Light, temperature, and nutrients as drivers for primary productivity in phytoplankton

PhD thesis

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List of papers

I

Thrane, J.-E., Hessen, D. O. and Andersen, T. (2016), The impact of irradiance on optimal and cellular nitrogen to phosphorus ratios in phytoplankton. *Ecology letters*. doi:10.1111/ele.12623

II

Thrane, J.-E., Hessen, D. O. and Andersen, T. Plasticity in algal stoichiometry: experimental evidence of a temperature-induced shift in optimal N:P ratio. *Manuscript,* submitted to *Limnology and Oceanography*

III

Thrane, J.E., Hessen, D. O., Andersen, T. (2014), The absorption of light in lakes: negative impact of dissolved organic carbon on primary productivity. *Ecosystems* **17**: 1040-1052

IV

Thrane J.-E., Kyle M., Striebel M., Haande S., Grung M., Rohrlack T., Andersen, T. (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. doi:10.1371/journal.pone.0137645

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Perspective

Phytoplankton and the earth system

The vast group of photoautotrophic unicellular organisms drifting passively around in the water-bodies of planet earth is collectively called phytoplankton. Although constituting members from highly different branches on the tree of life (Bhattacharya and Medlin 1998), the phytoplankton can broadly be divided into two main groups: the photosynthetic bacteria commonly known as cyanobacteria or blue-green algae, and the unicellular, autotrophic eukaryotes. Thriving both in fresh and salt waters, from the smallest ponds to the open oceans, the phytoplankton represents a highly diverse group both morphologically and physiologically. Considering a trait like cell volume, for example, one could fit about 130 million cells of a cyanobacteria like *Prochlorococcus* (~0.6 μm in diameter) within the cell of a large diatom like *Coscinodiscus* (~300 μm in diameter). While most phytoplankton are so minute that they are invisible to the human eye, they nevertheless play a crucial role in major biogeochemical cycles and climate regulation on earth – and have done so for the past couple of billion years.

Cyanobacteria evolved at least 2.7 billion years ago, when the earth's atmosphere was still anoxic (Bekker et al. 2004, Holland 2006). However, as the photosynthetic bacteria grew and photosynthesized, their photosystem II continuously split water to oxygen, hydrogen ions, and electrons, laying out the foundation for aerobic, heterotrophic life to evolve. Through time, the eukaryotic clades of phytoplankton, like the red and the green algae, and later diatoms and dinoflagellates, developed through endosymbiosis, contributing to both increased diversity and further O_2-release and carbon (C) fixation (Falkowski et al. 2004, 2008). When the first land plants evolved about 500 million years ago (Wellman et al. 2003), atmospheric oxygen concentration was already more than halfway to present day levels (Holland 2006).

As primary producers, phytoplankton take up CO_2 and convert it to organic C, thereby providing the basal energy input[1] to pelagic food chains. Hence, the phytoplankton supports higher trophic levels ranging from zooplankton to fish, eventually setting an upper limit to what can be harvested of many commercial fish stocks (Chassot et al. 2010). The marine phytoplankton are responsible for nearly 50 % of the global CO_2 uptake through primary production (Field et al. 1998). By weight, this represents about 50 billion tons C per year, which is equivalent to the C content of about 3800 billions human beings. Annually, some 15 % of this

[1] In lakes, allochthonous C may contribute significantly to the basal energy input (discussed later in this thesis).

photosynthetically fixed C is transported to the deep ocean via the "biological pump"[2] (Laws et al. 2000). Phytoplankton therefore removes large quantities of CO_2 from the upper ocean to deep-water storage as organic or mineral bound C. If all phytoplankton in the ocean would suddenly disappear, the atmospheric levels of CO_2 would instantly increase by 200 ppm (Falkowski 2012). Hence, the marine phytoplankton is one of the most important components in the global C cycle, acting as a buffer against changes in atmospheric CO_2 concentrations.

Phytoplankton do not only affect the C cycle, but other biogeochemical cycles as well (Litchman et al. 2015). On average, phytoplankton communities – both marine and in fresh water – contain C, nitrogen (N), and phosphorus (P) in an atomic ratio close to 106:16:1[3] (Redfield 1958, Copin-Montegut and Copin-Montegut 1983, Sterner et al. 2008). While the C:N and C:P ratios may be used to estimate carbon uptake or export production from nutrient (N or P) concentrations (Geider and La Roche 2002), the phytoplankton N:P ratio impacts aquatic ecosystems on (literally) deeper levels. Alfred C. Redfield was the first to notice the peculiar similarity between the N:P of phytoplankton (actually, seston) and the dissolved N:P ratio of the deep ocean waters, which both were around 16:1 (Redfield 1958). He suggested, and today we know that this was correct, that the pattern emerged because the N and P bound in phytoplankton biomass is released in the deep waters when the cells sink out and break down. Hence, the nutrient requirements and stoichiometry of phytoplankton profoundly shapes the chemical composition of the ocean at large (Falkowski 2000).

Due to the huge difference in global area covered by ocean (~71 %) and lakes (~3 %; Tranvik *et al.* 2009), the large-scale impacts of phytoplankton on climate and biogeochemical cycling are mainly due to the action of marine plankton. Still, the average concentrations of phytoplankton in lakes are generally higher (1-300 mg m^{-3} chlorophyll *a* (chl*a*)) than in the ocean (ca. 0.19 mg m^{-3} chl*a*; Field *et al.* 1998). Most lakes in the world are shallow – with plenty of light and nutrients – and should therefore be among the most productive aquatic systems on earth (Tranvik et al. 2009). In fact, lakes many places are "too productive" (i.e., eutrophic) due to anthropogenic nutrient inputs, and the detrimental effects associated with eutrophication range from harmful algal blooms to oxygen depletion and concomitant fish kills (Heisler et al. 2008,

[2] The downward flux of C from the atmosphere to the ocean interior, driven by the sinking of biological material (Longhurst and Glen Harrison 1989)

[3] But there is large variation between species and within species growing under different environmental conditions (discussed later in thesis).

Smith and Schindler 2009). Hence, much limnological research has focused on finding which factors that limit phytoplankton productivity, with the ultimate aim of reducing eutrophication (Schindler 2006). There are other major changes occurring primarily in many freshwater systems today, such as increasing concentrations of dissolved organic matter (DOM), or "browning" (Monteith et al. 2007), with potentially large impacts on light attenuation and nutrient inputs. Ultimately, this may alter the balance between pelagic and benthic primary production, and autotrophic *vs.* heterotrophic processes (Cole et al. 2000, Karlsson et al. 2009). Also anthropogenic N-deposition (Elser et al. 2009) and climate change (Adrian et al. 2009) is expected to affect lake ecosystems, which calls for a closer examination of freshwater phytoplankton responses.

Despite the differences between freshwater and marine systems, many of the basic aspects in the intersection between phytoplankton physiology, stoichiometry, and productivity are basically similar across salinity gradients. Ultimately, phytoplankton are single cells with a highly conserved photosynthetic machinery (Falkowski et al. 2008) that allows them to harvest solar energy and fix CO_2 to organic C. Regardless of the salinity of their habitat, they require the same basic nutrients (e.g. N, P, and Fe) to grow and build up their biomass (Sterner and Elser 2002). Moreover, their growth and physiology is affected by the same physical factors such as light and temperature. This view will be reflected in the synthesis-part of this PhD-thesis, where I will discuss both limnological and marine literature, trying to put my findings in a larger picture.

Outline of thesis

My PhD thesis evolves around the effects of light, temperature, and nutrients on primary production, resource requirements, and stoichiometry in phytoplankton. In paper **I**, my aim is to test the hypothesis that irradiance influences the N:P stoichiometry of phytoplankton by affecting the allocation to N-rich light harvesting machinery. In a controlled lab-experiment, we address how the tipping point between N and P limitation (the optimal N:P ratio) changes with irradiance for a single species. The results are combined with a meta-analysis of how N:P ratios varies with irradiance within species, to evaluate the generality of the hypothesis. Paper **II** takes a similar experimental approach, but here, we test how temperature-acclimation affects the optimal N:P ratio. Temperature is hypothesized to increase the optimal N:P through differential effects on

biosynthetic rates and light absorption. In paper **III,** we move from the lab to the field, where we aim at disentangling the effects light-availability related to the concentrations of dissolved organic C (DOC), and nutrients (P), on pelagic primary productivity in lakes. Paper **IV** takes a methodological approach. Here, we aimed at further developing a fast and cheap method for pigment analysis by absorbance measurements in microwell-plates.

In this synthesis, I discuss three key drivers for phytoplankton growth, namely nutrients (focusing on N and P), light, and temperature. I will review how these factors influence primary production, and discuss mechanisms through which light and temperature may alter resource allocation and eventually N:P stoichiometry in algae. When discussing light, I will also circle around the effects that DOM may have on light-climate, and eventually on primary production. The most important results from my own papers (particularly paper **I-III**) will be presented as a part of a more general literature review, and I refer to the actual manuscripts for the more detailed information. Paper **IV** will be presented as part of a methodological section at the end, where I highlight some of the methodological challenges related to the different papers. I will end the synthesis by discussing possible impacts of future environmental change on pelagic productivity and nutrient limitation.

Three key drivers for phytoplankton growth

Nutrients: A matter of demand and supply

To synthesize new biomass and divide, all organisms require certain quantities of different chemical elements. These are often grouped into either macronutrients (e.g. C, N, and P) or micronutrients (e.g. Fe, Mn, and Cu). In reality, however, the cellular requirements for the various chemical elements span a continuum, which itself varies both between and within species (Sterner and Elser 2002, Geider and La Roche 2002, Ho et al. 2003, Quigg et al. 2003). Based on precise measurements of the cellular elemental content of 15 different phytoplankton species grown under nutrient replete conditions and similar light and temperature, Ho *et al.* (2003) presented the molar stoichiometry of an average phytoplankter (normalized to P):

$$(C_{124}N_{16}P_{1}S_{1.3}K_{1.7}Mg_{0.56}Ca_{0.5})\times 1000 \quad \text{(Eq. 1)}$$
$$Sr_{5}Fe_{7.5}Mn_{3.8}Zn_{0.8}Cu_{0.38}Co_{0.19}Mo_{0.03}$$

Their values for C:N:P are close to the Redfield ratio ($C_{106}N_{16}P_1$), but Ho *et al*.'s equation takes Redfield a step further by also including trace elements. Strikingly, the range of cellular nutrient concentrations spans over six orders of magnitude, with about 4 million C atoms per atom of molybdenum (Mo). Yet, despite these huge differences in relative requirements, both macro- and micronutrients may limit phytoplankton growth in natural ecosystems.

