CHAPTER 4

NUTRIENT LIMITATION OF FRESHWATER CYANOBACTERIA

Tools to monitor phosphorus limitation at the individual level

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1 Introduction

Changes in species composition that are often observed in phytoplankton communities result from a complex interplay between physical and chemical properties of the aquatic environment on the one hand and the responsiveness of the individual species on the other hand. The potential role of nutrient limitation in accommodating these changes in species composition has since long been recognized. However, the interpretation of results, to the benefit of understanding nutrient competition between species in phytoplankton communities, is very much hampered by the limited knowledge that is gained by chemical assay methods for measurements of actual nutrient concentrations in surface waters. Release of limiting nutrients in lake water may even simply escape detection, because phytoplankton is able to sequester occasional nutrient pulses with high affinity. Although biological assays, which define the limiting nutrient from an increase in biomass following addition of that particular nutrient, are in common use, their reporting value is generally limited to the total phytoplankton community only. Furthermore, these bioassays require relatively long incubation times before results are known. Rapid detection of early changes in the phytoplankton community is required to combat the development of unwanted blooms of harmful cyanobacteria. For this purpose, we have invested in the development of fluorescent staining procedures that enable the recognition of nutrient limitation within individual phytoplankters. That way, more detailed information on the contribution of different species to phytoplankton community development is gained.

To detect nutrient limitation at the level of the individual cell, we have searched for marker proteins that indicate nutrient limitation. The choice of such marker proteins requires a sound knowledge of metabolic pathways and gene expression

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patterns involved. Furthermore, proteins that will be used as a marker should preferably be exposed on the periphery of the cell to facilitate fluorescent staining. At the same time they must be tightly connected to the cell to prevent excretion or loss. Cell wall proteins are the first choice; these are ideally located at the outer membrane surface, or else in the periplasmic space. Possibly, integral cytoplasmic membrane proteins would still provide an adequate target. Monitoring the expression levels of marker proteins in individual cells permits a characterization of nutrient availability to different phytoplankton species during a growth season. In this contribution the focus is on phosphorus, because this nutrient is often the limiting factor in freshwater communities. We provide an overview of what is known about phosphorus metabolism in cyanobacteria, with emphasis on the cell wall proteins. Initial results from combined application of flow cytometry and fluorescent staining of phosphatase activity are presented.

2 The nutrient status of phytoplankton

2.1 NUTRIENT LIMITATION VERSUS EUTROPHICATION

The presence and growth of phytoplankton in surface water is not only related to the availability of sunlight and carbon dioxide, but also depends on the availability of certain key nutrients. Of these nutrients, phosphorus (P) and nitrogen (N) have attracted the most attention. Low nutrient levels (oligotrophic conditions) allow biodiversity to be high because a variable flow of materials and energy through the ecosystem creates complex nutritional interrelationships and specialised niches. Competition processes in a complex community may generate non-equilibrium dynamics, which sustain a large number of coexisting phytoplankton species (Huisman and Weissing, 1999). This situation, of high biodiversity and low biomass, is associated with a high water quality. At higher levels of nutrients, however, cyanobacteria may become dominant, and may form dense surface blooms. These organisms can be toxic, and generally their over-abundance has a deteriorating effect on the aquatic ecosystem, resulting in low water quality. The increased availability of nutrients through eutrophication is mostly caused by our agricultural and industrial habits (Reynolds, 1998, and references therein). The reverse situation, in which measures are taken to reduce nutrient inputs into aquatic ecosystems (remediation), may eventually lead to nutrient limitation and water quality improvement.

2.2 HOW TO MONITOR THE NUTRIENT STATUS ?

Attempts to control algal problems have focused primarily on managing the reduction of phytoplankton biomass by, for example, reduced phosphorus loading or biomanipulation (e.g. Hosper, 1997). This approach was often appropriate to reduce the overall biomass production, yet in many cases failed to render complete restoration. A more balanced approach appears to be required, taking into account the interplay of various nutrients and the effect on community complexity. The ideas

of Hecky and Kilham (1988) are still relevant in this respect. According to these authors, we need to refine our understanding of nutrient limitation to be able to manage species composition. Therefore, indicators for the phytoplankton nutrient status are required. The term 'diagnostic tool' has been used to denote a signal or analytical procedure that empirically identifies the symptoms of an environmental constraint on phytoplankton growth rates (Falkowski et al., 1992; Mann et al., 1993). Assessment of the nutrient status down to the individual level is desirable to account for all aspects of heterogeneity in the phytoplankton community. A direct approach to address this challenge is to monitor the presence of those cell-constituents, which are specifically linked to the nutrient status of interest by fluorescent detection in a flow cytometer (Scanlan et al., 1997; Palenik and Wood, 1998). Obvious requirements for suitable markers of the nutrient status are that composition, accumulation, or activity of these markers (mRNA, proteins, lipids, carbohydrates, metabolites) depends on the physiological state of the cell and especially on the availability of specific nutrients. The results from whole-genome analysis and protein characterisation studies in model organisms help identify the genes involved in nutrient uptake. In the case of freshwater cyanobacteria, the complete genome of two model organisms is available: *Synechocystis* PCC 6803 and *Synechococcus elongatus* PCC 7942. Obviously, the general applicability of techniques that work for specific model organisms needs to be checked for the species actually present in ecosystems of interest. Fortunately, however, biochemical knowledge gained from model organisms can often be applied to a wider range of organisms in ecosystems as biochemical traits usually follow a common blue print.

3 Nutrient uptake through the cell envelope

The periphery of the cell is of interest for two reasons. Firstly, this is where contact with the environment takes place. The process of adaptation to changing conditions in the environment is likely to be well pronounced in this cell fraction. Secondly, the use of non-intrusive diagnostics for the nutrient status requires the target to be exposed at the cell surface, to minimise the extent of manipulation of the sample. Protection against osmosis and specific transport of nutrients are both function of the cell envelope. The thick multi-layered envelopes of cyanobacteria form a considerable mechanical and permeability barrier for mostly larger molecules (Hoiczyk and Hansel, 2000). The outermost surface of cyanobacteria is usually covered with a carbohydrate sheath and a two-dimensional crystalline, single-protein surface-layer (S-layer; Šmarda et al., 2002). The various sections of the cell wall are discussed next.

