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

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Chapter 7

A critical review of analytical procedures for improved understanding of the impact of phosphate availability on phytoplankton ecophysiology.

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About this chapter

The development of a diagnostic tool for investigating the phosphate status of phytoplankton as presented in this thesis mainly entailed work on the levels of biochemistry and microbial physiology. However, the application of this tool is connected with ecological and policy problems. Therefore, in this final chapter, we attempt to view analytical procedures and obtained results in the broader light of phytoplankton ecophysiology and lake restoration. Although the techniques proposed in this thesis are broadly applicable among various ecosystems, practicality has prompted us to focus on their application in Lake Loosdrecht. This nearby lake has been extensively studied in the 1980's, and has served as a model study to monitor the effects of nutrient reductions in other Dutch lakes under rehabilitation (Van Liere et al. 1991; Gulati and Van Donk 2002). After the intensive studies of the workgroup Water Quality research Loosdrecht lakes (WQL; Van Liere et al. 1992) had ended in 1992, routine water quality monitoring continued at individual organisations, but was not published. Therefore, we have also taken unpublished data into account.

Data were gathered from long-term routine measurements at Municipal Waterworks Amsterdam (Gemeente Waterleidingen Amsterdam; GWA), the Centre for Limnology (part of the Netherlands Institute for Ecology; NIOO-CL) and the regional Water Management and Sewerage Service (Dienst Waterbeheer en Riolering; DWR). I wish to thank Hans Hoogveld, Roel Pel, Jeannine Ebert, Maarten Ouboter, and Jeanette de Groot-Abbenes for their cooperation. $P_i$-concentrations were measured using a continuous photometric method in accordance with NEN6663. For total P measurements, P was liberated by destruction in sulphuric acid in accordance with NEN6645. Numbers of filamentous cyanobacteria were determined by microscopy (1988-1992) and flow cytometry (1997-2002).

§1 Role of phosphate in lake ecology

$P_i$-concentrations in lakes

In a classical experiment in 1956 the Canadian limnologist F.H. Rigler charged an entire lake with inorganic phosphate, partially presented as $^{32}P$-phosphate, which permitted tracking of the uptake of phosphate by the algal population. He showed that the algae maintained the phosphate concentration in the lake water at a constant level even after repeated additions of phosphate (Rigler, 1956). These observations were fundamental to appreciate the meaning of the very low phosphate concentration in freshwater ecosystems, and the scavenging effect of the algae.
According to Rigler (Rigler 1966, Shapiro 1988), P\textsubscript{i}-measurements using the Murphy-Riley technique (Murphy and Riley 1962), which lowers the pH to about 0.7, probably yield much more orthophosphate (P\textsubscript{i}) than seen by the organisms. This could lead to a gross overestimation of P\textsubscript{i}-concentrations, especially when they are very low. In the opinion of Shapiro (1988), there are two cases of phosphate presence in lakes to be distinguished: lakes in which the P\textsubscript{i}-concentrations are high where the Murphy-Riley method is legitimate, and those in which the P\textsubscript{i}-concentrations are low (less than 3 mg.l\textsuperscript{-1}; equivalent to 30 nM), where other methods are needed. Several improvements have been suggested to extend the sensitivity of the P\textsubscript{i}-measurements into the nanomolar range, for example by complexation with malachite green (Hess and Derr 1975; Van Veldhoven and Mannaerts 1987; Baykov et al. 1988) or rhodamine B (Debruyn 1983). In our hands none of these older methods gave a sufficient increase of the detection threshold. A newer useful method, called MAGIC, first concentrates the P\textsubscript{i} from a larger water sample by co-precipitation with Mg(OH)\textsubscript{2} before standard measurement with the Murphy-Riley method (Karl and Tien 1992). Other attempts to device more accurate ways for measurement of the very low P\textsubscript{i}-concentrations at stake include enzymatic determination (Petterson 1979; Webb 1992). However, regardless of the observations documented in the above reports that the concentration of P\textsubscript{i} in lakes is usually too low to be estimated with acceptable reliability, the methods are still widely used.

A new method for estimating phosphate concentrations was recently suggested by Hudson et al. (2000), referred to as steady state P\textsubscript{i}. Basic to their calculation are the assumptions that the turnover of P is rapid, and that the flux into (uptake) and out of (regeneration) the plankton must be approximately equal. From measured uptake rates for P\textsubscript{i} and estimated regeneration rates, the steady state P\textsubscript{i} concentrations were calculated for 56 lakes. The estimated steady state P\textsubscript{i} concentrations were largely in the picomolar and lower nanomolar range, and showed large discrepancies with earlier, measured P\textsubscript{i} concentrations. Thus the P\textsubscript{i}-concentration is a misleading parameter for measuring eutrophication potentials; the concentration of total P is more meaningful (Correll 1999). However, total P represents an indication for the amount of biomass, rather than the nutrient status. This lack of a direct P-status indicator has prompted us to develop a method that uses the response that phytoplankton cells themselves show to lack of P\textsubscript{i}.

It is important to emphasize that concentrations of P\textsubscript{i} are not nearly as important as rates of supply (Shapiro 1988). These supply-rates are in turn conditioned by the algal uptake rates. Dissolved P\textsubscript{i} in eutrophic surface waters has a turnover time of minutes, and a small pool size (1-2 mg.l\textsuperscript{-1}; 10-20 nM) is sufficient to saturate algal growth. Because the P-influx in lakes is a complex ensemble of factors related to the total water balance, we can calculate, but not directly measure it.
The P-cycle in an eutrophic lake is almost closed, and the continuous external P-load may be as low as only 3-5% of the total P-cycle. Sedimentation after adsorption to particulate matter or precipitation with calcium or magnesium is the major sink for this element, and the losses of P to deep bottom sediments approximately equal the external P-load, resulting in a sheer stagnating eutrophic situation (Sijde 1984). It can take centuries before the increased amounts of P are permanently fixed in the sediment. Any available P is rapidly incorporated into living cells. This leads to a circulating pool of P, incorporated in or adsorbed to organic compounds. Successive phytoplankton blooms develop mainly by utilising P regenerated from these organic compounds. Shallow lakes have a capacity to recycle the same nutrient over and over again, because, by definition, they cannot export products to depth. When the retention time is also high, enriched shallow lakes can maintain much higher average levels of biomass than the external load would appear to permit (Reynolds 1998).

