Phosphate uptake proteins as markers for the nutrient status of freshwater cyanobacteria
Dignum, M.

Citation for published version (APA):
Amsterdam: IBED, Universiteit van Amsterdam
Chapter 1

General Introduction
Contents chapter 1

§ 1 The nutrient status of phytoplankton
   Nutrient limitation vs. eutrophication
   The toolbox: how to monitor the nutrient status
   What type of information can we use to monitor the nutrient status?
§ 2 Cyanobacteria and their cell envelope
   General description
   The cell envelope
§ 3 Importance of phosphate
   Key roles of phosphate
   Phosphate incorporation
   Semantics
   Is $P_i$ the limiting factor for growth of phytoplankton in lakes?
   Methods to determine nutrient limitation in phytoplankton
§ 4 Adaptation to P-deficiency
   Adaptation strategies to P-deficiency
   The $pho$-operon: a high-affinity $P_i$ uptake system
§ 5 Technique and study site
   Flow cytometry applied to phytoplankton
   History of the Loosdrecht lakes
§ 6 Scope and outline of this thesis
§1 The nutrient status of phytoplankton

Nutrient limitation vs. eutrophication

The presence and growth of phototrophic microorganisms in surface water environments (phytoplankton) 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, phosphorous (P), nitrogen (N), and iron have attracted the most attention. Competitive exclusion theory states that the number of species on the same trophic level cannot exceed the number of limiting resources. This may be true for extremely low nutrient levels, low nutrient levels (oligotrophic conditions), however, allow biodiversity to be high because complex nutritional interrelationships are required to ensure a constant flow of materials and energy through the ecosystem. The competition process in such a complex community may generate non-equilibrium dynamics, sustaining a large number of coexisting phytoplankton species (Huisman and Weissing 1999). This situation, high biodiversity and low turbidity, is associated with a high water quality. At higher levels of nutrients, one type of phototrophic microorganism often becomes dominant: the cyanobacteria. Over-abundance of these organisms has a suffocating effect on the aquatic ecosystem. The increased availability of essential nutrients is mostly caused by our agricultural and industrial habits. This nutrient enrichment and its ecological consequences are summarised in the term eutrophication, which had its foundation around 1920. A simplified distinction was made between oligotrophic lakes (deep and clear) and eutrophic lakes (shallow and productive). The understanding of lake evolution was that, through progressive siltation and accumulation of catchment export, oligotrophic lakes slowly turned into eutrophic ones (Reynolds 1998 and references therein). When lakes and rivers became enriched with nitrogen and phosphate, this was regarded as accelerated eutrophication. The level of nutrient enrichment and consequent growth potential can be termed nutrient status.

The toolbox: how to monitor the nutrient status

Attempts to control algal blooms have focused primarily on managing the reduction of biomass (e.g. Hosper 1997). This initial approach was often appropriate, but in many cases restoration measures have failed to take immediate effect. Now a more balanced approach is 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. Indicators for the physiological and compositional response of phytoplankton cells to nutrient depletion reflect their nutrient status. The term ‘diagnostic tool’ has been used to denote a signal (or procedure) that empirically identifies
the symptoms of an environmental constraint on phytoplankton growth rates (Falkowski et al. 1992; Mann et al. 1994). To account for community complexity, assessment of the nutrient status of individual cyanobacterial populations is required. Taking population heterogeneity into account, measurements down to the single cell level are desirable. A direct approach to address this problem 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 (Palenik and Wood 1998; Scanlan et al. 1997).

What type of information can we use to monitor the nutrient status?
The common characteristics of molecular markers of the physiological status of the cell are that they are molecules (e.g. proteins, lipids, metabolites, mRNA) whose expression can be related to its general physiological state or to its physiological state relative to specific nutrients. The biochemical information available in organisms can be categorized into three major domains: macromolecules, small molecules, and isotope ratios (Brenna, 2001). The results from whole genome analysis reveal an overview of all the genes in model organisms (genomics). The next, elaborate, step is to identify the conditions under which certain genes are expressed, and to determine where in the cell the consequent proteins carry out their function (proteomics). The metabolic activity of proteins is reflected in the presence and turnover rates of metabolites; the study of these is referred to as metabolomics. Natural isotopic variability, particularly on an intramolecular level, gives additional physiological information. Naturally occurring elemental isotopes have fixed ratios. However, biochemical reactions bring about isotopic fractionation, dependent on enzymatic conversion rates. The changed isotope ratios therefore give information about the growth conditions of living cells (e.g. Laws et al. 1995; Popp et al. 1998). This science can be called isotope physiology, or simply isotopics (Brenna, 2001).

§2 Cyanobacteria and their cell envelope

General description
Phytoplankton consists of diverse groups of phototrophic microorganisms, such as green algae, diatoms, and cyanobacteria. Cyanobacteria (synonym: Oxyphotobacteria) are oxygenic photoautotrophic bacteria. They harbour a combination of typically prokaryotic cell morphology and a photosynthetic apparatus analogous to that of phototrophic eukaryotes. Like higher plants, cyanobacteria possess two distinct chlorophyll \(a\) containing photosystems in specialised membranes, the thylakoids. These thylakoid membranes also carry a full respiratory chain with a cytochrome
aa3 type terminal oxidase (Peschek 1981). The assembly permits ATP formation by cyanobacteria in the light through oxygenic photo-phosphorylation and substantial photosystem-driven cyclic photophosphorylation as well through oxidative phosphorylation ("respiration") in darkness.

