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How nitrogen and phosphorus supply to nutrient-limited autotroph communities affects herbivore growth: testing stoichiometric and co-limitation theory across trophic levels

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Primary producer communities are often growth-limited by essential nutrients such as nitrogen (N) and phosphorus (P). The magnitude of limitation and whether N, P or both elements are limiting autotroph growth depends on the supply and ratios of these essential nutrients. Previous studies identified single, serial or co-limitation as predominant limitation outcomes in autotroph communities by factorial nutrient additions. Little is known about potential consequences of such scenarios for herbivores and whether their growth is primarily affected by changes in autotroph quantity or nutritional quality. We grew a community of phytoplankton species differing in various food quality aspects in experimental microcosms at varying N and P concentrations resulting in three different N:P ratios. At carrying capacity, N, P, both nutrients or none were added to reveal which nutrients were limiting. The nutrient-supplied communities were fed to the generalist herbivorous rotifer *Brachionus calyciflorus* to investigate how changing phytoplankton biomass and community composition affect herbivore abundance. We found phytoplankton being growth-limited either by N alone (single limitation) or serially, i.e. primarily by N and secondarily by P, altering available food quantity for rotifers. Rotifer growth showed a different response pattern compared to phytoplankton, suggesting that apart from food quantity food quality aspects played a substantial role in the transfer from primary to secondary production. The combined addition of N and P to phytoplankton had generally a positive effect on herbivore growth, whereas adding non-limiting nutrients had a rather detrimental effect probably due to stoichiometrically imbalanced food in terms of nutrient excess. Our experiment shows that adding various nutrients to primary producer communities will not always lead to increased autotroph and herbivore growth, and that differences between autotroph and herbivore responses under co-limiting conditions can be partly well explained by concepts of ecological stoichiometry theory.

Keywords: *Brachionus*, experimental microcosms, food quality, food quantity, multiple resource limitation, phytoplankton, rotifers, stoichiometry, zooplankton

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Introduction

Nutrient co-limitation is an emerging concept that derives from and is somehow opposed to Justus von Liebig's law of the minimum (Von Liebig 1840, Paris 1992, Sperfeld et al. 2012). Liebig's law postulates that in a system, only a single nutrient can be limiting at a time and that the limiting nutrients change depending on their availability (de Baar 1994). This view has been questioned as organisms or communities are frequently limited simultaneously by more than one nutrient (Arrigo 2005, Elser et al. 2007, Saito et al. 2008, Martin-Creuzburg et al. 2009, Fay et al. 2015). Co-limitation is broadly defined as the simultaneous limitation of primary production or fitness-related responses such as growth by multiple nutrients (Tilman 1982, Harpole et al. 2011, Sperfeld et al. 2016).

Mainly two approaches have been used to study nutrient co-limitation: factorial limitation scenarios and response surfaces (Fig. 1, Sperfeld et al. 2016). The first approach characterises limitation outcomes from single and combined factorial supplies of potentially limiting nutrients (Allgeier et al. 2011, Harpole et al. 2011). Among these outcomes, a simultaneous co-limitation scenario is detected when only the addition of two nutrients together stimulates a positive response, whereas an independent co-limitation scenario arises when the addition of either nutrient alone and in combination produces a response (Fig. 1). If the addition of only one of the nutrients produces a response and the joint addition of nutrients does not result in further growth, single limitation occurs, whereas serial limitation occurs if the addition of both nutrients together results in further growth compared to single nutrient addition (Fig. 1). The two latter cases are conventionally not considered co-limitation, but rather conform to Liebig's minimum law (Harpole et al. 2011, Sperfeld et al. 2016). The second approach uses response surfaces measured as growth responses across nutrient gradients in a two or multi-dimensional nutrient space (Saito et al. 2008, Sperfeld et al. 2012). Different response surface types can emerge depending on whether nutrients are substitutable or essential (Tilman 1982, Saito et al. 2008, Sperfeld et al. 2016). For essential nutrients, the strictly essential resource type supports Liebig's minimum law, while the interactively essential resource type indicates co-limitation (Fig. 1, Sperfeld et al. 2012). Sperfeld et al. (2016) summarised and integrated the two approaches by developing a general co-limitation framework, which showed that different factorial nutrient (co-)limitation scenarios can emerge across the continuous multi-dimensional nutrient space, depending on the position within that space (Fig. 1).

The concept of co-limitation can be applied at different levels of biological organization, such as the organismal or community level (Arrigo 2005, Danger et al. 2008, Sperfeld et al. 2016). For instance, meta-analyses on experiments with factorial N and P additions showed that primary producer communities are often (co-)limited by nitrogen (N) and phosphorus (P) deficiency (Elser et al. 2007, Allgeier et al. 2011, Harpole et al. 2011). These studies also showed that the combined supply of N and P often yielded higher autotroph growth than single nutrient addition and that this pattern is similar in magnitude and occurrence across freshwater, marine and terrestrial systems. The hypothesized mechanism of nutrient co-limitation in communities is that species differ in their nutrient use efficiencies and are each limited by a different nutrient (Arrigo 2005, Sperfeld et al. 2016).

Co-limitation of consumers has been mainly studied at the organismal level of particular species. Generally, consumers can be co-limited by the quantity and quality of their food (Halvorson et al. 2017, Schälicke et al. 2019a, b) as well as by substitutable food sources in mixed diets (Tilman 1982, Rothhaupt 1988, Sperfeld et al. 2016). Macronutrients such as proteins, carbohydrates and lipids have been shown to co-limit growth and development of terrestrial insects (Simpson et al. 2004, Lee et al. 2008, Jensen et al. 2012). Freshwater consumers, such as cladocerans and rotifers, can be co-limited by essential micronutrients such as amino acids, sterols and polyunsaturated fatty acids (PUFAs) (Martin-Creuzburg et al. 2009, Wacker and Martin-Creuzburg 2012, Sperfeld et al. 2012). Also macro elements, such as carbon (C), P or calcium, can co-limit the growth of arthropods (Lukas et al. 2011, Halvorson et al. 2017, Jones et al. 2020). In the context of whole communities, including primary producers and consumers, it is not yet fully understood how the supply of multiple nutrients to primary producers affects consumer growth. Case studies on terrestrial communities experiencing nutrient additions have found, for instance, that the abundance of particular herbivorous arthropods is co-limited by N and P (Bishop et al. 2010) or that the abundance of prairie invertebrates was independently limited aboveground, but serially limited belowground by sodium (Na) and N+P (Kaspari et al. 2017). A recent meta-analysis found strong negative impacts of combined N and P additions to whole communities on invertebrate abundance, but weaker or inconclusive impacts on invertebrate biomass or species richness (Nessel et al. 2021).