To determine which elements that may most likely limit phytoplankton production, one could consider the ratio between the elements that are supplied and the elements that are demanded by phytoplankton for growth. The former would be represented by the elemental concentrations in the ambient water, and the latter by the elements in phytoplankton biomass. The elements with the lowest "supply-to-demand" ratios are the most likely candidates for becoming limiting. In this context, limitation refers to the Liebig-type of limitation, which usually means limitation of the biomass yield (de Baar 1994, Moore et al. 2013). Hence, the nutrient might not limit the rate of production at a given time, but will eventually limit for the total biomass that can be formed if biomass is allowed to accumulate (Beardall et al. 2001). Combining data on average elemental ratios in the ocean and rivers (from Hecky & Kilham 1988) with the average phytoplankton stoichiometry from Ho *et al.* (2003) reveals some hot candidates (fig. 1):

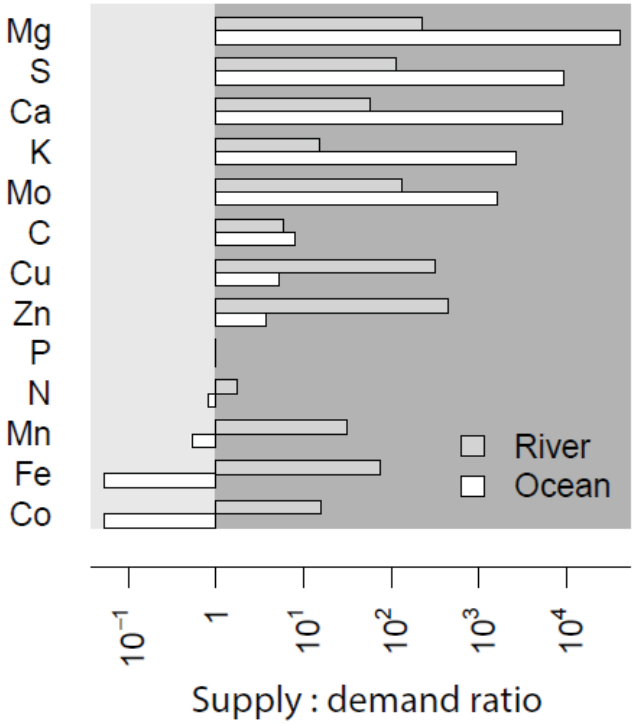


Fig. 1: Supply : demand ratios for different chemical elements required for phytoplankton growth. Supply:demand ratios were calculated by dividing the element:P ratio in average ocean and fresh water (actually, river water; data from table 2 in Hecky & Kilham 1988) by the element:P ratio in phytoplankton biomass growing under nutrient replete conditions (data from Ho *et al.* 2003). For example, if N:P is 30:1 in river water, and 16:1 in algal biomass, the supply:demand ratio is 30:16 ≈ 2. Supply:demand ratios close to or below unity represent potentially limiting nutrients. Ratios > 1 represent nutrients in supplied in surplus relative to phytoplankton demand.

In freshwater, the element with lowest supply : demand ratio is P followed by N. With the exception of inorganic C, which due to its pH-dependency anyways is not straight forward to judge in a simple Liebig-perspective, the other elements in fresh water have supply:demand ratios > 10 and is thus generally not likely to be limiting. According to fig. 1, N and P are also among the most potentially limiting elements in oceanic water, but with N in relative shortage compared to P. Trace elements like Fe, Co, and Mn, have even shorter supply : demand ratios than N and P; not surprising considering that trace element limitation is common in certain oceanic regions (Boyd et al. 2007).

The patterns of supply : demand in fig. 1, gives, of course, only a crude indication of potential limiting nutrients for freshwater and oceanic environments in general. As ambient nutrient concentrations varies strongly both spatially and temporally, and phytoplankton nutrient demand varies both inter- and intraspecifically (Geider and La Roche 2002, Finkel et al. 2006, Quigg et al. 2011), the nature of nutrient limitation will also vary across time and space. Still, the patterns are actually broadly consistent with the bulk of research on nutrient limitation in aquatic ecosystems. Although lakes often is thought to be P-limited (Vollenweider 1968, Edmondson 1970, Schindler 1974, 1977, Hecky and Kilham 1988), N-limitation is also common (Elser et al. 2007), especially in areas with low atmospheric N-deposition (Bergström et al. 2005, Elser et al. 2009) or in highly eutrophic lakes (Downing and McCauley 1992) In fact, the majority of lakes seem to be close to the tipping point between N and P-limitation, at least judging from a meta-analysis of nutrient addition experiments, where the phytoplankton in most lakes responded most strongly to simultaneous N and P addition (Elser et al. 2007). Trace metal limitation is, as far as we know, not that common in lakes, but there are indications that Fe could limit primary production in oligotrophic, low-humic systems (Vrede and Tranvik 2006). Iron is, however, estimated to limit primary production in about one third of the world ocean (Boyd et al. 2007). In most other areas, including much of the low-latitude ocean, N is the primary limiting nutrient (Moore et al. 2013). Still, P may also limit phytoplankton production, e.g. in Western North Atlantic, where inorganic P concentrations are depleted relative to inorganic N (Wu et al. 2000). Note that although P is relatively seldom proximately limiting in the ocean, it is generally considered the “ultimate” limiting nutrient, since N is available from the atmosphere through N-fixation (Tyrrell 1999).

Clearly, N and P are important elements often limiting primary productivity in both freshwater and marine systems. By and large, N and P are so commonly limiting because they are needed in relatively large proportions compared to what is available or supplied to the ambient water. An important question then emerges: what determines the requirements for N and P in phytoplankton?

Nutrient requirements are linked to biochemical composition

A proximate answer to this question is that the N and P demands of phytoplankton cells are a function of the cellular concentrations (quota) of essential macromolecules like proteins and nucleic acids (RNA and DNA). Amino acids – the building blocks of proteins – all contain one or two N atoms such that proteins, on average, contain about 16% N by weight (Sterner and Elser 2002). The average protein content of phytoplankton is, again, about 30-60% by weight (Geider and La Roche 2002). This leaves protein content the primary predictor of cellular N content (Lourenço et al. 1998). Judged by the size of their cell quota, the most important groups of proteins in phytoplankton are the light-harvesting proteins (likely between 18-50% of total protein; Geider & La Roche 2002) and certain enzymes, e.g. the enzyme catalyzing the primary chemical reaction by which inorganic C enters the biosphere; Rubisco[4] (2-6% of total protein; Losh *et al.* 2013). The degree of allocation to different protein pools, which may change depending on the environmental conditions, can therefore influence the demand for N, a topic I will return to later in this synthesis.

Nucleic acids are long chains of nucleotides, which again are characterized by containing a specific nitrogenous base. These bases contain on average 3.75 N atoms, leaving nucleic acids also quite N-rich. Specifically, both RNA and DNA contain 15-16 % N, and typically comprise from 2.5-13 % (RNA) and 0.5-3 % (DNA) of cellular dry weight depending on species and growth conditions (Geider and La Roche 2002). The nucleic acids, having a "backbone" of sugar and phosphate, are particularly rich in P. Both RNA and DNA contain around 9-10 % P by weight. Yet, since RNA is by far the most abundant and variable of these molecules within cells, the RNA content is what matters most for the variation in P-quota of phytoplankton (Sterner and Elser 2002). In fact, RNA usually accounts 50 % or more of non-storage P in algae (Raven 2013). At periods, autotrophs may store significant amounts of P as polyphosphate, especially if the

[4] Ribulose-1,5-bisphosphate carboxylase/oxygenase

supply of P is high and other factors than P limit growth (Rhee 1974, Siderius et al. 1996, Eixler et al. 2006). Other cellular pools of P are phospholipids, which may constitute up to ca. 30 % of cellular P, and energy carriers like ATP. This pool is general minor (Geider and La Roche 2002). The relative contribution of m(messenger)RNA, r(ribosomal)RNA, and t(transport)RNA to the total RNA-pool is variable, but it is clear that rRNA constitutes the largest fraction followed by tRNA and then mRNA (Raven 2013).

Eukaryotic ribosomes contain about 50 % rRNA by weight and have an N:P ratio of ~7.2:1 (Sterner and Elser 2002, Raven 2013). Hence, increasing allocation to these molecular "protein factories" may significantly decrease the relative N:P demand of a cell. This observation was the basis for one of the most fundamental hypotheses in the field of ecological stoichiometry (Sterner and Elser 2002), namely the growth rate hypothesis (GRH). The GRH states that "*differences in organismal C:N:P ratios are caused by differential allocations to RNA necessary to meet the protein synthesis demands of rapid rates of biomass growth and development*" (Sterner and Elser 2002). The rationale behind the GRH is that if growth rate is to be increased, then the number of ribosomes per cell has to increase, assuming that the ribosomes are synthesizing protein at the maximal possible rate. It was further deduced, due to the relatively high P content of rRNA, that the organismal N:P ratio also should decrease with growth rate (Sterner & Elser 2002). The GRH has support in different types of heterotrophic organisms, both within and between species (Elser et al. 2003, Makino et al. 2003, Acharya et al. 2004). For higher plants, N:P does not seem to decrease with growth rate (Matzek and Vitousek 2009). Whether the GRH is valid for phytoplankton is a matter of controversy (Flynn et al. 2010, Raven 2013). Still, the hypothesis has been adopted in models of phytoplankton physiology (Klausmeier *et al.* 2004) to explain why fast-growing species have low N:P requirement. Testing the GRH for phytoplankton, at least within species, requires some caution, because many phytoplankton species have large capacities for excess uptake of non-limiting nutrients (e.g. Rhee 1974; Elrifi & Turpin 1985) which may mask growth-rate effects on N:P requirement. I will return to this topic and the relationship between ribosome content and N:P ratio in the section about how temperature might influence N:P requirement in algae (paper **II**).

Because the biochemical composition eventually is an outcome of evolutionary adaptation, the contents of N and P, and hence the N:P ratio, may also be viewed from an ultimate perspective. Similarities in elemental stoichiometry within phyla and superfamilies

reflect this (Ho et al. 2003, Quigg et al. 2003). For example, cyanobacteria and algae from the green lineage (chl*b* containing) have consistently higher N:P than those from the red lineage (chl*c* containing; Quigg *et al.* 2003; Martiny *et al.* 2013). Patterns in N:P stoichiometry may also be viewed in light of the environmental conditions under which the algae grow and compete, which have selected for a certain optimal allocation to different cellular components (Klausmeier et al. 2004b). Using a modelling approach, Klausmeier *et al.* (2004a) show that depending on the environmental conditions, different allocation to nutrient-uptake proteins and light-harvesting components (both rich in N), and ribosomes (rich in P and N) may be optimal. For example, under nutrient replete conditions and exponential growth, maximizing the maximal growth rate is the optimal strategy. This involves a high allocation to ribosomes, which entails a low optimal N:P ratio (cf. the GRH). Indeed, diatoms, which grow fast and form blooms under nutrient replete conditions in temperate parts of the ocean, generally have a low N:P ratios (i.e., < 16; Arrigo *et al.* 1999; Quigg *et al.* 2003; Rembauville *et al.* 2016). Under equilibrium conditions with light or nutrient limitation, minimizing the break-even level of the limiting resource is the optimal strategy. According to the model, this would involve a low allocation to ribosomes, but a high allocation to light-harvesting proteins or nutrient-uptake proteins, respectively. Both these scenarios will yield quite high optimal N:P ratios. The fact that small marine cyanobacteria like *Prochlorococcus* and *Synechococcus* – which often dominate under low nutrient conditions at low latitudes – exhibits high N:P ratios (i.e., > 16; Martiny *et al.* 2013; Mouginot *et al.* 2015), may be explained by the mechanisms at work in Klausmeier et al. (2004a)'s model.

Optimal N:P stoichiometry

The optimal N:P ratio of a species defines the threshold between N and P limitation, and is equal to the ratio at which the cells *require* the nutrients to sustain growth at a given rate (Rhee and Gotham 1980, Terry et al. 1985). The optimal N:P ratio may differ significantly from the cellular N:P ratio, which due to excess storage of the non-limiting nutrient can take values higher or lower than optimal N:P depending on whether N or P is limiting (Rhee 1978, Klausmeier et al. 2004a, 2008, Hillebrand et al. 2013). Since the optimal N:P ratio is central in papers **I** and **II**, where we study how this ratio depends on light (**I**) and temperature (**II**), I will use some paragraphs to clarify the concept.

