UNIVERSITAT DE BARCELONA

Nutrients and algal growth in the oligotrophy: a field experimental approach in mountain lakes

Nutrients i creixement algal en la oligotròfia: una aproximació experimental de camp en estanys de muntanya

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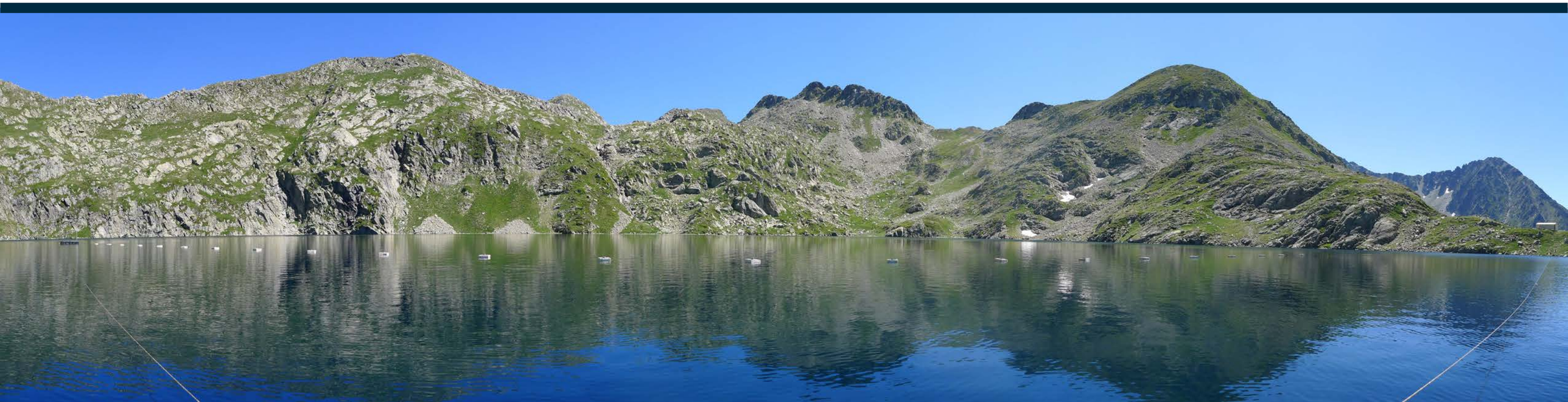

TESI DOCTORAL

Universitat de Barcelona

Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals

Programa de doctorat en Ecologia, Ciències Ambientals i Fisiologia Vegetal

Nutrients and algal growth in the oligotrophy: a field experimental approach in mountain lakes

Nutrients i creixement algal en la oligotròfia: una aproximació experimental de camp en estanys de muntanya

Memòria presentada per Pau Giménez Grau per optar al grau de doctor per la Universitat de Barcelona

Pau Giménez Grau

Cardedeu, setembre de 2016

Vist-i-plau dels directors de la tesi

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Dra. Marisol Felip Benach

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If I had my life to live over,

I'd try to make more mistakes next time

Don Herold

Agraïments

Aquesta tesi no hauria estat possible sense el suport econòmic que he rebut del Departament d'Economia i Coneixement de la Generalitat (beca FI, 1 any) i del Ministeri d'Educació (beca FPU, 3 anys). O sigui, gràcies també a tota la gent que paga impostos -en lloc d'evadir-los-, la majoria sense saber que estan finançant persones com jo, i investigacions com aquestes. Tota la infraestructura experimental tampoc hauria estat possible sense el "projecte de les vaques" (EGALA, OAPN: 124/2010) i el "projecte del nitrogen" (NITROPIR, MICT: CGL2010-19373).

Si aquesta tesi ha arribat a (bon) port també és gràcies a la persistència d'en Jordi i la Marisol. Gràcies per tot el que m'heu ensenyat. He après moltes coses durant aquests anys, coses que, fins i tot sense ser-ne conscient, hi són. En Pau d'avui està bastant més "curtit" científicament (i no científicament) que el que va començar el màster anys enrere. Gràcies!

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Pau

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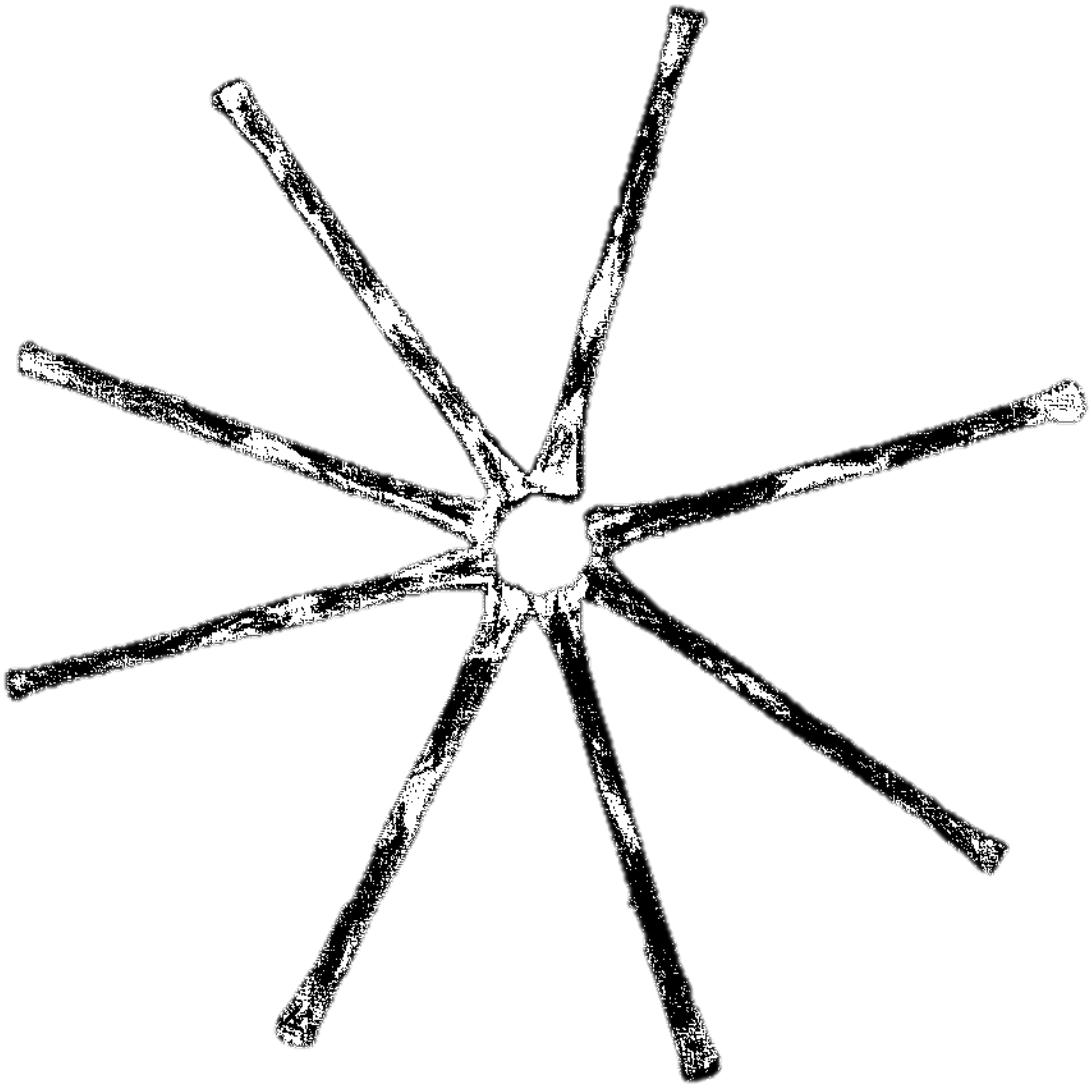


General introduction and objectives

General introduction

Global change and nutrient cycles

Humans are altering the natural processes of the Earth system in such a way that we could have already entered a new epoch called the "Anthropocene" (Waters *et al.*, 2016). Although global environment continually changes due to natural variability, the Holocene (~10000 years BP) has been a period of unusual stability. The human imprint was initially visible during the Industrial Revolution, but the previous stability was drastically and definitely altered about the mid-20th century with the "Great Acceleration" of population growth and industrialization (Steffen *et al.*, 2015a; Waters *et al.*, 2016). Among the multiple processes involved in the global change, the alterations of nitrogen (N) and phosphorus (P) biogeochemical flows are particularly threatening to the stability of the Earth system (Rockström *et al.*, 2009; Steffen *et al.*, 2015b).

Reactive nitrogen (N) has notably increased as a consequence of fossil fuel combustion and agricultural activities, mainly the utilization of N-rich fertilizers obtained from the Haber-Bosch reaction and the cultivation of N_2-fixing species (Galloway *et al.*, 2008; Gruber & Galloway, 2008). This anthropogenic N can be retained by living organisms, denitrified, accumulated in soils and water, or emitted to the atmosphere (Schlesinger, 2009; Canfield *et al.*, 2010). Once in the atmosphere, it is locally or regionally transported, and returned back to the ecosystems through deposition (Hietz *et al.*, 2011; Kim *et al.*, 2011). Thus, even the most remote and pristine ecosystems are exposed to this N fertilization (Bergström & Jansson, 2006; Holtgrieve *et al.*, 2011).

Phosphorus (P) has become a pollutant in some areas with intense pasture and excessive application of livestock slurry for land fertilization (Peñuelas *et al.*, 2012). Although P does not have a gaseous form, it can be found in the atmosphere associated with particles (e.g. dust, sea-salt, biogenic particles, combustion ashes). Desert and dusty regions are then primary sources of atmospheric P to nearby

ecosystems (Mahowald et al., 2008). Some human land uses (e.g. livestock grazing) favoured dust emission (Neff et al., 2008). Moreover, fuel combustion and fires have a greater contribution to global P emissions than previously thought (Wang et al., 2014). Even though, the rise of N emissions in Europe and North America during the 1960-1990 period notably exceeded the increase of P emissions, and, consequently, nutrient depositional loads have become N-enriched. These N:P imbalances have altered the elemental composition of organisms, the productivity, the community structure, and thus the overall ecosystem functioning (Peñuelas *et al.*, 2013).

Primary producers: who they are, where they come from

All complex life on Earth ultimately depends on a relatively small subset of metabolic pathways capable of reducing the inorganic carbon (i.e. CO_2, HCO_3^-) to complex organic compounds. The organisms that perform such reactions are called "primary producers" (or autotrophs) because they provide organic matter for all other organisms. Actually, in most ecosystems, the growth rate of primary producers determines the energy flow and the production of the upper trophic levels (Lindeman, 1942). Despite some Bacteria and Archaea can produce biomass from the oxidation of inorganic chemical compounds (i.e. chemoautotrophy), the most efficient and widespread way to accomplish the reduction of inorganic carbon is the photosynthesis (i.e. photoautotrophy) (Falkowski & Raven, 2007). More specifically, one kind of photosynthesis that requires water as electron donor and produces oxygen came to dominate all ecosystems, the oxygenic photosynthesis.

All oxygenic photoautotrophs share a similar photosynthetic machinery, composed of two photochemical reaction centres derived from two anoxygenic photosynthetic groups, purple bacteria and green sulfur bacteria (Falkowski & Knoll, 2007). The first oxygenic photosynthetic organism, an ancestor of the current cyanobacteria, probably expanded about 2.300 million years ago when oxygen levels in the atmosphere started to increase (Falkowski, 2006). The oxygenation of Earth's

atmosphere precipitated a huge increase of genomic and metabolic complexity, and, indeed, is considered a major transition in the history of life (Raymond & Segrè, 2006). Then, oxygenic photosynthesis might spread via endosymbiosis to a wide variety of eukaryotic clades (Falkowski *et al.*, 2004). The first endosymbiosis was, apparently, the engulfment of a cyanobacterium by a eukaryotic host cell that already contained a mitochondrion (Fig. 1). The engulfed cyanobacterium gradually lost functions, and ultimately became the plastid of the earliest photosynthetic eukaryote. Three clades directly evolved from this primary symbiont: glaucophytes, red algae (i.e. rhodophytes), and green algae and their descendants, the land plants. Glaucophytes currently constitute a quite small group of freshwater species that present plastids with only one kind of chlorophyll, chlorophyll a. Along the evolution of the green algae ancestor, a new accessory pigment appeared, the chlorophyll b, which characterizes the "green plastid lineage" (Falkowski *et al.*, 2004). This lineage includes groups rich in species such as green algae (e.g. chlorophytes and charophytes) and land plants, but also smaller groups derived from endosymbiotic events occurred a long time ago such as the euglenids and chlorarachniophytes, or more recently, as certain dinoflagellates. Although contemporary rhodophytes lack any chlorophyll apart from chlorophyll a, a second main plastid lineage might have born from the endosymbiosis of a red algae ancestor, and is now characterized by the presence of chlorophyll c (i.e. the "red plastid lineage"). This lineage includes a broad range of phylogenetically distant groups, and the endosymbiotic events that originated them are indeed quite uncertain (Lane & Archibald, 2008; Archibald, 2015). A recent study suggests that photosynthesis might have spread throughout the eukaryotic domain by some serial endosymbiosis: the incorporation of a red algae by a cryptophyte ancestor, followed by the adoption of this symbiont by an ochrophyte ancestor, and, finally, of this ochrophyte by a haptophyte (Fig. 1, Stiller *et al.*, 2014). Dinoflagellates are photosynthetically promiscuous; therefore, it is not surprising that a lineage of this group had acquired a "red" plastid, and now also have chlorophyll c.

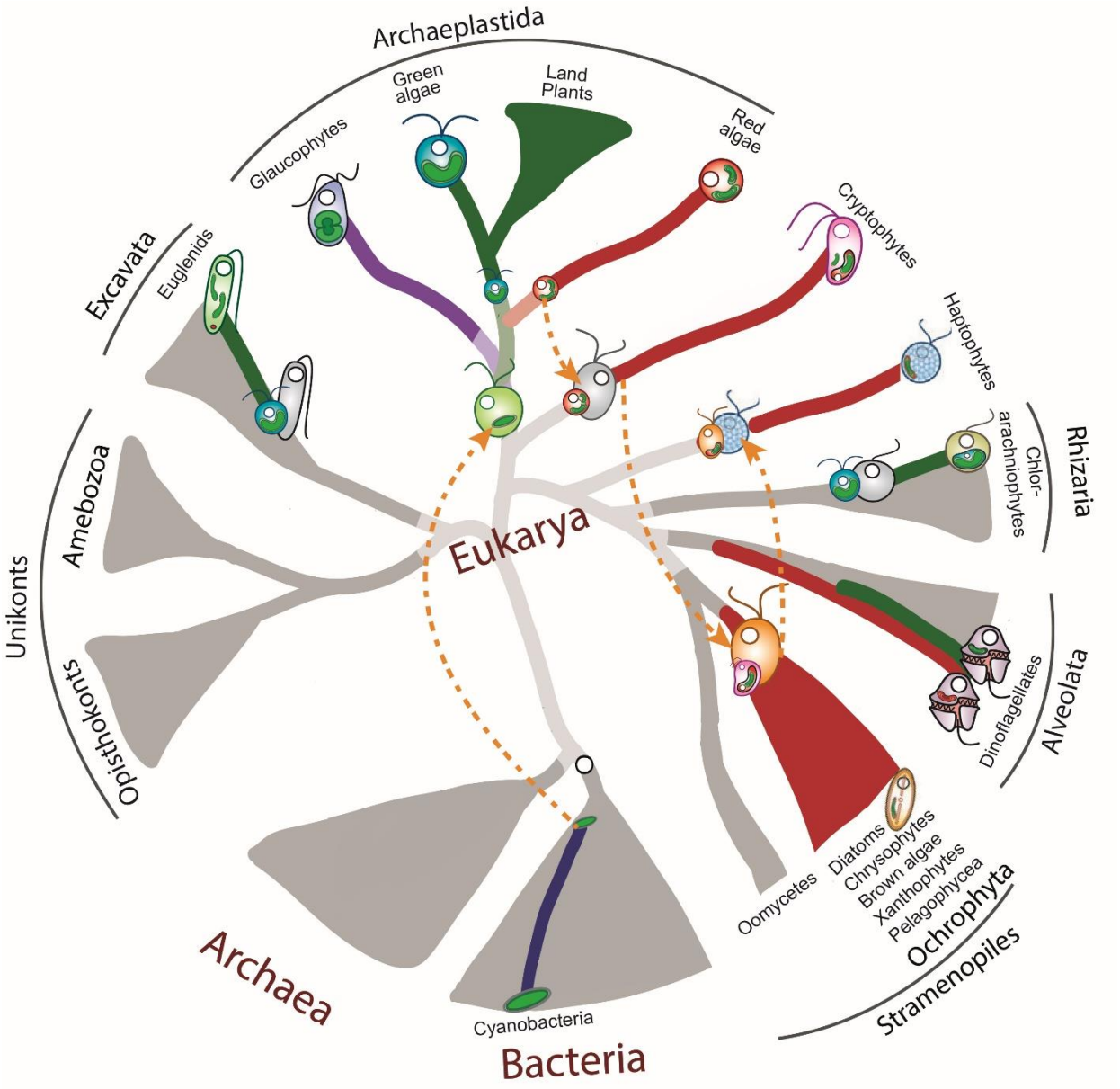


Figure 1 Distribution of oxygenic photosynthesis among the major phylogenetic groups of life. Heterotrophic lineages are represented in gray, while the autotrophic lineages are shown in different colours: blue for cyanobacteria, purple for glaucophytes, green for the "green plastid lineage" derived from a chlorophyte ancestor, and red for the "red plastid lineage", derived from a rhodophyte ancestor. Hypothetical endosymbiotic events in the "red plastid lineage" (Stiller et al., 2014) are represented by arrows. Drawn from diverse sources (Fehling et al., 2007; Reyes-Prieto et al., 2007).

While photoautotrophy on land is markedly dominated by a single clade (i.e. the embryophyte land plants), in marine and freshwater ecosystems there is a phylogenetically diverse set of organisms (Fig. 1), traditionally known as "algae". Algae come in many shapes and sizes (Graham et al., 2009), some being large enough to be seen with the unaided eye (macroalgae), while others being so small

that a microscope is needed (microalgae). Many microalgal species are unicellular, and always occur as solitary cells, but many others form colonies of few to several cells more or less organized (Fig.2). The size of organisms represents an additional difficulty in studying microalgae in comparison to macroalgae or land plants. The identification of microalgal species at the microscope based on morphological features is time-consuming and requires a high degree of expertise. Also, the estimation of algal biomass through the calculation of algal biovolume by assimilating cells to known geometric forms incorporates certain inaccuracy. In contrast, the pigments of an algal community can be readily analyzed at the laboratory (e.g. photometry, fluorimetry, chromatography). Hence, chlorophyll a has become a convenient proxy for algal biomass, and the concentrations of the accessory pigments provides taxonomic information about the sample (Buchaca, 2005; Roy *et al.*, 2011).

Microalgae can live suspended in water bodies as part of phytoplankton, or attached to various types of substrates in periphyton. Although the term "periphyton" has been used in different ways, it generally refers to a complex mixture of algae, bacteria, fungi, detritus and inorganic particles embedded in a mucilaginous matrix above submerged surfaces. Therefore, in general, the term does not just include the autotrophic component, in contrast to phytoplankton. Periphyton usually develops attached to inorganic substrates such as rocks (epilithic) or sand (episammic), but it can also grow attached to plants, macroalgae or animals. Periphyton is an important source of organic matter in streams and shallow zones of lakes, especially if terrestrial vegetation is not well developed and the light path is not obstructed. The autotrophic component of freshwater periphyton is mostly dominated by cyanobacteria, green algae, and diatoms, but there might also be some representatives of red algae, chrysophyceans, and xanthophyceans. In turn, freshwater phytoplankton mainly consists of chlorophytes, chrysophyceans, dinophytes, cryptophytes, diatoms, and cyanobacteria, and the dominance of one or another group is highly dependent on trophic status.

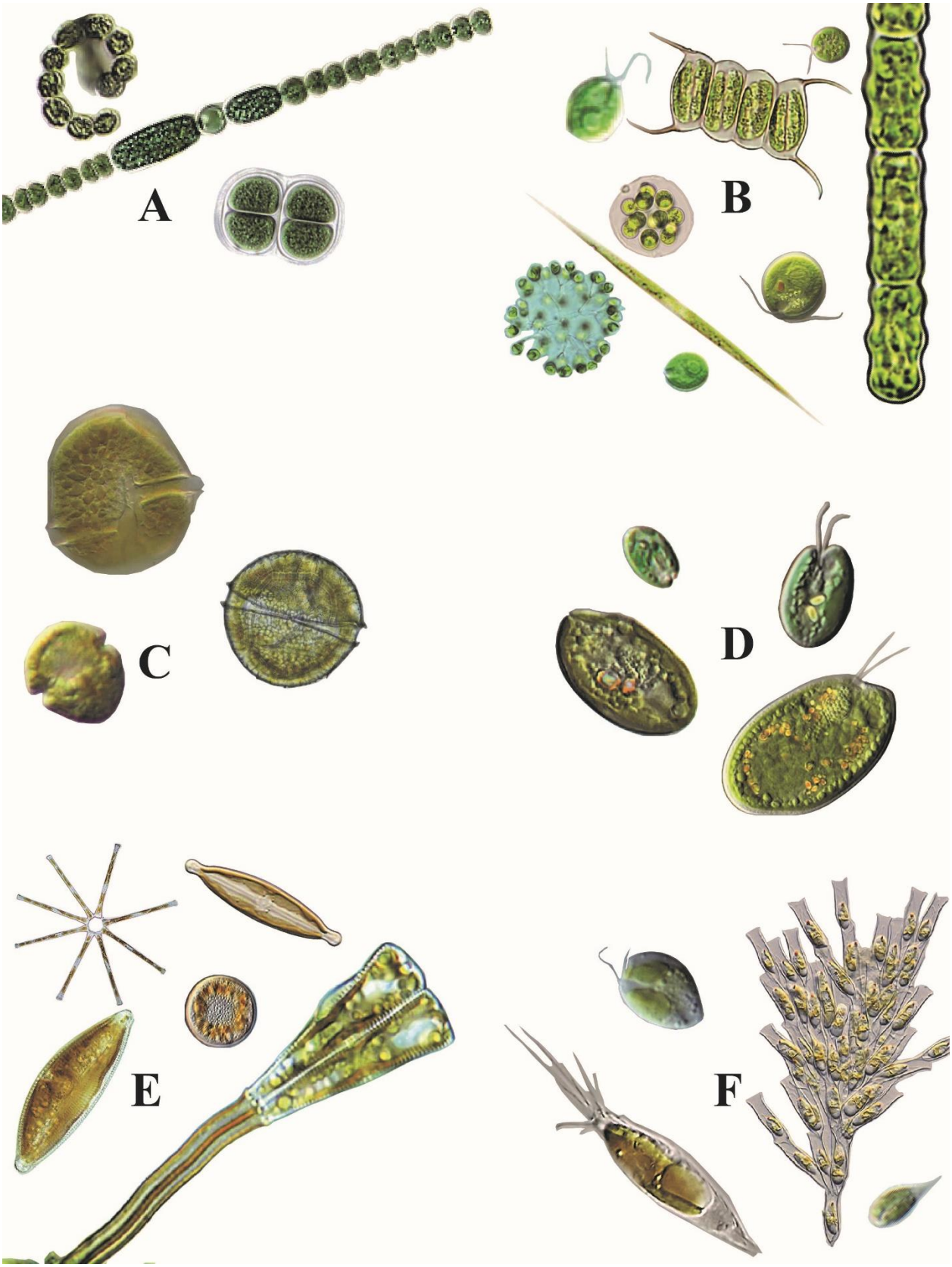


Figure 2 Diversity of microalgal body forms commonly found in phytoplankton and periphyton of mountain ecosystems, by major phylogenetic groups: (A-F) cyanobacteria, chlorophytes, dinophytes, cryptophytes, bacillariophytes, chrysophyceans, respectively. Organisms are shown at different scales. Photos by M. Plewka and Wikimedia Commons. A more detailed compilation of algal diversity in the Pyrenees is provided by Cambra (2003).

Homeostatic regulation of C:N:P composition

All living organisms are highly ordered systems in comparison to their surrounding environment, what is achieved through active, energetically costly, regulatory processes (e.g. negative feedbacks). Homeostasis is intrinsic to life, as metabolic processes only occur under certain physical and chemical conditions, and the external environment is rapidly changing. Homeostasis includes several regulatory processes, but one has taken particular interest in ecology: the control of the elemental composition, or "stoichiometric homeostasis" (Sterner & Elser 2002, Hessen *et al.* 2013). The elemental homeostasis is commonly evaluated as the effect of a change in resource elemental availability on the elemental composition of its consumer (i.e. the slope of the relationship, Fig. 3A). However, below or above certain levels of elemental imbalances, we do not expect any change in consumers' composition, and, therefore, the range under which a consumer can regulate its own composition is also a measure of homeostasis (Fig. 3B).

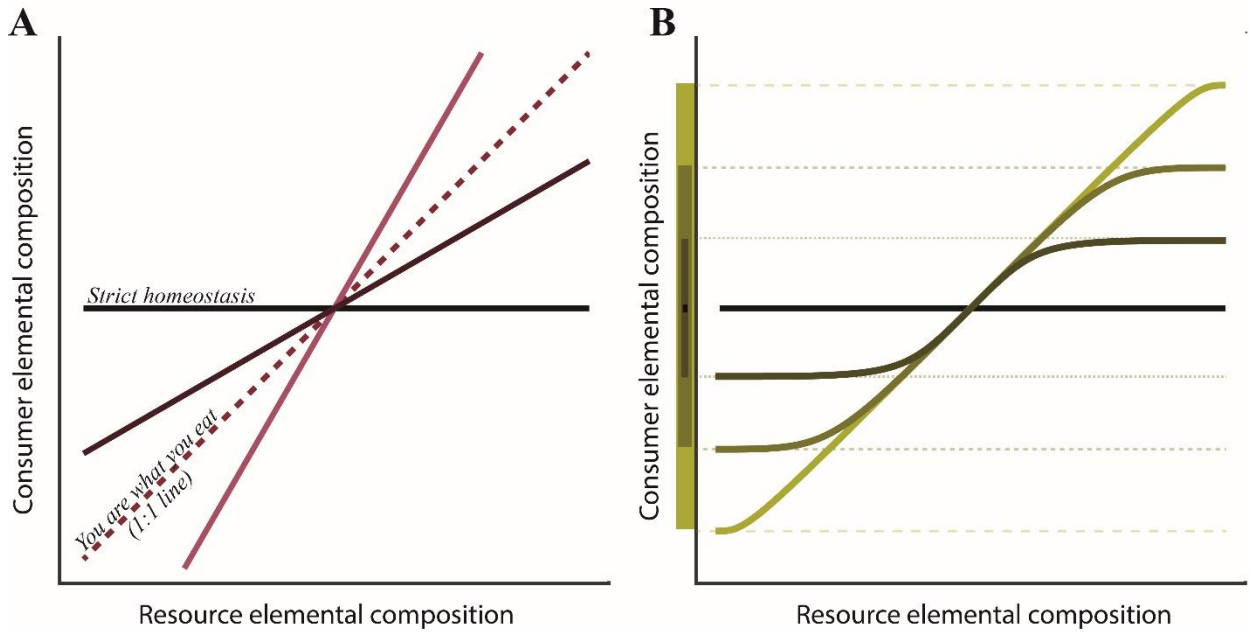


Figure 3 Alternative views of the elemental homeostasis (or flexibility) of an organism: as the rate of change in the consumer elemental composition produced by a change in the resource (A), or as the range of which the consumer elemental composition is affected by the resource elemental availability (B). The relationship between resources and consumers can be referred to nutrients and autotrophs, but also to autotrophs and herbivores, herbivores and predators, and so on. In the first case, the elemental ratio commonly used is the N:P, whereas, in the other cases, it is also possible to compare C:N, C:P, % N, % P, etc.

All living organisms share a core elemental "recipe" of ~20 elements, some in high proportions such as the macroelements (e.g. C, O, H, N, P, S), and others in lower amounts (e.g. Fe, Mg). Although all of them are in principle subject to homeostatic regulation, the focus has been overwhelmingly centred in just three elements, carbon (C), nitrogen (N), and phosphorus (P). They are considered surrogates of three main macromolecular components: carbohydrates, proteins, and nucleic acids. While C is the structural element of all organic compounds, N and P are particularly interesting because the demand for these nutrients by primary producers often exceeds the availability in the surrounding environment, what reduces or even stops their growth. Thus, N and P become quite often the "limiting" elements.

Redfield (1958) noted that the proportion of these elements in marine plankton was remarkably uniform around 106C:16N:1P. Since then, this proportion has become a reference to talk about algal composition, as well as a convenient constant for modelling biogeochemistry and plankton processes (Falkowski, 2000). However, reviews indicate that C:N:P of seston and algal cultures is far less constrained (Geider & La Roche, 2002), and even propose a new global ratio of 166C:20N:1P, and varying power laws C:N, C:P, N:P at regional scales (Sterner *et al.*, 2008). Some of this C:N:P variability in natural algal communities could be explained by the presence of species with different phylogenetic imprints in their elemental composition (Ho *et al.*, 2003; Quigg *et al.*, 2003). However, this phylogenetic signal seems rather small in comparison to the environmental signal. Studies with algal cultures show a considerable flexibility of some species (e.g. *Scenedesmus*; Rhee, 1978), and, in general, autotrophs are less homeostatic than heterotrophs (Persson *et al.*, 2010). This is mainly viewed as an adaptation of autotrophs to store elements when available in excess, and the possibility to use them later when external levels fall. The storage capacity of algal organisms has obviously certain limits (Fig. 3B, Hall *et al.*, 2005), though they are not fully defined yet.

Growth has been argued to be a relevant factor in homeostasis of microalgae, in that achieving the highest growth rates requires a more constrained C:N:P proportion

close to Redfield's (Goldman *et al.*, 1979; Klausmeier *et al.*, 2008). Recent evidence suggests that fast-growing phytoplankton has a more confined elemental composition, and is also more P-rich (Hillebrand *et al.*, 2013). Since the highest growth rates can only be achieved under nutrient excess conditions, which regulatory processes (e.g. ribosomes, P stores) do drive this pattern is a question not fully resolved.

Physiological traits and constraints on resource utilization and growth

The ecological niches of algal species are largely defined by their ability to acquire resources (nutrients, light), to convert them into growth and reproduction, and to avoid loss processes by grazing, infection, toxicity or sedimentation. Functional traits arise from the adoption of diverse ecological strategies among algal species, and physical and chemical constraints on physiological functions (Litchman & Klausmeier, 2008). The functional trait concept is indeed tightly linked to the trade-off concept, as traits usually confer an advantage for performing one function, and, simultaneously, a disadvantage for playing another. Then, selective pressures are not unidirectional but multidirectional, thus preventing the appearance of "superspecies", and promoting the coexistence and diversity.

The ability to acquire and utilize nutrients has been typically characterized by laboratory measurements on cultured algae (reviewed in Edwards *et al.*, 2012), and the subsequent fitting to just a few models:

$$\mu = \mu_{\infty}\left(1 - \frac{Q_{min}}{Q}\right) \quad (\mathbf{1})\ \boldsymbol{Droop\ model},\ \text{growth on internal resource quota}$$

$$\mu = \mu_{max}\ \frac{R}{K_s + R} \quad (\mathbf{2})\ \boldsymbol{Monod\ model},\ \text{growth on external resource conc.}$$

$$V = V_{max}\ \frac{R}{K_m + R} \quad (\mathbf{3})\ \boldsymbol{M - M},\ \text{resource uptake on external resource conc.}$$

The Droop model describes the algal growth on the cellular nutrient concentrations (Q), being μ_∞ the theoretical growth rate at infinite quota, and Q_{min} the internal resource concentration at which $\mu=0$ (Droop, 1973). Two Michaelis-Menten models describe the growth (Monod model) and the nutrient uptake depending on external nutrient concentrations, being the parameters μ_{max} and V_{max} the maximum growth and uptake rate, respectively, and K, the half-saturation constant (Fig. 4). Growth and uptake rates increase with the availability of the limiting nutrient, although all curves tend to saturation.

According to resource competition theory (Tilman, 1982), the competitive ability of species improves decreasing Q_{min}, K_s, and K_m, and increasing μ_∞, μ_{max}, and V_{max}. Classic experiments of resource competition demonstrated the differential ability of *Asterionella* and *Cyclotella* in using P and Si, and tendency towards the replacement of one or the other species, or towards the coexistence, depending on the particular nutrient availability in the medium (Tilman, 1977). A literature review later noted that physiological traits related with NO_3^- and NH_4^+ utilization can vary notably among algal groups, and could be of great utility to understand the distribution patterns of phytoplankton along environmental gradients (Litchman *et al.*, 2007). Later compilations have evidenced the significant influence of cell volume in explaining the variability of NO_3^- and PO_4^{-3} utilization among algal species, as well as in explaining the correlations among these traits (Edwards *et al.*, 2012).

Actually, cell size is considered a "master" trait because it constrains many key organismal characteristics related to diverse ecological functions. Smaller cells have a higher affinity for nutrients simply due to physical diffusion, and dominate the extensive and nutrient-poor regions of the open ocean; contrastingly, nutrient-rich coastal and upwelling zones are generally dominated by larger phytoplankton cells. Cell size is also involved in a trade-off between nutrient storage capacity (Q_{max}) and rapid growth since smaller cells can grow faster but show lower storage capacity (Grover, 1991). Thus, small and fast-growing marine diatoms firstly respond to nitrogen pulses, while larger diatoms can maintain growth at later pulse phases,

when nutrients get depleted, by using the nitrate stored in vacuoles (Raven, 1987). Being large may confer other advantages, such as the capacity to eat other organisms (e.g. mixotrophy in dinoflagellates), but can be risky due to increased grazing pressures and sedimentation rates.

Likely, the major trade-off in algae is that between equilibrium competitive ability (i.e., low K_s and Q_{min}) and maximum growth rate (i.e., high μ_∞ and μ_{max}), often referred as the K and r strategies (MacArthur & Wilson, 1967), or the gleaner-opportunist trade-off (Fig. 4, Grover, 1997). The "K" strategy dominate under conditions of low nutrient availability, whereas the "r" is advantageous when nutrient supply is high and fluctuate intensely. Slow-growing chrysophytes are often taken as an example of good nutrient competitors, and are found abundantly in most oligotrophic lakes (Reynolds, 2006); conversely, fast-growing chlorophytes tend to dominate in most eutrophic lakes, and can also be abundant in oligotrophic lakes during the relatively short and nutrient-rich mixing periods. The capacity to grow fast may somehow impair the ability to deal with low-nutrient conditions, and, frequently, it also comes at the cost of lower yield or efficiency (Litchman *et al.*, 2015). Moreover, growth capacity may be linked with the elemental composition of organisms. For instance, seston C:N and C:P tend to decline as seston abundance increase, and, hence, C sequestration per unit of nutrient decrease with productivity (Sterner *et al.*, 2008). Furthermore, the Growth Rate Hypothesis (GRH) states that achieving high growth rates requires high proportions of P-rich ribosomes, what reduces cellular N:P content in fast-growing organisms (Sterner & Elser, 2002; Vrede *et al.*, 2004). Contrastingly, good competitors for nutrients and light may invest more in N-rich proteins dedicated to nutrient uptake and light-harvesting structures (e.g. chlorophylls), thus increasing their N:P content. While GRH appears to be valid for heterotrophs, its applicability to autotrophs is currently under discussion (Flynn *et al.*, 2010; Hessen *et al.*, 2013; Hillebrand *et al.*, 2013). The key to this debate seems to be the influence of P storage compared to ribosomes in cellular P pools.

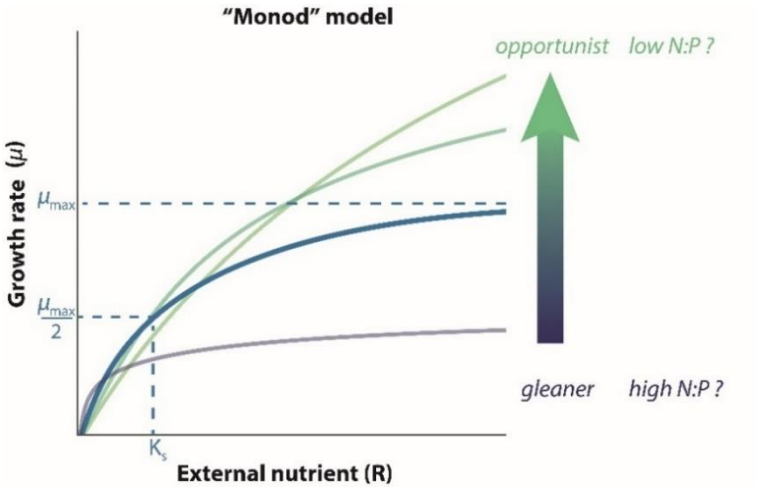


Figure 4 Monod model describing the algal growth based on the external availability of the limiting nutrient. Different nutrient utilization strategies can be found among algal species, basically from slow-growing good competitors (gleaners) to fast-growing bad competitors (opportunists). These strategies could have implications for the N:P content of organisms.

Within each of these two major strategies we can also found relevant physiological trade-offs, such as in the utilization of different resources. For instance, there are selective pressures in oligotrophic waters to diminish internal requirements for limiting nutrients, but, at once, this pressure is compromised with the ability to absorb light at low irradiances, what requires N and Fe (Rhee & Gotham, 1981; Strzepek & Harrison, 2004). A trade-off in the utilization of N and P have also been proposed (Edwards *et al.*, 2011), considering that cell surface and cell volume are limited, and, therefore, the uptake and structural machinery dedicated to one nutrient represents a drawback for the other. However, determining if a trait really constrain another trait can be rather complicated, especially for weak interactions.