Eutrophication problems have discredited cyanobacteria. They are ancient organisms, however, and an important prerequisite for the way life has developed on our planet, as they are largely responsible for the accumulation of oxygen in our atmosphere (Dismukes et al. 2001). Moreover, the endosymbiont hypothesis states that the chloroplasts, which harbour the photosynthetic apparatus in plants, are derived from internalised symbiotic cyanobacteria. On the other side, a distinct feature of cyanobacteria is that they lack the membrane embedded chlorophyll $a$ and $b$ containing light-harvesting complex II. Instead, they contain cytoplasm exposed hydrophilic phycobilisome antenna systems with pigmented proteins (blue phycocyanin, red phycoerythrin, and allophycocyanin). Prochlorothrix hollandica is a special case in this respect, as it contains both chlorophyll $a$ and $b$ (the latter is normally a pigment of eukaryotic algae), but lacks the accessory phycobilins. This type of cyanobacteria is sometimes referred to as Prochlorophytes, or Oxychlorobacteria (Burger-Wiersma and Matthijs 1990; Matthijs et al. 1993).

Free-living cyanobacteria may be unicellular or form small aggregates (the Chroococcales, e.g. *Microcystis* sp.). Another type of cyanobacteria forms linear associations (trichomes or filaments), these are referred to as filamentous cyanobacteria (the Hormogonales). In this thesis I would like to make a functional distinction of two types of cyanobacteria: on the one hand laboratory model organisms, and on the other hand organisms that are ecologically relevant for lakes in the Netherlands. At the start of this project, sequence information was available for the genome of one unicellular freshwater cyanobacterium, *Synechocystis* sp. strain PCC 6803 (Kaneko et al. 1996). This organism (hereafter referred to as *Synechocystis*) is our main study model. In the following I will mention characteristics of this organism where relevant. More recently, the genome sequence for *Anabaena* sp. PCC 7120 was published (Kaneko et al. 2001). Genome sequences are expected for *Thermosynechococcus elongatus* BP-1 (in press), *Gloeobacter violaceus* PCC 7421 (annotation phase). Sequencing of *Synechococcus elongatus* PCC 7942 (formerly called *Anacystis nidulans* R2) is also in progress, presently three cosmids with about 33 kb each have been deposited in Genbank. This strain is genetically very closely related to *Synechococcus* sp. strain PCC 6301 (Golden et al. 1989). The phosphate uptake system of *Synechococcus elongatus* is relatively well characterized. Therefore this strain serves as a reference for the identification of phosphate-related proteins in *Synechocystis*: it will be referred to as *Synechococcus*. These unicellular cyanobacteria are rare in the Netherlands however, therefore other species have also been subject of study in this thesis; eutrophied
Dutch lakes are often dominated by various filamentous cyanobacteria, predominantly *Anabaena* sp., *Aphanizomenon* sp., *Planktothrix agardhii*, *Planktolyngbya limnetica* (synonym *Oscillatoria cf. limnetica*), and *Prochlorothrix hollandica*.

**The cell envelope**

The work presented in this thesis has focused on the periphery of the cell 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 to the cell surface, to minimise the extent of manipulation of the sample. Both protection against osmosis and specific transport of nutrients are functions of the cell envelope, which acts as a molecular sieve. The thick multi-layered envelopes of cyanobacteria form a considerable mechanical and permeability barrier for most larger molecules (Hoiczyk and Hansel, 2000). Further functions associated with the outside of the cell are cell adhesion, motility, and protection against parasites. The cyanobacterial cell wall has features of both Gram-positive (a thick peptidoglycan layer with a high degree of cross-linking) and Gram-negative bacteria (an outer membrane) (Jürgens et al. 1983). In the following I will briefly describe the cell envelope from outside to inside (see Figure 1).

1. **Surface layers**

In many cases cyanobacteria are surrounded by external polysaccharide layers, such as slime, capsule or sheath (Weckesser and Jürgens, 1988). However, many unicellular cyanobacteria are devoid of layers outside of the outer membrane (Vaara 1982). The latter author urges the distinction between glycocalyx layer (thin insoluble polysaccharide layer closely associated with the outer membrane and with a diffuse outer boundary), and proper sheath (well-defined electron-dense layer loosely surrounding cells and cell groups). *Synechocystis* possesses a well-defined external layer as seen in electron microscopic images (Engels et al. 1997), referred to as slime by these authors. It is presently unclear, however, what comprises this layer. The few cyanobacterial exopolysaccharides that have been defined structurally show a remarkable resemblance to plant carbohydrates, like cellulose and pectin (Hoiczyk and Hansel, 2000). A permanent carbohydrate sheath may be produced for protection against unfavourable conditions such as desiccation (Hoiczyk 1998). Apart from protection, the cell surface has a role in gliding motility of some filamentous cyanobacteria (Hoiczyk and Baumeister, 1995), and the ‘swimming’ of marine *Synechococcus* strains (Brahamsha 1996). The cells are not propelled by flagella, which have never been found in cyanobacteria (Hoiczyk and Hansel, 2000). It is presently unknown how thrust for swimming is generated.
in these organisms, but one surface-associated protein, SwmA, definitely plays a role in it (Brahamsha 1996). Gliding is probably driven by the excretion of slime composed of carbohydrate fibrils (Hoiczyk 1998), guided into one direction by helically arranged surface fibrils, consisting of a single rod-shaped protein, called Oscillin. No homologues of this protein have been found in the *Synechocystis* genome (Hoiczyk and Baumeister, 1997). Pili-like structures have been observed on the surface of both unicellular and filamentous cyanobacteria. Various types of pili facilitate phototactic motility of *Synechocystis* (Bhaya et al. 1999).

The surface fibrils, if present, are arranged on top of a surface-layer called S-layer. These S-layers are two-dimensional crystalline arrays formed by a single species of (glyco)protein that covers the entire surface of a cell. They are involved in cell adhesion and surface recognition, and also function as protective coats, molecular sieves, and as molecule and ion traps. S-layers are common among both Bacteria and Archaea. In cyanobacteria, a sheath always covers the S-layers. The structure shows resemblance with the internal gas vesicle membranes of buoyant cyanobacteria. S-layers were found in several *Synechocystis*, *Synechococcus*, and *Microcystis* strains, but are more rare among filamentous cyanobacteria (a complete overview of publications from 1972-2000 is given in Šmarda et al. 2002). *Synechocystis* PCC 6803 has an S-layer (Vaara 1982), comprised of a hitherto unknown exocellular protein containing a surface layer homology (SLH) domain (Leibovitz et al. 1997). These domains connect the S-layer proteins to cell wall components, thereby stabilising the cell wall (Hoiczyk and Hansel, 2000).

