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Identity of the limiting nutrient (N vs. P) affects the competitive success of mixotrophs

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ABSTRACT: Empirical and theoretical evidence predicts that mixotrophic bacterivores dominate over specialized heterotrophic bacterivores and specialist photoautotrophs under conditions of high light and low loss rates. Here we extend this concept towards nutrient limitation and ask whether the identity of the limiting nutrient affects the competition of mixotrophs with their specialist competitors. Due to their photosynthetic machinery, mixotrophs should have higher cellular N contents than heterotrophs and, following this assumption, a higher demand for N. Conversely, heterotrophs, with their potential high growth rates compared to mixotrophs, may have a higher demand for P ('growth rate hypothesis'). Simplified, mixotrophs should be more prone to N-limitation, while heterotrophs should be more prone to P-limitation. We tested these predictions in artificial food webs studying the competitive success of mixotrophic bacterivores under a range of light intensities and loss rates and under either P- or N-limitation. Under low-light conditions, mixotrophs were more successful than heterotrophs under P-limitation, whereas the heterotrophs were more successful under N-limitation. At higher light intensity, mixotrophs had an advantage over photoautotrophs, due to the acquisition of nutrients ingested with prey. Overall, the effects of the limiting nutrient on the competitive success of mixotrophs were stronger under conditions already unfavorable for mixotrophs (low light). Further, our results suggest that communities dominated by mixotrophs might have low and relatively stable seston C:nutrient ratios. The results presented here supplement existing data well and help to define the ecological niche of mixotrophic protists.

KEY WORDS: Mixotrophy · Bacterivory · Nutrient limitation · Light · Loss rates · Competition · Microbial food webs

INTRODUCTION

In the traditional view, protist plankton is divided into phytoplankton and zooplankton. Phytoplankton (photoautotrophs) use light and inorganic nutrients for growth, while zooplankton (heterotrophs) obtain energy and nutrients from ingested prey. Mixotrophic protists combine these modes of nutrition in one organism. Several studies have reported that mixotrophic activity — in this context phagotrophy in organisms otherwise seen as primarily phototrophic — is driven or enhanced by P-limitation and/or N-limitation (Jones 1997, 2000, Stoecker 1998, Li et al. 2000, Smalley et al. 2003). However, the limitation through P and N does not always promote phagotrophy in mixotrophs (Skovgaard et al. 2003).

The flexible mode of nutrition of mixotrophs is linked to trade-offs and may be advantageous only under certain conditions. Mixotrophs need to synthesize and maintain cellular structures for photosynthesis and phagotrophy (photosynthetic organelles, enzyme systems for the assimilation of inorganic nutrients, and a feeding apparatus). Raven (1995) estimated that the photosynthetic apparatus can ac-

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count for up to 50% of the metabolic costs (energy and nutrients) of a cell, while the phagotrophic apparatus only accounts for up to 10% of the metabolic costs of a cell. Clearly, the costs for a protist with both a photosynthetic and a phagotrophic apparatus are greater than for a protist with either apparatus alone. Based on this consideration, Raven (1997) suggested that mixotrophs should have lower maximum growth rates than specialist photoautotrophs and heterotrophs. Further, mass balance constraints suggest that mixotrophs have lower affinities for dissolved nutrients than photoautotrophic algae (Flynn & Mitra 2009). Nevertheless, their prevalence in aquatic systems shows that mixotrophs are able to compete with specialist competitors under conditions of nutrient limitation (Nygaard & Tobiesen 1993, Rothhaupt 1996a, Flynn & Mitra 2009). Several recent studies have pointed out that mixotrophs may be increasingly dominant in situations of nutrient limitation (Caron et al. 1993, Christaki et al. 1998, Unrein et al. 2007, Zubkov & Tarran 2008, Frias-Lopez et al. 2009, Stukel et al. 2011, Hartmann et al. 2012). That is, their contribution to bacterivory is assumed to be high under high-light/low-nutrient conditions.

The supply rates of light and limiting nutrients affect C:nutrient ratios of algal biomass. High light: nutrient supply may result in phytoplankton with high C:nutrient ratios. This may result in the paradoxical situation where zooplankton 'starve' because food supply is high, yet quality is low ('light:nutrient hypothesis': Sterner et al. 1997, Sterner & Elser 2002). In contrast, a low light:nutrient ratio tends to cause overall low biomass with low photoautotroph C:nutrient ratios. Enhanced food quality may offset the negative effect of reduced food quantity.

In lakes and oceans, the C:nutrient ratio of the seston is a mixed signal of a multispecies community. Light-induced variations in sestonic C:nutrient ratios are based on the high plasticity of phototroph stoichiometry (cellular concentrations and ratios of C, N, and P) in response to the availability of light and nutrients. The actual flexibility of particular algal taxa is obviously species-dependent. Especially green algae and diatoms are known to exhibit an extreme variation in cellular C:nutrient ratios (see Andersen 1997, Sterner & Elser 2002, and references therein). The high stoichiometric plasticity of phototrophs can affect several ecosystem processes, such as secondary production and nutrient cycling.

While the rationale for the light:nutrient hypothesis is intuitive and well supported from experiments with monospecific algal cultures (see Sterner & Elser 2002 and references therein), some field studies and experiments (Sterner & Elser 2002, Hessen 2006) revealed variable and sometimes relatively weak effects of light and nutrients on phytoplankton stoichiometry.

Katechakis et al. (2005) proposed that mixotrophic protists might counterbalance extreme C:nutrient (P) ratios under high light:nutrient conditions. The presence of mixotrophs in natural communities therefore might buffer against extreme C:nutrient ratios at the community level. The authors based their hypothesis mainly on the assumption that, due to the flexible mode of nutrition of mixotrophs, their stoichiometric composition is less affected by light and dissolved nutrients than the stoichiometry of photoautotrophic specialists. Further, they argued that potentially limiting nutrients are several orders of magnitude more concentrated in the biomass of food organisms of mixotrophs than in the dissolved phase (Vadstein 2000). By ingestion of P-rich heterotrophic bacteria, mixotrophs may circumvent nutrient limitation under high light:nutrient conditions (Caron et al. 1993).