The trait-based approach to understanding algal communities (Litchman & Klausmeier, 2008) faces another problematic issue: the parameters estimated at the laboratory are not constant. For a single genotype, traits of resource utilization and growth vary depending on environmental factors and growth history (i.e., phenotypic plasticity). The kinetics of metabolic reactions tend to accelerate with temperature -up to an optimal level-, thus potentially affecting all estimates of uptake and growth (e.g., Reay *et al.*, 1999). Also, it is long recognized that organisms can "acclimate" their light-harvesting structures to irradiance (Falkowski & LaRoche, 1991), and the uptake machinery to varying resource levels in a more or less extended time span (Collos *et al.*, 2005). Recently, Van Mooy *et al.* (2009) showed that algae can also diminish their internal P requirements (Q_{min}) under conditions of P scarcity through the synthesis of non-phosphorus lipids.

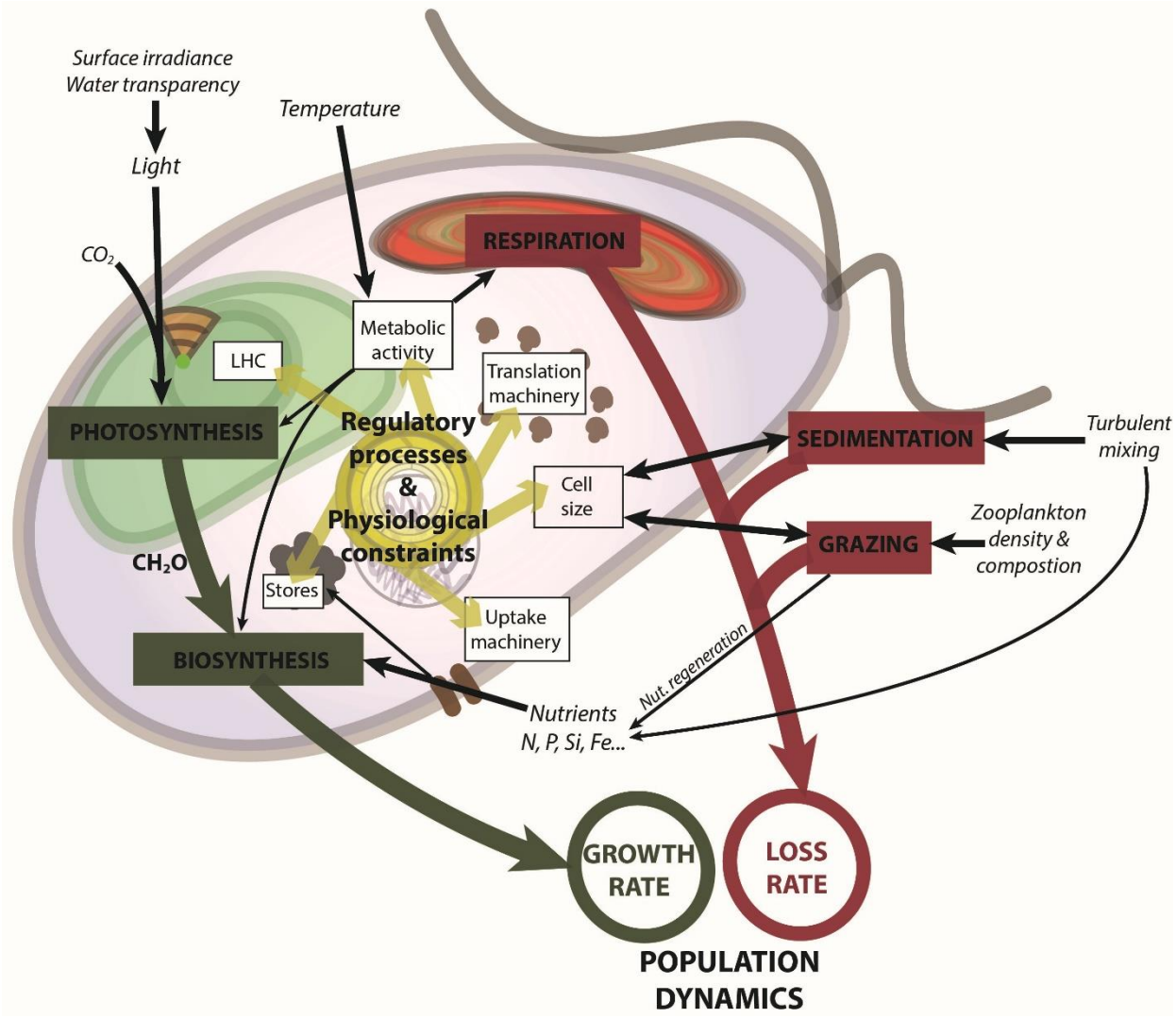


Figure 5 Conceptual model showing some of the physiological interactions occurring in algae and the influence of the environmental control; all together ends up determining population dynamics (modified from Capblancq & Catalan, 1994).

The physiological processes and constraints involved in the regulation of algae are still far to be totally deciphered (Fig. 5). Even if deeply understood, the prediction of algal communities would be unfeasible because they are not only driven by competence, but also by other ecological interactions (e.g. predation, mutualism, and commensalism) and physicochemical factors. Actually, studies that just consider competition and equilibrium conditions tend to result in the dominance of one or few species, and the exclusion of the others (Hardin, 1960), while in nature we commonly find a relatively high number of species. This question has been traditionally referred as the "paradox of the plankton" (Hutchinson, 1961), and its solution may precisely come from the influence of non-competitive ecological

interactions, fluctuations in environmental factors (Descamps-Julien & Gonzalez, 2005), and spatial and temporal heterogeneity in resources (Károlyi *et al.*, 2000). Aquatic ecosystems are indeed highly conditioned by its physical component. For instance, the stratification and mixing of the water column determine key processes such as the nutrient cycling, the sedimentation fluxes, and the interactions among organisms.

Effects of nutrient availability on aquatic ecosystems

By the late 1960's, evidences accumulated of lakes suffering, or that had suffered, processes of eutrophication (Vollenweider, 1968). The uncontrolled increase of algal biomass, the depletion of oxygen in water, the mortality of fishes and other organisms, and the appearance of toxic species had become serious problems in many lakes. Limnological studies then broadly expanded, with the objective to understand the causes of eutrophication, and find mechanisms to control it. Edmondson (1970) demonstrated that algal biomass could be reduced in a lake through sewage diversion, given the control of N and P availability on algal growth. Further studies pointed that controlling P pollution is the best way to revert eutrophication, as the mere reduction of N can still originate blooms of N-fixing cyanobacteria (Schindler, 1974, 1977). Then, management policies aimed at reducing P loads in freshwaters began to be applied (e.g. the regulation of P-containing detergents), and lakes became clearer and better oxygenated.

After whole-lake nutrient enrichments realized in the Experimental Lake Area (Ontario), a general consensus developed in that primary production of lakes was primarily limited by P (Schindler, 1977). Later on, compilations of numerous nutrient enrichment experiments questioned this P limitation paradigm, and indicated that N was as likely as P to be limiting algal growth (Elser *et al.*, 2007; Lewis & Wurtsbaugh, 2008). The relative importance of N, P, and other elements in autotrophic limitation is linked to some climatic, geologic and ecologic factors that determine their availability in a particular habitat; hence, a uniform and broad

picture of nutrient limitation is unfeasible. Actually, even the element (or elements) limiting primary production in the relatively homogeneous marine ecosystems markedly differ among regions (Moore *et al.*, 2013). Recently, some studies stress the importance of synergistic interactions between N and P, and suggest that co-limitation is more widespread than previously recognized (Harpole *et al.*, 2011). Nutrient co-limitation has been invoked in a number of situations, being the simplest cases (a) when, in a single species, one nutrient aids in the uptake of the other, (b) when different species of a community are limited by different nutrients, and (c) when the simultaneous addition of both nutrients is required to get a response because both nutrients are at low levels (Arrigo, 2005). Currently, the framework of single-resource limitation appears to be shifting to a more complex framework including multiple nutrients (Kaspari & Powers, 2016), and where the main focus is not just total algal growth, but also key regulatory processes of algae such as their internal N:P content (Bracken *et al.*, 2015).

Although the effects of nutrient fertilization are relatively well-known in eutrophic and human-impacted ecosystems, the effects on freshwater oligotrophic ecosystems have received less attention. Indeed, the recognition that several pristine and remote ecosystems are being fertilized via atmospheric deposition have aroused much interest (Elser *et al.*, 2009; Camarero & Catalan, 2012). The first and most direct effect of higher N and/or P availability may be the stimulus of primary production, what, in turn, may increase the transfer of energy and matter to upper trophic levels (i.e. a bottom-up effect). Moreover, the community of primary producers is expected to change due to a proportional increase of fast-growing species with high nutrient requirements. Algal N:P content could also be affected by these changes, particularly if nutrient inputs are unbalanced. Since both the "quantity" and the "quality" of primary producers affect the energy transfer to grazers, herbivores community and nutrient cycling may be altered as well (Hessen *et al.*, 2013). Therefore, an entire re-organization of trophic network and ecosystem functioning can be expected.

Objectives

The main objective of this dissertation was to determine the effects of N and P fertilization on the growth of major algal groups, and evaluate some indicators of the regulatory processes (e.g. N:P content, chlorophyll:biovolume) involved in the growth responses. To do so, we followed a field experimental approach of short-term nutrient enrichments (21-25 days), using nutrient-diffusing substrates in the case of epilithic periphyton (Chapter 1), and mesocosms in the case of phytoplankton (Chapter 2 and 3). This experimental approach increases the complexity of the oversimplified laboratory studies, and reduces the complexity of the whole-lake nutrient enrichments. These experiments were performed in the lake district of the Pyrenees, a region of particular interest because the average conditions are just on the threshold between the N and P limitation of algal growth (Camarero & Catalan, 2012). As well, most lakes are oligotrophic, and its nutrient availability is highly conditioned by N and P inputs of atmospheric origin, which appear to be changing due to human activities and global change. The specific objectives of the experiments were:

Objective 1 was to determine the main factors controlling the algal colonization of epilithic periphyton and examine the differences among major algal groups.

We selected 9 lakes and 2 streams to account for environmental gradients of trophic status and water renewal, while the influence of N and P availability was evaluated in each location using nutrient-diffusing substrata.

Objective 2 aimed to assess the influence of nutrient availability on the productivity, the composition of major phytoplankton groups, and the C:N:P proportions in seston, and evaluate the interrelationships among them.

Mesocosms were vertically deployed in Lake Redon and enriched with different amounts of P (phosphate) and N (ammonium or nitrate), so as to create a gradient of increasing P availability, another gradient of increasing N, and compare the effects produced by shifts in DIN dominant forms (ammonium vs. nitrate).

Objective 3 aimed at understanding the relationships among nutrient availability, growth, and chlorophyll content per biovolume in phytoplankton. Specifically, we evaluated to what extent intense growth is associated with high chlorophyll a contents per biovolume, or with a major plasticity of that content.

Pigment contents per biovolume and their plasticity were determined for all major algal groups by estimating non-linear, power-law based, models. The gradients of growth conditions were obtained from the nutrient-enriched mesocosms of the Objective 2.

1

Contrasting factors drive early periphyton colonization by diatoms and chlorophytes in oligotrophic lakes

Abstract

We conducted periphyton colonization experiments in nine oligotrophic lakes and two streams of a high-mountain catchment to determine the factors controlling the algal development, especially of the two major algal groups: chlorophytes and diatoms. We selected the sites to account for trophic and water renewal gradients, and used nutrient-diffusing substrates to study the local effect of nutrient availability (non-enriched, N-enriched, P-enriched and N+P-enriched substrates). We estimated the diatom and chlorophyte biomass using marker pigments combined with CHEMTAX. Algal growth was then related with environmental gradients and experimental enrichments using multilevel regression models. The variation in biomass accrual among sites was higher than the differences related with the respective enrichments, mainly due to high variation in the development of diatoms. The "site effect" was mainly associated with trophic status (e.g., dissolved organic carbon, total phosphorus) and hydrodynamics (e.g., average water renewal), both factors enhancing total algal growth, although chlorophytes were not significantly affected by hydrodynamics. Diatoms dominated the periphyton at high water renewal, and were responsible of the highest total algal biomasses. Enrichments inhibited the growth of diatoms, particularly nitrogen, although enrichment effects were not independent of the water renewal. High water renewal mitigated the inhibitory effects on diatoms, and stimulated chlorophytes growth, particularly in N-enriched substrates. Trophic status also conditioned the response of chlorophytes to P and N+P treatments. Overall, the findings add evidence to the suggestion that nutrient fertilization caused by atmospheric deposition would favour chlorophytes at the expense of diatoms in high mountain periphyton communities, and highlights water renewal as a key factor in the colonization of littoral periphyton in lakes.

Introduction

Periphyton communities are the primary source of energy for higher trophic levels in several freshwater ecosystems, particularly in unshaded streams with little allochthonous inputs (Biggs, 1996), and shallow oligotrophic lakes with poorly developed phytoplankton communities (Vadeboncoeur *et al.*, 2008). The latter might be the case of many high-mountain lakes located above or near the tree line. Periphyton in high-mountain lakes is periodically affected by harsh conditions and disturbances that can alter or even reset the community (Rott *et al.*, 2006; Uehlinger *et al.*, 2010). In winter, for instance, the presence of a snowpack that prevents the arrival of light, the littoral scouring effect by ice, and the low temperatures have an impact on periphyton communities. Storms can be severe throughout the year, and the wind and high flows can disturb the development of periphyton. Moreover, algal biomass can be constrained by high UV radiation in the shallower zones (Vinebrooke & Leavitt, 1996). Under these circumstances, periphyton assemblages are periodically reset, particularly in shallow littoral waters. Despite the relevance for the system, little is known about which factors drives the early periphyton colonization in oligotrophic mountain lakes.

Periphyton has long attracted research in freshwaters (Hoagland *et al.*, 1982), but most of the studies have focused on streams rather than lakes (Cantonati & Lowe, 2014). All in all, some factors can be anticipated as potentially relevant in mountain lakes. i) First, the size of the local pool of colonizers able to settle on the free substrates should accelerate the process. This pool is proportional to the lake trophic status and variables such as total phosphorus (TP) and dissolved organic carbon (DOC), which are correlated with carbon flow in mountain lakes (Catalan *et al.*, 2009a). ii) A second factor is water flow, which enhances the transport of colonizers from the pool sources to the substrates to be colonized. Water renewal, as a surrogate of average water flow, can range orders of magnitude in mountain lakes depending on the lake size and how it is connected to the surface drainage systems.

Energetic hydrodynamics might remove settlers, particularly if carrying mineral particles. This negative influence of water flow has been shown in some streams (Steinman & McIntire, 1990), and could be relevant in some mountain lakes exposed to strong winds and sandy littorals, in which case water flow would have an ambivalent role. iii) Finally, nutrient availability at a local scale (i.e. mesohabitat) compared with the average conditions of lake can favour some algal groups upon others. For instance, colonization of isolated large bare rocks should not proceed in the same way as in small pebbles close to organic sediments releasing nutrients. More generally, nutrient availability in the system and stoichiometric constraints may condition the colonization process interacting with other factors. P is usually the primary nutrient controlling lake productivity (Schindler, 1977), yet N limitation and N and P co-limitation conditions have also been reported (Maberly *et al.*, 2002; Nydick *et al.*, 2004; Elser *et al.*, 2007). The increase in N atmospheric deposition during the last decades have driven some remote lakes towards enhanced P limitation (Elser *et al.*, 2009); yet, P deposition may counteract such tendency in Pyrenean lakes, and lead them towards N limitation (Camarero & Catalan, 2012).

In this study, our primary aim is to determine the main factors controlling early periphyton colonization in oligotrophic lakes, and whether they differ among the main algal groups. We followed an experimental approach in which we selected nine lakes in a mountain catchment covering a broad range of trophic conditions (always within the oligotrophy) and water renewal. Two stream experimental sites were also included to break the high correlation usually found between trophic state and water renewal in mountain lakes. In each experimental site, we assessed the periphyton early colonization rates using nutrient-diffusing substrates (NDS). This system allows us to increase phosphorus (P), nitrogen (N) or both nutrients (N+P) at a scale of mesohabitat over the background nutrient concentrations of the site.

Periphyton includes a complex assemblage of evolutionarily diverse microalgal organisms (Stevenson, 1996a). Even experienced algologists cannot easily identify

all species present in a sample microscopically, and precise information about the community structure actually requires considerable effort and skill. For this reason, many studies only measure a surrogate of the total algal biomass (i.e. chlorophyll a) (Steinman *et al.*, 2006); thus simplifying the understanding of the colonization process. A compromise between effort and information is achieved using group-specific marker pigments determined by liquid chromatography (e.g., HPLC, UPLC) (Hagerthey *et al.*, 2006). Based on these measures, the relative contribution of the main algal groups to total biomass was estimated using the CHEMTAX algorithm (Mackey *et al.*, 1996). This procedure, originally developed for marine phytoplankton, has also been applied satisfactorily to phytoplankton and benthic algal assemblages in freshwater ecosystems (Buchaca *et al.*, 2005; Majdi *et al.*, 2011). The periphyton growth and the relative contribution of the main algal groups were finally related to the experimental and background conditions by statistically fitting multilevel/hierarchical models.

Methods

Study sites

The experiment was conducted in nine lakes and two stream sites from the St. Nicolau catchment of the Aigüestortes i Estany de St. Maurici National Park (Fig.1A), located in the Central Pyrenees. Siliceous bedrocks dominate the area and the lakes and streams are typically oligotrophic. The study sites expand from 1600 to 2500 m.a.s.l. and include five locations (T, E, I, S and L) below the tree line and six above. Table1 summarises the main characteristics of sites, and indicates the site codes used in figures and tables.

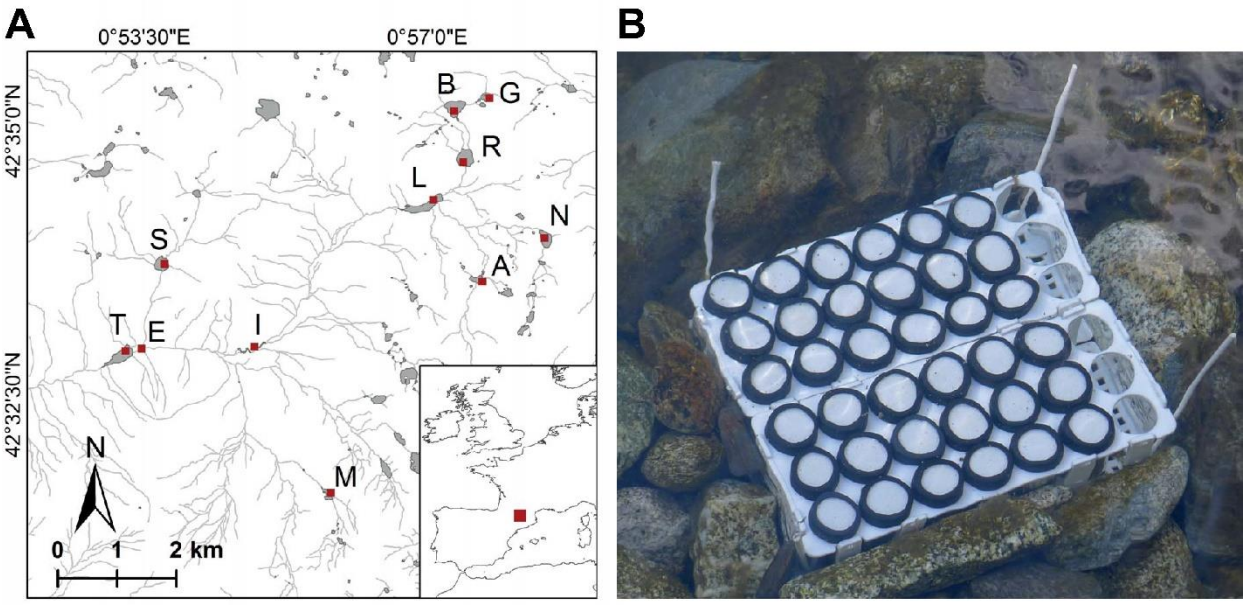


Figure 1 A) Map of St. Nicolau catchment with the study sites (codes as in Table 1). B) Picture of the NDS experimental setting.

Table 1 Location and morphometric characteristics of the study sites

Site	**Code**	**Type**	**Lat N**	**Long E**	**Altitude** (m)	**Watershed area** (ha)	**Lake area** (ha)	**Depth** (m)
Llebreta	T	lake	42.55065	0.89016	1620	5439	8	11.5
Planell Llebreta	E	stream	42.55053	0.89209	1622	5439	-	0.3
Planell Aigüestortes	I	stream	42.55306	0.92078	1826	3112	-	0.4
Llong	L	lake	42.57504	0.95304	2000	1131	7.1	12.5
Redó	R	lake	42.57984	0.95788	2116	307	6.3	12
Sarradé	S	lake	42.56268	0.89702	2124	427	4.2	15
Coma Amitges	A	lake	42.56243	0.96235	2272	183	1.8	10
Nere	N	lake	42.56920	0.97435	2298	160	4.2	38
Mussoles	M	lake	42.52927	0.93157	2359	169	1.5	10
Bergús	B	lake	42.58722	0.95534	2447	126	6.2	51
Gelat Bergús	G	lake	42.58989	0.96153	2494	24	1.4	8.5

Environmental conditions

We measured water temperature (Temp) and collected water samples from each study site two weeks before the beginning of the experiment. All samples were analysed for pH, conductivity, and alkalinity (Gran titration) immediately after collection. Total phosphorus (TP), dissolved reactive silica (DRSi), NH_4^+ and NO_2^- were determined using a segmented flow auto-analyser (AA3HR, Seal/Bran+Luebbe). TP was determined in samples previously digested by

persulphate oxidation (Grasshoff *et al.*, 1983), followed by colorimetry based on a Murphy & Riley's (1962) method (Bran+Luebbe G-175-96). DRSi was determined using a molibdo-silicate reduction to heteropoly blue (B+L G-177-96), NH_4^+ by the blue indophenol (Berthelot reaction) method (B+L G-171-96), NO_2^- by the Griess reaction (B+L G-173-96), and NO_3^- was determined by capillary electrophoresis (Quanta 4000, Waters). Dissolved inorganic nitrogen (DIN) was calculated as the sum of NO_3^-, NO_2^- and NH_4^+. DOC was determined by catalytic combustion and infrared spectrometric detection of the CO_2 produced (TOC5000 Shimadzu analyser). Water chemistry was analysed in five sites (T, E, I, L and R) three more times during and just after the experiment to estimate a coefficient of variation (CV) for each chemical variable during the incubation period.

We characterized water renewal at each site using two variables, namely, water flow and characteristic velocity. We estimated the average water flow (Flow, L s^{-1}) from the watershed area of the site, the rainfall during the experiment period (averaged from three weather stations within the valley), and the evapotranspiration estimated at two points in the catchment. In the case of T and E sites, we also took into account the amount of water diverted by a small dam. As similar water flows may be achieved through "channels" of different section (e.g., lake and stream), we estimated a characteristic mesohabitat velocity (Vel, cm s^{-1}) dividing the water flow by the lake or stream section at the point where the diffusive substrates were deployed.

Experimental substrates

Nutrient-diffusing substrates (NDS) were constructed using 36 ml plastic cups and lids with holes, following designs by Gibeau & Miller (1989) and Tank, Bernot & Rosi-Marshall (2006). The cups were filled with nutrient-enriched agar and a 2.5 cm fritted glass disc (Leco, #528-042, porous cover) placed on top of the agar, which eventually was the surface for periphyton growth. Four nutrient treatments were prepared: non-enriched, N-enriched, P-enriched and N+P-enriched. Nitrogen was

added to the agar as 0.8M KNO_3, and P was added as 0.025M K_2HPO_4 + 0.025M KH_2PO_4. Therefore, the N+P enrichment had a molar N:P ratio of 16:1. All treatments were buffered with 100µM $NaHCO_3$ to approach pH 7. The treatments were randomly distributed in the supporting trays, but following the same pattern for each site.

The experiments started between 6 and 9 September 2012 at the 11 study sites. A rack containing at least 12 randomly distributed NDS (3 replicates per treatment) was fixed to the ground at a depth of 0.46 ± 0.13 meters (Fig. 1B). After three weeks of colonization, the racks were collected, and the substrates retrieved. The discs were wrapped with aluminium foil and deep-frozen in liquid nitrogen to prevent pigment degradation.

To check the NDS performance during the experiment, in one lake we retrieved the substrates after 3, 10, 17 and 21 days, and determined the nutrient release in the laboratory. We submerged each NDS in a pre-rinsed plastic container filled with 500 ml deionized water for 2h, and subsequently analysed NO_3^- and SRP concentrations. We found a decline in NO_3^- and SRP release as the experiment proceeded, but nutrient release at the end of the experiment remained high, and the N:P ratio about 16 (Fig. 2).

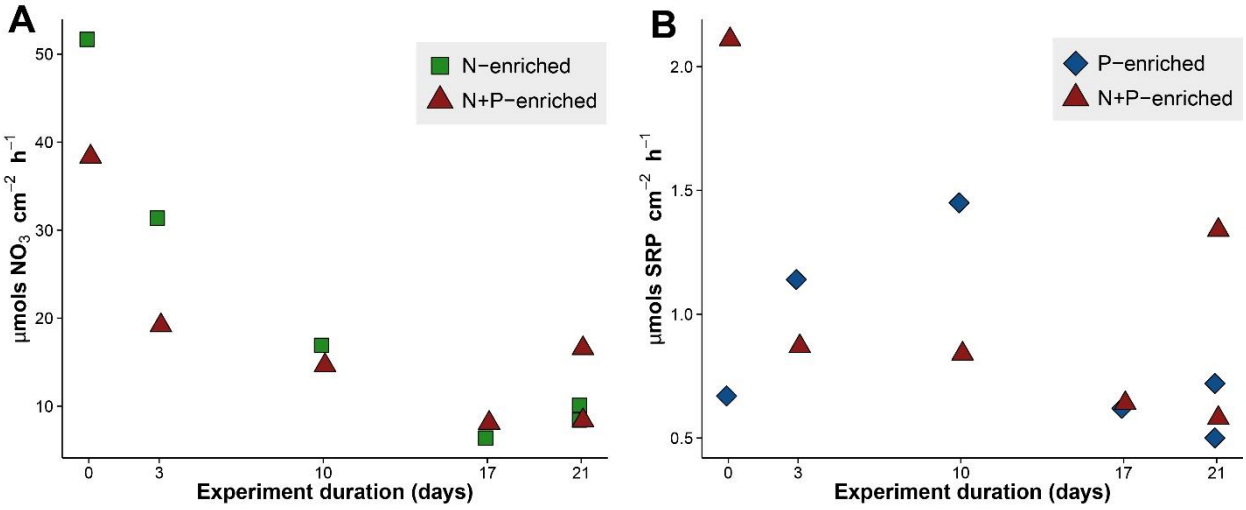


Figure 2 NDS diffusion rates for NO_3^- and SRP after 3, 10, 17 and 21 days of colonization.

Pigment analysis

The pigments were extracted in 3.5 ml 90% acetone with probe sonication (Sonopuls GM70 Delft, The Netherlands) (50W, 2 min). The extract was centrifuged (4 min at 3000 rpm, 4 ºC), filtered through Whatman Anodisc 25 (0.1 μm) and analysed by ultra-performance liquid chromatography (UPLC). The UPLC system (Acquity, Waters, Milford, MA, U.S.A.) was equipped with an Acquity UPLC HSS C18 SB column (dimensions: 100 x 2.1 mm, particle size: 1.8 μm) and photodiode array (λ 300-800 nm). Two channels, set at 440 and 660 nm, were recorded for better carotenoid and phorbin detection, respectively. After sample injection (4.5-7.5 μl), pigments were eluted by linear gradient from 100% solvent B (51:36:13 methanol : acetonitrile : MilliQ water, v/v/v 0.3 M ammonium acetate) to 75% B and 25% A (70:30 ethyl acetate : acetonitrile, v/v) for 3 min, followed by 0.45 min of isocratic hold at 75% B and 2 min of linear gradient to 99.9% solvent A. Initial conditions (100% B) were linearly recovered in 0.65 min. The flow rate was 0.7 ml min^{-1}. Pigments were identified checking retention times and absorption spectra against a library made based on standard commercial mixtures (DHI, PPS-MiX-1), and extracts from pure cultures of algae and bacteria available in our lab. Final quantification was made using specific absorption coefficients at 440 nm.

Contributions of the main algal groups to total algal biomass

The degree of algal colonization was estimated determining the amount of chlorophyll a (Chla) per experimental substrate. The relative contribution of the main algal groups was assessed using indicative auxiliary photosynthetic pigments for each group, and the CHEMTAX algorithm (version 1.95, Mackey *et al.*, 1996). The calculations were initialised using marker pigment to Chla ratios obtained from the literature (Majdi *et al.*, 2011). We anticipated finding diatoms, chlorophytes, and cyanobacteria as major groups in the samples. However, after analysis, we did not find any marker pigment exclusive of the cyanobacteria. Zeaxanthin, a pigment present in both cyanobacteria and chlorophytes, was below the detection limit in 58% of the samples, and at low concentrations in the rest. Zeaxanthin to chlorophyll

b ratios of the latter samples, and initial CHEMTAX calculations including cyanobacteria confirmed the negligible abundances of this algal group. Thus, we discarded the cyanobacteria contribution in the final CHEMTAX optimization, and focused the study on diatoms and chlorophytes. We estimated the Chla of these two groups using diadinoxanthin (Diadin), fucoxanthin (Fuco) and chlorophyll c (Chlc) as marker pigments of diatoms, and lutein (Lut) and chlorophyll b (Chlb) as marker pigments of chlorophytes. All available samples (n=132) were included at once to maximize CHEMTAX performance and comparability; no reasons for splitting them into different compositional sets were found.

Data analysis

The relationship between the response variables (total Chla, diatoms Chla, and chlorophytes Chla) and the experimental factors was established using regression and multilevel/hierarchical models (Gelman & Hill, 2007). The data analysis followed three phases with the aim of disentangling site vs. general variation, and the interactions among factors. The process led to three sets of models: site models, full models, and minimal models.

Site models

We modelled the effects of nutrient enrichment at each site separately using linear regression:

$$\ln Chla \sim b_I + b_N N + b_P P + b_{NxP} NxP \qquad (1)$$

where lnChla is the natural logarithm of diatoms, chlorophytes or total Chla; b_I, b_N, b_P, b_{NxP} are the intercept value and the coefficients of the N enrichment, P enrichment, and NxP interaction effect, respectively. Following, we determined the two most influential environmental factors explaining the variability of each coefficient. An automatic model selection was applied (MuMIn, Bartroń 2009), and the better models were ranked according to the smaller sample Akaike information criterion (AICc). All environmental variables were entered as candidate predictors.

Flow and Vel were log-transformed due to their skewed distribution (lnFlow, lnVel), and TP because we observed non-linear effects. The automatic variables selection was followed by a final supervised selection based on two criteria: 1) the models that included two highly correlated variables were discarded, and 2) the appearance of new unnecessary variables was limited by preferentially selecting the same variables for diatoms, chlorophytes, and total Chla, when similar models (ΔAICc<4) were found. This second criterion allowed us to compare the effects of the same variables on the distinct algal groups, and verify whether the effects on total algal biomass were coherent with the effects on diatoms and chlorophytes.

Full models

We built a mixed (or multilevel) linear model for each algal group with all the available samples (n=132). The environmental variables selected in the previous step were included as fixed terms, whereas the nutrient enrichment effects (N, P, NxP) were included as both fixed and random terms of the model. Thus, the model considered the hierarchical design of the experiment, and the site-related variability of the intercept and the enrichment effects were accounted in the random part of the model. The continuous environmental variables were standardized by subtracting the mean and dividing by two standard deviations to facilitate the comparison of their respective effects (Gelman & Hill, 2007). The enrichments (N, P, NxP) were coded as binary factors with values 0 or 1. The adequacy of the selected predictors was subsequently checked, adding or excluding components in the respective full models.

Minimal models

Full models allowed the comparison of treatment and environmental effects among algal groups. However, a simplified model including the respective most essential factors can be sufficient for prediction. Automatic model selection (MuMIn) was applied, starting with the previous full models and simplifying so that the model with minimal AICc ("Minimal model") was finally selected for diatoms,

chlorophytes, and total Chla. The enrichment effects were not forced to be in the fixed part of the model, and were discarded if appropriate.

We used the statistical software R version 3.1.2 for data analysis (R Core Team, 2014), and the extension package ggplot2 for graphical display (Wickham & Chang, 2015). All models were fitted with classical Restricted Maximum Likelihood (REML) methods using the lme4 package (Bates *et al.*, 2014). The estimation using Bayesian methods based on Markov chain Monte Carlo and uninformative priors gave nearly identical results (data not shown).

Results

Environmental gradients and correlations

The selected sites differed in trophic, chemical, and water renewal characteristics (Table 2). TP placed within the assumed oligotrophy range in all sites (Wetzel, 2001), ranging from 1.3 up to 4.9 $\mu g \cdot L^{-1}$. Lakes G and L were the sites with the lowest N availability (DIN:TP molar ratio ~30, DIN <70 $\mu g \cdot L^{-1}$), while the most P-deficient sites were the lake S and the stream E (DIN:TP molar ratio~250, DIN >200 $\mu g \cdot L^{-1}$). The two variables that showed more variability were TP and DOC, with a coefficient of variation ~20%.

Table 2 Physico-chemical characterization of the study sites

Site	Flow $L \cdot s^{-1}$	Vel $cm \cdot s^{-1}$	Temp ºC	pH	Alkalinity $\mu eq \cdot L^{-1}$	Conductivity $\mu S \cdot cm^{-1}$	DOC $mg \cdot L^{-1}$	DRSi $mg \cdot L^{-1}$	TP $\mu g \cdot L^{-1}$	DIN $\mu g \cdot L^{-1}$	DIN:TP molar
T	835	1.657	19	7.8	325	39.7	1.10	1.26	3.2	115	79
E	835	46.39	16	7.5	313	39.8	0.56	1.88	1.8	202	251
I	605	37.81	16	7.2	224	29.2	0.57	1.76	1.8	173	217
L	220	0.453	19	7.4	209	25.5	1.02	0.73	4.8	62	29
R	60	0.066	17	6.7	89	13.7	0.81	1.26	2.4	128	117
S	83	0.084	17	7.0	118	19.4	0.62	1.43	2.5	281	251
A	35	0.075	20	7.5	346	40.8	1.08	1.06	3.2	96	67
N	31	0.051	19	7.6	248	31.5	0.89	0.59	1.7	87	111
M	33	0.088	19	7.0	317	39.9	0.84	1.60	4.9	199	89
B	25	0.031	16	7.0	74	11.4	0.60	1.01	1.3	123	204
G	5	0.011	16	6.9	72	10.4	0.76	0.84	3.5	46	30
CV Mean				1.6%	3.5%	4.3%	18.1%	6.0%	18.7%	14.7%	22.3%

The correlation patterns among variables were examined by Principal Components Analysis (PCA) (Fig. 3). The two first principal components accounted for 76% of total variation. The first axis was defined by the positive load of DIN, and the negative load of TP and DOC, thus reflecting changes in trophic status. The second axis was associated with two groups of variables loading positively: water renewal (lnFlow and lnVel), and water acidity (pH, alkalinity). The inclusion of two stream sites in the experiment helped to decrease the correlation between trophic status and water renewal. Streams, which obviously had the highest water velocities, showed the lowest DOC values, and medium to low TP concentrations. The correlations between DOC and lnVel (r = - 0.27), and TP and lnVel (r= -0.22) were not significant; thus, we could consider them as independent variables in the subsequent analyses. There were sites present in the four quadrants defined by the two axes, and, hence, the main environmental variability was covered with representative sites (Fig. 3).

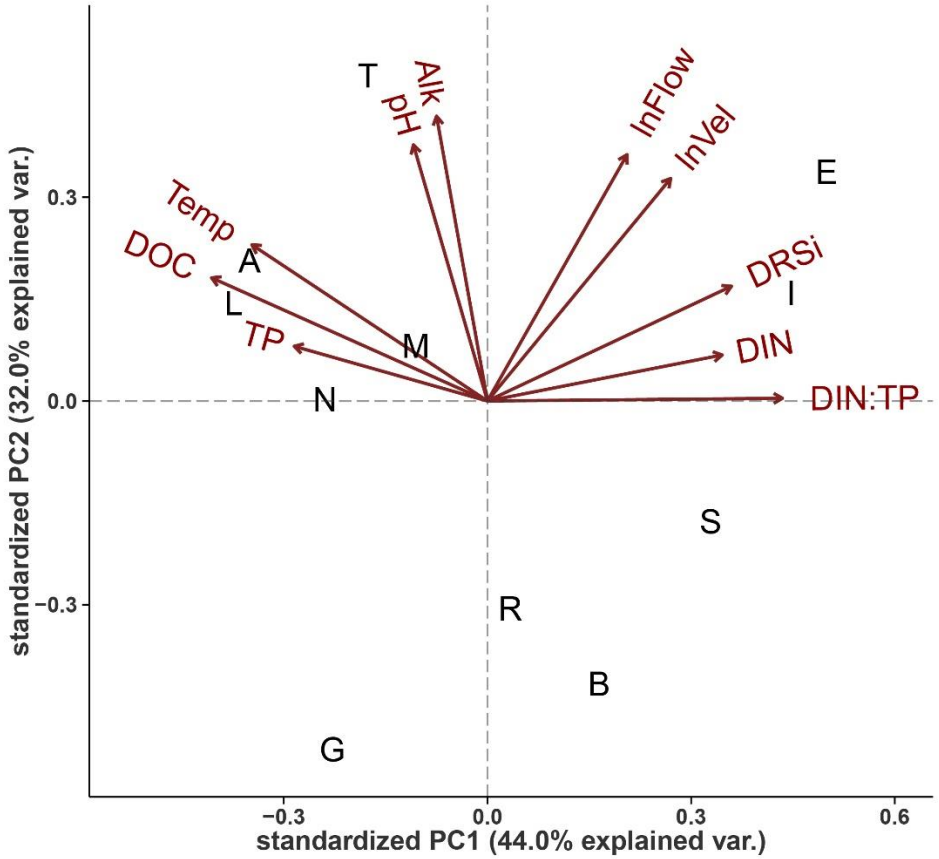


Figure 3 Principal component analysis biplot with the environmental variables (site codes as in Table 1).

