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### Impact of environmental factors on toxic and bioactive peptide production by harmful cyanobacteria

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**Publication date**

2007

**Document Version**

Final published version

[Link to publication](#)

**Citation for published version (APA):**

Tonk, L. (2007). *Impact of environmental factors on toxic and bioactive peptide production by harmful cyanobacteria*. [Thesis, fully internal, Universiteit van Amsterdam].

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2007

Tonk, L. Impact of environmental factors on toxic and bioactive peptide production by harmful cyanobacteria.

ISBN 978-90-76894-79-9

Layout and design

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Cover design

Berber van Beek

Cover pictures

Microscopic image of *Anabaena* sp. and research vessel in *Microcystis* bloom at lake 't Joppe, by Linda Tonk.

Printed

Gildeprint B.V., Enschede, The Netherlands.

The studies described in this thesis were performed at the Department of Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics (IBED), Universiteit van Amsterdam Nieuwe Achtergracht 127, 1018 WS, Amsterdam.

The investigations reported in this thesis were supported by:

- The project CYANOTOX, funded by the European Commission
- The project PEPCY, funded by the European Commission
- The project DYNATOX, funded by the Technology Foundation (STW)
- The Pioneer-project 'Dynamics and Species Composition of

Phytoplankton Communities', funded by the Earth and Life Sciences Foundation (ALW) of the Netherlands Organization for Scientific Research (NWO).



# Impact of environmental factors on toxic and bioactive peptide production by harmful cyanobacteria

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor  
aan de Universiteit van Amsterdam  
op gezag van de Rector Magnificus  
prof.dr. J.W. Zwemmer  
ten overstaan van een door het college voor promoties  
ingestelde commissie,  
in het openbaar te verdedigen in de Aula der Universiteit  
op vrijdag 28 september 2007, te 12:00 uur

door

**Linda Tonk**

geboren te Weert

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# Chapter 1

## INTRODUCTION



## 1.1. CYANOBACTERIA AND THE EVOLUTION OF LIFE

Cyanobacteria have been around for a long time. The fossil records of these photosynthetic microorganisms go back more than 3.5 billion years, making them the oldest known fossils in the world (Schopf 2000). Cyanobacteria use light, carbon dioxide and water to grow, and produce oxygen as a byproduct. Their most important contribution to life on Earth dates back to the Proterozoic Era (2500 - 570 million years ago), when the atmosphere primarily consisted of methane, carbon dioxide, water vapor and a little nitrogen. During this period, also referred to as the 'Age of Cyanobacteria', cyanobacteria became abundant and widespread (Schopf and Walter 1982). This resulted in an oxygen increase in the atmosphere from less than 1% to over 21%, which allowed the evolution of aerobic organisms such as ourselves (Kasting and Siefert 2002).

At present, cyanobacteria are widespread in both marine and freshwater environments. Additionally, they may be found in terrestrial ecosystems, for instance on exposed rocks, and in many extreme environments, from highly saline lakes (e.g., the halophilic *Aphanotece halophytica*) to extremely cold habitats (e.g., cryptobiotic cyanobacteria in Antarctica). Cyanobacteria contain the photosynthetic pigments phycocyanin (a blue pigment) and chlorophyll *a* (a green pigment). This resulted in the misleading name 'blue-green algae'. In reality, cyanobacteria are not algae but prokaryotes and form a large monophyletic group within the bacterial lineage. It is merely the chloroplast in eukaryotic algae and terrestrial plants, to which cyanobacteria are functionally and genetically related. Indeed, it is generally accepted that chloroplasts evolved from endosymbiotic cyanobacteria taken up by eukaryotic cells (Margulis 1970, Tomitani et al. 1999, McFadden 2001). This points out another essential role of cyanobacteria in the course of evolution, the origin of plants. Today, the symbiotic occurrence of cyanobacteria is still widespread. Plants, lichens, diatoms, and even animals such as sponges and corals can be inhabited by cyanobacteria (Drum and Pankratz 1965, Lesser et al. 2004).

## 1.2. HARMFUL CYANOBACTERIA

During warm summers, when weather conditions are stable and photon irradiance and temperature are most favorable, cyanobacterial blooms often develop in freshwater lakes and brackish waters. These blooms are undesirable, not only because of their smell and lack of appeal for recreational purposes, but also because some cyanobacteria produce toxins. Cyanobacteria produce a wide variety of bioactive compounds. Although many of these compounds are harmless, and some are in fact used for the development of medicines and even 'health products' (such as *Spirulina*), several of the secondary metabolites produced by cyanobacteria are indeed dangerous. Besides their toxic effect on bacteria and protists, these bioactive compounds, generally called cyanotoxins, may also be hazardous to both animals and humans. Depending on the concentration and identity of the cyanotoxins involved, health

risks may range from relatively minor inconvenience such as skin irritation and abdominal pain to severe intestinal damage and even death.

Throughout history, the death of livestock and wildlife has been associated with cyanobacterial blooms (Codd et al. 2005). One of these tragic incidents caused by harmful cyanobacteria is for instance the recurrent mass mortality of thousands of Lesser Flamingos at Lake Bogoria, Kenya (Krienitz et al. 2003), which was most likely caused by the neurotoxin anatoxin-a. Another severe incident with cyanotoxins was the death of 52 dialysis patients in a haemodialysis clinic in Brazil, caused by microcystins in the water reservoir of the clinic (Jochimsen et al. 1998). Consequently, in 1998, the World Health Organization (WHO) introduced provisional guideline values for the concentration of microcystin-LR, with maximum values of  $1.0 \mu\text{g microcystin-LR liter}^{-1}$  as a provisional guideline value for drinking-water quality and  $20 \mu\text{g liter}^{-1}$  as a provisional guideline value for recreational waters (WHO 1998).

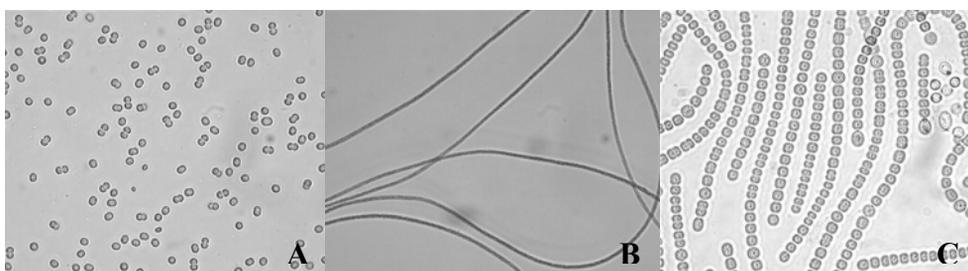


Figure 1: (A) *Microcystis aeruginosa*, (B) *Planktothrix agardhii* and (C) *Anabaena sp.*

### 1.3. CYANOTOXINS

The cyanobacterial genera *Microcystis*, *Planktothrix* and *Anabaena* (Fig. 1) occur world-wide. They can be found in freshwater or brackish waters, such as ponds, reservoirs, lakes and estuaries. Cyanobacteria are capable of producing a variety of cyanotoxins. Based on their chemical structure, cyanotoxins fall into three groups: oligopeptides (the largest group), alkaloids and lipopolysaccharides. Originally, cyanotoxins were classified into dermatotoxins, neurotoxins and hepatotoxins, according to their toxic effect. Dermatotoxins may cause acute and severe skin irritation and include the lipopolysaccharides (LPS), which are common among cyanobacteria (Moore et al. 1993). Neurotoxins, such as anatoxin-a (an alkaloid), damage the nerve system by inhibiting transmission of the nerve signal at the neuromuscular junction, while paralytic shellfish poisons (PSPs), which include the saxitoxins (also alkaloids), inhibit nerve conduction by blocking neuronal sodium channels, leading to respiratory arrest (Narahashi and Moore 1968, Spivak et al. 1980). Hepatotoxins, such as microcystins and nodularins (both cyclic oligopeptides), damage the liver through inhibition of protein

phosphatases 1 and 2a, and may also promote the development of liver tumors (MacKintosh et al. 1990, Nishiwaki-Matsushima et al. 1992). In addition to the well-known microcystins and nodularins, many other bioactive oligopeptide groups have been discovered. Recently, these peptides were grouped in seven main peptide classes based on their chemical structure (Welker and von Döhren 2006):

- (1) Aeruginosins (Fig. 2A, Murakami et al. 1995), a group of linear peptides.
- (2) Microginins (Fig. 2B, Okino et al. 1993), also linear peptides but characterized by different derivatives.
- (3) Anabaenopeptins, cyclic peptides with an ureido linkage (Fig. 2C, Harada et al. 1995).
- (4) Cyanopeptolins, cyclic depsipeptides with an amino-hydroxy piperidone (Ahp) (Fig. 2D, Martin et al. 1993). These include the anabaenopeptilides, which are structurally similar to cyanopeptolins and are produced by *Anabaena* (Rouhiainen et al. 2000).
- (5) Microcystins and nodularins, cyclic peptides characterized by an Adda moiety at position 5 and glutamate and aspartate derivatives at positions 6 and 3, respectively (Fig. 2E, Botes et al. 1984, Rinehart et al. 1988).
- (6) Microviridins, depsipeptides with a tricyclic ring system (Fig. 2F, Ishitsuka et al. 1990).
- (7) Cyclamides, cyclic structures characterized by thiazole and oxazole moieties (Fig. 2G, Todorova et al. 1995).

Each of these peptides may have a large number of structural variants. Of the hepatotoxin microcystin, for instance, at least 71 different structural variants have been found (Codd et al. 2005). In total, about 600 cyanobacterial peptides have been described thus far (Welker and von Döhren 2006). It is unknown to which limits peptide diversity extends. A single field study on the identification of peptides in *Microcystis* colonies from a Czech drinking water reservoir revealed 29 new structural variants, indicating the high potential for an even wider cyanobacterial peptide diversity than currently established (Welker et al. 2006).

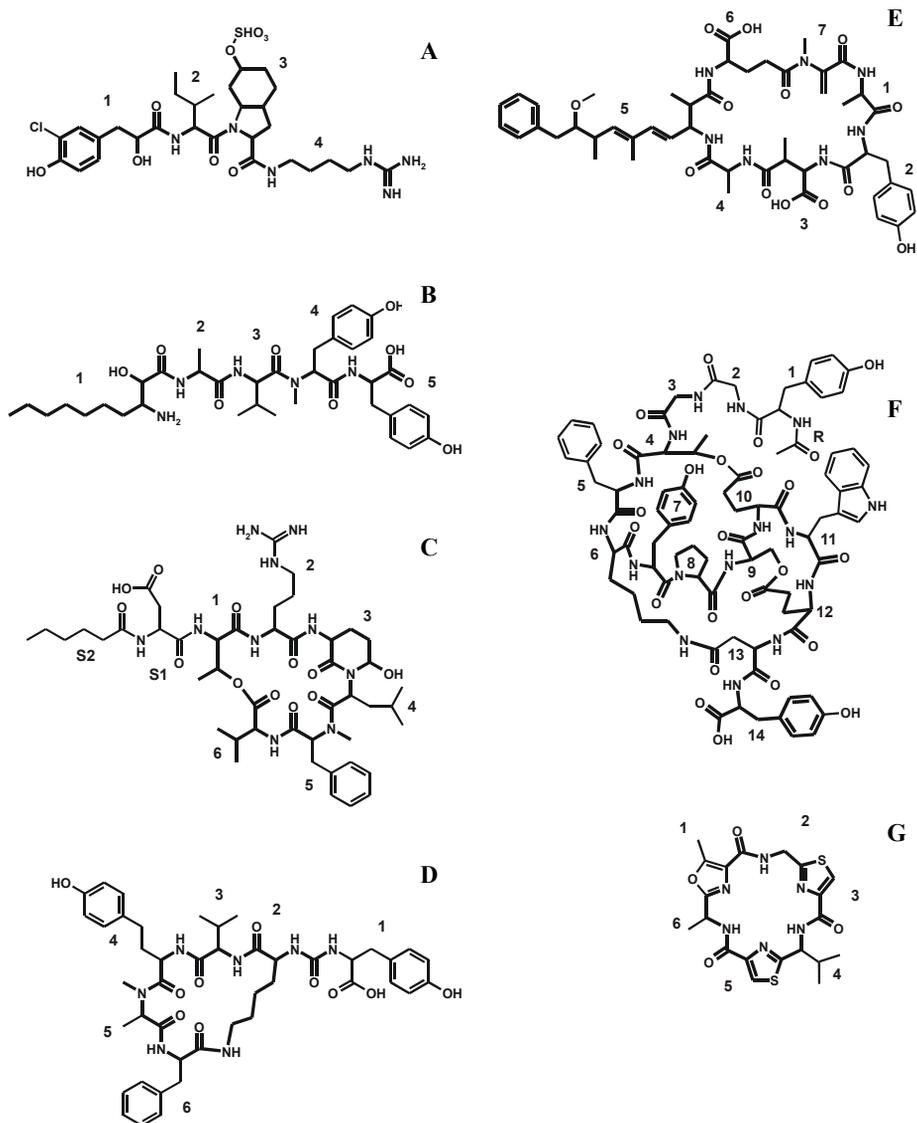


Figure 2: Structure of (A) Aeruginosin 98-A (Murakami et al. 1995), (B) microginin (Okino et al. 1993), (C) anaebaopeptin A (Harada et al. 1995), (D) cyanopeptolin A (Martin et al. 1993), (E) microcystin-LA (Botes et al. 1984), (F) microviridin A (Ishitsuka et al. 1990), and (G) Nostocyclamide (Todorova et al. 1995). Bold lines represent the conserved part of the molecule that can be found in all or most congeners; thin lines refer to variable parts. The numbering of amino acid and other residues is generally according to the (presumed) biosynthetic steps except in the structure of cyanopeptolin A, where the numbering in the sidechain is in reversed order, in microcystin-LA where the biosynthetic steps start with 5 and end with 4, and in nostocyclamide where the numbering is arbitrary due to unknown biosynthesis. Redraw from Welker and Von Döhren (2006).

#### 1.4. NON-RIBOSOMAL PEPTIDE SYNTHESIS

Many of the toxic oligopeptides produced by cyanobacteria are not gene encoded, but are synthesized non-ribosomally by large multifunctional protein complexes, called peptide synthetases (Marahiel et al. 1997). These peptide synthetases consist of non-ribosomal peptide synthetase- (NRPS) and polyketide synthase (PKS) modules. Each module consists of several domains which are responsible for the activation, modification and condensation of an individual amino acid (Kleinkauf and von Döhren 1990). The order of the modules together with the number and type of catalytic domains within each module determines the structure of the resulting polyketide or peptide product (Tillet et al. 2000).

The high structural diversity in cyanobacterial peptides points towards many different NRPS and PKS gene clusters. Some of these gene clusters have already been identified such as the microcystin synthetase gene cluster in *Microcystis* and *Planktothrix* (Tillet et al. 2000; Christiansen et al. 2003) and the anabaenopeptilide synthetase gene cluster in *Anabaena* 90 (Rouhiainen et al. 2000, 2004). It has been shown that *Anabaena* 90 has at least three peptide synthetase gene clusters for synthesis of microcystins, anabaenopeptilides and anabaenopeptins. However, the genes encoding for synthetases of anabaenopeptins have not yet been identified (Fujii et al. 2002). The relationships between multiple gene clusters within one species are still unknown and they seem to be independent of each other (Fujii et al. 2002). Rouhiainen et al. (2000) demonstrated that a mutant strain of *Anabaena* in which the *apdA* gene is interrupted did not produce the anabaenopeptilides 90A and 90B, whereas other cyclic peptides such as microcystins and anabaenopeptins were still present. Disruption of genes within the *mcy* cluster also stopped microcystin production without affecting the production of cyanopeptolins (Dittmann et al. 1997).

The production of different structural variants of microcystin can be explained by the multi-specificity of single domains of the microcystin biosynthesis complex (Neilan et al. 1999). *Planktothrix agardhii*, for instance, contains a non-specific binding pocket at the first module of the *mcyB* gene, which can bind either leucine or arginine. This binding pocket can incorporate a variety of different amino acids at the variable X position within the microcystin structure (Christiansen et al. 2003). The question remains, which factors determine which amino acid will be incorporated at this variable site? Since these are the factors that will determine the composition of microcystin variants within a single cyanobacterium.

## 1.5. FUNCTION OF CYANOBACTERIAL PEPTIDES

Until now, research on the potential function of cyanotoxins has mainly focused on microcystins. It has been suggested that microcystins play a role in cyanobacterial physiology, for instance as metal ion chelators (Utkilen and Gjørlme 1995), or as intraspecific signaling molecules (Dittmann et al. 2001a). The effect of microcystins on other organisms has led to the suggestion that these toxins may have an ecological function, by reducing grazing pressure from predators such as zooplankton (Koski et al. 1999, Rohrlack et al. 1999), or by suppressing competing phototrophic organisms (Keating 1977, Gross 2003). However, no conclusive evidence has been found to support these hypotheses. Thus far, the physiological and/or ecological function of microcystins remains unsolved.

Protease inhibition, which is used as a protective mechanism against grazing in terrestrial plants, is an effect which has been found for many cyanotoxins as well (Dittmann et al. 2001b, Welker and von Döhren 2006). A few examples of peptides exhibiting protease inhibition are microcystin, cyanopeptolin, aeruginosin, anabaenopeptin, microviridin and microginin (Table 1). None of these peptides seem to be essential for cyanobacteria, since there are many cyanobacterial strains that do not produce these peptides. Nonetheless, their importance is illustrated by the long evolutionary history of their biosynthetic pathway and the maintenance of peptide synthesis under long-term laboratory conditions (Welker and von Döhren 2006). The similarity between various peptide synthetase genes and suspected recombination events between these genes suggest a similar function for several peptides. What function that is, is thus far still unknown.

Table 1: Main classes of cyanobacterial peptides and their bioactive response

Peptide class	Bioactivity	Reference
Aeruginosins	Thrombin/trypsin inhibitor	Kodani et al. 1998
Microginins	Angiotensin-converting enzyme inhibitor	Neumann et al. 1997
Cyanopeptolins	Trypsine inhibitor	Martin et al. 1993
Anabaenopeptins	Carboxypeptidase A inhibitor	Itou et al. 1999
Microcystins	Protein phosphatase 1 and 2A inhibitor	MacKintosh et al. 1990
Microviridins	Elastase/trypsin-like proteases inhibitor	Rohrlack et al. 2003
Cyclamides	Anticyanobacterial/antialgal	Todorova et al. 1995

## 1.6. FACTORS AFFECTING GROWTH AND PEPTIDE PRODUCTION

1.6.1. *Field and laboratory studies:* Specific growth rates of cyanobacteria typically vary in the range of 0 to 2 d<sup>-1</sup>, depending on the environmental conditions and the specific requirements and characteristics of the species concerned. Elevated water temperature, low photon irradiance, high water-column stability, intense zooplankton grazing on competing species, high pH, and low nitrogen/phosphorus ratio are generally the most important factors stimulating the growth of harmful cyanobacteria (Kardinaal and Visser 2005). In the field, all these environmental factors may vary at the same time, making it difficult to assess which environmental factor causes differences in growth rate and microcystin production. Controlled laboratory experiments on the effect of light intensity (Watanabe and Oishi 1985, Wiedner et al. 2003), temperature (Watanabe and Oishi 1985), pH (Song et al. 1998), nitrogen (Long et al. 2001, Orr and Jones 1998, Watanabe and Oishi 1985), phosphorus (Oh et al. 2000, Watanabe and Oishi 1985), and iron (Utkilen and Gjørlme 1995) demonstrated that these environmental factors influence the growth rate and microcystin production. However, results from different studies may vary distinctively depending on culture technique, biomass estimate and cyanobacterial species and strain used, making it difficult to compare studies.

1.6.2. *Growth rate and production:* A review of microcystin production studies, published several years ago, indicated that conditions most favorable for growth seemed to result in highest microcystin production rates (Sivonen and Jones 1999). It has even been suggested that growth rate directly influences microcystin production rate, irrespective of the environmental factor limiting cell division (Orr and Jones 1998), and that the microcystin content can be predicted from the growth rate of nutrient-limited cyanobacteria (Long et al. 2001). However, these suggestions are not supported by more recent studies on microcystin production as a function of photon irradiance (Wiedner et al. 2003). Photon irradiance is suggested to act directly on transcription and/or transcript stability of microcystin synthetase genes (Kaebernick et al. 2000, Börner and Dittmann 2005). Furthermore, within a single strain, the production of different microcystin variants may respond to environmental factors in different ways (Rapala et al. 1997, Hesse and Kohl 2001, Tonk et al. 2005). This indicates that regulation of microcystin production is more complicated than previously thought.

1.6.3. *Salt concentration:* Freshwater ecosystems are confronted with increasing salinity on a world-wide scale. As a result, the species composition of phytoplankton communities may shift. Most freshwater phytoplankton does not survive in brackish or seawater. However, several species of harmful cyanobacteria, including species from the genera *Anabaena*, *Aphanizomenon*, and *Microcystis*, can be found in both freshwater ecosystems and brackish waters (Rai and Tiwari 1999, Laamanen et al. 2002, Tonk et al. 2007). Species able to maintain high growth rates when exposed to variation in `_salinity` may have a competitive advantage in phytoplankton communities. Thus, blooms of harmful cyanobacteria might actually be favored

in slightly brackish waters. However, little is known about the impact of elevated salinity on the microcystin production of cyanobacteria. This is of special interest in The Netherlands, characterized by large stretches of land reclaimed from sea (called polders), where blooms of harmful cyanobacteria are a widespread nuisance. Due to increasing demands for freshwater irrigation and slowly intruding sea water, many of the eutrophic lakes in these polder areas face elevated salinities during summer.

### 1.7. POPULATION DYNAMICS OF TOXIC VERSUS NONTOXIC STRAINS

Many cyanobacterial strains do not produce cyanotoxins. Consequently, cyanobacterial strains can be either toxic or nontoxic, even within species. These toxic and nontoxic strains coexist in the field (Fastner et al. 2001, Kurmayer et al. 2002, Janse et al. 2004). The strain composition of cyanobacterial blooms may change during the season. Laboratory experiments typically show 2-fold to 3-fold variation in microcystin content within isolated strains, whereas the variation in microcystin content of cyanobacterial blooms in lakes is often considerably higher (Kardinaal and Visser 2005). Increasing evidence suggests that microcystin dynamics in lakes mainly result from the succession of a large number of cyanobacterial genotypes that differ in their ability to produce microcystins (Welker et al. 2003, Kardinaal and Visser 2005). The factors driving population dynamics of these toxic and nontoxic strains, however, are not yet well understood.

## 1.8. THIS THESIS

Although the production of cyanotoxins is an extensively studied topic, many questions still remain unsolved. In this thesis, the results of several laboratory studies on the toxin production of three cyanobacterial genera will be described. The studies were carried out in association with the Dutch research program DYNATOX, and the two European research programs CYANOTOX and PEPCY. DYNATOX was initiated in order to study the dynamics of toxic cyanobacteria in Dutch lakes, and to create early-warning systems for harmful cyanobacteria. The aims of the CYANOTOX project were (1) to develop more sensitive analytical methods for cyanobacterial toxin analysis, (2) to characterize the molecular biological regulation of toxin synthesis, and (3) to determine the environmental conditions under which toxin synthesis is enhanced. The PEPCY project focused on a broad spectrum of peptides in cyanobacteria. Its central aim was to improve cyanotoxin risk assessment and risk management. Integration of the research aims of these three projects has led to the following chapters in this thesis:

In *Chapter 2* I investigate the effect of salinity on the microcystin production of the harmful cyanobacterium *Microcystis aeruginosa* PCC 7806. *M. aeruginosa* is known to tolerate small increases in salinity (Otsuka et al. 1999, Orr et al. 2004, Verspagen et al. 2006). However, it is not yet known how increasing salinity affects the microcystin production of *M. aeruginosa*. This issue is of considerable practical interest, since freshwater ecosystems are confronted with increasing salinity on a worldwide scale. In *Chapter 2*, we investigate the following questions: 1) What is the effect of gradually increasing salinity on the growth rate and microcystin production of *M. aeruginosa* PCC 7806? 2) What is the effect of a salt shock on growth rate and microcystin production of *M. aeruginosa* PCC 7806?