The relatively constant structural stoichiometry of the cells' 'functional machinery' determines the nutrient requirements of an organism (Sterner & Elser 2002, Klausmeier et al. 2004). Rhee & Gotham (1980) showed that the structural N:P ratio is species-specific. Mixotrophic and heterotrophic bacterivores probably show differences in their stoichiometric composition, due to the photosynthetic machinery of mixotrophs. Unfortunately, no systematic survey is available. Photosynthesis is an N-intensive process; this is especially due to the high involvement of N in CO_2 fixation (mainly RUBISCO), in light harvesting (chlorophyll and other pigments), and in biosynthesis (ribosomes). Consequently, mixotrophs (and obviously photoautotrophs) can be expected to have higher cellular N than heterotrophs and, following this assumption, a higher demand for N. Conversely, heterotrophs, with their potentially high growth rates (Weisse & Scheffel-Möser 1991, Eccleston-Parry & Leadbeater 1994) may have a higher demand for P. This is explained by the high amounts of (P-rich) RNA necessary to meet the demands of rapid protein synthesis in fast-growing organisms ('growth rate hypothesis'; Sterner & Elser 2002). Based on these considerations, it is conceivable that the relative contribution of mixotrophs and heterotrophs to community composition differs between N- and P-limited systems.

Fischer et al. (2016) proposed a concept which predicts that the relative success of mixotrophic versus heterotrophic bacterivores can be predicted by considering light intensity and loss rates. They showed that mixotrophic bacterivores are favored under high-

light/low-loss conditions, while heterotrophic bacterivores are favored under low-light/high-loss conditions. Here we extend their concept by considering different limiting nutrients (N- vs. P-limitation). We performed experiments where we studied the competitive success of mixotrophic bacterivores in artificial food webs, exposed to a factorial manipulation of light intensities, dilution (loss rates), and nutrient ratios (N- or P-limitation). The food webs consisted of a mixotrophic flagellate (*Ochromonas minima*), a heterotrophic flagellate (*Cafeteria* sp.), a photoautotrophic green alga (*Dunaliella* sp.), a single-celled pico-cyanobacterium (*Synechococcus* sp.), and heterotrophic bacteria. To answer the question whether the identity of the limiting nutrient (N or P) affects the competition of mixotrophs and their specialist competitors, we specifically tested the following hypotheses.

H_1: Under high-light conditions, neither mixotrophs nor photoautotrophs are energy-limited. While growth of photoautotrophs in high light is limited by the availability of inorganic nutrients, mixotrophs may take up nutrients through phagotrophy. Hence, the success of mixotrophs under different limiting nutrients depends on the effect of the respective limiting nutrient on the stoichiometric composition, i.e. the 'food quality' of their prey. Growth of bacterioplankton (including cyanobacteria) in the euphotic zone of oligotrophic waters is predominately P-limited (Vaulot et al. 1996, Sala et al. 2002), which directly affects their stoichiometric composition and hence their quality as prey for bacterivores. Therefore, we expect that mixotrophs in high light are less successful under P-limitation, as compared to N-limitation, due to lower quality of their prey.

Moreover, this should result in higher standing stocks of the prey under P-limitation relative to N-limitation. C:P ratios of P-limited bacteria can be assumed to be around 178:1, while the C:N ratios of N-limited bacteria may be around 7.5:1 (Vrede et al. 2002). The C:P ratio of P-limited bacteria diverges more strongly from the algal Redfield ratio (106:1) than does the C:N ratio of N-limited bacteria (6.6:1). Assuming that cellular stoichiometry of mixotrophs is similar to that of photoautotrophic algae, mixotrophs need to ingest more bacteria under P-limitation than under N-limitation in order to saturate their demand for the limiting nutrient. Given the above ratios, we can calculate the ratios of specific N in algae to specific N in bacteria (6.6:7.5 = 0.88) and specific P in algae to specific P in bacteria (106:178 = 0.60). Comparing both ratios (0.88:0.60) we can estimate that biomass of bacteria under P-limitation should be higher by a factor of about 1.5.

H_2: The cellular chlorophyll content depends on light availability (light acclimation). The requirements for N should generally increase with decreasing light. Therefore, at limiting light intensities, mixotrophs should be more prone to N-limitation than heterotrophic bacterivores. Conversely, heterotrophic consumers should be more prone to P-limitation due to the relatively high P demand necessary to meet rapid rates of biomass growth and development ('growth rate hypothesis)'.

H_3: The competitive advantage of heterotrophs over mixotrophs under conditions of increased loss rates/top-down control, as suggested by Fischer et al. (2016), is diminished when heterotrophic growth is limited due to P-limitation.

In the experiment, we manipulated dilution rate to create a gradient of loss rates. By this approach, we ensured equal loss rates for all organisms and avoided feedback loops that would have been introduced by manipulating grazer densities. By manipulating the dilution rate, we also manipulated the nutrient supply rate, while total nutrients were unaffected by dilution. Hence, the dilution gradient can be compared to a gradient of increasing top-down control by ('microzooplankton') grazers, with a parallel increase in remineralized nutrients. For reasons of comprehensibility, here we simply use the term 'loss rates' when we refer to the effects of the dilution rate.

MATERIALS AND METHODS

Microalgae and culture medium

The artificial food web consisted of 3 flagellates and 1 cyanobacterial species (plus heterotrophic bacteria, see below). The mixotrophic species used in the experiment was *Ochromonas minima* Throndsen 1969 (obtained from the Norwegian Institute for Water Research [NIVA], Norway). *O. minima* is able to ingest bacteria as well as photosynthetic picoplankton. However, *O. minima*, unlike some other *Ochromonas* strains (Rothhaupt 1996b, Sanders et al. 2001, Wilken et al. 2013), is unable to grow in the dark, even if particulate food is available (Flöder et al. 2006, our own preliminary experiments, data not shown). *Cafeteria* sp. (strain RCC 1077, Roscoff Culture Collection, France) was the heterotrophic bacterivore and competitor of the mixotroph for particulate food in the food web. *Synechococcus* sp. (strain RCC 744, Roscoff Culture Collection, France) was added as a prey organism for both the mixotroph and the heterotroph. To expose the mixotroph to simulta-

neous competition with specialized heterotrophic and photoautotrophic protists, the food web also contained a photoautotrophic green flagellate, *Dunaliella* sp. (obtained from the Alfred-Wegener-Institut [AWI] Helgoland, Germany).

Stock cultures were non-axenic and contained unidentified heterotrophic bacteria; hence, bacteria of unknown composition were also present in the experimental food webs and served as potential prey for the heterotroph and the mixotroph. No additional C source was added to the medium in the experiment. Exudates from algae therefore served as the main energy source for heterotrophic bacteria (Bratbak & Thingstad 1985).