Algal growth variability within and between sites

After three weeks of colonization, the algal biomass grown on the substrates ranged more than two orders of magnitude, from 0.04 up to 5.38 µg Chla·cm^{-2}. Higher variation was found among sites (CV = 90%) than among treatments in each single site (CV_{mean} = 32%; CV_{min} = 8% in site A; CV_{max} = 47% in site M). In most sites, the highest algal biomass was recovered in non-enriched substrates, and the lowest in the N-enriched (Fig. 4). The exceptions occurred in sites with high periphyton colonization, the two streams (E, I) and the lake L.

Visual inspection indicated that grazing effects by large organisms (e.g. snails) were negligible as a source of variation for sites and substrates. Furthermore, the detection of the grazing indicative pigment phaeophorbide a was limited to 5% of the samples, and in small amounts compared to Chla. Other Chla degradation products were undetected (phaeophytin) or extremely rare (chlorophyllide a).

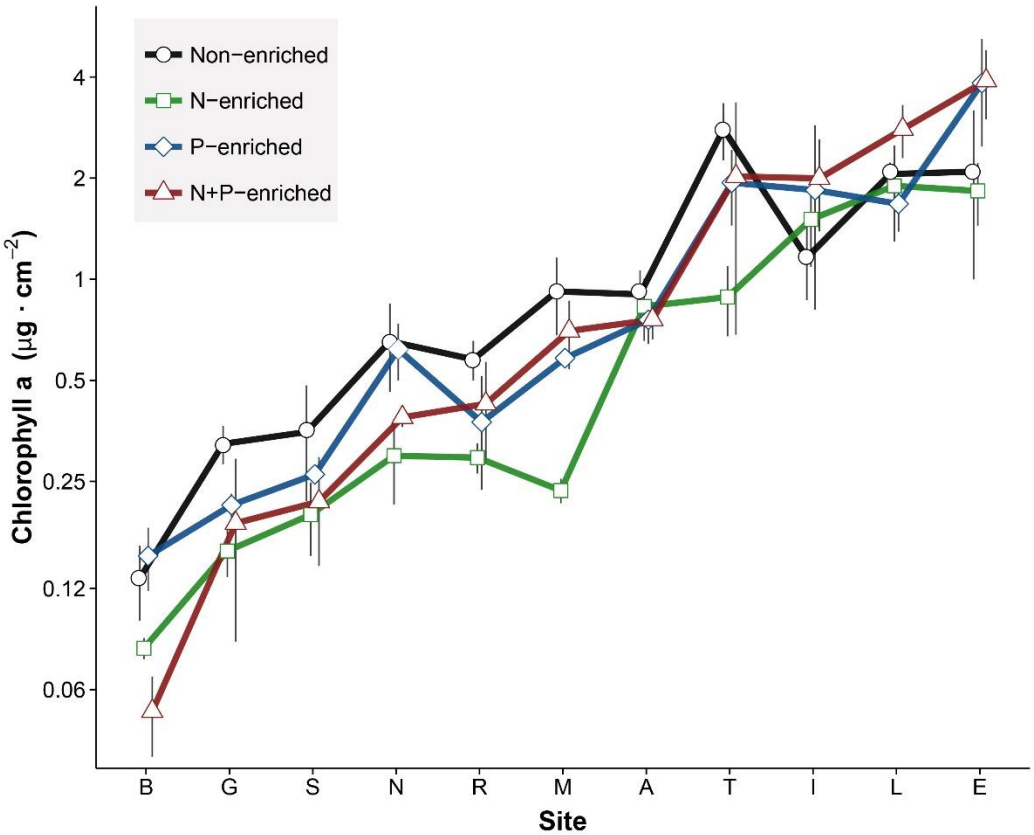


Figure 4 Chla obtained after three weeks of colonization for each experimental treatment (see legend), and for every site. Sites ranked by Chla levels in N+P treatment. Line ranges indicate ±1 standard deviations of 3 replicates. Note that Y-axis is logarithmic.

Diatoms and chlorophytes contributions to algal growth

The marker pigments of chlorophytes (Chlb and Lut) were detected in 95% of the samples, while the markers of diatoms (Diadin, Fuco and Chlc) were detected in >83% of the samples. Output marker pigment to Chla ratios of CHEMTAX placed within the typical range of these algal groups: 0.20 Chlb:Chla, 0.13 Lut:Chla, 0.12 Diadin:Chla, 0.36 Fuco:Chla, and 0.09 Chlc:Chla. High algal biomasses generally coincided with the dominance of diatoms, while the samples with low algal biomasses were generally dominated by chlorophytes. This pattern was particularly marked in non-enriched and P-enriched substrates (Fig. 5A, B). The dominance of chlorophytes accentuated in N- and N+P-enriched substrates because chlorophytes growth tended to increase, and diatoms growth to decline (Fig. 5C, D). Diatoms were leading the wide variation of algal biomass among locations, while the growth of chlorophytes was more uniform.

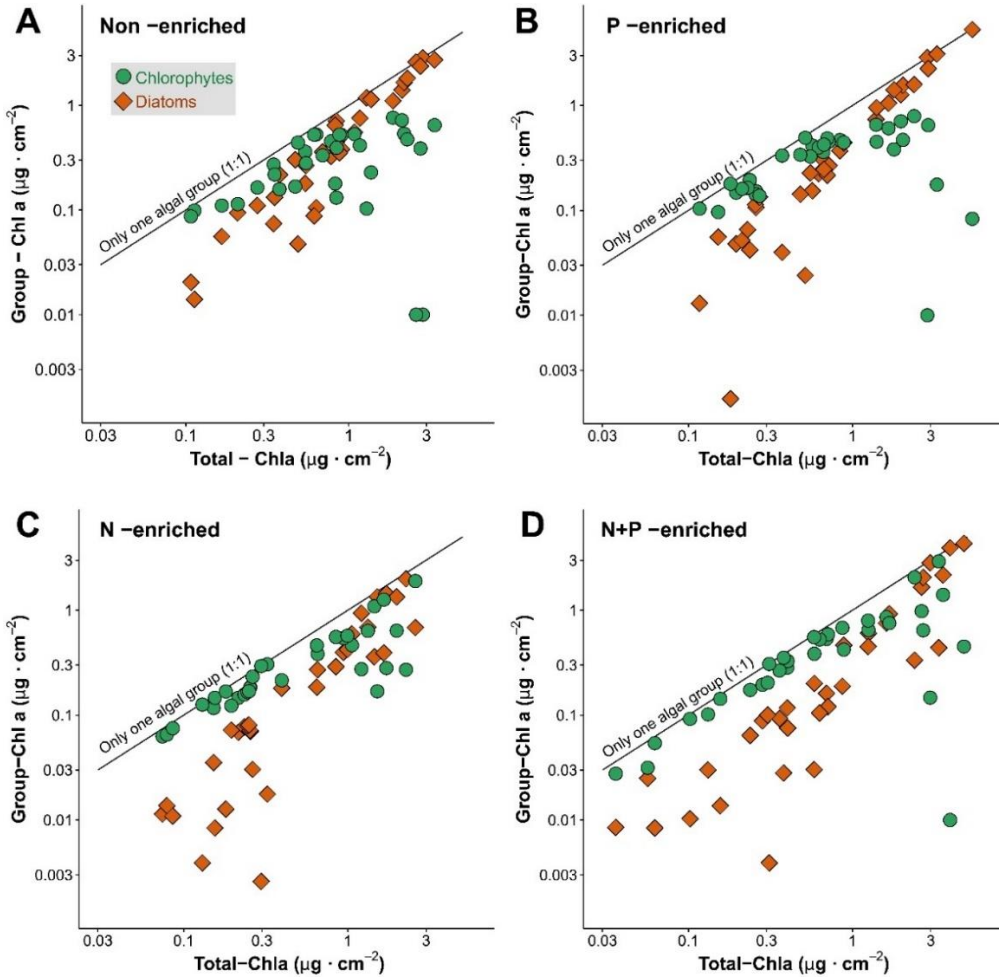


Figure 5 Estimated Chla of chlorophytes (circles) and diatoms (diamonds) in relation to total observed Chla in non-, P-, N-, and N+P-enriched substrates (A-D). For each treatment, n = 33.

Trophic status and water renewal determine algal growth and the response to nutrient enrichments

The coefficients b_I , b_N , b_P , and b_{NxP} were extracted from the "site models" to explore the relationships with the environmental variables. DOC, TP, and lnVel were the three variables that better explained the periphyton colonization and the enrichment effects (Fig. 6). The colonization of periphyton in the absence of nutrient enrichment effects (b_I) was well explained by DOC (mainly "lake" effects) and by lnVel (mostly "stream" effects) (R^2=91%, p-value<0.0001, Fig. 6A). These two variables were also selected in the model of diatoms (R^2=86%, p-value<0.0004). However, lnVel was not significant in chlorophytes, and, in this case, DOC alone explained the 66% of the variability (p=0.002). Consequently, lnVel was the environmental variable that better explained the changes in dominance between chlorophytes and diatoms (Fig. 7), which were predominant at low and high Vel, respectively. This shift occurred about 0.08 $cm \cdot s^{-1}$ in non-enriched and P-enriched substrates (Fig. 7A, B), and about 1.5 $cm \cdot s^{-1}$ in N- and N+P-enriched substrates (Fig. 7C, D).

N enrichment had a negative effect on algal growth (most b_N coefficients <0, Fig. 6B), but this adverse effect was stronger for diatoms than for chlorophytes (Fig. 7C). The factor that better explained b_N coefficients of diatoms, chlorophytes, and total Chla models was lnVel, indicating that the adverse effect could be mitigated at the highest velocities (e.g. the streams). The relationship was not significant for total Chla due to the influential site T (Fig. 6B, R^2=26%, p-value<0.1), marginally significant for diatoms (R^2=39%, p-value=0.04) and significant for chlorophytes (R^2=72%, p=0.001).

LnVel was also the top ranked variable explaining the b_P coefficients of all three models. In this case, however, the inclusion of log-transformed TP improved the models of chlorophytes and total Chla (R^2=85%, p=0.0005, and R^2=78%, p=0.002 respectively). The four sites with TP concentrations below 2 $\mu g\ L^{-1}$, including the

two streams, presented higher b_P coefficients than the other sites for total Chla model (Fig. 6C). This negative effect of TP on P-enrichment response appeared to be rather non-linear. In contrast, TP positively affected the NxP interaction for chlorophytes (R^2=44%, p=0.03), and total algal biomass (R^2=59%, p=0.006, Fig. 6D). No environmental variables significantly explained the NxP effects for diatoms.

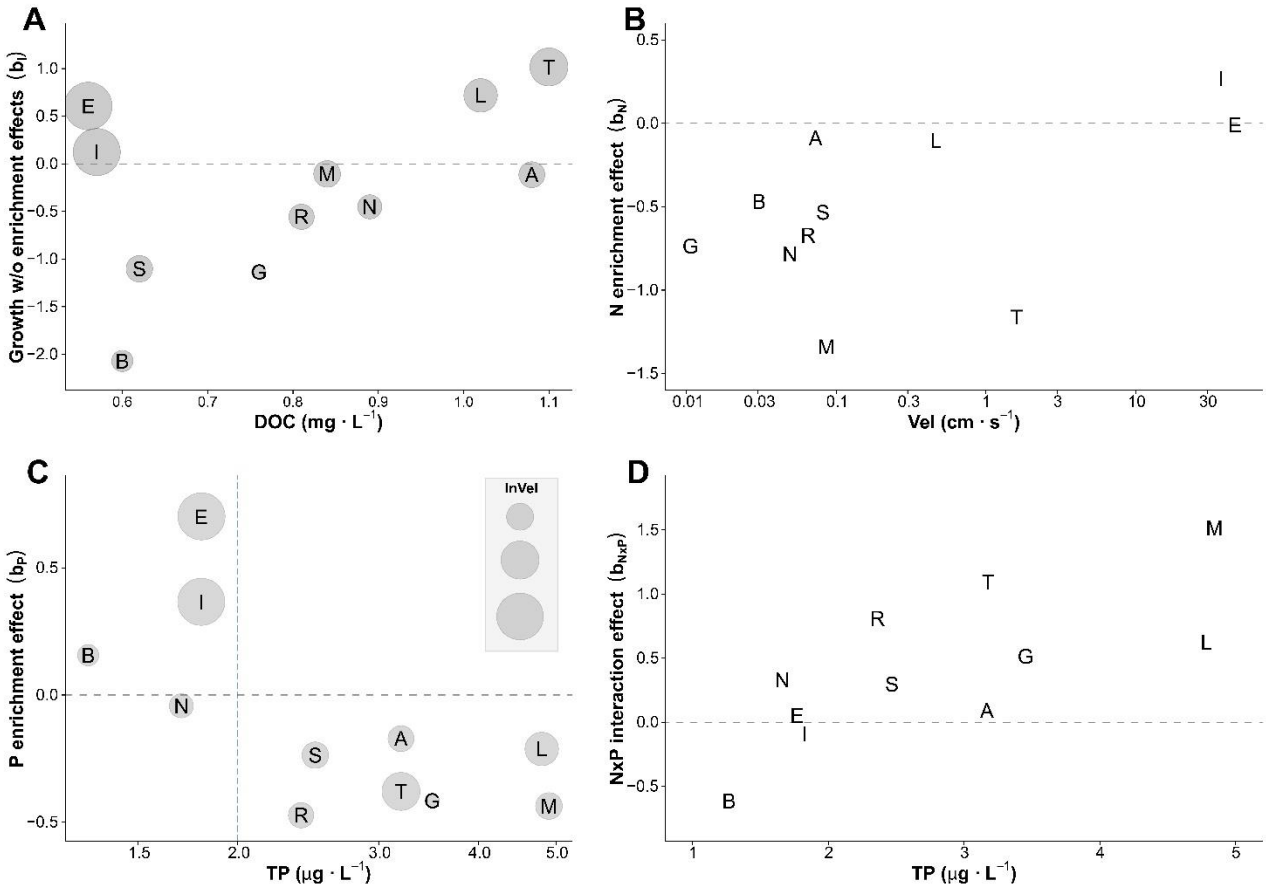


Figure 6 Relationship between the coefficients b_I, b_N, b_P, and b_{NxP} of the site models for total Chla (Equation 1), and the respective best environmental predictors (A, B, C, D). The bubble area is proportional to log-transformed water velocity in A and C. X-axes are logarithmic in B and C.

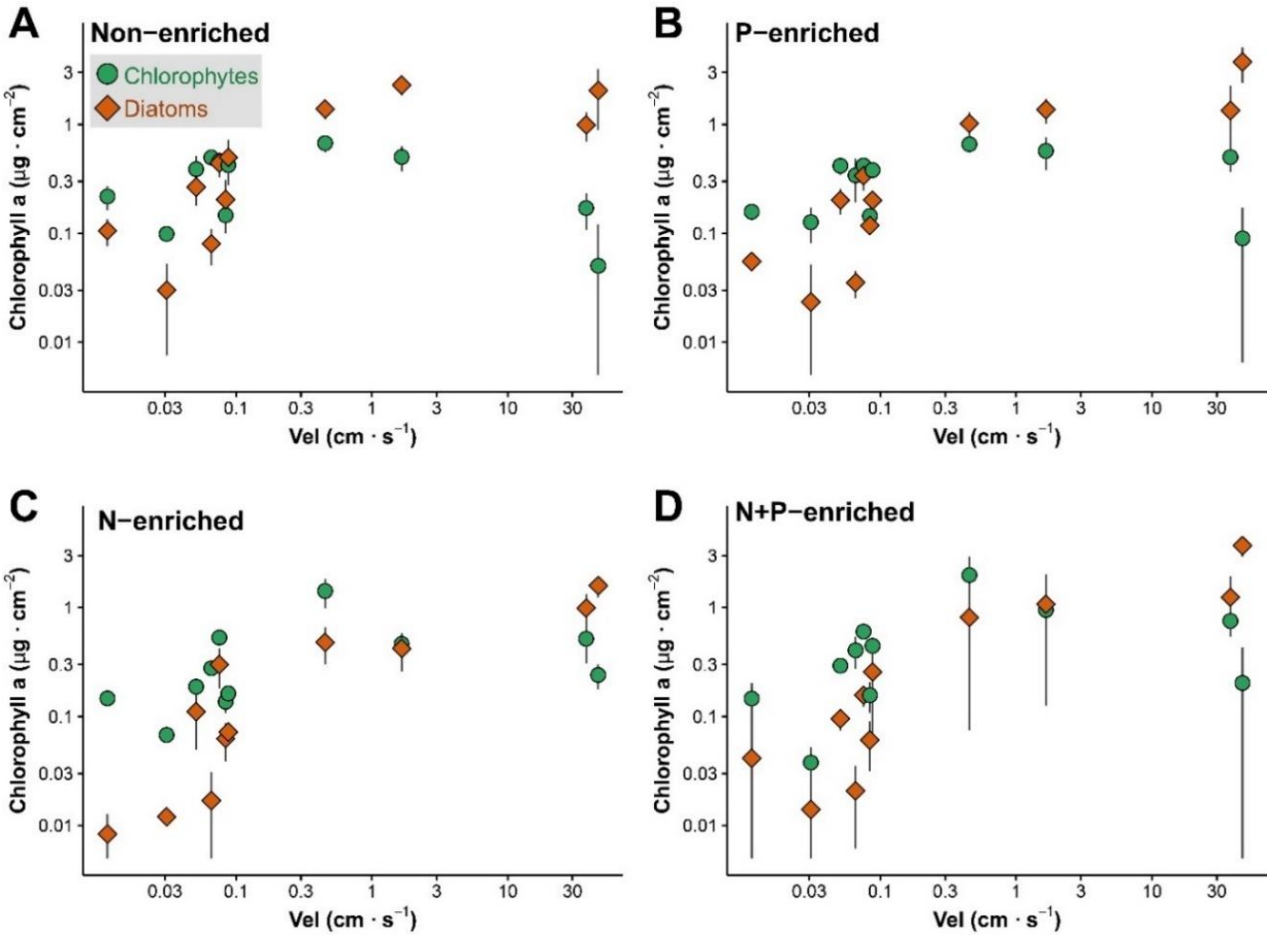


Figure 7 Group-specific Chla (chlorophytes in circles; diatoms in diamonds) against average water velocity (both in logarithmic scale), for each treatment (A, B, C, D). Line ranges are ±1 sd.

Quantifying the influence of the main factors driving algal growth

In the following step, we quantified the relative influence of all factors -within and among sites- on algal growth by building the "full models" for total algal biomass (total Chla), diatoms, and chlorophytes (Fig. 8). In general, the estimated coefficients for total Chla were more accurate (i.e., lower deviation) than for diatoms and chlorophytes, and they used to place in between both groups, or somewhat closer to diatoms. LnVel was the most influent variable for diatoms and total Chla, but it did not affect chlorophytes. DOC had a similar positive influence on chlorophytes and diatoms, and was the second major driver of total algal growth.

The effects of nutrient enrichments clearly differed between diatoms and chlorophytes. N enrichment had a substantial adverse effect on diatoms and total

Chla, but did not negatively affect chlorophytes (Fig. 8). LnVel interacted with N effects in both algal groups, mitigating the adverse effect of N enrichment on diatoms, and stimulating the growth of chlorophytes. P enrichment also hindered diatoms colonization, although with less intensity than N. Again, this negative effect on diatoms was reduced when lnVel was high. P enrichment did not affect chlorophytes growth or even produced a minimal positive effect, which was accentuated in sites with high lnVel and low TP concentrations. Finally, we detected that the interaction NxP had a positive effect on diatoms, which reduced the negative effects of N and P enrichments in N+P substrates. This positive effect of NxP interaction was only detected on chlorophytes in those sites with high TP concentrations.

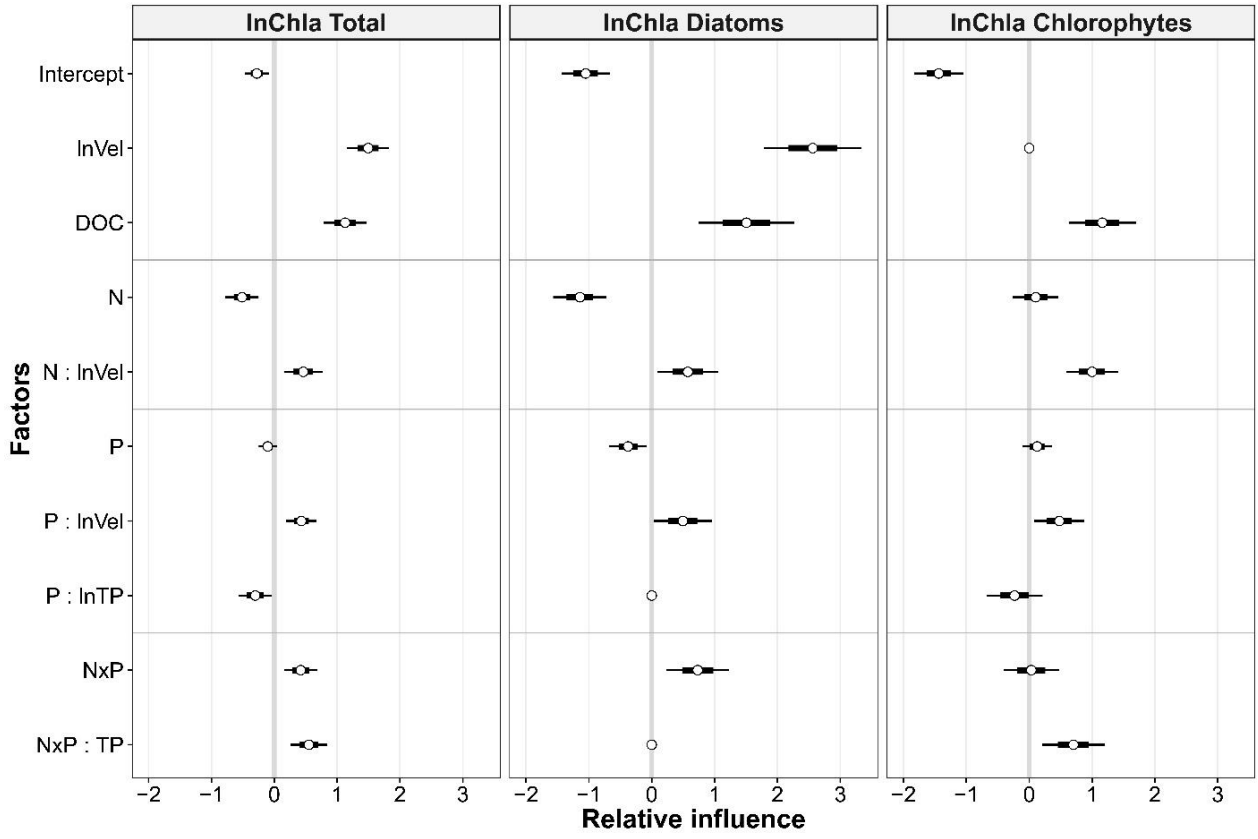


Figure 8 Estimated coefficients for the three "full models". As all environmental variables were standardized, the position in the X-axis represents the relative influence of each factor on log-transformed Chla (total, diatoms, chlorophytes), being the less influent factors closer to "0". Those factors not included in the model are shown as a dot without line range at the "0" position. The thick and thin line ranges represent ±1 sd and ±2 sd, respectively.":" and "x" indicate interaction, and "ln" indicate that the variable was log-transformed.

We then evaluated to what extent some factors could be ignored for each response variable through the AIC-based simplification of "full models" to "minimal models" (Table 3). The model for total algal colonization was reduced to three influential variables (DOC, lnVel and the interaction P:lnVel) with a change in AICc of 10 points. However, this simplification also produced a marked decline of the variance explained by fixed factors (Δmarginal-R^2 =10). The full model for diatoms was already good regarding AICc, and, consequently, the simplification barely changed the AICc (ΔAICc < 2), and several factors were still maintained (lnVel, DOC, N, N:lnVel, P:lnVel). In this case, the exclusion of P and NxP effects diminished the negative effects of N enrichment. The AICc was significantly reduced in the minimal model for chlorophytes (ΔAICc=13), though the factors maintained in the model (DOC, N:lnVel, P:lnVel, NxP:TP) presented similar coefficients than the full model. The colonization of chlorophytes was harder to explain than the colonization of diatoms, what is clear comparing the values of marginal and conditional R^2.

Table 3 Regression coefficients of the fixed terms of the full and minimal models

Response variable	**Intcpt**	**lnVel**	**DOC**	**N**	**N:lnVel**	**P**	**P:lnVel**	**P:lnTP**	**NxP**	**NxP:TP**	**AICc**	**Marg. R^2 (%)**	**Cond. R^2 (%)**
lnChla Total													
Full	-0.28	1.49	1.13	-0.51	0.46	-0.10	0.43	-0.30	0.42	0.55	148.1	83.5	93.8
Minimal	-0.41	1.42	0.94				0.50				137.9	73.2	92.2
lnChla Diatoms													
Full	-1.05	2.56	1.51	-1.14	0.57	-0.38	0.50		0.73		314.1	77.0	90.2
Minimal	-1.17	2.53	1.49	-0.61	0.56		0.48				312.8	74.6	89.7
lnChla Chlorophytes													
Full	-1.44		1.16	0.10	1.00	0.13	0.48	-0.23	0.03	0.70	271.2	45.7	77.9
Minimal	-1.44		1.12		1.02		0.51			0.56	257.8	46.0	77.5

AICc : small sample corrected AIC; **Marginal R^2**: variance explained by fixed factors; **Conditional R^2**: variance explained by both fixed and random factors; "**:**" and "**x**" indicate interaction and "**ln**" indicate that the variable was log-transformed.

Discussion

Periphyton colonization and development is a complex process influenced by a mixture of autogenic and allogenic factors (McCormick & Stevenson, 1991; Stevenson & Peterson, 1991; Villeneuve *et al.*, 2010). The increase in algal biomass during the experimental period results from the arrival and attachment of colonizing organisms (immigration), the reproduction of these organisms on the substrates

(growth), and the loss of individuals by export, death or grazing (Stevenson & Peterson, 1991). All these processes are affected by distinct environmental factors, but trophic status and water-mediated transport may play a significant role, according to our results.

Trophic status

The immigration rate in a site depends on the pool of colonizers in the surroundings, and other parts of the system (Hamilton & Duthie, 1987). The size of this pool increases with trophic status. This overarching term can be operatively approximated using several variables. TP is the typical surrogate of trophic status in freshwater ecosystems. In alpine lakes, DOC is commonly correlated with TP, and can be considered an indicator of trophic status that present lower seasonal fluctuations than TP (Catalan *et al.*, 2002, 2009a). Which of the two variables takes the leading explicative role in the statistical analysis of the periphyton colonization may be either circumstantial, due to specific values of data set, or it may be attributable to marginal differences among them. In our study, DOC was predominantly selected in the models, and we found a similar positive effect on both diatoms and chlorophytes. In addition to being a surrogate for the algal pool in the system, DOC may also correlate with the amount of prokaryotic colonizers, which may be crucial for preparing an initial biofilm in the new substrate, and thus facilitating the attachment of larger organisms (Jordan & Staley, 1976; Barranguet *et al.*, 2005). DOC could also have an adverse impact on benthic primary production because it attenuates the light (Godwin *et al.*, 2014). However, this is not the case of mountain lakes, where DOC is relatively low, and periphyton has to deal with high radiation, particularly UV (Laurion *et al.*, 2000). In fact, DOC absorption properties reduce photoinhibition and enhance primary production in shallow littoral biofilms.

Water renewal

In high-mountain environments, there is some correlation between water renewal and trophic status. Lakes located at the lower zones of the catchments show higher water flows, and higher nutrient and organic carbon loadings from the surrounding terrestrial systems than the upper lakes. The inclusion of the two streams in our experimental design broke the existing correlation, and permitted to differentiate the respective responses to both factors. Main streams and nearby lakes showed similar water flows but markedly different water renewal characteristic velocities (Vel). Selecting sites in streams revealed that water renewal was more relevant than trophic status (DOC, TP) for early periphyton colonization, yet exclusively mediated by diatoms growth. In fact, even when stream sites were excluded from the model, Vel and Flow were top ranked predictors for algal growth (data not shown). This indicates that the result was not statistically ill-conditioned by the stream leverage.

The enhancement of diatom accrual by water renewal may comprise some causes related to transport, settlement, and growth of colonizers. Research on water-mediated transport on lake periphytic communities is scarce (Lowe, 1996). In lotic systems, some studies reveal inverse relationships between water current and periphyton accumulation, likely because swift currents hinder the adherence and settlement of the organisms to the substrates (McIntire, 1966; Reisen & Spencer, 1970; Stevenson, 1983). Thus, pool zones may present greater initial colonization rates than riffle zones (Korte & Blinn, 1983; Oemke & Burton, 1986). However, this does not seem to be the case in the lakes and streams considered, given the relatively low water currents. Instead, higher average flows upon a substrate might have increased the probability of arrival of colonizers from the surrounding source areas. Our results demonstrate that water renewal cannot be ignored in colonization experiments in lakes. This would be a critical factor in comparisons among sites with contrasting water flows. Methods estimating mesoscale water renewal as better as possible should be included in colonization experiments.

In addition to enhancing the arrival of new individuals, water renewal can also improve the growth of the population. An increase in local water renewal produces a steeper diffusion gradient that enhances the exchange of materials, solutes and gasses between the periphyton and the water column (Whitford & Schumacher, 1961; McIntire, 1966; Horner & Welch, 1981). As water current also increases the shear effect and the drag of organisms (McIntire, 1966), moderate currents may be more beneficial than high velocities (Stevenson, 1996b). Our study demonstrates that diatoms are favoured upon chlorophytes by water velocity, at least during early colonization. McIntire (1966) described a dominance of diatoms on fast currents, and of chlorophyte filaments on slow currents, and argued that the latter could be controlled by shear stress. We observed that chlorophytes accrual in streams was relatively low under non-enriched conditions, but increased in enriched substrates, thus weakening this hypothetical shear stress influence.

Chlorophytes vs. diatoms development

The algal growth on the substrates varied considerably among sites. We observed that chlorophytes dominated the periphyton where total biomass was low, and diatoms where it was high. We may ask whether this was a final pattern of differentiation in periphyton, or the biofilms were at different successional stages. In fact, the pattern observed agrees with the succession of algal groups dominance described by Sekar et al. (2002, 2004) in a reservoir: initial chlorophytes dominance, followed by diatoms, and finally by cyanobacteria. Barranguet et al. (2005) also found a higher proportion of chlorophytes than diatoms during the first ten days of colonization, and negligible proportions of cyanobacteria until the fourth week of development. In streams, the presence of cyanobacteria in later stages of biofilm development (>2-3 weeks) was also observed by Korte & Blinn (1983), and could be related to their slow growth. According to previous studies, and our own observations, cyanobacteria may require more than three weeks to develop populations at the levels previously observed in these systems (Bartrons *et al.*, 2012). The different degree of biomass saturation among locations after 21 days of

colonization may be supported by a similar study performed the previous year, which only included six of the nine lakes, and lasted 52 days (Fig. 9). Chla concentrations at 21 days of colonization were close to the levels at 52 days in some lakes (e.g. Llong, L), whereas the differences between the two periods were still great in other lakes. Interestingly, the periphyton biomass obtained in the two studies was strongly correlated (r= 0.98), what reinforces the influence of the general environmental context associated with one site in determining algal growth.

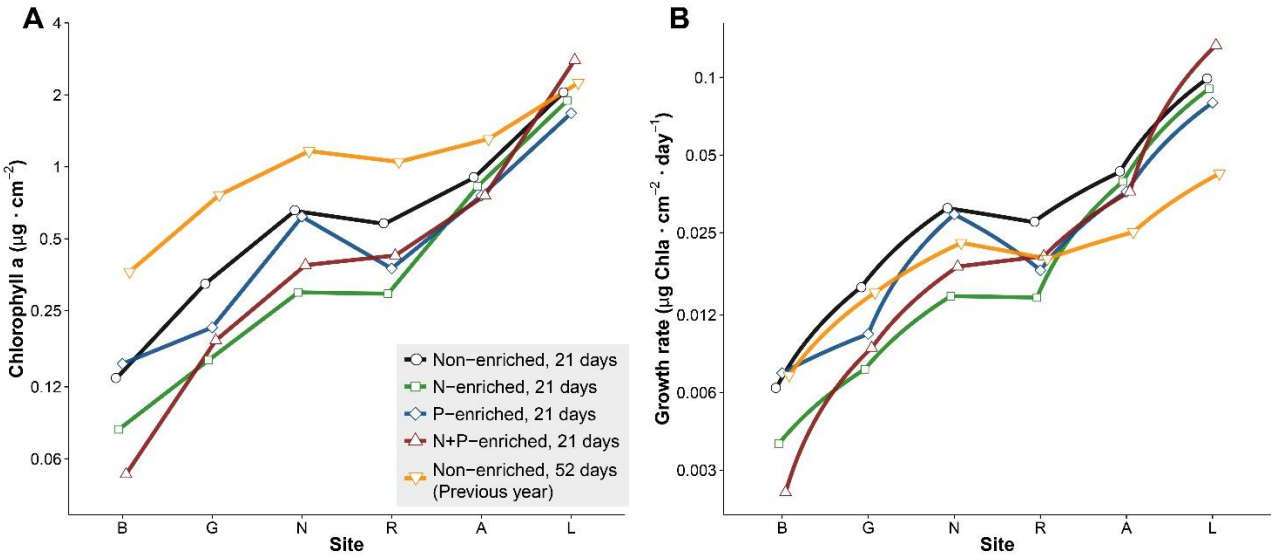


Figure 9 Chlorophyll a concentration (A) and increase rate (B) after 21 days of colonization (current experiment) compared with a previous study that lasted 52 days, and performed in only six lakes.

Nutrient enrichments

Diatoms suffered a severe inhibitory effect by N enrichment, and a slighter negative effect by P enrichment; contrastingly, chlorophytes were not so adversely affected by any nutrient. Water renewal clearly interacted with nutrient enrichment effects, since at the highest water renewal sites the inhibitory effects on diatoms were mitigated, and the slightly positive effects on chlorophytes were intensified. These results suggest that diatoms are especially sensitive to the nutrient perturbation produced by nutrient-enriched substrates, and that inhibitory effects are alleviated by high water renewal. Evidence of inhibitory effects produced by nutrient

enrichments have been previously reported in lakes and streams (Fairchild *et al.*, 1985; Tank & Dodds, 2003), and have been ascribed to different causes: an excess of the ion of interest (e.g. NO_3^-, NH_4^+, PO_4^-), an excess of the ion that balances the charge of the ion of interest (e.g. K^+, Na^+), and the generation of toxic by-products when preparing the agar medium (Tanaka *et al.*, 2014). Fairchild et al. (1985) observed a decline in algal biovolume on substrates enriched with 0.5M compared to 0.05M of K_2HPO_4, and hypothesized it could be related to an excess of phosphate (Rodhe, 1948) or potassium (Lehman, 1976). However, Luttenton & Lowe (2006) discarded the second hypothesis, as they used NaH_2PO_4 instead of K_2HPO_4 and also found P inhibition, especially when P was added in excess relative to N. They also discarded a negative effect of Na^+, as all treatments had similar concentrations of this ion. Instances of inhibitory effects produced by N enrichment are also relatively common in the literature (Francoeur, 2001; Bernhardt & Likens, 2004; Schiller *et al.*, 2007). Although our study does not allow us to determine the mechanisms causing the inhibitory effects, we recommend to enrich with lower concentrations (Maberly *et al.*, 2002; Hogan *et al.*, 2014), particularly in unproductive environments with low water renewal.

The tolerance to high nutrient levels might vary across algal species and classes, in the same way that the optimal nutrient concentrations vary among taxa. In a recent review, Collos & Harrison (2014) observed that chlorophytes were the most tolerant algal group to toxic levels of ammonium, and the group with higher ammonium optimal concentrations. Consistently, in our experiment, high nutrient levels were better tolerated by chlorophytes than diatoms, and even positive effects of nutrient enrichment were detected in chlorophytes at high water velocity. Under these conditions, the growth of chlorophytes was more stimulated by N than by P (i.e. the relative influence of N:lnVel was greater than P:lnVel). In contrast, diatoms growth was only stimulated by P and N+P treatments in the streams, and they generally better tolerated P than N excess conditions. Consistently, chlorophytes were more favoured by N enrichment than diatoms and cyanobacteria in a recent study in two

Alpine lakes (Lepori & Robin, 2014). This evidence suggests that deposition-driven N:P imbalances can substantially affect the periphyton communities of these ecosystems, and that N fertilization could specifically favour chlorophytes.

