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Stoichiometric constraints on phytoplankton resource use efficiency in monocultures and mixtures

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Abstract

A central concept for understanding the mechanisms linking diversity and primary production or more general, ecosystem functioning, is resource use efficiency (RUE). It quantifies the amount of biomass production over time relative to unit resource supplied, that is, represents a quota of matter use efficiency. Given anthropogenic alterations of biogeochemical cycles, the consequent changes in supply rate and especially supply ratio of nutrients will change. Using four species of freshwater phytoplankton, and their mixture, we asked how the RUE for nitrogen and phosphorus depends on the stoichiometry of resource supply and how this differs between single species and their mixture. We conducted a factorial laboratory experiment spanning 25 different nutrient supply treatments with differing absolute and relative nitrogen (N) and phosphorus (P) concentrations. N and P supply increased biomass production and decreased C : nutrient ratios and RUE for the respective nutrient, but always significantly affected by the supply of the respective other nutrient. Biomass peaked at molar N : P supply ratios above the Redfield ratio (18–22). Species tended to respond similarly to the resource gradients. Consequently, mixtures outperformed the component species only during early growth responses, but not regarding maximum biomass and RUE. Bioassays performed at the end of the main experiment revealed predominance of N-limitation, but again strongly depending on the interaction between both nutrient gradients. Our study suggests that stoichiometric constraints of resource incorporation and RUE need to be accounted for when studying the response of phytoplankton to natural and anthropogenic variation in resource availability.

The limitation of primary production is often discussed in the framework of Liebig's law of the minimum, making productivity dependent on the resource being in least supply compared to demand. For communities, Liebig's law of the minimum can explain part of the observed limitations scenarios (about 15% of the studies in Harpole et al. 2011 showed single limitation); however, a series of meta-analyses and experiments suggest that primary production on the level of communities is frequently limited by more than one nutrient or resource (Arrigo 2005; Elser et al. 2007; Harpole et al. 2011). In general, colimitation can occur on all organizational levels from intracellular processes up to communities. At the organismal level, biochemical processes can be actively and passively colimited by nutrients affecting each other, regulating each other's uptake rate, being equally essential for physiological processes or molecules competing for restricted membrane space (Saito et al. 2008; Bonachela et al. 2015; Thrane et al. 2017). Within populations, individuals at different growth stages can display different optimal resource preferences and limitation may vary with genotype (Klausmeier et al. 2004*a*; Ptacnik et al. 2010*b*; Hillebrand et al. 2013; Bonachela et al. 2015; Thrane et al. 2017). At the community level, colimitation can occur, for example, when species show trade-offs in their resource demand and therefore incorporation of individual populations is limited by individual resources (Danger et al. 2008).

How efficiently limiting nutrients are taken up and used for production, especially in context with biodiversity, has recently been assessed in terms of resource use efficiency (RUE), both in field situations (Ptacnik et al. 2008; Filstrup et al. 2014; Verbeek et al. 2018; Hodapp et al. 2019) and experimental resource gradients (Cardinale et al. 2009*a*; Hillebrand and Lehmpfuhl 2011). RUE has been defined as the production realized per unit of the

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limiting resource (Chapin 1997; Nijs and Impens 2000). It thereby normalizes realized production to potential production, which is set by the amount of resources available (Cardinale et al. 2009*a*). RUE has been found to increase with increasing diversity of the autotroph community, if species differ in their resource needs, as complementarity reduces the proportion of resources remaining unused (Ptacnik et al. 2008).

Bringing RUE into a multiple resource limitation perspective, metacommunity models (Gross and Cardinale 2007; Hodapp et al. 2016) and experiments (Gamfeldt and Hillebrand 2011; Hillebrand and Lehmpfuhl 2011; Gülzow et al. 2019) have shown that the stoichiometry of resource supply alters the efficiency of using available resources.

In aquatic systems, changes in the balance of resource ratios occur either naturally depending on the species present and their differences in uptake preferences and nutrient recycling (Sterner 1990; Elser and Urabe 1999; Evans-White and Lamberti 2006; Danger et al. 2007; Plum et al. 2015), or due anthropogenic changes in relative nutrient availability (e.g., Elser et al. 2009) with variable effects over spatiotemporal scales (Elser et al. 2009; Beusen et al. 2016; Greaver et al. 2016; Yan et al. 2016).

In systems, with highly unbalanced resource ratios, the superabundant resource is inefficiently transferred into biomass production—or even not be incorporated, if uptake depends on the resource in short supply (Hodapp et al. 2019).

The aim of this study is to investigate how RUE is affected by nonindependent interactions between multiple resources and if RUE is a useful measure in a multiple resource field. In order to analyze stoichiometric constraints of RUE more systematically, we conducted a factorial laboratory experiment, spanning a range of absolute and relative nitrogen (N) and phosphorus (P) concentrations, two of the most commonly limiting elements for primary production (Elser et al. 2007). We used a full factorial design of 5 N × 5 P concentrations, resulting in 25 different supply points, and analyzed biomass production, RUE and cellular stoichiometry for four algal species as well as their mixture. Additionally, we conducted bioassays once the stationary phase was reached to test whether the realized limitation of primary production changed in accordance to the relative supply of both nutrients. These experiments allowed testing core hypotheses on nutrient incorporation, RUE, and biomass production as well as effects of poly- over monocultures:

H1: Algal stoichiometry, biomass production, and RUE reflect absolute and relative nutrient supply. Concisely, we expect that increasing one nutrient decreases RUE and algal C : nutrient ratio, but increases biomass production for this nutrient respectively (H1a). Furthermore, C : nutrient ratio, biomass, and RUE will be constrained by the absolute and relative supply of the other potentially colimiting element, yielding significant interaction terms between N and P supply (H1b).

H2: Mixtures differ from the monocultures they are composed of in terms of nutrient incorporation, biomass production, and RUE. We expect higher RUE in the mixture than in the monocultures due to complementarity effects (H2a), and RUE to scale with realized diversity within the mixture (H2b). Realized diversity in turn is affected by the quantity and ratio of resource supply (H2c).

H3: Testing N and P limitation separately via bioassay, we predict that responses reflect (1) the relative nutrient demand of the species and (2) the stoichiometric constraints of growth. Therefore, we expect the growth response to adding one nutrient to decline with the prior availability of this nutrient, but to increase with the prior availability of the potentially colimiting nutrient (H3a). Consequently, we expect the effect of N addition to increase with increasing C : N and decreasing internal N : P ratios in the algae, and the effects of P addition to increase with increasing internal N : P and C : P (H3b).