Most commonly, the optimal N:P ratio has been defined according to Droop (1973)'s growth model, which relates the nutrient limited specific growth rate[5] (μ; time^{-1}) to the cellular concentration (quota; Q) of the limiting nutrient:

$$\mu = \mu_m(1 - Q_0/Q) \qquad \text{(Eq. 2)}$$

The parameter Q_0 (the "subsistence quota") represents the cell quota of the limiting nutrient at zero growth rate (or dilution rate, D), while μ_m is the theoretical maximum growth rate when Q approaches infinity. The relationship between Q and μ is exemplified in fig. 2, and further described in the figure legend.

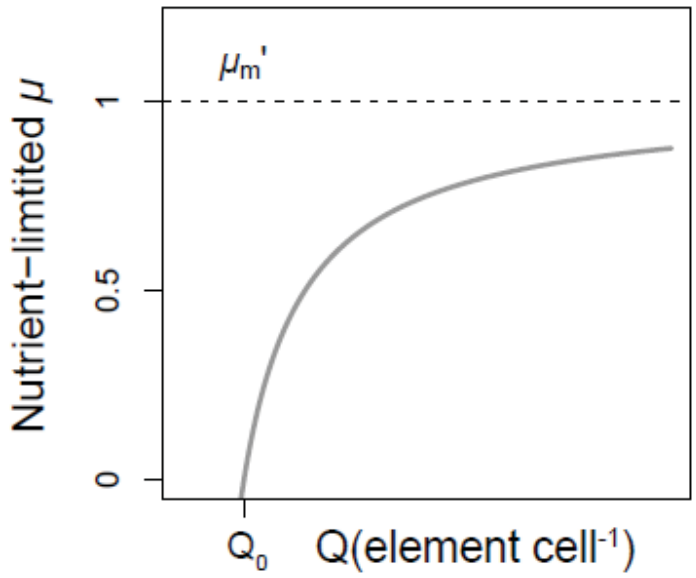


Fig. 2. Nutrient limited specific growth rate (or dilution rate, D, which equals the specific growth rate at steady state in a chemostat), often follows a hyperbolic relationship with the intracellular quota (Q) of the limiting nutrient. At very low growth rate, i.e., strong nutrient limitation, the Q of limiting nutrient is close to the minimum value that can support life, Q_0. As the Q increases, higher growth rate can be supported, eventually reaching a theoretical maximum growth rate, μ_m', when Q approaches infinity.

Note: Functionally, the Droop model is similar to the logistic growth model, which is the basis for density dependent growth. In the logistic model, specific growth rate can be described as a function of the biomass (X, here cell number) and the carrying capacity (K): $\mu = \mu_m(1 - X/K)$. If the total amount of nutrients, Nt, is approximately equal to QX (i.e., most nutrients is within algal cells), then $Q \sim Nt/X$. Recognizing that $K = Nt/Q_0$, then $\mu = \mu_m(1\text{-}Q0/Q)$ [the Droop equation] ~ $\mu = \mu_m(1\text{-}X/K)$ [the logistic growth model].

[5] Data on how μ and Q co-varies are often obtained using chemostats set to different dilution rates (D). In a chemostat at steady state, the specific growth rate equals the dilution rate.

For a pair of essential, non-interactive[6] resources such as N and P (Rhee 1978), the Droop model predicts that the element in biomass with the lowest ratio of cell quota to subsistence quota, i.e., Q/Q_0, will be the one which limits phytoplankton growth (Rhee and Gotham 1980).

Therefore, if

$$Q_N/Q_{0N} = Q_P/Q_{0P} \qquad \text{(Eq. 3)}$$

the cell is exactly co-limited by N and P, and the quota of both nutrients have to be increased to increase growth rate. Rearranging eq. 3, we find that the cellular N:P ratio at this point of co-limitation is equal to Q_{0N}/Q_{0P}. This is the ratio of the subsistence quotas for N and P and has been termed the an optimal N:P ratio (Rhee & Gotham 1980). The optimal N:P ratio may be visualized in a in a contour plot of a species' specific growth rate as a function of the cellular quota of N and P (fig. 3, see explanation in legend).

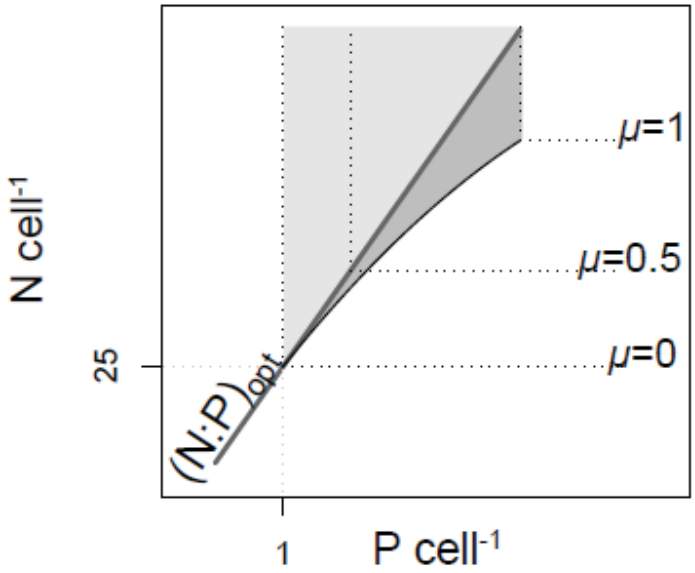


Fig. 3. Assuming that limitation by N and P follows a threshold model, a contour plot of the species' specific growth rate (or dilution rate, D) as a function of the cellular N and P quota will have isoclines running parallel to both axes (cf. Tilman 1980). The N and P limited regions of this plot can then be separated by a line drawn from the origin through the corners of the isoclines. This line, represented by the straight line in the figure, has a slope which is equal to the optimal N:P ratio ($(N:P)_{opt}$, in this example taking a value of 25 N:P). When cells contain N and P in this ratio, N and P are exactly co-limiting. If N:P is higher, the cells are P-limted; if N:P is lower, the cells are N-limited. Note that the line separating N from P limitation may bend as growth rate increases (the curved line in figure). This would imply that the optimal ratio of N to P is not constant, but depends on the growth rate. This seems to be the case for phytoplankton (discussed below).

[6] Limitation by N and P is often assumed to follow a threshold model, meaning that *only* the nutrient in shortest supply relative to demand limits growth. A wider zone of co-limitation might, however, occur (Bonachela et al. 2013), but there is little experimental data supporting this hypothesis.

If we define the optimal N:P as the ratio of subsistence quotas, we assume that the optimal N:P is the same for all growth rates (Rhee and Gotham 1980). However, the optimal N:P has been shown in experiments to vary with growth rate, usually in a unimodal or decreasing fashion (exemplified by the curved line in fig. 3; Elrifi & Turpin 1985; Healey 1985; Terry *et al.* 1985; Ågren 2004). This means that the tipping point between N and P limitation could depend on the growth rate, and that the ratio of subsistence quotas only is a good estimate of the tipping point between N and P limitation when growth rate is close to zero. According to the Droop model, however, we can express the optimal N:P in an alternative way, namely as the cellular N:P ratio representing the threshold between N and P limitation at any given growth rate:

$$Q_N(\mu)/Q_P(\mu) \qquad \text{(Eq. 4)}$$

Tis growth rate-dependent generalization of the optimal N:P ratio is sometimes called the "critical" N:P ratio (Terry et al. 1985). In the experiments for paper **I** and **II** we grew the algae in semi-continuous cultures diluted at the same rate (see *methods*). This yields the same steady state growth rate for all light and temperature levels. In that way, we "corrected" for potential confounding effects of growth rate on the optimal N:P ratio estimates.

Since the optimal N:P ratio represents the N:P ratio required for growth, it will depend on the macromolecular composition the cells (e.g. the content of P-rich ribosomes relative to N-rich proteins) which in turn is expected to be influenced by ambient environmental conditions (Geider and La Roche 2002, Leonardos and Geider 2004). In the next section I will discuss how irradiance might influence optimal N:P ratios, and thereby the degree to which a population of algae is N or P limited. First, I will briefly discuss the effect of irradiance on phytoplankton growth and physiology.

Light

Common to all photoautotrophs is the ability to absorb light and convert the electromagnetic energy to chemical energy, while extracting electrons from water to generate reducing power. Both the chemical energy (ATP) and the electrons (carried by NADPH) are used in the dark reactions to reduce CO_2 to organic C. Light, when discussed in connection with photosynthesis, usually means *photon irradiance* (E) – the number of photons impinging on a unit area per unit

time (e.g., μmol photons m^{-2} s^{-1}). Using photon irradiance (instead of energy, e.g. W m^{-2}) makes sense because as long as a photon gets absorbed by an alga, it can do the same amount of photochemical work regardless of the energy it contains (Kirk 2011). The probability of absorption, however, depends on the wavelength since the different photosynthetic pigments have absorbance spectra with distinct peaks and valleys. For photosynthesis, the relevant wavelength range spans from ca. 400 to 700 nm, and is commonly known as photosynthetic active radiation (PAR). These wavelengths contain an adequate amount of energy, meaning that they have enough energy to excite the electrons in chlorophyll *a* to its singlet level, but not so much energy that they start breaking chemical bonds (which starts happening in the UV region).

The relationship between photosynthetic rate (e.g. mg C mg chla^{-1} h^{-1}) and irradiance may be divided in two or three distinct regions (Falkowski and Raven 1997). In the light-limited region, photosynthesis increases linearly with irradiance, with a slope that is related to the maximum quantum yield of photosynthesis. At a certain irradiance, however, the curve starts levelling off. This point – the "onset of light saturation" (E_k) – is often quantified as the interception between the initial linear slope and the light-saturated, maximum photosynthetic rate (P_{max}). When photosynthesis is light saturated, the rate of light absorption exceeds the rate of electron transport, and photosynthesis cannot be increased further by increasing light absorption (Falkowski and Raven 1997). Actually, if irradiance or light absorption is increased further, photo-inhibition may occur, which would be manifested as a dip in the photosynthesis-irradiance (*P-E*) curve. The characteristics of the *P-E* relationship may be characterized by fitting empirical functions (e.g. Jassby & Platt 1976) to experimental data to estimate parameters like E_k and P_{max}. There are marked differences between species in the values of these parameters, and there may be significant differences between populations acclimated to different level of light, temperature, and nutrients (Kirk 2011).

Growth at different irradiances may involve different nutrient demands

The ability to photo-acclimate allows algae to photosynthesize and grow as efficient as possible at ambient irradiance, and involves the adjustment of cellular physiology and biochemical composition in response to changes in irradiance (Falkowski and LaRoche 1991, Brunet et al. 2011). Photo-acclimation is different from adaptation, which involves changes in allele-frequencies, but is related to it in the sense that the genetic makeup determines the potential for

phenotypic plasticity (Falkowski and Raven 1997). One may view photo-acclimation as responses serving to either increase growth rate at low light (relative to E_k), or to avoid photo-oxidative stress at high light (Geider et al. 1998). Both changes irradiance and spectral quality can cause photo-acclimation, but I will not consider spectral effects further in this thesis. Note, however, that spectral- and quantity-effects are inevitably linked through the spectral attenuation of PAR in a water column (Kirk 2011; paper **III**).

