THE ROLE OF NUTRIENT UTILISATION AND PHOTOSYNTHETIC CAPACITY IN MICRO-ALGAL BLOOM FORMATION AND THE PRODUCTION OF CYANOTOXINS

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Report to the Water Research Commission

by

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EXECUTIVE SUMMARY

THE ROLE OF NUTRIENT UTILISATION AND PHOTOSYNTHETIC CAPACITY IN MICRO-ALGAL BLOOM FORMATION AND THE PRODUCTION OF CYANOTOXINS

MOTIVATION

Freshwater is South Africa's most valuable natural resource. Seventy five percent of the available water is used for agriculture and very little is left for industrial and domestic uses. Furthermore, rivers are under constant pressure of pollution from agricultural, mining, industrial and domestic uses. This pollution enhances the process of eutrophication: the presence of high concentrations of organic and inorganic compounds, which enhance algal blooms and concomitantly decrease water quality. The regulation of most rivers in southern Africa by means of dams further contributed to the alteration of these rivers from natural waters to regulated and polluted watercourses.

Eutrophication leads to changes in the phytoplankton composition, often shifting the dominance toward cyanobacteria and other species forming algal blooms. These algal blooms, occurring widely in South African water sources, result in the production of unpleasant odours and tastes and a general decline in water quality. Some of the bloom-forming species are potentially toxic and as a result pose a serious health risk to humans and animals alike. In South Africa blooms of especially *Microcystis aeruginosa* can cause the release of high concentrations of toxins into the water. Scientists estimate that between 30-50% of all blooms are potentially toxic (http//www.hc-sc.gc.ca/hecs-sesc/water /factsheet/ blue_green_algae.htm March 2005). The environmental and metabolic variables leading to the formation of mass occurrences and eliciting toxin production in cells are, however, still unknown.

In order to explain harmful algal bloom (HAB) development, a more complete understanding of the physiological rationale for the occurrences of HABs is essential. Certain key questions need to be answered, such as:

- What makes certain species more competitive than others and allows them to displace other species of phytoplankton assemblages?
- How do these species maintain prolonged competitive dominance?
- Under what conditions do such blooms become toxic?

The most important nutrients for algal growth are nitrogen and phosphorus. These compounds occur in different forms in the aquatic environment, but only a few are readily available for phytoplanktonic use. Phosphatase and nitrate reductase are enzymes, which transform nitrate and phosphate compounds respectively into bioavailable forms. They are therefore essential parts of the nutrient recycling process. The activity of these enzymes gives us information about the nutrient utilisation in the aquatic environment. It has been suggested that key enzyme activities might vary with nutrient availability in the water. The literature, however, provides contradictory results for the relationship between nutrient concentrations and the enzymatic activities of the phytoplankton and bacteria in the water (Yiyong & Xinyu, 1997; Berman, Wynne & Kaplan, 1990; Elser & Kimmel, 1986; Spijkerman & Coesel, 1996; Pick, 1987; Dodds, 1995; Jamet, Lofti & Devaux, 1995; Jamet, Amblard & Devaux, 1997; Boavida & Marques, 1995). This uncertainty may be due to a connection between the nutrient concentrations and the enzyme activities which depends on the ratio of nutrients rather than their absolute concentration, as well as the species composition of the phytoplankton in the water. Therefore it is of importance to study the enzyme activities in the

phytoplankton community in combination with phytoplankton species composition determination. Such knowledge will enable us to manage a healthy and sustainable ecosystem by contributing to the development of appropriate early warning systems and the capacity to analyse and interpret fresh water variability and change.

OBJECTIVES

The objectives set out are as follow:

1. Determination of the effect of different N:P ratios as well as the combined effect of other environmental variables, namely light and temperature on:
- the growth dynamics,
- nitrate reductase and alkaline and acid phosphatase activities,
- ectoenzymatic phosphatase activities,
- performance index and physiological status of problematic bloom forming cyanobacteria isolated from the Vaal River namely, *O. simplicissima* and *M. aeruginosa.*

2. Initiate molecular studies to follow *in situ* the physiological changes occurring in problem taxa by developing cDNA probes and primers to investigate changes in the expression of key metabolic enzymes of specific harmful algal species.

RESULTS AND CONCLUSIONS

The main conclusions of this study, based on the results generated, are:

1. *O. simplicissima* grew better in all nutrient limited treatments than *M. aeruginosa* at optimum temperature and light, indicating adaptation to low, or fluctuating, N and P concentrations under these conditions.
2. The filament length decreased in N and P limitation in *O. 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 was induced in both species, being present in relatively high amounts under conditions deficient nutrient treatments.
4. Acid phosphatase activity was clearly induced in *M. aeruginosa* and *O. simplicissima*, and was present at lower levels than APA during nutrient limitation. This suggests that AcPA is similarly, if not more, inducible compared to APA in the cyanobacterial species studied.
5. Nitrate reductase activity was only present in *O. simplicissima*. The level of NR activity was high in all nutrient limited treatments.
6. Possible reasons explaining the increased abundance of *O. 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 *M. aeruginosa* were found in *O. simplicissima*. In addition, other environmental variables, such as increased turbidity, may benefit *O. simplicissima*.
7. *M. aeruginosa* can maintain growth at a range of different conditions and keep forming mass occurrences.
8. *M. aeruginosa* have high light requirements for photosynthesis and maintenance and the ability to tolerate a much higher light intensity without experiencing photo-inhibition.
9. *Oscillatoria simplicissima* is able to harvest the available light more efficiently at 25°C and 15 $\mu molm^{-2}s^{-1}$ giving it a competitive edge over *M. aeruginosa* at these

environmental conditions. That may explain why *O. simplicissima* succeeded *M. aeruginosa* in the Vaalriver.

10. There was a decrease in APA with an increase in temperature observed in *M. aeruginosa*, while an increase in temperature was correlated with an increase in growth and APA and AcPA in *O. simplicissima.*
11. The "*Oscillatoria simplicissima*" must be renamed to *Planktothrix pseudagardhii nodum nudum* under the order Oscillatoriales, family Phormidiaceae and subfamily Phormidioideae.

INFORMATION AND KNOWLEDGE DISSEMINATION

Workshops

During the period of funding, several workshops were attended, organised and hosted by the research group. These workshops were all directly related to the research in progress in this program. These workshops were funded mostly by other institutions.

The following visits and workshops presented by overseas collaborators were organised by the research team:
The head of a very successful research team on harmful cyanobacteria at the Department of Applied Chemistry and Microbiology, Viikki Biocenter Helsinki University, Finland, visited our laboratories from 11-16 November 2002. During this period Prof Sivonen presented several talks, at Potchefstroom and Rand Water, Johannesburg.

Once the collaboration was initiated with Prof Sivonen's research group, several other workshops followed:
- Phylogenetic studies: Obtaining, analyses and interpretation of data, presenter: Dr Ilona Oksanen. 23 November 2004, Potchefstroom
- Quantitative PCR : Presenters: Me U Gorniak and Dr Anne Rantala, 24-25 November 2004, Potchefstroom.

In order to keep up with the development of new techniques to better analyse and understand the environment, collaboration was initiated with Prof Friedrich Recknagel, a leader in the field of Ecological informatics. Prof Recknagel visited South Africa from 31 May – 3 June 2005 and immediately started working on the data that have been acquired during the past 5 years. He also presented several talks on Ecological informatics and status of eutrophication in South Africa.

Prof Strasser is a collaborator of Prof GHJ Kruger, at the North West University, and the research team benefited from this collaboration and discussed results generated on chlorophyll fluorescence during this study with Prof Strasser. Dr A Venter also attended a workshop held at the North-West University, namely: Chlorophyll-*a* fluorescence as a non-invasive tool to study plant stress and adaptation. Presenters: Prof Reto Strasser and Prof GHJ Kruger, 24-25 August 2005 Potchefstroom.

Conferences attended

During the time of funding members of the research group attended several national and international conferences where they presented papers and posters:

1. VENTER, A., DU PLESSIS, S: Growth dynamics and photosynthetic performance of *Microcystis aeruginosa* at different temperatures, light intensities and N:P ratios. SAIE&ES & SASAqS joint congress, Research, conservation and management of ecosystems in Southern Africa, CR Swart Auditorium UOVS, Bloemfontein, 30 June - 4 July 2002.
2. KRUSKOPF, M.M., STRASSER, R.J.; DU PLESSIS, S.; (paper) Induction of both acid and alkaline phosphatase activities in two green algae in low N and P concentrations. 18th Congress of the Phycological Society of Southern Africa, Cape Town, 20-23/1/2002
3. KRUSKOPF, M.M., STRASSER, R.J; DU PLESSIS, S. (poster): Photosynthetic performance and phosphatase activities in two green algae under nitrate and phosphate limiting conditions. 28th Annual Conference of the South African Association of Botanist, Grahamstown, 13 – 16/01/02
4. A.VENTER AND S DU PLESSIS Growth dynamics and photosynthetic performance of *Microcystis aeruginosa* at different temperatures, light intensities and N:P ratios. South African Association of Botany, Pretoria, 2003.
5. KRUSKOPF, MM AND DU PLESSIS, S. Ecophysiology of HAB species in the Vaal River (South Africa). Third Symposium for European Freshwater Sciences" at the University of Edinburgh, UK, 13-18 July 2003
6. DU PLESSIS & VENTER A. Growth dynamics and photosynthetic performance of *Microcystis aeruginosa* and *Oscillatoria simplicissima* at different temperatures, light intensities and N:P ratios. International Conference on Toxic Cyanobacteria, 20 June 2004, Bergen, Norway.
7. VENTER A, DU PLESSIS S & JANSE VAN VUUREN S. A comparison of the growth dynamics and photosynthetic performance of *Microcystis aeruginosa* and *Oscillatoria simplicissima* at different temperatures, light intensities and N:P ratios. XI International Conference on Harmful Algal Blooms, Cape Town, 14-19 November 2004

Papers published

The researchers are currently in the process of preparing several manuscripts that originated from this research program. The following papers have already been published or accepted for publication:

1. KRUSKOPF, M.M., DU PLESSIS, S (2004). Induction of both acid and alkaline phosphatase activity in two green-algae (chlorophyceae) in low N and P concentrations. *Hydrobiologia* 513 (1): 59-70.
2. KRUSKOPF, M.M., DU PLESSIS, S (2006). Growth and filament length of the bloomforming *Oscillatoria simplicissima* (Oscillatoriales, Cyanophyta) in varying N and P concentrations. *Hydrobiologia* 556 :357-362
3. CONRADIE, K.R., DU PLESSIS, S. & VENTER, A. (2006). Re-identification of *Oscillatoria simplicissima* isolated from the Vaal River, South Africa to *Planktothrix pseudagardhii* (Cyanophyta) *nomen nudum.* (in press)

PROJECT CAPACITY DEVELOPMENT

PhD degrees

Dr M Kruskopf (2003). Her thesis included a chapter on cyanobacteria that formed part of the research done for this program. The title of her thesis is: *Phosphatase activities of*

riverine phytoplankton in the Vaal River (South Africa): Physiological responses of nuisance species to different nutrient regimes.

Mrs K Conradie will complete the molecular research phase of her PhD study and will obtain her PhD degree in 2007. The title of her thesis is: *The development of cDNA primers of key metabolic enzymes of harmful cyanobacteria.*

Dr A Venter has completed her post doctoral study on the growth and photosynthetic performance of *Microcystis aeruginosa* and *Oscillatoria simplicissima* (in preparation).

ACKNOWLEDGEMENTS

We wish to thank the following people for their participation as steering committee members in this research program:

Mrs APM Moolman	Water Research Commission, Chair
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CHAPTER 1

INTRODUCTION

1.1 MOTIVATION

South Africa has an average rainfall of 497 mm per annum, which is clearly below the average rainfall of 860 mm for the rest of the world. This makes fresh water one of South Africa's most valuable and vulnerable natural resources. The Vaal River in semi-arid South Africa is a fairly large river, and of importance to the highly populated area of the South African economic and industrial heartland 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 industries, it has been under intensive pollution pressure.

Agricultural pollution and sewage effluent is particularly responsible for eutrophication (nutrient enrichment) of water. It has been suggested that excessive nutrient enrichment (eutrophication) can be a major factor in the increased occurrence of harmful algal blooms (HABs, Millie *et al.*, 1999). 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 (Harding & Paxton, 2001). The utilisation of these nutrients is of major importance in determining the competitive capabilities of different algae. Eutrophication leads to changes in the phytoplankton composition, often shifting the dominance toward cyanobacteria and other species that form algal blooms. Cyanobacterial blooms in the Vaal River consist predominantly of *Microcystis* species, which produce potent hepatotoxins. The costs incurred due to the necessity of purification of water containing large amounts of algae and toxins, runs into tens of millions of Rands annually.

Despite a considerable amount of ecological data which have been gathered in the field, there is still paucity in our understanding of how environmental variables impact on basic physiological mechanisms of harmful algae. Although physiologists have meticulously studied the different pathways of algal metabolism, such studies have not been done with those species common in the field. This current study will incorporate algal physiological studies to the ongoing ecological monitoring of the river. Thus, contributing to the fundamental understanding of the specific ecosystem's structure and function from a different perspective.

Such knowledge will enable us to manage a healthy and sustainable ecosystem by contributing to the development of appropriate early warning systems and the capacity to analyse and interpret fresh water variability and change.

In order to explain harmful algal bloom (HAB) development, a more complete understanding of the physiological rationale for the occurrences of HABs is essential. Certain key questions need to be answered, such as:

- What makes certain species more competitive than others and allows them to displace other species of phytoplankton assemblages?
- How do these species maintain prolonged competitive dominance?
- Under what conditions do such blooms become toxic?

The most important nutrients limiting algal growth in inland waters are nitrogen and phosphorus containing compounds. These nutrients occur in different forms in the aquatic environment, but only a few are readily available for phytoplanktonic use. Phosphatases and nitrate reductase are enzymes, which transform phosphate and nitrate containing compounds respectively, into bioavailable forms. These enzymes are therefore essential parts of the nutrient recycling process. Understanding the activity of these enzymes gives us information about nutrient utilisation and subsequent growth of phytoplankton. Molecular studies of metabolic pathways concerning these enzymes will enable us to apply the results generated during the *in vitro* study and help to verify findings in the aquatic environment. Currently our understanding of how environmental variables such as light, temperature and nutrient ratio's impact nutrient utilisation are still insufficient.