Methods

Phytoplankton species used

As algal phyla strongly differ in their pigment composition, using a wide range of algae would bear the risk that what appears to be differences in nutrient use actually reflects complementarity in light use (Striebel et al. 2009). Therefore, we restricted our selection of algae to a single phylum, Chlorophyta, with similar pigment composition. The four species chosen were *Ankistrodesmus* sp. (ANK, SAG 47.80, now *Monoraphidium contortum*), *Chlamydomonas reinhardtii* (CHLA, SAG 11–31), *Scenedesmus obliquus* (SCE, SAG 276–10, now *Acutodesmus obliquus*), and *Staurastrum* sp. (STAU, SAG 7.94). We obtained cultures from the culture collection of Algae at the University of Göttingen, Germany (SAG). Algae were pregrown in cell culture flasks for months, using sterile WC medium (Guillard and Lorenzen 1972) which was replaced every 2 weeks prior to the experiment, at 18°C with a 12:12 h light : dark cycle and 40.45 ± 15.00 μmol photons s^{-1} m^{-2}.

General experimental design

To test hypotheses 1 and 2, we conducted a 40 d laboratory factorial experiment in 250 mL culture flasks (T-75 with filter cap, Sarstedt, Germany) containing 150 mL of medium each. The medium was based on sterile WC medium (Guillard and Lorenzen 1972); however, we reduced the concentrations of N and P compared to the full medium. The supplied concentrations were 10.3, 20.6, 31.0, 41.3, and 51.6 μmol N L^{-1} and 0.6, 1.3, 1.9, 2.6, and 3.2 μmol P L^{-1}, yielding 25 possible combinations of absolute and relative N : P supply (Table 1). Later on we used the term "supply" for the nutrient concentrations that were initially supplied. Nutrient levels for N and P range from oligotrophic to eutrophic conditions, reflecting the observed availability of nutrients in freshwater ecosystems (Guildford and Hecky 2000). For all other nutrients, we used the concentrations of the full WC medium, which reduces the risk of limitation by other nutrients than N and P. Medium, nutrients, and algae were

Table 1. Experimental design with N and P supply (μmol L^{-1}, bold letters) and molar nutrient ratios for all 25 combinations of N and P supply.

	N (μmol L^{-1})				
	10.3	**20.6**	**31.0**	**41.3**	**51.6**
P (μmol L^{-1})					
3.2	3.2	6.5	9.7	12.9	16.1
2.6	4.0	7.9	11.9	15.9	19.9
1.9	5.4	10.9	16.3	21.7	27.2
1.3	7.9	15.9	23.8	31.8	39.7
0.6	17.2	34.4	51.6	68.8	86.0

added at the beginning of the experiment and afterward no addition (only sampling without refilling) took place.

In order to efficiently capture the nonlinear responses of algal growth and nutrient use in different stoichiometric contexts, we chose to maximize the gradient size and not to replicate each nutrient composition (Kreyling et al. 2018). The experiment followed a gradient design with orthogonal five-levels of N and P, generating 25 nutrient compositions (Table 1). We further added a categorical variable (five levels), which included each of the four phytoplankton species in monoculture as well as their mixture. The mixed community had to be sampled more frequently than the monocultures (see below), therefore duplicates were established for the polycultures allowing alternated sampling. The experiment thus comprised 150 flasks, 25 flasks per monoculture, and 50 for the mixture. Each flask was inoculated with the same amount of algae biomass, for which we estimated optical density (OD) (explained below) for each culture. Mixed cultures were composed of equal biovolume proportions of the four species and with the same total biovolume as used for the monocultures (48 mm^3 L^{-1}).

All units were kept in a climate chamber at 18°C with a 16:8 h light : dark cycle of 247.52 μmol photons s^{-1} m^{-2} ($\pm$ 101.65). We used LED aquarium light units (IT2040 Evergrow, Evergrow Lighting, China) and measured irradiance using a spherical quantum sensor (LI-193, LI-COR, Germany). Flasks were shaken and cleaned daily before measurements and randomly rearranged afterward to assure comparable light conditions during the experiment. The experiment was set up and sampled under sterile conditions. All materials used were acid-washed and either autoclaved or precombusted.

Sampling and analysis

Daily measurements of OD were used as a proxy for biomass. For this aim, we custom-tailored a sample holder for the 250 mL cell culture bottles, which were placed between a light source of distinct wavelength and a light detector. Following the Lambert–Beer law, absorption gives a proportional correlation to concentration changes of the cultures in the cell culture bottles, that is, decreasing intensity of detected light is correlated with increasing particle (phytoplankton) concentration (Wollschläger et al. 2016). Using this setup, we were able to measure the change in biomass on a daily basis without opening the bottles (removing the risk of possible contamination) and without sampling (maintaining the original volume and setup of the culture).

We used this measurement of OD to identify two sampling times for in-depth analyses. The first sampling of all cultures was on day 7, during the exponential growth phase. The second sampling took place once the respective culture entered stationary phase (SCE: day 14, ANK and CHLA day 18, STAU day 34, *see* Supporting Information Fig. A1). We abbreviate the first sampling in the exponential growth phase as EXP and the second—when communities reaching capacity—as CAP. At each of these time points, we also sampled the MIX in order to be able to test H2, and to assess changes of community composition in the mixed cultures (*see* Supporting Information Fig. A1). For community analyses, we preserved 7 mL samples with Lugol's solution (1% final concentration) stored in brown glass bottles. Subsamples were counted in cell culture wells (Sarstedt, Germany), using an inverted microscope (Leica, Germany) using the Utermöhl (1958) method with a minimum amount of 400 cells per algae. Abundance was converted to biovolume (Hillebrand et al. 1999). To obtain particulate nutrient concentrations, 20 mL samples were filtered onto acid-washed precombusted glass-fiber filters (Whatman GF/C, GE Healthcare UK, England) and stored at −20°C for CN and P, respectively. Particulate organic phosphorous was determined photometrically (AquaMate Plus, Thermo Fisher Scientific, U.S.A.) by molybdate reaction after sulfuric acid digestion (Wetzel and Likens 1991). Filters for particulate organic CN were oven-dried at 58°C for 48 h, placed in tin capsules and measured with an elemental analyzer (Euro EA 3000, HEKAtech GmbH, Germany). For dissolved nutrient analysis, 10 mL of filtrate were pipetted into polyethylene vials (Zinsser Analytic, Germany), stored over night at 4°C, and analyzed for nitrate, nitrite, ammonium, and phosphate by a continuous-flow analyzer (Skalar, The Netherlands).

The measures of biomass (OD, carbon, and biovolume) were highly correlated ($r > 0.74$, $p < 0.0001$); therefore, we only use carbon biomass (particulate organic carbon (POC) in μmol C L^{-1}) throughout the article. Resource use efficiencies for phosphorous (RUE_P) and nitrogen (RUE_N) were consequently calculated as the dimensionless ratio of attained carbon biomass (in μmol L^{-1}) over the amount of the respective nutrient supplied at the beginning of the experiment (P or N in μmol L^{-1}). Stoichiometry of algal biomass was expressed as molar ratios of C : N, C : P, and N : P, respectively.

