Phosphatase activities of riverine phytoplankton in the Vaal river (South Africa),

Physiological responses of nuisance species to different nutrient regimes

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PREFACE

This work has been done at the School of Environmental Sciences and Development, Potchefstroom University of Christian Higher Education, South Africa.

I hereby declare that this thesis presented for the degree *Philosophiae Doctor* at the Potchefstroom University of Christian Higher Education, consists exclusively of my own original research and has not previously been presented for a degree at any other university.

_____ Potchefstroom, 8 November 2002

ABSTRACT

PHOSPHATASE ACTIVITIES OF RIVERINE PHYTOPLANKTON IN THE VAAL RIVER (SOUTH AFRICA)

Physiological responses of nuisance species to different nutrient regimes

The theme of this thesis is the ecophysiological basis for development of mass-occurrences of phytoplankton species in aquatic environments. Research concerning the reasons for harmful algal bloom (HAB) formation has been intensifying during the past few decades. The need for physiological investigations concerning HAB species has been emphasised in recent literature.

The specific objectives of this investigation were to:

- study the Vaal River in respect to its phytoplankton species composition, emphasising HAB species, and the environmental as well as physiological characteristics of the river and its phytoplankton community,
- study the role of P assimilation by way of determining the magnitude, size fractionation, seasonal and spatial variation of alkaline and acid phosphatase activities (APA and AcPA) in the river,
- compare the composition of the phytoplankton community, and especially the occurrence of HAB species, in relation to both environmental and physiological parameters,
- study the growth, P and N metabolism and photochemistry, in response to varying N and P concentrations and ratios, of key bloom forming species in the river.

It was demonstrated that the eutrophic Vaal River has a high percentage of bloom forming species, algal blooms being present throughout the year. The bloom forming taxa occurred in a variety of environmental conditions, thus occupying separate (temporally or spatially) ecological niches in the river. The phosphatase activities in the river were high in comparison to literature, possibly being a physiological indicator of high trophic status. The bacterial and dissolved fraction contained most of the summed phosphatase activity, consisting dominantly of AcPA.

The phytoplankton, on the other hand, was dominated by APA. The bloom formers were distinguished physiologically from other phytoplankton species occurring in the river by a correlation with high alkaline phosphatase activity measured.

Three important bloom forming species from the Vaal River, *Chlamydomonas* sp., *Microcystis aeruginosa* and *Oscillatoria simplicissima,* were chosen for experimental studies, for further investigation of the physiological potential and adaptations of these species. As an example of a non-bloom forming species *Chlorella* sp. was chosen. Physiological responses, such as growth, cell density, chlorophyll-*a* content of the cells, endoenzymatic phosphatase activities, ectoenzymatic phosphatase activities, nitrate reductase activity, and photosynthetic performance of these species were studied *in vitro*. The cells were grown in nutrient replete growth medium (corresponding to a highly eutrophied environment), as well as N and P limited growth medium with varying N:P ratios.

Two different methods were applied for determination of phosphatase enzyme activity. The spectrophotometric method involves pNpp as enzyme substrate and detects endoenzymatic enzyme activity, while the fluorometric method uses fluorogenic MUF-substrates to detect ectoenzyme activity. These methods were used in parallel during the experimental studies, demonstrating the need of more detailed investigation into the origin and functions of phosphatase enzymes.

It was demonstrated that the bloom forming species indeed have higher constitutive APA levels, but also AcPA was present in nutrient replete conditions. In contrast, *Chlorella* sp., the non-bloom forming species, had very low constitutive APA and AcPA. After exposing cells to N and P deplete conditions both APA and AcPA were strongly induced in all species, suggesting that both enzyme groups are of importance in P-depleted situations.

The results indicate that the induction of both phosphatase and nitrate reductase activities function in most species as a general nutrient stress-response, probably preparing the cells for potential supply of organic phosphorus compounds (phosphatases) or nitrates (nitrate reductase), and enabling them to rapidly metabolise these nutrients.

Oscillatoria simplicissima was especially well equipped for N-limitation, maintaining a high growth rate despite N and P being supplied in very low concentrations. The filament length in *Oscillatoria simplicissima* decreased markedly in N limitation, indicating a morphological adaptation mechanism to low N concentrations in the environment. Compared to *Chlamydomonas* sp., the induction of phosphatases was not as strong, but both N and P limitation induced nitrate reductase activity considerably.

The results also implicate differing strategies in the studied species for coping with nutrient limitation of varying degree and type. All bloom formers showed K-strategic ("survival specialist") features either in their nutrient metabolism (*Chlamydomonas* sp.) or growth (*Oscillatoria simplicissima*), while the non-bloom forming *Chlorella* sp. showed characteristics of a r-strategist, having a high growth rate in nutrient replete conditions, whereas nutrient limitation had detrimental and rapid effects on its performance.

The use of phosphatases for ecophysiological studies, as well as the implications of the ecophysiological performance of the selected species are summarised and discussed in the concluding chapter.

KEY WORDS: algal (phytoplankton) blooms, *Chlamydomonas* sp., *Chlorella* sp., ecophysiology, eutrophication, *Microcystis aeruginosa*, nitrate, nitrate reductase, nutrient limitation, nutrient stress, *Oscillatoria simplicissima*, phosphatase, phosphate, photosynthetic vitality, PSII function, South Africa, Vaal River.

OPSOMMING

FOSFATASE AKTIWITEIT VAN RIVIERFITOPLANKTON IN DIE VAALRIVIER (SUID-AFRIKA)

Fisiologiese reaksies van probleemspesies op verskillende voedingstofregimes

Die tema van hierdie skripsie is die ekofisiologiese basis vir die ontwikkeling van opbloeie van fitoplanktonspesies in akwatiese omgewings. Gedurende die afgelope paar dekades is meer intensiewe navorsing rakende die redes vir die vorming van skadelike algopbloeie (HAB) gedoen. Die behoefte aan fisiologiese ondersoeke rakende HAB-spesies is in onlangse literatuur beklemtoon.

Die spesifieke doelstellings van hierdie ondersoek was om:

- die Vaalrivier te bestudeer ten opsigte van die fitoplankton-spesiesamestelling, met die klem op skadelike spesies, asook om die omgewings- en fisiologiese eienskappe van die rivier en sy fitoplanktongemeenskap te bestudeer,
- die rol van P-assimilering te bestudeer deur die omvang, grootte fraksionering, seisoenale en ruimtelike variasie van alkaliese- en suurfosfatase aktiwiteite (APA en AcPA) in die rivier te bepaal,
- die samestelling van die fitoplanktongemeenskap, en spesifiek die voorkoms van skadelike HAB-spesies, te vergelyk in verhouding tot beide omgewings- en fisiologiese parameters,
- die groei, N- en P-metabolisme en fotochemie in reaksie op variërende N- en P-konsentrasies en verhoudings van sleutel opbloeivormende spesies in die rivier te bestudeer.

Daar is gedemonstreer dat die eutrofe Vaalrivier 'n hoë persentasie opbloeivormende spesies het aangesien algopbloeie deur die hele jaar aanwesig is. Die opbloeivormende taksa het onder 'n verskeidenheid van omgewingstoestande voorgekom en beset dus afsonderlike (tydelike of ruimtelike) ekologiese nisse in die rivier. Die fosfatase-aktiwiteite in die rivier was hoog in vergelyking met diè in die literatuur genoem, wat waarskynlik 'n aanduiding is van 'n hoë trofiese status. Die bakteriële- en opgeloste fraksie het die meeste van die totale fosfatase-aktiwiteit bevat

wat hoofsaaklik uit AcPA bestaan het. Die fitoplanktonfraksie, aan die ander kant, is deur APA gedomineer. Die opbloeivormers is fisiologies van die ander fitoplanktonspesies wat in die rivier voorkom onderskei deur 'n korrelasie met hoë bepaalde alkaliese fosfatase aktiwiteit.

Drie belangrike opbloeivormende spesies uit die Vaalrivier, *Chlamydomonas* sp., *Microcystis aeruginosa* en *Oscillatoria simplicissima,* is gekies vir eksperimetele studies om die fisiologiese potensiaal en aanpassings van hierdie spesies verder te ondersoek. *Chlorella* sp. is gekies as voorbeeld van 'n nie-opbloeivormende spesie. Fisiologiese reaksies van hierdie spesies, soos groei, seldigtheid, chlorofil-*a* inhoud van die selle, endo-ensiematiese fosfatase-aktiwiteite, ekto-ensiematiese fosfatase-aktiwiteite, nitraatreduktase aktiwiteit en fotosintetiese vitaliteit is *in vitro* bestudeer. Die selle is gegroei in 'n voedingstofryke groeimedium (in ooreenstemming met 'n hoogs eutrofe omgewing), asook in N- en P-arme groeimedia met verskillende N:P verhoudings.

Twee verskillende metodes is toegepas vir die bepaling van fosfatase-ensiemaktiwiteit. Die spektrofotometriese metode gebruik pNpp as ensiemsubstraat en stel endo-ensiematiese ensiemaktiwiteit vas, terwyl die fluorometriese metode fluorogeniese MUF-substrate gebruik om ekto-ensiematiese aktiwiteit vas te stel. Hierdie metodes is in parallel gebruik gedurende die eksperimentele studies en het die behoefte aan meer in diepte ondersoeke na die oorsprong en funksies van fosfatase-ensieme na vore gebring.

Daar is gedemonstreer dat die opbloeivormende spesies wel hoër konstitutiewe APA-vlakke het, maar dat AcPA ook teenwoordig was onder voedingstofryke toestande. In teenstelling daarmee het *Chlorella* sp., die nie-opbloeivormende spesie, baie lae konstitutiewe APA en AcPA gehad. Nadat selle aan N- en P-arme toestande blootgestel is, is beide APA en AcPA sterk geïnduseer in al die spesies, wat daarop dui dat beide ensiemgroepe van belang is in P-arme situasies.

Die resultate dui aan dat die induksie van beide fosfatase- en nitraatredutase-aktiwiteite in die meeste spesies as 'n algemene voedingstofstresreaksie optree wat moontlik die selle voorberei vir 'n potensiële toevoer van organiese fosforverbindings (fosfatases) of nitrate (nitraatreduktase) en hulle in staat stel om hierdie voedingstowwe vinnig te metaboliseer. *Oscillatoria simplicissima* was besonder goed toegerus vir N-beperking en het 'n hoë groeitempo volgehou ten spyte daarvan dat N en P in baie laë konsentrasies voorsien is. Die filamentlengte by *Oscillatoria simplicissima* het beduidend afgeneem by N-beperking wat dui op 'n morfologiese aanpassing vir lae N-konsentrasies in die omgewing. In vergelyking met *Chlamydomonas* sp. was die induksie van fosfatases nie so sterk nie, maar beide N- en P-beperking het nitraatreduktase-aktiwiteit aansienlik geïnduseer.

Die resultate impliseer verskillende strategieë in die bestudeerde spesies om voedingstofbeperking van verskillende grade en tipes te kan hanteer. Alle opbloeivormers toon K-strategiese ("oorlewingspesialis") kenmerke in òf hul voedingstofmetabolisme (*Chlamydomonas* sp.) òf groei (*Oscillatoria simplicissima*), terwyl die nie-opbloeivormende *Chlorella* sp. eienskappe van 'n r-strategis getoon het wat 'n hoë groeitempo het in voedingstofryke toestande, terwyl voedingstofbeperking hul vitaliteit vinnig nadelig beïnvloed.

Die gebruik van fosfatases vir ekofisiologiese studies, asook die implikasies van die ekofisiologiese vitaliteit van die gekose spesies word in die slothoofstuk opgesom en bespreek.

SLEUTELWOORDE: algopbloeie (fitoplanktonopbloeie), *Chlamydomonas* sp., *Chlorella* sp., ekofisiologie, fosfatase, fosfaat, fotosintetiese vitaliteit, *Microcystis aeruginosa*, nitraat, nitraatreduktase, *Oscillatoria simplicissima,* PSII-funksie, Suid-Afrika, Vaalrivier, voedingstofbeperking, voedingstofstres

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LIST OF ABBREVIATIONS

ABS/RC	The specific energy flux (per PS II reaction centre) for absorption
AcPA	Acid phosphatase activity
AcP	Acid phosphatase
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APA	Alkaline phosphatase activity
ASP	Amnesic shellfish poisoning
CCA	Canonical correspondence analysis
chl a	chlorophyll *a*
D	Dilution rate
DCA	Detrended correspondence analysis
DI_0/RC	The specific energy flux (per PS II reaction centre) for heat dissipation
DSP	Diarrethic shellfish poisoning
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetraacetic acid
ELF	Enzyme-labelled fluorescence (fluorogenic substrate)
ET_0/RC	The specific energy flux (per PS II reaction centre) for electron transport
F	Fluorescence
GOGAT	Glutamate synthase
GS	Glutamine synthase
HAB	Harmful algal bloom
HPLC	High performance liquid chromatography
MUF	methylumbelliferyl (product)
MUP	4-methylumbelliferyl phosphate (substrate)
μ	growth rate
N:P	Nitrate to phosphorus ratio
NR	Nitrate reductase

PCA	Principal component analysis
φ_{E0}	Quantum yield of electron transport
φ_{P0}	Maximum quantum yield of primary photochemistry
PEA	Plant Efficiency Analyser
PI_{ABS}	Performance index expressed on absorption basis
P_i	Inorganic phosphorus
pNPP	p-nitrophenyl phosphate
P_o	Organic phosphorus
PSI	Photosystem I
PSII	Photosystem II
Ψ_0	Efficiency with which a trapped exciton, having triggered the reduction of Q_A to Q_A^-, can move an electron further than Q_A^- into the electron transport chain.
PSP	Paralytic shellfish poisoning
Q_A	Plastoquinone
Q_{A^-}	Reduced plastoquinone
RC	Photosystem II reaction centre
RDA	Redundancy Analysis
TR_0/RC	The specific energy flux (per PS II reaction centre) for trapping

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CHAPTER 1
INTRODUCTION

This study investigates algal nutrient physiology in connection with algal bloom formation. In this introductory chapter recent literature in connection to algal blooms is reviewed. Firstly algal blooms and their relationship to increasing eutrophication of water bodies is discussed, examples of bloom forming species and some of their ecological and physiological characteristics are considered, and finally the "nutrient physiology" of phytoplankton is reviewed, with emphasis on phosphorus (P) and nitrogen (N) utilisation. Secondly the functions and characteristics of phosphatase and nitrate reductase enzymes in relation to phosphorus and nitrogen utilisation, as well as their relationship with photosynthetic processes are reviewed. At the end of this chapter the objectives and the specific aims of this thesis are presented.

1.1. Algal blooms

An algal (also called microalgal or phytoplankton) bloom is the growth of one or a few algal species, which leads to an intensive increase in the biomass of the species in question. These blooms can be observed directly by their effects, such as visible discoloration of the water, foam production or toxicity to other organisms (Richardson 1997). It is estimated that the world's phytoplankton population consists of approximately 4000 different species, of which ca. 200 species (6%) have been identified as causing harmful algal blooms (HABs, Richardson 1997).

According to a widely cited review by Hallegraeff (1993) HABs have increased both in frequency as well as intensity during the last decades of the 20th century. A quantitative demonstration of a global increase in HABs is, however, difficult to prove. With the intensifying utilisation of marine and fresh waters for human activities (domestic, industrial or recreational purposes), the number of incidents of algal blooms that are perceived as harmful from a human point of view, has increased. Consequently, the early detection of the occurrence of, for instance, cyanobacterial blooms is increasingly prioritised in the monitoring and management of water bodies (e.g. Leppänen *et al.* 1995, Kahru and Brown 1997, Schofield *et al.* 1999, Codd 2000). This does not necessarily mean that these blooms have previously been absent, but that they now are recorded because of the problems they pose. Environmental monitoring programmes around the world have been expanding during the past decades, and these increased efforts in detecting

phytoplankton mass occurrences have contributed to the fact that more of them are being found (Richardson 1997).

A more complete understanding of the ecophysiological "rationale" for the occurrence of HAB's is urgently needed. What makes certain species superior to others, and allow them to displace other species in phytoplankton assemblages? How do these species maintain prolonged competitive dominance? The identification of key ecophysiological processes that permit a given species to occupy a defined ecological niche is a crucial step in modelling bloom dynamics and succession (Cembella 1998).

1.1.1. Examples of bloom forming species

The number of species known to form mass-occurrences has increased rapidly in recent decades, even though many algal species have been recognised to form blooms for centuries (Richardson 1997, Edvardssen and Paasche 1998). Bloom forming species occur in marine (e.g. Johnsen *et al.* 1997, Steidinger *et al.* 1998, Chang *et al.* 2001) brackish (e.g. Sivonen *et al.* 1989, Tanskanen 1990, Leppänen *et al.* 1995, Kankaanpää *et al.* 2001) and fresh water (e.g. Harding 1992, Jayatissa *et al.* 1998, Eynard *et al.* 2000). Dinoflagellates are mainly confined to marine environments, causing so called "red tides", whereas cyanobacteria are a bloom forming genera mainly found in fresh waters (Richardson 1997). HAB species are capable of exploiting an array of ecological niches, survival strategies, and nutritional modes (Cembella 1998). Table 1.1. gives some examples of species known to cause mass-occurrences, as well as their main feature responsible for causing "nuisance" conditions. The species in Table 1.1. are represented by their:

1. type of toxicity (Paralytic Shellfish Poisoning PSP, Amnesic Shellfish Poisoning ASP, Diarrethic Shellfish Poisoning DSP, toxic aerosols, cyanobacterial toxins such as variants of microcystins, anatoxins or nodularin),
2. ichtyotoxicity (toxic or harmful to fish, e.g. via mucus formation on the gills causing suffocation or irritation of tissues) or
3. other nuisance, such as filter clogging or irritation of skin, taste or odour production, high oxygen consumption.

Table 1.1. Examples of toxic and nuisance micro-algal and cyanobacterial species reported in the literature in the past 15 years.

Toxic, producing saxitoxins (causing PSP), hepatotoxins and ocadaic acid (causing DSP), domoic acid (causing ASP) or other toxic substances such as cyanobacterial toxins.	***Alexandrium*** complex	Taylor and Fukuyo 1998, Scholin 1998, Anderson 1997
	Ceratium furca and spp.	Johnsen *et al.* 1997
	Dinophysis spp.	Tanskanen 1990, Maestrini 1998
	Gymnodinium (***Gyrodinium***) spp	Hallegraeff and Fraga 1998, Steidinger *et al.* 1998, Gobler and Cosper 1996
	Prorocentrum minimum	Johnsen *et al.* 1997
	Pyrodinium bahamense	Usup and Azanza 1998
	Aureococcus anophagefferens **("brown tide")**	Bricelj and Lonsdale 1997
	Karenia brevisulcata (causing toxic aerosol syndrome)	Chang *et al.* 2001
	Pseudo-nitzschia spp.	Horner *et al.* 1997, Johnsen *et al.* 1997, Bates *et al.* 1998
	Nitzschia sp.	Kotaki *et al.* 2000
	Anabaena spp.	Tanskanen 1990, Leppänen *et al.* 1995, Eynard *et al.* 2000
	Aphanizomenon spp.	Tanskanen 1990, Leppänen *et al.* 1995, Eynard *et al.* 2000
	Lyngbya majuscula	Nagle and Paul 1999
	Microcystis spp.	Tanskanen 1990, Harding 1992, Leppänen *et al.* 1995, Yunes *et al.* 1998, Jayatissa *et al.* 1998, Eynard *et al.* 2000
	Nodularia spumigena	Sivonen *et al.* 1989, Tanskanen 1990, Leppänen *et al.* 1995, Kankaanpää *et al.* 2001
	Oscillatoria spp.	Tanskanen 1990, Leppänen *et al.* 1995
	Planktothrix spp.	Tanskanen 1990, Leppänen *et al.* 1995
Fish killers	***Ceratium*** spp.	Horner *et al.* 1997, van Ginkel *et al.* 2001
	Chaetoceros convolutus and spp.	Horner *et al.* 1997, Johnsen *et al.* 1997
	Chattonella spp.	Imai *et al.* 1998
	Chrysocromulina leadbeateri	Johnsen *et al.* 1997
	Chrysocromulina polylepis	Dahl *et al.* 1989, Edvardssen and Paasche 1998

	Cochlodinium polykrikoides	Park *et al.* 2001, Whyte *et al.* 2001
	Dictyohca speculum	Johnsen *et al.* 1997
	Heterosigma akashivo	Smayda 1998
	Pfiesteria piscicida and other ***Pfiesteria*** like species	Burkholder *et al.* 1998, 2001
	Prymnesium parvum	Johnsen *et al.* 1997, Edvardssen and Paasche 1998
	Prymnesium patelliferum	Johnsen *et al.* 1997
	Phaeocystis cf ***pouchetii***	Johnsen *et al.* 1997, Lancelot *et al.* 1998
	Rhizosolenia spp.	Johnsen *et al.* 1997
Others	***Emiliana huxleyi***	Johnsen *et al.* 1997
	Noctiluca scintillans	Elbrächter and Qi 1998
	Prorocentrum micans	Horner *et al.* 1997
	Protoperidinium spp.	Horner *et al.* 1997
	Rhizosolenia setigera	Horner *et al.* 1997

One species can, however, belong to more than one nuisance "type". Many of the examples of bloom forming algae belong to species complexes. The taxonomy of some species is still unclear and in a dynamic phase due to intensive research and new methods in molecular systematics, which constantly shed light on the taxonomy and systematic relationships. Some species have been known for a long time as non-toxic, normally occurring algae, but have in one or a few instances formed sudden intensive, highly noxious blooms (e.g. *Chrysocromulina* sp., Edvardssen and Paasche 1998). Others "attack" a variety of marine organisms, being able to consume bacteria, algae, microfauna, fish and mammalian tissues (e.g. *Pfiesteria piscicida*). This type of phytoplanktonic ambush-predator was unrecognised until the early 1980s (Burkholder *et al.* 2001). For a detailed review on nuisance and toxicity types see e.g. Richardson (1997) and references therein.

1.1.2. Causes of algal blooms, and ecophysiological characteristics of bloom formers

Although critical factors have been described for some species, especially for some marine dinoflagellates, we still have only a crude understanding of the ecophysiological mechanisms that promote and maintain HAB's. An algal mass-occurrence is, in a way, at odds with the normal coexistense of a multispecies phytoplankton assemblage (comprising of often more than 30 species) described by Hutchinson (1961) as the "Paradox of the Plankton". Which are the mechanisms that disrupt this coexistence of a large number of species? What makes HAB species capable of bloom forming, and developing near monocultures in an environment that normally exhibits a heterogenous range of co-occurring phytoplankton species? Many contributing environmental conditions causing increased occurrence of phytoplankton blooms have been suggested, including physical, chemical and biological conditions, as well as ecological and physiological features benefiting bloom forming phytoplanktonic species in these conditions.

1.1.2.1. Physical variables

Turbulence and mixing are critical factors for the growth and persistence of natural populations of phytoplankton (Reynolds 1984, Berman and Shteinman 1998). The stratification of the water column has been mentioned as one of the triggering factors for phytoplankton bloom development. Margalef (1978) suggested that one of the most important factors in the ecological selection of phytoplanktonic species is the mechanical energy of the water column.

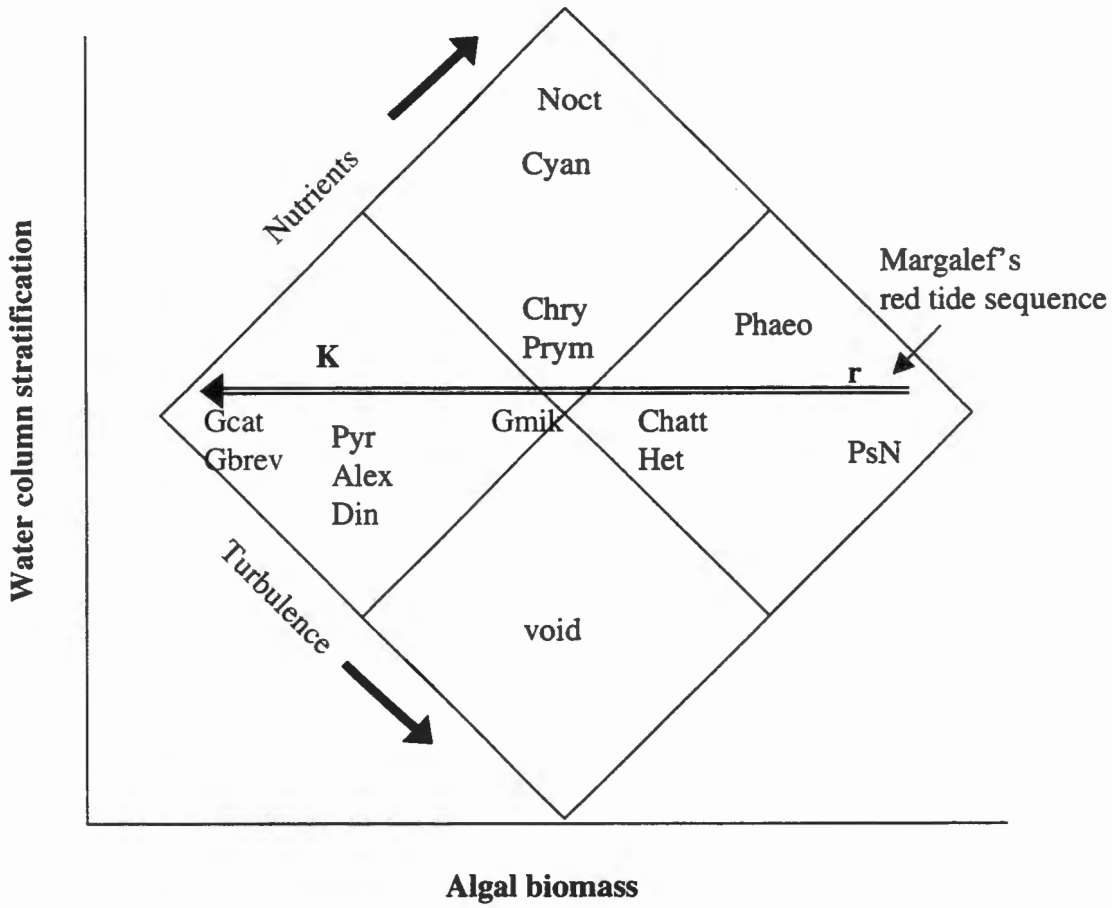


Figure 1.1. An adaptation of "Margalef's Phytoplankton Mandala", modified from Hallegraeff (1998), showing the preference of certain algal genera or species to different nutrient and turbulence conditions (square), which is superimposed on the plane defined by the coordinates production potential (algal biomass) and water column stratification. Margalef's original "red tide sequence" is marked with the horizontal arrow. K represents species that display K-strategic features, and r represents r-strategic species. Alex = Alexandrium, Chatt = Chattonella, Chrys = Chrysocromulina, Cyan = Cyanobacteria, Din = Dinophysis, Gbrev = Gymnodinium breve, Gcat = Gymnodinium catenatum, Gmik = Gymnodinium mikimotoi, Het = Heterosigma, Phaeo = Phaeocystis, Prym = Prymnesium, Noct = Noctiluca, PsN = Pseudo-Nitzschia, Pyr = Pyrodinium.

Margalef (1978) presented schematically how different life forms of microalgae in the phytoplankton would correspond to different regimes of turbulence and nutrient availability (Figure 1.1). A main axis in the model is a transfer between species adapted to low turbulence – low nutrient conditions (e.g. many dinoflagellates), and species adapted to high turbulence – high nutrient conditions (e.g. diatoms). The persistence of high nutrient conditions despite low turbulence can be associated with areas with external inputs of nutrients, creating conditions conducive to cyanobacterial or dinoflagellate blooms (red tides).

The adaptation of different species to varying turbulence or nutrient concentrations has been described by MacIntyre *et al.* (1997). The authors suggested, that species typical of turbulent conditions should have adaptations to exploit variable irradiances rather than to acquire nutrients effectively. As adaptations for conditions where turbulence decreases they suggest migration, sinking and alternative life-form formation or layer formation. Movement enables the algal cell to migrate to a favourable light, temperature or nutrient environment. Morphological and physiological adaptations, such as spine formation, movement by flagellae or buoyancy control can give some phytoplanktonic microalgae a competitive advantage. Certain cyanobacterial species (e.g. *Microcystis aeruginosa*, *Anabaena* sp., *Aphanizomenon* sp.) can regulate their buoyancy by gas vesicles, which can give them a competitive advantage in stable water columns (South and Wittick 1987). It has been suggested that buoyancy regulation is a mechanism by which cyanobacteria may overcome the vertical separation of light and nutrients (Brookes and Ganf 2001), through regulation of cell density as well as the molecular control of gas vesicle production. In low irradiance (but high nutrient concentrations) these species can produce gas vesicles, thereby increasing their buoyancy and thus migrate to higher light to enable efficient photosynthesis. When irradiation is too high, the vacuolation of the cells decreases as a consequence of increased turgor pressure from photosynthate, and the cells will sink (South and Wittick 1987). By regulating their buoyancy in this manner, these cyanobacterial species are capable of high primary production resulting in enhanced reproduction, thus forming mass occurrences that shade the rest of the phytoplankton population (Brookes and Ganf 2001). A study by Wallace and Hamilton (2000) showed another physiological mechanism for buoyancy regulation, which is based on carbohydrate accumulation and consumption. For example *Aphanizomenon ovalisporum* (Porat *et al.* 2001) and *Anabaena circinalis* (Brookes *et al.* 1999) increase the carbohydrate content in the cells during increasing irradiance, hence decreasing the buoyancy. However, according to Oliver (1994), *Microcystis aeruginosa* blooms occur when the

buoyancy regulating mechanisms fail to negate the buoyancy provided by the gas vesicles before the colonies reach the surface. Such failure of buoyancy regulation could be due to prolonged mixing of the water column, creating a variable light climate, which prevents *Microcystis aeruginosa* cells from adjusting their carbohydrate accumulation to necessary levels to prevent floating, the cells therefore forming a bloom when the mixing subsides (Wallace and Hamilton 2000).

In a study relating environmental factors to a bloom of *Microcystis aeruginosa* in Steilacoom Lake, Washington, Jacoby *et al.* (2000) found that physical characteristics associated with the bloom were increased turbidity (indicating decreased light), high water column stability, temperature and pH. In connection to cyanobacteria generally, low turbulence, high pH and high temperature are physical conditions often connected to the formation of blooms (Steinberg and Hartmann 1988, Shapiro 1990, Hyenstrand *et al.* 1998). Contrasting views exist especially in connection to light climate (irradiance) and turbidity, and the preference for either high or low turbidity (and irradiance) is probably highly species specific.

Other physical variables that may be of importance for bloom formation are e.g. water currents, mixing and eddies. Eddy formation has been shown to be an important factor in controlling the bloom initiation of *Gymnodinium breve* (Tester and Steidinger 1997).

1.1.2.2. Chemical variables

Eutrophication and nutrient availability: It has been implicated that excessive nutrient enrichment (eutrophication) via anthropogenic inputs can be a major factor in the increased prevalence of HABs (Millie *et al.* 1999). Differing views are being regularly published, however, suggesting that nutrient enrichment *per se* is only one, even minor, contributor to algal bloom formation (Richardson 1997), and especially certain cyanobacterial blooms have been suggested to form in mesotrophic, rather than eutrophic, environments (Steinberg and Hartmann 1988). As established at the International Association of Phytoplankton Taxonomy and Ecology in 1998: "few phytoplankton patterns or assemblies have been recognised or associated with the relevant dimensions of trophic state – much less any real basis for predicting structures." Quantitative responses of the phytoplankton biomass are more or less predictable on the basis of the nutrients supplied, excessive nutrients causing increased algal biomass. When it comes to

species composition, no such convenient surrogate is demonstrable (Reynolds *et al.* 2000). Eutrophication does, however, result in a reduction of species diversity in water bodies at all trophic levels (Codd 2000). In eutrophied water bodies a general shift from diatom-dominated phytoplankton assemblages to a green algae and cyanobacterial dominated species composition often takes place (Makarewicz and Baybutt 1981, South and Wittick 1987, Steinberg and Hartmann 1988). It is still unclear, however, which environmental conditions (such as differing trophic levels) affect the appearance of a certain species in the aquatic environment, and how (Reynolds *et al.* 2000).

P-enrichment is recognised as a major factor in freshwater eutrophication but it may also lead to a switch in the critical metabolic constraints to other factors or factor interactions (Reynolds *et al.* 2000). Lately it has been shown that N often is as likely to limit phytoplankton growth as P in freshwaters (Robertson 1999), and vice versa, P limiting phytoplankton growth in estuaries and coastal regions (e.g. Murrell *et al.* 2002). According to Riegman (1998) there has been no evidence that HABs would occur more often in either N or P enriched conditions, but rather it seems like HABs relate to a general macronutrient enrichment-induced increase in phytoplankton biomass.

Rapid growth is usually seen as a characteristic typical of opportunistic species (r-strategists) (Kilham and Kilham 1980). Despite the definition of an algal bloom seemingly being related to rapid growth, it has been hypothesised that bloom formers would rather be K-strategists than opportunistic r-strategists. According to a multialgal species foodweb (Riegman and Kuipers 1994) increasing eutrophication would lead to a replacement of 1) competitive specialists by 2) rapidly growing generalists (mesotrophy) and finally by 3) poorly edible primary producers (hypertrophy). Algae which invest part of their energy in grazing resistance (toxic substances, spines etc.) are not able to invest the same energy in producing new cells, as a result of which these species should be poor competitors compared to related edible species. Higher eutrophy, however, enhances the zooplankton biomass and selective grazing pressure channels nutrients to poorly edible, K-strategic species, that consequently will be able to dominate and form blooms.

Contrary to the view that enrichment would increase the biomass of bloom formers, e.g. blooms by *Aureococcus anophagefferens* seem not to occur in response to eutrophication, but has rather been related to its ability to grow at very low DIN levels which are known to limit the growth of

other phytoplanktonic species (Bricelj and Londsdale 1997). Similarly, species of *Oscillatoria* and *Aphanizomenon* have frequently been found to be dominating in nutrient poor systems (Steinberg and Hartmann 1988 and references therein). Delivery of micro-nutrients, such as iron and selenium, from watersheds has been implicated to be a contributory cause in bloom formation by the brown-tide alga *Aureococcus anophagefferens* (Cosper *et al.* 1993) and *Gymnodinium sanguineum* (Gobler and Cosper 1996).

Nutrient ratios and speciation: It has been suggested that the apparent global increase of harmful algal blooms is related to increased N:Si and P:Si supply ratios favouring non-siliceous microalgae in the phytoplankton (Smayda 1990), shifting the species composition to less diatom dominated systems. Increased runoff of phosphorus from anthropogenic sources has in many waters decreased the N:P ratio. Many cyanobacterial species are equipped for N-limited situations by being capable of fixing nitrogen with the aid of nitrogenase enzymes. Many (filamentous) species have specialised thick-walled cells called heterocysts, which develop in N-limited situations (South and Wittick 1987). Heterocysts lack the oxygen evolving photosystem II (Wolk 1982) and the heterocyst walls contain oxygen-binding glycolipids (Lambein and Wolk 1973), thereby maintaining the anaerobic conditions necessary for nitrogen fixation (Bothe 1982). In the heterocysts these species are able to fix atmospheric nitrogen, which has dissolved into the water, and transform it into NH_4^+ with the aid of nitrogenase enzyme (South and Wittick 1987). Since most phytoplanktonic species are dependent on the ambient NO_3^- pool, heterocystous cyanobacterial species will attain competitive advantage in NO_3^- poor situations, and may especially benefit from high phosphorus loading in combination with low NO_3^- supply. Stockner and Shortreed (1988) concluded, however, that the development of nitrogen fixing cyanobacterial blooms is dependent on both a low N:P supply ratio and a sufficient phosphorus supply.

A frequently quoted hypothesis is that cyanobacteria in general would benefit from low N:P ratios (Smith 1983), but has recently been questioned especially in connection to non-heterocystous cyanobacteria (Shapiro 1990, An and Jones 2000, Downing *et al.* 2001). Riegman (1998) states that: "The indirect effect of enrichment with macro-nutrients on algal species composition is probably more related to rigorous changes in the modulation of resource limitation... and the enhanced biogeochemical and biological impact of the sediments on the pelagic system". In other words, Riegman puts emphasis on the importance of changing nutrient

ratios and internal (within the lake/water system) nutrient enrichment due to mobilisation of nutrients from sediments.

Competition experiments have demonstrated how different nutrient ratios favour different species. Experiments including two cyanobacterial species showed that *Microcystis aeruginosa* was superior in low N:P ratios and high temperature, whereas *Phormidium tenue* was superior in high N:P ratios and low temperatures (Fujimoto *et al.* 1997). Another study showed, however, that *Microcystis aeruginosa* grown with N and P in the growth limiting range, grew optimally at a comparatively high N:P ratio of 100:1 (Nalewajko and Murphy 2001).

According to Riegman (1998) light or nutrients control growth during the initial stages of bloom formation. Basically an algal species will outcompete others when it is able to reduce the availability of the limiting factor to such an extent that the other competing species are not able to obtain enough of it to compensate for their natural population loss routes. Therefore not only nutrient ratios but also nutrient speciation is of importance. Certain species use NO_3^- better than other N-sources, while others grow faster on urea or humic acids (Riegman 1998, see also section 1.1.2.3 "Ecological clusters"). Movement plays a role also in nutrient acquirement. Direct retrieval of important nutrients by motile forms of microalgae may provide an important alternative mechanism of e.g. P transport (Salonen *et al.* 1984, Park *et al.* 2001). *Ceratium hirundinella* is an example of a species that can access multiple P sources through vertical migration (James *et al.* 1992), and thus attain competitive advantage e.g. in strongly stratified water columns.

As an example of a straightforward connection between survival mechanisms and certain environmental conditions, *Anabaena* and *Aphanizomenon* cyanobacteria are co-occurring in mesotrophic, stratified lakes especially in NO_3^- depleted situations (Stockner and Shortreed 1988, Reynolds *et al.* 2000). These species have the ability to stay in the surface mixed layer because of their capability of regulating their buoyancy by gas vesicles. Simultaneously they can enhance their C uptake in high pH, and fix N_2 in heterocysts.

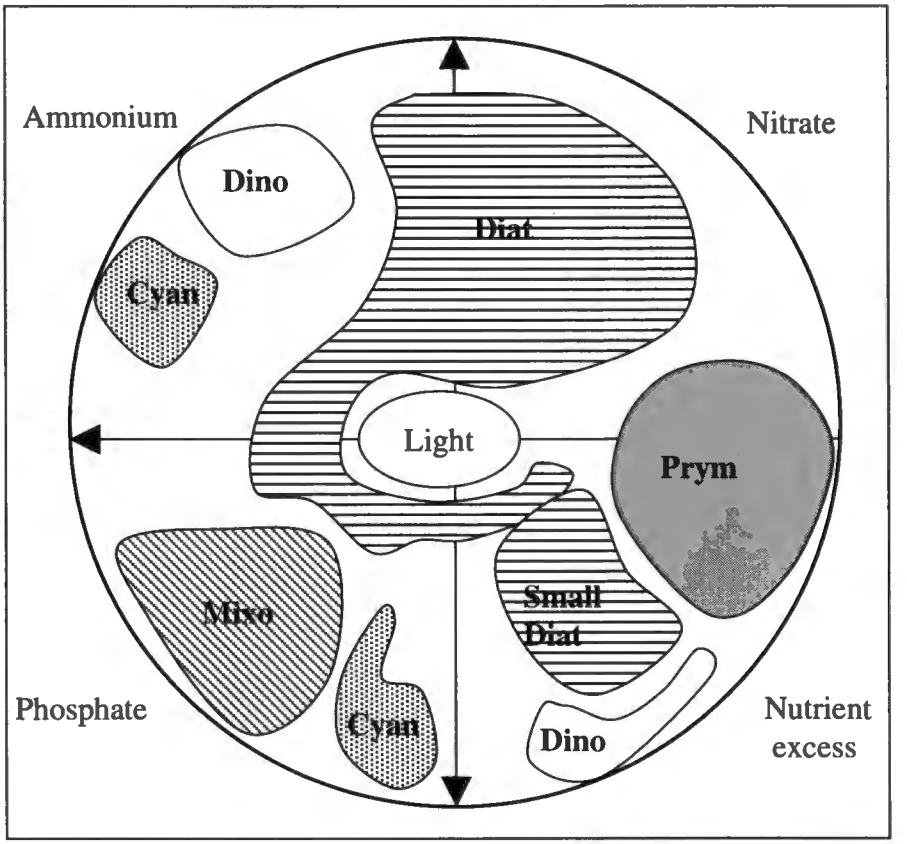


Figure 1.2. Ecological clusters as suggested by Riegman (1998), describing species clusters of dinoflagellates (Dino), diatoms (Diat), haptophyceae (Prym), cyanobacteria (Cyan) and mixotrophic algae (Mixo) based on their preference to differing light and nutrient conditions. The centre of the circle indicates light limiting conditions, the border of the circle light saturating levels and nutrient limitation or nutrient excess.

1.1.2.3. Biological variables

Cell size: Microalgae smaller than 5 µm in diameter have higher growth rates and are often better competitors for nutrients than larger cells, both in nutrient limiting conditions as well as under saturating irradiance and nutrient levels (South and Wittick 1987, Kjørboe 1993). The exception to this is diatoms, which have an advantage under pulsating N-limitation (Riegman 1998). Diatoms can store NO_3^- in the vacuole of the cell, and the vacuole volume:cytoplasm ratio increases with cell volume in diatoms. Nevertheless, larger algae (>5 µm) seem often to dominate algal blooms (Bentzen *et al.* 1992). The reason for this might be the short regeneration times for microzooplankton, which leads to an effective control of the biomass of the smaller microalgal fraction (Riegman 1998, Franks 2001).

Ecological clusters: Attempts have been made to combine larger taxonomic groups into ecological clusters based on their success in differing nutrient and light conditions. The model presented in Figure 1.2 (according to Riegman 1998) suggest that e.g. diatoms would appear to have a high growth rate under low-irradiance conditions, but are dependant on silica, whereas dinoflagellates seem to be more capable of competing for ammonium than for NO_3^-. In certain conditions a species from the appropriate cluster would be expected to form a bloom. Other similar clusters have been presented by e.g. Reynolds (1980, presented and quoted in Richardson 1997), with emphasis on different phytoplankton assemblages (consisting of 12 species) as a function of nutrient availability and turbulence. Such structures will not, however, allow predictions regarding the exact species that will emerge as dominant from each group – a crucial question when HAB species are concerned (Cembella 1998).

Toxin production: In the Gulf of Mexico more than 30 toxic microalgal species occur (Steidinger *et al.* 1998), and in the Baltic sea 22 potentially toxic phytoplanktonic species have been reported (Leppänen *et al.* 1995). At least twenty-five toxin producing cyanobacteria have been reported (Codd 1998) from fresh and brackish waters. Surveys conducted in Finland (Sivonen *et al.* 1990) and the UK (Codd and Bell 1996) have found that approximately half of all fresh and brackish water cyanobacterial blooms were producing toxins, in Slovenia the proportion was even higher, exceeding 80% (Sedmak and Kosi 1997). Over 60 structural variants of the cyanobacterial toxin microcystin are presently known (Rapala and Sivonen 1998, Namikoshi *et al.* 1998). Many of these toxins are potent inhibitors of protein phosphatase 1 and 2A from both plants and animals

(MacKintosh *et al.* 1990), as well as potent tumour promoters (Nishiwaki-Matsushima *et al.* 1992). The ecophysiological significance of phytotoxins has engendered much speculation (Cembella 1998), and an array of different reasons for toxin production have been suggested (the following discussion concerns freshwater toxins only):

- Grazer repellent. Toxicity against grazers has oftentimes been suggested as the reason for toxin production (Kirk and Gilbert 1999, Nagle and Paul 1999). Many of the toxins produced are, however, endogenous, and will therefore affect the grazer only after the toxin producer itself has been ingested. In a situation where the foodweb is dominated by several grazers, this might have an indirect benefit on the toxic species: if the foodweb includes a generalist grazer and a specialist herbivore, the generalist might be suppressed by ingesting toxic cells. Consequently the abundance of the specialist herbivore increases due to competitive release of the generalist grazer. The specialists ingest selectively non-toxic species, which compete with the toxic species for limiting nutrients, which in turn will benefit the toxic species (Kirk and Gilbert 1999). However, because of the large variety of toxins and their impact mechanisms, it is not probable that grazer repellence would be the only, or even the most important, reason for toxin production. In the case of cyanobacteria, according to Sedmak and Kosi (1998) the evolutionary age of the organisms (3.3-3.5 billion years) proves it unlikely that microcystin toxins would play the role of a defensive substance. It seems that the reasons are much more complex, and the mechanisms functioning in one instance of toxic phytoplankton – grazer interactions cannot usually be extrapolated to another (Turner and Tester 1997).
- Bioactivity against photosynthesis in other cyanobacteria, phytoplankton or higher plants. Microcystins and nodularins are examples of cyanobacterial toxins that, apart from being toxic to humans and animals, also are active against higher plants (MacKintosh *et al.* 1990) and can have direct action on cell membranes, thus being capable of affecting other biochemical processes, including photosynthetic ones (Smith and Doan 1999). Many cyanobacterial toxins have been proven to be directed against oxygenic photosyntethic processes – therefore they would probably be likely to be involved in regulating natural populations of other photosynthetic organisms (Schlegel *et al.* 1999). *Oscillatoria late-virens* has antibiotic activity against certain green algae and cyanobacteria, affecting the growth and photosynthesis of e.g. *Microcystis* species (Bagchi *et al.* 1993, Bagchi (1995). Similarly, toxins (microcystin-LR) produced by *Microcystis aeruginosa* are strongly algicidal against other cyanobacteria and green algae, causing inhibited photosynthetic and nutrient

acquisition processes (Singh *et al.* 2001), such as inhibition of nitrate reductase enzyme activity (MacKintosh 1992), and subsequent growth. The cyanobacterium *Trichormus doliolum* releases secondary metabolites that strongly inhibit the growth of other cyanobacteria, the major allelopathic compound inhibiting PSII mediated photosynthetic electron transport, especially in phosphorus limited systems (Von Elert and Jüttner 1997). Smith and Doan (1999) presented several cyanobacterial secondary metabolites that were found to be directed against oxygenic photosynthetic processes. Antibiotic activity of several other cyanobacterial species has been shown against green algae and other cyanobacteria (Schlegel *et al.* 1999). Sedmak and Kosi (1998) concluded that toxic cyanobacterial blooms strongly decrease the species diversity in the water, causing entire taxonomic groups to temporarily be inhibited or completely cleared from the environment. Studies concerning factors controlling or affecting toxin production have mainly concentrated on the cyanobacterial toxin microcystin. The results have been contradictory, with both positive and negative correlations being reported with e.g. microcystin production and light intensity (Sivonen 1990, Utkilen and Gjølme 1992, Hobson *et al.* 1999), temperature (Watanabe and Oishi 1985, Sivonen 1990, Rapala *et al.* 1997) and nutrient concentrations (Watanabe and Oishi 1985, Rapala *et al.* 1997, Rapala and Sivonen 1998). Also trace metal supply (Lukač and Aegerter 1993), especially iron (Utkilen and Gjølme 1995, Lyck *et al.* 1996), and pH (Song *et al.* 1998) have been shown to affect toxin production in batch cultures of e.g. *Microcystis aeruginosa*. On the other hand, positive allelopathic activity has also been shown. For example *Oscillatoria* sp. is stimulated by the presence of *Spirogyra* sp. (Mohamed 2002).

- Storage of nutrients. It has been suggested that toxin production or increase in the cellular content of toxins, may be a way to store excess organic carbon made available by photosynthesis, in a similar manner as e.g. lipids and carbohydrates are produced in excess by most phytoplanktonic cells when there is not enough N or P available to build up material for cell division (Anderson *et al.* 1990, Granéli *et al.* 1998). This is supported by the findings of Rapala *et al.* (1993), who showed higher anatoxin-*a* concentrations in N-fixing *Anabaena* sp. and *Aphanizomenon* sp. in NO_3^- free medium. In a recent study it was shown that fast-growing cells of N-limited *Microcystis aeruginosa* had higher microcystin concentrations than slower growing cells (Long *et al.* 2001). Sivonen (1990) found higher toxin production in *Oscillatoria agardhii* at high N concentrations. Lee *et al.* (2000) demonstrated highest microcystin contents at N:P ratios equalling 16:1, and a positive correlation with chlorophyll-

a concentration. Watanabe and Oishi (1985) showed a slight increase in toxin production under P-limitation. Oh and co-workers (2000) also found higher microcystin concentrations in *Microcystis aeruginosa* grown under P-limitation, as well as an increase in the number of different microcystin-toxin forms in more P-limited conditions. The opposite was noted during observations of environmental factors associated with toxic *Microcystis aeruginosa* blooms, which shows that microcystin concentrations increased with increasing soluble reactive phosphorus (Kotak *et al.* 2000), indicating that the toxin production may have been limited by phosphorus (Jacoby *et al.* 2000). The same trend was demonstrated by Rapala *et al.* (1997) in batch cultures of a microcystin producing *Anabaena* sp., where toxin concentrations increased with increasing phosphorus. In another study by Rapala *et al.* (1993) the toxin production of *Anabaena* sp. and *Aphanizomenon* sp. was not affected by PO_4^{3-} concentrations. Utkilen and Gjølme (1995) showed that neither nitrate nor phosphorus limitation had an effect on the toxin production of *Microcystis aeruginosa*. Thus no systematic pattern in regard to the connection between N, P, or other nutrients, and toxin production can be deduced from existing literature.

- Also other physiological functions for toxin production have been suggested, such as toxins functioning as intracellular chelators inactivating free cellular Fe^{2+} (Utkilen and Gjølme 1995) and other specific cell regulatory functions (Rapala *et al.* 1997).

In summary, a range of experiments have been done and field observations made in the attempt to relate toxin production to environmental factors, but to date the reasons for toxin production remain unsolved.

The food web: The development and persistence of an algal bloom may reflect failure of normal grazing control by zooplankton. Reduced grazing by planktonic and benthic communities contributed to the maintenance of a brown tide caused by *Aureococcus anophagefferens* (Bricelj and Londsdale 1997). Elser (1999) suggested that the food web might influence the development of cyanobacterial blooms not just by regulating the rate of mortality caused by grazing, but also through trophic interactions regulating cyanobacteria dynamics by altering the consumer-driven nutrient recycling regime in a way that shifts competitive advantage away from cyanobacteria. In a situation where N:P ratios are low, and cyanobacterial blooms frequent, in some circumstances the blooms might not occur despite favourable N:P ratios. This could be due to a shift in the zooplankton community towards a *Daphnia* dominated community. The reason for the

disappearance of the cyanobacteria is not entirely due to grazing by *Daphnia* (which might occur as well) but rather of the body elemental composition of *Daphnia*. The zooplankton family to which *Daphnia* belongs has an unusually low body N:P ratio, and therefore will tend to retain P in its biomass and release N, this way generating higher N:P ratios in the surrounding media. Thus, shifts in zooplankton community composition might also alter algal community composition via the nutrient resources. This shift in zooplankton community composition would also mean that consumer-driven nutrient cycling is a key internal mechanism operating in the pelagic zone, and we can move away from the debate surrounding the false dichotomy of "top-down" or "bottom-up" factors regulating pelagic ecosystem function (Elser 1999).

1.1.3. Conclusion

Little direct information is available on the eco-physiological causes of HAB's, but it is clear that physiological and metabolical processes of bloom forming micro-algae should be a central explanatory factor (Millie *et al.* 1999), considering that the presence or absence of nutrients affect the phytoplankton both directly as well as indirectly via changing nutrient ratios or nutrient release from sediments or other organisms. Physiological responses, such as photophysiological regulation, respiration, protein and enzyme kinetics, form the basis for cell growth, maintenance and proliferation of a phytoplankton bloom.

Nutrient concentrations play an important role in the growth of any phytoplanktonic species, and thus the utilisation of available nutrients is of major importance in determining the competitive capabilities of different algae. It is of importance to investigate how different species are equipped for e.g. adapting to rapidly changing conditions or nutrient plumes, or to effectively utilise and store nutrients that for other species will be limiting. Phosphorus constitutes the growth-limiting nutrient in fresh waters (Wetzel 1983, South and Wittick 1987), and recently phosphorus has been recognised as "the most essential of nutrients" also in marine environments (Karl 2000, Labry *et al.* 2002). Thus uptake and storage of inorganic phosphorus (P_i) and regeneration of phosphate from organic phosphorus (P_o) compounds is essential for plankton succession. Nitrogen is another essential macronutrient in freshwaters, which together with phosphorus often acts as a limiting nutrient (Robertson 1999). Exactly how these nutrients are utilised and circulated is, despite considerable effort, not yet completely understood, and

therefore studies concentrating on both phosphorus and nitrogen utilisation of important phytoplanktonic species are much needed.

1.2. Phosphorus and Phosphatase enzymes

The ecological interest in phosphorus stems from its major role in biological metabolism, and the relatively small amounts of phosphorus available in the hydrosphere (Wetzel 1983). In terrestrial plants, P-deficiency has been shown to result in an investment in e.g. the initiation of specialised root types with the sole purpose of P_i-acquisition (Miller *et al.* 2001). Raghothama (1999) lists a number of morphological, physiological, biochemical and molecular responses of plants to phosphate deficiency. Biochemical responses in plants include the induction of enzymes involved in the acquisition of P_i. In microalgae, similar functions of phosphatases have been shown (e.g. Pettersson 1980, Heath 1986, Jansson *et al.* 1988, Hantke *et al.* 1996).

1.2.1. Physiological attributes of phosphorus utilisation

Physiological attributes that are considered essential for determining the competitive ability of microalgae with respect to a limiting nutrient are according to Spijkerman and Coesel (1996):

1. Ability to acquire the resource in question (affinity)
2. Ability to use the resource for production of new cells (yield)
3. Ability to store the resource (storage capacity)

An example of a cyanobacterial species with some strategic features considering the phosphorus utilisation is *Cylindrospermopsis raciborskii* (Isvánovics *et al.* 2000). This species is rapidly expanding its geographical area of occurrence, and has produced extensive and very toxic blooms in water bodies world-wide (Chapman and Schelske 1997). Isvánovics *et al.* (2000) showed by P uptake kinetic studies that this species is opportunistic with respect to P. It has a high excess P storage capacity after saturating P pulses (e.g. pulses from the sediment in well-mixed environments or from catchment areas during high run-off). Its affinity of the P uptake system is also high, which will give it a competitive advantage also in P-depleted situations.

In general, small cells with a large surface-to-volume ratio (S/V) have higher maximum uptake rates (V_{max}) whereas larger cells, which have relatively large cell vacuoles, have higher storage

capacities (South and Wittick 1987, see also section 1.1.2.3). Lately it has been shown that a simple relation between S/V and V_{max} of uptake is not entirely true. Spijkerman and Coesel (1997) demonstrated that combinations of high V_{max} – low S/V and low V_{max} – high S/V occur also among algal species of comparable cell size but differing in the trophic state of their habitat. In two studies (Spijkerman and Coesel 1996, 1998) they investigated the P uptake in two desmid species from lakes differing in trophy. The authors compared *Cosmarium abbreviatum* (from an oligotrophic lake) and *Staurastrum pingue* (from a meso-eutrophic lake) and found that they differed distinctly in their P uptake and growth kinetics. *C. abbreviatum* had a high affinity for P for both uptake and growth, and also high long-term uptake rate and high storage capacity. This would give *C. abbreviatum* competitive advantage in P limited environments or when exposed to an infrequent but lasting P pulse. *S. pingue* had a high maximum P uptake rate and high maximum growth rate which will make it benefit in more dynamic environments where P release is in marked pulses (termed "velocity specialist"). Thus the P-uptake was not, in this case, related to size, but to the type of nutrient environment.

Orthophosphate (PO_4^{3-}), the most significant form of inorganic phosphorus (P_i, Wetzel 1983), is generally regarded to be directly available to, and most rapidly assimilated by, bacteria and phytoplankton (Currie and Kalff 1984). Ambient P_i concentrations in epilimnetic waters is often insufficient to satisfy planktonic demand (Wetzel 1983, Hantke *et al.* 1996). According to Currie *et al.* (1986) the PO_4^{3-} uptake is to a large extent dominated by bacterial uptake, algae being capable of competing for PO_4^{3-} only when the PO_4^{3-} concentration in the water is relatively high and the bacterial uptake has been saturated. Similarly, Bentzen and Taylor (1991) found that the bacterial fraction of the biota dominated P uptake only when PO_4^{3-} concentrations were low. In many lakes the concentration of dissolved organic phosphorus (DOP or P_o) can be up to double that of the soluble reactive P_i (Wetzel 1983). Therefore, regeneration mechanisms are needed to release P_i from colloidal P complexes or phosphomonoesters. The enzymatically hydrolyseable portion of DOP varies between 0-70% (Heath 1986). Hantke *et al.* (1996) calculated that approximately 60% of DOP can be hydrolysed by the enzyme alkaline phosphatase to release P_i, which can be used for growth, indicating the importance of DOP to the phytoplankton.

1.2.2. Function, structure, characteristics and origin of phosphatase enzymes

Most plants and algae need phosphatase enzymes to hydrolyse P_o to bioavailable P_i (Cembella *et al.* 1984). The ability to regulate the activity or production of these enzymes may therefore be of great importance in a fluctuating nutrient environment. Phosphatases (= phosphomonoesterases) are a group of enzymes that can catalyse the hydrolysis of a variety of organic phosphorus compounds into bioavailable P_i. Apart from phosphomonoesterases, phosphodiesterases also occur, and it has been suggested that these would be more constitutive, rather linked to species composition than phosphorus limitation (Jansson *et al.* 1988). Cyanobacteria in particular might be able to produce large amounts of these enzymes (Pettersson 1980).

Phosphatases have been demonstrated in free dissolved form, in bacteria, phytoplankton and in zooplankton. Phosphatases can also act as phosphotransferases if a suitable acceptor molecule other than water is available (Olsson 1990), and may thus play a very central role in both P-uptake as well as metabolism.

Both alkaline phosphatases (AP) and acid phosphatases (AcP) catalyse the hydrolysis of the phosphate-ester bond. The reaction mechanism according to Jansson *et al.* (1988) is the following:

Orthophosphate monoester + $H_2O \Rightarrow$ alcohol + P_i

The reactions at the active enzymatic site follow the subsequent order:

1. Non-covalent binding of the substrate to the enzyme
2. Alcohol release, PO_4^{-3} becomes covalently bound to the enzyme (phosphoryl-enzyme complex)
3. Conversion of the complex through uptake of water to a non-covalent complex.
4. Release of PO_4^{-3} and regeneration of enzyme.

The phosphatase activity will primarily depend on the type and concentration of the substrate and the enzyme. Other factors affecting phosphatase activity are temperature, ionic strength, pH and metal ions (Jansson *et al.* 1988).

Alkaline phosphatases have been characterised as metallo-enzymes with essential metal ions, most often two Zn^{2+} and one Mg^{2+} ion in each active site taking part in the reaction of the enzyme (Simopoulos and Jencks 1994).

In algae phosphatases are located on the cell surface (ectoenzymes), in the cytosol, in cell membranes, loosely associated within the periplasmic space (endoenzymes or intracellular enzymes) or secreted (free, dissolved extracellular enzymes, also called exoenzymes, Chróst *et al.* 1989) into the environment (Aaronson and Patni 1976, Bentzen *et al.* 1992). Phosphatase activities in fresh waters are often assumed to be of mainly algal origin, and many studies have found a large fraction to originate from phytoplankton (Jansson *et al.* 1981, Matavulj and Flint 1987, Chróst *et al.* 1989). According to Jamet *et al.* (1995 and 1997) no AP activity (APA) could be found in the dissolved fraction. Berman (1970), Pettersson (1980) and Currie *et al.* (1986) suggested that APA is predominantly algal. Labry *et al.* (2002) found that phytoplankton obtained its phosphorus mainly from the DOP-pool via APA whereas the bacteria completely dominated the P_i uptake. Wetzel (1983) on the other hand states that substantial quantities (up to 50%) of APA can be in the dissolved phase, and Stewart and Wetzel (1982) report that most phosphatase was either free in solution or associated with bacteria. Dodds (1995) claims that the 0.2-3µm size fraction of the biotic community was responsible for the majority of phosphatase activity. In oligotrophic environments where DOP levels were low, bacterial surface PA often dominated DOP hydrolysis (Hantke *et al.* 1996). Berman (1988) has demonstrated that bacterioplankton in Lake Kinneret used DOP to a higher degree than phytoplankton. Chróst *et al.* (1989) showed that during an algal bloom the APA was highest in the phytoplanktonic fraction whereas during the breakdown of the bloom >60 % of APA was confined to the bacterial fraction, while 20-30% originated from free dissolved enzymes. Rengefors *et al.* (2001), on the other hand, showed that the phytoplanktonic species dominating the biomass rarely or never showed any ectoenzymatic phosphatase activity. Thus no clear consensus exists as to the origin of phosphatase activities, but it seems to vary considerably between bacteria and larger phytoplankton, as well as interspecifically.

Zooplankton-excreted phosphatases might locally have a great impact on the total activity (Hantke *et al.* 1996). Dissolved enzymes are supplied by growing algae and zooplankton but perhaps not so much by bacteria (Jansson *et al.* 1988). Singh and Tiwari (2000) found that in

Anabaena oryzae grown in P-free medium extracellular phosphatase activity increased simultaneously with cellular phosphatase activity, but its activity was two-fold lower than the cellular activity. Wynne and Rhee (1988), on the other hand, found no correlation between extracellular and intracellular phosphatase activities in four different phytoplanktonic species. Another important source of dissolved enzymes should be losses from dying and disintegrating cells. It seems like it is not entirely clear how, or to which extent, active excretion of phosphatases takes place, and whether it is more beneficial for the algal cell to release the enzymes or to localise them on the external cell surface.

Phosphatases typically have maximum hydrolysing capacity at different pH (Jansson *et al.* 1988). Alkaline phosphatases have an pH optimum above 7, often between 9 and 10, while the acid phosphatases have an optimum below 7, generally between 4 and 6. The alkaline phosphatases have been the subject of most studies, because conditions, which would favour the activity of acid phosphatases, are not common in most lakes. Therefore it is believed that acid phosphatases are less important for *in situ* phosphorus mineralisation, with the exception of heavily acidified environments (Jansson *et al.* 1981). Four different acid phosphatases have been described from the acidified Lake Gårdsjon (Jansson *et al.* 1981), all produced by phytoplankton. It has also been established that algae and bacteria growing in alkaline environments produce more alkaline than acid phosphatases with external function (Jansson *et al.* 1988). The optimum temperature for APA is 35-40° (Rose and Axler 1997, Healey and Hendzel 1979, Pick 1987).

The synthesis of these enzymes is mostly influenced by substrate supply or reaction product. Repression occurs when a compound, often the end product of the enzyme-catalysed reaction, turns off the enzyme synthesis, e.g. when high levels of P_i represses phosphatase synthesis. Inhibition occurs when a compound reacts with the enzyme itself and stops its activity. P_i is also a common inhibitor, which competes with phosphate esters for the active site on the phosphatases (Jansson *et al.* 1988). Thus, P_i can both repress the synthesis, as well as inhibit the activity of phosphatase enzymes.

It has been hypothesised that APs are to a high extent repressible, having an external function and a synthesis mechanism, which depend on the ambient phosphorus nutrition. According to Jansson *et al.* (1988) AcPs are more often found within the cell than in contact with the surrounding media, and the synthesis of the acid phosphatases is generally not repressed by P_i.

AcPs are therefore often called constitutive enzymes (produced independently of an activator, i.e. more or less constantly synthesised in the cell), produced mainly to serve the internal phosphorus metabolism. Phosphatases excreted by zooplankton seem to be less inhibited by phosphate than algal phosphatases (Jansson *et al.* 1988).

In a study done by Quisel *et al.* (1996) it was found that P-deprived cells of terrestrial *Chlamydomonas reinhardtii* increase extracellular APA 300-fold relative to unstarved cells. At least two forms of APs were found:

1. a Ca^{2+} dependent enzyme composed of 190 kD glycoprotein subunits. This enzyme comprised 85-95% of total APA present, the optimal activity being at pH 9.5, and the best substrate being pNpp and
2. a non divalent, cation dependent enzyme was also found, the optimal activity being at pH 9. This form was responsible for 2-10% of total APA present.

According to Quisel *et al.* (1996), many more phosphatases are present in *Chlamydomonas reinhardtii*. Most of these have not been characterised with respect to polypeptide composition, substrate specificity, inhibition or cofactor requirements. Bachir and Loppes (1997) mention two constitutive enzymes and two highly expressed derepressible phosphatases in *Chlamydomonas reihardtii*. This is in accordance with earlier findings by Matagne *et al.* (1976), demonstrating two constitutive acid phosphatases and three derepressible neutral and alkaline phosphatases in the same species. In *Chlamydomonas acidophila* three acid and two alkaline phosphatases were found – of these one AcP was produced only in P-deficient medium. All phosphatase enzymes were produced in larger quantities under P-limitation (Boavida and Heath 1986).

Generally phosphatase enzymes are not considered to be substrate specific (Pettersson and Jansson 1978, Jansson *et al.* 1988). In *Escherichia coli* an AP with broad substrate specificity is regulated by the extracellular phosphate concentration through the action of the phosphate transporter and a "two-component regulatory system" (Quisel *et al.* 1996). Also some cyanobacteria (*Synechococcus* sp.) have a similar kind of regulatory mechanism. *Synechococcus* sp. has also several secreted acid phosphatases with broad substrate specificity, regulated by both intra and extracellular phosphate levels (Quisel *et al.* 1996).

The synthesis as well as the regulation of the activity of phosphatase enzymes is one strategy by which phytoplankton may influence nutrient dynamics in freshwaters. PAs hydrolyse a variety of P_o compounds into bio-available P_i. For example in low external P_i concentrations during summer algal blooms, the degree to which phytoplankton utilise organic phosphorus compounds can be decisive for competition (Spijkerman and Coesel 1998).

1.2.3. Relationship between APA and ambient P_i concentration, and phytoplanktonic species composition

1.2.3.1. Ambient phosphate concentration

According to many studies APA has been found to be inversely correlated to its product, namely P_i availability, *in situ* (Pettersson 1980, Boavida and Heath 1986, Currie *et al.* 1986, Elser and Kimmel 1986, Jansson *et al.* 1988, Nausch 1998) as well as *in vitro* (Yiyong and Xinyu 1997). These findings suggest that phosphatase activity would be induced during phosphate depletion. Healey and Hendzel (1979) found that in some phytoplanktonic species phosphatase activity was induced by P_i-depletion, whereas in others not. In other studies, species from typically eutrophic environments have been investigated, and Spijkerman and Coesel (1996, 1998) showed a clear relationship between P-limitation and APA especially for species from eutrophic lakes. Nausch (1998) showed a relationship between PO_4^{3-} concentrations and APA when PO_4^{3-} concentrations were lower than 1 µmol l^{-1} but no relationship when PO_4^{3-} concentrations were above this threshold value.

On the other hand, a multitude of studies exist where the relationship between ambient PO_4^{3-} concentration and APA has not been shown (Pick 1987, Wynne and Rhee 1988, James *et al.* 1992, Dodds 1995, Jamet *et al.* 1995, Boavida and Marques 1995, Jamet *et al.* 1997, 2001). Possible reasons for the lack in relationship could be attributed to differences in the specific maximal release velocity of PA by the prevailing plankton species (Pick 1987). The efficiency of P release by PA depends on the maximal release velocity of PA, the substrate affinity and substrate specificy. The situation gets even more complex, because DOP consists of two groups of compounds; the high molecular mass compounds (mainly nucleic acids) that are slowly downgraded by PA, and low molecular phosphomonoesterases that are easily hydrolysed by PA (Hantke *et al.* 1996). It has been suggested that an APA increase would require longer term P-

depletion in the environment (Dodds 1995) and that APA would not be repressed or inhibited by P_i concentrations ranging up to 0.32 mg l^{-1} (Reichardt *et al.* 1967, Jamet *et al.* 1997).

1.2.3.2. Trophic status

Some studies link phosphatase activities to the trophic status of the water (Hantke *et al.* 1996, Spijkerman and Coesel 1998). Hantke *et al.* (1996) concluded that algae in more eutrophied environments hydrolysed ambient DOP more effectively than algae in oligotrophic environments. Jones (1972) found that higher trophic status of lakes corresponds with increasing production of AP by phytoplankton, and with a prevailing role of APA in organic regeneration (Ammerman and Azam 1991, Cotner and Wetzel 1991).

Boavida and Marques (1995), on the other hand, found low activity of APA in two eutrophic reservoirs, probably because of soluble reactive phosphorus always being abundant. Spijkerman and Coesel (1998) showed that when comparing several physiological features of two desmid species typically occurring in environments with differing trophic status, the species typical for oligotrophic conditions had higher maximal APA than species from eutrophic lakes. The authors concluded that the degree to which phytoplankton may benefit from organic compounds may be decisive for competition. It seems like the function of phosphatases varies between different phytoplanktonic species, and only constitutes one of many mechanisms involved in the nutrient acquisition. It may, however, in connection to other growth parameters, give an idea of the nutrition acquisition strategy of the species.

1.2.3.3. Species composition

Some studies suggest that phosphatase activities might be more linked to species composition (Hino 1988, Olsson 1990, Rengefors *et al.* 2001) than phosphorus supply. Olsson (1990) found that in oligotrophic lakes the optimum phosphatase activity occurred at pH < 6. In eutrophic lakes (dominated by *Cyanophyceae* and *Bacillariophyceae*) the optimum phosphatase activity was at pH 7.5-8.5. Thus *Cyanophyceae* and *Bacillariophyceae* would be expected to have higher APA than AcPA, in contrast to species from oligotrophic environments. Partly contradictory results were reported by Romanowska-Duda (1999), finding that generally APA was characteristic for bacteria and diatoms, whereas for cyanobacteria AcPA was prevailing. Other

studies have found little influence of changes in community composition on APA in culture (Smith and Kalff 1981).

1.2.4. Endo- and ectoenzymatic phosphatase activity

Literature concerning phosphatase activities differ in the approach toward what type of enzyme activity is determined. Phosphatase activity can be measured using two basic methods, namely endoenzymatic phosphatase and ectoenzymatic phosphatase. Endoenzymatic phosphatase measurements are based on homogenising the algal cell material, and extracting the intracellular enzymes into a reaction buffer (Wynne 1977). The enzyme activity measured is thus diluted into the buffer and the resulting enzyme activity determined spectrophotometrically (see details in Chapter 2). Ectoenzymatic phosphatase activity is determined by using fluorogenic model substrates that are added to a solution containing intact algal cells. Fluorescence is observed after enzymatic splitting of the complex model substrate molecule (Hoppe 1993). The resulting fluorescing product is thus a result of only the enzymes that are located outside the cell wall, whereas any internal enzyme activity will not be detected (Healey and Hendzel 1979, Hoppe 1993). The newest addition to the study of phosphatases is the utilisation of insoluble fluorogenic ELF substrates, which yields a stable fluorescent precipitate at the site of enzyme activity on the cell (Gonzáles-Gil *et al.* 1998, Rengefors *et al.* 2001). This type of study will in future be of great significance to the study of P-nutrition of phytoplankton.

1.2.5. Conclusion

Numerous studies have investigated the role of phosphatases in fresh water environments, many with the purpose of relating phosphatase activity to nutrient sufficiency or deficiency. However, no clear consensus has been reached on the use of phosphatases as an indicator of nutrient stress. In connection to HAB research it seems important to include aspects of nutrient physiology of the phytoplanktonic cells, because nutrient concentration and speciation plays a key-role in the initiation and persistence of HABs. Only a limited number of studies (Rengefors *et al.* 2001) have investigated the differences in potential phosphatase activity between species capable of bloom formation. Another lack of consensus in the literature seems to be the variable use of endo- and ectoenzyme phosphatase activities, and only a few (e.g. Yelloly and Whitton 1996) studies have used both methods in parallel.

1.3. Nitrogen and Nitrate reductase enzyme

Nitrogen is a major constituent of phototrophic biomass, and the supply of this element can be, in addition or parallel with P, the primary factor limiting micro-algal growth rates and population size in the aquatic environment (Vincent 1992). Inorganic nitrogen in the biosphere is converted to a biologically useful form (organic nitrogen) by either the fixation of molecular nitrogen N_2 or the assimilation of nitrate (Solomonson and Barber 1990). Nitrogen assimilation involves three steps (Figure 1.3): import of nitrogen into the cell (transport), conversion of this nitrogen into NH_4^+ (reduction) and assimilation of NH_4^+ into N-containing macromolecules (biosynthesis, Vincent 1992), such as amino-acids, porphyrin, phycobilin and chlorophyll (Flores and Herrero 1994). Each of the steps involved in nitrogen assimilation have been widely studied (for recent reviews see Grossman and Takahashi 2001, Moir and Wood 2001 and Inokuchi *et al.* 2002).

The conversion of nitrate to ammonia (nitrate reduction) is an 8-electron reduction process that occurs in two steps. The first step is a 2-electron reduction of nitrate to nitrite, catalysed by the enzyme nitrate reductase (NR) catalysing the reaction:

$$NO_3^- + NADH \longrightarrow NO_2^- + NAD^+ + OH^-$$

NAD(P)H serves as the physiological electron donor for this reaction in eukaryotes (Campbell 1999). The second step is a 6-electron reduction of nitrite to ammonia, catalysed by the enzyme nitrite reductase (NiR). This step is coupled to photosynthetic electron transport in algae and higher plants via reduced ferredoxin, a product of the light reactions of photosynthesis, which serves as the physiological electron donor for NiR (Solomonson and Barber 1990). The final step is the conversion of ammonium into amino acids, in most plants mainly by the glutamine synthetase / glutamine-oxoglutarate amino-transferase synthase cycle (GS/GOGAT, Vincent 1992). As much as 25% of the energy of photosynthesis is consumed in driving nitrate assimilation (Solomonson and Barber 1990), and in addition to this requirement for ATP and reductant to drive nitrogen assimilation, it is also dependant on carbon skeletons generated by photosynthesis and respiration (Turpin *et al.* 1997).

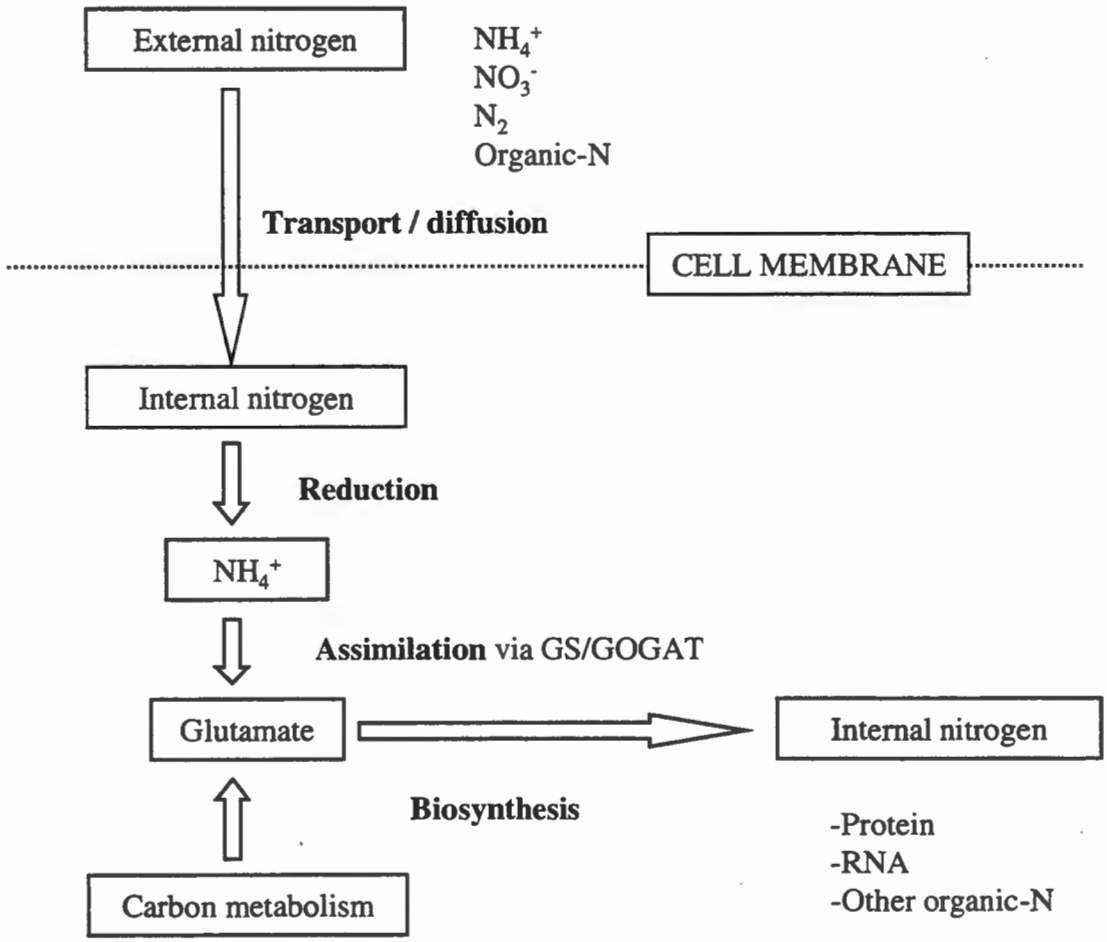


Figure 1.3. Major steps in phototrophic nitrogen uptake, assimilation and biosynthesis (according to Vincent 1992).

