ECOLOGY LETTERS

Ecology Letters, (2016) **19**: 880–888 doi: 10.1111/ele.12623

LETTER

The impact of irradiance on optimal and cellular nitrogen to phosphorus ratios in phytoplankton

Jan-Erik Thrane,* Dag O. Hessen and Tom Andersen

Section for aquatic biology and toxicology (AQUA) Department of Biosciences, University of Oslo, P.O. Box 1066, Blindern, 0316 Oslo, Norway

**Correspondence: E-mail: j.e.thrane@ibv.uio.no*

Abstract

Phytoplankton acclimates to irradiance by regulating the cellular content of light-harvesting complexes, which are nitrogen (N) rich and phosphorus (P) poor. Irradiance is thus hypothesised to influence the cellular N : P ratio and the N : P defining the threshold between N and P limitation (the 'optimal' N : P). We tested this hypothesis by first addressing the response of the optimal N : P to irradiance in a controlled experiment with *Chlamydomonas reinhardtii*. Then, we did a meta-analysis of experimental data on optimal and cellular N : P ratios across light gradients to test the generality of an N : P to light response within species. In both the experiment and the meta-analysis, N : P ratios decreased with irradiance, indicating that factors affecting underwater irradiance, like depth and the composition of the water, may influence the relative N : P requirement. The effect of irradiance did not differ between optimal and cellular N : P ratios, but observations of optimal N : P were on average 2.8 times higher than observations of cellular N : P.

Keywords

Chlamydomonas reinhardtii, ecological stoichiometry, irradiance, light, N : P ratio, nitrogen, optimal N : P, phosphorus, Redfield ratio.

Ecology Letters (2016) **19**: 880–888

INTRODUCTION

Since Redfield (1958) published data on plankton and deep water nitrogen (N) : phosphorus (P) ratios, suggesting a biological control of the deep ocean stoichiometry, phytoplankton has often been assumed to contain N and P in a molar ratio of 16 : 1 (the 'Redfield N : P'). This ratio is widely used as a reference to differentiate between N and P limitation of phytoplankton production (Geider & La Roche 2002; Ptacnik *et al*. 2010) and is thought to set an upper limit to the dissolved N : P in the deep ocean (Falkowski 1997).

Although the distributions of phytoplankton (or actually, seston) N : P average close to 16 : 1 across large regions of the ocean (Copin-Montegut & Copin-Montegut 1983), there is substantial variation in N : P both among marine and freshwater ecosystems (Sterner *et al*. 2008; Martiny *et al*. 2013). Apart from the variation caused by contributions from non-algal particles in the seston (Frigstad *et al*. 2011), the bulk N : P is influenced by community composition (Weber & Deutsch 2010; Martiny *et al*. 2013) because different lineages show distinct stoichiometric signatures (Quigg *et al*. 2003). Ambient nutrient concentrations also drive variation in N : P. Such effects may be seen in natural communities (Galbraith & Martiny 2015), but are typically much stronger if nutrients are manipulated in phytoplankton cultures. Experiments have shown that at low growth rates, the cellular N : P can match the supply N : P over a wide range of dissolved N : P ratios due to excess or 'luxury' uptake of the nonlimiting nutrient (Rhee 1978). At higher growth rates, the cellular N : P converges (Goldman *et al*. 1979; Elrifi & Turpin 1985), eventually reaching the species' 'optimal' N : P under exponential growth (Klausmeier *et al*. 2004; Hillebrand *et al*. 2013).

A less explored source of variation in N : P ratios is physical factors like light and temperature. When actively growing, phytoplankton assimilate N and P in a ratio that depends on the relative demand for these elements in the biochemical machinery that drives cellular processes like light harvesting and protein synthesis (Geider & La Roche 2002; Sterner & Elser 2002). As physical factors influence these processes, phytoplankton populations acclimating to different levels of, for example, irradiance or temperature might experience different requirements for N relative to P.

Gradients in irradiance can be strong both within (e.g. due to vertical mixing) and between (e.g. related to varying concentrations of dissolved organic matter in lakes, or distance from the coast in the ocean) aquatic ecosystems. Variation in irradiance leads to photoacclimation, which can affect the allocation of resources within the cell (Falkowski & LaRoche 1991; Leonardos & Geider 2004). Generally, photoacclimation is manifested as increased or decreased intracellular concentrations of light-harvesting components, either to increase growth rate at low light or to avoid photo-oxidative stress at high light (Falkowski & LaRoche 1991). A common acclimation response in eukaryotic algae, for example, is to adjust the number of photosystem II chlorophyll light-harvesting complexes (Falkowski & LaRoche 1991; Kirk 2011). Comparing high- and low-light-acclimated chlorophytes, one might find a 2- to 20-fold difference in the concentration of these complexes (Sukenik *et al*. 1990; Tanaka & Melis 1997). Other phytoplankton groups, like cyano- and cryptophytes, may acclimate by adjusting the cellular content of light-harvesting phycobiliproteins (MacIntyre *et al*. 2002). Light-harvesting complexes are rich in N because the pigments are bound to proteins (which contain ~ 17% N by weight; Sterner & Elser 2002). As these light-harvesting proteins generally constitute a large but variable fraction of total cellular protein (likely between 18 and 50%; Geider & La Roche 2002),

photoacclimation may induce significant variability in N : P requirement.

Based on these observations, one may hypothesise that the N : P requirement should correlate negatively with irradiance due to elevated allocation to N-rich light-harvesting components under low light (Geider & La Roche 2002; Leonardos & Geider 2004). When testing this hypothesis, one would ideally address the 'optimal' N : P ratio – the N : P reflecting the cell's actual physiological requirements at a given growth rate without the confounding effects of luxury uptake and storage. There are, however, some conceptual ambiguities on the interpretation of 'optimal' in a physiological context. Originally, the term optimal (or actually, 'optimum') N : P was defined as the cellular N : P at the threshold between N- and P-limited growth. In the context of the Droop model [which relates the nutrient-limited growth rate to the intracellular concentration (quota) of the limiting nutrient; Droop 1973], the optimum N : P can be calculated as the ratio of the subsistence quotas of N and P, assuming that the theoretical maximum growth rates for N- and P-limited growth are equal (Rhee & Gotham 1980). If these differ, then the optimum N : P is expected to deviate from the ratio of subsistence quotas when growth rate increases. The term 'critical' N : P is often used when referring to the optimum N : P at any given growth rate (Terry *et al*. 1985). Others, however, have used the term optimum N : P also in this context (Elrifi & Turpin 1985). In the remainder of this paper, we will for simplicity denote both these threshold-type N : P ratios as 'optimal' N : P ratios [$(N : P)_{opt}$] to distinguish them from cellular N : P ratios [$(N : P)_{cell}$]. Unfortunately, few studies have tested how the $(N : P)_{opt}$ responds to irradiance (but see Wynne & Rhee 1986; Leonardos & Geider 2004, 2005), likely because estimating the $(N : P)_{opt}$ requires growing phytoplankton at a range of dissolved N : P ratios where each nutrient, in turn, is limiting. To address the irradiance effect on N : P requirement, one could also measure the $(N : P)_{cell}$ over a gradient in irradiance (as in Finkel *et al*. 2006), but one might expect a signal from irradiance on the $(N : P)_{cell}$ to be harder to detect due to confounding effects of stored nutrients. Preferentially, the $(N : P)_{cell}$ should be addressed under exponential growth, where $(N : P)_{cell}$ is thought to be similar to the actual requirement ratio, that is the $(N : P)_{opt}$ (Klausmeier *et al*. 2004; Bonachela *et al*. 2013).