Photo-acclimation occurs at time-scales from seconds to days, but generally, most responses occur within the generation time of the cell (Falkowski and LaRoche 1991, Brunet et al. 2011). Among the short-term responses (i.e., seconds to minutes) to high light exposure is an increase in the non-photochemical quenching of excited chlorophyll, which involves the xanthophyll cycle and facilitates dissipation of excess excitation energy as heat (Brunet et al. 2011). With respect to this thesis, however, the more long-term photo-acclimation responses – those related to the regulation of cellular pigment concentrations – are more relevant. As we shall see, these may link the irradiance regime experienced by the cells to their nutrient demands (paper **I**).

When acclimating to low irradiance, phytoplankton generally increase their cellular concentration of light harvesting pigments (Geider 1987, MacIntyre et al. 2002, Brunet et al. 2011). This occurs within hours to days, and may involve 5-10 fold changes in the amount of chl*a* per cell compared to growth at high irradiance (Falkowski and Raven 1997). As pigments are bound to proteins in light harvesting complexes (LHCs), a change in cellular pigment content also brings along parallel changes in protein content (Anderson 1986, Masuda et al. 2003). Comparing low- and high-light acclimated green algae, large differences in the concentration of LHC II (the LHC associated with PSII in green algae; Sukenik *et al.* 1990; Tanaka & Melis 1997) and the expression of its mRNA (LaRoche et al. 1991) has been observed. Since the light harvesting pigment-proteins can constitute a large and variable fraction of a cell's total protein pool (Geider and La Roche 2002, Leonardos and Geider 2004), the differential allocation to LHCs as a function of irradiance may be a significant factor in the cellular N-budget. Eventually, it may lead to increasing N-demand under low light. Note that although the chlorophyll molecules themselves contain four N-atoms, the N in chlorophyll generally constitutes < 1% of total cellular N (Lourenço et al. 1998). It is therefore the N-content of the pigment-binding proteins that is the potential N-sink.

Assuming that about 80 % of total N in algae is protein-bound (Lourenço et al. 1998) and further that 18-50 % of total protein is associated with pigments (Geider and La Roche 2002), then around 16 – 40% of cellular N may be bound in pigment-proteins. Because proteins are rich in N, but contain little P, low-light acclimation has been hypothesized to increase the optimal N:P ratio (Geider and La Roche 2002, Leonardos and Geider 2004). As such, irradiance could affect the balance between N and P limitation (paper **I)**.

In paper **I**, we conducted a controlled experiment to assess the effect of irradiance on the optimal N:P ratio of *Chlamydomonas reinhardtii*. Although the hypothesis has been tested for a few species before (Wynne and Rhee 1986, Leonardos and Geider 2004, 2005), there was no clear consensus in the literature about the strength or the direction of any irradiance effect. Therefore, in paper **I**, we also included a meta-analysis of published experimental data on N:P ratios as a function of irradiance within species. Although we were mostly interested in the response of optimal N:P ratios, we also included data from studies measuring cellular N:P during exponential nutrient replete growth. Theoretical models (Klausmeier et al. 2004a, Bonachela et al. 2013) suggest that the cellular N:P at exponential growth should match the optimal N:P, but there are little experimental data testing this assumption. In total, the compiled dataset included 21 different subsets of N:P measured over an irradiance gradient from single-species experiments (see SI paper **I**). We analyzed the whole dataset with a linear mixed effect model, where each of the subsets were treated as a random sample from the whole "population" of N:P *vs.* irradiance datasets. The model then makes inference about the whole population (the *fixed* effects, representing the average response of N:P to irradiance), while also quantifying the variation in response between species (the *random* effects).

By and large, the results from paper **I** supported the hypothesis of an increasing N-to-P requirement with decreasing irradiance. In the experiment, the populations of *C. reinhardtii* acclimated to low irradiance had an optimal N:P ratio that was about 17 % higher than populations acclimated to high irradiance. Consistent with this, the meta-analysis revealed a significant negative relationship between irradiance and N:P ratio within species. The slope of the response varied between species, but 18 out of 22 species had a slope that was negative. On average, N:P decreased about 7 % per doubling of experimental irradiance. A relevant question to raise here is whether this effect is biologically relevant for phytoplankton in nature. On one hand, the effect-size in itself was not very large (7% per doubling of irradiance on average). On

the other hand, natural irradiance gradients experienced by algae *in situ* can be quite strong. In boreal lakes, for example, the depth of 1% of surface irradiance typically ranges from 3.5-6.5 m[7]. If algae are circulating in in this layer, they would also circulate through a 100-fold difference in irradiance, which would translate into a factor 1.67 higher N:P ratio at depth compared to the surface applying the effect size from our meta-analysis (see paper **I**). More generally, species or populations residing near the bottom of the mixed layer, where light is low, could experience higher optimal N:P ratios than populations residing closer to the surface. As such, they may also have a higher likelihood for N limitation at depths (or P limitation at the surface).

What determines the light-climate in lakes?

The attenuation (= absorption + scattering) of light in water is a function of the concentrations and properties of the light-absorbing and light-scattering components making up the medium. As a useful approximation, the absorption of incident solar irradiance can be attributed to four main components (Kirk 2011). These are the water molecules themselves (w), colored dissolved organic matter (CDOM), phytoplankton (phyto), and "non-algal" particulate matter (NAP). Expressed in terms of spectral absorption coefficients, the total absorption coefficient spectrum ($a_{tot}(\lambda)$, m^{-1}) equals the sum of the four component spectra[8]:

$$a_{tot}(\lambda) = a_w(\lambda) + a_{phyto}(\lambda) + a_{CDOM}(\lambda) + a_{NAP}(\lambda) \qquad \text{(Eq. 5)}$$

Note that each of the partial absorption coefficients further can be expressed a product of the concentration of the component (e.g. mg m^{-3}) and a specific absorption coefficient (m^{-2} mg).

By simple calculations based on the abovementioned relationships, Jones (1992) estimated the fraction of PAR that would be absorbed by phytoplankton under different theoretical concentrations of CDOM[9] and phytoplankton (chl*a*). He showed that for chl*a* concentrations of 10 μg L^{-1} or less, the fraction of PAR absorbed by phytoplankton would be low (< 23%) for all but the most clear lakes. Hence, he suggested that CDOM could compete with

[7] Calculated using the 1st and 3rd quartiles of a distribution of PAR attenuation coefficients from the 75 Norwegian and Swedish lakes sampled in paper **III**.

[8] A similar breakdown of components may be done for the total light scattering (although without the *CDOM* component, which mainly absorbs light; Kirk 2011), but I will not consider scattering in detail in this thesis. Scattering is, however, important for light attenuation, because it increases the path-length of the photons and hence the probability of absorption.

[9] He used water color measured as mg Pt L^{-1}.

phytoplankton for available irradiance, potentially reducing primary productivity. He did, however, not present any empirical data supporting his estimates.

In paper **III**, our main aim was to study the potential contrasting effects of CDOM or DOC and nutrients on pelagic primary productivity in lakes. As a first step in this assessment, we wanted to quantify the relative absorption of PAR in the water column to get a real impression of the degree of "competition" for photons between autotrophs and the other components, particularly CDOM. The 75 lakes we surveyed were chosen to span as orthogonal gradients as possible[10] in DOC and total P concentrations. By measuring the absorption spectra of phytoplankton, CDOM, and NAP, and combining these with a pure water absorption spectrum (see methods paper **III**), we constructed a total absorption spectrum from each lake by addition of the components (cf. eq. 7). Examples from an oligotrophic, a eutrophic, and a brownish "dystrophic" lake are shown in fig 5. A striking feature in these examples is the apparent dominance of absorption by CDOM (the light brown areas in fig 5) – even in the oligotrophic and the eutrophic lake.

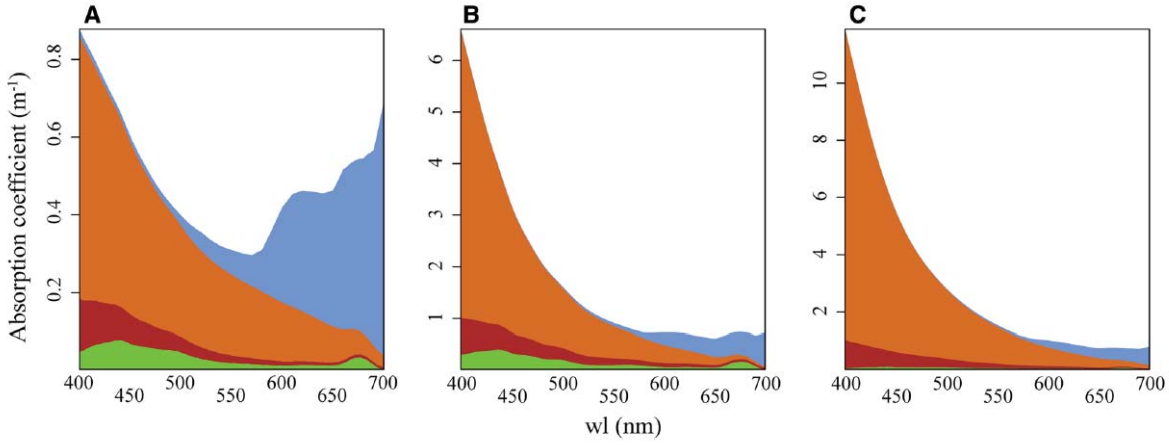


Fig. 5: Examples of partial absorption spectra from three lakes with different levels of DOC, TP, and Secchi disc depth (SD). Note that values on the y-axis differ. The total spectrum (a_{tot} [m^{-1}]) is represented by the intersection between white and colored area, and was created by stacking the component spectra (different colored areas) on top of each other. Green area: phytoplankton absorption, dark brown area: "non-algal particle" (NAP) absorption, light brown area: colored dissolved organic matter (CDOM) absorption, blue area: pure water absorption. A) Lake Jølstravannet (oligotrophic; SD = 10 m, TP < 1 μg L^{-1}, DOC = 0.6 mg L^{-1}), B) Lake Bergsvannet (eutrophic; SD = 1.05 m, TP = 17.9 μg L^{-1}, DOC = 3.7 mg L^{-1}), C Lake Rokosjøen (humic and mesotrophic; SD = 1.8 m, TP = 8.3 μg L^{-1}, DOC = 11.1 mg L^{-1}).

[10] Perfect orthogonality is difficult to obtain when studying natural ecosystems, since many variables are naturally correlated.

Combining the absorption spectra with a representative spectrum of incoming solar irradiance, we calculated the fraction of incoming solar irradiance absorbed by the different components in the 75 lakes. The distribution of "photon budgets" showed, strikingly, that the CDOM component absorbed between 37 % and 76 % of the incoming photons in the PAR region. The average fraction (57 %) was almost 9-fold higher than the average fraction absorbed by phytoplankton pigments (6.6 %), highlighting the potential shading effect of CDOM on autotroph productivity (Jones 1992, Carpenter et al. 1998, Karlsson et al. 2009). PAR absorption by phytoplankton ranged from about 2 % to 28 %, and correlated positively with TP and negatively with DOC.

DOC absorbs light, but is the net effect on primary productivity negative?