Prior to the experiment, all organisms were precultivated for several weeks. Algae were grown in a walk-in environmental chamber at a constant temperature of 18°C and a light:dark cycle of 12:12 h. Light was supplied in a non-limiting irradiance of ca. 100 µmol m^{-2} s^{-1}. The medium used for cultivation was the same as used in the experiment, a modified f/2-Si seawater medium (Guillard & Ryther 1962, Guillard 1975), based on aged seawater (more than 8 wk), with nutrient concentrations adjusted to an N:P ratio of about 1 (N: ~32 µmol l^{-1}, P: ~36 µmol l^{-1}) for the N-limited treatments or about 500 (N: ~1000 µmol l^{-1}, P: ~2 µmol l^{-1}) for the P-limited treatments. Hence, the non-limiting nutrient was available in excess. *Cafeteria* sp. stock cultures were kept in culture with food bacteria and an added barley grain to sustain bacterial growth in the same medium. Stock cultures were kept under exponential growth by frequent dilution (dilution rate: ~0.1 d^{-1}).

With the exception of heterotrophic bacteria, abundances of all organisms were converted into biovolume prior to data analysis. For the calculation of biovolume, the dimensions of 30 or more cells of each species were measured at 1000× magnification under a light microscope in samples fixed with glutaraldehyde (final concentration 1%). Biovolume was then approximated using conversion factors for corresponding geometrical bodies of each respective species (Hillebrand et al. 1999).

Experimental design and set-up

The experiment was carried out in a walk-in environmental chamber at a constant temperature of 18°C and a light:dark cycle of 12:12 h using a continuous culture method.

In a 3-factorial design, we manipulated the supply of light, the dilution rate, and the nutrient limitation (N-limitation, N:P ~1; and P-limitation, N:P ~500). We applied 3 light levels, 10, 35, and 60 µmol m^{-2} s^{-1}, and 2 dilution rates, 0.1 and 0.3 d^{-1}. Each of the 12 resulting treatments was replicated twice.

For the light levels, 4 wooden incubation boxes (width × height × depth: 125 × 36 × 28 cm) were used, and 6 culture flasks were placed into each box. Three boxes were used for the 3 light levels and the fourth box was partitioned with dividing walls into 3 compartments for the remaining 6 culture flasks, 2 flasks for each light level. Tubing was connected through slots in the top of each box. Light was adjusted by mounting a neutral-density filter to the open side of each box. For the illumination, fluorescent lamps (Lumilux Duo EL-F/R 2×36 W HF, Osram; length: 123.5 cm), equipped with 1 warm white fluorescent tube (Lumilux L 36 W, Osram) and 1 fluorescent tube designed for promoting photo-biological processes (Fluora L 36 W, Osram), were mounted at about mid-height of the culture flasks at the back side of the incubation box, resulting in an even horizontal illumination of all 6 culture flasks per box. As light intensity declines towards the outer ends of a fluorescent tube, we first measured the light intensity along the tube, and placed the culture flasks in each box in an arc in front of the tube such that all flasks of each light level received the same respective light intensity. The light intensity was measured with an LI-250A Light Meter equipped with a LI-190A Quantum Sensor (LI-COR®). The temperature within the incubation boxes was the same as in the walk-in environmental chamber in which the experiment was conducted.

We applied the 'exponentially fed batch' culture method (Fischer et al. 2014), which works like a chemostat without continuous outflow. To ensure a constant specific dilution rate, fresh medium is constantly supplied proportional to the current culture volume. Consequently, both the culture volume and the rate at which medium is added to the culture vessel must increase exponentially with time. At each sampling, the culture volume is set back to the initial volume. This provides the opportunity to take a fairly large sampling volume, relative to the culture volume, at regular intervals, while working with relatively small culture volumes. For details on the materials used and the set-up, see Fischer et al. (2014). The cultures were not aerated by bubbling; instead, mixing was ensured by gently shaking the flasks by hand, several times every day. In addition, the culture volume was mixed at each sampling by gently pumping up and down with a 10 ml pipette. For sampling, all tubing was disconnected from the cul-

ture flasks, and the open ends were kept in sterile 100 ml Erlenmeyer flasks, while the samples were then taken under a laminar flow hood. In order not to expose the low-light treatments to a high-light pulse during sampling, all sampling was conducted under light intensities <10 µmol m^{-2} s^{-1}.

The experimental cultures were started at low abundances (P-limitation treatments: *O. minima* ~480 cells ml^{-1}, *Cafeteria* sp. ~3000 cells ml^{-1}, *Dunaliella* sp. ~710 cells ml^{-1}, and *Synechococcus* sp. ~63 000 cells ml^{-1}; bacteria ~2 800 000 cells ml^{-1}; N-limitation treatments: *O. minima* ~500 cells ml^{-1}, *Cafeteria* sp. ~3500 cells ml^{-1}, *Dunaliella* sp. ~810 cells ml^{-1}, and *Synechococcus* sp. ~67 000 cells ml^{-1}; bacteria ~2 800 000 cells ml^{-1}). Note that differences in the starting abundances between the 2 limitations were caused by different abundances of the respective organisms in the pre-cultivated stock cultures for both media. The experiment was run for 18 d, with samples taken every other day.

To assess if the cultures were in steady state, at each sampling, the fluorescence, in terms of chlorophyll *a* (chl *a*) concentration, of each culture was measured with a fluorometer (TD-700 Turner Designs). The experiment was terminated after the chl *a* signal stabilized for several days (data not shown).

At one sampling, tubing of 2 flasks was permuted, resulting in 2 high-light/high-dilution flasks receiving the wrong medium for 24 h (light 60 µmol m^{-2} s^{-1}, dilution 0.3, N-limitation, replicate b; and light 60 µmol m^{-2} s^{-1}, dilution 0.3, P-limitation, replicate a). These 2 units clearly diverged from their corresponding replicates and were excluded from the statistical analysis.

Flow cytometrical analysis

Numbers of photoauto-, hetero-, and mixotrophic microalgae were determined by flow cytometry using the cell analyzer and sorter ARIAIII (Beckton Dickinson) equipped with 4 air-cooled fiber-launched solid-state lasers (excitations: UV 375 nm, blue 488 nm, yellow-green 561 nm, red 633 nm). Subsamples were fixed with glutaraldehyde (final concentration 1%) and subsequently stored at 4°C until further analysis (maximal 3 d). All cytometrical analyses were done following exactly the same protocol, keeping all settings constant. All samples were analyzed regarding planktonic autofluorescence using the cytometer configuration for the 70 µm nozzle. Populations of the different organisms were centered in the channels by adjusting the photomultiplier voltages. All parameters were collected as logarithmic signals. In order to remove background noise and to enhance processing speed, a threshold for the Texas-Red channel was set at 200. Analyses were performed at a flow rate of 3.4 µl m^{-1}, and the event rate ranged between 300 and 2500 events s^{-1}, while the data collection was done for 3 min. Samples with a higher event rate were diluted with sterile 1× phosphate-buffered saline.

