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Plasticity in algal stoichiometry: Experimental evidence of a temperature-induced shift in optimal supply N:P ratio

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Abstract

There is growing empirical and theoretical evidence for a positive relationship between the nitrogen (N)-to-phosphorus (P) ratio of phytoplankton and temperature. However, few have tested how the optimal supply N:P ratio; the dissolved N:P ratio at which nutrient limitation switches from one element to the other, responds to temperature. In this study, we conducted a factorial experiment crossing 12 temperature levels with 8 supply N:P ratios to determine the effect of temperature acclimation on the optimal supply N:P ratio of the microalgae *Chlamydomonas reinhardtii*. We found that the optimal supply N:P increased in a sigmoidal manner from 26.5 to 36.5 (atomic ratio) over a temperature gradient spanning from ~10 to 18°C, with the steepest change around 15°C. This result is in accordance with trends observed for cellular and seston N:P ratios, and indicates that phytoplankton populations may be shifted toward N-limitation in a scenario of warmer waters.

Nitrogen (N) and phosphorus (P) are the most commonly limiting nutrients for pelagic primary production (Hecky and Kilham 1988; Elser et al. 2007). While the cellular N:P ratio of phytoplankton can vary widely due to stored nutrients (Rhee 1978; Goldman et al. 1979; Elrifi and Turpin 1985), an actively growing population of phytoplankton requires N and P in a specific ratio that is related to the demands for these essential nutrients in macromolecules like N-rich proteins and P- and N-rich ribosomes (Geider and La Roche 2002; Sterner and Elser 2002). The N:P requirement ratio defines the threshold for the transition between N and P-limited growth, and has been termed a critical (Terry et al. 1985) or an optimal (Rhee and Gotham 1980; Klausmeier et al. 2008) N:P ratio. In this paper, we will for simplicity use the term "optimal N:P" when referring to threshold N:P ratios.

Species differ in their optimal N:P ratios (Rhee and Gotham 1980; Andersen 1997; Leonardos and Geider 2005). Moreover, a systematic difference between the red- and green algal lineages in cellular N:P ratios under exponential growth (Quigg et al. 2003) suggests that there could be differences in optimal N:P also at higher phylogenetic levels. While these variations, on a proximate level, are related to the biochemical composition of the nonstorage biomass (Geider and La Roche 2002), interspecific differences in optimal N:P may ultimately be explained by selection for different ecological strategies. Selection for fast growth, for example, may yield a low optimal N:P due to a high allocation of resources to P-rich ribosomes (Klausmeier et al. 2004b)

A highly unexplored question is how environmental factors affects a species' optimal N:P ratio. It is well known that environmental acclimation can induce a range of phenotypic responses in algae (Geider 1987; Davidson 1991; Falkowski and LaRoche 1991), and if these responses involve differential allocation to N and P-rich macromolecules, the optimal N:P ratio may also change. Understanding how ambient drivers can influence the optimal N:P is a core issue for linking changes in the environment to the balance between N and P-limitation of primary production, but also to the biogeochemical cycling of N and P since the optimal N:P defines the drawdown ratio of N to P, at least at high nutrient concentrations (Klausmeier et al. 2004a).

Temperature is an environmental factor that potentially can affect the optimal N:P because different processes involved in phytoplankton growth and photosynthesis vary in their response to temperature. The rates of protein synthesis by ribosomes and CO_2-fixation by Rubisco, for example, change by a factor of ~2 when temperature is changed by 10°C (i.e., the Q_{10} for these processes is around 2 (Shuter 1979; Raven and Geider 1988)). Light-harvesting by pigment-protein complexes, however, is independent of temperature. When acclimating to low temperature, phytoplankton cells

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may be hypothesized to produce more of the catalysts that are temperature-dependent (e.g., P-rich ribosomes), to compensate for the reduced specific reaction rate of the same components (Raven and Geider 1988). Moreover, cells may be expected to reduce the allocation to components involved in temperature independent processes (e.g., N-rich light harvesting complexes) if it does not compromise the growth rate (Shuter 1979). This implies that acclimating to different temperatures might affect the relative requirement for N versus P.