Many studies investigated the effects of N or P limitation of particular primary producer species on herbivorous consumers (Sterner and Hessen 1994, Rothhaupt 1995, Kilham et al. 1997, Van Donk et al. 1997, Elser et al. 2001, Lürling 2006, Zhou et al. 2018, Ruiz et al. 2020). N or P deficiency can first of all constrain primary production, leading to reduction in autotroph biomass and thus lower quantity of food available for consumers (Hessen 1992, Bledsoe et al. 2004, Xu et al. 2010). Nutrient limitation of autotrophs can also affect the quality of food for consumers, either directly by altering its stoichiometry or indirectly by changing other non-stoichiometric aspects (Van Donk et al. 1997, Boersma 2000, Ravet and Brett 2006, Zhou et al. 2018). Low N and P concentrations in food (i.e. high plant C:N and C:P ratios) limit herbivore growth, as N and P are associated with molecules, such as amino acids and RNA, which are important for growth by maintaining protein synthesis and ribosomal activity (Elser et al. 1996, Sterner and Elser 2002). Stoichiometric imbalance in terms of nutrient excess can also negatively affect consumer growth due to elevated metabolic

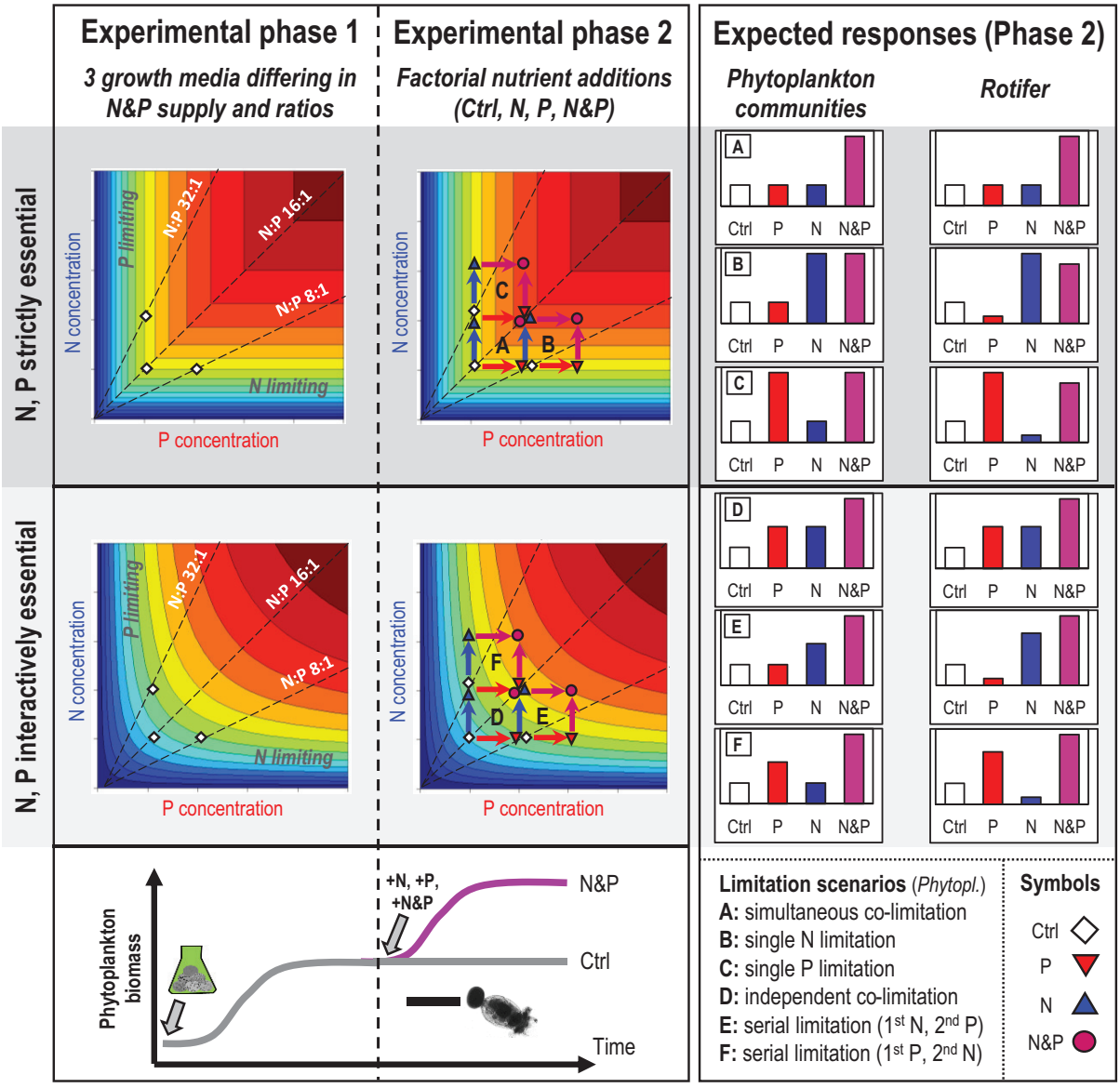

Figure 1. Overview about the experimental setup and expected growth responses of primary producers (phytoplankton communities) and the herbivore (rotifer) based on co-limitation theory regarding essential nutrients (Sperfeld et al. 2016). From the left: coloured response surfaces show predicted growth responses of phytoplankton across a gradient of both nitrogen (N) and phosphorus (P) concentrations based on whether N and P are considered strictly essential nutrients (in accordance with Liebig's minimum law, dark grey shaded) or interactively essential nutrients (in accordance with the multiple limitation hypothesis, light grey shaded) (Gleeson and Tilman 1992, Sperfeld et al. 2012). In the first experimental phase, the phytoplankton community grew on media with varying N and P concentrations (white diamonds) resulting in three different N:P ratios (dashed diagonal lines), one more 'balanced' ratio (16:1 = Redfield ratio) and two ratios where either N or P should become limiting (8:1 and 32:1, respectively). The second experimental phase started after phytoplankton growth reached stationary phase, by adding N, P, N and P together, or nothing (control = Ctrl) to the nutrient-limited communities. Nutrient additions changed the supply points within the two-dimensional nutrient space (blue triangle: increasing N concentration, red triangle: increasing P concentration, purple circle: increasing N as well as P concentration), and may lead to further phytoplankton growth depending on growth medium and nutrient addition treatment. The thick black horizontal bar in the experimental time course panel indicates the time period of the rotifer growth experiment, in which the animals were fed the nutrient-supplied phytoplankton communities in separate microplate wells. The bar plots on the right represent the expected growth responses of phytoplankton communities and the rotifer. According to co-limitation theory, different factorial limitation scenarios (A–E) can emerge in the phytoplankton communities depending on growth medium of phase 1, nutrient addition treatment of phase 2, and the underlying nutrient type (i.e. whether N and P are strictly or interactively essential) (Sperfeld et al. 2016). Predictions of the rotifer's responses are based on expected changes in quantity (biomass) and quality (stoichiometry, edibility, biochemical composition) aspects of the phytoplankton communities used as food. The experimental time course panel shows a grey line representing the expected growth trajectory of a phytoplankton community growing on a particular medium that did not receive nutrient addition in the second phase, whereas the purple line represents a hypothesized growth trajectory of a treatment with simultaneous N and P addition (N&P).

costs, leading together with nutrient deficiency to a unimodal 'stoichiometric knife-edge' pattern across elemental gradients (Boersma and Elser 2006, Elser et al. 2016, Sperfeld et al. 2017, Zhou and Declerck 2019). In addition to a direct stoichiometric effect, nutrient limitation or excess nutrient supply can lead to shifts in species composition of primary producers due to different use efficiencies and requirements (Chapin et al. 1986, Interlandi and Kilham 2001, Arrigo 2005). For instance, increasing nutrient inputs in aquatic systems, leading to eutrophication, can favour the occurrence of cyanobacteria (O'Neil et al. 2012), which are known to be of poor food quality for zooplankton consumers due to toxicity, colony/filament development hindering ingestion and the lack of micronutrients, such as phytosterols and PUFAs (Von Elert et al. 2003, Wilson et al. 2006, Martin-Creuzburg et al. 2009). Nutrient limitation can also reduce the digestibility or edibility of particular algae species, by causing thickened cell walls and colony formation (Van Donk et al. 1997), or alter their biochemical composition, e.g. by changing the cellular content of sterols and PUFAs (Müller-Navarra 1995, Spijkerman and Wacker 2011).