In boreal lakes, CDOM[11] is highly correlated with the concentration of DOC (paper **III**; Weyhenmeyer *et al.* 2014). While some of the DOC is autochthonous and originates from in-lake primary production, most of the DOC in boreal lakes is allochthonous, meaning that it is derived from terrestrial primary production in the catchment (Karlsson *et al.* 2003; Caraco & Cole 2004). Time-series of DOC from 1990 to 2004 show that over 70 % of the lakes, streams, and rivers surveyed in Scandinavia, UK, and North-America, experienced an increase in DOC concentration (Evans et al. 2006, Monteith et al. 2007). This phenomenon – often called "browning" (Roulet and Moore 2006) – is likely caused by a combination of mechanisms related to increased productivity in catchments, increasing pH and organic C solubility resulting from reduced sulfate deposition, and variation in hydrologic transport of DOC in surface waters (reviewed in Solomon *et al.* 2015). Still, reduced sulfate deposition and increased solubility of organic C is thought to be the main driver behind the last decades' increases in DOC (Evans et al. 2006, Monteith et al. 2007). To what extent this factor will affect DOC concentrations in the future is, however, uncertain. In North-America and Europe, it is likely that the importance will cease as soils recover from the earlier sulfate emissions that now are being increasingly controlled (Solomon et al. 2015). Nevertheless, it is likely that DOC concentrations will continue to change in the future, driven by mechanisms like increased plant biomass in catchments ("greening") in response to a warmer and wetter future (Larsen et al. 2011a) with higher atmospheric CO_2 (Zhu et al. 2016). Changing DOC concentrations have numerous impacts on

[11] Which often is quantified as the absorption coefficient (m^{-1}) of the dissolved fraction at 440 nm.

aquatic ecosystems (reviewed in Williamson & Morris 1999; Solomon *et al.* 2015), but in the following section, I will discuss the possible effects of DOC on primary productivity (PP).

CDOM absorbs the majority of photons in many boreal lakes (paper **III**). Increasing allocthonous DOC concentrations will increase the absorption due to CDOM and thereby reduce irradiance at depth. Moreover, the absorption spectrum of CDOM increases exponentially towards the blue part of the PAR spectrum (Bricaud et al. 1981), overlapping with the Soret peak of the chlorophylls and the absorption maxima of many carotenoids. Hence, one may hypothesize CDOM or DOC to reduce PP through shading.

For benthic PP by microalgae in small (0.02-0.17 km^2) and shallow boreal lakes, this hypothesis has considerable support (Ask et al. 2009, Karlsson et al. 2009, Seekell et al. 2015a). In these types of lakes, benthic PP is usually much higher than pelagic PP, causing whole-lake PP to decline with increasing DOC due to shading of the benthic habitat. Interestingly, reduced benthic PP may actually benefit pelagic producers in such shallow lakes by reducing nutrient uptake at the sediment surface, hence alleviating pelagic producers from nutrient limitation (Vasconcelos et al. 2016). Indeed, in small and shallow lakes, pelagic PP may correlate positively with DOC (Karlsson et al. 2009, Seekell et al. 2015a), likely reflecting a stimulating effect of nutrients brought along with allochthonous organic matter.

While pelagic PP may be stimulated by DOC in small, shallow, and nutrient poor lakes, the net effect of DOC on PP in larger and deeper lakes is less known. In paper **III**, we studied this question in lakes > 1 km^2, where pelagic production presumably constitutes a larger fraction of total PP. The lakes (n = 75) sampled for this study were deliberately chosen to span wide and weakly correlated gradients in DOC and total P (fig. 6), which allowed potential contrasting effects of DOC and nutrients on pelagic PP to be disentangled. As paper **III** was part of a larger project looking at the effect of

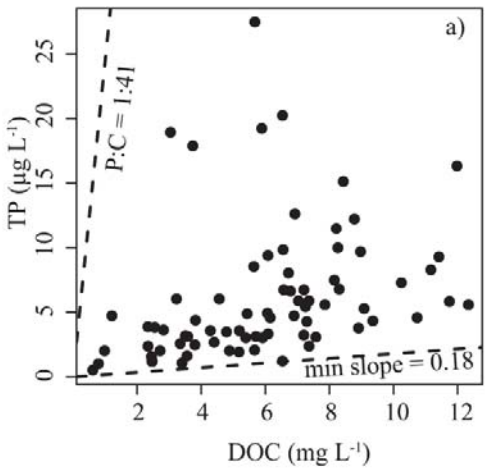


Fig. 6. The concentration of total phosphorus (TP) in the 75 lakes surveyed in paper III spanned from 0.5-27.5 μg L^{-1}, while dissolved organic carbon (DOC) spanned from 0.25-12.3 mg L^{-1}. Although correlated (r = 0.41), the variables were fairly orthogonal.

biodiversity on ecosystem function[12], the lakes were also chosen to span a longitudinal gradient in phytoplankton species richness from western Norway to eastern Sweden. We used a hydroplane to manage sampling all the lakes within a relatively short time-frame, and with a limited amount of time per lake, it was not feasible to carry out depth integrated measurements of pelagic PP using standard ^{14}C methods. Instead, we used a bio-optical model (see *methods*) based on vertical profiles of PAR, the bulk phytoplankton absorption coefficient, and measurements of photosynthetic efficiency by active fluorescence. Although the absolute estimates of PP obtained by such methods are uncertain (Johnsen and Sakshaug 2007, Suggett et al. 2011), our estimates should represent a maximum estimate of the gross area-specific PP (PP_A) that could be achieved by the phytoplankton community in each lake under the given light-conditions. As we were mostly interested in the relative difference between lakes of different DOC and total P concentrations, some uncertainty in the absolute values is acceptable.

As suspected from the strong importance of CDOM for PAR absorption, PP_A related negatively to DOC (and CDOM) in paper **III**. The effect was, however, only apparent when also including total P in the regression model. Therefore, our results may be interpreted as when comparing lakes of similar limiting nutrient concentrations (P for these lakes), higher DOC yields lower PP_A. Note that our estimates of PP_A were derived based on the standing stock of phytoplankton (which more or less determines the phytoplankton absorption coefficient, see *methods*). Phytoplankton standing stock is the result of past influence of factors like nutrient availability, grazing, and sinking, hence the positive effect of total P on PP_A should be interpreted as a positive effect on phytoplankton standing stock. The negative effect of DOC on PP_A likely results from the strong influence that DOC had on vertical PAR attenuation (see paper **III**), which caused lower irradiance at depth and a shallower euphotic zone in lakes with high DOC. However, the ratio of chl*a* to total P also correlated negatively to DOC, indicating a negative influence of DOC on phytoplankton standing stock as well (unpublished results).

A few other studies have investigated the effect of DOC on pelagic PP or photosynthesis for lakes spanning wide gradients in DOC and nutrients. In 20 Quebec lakes[13], the ratio of pelagic photosynthesis to respiration (the P:R ratio) related negatively to DOC (del Giorgio and Peters 1994). The negative effect on P:R was almost exclusively due to the depressing effect of

[12] The EU project "Biodiversity, community saturation, and ecosystem function in lakes" (COMSAT)
[13] TP ranged from 5-46 $\mu g\ L^{-1}$ and DOC from 3-8 $mg\ L^{-1}$.

DOC on photosynthesis; an effect mainly caused by light absorption, which reduced the depth of the euphotic layer relative to the mixing depth. Interestingly, they also observed that the chl*a*-specific maximum rate of photosynthesis decreased for lakes with DOC concentration above ca 6 mg L^{-1}. No clear negative effect of DOC on gross PP was found in 25 Wisconsin lakes[14] (Hanson et al. 2003). However, the authors noticed that for lakes with low DOC (< 10 mg L^{-1}), gross PP correlated positively with DOC. For lakes above this "threshold", DOC influenced PP negatively. A unimodal relationship between DOC and PP was also apparent when comparing DOC vs. PP relationships in regions of low DOC (arctic lakes) and high DOC (boreal lakes; Seekell *et al.* 2015a). Here, the unimodal pattern was explained by a stimulating effect of nutrients (nitrogen, which is limiting in this area) bound to organic matter the in arctic lakes, which changed to a negative effect of shading in the boreal lakes. They identified a threshold around 5 mg L^{-1} DOC for the switch between net nutrient stimulation and net light limitation (Seekell et al. 2015a, 2015b) The trend, however, was mainly caused by changes in benthic PP, which made up the bulk of total PP in these lakes (Ask et al. 2009, Seekell et al. 2015a).

The idea that DOC can stimulate PP when the background DOC concentration is below a certain threshold is supported by a mesocosm-study from an alpine lake (Kissman et al. 2013). Addition of about 1 mg L^{-1} DOC to water that contained 0.5 mg L^{-1} resulted in higher biomass and growth rate of phytoplankton (Kissman et al. 2013). The positive effect was attributed to P brought along with the DOC. A unimodal response to DOC has also been observed for the biomass of fish (brown trout), suggesting that the dual effects of DOC can propagate up the trophic ladder (Finstad et al. 2014).

In whole-lake manipulation experiments, a decline in both phytoplankton biomass (Carpenter et al. 2016) and pelagic PP (Carpenter et al. 1998)[15] was observed as a response to increasing DOC concentration. Shading by DOC was a probable cause of the decline in PP because the light absorption *not* due to chl*a* was linearly related to DOC (Carpenter et al. 1998). Similarly, gross PP (bentic + pelagic) declined linearly with DOC along a gradient from 25-200 mg L^{-1} in large experimental ponds (Jones and Lennon 2015). A reciprocal transplant experiment incubating low-DOC water in high DOC ponds, and vice versa, identified light limitation as the cause of this trend.

[14] TP ranged from 4-105 μg L^{-1} and DOC from 2-25 mg L^{-1}.
[15] DOC ranged between 4-17 mg L^{-1} in the four lakes studied

Clearly, the net effect of DOC on PP may be both negative and positive, and seem to depend on factors like the background DOC concentration and whether one considers benthic or pelagic production. But DOC may also influence PP in other ways. Jones (1992) suggested that the negative effect of light absorption by DOC may be counteracted if the phytoplankton relocate themselves closer to the surface. This has also been predicted in a marine ecosystem model, which suggests that increased DOC or CDOM may cause a eutrophication-like response, by "pushing" the phytoplankton towards the surface (Urtizberea et al. 2013). To my knowledge, however, there only exists anecdotal evidence from a single lake indicating that this could happen in nature (Christensen and Carpenter 1996). Controlled experiments need to be carried out to test this hypothesis. DOC might also affect PP positively via changing the partial pressure of CO_2: By measuring volumetric rates of PP in lake-water naturally supersaturated with CO_2 and in water from the same source, but equilibrated with air, Jansson *et al.* (2012) found a strong reduction in PP after CO_2 was reduced (down to 15% of the rates for supersaturated water). Since pCO_2 generally is tightly linked to DOC (Larsen et al. 2011b), this may imply an indirect positive effect of DOC on PP. At least, it suggests that a light-related reduction in PP due to increasing DOC could be partially counteracted by a stimulating effect of CO_2.

Temperature

Apart from indirectly affecting phytoplankton through physical phenomena like vertical stratification and the solubility of CO_2 in water, temperature is a master variable controlling the speed of physiological processes. Within the tolerance range of a species, the relationship between a biological rate and temperature is often well described by an Arrhenius function[16] (Kooijman 2009). Commonly, this temperature dependency expressed in terms of a Q_{10}-value, which denotes the relative increase of a rate if temperature is raised by 10°C (Kooijman 2009). With no prior acclimation, most processes relevant to the growth of phytoplankton have Q_{10} values between 1.5 and 3 (Raven and Geider 1988). The rate of protein synthesis per ribosome, for example, exhibits a Q_{10} around to 2 (Shuter 1979). This is also the case for the light saturated rate of photosynthesis, a rate which itself is controlled by the temperature dependency of sub-

[16] $r(T) = r_1 \times \exp((T_A/T_1) - (TA/T))$, where r is a biological rate, T is the temperature in Kelvin, T_A the Arrhenius temperature, and T_1 a reference temperature where $r = r_1$. This function behaves almost like an exponential function of temperature within the tolerance range of most rates, but is not strictly an exponential function of temperature since the exponent contains the inverse of T.

processes like the Rubisco-catalyzed binding of CO_2 and the rate of electron transport in the photosystems (Davidson 1991). In contrast, the rate of light absorption by photosynthetic pigments (which may be considered a pure physical process; the interception between photons and pigments) is independent of temperature ($Q_{10} = 1$).