3.1 THE OUTER MEMBRANE

The outer membrane (OM) protects the cell from harmful agents such as proteases, bile salts, antibiotics, toxins and phage, and against drastic changes in osmotic pressure (Cowan et al., 1992). The OM is an asymmetric lipid bilayer. The outer monolayer contains lipopolysaccharide (LPS) as major lipid, whereas the inner

monolayer contains phospholipids. In addition to lipids and proteins, carotenoids are components of the isolated OM fractions of cyanobacteria (Resch and Gibson, 1983; Jürgens and Weckesser, 1985). The function of these pigments is to protect the cells from oxidative stress, by shielding them from excessive light (Hirschberg and Chamovitz, 1994; Miller et al., 2002). The asymmetry of the outer membrane relies on an intact peptidoglycan layer in the periplasmic space. The outer membrane contains structural lipoprotein, which links to the underlying peptidoglycan either covalently, or via ion-bridges. Pore-forming proteins, called porins, mainly determine the permeability of the outer membrane. The *Synechocystis* PCC 6803 genome encodes six homologues to the outer membrane porins SomA and SomB with highly conserved surface layer homologous domains (Hoiczyk and Hansel, 2000). The predicted structure of the translated proteins shows all characteristics of porins, and furthermore, the expression pattern of several of these proteins is modified by nutrient availability (Dignum, 2003). The fact that these porins are responsive to nutrient availability and have regions that are exposed at the periphery of the cell makes this class of proteins of keen interest for further research.

3.2 THE PERIPLASMIC SPACE AND THE PEPTIDOGLYCAN LAYER

The layer between the inner and outer membrane of Gram-negative bacteria is defined as the periplasmic space, and is estimated to contribute about 7% of the total volume of the cell. The osmotic pressure in the water-filled periplasm is only slightly higher than that of the medium (Koch, 1998). Close contact with the exterior through the semi-permeable outer membrane allows (small-molecule) tags to be used to visualise periplasmic proteins as diagnostic for the nutrient status. The periplasmic space is much more viscous and dense with proteins than the cytoplasm (Raivio and Silhavy, 2001). Soluble proteins in the periplasm are released by applying cold osmotic shock (Heppel, 1967; Fulda et al., 1999). The periplasm contains other proteins than the cytoplasm, including binding proteins and hydrolytic enzymes that degrade substances for nutrition (Koch, 1998). Most of the proteins identified in the periplasm of *Synechocystis* represent 'hypothetical proteins' with unknown function (Fulda et al., 2000). The majority of the assigned proteins are involved in the generation and modification of the external cell layers. About 10% of the assigned proteins belong to the family of proteases (Fulda et al., 2000). The periplasmic space also contains the peptidoglycan layer, which constitutes the actual cell wall in mechanical terms. The peptidoglycan (synonym: murein) layer primarily functions in maintaining the cell shape and withstands the very high internal osmotic pressure that the cytoplasm exerts in dilute environments. It consists of a relatively open network of amino sugars and amino acids. Long strands of amino sugars are covalently linked by pentapeptides, forming a giant, hollow, net-like molecule. The high degree of cross-linking found in cyanobacteria is similar to that in Gram-positive bacteria. However, teichoic acid, a typical constituent of Gram-positive bacteria is missing in cyanobacterial cell walls. But then again, the cyanobacterial peptidoglycan forms complexes with polysaccharides, similar to Gram-positive peptidoglycan.

3.3 THE CYTOPLASMIC MEMBRANE

The cytoplasmic membrane acts as a real diffusion barrier, and contains a large number of uptake systems for solutes. An interesting approach to assess the transport capability of the cytoplasmic membrane of *Synechocystis* was conducted by microbial genome analysis (Paulsen et al., 1998). The *Synechocystis* genome encodes 92 transporters. The number of ATP-dependent transporters (see below) was ten times the number of proton-motive-force dependent transporters. This in contrast with *E. coli*, which has equal numbers of the two types. The special status of the thylakoid membranes that are embedded in the cytoplasm and add ATP through photophosphorylation for use at the cytoplasmic membrane can explain this difference. The transport proteins may render a focus for our interest in characterisation of proteins with a reporting value for nutrient limitation. Another relevant feature of the cytoplasmic membrane is its role in registration of fluctuations in the environment. Two-component regulatory systems with sensor proteins located in the cytoplasmic membrane function to sense specific changes in the surroundings (Ronson et al., 1987; Parkinson and Kofoid, 1992; Bijisma and Groisman, 2003). The signal is transferred over the membrane and passed on to the transcription apparatus by corresponding regulatory proteins.

4 Importance of phosphate

The important role that phosphate plays in cells is apparent on three levels.

Structure. Phosphate forms covalent ester links between carbohydrate monomers, creating a rigid structure. Organically bound phosphate is found in the peptidoglycan fraction of the cell wall. Its probable function is to bind polysaccharide covalently to the peptidoglycan matrix (Jürgens et al., 1983). The role of these compounds in giving the cell its shape and strength was discussed above. Phosphate is also a structure determining part of nucleic acids: it connects the ribonucleotide monomers that form the DNA or RNA backbone. Another way in which phosphate gives structure to cell components is by acting as an intermediate for ionic binding. The inner leaflet of the outer membrane consists of phospholipids, which stick together by binding to intermediate divalent cations. The lipopolysaccharides of cyanobacteria also contain small amounts of bound P (Hoiczek and Hansel, 2000).

Energy carrier. Energy-rich phosphates are involved in the cells' metabolism as universal free energy carriers. The most important energy carrier is adenosine triphosphate (ATP). Energy can be directly stored inside the cell by conversion to poly-phosphate (polyP), or indirectly by generating carbohydrates. The biosynthesis of many macromolecules is accompanied by liberation of pyrophosphate (PP_i) as waste product (Lahti, 1983). More than one PP_i molecule is liberated for every monomer in protein, nucleic acid and polysaccharide (Klemme, 1976). Both PP_i and ATP can be synthesised phototrophically.

Information carrier. Environmental and internal signals provide important information for the adaptive responses of bacteria. Prokaryotic signalling systems are complex, with multiple components, connections to other regulatory circuits, and

feedback loops (Parkinson and Kofoid, 1992). These networks commonly contain transmitter and receiver modules. The communication between transmitter and receiver usually involves activation by phosphorylation (kinase activity), deactivation by dephosphorylation (phosphatase activity; Ronson et al., 1987). Well-conserved two-component regulatory systems (TCRS) function to sense specific changes in the environment (sensory component) and transduce that information to the transcriptional apparatus (regulatory component). The sensor is usually a transmembrane protein, which binds to ligands with a variable periplasmic domain, and transmits the signal to a conserved cytoplasmic domain (transmitter), through allosteric alteration. The activated sensor interacts with the N-terminal part of the regulator (receiver). The response regulator is usually a DNA binding protein, facilitating transcription by activating the promoter. The phosphoryl transfer pathways may diverge (more than one regulator phosphorylated by one kinase), or converge (more than one kinase phosphorylates one response regulator) (Hellingwerf et al., 1995, 1998). Accordingly, signal transduction pathways by phosphoryl transfer meet all the criteria of a neural network. In *Synechocystis*, at least 80 TCRS pairs have been found in the genome (Mizuno et al., 1996).