The underlying problem directing the study was the question: Why do certain phytoplankton species form mass-occurrences? The choice of approach to this question as from a physiological point of view came from a lack of consensus in the literature (Yiyong & Xinyu, 1997; Berman *et al.*, 1990; Elser & Kimmel, 1986; Pick, 1987; Dodds, 1995; Jamet *et al.*, 1995; Jamet *et al.*, 1997; Boavida & Marques, 1995). 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.

A field study comprising of four localities in the Vaal River was carried out, to determine phytoplankton species composition 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*. These three species were grown in N and P concentrations corresponding to both nutrient replete conditions, as well as low nutrient concentrations comparable to levels measured in the Vaal River *in situ*. The species were grown in differing N:P ratios, light and temperature regimes, and the physiological responses of the cells were determined. Physiological responses studied were changes in growth, phosphatase and nitrate reductase activity, as well as changes in the function of PSII, indicating the level of (photosynthetic) stress of the cell.

The following aims were set for this study:

1. Determination of the effect of different N:P ratios as well as the combined effect of other environmental variables, namely light and temperature on:
- the growth dynamics,
- nitrate reductase and alkaline and acid phosphatase activities,
- ectoenzymatic phosphatase activities,
- performance index and physiological status of problematic bloom forming cyanobacteria isolated from the Vaal River namely, *O. simplicissima* and *M. aeruginosa.*

2. Initiate molecular studies to follow *in situ* the physiological changes occurring in problem taxa by developing cDNA probes and primers to investigate changes in the expression of key metabolic enzymes of specific harmful algal species.

1.2 REFERENCES

See Appendix B on CD.

CHAPTER 2
LITERATURE REVIEW ON THE HARMFUL ALGAL BLOOMS

2.1 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 considered, and finally the "nutrient physiology" of phytoplankton is reviewed, with emphasis on phosphorus and nitrogen utilisation. Secondly the functions and characteristics of phosphatase enzymes in relation to phosphorus and nitrogen utilisation, as well as their relationship with photosynthetic processes are reviewed.

2.2 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 & 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 more competitive than 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 which permit a given species to occupy a defined ecological niche is a crucial step in modelling bloom dynamics and succession (Cembella, 1998).

2.2.1 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 bloom-forming genera mainly found in fresh waters (Richardson, 1997). HAB species are capable of exploiting a bewildering array of ecological niches, survival strategies, and nutritional modes (Cembella, 1998). Table 2.1 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 2.1 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.

One species could, 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 (*Pfiesteria piscicida*). These types of phytoplanktonic ambush-predators were 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.

2.2.2 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 coexistence 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 to disrupt this coexistence of a large number of species? What makes HAB species capable of bloom forming, and developing nearly monocultures in an environment that normally exhibits a heterogeneous 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 phytoplankton species in these conditions.

2.2.2.1. 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 phytoplankton species is the mechanical energy of the water column. Margalef presented schematically how different life forms of phytoplankton would correspond to different regimes of turbulence and nutrient availability (Figure 2.1). 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 to 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 phytoplankton a competitive advantage. Certain cyanobacterial species (e.g. *Microcystis aeruginosa*, *Anabaena* sp., *Aphanizomenon* sp.) can regulate their buoyancy by gas vacuoles, which can give them a competitive advantage in stable water columns (South & 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 & 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 & 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) show 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 to adjust 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 & 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 & Steidinger, 1997).

2.2.2.2. 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 & 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 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 & Baybutt, 1981; South & Wittick, 1987; Steinberg & 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 & 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 & 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 phytoplankton species (Bricelj and Londsdale, 1997). Similarly, species of *Oscillatoria* and *Aphanizomenon* have frequently been found to be dominating in nutrient poor systems (Steinberg & 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 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 phytoplankton 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 out compete 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 phytoplankton 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.

2.2.2.3. 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: Attempt has 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 2.2 (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 phytoplankton 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 part 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 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 has 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 photosynthetic 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 which 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 & 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 & 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, also positive allelopathic activity has 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 phytoplankton 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 high N concentrations in *Oscillatoria agardhii*. 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 in P-limitation. Oh and co-workers (2000) also found higher microcystin concentrations in *M. 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 and field observations have been done 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*. This zooplankton family 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).

2.2.3 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 phytoplankton, 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 (P) 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 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 phytoplankton species are much needed.

2.3 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 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 phytoplankton, similar functions of phosphatases have been shown (e.g. Pettersson, 1980; Heath, 1986; Jansson *et al.*, 1988; Hantke *et al.*, 1996).

2.3.1 Physiological attributes of phosphorus utilisation

Physiological attributes that are considered essential for determining the competitive ability of phytoplankton 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 form 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 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). 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 & 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 are 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 PO_4^{-3} concentrations 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 of 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 and used for growth, indicating the importance of DOP to the phytoplankton, in relation to P_i.

2.3.2 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, which 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). Especially cyanobacteria 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 (APA) and acid phosphatases (AcPA) catalyse the hydrolysis of the phosphate-ester bond. The reaction mechanism is according to Jansson *et al.* (1988) the following:

Ortophosphate 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+} ions 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 & 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 & Flint, 1987; Chróst *et al.*, 1989). According to Jamet *et al.* (1995 and 1997) no APA 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 dissolved phase, and Stewart and Wetzel (1982) report that most phosphatase was either free in solution 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 phytoplankton 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.

Also 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 phytoplankton 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 a 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 repress 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 APAs 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) AcPAs 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. AcPAs 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 terrestrial *Chlamydomonas reinhardtii* P-deprived cells increase extracellular APA 300-fold relative to unstarved cells. At least two forms of APAs 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.
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 AcPA 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 APA 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).

2.3.3 Relationship between APA and ambient P_i concentration, and phytoplankton species composition

2.3.3.1. 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 phytoplankton species phosphatase activity was induced by P-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 exists where the relationship between ambient PO_4^{-3} concentrations 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 compounds (mainly nucleic acids) which 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).

2.3.3.2. 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 APA by phytoplankton, and with a prevailing role of APA in organic regeneration (Ammerman and Azam, 1991; Cotner and Wetzel, 1991).

Boavida & 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 phytoplankton 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.

2.3.3.3. 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 & Kalff, 1981).

2.3.4 Endo- and ectoenzymatic phosphatase activity

Literature concerning phosphatase activities differs 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, which 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 studies will in future be of great significance to the study of P-nutrition of phytoplankton.

2.3.5 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. No clear consensus has been outlined for the use of phosphatases as indicator of nutrient stress, however. In connection to HAB research it seems important to include aspects of nutrient physiology of the phytoplankton cells, because nutrient concentrations 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 (Yelloly and Whitton, 1996) studies have used both methods in parallel.

2.4 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 2.3): 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 has been widely studied (for recent reviews see Grossman & Takahashi, 2001; Moir & 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).

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 2.4). 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 & 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 well standardised.

2.5 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, as well as a role in the partitioning and export of photoassimilates (Rao & Terry, 2000). Phosphorus has a role in the cellular energetics, co-enzyme regulation and, similarly to nitrogen, partitioning and export of photoassimilates (Rao & 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 is 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-Cristobal *et al.*, 2002). Therefore it is advantageous to study phosphorus and nitrogen assimilation simultaneously, and in association with photosynthesis or 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 & 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 & Tiwari, 2000) it is of importance 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 project.

2.6 REFERENCES

See Appendix B on CD.

Table 2.1: 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
	Notiluca scintillans	Elbrächter and Qi 1998
	Prorcentrum micans	Horner *et al.*, 1997
	Protoperidinium spp.	Horner *et al.*, 1997
	Rhizosolenia setigera	Horner *et al.*, 1997

CHAPTER 3

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

3.1 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 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 & 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 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).

In the Vaal River, several bloom-forming cyanobacterial species occur. A PCA analyses of previous ecological work showed that the main bloom-forming cyanobacterial species were scattered over varying environmental conditions, however showing slight negative correlation with N:P ratios and favoured higher temperature (Kruskopf, 2001). A strong positive correlation with APA:AcPA ratios were shown for the three main bloom-forming cyanobacteria, *O. simplicissima, M. aeruginosa* and *Spirulina* sp. in the Vaal River. *O. simplicissima* has in the past decades replaced *M. aeruginosa* as the dominant bloom forming cyanobacteria in the Loch Vaal catchment area in the Vaal River

(Pieterse and Steynberg, 1993; Venter, 2000; Janse van Vuuren, 2001). The reported replacement of *M. aeruginosa* by *O. simplicissima* has been suggested to be a consequence of an increase in inorganic phosphorus supply (from 0.2 mg l^{-1} to 0.9 mg l^{-1}) under conditions of relatively low inorganic nitrogen (0.2 mg l^{-1}) availability (Pieterse and Steynberg, 1993). The underlying physiological mechanisms operating in connection, and as a possible cause to 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*.

M. aeruginosa (Kütz) is a unicellular, colony-forming cyanobacterium, which commonly forms blooms in eutrophic, warm brackish and freshwaters (e.g. Brookes & 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 *M. 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).

Oscillatoria simplicissima Gomont is a filamentous, non-heterocystous cyanobacterium, described in detail by Venter (2000; 2003). Apart from its lack of red pigmentation, it is morphologically very similar to *O. rubescens* (Desikachary, 1959), with undifferentiated filaments and no branches, but it has not been conclusively determined whether *O. simplicissima* and *O. rubescens* may be a variants of the same species or not (Venter, 2000). *O. simplicissima* is a bloom forming species. The main problem with *O. simplicissima* blooms is the rapid clogging of water purification filters (Juanico *et al.*, 1995; Venter, 2000). Species of the family 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 toxic in 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 & Paxton, 2001), but *O. simplicissima* has to date, to the authors' knowledge, not been reported 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 *M. aeruginosa* and *O. 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.

3.2 MATERIALS AND METHODS

3.2.1 Growth conditions in experimental studies

Unialgal, but not axenic, cultures of *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 section 7.3. Cultures of these algae were grown in GBG-11 (Krüger, 1978) medium or EM medium (*O. simplicissima*, Venter, 2000, Table 3.1) in constant irradiance (18-22 µmol m^{-2} s^{-1}) and temperature (22°C).

Table 3.1: Modified GBG-11 (Krüger, 1978) 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 exponential growth was attained, about 12 days (8 days for *O. simplicissima*) after inoculation. The algal cultures were then exposed to semi-continuous culturing 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, 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). Trace metals, other nutrients, and EDTA were 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.

3.2.2. Monitoring of growth during the experiments

Only chlorophyll-*a* was used for growth rate determination. The growth rate (per day) was determined for the last 2 days before the termination of the experiment using the exponential growth equation (Isvánovics *et al.*, 2000),

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

where
B = chlorophyll-*a* concentrations,
t = time in days, and
D = dilution rate (0.4 per day)

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

3.2.3. Measurement of phosphatase activity

3.2.3.1. 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. 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 of 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. The homogenised material was centrifuged (12 000 rpm JA 20 rotor for 20 minutes) and the supernatant was used for determining the phosphatase activity. Hundred µl pNpp (10 mM) was added to 1.5 ml supernatant, and the reaction mixture was incubated in 30°C for 2 hours. The reaction was stopped by adding 300µl NaOH (1M), and the concentration of the resulting 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.

3.2.3.2. Fluorometric method (ectoenzymes):

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 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 *O. simplicissima*). *M. aeruginosa* did not exhibit any ectoenzymatic phosphatase activity despite a range of 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 in 30° and in the dark. The fluorescence of the resulting fluorescing product MUF was measured initially (to determine background fluorescence) and after the incubation period. The fluorescence of MUF was measured after adjusting the pH to 10.3 with Tris/HCl buffer. Calibration was done by standard additions of fluorochrome standard solution (0.1 µM) after last measurement.

3.2.4 Measurement of nitrate reductase activity

Nitrate is actively transported across the cell membrane by either an ATP-binding cassette transporter of a nitrate permease. Once inside the cell, it is reduced to nitrite and ammonium through the activities of nitrate reductase and nitrite reductase, respectively (Miller & 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 200mM 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 1 mM KNO_3 acting as substrate. The reaction mixture was incubated in 30°C for 30 minutes. The reaction was terminated by addition of 60 mM $ZnSO_4$ and 0.05N NaOH. After centrifugation in 7500 rpm for 10 minutes 0.1% sulfanylamide and 0.008% NED was added, and the NO_2^- was measured spectrophotometrically at 540 nm.

3.2.5 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 Kühn (1970). During this experimental work (Chapter 3) NH_4^+ was determined mainly to monitor potential release of NH_4^+ from algal cultures. From river samples also nitrate NO_3^- was determined according to Cataldo *et al.* (1975). The NO_3^- determination method was unfortunately not possible to use due to interference by some substances in the growth medium, and therefore no NO_3^- determinations were done during the experiments. In some instances HPLC measurements were done using external consultant 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.

3.2.6 Direct chlorophyll-*a* fluorescence

In all dark-adapted photosynthetic samples, 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.

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 antennae absorb light but before the excitons have been trapped. F_0 is measured with 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 Rensburg *et al.*, 1996) or chemical changes (Ouzounidou *et al.*, 1997).

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).

Chlorophyll-*a* fluorescence 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 from experimental cultures (*M. aeruginosa* and *O. simplicissima*) at the end of each experiment. Samples (5-20 m*l*) of the culture were filtered onto GF/C filters and the filters consequently treated as "leaves". For each treatment the chlorophyll-*a* fluorescence transients of 18-30 replicates were measured and recorded up to 1s, with a 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 samples were dark adapted for at least an hour prior to measurement.

3.2.6.1. 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). 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 3.2). A set of biophysical parameters are then calculated based on the recorded parameters (Table 3.3).

