1,27	2,93	0,00	0,00	7,57	0,00	0,00	0,00	1,65	0,00	0,00	28,85	3,82	0,00	0,00	0,00	0,00	0,00	12,08
Klostersee	Spring17	7124,03	0,00	0,00	0,00	0,00	7,94	0,00	1,56	0,00	0,00	0,00	0,00	27,47	5,36	0,00	0,00	0,00	0,00	2,03	9,86
Königssee	Spring16	12793,92	0,00	0,00	0,50	0,00	11,13	0,46	0,00	0,00	0,70	0,00	0,00	19,94	20,44	0,00	0,00	2,29	0,00	0,00	4,24
Königssee	Spring17	5720,12	0,00	0,00	1,03	0,39	11,70	0,44	0,73	0,46	2,03	0,00	0,00	31,39	3,03	0,00	0,00	2,66	0,00	0,00	0,00
Königssee	Autumn16	3279,69	0,00	0,00	0,00	0,00	9,19	0,00	0,00	0,00	0,00	0,00	0,00	32,40	7,59	0,00	0,00	0,00	0,00	0,00	16,66
Riegsee	Spring16	4151,22	0,00	0,00	0,00	0,00	5,55	0,00	0,00	0,00	0,00	0,00	0,00	40,17	3,26	3,57	0,00	0,00	0,00	0,00	18,59
Riegsee	Spring17	10270,84	0,00	0,00	1,75	0,00	14,98	0,00	1,11	0,77	0,64	0,00	0,68	21,11	5,25	0,82	0,93	0,88	0,00	0,52	4,99
Simssee	Spring16	11359,86	0,00	0,00	1,29	0,00	9,92	0,00	1,13	0,00	1,51	0,00	0,00	29,61	1,23	0,00	0,00	1,93	1,37	0,00	11,50
Simssee	Spring17	45374,22	0,00	0,00	0,81	0,00	14,57	0,00	0,40	0,00	0,52	0,00	0,43	19,00	21,76	0,00	0,72	2,53	3,44	0,00	2,82
Simssee	Autumn16	12469,09	0,00	0,00	0,00	0,00	7,81	0,00	0,00	0,00	0,00	0,00	0,00	31,36	3,92	0,00	0,00	0,00	0,00	0,00	11,97
Staffelsee	Spring16	6313,53	0,00	0,00	0,00	0,00	10,45	0,00	0,00	0,00	0,00	0,00	0,00	38,63	7,48	0,00	0,00	0,00	0,00	0,00	21,19
Staffelsee	Spring17	7246,03	0,00	0,00	1,27	0,37	10,67	0,00	1,11	0,44	1,52	0,00	0,43	27,96	5,67	0,00	0,39	1,08	0,00	0,37	11,42
Starnberger See	Spring16	4817,76	0,00	2,03	2,13	0,00	8,20	0,00	0,00	0,00	1,86	0,00	0,00	33,26	0,00	0,00	0,00	0,00	0,00	0,00	19,69
Starnberger See	Spring17	10208,02	0,00	0,00	0,00	0,00	12,74	0,00	1,00	0,00	0,91	0,00	0,00	25,42	11,61	1,14	0,00	1,69	0,00	0,00	15,50
Tegernsee	Spring16	6300,47	0,00	0,00	0,00	0,00	12,32	0,00	0,00	0,00	1,54	0,00	0,00	30,53	7,67	0,00	0,00	0,00	0,00	0,00	13,52
Tegernsee	Spring17	7945,73	0,00	0,00	0,00	0,00	12,01	0,00	0,00	0,00	0,00	0,00	0,00	30,38	6,89	0,00	0,00	0,00	0,00	0,00	17,97
Tegernsee	Autumn16	17905,56	0,00	0,00	0,00	0,00	5,51	0,00	0,00	0,00	0,00	1,74	0,00	29,63	2,28	0,00	0,00	0,00	0,00	0,00	16,89
Walchensee	Spring16	5581,07	0,00	0,00	0,00	0,00	9,07	0,00	0,00	0,00	1,57	0,00	0,00	32,58	5,62	0,00	0,00	0,00	0,00	0,00	13,37
Walchensee	Spring17	9216,63	0,00	0,00	0,78	0,00	10,20	0,00	0,82	0,43	1,15	0,00	0,00	30,67	4,98	0,00	0,00	0,00	0,00	0,00	16,95
Walchensee	Autumn16	13945,93	0,00	0,00	0,87	0,00	7,30	0,00	0,00	0,00	1,00	0,00	0,00	34,15	4,17	0,00	0,00	0,00	0,00	0,00	28,46

Continued:

Lake	Season	18:1ω9c, 18:1ω7	18:2ω6c	cis-9,10-C19:0	18:3ω6	3-OH-14:0	18:3ω3	2OH-C16:0;18:4ω3	20:0	20:1ω9c	20:3ω6	20:4ω6c	20:3ω3	22:0	20:5ω3c	22:1ω9c	24:0	22:6ω3c
Ammersee	Spring16	7,63	0,00	0,00	0,00	0,00	10,55	3,47	0,00	3,43	0,00	0,00	0,00	3,03	0,00	0,00	3,39	0,00
Ammersee	Spring17	5,69	0,00	2,99	0,00	0,84	3,47	0,00	3,67	0,00	0,00	0,34	0,98	0,31	0,73	2,80	0,81	4,30
Ammersee	Autumn16	8,45	3,15	0,00	0,00	0,00	9,25	6,09	0,00	0,00	0,00	0,00	0,00	0,00	5,50	0,00	0,00	6,88
Lake Constance	Spring16	9,21	3,61	0,00	0,00	4,19	6,98	3,93	0,00	0,00	0,00	0,00	0,00	0,00	3,21	0,00	0,00	0,00
Lake Constance	Spring17	8,61	0,00	5,64	0,00	0,00	6,50	0,00	9,35	0,00	0,00	0,00	1,09	1,30	3,35	0,00	3,20	4,14
Lake Constance	Autumn16	6,16	3,87	0,00	0,00	0,00	4,52	3,26	0,00	0,00	0,00	0,00	0,00	0,00	4,97	0,00	0,00	3,07
Chiemsee	Spring16	8,73	3,68	0,00	0,00	3,66	6,18	0,00	4,21	0,00	0,00	0,00	0,00	0,00	5,44	0,00	0,00	2,76
Chiemsee	Spring17	5,74	2,59	0,00	0,00	1,34	5,48	0,00	0,77	0,00	0,00	0,00	0,80	0,00	4,49	0,00	0,72	3,95
Chiemsee	Autumn16	8,77	2,33	0,00	0,00	2,01	2,37	2,35	0,00	3,50	2,73	0,00	0,00	3,71	2,57	0,00	4,18	3,32
Klostersee	Spring17	6,83	4,08	0,00	0,00	0,00	9,00	0,00	6,63	0,00	0,00	1,80	0,00	2,03	5,12	0,00	2,04	8,26
Königssee	Spring16	6,17	3,73	0,00	3,43	0,00	3,92	0,00	4,67	0,00	0,00	1,09	0,00	0,00	10,18	0,00	1,01	6,10
Königssee	Spring17	21,32	3,33	0,00	0,00	0,92	5,05	0,00	0,90	0,00	0,78	0,90	0,00	0,78	5,53	0,00	1,18	5,48
Königssee	Autumn16	7,86	4,23	0,00	0,00	0,00	5,41	5,45	0,00	2,52	0,00	0,00	0,00	0,00	4,52	0,00	0,00	4,17
Riegsee	Spring16	10,63	5,72	0,00	0,00	0,00	6,12	3,73	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	2,66	0,00
Riegsee	Spring17	6,69	0,00	3,34	1,25	0,00	6,17	0,00	8,08	0,00	0,00	0,00	2,03	1,62	5,61	0,00	3,83	6,95
Simssee	Spring16	5,29	2,91	0,00	0,00	0,00	7,80	4,98	0,00	0,00	0,00	2,23	0,00	0,00	12,00	0,00	0,00	5,30
Simssee	Spring17	2,70	0,00	1,94	1,65	0,00	2,16	0,00	5,04	0,00	0,00	0,00	2,03	0,59	12,44	0,00	0,30	4,13
Simssee	Autumn16	19,62	7,56	0,00	0,00	0,00	3,72	3,06	0,00	1,47	0,00	0,00	0,00	0,00	3,16	0,00	0,80	5,54
Staffelsee	Spring16	5,07	3,44	0,00	0,00	0,00	2,72	3,50	0,00	0,00	0,00	0,00	0,00	0,00	4,94	0,00	0,00	2,57
Staffelsee	Spring17	5,35	0,00	2,77	0,63	0,65	6,29	0,00	6,35	0,00	0,00	0,00	1,33	0,77	5,90	0,00	0,78	6,47
Starnberger See	Spring16	11,94	4,64	0,00	0,00	2,93	2,71	2,26	0,00	3,06	2,27	0,00	0,00	0,00	3,02	0,00	0,00	0,00
Starnberger See	Spring17	3,78	2,41	0,00	0,00	0,00	4,01	0,00	5,29	0,00	0,00	0,00	1,11	0,00	0,00	6,40	1,44	5,53
Tegernsee	Spring16	5,50	4,48	0,00	0,00	0,00	5,26	0,00	5,90	0,00	0,00	1,33	0,00	0,00	5,37	0,00	1,09	5,48
Tegernsee	Spring17	5,60	2,97	0,00	0,00	0,00	4,56	0,00	7,37	0,00	0,00	0,00	0,00	0,00	6,47	0,00	0,00	5,78
Tegernsee	Autumn16	33,44	3,45	0,00	0,00	0,00	1,87	2,21	0,00	0,00	0,00	0,00	0,00	0,00	1,50	0,00	0,00	1,47
Walchensee	Spring16	9,46	4,78	0,00	0,00	1,68	5,43	5,45	0,00	0,00	0,00	0,00	0,00	0,00	5,13	0,00	0,00	5,85
Walchensee	Spring17	6,74	0,00	4,02	0,00	0,85	4,56	0,00	5,31	0,00	0,00	0,00	0,95	0,00	0,61	3,76	0,76	6,45
Walchensee	Autumn16	5,08	2,64	0,00	3,08	0,00	0,00	0,00	4,15	0,00	0,00	0,00	0,00	0,00	0,00	4,23	0,00	4,86