Two modes of temperature-acclimation

The temperature dependency and independency, respectively, of biosynthetic- and light absorption processes, are associated with two different modes of temperature acclimation in algae (Raven and Geider 1988, Davidson 1991). One, which is termed *photosynthetic temperature acclimation*, concerns adjustments of the photosynthetic machinery in response to temperature (Öquist 1983). Phytoplankton have to maintain a balance between supply of energy (ATP) and reducing power (NADPH) through light absorption, and consumption of the same substances in the dark reactions. If the absorbed light energy exceeds what the alga can use, the likelihood photo-oxidative stress increases (Davidson 1991). When the temperature of an algal cell decreases, the rate of "consumption" slows down relative to light absorption because the dark reactions are temperature dependent (Raven and Geider 1988). To maintain a balance, however, experiments have shown that phytoplankton can acclimate to low temperature in a way similar high-light acclimation. That is, by reducing cellular chl*a* and light harvesting protein concentrations, while increasing the concentration of photo-protective carotenoids (Davidson 1991, Maxwell et al. 1994, Anning et al. 2001). Indeed, a general response in phytoplankton seems to be a reduction of chl*a* : C when temperature is decreased (Geider 1987, Thompson et al. 1992). This response may reflect a mechanism to reduce photo-oxidative stress at low light (Raven and Geider 1988), but could also reflect other mechanism such as altered lipid content (i.e., C) of thylakoid membranes (Geider 1987).

Another type of temperature-acclimation responses are so-called *compensatory* responses (Hochachka and Somero 1984). When growing at low temperature, poikilothermic[17] organisms may compensate – either fully or partially – for reduced specific reaction rates by increasing the concentration of the macromolecules involved in the temperature dependent processes (Hochachka and Somero 1984, Raven and Geider 1988, Woods et al. 2003). For phytoplankton, there are e.g. evidence that the cellular concentration or activity of Rubisco increases at low

[17] Organisms which body temperature adjusts depending of the environment

temperature (William and Morris 1982), although this seems not to be a universal response (Davidson 1991, Anning et al. 2001). Cellular ribosome content may also be affected, as indicated by a recent study of marine phytoplankton from the global ocean (Toseland et al. 2013). Here, temperature was found to explain a large fraction of the latitudinal variation in the expression of ribosomal genes. The expression was significantly lower in warm relative to cold areas. Moreover, the rate of protein synthesis strongly increased at high temperature, even though the concentration of ribosomes decreased. This suggests that phytoplankton require fewer ribosomes to synthesize the same amount of protein at high temperature (Toseland et al. 2013, Daines et al. 2014).

Temperature acclimation may alter the relative N:P requirement

The phenotypic acclimation responses may have consequences for the N and P requirements of algae acclimating to different temperatures. For example, if ribosomal content is lowered at high temperature, P-demand will decrease since ribosomes contain the majority of the cellular RNA (Raven 2013). Moreover, a reduction of light harvesting pigments and proteins at low temperature will lead to a lower N-demand, as discussed in relation to paper **I**. Temperature-related changes in Rubisco content may also affect N-demand, but as a recent study found that Rubisco only constitutes between 2 and 6 % of cellular protein under nutrient replete, exponential growth, changes in Rubisco content may be less important for the N-budget than e.g. changes in light harvesting protein content, which may constitute a way larger fraction of the total cellular protein pool (Geider and La Roche 2002). There are, however, not many experimental studies testing the effect of temperature on the net requirement for N vs P, and hence on the threshold between N and P limitation (but see Rhee & Gotham 1981).

In paper **II**, we addressed how the optimal N:P ratio of *Chlamydomonas reinhardtii* responded to several generations of growth at different temperatures. Using a microwell-plate setup and a custom-made temperature incubator (see *methods*), we were able to estimate the optimal N:P ratio over a 12-step temperature gradient under saturating irradiance levels. As in paper **I**, we determined the optimal N:P at each temperature level as the supply N:P ratio at which biomass switched from N to P limitation (see *methods*).

Over the temperature gradient, we found that the optimal N:P ratio increased in a sigmoidal manner from an N:P of ca. 26.5 to an N:P of ca. 36.5 (fig 8). This implies that the temperature of growth can influence whether a population of algae is N or P-limited. For example, our results indicates that given an ambient N:P ratio of 30, *C. reinhardtii* will be N-limited at high temperature, but P-limited at low temperature.

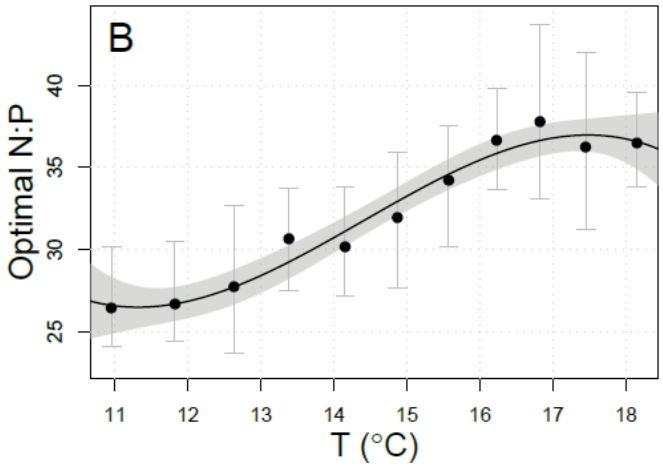


Fig. 8. Estimates of the optimal N:P ratio of *Chlamydomonas reinhardtii* plotted as a function of temperature.

The microplate design has advantages with respect to the number of experimental combinations and replication. However, it comes with the drawback of small sample volumes and constraints on which parameters that can be measured. We were therefore not able to measure cellular RNA or protein to address what changes in macromolecular composition that led to the increase in optimal N:P ratio. Nevertheless, net result is consistent with an hypothesis of increasing demand for P to ribosomes at low temperature and/or a decreasing demand for N to light harvesting machinery at low temperature.

Closing thoughts

Possible effects of future changes in light, temperature, and nutrient regimes

Phytoplankton are affected both directly and indirectly by large scale phenomena such as climate change, browning, and atmospheric N-deposition. Apart from increasing temperature, which itself has numerous impacts on aquatic ecosystems (Adrian et al. 2009, Hoegh-Guldberg and Bruno 2010), climate change may also alter light and nutrient regimes in lakes due to indirect effects of climate on the catchment (Adrian et al. 2009). In the boreal zone, many inland waters may continue to experience increasing levels of DOC (Larsen et al. 2011b) and iron (Sarkkola et al. 2013, Weyhenmeyer et al. 2014), with concomitant effects on water color and light-climate (paper **III**). If this browning continues, the production due to benthic autotrophs will likely

suffer (Karlsson et al. 2009, Vasconcelos et al. 2016). Also pelagic productivity may be reduced, especially in larger and deeper lakes (paper **III**) and lakes with relatively high background concentration of DOC (Seekell et al. 2015a). In shallow and clear lakes, however, browning may in fact increase pelagic production due to DOC-bound nutrient and lessened competition for nutrients with benthic algae (Seekell et al. 2015a, Vasconcelos et al. 2016). The effects of browning could also impact the parts of marine systems that are influenced by runoff from fresh waters (Aksnes et al. 2009, Urtizberea et al. 2013).

Darker waters may also increase the algae's physiological demand for N relative to P due to increased allocation to light harvesting components (paper **I**). If this occurs, optimal N:P ratios may increase, and phytoplankton become N-limited at higher supply N:P ratios. Importantly, the effects studied in the experimental papers (**I** and **II**) represent relatively short term acclimation responses. Changes in light-climate due to e.g. browning will take long time, and allow populations to adapt through evolutionary changes. Hence, acclimation responses may be replaced by adaptive changes that may involve other mechanisms, e.g. pigment packaging. Such responses could increase light absorption efficiency without increasing N-demand (Kirk 2011). Moreover, changing light-climate will alter the competition between species, possibly favoring species that are efficient light-absorbers or have low critical irradiances (Huisman et al. 1999). One may also imagine that darker waters could cause phytoplankton to concentrate in the upper parts of the water column (Jones 1992), possibly causing a "eutrophication" of the surface layer (Urtizberea et al. 2013). However, such a response would only be relevant to motile phytoplankton.

Increasing temperatures may also affect N:P requirement (paper **II**, Yvon-Durocher *et al.* 2015). In fact, the differential effects of temperature on biosynthesis and light absorption have been suggested to increase phytoplankton N:P ratios in scenario a warmer waters (Toseland et al. 2013, Daines et al. 2014). Again, this may cause phytoplankton to be become N-limited at higher N:P ratios. Changes in phytoplankton N:P stoichiometry could also influence biogeochemical cycling of N and P, as these cycles largely are driven by biological uptake and release by phytoplankton (Redfield 1958, Litchman et al. 2015).

In the global ocean today, bulk N:P ratios increases from the cold, nutrient rich, high-latitude regions, towards the warm, nutrient-depleted low-latitude regions (Martiny et al. 2013, Yvon-Durocher et al. 2015). While temperature likely plays a role in this trend (Yvon-Durocher

et al. 2015), the importance of "direct" temperature effects through physiological acclimation (as indicated in paper **II**) relative to other factors like community composition and ambient nutrient concentrations, is still under scrutiny. Indeed, much of the stoichiometric variation at the global scale is related to community composition, as low N:P diatoms dominate at cold, high latitudes, and high N:P cyanobacteria dominate in warm, low latitudes (Martiny et al. 2013). Hence, the correlation between N:P and temperature may be an indirect effect of co-varying temperature and community composition. Still, temperature ultimately drives patterns in stratification and mixing, which again determines the length of the growing season and the mixed layer nutrient levels. These factors ultimately selects for different ecological strategies, which involves different allocation to cellular macromolecules, eventually influencing N:P stoichiometry (Klausmeier et al. 2004b)

Environmental effects on algal N and P requirements may alter the balance between N and P limitation, but this balance also depends strongly on the relative supply of N and P to the ecosystem. Due to anthropogenic activities, atmospheric N-deposition has increased dramatically since the 1950s (Galloway and Cowling 2002), and in fact seem to have shifted lakes is high-deposition areas from an N- to a P-limited state (Bergström et al. 2005, Bergström and Jansson 2006, Elser et al. 2009). It is possible that many marine and terrestrial areas will experience a similar shift towards P-limitation if the high N:P input continues in the future (Peñuelas et al. 2013).