2. The outer membrane

![Diagram of outer membrane components](image)

**Figure 1.** Electron microscopic photograph of *Synechocystis* sp. PCC 6803; Uranyl acetate stained thin section of glutaraldehyde fixed cell (Photograph K. A. Sjollema, by courtesy of J. J. van Thor).
The outer membrane (OM) protects the cell from harmful agents such as proteases, bile salts, antibiotics, toxins and phages, 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. The LPS of photosynthetic bacteria frequently contain O-methyl sugars, in contrast with their rare occurrence in Enterobacteria (Rudrapatnam et al., 1978). The LPS is anchored in the outer membrane by binding to outer membrane proteins, and by non-covalent cross-bridging of adjacent LPS molecules with divalent cations (Benz and Bauer, 1988). Pore-forming proteins, called porins, mainly determine the permeability of the outer membrane. The *Synechocystis* genome encodes six homologues to the outer membrane porins SomA and SomB with highly conserved SLH domains (Hoiczyk and Hansel, 2000). The function and expression pattern of these genes is yet unknown. 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 depends on an intact peptidoglycan layer in the periplasmic space. The outer membrane contains structural lipoprotein, which links to the underlying peptidoglycan.

3. The periplasmic space and the peptidoglycan layer
The layer between inner and outer membrane of Gram-negative bacteria is defined as the periplasmic space, and is estimated to have a volume of 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). The periplasmic space is highly anionic as compared to the external medium, however, mainly due to the anionic membrane-derived oligosaccharides (Hagge et al. 2002). 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 into the medium by applying cold osmotic shock (Heppel 1967, Fulda et al. 1999). The periplasm contains different 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. 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 contains the peptidoglycan layer, or actual cell wall. The peptidoglycan (synonym: murein) layer primarily functions in maintaining the cell shape and withstands the very high internal osmotic pressure in dilute environ-
ments. It consists of a network of amino sugars and amino acids. Long strands of amino sugars are covalently cross-linked by pentapeptides, thus forming a giant, hollow, net-like molecule. Regarding the high degree of cross-linking, cyanobacteria are similar to Gram-positive bacteria. Teichoic acid, however, a phosphate-rich constituent of the cell walls of Gram-positive bacteria is missing in cyanobacteria.

4. The cytoplasmic membrane
The cytoplasmic membrane acts as a real diffusion barrier, and contains a large number of uptake systems for hydrophilic substrates. 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 was found to encode 92 transporters. The number of ATP-dependent (see below) transporters was ten times the number of proton motive force dependent transporters. This in contrast to *E. coli*, which has equal numbers of the two types. Another relevant feature of the cytoplasmic membrane is its role in responding to fluctuations in the environment. Two-component regulatory systems allow bacterial cells to sense specific changes in their surroundings, with sensor proteins located in the cytoplasmic membrane (Ronson et al. 1987; Parkinson and Kofoid, 1992). The signal is transferred over the membrane and passed on to the transcriptional apparatus by corresponding regulatory proteins.

§3 Importance of phosphate

*Key roles of phosphate*

The notion that phosphate may be a limiting factor for algal growth goes back more than a century. For the highlights in the early research on the role of P in freshwater ecosystems I refer to an excellent summary in a lecture by Shapiro (1988). A more recent review has been written by Correll (1999). I do not attempt here to give a review on the literature, but rather sketch the function that P has in phytoplankton cells. The important role that phosphate plays in cells is apparent on three levels:

1. **Structure**

Phosphate forms covalent ester links between carbohydrate monomers, creating a rigid structure. Organically bound P is found in the peptidoglycan fraction of cell wall, probably to covalently bind polysaccharide to the peptidoglycan matrix (Jürgens et al. 1983). These compounds give the cell its shape and strength. Phosphate is also part of the structure of nucleic acids: it connects the ribonucleoside monomers to 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 LPS of cyanobacteria also contains small amounts of bound P (Schmidt et al. 1980; Hoiczek and Hansel 2000).

2. Energy
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. Also, many intracellular metabolites contain a phosphoryl-group.

3. Information
Environmental and internal variables provide important information for the central adaptive responses of bacteria. Prokaryotic signalling systems are very complex, having multiple components, interconnections with other regulatory circuits, and feedback loops (Parkinson and Kofoid, 1992). Nevertheless, these networks contain transmitter and receiver modules. The communication between transmitter and receiver 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 of enzyme systems (regulatory component). The sensor is usually a transmembrane protein, which binds to ligands with a 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 a promoter. In Synechocystis at least 80 TCRS pairs have been found in the genome (Mizuno et al. 1996). About ten major polypeptides can be \(^{32}\text{P}\)-phosphorylated in Synechococcus and Synechocystis, and many more when \(^{32}\text{P}\)-labeled ATP is used as a labelling agent (Mann 1994). 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). According to these authors, signal transduction pathways by phosphoryl transfer may meet all the criteria of a
neural network. Recent work to detect in vivo cross-talk in *E. coli*, however, has not confirmed these ideas experimentally (Verhamme et al. 2002).

**Phosphate incorporation**
Orthophosphate (H$_2$PO$_4^-$, HPO$_4^{2-}$, or PO$_4^{3-}$), synonymous with inorganic phosphate (abbreviated P$_i$), is the only directly available P source for phytoplankton. 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$_i$ in- and outside the cells, and the capacity to use a variety of phosphorylated compounds. P$_i$ incorporation in cyanobacteria takes place in several steps (Falkner et al. 1989), schematised in figure 2.