Based on a mechanistic cell model incorporating different temperature dependencies for biosynthesis and light harvesting (Daines et al. 2014), and meta-transcriptomic data showing a general upregulation of ribosomal genes at low temperature, Toseland et al. (2013) predict that the N:P stoichiometry of phytoplankton will increase in response to global warming. They suggest that a lower cellular density of P-rich ribosomes, and hence less P relative to N, will be needed to maintain a certain level of protein synthesis at higher temperatures. Their prediction is supported by trends in the N:P ratio of marine particulate matter (Martiny et al. 2013; Yvon-Durocher et al. 2015) and the tissue N:P of plants (Reich and Oleksyn 2004) and macroalgae (Borer et al. 2013), which correlates positively with temperature (or latitude) globally. The variation in N:P across these geographical temperature gradients is, however, likely caused by a combination of direct temperature effects on resource allocation, differences in community composition, and differences in ambient nutrient concentrations. Although a recent meta-analysis show that cellular (bulk) N:P ratios on average increases with temperature for cultured phytoplankton (Yvon-Durocher et al. 2015), only few experimental studies have rigorously tested the net effect of temperature acclimation on the optimal N:P. In doing so, we can test for the temperature effects on resource requirements for growth, excluding the potential confounding effects of nutrient storage.

Here, we test the hypothesis that the optimal N:P ratio increases with temperature in the unicellular green alga *Chlamydomonas reinhardtii*. For our experiment, we define the optimal N:P as the supply N:P ratio at which the biomass yield shifts from limitation by one element to the other, i.e., the supply N:P ratio where N and P are co-limiting. We will use the term "optimal supply N:P" to designate this ratio.

Materials and methods

Experimental design

As basic units in the experiment, we used 96-well microplates (white walls, clear bottom, Greiner Bio One, Kremsmünster, Austria). This facilitated a factorial design that crossed twelve temperature levels (one temperature level per column) with eight supply N:P ratios (one ratio per row), resulting in 96 unique experimental combinations per plate (Fig. 1a). Two replicates were run in parallel.