Table 3.2: 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 3.3: 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	
ν_{P0} or TR_0/ABS	$[1-(F_0/F_M)]$ or F_V/F_M
ν_{E0} or ET_0/ABS	$[1-(F_0/F_M)]* \Theta_0$
Θ_0 or ET_0/TR_0	$(1-V_J)$
Specific energy fluxes or specific activities	
ABS/RC	$M_0*(1/V_J)*(1/\nu_{P0})$
TR_0/RC	$M_0*(1/V_J)$
ET_0/RC	$M_0*(1/V_J)* \Theta_0$
DI_0/RC	$(ABS/CS_0)-(TR_0/RC)$
Phenomenological fluxes or phenomenological activities	
ABS/CS_0	F_0
TR_0/CS_0	$\nu_{P0}*(ABS/CS_0)$
ET_0/CS_0	$\nu_{P0}*\Theta_0*(ABS/CS_0)$
DI_0/CS_0	$(ABS/CS_0)-(TR_0/CS_0)$
Density of reaction centres	
RC/CS_0	$\nu_{PO}*(V_J/M_0)*F_0$
Vitality indexes	
SFI_{P0}	$(RC/ABS)* \nu_{PO}*\Theta_0$
SFI_{N0}	$(1-\nu_{P0})*(1-\Theta_0)$
PI_{ABS}	$(RC/ABS)*[\nu_{PO}/(1-\nu_{P0})]*[\Theta_0/(1-\Theta_0)]$
PI_{N0}	$1/PI_{P0}$
Driving forces	
DF_{P0}	$Log(PI_{P0})$
DF_{N0}	$Log(PI_{N0}) = -log(PI_{P0})$

3.3 RESULTS

3.3.1 Growth

M. aeruginosa was grown in GBG-11 medium until exponential growth was achieved after 11 days, whereas *O. simplicissima* grown in EM medium reached exponential phase after only 8 days. After the start of dilutions the chlorophyll *a* concentration decreased rapidly in *M. aeruginosa*, while *O. simplicissima* increased its chlorophyll *a* concentration for one day after the dilutions and only after two dilutions started decreasing (Figure 3.1.). At the end of the experiment *M. aeruginosa* had not reached steady-state but the cells were being washed away from the medium. Only in the external control did the growth of *M. aeruginosa* reach steady state and the biomass was even increasing slightly (Table 3.4.). *O. simplicissima* reached clear steady state in all treatments, with lowest growth rates in the N-limited treatment.

3.3.2 Chlorophyll-*a* concentration

The chlorophyll *a* concentration was initially (during exponential phase growth and before the start of dilutions) higher in *M. aeruginosa* than *O. simplicissima* (ANOVA $F_{(1,46)}$ $p = 0.00026$, Figure 3.2). The external controls kept to initial chlorophyll *a* concentration in both species and even increased in *O. simplicissima* during the dilutions. At the end of the experiment the chlorophyll *a* concentrations were higher in *M. aeruginosa* cultures, as was the cell density (Table 3.4). It must be borne in mind that cell density is based on single cells for *M. aeruginosa*, while it is based on number of filaments for *O. 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 3.3). The cell density in *M. 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 3.4). 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 3.2). In *O. simplicissima* the number of filaments per litre did not vary significantly between the treatments. A comparison between the lengths of the filaments was done, however, and the different treatments had clear differences in the composition of size classes in the filaments (Figure 3.5.). 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 3.5). The difference between the treatments was not statistically significant (ANOVA $F_{(2,15)}$ $p = 0.1534$).

3.3.3 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 3.5). 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 3.6). 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 *O. 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 *M. 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 3.3).

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 *M. aeruginosa* or *O. 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 *M. 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 *O. simplicissima* cultures at any time throughout the experiment (Table 3.4).

3.3.4 Alkaline phosphatase activity

3.3.4.1. Specific activity:

The APA at the beginning of the experiment was significantly higher in *M. aeruginosa* compared to *O. 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 3.7.).

The nutrient limitation induced APA in *M. aeruginosa* in all treatments. The increase in APA was between 1.3 (P-limited) and 2 times (N-limited and internal control). In *O. 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 *O. simplicissima* (Kruskal-Wallis $H_{(2,17)}$ $p = 0.0046$, Figure 3.8).

3.3.4.2. 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 3.9.). In *M. aeruginosa* no statistically significant differences between the treatments could be detected (Kruskal-Wallis $H_{(2,18)}$ $p = 0.441$), whereas in *O. simplicissima* the internal control exhibited the lowest total APA and the N-limited treatment the highest total APA (Kruskal-Wallis $H_{(2,18)}$ $p = 0.0114$).

3.3.5 Acid phosphatase activity

3.3.5.1. Specific activity:

Initially a low level of AcPA was present in both species (Figure 3.10), with no statistically significant difference between the species (Kruskal-Wallis $H_{(1,46)}$ $p = 0.3223$). In *M. 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 *O. 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 *M. aeruginosa* (Kruskal-Wallis $H_{(2,18)}$ $p = 0.53$), whereas in *O. simplicissima* the treatments differed significantly (Kruskal-Wallis $H_{(2,17)}$ $p = 0.0046$, Figure 3.11.). 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.

3.3.5.2. Total AcPA:

The total AcPA followed the same pattern as the specific (Figure 3.12), *M. aeruginosa* showing no statistically significant differences between the treatments (Kruskal-Wallis $H_{(2,18)}$ $p = 0.087$) whereas in *O. 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.

3.3.6 Temporal changes in phosphatase activity

In *O. simplicissima* both APA and AcPA was determined halfway through the experiment (Figure 3.13). 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.

3.3.7 Ectoenzyme activities

Ectoenzymatic phosphatase activity was not detected in *M. aeruginosa* at all. In *O. simplicissima* the specific ectoenzymatic phosphatase activity was highest in the N-limited treatment, but the difference between the treatments was not statistically significant (Figure 3.14). 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.

3.3.8 Nitrate reductase activity

Nitrate reductase activity was not detected in *M. aeruginosa*, while *O. simplicissima* had relatively high amounts of nitrate reductase both initially, as well as after the experiment (Figure 3.15). 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.

At the end of the experiment the P-limited treatment had the lowest specific and total NR activity (Figure 3.16), 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.

3.3.9 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 3.17 and Table 3.5 the correlations for *M. 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 *O. simplicissima* cultures (Figure 3.18 and Table 3.6) 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.

3.3.10 Summary of enzyme activities

Table 3.7 summarises the mean enzyme activities in the two cyanobacteria, *M. aeruginosa* and *O. simplicissima*. In general both APA and AcPA were clearly higher in *M. aeruginosa* compared to *O. simplicissima*, whereas NR activity was very high in *O. simplicissima*. *O. simplicissima* also had some, albeit low, ectoenzymatic phosphatase activity, which was not at all detected in *M. aeruginosa*.

At the end of the experiment the summed (APA + AcPA) phosphatase activity was fairly evenly distributed between the different treatments in both *M. aeruginosa* and *O. simplicissima* (Figure 3.19). In *M. aeruginosa* AcPA dominated all treatments, while in *O. simplicissima* APA dominated in the N-limited treatment. Ectoenzymatic phosphatase activity constituted only 1% of the overall phosphatase activity in *O. simplicissima*.

3.3.11 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 3.8). The performance index was significantly higher in *M. aeruginosa* compared to *O. 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 *M. 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 (δP_0), F_V/F_0, dark reaction function (ψ_0), electron transport (δE_0 and ET_0/RC) as well as performance index (PI_{ABS}). Heat dissipation (δD_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 3.20 and Table 3.9).

In *O. simplicissima* (Table 3.10) 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 3.20).

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 *M. aeruginosa* lowest in the N-limited treatment and in *O. simplicissima* in the P-limited treatment. The effect of N-limitation was more severe in *O. simplicissima* cells compared to *M. aeruginosa* (Figure 3.20).

M. aeruginosa exhibited no K-peak or J-peak in the fast fluorescence transient in either treatment, compared to the internal control. *O. 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 *O. simplicissima* cells.

3.4 DISCUSSION

O. simplicissima reached exponential growth faster compared to *M. aeruginosa*, and also continued its growth after the dilutions had started, which suggests that the level of nutrients in the *O. 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 *O.* sp. was 0.1mM. During this experiment, however, *O. simplicissima* grew well and attained steady-state with significantly lower PO_4^{-3} concentrations (0.2 µM).

In the low nutrient concentrations used, *M. 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 *M. 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 *M. 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.

M. 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 *M. aeruginosa* is capable of storing

nitrate in this form. *O. simplicissima* cells also contain polyphosphate granules storing phosphorus especially during the lag and exponential 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. *O. simplicissima* also possesses cyanophycin granules in all growth stages, storing especially nitrogen (Venter, 2000). The probable presence of nitrate stores in *O. 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 & Takahashi, 2001), and this substrate may, in this case, have been intracellular. Larger cell size in *O. 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 *M. 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 *O. 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 *M. 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 *M. 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 *O. 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 *O. 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. the initial levels were significantly higher for the cyanobacteria studied (Kruskopf, 2003). Similar results were found in a previous study by Du Plessis *et al.* (2002), where constitutive APA was > 10 times higher in *O. 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 *M. 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 *M. aeruginosa* nutrient limitation, irrespective of the N:P ratio, induced some increase in the APA, whereas in *O. 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 *O. 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 *O. 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 *M. 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 green algae (Kruskopf, 2003). However, in a study by Olsen *et al.* (1989) *M. 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 (Kruskopf, 2003). 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 *M. aeruginosa*, and to a lesser extent in the P-limited and internal control treatments of *O. 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 *O. 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 *M. aeruginosa* and *O. simplicissima*, thus confirming the observations made during the field-studies in the Vaal River (Kruskopf, 2003).

The ectoenzymatic phosphatase activity was, as in *Chlamydomonas* sp., quantitatively unimportant compared to the intracellular phosphatase activities (Kruskopf, 2003).

To summarise, the level of APA and AcPA induction seems to be more arbitrary compared to the e.g. green algae studied (Kruskopf, 2003), both temporally as well as quantitatively.

Nitrate reductase activity was only induced in *O. simplicissima*, while no NR activity was detected in *M. 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 the *Chlamydomonas* sp. studied (Kruskopf, 2003), 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 *O. simplicissima* (Du Plessis *et al.*, 2002). During these experiments the NR activity in *O. 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 *M. 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 (δP_0) and electron transport (δE_0) indicates increased damage and loss of both energy trapping and electron transport capabilities with decreasing N-concentration in both *M. aeruginosa* and *O. simplicissima*, also seen as a J-peak in *O. 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 *O. simplicissima*, which in general showed a severe decrease in photosynthetic vitality (Figure 3.20). 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 *M. aeruginosa* with *O. 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, *O. simplicissima* consumed the available PO_4^{3-} more efficiently compared to *M. aeruginosa*, indicating effective storage of this nutrient. Concurrently, *O. 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 *O. simplicissima* to a lesser extent compared to *M. aeruginosa*. This could also be seen as a slight negative correlation between *O. simplicissima* and NO_3^-, as well as N:P ratio, in the Vaal River (Kruskopf, 2003). 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 *O. simplicissima* and *M. 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 *M. 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 *M. 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 *M. aeruginosa* and *O. simplicissima*. *O. simplicissima* seems more capable of utilising stored N reserves (high NR activity) and may therefore have an advantage in a N-limited environment, whereas *M. 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 *O. 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 exponential 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 occurs 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 *M.*-dominated community to intensified *O. simplicissima* blooms. However, also *M. 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. *O. simplicissima* grew better in all nutrient limited treatments than *M. aeruginosa*, indicating adaptation to low, or fluctuating, N and P concentrations.
2. The filament length decreased in N and P limitation in *O. 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 *M. 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 *O. simplicissima*. The level of NR activity was high in all nutrient limited treatments.
7. Possible reasons explaining the increased abundance of *O. 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 *M. aeruginosa* were found in *O. simplicissima*. In addition, other environmental variables, such as increased turbidity, may benefit *O. simplicissima*.

3.5 REFERENCES

See Appendix B on CD.

Table 3.4: 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.

	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
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

Table 3.5: Biomass (as klett, chlorophyll-a and cell density) and chlorophyll-a per cell (*Microcystis aeruginosa* only) in the cultures of *Microcystis aeruginosa* and *Oscillatoira 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	Control	P-limited	**N-limited**	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

Table 3.6: 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 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 = 0.1 µmol l^{-1}.

	PO_4^{-3} concentration in medium PO_4^{-3} µmol l^{-1}					
	Microcystis aeruginosa			*Oscillatoria simplicissima*		
Measurement	**N-limited**	Control	P-limited	N-limited	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	Control	P-limited	N-limited	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

Table 3.7: 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. *Method only tested for values as low as 7 µmol l^{-1} (for single determination), most values are therefore close to detection limit.*

	Microcystis aeruginosa NH_4^+ µmol l^{-1}			*Oscillatoria simplicissima* NH_4^+ µmol l^{-1}		
Measurement	N-limited	Control	P-limited	N-limited	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

Table 3.8: 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 (*Microcycstis aeruginosa*) and day 17 (*Oscillatoria simplicissima*).

		Microcystis aeruginosa			*Oscillatoria simplicissima*		
		N-limited	Control	P-limited	**N-limited**	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	—	0.2 ± 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

Table 3.9: 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).

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)					
$*P_0 (= F_V/F_M)$	0.474	0.03	0.444	0.02	p=0.0021
Θ_0	0.609	0.02	0.409	0.02	p<0.001
$*E_0$	0.288	0.01	0.181	0.01	p<0.001
$*D_0$	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 3.10: 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 Table 3.2 and 3.3. 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)							
$*P_0 (= F_V/F_M)$	0.313	0.05	0.352	0.03	0.386	0.01	p=0.0000
Θ_0	0.497	0.04	0.512	0.02	0.546	0.03	p=0.0001
$*E_0$	0.156	0.03	0.180	0.02	0.211	0.01	p=0.0000
$*D_0$	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 3.11: Differences between the fluorescence signal and derivations thereof in the three experimental treatments at the end of the experiment in *O. simplicissima*. For explanations of the abbreviations in the first column, refer to Table 3.2 and 3.3. 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)							
$*P_0 (= F_V/F_M)$	0.109	0.01	0.206	0.02	0.221	0.02	p=0.0000
Θ_0	0.493	0.06	0.478	0.05	0.430	0.06	p=0.0081
$*E_0$	0.054	0.01	0.098	0.01	0.096	0.02	p=0.0000
$*D_0$	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

CHAPTER 4

COMPARISON OF THE GROWTH DYNAMICS OF THE CYANOBACTERIA MICROCYSTIS AERUGINOSA KÜTZING EMEND ELENKIN (1846) AND OSCILLATORIA SIMPLICISSIMA GOMONT (1982) AT DIFFERENT TEMPERATURES, LIGHT INTENSITIES AND N:P RATIOS

4.1 INTRODUCTION

In eutrophic lakes cyanobacteria are favoured relative to other phytoplankton, both under stratified and mixed conditions. The potential for buoyancy regulation by the formation of gas vacuoles and an increased capability to harvest green light by phycobilisomes are some of the capabilities that are instrumental in the competitive success of cyanobacteria (Tilzer, 1987).