5 Phosphate incorporation

Orthophosphate ($H_2PO_4^-$, HPO_4^{2-}, or PO_4^{3-}), synonym inorganic phosphate (abbreviated P_i), some phosphorylated sugars and phosphonate are the only directly available P sources for phytoplankton (Palenik et al., 2003). Bio-available P can be defined as the sum of immediately available P (P_i), and P that can be transformed by naturally occurring physical (e.g. desorption), chemical (e.g. dissolution) and biological processes (e.g. enzymatic degradation; Boström et al., 1988). Three factors determine the efficiency of P_i-uptake: The permeability of the cell membranes, the relative concentrations of P_i inside and outside the cells, and the capacity to use a variety of phosphorylated compounds. P_i incorporation takes place in several steps (Falkner et al., 1989), which are schematically indicated in Figure 1. Organic or inorganic phospho-esters are converted into P_i by the activity of alkaline phosphatase enzymes outside the cell, at the cell-surface or in the periplasm. Transport through the cytoplasmic membrane is the rate-limiting step of P_i incorporation (e.g. Falkner et al., 1989). At low concentrations many organisms can induce a high-affinity uptake system, which transports P_i at the expense of ATP. Uptake of P_i ceases at a threshold concentration because the energy available for the uptake process is then insufficient. P_i is subsequently converted into energy-rich ATP. The energy required to drive the process of ATP formation from ADP and sequestered P_i is provided by photo-phosphorylation, which is coupled to the proton flux across the thylakoid membranes in the light reactions of photosynthesis (Simonis and Urbach, 1973). Excess P_i is stored inside the cells as polyphosphate granules (Poly-P). PolyP-bodies are also referred to as metachromatic granules or volutin (Kornberg 1995), and are similar to acidocalcisomes (Marchesini et al., 2002). PolyP is present in these granules in microcrystalline aggregates, consistent with their very high electron density (Ruiz et al., 2001). Many functions have been attributed to polyP. It is obviously a reservoir for P_i, and connected to the polyP-

overplus phenomenon: the rapid and extensive polyP accumulation, when P_i is added to cells previously subjected to P_i-depletion. As expected for a polyanion, polyP is a strong chelator of metal ions. Chelation of various metals (e.g. Zn, Fe, Cu, and Cd) may reduce their toxicity or affect their functions (Kornberg, 1995). Other functions reported are as a buffer against alkali ions (Pick and Weiss, 1991), and as a channel for DNA entry (Kornberg, 1995). Furthermore, polyP has a function in physiological adjustments to growth, development, and deprivations. A function as phosphagen, a store of P-bound energy is disputable. Although high amounts of polyP can be stored in the cells, even highly elevated levels of polyP could supply the cell with ATP for only a few seconds (Rao et al., 1998). PolyP is coupled to the energy charge of the cell, though, because it is reversibly synthesized from ATP. PolyP formation is an equilibrium reaction that does not require anything but the energy gained from the hydrolysis of ATP into ADP and P_i.

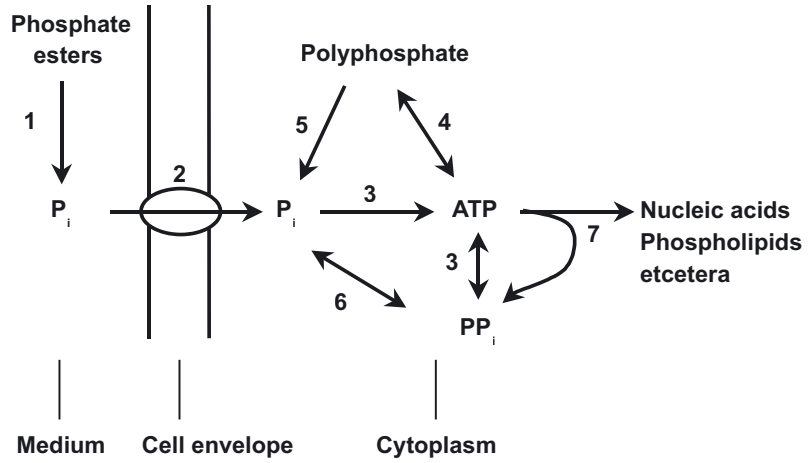

Figure 1. Cellular phosphate incorporation. The numbers indicate the following enzymes: 1: Alkaline phosphatase, 2: P_i-transport system, 3: ATP synthase, 4: Polyphosphate kinase, 5: Polyphosphatase, 6: Pyrophosphatase, and 7: Anabolic enzymes.

5.1 IS P_i THE LIMITING FACTOR FOR PHYTOPLANKTON IN LAKES ?

The notion that phosphate may be a limiting factor for algal growth goes back more than a century (see Shapiro, 1988; Correll, 1999). Since the beginning of eutrophication management in the 1970s there has been general agreement that phytoplankton biomass production is determined by availability of phosphate in a wide variety of lakes. In lake surveys as well as experimental lakes the concentration of chlorophyll and the concentration of carbon in the seston fraction were proportional to the concentration of total phosphorus in the water column (Vollenweider, 1975; Schindler, 1977). The plausible explanation for this general correlational pattern is that organisms can draw on the massive atmospheric sources

and sinks for carbon and nitrogen to maintain, on average, the C:N:P ratios that reflect their requirements for growth. However, phosphorus cannot be replenished in a similar way from atmospheric resources. It follows that phosphorus ultimately controls phytoplankton abundance in many aquatic environments. Even while a sudden increase in the phosphate input (pulse) may cause algae to enter N- or C-limitation, there are long-term processes at work in the environment, which cause these deficiencies to be corrected (Schindler, 1977; Hecky and Kilham, 1988). In freshwater situations phosphate is thus the most likely macronutrient to become limiting to phytoplankton production. It would be simplistic, though, to assume that all the species in a community are limited by the same factor. Different species within the same phytoplankton community may have different resource requirements, and consequently may be limited by different factors. In fact, multiple resource limitation of phytoplankton communities has often been demonstrated (Hecky and Kilham, 1988; Elser et al., 1990; Huisman and Weissing, 2001).