Diversity in mixtures at each of the two samplings was measured as effective number of species, ENS, which is a measure of diversity less affected by sampling issues (Chase and Knight 2013). ENS is equivalent to the inverse Simpson dominance index and quantifies the number of species if they had equal proportions.

In order to compare mixture performance with monoculture results, we calculated a log response ratio (LRR_{mix}) between the

biomass attained of the mixture compared to the monoculture average attained during the same sampling day. Doing so for all N × P supply treatments allowed visualizing the performance of the assemblage over the expectation from the monocultures.

Design and analysis of bioassays

To test H3, we conducted bioassays after each final sampling. The flasks, holding the remaining algal suspension (≈ 50 mL), were refilled with 150 mL of fresh growth medium lacking N and P, and thoroughly shaken, and then split equally to yield two flasks for each treatment combination, with 100 mL each. One set was then amended with 50 μmol K_2HPO_4 (per 100 mL), the other with 1 mmol $NaNO_3$ (per 100 mL), supplying the algae with an excess of either N or P. OD was assessed daily over 5 d and converted to carbon units as a measurement of biomass increase. We used the slope of the regression of ln-transformed biomass over time as a measure of growth induced by each of the nutrients.

Statistical analysis

All analyses were done using R (R Development Core Team 2018). Visualizations were based on packages ggplot2 (Wickham 2016) and lattice/latticeExtra (Sarkar 2008). Testing hypothesis 1 for each of the response variables (C : N, C : P, N : P, biomass, RUE_N, and RUE_P), the primary model included N_{supply} and P_{supply} as interactive continuous variables and species (four monocultures + mix) as additive categorical variable. Since the second sampling date differed between monocultures, we opted for separate analyses of stoichiometry, biomass, and RUE for the exponential (exp) and stationary (cap) phase instead of repeated measure approach. Whereas this slightly increased the probability of a type I error, the significance of the effects was not altered if p levels were adjusted for $k = 2$ tests (*see* "Results" section). Significant main effects support hypothesis H1a, whereas significant interaction terms for both nutrients support H1b.

In Supporting Information Table A1, we present an alternative statistical analysis which uses a 2nd order polynomial regression of supplied N : P ratios instead of the single nutrient gradients, and again adds species (four monocultures + mix) as categorical factor. In case of a positive linear and negative quadratic term for N : P supply on algal biomass, RUE, and stoichiometry, this model allowed to test for the location of a significant hump (or pit) in the unimodal response to N : P. For this, we used the MOS test (Mitchell-Olds and Shaw 1987) as implemented in the vegan package in R (Oksanen et al. 2015).

Table 2. Statistical results for the analysis of algal stoichiometry (C : N, C : P, N : P), biomass, and RUE for N and P, separated for the exponential (EXP) and stationary (CAP) phases. For each of the models, we give the overall explained variance (R^2_{adj}), the F ratio, and the significance of the model. For each of the continuous factors, we give the slopes (standard errors) and their significance, for the species, we give the contrast to the mixture.

Time/factor	CN	CP	NP	Biomass	RUEN	RUEP
EXP	R^2_{adj} = 0.7804	R^2_{adj} = 0.6163	R^2_{adj} = 0.5204	R^2_{adj} = 0.8463	R^2_{adj} = 0.8734	R^2_{adj} = 0.8068
	F = 63.96 ***	F = 29.45 ***	F = 20.22 ***	F = 98.56 ***	F = 123.2 ***	F = 74.97 ***
Intercept	22.12 (1.19) ***	188.04 (33.85) ***	7.02 (2.59) **	447.59 (51.35) ***	38.82 (1.99) ***	628.35 (56.57) ***
N	−0.18 (0.03) ***	ns	0.26 (0.07) ***	ns	−0.59 (0.05) ***	4.15 (1.55) **
P	ns	−54.01 (14.92) ***	ns	ns	ns	−178.57 (24.93) ***
N×P	0.06 (0.02) ***	ns	ns	2.69 (0.66) ***	0.06 (0.03) *	ns
Species		ANK>MIX(***)	ANK>MIX(***)	ANK>MIX(***)	ANK>MIX(***)	ANK>MIX(*)
	CHLA<MIX(***)		CHLA>MIX(**)	CHLA<MIX(***)	CHLA<MIX(***)	CHLA<MIX(***)
	SCE>MIX(***)	SCE>MIX(***)	SCE>MIX(**)	SCE>MIX(***)	SCE>MIX(**)	SCE>MIX(*)
	STAU<MIX(***)	STAU<MIX(***)		STAU<MIX(***)	STAU<MIX(***)	STAU<MIX(***)
CAP	R^2_{adj} = 0.4595	R^2_{adj} = 0.3743	R^2_{adj} = 0.4674	R^2_{adj} = 0.8781	R^2_{adj} = 0.6414	R^2_{adj} = 0.7811
	F = 22.13 ***	F = 15.87 ***	F = 22.81 ***	F = 180 ***	F = 45.47 ***	F = 89.72 ***
Intercept	30.13 (1.81) ***	475.4 (57.89) ***	13.42 (2.03) ***	531.47 (52.67) ***	58.52 (3.81) ***	834.64 (92.27) ***
N	−0.2 (0.05) ***	ns	0.2 (0.06) ***	10.18 (1.5) ***	−0.84 (0.11) ***	19.23 (2.64) ***
P	ns	−146.77 (26.6) ***	−3.83 (0.93) ***	−79.27 (24.2) **	ns	−265.64 (42.39) ***
N×P	0.11 (0.02) ***	2.11 (0.78) **	ns	4.89 (0.71) ***	0.11 (0.05) *	−3.83 (1.24) **
Species				ANK>MIX(*)		
	CHLA<MIX(***)	CHLA<MIX(*)		CHLA<MIX(***)	CHLA<MIX(***)	CHLA<MIX(***)
				SCE<MIX(**)		
	STAU>MIX(***)			STAU>MIX(***)	STAU>MIX(***)	STAU>MIX(***)

ns, not significant.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Significant effects of species identity and mixture in the main model described above already address hypothesis H2, but to more explicitly test this hypothesis, we compared each monoculture to the mix as reference in the form of a log response ratio, where LRR_{mix} is the natural log of mixture biomass compared to the monoculture biomass. For hypothesis H2a, we