Although there is strong evidence of reduced consumer performance when fed nutrient-limited food in laboratory experiments, either due to its imbalanced stoichiometry or other food quality aspects, studies have used mainly single species of autotrophs as food source and mostly investigated effects of single nutrients. Therefore, there is a need to understand how N and P co-limitation of primary producer communities affect herbivore growth and the associated potential mechanisms. In this study, we investigated how a phytoplankton community differently supplied with N and P affects the population growth of a generalist herbivorous consumer. First, we grew a freshwater phytoplankton community, composed of species differing in their biochemical composition and edibility, in microcosms under varying N:P supply ratios until limiting conditions. Subsequently, we assessed the types of factorial limitation by adding N, P or both to the phytoplankton communities and hypothesized to detect single or serial limitation of the nutrient in deficit, and co-limitation scenarios under a more balanced N:P ratio (Fig. 1). We also expected changes in phytoplankton community composition during growth with communities dominated by the most efficient species in regard to the use of the supplied nutrient. We then measured the abundance development of the rotifer *Brachionus calyciflorus* fed these nutrient-supplied communities. We hypothesized enhanced rotifer growth in cases where phytoplankton communities are released from limitation by addition of the deficient nutrient, thereby increasing autotroph biomass (i.e. food quantity). Therefore, the highest herbivore growth is expected at combined N and P addition or where the addition of the most limiting nutrient results in high phytoplankton growth (Fig. 1). In contrast, we hypothesized reduced rotifer growth when the non-limiting nutrient is added to the phytoplankton community (Fig. 1), which may lead to adverse effects of excess nutrients on herbivore performance. We expected that rotifer abundance is primarily constrained by food quantity and secondarily by food quality aspects in terms of stoichiometric imbalance or the availability of micronutrients (e.g. sterols and PUFAs) that alter with changing phytoplankton community composition.

Methods

Organisms and general culture conditions

We used six representative freshwater phytoplankton species, varying in edibility and biochemical composition, from four major taxonomic groups to compose the phytoplankton community (Table 1). Stock cultures of phytoplankton strains were maintained in axenic batch cultures in modified WC medium with vitamins (Guillard 1975). The rotifer *Brachionus calyciflorus* s. str. (Monogononta) (strain 'Cornell', provided by Lutz Becks and used in other studies, Schälicke et al. 2019b) was used as herbivorous zooplankton species and grown on WC medium with *M. minutum* as food algae. The phytoplankton and rotifer cultures were kept in a walk-in climate chamber at 20°C on low light levels with a 16:8 h light:dark cycle.

Experimental design and protocol of the phytoplankton growth experiment

The experiment was set up in two time periods, a first pregrowing phase, in which phytoplankton communities grew on media with different N and P concentrations resulting in varying N:P ratios until they reached the stationary phase

Table 1. Species of the phytoplankton community and their main features in terms of food quality (FQ).

Species	Strain	Family	Edibility	Fatty acids	Sterols	Overall FQ	Ref.
Synechococcus elongatus	SAG 89.79	Cyanophyceae	well-edible	no PUFAs	absent	low	1
Anabaena variabilis	ATCC 29413	Cyanophyceae	less-edible	PUFAs ≤ C18	absent	low	1
Acutodesmus obliquus	SAG 276-3a	Chlorophyceae	well-edible	rich in PUFAs ≤ C18	present	moderate	2, 3, 4
Monoraphidium minutum	SAG 243-1	Chlorophyceae	well-edible	rich in PUFAs ≤ C18	present	moderate	2, 5, 6
Cryptomonas ovata	SAG 979-3	Cryptophyceae	well-edible	rich in long-chain PUFAs (≥ C20)	present	high	1, 2
Nannochloropsis limnetica	SAG 18.99	Eustigmatophyceae	well-edible	rich in long-chain PUFAs (≥ C20)	present	high	4, 5, 7

SAG = culture collection Göttingen (Germany), ATCC = American Type Culture Collection (USA). PUFAs = polyunsaturated fatty acids with less than 20 carbon atoms (≤ C18) and more than 18 carbon atoms (≥ C20). References (Ref.) 1: Martin-Creuzburg et al. 2008, 2: Sperfeld et al. 2010, 3: Basen et al. 2012, 4: Martin-Creuzburg et al. 2012, 5: Martin-Creuzburg and Merkel 2016, 6: Schälicke et al. 2019b, 7: Marzetz et al. 2017.

(i.e. were nutrient limited), and a second phase, in which growth of the communities was monitored after the addition of different amounts of N, P or both nutrients (Fig. 1). The development of phytoplankton biomass in the batch cultures was regularly monitored over time to determine when the communities showed stationary growth, i.e. reached their carrying capacity. In the first phase, the timing of reaching carrying capacity indicates when phytoplankton communities have used up N or P, or both nutrients (depending on N:P supply ratio) to limiting levels in the medium. In the subsequent second experimental phase, addition of P or N alone, or in combination should reveal which nutrient is limiting the communities by observation of further growth responses (Fig. 1). For instance, in a control treatment without nutrient addition, the community should not show further growth in the second phase, whereas additional growth might be observed in a treatment with combined N and P addition if N, P or both are limiting (Fig. 1).

COMBO medium was used in the experiment, which is designed for culturing both phytoplankton and zooplankton as well as modification of N and P concentrations (Kilham et al. 1998). We prepared COMBO media of three different N:P ratios, intended to simulate limitation by N, by P or by both nutrients simultaneously at the end of the first growth phase. One medium was prepared with low concentrations of N and P (N&P-low: 2.58 µM P, 41.33 µM N), resulting in the Redfield ratio (molar N:P = 16), to provide phytoplankton with a balanced nutrient supply in terms of N and P, where the community might be limited by both nutrients after reaching carry capacity. The other two media were prepared to have ratios deviating from the Redfield ratio, N-low medium (molar N:P = 8:1, 5.17 µM P, 41.33 µM N), where the community might be limited by N after reaching carrying capacity, and P-low medium (molar N:P = 32:1, 2.58 µM P, 82.66 µM N), where P limitation might prevail after growth to carrying capacity in the first phase. Potassium chloride (KCl) was added to the P-reduced media to avoid a limitation by potassium (K) due to the reduction of the K_2HPO_4 stock solution (Kilham et al. 1998). All working steps involving media and phytoplankton cultures were carried out under sterile conditions. Twelve 300-ml Erlenmeyer flasks were filled with 150 ml of each of the three N- and P-reduced COMBO media, resulting in a total of 36 flasks/microcosms.

Monocultures of each phytoplankton species have been grown in full COMBO medium (50 µM P, 1000 µM N, molar N:P = 20) until carrying capacity before use in the experiment. By reaching carrying capacity, most of the nutrients are bound in phytoplankton biomass and residues of dissolved nutrients in the medium should be very low. The starting community was composed by combining the monocultures of all six phytoplankton species with equal relative biomass (i.e. 16.67% per species). Each of the 36 microcosms were inoculated only with a small amount (1 ml) of this starting community to avoid adding significant amounts of potential residual nutrients from monocultures to the flasks. Microcosms were placed randomly on a shelf in a climate chamber (20°C) and illuminated in a 16:8 h light:dark cycle with LED lamps at a photosynthetic photon flux density (400–700 nm) of 53.2 ± 8.6 µmol m^{-2} s^{-1} (mean ± SD). After start of the experiment (day 0), the development of phytoplankton community biomass in each microcosm was monitored by measuring optical density (OD) on days 1, 4, 5 and every second day thereafter. At each measurement day, a 3 ml subsample of each microcosm has been taken under sterile conditions and OD was measured by photometric light extinction at a wavelength of 800 nm using a spectrophotometer (Lukas et al. 2011, Sperfeld et al. 2012). Microcosms were placed randomly back on the shelf in the climate chamber after each day of subsampling and placed differently on the shelf when manually shaken each day. The first growth phase was ended after 22 days, as OD did not increase anymore for several days in all microcosms (Fig. 2). Subsamples (10 ml) of each microcosm were taken on day 22 and fixed with Lugol's iodine solution for later determination of phytoplankton biovolume. After taking subsamples, each remaining culture was transferred to a new sterilized Erlenmeyer flask to avoid buildup of wall growth in the further course of the experiment.