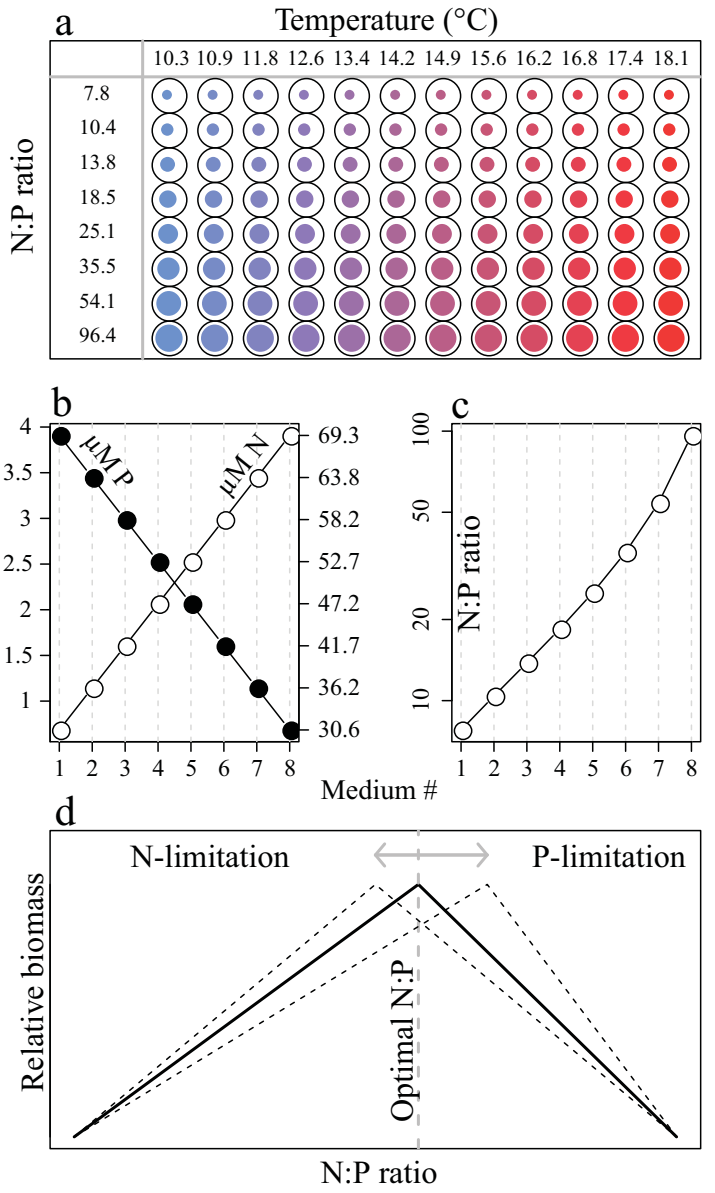

Fig. 1. Overview of the experiment. A) Schematic outline of the 12 × 8 factorial microplate design. The temperature gradient spanned the columns of the microplate, from left ("cold," blue) to right ("warm," red). The supply N:P gradient spanned from top to bottom, across the rows of the plate. B) Concentrations of N and P (μM) in the 8 different media. An increasing concentration of N was mixed with a decreasing concentration of P, resulting in the supply N:P ratio gradient shown in C) (atomic, y-axis log transformed). D) Experimental predictions. We expected steady-state biomass (solid line) to increase with supply N:P ratio under N-limitation, reach a peak at the optimal supply N:P ratio (where N and P are co-limiting), and then decrease as P became limiting. We predicted that any effect of temperature on the relative requirements for N and P should shift the position of the peak (dotted lines).

We created the temperature gradient using an incubator custom-made for microplates. The incubator consisted of a stainless steel plate that was heated at one side by a high power resistor, and cooled at the opposite side by Peltier

Table 1. Experimental nutrient- and temperature-conditions. N and P concentrations (μM) in each of the 8 media and the corresponding supply N:P ratios (atomic). The average temperature (T, °C) measured in each microplate column is shown in the bottom row.

Medium #	N (μM)	P (μM)	N:P ratio
1	30.64	3.94	7.8
2	36.17	3.48	10.4
3	41.69	3.02	13.8
4	47.2	2.56	18.5
5	52.72	2.1	25.1
6	58.24	1.64	35.5
7	63.76	1.18	54.1
8	69.28	0.72	96.4

Column #	1	2	3	4	5	6	7	8	9	10	11	12
Mean column T (°C)	10.3	10.9	11.8	12.6	13.4	14.2	14.9	15.6	16.2	16.8	17.4	18.1

elements. As predicted by thermodynamics, this creates a linear temperature gradient across the steel plate. The range of the gradient was controlled by a microcontroller, which maintained a constant temperature at each side. The gradient ranged from ca. 10°C to 18°C with 12 temperature steps (Table 1 and Fig. 1a). The exact temperatures were measured in water-filled wells with a K-Type thermocouple (Phidgets Inc., Calgary, Alberta, Canada).

To produce the eight-step gradient in supply N:P ratios, we first prepared two different stock media, A and B. Both media were based on natural lake water, with background concentrations of dissolved inorganic N (DIN) and dissolved inorganic P (DIP) of 17.9 μM and below the detection limit (i.e., <0.03 μM P), respectively. To medium A, we added 60 μM N (as $NaNO_3$) and no P, yielding a final concentration of 77.9 μM N and 0.0 μM P. To medium B, we added 5 μM P (as K_2HPO_4) and no N, yielding final concentrations of 5.0 μM P and 17.9 μM N. We added a standard trace metal and vitamin mix according to the WC medium of Guillard and Lorenzen (1972) to ensure that these elements were nonlimiting. The eight different supply N:P ratios (atomic) were created by mixing an increasing volume fraction of medium A with a decreasing volume fraction of medium B in 100-ml glass bottles (Table 1 and Fig. 1b). The resulting gradient was centered around an N:P of 20 and ranged from N:P = 7.8 to N:P = 96.4 (Table 1 and Fig. 1c). All media were sterile-filtered (0.2 μm pore size) before use and stored at 4°C.