Earlier, Wiedner et al. (2003) presented an extensive study of the effects of photon irradiance on the microcystin production of *Microcystis aeruginosa* PCC 7806. However, it is not known how photon irradiance affects the microcystin production of the filamentous cyanobacterium *Planktothrix agardhii*. In *Chapter 3*, we investigate the effect of photon irradiance on the *mcyA* transcript, total microcystin production, and microcystin composition of *P. agardhii* strain 126/3, using light-limited turbidostats. *P. agardhii* strain 126/3 produces two microcystin variants. Surprisingly, we found that the total microcystin content of *P. agardhii* was not affected by irradiance, while the relative abundances of the two microcystin variants changed with the light conditions.

It was not exactly clear what caused the shift in microcystin composition reported in *Chapter 3*. In *Chapter 4*, we therefore set out to investigate how amino acid availability influences the synthesis of microcystin variants by *P. agardhii*. We hypothesize that differences in availability of the amino acids arginine and leucine might explain why *Planktothrix* should produce less of the arginine-based microcystin-RR variant and more of the leucine-based microcystin-LR variant at higher light intensities. Furthermore, we address the question whether the synthesis of [Asp<sup>3</sup>]microcystin-RR is hampered by nitrogen limitation in *P. agardhii* filaments.

Until recently, research on physiological regulation of cyanotoxin production mainly focused on microcystins. Other oligopeptides produced by cyanobacteria have received much less attention. In *Chapter 5*, the physiological regulation of the microcystins, cyanopeptolins and anabaenopeptins produced by *Anabaena 90* and *Microcystis* PCC 7806 are investigated. The following questions will be addressed: 1) What is the effect of phosphorus availability, photon irradiance and temperature on the peptide content and peptide production of *M. aeruginosa* PCC 7806 and *Anabaena 90*? 2) Are these peptides constitutively produced? 3) Is there a relationship between the peptide production rates and the growth rates of *M. aeruginosa* PCC 7806 and *Anabaena 90*?

In *Microcystis*-dominated lakes, microcystin-producing and non-microcystin-producing strains coexist. The dynamics of these toxic and nontoxic strains are not yet well understood. In *Chapter 6*, we investigate competition for light between toxic and nontoxic *Microcystis* strains. Since the toxic and nontoxic strains cannot be distinguished by microscope, we use light absorption spectra and molecular techniques to identify the different strains and to estimate their relative abundances. The results of our laboratory competition experiments suggest that nontoxic strains are stronger competitors for light, which offers a plausible explanation for the seasonal changes in microcystin concentrations of *Microcystis*-dominated lakes.

In *Chapter 7*, the main outcomes of the topics investigated in this thesis are placed in perspective with current knowledge and where needed, further discussed. Additionally, the ecological implications of my findings are presented.

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# Chapter 2

## SALT TOLERANCE OF THE HARMFUL CYANOBACTERIUM *MICROCYSTIS AERUGINOSA*

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(2007) AQUATIC MICROBIAL ECOLOGY VOL. 46, P. 117-123

*ABSTRACT* Increasing salinities in freshwater ecosystems, caused by agricultural practices, droughts, or sea level rise, are likely to affect the species composition of phototrophic microorganisms. Cosmopolitan freshwater cyanobacteria of the *Microcystis* genus can produce the toxin microcystin, and present a potential health risk in many eutrophic lakes. In this study, *Microcystis aeruginosa* strain PCC 7806 was grown in semi-continuous turbidostats to investigate the effect of rising salinity on growth rate, microcystin cell quota, microcystin production and extracellular microcystin concentration. Specific growth rate, microcystin cell quota and microcystin production remained more or less unaffected by salinity levels up to 10 g liter<sup>-1</sup>. Specific growth rate collapsed when salinity was increased beyond 10 g liter<sup>-1</sup> for several weeks. Cell size and microcystin cell quota decreased while extracellular microcystin concentrations increased at salinities beyond 10 g liter<sup>-1</sup>, indicating leakage and/or cell lysis. Salt-shock experiments revealed that *Microcystis* can temporarily endure salinities as high as 17.5 g liter<sup>-1</sup>. These results indicate that *Microcystis* has a high salt tolerance for a freshwater species. Rising salinities in freshwater ecosystems are therefore unlikely to suppress *Microcystis* blooms, and may in fact enhance the exposure of aquatic organisms to elevated concentrations of extracellular microcystins.

## INTRODUCTION

Freshwater ecosystems are confronted with increasing salinity on a world-wide scale, due to a variety of different processes, including long-term droughts, rising seawater levels, agricultural practices, or specific water management strategies (Williams 2001, Nielsen et al. 2003). Rising salt concentrations are known to affect numerous freshwater biota (Hart et al. 1991, 2003, James et al. 2003), and may shift the species composition of phytoplankton communities (Ahmed et al. 1985, Mohapatra et al. 1998, Wilson et al. 1994, Muylaert et al. 2000, Moisander et al. 2002, Bordalo & Vieira 2005).

Cyanobacteria are found across a wide range of different salinities, including hypersaline waters (DasSarma & Arora 2001). Most freshwater cyanobacteria, however, do not survive in brackish or seawater. *Microcystis aeruginosa* is a harmful cyanobacterium that occurs worldwide in freshwater lakes. The toxins produced by *Microcystis* are commonly known as microcystins, and belong to an extensive group of cyclic heptapeptides comprising at least 71 described variants (Codd et al. 2005). Microcystins can cause liver damage through inhibition of protein phosphatases (MacKintosh et al. 1990). Usually, microcystins are concentrated inside *Microcystis* cells, whereas extracellular microcystin concentrations remain low. The occurrence of *Microcystis* is related to environmental factors such as excess nutrient availability, high temperature and a stable water column with little vertical mixing (Reynolds & Walsby 1975, Huisman et al. 2004, Visser et al. 2005). During calm summer weather, *Microcystis* floats towards the surface and forms dense scums, thereby causing serious health risks (Chorus & Bartram 1999, Carmichael et al. 2001, Huisman et al. 2005).

In The Netherlands, characterized by large stretches of land reclaimed from sea (called polders), *Microcystis* blooms are a widespread nuisance. Due to increasing demands for freshwater irrigation and slowly intruding sea water, many of the eutrophic lakes in these polder areas face elevated salinities during summer. Furthermore, the possibility is considered to convert freshwater lakes into brackish waters to reduce *Microcystis* growth and to restore former estuarine ecosystems (Verspagen et al. 2006). As a result, the salinity in many *Microcystis*-dominated lakes in The Netherlands is slowly increasing. However, little is known about the impact of elevated salinity on the growth rate and microcystin production of *Microcystis*.

Preliminary studies in batch cultures suggest that the growth rate of *Microcystis aeruginosa* might be stimulated by rising salinities, at least up to a salinity of  $\sim 2$  g liter<sup>-1</sup> (recalculated data from Prinsloo & Pieterse 1994). Furthermore, *Microcystis aeruginosa* seems to have a relatively high salt tolerance, estimated at 7 g liter<sup>-1</sup> by Otsuka et al. (1999), 10 g liter<sup>-1</sup> by Orr et al. (2004) and 14 g liter<sup>-1</sup> by Verspagen et al. (2006). If these observations are correct, *Microcystis* blooms might actually be favored in slightly brackish waters. In this study, the strain *Microcystis aeruginosa* PCC 7806 was grown in controlled semi-continuous turbidostats to investigate in detail how elevated salt concentrations affect the growth rate, intracellular and extracellular microcystin content of *Microcystis*. We followed two approaches. In one set of experiments, cells were exposed to slowly rising levels of salinity, providing sufficient time for cells to

adjust their physiology to the prevailing osmotic conditions. In the other set of experiments, *Microcystis* cells were exposed to a sudden salt shock to examine whether cells would respond differently when confronted with a rapid rise in salinity.

## MATERIALS & METHODS

*Organism and culture conditions.* *Microcystis aeruginosa* strain PCC 7806 was provided by the Pasteur Institute, in Paris. The strain was originally isolated from the Braakman Reservoir, The Netherlands, in 1972. We cultured this *Microcystis* strain as single cells in semi-continuous cultures, using a turbidostat approach (Huisman et al. 2002). The optical density (750 nm) of the semi-continuous turbidostats was kept constant between 0.1 and 0.2 cm<sup>-1</sup> by diluting the culture with nutrient-rich mineral medium once every two days. Flat culture vessels with a working volume of 400 ml were used in combination with a constant aeration of filtered and moistened air to ensure homogeneous mixing and to provide sufficient amounts of inorganic carbon. A cooling vessel of the same dimensions as the culture vessel was placed between the culture vessel and the light source to maintain a constant temperature of 23°C (± 1°C). Light was provided by white fluorescent tubes (Philips PL-L 24W/840/4P), directed towards the front surface of the culture vessel. Incident irradiance ( $I_{in}$ ) and outgoing irradiance ( $I_{out}$ ) were measured with a LI-COR LI-250 quantum photometer at 7 points on the front surface and back surface of the culture vessel, respectively. The average photon irradiance inside the culture vessel was calculated as  $I_{avg} = (I_{in} - I_{out}) / (\ln I_{in} - \ln I_{out})$  (Huisman et al. 2002). Cultures were run in triplicate on a 12 h light: 12 h dark cycle, with a depth-averaged photon irradiance of  $I_{avg} = 110 \pm 20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  during the light period.

*Experimental outline.* Our mineral medium was based on the O<sub>2</sub> medium, which contains the macronutrients MgSO<sub>4</sub> · 7H<sub>2</sub>O (50 mg liter<sup>-1</sup>), NaNO<sub>3</sub> (500 mg liter<sup>-1</sup>), K<sub>2</sub>HPO<sub>4</sub> (25 mg liter<sup>-1</sup>), CaCl<sub>2</sub> · 2H<sub>2</sub>O (13 mg liter<sup>-1</sup>), NaHCO<sub>3</sub> (200 mg liter<sup>-1</sup>) as well as a FeCl<sub>2</sub> · 4H<sub>2</sub>O (2.2 mg liter<sup>-1</sup>) and a variety of trace metals (van Liere & Mur 1978). O<sub>2</sub> medium has a total salinity of 0.6 g liter<sup>-1</sup>. In order to detect the effect of slowly rising salinity NaCl was added to this mineral medium to increase the salinity in a stepwise fashion to the following levels: from 0.6 (O<sub>2</sub> medium) to 0.7, 0.85, 1.1, 1.35, 1.6, 2.5, 3.6, 4.6, 5, 7.5, 10, 12.5, 15 and 17.5 g liter<sup>-1</sup>. For comparison, sea water has a salinity of ~35 g liter<sup>-1</sup>. Cultures were grown in triplicate at a constant salinity level for a period of two weeks, one week for acclimation and one week for sampling. After each two-week period the salinity of the cultures was increased to the next level by adding the required amount of salt during a dilution event. Additionally, three salt-shock experiments were performed in which *Microcystis* cells, pregrown in O<sub>2</sub> medium, were directly exposed to salinities of 10, 15 and 17.5 g liter<sup>-1</sup>, without the opportunity of gradually adjusting to intermediate salinities.

The salt-shock experiments were again carried out in semi-continuous turbidostats, and the required amount of salt was added during a dilution event. The salt-shock experiments were run in triplicate using the same conditions as described above.

*Sampling and microcystin analysis.* The experiments with gradually rising salinities were sampled four times during each two-week period, on days 8, 10, 12 and 14 after the imposed salinity rise. The salt-shock experiments were monitored for a period of 10 days and sampled every other day. Samples were all taken on the same time of day to reduce possible variation in the data due to the light-dark cycle. Aliquots of all samples were analyzed in triplicate for cell concentration and intra- and extracellular microcystin concentration. Intracellular microcystin concentrations were expressed per cell (cell quota). Cell concentrations and cell size were measured with a cell counter (Casy 1 TTC, Schärfe System). Since the semi-continuous turbidostats were diluted once every two days, the specific growth rate,  $\mu$ , can be calculated according to the following equation:

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} \quad (1)$$

where  $t_1$  is the time directly after a dilution event,  $t_2$  is the time directly before the next dilution event (two days later), and  $x_1$  and  $x_2$  are the cell concentrations at time  $t_1$  and  $t_2$ , respectively. Microcystin production was calculated according to Long et al. (2001), as the product of microcystin cell quota and specific growth rate. For intracellular microcystin analysis, 10 ml of the culture suspension was filtered in triplicate using Whatman GF/C filters (pore size  $\sim 1.2 \mu\text{m}$ ). Filters were freeze dried and stored at  $-20^\circ\text{C}$ . Microcystin was extracted in 75% methanol (three extraction rounds) as described by Fastner et al. (1998) with an extra step for grinding of the filters in a Mini Beadbeater (Biospec products, Bartlesville, Okla.) with 0.5 mm silica beads (Tonk et al. 2005). Dried extracts were stored at  $-20^\circ\text{C}$  and dissolved in 50% MeOH for analysis of microcystin content using high performance liquid chromatography (HPLC) with photodiode array detection (Kontron instruments). The extracts were separated using a 30 to 70% acetonitrile gradient with 0.05% trifluoroacetic acid at a flow of  $1 \text{ ml min}^{-1}$  and a LiChrospher 100 ODS  $5 \mu\text{m}$  LiChorCART 250-4 cartridge system (Merck). The different microcystin variants were identified based on their characteristic UV-spectra and quantified by means of a MC-LR gravimetric standard kindly provided by the Laboratory of Microbiology of the University of Dundee.

For extracellular microcystin analysis, the effluent from the filtered culture suspension used for intracellular microcystin analysis was freeze dried and resuspended in 1.5 ml milliQ. We expected that extracellular microcystin concentrations would be below the detection limit of the HPLC. Therefore, extracellular microcystin concentrations were determined using an Enzyme-Linked Immuno Sorbent Assay (ELISA), according to the protocol of the Microcystin Plate Kit (EnviroLogix Inc. Catalog No. EP 022).

*Data analysis.* We used ANOVA to test for significant differences between the three salt-shock experiments (10, 15 and 17.5 g liter<sup>-1</sup>) in combination with Tukey's (HSD) Post-Hoc test to compare the means. Homogeneity of the variances was checked by Levene's test. The statistical tests were carried out in SPSS version 11.

## RESULTS

## GRADUALLY RISING SALINITIES

*Growth rates.* The specific growth rate ( $\mu$ ) of *M. aeruginosa* strain PCC 7806 remained constant around  $0.40 \text{ d}^{-1}$  (s.d. = 0.5;  $N = 12$ ), up to a salinity of  $10 \text{ g liter}^{-1}$  (Fig. 1A). This was followed by an abrupt collapse to zero growth at  $12.5$  and  $15 \text{ g liter}^{-1}$ .

*Cell size.* Mean cell size was  $34 \mu\text{m}^3$  (s.d. = 1.6;  $N = 5$ ) in the salinity range of  $0.6$  to  $10 \text{ g liter}^{-1}$ . At higher salinity, cell size decreased (Fig. 1B). Smallest cell size was approximately  $20 \mu\text{m}^3$  at a salinity of  $15 \text{ g liter}^{-1}$  (Fig. 1B).

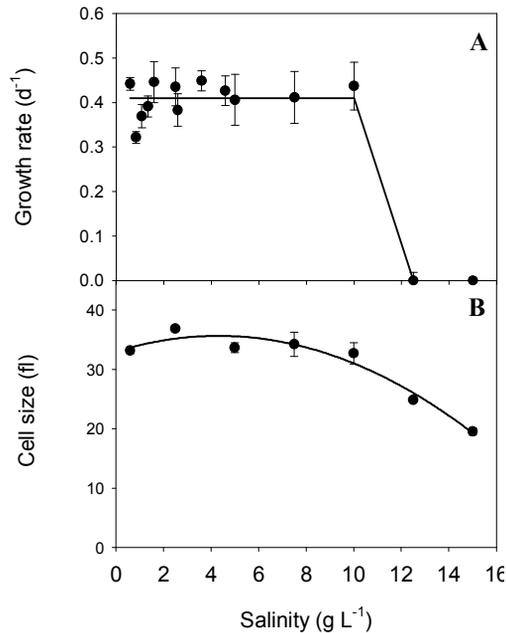


Figure 1: Specific growth rate (A) and average cell size (B) as a function of salinity. Error bars denote standard deviation ( $N=3$ ). Solid line in B is based on multiple regression.

*Intracellular microcystin.* *M. aeruginosa* strain PCC 7806 produced microcystin (MC) variants MC-LR and [Asp<sup>3</sup>]MC-LR. The peaks of [Asp<sup>3</sup>]MC-LR and MC-LR could not be separated completely by means of HPLC. Therefore both peaks were merged and referred to as total microcystin. The total microcystin cell quota was on average  $45 \text{ fg cell}^{-1}$  (s.d. = 6.7;  $N = 10$ ) for salinity levels up to  $8 \text{ g liter}^{-1}$ . Above  $8 \text{ g liter}^{-1}$  the total microcystin content decreased (Fig. 2A). The total microcystin production was on average  $18 \text{ fg cell}^{-1} \text{ d}^{-1}$  (s.d. = 3.8;  $N = 11$ ) in the salinity range from  $0.6$  to  $10 \text{ g liter}^{-1}$ , and was zero at higher salinities (Fig. 2B).

*Extracellular microcystin.* The extracellular microcystin concentration was not affected by salinity levels up to  $10 \text{ g liter}^{-1}$ , where it amounted to approximately 16% of the total (intra- + extracellular) microcystin concentration in the culture vessels (s.d. = 1.6;  $N = 5$ ). At  $15 \text{ g liter}^{-1}$ , the extracellular microcystin concentration increased to almost 100% of the total microcystin concentration (Fig. 2C).

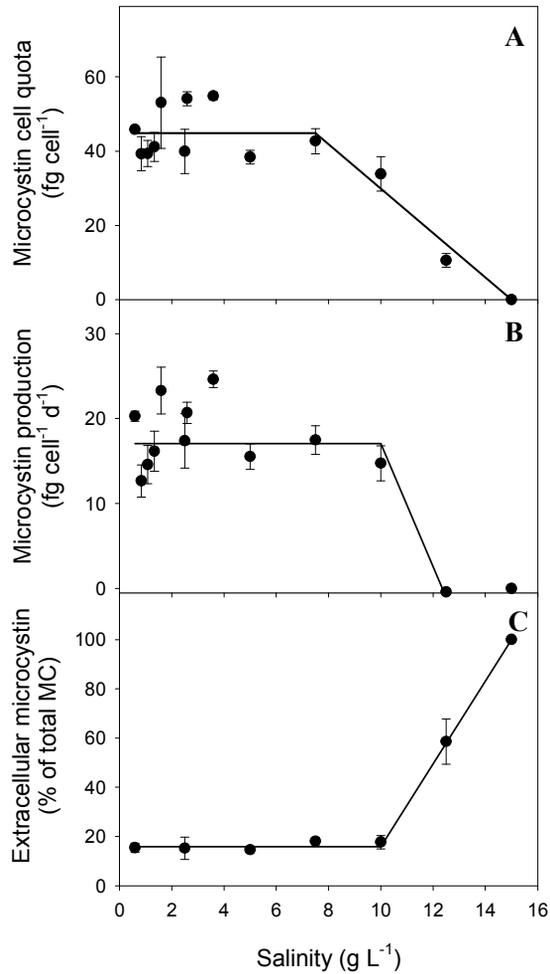


Figure 2: Total intracellular microcystin cell quota (A), microcystin production (B), and total extracellular microcystin concentration (C) as a function of salinity. Error bars denote standard deviation ( $N=3$ ). Solid lines represent the mean values at low salinities, and indicate the steep changes at high salinities.

## SALT-SHOCK EXPERIMENTS

*Growth rates.* The specific growth rate ( $\mu$ ) of *Microcystis* was reduced when exposed to a sudden increase in salinity in the salt-shock experiments (Fig. 3A). The specific growth rate recovered within a week in the salt-shock experiment with 10 g liter<sup>-1</sup>. The *Microcystis* population was clearly negatively affected by the salt-shock experiments with higher salinities, but was nevertheless still able to grow for 9 days when suddenly exposed to salinities as high as 17.5 g liter<sup>-1</sup>. At day 9, the growth rates at salinities 10, 15 and 17.5 g liter<sup>-1</sup> differed significantly from each other, decreasing with higher salinities (Fig. 3A; ANOVA:  $F_{2,6} = 121, P < 0.001$ ).

*Cell size.* Mean cell size was 35  $\mu\text{m}^3$  (s.d. = 0.2; N = 54) throughout the salt-shock experiment, with a slight increase towards the end of the experiment (Fig. 3B). At day 9, the cell size of cells grown at a salinity of 17.5 g liter<sup>-1</sup> were significantly larger than those cultured at 10 and 15 g liter<sup>-1</sup>, cell sizes at 10 and 15 g liter<sup>-1</sup> were not significantly different (Fig. 3B; ANOVA:  $F_{2,6} = 11.6, P < 0.01$ ).

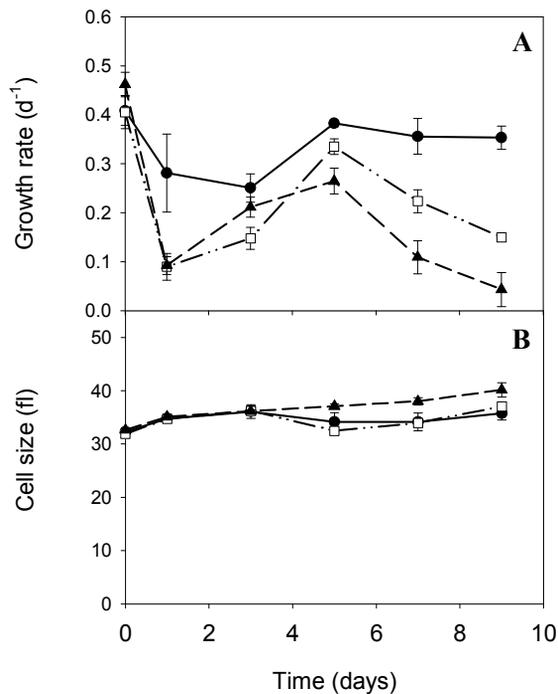


Figure 3: Time course of specific growth rate (A) and cell size (B) of *Microcystis* cells during the salt-shock experiments (●, solid line: 10 g liter<sup>-1</sup>; □, dotted line: 15 g liter<sup>-1</sup>; ▲, dashed line: 17.5 g liter<sup>-1</sup>). Error bars denote standard deviation (N = 3).

*Intracellular microcystin.* The microcystin cell quota of cells exposed to salinities of 10, 15 and 17.5 g liter<sup>-1</sup> initially increased (Fig. 4A). After three days, the microcystin cell quota of cells exposed to 10 g liter<sup>-1</sup> remained high, while the microcystin cell quota at 15 and 17.5 g liter<sup>-1</sup> decreased. At day 9, microcystin cell quota was significantly higher at a salinity of 10 g liter<sup>-1</sup> than at the salinities of 15 and 17.5 g liter<sup>-1</sup> (Fig. 4A; ANOVA:  $F_{2,6} = 28, P < 0.01$ ).

Microcystin production of cells exposed to a salt shock of 10 g liter<sup>-1</sup> decreased during the first day, but fully recovered in the following days (Fig. 4B). Total microcystin production of cells exposed to salt shocks of 15 and 17.5 g liter<sup>-1</sup> also decreased during the first day, then increased during the next few days, after which the microcystin production collapsed. At day 9, microcystin production at salinities 10, 15 and 17.5 g liter<sup>-1</sup> significantly differed from each other and significantly decreased with higher salinities (Fig. 4B; ANOVA:  $F_{2,6} = 121, P < 0.001$ ).