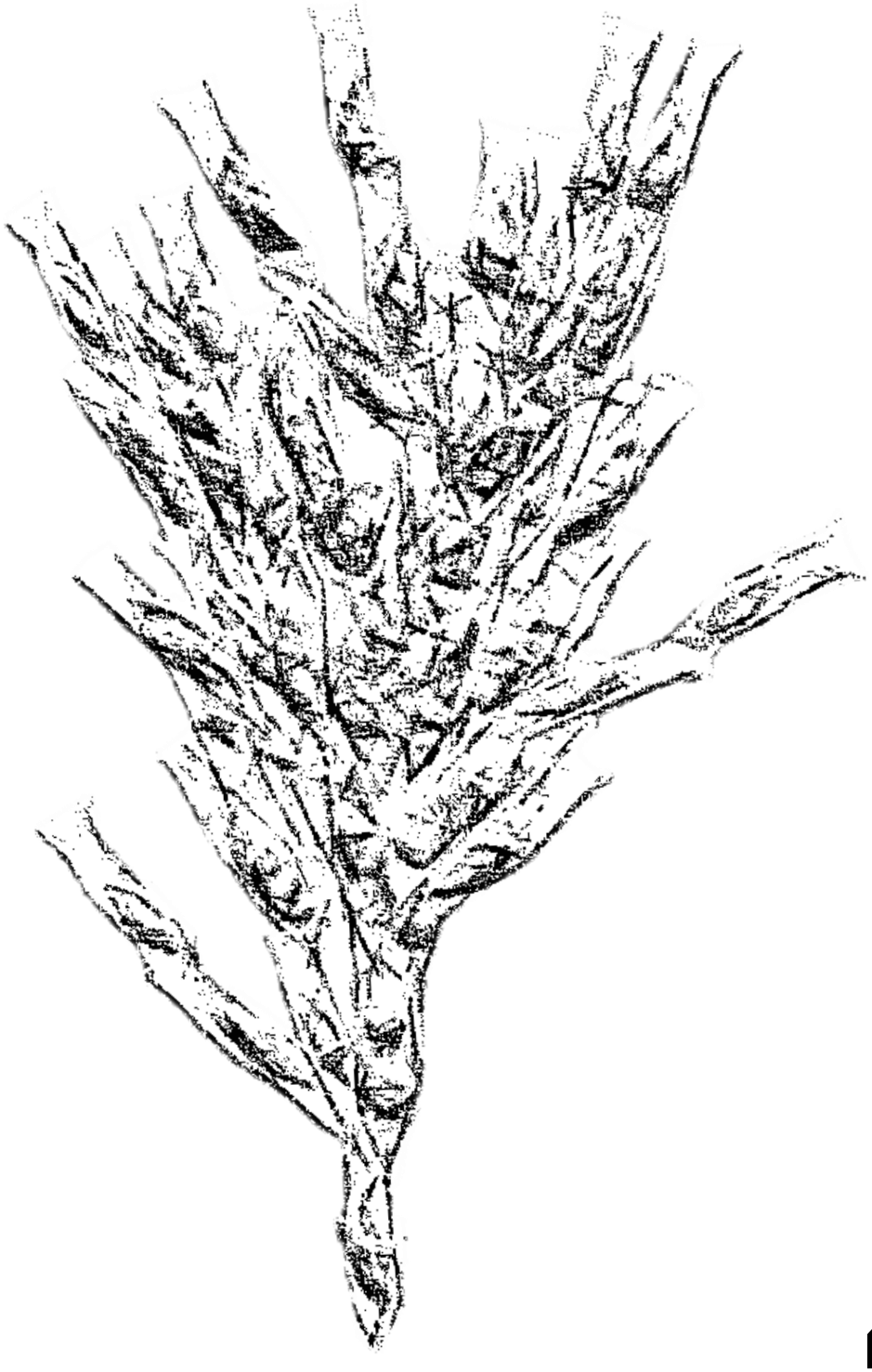
The effect of P enrichment appeared to be conditioned to TP concentrations, since the algal growth (and particularly the chlorophytes growth) responded more in locations with TP <2 $\mu g \cdot L^{-1}$ (Fig. 5C). Catalan et al. (2009) identified an ecological threshold in European alpine lakes at ~0.6 mgDOC L^{-1} or ~3 µgTP L^{-1}, which fairly corresponds to the observed threshold. However, this P:lnTP factor was weak, and actually excluded of minimal models, possibly because the effect was rather non-linear (Fig. 5C). The NxP interaction was also related with TP in chlorophytes, but, in this case, positively. A possible explanation may be that species with high requirements for both N and P are more abundant in the colonizer pool of locations with relatively high trophic status. In contrast to chlorophytes, the response of diatoms to nutrient enrichments seemed poorly conditioned by trophic status.

Concluding remarks

The early periphyton growth was mostly related to lake trophic status and water renewal, both accelerating it. The role of water renewal on periphyton development has been largely ignored in lakes; and this study highlights its relevance, especially for diatoms. Further research should aim to disentangle the mechanistic processes behind the observed phenomenological patterns. For instance, some remaining questions are: is there a higher dependence on water transport for diatoms dispersal and immigration than for chlorophytes? Does water velocity stimulate diatom growth? Which are the autogenetic and successional changes that favours the dominance of chlorophytes in the first stages, and of diatoms in the advanced ones?

Grazing was not relevant in our experiment, yet it can cause sharp changes on periphyton communities (Feminella *et al.*, 1995; Steinman, 1996). Since periphyton accumulation may increase the attraction of grazers, it might be interesting to

determine whether there is a tipping point in periphyton development at which herbivore effects become more likely, and how it depends on the kind of herbivores. In our experiment, N enrichments, and to lower extent also P enrichments, inhibited the growth of diatoms. Therefore, the fertilization of oligotrophic lakes does not directly imply enhanced algal growth, as unusually high levels may lead to toxicity for the existing communities. Regular nutrient dosage at low concentrations may be indeed more efficient than punctual high loadings, and much experimentation is already required in that respect in natural communities. From our experiment and other studies, there is increasing evidence that N fertilization comparatively favours chlorophytes over the other main algal groups in high-mountain benthic ecosystems. This is especially relevant in a context of high N atmospheric emissions, and discussion on effects of climate warming in remote ecosystems (Holtgrieve *et al.*, 2011; Catalan *et al.*, 2013).



2

Decoupled responses of productivity, community structure and seston C:N:P composition to experimental gradients of P limitation in an oligotrophic lake

Abstract

Human activities have increased nutrient supply and unbalanced N:P availability in aquatic ecosystems. Effects of changed nutrient availability on the productivity, C:N:P stoichiometry and structure of planktonic communities have been already reported, but there is a lack of studies that simultaneously compare their responses and patterns of change. We performed P (PO_4^{3-}) and N (NH_4^+ or NO_3^-) additions to 20 m deep and ~100 L tubular mesocosms in a P-limited lake, and analyzed the changes when the system is released from that limitation ("P enrichment"), when is supplied with N excess ("N:P imbalance"), and when NH_4^+ becomes the dominant source of DIN. N excess conditions reduced the growth of autotrophs -especially chrysophyceans- and enhanced the differentiation of autotrophic community, but did not affect seston C:N:P. NH_4^+ dominance tended to increase seston C:P and N:P ratios and stimulated the productivity and the differentiation of the autotrophic community when P limitation diminished. Particulate matter recovered from sediment traps was N-impoverished and P-enriched in relation to seston, thus strengthening P limitation of the system. Productivity was highly sensitive to low and medium P additions, but the response decelerated at high P additions. In contrast, the autotrophic community differentiated almost linearly, driven by the progressive substitution of chrysophyceans by diatoms and cryptophytes. Seston C:N:P remained fairly unchanged to low and medium P additions, but fell drastically and approached to Redfield's at the highest P additions. The change of seston structure was not sufficient to explain this stoichiometric shift, that, instead, may be more related with a reorganization of macromolecular components in organisms. Overall, this study evidences that responses of productivity, community structure, and C:N:P composition to changed nutrient availability can be markedly decoupled, and reinforces the importance of studying them together to deepen their connections.

Introduction

The biomass of all living organisms consists of more than 20 essential elements in quite defined proportions, and whichever of these elements is in shortest supply than demand may be limiting their growth (Hessen *et al.*, 2013). Demand for nitrogen (N) and phosphorus (P) is high in all organisms (e.g. synthesis of proteins and nucleic acids) and, indeed, typically limit productivity in aquatic ecosystems (Elser *et al.*, 2007). Human activities have not only increased the nutrient availability and productivity in many ecosystems, but also unbalanced natural N:P supplies and changed limiting conditions. Inputs of N and P into ecosystems through atmospheric deposition can be particularly unbalanced, as P -unlike N- has no gaseous phase and its presence in the atmosphere is necessarily associated with particles (e.g. dust, sea-salt, biogenic particles, combustion ashes) (Mahowald *et al.*, 2008). In general, non-dusty regions of Europe and North America show N:P depositional ratios several-fold higher than Redfield ratio (Peñuelas *et al.*, 2013; Wang *et al.*, 2014), what it is decreasing N-limitation and intensifying P-limitation in aquatic ecosystems (Elser *et al.*, 2009).

Enhanced P deficiency may not just alter the quantity but also the quality of the biomass (i.e. the C:N:P coupling). We now know that the elemental composition of autotrophs and seston in aquatic ecosystems is less constrained to 106C:16N:1P than previously thought (Geider & La Roche, 2002; Sterner *et al.*, 2008). N and P availability in the environment could affect organisms C:N:P in two ways, through changes in growth rate, and in nutrient storage. Fast-growing phytoplankton present a more rigid and P-rich elemental composition, with N:P ratios frequently below 16N:1P (Hillebrand *et al.*, 2013). The growth rate hypothesis states that achieving high growth rates requires high concentrations of ribosomes, which are P-rich and increase the P content of organisms (Sterner & Elser, 2002). This hypothesis has been recently questioned for autotrophs (Flynn *et al.*, 2010), and the role of different macromolecular components in C:N:P composition is still an open debate. The trend

of N:P on growth may also be explained by changes in P storage pools, if organisms accumulate P under optimal nutrient-replete growth conditions, and the reserves of P diminish as environmental conditions become P limited (Hillebrand *et al.*, 2013). The increase of N:P and C:P ratios with P deficiency may stop at certain point, due to limits in the stoichiometric plasticity of the organisms, i.e. their maximum storage capacity and minimum P quota (Hall *et al.*, 2005). However, there are still few pieces of evidence about this hypothetical stoichiometric threshold and its specific values. Determining to what extent P deficiency is translated to autotrophic C:N:P composition is of major relevance since the presence of P-poor phytoplankton affects the productivity of higher trophic levels, nutrient cycling, and carbon sequestration (Sterner & Elser, 2002; Hessen *et al.*, 2013).

One difficulty of stoichiometric studies in lakes and oceans is that seston is a mixture of autotrophs, heterotrophs and detritus that can be hardly separated. Since autotrophs are frequently the dominant fraction, and the major contributor to detritus, the C:N:P composition of seston is commonly assigned to primary producers. Heterotrophic C:N:P composition tends to be more homeostatic than the autotrophic (Persson *et al.*, 2010), which supports the idea that changes in seston elemental composition are mainly related to autotrophs. Nevertheless, changes in nutrient availability and productivity may affect the proportion of different seston fractions, and the relative abundance of autotrophic groups. Major algal groups present distinguishable C:N:P compositions (Quigg et al., 2003), and, therefore, changes in the phytoplankton community could directly affect the seston C:N:P proportions. Despite the recognised connection among community structure, C:N:P composition and productivity, studies that simultaneously compare their patterns of change are scarce.

An important issue in the context of P limitation is the degree of N:P imbalance. Higher atmospheric N inputs over already P-limited aquatic systems increase dissolved inorganic nitrogen (DIN) concentrations in water. Apparently, this excess

of N availability may produce minimum effects on productivity and community structure, although examples of enhanced release of extracellular phosphatases and toxins under N:P imbalanced conditions are reported (Raven, 2010). A greater availability of N may affect more importantly the elemental composition of seston (i.e. lower C:N and higher N:P ratios), as N can be stored in the organisms. Actually, stoichiometric studies have traditionally analysed the homeostasis of organisms linking seston N:P to supplies N:P (Hall *et al.*, 2005; Persson *et al.*, 2010). Yet, it has not been sufficiently analysed whether the simultaneous increase of N and P availability -maintaining the supply N:P unchanged- cause any change in seston N:P ratio when P is limiting.

Human-induced changes in N cycle not solely affect the amount of N available for organisms, but also the relative abundance of N forms. For instance, the emissions of oxidized nitrogen into the atmosphere are typically associated with combustion processes, whereas the emissions of reduced nitrogen come primarily from agricultural activities (Fowler *et al.*, 2013). Thus, the proportion of both forms in atmospheric deposition changes regionally and over time, depending on the extent of human activities and other meteorological conditioners (Dentener *et al.*, 2006; Zhang *et al.*, 2012; Liu *et al.*, 2013). Although both nitrate (NO_3^-) and ammonium (NH_4^+) can be assimilated by primary producers, NH_4^+ assimilation is energetically cheaper (Syrett, 1981) and preferentially used (Dortch, 1990; Harrison *et al.*, 1996). The preference for NH_4^+ contributes to lower NH_4^+ concentrations in oceans, lakes, and streams compared to NO_3^- (Berman & Bronk, 2003; Catalan *et al.*, 2009b; Durand *et al.*, 2011). As NH_4^+ becomes more and more scarce, the pressures of organisms to assimilate NO_3^- increase. These selection pressures on N assimilation might have evolutionary resulted in a broad range of NH_4^+ and NO_3^- uptake traits (and trade-offs) among phytoplankton groups (Litchman *et al.*, 2007). Therefore, we may expect significant changes in the autotrophic community if NH_4^+ becomes the dominant form of DIN (Donald *et al.*, 2013). Lower costs in NH_4^+ assimilation could also enhance primary production in such conditions, especially if P limitation

is not severe. Possible effects of NH_4^+:NO_3^- availability on seston C:N:P ratios are uncertain.

In short, there is a growing interest in understanding the effects of P limitation on productivity, planktonic community and seston C:N:P composition of pelagic systems. We expected that absolute P availability would drive the main changes, though the excess of N availability (hereafter, the N:P imbalance) and changes in DIN dominant forms (NH_4^+:NO_3^-) could also have a role. To solve some of the unknowns stated above, we performed a perturbation experiment in a P-limited oligotrophic lake (Lake Redon). The experiment consisted in enrichments of phosphorus (PO_4^{3-}) and nitrogen (NO_3^- or NH_4^+) using 20-m long columnar mesocosms installed during 25 days. The experiment was performed shortly after the onset of summer stratification. At this transition period, there is a high diversity of planktonic organisms in the water column, which provide enough seeding elements for potential contrasting responses to mesocosms treatments. Located in the Pyrenees, Lake Redon is quite sensitive to changes in atmospheric nutrient inputs, such as the human-induced increase in N deposition, and the events of P-enrichment caused by northern Africa dust deposition (Camarero & Catalan, 2012). However, the experiment was not intended as a simulation of new conditions in this lake. Rather, the aim was to deepen in the directions and modes of response of planktonic communities in oligotrophic freshwaters to changes in nutrient availability, and better understanding the coupling of productivity, community structure, and seston C:N:P.

Methods

Study Site

The experiment was performed in Lake Redon, an oligotrophic high-mountain lake located in the Central Pyrenees (42º38'33"N, 0º36'13"E, 2232 m asl). This lake has been the centre of intense limnological research for over 30 years (Catalan *et al.*,

2006). It is a dimictic lake with a surface area of 24 ha, and maximum and mean depths of 73 and 32 m, respectively (Catalan, 1988). The lake is ice-covered about six months a year. During the ice-free period, the penetration of solar radiation is high, and the photic zone (40-50 m) extends beyond the seasonal thermocline (15-20 m). Phytoplankton is the main fraction of planktonic biomass and is usually dominated by chrysophyceans (Felip *et al.*, 1999). Others groups can be occasionally relevant during the mixing period (chlorophytes, diatoms), during summer stratification (dinoflagellates) or at greater depth (cryptophytes).

Mesocosm installation and field procedures

Mesocosms were constructed using tubular-shaped polythene bags (diameter: 8.5 cm; length: 20 m) and two polyvinyl chloride (PVC) tubes, attached one at each extreme of the bag (Fig. 1). The PVC tube at the lower end (length: 0.5 m) was closed and served as a sediment trap, while the upper tube (length: 1.5 m) enabled the gaseous exchange with the atmosphere. An expanded polystyrene float was attached to the upper tube to hold the mesocosms at the water surface, and weight was tied at the sediment trap to stretch the bag. The mesocosm was filled with ~100 L of water from 0 to 20 m lake depth, avoiding high disturbances for the organisms. The installation of the mesocosms consisted in three steps. First, the mesocosm was mounted: the polythene bag was folded around the upper tube, and this tube was held together with the sediment trap by a rope lacing. Second, the folded mesocosm was placed horizontally on the lake surface and filled with water. Third, the folded mesocosm began to sink to a depth of 20 meters, where it was stopped by a rope. At the moment the rope tensioned, the lacing that held the tubes together was released, and the upper tube started to float up to the lake surface, thus filling the bag with water from the upper 20 meters (Fig. 1). Once the mesocosms were installed, we proceeded to the specific enrichments. For each mesocosm, a 20 m long thin plastic tube was filled with 0.9 L of nutrient-enriched water. This thin tube was introduced inside the mesocosms, and its solution was released homogeneously along the water column as the tube was withdrawn from the mesocosm.

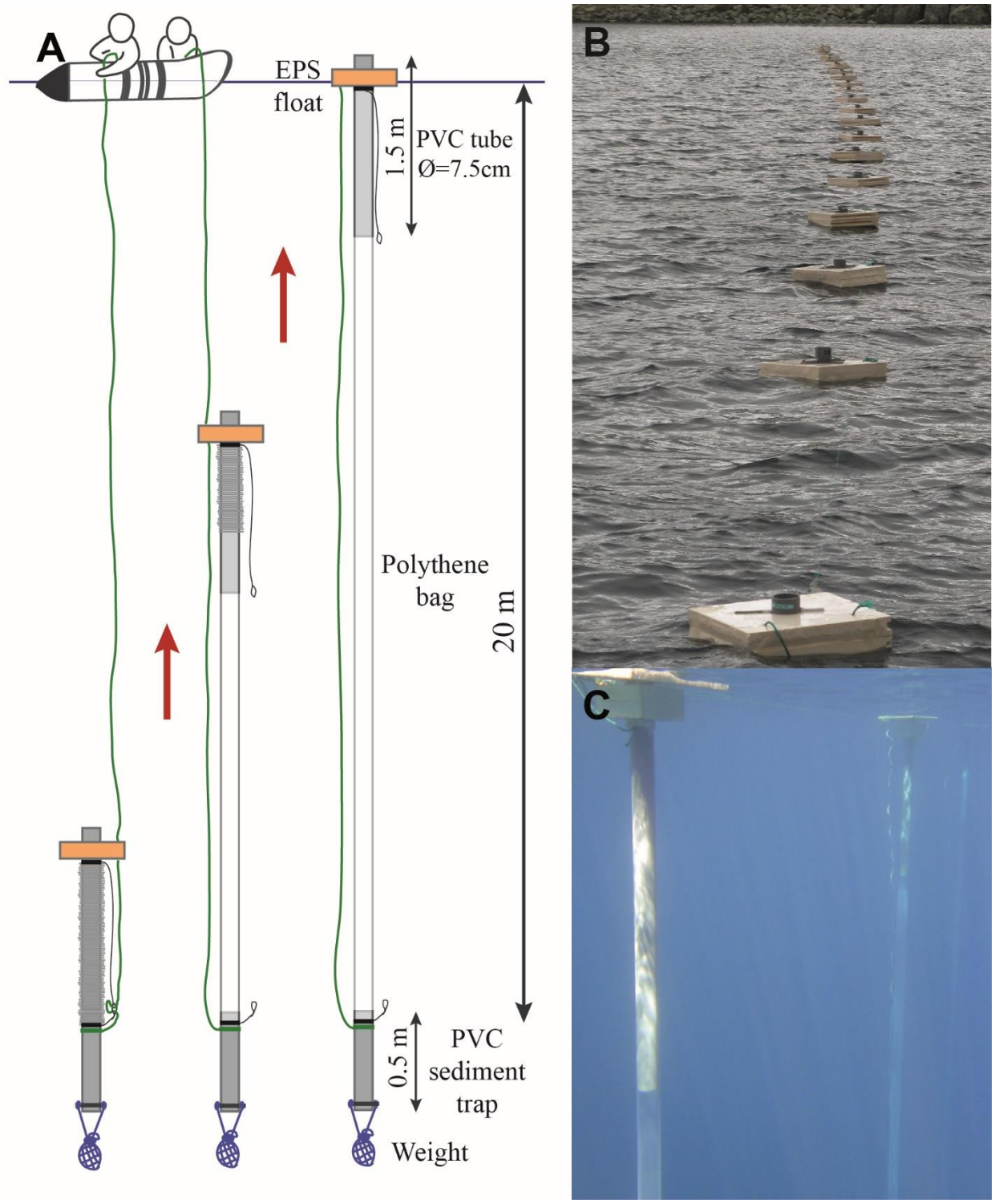


Figure 1 Mesocosms installation. A) Mesocosms were gradually filled with water as the upper tubes ascended from 20 m deep and unfolded the polythene bags. B) Mesocosms were subjected to a rope that went from the shore to a platform placed at the centre of the lake. C) Underwater view of the upper part of mesocosms.

The mesocosms were deployed on 5-6 August 2013 and recovered 25 days later. An integrated water sample (i.e. ~5 L, from 0 to 20 m deep) was obtained from each mesocosm at the end of the experiment, using 20 m long plastic tubes and pumping

the water volume inside these tubes. The water sample was immediately filtered through a 250 μm pore size mesh to discard large zooplankton, which will not be considered in this study (its abundance was low and did not vary among mesocosms). The sediment trap was collected just before removing the mesocosm, and its water volume was decanted into a plastic bottle and kept until filtration in the laboratory. Once on the land, water samples were filtered for dissolved nutrient analyses through precombusted (5h, 450ºC) glass fiber filters (GF/F, Whatman), and the material on the filters was used for particulate analyses. Samples were frozen stored until analyses in the laboratory. Between one and two litres of water samples were also filtered on glass fibre filters for chlorophyll a (Chla) analyses, wrapped in aluminium foil and frozen in liquid nitrogen to prevent degradation. Two subsamples were fixed to estimate microbial abundance: a 10 ml subsample was processed for prokaryotes following Medina-Sánchez *et al.* (2005), whereas a 200 ml subsample was preserved with 0.5% (vol/vol) alkaline Lugol's solution for protists (Sournia, 1978). An integrated sample was collected on 6 August 2013, and processed as previously described, to assess Chla and microbial abundance at the beginning of the experiment. Regarding the initial levels of C, N and P in the dissolved and particulate fractions, we used a sample collected on 8 August 2013, coinciding with the monthly monitoring in Lake Redon.

Experimental design

To assess the effects of P limitation on planktonic communities we created one gradient of increasing P availability ("P enrichment"), and another gradient of increasing N availability ("N:P imbalance"). Both P and N were added in all enriched treatments because the concentrations were initially low for both nutrients, and the addition of only one nutrient would produce the other to become limiting quite fast. N and P were added in three different levels: low, intermediate and high (Fig. 2A). The concentrations of total dissolved phosphorus (TDP) and dissolved inorganic nitrogen (DIN) just after nutrient addition were estimated using the added mass of P and N, the water volume of the mesocosms, and the initial concentrations

of TDP and DIN in lake (TDP: 0.022 µmols L^{-1}; DIN: 4.4 µmols L^{-1}). Thus, P additions resulted in rounded TDP initial concentrations of 0.06, 0.21 and 1.90 µmols L^{-1}, whereas N additions resulted in rounded DIN initial concentrations of 17, 35 and 73µmols L^{-1}. The low P and N-enriched condition (N_P) intended to simulate the original DIN:TDP ratio (223:1), but with higher absolute TDP and DIN concentrations (Fig 2B). From the N_P condition, the P enrichment was obtained maintaining the DIN levels and increasing the P addition to medium (N_P+) or high levels (N_P++). Likewise, the N:P imbalance was obtained maintaining low TDP levels, and increasing the N addition to medium (N+_P) or high levels (N++_P).

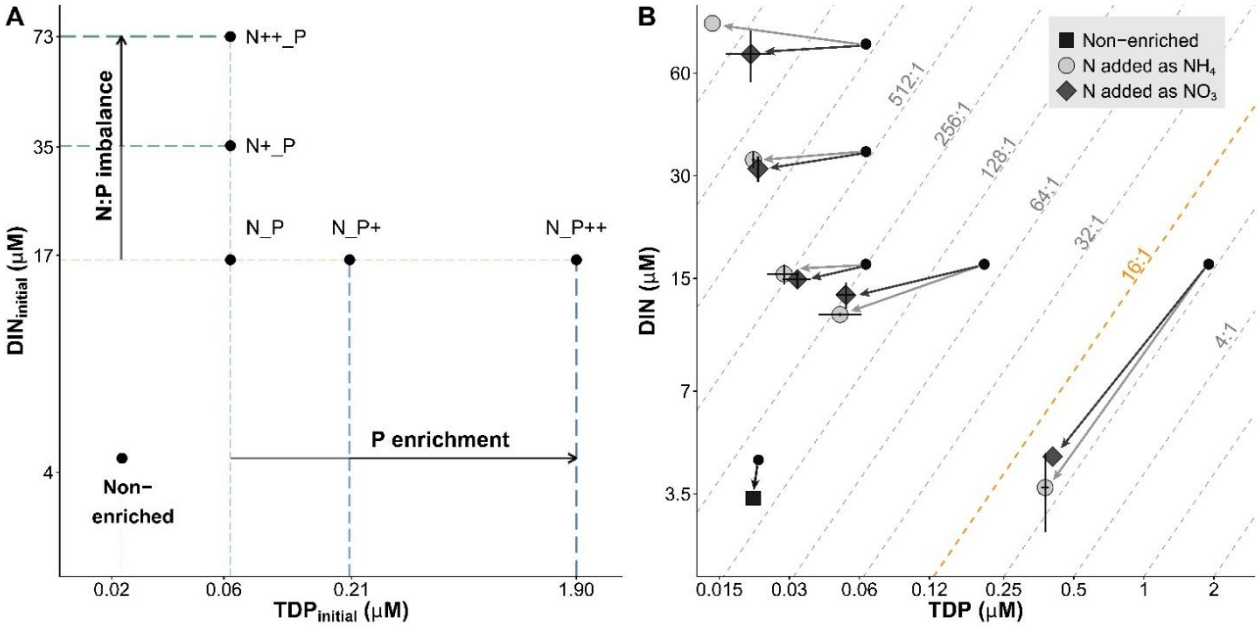


Figure 2 Experimental design and nutrient concentrations. A) Estimated initial DIN and TDP concentrations just after nutrient addition for each experimental condition (black dot). B) Change of DIN and TDP concentrations from the beginning (black dot) to the end of the experiment (squares, circles, diamonds). Squares, circles, and diamonds stand for non-enriched, NH_4-enriched and NO_3-enriched conditions, respectively. Dashed lines indicate DIN:TDP molar ratios. Note that axes are shown in logarithmic scale.

In lake, DIN concentrations were clearly dominated by nitrate (NO_3^-: 4.2 µmol L^{-1}; NH_4^+: 0.2 µmol L^{-1}). To determine the effects of changed NH_4^+:NO_3^- dominance on planktonic communities ("DIN form" effects), N was added as NH_4Cl in five treatments and as KNO_3 in the other five. P was always added as K_2HPO_4. To specify which form of DIN was added, the code include an "H" after the "N" when this form was NH_4^+ (NH++_P, NH+_P, NH_P, NH_P+, NH_P++) and an "O" after

the "N" when this form was NO_3^- (NO++_P, NO+_P, NO_P, NO_P+, NO_P++). A total of 22 mesocosms were installed, with two replicates for each treatment, plus two non-enriched control mesocosms. Unfortunately, we had technical incidences with some mesocosms (i.e. non-enriched, NO_P++, NH++_P), and we lost its replication in this study. When two replicates were available, we draw a line range among them and a symbol with its average in figures (e.g. Fig. 2B).

Chemical analyses

As regards the dissolved fraction, total dissolved phosphorus (TDP) was determined by colorimetry using a segmented flow auto analyser (AA3HR, Seal/Bran+Luebbe) with an automated method based on Murphy & Riley's (1962) method (Bran+Luebbe method G-175-96), with samples previously digested by the acid persulphate oxidation (Grasshoff *et al.*, 1983). NH_4^+ and NO_2^- were determined by automated versions of the blue indophenol (Berthelot reaction) method (B+L G-171-96) and the Griess reaction (B+L G-173-96), respectively, and analysed by colorimetry using the segmented flow auto analyser. NO_3^- was measured by capillary electrophoresis (Quanta 4000, Waters). Dissolved inorganic nitrogen (DIN) was calculated as the sum of NO_3^-, NO_2^- and NH_4^+. Dissolved organic carbon (DOC) was determined by catalytic combustion and infrared spectrometric detection of the CO_2 produced (TOC5000 Shimadzu analyser). Regarding the particulate fraction, particulate C and particulate N were determined using a Carlo Erba elemental analyser. Filters for particulate P analyses were firstly digested using the acid persulphate wet oxidation, and the extracts analysed by the same colorimetric method already specified for TDP.

Chlorophyll a (Chla) was firstly extracted in 5 ml 90% acetone with a probe sonicator (Sonopuls GM70 Delft, The Netherlands) (50W, 2 min), and the extracts were subsequently centrifuged (4 min at 3000 rpm, 4 ºC) and filtered through a Whatman Anodisc 25 (0.1 μm). Chla was analysed by ultra-performance liquid chromatography (UPLC, Acquity, Waters, Milford, MA, U.S.A.), as similarly reported in Buchaca *et al.* (2005).

Planktonic community and seston fractions

The abundance of living protists (eukaryotic autotrophs and heterotrophs), cysts, resting stages and other particles (e.g. pollen, thecae) were estimated using the Utermöhl method (Sournia, 1978). Biovolume was determined by measuring the main cell dimensions, and assimilating its shape to known geometric forms (Hillebrand et al., 1999). The abundance of prokaryotic heterotrophs was estimated as DAPI counts. Cells were filtered through 0.2 µm polycarbonate filters (Millipore, GTTP, 25 mm filter diameter), stained with DAPI (4´,6´-diamidino-2-phenylindole; 1µg mL^{-1} final concentration), and mounted on glass slides using Citifluor (Citifluor Ltd., UK). Slides were stored at -20 °C in the dark until counting at the epifluorescence microscope at x1000 magnification (>2000 cells per sample). We also examined the presence of autotrophic picoplankton at the chlorophyll a channel of the epifluorescence microscope, but its abundance was negligible, in accordance with previous studies in Lake Redon (Felip et al., 1999).

We estimated C biomass of seston components with conversion factors previously applied in this lake (Felip et al., 1999). The biovolume of planktonic organisms and large particles was transformed to C using a conversion factor of 0.2 pg C μm^{-3}. Although this factor could vary depending on biovolume, we decided to maintain it constant because the range of size was small compared to marine systems (Mullin et al., 1966; Menden-Deuer & Lessard, 2000). According to their mean biovolume (0.051 μm^3), and the allometric equation proposed by Norland (1993), we transformed the abundance of prokaryotic cells to C biomass using a conversion factor of 0.014 pg C $cell^{-1}$. The sum of prokaryotes, eukaryotes, and large particles was referred as "cellular" particulate C, while the difference between cellular C and total particulate C was referred as "extracellular" particulate C.

Parameter calculations

Net primary production (NetPP) was estimated by the change (final minus initial) of total organic carbon in the mesocosms. The carbon budget included the dissolved

(DOC) and particulate fractions in water, and the particulate C accumulated in sediment traps. We estimated the changes in the autotrophic community composition as the Hellinger distance (Legendre & Gallagher, 2001; Borcard *et al.*, 2011; Oksanen *et al.*, 2016) between each enriched mesocosm and the non-enriched condition, using the biovolumes of major phylogenetic groups of autotrophs. More details on the planktonic community will be provided and analysed elsewhere.

Results

Productivity and export of C

Net primary production (NetPP) increased with P enrichment (Fig. 3). The increase to P additions was more marked with NH_4^+ (~2x) than NO_3^- (~1.4x) treatments (Fig. 3A) and was mainly driven by higher particulate C in water (~3.7x with NH_4^+, and ~2.8x with NO_3^-, Fig. 3B). Taking into account the X-axes on Fig. 3 are shown in a log scale, the proportional increase of NetPP and particulate C was higher at N_P+ than at N_P++ conditions, and, therefore, the response to P enrichment tended to decelerate.

Contrastingly, the response of NetPP to N:P imbalance paralleled the response of DOC, which was the dominant organic carbon fraction in water (Fig. 3D). DOC tended to decline at NO_3:P imbalanced conditions, what resulted in a slight decline of NetPP in such conditions. In turn, DOC was similar -or even higher- at NH_4^+:P imbalanced than at N_P conditions, and NetPP did not change.

Between 3 and 13% of NetPP was exported to sediment traps during the experiment. Despite NetPP clearly increased at N_P+ and N_P++, the amount of particulate C accumulated in sediment traps did not increase (N_P+), or even decreased (N_P++) in such conditions (Fig. 3C). Therefore, the percentage of exported C was lower in the more productive conditions (6-8% at N_P+, and 3-4% at N_P++).

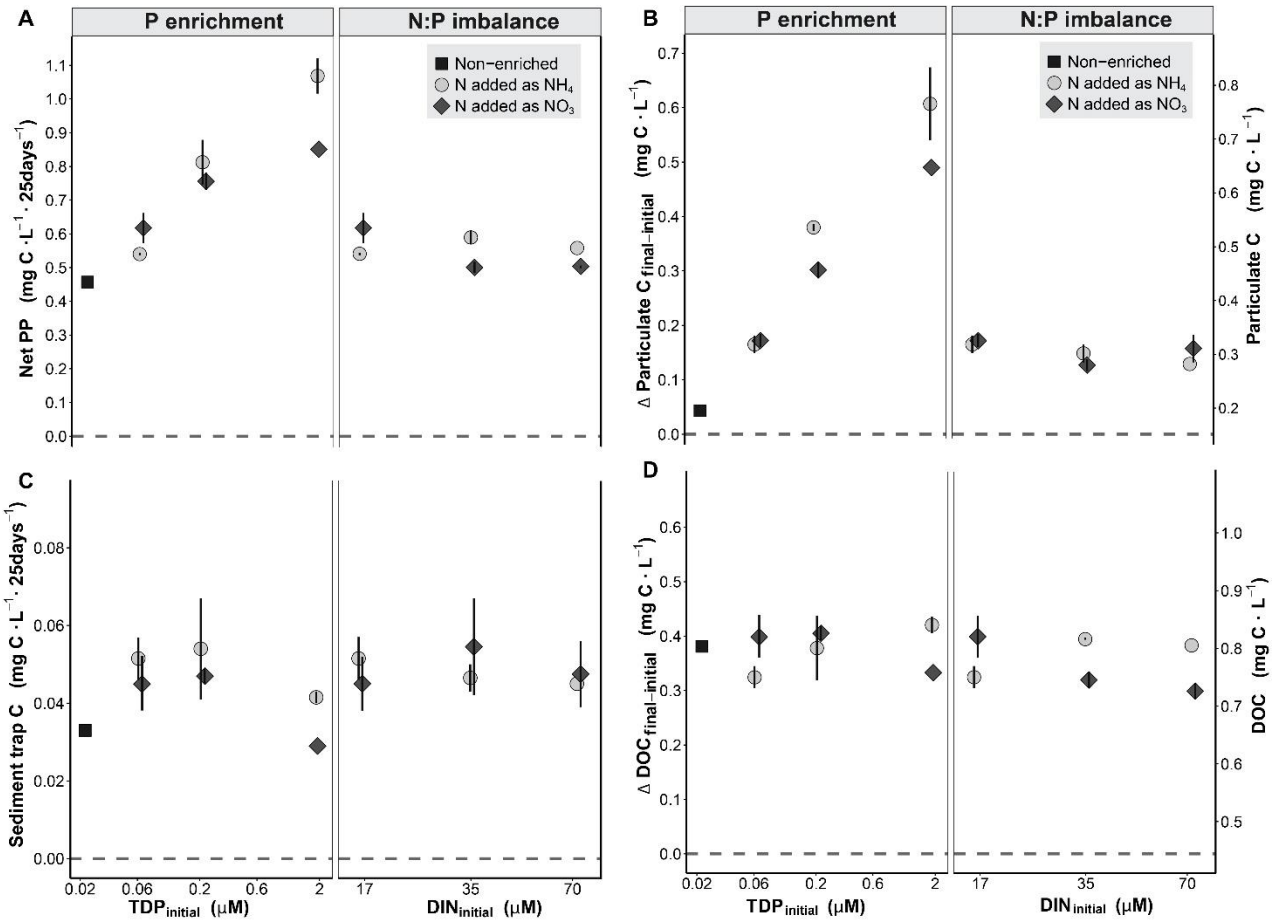


Figure 3 Effects of P enrichment and N:P imbalance on Net primary production (A), particulate C (B), sediment trap particulate C (C), and DOC (D). Squares, circles and diamonds stand for non-enriched, NH_4-enriched and NO_3-enriched conditions, respectively. Dashed lines indicate lake initial concentrations. Note that left y-axes on C and D indicate the relative change from initial concentrations, whereas right y-axes refer to the absolute concentrations.

Seston components

NetPP was highly correlated with the biovolume of autotrophs (n=19, $p<0.0001$, $R^2=91\%$). Indeed, they showed similar patterns of response to P enrichment: a steep increase at medium P additions and a decelerated increase at high P additions (Fig. 4A). The increase of autotrophs at high P additions was also more intense with NH_4^+ (~4.3x) than with NO_3^- (~2.1x) dominance. Yet, the patterns of response to N:P imbalance differed slightly: the negative effect of NO_3^-:P imbalance was more marked on the autotrophic biovolume than on NetPP, and the autotrophic biovolume declined with NH_4^+:P imbalance, although NetPP did not. The autotrophic biovolumes achieved at N:P imbalanced conditions were similar to those initially present in the lake and in non-enriched conditions. Autotrophic biovolume and

chlorophyll showed extremely similar patterns (Fig. 4B), and thus were highly correlated (n=19, $p<0.0001$, $R^2 = 95\%$), indicating that the biovolume estimation was accurate.

The biovolume of eukaryotic heterotrophs declined under non-enriched and low P-enriched conditions compared to initial values (Fig. 4C). High P additions increased the biovolume of eukaryotic heterotrophs ~5x under NH_4^+ dominance and ~2.3x under NO_3^-. Broadly, the pattern of eukaryotic heterotrophs resembled that of autotrophs, though eukaryotic heterotrophs were not negatively affected by NH_4^+:P imbalance. In contrast, the pattern of prokaryotic heterotrophs was markedly different (Fig. 4D). First, the response of prokaryotes to medium P additions was much smaller than to high P additions, and NH_4^+ dominance did not enhance their growth (~2.4x under NH_4^+ dominance; ~2.7x under NO_3^- dominance). Moreover, higher prokaryotic abundances than initially were observed at all experimental conditions, and the effect of N:P imbalance on prokaryotic abundance was slightly positive.

The biomass of autotrophs was always higher than that of heterotrophs (prokaryotes plus eukaryotes). The percentage of autotrophs to all these living organisms ranged from 55% up to 88%. Initially, this percentage was of 66%, and slightly declined under non-enriched conditions (62%). Lower percentages were observed at N:P imbalanced conditions ($N{+}{+}\_P_{mean} = 59\%$; $N{+}\_P_{mean} = 64\%$) than at the rest of treatments ($N\_P_{mean} = 79\%$; $N\_P{+}_{mean} = 86\%$; $N\_P{+}{+}_{mean} = 81\%$). The biomass associated with cysts and thecae was comparatively low (data not shown), and, hence, autotrophs were also the dominant fraction of cellular particulate biomass (48-86%). The high influence of autotrophs to cellular particulate was reflected in nearly identical patterns to P enrichment and N:P imbalance (Fig. 4E).