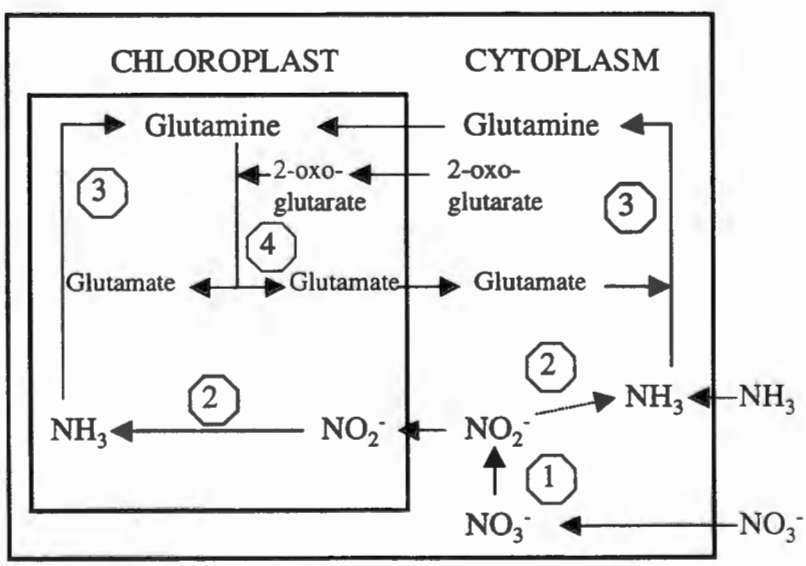


Figure 1.4. Compartmentation of the flow of compounds during the assimilation of nitrate and ammonium in *Chlamydomonas reinhardtii*, according to Fischer and Klein (1988).

1. Nitrate reductase, 2. Plastidic and cytoplasmic nitrite reductase, 3. Plastidic and cytoplasmic glutamine synthetase (GS), 4. GOGAT.

The rate-limiting and regulated step of nitrate assimilation appears to be the initial reaction, catalysed by NR. This enzyme is considered to be a limiting factor for growth, development, and protein production in plants and other nitrate-assimilating organisms (Solomonson and Barber 1990).

Nitrate reductase is a complex enzyme containing several different redox-active prosthetic groups (Solomonson and Barber 1990). The nitrate reductase enzyme is a homodimer composed of two identical 100-kD subunits, each containing one flavin adenine dinucleotide (FAD), heme-iron (cytochrom b_{557}) and molybdenum-molybdopterin prosthetic groups (Campbell 1999). NR is usually located in the cytoplasm (Fischer and Klein 1988), where it reduces nitrate to nitrite (Figure 1.4). Most of this nitrite is then transferred to the chloroplast, where it is further reduced to ammonia by nitrite reductase (NiR). A small part of the nitrite may be reduced to ammonia in the cytoplasm. Ammonia, in turn, may be assimilated further in the chloroplast or in the cytoplasm to glutamine by GS, and glutamate by GOGAT (Fischer and Klein 1988).

NR has also been found in the pyrenoid of several green algae, such as *Monoraphidium braunii*, *Chlamydomonas reinhardii*, *Chlorella fusca*, *Dunaliella salina* and *Scenedesmus obliquus* (Lopez-Ruiz *et al.* 1985). The function of pyrenoidal NR has been suggested to represent a protein pool for the cell that can be mobilised when needed, for example under nitrogen limited conditions (Fischer and Klein 1988). Later studies have shown, however, that NR in the pyrenoids of *Bryopsis maxima* is functional and even concentrated, suggesting an important physiological role for pyrenoids in the assimilation of nitrate (Okabe and Okada 1990).

NR is repressed by ammonia or amino acid, and activated by nitrate and light (Velasco *et al.* 1989). NR shows a circadian rhythm in many algal species (Ramalho *et al.* 1995). The activation of the enzyme during light periods is attributed to *de novo* NR protein synthesis rather than activation of a constitutive enzyme (Velsaco *et al.* 1989, Ramalho *et al.* 1995).

Nitrate reductase structure, function and regulation in plants and bacteria has been thoroughly investigated (for recent reviews see Campbell 1999 and Inokuchi *et al.* 2002), and methods for measuring its activity are well standardised.

1.4. Direct chlorophyll-a fluorescence as an indicator of stress

Photosynthesis is the most fundamental reaction in all plants, and therefore a measure of the photosynthetic process is of great importance when studying any aspect of plant physiology. Nutrients are a central element in the function of photosynthesis. Nitrogen is an important constituent of the photosynthetic apparatus, protein synthesis and enzyme regulation, and it plays a role in the partitioning and export of photoassimilates (Rao and Terry 2000). Phosphorus has a role in the cellular energetics, co-enzyme regulation and, similarly to nitrogen, partitioning and export of photoassimilates (Rao and Terry 2000). Nitrogen metabolism is tightly co-ordinated with carbon metabolism, since a large amount of the photosynthetically produced energy as well as carbon skeletons provided are committed to nitrogen assimilation (Inokuchi *et al.* 2002). Recent findings have also suggested that nitrogen assimilation is coupled to the presence of P_i (Camacho-Cristóbal *et al.* 2002). Therefore it is advantageous to study phosphorus and nitrogen assimilation simultaneously, and in association with photosynthesis and respiration.

Photosynthetic performance, monitored via the function of photosystem II (PSII) has been used as an indicator of the vitality of the cell. F_V/F_M values, indicating initial and maximal fluorescence of a cell exposed to a saturating light pulse, have frequently been used as indicators of the photosynthetic efficiency. It has been suggested that F_V/F_M could be used to indicate nutrient stress in phytoplankton (Kolber *et al.* 1988, Cleveland and Perry 1987) but serious critique against this hypothesis has recently been raised (Parkhill *et al.* 2001), recommending that F_V/F_M alone should not be used to measure nutrient stress. Fluorescence transients recorded with high time-resolution fluorimeters, such as the PEA- instrument (Plant Efficiency Analyser, Hansatech Instruments Ltd., UK) have, however, provided additional and more accurate information regarding PSII related functions (Strasser and Govindjee 1992, Strasser *et al.* 1995). Chlorophyll-*a* fluorescence measurements have proved to be a sensitive tool in comparing the effects of different types of environmental stress on PSII function (first described by Kautsky and Hirsh 1931, quoted in Strasser *et al.* 2000). Measurement of chlorophyll *a* fluorescence provides information on the energy fluxes of absorption, trapping and electron transport through PSII (Krüger *et al.* 1997). Because of the interactions between photosynthesis, respiration and nitrate assimilation (Turpin *et al.* 1997), as well as the requirement of photo-energy for the synthesis of phosphatases (Singh and Tiwari 2000) it is important to include a measure of photosynthetic performance in studies concerning nutrient physiology of phytoplankton.

Presuming that the assumptions concerning the mathematical derivations of the JIP-test are valid (Strasser *et al.* 1995), this test gives much information about the photosynthetic status of a cell, and was therefore used in connection to all experimental work presented in this thesis.

1.5. Objectives of the study

The underlying problem directing the study was the question: Why do certain phytoplanktonic species form mass-occurrences? The choice to approach this question from a physiological point of view came from a lack of consensus in the literature. This study is an attempt to contribute to the pool of information concerning the two most important growth-limiting nutrients in aquatic environments, phosphorus and nitrogen. The utilisation of these nutrients was studied via phosphatase and nitrate reductase activities, firstly of a eutrophied system as a whole, and secondly in selected species from this system under different nutrient conditions.

The study is divided in two main parts, namely *in situ* studies in the Vaal River (1999-2001, Chapter 3), and *in vitro* studies concerning the physiological responses of two green algal and two cyanobacterial species to varying N and P stress (Chapters 4 and 5).

Hardly any investigations concerning phosphatase activities in rivers, especially on the African continent, can be found in the literature. Riverine environments are not considered to be typical environments for algal bloom development, because of high flow rates and rapidly changing environmental conditions. Large, slow-flowing rivers have, however, been reported to host algal blooms (e.g. Descy 1987, Prygiel and Leitao 1994).

The Vaal River in a semi-arid region of South Africa is a fairly large river, and of importance to the highly populated area of the South African economic and industrial heartland, the Gauteng Province surrounding Johannesburg. Due to heavy utilisation of the river as both potable water source as well as receiver of wastewater from agriculture, settlements, mining and other industry, it has been under intensive pollution pressure. The regulation of most rivers in southern Africa also contributes to the alteration of the rivers from natural waters to regulated and polluted watercourses. Much limnological work has been done on the Vaal River (Janse van Vuuren 2001 and references therein) and it has been established that the river is eutrophied with several bloom forming genera present throughout the year. Intensive phytoplankton blooms are a yearly

phenomenon in the Vaal River (Janse van Vuuren 2001). Most bloom formers belong to the green algae (e.g. *Chlamydomonas* sp. and *Pandorina morum*), cyanobacteria (*Microcystis aeruginosa*, *Oscillatoria simplicissima* and *Spirulina* sp.) and diatoms (Janse van Vuuren 2001). In the Vaal River *Microcystis aeruginosa* has recently been replaced by *Oscillatoria simplicissima* as the main bloom forming cyanobacterium (Pieterse and Steynberg 1993). Later it was suggested that a further shift from *Oscillatoria simplicissima* to *Anabaena circinalis* might be under way (Janse van Vuuren 2001).

The mass-occurrence of nuisance phytoplanktonic species in the Vaal River has caused concern from a public health perspective. The water purification industry has been forced to invest considerable finances in combating the effects of algal clogging of filters and removal of algal residues and algal produced compounds (odours, tastes and toxins) from the water (Visser 1997, Venter 2000). Algal blooms are reported to occur frequently in South African fresh waters, including toxic blooms that have caused death of cattle, sheep, dogs, giraffes, black wildebeest and white rhinoceros (see Harding and Paxton 2001 for review).

Increasing eutrophication, and occurrence of algal blooms, is not a problem in South Africa alone, but has received much attention globally. Research in the past has to a great extent focused on the ecological aspects of nuisance species, but recently the focus is shifting to ecophysiological investigations emphasising the reasons for and mechanisms of bloom formation.

It was therefore of interest to investigate how the bloom formers in the Vaal River respond to varying nutrient concentrations and changing nutrient regimes. A field study comprising of four localities in the Vaal River was carried out, to determine the species composition of the phytoplankton and the fluctuations in nutrient concentrations, other environmental variables and phosphatase activities in the river over a period of two years. Based on these field studies three common bloom forming species were selected for *in vitro* studies, namely the green alga *Chlamydomonas* sp., and the cyanobacteria *Microcystis aeruginosa* and *Oscillatoria simplicissima*. *Chlorella* sp. was also chosen, to represent a green alga that has not been reported to form blooms in the Vaal River. These four species were grown in media whose N and P concentrations corresponded to both nutrient replete conditions, as well as low nutrient concentrations comparable to levels measured in the Vaal River. The species were also grown in

media with different N:P ratios, and the physiological responses of the cells were determined. Physiological responses studied were changes in growth rate, phosphatase and nitrate reductase activity, as well as changes in the function of PSII, indicating the level of (photosynthetic) stress of the cell. In the literature phosphatase activity is commonly measured as endo- and ectoenzymatic phosphatase activity, and therefore both endo- and ectophosphatase activities were determined, to make the results comparable with those in the literature. Also it is of interest to compare the partitioning between ectoenzymatic and endoenzymatic phosphatase activity.

To summarise, the primary aims of the research were:

- To study both acid and alkaline phosphatase activity in the Vaal River and establish the magnitude, ratios, seasonal and spatial variation, and fractionation of the activity between bacteria and phytoplankton.
- To study the phytoplankton composition in the Vaal River in association with basic environmental variables, as well as phosphatase activities, and to establish the possible relationship between environment and enzyme activity in the Vaal River,
- To study differences in the physiology between bloom forming and non-bloom forming green algal species isolated from the Vaal River,
- To study differences in the physiology between two bloom forming cyanobacterial species isolated from the Vaal River, with emphasis on the possible reasons for the increased occurrence of *Oscillatoria simplicissima* blooms compared to *Microcystis aeruginosa*, and
- To compare green algal and cyanobacterial bloom formers in aspects of their nutrient physiology.

Each chapter has a concluding paragraph, and Chapter 6 presents the general conclusions and an overall summary of the work.

CHAPTER 2
STUDY AREA, MATERIAL AND METHODS

2.1. Study area and sampling regime

2.1.1. The Vaal River

The Vaal River is one of the largest rivers in South Africa, and originates from the western slopes of the Drakensberg escarpment. It flows about 900 km through the Freestate province before joining the Orange River near Douglas. The major tributaries of the Vaal River are from the Drakensberg area, the heavily populated and industrialised Gauteng, as well as the Maluti Mountains. The catchment area of the Vaal River comprises 192 000 km^{-2} (Braune and Rogers 1987) and is divided into the upper, middle and lower Vaal. This study was confined to the Middle Vaal River. The river flow is controlled from the Vaal Dam with numerous weirs and dams along its route downstream. One of the main weirs in the Vaal River is the Barrage, which constitutes one of the sampling localities. The four sampling localities are shown in Figure 2.1. The localities Balkfontein (27°23'45''S, 26°30'30''E) and Stilfontein (26°56'01"S, 26° 55' 20"E) represent "true" riverine localities, whereas the sampling localities at the Barrage (26°46'00'', 27°40'00''E) and Loch Vaal (26°44'80''S, 27°42'00''E) are located in an impoundment, representing a "shallow lake", flowing into the Vaal River.

2.1.2. Sampling regime

Sampling for *in situ* studies (Chapter 3) was performed on a monthly basis for two years (February 1999 – March 2001) at the four localities. The following variables were measured *in situ*: transparency (using a Secchi disc), temperature, turbidity (HACH company Model 2100P turbidimeter) dissolved and saturated oxygen (YSI incorporated oxygen meter Model 50B), pH (WTW pH 330 meter) and conductivity (HI9033 HANNA Instruments). Water samples (2 – 3 l) were collected in six replicates (separate samples) from the surface water for enzyme assays, phytoplankton composition, nutrient and chlorophyll-*a* determination. Phytoplankton samples were taken into separate bottles including 150 ml of sample mixed with two percent formaldehyde (final concentration). Samples for enzyme activity assays were, when possible, filtrated in the field and the filters and filtrates were stored in liquid nitrogen until analysing. The rest of the water samples were transported to the laboratory, kept cool and in the dark.

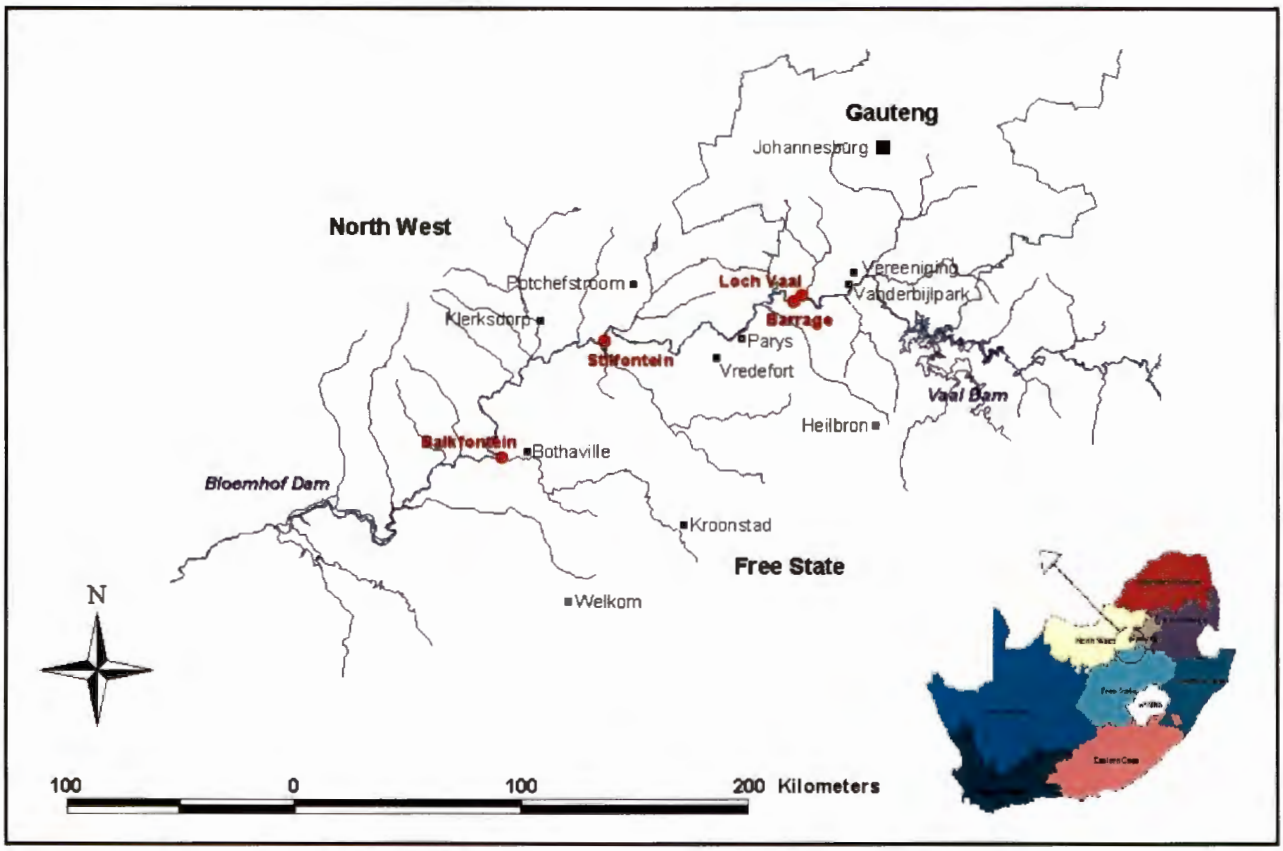


Figure 2.1. The Mid-Vaal region is situated between the Vaal Dam and the Bloemhof Dam, and forms the border between the North West and the Free State Provinces in South Africa. The two sampling localities furthest upstream, namely Loch Vaal and Barrage, are situated in the Gauteng province and represent "shallow lake" type localities. Stilfontein and Balkfontein further downstream represent the true "riverine" localities.

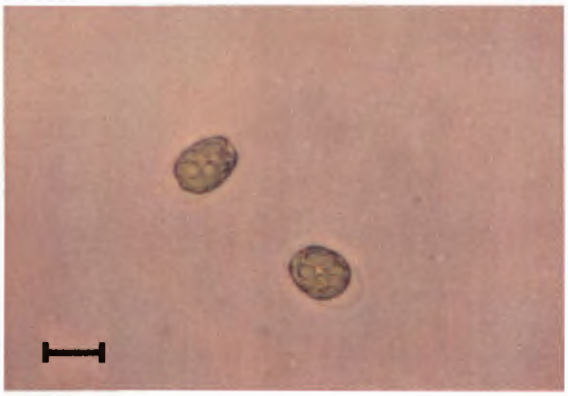
During parts of the study basic physical and chemical parameters were measured by water companies performing routine monitoring of the water quality in the river (Rand Water for the Loch Vaal and Barrage localities, and MidVaal Water Company for the Stilfontein locality). The main purpose of this data was to serve as background information for the enzymatic studies, and to be included in multivariate analyses in combination with phytoplanktonic species composition and enzyme activities.

2.2. Fractionation of water samples for enzyme activity measurements

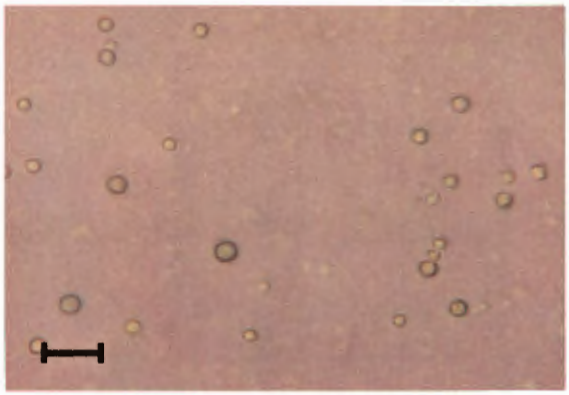
Enzyme activities were measured in two size fractions. The fractionation was done by filtering the sample through a GF 52 filter (pore size 1.2 µm). It has been shown that a 1.2 µm filter retains 96% or more of the chlorophyll-*a* content of a sample (Elser *et al.* 1986) thus retaining most of the phytoplankton (referred to as the phytoplanktonic fraction, or the > 1.2 µm fraction). The filtrate will include dissolved and soluble enzymes as well as bacteria (referred to as the bacterial / dissolved fraction, or the < 1.2 µm fraction). In the bacterial / dissolved fraction also picoplankton, i.e. autotrophic phytoplankton less than approximately one µm in diameter / length, are included.

2.3. Growth conditions in experimental studies

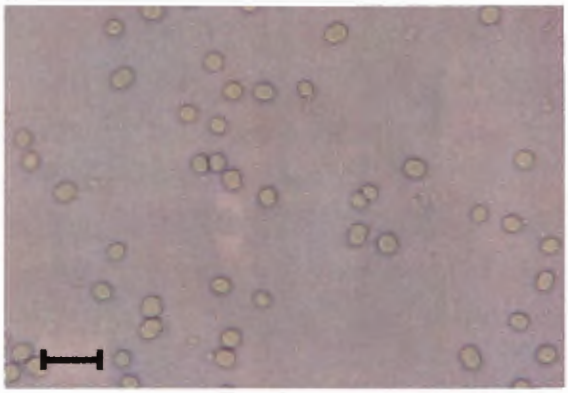
In experimental studies algal cultures (Figure 2.2) were used (Chapters 4 and 5). Unialgal, but not axenic, cultures of *Chlamydomonas* sp. Pasch., (source Balkfontein), *Chlorella* sp. Beyerinck, (source Balkfontein), *Microcystis aeruginosa* Kütz., (source Balkfontein) and *Oscillatoria simplicissima* Gomont, (source Loch Vaal) have been isolated from the Vaal River (Steynberg 1994, available at the University of Potchefstroom). Closer description of the species is given in Chapters 4 and 5. Cultures of these algae were grown in GBG-11 (Krüger 1978) medium or EM medium (*Oscillatoria simplicissima*, Venter 2000, Table 2.1) in constant irradiance (16 µmol m^{-2} s^{-1} for the green algae, and 18-22 µmol m^{-2} s^{-1} for the cyanobacteria) and temperature (18° for green algae and 22° for cyanobacteria).



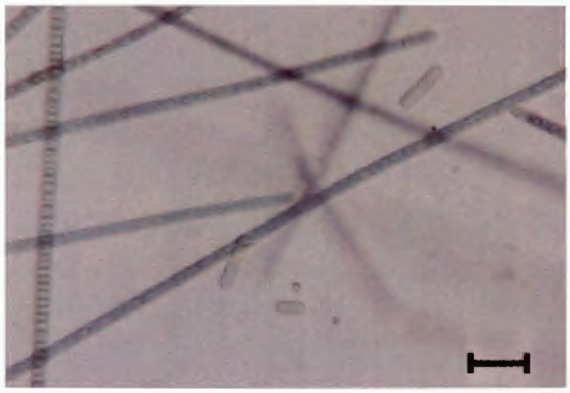
***Chlamydomonas* sp.**



***Chlorella* sp.**



Microcystis aeruginosa



Oscillatoria simplicissima

Figure 2.2. Photomicrographs of the four species used in the experimental studies (Chapters 4 and 5). The scale bar represents 10 µm, except in *Oscillatoria simplicissima* where it represents 20 µm.

Table 2.1. Modified GBG-11 (Krüger1978) and EM (Venter 2000) media used for all algal cultures during experimental work. Only the concentration of $NaNO_3$ and K_2HPO_4 were varied in the experimental treatments (for details see text).

Macro-nutrients	**Final concentration GBG-11**	**Final concentration EM**
1. $NaNO_3$	150.5 mg l^{-1}	270 mg l^{-1}
2. $K_2HPO_4 \cdot 3\ H_2O$	69.3 mg l^{-1}	67.2 mg l^{-1}
3. $MgSO_4 \cdot 7\ H_2O$	75.0 mg l^{-1}	77.0 mg l^{-1}
4. $CaCl_2 \cdot 2\ H_2O$	36.0 mg l^{-1}	36.0 mg l^{-1}
6. Na_2CO_3	20.0 mg l^{-1}	40.0 mg l^{-1}
7. EDTA	1.0 mg l^{-1}	0 mg l^{-1}
8. Citric acid	12.0 mg l^{-1}	12.0 mg l^{-1}
9. $FeSO_4 \cdot 7\ H_2O$	10.0 mg l^{-1}	10.0 mg l^{-1}
Micro-nutrients		
1. H_3BO_3	1.43 µg l^{-1}	1.43 µg l^{-1}
2. $MnCl_2 \cdot 4\ H_2O$	0.57 µg l^{-1}	0.57 µg l^{-1}
3. $ZnSO_4 \cdot 7\ H_2O$	0.11 µg l^{-1}	0.11 µg l^{-1}
4. $NaMoO_4 \cdot 5\ H_2O$	0.20 µg l^{-1}	0.20 µg l^{-1}
5. $Co(NO_3)_2 \cdot 6\ H_2O$	0.02 µg l^{-1}	0.02 µg l^{-1}
6. $CuSO_4 \cdot 5\ H_2O$	0.04 µg l^{-1}	0.04 µg l^{-1}
Vitamins		
1. Cyanocobalamin (B_{12})	0 µg l^{-1}	33.0 µg l^{-1}
2. Thiamine – HCl	0 µg l^{-1}	33.0 µg l^{-1}
3. Biotin	0 µg l^{-1}	330.0 µg l^{-1}

The algae were grown in batch-cultures in GBG-11 or EM media until fast growth was attained, about 12 days (8 days for *Oscillatoria simplicissima*) after inoculation. The algae did not reach exponential growth, but the experiment was started at a phase where they grew fast, but the growth had not yet levelled out (based on previous growth studies using the same species). The algal cultures were then exposed to semi-continuous culturing (experimental set-up and method according to Johansson and Granéli 1999) of four different treatments: one set of external control

(GBG-11 or EM) and three low-nutrient treatments with differing N:P ratios. Each treatment consisted of six 500 m*l* Erlenmayer flasks with 200 m*l* of culture. After mid-log phase, 40% of the culture volume (80 m*l*) was sampled every day (08:00 am) and replaced with an equal volume of new media containing the appropriate nutrient concentrations and ratios. The first treatment consisted of the original GBG-11 or EM medium (external control – disturbance effect) representing nutrient replete conditions (1760µM N, 361µM P, N:P = 5:1) whereas the three experimental treatments consisted of media nearly depleted of N and P nutrients. The daily additions of nutrients were as follows: N-limited, N:P = 1:1 (2.0 µM NO_3^-, 2.0 µM PO_4^{3-}), "non-limited" or internal control, N:P = 16:1 (32 µM NO_3^-, 2 µM PO_4^{3-}) and P-limited, N:P = 160:1 (32 µM NO_3^-, 0.2 µM PO_4^{3-}) conditions. These concentrations are in the range of naturally occurring concentrations of N and P in the Vaal River (Pieterse and Janse van Vuuren 1997). The internal control was used to compare the treatments with severe N- or P-limitation with a treatment in which the N:P ratio is closer to "optimal" for microalgal growth. Trace metals, other nutrients, and EDTA was added to all cultures at levels corresponding to the normal GBG-11 or EM medium. Culture purity was checked throughout the experiment using light microscopy. After the cultures reached steady-state (9 – 11 days after start of dilutions, steady-state determined as µ (growth rate) ≈ D (dilution rate), the experiment was terminated and samples were harvested for enzyme, chlorophyll-*a* concentration, chlorophyll-*a* fluorescence, cell density and nutrient (PO_4^{3-} and NH_4^+ concentrations) analyses.

2.4. Monitoring of growth during the experiments

During the dilutions, turbidity was recorded each day to measure the growth rate (Klett-Summerson colorimeter, equipped with a filter at 450nm (green)) during the green algal experiments. The flasks were fitted with a side arm, which could be filled with culture and inserted into the colorimeter without opening the flasks, thus reducing the contamination risk. Growth was also monitored by chlorophyll-*a* extractions. The equipment for turbidity measurements was not available during the cyanobacterial experiments, and therefore only chlorophyll-*a* was used for growth rate determination. The growth rate (per day) was determined for the last 4 days (green algae) or the last 1-2 days (cyanobacteria) before the termination of the experiment using the exponential growth equation (Pirt 1975) including a factor for the dilution of the cultures (Brock *et al.* 1994), applied as in Isvánovics *et al.* (2000);

$$\mu = \frac{\ln\left(\frac{B_t}{B_{t-1}}\right)}{\Delta t} + D_t$$

where B = klett-values or chlorophyll-*a* concentrations,

t = time in days, and

D = dilution rate (0.4 per day)

Chlorophyll-*a* concentrations were determined (every third (or second) day of the experiment) according to methods described by Sartory (1982), using ethanol and a boiling water bath as extraction method. At the end of the experiment cell counts were determined. Samples from the cultures were preserved in formalin, and cell concentration was determined using a Zeiss inverted light microscope.

2.5. Measurement of phosphatase activity

2.5.1. Spectrophotometric method

It has been recommended that especially in connection to studies where the plankton species composition is expected to change (such as in most *in situ* studies), the phosphatase activity should be measured at least at one low and one high pH value (Olsson, 1990). During this study both acid and alkaline phosphatase activities were measured from river samples as well as during experimental work. Phosphatase activities were determined spectrophotometrically according to Wynne (1977), using p-nitrophenyl phosphate (pNpp) as substrate. The substrate pNpp has been commonly used in phosphatase activity studies, and has been shown to be the best substrate for e.g. *Chlamydomonas reinhardtii* phosphatase enzymes (Quisel *et al.* 1996).

Cells were concentrated on GF/C filters (1.2 µm pore size) and homogenised in the buffer medium using a polytron. For the acid phosphatase (AcPA) Na-acetate–acetic acid, (pH 5) and for the alkaline phosphatase (APA) TRIS-HCl (pH 9) was used as buffer, both buffers including 0.1M $MgCl_2$ as the required divalent metal component. The homogenised material was centrifuged (12 000 rpm, 20 minutes) and the supernatant was used for determining the phosphatase activity. One hundred µl pNPP (10 mM) was added to 1.5 ml supernatant, and the reaction mixture incubated at 30°C for 2 hours. The reaction was stopped by adding 300µl

NaOH (1M), and the concentration of the product was measured spectrophotometrically (410 nm). The enzyme activity is reported as specific activity per µg chlorophyll-*a* or as total activity per litre of sample water (or culture medium). Generally specific activities are more suited for physiological work since it reflects the enzyme activity calculated against a biomass indicator (in this case chlorophyll-*a*). Total activity has been calculated and presented in this work mainly to confirm trends observed in the specific activities, as well as to facilitate comparisons with literature, where total phosphatase activities are commonly reported.

2.5.2. Fluorometric method

Ectoenzyme phosphatase activity was determined according to Hoppe (1993), using 4-methylumbelliferyl phosphate (MUP) as substrate and 4-methylumbelliferone (MUF) as standard. The rate of hydrolysis of MUP as a function of substrate concentration was measured for all species. A kinetic curve for each species was constructed on the basis of measurements done using various substrate concentrations, usually between 0.05-2500 µM MUP. *Chlamydomonas* sp. and *Oscillatoria simplicissima* phosphatase exhibited Michaelis-Menten kinetics, and the MUP substrate concentration yielding ½ V_{MAX} was subsequently used for determination of the phosphatase activity in the samples (1 µM for both *Chlamydomonas* sp. and *Oscillatoria simplicissima*). *Microcystis aeruginosa* did not exhibit ectoenzymatic phosphatase activity at any of the substrate concentrations tested. The ectophophatase activity was determined in the samples by mixing 1.25 ml of algal culture with 62.5 µl MUP substrate (1 µM), and incubating for 2 hours at 30° and in the dark. The fluorescence of the resulting product MUF was measured initially (to determine background fluorescence) and after the incubation. The fluorescence of MUF was measured after adjusting the pH to 10.3 with Tris/HCl buffer. Calibration was done using standard additions of fluorochrome standard solution (0.1 µM) after last measurement.

2.6. Measurement of nitrate reductase activity

Nitrate is actively transported across the cell membrane by either an ATP-binding cassette transporter or a nitrate permease. Once inside the cell, it is reduced to nitrite and subsequently ammonium through the activities of nitrate reductase and nitrite reductase, respectively (Miller and Castenholz 2001). The activity of nitrate reductase was determined according to Hochman *et al.* (1986), using KNO_3 as substrate. The cells were homogenised in the same way as for the

phosphatase activity assay, in a buffer consisting of 200 mM KPO_4, 1 mM DTT and 1 mM EDTA. After centrifugation 500 µl of the supernatant was used for subsequent measurements. The supernatant was mixed with 1 mM NADH and KNO_3 acting as substrate. The reaction mixture was incubated at 30°C for 30 minutes. The reaction was terminated by the addition of 60 mM $ZnSO_4$ and 0.05N NaOH. After centrifugation at 7500 rpm for 10 minutes 0.1% sulfanylamide and 0.008% NED was added, and the NO_2^- was measured spectrophotometrically at 540 nm.

2.7. Nutrient analyses

PO_4^{3-} concentrations were determined spectrophotmetrically using the stannous chloride method according to Taras *et al.* (1971). NH_4^+ concentrations were determined spectrophotmetrically using the phenol alcohol method according to Hardwood and Kűhn (1970). During experimental work (Chapters 4 and 5) NH_4^+ was determined mainly to monitor potential release of NH_4^+ from algal cultures. Nitrate NO_3^- was determined in river samples according to Cataldo *et al.* (1975). The NO_3^- determination method could not be used due to interference by some substances in the growth medium, and therefore no NO_3^- determinations were done for the experiments described in Chapters 4 and 5. In some instances HPLC measurements were done using external consult agencies (Viridus Technologies (Pty) Ltd. EKOREHAB, Potchefstroom), to attain information on nitrate concentrations in the experimental cultures, as well as for checking/comparing the results obtained during the experimental work. Ratios of N:P in this work refer to the ratio between NO_3^-: PO_4^{3-} unless stated otherwise.

2.8. Phytoplankton species composition

The composition of the microalgal species in the phytoplankton (Chapter 3) was analysed using a Zeiss inverted light microscope according to Utermöhl (1958) and Lund *et al.* (1958). Gas vesicles of cyanobacteria were pressure-deflated in a steel-container. Between 1 and 5 m*l* of formalin-preserved river water was pipetted into sedimentation tubes and filled with distilled water and covered with glass slides. The sedimentation tubes were left for two days in a dessicator before analysing the sample. The phytoplanktonic species present were identified and quantified as cells, filaments or colonies (Table 3.7). Results from phytoplanktonic species

composition analyses are presented in Chapter 3. Algal counts in connection with the experimental work (Chapters 4 and 5) were done using the same technique.

2.9. Direct chlorophyll-a fluorescence

In all dark-adapted photosynthetic organisms, the intensity of chlorophyll-*a* fluorescence emission shows a fast rise over time, known as the fluorescence transient (e.g. Strasser *et al.* 1995). This variable chlorophyll-*a* fluorescence yield is related to the photochemical activity of PSII. When illuminated with high light intensity, the fluorescence rise follows a fast polyphasic sequence with the main steps labelled as O-J-I-P (Strasser and Govindjee 1992). Additional steps, such as the K-step at approximately 300 μs, have been demonstrated in e.g. heat-stressed samples (Srivastava *et al.* 1997) and later also in N-deficiency (Strasser pers. comm.). On illumination there is an immediate rise in chlorophyll *a* fluorescence to an initial level, termed F_0. This is attained when the chlorophyll antenna absorbs light but before the excitons have been trapped. F_0 is measured when the majority of PSII reaction centres (RC) are open for primary photochemistry and both the potential for photochemical use of excitation energy and photochemical quenching of fluorescence are maximal. The O-J phase is the photochemical phase leading to the reduction of Q_A to Q_A^-. The J-P phase is non-photochemical. The I-phase is related to heterogeneity in the filling-up of the plastoquinone pool. The P phase is reached when all the plastoquinone is reduced to PQH_2 and all the RCs are closed (Strasser *et al.* 1995). The OJIP transient (Figure 2.3 demonstrates an example of a fast fluorescence rise curve) changes its shape according to many environmental conditions, such as light intensity (Srivastava and Strasser 1996, Krüger *et al.* 1997), temperature (Srivastava *et al.* 1997), drought (Van Rensberg *et al.* 1996) or chemical changes (Ouzounidou *et al.* 1997).

Performance Index, or PI, is an index that expresses the independent parameters or the density of RC, the quantum yield of trapping and the probability that a trapped exciton will move an electron into the electron transport chain beyond Q_A. This overall performance of PSII, quantified by the performance index PI, can be used as a vitality index of any stressed organism (Strasser *et al.* 2000 and references therein).

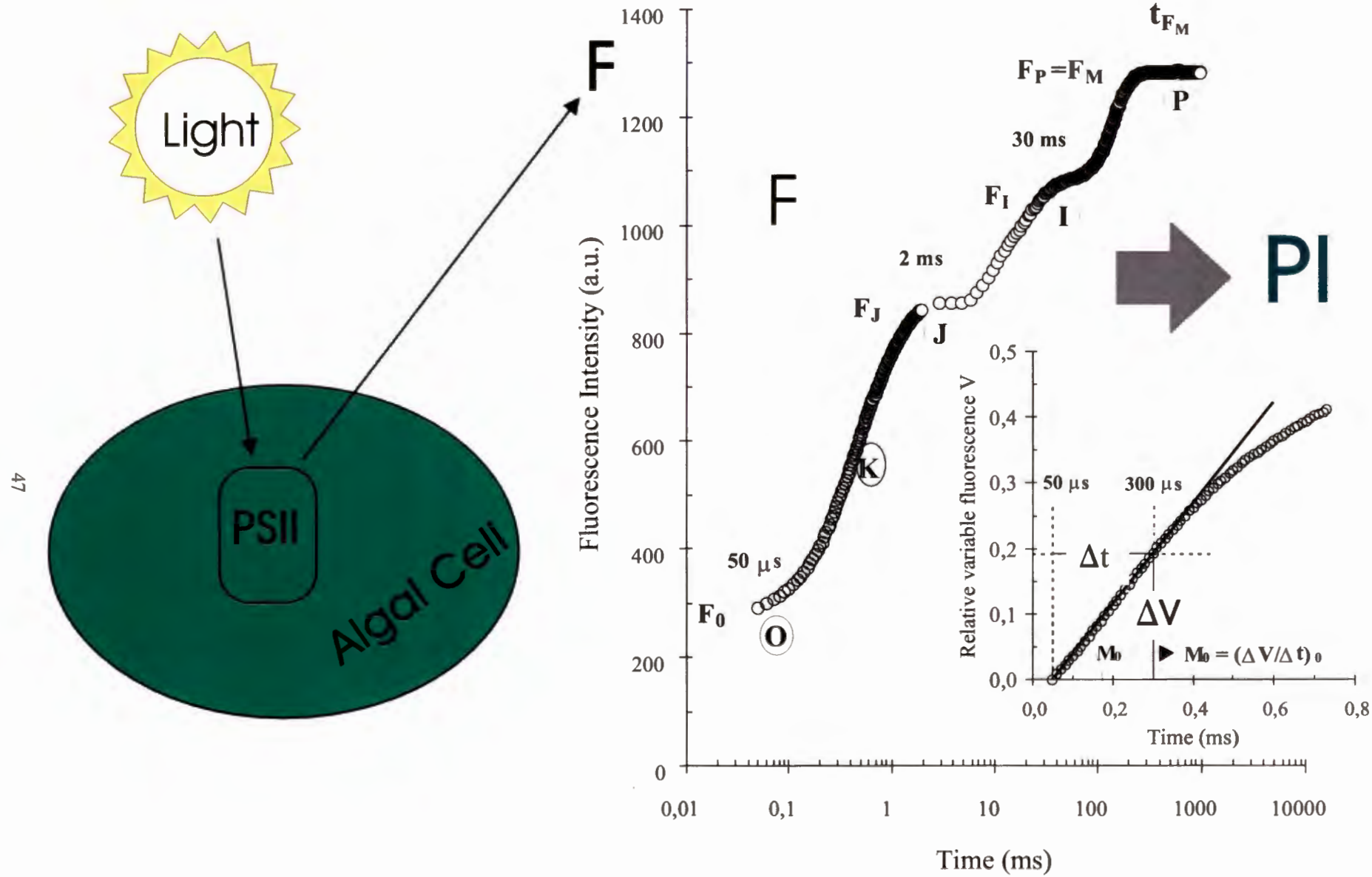


Figure 2.3. Principle for measurement of the fast fluorescence rise transient, and an example of the resulting O-J-I-P curve, from which energy fluxes and vitality indexes (PI) are derived. F = fluorescence, PSII = photosystem II. For fluorescence abbreviations please refer to Chapter 2.

Chlorophyll-*a* fluorescence, in connection to experimental work reported in Chapters 4 and 5, was measured using a method developed by Strasser *et al.* (1995). Using the PEA-instrument (Plant Efficiency Analyser) a precise detection of the initial fluorescence, the initial slope – which offers a link to the maximum rate of primary photochemistry per reaction centre - and of the amplitude and appearance time of the intermediate steps, can be recorded. The procedure is used for quantification of photosystem II function by analysing the polyphasic fast fluorescence rise, using a fluorometer providing high time resolution and large data acquisition capacity.

Chlorophyll-*a* fluorescence measurements were conducted using experimental cultures (*Chlorella* sp., *Chlamydomonas* sp., *Microcystis aeruginosa* and *Oscillatoria simplicissima*) at the end of each experiment (Chapters 4 and 5). Samples (5-20 m*l*) of the culture were, at the end of the experiment, dark adapted for one hour. After this the algal culture was filtered onto GF/C filters and the filters subsequently treated as "leaves" (Strasser pers. comm. and Venter 2000). For each treatment the chlorophyll-*a* fluorescence transients of 18-30 replicates were measured and recorded up to 1s, with data acquisition every 10 μs for the first 2 ms and every 1 ms thereafter (Strasser *et al.* 1995). The fluorescence transients were induced by a red light (peak at 650 nm) of 600 W m^{-2} provided by an array of six light-emitting diodes. All measurements were performed immediately after filtering the cells onto filterpapers, in darkness except for a green light.

2.9.1. The JIP-test

The fluorescence value F_0 at the onset of illumination indicates fluorescence when all the reaction centres (RCs) are open. Between the initial (F_0) and the maximum (F_M) fluorescence two more steps, J and I can be seen when fluorescence is plotted on a logarithm time scale (Strasser and Govindjee 1992, Figure 2.3). These O-J-I-P fluorescence transients can be analysed using the JIP-test (for details on the method see e.g. Strasser and Strasser 1995, Strasser *et al.* 1995, Krüger *et al.* 1997, Strasser *et al.* 1999). The test records a number of variables from the fast fluorescence transient O-J-I-P (Table 2.2). A set of biophysical parameters are then calculated based on the recorded parameters (Table 2.3).

Table 2.2. Variables recorded during measurements using the Plant Efficiency Analyser.

Abbreviation	Explanation
F_0	F50μs, fluorescence intensity at 50 μs
F_{150}	Fluorescence intensity at 150 μs
F_{300}	Fluorescence intensity at 300 μs
F_J	Fluorescence intensity at the "J-step" (2 ms)
F_I	Fluorescence intensity at the "I-step" (30 ms)
F_M	Maximal fluorescence intensity
t_{Fmax}	Time to reach F_M, in ms
V_J	$(F_{2ms}-F_0)/(F_M-F_0)$
Area	Area between the fluorescence curve and F_M
M_0	$4*(F_{300}-F_0)/(F_M-F_0)$

Table 2.3. Biophysical parameters calculated based on the recorded parameters, and their derivations.

Abbreviation	**Explanation**
S_m	$Area/(F_M-F_0)$
B_{av}	$1-(S_m/t_{Fmax})$
N	$S_m*M_0*(1-V_J)$ turn over number of Q_A
Quantum efficiencies or flux ratios	
φ_{P0} or TR_0/ABS	$[1-(F_0/F_M)]$ or F_V/F_M
φ_{E0} or ET_0/ABS	$[1-(F_0/F_M)]*\Psi_0$
Ψ_0 or ET_0/TR_0	$(1-V_J)$
Specific energy fluxes or specific activities	
ABS/RC	$M_0*(1/V_J)*(1/\varphi_{P0})$
TR_0/RC	$M_0*(1/V_J)$
ET_0/RC	$M_0*(1/V_J)*\Psi_0$
DI_0/RC	$(ABS/CS_0)-(TR_0/RC)$
Phenomenological fluxes or phenomenological activities	
ABS/CS_0	F_0
TR_0/CS_0	$\varphi_{P0}*(ABS/CS_0)$
ET_0/CS_0	$\varphi_{P0}*\Psi_0*(ABS/CS_0)$
DI_0/CS_0	$(ABS/CS_0)-(TR_0/CS_0)$

Density of reaction centres	
RC/CS_0	$\varphi_{P0}*(V_J/M_0)*F_0$
Vitality indexes	
SFI_{P0}	$(RC/ABS)*\varphi_{P0}*\Psi_0$
SFI_{N0}	$(1-\varphi_{P0})*(1-\Psi_0)$
PI_{ABS}	$(RC/ABS)*[\varphi_{P0}/(1-\varphi_{P0})]*[\Psi_0/(1-\Psi_0)]$
PI_{N0}	$1/PI_{P0}$
Driving forces	
DF_{P0}	$Log(PI_{P0})$
DF_{N0}	$Log(PI_{N0}) = -log(PI_{P0})$

2.10. Data analysis and presentation

The data were analysed using several statistical programs. Statistical analyses were done in Statistica for Windows version 5.5, but occasionally (especially for data requiring log-transformation) SYSTAT version 6.0.1 was used. Normality of the data was tested using Shapiro-Wilk's W and Kolmogorov-Smirnov & Lilliefors test for normality, and the homogeneity of the variances was tested by using the Levenes test tests (STATISTICA for Windows, Statsoft Inc. 1999). Since most data-sets were not normally distributed, and normal distribution was not achieved using log-transformation, non-parametric tests were often used. Spearman Rank correlation tests (STATISTICA for Windows, Statsoft Inc. 1999) were performed instead of parametric Pearsons correlation, and Kruskal Wallis ANOVA instead of parametric ANOVA. When *post hoc* tests were appropriate, Kolmogorov Smirnoff test for comparing two independent samples was used in the case of non-parametric data. In the case of parametric data, Tukey's *post hoc* test for equal or unequal N, as appropriate, was used. The statistical significance is reported as p-values, as received from the result tables in the statistical computer programs. In the case of ANOVA and Kruskal-Wallis ANOVA p-values are given exactly to the fourth or fifth decimal. When using other tests, such as Kolmogorov Smirnov test, p-values are reported only as $p > 0.05$ (not statistically significant), $p < 0.05$ (significant at the 95% confidence level), $p < 0.01$ (significant at the 99% confidence level) and $p < 0.001$ (significant at the 99.9% confidence level).

For multivariate analyses (Chapter 3) Canoco for Windows version 4 was used (Ter Braak 1986, Ter Braak and Šmilauer 1998).

In all statistical analyses concerning the experimental work (Chapters 4 and 5) only the three treatments exposed to nutrient limitation (N-limited, internal control and P-limited) were compared, excluding the external control treatment. The reasons for this is that the algae in the external control treatment were grown in optimal conditions and therefore express largely different physiology compared to the nutrient limited cells. Comparisons including the control would therefore be strongly affected by extreme values in the control treatment and these would overshadow differences between the actual treatments. In graphs the external controls have been included to demonstrate e.g. any responses caused by the disturbance effect.

Graphical presentations have been done using SigmaPlot 2000 and Excel 97 for Windows version 6.10 and CorelDRAW 7 version 7.467.

CHAPTER 3

ENVIRONMENTAL VARIABLES, PHYTOPLANKTONIC SPECIES COMPOSITION AND ACID AND ALKALINE PHOSPHATASE ACTIVITIES IN PHYTOPLANKTON AND BACTERIA IN THE VAAL RIVER

3.1. Introduction

The studies of phytoplankton nutrient physiology has become a central topic in aquatic research, especially in connection with eutrophication of water bodies and increasing incidents of harmful algal blooms. As orthophosphate (PO_4^{3-}) often constitutes the growth-limiting nutrient in fresh waters, regeneration of bioavailable PO_4^{3-} from organic P (P_o) compounds is essential for plankton growth and succession.

The synthesis as well as the regulation of the activity of phosphatase enzymes is one strategy by which phytoplankton may influence nutrient dynamics in lakes. Phosphatases hydrolyse a variety of organic phosphorus compounds into bio-available inorganic PO_4^{3-}. In many earlier studies alkaline phosphatase activity (APA) has been found to be inversely correlated to PO_4^{3-} availability in the water (Pettersson 1980, Currie *et al.* 1986, Elser and Kimmel 1986, Jansson *et al.* 1988) suggesting that phosphatase activity is induced during PO_4^{3-} depletion. On the other hand, a number of studies exist where this relationship between APA and PO_4^{3-} has not been shown (Pick 1987, Dodds 1995, Jamet *et al.* 1995, Boavida and Marques 1995, Jamet *et al.* 1997).

Some studies suggest that phosphatase activity might be linked to species composition (Hino 1988, Olsson 1990, Rengefors *et al.* 2001) or trophic (enrichment) status of the water (Hantke *et al.* 1996, Spijkerman and Coesel 1998). Thus the origin and function of phosphatases in aquatic communities is still unclear.

Not much work has been done concerning phosphatase activity in rivers, especially on the African continent. The Vaal River in South Africa is a fairly large river, and of great importance for the highly populated area around Johannesburg. Eutrophication, algal blooms and salinisation of the water are the major environmental problems in this river (Pieterse and Janse Van Vuuren 1997).

Multivariate analysis has recently become a routine analysis tool for studying the relationship between phytoplankton species composition and environmental variables (see e.g. Varis *et al.* 1989, Varis 1991, Romo and van Tongeren 1995). This study analyses, in addition to the species composition and environmental variables, the phosphatase activities produced by the phytoplankton community in relation to the species composition, using multivariate tools. Such an integration of ecophysiological processes is not the conventional method in multivariate analyses within environmental research, but may be a useful approach in order to recognise important relationships between environment, species composition and the physiological adaptations and processes of the dominant species.

The aim of this study was, during a period of 24 months, and at four different localities in the Vaal River, to

1. establish the presence, magnitude, seasonal and spatial variation in potential acid and alkaline phosphatase activities (AcPA and APA),
2. study the partitioning of APA and AcPA accounted for by the phytoplankton compared to APA and AcPA in the dissolved / bacterial fraction in the water,
3. investigate the partitioning between APA and AcPA in the different size fractions
4. measure basic physical and chemical growth factors and analyse the phytoplankton composition (one year only), and
5. determine potential interactions between the phosphatase activity, phytoplankton species composition and environmental variables, using multivariate Redundancy Analysis.

The study was done at four localities in the Vaal River from February 1999 to March 2001. The study area, study sites as well as material and methods are described in Chapter 2.

3.2. Results

3.2.1. Environmental and biological variables

Chlorophyll-*a* concentrations were measured in the Vaal River monthly from February 1999 to March 2001. The annual variation in chlorophyll-*a* concentration is presented in Figure 3.1A. Highest chlorophyll-*a* concentrations were measured in spring and early summer at all localities.

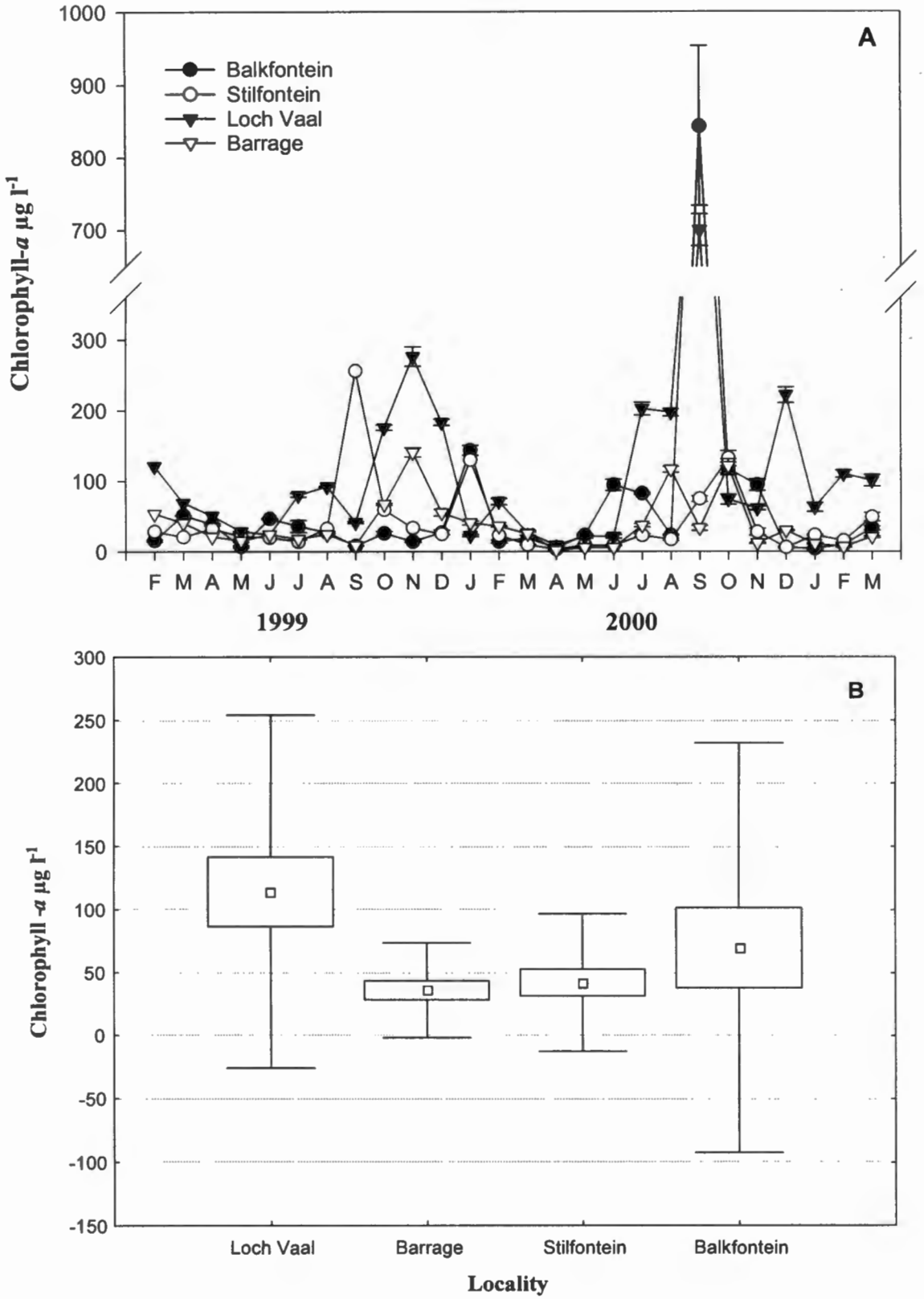


Figure 3.1. Chlorophyll-*a* concentrations (mean and standard error of six samples) during the study period at the four localities in the Vaal River (A) and box and whisker plot indicating differences in the average concentrations between the localities (B). Loch Vaal had the highest average chlorophyll-a concentration ($p < 0.01$).

A cyanobacterial bloom consisting mainly of *Microcystis aeruginosa* and *Oscillatoria simplicissima* caused high chlorophyll-*a* levels in February 1999 at Loch Vaal. In September 1999 at Stilfontein high chlorophyll-*a* concentrations were due to *Chlamydomonas incerta*. *Cryptomonas major*, in combination with centric diatoms, caused elevated chlorophyll-*a* levels at Loch Vaal in October 1999. High numbers of diatoms occurred in the Loch Vaal and Barrage in November 1999, in Loch Vaal combined with high numbers of *Chlamydomonas incerta*. This was followed by a *Microcystis aeruginosa* bloom at Loch Vaal in December 1999. In January 2000 the riverine locations experienced mass occurrences of *Oscillatoria simplicissima* combined with centric diatoms. At Balkfontein *Trochiscia prescottii* dominated in June – July 2000 causing high biomass values. Between July and August 2000 elevated chlorophyll-*a* levels were a consequence of centric diatoms at Loch Vaal. The highest chlorophyll-*a* concentrations registered during this study (nearly 900 $\mu g\ l^{-1}$) were measured in September at Balkfontein, caused by *Pandorina morum*. Simultaneously *Cryptomonas major* formed a mass-occurrence upstream at Loch Vaal, causing the chlorophyll-*a* levels to exceed 650 $\mu g\ l^{-1}$. In December *Melosira granulata* was abundant in Loch Vaal, followed by a bloom of *Spirulina* sp. (sometimes called *Arthrospira* sp., see e.g. Canter-Lund and Lund 1995 and Fox 1996) in February 2001 and at the end of the study period a *Ceratium hirundinella* bloom in March 2001. For a more detailed description of the species composition refer to section 3.2.3.1.

The mean chlorophyll-*a* concentration differed significantly between the localities (Kruskal Wallis ANOVA $p = 0.0012$, Figure 3.1 B), and were, during the study period, on average between 31 and 139 $\mu g\ l^{-1}$ (Tables 3.1 and 3.2). The highest mean chlorophyll-*a* concentration was in Loch Vaal (Figure 3.1.B), being significantly higher than that of Balkfontein (Kolmogorov Smirnoff $p < 0.025$), Stilfontein (Kolmogorov Smirnoff $p < 0.005$) and Barrage (Kolmogorov Smirnoff $p < 0.005$). Balkfontein had the second highest mean chlorophyll-*a* concentration, partly caused by an extremely dense bloom of *Pandorina morum* (Chlorophyceae) in September 2000, which caused the chlorophyll-*a* levels to exceed 800 $\mu g\ l^{-1}$.

The PO_4^{3-}, NO_3^- and NH_4^+ concentrations were measured at the Potchefstroom University for the last year of the study period, between February 2000 and March 2001 (Figure 3.2). For the first year of the study results for PO_4^{3-}, NO_3^- and NH_4^+ were acquired from Rand Water and MidVaal Water companies (Table 3.1).

Table 3.1. Environmental variables at four locations in the Vaal River between February 1999 and February 2000. Means, minimum and maximum values are given for each parameter.

	LOCH VAAL			BARRAGE			STILFONTEIN			BALKFONTEIN		
	mean	min	max	mean	min	max	mean	min	max	mean	min	max
TEMPERATURE °C	20.9	12.6	27.4	19.8	12.0	25.8	18.8	10.0	26.0	21.2	11.8	30.0
pH	9.0	7.9	9.4	8.6	8.0	9.0	8.6	7.9	9.6	8.9	7.8	9.6
CONDUCTIVITY mS M^{-1}	85.5	67.0	96.0	76.7	60.0	86.0	79.6	33.0	99.0	41.4	16.4	59.7
TURBIDITY NTU	30.8	5.7	60.0	8.9	2.4	19.0	18.8	7.3	91.0	23.2	7.6	100
ALKALINITY CaCO3	101.6	86.0	127.1	116.9	96.0	132.3	128.0	88.0	146.0	166.0	108.0	239.0
CHLOROPHYLL-a µg l^{-1}	94.3	22.9	276.6	40.3	4.1	140.8	53.5	14.0	256.0	35.1	7.2	143.7
DISSOLVED O_2 mg l^{-1}	8.7	0.0	15.2	6.5	0.0	12.6	7.1	5.0	9.7	9.4	5.7	12.8
SATURATED O_2 %							88.2	72.0	98.0	104.9	67.0	137.1
SECCHI cm	35.7	23.0	70.0	81.2	44.0	162.0				51.2	18.0	84.0
LIGHT (surface)										801.9	266.0	1485.0
LIGHT (subsurface)										577.6	360.0	810.0
NO_3^- µmol l^{-1}	132.4	3.6	342.6	104.0	31.4	192.7	26.8	1.4	94.9	14.3	n.d.	35.3
NH_4^+ µmol l^{-1}	38.8	0.0	156.4	6.8	0.0	22.1	6.1	0.4	13.6	3.5	n.d.	9.8
PO_4^{3-} µmol l^{-1}	10.4	1.2	23.6	5.3	1.5	8.7	4.7	1.9	8.7	0.5	n.d.	2.9
NO_3:PO_4 (mol:mol)	20.1	0.6	76.6	27.5	0.6	82.1	6.2	0.7	19.6	13.1	3.1	74.2

n.d. = not detected

Table 3.2. Environmental variables at four locations in the Vaal River between February 2000 and March 2001. Means, minimum and maximum values are given for each parameter.

	LOCH VAAL			BARRAGE			STILFONTEIN			BALKFONTEIN		
	mean	min	max	mean	min	max	mean	min	max	mean	min	max
TEMPERATURE °C	18.8	10.4	24.6	18.3	10.8	23.7	18.0	10.0	25.0	20.5	11.4	26.8
pH	8.6	7.4	9.4	8.2	7.7	8.9	8.5	8.0	9.6	8.5	7.4	10.0
CONDUCTIVITY mS M^{-1}	121.5	23.0	687.0	115.7	23.0	600.0	56.5	26.0	89.0	38.5	16.9	54.9
TURBIDITY NTU	96.6	10.0	689.0	43.3	5.3	105.0	45.1	5.8	99.0	39.4	9.1	109.0
ALKALINITY CaCO3	100.4	85.0	115.0	91.2	61.0	115.0	112.5	76.0	152.0	125.2	89.3	181.7
CHLOROPHYLL-a µg l^{-1}	139.3	9.1	700.3	33.0	2.4	121.3	30.8	3.3	132.3	104.4	4.3	843.6
DISSOLVED O_2 mg l^{-1}	9.6	0.00	17.7	7.7	0.03	12.0	7.1	4.9	9.0	9.8	6.5	20.0
SATURATED O_2 %										108.9	67.3	200.0
SECCHI cm	32.9	19.0	49.0	50.5	15.0	110.0				41.7	16.0	70.0
LIGHT (surface)										576.5	40.0	2584.0
LIGHT (subsurface)										339.7	26.0	1308.0
NO_3^- µmol l^{-1}	102.4	8.7	316.5	64.9	1.8	251.9	44.7	0.3	162.2	38.8	0.1	123.7
NH_4^+ µmol l^{-1}	11.5	0.4	53.6	5.5	1.0	22.6	6.7	2.0	20.9	4.5	0.3	12.5
PO_4^{3-} µmol l^{-1}	16.8	3.9	23.1	8.4	1.7	23.3	5.3	2.4	9.1	3.7	0.7	6.2
NO_3:PO_4 (mol:mol)	5.6	0.6	15.5	7.7	0.6	25.4	8.1	0.1	30.6	13.1	0.1	74.2

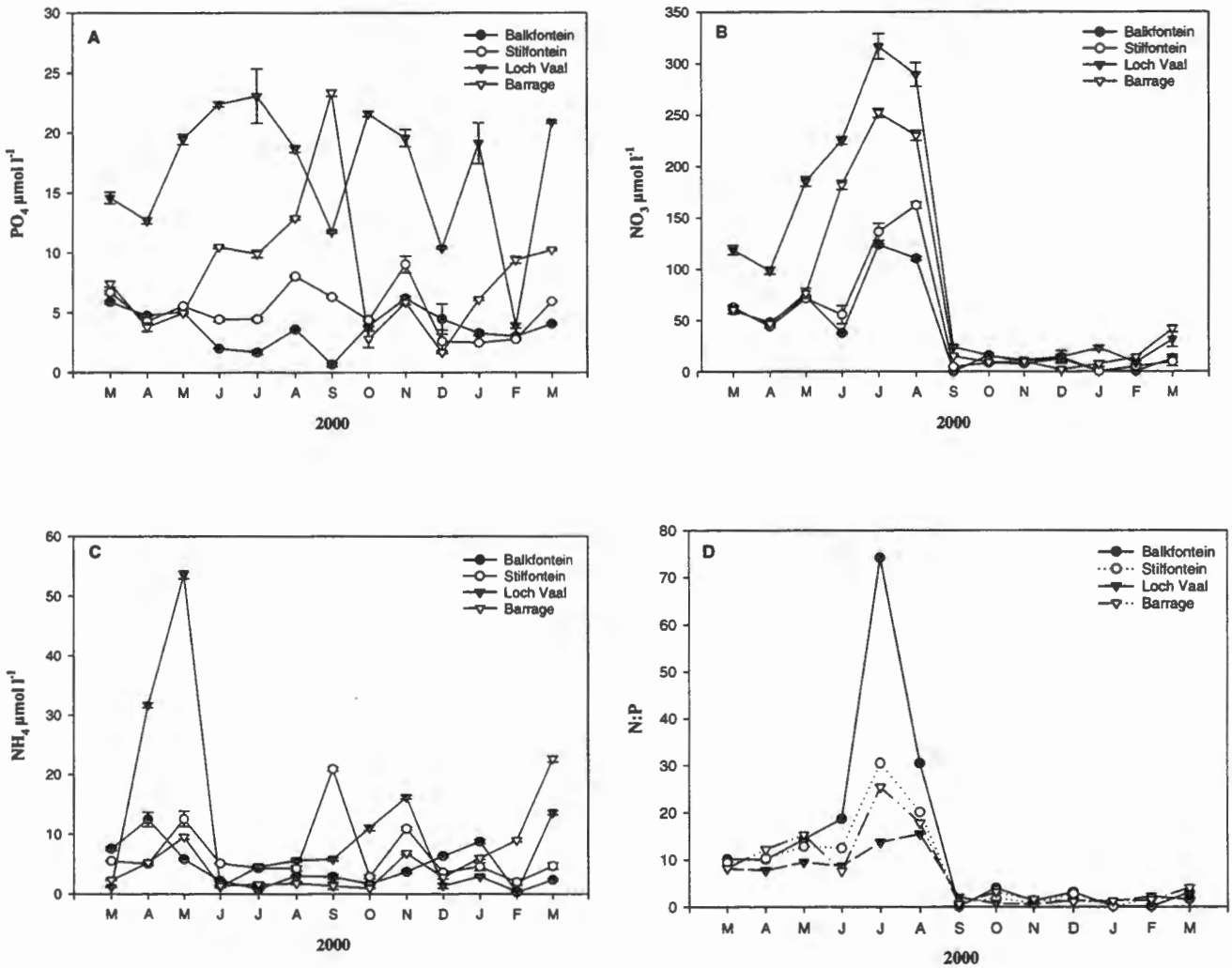


Figure 3.2. Concentration (mean and standard error of six samples) of PO_4^{3-} (A), NO_3^- (B), NH_4^+ (C) and NO_3^-:PO_4^{3-} ratio (mol:mol, D) at the four sampling localities in the Vaal River between March 2000 and March 2001.

The mean PO_4^{3-} concentrations varied between 0.5 and 17 µmol l^{-1} (Tables 3.1 and 3.2). The annual variation was large with no clear seasonal pattern (Figure 3.2. A). The mean PO_4^{3-} concentration was highest in the Loch Vaal, decreasing in a down-stream direction (Figure 3.3 A). The localities differed significantly in the PO_4^{3-} concentration (Kruskal Wallis ANOVA $p = 0.0000$). A log-transformation of the PO_4^{3-} data resulted in normally distributed data-set, and *post hoc* tests were performed to detect differences between the localities. Loch Vaal had the highest concentrations, differing from the Barrage (Tukey's $p = 0.00569$), Stilfontein (Tukey's $p = 0.00029$) and Balkfontein (Tukey's $p < 0.00015$). No statistically significant difference was detected between the other localities.

The seasonal variation in NO_3^- concentration was strong, with high concentrations occurring in late autumn and winter, and lower concentrations in spring and summer months (Figure 3.2 B). The NO_3^- concentrations showed a similar trend to PO_4^{3-} in distribution between the localities, Loch Vaal and Barrage showing highest concentrations (Figure 3.3. B). The differences between the localities were statistically significant (Kruskal Wallis ANOVA $p = 0.0000$). Normally distributed data was not achieved by log-transformation, therefore differences between the localities were tested using a Kolmogorov Smirnoff test for two independent samples (instead of parametric *post hoc* test). According to the results the mean NO_3^- concentration in Loch Vaal was significantly higher compared to the concentrations at Balkfontein (Kolmogorov Smirnoff $p < 0.05$) and Stilfontein (Kolmogorov Smirnoff $p < 0.025$), and significantly higher at the Barrage than at Balkfontein (Kolmogorov Smirnoff $p < 0.01$).

The NH_4^+ concentrations were low, close to the detection limit of the method (approximately 7 µmol l^{-1}), throughout the year, with occasional peaks at all four localities (Figure 3.2. C). The differences between the mean concentrations at the localities once again showed the same pattern as the other nutrients, with Loch Vaal having the highest concentration and Balkfontein the lowest (Figure 3.3.C, Kruskal Wallis ANOVA $p = 0.0034$). Normally distributed data was not achieved by log-transformation. Non-parametric comparisons were performed, and it was shown that Balkfontein had a significantly lower concentration compared to Stilfontein (Kolmogorov Smirnoff $p < 0.005$), Barrage (Kolmogorov Smirnoff $p < 0.025$) and Loch Vaal (Kolmogorov Smirnoff $p < 0.05$). The differences between the other stations were not statistically significant.

The N:P ratio is of importance for phytoplankton and is often regarded as more important for algal growth than nutrient concentrations as such (Boulton and Brock 1999). Generally the N:P ratios in the Vaal River (Figure 3.2) during the study period were lower than the Redfield ratio of N:P = 16:1 (Redfield 1958). The mean N:P ratios did not differ between the localities (Figure 3.3 D, Kruskal Wallis $H_{(3,89)}$ $p > 0.05$). High N:P ratios were observed in winter 2000 at Balkfontein, Stilfontein and Barrage, indicating that phytoplankton growth may have been phosphorus limited during these periods. In Loch Vaal the ratio was constantly less than 16:1, indicating N limitation. During spring and summer the N:P ratio indicated severe N-limitation at all localities.

Alkalinity of the water increased downstream, and was higher at the more riverine localities (Figure 3.4.A). The difference between the localities was statistically significant (Kruskal Wallis ANOVA $p = 0.000$). Balkfontein had highest mean alkalinity, being significantly higher than at Stilfontein (Kolmogorov Smirnoff $p < 0.005$), Barrage (Kolmogorov Smirnoff $p < 0.001$) and Loch Vaal (Kolmogorov Smirnoff $p < 0.001$). Loch Vaal had also significantly lower alkalinity than Barrage (Kolmogorov Smirnoff $p < 0.005$), and Stilfontein (Kolmogorov Smirnoff $p < 0.001$), and Barrage had significantly lower mean alkalinity than Stilfontein (Kolmogorov Smirnoff $p < 0.005$).

The pH of the Vaal River is high, mean values during the study period varied between 8.2 and 9.0. The mean pH was lowest at the Barrage (Figure 3.4.B), being significantly lower only compared to Loch Vaal (Kolmogorov Smirnoff $p < 0.005$).

The mean turbidity of the water was higher at Loch Vaal, where the variation in turbidity also was greatest (Figure 3.4.C). Loch Vaal had significantly higher turbidity compared to Barrage (Kolmogorov Smirnoff $p < 0.005$) and Stilfontein and Balkfontein (Kolmogorov Smirnoff $p < 0.025$). Generally, the water can be regarded as very turbid in the Vaal River. The high turbidity may partly be due to heavy floods, which were frequent and intensive during the study period (January – April 2000).

The mean temperature of the water did not vary significantly between the locations (Kruskal Wallis ANOVA $p = 0.3249$). The mean temperature was typical for temperate climates, varying between 18°C and 21°C (Figure 3.4.D).

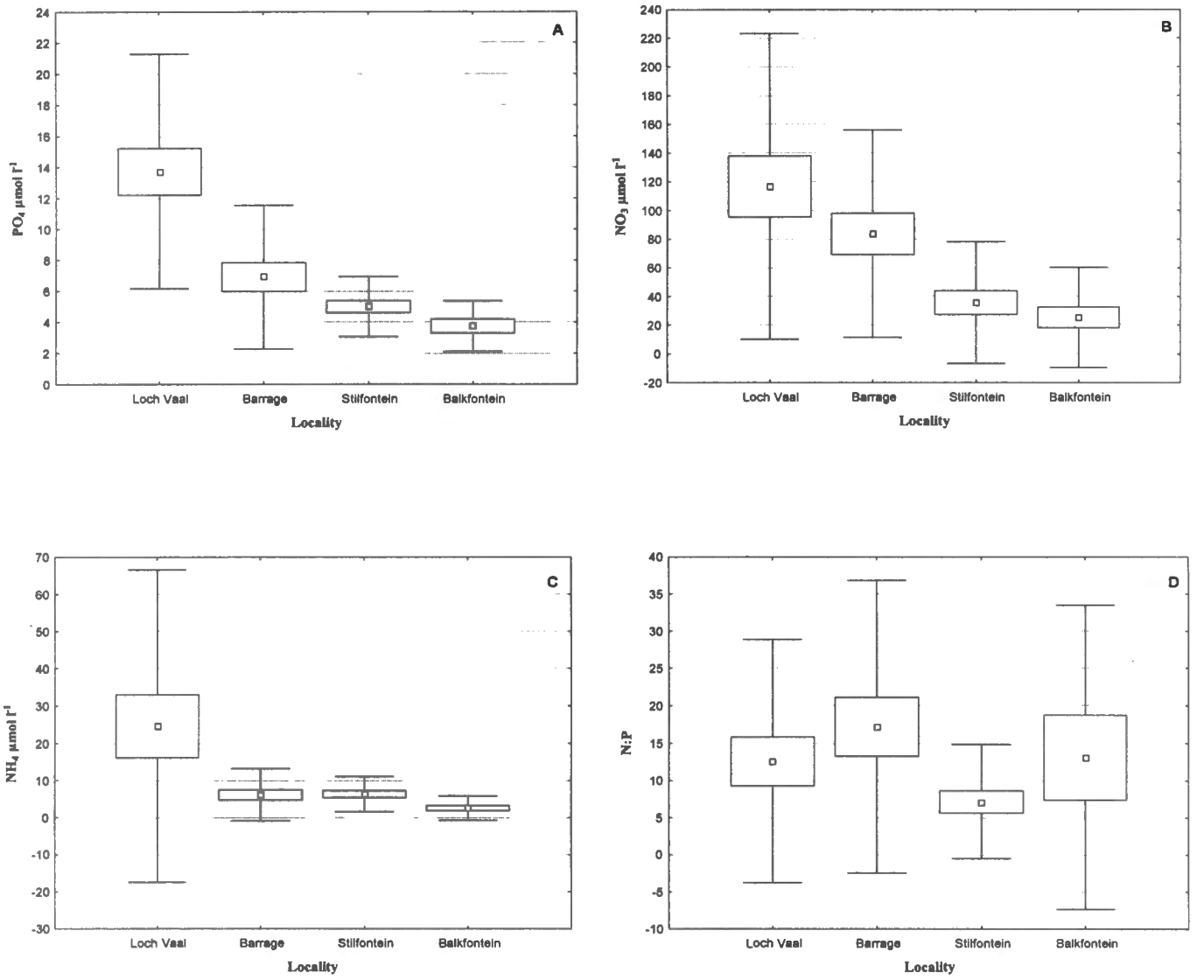


Figure 3.3. Mean (middle point), standard error (box value) and standard deviation (whisker value) of PO_4^{3-} (A), NO_3^- (B), NH_4^+ (C) and NO_3^-:PO_4^{3-} ratio (mol:mol, D) at the four sampling localities in the Vaal River between February 1999 and March 2001. The difference between the nutrient concentrations were statistically significant between the four localities, but the N:P ratios did not differ significantly.