![Diagram of phosphate incorporation](image)

**Figure 2.** Cellular phosphate incorporation, numbers indicate the following enzymes:

Organic or inorganic phospho-esters are converted into P$_i$ by the activity of Alkaline Phosphatases (AP) outside the cell, at the cell-surface or in the periplasm. Transport through the cell membrane is an active, energy dependent process, but net fluxes are low compared to sodium or bicarbonate uptake, and are not likely to impose a large metabolic cost to the cells (Ritchie et al. 1997). 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 then becomes insufficient. $P_i$ is subsequently converted into ATP. The energy required to drive this process is provided by photo-phosphorylation coupled to proton flux across the thylakoid membranes (Simonis and Urbach, 1973). Excess $P_i$ is stored inside the cells as polyphosphate granules (PolyP), providing enough for growth of about 6 generations (Falkner et al. 1989). PolyP formation is a close-to-equilibrium reaction that does not require an energy source apart from that provided by ATP itself.

**Semantics**

The terminology in studies concerned with bacterial responses to lack of nutrients has different connotations, and can therefore be confusing. For clarity, I wish to define the terms deficiency, depletion, deprivation, limitation, starvation and stress. The term nutrient deficiency has long been used as a general term to describe the physiology of cells with a lack of a certain nutrient (e.g. Healey, 1973).

Alternatively, nutrient deprivation is used as a general term (Matis et al. 1989). Nutrient depletion is specifically defined from an experimental point of view, meaning a batch culture with a reduced amount of one nutrient. In a batch culture, the physiology of the cells rapidly progresses through different stages of response to the lack of nutrient (Healey and Hendzel, 1975; Graziano et al. 1996). The term ‘nutrient stress’ is often used for the general response that can be observed in the metabolism or morphology of the organisms when confronted with a low level of nutrients; this term seems to be anthropomorphic, and does not consider the fact that sub-optimal growth is the natural condition for most microorganisms, while growing at a maximal rate could also be seen as causing ‘stress’. Similarly, the term ‘hunger state’ was recently proposed for the specific cellular responses that accompany growth at nutrient levels in between excess (replete) and starvation (Ferenci 2001). Nutrient starvation is a case of severe shortage of nutrient, eventually causing cell death and survival responses. Nutrient limitation was defined to specifically refer to situations where the growth rate is balanced, but controlled by a well known, sub-maximal availability of a nutrient in question, especially in continuous culture (Herbert et al 1956; Droop 1973; Matis et al. 1989).

The term nutrient limitation itself is subject to some confusion in the ecological literature (Hecky and Kilham 1988). It is important to distinguish between limitation of biomass production (Liebig’s definition) and limitation of instantaneous growth rate (Falkowski et al. 1992; Beardall et al. 2001). This will be explained in more detail in the next paragraphs. To contemplate another potential source of confusion, consider the following situation: cells can sense a lack of $P_i$ in the environment, while at the same time they have sufficient $P_i$ for growth inside the cells, due to internal reserves. Some other factor could then be limiting the growth rate, while at the same time the $P_i$-concentration is under the threshold concentration at
which specific hunger responses are induced. This leads to another set of confusing terms. Cells constantly produce constitutive enzymes, independent of an activator or deactivator. Induction means that synthesis starts in the presence of suitable inducers. Repression occurs when a compound, often the end product of the enzyme-catalysed reaction pathway, turns off the enzyme synthesis. Consequently, derepression is the onset of enzyme production after the depletion of the repressor (Jansson et al. 1988). High levels of $P_i$ repress the synthesis of proteins involved in high affinity $P_i$ uptake; hence the expression of this system when $P_i$ is depleted should be referred to as derepression. In many articles, however, the term induction is used synonymously with the term derepression. Inhibition takes place when a compound reacts with the enzyme itself and stops its activity. For example $P_i$ is a common inhibitor of alkaline phosphatase (AP), and competes with phosphate esters for the active sites of the enzyme, a mechanism called competitive inhibition (Jansson et al. 1988).

**Is $P_i$ the limiting factor for growth of phytoplankton in lakes?**

Since the beginning of eutrophication management in the 1970’s there has been general agreement that phytoplankton biomass production is limited by the total availability of phosphate in the majority of lakes. In experimental lakes both concentrations of chlorophyll and carbon varied proportional to the $P_i$ concentrations (e.g. Schindler 1977). The reason for this is that organisms can draw on the massive atmospheric sources and sinks for carbon and nitrogen to maintain, on average, C:N:P ratios to meet their requirements for growth. The energy requirements of phototrophic organisms are met by light irradiance, which is ubiquitous in summer, but can be limiting in winter. No external mechanisms exist for phosphorus, which has no gaseous atmospheric cycle. It follows that $P$ ultimately controls phytoplankton abundance in aquatic environments. Even while a sudden increase in the phosphate input (P-pulse) may cause algae to show symptoms of N- or C-limitation, there are long-term processes at work in the environment which cause these deficiencies to be corrected, leaving phytoplankton growth proportional to the concentration of phosphate (Schindler 1977; Hecky and Kilham 1988). Any single species within a community however, can be limited by its growth coefficient, its loss terms (dilution, sedimentation, physiological death and grazing), or by both. I.e. when a population is prevented from increasing, presenting a limitation of primary production, this does not automatically mean a growth rate limitation. Population increase can occur at both low and high cellular growth rate; therefore a low abundance does not mean that the cellular growth rate is limited in any way. Based on the potential complexity of population dynamics, all the phytoplankton populations are - or given their capacity for exponential increase, soon must be - limited by something. It is simplistic to assume that all the species in a community
are limited by a single factor. In fact, multiple resource limitation of phytoplankton growth has been demonstrated both theoretically and empirically (Hecky and Kilham, 1988; Van den Berg, 1998; Huisman and Weissing 2001).