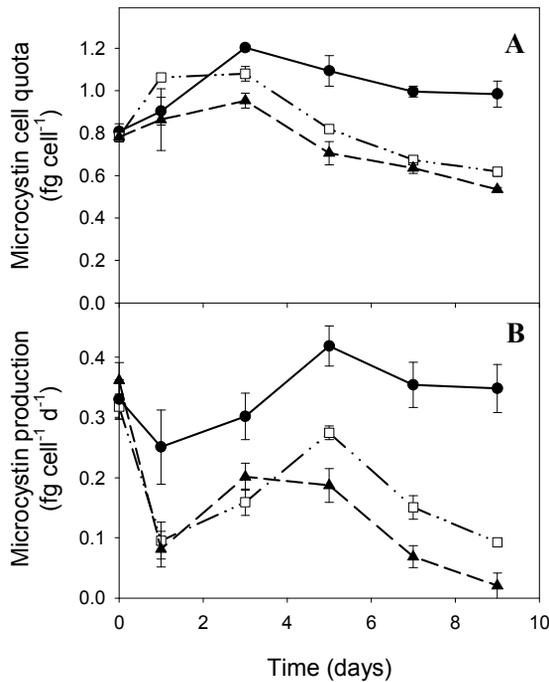


Figure 4: Time course of total microcystin cell quota (A) and microcystin production (B) during the salt-shock experiments (●, solid line: 10 g liter<sup>-1</sup>; □, dotted line: 15 g liter<sup>-1</sup>; ▲, dashed line: 17.5 g liter<sup>-1</sup>). Error bars denote standard deviation (N=3).

*Extracellular microcystin.* Extracellular microcystin concentrations measured at day 7 were 17%, 15% and 30% of the total microcystin concentration (intracellular + extracellular) in the cultures exposed to salinities of 10, 15 and 17.5 g liter<sup>-1</sup>, respectively.

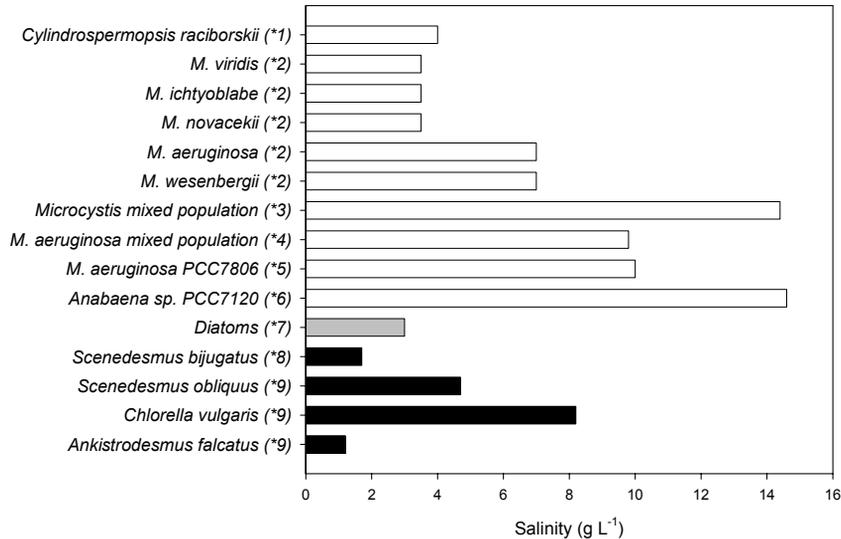


Figure 5: Salt tolerance in some freshwater cyanobacteria (white), green algae (black) and diatoms (grey). The data are based on the following references: 1, Moisaner et al. (2002); 2, Otsuka et al. (1999); 3, Verspagen et al. (2006); 4, Orr et al. (2004); 5, present study; 6, Rai & Tiwari (1999); 7, Wilson et al. (1994); 8, Mohapatra (1998); 9, Ahmed et al. (1984).

## DISCUSSION

The growth rate, microcystin cell quota, and microcystin production of the harmful cyanobacterium *Microcystis* PCC 7806 remained unaffected up to a salinity of 10 g liter<sup>-1</sup> (Fig. 1). These results from controlled semi-continuous turbidostats confirm earlier findings in batch cultures (Otsuka et al. 1999, Orr et al. 2004, Verspagen et al. 2006) that *Microcystis* has a high salt tolerance for a freshwater species. We investigated only a single *Microcystis* strain. Therefore, it is not clear to what extent our findings can be extrapolated to other *Microcystis* strains. However, Otsuka et al. (1999) reported a rather similar salt tolerance of 4 different *Microcystis aeruginosa* strains. Moreover, the observed salt tolerance is consistent with observations of *Microcystis* blooms in brackish waters, for example in the Patos Lagoon Estuary in Brazil (Matthiensen et al. 2000), the Oued Mellah reservoir, Morocco (Sabour et al. 2002), the Swan River Estuary, Australia (Robson & Hamilton 2003), San Francisco Bay, USA (Lehman et al. 2005), and in the Kucukcekmece Lagoon, Turkey (Albay et al. 2005). The growth rate was not stimulated by salinity in our experiments. Hence, our results do not support preliminary observations of Prinsloo and Pieterse (1994) that the growth rate of

*Microcystis* may actually benefit from a slightly brackish environment. Cell size was reduced above  $10 \text{ g liter}^{-1}$  (Fig. 1B), indicating that osmoregulation capacity was exceeded and cells were no longer able to uphold turgor, and started leaking and shrank when the salinity became too high. The reduction in cell size in response to osmotic stress is known as plasmolysis, and is also widely reported for other bacteria (Koch 1984, Csonka 1989, Shapiguzov et al. 2005). The combination of leakage and cell lysis most likely resulted in the high extracellular microcystin concentrations observed at salinities exceeding  $10 \text{ g liter}^{-1}$  (Fig. 2C). The growth rate and microcystin production rate collapsed when cells were exposed to salinities exceeding  $10 \text{ g liter}^{-1}$  for several weeks.

The salt-shock experiments show that *Microcystis* may temporarily withstand even higher salinities. The microcystin cell quota and microcystin production in the salt-shock experiment of  $10 \text{ g liter}^{-1}$  match the values found in the experiments with gradually rising salinity. When *Microcystis* was exposed to stronger salt shocks of 15 and  $17.5 \text{ g liter}^{-1}$ , the growth rates and microcystin production declined, but *Microcystis* was still able to grow and produce microcystins for at least a week. During this period the average cell size did not decrease, indicating that the surviving cells were still able to withstand the high turgor pressure. However, the extracellular microcystin concentration increased to 30% of the total microcystin concentration in the cultures exposed to a salinity of  $17.5 \text{ g liter}^{-1}$ . These findings are consistent with recent salt-shock studies with a mixed *Microcystis* population from the Swan River estuary in Western Australia, which revealed cell lysis and increased concentrations of extracellular microcystins when cells were exposed to a salinity of  $21 \text{ g liter}^{-1}$  (Orr et al. 2004).

What are the environmental implications? In the literature, surprisingly few data are available on the salt tolerance of freshwater phytoplankton species. A compilation of available data is shown in Figure 5. In line with earlier suggestions (Hart et al. 1991, Nielsen et al. 2003), these data show that many of the freshwater diatoms and green algae thus far investigated have a relatively low salt tolerance. Accordingly, cyanobacteria with a higher salt tolerance, like *Microcystis aeruginosa* and *Anabaena* sp., may profit from rising salinities in freshwater ecosystems by gaining a competitive advantage over other freshwater phytoplankton. Conversely, when estuaries face a sudden input of fresh water, *Microcystis* may gain an advantage over resident marine phytoplankton (Robson & Hamilton 2003). Current management evaluations consider whether several Dutch freshwater areas should be converted into brackish waters (e.g., Lake Volkerak; Verspagen et al. 2006). Our results indicate that these large-scale changes in water management may facilitate a competitive advantage of salt-tolerant cyanobacteria over salt-sensitive freshwater phytoplankton such as green algae and diatoms. Moreover, our results show that temporary exposure to salinities between 10 and  $20 \text{ g liter}^{-1}$  allows survival of *Microcystis* populations, while extracellular microcystin concentrations will increase. This implies that salinity fluctuations in brackish waters may not only favor *Microcystis* over other freshwater phytoplankton species, but may also increase the exposure of many aquatic organisms to elevated microcystin concentrations. In conclusion, a transition of freshwater ecosystems into brackish waters is unlikely to prevent

*Microcystis* blooms, unless salinities increase to sufficiently high levels at which *Microcystis* no longer survives (i.e., beyond 17.5 g liter<sup>-1</sup>). Increases in salinity in freshwater ecosystems that remain below this threshold are more likely to enhance the risk of *Microcystis* blooms and the exposure to elevated concentrations of microcystins.

*Acknowledgements.* We thank Prof. G.A. Codd for kindly providing their MC-LR gravimetric standard. The research of LT, PMV and JH was supported by the Earth and Life Sciences Foundation (ALW), which is subsidized by the Netherlands Organization for Scientific Research (NWO). LT and PMV were additionally supported by a European Union grant within the program PEPCY.

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## Chapter 3

THE MICROCYSTIN COMPOSITION OF THE  
CYANOBACTERIUM *PLANKTOTHRIX AGARDHII*  
CHANGES TOWARD A MORE TOXIC VARIANT  
WITH INCREASING LIGHT INTENSITY

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HUISMAN. (2005) APPLIED AND ENVIRONMENTAL MICROBIOLOGY  
VOL. 71, P. 5177-5181

*ABSTRACT* The cyanobacterium *Planktothrix agardhii*, which is dominant in many shallow eutrophic lakes, can produce hepatotoxic microcystins. Currently, more than 70 different microcystin variants have been described, which differ in their toxicity. In this study, the effect of photon irradiance on the production of different microcystin variants in *P. agardhii* was investigated using light-limited turbidostats. Both the amount of mRNA transcript of the *mcxA* gene and the total microcystin production rate increased with photon irradiance up to  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ , but started to decrease with irradiance greater than  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The cellular content of total microcystin remained constant, independent of irradiance. However, of the two main microcystin variants detected in *P. agardhii*, the [ $\text{Asp}^3$ ]microcystin-RR content decreased twofold with increased photon irradiance, whereas the [ $\text{Asp}^3$ ]microcystin-LR content increased threefold. Since [ $\text{Asp}^3$ ]microcystin-LR is considerably more toxic than [ $\text{Asp}^3$ ]microcystin-RR, this implies that *P. agardhii* becomes more toxic at high light intensities.

## INTRODUCTION

Cyanobacteria can produce a wide variety of toxic compounds, including microcystins (Botes et al. 1984, Carmichael 1997, Carmichael et al. 2001). Microcystins are cyclic heptapeptides that can cause liver damage through inhibition of protein phosphatases (MacKintosh et al. 1990) and may promote the development of liver tumors (Nishiwaki-Matsushima et al. 1992). So far, more than 70 different microcystin variants have been described (Codd et al. 2005). These microcystin (MC) variants may differ in their toxicological effect. For instance, LD-50 experiments with mice indicate that MC-LR is about four times more toxic than MC-RR (Harada 1996, Kotak et al. 1995).

Microcystin producing cyanobacteria usually generate multiple microcystin variants per strain (Sivonen and Jones 1999). Relatively few studies (Oh et al. 2000, Rapala et al. 1997) have investigated which factors determine the specific composition of microcystin variants. Yet, such studies are highly relevant given the dissimilar toxicities of the different microcystin variants. Basically, one *mcy* gene cluster is responsible for the synthesis of the molecular core of all microcystins in a given strain (Dittmann et al. 1997, Nishizawa 2000). The different microcystin variants are synthesized non-ribosomally, as variations on the same molecular core, catalyzed by a large multifunctional enzyme complex consisting of peptide synthetase and polyketide synthase modules (Börner and Dittmann 2005). The specificity of modules and various functional domains within this large enzyme complex determines the complement of microcystin variants that are produced (Neilan et al. 1999). How the synthesis and activity of the entire enzyme complex is regulated is not well understood.

Several studies have revealed that the total microcystin production of cyanobacteria may vary considerably with environmental factors (Nixdorf et al. 2003, Sivonen 1990, Watanabe and Oishi 1985). Previous work has shown that the total microcystin production in *Microcystis aeruginosa* responds to iron (Utkilen and Gjølme 1995), phosphorus (Oh et al. 2000), nitrogen (Long et al. 2001), and photon irradiance (Utkilen and Gjølme 1992, Wiedner et al. 2003). Orr and Jones (1998) and Long et al. (2001) hypothesized that the growth rate determines the microcystin production of cyanobacteria, which offers an explanation for the impact of so many environmental factors on microcystin production. Wiedner et al. (2003) however, showed that for *Microcystis* this relation indeed applied to light-limited conditions, but not to light-saturated conditions. Genetic regulation studies have shown an increased microcystin peptide synthetase and polyketide synthase gene transcription in *Microcystis* as a result of increasing light intensities (Kaebnick et al. 2000). Hence, we hypothesize that light is a major factor in microcystin production.

In the present study we aimed to investigate the effect of photon irradiance on the *mcyA* transcript, total microcystin production, and microcystin composition of the filamentous cyanobacterium *Planktothrix agardhii* Anagn. et Kom. We selected the *mcyA* gene as a representative of the microcystin biosynthesis gene cluster, to link expression of the *mcy* gene to total microcystin production as measured by HPLC.

The experiments were carried out in continuous cultures specifically tailored to study light limitation under highly controlled conditions (Huisman et al. 2002). *Planktothrix* was chosen because it is a widespread nuisance species (Fastner et al. 1999, Scheffer et al. 1997, Sivonen et al. 1990), particularly in shallow turbid lakes where light is a major limiting factor (Mur and Schreurs 1995, Nixdorf et al. 2003, Wiedner et al. 2002). Lakes dominated by *Planktothrix* have significantly higher concentrations of microcystin per unit cyanobacterial biomass than lakes dominated by other cyanobacterial species (Fastner et al. 1999). Yet, little is known about the impact of light on the microcystin production and composition of *Planktothrix*.

## MATERIAL & METHODS

*Organism and culture set-up.* *Planktothrix agardhii* strain 126/3 was kindly provided by Dr. K. Sivonen from the Department of Applied Chemistry and Microbiology, University of Helsinki (Sivonen 1990). Cultures were checked frequently for bacterial contamination by microscope. All experiments were performed in continuous cultures with flat culture vessels specifically designed to study light-limited growth (Huisman et al. 2002, Matthijs et al. 1996), using a working volume of 1.85 liters. Temperature was kept constant at  $23 \pm 1$  °C by means of a cooling element placed between the culture vessel and the light source. A continuous flow of nutrient-saturated O<sub>2</sub>-medium (Van Liere and Mur 1978) was added to the culture to prevent nutrient limitation. Furthermore, the culture was aerated by a continuous airflow to ensure homogeneous mixing and to provide sufficient amounts of carbon dioxide for photosynthesis. The turbidostat technique was used. The pump was adjusted to keep the optical density (750 nm) constant at 0.10–0.15 cm<sup>-1</sup>.

*Light conditions.* The light source consisted of four white fluorescent tubes (Philips PL-L/24W/840/4P) placed in front of the culture vessel. A day-night cycle consisting of 12 h of light and 12 h of darkness was used, while all other factors were kept constant. Photon irradiance was measured using a LI-COR SA 198 quantum sensor.

The specific design of the flat culture vessels allowed accurate estimation of the average photon irradiance (Huisman et al. 2002). The incident irradiance ( $I_{in}$ ) and the irradiance penetrating through the vessel ( $I_{out}$ ) were measured at 10 evenly spread points on the front and back surface of the culture vessel, respectively. The depth-averaged photon irradiance in the culture vessel was calculated as follows:  $I_{avg} = (I_{in} - I_{out}) / (\ln I_{in} - \ln I_{out})$ .

*Experiments and sampling.* We studied the microcystin production of *P. agardhii* using three time scales. First, we investigated short-term changes in microcystin composition in two diurnal experiments with a cycle consisting of 12 h light and 12 h dark. In one experiment we used low light (depth-averaged irradiance of  $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), and in the other experiment we used high light ( $110 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) during the light period. Second, on a time scale of days, we studied the transient dynamics of microcystin composition in cultures that were transferred from 25 to  $65 \mu\text{mol m}^{-2} \text{s}^{-1}$  and from 110 to  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$  during the light period. Third, on an even longer time scale, we performed a series of steady-state experiments with depth-averaged photon irradiances of 6, 9, 17, 25, 36, 48, 74, 79, and  $112 \mu\text{mol m}^{-2} \text{s}^{-1}$  during the light period. The optical density of 750 nm was monitored on a daily basis. When the optical density remained constant ( $\pm 15\%$ ) for more than a week, the culture was considered to be in steady state. During a steady state, samples were taken daily for five days. Samples were always taken one hour after the light was switched on. Each sample was divided in two subsamples for the estimation of biovolume and microcystin analysis.

*Microcystin analysis.* Intracellular microcystin contents were analyzed by filtering 10 ml of the culture suspension in triplicate using Whatman GF/C filters (pore size  $\sim 1.2 \mu\text{m}$ ). Filters were freeze dried and stored at  $-20^\circ\text{C}$ . Microcystin was extracted in 75% MeOH (three extraction rounds) according to Fastner et al. (1998) with an extra step for grinding of the filters in a Mini Beadbeater (Biospec Products, Bartlesville Okla) with 0.5 mm silica beads. Dried extracts were stored at  $-20^\circ\text{C}$  and dissolved in 50% MeOH for analysis of microcystin using high performance liquid chromatography (HPLC) with photodiode array detection (Kontron instruments). The extracts were separated using a 30 to 70% acetonitrile gradient with 0.05% trifluoroacetic acid at a flow of  $1 \text{ ml min}^{-1}$  and a LiChrospher 100 ODS  $5 \mu\text{m}$  LiChorCART 250-4 cartridge system (Merck). The different microcystin variants were identified on the basis of their characteristic UV-spectra and quantified by means of a MC-LR gravimetric standard provided by the University of Dundee.

The extracellular microcystin concentrations were below the detection limit of the HPLC (2.5 ng of microcystin). Therefore, extracellular microcystin concentrations were determined using a Enzyme-Linked Immuno Sorbent Assay (ELISA), according to the protocol of the Microcystin Plate Kit (EnviroLogix Inc. Catalog No. EP 022). Since the ELISA approach does not distinguish between different microcystin variants, we were unable to quantify the composition of the extracellular microcystins.

*Biovolume and growth rate.* The lengths of the filaments were measured using Image Analysis (Qwin, Leica microsystems) from samples stored in Lugol's iodine. The average diameter of *P. agardhii* filaments was estimated to be  $3.3 \mu\text{m}$  (s.d. = 0.3;  $N = 147$ ). The biovolume of the filaments was calculated as  $BV = L\pi r^2$ , where  $L$  is filament length and  $r$  is the filament diameter divided by two (radius).

The specific growth rate,  $\mu$ , was calculated according to the following equation:

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} + D \quad (1)$$

where  $x_1$  and  $x_2$  are the estimated biomasses (measured as biovolume) at times  $t_1$  and  $t_2$ , respectively, and  $D$  is the dilution rate (in  $\text{h}^{-1}$ ). The microcystin production rate was calculated by multiplying the microcystin content by the specific growth rate.

*RNA extraction and quantification.* Samples (25 ml) were taken from four continuous cultures in steady state, grown at different photon irradiance. These samples (3 per continuous culture) were added to tubes filled up to 25 ml with ice. The tubes were centrifuged, and pellets were frozen with liquid nitrogen and stored at  $-20^\circ \text{C}$  until RNA extraction. Total RNA was extracted similar to Kaebnick et al. (2000) using Trizol reagent (Gibco BRL, Life Technologies, Rockville, Md.) after crushing the pellet in a precooled mortar. Phenol extraction and precipitation were performed according to the manufacturer's protocol. RNA was quantified using northern analysis.

*mRNA protection assay (RPA).* Primers McyA-Cd rR (5'-AAAAGTGTTTT ATTAGCGGCTCAT-3') and McyA-Cd rF (5'-AAAATTAAAAGCCGTATCAAA-3') were used to amplify a 300 bp fragment of the *mcyA* gene of *P. agardhii* (Christiansen et al 2003) as described by Hisbergues et al. (2003). This fragment was ligated into the pGEM-T cloning vector (Promega). The probe for the RPA was prepared by in vitro transcription (Maxiscript, Ambion, Austin, Texas) and labeled with [ $\alpha$ - $^{32}\text{P}$ ] UTP. The RPA was performed similar to Kaebnick et al. (2000) according to the manufacturer's instructions (Boehringer). The probe was mixed with different amounts of mRNA standardized by 16S RNA gel electrophoresis. The transcripts were analyzed by polyacrylamide gel electrophoresis, using 12.5% Gel 40 and 16.8 gr Urea in a total volume of 40 ml combined with a Tris-borate-EDTA buffer. The gel was run for 2.5 hours at 30 mA, and subsequently exposed to X-ray film overnight at  $-80^\circ \text{C}$ . The pixel densities of the bands were estimated with image analysis software (Qwin, Leica microsystems). The background density was subtracted from the density of the actual band itself, and the highest pixel density was set at 100%.

*Data analysis.* The data were analyzed by means of stepwise multiple regression, using polynomials to estimate higher-order terms. Higher-order terms were added only if they improved the fit significantly at the 0.05 level.

## RESULTS

*Diurnal and transient dynamics.* The microcystins of *P. agardhii* consisted mainly of [Asp<sup>3</sup>]MC-RR and lower concentrations of [Asp<sup>3</sup>]MC-LR. Traces of other microcystin variants were observed but their concentrations barely exceeded the detection level. The [Asp<sup>3</sup>]MC-RR and [Asp<sup>3</sup>]MC-LR content showed no distinct diurnal fluctuations, despite the imposed day-night cycles (Fig. 1A). In the transient-state experiments, where cultures were transferred from low light to high light, and vice versa, the [Asp<sup>3</sup>]MC-RR and [Asp<sup>3</sup>]MC-LR contents adjusted to the new light conditions within a time span of 6 to 8 days (Fig. 1B,C). This shows that the relevant time scale for changes in microcystin composition is in the order of several days.

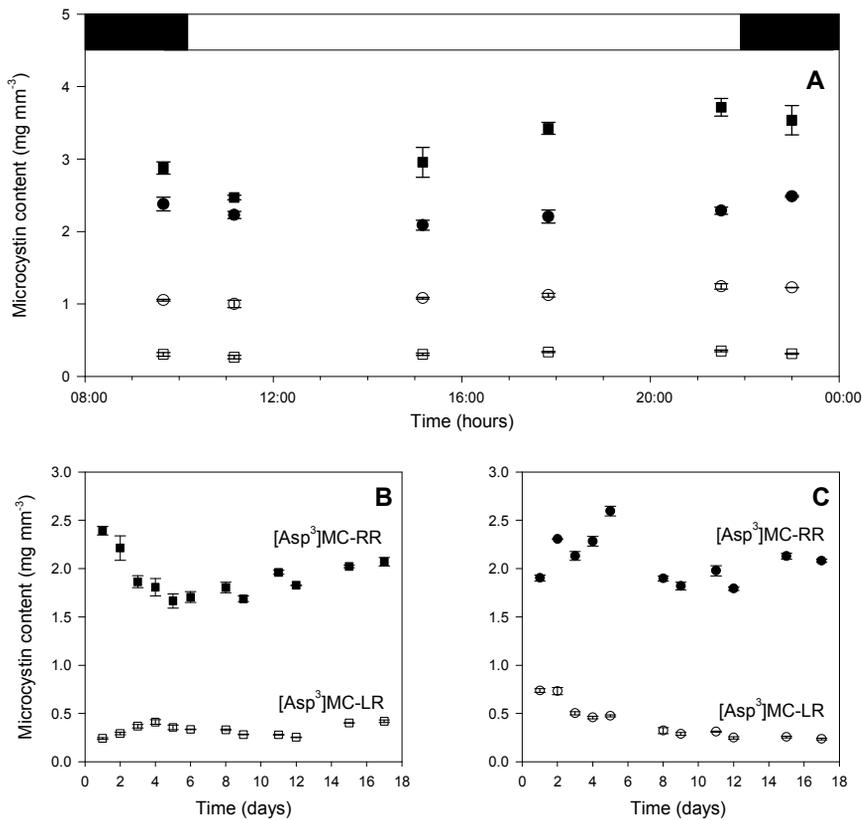


Figure 1: Time scales of microcystin dynamics. (A) Diurnal changes in microcystin contents at two different photon irradiances ([Asp<sup>3</sup>]MC-RR 25 μmol m<sup>-2</sup> s<sup>-1</sup> = ■, [Asp<sup>3</sup>]MC-LR 25 μmol m<sup>-2</sup> s<sup>-1</sup> = □, [Asp<sup>3</sup>]MC-RR 110 μmol m<sup>-2</sup> s<sup>-1</sup> = ● and [Asp<sup>3</sup>]MC-LR 110 μmol m<sup>-2</sup> s<sup>-1</sup> = ○). (B,C) Changes in [Asp<sup>3</sup>]MC-RR content (black) and [Asp<sup>3</sup>]MC-LR content (white) during transient states (B) from 25 to 65 μmol m<sup>-2</sup> s<sup>-1</sup> and (C) from 110 to 40 μmol m<sup>-2</sup> s<sup>-1</sup>. Error bars indicate standard deviation (N = 3).