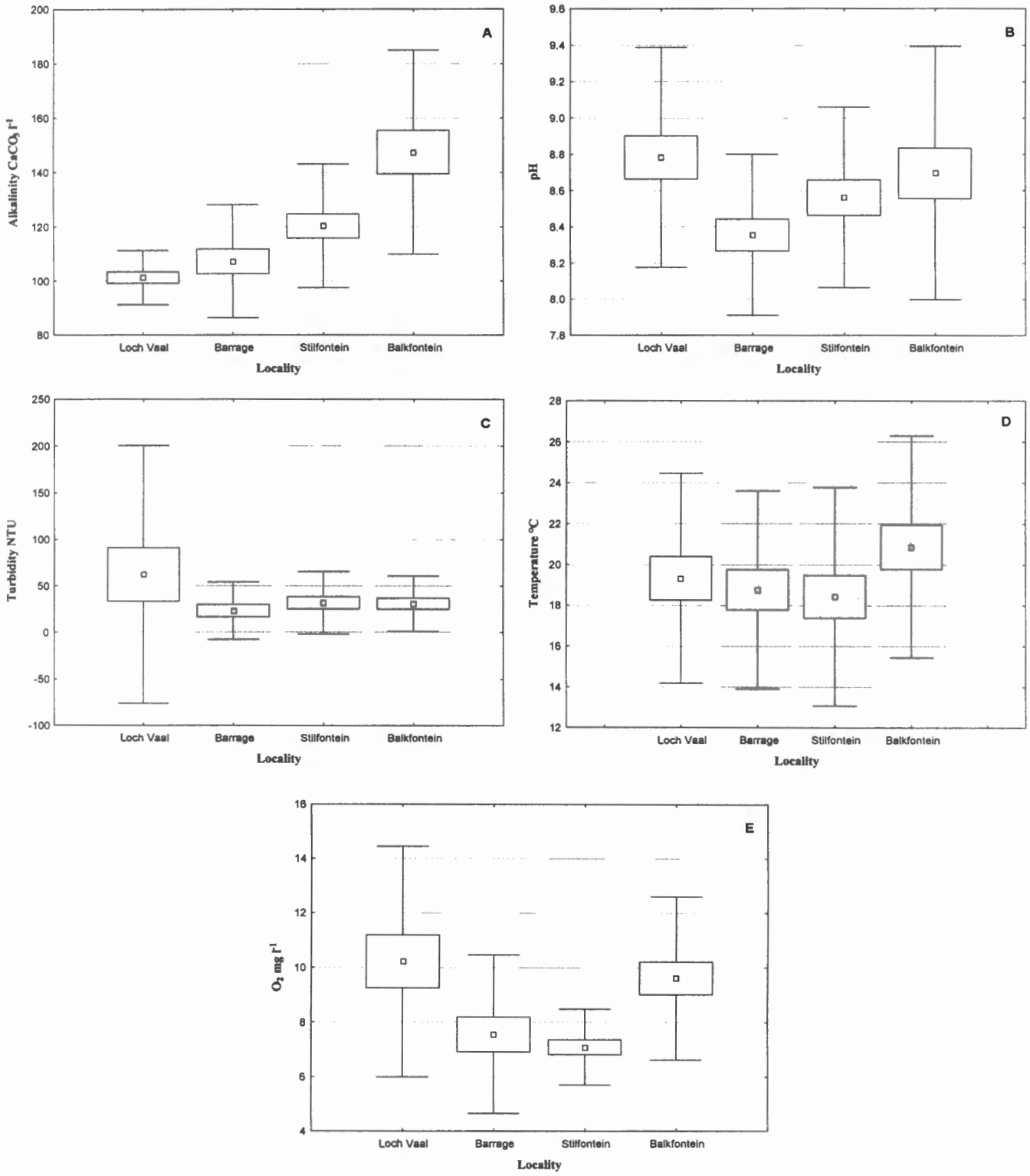


Figure 3.4. Mean (middle point), standard error (box value) and standard deviation (whisker value) of alkalinity (A), pH (B), turbidity (C), temperature (D) and oxygen concentration (E) at the four sampling localities in the Vaal River between February 1999 and March 2001. The difference between the four localities were statistically significant for all variables except for temperature.

The oxygen concentration of the water was highest in Loch Vaal and at Balkfontein, which were also the localities that exhibited the highest mean chlorophyll-*a* concentrations (Figure 3.4.E).

The dissolved oxygen concentration followed the same pattern between the localities as the chlorophyll-*a* concentrations, and highest O_2 concentrations were observed during peak chlorophyll-*a* levels. The O_2 concentrations at Stilfontein were significantly lower compared to Loch Vaal (Kolmogorov Smirnoff $p < 0.01$) and Balkfontein (Kolmogorov Smirnoff $p < 0.005$).

3.2.2. Phosphatase activity

Both acid and alkaline phosphatase activities were recorded at all locations, and in both size fractions, throughout the study period. Phosphatase activities were, when compared to values reported in the literature, relatively high in both size fractions (Tables 3.3, 3.4 and 3.5). Exeptionally high values were recorded occasionally (Figures 3.5 and 3.6) at all localities.

3.2.2.1 Phytoplanktonic fraction

Total and specific APA followed the same annual pattern with highest peaks in summer months, between January and April (Figure 3.5), the highest specific activities measured were at Loch Vaal and Balkfontein. Contrary to this, the highest AcPA were measured in autumn and spring, and the highest specific activity was measured at Balkfontein (Figure 3.5). The differences between the localities (Figure 3.6) were statistically significant for total APA (Kruskal Wallis ANOVA $p = 0.0007$) and specific AcPA (Kruskal Wallis ANOVA $p = 0.0105$), and nearly significant for specific APA (Kruskal Wallis $p = 0.062$). Comparing two localities at a time (non-parametrically) revealed that total APA was significantly higher in Loch Vaal compared to Stilfontein (Kolmogorov Smirnoff $p < 0.005$) and Balkfontein (Kolmogorov Smirnoff $p < 0.025$). Contrary to this, specific AcPA was significantly lower in Loch Vaal compared to Barrage (Kolmogorov Smirnoff $p < 0.01$), Stilfontein (Kolmogorov Smirnoff $p < 0.025$) and Balkfontein (Kolmogorov Smirnoff $p < 0.05$).
In general, Loch Vaal and Barrage had higher mean APA than the more riverine localities, whereas the AcPA was more or less the same at all four localities (Figure 3.6).

Table 3.3. Specific and total acid and alkaline phosphatase activities (AcPA and APA) of the phytoplanktonic fraction (> 1.2µm) and bacterial / dissolved fraction (< 1.2µm) in the Vaal River (pooled data for four investigated locations) for the whole study period from February 1999 to March 2001. The specific enzyme activities are given in nmol P µg chl a^{-1} min^{-1}. The total enzyme activities are given in µmol P l^{-1} min^{-1}. Number of samples = n.

	n	MEAN	MIN	MAX
Specific				
AcPA >1.2µm	101	35.8	4.9	239.2
AcPA <1.2µm	99	781.9	1.8	7105.5
APA >1.2µm	101	40.4	3.3	379.0
APA <1.2µm	99	462.0	18.4	2462.4
Total				
AcPA >1.2µm	101	1.3	0.1	23.7
AcPA <1.2µm	99	11.2	0.4	36.6
APA >1.2µm	101	1.9	0.1	17.9
APA <1.2µm	99	9.1	0.1	32.1

Table 3.4. Specific and total acid and alkaline phosphatase activities (AcPA and APA) of the phytoplanktonic fraction (> 1.2µm) and bacterial / dissolved fraction (< 1.2µm) in the Vaal River during February 1999 to February 2000. The specific enzyme activities are given in nmol P µg chl a^{-1} min^{-1}. The total enzyme activities are given in µmol P l^{-1} min^{-1}. Total number of samples is 12.

	LOCH VAAL			BARRAGE			STILFONTEIN			BALKFONTEIN		
	mean	min	max	mean	min	max	mean	min	max	mean	min	max
Specific												
APA >1.2µm	59.28	8.35	378.97	77.0	9.2	258.8	24.7	4.3	95.9	30.9	3.3	150.3
APA<1.2µm	160.57	64.08	418.60	371.3	55.2	1449.4	376.9	50.7	875.8	444.8	73.6	907.9
AcPA>1.2µm	12.04	5.33	37.18	21.6	7.6	58.8	28.9	5.7	72.6	24.9	4.9	70.2
AcPA<1.2µm	140.32	31.80	376.16	379.6	47.7	1285.2	371.6	28.3	988.6	688.1	66.9	2605
Total												
APA >1.2µm	3.91	0.56	12.88	3.1	0.3	10.5	0.9	0.1	2.7	0.6	0.1	2.4
APA<1.2µm	11.61	5.86	32.06	11.2	1.4	22.3	9.2	1.4	16.7	9.4	3.1	20.6
AcPA>1.2µm	0.88	0.40	1.62	1.0	0.2	3.9	1.1	0.1	4.2	0.6	0.1	2.5
AcPA<1.2µm	9.84	1.70	22.46	8.9	2.3	19.7	9.4	2.8	28.4	9.4	6.6	18.4

Table 3.5. Specific and total acid and alkaline phosphatase activities (AcPA and APA) of the phytoplanktonic fraction (> 1.2µm) and bacterial / dissolved fraction (< 1.2µm) in the Vaal River during March 2000 to February 2001. The specific enzyme activities are given in nmol P µg chl a^{-1} min^{-1}. The total enzyme activities are given in µmol P l^{-1} min^{-1}. Total number of samples is 11.

	LOCH VAAL			BARRAGE			STILFONTEIN			BALKFONTEIN		
	mean	min	max	mean	min	max	mean	min	max	mean	min	max
Specific												
APA >1.2µm	31.1	5.2	163.1	47.1	9.5	159.4	25.7	9.7	63.7	214.7	7.2	371.0
APA<1.2µm	276.8	20.0	1274.4	761.1	86.5	2153.2	688.3	18.4	2056.6	569.8	21.3	2462.4
AcPA>1.2µm	29.0	5.4	111.1	66.7	14.0	239.2	50.0	13.4	101.3	51.6	11.9	116.2
AcPA<1.2µm	442.2	1.9	2167.0	1411.8	106.3	4941.3	1865.0	101.2	7105.5	822.7	21.5	2948.2
Total												
APA >1.2µm	2.9	0.3	17.9	0.9	0.1	2.2	0.5	0.1	1.2	2.4	0.2	12.9
APA<1.2µm	8.8	5.9	12.1	8.1	3.3	15.1	8.0	0.1	15.8	7.3	2.1	14.4
AcPA>1.2µm	1.8	0.4	8.8	1.2	0.3	5.7	0.9	0.2	2.6	3.0	0.5	23.7
AcPA<1.2µm	11.4	0.4	25.5	13.3	1.1	24.3	15.6	4.5	36.6	10.9	3.8	16.6

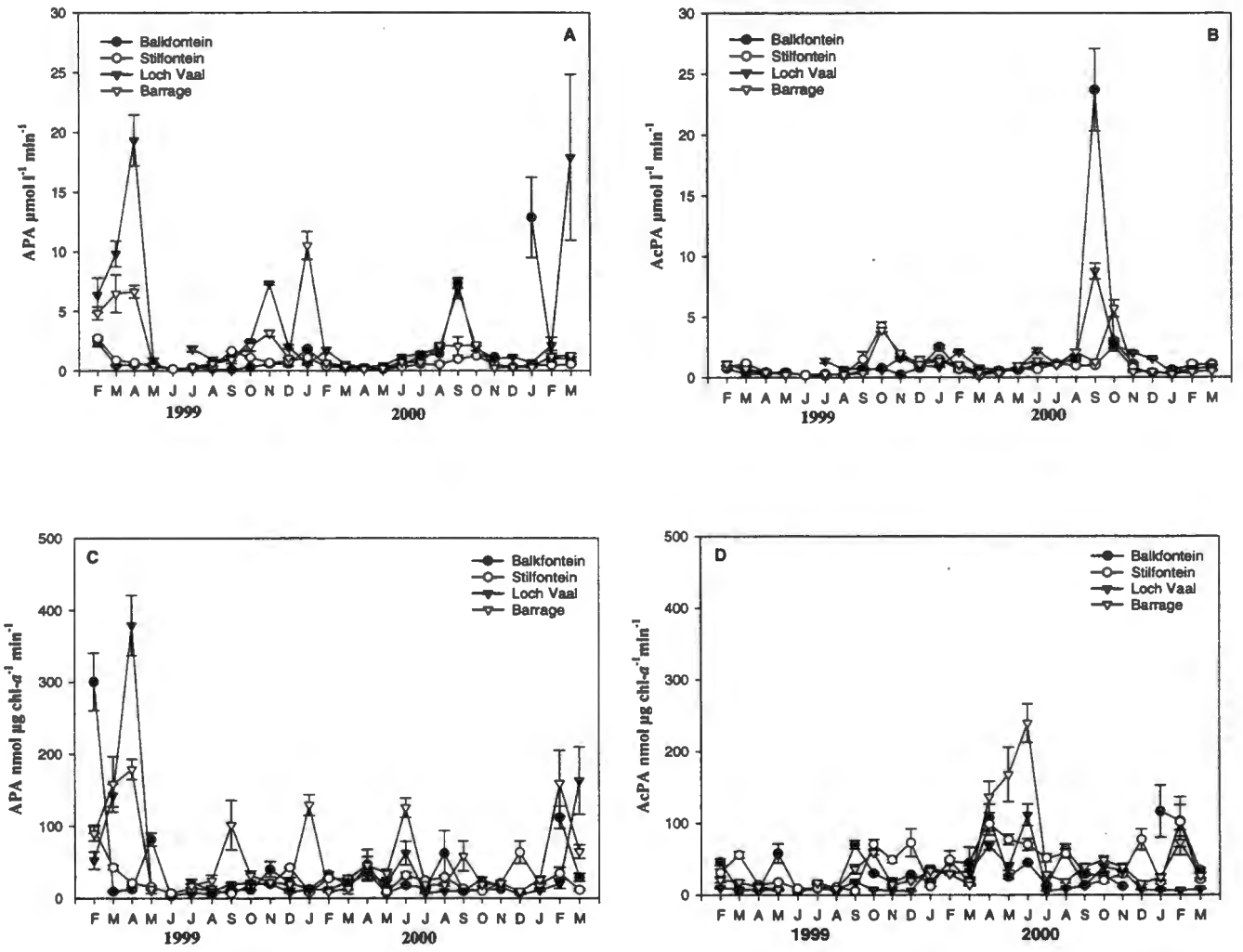


Figure 3.5. Annual variation (mean and standard error of six samples) of total APA (A), total AcPA (B), specific APA (C) and specific AcPA (D) in the phytoplanktonic fraction (> 1.2 µm) at the four sampling localities in the Vaal River between February 1999 and March 2001.

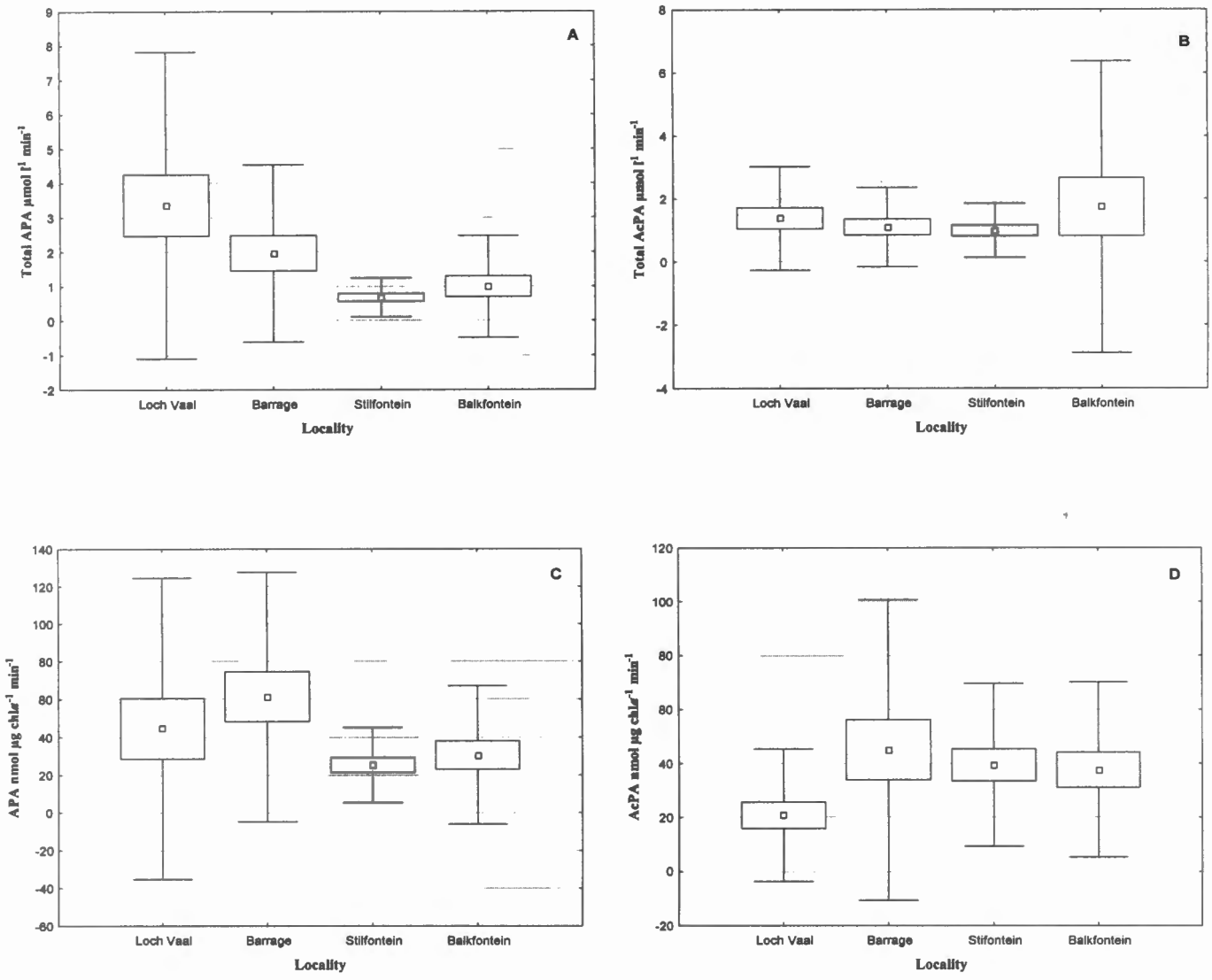


Figure 3.6. Variation of total APA (A), total AcPA (B), specific APA (C) and specific AcPA (D) in the phytoplanktonic fraction (> 1.2 µm) at the four sampling localities in the Vaal River between February 1999 and March 2001. Middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment.

3.2.2.2. Bacterial / dissolved fraction

No seasonal patterns could be shown in the distribution of bacterial / dissolved APA or AcPA during the study period (Figure 3.7). The activities were highly variable throughout the year, and at all localities. Little difference could be detected in the total activity between the localities (Figure 3.8), but the mean specific activities of both APA and AcPA were significantly lower at Loch Vaal, APA being significantly lower compared to Barrage (Kolmogorov Smirnoff $p < 0.025$), Stilfontein (Kolmogorov Smirnoff $p < 0.025$) and Balkfontein (Kolmogorov Smirnoff $p < 0.05$). AcPA was similarly lower at Loch Vaal compared to Stilfontein (Kolmogorov Smirnoff $p < 0.005$) and Balkfontein (Kolmogorov Smirnoff $p < 0.025$).

3.2.2.3. Seasonal differences in APA and AcPA

The specific APA and AcPA data were analysed using Kruskal Wallis ANOVA, rank tests for detecting differences between seasons (Figures 3.9 – 3.12). At all localities phytoplanktonic APA was higher during summer and / or autumn (Figure 3.9), but the difference between the seasons was not statistically significant. The AcPA (Figure 3.10) was homogeneously distributed between the seasons at the more riverine localities but at Loch Vaal and Barrage appeared to be higher in autumn and winter. Also in this case the differences were not statistically significant. The APA and AcPA in the bacterial/dissolved fraction varied throughout the year, and no statistically significant differences could be detected between the seasons (Figures 3.11 and 3.12). In summary both APA and AcPA were present throughout the year, and in both size fractions at fairly consistent levels. In autumn both APA and AcPA were high at all localities.

3.2.2.4. Ratio of phytoplanktonic and bacterial APA and AcPA

The bacterial/dissolved fraction contained most of both acid and alkaline phosphatase activity (Figure 3.13). The highest AcPA was always located in the bacterial / dissolved fraction. The phytoplanktonic fraction accounted for slightly more of the APA compared to the AcPA (Figure 3.13). In more than 90% of the measurements the bacterial / dissolved fraction contained more than 75%, frequently more than 95%, of the activity. The bacterial / dissolved activity was even higher in 2000 compared to 1999, both in AcPA and APA.

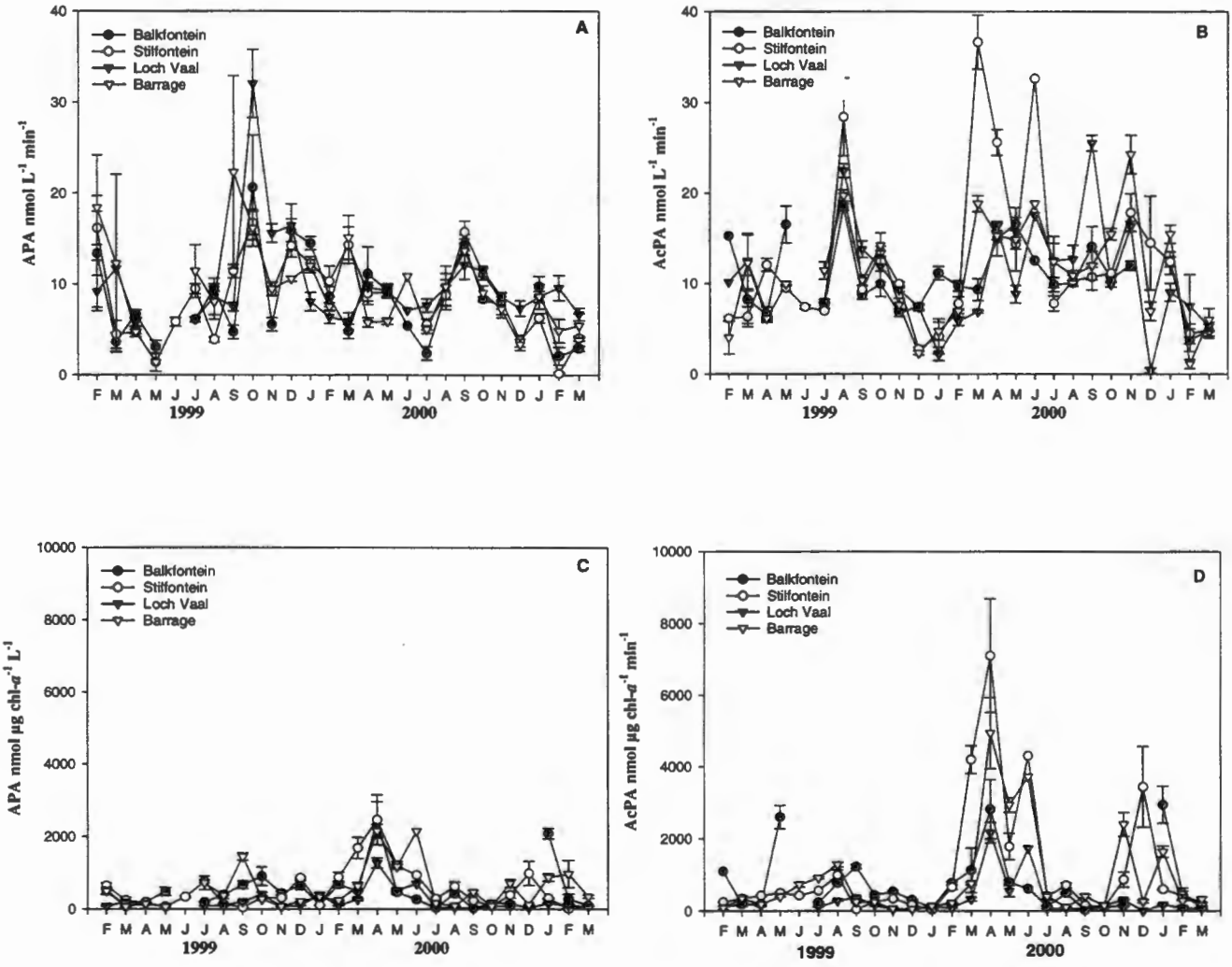


Figure 3.7. Annual variation (mean and standard error of six samples) of total APA (A), total AcPA (B), specific APA (C) and specific AcPA (D) in the bacterial / dissolved fraction (< 1.2 µm) at the four sampling localities in the Vaal River between February 1999 and March 2001.

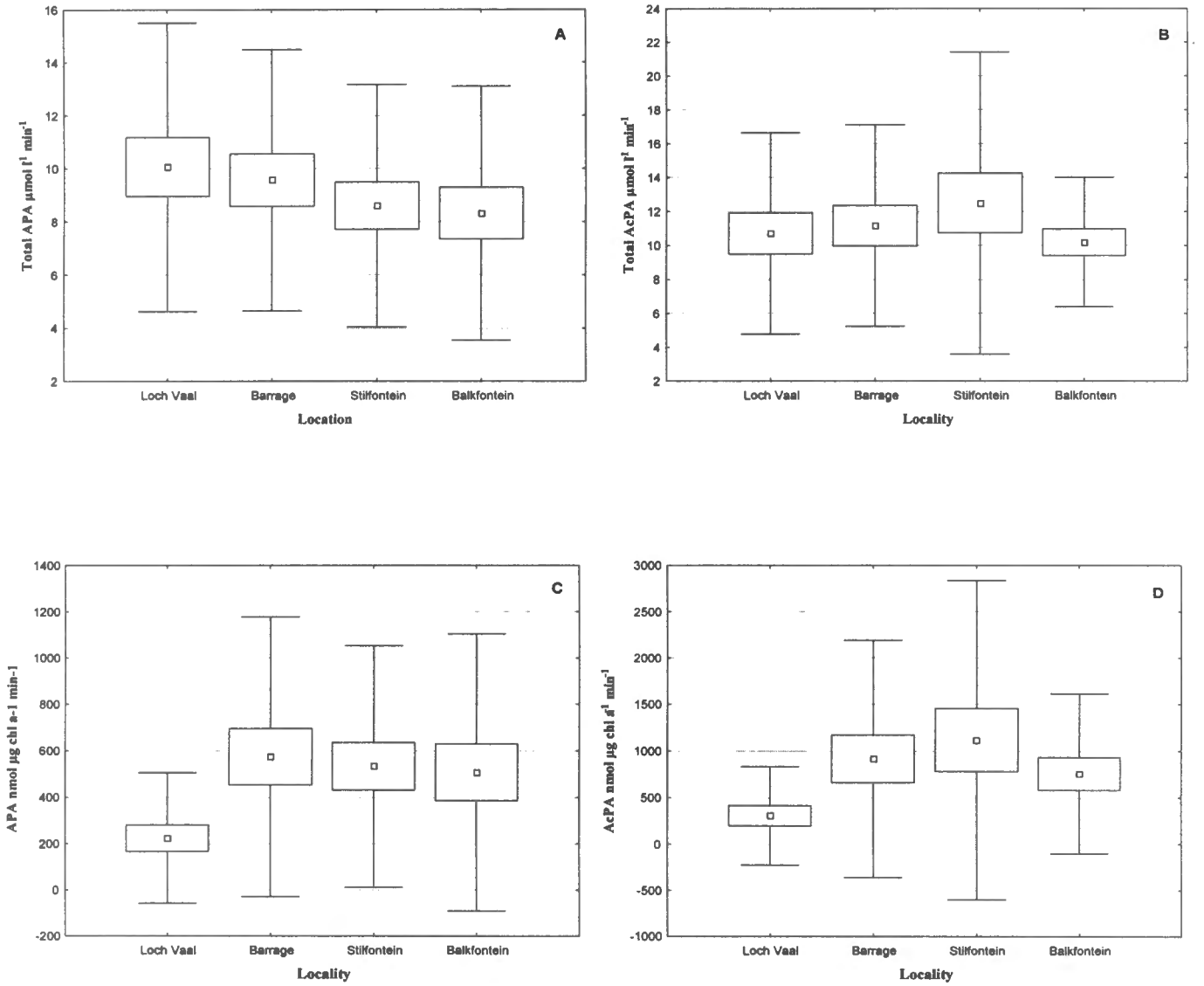


Figure 3.8. Variation of total APA (A), total AcPA (B), specific APA (C) and specific AcPA (D) in the bacterial / dissolved fraction (< 1.2 µm) at the four sampling localities in the Vaal River between February 1999 and March 2001. Middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment.

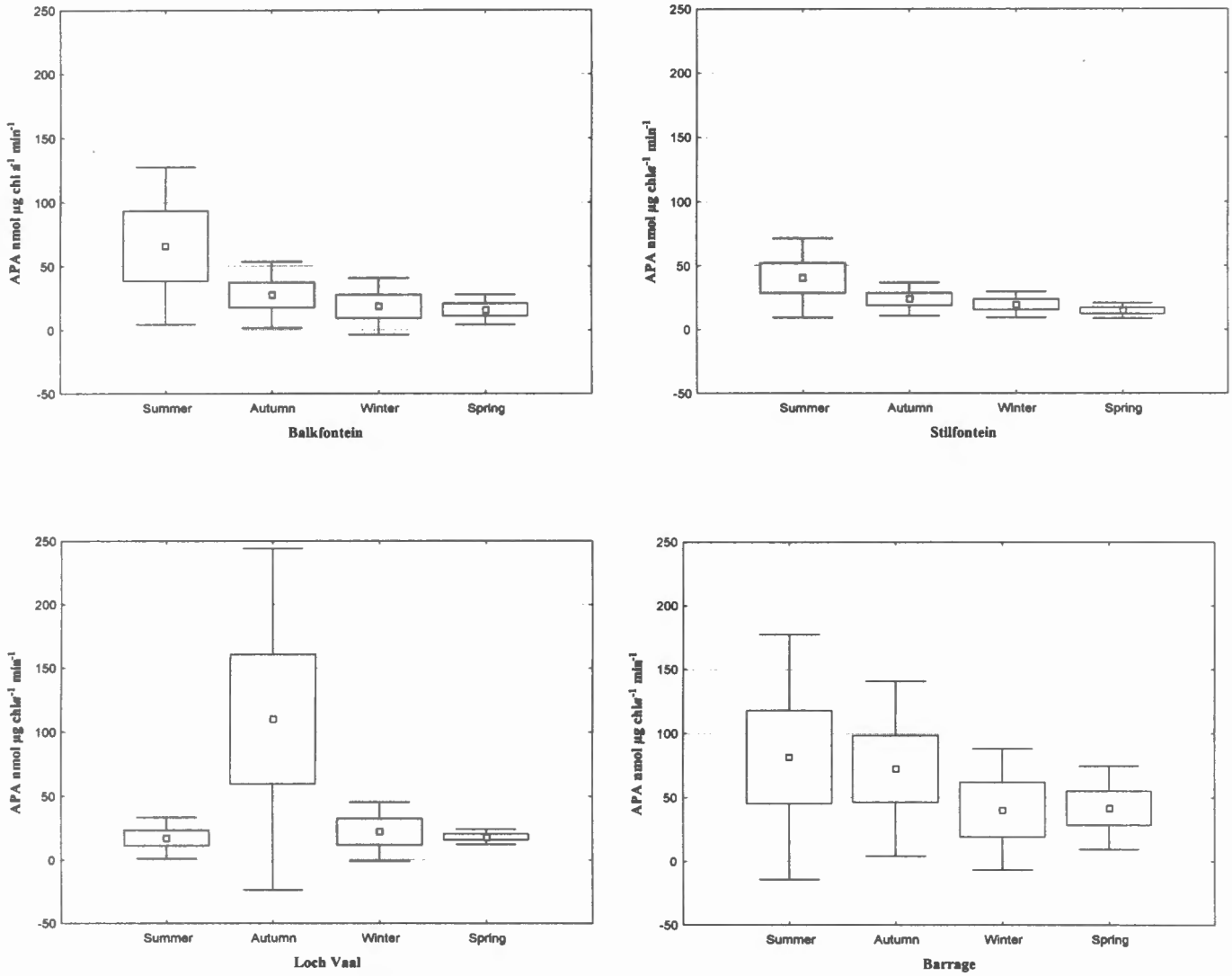


Figure 3.9. Alkaline phosphatase activity in the phytoplanktonic fraction (> 1.2 µm) at the four localities in the Vaal River during February 1999 to March 2001, grouped by season. Middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment. No statistically significant differences could be detected between the seasons (Kruskal Wallis ANOVA $p > 0.05$ in all cases).

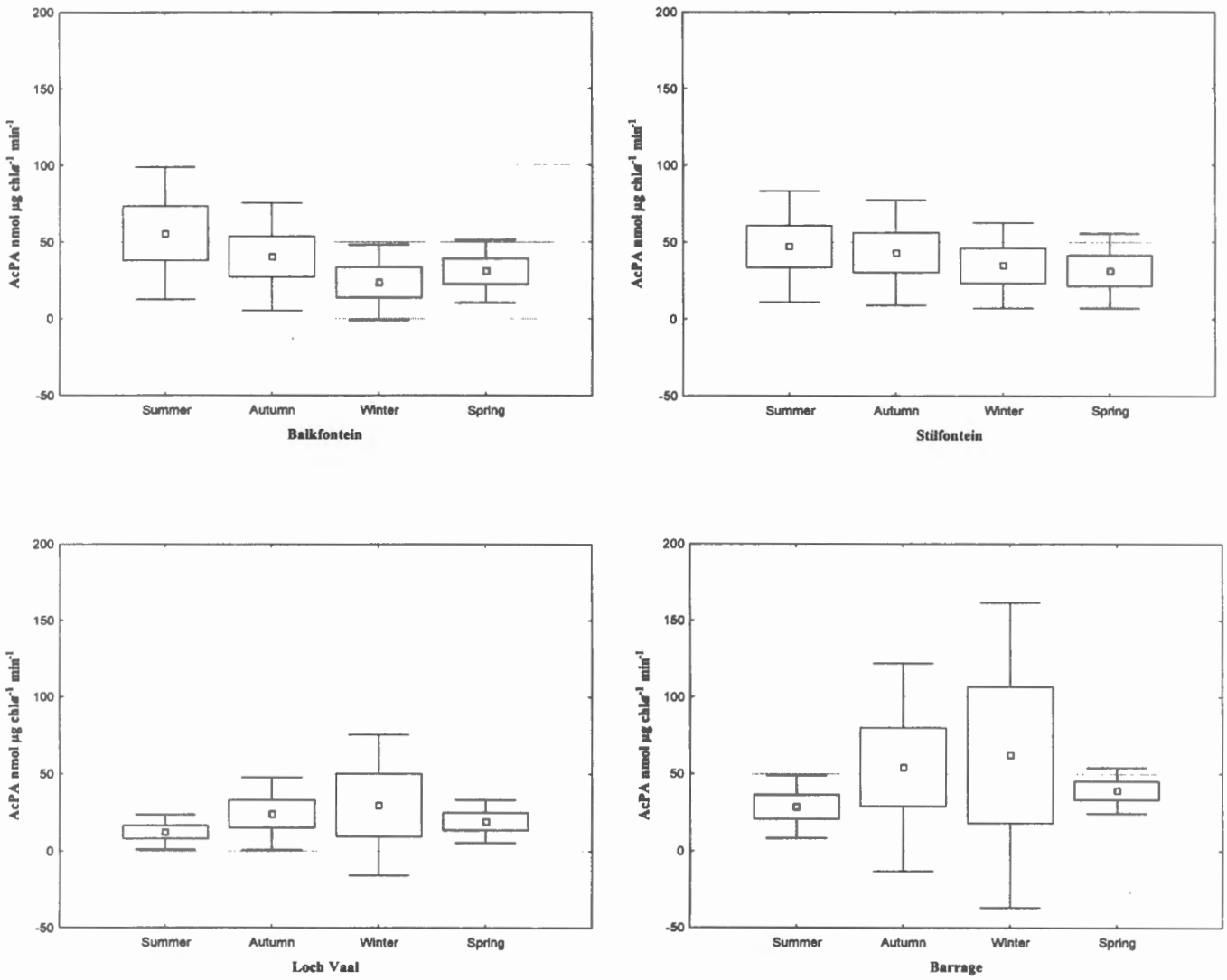


Figure 3.10. Acid phosphatase activity in the phytoplanktonic fraction (> 1.2 µm) at the four localities in the Vaal River during February 1999 to March 2001, grouped by season. Middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment. No statistically significant differences could be detected between the seasons (Kruskal Wallis ANOVA $p > 0.05$ in all cases).

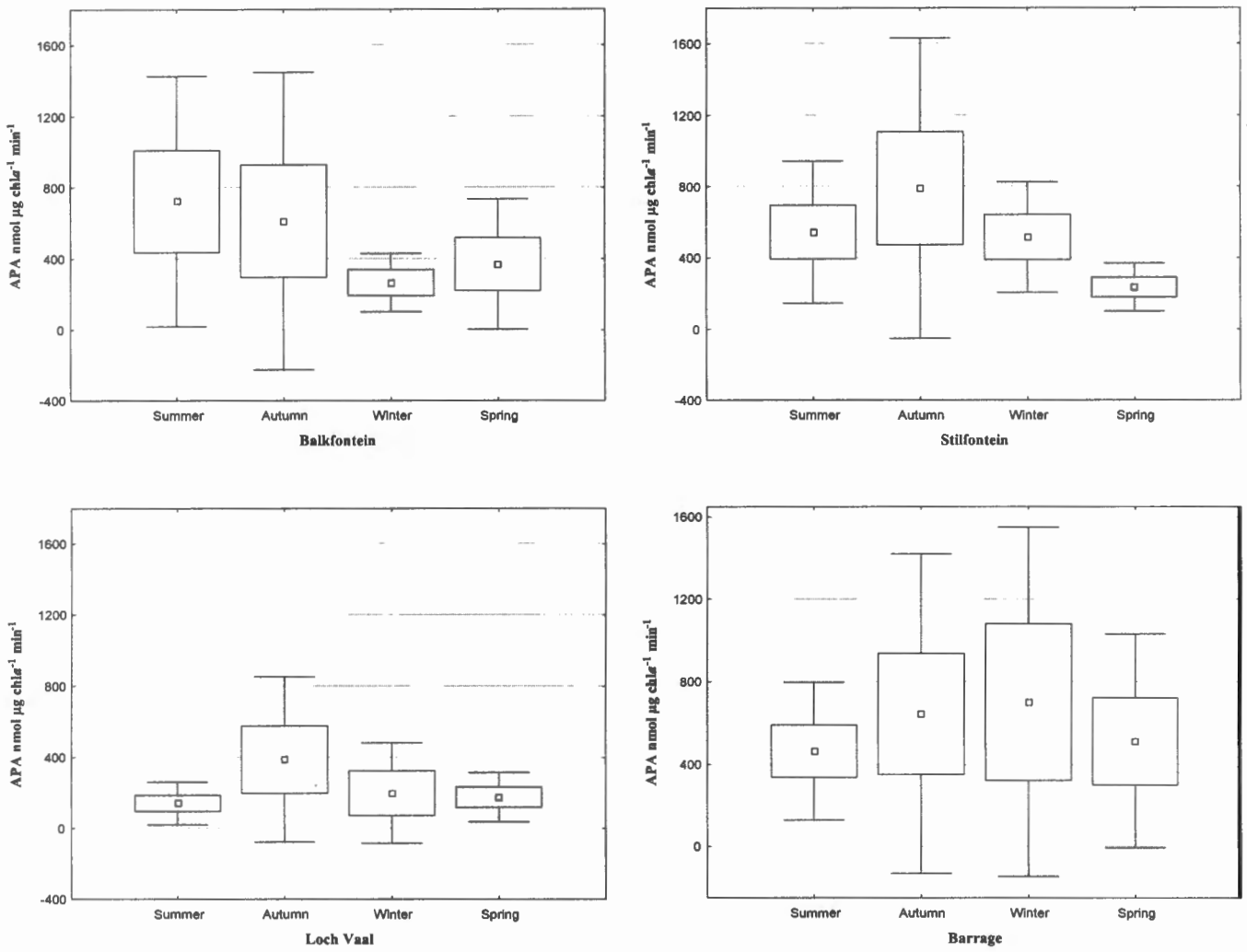


Figure 3.11. Alkaline phosphatase activity in the bacterial / dissolved fraction (< 1.2 µm) at the four localities in the Vaal River during February 1999 to March 2001, grouped by season. Middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment. No statistically significant differences could be detected between the seasons (Kruskal Wallis ANOVA $p > 0.05$ in all cases).

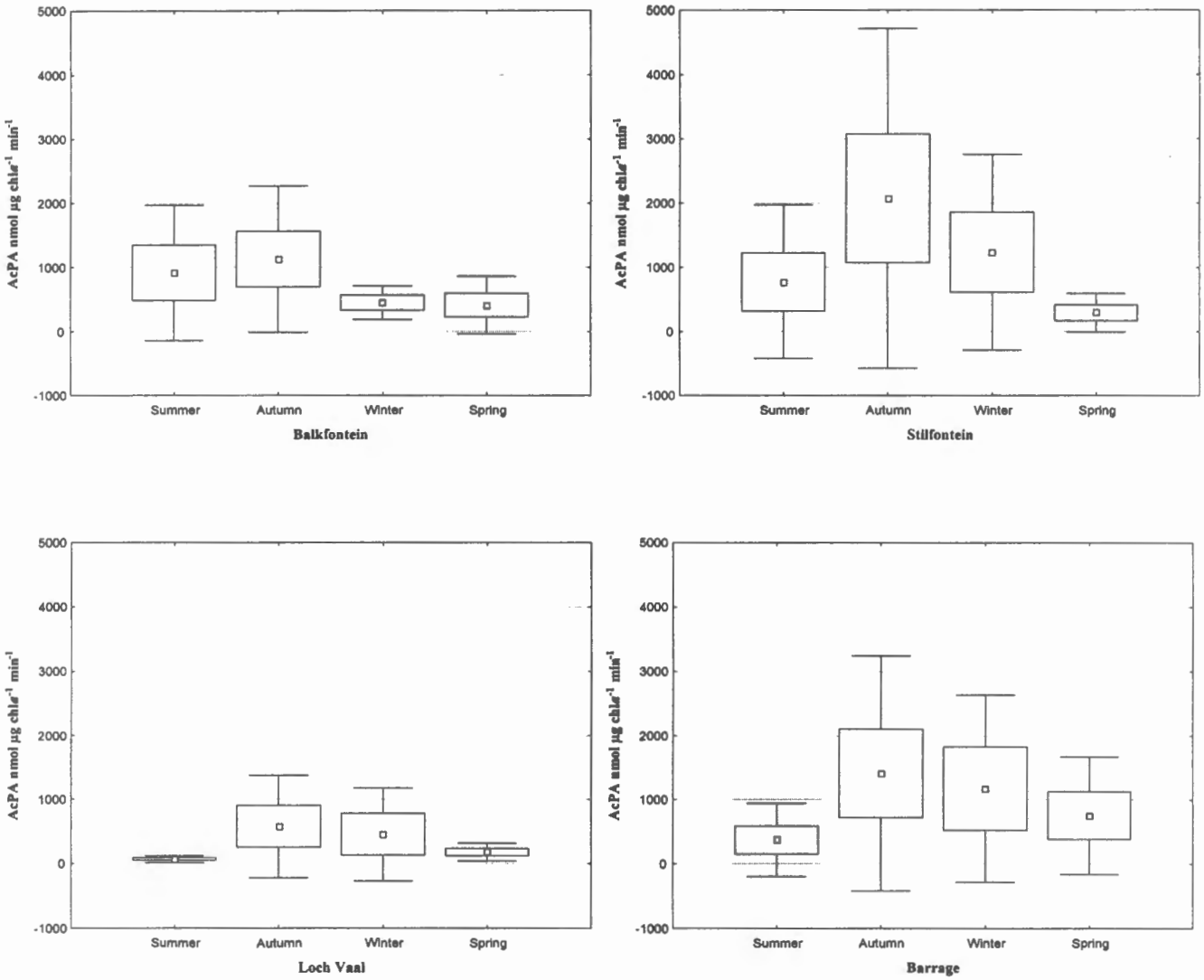
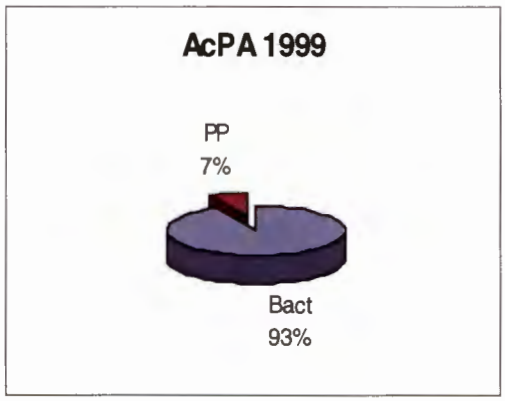
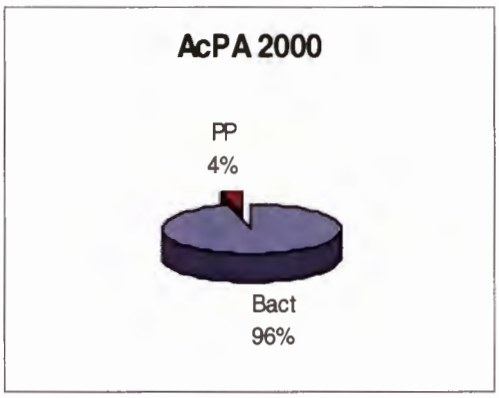
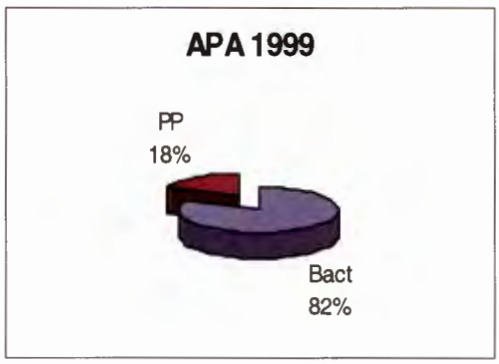
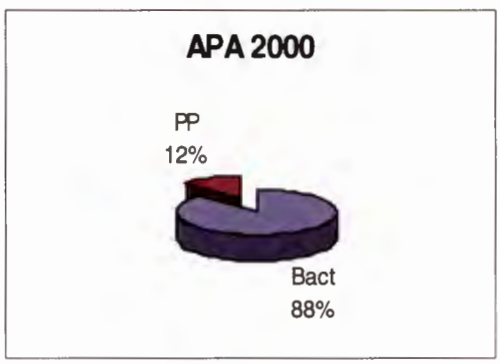


Figure 3.12. Acid phosphatase activity in the bacterial / dissolved fraction (< 1.2 µm) at the four localities in the Vaal River during February 1999 to March 2001, grouped by season. Middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment. No statistically significant differences could be detected between the seasons (Kruskal Wallis ANOVA $p > 0.05$ in all cases).









Year	Enzyme	Fraction	Loch Vaal	Barrage	Balkfontein	Stilfontein
1999	AcPA	<1.2	91.4	94.6	96.2	92.8
		>1.2	8.6	5.4	3.8	7.2
	APA	<1.2	71.3	82.8	90.6	93.8
		>1.2	28.7	17.2	9.4	6.2
2000	AcPA	<1.2	94.0	95.5	94.1	97.4
		>1.2	6.0	4.5	5.9	2.6
	APA	<1.2	89.9	94.2	72.6	96.4
		>1.2	10.1	5.8	27.4	3.6

Figure 3.13. Percentage distribution of acid (AcPA) and alkaline (APA) phosphatase activity accounted for by phytoplankton (>1.2µm, "PP") and bacteria (<1.2 µm "Bact") in the Vaal River, in 1999 and 2000. The fractions at each of the four locations in the river is given separately in the table.

Phosphatase activities were clearly dominated by the dissolved phosphatase enzymes, or phosphatase enzymes associated with bacteria or picoplankton, during the entire study period in the Vaal River.

3.2.2.5. Ratio between specific APA and AcPA

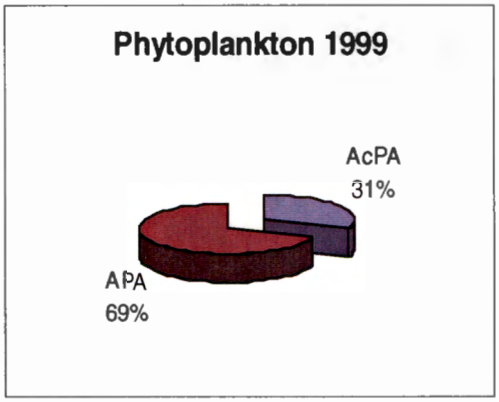
Generally, APA was dominating in the phytoplanktonic fraction, whereas the bacterial / dissolved fraction was dominated by AcPA (Figure 3.14). In 1999 the portion of APA was higher than the mean value at Loch Vaal and Barrage, both in the phytoplanktonic and the bacterial / dissolved fraction. In 2000 Balkfontein had higher APA than the mean, also in both size fractions.

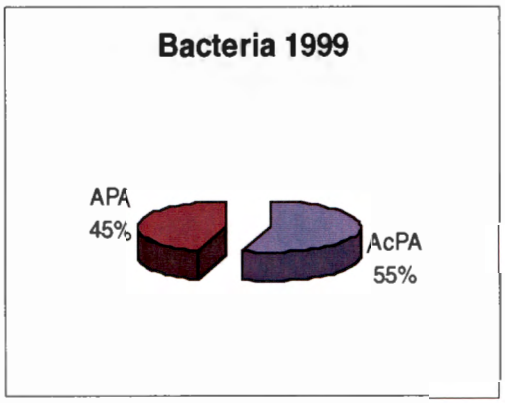
3.2.2.6. Inter-annual variation

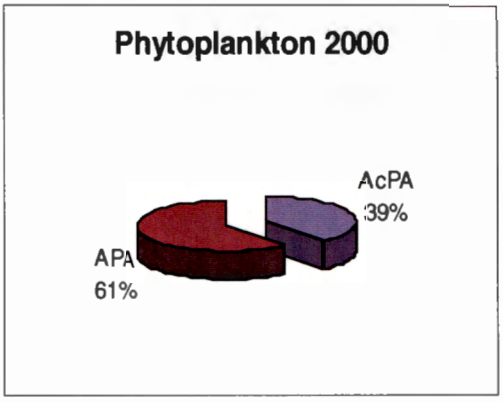
In the phytoplanktonic fraction the APA was at the same level during both years (Figure 3.15), but the AcPA (Figure 3.16) was higher in 2000 at Barrage (Kruskal Wallis ANOVA $p = 0.014$), Stilfontein (Kruskal Wallis ANOVA $p = 0.048$) and Balkfontein (Kruskal Wallis ANOVA $p = 0.04$). In the bacterial / dissolved fraction (Figure 3.17 and 3.18) no differences in the mean enzyme activities were observed, except at the Barrage, where the amount of AcPA was significantly higher in 2000 (Kruskal Wallis ANOVA $p = 0.03$). Thus APA did not vary notably between the years, whereas AcPA in general were higher in 2000 compared to 1999.

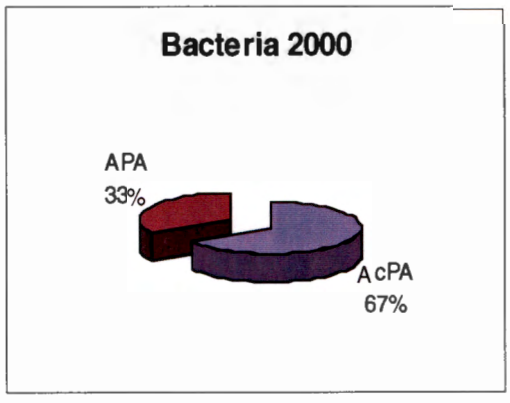
3.2.2.7. Correlation with other environmental variables

Spearman Rank correlation tests were performed between the specific and total APA and AcPA, and the other environmental variables using pooled data from all localities and both years (Table 3.6). In both the phytoplanktonic and the bacterial / dissolved fraction both specific APA and specific AcPA were higher during lower pH and dissolved oxygen values. APA (specific and total) increased with increasing water temperature, but only in the phytoplanktonic fraction. Total APA and AcPA were strongly correlated with chlorophyll-*a* in the phytoplanktonic fraction, indicating higher enzyme activity in more eutrophic conditions. No correlation between PO_4^{-3}, or any other nutrients, and any form of APA or AcPA was recorded during this study in the river.









Year	Enzyme	Fraction	Loch Vaal	Barrage	Balkfontein	Stilfontein
1999	AcPA	<1.2	46.6	50.6	60.7	49.7
		>1.2	16.9	21.9	37.0	53.9
	APA	<1.2	53.4	49.4	39.3	50.3
		>1.2	83.1	78.1	63.0	46.1
2000	AcPA	<1.2	61.5	65.0	59.1	73.0
		>1.2	47.8	58.6	19.4	66.0
	APA	<1.2	38.5	35.0	40.9	27.0
		>1.2	52.2	41.4	80.6	34.0

Figure 3.14. Percentage distribution of acid (AcPA) and alkaline (APA) phosphatase activity in the river for the bacterial (<1.2 µm) and phytoplanktonic (> 1.2 µm) size fractions in 1999 and 2000. The fractions at each of the four locations in the river are given separately in the table.

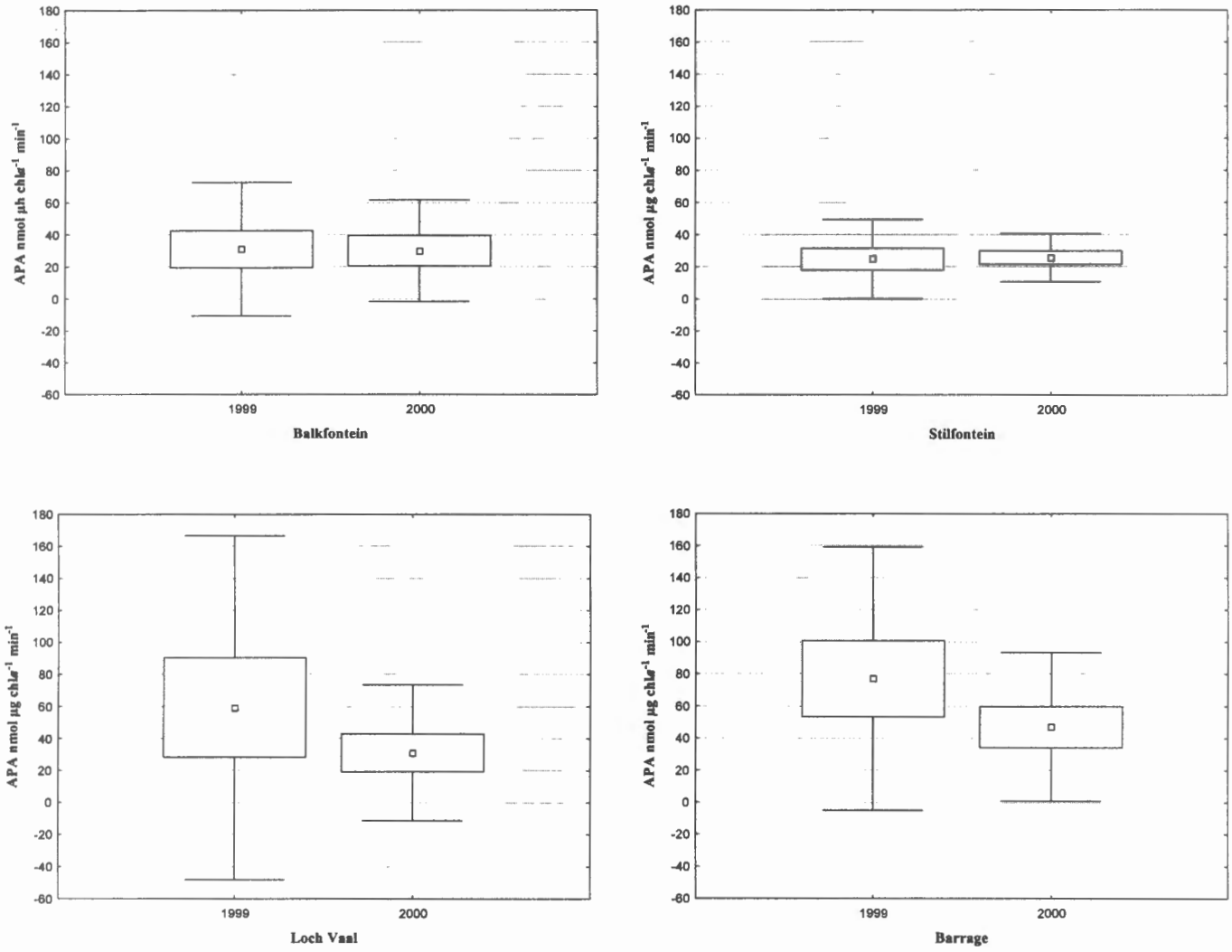


Figure 3.15. Alkaline phosphatase activity at the four different localities in the river in the phytoplanktonic (> 1.2 µm) size fraction in 1999 and 2000. Middle point indicates mean, box value standard error and whisker value standard deviation, n = 12 in each treatment. No statistically significant differences were detected for any of the localities (Kruskal Wallis ANOVA > 0.05 in all cases).

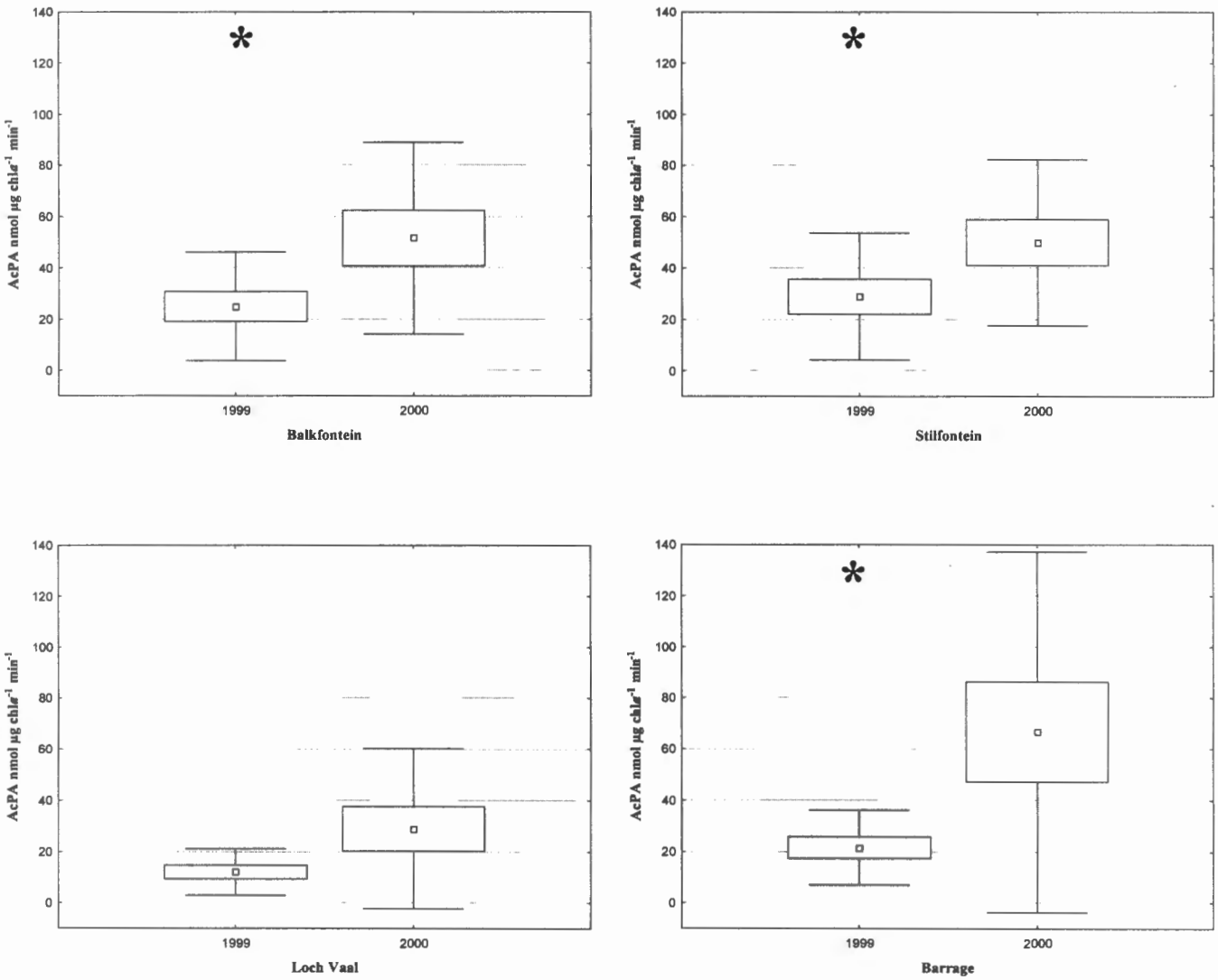


Figure 3.16. Acid phosphatase activity at the four different localities in the river in the phytoplanktonic (> 1.2 µm) size fraction in 1999 and 2000. Middle point indicates mean, box value standard error and whisker value standard deviation, n = 12 in each treatment. Statistically significant differences (Kruskal Wallis ANOVA) are indicated with asterisk (* = significant at $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

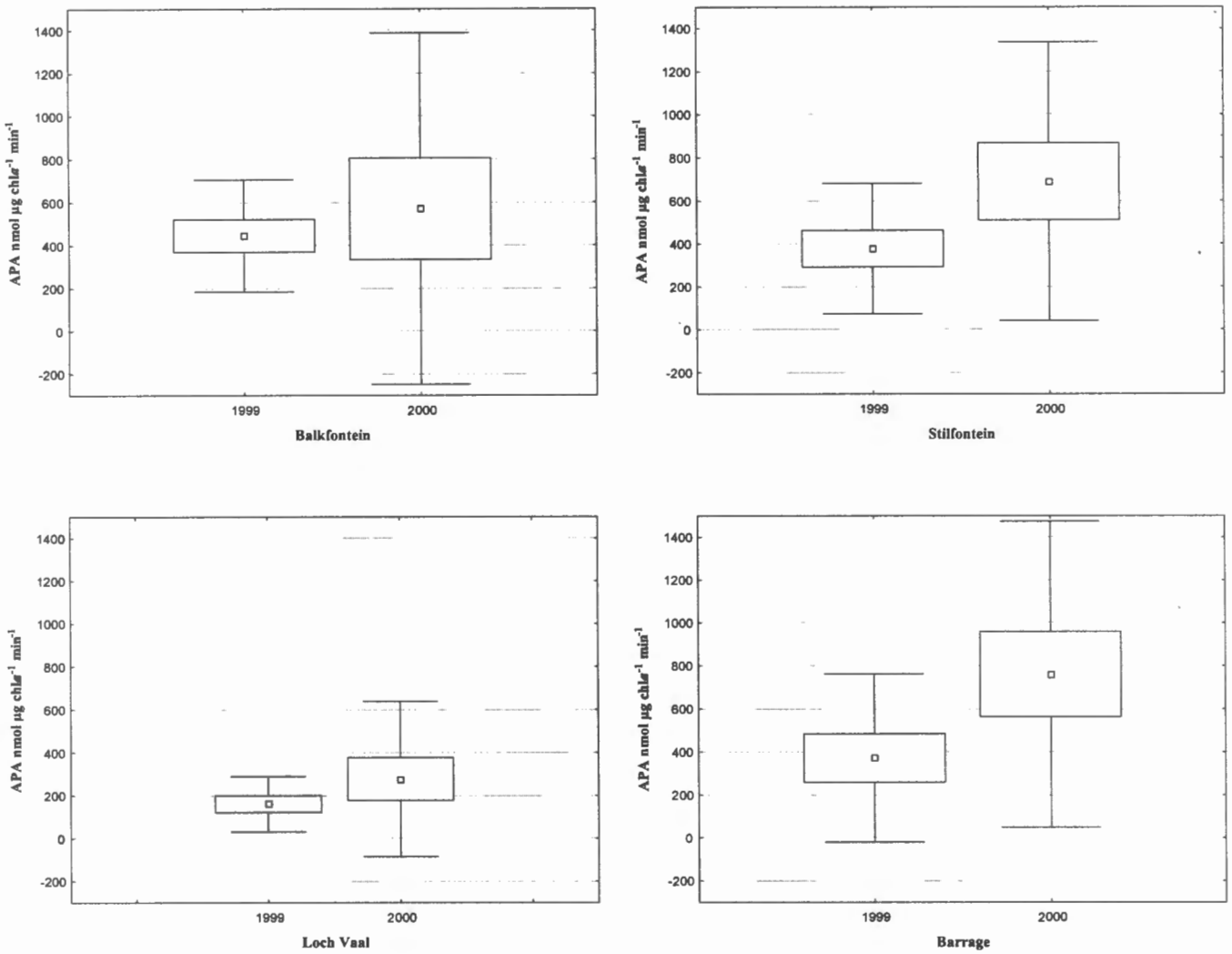


Figure 3.17. Alkaline phosphatase activity at the four different localities in the river in the bacterial / dissolved (< 1.2 µm) size fraction in 1999 and 2000. Middle point indicates mean, box value standard error and whisker value standard deviation, n = 12 in each treatment. No statistically significant differences were detected for any of the localities (Kruskal Wallis ANOVA > 0.05 in all cases).

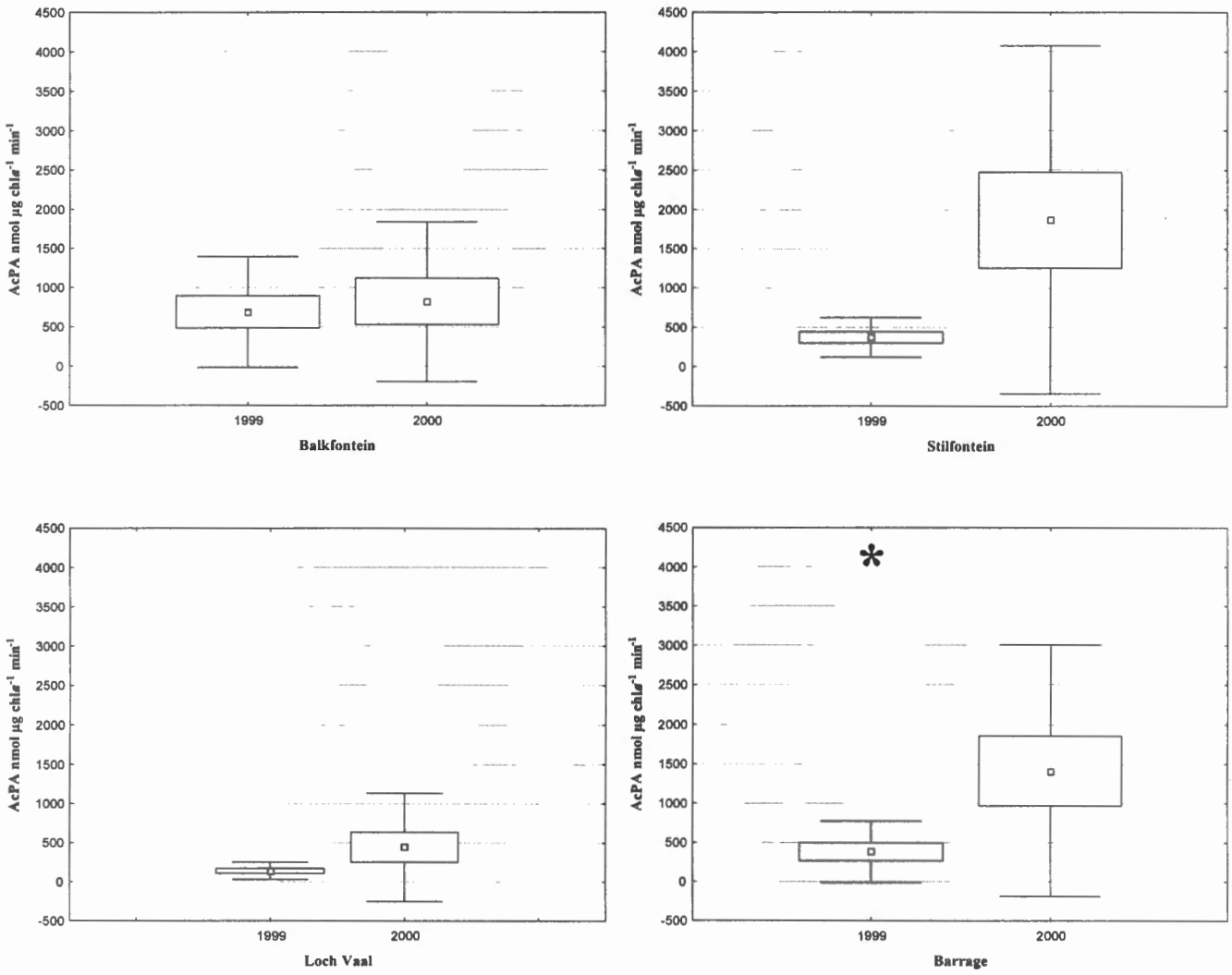


Figure 3.18 Acid phosphatase activity at the four different localities in the river in the bacterial / dissolved (< 1.2 µm) size fraction in 1999 and 2000. Middle point indicates mean, box value standard error and whisker value standard deviation, n = 12 in each treatment. Statistically significant differences (Kruskal Wallis ANOVA) are indicated with asterisk (* = significant at $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

Table 3.6. Correlations between phytoplanktonic (> 1.2 µm) and bacterial / dissolved (< 1.2 µm) specific and total APA and AcPA, and environmental parameters measured at four locations in the river (pooled data). Spearman Rank correlation coefficients (R), p-values and number of observations (n) are given for each locality where the correlation was significant at the p < 0.05 level. Brackets indicate false correlation: chlorophyll-a and specific activities are negatively correlated, as chlorophyll-a has been used for the calculation of specific enzyme activity.

	APA						AcPA					
	Specific			Total			Specific			Total		
	R	p	n	R	p	n	R	p	n	R	p	n
Phytoplanktonic												
pH	-0.330050	0.000849	99	0.311184	0.001625	100	-0.433087	0.000007	100	0.220477	0.027507	100
CHLOROPHYLL	(-0.424239)	0.000011	100	0.607551	0.000000	101	(-0.563320)	0.000000	101	0.587180	0.000000	101
DISSOLVED O_2	-0.390244	0.000156	89				-0.264373	0.011803	90			
TEMPERATURE	0.252311	0.014154	94	0.251719	0.013865	95						
Bacterial / dissolved												
pH	-0.437899	0.000007	98	0.245278	0.014921	98	-0.447087	0.000004	98			
CHLOROPHYLL	(-0.764716)	0.000000	99	0.304131	0.002210	99	(-0.868436)	0.000000	99			
DISSOLVED O_2	-0.285204	0.006747	89				-0.340358	0.001100	89			
TEMPERATURE										-0.465085	0.000003	93

3.2.3. Species composition

Microalgal species composition was determined for samples taken between March 2000 and March 2001 at all localities. The samples used were the same as those from which the environmental and enzyme data was obtained. The main objective of recording the species composition was to determine whether there is any relationship between phytoplanktonic species occurring in the river and the speciation of phosphatase enzymes. To achieve this, species composition data in association with enzyme activity was analysed using multivariate methods (section 3.2.4). In addition, the more conventional comparison of environmental variables with the species composition, using multivariate analysis methods, was also performed.

Seven major algal groups were present in the river during the study period (Table 3.7), Cyanophyceae (cyanobacteria), Bacillariophyceae (diatoms), Chlorophyceae (green algae), Cryptophyceae (cryptophytes), Dinophyceae (dinoflagellates) and Euglenophyceae (euglenophytes). Chrysophyceae (golden algae) were present in small numbers in samples from Barrage, Stilfontein and Balkfontein (data not shown in Table 3.7).

The number of species recorded during the study period was 78 (out of which 11 were only identified to genus level). Cyanophyceae were represented by 5 species and 6 genera that were identified only to genus level. For the Bacillariophyceae only *Melosira granulata* was identified to species level because of complicated species identification techniques within this group, often requiring scanning electron microscopy. Diatoms were therefore registered as either "centric" or "pennate" diatoms. Chlorophyceae had the greatest species diversity during the study, 44 species and 2 genera were identified. In the Cryptophyceae only two "cf" groups (*Cryptomonas major* and *C. minor*) were identified, and in Dinophyceae three species were present. The Euglenophyceae were the second richest in diversity, with 14 species being identified.

Of the Cyanophyceae species *Oscillatoria simplicissima*, *Spirulina* sp. and *Synechocystis* sp. occurred at all localities. The diatom *Melosira granulata* also occurred at all localities, as well as most of the Chlorophytes.

Table 3.7. List of algal species identified from water samples collected at Balkfontein (Balk), Stilfontein (Stil), Loch Vaal (LV) and Barrage (BRG) in 2000, and their presence at the different sampling localities. Column "Unit" indicates whether the species has been quantified by counting cells (cell), filaments (fil) or colonies (col).

	LV	BRG	Stil	Balk	Unit
CYANOPHYCEAE					
Anabaena circinalis Rabenhorst		X		X	fil
Aphanocapsa littoralis Hansgirg		X		X	cell
Aphanocapsa sp. Näg.					cell
Chroococcus sp. Näg.	X	X		X	cell
Cyanodictyon sp. Pascher				X	cell
Microcystis aeruginosa Kützing	X	X		X	col
Oscillatoria simplicissima Gomont	X	X	X	X	fil
Oscillatoria sp. Vaucher		X		X	fil
Spirulina sp. (Nordst.) Gom.	X	X	X	X	fil
Synechococcus cedrorum Sauvageau			X	X	cell
Synechocystis sp. Skuja	X	X	X	X	cell
BACILLARIOPHYCEAE					
Melosira granulata (Ehr.) Ralfs.	X	X	X	X	cell
Asterionella formosa Hass.	X	X			cell
CHOLORPHYCEAE					
Actinastrum hantzschii Wolosz.	X	X	X	X	cell
Ankistrodesmus stipitatus (Chod.) Kom. –Legn.	X				cell
Carteria fornicata Nyg.	X	X	X	X	cell
Carteria simplicissima Pasch.	X	X	X	X	cell
Carteria peterhofiensii Kiss	X	X	X		cell
Characium ensiforme Hermann.	X	X			cell
Characium limneticum Lemm.	X	X	X		cell
Chlamydomonas bicocca Pasch.	X	X	X	X	cell
Chlamydomonas incerta Pasch.	X	X	X	X	cell
Chlamydomonas ulla Skuja	X	X	X	X	cell
Chlorococcum infusionum (Schrank) Menegh.	X		X	X	cell
Closterium cornu Ehr. *ex* Ralfs	X	X	X	X	cell
Coelastrum carpaticum Hind.		X			cell
Coelastrum pseudomicroporum Kors.	X	X	X	X	cell
Crusigenia lauterbornii (Schmidle) Schmidle	X	X	X	X	cell
Crusigenia tetrapedia (Kirsch.) West et West		X	X		cell
Crusigeniella rectangularis (Näg.) Kom.				X	cell
Dictyosphaerium elegans Bachm.	X				cell
Eudorina elegans (Ehr.)	X		X	X	cell
Golenkinia radiata Chod.	X	X		X	cell
Kircheriniella sp. Schmidle			X		cell
Lepocinclis sp. Perty	X	X	X		cell
Micractinium pusillum Fres.	X	X	X	X	cell
Monoraphidium arcuatum (Kors.) Hind.	X	X	X	X	cell

Monoraphidium circinale (Nyg.) Nyg.	X	X	X	X	cell
Monoraphidium griffithii (Berk.) Kom.-Legn.			X	X	cell
Monoraphidium cf. *mirabile* (Nyg.) Kom.-Legn.	X				cell
Oocystis lacustris Chod.	X	X	X	X	cell
Oocystis marsonii Lemm.	X			X	cell
Oocystis pusilla Hansgirg				X	cell
Pandorina morum (Müller) Bory	X	X	X	X	cell
Pediastrum duplex Meyen	X		X	X	cell
Pediastrum simplex (Meyen) Lemm.			X	X	cell
Pediastrum tetras (Ehr.) Ralfs.				X	cell
Pteromonas aculeata var. *lemmermannii* Skuja	X		X		cell
Scenedesmus acuminatus (Lag.) Chodat	X	X	X	X	cell
Scenedesmus disciformis (Chod.) Fott & Kom.	X	X	X	X	cell
Scenedesmus intermedius var. *balatonicus* Hortob.	X	X			cell
Scenedesmus lefevrii Lefév. *et* Bourr.	X	X	X	X	cell
Scenedesmus opoliensis var. *opoliensis* P. Richt.	X	X	X	X	cell
Tetraedron mediocris Hind.	X	X	X	X	cell
Tetraedron planktonica G.M.Smith			X		cell
Tetraedron regulare var. *torsum* (Turner) Brunnthaler				X	cell
Tetrastrum heteracanthum (Nordstedt) Chodat				X	cell
Thorakomonas feldmannii Bourr.	X	X			cell
Trochiscia prescottii Sieminska				X	cell
CRYPTOPHYCEAE					
Cryptomonas cf. major	X	X	X	X	cell
Cryptomonas cf. minor	X	X	X	X	cell
DINOPHYCEAE					
Peridinium penardiforme Lindem.	X	X	X	X	cell
Ceratium hirundinella (O.F.Müller) Schrank	X	X		X	cell
Sphaerodinium ravumfluvium Pieterse *et* Theron	X	X		X	cell
EUGLENOPHYCEAE					
Euglena acus Ehr.				X	cell
Euglena clavata Skuja	X	X			cell
Euglena elastica Prescott	X	X			cell
Euglena hemicromata Skuja	X			X	cell
Euglena pusilla var *longa* Playf.	X	X			cell
Phacus acuminatus Stokes	X				cell
Phacus orbicularis Hübner		X			cell
Phacus pyrum (Her.) Stein				X	cell
Strombomonas fluviatilis (Lemm.) Defl.	X	X	X	X	cell
Strombomonas jaculata (Palmer) Defl.	X				cell
Strombomonas verrucosa var. *borystheniensis* (Roll.) Defl.				X	cell
Trachelomonas hispida (Perty) Stein *emend* Defl.	X	X	X	X	cell
Trachelomonas intermedia Dangeard	X				cell
Trachelomonas scabra Playf.	X			X	cell

Species only occurring at Loch Vaal were the Chlorophytes *Ankistrodesmus stipitatus*, *Dictyosphaerium elegans*, *Monoraphidium* cf. *mirabile*, and the Euglenophytes *Phacus acuminatus*, *Strombomonas jaculata* and *Trachelomonas intermedia*. Species only occurring at the Barrage were *Coelastrum carpaticum* (Chlorophyceae) and *Phacus orbicularis* (Euglenophyeae), and at Stilfontein *Kircheriniella* sp. and *Tetraedron planctonica* (Chlorophyceae). Balkfontein had the greatest species diversity observed, with *Oocystis pusilla*, *Pediastrum tetras*, *Tetraedron regulare*, *Tetrastrum heteracanthum*, *Trochiscia prescottii*, *Euglena acus*, *Phacus pyrum* and *Strombomonas verrucosa* being species observed only at this locality. The fact that these species were not recorded at the other sampling localities will naturally not rule out their potential occurrence – it may be that their occurrence was just more scarce at the other localities.