Manual gating was used to detect populations of *O. minima*, *Dunaliella* sp. (detection: 695 ± 40 nm vs. side scatter, 585 ± 42 nm, 616 ± 23 nm, 660 ± 20 nm, respectively, and 585 ± 42 nm vs. 660 ± 20 nm), *Synechococcus* sp. (detection: 585 ± 42 nm vs. 660 ± 20 nm), and *Cafeteria* sp. (detected by scattered and specific diffracted light: detection: 530 ± 30 nm vs. 695 ± 40 nm, 670 ± 14 nm vs. side scatter) after visual inspection of dot plots from different channels and gating as a histogram plot.

Absolute volume calculation and intercalibration were done for each analysis day using TruCount beads (Beckton Dickinson) following Giebel et al. (2009). Multicolored latex beads (1 µm diameter, Polysciences) served as an internal standard for the volume normalization of counted events. Bead doublets and triplets were neglected during the analyses. Details were described by del Giorgio et al. (1996) and Gasol & del Giorgio (2000). Operation of the ARIAIII flow cytometer and data acquisition and analysis (batch mode) were done using FACS DIVA Software 6.1.3.

Subsamples for bacterial cell numbers were fixed with glutaraldehyde (final concentration 1%) and subsequently stored at −20°C until further analysis. Bacteria were stained with the intercalating DNA dye Sybr Green I (SGI, Invitrogen). SGI working solution was prepared fresh every day by diluting the stock solution (concentration 10 000×) 1:50 with dimethyl sulfoxide (Sigma). Ten µl of the SGI working solution were added to the sample at a ratio of 1:22. After 30 min of incubation in the dark, the samples were analyzed by flow cytometry as mentioned above. Analyses were performed at a flow rate of 3.4 µl min^{-1} with a set-up for the 70 µm nozzle. Event range was between 100 and 1500 s^{-1}. Green fluorescence was detected with the standard filter set-up (emission 530 ± 15 nm) as FITC-Sybr channel with a threshold of 200. Bacteria were detected using manual gating after visual inspection of the dot plot of green fluorescence (FITC-Sybr) vs. right angle side scatter (SSC-H) and its histogram plot of FITC-Sybr, respectively. Data collection and analyses corre-

sponded to the approach applied as mentioned above for autofluorescence set-up.

Nutrient analysis

For particulate nutrient samples, a defined volume (60 ml) per sample was filtered on pre-combusted Whatman GF/F filters. Separate filters were prepared for C and N vs. P. Prior to measurements, C, N, and P filters were dried for at least 48 h in a drying chamber at 60°C and stored until analysis. Particulate C and N was measured with a CHN analyzer (Flash EA 1112; Thermo Fisher). Particulate phosphate was measured as orthophosphate by a molybdate reaction after sulfuric acid digestion (Wetzel & Likens 2000).

Statistical analysis

To illustrate the temporal development of the communities, we fitted smooth terms over the ln-transformed temporal data of each species using generalized additive models (package 'mgcv' in R; Wood 2008). By fitting the time trends to daily intervals, we could approximate net growth rates for all groups. As the flagellates did not experience grazing, we estimated their respective gross growth rates by adding the corresponding dilution rate to the growth rates.

To assess the relative competitive success of the mixotroph, heterotroph, and photoautotroph, we calculated the fraction of mixotrophic biovolume to total bacterivore biovolume (mixotroph:[mixotroph + heterotroph]) and the fraction of mixotrophic biovolume to total pigmented protist biovolume (mixotroph:[mixotroph + photoautotroph]).

While all 3 light levels were considered in all statistical analyses, throughout the text we distinguish only between low and high light, as the results for both higher light intensities (35 and 60 µmol m^{-2} s^{-1}) were overall very similar.

All statistical analyses were done using R (version 2.11.1; R Core Team 2014).

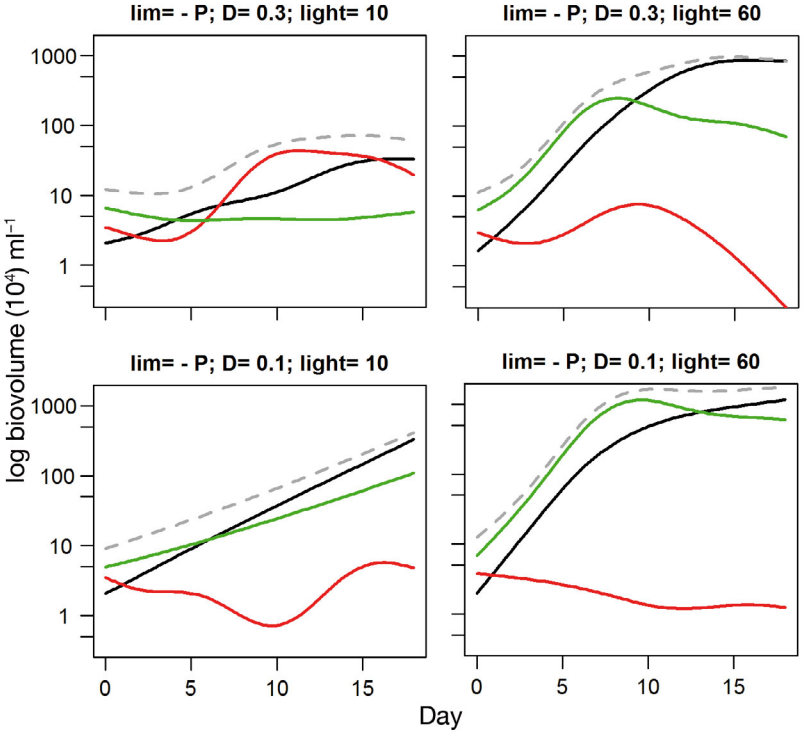

Fig. 1. Abundances of functional protist groups over time for the 4 'extreme' combinations of dilution rates (D; 0.1 or 0.3 d^{-1}) and light intensity (10 or 60 µmol m^{-2} s^{-1}), under P-limitation (smoothed fit; calculated average of the replicates). Mixotrophic flagellate/*Ochromonas minima*: black; heterotrophic flagellate/*Cafeteria* sp.: red; photoautotrophic flagellate/*Dunaliella* sp.: green; total protist biovolume: grey

RESULTS

Development over time

Total community biovolume increased over time in all treatments. Total biomass generally increased with light intensity (Figs. 1 & 2; see also Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m563p051_supp.pdf). Biovolume saturated earlier in the N-limited treatments and with increasing light intensity. Overall, the increase was slower in the high-dilution treatments (Fig. S1).