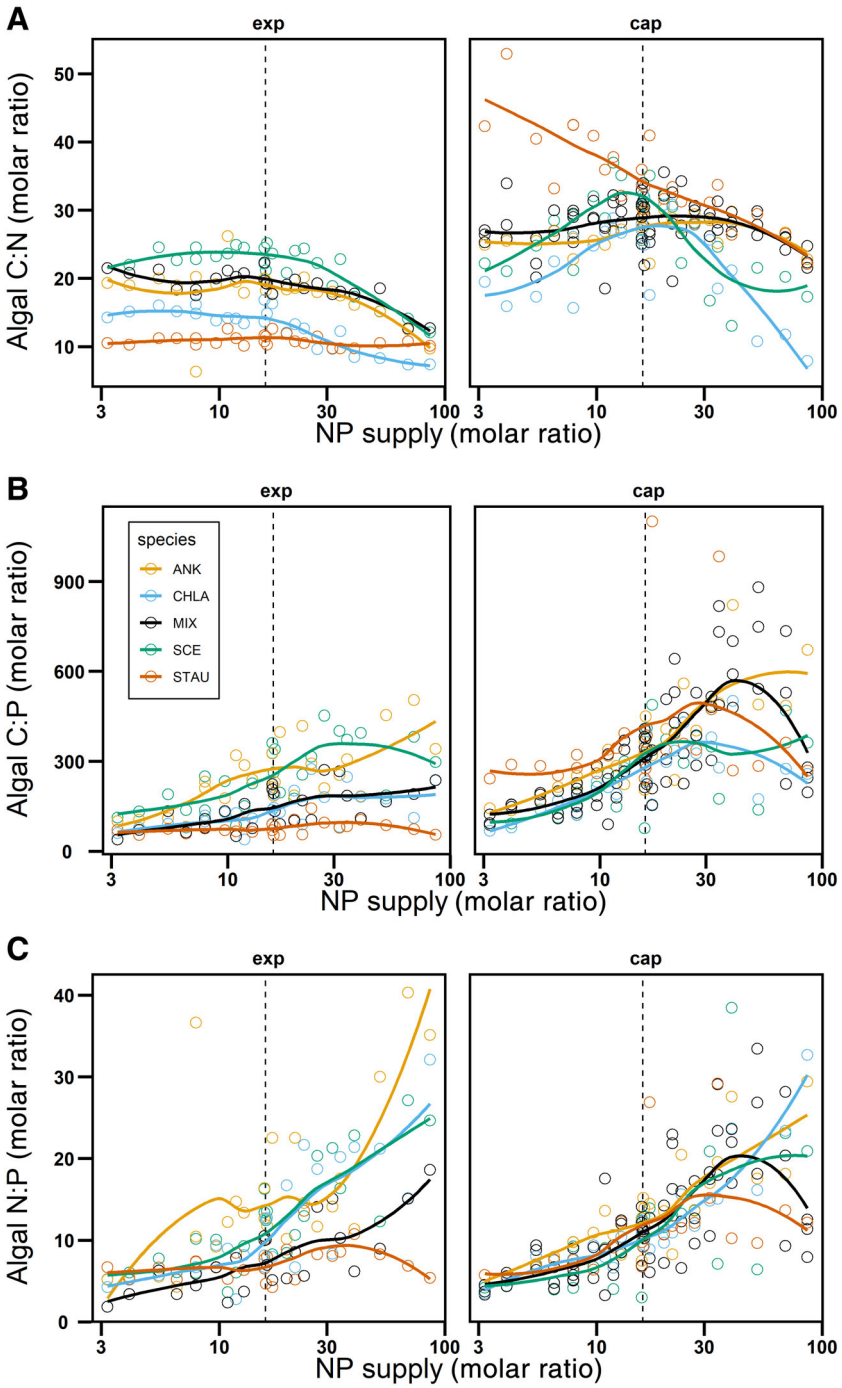

Fig. 1. Algal stoichiometry in response to initial N : P supply ratio for exponential and stationary growth phase (left and right column, respectively) of monocultures (colored symbols) and mixture (black symbols) of freshwater phytoplankton. Curves are loess-fits added to visualize the trend of algal C : N (**A**), algal C : P (**B**), and algal N : P (**C**) over supply N : P. Vertical dashed line represents Redfield N : P of 16. Significant humps in C : N ratio within the range of supplied N : P were for MIX (at molar N : P supply = 15.8), CHLA (at molar N : P supply = 14.30), and SCE (at molar N : P supply = 12.68) (in all cases $p < 0.01$, MOS-test). The apparent hump in the response of the other ratios for MIX (**B**, **C**) was marginally nonsignificant for N : P ($p = 0.09$) and nonsignificant for C : P ($p = 0.353$).

performed a *t*-test of LRR_{mix} against zero, where a significant positive deviation from zero indicates that the mixture outperformed the biomass production in the monoculture for the EXP and CAP phases, respectively. For H2b, we correlated LRR_{mix} against ENS. For H2c, we tested the response of ENS in the mixture to N_{supply} and P_{supply} as interactive continuous variables.

Finally, with regard to hypothesis H3, we used separate models for growth rate in response to N and P addition,