In this study, we use a two-way approach to test the hypothesis that the N : P stoichiometry of phytoplankton relates negatively to irradiance. First, we carry out a controlled experiment with a green alga (*Chlamydomonas reinhardtii* P.A. Dangeard), examining the effect of irradiance on the $(N : P)_{opt}$ under steady-state growth. Next, we carry out a meta-analysis of published experimental data on $(N : P)_{opt}$ and $(N : P)_{cell}$ measured across irradiance gradients, to assess the generality of an N : P to light response within species.

MATERIALS AND METHODS

Experimental design and implementation

We used white 96-well microplates (μClear bottom, Greiner bio-one, Kremsmünster, Austria) as basic units in the experiment. This allowed us to run a factorial design crossing six levels of irradiance with 16 supply N : P ratios, resulting in 96 combinations of irradiance and N : P per microplate (Fig. 1a). Three replicates were run in parallel.

A pilot experiment showed that the specific growth rate for *C. reinhardtii* was light limited below ~ 50 μmol photons m^{-2} s^{-1}, hence we chose an irradiance gradient with six equally spaced steps ranging from 10 to 60 μmol photons

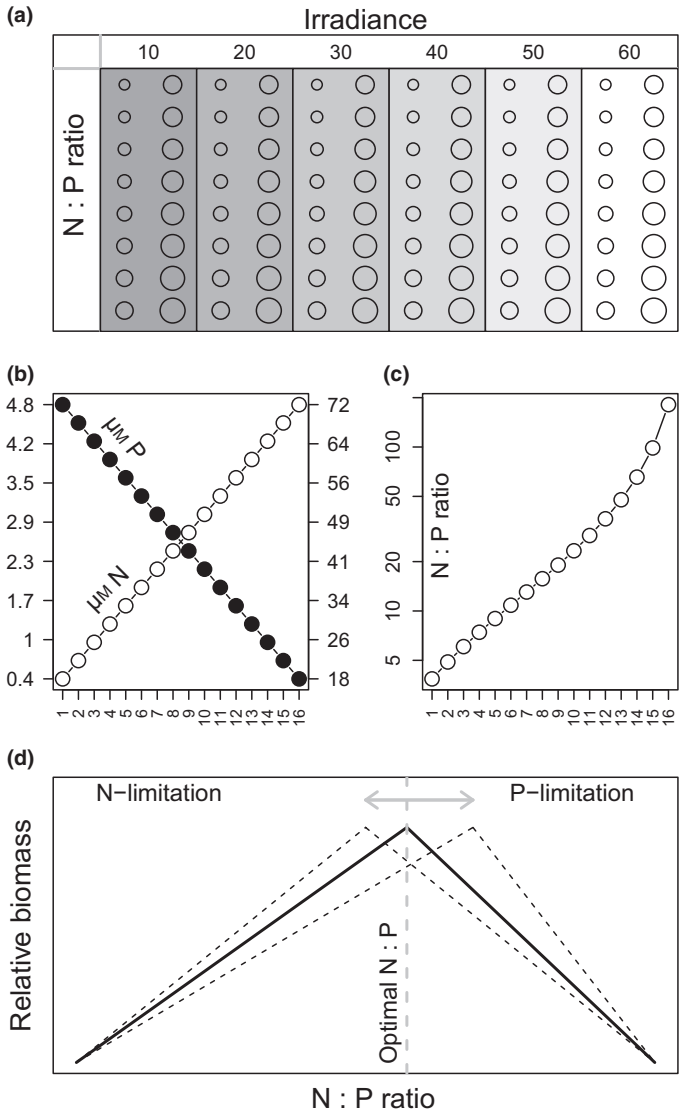

Figure 1 Experimental design. (a) Layout of the 96-well microplate. Two and two columns received the same irradiance (μmol photons m^{-2} s^{-1}). Within each irradiance level, each of the 16 wells received one of 16 different growth media in a gradient of increasing supply N : P ratios (represented by circles of increasing size). (b) μmolar concentrations of P (black dots) and N (white dots) in the 16 different supply N : P ratios, which are shown in (c). (d) Predicted development of steady-state biomass as a function of supply N : P ratio. Biomass (chl*a* concentration) is expected to increase with N : P in the N-limited part of the gradient, reach a peak at the optimal N : P and then decrease as P becomes limiting. Any effect of irradiance on the relative requirement for N and P would shift the peak's position along the *x*-axis. We hypothesise that the peak should shift to a higher N : P as irradiance decrease.

m^{-2} s^{-1} (Fig. 1a). The irradiance gradient was generated using a custom-made incubator with 96 white light-emitting diodes (LEDs) fitted to the geometry of a 96-well microplate. The LEDs were individually controlled with an Arduino microcontroller, using calibration factors determined with a miniaturised spherical irradiance sensor (Walz GmbH, Effeltrich, Germany). When calculating the intensity to programme each LED, we used a set of linear equations incorporating the translucency of the well material and the number of neighbouring LEDs. That way, we were able to correct for cross-contamination of light between wells. Unfortunately, data from the lowest irradiance level had to be omitted because population growth was too slow relative to the dilution rate (described below).

To obtain a gradient in supply N : P ratios, we first prepared two different stock media A and B. Both media were based on the same natural lake water, which had background concentrations of dissolved inorganic N of 15 μM and dissolved inorganic P below the detection limit (< 0.03 μM P). To medium A, we added 60 μM N (as $NaNO_3$), yielding N and P concentrations of 75 μM and 0.0 μM respectively. To medium B, we added 5 μM P (as K_2HPO_4), yielding P and N concentrations of 5.0 μM and 15 μM respectively. We added a standard trace metal and vitamin mix according to the WC medium of Guillard & Lorenzen (1972) to ensure that these elements were nonlimiting. The 16 supply N : P ratios were made by mixing an increasing fraction of medium A with a decreasing fraction of medium B (Fig. 1b) in 100 mL glass bottles. The resulting N : P gradient (atomic) was centred around the Redfield N : P (16 : 1 by moles) and spanned from 4 to 181 (Table S1, Fig. 1c). All media were titrated to pH = 7.0 using CO_2-enriched water before sterile filtration (0.2 μm pore size) and storage at 4 °C.