The sampling localities with the greatest species diversity were Loch Vaal and Balkfontein (56 and 55 species respectively), the least species were found at Stilfontein (41).

At all sampling localities Bacillariophyceae and Chlorophyceae dominated the phytoplankton (Figure 3.19). At Balkfontein, the percentile fraction of Bacillariophyceae was much less than at the other localities, whereas the fraction of Chlorophyceae was clearly higher. The percentile fraction of Cyanophyceae was nearly 20% at Loch Vaal and Stilfontein, compared to approximately 10% at Barrage and Balkfontein (Figure 3.19). A small percentage of the total phytoplanktonic cell numbers was composed of Dinophyceae, the highest percentage being recorded at Stilfontein (1.6%) and at Balkfontein (0.7%). In Loch Vaal and Barrage the dinoflagellate fraction was mainly comprised of *Ceratium hirundinella* in April 2001, forming a mass-occurrence in combination with the cyanobacteria *Oscillatoria simplicissima*, *Spirulina* sp. and *Microcystis aeruginosa*. Cyanophyceae mainly occurred during warm summer months (Figure 3.20) while diatoms dominated during winter and spring. Green algae were present throughout the year, but developed the highest numbers during spring.

In Table 3.8 dominant species (occurring in numbers exceeding 30% of the total cell number) are presented. Centric diatoms had the highest incidence of dominating the species composition, being dominant in 23 samples (of the 77 samples).

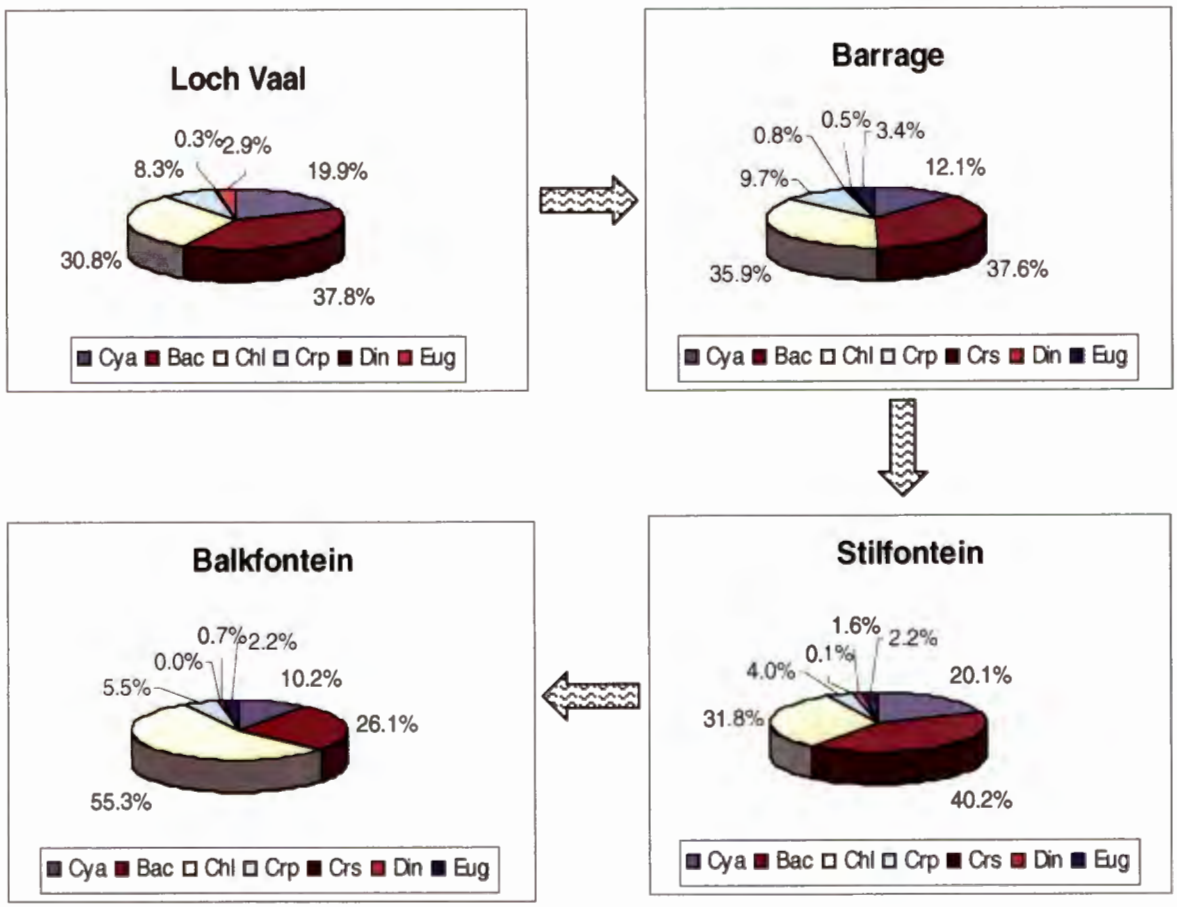


Figure 3.19. Percentage composition (based on cell counts) of algal groups at the different localities in the Vaal River in 2000. Arrows indicate downstream direction. (Cya = Cyanophyceae, Bac = Bacillariophyceae, Chl = Chlorophyceae, Crp = Cryptophyceae, rs = Chrysophyceae, Din = Dinophyceae, Eug = Euglenophyceae).

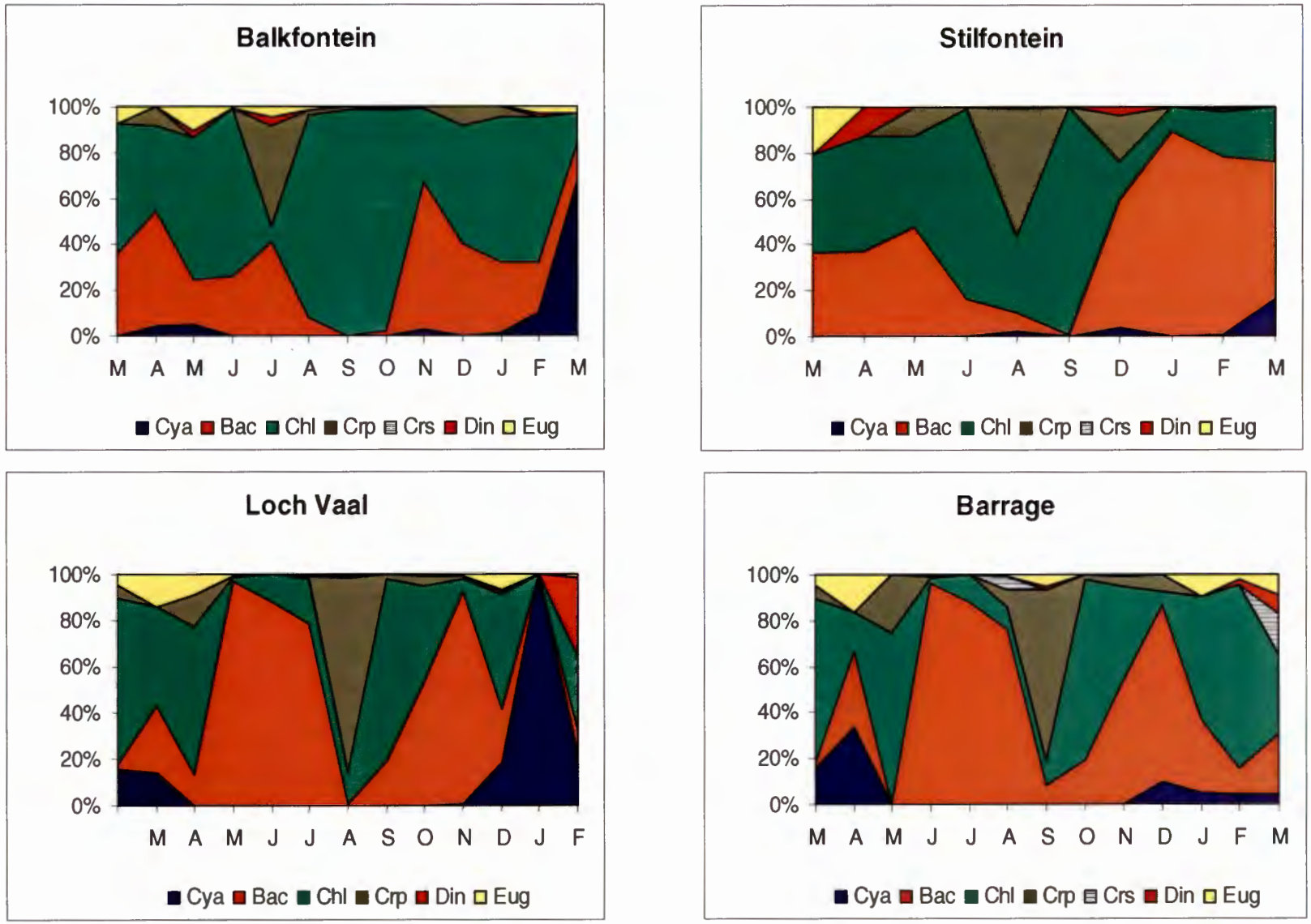


Figure 3.20 Percentile variation in the phytoplankton composition by major taxonomic group at four locations in the Vaal River during 2000. Note that data is missing for Stilfontein in June, October and November. Chrysophyceae were not detected at all sampling locations. (Cya = Cyanophyceae, Bac = Bacillariophyceae, Chl = Chlorophyceae, Crp = Cryptophyceae, Crs = Chrysophyceae, Din = Dinophyceae, Eug = Euglenophyceae.)

Table 3.8. Dominating (≥30% of algal count) phytoplanktonic species or groups in the Vaal River, and their number of occurrences. LV = Loch Vaal (data for 1999 and 2000), BRG = Barrage (data for 1999 and 2000), Stil = Stilfontein (data for 2000 only) and Balk = Balkfontein (data for 2000 only).

	LV	BRG	STIL	BALK	TOT
CYANOPHYCEAE					
Microcystis aeruginosa	3	2		1	6
Oscillatoria simplicissima	4	3	1	1	9
Spirulina sp.	1				1
Chroococcus sp.		1			1
Total Cyanophyceae					**17**
BACILLARIOPHYCEAE					
Centric diatoms	9	8	4	4	25
Melosira granulata	1	4	1		6
Total Bacillariophyceae					**31**
CHOLORPHYCEAE					
Chlamydomonas bicocca		1			1
Chlamydomonas incerta	1		1		2
Micractinium pusillum	1	1			2
Monoraphidium arcuatum		1			1
Monoraphidium circinale		1		4	5
Monoraphidium griffithii			1		1
Monoraphidium cf. *mirabile*				1	1
Oocystis lacustris		2			2
Pandorina morum				1	1
Scenedesmus opoliensis var. *opoliensis*			1		1
Thorakomonas feldmannii		1			1
Trochiscia prescottii				3	3
Total Chlorophyceae					**21**
CRYPTOPHYCEAE					
Cryptomonas cf. major	2	2	1		5
Total Cryptophyceae					**5**
DINOPHYCEAE					
Ceratium hirundinella	1				1
Total Dinophyceae					**1**

The diatom *Melosira granulata* was dominant in a further 6 samples – thus the diatoms dominated in 39% of all samples counted. The cyanobacteria *Oscillatoria simplicissima* and *Microcystis aeruginosa* were dominant in 7 and 6 samples, respectively. A variety of green algae reached dominating numbers, *Monoraphidium circinale* being present in high numbers in 5 samples. *Cryptomonas* cf. *major* also dominated in 5 samples. *Ceratium hirundinella*, which was observed at these localities for the first time, was the only dinoflagellate to become dominant and then only at Loch Vaal in late March 2001.

3.2.4. Multivariate analysis

3.2.4.1. Principal Component Analyses

Principal Component Analyses (PCAs) were performed on environmental and enzyme data to detect relationships and potential collinearity between

1. environmental variables and
2. different enzyme "types".

Only environmental variables that were measured at all localities were included in the analyses, and chlorophyll-*a* concentrations were also included (Table 3.9). Because most data-sets did not follow the normal distribution, all variables were normalized using the $y' = ln\ (y + 1)$ transformation. Abbreviations for environmental, enzyme and phytoplankton data are given in Table 3.9.

Correlation between different environmental variables is shown in Figure 3.21, with a biplot ordination including sampling localities. NO_3^- and N:P ratios had the greatest influence on the first ordination axis, whereas conductivity, chlorophyll-*a*, alkalinity and pH were associated with the second ordination axis. Chlorophyll-*a*, alkalinity and pH were positively correlated, indicating higher pH during higher primary productivity. Temperature and dissolved oxygen were positively correlated, probably also a consequence of higher primary production at warmer temperatures. No clear negative correlations were found, with the possible exception of temperature and N:P (higher N:P ratios occurring in winter months), turbidity and PO_4^{3-}, and ammonium and dissolved oxygen.

Table 3.9. List of abbreviations and units of measurement used for environmental variables and microalgal species and higher taxonomic groups in ordination diagrams.

DESCRIPTION	UNIT	ABBREVIATION
ENVIRONMENTAL VARIABLES		
Turbidity	NTU	Turb
Temperature	°C	Temp
Conductivity	mS m^{-1}	Cond
Ortho-phosphate / PO_4	µmol l^{-1}	PO4
Nitrate / NO_3	µmol l^{-1}	NO3
Nitrate:Phosphate ratio / N:P molar ratio		N:P
Ammonium / NH_4	µmol l^{-1}	NH4
pH		pH
Alkalinity $CaCO_3$	mg l^{-1}	Alk
Dissolved oxygen	mg l^{-1}	O2
Chlorophyll-*a*	µg l^{-1}	Chl
ENZYMES		
Specific		
Alkaline Phosphatase Activity - > 1.2 µm	nmol µg chl-*a*$^{-1}$ min^{-1}	APA
Acid Phosphatase Activity- > 1.2 µm	as above	AcPA
Alkaline Phosphatase Activity - < 1.2 µm	as above	APAb
Alkaline Phosphatase Activity - < 1.2 µm	as above	AcPAb
Total		
Alkaline Phosphatase Activity - > 1.2 µm	µmol l^{-1} min^{-1}	TAPA
Acid Phosphatase Activity- > 1.2 µm	as above	TAcPA
Alkaline Phosphatase Activity - < 1.2 µm	as above	TAPAb
Alkaline Phosphatase Activity - < 1.2 µm	as above	TAcPAb
Ratios		
Alkaline:Acid phosphatase activity - > 1.2 µm		AP:AcP
Alkaline:Acid phosphatase activity < 1.2 µm		APb:AcPb
ALGAE		
Cyanophyceae		**Cyano**
Microcystis aeruginosa		Micro
Oscillatoria simplicissima		Osci
Spirulina sp		Spir
Anabaena circinalis		Anab
Chroococus dispersus		Chro
Baciallariophyceae		**Bacil**
Centric diatoms		Cent
Melosira granulata		Melo
Pennate diatoms		Penn

DESCRIPTION	UNIT	ABBREVIATION
Chlorophyceae		**Chlor**
Actinastrum hantzchii		Acti
Carteria spp.		Cart
Characium spp.		Char
Chlamydomonas spp.		Chla
Closterium cornu		Clos
Coelastrum spp.		Coel
Crusigenia spp.		Crus
Eudorina elegans		Eudo
Micractinium pusillum		Micra
Monoraphidium spp.		Mono
Oocystis spp.		Oocy
Pandorina morum		Pand
Pediastrum spp		Pedi
Scenedesmus spp.		Scen
Tetraedron spp.		Tetre
Tetrastrum spp.		Tetra
Thorakomonas feldmannii		Thor
Trochischia prescotii		Troc
Cryptophyceae		**Crypt**
Cryptomonas major		CryMa
Cryptomonas minor		CryMi
Dinophyceae		**Dino**
Peridinium penardiforme		Peri
Ceratium hirundinella		Cera
Euglenophyceae		**Eugle**
Euglena spp.		Eugl
Phacus spp.		Phac
Strombononas spp.		Stro
Trachelomonas spp.		Trac

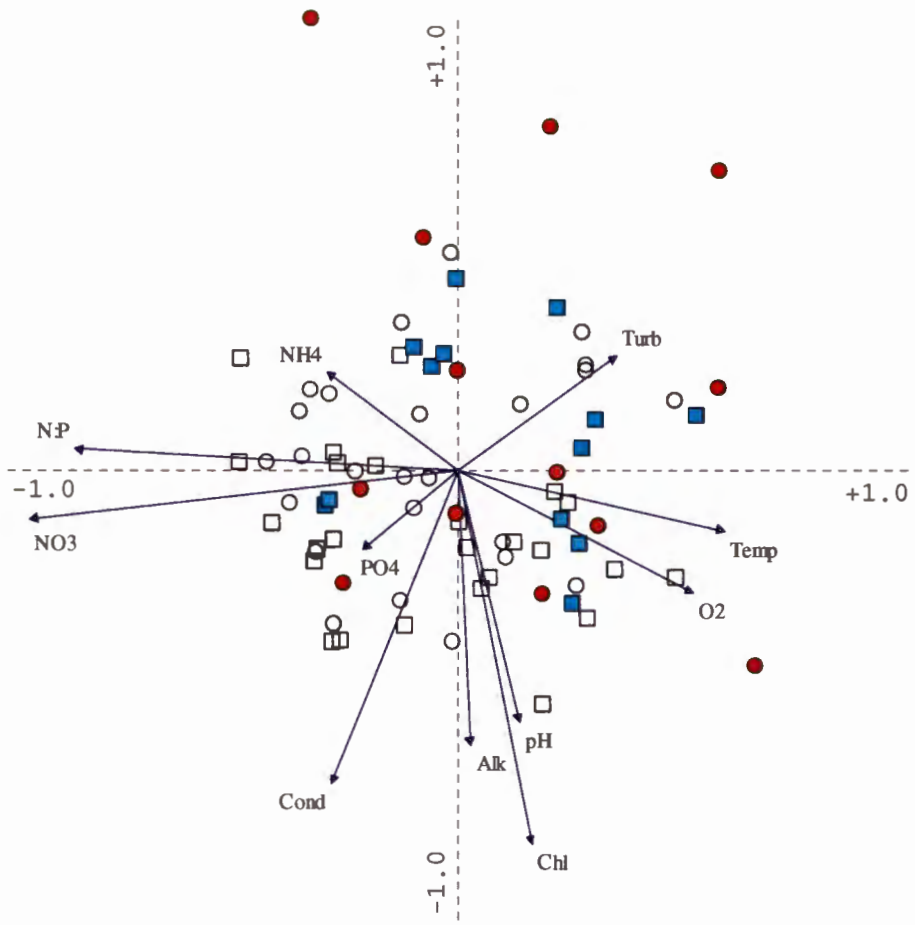


Figure 3.21. PCA ordination biplot showing sampling localities and environmental variables measured at Loch Vaal (open square), Barrage (open circle), Stilfontein (filled square) and Balkfontein (filled circle). Explanations for abbreviations used are given in Table 3.9.

Table 3.10. *Results from the PCA analysis for environmental variables.*

Axes	1	2	3	4	Total variance
Eigenvalues	0.317	0.150	0.121	0.108	1.000
Cumulative % variance of environmental data	31.7	50.7	62.8	73.7	
Sum of all unconstrained eigenvalues					1.000

Sampling localities were scattered homogeneously in the ordination, except for Loch Vaal, which grouped mostly in the two lower quadrants reflecting higher nutrient concentrations as well as higher conductivity, alkalinity, pH and chlorophyll-*a* concentrations. No collinearity could be detected, and thus all environmental variables were used in the RDA analyses.

The eigenvalue for the first ordination axis was 0.317, indicating strong gradients in the data (Table 3.10). The first axis explained approximately 32% of the variation in the data, whereas the two first axes explained approximately half of the variation in the environmental data.

In Figure 3.22 enzyme types (treated similarly to environmental variables) are shown in a biplot ordination. From the ordination one can see that all acid phosphatase fractions were strongly associated with the first ordination axis, and correlated positively with each other, except for total AcPA, which was negatively correlated with the other AcPA fractions. All alkaline phosphatase fractions were associated with the second ordination axis, but the correlation between the different APA types were not as strongly correlated.

Interestingly, the spatial grouping of the sampling locations shows that the riverine localities were mostly situated in the two lower quadrants of the ordination diagram. This suggests generally lower AP activities found at samples taken from the riverine localities compared to the other localities (also seen in Figure 3.6). The shallow-lake-type localities were more scattered over the ordination diagram. In several instances, Loch Vaal was strongly associated with APA:AcPA ratio, APA or total APA.

The eigenvalue for the first ordination axis was 0.619, indicating very strong gradients in the data (Table 3.11). The first axis alone explained more than half of the variation in the data. The two first axes explained more than 80% of the variation. The strong gradients are probably partly an artifact originating from the inter-related nature of the physiological enzyme data, but does give an indication of the interrelationships.

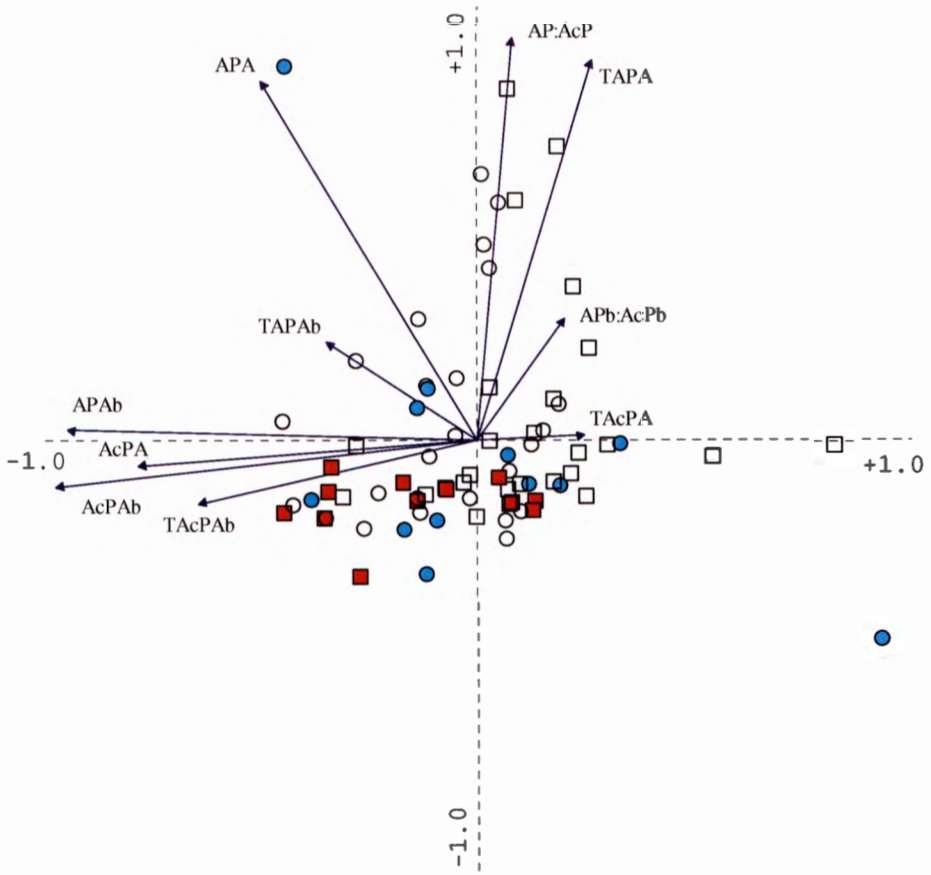


Figure 3.22. PCA ordination biplot showing sampling localities and enzyme types measured at Loch Vaal (open square), Barrage (open circle), Stilfontein (filled square) and Balkfontein (filled circle). Explanations for abbreviations used are given in Table 3.9.

Table 3.11. Results from the PCA analysis for enzyme type.

Axes	1	2	3	4	Total variance
Eigenvalues	0.619	0.184	0.071	0.064	1.000
Cumulative % variance of environmental data	61.9	80.3	87.5	93.8	
Sum of all unconstrained eigenvalues					1.000

3.2.4.2. Detrended Correspondence Analyses

Species abundance data was first analysed using Detrended Correspondence Analyses (DCA), which is comparable to PCA for environmental data and searches for major gradients in the species data. DCA determines the length of the gradients, which will give an indication of which multivariate technique will be suitable to apply to the data.

Two DCAs were done, the first one using only major phytoplankton groups and the second including all algal genera present in more than 5% of all samples (see Table 3.9 for species, groups and abbreviations).

In Figure 3.23 the arrangement of the major taxonomic groups in the ordination diagram can be seen. All taxonomic groups were situated in the upper right quadrant of the ordination diagram. Euglenophytes, Chlorophytes and diatoms grouped relatively close to each other, suggesting similarity between these groups, whereas dinoflagellates, Cryptophytes and cyanobacteria were situated far apart. Typical of these groups was infrequent, but intensive mass-occurrences in the river, and therefore their position on the ordination diagram may be influenced by few outlier values.

The eigenvalues for the axes were low, < 0.3 for all axes. Nevertheless, the two first axes explained more than half of the variation in the species data. The length of the gradients marginally exceeded 2.0, therefore both redundancy analysis (RDA) and canonical correspondence analysis (CCA) can be used for further analyses of the data (Ter Braak and Šmilauer 1998).

Figure 3.24 illustrates the grouping of different genera in the DCA ordination diagram. All genera occurred in the two right hand quadrants of the ordination diagram. The cyanobacteria seem to be associated with the second ordination axis. All other genera were homogeneously distributed over especially the top quadrant, with only a few species in the lower quadrant. A "wedge-effect" can be seen in the data (toward the right pointing arrow shape of the data), indicating that both the 1st and the 2nd axes have a strong influence on the species present (Ter Braak and Šmilauer 1998).

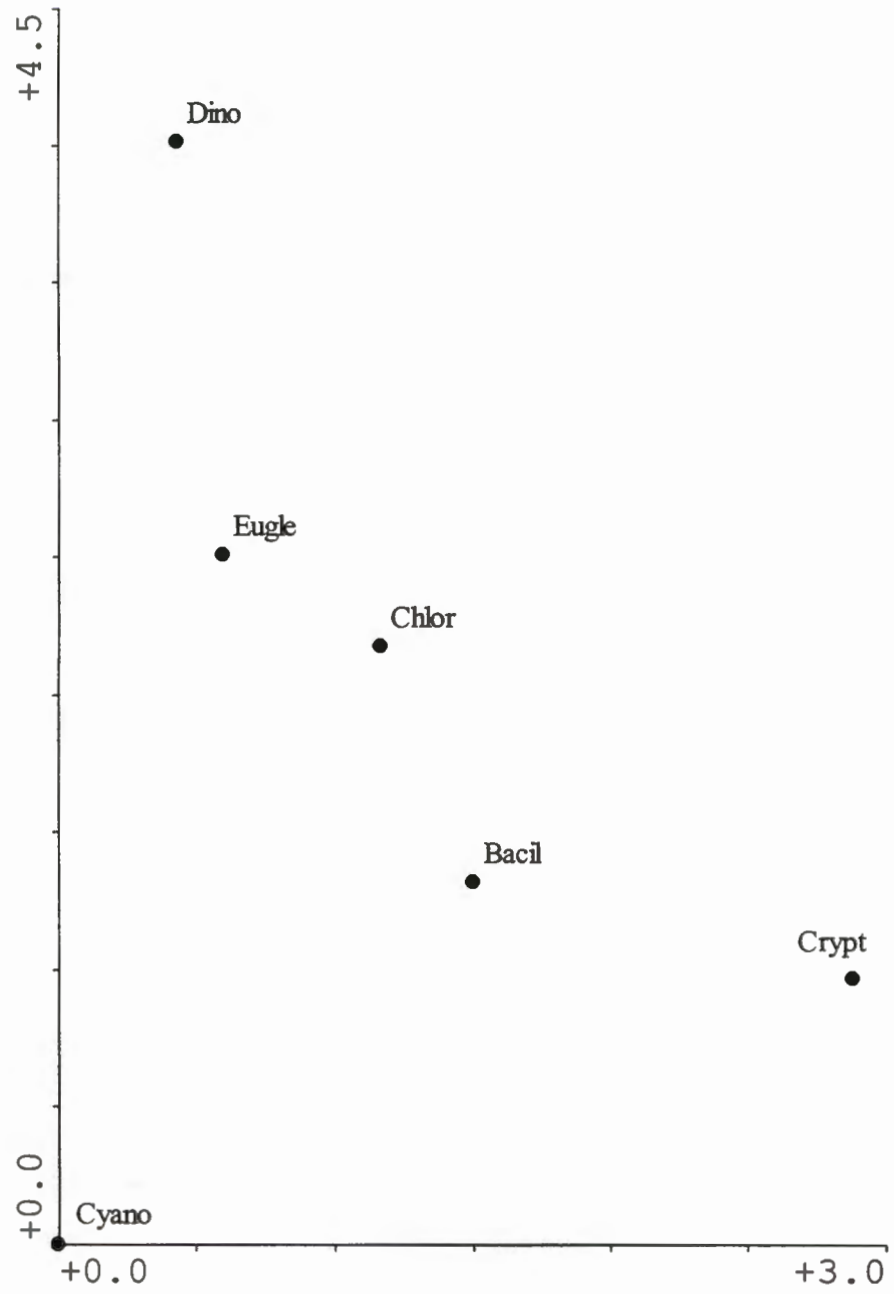


Figure 3.23. DCA ordination scatter plot showing major taxonomic algal groups. Explanations for abbreviations used are given in Table 3.9.

Table 3.12. Results from the DCA analysis for algal data including only major taxonomic groups.

Axes	1	2	3	4	Total inertia
Eigenvalues	0.281	0.114	0.089	0.065	0.7
Length of gradient	2.070	2.339	1.563	1.320	
Cumulative % variance of species data	40.2	56.5	69.3	78.6	
Sum of all unconstrained eigenvalues					0.7

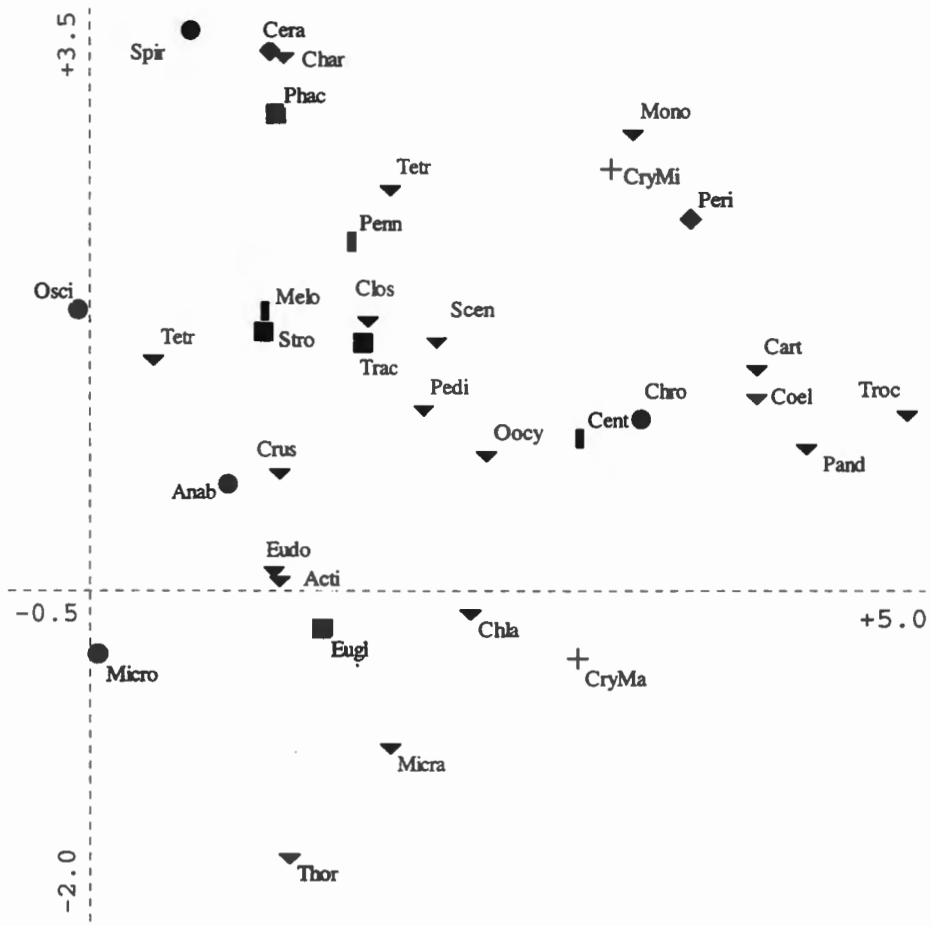


Figure 3.24. DCA ordination scatter plot showing algal genera present in more than 5% of the samples. Members of cyanobacteria are marked with circles, diatoms with boxes, chlorophytes with triangles, cryptophytes with crosses, dinoflagellates with diamonds and euglenophytes with squares. Explanations for abbreviations used are given in Table 3.9.

Table 3.13. *Results from the DCA analysis for algal groups present in more than 5% of the samples.*

Axes	1	2	3	4	Total inertia
Eigenvalues	0.458	0.333	0.192	0.155	3.961
Length of gradient	3.652	2.861	2.425	2.418	
Cumulative % variance of species data	11.6	20	24.8	28.7	
Sum of all unconstrained eigenvalues					3.961

The eigenvalues (Table 3.13) were rather high for the two first axes, however, the four first axes together could only explain 29% of the variation in the data. For abundance data these percentages are usually quite low, due to "noisy" species data (Ter Braak and Šmilauer 1998).

The lengths of the gradients were over 2.0 – suggesting that CCA analyses may be suitable for the data. Because many other studies have used RDA rather than CCA, RDA results have been presented in this chapter. CCAs were also performed, however, all main conclusions remained the same, and therefore further presentation of CCA results have been omitted from this chapter.

3.2.4.3. Redundancy Analyses

Redundancy Analyses (RDAs) were chosen as the multivariate analysis tool to use to perform the ordinations on environmental and species abundance data. Enzyme data was treated as environmental data and analysed in combination with the species data, to see possible interaction between enzyme speciation and phytoplanktonic species.

In Figure 3.25 the ordination of the environmental variables and the algal groups is displayed. The most important environmental variables (indicated by the length of the arrow) were conductivity and turbidity on the first axis, as well as NO_3^-, alkalinity and pH. Genera grouped close together were those belonging to green algae and euglenophytes, and diatoms and cryptophytes. These groups are therefore similar in their response to the environment. Cyanobacteria correlated positively with N:P ratios and temperature, indicating higher cyanobacterial biomass in warmer seasons and higher N:P ratios. Euglenophytes correlated positively with turbidity, and negatively with conductivity, indicating preference to fresh water with lower ion concentrations. Green algae correlated positively with NH_4^+ concentrations, and negatively with conductivity. Diatoms were negatively correlated with N:P ratios, and weakly positively correlated with oxygen concentration.

Algal groups with long arrows in the ordination diagram have a greater proportion of their variability explained by the measured environmental variables – the groups having the longest arrows in this study were the cyanobacteria, euglenophytes and green algae, whereas dinoflagellates and cryptophytes had the shortest arrows.

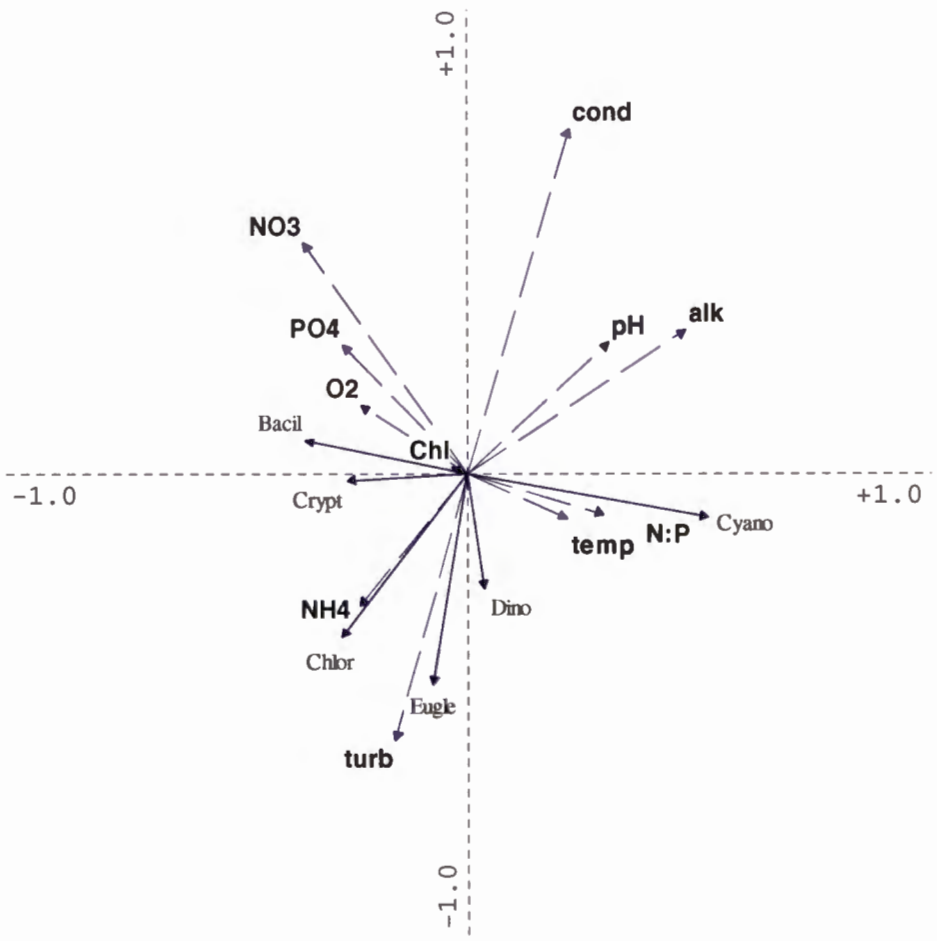


Figure 3.25. RDA ordination biplot showing environmental variables and major algal groups. Explanations for abbreviations used are given in Table 3.9.

Table 3.14. *Results from the RDA analysis for environmental variables and major algal groups.*

Axes	1	2	3	4	Total variance
Eigenvalues	0.142	0.046	0.033	0.009	1.000
Species-environment correlations	0.551	0.544	0.434	0.413	
Cumulative % variance of species data of species-environment relation	 14.2 59.9	 18.8 79.2	 22.1 93.1	 23.1 97.0	
Sum of all unconstrained eigenvalues					1.000
Sum of all canonical eigenvalues					0.238

In Table 3.14 the results from the RDA on environmental data and major taxonomic algal groups is shown. The first four canonical axes explained 23.1% of the variance within the algal groups, and 97% of the variance in the species-environment relationship.

In Figure 3.26 the RDA including all algal genera occurring in more than 5% of the samples is shown. Conductivity, PO_4^{3-}, O_2 and temperature are the most important environmental variables. The majority of the algal genera were situated in the two lower quadrants, indicating preference for environments with low conductivity, pH and nutrients. The genera with the strongest response to the environmental variables were *Microcystis aeruginosa, Oscillatoria simplicissima, Ceratium hirundinella, Cryptomonas major, Carteria* spp., *Pandorina morum, Monoraphidium* spp. *Ceratium hirundinella* correlated positively with pH and nutrient concentrations. *Spirulina* spp. correlated with high N:P ratio, while *Microcystis aeruginosa* was negatively correlated to N:P ratio. *Oscillatoria simplicissima* was negatively correlated with O_2 concentration. Interestingly, the bloom formers were scattered over all four quadrants of the ordination, indicating very diverse preferences in relation to environmental conditions.

Table 3.15 shows the results of the RDA including algal genera. The first four canonical axes explained nearly 15% of the variance within the algal groups, and 83% of the variance in the species-environment relationship.

One of the objectives of recording the species abundance in the samples was to relate species composition to the phosphatase enzyme activity in the phytoplankton community. In Figure 3.27 it can be seen that the bacterial AcPA activities as well as the APA:AcPA ratio in the phytoplanktonic fraction have the longest arrows, indicting importance of these enzymatic fractions in explaining the variation in the species data. Cryptophytes were positively correlated with APA, and negatively with APA:AcPA in the bacterial fraction. Cyanobacteria were positively correlated with the APA:AcPA ratio in the phytoplanktonic fraction, and diatoms and Euglenophytes with AcPA in the phytoplanktonic fraction. Euglenophytes and diatoms were situated close to each other in the ordination diagram, indicating similarity in their "response" to enzyme speciation.

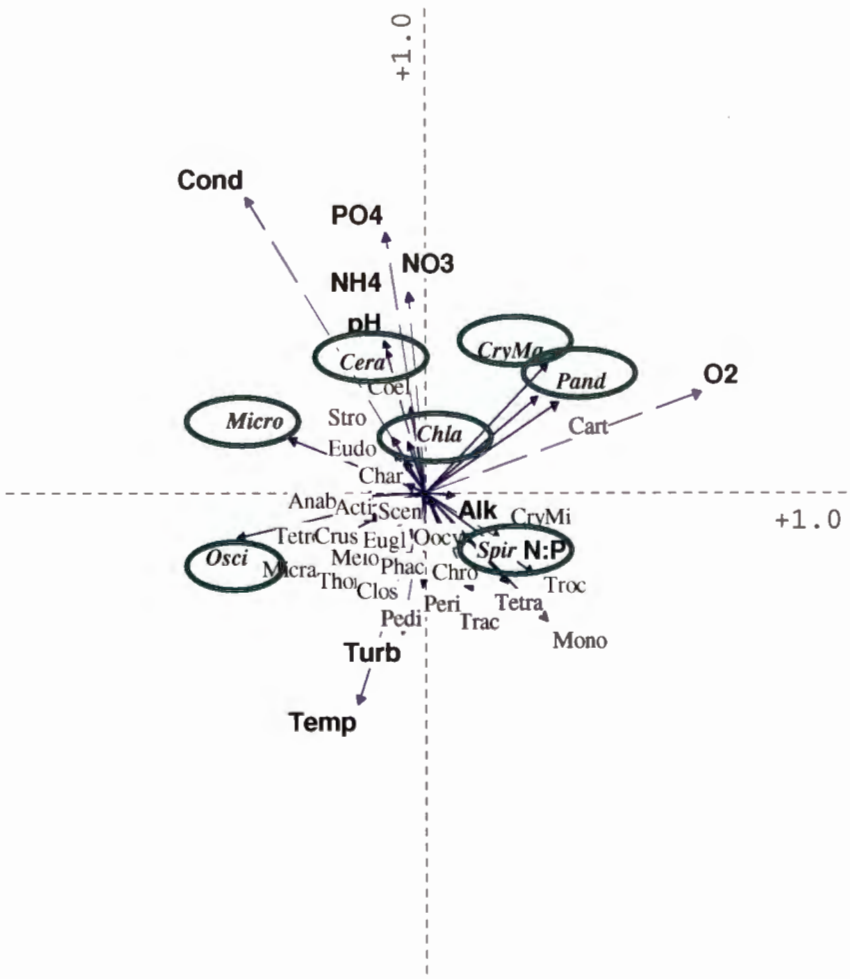


Figure 3.26. Figure 3.26. RDA ordination biplot showing environmental variables and algal genera present in more than 5% of the samples. Bloom forming species are indicated with Italic fonts and encircled. Explanations for abbreviations used are given in Table 3.9.

Table 3.15. *Results from the RDA analysis for environmental variables and algal genera present in more than 5% of the samples.*

Axes	1	2	3	4	Total variance
Eigenvalues	0.078	0.027	0.022	0.017	1.000
Species-environment correlations	0.635	0.442	0.555	0.493	
Cumulative % variance of species data of species-environment relation	 7.8 44.9	 10.5 60.3	 12.7 73.0	 14.5 83.0	
Sum of all unconstrained eigenvalues					1.000
Sum of all canonical eigenvalues					0.174

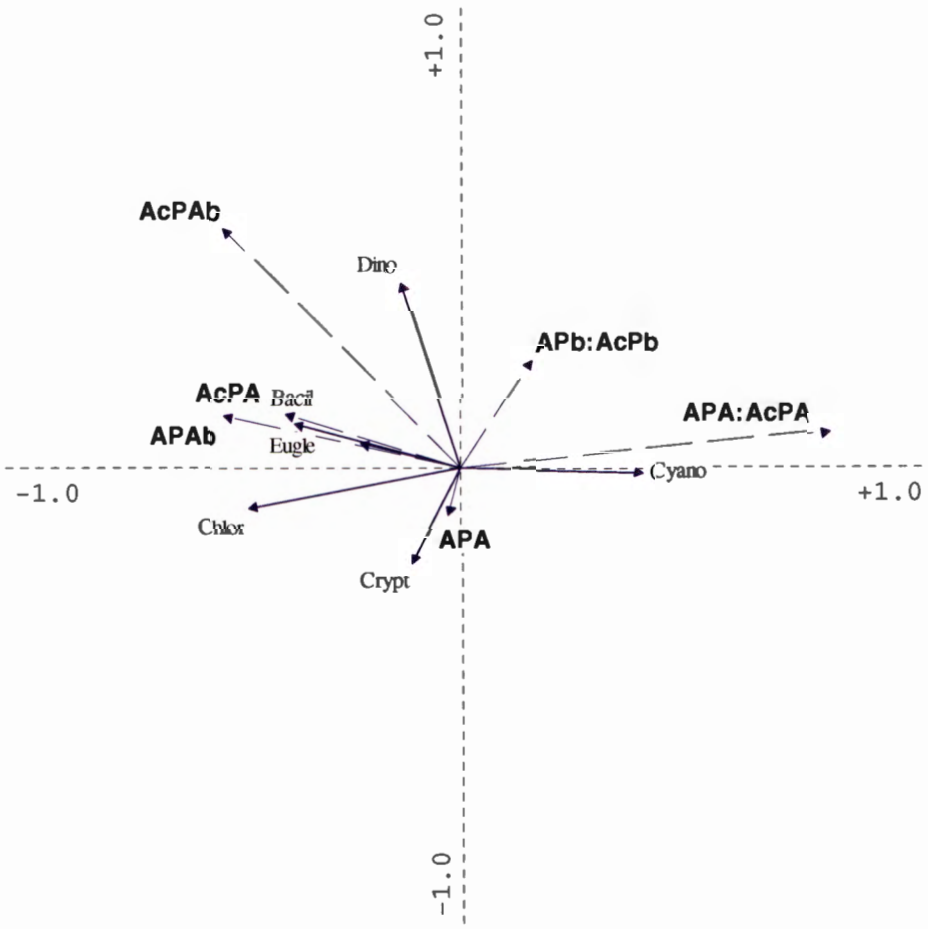


Figure 3.27. RDA ordination biplot showing phosphatase enzyme speciation and major algal groups. Explanations for abbreviations used are given in Table 3.9.

Table 3.16. *Results from the RDA analysis for enzyme speciation and major algal groups.*

Axes	1	2	3	4	Total variance
Eigenvalues	0.127	0.014	0.009	0.003	1.000
Species-environment correlations	0.544	0.337	0.343	0.158	
Cumulative % variance					
of species data	12.7	14.2	15.0	15.3	
of species-environment relation	82.7	92.0	97.6	99.6	
Sum of all unconstrained eigenvalues					1.000
Sum of all canonical eigenvalues					0.154

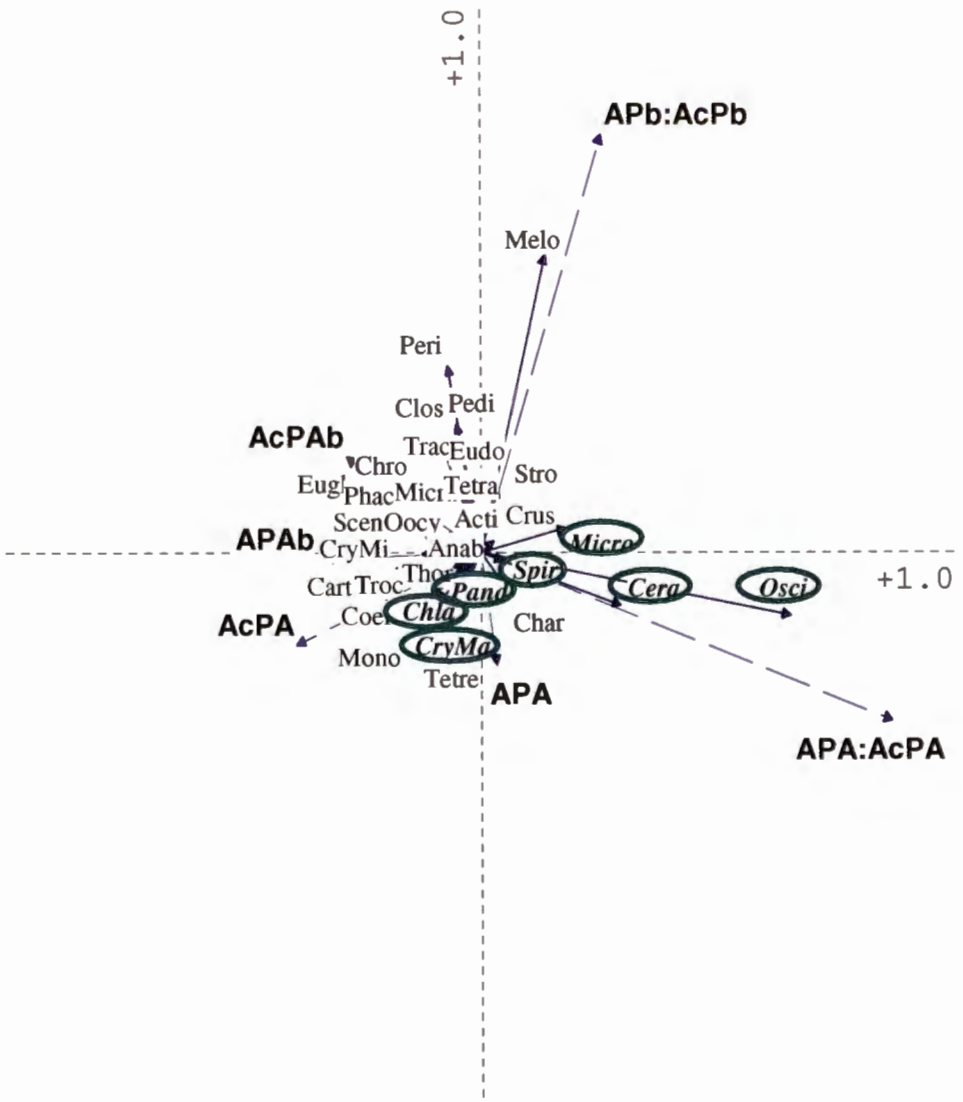


Figure 3.28. RDA ordination biplot showing phosphatase enzyme speciation and algal genera occurring in more than 5% of all samples. Bloom forming species are shown in italic fonts and encircled. Explanations for abbreviations used are given in Table 3.9. Bloom forming species are indicated with italic fonts.

Table 3.17. *Results from the RDA analysis for environmental variables and algal genera present in more than 5% of the samples.*

Axes	1	2	3	4	Total variance
Eigenvalues	0.091	0.041	0.010	0.006	1.000
Species-environment correlations	0.660	0.576	0.286	0.307	
Cumulative % variance					
of species data	9.1	13.3	14.2	14.8	
of species-environment relation	59.1	85.7	92.1	96.0	
Sum of all unconstrained eigenvalues					1.000
Sum of all canonical eigenvalues					0.155

The RDA results show (Table 3.16) that approximately 15% of the variation in the species data could be explained by the four first axes, and 99.6% of the species – enzyme relationship.

When all genera were included (Figure 3.28), some interesting aspects emerged. Of the cyanobacteria, *Microcystis aeruginosa, Oscillatoria simplicissima* and *Spirulina* spp. were positively correlated with the ratio of APA:AcPA in the phytoplanktonic fraction. All other bloom formers recorded during the study similarly grouped in the same two lower quadrants, thus all being associated with high APA and APA:AcPA. Nearly 15 percent of the variation in the species data could be explained by the four first axes, and 96% of the species – enzyme relationship (Table 3.17).

3.3. Discussion and conclusion

3.3.1. Environmental parameters

The Vaal River can be characterised as a clearly eutrophic ecosystem, with very high chlorophyll-*a* concentrations and nutrient levels. The chlorophyll-*a* concentrations (on average 31 – 139 µg l^{-1} at the four sampling sites, maximum 843 µg l^{-1} at Balkfontein) were extremely high in comparison to other rivers. As examples of chlorophyll-*a* concentrations in other rivers the following can be mentioned: the River Meuse with chlorophyll-*a* concentrations varying between 0.2 and 120 µg l^{-1}, (Descy 1987), Luján River with chlorophyll-*a* concentrations varying between 1 and 316 µg l^{-1}, (O'Farrell *et al.* 2002), Paraná River with chlorophyll-*a* concentrations varying between 2 and 22 µg l^{-1}, (Gomes *et al.* 2002), Columbia River with chlorophyll-*a* concentrations varying between 1 and 50 µg l^{-1}, (Sullivan *et al.* 2001). The chlorophyll-*a* concentrations were also higher than other South African rivers (e.g. the Orange River with a mean of 10.5 µg l^{-1} and maximum of 32 µg l^{-1} in 2000 (van Ginkel, pers. comm. 2002) and the Modder River with maximum chlorophyll-*a* concentrations of 180 µg l^{-1}, (Koning and Roos 1999). In addition the number of species typical of eutrophic waters was high, out of the 44 genera observed, 25 (i.e. more than 50%) are listed as indicators of eutrophic waters (Järnefelt 1952, Kwandrans *et al.* 1998, Wu 1999, see Janse van Vuuren 2001 for overview). Euglenophyceae, of which many species have been used as indicators of eutrophic conditions (Järnefelt 1952), occurred the least at Stilfontein, which also had lowest nutrient concentrations – indicating the relative oligotrophy of this locality compared to the others. Janse van Vuuren (2001) observed a possible shift in cyanobacteria abundance from *Oscillatoria simplicissima*

towards *Anabaena circinalis*. The main cyanobacterial species forming blooms during this study were *Oscillatoria simplicissima* and *Microcystis aeruginosa*, whereas *Anabaena circinalis* was not observed forming mass-occurrences. Thus the results generated during this study could not lend support to the hypothesis of increasing abundance of *Anabaena circinalis*. To summarise, the bulk of bloom formers in the Vaal River was composed mainly of cyanobacteria, green algae (*Pandorina morum, Chlamydomonas incerta*) and diatoms – displaying a normal species composition for highly eutrophic riverine environments (Descy 1987) apart from an unusually high abundance of cyanobacteria.

Apart from being eutrophied, the Vaal River also has high total dissolved salt concentration (TDS) and high conductivity (reflecting the TDS of the water, for relationship between conductivity and TDS see Wetzel 1983), decreasing downstream. The water is also alkaline with comparatively high pH values (8.2 being the lowest annual mean value). High pH values are typical of eutrophied environments because of high primary production causing rapid utilisation of dissolved CO_2, and thus increasing the pH of the water during daytime (Dallas and Day 1993). The high primary productivity is also reflected by the high O_2 concentrations measured in the river. The turbidity observed during the study period was in the same range or marginally lower than reported previously for the Vaal River (Janse van Vuuren 2001).

The PO_4^{3-} concentrations in the Vaal during 1999 – 2001 were similar to those reported earlier for the Vaal River in 1992-1996 (Janse van Vuuren 2001). The highest PO_4^{3-} concentrations occurred upstream at Loch Vaal and Barrage during 2000 (8.4 – 16.8 µmol l^{-1}), being higher than average concentrations reported from e.g. the South African rivers Modder (8 µmol l^{-1}, Koning and Roos 1999), Orange (1.9 µmol l^{-1}, van Ginkel pers. comm. 2002) and Caledon (2.1µmol l^{-1}, Keulder 1979). In the Loch Vaal nutrients are supplied also from the bottom sediments due to the shallowness of the lake (Pieterse and Steynberg 1993) and thus contributes to the internal loading and further eutrophication of the system. Shallow lakes have been reported to be more efficient in converting the available phosphorus into phytoplankton biomass because of the constant and sufficient underwater light climate, as well as a regular mixing regime (Nixdorf and Deneke 1997). This may partly explain the higher nutrient and biomass concentrations at the Loch Vaal, compared to the other localities.

The NO_3^- concentrations were between 14 and 132 µmol l^{-1}, in the range of previously reported values for the Vaal River (28 µmol l^{-1} (Roos and Pieterse 1995), 11 – 206 µmol l^{-1} (Janse van Vuuren 2001)). Other South African rivers have been reported to have NO_3^- concentrations in a similar range, e.g. 16 µmol l^{-1}in the Modder and 61 µmol l^{-1} in the Klein Modder river (Koning and Roos 1999), 67 µmol l^{-1} in the Orange River and 63 µmol l^{-1} in the Caledon river (Keulder 1979).

The N:P (in this study mole/mole NO_3^-: PO_4^{3-}) ratios were low at all localities in the Vaal River during this study, indicating N-limitation of phytoplankton growth. The N:P ratio in the Loch Vaal decreased from an average of 35-50 in 1986-1990 to an average of 10-20 in 1990-1993 (Pieterse and Steynberg 1993). This decrease was mainly the result of decreasing inorganic N loading concurrently with a slight increase in the inorganic P loading. Low N:P ratios in the Vaal River have been shown previously (Janse van Vuuren 2001), especially in spring and summer. During this study only occasional peaks of high N:P ratios occurred, especially in summer months. The mean N:P ratios were lower compared to previous studies, and decreased further during the study period, except for Stilfontein where low N:P ratios were recorded throughout the study period.

The optimum N:P ratio varies widely between different phytoplanktonic species (Rhee and Gotham 1980). It has been suggested that cyanobacteria, as well as dinoflagellates causing Red Tides, may be favoured by low N:P ratios (Smith 1983, Varis 1991, Hendzel *et al.* 1994, Hodgkiss and Ho 1997). The "low N:P ratio – high cyanobacterial dominance" hypothesis is supported by the fact that many cyanobacterial species are able to fix atmospheric N_2 (Schindler 1977). However, Canfield *et al.* (1989) showed that cyanobacteria occur in large numbers over a wide range of N:P ratios while McQueen and Lean (1987) found no correlation between TN:TP ratio and cyanobacterial dominance, but did show an inverse relation between NO_3^-:TP and cyanobacterial biomass. Several other studies have questioned the validity of the "dogma" of low N:P ratios favouring cyanobacterial dominance (Shapiro 1990, An and Jones 2000, Downing *et al.* 2001).

The mean N:P ratios during this study ranged between 5.6 (Loch Vaal 2000) and 27.5 (Barrage 1999), being comparatively low, which is typical of eutrophied water bodies (Downing and McCauley 1992). In 2000 all localities had mean N:P ratios of less than 9, being clearly lower

than the suggested limit for "cyanobacterial favourable N:P" of < 29 (Smith 1983). The results from the RDA plotting showed that cyanobacteria were, however, associated with high N:P ratios (Figure 3.24). When examining the data in detail, it was clear that during this study in the Vaal River cyanobacteria occurred in periods when N:P ratios < 16 in all occasions, largest cyanobacterial biomasses were recorded during very low N:P ratios of < 5 (January to March 2001). Similar results were found earlier in the Vaal River (Pieterse and Janse van Vuuren 1997, Janse van Vuuren 2001), reporting highest cyanobacterial concentrations when N:P ratios were < 25. During this study diatoms were also observed in high numbers during periods of low N:P. It has been previously suggested by Sommer (1985) that diatoms would also be favoured by low N:P ratios, whereas green algae would be more abundant in higher N:P ratios (Sommer 1985, Barica *et al.* 1980, Shapiro 1990). During this study no correlation between high N:P ratios and green algae concentrations could be shown, and thus does not support the hypothesis suggesting high green algal abundance in high N:P ratios.

3.3.2. Phosphatase activity in the phytoplanktonic fraction

Compared with APA reported in literature (*e.g.* Elser and Kimmel 1986, Boavida and Marques 1995, Dodds 1995, Jamet *et al.* 1995 and 1997, Yiyong and Xinyu 1997), the activities in the Vaal River were relatively high. The phytoplanktonic APA was highest at the Barrage and Loch Vaal, the localities with the highest nutrient concentrations. Contradictory reports exist concerning the relationship between phosphatase activity and trophic status of water bodies. Hantke *et al.* (1996) showed that the substrate affinity, and consequently the efficiency of AP to hydrolyse organic P compounds, increased with increasing trophic level of the water. Opposite results have also been reported; Stockner (1988) suggested that generally the contribution of AP production reaches higher values in oligotrophic water and decreases with eutrophication. Spijkerman and Coesel (1998) showed that a desmid species from oligotrophic lakes had higher maximum APA compared to a desmid species from eutrophic lakes.

In this study, the total phosphatases (both acid and alkaline) correlated significantly with chlorophyll-*a*. It seems likely that conditions of elevated biomass, are related to higher APA. This supports the notion that phosphatase activities are higher in eutrophied environments compared to oligotrophic waters (Hantke *et al.* 1996).

It has been suggested that APA is predominantly phytoplanktonic in origin, or characteristic of species in eutrophied conditions whereas AcPA occurs predominantly in bacteria or as dissolved enzymes (Olsson 1990). This hypothesis was supported by the fact that in this study in the Vaal River APA dominated over AcPA in the phytoplanktonic fraction, especially at the more eutrophied localities. Also the RDA plot (Figure 3.28) reveals that all bloom forming species, as well as the majority of all recorded species, group in the same lower quadrants as APA and APA:AcPA, indicating that these species indeed occur associated with high APA compared to AcPA. The pH in the Vaal River is very high (8.2-9.0 on average) and thus one could expect a clear dominance of APA as an adaptation to highly alkaline conditions; this, however, was not supported by the fact that APA in this study decreased with increasing pH values.

The APA in the phytoplankton was highest in summer and autumn, summer being the season when P limitation is normally the most severe in fresh waters (Pettersson 1980, Nausch 1998). Based on the very low N:P ratios, the summer months in the Vaal River could at times also be N-limiting. This suggests that there is no obvious relationship between phosphatase activity and P-limitation in the river. Also the lack of correlation between PO_4^{3-} concentrations and APA suggests that PO_4^{3-} as such (as well as N:P ratios) cannot be used as an indicator of P deficiency – especially in an environment where N and P successively limit the phytoplankton growth, which is the case in the Vaal River (Janse van Vuuren 2001). This is supported by Rose and Axler (1997), who reached the same conclusion after studies done in four Minnesota lakes.

The negative correlation between O_2 concentration and APA might suggest higher enzyme activity during times of lower primary production. Smith and Kalff (1981) showed that in times of low growth APA was high, but low growth does not necessarily result in lower O_2 concentration in the water. The solubility of oxygen decreases with increasing temperature (Wetzel 1983), and therefore the negative correlation might also be a result of higher APA in summer months when O_2 is less soluble due to higher water temperatures.

Species composition is of importance when studying the nutrient dynamics as it introduces the interesting aspect of competitive status of different phytoplanktonic species in relation to nutrient bioavailability. The species composition of the phytoplankton was typical of temperate, nutrient-rich rivers (Descy 1987) dominated by diatoms and green algae. Cyanobacteria were abundant

during the warm-water season. Few studies have been made on cyanobacteria in running waters, but generally cyanobacteria are more typical in large, slow-flowing rivers (such as the Vaal) than in smaller streams (Fogg *et al.* 1973). In this study the cyanobacteria accounted for 10 – 20% of the total phytoplankton cell-concentration, and the samples with the most cyanobacteria were collected from the shallow-lake-type localities Loch Vaal and Barrage (Table 3.8). Loch Vaal and Balkfontein had the highest overall species diversity despite their high trophic status, which is in contradiction to earlier reports suggesting decreasing biodiversity of phytoplankton in increasing trophic status (Codd 2000).

A bloom caused by the dinoflagellate *Ceratium hirundinella* was observed during this study in early autumn 2001 (March) in Loch Vaal. The species has been recorded in South Africa before in the hypereutrophic Hartebeesport Dam in spring 1999 (van Ginkel *et al.* 2001). The Hartebeesport Dam is an impoundment at the confluence of the Crocodile and the Magalies Rivers, approximately 120 km north of Loch Vaal and Barrage, but in a different drainage region (DWAF 1993). Van Ginkel *et al.* (2001) observed that the start of the bloom in Hartebeesport Dam coincided with comparatively low PO_4^{-3} concentrations. Similarly, the *Ceratium hirundinella* bloom in the Loch Vaal was preceded by low PO_4^{-3} concentrations in February 2001. *Ceratium hirundinella* has been shown to be able to assimilate both organic and inorganic phosphorus (Bucka 1989, James *et al.* 1992) and this may explain its rapid increase despite low PO_4^{-3} concentrations. During the bloom high APA (> 150 nmol μg chl-a^{-1} min^{-1}) was measured from the samples from Loch Vaal, whereas the AcPA was low at the time of the bloom. James *et al.* (1992) also showed APA during occurrence of *Ceratium hirundinella* in Eau Galle Reservoir, indicating that apart from its capacity to physically migrate in the water-column "scavenging" phosphorus, it can also effectively use organic phosphorus compounds. High APA compared to AcPA is contradictory to the findings by Olsson (1990), who measured higher AcPA than APA when species of dinoflagellates dominated the phytoplankton biomass. Using novel methods to measure phosphatase activity Rengefors *et al.* (2001) have, however, in recent studies been able to show considerable interspecies, as well as intraspecies, differences with respect to phosphatase activity. Therefore studies done on a group of species does not necessarily reflect the adaptations of one specific species.

Another noteworthy mass-occurrence was that of the green alga *Pandorina morum* at Balkfontein in early spring (September) 2000. *Pandorina morum* is a colonial green algal species

(Volvocales) that resembles *Chlamydomonas* species, especially on molecular criteria (Harris 2001). On a global scale, blooms of this species are not frequently observed, but *Pandorina morum* usually occurs at low densities (Pierre 1987). The occurrence of the species is of great interest also because *Pandorina morum* has been reported to produce a toxic substance that strongly inhibits the photosystem II electron flow in chloroplasts (Patterson *et al.* 1979). The bloom also coincided with the lowest measured phosphorus concentrations in the water, but whether the low P was a reason or a consequence of the bloom is not possible to say. The total AcPA was markedly high during the same time, but the specific phosphatase activities were not higher than normal.

Olsson (1990) showed that the type of ambient plankton is important for the pH optimum and the activity of lake water phosphatases. He further demonstrated that P-deficient *Chlamydomonas* sp. showed high phosphatase activity at high pH, whereas several *Peridinium* species had highest phosphatase activity at low pH. During this study the occurrence of *Peridinium* was negatively correlated with pH, suggesting adaptation to low pH environments, but it was not correlated with AcPA (Figures 3.26 and 3.28). *Chlamydomonas* sp. on the other hand, grouped together with APA in the RDA ordination, supporting Olsson's findings. Olsson (1990) further suggests that high AcPA would be expected in conditions where Ochromonadaceae and dinoflagellates dominate the species composition, whereas high APA would be more typical of cyanobacterial and diatom dominated lakes. In this study euglenophytes and diatoms (pennates especially) were associated with high AcPA whereas cryptophytes and a wide array of green algae were associated with high APA. Green algae as a group were strongly negatively correlated with APA:AcPA ratio, suggesting higher levels of AcPA, while Cyanobacteria were strongly positively correlated with a high APA:AcPA ratio, especially *Oscillatoria* sp., *Microcystis aeruginosa* and *Spirulina* sp. (Figures 3.27 and 3.28). Thus cyanobacteria in the Vaal River seem to have comparatively high APA levels.

3.3.3. Bacterial / dissolved fraction

The localities with highest concentrations of chlorophyll-*a*, Loch Vaal and Balkfontein, had the lowest APA and AcPA in the bacterial / dissolved fraction. No seasonal variation could be shown at any of the localities, indicating a fairly homogenous production of the enzyme in the bacterial population in the river.

The fact that the bacterial / dissolved APA and AcPA were so much higher than the phytoplanktonic fraction was not expected. The bacterial / dissolved fraction included the picoplankton (phytoplankton species less than 1.2 µm in size)– which was not quantified during this study, but the presence of occasionally high numbers of picoplankton could be observed during light-microscope determination of quality and quantity of microphytoplankton. It has been reported, that autotrophic picoplankton can provide 16 – 70% of the carbon fixation in freshwaters (Stockner 1988). Thus it is perhaps not surprising that a large fraction on the enzyme activity would originate from the picoplankton or bacteria. Contradictory results have been reported in literature concerning APA partitioning between phytoplankton and bacteria in water. Jamet *et al.* (1995 and 1997) found no APA in the dissolved fraction. Berman (1970), Pettersson (1980) and Currie *et al.* (1986) suggested that APA is predominantly algal. Wetzel (1983) on the other hand states that substantial quantities (up to 50%) of APA can be in the dissolved phase, and Stewart and Wetzel (1982) report that most phosphatase was either free in solution or associated with bacteria. Also Rose and Axler (1997) found that dissolved activity was an important component of APA in Lake Superior, accounting for 33 – 79% of the total activity.

Little information is available concerning the role of AcPA in aquatic environments, and even less on its partitioning between phytoplanktonic and bacterial / dissolved fractions. In this study the bacterial / dissolved fraction often accounted for over 95% of the overall activity, of which the major part was AcPA. A possible explanation could be that species in eutrophied environments release more readily extracellular enzymes compared to species in nutrient poor environments, as demonstrated by Spijkerman and Coesel (1998). Therefore the high enzyme activities in the river might be due to high amounts of released enzymes (of phytoplanktonic origin) rather than bacterial enzyme activity *per se*. Unfortunately the data cannot provide evidence for or against this hypothesis, but it should be studied in more detail. Studies concerning any phosphatase activities in rivers are also very scarce, making comparisons impossible at this time.

3.3.4. Multivariate analyses

From the PCA ordinations it was clear that the riverine localities Balkfontein and Stilfontein grouped together, especially with respect to the enzyme speciation (Figure 3.22), indicating that the riverine localities "behave" differently from the more shallow-lake-like localities.

In previous studies it has been shown that green algae make a significant contribution to the phytoplanktonic species composition (Sedmak and Kosi 1998), and e.g. the genera *Scenedesmus* and *Staurastrum* are frequently associated with cyanobacterial blooms (Jensen *et al.* 1994, Komarkova and Hejzlar 1996). In this study, *Staurastrum* sp. was not observed in the samples, even though the genus has been observed in the river previously (Janse van Vuuren 2001) and *Scenedesmus* seemed to rather group with diatoms and euglenophytes (Figure 3.24). Several other green algal genera, on the other hand, grouped close to the cyanobacteria, e.g. *Tetrastrum*, *Actinastrum*, *Crusigenia*, *Eudorina*, *Micractinium* and *Thorakomonas*. *Chroococcus*, which was the only cyanobacterial genus displaying a very different trend in the DC-analyses, grouped together with green algae of the genera *Carteria*, *Coelastrum*, *Pandorina* and *Trochischia*. The "wedge-effect" in the DCA ordination in Figure 3.24 indicates that both the first and the second ordination axes are very strong in reflecting the species occurrence. Green algae are scattered homogeneously over the ordination plot, indicating large variation in this group. This variation can also be seen in the river as spatial and temporal variability, green algae being present at all localities and throughout the year, sometimes causing blooms, which were also recorded at all localities.

Microcystis aeruginosa seems to be separated in the ordination from other cyanobacteria, such as *Spirulina* sp. and *Oscillatoria* sp., which are both filamentous cyanobacteria often referred to as belonging to the same species group (Canter-Lund and Lund 1995). It has been reported that *Oscillatoria* species can produce compounds with antibiotic activity against *Microcystis aeruginosa* (Bagchi *et al.* 1993), which might partly explain the separate occurrence of these two species.

It is interesting that the cyanobacteria in this study correlated with high N:P ratios as shown by the RDA ordinations, rather than low N:P ratios as often reported elsewhere (Smith 1983, Varis 1991, Hendzel *et al.* 1994, Hodgkiss and Ho 1997). This may be a result of the overall low N:P

ratios in the river, with only a few peaks when N:P exceeded 10. Also most cyanobacteria identified during this study were species lacking heterocysts, therefore not being as much advantaged by low N:P ratios. The paucity of heterocystous species in a predominantly low N:P environment can possibly be explained by the overall nutrient richness of the water (Shapiro 1990, Romo and Miracle 1994, Scheffer *et al.* 1997). The green algae as a group were strongly correlated with NH_4^+ concentration (Figure 3.25), indicating that this group may grow well on this N substrate. The specific green algal species, however, were very scattered (Figure 3.26) over the ordination showing interspecific differences in environmental preferences. *Chlamydomonas* sp. was correlated to high NO_3^- concentrations, indicating preference for this N substrate, in contradiction to the findings by Chróst *et al.* (1989). They found *Chlamydomonas* spp. to dominate when NO_3^- concentrations in the environment were low.

Cyanobacteria have been suggested to be favoured by high pH and / or low CO_2 availability, while green algae would be favoured by the opposite conditions (Shapiro 1990). In the ordination diagrams no relationship between cyanobacteria and high pH could be detected, whereas green algae (as a group) were negatively correlated to pH, lending support to the hypothesis of low pH favouring green algae. The river as such is a comparatively high pH environment, and may therefore be expected to support cyanobacterial development in favour of green algae.

The ordination of species along environmental gradients (presented in Figure 3.26) brings forward the fact that bloom formers were scattered, indicating preference for very different types of environmental conditions. This "scattering" emphasises the fact that bloom formation is an event during which one species can form near monocultures, replacing the normal species diversity associated with healthy aquatic environments. The scattered ordination pattern also illustrates the fact that bloom forming species are capable of exploiting a wide array of ecological niches – which is why attempts at dynamic modelling using conventional input parameters have been of little use for predicting HAB formation (Cembella 1998).

RDA ordinations confirmed the association of cyanobacteria with high APA:AcPA, being valid for especially the bloom forming cyanobacterial species (Figure 3.28). It must be emphasised, that it seems like the common bloom formers in the river are all grouped in the two lower quadrants in the RDA ordination, associated with high APA and APA:AcPA ratio. This grouping

suggests that a common factor for the bloom formers can indeed be a capability to maintain high alkaline phosphatase activity. Thus the bloom formers seem to have adapted ecologically to different niches (as seen in Figure 3.26) but have a common physiological advantage in high APA production (Figure 3.28), which is not connected to the environmental conditions but rather to the physiological fitness of the bloom formers.

3.3.5. Conclusions

The main conclusions of the *in situ* study in the Vaal River in 1999-2001 are summarised as follows:

1. High enzyme activities in the eutrophied Vaal River support the hypothesis that APA increases with increasing trophic level. AcPA follows the same pattern.
2. The largest part of APA and AcPA was found in the smaller size fraction, including dissolved enzymes, as well as enzymes originating from bacteria and picoplankton.
3. APA dominated in the phytoplanktonic fraction, whereas AcPA dominated in the bacterial / dissolved fraction, however,
4. AcPA comprised a significant portion (>30%) of the phosphatase activities in both size fractions, suggesting that the significance of these enzymes for aquatic phosphorus metabolism may have been underestimated.
5. In this study no relationship could be detected between APA and PO_4^{3-} concentrations or N:P ratios, thus APA is not suitable as an indicator of P-deficiency in a phytoplankton population *in situ.*
6. All common bloom formers in the river (*Pandorina morum, Chlamydomonas* sp., *Cryptomonas major, Spirulina* sp., *Microcystis aeruginosa* and *Oscillatoria* sp., as well as centric diatoms) corresponded with high APA or APA:AcPA ratio, suggesting that bloom formers have a physiological advantage in being capable of producing more, and / or having higher APA than other groups present in the river. Ecologically, the bloom formers are adapted to a wide variety of different environmental conditions, making possible the formation of near monocultures.

CHAPTER 4

GROWTH, PHOSPHATASE ACTIVITY AND PHOTOSYNTHETIC VITALITY IN TWO GREEN-ALGAL (CHLOROPHYCEAE) SPECIES UNDER DIFFERENT NUTRIENT CONDITIONS

4.1. Introduction

The Vaal River can be described as a eutrophic system, but because of the dynamic nature of any riverine system, the planktonic microalgae in the river must be capable of adjusting to rapidly changing physical and chemical conditions. In the Vaal River annual blooms caused by several green algal genera occur (Janse van Vuuren 2001, Chapter 3 in this work). The most regularly bloom forming green alga in the Vaal River, *Chlamydomonas* spp., form intensive blooms throughout the year, but especially in spring and autumn (Janse van Vuuren 2001, Chapter 3 in this work). In Chapter 3 *Chlamydomonas* sp. was positively correlated with NO_3^- and N:P ratios, and showed a preference for lower water temperatures. It was also positively correlated with high alkaline phosphatase activity (APA) measured in the river.