**Methods to determine nutrient limitation in phytoplankton**

Several classical approaches for detection of nutrient limitation in phytoplankton have been described (e.g. reviewed in Hecky and Kilham 1988; Beardall et al. 2001). **Dissolved nutrient concentrations** present negative evidence: low nutrient concentrations are likely to limit algal production. However, phytoplankton species and communities have such high affinity for N and P that nutrient limitations occur at concentrations not analytically detectable. **Cellular elemental ratios** have often been used to indicate nutrient limitation. However, these ratios are fortuitous consequences of the absolute availabilities. They are not specific for one element, but are related to carbon. Changes in light intensity can considerably influence the C-content of the cells (Riegman and Mur 1986). Light conditions also significantly influence the N-content of the cells (e.g. Zevenboom et al. 1980). **Nutrient enrichment bioassays** are operational tests in which one or more nutrients are added to a volume of water to determine if algal growth is stimulated. The test result is likely to be dependent on the complexity of the natural components used in the system (whole lake nutrient enrichment; laboratory scale enclosure; continuous culture). Higher complexity-level systems, especially natural systems are capable of much more complex responses at longer time scales. The only level of concern to aquatic resource managers is the highest level, but inferences are often made based on evidence from lower complexity-level test systems. Furthermore, these experiments reflect a ‘Liebig’ limitation, indicating that if growth continues, one nutrient will eventually limit the total amount of biomass (Beardall et al. 2001). 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. Note the analogy with a continuous culture: in steady state the growth rate of the cells equals the dilution rate of the culture. Problem is that the system must be very well defined and all the nutrient inputs must be measured, which is difficult to apply for a natural system. **Physiological responses to nutrient limitation** can be used as direct 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), immuno-fluorescent detection of specific markers (Scanlan et al. 1997; see also the reviews by La Roche et al. 1999; Jochem 2000; Beardall et al. 2001), and enzymatic assays.
Adaptation to P-deficiency

Adaptation strategies
As P$_i$ is essential for the growth of microorganisms, and is required at relatively high levels, microorganisms will most probably have developed strategies to be able to cope with conditions of (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 evoked experimentally (Reynolds 1998). Generally, there are three functions that help to survive P$_i$-deficiency (Falkner et al. 1998): Firstly, the cells can extend the range of P-forms that are utilised. Secondly, the cells can activate uptake systems that operate efficiently at very low P$_i$ concentrations. Thirdly, P$_i$ can be stored inside the cells to secure the availability in times when P$_i$-uptake ceases, the cellular growth rate can then be independent of external concentrations, but proportional to the amount of P$_i$ stored in the cells. In the following, I explore well-known models for cellular growth to survey the possibilities that a cell has to adapt to low nutrient concentrations. In the Monod model, the cellular growth rate ($\mu$) depends on the external nutrient-concentration ($S$) and an intrinsic saturation constant ($K_S$), as expressed in the equation: $\mu = \mu_{\text{max}} \cdot S / (S + K_S)$. Growth can be seen as a complex interplay of enzymatic reactions and transport processes. Hence a relation exists between growth- and enzyme-kinetics. If a nutrient is available in growth-limiting concentration, then the uptake rate for that nutrient will be limiting the growth rate. The kinetics of enzymatic reaction or transport rate ($v$) is described by the Michaelis-Menten equation: $v = v_{\text{max}} \cdot S / (S + K_m)$, in which $v_{\text{max}}$ is the maximal uptake rate, and $K_m$ an affinity constant. A restricted amount of biomass can be formed from a certain amount of nutrients, which are anabolic substrates. This is expressed in $\mu = Y_x \cdot v$, in which $Y_x$ is the specific growth yield on nutrient $x$. This gives a new relation between the specific growth rate and nutrient concentration, resembling the Monod equation: $\mu = Y_x \cdot v_{\text{max}} \cdot S / (S + K_m)$. The problem with this equation is that $\mu_{\text{max}}$ is a constant, but $v_{\text{max}}$ is not. Furthermore, $K_S$ does not equal $K_m$. The model for uptake based on the Michaelis-Menten enzyme equation should be replaced by one that contains an element of product control (Caperon and Meyer 1972). The observable consequence is that it is not possible for an organism to completely deplete its environment of a limiting nutrient. In other words, a (thermodynamic) threshold exists where substrate uptake equals substrate leakage. The potential of a body of water for supporting further growth may depend as much on the nutrient already inside the cells, as that yet to be taken up (Droop 1974). Furthermore, Michaelis-Menten kinetics solely considers the present external substrate conditions, and not previous states (Droop 1974). The
Monod equation can be adapted for considerations on the internal $P_i$-concentration (Van Dien and Keasling 1998; 1999). Alternatively, the Droop model describes the dependence of the cellular growth rate on cell quota of the nutrient. In the Droop model, the growth rate depends on the cell quota (concentration of the limiting nutrient in the cell; $q$) and minimal cell quota (minimal concentration of the limiting nutrient in the cell; $q_0$) of the nutrient, thus taking internal storage into account: $\mu = \mu_{\text{max}} \cdot (1 - q_0 / q)$.

In phytoplankton ecology three different strategies to cope with temporary nutrient limitations are usually distinguished (Sommers 1989; Ducobu, 1998). These strategies may operate separately or in co-operation. Cells with a **growth strategy** use transient nutrient enrichment 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 but excess carboxylic acid groups (e.g. Lahooti et al. 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.