*Growth rates.* The specific growth rate of *P. agardhii* strain 126/3, as measured during steady-state conditions in continuous cultures, is a unimodal function of photon irradiance (Fig. 2; multiple regression:  $\mu = -0.056 + 0.016 I - 1 \cdot 10^{-4} I^2$ ;  $R^2 = 0.97$ ,  $N = 9$ ,  $P < 0.01$ ). The specific growth rate was limited by photon irradiance at least until  $\sim 60 \mu\text{mol m}^{-2} \text{s}^{-1}$ , whereas beyond  $\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$  growth seemed to be inhibited by photon irradiance.

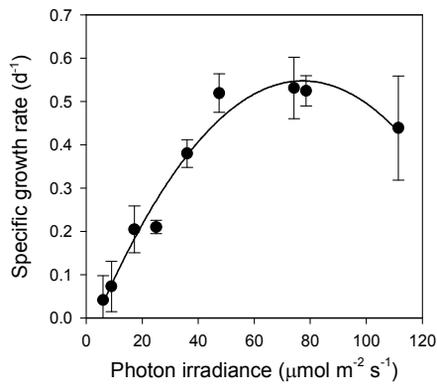


Figure 2: Specific growth rate of *P. agardhii* grown under light-limited conditions as a function of photon irradiance. Solid line is based on multiple regression. Error bars indicate standard deviation ( $N = 5$ ).

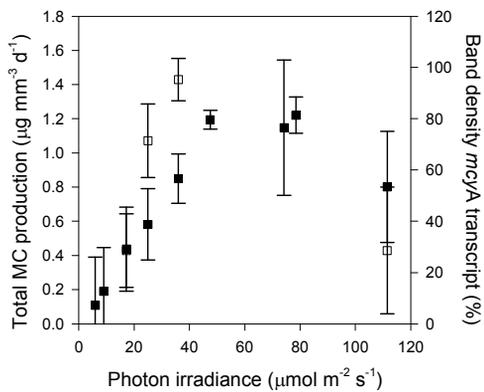


Figure 3: Total microcystin production (■) and the amount of *mcyA* transcript (□) of *P. agardhii* plotted against photon irradiance. Error bars indicate standard deviation ( $N = 5$ ).

*mRNA transcripts and microcystin production.* Samples were taken from four of the steady-state cultures in order to perform mRNA protection assays to estimate the effect of different light intensities on the amount of *mcyA* transcript. This experiment revealed a pattern similar to the specific growth rate pattern. The transcriptional response of the *mcyA* gene increased with photon irradiance up to  $\sim 60 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Fig. 3). Beyond  $\sim 100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  the *mcyA* gene produced less mRNA transcript. This pattern was further matched by the total microcystin production, which also increased with photon irradiance up to  $\sim 60 \mu\text{mol m}^{-2} \text{s}^{-1}$  and decreased at  $\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Fig. 3).

*Microcystin composition.* In all experiments, the extracellular microcystin concentration was less than 1% of the total microcystin concentration in the cultures. Thus, almost all microcystin was intracellular. The total microcystin content of *P. agardhii* in the steady-state experiments did not change significantly with photon irradiance (Fig. 4; linear regression:  $R^2 = 0.39$ ,  $N = 9$ ,  $P > 0.05$ ). However, the composition of the microcystin variants did change with photon irradiance. The  $[Asp^3]MC-RR$  content decreased with photon irradiance (Fig. 4; linear regression:  $[Asp^3]MC-RR = 2.37 - 0.008 I$ ;  $R^2 = 0.81$ ,  $N = 9$ ,  $P < 0.01$ ), whereas the  $[Asp^3]MC-LR$  content increased with photon irradiance (Fig. 4; linear regression:  $[Asp^3]MC-LR = 0.162 + 0.004 I$ ;  $R^2 = 0.93$ ,  $N = 9$ ,  $P < 0.01$ ). As a result, the ratio of  $[Asp^3]MC-LR$  over  $[Asp^3]MC-RR$  increased more than 6-fold with photon irradiance, as accurately described by a linear relation (linear regression:  $LR/RR \text{ ratio} = 0.044 + 0.0034 I$ ;  $R^2 = 0.97$ ,  $N = 9$ ,  $P < 0.001$ ).

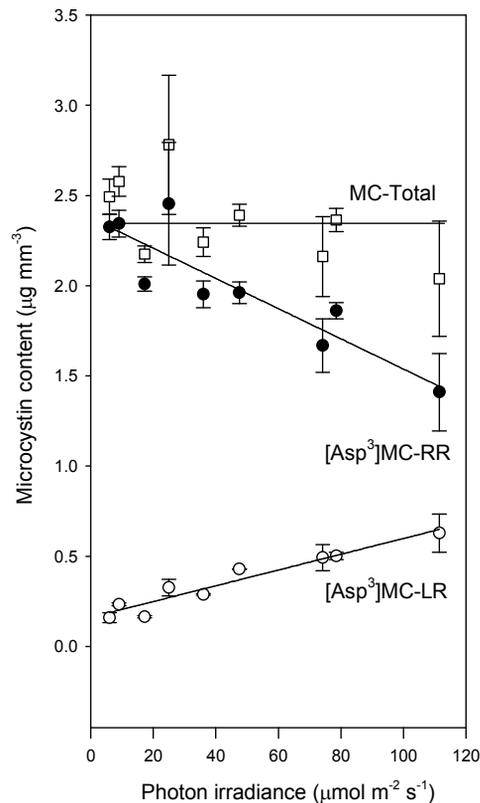


Figure 4: Total microcystin content ( $\square$ ),  $[Asp^3]MC-RR$  content ( $\bullet$ ) and  $[Asp^3]MC-LR$  content ( $\circ$ ) of *P. agardhii* as a function of photon irradiance. Error bars indicate standard deviation ( $N = 5$ ). Solid lines  $[Asp^3]MC-RR$  and  $[Asp^3]MC-LR$  are based on linear regression.

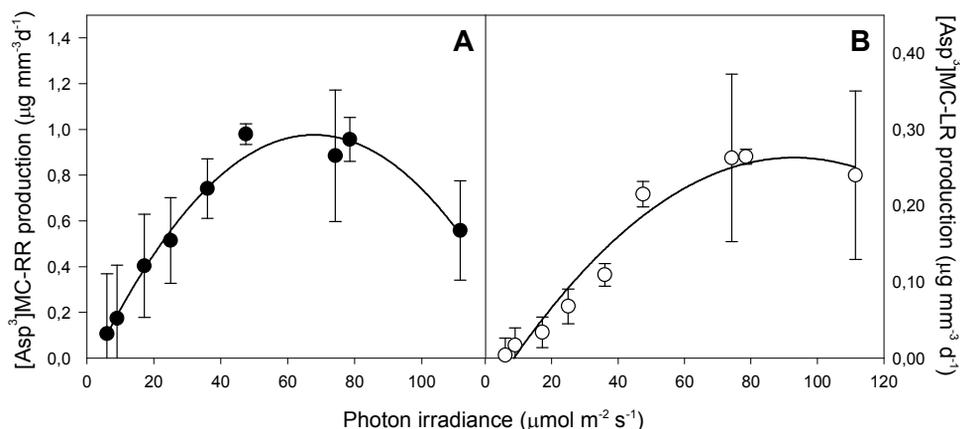


Figure 5: Production of [Asp<sup>3</sup>]MC-RR (A) and [Asp<sup>3</sup>]MC-LR (B) in *P. agardhii* as a function of photon irradiance. Error bars indicate standard deviation (N=5). Solid lines are based on multiple regression.

The [Asp<sup>3</sup>]MC-RR production was a unimodal function of photon irradiance, with a maximum production at a photon irradiance in the range 50–80  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (Fig. 5A; multiple regression:  $P_{[\text{Asp}^3]\text{MC-RR}} = -0.076 + 0.031 I - 2 \cdot 10^{-4} I^2$ ;  $R^2 = 0.98$ ,  $N = 9$ ,  $P < 0.01$ ). The production of [Asp<sup>3</sup>]MC-LR increased but saturated with increasing photon irradiance (Fig. 5B; multiple regression:  $P_{[\text{Asp}^3]\text{MC-LR}} = -0.057 + 0.007 I - 2 \cdot 10^{-6} I^2$ ;  $R^2 = 0.96$ ,  $N = 9$ ,  $P < 0.01$ ).

## DISCUSSION

Our results reveal a direct relationship between the transcription rate of the *mcyA* gene and the total microcystin production of *P. agardhii* (Fig. 3). Both gene transcription and total microcystin production increased with increasing photon irradiance up to  $\sim 60 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Beyond  $\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$  both rates decreased with photon irradiance. This result confirms earlier findings with *Microcystis* that light exerts a major control over the transcription of the *mcy* gene complex (Kaebernick et al. 2000). The close correspondence between *mcyA* transcription and microcystin production is consistent with earlier findings (Dittmann et al. 1997, Nishizawa et al. 1999, 2000) that one *mcy* gene cluster is responsible for the synthesis of the core of all microcystin variants.

Our observation that an isolated strain of *Planktothrix* produces different structural variants of microcystin can be explained by the multispecificity of single domains of the microcystin biosynthesis complex. In particular, the first module of McyB can incorporate a variety of different amino acids at the variable X position within the microcystin structure (Christiansen et al. 2003). At this variable position, [Asp<sup>3</sup>]microcystin-RR contains arginine whereas [Asp<sup>3</sup>]microcystin-LR contains leucine. Strikingly, our results show that the composition of microcystin variants may change substantially with light conditions. The content of [Asp<sup>3</sup>]MC-RR decreased with photon irradiance, whereas the content of [Asp<sup>3</sup>]MC-LR increased (Fig. 4). Transient-state experiments confirmed these results (Fig. 1B,C). Why does *Planktothrix* produce less arginine-based microcystins but more leucine-based microcystins at higher irradiances? Perhaps a conformational change in the substrate-binding pocket of the first module of the McyB enzyme could lead to a change in the substrate specificity of the module. Biochemical assays with the McyB enzyme could elucidate this possibility. A plausible alternative explanation is that different light conditions induce changes in the composition of available amino acids. Increased photosynthesis at a high light intensity may raise the C:N ratio of the cells. This would favor leucine over arginine, since relatively less nitrogen would be available to synthesize the nitrogen-rich arginine molecule, resulting in a shift in microcystin synthesis from [Asp<sup>3</sup>]MC-RR to [Asp<sup>3</sup>]MC-LR. This hypothesis can be tested by measuring the amino acid concentrations in cells grown at different levels of photon irradiance.

Previous studies with *Microcystis aeruginosa* PCC 7806 showed that its total microcystin content increased with irradiance under light-limited conditions (Wiedner et al. 2003). *Microcystis aeruginosa* mainly contains the microcystin variants microcystin-LR and [Asp<sup>3</sup>]microcystin-LR. Our findings with *Planktothrix* revealed patterns similar to the *Microcystis* patterns for [Asp<sup>3</sup>]MC-LR, as the [Asp<sup>3</sup>]MC-LR content of *Planktothrix* increased with increased irradiance. However, in *Planktothrix* the increase in [Asp<sup>3</sup>]MC-LR was accompanied by a decrease in [Asp<sup>3</sup>]MC-RR. As a result, in contrast to *Microcystis*, the total microcystin content of *P. agardhii* remained constant while the ratio of [Asp<sup>3</sup>]microcystin-LR to [Asp<sup>3</sup>]microcystin-RR increased more than sixfold with increased irradiance. According to mouse bioassays, [Asp<sup>3</sup>]MC-LR is at least four times more toxic than [Asp<sup>3</sup>]MC-RR (Harada 1996, Kotak et al. 1995). Thus, while the total microcystin content of *P. agardhii* was not affected by light, the toxicity of *P. agardhii* actually increased with increased photon irradiance.

Lakes dominated by microcystin-producing cyanobacteria may show considerable seasonal variability in total microcystin concentrations (Briand et al. 2002, Fastner et al. 1999, Kardinaal and Visser 2005). It could be suggested that the observed variability results from physiological changes in the total microcystin content of *P. agardhii* (for instance, changes induced by changing light conditions). However, our results do not support this hypothesis, as photon irradiance had no significant influence on the total microcystin content of *P. agardhii*. Likewise, the diurnal experiments showed that short-term changes in photon irradiance have little effect on total microcystin content. It is more likely that the variability in total microcystin

concentration commonly observed in *Planktothrix*-dominated lakes results from a succession of closely related *Planktothrix* genotypes that differ in microcystin production. This is consistent with recent findings of Kurmayer et al. (2004) on the coexistence of toxic and non-toxic *P. agardhii* strains in shallow lakes. However, our results show that monitoring of the different strains of *P. agardhii* is not sufficient to predict the toxicity of a *P. agardhii* bloom. Even the toxicity of a single *P. agardhii* strain is quite variable, since the composition of intracellular microcystin variants may change in response to environmental conditions.

In conclusion, *P. agardhii* is a widespread cyanobacterium of shallow, turbid lakes. Light conditions in shallow lakes may change on a time scale of days to weeks due to changes in cloudiness or wind-induced resuspension of sediments. Our findings show that such changes in light conditions may profoundly affect the microcystin composition and thereby the toxicity of *P. agardhii*. The harmful cyanobacterium *Planktothrix agardhii* produces a more toxic variant during periods of sunny weather, when recreational activities in lakes are most attractive.

*Acknowledgements* This study was supported by grants from the European Union within the programmes CYANOTOX, TOPIC, and PEPCY. The investigations of PMV and JH were supported by the Earth and Life Science Foundation (ALW), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

We thank Kaarina Sivonen for providing the *Planktothrix agardhii* strain used in this study, and Geoff Codd and co-workers of the University of Dundee for providing the MC-LR gravimetric standard. Furthermore, we are grateful to Jan-Christoph Kehr and Michael Hisbergues for their help with the mRNA protection assay, to Eugenia Sampayo-Garrido and Mariska Weijerman for their contributions to the experimental work, and to Hans Matthijs, Jolanda Verspagen and Edwin Kardinaal for discussions on the topic.

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# Chapter 4

## AMINO ACID AVAILABILITY DETERMINES THE COMPOSITION OF MICROCYSTIN VARIANTS IN THE CYANOBACTERIUM *PLANKTOTHRIX AGARDHII*

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PETRA M. VISSER. SUBMITTED TO APPLIED AND ENVIRONMENTAL MICROBIOLOGY

*ABSTRACT* Cyanobacteria are capable of producing multiple microcystin variants simultaneously. The mechanisms that determine the composition of microcystin variants in cyanobacteria are still debated. [Asp<sup>3</sup>]Microcystin-RR (abbreviated as RR) contains arginine (R) at the position where the more toxic [Asp<sup>3</sup>]microcystin-LR (LR) incorporates leucine (L). We cultured the filamentous cyanobacterium *Planktothrix agardhii* strain 126/3 with and without external addition of the amino acids L and R. Addition of L to the growth medium resulted in a strong increase of the microcystin LR/RR ratio, while addition of R resulted in a decrease of the microcystin LR/RR ratio. This demonstrates that, the availability of these amino acids is a major factor in the synthesis of different microcystin variants. Environmental changes affecting cell metabolism may render differences in the intracellular availability of L and R, which can affect the production of microcystin-LR and/or RR. Since L contains one N atom while R contains four N atoms, we hypothesized that low nitrogen availability might shift the amino acid composition in favor of L. However, when a continuous culture of *P. agardhii* was shifted from nitrogen-saturated to nitrogen-limited mineral medium, the cellular concentrations of both L and R decreased, but the L/R ratio did not change in favor of L. Accordingly, while the total microcystin concentration of the cells decreased, we did not observe changes in the microcystin LR/RR ratio in response to nitrogen limitation. How interactive effects between nitrogen limitation and light limitation will affect the microcystin LR/RR ratio remains an interesting open question.

## INTRODUCTION

Cyanobacteria produce a wide variety of bioactive compounds, including the hepatotoxic microcystins. Many cyanobacteria are capable of producing multiple microcystin (MC) variants simultaneously. Currently, more than 70 microcystin variants have been described that differ considerably in their toxicity (Harada 1996, Sivonen and Jones 1999, Codd et al. 2005). Factors that affect the total microcystin production of cyanobacteria have been studied extensively, and include light intensity (Watanabe and Oishi 1985, Wiedner et al. 2003), temperature (Watanabe and Oishi 1985), nitrogen (Watanabe and Oishi 1985, Orr and Jones 1998, Long et al. 2001), phosphorus (Watanabe and Oishi 1985, Oh et al. 2000), and iron (Utkilen and Gjørlme 1995).

Only a few studies have focused on the production of different microcystin variants, and their relative abundances. These studies show that the cellular composition of microcystin variants may change in response to changing environmental conditions. For instance, the relative abundances of different microcystin variants are affected by temperature in *Anabaena* 90 (Rapala et al. 1997) and by light intensity and nutrient supply in *Microcystis aeruginosa* HUB 5-2-4 (Hesse and Kohl 2001). Recent experiments with the filamentous cyanobacterium *Planktothrix agardhii* have shown that the prevalence of the two microcystin variants [Asp<sup>3</sup>]MC-LR and [Asp<sup>3</sup>]MC-RR (abbreviated as microcystin-LR and microcystin-RR, respectively) changed as a function of photon irradiance (Tonk et al. 2005). Microcystin-LR molecules contain leucine (L) at the position where microcystin-RR contains arginine (R). An increased cellular content of microcystin-LR in high light coincided with a decreased content of the RR variant, resulting in a change in the microcystin LR/RR ratio from 0.1 to 0.4 (Fig. 1). Since mouse assays indicate that the microcystin-LR variant is four times more toxic than its RR counterpart, this change is of significance and may have ecological implications (Kotak et al. 1995, Harada 1996).

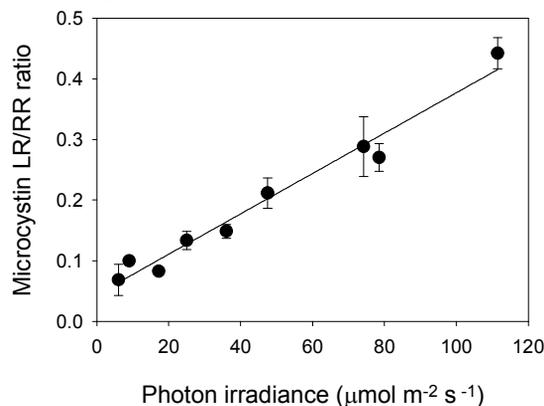


Figure 1: Microcystin LR/RR ratio of *Planktothrix agardhii* versus photon irradiance. Solid line is based on linear regression. Error bars denote standard deviation ( $n = 5$ ). Modified from Tonk et al. (2005).

The molecular mechanisms that direct changes in the pattern of synthesis and the composition of microcystin variants have not yet been resolved (Rapala et al. 1997, Tonk et al. 2005). Although microcystins are polypeptides, their synthesis does not involve the ribosomal protein synthesis machinery. Instead, an elaborate chain of enzymes functions as synthetase for microcystins (Dittmann et al. 1997, Nishizawa et al. 1999, 2000, Tillet et al. 2000). The enzymatic sites of the microcystin synthetase complex are arranged in a modular way (Dittmann et al. 1997, Marahiel et al. 1997). The order of the modules together with the number and type of catalytic domains present, determines the structure of the resulting polyketide (polypeptide) product (Tillet et al. 2000). The production of different structural variants of microcystin can be explained by the multi-specificity of domains of the microcystin biosynthesis complex (Neilan et al. 1999). The first module of the McyB enzyme of *Planktothrix agardhii* contains a binding pocket which can incorporate a variety of different amino acids at the variable X position within the microcystin structure (Christiansen et al. 2003). Questions remain about the factors that control the relative abundance of different microcystin variants within a single cyanobacterium.

Several mechanisms have been proposed that might steer the relative abundances of microcystin variants at the level of synthesis (Tonk et al. 2005). A conformational change of the substrate-binding pocket at the first module of the McyB enzyme could lead to a change in the substrate specificity of the module. Alternatively, changing environmental conditions (e.g., light, nutrients, temperature) could lead to differences in the availability of amino acids that can be used for incorporation during microcystin synthesis.

Here, we hypothesize that the relative availability of different amino acids directs the specificity of microcystin synthesis. Cyanobacteria possess mechanisms for the direct uptake of amino acids such as leucine (L) and arginine (R) from their environment (Herrero and Flores 1990, Kamjunke and Jahnichen 2000). L incorporation in *Microcystis aeruginosa* cells is known to increase with increasing L concentration in the medium (Kamjunke and Jahnichen 2000). Hence, our first hypothesis is that addition of L will increase the microcystin LR/RR ratio, while addition of R will decrease the microcystin LR/RR ratio. Furthermore, R is a very nitrogen-rich amino acid, since it has 4 N atoms instead of the 1 N atom present in most other amino acids including L. Upon nitrogen limitation the synthesis of R likely decreases to a minimum due to the low abundance of ammonium ions needed for incorporation in amino acid synthesis. Thus, our second hypothesis is that nitrogen limitation will increase the microcystin LR/RR ratio.

In this study, we tested the first hypothesis that amino acid availability determines the composition of microcystin variants, by adding the amino acids L and R to the growth medium of *P. agardhii*. Since earlier studies had shown that the microcystin LR/RR ratio depends on the light conditions (Tonk et al. 2005), we performed these addition experiments at both low and high light intensities. To investigate the second hypothesis, we examined whether a shift from nitrogen saturation to nitrogen limitation in a chemostat would change the prevalence of available amino acids in *P. agardhii* cells, and thereby would change the microcystin LR/RR ratio.

## MATERIAL &amp; METHODS

*Organism.* *Planktothrix agardhii* strain 126/3 was provided by the Division of Microbiology, University of Helsinki. The strain was grown in a nutrient-rich mineral medium originally defined for growth of freshwater *Oscillatoria* species, and known as O<sub>2</sub> medium (van Liere and Mur 1978)

*Semi-continuous turbidostats.* *P. agardhii* was grown in semi-continuous cultures, using a turbidostat approach. The optical density (750 nm) was kept constant between 0.1 and 0.2 cm<sup>-1</sup> by diluting the culture with O<sub>2</sub> medium once every two days. Flat culture vessels were used with a working volume of 400 ml and constant aeration with compressed filter-sterilized air (0.2 µm membrane, Millipore) to ensure homogeneous mixing and to provide sufficient amounts of carbon dioxide. Temperature was maintained at 22°C (± 1°C) by leading the compressed air used for aeration through a temperature-controlled water bath (Colora, thermocryostat). In addition, a ventilator was used to diffuse the warmth emitted by the light source. Light was provided by white fluorescent tubes (Philips PL-L 24W/840/4P), directed towards the front surface of the culture vessel. Incident irradiance ( $I_{in}$ ) and outgoing irradiance ( $I_{out}$ ) were measured with a LI-COR LI-250 quantum photometer at 7 points on the front surface and back surface of the culture vessel, respectively. The average photon irradiance in the culture vessel was calculated according to the following equation (Huisman et al. 2002):

$$I_{avg} = (I_{in} - I_{out}) / (\ln I_{in} - \ln I_{out}) \quad (1)$$

We applied a cycle of 12 h light and 12 h darkness, with an incident irradiance of  $I_{in} = 26 \pm 0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$  ( $I_{avg} = 21 \pm 1 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for the low light cultures, and  $I_{in} = 104 \pm 0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$  for the high light cultures ( $I_{avg} = 84 \pm 4 \mu\text{mol m}^{-2} \text{s}^{-1}$ ).

Experiments were performed in triplicate. After two weeks of acclimation to the imposed light conditions, the triplicates were split: 10 mM L-leucine was added to one culture vessel, 10 mM L-arginine was added to the second culture vessel, while the third culture vessel served as a control.