Many factors affect the growth of cyanobacteria including light attenuation, water temperature and nutrient enrichment. Light fuels photosynthetic electron transport and CO_2 fixation, it is the primary determinant of levels of NADP/NADPH, ATP and carbon metabolites, all of which can serve to modulate cellular processes (Grossman *et al.*, 2001). The available light quanta are controlled by the harvesting of light by the photosynthetic pigment apparatus of the organism and the efficiency of the photosynthetic machinery as defined by the quantum yield of photosynthesis (Tilzer, 1987). The efficiency of the photosynthetic machinery depends on light intensity.

The term eutrophication is synonymous with increase productivity and under most water environments the most important nutrient factors causing the shift from a lesser to more productive state are phosphorous and nitrogen (Wetzel, 2001). Phosphorus and secondly nitrogen, are generally the first to impose limitations on the system. Nitrogen limitations usually result in reduced protein content and relatively enhanced carbohydrate or lipid storage (Stehfest *et al.*, 2005). Phosphate limitation can also lead to a shift in the proportions of proteins, lipids and carbohydrates (Stehfest *et al.*, 2005). Therefore, the biochemical composition of phytoplankton is correlated with growth rate and reflects the physiological potential of primary productivity.

Light represents a single environmental cue and other signals such as temperature, nitrogen and phosphorous, interact with light through a web of regulatory circuits that result in dynamic acclamatory responses (Grossman *et al.*, 2001). Therefore, in this study the effects of different light regimes as well as the combined effect of temperature and N:P ratios on growth dynamics, phosphatase activities and photosynthetic performance of the cyanobacteria *Microcystis aeruginosa* Kützing emend Elenkin (1846) and *Oscillatoria simplicissima* Gomont (1982) further investigated.

4.2 METHODS

4.2.1 Growth conditions and measurement of growth

M. aeruginosa and *O. simplicissima* were grown in batch cultures in GBG 11 and EM growth medium (Venter *et al.*, 2003) (see section 3.2.1, Table 3.1). In contrast to the previous chapter (3) these cultures were grown from the onset in medium with N:P ratios of 1:1 (2mmol N and 2 mmol P), 5:1 (0.32 mol N and 0,02 mol P), 16:1 (32 mmol N and 2 mmol P) and 160:1 (3.2 mmol N and 0.02 mmol P). The 16:1 treatment is the internal control and the 5:1 treatment is the external

control. The concentrations used in the 5:1 treatment are that of the EM medium as described by (Venter *et al.*, 2003).

Cultures were incubated at temperatures of 18, 25 and 32°C in continuous light with light intensities of 5, 15 and 20 $\mu molm^{-2}s^{-1}$. Six replicates of each treatment were used. Chlorophyll-*a* concentrations were measured every second day. Growth rates were determined from the onset of the exponential growth phase to midlog. Only chlorophyll-*a* was used for growth rate determination. The growth rate (per day) was determined for the last 2 days before the termination of the experiment using the exponential growth equation. Chlorophyll-*a* concentrations were determined according to methods described by Sartory (1982), using ethanol and a boiling water bath as extraction method, second day of the experiment.

The algal cells harvested during midlog were subsequently used to measure, alkaline phosphatase activity (APA), acid phosphatase activity (AcPA) and chlorophyll-*a* fluorescence. Ten millilitres of each replicate were harvested on a 47 mm GF/C glass fibre filter and stored in liquid nitrogen for measurement of the enzyme activities, while 10 millilitres of each treatment were placed in the dark to determine the chlorophyll-*a* fluorescence transients with a Plant Efficiency Analyzer (PEA, Hansatech Ltd. King's Lynn, Norfolk, UK).

4.3 RESULTS OF THE GROWTH DYNAMICS OF *MICROCYSTIS AERUGINOSA AND OSCILLATORIA SIMPLICISSIMA*

From figures 4.1 and 4.3 it is clear temperature had a significant effect on the growth of the two species studied. *M. aeruginosa* is able to tolerate a wider range of temperatures than *O. simplicissima*. The latter did not grow in any of the treatments grown at 18°C. *M. aeruginosa* exhibited the highest growth rate at 25°C, 20 $\mu mol.m^{-2}.s^{-1}$, while *O. simplicissima* had the highest growth rate at 32°C, 15 and 20 $\mu mol\ m^{-2}\ s^{-1}$, respectively. It is well known that *M. aeruginosa* can grow at much higher light intensities *in vitro*, but *O. simplicissima* cannot and in order to compare the physiological parameters we did not use higher light intensities.

M. aeruginosa showed higher growth rates and chlorophyll-*a* concentrations than *O. simplicissima* even at the 32°C temperature treatment. The highest chlorophyll-*a* concentration (Figure 4.1) of *M. aeruginosa* was measured for the non-deficient treatments, incubated at 32°C and 20 $\mu molm^{-2}s^{-1}$. *M. aeruginosa* did not grow in any of the N:P 1:1 treatments, except at the 32°C temperature treatment and here growth rate increased with an increase in light intensity. High growth rates were observed in all the N:P 16:1 and 160:1 treatments. These also showed an increase with an increase in light intensity at each temperature treatment. It is also important to note that the growth rate of *M. aeruginosa* did not vary significantly between the N:P treatments 16:1 and 160:1.

In contrast *O. simplicissima* showed a significantly higher growth rate in all the N:P 16:1 treatments. It only exhibited better growth rates compared to *M. aeruginosa*, in the N:P 1:1 treatments indicating that it will grow better than *M. aeruginosa* in an environment where nitrogen is deficient. This is supported by its higher nitrate reductase activity as reported by Du Plessis *et al.* (2002).

The highest growth rates for the external control (Figure 4.2) were also measured at 18 and 25°C at 20 $\mu molm^{-2}s^{-1}$, while the highest chlorophyll-*a* concentration for the external control (Figure 4.2) was measured at the same conditions. Chlorophyll-*a* concentrations of the external control similar to the non-deficient and P-deficient treatments (Figure 4.1 and Figure 4.2) increased with an increase in light intensity for the treatments incubated in 25°C and 32°C.

None of the treatments for the external control of *O. simplicissima* (Figure 4.4) grew at 18°C. The growth rate per day, and the chlorophyll-*a* concentrations of the external control (Figure 4.4) were the lowest for those incubated at 25°C and 20 $\mu molm^{-2}s^{-1}$ and 32 °C and 5 $\mu molm^{-2}s^{-1}$. The chlorophyll-*a* concentrations of followed the same trend.

4.4 DISCUSSION

These results suggest that although *O. simplicissima* was more abundant in the Vaal River it only fills a specific environmental niche, and should that environmental conditions change *O. simplicissima* will no longer form mass occurrences. *M. aeruginosa* can maintain growth at a range of different conditions and keep forming mass occurrences. This is already evident in the Vaal River where *Anabaena* has begun to become more abundant than *O. simplicissima* in 2004.

Guerrero and Lara (1987) stated that the utilization of any form of inorganic nitrogen by blue-green algae is strictly dependent on the availability of light and CO_2. Furthermore, phosphates are essential for primary metabolic pathways such as photosynthesis and respiration. *M. aeruginosa* did not grow well in the N-deficient media at 18 and 25°C (Figure 4.1). No nitrate reductase activity was previously detected (Chapter 3) in *M. aeruginosa* cells, possibly suggesting that a certain threshold level of NO_3^- must be present to activate NR and make possible NO_3^- assimilation.). The lack of NR activity in *M. aeruginosa* cells could also be a result of preferred growth on NH_4^+. *M. aeruginosa* cyanophycin granules in its cells (Barlow *et al.*, 1979). Cyanophycin granules most probably consists of proteinaceous reserve material (Carr & Whitton, 1973), the presence of which suggest that *M. aeruginosa* is capable of storing nitrate in this form. *O. simplicissima* did not grow well in the EM growth medium with NH_4^+as a nitrogen source (Venter, 2000) but exhibited high NR activities as shown in section 3.3.2.8 and by Du Plessis *et al.* (2002). The chlorophyll-*a* concentrations and growth rates of the N-deficient treatment were, however, higher at 32°C, with the highest growth rate for the N-deficient treatment measured at 32°C and 20 $\mu molm^{-2}s^{-1}$ (Figure 4.1). This suggests that with an increase in temperature and light intensity increased growth of this organism will follow in N-deficient conditions.

The chlorophyll-*a* concentrations of *M. aeruginosa* growing in the non-deficient and P-deficient media showed an increase with an increase in light intensity. The light-dependence corresponds to the requirement of reductant and ATP in the assimilatory processes (Guerrero and Lara, 1987). No such trend can be observed for *O. simplicissima*. Robarts and Zohary (1987) found that *M. aeruginosa* was the dominant primary producer in Hartbeespoort dam because it was living in an environment in which N and P were in excess of algal requirements and where a major factor limiting growth, was light. They found that low temperatures limited *M. aeruginosa* more severely than other genera, with sharp declines in growth rates below a temperature of 15°C (Robarts & Zohary, 1987).

O. simplicissima grew better than *M. aeruginosa* at most N-deficient treatments at 25 and 32°C. Kruskopf & du Plessis (2006) found that *O. simplicissima also* adapt morphologically to low NO_3-N concentrations by decreasing the length of its filaments thereby increasing the surface area to volume ratio for improved nutrient uptake.

Pieterse and Steynberg (1993) suspected that *O. simplicissima* replaced *M. aeruginosa* due to an increase in the phosphorous concentration; however the results of this study indicate that the main factor might have been the low N:P ratios. This is supported by the fact that *M. aeruginosa* grew well in the P-deficient media but failed to flourish under in the N-deficient medium. The balance between nitrogen and phosphorus deficiency in the phytoplankton communities of natural lakes

implies that phosphorus dynamics and deficiency may commonly be affected by nitrogen availability, with more acute phosphorus deficiency as nitrogen availability increases (Elser, *et al.*, 1990; Dodds, 1995). According to Wetzel (2001) this contradict the fact that phosphorus are the foremost macronutrient to limit algal photosynthesis and stated that when phosphorus is available in large quantities adequate to support metabolism, nitrogen availability can become limited to phytoplankton. Jungia *et al.* (2004) also found that a significant decrease in phosphate-phosphorous did not always lead to a significant decrease in chlorophyll-*a* concentrations of *M. aeruginosa.*

4.5 REFERENCES

See Appendix B on CD.

CHAPTER 5

COMPARISON OF THE PHOSPHATASE ACTIVITY OF THE CYANOBACTERIA MICROCYSTIS AERUGINOSA KÜTZING EMEND ELENKIN (1846) AND OSCILLATORIA SIMPLICISSIMA GOMONT (1982) AT DIFFERENT TEMPERATURES, LIGHT INTENSITIES AND N:P RATIOS

5.1 INTRODUCTION

In comparison with other major nutritional and structural components of the biota such as carbon, hydrogen, nitrogen, oxygen and sulphur, phosphorus is least abundant and most commonly limits biological productivity (Wetzel, 1983). Compounds containing phosphorus influence nearly all phases of metabolism, particularly in the energy transformation of phosphorylation reactions during photosynthesis. Phosphorus is present in nucleic acids, phospholipids and a number of biochemically important substances such as ATP (Block & Grossman, 1988). Inorganic orthophosphate (PO_4-P) is the main phosphorus source directly available to phytoplankton and the uptake and transport through the cell membrane in cyanobacteria is strongly regulated by calcium and magnesium cations (Falkner *et al.*, 1980). When phosphate supply is in excess, the amount in excess of physiological need is often incorporated and stored as polyphosphates (Wetzel, 2001). Phosphate deprivation can stop growth or discoloration of the cells can occur as phycobilisome complexes are degraded and chlorophyll levels decline. During periods of phosphate limitation, many micro-organisms employ emergency systems that aid in the acquisition of phosphorus such as the production of the enzyme phosphatase (Wynne & Bergstein Ben Dan, 1995). Algal cells produce different phosphatases such as acid (AcPA) and alkaline phosphatase (APA). These enzymes can be secreted through the cell membrane and appear to be firmly bound near the cell surface and enable phosphorus deficient algae to obtain phosphorus from phosphorylated compounds present in natural water (Kuhl, 1974). Phosphatases typically have maximum hydrolyzing capacity at different pH values, hence the division into APA and AcPA (Jansson, *et al.*, 1988). External lake water phosphatases usually have pH optima in the alkaline region, while acid phosphatases generally seem to be active in the internal cell metabolism (Jansson, *et al.*, 1988).

APA is a non-specific phosphomonoesterase that is found in both prokaryotes and eukaryotes. It is a dimeric metalloprotein that has two Zn^{2+} ions and a Mg^{2+} ion in each active site (Simopoulos & Jencks, 1994). Cells are able to synthesise APA to hydrolyse organophosphate esters, thereby liberating orthophosphate for metabolism by algal cells (Wynne & Bergstein Ben Dan, 1995). This enzyme occurs around the perimeter of polyphosphate bodies with some localised in the cytoplasm (Simon, 1987).

Low orthophosphate concentrations commonly occur in the photic zone of eutrophic freshwater lakes during the growing season (Boavida *et al.*, 1997). Therefore, it should be possible to find an inverse relationship between the activity of the enzyme, phosphatase and the concentration of the product of hydrolysis, orthophosphate. The lower the amount of orthophosphate in lake water, the more phosphatase would be produced by algae (Boavida *et al.*, 1997). Switzer (1977) found that the addition of inorganic phosphate led to the inactivation of AP.

5.2. METHOD TO DETERMINE THE ALKALINE PHOSPHATASE ACTIVITY OF *MICROCYSTIS AERUGINOSA AND OSCILLATORIA SIMPLICISSIMA*

Ten millilitres of the algal suspension (section 4.2) were harvested on a 47 mm GF/C glass fibre filter and stored in liquid nitrogen. Frozen glass fibre filters with the algae were homogenised in extraction buffer. 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. The homogenised material was centrifuged (12 000 rpm JA 20 rotor for 20 minutes) and the supernatant was used for determining the phosphatase activity. Hundred µl pNpp (10 mM) was added to 1.5 ml supernatant, and the reaction mixture was incubated in 30°C for 2 hours. The reaction was stopped by adding 300µl NaOH (1M), and the concentration of the resulting 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). (Block & Grossman, 1988; Coetzee & Pieterse, 1997; Wynne, 1977).