Selected methods

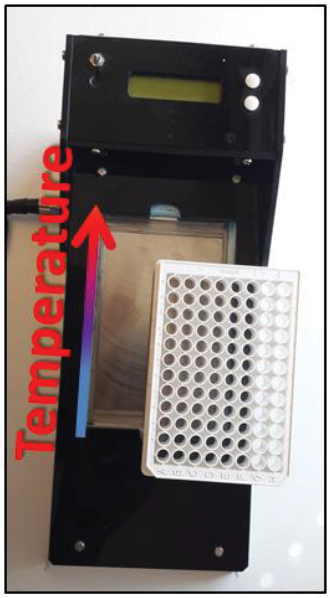
Paper I and II: Experimental determination of optimal N:P ratios

In paper I and II we determined the optimal N:P ratio, at which N and P are co-limiting, and addressed whether this ratio varied as a function of irradiance (E, paper **I**) and temperature (T, paper **II**). To determine the optimal N:P ratio, we did experiments where N and P in turn were limiting under otherwise similar levels of E and T. Such experiments require a large number of experimental treatment combinations, because gradients in E or T must be crossed with a gradient in algal growth media spanning from N- to P-limitation (i.e., a gradient of "supply" N:P ratios). To acquire this, we used a design involving semi-continuous cultures grown in 96-well

microplates. The microplate design has the advantage of allowing high number of experimental treatment combinations that also can be replicated.

We used custom made equipment fitted to 96-well microplates to generate the gradients in E and T (fig. 9 & 10a). For the E-gradient (**I**), we used a device[18] with 96 surface-mounted white light emitting diodes (LEDs; fig 9a). These were individually controlled with an Arduino[19] microcontroller, and calibrated to span irradiances ranging from light limitation to light saturation for *C. reinhardtii.* When programming the irradiance for each LED, we accounted for cross contamination of light between wells. This was done by modelling the stray light based on the number of neighboring wells and the translucence of the well material.

A



B

Fig. 9. Equipment for generating light and temperature gradients. A) Device with 96 white light emitting diodes (LEDs) programmed to span a gradient in irradiance. For the experiment, this device was placed on top of a microwell plate containing growth medium and algae. B) Temperature incubator consisting of a stainless steel plate over which a linear temperature gradient was generated. The microwell plate with algae was placed on top of this steel plate during the experiment.

The board was placed on top of the microwell plate, illuminating the algae from the top with a unique irradiance for each well.

For the gradient in T (**II**), we used an incubator consisting of a stainless steel plate heated at one side by a high power resistor, and cooled at the opposite side by Peltier elements (fig 9b). As predicted by thermodynamics, this creates a linear temperature gradient across the steel plate.

[18] Produced by Geir Andersen (https://www.tindie.com/products/Dead_Bug_Prototypes/microwell-96-led-controller/)
[19] https://www.arduino.cc/

We placed the microwell plate on top of the steel plate and obtained a temperature gradient spanning from ca. 11 to ca. 18 degrees C.

Within each level of irradiance (**I**) or temperature (**II**), algae were grown along a gradient from N- to P-limitation represented by growth media of increasing supply N:P ratios (fig. 10 a-c). The gradient of supply N:P ratios (fig. 10 c) was generated by mixing a decreasing fraction of a P-rich medium (added inorganic P, but no N) with an increasing fraction of an N-rich medium (added inorganic N, but no P, fig. 10 b).

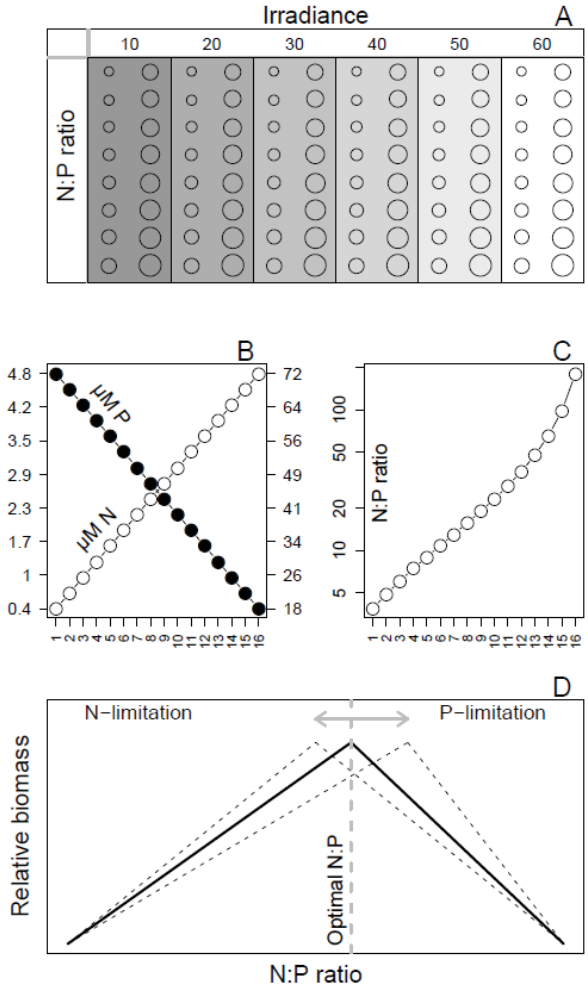


Fig. 10. Overview of the experimental microplate-design from paper **I**. A similar design was applied for paper **II**, where temperature was varied instead of irradiance.

A) Two and two columns received the same irradiance. Within each irradiance level (represented by a unique shade of gray), each of the wells received one of 16 different supply N:P ratios (represented by circles of increasing size). B) The 16 supply N:P ratios (shown in C) were generated by decreasing the μmolar concentration of P (left y-axis, black dots) while simultaneously increasing the μmolar concentration of N (right y-axis, white dots). 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 N:P ratio (where N and P are co-limiting), and decrease as P became limiting. Any effect of irradiance on the relative demands for N and P should shift the position of the peak (dotted lines).

In the growth media with low N:P ratio, the biomass yield will be limited by N (fig 10 b-d). The yield is therefore expected to increase with the concentration of N, and hence with the supply N:P ratio (fig 10 d). At a certain supply N:P ratio, which corresponds to the optimal N:P ratio of the species under the given E or T level, N and P will become exactly co-limiting. At this point, the biomass yield will peak. At supply N:P ratios above the optimal N:P, the yield

becomes P-limited and is expected to decrease with further increases in supply N:P ratio (fig. 10 d). We used either piecewise regression (paper **I**) or a generalized additive model (GAM; paper **II**) to determine the exact supply N:P ratio where the yield turned from N to P limited, i.e., the optimal N:P ratio. Note that this ratio differs slightly from the optimal N:P ratio as defined according to the Droop model. We use the N:P ratio of the medium at the point of co-limitation, not the cellular N:P at the point of co-limitation.

We grew the algae semi-continuously, meaning that each micro-well culture was diluted with a certain constant volume fraction at regular time intervals. We applied the same dilution rate for all cultures. This mode of culturing yields a saw-toothed development of biomass over time, eventually reaching a steady-state biomass that depends mainly of the concentration of the limiting nutrient (fig. 11). This steady-state biomass is the yield we used to determine the optimal N:P within each E or T level.

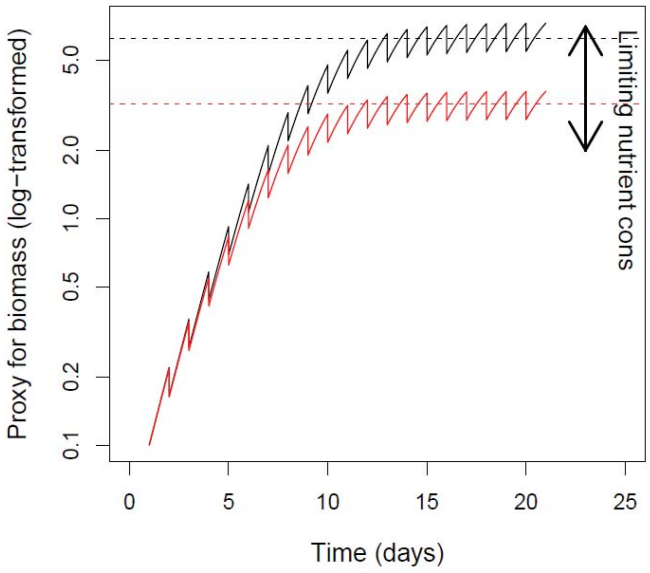


Fig. 11. Simulated development of biomass over time for a semi-continuous culture of algae growing logistically between dilutions. The dilution rate (D, day^{-1}) was set to 0.25; the maximum specific growth rate was set to 0.8 day^{-1}. The dashed lines represent steady-state biomass for a relatively low concentration of limiting nutrient (red) and a relatively high concentration of limiting nutrient (black). Note that the y-axis is log-transformed.

An advantage of using semi-continuous cultures is that the growth period is "extended" compared to growth in batch culture. This gives the algae a longer time to acclimate to the given ambient conditions. Moreover, when the cultures are diluted with the same dilution rate, they obtain the same steady-state growth rate. In that way, we "correct" for the fact that the optimal N:P ratio may vary with growth rate.

Due to the small culture volumes (~ 300 μL per well), we were constrained with regards to which parameters we could measure. We therefore used the concentration of chlorophyll *a*

(chl*a*) as a proxy for biomass in these experiments. This variable could be measured with high sensitivity in the small sample volumes. While the amount of chl*a* per biomass (e.g. chl*a*:C, or chl*a*:cell) undoubtedly varies as a function of irradiance (and temperature), this should not be a confounding factor for our optimal N:P ratio estimates. A higher chl*a* per biomass at low light, for example, will only result in an overall higher chl*a* concentration for all supply N:P ratios. It will not change the position of peak chl*a* along the supply N:P ratios *per se*. Note, however, that if the higher chl*a* content also is associated with a significantly higher demand for N relative to P, the optimal N:P may change. But this would be due to an altered biochemical composition, not the use of chl*a* as a biomass proxy.

Paper III: Estimation of area-specific primary productivity with a bio-optical model

The bio-optical model used to estimate area-specific primary productivity (PP_A) was based on measurements vertical PAR attenuation, data on incoming PAR from each lake, measurements of phytoplankton absorption coefficients in the PAR-region, and estimates of the effective quantum yield of photosystem II obtained from a PAM-fluorometer.

In fig. 12, I have visualized some steps in the procedure: Data on incoming solar irradiance (E(0)) over the day from each lake[20] (fig. 12 a) was combined with lake-specific measurements of the diffuse attenuation coefficient for PAR, to obtain vertical profiles of PAR over the course of the day. An example from a time point *t* (noon) is shown by the black line in fig 12b. By multiplying the depth-specific irradiance (μmol photons m^{-2} s^{-1}) with the phytoplankton absorption coefficient (m^{-1}), we estimated the depth-specific rate of photon absorption by the phytoplankton community (μmol photons m^{-3} s^{-1}; green line in fig. 12b). Multiplying this rate with the irradiance-specific effective quantum yield of photosystem II (PSII) measured by a PAM fluorometer and assuming that 50 % of the irradiance was absorbed by PSII, we obtained the rate of electron transport through PSII (ETR_{PSII}; μmol electrons m^{-3} s^{-1}). Due to the negative relationship between quantum yield and irradiance, this curve (C) typically had a maximum a bit below the surface. To convert from electron transport rate to oxygen evolution, we assumed a constant number of O_2 produced per electron shuttled through PSII. We further assumed a constant number of CO_2 fixed per O_2 produced, to obtain volume-specific estimates of PP in terms of C (μg C m^{-3} s^{-1}). Further, we integrated these estimates over depth (D), obtaining

[20] Downloaded from the STRÅNG database (http://strang.smhi.se/)

area-specific rates of PP (μg C m^{-2} s^{-1}). Finally, these rates were integrated from sunrise to sunset, obtaining daily rates of PP (μg C m^{-2} day^{-1}).