*Chemostat experiment.* *P. agardhii* was grown in continuous culture using a flat culture vessel with a working volume of 1.85 liters in combination with constant aeration of filtered and moistened air to ensure homogeneous mixing and to provide sufficient amounts of inorganic carbon (Matthijs et al. 1996, Huisman et al. 2002). We used a chemostat approach, in which the dilution rate was fixed at  $D = 0.34 \text{ d}^{-1}$ . Temperature was kept constant at 21°C (± 1°C) by means of a transparent water jacket, connected to a Colora thermocryostat, that was placed between the light source and the culture vessel. The incident irradiance was  $I_{in} = 38 \pm 4 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The average irradiance was calculated according to Eqn.1, using 10 points on the front surface and back surface of the culture vessel. A 12 h light: 12 h dark cycle was used.

After reaching a steady state, the N-saturated O<sub>2</sub> medium (6 mM nitrate) was replaced by N-limited O<sub>2</sub> medium with a nitrate concentration of 200 μM. The changes in culture properties were recorded until full N depletion of the cells was reached.

*Sampling.* Samples were taken from the semi-continuous cultures at Day 0 (before addition of L and R) and at Day 1, 3 and 5 (after addition of L and R). The continuous culture was sampled during the N-saturated steady state (Day 1-5), and every other day during the transient state caused by the onset of N-limitation (Day 7-23). Samples were always taken 1 hour after the light was switched on. Aliquots of all samples were analyzed in triplicate for intracellular microcystin content, biovolume and C/N concentration.

*Microcystin analysis.* For intracellular microcystin analysis 10 ml of the culture suspension was filtered in triplicate using Whatman GF/C filters (pore size ~1.2 μm). Filters were freeze dried and stored at -20°C. Microcystin was extracted in 75% MeOH (three extraction rounds) according to Fastner et al. (1998) with an extra step for grinding of the filters in a Mini Beadbeater (Biospec products, Bartlesville, Okla.) with 0.5 mm silica beads (Tonk et al. 2005). Dried extracts were stored at -20°C and dissolved in 50% MeOH for analysis of microcystin using high performance liquid chromatography (HPLC) with photodiode array detection (Kontron instruments). The extracts were separated using a LiChrospher 100 ODS 5 μm LiChorCART 250-4 cartridge system (Merck) and a 30 to 70% acetonitrile gradient in water with 0.05% trifluoroacetic acid at a flow rate of 1 ml min<sup>-1</sup>. The different microcystin variants were identified based on their characteristic UV-spectra and quantified by means of a MC-LR gravimetric standard provided by the University of Dundee. The extracellular microcystin concentrations were below the detection limit of the HPLC.

*Biovolume.* Biovolume was measured using a Casy counter (Casy 1 TTC, Schärfe System with a 150 μm capillary). The specific growth rate, μ, was calculated according to the following equation:

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} + D, \quad (2)$$

where  $x_1$  and  $x_2$  represent biovolumes of the cultures at times  $t_1$  and  $t_2$  respectively, and  $D$  represents the dilution rate of the culture.

*Analysis of C and N content.* For determination of the intracellular carbon and nitrogen content 10 ml of the culture suspension was concentrated over a 0.45 μm HA membrane filter (Millipore) in triplicate. The residue on the filters was collected into 2 ml Eppendorf tubes, these were centrifuged (5 min, 15000 rpm), and pellets were stored at -20°C and subsequently freeze dried. Carbon and nitrogen content was quantitatively analyzed in 70 μg of the freeze dried cell powder using a Vario EL Elemental Analyzer (Elementar Analysensysteme GmbH).

*Amino acid analysis.* The continuous culture was sampled at Day 8 (at the onset of N limitation) and at Day 22 to Day 29 (during N limitation), for analysis of the total amino acid composition of the cells. Samples, containing 40 ml of culture material, were centrifuged and freeze dried. In order to split the proteins into constituting amino acids  $\pm$  8 mg of each freeze-dried sample was hydrolyzed in 6 M HCl for 24 hours at 105-110°C. Separation of the amino acids was carried out with an amino acid analyzer (Biochrom Alpha II Plus, Cambridge), which separated the amino acids by differential binding to a column filled with a weak cation exchanger bound to a solid matrix; subsequent elution was based on citric acid to which an increasing amount of lithium hydroxide was added. Detection and quantification of the amino acids was based on post-column derivatization with Ninhydrin at 570 nm (primary amines) or 440 nm (secondary amines).

*Data analysis.* Independent samples *t*-tests were performed, using SPSS version 11, to detect significant differences in microcystin contents between two treatments (i.e., low versus high light).

## RESULTS

*Effects of light intensity in semi-continuous turbidostats.* The specific growth rates of *P. agardhii* grown in nutrient-saturated mineral medium were  $\mu = 0.25 \text{ d}^{-1}$  (sd = 0.06; n = 3) and  $\mu = 0.37 \text{ d}^{-1}$  (sd = 0.11; n = 3) for low light and high light conditions, respectively. In the control cultures, the average microcystin-RR content was significantly lower at high light intensity than at low light intensity (Fig. 2A,B; comparison of the means using  $t$ -test:  $t_6 = 6$ ,  $P < 0.001$ ), while the average microcystin-LR content was significantly higher (Fig. 2A,B;  $t$ -test:  $t_6 = 5.9$ ,  $P < 0.001$ ). As a consequence, the microcystin LR/RR ratio was much higher at high light than at low light conditions (Fig. 3A,B). The same response to light intensity was also found in previous experiments (Tonk et al. 2005; see Fig.1), which demonstrates that patterns of microcystin composition can be reproduced in different experiments.

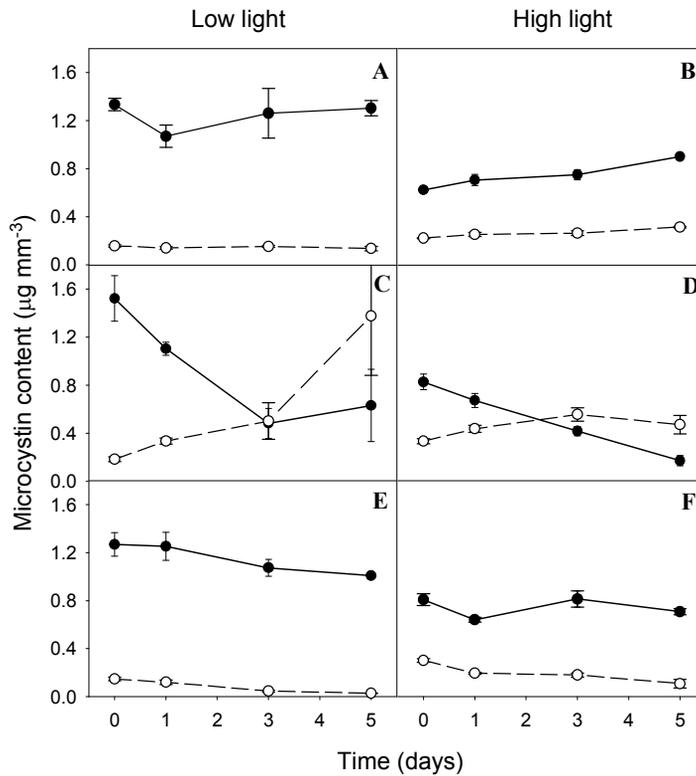


Figure 2: Microcystin-RR (●) and microcystin-LR (○) contents of *P. agardhii* grown in semi-continuous turbidostats: (A,B) with nutrient-saturated medium, (C,D) after addition of leucine, and (E,F) after addition of arginine. Panels on the left show results at low light ( $I_{\text{avg}} = 21 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ), while panels on the right show results at high light ( $I_{\text{avg}} = 84 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ). Error bars denote standard deviation (n = 3).

*Addition of L and R.* In cultures with L addition, the decline of microcystin-RR and associated increase of microcystin-LR were evident under both light conditions (Fig. 2C,D). Accordingly, the microcystin LR/RR ratio showed a strong increase after L addition (Fig. 3C,D). In cultures supplied with R, the microcystin-RR content remained approximately the same as in the control, while the microcystin-LR content was reduced (Fig. 2E,F). Hence, the microcystin LR/RR ratio was reduced after R addition (Fig. 3E,F).

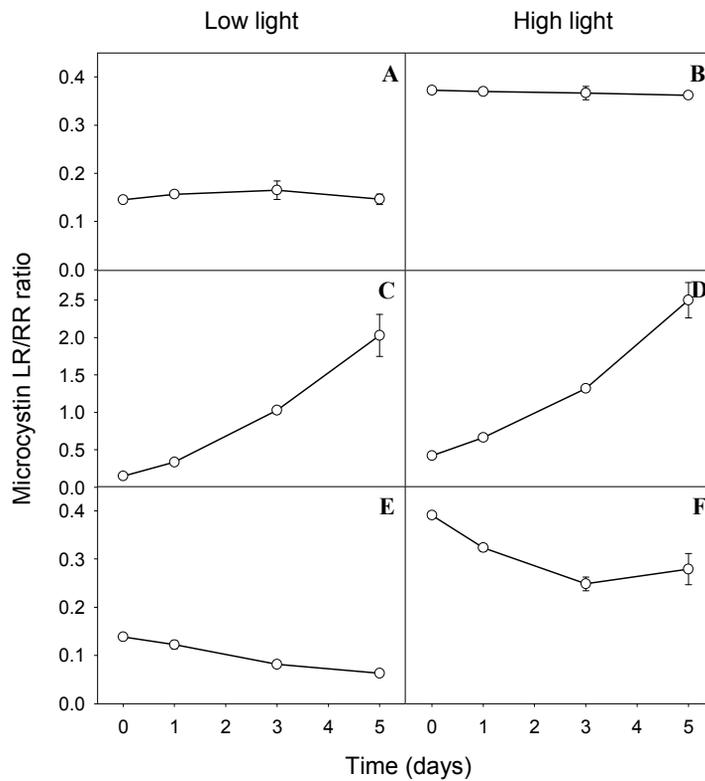


Figure 3: Microcystin LR/RR ratios of *P. agardhii* grown in semi-continuous turbidostats: (A,B) with nutrient-saturated medium, (C,D) after addition of leucine, and (E,F) after addition of arginine. Panels on the left show results at low light ( $I_{\text{avg}} = 21 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), while panels on the right show results at high light ( $I_{\text{avg}} = 84 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Error bars denote standard deviation ( $n = 3$ ).

*Chemostat experiment.* In this experiment, the N-saturated medium of a continuous culture containing *P. agardhii* grown at a relatively low light intensity ( $I_{\text{in}} = 38 \pm 4 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was replaced by N-limited medium at Day 5, thereby slowly decreasing the N availability in the culture vessel from Day 5 onwards. The dilution rate of the chemostat was maintained at  $D = 0.34 \text{ d}^{-1}$  throughout the experiment. The microcystin-RR content, microcystin-LR content, nitrogen content in the cells, and the average light intensity in the culture were judged stable during the initial steady state (Day 1 to 5, Fig. 4). When provided with N-limited medium, the biovolume started to decrease from the default level of  $\sim 1.0 \text{ mm}^3 \text{ ml}^{-1}$  during the first 5 days to  $0.4 \text{ mm}^3 \text{ ml}^{-1}$  on Day 24 (Fig. 4A). The average light intensity in the culture increased (Fig. 4C) as a result of both a lower population density and a lower pigment content of the remaining cells under N-limited conditions. A decrease in the cellular nitrogen content was observed from Day 10 onwards (Fig. 4B).

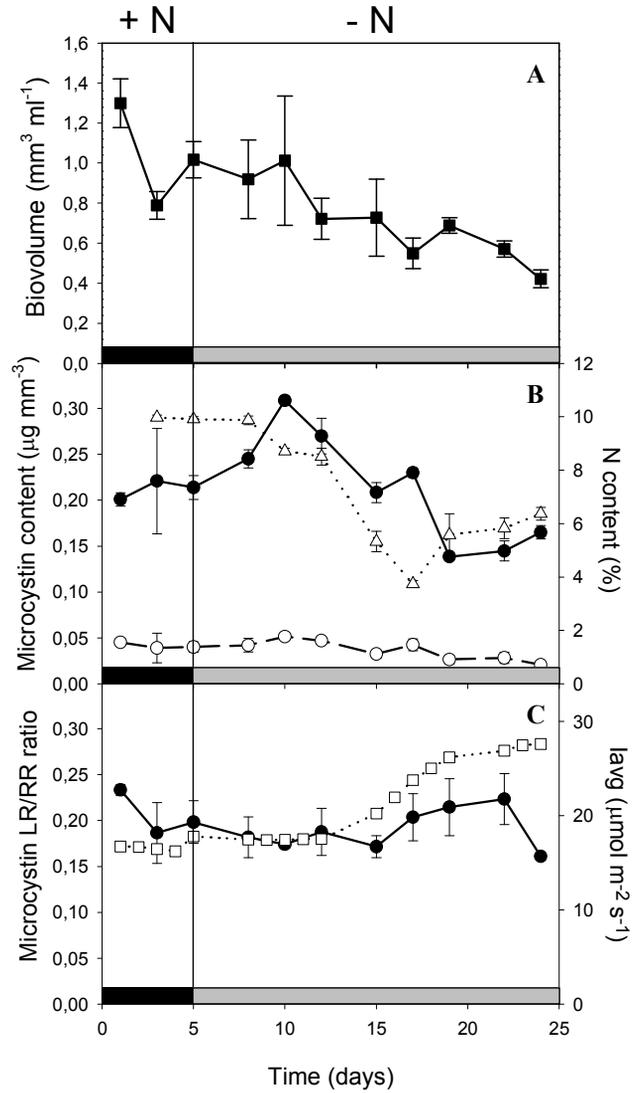


Figure 4: Time course of changes in selected properties of *P. agardhii* grown in continuous culture. (A) Biovolume (■). (B) Microcystin-RR content (●), microcystin-LR content (○), and N content (Δ), where N content is expressed as percentage of the dry weight. (C) Microcystin LR/RR ratio (●) and depth-averaged light intensity in the culture (□). Error bars indicate standard deviation (n = 3). The black bar along the x-axis indicates the use of N-saturated medium, and the grey bar the use of N-limited medium.

At Day 17, cells started to appear pale, a common phenomenon in N-starved cyanobacteria caused by the breakdown of phycobilisomes and chlorophyll a. The microcystin-RR content showed an initial increase, but then also started to decrease from Day 10 onwards (Fig. 4B). The initial increase in microcystin-RR, from Day 5 to Day 10, might be the consequence of the release of nitrogen stocks from phycobilin and cyanophycin degradation. During the transition to N limitation both the microcystin-RR and microcystin-LR content decreased, while the ratio of the two microcystin variants remained constant (Fig. 4C). As a consequence, the expected shift towards microcystin-LR under N-limiting conditions was not found.

Total amino acid analysis was performed on samples taken from the chemostat at Day 8 (when the cellular N content was still high) and at Days 22-29 (during N-limitation). The expected increase of the L/R ratio in response to N limitation was not observed, and likewise the expected increase in the microcystin LR/RR ratio was not found. In fact, all of the amino acids leucine, arginine, and aspartic acid decreased proportionally under N-limited conditions, such that the cells maintained a constant L/R ratio and, thus, a constant microcystin LR/RR ratio (Table 1).

Table 1: Amino acid content and microcystin content of *P. agardhii* grown in the chemostat experiment<sup>a</sup>

	N-saturated	N-limited
<b>Amino acid content (<math>\mu\text{g mg}^{-1}</math>)</b>		
Aspartic acid	73.8	53.5
Leucine	67.7	45.9
Arginine	41.8	30.7
L/R ratio <sup>b</sup>	1.62	1.50
D/R ratio <sup>b</sup>	1.77	1.74
<b>Microcystin content (<math>\mu\text{g mm}^{-3}</math>)</b>		
Microcystin-LR	0.042	0.025
Microcystin-RR	0.245	0.149
Microcystin LR/RR ratio	0.171	0.167

<sup>a</sup> N-saturated samples were taken on Day 8, and N-limited samples on Days 22-29.

<sup>b</sup> D = aspartic acid; L = leucine; R = arginine.

## DISCUSSION

In this study, we provided evidence that amino acid availability determines the relative composition of microcystin variants in cyanobacteria, here shown for the filamentous cyanobacterium *Planktothrix agardhii* strain 126/3. Addition of leucine (L) resulted in an increase in the microcystin LR/RR ratio (Fig. 3C,D), while addition of arginine (R) resulted in a decrease of that ratio (Fig. 3E,F). Remarkably, while addition of L resulted in a higher microcystin-LR content (Fig. 2C,D), addition of R did not yield a higher microcystin-RR content (Fig. 2E,F) compared to the control. Instead, addition of R suppressed the microcystin-LR content substantially and thereby lowered the microcystin LR/RR ratio. Arginine can be taken up by several cyanobacteria but has also been judged inhibitory or even non-permissive in quite a few cases (Montesinas et al. 1997, Stephan et al. 2000, K-P Michel pers. commun.). This might explain why addition of R did not increase the microcystin-RR content. We conclude from these amino acid addition experiments that the availability of amino acids during microcystin polyketide synthesis is a major determinant of the composition of microcystin variants in cyanobacteria. Our findings do not rule out other mechanisms that could control the synthesis of different microcystin variants. For instance, in addition to the role of amino acid availability reported here, conformational changes in synthetase enzymes could also contribute to changes in the production of different microcystin variants.

Speculations on the function of microcystins are multiple, and include physiological functions such as metal ion chelators (Utkilen and Gjølme 1995) or intraspecific signaling molecules (Dittmann et al. 2001), as well as protection against predators such as zooplankton (Koski et al. 1999, Rohrlack et al. 1999, 2001). It has been suggested that cyanobacterial toxins also play a role in the competition between cyanobacteria and other phototrophic organisms (Keating 1977, Gross 2003), but recent studies have questioned whether microcystins have significant allelopathic effects (Babica et al. 2006, Kardinaal et al. 2007). In analogy with antibiotics made by bacteria in a similar non-ribosomal way, variation in microcystin composition may broaden the effectiveness of action. Assembling different microcystin variants may thus be a strategy of cyanobacteria to sustain continued production of microcystins under different environmental conditions.

Now that we have observed that the availability of amino acids is indeed important for the production of the two microcystin variants, the question remains what factor initiates changes in the free amino acid pool. We hypothesized that N limitation can decrease the relative availability of the N-rich amino acid R in the free amino acid pool or that N-surplus after a period of limitation might increase R presence. Subsequently, according to our findings on L and R addition, we expected that the decrease in R relative to L would favor microcystin-LR synthesis over microcystin-RR synthesis. However, when N-saturated medium was replaced by N-limited medium in our chemostat experiment, the anticipated suppression of R content and increase in microcystin LR/RR ratio were not observed (Table 1, Fig. 4C). Instead, N limitation induced an overall decrease of the cellular amino acid content and microcystin content (Table 1, Fig. 4B).

Our experiments did show a significantly higher microcystin LR/RR ratio at high light than at low light (Fig. 3A,B). The same response to light intensity was also found in previous experiments with *P. agardhii* (2005), which demonstrates that patterns of microcystin composition can be reproduced in different experiments. Discrepancies between the absolute values in microcystin contents reported by Tonk et al. (2005) and the microcystin contents reported in this article probably stem from different methods used for biovolume determination. In Tonk et al. (2005) biovolume was determined by means of microscope countings on lugol-fixed samples, while in the present study we determined biovolumes of fresh samples using a Casy cell counter. The established strong response of the microcystin LR/RR ratio to light made us question why this ratio failed to respond to N limitation? It might be that in our experiment an alternative N source compensated for N depletion. For instance, cyanobacteria might re-allocate N obtained from the breakdown of cyanophycin or the degradation of phycocyanin pigments to microcystin production. During N excess, cyanobacteria can store the R surplus in cyanophycin, a nitrogen-rich polypeptide consisting of arginine and aspartic acid (Oppermann-Sanio and Steinbüchel 2002). In rapidly growing cyanobacteria that are in physiological balance with their environment, cyanophycin is present only in small amounts (Allen 1988). During periods of nitrogen deficiency, however, R stored in cyanophycin can play an important role in the balance between C and N metabolism of cyanobacteria (Maheswaran et al. 2006). Thus, N storage in cyanophycin might provide a buffer against changes in external N availability, which might explain the absence of the expected shift in microcystin LR/RR ratio.

In conclusion, we have demonstrated that the relative availability of different amino acids is an important factor in the non-ribosomal synthesis of different microcystin variants, and thereby affects the composition of microcystin variants in cyanobacteria. Under low light conditions, however, N limitation induced neither obvious changes in the relative availability of the amino acids L and R, nor changes in microcystin variant composition. The interactive effects of nitrogen limitation and light limitation on the production of different microcystin variants and the potential role of cyanophycin as a major store of R deserve further investigation.

*Acknowledgements.* The research of LT, DvdW, JH and PMV was supported by the Earth and Life Sciences Foundation (ALW), which is subsidized by the Netherlands Organization for Scientific Research (NWO). In addition, LT and PMV were supported by a European Union grant within the program PEPCY. We thank Kaarina Sivonen of the University of Helsinki for providing the *Planktothrix agardhii* strain used in this study, and Geoff Codd and co-workers of the University of Dundee for providing the MC-LR gravimetric standard.

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# Chapter 5

## PRODUCTION OF CYANOPEPTOLINS, ANABAENOPEPTINS AND MICROCYSTINS BY THE HARMFUL CYANOBACTERIA *ANABAENA* 90 AND *MICROCYSTIS* PCC 7806

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SUBMITTED TO HARMFUL ALGAE

*ABSTRACT* During recent years, various new bioactive oligopeptides have been discovered in cyanobacteria, and more information on their production rates is required to improve our understanding of what drives peptide production and to guide cyanotoxin risk assessment. In this study, we investigated effects of light intensity, temperature, and phosphorus limitation on the peptide production of the cyanobacteria *Microcystis* PCC 7806 and *Anabaena* 90. *Microcystis* PCC 7806 produced two microcystin variants and three cyanopeptolins, whereas *Anabaena* 90 produced four microcystin variants, three anabaenopeptins, and two anabaenopeptilides. Microcystin and cyanopeptolin contents varied by a factor 2 to 3, whereas the anabaenopeptins and anabaenopeptilides of *Anabaena* varied more strongly. Under phosphorus limitation, peptide production rates increased with the specific growth rate. The response of peptide production to light intensity and temperature was more complex, and in many cases peptide production decreased with specific growth rate. We observed compensatory changes of different peptide variants. For instance, decreased cyanopeptolin A and C contents were accompanied by increased cyanopeptolin 970 contents. Likewise, decreased anabaenopeptin A and C contents were accompanied by increased anabaenopeptilide 90B contents. Compensatory dynamics in peptide production may enable cyanobacteria to sustain stable peptide levels in a variable environment.

## INTRODUCTION

Blooms of harmful cyanobacteria are reported worldwide in freshwater ecosystems and brackish waters (Lehtimäki et al., 1997; Matthiensen et al., 2000; Vanderploeg et al., 2001; Robson and Hamilton, 2003; Verspagen et al., 2006), and have caused numerous animal and human poisonings (Kuiper-Goodman et al., 1999; Carmichael et al., 2001; Codd et al., 2005). During the past two decades, several cyanotoxins have been studied extensively, particularly the microcystins, nodularins (both hepatotoxins) and anatoxins (a neurotoxin). In addition, other bioactive peptide groups have been discovered, including linear peptides like aeruginosins (Murakami et al., 1994) and microginins (Okino et al., 1993), cyclic depsipeptides with an amino-hydroxy piperidone (Ahp) like cyanopeptolins (Martin et al., 1993) and anabaenopeptilides (Rouhiainen et al., 2000), depsipeptides with a tricyclic ring system (microviridins; Ishitsuka et al., 1990), cyclic peptides with a ureido linkage (anabaenopeptins; Harada et al., 1995), and cyclic peptides with a  $\beta$  amino acid (nostophycins; Fujii et al., 1999). Details on structural diversity, taxonomic distribution, and biosynthesis of these cyanobacterial oligopeptides can be found in Welker and von Döhren (2006). Whereas the production and toxicity of microcystins, nodularins and anatoxins have been investigated extensively, only few studies (e.g., Repka et al., 2004) focused on other bioactive peptides produced by cyanobacteria. Yet, a better understanding of the production and toxicity of these peptides is essential to improve our understanding of what drives peptide production and to guide cyanotoxin risk assessment.