The function of phosphatases probably varies between different phytoplanktonic species, and only constitutes one of many mechanisms involved in nutrient acquistion. It may, however, in connection to other growth parameters, give an idea of the nutrition acquisition strategy of the species. It may be hypothesised, that if a bloom forming alga from predominantly eutrophic environments has the capability of utilising organic phosphorus (P_o) compounds to enhance its growth in fluctuating nutrient conditions, it might have a competitive advantage compared to other, non-bloom forming species also occurring in the same eutrophic conditions.

In order to examine this hypothesis differences in physiological responses to differing nutrient conditions are in this chapter compared between two green algae isolated from the Vaal River, namely the bloom forming *Chlamydomonas* sp. and the non-bloom forming *Chlorella* sp.

The *Chlamydomonas* family consists of over 600 species occurring world-wide (Van den Hoek *et al.* 1995). The *Chlamydomonas* genus comprises unicellular, ellipsoidal chlorophyte algae with two anterior flagella, a basal chloroplast surrounding one or more pyrenoids, and a distinct cell wall (John *et al.* 2002). Most *Chlamydomonas* species have an eyespot, which enables

swimming cells to orient themselves with respect to light (Harris 2001). *Chlamydomonas* species, especially *Chlamydomonas reinhardtii*, has been extensively used since the 1940's as a model organism for investigations on e.g. flagellar function and structure, genetics, chloroplast biogenesis, photosynthesis, light perception and cell cycle control (Harris 2001). The *Chlamydomonas* sp. used in this study was isolated from the Vaal River (Steynberg 1994), where it frequently forms blooms (Janse van Vuuren 2001).

The *Chlorella* genus has more than 100 species, but the absence of morphological characteristics has led to considerable difficulty in identifying them. The genus is cosmopolitan, occurring in both marine and freshwater habitats as well as in soil, and as endosymbionts of invertebrate animals and in symbiosis with ascomycete fungi in lichens (John *et al.* 2002). The *Chlorella* species used in this work is spherical and somewhat smaller when compared to *Chlamydomonas* sp. In contrast to *Chlamydomonas* sp. it has not been reported to form blooms in the Vaal River, but is present in the river in low numbers throughout the year (Pieterse and Janse van Vuuren 1997). It has been isolated for studies concerning algal related water quality and purification problems, as an "algal standard" but also due to its small size and subsequent penetration of filters used in water purification (Steynberg 1994).

Photophysiological responses and enzyme activities taking part in N and P assimilation or uptake are important parts of cell growth, maintenance and proliferation of a phytoplankton bloom. Therefore comparisons of these physiological aspects in regulated and controlled laboratory studies are required to understand the underlying physiological causes of bloom formation. *Chlamydomonas* sp. is useful as an experimental organism also because of the large amount of physiological studies done on another *Chlamydomonas* species, *C. reinhardtii* (see e.g. Harris 2001 and references therein), thus allowing comparisons and the formulation of hypotheses based on physiological functions found in *Chlamydomonas* sp.

The aim of this study was to compare certain physiological traits, namely the growth, induction and level of phosphatase and nitrate reductase activities, and photosynthetic vitality (chlorophyll-*a* fluorescence, PSII function) in the chosen green algae, *Chlamydomonas* sp. and *Chlorella* sp. in:

1. nutrient sufficient growth medium (GBG-11),

2. N or P deficiency and (GBG-11 – N/P) and
3. different N:P ratios.

For detailed description of the experimental design and the analytical methods used, please refer to Chapter 2.

4.2. Results

4.2.1. Growth

Chlorella sp. was grown in batch cultures for 12 days and *Chlamydomonas* sp. for 13 days before the dilutions (semi-continuous culturing or fed batch cultures, for explanations of experimental set-up, treatments and methods, refer to Chapter 2) were started. Klett (turbidity) values decreased rapidly after the commencement of dilutions, but levelled off after eight days, indicating steady-state growth and adaptation to the low-nutrient regime in the cultures (Figure 4.1). At the end of the experiment the average specific growth rate was slower for *Chlorella* sp. compared to *Chlamydomonas* sp. for the low-nutrient treatments, but remained at steady-state level for the external control (μ = 0.43 – 0.44 dilution rate D = 0.4, Table 4.1). The internal control grew the slowest in *Chlorella* sp. (μ = 0.21 day^{-1}) whereas the N-limited cultures grew the slowest in *Chlamydomonas* sp. (μ = 0.35 day^{-1}). Highest growth rates were recorded in the external control treatments.

4.2.2. Chlorophyll-*a* concentration

Initially the chlorophyll-*a* concentrations in the *Chlorella* sp. cultures were (during fast growth phase) significantly higher ($F_{(1,34)}$ $p<0.0001$) when compared with those in the *Chlamydomonas* sp. cultures (Figure 4.2). At the end of the experiment the biomass, measured as chlorophyll-*a*, as well as cell densities and Klett values (Table 4.2), were higher in *Chlorella* sp. cultures, but the significance of the difference was slightly weaker ($F_{(11,34)}$ $p<0.05$). The chlorophyll-*a* concentration in the external control treatment remained high in *Chlorella* sp. but decreased in *Chlamydomonas* sp.

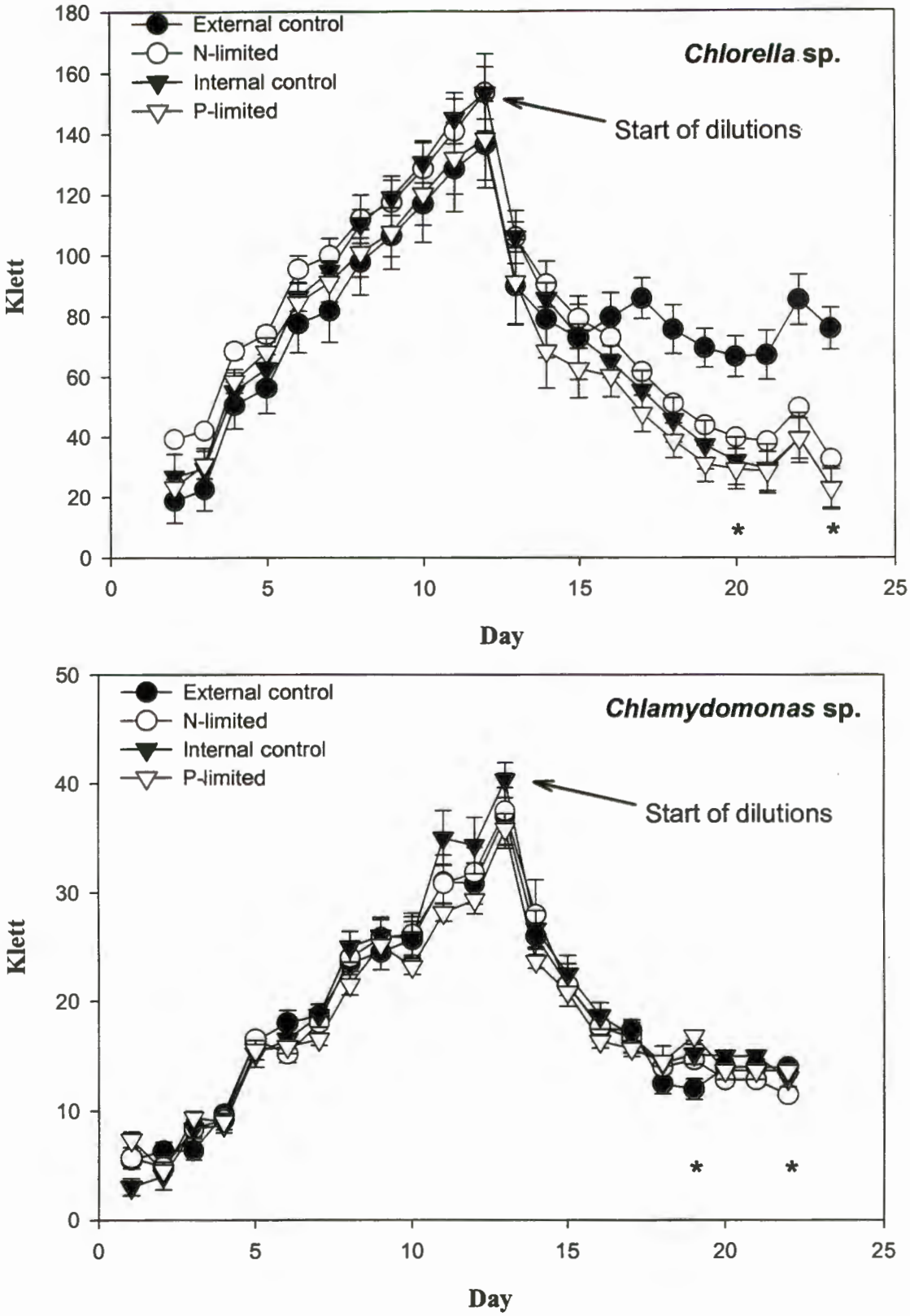


Figure 4.1. Growth (mean and standard error) of *Chlorella* sp. and *Chlamydomonas* sp. throughout the experiment. The dilutions were started on day 12 and 13 respectively, and the experiment was terminated on day 24 and 22 respectively. Note different scale on y-axis. Asterisks indicate the values used for growth rate analyses.

Table 4.1. Specific growth rates (μ day^{-1}) of Chlorella *sp. and* Chlamydomonas *sp. at the end of the experiment (mean values ± standard deviations of six replicates). The growth rate has been calculated to determine steady-state, using Klett colorimeter values for the four days before the termination of the experiment (see Figure 1).*

	Chlorella sp. μ day^{-1}	*Chlamydomonas* sp. μ day^{-1}
External control	0.44 ± 0.03	0.43 ± 0.04
N-limited (N:P = 1:1)	0.33 ± 0.01	0.35 ± 0.02
Internal Control (N:P = 16:1)	0.21 ± 0.16	0.38 ± 0.06
P-limited (N:P = 160:1)	0.28 ± 0.08	0.38 ± 0.02

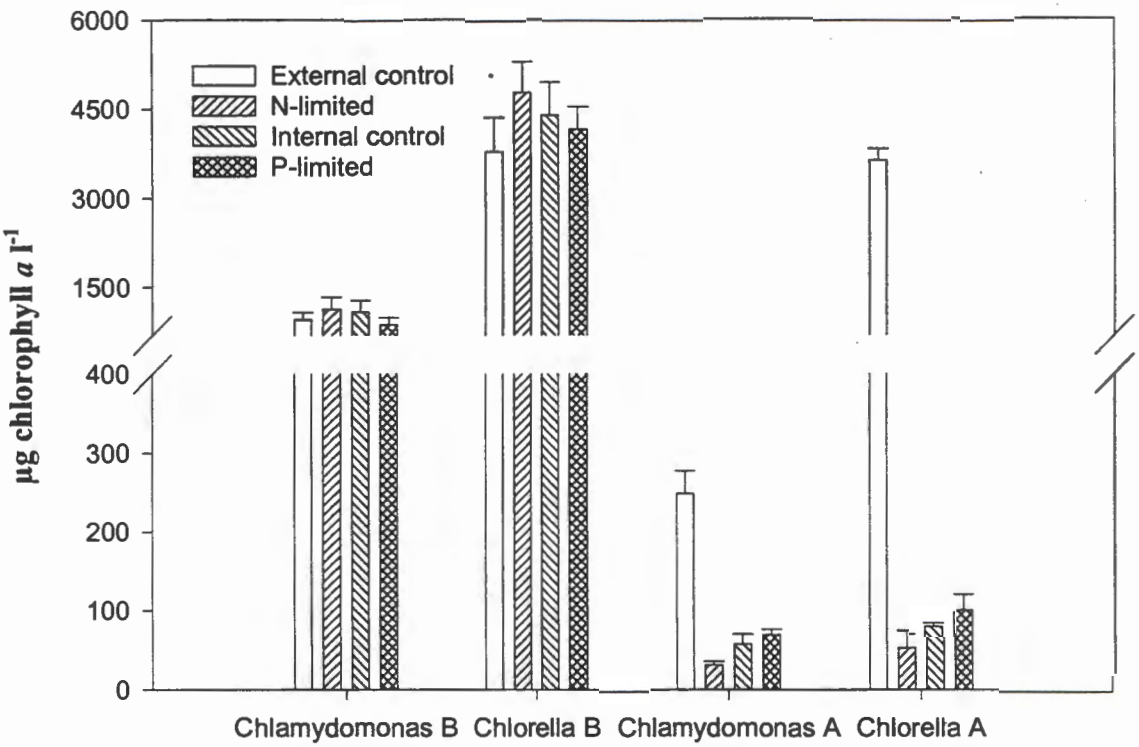


Figure 4.2. Chlorophyll-*a* concentration (mean and standard error for six samples) for *Chlamydomonas* sp. and *Chlorella* sp. initially (B = before treatment, samples taken on day 13) and after (A = after treatment, samples taken on day 23 and 22 respectively) being exposed to varying N:P ratios (External control = GBG-11 medium).

*Table 4.2. Biomass (as klett, chlorophyll-*a *and cell density) and chlorophyll-*a *per cell in the removed media of* Chlorella *sp. and* Chlamydomonas *sp. in treatments with different N:P ratios (mean values ± standard error of six replicates). Samples from removed media were taken at day 24 (*Chlorella *sp.) and day 22 (*Chlamydomonas *sp.).*

	N:P ratio, *Chlorella* sp.			N:P ratio, *Chlamydomonas* sp.		
	N-limited	Internal Control	P-limited	N-limited	Internal Control	P-limited
Klett	32.5 ± 1.2	23.0 ± 7.1	22.8 ± 6.4	11.5 ± 0.43	13.0 ± 0.77	13.7 ± 0.61
Chlorophyll-*a* (µg l^{-1})	52.5 ± 53.4	83.6 ± 10.8	100.3 ± 48.0	31.0 ± 4.4	57.3 ± 12.3	69.3 ± 6.8
Cell density (10^6 cells l^{-1})	2.85 ± 0.07	1.71 ± 0.51	0.84 ± 0.99	0.11 ± 0.02	0.21 ± 0.04	0.25 ± 0.02
Chl-*a* $cell^{-1}$ (ng $cell^{-1}$)	0.03 ± 0.04	0.05 ± 0.02	0.15 ± 0.09	0.33 ± 0.05	0.30 ± 0.06	0.28 ± 0.04

Throughout the experiment the average cell count in the *Chlorella* sp. cultures was approximately 10 times higher than in the *Chlamydomonas* sp. cultures. *Chlamydomonas* sp. cultures were, however, less affected by the nutrient limitation than *Chlorella* sp. measured in percentage decrease from initial chlorophyll-*a* values. The average chlorophyll-*a* content in the three treatments in *Chlamydomonas* sp. decreased to approximately 5% of the initial (average between the three treatments) level, whereas in *Chlorella* sp. the corresponding decrease was to 1% of the initial level (Figure 4.2).

At the end of the experiment the chlorophyll-*a* concentration was highest in the P-limited treatments and lowest in the N-limited treatment in both species, but the differences between the treatments were statistically significant only in *Chlamydomonas* sp. (ANOVA, $F_{(2,15)}$ $p<0.05$, Figure 4.3). A pair-wise comparison showed that the chlorophyll-*a* concentrations in *Chlamydomonas* sp. cultures grown in N-limited medium were significantly lower than the concentrations measured under P-limited conditions (Tukey's $p<0.05$). No statistically significant difference could be shown between the chlorophyll-*a* concentrations in N-limited and internal control cultures, or the P-limited and internal control cultures.

In *Chlamydomonas* sp. the chlorophyll-*a* content per cell unit decreased when the cells were exposed to nutrient stress (Table 4.2), but the difference between the stressed cultures were not significant. The cells in the external control had significantly more chlorophyll-*a* per cell than the cells in the internal control (Tukey's $p<0.05$) and N and P-limitation (Tukey's $p<0.01$). In *Chlorella* sp. cell counts were done from two samples per treatment only, which does not allow statistical comparison. These results, however, indicate that the N-limited cells had the least chlorophyll-*a* per cell unit.

4.2.3. Inorganic nutrients

The PO_4^{-3} concentrations decreased steadily after the commencement of dilutions to very low levels in all treatments except the external control (data not shown), in which all nutrients were kept at initial concentrations (Table 4.3). At the end of the experiment the PO_4^{-3} in all nutrient limited treatments was nearly depleted. *Chlorella* sp. cells used the PO_4^{-3} in the internal control and P-limited treatments very efficiently (PO_4^{-3} concentrations below detection limit) whereas *Chlamydomonas* sp. did not utilise the PO_4^{-3} equally efficient. In both species the lowest residual PO_4^{-3} was measured in the P-limited cultures.

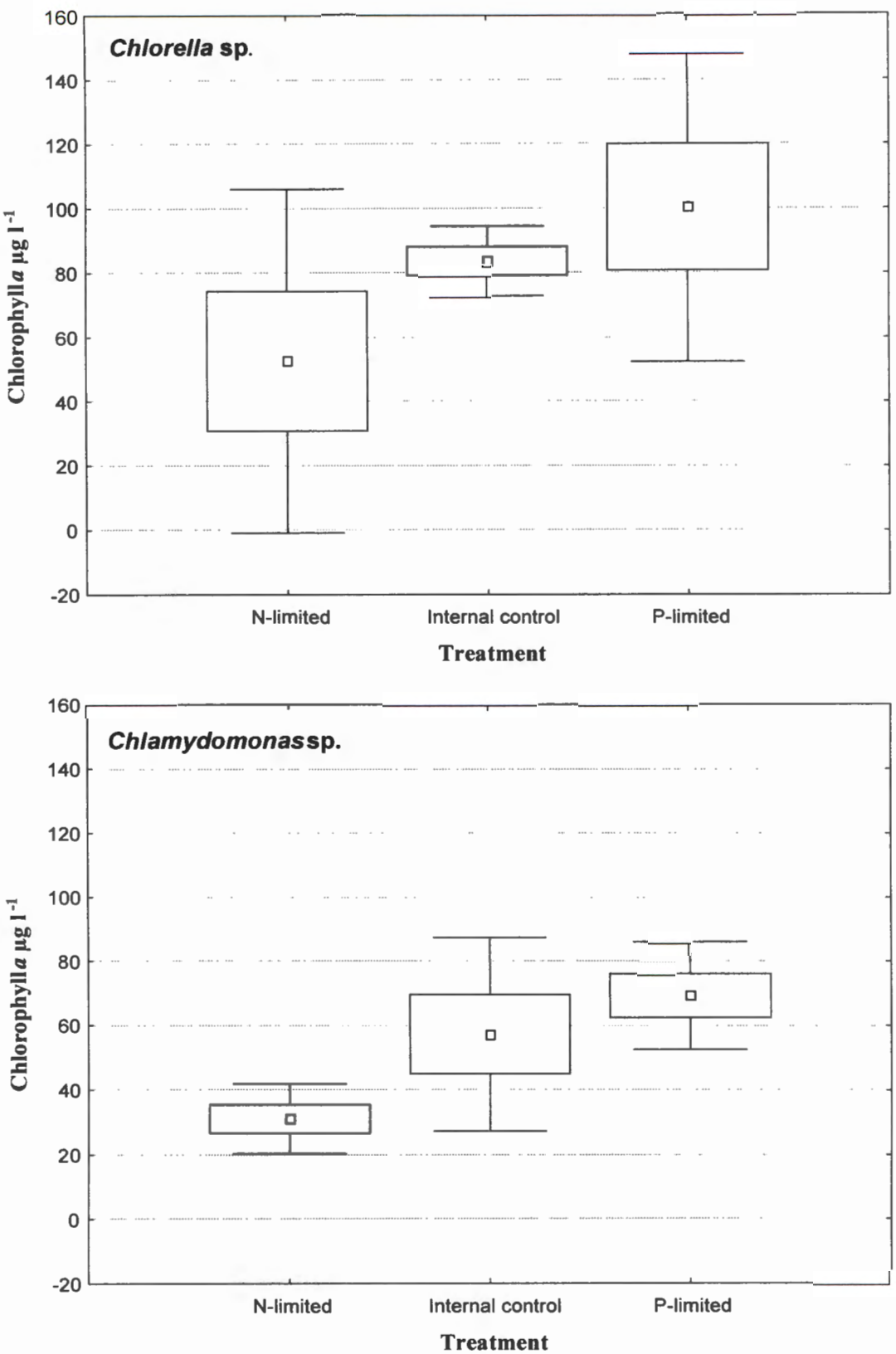


Figure 4.3. Chlorophyll-*a* concentrations (middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment) of *Chlorella* sp. and *Chlamydomonas* sp. at the end of the experiment in the three different experimental treatments.

Table 4.3. PO_4^{-3} concentrations expressed as µmol l^{-1} (mean values ± standard deviation of six replicates) in Chlorella *sp. and* Chlamydomonas *sp. culture medium during the experiment. The 1st measurement refers to batch-culture conditions before the start of dilutions. The 2nd and 3rd measurements were determined with 3-4 days intervals during the course of the experiment. The 4th measurement was made on the day of termination of the experiment. Detection limit = 0.1 µmol l^{-1}.*

	Chlorella sp. PO_4^{-3} µmol l^{-1}			*Chlamydomonas* sp. PO_4^{-3} µmol l^{-1}		
Measurement	N-limited	Internal Control	P-limited	N-limited	Internal Control	P-limited
1st	60.8 ± 7.7	61.8 ± 8.2	63.7 ± 4.7	159.8 ± 7.5	163.3 ± 15.5	133.7 ± 21.8
2nd	6.7 ± 1.2	6.3 ± 1.0	7.4 ± 1.2	17.3 ± 3.6	15.1 ± 3.0	14.0 ± 2.4
3rd	1.7 ± 0.7	1.1 ± 1.4	0.4 ± 0.2	1.2 ± 0.4	1.2 ± 0.5	0.7 ± 0.3
4th	0.4 ± 0.3	—	—	1.1 ± 0.9	0.8 ± 1.3	0.2 ± 0.1

— = not detected

All NH_4^+ concentrations were low throughout the experiment, sometimes close to the detection limit (0.1 µg l^{-1} = 0.007 µmol l^{-1} for a single determination, Strickland and Parsons 1972). Marginally higher concentrations were detected in all *Chlamydomonas* sp. cultures compared to *Chlorella* sp. cultures (Table 4.4), except in the first half of the experiment when both species had the highest concentrations of ammonium in the medium.

4.2.4. Alkaline phosphatase activity

4.2.4.1. Specific activity

The initial APA levels, reflecting "normal" conditions with no nutrient limitation, were quite different in the two species. *Chlamydomonas* sp. had significantly higher APA levels ($F_{(1,32)}$ $p<0.0001$) compared to *Chlorella* sp. (Figure 4.4) under comparable nutrient replete conditions.

With the gradually increasing nutrient limitation the APA increased for both species, and at the end of the experiment both species reached the same level of APA (averaged for all treatments, $F_{(1,32)}$ $p>0.05$). In *Chlorella* sp. the external control treatments kept to the initial APA level throughout the experiment, whereas P-limitation triggered the greatest response, exceeding a 100-fold increase (compared to initial levels) in APA for this alga (Figure 4.4). At the end of the experiment, the highest APA was recorded in the P-limited treatment, but there were no significant differences between the treatments (Kruskal Wallis ANOVA $F_{(2,15)}$ $p>0.05$). The average values for the APA do, however, increase with increasing N:P ratio (Figure 4.5).

Chlamydomonas sp. showed an increase in APA also in the control treatment, which was not exposed to nutrient limitation (Figure 4.4). This might indicate that the cells were exposed to some other stress, e.g. osmotic stress, caused by the replacement of culture with fresh medium and possibly causing an indirect induction of APA. The N-limited cells showed the largest response, exhibiting a 20-fold increase in APA. The difference between treatments was statistically significant (ANOVA $F_{(2,13)}$ $p<0.01$, data log-transformed) in respect to APA. A pair-wise comparison showed that the APA in cultures grown in N-limited conditions was significantly higher compared to the cultures grown in P-limitation (Tukey's $p<0.05$) and in non-limiting conditions (Tukey's $p<0.01$). The cultures grown in P-limitation did not differ significantly from internal control cultures (Figure 4.5).

Table 4.4. NH_4^+ concentrations (µmol l^{-1}, mean of six replicate samples, ± standard deviation) in Chlorella *sp. and* Chlamydomonas *sp. cultures during the experiment. 1st measurement refers to batch-culture conditions before the start of dilutions. 2nd and 3rd measurements were determined with 3-4 days intervals during the course of the experiment. The 4th measurement was made on the day of termination of the experiment. Detection limit of the method is 0.007 µmol l^{-1} (for single determination).*

	Chlorella sp. NH_4^+ µmol l^{-1}			*Chlamydomonas* sp. NH_4^+ µmol l^{-1}		
Measurement	N-limited	Internal Control	P-limited	N-limited	Internal Control	P-limited
1st	n.d.	n.d.	n.d.	–	–	–
2nd	3.6 ± 1.5	4.9 ± 2.7	8.1 ± 3.8	5.7 ± 1.3	4.9 ± 1.0	6.1 ± 2.0
3rd	0.8 ± 0.2	1.3 ± 0.7	1.3 ± 0.6	3.8 ± 0.8	3.4 ± 0.3	3.6 ± 0.5
4th	1.6 ± 0.4	2.0 ± 0.5	4.3 ± 2.9	4.6 ± 0.8	4.5 ± 0.7	4.9 ± 0.5

n.d. = not determined
— = not detected

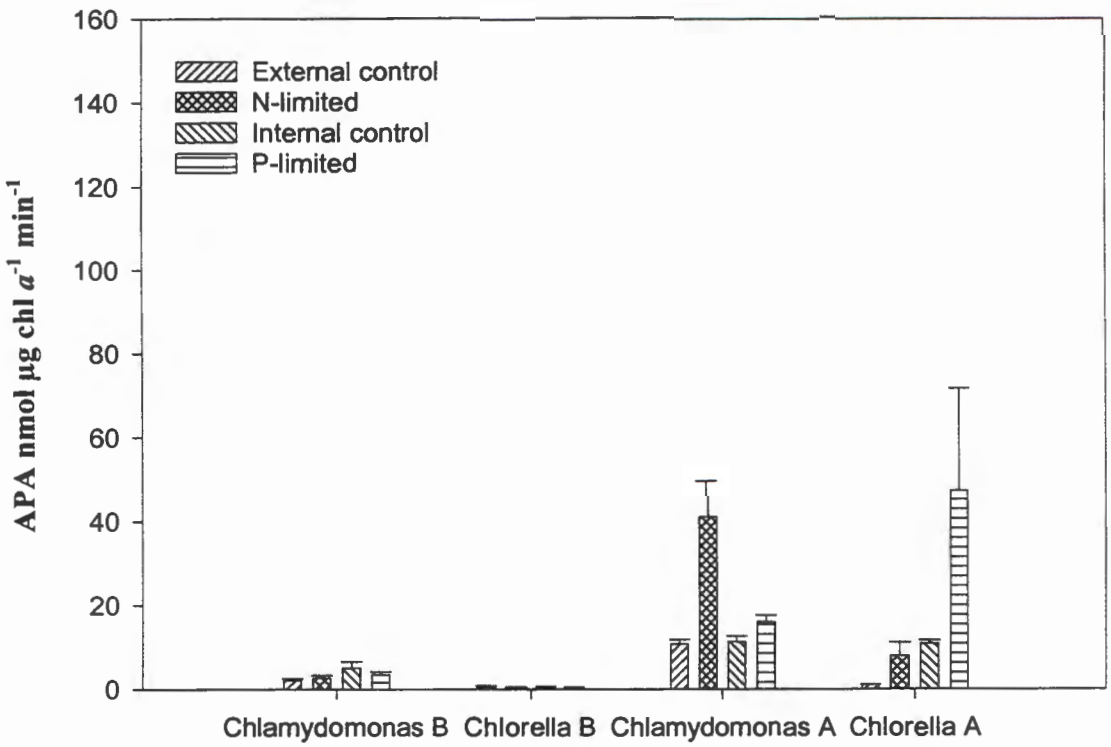


Figure 4.4. Alkaline phosphatase activity (mean and standard error for six samples) for *Chlamydomonas* sp. and *Chlorella* sp. initially (B = before treatment, samples taken on day 13) and after (A = after treatment, samples taken on day 23 and 22 respectively) being exposed to varying N:P ratios (External control = GBG-11 medium).

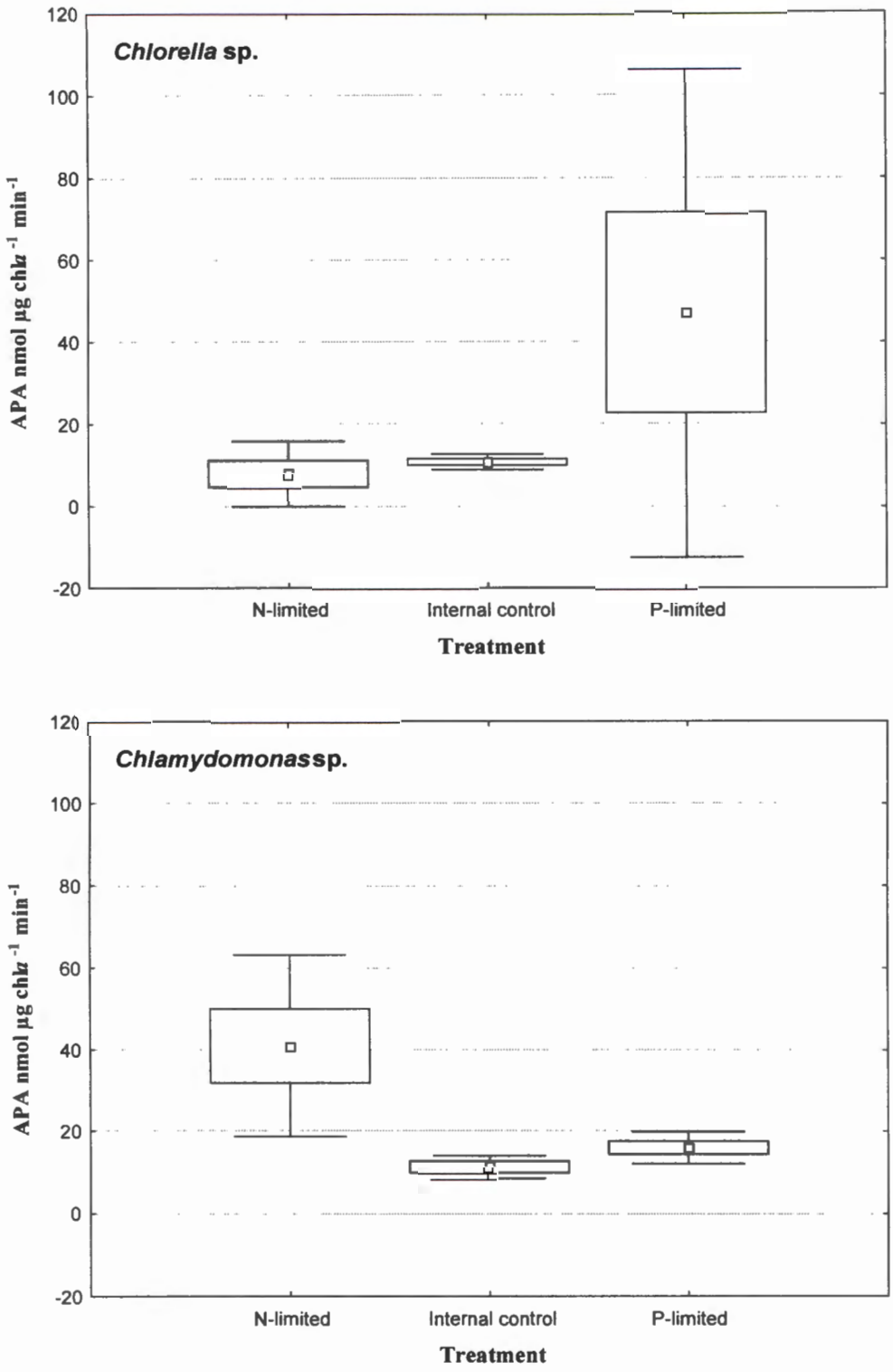


Figure 4.5. Specific alkaline phosphatase activity (middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment) of *Chlorella* sp. and *Chlamydomonas* sp. at the end of the experiment in the three different experimental treatments.

4.2.4.2. Total APA

In *Chlorella* sp. the total APA (µmol l^{-1} min^{-1}) showed the same tendency as the specific APA, but the differences were statistically significant (Figure 4.6). The total APA differed significantly between the treatments (Kruskal Wallis ANOVA, $F_{(2,15)}$ $p=0.01$). When log-transformed, a pair-wise comparison showed that the total APA in cultures grown in N-limited medium was significantly lower than the activity measured under P-limited conditions (Tukey's $p<0.01$) and internal control conditions (Tukey's $p<0.05$). Total APA in the P-limited cells was on average higher than in internal control cells, but no statistically significant difference (Tukey's $p>0.05$) could be shown.

In *Chlamydomonas* sp. the highest total APA were recorded in the N-limited treatment but the activity did not differ significantly between the treatments (Kruskal Wallis ANOVA $F_{(2,14)}$ $p=0.59$).

4.2.5. Acid phosphatase activity

4.2.5.1. Specific activity

The initial AcPA levels behaved in a very similar fashion as the APA, *Chlamydomonas* sp. having significantly ($F_{(1,34)}$ $p<0.0001$) higher initial AcPA than *Chlorella* sp. (Figure 4.7). In *Chlamydomonas* sp. the initial AcPA was higher than the initial APA, and also increased to a higher level than APA in the nutrient limited cultures. Also after the treatments the average level (of all treatments) of AcPA was significantly higher in *Chlamydomonas* sp. ($F_{(1,33)}$ $p<0.0001$) compared to *Chlorella* sp., even though the AcPA in the N-limited treatment in *Chlorella* sp. was higher than AcPA in the internal control and P-limited treatments in *Chlamydomonas* sp. (Figure 4.7). In the *Chlorella* sp. cultures the amount of AcPA was approximately equal to the amount of APA both initially and after stress. Both *Chlamydomonas* sp. and *Chlorella* sp. exhibited highest levels of AcPA in the N-stressed treatments (14-fold and 118-fold increase, respectively), whereas the two other treatments had lesser effects on the AcPA. In *Chlorella* sp., the treatments differed significantly (Kruskal Wallis ANOVA $F_{(2,15)}$ $p<0.01$, Figure 4.8).

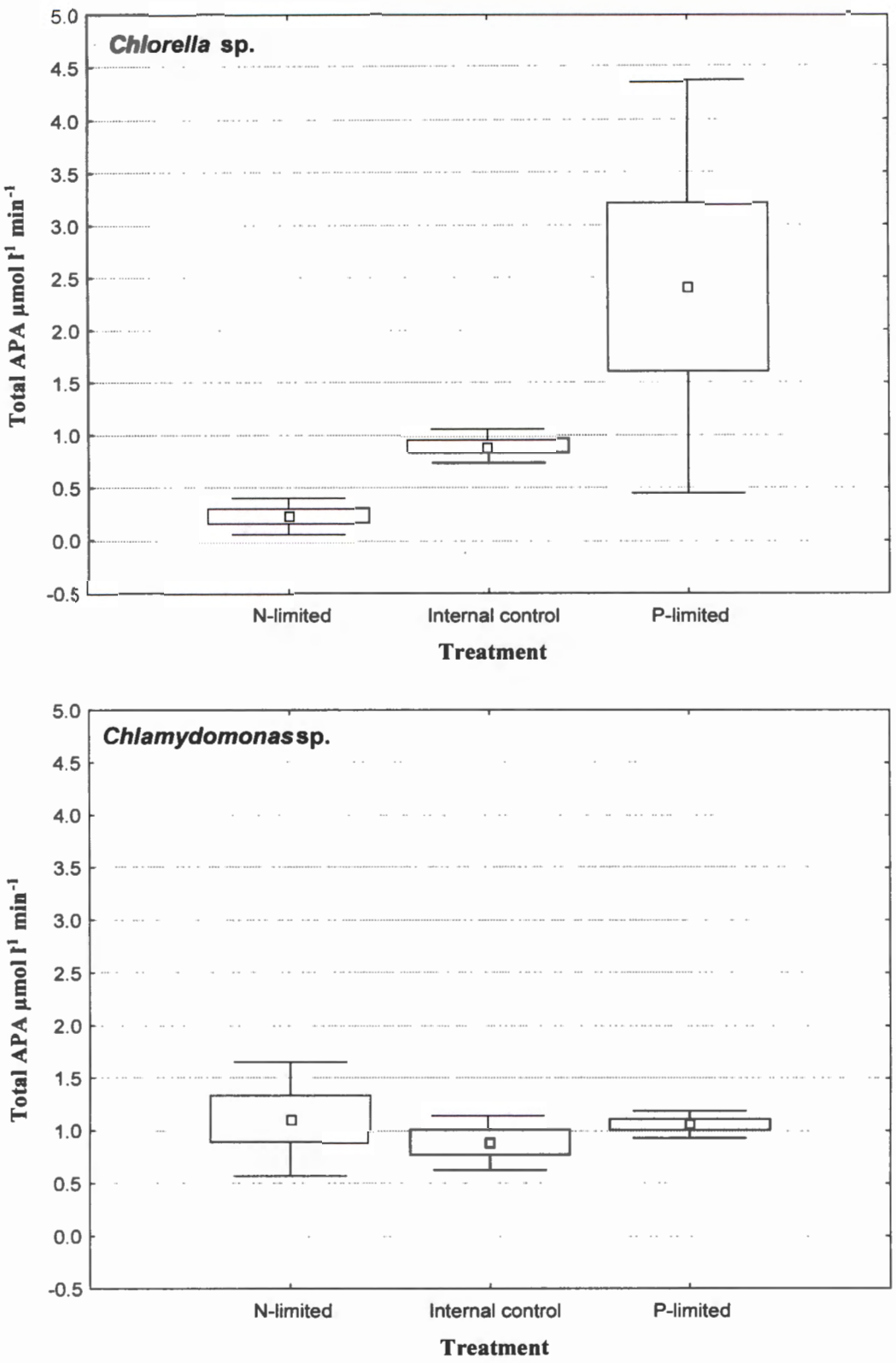


Figure 4.6. Total alkaline phosphatase activity (middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment) of *Chlorella* sp. and *Chlamydomonas* sp. at the end of the experiment in the three different experimental treatments.

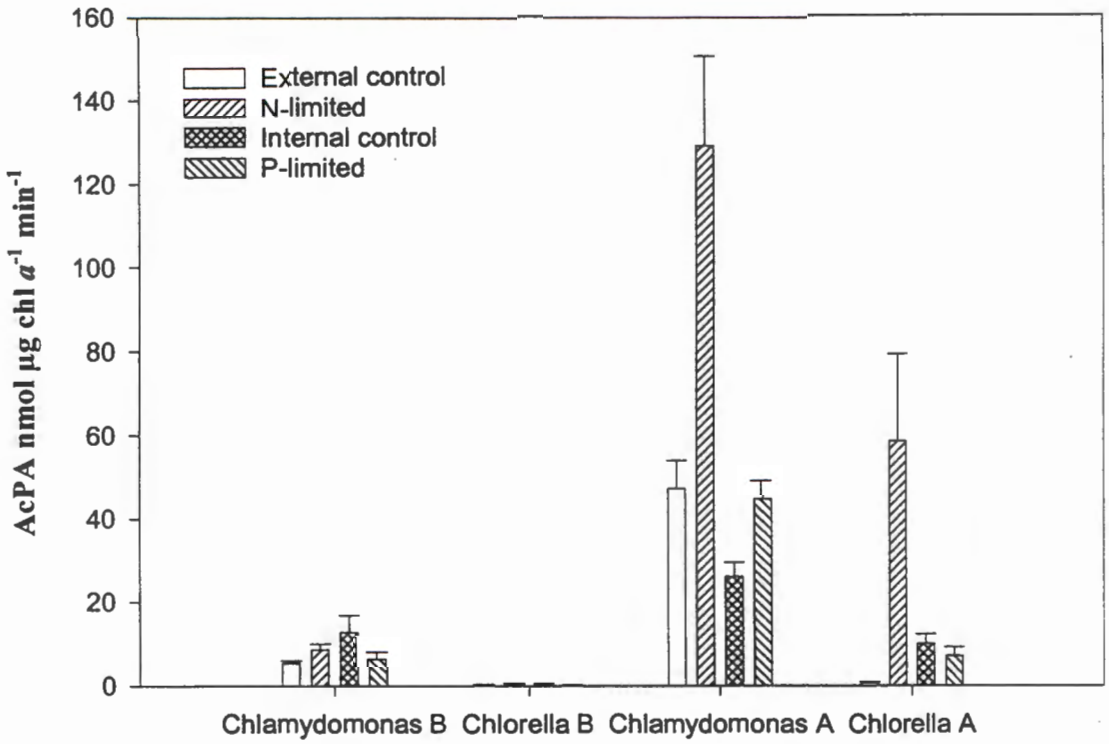


Figure 4.7. Acid phosphatase activity (mean and standard error for six samples) for *Chlamydomonas* sp. and *Chlorella* sp. initially and after being exposed to varying N:P ratios. B = before treatment, samples taken on day 13, A = after treatment, samples taken on day 23 and 22 respectively.

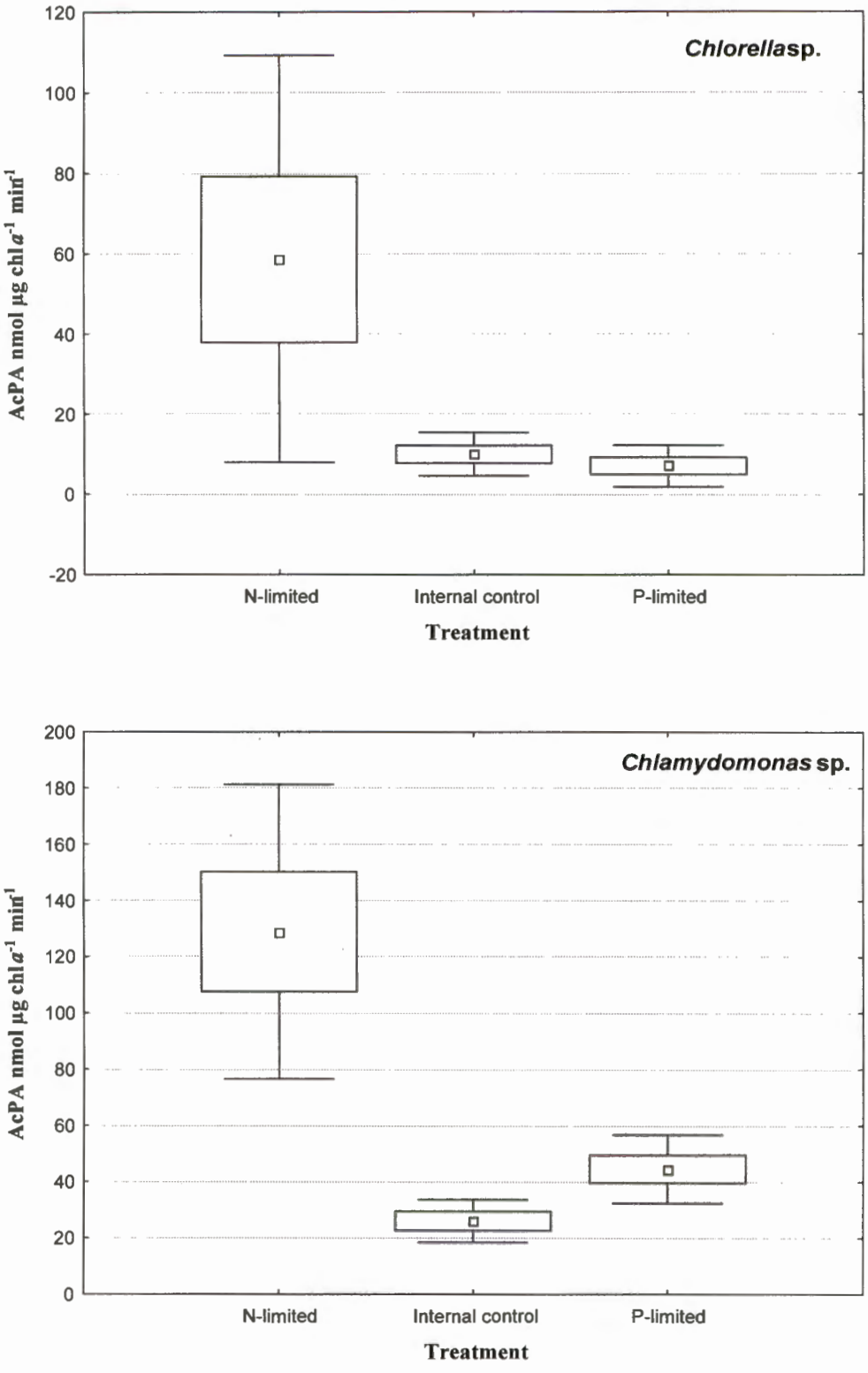


Figure 4.8. Specific acid phosphatase activity (middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment) of *Chlorella* sp. and *Chlamydomonas* sp. at the end of the experiment in the three different experimental treatments. Note different scales on y-axis.

With log-transformed data, a pair-wise comparison showed that the AcPA in cultures grown in N-limited conditions was significantly higher compared to cultures grown in P-limited conditions (Tukey's $p<0.001$) and internal control cultures (Tukey's $p<0.05$). The cultures grown in P-limited conditions did not differ significantly from the internal control cultures (Tukey's $p>0.05$). The same pattern could be seen in *Chlamydomonas* sp. cultures. When the data was log transformed the treatments differed significantly (ANOVA $F_{(2,14)}$ $p<0.001$) in respect to AcPA. A pair-wise comparison showed that the AcPA in cultures grown in N-limited conditions was significantly higher compared to cultures grown in P-limited conditions (Tukey's $p < 0.01$) and internal control cultures (Tukey's $p < 0.001$). The cultures grown in P-limited conditions did not differ significantly from the internal control cultures (Tukey's $p > 0.05$) (Figure 4.8).

4.2.5.2. Total AcPA

In both species the total AcPA varied in the same fashion as the specific AcPA, the highest activities were recorded in the N-limited cultures (Figure 4.9). In *Chlamydomonas* sp. the difference between treatments was not significant ($F_{(2,14)}$ $p = 0.059$), whereas in *Chlorella* sp. the results of the treatments differed significantly (Kruskal Wallis ANOVA $F_{(2,15)}$ $p<0.01$). With log-transformed data a pair-wise comparison showed that the AcPA in cultures grown in N-limited conditions was significantly higher compared to cultures grown in P-limited conditions (Tukey's $p<0.001$) and internal control cultures (Tukey's $p<0.05$). The cultures grown in P-limited conditions did not differ significantly from the internal control cultures (Tukey's $p>0.05$). Thus the total activities, for both APA and AcPA, support the findings in the specific fraction.

4.2.6. Temporal changes in enzyme activity

In *Chlorella* sp. both APA and AcPA was monitored half-way (day 18) through the experiment (Figure 4.10). There was a slight, albeit noticeable, difference between APA and AcPA; APA increasing in an earlier stage of the nutrient-stress-treatment, whereas AcPA increased intensively at the end.

4.2.7. Ectoenzyme activities

Ectoenzyme activity was only measured in *Chlamydomonas* sp., because the equipment for ectoenzyme activity measurement was not available at the time of the *Chlorella* sp. experiment.

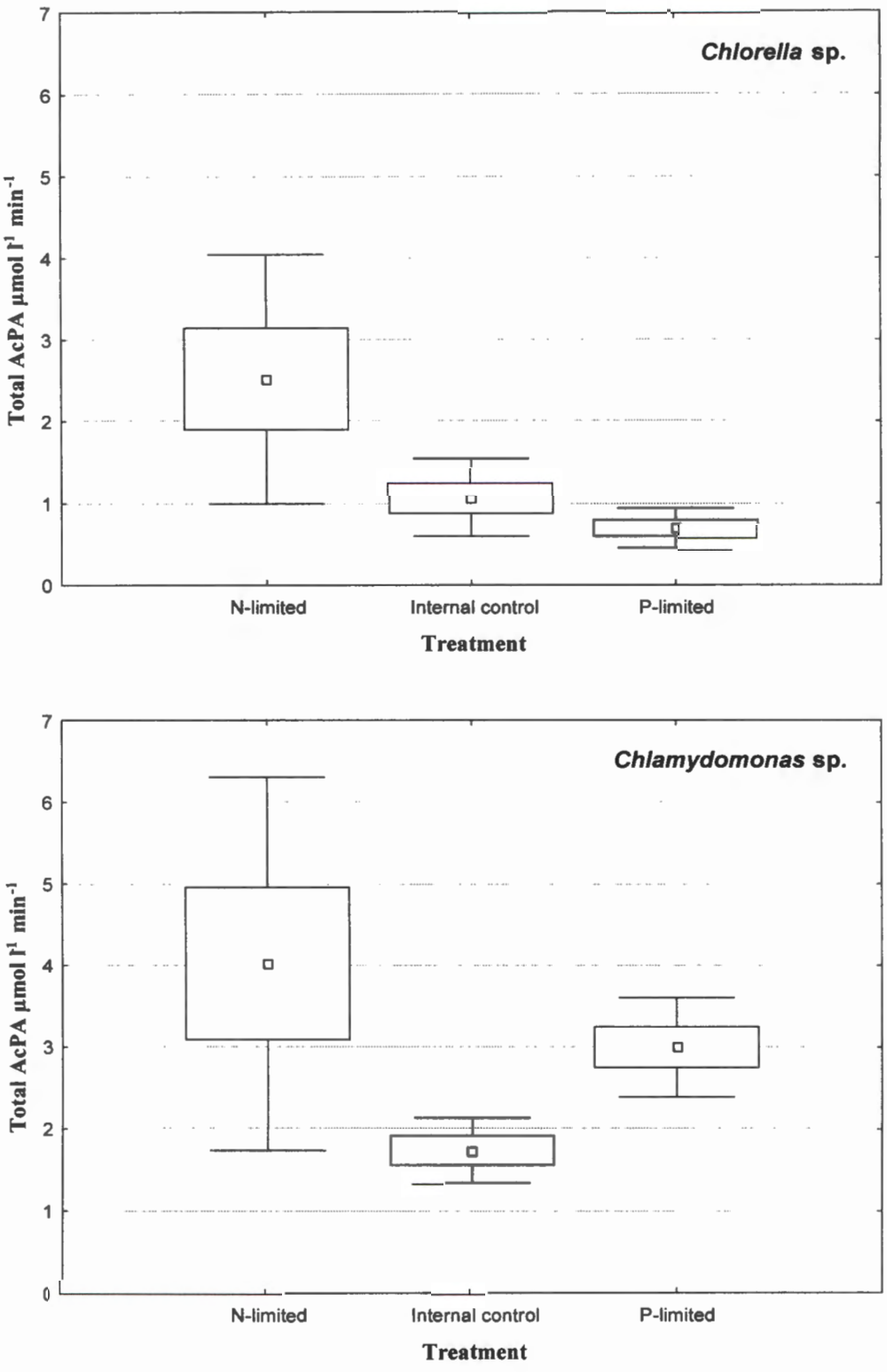


Figure 4.9. Total acid phosphatase activity (middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment) of *Chlorella* sp. and *Chlamydomonas* sp. at the end of the experiment in the three different experimental treatments.

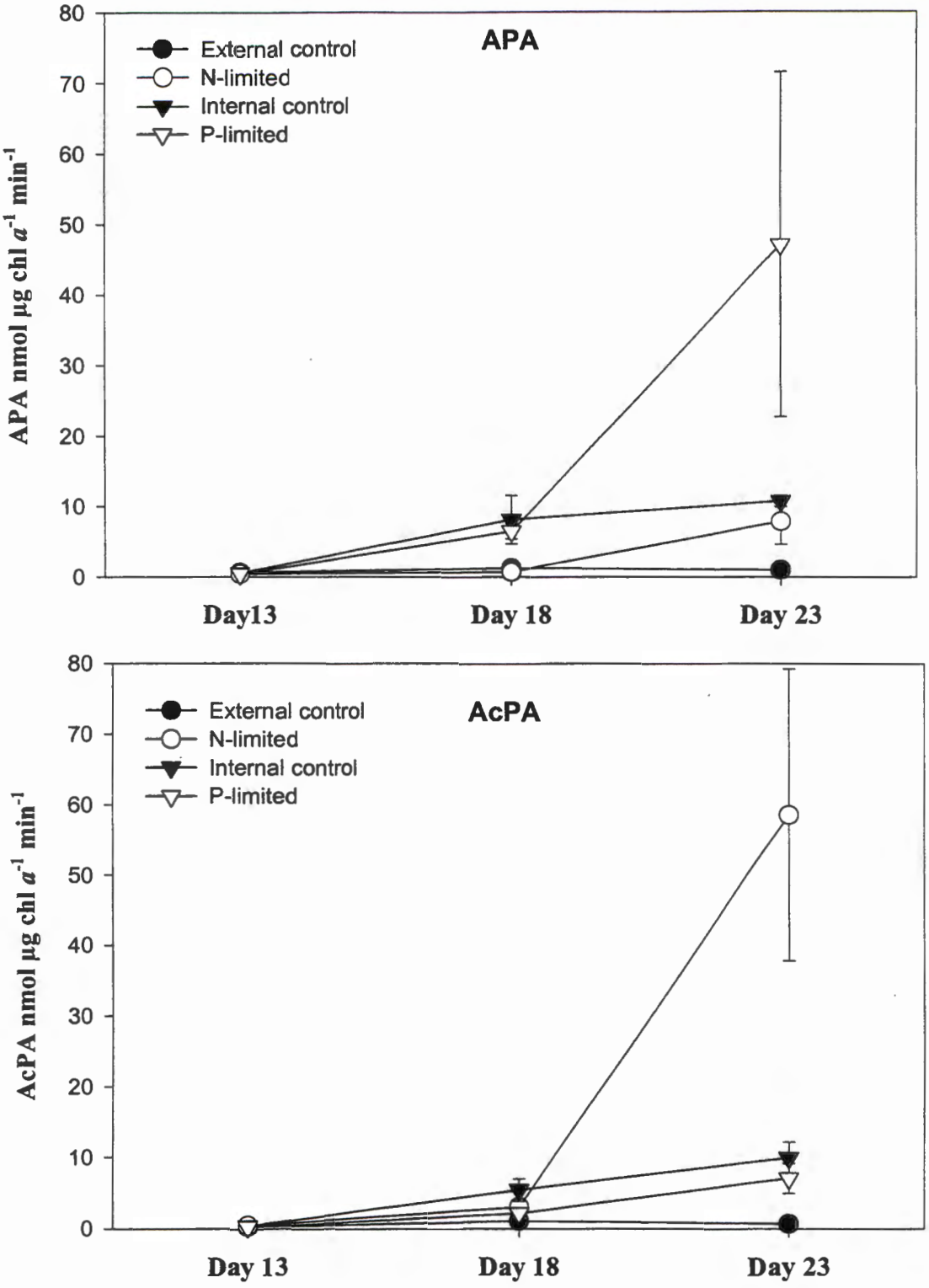


Figure 4.10. Alkaline (APA) and acid (AcPA) phosphatase activity (mean and standard error, n = 6) in *Chlorella* sp. cultures before (Day 13), in the middle of (Day 18) and after (Day 23) treatments during the experiment.

The total ecto-enzymatic phosphatase activities increased significantly in the nutrient stressed treatments compared to the external control treatment (Kruskal Wallis ANOVA p=0.0047, data not shown), even though some activity existed also in the control treatments. Between the treatments (Figure 4.11) no significant difference was detected (Kruskal Wallis ANOVA $F_{(2,15)}$ $p>0.05$).

The specific ectoenzymatic phosphatase activities also increased significantly in the treatments compared to the external control (Kruskal Wallis ANOVA $p = 0.0003$, data not shown) and the N-limited treatment exhibited significantly higher ecto-phosphatase activities (Figure 4.11) compared to the other treatments (Kruskal Wallis ANOVA $p = 0.004$).

4.2.8. Nitrate reductase activity

Nitrate reductase (NR) activity was only detected in *Chlamydomonas* sp. (Figure 4.12). Initially, in high N and P concentrations, the NR levels were low – indicating little need for uptake of NO_3^-. The NR activity increased markedly when NO_3^- became scarce at the end of the experiment. There was no statistically significant difference in the NR activities (Figure 4.13) between the treatments (Specific NR ANOVA $F_{(2,14)}$ $p = 0.428$, total NR ANOVA $F_{(2,14)}$ $p = 0.874$).

4.2.9. Correlation between enzyme activity and nutrient concentration

All enzyme activities are summarised in Table 4.5. Since most data were not normally distributed, and for example PO_4^{-3} data, because of the set-up of the experiment, was concentrated to two groups (0.2 and 2.0 µmol l^{-1} respectively), only non-parametric Spearman Rank Correlations were performed. Scatterplots between specific enzyme activities and nutrient concentrations in *Chlorella* sp. are shown in Figure 4.14, with results of the correlation in a correlation matrix in Table 4.6. No correlation between AcPA and APA could be detected. APA decreased with increasing PO_4^{-3} concentration in the medium, whereas in AcPA a positive correlation was shown. AcPA decreased with increasing NH^+_4 concentrations.

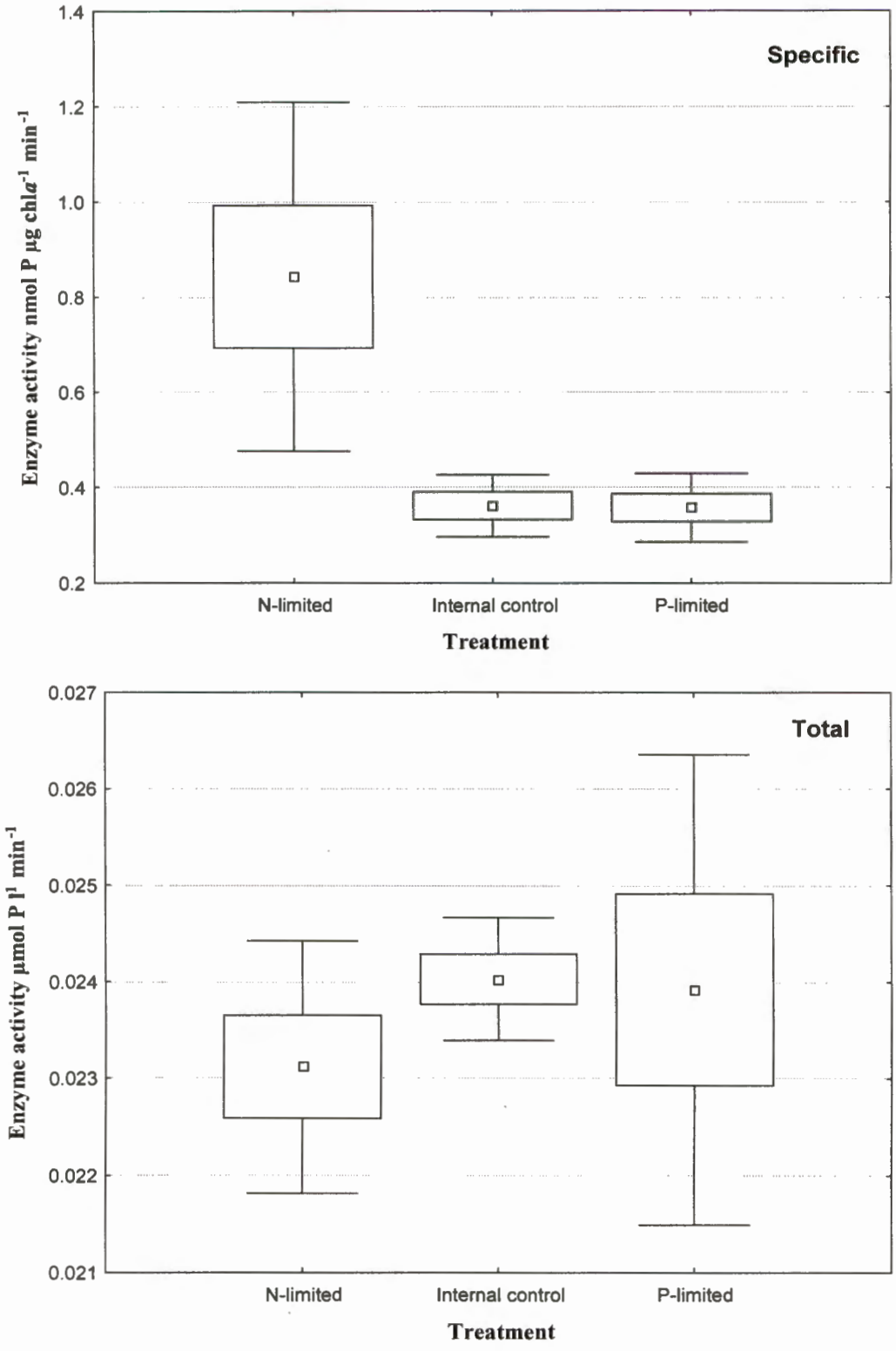


Figure 4.11. Specific and total ecto-enzymatic phosphatase activity (middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment) in *Chlamydomonas* sp. in the three experimental treatments at the end of the experiment.

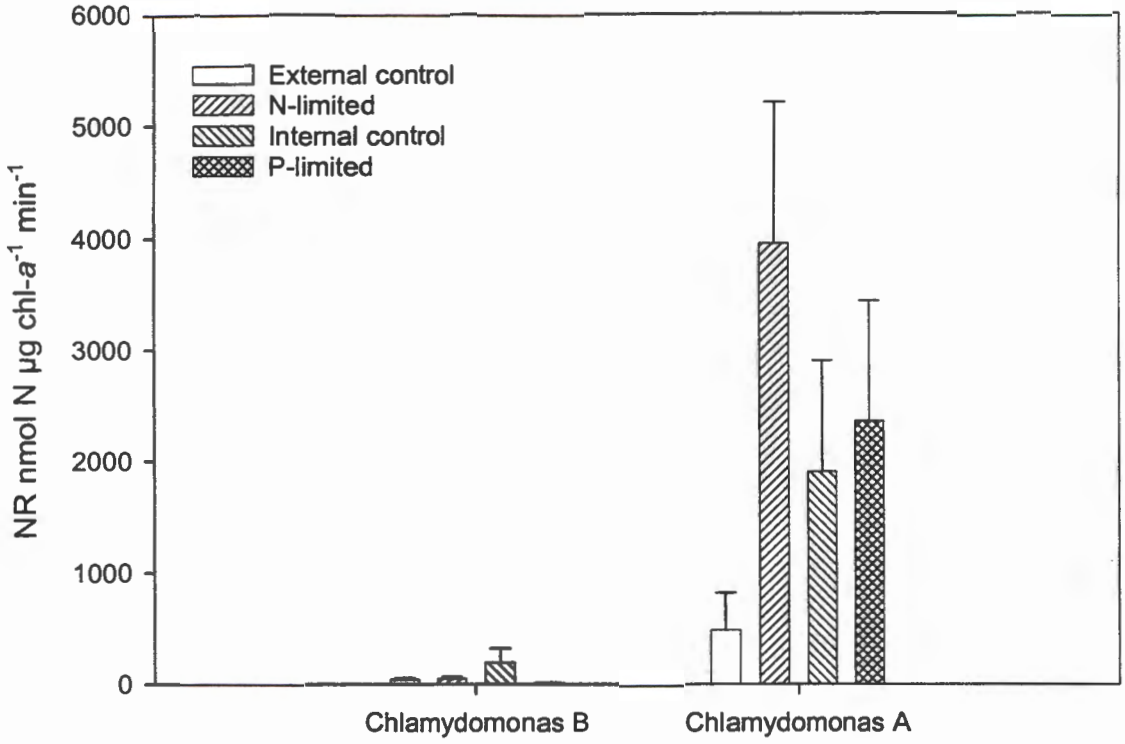


Figure 4.12. Nitrate reductase activity (mean and standard error for six samples) for *Chlamydomonas* sp. initially and after being exposed to varying N:P ratios. B = before treatment (samples taken on day 13), A = after treatment (samples taken on day 22).

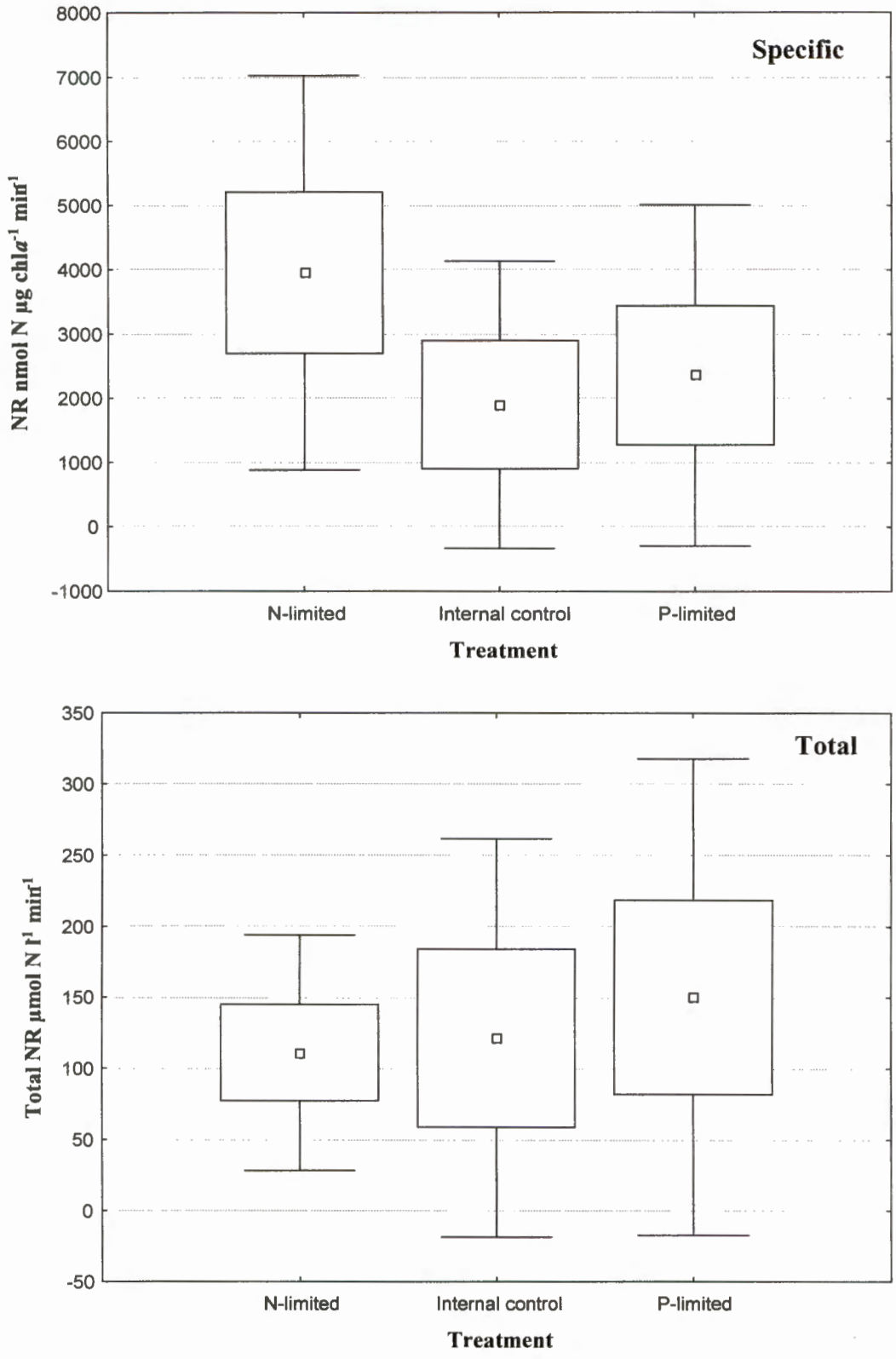
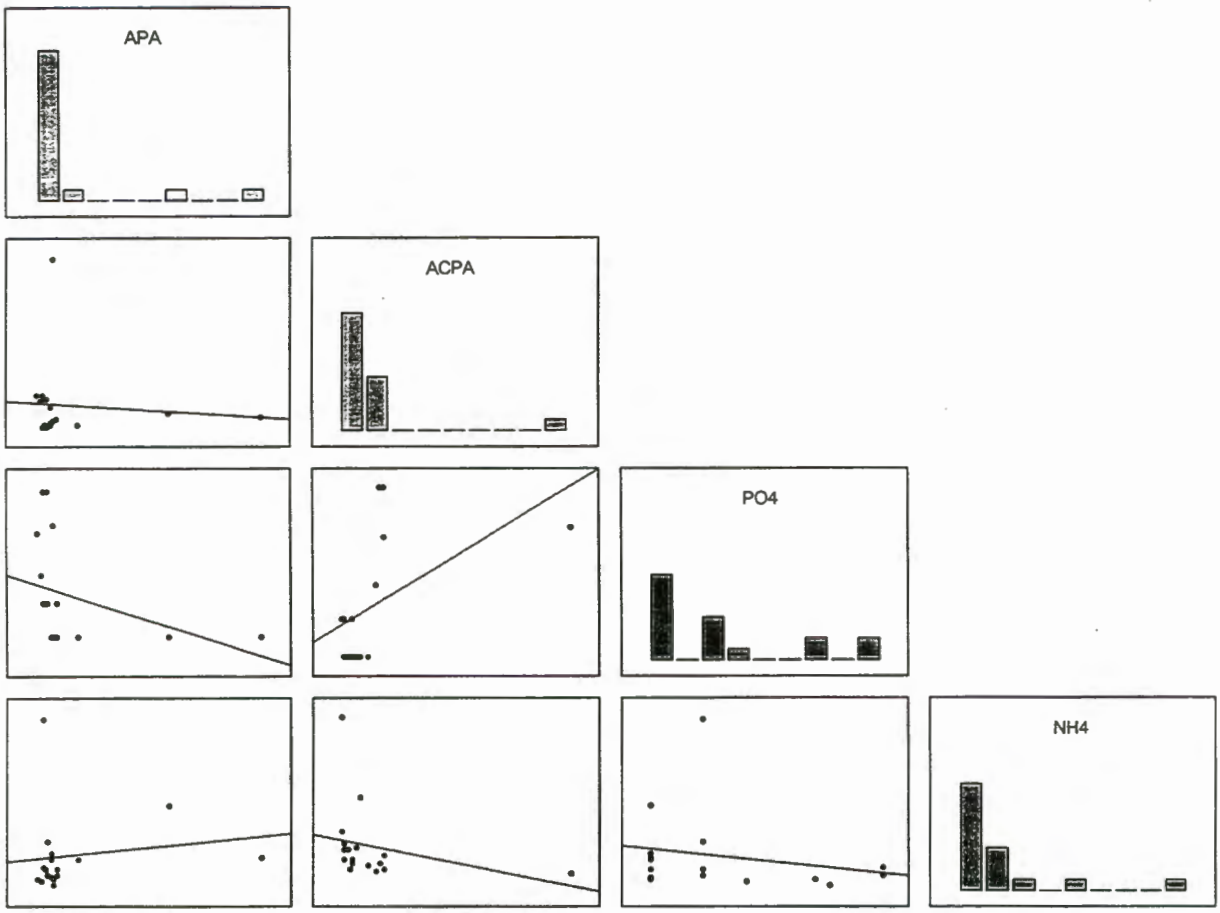


Figure 4.13. Specific and total nitrate reductase (NR) activity (middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment) in *Chlamydomonas* sp. in the three experimental treatments at the end of the experiment.

Table 4.5. Specific (nmol P µg chl a^{-1} min^{-1}) and total (µmol P l^{-1} min^{-1}) enzyme activities (alkaline phosphatase activity (APA), acid phosphatase activity (AcPA), ectoenzymatic PA (ecto) and nitrate reductase (NR)) in Chlorella *sp. and* Chlamydomonas *sp. in treatments with varying N:P ratios (mean values ± standard error of six replicates). Samples from removed media were taken at day 23 (*Chlorella *sp.) and day 22 (*Chlamydomonas *sp.).*

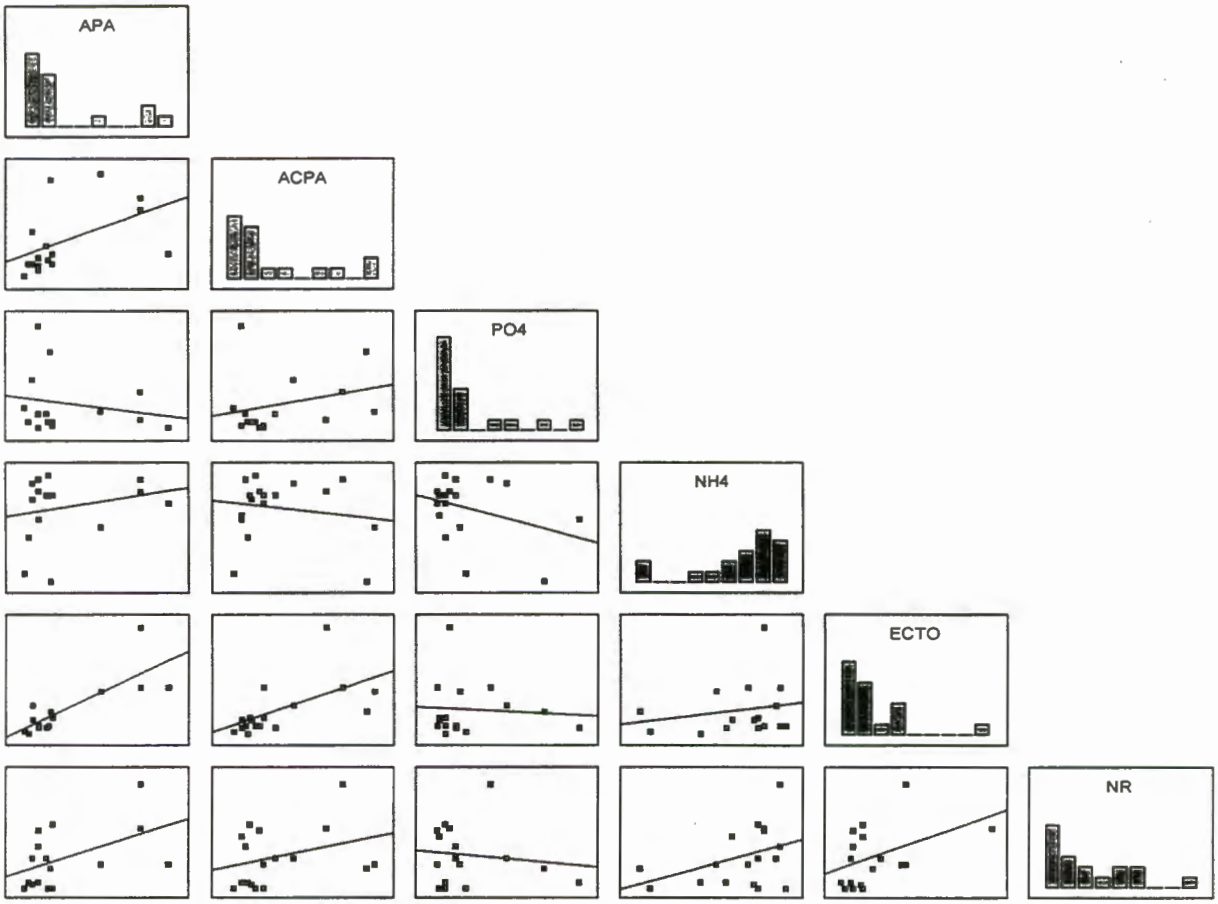
		***Chlorella* sp.**			***Chlamydomonas* sp.**		
		N-limited	Internal Control	P-limited	N-limited	Internal Control	P-limited
APA	specific	7.89 ± 7.97	10.81 ± 1.93	47.13 ± 59.65	40.92 ± 22.23	11.26 ± 2.79	15.98 ± 3.90
	total	0.2 ± 0.2	0.9 ± 0.2	2.4 ± 2.0	1.1 ± 0.5	0.9 ± 0.3	1.1 ± 0.1
AcPA	specific	58.59 ± 50.64	10.00 ± 5.44	7.09 ± 5.19	128.85 ± 52.37	25.99 ± 7.66	44.63 ± 12.21
	total	2.5 ± 1.5	1.1 ± 0.5	0.7 ± 0.2	4.0 ± 2.3	1.7 ± 0.4	3.0 ± 0.6
Ecto	specific	n.d.	n.d.	n.d.	0.027 ± 0.01	0.01 ± 0.005	0.012 ± 0.002
	total	n.d.	n.d.	n.d.	0.0007 ± 0	0.0008 ± 0	0.0008 ± 0
NR	specific	—	—	—	3953 ± 3072	1902 ± 2233	2357 ± 2652
	total	—	—	—	111 ± 83	122 ± 140	150 ± 168

n.d. = not determined
— = not detected



	APA	AcPA	PO_4	NH_4
APA	1.0000			
AcPA	-0.1227	1.0000		
PO_4	***-0.6740***	***0.4928***	1.0000	
NH_4	0.2297	***-0.6408***	-0.3862	1.0000

Figure 4.14 and Table 4.6. Scatterplots illustrating the presence and absence of correlation between the measured specific enzyme activities and chemical variables in the three experimental treatments in *Chlorella* sp. at the end of the experiment. Scatterplots present the variables shown (as histograms presenting the distribution of the data) on top (y-axis) and to the right (x-axis) of each plot. Spearman correlation coefficients (R) are given in the table, statistically significant ($p < 0.05$) values are indicated with bold italic numbers. N = 18.