5.2 ACQUIRING EVIDENCE FOR NUTRIENT LIMITATION

Several classical approaches for detection of nutrient limitation have been described (e.g. reviewed in Hecky and Kilham, 1988; Beardall et al., 2001). ***Dissolved nutrient concentrations*** present negative evidence: when a nutrient concentration becomes very low, it is likely to limit algal production. However, phytoplankton species and communities have such high affinity for N and P that nutrient limitations occur at concentrations that are generally not analytically detectable, or otherwise, in case of pulsed supply, may be missed due to low sampling frequency. ***Elemental ratios*** have often been used to indicate which nutrient will eventually limit biomass production ('Liebig' limitation). They are less useful, however, to ascertain whether a certain phytoplankton population is nutrient limited at a given point in time (Beardall et al., 2001). ***Nutrient enrichment bioassays*** are operational tests in which one or more nutrients are added to a volume of water to determine to what extent algal growth is stimulated. The test result of these bioassays is likely to be dependent on the level of complexity of the assay system used (whole lake nutrient enrichment; laboratory scale enclosure; continuous culture). Especially natural systems are capable of much more complex responses at longer time scales. The only concern to aquatic resource managers is the highest level, the natural system, whereas inferences are often made based on evidence from lower level test systems. For instance, the artificial system in which the nutrient enrichment bioassay is carried out may seriously underestimate the role of tight nutrient recycling in the aquatic ecosystem that it is presumed to represent. If ***calculated fluxes of nutrients*** can show that growth is dependent on one nutrient rather than any other, then that nutrient may limit algal growth. The problem is that the system must be very well defined and all nutrient inputs must be measured with significant accuracy, which is difficult to apply in a natural system. ***Physiological responses to nutrient limitation*** can be used instantaneously as indicators for the nutrient status. Prolonged incubations are not required, but repetitive sampling to characterize the general state of an ecosystem is. Examples of nutrient status indicators are: variation in cell contents, maximum uptake rate, maximal growth yield estimations based on variable

fluorescence (Healey and Hendzel, 1980), bio-availability to reporter strains (e.g. Pat et al., 2001), immunofluorescence detection of specific markers (Dyhrman and Palenik, 1999), and enzymatic assays (Rengefors et al., 2003; Nedoma et al., 2003). The search in our present work focuses on the identification of biomolecules or processes that are useful as physiological indicator for nutrient limitation.

6 Adaptation strategies to P-deficiency

6.1 CLASSIFICATION OF ADAPTATION STRATEGIES

As P_i is essential for the growth of phytoplankton, they have developed strategies to cope with (temporary) P_i-depletion. These growth strategies underlie the ecological patterns that allow us to interpret and understand the processes of community assembly. They are pre-adaptations that are permanent, quantifiable features of the organism, which can be invoked experimentally (Reynolds, 1998). It is important to distinguish general from specific responses. P-depleted cyanobacteria display several phenomenological changes as compared to nutrient-replete cultures. The colour changes from intense blue-green to yellowish (bleaching, chlorosis), the cells appear smaller, and sometimes foam appears on the culture (cell lysis). At the physiological level, there are three prerequisites to survive P_i-deficiency (Falkner et al., 1998). Firstly, the cells must possess uptake systems that operate efficiently at very low and fluctuating P_i concentrations. Secondly, P_i must be stored inside the cells to secure the availability in times when P_i-uptake ceases. Thirdly, the cellular processes must be largely independent of external P_i-concentrations, but proportional to the amount of P_i stored in the cells. According to La Roche et al. (1999), phytoplankton cells exhibit three major categories of responses to nutrient limitation: retrenchment, compensation and acquisition. Retrenchment, or down-regulation of physiological rates is a progressive and reversible response, resulting in a modulation of the overall growth rate and changes in biochemical composition of the cells (proper growth rate limitation). Examples are: changes in the relative amounts of photo-pigments, a marked decrease in RNA content in P_i-depleted cells (N. Yeremenko, personal communication). This last observation is in line with results from a study with *Synechococcus* cells that are growing with a P-limitation, in which the RNA contents varies directly with the growth rate (Grillo and Gibson, 1979). Compensation includes all cellular responses that alleviate the effects that the lack of nutrients imposes (La Roche et al., 1999), exemplifying a general response. For example, lack of P_i in the cells obstructs photophosphorylation. This may lead to photo-inhibitory stress, for which the cells have to compensate as if they were experiencing excessive light conditions, e.g. by increased synthesis of carotenoids in the outer membrane. Acquisition is the development of more efficient uptake systems, which is a specific response. This involves synthesis of both high-affinity P_i-uptake system and alkaline phosphatase enzymes that convert alternate chemical forms of P.

Similarly, in phytoplankton ecology three different strategies are usually distinguished to cope with temporary nutrient limitations (Sommer, 1989; Ducobu et al., 1998). These strategies may operate separately or in co-operation. Cells with a

growth strategy use transient nutrient enrichments to achieve a high growth rate by optimising their specific yield (Y_x). A well-known example of the growth strategy to cope with shortage of phosphate is the replacement of the P-containing cell wall component teichoic acid by teichuronic acid in Gram-positive bacteria. In phosphate-rich conditions 15% of the cellular phosphate of *Bacillus subtilis* is stored in the cell wall polymer teichoic acid. During P-deficiency this compound is replaced by teichuronic acid, which contains no phosphate (e.g. Lahooti and Harwood, 1999). The result is a higher amount of biomass on the same amount of P, and also a higher growth rate with a lower P-concentration. Although the cyanobacterial peptidoglycan has phosphate links, similar use of this potential transient nutrient enrichment has as far as the authors know never been described for cyanobacteria. Alternatively, cells can increase the uptake rate, by making more uptake proteins or changing the conformation of uptake proteins. This is connected to the ***affinity strategy***, in which new synthesis of an uptake system with higher affinity for the nutrient causes a decrease of the saturation constant (K_m). Affinity strategists are efficient users of low external nutrient concentrations. A potential response is the induced synthesis of a high-affinity uptake system for P_i. Cells with a ***storage strategy*** secure the P_i-availability in times when uptake ceases, by build-up of internal supplies. This implies synthesis of enzymes that transform P_i into insoluble macromolecules (polyphosphates) inside the cell. This internal storage may enable the cells to produce several generations when external nutrient concentrations are low. To these three strategies, a fourth can be added that may be referred to as the ***scavenging strategy***. This strategy involves the induced synthesis of enzymes that transform generally inaccessible phosphate-containing compounds into a form that the cell can use, or the excretion of compounds that liberate inaccessible forms of phosphate. Organisms employing this strategy are able to use organic molecules that contain the required nutrient (e.g., P-esters), or they may use alternative inorganic forms of the nutrient.