The cellular microcystin content of cyanobacteria is known to be affected by a variety of environmental factors, including light intensity (Watanabe and Oishi, 1985; Wiedner et al., 2003), temperature (Watanabe and Oishi, 1985), nitrogen (Long et al., 2001; Orr and Jones, 1998; Watanabe and Oishi, 1985), phosphorus (Oh et al., 2000; Watanabe and Oishi, 1985), and iron (Utkilen and Gjølme, 1995). It has been suggested that growth-limiting factors indirectly control microcystin production of cyanobacteria through their effect on growth rate (Orr and Jones, 1998), and that, at least under nutrient-limited conditions, microcystin content can be predicted from growth rate (Long et al., 2001). However, other studies have revealed that microcystin production shows a more complicated, nonlinear response to photon irradiance (Wiedner et al., 2003). Photon irradiance is suggested to act directly on the transcription and/or transcript stability of microcystin synthetase genes (Börner and Dittmann, 2005). Furthermore, within a single strain, the production of different microcystin variants may respond to environmental factors in different ways (Rapala et al., 1997; Hesse and Kohl, 2001; Tonk et al., 2005). For instance, Tonk et al. (2005) observed that the [Asp<sup>3</sup>]microcystin-RR content of *Planktothrix agardhii* decreased twofold while its [Asp<sup>3</sup>]microcystin-LR content increased threefold with increasing photon irradiance.

The striking variability in peptide production can probably be attributed to the process of oligopeptide biosynthesis. Most oligopeptides produced by cyanobacteria are synthesized by large multifunctional enzyme complexes consisting of non-ribosomal peptide synthetases

(NRPS) and polyketide synthase modules (PKS) (Börner and Dittmann, 2005; Welker and von Döhren, 2006). Numerous different structural variants can be produced by homologue NRPS-systems present in different cyanobacterial strains. Some NRPS/PKS gene clusters have already been identified, such as the microcystin synthetase gene clusters in *Microcystis* and *Planktothrix* (Tillet et al., 2000; Christiansen et al., 2003) and the anabaenopeptilide (a cyanopeptolin congener) synthetase gene cluster in *Anabaena* 90 (Rouhiainen et al., 2000). There is evidence that *Anabaena* 90 harbors at least three peptide synthetase gene clusters for synthesis of microcystins, anabaenopeptilides, and anabaenopeptins, respectively. However, the genes encoding the anabaenopeptin synthetase have not yet been sequenced (Fujii et al., 2002). The relationships between multiple gene clusters within a single strain are still unknown, but they seem to operate largely independently of each other (Fujii et al., 2002). Rouhiainen et al. (2000) demonstrated that a mutant strain of *Anabaena*, in which the *apdA* gene is interrupted, did not produce the anabaenopeptilides 90A and 90B, whereas other cyclic peptides such as microcystins and anabaenopeptins were still present. Similarly, disruption of genes within the *mcy* cluster of *Microcystis* blocked microcystin production without affecting the production of cyanopeptolins (Dittmann et al., 1997). The observation that different cyanobacterial peptides can be synthesized largely independently suggests that the production of, for instance, cyanopeptolins and anabaenopeptilides may respond differently to environmental factors than the production of, say, microcystins.

To investigate this hypothesis in further detail, we focused on the effects of different environmental factors on the peptide production of two widespread freshwater cyanobacteria: *Microcystis* and *Anabaena*. Both *Microcystis* and *Anabaena* can form dense surface blooms in eutrophic lakes (Reynolds, 2006), and have relatively high phosphorus requirements (De Nobel et al., 1997; Fujimoto et al., 1997). *Microcystis aeruginosa* PCC 7806 is a single-celled cyanobacterium with optimal growth at relatively high temperatures and light intensities (Van der Westhuizen and Eloff, 1985; Wiedner et al., 2003). *Anabaena* 90 is a filamentous heterocyst-forming species capable of nitrogen fixation, with optimal growth at relatively low temperature and light intensity (Rapala et al., 1997) compared to *Microcystis*. Both species produce multiple toxic and non-toxic peptides. In addition to microcystins, some *Microcystis* strains also produce other bioactive depsipeptides such as cyanopeptolins (trypsin inhibitors), aeruginosin (thrombin/trypsin inhibitor), anabaenopeptin (inhibitor of carboxypeptidase A) and microginin (inhibitor of angiotensin-converting enzyme) (Martin et al., 1993; Dittmann et al., 2001; Welker et al., 2006). *Anabaena* strains are known to produce, among others, microcystins, anatoxins (nicotinic agonists), anabaenopeptilides (which function as protease inhibitors) and anabaenopeptins (Dittmann et al., 2001; Fujii et al., 2002; Repka et al., 2004). These peptides are frequently found in cyanobacterial blooms, along with numerous other not yet identified peptides (Fastner et al., 2001; Welker et al., 2006).

In this study, we investigate how light, phosphorus availability, and temperature affect the peptide content and peptide production of *Microcystis aeruginosa* PCC 7806 and *Anabaena* 90.

We set out to explore whether peptides are continuously produced and whether variation in peptide production is found between and within peptide classes. Additionally, we investigate the relation between peptide contents and growth rate of *Microcystis* PCC7806 and *Anabaena* 90.

## MATERIAL & METHODS

*Strains.* *Microcystis aeruginosa* strain PCC 7806 was provided by the Pasteur Institute, in Paris. The strain was originally isolated from the Braakman Reservoir, The Netherlands, in 1972 and grows as single cells. *Anabaena* 90 was provided by the University of Helsinki. The strain was originally isolated from Lake Vesijärvi, Finland, in 1986 (Sivonen et al., 1992). Experimental conditions were chosen according to the specific needs of each organism.

*Light experiments.* *Microcystis* PCC 7806 and *Anabaena* 90 were grown in semi-continuous cultures consisting of flat plastic vessels (400 ml) aerated by a continuous airflow. The cultures were provided with a nutrient-rich mineral medium (O<sub>2</sub> medium; van Liere and Mur, 1978), to avoid nutrient limitation. The phosphorus concentration in O<sub>2</sub> medium was 144 µM. Temperature was kept constant at 20 ± 2°C by leading the compressed air used for aeration through a temperature-controlled water bath (Colora thermocryostat). In addition, a ventilator was used to diffuse the warmth emitted by the light source. Light was provided by white fluorescent tubes (Philips PL-L 24W/840/4P), directed towards the front surface of the culture vessel. Incident irradiance ( $I_{in}$ ) and outgoing irradiance ( $I_{out}$ ) were measured with a LI-COR LI-250 quantum photometer at 7 points on the front surface and back surface of the culture vessel, respectively. The average photon irradiance inside the culture vessel,  $I_{avg}$ , was calculated as follows (Huisman et al., 2002):

$$I_{avg} = \frac{I_{in} - I_{out}}{\ln I_{in} - \ln I_{out}} \quad (1)$$

*Microcystis* and *Anabaena* cultures were placed at two different average light intensities: a light-saturated photon irradiance (*Microcystis* PCC 7806:  $I_{in} = 175 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $I_{avg} = 150 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; *Anabaena* 90:  $I_{in} = 75 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $I_{avg} = 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and a light-limited photon irradiance (*Microcystis* PCC 7806:  $I_{in} = 58 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $I_{avg} = 40 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; *Anabaena* 90:  $I_{in} = 30 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $I_{avg} = 22 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). The experiments were performed in triplicate at both light intensities. A day-night cycle of 12 hours light/12 hours dark was imposed. The optical densities (at 750 nm) of the semi-continuous cultures were kept constant between 0.1 and 0.2 cm<sup>-1</sup> by diluting the culture material every other day with mineral medium. After a period of acclimatisation, for at least two weeks, each culture was sampled four times during one week. The data were averaged over these 4 samples, and these average values per culture were used in the statistical analysis.

*Phosphate experiments.* *Microcystis* PCC 7806 and *Anabaena* 90 were grown in continuous cultures under phosphorus-limited conditions. The continuous cultures consisted of flat culture vessels, based on the design of Huisman et al. (2002), with a working volume of 1.85 L and a continuous supply of phosphorus-limited O<sub>2</sub> medium containing 6 μM phosphate. Temperature was kept constant at 22 ± 1°C by a water jacket placed between the culture vessel and the light source. The light source consisted of four white fluorescent tubes (Philips PL-L/24W/840/4P) directed towards the front of the culture vessel. The average photon irradiance was calculated according to Eq.1. Average photon irradiance was  $I_{avg} = 100 \mu\text{mol m}^{-2} \text{s}^{-1}$  ( $I_{in} = 150 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for the *Microcystis* cultures, and  $I_{avg} = 22.5 \mu\text{mol m}^{-2} \text{s}^{-1}$  ( $I_{in} = 44 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for the *Anabaena* cultures. A day-night cycle of 12 hours light/12 hours dark was imposed. The cultures were aerated with a continuous airflow to ensure homogeneous mixing and to provide sufficient amounts of carbon dioxide for photosynthesis. The dilution rates of two continuous cultures with *Microcystis* were set at  $D = 0.21$  and  $D = 0.35 \text{ d}^{-1}$ , respectively, while the dilution rates of two continuous cultures with *Anabaena* were set at  $D = 0.20$  and  $D = 0.26 \text{ d}^{-1}$ . A higher dilution rate implies that, at steady state, phosphate concentrations in the culture vessel are typically slightly higher. Once the cultures reached steady state, for at least two weeks, each culture was sampled four times during one week. Phosphate concentrations were analyzed according to Murphy and Riley (1962).

*Temperature experiments.* The effect of temperature on the peptide production of *Microcystis* PCC 7806 and *Anabaena* 90 was investigated in batch cultures at 20 ± 1°C and 30 ± 0.5°C, respectively. Temperature was kept constant by performing the experiment in an orbital incubator (Gallenkamp). The batch cultures were run in triplicate, and were inoculated with *Microcystis* and *Anabaena* at an optical density of  $\text{OD}_{750} = 0.025 \text{ cm}^{-1}$ . The batch cultures were sampled during the exponential growth phase, at an optical density of  $\text{OD}_{750} = 0.1 \text{ cm}^{-1}$ . Standard O<sub>2</sub> medium was used. A day-night cycle of 12 hours light/12 hours dark was imposed, with an incident irradiance of  $I_{in} = 40 \mu\text{mol m}^{-2} \text{s}^{-1}$  during the day period.

*Growth rates and toxin production rates.* To quantify cyanobacterial biomass in the experiments, biovolumes were measured using a Casy cell counter (Casy 1 TTC, Schärfe System), with a 60 μm capillary for *Microcystis* cells and a 150 μm capillary for *Anabaena* filaments. The specific growth rate,  $\mu$ , was calculated according to the following equation:

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} + D \quad (2)$$

where  $x_1$  and  $x_2$  are the biovolumes measured at time  $t_1$  and  $t_2$ , respectively, and  $D$  is the dilution rate.

The peptide production rate was calculated as the product of peptide content and specific growth rate.

*Peptide analysis.* For analyses of cell-bound peptides, 15 ml of culture suspension was filtered in triplicate using Whatman GF/C filters (pore size  $\sim 1.2 \mu\text{m}$ ). Filters were freeze dried and stored at  $-20^\circ\text{C}$ . Peptides were extracted in 50% methanol (three extraction rounds) as described by Fastner et al. (1998) with an extra step for grinding of the filters in a Mini Beadbeater (Biospec products) with 0.5 mm silica beads (Tonk et al., 2005). Dried extracts were stored at  $-20^\circ\text{C}$  and dissolved in 50% MeOH for analysis of peptide contents using high performance liquid chromatography (HPLC) with photodiode array detection (Kontron instruments). The *Microcystis* PCC 7806 extracts were separated using a 30 to 70% acetonitrile gradient, and the *Anabaena* 90 extracts were separated using a 20 to 60% acetonitrile gradient, both with 0.05% v/v trifluoroacetic acid at a flow of  $1 \text{ ml min}^{-1}$  applied to a LiChrospher 100 ODS  $5 \mu\text{m}$  LiChorCART 250-4 cartridge system (Merck). Peptides were identified in HPLC-fractions by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Voyager DE Pro, Applied Biosystems), with post-source decay and collision-induced dissociation (PSD-CID) fragment analysis. Fragment patterns of individual peptides were analysed as described by Welker et al. (2006). Microcystins were quantified by peak area at  $\lambda = 238 \text{ nm}$ . Microcystin concentrations were calibrated with MC-LR as gravimetric standard, which was kindly provided by the Laboratory of Microbiology of the University of Dundee. Extracellular microcystin concentrations were below the detection limit of the HPLC ( $7.5 \mu\text{g l}^{-1}$  for extracellular microcystin). Cyanopeptolins and anabaenopeptins were quantified by peak area at  $\lambda = 220 \text{ nm}$ . At this wavelength, peptide bonds have an absorption band that can be used for quantification. However, since absorption coefficients of different peptides are most likely not identical, our quantitative data of cyanopeptolins and anabaenopeptins should be taken only as approximate peptide contents and results should therefore be interpreted as relative changes in peptide concentration. Purified gravimetric standards were not available for peptides other than MC-LR.

*Data analysis.* Independent samples *t*-tests were performed in SPSS version 11 to detect significant differences between the means of two treatments (i.e., low versus high resource availability, or low versus high temperature). The data were additionally subjected to Levene's test for homogeneity of variances.

## RESULTS

*Peptide identity.* In *Microcystis* PCC 7806, two microcystin variants ([Asp<sup>3</sup>]MC-LR and MC-LR) and three cyanopeptolins (cyanopeptolin A, C and 970) were identified by MALDI-TOF mass spectral analysis and PSD-CID fragment analysis (Table 1). The peaks of [Asp<sup>3</sup>]MC-LR and MC-LR could not be completely separated under the actual HPLC conditions. Therefore, both peaks were summed in quantitative analyses, and are referred to as total MC-LR. Cyanopeptolins A and C have been reported for *Microcystis* PCC 7806 previously (Martin et al., 1993), while cyanopeptolin 970 is a new variant with an isoleucine

moiety exchanged for a valine moiety compared to cyanopeptolin A. Two other peaks were observed which contained traces of multiple cyanopeptolins and other peptides that could not be separated and unambiguously identified.

In *Anabaena* 90, four microcystins (MC-LR, [Asp<sup>3</sup>]MC-LR, MC-RR and [Asp<sup>3</sup>]MC-RR), three anabaenopeptins (anabaenopeptin A, B and C) and two anabaenopeptilides (anabaenopeptilide 90A and 90B) were identified (Table 1). The peaks of anabaenopeptilide 90A and anabaenopeptin B overlapped. Since they belong to two different peptide groups, they were not included in the quantitative analyses.

Table 1: Identity and mono-isotopic protonated molecular mass (M+H) of peptides detected in *Microcystis* PCC 7806 and *Anabaena* 90.

Strain	Peptide identity	M+H
<i>Microcystis</i> PCC 7806	[Asp <sup>3</sup> ]Microcystin-LR <sup>b</sup>	981.5
	Microcystin-LR <sup>b</sup>	995.8
	Cyanopeptolin C	943.5
	Cyanopeptolin A	957.5
	Cyanopeptolin 970	971.5
<i>Anabaena</i> 90	Anabaenopeptilide 90B <sup>a</sup>	973.4
	Anabaenopeptin C	809.5
	Anabaenopeptilide 90A <sup>c</sup>	953.5
	Anabaenopeptin B <sup>c</sup>	837.5 <sup>b</sup>
	Anabaenopeptin A	844.4
	[Asp <sup>3</sup> ]Microcystin-RR	1024.6
	Microcystin-RR	1038.6
	[Asp <sup>3</sup> ]Microcystin-LR <sup>d</sup>	981.5
Microcystin-LR <sup>d</sup>	995.6 <sup>b</sup>	

a: detected with highest peak intensities as M+Na<sup>+</sup> ( $\Delta m/z = 22$  Da) and M+Na-H<sub>2</sub>O<sup>+</sup> ( $\Delta m/z = 4$  Da)

b,c,d: peptides co-eluting in HPLC under given conditions

Table 2: Specific growth rates (mean  $\pm$  standard deviation; in  $d^{-1}$ ) of *Microcystis* PCC 7806 and *Anabaena* 90 under different experimental conditions.

<b>Experimental variable</b>	<b><i>Microcystis</i> PCC 7806</b>		<b><i>Anabaena</i> 90</b>	
	<b>low</b>	<b>high</b>	<b>low</b>	<b>high</b>
Light	0.30 $\pm$ 0.01	0.44 $\pm$ 0.05	0.22 $\pm$ 0.03	0.31 $\pm$ 0.02
Phosphate	0.21 $\pm$ 0.02	0.35 $\pm$ 0.02	0.20 $\pm$ 0.02	0.26 $\pm$ 0.02
Temperature	0.41 $\pm$ 0.02	0.99 $\pm$ 0.09	0.50 $\pm$ 0.04	0.38 $\pm$ 0.05

*Light experiments.* The specific growth rates of both *Microcystis* PCC 7806 and *Anabaena* 90 were higher under light-saturating conditions than under light-limiting conditions (Table 2). Under light-saturating conditions, the contents of the microcystins and cyanopeptolins produced by *Microcystis* PCC 7806 were significantly lower than under light-limited conditions (Fig. 1A). Likewise, the production rates of these peptides were lower under light-saturated than under light-limited conditions, except for cyanopeptolin C (Fig. 1C).

In *Anabaena* 90 only the anabaenopeptin contents were significantly lower under light-saturating than under light-limited conditions (Fig. 1B). The peptide production rates of *Anabaena* 90 were not significantly affected by the applied light conditions (Fig. 1D).

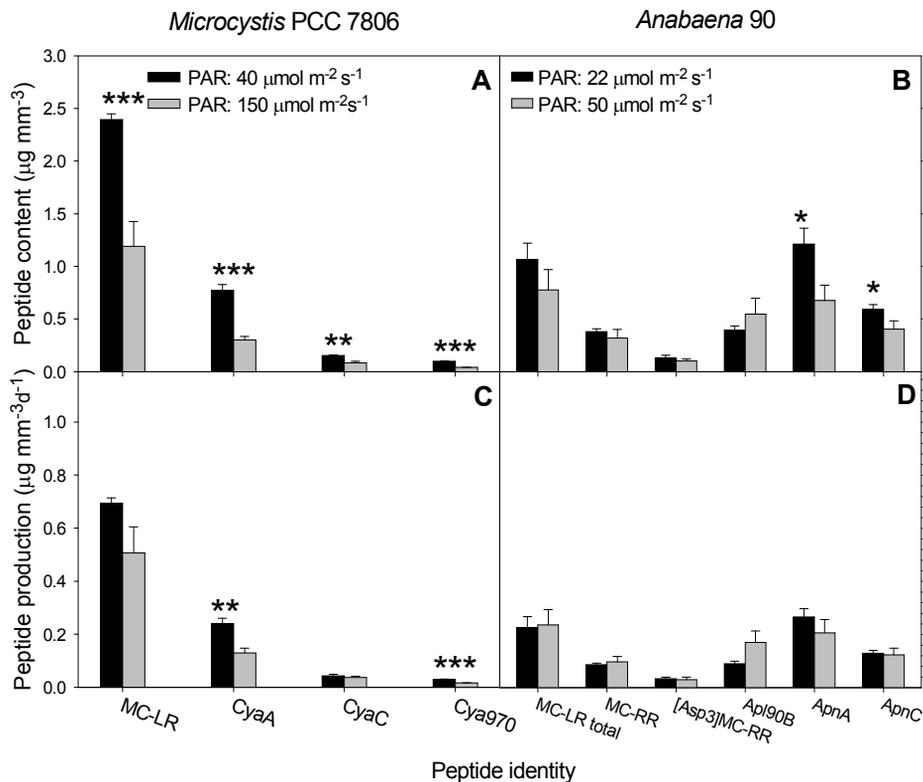


Figure 1: Peptide contents of (A) *Microcystis* PCC 7806, and (B) *Anabaena* 90 under light-limiting conditions (black bars) and light-saturating conditions (grey bars). Peptide production rates of (C) *Microcystis* PCC 7806, and (D) *Anabaena* 90. Error bars denote standard deviations (N = 3). Significant differences between means were tested by the independent samples *t*-test (df = 4; \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ ). Microcystin is denoted as MC, cyanopeptolin is denoted as Cya, anabaenopeptin as Apl, and anabaenopeptin as Apn.

*Phosphate experiments.* In the phosphate experiments, which were carried out in continuous cultures, the specific growth rates were imposed by the dilution rates. That is, at equilibrium the specific growth rate equals the dilution rate (Table 2). The phosphate concentrations measured in the culture vessels during steady state were low (*Microcystis* PCC 7806: at  $D = 0.21 \text{ d}^{-1}$ ,  $P = 0.03 \pm 0.00 \text{ }\mu\text{M}$ ; at  $D = 0.35 \text{ d}^{-1}$ ,  $P = 0.29 \pm 0.10 \text{ }\mu\text{M}$ ; *Anabaena* 90: at  $D = 0.20 \text{ d}^{-1}$ ,  $P = 0.10 \pm 0.03 \text{ }\mu\text{M}$ ; at  $D = 0.26 \text{ d}^{-1}$ ,  $P = 0.18 \text{ }\mu\text{M} \pm 0.13$ ). As a result, the phosphate availability at steady state slightly increased with a higher dilution rate.

In *Microcystis* PCC 7806, the contents of MC-LR and cyanopeptolin 970 were significantly higher at higher dilution rate (Fig. 2A). Conversely, cyanopeptolin A and C contents were significantly lower at higher dilution rate, although differences in cyanopeptolin content were relatively small (Fig. 2A). Production rates of all peptides of *Microcystis* PCC 7806 were significantly higher at higher dilution rate (Fig. 2C).

In *Anabaena* 90, the contents of all peptides investigated were also significantly higher at higher dilution rate (Fig. 2B). Similar patterns were found in the peptide production rates (Fig. 2D).

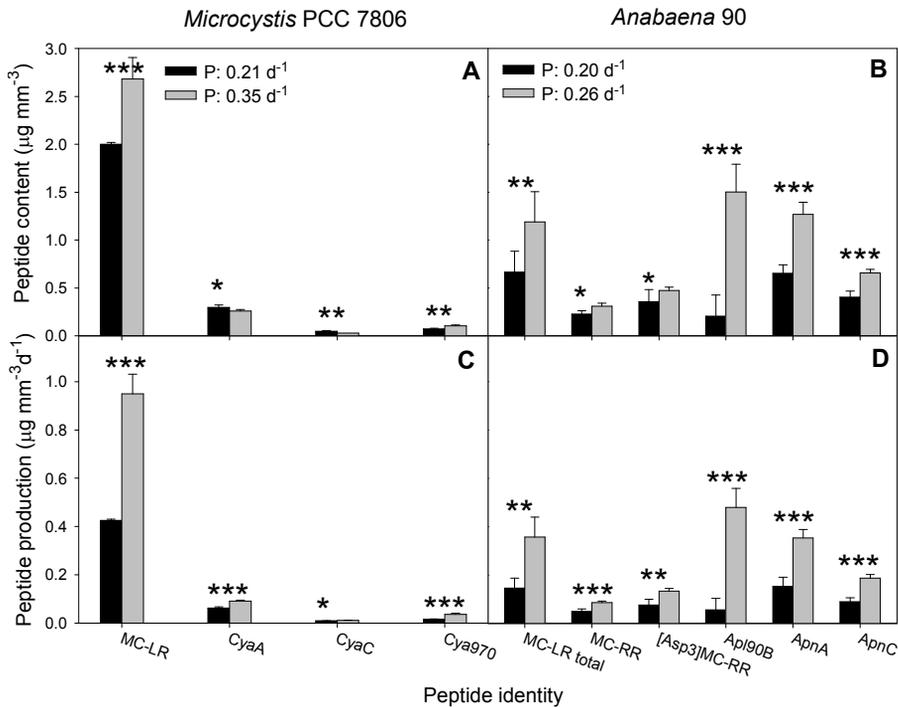


Figure 2: Peptide contents under phosphorus-limited conditions of (A) *Microcystis* PCC 7806, and (B) *Anabaena* 90 at low dilution rate (black bars) and high dilution rate (grey bars). Peptide production rates of (C) *Microcystis* PCC 7806, and (D) *Anabaena* 90. Error bars denote standard deviations ( $N = 4$ ). Significant differences between means were tested by the independent samples t-test ( $df = 6$ ; \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ ). Microcystin is denoted as MC, cyanopeptolin is denoted as Cya, anabaenopeptilide as Apl, and anabaenopeptin as Apn.