Cyanobacteria do not have these Gram-positive cell wall compounds. Although cyanobacterial peptidoglycan has phosphate links, as far as I know this strategy has never been described for cyanobacteria. Alternatively, cells can increase the maximum uptake rate ($v_{\text{max}}$), by making more uptake proteins, or changing the affinity of uptake proteins for their substrate. 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 affinity constant ($K_m$). Affinity strategists efficiently grow at low external nutrient concentrations. A potential response is the induced synthesis of a high-affinity uptake system for $P_i$. Directional attraction toward a nutrient could be considered as an extremely high affinity for this nutrient, or as a way the cells change the external nutrient-concentration. Although chemotaxis toward $P_i$ is known in several heterotrophic bacteria (e.g. Kato et al. 1994; Kusaka et al. 1997), I have found no literature about this phenomenon in cyanobacteria. The internal status of an algal cell with respect to the various essential nutrients can be as important as, or even more important, than the concentration of the nutrient in the environment (Droop 1973). 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 inside the cell, to control osmotic pressure. The internal supply may enable the cells to produce significant offspring when external nutrient concentrations are low. *Gloeotrichia echinulata* (an $N_2$-fixing, filamentous cyanobacterium
which forms spherical colonies) has a unique growth strategy and occupies an extreme position in the storage strategy. There is evidence that large *G. echinulata* colonies (diameter 1-2 mm) assimilate P in the sediments prior to their migration to the water column, and use only their internal P stores during epilimnetic growth. Alkaline phosphatase activity of the colonies was much lower than that of the rest of the phytoplankton in lake Erken (a moderately eutrophic, stratified lake in south-eastern Sweden). Organic P is thus not an important P source for this organism. On the contrary, organic P seemed to be released from the cells, which even might benefit other bacteria and algae (Istvánovics et al. 1993). To these strategies, a fourth can be added: the *scavenging strategy*. This strategy involves the induced synthesis of enzymes that transform phosphate-containing compounds into a form that the cell can use. The above-mentioned alkaline phosphatase activity is an example of this strategy. Organisms using the scavenging strategy are able to use organic derivatives of the required nutrient, or they may be able to use alternative inorganic forms of the nutrient.

**The pho-operon: a high-affinity $P_I$ uptake system**
Mechanistic information on $P_I$-uptake in cyanobacteria is relatively scarce. However, $P_I$-uptake has been extensively studied for some Gram-negative bacteria. As was discussed before, there are some important similarities between the Gram-negative bacteria and cyanobacteria. Therefore, studies for some Enterobacteria and Pseudomonads (specifically *Escherichia coli* and *Pseudomonas aeruginosa*) are discussed in the following. While *E. coli* is an intestinal bacterium that encounters high nutrient concentrations inside animals and low nutrient concentrations when excreted, freshwater cyanobacteria permanently live in nutrient-poor surroundings. Therefore most cyanobacteria have a greater need for high-affinity uptake systems. The hypothesis is that in $P_I$-deficient conditions, cyanobacteria will express a high-affinity $P_I$-uptake system, analogous to that of Enterobacteria and Pseudomonads.

The uptake of $P_I$ in *E. coli* occurs by kinetically distinct types of systems, with low affinity for $P_I$ (Pit systems; Harris et al. 2001), and with high affinity for $P_I$ (Pst system). *E. coli* cells grown in media devoid of $P_I$ deplete an internal $P_I$-pool, which is rapidly refilled when cells are presented with $P_I$. The two systems function simultaneously during the filling of the pool, but thereafter uptake takes place only by the low-affinity system. The $P_I$ is taken up as such, and esterification of $P_I$ starts soon after it has entered the cell. In a $P_I$-depleted environment, $P_I$ is taken up against a steep concentration gradient in an energy-dependent process (Medveczky and Rosenberg, 1971). Two single-gene Pit systems are currently known, of which PitA is constitutively expressed, whereas PitB is repressed in low $P_I$-concentrations (Harris et al. 2001). $P_I$-uptake by the Pit system depends on coupling to the
energised membrane state. Because of its constitutive expression, PitA is not a useful marker for the $P_1$-sensing status. The Pst system, however, is carefully regulated, and is ATP-driven (Rosenberg et al., 1977). Transport systems may be classified in different groups, as distinguished by biochemical and genetic methods, as well as by their mode of action and energization. The Pst system is an example of an active transport system sensitive to a cold osmotic shock. These active transport systems are equipped with periplasmic substrate binding proteins, which are released by the osmotic shock procedure (in Gram-negative organisms). Binding of the solute on the outside of the osmotic barrier may be regarded as the first step in the transport process (Boos, 1974). Periplasmic and outer membrane proteins that are $P_1$-depletion derepressible will be useful in this research, and therefore, the Pst system is of major interest.

During periods of $P_1$-depletion, alkaline phosphatase (AP) serves to scavenge $P_1$ from organic phosphomonoesters and polyP in the medium. The efficiency of $P_1$-uptake from environments with a low $P_1$-concentration is considerably enhanced by: channels through the outer membrane that exhibit strong anion selectivity, specific $P_1$-binding sites in these channels, and a high affinity $P_1$-binding protein in the periplasmic space (the affinity must be higher than that of the channels). Indeed, a high-affinity $P_1$-binding protein (PBP), which is localised in the periplasm, is synthesised at low $P_1$ concentrations in $E. coli$. Furthermore, in the outer membrane of $E. coli$ two proteins, OmpF and OmpC, are involved in the formation of non-specific aqueous pores through which hydrophilic molecules with a molecular weight of up to approximately 600 can pass via a diffusion-like process (Overbeek et al., 1983). At low external $P_1$ concentrations, an additional, $P_1$-selective pore protein is synthesised (PhoE), which is also co-regulated with AP (Tomassen and Lugtenberg, 1980).

The pathway for $P_1$-uptake into cells grown under $P_1$-depleting conditions is summarised in figure 3. It starts with the passage of $P_1$ or phosphorylated compounds through an outer membrane pore protein channel (PhoE or a non-specific porin) into the periplasmic space. In the periplasm, the phosphorylated compounds will be hydrolysed by AP, and $P_1$ will be captured by the pstS-encoded PBP, 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).