The concentrations of the limiting nutrient along the gradient had to be carefully considered to avoid self-shading and dissolved inorganic carbon (DIC) limitation. Despite a large surface to volume ratio in these shallow wells and thus likely a good exchange with atmosphere, DIC limitation could in principle still occur because bubbling with air was not feasible with the well-plate setup. To minimise the risk of DIC limitation, we used a medium based on natural high-alkalinity lake water (alkalinity = 2.05 mEq L^{-1}, pH = 8.4). The carbonate fraction of DIC increases rapidly as pH exceeds 9 (Stumm & Morgan 1996), at which point DIC limitation becomes likely because no alga can utilise carbonate for C fixation (Maberly & Spence 1983). Using the *AquaEnv* package (Hofmann *et al.* 2010) in R (R Development Core Team 2013), we calculated that 0.58 mM DIC needed to be taken up by the algae for pH to increase from 7 to 9. Assuming Redfield proportions, a consumption of 0.58 mM DIC would require 5.5 μM of P or 87.5 μM N. Hence, to avoid DIC limitation, the concentrations of P and N (when limiting) were held below these levels (Table S1). As an additional test of DIC limitation, we carried out a batch culture experiment assessing the biomass development over a nutrient gradient. The results indicated that DIC would not be limiting under the experimental conditions applied in the main experiment (see Appendix S1).

At the start of the experiment each well was filled with 320 μL of medium and inoculated with 2 μL (~1000 cells) of *Chlamydomonas reinhardtii* (strain CC-1690 21 gr mt+) stock culture. The stock culture was grown under 50 μmol photons m^{-2} s^{-1} in the same medium as used in the well plates, but with N and P concentrations of 100 and 5 μM respectively. Transparent sealing tape (BarSeal, Thermo scientific Nunc, Waltham, MA, USA) was used to reduce evaporation, but to allow transmission of CO_2. Temperature was kept stable at 19 °C (in a climate room) and we applied a 12/12-h light/dark cycle. The cultures were grown semi-continuously with a dilution rate (D) of 0.25 d^{-1}, which was achieved by replacing 50% of the well volume with fresh medium every second day using a multichannel pipette. This regime assured that all experimental units ended up at the same (quasi) steady-state growth rate. We ended the experiment when all units had reached steady state.

Response variables

To determine when the cultures reached steady state, we measured the chl*a* *in vivo* fluorescence (IVF; excitation at 460 nm, emission at 680 nm, BioTek synergy MX plate reader, Winooski, VT, USA) prior to every dilution. At day 26, all units had reached steady state and the cultures were harvested for sampling. As a proxy for steady-state biomass we used extracted chl*a* concentration ([chl*a*]; μg L^{-1}). Although the ratio of chl*a* to carbon (C) or biomass varies with irradiance, it should not pose a problem for the estimation of $(N : P)_{opt}$ (see next section). We measured [chl*a*] by freeze-drying the whole well plate for 24 h to remove water and improve extraction yield (Hagerthey *et al.* 2006). Subsequently, we added 96% ethanol to the wells, and extracted the pigments for 20 h in the dark at 4 °C. The [chl*a*] in each well was measured fluorometrically (excitation at 430 nm, emission at 675 nm) using the aforementioned plate reader. Method testing has shown that this method yields concentrations that are well correlated with traditional chl*a* measurements on filter-collected cells (data not shown), but with the advantage of requiring only small sample volumes. We also measured the absorbance spectra of the extracts from 400 to 700 nm (1 nm resolution).

To assess photoacclimation, we calculated the ratio of [chl*a*] to growth medium N concentration in the N-limited part of the N : P gradient ($chla_N$; μg chl*a* (μg N)$^{-1}$ for supply N : P < 30) at each irradiance level. This parameter should reflect the amount of chl*a* per cell assuming that the cell number is proportional to the concentration of the limiting nutrient. Further, we utilised the absorbance spectra to estimate the ratio between total chlorophylls (chl = the sum of chl*a*, chl*b*, pheophytin *a* and pheophytin *b*) and total carotenoids (car = the sum of β carotene, neoxanthin, lutein, zeaxanthin, antheraxanthin and violaxanthin) by spectral deconvolution (Küpper *et al.* 2007; Thrane *et al.* 2015). To ensure that our experimental irradiance levels were actually spanning the light-limited part of *C. reinhardtii*'s growth curve, we calculated exponential growth rates for each treatment using the IVF data from the initial days of the experiment. These were modelled as a function of irradiance (E) to estimate the onset of light saturation (E_k). As the experiment was run semi-continuously with dilution every second day, we calculated specific growth rate (μ, day^{-1}) between each dilution event as $\mu = \log(IVF_t/IVF_{t-2})/2$, where IVF_{t-2} is the IVF at the

beginning of the 2-day growth interval and IVF_t is the IVF at the end of the growth interval. The mean of μ's from day 2 to 4 and day 4 to 6 (when growth was exponential) were fitted to the function $\mu(E) = \mu_m(1 - \exp[-(E-E_0)/(E_k-E_0)])$ (Peterson *et al*. 1987), and the parameters (μ_m, the light saturated growth rate; E_k, the onset of light saturation and E_0, the compensation irradiance) for each supply N : P estimated with a nonlinear mixed-effect model (Pinheiro & Bates 2000).

Assessment of the optimal N : P ratio

The design of the experimental N : P gradient (Fig. 1b,c) predicts that the steady-state biomass should increase with supply N : P ratio when N is limiting, reach a peak at the $(N : P)_{opt}$ – where N and P are co-limiting – and decrease with supply N : P as P becomes limiting (Fig. 1d). Our data suggested that the flanks of the relationship between biomass and supply N : P were close to linear when plotting log(N : P) on the *x*-axis (see Results). Hence, we used piecewise regression, which estimates the slopes of the two linear relationships and the breakpoint between them to estimate the $(N : P)_{opt}$. Under a null hypothesis of no effect of irradiance on $(N : P)_{opt}$, the breakpoint will occur at the same supply N : P ratio for all irradiance levels. Alternatively, the breakpoint could shift to a higher N : P as irradiance decreases (Fig. 1d, dashed lines). We tested this hypothesis by fitting a piecewise regression (using the R-package *SiZer*; Sonderegger *et al*. 2009) to each of the irradiance-specific relationships between steady-state [chl*a*] and log(N : P). The breakpoint estimates [i.e. the $(N : P)_{opt}$ estimates] from these regressions were then modelled as a simple linear function of irradiance. We estimated the uncertainty in the breakpoints by bootstrapping the original data (sampling with replacement from the three replicates at each supply N : P), fitting piecewise regressions to the bootstrapped data set, and regressing the bootstrapped breakpoints against irradiance. The 2.5 and 97.5 percentiles of the distribution of regression slopes was used as a confidence interval for the effect of irradiance on $(N : P)_{opt}$.

Meta-analysis of the effect of irradiance on N : P within species

We identified relevant studies by searching ISI Web of Science for various combinations of keywords like nitrogen, phosphorus, elemental composition, optimum and critical nitrogen to phosphorus ratio, in combination with light or irradiance and phytoplankton or algae. Studies were included if they contained observations of $(N : P)_{cell}$ (or cellular N and P from which the ratio could be calculated) measured during exponential growth, or optimal or critical N : P [both denoted as $(N : P)_{opt}$ in our analysis] measured at two or more irradiance levels in single-species experiments. The compiled data set (Table S2) contained 116 observations of N : P measured over an irradiance gradient (median irradiance levels = 5) from 22 unique combinations of study (n = 11), species/strain (n = 20; 4 freshwater, 16 marine, covering 7 taxonomic classes) and experimental condition (one species was measured at three temperatures).