	APA	AcPA	PO_4	NH_4	Ecto	NR
APA	1.0000					
AcPA	***0.6393***	1.0000				
PO_4	-0.2401	0.2249	1.0000			
NH_4	0.2047	0.1612	-0.1514	1.0000		
Ecto	***0.7873***	***0.7075***	-0.0414	0.2017	1.0000	
NR	0.4240	0.2528	-0.0705	0.4010	0.4401	1.0000

Figure 4.15 and Table 4.7. Scatterplots illustrating the presence and absence of correlation between the measured specific enzyme activities and chemical variables in the three experimental treatments in *Chlamydomonas* sp. at the end of the experiment. Scatterplots present the variables shown (as histograms presenting the distribution of the data) on top (y-axis) and to the right (x-axis) of each plot. Spearman correlation coefficients (R) are given in the table, statistically significant ($p < 0.05$) values are indicated with bold italic numbers. N = 18.

In *Chlamydomonas* sp. (Figure 4.15 and Table 4.7) all enzymes (APA, AcPA, ectoenzymatic phosphatase activities and NR) were positively correlated, but none were significantly correlated to PO_4^{-3} concentration. The scatterplots show, however, that the highest ectoenzyme and APA did coincide with low PO_4^{-3} concentrations, whereas no such trend could be shown in AcPA.

Chlorophyll-*a* concentrations were, as expected, negatively correlated with all enzyme activities, but are not shown because of the interrelationship between chlorophyll-*a* and specific enzyme activities. All correlations shown were done using only the data from the three treatments, excluding the external control because of considerably higher nutrient concentrations in these cultures, which would affect the results significantly. The results (not shown) from correlations including the external control, however, showed the same patterns in the relationships between the parameters, and the statistical significance of the correlations was higher.

4.2.10. Chlorophyll-*a* fluorescence

The *in vivo* chlorophyll-*a* fluorescence kinetics allows a description of the vitality and photosynthetic performance of the studied green algae (Strasser *et al.* 1999). Comparison of fluxes and yields derived from the values provided from the JIP-test, showed significant differences between most derivatives, including fluorescence signals and their ratios, antenna size (ABS/RC), primary photochemistry (φP_0, light reaction = F_V/F_M), electron transport (φE_0, dark reaction) and performance index between the two species in the external control (Table 4.8). The performance index was higher in *Chlorella* sp. than in *Chlamydomonas incerta* in the GBG-11 medium.

In Tables 4.9 and 4.10 differences between the treatments in photosynthetic parameters can be seen. The growth in N or P limited medium also affected the energy yields in both *Chlorella* sp. and *Chlamydomonas* sp. (Tables 4.8. and 4.9). The primary photochemistry (light reactions = F_V/F_M ratio, often used as an indicator of nutrient stress) in *Chlamydomonas* sp. cells differed between treatments (ANOVA $F_{(3,113)}$ p = 0.0000) – being significantly lower in the P-limited treatment compared to the other treatments (Tukey's *post hoc* test for unequal N p = 0.0000). In *Chlorella* sp. the N-limited cells showed lowest values for primary photochemistry (Kruskal Wallis ANOVA $H_{(2,27)}$ p = 0.0000).

Table 4.8. Differences between the fluorescence signal and derivations thereof in the external control treatment (grown in GBG-11 medium) of Chlorella *sp. (n = 18) and* Chlamydomonas *sp. (n = 27) (p-values based on ANOVA or Kruskal-Wallis ANOVA, depending on normality of the data). For explanations of the abbreviations in the first column, refer to Chapter 2.*

Fluorescence signal / yields / derivations	***Chlorella* sp.**		***Chlamydomonas* sp.**		**ANOVA**
	Average	St. deviation	Average	St. deviation	
F_0/F_M	0.377	0.02	0.495	0.04	p=0.0000
F_V/F_0	1.661	0.17	1.033	0.17	p=0.0000
Quantum efficiencies (flux ratios)					
φP_0 (= F_V/F_M)	0.623	0.02	0.505	0.04	p=0.0000
Ψ_0	0.493	0.01	0.619	0.04	p=0.0000
φE_0	0.307	0.02	0.311	0.02	p=0.467
φD_0	0.377	0.02	0.495	0.04	p=0.0000
Specific fluxes					
ABS/RC	4.811	0.35	7.422	0.64	p=0.0000
TR/RC	2.988	0.10	3.723	0.18	p=0.0000
ET/RC	1.472	0.04	2.300	0.11	p=0.0000
DI/RC	1.823	0.25	3.699	0.61	p=0.0000
Vitality indexes					
PI_{ABS}	3.417	0.67	2.286	0.43	p=0.0000

Table 4.9. Differences between the fluorescence signal and derivations thereof in the three experimental treatments at the end of the experiment in Chlorella *sp. (p-values based on Kruskal-Wallis ANOVA). For explanations of the abbreviations in the first column, refer to Chapter 2. For results concerning statistical differences between the treatments, refer to text.*

	N-limited		Internal Control		P-limited		ANOVA
	Average	St. Deviation	Average	St. Deviation	Average	St. Deviation	
F_0/F_M	0.762	0.01	0.698	0.02	0.725	0.02	p=0.0001
F_V/F_0	0.312	0.02	0.432	0.04	0.381	0.04	p=0.0001
Quantum efficiencies (flux ratios)							
$\varphi P_0 (= F_V/F_M)$	0.238	0.01	0.301	0.02	0.275	0.02	p=0.0001
Ψ_0	0.546	0.02	0.559	0.02	0.588	0.02	p=0.0027
φE_0	0.130	0.01	0.168	0.01	0.161	0.01	p=0.0001
φD_0	0.762	0.01	0.699	0.02	0.725	0.02	p=0.0001
Specific fluxes							
ABS/RC	10.492	0.37	9.217	0.32	9.410	0.26	p=0.0000
TR/RC	2.494	0.09	2.773	0.08	2.586	0.13	p=0.0003
ET/RC	1.361	0.05	1.550	0.02	1.518	0.03	p=0.0000
DI/RC	7.998	0.38	6.444	0.38	6.824	0.37	p=0.0001
Vitality indexes							
PI_{ABS}	0.359	0.03	0.595	0.04	0.576	0.03	p=0.0001

Table 4.10. Differences between the fluorescence signal and derivations thereof in the three experimental treatments at the end of the experiment in Chlamydomonas *sp. (p-values based on ANOVA or Kruskal-Wallis ANOVA, depending on normality of the data). For explanations of the abbreviations in the first column, refer to Chapter 2. For results concerning statistical differences between the treatments, refer to text.*

	N-limited		Internal Control		P-limited		ANOVA
	Average	St. Deviation	Average	St. Deviation	Average	St. Deviation	
F_0/F_M	0.590	0.03	0.573	0.03	0.628	0.03	p=0.0000
F_V/F_0	0.698	0.09	0.748	0.09	0.596	0.07	p=0.0000
Quantum efficiencies (flux ratios)							
φP_0 (= F_V/F_M)	0.410	0.03	0.426	0.03	0.372	0.03	p=0.0000
Ψ_0	0.616	0.04	0.636	0.03	0.654	0.05	p=0.0009
φE_0	0.253	0.03	0.271	0.02	0.244	0.03	p=0.0004
φD_0	0.590	0.03	0.574	0.03	0.628	0.03	p=0.0000
Specific fluxes							
ABS/RC	8.855	1.20	8.485	1.09	11.033	1.81	p=0.0000
TR/RC	3.596	0.28	3.590	0.25	4.061	0.41	p=0.0000
ET/RC	2.211	0.16	2.279	0.16	2.654	0.33	p=0.0000
DI/RC	5.259	0.95	4.895	0.86	6.972	1.42	p=0.0000
Vitality indexes							
PI_{ABS}	1.356	0.47	1.601	0.41	1.114	0.43	p=0.0002

In both species, P-limited cells showed highest values for the dark reaction, in *Chlamydomonas* sp. differing significantly from the N-limited treatment (Tukey's *post hoc* test for unequal N p=0.0046) but not from the internal control (Tukey's *post hoc* test for unequal N $p > 0.3$). In *Chlorella* sp. the treatments differed significantly (Kruskal Wallis ANOVA $H_{(2,27)}$ $p < 0.01$) in dark reaction function. The maximum yield for electron transport was highest in the internal control in both species. In *Chlamydomonas* sp. it differed significantly from the N and P-limited treatments (Tukey's *post hoc* test for unequal N $p = 0.0312$ and $p = 0.0004$, respectively), in *Chlorella* sp. the treatments differed significantly (Kruskal Wallis ANOVA $H_{(2,27)}$ $p < 0.001$). Similarly, in both species dissipation showed the inverse, being lowest in the internal control. In *Chlamydomonas* sp. the dissipation in the N-limited treatment was significantly lower than in the P-limitation (Tukey's *post hoc* test for unequal N $p < 0.001$), in *Chlorella* sp. the treatments differed significantly (Kruskal Wallis ANOVA $H_{(2,27)}$ $p < 0.001$).

Specific energy fluxes varied between the treatments. In *Chlamydomonas* sp. the antenna size (ABS/RC) was highest in P-limited cells (Kruskal Wallis ANOVA $H_{(3,117)}$ $p < 0.001$), whereas in *Chlorella* sp. the N-limited cells had largest antenna size, the treatments differing significantly (Kruskal Wallis ANOVA $H_{(2,27)}$ $p < 0.001$). In *Chlamydomonas* sp. P-limited cells had more efficient trapping of energy (TR/RC) (ANOVA $F_{(3,113)}$ $p < 0.001$) compared to the other treatments (Tukey's *post hoc* test for unequal N $p < 0.001$) whereas the N-limited cells did not differ significantly from the internal control. In *Chlorella* sp. the internal control exhibited most efficient trapping of energy (Kruskal Wallis ANOVA $H_{(2,27)}$ $p < 0.001$). In *Chlamydomonas* sp. the dissipation and electron transport were also the highest in the P-limited cells (Kruskal Wallis ANOVA $H_{(3,117)}$ $p < 0.001$). In *Chlorella* sp. dissipation was lowest in the internal control, and highest in the N-limited treatment (Kruskal Wallis ANOVA $H_{(2,27)}$ $p < 0.001$) whereas electron transport was highest in the internal control, and lowest in the N-limited treatment (Kruskal Wallis ANOVA $H_{(2,27)}$ $p < 0.001$).

The vitality of the cells, measured by PI_{ABS}, express the accumulation of all responses of the PSII apparatus, and can be regarded as the most reliable criterion for the evaluation of the effects of stress treatment (Srivastava *et al.* 1999, Strasser *et al.* 1999). In *Chlamydomonas* sp. PI_{ABS} was the highest in the internal control, which differed significantly from P-limitation (expressing lowest PI_{ABS}) (Tukey's *post hoc* test for unequal N $p < 0.001$).

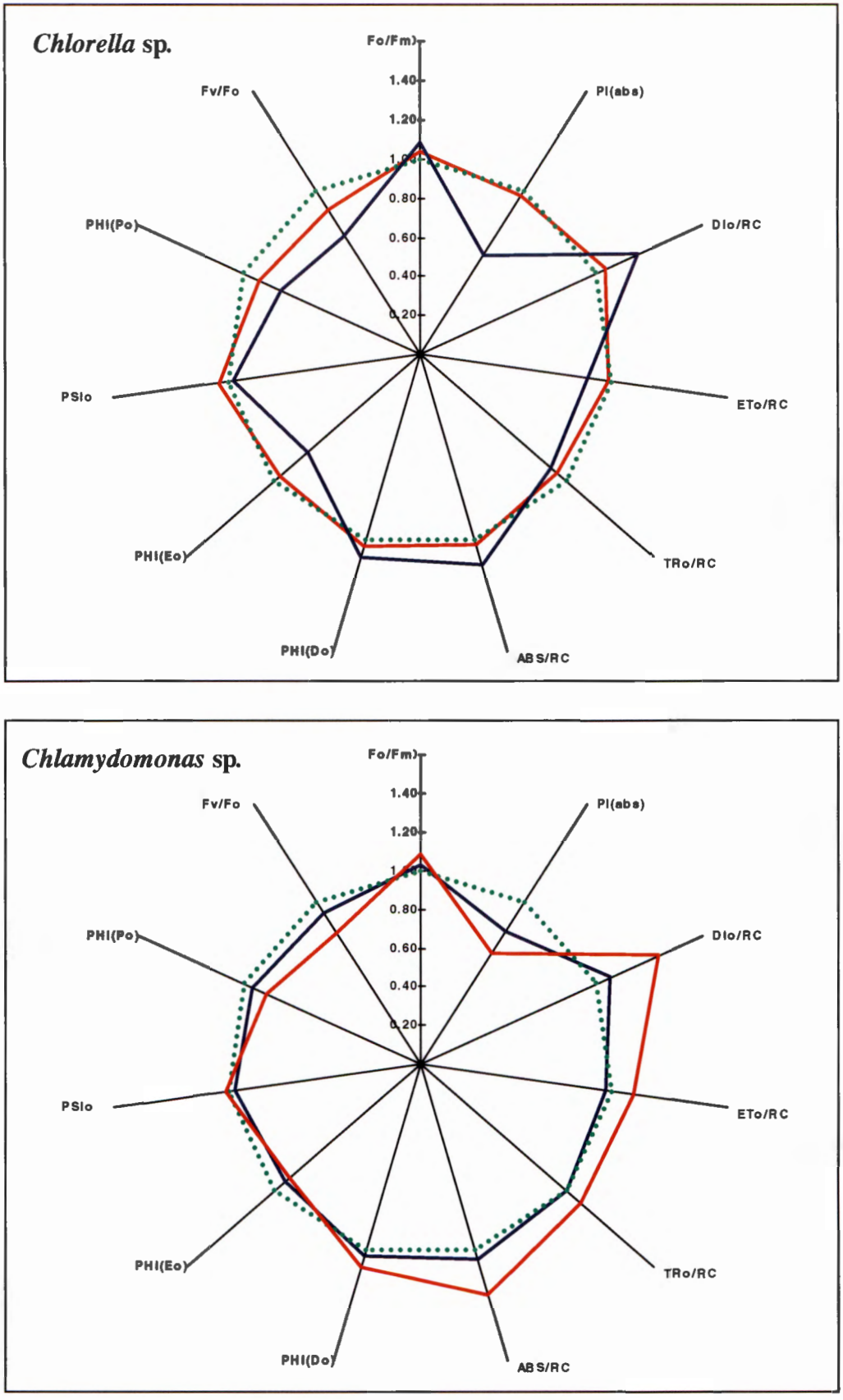


Figure 4.16. Flux ratios, specific energy fluxes and vitality of PSII functions in *Chlorella* sp. and *Chlamydomonas* sp. grown in different N:P ratios. Red line = N-limited treatment, blue line = P-limited treatment and green line = Internal control.

There was, however, no significant difference between N-limitation and P-limitation, or N-limitation and internal control (Tukey's *post hoc* test for unequal N $p > 0.05$). Also in *Chlorella* sp. the PI_{ABS} was highest in the internal control, but lowest in the N-limited treatment (Kruskal Wallis ANOVA $H_{(2,27)}$ $p < 0.001$).

The specific and phenomenological energy flux ratios are summarised in the form of a spider plot in Figure 4.16, illustrating especially the detrimental effects of N-limitation on the specific flux ratios and the PI in *Chlamydomonas* sp. cells.

4.2.11. Summary of phosphatase activities and photosynthetic vitality

The results on the photosynthetic vitality observations were compared with phosphatase activity values. All values were normalised against the internal control, to investigate the effect of N and P-limitation. The decrease of the vitality can be presented as "biophysical stress" whereas the increase in phosphatase activity may be described as "biochemical stress".

A synthesis of the chlorophyll-*a* fluorescence APA and AcPA results is shown schematically in Figure 4.17, and three main results have been indicated in the schematic presentation:

A. A decrease in photosynthetic performance is related with an increase in the AcPA of *Chlorella* sp. and *Chlamydomonas* sp. in both N and P-limitation. This suggests that AcPA may be used as an indicator of the decrease of vitality of the cells, because of the applied nutritional stress condition. In *Chlamydomonas* sp. the activity of the APA responded to nutritional stress similar to that of the AcPA.
B. N-limitation severely decreased the photosynthetic performance in both algae, especially in *Chlorella* sp., in which AcPA responded as described in A (above), whereas no induction of APA was detected.
C. In *Chlorella* sp. the APA increased more than 4-fold under P-limitation while the photosynthetic performance (fitness of the cells) was nearly unchanged. This probably illustrates the capacity of this species to compensate for the P-limitation (by increasing its APA) efficiently enough to maintain it's fitness in this condition.

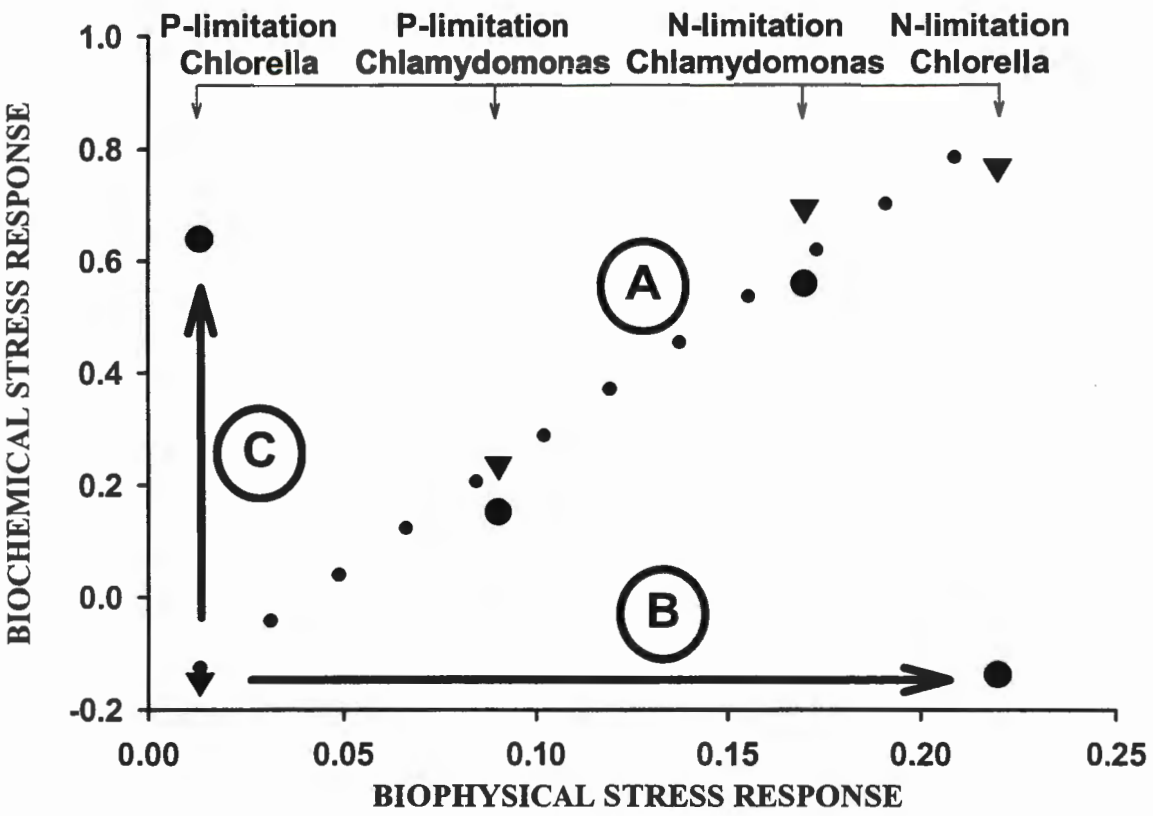


Figure 4.17. Relationship (dotted line) between the photosynthetic performance index (x-axis) and enzyme activities (y-axis) in *Chlorella* sp. and *Chlamydomonas* sp. grown in N or P-limitation (arrows on top). APA is indicated with filled circles, AcPA with filled triangles. Processes indicated by A, B and C are discussed in the text.

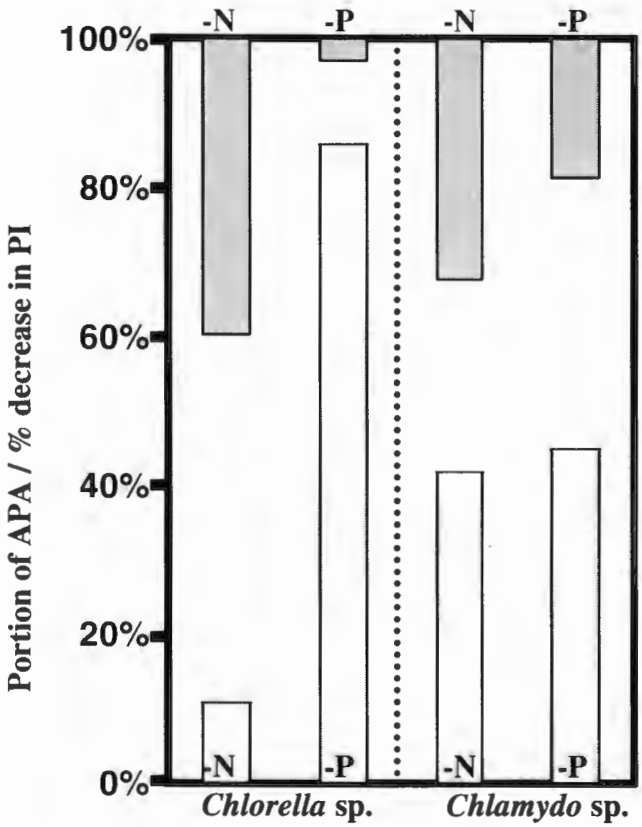


Figure 4.18. The percentage of APA of the total (APA+AcPA) activity (lower bars) and percentage decrease of the PI or vitality (hanging bars) in comparison to the internal control, in N- and P-limited treatments of *Chlorella* sp. and *Chlamydomonas* sp. (*Chlamydo* sp.).

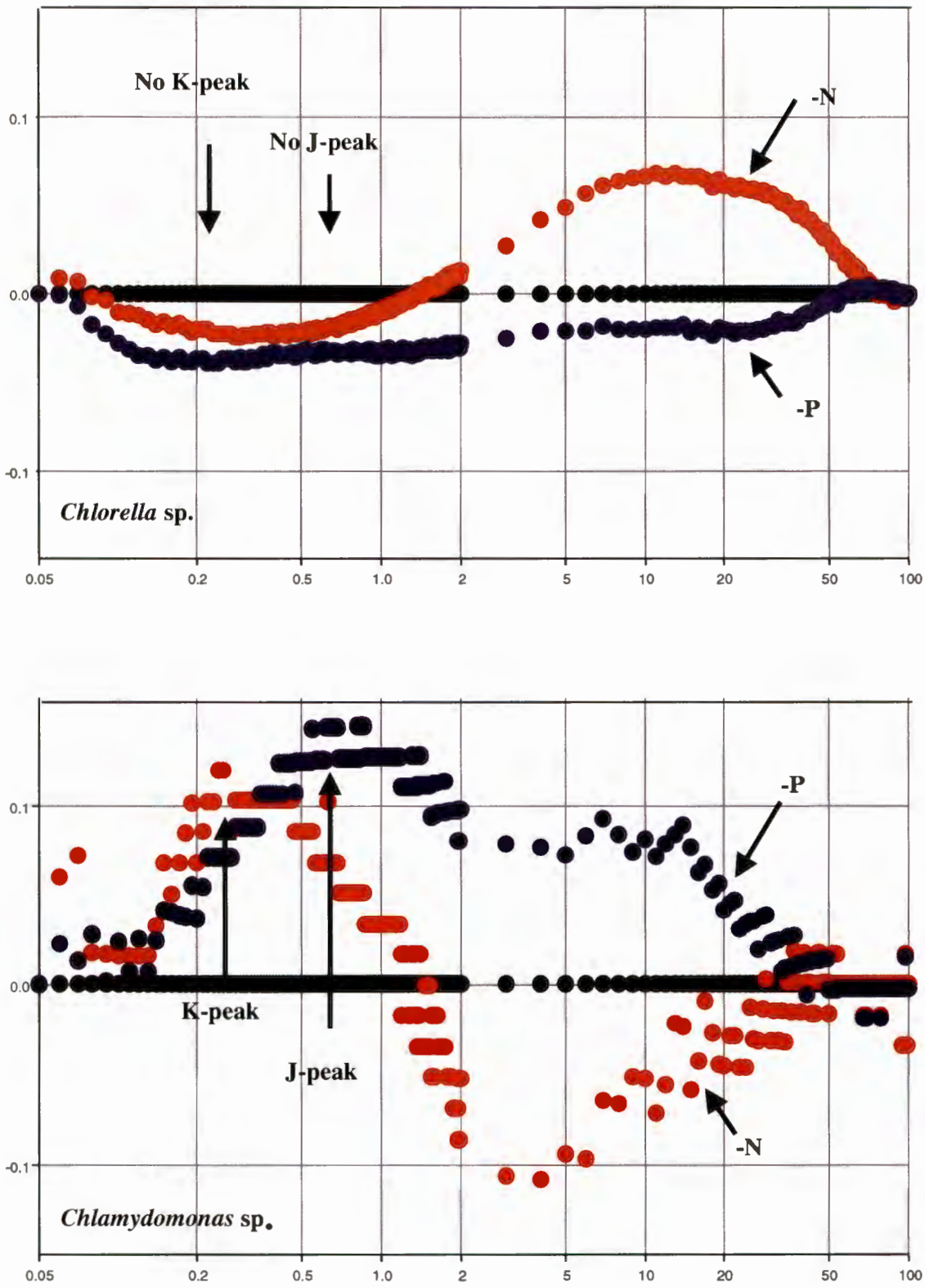


Figure 4.19. Normalised fluorescence rise curves (N and P-limited treatments as compared to the internal control, deviation from control on y-axis) demonstrating the presence of a pronounced J and K-peak in *Chlamydomonas* sp., whereas no such peaks were present in *Chlorella* sp.

The percentage of APA of the summed (APA + AcPA) activity was clearly highest in *Chlorella* sp. under P-limitation. N-limitation caused an opposite effect; AcPA dominated, combined with the highest decrease in PI. In *Chlamydomonas* sp. the ratio of APA/AcPA was close to 1 in both treatments (Figure 4.18).

Further calculations using normalisation of the fluorescence data against the internal control showed that N-limitation inhibits the metabolism in both species (indicated by deviation compared to the internal control in the later parts of the fast fluorescence rise curve, Figure 4.19).

In addition, the presence of a "K-peak" was detected in *Chlamydomonas* sp., indicating the inactivation of the oxygen–evolving–complex (Strasser 1997). This suggests a block in the water splitting system, indicating rapid, detrimental effects of N-limitation on this alga.

P-limitation blocked the electron transport (indicated by a pronounced J-peak in the normalised fluorescence curve, Figure 4.19) between PS II and PS I in *Chlamydomonas* sp. but not in *Chlorella* sp., probably because of its capability to increase the APA, and thereby supply the cells with sufficient inorganic P for continuing ATP synthesis.

4.3. Discussion and conclusions

Chlorella sp. was observed to use more of the supplied PO_4^{-3} in all treatments than *Chlamydomonas* sp. It has been previously shown that *Chlamydomonas eugamentos* stores phosphate in polyphosphate bodies in the cytoplasm (Siderius *et al.* 1996) and *Chlamydomonas reinhardtii* has polyphosphate granules in vacuoles inside the cell (Komine *et al.* 2000), suggesting the potential for luxury uptake. Whether this is true for the studied *Chlamydomonas* sp. is not known, but if this is the case it might partly explain the lesser need for P-uptake for this species.

Chlamydomonas sp. had clearly lower chlorophyll-*a* concentrations than *Chlorella* sp. during the fast growth phase. During the experiment *Chlamydomonas* sp. cells grown in the external control medium were not capable of restoring the chlorophyll-*a* levels within one day after the 40% dilution, as *Chlorella* sp. did. Nevertheless, *Chlamydomonas* sp. survived and, although the chlorophyll-*a* concentrations were low, it maintained a higher growth rate than *Chlorella* sp.,

despite low nutrient concentrations. According to Parkhill *et al.* (2001) nutrient stress refers to both nutrient limitation and nutrient starvation. Nutrient limitation refers to balanced (steady-state) growth – which in this study was achieved by *Chlamydomonas* sp. – indicating that the cells were fully acclimated to the nutrient restriction. Nutrient starvation, on the other hand, refers to unbalanced growth with growth rates less than steady-state. Thus, based on the growth rates in *Chlorella* sp., signs of nutrient starvation, rather than limitation only, could be observed. *Chlamydomonas* sp. showed the capacity to endure nutrient poor environments, and used less of the PO_4^{-3} supplied in the medium, measured as higher residual PO_4^{-3} in the medium. The lesser PO_4^{-3} use may partly be explained by *Chlamydomonas* sp. potential capacity to store polyphosphates, which would be of great importance in P-limiting conditions (Komine *et al.* 2000). *Chlamydomonas* sp. has previously been shown to be a good competitor for P, compared to e.g. *Chlorella* sp. and *Oscillatoria* sp. and several diatom species (Grover 1989). It is clear that inter-species differences exist, and these may be of importance in explaining differences in physiological fitness.

In both species N-limited cultures had least chlorophyll-*a*. Figure 4.20 summarises how nitrate limitation induced most enzyme activity in both species (47% and 63% in *Chlorella* sp. and *Chlamydomonas* sp. respectively), whereas internal control cultures exhibited the lowest summed phosphatase activity. In *Chlamydomonas* sp. AcPA dominated all treatments, whereas in *Chlorella* sp. AcPA dominated only N-limitation. In *Chlorella* sp. non-limitation caused an equal distribution between APA and AcPA, whereas APA constituted for nearly 90% of the entire phosphatase activity in P-limitation. This suggests that either the phosphatase enzymes in the different algal species are different, or that their regulation or functions are different. It might even be speculated that *Chlamydomonas* sp. does not possess alkaline phosphatases, but that the activity measured in high pH is a result from acid phosphatases functioning in non-optimal pH. Other *Chlamydomonas* species have, however, been shown to have at least one (Patni *et al.* 1977, Bachir and Loppes 1997 for *C. reinhardtii*) or even two alkaline phosphatases (Matagne *et al.* 1976 for *C. reinhardi*), therefore an absence of alkaline phosphatases in the studied *Chlamydomonas* sp. seems unlikely. In a study concerning four different phytoplankton species in low P_i, and several lakes in Sweden, Olsson (1990) found *Chlamydomonas* sp. to exhibit highest phosphatase activity compared to both in the other species and in the lake-water. The highest activity also occurred in pH 8.5, rather than in lower pH, contrary to what was found in this study.

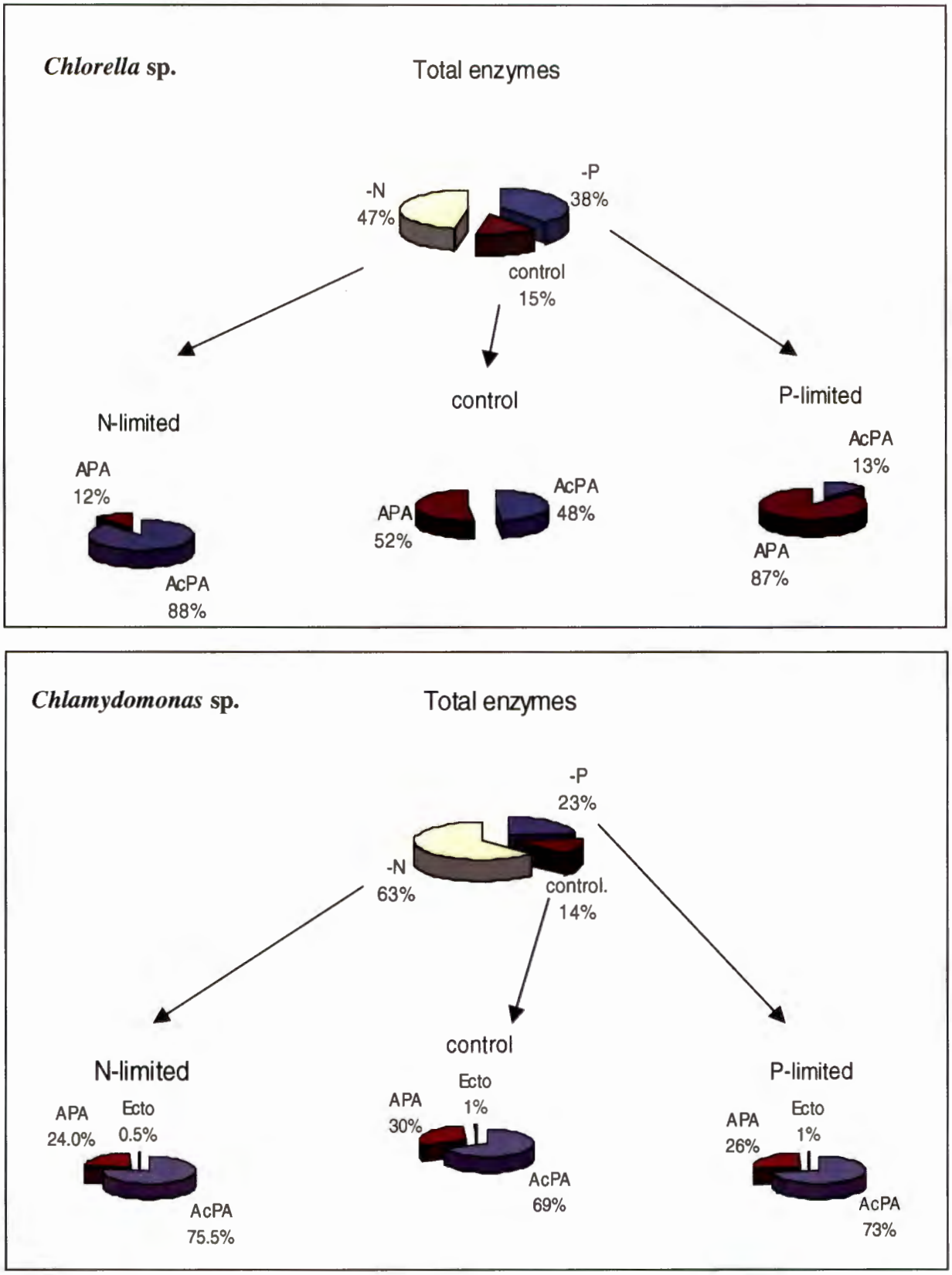


Figure 4.20. Percentage distribution of total enzyme activity (APA, AcPA and ectoenzymatic phosphatase activity (*Chlamydomonas* sp. only)) in the different treatments, and percentage distribution of enzyme type in each treatment in *Chlorella* sp. (above) and *Chlamydomonas* sp. (below).

The percentage of ectoenzymatic activity in *Chlamydomonas* sp. was very low (0.5 – 1%) in all treatments. It has been reported that EDTA inhibit the activity of these enzymes in *C. reinhardtii* (Quisel *et al.* 1996) and if this applies also to *Chlamydomonas* sp., the low ectoenzymatic activity can be due to inhibition by EDTA in the GBG-11 growth media. However, addition of Mg^{2+} to the buffer in which the enzyme activity assays were carried out should have counteracted the inactivation caused by the EDTA.

The constitutive (initial) level of APA was higher in *Chlamydomonas* sp. compared to *Chlorella* sp. – supporting the findings from the Vaal River suggesting high APA in bloom forming species. Initially *Chlamydomonas* sp. had even higher AcPA than APA levels, whereas *Chlorella* sp. had approximately the same level of AcPA and APA (Figure 4.10). The measured APA and AcPA reflect the activity of intracellular enzymes only – both acid (Matagne *et al.* 1976) and inducible alkaline phosphatases have been found to be primarily intracellular (Quisel *et al.* 1996). Alkaline phosphatase has been found to be bound to the cell wall or retained in the periplasm in *Chlamydomonas reinhardtii* (Quisel *et al.* 1996). Alkaline phosphatases are generally characterised as inducible enzymes in phytoplankton (Cembella *et al.* 1984, Jansson *et al.* 1988, Quisel *et al.* 1996, Riegman *et al.* 2000, Rengefors *et al.* 2001). Acid phosphatase enzymes have been found to occur within the cell (Jansson *et al.* 1988), for example in the vacuoles of *C. reinhardtii* (Matagne *et al.* 1976). The algal cell, being a highly acid environment, is conducive to the higher activity of acid phosphatases, and may thus contribute to the difference between the initial levels of AcPA and APA in *Chlamydomonas* sp. cells. Acid phosphatases have been described as constitutive enzymes, and only a few microalgal species (*Oochromonas danica*, *Gymnodinium nelsoni* and *Euglena gracilis*) have been reported to show increased AcPA with increasing phosphorus deficiency (see Olsson 1990 for summary). On the other hand, several publications exist reporting inducible acid phosphatases in terrestrial plants, such as lupin (Miller *et al.* 2001), Indian mustard (Haran *et al.* 2000) and tomato (Baldwin *et al.* 2001, for review see Duff *et al.* 1994), as well as the green macroalga *Ulva lactuca* (Lee 2000). Therefore, the shortage on information concerning inducible acid phosphatases in phytoplankton may simply be the result of a lack of investigations. Olsson (1990) showed that *Chlamydomonas* sp. had low AcPA in cultures grown in low (1 mg l^{-1}) PO_4^{-3} concentrations. Contrary to this, it was found during the present experiments, that acid phosphatase enzyme is inducible in both *Chlamydomonas* sp. and *Chlorella* sp. During the nutrient limitation treatments, which was more severe than that of the experiments reported by Olsson (1990), both species showed a strong

increase in all enzyme activities. After the N-limitation treatment both species had higher AcPA than APA (Figures 4.4 and 4.7).

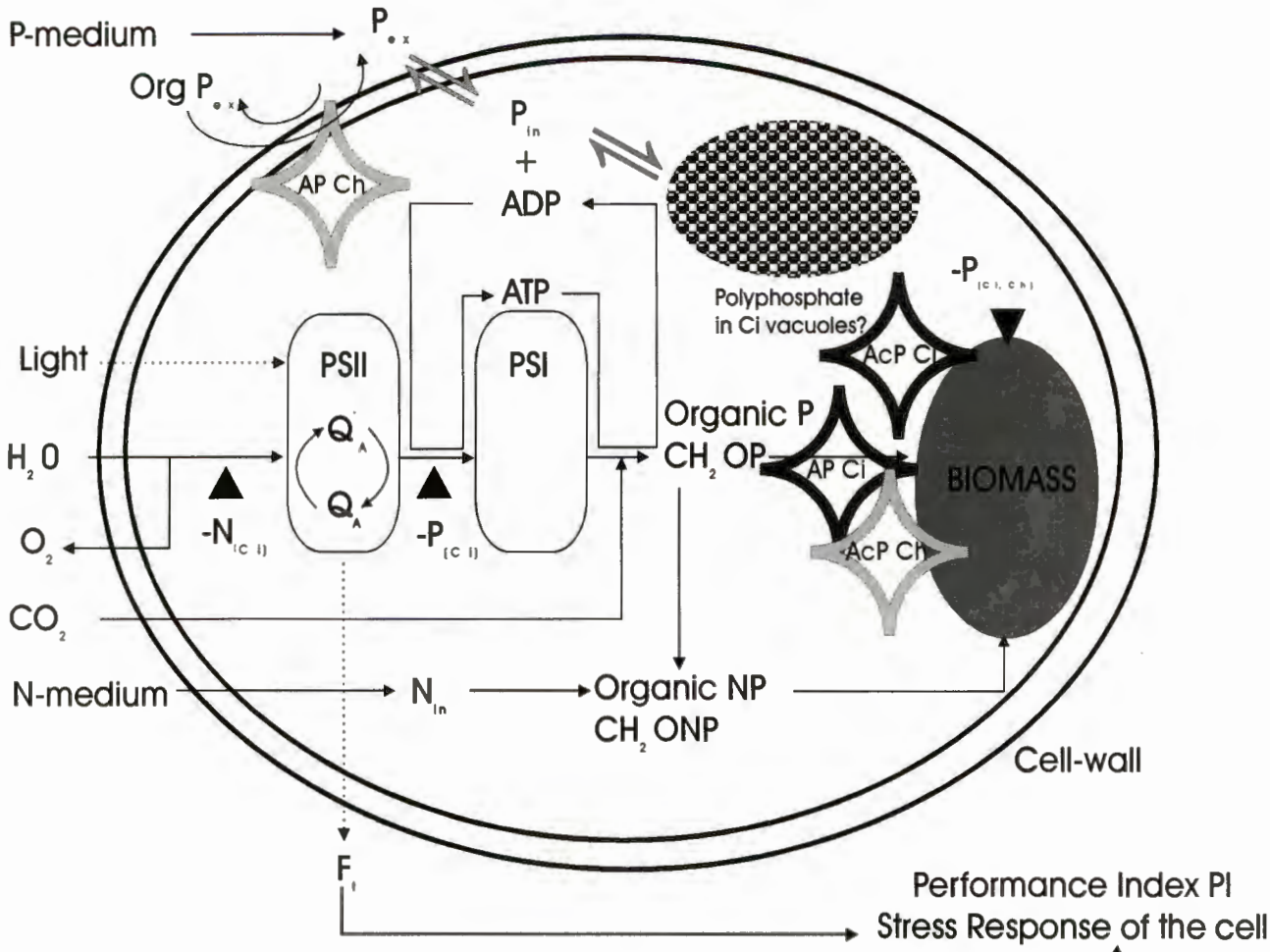
The results lead to the hypothesis, that the degree of nutrient limitation experienced by the algae in the present experiments were severe enough to deplete not only external PO_4^{-3} and NO_3^- pools (as also indicated by induction of APA and ectoenzyme activity), but also lead to a decrease in intracellular PO_4^{-3} and NO_3^- pools. As a consequence of the lack of PO_4^{-3}, the need for mobilisation of stored phosphate resources induces intracellular (acid) phosphatase activity, as well as NR activity for utilisation of potential NO_3^- pools. To take this train of thought one step further, one can hypothesise that there is a time lag in the induction of the alkaline and acid phosphatases. APA in *Chlorella* sp. was induced in the first half of the nutrient limitation experiment (Figure 4.10), whereas AcPA had a sharper increase in its activity at a later, more severe stage of the nutrient limitation (Figure 4.10). This is in opposition to the suggestion that an APA increase would require longer-term P-depletion in the environment (Dodds 1995). Thus, AcPs are, during prolonged N and P limitation, important and clearly inducible in the studied species.

It has been suggested that phosphatases may have transferase-activity in addition to hydrolysation of monoesters (Feder 1973), but generally little is known about the nature and function of P_i transporters in plants, compared to for example N and S transporters (Ragothama 1999, Grossman and Takashi 2001). The induction of APA and AcPA may, therefore, also be dependent on potential other functions of these enzymes. In connection to the preparation of this work, however, no recent publications could be found confirming the action of phosphatases as transferases.

The reason for high PAs in algae limited primarily by nitrogen, rather than phosphorus, is unclear. Opposite results reporting increased PA in the presence of NO_3^- have been previously reported (Bone 1971). An explanation for high PAs in algae grown in low nitrogen might be the chlorophyll-*a* concentrations, which have been used for calculating the specific enzyme activities. The chlorophyll-*a* concentrations in the N-limited cultures were very low, as were the growth rates. The total activity (nmol l^{-1} min^{-1}) of the enzymes were, however, also higher than in the other treatments (Figure 4.9), with the exception of APA in *Chlorella* sp., indicating that the high phosphatase activities in N-limitation are real and not artefacts.

Another possibility may be a lower content of chlorophyll-*a* in each individual cell, which could be mirrored in an apparent increase in enzyme activities per chlorophyll-*a*, but not necessarily per cell. The ratio between the cell counts for *Chlamydomonas* sp. and chlorophyll-*a* in the samples showed that there was no significant difference ($F_{(2,15)}$ p=0.67) between the amount of chlorophyll-*a* per cell between the treatments (concentration on average between 0.25 and 0.33 ng chlorophyll-*a* $cell^{-1}$). Therefore, also the activity of the enzymes was in reality higher, also on a per cell basis, in this species. For *Chlorella* sp., the cell counts were made from only two samples in each treatment – the data not allowing statistical comparison. The amount of chlorophyll-*a* per cell, based on this data, was approximately 10 times lower than in *Chlamydomonas* sp. The ratio between the cell concentration and chlorophyll-*a* concentration in the counted samples was lowest in the N-limited treatment (0.03 ng chlorophyll-*a* $cell^{-1}$), whereas the internal control cells had an average of 0.05 ng chlorophyll-*a* $cell^{-1}$ and the P-limited an average of 0.15 ng chlorophyll-*a* $cell^{-1}$. In *Chlorella* sp. the APA was highest in the P-limitation, in which the chlorophyll-*a* concentration also was highest - both in the cultures and per individual cell. Therefore, the chlorophyll-*a* concentration did not have an artificial increasing effect on the APA values, but in this species P-limitation resulted in highest growth simultaneously with enhanced APA. Hence only the AcPA in *Chlorella* sp., which was the highest in N-limitation, could to some extent be explained by lower chlorophyll-*a* concentrations. The chlorophyll-*a* concentration calculated per cell does indicate, however, that *Chlamydomonas* sp. was able to maintain its chlorophyll-*a* content constant in all cells, irrespective of the treatment and nutrient stress. *Chlorella* sp. reacted to the N-limitation especially, also by decreasing the concentration of chlorophyll-*a* in each surviving cell.

In Figure 4.21 the hypothesised enzymatic functions (based on the results of the present experiments and discussions above), as well as the measured effects in different phases of the photosynthetic pathways are presented. Based on the measured effects of N and P limitation during the present experiments, it may be hypothesised, that the limitation treatments lead to such severe nutrient depletion, that the cells were forced to mobilise any stored nutrients intracellularly. Nitrogen reduction is a reaction that requires high amounts of energy (Turpin *et al.* 1997) and would entail a great need for extra energy in the form of ATP. Most reducing equivalents used for the reduction of NO_3^- to NH_4^+ are derived from photosynthetic electron transport (Grossman and Takahashi 2001).



Organism	Treatment	To the outside oriented APA	Cytosol APA	Cytosol AcPA	ΔK-peak	ΔJ-peak	Stress response	Performance Index
Chlorella sp.	Control	+		+	-	-	-	+++
	-N	+		+++	+	-	+++	-
	-P	++		+	-	-	-	+++
Chlamydo-monas sp.	Control		+	+	-	-	-	+++
	-N		+++	+++	+	-	+++	+
	-P		++	++	-	+	++	++

Figure 4.21 and Table 4.11. Presentation of the suggested functions of the different enzymes, as well as their connection to photosynthetic pathways, in a hypothesised algal cell. Filled triangles indicate the suggested site of effect by N-limitation (-N) and P-limitation (-P) in either *Chlamydomonas* sp. (Ci) or *Chlorella* sp. (Ch). Explanations: Org P_{ex} = external organic phosphorus, P_{ex} = inorganic external PO_4^{3-}, P_{in} = inorganic intracellular PO_4^{3-}, AP = alkaline phosphatase, AcP = Acid phosphatase, ADP = adenosin di-phosphate, ATP = adenosin tri-phosphate, PSII = photosystem II, PSI = photosystem I, Q_A = plastoquinone, Q_{A-} = reduced plastoquinone, F = fluorescence from PSII. The Table summarises the strength of the effects (on a scale of + to +++) in the different species and treatments.

The chlorophyll *a* fluorescence results showed that the electron transport was significantly reduced in N and P-limited treatments compared to the internal control especially in *Chlorella* sp. and the specific electron transport per reaction centre was lowest in the N-limited cells. During the experiment no nitrate reductase activity was detected in *Chlorella* sp., which could be explained by the lack of ATP energy (due to the reduced electron transport) to drive the enzyme reaction. Therefore, the low N concentration might have triggered a need for mobilising intracellular NO_3^- stores, which requires energy both as reducing power as electrons, as well as in the form of ATP, which requires inorganic P.

Despite N-limitation also P is present in very low concentrations (2μM), and this high energy-need might be the reason for sharp increases in phosphatase activity. However, in the presence of NH_4^+, N-deficiency would most probably be corrected by primarily an uptake of the available NH_4^+, because in comparison with the utilisation of NO_2^- and NO_3^-, the assimilation of NH_4^+ is metabolically inexpensive (Grossman and Takahashi 2001). In this study all treatments had low NH_4^+ concentrations, and *Chlorella* sp. cultures had the lowest levels of NH_4^+ in the N-limitation (Table 4.3), which may suggest a higher uptake of NH_4^+, but may also be due to lesser NH_4^+ produced in the culture. To confirm the dynamics and differences in NH_4^+ uptake and utilisation more detailed analyses concerning the N and P assimilation would be required. *Chlamydomonas reinhardtii* has been shown to grow readily on NH_4^+ as its sole source of N (Franco *et al.* 1988), and therefore it was surprising that *Chlamydomonas* sp. did not utilise a larger part of the NH_4^+ measured in the culture medium. In *Chlamydomonas reinhardtii* the ability to synthesise extracellular L-amino acid oxidase, and thereby release NH_4^+ from amino acids in the N-limited medium, have been reported (Grossman and Takahashi 2001). At the end of the experiment amino acids were probably present in the cultures in abundance, originating in dying and disintegrating cells. If an extracellular L-amino acid oxidase similar to the one described from *Chlamydomonas reinhardtii* exists in this *Chlamydomonas* sp., it could explain the higher amount of NH_4^+ in the medium, and it could be assumed that the cells in fact took up a large part of the NH_4^+ pool.

Negative correlation between APA and PO_4^{3-} concentrations in the growth media have been shown in a number of studies (e.g. Pettersson 1980, Currie *et al.* 1986, Elser and Kimmel 1986, Jansson *et al.* 1988). Generally *Chlorella* sp., the non-bloom forming species, behaved like the "text-book" example in respect to inducible APA. In addition to increasing APA levels with

decreasing P-concentrations in the media, *Chlorella* sp. also used the P supplied in the medium more efficiently than *Chlamydomonas* sp.

Reduced F_V/F_M values have been reported to indicate nutrient stress in phytoplankton (Cleveland and Perry 1987, Kolber *et al.* 1988), but later studies reporting no effect of N-limitation on F_V/F_M ratios (Cullen *et al.* 1992, MacIntyre *et al.* 1997) question these findings. Also Parkhill *et al.* (2001) concluded that F_V/F_M is not a good measure of nutrient limitation under balanced (steady-state) growth conditions. Therefore, it is of importance to consider more detailed calculations derived from photosynthetic efficiency measurements, such as specific fluxes and vitality indexes as described in Strasser *et al.* (1995, 1999). During the present study, all nutrient stressed cultures exhibited reduced F_V/F_M ratios, but further calculations of the derivatives of the fluorescence signals shed more light on the pathways that were affected. Phosphatase activities in *Chlamydomonas* sp. appeared to be antiparallel to the vitality parameters of the fluorescence measurements. In *Chlorella* sp. AcPA followed the same antiparallel pattern. Thus the stress caused by N and P limitation can in *Chlamydomonas* sp. be detected by both biochemical and biophysical stress responses. *Chlorella* sp. was highly stressed by N-limitation as seen by the severe decrease in photosynthetic performance, but the APA was unaffected by this stress. However, under P-limitation APA was strongly induced and the applied stress is apparently compensated for. Thus the cells perform nearly as good as the control treatment, as confirmed by the high fitness measured as photosynthetic intensity.

In conclusion of the fluorescence results, *Chlamydomonas* sp. showed a moderate stress response in comparison to *Chlorella* sp., which seemed to have developed survival strategies counteracting some stress situations such as P-deficiency, while it was severely affected by N-deficiency.

The main results from this study indicate that

1. *Chlorella* sp. exhibits an r-strategy in respect to low nutrient conditions. It had a relatively high specific growth rate in optimal conditions, but very low growth rates in nutrient limitation, especially when N was limiting. N-limitation also severely damaged its photosynthetic vitality. It was able to rapidly increase its APA, and its phosphatase activity correlated strongly with decreasing PO_4^{-3} concentration in the medium.

2. *Chlamydomonas* sp. can rather be defined as a K-strategist, based on the slower growth rates in control treatments during the experiment. Under limiting conditions *Chlamydomonas* sp. was able to keep its growth rates on a higher level than *Chlorella* sp., and also the ratio of chlorophyll-*a* per cell kept constant despite the treatment. The phosphatase activity in *Chlamydomonas* sp. did show a decreasing trend with increasing PO_4^{-3} concentration of the media, albeit the correlation was not significant, as was the case in *Chlorella* sp. Instead *Chlamydomonas* sp. had higher constitutive enzyme activities compared to *Chlorella* sp., corresponding to the observed pattern in the Vaal River (Chapter 3). High constitutive PA may indicate that this species is at all times "prepared" for low PO_4^{-3} conditions. It might also indicate a more efficient P_o utilisation also in PO_4^{-3} surplus or possible storage of PO_4^{-3} in favourable conditions, which would increase efficiency and competitive ability in mixed culture, or sudden low nutrient conditions. Furthermore, the high AcPA also indicate an efficient mobilisation of intracellular PO_4^{-3} stores. In both species the AcPA was highly induced and therefore the function of acid phosphatases needs to be further investigated.

3. The reason for *Chlamydomonas* sp. forming blooms in the Vaal River may, as indicated by the results from this study, be because of a strategy that will allow it to maintain a prolonged competitive advantage, and thereby create blooms under circumstances less favourable for other competing species. Competitive ability and greater resource utilisation define K-strategists (Kilham and Kilham 1980) and with regard to phosphorus nutrition, *Chlamydomonas* sp. seems to be able to maintain a higher level of phosphatase activities, and may therefore invest in nutrient storage, rather than high growth. Other studies have also indicated that bloom forming algae typically show traits of K-strategy rather than r-strategy (Riegman and Kuipers 1994), whereas rapidly growing generalists would be more indicative of mesotrophic environments where bloom formers tend to be scarce. *Chlorella* sp. is capable of forming high biomass in uni-algal cultures and in optimal conditions, but would probably be easily out-competed by other phytoplankton species in fluctuating nutrient conditions.

CHAPTER 5

GROWTH, PHOSPHATASE ACTIVITY AND PHOTOSYNTHETIC VITALITY OF TWO CYANOBACTERIA SPECIES UNDER DIFFERENT NUTRIENT CONDITIONS

5.1. Introduction

Cyanobacteria are the oldest autotrophic organisms, and made their appearance in the Archaean (3.8-2.6 Ga BP, Schopf 1992). Today they are the only prokaryotes that carry out oxygenic photosynthesis (Golden *et al.* 1997). In the Precambrian (2.6-0.58 Ga BP) they were ubiquitous and fossil records of most present-day cyanobacterial orders have been made from this period (Schopf and Walter 1982). Due to their early development in evolutionary history, cyanobacteria occur abundantly all over the globe and in a wide range of habitats (South and Wittick 1987). Many cyanobacteria are capable of fixing N_2 (termed diatzotrophic cyanobacteria) with the help of nitrogenase enzymes (South and Wittick 1987). Nitrogenase is an oxygen sensitive enzyme that can function only under anaerobic conditions. Many filamentous cyanobacteria have developed special thick-walled cells, heterocysts, in which the oxygen evolving part of photosynthesis, PSII can be turned off, and anaerobic conditions sustained during nitrogenase-expression (Gallon 1992). Also some non-heterocystous species, such as *Synechococcus* sp. (Mitsui *et al.* 1986) and *Oscillatoria* sp. (Stal and Krumbein 1987) can be diazotrophic. In these species nitrogenase and PSII activities have been found to be temporally separated, thus exhibiting circadian rhythms with N_2 fixation taking place chiefly during darkness (Golden *et al.* 1997).

Cyanobacterial blooms have in recent times received much attention in fresh water research and water quality monitoring (Sivonen 1996, Scheffer *et al.* 1997, Codd 2000). Large or colony-forming cyanobacteria genera like *Microcystis*, *Oscillatoria*, *Anabaena* and *Aphanizomenon* have received particular attention because of their frequent dominance of the plankton in eutrophic fresh waters (Scheffer *et al.* 1997). In addition to increased nutrient concentrations several different factors have been presented to explain cyanobacterial success in aquatic environments. According to a review by Hyenstrand *et al.* (1998) cyanobacteria have been observed to be favoured by a number of environmental conditions. Low N:P ratios and high water temperatures increase the probability of cyanobacterial bloom development. High NH_4^+ levels favour non-nitrogen fixing cyanobacteria while a scarcity of N benefits the development

of nitrogen fixing cyanobacteria, which also need high levels of trace elements. Furthermore, cyanobacteria can store P, regulate their buoyancy in the water column, and they have a competitive advantage over eukaryotic phytoplankton in high pH or low CO_2 situations, and under low light conditions. Cyanobacterial dominance leads to a decrease in the phytoplanktonic species diversity, and hence affects the overall ecology of the community (Codd 2000). Increases in cyanobacterial biomass are of additional concern because of the common ability of these organisms to produce toxins (Codd *et al.* 1989, Codd 1995, 2000, Sivonen 1996, Carmichael 1997).

Several bloom forming cyanobacterial species were found in the Vaal River in this study (Chapter 3). The main bloom forming cyanobacterial species were found in varying environmental conditions, however showing slight negative correlation with N:P ratios and favoured higher temperatures. A strong positive correlation with APA:AcPA ratios was shown for the three main bloom forming cyanobacteria, *Oscillatoria simplicissima, Microcystis aeruginosa* and *Spirulina* sp. *Oscillatoria simplicissima* has in the past decades replaced *Microcystis aeruginosa* as the dominant bloom forming cyanobacteria species in the Loch Vaal catchment area in the Vaal River (Pieterse and Steynberg 1993, Venter 2000, Janse van Vuuren 2001). The reported replacement of *Microcystis aeruginosa* by *Oscillatoria simplicissima* has been suggested to be a consequence of an increase in inorganic phosphorus supply (from 0.2 mg P l^{-1} to 0.9 mg P l^{-1}) under conditions of relatively low inorganic nitrogen (0.2 mg N l^{-1}) availability (Pieterse and Steynberg 1993). The underlying physiological mechanisms operating in connection with, and as possible causes of this switch in dominant bloom formers, are still unclear. Therefore it was of interest to study the differences in growth and vitality of the two species under differing N:P ratios and concentrations *in vitro*.

Microcystis aeruginosa Kütz. is a unicellular, colony-forming cyanobacterium, which commonly forms blooms in eutrophic, warm brackish and freshwaters (e.g. Brookes and Ganf 2001). It has received much attention due to its production of potent toxins, microcystins (Oh *et al.* 2000 and references therein). Toxic blooms of *Microcystis aeruginosa* are a common phenomenon, and numerous incidents of e.g. livestock poisoning as a consequence of ingesting microcystin toxin, have been reported (Codd 1995, Codd *et al.* 1989, Sivonen 1996). *Microcystis aeruginosa* has caused numerous toxic blooms leading to death of cattle, sheep and dogs, as well as giraffes, black wildebeest and possibly white rhinoceros in South Africa (Harding and Paxton 2001).

Oscillatoria simplicissima Gomont is a filamentous, non-heterocystous cyanobacterium, described in detail by Venter (2000, 2002). Apart from its lack of red pigmentation, it is morphologically very similar to *Oscillatoria rubescens* (Desikachary 1959), with undifferentiated filaments and no branches, but it has not been conclusively determined whether *Oscillatoria simplicissima* and *Oscillatoria rubescens* may be a variants of the same species or not (Venter 2000). *Oscillatoria simplicissima* is a bloom forming species. The main problem with *Oscillatoria simplicissima* blooms is the rapid clogging of water purification filters (Juanico *et al.* 1995, Venter 2002). Species of the *Oscillatoriaceae* have been reported to produce various toxins, such as microcystins, anatoxin-*a* and aplysiatoxin (Luukkainen *et al.* 1993, Mez *et al.* 1997, Codd 2000). The importance of monitoring the occurrence of *Oscillatoria* species lies in their potential toxicity (Sivonen 1990). *Planktothrix* (formerly *Oscillatoria*) *agardhii* has been found to be toxic on numerous occasions (e.g. Leeuwangh *et al.* 1983, Berg *et al.* 1986, Sivonen 1990), as well as *Planktothrix* (formerly *Oscillatoria) rubescens* (Loizzo *et al.* 1988, Kurmayer and Jüttner 1999). In South Africa, incidents of toxic *Oscillatoria* sp. have been reported to cause death of goats, caused by an unidentified hydrophobic microcystin (Harding and Paxton 2001), but *Oscillatoria simplicissima* has to date, to the authors' knowledge, not been reported to be toxic.

The aim of this study was to compare the growth, induction and level of phosphatase activities and photosynthetic vitality (measured as chlorophyll-*a* fluorescence and PSII function) in *Microcystis aeruginosa* and *Oscillatoria simplicissima* in

1. nutrient sufficient growth medium (GBG-11 or EM),
2. N or P deficiency (GBG/EM – N/P) and
3. varying N:P ratios.

The species used in the experiments were isolated from the Vaal River, South Africa. The experimental design, as well as all analytical methods used are described in detail in Chapter 2.

5.2. Results

5.2.1. Growth

Microcystis aeruginosa was grown in GBG-11 medium until fast growth was achieved after 11 days, whereas *Oscillatoria simplicissima* grown in EM medium reached fast growth phase after only 8 days. After the start of dilutions the chlorophyll *a* concentration decreased rapidly in the *Microcystis aeruginosa* cultures, while the *Oscillatoria simplicissima* cultures increased its chlorophyll *a* concentration for one day after the dilutions and only after two dilutions started decreasing (Figure 5.1.). At the end of the experiment *Microcystis aeruginosa* had not reached steady-state but the cells were being washed out of the medium. Only in the external control did the growth of *Microcystis aeruginosa* reach steady state and the biomass even increased slightly (Table 5.1.). *Oscillatoria simplicissima* reached clear steady state in all treatments, with lowest growth rates in the N-limited treatment.

5.2.2. Chlorophyll-*a* concentration

The chlorophyll *a* concentration was initially (during fast growth phase and before the start of dilutions) higher in *Microcystis aeruginosa* than *Oscillatoria simplicissima* (ANOVA $F_{(1,46)}$ p = 0.00026, Figure 5.2). The external controls kept to initial chlorophyll *a* concentration in both species and even increased in *Oscillatoria simplicissima* during the dilutions. At the end of the experiment the chlorophyll *a* concentrations were higher in *Microcystis aeruginosa* cultures, as was the cell density (Table 5.2). It must be borne in mind that cell density is based on single cells for *Microcystis aeruginosa*, while it is based on number of filaments for *Oscillatoria simplicissima*, and the numbers are therefore not directly comparable.

At the end of the experiment the chlorophyll *a* concentrations were lowest in the N-limited treatments in both species, but the difference between the treatments was not statistically significant for either of the species (Figure 5.3). The cell density in *Microcystis aeruginosa* was significantly lower in the N-limited treatment compared to the P-limited (Tukey's post hoc p = 0.05) and the internal control (Tukey's post hoc $p < 0.0119$, Figure 5.4). The amount of chlorophyll *a* per cell was not statistically significant between the treatments, but the highest values were recorded in the nitrate-limited treatment (Table 5.2).

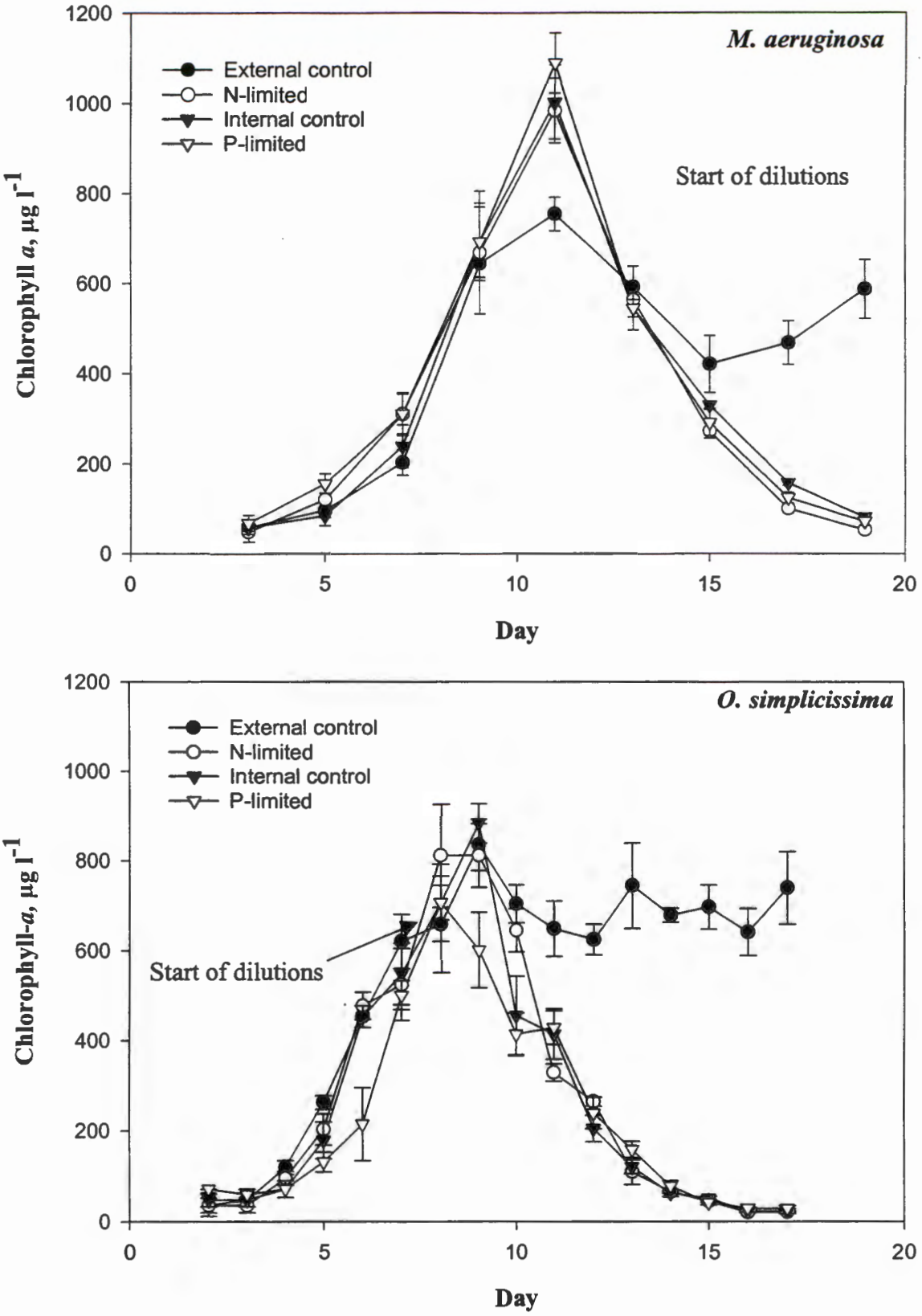


Figure 5.1. Growth of *Microcystis aeruginosa* and *Oscillatoria simplicissima* throughout the experiment (means and standard error of chlorophyll-*a* concentrations). The dilutions were started on day 11 and 8 respectively. The experiment was finished on day 18 and 17 respectively.

Table 5.1. Specific growth rates (μ day^{-1}) of Microcystis aeruginosa *and* Oscillatoria simplicissima *at the end of the experiment (mean values ± standard deviations of six replicates). The growth rate has been calculated to determine steady-state, using chlorophyll* a *values for the two last days* (Microcystis aeruginosa) *or the last day* (Oscillatoria simplicissima) *before the termination of the experiment (see Figure 1).*

	Microcystis aeruginosa μ day^{-1}	*Oscillatoria simplicissima* μ day^{-1}
External control	0.51 ± 0.06	0.53 ± 0.43
N-limited (N:P = 1:1)	0.08 ± 0.18	0.38 ± 0.43
Internal Control (N:P = 16:1)	0.06 ± 0.13	0.55 ± 0.39
P-limited (N:P = 160:1)	0.12 ± 0.12	0.56 ± 0.24

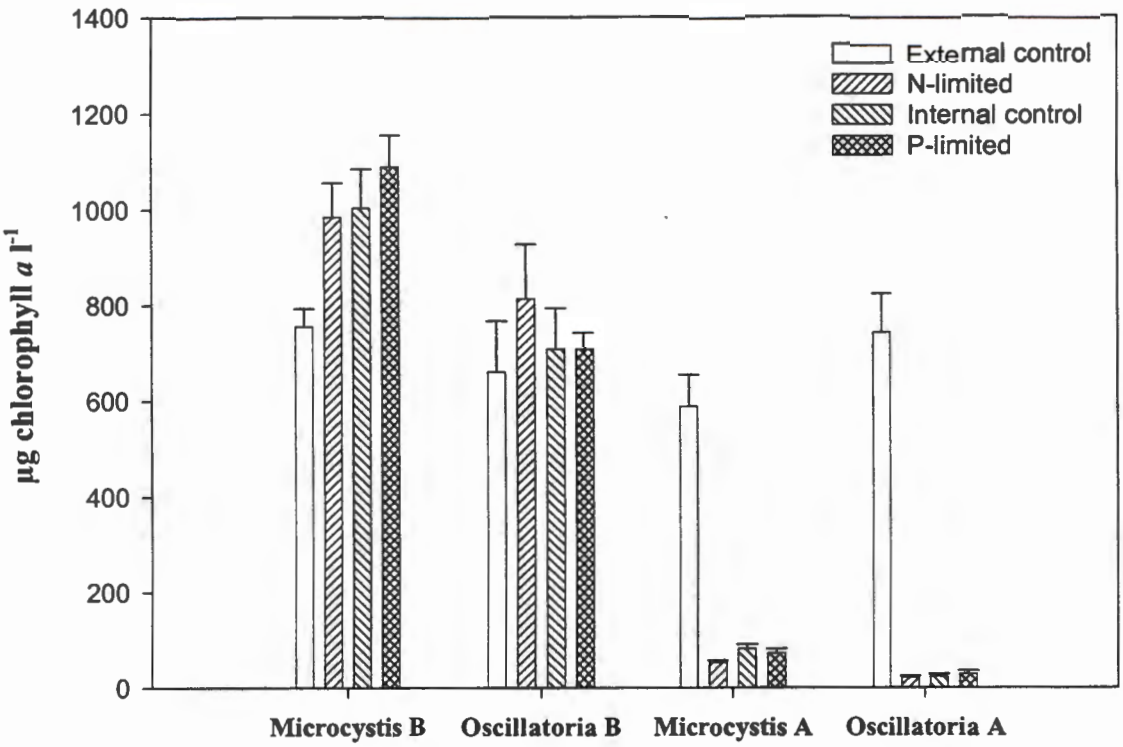


Figure 5.2. Chlorophyll-*a* concentration (mean and standard error for six samples) for *Microcystis aeruginosa* and *Oscillatoria simplicissima* initially (B = before treatment, day 11 and 8 respectively) and after (A = after treatment, day 18 and 17 respectively) being exposed to varying N:P ratios (External control = EM medium).

*Table 5.2. Biomass (as klett, chlorophyll-*a *and cell density) and chlorophyll-*a *per cell (*Microcystis aeruginosa *only) in the cultures of* Microcystis aeruginosa *and* Oscillatoria simplicissima *in treatments with varying N:P ratios (mean values ± standard error of six replicates). Cell density is given as number of cells (*Microcystis aeruginosa*) or number of filaments irrespective of filament length (*Oscillatoria simplicissima*). Samples from removed media were taken at day 19 (*Microcystis aeruginosa*) and day 17 (*Oscillatoria simplicissima*).*

	Microcystis aeruginosa			*Oscillatoria simplicissima*		
	N-limited	Internal Control	P-limited	N-limited	Internal Control	P-limited
Chlorophyll-*a* (µg l^{-1})	52.5 ± 11.7	81.2 ± 21.6	71.7 ± 24.0	21.5 ± 7.8	23.1 ± 13.6	28.7 ± 15.7
Cell density (10^9 cells l^{-1})	0.57 ± 0.29	1.40 ± 0.65	1.22 ± 0.26	0.009 ± 0.002	0.007 ± 0.002	0.007 ± 0.002
Chl-*a* $cell^{-1}$ (pg $cell^{-1}$)	0.015 ± 0.016	0.009 ± 0.009	0.006 ± 0.002	n.d.	n.d.	n.d.

n.d. = not determined

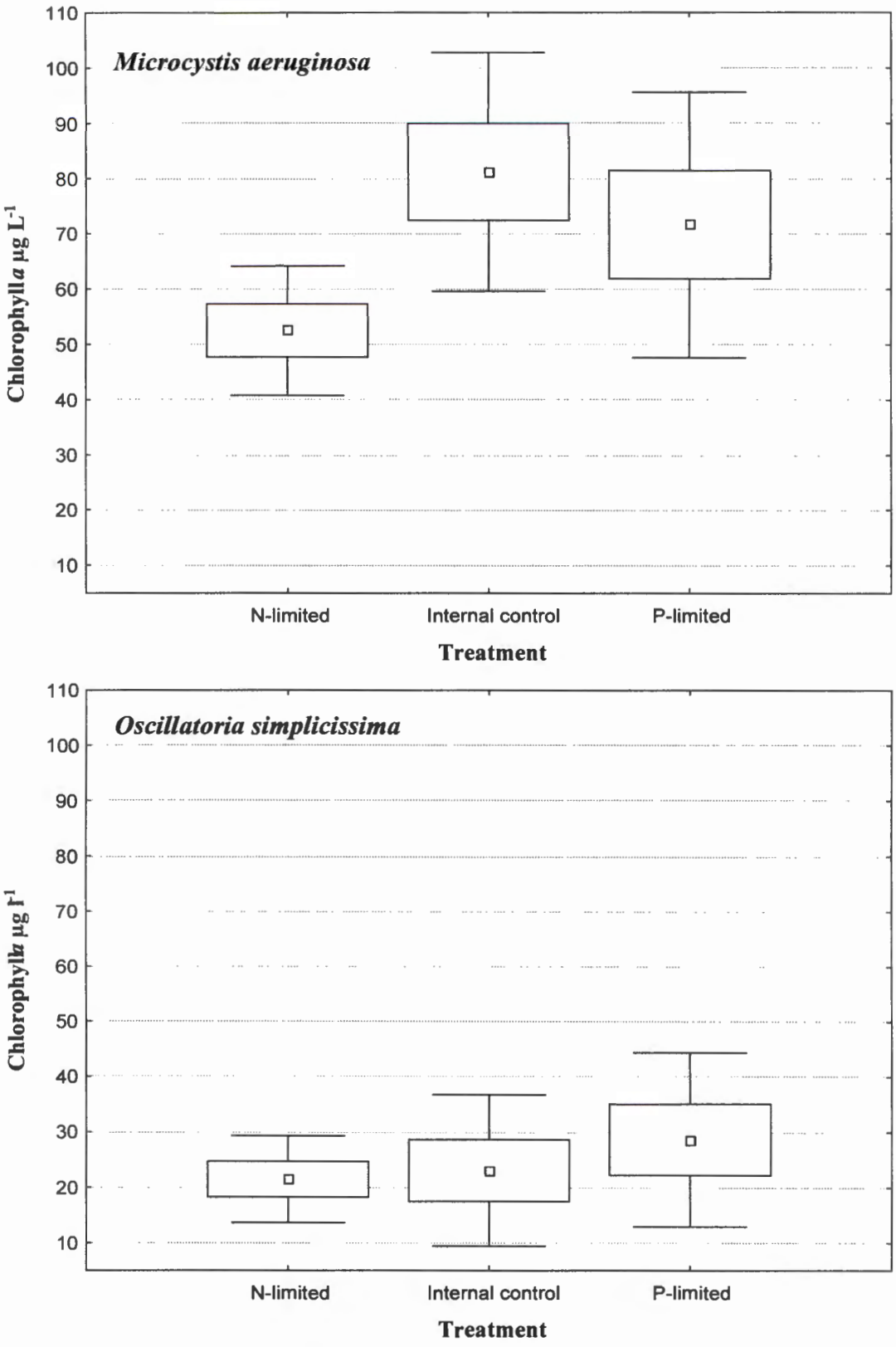


Figure 5.3. Chlorophyll-*a* concentrations of *Microcystis aeruginosa* and *Oscillatoria simplicissima* at the end of the experiment in the three experimental treatments. Averages marked by rectangle, boxes include standard error and whiskers mark standard deviation, n = 6 in each treatment.