In the low-light/low-dilution treatments, abundances of *Cafeteria* sp. only increased under N-limitation (Fig. S2). After a first increase, the subsequent decrease in the abundance of *Cafeteria* sp. coincided with a drop in prey abundances (Figs. S2–S4). The mixotroph *Ochromonas minima* was the only protist that increased over the entire period in all treatments (Fig. S5), eventually exceeding biovolume of the heterotroph *Cafeteria* sp. in all but the low-light/high-dilution treatment under N-limitation. The increase in the abun-

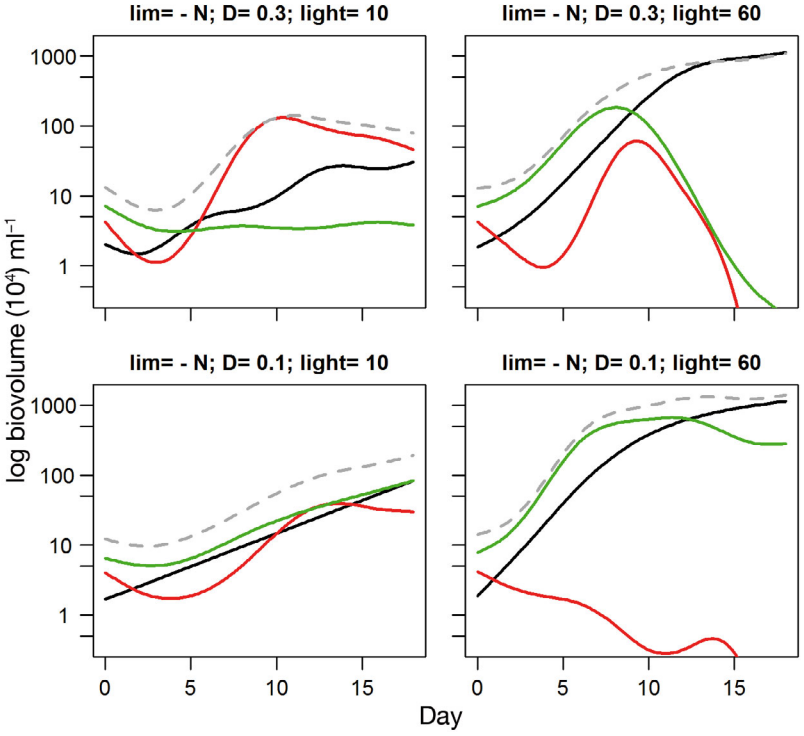

Fig. 2. Abundances of functional protist groups over time for the 4 'extreme' combinations of dilution rates (D; 0.1 or 0.3 d^{-1}) and light intensity (10 or 60 µmol m^{-2} s^{-1}), under N-limitation (smoothed fit; calculated average of the replicates). Mixotrophic flagellate/*Ochromonas minima:* black; heterotrophic flagellate/*Cafeteria* sp.: red; photoautotrophic flagellate/*Dunaliella* sp.: green; total protist biovolume: grey

dance of *O. minima* was slower under N-limitation, and this was most pronounced in the low-light treatments (Fig. S5). The strong increase in the abundance of *Cafeteria* sp. in the early period of the experiment (in the high-dilution treatments) illustrated the potential high growth rates of heterotrophic nanoflagellates as compared to the mixotroph (Table S1 in the Supplement). The increase in biovolume of *Dunaliella* sp. was slower under N-limitation. An initial increase in biovolume in the high-dilution/N-limitation treatments was followed by a decrease in biovolume, which was faster with increasing light intensity (Fig. S6).

Relative success of the different functional groups depending on light, dilution, and identity of the limiting nutrient

The experiment did not last long enough to allow the communities to fully equilibrate. Nevertheless, we assumed that the trends visible towards the end of the experiments reflected the trajectories if the experiment had run longer, and correspond to a 'quasi steady state.' At this time, the community chl *a* signal had equilibrated. To compare the relative success of the different functional groups for the given experimental conditions, we calculated average abundances for the last 3 sampling days for both nutrient regimes (Fig. 3).

Under both nutrient limitations, abundances of the mixotroph *O. minima* were positively related to light, while dilution had no significant effect (Table S2). In the low-light/low-dilution treatments, the abundances of *O. minima* were lower in the N-limited treatments (Fig. 3C).

Final abundances of *Cafeteria* sp. were independent of light and dilution in the N-limited treatments. Under P-limitation, dilution had a positive effect on the abundances of *Cafeteria* sp., whereas the combination of light and dilution had a negative effect. In the high-light treatments, *Cafeteria* sp. had lower abundances under N-limitation, while under P-limitation the abundances were lower in the low-light treatments (Table S2). Abundances of the shared prey for *O. minima* and *Cafeteria* sp., i.e. heterotrophic bacteria and *Synechococcus* sp., were highest in the low-light treatments. In these treatments, both prey groups had higher abundances under N-limitation, which was more pronounced for *Synechococcus* sp. Under both limitations, *Synechococcus* sp. abundances were significantly negatively related to light. Conversely, light had no significant effect on the abundances of heterotrophic bacteria. Abundances of the photoautotroph *Dunaliella* sp. were positively related to light, while under N-limitation the combination of light and dilution had a negative effect on its abundance (Table S2).

Relative contribution of the mixotroph to total bacterivores and total pigmented protists

In the high-light treatments, the mixotroph made up the highest proportion of total bacterivores independent of the dilution rate ($F_{1,12}$ = 1.28, p = 0.281), with only a slightly, but still significantly, higher proportion under N-limitation ($F_{1,12}$ = 29.2, p < 0.001). In the low-light treatments, the proportions of the mixo-