**Influence of biota**

In a P-limited ecosystem, living cells rapidly absorb all incoming P. Currie and Kalff (1984) found that by far the greatest part (more than 90%) of $P_1$ uptake is due to bacteria (cells smaller than 3.0 mm), which have much higher uptake rates of $P_1$ than any other organism. These authors found that algae utilise primarily organic P from bacterial excretion, and are responsible for uptake of 99% of this fraction. The total fluxes of $P_1$ and organic P are of similar magnitude. Dissolved organic P arises from plankton excretion, death, and decomposition. The bacterioplankton is probably not P-limited, but rather limited by the supply of reduced carbon, which they obtain to a large extent from the phytoplankton. The phytoplankton and bacterioplankton dynamics are therefore probably tightly coupled (Currie and Kalff 1984a). Variation of phytoplankton abundance among lakes depends on total P, and is also related to bacterial activity (and not bacterial abundance per se). A complicating factor is that $P_1$ uptake is basically an indicator of bacterial activity, and only secondarily determines phytoplankton abundance. As the total P concentration increases above a level of 5 to 7 mg P L$^{-1}$ (corresponding to 160-225 nM inorganic phosphate equivalents), partitioning of uptake between algae and bacteria increases in favour of the algae (Currie 1990). Rapid initial P uptake by bacteria, followed by more gradual but steady P uptake by cyanobacteria may reflect mutually compatible P utilisation strategies aimed at avoiding direct competition for this resource. Bacteria are often closely associated with the phycosphere surrounding cyanobacterial cells, and exchange of several nutrients takes place (Pearl 1996). Low molecular weight forms of P exist in lake water. These might result from initial bacterial uptake of $P_1$, followed by excretion of an organic P-ester that can be assimilated by the host cyanobacteria. Organisms higher up in the
food-chain, such as zooplankton and fish are also a sink for P, and higher plants fix P by absorption through their leaves and by holding sediment particles with their roots (e.g. Van Liere and Janse 1992; Havens and Schelke 2001; Gulati and Van Donk 2002). However, these higher organisms are outside the scope of this study, which focuses on microbial ecology.

Viruses or phages may contribute to the recycling of P. Bacterial cell lysis by phage infection gives very high release rates for dissolved organic compounds (Scanlan and Wilson 1999). For marine systems these authors argue that both the product of viral lysis and the viral DNA itself may become components of the organic P pool. Conversely, the P status of the cells was shown to exert control over the ultimate outcome of cyanophage-host interactions, presumably reflecting the high P demand of cyanophage replication in the host cells. Thus, a fast-growing abundant population that is controlled by viral lysis may depend on its own organic P as a source for P. For coastal waters, it has been established that viral lysis may account for up to 50% of the mortality of heterotrophic bacteria, and up to 100% of the mortality of algae (Fuhrman 1999). Populations of cyanobacteria in laboratory scale enclosures (LSE) of Lake Loosdrecht water (cf. chapter 6, this thesis) repeatedly showed mass mortality (Gons et al. 2002). Phage-like particles were found in the water and on dead cyanobacterial cells. The lysis was probably aided by the increased P-availability (Scanlan and Wilson 1999), and washout of detritus particles that compete for phage binding. Mass lysis was not observed in LSE-experiments in which growth was P-limited.

Effect of abiota

Another very important notion, pioneered by Mortimer (1941, reference in Shapiro 1988), is the release of P from sediment and suspended organic particles (detritus). Particulate P and dissolved organic P are not inert, but these forms of P can be converted to dissolved P$_i$ under appropriate conditions. These dynamic equilibria are known as the P buffer mechanism (e.g. Rijkeboer et al.1991), and include adsorption/desorption, phosphatase activity and mineralization in bottom sediment. In eutrophic lakes, the bottom waters become anoxic during growing season. This brings about a more rapid regeneration of P$_i$ from the sediments, thus generating substantial P-pulses in warm windless weather (Shapiro 1988; Correll 1999).
§2 Phosphate in the Loosdrecht Lakes

**P-loading rates in the Loosdrecht Lakes**

Eutrophication of the Loosdrecht lakes area is a result of prolonged external nutrient loading in the twentieth century (for a recent review, see Gulati and Van Donk 2002). Due to measures taken by The Municipal Waterworks Amsterdam (GWA) and The Water Authorities of the Province of Utrecht the external P-load was reduced in 1984 (Hofstra and Van Lier 1992). Calculation of P-loading rates depends on assumptions in the water supply from various sources. For example, mass balance calculations (Engelen et al. 1992) in the period after dephosphorylation of intake water had started (1984-1987) gave external P-loading rates of 0.8 mg P.m\(^{-2}\).d\(^{-1}\) (= 0.29 g P.m\(^{-2}\).y\(^{-1}\)), of which 30% came from the Gooi catchment, 30% was intake from the Bethune polder, 10% supply from Amsterdam-Rhine canal, and 30% leakage from locks, precipitation, tourism, and unsanwered housing. On the basis of the same data, with different assumptions about the water supply, M. Ouboter at DWR calculated P-loading rates of 1.25 mg P.m\(^{-2}\).d\(^{-1}\) (= 0.46 g P.m\(^{-2}\).y\(^{-1}\)) (M. Ouboter, personal communication). An overview of calculated P-loading rates in Lake Loosdrecht is given in Table 1 (values were taken from several sources, e.g. Van Lier et al. 1989; Van Lier et al. 1990; Van Lier and Janse 1992; Hofstra and Van Lier 1992). From these studies, the P-loading rates over the period 1984-1987 comes to an average of 0.41 g P.m\(^{-2}\).y\(^{-1}\), which is in better agreement with the value calculated by M. Ouboter (see above). Reduction of the P-load after 1984 is evident; the relative increase in 1986-1987 was attributed to leakage of sluices (Van Lier and Janse 1992), which were later repaired. A recent series of wet winters has probably led to increased P-loads.

In the period 1984-1987, the internal P-load was about 0.11 g P.m\(^{-2}\).y\(^{-1}\), mineralization was 1.1 g P.m\(^{-2}\).y\(^{-1}\), and diffusive release was 0.11 g P.m\(^{-2}\).y\(^{-1}\) (Engelen et al. 1992). According to Engelen et al. (1992), downward seepage (0.29 g P.m\(^{-2}\).y\(^{-1}\)) balanced the major part of the external P-load. The higher estimations for the external P-load mentioned earlier would mean that P was still accumulating.