To assess the general effect of irradiance on N : P within species, we analysed the whole data set using a linear mixed-effect model. Such a model is appropriate for meta-analysis of data sets containing variance at the level of experimental unit nested within the full data set, and an uneven number of observations per experimental unit (Pinheiro & Bates 2000; Yvon-Durocher *et al*. 2015). Essentially, we fitted log(N : P) as a linear function of log(irradiance), but treated each of the N : P vs. irradiance relationships as a random sample from a larger 'population' of relationships. In the mixed model, inference is made about the larger population (i.e. the fixed effects; the average slope and intercept), but each unit is allowed to deviate randomly from the population averages by random effects. We also included an additive effect of 'ratio type' [Rt, a factor variable with two levels: $(N : P)_{cell}$ and $(N : P)_{opt}$] to quantify differences between observations of cellular and optimal N : P ratios. As we mainly were interested in the effect of irradiance (E) *within* species (i.e. the acclimation response), we subtracted the mean log(E) in each experimental unit *j* from each of the *i* log(E) values in unit *j*, creating a centred log-transformed irradiance variable $Ec_{ij} = 1\log(E_{ij}) - \overline{\log(E_j)}$ (Van de Pol & Wright 2009). Apart from removing between-unit variation, centring also serves to reduce the correlation between slopes and intercepts (Pinheiro & Bates 2000). The mixed-effect model may then be written as:

$$\log(N:P)_{ij} = (\beta_0 + u_{0j}) + (\beta_1 \times Rt) + (\beta_2 + u_{2j})Ec_{ij} + \varepsilon_{ij}$$

where $\log(N : P)_{ij}$ is the logarithm of the N : P ratio at Ec value *i* in experimental unit *j*, Rt a fixed factor variable taking the value 0 for $(N : P)_{cell}$ and 1 for $(N : P)_{opt}$, Ec the centred log-transformed irradiance and ε_{ij} the residual error term. The parameters are modelled as mixed effects where the β's represent the fixed effects [the average parameter estimates in the whole population of log(N : P) vs. Ec relationships], and u_{0i} and u_{2i} the random effects (deviations from the fixed intercept and slope, respectively, in each unit *j*). Due to the centring, the fixed intercept (β_0) represents the average log(N : P) at mean log irradiance (Ec = 0) for observations of $(N : P)_{cell}$. For observations of $(N : P)_{opt}$, average log(N : P) at mean log irradiance is $(\beta_0 + \beta_1)$.

To determine the best mixed-effect model structure, we followed Zuur *et al*. (2009, p. 127–129). We first fitted a model with only fixed effects of Ec, Rt and an interaction (not shown in the above equation) between the two. Then, we tested this model against one with a random intercept, and one with both random intercept and slope. After finding the best random structure, we excluded fixed terms until all were significant at significance level $\alpha = 0.05$. Comparison of models were done using Akaike's information criterion (AIC) and likelihood ratio (LR) tests on models fitted with restricted maximum likelihood (REML) for comparison of random effects, and maximum likelihood for comparison of fixed effects (Zuur *et al*. 2009, p. 121–122). The best model was refitted using REML. Finally, we assessed the model for homogeneity of variance, normality of residuals and normality of random effects.

RESULTS

Experimental results

For all irradiance levels, steady-state [chl*a*] followed a piecewise linear relationship with log supply N : P ratio (Fig. 2).

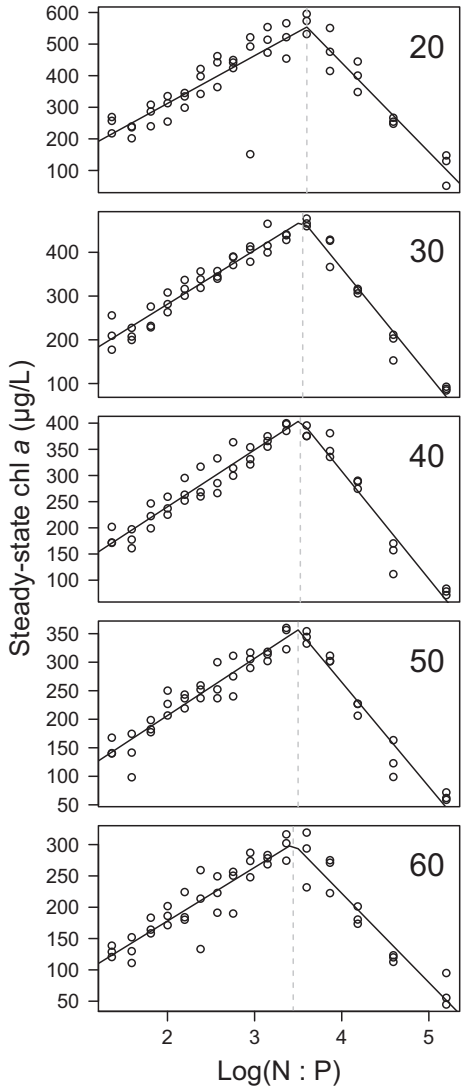

Figure 2 The concentration of steady-state chl*a* (µg/L) vs. supply N : P ratio (log-transformed) plotted for each irradiance level (µmol photons m^{-2} s^{-1}; noted in upper right corner). The three dots at each N : P ratio represent the three replicates, the vertical line and the estimated $(N : P)_{opt}$ from each piecewise regression.

The estimates of $(N : P)_{opt}$ (the breakpoints estimated by piecewise regression) decreased linearly with irradiance (Fig. 3) and values were as follows: 36.6 [irradiance (E) = 20 µmol m^{-2} s^{-1}], 34.9 (E = 30), 33.9 (E = 40), 33.1 (E = 50) and 31.3 (E = 60). The slope of the linear relationship between $(N : P)_{opt}$ and irradiance (Fig. 3) was −0.135 and significantly different from zero (95% bootstrap interval: [−0.27, −0.016]).

The other response variables showed distinct relationships with irradiance (Fig. 4). Specific growth rate (µ) increased asymptotically with irradiance (Fig. 4a), with an onset of light saturation (E_k) of 31.2 ± 1.13 µmol m^{-2} s^{-1} (± SE). Chla_N decreased linearly with irradiance (Fig. 4b), while the ratio of car:chl (w/w) increased monotonously from 0.21 at the lowest to 0.30 at the highest irradiance (Fig. 4c).