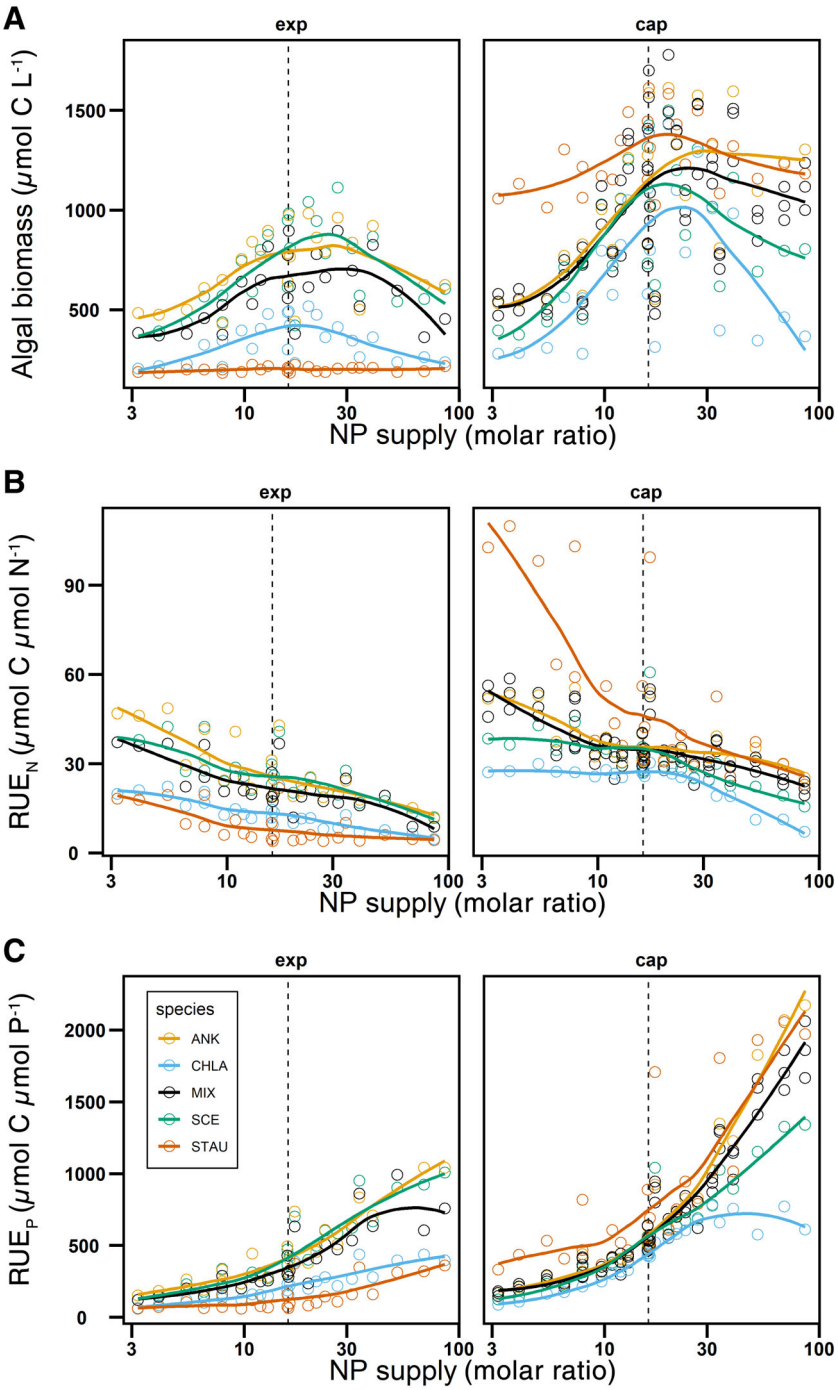

Fig. 2. Algal biomass and RUE in response to initial N : P supply ratio for exponential (exp) and stationary (cap) growth phase (left and right column, respectively) of monocultures (colored symbols) and mixture (black symbols) of freshwater phytoplankton. Curves are loess-fits added to visualize the trend of algal biomass (**A**), RUE for N (**B**) or RUE for P (**C**) over molar N : P supply ratio. Vertical dashed line represents Redfield N : P of 16. Significant humps within the range of molar supplied N : P were observed for biomass in the exponential phase (at molar N : P supply of 21.5 for MIX, 22.2 for ANK, 18.0 for CHLA, and 22.4 for SCE) and in the stationary phase (at molar N : P supply of 35.2 for MIX, 19.9 for CHLA, and 23.1 for SCE).

respectively. We used the main model as described above (continuous factorial gradients of intitial N_{supply} and P_{supply} plus species as categorical factor) as well as the alternative model with initial N : P supply ratio as 2nd order polynomial and species as independent factors. Both models provide a test of hypothesis H3a, whereas H3b was tested by a correlation analysis between algal stoichiometry before the bioassay and the growth rate response to N and P addition.

Results

Algal stoichiometry

During exponential growth, nutrient incorporation reflected mainly the absolute availability of the respective nutrient, that is, C : N decreased and N : P increased with N_{supply}, C : P decreased with P_{supply} (Table 2, Supporting Information Fig. A2). A significant positive interaction term between supplied nutrients appeared for C : N, which became stronger during stationary phase, indicating higher C : N if both N and P were high (Table 2, Supporting Information Fig. A2). By contrast, high N_{supply} coupled to low P_{supply} yielded very low C : N ratios, indicating that N was stored internally (Table 2, Supporting Information Fig. A2). The same interaction was also found for C : P during stationary phase, with a significant interaction term reflecting low C : P coupled to high P_{supply} but low N_{supply} (Table 2, Supporting Information Fig. A2). N : P during stationary phase increased with N_{supply} and decreased with P_{supply}, with linear responses to both supply gradients and without significant interactions (Table 2, Supporting Information Fig. A2).

Consequently, algal stoichiometry monotonically followed the N : P ratio of the supply during the exponential phase, that is, C : P and N : P increased and C : N decrease with increasing

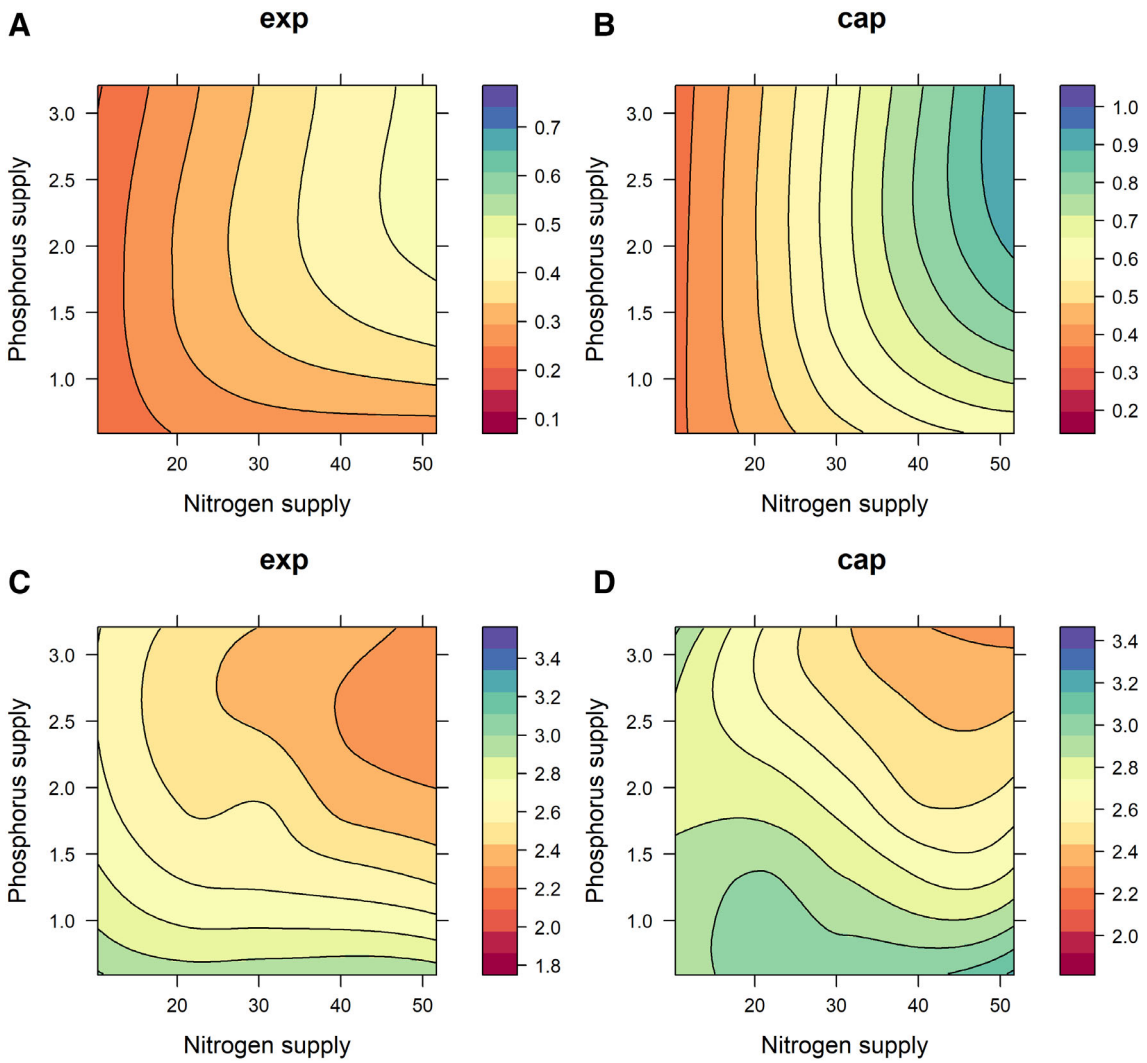

Fig. 3. Contour plots of relative biomass (**A**, **B**) and effective number (**C**, **D**) of species along the axes of P and N supply (in μmol L^{-1}). Relative biomass was scaled to the maximum biomass obtained by the species or the mixture in the experiment. (**A**, **C**) During exponential (exp) and (**B**, **D**) during stationary (cap) growth phase.