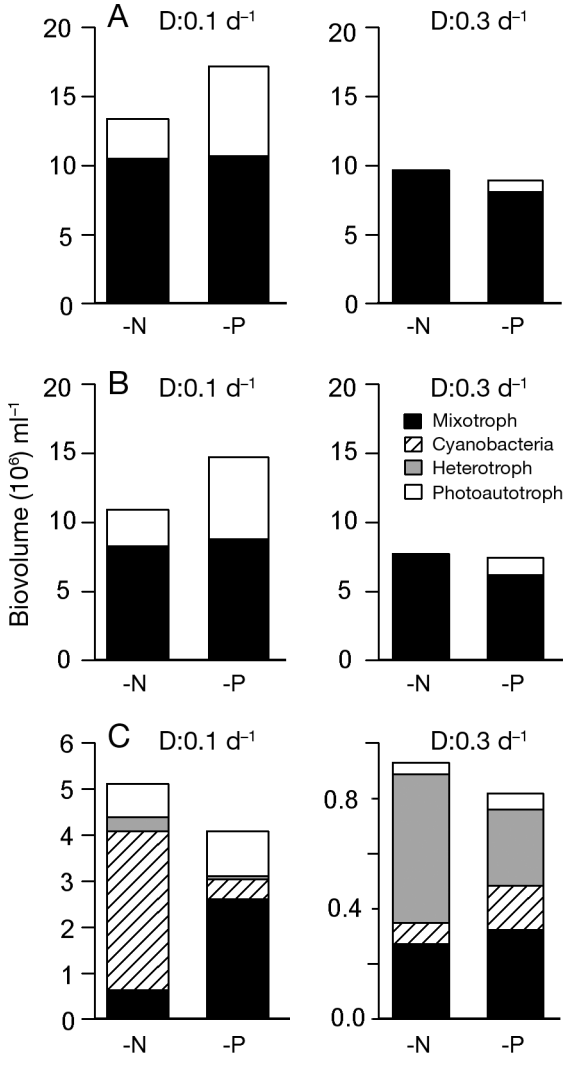

Fig. 3. Abundances of the different species in the food web, excluding heterotrophic bacteria, at the end of the experiment (averages of both replicates and of the last 3 sampling days, Days 14–18) depending on light intensity: (A) 60 µmol m^{-2} s^{-1}, (B) 35 µmol m^{-2} s^{-1}, (C) 10 µmol m^{-2} s^{-1}, and dilution rate (D; 0.1 or 0.3 d^{-1}; note the differences in axis scales)

troph to total bacterivores was lower and was significantly lower under N-limitation compared to P-limitation (low dilution: $F_{1,2}$ = 105.3, p = 0.0094; high dilution: $F_{1,2}$ = 41.5, p = 0.0232). This was more pronounced in the low-dilution treatments. Only in the high-dilution/N-limitation treatment was the heterotroph superior in terms of achieving a higher biovolume than the mixotroph (Fig. 4).

The proportion of mixotrophs to total pigmented protists in the high-light treatments was higher under N-limitation ($F_{1,12}$ = 4.1, p = 0.0653). Compared to the high-dilution treatments, the proportion of the mixotroph to total pigmented protists in the low-dilution treatments was lower ($F_{1,6}$ = 17.03, p = 0.0014). In the low-light/low-dilution treatments, the proportion of the mixotroph to total pigmented protists was lower under N-limitation than P-limitation ($F_{1,2}$ = 29.03, p = 0.0328), while in the high-dilution treatments, the proportion of total pigmented protists was independent of the limiting nutrient ($F_{1,2}$ = 0.18, p = 0.711; Fig. 4).

Seston stoichiometry

In agreement with basic considerations of ecological stoichiometry, seston C:N ratios significantly increased with increasing light under N-limitation, while seston C:P ratios significantly increased with increasing light under P-limitation (Fig. S1 in the Supplement). Across treatments, seston C:P ratios were relatively low. The C:P ratio was lowest in the low-light/high-dilution treatment under N-limitation, where the heterotrophic flagellate *Cafeteria* sp. peaked in abundance relative to the other organisms (Fig. 4). Seston C:N ratio was higher and C:P ratio was lower in the low-dilution treatments, where the phototroph persisted, compared to the high-dilution treatments, where the mixotroph almost completely outcompeted all other protists (Table 1). Under P-limitation in the high-light treatments, the seston C:P ratio was lower at higher dilution, where the mixotroph made up a higher proportion of total pigmented protists as compared to the low-dilution treatments, while seston C:N ratios were about even at both dilution rates.

DISCUSSION

In accordance with expectations (Tittel et al. 2003, Fischer et al. 2016, Ptacnik et al. 2016), the mixotroph was more successful in high-light compared to low-light conditions. However, in contrast to predictions and the results of Fischer et al. (2016), the relative success of the mixotroph increased with loss (dilution) rate. This might indicate that there is a critical loss rate above which the specialist competitors of mixotrophs should be able to outcompete the mixotrophs, due to their relatively higher maximal growth rates. Thus, the high dilution we applied in this study might not have been high enough for the specialist competitors of the mixotroph to play out their advantages under these conditions. Hence, we argue that our results are not in conflict with our general predications regarding the combined effect of light and loss rates on the competitive success of mixotrophs

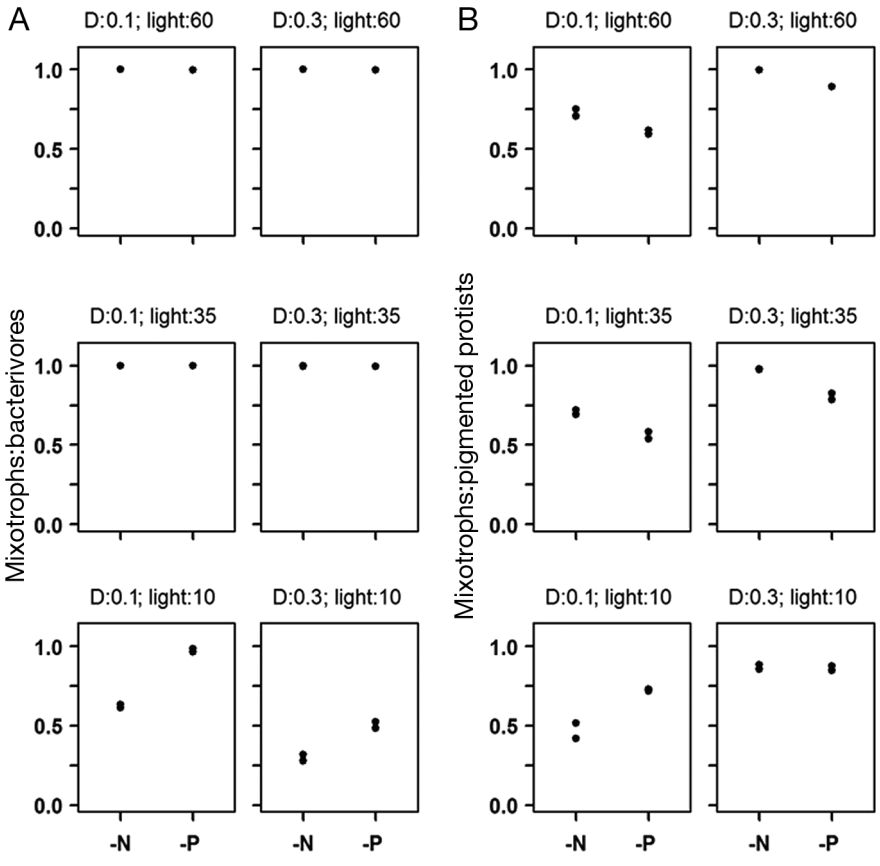

Fig. 4. Contribution of mixotrophs to (A) total bacterivores and (B) total pigmented protists at the end of the experiment (averages of both replicates over the last 3 sampling days, Days 14–18) depending on light intensity (10, 35, or 60 µmol m^{-2} s^{-1}) and dilution rate (D; 0.1 or 0.3 d^{-1})