MANUSCRIPT II

Ammonium exposure of *Daphnia hyalina* induces life-history shifts similar to fish kairomones

Patrick Lorenz, Gabriele Trommer, Anna Hofmeister and Herwig Stibor

In preparation

Ammonium exposure of *Daphnia hyalina* induces life-history shifts similar to fish kairomones

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Running title: *Daphnia hyalina* response to ammonium

***Keywords*:** ammonium, toxicity, *Daphnia hyalina*, life history, fish kairomones

Summary

Due to globally intensive fertilizer application, the supply of ammonium (NH_4^+) in freshwater lakes is proposed to increase in the future. Potential effects on ecosystems and organisms are still not completely understood. The aim of this study was to investigate effects of chronic NH_4^+ exposure on survival and life history patterns of *Daphnia hyalina*. In a laboratory experiment synchronized, neonate *D. hyalina* were exposed for 24 days to a gradient of NH_4^+ concentrations (0–87.8 mg L^{-1}). Days of survival, age at first reproduction (AFR), size at first reproduction (SFR), size of neonates and clutch sizes were measured. Above a threshold concentration of 4.22 mg L^{-1}, NH_4^+ had acute toxic effects and the days of survival of daphnids decreased significantly. At highest, non-lethal NH_4^+ concentrations (4.22 mg L^{-1}), reproduction of *Daphnia* happened earlier and adult *Daphnia* were smaller at first reproduction. Additionally, neonates at a concentration of 4.22 mg L^{-1} NH_4^+ were smaller but clutch sizes were significantly larger. The observed responses of *Daphnia hyalina* to NH_4^+ are similar to those that are known to be induced by fish kairomones.

1. Introduction

A continued increase in reactive nitrogen (Nr) emissions will intensify future Nr loadings into ecosystems (Galloway et al. 2008, Fowler et al. 2013). Reactive nitrogen emissions derive mainly from anthropogenic activities and comprise nitrogen (N) compounds with oxygen such as nitrogen oxides (NO_x), nitrous oxide (N_2O) and N compounds with hydrogen (H) in the form of NH_x. Dissolved in water and in gaseous forms, Nr is globally distributed via the atmosphere, with the result that even ecosystems in remote areas are affected by increasing Nr depositions (Schlesinger 2009, Cias et al. 2013). Projected scenarios for the future primarily estimate an increase in the depositions of NH_x compounds (Cias et al. 2013). The models applied for the IPCC 2013 report predict a worldwide increase in NH_x-N depositions from 50 Mt NH_x-N yr^{-1}in the year 2000, to 55–70 Mt NH_x-N yr^{-1} in the year 2050. Reduced N compounds in the form of NH_x include ammonia (NH_3) as well as ammonium (NH_4^+). Both can act directly as toxicant for organisms in freshwater and marine systems, even though NH_3 is considered to be the more toxic compound (Camargo and Alonso 2006). In general, NH_3 and NH_4^+ in aquatic ecosystems exist in equilibrium and the relative concentration of each compound is determined by the pH, temperature and salinity (Whitfield 1974, Bower and Bidwell 1978, Spotte and Adams 1983, Collos and Harrison 2014). However, the main factor that determines the ratio of NH_3 to NH_4^+ is pH. Collos and Harrison (2014) gave an example of

a freshwater fishpond at 30°C, were an increase in pH from 7.0 to 9.0 generated an over 60-fold increase in NH_3 concentration. Despite a significant NH_3 increase, the relative concentration of NH_3 was only 45% of the total NH_x (NH_3 + NH_4^+) concentration in the pond. Overall, the balance in the NH_3–NH_4^+ equilibrium of freshwater systems is principally on the side of NH_4^+, even at relatively high pH. However, regardless of the NH_x compound, the predicted increases of NH_3 and NH_4^+ concentrations in freshwater lakes may have severe consequences for various aquatic organisms and potentially affect the functioning of entire trophic lake systems (Camargo and Alonso 2006).

In lake ecosystems, trophic interactions between phytoplankton and zooplankton form the basis for the upwards transfer of energy towards animals of higher trophic levels (Winder and Schindler 2004). In zooplankton communities cladoceran taxa are often the dominant group and contribute up to 80% to the secondary production in lakes (Mangas-Ramírez et al. 2001). Hereby, cladocerans and mainly *Daphnia* species play an important role for manifold ecological interactions and take up a central position in pelagic food webs (Lampert 2006). In most lakes *Daphnia* act as important prey organisms for planktivores, in particular for the great majority of fish species, at least during juvenile phases (Bergstrand 1982, Lampert and Sommer 1999, Kahilainen et al. 2004, Eckmann 2013). Hence, it can be assumed that any significant alterations in *Daphnia* abundances, growth performance or reproductive success would have severe consequences for the majority of lake ecosystems. The predicted increases in NH_x depositions as well as the fact that NH_3 can have negative effects on *Daphnia* survival and reproduction (Gersich and Hopkins 1986, Adamsson et al. 1998, Mangas-Ramírez et al. 2001), underline the potential of NH_x compounds to de facto influence *Daphnia* species in the near future. In general, the magnitudes of effects toxins have on organisms vary with concentration and exposure time to the toxicant. Depending on the experimental focus, in toxicity studies usually “acute” or “chronic” toxicity tests are applied. Acute toxicity tests are carried out over short time periods such as 48 h or 72 h and are mainly designed to reveal respective lethal concentrations (Johnson 1995). Whereas, for chronic toxicity tests exposure times are longer and in general more than one life stage of the test animal are exposed to the tested chemical. Chronic tests aim to assess effects on survival, growth and reproductive output of test organisms (Johnson 1995).

Today and in the near future, various aquatic organisms in freshwater lakes will have to cope with continuously increasing NH_4^+ concentrations. Hence, organisms in these lakes are faced to a chronic exposure of slowly rising NH_4^+ concentrations. To evaluate the consequences we

performed an experiment where *Daphnia*, a typical key stone species for lake food webs (Gaedke and Straile 1998), was exposed to rising concentrations of NH_4^+. The aim of this study was to investigate potential effects of increasing ambient NH_4^+ concentrations on the duration of life time and on life history traits of *Daphnia hyalina*. The *D. hyalina* clone used in this experiment was obtained from Lake Brunnsee in southern Germany where it is the predominant cladoceran of the natural zooplankton community. Lake Brunnsee is groundwater-fed and has usually high concentrations of dissolved N, especially of nitrate (NO_3^-). Therefore, the studied *D. hyalina* clone has naturally high tolerance limits for dissolved N compounds. In the experiment, daphnids were exposed to a gradient of NH_4^+ concentrations, reflecting possible future NH_4^+ concentrations in freshwater lakes. Besides the determination of lethal NH_4^+ concentrations, further parameters of interest were the length of life span, the age at first reproduction (AFR), the body size at first reproduction (SFR), clutch size and body size of neonate *Daphnia*.

2. Material and Methods

2.1 *Origin and culture conditions of Daphnia hyalina*

In January 2016 *Daphnia hyalina* was collected from the oligotrophic Lake Brunnsee (N 47°98′41′′, E 12°43′65′′) in upper Bavaria (Germany). Lake Brunnsee has an area of 5.8 hectares, a maximum depth of 19 m and an average depth of 8.5 m. The lake water has average concentrations of total phosphorus ~ 8 µg L^{-1}, of NO_3^- ~ 13 mg L^{-1} and of NH_4^+ ~ 65 µg L^{-1}. In the laboratory *Daphnia* were raised and synchronized (~ 3 months) in 330 ml bottles filled with artificial *Daphnia* medium (330 ml L^{-1} ultrapure water, 660 ml L^{-1} well water, 10 ml L^{-1} SMB buffer (Miyake 1981), 1.5 ml L^{-1} sea salt, 100 µl L^{-1} SeO_2). The bottles were placed in a climate chamber and set to 24 h light at an intensity of ≤ 2.5 µmol s^{-1} m^{-1} (Light Meter LI-250A; LI-COR, USA). The temperature conditions were kept constant at 19°C. Five times per week (Monday–Friday), *Daphnia* were fed with green algae *Scenedesmus obliquus* (1 mg C L^{-1}) which was cultivated in WC Medium modified after Guillard and Lorenzen (1972). Before *S. obliquus* was fed to *Daphnia*, the algae were centrifuged, WC medium was discarded and algal pellets were resuspended in well water.

2.2 Experimental design

To assess the effects of chronic NH_4^+ exposure on *Daphnia hyalina*, a semi-static test was performed over a period of 24 days. The experiment was carried out at 19°C in 50 ml glass

bottle microcosms filled with test solutions. Test solutions contained ~ 35 ml *Daphnia* medium, the respective concentrations of NH_4^+ and in well water resuspended food algae *S. obliquus* (1 mg C L^{-1}). Eight treatments were applied with NH_4^+ concentrations reaching from 0-87.8 mg L^{-1} (Tab. 1). These concentrations were chosen to cover a wide range starting from present, natural NH_4^+ concentrations in lakes, over estimated future NH_4^+ concentrations towards high and most likely lethal NH_4^+ concentrations (Gersich and Hopkins 1986). To establish the NH_4^+ gradient throughout different treatments a stock solution of ammonium chloride (NH_4Cl) (3.34 g L^{-1} NH_4Cl in ultrapure water) was diluted with respective volumes of *Daphnia* medium.