The concentrations of the limiting nutrient in each well had to be carefully considered to avoid dissolved inorganic carbon (DIC) limitation. Despite a large surface-to-volume ratio in the wells and thus, a good exchange with the atmosphere, DIC-limitation might in principle still occur, since bubbling with air is impossible due to the small volumes and the high number of wells. To overcome DIC limitation, we deliberately based the media on lake water with a naturally high alkalinity (i.e., a high DIC concentration) and high pH. Specifically, we measured an alkalinity of 2.05 mEq l^{-1} by titration with HCl, and a pH of 8.4. We know that the carbonate fraction of DIC begins to increase greatly as the pH exceeds ca. 9.0 (Stumm and Morgan 1996). At this point, DIC limitation is likely, because algae cannot utilize carbonate. Using the *AquaEnv* package (Hofmann et al. 2010) in R (R Core Team 2014), we did a simulation of the relationship between pH and DIC at the given alkalinity. Based on this, we calculated the maximum amount of DIC that could be taken up by the algae before the pH reached 9.0. We found that if the medium was titrated down to pH = 7 with CO_2-enriched water, 6.9 mg DIC l^{-1} (=575 μM DIC) had to be taken up to reach this critical point (see supporting information Fig. S1). After preparing the eight media, we thus titrated the pH down to 7.0 with CO_2-enriched water. Assuming Redfield proportions (C:P = 106, C:N = 6.625), a consumption of 575 μM DIC by the phytoplankton would require 5.4 μM of P or 86.8 μM N. Hence, to avoid DIC limitation, the concentrations of P and N (when limiting) were maintained well below these levels (specifically, P was below 2.1 for P limited cultures, N below 58.2 for N-limited cultures, see Table 1).

Execution of experiment

At the start of the experiment (day 0), each well was filled with 320 μL medium and inoculated with 10 μL *Chlamydomonas reinhardtii* (strain *CC-1690 21 gr mt+*) stock culture (approximately containing 2,000 cells, as estimated with a CASY cell counter, Schärfe system GmbH). The stock culture had been grown at 17°C under ca. 70 μmoles photons $m^{-2}s^{-1}$ in the same lake-water medium as used in the main experiment, but with N and P concentrations of 100 and 5 μM, respectively. We then incubated the plates in the temperature-units, which again were located in a temperature-controlled room at 19°C. Transparent sealing-tape (BarSeal, Thermo scientific Nunc, Waltham, Massachusetts, U.S.A.) was used to reduce evaporation but to allow the exchange of gases. The

plates were illuminated from above using white fluorescent light. The irradiance, as measured with a spherical quantum sensor close to the white plate surface, was ca. 70 μmoles photons $m^{-2}s^{-1}$. A 12 h: 12 h, light: dark cycle was applied.

We ran the experiment as a semi-continuous culture, ensuring that all experimental units reached the same (quasi) steady-state growth rate after an initial period where growth was not limited by nutrients. We applied a semi-continuous dilution rate (D) of 0.25 d^{-1}, and removal and replacement of culture and medium was performed using a multichannel pipette under laminar air flow hood to minimize contamination. Growth was monitored prior to each dilution by measuring the in vivo fluorescence (IVF) of Chl *a* in each well (excitation at 480 nm; emission at 680 nm; Bio-Tek synergy MX plate reader, Winooski, Vermont, U.S.A.). We used these data (supporting information Fig. S2) to determine when all the cultures had reached quasi steady-state (which varied depending on growth temperature), and ended the experiment at this point (21 days).

Biomass measurements

Due to the small culture volumes and high number of experimental units, we were not able measure biomass directly, but used extracted Chl *a* as a proxy for biomass. This was measured as follows: the sample volume that was removed from each well during dilution was pipetted directly into a new 96 well plate, which was covered with sealing tape and frozen at −20°C. Following the experiment, the plates were freeze-dried (for 15–20 h) to remove the water, such that ethanol (96%) could be added directly to the wells, and pigments extracted for 4 h at 4°C in the dark. Method testing has shown that extraction yield was practically the same after 4 h compared as after 24 h (data not shown). The extracted Chl *a* was quantified by fluorescence (using the plate reader; excitation at 425 nm and emission 675 nm).