supplied N : P ratio (Fig. 1). However, in the stationary phase, deviations between supply and incorporation reflected that maximum algal nutrient ratios did not necessarily occur at extreme supply ratios. Significant intermediate maxima of C : N at intermediate N : P supply were detected by the MOS test for MIX, CHLA, and SCE in the stationary phase (Fig. 1).

In both models (Table 2, Supporting Information Table A1), the same significant differences between single species and the mixture became apparent. The mixture showed intermediate stoichiometry compared to the component species, with some species showing higher and other lower internal C : nutrient and N : P ratios (Fig. 1, Supporting Information Fig. A2, Table 2, see also Supporting Information Table A1). These differences were more pronounced during exponential phase, as for each ratio three out of four species significantly differed from MIX. After reaching capacity, only a few significant differences persisted with the MIX showing higher C : N and C : P than CHLA and lower C : N than STAU.

Biomass

During both phases, EXP and CAP, algal biomass increased as both N_{supply} and P_{supply} increased (significantly positive interaction term, Table 2, Supporting Information Fig. A3A,B). The maximum biomass attained by each species or mixture was strongly linked to the highest absolute balanced supply of N and P (Figs. 2A, 3A,B). Consequently, algal biomass followed a significant unimodal relationship with supplied molar N : P ratio (Supporting Information Table A1, positive linear and negative quadratic term in the polynomial model). Significant peaks of biomass at intermediate supply ratios were found for ANK, CHLA, SCE, and MIX in the EXP phase and the same (except ANK) at the CAP phase (Fig. 3A). Peaks occurred at molar N : P larger than Redfield proportions (18–35, mean 23.2, Fig. 2).

Initially, mixtures grew faster than CHLA and STAU, but slower than SCE and ANK (Fig. 2A, Supporting Information Fig. A3A). In the stationary phase, this difference remained

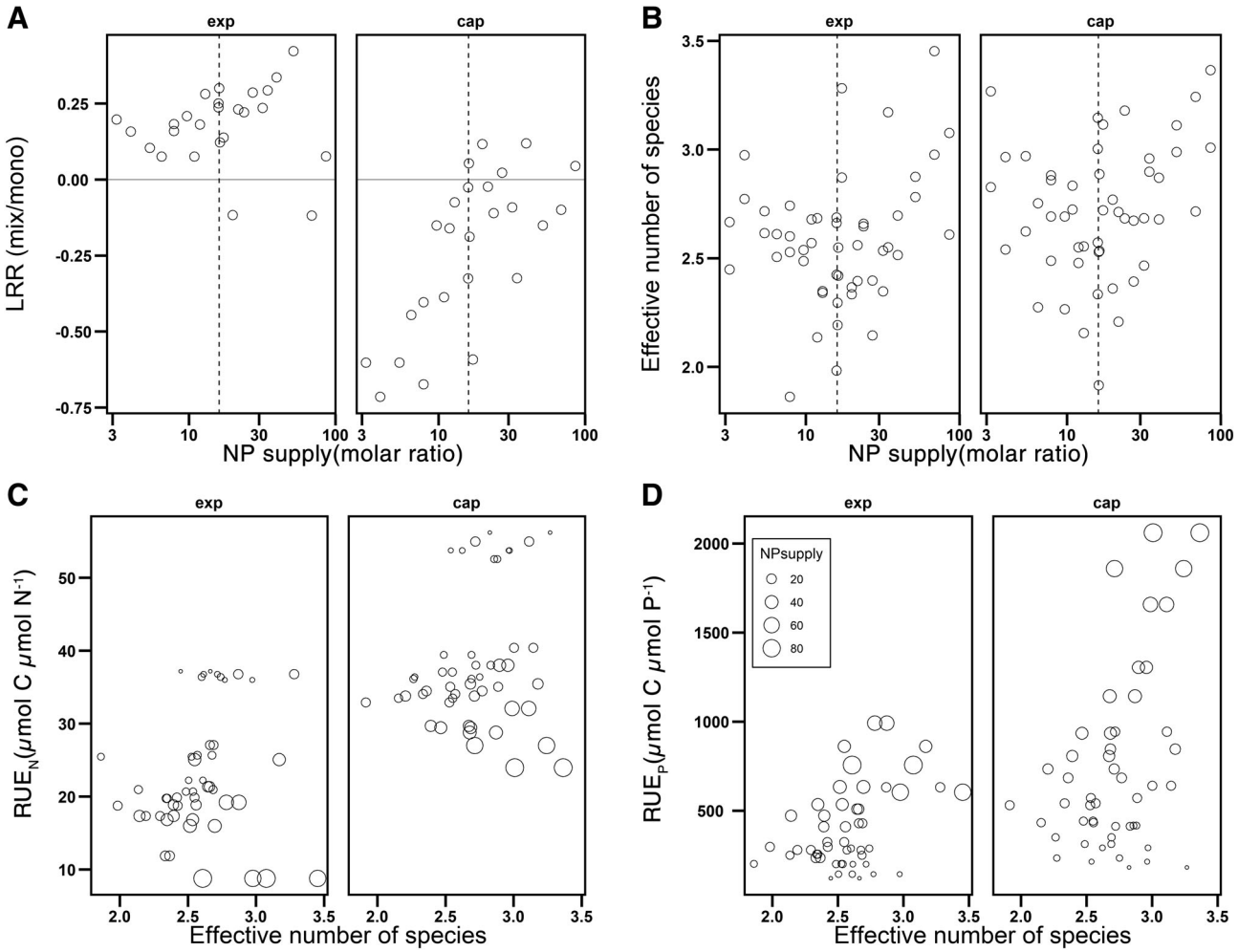

Fig. 4. The biomass production (**A**) of mixtures against the average monoculture sampled (LRR mix/mono: log response ratios of mixture over average monoculture biomass; zero indicates no difference, gray horizontal line) and the ENS (**B**) during the exponential (exp, left panel) and stationary (cap, right panel) phases of the experiment in relation to N : P supply ratios. Vertical dashed line represents Redfield N : P of 16. RUE_N (**C**) and RUE_P (**D**) during the exponential (exp, left panel) and stationary (cap, right panel) phases of the experiment in relation to ENS. Symbol size scales to the molar N : P supply ratio.

consistent for CHLA and ANK, but reversed for the other two species with lower biomass in mixtures compared to STAU and larger compared to SCE. Compared to the average performance of the monocultures, the mixture showed higher biomass production in the EXP phase (Fig. 4A, mean LRR_{mix} = 0.18, $t_{[x;0]}$ = 10.43, p value < 0.001). This outperformance of the average monoculture disappeared and turned into the opposite in the stationary phase (CAP, Fig. 4A, mean LRR_{mix} = −0.23, $t_{[x;0]}$ = −6.42, p value < 0.001).