6.2 P_i-UPTAKE NEAR THE THERMODYNAMIC LIMIT

Microorganisms cannot completely deplete their environment of phosphate; a threshold concentration exists, below which incorporation is thermodynamically impossible (typically 3-10 nM: Falkner et al., 1989). The threshold concentration, however, is influenced by the affinity of the uptake system. P_i-uptake of *Synechococcus elongatus* PCC 7942 efficiently takes place near the threshold concentration (Wagner et al., 2000, and references therein). The P_i-uptake rate by *Synechococcus* near the thermodynamic equilibrium was found to be linearly dependent on the free energy of polyphosphate formation and the pH-gradient at the thylakoid membrane (Wagner et al., 1995). Cells were able to independently change their kinetic and energetic properties, and seemed to optimise the efficiency of P_i-uptake, rather than absorb as much as possible. In this situation, P_i-uptake and growth were not directly coupled; the growth rate was rather related to the amount of stored phosphate, as intuited by Droop (1973). Wagner and co-authors suggest that cyanobacteria have a capacity to “memorise” nutrient fluctuations for several hours, which provides a means to adjust growth rate to P_i-availability. The nature of

this adaptive response to changes in the external concentrations appears to be an index of previous growth conditions, and reflect the whole concentration range the population has previously experienced (Falkner et al., 1993; Wagner et al., 1995). The information about previous P-supply is lost, however, after complete growth arrest (Falkner et al., 1995). Analysis of the uptake activity of phytoplankton by the methods developed in these studies may also serve as a proper tool for monitoring P_i inflow into lakes, and to establish threshold concentrations below the normal measurable range (Falkner et al., 1993; Aubriot et al., 2000). The observed adaptive flexibility towards P_i concentrations fluctuating around the thermodynamic threshold may be provided by staggered interplay of two parallel uptake systems with distinct high affinities (see below).

7 The *pho*-operon: a high-affinity P_i uptake system

In a P_i-depleted environment, P_i is taken up along a steep concentration gradient in an energy-dependent process. In these conditions, the efficiency of P_i-uptake from environments with a low P_i concentration is considerably enhanced by channels through the outer membrane that exhibit strong anion selectivity or bear specific P_i-binding sites, and by a periplasmic space that possesses high affinity P_i-binding proteins. The high-affinity P_i-uptake system, Pst, known from early research on *Escherichia coli* (Enterobacteriaceae), has all these characteristics. The Pst-system is carefully regulated, and is directly ATP-driven (Horiuchi et al., 1959; Rosenberg et al., 1977). The pathway for P_i-uptake into cells grown under P_i-depleting conditions is summarised in Figure 2. It starts with the passage of P_i or phosphorylated compounds through an outer membrane porin protein channel (phosphate-specific PhoE or a non-specific porin) into the periplasmic space. In the periplasm, the phosphorylated compounds are hydrolysed by alkaline phosphatases; P_i is captured by the P_i-binding protein PstS, and directed to the ATP-binding cassette-dependent (ABC) transporter in the cytoplasmic membrane. This transporter consists of two integral membrane-bound proteins, PstA and PstC, and a cytoplasmic peripheral membrane protein, PstB (Silver and Walderhaug, 1992). Homologous high-affinity P_i-uptake systems have been found in cyanobacteria, and have most extensively been studied for *Synechococcus*. For this cyanobacterium, the P_i-binding protein SphX (Scanlan et al., 1993; Mann and Scanlan, 1994), two alkaline phosphatases PhoA (Ray et al., 1991) and PhoV (Wagner et al., 1995), and the sensor/regulator pair SphS/SphR (Aiba et al., 1993) have been described. A similar P_i-uptake system has been identified in *Synechocystis*. The genome of *Synechocystis* contains two operons (sll0679-0684 and slr1247-1250) that are homologous to the Pst-system (Kaneko et al., 1996; Dignum, 2003; Suzuki et al., 2004; Yeremenko, 2004). The first system is involved in an initial, fast response toward P_i-depleting conditions; the second system is the major high-affinity P_i-uptake system, but takes longer to be derepressed (Suzuki et al., 2004). The presence of two high-affinity P_i-uptake systems with different sensitivity for P_i-concentrations may be important in providing a flexible response near the thermodynamic threshold concentration, as described above for *Synechococcus* (Falkner et al., 1998). Regulation of several porins by P_i-availability also occurs in *Synechocystis* (Dignum, 2003). It is presently

unknown whether these porins are selective towards P_i. The genes encoding alkaline phosphatase *pho*A (sll0654), P_i-sensing histidine kinase *pho*R (sll0337) and response regulator *pho*B (slr0081) have been identified (Hirani et al., 2001). The regulation of the Pst-system is highly specific for P_i-availability, and the proteins are located in the cell periphery. Therefore, the Pst-system is of major interest as potential markers for the nutrient status of cyanobacteria.

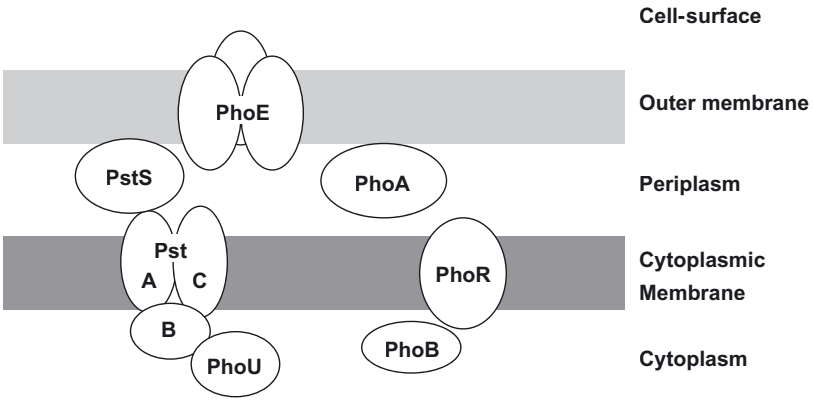

Figure 2. High-affinity phosphate uptake by the Escherichia coli pho-*operon (after Silver and Walderhaug, 1992), PhoA: Alkaline phosphatase, PhoE: phosphate-selective outer membrane porin, PstS: phosphate-binding protein, PstABC: ATP-binding cassette transporter, PhoR: sensory kinase, PhoB: transcription regulator, PhoU: tentative inhibitor of pho-expression.*