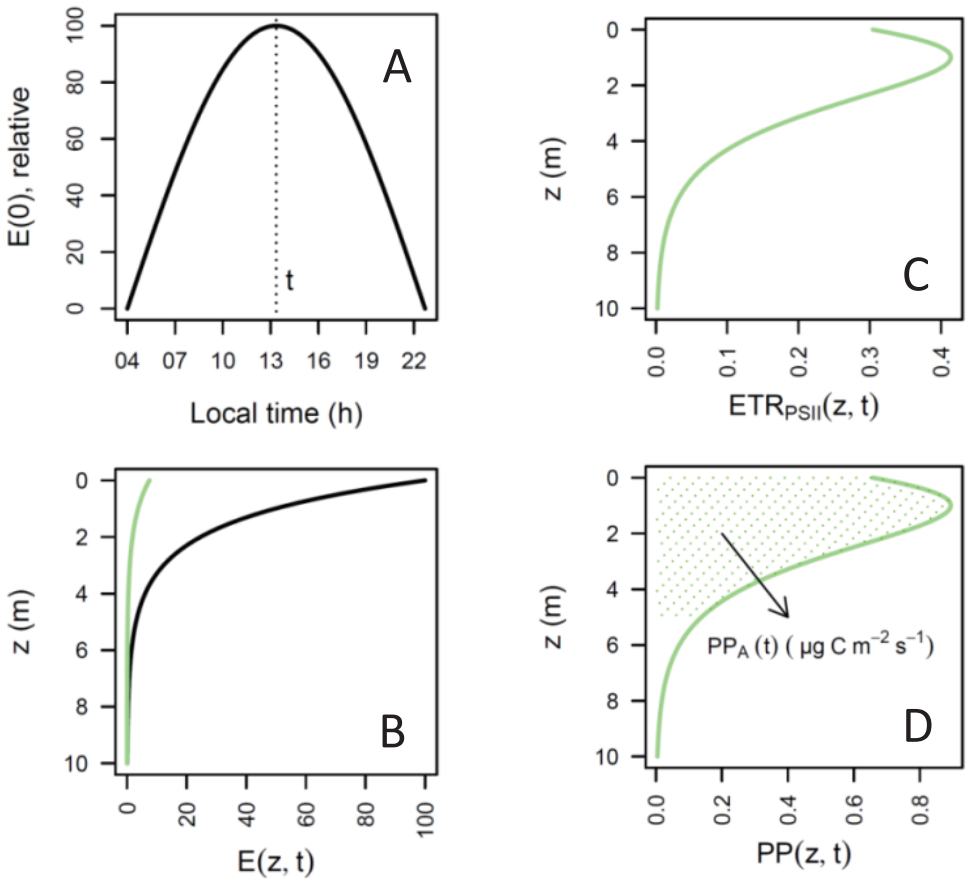


Fig. 12. A sketch of the main steps in the bio-optical estimation of area-specific primary productivity (PP_A). A) Surface solar irradiance in the PAR region, E(0), plotted as a sinuous function of local time. Local noon is shown as a time point *t*. B) Vertical profiles (z = depth in meters) of the irradiance at time *t* (black curve) and the rate of light absorption by phytoplankton (green line) from a hypothetical lake. C) Vertical profile of electron transport through photosystem II (ETR_{PSII}). D) Vertical profile of PP in terms of C, obtained by converting from ETR to O_2-production and finally to C-fixation. The dotted green area represents the area-specific PP from 0 to 5 meter depth.

The estimates of PP obtained by this method are most closely related to gross PP because they are directly linked to the rate of electron flow through PSII. The values we assumed to convert from ETR to PP were close to the maximum efficiencies; hence our values should be interpreted

as estimates of the maximum gross PP that can be achieved by the phytoplankton community under the given light conditions. We acknowledge that the absolute estimates of PP obtained by this method has uncertainties (e.g. related to measurements of phytoplankton absorption and the factors assumed for PSII absorption, the number of O_2 produced per photon absorbed etc.; Johnsen & Sakshaug 2007; Suggett *et al.* 2011). However, since we were most interested in the relative difference between lakes of different DOC and total P concentrations, we believe that uncertainties regarding the absolute estimates should not confound our main conclusions.

Paper IV: Development of a high-throughput method for analysis of algal pigment extracts

The aim of paper **IV** was to further develop and critically test a fast and cheap method for analysis of algal pigment extracts. We took starting point in a method published by Küpper *et al.* (2007). Their method was based on spectral deconvolution of pigment extracts and implemented in the commercial software SigmaPlot[21]. To make the method more generally applicable, we implemented a modified version of the method in the free open source statistical software *R* [22]. In this process, we made several improvements including fast and efficient modelling of pigment and background spectra by non-negative least squares (NNLS), inclusion of a wider range of ecologically relevant pigments, and adaptation to plate reader technology and microwell plates. The latter allows the method to potentially be used in high-throughput microplate experiments, such as the ones described in paper **I** and **II**.

In short, the method involves using NNLS to model the total absorbance spectrum of a pigment extract as a weighted sum of individual pigment spectra, which themselves are represented as sums of Gaussian peaks. Simultaneously, we correct for background attenuation due to e.g. non-algal pigment absorption or scattering, by modelling the background spectrum as a weighted sum of background components that are power functions of decreasing wavelength. The procedure is exemplified in the figure below (but sees paper **IV** for a detailed description):

[21] https://systatsoftware.com/
[22] https://www.r-project.org/

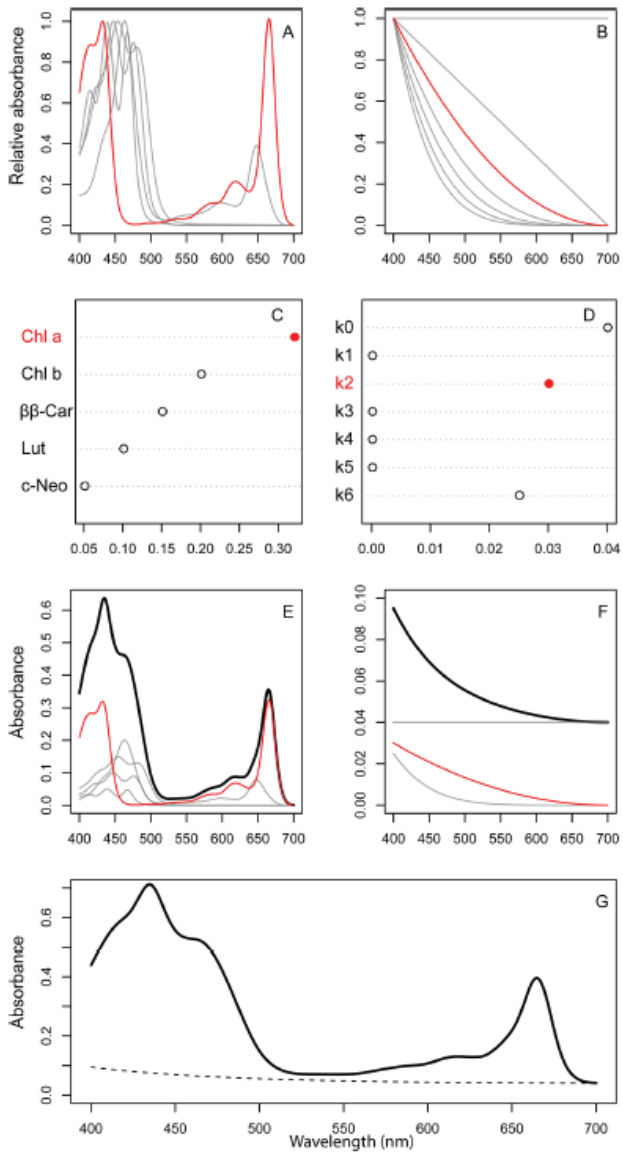


Fig. 13. The method comes with 28 different pigment spectra (five shown in A) and different background component spectra (six shown in B) that all have a peak absorbance of 1. Presenting the method with an absorbance spectrum from a pigment extract of unknown pigment composition (G), the algorithm tries to model the total spectrum as a weighted sum of pigment (A) and background (B) components. This is done by none-negative least squares, such that each pigment that is judged to be present in the unknown sample will be given a non-negative weight (C). The same is done for the background components (D). This weight is proportional to the contribution of that component to the total absorbance spectrum. In fig. E) we have plotted the absorbance spectra of the pigments that were estimated to be present in this unknown sample (thin lines). The height of each spectrum is equal to the component spectrum in A) multiplied with the weight from C). A similar plot for the background components is shown in F). The thick lines in E) and F) represent the sums of the individual pigment (E) and background (F) component spectra. Adding these contributions together yields the total spectrum of the extract (thick line in G). The modelled background spectrum is shown as a dotted line.

Individual pigment concentrations are obtained by diving each pigment's estimated weight (absorbance; cm^{-1}) by its specific absorption coefficient (L g^{-1} cm^{-1}).

The method was tested on extracts from phytoplankton cultures, natural community samples, and sediment samples, and compared to pigment concentrations measured by high performance

liquid chromatography (HPLC). Generally, the modelled absorbance spectra were almost indistinguishable from the measured spectra (shown in fig. 14 for natural lake samples). Individual pigment concentrations, however, were best quantified in single-species cultures, where the number of component spectra to include could be restricted to the known pigment diversity of the species. In natural samples, more candidate pigments must be included to cover the potential pigment diversity of the unknown sample. This caused problems with aliasing of carotenoids because many of the carotenoids have largely similar absorbance spectra. However, robust estimates of total carotenoids and total chlorophylls could be obtained from both lake- and sediment samples.

In conclusion, our method provides a fast and cheap way of quantifying various pigments in cultures with known pigment diversity, and robust estimates of total chlorophylls and total carotenoids from natural lake and sediment samples. The adaptation to microwell plates allows many samples to be run simultaneously, making the method particularly relevant to microplate experiments. The method, however, need further development to be able to separate individual pigments with similar absorbance spectra.

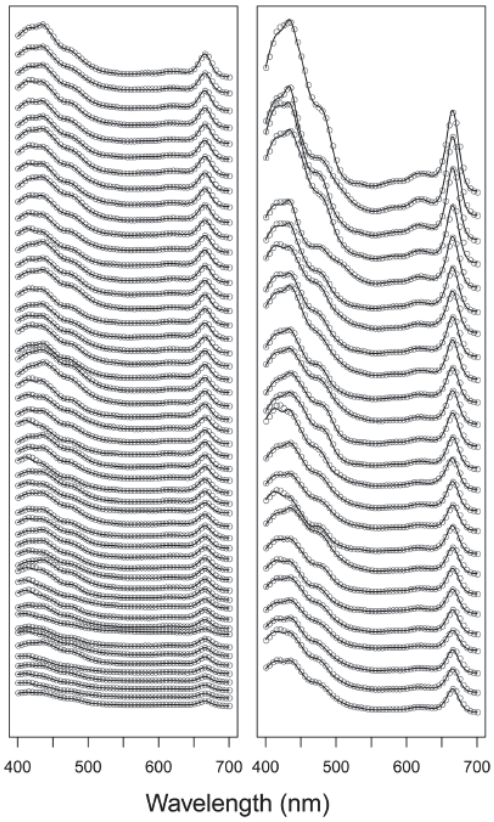


Fig. 14. Absorbance spectra of ethanol extracts from 75 lake seston samples. Measured spectra are shown as dots (only every 5th nm is plotted for clarity), while modelled spectra are shown as lines. For visualization, the spectra are stacked on top of each other by adding a vertical offset between each curve. Hence, there are no units on the y-axis. Spectra are sorted from the lowest (bottom left) to highest (top right) peak absorbance.

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