Enzyme activity was calculated using the following equation:

$$\text{Enzyme activity} = \left\{\left(\frac{\Delta A}{Et}\right)\frac{V_f}{V_s} * 1000\right\}\frac{V_t}{[Chla]}$$

A = absorbency (nm)
E = extinction coefficient (13.3 m mol/ml)
t = time of reaction (min)
V_f = final volume in the cuvette (ml)
V_s = volume of the homogenate of the sample (ml)
V_t = volume of extraction buffer (ml)
[Chla] = chlorophyll-*a* concentration (µg/ml)

5.3. METHOD TO DETERMINE THE ACID PHOSPHATASE ACTIVITY OF *MICROCYSTIS AERUGINOSA AND OSCILLATORIA SIMPLICISSIMA*

Ten millilitres of the algal suspension (see paragraph 4.2) were harvested on a 47 mm GF/C glass fibre filter and stored in liquid nitrogen. Frozen glass fibre filters with the algae were homogenised in 3 ml 0.2 M acetate buffer (pH = 5) consisting of acetic acid and sodium acetate. Homogenates were clarified by centrifugation at 10 000 rpm (JA 20 Beckman rotor) for 20 min and the supernatant collected. 0.1 ml 10 mM P-nitrophenyl phosphate (pNpp) was added prior to incubation of 2 hours at 30°C. The reaction was terminated by the addition of 0.3 ml 1 M NaOH and APA/AcPA were calculated from the absorbency of released NPP read at 410 nm with a Genesis 2 spectrophotometer according to Wynne (1977).

Enzyme activity was calculated using the following equation:

$$\text{Enzyme activity} = \left\{\left(\frac{\Delta A}{Et}\right)\frac{V_f}{V_s} * 1000\right\}\frac{V_t}{[Chla]}$$

A = absorbency (nm)
E = extinction coefficient (13.3 m mol/ml)
t = time of reaction (min)
V_f = final volume in the cuvette (ml)
V_s = volume of the homogenate of the sample (ml)
V_t = volume of extraction buffer (ml)
[Chla] = chlorophyll-*a* concentration (µg/ml)

5.4 RESULTS

The nutrient limitation induced APA in *M. aeruginosa* in all the 16:1 and 1:160 treatments. Figure 5.1 compare the APA of the different treatments of *M. aeruginosa* and *O. simplicissima* harvested at midlog phase. Looking at the temperature effect, the APA of *M. aeruginosa* in general show an increase with increased temperature if grown in the non-deficient medium but decreased with temperature if grown in the P-deficient medium. This was particularly evident when incubated at 5 $\mu molm^{-2}s^{-1}$. The APA of *M. aeruginosa* incubated at 15 $\mu molm^{-2}s^{-1}$, grown in the P-deficient medium was the highest at 18°C but was significantly lower at 25 and 32°C. The APA of *M. aeruginosa* incubated at 15 $\mu molm^{-2}s^{-1}$, grown in the non-deficient medium was the highest at 25°C. The APA of *M. aeruginosa* incubated at 20 $\mu molm^{-2}s^{-1}$ stayed low for all treatments and temperatures. The APA of those grown in the P-deficient decreased with an increase in light intensity. The APA activities of *M. aeruginosa* incubated at 32°C, grown in the N-deficient and P-deficient medium was the highest at 5 and 15 $\mu molm^{-2}s^{-1}$ and significantly lower at 20 $\mu molm^{-2}s^{-1}$. The APA of the external control of *M. aeruginosa* also stayed low for all treatments (Figure 5.2).

Figure 5.3 compare the AcPA activity of the different treatments of *M. aeruginosa* and *O. simplicissima* harvested at midlog phase and Figure 5.4 compare the AcPA activity of the external controls of these treatments. No results could be obtained for *M. aeruginosa* grown in the N-deficient medium and incubated at 5, 15 and 20 $\mu molm^{-2}s^{-1}$ for 18°C and 5 and 15 $\mu molm^{-2}s^{-1}$ for 25°C. No results could be obtained for *O. simplicissima* incubated at 5 and 15 $\mu molm^{-2}s^{-1}$ for 18 and 25°C.

O. simplicissima exhibited higher activity for both APA and AcPA at 32°C. The APA activity of *O. simplicissima* incubated at 32°C grown in the P-deficient medium was the highest at 15 $\mu molm^{-2}s^{-1}$. The APA activity of *O. simplicissima* incubated at 32°C grown in the non-deficient medium decreased with an increase in light intensity, while those grown in the N-deficient medium stayed the same for all the light intensities. The APA activity of *O. simplicissima* incubated at 20 $\mu molm^{-2}s^{-1}$, grown in the N-deficient and non-deficient medium was the highest at 25°C, however, those grown in the P-deficient medium had the highest APA activity at 18°C. The APA activity of the external control of *O. simplicissima* incubated at 5 $\mu molm^{-2}s^{-1}$, 32°C was significantly higher than those of *M. aeruginosa* at the same conditions.

AcPA of *M. aeruginosa* was lower than the APA for most of the treatments. The treatments incubated at 5 $\mu molm^{-2}s^{-1}$ stayed the same for the different temperatures. The AcPA of *M. aeruginosa* incubated at 15 $\mu molm^{-2}s^{-1}$, grown in the non-deficient medium was higher at 25°C, while those grown in the P-deficient medium was the highest at 18 and 25°C but significantly lower at 32°C. The treatments incubated at 20 $\mu molm^{-2}s^{-1}$ stayed low for the different temperatures.

The AcPA of *O. simplicissima* incubated at 20 $\mu molm^{-2}s^{-1}$, grown in the N-deficient decreased with an increase in temperature. Those grown in the non-deficient medium stayed low, while those grown in the P-deficient medium had the highest AcPA at 18°C. The AcPA of the external control of *O. simplicissima* incubated at 5 and 20 $\mu molm^{-2}s^{-1}$ at 32°C was significantly higher than those of *M. aeruginosa* at the same conditions.

Looking at the light effect, the AcPA activities of *M. aeruginosa* incubated at 18°C, grown in the non-deficient medium stayed low at all the light intensities while those grown in the P-deficient medium was high at 5 and 15 $\mu molm^{-2}s^{-1}$ but significantly lower at 20 $\mu molm^{-2}s^{-1}$. The AcPA of *M. aeruginosa* incubated at 25°C, grown in the non-deficient and P-deficient medium was the highest at 15 $\mu molm^{-2}s^{-1}$. The treatments incubated at 32°C stayed low for all the light intensities.

The AcPA of *O. simplicissima* incubated at 32°C grown in the N-deficient and non-deficient medium decreased with an increase in light intensity, those grown in the P-deficient medium was the same for 5 and 20 $\mu molm^{-2}s^{-1}$ but significantly lower at 15 $\mu molm^{-2}s^{-1}$.

5.5 DISCUSSION

As expected the APA as well as the AcPA of the external controls of both organisms stayed very low (Figures 5.2 and 5.4) as phosphorus was not deficient in the growth medium. What was surprising was that the enzyme activity of the organisms grown in the P-deficient medium was not always the highest, especially at the higher temperature and higher light treatments. Dodds (1995) also observed that increasing the degree of P-deficiency had little effect upon the phosphatase activity although; elevated phosphatase activity is generally thought to indicate conditions of P-deficiency (Jones, 1972). Pick (1987) suggested that APA is not a sensitive indicator of P-deficiency and that some of the APA measured represents constitutive enzymes rather than P-suppressible enzymes. Constitutive enzymes are produced independently of an activator and are more or less constantly synthesized in the cell (Jansson, *et al.*, 1988).

As shown previously in Chapter 3, APA of *O. simplicissima* did not respond to nutrient limitation, except at high temperature. The APA of the different treatments for both *M. aeruginosa* and *O. simplicissima* were higher than the AcPA. According Jansson, *et al.* (1988) algae and bacteria growing in alkaline environments such as the Mid-Vaal River, generally produce more alkaline than acid phosphatases. The synthesis of internal acid phosphatases is generally not repressed by orthophosphate (Wynn, 1977); it is therefore possible that acid phosphatases are constitutive enzymes produced mainly to serve the internal phosphorous metabolism while alkaline phosphatases have external functions and a synthesis which depend on ambient phosphorous nutrition (Jansson, *et al.*, 1988). *M. aeruginosa* has polyphosphate granules as well (Barlow *et al.*, 1979), and has been shown to use its stored polyphosphate for metabolism during the beginning of phosphate limitation. The increase in AcPA may indicate release of these internal P reserves, with subsequent AcPA increase to facilitate its hydrolysation to bioavailable monophosphate. *O. simplicissima* cells also contain polyphosphate granules storing phosphorus especially during the lag and exponential phases of the growth cycle (Venter, 2000), *O. simplicissima* also possesses cyanophycin granules in all growth stages, storing especially nitrogen (Venter, 2000).

According to Wetzel (2001) the activity of APA exhibited a relation that was inversely related to growth rate and was correlated with both polyphosphates and internally stored surplus phosphorus. In this study high growth rates of *M. aeruginosa* and *O. simplicissima* were found to be associated with low APA activity and vice versa (Figures 5.5 - 5.8 respectively) for most of the treatments. High performance indexes were also found to be associated with low APA activity and vice versa (see Figures 5.9 – 5.12 respectively) for most of the treatments.

The APA activity of *M. aeruginosa* grown in the P-deficient medium and incubated at 5 $\mu molm^{-2}s^{-1}$ decreased with an increase in temperature while those grown in the non-deficient medium at the same light intensity increased with increase in temperature. According to McComb *et al.* (1979) phosphatase activity is affected by temperature, pH and metal ions. Huber & Kidby (1984) presented data on natural waters and algal cultures showing phosphatase activity maxima mostly in the range of 25-50°C. There was also a decrease in the APA activity of *M. aeruginosa* grown in the P-deficient medium with an increase in light intensity at a specific temperature. The same was observed for *O. simplicissima* grown in a non-deficient medium at 32°C.

The AcPA activity of *O. simplicissima* grown in a N-deficient medium and incubated at 20 $\mu molm^{-2}s^{-1}$ decreased with an increase in temperature as well as with an increase in light intensity when incubated at 32°C.

According to Wetzel (2001) the chemical mass of inorganic phosphorous in water has little relation to the growth kinetics of algae. Of overriding importance is the rapidity with which phosphorus is cycled and exchanged between the particulate phosphorus and soluble inorganic and organic phases. The minimal phosphorus requirements per unit cell volume of *M. aeruginosa* and *O. simplicissima* are less than 0.5 $\mu g\ mm^{-3}$ (Wetzel, 2001). Organism's response to nutrient deficiency include metabolic changes enabling organisms to efficiently scavenge the limiting nutrient; these responses may include synthesis of high affinity transport systems and production of hydrolytic enzymes that facilitate utilization of alternate forms of the limiting nutrient (Grossman *et al.*, 2001).

5.6 REFERENCES

See Appendix B on CD.

CHAPTER 6

COMPARISON OF THE PHOTOSYNTHETIC PERFORMANCE OF THE CYANOBACTERIA MICROCYSTIS AERUGINOSA KÜTZING EMEND ELENKIN (1846) AND OSCILLATORIA SIMPLICISSIMA GOMONT (1982) AT DIFFERENT TEMPERATURES, LIGHT INTENSITIES AND N:P RATIOS

6.1 INTRODUCTION

The photosynthetic apparatus of cyanobacteria is located on thylakoids that may be either single of stacked (Sherman, *et al.*, 1987). Most strains lack chlorophyll *b* and phycobilins are the major light-harvesting pigment. The major phycobiliproteins are phycoerythrin, phycocyanin, and allophycocyanin (Grossman, *et al.*, 1994). These pigments are organized in phycobilisomes, which are located on the external surface of the thylakoid membrane (Wildeman & Bowen, 1973). The excitation energy absorbed by phycoerythrin is transferred sequentially to phycocyanin to allophycocyanin and then to the chlorophyll molecules associated with the reaction centres of photosynthesis (Grossman, *et al.*, 1994).

Photosystem II (PSII) of cyanobacteria is an integral membrane complex that supports light – mediated electron transfer from water to mobile plastoquinine molecules inside the thylakoid. This complex contains a reaction center with the specialized chlorophyll P680 that absorbs light quanta, passes electrons sequentially to a pheophytin molecule and then to a stable primary acceptor quinone (Q_A) and finally to a second acceptor quinone (Q_B) (Wraight, 1982).

In a given habitat, temporary and cyclic fluctuations of light, temperature and availability of nutrients influence PSII function (Golden, 1994). Chlorophyll-*a* fluorescence measurement has proved to be a sensitive tool in comparing effects of stress on PSII function and a powerful analytical tool for the investigation of mechanisms for stress damage (Strasser and Strasser, 1995). Measurement of chlorophyll-*a* fluorescence is rapid and non-destructive, providing information on the energy fluxes of absorption, trapping and electron transport through PSII and is therefore a sensitive indicator of photo inhibition (Krüger *et al.*, 1997).

In all dark-adapted oxygen-evolving systems, the intensity of chlorophyll-*a* fluorescence emission shows a characteristic variation over time, known as the fluorescence transient. The variable chlorophyll-*a* fluorescence yield is related to the photochemical activity of PSII. The kinetics of the fluorescence rise from the minimum yield F_0 to the maximum yield F_M, is a measure of the accumulation of net reduced primary bound Q_A in all the PSII reaction centres (RC's) with time. With high light intensity, the fluorescence rise follows a fast polyphasic sequence with the main steps labelled as O-J-I-P (Strasser *et al.*, 1995).

On illumination there is an immediate rise in fluorescence to an initial O level, termed F_0. This is attained when the chlorophyll antennas absorb light but before the excitons have been trapped. F_0 is measured when the majority of PSII RC's 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^-, while the J-P phase is non-photochemical. The intermediate I phase is related to heterogeneity in the filling-up of the plastoquinone pool, which is due to the existence of fast and slow reducing PQ

centres as well as different redox states of Q. The electrons are transferred via Q_B to the plastoquinone pool, so that reoxidation of Q_A occurs. The P phase is reached when all the plastoquinone (PQ) is reduced to PQH_2 and all the RC's are closed (Bolhar-Nordenkampf & Öquist, 1993; Strasser *et al.*, 1995). The OJIP transient changes its shape according to many environmental conditions, such as light intensity, temperature, drought or chemical influences (Krüger *et al.*, 1997).