For the experiment, age-synchronized neonates (<24 h) from the third clutch of one single clonal line were used. Each of the 8 treatments was replicated 3 times with 5 randomly selected *Daphnia* per replicate. Three times per week (Monday, Wednesday, Friday) *Daphnia* were transferred with a glass pipette into new 50 ml glass bottles with freshly prepared test solutions. Thereby, *Daphnia* were supplied with freshly prepared food in intervals of 48 h or 72 h. In general, NH_4^+ is the preferred N source for algae growth (McCarthy 1981) and can be relatively quickly taken up by algae. In order to avoid algal growth and an accompanied NH_4^+ loss due to algal uptake, the microcosms were kept in darkness during the experimental period.

2.3 *Sampling procedures and analyses*

2.3.1 *Abiotic factors*

Once a week, the concentrations of NH_4^+, oxygen (O_2), calcium carbonate ($CaCO_3$) and pH were measured. To detect potential, temporal variations all measurements were carried out in both, the freshly prepared test solutions as well as in the old test solutions ("old test solutions" from here on referred to as "used test solutions/media") to which *Daphnia* have already been exposed to for 48 h - 72 h intervals. Ammonium concentrations in test solutions were measured fluorometrically using a method modified after Holmes and colleagues (Holmes et al. 1999). An aliquot of 2.5 ml microcosm water was mixed with 10 ml of working reagent in scintillation vials. The working reagent was composed of borate buffer (40 g L^{-1}), sodium sulfite (40 mg L^{-1}) and orthophthaldialdehyde in ethanol (50 ml L^{-1}). After an incubation time of 2 h in darkness, the samples were measured with a fluorometer (Trilogy Laboratory Fluorometer Module CDOM/NH4; Turner Designs, USA). The concentrations of O_2 were measured with an oxygen probe by WTW (Oxi 340/SET; WTW GmbH, Germany). Concentrations of $CaCO_3$ were measured by using semi-quantitative test stripes (Quantofix®

Carbonate hardness; MACHEREY-NAGEL GmbH & Co. KG, Germany). Ammonium dissolved in water exists in equilibrium with NH_3 and the amount of free NH_3 depends strongly on the pH. To calculate concentrations of NH_3, the pH was measured using a pH meter (FiveEasy; Mettier-Toledo AG, Switzerland). Respective concentrations of NH_3 were calculated by applying equation (1) and (2) (Hobiger 1996):

$$[NH_3] = \frac{0.94412 \times [NH_4^+]}{1 + 10^{(pKA\ -\ pH)}} \quad (1)$$

$$pKA = \frac{0.0925 + 2728.795}{T + 273{,}15} \quad (2)$$

where $[NH_3]$ is the concentration of NH_3 in mg L^{-1}, $[NH_4^+]$ is the concentration of NH_4^+ in mg L^{-1} and T is temperature in °C.

2.3.2 Daphnia hyalina

At the start of the experiment, the average size of daphnids was determined by measuring 25 neonates of the tested clutch. For this purpose, neonate *Daphnia* were fixated in sugar - ethanol (714 ml L^{-1} ethanol 98%, 285 ml L^{-1} tab water, 40 ml L^{-1} glycerin, 40 g L^{-1} sugar) and stored at 4° C for subsequent analysis. Length measurements of adult *Daphnia* were done in vivo. Body length of *Daphnia* was defined as the distance from the upper edge of the compound eye to the base of the tail spine. Measurements were carried out by using a light stereomicroscope (LEICA MZ26; Leica Microsystems CMS GmbH, Germany) combined with a microscope camera (AM7023CT Dino-Eye C-Mount Camera; AnMo Electronics Corporation, Taiwan) and the camera software DinoCapture (DinoCapture 2.0; Dino-Lite Europe/IDCP B.V., Netherlands). Microcosms were checked every 24 h for dead *Daphnia* and neonates. The term "first reproduction" was defined as the time point when neonates were found in respective replicates for the first time.

2.4 *Statistical analyses*

Statistical analyses were carried out with the software SigmaPlot (SigmaPlot 11.0; Systat Software GmbH, Germany). Based on our experimental design, data were either analyzed by using analyses of variance (ANOVA) or regression models. If data for ANOVAs did not meet the criteria of normal distribution (failed normality test: "*Shapiro-Wilk*"), the nonparametric "Kruskal-Wallis ANOVA on Ranks" was applied. For "Post-Hoc" tests, either "Dunn´s Method", "Dunnett´s Method" or "Fisher-LSD Method" was used. Functions for regression

models, were either polynomial, linear (equation: $f = y_0 + a \times x$) or polynomial, quadratic (equation: $f = y_0 + a \times x + b \times x^2$). Regressions were calculated by using mean values of all living daphnids per replicate. Standard deviations in plots display scatters among the three replicates of respective treatments. If necessary data were log (x) or log (x + 1) transformed. To estimate NH_4^+ effective concentrations for the survival time of adult *Daphnia*, the "No Observed Effect Concentration" (NOEC) and the "Lowest Observed Effect Concentration" (LOEC) were evaluated. Statistical tests applied for evaluations of NOEC and LOEC were ANOVAs with post-Hoc tests. The LOEC was defined as the lowest concentration for which a statistically significant difference from the control group was observed. No Observed Effect Concentration was defined as the next concentration just below LOEC (OECD 2006).

3. Results

3.1 *Ammonium (NH_4^+)*

The respective NH_4^+ concentrations in fresh media of all treatments could be kept constant over the entire experimental duration (Tab. 1). Similar to the NH_4^+ concentrations in used media of treatments 1–7 in which also no significant temporal variations in NH_4^+ concentrations were found. Whereas, the NH_4^+ concentrations in used media of treatment 8 (average $NH_4^+ = 33.57$ mg L^{-1}) were significantly lower (ANOVA on Ranks, df = 8; Dunn´s Method, $p < 0.05$) than NH_4^+ concentrations in the freshly prepared media ($NH_4^+ = 87.8$ mg L^{-1}) (Fig. S1a).

3.2 *Oxygen (O_2), pH and carbonate hardness ($CaCO_3$)*

During the experimental period the freshly prepared media had a mean O_2 concentration of 7.61 ± 0.12 mg L^{-1} (± 1 SD), an average pH of 8.05 ± 0.08 and an average carbonate hardness of 10 ± 2 °d. After 48–72 h *Daphnia* exposure, the used media had a mean pH of 8.26 ± 0.07, a mean O_2 concentration of 7.1 ± 0.28 mg L^{-1} and a mean carbonate hardness of 10 ± 1 °d.

3.3 *Ammonia (NH_3)*

According to the O_2 concentrations and pH values stated in the section above, NH_3 concentrations in fresh media reached from 0 mg L^{-1} NH_3 in the control treatment to 2.76 mg L^{-1} NH_3 in treatment 8. Detailed NH_3 concentrations of all treatments are shown in table 2. In treatments 1–7, we found no significant differences in NH_3 concentrations of fresh media and used media. Whereas, in treatment 8 the NH_3 concentrations decreased significantly (ANOVA on Ranks, df = 8; Dunn´s Method, $p < 0.05$) within 48 h from $NH_3 = 2.76$ mg L^{-1} in fresh

media to NH_3 = 2.18 mg L^{-1} in used media. This was in accordance with the respective NH_4^+ concentrations found in treatment 8 (Fig. S1b).

3.4 *Survival of adult Daphnia hyalina*

Overall, the effects of increasing NH_4^+ concentrations on the live time duration of adult *Daphnia* were distinct and evident. We found significant differences in live time lengths along the NH_4^+ gradient. Adult daphnids in treatments 1 - 4, with NH_4^+ concentrations of 0–2.81 mg L^{-1} survived significantly longer than daphnids in treatments 5–8 with NH_4^+ concentrations reaching from 4.22 to 87.8 mg L^{-1} (ANOVA on Ranks, df = 7; Dunnett´s Method, $p < 0.05$) (Fig. 1a). The live time of adult *Daphnia* in treatments 1 - 4 reached from 4–24 days (mean 20 ± 4 days (± 1 SD)). In treatments 5 - 8 live time lengths of adult *Daphnia* were between 1–23 days (mean 4 ± 4 days). The NH_4^+ concentration of 4.22 mg L^{-1}, applied in treatment 5, was the lowest NH_4^+ concentration at which live time lengths of daphnids were significant shorter if compared to the control group.

3.5 *Age at first reproduction (AFR)*

Daphnia have reproduced only in treatments 1 - 5. In treatments 6–8 life time of adult *Daphnia* was too short to reach maturity. In treatments 1–5 AFR was unimodal distributed along the NH_4^+ gradient (regression: polynomial, quadratic, $p = 0.001$, $R^2 = 0.78$, df = 11) (Fig. 1b). Animals in all three replicates of treatments 1 and 2 with NH_4^+ concentrations of 0 mg L^{-1} and 0.59 mg L^{-1} reproduced at day 22. *Daphnia* in all replicates of treatment 3 (NH_4^+ = 1.23 mg L^{-1}) reproduced between day 22 and 23. In treatment 4 at NH_4^+ concentrations of 2.81 mg L^{-1} reproduction happened only in one replicate at day 22. *Daphnia* in two replicates of treatment 5 (NH_4^+ = 4.22 mg L^{-1}) were the first to have reproduced, at days 15 and 18.

3.6 *Size at first reproduction (SFR)*

In contrast to the survival of adult *Daphnia* and to the AFR, SFR was not significantly affected by increasing NH_4^+ concentrations. However, there was a trend for a unimodal distribution of SFR along the gradient of NH_4^+ concentrations (regression: polynomial, quadratic, $p = 0.12$, $R^2 = 0.38$, df = 11) (Fig 1c). The mean SFR in the control group was 1.40 ± 0.04 mm (± 1 SD), in treatment 2 (NH_4^+ = 0.59 mg L^{-1}) 1.44 ± 0.05 mm, in treatment 3 (NH_4^+ = 1.23 mg L^{-1}) 1.50 ± 0.02 mm, in treatment 4 (NH_4^+ = 2.81 mg L^{-1}) 1.32 ± 0 mm and in treatment 5 (NH_4^+ = 4.22 mg L^{-1}) 1.36 ± 0.14 mm.