<table>
<thead>
<tr>
<th>Period (year)</th>
<th>P-loading rate (g P.m(^{-2}).y(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1932-1944</td>
<td>0.57</td>
</tr>
<tr>
<td>1947-1957</td>
<td>1.12</td>
</tr>
<tr>
<td>1959-1970</td>
<td>0.98</td>
</tr>
<tr>
<td>1970-1975</td>
<td>1.23</td>
</tr>
<tr>
<td>1976</td>
<td>4.03</td>
</tr>
<tr>
<td>1977-1983</td>
<td>1.10</td>
</tr>
<tr>
<td>1984-1985</td>
<td>0.35</td>
</tr>
<tr>
<td>1986-1987</td>
<td>0.47</td>
</tr>
<tr>
<td>1988-1992</td>
<td>0.35</td>
</tr>
<tr>
<td>1996-2001</td>
<td>0.36-0.58*</td>
</tr>
</tbody>
</table>

*: M. Ouboter, personal communication. This estimation were calculated from hydrological data and checked with the chloride balance, but not corrected for the development of biomass.
in the system in this period. However, further reduction of external P-loading due to decrease of P-loading from leakage, tourism, and unsewered housing in later years must have reduced the total amount of P in the system (M. Ouboter, personal communication). Settling of particulate matter is a major loss process for P from the water column of lakes, but the sediments is also a potential source of P, especially after resuspension during storms. The amount of potentially bio-available P in the upper 10 cm of the sediment of lake Loosdrecht was less than 15% of the total P content of the sediment, but was still 3 times larger than the annual external P-load (Keizer and Sinke 1992). The uptake capacity of the aerobic sediment was 0.2 g P.m$^{-2}$.y$^{-1}$. Recycling of P also takes place within the water column itself. Dissolved organic matter in the water mainly comes from lysis and further decomposition of phytoplankton cells (Otten et al. 1992). P in the water column (both algae and detritus) comprised about 16% of the total amount of P in the P cycle of Lake Loosdrecht. It is difficult, however, to separately quantify adsorption/desorption processes between living and dead material within the complex resuspension-sedimentation compartment (Van Lier e and Janse 1992). P recycling further depends on grazing by zooplankton. In Lake Loosdrecht, zooplankton daily removed 4-6% of the P-stock (years 1987-1990), but returned more than half as egestion, mostly detrital P (Van Lier e and Janse 1992). Despite the relevant internal P-load in the lake, growth of phytoplankton has shifted from being P-saturated (light-limited) in 1985 (Rie gman and Mur 1986) to being P-limited in 1987 (M. Rijkeboer, internal report; see Rijkeboer and Gons 1991).

**Biomass and phosphate concentrations**

The preferred trophic status of a lake is not necessarily oligotrophic. To indicate the natural trophic level of polluted lakes, Vighi and ChiAUDani (1985) used a morphoedaphic index (MEI), the ratio between total dissolved solids and mean depth. According to these authors, the ratio between conductivity (mS.l$^{-1}$), alkalinity (mequiv.l$^{-1}$), and mean depth is a measure for the natural background loading. Data from 53 lakes with various depths and with negligible P-load due to human influence were analysed to derive an empirical equation (Vighi and ChiAUDani 1985). In Lake Loosdrecht, the average conductivity in the years 1999-2001, measured at temperatures around 20°C, was 419 ± 70 μS/cm (9 df, 95% CI; data were kindly provided by M. Ouboter from DWR, see also fig 1C). This gives an estimated background total P value of 4.7 ± 1.2 μg Pl$^{-1}$ (50 df, 95% CI). This value may give an indication for the highest possible level of water quality improvement obtainable. For practical reasons, however, this value seems extremely low. Furthermore, the ecological target for the Loosdrecht lakes, clear water with submerged plants, refers to the (mesotrophic) situation around 1940, and not to the original oligotrophic conditions before 1920 (Hofstra and Van Lier e 1992).
Figure 1. Routine measurements of nutrient and biomass parameters in Lake Loosdrecht. A: Orthophosphate concentration (P<sub>i</sub>), the broken line marks the detection limit. B: Total phosphorus concentration (including particulate matter), the broken line marks the target value. C: conductivity.
Figure 1 continued. Routine measurements of biomass parameters in Lake Loosdrecht.

D: Chlorophyll $a$ concentration, the broken line marks the desired ecological quality. E: Transparency (Secchi-disc depth), the broken line marks the desired ecological quality. F: Numbers of filamentous cyanobacteria, separated into phycobilin-containing trichomes, predominantly $P. \text{limnetica}$ (closed symbols), and phycobilin-less trichomes, predominantly $P. \text{hollandica}$ (open symbols). Data in the series 1988-1992 were calculated from the total number of cyanobacterial trichomes as determined by light microscopy (see Fig. 2), and the fractions of $P. \text{limnetica}/P. \text{hollandica}$ as determined by red/orange fluorescence microscopy (Ex. 546 nm, Em. $>590$ nm; Van Lier et al. 1989). Data in the series 1997-2002 were determined by flow cytometry (chapter 5 and 6, this thesis).
Figure 2. Total number of filamentous cyanobacteria in Lake Loosdrecht. Data in the series 1984-1993 were determined by light microscopy, data in the series 1997-2002 are flow cytometric measurements.
Generally, the maximum tolerable concentration (MTC) for nutrients in shallow lakes in the Netherlands is based on ‘desired ecological quality’, described by 100 μg chlorophyll a•l⁻¹ (Van Lieren and Jonkers 2002). In 1980, the critical summer-average concentration of total P was chosen at 150 μg P•l⁻¹, derived from a data set of 80 lakes, mainly dominated by green algae (CUWVO 1980). A data set of 120 lakes showed that in 1987 many of these lakes were dominated by cyanobacteria (CUWVO 1987). The critical summer-average concentration found in the latter study was much lower (70 μg P•l⁻¹), but this has not led to any policy change (Verkeer en Waterstaat 1999). For heavily eutrophied shallow lakes dominated by cyanobacteria the ecological target, clear water, will probably only be met at total P concentrations lower than 50 μg P•l⁻¹. In lake Loosdrecht the summer-average concentrations of total P (Fig. 1B) decreased from over 100 μg P•l⁻¹ in the years 1982-1990 to about 60 μg P•l⁻¹ in the last ten years. The chlorophyll a concentration (Fig. 1D) decreased from over 100 μg•l⁻¹ in the years 1982-1990 to about 60-80 μg•l⁻¹ in the last ten years. The Secchi-disc depth (Fig. 1E) increased from 0.35 in the years 1982-1990 to 0.40 in the last ten years. A change has taken place in these values in the years 1990-1992, bringing the indicators within the range of the standard values. Measurements of P₄-concentrations (Fig. 1A) in Lake Loosdrecht were found to be around the detection level of the photometric methods (10 μg P•l⁻¹ or 0.32 μM; NEN standard 6663:1983), and are thus not very useful.