The physiological stress levels of PSII of *M. aeruginosa* and *O. simplicissima* grown at different temperatures, light intensities and N:P ratios were compared by using a fluorometer with a very high time resolution and data acquisition capacity for measuring the fast fluorescence rise from O – P of dark-adapted algal cells. The fluorescence transients were quantified by JIP-test (Strasser & Strasser, 1995).

6.2 METHODS

The JIP-test (Strasser & Strasser, 1995; Krüger *et al.*, 1997) was used to measure and quantify chlorophyll-*a* fluorescence transients of 18 replicates of the different treatments. Ten-millilitre growth suspension (in midlog phase) was filtered through a GF/C glass fibre filter. Chlorophyll-*a* fluorescence transients of the filaments on the filter were measured with a Plant Efficiency Analyzer (PEA, Hansatech Ltd. King's Lynn, Norfolk, UK).

The data acquisition rate of the fluorimeter is 10 µs for the first 2 ms and 1 ms thereafter for a 1 s measurement, which allows for the accurate estimation of F_0. The transients were induced by a red light of 600 W m^{-2} intensity provided by an array of 6 light-emitting diodes, allowing true F_M to be reached. All measurements were done on dark-adapted (1 h period) suspensions. The fluorescence intensities at 50 µs (F_0), 100 µs, 300 µs, 2 ms (F_J), 30 ms (F_I) and 1s (F_M) as well as the time to reach F_M (t_{Fmax}) and the area above the curve from F_0 to F_M were retained and used for the calculation of several phenomenological and biophysical expressions shown in Table 3.1 and 3.2.

The mean values of the different parameters were compared. One of the parameters is the performance index that can be used as a vitality index of the organism. PI(abs) is an index that expresses the independent parameters of the density of RC (ABS/RC), the quantum yield of trapping ($\varphi_{P0}/(1 - \varphi_{P0})$) and the probability that a trapped exciton will move an electron into the electron transport chain beyond Q_A ($\psi_0/(1 - \psi_0)$). It is a multi-parametric expression of three independent parameters.

$$PI(abs) = 10 \cdot ABS/RC \cdot (\varphi_{P0}/(1 - \varphi_{P0})) \cdot (\psi_0/(1 - \psi_0))$$

6.3 RESULTS

The performance indexes of *O. simplicissima* and *M. aeuginosa* are compared in Figure 6.1. Photosystem II of *O. simplicissima* performed better than *M. aeuginosa* at 25°C and 32°C, this was also reflected in the external control (Figure 6.2). The highest performance index for *O. simplicissima* was calculated for the non-deficient treatments incubated at 25°C and light intensities of 15 and 20 µmol$m^{-2}s^{-1}$. The performance index of the non-deficient treatments of *O. simplicissima* was the highest for a specific light intensity except for those incubated at 32°C and 5 µmol$m^{-2}s^{-1}$. The highest performance for *M. aeruginosa* was calculated for the N-deficient treatments incubated at 25°C and 20 µmol$m^{-2}s^{-1}$. The performance index of the external control of *O. simplicissima* was the highest for treatments incubated at 32°C and 20 µmol$m^{-2}s^{-1}$, while the external control of *M. aeruginosa* performed best at 25°C and 20 µmol$m^{-2}s^{-1}$.

Other parameters of the JIP-analysis can clarify the stress mechanisms reflected in the performance index (Figures 6.1 and 6.2).

ABS/RC (Figure 6.3) for *M. aeruginosa* incubated at 18°C and 5 $\mu molm^{-2}s^{-1}$ was the highest for the P-deficient medium. A high ABS/RC represents an increase in antenna size (Hillier *et al.*, 1992) that can be viewed as an effort of the stressed organism to compensate for the deactivation of active RC's, which are transformed to quenching sinks that do not reduce Q_A (Krüger *et al.*, 1997). Through regrouping, the antenna molecules of deactivated RC's can be transferred to active RC's (Strasser *et al.*, 1995), resulting in the observed ABS/RC increase (Figure 6.3). TR/RC (Figure 6.4) for the P-deficient treatment was also the highest but the higher ET/RC (Figure 6.5) of this treatment usually oppose this effect leading to an increase in the rate of RC closure and therefore a decrease in the fraction of active RC/CS (Figure 6.6). Deactivation of RC's ultimately results in the observed decrease in the phenomenological fluxes TR/CS (Figure 6.7) and ET/CS (Figure 6.8) as well as the decrease in the quantum yield of primary photochemistry (φ_{PO}) shown in Figure 6.9. A decrease in φ_{PO} indicates increased damage and loss of energy trapping while a decrease in φ_{EO} (Figure 6.10) indicate a loss of electron transport capabilities. The observed increase in Ψ_0 (Figure 6.11) indicates energy loss at antenna level. Photo system II of the P-deficient treatment of *M. aeruginosa* incubated at 18°C and 5 $\mu molm^{-2}s^{-1}$ was more stressed than treatments growing in N-deficient and non-deficient media because of damage at antenna level and a loss of electron transport capabilities. This correlated with the low calculated performance index (Fig 6.1) of this treatment.

M. aeruginosa grown in the N-deficient medium incubated at 18°C and 15 $\mu molm^{-2}s^{-1}$ did not grow but the non-deficient and P-deficient treatments followed the same trend as those grown at 5 $\mu molm^{-2}s^{-1}$ (Figures 6.3 – 6.11). Therefore, Photo system II of the P-deficient treatment of *M. aeruginosa* growing at 18°C and 15 $\mu molm^{-2}s^{-1}$ was more stressed than treatments growing in the non-deficient media because of damage at antenna level and a loss of electron transport capabilities. This correlated with the low calculated performance index (Figure 6.1) of this treatment.

The PSII of non-deficient *M. aeruginosa* treatment incubated at 18°C and 20$\mu molm^{-2}s^{-1}$ had the lowest performance index (Figure 6.1) of the three treatments. This can be explained by the decrease in φ_{PO} (Figure 6.9) of this treatment that indicates increased damage and loss of energy trapping while the decrease in φ_{EO} (Figure 6.10) indicate a loss of electron transport capabilities.

The high ABS/RC (Figure 6.3) of *M. aeruginosa* grown in the P-deficient medium incubated at 25°C and 5 $\mu molm^{-2}s^{-1}$ is not enough to compensate for the effects caused by deactivation of the RC's (Figure 6.6). This resulted in a decrease in the TR/CS (Figure 6.7). However, there is a slight increase in the ET/CS (Figure 6.8) of the P-deficient treatments if compared to the non-deficient treatment. This is reflected in φ_{EO} (Figure 6.10). A decrease in φ_{PO} (Figure 6.9) of the P-deficient treatment indicates increased damage and loss of energy trapping while an increase in Ψ_0 (Figure 6.11) indicates energy loss at antenna level. Photosystem 11 of the P-deficient treatment of *M. aeruginosa* growing at 25°C and 5 $\mu molm^{-2}s^{-1}$ did not capture light as efficiently as the N-deficient or non-deficient treatment. However, its electron transport capabilities were slightly better than those of the non-deficient medium. This explains why the performance index (Figure 6.1) of *M. aeruginosa* growing in the non-deficient and P-deficient medium at 25°C and 5 $\mu molm^{-2}s^{-1}$ are the same.

The N-deficient treatments of *M. aeruginosa* incubated at 25°C and 15 $\mu molm^{-2}s^{-1}$ did not grow. φ_{PO} (Figure 6.9) of the P-deficient treatments of *M. aeruginosa* incubated at 25°C and

15 $\mu molm^{-2}s^{-1}$ are slightly lower than the other treatments indicating a loss of energy trapping. This is also reflected in Ψ_0 (Figure 6.11) that indicates energy loss at antenna level of this treatment.

The performance index of *M. aeruginosa* incubated at 25°C and 20 $\mu molm^{-2}s^{-1}$ in a N-deficient medium was the highest for treatments incubated at this temperature and light intensity. The quantum yield of trapping φ_{P0} (Figure 6.9) was the highest while there was no statistical difference between the quantum yields of electron transport φ_{E0} (Figure 6.10) of the treatments at this temperature and light intensity.

M. aeruginosa growing in the P-deficient medium at 32°C and 5 $\mu molm^{-2}s^{-1}$ had the lowest performance index. Although there was an increase in antenna size (ABS/RC in Figure 6.3), the amount of active RC/CS (Figure 6.6) decreased. The deactivation of RC's ultimately results in the observed decrease in the phenomenological fluxes TR/CS (Figure 6.7) and ET/CS (Figure 6.8) as well as the decrease in the quantum yield of trapping (φ_{P0}) shown in Figure 6.9 and the quantum yield of electron transport φ_{E0} (Figure 6.10) that indicate a loss of electron transport capabilities.

The performance indexes of all the treatments of *M. aeruginosa* incubated at 32°C and a light intensity of 15 and 20 $\mu molm^{-2}s^{-1}$ are low. The low quantum yields of trapping φ_{P0} (Figure 6.9) and electron transport φ_{E0} (Figure 6.10) indicated damage at antenna level as well as a loss of electron transport capabilities.

For *O. simplicissima* incubated at 25°C and 5 $\mu molm^{-2}s^{-1}$. ABS/RC (Figure 6.3) was slightly higher for the P-deficient medium. TR/RC (Figure 6.4) for the P-deficient treatment was also the highest but the higher ET/RC (Figure 6.5) of this treatment opposes this effect leading to the deactivation of the RC/CS (Figure 6.6). Deactivation of RC's ultimately results in the observed decrease in the phenomenological fluxes TR/CS (Figure 6.7) and ET/CS (Figure 6.8) as well as the decrease in the quantum yield of trapping (φ_{P0}) shown in Figure 6.9. A decrease in φ_{P0} indicates increased damage and loss of energy trapping while a decrease in φ_{E0} (Figure 6.10) indicate a loss of electron transport capabilities. The observed increase in Ψ_0 (Figure 6.11) indicates energy loss at antenna level. Photosystem 11 of the P-deficient treatment of *O. simplicissima* incubated 25°C and 5 $\mu molm^{-2}s^{-1}$ is more stressed than treatments growing in N-deficient and non-deficient media because of damage at antenna level and a loss of electron transport capabilities. This correlated with the calculated performance index (Figure 6.1) of the treatments.

O. simplicissima incubated at 25°C and 15 $\mu molm^{-2}s^{-1}$ growing in the non-deficient medium outperformed the other treatments (Figure 6.1). ABS/RC (Figure 6.3), TR/RC (Figure 6.4) and ET/RC (Figure 6.5) of the treatments incubated at 25°C and 15 $\mu molm^{-2}s^{-1}$ do not differ that much. However, the high fraction of active RC/CS (Figure 6.6) for the non-deficient treatment results in the observed increase in the phenomenological fluxes TR/CS (Figure 6.7) and ET/CS (Figure 6.8) as well as the increase in the quantum yield of trapping (φ_{P0}) shown in Figure 6.9. The increase in φ_{E0} (Figure 6.10) that indicate better electron transport capabilities of the non-deficient treatment.

The quantum yield of trapping (Figure 6.9) and electron transport of *O. simplicissima* incubated at 25°C and 20 $\mu molm^{-2}s^{-1}$ followed the same trends as those incubated at 25°C and 15 $\mu molm^{-2}s^{-1}$.

There was no statistical difference in the performance indexes (Figure 6.1) of treatments of *O. simplicissima* incubated at 32°C and 5 $\mu molm^{-2}s^{-1}$ and that is reflected in the JIP-parameters (Figure 6.3 – 6.11).

O. simplicissima incubated at 32°C and 15 $\mu molm^{-2}s^{-1}$ growing in the N-deficient medium had the lowest performance index (Figure 6.1). Although there was an increase in antenna size (ABS/RC in Figure 6.3), the amount of active RC/CS (Figure 6.6) decreased. The deactivation of RC's ultimately results in the observed decrease in the phenomenological fluxes TR/CS (Figure 6.7) and ET/CS (Figure 6.8) as well as the decrease in the quantum yield of trapping (φ_{P0}) shown in Figure 6.9 and the quantum yield of electron transport φ_{E0} (Figure 6.10) that indicate a loss of electron transport capabilities.

There is no statistical difference between the performance indexes of *O. simplicissima* growing in the N-deficient and P-deficient media at 32°C and 20 $\mu molm^{-2}s^{-1}$. A decrease in φ_{P0} (Figure 6.9) indicates increased damage and loss of energy trapping while a decrease in φ_{E0} (Figure 6.10) indicate a loss of electron transport capabilities for both these treatments.

6.4 DISCUSSION

Light fuels photosynthetic electron transport and CO_2 fixation and is the primary determinant of levels of NADP/NADPH, ATP and carbon metabolites, all of which can serve to modulate cellular processes (Grossman, *et al.*, 2001).

Despite the higher growth rates of *M. aeruginosa, O. simplicissima* performed metabolically better at 25 and 32°C. The low performance (Figure 6.1) of *M. aeruginosa* incubated at 32°C was unexpected as the chlorophyll-*a* (Figure 4.1) concentrations of these treatments showed an upward trend with temperature and light intensity. According to Krüger & Eloff (1981) the cessation of growth in *M. aeruginosa* batch cultures grown at high light intensities was the depletion of CO_2 due to photosynthetic activity and insufficient replenishment of CO_2 by diffusion. At low CO_2 concentrations and high pH, ribulose biphosphate caboxylase would not be able to assimilate CO_2.

The only pattern that emerges from the performance indexes (Figure 6.1) was that the non-deficient treatments of *O. simplicissima* performed the best for a specific temperature and light intensity. The highest performance indexes were calculated for the non-deficient treatments of *O. simplicissima* incubated at a temperature of 25°C and light intensities of 15 and 20 $\mu molm^{-2}s^{-1}$.

According to Strasser *et al.* (2004) a step at about 300 µs (K-step) in the fluorescence transients was caused by N-deficiency. This can be related to the inactivation of the oxygen-evolving complex leading to an imbalance between the electron flow leaving the RC towards the acceptor side and the electron flow coming to the RC from the donor side of PSII, associated with the accumulation of a high fluorescence yield species, possibly $Pheo^-$, acting as precursor of Q_A^-. Such a step was not observed in the fluorescence transience of either *M. aeruginosa* or *O. simplicissima*.