After transfer to new flasks, four treatments, each in triplicates, were established for each of the three media: a control without N and P addition (Ctrl), N addition (N, 62.00 µM N), P addition (P, 3.875 µM P) and N and P addition (N&P, 3.875 µM P and 62.00 µM N). After nutrient additions, OD has been monitored further every second day to observe when the communities that have been released from nutrient limitation reach carrying capacity a second time. The experiment was stopped on day 49 when maximum community biomass had been reached in the second phase. Subsamples of each microcosm were fixed with Lugol's iodine solution for the determination of phytoplankton biovolume.

The biovolume of all phytoplankton species was determined from the fixed subsamples at days 22 and 49 by counting and measuring the cells using an inverted microscope equipped with a computer-aided camera and measurement software (Leica Application Suite software, LAS X, ver. 3.7.1.21655). The calculation of individual cell biovolume was based on geometric forms that closely matched the shapes of phytoplankton species (Hillebrand et al. 1999). The sum of biovolumes per replicate (biovolume of whole community) and respective OD measurements were highly correlated (Pearson coefficient, $r = 0.85$, $p < 0.001$). However, we use the OD measurements as estimates of overall phytoplankton biomass rather than calculated biovolumes, because biovolumes usually show lower measurement precision due to counting and cell measuring errors.

Rotifer growth experiment

On day 23, one day after nutrient additions to phytoplankton communities, 4 ml of each microcosm were subsampled and distributed into four wells of a 24-well plate with 1 ml each. Each well was stocked with two asexually reproducing females of *B. calyciflorus*, resulting in sum in 288 rotifers (3 media × 4 treatments × 3 replicate microcosms × 4 wells × 2 rotifers = 288). The two individuals were randomly chosen from

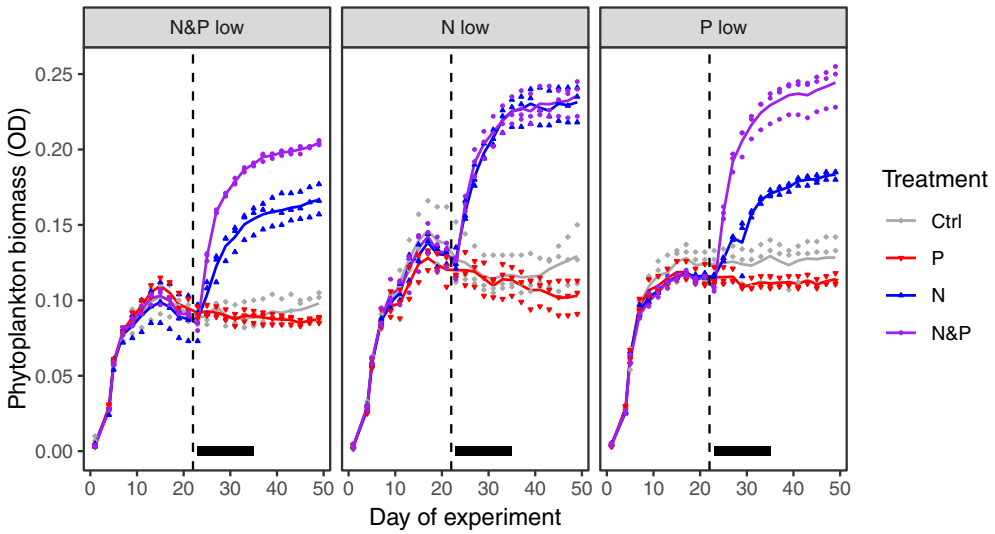

Figure 2. Development of phytoplankton biomass on different media and treatments during the two phases of the experiment. Biomass was estimated by measuring optical density (OD) every second day. Colored lines indicate means of treatment triplicates. The vertical dashed line separates the first and second phase of the experiment. In the first phase, phytoplankton was grown in medium with low N concentration (N-low), low P concentration (P-low) and low concentration of N and P (N&P-low). On day 22, N, P, N and P, or nothing (C = control) has been added. The black horizontal bar indicates the time period when the rotifer growth experiment was carried out, i.e. days 23–35.

the rotifer stock culture and pipetted into each well. Setting up four rotifer wells per phytoplankton microcosm replicate required the application of a mixed model design for statistical analysis. After starting the rotifer experiment (day 23), the abundance of live rotifers was counted daily for 11 days using a stereomicroscope. Every second day, when OD of phytoplankton flasks was measured, 0.5 ml of each phytoplankton community was pipetted to the wells of the respective replicate until each well contained 3 ml (eight days after start of the rotifer experiment). Rotifers died out or did not grow in several wells (Supporting information) probably due to the low starting abundance of only two rotifers per well, which may have resulted in stochastic extinction events caused by accidentally choosing old females that were not able to reproduce anymore or were in a state shortly before age-related death.

The abundance of live rotifers after 11 days (= experiment day 34) was difficult to count due to high numbers of moving animals in 3 ml, and thus the number of individuals was likely underestimated. Therefore, the rotifers were fixed with Lugol's iodine solution after 12 days (= experiment day 35) before counting. The counts of the fixed rotifers may also include recently died individuals and thus may overestimate abundance of live individuals after 12 days somewhat. However, we believe that the counts of fixed individuals on day 35 give a more reliable estimate of rotifer abundance at the end of the experiment than the counts of live individuals on day 34. Moreover, the statistical outcomes were very similar regardless of whether fixed or live counts were used.

Statistical analyses

All statistical analyses and tests of their assumptions were performed using the statistical software R, ver. 4.0.2 (<www.r-project.org>). Data were visualized using the ggplot2 package. General linear models (*lm* function) were used to test the effects of medium and treatment on phytoplankton biomass on days 21 and 49, estimated by OD measurements. Tukey's HSD post hoc tests were used to assess differences between treatments within each media group. Post hoc tests were applied for each medium separately to readily identify predicted factorial limitation scenarios in phytoplankton (Fig. 1). For the analyses of rotifer abundance, we excluded wells in which the rotifers died out or did not grow (exclusion criterion: cumulative daily counts were less than the number of counting days multiplied by the starting density, i.e. cumulative counts $< 24 = 12\ d \times 2$ rotifers, Supporting information) to avoid introducing a potential bias likely caused by stochastic extinction events (above). Furthermore, there was no clear relationship between the number of excluded wells and media or treatment (Supporting information), suggesting no systematic effects of medium or treatment on mortality. Linear mixed-effects (LME) models were applied to test the fixed effects of phytoplankton growth medium and nutrient addition treatment on rotifer abundance counted at day 34 (alive) and at day 35 (Lugol-fixed) using the *lmer* function of the lme4 package (model 1). Phytoplankton replicate identity was used as random effect to account for potential non-treatment effects by using multiple rotifer wells per phytoplankton community replicate. p-values for ANOVA tables were computed using the *anova* function of the lmerTest package. Marginal and conditional R^2 were calculated using the *r2_nakagawa* function of the performance package to assess the variance explained by fixed effects and the entire model, respectively. Multiple comparisons between treatments within each media category were conducted using the *glht* function of the multcomp package with p-values adjusted according to Holm, using log-transformed abundance data to mitigate heteroscedasticity. We applied additional LME

models to investigate whether phytoplankton biomass and biovolume of particular phytoplankton species have a significant effect on rotifer abundance and how much variance these fixed variables explained. We added OD measured at the end of the rotifer experiment (day 35) as sole explanatory variable or together with the fixed factor medium to the models (model 2 and 3, respectively). We explored the role of biovolume of particular phytoplankton species with LME models. Because only green algae showed substantial treatment differences in the second phase after nutrient addition, we used the sum of biovolumes of the two green algae (*A. obliquus* and *M. minutum*) on day 49 as fixed variable in the model together with the fixed factor medium (model 4).