In an effort to estimate actual P₄-concentrations, Hudson et al. (2000) calculated steady state P₄ concentrations from measured uptake constants for P₄ and estimated regeneration rates of P from algal biomass. According to these authors, actual P₄-concentrations are thus related to the biomass indicator total P, and this relation can be described by an empirical relation between the two. For a total P value of 100 μg P•l⁻¹ (3.2 μM P) this gave a steady state P₄ concentration of 0.55 nM. Measured total P values in lake Loosdrecht (Fig 1B), averaged over the summers 2000-2002, are: 62 ± 13 μg P•l⁻¹ (13 df., 95% CI). This total P value equals 2.00 ± 0.20 μM P (13 df., 95% CI), and gives a steady state P₄ concentration of 0.38 nM. These extremely low values (picomolar range) are only approximations (Hudson et al. 2000), and should be corrected for the detritus/live cell ratio, but nevertheless illustrate the great difficulty of estimating real P₄-concentrations.

The densities of the cyanobacterial populations in lake Loosdrecht fluctuate with the seasonal temperature and light variations (Fig. 2). The total number of filamentous cyanobacteria in the lake water increased from 75 to 250.10³ ml⁻¹ in the years 1984-1990, and varied from year to year between 120 and 250.10³ ml⁻¹. This increase in the size of the cyanobacterial community after the implementation of restoration measures in 1984 implies that the filamentous cyanobacteria have profited from the decreased P-loading rates. During the interdisciplinary project for monitoring the water quality in Lake Loosdrecht (Water Quality Research
Loosdrecht Lakes (WQL); e.g. Van Liere et al. 1992), which stopped in 1993, elaborate microscopic counts of phytoplankton populations were conducted. In 1985, the phycobilin-less filamentous cyanobacterium *Prochlorothrix hollandica* was discovered in lake Loosdrecht (Burger-Wiersma et al. 1986). The relative numbers of phycobilin-containing filamentous cyanobacteria (predominantly *P. limnetica*) and the phycobilin-less filaments (predominantly *P. hollandica*) were determined in the period 1988-1993 by fluorescence microscopy (Van Liere et al. 1989). Flow cytometric monitoring of the cyanobacterial community in Lake Loosdrecht started halfway through 1997; this leaves a gap of three and a half years in the determination of the numbers of filamentous cyanobacteria *P. hollandica* and *P. limnetica* (Fig 1F). Although the two data series were determined with different methods (fluorescence microscopy and flow cytometry respectively), deviations between the two methods were less than 5% (H. Hoogveld, personal communication). Comparison of summer averages of the relative amounts of *P. hollandica* in the total number of filamentous cyanobacteria in the two periods (Table 2) revealed that this species was relatively less abundant in 1997-2002 than in 1988-1993. Despite these subtle changes in dominance, the populations are remarkably stable (J. Huisman, personal communication).

**Table 2.** Summer averages of the fraction of *P. hollandica* in the total of filamentous cyanobacteria in Lake Loosdrecht.

<table>
<thead>
<tr>
<th>Year</th>
<th>Fraction <em>P. hollandica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1988</td>
<td>0.41 +/- 0.19 (5)</td>
</tr>
<tr>
<td>1989</td>
<td>0.30 +/- 0.12 (8)</td>
</tr>
<tr>
<td>1990</td>
<td>0.38 +/- 0.12 (9)</td>
</tr>
<tr>
<td>1991</td>
<td>0.24 +/- 0.26 (6)</td>
</tr>
<tr>
<td>1992</td>
<td>0.31 +/- 0.14 (6)</td>
</tr>
<tr>
<td>1993</td>
<td>0.33 +/- 0.14 (8)</td>
</tr>
<tr>
<td>1997</td>
<td>0.109 +/- 0.024 (19)</td>
</tr>
<tr>
<td>1998</td>
<td>0.188 +/- 0.050 (21)</td>
</tr>
<tr>
<td>1999</td>
<td>0.175 +/- 0.028 (46)</td>
</tr>
<tr>
<td>2000</td>
<td>0.170 +/- 0.047 (39)</td>
</tr>
<tr>
<td>2001</td>
<td>0.208 +/- 0.036 (24)</td>
</tr>
<tr>
<td>2002</td>
<td>0.216 +/- 0.033 (14)</td>
</tr>
</tbody>
</table>

*Confidence intervals (95%) with degrees of freedom in brackets.*
§3 Physiological and bio-energetic considerations

Classification of the adaptive strategies

Microorganisms growing at maximal rate are probably rare in nature; the physiological conditions of a laboratory culture in exponential phase therefore represent an unrealistic point of reference. Sub-optimal growth rates always affect the physiological state of cells, regardless of which factor is limiting. It is therefore important to distinguish general from specific responses. P-depleted cyanobacteria display several phenomenological changes compared to when they are growing exponentially. The colour of cultures changes from intense blue-green to yellowish (bleaching, chlorosis), the cells look smaller through a microscope (metabolic changes), and sometimes foam appears on the culture (cell lysis). 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 (instantaneous growth rate limitation). We observed changes in the relative amounts of photo-pigments in absorption spectra and 77K-fluorescence spectra (chapter 2, this thesis), and also observed a decrease in phycocyanin concentration in SDS-PAGE, albeit to a lesser extent and at a later stage than in N-deficient cultures (not shown). A marked decrease in RNA content in P$_i$-depleted cells that very much hampered the differential array experiments shown in chapter 3 (N. Yeremenko, personal communication) is another example of retrenchment observed in our studies. 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, the lack of P$_i$ in the cells obstructs energy transfer from chemiosmotic photosynthetic electron transfer to the ATP pool. It is not the light conditions that have changed as such, but the cells compensate for photo-inhibitory stress as if they were experiencing excessive light conditions. This results in the increased synthesis of carotenoids in the outer membrane as was seen in the sucrose gradients that were described in chapter 3.

Acquisition is the development of more efficient uptake systems, which is a specific response. This is the main focus of this thesis, and involves synthesis of both high-affinity P$_i$-uptake system and alkaline phosphatase enzymes that convert alternate chemical forms of P. To explain the kinetic behaviour of P in continuous cultures, Monod had already mentioned sequestration of P$_i$ by cell excretions, but
he hesitated to postulate protein binding of $P_i$ (Droop 1974). It is now clear that this sequestration indeed takes place by $P_i$-binding proteins in the periplasmic space of cyanobacteria (chapter 2 and 3, this thesis). The derepression of the Pst system in cyanobacteria described in this thesis is a clear example of an affinity strategy. The synthesis of AP to liberate $P_i$ from organic sources can be referred to as a scavenging strategy, which the cells use to influence the external substrate concentration. The storage strategy does not fit in this classification very well, but can be placed between compensation and acquisition.