The JIP-test revealed a down regulation of PSII function of *O. simplicissima* growing in N-deficient as well as P-deficient media. A decrease in and electron transport, the source of ATP and NADPH, could limit reduction of PGA and RuBP regeneration in the Calvin cycle resulting in reduced CO_2 assimilation during photosynthesis and therefore growth.

Robarts and Zohary (1984) noted that nitrogen and phosphorus were in excess of algal requirements throughout the year and that light was probably a major factor limiting algal growth, especially during the summer (high temperatures). Scheffer *et al.* (1997) did not find any significant relationship between the abundance of Oscillatoriaceae and the concentration of phosphorus or N:P ratios in shallow lakes. However, these authors found that the relative abundance of Oscillatoriaceae is related to the light conditions under water.

Cyanobacterial development is favoured by low to moderate levels of turbidity (50-100 NTU) (Harding and Paxton, 2001; Janse van Vuuren, 2001) but absent in relatively clear water (Van Liere & Walsby, 1982). This is due to the fact that many cyanobacterial genera have light harvesting mechanisms that impart to them a selective growth advantage in light-limited situations. The large cross section of the photosynthetic antennae in cyanobacteria is caused mainly by the large antennae systems, the phycobilisomes that are arranged on the thylakoid membranes. All phycobilisomes contain the phycobiliproteins allophycocyanin (Grossman *et al.*, 2001) and phycocyanin and sometimes phycoerythrin that absorb light in the spectral region of 680 nm and 550 nm respectively while chlorophyll-*a* has absorption maxims at 380 nm and 700 nm (Mur & Schreurs, 1995). Many environmental parameters can significantly alter the composition or abundance of the phycobilisomes (Grossman, *et al.*, 1994).

Low light intensity may stimulate the synthesis of phycobilisomes and cause an increase in the size of the rod structure but in contrast macronutrient limitation can result in extensive phycobilisome breakdown during a response known as chlorosis. The degradation of phycobilisomes under these conditions may allow for the recycling of amino acids into proteins that aid in acclimation of the cell nutrient-limited growth and may be important in preventing cellular damage via photo-oxidation (Grossman, *et al.*, 1994). Cell colour changes from normal blue-green to yellow when *Synechococcus* sp. is deprived of N and P (Collier & Grossman, 1992). Chlorophyll accumulation stopped almost immediately upon N deprivation but continued for several hours after P deprivation. Levels of phycobilisomes declined dramatically in response to N deprivation but in contrast, P-deprived cultures continued to accumulate phycobilisomes for several hours (Collier & Grossman, 1992).

Zevenboom & Mur (1984) found that in *M. aeruginosa* the C-phycocyanin: chlorophyll-*a* ratios remained constant over a range of growth irradiances whereas they rose in *O. simplicissima* in low light. Post *et al.* (1985) observed that the apparent photosynthetic efficiencies of cyanobacterial cultures grown at 20 $\mu molm^{-2}s^{-1}$ were higher than those of highlight (130 $\mu molm^{-2}s^{-1}$) cultures at the same temperature, due to an enhancement in the C-phycocyanin content. The cellular content of chlorophyll *a* was up to three times greater than in the highlight cultures (Post *et al.*, 1985). However the synthesis of both chlorophyll and phycocyanin can be restricted by short nitrogen supply (Van Liere *et al.*, 1979). Steglich *et al.* (2001) observed that nitrogen deprivation in cyanobacteria causes rapid proteolytic degradation of major light –harvesting pigments and a loss in photosynthetic capacity. Low PI values were observed for the N-deficient treatments for both *Microcystis* and *Oscillatoria*. According to Grossman, *et al.*, (1994) the phycocyanin contents of N-deprived cultures can decrease to virtually undetectable levels, while the chlorophyll contents are often less affected. PSII activities usually also decline during nutrient deprivation. According to Bodemer (2004) light stress decrease the efficiency of charge separation due to damage of reaction centre protein D1.

Studies on an isolate from Balkfontein revealed that *O. simplicissima* did not grow at 20°C and 38°C and that light intensities of 5 and 20 $\mu molm^{-2}s^{-1}$ inhibits its growth. The mean fluorescence transients of *O. simplicissima* grown at light intensities of 10 and 20 $\mu molm^{-2}s^{-1}$ revealed a down regulation of photosystem 11 at 20 $\mu molm^{-2}s^{-1}$ and 28°C (Venter, 2003). In this study *O. simplicissima* did performed (Figure 6.1) slightly better at 25°C at 15 $\mu molm^{-2}s^{-1}$ than 20 $\mu molm^{-2}s^{-1}$. This was more prominent in the chlorophyll-*a* concentrations (Figure 4.3) and the same trend was observed in the growth rates of this temperature. This was also reflected in the external control (Figures 4.4 and 6.1) of *O. simplicissima* growing at 25°C. The chlorophyll-*a* concentrations (Figure 4.4) of the external control (optimum growth medium) were the highest for cultures incubated at 15 $\mu molm^{-2}s^{-1}$ at both 25 and 32°C. The performance indexes (Figure 6.1) at

25°C was high for the lower light intensities but gave an over scale error for the higher light intensity. However at 32°C the performance indexes for the external control incubated at 12 and 20 $\mu molm^{-2}s^{-1}$ was the highest.

Results observed by Post *et al.* (1985), Konopka (1981) and Foy (1983) indicated that temperature affected only the light-saturated growth and photosynthetic rates and that light limited growth and photosynthetic rates was temperature independent. This is because light–limited photosynthesis is controlled by light harvesting antenna pigments and by the maximum quantum yield of photosynthesis – both of which are temperature independent (Tilzer, 1987). Light-saturated photosynthesis by contrast is temperature dependent because it is controlled, among other things, by the photochemical turnover time, which is a function of temperature (Post *et al.*, 1985).

Acclimation responses observed in photosynthetic organisms include the alternation of light harvesting complex synthesis and degradation responses to changes in light quality, light intensity and nutrient availability. Such alterations help these organisms efficiently balance the absorption of excitation energy and the production of NADPH and ATP with their utilization for cell maintenance and growth. It is critical to maintain this balance because excess excitation of photosynthetic reaction centers could result in the production of toxic oxygen species that could be damaging too many cellular processes (Schwarz & Grossman, 1998).

6.5 REFERENCES

See Appendix B on CD.

CHAPTER 7
RE-IDENTIFICATION OF OSCILLATORIA SIMPLICISSIMA ISOLATED FROM THE VAAL RIVER, SOUTH AFRICA, AS [#]PLANKTOTHRIX PSEUDAGARDHII

7.1 RESULTS AND DISCUSSION

Pieterse & Steynberg (1993) as well as Venter *et al.* (2003) originally classified the organism "*Oscillatoria simplicissima*" under the order Nostocales, family Oscillatoriaceae, and species *Oscillatoria simplicissima* according to Desikachary (1959).

A cladogram (Figure 7.1) based on the 16S rDNA sequences revealed that two distinct groups could be recognised, the filamentous and the single cell cyanobacteria. The filamentous group are divided in four distinct clusters, namely, a cluster of *Oscillatoria/Spirulina/Leptol*yngbya, a cluster with *Oscillatoria princeps*, a cluster with *Arthruspira/ Lyngbya/ Spirulina* as well as a cluster with *Planktothrix* species. This cluster is clearly divided into two subclusters, *Planktothrix pseudagardhii* and *Planktothrix agardhii/rubescens*.

Oscillatoria princeps (AB045961^{T}) and *Planktothrix pseudagardhii* (AB045968^{T}) were selected as type representatives of the genera *Oscillatoria* and *Planktothrix* respectively (Suda, personal communication) and according to other findings of Suda (2002). Furthermore similar to Suda (2002) we found that other species of *Oscillatoria* were placed in different clusters from the type species of *Oscillatoria*.

Sequence results obtained revealed that the isolate from the Vaal River, had nearly identical sequence of 16S rDNA with that of the type species *Planktothrix pseudagardhii* NIES 845 and forms part of a distinct *Planktothrix pseudagardhii* clade (Figure 7.1). Computation was performed at NCBI using the BLAST network service (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and the sequences of the 16S rDNA of this clade shares 99% similarity. The high bootstrap values for that clade strongly supports the re-identification of the organism formerly known as "*Oscillatoria simplicissima*" found in the Vaal River, South Africa.

Use of 16S rDNA sequence analyses is a powerful tool for taxonomy; however, characterization based on the sequence alone is not enough for classification of species of some organisms, especially for closely related species (Suda *et al.*, 2002). Interestingly however, Suda *et al.* (2002) then reported that closely related species *Planktothrix agardhii* and *Planktothrix pseudagardhii* were well defined by 16S rDNA sequence analysis, although they shared the same morphology as well as fatty acid composition, phycobilin pigment and G+C content. This was again confirmed by the results (Figure 7.1) generated during this study, where *P. agardhii* clearly clustered separately from the group *Planktothrix pseudagardhii*

To verify the molecular results, a comparative morphological study was also done between "*Oscillatoria simplicissima*" and *Planktothrix pseudagardhii* NIVA CYA 153 under different environmental conditions.

[#] The EMBL accession number for the partial 16S rRNA gene sequence of *Planktothrix pseudagardhii* is AM236076 Du Plessis S, Conradie, KR & Venter, A. Re-identification of *Oscillatoria simplicissima* isolated from the Vaal River, South Africa, as "*Planktothrix pseudagardhi*". South African Journal of Botany (in press).

Venter *et al.* (2003) found that "*Oscillatoria simplicissima*", isolated from the Vaal River, has typical characteristics of the Oscillatoriaceae as described by Geitler (1932) and Desikachary (1959). This namely states that *Oscillatoria simplicissima* is a filamentous bacterium with uniserially arranged cells that are not constricted at the cross walls. Terminal cells are hemispherical with a slightly thickened membrane on the outer cell envelope. The trichomes are not attenuated or capitated at the apice. Venter *et al.* (2003) found gas vesicles present in the trichome and found that the straight, unbranched trichome is covered with a thin hyaline sheath, which is in contrast with the description of Anagnostidis & Komárek (1988) for the genus *Oscillatoria*.

The family Oscillatoriaceae, according to Anagnostidis & Komárek (1988), is characterized by straight or slightly waved trichomes. One or more trichomes can be present in a sheath. The trichomes have a thicker outer cell wall sometimes with a calyptra and the trichomes are rarely solitary, mainly compact. In the genus *Oscillatoria* the trichomes are usually without sheaths, but a sheath can occur in suboptimal conditions, no gas vesicles are present. According to our results the trichomes of our isolate are always straight, only one trichome occur in each sheath, and the trichomes are solitary. Gas vesicles are present. On the basis of these findings, this species cannot be classified under the family Oscillatoriaceae, genus *Oscillatoria* according to Anagnostidis & Komárek (1988).

Anagnostidis & Komárek (1988) however renamed the organism *Oscillatoria simplicissima* to *Phormidium simplicissimum*. According to Anagnostidis & Komárek (1988) the family Phormidiaceae is characterized by slight or intensely waved or coiled trichomes forming mats. No gas vesicles are present in the trichomes. Sheath production is dependent on environmental conditions and the sheath remains open at the ends and contains one or more trichomes. In the genus *Phormidium* the trichomes are slightly too intensely waved or coiled. These phenotypical characteristics clearly exclude the isolate "*Oscillatoria simplicissima*" from the family Phormidiaceae and the genus *Phormidium*.

Anagnostidis & Komárek (1988) established the new genus *Planktothrix* for gas-vacuolated *Oscillatoria* species. According to Suda *et al.* (2002), gas vesicles in *P. pseudagardhii* are relatively large and scattered at the periphery of the cells. Venter *et al.* (2003) found that the gas vesicles of *Oscillatoria simplicissima* are conspicuous beehive-like structures observed in the older cells of filaments and that filaments grown in culture for a long time do not have any gas vesicles. Our finding showed that gas vesicles are present in both "*Oscillatoria simplicissima*" (Figure 7.2 (1)) as well as *Planktothrix pseudagardhii* under different light and temperature conditions (Figure 7.2 (2)).

According to Suda *et al.* (2002) the trichomes of *P. pseudagardhii* are not surrounded by a sheath under conditions favourable for growth. Anagnostidis & Komárek (1988) stated that the ability of *Oscillatoria simplicissima* to form sheaths around the trichome depends on environmental conditions. Venter *et al.* (2003) found that the straight, unbranched trichome is always covered with a thin hyaline sheath.

The results also indicate that the average width of *Planktothrix pseudagardhii* was less than that of "*Oscillatoria simplicissima*" (3.05 and 3.65 µm) as is also seen in figure 7.2 and 7.3. Suda *et al.* (2002) found that the cell dimensions of *Planktothrix pseudagardhii* is 3.0-6.4 µm wide and 1.2-4.2 µm long. Desikachary (1959), Drouet (1968) and Anagnostidis & Komárek (1988) included organisms with a width of 8 to 9 µm, 1.5 to 8 µm and 1 to 12 µm in this species respectively. It is thus clear that no specific width is absolute for this species. Venter *et al.* (2003) found that the width of *Oscillatoria simplicissima* cells varies between 8.0-12.0 µm. According to our results

"*Oscillatoria simplicissima*" have an average width of 3-5 µm, and *Planktothrix pseudagardhii* NIVA 153 a width of 2–4 µm.

According to Suda *et al.* (2002), the trichomes of *Planktothrix* are sometimes attenuated towards the end and apical cells show variable shapes, such as rounded, tapered, bluntly conical and occasionally capitate, with and without calyptra. Anagnostidis & Komárek (1988) found that trichomes of *Planktothrix* are slightly tapering or not tapering to the end, end cells (when fully developed) with calyptra or with thickened outer cell. According to Venter *et al.* (2003) the trichome of *Oscillatoria simplicissima* is not attenuated or capitated at the apice and the terminal cells are hemispherical with a slightly thickened membrane on the outer cell envelope of *Oscillatoria simplicissima*. Our results indicate that the trichomes are hemispherical to the end without a calyptra. No transient forms have been observed.

According to Suda *et al.* (2002) and Anagnostidis & Komárek (1988), the cross walls of *P. pseudagardhii* may be slightly constricted or not constricted at the end. Venter *et al.* (2003) found that the cells are not constricted at the cross walls. Our results indicate that filaments may be constricted or not constricted under different environmental conditions. Figure 7.3 shows a filament of "*Oscillatoria simplicissima*" of which the cells are constricted at the cross walls. The filaments were grown at 18°C and a light intensity of 20 µmol.$m^{-2}s^{-1}$.