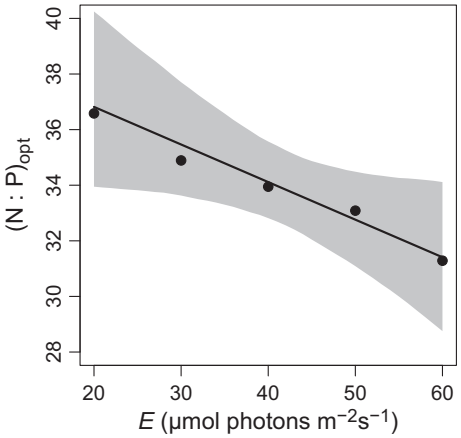

Figure 3 The estimates of optimal N : P ratio $[(N : P)_{opt}]$ for *Chlamydomonas reinhardtii* as a function of irradiance. The grey band is the 95% prediction interval obtained by 5000 bootstrap iterations of the data (see methods), while the solid line represents the average regression line from these iterations.

Results from the meta-analysis of the effect of irradiance on N : P within species

The best mixed-effect model (Table 1) revealed a significant negative effect of Ec on log(N : P) within species ($p < 0.01$, Fig. 5). The slope for this relationship was −0.11 (the fixed effect of Ec), indicating that a doubling of irradiance would lead to ~7% ($1–2^{-0.11}$) decrease in N : P, on average. There was considerable variation between experimental units in the effect of irradiance on N : P, as apparent from the large standard deviation of the random slope effect (0.17) which amounted to 155% of the fixed slope estimate (Table 1). In total, however, 18 of the 22 experimental units had negative slopes (shown in Fig. S1). Observations of $(N : P)_{cell}$ and $(N : P)_{opt}$ were highly different ($p < 0.0001$), with optimal N : P ratios being on average 2.8 times higher than cellular N : P ratios (Table 1, Fig. 5). At mean experimental irradiance (Ec = 0), the average observation of $(N : P)_{cell}$ was 11.9 (95% CI: [9.7, 14.8]), while it was 33.4 (95% CI: [25.4, 44.1]) for $(N : P)_{opt}$ (Table 1). There was no interaction between Ec and Rt ($p = 0.75$, LR test), hence the response of N : P to irradiance was similar for observations of both ratio types.

DISCUSSION

The $(N : P)_{opt}$ decreased with increasing irradiance in *C. reinhardtii*, supporting the hypothesis of an increased N requirement relative to P when acclimating to low-light conditions. We are confident that the change in $(N : P)_{opt}$ truly reflects a light acclimation response because the cells were given long time to acclimate (> 3 weeks), and because the response of chla_N (Fig. 4b) and the car:chl ratio (Fig. 4c) to irradiance showed that the cells adjusted their pigment content as expected under different degrees of light limitation (MacIntyre *et al*. 2002). As chl*a* is associated with proteins in light-harvesting complexes, the higher chla_N at low light is consistent with increased $(N : P)_{opt}$ at low light, although this should be

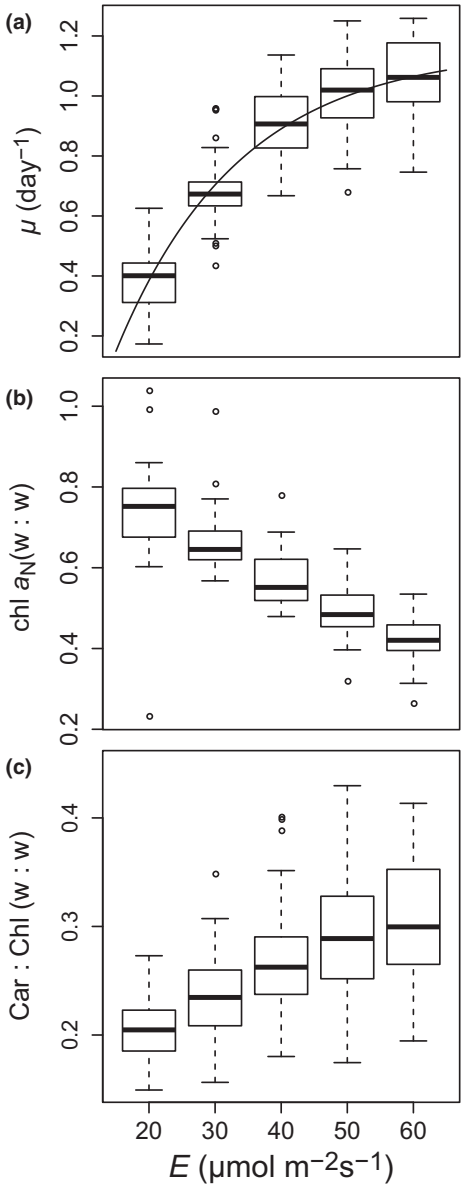

Figure 4 The response of auxiliary variables to irradiance (E). (a) Specific growth rate (μ, d^{-1}). The line is the fitted light response curve drawn using the fixed effect estimates of E_k, E_0 and μ_m. (b) The ratio of chl*a* concentration (μg/L) to N concentration (μg N/L in the medium) for the N-limited cultures (i.e. supply N : P < 30). (c) The ratio between the concentrations (w/w) of total carotenoids (Car) and total chlorophylls (Chl). In all box plots, data from the 16 supply N : P ratios are pooled within each irradiance level.

verified by analysing cellular protein and RNA content (which we were not able to do because of small sample volumes). Further, the estimate of E_k fell in the middle of our experimental irradiance gradient, showing that we captured both the light-limited range and the onset of light saturation for this species.

Growth rate may influence the requirement for P relative to N (cf. the growth rate hypothesis; Sterner & Elser 2002), and there are studies indicating that the $(N : P)_{opt}$ varies unimodally with growth rate in algae (Terry *et al*. 1985; Agren 2004). We did, however, account for this potential confounding factor by applying a single fixed dilution rate for all experimental units. This caused all irradiance treatments to converge at the same (quasi) steady-state growth rate after an initial period of exponential growth, minimising the influence of growth rate on the $(N : P)_{opt}$.

So how universal is an N : P to light response? Our meta-analysis revealed that for the whole 'population' of 22 different N : P vs. irradiance data sets, the average response was negative. Hence, the average species had a higher N : P ratio when acclimated to low (in a relative sense) irradiance, a trend that held true for both for cellular and optimal N : P ratios. One might therefore expect factors affecting the irradiance experienced by a phytoplankton community, like vertical mixing and the composition of the water with regard to other light-absorbing components, to affect the relative N : P requirement. This could also influence coexistence between species if light-related changes in the optimal N : P cause species to be limited by different nutrients (Wynne & Rhee 1986).

Although significant, the size of the irradiance effect estimated from the mixed-effect model appeared relatively modest (an average 7% decrease per doubling of experimental irradiance). Is this effect biologically relevant? Compared to the effect of temperature on N : P, which recently was quantified based on both experimental and natural community data (Yvon-Durocher *et al*. 2015), the light effect might seem small: with temperature, cellular N : P increased at rates of 0.02 $°C^{-1}$ in nutrient replete cultures and 0.032 $°C^{-1}$ in natural samples from the global ocean, leading to a 1.5-fold (over a 20 °C temperature range) and 2.6-fold (over a 30 °C temperature range) change in N : P respectively. On the other hand, irradiance gradients experienced by algae are often strong; one could easily have a 100-fold difference in irradiance between the upper and lower part of a mixed layer (e.g. 1000 μmol m^{-2} s^{-1} at the surface and 10 or less at depths). A 100-fold difference in irradiance would imply a factor 1.67 higher N : P at depth compared to the surface applying our estimated fixed irradiance effect ($1/100^{-0.11}$), hinting that vertical irradiance gradients indeed could have relevance for N vs. P requirement in natural systems.