Results

Phytoplankton growth responses (factorial limitation scenarios) and community composition

Phytoplankton biomass increased in all three media until reaching stationary phase approximately on day 17 (Fig. 2). Shortly after the application of nutrient treatments on day 22, phytoplankton biomass increased further only in treatments with N and N&P addition. Biomass in control and P treatments did not increase further and remained more or less constant during the rest of the experiment (Fig. 2). At the end of the first phase (day 21), phytoplankton biomass was lower in the N&P-low medium compared to either the N-low or P-low media (Fig. 3a, Table 2) and phytoplankton communities differed somewhat in their species composition depending on the medium they grew on (Supporting information). Within each medium category, there were no significant differences in overall phytoplankton biomass at day 21 between microcosms that have been assigned afterwards to different nutrient addition treatments (Fig. 3a). Also, the biovolumes of the different phytoplankton species at the end of the first phase showed no significant differences between subsequently assigned nutrient treatments (Supporting information). These were excellent starting conditions for the second experimental phase with nutrient additions.

Phytoplankton biomass differed significantly among nutrient addition treatments at the end of the second phase (Fig. 3b, day 49). Additionally, phytoplankton communities showed different growth response patterns to nutrient addition in the three media (Fig. 3b), i.e. different factorial limitation scenarios, indicated by a significant interaction between medium and treatment (Table 2). Phytoplankton communities grown in N-low medium were solely limited by N (single limitation scenario), because P addition did not stimulate growth, N addition resulted in a substantial growth response, and the combined addition of N and P did not result in a larger growth response compared to the single N addition treatment (Fig. 3b, cf. Fig. 1). By contrast, the communities grown in P-low and N&P-low medium were primarily limited by N and secondarily by P (serial limitation scenario), because the combined addition of N and P resulted in larger

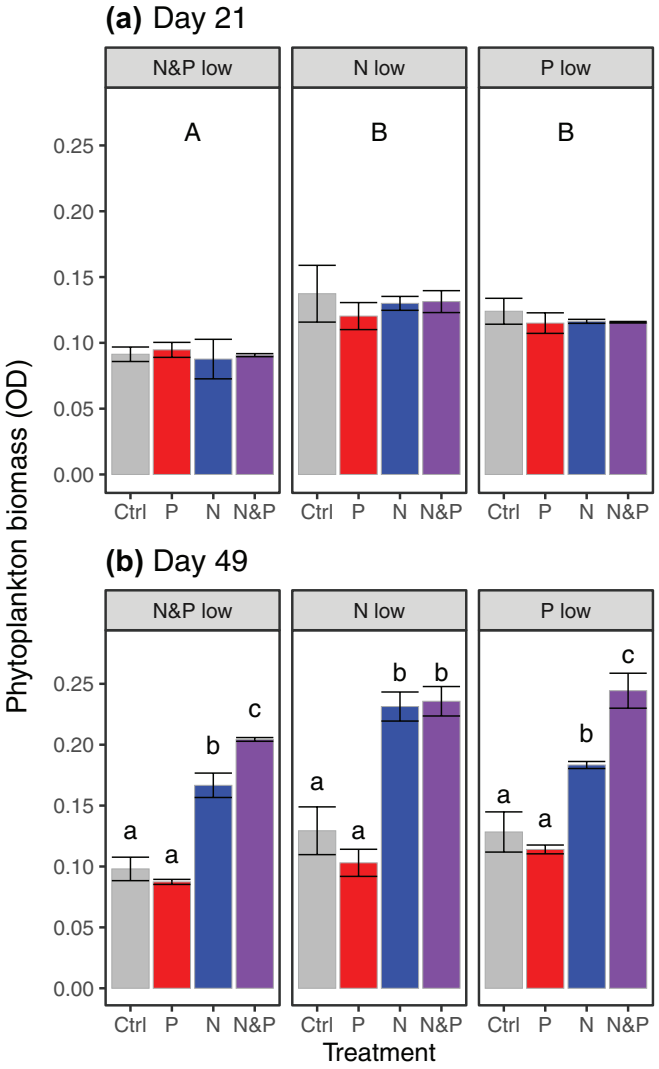

Figure 3. Phytoplankton biomass at the end of the (a) first phase (day 21) and (b) second phase (day 49) of the experiment. Biomass was estimated by measuring optical density (OD). In the first phase, phytoplankton was grown in medium with low N concentration (N low), low P concentration (P low) and low concentration of N and P (N&P low). On day 22, N, P, N and P, or nothing (Ctrl = control) has been added. Mean ± SD are shown (n = 3). In A, capital letters indicate significant differences between media (Tukey HSD post hoc tests, $p < 0.05$; there were no sign. differences between treatments after 21 days within each medium category). In B, small letters indicate significant differences between treatments within media categories to assess factorial limitation scenarios (Tukey HSD post hoc tests, $p < 0.05$, Supporting information; post hoc comparisons between media groups could not be performed due to a significant M × T interaction).

growth responses compared to sole N addition (Fig. 3b). Treatments with N and N&P addition were characterized by higher abundances of the green algae *M. minutum* and *A. obliquus* compared to control and P addition treatments at the end of the second phase, whereas all other phytoplankton species did not show substantial treatment differences (Fig. 4). Because of this, variability in OD on day 49 was

Table 2. ANOVA results of general linear models testing the effects of medium and treatment on phytoplankton biomass (estimated as optical density, OD) measured on day 21 (after first growth phase) and day 49 (after second growth phase).

		OD day 21		OD day 49	
	df	F	p	F	p
Medium (M)	2	50.45	< 0.001***	34.30	< 0.001***
Treatment (T)	3	1.05	0.388	262.74	< 0.001***
M × T	6	0.69	0.660	5.13	0.002**

explained to ~90% by biovolumes of the two green algae (i.e. sum of *A. obliquus* and *M. minutum*).

Rotifer growth patterns

The development of rotifer abundance showed very high variability, but generally increased with time depending on media type and treatment (Fig. 5). Average rotifer abundance increased similarly at the beginning across all treatments, with treatment differences starting to emerge after four days. Most treatments showed a transition towards reaching carrying