3.7 *Clutch size*

The number of neonates per female *Daphnia* increased significantly with rising NH_4^+ concentrations (regression: polynomial, quadratic, $p < 0.001$, $R^2 = 0.80$, df = 11) (Fig. 2a). Female daphnids in treatments 1 - 4 ($NH_4^+ = 0$ - 2.81 mg L^{-1}) produced on average 0.58 ± 0.24 (± 1 SD) neonates. Whereas, daphnids in treatment 5 ($NH_4^+ = 4.22$ mg L^{-1}) produced with 2.75 ± 1.06 neonates per female almost five times more offspring.

3.8 *Size of neonate Daphnia hyalina*

The size of neonate *Daphnia* followed a unimodal distribution along increasing NH_4^+ concentrations of treatments 1–5 (regression: polynomial, quadratic, $p < 0.0001$, $R^2 = 0.79$, df = 31) (Fig. 2b). Neonates out of treatment 3 ($NH_4^+ = 1.23$ mg L^{-1}) had largest body sizes with a mean size of 0.68 ± 0.03 (± 1 SD). Body sizes of neonates out of treatments 1, 2 and 4 were in the range of 0.62–0.7 mm with a mean size of 0.65 ± 0.02 mm. Neonates out of treatment 5 had an average size of 0.55 ± 0.03 mm and were significantly smaller than neonates out of treatments 1–4 (ANOVA, df = 30; Fisher-LSD Method, $p < 0.001$).

4. Discussion

4.1 *Effects of NH_4^+ on survival time of Daphnia hyalina*

Models for future N emissions predict increasing NH_4^+ depositions in lake ecosystems. Effects on aquatic organisms and lake food webs are to be expected. To estimate toxic NH_4^+ threshold concentrations for *D. hyalina*, a keystone species for lake food webs, we determined the no effect concentration (NOEC) and the lowest effect concentration (LOEC) of NH_4^+. After an exposure time of 24 days we found no or only weak effects of NH_4^+ on the survival time of *Daphnia* in the treatments 1–4 with NH_4^+ concentrations reaching from 0 - 2.81 mg L^{-1}. The lowest NH_4^+ concentration that shortened survival time of daphnids significantly in comparison to the control, was found in treatment 5 at $NH_4^+ = 4.22$ mg L^{-1}. Therefore, according to the OECD guidelines for ecotoxicological studies (OECD 2006), we could identify the LOEC for survival of *D. hyalina* at the NH_4^+ concentration of 4.22 mg L^{-1} of treatment 5 (Fig. 1a). The no effect concentration (NOEC) is defined as the next lower test concentration before LOEC. In this experiment it would be $NH_4^+ = 2.81$ mg L^{-1} of treatment 4. Thus, we could determine NOEC of NH_4^+ for the survival of *D. hyalina* at 2.81 mg L^{-1} (Fig. 1a). We had an average pH of 8 in our microcosms and accordingly in the NOEC treatment 4, a concentration of free NH_3

of 0.13 mg L^{-1}. This is, in comparison to NH_3 - NOECs for other *Daphnia* species a relatively low value. For *Daphnia magna*, Gersich and Hopkins (1986) found for an exposure time of 21 days a maximum acceptable toxic concentration of 0.6 mg L^{-1} NH_3 - N (0.73 mg L^{-1} NH_3). Furthermore, for *Daphnia hyalina* the determined LOEC at NH_4^+ = 4.22 mg L^{-1} seemed to be a critical concentration, beyond which the lethal effects of NH_4^+ started to increase drastically (Fig. 1a). In the treatments with NH_4^+ concentrations of 4.22 mg L^{-1} and higher the mean survival time of *D. hyalina* was 4 days. In contrast, daphnids in NH_4^+ concentrations between 0–2.81 mg L^{-1} survived on average 21 days which was significantly longer. This indicates that a concentration of 4.22 mg L^{-1} NH_4^+ delineates a certain threshold for *D. hyalina* above which NH_4^+ toxicity seems to increase considerably. Focusing on the reproductive success, it is important to note that all daphnids in NH_4^+ concentrations higher than 4.22 mg L^{-1} died prior to maturity without producing living offspring. Hence, below 4.22 mg L^{-1} NH_4^+, the majority of *Daphnia* lived long enough to reach fertility, and had at least one reproduction event.

4.2 *Effects of NH_4^+ on life history of Daphnia hyalina*

In the present study we also have tested to what extent increasing NH_4^+ concentrations could affect life history patterns of *D. hyalina*. Interestingly, *D. hyalina* responded with evident shifts in reproductive traits to increasing NH_4^+ concentrations. Even though, O_2 concentration, pH, temperature, carbonate hardness as well as food supply were similar for all treatments, we found a distinct tendency for daphnids to reproduce earlier (Fig. 1b) and at smaller body size with increasing NH_4^+ (Fig. 1c). Additionally, clutch sizes in the LOEC treatment (NH_4^+ = 4.22 mg L^{-1}), which had the highest NH_4^+ concentration where *D. hyalina* reached maturity at all, were 4–5 times larger than clutch sizes in lower NH_4^+ concentrations (Fig. 2a). Regarding the size of neonate *Daphnia*, we found a unimodal relationship with increasing NH_4^+ with significantly smaller neonates in the LOEC at 4.22 mg L^{-1} NH_4^+ (Fig. 2b). These results were unexpected and are remarkable, because similar types of responses have been reported when *Daphnia* was exposed to chemical cues released by pelagic predators (Riessen 1999 and references therein).

It is known from a number of inducible defense experiments with different *Daphnia* species that shifts in life history patterns are a typical response of *Daphnia* to the presence of chemical alarm signals released by fish (reviewed in Tollrian and Harvell 1999). The term “inducible defense” refers to the ability of *Daphnia* species to develop a variety of different defense mechanisms in order to reduce predation risk at times of high predation pressure (Lass and

Spaak 2003). To avoid unnecessary costs, the developments of defense mechanisms are triggered by predator-released chemicals, named kairomones (Brown et al. 1970), which act as alarm signals for daphnids if certain predators are present. However, different predators release different kairomones which induce distinct and predator-specific defense mechanisms (Brett 1992, Kusch 1993, Stibor and Lüning 1994, Lass and Spaak 2003). The ability to identify predators by their individual chemical signals enables *Daphnia* to precisely adjust the defense response based on predator specific traits. Thus, it becomes possible for daphnids, to increase the effectiveness of the respective defense mechanisms. Depending on the type of the kairomone, defense responses can be the formation of morphological structures to impede predator handling, changes in behavior such as diel vertical migration for avoidance of predator encounter as well as shifts in life history traits to avoid being preyed upon and to compensate predation losses (Lass and Spaak 2003 and references therein).

Daphnia species preyed upon by planktivorous fish respond in general with shifts in life history patterns to fish presence (Dodson 1988). An example for this behavior was provided by Stibor (1992) when he had exposed *Daphnia hyalina* to water formerly inhabited by fish. *D. hyalina* responded with shifts in life history patterns towards an earlier reproduction at lower body size and with higher reproductive effort. These life history changes were directed responses to a predation pressure of planktivorous fish, triggered by predator kairomones. Planktivorous fish are known to be visually oriented predators and to feed selectively on larger *Daphnia* (Zaret 1980, Eggers 1982). Therefore, the strategy to increase the reproductive output, in combination with an early reproduction at a small body size, reduces the risk for *Daphnia* of being killed without having reproduced once and guarantees the maintenance of a certain population size (Stibor 1992). Stibor´s observations are in accordance with the results of numerous other studies (Dodson 1989, Machácek 1991, Weider and Pijanowska 1993, Stibor and Lüning 1994, Stibor and Müller-Navarra 2000) and consistent with our findings when *D. hyalina* was exposed to high, but not lethal NH_4^+ concentrations.

Nevertheless, to our knowledge the chemical structures of fish kairomones have not been identified completely yet. Furthermore, we did not intend to investigate the chemical compositions of fish kairomones in this study. Hence, we do not know if NH_4^+ is one compound of a fish kairomone and we do also not want to claim that NH_4^+ is the fish kairomone. Even so, the similarity of *Daphnia* responses to either fish kairomone exposure or to NH_4^+ exposure, underlines the possibility that observed effects in fish kairomone experiments might not be kept sufficiently distinct from the effects deriving out of naturally

elevated NH_4^+ concentrations in the fish water. This assumption is further supported by the fact that fish are known to excrete high amounts of NH_4^+ and NH_3 (Randall and Wright 1987, Collos and Harrison 2014). Therefore, we would like to point out that there exists a certain potential to confound interpretations in experiments investigating responses to fish kairomones by using water from fish tanks, as it is usually done in kairomone experiments. However, this hypothesis is currently hard to proof or disproof, since no data about NH_4^+ concentrations of fish kairomone experiments are available so far. The majority of studies that reported adaptive life history responses of *Daphnia* to fish kairomones have used aquarium water that had been inhabited by high densities of fish for 24 h or longer. This aquarium water that *Daphnia* were exposed to was either untreated or only filtered in order to remove larger particles prior to use (e.g. Stibor and Lüning 1994, Hanazato et al. 2001, Von Elert and Loose 1996, Reede 1997). One exception is the rarely used step of solid-phase extraction, in order to concentrate chemical compounds (Von Elert and Pohnert 2000) prior to use. Therefore, along with fish kairomones, also dissolved chemicals should have remained in the fish water to which daphnids were exposed to.