While increasing the intracellular concentration of light-harvesting pigments and associated proteins at low light is such a widespread response in algae (Falkowski *et al*. 1985; Geider 1987; Tanaka & Melis 1997), it is perhaps unexpected that the slope of the relationship between N : P and irradiance varied so much between experimental units (cf. Fig. S1). Especially when considering that every gram of chl*a* typically is associated with about 5.6 g of protein (Geider & La Roche 2002), equivalent to 0.95 g N (g chl*a*)$^{-1}$ if assuming 0.17 g N (g protein)$^{-1}$. The random slope effects from the mixed model accounts for factors not specifically included in the model, and hence the varying response could be related both to differences in experimental conditions between studies and evolutionary or physiological differences between species. Regarding the latter, phytoplankton species may vary in their ability to adjust the cellular content of light-harvesting components in response to irradiance (Geider 1987; Rodríguez *et al*. 2006). For example, species adapted to fluctuating light environments seem to have narrower ranges of chl*a*:C

Table 1 Results from the best mixed-effect model describing the effect of irradiance (log-transformed and centred; Ec) on log(N : P) within species

Coefficient	Est [95% CI]	*p*	N : P at Ec = 0	Sd RE [95% CI]
Intercept (β_0)	2.48 [2.27, 2.7]	< 0.0001	$\exp(\beta_0) = 11.9$	0.39 [0.28, 0.54]
Rt [$(N : P)_{opt}$] (β_1)	1.03 [0.67, 1.39]	< 0.0001	$\exp(\beta_0 + \beta_1) = 33.4$	0.39 [0.28, 0.54]
Ec (β_2)	−0.11 [−0.2, −0.03]	0.0087	–	0.17 [0.11, 0.26]

Residual standard error: 0.265. The coefficient β_2 represents the fixed effect of Ec on log(N : P). While ratio type (Rt) is included as a fixed factor with levels $(N : P)_{cell}$ and $(N : P)_{opt}$, the intercept (β_0) represents the average log(N : P) at mean irradiance (Ec = 0) across species for observations of $(N : P)_{cell}$. The coefficient β_1 represents the additive effect of $(N : P)_{opt}$ compared to $(N : P)_{cell}$. Transforming to linear scale, $\exp(\beta_0)$ and $\exp(\beta_0 + \beta_1)$ represents average N : P for cellular and optimal N : P ratios, respectively, at mean irradiance. The last column shows the standard deviations of the random effects (RE), which indicate the magnitudes of deviations from the fixed effects in the different experimental units. The best model included REs for both the intercept and the slope.

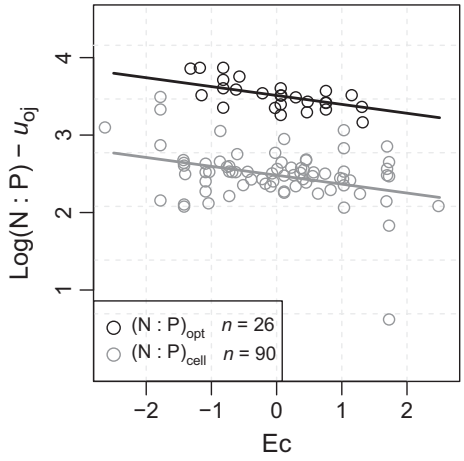

Figure 5 The relationship between log-transformed N : P ratio and centred log-transformed irradiance (Ec) within species. Black dots represent observations of optimal N : P [$(N : P)_{opt}$], grey dots observations of cellular N : P [$(N : P)_{cell}$]. Regression lines are drawn using the estimated fixed effects (Table 1). For both ratio types, log (N : P) decreased significantly with Ec ($p < 0.01$). Observations of $(N : P)_{opt}$, however, were higher on average than values of $(N : P)_{cell}$, as shown by the significantly higher intercept of the regression line for $(N : P)_{opt}$ (black line) compared to $(N : P)_{cell}$ (grey line). Note that the N : P values have been centred by subtracting from each value the deviation in the given experimental unit from the average intercept, that is the random intercept (u_{0j}) estimated by the mixed model. This was done for visualisation only. Figure S1 shows the response in each experimental unit without this centring.

compared to species adapted to more stable light environments (Talmy *et al*. 2013), which would lead to less variation in N demand (and hence N : P) when acclimating to different irradiances. In a marine diatom, Li *et al*. (2015) observed that an increased N requirement for light-harvesting machinery at low light was offset by a lesser N requirement for Rubisco, leading to a small net change in N : P. Such re-distribution of N between biochemical pools might explain some of the variation in the response of N : P to irradiance between species, since the relationship between Rubisco content and irradiance also differs between species (Talmy *et al*. 2013; Li *et al*. 2015; Vandenhecke *et al*. 2015). That the studies included in the meta-analysis differed in their experimental irradiance values also adds to the variation in N : P response. The physiological response of an alga acclimating to a gradient spanning from 20 to 100 μmol photons m^{-2} s^{-1} would likely differ from an alga acclimating to a gradient spanning from 50 to 500. When acclimating to low irradiance (relative to the species' E_k), cells typically produce more light-harvesting machinery to increase light absorption and maintain high growth rate (Falkowski & LaRoche 1991). At irradiances above E_k, however, allocation to light harvesting is expected to go down while photoprotective mechanisms gradually become more important. Processes such as the repair of photosystem II proteins, for example the D1 protein (Demmig-Adams & Adams 1992), could impose significant costs in terms of N when irradiance is high (Li *et al*. 2015; Talmy *et al*. 2013) and might explain why the N : P did not decrease with irradiance for all species (Fig. S1). Finally, while the N : P ratio also depends on the specific P content, a concerted increase in both N and P at low irradiance will cause no change in N : P. Increased cellular content of P have been observed at low light in some species (Rhee & Gotham 1981; Floder *et al*. 2006).

The relationship between N : P and irradiance was not significantly different between observations of $(N : P)_{cell}$ and $(N : P)_{opt}$. Interestingly, however, optimal N : P ratios were 2.8 times higher than cellular N : P ratios on average [mean $(N : P)_{opt} = 33.4$, mean $(N : P)_{cell} = 11.9$]. The cellular N : P ratios included in the meta-analysis were measured under exponential (nutrient replete) growth, where phytoplankton often is assumed to take up nutrients in an optimal ratio (Klausmeier *et al*. 2004). Therefore, the large difference was quite unexpected. The lower cellular N : P ratios, however, might be related to the generally higher capacity for excess uptake and storage of P compared to N (Rhee & Gotham 1980; Elrifi & Turpin 1985). It could also result from differences in uptake rates during exponential growth, if uptake of P exceeds optimal requirements, but whether this is common in algae is poorly understood (Klausmeier *et al*. 2004). Note also that the optimal N : P ratios included here are significantly higher than the Redfield N : P of 16, suggesting that the representation of N : P ratios and requirements is far from trivial, and that the threshold between N and P limitation in many cases might be way above Redfield N : P (see also Leonardos & Geider 2004).