8 P-availability and the importance of phosphatases

Although P_i and some small organic phosphate esters are readily available for phytoplankton, these compounds are usually present in only minute amounts in lakes. Dissolved organic phosphates are predominantly of large molecular weight or colloidal material (Bentzen et al., 1992). These are not ubiquitous (Jansson et al., 1988; Rijkeboer et al., 1991), and the colloidal-P is very stable and resistant against dephosphorylation (Olsson and Jansson, 1984). Nonetheless, the available fraction is actively utilised by bacteria and algae (Bentzen et al., 1992; Cotner and Wetzel, 1992). Particulate organic P is also found to be available to phytoplankton to a certain extent (Boström et al., 1988). In other words, when P_i is released from dead cells (detritus), or from dissolved substrates, it is quickly reabsorbed by living cells (Jansson et al., 1988). Most naturally produced organic phosphorus compounds, except phosphonate, are esters of orthophosphoric acid. The main forms of P in detrital matter are probably adsorbed P_i, sugar phosphates, glycerophosphate, polynucleotides and phospholipids (e.g. Pant and Reddy, 2001). The P-moiety in these compounds is only available after hydrolysis of the phosphate ester bonds. This reaction is carried out mainly by phosphohydrolases. These can be classified as phosphomonoesterases, phosphodiesterases, triphosphoric monoester hydrolases, hydrolases splitting anhydride bonds in phosphoryl-containing anhydrides, and

hydrolases splitting P-N bonds (Siuda, 1984). The term 'phosphatases' is mostly used synonymously with non-specific phosphomonoesterases, which have broad specificity towards different substrates. In addition to phosphomonoesters, nucleic acids may become available as P-source, although at a slower rate than phosphomonoesters (Boström et al., 1988). The phosphate forms a diester bond between nucleotide monomers, which can be hydrolysed by either alkaline phosphatases or the more specific 5'-nucleotidase (Bentzen et al., 1992). Other potential substrates for phosphatases are inorganic pyrophosphate, polyP, and short chain metapolyP (Siuda, 1984).

Acid phosphatases, on the one hand, have mostly been found inside cells, and are generally not repressed by orthophosphate (Jansson et al., 1988). Alkaline phosphatases, on the other hand, usually have an extracellular function, and their expression is sensitive to phosphate availability. The fact that alkaline phosphatases are synthesized under P-deficiency, in combination with the notion that the product of phosphatase activity, P_i, is readily assimilated by phytoplankton, provides the basis for the hypothesis that phosphatases have an essential function in the P dynamics of lakes (Jansson et al., 1988). According to Currie and Kalff (1984), a consequence of the hypothesis that algal P comes predominantly from organic P, is that there is no theoretical reason to expect algal growth to depend on ambient P_i-concentrations, nor to expect that resource competition among freshwater phytoplankton should depend on interspecific differences in P_i-uptake kinetics alone. Rather, resource competition depends on the combination of uptake-, storage-, and conversion-capacities in relation to the availability of P. In P_i-depleted conditions, the factor that limits P-availability is probably the rate at which organic P becomes available as substrate for alkaline phosphatases. Ubiquity of inducible alkaline phosphatases among phytoplankton supports these conclusions for subsaturating P-loads. In general, alkaline phosphatase activity reduces the residence time of P in the water phase.

9 Alkaline phosphatases as indicator for P-deficiency

The presence and activity of alkaline phosphatases can be used as an indicator for P-deficiency, whereas direct measurements of the P_i-concentration do not give the relevant information (Dignum, 2003). Theoretically, the production rate of derepressible phosphatases should give the best measurement of P-deficiency. In practice, the potential phosphatase activity has been used as an indicator of P-deficiency. Potential phosphatase activity is assayed with a suitable artificial substrate (e.g., a P-ester giving a coloured product upon dephosphorylation), at substrate concentrations near the saturation concentration to allow the reaction to proceed at maximum rate. The phosphatase activity measured in routine assays cannot be used for predictions of in situ hydrolytic activity, for the following reasons (Jansson et al., 1988). Firstly, natural substrate concentrations, and thus conversion rates may be different from those used in the assays. Secondly, pH and temperature used in the assay are not comparable to those in lake waters. Thirdly, the artificial substrates may not be representative of the natural substrates. Although bulk phosphatase activity has been widely used as a means of diagnosing P-deficiency, an

important flaw in its use as a P-deficiency indicator is the uncertainty about the origin of the enzymes (Jansson et al., 1988). Cells may actively excrete dissolved enzymes, and another important but non-specific contribution is delivered from the cytoplasm of dying and disintegrating cells (Jansson et al., 1988). Also, there is uncertainty whether the activity comes from (constitutive) acid phosphatases, or (derepressible) alkaline phosphatases. It is therefore crucial to trace phosphatase activity back to the cells that produced the enzyme. The ELF-97^{TM} phosphate (ELF-P) substrate yields intensely green fluorescent precipitates of ELF-97^{TM} alcohol (ELF-A) upon enzymatic dephosphorylation. Applications of the ELF substrate to study the phosphatase activity of freshwater phytoplankton were recently published (Rengefors et al., 2003; Nedoma et al., 2003; Dignum et al., 2004). Figure 3 shows some examples. The cells show intense fluorescence of ELF-A only under P_i-limited conditions (Fig. 3B,D).

10 Flow cytometry to detect P-deficiency

Flow cytometry offers several advantages over traditional methods used in aquatic microbial ecology: phytoplankton data on large numbers of samples can be obtained in a short time, on a scale relevant for individual cells or colonies. Various parameters are analysed simultaneously, and gated amplification allows separate investigation of species groups. Furthermore, the sorting capacity of some flow cytometers offers the possibility to select (sub)populations for further analysis elsewhere (Vrieling and Anderson, 1996; Collier and Campbell, 1999; Vives-Rego et al., 2000). Spectral characteristics of endogenous pigments (autofluorescence) provide the means to discriminate between phytoplankton groups. The emission spectra of phytoplankton show red fluorescence of the photosynthetic pigment chlorophyll *a*, with a maximum at about 685 nm. In addition, cyanobacteria have accessory pigments, the phycobiliproteins, which emit in the orange and red regions of the spectrum. Phycoerythrin has an emission maximum in the 560-590 nm or in the 620-650 nm range, depending on the kind of chromophore it carries. Phycocyanin has an emission maximum at about 652 nm. The orange-red autofluorescence is therefore a highly selective property for the detection of cyanobacteria (Hofstraat et al., 1991). An example of the flow-cytometric separation of several phytoplankton groups from Lake Loosdrecht (The Netherlands) is shown in Figure 4A. The lake's phytoplankton community consists for more than 90% of filamentous cyanobacteria with very similar morphological characteristics. Due to differences in pigmentation, clusters of the dominant species *Limnothrix* sp. (with phycocyanin; cluster III in Fig. 4A) and the second-most abundant species in the lake, *Prochlorothrix hollandica* (without phycocyanin; cluster II in Fig. 4A), are separated. Application of the ELF-P staining method to phytoplankton extends the use of flow cytometry (González-Gil et al., 1998; Rengefors et al., 2001; Nedoma et al., 2003; Rengefors et al., 2003; Dignum et al., 2004). The green ELF-A fluorescence is maximal at 520-530 nm. In P-depleted batch-cultures of *Limnothrix* sp. isolate MR1 about 50% of the trichomes showed ELF-A fluorescence, whereas only a few trichomes fluoresced in nutrient-replete cultures (Dignum et al., 2004). In a laboratory scale enclosure initially started up with natural lake water in spring