The amount of cellular C was frequently exceeded by the amount of extracellular C, which was, however, less variable (Fig. 4F). The extracellular fraction ranged from 33% to 82% of total particulate C. From an initial percentage of 41%, it

increased up to 66% under non-enriched conditions. The extracellular fraction was proportionally higher at N:P imbalanced than P-enriched conditions ($N{+}{+}\_P_{mean}$ = 77%; $N{+}\_P_{mean}$ = 72%; N_P_{mean} = 60%; $N\_P{+}_{mean}$ = 46%; $N\_P{+}{+}_{mean}$ = 45%). However, in absolute terms, the highest levels of extracellular C were found at the most P-enriched conditions. Extracellular particulate C tended to increase slightly with N:P imbalance, in particular, at the most NO_3^-:P imbalanced condition.

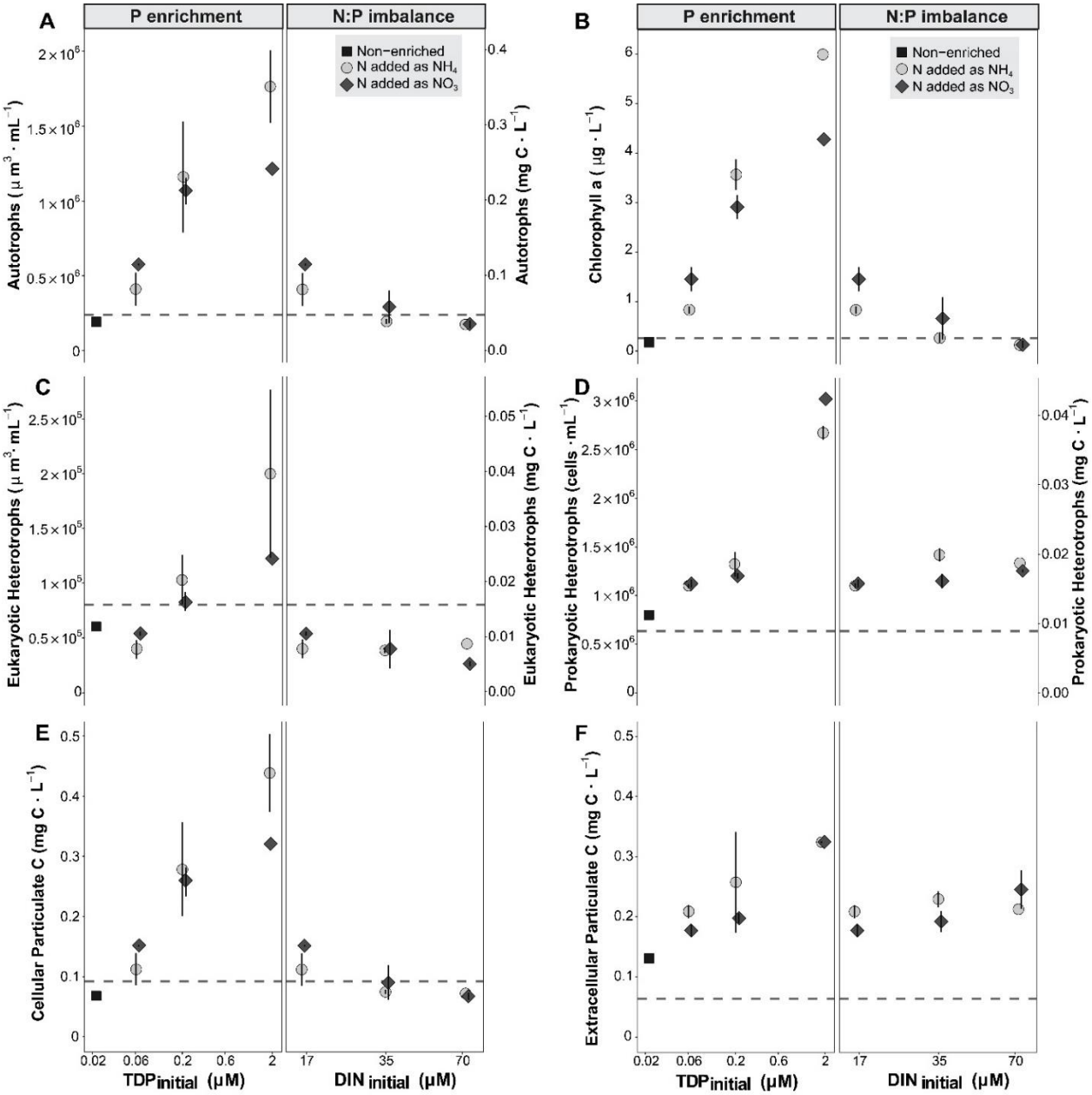


Figure 4 Effects of P enrichment and N:P imbalance on different seston components: autotrophs (A), eukaryotic heterotrophs (C), prokaryotic heterotrophs (D), cellular particulate C (E) and extracellular particulate C (F). In B, chlorophyll a levels are shown for comparison with autotrophic biovolumes. In A and C, left Y-axes show biovolume units, whereas right Y-axes show biomass units. In D, left Y-axis shows abundance units, whereas right Y-axis shows biomass units. In E and F, Y-axes just show biomass units. Squares, circles, and diamonds stand for non-, NH_4-, and NO_3-enriched conditions, respectively. Dashed lines indicate lake initial levels.

Structure of the autotrophic community

Both P enrichment and N:P imbalance differentiated the autotrophic community in relation to the non-enriched mesocosm, though the effects produced by P enrichment were higher (Fig. 5A). NH_4^+ addition increased the differentiation of autotrophic community at medium and high P additions. The abundance of chrysophyceans was a primary driver of changes in the autotrophic community since it was the dominant phytoplankton group (Fig. 5B). The relative abundance of chrysophyceans reached maximum values at N_P (61-71%) and declined with increasing P and N availability. Cryptophytes and diatoms became relevant groups and co-dominated the autotrophic community at the most P-enriched conditions; in the case of diatoms, the growth was more intense with NH_4^+ than with NO_3^-. Diatoms also became relevant at the most N:P imbalanced conditions.

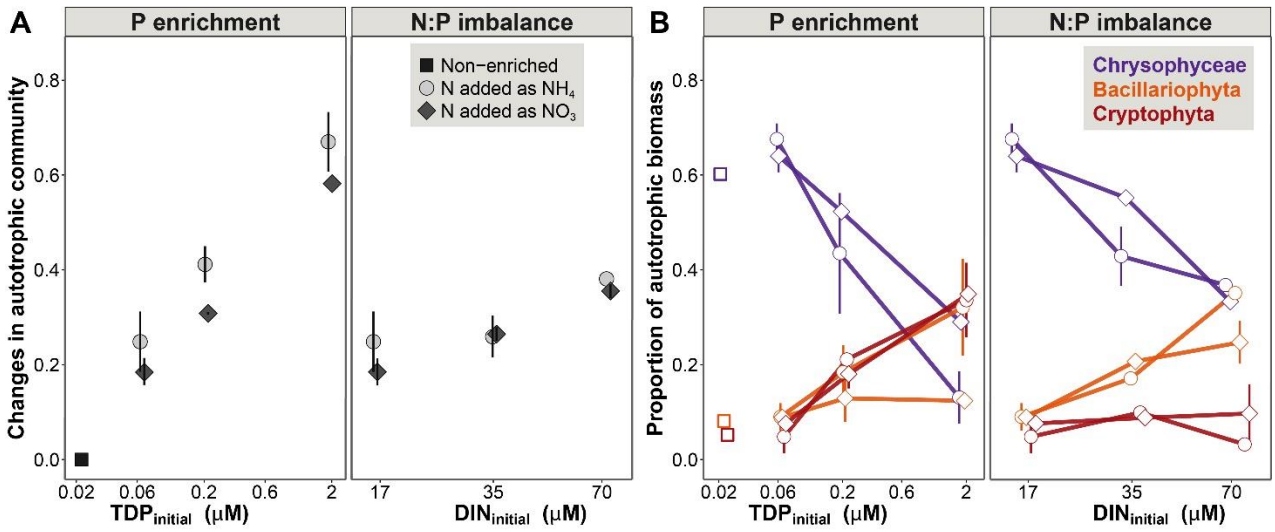


Figure 5 Effects of P enrichment and N:P imbalance on the structure of the autotrophic community. A) The Hellinger distance of each enriched mesocosm from the non-enriched condition is shown. B) Changes in the biomass proportion of the main phytoplankton groups: Chrysophyceae (purple), Bacillariophyta (orange) and Cryptophyta (red). The sum of these three groups represented from 66% up to 91% of total autotrophic biomass. Squares, circles, and diamonds stand for non-enriched, NH_4-enriched and NO_3-enriched conditions, respectively.

C:nutrient composition

Seston C:N ratio declined in all experimental conditions in relation to an initial 9.6C:1N (Fig. 6A). Seston C:N was highly constant at the end of the experiment, ranging from 8.1 up to 8.9, with the only exception of the most P-enriched condition, which ratios were lower (6.1 – 6.4). Conversely, seston C:P tended to increase at most experimental conditions in relation to an initial value of 285 (Fig. 6B), and typically ranged from 280 to 520. The most P-enriched conditions showed seston C:P ratios markedly lower (80 – 110).

Since extracellular particulate matter could be particularly C-rich and nutrient-poor, we calculated the C:nutrient ratios using the "cellular" particulate C (C_{cell}) instead of total particulate C. C_{cell}:N ranged from 1.5 to 5.6, while C_{cell}:P ranged from 40 to 260 (Fig. 6A and 6B). Both ratios tended to increase up to medium P additions, but at high P additions the tendency changed, and the ratios declined. C_{cell}:nutrient ratios also declined with increasing N:P imbalance.

Particulate matter exported to sediment traps was markedly N impoverished under non-enriched conditions (C:N = 12.3) in relation to initial and final seston ratios (Fig. 6C). As observed for seston, the C:N ratio of sedimented matter markedly declined at high P additions. NO_3^-:P imbalance did not affect the C:N of sediment trap matter, but NH_4^+:P imbalance did lower slightly the C:N ratio. C:P of sediment trap matter was between the initial and final seston ratios under non-enriched conditions (C:P = 295, Fig. 6D). The sediment trap matter was P-richer (or C-poorer) than seston at N_P and N_P+ conditions, but the differences between sediment and seston were considerably smaller at N_P++.

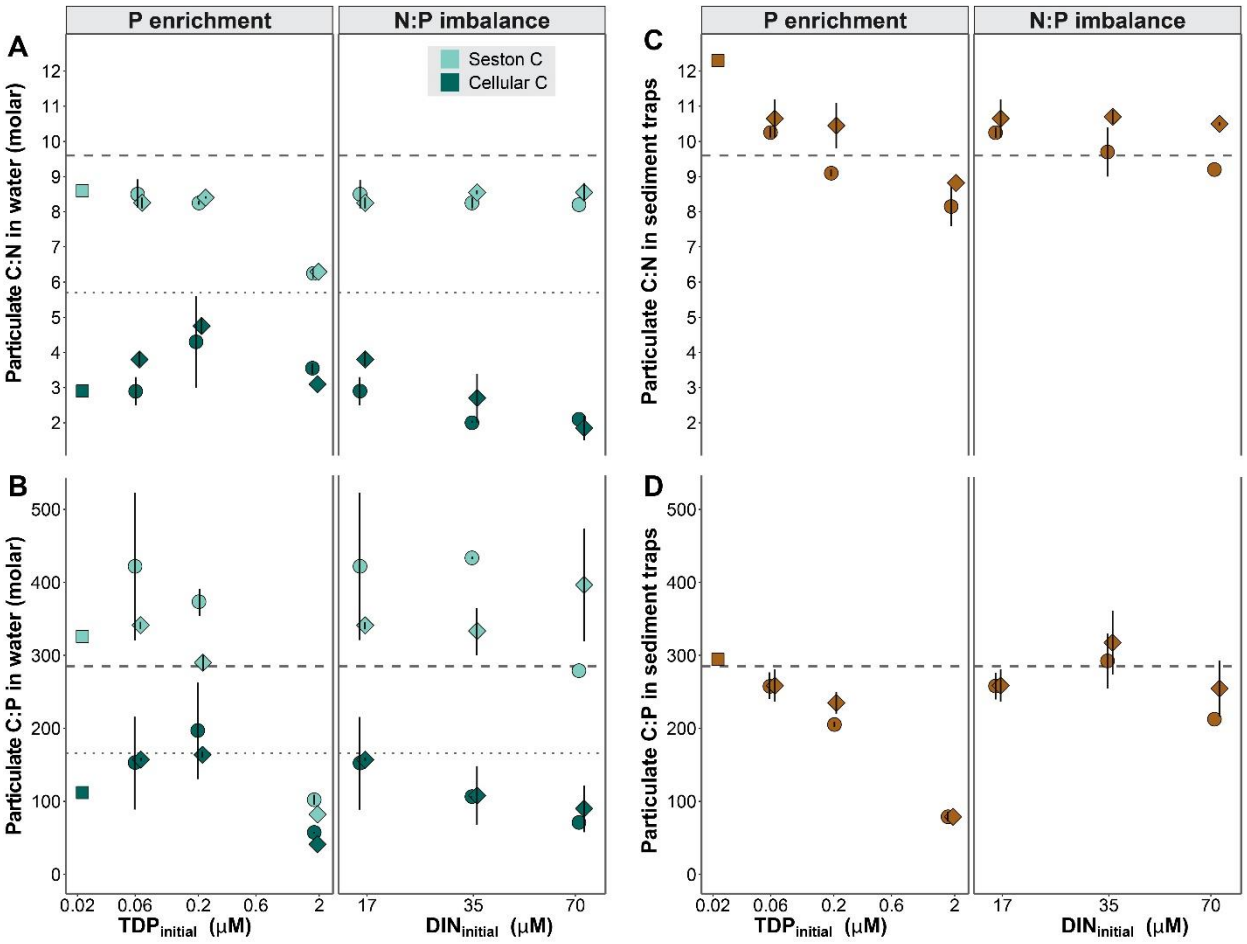


Figure 6 Effects of P enrichment and N:P imbalance on C:N and C:P ratios of particulate matter in water (A, B) and sediment trap matter (C, D). In A and B, C:nutrient ratios were calculated using the total particulate C (light blue) and the cellular particulate C (dark blue). Dashed lines indicate initial lake ratios calculated with particulate C, and dotted lines with cellular particulate C. Squares, circles and diamonds stand for non-enriched, NH_4-enriched and NO_3-enriched conditions, respectively.

N:P stoichiometry

The concentrations of DIN and TDP in mesocosms remained almost unchanged under non-enriched conditions (Fig. 2B). TDP concentrations declined importantly at low and medium P additions, but the decline of DIN was proportionately small. Consequently, DIN:TDP increased markedly at these conditions and P limitation accentuated. Net assimilation of TDP increased when DIN availability was higher (N:P imbalance). At high P additions, TDP fell even more sharply, but DIN also declined importantly, and DIN:TDP ratios barely changed (~10:1). The pattern of net DIN:TDP assimilation shifted between N_P+ and N_P++ treatments, when the initial DIN:TDP availability was experimentally reduced from ~64 to ~10.

The stoichiometric shift between medium and high P additions was also evident in the N:P ratios of seston and the sediment trap matter (Fig. 7). Seston N:P increased from an initial value of ~30 up to ~33-65 at most experimental conditions, but seston N:P decreased at N_P++ (~13-17, Fig.7A). The comparison between non-enriched and NO_P conditions -which had similar initial DIN:TDP but different absolute concentrations of DIN and TDP- showed that seston of NO_P did not become P-richer than that of non-enriched treatment. As observed for seston C:P, seston N:P tended to increase when NH_4^+ was the dominant form of DIN, though this may not apply for highly N:P imbalanced conditions. Contrastingly, DIN form did not affect the N:P ratio of the sediment trap matter (Fig. 7B). The sediment trap matter often became N-poorer (or P-richer) than initial seston N:P, and always N-poorer than final seston N:P.

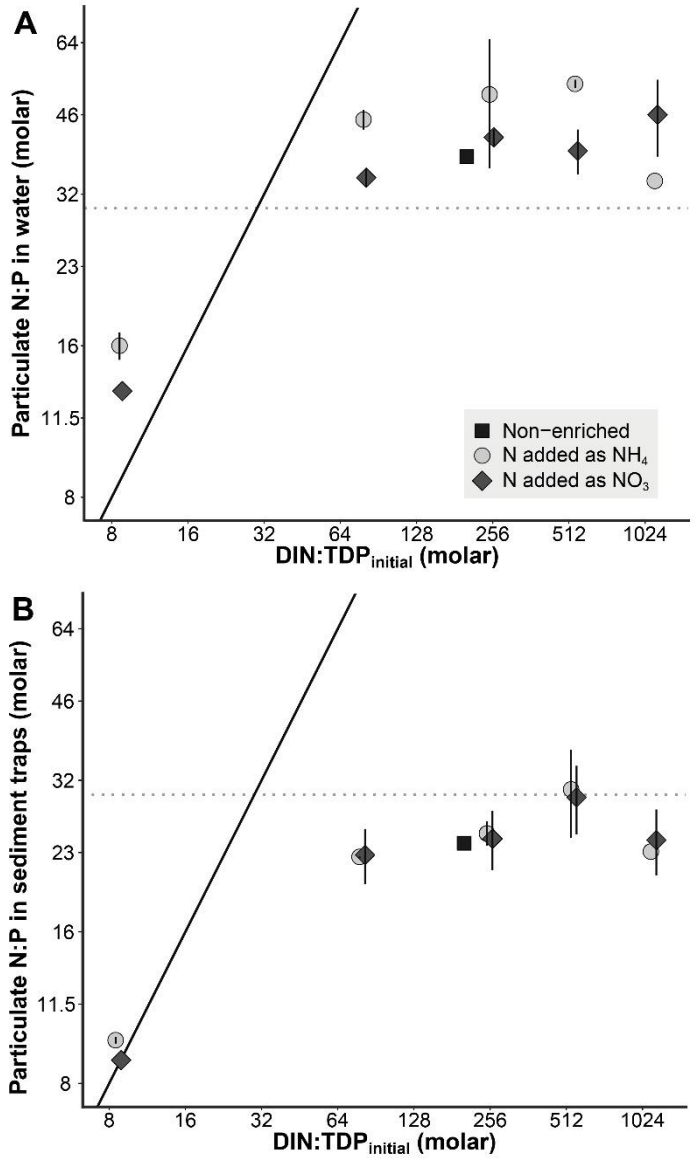


Figure 7 N:P stoichiometry of seston (A) and sediment trap matter (B). Strict stoichiometric flexibility is accomplished when N:P of consumers reflects N:P of supplies, following the same slope that the solid black line. In contrast, strict homeostasis appears when N:P of consumers stays constant, independently of supply (an horizontal line). Horizontal dotted line indicates the seston N:P ratio at the beginning of the experiment. Squares, circles and diamonds stand for non-enriched, NH_4-enriched and NO_3-enriched conditions.

Discussion

In this study, we described changes in productivity, community structure and C:N:P composition when a P-deficient system is released from limitation ("P enrichment"), when is supplied with N excess ("N: P imbalance"), and when NH_4^+ becomes the dominant source of DIN ("DIN form"). We found that the responses of productivity, community structure, and seston C:N:P to varying levels of P enrichment can be markedly decoupled (Fig. 8). Productivity was highly sensitive to low P additions but tended to saturate at high P additions, whereas seston C:N:P remained fairly unchanged to low and medium P additions, but changed drastically with high P additions. The autotrophic community showed a pattern between both, since the community already differentiated at medium P additions, and the rate of change was maintained at high P additions (Fig.8).

Potential drivers of the stoichiometric shift

Seston C:N:P shifted drastically and approached the Redfield ratio (106C:16N:1P) when we added high amounts of P ($TDP_{initial}$ ~2μM) and source DIN:TDP ratio placed below Redfield. This stoichiometric shift was the most unexpected and remarkable result of this study, since, to our knowledge, so marked shifts on N:P stoichiometry have never been reported (see, for instance, Hall *et al.*, 2005). Given that seston components can have different elemental compositions, we may first ask whether this shift is related to an alteration of the relative abundance of these components, or to a change of the elemental composition of each seston component. Neither the proportion of cellular to total particulate nor the proportion of autotrophic to living biomass changed substantially between the treatments N_P+ and N_P++. The increase of prokaryotic abundance between these treatments was quite drastic, but prokaryotes only represented a small fraction of living biomass (from 5-8% in N_P+ up to 10-14% in N_P++). As living matter was clearly dominated by autotrophs (~80%), we may suspect that changes in the autotrophic community had a greater effect.

In general, diatoms present lower N:P ratios than the rest of phytoplankton groups (Quigg *et al.*, 2003; Weber & Deutsch, 2010), although the variation among species of the same phylogenetic group is considerable. The relative abundance of chrysophyceans, diatoms and cryptophytes already changed at N_P+ compared to N_P, while the change of seston C:N:P between these treatments was small. Moreover, if chrysophyceans had higher N:P ratios than diatoms and cryptophytes, we would have expected higher seston N:P at NO_P++ (where they represented ~29% of autotrophic biomass) than at NH_P++ conditions (~14% of autotrophic biomass), but the opposite was the case. Therefore, all evidence point that the stoichiometric shift was not driven by a change in seston structure, but, rather, by a change of C:N:P composition of the organisms (or most of them).

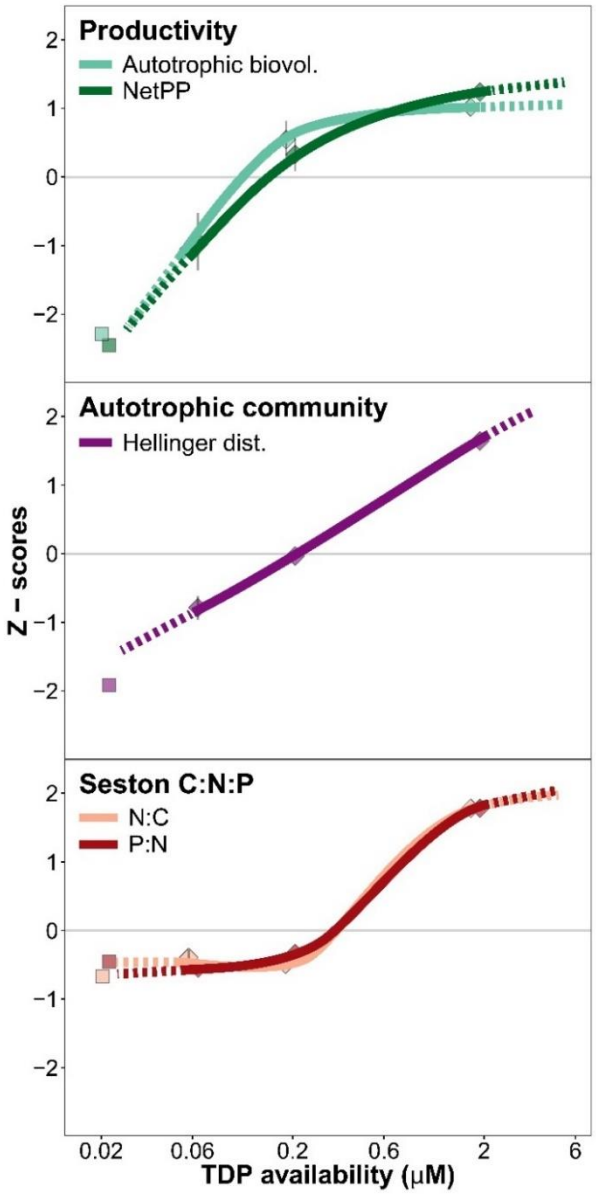


Figure 8 Patterns of change of productivity, autotrophic community (estimated as Hellinger distance) and seston C:N:P to P enrichment. Note that the elemental composition of seston is shown as N:C and P:N ratios (instead of C:N and N:P ratios) to easily compare the patterns of change in productivity and community structure. Only NO_3^--dominated conditions are shown for simplicity. Original values were standardized using the mean and standard deviation of NO_P, NO_P+ and NO_P++ mesocosms. Solid lines indicate the tendency within the study range, whereas dashed lines extend that tendency to lower and higher P availability. The non-enriched condition (squares) had lower DIN concentrations initially than the other conditions (diamonds), and, consequently, it is only shown as a reference.

N and P availability control the elemental composition of autotrophs through changes in the relative abundance of structural macromolecules and nutrient stores (Rhee, 1978; Elrifi & Turpin, 1985). Ribosomes have received much attention because they are a relevant pool of P in organisms (Geider & La Roche, 2002), and are related to the growth capacity of organisms. According to the "Growth rate hypothesis" (GRH), organisms upregulate ribosomes synthesis under non-limiting conditions and maximum growth rates, what increases their P content (Sterner & Elser, 2002). Thus, GRH offers a framework to understand the decline of phytoplankton N:P ratio at increasing growth rates (Goldman *et al.*, 1979; Hillebrand *et al.*, 2013). However, in our study, productivity and seston N:P were clearly decoupled (Fig.8), as seston N:P declined substantially at N_P++ compared to N_P+, but the increase of NetPP and autotrophic growth between these treatments was rather low. Some theoretical models predict non-linear and drastic transitions from the "optimal" N:P states at N- and P-limited conditions, towards the "optimal" N:P state at exponential growth conditions (Klausmeier *et al.*, 2004). Our results also suggest phytoplankton can shift its C:N:P content in that nonlinear way when released from P-deficiency. However, whether this shift is driven by higher growth rates and ribosomes upregulation is more uncertain.

GRH have been recently questioned for phytoplankton (Flynn *et al.*, 2010), among other reasons, because most phytoplankton accumulates P in non-ribosomic pools under optimal nutrient-replete conditions, and lose these reserves under P stress. Then, changes in "other" P-pools, more related to nutrient availability, could actually underlie the pattern of N:P with growth (Hillebrand *et al.*, 2013). The role of other P pools and their regulation by phytoplankton is increasingly addressed. For instance, it has been shown that phytoplankton can use non-phosphorus lipids under P deficiency, but increase phospholipid synthesis when it is more available (Van Mooy *et al.*, 2009). Besides, a considerable amount of P can be adsorbed at the cellular surface of phytoplankton (Sañudo-Wilhelmy *et al.*, 2004; Fu *et al.*, 2005), what may directly link P availability in the medium and P content of

organisms. However, seston C:N also declined substantially at high P additions, and, therefore, the stoichiometric shift was not only related to a change of P content. The regulation of P content in phytoplankton appears to be connected with C and N content, but, unfortunately, we still do not know the macromolecular reorganizations involved.

Effects of N availability on seston C:N:P

Non-enriched and NO_P conditions were compared because they had similar DIN:TDP ratios at the beginning of the experiment, but different concentrations of DIN and TDP. Considering the P deficiency of the lake, we expected that seston of NO_P treatment would become P-richer than seston of non-enriched treatment. Although seston P increased at NO_P compared to non-enriched conditions, the increase of seston N was proportionately similar, or even higher, and seston N:P actually increased slightly. In contrast, productivity was considerably higher at NO_P than at non-enriched conditions, and, thus, the responses of productivity and seston N:P were also decoupled at higher DIN:TDP availability. The increase in productivity between non-enriched and NO_P conditions had a similar rate of change than between NO_P and NO_P+ (Fig. 8), indicating that such increase was more affected by the addition of P than N. While productivity was mainly controlled by P availability, seston C:N:P depended on both P and N availability (DIN:TDP).

High DIN additions (>17μM $DIN_{initial}$; >256:1 molar $DIN:TDP_{initial}$) did not produce any effect on seston N:P. Increasing seston N:P beyond ~60 appears unlikely, regarding the maximum values annually detected in this lake (Ventura & Catalan, 2005). In a recent meta-analysis of seston C:N:P, more than 90% of N:P ratios also placed below ~60 (Sterner *et al.*, 2008). Some constraints may prevent higher seston N:P imbalances, such as the minimum P quotas and maximum N storage capacity of organisms (Hall *et al.*, 2005). N excess availability did not lower seston C:N either. Indeed, seston C:N:P was remarkably insensitive to varying DIN and TDP levels above ~64DIN:1TDP. Wider ranges for seston C:N:P are reported in Lake Redon along the year (Ventura & Catalan, 2005), suggesting that other factors (e.g.

light) may also affect seston C:N:P importantly (Hessen *et al.*, 2013).

Interestingly, this study reveals that seston C:N:P composition can be affected by the relative abundance of NH_4^+:NO_3^-. Specifically, NH_4^+ dominance tended to increase seston C:P and N:P without altering seston C:N. There is a lack of studies addressing the influence of DIN forms on seston C:N:P, and, thus, the mechanisms underlying this pattern are fairly unknown. The fact that increased seston C:P and N:P ratios were more preferentially associated with higher concentrations of seston C and N than to lower seston P suggests that protein synthesis could have been stimulated under NH_4^+dominance. Even though, this change might not be only attributed to an internal increase of C and N in the organisms. The differential increase of seston C between NH_4^+- and NO_3^--dominated conditions was associated with the cellular fraction at N_P++, but with the extracellular fraction at the other conditions (Fig. 4).

Sedimentation of particulate matter and mineralization

C:P and N:P ratios of sedimented matter were not higher at NH_4^+- than NO_3^--dominated conditions, and frequently differed from final seston ratios. The observed differences among seston and sediment trap C:N:P cannot be directly associated with mineralization (e.g. higher decomposition of C and N compounds under NH_4^+ dominance) because sediment traps integrated what occurred during all the 25 days of experiment, and we may speculate whether its composition was more related with the seston of the first stages of the experiment than to the final. For instance, it cannot be discarded that seston became C- and N-enriched at the final stages of the experiment, and that this material had not already reached sediment traps. Temporal changes of seston C:N:P were presumably smaller under non-enriched conditions (e.g. DIN and TDP levels barely changed between the beginning and the end of the experiment), and the initial seston C:N:P may be representative of the first stages of the experiment. Under non-enriched conditions, sediment trap matter was markedly N-impoverished in relation to the initial and final C:N:P composition, indicating that mineralization of particulate N could be higher than that of C and P. In turn,

C:P of sediment trap matter placed between the initial and final seston C:P under non-enriched conditions, suggesting that mineralization of both elements was similar. The low mineralization of P in comparison to N (or even C) has been previously described for lakes (Elser & Foster, 1998). This suggests that some P-containing molecules and aggregates (e.g. polyphosphates) could be difficult to mineralize in the water column (Diaz *et al.*, 2008), and thus contribute to P deficiency in the ecosystem.

Sedimentation rates within the mesocosms ranged from ~0.63 to 1.26 g C m^{-2} $month^{-1}$, which are slightly higher than previously estimated for the entire water column in this lake (Camarero *et al.*, 1999). Absolute C sedimentation did not substantially vary among experimental conditions, and, hence, the percentages of sedimented C to NetPP declined as P availability and productivity increased (from ~10% up to ~3% at N_P++). Lower organisms' mortality and enhanced mineralization of dead particulate matter under P-enriched conditions may drive that tendency.

Responses of productivity and autotrophic community to nutrient enrichments

NetPP and autotrophic growth were particularly sensitive to low P additions, but the response decelerated at high P additions (2µM of $TDP_{initial}$) (Fig. 8). Chrysophyceans were the dominant phytoplankton group and mostly responsible for the observed pattern. The highest chrysophyceans biovolumes were found at medium instead of high P additions, what contributed to the saturation shape of productivity (Fig. 8). However, the factors that impaired the growth of chrysophyceans at N_P++ are quite unknown (e.g. higher grazing pressures, P toxicity). In relative terms, the proportion of chrysophyceans diminished progressively from N_P to N_P++ conditions (Fig. 5). Thus chrysophyceans appeared to be less favoured by higher P availability than other phytoplankton groups (e.g. diatoms, cryptophytes). The progressive substitution of

chrysophyceans was the primary driver of changes in the autotrophic community. The differentiation of autotrophic community increased almost linearly with P addition (Fig. 8), and thus followed a different pattern than the observed for productivity. Community structure is expected to be more stable and responds more slowly to changes in nutrient availability than productivity, but if new conditions are sufficiently different and maintained over time, changes in community structure may end up being more relevant.

Higher differentiation of autotrophic community under NH_4^+ dominance was previously expected since NO_3^- typically dominates DIN in Lake Redon. Our study shows that DIN form effect is inexistent under severe P limitation, but increases when the system gets released from that limitation (Fig. 5A). Productivity was also stimulated by NH_4^+ dominance at the most P-enriched conditions. Diatoms were fairly responsible of DIN form effects observed on community structure and productivity because increased more importantly at N_P++ when NH_4^+ was added. This could be an attribute of freshwater or mountain lake diatoms, given the general idea is that marine diatoms are good competitors for NO_3^- (Berg *et al.*, 2003; Litchman *et al.*, 2007; Glibert *et al.*, 2014).

N excess availability (N:P imbalance) resulted in the decline of total phytoplankton biomass, and the alteration of the community structure (Fig. 4A-B and 5A-B). The positive effect of low P additions was offset by this negative effect, and the autotrophic biovolume of N:P imbalanced conditions approached the levels of non-enriched conditions. A possible increase of grazing under N:P imbalanced conditions was discarded, since the abundance of eukaryotic heterotrophs rather declined. More likely, high DIN concentrations could be toxic for some phytoplankton species. High toxins release by cyanobacteria is reported under N:P imbalanced conditions (Raven, 2010), but the abundance of this group in mesocosms was low. The decline of autotrophic biomass was not paralleled by a similar reduction of particulate C, due to slight increases of prokaryotic and extracellular C. Indeed, these planktonic compartments are expected to respond to

the presence of dead organisms. Lower TDP levels at the end of the experiment under N:P imbalanced conditions (Fig. 2B) might be regarded as a consequence of increased prokaryotic activity, or even, a cause of the autotrophic decline. On the other hand, high N availability may favour the growth of prokaryotes associated with DIN transformations (e.g. nitrifiers). Accordingly, final DOC levels were slightly higher under NH_4^+:P than NO_3^-:P imbalanced conditions, and prokaryotic abundance was also higher at NH+_P.

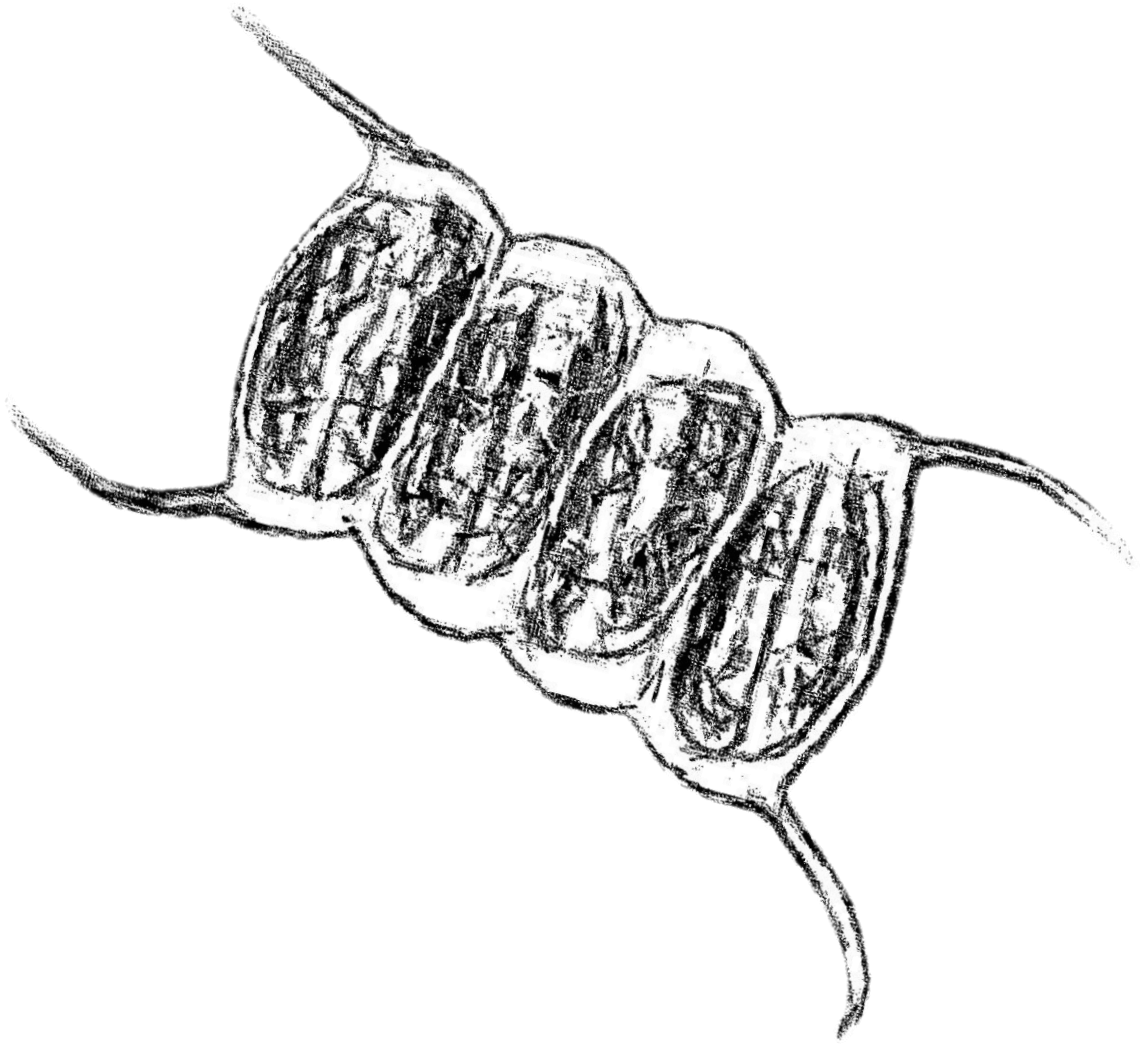
Dissolved and extracellular organic matter

In general, DOC was quite insensitive to different nutrient enrichments (ranging from 0.7 up to 0.9 mg C L^{-1}). In relation to initial lake levels, DOC almost doubled in all mesocosms, what could be a side effect of water enclosures. Extracellular particulate C also increased under non-enriched conditions, what could be related to higher DOC levels. It has been shown that DOC is highly correlated with particulate C, probably through a link with the extracellular -or detrital- fraction (Camarero *et al.*, 1999). Flocculation of DOC molecules in large aggregates could explain this correlation, and, besides, the considerable amount of "invisible" extracellular particulate (about half the total particulate C). On the other hand, microscope observation shows that debris of death organisms, prokaryotes and also eukaryotes are often found within mucilaginous lumps or aggregates, what could also contribute to extracellular particulate matter. Higher amounts of extracellular particulate were detected at the most P-enriched conditions, when the visual detection of these mucilaginous aggregates also increased. Prokaryotic abundance and extracellular particulate presented similar patterns to experimental conditions (Fig. 4), which suggests that prokaryotes could be favoured by the presence of these mucilaginous aggregates, or, as occurs in "marine snow", actively contribute to their formation (Azam & Malfatti, 2007). The main components that comprise extracellular biomass are not well-known, even though it is a considerable fraction of particulate, and its influence on seston C:N:P composition is relevant (Frigstad *et al.*, 2011). The hypothesis that extracellular matter is only constituted by C

(totally lacking N and P) was evaluated: we calculated the C:N and C:P ratios of planktonic organisms using the cellular particulate C (i.e. C_{cell}:N and C_{cell}:P), and checked if the resulting values were reliable or not. C_{cell}:N placed below the minimal C:N ratios commonly reported for seston (~4C:1N, Sterner *et al.*, 2008), and most algal cultures (Geider & La Roche, 2002). C_{cell}:P of non-enriched treatment placed close to Redfield ratio (106C:1P), which is relatively low for lake seston (Sterner *et al.*, 2008). The minimum C_{cell}:P ratios of N_P++ treatment (~50C:1P) are considerably low, and, as occurred for C_{cell}:N, appeared unrealistic for natural organisms. Therefore, the extracellular matter was not entirely constituted by C, and organisms' C to nutrient ratios should place between the C_{cell}:nutrient and the C:nutrient ratios.