RUE and diversity

The negative effect of N_{supply} on RUE_N and of P_{supply} on RUE_P was significant in both phases (EXP and CAP, Table 2, Supporting Information Fig. A3C,D). However, RUE of both elements increased with the availability of the other resource, as reflected by significant positive effects of N_{supply} on RUE_P and a significant positive interaction effect of N and P on RUE_N. Likewise, RUE_N decreased with increasing N : P supply ratio, whereas RUE_P increased with increasing N : P supply ratio (Fig. 4C,D, Supporting Information Table A1).

ENS peaked at lowest combined supply of N and P and decreased with increasing supply of both nutrients (Figs. 3C, D, 4B). This was reflected by a significant negative interaction term N×P during stationary phase (slope = −0.007 ± 0.002, p = 0.005) and a similar, yet nonsignificant interaction during EXP (p = 0.189).

ENS was weakly positively correlated to RUE_N but significantly to RUE_P during EXP (r = 0.444, p = 0.001) and CAP (r = 0.425, p = 0.002).

In order to test whether this correlation was only because of the common effect of N : P supply on RUE and ENS (cf. Figs. 2, 4), we amended the previous analysis of RUE in response to the N : P supply gradient (Supporting Information Table A1) with ENS as additional covariate at the end of the experiment. This model, by definition restricted to the mixture, explained 65.4% and 97.2% of the variance in RUE_N and RUE_P, respectively, and included a highly significant positive effect of ENS (RUE_N = −51.99 * NP + 14.2 * $(N:P)^2$ + 13.2 * ENS + 2.1, full model F = 31.92; RUE_P = 3320.2 * NP − 570.8 * $(N:P)^2$ + 165.9 * ENS + 290.2, full model F = 559.2; in both models all parameters and the full model were significant at p < 0.001).

Bioassay

Adding N and P individually to each experimental unit after reaching the stationary phase yielded positive growth responses overall, but growth rates were more consistently positive and overall stronger for the addition of N compared to P (Supporting Information Fig. A4). The effect of adding N increased significantly with increasing P_{supply} and decreasing N : P ratio (Table 3). The latter was reflected by a significant negative N×P interaction in the main model as well as by a strong linear decline with increasing supplied N : P (Fig. 5). Likewise, the growth response to P increased with increasing N_{supply} and increasing N : P ratios (Supporting Information Fig. A4, Fig. 5). Interestingly, the growth rate in the bioassay was constrained by the other nutrient and the nutrient ratio, but not by the previous concentration of the nutrient itself (no significant main effects of the target nutrient, Table 3).

Mixtures tended to have lower growth responses than single species which was significant for three out of four species for P addition (ANK, CHLA, STAU) and two species for N addition (CHLA, SCE); only STAU responded significantly less to N addition than the mixture (Table 3).

Table 3. Statistical results for the analysis of growth response to N and P addition in the biomass. We used both the factorial model of N and P supply (as in Table 2) and the polynomial model of N : P ratios (as in Table B1). For each of the models, we give the overall explained variance (R^2_{adj}), the F ratio, and the significance of the model. For each of the continuous factors, we give the slopes (standard errors) and their significance, for the species, we give the contrast to the mixture.

Factorial model of N and P supply			Polynomial model of N : P ratios		
Factor	**N-addition**	**P-addition**	**Factor**	**N-addition**	**P-addition**
	R^2_{adj} = 0.7868	**R^2_{adj} = 0.4227**		**R^2_{adj} = 0.7776**	**R^2_{adj} = 0.3952**
	F = 61.11 ***	**F = 12.92 *****		**F = 62.42 *****	**F = 13.42 *****
Intercept	0.026 (0.024)	0.042 (0.016) **	Intercept	0.098 (0.009)***	−0.004 (0.006)
N	−0.001 (0.001)	0.001 (0.0004) **	NP	−0.665 (0.044)***	0.090 (0.028) **
P	0.083 (0.010) ***	0.006 (0.006)	NP^2	0.197 (0.044)**	−0.009 (0.028)
N×P	−0.001 (0.0002) ***	−0.0003 (0.0002)			
Species		ANK>MIX(***)	Species		ANK>MIX(**)
	CHLA>MIX(**)	CHLA>MIX(***)		CHLA>MIX(**)	CHLA>MIX(***)
	SCE>MIX(***)			SCE>MIX(***)	
	STAU<MIX(***)	STAU>MIX(***)		STAU<MIX(***)	STAU>MIX(***)

ns, not significant.
*p < 0.05, **p < 0.01, ***p < 0.001.

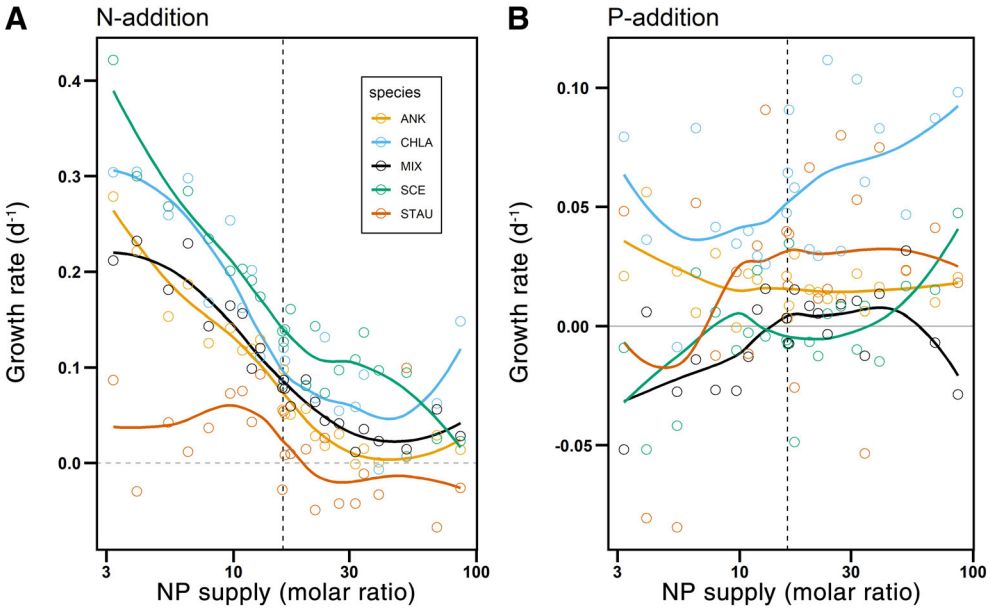

Fig. 5. Growth rate of stationary phase phytoplankton after adding N (**A**) or P (**B**) in bioassays in relation to the original molar N : P supply ratio in the main experiment. For monocultures (colored symbols) and mixture (black symbols), loess-fits are added to visualize the trend of over supply N : P. Vertical dashed line represents Redfield N : P of 16.