*Temperature experiments.* Higher temperature resulted in a higher specific growth rate of *Microcystis* PCC 7806, but a lower specific growth rate of *Anabaena* 90 (Table 2). Higher temperature had a significant negative effect on the contents of MC-LR and cyanopeptolin C in *Microcystis* PCC 7806, while the contents of cyanopeptolin A and cyanopeptolin 970 were not significantly affected (Fig. 3A). The production rates of cyanopeptolin A and cyanopeptolin 970, however, were significantly higher at higher temperature (Fig. 3C). At 30°C, cyanopeptolin C did not exceed the detection limit.

In *Anabaena* 90, the contents of both anabaenopeptins (A and C) and both microcystin-RR variants ([Asp<sup>3</sup>]MC-RR and MC-RR) were significantly higher at higher temperature (Fig. 3B). Likewise, the production rates of anabaenopeptin A, anabaenopeptin C and [Asp<sup>3</sup>]MC-RR were higher at higher temperature (Fig. 3D).

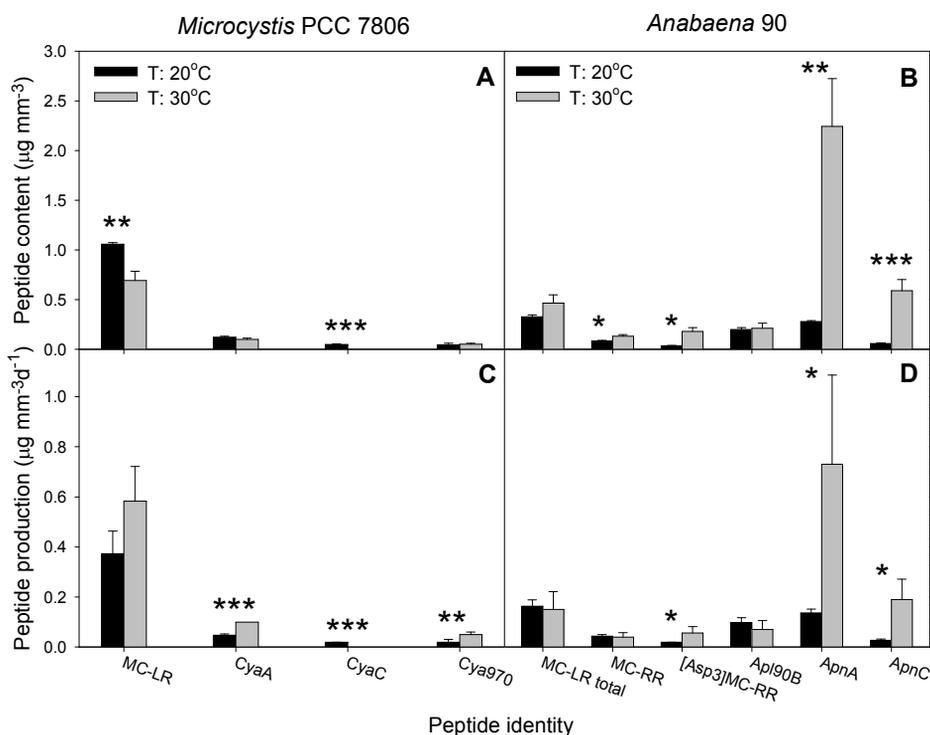


Figure 3: Peptide contents of (A) *Microcystis* PCC 7806, and (B) *Anabaena* 90 at 20°C (black bars) and 30°C (grey bars). Peptide production rates of (C) *Microcystis* PCC 7806, and (D) *Anabaena* 90. Error bars denote standard deviations (N = 3). Significant differences between means were tested by the independent samples *t*-test (df = 4; \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ ). Microcystin is denoted as MC, cyanopeptolin is denoted as Cya, anabaenopeptilide as Apl, and anabaenopeptin as Apn.

## DISCUSSION

Except for cyanopeptolin C in *Microcystis* at 30°C, which was found in very low concentrations only, the microcystins, cyanopeptolins, anabaenopeptins and anabaenopeptilides produced by *Microcystis* PCC 7806 and *Anabaena* 90 were above the detection level under all investigated conditions. Apparently, the production of these peptides is not switched on or off under the imposed culture conditions. Since we tested a variety of different experimental conditions, including phosphorus limitation, light limitation, and two different temperatures, we conclude that cyanopeptolins, anabaenopeptins and anabaenopeptilides are constitutively produced, meaning that these peptides are always present in these cyanobacterial strains. In this sense, the production of cyanopeptolins, anabaenopeptins, and anabaenopeptilides resembles the constitutive production of microcystins established by numerous earlier laboratory studies (reviewed by Kardinaal and Visser, 2005).

Microcystin contents of *Microcystis* PCC 7806 and *Anabaena* 90 varied by a factor 2 or 3 across the different experimental conditions (Figs. 1-3). This is in agreement with many other experimental studies, which typically found a similar magnitude of variation in microcystin contents within cyanobacterial strains (reviewed by Kardinaal and Visser, 2005). The cyanopeptolin contents of *Microcystis* also showed a similar magnitude of variation in our experiments. Several peptides of *Anabaena* 90, including anabaenopeptilide 90B (Fig. 2B), [Asp<sup>3</sup>]MC-RR and anabaenopeptin A and C (Fig. 3B) varied more strongly, by a factor 6 to 10 across different experimental conditions.

Under phosphorus-limited conditions, the dilution rate had a positive effect on the contents of all identified peptides in *Anabaena* 90, and of total microcystin-LR and cyanopeptolin 970 in *Microcystis* PCC 7806 (Fig. 2). The measurements in these experiments were made in continuous cultures at steady state. In this case, the dilution rate determines the specific growth rate. In other words, under phosphorus-limited conditions, the contents of nearly all peptides increased with the specific growth rate. This is consistent with earlier findings that the microcystin content of *Microcystis* increases with growth rate under nutrient-limited conditions (Long et al., 2001).

Light intensity had a negative effect on all peptide contents in *Microcystis* PCC 7806 (Fig. 1A,C). Our microcystin data match the results of Wiedner et al. (2003), who investigated the same *Microcystis* strain and found that microcystin contents per unit biovolume decreased with light intensity for light intensities above 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The effect of light intensity on the peptide contents in *Anabaena* 90 seemed less consistent. Only the anabaenopeptins A and C of *Anabaena* decreased significantly with light intensity, whereas anabaenopeptilide 90B seemed to increase (Fig. 1B). These results confirm findings of Repka et al. (2004), who showed that the contents of anabaenopeptilide 90B and anabaenopeptin reacted in an opposite manner to increasing light intensity. To the best of our knowledge, Repka's study on the *Anabaena* 90 wild type and its anabaenopeptilide deficient mutant has thus far been the only other study that investigated effects of light on peptides other than microcystins.

Repka et al. (2004) suggested that anabaenopeptins and anabaenopeptilides, which are both serine protease inhibitors, have similar functions in the cell. The anabaenopeptins possibly increased in content in the mutant strain to compensate for the lack of anabaenopeptilides. Similar compensatory dynamics within the microcystins were reported by Tonk et al. (2005), who described that a decrease of [Asp<sup>3</sup>]microcystin-RR was accompanied by an increase of [Asp<sup>3</sup>]microcystin-LR in *Planktothrix agardhii*. Likewise, in our phosphorus-limited experiments with *Microcystis* PCC 7806, cyanopeptolin A and C contents decreased with dilution rate, whereas cyanopeptolin 970 increased significantly (Fig. 2A). Each of these three examples shows compensatory dynamics within a peptide class. Variation in environmental conditions poses multiple challenges in cellular metabolism. The production of a range of peptide variants with similar functions might therefore provide cyanobacteria with a better chance to sustain the constitutive production of the peptide class in a variable environment.

In cyanobacteria, each peptide class is most likely produced by a homologue NRPS or NRPS/PKS enzyme complex (Welker and von Döhren, 2006). The relationships between the different NRPS and NRPS/PKS clusters are still unknown. Those clusters that have been investigated seem to operate independently of each other (Dittmann et al., 1997; Rouhiainen et al., 2000), apparently leading to variation in production among peptide classes. Individual enzyme complexes can produce a series of peptide variants. For instance, the *mcy* gene cluster encodes a family of enzymes responsible for the synthesis of the different microcystin variants produced by a given strain (Dittmann et al., 1997). The synthesis of different microcystin variants is made possible due to the flexible substrate specificity of particular adenylation domains of the enzyme complex, allowing the activation and hence incorporation of different amino acids in the peptide (Börner and Dittmann, 2005). It is not yet known which factors determine substrate specificity. However, recent investigations indicate that the relative availability of the amino acids leucine (L) and arginine (R) determine the [Asp<sup>3</sup>]MC-LR / [Asp<sup>3</sup>]MC-RR ratio in *Planktothrix agardhii* (L. Tonk et al., in preparation). The amino-acid availability in cells is influenced by environmental conditions. If the synthesis of different peptide variants within the same peptide class is determined by amino-acid availability, this provides a simple mechanism whereby cells might produce different peptide variants depending on the environmental conditions, thus explaining the observed compensatory dynamics. Other suggested regulators of peptide content may act on transcript stability, enzyme activity or peptide turnover rate (Börner and Dittmann, 2005).

Microcystin production has been investigated in numerous laboratory experiments. In a review of this work, Sivonen and Jones (1999) concluded that the majority of these studies indicated that cyanobacteria produce the highest amount of toxins under conditions that are most favourable for growth. Growth rate has even been proposed as a master factor steering microcystin production regardless of the environmental conditions (Orr and Jones, 1998). Our phosphorus-limited experiments support this view, since peptide production generally increased with dilution rate (Fig. 2). However, under light-limited conditions, higher light intensities resulted in higher growth rates (Table 1), but in a reduced peptide production,

at least in *Microcystis* (Fig. 1). Moreover, higher temperatures resulted in higher growth rates but lower peptide contents in *Microcystis*, and in lower growth rates but higher peptide contents in *Anabaena* (Table 1, Fig. 3). All in all, these results do not support the general contention that toxin production of cyanobacteria is highest under conditions that are most favourable for growth. Instead, we suggest that transcriptional regulation of individual gene clusters and the availability of different substrates, like amino acids, result in different patterns of toxin production under different environmental conditions.

## CONCLUSIONS

Our results show that the microcystins, cyanopeptolins and anabaenopeptins identified in *Microcystis* PCC 7806 and *Anabaena* 90 were constitutively produced under the different environmental conditions tested. On average, contents of microcystin and cyanopeptolin in *Microcystis* and *Anabaena* varied by a factor 2 to 3, whereas the anabaenopeptins and anabaenopeptilides of *Anabaena* varied more strongly. Our results do not support the hypothesis that favourable growth conditions generally lead to higher toxin production rates in cyanobacteria. Instead, we suggest that transcriptional regulation and the cellular availability of different substrates have a major impact on toxin production. This may also explain the observed compensatory dynamics of different peptide variants within the same peptide class. In total, our results illustrate that examination of the complete array of peptides produced, instead of a single peptide class, yields a more comprehensive overview of the effects of environmental conditions on peptide production and thereby provides more insight into the factors governing peptide production. The variation in peptide production revealed by this study emphasizes the complexity of toxin production by harmful cyanobacteria.

*Acknowledgements.* The research of LT, PMV and JH was supported by the Earth and Life Sciences Foundation (ALW), which is subsidized by the Netherlands Organization for Scientific Research (NWO). LT, PMV and MW were additionally supported by the EU project PEPCY (European commission research grant QLRT-2001-02634).

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# Chapter 6

## COMPETITION FOR LIGHT BETWEEN TOXIC AND NON-TOXIC STRAINS OF THE HARMFUL CYANOBACTERIUM *MICROCYSTIS*

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HUISMAN AND PETRA M. VISSER. (2007) APPLIED AND ENVIRONMENTAL MICROBIOLOGY  
VOL. 73, P. 2939-2946

*\*BOTH AUTHORS CONTRIBUTED EQUALLY TO THIS WORK*

*ABSTRACT* The cyanobacterium *Microcystis* can produce microcystins, a family of toxins that are of major concern in water management. In several lakes the average microcystin content per cell gradually declines from high levels at the onset of *Microcystis* blooms towards low levels at the height of the bloom. Such seasonal dynamics might result from a succession of toxic to non-toxic strains. To investigate this hypothesis, we ran competition experiments with two toxic and two non-toxic *Microcystis* strains using light-limited chemostats. The population dynamics of these closely related strains were monitored by means of characteristic changes in light absorbance spectra and by PCR amplification of the rRNA ITS region in combination with denaturing gradient gel electrophoresis (DGGE), which allowed identification and semi-quantification of the competing strains. In all experiments the toxic strains lost competition for light from non-toxic strains. As a consequence, the total microcystin concentrations in the competition experiments gradually declined. We did not find evidence for allelopathic interactions, as non-toxic strains became dominant even when toxic strains were given a major initial advantage. These findings show that, in our experiments, non-toxic strains of *Microcystis* were better competitors for light than toxic strains. The generality of this finding deserves further investigation with other *Microcystis* strains. The competitive replacement of toxic by non-toxic strains offers a plausible explanation for the gradual decrease in average toxicity per cell during the development of dense *Microcystis* blooms.

## INTRODUCTION

Blooms of the cyanobacterium *Microcystis* can be a major hazard in recreational lakes, drinking water reservoirs, and protected wetland areas (Chorus and Bartram 1999, Visser et al. 2005, Verspagen et al. 2006). *Microcystis* often forms dense blooms that may cause anoxia when cells die-off massively. Moreover, *Microcystis* can produce the toxin microcystin. This hepatotoxin poses serious health risks for animals and humans (Carmichael 2001, Codd et al. 2005). Especially in dense scums, the concentration of microcystins may increase dramatically. Microcystin concentrations up to 25,000  $\mu\text{g liter}^{-1}$  have been reported (Kardinaal and Visser 2005), exceeding the guideline values for recreational waters, of 20  $\mu\text{g liter}^{-1}$ , by more than three orders of magnitude (Chorus 2005).

*Microcystis* populations often consist of mixtures of microcystin producing and non-microcystin producing strains (Fastner et al. 2001, Kurmayer et al. 2002, Via-Ordorika 2004, Welker et al. 2004). Interestingly, several studies show that the average microcystin content expressed per cell is typically high at the onset of *Microcystis* blooms but much lower at the height of these blooms (Welker et al. 2004, Kardinaal and Visser 2005). In other words, with increasing *Microcystis* biomass the *Microcystis* cells become, on average, less toxic. Examples from three *Microcystis*-dominated Dutch lakes are shown in Figure 1. This striking seasonal variability in microcystin content of *Microcystis* blooms exceeds the physiological variability in cellular microcystin content reported for isolated *Microcystis* strains in laboratory experiments (Oh et al. 2000, Hesse and Kohl 2001, Wiedner et al. 2003). Thus, it seems that the changes in microcystin contents during the development of *Microcystis* blooms are due to a seasonal succession of toxic and non-toxic strains, in which non-toxic strains prevail at the height of the *Microcystis* bloom. A seasonal succession of toxic and non-toxic *Microcystis* genotypes has indeed been observed in several lakes (Fastner et al. 2001, Welker et al. 2003).

Competition for light is an important selective factor in phytoplankton communities of eutrophic waters (Mur and Schreurs 1995, Huisman et al. 1999, 2004). Competition models predict that the species (or genotype) with the lowest 'critical light intensity' is the best competitor for light, as it can withstand the shading cast by its competitors (Huisman and Weissing 1994, Weissing and Huisman 1994). This model prediction is confirmed by laboratory competition experiments with light-limited phytoplankton (Huisman et al 1999, Litchman 2003, Passarge et al. 2006). Competition for light might play a key role in the seasonal succession of toxic and non-toxic genotypes in *Microcystis* blooms. The gradual increase in *Microcystis* biomass during bloom development may cause substantial shading and thereby limits the light available for growth. We therefore hypothesize that the best competitor for light among the *Microcystis* genotypes present may increase its relative abundance during bloom development, and the toxicity of this strain will then largely determine the overall microcystin content of the *Microcystis* bloom.

Here, we use competition experiments to investigate competition for light between toxic and non-toxic *Microcystis* strains. The experiments were carried out in laboratory chemostats specifically designed to study competition for light (Huisman et al 1999, 2002, Stomp et al. 2004, Passarge et al. 2006). Toxic and non-toxic strains cannot be distinguished by traditional light microscopic techniques. Therefore, we use two alternative approaches to distinguish the different strains in our competition experiments. In one competition experiment, we use observed differences in pigment composition to monitor the competing strains. In the other competition experiments we apply recently developed molecular tools based on denaturing gradient gel electrophoresis (DGGE) of the PCR amplified ITS region of the rRNA operon (Janse et al. 2003, 2004) to monitor competition between the *Microcystis* strains.

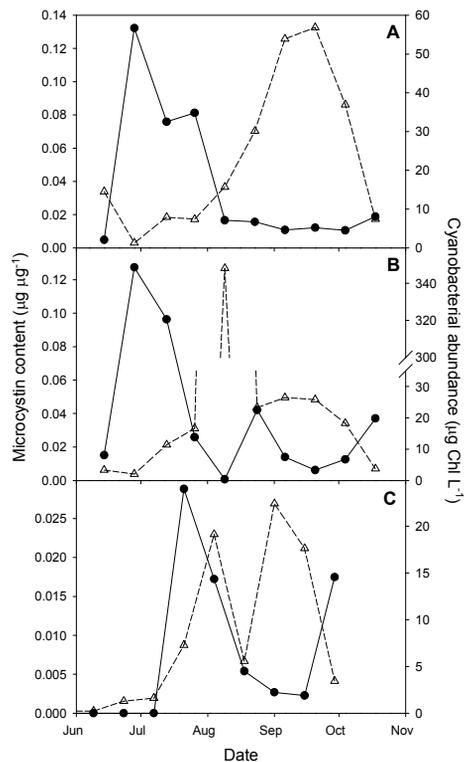


FIG. 1. Seasonal dynamics of cellular microcystin content (closed circles) and cyanobacterial abundance (open triangles) in three eutrophic Dutch lakes: (A) t Joppe, (B) Sloterplas and (C) De Gouden Ham. All three lakes were dominated by *Microcystis*. Microcystin contents are expressed per unit of cyanobacterial abundance. Cyanobacterial abundance is expressed as cyanobacteria-bound chlorophyll, which was determined by flow cytometry with lasers specific for phycocyanin and chlorophyll fluorescence. All data are from the summer season of 1999, and were kindly provided by the Dutch Foundation for Applied Water Research (STOWA).

## MATERIALS & METHODS

*Organisms.* The experiments were performed with two recently isolated *Microcystis* spp. strains from Lake Volkerak, The Netherlands, and with two *Microcystis aeruginosa* laboratory strains originating from the NIVA culture collection (Table 1). Volkerak strain V163 and NIVA strain CYA140 both produce microcystin-LR. Furthermore, strain V163 produces three other, unidentified, microcystin variants in much lower concentration. Volkerak strain V145 and NIVA strain CYA43 do not produce microcystins. The two NIVA strains and strain V145 contain relative high amounts of the pigment phycocyanin, which gives these strains a bluegreen color. Conversely, strain V163 has a greenish brown appearance as it contains the pigment phycoerythrin and relatively low amounts of phycocyanin. Thus far phycoerythrin has been found only in one other *Microcystis* strain (Schatz et al. 2005).

*Light-limited chemostats.* Experiments were conducted in light-limited chemostats (Huisman et al. 1999, 2002), using flat culture vessels with a working volume of 1.85 liters and an optical path length ('mixing depth') of 5 cm. The dilution rate was fixed at  $D = 0.011 \text{ h}^{-1}$  by a continuous inflow of nutrient-saturated mineral medium (Van Liere and Mur 1978). The chemostats were bubbled with a constant inflow of filtered and moistened air to ensure homogeneous mixing and to provide sufficient amounts of inorganic carbon. Temperature was kept constant at  $20 \pm 1^\circ \text{ C}$  by means of a stainless steel cooling element placed within the culture vessel. The specific design of the chemostats allowed an accurate definition of the light conditions (Huisman et al. 1999). The light source consisted of four white fluorescent tubes (Philips PL-L/24W/840/4P) directed towards the front surface of the culture vessel. Incident light intensity ( $I_{\text{in}}$ ) and the light intensity penetrating through the vessel ( $I_{\text{out}}$ ) were measured with a LI-COR SA 190 quantum sensor at 10 evenly spread points on the front surface and back surface of the culture vessel, respectively. The incident light intensity was set at  $I_{\text{in}} = 25 \pm 1 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  for all experiments. The light intensity  $I_{\text{out}}$  penetrating through the vessel was measured every three to four days.

*Experiments.* The *Microcystis* strains were grown in monoculture experiments and competition experiments. The monoculture experiments were performed for four reasons: 1) to ensure that the strains could all survive in monoculture under the imposed experimental conditions; 2) to determine the microcystin content of the toxic strains under the imposed experimental conditions; 3) to assess changes in pigment concentration during the experiments; and 4) to determine the critical light intensities of the strains. The critical light intensity ( $I_{\text{out}}^*$ ) of each strain was measured as the light intensity penetrating through the monoculture, once the monoculture had reached a steady state (Huisman et al 1994, 1999, Passarge et al. 2006). We ran three competition experiments. In competition experiment 1 the toxic strain V163 and non-toxic strain V145 were inoculated at low initial population densities with a cell ratio of 1:1. Likewise, in competition experiment 2 the toxic strain CYA140 and non-toxic CYA43

were inoculated with a cell ratio of 1:1. In competition experiment 3 the toxic strain CYA140 and non-toxic strain CYA43 were inoculated with a cell ratio of 9:1, to give the toxic strain an initial advantage.

*Sampling.* Cultures were not completely axenic. However, frequent examination by phase contrast microscopy indicated that heterotrophic bacteria amounted to less than 1% of the total biovolume. Furthermore, we used our DGGE analysis (see below) to check for contamination with other cyanobacterial species: the primers used for PCR and DGGE were cyanobacteria specific and contamination by other cyanobacteria would have been detected as an additional band in the DGGE profiles. Contamination by other cyanobacteria was not detected.

Samples were taken from day 1 (inoculation) until the cultures had maintained a steady state (constant population density and constant  $I_{out}$ ) for at least one week. During the entire experimental period, samples were taken once every four days from the monocultures and once every two days from the competition experiments. Samples were divided in subsamples for analysis of cell counts (Casycounter, type Casy 1 TTC, Schärfe System, Germany), light absorption spectra, DGGE profiles, and intracellular and extracellular microcystin concentrations.

*Microcystin analysis.* For intracellular microcystin analysis, 10 ml of culture suspension was filtered in triplicate using GF/C filters (pore size  $\sim 1.2 \mu\text{m}$ , 25 mm diameter, Whatman, Maidstone, UK). The filters were lyophilized, and subsequently 1.5 ml 75 % (vol/vol) aqueous methanol was added for extraction of microcystins according to Fastner et al. (1998) with an extra step for grinding of the filters in a Mini Beadbeater (Biospec products, Bartlesville, Oklahoma) with 0.5 mm silica beads (Tonk et al. 2005). Dried extracts were stored at  $-20^\circ\text{C}$  and dissolved in 50% MeOH for analysis of microcystin content using high performance liquid chromatography (HPLC) with photodiode array detection (KONTRON instruments, Watford, UK). Extracts were separated on a LiChrospher 100 RP-18 ( $5 \mu\text{m}$ ) LichorChart 250-4 cartridge system (Merck, Darmstadt, Germany), using a gradient of 30 to 70 % (vol/vol) aqueous acetonitrile (with 0.05 % vol/vol trifluoroacetic acid) at a flow rate of  $1 \text{ ml min}^{-1}$ . Microcystins were identified using their typical UV spectra (Lawton et al. 1994). Total microcystin concentrations were quantified as the sum of all microcystin peaks using a MC-LR gravimetric standard provided by the Laboratory of Microbiology of the University of Dundee.