Concerning the amounts of fish released NH_4^+, Whiles and colleagues (Whiles et al. 2009) measured the direct NH_4^+ excretion rates of brook trout (*Salvelinus fontinalis*) and reported an excretion between 0.2 and 0.34 µg NH_4^+ per g wet weight per minute. This would mean that within 24 h a brook trout can release up to 0.49 mg NH_4^+ per g wet weight. Fish densities in kairomone studies reached in general from 0.1–0.25 fish per liter (e.g. Dodson 1989, Machácek 1991, Stibor 1992, Weider and Pijanowska 1993, Stibor and Machácek 1998). Thus, after 24 h of incubation, fish excreted NH_4^+ should be in the range of 0.05–0.12 mg per g wet weight per liter. Assuming wet weights of tested fish to be between 1–15 g, after one day of fish incubation, NH_4^+ should be present at concentrations of 0.05–1.8 mg L^{-1}. This estimation of NH_4^+ levels produced by fish excretion indicates that NH_4^+ concentrations in fish water of kairomone experiments could indeed have been comparable to those we have applied in this study. However, to our knowledge, brook trout has not been used for kairomone studies yet; the utilized fish species were either Perciformes (e.g. perch, bluegill sunfish) or Cypriniformes (e.g. roach, belica, ide). Furthermore, NH_3 and NH_4^+ excretion rates differ among fish species and these intraspecific variations are determined by body weight and by the amount, respectively the protein content, of consumed food (Stanley 1974, Carter and Brafield 1992). Thus, particular excretion rates of fish that have been used in former kairomone experiments may differ from the above calculated NH_4^+ excretion rate of brook trout (Whiles et al. 2009).

A more general and unspecific approach to estimate NH_4^+ concentrations in fish kairomone studies was provided by Carter and Brafield (1992). The authors suggested a nitrogen excretion rate for a 15 g "standard" fish of 0.11 mg total N (TN) per g wet weight, per day. By utilizing this generalized "standard" TN excretion rate, an overall estimation of NH_4^+ excretion for different fish species is possible. Basically, the main component of TN released by freshwater teleosts is NH_3 (~ 90%), the rest is urea (Wright and Andersen 2001). This means that about 0.1 mg of released TN per g wet weight per day is NH_3. In freshwater, at a pH of 7.3, 99% of excreted NH_3 would be present as NH_4^+ (Hargreaves 1998). Supposing that fish densities in kairomone experiments reached from 0.1–0.25 fish per liter and fish wet weights were between 1–15 g, the estimated NH_4^+ end concentrations in fish water would have be between 0.01–1.5 mg L^{-1}. Although, these approaches to assess NH_4^+ concentrations in former fish kairomone experiments are only rough estimations, the actual NH_4^+ concentrations in those experiments can be considered to have had roughly the same order of magnitude at which we observed life history shifts of *Daphnia hyalina*. This supports our assumption that NH_4^+ can potentially be associated with *Daphnia* responses to fish kairomones. Based on our results, we propose that the observed effects on *Daphnia* life history in fish kairomone experiments, could also have been directly induced by fish excreted NH_4^+.

5. Conclusions

This study demonstrates that increasing ambient NH_4^+ concentrations have the potential to change life history patterns of *Daphnia hyalina*. The observed life history shifts are similar to those when daphnids are exposed to fish kairomones. Associated shifts in body size and reproductive effort may further influence food web dynamics since the body size of daphnids can largely influence their feeding performance and their risk of predation. Particular threshold concentrations at which those shifts are induced may be found between 2.81–4.22 mg L^{-1} NH_4^+. Against the background of recent and future nitrogen loading scenarios these NH_4^+ concentrations are not completely unrealistic for natural lake ecosystems. There is a need for further experiments to define this threshold NH_4^+ concentration more precisely and for more cladocerans or rather, also for other zooplankton species.

Our findings also indicate a potential association of NH_4^+ and fish kairomones. To clarify the role of NH_4^+ for fish kairomones, it is important to identify the chemical compounds and structures of molecules composing fish kairomones. Until then, it is necessary and advisable to monitor NH_4^+ concentrations in future experiments dealing with fish kairomones. Otherwise, it

will be difficult to argue that observed adaptive responses of *Daphnia* are responses to the presence of fish kairomones alone. This is especially suggested for experiments in which life history shifts of daphnids are induced directly by using water from fish tanks, stocked with high numbers of fish.

In conclusion, the effects of fish kairomones and of ammonium on the life history of *Daphnia hyalina* are very similar, just as the result of the presence of either one is the same, an increased risk for an earlier death. The ability to perform life history changes towards fast reproduction, reproduction at smaller body size and as early as possible - thus, investing all resources into reproduction, is an advantageous strategy when facing an earlier death.

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Conflict of Interest

None declared.

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Tables

Table 1: For all treatments, the average NH_4^+ concentrations ± 1 standard deviation (SD) [mg L^{-1}] calculated over the entire experimental period of 24 days.

Treatment	1	2	3	4	5	6	7	8
Average NH_4^+ [mg L^{-1}]	0.0	0.59	1.23	2.81	4.22	6.7	13.5	87.8
± 1 SD [mg L^{-1}]	0.0	0.03	0.04	0.24	0.38	0.0	0.0	0.0

Table 2: For all treatments, the average NH_3 concentrations ± 1 standard deviation (SD) [mg L^{-1}] calculated over the entire experimental period of 24 days.

Treatment	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Average NH_3 [mg L^{-1}]	0.0	0.02	0.06	0.13	0.19	0.26	0.54	2.76
± 1 SD [mg L^{-1}]	0.0	0.01	0.03	0.08	0.11	0.09	0.21	0.0

Figure Legends

Figure 1: a) Boxplot displaying the survival time [days] of adult *Daphnia* in different NH_4^+ concentrations. Different numbers of asterisks display significantly different treatments ($p < 0.05$). The abbreviations "NOEC" (no observed effect concentration) at 2.18 mg L^{-1} NH_4^+ (treatment 4) and "LOEC" (lowest observed effect concentration) at 4.22 mg L^{-1} NH_4^+ (treatment 5), indicate the respective concentrations. Relationship of log (x + 1) transformed NH_4^+ concentrations with **b)** age at first reproduction (AFR) [days] ($p = 0.001$) and **c)** size at first reproduction (SFR) ($p = 0.12$). Gray lines display 95% confidence interval, error bars in **b)** and **c)** display ± 1 standard deviation from mean values (N = 3). Missing error bars denote identical responses without deviations in all 3 replicates. In **b)** and **c)** only treatments where *Daphnia* have reproduced (treatments: 1 - 5, NH_4^+ = 0 - 4.22 mg L^{-1}) are shown.

Figure 2: Relationships of log (x + 1) transformed NH_4^+ concentrations (0–4.22 mg L^{-1}) with **a)** number of neonates per female *Daphnia* and **b)** size of neonates. Regression lines indicate significance on the level of **a)** $p < 0.01$ and b) $p = 0.01$. Gray lines display 95% confidence intervals and error bars display ± 1 standard deviation.

Supplementary Figure S1: Average concentrations of **a)** NH_3 and **b)** NH_4^+ for all treatments during the experimental period. Black circles display respective concentrations in freshly prepared media, right before *Daphnia* were introduced. Gray triangles display respective concentrations in used media, after 48 - 72 h of *Daphnia* exposure. Y–Axis are log scaled, error bars display ± 1 standard deviation.