Statistical analysis and hypothesis

The design of the supply N:P ratio gradient (Fig. 1b) predicts that the biomass yield at (quasi) steady-state should increase monotonously with supply N:P ratio as long as N is limiting, reach a peak at the optimal supply N:P ratio, and decrease as P becomes limiting (Fig. 1d). Under a null hypothesis (H_0) of no effect of temperature on the optimal supply N:P, the biomass peak would occur at the same supply N:P ratio for all temperatures. The alternative hypothesis (H_a) predicts that the peak's position should occur at higher supply N:P ratio at higher temperature (Fig. 1d, dashed lines). To test H_a against H_0, we used the extracted Chl *a* as a proxy for biomass. Ideally, we would have used the steady-state Chl *a* from the end of the experiment as the response variable; however, we experienced a tendency for the cultures growing at the highest supply N:P ratios (N:P = 96 and to some degree N:P = 54) to wash out 4-10 days after reaching asymptotic biomass (supporting information Fig. S2). Thus, since some of the cultures in these small volumes did not maintain steady-state for an extended period, we used "realized" steady-state Chl *a* as the response variable. This was calculated as the mean of the three highest Chl *a* concentrations during the growth period from each experimental unit.

We estimated the optimal supply N:P at each temperature using generalized additive models (GAMs; Wood 2011). A GAM allows the response variable to be fitted as a smooth function of the predictor variable, and is thus well suited for modelling nonlinear relationships like the "peak-shaped" relationship between steady-state Chl *a* and supply N:P ratio expected from our experimental design. At each temperature level, we fitted a GAM to the relationship between steady-state Chl *a* and log-transformed N:P ratio (supporting information S1). Optimal supply N:P was taken as the supply N:P ratio where the function had its maximum value. Confidence intervals for the optimal supply N:P values were estimated by means of sampling from the posterior distribution of the coefficient estimates for each GAM (Wood 2010; Orr et al. 2015; described in supporting information S1). Models were fitted using the *gam* function in the *mgcv* package (Wood 2011) in R (R Core Team, 2014). The effect of temperature on the optimal supply N:P estimates was tested by linear regression.

Results

For all temperatures steady-state (or maximum realized) Chl *a* increased with supply N:P ratio when N was limiting, reached a peak at the optimal supply N:P, and decreased with supply N:P ratio as P became limiting (Fig. 2A). Data from the lowest temperature (10.3°C) had to be omitted because growth rate was too low relative to the dilution rate for the cultures to reach steady state.

The optimal supply N:P ratio (atomic) increased with temperature from ca. 26.5 at the lowest temperatures to ca. 36.5 at the highest (Fig. 2B; the error bars represent 95% confidence intervals estimated by the posterior simulation; exact estimates and confidence limits are given in supporting information Table S1). This represents a change in N:P requirement of ∼ 38%. The data were best described by a third degree polynomial ($R^2 = 0.96$, cross-validation prediction error = 1.1), indicating that the relationship with temperature was closer to s-shaped than linear. A linear fit was, however, also highly significant ($p < 0.0001$), but explained slightly less of the variation in optimal N:P ($R^2 = 0.92$, cross-validation prediction error = 1.95). Judging from the polynomial regression fit, the change in optimal supply N:P was steepest close to 15°C. Peak Chl *a* concentration, taken as the Chl *a* concentration at the optimal supply N:P ratio, increased with temperature (Fig. 2C), suggesting higher Chl *a* content of cells grown at high temperature and/or higher carrying capacity at high temperature.