The general negative effect of irradiance on N : P indicates that phenomena affecting light attenuation such as increasing loads of terrestrially derived organic carbon ('browning') in boreal lakes (Monteith *et al*. 2007; Larsen *et al*. 2011) could raise the autotroph demand for N relative to P and hence the

likelihood of N limitation. However, the considerable variation in N : P response between species and the relatively modest average effect of 7% change per doubling of irradiance suggests that factors like increasing in N deposition (Peñuelas *et al*. 2013) may have a stronger impact on N vs. P limitation of the phytoplankton community (driving systems towards P limitation; Elser *et al*. 2009; Peñuelas *et al*. 2013) than changes in light climate as a response to, for example, browning. However, as vertical gradients in irradiance are strong, it is likely that irradiance would influence the N : P requirement of species residing at different depths, possibly also affecting coexistence between species (Rhee & Gotham 1980; Wynne & Rhee 1986).

AUTHOR CONTRIBUTIONS

TA designed and conceived the laboratory experiment. JET did the laboratory work, data analysis, meta-analysis and wrote the first draft of the manuscript. TA and DOH contributed significantly to revisions of the manuscript.

REFERENCES

Agren, G.I. (2004). The C:N: P stoichiometry of autotrophs – theory and observations. *Ecol. Lett*., 7, 185–191.

Bonachela, J.A., Allison, S.D., Martiny, A.C. & Levin, S.A. (2013). A model for variable phytoplankton stoichiometry based on cell protein regulation. *Biogeosciences*, 10, 4341–4356.

Copin-Montegut, C. & Copin-Montegut, G. (1983). Stoichiometry of carbon, nitrogen, and phosphorus in marine particulate matter. *Deep Sea Res. Part A Oceanogr. Res. Pap*., 30, 31–46.

Demmig-Adams, B. & Adams, W.W. (1992). Photoprotection and other responses of plants to high light stress. *Annu. Rev. Plant Physiol. Plant Mol. Biol*., 43, 599–626.

Droop, M.R. (1973). Some thoughts on nutrient limitation in algae. *J. Phycol*., 9, 264–272.

Elrifi, I.R. & Turpin, D.H. (1985). Steady-state luxury consumption and the concept of optimum nutrient ratios: a study with phosphate and nitrate limited Selenastrum minutum (Chlorophyta). *J. Phycol*., 21, 592–602.

Elser, J.J., Andersen, T., Baron, J.S., Bergström, A.-K., Jansson, M., Kyle, M. *et al*. (2009). Shifts in lake N: P stoichiometry and nutrient limitation driven by atmospheric nitrogen deposition. *Science*, 326, 835–837.

Falkowski, P.G. (1997). Evolution of the nitrogen cycle and its influence on the biological sequestration of CO2 in the ocean. *Nature*, 387, 272–275.

Falkowski, P.G. & LaRoche, J. (1991). Acclimation to spectral irradiance in algae. *J. Phycol*., 27, 8–14.

Falkowski, P.G., Dubinsky, Z. & Wyman, K. (1985). Growth-irradiance relationships in phytoplankton. *Limnol. Ocean*., 30, 311–321.

Finkel, Z.V., Quigg, A., Raven, J.A. & Schofield, O.E. (2006). Irradiance and the elemental stoichiometry of marine phytoplankton. *Limnology and Oceanography*, 51, 2690–2701.

Floder, S., Combuchen, A., Pasternak, A., Hillebrand, H., Flöder, S. & Combüchen, A. (2006). Competition between pelagic and benthic microalgae for phosphorus and light. *Aquat. Sci*., 68, 425–433.

Frigstad, H., Andersen, T., Hessen, D.O., Naustvoll, L.J., Johnsen, T.M. & Bellerby, R.G.J. (2011). Seasonal variation in marine C:N: P stoichiometry: can the composition of seston explain stable Redfield ratios? *Biogeosciences*, 8, 2917–2933.

Galbraith, E.D. & Martiny, A.C. (2015). A simple nutrient-dependence mechanism for predicting the stoichiometry of marine ecosystems. *Proc. Natl Acad. Sci*., 112, 8199–8204.

Geider, R.J. (1987). Light and temperature dependence of the carbon to chlorophyll a ratio in microalagae and cyanobacteria: implications for physiology and growth of phytoplankton. *New Phytol*., 106, 1–34.

Geider, R.J. & La Roche, J. (2002). Redfield revisited: variability of C:N: P in marine microalgae and its biochemical basis. *Eur. J. Phycol*., 37, 1–17.

Goldman, J.C., McCarthy, J.J. & Peavey, D.G. (1979). Growth rate influence on the chemical composition of phytoplankton in oceanic waters. *Nature*, 279, 210–215.

Guillard, R.R.L. & Lorenzen, C.J. (1972). Yellow-green algae with chlorophyllide C. *J. Phycol*., 8, 10–14.

Hagerthey, S.E., William Louda, J. & Mongkronsri, P. (2006). Evaluation of pigment extraction methods and recommended protocol for periphyton chlorophyll a determination and chemotaxonomic assessment. *J. Phycol*., 42, 1125–1136.

Hillebrand, H., Steinert, G., Boersma, M., Malzahn, A., Léo Meunier, C., Plum, C. *et al*. (2013). Goldman revisited: faster growing phytoplankton has lower N: P and lower stoichiometric flexibility. *Limnol. Oceanogr*., 58, 2076–2088.

Hofmann, A.F., Soetaert, K., Middelburg, J.J. & Meysman, F.J.R. (2010). AquaEnv: an aquatic acid-base modelling environment in R. *Aquat. Geochem*., 16, 507–546.

Kirk, J.T.O. (2011). *Light and Photosynthesis in Aquatic Ecosystems*, 3rd edn. Cambridge University Press, New York.

Klausmeier, C.A., Litchman, E. & Levin, S.A. (2004). Phytoplankton growth and stoichiometry under multiple nutrient limitation. *Limnol. Oceanogr*., 49, 1463–1470.

Küpper, H., Seibert, S. & Parameswaran, A. (2007). Fast, sensitive, and inexpensive alternative to analytical pigment HPLC: quantification of chlorophylls and carotenoids in crude extracts by fitting with Gauss peak spectra. *Anal. Chem*., 79, 7611–7627.

Larsen, S., Andersen, T. & Hessen, D.O. (2011). Climate change predicted to cause severe increase of organic carbon in lakes. *Glob. Chang. Biol*., 17, 1186–1192.

Leonardos, N. & Geider, R.J. (2004). Responses of elemental and biochemical composition of Chaetoceros muelleri to growth under varying light and nitrate:phosphate supply ratios and their influence on critical N:P. *Limnol. Oceanogr*., 49, 2105–2114.