Concluding remarks

This study points that even small increases of P atmospheric inputs can substantially increase productivity and phytoplankton growth in this P-deficient oligotrophic lake. With some delay, these changes may eventually translate to the structure of the autotrophic community. In turn, low and medium nutrient additions may not produce great changes in seston C:N:P. Drastic changes of seston C:N:P were only detected when the system was brought out of the typical TDP levels in the lake, and DIN:TDP approached the Redfield ratio. Interestingly, we observed that this stoichiometric shift was clearly decoupled from productivity. The change appears more related to the readjustment of macromolecular components of the organisms than to changes in the structure of the planktonic community.



3

Chlorophyll to biovolume ratio and growth rate of phytoplankton groups: an experimental approach in a P-limited oligotrophic lake

Abstract

The transition from nutrient-deficient to nutrient-replete conditions precipitate a number of changes in the regulation of phytoplankton that ultimately alter their growth rate. Some laboratory studies show that chlorophyll a (Chla) content is one of these altered physiological traits, since Chla is generally upregulated under nutrient-replete conditions (or downregulated under nutrient-limitation). Although this plasticity may vary among algal taxa, the reported evidence is rather scarce. Here, we evaluate the relationships between growth conditions and Chla content per biovolume (BV) of the major phytoplankton groups in a high-mountain oligotrophic lake. We installed 20 m deep mesocosms (~100L) and enriched them with different amounts of P (phosphate) and N (nitrate or ammonium), thus creating gradients of growth conditions. Chla contents per BV were estimated using power law-based equations and Bayesian methods that allowed us to include a priori information on accessory pigment to Chla ratios. The power law between Chla and total phytoplankton BV gave a scaling exponent 1.26, indicating that Chla content tended to increase with P supply. The exponents were slightly higher than 1 in Chrysophyceae, Dinophyceae and Cryptophyta, 1.22 in Bacillariophyta, and 1.97 in Chlorophyta. These two last groups also showed the highest Chla contents per BV under P-replete conditions, and the highest increases of BV in relation to the initial levels of the lake. The study suggests that the high growth capacity of diatoms and chlorophytes could be associated with their ability to increase the Chla content per BV when nutrient supply increases.

Introduction

Chlorophyll a (Chla) is shared by all oxygenic photoautotrophs due to its key role in photosynthesis, both as a major constituent of light-harvesting antennas and as a primary electron donor in reaction centres. In addition, Chla can be easily analysed by different techniques, and has actually become a convenient surrogate for algal biomass. However, the role of Chla as indicator of biomass is inaccurate (Kruskopf & Flynn, 2006) because the Chla content per biovolume (or per cell, or per carbon) can vary considerably between species, and also within a single species (Reynolds, 1984, 2006).

Light-harvesting complexes in oxygenic photoautotrophs have gradually diverged from a common ancestor. This evolutionary process becomes apparent in that main phylogenetic groups now differ in the composition and proportions of auxiliary pigments. One may expect that other physiological traits such as the Chla content per biovolume (BV) and its plasticity could also be shared among species within major algal groups. A certain phylogenetic imprint has been noted in that chlorophytes generally show higher Chla content per BV than diatoms and cyanobacteria (Reynolds, 1984). However, Chla:BV ratios are still only reported for a relatively small number of species, and the intra-groups variability appears to be quite high (Llewellyn & Gibb, 2000). Thus, distinguishing group-specific traits from general variation among species is rather complicated. In addition to between species variation, “acclimation” have a major influence on Chla:BV variability. There is broad evidence that individuals of a species can regulate the synthesis of Chla and other pigments depending on external factors such as irradiance and nutrient availability (Rodríguez *et al.*, 2006; Ruivo *et al.*, 2011; Zhang *et al.*, 2015).

In natural systems, the peak of Chla may not correspond with the peak of phytoplankton BV because the phytoplankton of deeper depths, or under an ice-cover, contain more Chla per BV to offset the lower irradiance (Felip & Catalan, 2000). In contrast, Chla per BV tend to decline with nitrogen (N) and phosphorus

(P) deficiency due to down-regulation of Chla synthesis both pre- and post-translationally (Latasa & Berdalet, 1994; Juergens *et al.*, 2015). Physiological processes influenced by nutrient deficiency are not just limited to pigment content, but the overall regulation and the growth rate is ultimately altered. The plasticity of Chla content to growth conditions is usually determined at the laboratory comparing samples taken at the exponential and stationary phases. For instance, Ruivo *et al.* (2011) observed that the Chla cell content reduced 1.3 to 4.4 times from the exponential to the stationary phase, depending on the algal species. Goericke & Montoya (1998) also found marked changes in Chla:cell, and observed that it increased linearly with growth rate in continuous algal cultures.

In the field, determining the interdependence between growth and Chla content is far more complicated. One possible way is through the estimation of power law (or allometric) equations between Chla and BV. If all samples come from the same initial condition, and the effect of grazing is low, BV change can be taken as a measure of growth. Thus, in a Chla to BV log-log plot, a slope of one would indicate that Chla:BV remains constant regardless of growth, whereas a slope higher than one would mean that Chla increases faster than BV. In the latter case, higher growth rates would be achieved, as more energy will be available for a faster biosynthesis and reproduction. Concluding about the relationship between Chla:BV and growth rates from pure field observations is, however, extremely difficult because common initial conditions and different growth gradients are rare to be clearly distinguished.

Field experimental mesocosms provide a useful alternative that combines the advantages of lab manipulations and the realism of complex field communities. Starting with an epilimnetic plankton community of an oligotrophic lake (Lake Redon, Pyrenees), we used mesocosms and enriched them with different amounts of N and P, thus creating a wide range of growth conditions for phytoplankton. We estimated the power law between Chla and BV using a recently developed program based on Bayesian statistics (Bürkner, 2015), which allows the incorporation of prior knowledge about the parameters and thus restrict the possible outcomes to

those with biological sense. Although the available information about the Chla:BV ratios of the major algal groups is scarce, or presents wide ranges of variation, priors can be established based on the information provided by the accessory pigments. The knowledge about the presence of each accessory pigment on each algal group, and the proportion of these pigments to Chla have notably increased in recent years (Roy *et al.*, 2011), and is worth considering it to estimate Chla content of major phytoplankton groups.

Methods

Experimental procedures and design

We installed the mesocosms in Lake Redon, an oligotrophic lake located in the Central Pyrenees (42º38'33"N, 0º36'13"E, 2232 m asl) (see further information in Chapter 2; Catalan, 1988; Catalan *et al.*, 2006). Chrysophyceae are usually the dominant group in the lake's phytoplankton, whereas other groups can be also relevant during the mixing period (Chlorophyta, Bacillariophyta), during summer stratification (Dinophyceae) or at greater depths (Cryptophyta) (Felip *et al.*, 1999). Mesocosms consisted of tubular-shaped polythene bags (diameter: 8.5 cm; length: 20 m, water volume: ~100L) (see a further description of mesocosms and installation process in Chapter 2). Mesocosms were placed in the lake on 5-6 August 2013 and recovered 25 days after. We obtained integrated water samples (i.e. ~5 L, from 0 to 20 m depths) from the lake at the beginning of the experiment, and from each mesocosm at the end of the experiment. Water samples were immediately filtered through a 250 µm pore size mesh to discard large zooplankton. Once in the shore, between one and two litres of water sample were filtered on glass fibre filters for pigment analyses, wrapped in aluminium foil and frozen in liquid nitrogen to prevent degradation. A 200 ml subsample was preserved with 0.5% (vol/vol) alkaline Lugol's solution to estimate protists abundance (Sournia, 1978).

The experimental design consisted in one gradient of increasing P availability, and another gradient of increasing N availability, that together created a gradient of 5 N:P conditions (see further details in Fig. 2 of Chapter 2). The low P- and N-enriched condition (N_P) intended to simulate the original DIN:TDP ratio of the lake, but with higher absolute TDP and DIN concentrations. From the N_P condition, we maintained the N addition but increased the P addition to medium (N_P+) or high levels (N_P++), and maintained the P addition but increased the N addition to medium (N+_P) or high concentrations (N++_P). P was always added as K_2HPO_4, but N was added as NH_4Cl in five treatments (NH++_P, NH+_P, NH_P, NH_P+, NH_P++), and as KNO_3 in another five (NO++_P, NO+_P, NO_P, NO_P+, NO_P++). Thus, with two replicates for each treatment, and two non-enriched control mesocosms, we installed a total of 22 mesocosms. We had technical incidences with some mesocosms (i.e. non-enriched, NO_P++, NH++_P) and with one pigment sample (NH_P+), and these replicates were discarded in this study, making a final number of 18 observations plus the initial.

Pigment analyses

Pigments were extracted in 5 ml of 90% acetone with a probe sonicator (Sonopuls GM70 Delft, The Netherlands) (50W, 2 min). The extract was centrifuged (4 min at 3000 rpm, 4 ºC) and filtered through Whatman Anodisc 25 (0.1 μm) and analysed by ultra-performance liquid chromatography (UPLC). The UPLC system (Acquity, Waters, Milford, MA, U.S.A.) was equipped with an Acquity UPLC HSS C18 SB column (dimensions: 100 x 2.1 mm, particle size: 1.8 μm) and photodiode array (λ 300-800 nm) and fluorescence (λ excitation 440 nm, emission 660 nm) detectors. The detector was set at 440 and 660 nm for carotenoid and phorbin peak integration, respectively. After sample injection (7.5 μL), pigments were eluted by linear gradient from 100% solvent B (51 : 36 : 13 methanol : acetonitrile : MilliQ water, v/v/v 0.3 M ammonium acetate) to 75% B and 25% A (70 : 30 ethyl acetate : acetonitrile, v/v) for 3 min, followed by 0.45 min of isocratic hold at 75% B and 2 min of linear gradient to 99.9% solvent A. Initial conditions (100% B) were linearly

recovered in 0.65 min. The flow rate was 0.7 ml min^{-1}. Pigments were identified checking retention times and absorption spectra against a library based on standard commercial mixtures (DHI, PPS-MiX-1) and extracts from pure cultures of algae and bacteria.

Biovolume estimation

The abundance of living protists was estimated using the Utermöhl method (Sournia, 1978). Biovolume was determined by measuring the main cell dimensions, and assimilating its shape to known geometric forms (Hillebrand et al., 1999). We also examined the presence of autotrophic picoplankton at the chlorophyll a channel of the epifluorescence microscope, but its abundance was negligible, in accordance with previous studies in Lake Redon (Felip et al., 1999).

Chla-BV relationship

The estimation of the Chla-BV relationships for the various algal groups was performed in a stepwise procedure. We constrained the estimation of Chla to be consistent with the data obtained for the accessory pigments. In general, accessory pigments are not shared by all algal groups, so the estimation of the power laws between accessory pigments and biovolumes are simpler than Chla-BV estimations. We firstly estimated these simpler models, and then we estimated more and more complex models up to the final Chla model. The estimated models were based on power law equations:

$$P = \sum_{i=1}^{n} (k_i \cdot {B_i}^{a_i}) \qquad (Eq.1)$$

where *"P"* is the pigment concentration in µg/L; *"B"* is the biovolume of the algal group in mm^3/L; *"k"* is a parameter related to pigment content per BV (µg /mm^3), and *"a"* is the scaling exponent. The *"i"* goes from 1 to *"n"*, the number of algal groups with that pigment.

The models were simple power law equations when n=1, such as for accessory pigments exclusive of one algal group (e.g. alloxanthin (Allo) and lutein (Lut)), and for the relationship Chla-total autotrophic BV: In the case of violaxanthin (Viol) n was 2 (i.e. Chlorophyta and Chrysophyceae); for fucoxanthin (Fuco) n was also 2 (i.e. Chrysophyceae and Bacillariophyta); for zeaxanthin (Zea) n was 3 (i.e. Chlorophyta, Chrysophyceae and Bacillariophyta) and for chlorophyll c (Chlc) n was 4 (i.e. Chrysophyceae, Bacillariophyta, Cryptophyta and Dinophyceae). Finally, this equation was also used to estimate the Chla of the five mentioned phytoplankton groups.

All models were estimated using Bayesian methods with the R package "brms" (Bürkner, 2015), that offers the advantage to support non-lineal models. We used the family "Gaussian", the link "identity", and the default settings for the simulations: 4 chains of 2000 iterations, that, after discarding the first 1000 iterations (warm up), produced a total of 4000 posterior samples. Entering appropriate priors for the parameters is required to estimate non-lineal models with "brms". We always used normal priors for the parameters k and a, but we forced the parameters to be positive. We entered weakly informative priors for k and a (mean = 1, SD >0.5) when the knowledge about the expected values of the parameters was low, but, as the simpler models were estimated, the knowledge about these possible values increased. The scaling exponents are expected to be similar for all pigments of an algal group, given that they are found in light-harvesting antennas in certain proportions. On the other hand, the mean of k priors in complex models can also be refined multiplying the parameters k estimated in simpler models by pigment:Chla ratios described in the literature: for instance, refined k prior for Chlorophyta Viol = $(\text{lutein:BV})_{\text{estimated}}$ x $(\text{Chla:lutein})_{\text{Chlorophyta}}$ x $(\text{Viol:Chla})_{\text{Chlorophyta}}$. We performed exploratory runs with fairly unconstrained priors (i.e. relatively high SD) at the beginning, and used more constrained priors (SD_a=0.1; SD_k=0.5) for the definitive runs. The criterion used to evaluate the model fit and compare models was the WAIC (Watanabe, 2010), which is directly

calculated by the "brms" package. Definitive models were selected on the basis of minimum WAIC, with the constraint that the estimated accessory pigment to Chla ratios had to be consistent with the ratios reported in the literature. Specifically, the estimated accessory pigment to Chla ratios were not allowed to be 2-fold higher or lower than the maximum and minimum ratios reported in Schlüter *et al.* (2006) and Lauridsen *et al.* (2011), which include freshwater algal cultures under stationary and exponential phases, and under different levels of irradiance.

Results

Biovolume of phytoplankton groups

The biovolume of all phytoplankton groups was higher at P-rich conditions than at N-rich conditions (Fig. 1). N enrichment (i.e. N+_P and N++_P) had an adverse effect on the growth of Chrysophyceae, Dinophyceae, and Cryptophyta, but barely affected Bacillariophyta and Chlorophyta. The BV of these two groups, in particular Bacillariophyta, increased markedly in all mesocosms compared with the initial lake levels. The response of phytoplankton to the most P-enriched conditions also varied among algal groups: the BV kept increasing at N_P++ compared to N_P+ in Chlorophyta and Cryptophyta; no longer increased (or even declined) in Chrysophyceae; and only kept increasing when NH_4^+ was the added form of N in Bacillariophyta and Dinophyceae.

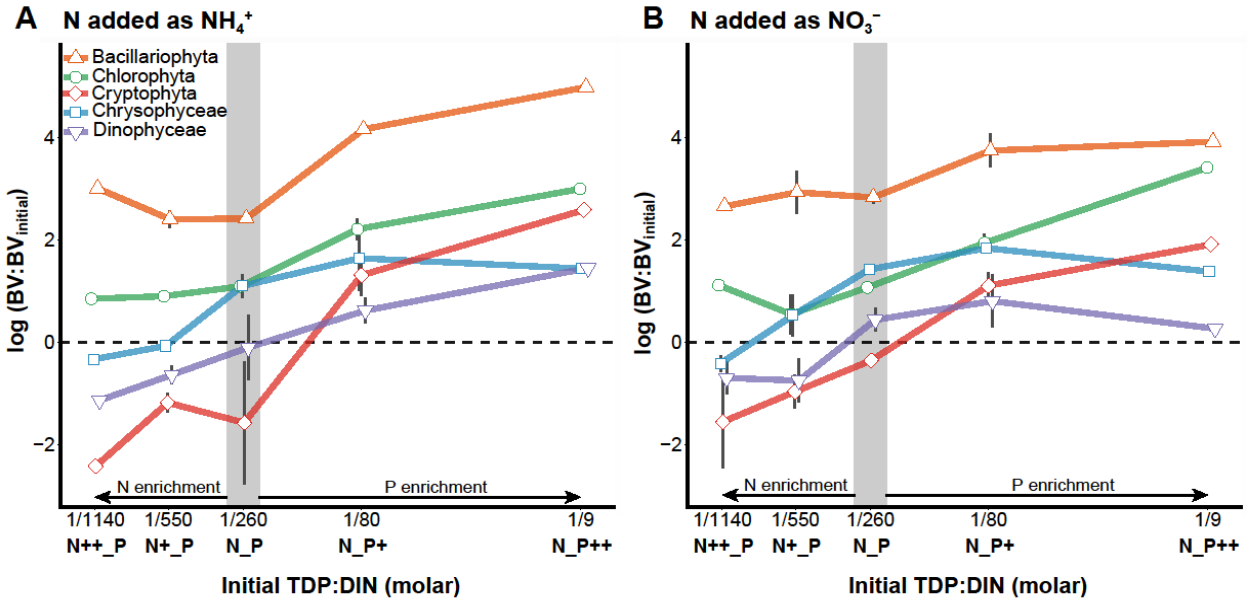


Figure 1 Relative BV change of the phytoplankton groups in relation to the initial lake levels. The mesocosms that were enriched with NH_4^+ are shown in A, whereas the mesocosms enriched with NO_3^- are shown in B. The symbols (see legend in A) show the mean of two mesocosms -when available-, whereas the line range goes from the value of one to the other mesocosm. The relative BV change of the non-enriched mesocosm is not shown in figures for simplicity: the values were 1.65 in Bacillariophyta, 0.33 in Chlorophyta, -1.83 in Cryptophyta, 0.27 in Chrysophyceae and -0.55 in Dinophyceae.

Accessory pigments content

The estimated power law between alloxanthin and BV of Cryptophyta gave parameters *k* and *a* of 1.40 and 1.08, respectively (Fig. 2). This relationship was particularly robust for high biovolumes (Fig. 3A), and, consequently, the *k* parameter presented a relatively narrow credible interval. The observed Allo:BV ratio ranged from 0.25 to 3 µg/mm^3, whereas the estimated content went from ~0.9 µg/mm^3 at minimum growth rates up to ~1.4 µg/mm^3 at maximum growth rates (Fig. 3A). Lutein was detected in P-enriched mesocosms (N_P+ and N_P++), but fell below the detection limit in the other mesocosms. We used lutein instead of chlorophyll b as marker pigment of Chlorophyta precisely because it was better detected in N_P+. The exponent of the power law between Lut and the BV of Chlorophyta was clearly higher than 1 (~1.8, Fig. 2). Therefore, Lut:BV ratio more than doubled from N_P+ (~0.25-0.5 µg/mm^3) to N_P++ (~0.8-1.4 µg/mm^3) (Fig. 3B).

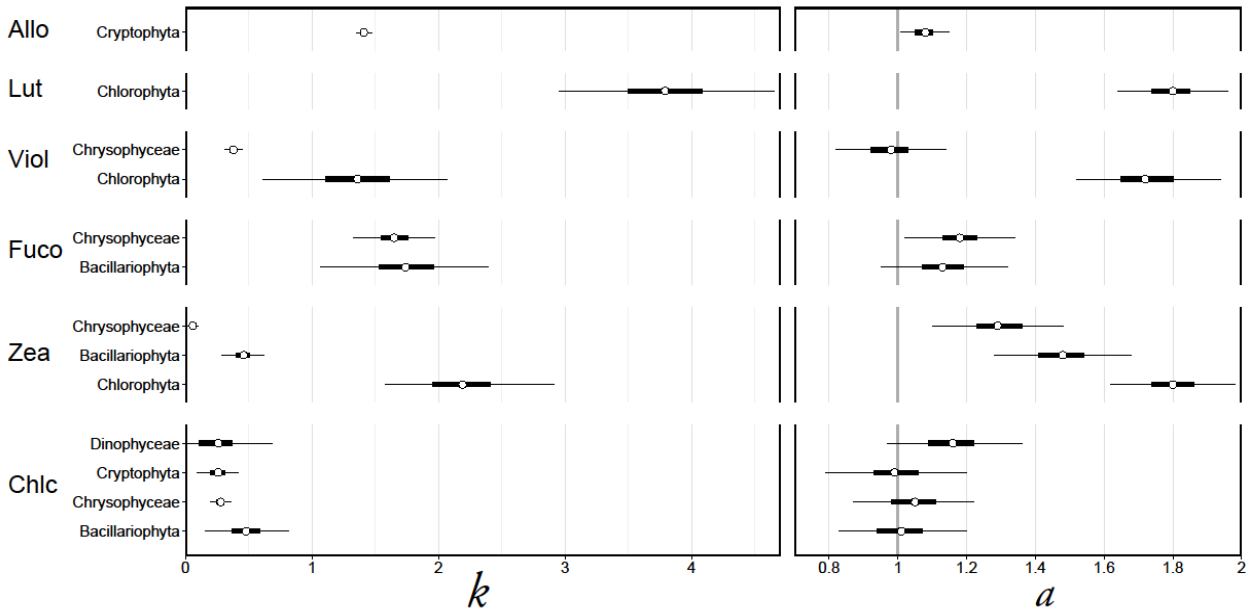


Figure 2 Parameters *k* and *a* of the six estimated models for the accessory pigments: alloxanthin, lutein, violaxanthin, fucoxanthin, zeaxanthin and chlorophyll c. The white point indicates the mean of the posterior distribution, the thick line range indicates the 50% credible interval, and the thin line range indicates the 95% credible interval.

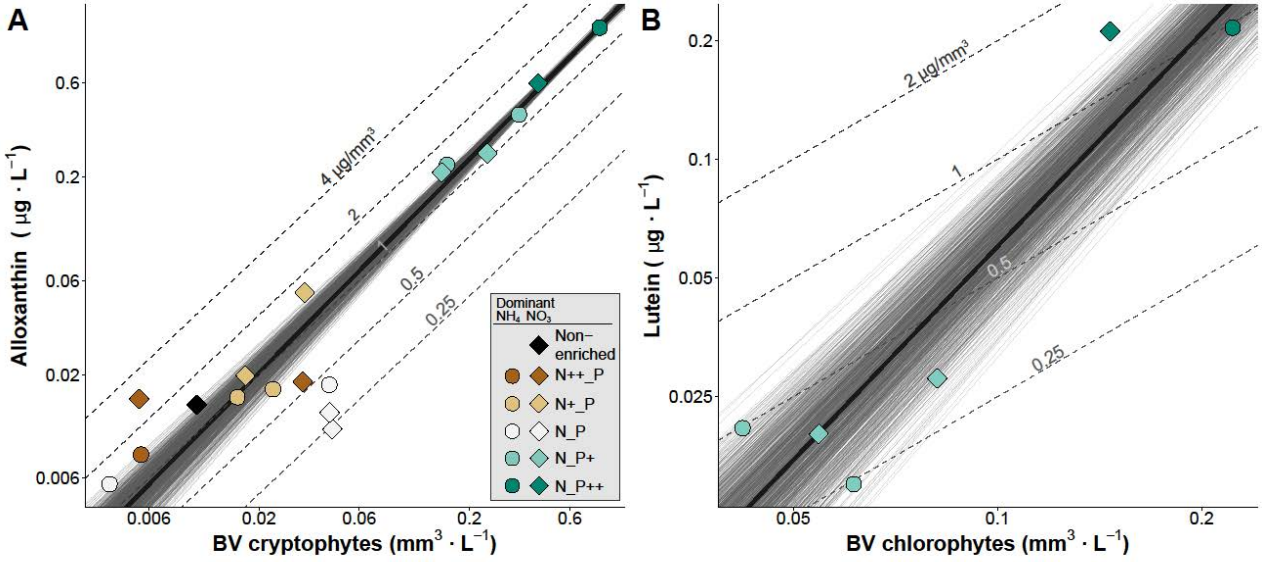


Figure 3 Relationships between alloxanthin and biovolume of cryptophytes (A), and between lutein and biovolume of chlorophytes (B) in log-log space. Black solid lines are the slopes obtained from the punctual estimations of parameters *k* and *a*. Grey solid lines indicate the uncertainty of the estimation; 1000 slopes are drawn from the 4000 available in the posterior distribution. Dashed lines indicate the pigment content per BV (µg/mm^3), as the slope is 1. The treatment of each sample is indicated by different symbols (circles, NH_4^+ was dominant; diamonds, NO_3^- was dominant) and colors, as shown in the legend.

The estimated exponents of the violaxanthin model were 0.98 in Chrysophyceae and 1.72 in Chlorophyta, while the estimated *k* parameters were 0.38 in Chrysophyceae and 1.36 in Chlorophyta (Fig. 2). Considering these estimations and the observed BV ranges, the Viol:BV ratios were 0.38-0.40 μg/mm^3 in Chrysophyceae and 0.04-0.46 μg/mm^3 in Chlorophyta.

The estimated exponents of the fucoxanthin model were 1.18 in Chrysophyceae and 1.13 in Bacillariophyta, whereas the estimated *k* parameters were 1.65 in Chrysophyceae and 1.74 in Bacillariophyta (Fig. 2). These estimations resulted in similar Fuco:BV ratios for both groups within the observed BV range: 0.96-1.61 μg/mm^3 in Chrysophyceae, and 1.1-1.56 μg/mm^3 in Bacillariophyta.

Even though zeaxanthin is a pigment found in some species of Chrysophyceae and Bacillariophyta but not in others, the model that incorporated these groups had a better adjust than the model without these groups. The estimated exponents of the zeaxanthin model were 1.29 in Chrysophyceae, 1.48 in Bacillariophyta and 1.80 in Chlorophyta, while the estimated *k* parameters were 0.06 in Chrysophyceae, 0.46 in Bacillariophyta and 2.19 in Chlorophyta (Fig. 2). These parameters resulted in Zea:BV ratios of 0.03-0.06 μg/mm^3 in Chrysophyceae, 0.06-0.31 μg/mm^3 in Bacillariophyta, and 0.05-0.66 μg/mm^3 in Chlorophyta for the observed ranges of BV.

The exponents of the chlorophyll c model were estimated close to 1 in Chrysophyceae, Cryptophyta and Bacillariophyta; hence, the Chlc:BV ratios were almost constant within the observed BV range: 0.24-0.28 μg/mm^3 in Chrysophyceae, 0.26-0.27 μg/mm^3 in Cryptophyta and 0.46-0.48 μg/mm^3 in Bacillariophyta (Fig.2). The estimated exponent of Dinophyceae was slightly higher (~1.16), what resulted in Chlc:BV ratios of 0.13-0.20 μg/mm^3.

Chlorophyll a content

The power law between Chla and BV of all autotrophs gave an exponent of 1.26, and a parameter *k* of 2.93 (Fig. 4). The Chla:BV ratio was close to 2 µg/mm^3 in those mesocosms with lower growth (N++_P, N+_P, non-enriched), but increased up to 3-4 µg/mm^3 in those mesocosms with higher growth (N_P+, N_P++) (Fig. 5A).

Finally, the model that related Chla with the BV of the phytoplankton groups resulted in exponents slightly higher than 1 in Dinophyceae, Cryptophyta and Chrysophyceae (1.08, 1.08, 1.05, respectively), 1.22 in Bacillariophyta, and 1.97 in Chlorophyta (Fig. 4). The parameter *k* was estimated at 2.10 in Dinophyceae, 2.38 in Cryptophyta, 2.98 in Chrysophyceae, 5.78 in Bacillariophyta and 34.99 in Chlorophyta. The estimated Chla:BV ratio of Dinophyceae went from 1.5 µg/mm^3 when the growth was minimum up to 1.8 µg/mm^3 when it was maximum (Fig. 5B). Cryptophyta presented similar Chla contents, which ranged from 1.5 µg/mm^3 up to 2.3 µg/mm^3 (Fig. 5C). Chrysophyceae had slightly higher Chla:BV ratios, which moved from 2.6 to 3.0 µg/mm^3 (Fig. 5D).

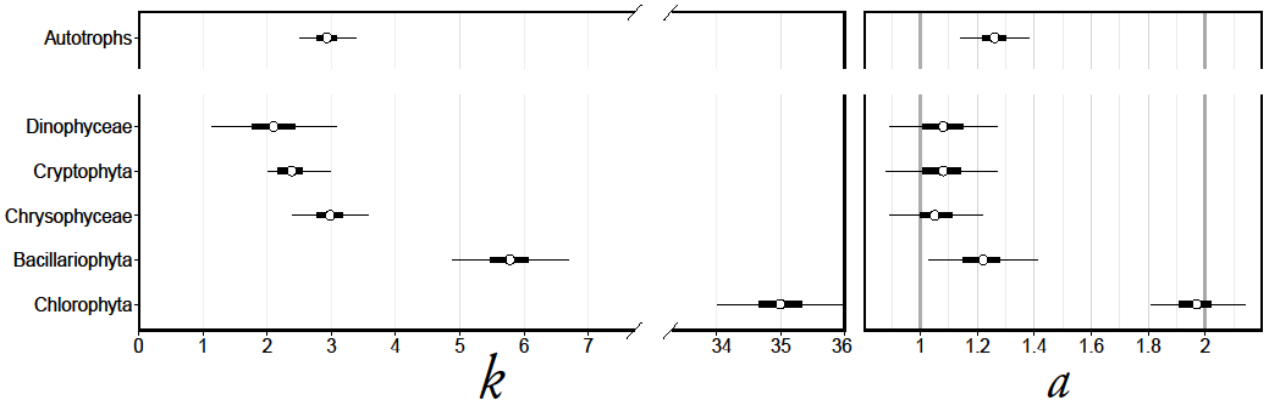


Figure 4 Parameters *k* and *a* of the two estimated models for Chla: the first including the whole autotrophic biovolume as a predictor, and the second with the biovolumes of the five major algal groups as predictors. The white point indicates the mean of the posterior distribution, the thick line range indicates the 50% credible interval, and the thin line range indicates the 95% credible interval.

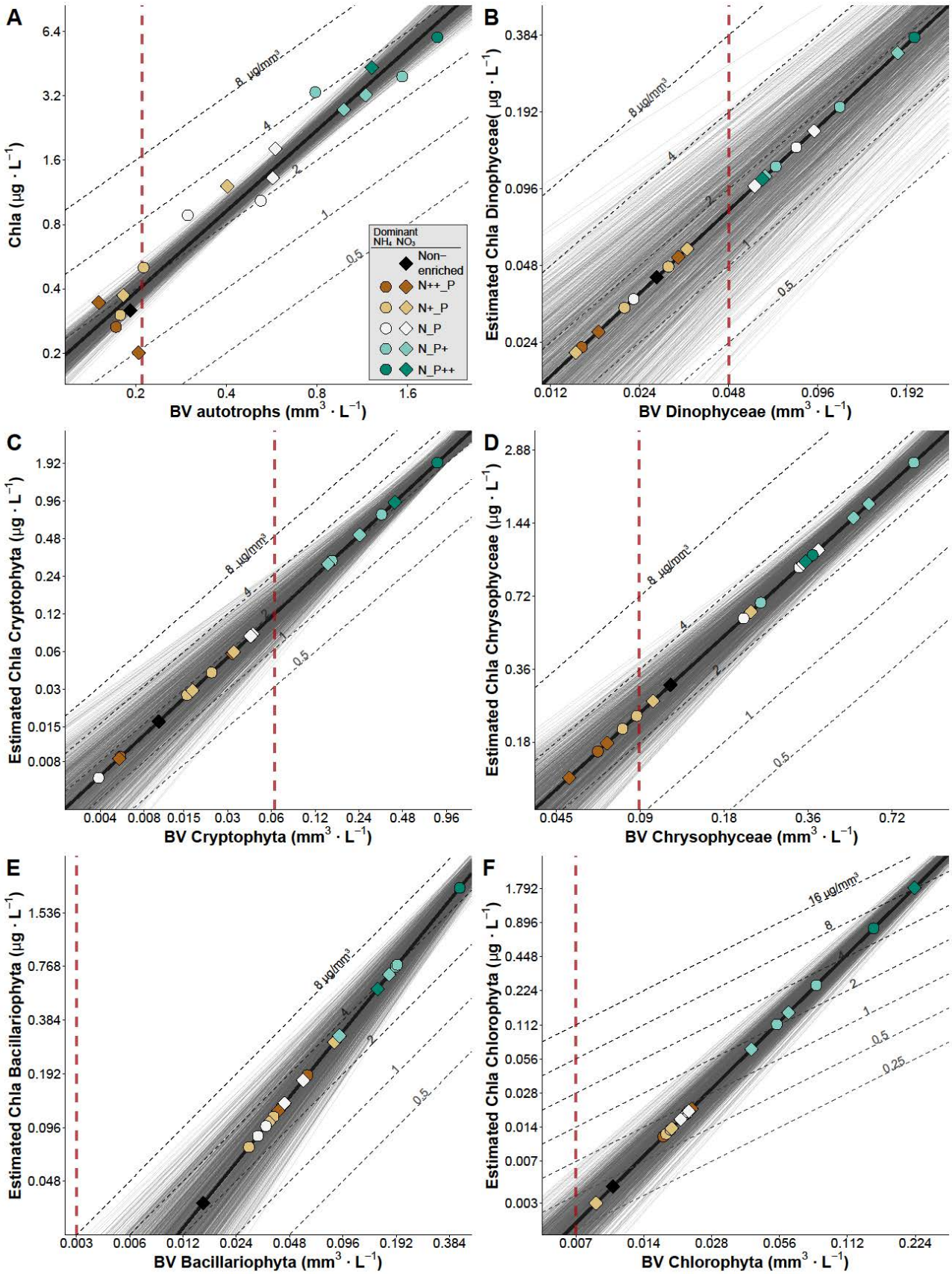


Figure 5 Relationships between the observed Chla and the BV of all autotrophs (A), and between the estimated Chla and the BV of the major algal groups (B, C, D, E, F) in log-log space. The vertical dashed red lines indicate the BV at the beginning of the experiment, and the ticks on axes represent duplications or divisions from this initial BV. Other specifications as in Fig. 2.

The estimated Chla content changed more importantly in Bacillariophyta (Fig. 5E), with Chla:BV ratios that increased from 2.3 μg/mm^3 when the growth was minimum (i.e. non-enriched mesocosm), up to 4.8 μg/mm^3 when it was maximum (i.e. N_P++ mesocosm enriched with NH_4^+). Even more marked was the increase of Chlorophyta, since the Chla:BV was below 1 μg/mm^3 under non-enriched, N_P, N+_P, and N++_P conditions, but increased up to 1.6-3.1 μg/mm^3 at N_P+, and further increased up to 5.4-8.1 μg/mm^3 at N_P++ (Fig. 5F).

Accessory pigment to Chla ratios

We calculated the accessory pigment to Chla ratios using the estimated parameters shown in Fig. 2 and Fig. 4. Since the exponents of the accessory pigments and the Chla models of an algal group were not necessarily the same, accessory pigment to Chla ratios could change depending on the BV (i.e. the growth) of that algal group. In the case of Cryptophyta, the exponents of Allo and Chla were the same, and, therefore, the estimated Allo:Chla ratio was constant at 0.59 (Fig. 6). In contrast, the exponent of Lut in chlorophytes was lower than the exponent of Chla, and, consequently, the Lut:Chla ratio tended to decline as growth increased (from 0.24 to 0.14). Most of the accessory pigment to Chla ratios tended to decline with growth, or remained practically unchanged along the BV range (Fig. 6). The only exception was the Fuco:Chla ratio of Chrysophyceae, which tended to increase as BV increased (from 0.41 to 0.54).