Figures

Figure 1

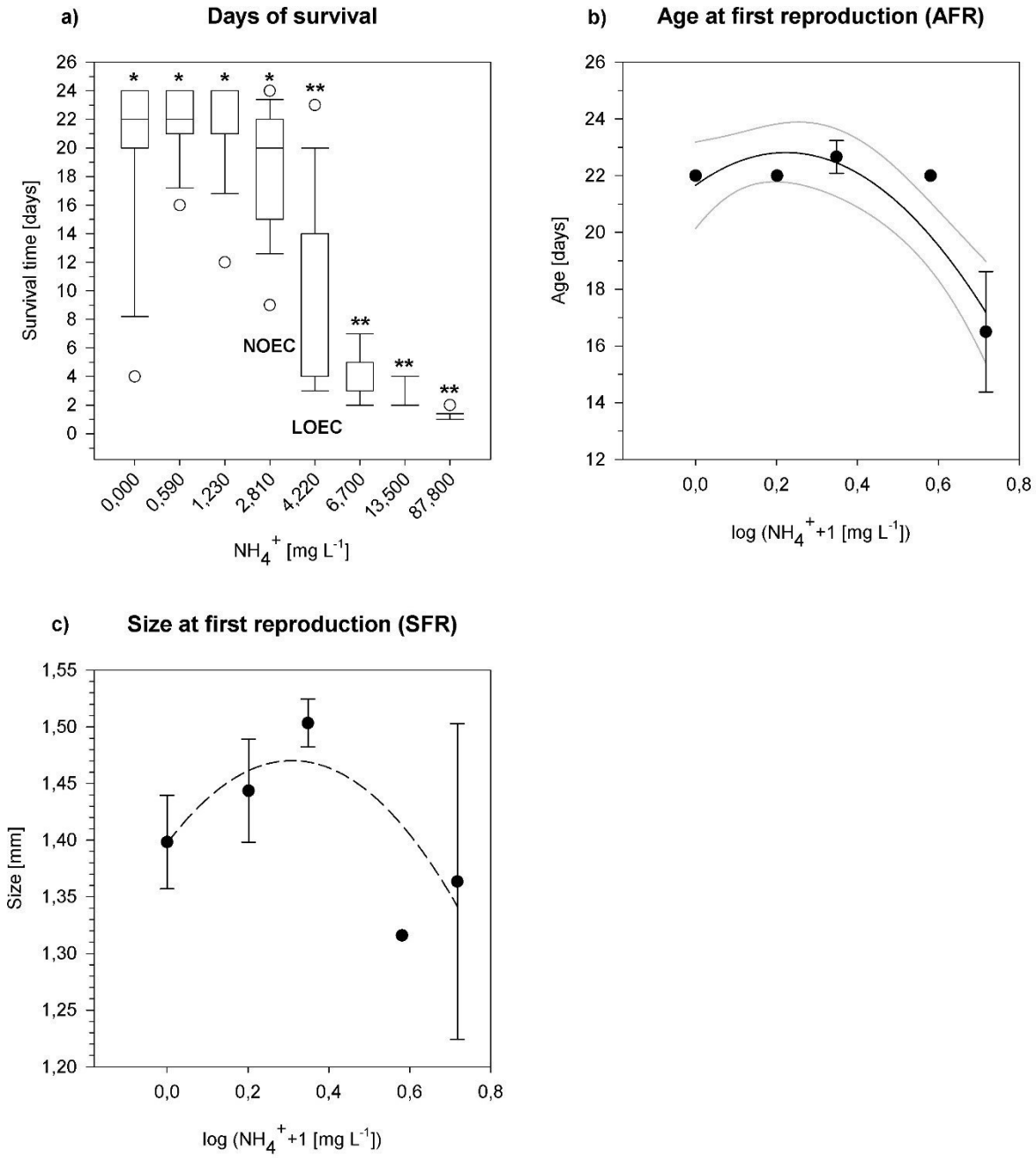
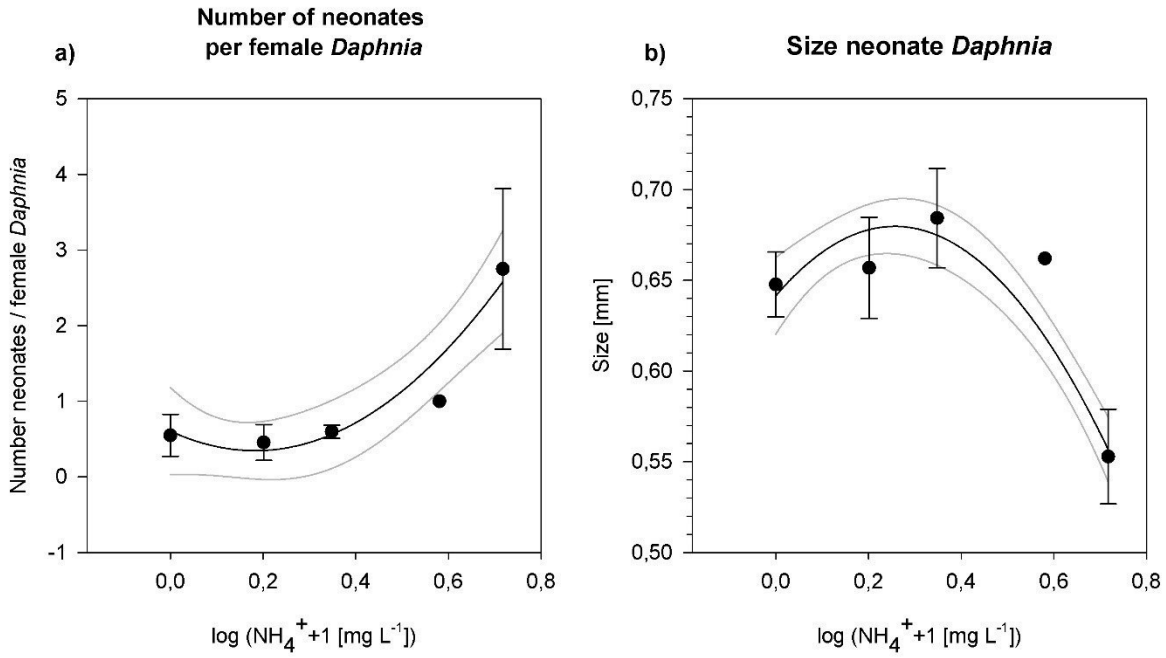
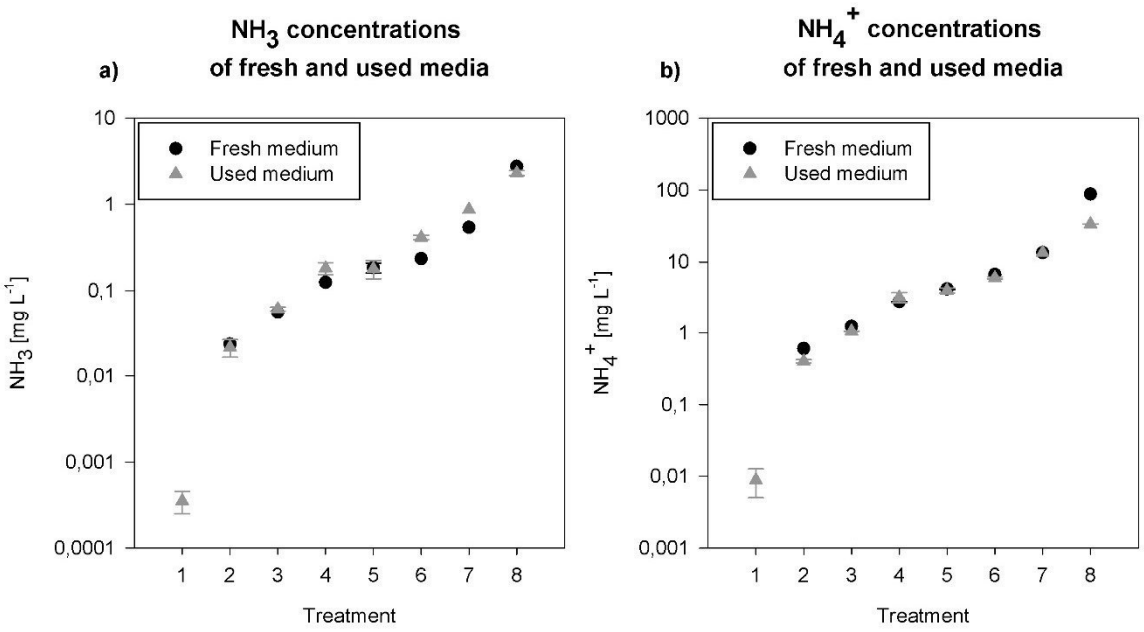


Figure 2


a)
Number of neonates per female Daphnia
Number neonates / female Daphnia
5
4
3
2
1
0
-1
0,0
0,2
0,4
0,6
0,8
log (NH4+ +1 [mg L-1])
b)
Size neonate Daphnia
Size [mm]
0,75
0,70
0,65
0,60
0,55
0,50
0,0
0,2
0,4
0,6
0,8
log (NH4+ +1 [mg L-1])

Supplementary Figure S1



4. General Discussion

The overarching objective of my work was to study the consequences to the food web of increasing N loading in lake ecosystems. To achieve a general and causal insight into the potential effects of N enrichment, I used different scientific approaches. By applying laboratory experiments, field experiments and a large-scale monitoring of 11 lakes over a period of three years, I investigated if rising N inputs have effects on the phytoplankton, zooplankton and whitefish communities of pre-alpine lakes. My results strongly suggest that increasing N loading has significant effects on the dynamics of entire food webs, not only on single trophic levels. These effects were also surprisingly strong in P-limited lakes, and it seems that the effects were not linked to specific conditions, as they were measurable over different years and in different lakes. Moreover, most of the effects had a negative influence on food-web transfer efficiencies as they decreased the transfer of energy and matter from primary producers to consumer levels in the lake food webs (Fig.7).

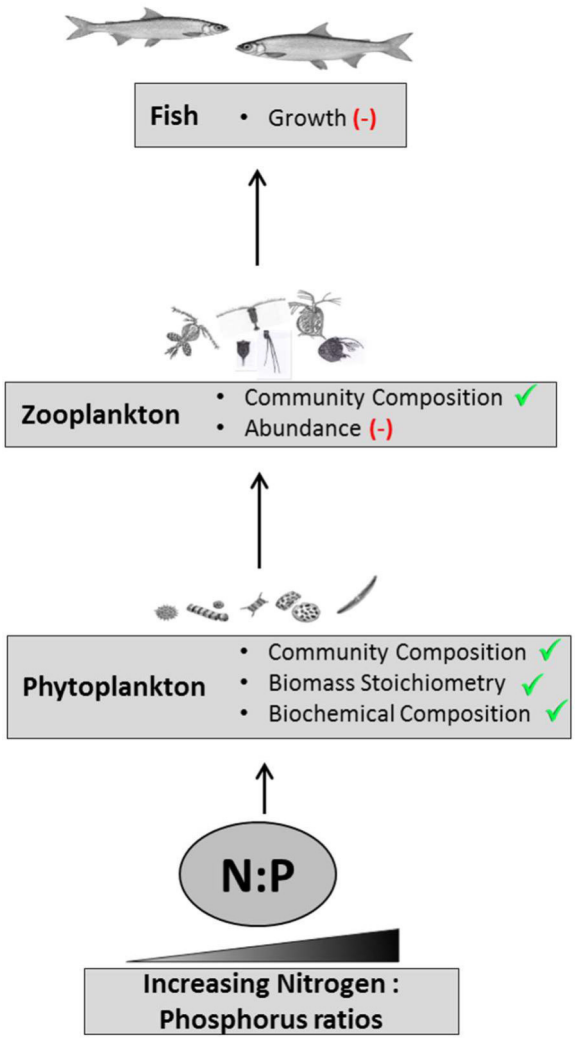


Fig. 7: This figure (together with Fig. 6) illustrates that the qualitative changes on the primary-producer level of phytoplankton are transferred into quantitative changes in zooplankton communities and fish growth. My publications indicate that in pre-alpine lakes these bottom-up effects, triggered by an increase in N:P ratios, result in generally lower food-web efficiency along the N:P gradient. For the respective terms, green hooks show the qualitative effect and red minuses in brackets indicate the negative quantitative effects caused by increasing N loading and accompanying rising N:P ratios.

From my publications I–IV and the manuscripts I and II, the following mechanisms resulting in lower food-web transfer efficiency became clear.