**$P_i$-uptake kinetics and its implications for adaptive behaviour**

$P_i$-uptake of *Synechococcus elongatus* PCC 7942 efficiently takes place near the thermodynamic threshold concentration, below which incorporation is energetically impossible (chapter 4, this thesis; Wagner et al. 2000 and references therein). A thermodynamic threshold in the lower nanomolar range seems contradictory to the steady state $P_i$-concentrations mentioned earlier, which were estimated to be in the picomolar range. There are few explanations for this discrepancy. Either the affinity of natural populations for $P_i$ is so high that the threshold is even lower than for *Synechococcus* in laboratory conditions, the estimations made by Hudson et al. (2000) are invalid, or the threshold determinations by Falkner et al. (1989) are overrated. Interestingly, *Synechococcus* had different threshold concentrations for dense and more dilute cell suspensions. This could be explained as follows: in the more dense suspension the external $P_i$ concentration following a pulse decreases so rapidly that the change in uptake behaviour does not occur. In the more dilute suspension, external $P_i$ is less rapidly incorporated and as a result the cyanobacteria are exposed to higher substrate concentrations for a period of time long enough for them to adapt. This situation then provokes a less active state of the high-affinity uptake system. As a consequence, under natural conditions of fluctuating phosphate supply the uptake behaviour ceases to be an objective property of the individual organism but becomes a function of the activity of the whole population (Wagner et al. 1995; Wagner et al. 2000). The authors suggest that cyanobacteria have a capacity to “memorise” nutrient fluctuations for several hours and 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 with a complete growth arrest (Falkner et al. 1996). Experiments have clearly shown that the absolute external $P_i$-concentration that was offered did not determine the adaptive alteration of the properties of the uptake system (Wagner et al. 2000). Rather, the relative concentration change during $P_i$-pulses contained the relevant information, independent of the amount of $P_i$ inside the cells. The pulse pattern also affected the
cytoplasmic P\textsubscript{i}-concentrations. The cells were able to independently change their kinetic and energetic properties, and seemed to optimise the efficiency of P\textsubscript{i}-uptake, rather than absorb as much as possible. Moreover, the authors concluded that the memory effect of previous P-supply patterns potentially provides a means to adjust growth rate to P\textsubscript{i}-availability. Analysis of the uptake activity of phytoplankton by the methods developed in these studies may also serve as a proper tool for monitoring P\textsubscript{i} inflow into lakes, and to establish threshold concentrations below the normal measurable range (Falkner et al. 1993; Aubriot et al. 2000).

**Regulation of the response to P\textsubscript{i}-deficiency**

The specific response of cyanobacteria involves a high-affinity uptake system (Pst) and a co-regulated alkaline phosphatase (AP). Enterobacteria and cyanobacteria have highly homologous Pst-systems (chapter 3 and 4). The regulation of the *pho* system also appears to be similar, with the two-component regulatory system PstRS (PhoBR in *E. coli*) and putative inhibitor PhoU (chapter 4). The analogy with Enterobacteria only holds to a certain level, as cyanobacteria do not seem to contain a low-affinity P\textsubscript{i}-uptake system similar to that of Enterobacteria (the Pit-system), but instead possess parallel high-affinity systems with different expression patterns. This is consistent with the environmental conditions of cyanobacteria, which continually live in water with nutrient concentrations around the thermodynamic threshold, as opposed to Enterobacteria, which spend most part of their existence in the gut with plenty of phosphate. The signal that triggers the up-regulation of the specific response (external P\textsubscript{i} concentration, sensed by PstRS) probably differs from the signal that triggers the down-regulation (internal P\textsubscript{i} concentration, sensed by repression complex involving the Pst system and PhoU; chapter 4, this thesis). The signal that starts the general response, summarised as bleaching (chapter 2, this thesis), is unknown, but probably involves cross-regulation via other regulatory systems. Potential signal inputs are energy charge ([ATP]/[ADP] ratio’s), redox state (pyridine nucleotide pools), or concentrations of key metabolites of photosynthetic and respiratory metabolism (chapter 4, this thesis). In this respect, the response to P\textsubscript{i}-deficiency resembles the response to high light conditions, even to the extent that the Pst system and AP are up-regulated in high light stress in *Synechocystis* (Hihara et al. 2001). Cyanobacteria seem to be adapted to fluctuating P\textsubscript{i}-concentrations more than low P\textsubscript{i}-concentrations. Internal P-stores cannot provide the signal for growth rate changes directly, because polyP itself is inert. From the studies reviewed in this paragraph it became apparent that previous P-supply patterns potentially provide a means to adjust growth rate to P\textsubscript{i}-availability. The regulation of parallel high-affinity uptake systems, attenuation of photosynthetic activity and growth rate is very complex and provides an area for future research.
§4 Ecological role of phosphatases

P-availability: importance of AP

Although P_i and some small organic phosphate esters are readily available for phytoplankton, these compounds are usually present in lakes in only minute amounts (see paragraphs 1 and 2, this chapter). 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), but the available fraction is actively utilised by the algae (Bentzen et al. 1992; Cotner and Wetzel 1992). The colloidal-P is very stable and resistant against dephosphorylation (Olsson and Jansson 1992). Particulate matter is another welcome source of substrates. Particulate organic P is found to be available to phytoplankton to a certain extent (see Boström et al. 1988). In other words, P_i is released either directly from dead cells, or from dissolved substrates supplied from living or dead particulate matter (Jansson et al. 1988). The main forms of P in this detrital matter are probably adsorbed P_j, sugar phosphates, glycerophosphate, polynucleotides and phospholipids (e.g. Pant and Reddy 2001). All naturally produced organic phosphorus compounds are esters of orthophosphoric acid. Potential substrates for AP are phospho-monoesters, but also phospho-ester bonds of polynucleotides as well as inorganic pyrophosphate, polyP, and short chain metapolyP (Siuda 1984). The P-moiety in these compounds is only available after hydrolysis of the phosphate ester bond. 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 phosphomonoesterase. These phosphatases are broad in specificity towards different substrates, their activity is only restricted to the P-O bond on the phosphomonoesters. The fact that phosphatases are synthesized in large amounts in situations of P-deficiency, in combination with the notion that the end product of the reaction catalysed by these enzymes, P_j, is readily assimilated by phytoplankton, provides the basis for the hypothesis that phosphatases have an essential function in the nutrient dynamics of lakes (Jansson et al. 1988). According to Currie and Kalff (1984b), 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-kinetics alone. Probably, resource competition depends on the combination of uptake-, storage-, and conversion-capacities in relation to the availability of P. The ubiquity of inducible AP among phytoplankton (chapter 5 and 6, this thesis) supports these conclusions for sub-saturating P-loads.
Shortcomings of AP as indicator for P-deficiency

The activity of AP can be used as an indicator for P-deficiency, where direct measurements of the Pi-concentration do not suffice (chapter 5 and 6, this thesis).