2001, but with continuously added P_i in excess, about 45% of the *Limnothrix*-trichomes initially showed ELF-A fluorescence. While the number of *Limnothrix*-trichomes increased quickly, about 15% had ELF-A fluorescence after one week, and only 5.5% after two weeks (Dignum, 2003). These results show that growth of the *Limnothrix*-population was P_i-limited, and that the percentage of trichomes with ELF-A fluorescence reflects the extent of P_i-limitation for a natural population of filamentous cyanobacteria.

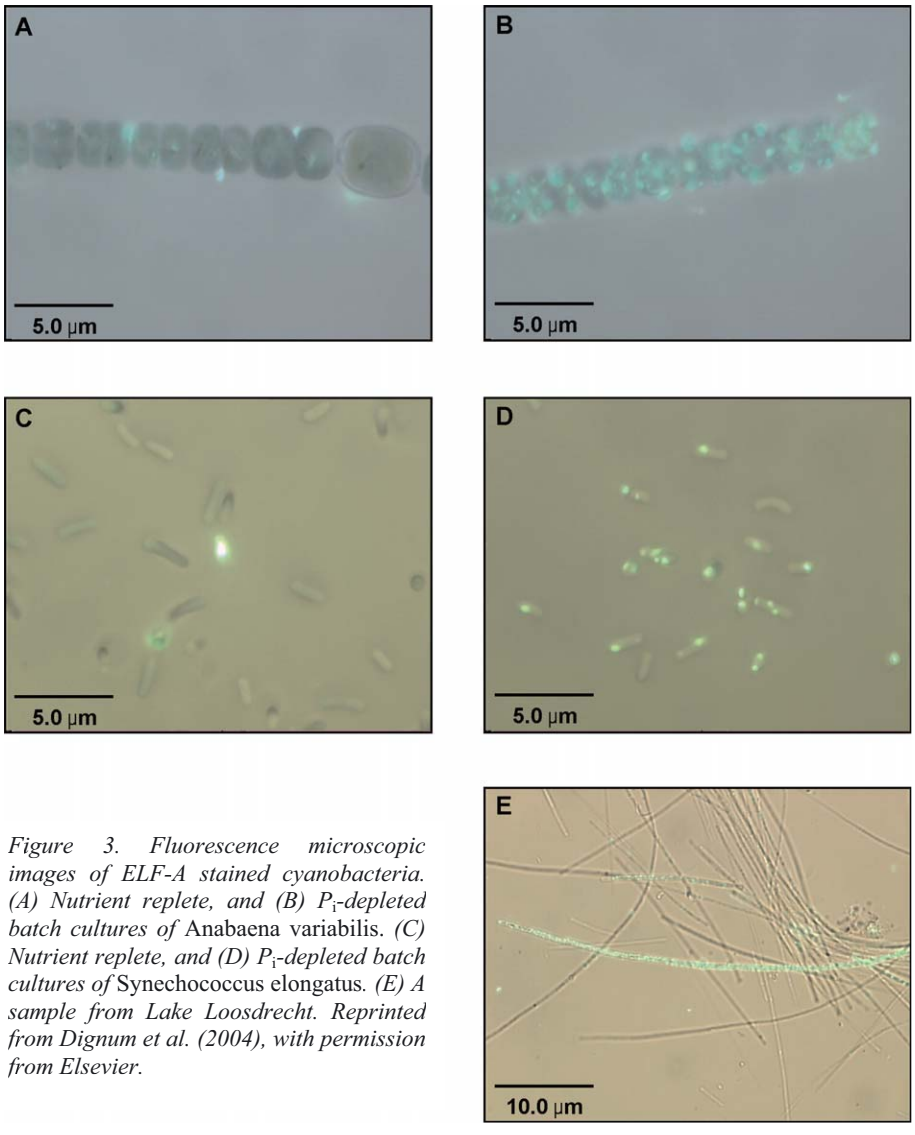

Figure 3. Fluorescence microscopic images of ELF-A stained cyanobacteria. (A) Nutrient replete, and (B) P_i-depleted batch cultures of Anabaena variabilis. *(C) Nutrient replete, and (D) P_i-depleted batch cultures of* Synechococcus elongatus. *(E) A sample from Lake Loosdrecht. Reprinted from Dignum et al. (2004), with permission from Elsevier.*

Figure 4 shows flow-cytometric analysis of a sample from Lake Loosdrecht with and without ELF-staining. The additional cluster in Figure 4B is the cyanobacterium *Aphanizomenon*, which was not visible without ELF-staining (Fig. 4A). The ELF-A versus phycocyanin fluorescence histogram of the ELF-stained sample (Fig. 4D) shows that about 25% of the *Limnothrix*-trichomes had ELF-A fluorescence (intense fluorescence at 525 nm) at that moment. Further ELF-measurements with lake samples revealed that the growth of *Limnothrix* sp. in Lake Loosdrecht was P_i-limited during part of 2001, that there was distinct seasonal variation in the extent of P_i-limitation, and that there was heterogeneity in P_i-limitation within the *Limnothrix* population (Dignum, 2003). Furthermore, about 15% of the *Prochlorothrix hollandica*-trichomes in Figure 4 show ELF-A fluorescence. Batch cultures of *P. hollandica* PCC 9006 were shown to have low, non-inducible phosphatase activity (Dignum et al., 2004). The two species thus seem to employ different strategies towards low P_i-availability. *Limnothrix* can be described as a scavenging and affinity strategists, whilst *Prochlorothrix* can be described as an affinity and storage strategist (Ducobu et al., 1998). In conclusion, the ELF-method is a useful indicator for rapid monitoring of alkaline phosphatase activity at the level of the individual cell by means of flow cytometry.