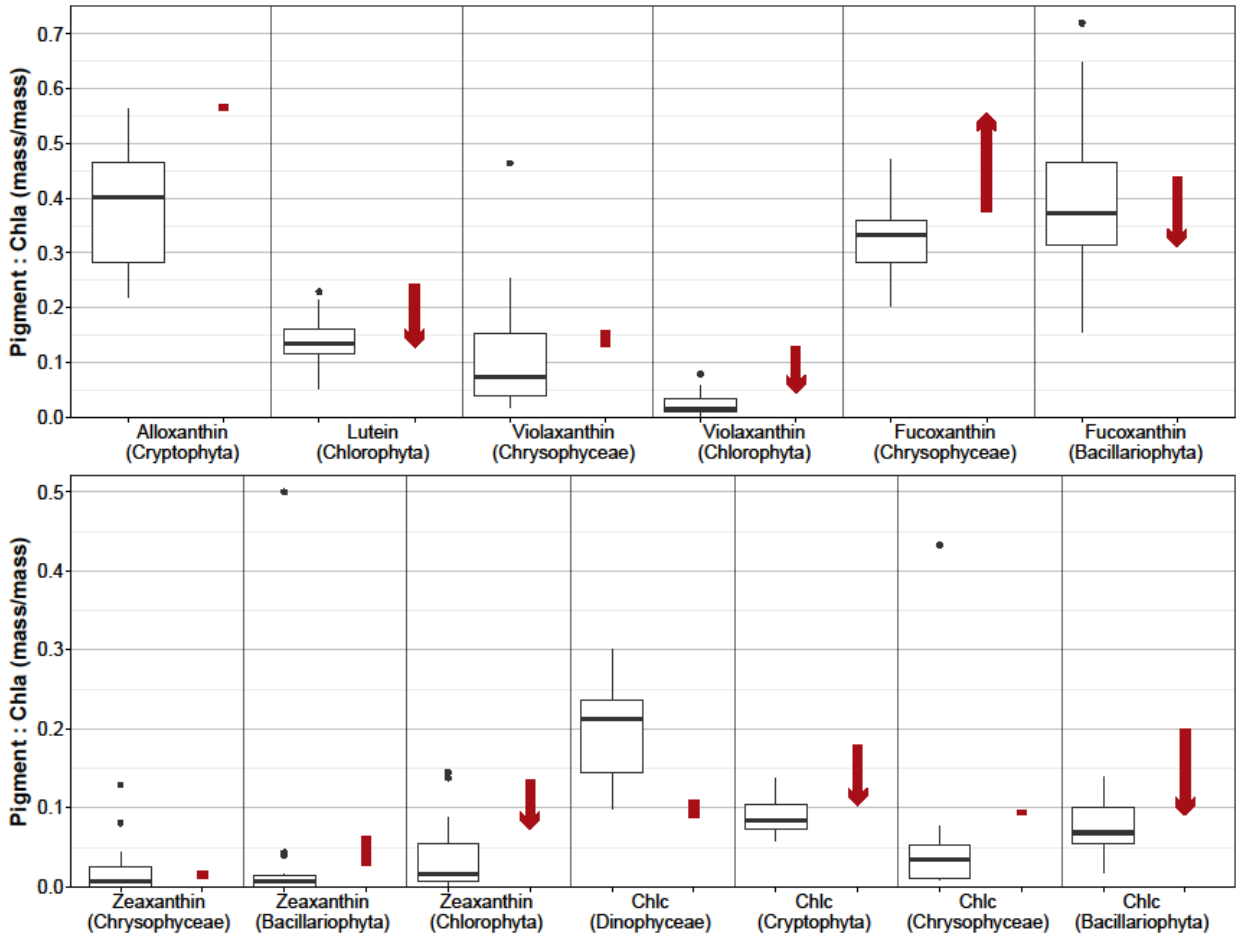


Figure 6 Accessory pigment to Chla ratios (mass/mass). Boxplots correspond to ratios reported in the literature and taken as a reference in this study (Schlüter *et al.*, 2006; Lauridsen *et al.*, 2011). Red ranges are the ratios estimated in this study, calculated with the means of parameters *k* and *a* shown in Fig. 1 and Fig. 3, and the minimum and maximum biovolumes observed for each algal group. When the exponents of accessory pigments and Chla were rather different, the pigment:Chla ratio depended on the BV of the algal group, what is represented as an arrow that goes from the minimum observed BV (lower growth) towards the maximum observed BV (higher growth).

Discussion

Effects of nutrient availability on phytoplankton growth

During the study period, P was limiting phytoplankton growth, what was evidenced by significant changes in the biovolume of all algal groups along the P enrichment gradient. However, not only P availability differentiated the biovolumes collected at the end of the experiment, but DIN availability also played a role. N enrichment (i.e. higher N:P imbalance) negatively affected the growth of Chrysophyceae, Dinophyceae and Cryptophyta, but barely affected the growth of Bacillariophyta

and Chlorophyta. One possible explanation might be that the concentrations of DIN added to the mesocosms (N+_P = 37μM, N++_P = 73uM) were toxic for the three mentioned groups (as already discussed in Chapter 2). In this case, the species of Bacillariophyta and Chlorophyta of lake Redon might be more tolerant to high NO_3^- and NH_4^+ concentrations. Collos & Harrison (2014) reviewed the effects of NH_4^+ on the growth of different algal groups and found that Chlorophyta are especially tolerant to high NH_4^+ concentrations. However, they also pointed out that an adverse effect of NH_4^+ on any microalgae species would be unlikely with field concentrations below 100 μM. We cannot discard the effect of other interactions to explain these results, but the toxic effect still seems the most parsimonious hypothesis.

The response to the most P-enriched condition also varied among phytoplankton groups. While the BV of Chlorophytes and Cryptophyta kept increasing at N_P++ respect to N_P+, the response of Chrysophyceae declined at N_P++, and Bacillariophyta and Dinophyceae only kept increasing when NH_4^+ was the dominant form of DIN. Phytoplankton taxa have evolved different capacities of nutrient utilization and growth (Litchman *et al.*, 2007), consequently covering the wide range of environmental conditions present in a lake. Indeed, the enhanced Chlorophyta growth at P-enriched conditions is consistent with a greater abundance of this group during the autumn mixing period (Felip *et al.*, 1999), when part of the P of the deepest layers and sediments distributes all over the water column. The abundance of Cryptophyta is proportionately higher at the deepest layers (Felip *et al.*, 1999), what could also be related to P availability given the results shown here. Bacillariophyta may take advantage of their high growth capacity during the spring bloom of phytoplankton, a short period before the stratification of the lake when the nutrient availability is already quite high. In contrast, Dinophyceae, the most representative group of summer stratification, might be more adapted to withstand nutrient scarcity, since it was the most insensitive group to nutrient enrichments. The limited response of Chrysophyceae -usually the dominant group in this lake- to

N_P++ conditions may respond to and adaptive trade-off, as having high competitive abilities for low nutrient concentrations may compromise the maximum growth capacity at high concentrations (Litchman *et al.*, 2007).

Chla content per BV and growth conditions

Chla content per biovolume of the entire phytoplankton community was previously estimated in Lake Redon both spatially and temporally (Felip & Catalan, 2000), giving ratios of 9.1 ± 5 μg/mm^3 (mean ± SD), and minimum and maximum ratios of 0.5 and 23.6 μg/mm^3, respectively. Therefore, the ratios presented here (1-4 μg/mm^3) were in the lower end of that range. This experiment included the upper layer of the water column (0-20 meters), while the previous observational study also considered situations where the light was limiting phytoplankton growth, and, hence, increasing the Chla content (e.g. deeper layers, ice-covered layers). If we only consider the most superficial layers and the ice-free period, the differences between both studies are much lower, and concentrated about the autumn phytoplankton bloom, when the Chla:BV ratios are higher (5-10 μg/mm^3) and chlorophytes are intensely growing. We observed that Chlorophyta was the phytoplankton group with the highest Chla content at the most P-enriched conditions (5-8 μg/mm^3), what may contribute to explain those ratios. Reynolds (1984, 2006) also reported comparatively high Chla:BV ratios for Chlorophyta species (4.5-20 μg/mm^3). Bacillariophyta also showed a relatively high Chla content at maximum growth conditions (≥ 4 μg/mm^3) in comparison to the other groups. The lowest Chla:BV ratio for Dinophyceae is also in agreement with a previous study in this lake that estimated the Chla of the groups using the CHEMTAX program (Buchaca *et al.*, 2005). Therefore, we notice that the groups that grew more intensely under P-replete conditions (e.g. Chlorophyta and Bacillariophyta) tended to show higher Chla contents per BV than the groups with lower growth (e.g. Dinophyceae). Indeed, greater capacity to absorb light energy may provide the chemical energy necessary to sustain high growth rates.

Interestingly, we obtained a good allometric relationship between Chla and the biovolume of all autotrophs, with a scaling exponent of 1.26 (i.e. the higher the algal growth, the higher the Chla:BV). In principle, if the different phytoplankton groups have different Chla:BV ratios, a change of the phytoplankton community structure could itself explain that change in Chla:BV ratios (e.g. a higher proportion of those algal groups with high Chla contents as P is more available). However, we found that allowing the Chla:BV of algal groups to change undoubtedly improved the fitting of the model. Indeed, none of all five scaling exponents was exactly estimated at 1, what would have mean constancy in the Chla content per BV. Although all groups shared the tendency to increase the Chla content at higher growth conditions, such tendency was stronger in Bacillariophyta, and, particularly, in Chlorophyta, than in the other groups. Within these two groups, the proportional increase of species with high Chla contents at P-enriched conditions could in principle explain the estimated exponents. However, the changes in the proportion of Chlorophyta and Bacillariophyta species do not seem enough to fully explain these exponents (Zufiaurre et al., *in prep.*), and, consequently, a change of the pigment ratios within the species (i.e. plasticity) is the most plausible explanation for our observations. Actually, 1.3- to 4.5-fold changes in Chla:BV ratios are reported between the exponential and the stationary phases of cultured phytoplankton species (Ruivo *et al.*, 2011), and even higher changes in the Chla content per cell of some species across gradients of N limitation (Goericke & Montoya, 1998). In contrast to the mentioned studies, our results suggest that the Chla content of Chlorophyta, and to lower extent also Bacillariophyta, are more plastic than the other groups, what may be convenient for life forms based on episodic and intense growth rates when the environmental conditions are appropriate.

With the only exception of the fucoxanthin of Chrysophyceae, the scaling exponent of the Chla was similar or higher than that of the accessory pigment. Therefore, in general, the accessory pigment to Chla ratios remained unchanged or decreased at high growth conditions, which is consistent with previous studies (Latasa &

Berdalet, 1994; Schlüter *et al.*, 2000; Henriksen *et al.*, 2002; Ruivo *et al.*, 2011). The increase of accessory pigment to Chla ratios from the exponential phase to the stationary phase has been interpreted as a change in the proportion between light harvesting complexes and reaction centres in the structure of photosystems (Ruivo *et al.*, 2011).

Methodological considerations

The CHEMTAX program (Mackey *et al.*, 1996) is, at the moment, the most widely used method to estimate the Chla of phytoplankton groups from accessory pigments. The initial weaknesses of the program such as its sensibility to the seed ratios could be apparently overcome providing several initial seed ratios and running the simulations successively (Latasa, 2007). A requirement of the method is that marker pigments to Chla ratios have to be relatively constant; otherwise, we should run predefined subsets of samples separately (e.g. if we suspect light is affecting the ratios, we may subset the samples by depth). However, in this study, the factor expected to influence the pigment to Chla ratios was growth in a gradient of nutrient limitation hardly separable in subsets. For this reason, we used a new method to estimate the Chla of phytoplankton groups based on fitting power law equations, using biovolumes as predictors and pigments as the response variable. The R package "brms" facilitated the estimation of the non-lineal equations and the incorporation of the prior knowledge (Bürkner, 2015). The possibility to fit models with multiple response variables would significantly improve the capabilities of this routine for chemotaxonomy purposes.

Concluding remarks

Our study evidenced that Chla content per BV increases when the system is released from P limitation. Chlorophyta and Bacillariophyta were the main responsible for this trend, although Chla content of the other phytoplankton groups also tended to increase at high growth conditions. Studies that use Chla as a proxy of phytoplankton biomass should be aware of this tendency, particularly those focused

on periods of high variability in growth conditions (e.g. during and after a phytoplankton bloom). Failure to consider that variation in Chla:BV may result in the overestimation of phytoplankton biomass during high growth conditions, and the underestimation during low growth conditions. Biovolume estimates of algal species are hardly found in most studies working with pigments, yet the pigment content per biovolume is valuable information to deepen in the regulatory processes occurring in phytoplankton.

General discussion

Experimental considerations

The eco-physiological responses of major algal groups to external nutrient availability in oligotrophic freshwater ecosystems is the leitmotif of this dissertation. The change of nutrient availability triggers a number of physiological processes in algae to acclimate to new conditions. As internal N and P availability constrains fundamental processes of organisms, such as the synthesis of proteins or nucleic acids, this physiological re-organization often implies the alteration of the growth rate. The response period of 21-25 days in our experiments was long enough to allow the differentiation of algal biomass among treatments and species, and, at the same time, it was short enough to avoid a significant influence of experimental side-effects.

Although algal cells can regulate their internal functioning depending on the features of their surrounding environment, such plasticity has certain limits, and each genotype is specifically adapted to a range of nutrient concentrations. The influence of nutrient availability on algal growth is generally described by a Michaelis-Menten curve (Fig. 4 in general introduction) so that growth is stimulated up to certain nutrient concentrations, and, then, the response tends to saturate (i.e. the specific nutrient no longer "limits" the algal growth). In some cases, this saturation phase is followed by a new phase where growth rate decreases due to harmful effects of nutrient excess (Fairchild *et al.*, 1985). In our mesocosms experiment, for instance, the highest growth of chrysophyceans was not observed at the most P-enriched conditions, but at intermediate P enrichments (Fig. 1 in chapter 3). This kind of response could not have been detected if we had designed the experiment with discrete treatments of nutrient excess, such as in the case of four treatments (control, N, P, N+P) applied to nutrient-diffusing substrates (NDS). Given that growth inhibition by nutrient excess may be more common than we previously expected, an experimental design based on nutrient enrichment gradients appears more convenient to unveil the eco-physiological responses of algal taxa accurately. This approach of enrichment gradients is, at the moment, not so

widespread in the research field of nutrient enrichment bioassays (Tank & Dodds, 2003; Marcarelli & Wurtsbaugh, 2007; Elser *et al.*, 2009; Smith *et al.*, 2009). Although using enrichment gradients represent an extra cost of sampling and analysis, given the adverse effects produced by not sufficiently adequate nutrient concentrations, it seems worth paying this cost. Future studies with benthic substrates should consider applying experimental designs based on gradients of nutrient availability, as well as performing relatively small alterations of nutrient levels, especially in oligotrophic systems. The amounts of nutrients added to NDS should be in accordance with the basal concentrations of the site instead of using the same enrichments everywhere, which is the typical practice (Tank *et al.*, 2006).

The adverse effects caused by nutrient enrichments lead us to ask how it is possible *too much of a good thing*. This question has been already formulated in the case of grazers, as multiple observations indicate that P excess in the elemental content of autotrophs has a negative effect on their growth (Boersma & Elser, 2006; Hessen *et al.*, 2013). The physiological basis involved in the impaired performance of algae (and other organisms) under nutrient excess is still not well understood. In principle, adverse effects may come from higher metabolic costs of maintaining the internal homeostasis (e.g. increased excretion, pH control), or from overt toxicity (e.g. oxidizing radicals). Collos & Harrison (2014) reviewed observations of ammonium toxicity in unicellular algae, and noticed that tolerance to toxic levels varied considerably among algal groups. Chlorophytes are especially tolerant to high ammonium levels, and, likely, also to nitrate and phosphate considering the results of this dissertation. The growth of phytoplankton diatoms was not negatively affected by N excess (Fig. 1 in chapter 3). Contrastingly, the growth of periphytic diatoms was clearly inhibited by N, likely because nutrient enrichments in NDS were higher than in mesocosms. The decline of algal biomass at N++_P and N+_P treatments in relation to N_P was driven by chrysophyceans, cryptophytes, and dinoflagellates, which appear to be the least tolerant groups to high N concentrations. Dinoflagellates were also particularly susceptible to high ammonium concentrations (Collos & Harrison, 2014).

Physiological acclimation to nutrient availability

Algae can acclimate to changes in nutrient availability up- or down-regulating several physiological traits and processes, such as the nutrient uptake machinery (e.g. membrane transporters and ecto-phosphatases), the nutrient stores, the transcriptional machinery, or the metabolic activity (Fig. 5 in general introduction). In this dissertation, we have focused in two of them: the C:N:P content, and the chlorophyll a (Chla) content per biovolume.

One of the most interesting results of this thesis has been the evidence that the seston N:P ratio can abruptly approach the Redfield ratio at a certain P supply, in line with the transition from oligotrophic to eutrophic conditions (Fig. 7 in chapter 2). This result indicates that the regulation of N:P content does not follow DIN:TDP in a linear and gradual way; rather, there might be preferential "homeostatic states", and stepwise transitions among them. Future studies should characterize this transition in more detail: first, including DIN:TDP treatments in between our N_P+ and N_P++ treatment concentrations, and also beyond the N_P++ levels (lower DIN:TDP ratios); and second, performing similar DIN:TDP gradients with different DIN and TDP absolute concentrations, so as to disentangle the strict "DIN:TDP" effect, from the axis "deficiency – no deficiency" associated with growth. Actually, both factors are known to affect the N:P content of phytoplankton (Klausmeier *et al.*, 2008), being the first more associated with the regulation of nutrient stores and non-structural components, and the second with the regulation of structural components such as the proportion of proteins to ribosomes (e.g. the Growth Rate Hypothesis, Sterner & Elser, 2002). The relative influence of both factors in explaining algal N:P variability is a question under current discussion (Hillebrand *et al.*, 2013). A recent study suggests that the observed phytoplankton N:P ratios cannot be explained uniquely considering the rRNA:protein allocation and additional contributions from phospholipids and P storage compounds may also play a role (Daines *et al.*, 2014). Thus, understanding the regulatory mechanisms used by algae to acclimate and adapt to changes in P availability proves to be an

interesting direction for future research (Lin *et al.*, 2016).

Determining the N:P content of single algal groups in periphyton or plankton is unfeasible with the current techniques. Contrastingly, we were able to estimate the Chla contents per biovolume (BV) of major phytoplankton groups, and evaluate its plasticity depending on growth conditions. We observed that Chla content per BV tended to increase with P availability and growth. All algal groups showed this pattern, but it was more marked in diatoms, and, especially, in chlorophytes. The ability to increase the Chla content per cell when nutrient conditions are favourable may allow these groups to increase the chemical energy available for metabolic reactions, and, ultimately, for achieving high growth rates (Geider *et al.*, 1997, 1998). Thus, our results suggest the greater plasticity of chlorophytes and diatoms is an adaptation that allows them to rapidly rearrange the photosynthetic capacity to obtain fast growth rates when nutrient availability increases.

A great leap forward in the understanding of the eco-physiological regulation of organisms is expected with the development of meta'omic approaches: metagenomics, metatranscriptomics, metaproteomics, and metametabolomics. These methods are rapidly progressing, but the experimental and computational techniques have not attained a standardized pipeline yet (Segata *et al.*, 2013). It is also true that many genomic and transcriptomic data cannot be related to known physiological functions at the moment. Nevertheless, the potential shown by these techniques, and the rapid development, seem to indicate that these drawbacks may be overcome in the next few years. For instance, comparative metatranscriptomics has been satisfactorily used to understand changes in gene expression of phytoplankton after an iron-enrichment (Marchetti *et al.*, 2012). Metabolomics open the opportunity to determine how macromolecular components affect the elemental stoichiometry (or other physiological traits) of organisms in response to changing environmental conditions (Rivas-Ubach *et al.*, 2012). These techniques may also help to understand how the evolutionary pressures in diminishing the requirements for limiting elements are coded in genomes (Elser *et al.*, 2011).

Growth of the major algal groups

The response of total algal biomass to nutrient enrichments is an integration of the multiple physiological responses of the initial pool of organisms, which, in our case, were adapted and acclimated to low nutrient concentrations. Species of an algal group may obviously share many physiological traits due to their common past; but, beyond this phylogenetical imprint, species have evolved and adapted to different environmental conditions. A recent compilation of phytoplankton communities in European lakes showed that Cyanobacteria are typical of eutrophic and hypereutrophic conditions, Chrysophyceae are found in oligotrophic conditions, while Cryptophyceae, Chlorophyceae, and Bacillariophyceae show a wider range of distribution along the trophic status gradient (Phillips *et al.*, 2013). Chlorophytes and cyanobacteria are known to generate the highest algal blooms under eutrophic and hypereutrophic conditions, the latter particularly when N:P availability is rather low, and N-fixation is favourable (Vrede *et al.*, 2009). Given that the initial pool of cyanobacteria in Lake Redon is negligible (Felip *et al.*, 1999), we did not expect a noticeable response of this group in the mesocosms. We did expect a response of cyanobacteria to NDS because they are abundant in the epilithic communities of mountain lakes (Bartrons *et al.*, 2012). However, in view of our results and previous studies, it seems that this group would appear at later phases of the periphyton colonization process (Korte & Blinn, 1983; Sekar *et al.*, 2004). Chlorophytes were in low abundances at the beginning of mesocosms experiment but increased markedly with P supply (Fig. 1 in chapter 3). This result is in agreement with previous P enrichments in mountain lakes (Gardner *et al.*, 2008). The growth of periphytic chlorophytes was also stimulated by nutrient-enriched substrates when water renewal was high. Therefore, chlorophytes may present the ability to grow fast when nutrient availability is sufficiently high (Fig. 4 in general introduction). The biovolume of planktonic diatoms increased even more than planktonic chlorophytes, and may also have high growth capacities at the expense of high nutrient requirements. Dinoflagellates may be the opposite case, as their growth was the least stimulated by P additions. This group could then achieve optimal growth

rates at relatively low nutrient supplies. Cryptophytes may require relatively high P concentrations to grow fast, as their biovolumes notably increased from low to high P availability. In contrast, chrysophyceans were proportionally more stimulated at low and intermediate P additions, and the response saturated (or even declined) at high P supply. Then, chrysophyceans may be able to attain relatively high growth rates with rather low nutrient concentrations. Which are the physiological, ecological and evolutionary bases for these empirical patterns constitute an exciting field for coming research.

Temporal patterns of phytoplankton composition and nutrient cycling

The responses of phytoplankton groups to nutrient enrichments were consistent with the seasonal patterns of phytoplankton composition in Lake Redon (Felip, 1997; Felip *et al.*, 1999). Planktonic diatoms are poorly represented in Lake Redon, and their biovolumes only occur in significant numbers during the mixing periods. Chlorophytes are also characteristic of mixing periods, especially of autumn, when they can achieve quite high biovolumes, and co-dominate the phytoplankton community. The competitive abilities of these two groups may allow them to take advantage of nutrient pulses during the mixing periods, but strongly decline when P availability is low. Cryptophytes often co-dominate the phytoplankton biomass during -or close to- the mixing periods at deep layers (>45 meters). P availability is there relatively high due to the proximity to sediments, what may explain their sensitivity to P enrichments. Although chrysophyceans are also responsible for spring and autumn phytoplankton blooms, it is during the months of July and August when their dominance is more accentuated due to the decline of other phytoplankton groups. Dinoflagellates are also relevant during summer months, particularly in the epilimnetic layer. Then, these two last groups may be the better adapted to cope with low-P stress.

The seasonal ecosystem dynamics of Lake Redon is highly-conditioned by weather conditions and physical processes that determine, among others, the intensity of the mixing events, and the duration of ice cover and water stratification (Catalan, 1988). In 2012, we monitored Lake Redon from the ice-cover melting to the establishment of summer stratification (Fig. 1). Just after the ice-cover melting, the water column became totally mixed; some sediments were resuspended, and the deep nutrient-rich waters were mixed throughout the water column (Fig. 1D). During this period phytoplankton has to deal with rapidly changing light conditions, going from light excess at the surface layers to light limitation at the deep layers. Thus, the phytoplankton bloom generally occurs at the very beginning of thermal stratification. In our monitoring, the phytoplankton bloom was observed at a depth of ~20 m, June 20th (Fig. 1C), associated with an increase in primary production, and a decline of seston $\delta\ ^{13}C$ (Fig. 1D). Simultaneously, the concentrations of total phosphorus (but not total nitrogen) peaked at the deepest layers (Fig. 1F). This peak was in the particulate fraction, and may be associated with the sedimentation of mineral particles, detritus, and organisms, although we cannot discard a certain resuspension of sediments. The pattern of some pigments, such as the diatoxanthin (marker pigment of diatoms), suggests that the sedimentation of organisms could be important in originating this peak (Fig. 1E), though this point should be confirmed in future studies.

In any case, after two weeks, the thermocline was already entirely constituted, TP concentrations had declined throughout the water column, and seston was clearly P-impoverished (Fig. 1G-H). This tendency towards increased P deficiency as summer advanced might have influenced the great response of phytoplankton in P-enriched mesocosms (installed in August 2013). The decline of TP during summer stratification was not linked to a similar decline of TN, what may be in agreement with the observation of mesocosms that particulate matter recovered from sediment traps was P-enriched and N-impoverished in relation to seston. This evidence suggests that N recycling in the water column may be more efficient than P

recycling, in agreement with previous studies of sedimentation in lakes (Elser & Foster, 1998). The mechanisms driving this process are not deeply understood, but, apparently, N-containing compounds may be more soluble, and rapidly released from dead organisms than certain P-containing compounds (e.g. polyphosphates) (Diaz *et al.*, 2008). Moreover, adsorption of P onto mineral surfaces (e.g. iron oxides) and cell membranes could also be relevant in promoting this N:P cycling imbalance (Fu *et al.*, 2005; Lin *et al.*, 2016).

Although not contemplated in our short-term nutrient enrichments, grazers are key determinants of nutrient regeneration at somewhat larger temporal scales. The dynamic relationship between autotrophs and grazers often lead to two big scenarios (Hessen *et al.*, 2013): periods of high grazing pressures and rapid nutrient regeneration maintain autotrophic biomass at low levels, but this biomass is of high quality (e.g., low C:P) and grow fast; conversely, periods of low grazing pressures and slow nutrient recycling are associated with high autotrophic biomass of low quality (e.g., high C:P). Then, grazers are mainly controlled by the "quantity" of autotrophic biomass in the first case, and by the "quality" in the second (Sommer, 1992). Zooplankton species of Lake Redon typically reproduce only once per year (Ventura & Catalan, 2005); as a consequence, their capacity to control the seasonal patterns of phytoplankton is limited, and their responses always come with a temporal delay. Given that major zooplankton groups have varying nutrient requirements (e.g. cladocerans are P-rich in comparison to copepods, Ventura & Catalan, 2005), changes in zooplankton structure (and, hence, in nutrient cycling) can induce trophic cascades and shift nutrient limitation of autotrophs (Sterner *et al.*, 1992).

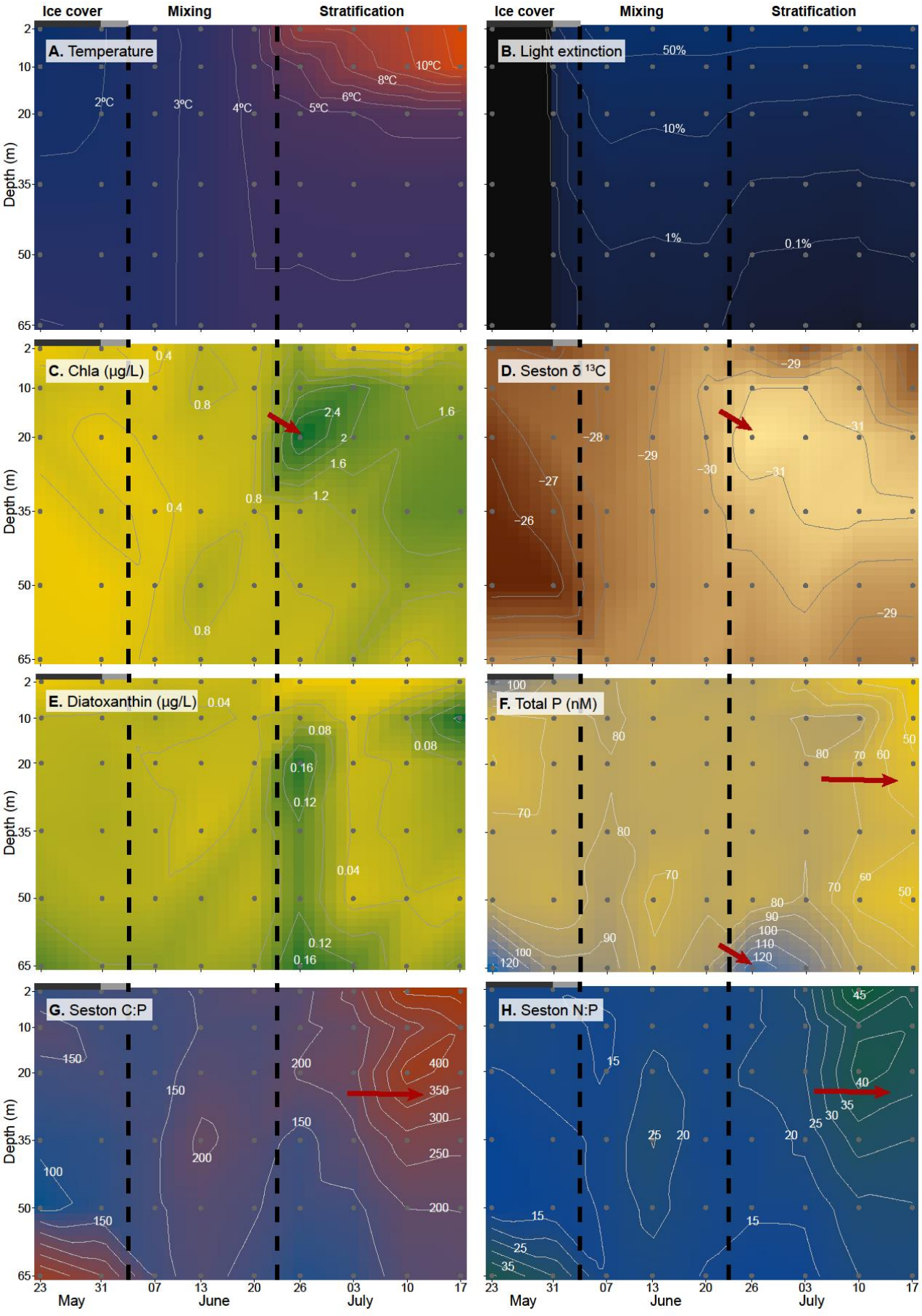


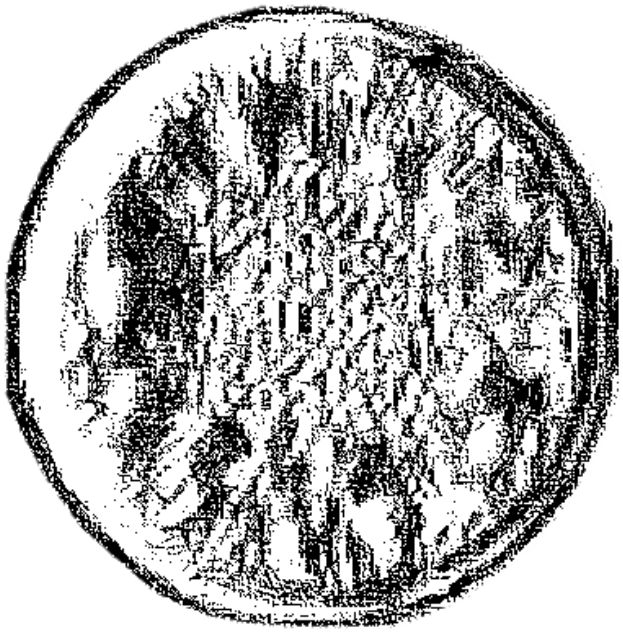
Figure 1 Representative variables of the period going from the ice-cover melting until the plenty establishment of summer stratification in Lake Redon (May 27 – July 17, 2012).

Nutrient availability and productivity in Pyrenean lakes

The beginning of this thesis coincided with the recognition that DIN levels in Pyrenean lakes had markedly declined so that average concentrations in 1987 reduced by half in 2011 (Camarero & Catalan, 2012). According to the Chla:TP to DIN relationship, the lake district of Pyrenees was on the boundary between the N- and P-limitation domains at that moment, and, hence, the continued decrease in DIN could lead these ecosystems towards N-limitation. Thus, a notable response of algal growth to N enrichments was initially expected in our experiments. Our results showed that phytoplankton growth in Lake Redon was constrained by P, and periphytic algal growth in other lakes was also higher in P- than N-enriched substrates. Although N deficiency may condition algal growth in next future, the productivity still seems to be mainly controlled by P in the study lakes, and, likely, in many lakes of the Pyrenees.

Atmospheric P deposition then arises as a major driver of productivity variation in these lakes, as occurs in several mountain lake districts around the planet (Brahney *et al.*, 2015). Here, atmospheric P deposition is primarily related to certain weather conditions that favour dust transport from northern Africa and Iberian Peninsula (Camarero & Catalan, 2012). Land uses and climate change could accentuate the severity of droughts, soil erosion, and desertification in these regions (Rodriguez-Lloveras *et al.*, 2016). Therefore, increased dust emissions can be reasonably expected in the near future, as well as a higher risk of wildfires and combustion-derived P emissions (Pausas, 2012). Higher P deposition may enhance the algal growth and N consumption in lakes, thus lowering DIN availability in waters (Camarero & Catalan, 2012). Moreover, higher P availability and productivity could also stimulate denitrification and N export in sediments (Finlay *et al.*, 2013). Although N emissions notably exceeded P emissions during the 20th century, current N emissions are stabilized or even declining in Europe and North America (Monks *et al.*, 2009).

Taken together all this evidence suggests that Pyrenean lakes could become more productive, and present lower N concentrations in next years. This prediction is naturally conditioned by climate change, as well as by several smaller-scale processes and interactions within the ecosystems, such as the whole-ecosystem N:P recycling, the re-organization of community structures, and the regulation of the C:N:P composition of the organisms. In this future scenario, our results suggest that chlorophytes, diatoms, and cryptophytes may be particularly favoured in plankton, while chlorophytes should increase in periphyton. Higher growth rates may increase the Chla content, and decrease the N:P and C:P ratios of autotrophs. In turn, these changes may alter grazers community (e.g. a hypothetical increase of cladocerans), the nutrient cycling, and, hence, the matter and energy fluxes of these ecosystems.



Conclusions

1. Water renewal has a significant positive effect on the colonization of epilithic periphyton in mountain lakes. Water renewal facilitated diatoms colonization but does not affect chlorophytes colonization. Lake trophic status (i.e. DOC) is the second most influential factor in the algal colonization.

2. High N:P availability tend to favour chlorophytes over diatoms in the colonization of periphyton in oligotrophic lakes.

3. Experiments with mesocosms and artificial substrates in oligotrophic lakes should apply gradients of nutrient availability rather than "saturating concentrations". The usual nutrient levels added in bioassays can produce adverse effects. Chlorophytes are the algal group that better tolerate high nutrient levels.

4. The response of productivity, community structure, and seston C:N:P to increasing P availability is not strictly coherent in oligotrophic conditions. Productivity is more sensitive to low and intermediate P additions, while seston stoichiometry is only markedly changed under high P supply.

5. The P-enrichment lead seston C:N:P toward Redfield proportions in a non-linear way. This shift was more associated with a change in the regulation of phytoplankton than with a reorganization of the community structure. We suggest alternative homeostatic states as the response of phytoplankton to a continuum of nutrient availability.

6. High NH_4 availability tends to increase seston C:P and N:P ratios. It can also stimulate productivity and alter phytoplankton structure when P stress diminishes.

7. Diatoms and chlorophytes are especially favoured by N and P pulses in deep mountain lakes due to their high growth capacity. The other phytoplankton groups are also stimulated by P enrichment, but to a lower extent. Cryptophytes may present comparatively higher P requirements than chrysophyceans and dinoflagellates.

8. The high growth capacity of diatoms and chlorophytes could be associated with their ability to increase the chlorophyll content per biovolume when nutrient supply increases.

References

Archibald JM (2015) Genomic perspectives on the birth and spread of plastids. *PNAS*, **112**, 10147–10153.

Arrigo KR (2005) Marine microorganisms and global nutrient cycles. *Nature*, **437**, 349–355.

Azam F, Malfatti F (2007) Microbial structuring of marine ecosystems. *Nature Reviews Microbiology*, **5**, 782–791.

Barranguet C, Veuger B, Van Beusekom SAM, Marvan P, Sinke JJ, Admiraal W (2005) Divergent composition of algal-bacterial biofilms developing under various external factors. *European Journal of Phycology*, **40**, 1–8.

Bartron K (2009) MuMIn: multi-model inference. R package version 1.10.5. URL: http://cran.r-project.org/package=MuMIn.

Bartrons M, Catalan J, Casamayor EO (2012) High bacterial diversity in epilithic biofilms of oligotrophic mountain lakes. *Microbial Ecology*, **64**, 860–869.

Bates D, Maechler M, Bolker B, Walker S (2014) lme4: Linear mixed-effects models using Eigen and S4. R package version 1.1-7. URL: http://cran.r-project.org/package=lme4.

Berg GM, Balode M, Purina I, Bekere S, Béchemin C, Maestrini SY (2003) Plankton community composition in relation to availability and uptake of oxidized and reduced nitrogen. *Aquatic Microbial Ecology*, **30**, 263–274.

Bergström A-K, Jansson M (2006) Atmospheric nitrogen deposition has caused nitrogen enrichment and eutrophication of lakes in the northern hemisphere. *Global Change Biology*, **12**, 635–643.

Berman T, Bronk DA (2003) Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. *Aquatic Microbial Ecology*, **31**, 279–305.

Bernhardt ES, Likens GE (2004) Controls on periphyton biomass in heterotrophic streams. *Freshwater Biology*, **49**, 14–27.

Biggs BJF (1996) Patterns in benthic algae of streams. In: *Algal ecology: freshwater benthic ecosystems* (eds Stevenson RJ, Bothwell ML, Lowe RL), pp. 31–56. Academic Press, San Diego, CA.

Boersma M, Elser JJ (2006) Too much of a good thing: on stoichiometrically balanced diets and maximal growth. *Ecology*, **87**, 1325–1330.

Borcard D, Gillet F, Legendre P (2011) *Numerical ecology with R*. Springer.

Bracken MES, Hillebrand H, Borer ET et al. (2015) Signatures of nutrient limitation and co-limitation: responses of autotroph internal nutrient concentrations to nitrogen and phosphorus additions. *Oikos*, **124**, 113–121.

Brahney J, Mahowald N, Ward DS, Ballantyne AP, Neff J (2015) Is atmospheric phosphorus pollution altering global alpine lake stoichiometry? *Global Biogeochemical Cycles*, **29**.