4.1 Effects on Phytoplankton Community Composition

In my first paper (Publication I), I show that rising N concentrations can change the taxonomical composition of phytoplankton communities in lakes. This result is consistent with the findings of other scientists (Sommer 1994b, Galloway et al. 2003, Roberts et al. 2003) and may be a consequence of the differing demands for N and P of the different algae taxa. The findings of Bulgakow and Levich (1999) support this hypothesis. The authors showed in a laboratory experiment that high N:P ratios favor the growth of green algae, while low N:P ratios are particularly beneficial for cyanobacteria (blue-green algae). Transferred to lake systems, this means that species with high N demands can then gain a competitive advantage.
However, my results indicate that the taxonomical changes in algal communities derived from a different mechanism. In the mesocosm experiment described in publication I, we found an increasing number of heterotrophic protists and mixotrophic algae along the artificially established N loading gradient. We also found increasing C:P ratios in algae biomass, indicating a rising P limitation of algae with increasing N (Carpenter et al. 1992, Hessen et al. 1997, Bergström et al. 2005, Carpenter and Brock 2006, Elser et al. 2009). Since mixotrophic phytoplankton taxa can also use bacteria for nutrition, they may have compensated for their lack of P by consuming prokaryotes that increased with increasing N loads. Increasing N loads can therefore result in a shift towards mixotrophic algae, which are often known to be of bad food quality for zooplankton (Katechakis et al. 2005, Taipale et al. 2013).

4.2 Effects on Phytoplankton Stoichiometry

My results also provide evidence that the stoichiometric composition of algal cells changes with increasing N loading. This was observed in three different mesocosm experiments, in which seston C:P and N:P ratios increased with rising N enrichment (Publications I-III). In addition, I also found evidence that high N concentrations can alter phytoplankton stoichiometry in lakes under natural nutrient regimes, as shown by the decreasing C:N ratios along an NO_3^- gradient (Manuscript I). These patterns are a clear indication of a rising P limitation in algal growth with increasing N enrichment. Those stoichiometric variations in primary producers are possible because phytoplankton is highly flexible in its stoichiometry (Hecky and Kilham 1988, Sommer 1989) and able to adjust its stoichiometric body C:N:P

ratios to follow ambient nutrient conditions (Diehl et al. 2005, Striebel et al. 2009). Phytoplankton prefers to absorb the nutrients C:N:P in a relatively constant ratio of about 106:16:1 (Redfield 1958, Klausmeier et al. 2004). However, if N concentrations increase in the lake water, the additional N that is not directly needed for growth is stored, leading to an increase in phytoplankton biomass N (Sterner and Elser 2002). The future consequences of these stoichiometric changes for phytoplankton communities in lakes may not be severe, due to their flexibility in elemental composition. In contrast, for herbivorous zooplankton taxa, these changes would mean a shift in food quality that may significantly affect the growth and composition of zooplankton communities.

4.3 Effects on Phytoplankton Biochemical Composition

The third possible mechanism by which phytoplankton communities may be altered by increasing N input is through changes in phytoplankton biochemical composition, for example, in the composition of fatty acids (Publication IV, Manuscript I). The substance class of fatty acids, which comprises the main components of animal cell membranes and animal hormones (Brett and Müller-Navarra 1997), is considered to be of special importance for aquatic animals. The essential fatty acids (ω3 and ω6 fatty acids) in particular play a crucial role because animals cannot synthesize these classes of fatty acids themselves and must absorb them from their food (Cook 1996, Gulatti and DeMott 1997, Wacker and Von Elert 2001).

A group of unsaturated fatty acids that has received special attention in the past 20 years is the group of PUFAs. Polyunsaturated fatty acids have two or more double bonds and are essential food-components for animals. In this thesis, I focus mainly on four PUFAs that are known to be able to substantially limit zooplankton and whitefish growth. These are DHA C22:6ω3, EPA 20:5ω3, LA C18:2ω6 and ALA C18:3ω3 (Brett and Müller-Navarra 1997, Müller-Navarra 1997, Wacker and Von Elert 2001). The nomenclature in the form X:YωZ describes the chemical structure of a fatty acid: X = the number of C atoms, :Y = the number of double bonds and ωZ = the location of the first double bond, counting from the methyl end of the fatty acid (Brett and Müller-Navarra 1997) (Fig. 8).

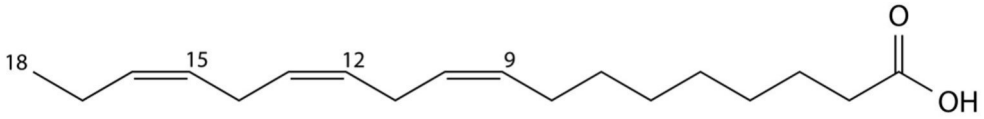


Fig. 8: The chemical structure of the polyunsaturated fatty acid alpha-linolenic acid (ALA). The morphology of ALA can be described as C18:3ω3, where "C18" indicates the 18 C atoms, ":3" indicates the three double bonds and "ω3" specifies the position of the first double bond (counted from the methyl end (left)) (Brett and Müller-Navarra 1997).

It is well known that algae change the quantity and quality (composition) of their fatty acids when grown under nutrient stress (Piorreck et al. 1984, Müller-Navarra 1995, Gulati and DeMott 1997, others). My results, shown in publication IV and in manuscript I, indicate that the essential PUFAs DHA, EPA, LA and ALA decrease in phytoplankton biomass with rising N concentrations. As with the effects I found for phytoplankton stoichiometry, the effects on PUFA content were significant for both the artificial N gradient in the mesocosm experiments (Publication IV) and for the natural N gradient in the monitored lake systems (Manuscript I). To my knowledge, this is the first causal evidence that increasing N concentrations can decrease algal PUFA contents that is supported by experimental analyses from mesocosm experiments (Publication IV) and by observational data from natural lake ecosystems (Manuscript I).

4.4 Effects on Zooplankton via Shifts in Phytoplankton Community Composition

Phytoplankton community composition is known to be an important parameter that determines the food quality of phytoplankton for zooplankton (Müller-Navarra and Lampert 1996). Changes in the community composition of algae can have severe consequences for zooplankton taxa, as this often also means changes in the edibility of algae. Since many zooplankters are filter feeders, the food-particle size, cell morphology, presence or absence of secondary metabolites and colony architecture of phytoplankton communities play a major role for herbivorous zooplankton taxa (Porter 1973, Lampert 1981, Lampert 1987, Brett and Müller-Navarra 1997). However, my results cannot support this hypothesis. Although I found indications that increasing N concentrations can change the composition of phytoplankton communities (Publication I), the experimental design did not fully allow the quantification of its impact on zooplankton communities. Additionally, publications III and IV show that strong

effects caused by N enrichment can be observed in zooplankton without accompanying qualitative changes in the composition of phytoplankton.

4.5 Effects on Zooplankton via Shifts in Phytoplankton Stoichiometry

In contrast to taxonomical shifts in phytoplankton communities, changes in phytoplankton stoichiometry induced by increasing N concentrations seem to have a greater influence on zooplankton communities (Publications I–III). It is known that animals obtain the majority of their essential elements from their food (Wacker and Von Elert 2001). Therefore, the elemental composition of food items is an important parameter that determines the quality of a diet. For zooplankton, it is the stoichiometry (mainly the C:N:P ratio) of food algae that potentially limits growth and reproduction. As mentioned above, phytoplankton in general is highly flexible in its elemental composition, and the variation in biomass C:N:P ratios can be immense. In contrast, zooplankton is much more homeostatic in its stoichiometry (Hessen 1990, Sterner 1990, Andersen and Hessen 1991, Hessen and Lyche 1991, Hessen 1992) and needs a rather constant C:N:P ratio in food (Hessen and Lyche 1991). For example, Sterner et al. (1993) found that zooplankton biomass varied only 10% when exposed to food differing by >700% in N:P ratio. This results in severe nutritional mismatches between zooplankton and phytoplankton if the C:N:P ratios of algal biomass do not fit the C:N:P demands of the respective zooplankton taxa. It is also known that such nutritional mismatches can cause major deficits in zooplankton growth and reproduction (Checkley 1980, Sommer 1992, Urabe and Watanabe 1992). The magnitude of detractions for zooplankton is taxon-specific and depends on the life-history traits of respective taxa (Elser et al. 1996). Species with a rapid-growth life history, such as cladocerans, require a high P supply in their diet. Copepods, in contrast, have a much longer generation time and a lower P demand in their diet (Sterner and Elser 2002). One biochemical basis for the divergence in P demands of rapid-growth and slow-growth life-history patterns arises from varying needs for ribosomal RNA (Elser et al. 1996).

I found strong evidence for such mismatch scenarios (Publication III) that are consistent with the literature and strongly suggest that N-induced shifts in phytoplankton stoichiometry are one important cause for the observed changes in the taxonomical compositions of zooplankton communities.

4.6 Effects on Zooplankton via Shifts in Phytoplankton Biochemical Composition

However, elemental limitation in diet is not the only chemical constraint that potentially limits zooplankton growth and reproduction. Besides C, N and P, there are also the PUFAs, which are essential biochemical compounds that cannot be synthesized by herbivorous zooplankton and must be absorbed with food (Wacker and Von Elert 2001). The PUFA content in phytoplankton depends on the availability of nutrients (e.g., Müller-Navarra 1995, Piorreck et al. 1984, El-Fouly et al. 1985). Therefore, I placed one focus of my research on the influence of increasing N depositions on phytoplankton PUFA composition and accompanying consequences for zooplankton communities.

In my publication IV and in the manuscript I, I show significantly decreasing proportions of total PUFAs and of ω-3 PUFAs (e.g., ALA, EPA, DHA) in seston biomass with increasing N concentrations. The zooplankton communities responded to the lowered amounts of ω-3 PUFAs in algal food with decreases in *Daphnia* biomass. This is the first time that both experimental and observational studies have shown this clear link between N enrichment and phytoplankton food quality. The fact that similar responses to increasing N concentrations were observed in both a mesocosm experiment with a duration of three months and a large-scale lake-monitoring exercise over three years including 11 natural lake systems highlights the robustness and the general relevance of my findings.

4.7 Toxic Effects on Zooplankton

Increasing N loading can affect the zooplankton communities of pre-alpine lakes not only via trophic effects, but also via toxic effects. For *Daphnia,* for example, the increasing concentrations of NH_x compounds (NH_3 and NH_4^+) can be a threat to survival, growth and reproduction (Gersich and Hopkins 1986, Adamsson et al. 1998, Mangas-Ramírez et al. 2001, Camargo and Alonso 2006).