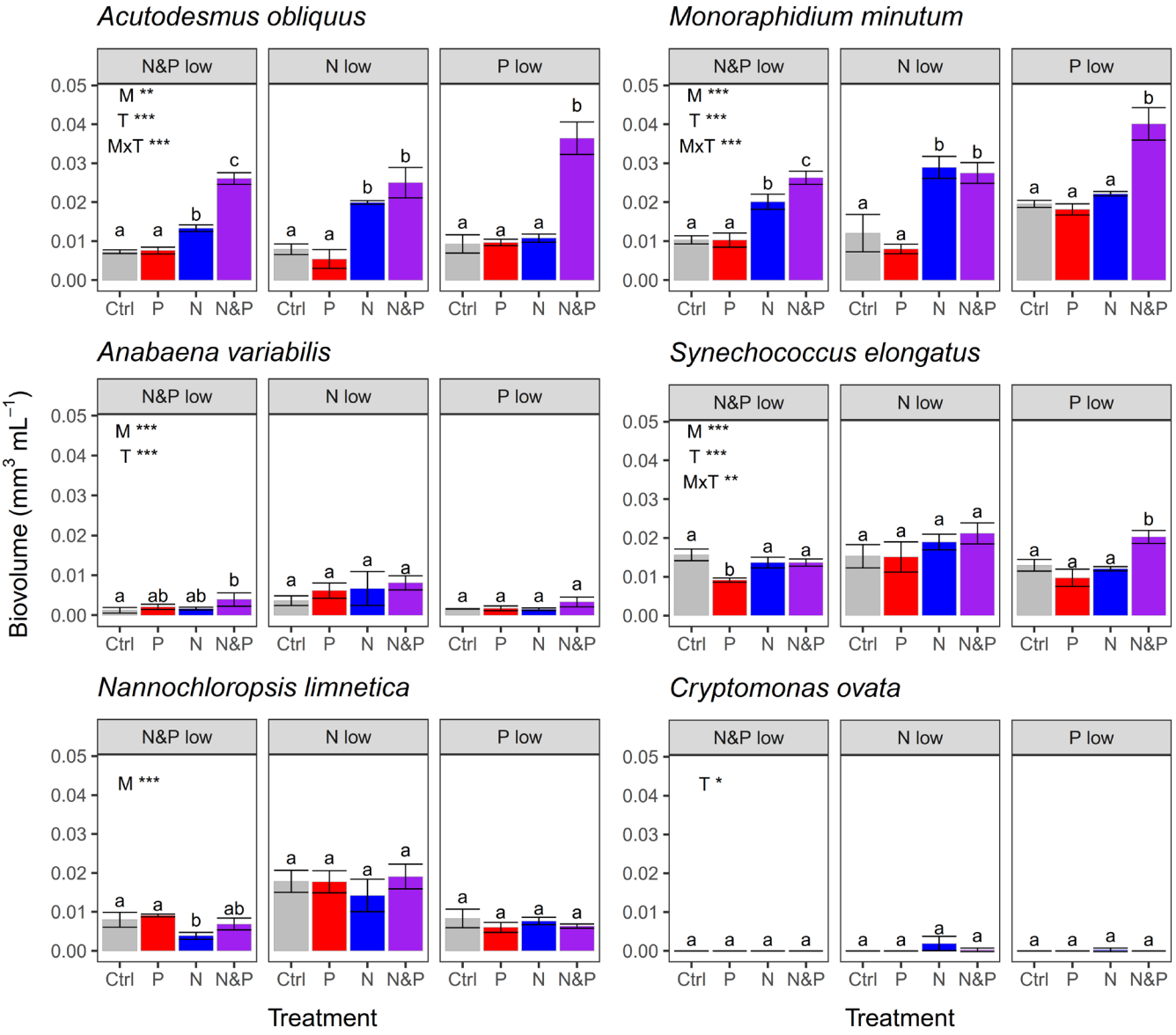

Figure 4. Biovolume of phytoplankton species at the end of the second experimental phase (day 49). Mean ± SD are shown (n = 3). After the first experimental phase (on day 22), N, P, N and P, or nothing (Ctrl = control) has been added to phytoplankton communities to establish different treatments (x-axes). Asterisks indicate significance levels (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) of ANOVA results for each phytoplankton species of a general linear model with the factors medium (M) and treatment (T), and their interaction (M × T) (non-significant terms are not shown). Small letters indicate significant differences between treatments within media categories (Tukey HSD post hoc tests, $p < 0.05$). Post hoc tests for testing differences between media were not performed due to significant M × T interactions for some phytoplankton species.

capacity, except for the N&P addition treatment in the P-low medium (Fig. 5). Counts of live rotifers after 11 days (Fig. 5, Supporting information) were lower than counts of fixed rotifers (Fig. 6), likely due to underestimating high numbers of moving individuals. Rotifer abundance at the end showed different response patterns among phytoplankton growth media, i.e. rotifer growth was differently affected by the treatment of nutrient addition to phytoplankton in the three media (Fig. 6, Supporting information). Under the N&P-low medium, rotifer abundance was similar in the control, P, and N addition treatments, and abundance in these treatments was much lower than in the N&P addition treatment (Fig. 6), which is according to expectations for strictly essential nutrients (Fig. 1). Under the N-low medium, abundances in the N and N&P addition treatments were similar to the control (Fig. 6), which is in contrast to expectations (Fig. 1), whereas the P addition treatment showed lower abundance as expected. Under the P-low medium, abundance in the N addition treatment was lower than in the control treatment as expected, whereas abundance tended to be higher in the P addition treatment and was much higher in the N&P addition treatment compared to the control (Fig. 6), which is according to expectations for interactively essential nutrients (Fig. 1).

Medium and treatment, applied as fixed effects in a mixed model, and their interaction explained a substantial part of the variance in the rotifer abundance data at the end of the experiment (47.3% on day 35, Table 3, model 1; 49.3% on day 34, Supporting information, model 1). Phytoplankton biomass at the end of the rotifer experiment explained only ~10% of the variance in rotifer abundance when considered as sole explanatory variable (Table 3, model 2; Supporting information, model 2) and only ~15% in a model including also medium (Table 3, model 3; Supporting information, model 3). Comparing the variance explained by model 1 and models 2 and 3 suggests that besides food quantity other factors, such as food quality aspects, played an important role. The inclusion of green algae (*M. minutum* and *A. obliquus*; moderate food quality, Table 1), which were the main driver of treatment differences in phytoplankton biomass at the end (Fig. 4), in a model with medium also explained a substantial part of the variance in rotifer abundance (26.6% on day 35, Table 3, model 4; 27.9% on day 34, Supporting information, model 4). However, the difference in explained variance by fixed effects between model 1 and model 4 (47.3% and 26.6%, respectively, Table 3) still suggests a role of other food quality aspects, such as stoichiometric imbalance.

Discussion

In the first experimental phase, we intended to create phytoplankton communities that were either limited by N, P or both nutrients simultaneously, by growing the communities in media of varying N and P concentrations resulting in substantially different N:P ratios. In the second experimental phase, factorial pulse additions of N and P revealed the type and severity of nutrient limitation. If limiting, N or P pulse additions stimulated a growth response of phytoplankton that 1) elevated food quantity for herbivores compared to the control that did not received nutrient additions and 2) increased stoichiometric food quality for consumers by shifting food C:nutrient ratios from limiting to more balanced levels. If not limiting, N or P pulse additions did not stimulate

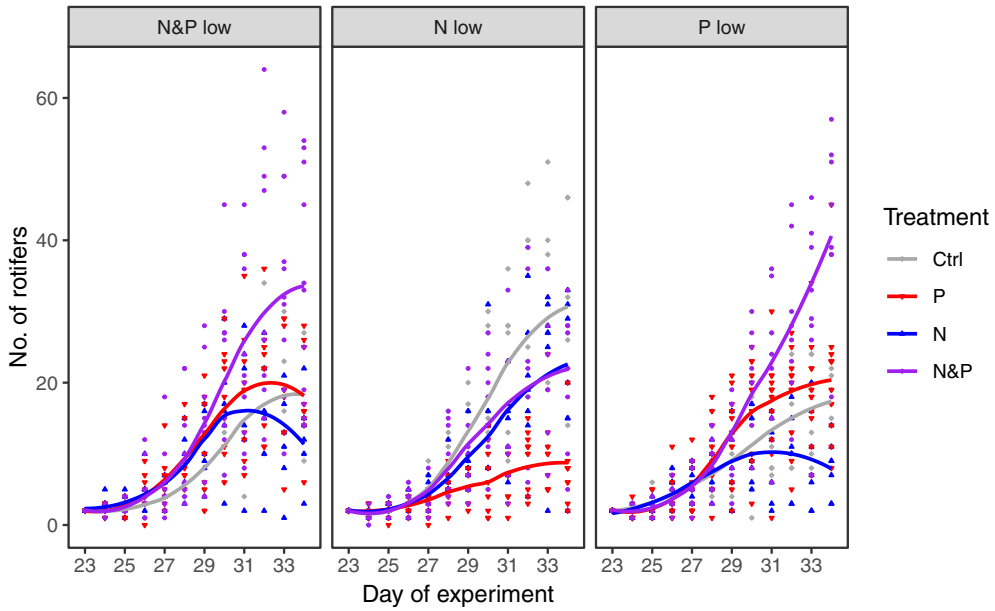

Figure 5. Development of rotifer abundance over time in the different nutrient addition treatments of each medium category. Counts of live *B. calyciflorus* from days 23 to 34 are shown. Lines indicate smoothed conditional means for each treatment based on local polynomial regression fitting.