When comparing "*Oscillatoria simplicissima*" and *Planktothrix pseudagardhii* NIVA CYA 153 with light microscopy and confocal microscopy no clear differences between these two strains were found, which further supports results obtained from the 16S rDNA study.

According to these findings, "*Oscillatoria simplicissima*" (Venter *et al.*, 2003) as originally identified by Pieterse & Steynberg (1993), should be renamed to *Planktothrix pseudagardhii nodum nudum* (Suda – personal communication).

In conclusion, typical features of *Planktothrix pseudagardhii*, such as the presence of gas vesicles and the solitary trichomes as described by Anagnostidis & Komárek (1988) and Suda *et al.* (2002), were observed in this investigation for both *Planktothrix pseudagardhii* NIVA CYA 153 and "*Oscillatoria simplicissima*". Venter *et al.* (2003) observed that "*Oscillatoria simplicissima*" grown in culture for a long time does not have gas vesicles, where strains grown in culture for only 6 months do have gas vesicles. The classification of this organism under the order Nostocales, the family Oscillatoriaceae and the species *Oscillatoria simplicissima* under the classification of Geitler (1932) and Desikachary (1959) by Venter *et al.* (2003) is in contrast with the description of Anagnostidis & Komárek (1988) which suggests that *Planktothrix* can be distinguished from other Oscillatoriaceae by the presence of gas vesicles.

The presented morphological and molecular results clearly indicate that the isolate formerly known as "*Oscillatoria simplicissima*" grouped with the taxon of *Planktothrix pseudagardhii*, with 99 % homology, and it is therefore suggested that "*Oscillatoria simplicissima*" must be renamed to *Planktothrix pseudagardhii nodum nudum* under the order Oscillatoriales, family Phormidiaceae and subfamily Phormidioideae.

7.2 REFERENCES

See Appendix B on CD.

Table 7.1: Strains used in the cladistic analysis and listed in the cladogram

Taxon	Accession number	Location of isolation	Reference	Base pairs
Arthrospira	-	Carolina	Carolina™ Science & Math. ER-15-1721. www.carolina.com	1350
Arthrospira fusiformis	AF260510	taxon: 54297 Strain Ethi-B2	Li, R., Debella, H.J. & Carmichael, W.W. Taxonomic re-evaluation of species of *Arthrospira* (Cyanobacteria). Unpublished.	1294
Arthrospira maxima	AF260509	taxon: 129910 Strain Ethi-A2	Li, R., Debella, H.J. & Carmichael, W.W. Taxonomic re-evaluation of species of *Arthrospira* (Cyanobacteria). Unpublished.	1293
Arthrospira sp.	X70769	taxon: 35824 Strain PCC 8005	Nelissen *et al.* (1994)	1959
Arthrospira sp.	AF329392	taxon: 153965 Strain FACHB438	Mao, Y., Yang, G., Zhang, B. & Zhang, X. Application of the sequences analysis of the 16S rRNA gene and ITS of 16S-23S rRNA to the systematic study of the genus *Arthrospira* and *Spirulina*. Unpublished.	1963
Gloeobacter violaceus	AF132790	Strain PCC 7421	Turner *et al.* (1999)	1407
Leptolyngbya sp.	AB039012	Taxon: 118166 Strain: PCC 7104	Ishida *et al.* (2001)	1440
Lyngbya aestuarii	AJ000714	taxon: 65095 Strain PCC 7419	Nübel *et al.* (1997) Garcia-Pichel *et al.* (1998)	1451
Lyngbya aestuarii	AB039013	taxon: 118322 Strain PCC 7419	Ishida *et al.* (2001)	1432
Lyngbya hieronymusii	AB045906	China: Lake Dalai, Inner Mongolia Strain CN4-3	Suda *et al.* (2002)	1368
Microcystis (Loch Vaal)	-	Loch Vaal: South Africa		1485
Microcystis sp (UV027)	-	Strain UV 027		1290
Oscillatoria neglecta	AB003168	taxon: 71189 strain="M-82"	Ishida *et al.* (1997)	1438
Oscillatoria princepsT	AB045961	Thailand:Chao Phya, Bangkok Strain NIVA CYA 150	Suda *et al.* (2002)	1367
Oscillatoria simplissisima	AM236076	Vaal River	Venter (2000)	1350
Oscillatoria sp.	AJ133106	Netherlands:Lake Loosdrecht	Zwart G., Van Agterveld M., Gons H., van der Werff I. & Hagen F. yanobacterial diversity in a shallow eutrophic lake. Unpublished	1410
Planktothrix sp.	AB045914	Finland:Lake Langsjon, Aland NIVA CYA 126	Suda *et al.* (2002)	1494
Planktothrix agardhii	X84811	taxon:1160 Strain CYA 18	Nelissen *et al.* (1996)	1463
Planktothrix agardhii	AB045958	Netherlands: Veluwemeer Strain NIES 596	Suda *et al.* (2002)	1373
Planktothrix agardhii	AB045915	Finland: Lake Vesijari, Lahti Strain NIVA CYA 127	Suda *et al.* (2002)	1371

Planktothrix agardhii	AB045918	Norway: Lake Ogderen, Akershus Strain NIVA CYA 133	Suda *et al.* (2002)	1369
Planktothrix agardhii	AB045938	Norway: Lake Steinsfjorden, Buskerud Strain NIVA CYA 56/3	Suda *et al.* (2002)	1369
Planktothrix agardhii	AB045935	Norway: Lake Kolbotnvatnet, Akershus Strain NIVA CYA 34	Suda *et al.* (2002)	1369
Planktothrix agardhii	AB045947	Sweden: Lake Oren, Atvidanberg, Ostergotland Strain NIVA CYA 88/3	Suda *et al.* (2002)	1371
Planktothrix agardhii	AB045924	United Kingdom: Windermere, Cumbria Strain NIVA CYA 168	Suda *et al.* (2002)	1370
Planktothrix mougeotii	AB045971	Thailand: Nakhon Pathon Strain TR1-5	Suda *et al.* (2002)	1360
Planktothrix mougeotii	AB045969	Thailand: Bangkok Strain TK4-5	Suda *et al.* (2002)	1370
Planktothrix pseudagardhii	ABO45922	Chao Phya, Bangkok, Thailand by R. *StrainNIVA CYA 153*	Suda *et al.* (2002)	1544
Planktothrix pseudagardhii (NIVA 153)	ABO45922	Thiland: Chao Phya, Bangkok Strain NIVA CYA 153	Suda *et al.* (2002)	1352
Planktothrix pseudagardhii	AB045907	China: Lake Dalai, Inner Mongolia Strain CW4-5	Suda *et al.* (2002)	1355
Planktothrix pseudagardhii	AB045966	Thailand: King Palace, Bangkok Strain T19-6'-8	Suda *et al.* (2002)	1355
Planktothrix pseudagardhiiT	AB045968	Thailand:Bangkok Strain "T1-8-4"	Suda *et al.* (2002)	1355
Planktothrix rubescens	AB045921	Denmark: Lake almind so Strain NIVA CYA 151	Suda *et al.* (2002)	1370
Planktothrix rubescens	AB045959	Norway: Lake Gjersjoen Strain NIES 610	Suda *et al.* (2002)	1369
Planktothrix rubescens	AB045946	Sweden: Lake Levrasjon, Skane Strain NIVA CYA 87	Suda *et al.* (2002)	1369
Planktothrix rubescens	AB045950	Norway: Lake steinsfjorden, Buskerud Strain NIVA CYA 97/5	Suda *et al.* (2002)	1370
Planktothrix rubescens	AB045937	Norway: Lake Steubsfjorden, Buskerud Strain NIVA CYA 55	Suda *et al.* (2002)	1369
Planktothrix rubescens	AB045934	Norway: Lake Kolbotnvatnet, Akershus Strain NIVA CYA 320	Suda *et al.* (2002)	1370
Planktothrix rubescens	AJ132250	Switzerland Strain BC-Pla 9401	Beard *et al.* (1999)	1443
Planktothrix sp	AJ133168	taxon: 213625 Strain NIVA-CYA 127	Lyra *et al.* (2001)	1446
Planktothrix sp.	AJ133166	taxon 213624 Strain NIVA CYA 126	Lyra *et al.* (2001)	1448
Planktothrix sp.	AJ133169	taxon: 213626 Strain NIVA CYA 128/R	Lyra *et al.* (2001)	1446

Spirulina platensis	AB074508	taxon: 1156 Strain IAM M-135	Seo, P. & Yokota, A. The phylogenetic relationships of cyanobacteria inferred from gyrB sequences. Unpublished.	1441
Spirulina sp. (BFN)	-	Kamferpan, Kimberley	Roos, J. (Unpublished)	1393
Spirulina sp. (G)	-	Kamferpan, Kimberley	Jordaan, G. (Unpublished)	1356
Synechococcus sp.	AF330251	Germany: Lake Constance Strain BO0014	Ernst *et al.* (2003)	2309
Synechococcus sp.	AY172832	taxon: 69042 Strain WH5701	Fuller *et al.* (2003)	1440
Synechococcus sp.	AY151249	taxon: 210768 Isolate MW101C3	Crosbie *et al.* (2003)	1552
Synechococcus sp.	AY151251	taxon: 210717 Isolate MW97C4	Crosbie *et al.* (2003)	1554

Table 7.2: The primers used for amplification of the cyanobacterial 16S rRNA plus ITS

Primer	Sequence (5' to 3')	Target site	Reference
16S27F	AGA GTT TGA TCC TGG CTC	7-27	Wilmotte *et al.* (1993)
23S30R	AG CTT CGC CTC TGT GTG CCT AGG T	30-52	Taton *et al.* (2003) and references therein

Table 7.3: The primers used for sequencing the 16S rRNA genomic fragment

Primer	Sequence (5' to 3')	Target site	Reference
16S27F	AGA GTT TGA TCC TGG	7-27	Wilmotte *et al.* (1993)
CYA781R(a)	CTC AG	781-805	Nübel *et al.* (1997)
CYA781R(b)	GAC TAC TGG GGT ATC	781-805	Nübel *et al.* (1997)
16S727R	TAA TCC CAT T	727-741	Wilmotte *et al.* (1993)
CYA1514R	GAC TAC AGG GGT ATC	1494-1514	Taton *et al.* (2003) and
	TAA TCC CTT T		references therein
	RGG ATT AGA TAC CCC		
	GTA CGG CTA CCT TGT		
	TAC GAC		

CHAPTER 8

CONCLUSIONS

The majority of limnetic environments fall short of the conceived ideal for cyanobacterial growth. The accretion of biomass increases the absorbance of light and reduces the depth of its penetration. It also represents an increasing demand upon the supply of nutrient resources. The response of phytoplankton to environmental deficiencies is many and complex. The dominance of cyanobacteria can result from a variety of interactions among constraining environmental factors (Reynolds, 1987). We therefore made use of multivariate analyses Canoco for Windows, version 4.5 (Ter Braak, 1986; Ter Braak and Šmilauer, 1998), to emphasise the conclusions made from chapters 4, 5, 6, and 7, where we investigated the influence of light, temperature and N:P ratio's on the growth, chlorophyll *a* levels, APA and AcPA, and chlorophyll *a* fluorescence.

Redundancy Analyses (RDAs) were chosen as the multivariate analysis tool to perform the ordinations on environmental and physiological data. The RDA was statistically significant ($p=0.026$). Similarly to what was found in literature light and then temperature had the biggest influence on the physiological parameters such as growth and photosinthetic performance (Figure 8.1). Light explained 48% of the variation in the data on the first axis and temperature 41% of the variation in the data on the second axis.

Van Liere & Mur (1979) showed that under limiting light, maintenance requirements in cyanobacteria are lower than in eukaryotes. In addition, phycobilins improve harvesting of green light and energy distribution between the two photosystems creating a selective advantage of cyanobacteria over eukaryotes under low-light conditions (Post *et al.*, 1986). According to Scheffer *et al.* (1997) cyanobacteria are superior competitors under conditions of low light, but also promote such conditions, as they cause a higher turbidity per unit of phosphorous than other algae. *M. aeruginosa* have high light requirements for photosynthesis and maintenance and the ability to tolerate a much higher light intensity without experiencing photo-inhibition than low light adapted taxa (Reynolds, 1987). *O. simplicissima* is able to harvest the available light more efficiently at 25°C and 15 $\mu molm^{-2}s^{-1}$ giving it a competitive edge over *M. aeruginosa* at these environmental conditions. That may explain why *O. simplicissima* succeeded *M. aeruginosa* in the Vaal River.

Principal Component Analyses (PCA Figure 8.2) were performed on environmental variables and growth, chlorophyll-*a*, enzyme and photosynthesis data to detect relationships and potential collinearity between different physiological parameters. The First two axes of this ordination explained 55% of the variation in the data. The most important physiological parameters were growth rate and APA, as indicated by the length of the arrows. There was a decrease in APA with an increase in temperature observed in *M. aeruginosa*, while an increase in temperature was correlated with an increase in growth and APA and AcPA in *O. simplicissima*. The results further emphasise that *O. simplicissima* performed photosynthetically better than *M. aeruginosa* at high temperatures, while *M. aeruginosa* grew better at lower temperatures with higher APA in the P-deficient treatment.

General responses to nutrient-limited growth include changes in cell morphology and metabolism (Grossman *et al.*, 2001). This was also evident by the positive correlation of APA and nutrient limitation (Figure 8.2) and the morphological changes in *O. simplicissima* (Chapter 3) during nutrient deficiency. Furthermore, a negative correlation was shown between the chlorophyll *a*

concentrations and the ABS/RC. The ABS/RC increase with a decrease in chlorophyll *a* concentrations in order to try and compensate for lower photosynthetic activity. Results obtained during nutrient limited growth showed low levels of thylakoid membranes, phycobilisomes, ribosomes (Sherman & Sherman, 1983) and a decline in PSII function (Collier *et al.*, 1994). Degradation of the phycobilisomes could provide amino acids or carbon skeletons for the production of other cellular constituents required during nutrient deprivation (Grossman *et al.*, 2001).

8.1 REFERENCES

See Appendix B on CD.