Theoretically, the production rate of derepressible phosphatases should give the best measurement of P-deficiency. In practice, the potential phosphatase activity and its variations have been used as an indicator of P-deficiency. Potential phosphatase activity is assayed with a suitable artificial substrate, at substrate concentrations near the saturation concentration, to allow the reaction to proceed at maximum rate. The AP activity measured in routine assays cannot be used for predictions of in situ hydrolytic activity, for the following reasons (Jansson et al. 1988):

First, natural substrate concentrations, and thus conversion rates are much lower than those used in the assays; Second, standardised pH and temperature are not realistic compared to those in lake waters; And third, the artificial substrates may not be representative of the structure of the natural substrates. Although bulk AP activity has widely been used as a means of diagnosing P-deficiency, an important flaw in the use of AP activity as a P-deficiency indicator is the uncertainty about the origin of the enzymes. Cells may actively excrete dissolved enzymes, although another important but a-specific contribution is delivered from the cytoplasm of dying and disintegrating cells (Jansson et al. 1988). Apart from this, confusion may also be caused by the persistence of AP in supply water.

In most cases, the de-repression of AP takes place at the onset of the depletion. This is not always the case. The chlorophyte *Dunaliella tertiolecta*, for example, only induces AP in starved cultures, not in steady state, limiting conditions. For this organism the AP activity responds only to extreme nutrient stress (Graziano et al. 1996). Another problem is that the constitutive activity can be highly variable among species (see chapter 5, this thesis), and increased phosphatase activities can be induced by other conditions than just low P-supply (Jansson et al. 1988). An important deviation from the pattern of AP induction is the loss of activity in case of extreme P-deficiency, due to requirement of some level of metabolic activity for the maintenance of enzymatic binding sites and production of enzymes (Healey and Hendzel 1975). The absence of AP induced fluorescence in a fraction of the cyanobacterial population seen in chapter 6 of this thesis may be an example of this phenomenon. The exact nature of the natural substrates for phosphatases remains obscure. The greatest difficulty is that in lake water the phosphatases hydrolyse substrates immediately after their exposure to the enzymes. In a nutrient dynamical approach, effort should be spent in studying both the connection between the enzymes and their producers, and between the enzymes and their substrates (Jansson et al. 1988). Two promising methods for the characterisation of organic P were recently described: $^{31}$P nuclear magnetic resonance (NMR) spectroscopy (Pant and Reddy 2001), and combined assays with the P$_{i}$-releasing
enzymes, phytase, AP, nuclease, and nucleotide pyrophosphatase (Olsson and Jansson 1992; He and Honeycutt 2001). Another problem is that polynucleotides are degraded by 5'-nucleotidase (5PN). The phosphate forms a diester bond between nucleotide monomers, which can be hydrolysed by either AP or 5PN (Bentzen et al. 1992). The mechanism of 5PN is more specific, and recognizes the carbon moiety of 5'-nucleotides. DNA was not reported as a bioavailable P-source by Cembella et al. (1984); other studies however, report that nucleic acids may become available, be it at a slower rate than phosphomonoesters (Boström et al. 1988).

In conclusion, AP induced fluorescence (from conversion of the ELF-97 phosphatase substrate) is a useful indicator for monitoring the origin of AP activity. However, this method does not allow conclusion about the actual conversion rate of organic P in the lake. In general, AP activity extends the residence time of P in the water phase; the rate at which organic P becomes available probably does not depend on enzymatic rates, but rather on the rate at which the organic compounds become available. The absence of knowledge about the nature of AP substrates, and the rates at which they become available is an important flaw in aquatic microbiological research.

§5 Implications of changing P supply to growth and community structure of phytoplankton

Phytoplankton species composition
In a nutrient-poor (oligotrophic) lake complex, negative feedback mechanisms exist that keep the water clear (Havens and Schelke 2001). Plants fix P directly (through the leaves) and indirectly (by holding sediment particles with their roots). Increased P-loading rates lead to increased growth of submerged plants. Algal biomass also increases, but zooplankters find sufficient shelter from fish among the plants, and are able to keep the algae under control. At a certain critical P-loading rate, algae are able to overgrow the plants, which then disappear because light is blocked by the suspended algae. In such a eutrophied lake, negative feedback mechanisms exist that keep the water turbid. These complex mechanisms include biological, physical and chemical processes, such as uptake and storage by algae, sorption /desorption of P to sediment, and high turnover rates of P because of mineralization of high biomass density, aided by AP activity. Even when the external P-loading rate is decreased, P will be delivered from the sediment for a long time (internal loading). A large build-up of dead cell material (detritus) adds to the maintenance of the turbid state. Moreover, the phytoplankton biomass density itself does not increase gradually with the P levels in a lake due to species succession taking place. When cyanobacteria dominate, the turbidity is higher at the
same nutrient concentration than when eukaryotic algae are dominant, leading to a
discontinuous response to changes in nutrient level (Mur et al. 1989). In lakes
where the $P_i$ concentration is continually and substantially below 1-2 mg P.L$^{-1}$
(30-60 nM), the phytoplankton is likely to tend quickly towards dominance by
high-affinity species (Reynolds 1998).
The above-mentioned negative feedback mechanisms cause forward and backward
switches between clear and turbid states to occur at different critical conditions, as
illustrated in an ecosystem response curve for a fictitious example in Fig. 3 (Janse
and Van Liere 1995). This pattern, called hysteresis, is thought to occur pro-
nouncedly in shallow lakes (Scheffer et al 1997), and is a probably a result of
complex interactions of several feedback mechanisms, and not due to unstable
equilibria in the competition for $P_i$ and light only (J Huisman, personal communi-
cation). The hysteretic effect has several implications. When one monitors the sys-
tem on a stable branch before a switch, little change in its state is observed.
Gradually changing conditions may have little effect on the state of an ecosystem,
but nevertheless reduce its resilience, the maximum perturbation that can be taken
without causing a switch to the alternative state. At some critical threshold, howev-
er, a ‘catastrophic’ transition to another state occurs. An important issue for water
quality managers is that to induce a switch back, it is not sufficient to restore the
environmental conditions of before the collapse, but one needs to go back to a
much lower P-loading rate.