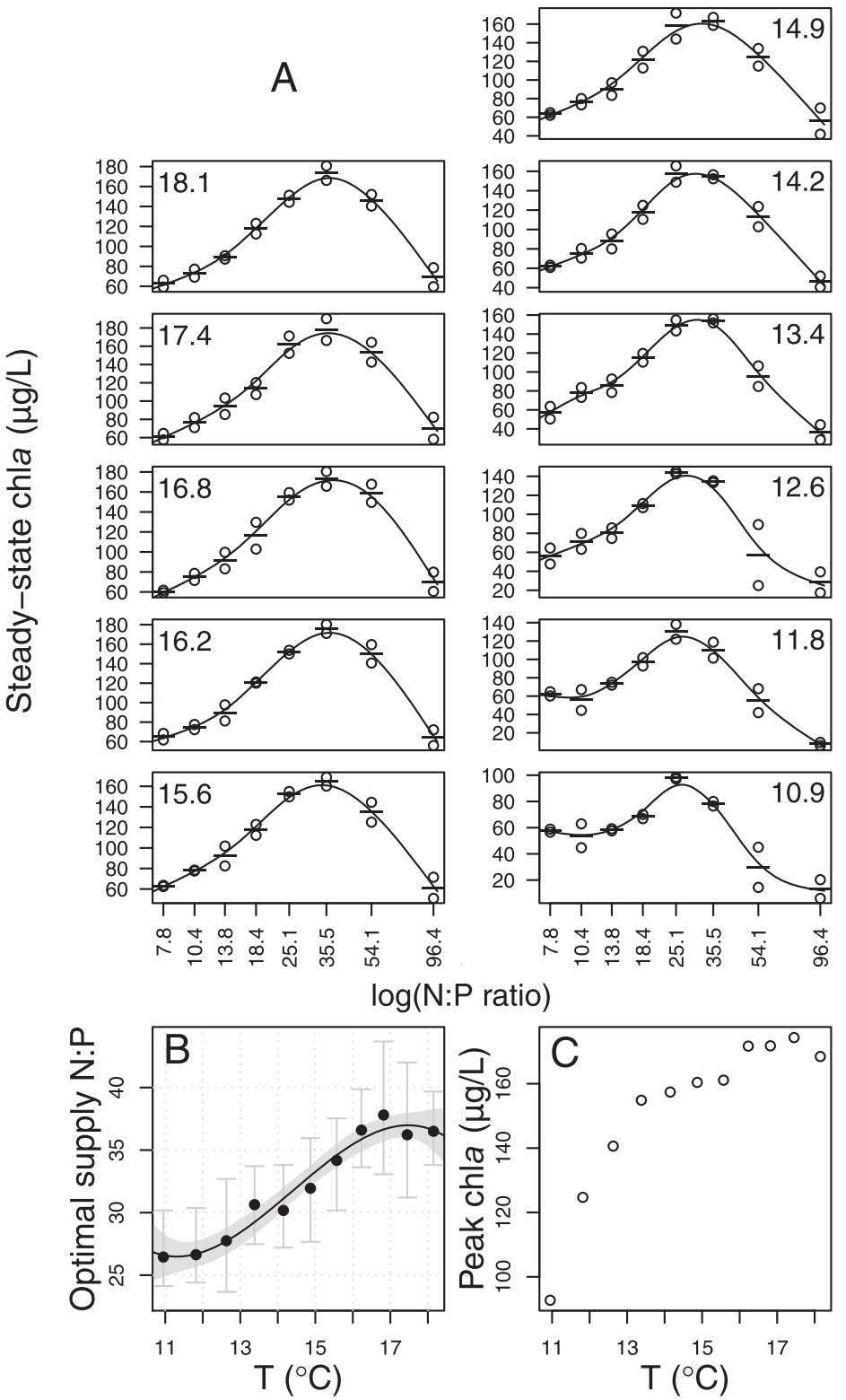

Fig. 2. (A) The response of steady-state Chl *a* concentration (µg/L) to supply N:P ratio for each temperature level. Note the logarithmic x-axis. Temperatures (°C) are given in the upper corner of each sub-plot. The dots represent replicates, while the smooth lines represent the fitted GAMs. (B) Estimates of optimal supply N:P ratio plotted as a function of temperature. Error bars represent 95% confidence intervals for the optimal supply N:P based on a posterior simulation (see supporting information S1). The black line is a third degree polynomial regression fitted to the point estimates of the optimal supply N:P ratios, with a 95% confidence bands in grey. (C) Peak Chl *a* concentration versus temperature.