Buchaca T (2005) *Pigments indicadors: estudi del senyal en estanys dels Pirineus i de la seva aplicació en paleolimnologia. (PhD thesis)*. University of Barcelona.

Buchaca T, Felip M, Catalan J (2005) A comparison of HPLC pigment analyses and biovolume estimates of phytoplankton groups in an oligotrophic lake. *Journal of Plankton Research*, **27**, 91–101.

Bürkner P-C (2015) brms : An R package for Bayesian Generalized Linear Mixed Models using Stan. URL: https://cran.r-project.org/web/packages/brms/ vignettes/brms.pdf.

Camarero L, Felip M, Ventura M, Bartumeus F, Catalan J (1999) The relative importance of the planktonic food web in the carbon cycle of an oligotrophic mountain lake in a poorly vegetated catchment (Redó, Pyrenees). *Journal of Limnology*, **58**, 203–212.

Camarero L, Catalan J (2012) Atmospheric phosphorus deposition may cause lakes to revert from phosphorus limitation back to nitrogen limitation. *Nature Communications*, **3**, 1118.

Cambra J (2003) *Algues del Parc Nacional d'Aigüestortes i Estany de Sant Maurici* (ed P. N. d'Aigüestortes i Estany de Sant Maurici).

Canfield DE, Glazer AN, Falkowski PG (2010) The evolution and future of Earth's nitrogen cycle. *Science*, **330**, 192–196.

Cantonati M, Lowe RL (2014) Lake benthic algae: toward an understanding of their ecology. *Freshwater Science*, **33**, 475–486.

Capblancq J, Catalan J (1994) Phytoplankton : which, and how much? In: *Limnology Now: A Paradigm of Planetary Problems* (ed Margalef R). Elsevier Science, New York.

Catalan J (1988) Physical properties of the environment relevant to the pelagic ecosystem of a deep high-mountain lake (Estany Redó , Central Pyrenees). *Oecologia Aquatica*, 89–123.

Catalan J, Ventura M, Brancelj A et al. (2002) Seasonal ecosystem variability in remote mountain lakes: implications for detecting climatic signals in sediment records. *Journal of Paleolimnology*, **28**, 25–46.

Catalan J, Camarero L, Felip M et al. (2006) High mountain lakes : extreme habitats and witnesses of environmental changes. *Limnetica*, **25**, 551–584.

Catalan J, Barbieri MG, Bartumeus F et al. (2009a) Ecological thresholds in European alpine lakes. *Freshwater Biology*, **54**, 2494–2517.

Catalan J, Curtis CJ, Kernan M (2009b) Remote European mountain lake ecosystems: regionalisation and ecological status. *Freshwater Biology*, **54**, 2419–2432.

Catalan J, Pla-Rabés S, Wolfe AP et al. (2013) Global change revealed by palaeolimnological records from remote lakes: a review. *Journal of Paleolimnology*, **49**, 513–535.

Collos Y, Harrison PJ (2014) Acclimation and toxicity of high ammonium concentrations to unicellular algae. *Marine Pollution Bulletin*, **80**, 8–23.

Collos Y, Vaquer A, Souchu P (2005) Acclimation of nitrate uptake by phytoplankton to high substrate levels. *Journal of Phycology*, **41**, 466–478.

Daines SJ, Clark JR, Lenton TM (2014) Multiple environmental controls on phytoplankton growth strategies determine adaptive responses of the N:P ratio. *Ecology Letters*, **17**, 414–425.

Dentener F, Drevet J, Lamarque JF et al. (2006) Nitrogen and sulfur deposition on regional and global scales: a multimodel evaluation. *Global Biogeochemical Cycles*, **20**.

Descamps-Julien B, Gonzalez A (2005) Stable coexistence in a fluctuating environment : an experimental demonstration. *Ecology*, **86**, 2815–2824.

Diaz J, Ingall E, Benitez-Nelson C, Paterson D, de Jonge MD, McNulty I, Brandes JA (2008) Marine polyphosphate: a key player in geologic phosphorus sequestration. *Science*, **320**, 652–655.

Donald DB, Bogard MJ, Finlay K, Bunting L, Leavitt PR (2013) Phytoplankton-specific response to enrichment of phosphorus-rich surface waters with ammonium, nitrate, and urea. *PloS one*, **8**, e53277.

Dortch Q (1990) The interaction between ammonium and nitrate uptake in phytoplankton. *Marine Ecology Progress Series*, **61**, 183–201.

Droop MR (1973) Some thoughts on nutrient limitation in algae. *Journal of Phycology*, **9**, 264–272.

Durand P, Breuer L, Johnes PJ (2011) Nitrogen processes in aquatic ecosystems. In: *The European nitrogen assessment* (eds A.Sutton M, Howard CM, Erisman JW, Billen G, Bleeker A, Grennfelt P, Grinsven H van, Grizzetti B). Cambridge University Press.

Edmondson WT (1970) Phosphorus, nitrogen, and algae in Lake Washington after diversion of sewage. *Science*, **169**, 690–691.

Edwards KF, Klausmeier C A, Litchman E (2011) Evidence for a three-way trade-off between nitrogen and phosphorus competitive abilities and cell size in phytoplankton. *Ecology*, **92**, 2085–2095.

Edwards KF, Thomas MK, Klausmeier CA, Litchman E (2012) Allometric scaling and taxonomic variation in nutrient utilization traits and maximum growth rate of phytoplankton. *Limnology and Oceanography*, **57**, 554–566.

Elrifi IR, Turpin DH (1985) Steady-state luxury consumption and the concept of optimum nutrient ratios: a study with phosphate and nitrate limited *Selenastrum minutum* (Chlorophyta). **21**, 592–602.

Elser JJ, Foster DK (1998) N:P stoichiometry of sedimentation in lakes of the Canadian shield: relationships with seston and zooplankton elemental composition. *EcoScience*, **5**, 56–63.

Elser JJ, Bracken MES, Cleland EE et al. (2007) Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecology Letters*, **10**, 1135–1142.

Elser JJ, Andersen T, Baron JS et al. (2009) Shifts in lake N:P stoichiometry and nutrient limitation driven by atmospheric nitrogen deposition. *Science*, **326**, 835–837.

Elser JJ, Acquisti C, Kumar S (2011) Stoichiogenomics: the evolutionary ecology of macromolecular elemental composition. *Trends in Ecology & Evolution*, **26**, 38–44.

Fairchild G. W, Lowe RL, Richardson WB. (1985) Algal periphyton growth on nutrient-diffusing substrates : an in situ bioassay. *Ecology*, **66**, 465–472.

Falkowski PG (2000) Rationalizing elemental ratios in unicellular algae. *Journal of Phycology*, **36**, 3–6.

Falkowski PG (2006) Tracing oxygen's imprint on Earth's metabolic evolution. *Science*, 1724–1725.

Falkowski PG, LaRoche J (1991) Acclimation to spectral irradiance in algae. *Journal of Phycology*, **27**, 8–14.

Falkowski PG, Katz ME, Knoll AH, Quigg A, Raven JA., Schofield OM, Taylor FJR (2004) The evolution of modern eukaryotic phytoplankton. *Science*, **305**, 354–360.

Falkowski PG, Knoll AH (2007) *Evolution of primary producers in the sea*. Elsevier Academic Press.

Falkowski P, Raven JA (2007) *Aquatic photosynthesis*, 2nd edn. Princeton University Press, New Jersey, USA.

Fehling J, Stoecker D, Baldauf SL (2007) Photosynthesis and the eukaryote tree of life. In: *Evolution of primary producers in the sea* (eds Falkowski PG, Knoll AH). Elsevier Academic Press.

Felip M (1997) *Ecologia del microplàncton d'un estany profund d'alta muntanya (Redó, Pirineus). (PhD thesis)*. University of Barcelona.

Felip M, Bartumeus F, Halac S, Catalan J (1999) Microbial plankton assemblages, composition and biomass, during two ice-free periods in a deep high mountain lake (Estany Redó, Pyrenees). *Journal of Limnology*, **58**, 193–202.

Felip M, Catalan C (2000) The relationship between phytoplankton biovolume and chlorophyll in a deep oligotrophic lake: decoupling in their spatial and temporal maxima. *Journal of Plankton Research*, **22**, 91–105.

Feminella JW, Hawkins CP, Benthological A, Dec N (1995) Interactions between stream herbivores and periphyton: a quantitative analysis of past experiments. *Journal of the North American Benthological Society*, **14**, 465–509.

Finlay JC, Small GE, Sterner RW (2013) Human influences on nitrogen removal in lakes. *Science*, **342**, 247–250.

Flynn KJ, Raven JA., Rees TAV., Finkel ZV, Quigg A, Beardall J (2010) Is the Growth Rate Hypothesis applicable to microalgae? *Journal of Phycology*, **46**, 1–12.

Fowler D, Coyle M, Skiba U et al. (2013) The global nitrogen cycle in the twenty-first century. *Philosophical Transactions of the Royal Society of London B*, **368**.

Francoeur SN (2001) Meta-analysis of lotic nutrient amendment experiments : detecting and quantifying subtle responses. *Journal of the North American Benthological Society*, **20**, 358–368.

Frigstad H, Andersen T, Hessen DO, Naustvoll LJ, Johnsen TM, Bellerby RGJ (2011) Seasonal variation in marine C:N:P stoichiometry: can the composition of seston explain stable Redfield ratios? *Biogeosciences*, **8**, 2917–2933.

Fu F-X, Zhang Y, Leblanc K, Sañudo-Wilhelmy SA, Hutchins DA (2005) The biological and biogeochemical consequences of phosphate scavenging onto phytoplankton cell surfaces. *Limnology and Oceanography*, **50**, 1459–1472.

Galloway JN, Townsend AR, Erisman JW et al. (2008) Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science*, **320**, 889–892.

Gardner EM, Mcknight DM, Lewis Jr WM, Miller MP (2008) Effects of nutrient enrichment on phytoplankton in an alpine lake , Colorado, USA. *Arctic, Antarctic, and Alpine Research*, **40**, 55–64.

Geider RJ, Macintyre HL, Kana TM (1997) Dynamic model of phytoplankton growth and acclimation: responses of the balanced growth rate and the chlorophyll a:carbon ratio to light, nutrient-limitation and temperature. *Marine Ecology Progress Series*, **148**, 187–200.

Geider RJ, Maclntyre HL, Kana TM (1998) A dynamic regulatory model of phytoplanktonic acclimation to light, nutrients, and temperature. *Limnology and Oceanography*, **43**, 679–694.

Geider RJ, La Roche J (2002) Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *European Journal of Phycology*, 1–17.

Gelman A, Hill J (2007) *Data analysis using regression and multilevel/hierarchical models*. Cambridge University Press, New York.

Gibeau GG, Miller MC (1989) A micro-bioassay for epilithon using nutrient-diffusing artificial substrata. *Journal of Freshwater Ecology*, **5**, 171–176.

Glibert PM, Wilkerson FP, Dugdale RC, Parker AE, Alexander J, Blaser S, Murasko S (2014) Phytoplankton communities from San Francisco Bay Delta respond differently to oxidized and reduced nitrogen substrates -even under conditions that would otherwise suggest nitrogen sufficiency. *Frontiers in Marine Science*, **1**, 1–16.

Godwin SC, Jones SE, Weidel BC, Solomon CT (2014) Dissolved organic carbon concentration controls benthic primary production: results from in situ chambers in north-temperate lakes. *Limnology and Oceanography*, **59**, 2112–2120.

Goericke R, Montoya JP (1998) Estimating the contribution of microalgal taxa to chlorophyll a in the field - Variations of pigment ratios under nutrient- and light-limited growth. *Marine Ecology Progress Series*, **169**, 97–112.

Goldman JC, McCarthy JJ, Peavey DG (1979) Growth rate influence on the chemical composition of phytoplankton in oceanic waters. *Nature*, **279**, 210–215.

Graham LE, Graham JM, Wilcox LW (2009) *Algae*, 2nd edn. Benjamin Cummings (Pearson), San Francisco.

Grasshoff K, Ehrhardt M, Kremling K (1983) *Methods of seawater analysis*, 2nd edn. Verlag Chemie.

Grover JP (1991) Resource competition in a variable environment : phytoplankton growing according to the Variable-Internal-Stores Model. **138**, 811–835.

Grover JP (1997) *Resource competition*. Chapman and Hall, London.

Gruber N, Galloway JN (2008) An Earth-system perspective of the global nitrogen cycle. *Nature*, **451**, 293–296.

Hagerthey SE, Louda JW, Mongkronsri P (2006) Evaluation of pigment extraction methods and a recommended protocol for periphyton chlorophyll a determination and chemotaxonomic assessment. *Journal of Phycology*, **42**, 1125–1136.

Hall SR, Smith VH, Lytle DA, Leibold MA (2005) Constraints on primary producer N:P stoichiometry along N:P supply ratio gradients. *Ecology*, **86**, 1894–1904.

Hamilton PB, Duthie HC (1987) Relationship between algal drift, discharge and stream order in a boreal forest watershed. *Archiv für Hydrobiologie*, **110**, 275–289.

Hardin G (1960) The competitive exclusion principle. *Science*, **131**, 1292–1297.

Harpole WS, Ngai JT, Cleland EE et al. (2011) Nutrient co-limitation of primary producer communities. *Ecology Letters*, **14**, 852–62.

Harrison WG, Harris LR, Irwin BD (1996) The kinetics of nitrogen utilization in the oceanic mixed layer: nitrate and ammonium interactions at nanomolar concentrations. *Limnology and Oceanography*, **41**, 16–32.

Henriksen P, Riemann B, Kaas H, Sørensen HM, Sørensen HL (2002) Effects of nutrient-limitation and irradiance on marine phytoplankton pigments. *Journal of Plankton Research*, **24**, 835–858.

Hessen DO, Elser JJ, Sterner RW, Urabe J (2013) Ecological stoichiometry: an elementary approach using basic principles. *Limnology and Oceanography*, **58**, 2219–2236.

Hietz P, Turner BL, Wanek W, Richter A, Nock CA, Wright SJ (2011) Long-term change in the nitrogen cycle of tropical forests. *Science*, **334**, 664–666.

Hillebrand H, Dürselen C-D, Kirschtel D, Pollingher U, Zohary T (1999) Biovolume calculation for pelagic and benthic microalgae. *Journal of Phycology*, **35**, 403–424.

Hillebrand H, Steinert G, Boersma M, Malzahn A, Meunier CL, Plum C, Ptacnik R (2013) Goldman revisited: faster-growing phytoplankton has lower N:P and lower stoichiometric flexibility. *Limnology and Oceanography*, **58**, 2076–2088.

Ho T-Y, Quigg A, Finkel Z V., Milligan AJ, Wyman K, Falkowski PG, Morel FMM (2003) The elemental composition of some marine phytoplankton. **39**, 1145–1159.

Hoagland KD, Roemer SC, Rosowski JR (1982) Colonization and community structure of two periphyton assemblages, with emphasis on the diatoms (Bacillariophyceae). *American Journal of Botany*, **69**, 188.

Hogan EJ, McGowan S, Anderson NJ (2014) Nutrient limitation of periphyton growth in Arctic lakes in south-west Greenland. *Polar Biology*, **37**, 1331–1342.

Holtgrieve GW, Schindler DE, Hobbs WO et al. (2011) A coherent signature of anthropogenic nitrogen deposition to remote watersheds of the Northern Hemisphere. *Science*, **334**, 1545–1548.

Horner RR, Welch EB (1981) Stream periphyton development in relation to current velocity and nutrients. *Canadian Journal of Fisheries and Aquatic Sciences*, **38**, 449–457.

Hutchinson GE (1961) The pararadox of the plankton. *American Naturalist*, **95**, 137–145.

Jordan TL, Staley JT (1976) Electron microscopic study of succession in the periphyton community of Lake Washington. *Microbial Ecology*, **2**, 241–251.

Juergens MT, Deshpande RR, Lucker BF et al. (2015) The regulation of photosynthetic structure and function during nitrogen deprivation in *Chlamydomonas reinhardtii*. *Plant Physiology*, **167**, 558–573.

Károlyi G, Péntek A, Scheuring I, Tél T, Toroczkai Z (2000) Chaotic flow: the physics of species coexistence. *PNAS*, **97**, 13661–13665.

Kaspari M, Powers JS (2016) Biogeochemistry and geographical ecology: embracing all twenty-five elements required to build organisms. *The American Naturalist*, **188**, S62–S72.

Kim T-W, Lee K, Najjar RG, Jeong H-D, Jeong HJ (2011) Increasing N abundance in the northwestern Pacific Ocean due to atmospheric nitrogen deposition. *Science*, **334**, 505–510.

Klausmeier CA, Litchman E, Drive F, Levin SA (2004) Phytoplankton growth and stoichiometry under multiple nutrient limitation. *Limnology and Oceanography*, **49**, 1463–1470.

Klausmeier CA, Litchman E, Daufresne T, Levin SA (2008) Phytoplankton stoichiometry. *Ecological Research*, **23**, 479–485.

Korte VL, Blinn DW (1983) Diatom colonization on artificial substrata in pool and riffle zones studied by light and scanning electron microscopy. *Journal of Phycology*, **19**, 332–341.

Kruskopf M, Flynn KJ (2006) Chlorophyll content and fluorescence responses cannot be used to gauge reliably phytoplankton biomass, nutrient status or growth rate. *New Phytologist*, **169**, 525–536.

Lane CE, Archibald JM (2008) The eukaryotic tree of life: endosymbiosis takes its TOL. *Trends in Ecology & Evolution*, **23**, 268–75.

Latasa M (2007) Improving estimations of phytoplankton class abundances using CHEMTAX. *Marine Ecology Progress Series*, **329**, 13–21.

Latasa M, Berdalet E (1994) Effect of nitrogen or phosphorus starvation on pigment composition of cultured Heterocapsa sp. *Journal of Plankton Research*, **16**, 83–94.

Lauridsen TL, Schlüter L, Johansson LS (2011) Determining algal assemblages in oligotrophic lakes and streams: comparing information from newly developed pigment/chlorophyll a ratios with direct microscopy. *Freshwater Biology*, **56**, 1638–1651.

Laurion I, Ventura M, Catalan J, Psenner R, Sommaruga R (2000) Attenuation of ultraviolet radiation in mountain lakes: factors controlling the among- and within-lake variability. *Limnology and Oceanography*, **45**, 1274–1288.

Legendre P, Gallagher ED (2001) Ecologically meaningful transformations for ordination of species data. *Oecologia*, **129**, 271–280.

Lehman JT (1976) Ecological and nutritional studies on *Dinobryon* Ehrenb.: seasonal periodicity and the phosphate toxicity problem. *Limnology and Oceanography*, **21**, 646–658.

Lepori F, Robin J (2014) Nitrogen limitation of the phytobenthos in Alpine lakes: results from nutrient-diffusing substrata. *Freshwater Biology*, **59**, 1633–1645.

Lewis WM, Wurtsbaugh WA (2008) Control of lacustrine phytoplankton by nutrients: erosion of the phosphorus paradigm. *International Review of Hydrobiology*, **93**, 446–465.

Lin S, Litaker RW, Sunda WG (2016) Phosphorus physiological ecology and molecular mechanisms in marine phytoplankton. *Journal of Phycology*, **52**, 10–36.

Lindeman RL. (1942) The trophic-dynamic aspect of ecology. *Ecology*, **23**, 399–417.

Litchman E, Klausmeier CA (2008) Trait-based community ecology of phytoplankton. *Annual Review of Ecology, Evolution, and Systematics*, **39**, 615–639.

Litchman E, Klausmeier CA, Schofield OM, Falkowski PG (2007) The role of functional traits and trade-offs in structuring phytoplankton communities: scaling from cellular to ecosystem level. *Ecology Letters*, **10**, 1170–1181.

Litchman E, Edwards KF, Klausmeier CA (2015) Microbial resource utilization traits and trade-offs: implications for community structure, functioning, and biogeochemical impacts at present and in the future. *Frontiers in Microbiology*, **6**, 1–10.

Liu X, Zhang Y, Han W et al. (2013) Enhanced nitrogen deposition over China. *Nature*, **494**, 459–462.

Llewellyn CA, Gibb SW (2000) Intra-class variability in the carbon, pigment and biomineral content of prymnesiophytes and diatoms. *Marine Ecology Progress Series*, **193**, 33–44.

Lowe RL (1996) Periphyton patterns in lakes. In: *Algal ecology: freshwater benthic ecosystems* (eds Stevenson RJ, Bothwell ML, Lowe RL), pp. 57–74. Academic Press, San Diego, CA.

Luttenton MR, Lowe RL (2006) Response of a lentic periphyton community to nutrient enrichment at low N:P ratios. *Journal of Phycology*, **42**, 1007–1015.

Maberly SC, King L, Dent MM, Jones RI, Gibson CE (2002) Nutrient limitation of phytoplankton and periphyton growth in upland lakes. *Freshwater Biology*, **47**, 2136–2152.

MacArthur RH, Wilson EO (1967) *The theory of island biogeography*. Princeton University Press, Princeton, NJ.

Mackey MD, Mackey DJ, Higgins HW, Wright SW (1996) CHEMTAX - a program for estimating class abundances from chemical markers: application to HPLC measurements of phytoplankton. *Marine Ecology Progress Series*, **144**, 265–283.

Mahowald N, Jickells TD, Baker AR et al. (2008) Global distribution of atmospheric phosphorus sources, concentrations and deposition rates, and anthropogenic impacts. *Global Biogeochemical Cycles*, **22**, 1–19.

Majdi N, Traunspurger W, Boyer S et al. (2011) Response of biofilm-dwelling nematodes to habitat changes in the Garonne River, France: influence of hydrodynamics and microalgal availability. *Hydrobiologia*, **673**, 229–244.

Marcarelli AM, Wurtsbaugh WA (2007) Effects of upstream lakes and nutrient limitation on periphytic biomass and nitrogen fixation in oligotrophic, subalpine streams. *Freshwater Biology*, **52**, 2211–2225.

Marchetti A, Schruth DM, Durkin CA et al. (2012) Comparative metatranscriptomics identifies molecular bases for the physiological responses of phytoplankton to varying iron availability. *PNAS*, **109**, E317-25.

McCormick PV, Stevenson RJ (1991) Mechanisms of benthic algal succession in lotic environments. *Ecology*, **72**, 1835–1848.

McIntire CD (1966) Some effects of current velocity on periphyton communities in laboratory streams. *Hydrobiologia*, **27**, 559–570.

Medina-Sánchez JM, Felip M, Casamayor EO (2005) Catalyzed reported deposition-fluorescence in situ hybridization protocol to evaluate phagotrophy in mixotrophic protists. *Applied and Environmental Microbiology*, **71**, 7321–7326.

Menden-Deuer S, Lessard EJ (2000) Carbon to volume relationships for dinoflagellates, diatoms, and other protist plankton. *Limnology and Oceanography*, **45**, 569–579.

Monks PS, Granier C, Fuzzi S et al. (2009) Atmospheric composition change – global and regional air quality. *Atmospheric Environment*, **43**, 5268–5350.

Moore CM, Mills MM, Arrigo KR et al. (2013) Processes and patterns of oceanic nutrient limitation. *Nature Geoscience*, **6**, 701–710.

Mullin MM, Sloan PR, Eppley RW (1966) Relationship between carbon content, cell volume, and area in phytoplankton. *Limnology and Oceanography*, **11**, 307–311.

Murphy J, Riley JP (1962) A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*, **27**, 31–36.

Neff JC, Ballantyne AP, Farmer GL et al. (2008) Increasing eolian dust deposition in the western United States linked to human activity. *Nature Geoscience*, **1**, 189–195.

Norland S (1993) The relationship between biomass and volume of bacteria. In: *Handbook of methods in aquatic microbial ecology* (eds Kemp PF, Sherr BF, Sherr EB, Cole JJ), pp. 303–307. Lewis Publishers, Boca Raton.

Nydick KR, Lafrancois BM, Baron JS, Johnson BM (2004) Nitrogen regulation of algal biomass, productivity, and composition in shallow mountain lakes, Snowy Range, Wyoming, USA. *Canadian Journal of Fisheries and Aquatic Sciences*, **61**, 1256–1268.

Oemke MP, Burton TM (1986) Diatom colonization dynamics in a lotic system. *Hydrobiologia*, **139**, 153–166.

Oksanen J, Blanchet FG, Kindt R et al. (2016) Community ecology package "vegan." R Repository.

Pausas JG (2012) Fire regime changes in the western Mediterranean Basin : fire regimes are not constant. **110**, 215–226.

Peñuelas J, Sardans J, Rivas-Ubach A, Janssens IA (2012) The human-induced imbalance between C, N and P in Earth's life system. *Global Change Biology*, **18**, 3–6.

Peñuelas J, Poulter B, Sardans J et al. (2013) Human-induced nitrogen-phosphorus imbalances alter natural and managed ecosystems across the globe. *Nature Communications*, **4**, 2934.

Persson J, Fink P, Goto A, Hood JM, Jonas J, Kato S (2010) To be or not to be what you eat: regulation of stoichiometric homeostasis among autotrophs and heterotrophs. *Oikos*, **119**, 741–751.

Phillips G, Lyche-Solheim A, Skjelbred B et al. (2013) A phytoplankton trophic index to assess the status of lakes for the Water Framework Directive. *Hydrobiologia*, **704**, 75–95.

Plewka M Life in water. URL: http://www.plingfactory.de/.

Quigg A, Finkel Z V, Irwin AJ et al. (2003) The evolutionary inheritance of elemental stoichiometry in marine phytoplankton. *Nature*, **425**, 291–294.

R Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. URL: http://www.r-project.org/.

Raven JA (1987) The role of vacuoles. *New Phytologist*, **106**, 357–422.

Raven JA (2010) Cyanotoxins: a poison that frees phosphate. *Current Biology*, **20**, R850-R852.

Raymond J, Segrè D (2006) The effect of oxygen on biochemical networks and the evolution of complex life. *Science*, **311**, 1764–1767.

Reay DS, Nedwell DB, Priddle J, Ellis-Evans JC (1999) Temperature dependence of inorganic nitrogen uptake : reduced affinity for nitrate at suboptimal temperatures in both algae and bacteria. *Applied and Environmental Microbiology*, **65**, 2577–2584.

Redfield AC (1958) The biological control of chemical factors in the environment. **46**, 205–221.

Reisen WK, Spencer DJ (1970) Succession and current demand relationships of diatoms on artificial substrates in Prater's Creek, South Carolina. *Journal of Phycology*, **6**, 117–121.

Reyes-Prieto A, Weber APM, Bhattacharya D (2007) The origin and establishment of the plastid in algae and plants. *Annual Review of Genetics*, **41**, 147–168.

Reynolds CS (1984) *The ecology of freshwater phytoplankton*. Cambridge University Press.

Reynolds CS (2006) *Ecology of phytoplankton*. Cambridge University Press.

Rhee G-Y (1978) Effects of N:P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake. *Limnology and Oceanography*, **23**, 10–25.

Rhee G-Y, Gotham IJ (1981) The effect of environmental factors on growth: light and the interactions of light with nitrate limitation. *Limnology and Oceanography*, **26**, 649–659.

Rivas-Ubach A, Sardans J, Perez-Trujillo M, Estiarte M, Peñuelas J (2012) Strong relationship between elemental stoichiometry and metabolome in plants. *PNAS*, **109**, 4181–4186.

Rockström J, Steffen W, Noone K et al. (2009) A safe operating space for humanity. *Nature*, **461**.

Rodhe W (1948) Environmental requeriments of freshwater plankton algae. Experimental studies in the ecology of phytoplankton. *Symbolae Botanicae Upsaliensis*, **10**, 1–149.

Rodríguez F, Chauton M, Johnsen G, Andresen K, Olsen LM, Zapata M (2006) Photoacclimation in phytoplankton: implications for biomass estimates, pigment functionality and chemotaxonomy. *Marine Biology*, **148**, 963–971.

Rodriguez-Lloveras X, Buytaert W, Benito G (2016) Land use can offset climate change induced increases in erosion in Mediterranean watersheds. *Catena*, **143**, 244–255.

Rott E, Cantonati M, Füreder L, Pfister P (2006) Benthic algae in high altitude streams of the Alps – a neglected component of the aquatic biota. *Hydrobiologia*, **562**, 195–216.

Roy S, Llewellyn C, Egeland ES, Johnsen G (2011) *Phytoplankton pigments: characterization, chemotaxonomy and applications in oceanography*. Cambridge University Press.

Ruivo M, Amorim A, Cartaxana P (2011) Effects of growth phase and irradiance on phytoplankton pigment ratios: implications for chemotaxonomy in coastal waters. *Journal of Plankton Research*, **33**, 1012–1022.

Sañudo-Wilhelmy SA, Tovar-Sanchez A, Fu F-X, Capone DG, Carpenter EJ, Hutchins DA (2004) The impact of surface-adsorbed phosphorus on phytoplankton Redfield stoichiometry. *Nature*, **432**, 897–901.

Schiller D Von, Martí E, Riera JL, Sabater F (2007) Effects of nutrients and light on periphyton biomass and nitrogen uptake in Mediterranean streams with contrasting land uses. *Freshwater Biology*, **52**, 891–906.

Schindler DW (1974) Eutrophication and recovery in experimental lakes: implications for lake management. *Science*, **184**, 897–898.

Schindler DW (1977) Evolution of phosphorus limitation in lakes. *Science*, **195**, 260–262.

Schlesinger WH (2009) On the fate of anthropogenic nitrogen. *PNAS*, **106**, 203–8.

Schlüter L, Møhlenberg F, Havskum H, Larsen S (2000) The use of phytoplankton pigments for identifying and quantifying phytoplankton groups in coastal areas: testing the influence of light and nutrients on pigment/chlorophyll a ratios. *Marine Ecology Progress Series*, **192**, 49–63.

Schlüter L, Lauridsen TL, Krogh G, Jørgensen T (2006) Identification and quantification of phytoplankton groups in lakes using new pigment ratios - A comparison between pigment analysis by HPLC and microscopy. *Freshwater Biology*, **51**, 1474–1485.

Segata N, Boernigen D, Tickle TL, Morgan XC, Garrett WS, Huttenhower C (2013) Computational meta'omics for microbial community studies. *Molecular Systems Biology*, **9**.

Sekar R, Nair KVK, Rao VNR, Venugopalan VP (2002) Nutrient dynamics and successional changes in a lentic freshwater biofilm. *Freshwater Biology*, **47**, 1893–1907.

Sekar R, Venugopalan VP, Nandakumar K, Nair KVK, Rao VNR (2004) Early stages of biofilm succession in a lentic freshwater environment. *Hydrobiologia*, **512**, 97–108.

Smith R, Johnson LT, Tank JL, Dodds WK (2009) The influence of land use on stream biofilm nutrient limitation across eight North American ecoregions. *Canadian Journal of Fisheries and Aquatic Sciences*, **66**, 1081–1094.

Sommer U (1992) Phosphorus-limited *Daphnia*: intraspecific facilitation instead of competition. *Limnology and Oceanography*, **37**, 966–973.

Sournia A (1978) *Phytoplankton manual*. UNESCO, Paris.

Steffen W, Broadgate W, Deutsch L, Gaffney O, Ludwig C (2015a) The trajectory of the Anthropocene: the Great Acceleration. *The Anthropocene Review*, **2**, 81–98.

Steffen W, Richardson K, Rockstrom J et al. (2015b) Planetary boundaries: guiding human development on a changing planet. *Science*, **347**, 1259855.

Steinman AD (1996) Effects of grazers on freshwater benthic algae. In: *Algal ecology: freshwater benthic ecosystems* (eds Stevenson RJ, Bothwell ML, Lowe RL), pp. 341–373. Academic Press, San Diego, CA.

Steinman AD, McIntire CD (1990) Recovery of lotic periphyton communities after disturbance. *Environmental Management*, **14**, 589–604.

Steinman AD, Lamberti GA, Leavitt PR (2006) Biomass and pigments of benthic algae. In: *Methods in stream ecology*, 2nd edn (eds Hauer FR, Lamberti GA), pp. 357–380. Academic Press, New York.

Sterner RW, Elser JJ, Hessen DO (1992) Stoichiometric relationships among producers, consumers and nutrient cycling in pelagic ecosystems. *Biogeochemistry*, **17**, 49–67.

Sterner RW, Elser JJ (2002) *Ecological stoichiometry: the biology of elements from molecules to the biosphere*. Princeton University Press.

Sterner RW, Andersen T, Elser JJ, Hessen DO, Hood JM, McCauley E, Urabe J (2008) Scale-dependent carbon:nitrogen:phosphorus seston stoichiometry in marine and freshwaters. *Limnology and Oceanography*, **53**, 1169–1180.

Stevenson RJ (1983) Effects of current and conditions simulating autogenically changing microhabitats on benthic diatom immigration. *Ecology*, **64**, 1514–1524.

Stevenson RJ (1996a) An introduction to algal ecology in freshwater benthic habitats. In: *Algal ecology: freshwater benthic ecosystems* (eds Stevenson RJ, Bothwell ML, Lowe RL), pp. 3–30. Academic Press, San Diego, CA.

Stevenson RJ (1996b) The stimulation and drag of current. In: *Algal ecology: freshwater benthic ecosystems* (eds Stevenson RJ, Bothwell ML, Lowe RL), pp. 321–338. Academic Press, San Diego, CA.

Stevenson RJ, Peterson CG (1991) Emigration and immigration can be important determinants of benthic diatom assemblages in streams. *Freshwater Biology*, **26**, 279–294.

Stiller JW, Schreiber J, Yue J, Guo H, Ding Q, Huang J (2014) The evolution of photosynthesis in chromist algae through serial endosymbioses. *Nature Communications*, **5**, 5764.

Strzepek RF, Harrison PJ (2004) Photosynthetic architecture differs in coastal and oceanic diatoms. *Nature*, **431**, 689–692.

Syrett PJ (1981) Nitrogen metabolism of microalgae. In: *Physiological bases of phytoplankton ecology, Bull. No. 210*, pp. 182–210. Canadian Government Publishing Center, Quebec.

Tanaka T, Kawasaki K, Daimon S et al. (2014) A hidden pitfall in the preparation of agar media undermines microorganism cultivability. *Applied and Environmental Microbiology*, **80**, 7659–7666.

Tank JL, Dodds WK (2003) Nutrient limitation of epilithic and epixylic biofilms in ten North American streams. *Freshwater Biology*, **48**, 1031–1049.

Tank JL, Bernot MJ, Rosi-Marshall EJ (2006) Nitrogen limitation and uptake. In: *Methods in stream ecology*, 2nd edn (eds Hauer FR, Lamberti GA), pp. 213–238. Academic Press, San Diego, California.

Tilman D (1977) Resource competition between plankton algae : an experimental and theoretical approach. *Ecology*, **58**, 338–348.

Tilman D (1982) *Resource competition and community structure*. Princeton University Press, Princeton, NJ.

Uehlinger U, Robinson CT, Hieber M, Zah R (2010) The physico-chemical habitat template for periphyton in Alpine glacial streams under a changing climate. *Hydrobiologia*, **657**, 107–121.

Vadeboncoeur Y, Peterson G, Vander Zanden MJ, Kalff J (2008) Benthic algal production across lake size gradients : interactions among morphometry, nutrients, and light. *Ecology*, **89**, 2542–2552.

Van Mooy BAS, Fredricks HF, Pedler BE et al. (2009) Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature*, **458**, 69–72.

Ventura M, Catalan J (2005) Reproduction as one of the main causes of temporal variability in the elemental composition of zooplankton. *Limnology and Oceanography*, **50**, 2043–2056.

Villeneuve A, Montuelle B, Bouchez A (2010) Influence of slight differences in environmental conditions (light, hydrodynamics) on the structure and function of periphyton. *Aquatic Sciences*, **72**, 33–44.

Vinebrooke RD, Leavitt PR (1996) Effects of ultraviolet radiation on periphyton in an Alpine lake. *Limnology and Oceanography*, **41**, 1035–1040.

Vollenweider RA (1968) *Scientific fundamental of the eutrophication of lakes and flowing waters, with particular reference to nitrogen and phosphorus as factors in eutrophication*. OECD, Paris.

Vrede T, Dobberfuhl DR, Kooijman SALM, Elser JJ (2004) Fundamental connections among organism C:N:P stoichiometry, macromolecular composition, and growth. *Ecology*, **85**, 1217–1229.

Vrede T, Ballantyne A, Mille-Lindblom C, Algesten G, Gudasz C, Lindahl S, Brunberg AK (2009) Effects of N:P loading ratios on phytoplankton community composition, primary production and N fixation in a eutrophic lake. *Freshwater Biology*, **54**, 331–344.

Wang R, Balkanski Y, Boucher O, Ciais P, Peñuelas J, Tao S (2014) Significant contribution of combustion-related emissions to the atmospheric phosphorus budget. *Nature Geoscience*, **8**, 48–54.

Watanabe S (2010) Asymptotic equivalence of Bayes cross validation and widely applicable information criterion in singular learning theory. *Journal of Machine Learning Research*, **11**, 3571–3594.

Waters CN, Zalasiewicz J, Summerhayes C et al. (2016) The Anthropocene is functionally and stratigraphically distinct from the Holocene. *Science*, **351**, aad2622.

Weber TS, Deutsch C (2010) Ocean nutrient ratios governed by plankton biogeography. *Nature*, **467**, 550–554.

Wetzel RG (2001) *Limnology*. Academic Press, New York.

Whitford LA, Schumacher GJ (1961) Effect of current on mineral uptake and respiration by a freshwater alga. *Limnology and Oceanography*, **6**, 423–425.

Wickham H, Chang W (2015) ggplot2: an implementation of the grammar of graphics. R package version 2.1.0. URL: www.ggplot2.org

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Zhang L, Jacob DJ, Knipping EM et al. (2012) Nitrogen deposition to the United States: distribution, sources, and processes. *Atmospheric Chemistry and Physics*, **12**, 4539–4554.

Zhang Y-J, Zhang S-F, He Z-P, Lin L, Wang D-Z (2015) Proteomic analysis provides new insights into the adaptive response of a dinoflagellate *Prorocentrum donghaiense* to changing ambient nitrogen. *Plant, Cell and Environment*, **38**, 2128–2142.