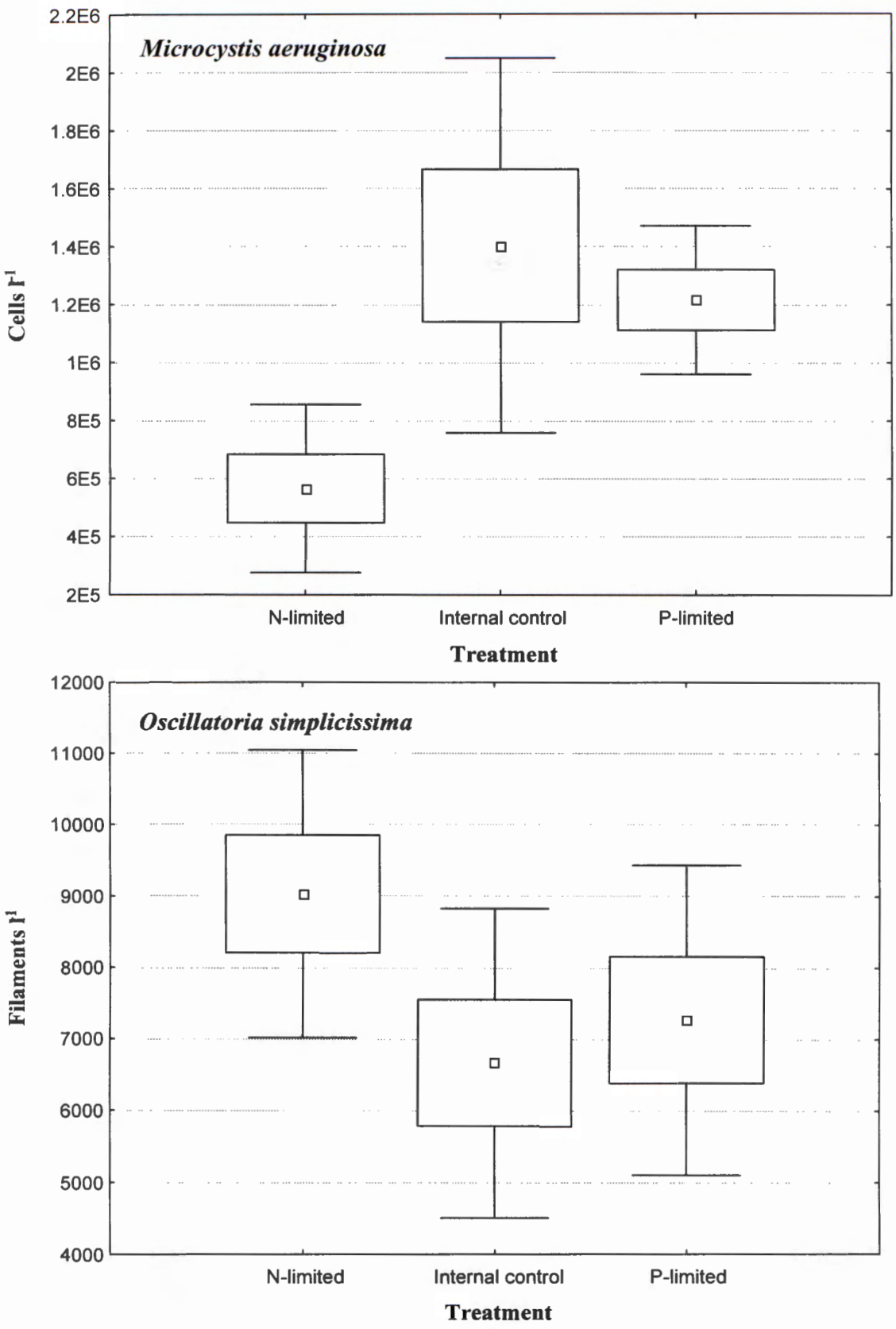
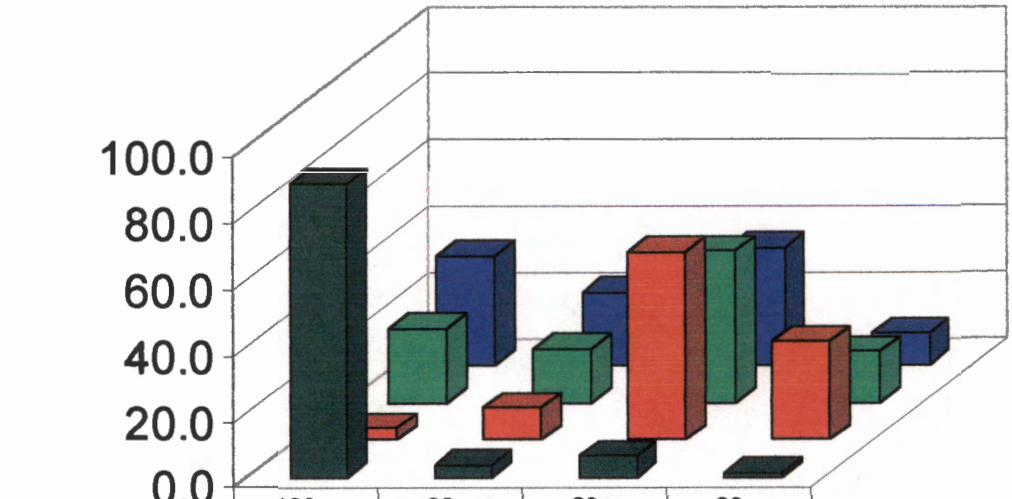


Figure 5.4. Number of cells / filaments of *Microcystis aeruginosa* and *Oscillatoria simplicissima* at the end of the experiment in the three experimental treatments. Averages marked by rectangle, boxes include standard error and whiskers mark standard deviation, n = 6 in each treatment.



	> 180 µm	> 90 µm	> 20 µm	< 20 µm
External Control	88.7	3.7	6.4	1.2
N-limited	3.7	9.6	56.8	29.9
Internal Control	22.3	16.2	46.0	15.4
P-limited	33.0	22.0	35.5	9.5

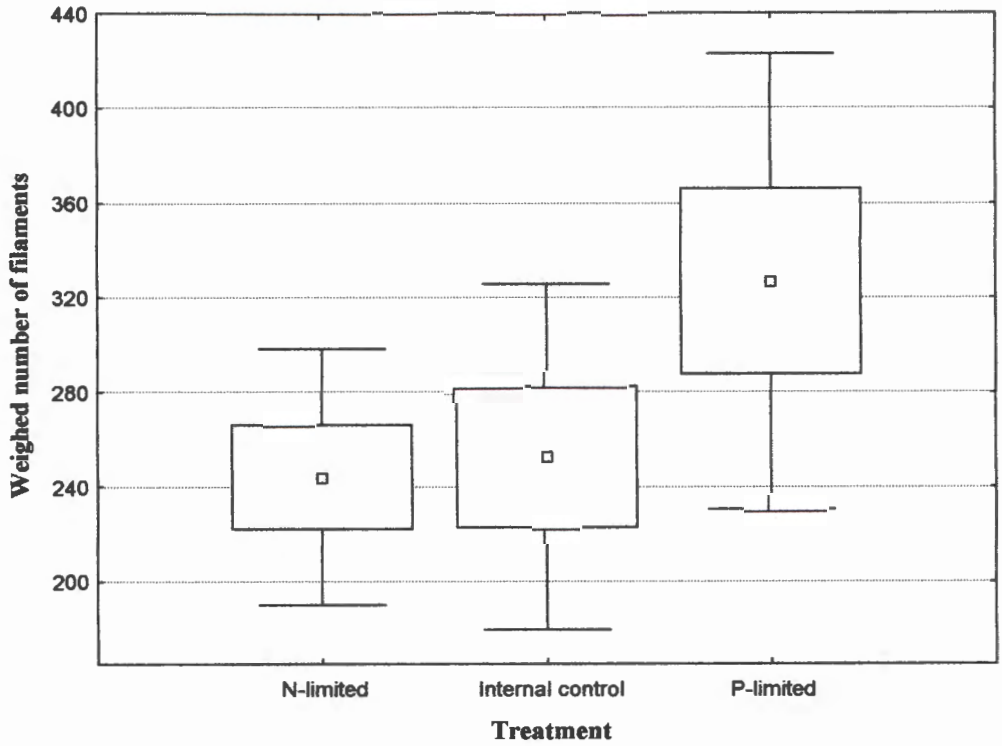


Figure 5.5. Variation in the size classes of *Oscillatoria simplicissima* grown under varying nutrient regimes. The uppermost figure presents the percentage distribution of filaments in four size classes in all treatments including the external control (EM medium). The box and whisker plot below presents the mean (middle point), standard error (box value) and standard deviation (whisker value) of weighed number of filaments to indicate biomass in the samples.

In *Oscillatoria simplicissima* the number of filaments per litre did not vary significantly between the treatments. A comparison between the length of the filaments was done, however, and the different treatments had clear differences in the composition of size classes in the filaments (Figure 5.5.). In the external control the majority of the filaments were 180 µm or longer, whereas the nutrient limited treatments had a greater number of shorter filaments. Especially in the N-limited treatment the number of filaments shorter than 90 µm accounted for more than 85% of all filaments present. The smallest size-class counted consisted of short filaments (or hormogonia) between one and three cells, these comprising nearly 30% of all filaments in the N-limited treatment. Visual observation could also confirm the presence of high number of hormogonia in the nutrient limited samples. For biomass calculations the different filament size classes were given increasing "weight" corresponding to increasing length of the filament (>180 = 5, >90 = 4, >20 = 2 and <20 = 1). When the biomass was calculated using this weighted number of filaments, the P-limited treatment seemed to have the highest biomass compared to the other treatments (Figure 5.5). The difference between the treatments was not statistically significant (ANOVA $F_{(2,15)}$ $p = 0.1534$).

5.2.3. Inorganic nutrient concentrations

The measured PO_4^{-3} concentrations were clearly lower (< 55 µmol l^{-1}) than the concentration supplied in the culture medium (290 µmol l^{-1} in both GBG-11 and EM medium) before the dilutions, which suggests that both algae utilised the inorganic phosphorus available efficiently (Table 5.3). The PO_4^{-3} concentrations decreased steadily after the start of dilutions, and reached lowest residual concentrations in the P-limited treatments in both species (Figure 5.6). Neither species used all the inorganic phosphorus available, suggesting that this nutrient was not limiting the growth of the cells in any of the treatments. The estimated PO_4^{-3} consumption was higher in *Oscillatoria simplicissima* (external control 6.9 ± 1.0 nmol PO_4^{-3} chl a^{-1} h^{-1}, nutrient limited treatments 1-1.9 nmol PO_4^{-3} chl a^{-1} h^{-1}) compared to *Microcystis aeruginosa* (external control 4.8 ± 0.6 nmol PO_4^{-3} chl a^{-1} h^{-1}, nutrient limited treatments 0.3-0.7 nmol PO_4^{-3} chl a^{-1} h^{-1}), whereas between treatments no statistically significant differences could be observed in either species (Table 5.3).

Table 5.3. PO_4^{3-} concentrations of the culture medium (expressed as µmol l^{-1}) and PO_4^{3-} consumption (expressed as nmol PO_4^{3-} chl a^{-1} h^{-1}) in Microcystis aeruginosa *and* Oscillatoria simplicissima *during the experiment (mean values ± standard deviation of six replicates). The 1st measurement refers to batch-culture conditions before the start of dilutions. The 2nd and 3rd measurements were determined with 3-4 days intervals during the course of the experiment. The 4th measurement was made on the day of termination of the experiment. Detection limit = 0.1 µmol l^{-1}.*

	PO_4^{3-} concentration in medium PO_4^{3-} µmol l^{-1}.					
	Microcystis aeruginosa			*Oscillatoria simplicissima*		
Measurement	N-limited	Internal Control	P-limited	N-limited	Internal Control	P-limited
1st	49.4 ± 6.8	46.5 ± 4.2	50.5 ± 13.3	20.2 ± 3.8	19.3 ± 2.3	21.6 ± 3.1
2nd	19.2 ± 5.4	19.3 ± 6.4	15.7 ± 3.7	17.5 ± 3.8	16.3 ± 1.8	13.2 ± 1.5
3rd	11.6 ± 2.1	8.0 ± 1.2	6.03 ± 1.7	11.2 ± 3.6	10.2 ± 1.9	9.5 ± 2.5
4th	5.7 ± 1.6	3.9 ± 1.4	2.2 ± 1.3	4.1 ± 1.5	2.6 ± 0.3	1.5 ± 0.8
	PO_4^{3-} consumption *nmol PO_4^{3-} chl a^{-1} h^{-1}*					
	Microcystis aeruginosa			*Oscillatoria simplicissima*		
	N-limited	Internal Control	P-limited	N-limited	Internal Control	P-limited
	0.7 ± 0.4	0.3 ± 0.2	0.3 ± 0.2	1.0 ± 1.7	1.9 ± 1.5	1.6 ± 0.9

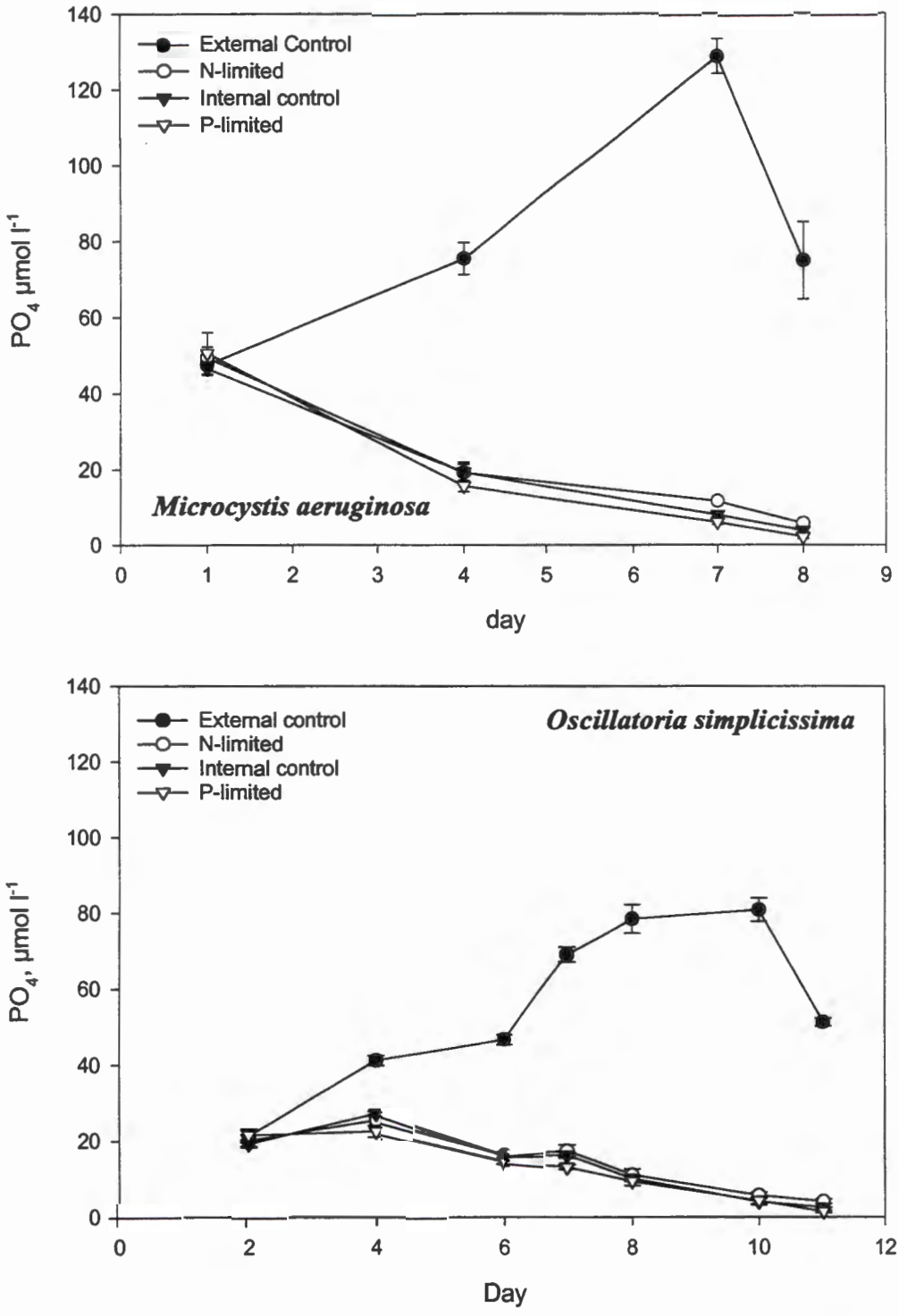


Figure 5.6. Gradual change in the phosphate concentrations (mean and standard error for six samples) in the culture medium of *Microcystis aeruginosa* and *Oscillatoria simplicissima* throughout the experiment (External control = EM medium). Samples are taken every second - third day after the start of dilutions.

Table 5.4. NH_4^+ concentrations (µmol l^{-1}, mean of six replicate samples, ± standard deviation) in Microcystis aeruginosa *and* Oscillatoria simplicissima *cultures during the experiment. 1st measurement refers to batch-culture conditions before the start of dilutions. 2nd and 3rd measurements are determined with 3-4 days intervals during the course of the experiment. The 4th measurement is made on the day of termination of the experiment. Detection limit for the method is 0.007 µmol l^{-1} (for single determination).*

	Microcystis aeruginosa NH_4^+ µmol l^{-1}			*Oscillatoria simplicissima* NH_4^+ µmol l^{-1}		
Measurement	N-limited	Internal Control	P-limited	N-limited	Internal Control	P-limited
1st	33.4 ± 32.5	52.6 ± 41.8	67.1 ± 42.2	—	—	—
2nd	1.2 ± 0.1	1.3 ± 0.2	2.5 ± 1.3	—	—	—
3rd	1.4 ± 0.4	1.8 ± 1.4	1.6 ± 0.4	2.1 ± 0.1	2.4 ± 0.5	2.1 ± 1.3
4th	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.2	0.9 ± 0	1.0 ± 0.2	1.0 ± 0.8

— = not detected

Since NO_3^- could not be measured during the experiments, samples were sent for NO_3^- measurements at an outside consultant at the end of the experiments. Measurements using a Metrohm IC analyser could not detect any residual NO_3^- present in any limited treatments in either *Microcystis aeruginosa* or *Oscillatoria simplicissima* cultures, indicating that in all treatments NO_3^- was limiting and efficiently taken up by the cells.

The NH_4^+ concentrations were high in *Microcystis aeruginosa* cultures before the start of dilutions but decreased rapidly during the dilutions, indicating that this NH_4^+ was rapidly taken up in addition to the diminishing NO_3^- pool. Nearly no NH_4^+ could be detected in *Oscillatoria simplicissima* cultures at any time throughout the experiment (Table 5.4).

5.2.4. Alkaline phosphatase activity

5.2.4.1. Specific activity

The APA at the beginning of the experiment was significantly higher in *Microcystis aeruginosa* compared to *Oscillatoria simplicissima* (Kruskal-Wallis $H_{(1,47)}$ $p = 0.0000$), but both species exhibited a clearly detectable level of activity in the pre-experiment nutrient replete condition (Figure 5.7.).

The nutrient limitation induced APA in *Microcystis aeruginosa* in all treatments. The increase in APA was between 1.3 (P-limited) and 2 times (N-limited and internal control). In *Oscillatoria simplicissima* the APA increased only in the N-limited treatment (approximately four-fold) whereas all other treatments stayed at the same level or even decreased.

At the end of the experiment the highest APA was registered in the N-limited treatments in both species, but the difference between the treatments was statistically significant only in *Oscillatoria simplicissima* (Kruskal-Wallis $H_{(2,17)}$ $p = 0.0046$, Figure 5.8).

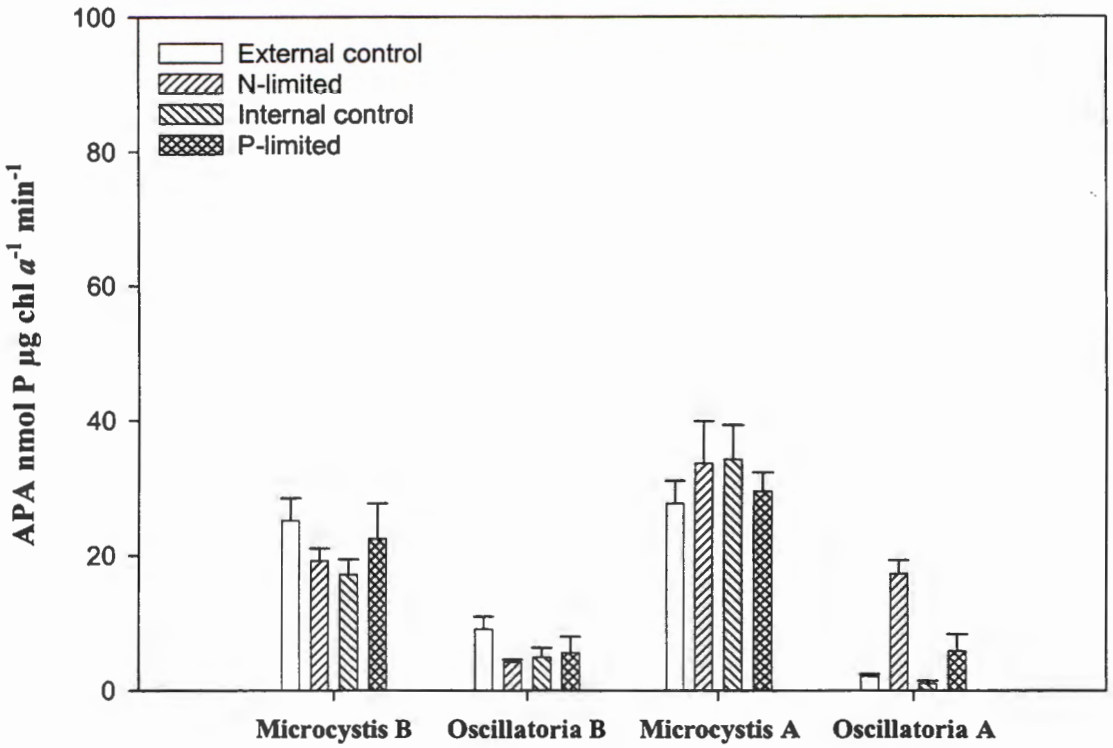


Figure 5.7. Alkaline phosphatase activity (mean and standard error for six samples) for *Microcystis aeruginosa* and *Oscillatoria simplicissima* initially (B = before treatment, day 11 and 8 respectively) and after (A = after treatment, day 18 and 17 respectively) being exposed to varying N:P ratios (External control = EM medium).

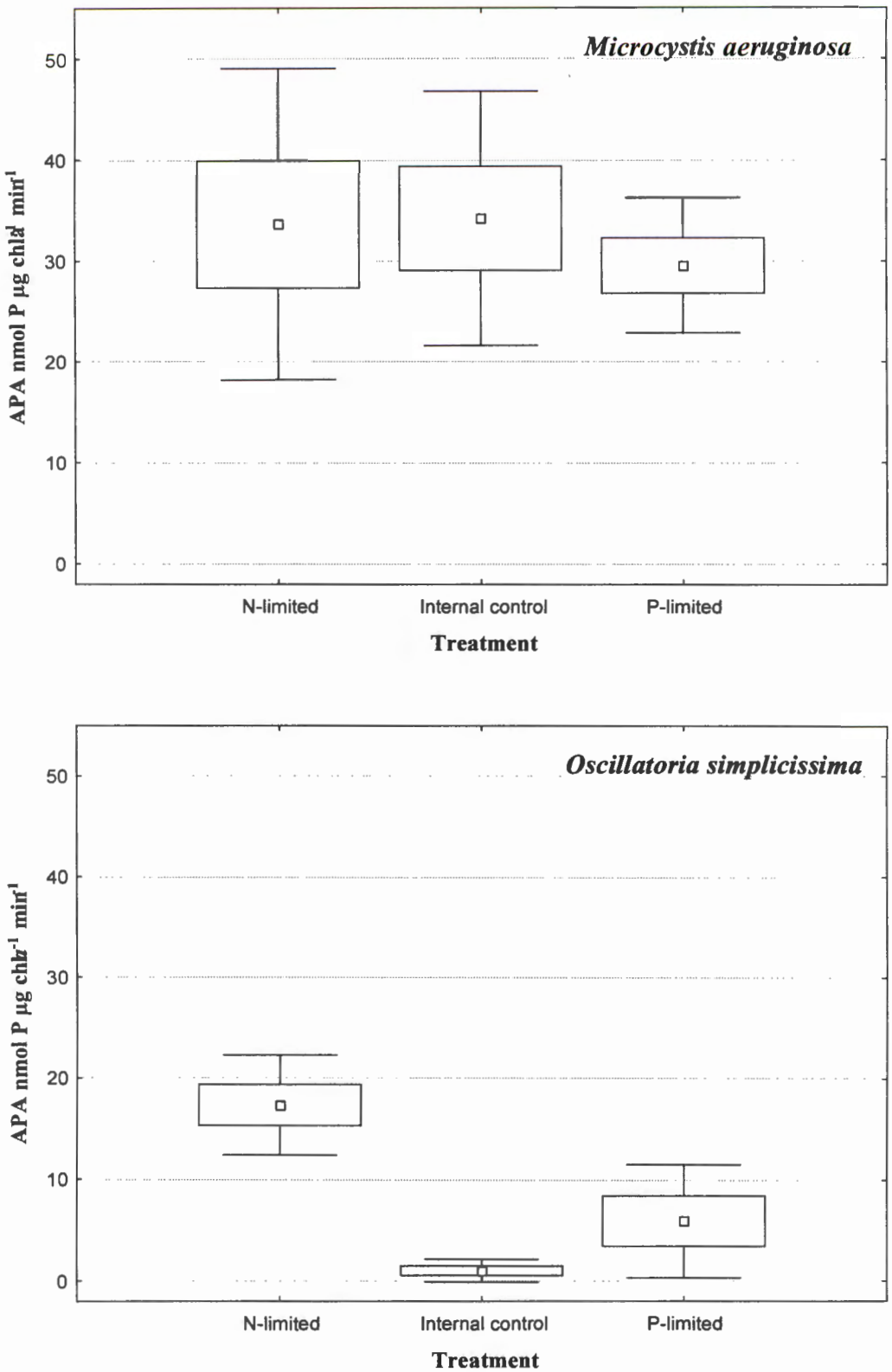


Figure 5.8. Specific alkaline phosphatase activity of *Microcystis aeruginosa* and *Oscillatoria simplicissima* at the end of the experiment in the three experimental treatments. Averages marked by rectangle, boxes include standard error and whiskers mark standard deviation, n = 6 in each treatment.

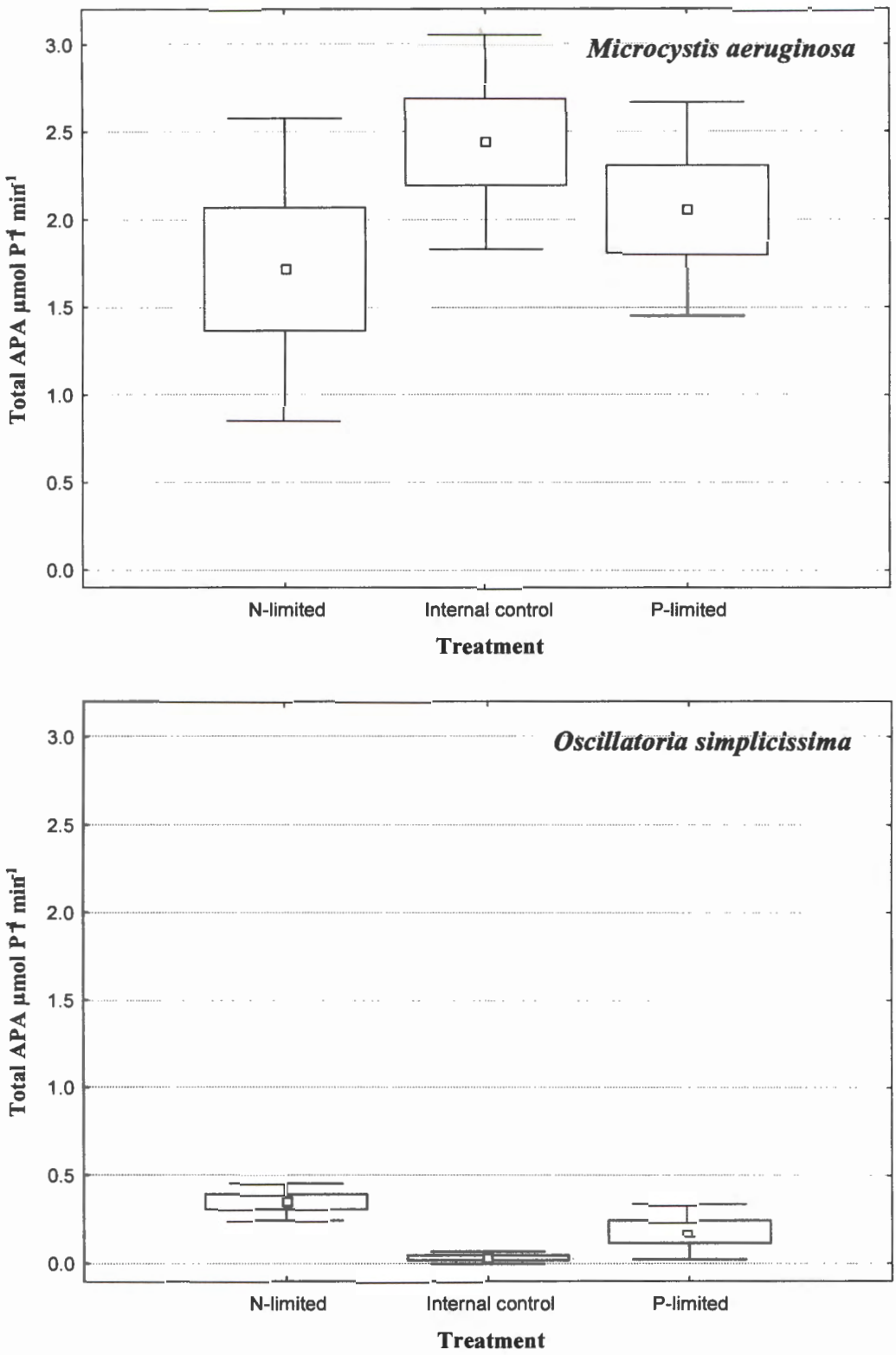


Figure 5.9. Total alkaline phosphatase activity of *Microcystis aeruginosa* and *Oscillatoria simplicissima* at the end of the experiment in the three experimental treatments. Averages marked by rectangle, boxes include standard error and whiskers mark standard deviation, n = 6 in each treatment.

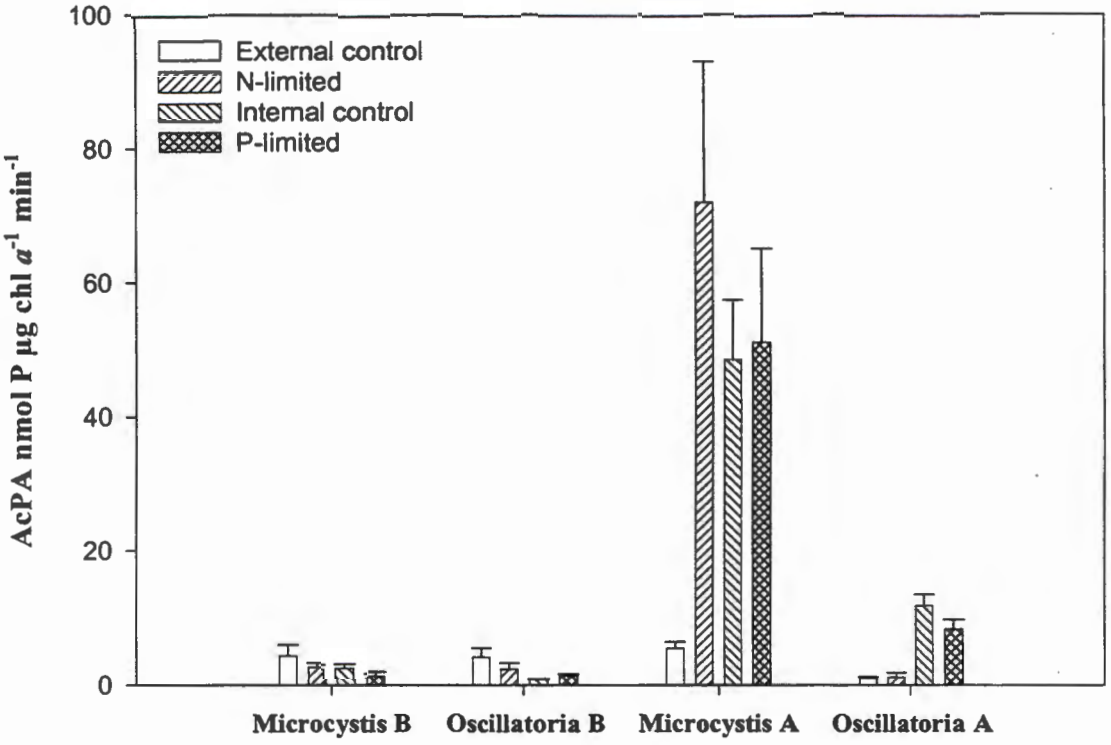


Figure 5.10. Acid phosphatase activity (mean and standard error for six samples) for *Microcystis aeruginosa* and *Oscillatoria simplicissima* initially (B = before treatment, day 11 and 8 respectively) and after (A = after treatment, day 18 and 17 respectively) being exposed to varying N:P ratios (External control = EM medium).

5.2.4.2. Total activity

The distribution of total APA between the treatments at the end of the experiment did not differ markedly from the specific activities (Figure 5.9.). In *Microcystis aeruginosa* no statistically significant differences between the treatments could be detected (Kruskal-Wallis $H_{(2,18)}$ p = 0.441), whereas in *Oscillatoria simplicissima* the internal control exhibited the lowest total APA and the N-limited treatment the highest total APA (Kruskal-Wallis $H_{(2,18)}$ p = 0.0114).

5.2.5. Acid phosphatase activity

5.2.5.1. Specific activity

Initially a low level of AcPA was present in both species (Figure 5.10), with no statistically significant difference between the species (Kruskal-Wallis $H_{(1,46)}$ p = 0.3223). In *Microcystis aeruginosa* the AcPA increased markedly with the nutrient limitation, exhibiting 17-fold increase in the internal control, 27-fold increase in the N-limited treatment and 38-fold increase in the P-limited treatment. The external control kept to the initial, low, level. In *Oscillatoria simplicissima* the external control and the N-limited treatment decreased the level of AcPA after nutrient limitation, whereas the internal control increased 13-fold, and the P-limited treatment increased 6-fold compared to initial levels.

At the end of the experiment there was no significant difference in the specific AcPA between the treatments in *Microcystis aeruginosa* (Kruskal-Wallis $H_{(2,18)}$ p = 0.53), whereas in *Oscillatoria simplicissima* the treatments differed significantly (Kruskal-Wallis $H_{(2,17)}$ p = 0.0046, Figure 5.11.). With log-transformed data a pair-wise comparison showed that the N-limited treatment had significantly lower AcPA compared to the internal control (Tukey's post hoc p = 0.0137), whereas the other treatments did not differ significantly.

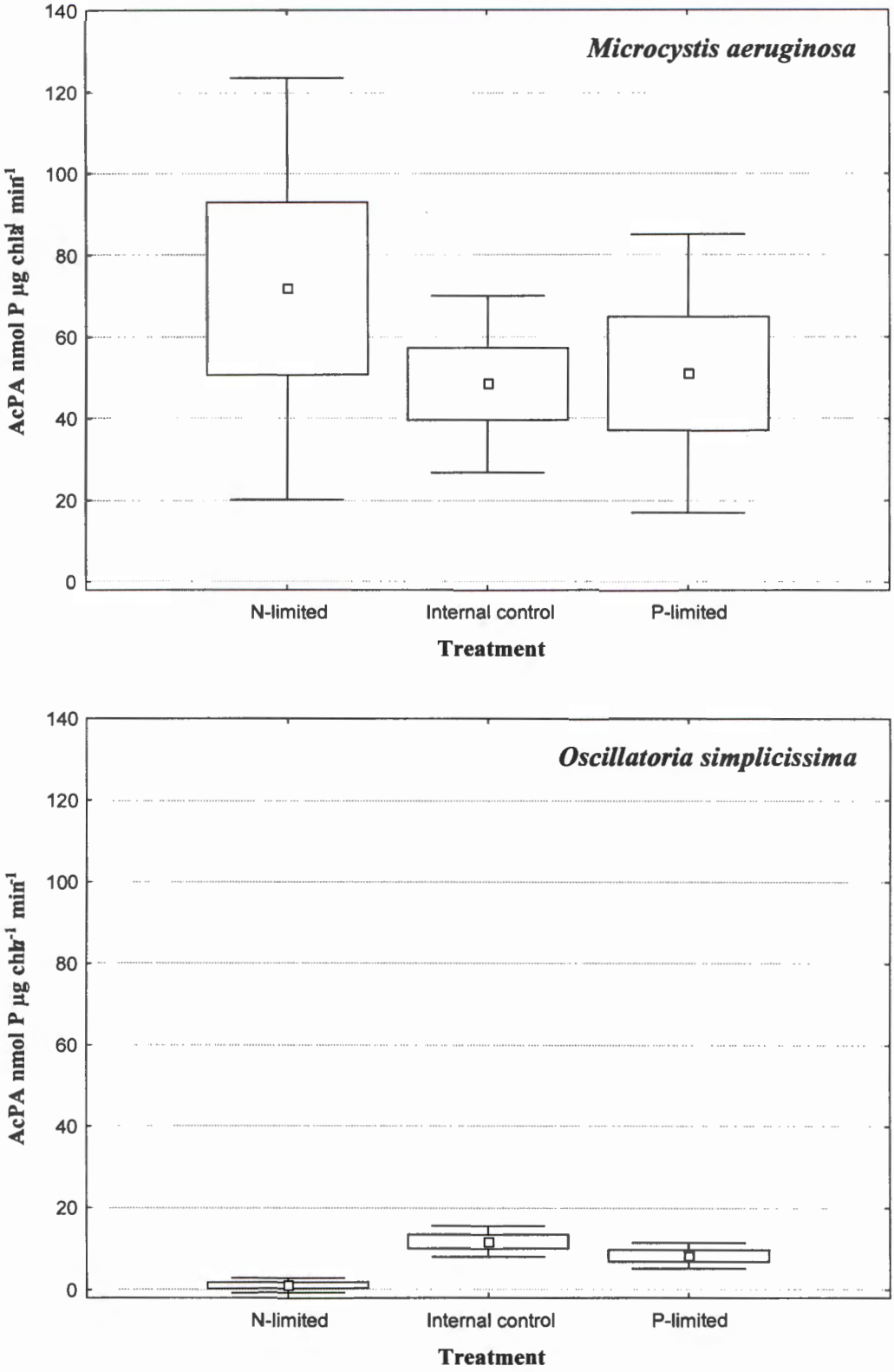


Figure 5.11. Specific acid phosphatase activity of *Microcystis aeruginosa* and *Oscillatoria simplicissima* at the end of the experiment in the three experimental treatments. Averages marked by rectangle, boxes include standard error and whiskers mark standard deviation, n = 6 in each treatment.

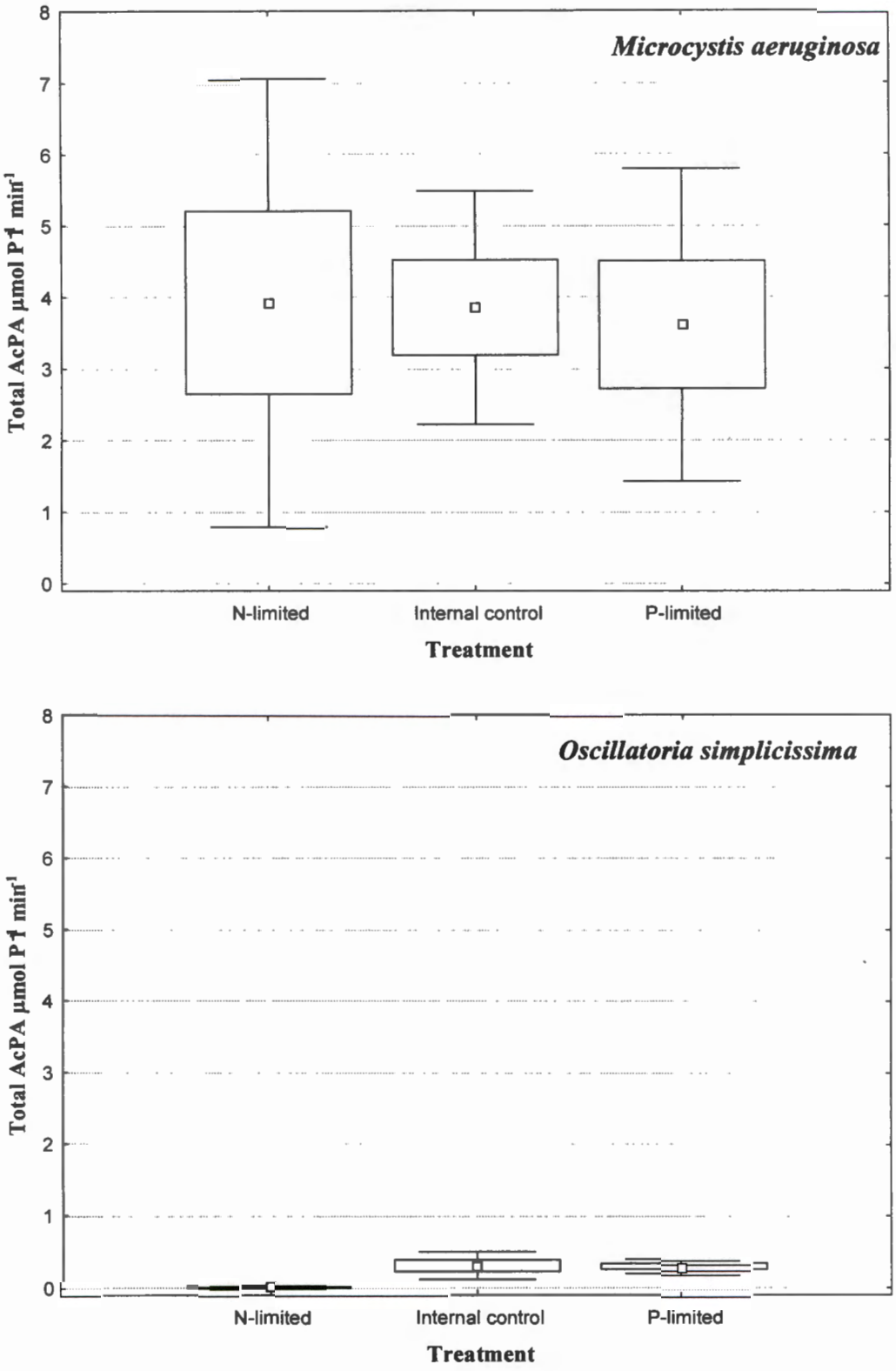


Figure 5.12. Total acid phosphatase activity of *Microcystis aeruginosa* and *Oscillatoria simplicissima* at the end of the experiment in the three experimental treatments. Averages marked by rectangle, boxes include standard error and whiskers mark standard deviation, n = 6 in each treatment.

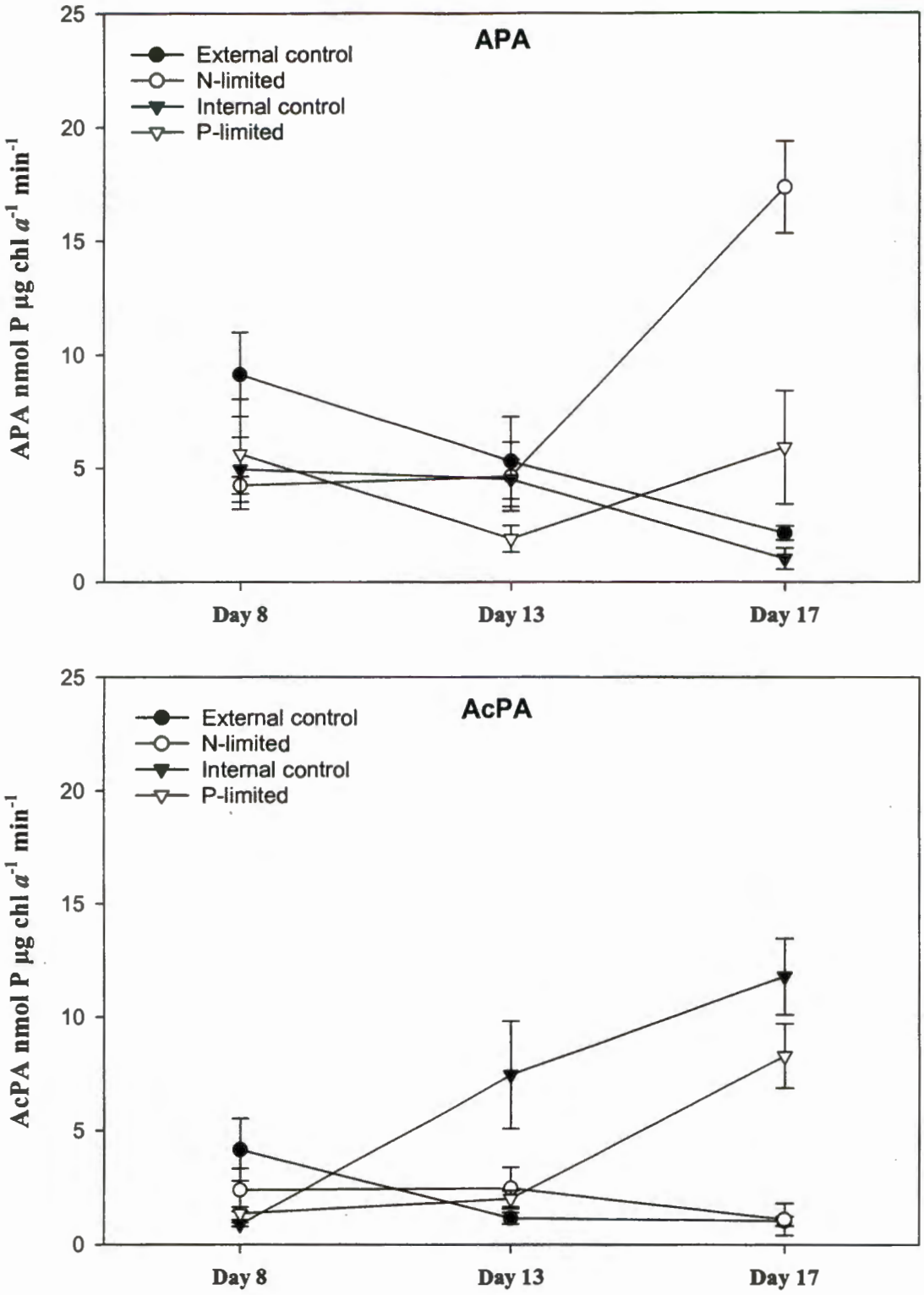


Figure 5.13. Alkaline (APA) and acid (AcPA) phosphatase activity (mean and standard error, n = 6) in *Oscillatoria simplicissima* before the dilutions started (day 8), halfway through the experiments (day 13) and when steady state was reached (day 17).

5.2.5.2. Total AcPA

The total AcPA followed the same pattern as the specific (Figure 5.12), *Microcystis aeruginosa* showing no statistically significant differences between the treatments (Kruskal-Wallis $H_{(2,18)}$ p = 0.087) whereas in *Oscillatoria simplicissima* the N-limited treatment had significantly lower total AcPA compared to both the internal control (Tukey's p = 0.0033) and the P-limited treatment (Tukey's p = 0.0025). Therefore the findings in the specific activities are supported by the results based on total activities.

5.2.6. Temporal changes in phosphatase activity

In *Oscillatoria simplicissima* both APA and AcPA was determined halfway through the experiment (Figure 5.13). APA decreased during the first part of the experiment, and started increasing thereafter, except for the external and the internal controls, which decreased throughout the experiment. AcPA increased in the internal control and P limited treatments steadily, whereas the external control and the N-limited treatment kept to approximately the same level throughout the experiment.

5.2.7. Ectoenzyme activities

Ectoenzymatic phosphatase activity was not detected in *Microcystis aeruginosa* at all. In *Oscillatoria simplicissima* the specific ectoenzymatic phosphatase activity was highest in the N-limited treatment, but the difference between the treatments was not statistically significant (Figure 5.14). Total ectoenzymatic phosphatase activity was significantly lower in the N-limited treatment (Kruskal Wallis $H_{(2,16)}$ p = 0.0046) compared to the other treatments.

5.2.8. Nitrate reductase activity

Nitrate reductase activity was not detected in *Microcystis aeruginosa*, while *Oscillatoria simplicissima* had relatively high amounts of nitrate reductase both initially, as well as after the experiment (Figure 5.15). At the end of the experiment, once steady-state was achieved, the nitrate reductase activity increased to 13 times the initial activities in the N-limited treatment, 11 times the initial activity in the internal control and 9 times the initial activity in the P-limited treatment. In the external control the nitrate reductase activity decreased four-fold.

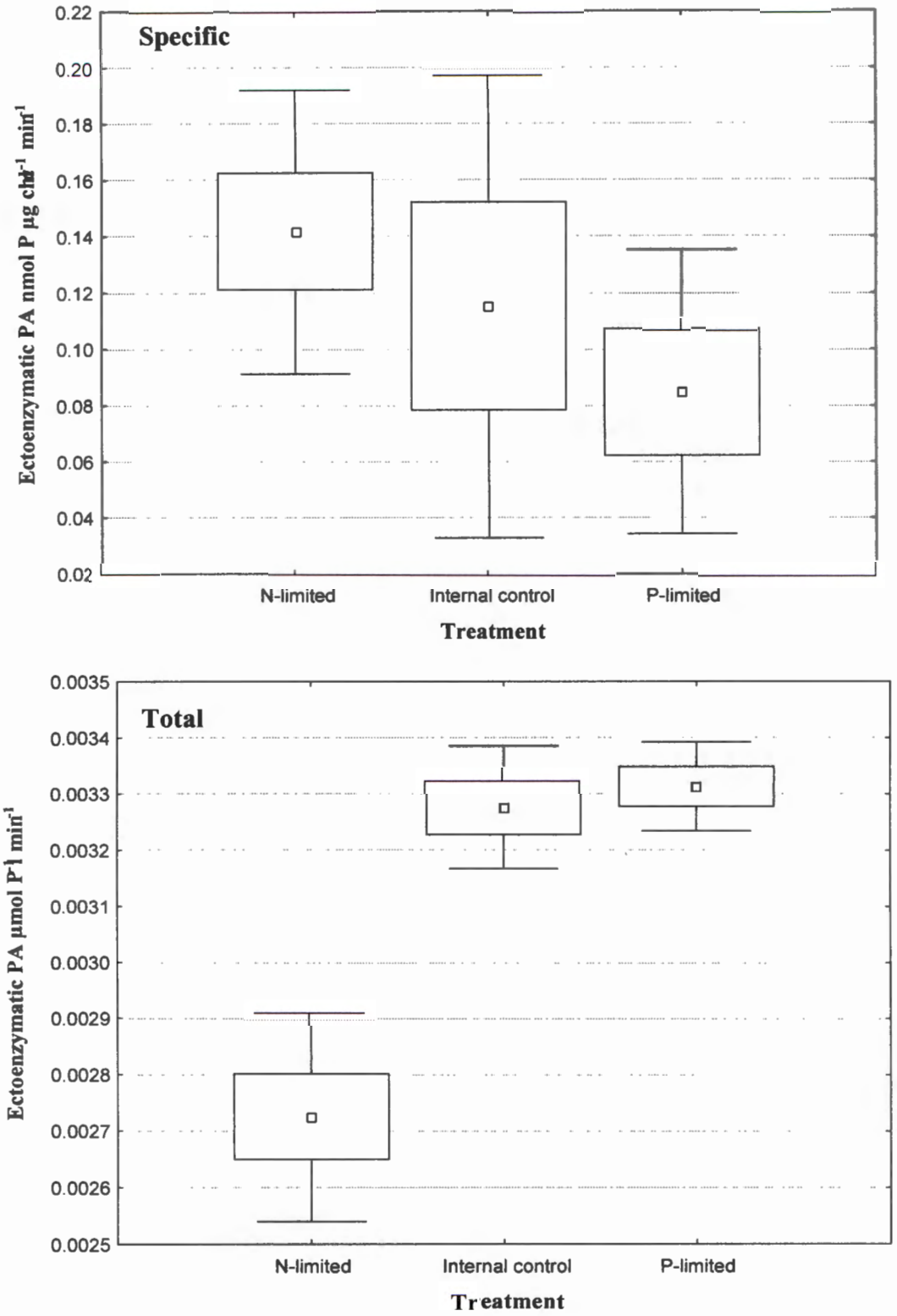


Figure 5.14. Specific and total ectoenzymatic phosphatase activity (middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment) of *Oscillatoria simplicissima* at the end of the experiment in the three different experimental treatments.

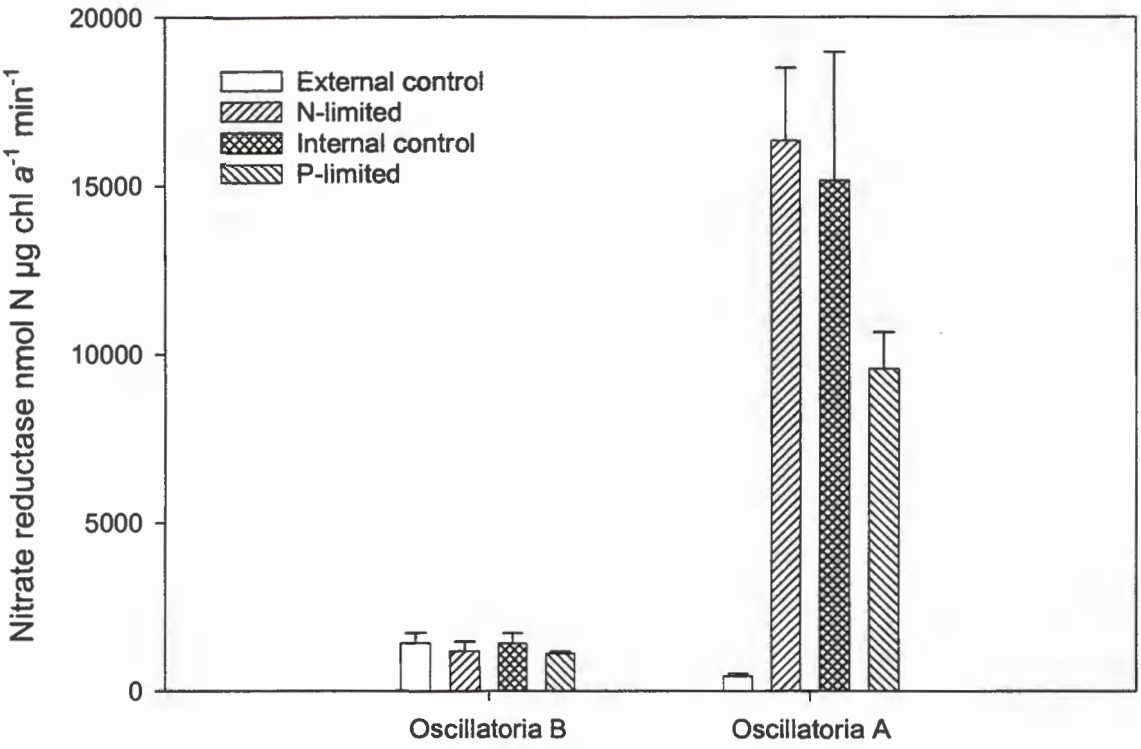


Figure 5.15. Specific nitrate reductase (mean and standard error for six samples) in Oscillatoria simplicissima initially (B = before treatment, day 11 and 8 respectively) and after (A = after treatment, day 18 and 17 respectively) being exposed to varying N:P ratios (External control = EM medium).

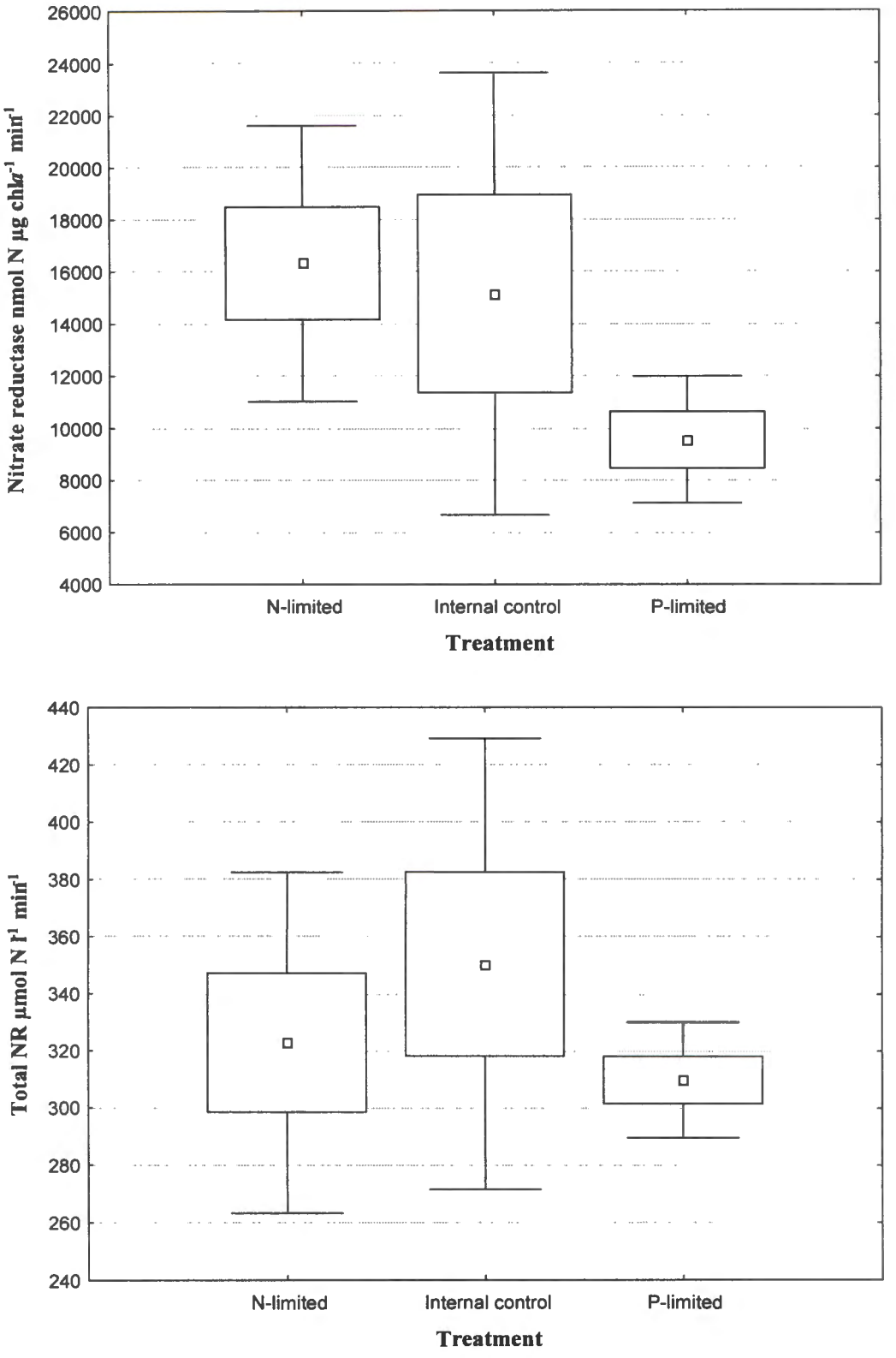
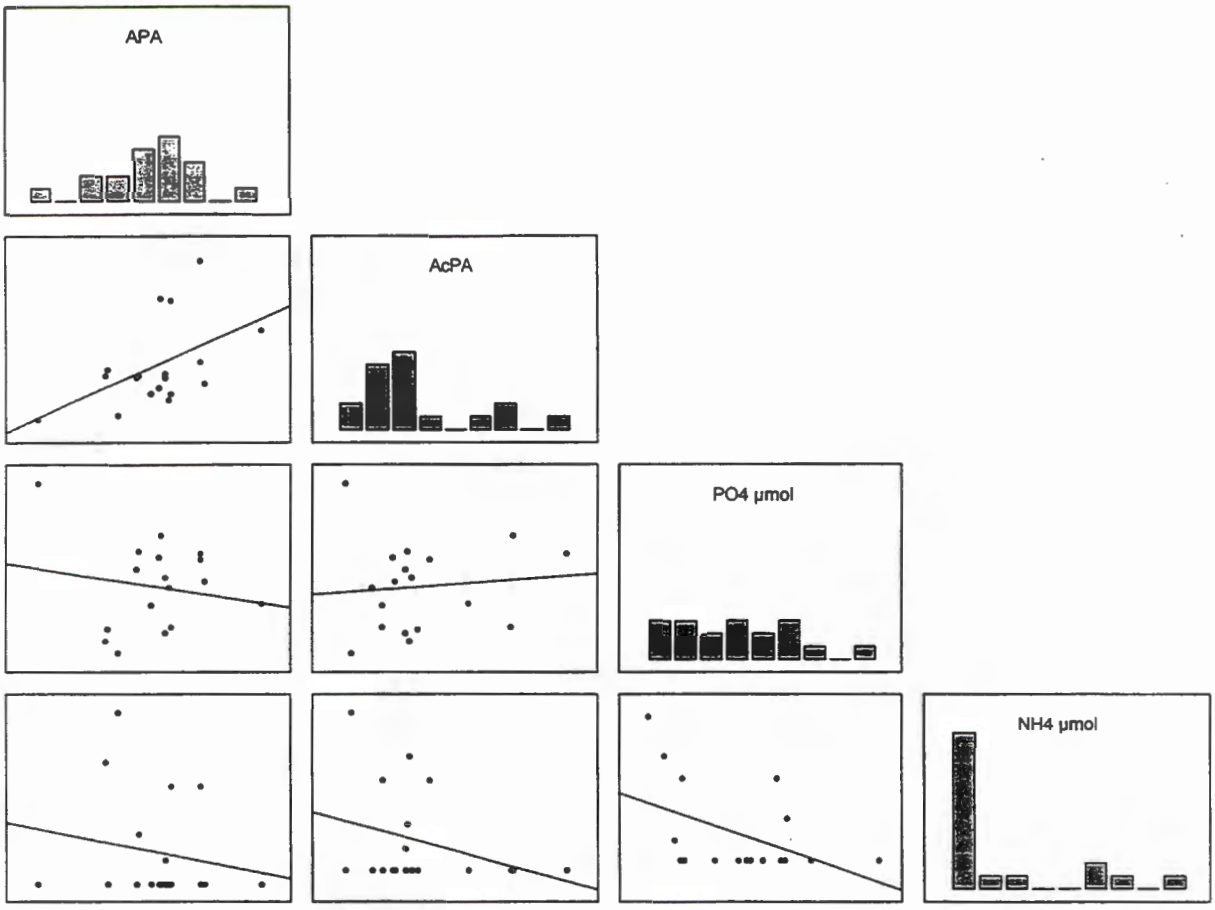
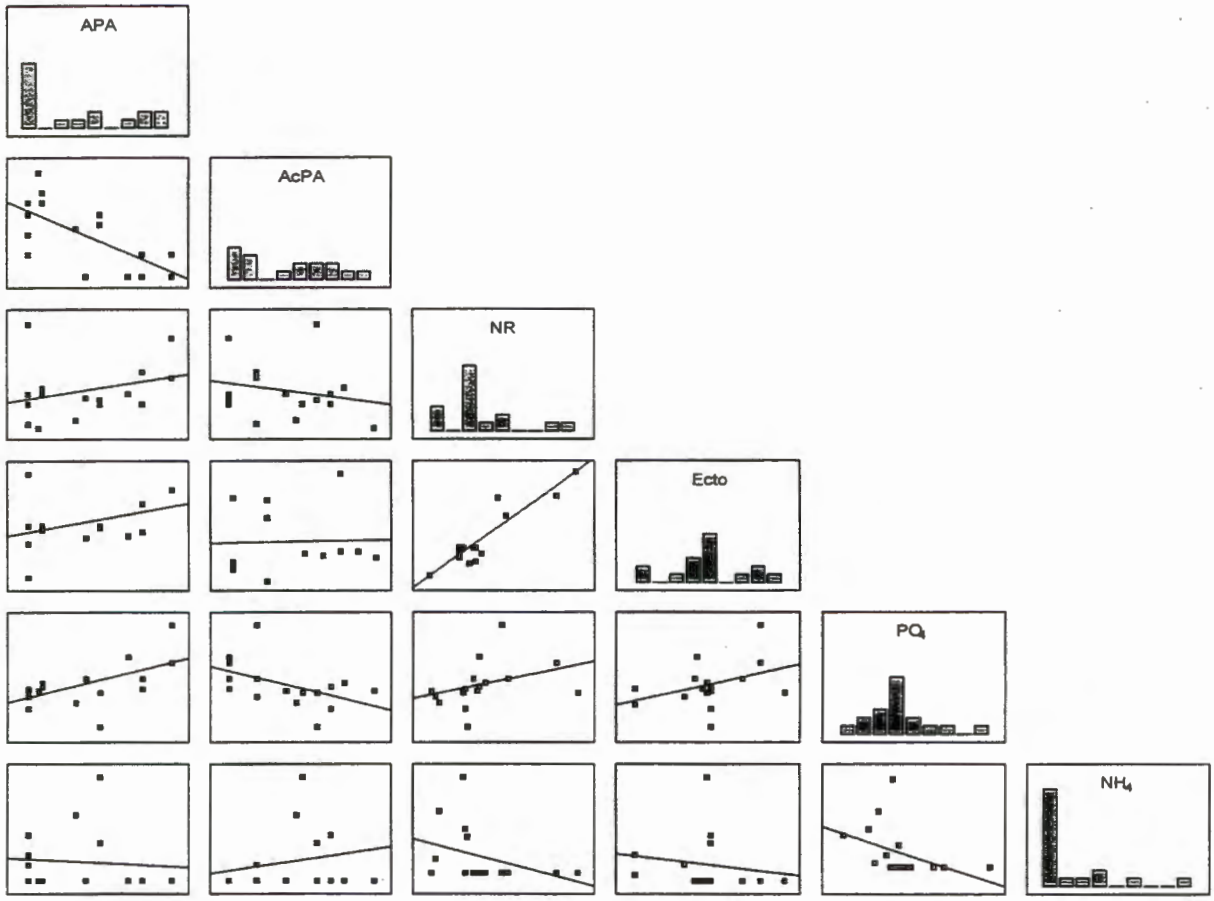


Figure 5.16. Specific and total nitrate reductase (middle point indicates mean, box value standard error and whisker value standard deviation, n = 6 in each treatment) of *Oscillatoria simplicissima* at the end of the experiment in the three different experimental treatments.



	APA	AcPA	PO4	NH4
APA	1.0000			
AcPA	***0.5***	1.0000		
PO4	-0.17	0.1	1.0000	
NH4	-0.17	-0.32	-0.45	1.0000

Figure 5.17 and Table 5.5. Scatterplots illustrating the presence and absence of correlation between the measured specific enzyme activities and chemical variables in the three experimental treatments in *Microcystis aeruginosa* sp. at the end of the experiment. Scatterplots present the variables shown (as histograms presenting the distribution of the data) on top (y-axis) and to the right (x-axis) of each plot. Spearman correlation coefficients (R, n = 18) are given in the table, statistically significant ($p < 0.05$) values are indicated with bold italic numbers.



	APA	AcPA	NR	Ecto	PO_4	NH_4
APA	1.0000					
AcPA	***-0.6256***	1.0000				
NR	0.3311	-0.2394	1.0000			
Ecto	0.3358	0.2029	***0.7026***	1.0000		
PO_4	***0.6034***	***-0.5413***	***0.5752***	0.1708	1.0000	
NH_4	-0.2459	0.2582	***-0.6017***	-0.2976	***-0.7250***	1.000

Figure 5.18 and Table 5.6. Scatterplots illustrating the presence and absence of correlation between the measured specific enzyme activities and chemical variables in the three experimental treatments in *Oscillatoria simplicissima* at the end of the experiment. Scatterplots present the variables shown (as histograms presenting the distribution of the data) on top (y-axis) and to the right (x-axis) of each plot. Spearman correlation coefficients (R, n = 18) are given in the table, statistically significant ($p < 0.05$) values are indicated with bold italic numbers.

At the end of the experiment the P-limited treatment had the lowest specific and total NR activity (Figure 5.16), but the difference between the treatments was not statistically significant (Kruskal-Wallis $H_{(2,16)}$ $p = 0.0529$). The highest NR activity was measured in the N-limited treatment and the internal control, which both contained 2 µmol NO_3^-, as opposed to the 32 µmol NO_3^- in the P-limited treatment.

5.2.9. Correlation between enzyme activities and nutrient concentrations

Spearman Rank correlations were performed to detect any linear relationships between the different types of enzyme activity and nutrient concentrations. In Figure 5.17 and Table 5.5 the correlations for *Microcystis aeruginosa* are shown. The only statistically significant correlation was between APA and AcPA, indicating that both enzymes increased simultaneously. PO_4^{3-} concentration in the media did not correlate with either APA or AcPA.

In *Oscillatoria simplicissima* cultures (Figure 5.18 and Table 5.6) AcPA decreased with increasing APA, as well as increasing PO_4^{3-} concentration. APA and NR activity increased in higher PO_4^{3-} concentration, while NR activity was negatively correlated with the NH_4^+ concentration in the medium. NR activity correlated strongly with higher ectophosphatase activity.

5.2.10. Summary of enzyme activities

Table 5.7 summarises the mean enzyme activities in the two cyanobacteria, *Microcystis aeruginosa* and *Oscillatoria simplicissima*. In general both APA and AcPA was clearly higher in *Microcystis aeruginosa* compared to *Oscillatoria simplicissima*, whereas NR activity was very high in *Oscillatoria simplicissima*. *Oscillatoria simplicissima* also had some, albeit low, ectoenzymatic phosphatase activity, which was not at all detected in *Microcystis aeruginosa*.

At the end of the experiment the summed (APA + AcPA) phosphatase activity was fairly evenly distributed between the different treatments in both *Microcystis aeruginosa* and *Oscillatoria simplicissima* (Figure 5.19). In *Microcystis aeruginosa* AcPA dominated all treatments, while in *Oscillatoria simplicissima* APA dominated in the N-limited treatment.

Table 5.7. Specific (nmol P µg chl a^{-1} min^{-1}) and total (µmol P l^{-1} min^{-1}) enzyme activities (alkaline phosphatase activity (APA), acid phosphatase activity (AcPA), ectoenzymatic PA (ecto) and nitrate reductase (NR)) in Microcystis aeruginosa *and* Oscillatoria simplicissima *in treatments with varying N:P ratios (mean values ± standard error of six replicates). Samples were taken at day 19* (M. aeruginosa) *and day 17* (O. simplicissima).

		Microcystis aeruginosa			*Oscillatoria simplicissima*		
		N-limited	Internal Control	P-limited	N-limited	Internal Control	P-limited
APA	specific	33.64 ± 15.42	34.23 ± 12.60	29.57 ± 6.67	17.35 ± 4.94	1.02 ± 1.13	5.91 ± 5.60
	total	1.7 ± 0.9	2.4 ± 0.6	2.1 ± 0.6	0.3 ± 0.1	0.03 ± 0.03	0.2 ± 0.2
AcPA	specific	71.86 ± 51.64	48.53 ± 21.61	51.08 ± 34.06	1.12 ± 1.74	11.79 ± 3.76	8.31 ± 3.16
	total	3.9 ± 3.1	3.9 ± 1.6	3.6 ± 2.2	—	0.3 ± 0.2	0.3 ± 0.1
Ecto	specific	—	—	—	0.14 ± 0.05	0.12 ± 0.08	0.08 ± 0.05
	total	—	—	—	0.003 ± 0.0002	0.003 ± 0.0002	0.003 ± 0
NR	specific	—	—	—	16329 ± 5297	15160 ± 8485	9560 ± 2430
	total	—	—	—	323 ± 60	350 ± 79	310 ± 20

— = not detected

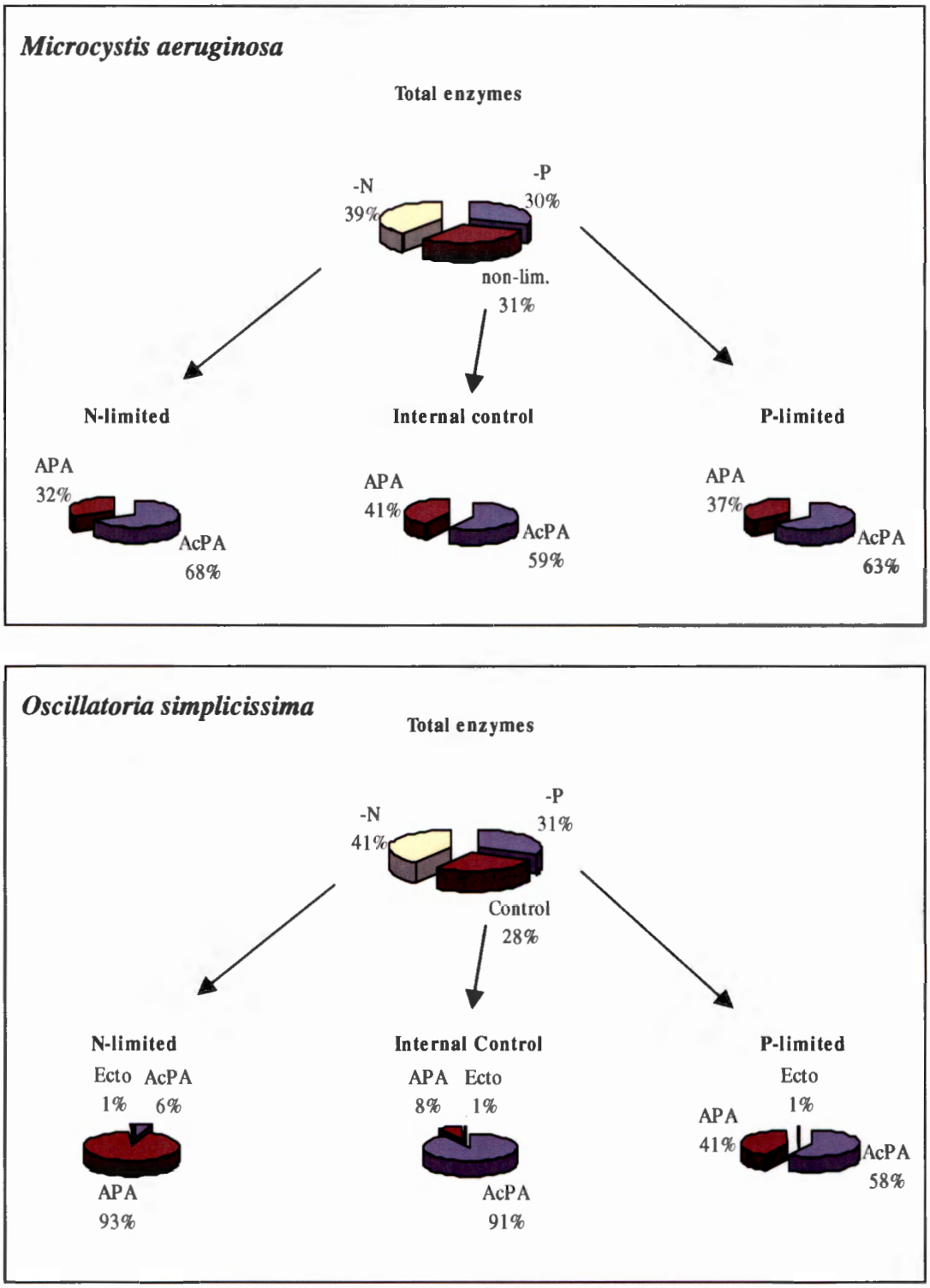


Figure 5.19. Percentage distribution of total enzyme activity (APA, AcPA and ectoenzymatic phosphatase activity (*Oscillatoria simplicissima* only)) in the different treatments, and percentage distribution of enzyme type in each treatment in *Microcystis aeruginosa* (above) and *Oscillatoria simplicissima* (below).