Participation of $P_1$ in the regulation of AP has been recognised since the first description of this enzyme in $E. coli$. Because the formation of AP stopped immediately after addition of $P_1$ to the medium, a negative feedback mechanism was suggested to occur with $P_1$ as a repressor (Horiuchi et al., 1959). Elucidation of the regulatory system for $P_1$-uptake started in the early 1960's, and at least three regu-
latory genes were mentioned to be required for repression of AP synthesis (Garen and Otsuji, 1964). Regulation of expression of the \textit{pst} and \textit{pho} genes is dependent on the regulatory proteins PhoR, PhoB, and PhoU. PhoR and PhoB are members of a family of bacterial two-component regulatory systems (TCRS). PhoR is an integral membrane sensor protein and PhoB is a soluble cytoplasmic DNA-binding effector protein. PhoU connects the ABC transporter with the TCRS, and is probably a regulatory inhibitor (Kim et al. 1996; Haldimann et al. 1998).

\begin{center}
\begin{tikzpicture}
  \node (PhoE) at (0,0) {PhoE};
  \node (PstS) at (-2,0) {PstS};
  \node (PhoA) at (2,0) {PhoA};
  \node (Pst) at (0,-2) {Pst};
  \node (PstABC) at (0,-4) {PstABC};
  \node (PhoR) at (2,-2) {PhoR};
  \node (PhoU) at (0,-4) {PhoU};
  \node (PhoB) at (2,-2) {PhoB};

  \draw[->] (PhoE) -- (PstS);
  \draw[->] (PstS) -- (PhoA);
  \draw[->] (Pst) -- (PstABC);
  \draw[->] (PstABC) -- (PhoR);
  \draw[->] (PhoR) -- (PhoU);
  \draw[->] (PhoU) -- (PhoB);

  \node[draw=none,fill=gray!30] at (-2,-2) {Cell-surface};
  \node[draw=none,fill=gray!30] at (0,-6) {Cytoplasmic Membrane};
  \node[draw=none,fill=gray!30] at (0,-6) {Cytoplasm};
  \node[draw=none,fill=gray!30] at (0,-3) {Periplasm};
  \node[draw=none,fill=gray!30] at (0,-1) {Outer membrane};
  \node[draw=none,fill=gray!30] at (-2,-1) {Cell-surface};
\end{tikzpicture}
\end{center}


Similar \textit{Pj}-uptake systems have been found in cyanobacteria, and have most extensively been studied for \textit{Synechococcus}. For this cyanobacterium the \textit{Pj}-binding protein SphX (Scanlan et al. 1993; Mann and Scanlan 1994), two AP enzymes 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. The genome of \textit{Synechocystis} contains two \textit{pst}-like operons, sll0679-0684 and slr1247-1250 (Kaneko et al. 1996). Recently, the AP encoding gene (\textit{phoA}) has been identified (sll0654), and the genes encoding regulatory proteins (\textit{Pj}-sensing histidine kinase \textit{phoR} (sll0337) and response regulator \textit{phoB} (slr0081)) have been described (Hirani et al. 2001). Expression patterns and location of components of the Pst systems in \textit{Synechocystis} are discussed in detail in chapters 3 and 4 (this thesis).
§5  Technique and study site

Flow cytometry applied to phytoplankton

Flow cytometry offers several advantages for aquatic microbial ecology: data on large numbers of cells can be obtained in short time, on a scale relevant for individual cells or colonies. The instrumental set-up allows multi-parametric analysis, and separate investigation of groups of cells (clusters) by gated amplification. Furthermore, the sorting capacity of the flow cytometer offers the possibility to transfer cells for further analysis elsewhere (see Vrielings and Anderson 1996; Davey and Kell 1996; Collier and Campbell 1999; Vives-Rego et al. 2000; Reckermann and Colijn 2000). Flow cytometry can on the one hand estimate the fraction of a population with a positive reaction of an indicator. On the other hand, it can provide a quantitative measure in terms of relative emission intensity (Jochem 2000). Furthermore, flow cytometry allows assessment of population heterogeneity (Davey and Kell 1996).

Central to flow cytometric phytoplankton analysis is the detection of inherent cell properties (e.g. Yentsch 1990; Jonker et al. 1995). Forward angle scatter of the excitation light is a measure for cell size, whereas side angle light scatter depends on cell shape. Moreover, spectral characteristics due to specific endogenous pigmentation (autofluorescence) provide the means to discriminate phytoplankton species (Hofstraat et al. 1991; Becker et al. 2002). 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 type of pigment present. Phycocyanin has an emission maximum of about 652 nm. Orange-red autofluorescence is therefore a highly selective property for the detection of cyanobacteria (Hofstraat et al. 1991).

Currently, efforts focus on determining nutrient availability on the individual cell level (Jochem 2000; Beardall et al. 2001) to improve the discriminative power of flow cytometry, by including physiology-related optical attributes. Such diagnostic tools should comprise fluorescent stains that are optimised for broad applicability, nutrient status specificity, and non-interference with the autofluorescence.

Furthermore, to be useful, they must identify those processes that 1) impose a truly physiological limitation, 2) are uniquely affected by a specific limiting factor, 3) are broadly applicable across phylogenetic lines, and 4) can be used in the field (Falkowski et al. 1992). One approach is the development of antibodies against antigens that are accessible in intact cells and are specifically under control of $P$-availability (e.g. Graziano et al., 1996; Scanlan et al. 1997; see also the review by La Roche et al. 1999). An alternative is the application of non-fluorescent sub-
strates that are processed by metabolic reactions of interest, and release strong fluorogens upon cleavage (Jochem 2000). In contrast to antibodies with mostly species-specific cross-reactivity, these enzymatic assays are applicable to a wide variety of species (e.g. González-Gil et al. 1998), and may have an enormous amplification factor.