11 Discussion

Diagnostic tools for the phytoplankton nutrient status should comprise fluorescent stains that are optimised for broad applicability, nutrient status specificity, and non-interference with the autofluorescence. Cell surface marker proteins are adequate indicators of nutrient limitation because their expression is highly sensitive to changes in nutrient concentration; their expression is specific for each nutrient, can potentially be quantified per cell, and is possibly taxon specific (Scanlan and Wilson, 1999). Compared to the immunological approach (Scanlan et al., 1997; La Roche et al., 1999), enzymatic assays are applicable to a wide variety of species and may have an enormous amplification factor. The ELF-method satisfies the criteria for diagnostic tools proposed by Falkowski et al. (1992): the tool is broadly applicable in the field across phylogenetic lines, it identifies a process that imposes a truly physiological limitation, and it is uniquely affected by a specific limiting factor. The ELF-method also has some important drawbacks, however: Not all species are responsive, even when they have inducible phosphatase activity. Distinction between inducible, extracellular alkaline phosphatases and constitutive, intracellular acid phosphatases is not possible. Furthermore, ELF-A fluorescence cannot be quantified in terms of the production rate of derepressible phosphatases, which would provide the best measurement of P-deficiency. It is not sure if a decrease in ELF-A fluorescence is caused by an alleviation of the P-limitation, or conversely, by an extreme P-limitation hampering de novo synthesis or accessibility of alkaline phosphatase. Other aspects of the physiological status should be included in order to get a complete picture of the present conditions and the nutrient history. The affinity, scavenging, and storage status give complementary information, reflecting the present growth conditions and the cells' nutrient history.

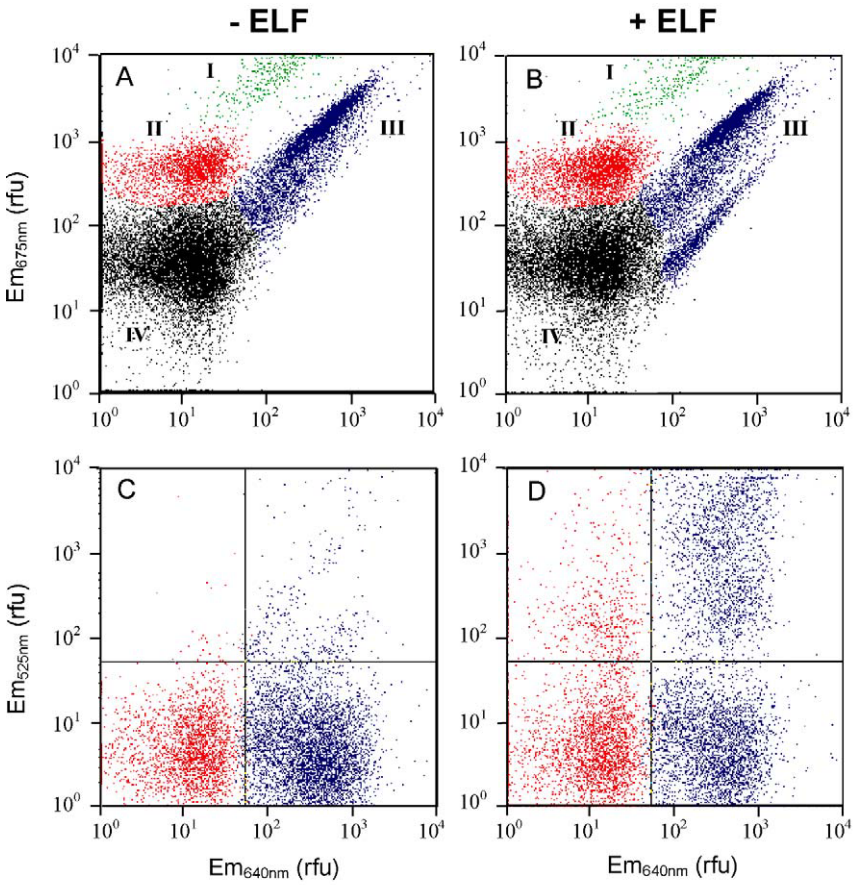

Figure 4. Flow-cytometric separation and ELF-staining of major phytoplankton groups from Lake Loosdrecht (The Netherlands). (A,B) Two-dimensional histograms showing chlorophyll fluorescence (675 nm) versus phycocyanin fluorescence (640 nm), in (A) samples without ELF-staining, and (B) samples with ELF-staining. Species clusters are: I, green algae and diatoms; II, prochlorophytes (here the filamentous Prochlorothrix hollandica*); III, cyanobacteria (predominantly filamentous* Limnothrix *sp.); IV, detritus (dead cells). (C,D) ELF-fluorescence (525 nm) versus phycocyanin fluorescence (640 nm) histograms of species clusters II and III, in (C) samples without ELF-staining, and (D) samples with ELF staining.*

Future work should involve development of indicators for the P-affinity status and the P-storage status, for example by creating antibodies against PstS or porins, and by optimising quantitative staining of internal polyP-stores. Furthermore, to study growth limitation, detailed knowledge about population-specific growth rates is required. By linking fluorescence-activated cell sorting (FACS) and isotope-ratio mass spectrometry through in-line pyrolytic methylation, phytoplankton can be probed for their population-specific $\delta^{13}C$ signature. This novel method allows assessment of population-specific growth rates from phytoplankton cells that are

labelled with ^{13}C-CO_2 (Pel et al., 2003). Alternatively, the cell division cycle can be monitored by fluorogenic staining of DNA as a measure for the growth rate. Parpais et al. (1996) have reported a remarkable, irreversible arrest of the marine cyanobacterium *Prochlorococcus* sp. in the DNA replication (S) phase, when subjected to P-starvation. Moreover, these authors suggested that examination of the cell cycle of natural populations could be used to determine whether they are limited by P (Parpais et al., 1996; Veldhuis et al., 2001).

To make these fluorescent stains successful tools in water management, affordable and amenable flow cytometers are required (e.g. Dubelaar et al., 1999). In conjunction with the possibility to separate populations according to their contents of endogenous fluorescent pigments, these tools provide insight in factors that constrain productivity, and also affect community structure and species succession (e.g. La Roche et al., 1999). This approach allows rapid assessment of the limiting factors that determine the growth of harmful cyanobacteria.

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