Table 1. Seston stoichiometry (molar) at the end of the experiment for each treatment (average values of the replicates and of the last 3 sampling dates ± SD)

Light intensity (µmol m^{-2} s^{-1})	Dilution rate (d^{-1})	Limitation	C:N	C:P	N:P
10	0.1	N	7.9 ± 0.2	28.9 ± 0.9	3.7 ± 0.2
35	0.1	N	17.0 ± 0.4	31.5 ± 2.7	1.9 ± 0.1
60	0.1	N	17.5 ± 0.3	29.8 ± 3.6	1.7 ± 0.2
10	0.3	N	7.1 ± 0.2	25.3 ± 1.6	3.6 ± 0.2
35	0.3	N	11.3 ± 1.1	43.1 ± 4.9	3.8 ± 0.3
60	0.3	N	12.8 ± 0.9	42.1 ± 2.8	3.3 ± 0.1
10	0.1	P	7.1 ± 0.2	52.1 ± 22.3	7.3 ± 3.1
35	0.1	P	9.2 ± 0.5	230.5 ± 11.7	25.2 ± 1.7
60	0.1	P	9.0 ± 0.5	252.6 ± 29.9	27.9 ± 2.2
10	0.3	P	7.0 ± 0.4	53.7 ± 4.9	7.7 ± 0.7
35	0.3	P	9.7 ± 0.4	147.1 ± 14.9	15.1 ± 1.0
60	0.3	P	10.6 ± 0.3	152.9 ± 13.1	14.5 ± 0.9

(Fischer et al. 2016, where a wider gradient in loss rates was applied).

The mixotrophs under high-light conditions were relatively more successful under N-limitation, due to higher growth efficiency on 'N-starved' prey. This might indicate that the stoichiometric composition of the prey was more suitable for the growth requirements of the mixotroph under N-limitation compared to P-limitation. Under low light, where the mixotroph can be assumed to be energy-limited, the mixotroph was less successful under N-limitation, likely due to a higher investment in N-rich pigments and therefore higher requirements for N under these conditions.

We manipulated dilution rates in our systems in order to create a non-selective gradient of loss rates. In natural systems, loss rates experienced by protists depend on many factors. By affecting organism abundances, nutrient levels play an obvious role for top-down control, as encounter rates of prey by grazers and parasites depend on the abundances of each. Implications of enrichment for top-down control have been addressed in numerous studies (e.g. Murdoch & McCauley 1985, McCauley et al. 1988). In agreement with our assumptions, enhanced herbivory selects for fast-growing species or grazing-resistant forms (Sarnelle 1992).

Our experiment did not last long enough to allow the communities to fully equilibrate. We cannot exclude the possibility that the communities would not have equilibrated at all. Hence, our results and conclusions must be interpreted with some care. Nonetheless, the abundances at the end of the experiment are consistent among replicates and seem to represent the trajectories of the

experimental systems under the given experimental conditions. Especially for *Ochromonas minima*, final abundances match well with the ongoing rates of change towards the end of the experiment. In other words, while we cannot make statements whether a certain taxon would eventually have disappeared, we feel certain that the dynamics seen towards the end of the experiment reflect the success of a given species and thereby indicate the outcome if the experiment were performed longer. Moreover, the focus of our study was not on stable co-existence, but on competitive abilities.

High light

In high light, the mixotroph outcompeted the heterotroph for prey independent of the limiting nutrient, which is in accordance with general assumptions regarding the competition between mixotrophs and heterotrophs in dependence of light (Tittel et al. 2003, Fischer et al. 2016). It is worthwhile to note that, in the competition between the mixotroph and the heterotroph, the heterotroph was outcompeted faster under N-limitation than under P-limitation (where it persisted at very low abundances, but probably would have completely disappeared with longer duration of the experiment). This is explained by the lower bacterial abundances under N-limitation as compared to P-limitation, when the system reached a ('quasi') steady state (Fig. S1 in the Supplement). In high-light conditions, heterotroph abundances were too low (<1% of total bacterivore biovolume) to affect the abundances of the prey organisms. Hence, here the mixotroph controlled prey abundances. However, the mixotroph had lower relative abundances under P-limitation, indicating lower growth efficiency of the mixotroph with 'P-starved' prey and eventually resulting in a higher standing stock of bacterial prey at ('quasi') steady state, by a factor of 2.5 at low dilution and 2.1 at high dilution. These numbers are even higher than the factor (1.5) presented in H_1. However, the calculation of the factor is quite generic and does not consider other factors, such as compositional changes in the bacterial community and prey selectivity. Indeed, the differences in final standing stock between the different prey organisms depending on the limiting nutrient (Figs. S3 & S4) might indicate selective grazing of the mixotroph on prey of different nutritional quality.

Conversely, the photoautotroph was relatively more successful under P-limitation than under N-limitation. In accordance with our stoichiometric considerations (H_1), the greater success of the mixotroph under N-limitation is matched by lower bacterial abundances under N-limitation. In our systems, mixotrophic and heterotrophic flagellates acted as top consumers. Assuming quasi-steady state, the reduced predator:prey ratio (mixotroph:bacteria) in P-limited conditions points at reduced prey conversion efficiency. Higher conversion efficiency with regard to N has been observed in the mixotroph *O. danica*, when grown on either N- or P-limited bacterial prey (Grover & Chrzanowski 2009). For a high light:nutrient environment, a subtropical coral reef (Jeju Island, Korea), Jeong et al. (2012) showed that the free-living *Symbiodinium* sp. (an important symbiotic dinoflagellate of zooxanthellate corals) ingested algal prey to sustain growth under N-depleted conditions, which tend to prevail in coral habitats. The authors further demonstrated that ingestion rates of *Symbiodinium* sp. were significantly higher under N-depleted conditions, as compared to P-depleted or nutrient-enriched conditions, and concluded that N-depletion stimulates feeding by *Symbiodinium* sp. on algal cells. Moreover, Jeong et al. (2012) reported that *Symbiodinium* sp. is highly efficient at retaining N from ingested prey, and is able to remove substantial portions of *Synechococcus* populations from coral reef waters.

The success of the mixotroph under N-limitation was pronounced in the high dilution treatment, where the mixotroph almost completely outcompeted the photoautotroph. At first glance, this is in contrast to assumptions, as the photoautotroph should generally be favored in the light at a higher nutrient supply rate (high dilution), due to its higher growth rate. However, it is conceivable that the loss rate might not have been high enough for the photoautotroph to play out its advantages over the mixotroph, as illustrated by the relatively similar maximal growth rates of the mixotroph and photoautotroph under these conditions (Table S1).