**History of the Loosdrecht lakes**

To test applicability of an indicator for the P-status in the field a case study is presented in this thesis. The Loosdrecht lakes (52°11'N, 5°3'E) form a system of shallow interconnected lakes with a total water area of 14.5 km², and a mean depth of 1.85 m. The object of our studies is the central section of this lake area, called Lake Loosdrecht. The lakes originate from peat mining that started in 1633, and reached their present morphometry by the end of the 18th century. In that period the water was clear, strongly nutrient limited, without submerged plants (period 1800-1920). In 1932 the Amsterdam Municipal Waterworks started to use water from one of the adjacent polders for drinking water supply. Water from the adjacent Polder Bethune and the River Vecht was let in to supplement the water that was withdrawn. The river water was highly polluted with organic waste and nutrients from the city of Utrecht. The initial inflow of nutrients allowed submerged macrophytes to thrive (period 1930-1955). After continued nutrient loading, the trophic status changed to domination by phytoplankton (period 1960-1980), leading to low transparency of the water (Secchi depth < 0.4 m; Hofstra and Van Liere 1992). Remedial measures were then taken to reduce the external P loading rate. Construction of sewage systems in the catchment area, and installation of sanitary facilities in recreational areas gave some reduction in P input. In 1984 the inlet of water was replaced by chemically dephosphorylised (P₇-depleted) water from the Amsterdam-Rhine Canal, considerably reducing the P loading rate. RepARATION of a lock gave a further reduction of leakage of P-rich water from the river (Van Liere et al. 1991; Engelen et al. 1992; Van Liere and Janse 1992). Since 1984, these measures have only resulted in minor visible improvement of the water quality in the Loosdrecht lakes (for a recent review, see Gulati and Van Donk 2002). Additional measures are planned to dredge deeper areas were particulate matter can settle, and rerouting of nutrient rich water from adjacent polders (M. Ouboter, personal communication).
§ 6 Scope and outline of this thesis

Literature study and experiments at the Centre for Limnology at the Netherlands Institute of Ecology (NIOO) have shown that the availability of phosphorus for phytoplankton growth in Lake Loosdrecht is currently low. Restoration measures have probably led to growth-limiting $P_I$-concentrations, but the number of cyanobacteria has not declined over the years. The formulation of the problem, "How does phosphate availability determine the persistence of cyanobacteria in eutrophied freshwater systems?" is thus related to a regional social issue, "How do we get Lake Loosdrecht clear again?", pursued by local water managers such as the Water Management and Sewerage Service (Dienst Waterbeheer en Riolering; DWR), and the Municipal Waterworks Amsterdam (Gemeente Waterleidingen Amsterdam; GWA). The answer to the first question probably lies in the notion that cyanobacteria are very resilient organisms that can readily adapt to changing environmental conditions. Their adaptation strategies can be inferred from growth theory, and have their parallel on the molecular level. The cell envelope forms the cellular interface with the environment, and is therefore considered as the key compartment where adaptive responses to fluctuating P-availability are most strongly expressed. The over-all objective of the current line of research in the laboratory of Aquatic Microbiology at the University of Amsterdam in which this thesis is embedded is to design non-intrusive diagnostics as components of a ‘molecular toolbox’ to monitor the nutrient status of phytoplankton in lakes. The work presented in this thesis is thus based on the integration of knowledge about bio-molecules with microbial physiology and their relevance in answering ecological questions. This is a discipline that can be referred to as molecular ecophysiology.

In chapter 1, I have shown that phosphorus (P) is a key element that determines the growth rate of algae in lakes; the efficiency of P incorporation depends on the conversion of organic P-esters ($P_O$) outside the cells (scavenging strategy), transport of inorganic phosphate ($P_I$) through the cell envelope (affinity strategy), and storage of surplus P (polyP) inside the cells (storage strategy).

In chapter 2, we describe preparatory work to define the growth conditions that are optimal to get the type of information needed to study the onset of a $P_I$-deficiency in cyanobacteria. We show that the model cyanobacterium *Synechocystis* has a specific adaptive response (variation of protein synthesis) as well as a general response (attenuation of photosynthetic activity and growth rate) to changes in P availability.
In chapter 3, we identify potential targets for a diagnostic tool for the P-sensing status: Proteins in the cell wall of the model cyanobacterium *Synechocystis* sp. PCC 6803 that are controlled by P$_1$-availability. Starting point is the published genome of this organism, and the approach is to label cell-surface accessible proteins. After cell fractionation and protein separation, we use mass spectrometric analysis to identify the proteins. Results are compared with a parallel experiment with DNA arrays to study gene expression at the transcriptional level.

In chapter 4, candidate marker proteins for diagnosing P$_1$-deficiency in algae that have been identified in chapter 3 are further dissected to analyse their localisation, topology and expression patterns. In addition to biochemical localisation, we have used in silico techniques like multiple sequence alignments and hydrophobicity plots, and present a literature study on the regulation of responses to P$_1$-deficiency.

In chapter 5, the activity of one of the identified proteins, alkaline phosphatase, provides a biochemical means for fluorescent detection of the response to P$_1$-deficiency. The ELF-97 phosphatase substrate yields highly fluorescent precipitates at the site of enzymatic activity (AP induced fluorescence), which allows recognition of the nutrient status of cells in conjunction with separation of phytoplankton groups according to content of endogenous fluorescent pigments. Optimisation of the use of this detection method in flow cytometry is described, along with a survey of the strain specificity. We evaluate the method on a technical and a physiological level.

In chapter 6, we apply ELF-97 as a diagnostic tool in conjunction with isotope ratio mass spectrometric assessment of in situ growth rates for a specific case study of a Dutch lake, Lake Loosdrecht. The dominant filamentous cyanobacterium *Planktolyngbya limnetica* in this lake shows seasonal variation and population heterogeneity in AP induced fluorescence with ELF-97 as a substrate. We evaluate the ELF-method on the physiological and the ecological level.

In chapter 7, I discuss the ecophysiological perspective of my work in the light of the literature, with additional data from NIOO-CL and DWR on a longer timescale, concerning the P-concentrations and -fluxes in Lake Loosdrecht. The resilience of the present state of Lake Loosdrecht is probably largely based on self-shading and self-feeding capacities of the dominant filamentous cyanobacteria, resulting from the prior switch to eutrophied conditions; the switch back requires additional measures. I evaluate my work and sketch some topics for future research.