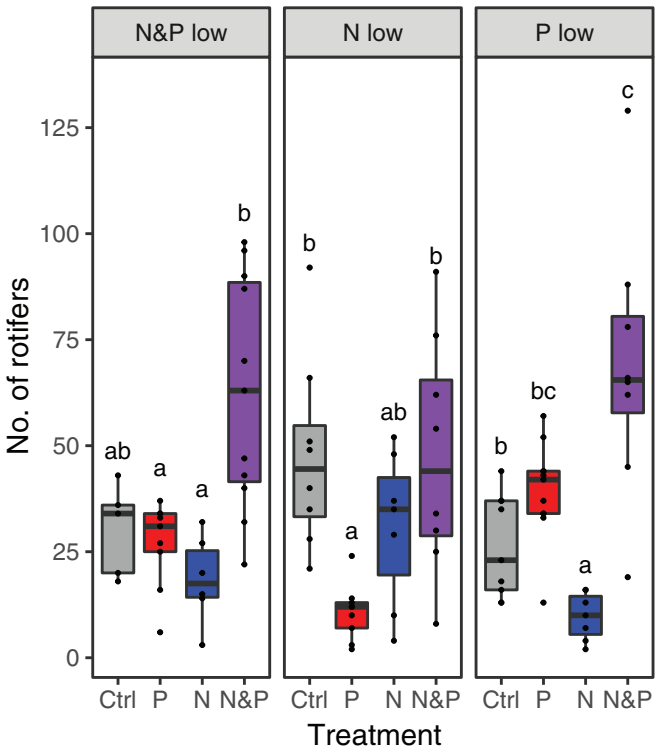

Figure 6. Rotifer abundance after 12 days of growth (i.e. on day 35). Rotifers were fixed on day 35 before counting. Letters indicate significant differences between treatments within media categories (Tukey contrasts on log-transformed data with p-values adjusted according to Holm, $p < 0.05$, Supporting information).

phytoplankton growth and likely reduced C:nutrient ratios to levels that were detrimental for herbivores due to stoichiometrically imbalanced food quality caused by excess nutrients in their food. The combined N and P addition treatment revealed whether the nutrients were singly or serially limiting phytoplankton growth, or whether they were co-limiting, and resulted generally in the highest herbivore growth.

Another aim of our study was to investigate whether changes in phytoplankton community structure, caused by the different nutrient supply regimes, had a food quality-mediated effect on herbivore growth. The nutrient pulse additions caused treatment-specific changes in the composition of phytoplankton species, which differed in their edibility and biochemical composition (cf. Table 1). However, these changes were not large enough to have a substantial effect on herbivore growth, because the abundance of cyanobacteria, which are of low quality due to lack of essential lipids and buildup of filaments, as well as the abundance of high food quality taxa, did not change substantially between treatments (Fig. 4). Instead, changes in phytoplankton community composition were mainly driven by changes in the biomass of green algae characterized as moderate food quality (Table 1). Because the responses of green algae were also the main driver of changes in the overall phytoplankton biomass after nutrient additions, the effect of green algae can be interpreted as mainly increasing the quantity of food in a quality that is sufficient for proper herbivore growth.

After nutrient additions in the second phase, phytoplankton biomass increased only when N or N and P were provided, indicating N as the primary limiting nutrient for the phytoplankton communities that established after the first experimental phase. This is partially in contrast to the general trend observed in freshwater systems, in which primary producer communities are mostly equally limited by both N and P (Elser et al. 2007). Our simultaneous addition of N and P produced a higher growth response than single nutrient addition in the N&P-low and P-low media, a frequent pattern observed in autotroph communities as shown by comprehensive meta-analyses (Elser et al. 2007, Allgeier et al. 2011, Harpole et al. 2011). Simultaneous co-limitation as well as single and serial limitation are common factorial limitation scenarios found for primary producer communities across ecosystems (Harpole et al. 2011). We predicted simultaneous or independent co-limitation for the N&P-low medium and single or serial limitation in the other two media deviating from the Redfield ratio (Fig. 1). For the N-low medium, we observed single N limitation of phytoplankton growth according to expectations. For P-low and N&P-low media, we observed serial limitation with N as primary and P as secondary limiting nutrient, because the addition of N, but not P, showed a positive response and the combined addition of N

Table 3. ANOVA (type III, Satterthwaite's method) results of linear mixed-effects models testing the effects of the fixed factors medium (M), treatment (T), phytoplankton biomass (OD, measured on day 35) and biovolume of green algae (BV_{GA}, sum of *M. minutum* and *A. obliquus* at day 49) on rotifer abundance counted on day 35 (fixed samples). Replicate identity was used as random effect in all models. Marginal R^2 (mar. R^2) indicates the variance explained by the fixed effects and conditional R^2 (con. R^2) indicates the variance explained by the entire model (including fixed and random effects).

Model		df_{Num}	F	p	mar. R^2	con. R^2	AIC
M1	M	2	0.08	0.922	0.473	0.548	800.9
	T	3	15.01	< 0.001***			
	M × T	6	3.59	0.013*			
M2	OD	1	6.24	0.018*	0.106	0.504	875.6
M3	M	2	0.33	0.721	0.151	0.541	844.9
	OD	1	8.25	0.008**			
	M × OD	2	0.73	0.490			
M4	M	2	0.72	0.496	0.266	0.537	831.5
	BV_{GA}	1	16.93	< 0.001***			
	M × BV_{GA}	2	0.92	0.408			

and P resulted in even higher growth. These growth patterns suggest N and P as interactively essential nutrients, but are in contrast to our expectations (Fig. 1). Serial limitation in the N&P-low medium, instead of the predicted simultaneous or independent co-limitation, can arise if the Redfield ratio is not corresponding to the balanced ratio for the used phytoplankton community. Serial limitation with N as the primary limiting nutrient in the P-low medium was unexpected, as we predicted that growth would be primarily limited by P. However, this contrasting outcome can be reconciled by theory if N limitation was severe, and thus the 'controls' of the factorial limitation scenarios of all media (white diamonds on surfaces in Fig. 1) started under nutrient ratios at which only N is strongly limiting.

At the end of the second experimental phase, nutrient additions affected community composition by mainly promoting green algae growth. While cyanobacteria and *N. limnetica* did not show a response to factorial nutrient additions, N supply in the N and N&P treatments supported high growth of the two green algae. P addition did not stimulate growth of any of the phytoplankton species. A similar outcome was observed by Frank et al. (2020), who noted that green algae in single cultures and communities were mostly N-limited and responded stronger to N than P addition. The species-specific responses in our communities showed that the overall response patterns to N and P addition were driven by the green algae.

The core part of this study was to assess the consequences of nutrient supplies to primary producers, which were limited by N or P or co-limited by both, on the growth of an herbivorous consumer. Phytoplankton community biomass towards the end of the second phase and final rotifer abundance did not show the same treatment response patterns within the same media category. Compared to phytoplankton, rotifer abundance did not increase primarily in the N addition treatments, but rather showed signs of decrease compared to the controls. Even though rotifer abundance was highest in N&P addition treatments in the N&P-low and P-low media, this was not the case in the N-low medium. These results indicate that primary production was not transferred with the same efficiency to secondary production in all treatments.