Leonardos, N. & Geider, R.J. (2005). Elemental and biochemical composition of *Rhinomonas reticulata* (Cryptophyta) in relation to light and nitrate-to-phosphate supply ratios. *J. Phycol*., 41, 567–576.

Li, G., Brown, C.M., Jeans, J.A., Donaher, N.A., McCarthy, A. & Campbell, D.A. (2015). The nitrogen costs of photosynthesis in a diatom under current and future pCO2. *New Phytol*., 205, 533–543.

Maberly, S.C. & Spence, D.H.N. (1983). Photosynthetic inorganic carbon use by freshwater plants. *J. Ecol*., 71, 705–724.

MacIntyre, H.L., Kana, T.M., Anning, T. & Geider, R.J. (2002). Photoacclimation of photosynthesis irradiance response curves and photosynthetic pigments in microalgae and cyanobacteria. *J. Phycol*., 38, 17–38.

Martiny, A.C., Pham, C.T.A., Primeau, F.W., Vrugt, J.A., Moore, J.K., Levin, S.A. *et al*. (2013). Strong latitudinal patterns in the elemental ratios of marine plankton and organic matter. *Nat. Geosci*., 6, 279–283.

Monteith, D.T., Stoddard, J.L., Evans, C.D., de Wit, H.A., Forsius, M., Høgåsen, T. *et al*. (2007). Dissolved organic carbon trends resulting from changes in atmospheric deposition chemistry. *Nature*, 450, 537–540.

Peñuelas, J., Poulter, B., Sardans, J., Ciais, P., van der Velde, M., Bopp, L. *et al*. (2013). Human-induced nitrogen-phosphorus imbalances alter natural and managed ecosystems across the globe. *Nat. Commun*., 4, 2934.

Peterson, D.H., Perry, M.J., Bencala, K.E. & Talbot, M.C. (1987). Phytoplankton productivity in relation to light intensity: a simple equation. *Estuar. Coast. Shelf Sci*., 24, 813–832.

Pinheiro, J.C. & Bates, D.M. (2000). *Mixed-Effects Models in S and S-plus*, 1st edn. Springer, New York.

Ptacnik, R., Andersen, T. & Tamminen, T. (2010). Performance of the Redfield ratio and a family of nutrient limitation indicators as

thresholds for phytoplankton N vs. P limitation. *Ecosystems*, 13, 1201–1214.
Quigg, A., Finkel, Z.V., Irwin, A.J., Rosenthal, Y., Ho, T.-Y., Reinfelder, J.R. *et al.* (2003). The evolutionary inheritance of elemental stoichiometry in phytoplankton. *Nature*, 425, 291–294.
R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2012; ISBN 3-900051-07-0, URL http://www.R-project.org
Redfield, A.C. (1958). The biological control of chemical factors in the environment. *Am. Sci.*, 46, 205–221.
Rhee, G.-Y. (1978). Effects of N: P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake. *Limnol. Oceanogr.*, 23, 10–25.
Rhee, G.-Y. & Gotham, I.J. (1980). Optimum N: P ratios and coexistence of planktonic algae. *J. Phycol.*, 16, 486–490.
Rhee, G.-Y. & Gotham, I.J. (1981). The effect of environmental factors on phytoplankton growth: light and the interactions of light with nitrate limitation. *Limnol. Oceanogr.*, 26, 649–659.
Rodríguez, F., Chauton, M., Johnsen, G., Andresen, K., Olsen, L.M. & Zapata, M. (2006). Photoacclimation in phytoplankton: implications for biomass estimates, pigment functionality and chemotaxonomy. *Mar. Biol.*, 148, 963–971.
Sonderegger, D.L., Wang, H., Clements, W.H. & Noon, B.R. (2009). Using SiZer to detect thresholds in ecological data. *Front. Ecol. Environ.*, 7, 190–195.
Sterner, R. & Elser, J. (2002). *Ecological Stoichiometry: the Biology of Elements from Molecules to the Biosphere*, 1st edn. Princeton University Press, Princeton, New Jersey.
Sterner, R.W., Andersen, T., Elser, J.J., Hessen, D.O., Hood, J.M., McCauley, E. *et al.* (2008). Scale-dependent carbon:nitrogen: phosphorus seston stoichiometry in marine and freshwaters. *Limnol. Oceanogr.*, 53, 1169–1180.
Stumm, W. & Morgan, J.J. (1996). *Aquatic Chemistry: Chemical Equilibria and Rates in Natural Waters*, 3rd edn. Wiley, New York.
Sukenik, A., Bennett, J., Mortain-Bertrand, A. & Falkowski, P.G. (1990). Adaptation of the photosynthetic apparatus to irradiance in Dunaliella tertiolecta: a kinetic study. *Plant Physiol.*, 92, 891–898.
Talmy, D., Blackford, J., Hardman-Mountford, N.J., Dumbrell, A.J. & Geider, R.J. (2013). An optimality model of photoadaptation in contrasting aquatic light regimes. *Limnol. Oceanogr.*, 58, 1802–1818.
Tanaka, A. & Melis, A. (1997). Irradiance-dependent changes in the size and composition of the chlorophyll a-b light-harvesting complex in the green alga *Dunaliella salina*. *Plant Cell Physiol.*, 38, 17–24.
Terry, K.L., Laws, E.A. & Burns, D.J. (1985). Growth rate variation in the N: P requirement ratio of phytoplankton. *J. Phycol.*, 21, 323–329.
Thrane, J.-E., Kyle, M., Striebel, M., Haande, S., Grung, M., Rohrlack, T. *et al.* (2015). Spectrophotometric analysis of pigments: a critical assessment of a high-throughput method for analysis of algal pigment mixtures by spectral deconvolution. *PLoS ONE*, 10, 1–24.
Van de Pol, M. & Wright, J. (2009). A simple method for distinguishing within- versus between-subject effects using mixed models. *Anim. Behav.*, 77, 753–758.
Vandenhecke, J.M.-R., Bastedo, J., Cockshutt, A.M., Campbell, D.A. & Huot, Y. (2015). Changes in the Rubisco to photosystem ratio dominates photoacclimation across phytoplankton taxa. *Photosynth. Res.*, 124, 275–291.
Weber, T.S. & Deutsch, C. (2010). Ocean nutrient ratios governed by plankton biogeography. *Nature*, 467, 550–554.
Wynne, D. & Rhee, G.-Y. (1986). Effects of light intensity and quality on the relative N and P requirement (the optimum N: P ratio) of marine planktonic algae. *J. Plankton Res.*, 8, 91–103.
Yvon-Durocher, G., Dossena, M., Trimmer, M., Woodward, G. & Allen, A.P. (2015). Temperature and the biogeography of algal stoichiometry. *Glob. Ecol. Biogeogr.*, 24, 562–570.
Zuur, A.F., Ieno, E.N., Walker, N.J., Seveliev, A.A. & Smith, G.M. (2009). *Mixed Effect Models and Extensions in Ecology with R*. Springer, New York.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Editor, Helmut Hillebrand
Manuscript received 21 March 2016
First decision made 20 April 2016
Manuscript accepted 30 April 2016