Low light

O. minima cannot compensate lack of light by phagotrophic nutrition (Flöder et al. 2006, R. Fischer unpubl. data). In agreement with this, the lower maximum growth rates indicate energy limitation at low light (Table S1). We therefore expected overall stronger success of the heterotroph at low light (Fischer et al. 2016) as well as a stronger signal with respect to the identity of the limiting nutrient (H_2). As predicted, the heterotroph reached lower relative

abundances under P-limitation, and the mixotroph reached lower relative abundances under N-limitation. Under low-light conditions, photoautotrophic organisms have a higher investment in pigments in order to increase their efficiencies in light (energy) utilization (light adaptation) (e.g. Richardson et al. 1983). Hence, low light should result in a relatively higher demand for N in photoautotrophs and mixotrophs, as pigments, as well as enzymes (e.g. RUBISO) associated with photosynthesis are rich in N. In line with this, in our experiment the energy-limited mixotroph is more affected by N-limitation compared to P-limitation. In a study of the plankton of a humic lake (Lake Öträsket, northern Sweden; an environment generally characterized by a poor light climate), Jansson et al. (1996) found mixotrophs to be N-limited, illustrated by the strong growth response of mixotrophs after N addition. The relatively higher success of the photoautotroph under N-limitation at low loss rates is likely to be explained by the generally more efficient photosystems of the photoautotroph compared to the mixotroph. As under high-light conditions, the mixotroph was relatively more successful at higher dilution (i.e. elevated nutrient supply rate).

The heterotroph was relatively more successful in the high-dilution treatments, independent of the limiting nutrient, illustrating the competitive edge of the heterotroph over the mixotroph at higher loss rates (Fischer et al. 2016).

Seston stoichiometry

The lowest C:P ratio coincided with peak abundances of the heterotrophic flagellate *Cafeteria* sp. relative to the other organisms. This matches our assumption that heterotrophs generally have a higher cellular P content to meet rapid rates of biomass growth and development. This is supported by a mesocosm study of Calbet et al. (2012), in which the authors investigated an oligotrophic plankton food web in eastern Mediterranean waters. While the sestonic C:P ratios they found ranged between ~94 and ~135, the lowest C:P ratios coincided with the peak in total heterotrophic biomass, pointing at the potentially high P content of heterotrophs.

The relatively higher seston C:N ratio (N-limitation) in the high-light/low-dilution treatment, as compared to the high-light/high-dilution treatment, might be explained by the proportionally higher abundances of the photoautotroph, due to its likely higher cellular C:N ratio.

The low seston C:N and C:P ratios under low-light conditions and the increase in seston C:N and C:P ratios with increasing light, depending on the respective limiting nutrient, are in line with the predictions of the light:nutrient hypothesis. However, absolute seston C:N and C:P ratios are lower than values described for photoautotrophic species or communities dominated by photoautotrophs (Sterner et al. 1997, Sterner & Elser 2002). This indicates that mixotrophic protists might counterbalance extreme C:nutrient ratios under high light:nutrient conditions, which is in accordance with the findings of Katechakis et al. (2005). In their work, they found remarkably stable C:P ratios between 100 and 300. However, the C:P ratios we found represent the whole community, including heterotrophic bacteria, not just a single species. In a study of 2 ultraoligotrophic Andean lakes, Modenutti & Balseiro (2002) estimated sestonic C:P ratios based on the light:nutrient model of Sterner et al. (1997). In both lakes, protist plankton was dominated by ciliates and nanoflagellates, where ciliates were dominated by the mixotroph *Ophrydium naumanni*, and nanoflagellates were dominated by mixotrophic species such as *Chrysochromulina parva, Rhodomonas lacustris*, or *Ochromonas* spp. For the first lake, Lake Moreno, they estimated a sestonic C:P ratio of 435 ± 42 (mean ± SD) and for the second lake, Lake Rivadavia, a sestonic C:P ratio of 316 ± 24. The higher sestonic C:P ratio of Lake Moreno might be explained by the higher proportion of the ciliate *O. naumanni*, as this pelagic ciliate has endosymbiotic *Chlorella* and therefore might be considered more like a photoautotrophic alga in terms of cellular stoichiometry.

In conclusion, communities dominated by mixotrophs, despite having an overall lower productivity, might show reduced and less variable seston C:nutrient ratios, as compared to communities dominated by specialist photoautotrophs. Notably, of all species in the food web, the mixotroph showed the lowest temporal variations in biovolume depending on P- or N-limitation, respectively (Fig. 5), which points to more balanced nutrition due to the utilization of nutrients ingested with their prey (bacteria and cyanobacteria; Jeong et al. 2010), independent of the nutrient limitation and by its overall low growth rate. Therefore, mixotrophs might enhance food quality for higher trophic levels in terms of stoichiometric composition. Our data further suggest that the identity of the limiting nutrient has direct implications on the balance between bacteria and their consumers. Systematic comparisons among provinces with prevailing N- and P-limitation are scarce.

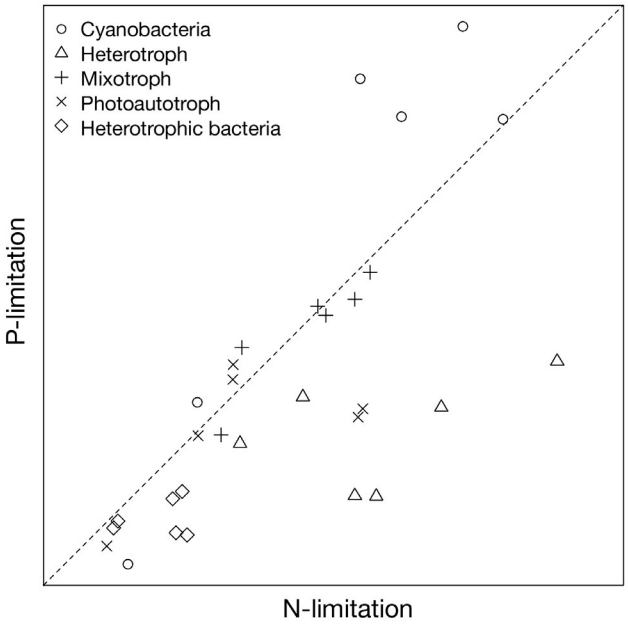

Fig. 5. Temporal variations in biovolume for each functional group under N- and P-limitation. The diagonal dashed line indicates identical variation in both situations

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