Table 5.8. *Differences between the fluorescence signal and derivations thereof in the external control treatment (grown in GBG-11 medium) of* Microcystis aeruginosa *(n = 16) and* Oscillatoria simplicissima *(n = 18) (p-values based on ANOVA or Kruskal-Wallis ANOVA, depending on normality of the data). For explanations of the abbreviations in the first column, refer to Chapter 2.*

Fluorescence signal / yields / derivations	***Microcystis aeruginosa***		***Oscillatoria simplicissima***		**ANOVA**
	Average	St. deviation	Average	St. deviation	
F_0/F_M	0.526	0.03	0.556	0.02	p=0.0021
F_V/F_0	0.907	0.11	0.801	0.07	p=0.0013
Quantum efficiencies (flux ratios)					
φP_0 (= F_V/F_M)	0.474	0.03	0.444	0.02	p=0.0021
Ψ_0	0.609	0.02	0.409	0.02	p<0.001
φE_0	0.288	0.01	0.181	0.01	p<0.001
φD_0	0.526	0.03	0.556	0.02	p=0.0021
Specific fluxes					
ABS/RC	10.039	1.11	8.862	0.80	p<0.001
TR/RC	4.729	0.22	3.917	0.15	p<0.001
ET/RC	2.883	0.23	1.606	0.15	p<0.001
DI/RC	5.309	0.90	4.946	0.66	p>0.10
Vitality indexes					
PI_{ABS}	1.423	0.22	0.631	0.08	p<0.001

Table 5.9. *Differences between the fluorescence signal and derivations thereof in the three experimental treatments at the end of the experiment in* Microcystis aeruginosa. *For explanations of the abbreviations in the first column, refer to Chapter 2. For results concerning statistical differences between the treatments, refer to text.*

	N-limited		Internal Control		P-limited		ANOVA
	Average	St. Deviation	Average	St. Deviation	Average	St. Deviation	
F_0/F_M	0.687	0.05	0.648	0.03	0.614	0.01	p=0.0000
F_V/F_0	0.461	0.10	0.546	0.06	0.628	0.03	p=0.0000
Quantum efficiencies (flux ratios)							
$\varphi P_0 (= F_V/F_M)$	0.313	0.05	0.352	0.03	0.386	0.01	p=0.0000
Ψ_0	0.497	0.04	0.512	0.02	0.546	0.03	p=0.0001
φE_0	0.156	0.03	0.180	0.02	0.211	0.01	p=0.0000
φD_0	0.687	0.05	0.648	0.03	0.614	0.01	p=0.0000
Specific fluxes							
ABS/RC	12.590	2.03	11.857	0.91	10.952	0.42	p=0.0055
TR/RC	3.863	0.351	4.153	0.11	4.220	0.10	p=0.0001
ET/RC	1.928	0.31	2.129	0.12	2.306	0.16	p=0.0000
DI/RC	8.727	1.92	7.704	0.90	6.732	0.37	p=0.0003
Vitality indexes							
PI_{ABS}	0.387	0.17	0.492	0.10	0.697	0.10	p=0.0000

Table 5.10. *Differences between the fluorescence signal and derivations thereof in the three experimental treatments at the end of the experiment in* Oscillatoria simplicissima. *For explanations of the abbreviations in the first column, refer to Chapter 2. For results concerning statistical differences between the treatments, refer to text.*

	N-limited		Internal Control		P-limited		ANOVA
	Average	St. Deviation	Average	St. Deviation	Average	St. Deviation	
F_0/F_M	0.892	0.01	0.794	0.02	0.779	0.02	p=0.0000
F_V/F_0	0.122	0.01	0.260	0.03	0.285	0.03	p=0.0000
Quantum efficiencies (flux ratios)							
φP_0 (= F_V/F_M)	0.109	0.01	0.206	0.02	0.221	0.02	p=0.0000
Ψ_0	0.493	0.06	0.478	0.05	0.430	0.06	p=0.0081
φE_0	0.054	0.01	0.098	0.01	0.096	0.02	p=0.0000
φD_0	0.892	0.01	0.794	0.02	0.779	0.02	p=0.0000
Specific fluxes							
ABS/RC	44.282	3.62	21.024	3.10	18.640	1.656	p=0.0000
TR/RC	4.799	0.44	4.285	0.31	4.100	0.17	p=0.0002
ET/RC	2.383	0.47	2.057	0.33	1.766	0.29	p=0.0003
DI/RC	39.483	3.30	16.739	2.843	14.541	1.58	p=0.0000
Vitality indexes							
PI_{ABS}	0.027	0.01	0.116	0.03	0.121	0.04	p=0.0000

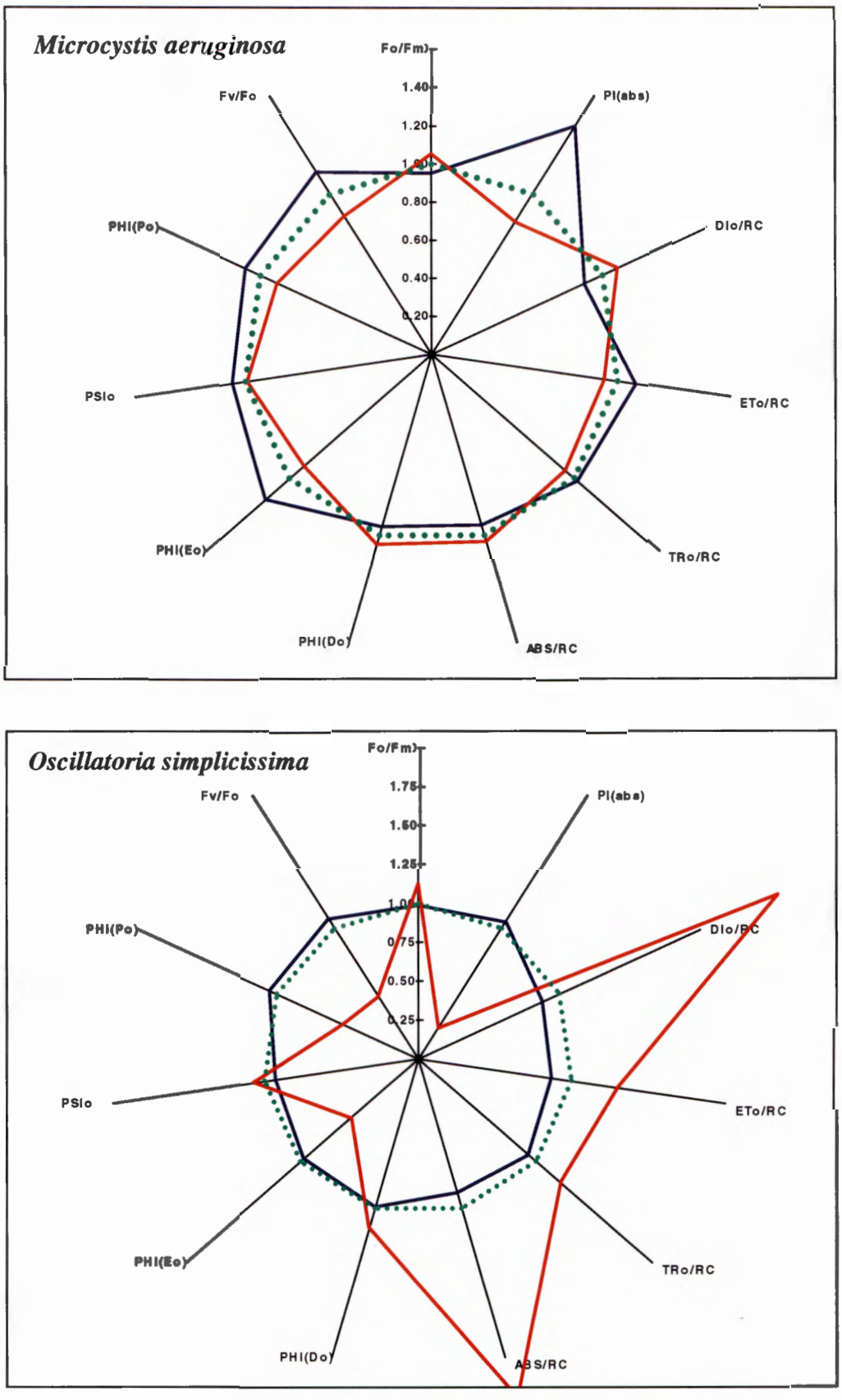


Figure 5.20. Flux ratios, specific energy fluxes and vitality of PSII functions in *Microcystis aeruginosa* and *Oscillatoria simplicissima* grown in different N:P ratios. Red line = N-limited treatment, blue line = P-limited treatment and green line = Internal control.

Ectoenzymatic phosphatase activity constituted only 1% of the overall phosphatase activity in *Oscillatoria simplicissima*.

5.2.11. Chlorophyll-*a* fluorescence

The vitality and photosynthetic performance of the cells were monitored using *in vivo* fluorescence kinetics and the JIP-test. When comparing the external controls of the different species, both flux ratios, such as the function of the light reaction, the dark reaction, electron transport and heat dissipation, as well as specific fluxes, such as the antenna size, trapping and electron transport per reaction centre, differed significantly between the species (Table 5.8). The performance index was significantly higher in *Microcystis aeruginosa* compared to *Oscillatoria simplicissima*.

In Tables 5.9 and 5.10 differences between the treatments in photosynthetic parameters are presented for both species separately.

When interpreting the information received from the JIP-test the following effects can be seen. Growth in N or P limited medium affected most other derivations in *Microcystis aeruginosa*. The JIP test revealed a down regulation of the PSII function in the N-limited cells, through reductions in both primary photochemistry, which indicates according to Parkhill *et al.* (2001) a higher level of nutrient stress in the cells. Also the electron transport and dark reaction functions were reduced. The N-limited cells exhibited significantly lower trapping of energy per reaction centre (Tr_0/RC), light reaction (φP_0), F_V/F_0, dark reaction function (ψ_0), electron transport (φE_0 and ET_0/RC) as well as performance index (PI_{ABS}). Heat dissipation (φD_0 and DI/RC), antenna size (ABS/RC), F_0/F_M were significantly higher in the N-limited cells compared to the other treatments (Figure 5.20 and Table 5.9).

In *Oscillatoria simplicissima* (Table 5.10) the N-limited cells once again differed from the other treatments. The N-limited cells had significantly lower F_V/F_0, light reaction function, electron transport and performance index compared to the other treatments, while the heat dissipation, antenna size, dark reaction functions and specific electron transport (per reaction centre) were significantly higher compared to the other treatments. P-limitation did not seem to affect the PSII

system nearly as severely as N-limitation, and only few differences can be seen between the PSII functions in the internal control and P-limited cells (Figure 5.20).

In summary, the two species reacted similarly to the nutrient limitation treatments with respect to PSII function with the exception of trapping and electron transport per reaction centre, and ψ_0 which is a derivation of the two. These parameters were in *Microcystis aeruginosa* lowest in the N-limited treatment and in *Oscillatoria simplicissima* in the P-limited treatment. The effect of N-limitation was more severe in *Oscillatoria simplicissima* cells compared to *Microcystis aeruginosa* (Figure 5.20).

Microcystis aeruginosa exhibited no K-peak or J-peak in the fast fluorescence transient in either treatment, compared to the internal control. *Oscillatoria simplicissima* cells increased their fluorescence in both the K-band and the J-band, and in both N and P-limited treatments. The K-peak was more pronounced in the N-limited *Oscillatoria simplicissima* cells.

5.3. Discussion and conclusions

Oscillatoria simplicissima reached fast growth faster compared to *Microcystis aeruginosa*, and also continued its growth after the dilutions had started, which suggests that the level of nutrients in the *Oscillatoria simplicissima* cultures were so low after only 8 days that they were already limiting the growth of this alga. The first "dilution" in this case may have been a replenishment of a limiting nutrient, other than N or P, such as a micronutrient or iron, which has been shown to be the of great importance for cyanobacterial growth (Lyck *et al.* 1996). In a study by Marco and Orús (1988) the lowest concentration of phosphate allowing the maintenance of a batch culture of *Oscillatoria* sp. was 0.1mM. During this experiment, however, *Oscillatoria simplicissima* grew well and attained steady-state with significantly lower PO_4^{-3} concentrations (0.2 µM).

In the low nutrient concentrations used, *Microcystis aeruginosa* did not grow fast enough to attain steady-state in the culture vessels, and was thus washed out of the medium. Work done elsewhere on *Microcystis aeruginosa* growth has shown that the alga is limited by NO_3^- at concentrations of 0.2 mM (Long *et al.* 2001), which is ten times higher compared to the highest concentrations used in this experiment, and was most probably restricting the growth of this alga. No nitrate reductase activity was detected during the experiments, possibly suggesting that a

certain threshold level of NO_3^- must be present to activate NR and make possible NO_3^- assimilation. P concentrations of 6 µM have been used for P-limited experiments on *Microcystis aeruginosa* (Oh *et al.* 2000), therefore both N and P was available in very low concentrations compared to the requirements of the alga. Low concentrations were, however, crucial in order to attain detectable increases in phosphatase activity.

Microcystis aeruginosa has polyphosphate granules as well as cyanophycin granules in its cells (Barlow *et al.* 1979), and has been shown to use its stored polyphosphate for metabolism during the beginning of phosphate limitation. The sharp increase in AcPA may indicate release of these internal P reserves at the end of the experiment, with subsequent AcPA increase to facilitate its hydrolysation to bioavailable monophosphate. It is thus equipped for adapting for at least short periods of P-starvation. Cyanophycin granules are most probably consisted of proteinaceous reserve material (Carr and Whitton 1973), the presence of which suggest that *Microcystis aeruginosa* is capable of storing nitrate in this form. *Oscillatoria simplicissima* cells also contain polyphosphate granules storing phosphorus especially during the lag and fast growth phases of the growth cycle (Venter 2000), and its comparatively high PO_4^{3-} consumption suggests that P indeed was being stored during the experiment. *Oscillatoria simplicissima* also possesses cyanophycin granules in all growth stages, storing especially nitrogen (Venter 2000). The probable presence of nitrate stores in *Oscillatoria simplicissima* cells may explain the high NR activities despite the absence of detectable NO_3^- in the surrounding medium. NR needs the substrate NO_3^- to activate the enzyme (Grossman and Takahashi 2001), and this substrate may, in this case, have been intracellular. Larger cell size in *Oscillatoria simplicissima* will probably allow this species to store more nutrients via luxury uptake during nutrient excess, which would provide it with a mechanism to maintain better growth than the smaller *Microcystis aeruginosa* cells during nutrient depleted conditions (Malone 1980).

It must be emphasised that no NH_4^+ was added to the growth medium but some NH_4^+can be expected to enter the medium from algal excretion and during cell lysis (Wetzel 1983). It has been previously shown, that many cyanobacteria in general are able to grow with NH_4^+as a nitrogen source (Flores and Herrero 1994), but *Oscillatoria simplicissima* did not grow well in the EM growth medium with NH_4^+as a nitrogen source (Venter 2000). The lack of NR activity in *Microcystis aeruginosa* cells could be a result of preferred growth on NH_4^+, this also being suggested by the rapid disappearance of the detected NH_4^+ present before the dilutions started.

Chlorophyll-*a* concentration and cell number was lowest in the N-limited treatment in *Microcystis aeruginosa*, whereas the amount of chlorophyll-*a* per cell increased in the N-limitation compared to the other treatments. Thus it seems like fewer cells equipped with higher amount of chlorophyll will be a response mechanism by this unicellular alga to severe N-limitation. The biomass, determined as chlorophyll-*a* concentrations, did not vary between the treatments in *Oscillatoria simplicissima*, whereas the filaments decreased in length with decreasing concentration of nutrients. N-limited cells had the highest percentage of short filaments (<20 µm), whereas the external control was dominated by long (>180 µm) filaments. In a study investigating the seasonal variation in trichome length in some filamentous cyanobacteria it was found that *Planktothrix agardhii* (previously known as *Oscillatoria agardhii*) had shorter filaments in lower ambient dissolved N, even though the most important variable influencing the trichome length was found to be temperature (Romo 1994). Similarly to the findings of Romo (1994), this work demonstrated that a pronounced decrease in size of the filaments is a consequence of lower NO_3^- concentrations in the filamentous *Oscillatoria simplicissima*. Smaller size of the filaments will increase the surface area taking up nutrients, which is a well-known coping mechanism of algae in nutrient depleted environments (e.g. Malone 1980).

The alkaline phosphatase activities were initially high in both cyanobacteria, indicating that alkaline phosphatase may be a constitutive enzyme. Compared to the initial levels of the green algae *Chlorella* sp. and *Chlamydomonas* sp. (Chapter 4) the initial levels were significantly higher for the cyanobacteria studied. Similar results were found in a previous study by Du Plessis *et al.* (2002), where constitutive APA was > 10 times higher in *Oscillatoria simplicissima* compared to *Chlamydomonas incerta*. Contrary to this, Marco and Orús (1988) demonstrated that in some other cyanobacteria, such as *Oscillatoria* sp. and *Anabaena* sp., constitutive levels of alkaline phosphatase were not significant. Hino (1988) found the highest constitutive level of APA in *Anabaena* sp. and *Melosira granulata*, whereas the constitutive level of APA was lowest in other cyanobacteria such as *Aphanizomenon flos-aquae* and *Microcystis aeruginosa*, as well as in the green algae *Oocystis* sp. and *Scenedesmus bijunga*. Thus great variability exists between species of algae and the constitutive level of APA.

In *Microcystis aeruginosa* nutrient limitation, irrespective of the N:P ratio, induced some increase in the APA, whereas in *Oscillatoria simplicissima* APA did not respond to nutrient

limitation at all, except in the N-limited treatment. The reason for this could be the presence of stored phosphates, which could be used directly for growth without phosphatase activity necessary, or that P uptake was simply not required in these circumstances. A positive correlation between APA and PO_4^{3-} existed in *Oscillatoria simplicissima* cultures, and may indicate that as a consequence of a moderately increased APA the ambient, bioavailable PO_4^{3-} increases in the surrounding medium. Nitrate, however, seemed to be the most critical nutrient, and it is possible that instead of increasing APA the cells used most of their energy for nitrate uptake and utilisation. Doonan and Jensen (1980) reached similar results for *Oscillatoria spiroides* and *O. prolifera,* concluding that APA was not affected by P-deficiency. Hino (1988) studied 8 different phytoplankton species and found a comparatively low induction of APA in *Microcystis aeruginosa* in P_i depleted conditions, compared to *Aphanizomenon flos-aquae, Anabaena* sp., 3 green algae including *Chlorella pyrenoidosa* and 2 diatom species. Induction of APA in P-deficiency has, however, been demonstrated in several other studies concerning cyanobacteria, such as *Oscillatoria* sp. and *Anabaena* sp. (Marco and Orús 1988), *Anabaena flos-aquae* (Bone 1971), *A. variabilis* (Healey and Hendzel 1979), *A. spiroides* and *A. cylindrica* (Doonan and Jensen 1980). The response of APA seems therefore to be highly species-specific.

It has been shown that cyanobacteria might be able to produce large amounts of phosphodiesterases (Pettersson 1980), which were not measured during this study, but may explain a lesser induced amount of phosphatase (= phosphomonoesterase) activity measured in comparison to the green algae (Chapter 4). However, in a study by Olsen *et al.* (1989) *Microcystis aeruginosa* was shown to have higher alkaline phosphatase activity compared to a green alga, *Staurastrum luetkemuellerii*, when grown under pulsating phosphorus supply. Another *Oscillatoria* sp. (strain BA 010) grew well on organic glucose-6-phosphate (Marco and Orús 1988), suggesting the potential to utilise organic phosphorus efficiently and thus indicating strong phosphatase activity. Therefore it is unclear why the cyanobacteria studied in this work exhibited lower APA in nutrient limitation compared to the green algae studied in Chapter 4. A possible explanation may be a channelling of energy and resources toward N regeneration and utilisation rather than P-uptake.

The AcPA, on the contrary to the APA, was at noticeably lower levels initially, and was strongly induced with N and P limitation irrespective of N:P ratio in *Microcystis aeruginosa*, and to a lesser extent in the P-limited and internal control treatments of *Oscillatoria simplicissima*. Thus

AcPA, contrary to common assumption (e.g. Jansson *et al.* 1988), exhibited less constitutive features, and was clearly induced by the nutrient limitation. During the first part of the study most phosphatase activities in *Oscillatoria simplicissima* were decreasing, instead of a steady temporal increase with decreasing ambient P-concentration. Late activation (or production) of phosphatase activity indicates that other strategies are present to manage moderate P-limitation, and phosphatases are induced only in the case of severe P-limitation, if at all.

The constitutive APA:AcPA ration was high in both *Microcystis aeruginosa* and *Oscillatoria simplicissima*, thus confirming the observations made during the field-studies in the Vaal River (Chapter 3).

The ectoenzymatic phosphatase activity was, as in *Chlamydomonas* sp., quantitatively unimportant compared to the intracellular phosphatase activities.

To summarise, the level of APA and AcPA induction seems to be more arbitrary compared to the e.g. green algae studied in Chapter 4, both temporally as well as quantitatively.

Nitrate reductase activity was only induced in *Oscillatoria simplicissima*, while no NR activity was detected in *Microcystis aeruginosa*. In prokaryotes, three types of NR have been identified, two of which (termed NAR and NAP) are associated with anaerobic NO_3 respiration, and one (NAS) participates in nitrogen assimilation (Richardson *et al.* 2001). Only the NAS-type NR has been identified in cyanobacteria (Richardson *et al.* 2001) and thus it can be assumed that all NR activity detected during the present work was directly associated with nitrate utilisation. Compared to *Chlamydomonas* sp. (Chapter 4) the specific NR activity was initially approximately 10 times higher, and once steady-state was achieved in the nutrient depleted medium 4-7 times higher. This is contradictory to earlier findings, where the constitutive level of NR activity in *Chlamydomonas incerta* was found to be significantly higher than in *Oscillatoria simplicissima* (Du Plessis *et al.* 2002). During these experiments the NR activity in *Oscillatoria simplicissima* was strongly induced by low NO_3^- concentrations in the medium, but showed a strong negative correlation with NH_4^+ concentration, possibly indicating inhibitory effect of NH_4^+ for NR activity. The presence of high NR activity in absence of ambient NO_3^- may be explained as either

1. a reduction in ambient NO_3^- as a consequence of high NR activity and subsequent uptake, or
2. enhanced mobilisation of intracellular N reserves causing an increased NR activity.
3. since the measurement of NR activity involves optimal NR activity (the substrate being present in abundance during the measurement) the high activity may also reflect that the activity level of the enzyme is high and the enzyme is ready to quickly produce NH_4 for the cell. Jones *et al.* (1998) has showed different activation states of NR enzyme in e.g. tomatoes.

Due to the prolonged deprivation of N during the experiments, the second and third alternatives seem most likely, thus suggesting efficient use of N reserves and potential to respond fast to increased NO_3^- supply.

Apart from enzyme activities the effect of low nutrient treatments could be clearly seen also in the photosynthetic efficiency and vitality of the cells. The increased ABS/RC in N-limited cells represents an increase in the chlorophyll antenna size (Krüger *et al.* 1997). In *Microcystis aeruginosa* this increase in antenna size was accompanied by an increase in chlorophyll *a* per cell. The increased antenna size can be viewed as a compensation for a greater number of deactivated reaction centres in the stressed organism (Hillier *et al.* 1992). Antenna molecules from deactivated reaction centres can be transferred to active reaction centres (Strasser *et al.* 1995) resulting in the observed ABS/RC increase. The decrease in primary photochemistry (φP_0) and electron transport (φE_0) indicates increased damage and loss of both energy trapping and electron transport capabilities with decreasing N-concentration in both *Microcystis aeruginosa* and *Oscillatoria simplicissima,* also seen as a J-peak in *Oscillatoria simplicissima.* The presence of a K-peak in the fast fluorescence rise transient also indicates damage to the light reaction and the oxygen evolving processes in *Oscillatoria simplicissima,* which in general showed a severe decrease in photosynthetic vitality (Figure 5.20). N-limitation can be expected to have severe effects on the photochemical processes, since it is one of the fundamental components of the photosynthetic apparatus (Rao and Terry 2000).

The replacement of *Microcystis aeruginosa* with *Oscillatoria simplicissima* in the river in conditions of elevated PO_4^{3-} concentrations in concert with relatively low NO_3^- concentrations, such as reported in the Vaal River (Pieterse and Steynberg 1993) can be supported by the findings of the experiments reported for in this chapter. Both species adapted best to P-limited treatments, indicating that an increase in P will probably not be the most significant change in

the environmental conditions affecting these species. However, *Oscillatoria simplicissima* consumed the available PO_4^{3-} more efficiently compared to *Microcystis aeruginosa,* indicating effective storage of this nutrient. Concurrently, *Oscillatoria simplicissima* had a higher NR activity, irrespective of the N:P ratio, indicating effective NO_3^- utilisation, transport and uptake. Therefore a relative scarcity in N would probably affect *Oscillatoria simplicissima* to a lesser extent compared to *Microcystis aeruginosa*. This could also be seen as a slight negative correlation between *Oscillatoria simplicissima* and NO_3^-, as well as N:P ratio, in the Vaal River (Chapter 3). Both certain unicellular cyanobacteria as well as *Oscillatoria* sp. have been shown to exhibit nitrogenase enzyme activity, which makes possible N_2 fixation in N-limited systems (Golden *et al.* 1997). Whether or not the used strains of *Oscillatoria simplicissima* and *Microcystis aeruginosa* possess nitrogenase is, to the author's knowledge, not known.

Nutrient limitation or excess has also been suggested to promote or decrease buoyancy and subsequent bloom formation. Oliver (1994) and Brookes and Ganf (2001) suggest that abundant nutrients promote buoyancy in *Microcystis aeruginosa*, whereas nutrient limitation decreases buoyancy in order for colonies to migrate and scavenge for nutrients. Brookes and Ganf (2001) also suggest that apart from carbohydrates, other cell constituents such as polyphosphate may also affect cell buoyancy by acting as ballast in the cells. Relative N-limitation (in the presence of abundant PO_4^{3-} and subsequent stored polyphosphates) may thus enhance downward movement in *Microcystis aeruginosa* to scavenge for needed nitrogen. The results from the present experimental work clearly demonstrate the detrimental effects of N-limitation relative to P-limitation to growth and photosynthetic vitality of both *Microcystis aeruginosa* and *Oscillatoria simplicissima. Oscillatoria simplicissima* seems more capable of utilising stored N reserves (high NR activity) and may therefore have an advantage in a N-limited environment, whereas *Microcystis aeruginosa* may have moved to deeper water layers, and would thus not be forming surface blooms or even be detected in routine surface water sampling.

One must also take into account that other limiting or growth-enhancing nutrients, besides N or P, may be affecting the presence and absence of the investigated species in the river. In fact, the increase in *Oscillatoria simplicissima* after the first dilution with N and P-limited media suggests that indeed some other nutrient may have been growth limiting also during the fast growth phase. Especially iron has been shown to be of importance for the growth of cyanobacteria (Lyck *et al.* 1996 and references therein).

Lakes dominated by *Oscillatoriaceae* are typically very turbid, and high abundance of *Oscillatoriaceae* occur predominantly under shady conditions (Scheffer *et al.* 1997). Pieterse and Van Vuuren (1997) reported increased turbidity in the Vaal River in the early 1990s, and therefore changes in the light climate of the river may be a partial reason for the change from a *Microcystis*-dominated community to intensified *Oscillatoria simplicissima* blooms. However, also *Microcystis aeruginosa* has been reported to be favoured by high turbidity (Jacoby *et al.* 2000).

The main conclusions based on the results from the experiments reported for in this chapter are:

1. *Oscillatoria simplicissima* grew better in all nutrient limited treatments than *Microcystis aeruginosa*, indicating adaptation to low, or fluctuating, N and P concentrations.
2. The filament length decreased in N and P limitation in *Oscillatoria simplicissima*, and a significant increase in the number of hormogonia produced was observed in the N-limited treatment. This is probably a response to low nutrient concentrations by an increase in the surface / volume ratio of the cells.
3. Alkaline phosphatase activity acted as a constitutive enzyme in both species, being present in relatively high amounts before nutrient limitation.
4. Acid phosphatase activity was clearly induced in *Microcystis aeruginosa*, and was present at lower levels than APA before nutrient limitation. This suggests that AcPA is similarly, if not more, inducible compared to APA in the cyanobacterial species studied.
5. Constitutive APA:AcPA ratio was high in both species, supporting the observations done in the Vaal River.
6. Nitrate reductase activity was only present in *Oscillatoria simplicissima*. The level of NR activity was high in all nutrient limited treatments.
7. Possible reasons explaining the increased abundance of *Oscillatoria simplicissima* in the Vaal River includes several physiological aspects. Better P storing capacity, more effective P consumption during both P excess and limitation, and more effective N transport, uptake and utilisation (based on higher NR activity), compared to *Microcystis aeruginosa* were found in *Oscillatoria simplicissima*. In addition, other environmental variables, such as increased turbidity, may benefit *Oscillatoria simplicissima*.

CHAPTER 6
GENERAL CONCLUSIONS AND DISCUSSION

The underlying theme for this work was the ecophysiological basis for development of mass-occurrences of certain phytoplanktonic species in fresh water environments. A lack of consensus concerning reasons for harmful algal bloom (HAB) formation can be found in literature (Chapter 1), and the research concerning bloom formers in aquatic environments has been intensifying during the past few decades. A number of recent studies have pointed out the need for physiological investigations concerning HAB species, and emphasise the need for understanding the ecophysiological rationale for bloom formation.

The specific aims for this thesis were to study the Vaal River in respect to its phytoplanktonic species composition, emphasising HAB species, and the environmental as well as physiological characteristics of the river and its phytoplankton community. P assimilation was studied by way of determining the magnitude, and seasonal and spatial variation of phosphatase enzyme activities in the river. The composition of the phytoplankton community, and especially the occurrence of HAB species, was studied in relation to both environmental and physiological parameters, in order to identify common features promoting or facilitating bloom formation. Enzyme activities of the phytoplankton community were compared with the species composition in order to trace such potential physiological attributes. Based on the findings from the field studies (Chapter 3) a selection of phytoplanktonic species was subsequently studied *in vitro* (Chapters 4 and 5) with respect to their growth, P and N metabolism and photochemistry, in response to varying N and P concentrations. The ecophysiological performance of the selected species are summarised and implications considered in this concluding chapter.

6.1. High phosphatase activities in the Vaal River

The results of the mean phosphatase activities in the Vaal River as well as in the selected species under experimental conditions are summarised in Table 6.1, together with phosphatase activities reported in previously published literature. To facilitate comparison, all activities found in the literature have been transformed to either specific activity (nmol P µg chl a^{-1} min^{-1}) or total activity (nmol P l^{-1} min^{-1}), when possible. It must be borne in mind, that most field studies use

different fractionation methods for separating the studied organisms into size classes and therefore care must be taken when comparing values.

The ecological field studies confirmed the Vaal River to be a highly eutrophic environment with large annual fluctuations in biomass, nutrient concentrations and species composition (Chapter 3). The analysis of the species composition of the phytoplankton demonstrated the presence of a number of phytoplanktonic species indicating eutrophication (Järnefelt 1952), and capable of forming mass-occurrences. Despite comparatively low N:P ratios in the river, suggesting relative N-limitation of the system, nitrogen fixing cyanobacteria, such as *Anabaena* and *Aphanizomenon* species, occurred sparsely. The phosphatase activities, on the other hand, were high in both algal and bacterial / dissolved size fractions. The relatively low abundance of nitrogen fixing species combined with high phosphatase activities suggest that the river, despite low N:P ratios, is predominantly P-limited.

Compared to values in the literature, the APA was very high in the river (Table 6.1), supporting the work of Hantke *et al.* (1996), who found increased APA in higher trophic level. Phosphatase activities of this magnitude have only been reported in a few other studies, all in highly eutrophic / hypertrophic environments (e.g. Villerest reservoir (Jamet *et al.* 1995, 2001) and in ponds (Matavulj and Flint 1987)). In this work the spatial variation in APA and AcPA in the Vaal River also reflected this pattern, highest activities (both total and specific) being found at the localities with highest nutrient concentrations, also mirrored by the positive correlation between total phosphatases and chlorophyll *a*. The level of AcPA in the Vaal River was high, and the few other field studies in which both AcPA and APA have been determined (Table 6.1) also confirm the importance of AcPA in comparison to APA (Berman *et al.* 1990, Lee 2000, Matavulj and Flint 1987 and Wynne 1977). Only Dodds (1995) report AcPA clearly lower compared to APA. Also studies investigating specific species in laboratory conditions (Table 6.1) have confirmed the presence of high AcPA in e.g. *Chlamydomonas reinhardi* (Patni *et al.* 1977) and the macroalga *Ulva lactuca* (Lee 2000). During this work the AcPA was clearly higher than APA in *Chlamydomonas* sp. (Chapter 4) and *Microcystis aeruginosa* (Chapter 5) in nutrient depleted cultures.

Table 6.1. Phosphatase activities from phytoplankton reported in the literature. If possible, activities have been transformed to nmol P min^{-1} µg chl a^{-1} (or l^{-1}) to facilitate comparisons with the results of this work (in table referred to as Kruskopf 2002). All activities refer to APA, unless AcPA is specifically mentioned.

Enzyme activity	Enzyme activity in nmol min^{-1} µg chl a^{-1} (or l^{-1})	Location / species	Reference
Method: spectrophotometric (pNPP)			
APA: 0-8.2 µmol pNP l^{-1} h^{-1}	*0-137 (l^{-1})*		Berman *et al.* 1990
AcPA: 3.6-21.4 µmol pNP l^{-1} h^{-1}	*60-357 (l^{-1})*		
0-542 µg P l^{-1} day^{-1}	1.26-12.15 (l^{-1})	Lake Kinneret	Berman 1970
0-2321 nmol mg dry $weight^{-1}$ h^{-1}		*Anabaena flos-aquae*	Bone 1971
0-30 nmol l^{-1} min^{-1}	0-30 (l^{-1})	Reservoirs in River Tejo, Portugal	Boavida and Marques 1995
APA: 11-27 nmol l^{-1} min^{-1}	11-27 (l^{-1})	Milford Lake, Kansas 0.2 - >100 µm	Dodds 1995
AcPA: 5-8 nmol l^{-1} min^{-1}	5-8 (l^{-1})	Milford Lake, Kansas 0.2 - >100 µm	
5-25 nmol chl a^{-1} min^{-1}	5-25 (chl a^{-1})	*Chlamydomonas incerta*, diel variation	Du Plessis *et al.* 2002
330-470 nmol chl a^{-1} min^{-1}	330-470 (chl a^{-1})	*Oscillatoria simplicissima*, diel variation	Du Plessis *et al.* 2002
0.05-0.34 µg PNP µg chl a-*1* h^{-1}		Lake Nantua, France	Feuillade *et al.* 1990
5-360 nmol PO_4 mg POM^{-1} h^{-1},		Minnesotan lakes (approximate)	Gage and Gorham 1985
0-10.74 µmol PNPP min^{-1} mg^{-1} protein		Extracellular matrix of *Volvox*	Hallman 1999
0.2-1.1 µmol mg $protein^{-1}$ h^{-1}		Lake Baratao	Hino 1988
0-2.6 µmol l^{-1} h^{-1}	0-43.3 (l^{-1})	Lake Baratao	
0.015-0.315 µmol mg $protein^{-1}$ h^{-1}		*Microcystis aeruginosa* in P_i replete and deplete medium	Hino 1988
0.235-1.73 µmol mg $protein^{-1}$ h^{-1}		*Chlorella pyrenoidosa* in P_i replete and deplete medium	Hino 1988
0.036-4.73 µmol mg $protein^{-1}$ h^{-1}		In 6 other phytoplankton species	Hino 1988
0.005-0.797 mmol pNP h^{-1} (l^{-1})	83-13283 (l^{-1})	Villerest reservoir, total (8-100µm)	Jamet *et al.* 1995
0.045-1.22 mmol pNP h^{-1} (l^{-1})	750-20333 (l^{-1})	Villerest reservoir, total (0.45-100µm)	Jamet *et al.* 1995

0.218-7.05 µmol pNP h^{-1} µg chl a^{-1}	3.6-117.5 (chl a^{-1})	Villerest reservoir, specific (0.45-100µm)	Jamet *et al.* 1995
2.6-45.8 µmol PO_4 l^{-1} h^{-1}	43.3-763 nmol (l^{-1})	Lake Pavin, particulate matter 0.22-100µm	Jamet *et al.* 1997
0-2.083 µm PO_4 h^{-1} µg $protein^{-1}$		Lake Pavin, specific	Jamet *et al.* 1997
11.1-301.7 µM PO_4 l^{-1} h^{-1}	185-5028 (l^{-1})	In Villerest reservoir, particulate matter total (0.22-100µm)	Jamet *et al.* 2001
0-1.6 µM PO_4 µg $protein^{-1}$ h^{-1}		specific (0.22-100 µm)	Jamet *et al.* 2001
2.4-57.5 µM PO_4 l^{-1} h^{-1}	40-958 (l^{-1})	Dissolved <0.22 µm	Jamet *et al.* 2001
0.99-1.92 µmol pNp l^{-1} h^{-1}	16.5-32 (l^{-1})	Lake Łuknajo, algal (>1.2 µm)	Kalinowska 1997
0.006-0.5 µmol pNp l^{-1} h^{-1}	0.1-8.3 (l^{-1})	bacterial (0.45-1.2 µm)	Kalinowska 1997
0.25-0.45 µmol pNp l^{-1} h^{-1}	4.2-7.5 (l^{-1})	dissolved (<0.45)	Kalinowska 1997
APA: 0.1-17.9 µmol min^{-1} l^{-1}	100-17900 (l^{-1})	Vaal River, algal fraction (>1.2 µm)	Kruskopf 2002
APA: 3.3-379 nmol min^{-1} chl a^{-1}	3.3-379 (chl a^{-1})		
AcPA: 0.1-23.7 µmol min^{-1} l^{-1}	100-23700 (l^{-1})		
AcPA: 4.9-239.2 nmol min^{-1} chl a^{-1}	4.9-239.2 (chl a^{-1})		
APA: 0.1-32.1 µmol min^{-1} l^{-1}	100-32100 (l^{-1})	Vaal River, bacterial and dissolved fraction (<1.2 µm)	Kruskopf 2002
APA: 18.4-2462.4 nmol min^{-1} chl a^{-1}	18.4-2462.4 (chl a^{-1})		
AcPA: 0.4-36.6 µmol min^{-1} l^{-1}	400-36600 (l^{-1})		
AcPA: 1.8-7105.5 nmol min^{-1} chl a^{-1}	1.8-7105.5 (chl a^{-1})		
APA: 0.2-2.4 µmol min^{-1} l^{-1}	200-2400 (l^{-1})	*Chlorella* sp.	Kruskopf 2002
APA: 7.9-47.1 nmol min^{-1} chl a^{-1}	7.9-47.1 (chl a^{-1})		
AcPA: 1.7-2.5 µmol min^{-1} l^{-1}	1700-2500 (l^{-1})		
AcPA: 7.1-58.6 nmol min^{-1} chl a^{-1}	7.1-58.6 (chl a^{-1})		
APA: 0.9-1.1 µmol min^{-1} l^{-1}	900-1100 (l^{-1})	*Chlamydomonas* sp.	Kruskopf 2002
APA: 11.3-41.0 nmol min^{-1} chl a^{-1}	11.3-41 (chl a^{-1})		
AcPA: 1.7-4.0 µmol min^{-1} l^{-1}	1700-4000 (l^{-1})		
AcPA: 26.0-128.9 nmol min^{-1} chl a^{-1}	26-128.9 (chl a^{-1})		
APA: 1.7-2.4 µmol min^{-1} l^{-1}	1700-2400 (l^{-1})	*Microcystis aeruginosa*	Kruskopf 2002
APA: 29.6-34.2 nmol min^{-1} chl a^{-1}	29.6-34.2 (chl a^{-1})		

AcPA: 3.6-3.9 µmol min^{-1} l^{-1}	3600-3900 (l^{-1})		
AcPA: 48.5-71.8 nmol min^{-1} chl a^{-1}	48.5-71.8 (chl a^{-1})		
APA: 0-0.3 µmol min^{-1} l^{-1}	0-300 (l^{-1})	*Oscillatoria simplicissima*	Kruskopf 2002
APA: 1.0-17.4 nmol min^{-1} chl a^{-1}	1.0-17.4 (chl a^{-1})		
AcPA: 0-0.3 µmol min^{-1} l^{-1}	0-300 (l^{-1})		
AcPA: 1.1-11.8 nmol min^{-1} chl a^{-1}	1100-11800 (chl a^{-1})		
APA: 1.4 µmol PNP mg $protein^{-1}$ min^{-1}		*Ulva lactuca,* total	Lee 2000
AcPA: 11.7 µmol PNP mg $protein^{-1}$ min^{-1}		*Ulva lactuca,* specific	
50-150 nmol pNP l^{-1} h^{-1}	0.8-2.5 (l^{-1})	Red Sea phytoplankton, 0.2-100 µm	Li *et al.* 1998,
125-150 nmol pNP l^{-1} h^{-1}	2.1-2.5 (l^{-1})	Red Sea dissolved <0.2 µm	(approximate)
<0.2-6.51 µmol pNP mg dry $weight^{-1}$ h^{-1}		*Calothrix viguieri* grown with various P sources	Mahasneh *et al.* 1990
APA: 1-35 µM pNP ml^{-1} h^{-1}	16667-583333 (l^{-1})	Pond in Warwick	Matavulj and Flint 1987, (approximate)
AcPA: 0-18 µM pNP ml^{-1} h^{-1}	0-300000 (l^{-1})	Pond in Warwick	Matavulj and Flint 1987, (approximate)
APA: 0.12-1.28 µmol PNPP hydrolysed ml^{-1} h^{-1}	2000-21333 (l^{-1})	*Chlamydomonas reinhardi* in varying light	Patni *et al.* 1977
AcPA: 0.04-2.02 µmol PNPP hydrolysed ml^{-1} h^{-1}	667-33667 (l^{-1})		
12.5-18 µmol PNPP min^{-1} mg^{-1} protein		190-kD phosphatase from *Chlamydomonas reinhardtii*	Quisel *et al.* 1996
0-14 µmol PNPP min^{-1} mg^{-1} protein		smaller phosphatase-enzyme from *Chlamydomonas reinhardtii*	Quisel *et al.* 1996
0.01-0.22 µmol PNPP µg chl a^{-1} h^{-1}	0.17-3.67 (chl a^{-1})	Red Sea planktonic <100µm	Stihl *et al.* 2001
0.15-31.6 µmol PNPP µg chl a^{-1} h^{-1}	2.5-526.7 (chl a^{-1})	*Trichodesmium* sp.	Stihl *et al.* 2001
*1-70 *10 nmol l^{-1} h^{-1} (peak >300 nmol l^{-1} h^{-1})*	*0.2-11.7 (l^{-1})*		*Vrba et al. 1995*
APA: 200-4000 µmol PNP mg $protein^{-1}$ h^{-1}		*Peridinium* sp. bloom in Lake Kinneret	Wynne 1977 (approximate)
AcPA: 250-5000 µmol PNP mg $protein^{-1}$ h^{-1}			
0-15 nmol pNpp $cell^{-1}$ h^{-1}		extracellular, 4 phytoplankton species in P-limitation	Wynne and Rhee 1988
0-9 nmol PNPP $cell^{-1}$ h^{-1}		intracellular, 4 phytoplankton species in	Wynne and Rhee 1988

		P-limitation	
5.06 µmol product µg l^{-1} h^{-1}	84.3 (l^{-1})	sea water	Yelloly and Whitton 1996
10.9 µmol product µg l^{-1} h^{-1}	181.7 (l^{-1})	drift seaweed pools	Yelloly and Whitton 1996
8.36 µmol product l^{-1} h^{-1}	139.3 (l^{-1})	*Rivularia atra* pools	Yelloly and Whitton 1996
0-0.3 µmol product chl a^{-1} h^{-1}	0-5 (chl a^{-1})		
0 – 80 nmol l^{-1} min^{-1}	0 – 80 (l^{-1})	Donghu Lake, total	Yiyong and Xinyu 1997 (approximate)
0.3-6 nmol µg chl a^{-1} min^{-1}	0.3-6 (chl a^{-1})	Donghu Lake, specific	Yiyong and Xinyu 1997 (approximate)
Method: fluorometric (MUF/MUP)			
0.5-3.0 nmol min^{-1} (50 µM MUP)		River Rheine (approximate)	Admiraal and Tubbing 1991
0.07-5.85 pmol PO_4 ng ATP^{-1} min^{-1}		Lake Plußsee, specific	Chròst and Overbeck 1987
27-124 ng PO_4 l^{-1} h^{-1}	0.01-0.07 (l^{-1})	total microplankton	
15-105 ng PO_4 l^{-1} h^{-1}	0.008-0.06 (l^{-1})	phytoplankton	
10-90 ng PO_4 l^{-1} h^{-1}	0.005-0.05 (l^{-1})	bacteria	
1-76.9 ng MUP hydrolysed h^{-1} ml^{-1}		Lakes in Quebec – Vermont – New York	Currie *et al.* 1986
0-3.5 nmol PO_4 µg chl a^{-1} min^{-1}	0-3.5 (chl a^{-1})	Lakes in Michigan	Elser *et al.* 1986
0-4.5 nmol PO_4 μg^{-1} chl a^{-1} min^{-1}	0-4 (chl a^{-1})	Reservoirs in Tennessee	Elser and Kimmel 1986
4-35 nmol PO4 l^{-1} min^{-1}	4-35 (l^{-1})	Reservoirs in Tennessee	
0-3.2 nM P min^{-1}		Hardwater lakes in Germany	Hantke *et al.* 1996
0-0.45 µmol organic dry wt^{-1} h^{-1} (10 µM MUP)		In 4 phytoplankton species	Healey and Hendzel 1979
0.013-0.66 µmol MUF hydrolysed h^{-1} µg particulate ATP^{-1} (10 µM MUP)		Canadian lakes	Healey and Hendzel 1980
0.01-0.15 µmol PO_4 g dry $weight^{-1}$ min^{-1}		*Ceratium hirundinella*	James *et al.* 1992
0.4-25.9 nmol PO_4 l^{-1} min^{-1}	0.4-25.9 (l^{-1})	In Swedish lakes	Jansson *et al.* 1981
0.01-0.03 nmol product µg chl a^{-1} min^{-1} (1 µM MUP)	0.01-0.03 (chl a^{-1})	*Chlamydomonas sp.*	Kruskopf 2002

0.0007-0.0008 µmol product l^{-1} min^{-1} (1 µM MUP)	0.7-0.8 (l^{-1})	*Chlamydomonas sp.*	
0.08-0.14 nmol product µg chl a^{-1} min^{-1} (1 µM MUP)	0.08-0.14 (chl a^{-1})	*Oscillatoria simplicissima*	Kruskopf 2002
0.003 µmol product l^{-1} min^{-1} (1 µM MUP)	3 (l^{-1})	*Oscillatoria simplicissima*	
1.9-128.4 nM h^{-1} (250 µM MUP)		Bay of Biscay	Labry *et al.* 2002
0-1100 nM h^{-1} (100 µM MUP)		Pomeranian Bight	Nausch 1998 (approximate)
0-67 nM µg chl a^{-1} h^{-1}	0-1.1 (chl a^{-1})		
0-16.4 nmol l^{-1} min^{-1}	0-16.4 (l^{-1})	Swedish lakes	Olsson 1990
5.6-45.9 nmol l^{-1} min^{-1}	5.6-45.9 (l^{-1})	*Chlamydomonas* sp. in low P_i	Olsson 1990
30.4 nmol l^{-1} min^{-1}	30.4 (l^{-1})	Other phytoplankton species in low P_i	Olsson 1990
0.1 – 2.5 nmol l^{-1} min^{-1} (0.1 µM MUP)	0.1 – 2.5 (l^{-1})	Lake Erken	Pettersson 1980
0.03-0.63 nmol min^{-1} chl a^{-1} (0.1 µM MUP)	0.03-0.63 (chl a^{-1})		
0-0.25 µM h^{-1} (1 µM MUF)		Lake Ontario	Pick 1987 (approximate)
0.06-0.6 nmol l^{-1} min^{-1}	0.06-0.6 (l^{-1})	Lake Erken, spring community	Rengefors *et al.* 2001
0.01-0.2 nmol µg chl a^{-1} min^{-1}	0.01-0.2 (chl a^{-1})		
0.2-78 nmol MF µg chl a^{-1} h^{-1}	0.003-1.3 (chl a^{-1})	Minnesota lakes	Rose and Axler 1997
1-4.9 µg MF µg chl a^{-1} h^{-1}		Lake Memphremagog	Smith and Kalff 1981, (approximate)
44.1-55.9 fmol P $cell^{-1}$ h^{-1} (2.25 µM MUP)		*Cosmarium abbreviatum*	Spijkerman and Coesel 1998
43.2-44.5 fmol P $cell^{-1}$ h^{-1}		*Staurastrum chaetoceros*	Spijkerman and Coesel 1998
0.07 µmol product l^{-1} h^{-1} (1 µM MUP)	1.17 (l^{-1})	seawater	Yelloly and Whitton 1996
0.339 µmol product l^{-1} h^{-1} (250 µM MUP)	5.65 (l^{-1})		
0.097 µmol product l^{-1} h^{-1} (1 µM MUP)	1.62 (l^{-1})	drift seaweed pool	Yelloly and Whitton 1996
0.879 µmol product l^{-1} h^{-1} (250 µM MUP)	14.65 (l^{-1})		
0.109 µmol product l^{-1} h^{-1} (1 µM MUP)	1.82 (l^{-1})	*Rivularia atra* pool	Yelloly and Whitton 1996
0.648 µmol product l^{-1} h^{-1} (250 µM MUP)	10.8 (l^{-1})		
0-0.013 µmol MUP µg chl a^{-1} h^{-1}	0-0.22 (chl a^{-1})		

Other Methods: ELF-staining			
0-201 µmol naphthol mg protein^{-1} h^{-1} (substrate α-naphthylphosphate)		*Chlamydomonas reinhardtii*	Bachir *et al.* 1996
ELF activity only in P-stressed cells, except no ELF activity in *A. fundyense*		*Alexandrium fundyense*, *Amphidinium* sp., *Isochrysis galbana*	Gonzáles-Gil *et al.* 1998
0.12-0.48 µmol naphthol mg protein^{-1} h^{-1}(substrate α-naphthylphosphate)		*Chlamydomonas reinhardi* WT strain and mutant deprived of cell wall	Loppes 1976
ELF activity detected in diatoms, dinoflagellates and chlorophytes		*Lake Erken, spring community*	Rengefors *et al.* 2001

Both APA and AcPA occurred predominantly in the bacterial / dissolved fraction in the river (Table 6.1). However, APA dominated in the algal fraction whereas AcPA dominated the dissolved / bacterial fraction (Chapter 3), suggesting that the pool of dissolved enzymes was predominantly originating from bacteria, rather than dissolved enzymes originating from algae (with a higher ratio of APA). Unfortunately this can not be proven based on the determinations done during this study, but should be tested e.g. through further size fractionation of *in situ* samples, in combination with analysis of both phytoplanktonic species composition as well as bacterial numbers. Previous studies have shown that during an algal bloom the algal fraction dominated the APA, whereas during the breakdown of a bloom the bacterial / dissolved fraction dominated APA (Chróst *et al.* 1989) – partly as a consequence of an increased number of bacteria at the breakdown of a bloom. This pattern could not, however, be seen in the temporal variation in APA in the Vaal River (Figures 3.1, 3.5 and 3.7).

Ecologically the bloom formers were associated (Figure 3.26) with a variety of environmental conditions. This scattered occurrence indicates that each bloom former has a specialised ecological niche, within which it has a competitive advantage over other bloom forming species, and thus the capability of forming a mass occurrence. The bloom forming species found in the Vaal River could not be integrated into the "ecological cluster" suggested by Riegman (1988, presented in Chapter 1, Figure 1.2), providing a model for broad taxonomic groupings according to their macronutrient and light preferences. In fact, rather the opposite was suggested by the RDA plot (Figure 3.26) revealing scattered occurrence of species belonging to the same taxonomic group. Physiologically, on the other hand, the bloom formers tended to have high APA and APA/AcPA ratios, indicating a common physiological advantage compared to other genera present in the river (Figure 3.28). These results strongly support the continuation of physiological studies concerning bloom forming genera, as well as the integration of physiological studies in ecological work.

6.2. Physiological response to nutrient limitation or excess

The results of the *in vitro* work (Chapters 4 and 5) have been summarised in the carpet plots (equivalent of a pyramid plot seen from above) in Figures 6.1 and 6.2, introducing the comparative aspect between the green algae and cyanobacteria.

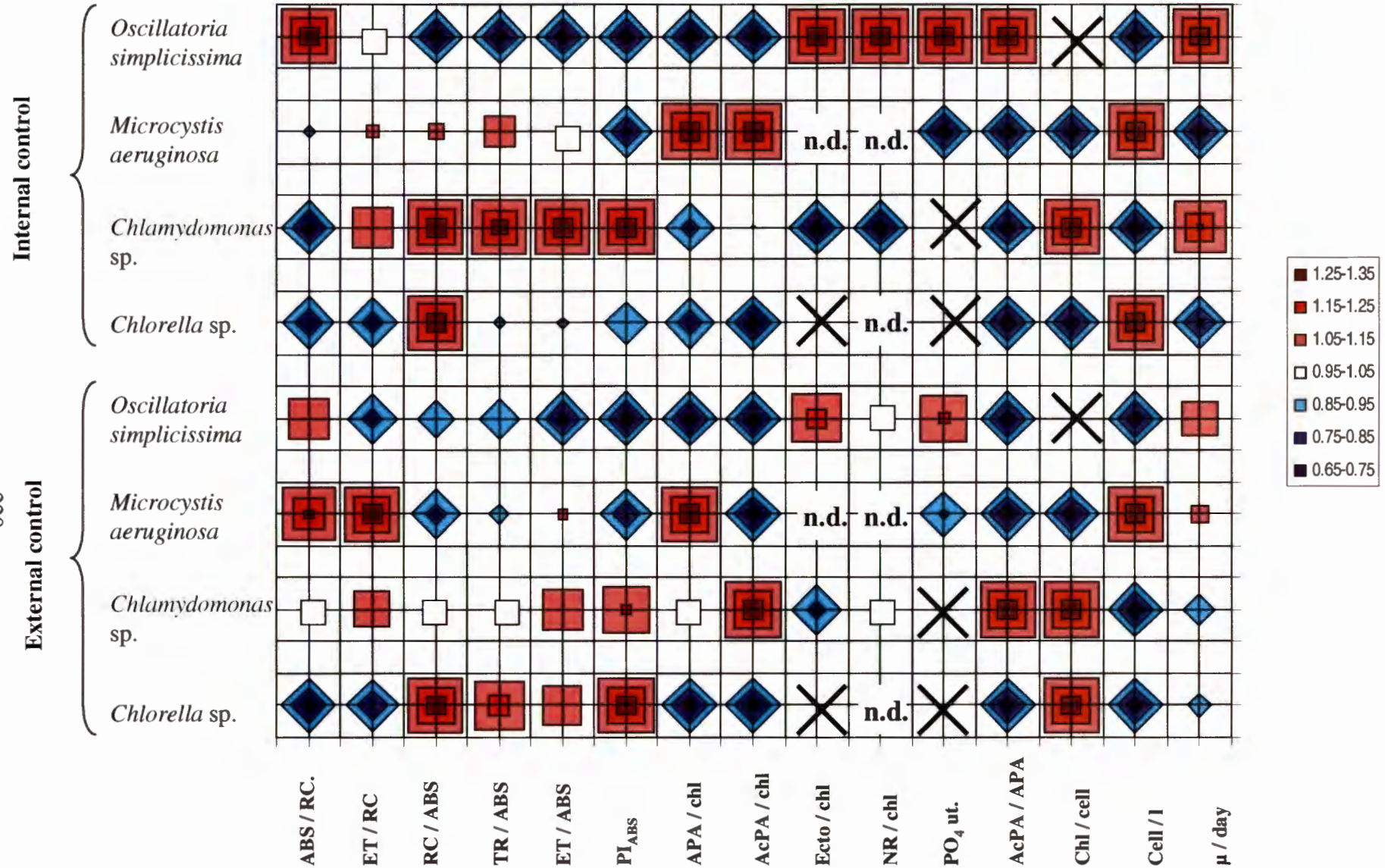


Figure 6.1. Carpet plot showing relative differences between the species in the internal controls (above) and the external controls (below). Crosses = analysis not done, n.d. = not detected. For abbreviations and detailed discussion concerning the plot, please refer to text.

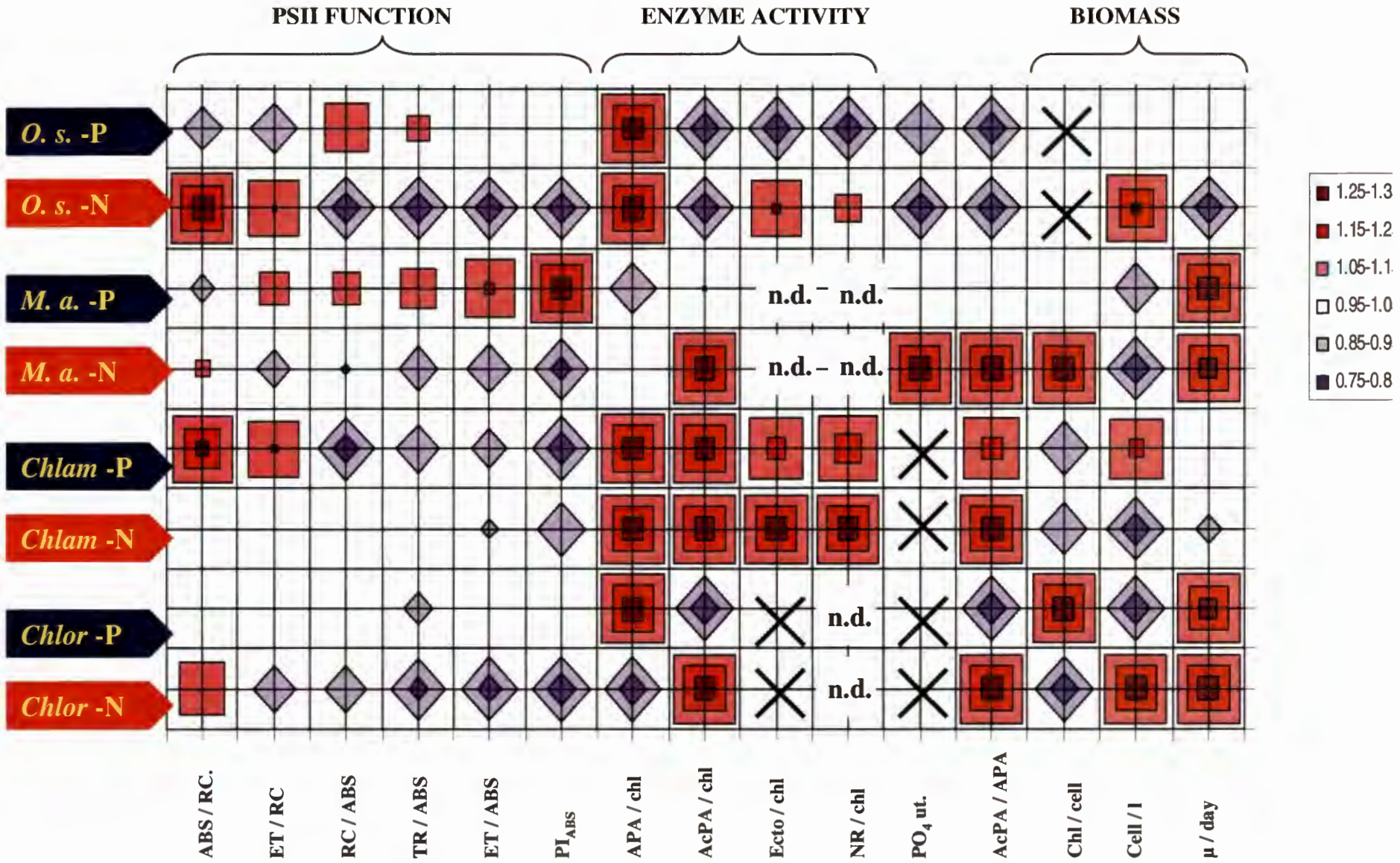


Figure 6.2. Carpet plot showing relative differences between the P and N-limited treatments relative to the internal control, in *Oscillatoria simplicissima* (*O. s.*), *Microcystis aeruginosa* (*M. a.*), *Chlamydomonas* sp. (*Chlam*) and *Chlorella* sp. (*Chlor*). Black crosses = not determined, n.d. = not detected. No symbol indicates that the deviation from the internal control is less than ± 5%. For further abbreviations and detailed discussion concerning the plot, please refer to text.

The plot in Figure 6.1 shows interspecific differences, presenting deviations from the mean calculated for all four species for different photochemical, enzymatic and growth parameters. Studying this plot allows comparisons between the four species studied, grown both in nutrient replete conditions (= external control) and in nutrient limitation (represented by N:P = 16:1 = internal control). The plot in Figure 6.2 summarises the results of nutrient limitation experiments presented in Chapters 4 and 5, indicating the relative deviation of the cells grown in N or P-limitation related to the internal control, thus showing differences caused by the N:P ratio for each species separately. Red squares indicate that the variable for the species in question was higher than the mean for the 4 species (Figure 6.1) or the internal control for the respective species (Figure 6.2) for each separate variable, whereas blue diamonds indicate a deviation below mean values. The colour and number of lines inside the squares or diamonds symbolises level of deviation, the deviation increasing with the number of lines. The left part of the carpet plot show the variation in photochemical variable – thereby indicating differences in the photosynthetic vitality, whereas the right part of the carpet plot summarises the enzymatic and growth reactions of the cells. As also shown in Chapter 4, it seems as if the photochemistry and phosphatase enzymes have opposing responses (Figure 6.2), nutrient stress decreasing the photosynthetic vitality, and simultaneously increasing the activity of phosphatase enzymes – the enzymatic activity thus operating as a "physiological stress-response" of the cell.

The growth rate (μ/day) represents, in certain respects, the "summed effect" of most of the measured physiological (photochemistry, enzyme activities, chlorophyll *a* concentrations and cell density) parameters. The external control represents nutrient replete conditions, thus corresponding to a highly eutrophic environment. In this optimal environment the PSII functions were on average higher, indicating more effective photochemical processes, in *Chlorella* sp., emphasising the capability of this species to grow well under "optimal" conditions (see Chapter 4 for discussion). *Oscillatoria simplicissima* had the lowest photosynthetic vitality in comparison to the other studied species. However, when examining the results of the chlorophyll *a* fluorescence studies, it must be emphasised that the cyanobacteria possess very efficient light-capturing mechanisms besides of chlorophyll *a* (Carr and Whitton 1973), and therefore the low performance measured based on the JIP test may be outweighed by other photochemical processes present. For instance, it has been found that part of the PSI variable fluorescence is due to photo-oxidation of P700 in cyanobacteria, which may affect the interpretation of the origins of PSII fluorescence (Karapetyan *et al.* 1999).

The results from the field investigations, suggesting higher APA in bloom formers compared to non-bloom forming genera, could be partly confirmed during the *in vitro* work. *Chlorella* sp., a non-bloom forming species grown in nutrient replete medium (= external control), had a lower constitutive level of APA in comparison to two other (bloom forming) species (Figure 6.1). However, *Oscillatoria simplicissima*, the main bloom forming cyanobacteria in the Vaal River, also had low APA in both nutrient replete and depleted conditions. *Microcystis aeruginosa* exhibited high constitutive APA, and *Chlamydomonas* sp. high constitutive AcPA – demonstrating the strong interspecific differences in type and level of constitutive phosphatase activity. Differences in the constitutive levels of APA was also demonstrated by Hino (1988), who reported low constitutive levels of APA in *Microcystis aeruginosa*, while the constitutive level in *Chlorella pyrenoidosa* was clearly higher. Unfortunately the exact amounts of activity cannot be compared due to differing methods of reporting specific enzyme activity. There were no marked interspecies differences in the level of constitutive ectoenzymatic phosphatase or NR activity between *Chlamydomonas* sp. and *Oscillatoria simplicissima* (Figure 6.1).

The growth rates in the external controls were very similar, only *Oscillatoria simplicissima* performing marginally better than the other species – reflecting the ability of *Oscillatoria* species to benefit from hypertrophic conditions (Köhler and Hoeg 2000) and having well-developed nutrient storage capacity (Steinberg and Hartmann 1988 and references therein).

Reports of *Oscillatoria* species also dominating in nutrient poor systems have been published (Steinberg and Hartmann 1988 and references therein), the potential for which could be verified in the experimental studies in Chapter 5. In the nutrient limited treatment *Oscillatoria simplicissima* still had the highest growth rates, despite clearly lower photosynthetic vitality compared to the other species. The antenna size (ABS/RC) was higher in *Oscillatoria simplicissima* compared to the other species, which, as mentioned earlier, may have connection to the presence of other light harvesting pigments besides chlorophyll *a* in cyanobacteria (Carr and Whitton 1973). *Oscillatoria* species have been shown to be able to grow in low irradiance (Scheffer *et al.* 1997), also suggesting alternative methods of effective light capturing. The level of NR activity and utilisation of PO_4^{3-} from the medium were higher in comparison to the other species, suggesting efficient N and P utilisation also under nutrient limited conditions in *Oscillatoria simplicissima*. In this regard it was also of interest to find that in connection to the experimental studies in Chapter 5, *Oscillatoria simplicissima* clearly responded to especially N-

depletion by a strongly increased production of hormogonia, also reflected by the increase in the cell/l in the N-limited treatment (Figure 6.2). Such a decrease in filament length probably increases the efficiency of nutrient uptake by a reduction of cell volume / surface ratio (South and Wittick 1987), making high growth rates possible. The RDA analyses presented in Chapter 3 also indicate that *Oscillatoria simplicissima* has an advantage in NO_3^- depleted environments, revealing a slight negative correlation between the occurrence of *Oscillatoria simplicissima* and ambient NO_3^- concentration.

Chlamydomonas sp. exhibited efficient function of all steps in the PSII processes despite smaller antenna size, and also maintained its chlorophyll *a* contents per cell better than the other species (see also Chapter 4 for discussion), thus exhibiting a strikingly different strategy for coping in nutrient depletion. In the RDA analyses in Chapter 3 *Chlamydomonas* sp. shows an occurrence opposite of *Oscillatoria simplicissima*, and a slight positive correlation with NO_3^- concentrations. In Figure 6.2 it can be seen that *Chlamydomonas* sp. had a slower growth rate, and a clearly decreased number of cells in the N-limited cultures. In P-limitation, on the other hand, *Chlamydomonas* sp. managed to sustain its growth rate and cell numbers, despite the negative effects of P-limitation on the PSII functions. Also in previous studies based on phosphorus dependent growth kinetics (Grover 1989) it has been shown that *Chlamydomonas* sp. is a better competitor for P compared to both *Chlorella* sp. and *Oscillatoria* sp.

In Figure 6.2 it can be seen that the N-limited treatment affected especially the PSII functions, the photosynthetic vitality (PI_{ABS}) being lower in N-limitation compared to the internal control in all four species. Considering the central role of N as a constituent of the photosynthetic apparatus (Rao and Terry 2000), the severe effects of N-limitation was not surprising.

As a general conclusion of the comparisons between the species it seems as if the important bloom formers in the Vaal River, *Oscillatoria simplicissima* and *Chlamydomonas* sp., maintained competitive advantage by ultimately K-strategic mechanisms (competitive survival), but employing completely different physiological coping mechanisms – reflected also by a separate occurrence in the field. This is in accordance with the suggested "multialgal species food web" (Riegman and Kuipers 1994, see discussion in section 1.1.2.2) indicating that bloom forming species often have K-strategic features, being "survival specialists". *Chlorella* sp., the

"non-bloom forming" species, appeared more opportunistic (r-selected with high intrinsic growth rate) in optimal conditions, but was physiologically severely influenced by the nutrient stress.

6.3. Phosphatases –general discussion

Healey and Hendzel suggested as early as in 1979, that APA could be used as an indicator of P-deficiency in phytoplanktonic species, which have been shown to exhibit inducible phosphatase activity. The authors tested seven species out of which two, namely *Pseudoanabaena catenata* and *Oochromonas vallesiaca*, did not have inducible APA. Based on their studies Healey and Hendzel suggest that an APA of < 0.05 nmol P μg chl a^{-1} min^{-1} indicates no P-deficiency, 0.05-0.08 nmol P μg chl a^{-1} min^{-1} slight and > 0.08 nmol P μg chl a^{-1} min^{-1} severe P-deficiency. These calculations were, however, based on the use of fluorogenic model substrates and intact phytoplankton cells, hence only determining the ectoenzymatic phosphatase activity. It seems logical to expect enzymes located on the cell wall, and thus in direct contact with the surrounding environment, to be capable of rapid reaction to the ambient nutrient concentrations, whereas enzymes located inside the cell would have a more constitutive function. Thus the location of the enzyme in the cell would be more important than the type of enzyme (as suggested by e.g. Jansson *et al.* 1988), when determining the constitutive versus inducible characteristics of the enzyme. The entire discussion on the use of APA as an indicator of P-deficiency may have been confused by the use of different methods to determine phosphatase activities. Some authors have measured phosphatases using mostly pNPP as a substrate (but also e.g. naphtylphosphate), whereas others have measured ectoenzyme activities using fluorogenic substrates such as MUP. In Table 6.1 studies concerning phosphatase activities have been reported, and divided according to method. Most studies, in which a negative correlation between phosphatase activity and PO_4^{3-} concentration has been found, indeed fall under the category ectophosphatases. Exceptions do, however, occur: Jamet *et al.* (1995, 1997) and Yiyong and Xinyu (1997) report negative correlation between endoenzyme (using pNPP as substrate) activity and ambient PO_4 concentration, while Healey and Hendzel (1979), James *et al.* (1992) and Pick (1987) report no relationship between ectophosphatase activity and ambient PO_4^{3-} concentration (using MUP as substrate). Apart from this work only Yelloly and Whitton (1996) report results for both pNpp and MUP based phosphatase activity, also stressing the insufficiency of neither substrate alone.

In the experimental studies in this work the ectoenzyme activities determined for *Oscillatoria simplicissima* (0.08 – 0.14 nmol P µg chl a^{-1} min^{-1}, Table 5.7) would, according to Healey and Hendzel (1979), indicate severe P-deficiency in all treatments, but no P-deficiency in any of the *Chlamydomonas* sp. cultures (0.01 – 0.03 nmol P µg chl a^{-1} min^{-1}, Table 4.5). The activities indicated by Healey and Hendzel are, however, based on measurements conducted using 10µM fluorogenic substrate (MUP), whereas the enzyme kinetics for *Oscillatoria simplicissima* and *Chlamydomonas* sp. suggested the use 1 µM concentration of MUP. The endoenzymatic phosphatase activities, however, indicate a very different result especially for *Oscillatoria simplicissima*, which had a negligible induction of APA and AcPA in nutrient limitation (Figures 5.7 and 5.10), thus suggesting an absence of inducible endoenzymatic phosphatases, but a presence of inducible ectoenzymatic phosphatases. In *Chlamydomonas* sp., on the other hand, the situation was the reverse, highly induced endoenzymatic phosphatases being detected (Figures 4.4 and 4.7) while the ectoenzyme activity responded less to nutrient depletion. In previous studies concerning *Chlamydomonas* sp. grown in low P_i a markedly (10-15 times) higher ectophosphatase activity was detected (Olsson 1990) compared to the *Chlamydomonas* sp. from the Vaal River – further illustrating the variability between species.

During the work performed for this thesis it became apparent that the conception of acid phosphatase having a constitutive nature, whereas alkaline phosphatases are the inducible enzymes being regulated by ambient P_i availability (Jansson *et al.* 1988), is untenable for many phytoplanktonic species. As shown e.g. in Chapter 5, alkaline phosphatases were present in phosphorus-replete cultures of *Microcystis aeruginosa* at comparatively high (constitutive) levels, and were only moderately induced after severe P_i limitation. Acid phosphatases, on the contrary, were significantly less active in pre-P_i-limited cells, whereas they were highly induced after P_i limitation. Furthermore it became evident that AcPA (in addition to, or sometimes instead of APA) in both field studies as well as in the four species studied was present at high levels, was highly induced and probably is of great physiological importance.

As a general conclusion it can be stated that endoenzymes are not suited for indicating phosphorus stress as direct consequence of ambient P depletion, but seem rather to function as a general "nutrient-stress response" in the cell (see Chapter 4 for discussion). The phosphatase activity as a response to nutrient limitation is very species specific (Figures 6.1 and 6.2, Healey and Hendzel 1979, Rengefors *et al.* 2001), and therefore best suited to *in vitro* studies using

selected species. Thus it is strongly recommended to include measurements of phosphatase activities in studies concerning P-utilisation, however using both pNPP as well as MUP as substrates. In connection to field studies ELF staining is suggested (Rengefors *et al.* 2001) in addition to enzyme activity measurements, to localise the site of action of the enzyme.

6.4. Ecophysiological studies as a tool to understand algal mass-occurrences

A problem in finding reasons for "bloom forming capacity", as a function of growth and competitive advantage, is the difficulty to separate between the cause and the consequence when operating on cellular level, and studying interactions between the cell, its physiological functions and its surrounding environment. Do certain species benefit from the excessive availability of a certain nutrient, and therefore form a bloom, or is it rather a response to nutrient limitation and subsequent stress by a species possessing the needed coping mechanism for this particular type of stress? Are moderate nutrient concentrations in the environment regulated by the uptake and transformations by an algal population, or are the physiological mechanisms in the cell dictated by the availability or lack of nutrient? Ultimately the questions boil down to the fundamental difficulty in separating cause and consequence in naturally dynamic, circular and strongly interrelated processes.

Further complications rise from the separation of physiological processes, which in reality are intricately interlinked and dependent on each other. It is important to realise the function of different nutrients in e.g. photosynthesis (Rao and Terry 2000). Measurements of both phosphorus and nitrogen utilisation should be closely related to concurrent photosynthesis and respiration in the cells. Nutrients may be taken up, distributed and utilised along several different pathways both within the cell as well as outside it. The understanding of physiological processes involved in the acclimation of phytoplanktonic organisms subjected to variable nutrient environment requires simultaneous studies of C, N and P assimilation pathways (Sciandra *et al.* 1997, see also section 6.5).

Another aspect complicating studies of an ecophysiological kind is the nature of *in vitro* growth conditions: cells are grown in artificial media and regulated physical conditions, described by measuring physiological processes in which artificial surrogates (e.g. pNPP) for natural substances are supplied in optimal concentrations. Responses detected are based rather on optimal potential of each physiological process, rather than factual, or even probable, processes

in nature. Furthermore, the exact nature of an optimal state is hard to define: it is not a static condition, but a constant dynamic movement and change in the physiology of the cell, responding to changes in its surroundings, as well as to internal fluctuations within the cell. In the experiments performed during this work, the optimal stage for each experimental species was defined by the stage at which it grew the fastest (hence the cells were in a similar, and thus comparable, physiological state) in a medium that was developed to meet all its nutritional needs. In nature, however, exponential growth may not be optimal in the context of species survival – an exponentially increasing population will soon reach the carrying capacity of the environment and cause its own collapse. This leads us to the question of a species' strategy in competing firstly with other species, but secondly also with individuals of its own kind in a rapidly increasing population. When and where does interspecific competition end, and intraspecific begin? And to which extent do these types of competition occur during a phytoplankton or cyanobacterial bloom? Is e.g. toxin formation a tool for either inter- or intraspecific competition, or has it perhaps some other function for the species in question (see section 1.1.2.3 for discussion)?

However, without fundamental investigations of the physiological potential of ecologically significant phytoplanktonic species attempts of e.g. modelling and early warning of HABs in varying ecosystems will remain an impossible task. Studies concerning the physiology of naturally occurring phytoplankton populations in the field are also of importance. Despite the complexity of an *in situ* community, dominating and important physiological aspects will emerge. An example of such a relationship is the high phosphatase activities detected in the Vaal River, indicating that high activities measured *in vitro* are not only a consequence of optimised laboratory conditions but a true reflection of the species' potential also in the river.

6.5. Future research

Ecophysiological studies concerning HAB species are still scarce, but much needed for the understanding of HAB dynamics and processes. In chapter 3 species dominating the species composition in the Vaal River were presented. It will be of importance to follow the changes in dominating species and compare these changes with recorded changes in the environment, and include simultaneous studies concerning the physiology of the dominating species. The introduction of new bloom forming species, such as *Ceratium hirundinella, Spirulina* sp. and the

increasing number of green algae forming mass-occurrences (*Chlamydomonas* sp., *Pandorina morum*) are of special interest. Knowledge of the physiology of bloom forming taxa is the key to our understanding of HAB phenomena (Millie *et al.* 1999) and therefore the physiology of these species should be investigated in detail.

In studies concerning algal physiology an experimental set-up consisting of continuous culture conditions would most probably facilitate greatly the careful manipulation of e.g. nutrient ratios and conditions. During this study a combination of batch and semi-continuous cultures were employed, which is a very laborious method, and leads to a limited sample-size and only once a day. Another drawback of batch cultures is that other variables, such as pH, will not keep constant. Continuous cultures will also make possible more gradual dilution of the cultures to bring them to the experimental nutrient concentrations and the possibility to monitor e.g. the change in nutrient concentrations more frequently. This will greatly facilitate the detection of steady state conditions in the cultures.

During this study it was found that many methods to measure NO_3^- were disturbed by components in the growth medium, and therefore proved to be incapable of detecting residual NO_3^-. In similar studies, it is recommended that HPLC or GCMS measurements be done for determination of nutrient concentrations.

In connection to studies involving phosphatase activities, combined use of endoenzyme, ectoenzyme and extracellular enzyme activity is recommended. An introduction of mRNA studies to investigate the regulation of these enzymes is also of importance, especially in context of determining the inducibility of AcPA in low P_i. Additionally, a combination of phosphatase activities and ELF labelling of the cells to locate the site of enzyme activity may bring much needed information on the interspecific differences in phosphatase activities. In connection to field studies careful size fractionation is of importance for defining the origin of the detected enzyme activities. The possible time lag between the induction of AcPA and APA would also be of interest to characterise these enzyme groups further.

In unicellular algae, respiration, photosynthesis and nitrogen assimilation are in tight interaction with each other due to the activation of several metabolic pathways with the factors sustaining them (Inokuchi *et al.* 2002), and these metabolic pathways should therefore be studied in

connection to each other. Also the findings reported by Camacho-Cristobal and co-workers (2002) concerning P_i activation of NR enzyme in tobacco leaves supports the connected study of phosphorus and nitrogen metabolic processes. Therefore investigations including the main steps of N, P and C uptake, assimilation, storage, and interactions between these pathways (for an example see Sciandra *et al.* 1997) should be carried out using ecologically important species.

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