Discussion

Our experimental data from *C. reinhardtii* supported the hypothesis that the optimal supply N:P ratio increases with temperature. The optimal supply N:P ratio, as quantified here, refers to the dissolved N:P ratio in the growth medium at the tipping point between N and P limitation (i.e., the dissolved N:P ratio yielding N and P co-limitation). Hence, it differs from an optimal N:P ratio based on cellular N:P. Whether or not the cellular N:P ratio matches the supply N:P at the tipping point between N and P limitation is not well known, mainly due to lack of good data. It seems likely, however, that cells growing in a medium with a dissolved N:P yielding co-limitation would contain N and P in the same proportion as the medium. Under co-limitation, neither N nor P are supplied in surplus relative to the cellular requirements, and the cells have little possibility for "luxury uptake" and storage of N or P that could drive the cellular N:P away from the optimum ratio. Along the supply N:P gradient in our experiment, however, we would expect that the N:P ratio of the cells followed some kind of relationship with the supply N:P due to luxury consumption of the non-limiting nutrient (especially P). Phytoplankton are generally flexible in their cellular N:P stoichiometry, which depends strongly on the N:P ratio of the growth medium under nutrient limited conditions (Rhee 1978; Geider and La Roche 2002; Hillebrand et al. 2013).

An increasing optimal supply N:P with temperature suggests that at high temperature, the cells require more N relative to P, or vice versa at low temperature. An increasing ratio, however, can be related either to a higher cellular requirement for N or to a lower requirement for P. What cellular mechanism may explain the changing ratio in our study? Although we were not able to directly measure the cellular concentrations of ribosomes due to small culture-volumes, the change in optimal N:P is consistent with the hypothesis of increased ribosome content at low temperature (Toseland et al. 2013). Increasing the intracellular concentration of ribosomes may, at least partially, compensate for the reduction in ribosome-specific protein synthesis rate caused by a lower growth temperature (Raven and Geider 1988; Woods et al. 2003; Toseland et al. 2013). Since ribosomes have a low N:P ratio (7.2:1 by atoms; Sterner and Elser 2002) compared to the cell as a whole, increased ribosome content would reduce the optimal N:P ratio. It is also possible that the shift in optimal N:P was related to the content of light harvesting pigment-protein complexes. These complexes are rich in N and comprise a large and variable fraction of total cellular protein (18-50% of total protein in algae is likely associated with pigments (Geider and La Roche 2002)). Since light absorption is independent of temperature, but the rate of downstream enzyme-catalyzed processes like CO_2-fixation decreases as it gets colder (Raven and Geider 1988), low growth temperature can result in excess light energy and increased likelihood of photo-oxidative stress (Davidson 1991). To avoid such harmful effects, algae typically decrease the concentration of light harvesting pigments when growing at low temperature (Geider 1987; Maxwell et al. 1994). That we observed a positive relationship between peak Chl *a* concentration and temperature (Fig. 2C) suggests that higher light harvesting complex content may have contributed to increased optimal N:P in our study. The positive relationship between Chl *a* and temperature may also be related to higher carrying capacity at high temperature, which would be expected if resource use efficiency (i.e., biomass per limiting nutrient) increases with temperature. This pattern was observed in a mesocosm study of natural communities dominated by green algae (De Senerpont Domis et al. 2014).

Since the carboxylase activity of Rubisco has a similar temperature dependency as protein synthesis (a Q_{10} of $\sim$ 2; Raven and Geider 1988), an increase in the cellular concentration (or activity) of Rubisco may also be expected during acclimation to lower temperatures. This would entail a higher demand for N, potentially contributing to increased optimal N:P at low temperature. Still, while a negative relationship between cellular Rubisco-content and temperature is indicated in a few studies (e.g., Mortain-Bertrand et al. 1988; Devos et al. 1998), there is little experimental support for a clear relationship between Rubisco concentration and temperature. Moreover, since Rubisco generally contributes to a relatively small fraction of total cellular protein ($<$ 6%; Losh *et al*. 2013), a significant relative change in Rubisco content with temperature would likely only impose a relatively small absolute effect on the total N-requirement of the cell.

We had no a priori expectation about the shape of the relationship between optimal N:P ratio and temperature, but found that an s-shaped relationship fitted the data best. Such a relationship may reflect that there must be a lower and an upper limit to the optimal N:P ratio of a species, which again is set by physiological constraints on the ratio of nonstorage N- and P-rich macromolecules, e.g., the ratio of protein to (RNA + DNA + phospholipid; Geider and La Roche 2002). It is worth noticing that the change in optimal N:P with temperature was highest at intermediate temperatures (around 15°C). Hence, a rather small temperature change in this range could cause a relatively large change in optimal supply N:P, and thus in the threshold between N and P limitation.