To clarify if the NH_x concentrations in the mesocosm experiments and those found in the monitored lakes had any toxic effects on *Daphnia,* I conducted a microcosm experiment (Manuscript II). In this experiment, a *Daphnia hyalina* clone from Lake Brunnsee (one of my study sites) was exposed for 24 days to increasing NH_3/NH_4^+ concentrations under controlled conditions in the laboratory. The observed lowest concentration that had an effect on the survival of *D. hyalina* was $NH_4^+ = 4.22$ mg L^{-1} (Manuscript II). This indicates that the decrease

of *Daphnia* biomass along the N gradients in the mesocosms ($NH_4^+{}_{max}$ = 1.88 mg L^{-1}) and in the monitored lakes ($NH_4^+{}_{max}$ = 0.32 mg L^{-1}) did not result from toxic NH_4^+ effects, but rather was a response of *Daphnia* to trophic mismatches caused by P- and PUFA-deficient food algae, as described above.

4.8 Effects on Whitefish

Overall, I found a negative effect of increasing N enrichment on the growth of juvenile whitefish. I was able to show for the first time that qualitative changes induced by N at the base of the pelagic food web travel up the food chain and result in strong quantitative changes at the top. My results suggest that this results from decreasing zooplankton densities (in particular decreasing *Daphnia* densities) with rising N loading, caused by the mechanisms described above (Publication III, Manuscript I). However, there is ample evidence that the development of whitefish stocks is a multifactorial process and not a simple function of N and P concentrations in lakes alone. The significantly decreasing proportions of C-18 PUFAs in phytoplankton biomass with increasing N concentrations (Manuscript I) could be an indication that zooplankton also had lower C-18 PUFA concentrations in their biomass under high N conditions. Recent scientific studies (Jardine et al. 2020) and results from aquaculture (Ahlgren et al. 1998 and references therein) show strong relationships between C-18 PUFAs and the growth and performance of fish. Hence, C-18 PUFAs especially are very important for fish and are needed in certain amounts.

Therefore, the reduced growth of whitefish in high N regimes found in the mesocosms and pre-alpine lakes may have been to some extent a result of biochemical limitation induced by the insufficient supply of C-18 PUFAs. However, fishing pressure, standing-stock management and the resulting intraspecific competition between fish may also strongly influence the growth conditions of whitefish in pre-alpine lakes (Mayr 2001) and thereby mask clear nutrient–fish growth relationships. Additionally, I was only able to investigate the short-term growth responses of individual fish to N manipulation; long-term observations on total fish stocks would need a minimum of ten years.

5. Future Directions

The divergence between N and P concentrations in lakes, resulting in food-web consequences as described above, may further increase in the future. Hence, deep pre-alpine lakes may be more affected than shallow lakes. Within the pelagic zone, P will sediment with dead biomass to deep water layers: only full mixing events will redistribute P, in spring and autumn. However, Yankova and colleagues (2017) found that the warming of lakes has started to decrease the mixing-depths of lakes and thereby strongly reduce P fluxes between deep waters and surface layers. Increasing atmospheric N loading and N inputs from ground and surface waters could therefore result in even larger dissolved N:P ratios in the epilimnion of deep, pre-alpine lakes.

In future studies, it would also be important to perform similar mesocosm experiments to those described in my thesis in different seasons. This is because the taxonomical community composition of phytoplankton changes during the year (Sommer et al. 1986, Sommer et al. 2012), and it would be interesting to know if different phytoplankton communities respond differently to increasing N loading. For example, spring communities with fast-growing and small phytoplankton taxa may respond differently to rising N concentrations than late summer communities (which usually include more large and slow-growing algal species) or winter communities (with mixotrophic phytoplankton species) (Sommer et al. 1986, Sommer et al. 2012).

I would further recommend that such N-addition mesocosm experiments should also be conducted in marine ecosystems. Evidence exists that changing N:P ratios can also alter the FA compositions of marine phytoplankton communities (Bi et al. 2014) and thus may also have a negative influence on marine zooplankton communities. In addition, marine phytoplankton communities are responsible for the great majority of global primary production. Their influence on economically important marine fisheries is immense, which further highlights the need for future work in this direction. Additionally, most marine systems are N-limited, so the addition of N may also add quantitative effects to the qualitative effects described above.In general, my results underline the need for a paradigm shift in the understanding of the role of N and P for the food webs of lakes but also for those of marine systems. Although this discussion started in the USA more than a decade ago (see: Schindler 1977, Downing and McCauley 1992, Elser et al. 2007, Luis and Wurtsbaugh 2008, Schindler et al. 2008, Sterner 2008), it seems that P still receives the most attention in lake management. However, my work, together

with other studies, indicates that both P and N, and their complex interplay (N:P ratios), must be considered in the future management strategies of lakes.

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Statutory Declaration (Eidesstattliche Versicherung)

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und ohne erlaubte Hilfe angefertigt wurde.

München, den 07.06.21 …….. Patrick Lorenz ……

(Unterschrift)

Statement (Erklärung)

Ich erkläre hiermit, dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist und dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

München, den 07.06.21 …….. Patrick Lorenz ……

(Unterschrift)

List of Publications and Manuscripts

Publication I

Poxleitner, M., Trommer, G., **Lorenz, P.**, & Stibor, H. (2016). The effect of increased nitrogen load on phytoplankton in a phosphorus-limited lake. *Freshwater Biology*, **61**(11), 1966–1980. https://doi.org/10.1111/fwb.12829

Publication II

Trommer, G., Poxleitner, M., **Lorenz, P.**, Bitzilekis, E., Gogaladze, A., Schultes, S., & Stibor, H. (2017). Altered food web dynamics under increased nitrogen load in phosphorus deficient lakes. *Aquatic Sciences*, **79**(4), 1009–1021. https://doi.org/10.1007/s00027-017-0551-2

Publication III

Lorenz, P., Trommer, G., & Stibor, H. (2019). Impacts of increasing nitrogen:phosphorus ratios on zooplankton community composition and whitefish (*Coregonus macrophthalmus*) growth in a pre-alpine lake. *Freshwater Biology,* **64**(6), 1210–1225. https://doi.org/10.1111/fwb.13296

Publication IV

Trommer, G., **Lorenz, P.**, Lentz, A., Fink, P., & Stibor, H. (2019). Nitrogen enrichment leads to changing fatty acid composition of phytoplankton and negatively affects zooplankton in a natural lake community. *Scientific Reports*, **9**(1), 16805. https://doi.org/10.1038/s41598-019-53250-x

Manuscript I

Lorenz, P., Spoerl, G., Trommer, T., Gum, B., Schubert, M. & Stibor H. (*in preparation*). Increasing N:P misbalances change seston fatty acids, zooplankton growth and whitefish growth conditions in pre-alpine lakes.

Manuscript II

Lorenz, P., Trommer, G., Hofmeister A. & Stibor H. (*in preparation*). Ammonium exposure of *Daphnia hyalina* induces life history shifts similar to fish kairomones.

Declaration of Contribution as a Co-Author

Publication I

I was substantially involved in performing the experiment and the laboratory analyses. I carried out the zooplankton counting and the statistical analyses of zooplankton data. I also contributed to the writing of the manuscript.

Publication II

I was substantially involved in performing the experiment and the laboratory analyses. I partly carried out data and statistical analyses and contributed to the interpretation of the results. I was involved in writing the manuscript and provided critical feedback on the manuscript.

Publication IV

I was significantly involved in conceiving, planning and performing the experiment. I helped carrying out laboratory, data and statistical analyses. Furthermore, I was responsible for parts of the graphical interpretation of the data. I wrote the zooplankton part in the manuscript, provided critical feedback and helped writing the rest of the manuscript.

München, den 07.06.21 Patrick Lorenz

(Unterschrift Patrick Lorenz)

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(Unterschrift Prof. Dr. Herwig Stibor)

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Conference Contributions

- **P. Lorenz**, G. Trommer, A. Hofmeister and H. Stibor (2017), "Ammonium exposure of *Daphnia* (*Daphnia hyalina*) induces similar life history shifts as fish kairomones." Deutsche Gesellschaft für Limnologie (DGL), Cottbus, Germany.

- G. Trommer, **P. Lorenz**, A. Lentz, P. Fink and H. Stibor (2016), "Nitrogen supply in lakes leads to changing fatty acid composition of phytoplankton and negatively affects zooplankton." International Society of Limnology Austria (SIL Austria) - Deutsche Gesellschaft für Limnologie (DGL), Wien, Austria.

- **P. Lorenz**, G. Trommer and H. Stibor (2015), "Increased atmospheric N-deposition can lead to shifts in zooplankton densities in P-limited lakes." Fresh Blood for Fresh Water (FBFW), Mondsee, Austria.

- **P. Lorenz**, G. Trommer and H. Stibor (2015),"Influences of N-deposition on a food web of a P-limited lake." Association for the Sciences of Limnology and Oceanography (ASLO), Granada, Spain.

- **P. Lorenz**, G. Trommer and H. Stibor (2014), "Increased N-input alters zooplankton densities in P-limited lakes." Deutsche Gesellschaft für Limnologie (DGL), Magdeburg, Germany.

- G. Trommer, M. Poxleitner, E. Bitzilekis, A. Gogaladze, **P. Lorenz** and H. Stibor (2014), "Nitrogen deposition on phosphorus limited lakes: Effects of increasing phosphorus limitation." International Society of Limnology Austria (SIL Austria), Lunz, Austria.

- G. Trommer, M. Poxleitner, E. Bitzilekis, A. Gogaladze, **P. Lorenz**, H. Stibor (2013), „Einfluss von erhöhter Stickstoffzufuhr auf Seeökosysteme - ein experimenteller Ansatz". Deutsche Gesellschaft für Limnologie (DGL), Potsdam, Germany.