Figure 3. Hysteretic effect of P-loading rate on chlorophyll concentration in a shallow lake
(fictitious example based on Janse and Van Liere 1995, by courtesy of L. van Liere, RIVM).
Our case study, Lake Loosdrecht, has dramatically switched from clear water with submerged vegetation to a turbid state dominated by filamentous cyanobacteria, accompanied by loss of transparency and vegetation. Although the lake encounters frequent temporary turbid phases due to resuspension of peat particles from the sediment during storms (Gons et al. 1991), the present, continuous turbidity is mainly caused by cyanobacterial detritus, which sink much more slowly than peat particles. Restorative measures (70% reduction in total P-load since 1984) have led to only a minor reduction of the phytoplankton biomass, as was discussed in paragraph 2 of this chapter. The current condition of the Loosdrecht lakes is therefore an example of persistence of an undesired ecosystem, maintained by the resilience of the current dominant phytoplankton community. From the hysteresis effect discussed above we infer that restoration of the clear water state may happen at substantially lower nutrient levels than those at which the collapse of the vegetation occurred.

**Competition in Lake Loosdrecht**

Generally, filamentous cyanobacteria are either a minor component of the phytoplankton community in lakes, or strongly dominant. The fact that the filamentous cyanobacteria that dominate the eutrophic Lake Loosdrecht contain high-affinity P\(_i\)-uptake systems (H. Ducodu, 1998) implies that another reason than inability to compete for P\(_i\) must account for their absence in oligotrophic systems (Molot and Brown 1986). Indeed, high abundance of filamentous cyanobacteria occurs predominantly under shady condition, whereas no correlation with total P was found (Jensen et al. 1994; Scheffer et al. 1997). Cyanobacteria are generally good competitors for both light and P\(_i\) (J. Passarge, manuscript in preparation). The dominance of filamentous cyanobacteria in Lake Loosdrecht has thus probably emerged from a combination of low light levels due to shading by other algae and lowered P\(_i\) concentrations.

From competition experiments between planktonic desmid species, Spijkerman (1998) concluded that in continuous limitation of P species with high AP activity as well as a high affinity uptake system have the advantage; under pulsed P\(_o\) conditions, however, P\(_i\) uptake kinetics are more decisive to the outcome of competition than AP activity characteristics. Taking into account that large organic phosphorylated compounds cannot be taken up by cells (paragraph 4, this chapter), it is clear that in case of a low P\(_i\) load, most of the available P\(_i\) will be quickly absorbed by organisms with high affinity for P\(_i\). Most of the P-stock in the water will then be organic (in biomass). This benefits scavengers for P\(_o\) that also have a high affinity for P\(_i\). Furthermore, a dense population responds differently to a P-pulse than a dilute population (paragraph 3, this chapter; Wagner et al, 1995; Wagner et al 2000). A short P-pulse is absorbed rapidly by the most abundant pop-
ulation and by the population with the highest affinity. These notions offer insight in the abundance of different species in Lake Loosdrecht. Resource competition in Lake Loosdrecht occurs mainly between the dominant populations of filamentous cyanobacteria. Eukaryotic algae are less abundant, although their in situ growth rate is high (Pel et al. 2002a + b, submitted). This is probably due to the preference that grazers have for the soft and single-celled eukaryotic cells over the filamentous and tough cyanobacteria. Although diatoms are good competitors for $P_i$, and also possess AP (Chapter 6, this thesis), they are only a minor component of the phytoplankton community in Lake Loosdrecht.

The phytoplankton community in Lake Loosdrecht is dominated by filamentous cyanobacteria. The phycobilin-less filamentous cyanobacterium *P. hollandica* is an affinity and a storage strategist sensu Sommers (1989), and a very good competitor for $P_i$ under regimes of pulsed $P$ supply (Ducobu, 1998). Its niche is probably a $P$-limited ecosystem in which $P$ is delivered in distinct pulses and in which the presence of large amounts of particles (e.g. peat and detritus) reduces light penetration (Ducobu et al. 1998). The combined affinity and storage strategy worked well when the internal $P$ loading rate in Lake Loosdrecht was very high due to the presence of a very high amount of $P$ in the sediment (Keizer and Sinke 1992).

*Planktothrix agardhii* has a higher maximal growth rate ($\mu_{\text{max}} = 0.036 \text{ h}^{-1}$) than *P. hollandica* ($\mu_{\text{max}} = 0.025 \text{ h}^{-1}$), but needs a higher external $P$-load to compensate for its low affinity for $P$-uptake (Ducobu et al. 1998). *Anabaena* sp. and *Aphanizomenon flos-aquae* are both heterocystous diazotrophs, having the advantage in $N$-limiting conditions. Because $N_2$-fixing cyanobacteria have a lower yield on light compared with non-diazotrophs, they will be outgrown in $N$-sufficient, light-limiting conditions. *Anabaena* sp. is a growth strategist for light (sun species) and an affinity strategist for $P$; better competitor for rest concentrations of $P$ than *Aphanizomenon flos-aquae*. If the two tested strains are representative for the behaviour of the two genera, the presence of an *Anabaena* bloom may indicate light sufficient, $N_2$-fixing, $P$-limited conditions, or conditions with all nutrients in excess. The growth conditions favouring *Aphanizomenon* blooms are not clear, but its presence may indicate light-limiting conditions, because of its higher photosynthesis efficiency combined with $N_2$-fixing and $P$-sufficient conditions (De Nobel et al, 1997a+b). However, *Aphanizomenon flos-aquae* may be a better competitor for organic $P$, due to its capacity to make large amounts of AP (chapter 5 and 6).

These species are only a minor part of the cyanobacterial community in Lake Loosdrecht. The ecophysiological characteristics of dominant *P. limnetica* are less well studied. Nevertheless, in chapters 5 and 6 of this thesis we have shown that this species is capable of responding to changes in $P$-availability with differential expression of AP, representing a scavenging strategy. The differences in adaptation strategies between the two most ubiquitous algal species in Lake Loosdrecht offer interesting subjects for further study.
Changes in biomass indicators were observed in Lake Loosdrecht around 1990 (paragraph 2, this thesis). In the 1980's, the cyanobacterial community seemed to be growing, but the populations of *P. limnetica* and *P. hollandica* have become remarkably stable since 1990. By comparing the fraction of *P. hollandica* in the period 1988-1993 to that in the period 1997-2002 (Fig 1F and Table 2), this species seems less abundant in recent years. Frequent resuspension of the upper sediment layers in Lake Loosdrecht by wind action may have benefited the *P. hollandica* population in the 1980's by providing a variable P availability originating from suspended sediment particles (Marsden 1989). The amount of P in the water phase (seston) is currently below the detection limit, and negligible as a P pool.