Our experimental results regarding the optimal supply N:P are in accordance with the positive correlations observed between temperature and the N:P ratio of marine seston (Martiny et al. 2013, 2016; Yvon-Durocher et al. 2015), and temperature and cellular N:P ratio within species (Yvon-Durocher et al. 2015). While the trends in seston N:P ratios likely are influenced by both differences in community composition, ambient nutrient concentrations, and direct temperature effects (Martiny et al. 2013; Galbraith and Martiny 2015; Yvon-Durocher et al. 2015), our study focused

solely on the temperature-related plasticity response. Since we addressed the optimal supply N:P, our results indicate that there are direct temperature effects on the N:P requirement ratio (excluding the influence of excess storage products such as P in polyphosphate, which may affect the cellular N:P strongly), supporting the idea that the threshold between N and P limitation could increase with warming (Toseland et al. 2013).

Since the cultures were grown semi-continuously over a three week period, we did not consider it necessary to pre-acclimate the algae to the given supply N:P and temperature regime before the experiment started – the cells should have had enough time to acclimate given that many acclimation responses happens with one or a few generations. Still, it is possible that the tendency for the cultures at the highest N:P ratios to wash out after 4-10 days after reaching asymptotic biomass could have been avoided with a proper pre-acclimation period. However, the decline in biomass may also have been caused by another, unknown factor.

We cannot exclude the possibility that adaptation also may have played a role in the change in optimal supply N:P with temperature. I.e., if the stock culture contained different genotypes differing in their N:P requirement, these may have experienced differential fitness along the temperature gradient, thereby contributing to the variation in optimal supply N:P. The stock culture used for inoculating was, however, based on a single *C. reinhardtii* strain, which genetic makeup presumably should be rather uniform. Hence, it is most likely that plastic responses in N:P requirement caused the shift.

While the microplate-design allows a high number of experimental combinations and replication, it comes with the downside of small volumes available for measurements. Since Chl *a* could be determined accurately from small volumes, we used this variable as a proxy for biomass throughout the experiment. One could argue that another measure, like particulate C concentration, would have been a better estimate of biomass because the amount Chl *a* per biomass can vary with temperature (Geider 1987) and the degree of nutrient limitation (Kruskopf and Flynn 2006). Still, we believe that using Chl *a* should be adequate to estimate the optimal supply N:P ratio because this parameter only depends on the relative response of a proxy for biomass to supply N:P ratio within each temperature level such that the position of the biomass peak can be identified. As long as Chl *a* per biomass is constant within each temperature level, optimal supply N:P can confidently be estimated. We are aware that Chl *a* per biomass can be affected by the degree of N-limitation, and that the concentration of N in the medium changed with supply N:P ratio within each temperature level. Still, since the algae were grown as semi-continuous cultures with the same dilution rate for all experimental units, the degree of nutrient limitation should be similar for the different supply N:P ratios. The experiment was designed to obtain differences in biomass along the supply N:P gradient, but at quasi steady state, the dilution rate is what matters for the degree of nutrient limitation – not the biomass. We are therefore confident that Chl *a* per biomass was little affected by differences in N-limitation within each temperature level.

Our estimates of the optimal supply N:P were well above the Redfield N:P of 16:1. However, optimal N:P ratios are often found to exceed Redfield proportions (see e.g., Table 5 in Leonardos and Geider 2005), which implies that microalgae may experience N-limitation for N:P ratios above the commonly used threshold of 16:1. Our results further suggest that the threshold between N and P limitation for a given species can be influenced by temperature, with higher temperature leading to increased N-demand relative to P. Hence, judging from our results with *C. reinhardtii*, higher temperatures could shift a phytoplankton population toward N-limitation, and if this is a general phenomenon (yet likely with different optimal N:P ratios and temperature responses between taxa), it could have major impacts in ecosystems undergoing both changes in N:P-ratios (Elser et al. 2009) and global warming.

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Conflict of Interest

None declared.

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