The magnitude of the N-addition effect was strongly related to the nutrient content of the algae, especially the P content: Strong growth responses to N addition were restricted to low algal C : P and N : P, that is, high relative P content (Supporting Information Fig. A5). A weaker, negative correlation between algal C : N and the response to N addition was strongly reflecting the high C : N and low responsiveness of one species, STAU. The growth response to P-addition was also negatively correlated to the C : N ratio of the algae only, again reflecting that highest P-addition effects required high relative N-content.

Discussion

H1: Nutrient incorporation, biomass production, and RUE reflect both the rate and ratio of resource supply

Algal stoichiometry, biomass production, and RUE were constrained by the stoichiometric interplay of both N and P. On both levels of organization, single species population, and mixed communities, nutrient incorporation responded not only to the supply of that nutrient (not refuting hypothesis H1a), but also to the relative availability of the other nutrient (H1b). This stoichiometric constraint was reflected by a significant N×P interaction term when analyzing algal C : N and C : P in the main experiment as well as a nonlinear response of algal stoichiometry to the gradient of N : P supply ratios. This outcome was independent of the model chosen to analyze the data, that is, whether the orthogonal factors reflected N_{supply} and P_{supply} or the supply ratio. Our results corroborate previous findings showing that more balanced supply of resources leads to more efficient resource use as less resources remain unconsumed (Gross and Cardinale 2007; Cardinale et al. 2009*b*; Hodapp et al. 2016).

We found species specific differences in the optimal N : P supply ratio sustaining maximum growth and biomass yield; however, consistently these ratios were larger than the Redfield ratio of N : P = 16 (Redfield 1958; Hillebrand et al. 2013). Consequently, the efficiency of nutrient transfer into biomass was stoichiometrically constrained as well (not refuting H1a and b), such that RUE for N was enhanced by P_{supply} and vice versa, this pattern being most pronounced in the stationary phase of the experiment. Thus, RUE for a given resource does not only decline with increasing supply, but also depends on the availability of other resources. Still, most studies—including some of our own—base RUE on a single resource (Ptacnik et al. 2008; Hillebrand and Lehmpfuhl 2011; Filstrup et al. 2014), whereas only few worked on ways to address RUE in a stoichiometric context (Cardinale et al. 2009*b*; Lewandowska et al. 2016).

H2: Mixtures differ from the monocultures in terms of nutrient incorporation, biomass production, and RUE

Overall, we found frequent and strong differences between mixtures and single species, but with different signs of the differences. Thus, the mixture was not consistently different from all monocultures, and no overyielding regarding biomass and RUE was found (refuting H2a). Only for the exponential growth phases, mixtures differed from all component species by showing faster growth. However, within the mixtures, a higher realized diversity (in terms of ENS) was correlated to higher RUE_P, and this relationship remained significant when

accounting for the common response of RUE_P and ENS to the nutrient supply treatments (not refuting H2b). The effect of nutrients on ENS was, as predicted, dependent of the interactive supply of both nutrients, which selected for high dominance and low ENS. In these cases, two species, ANK and SCE, dominated the polyculture.

The relative similarity of the nutrient demands of the four species can explain the lack of an overall difference between mixture and monoculture. In an experiment similar to ours, Weis et al. (2008) examined diversity effects on production for green algae grown on a similar range of N : P supply ratios from 4 to 64. They found that nutrient supply balance affected biomass but not diversity. None of the species varied in optimal ratio for production, and therefore no complementarity effects were visible in their polycultures. Contrary to our results though, Behl et al. (2011) found that increasing algal diversity leads to transgressive overyielding caused by trait complementarity, but their study used a much broader phylogenetic diversity. Thus, our results corroborate model predictions (Ptacnik et al. 2010*a*; Hodapp et al. 2016) that complementarity requires both the variability in traits and in environmental heterogeneity.

Still, the species showed some differences under which N : P supply ratios they produced most biomass, which explains why low ENS (= high dominance of ANK and SCE) was related to low RUE, as both species were not producing most carbon biomass in absolute terms.

H3: The limitation by N and P reflects the nutrient content of the algae and the stoichiometric constraints of growth

Adding N or P to the nutrient-deficient cultures at the end of the main experiment, we found that the algae were rather N-limited, responding more strongly to N than to P addition. This can be linked to high N demand and high optimal N : P ratios in green algae (Quigg et al. 2003; Klausmeier et al. 2004*b*; Hillebrand et al. 2013). In fact, the realized N : P ratios in the algae at the end of the experiment was consistently lower (i.e., poorer in N) than the supply ratios sustaining maximum biomass yield (cf. Figs. 1, 5).

We found strong support for the hypotheses that the response to adding one nutrient depended strongly on the stoichiometry of the previous nutrient supply (not refuting H3a) and reflected the limitation status indicated by the internal nutrient concentrations (not refuting H3b). The magnitude of the growth responses to N depended more on previous P_{supply} and N : P/C : P concentrations than on the N_{supply} and incorporation, and vice versa for responses to P. Mono- and polycultures tended to differ in their growth response to the nutrient spike, with the MIX most often being less responsive than the single species.

Conclusions

Our lab experiment provides evidence for clear stoichiometric constraints for incorporating nutrients and transferring them into new production across a group of chlorophyte species. Human interventions alter biogeochemical cycles through anthropogenic mobilization of nitrogen from fossil fuels and N_2-fixation for fertilizer production (Canfield et al. 2010) and P-mining (Filippelli 2008), leading to global changes in the absolute but also relative availabilities of these nutrients (Falkowski et al. 2000; Elser et al. 2009). Climate change further enhances the spatial and temporal variability of nutrient supplies via changed precipitation patterns, enhanced stratification of waterbodies, growing anoxic zones, and other mechanisms (Tilman and Lehman 2001; Hessen et al. 2009). These changes culminate to expose autotrophs in different ecosystems to a stoichiometry of resource supply that they did not encounter during their postglacial history (Peñuelas et al. 2012). Consequently, a stoichiometric perspective on the responses of primary producers and the subsequent food web supported by them appears as an important prerequisite to make predictions on the future of various ecosystem processes (Peñuelas et al. 2012; Hillebrand et al. 2014; Harpole et al. 2016).

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Conflict of Interest

None declared.

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