Carbon concentrations in all treatments (range 20–60 mg C l^{-1}, estimated from previously established OD-carbon-measurement regressions) were well above food levels limiting rotifer population growth (incipient limiting levels ~1–3 mg C l^{-1}, Stemberger and Gilbert 1985, Walz 1993). However, we did not measure phytoplankton biomass (i.e. OD) in the rotifer wells. Rotifers might have grazed down phytoplankton when they reached high abundances, and thus may have been partly food limited towards the end of the experiment, even though we compensated for this by providing food every two days. An indication of limiting food levels might be that rotifers approached carrying capacity towards the end (Fig. 5). However, also other factors might be responsible for this observation. In general, higher phytoplankton biomass did not always result in higher rotifer abundance, and overall biomass explained only a relatively small proportion of variance in the herbivore abundance data (Table 3). Therefore, food quantity, which is an important factor limiting rotifer growth (Rothhaupt 1990, Schälicke et al. 2019a), did not seem to be the only factor explaining treatment differences in rotifer abundance in our experiment.

Apart from food quantity, food quality aspects have likely influenced rotifer growth in our study. Biochemical food quality aspects in terms of sterol and PUFA content can be a key attribute affecting zooplankton performance (Müller-Navarra et al. 2000, Martin-Creuzburg et al. 2009, Wacker and Martin-Creuzburg 2012). For instance, dietary cholesterol supply positively affects population growth of rotifers along a food quantity gradient (Schälicke et al. 2019a). The communities we fed the rotifers were characterized by high amounts of green algae (moderate food quality) and *N. limnetica* (high food quality), and cyanobacteria (low food quality) did not become dominant during the second experimental phase. Therefore, biochemical food quality limitation of rotifer growth due to a lack of sterols, PUFAs or both seems unlikely. Furthermore, no nutrient addition treatment differences developed for the low and high food quality phytoplankton taxa, making biochemical food quality unlikely to explain treatment differences in rotifer growth. Poor edibility of phytoplankton caused by colony-forming and filamentous cyanobacteria is another aspect of food quality that can adversely affect zooplankton (de Bernardi and Giussani 1990, Wilson et al. 2006). For instance, the rotifer *B. calyciflorus* can ingest the cyanobacterium *Anabaena flos-aque* only at a moderate rate (Rothhaupt 1991). In our study, the only filamentous species, *A. variabilis*, had very low biomasses in the communities, especially during the second phase. Therefore, a food quality effect from poor edibility due to filamentous cyanobacteria seems unlikely to have affected rotifer abundance. Thus, other food quality aspects besides biochemical composition and edibility may have played a role in causing treatment differences of rotifer growth.

Stoichiometric imbalance in terms of autotroph C:N:P ratios is another food quality aspect affecting growth of herbivores (Sterner and Elser 2002, Hessen et al. 2013, Sperfeld et al. 2017). Imbalance in terms of both low and high C:nutrient ratios (i.e. nutrients in excess and deficiency, respectively) can reduce growth of herbivorous consumers (Boersma and Elser 2006, Bullejos et al. 2014, Elser et al. 2016, Zhou and Declerck 2019). For instance, it has been shown that a surplus supply of elemental nutrients to autotrophs negatively affects the growth of consumers (Boersma and Elser 2006, Elser et al. 2016, Zhou and Declerck 2019). In our experiment, we also observed inhibiting effects on rotifer growth in treatments where P was added to phytoplankton grown in the N-low medium and where N was added to phytoplankton grown in the P-low medium. We did not measure elemental composition of the phytoplankton used to feed rotifers. Nevertheless, we can suppose that for N-low and P-low media in P and N addition treatments, respectively, phytoplankton biomass might have had very low C:P and C:N ratios. These elemental food ratios could have been very imbalanced in terms of nutrient excess, and in this way caused inhibition of rotifer population growth. This is

supported by growth response curves of *B. calyciflorus* measured along a food C:P gradient (Zhou and Declerck 2019). Phytoplankton that is not P limited, as it is the case in the N-low medium, show typically C:P ratios in the range of 100–150, ratios where *B. calyciflorus* shows its highest growth rates (Zhou and Declerck 2019). Adding P to the N-low medium will reduce C:P ratios to levels that inhibit rotifer growth by excess P (Zhou and Declerck 2019). P addition to phytoplankton grown in P-low media and N addition at N-low media did not inhibit rotifer growth compared to controls. Instead, a slight increase in rotifer abundance could be observed in the P addition treatment of the P-low medium. This is also supported by the 'knife-edge' response of *B. calyciflorus* measured along the food C:P gradient, in that high C:P ratios of P limited phytoplankton will be reduced by P addition to levels that allow increased rotifer growth (Zhou and Declerck 2019).

For the N&P-low medium, a negative food quality effect due to nutrient surplus seems unlikely, because nutrient supply to phytoplankton was more balanced in the first growth phase. Moreover, rotifer abundance of the single nutrient addition treatments in the N&P-low medium was low and similar to the control treatment, whereas it was much higher in the N&P addition treatment than in the single or no nutrient addition treatments (Fig. 6). At the beginning, rotifer growth in this treatment probably benefitted by the nutrient spike of N and P, and later by the increased quantity of green algae. This is also in agreement with previous studies, showing that both low food quantity as well as N- and P-limited food resulted in reduced growth rates of rotifers (Jensen and Verschoor 2004, Lürling 2006, Strojsova et al. 2009, Schälicke et al. 2019a), and that nutrient, especially P, spikes to nutrient limited algae increased rotifer growth (Jensen and Verschoor 2004, Wojewodzic et al. 2011, Zhou et al. 2018, Zhou and Declerck 2020).

To conclude, we showed that the response of nutrient limited primary producer communities to factorial pulse additions of N and P did not translate one-to-one to the herbivore level, probably caused by a complex interplay between effects of food quantity and different aspects of food quality. In particular, we found that N and P additions to nutrient limited phytoplankton was only beneficial for zooplankton when increased food quantity is stoichiometrically balanced and at least of moderate biochemical quality. Furthermore, supply of a non-limiting nutrient can lead to stoichiometrically imbalanced food in terms of nutrient excess, which negatively affected the herbivore's growth. In the context of increasing nutrient loads to freshwaters, and the efforts to either limit or cut down such inputs, it is necessary to assess impacts of eutrophication and re-oligotrophication across trophic levels as well as in the whole ecosystem. With our study, we demonstrated the multifaceted effects of nutrient additions to freshwater autotrophs on herbivores, and that there is a need to better understand how its consequences vary not only between these two basal trophic levels but also in the whole food web or in different ecosystems.

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Author contributions

Andrea Redoglio: Formal analysis (supporting); Investigation (supporting); Validation (equal); Visualization (equal); Writing – original draft (equal); Writing – review and editing (equal). **Kassandra Radtke**: Conceptualization (supporting); Data curation (lead); Formal analysis (equal); Investigation (lead); Methodology (lead); Validation (equal); Writing – original draft (supporting). **Erik Sperfeld**: Conceptualization (lead); Data curation (supporting); Formal analysis (equal); Funding acquisition (lead); Investigation (supporting); Methodology (supporting); Project administration (lead); Resources (lead); Supervision (lead); Validation (equal); Visualization (equal); Writing – original draft (equal); Writing – review and editing (equal).

Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.dz08kps0p> (Redoglio et al. 2022).

Supporting information

The Supporting information associated with this article is available with the online version.

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