Detritus, however, comprises an important P pool (Rijkeboer et al. 1991). The sestonic detritus consists of peat fragments and fine-sized (< 15 mm) particles derived from the phytoplankton. Lake Loosdrecht contains more detritus than phytoplankton cells; the phytoplankton to particulate detritus ratio was estimated to be 0.3 in 1988 (Gons et al. 1992). Dissolved organic matter mainly originates from lysis and further decomposition of phytoplankton cells. The phytoplankton detritus is about equally distributed between seston and the sediment/water interface (epipelon). The detritus is resuspended frequently, and fine-sized particles remain in the water column for several weeks (Otten et al. 1992). It is difficult to separately quantify adsorption/desorption processes between living and dead material within the complex resuspension-sedimentation compartment (Van Lieren and Janse 1992). Part of the organic P in the detritus must be bio-available, however (Rijkeboer et al. 1991; He and Honeycutt 2001). In contrast to *P. hollandica*, *P. limnetica* expresses AP, and can therefore use this pool of P. The population of *P. limnetica* has remained dominant and relatively constant throughout recent years. The increased dominance over *P. hollandica* may well be a result of the scavenging strategy (expression of AP combined with a high affinity uptake system) of this species. Although the overall AP linked fluorescence indicates an all-year P-limitation, the capability of *P. limnetica* to recycle phosphate by combining AP activity and high P-affinity, ensures the availability of P. Cells quickly reabsorb P that is liberated from detritus, and the dominant species with the highest affinity will get the greatest share. Coincidence of relapse in population size and activity of the grazer *Euchlanis dilatata* that has a preference for filamentous cyanobacteria (Pel et al. 2002), is an indication that *P. limnetica* further benefits from this property by allowing part of the slowly growing population in Lake Loosdrecht to die so that other parts can continue to grow more vitally (R. Pel, personal communication).
§6 General conclusions and future prospects

Cyanobacteria are able to efficiently scavenge and transport phosphate at concentrations in the nanomolar range, below the limits of the current detection methods, due to the use of parallel high-affinity uptake systems. This implies that turnover rates are more important than actual concentrations. Turnover rates, however, cannot be measured directly in lakes, without intrusive methods such as $^{32}$P-charging. Therefore, we have developed a different approach to detecting P$_i$-deficiency in phytoplankton, involving direct interrogation of phytoplankton cells on their nutrient status. Three factors determine the efficiency of P$_i$-uptake: the capacity to use a variety of phosphorylated compounds, the permeability of the cell membranes, and the relative concentrations of P$_i$ in- and outside the cells. Phytoplankton has strategies to deal with each of these factors using the following processes: conversion of P$_0$ (scavenging strategy; AP), high affinity P$_i$-uptake (affinity strategy; Pst system), and P-storage (storage strategy; polyP). The use of two complementary shotgun methods (La Roche et al. 1999) for expression analysis on protein and RNA levels, combined with sequence analysis, has revealed a wealth of information allowing a funded choice for markers of the P-status (chapter 3, this thesis).

We have focused on the targeting of marker proteins for P-deficiency and the application of a suitable diagnostic tool to detect these proteins. 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). Alkaline phosphatases (AP) play a key role in the recycling of organic P in lakes. Relevant components contributing to the dynamics of the internal P-cycle are sediment, detritus, bacteria, and phages. In spite of its shortcomings, the ELF-97 AP substrate was proven to be a useful indicator for P-deficiency (chapters 5 and 6, this thesis). To study the nutrient status of the phytoplankton in more detail, we have applied the ELF-97 AP substrate in flow cytometry. This method satisfies the criteria for diagnostic tools proposed by Falkowski et al. (1992): to be useful the tools must be broadly applicable in the field across phylogenetic lines, they must identify those processes that impose a truly physiological limitation, and they must be uniquely affected by a specific limiting factor.

After the development and testing stage presented in this thesis, the issues of calibration, quantification, and normalisation in routine research all lie ahead.

To get a complete picture of the present conditions and the nutrient history, more aspects of the P-status should be monitored. In conjunction with probes for species-specific resolution, these provide insight in factors that constrain productivity, and also affect community structure and species succession (Fitzgerald and Nelson 1966; La Roche et al. 1999).
The affinity, scavenging, and storage status give complementary information, reflecting the present growth conditions and the cells’ nutrient history. Future work should therefore 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 polyP staining (e.g. Ault-Riché et al. 1998). To answer the question which organic phosphomonoesters can be hydrolysed and assimilated by cyanobacteria, several compounds could be tested in enzymatic assays (Plant and Reddy 2001) as well as in growth experiments.

We have presented a case study (Lake Loosdrecht) and applied our diagnostic tool in this lake. Due to restoration measures, the chlorophyll $a$ concentrations in Lake Loosdrecht are currently below 100 µg.l$^{-1}$, giving a transparency of about 0.5 m. The phytoplankton community is growing under P-limitation, with a total P concentration of about 50 µg P.l$^{-1}$. These slight improvements of the major biomass indicators have made the water quality acceptable by the national standards (Vermeer en Waterstaat 1999), but are not sufficient to reach the ecological target for the lake: clear water with submerged plants. On the contrary, the measures have led to the adverse effect of increased cyanobacterial dominance. This dominance is likely due to the excellent adaptation capabilities of the cyanobacteria, allowing them to thrive at extremely low P$_i$-concentrations and light intensities. A more flexible approach to nutrient standards is therefore required, oriented to specific regions (see also Gulati and Van Donk 2002). This flexibility is provided with the establishment of the European Water Framework Directive (Commissie Integraal Waterbeheer 2002).

The presented results have revealed differences in adaptation strategies between the two dominant populations in Lake Loosdrecht: *Planktolyngbya limnetica* and *Prochlorothrix hollandica*. The idea that phosphatases play a key role in the recycling of P in the lake gives insight in the resilience to recovery of the water quality: a dense community of phytoplankton is inert to changes, owing to ‘self-shading’ and ‘self-feeding’. Additional measures are required to further improve the water quality, along with continued monitoring of species composition, nutrient status, and light climate in the lake. Finally, to get full benefit from the newly developed methods to address questions concerning the P-status in Dutch lakes, conclusive random sampling strategies are required instead of explorative judgment sampling used in this study. Therefore, spatio-temporal analysis of the nutrient status needs to be implemented in routine measurements. Currently, a new monitoring programme is being set up in the Loosdrecht lakes, together with additional measures to restore the water quality. Monitoring the phytoplankton nutrient status over the coming years is therefore of interest, both from a water management and a scientific point of view, as this might be a unique chance to monitor the switch from cyanobacterial dominance to clear water.