Extracellular microcystins were obtained from the 10 ml of filtered culture suspension mentioned above. The filtrate was lyophilized and subsequently resuspended in  $150 \mu\text{l}$  Milli-Q water. Prior to analysis the samples were vortexed, boiled in a water bath for 1 hour (Metcalf and Codd 2000) and centrifuged for 3 min at  $18\,300 \text{ g}$ . Extracellular microcystin concentrations were below the detection limit of the HPLC (2.5 ng microcystin). Therefore, they were determined using an Enzyme-Linked Immuno Sorbent Assay (ELISA). The ELISA was performed according to the protocol of the Microcystin Plate Kit (EnviroLogix Inc. Catalog No. EP 022).

*Light absorbance spectra.* Because strain V<sub>I45</sub> has a higher content of the pigment phycocyanin than strain V<sub>I63</sub>, we could deduce the population dynamics of the two strains in competition experiment 1 from the relative concentration of phycocyanin. For this purpose, 2 ml of culture suspension was pressurized at 10 Bar to collapse the gas vesicles of the cells. Next, the culture suspension was transferred to a quartz cuvet (10 mm width) and its light absorbance spectrum was scanned from 350 to 700 nm with a bandwidth of 0.4 nm using an Aminco DW-2000 double-beam spectrophotometer. Mineral medium without *Microcystis* was used for baseline measurements. After baseline correction, the relative concentration of phycocyanin in the culture was estimated by expressing light absorption by phycocyanin (at 627 nm) as a percentage of the light absorption by the first chlorophyll peak (at 438 nm).

*DGGE profiling.* Strain CYA<sub>I40</sub> and strain CYA<sub>43</sub> have a very similar pigment composition. Previous work, however, has shown that different *Microcystis* strains can be differentiated at high resolution using DGGE analysis of the ITS region (Janse et al. 2003, 2004). Therefore, we prepared a range of different mixtures of the two strains, to assess whether the relative abundances of the two strains could be quantified using the relative band intensities of strain-specific bands in DGGE profiles. Since this worked out very well, we decided to monitor the population dynamics of *Microcystis* strains CYA<sub>I40</sub> and CYA<sub>43</sub> in competition experiments 2 and 3 using their relative band intensities in DGGE profiles of the samples. After sampling, 2 ml of the culture suspension was transferred to Eppendorf tubes and put under pressure (10 Bar) to collapse the gas vesicles of the cells. Subsequently, the Eppendorf tubes were centrifuged at 18,300 g and the supernatants were removed. The Eppendorf tubes were stored at -20 °C until further processing. We used a Xanthogenate-based protocol for DNA isolation (Tillet and Neilan 2000). We applied DGGE analysis to sections of the internal transcribed spacer (ITS) between the 16S and 23S rRNA genes. The PCR amplification protocol and primers used for the ITS region were based on Janse et al. (2003). PCR products were separated on a 1.5 mm thick, vertical DGGE gel containing 8% (w/v) polyacrylamide (37.5:1 acrylamide: bisacrylamide) and a linear gradient of the denaturants urea and formamide. After staining of the gel in water containing 0.5 µg ml<sup>-1</sup> ethidium bromide, an image of the gel was recorded with a CCD camera system (Imago, B&L Systems, The Netherlands). DGGE gel pictures were analyzed using the Phoretics-ID package (Nonlinear Dynamics, UK). Lanes were created manually with a fixed width. Subsequent lanes represented subsequent sampling days. Peaks smaller than 1% of the maximum peak were discarded. Relative densities of *Microcystis* bands were calculated by dividing the peak intensity of the band concerned by the sum of the peak intensities from all *Microcystis* bands in that lane. Here, peak intensity is defined as the sum of all pixel values within the band boundaries. The DGGE profiles were run in duplicate to check the consistency of the results.

Table 1. Characteristics of the *Microcystis* strains used in the experiments.

Code	Origin	Source of reference	Microcystins produced	Phycoerythrin	Competition experiment
V145	Lake Volkerak, The Netherlands	UvA*	None	-	1
V163	Lake Volkerak, The Netherlands	UvA*	MC-LR & 3 unidentified variants	+	1
CYA43	USA	NIVA**	None	-	2,3
CYA140	Bendig's pond, Bruno, Canada	NIVA**	MC-LR	-	2,3

\*UvA: Algal culture collection of the University of Amsterdam, The Netherlands

\*\*NIVA: Algal culture collection of the Norwegian Institute for Water Research, Norway

## RESULTS

### MONOCULTURE EXPERIMENTS

All strains were able to grow well in monoculture. Figure 2 shows examples of monoculture experiments of strain V145 and strain CYA140. Cell densities increased to a steady-state value of about 6 million cells ml<sup>-1</sup> for strain V145 and 23 million cells ml<sup>-1</sup> for strain CYA140. This difference in steady-state cell density can be attributed to a difference in cell size, since strain V145 had an average cell diameter of 5.2 µm while strain CYA140 had an average cell diameter of only 3.9 µm. Hence, in terms of biovolume, the steady states of the two strains were quite similar. With increasing cell density, the light intensity  $I_{out}$  penetrating through the cultures decreased. The critical light intensities ( $I_{out}^*$ ) of strain V145 and strain CYA140 were both around 1.3 µmol photons m<sup>-2</sup> s<sup>-1</sup> (Fig. 2). Strain CYA43 reached a similar critical light intensity of about 1.3 µmol photons m<sup>-2</sup> s<sup>-1</sup>, whereas the critical light intensity of the toxic strain V163 was higher, at 4.6 µmol photons m<sup>-2</sup> s<sup>-1</sup>. The steady-state microcystin content in the monoculture experiments was around 24 fg cell<sup>-1</sup> in strain V163 (s.d. = 11, N = 4) and around 40 fg cell<sup>-1</sup> in strain CYA140 (s.d. = 6, N = 11).

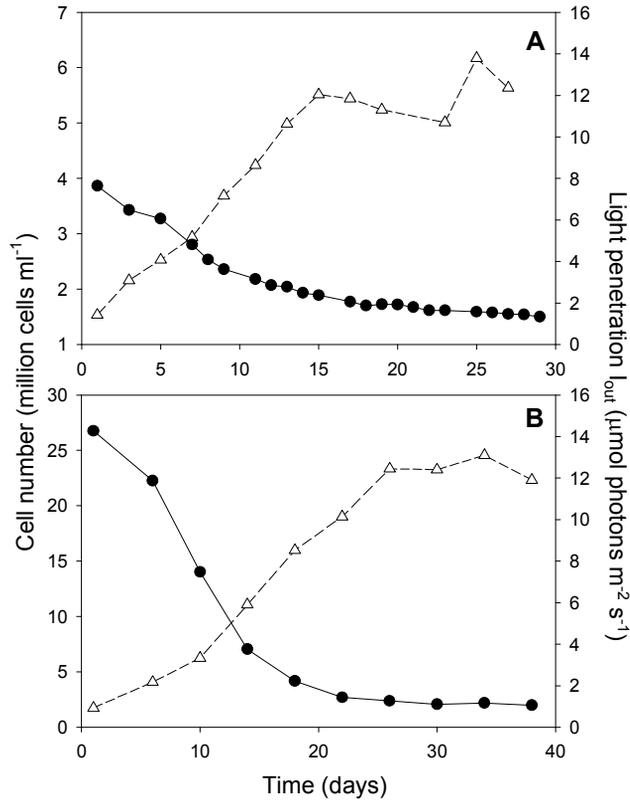


FIG. 2. Time course of cell number (open triangles) and light penetration through the culture vessel ( $I_{out}$ ; closed circles) in monoculture experiments of (A) the non-toxic strain *VI45* and (B) the toxic strain *CYA140*. A steady state was reached in about 20-30 days.

#### COMPETITION BETWEEN TOXIC STRAIN *VI63* AND NON-TOXIC STRAIN *VI45*

Strains *VI63* and *VI45* used in competition experiment 1 differed in their pigment composition. Toxic strain *VI63* contains accessory pigments absorbing light between 350 and 400 nm, probably playing a role in UV-protection, the red pigment phycoerythrin (peak absorbance at 570 nm), and a relatively low content of the bluegreen pigment phycocyanin (peak absorbance at 627 nm) (Fig. 3A). Non-toxic strain *VI45* lacks phycoerythrin but has a much higher content of the pigment phycocyanin (peak absorbance at 627 nm) (Fig. 3B).

*Population dynamics.* The differences in pigment composition between the two strains were used to monitor the competition experiment. The absorbance spectrum of the mixture in the competition experiment shifted from a spectrum quite similar to toxic strain V<sub>163</sub> at day 4 (Fig. 3C) towards a spectrum similar to non-toxic strain V<sub>145</sub> at the end of the competition experiment (Fig. 3D). In fact, the changes in the light absorbance spectra of the competition experiment indicated that the toxic strain V<sub>163</sub> was competitively replaced by the non-toxic strain V<sub>145</sub> within about two weeks (Fig. 3E).

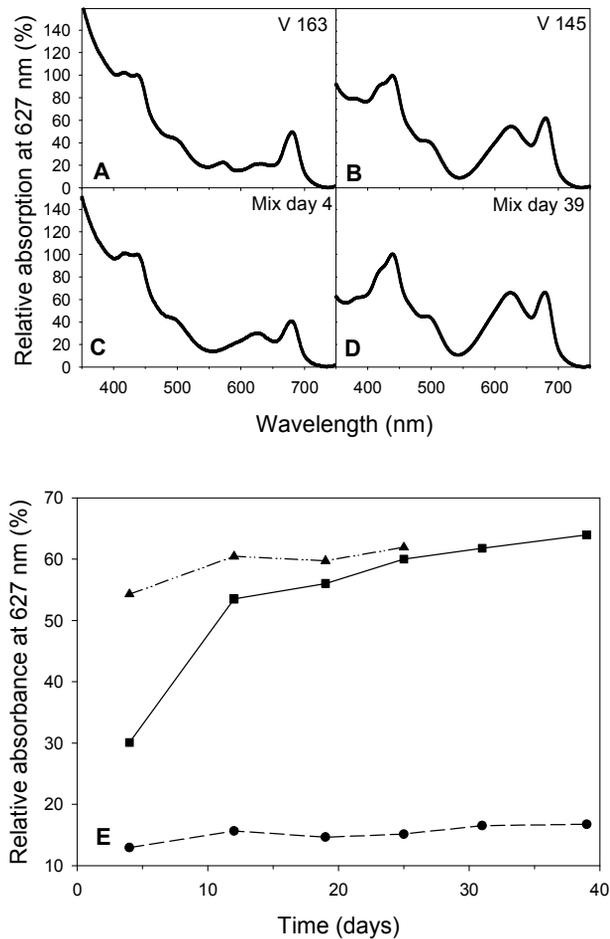


FIG. 3. Light absorption spectra, normalized on the first chlorophyll peak at 438 nm, of the monoculture experiments of (A) toxic strain V<sub>163</sub>, and (B) non-toxic strain V<sub>145</sub>, and of the competition experiment between these two strains on (C) day 4, and (D) day 39. (E) Changes in the relative absorbance at 627 nm, the characteristic wavelength for phycocyanin, show the displacement of the toxic strain V<sub>163</sub> by the non-toxic strain V<sub>145</sub> during the competition experiment (squares connected by solid line). Changes in the relative absorbance at 627 nm during the monoculture experiments of strain V<sub>145</sub> (triangles connected by dash-dotted line) and strain V<sub>163</sub> (circles connected by dashed line) are also indicated.

*Microcystin concentration and Microcystis biomass.* The increasing dominance of the non-toxic strain  $V_{I45}$  was confirmed by changes in microcystin concentration. While the total *Microcystis* population increased more than four-fold, the total microcystin concentration in the competition experiment decreased to nearly zero in 10 days (Fig. 4).

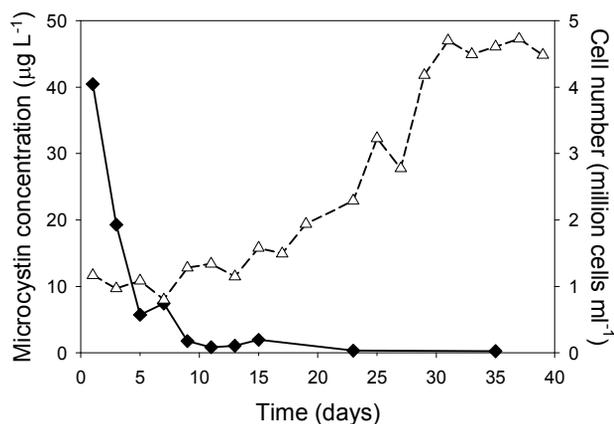


FIG. 4. Time course of the total microcystin concentration (closed diamonds) and cell number (open triangles) in competition experiment 1, between the non-toxic strain  $V_{I45}$  and the toxic strain  $V_{I63}$ . Data present the mean of three replicate measurements.

#### COMPETITION BETWEEN TOXIC STRAIN $CYA_{I40}$ AND NON-TOXIC STRAIN $CYA_{43}$

In the next two competition experiments, we used two strains (non-toxic  $CYA_{43}$  and toxic  $CYA_{I40}$ ) with a similar pigment composition. We therefore used DGGE analysis of the ITSa region to distinguish the two strains. DGGE profiles from the monocultures of strain  $CYA_{43}$  and  $CYA_{I40}$  each yielded a single band when amplified with rRNA ITSa primers. The position of the bands on the gels differed clearly, thus allowing identification of the two strains in the competition experiments. To test whether the DGGE profiles also allowed quantification of the relative abundances of the two strains, we prepared mixtures of the two strains in a range of different relative abundances. This yielded corresponding ratios of the band intensities in rRNA-ITS DGGE profiles (Fig. 5A). Furthermore, all DGGE profiles were run in duplicate, and the duplicates always showed very similar results. Therefore, we conclude that the relative band intensities indeed enable semi-quantitative monitoring of competition between the two strains (Fig. 5B).

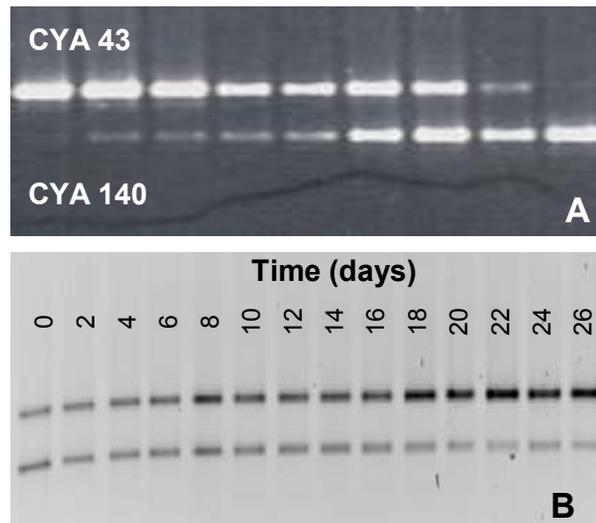


FIG. 5. DGGE gels loaded with PCR products after amplification with ITSa primers of the rRNA ITS region, for different mixtures of strain *CYA*<sub>43</sub> and strain *CYA*<sub>140</sub>. (A) DGGE gel of strains *CYA*<sub>43</sub> and *CYA*<sub>140</sub> mixed in the following ratios, from left to right: 99:1, 5.67:1, 2.45:1, 1.33:1, 0.89:1, 0.59:1, 0.32:1, 0.14:1 and 0.01:1. (B) DGGE gel of competition experiment 2, in which the toxic strain *CYA*<sub>140</sub> is gradually displaced by the non-toxic strain *CYA*<sub>43</sub>.

*Population dynamics.* We carried out two experiments, with different initial ratios of toxic versus non-toxic cells. Experiment 2 was started with equal amounts of toxic and non-toxic cells (1:1), while experiment 3 was started with many more toxic than non-toxic cells (9:1) to give the toxic strain an initial advantage. Analysis of the relative band intensities in the two competition experiments revealed that the ratio between strains *CYA*<sub>140</sub> and *CYA*<sub>43</sub> changed towards dominance of the non-toxic strain *CYA*<sub>43</sub> in both experiments (Fig. 6A and 6B). In experiment 2, the relative band intensity of toxic strain *CYA*<sub>140</sub> was reduced to less than 20% within 25 days (Fig. 6A). In experiment 3, which started with a high initial density of toxic cells, the toxic strain also gradually declined but it took much longer, around 120 days, before the toxic strain was reduced to less than 20% of the total *Microcystis* population (Fig. 6B).

*Microcystin concentration and Microcystis biomass.* The total microcystin concentration increased during the first 15 days of competition experiment 3, in parallel with the increase of the total *Microcystis* population (Fig. 6C). However, once the experiment reached its highest cell densities (approximately 25 million cells ml<sup>-1</sup>) the total microcystin concentration started to decline. After about 140 days, the total microcystin concentration was reduced to less than 20% of its highest value, reflecting the competitive replacement of the toxic strain *CYA*<sub>140</sub> by the non-toxic strain *CYA*<sub>43</sub>.

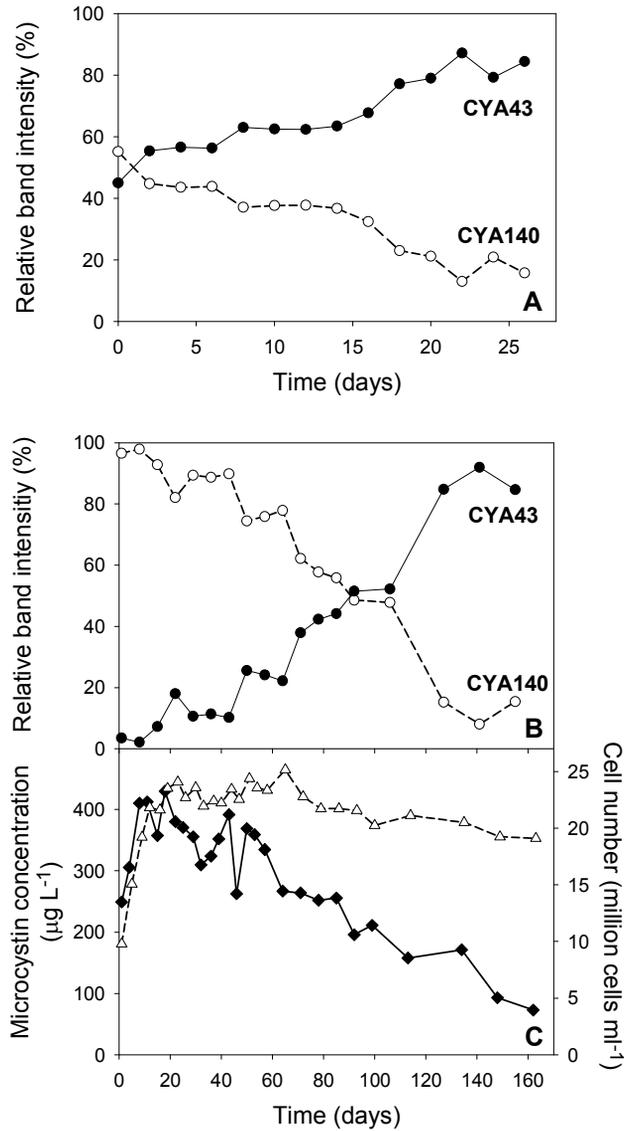


FIG. 6. Time courses of competition between the toxic strain *CYA140* (open circles) and the non-toxic strain *CYA43* (closed circles), deduced from the relative band intensities on the DGGE gels. (A) At the start of competition experiment 2, the competing strains *CYA43* and *CYA140* were inoculated in a 1:1 ratio. (B) At the start of competition experiment 3, the competing strains *CYA43* and *CYA140* were inoculated in a 1:9 ratio to give the toxic strain *CYA140* an initial advantage. (C) Time course of the total microcystin concentration (closed diamonds) and total cell density (open triangles) in the latter competition experiment shown in (B). Data of total microcystin concentration and total cell density present the mean of three replicate measurements. Data of the relative band intensities of the two strains are based on duplicate DGGE profiles.

## DISCUSSION

In this study, we investigated competition for light between different strains of the *Microcystis* genus. Traditionally, phytoplankton competition studies make use of microscopy and/or flow cytometry to monitor the population dynamics of competing species (e.g., Tilman 1977, Sommer 1986, Grover 1991, Huisman et al. 1999, Stomp et al. 2004, Passarge et al. 2006). However, the *Microcystis* strains in our competition experiments could not be distinguished microscopically. Therefore, in our experiment 1 we made use of the observation that the two strains differ in their pigment composition (Fig. 3), which allowed monitoring of the relative abundances of the two strains during the competition experiment. In experiment 2 and 3 we applied a recently developed molecular approach that can distinguish different strains by DGGE profiles of the ITS region of the rRNA operon (Janse et al. 2003, 2004). This molecular technique allowed recognition of the different strains, and we showed that in our experiments the relative band intensities in the DGGE profile also enabled semi-quantitative estimates of the abundances of these strains (Fig. 5). The population dynamics deduced from the DGGE analysis were confirmed by independent measurements of changes in the total microcystin concentration in the competition experiments (Fig. 6).

*Competition for light.* Earlier laboratory studies revealed subtle differences in the light-dependent growth response of various *Microcystis* strains (Böttcher et al. 2001, Hesse and Kohl 2001). Nevertheless, our results show that these subtle differences among *Microcystis* strains are sufficient to cause competitive replacement (Fig. 3, Fig. 6). Competition theory predicts that, in well-mixed waters, the phytoplankton species with the lowest critical light intensity will be the best competitor for light (Huisman and Weissing 1994, Weissing and Huisman 1994). This prediction is confirmed by a series of competition experiments (Huisman et al. 1999, Litchman 2003, Passarge et al. 2006). In our study, the critical light intensity of toxic strain V163 was higher than the critical light intensity of non-toxic strain V145. As predicted by theory, the toxic strain V163 was indeed competitively displaced by the non-toxic strain V145 (Fig. 3). Competitive displacement took less than two weeks. The critical light intensities of strains CYA140 and CYA43 were very similar. Therefore, competition theory predicts that these two strains should be more or less equal competitors for light. Yet, the non-toxic strain CYA43 became dominant in the competition experiments. Even in competition experiment 3, where toxic strain CYA140 was given a strong initial advantage, the non-toxic strain CYA43 became dominant in the end. Competitive displacement of CYA140 by CYA43 took much longer, however, than competitive displacement of V163 by V145 (compare the time scales of Fig. 3 and Fig. 6), which is consistent with the finding that CYA140 and CYA43 are more or less equal competitors for light, which indicates that the difference in competitive ability between these two strains must have been small.

Our competition experiments suggest that non-toxic strains are better competitors for light than toxic strains. In each of the competition experiments, toxic strains were competitively excluded by non-toxic strains. One might expect a trade-off between the costs and benefits of toxin production (Riley and Gordon 1999).

Strains that invest their resources in microcystin production and the microcystin synthetase complex may have less resources available to invest in other cellular functions. Although the physiological costs of microcystin production have not yet been fully elucidated, this might indeed imply that toxic strains are usually poorer competitors than non-toxic strains. However, the number of toxic and non-toxic *Microcystis* strains that we investigated is relatively small, and we explored only one set of environmental conditions. Further research, with more strains competing under a wide range of different environmental conditions, will be needed to shed more light on the generality of this finding.

*Allelopathic interactions?* Several studies have suggested that microcystins and other toxic peptides produced by cyanobacteria may have allelopathic effects on other phytoplankton and plants (Sedmak and Kosi 1998, 2005, Schagerl et al. 2002, Pflugmacher 2003, Schatz et al. 2005, but see LeBlanc et al. 2005). In particular, microcystins can function as inhibitors of photosynthetic activity (Smith and Doan 1999, Sukenik et al. 2002, Hu et al. 2004). Mathematical theory predicts that, at least in well-mixed chemostats, the winner of allelopathic interactions between toxin-producing and toxin-sensitive strains will depend on the initial abundances of these strains (Chao and Levin 1981, Durett and Levin 1997, Hulot and Huisman 2004). That is, allelopathic interactions will be effective only if toxin-producing strains are sufficiently abundant and produce enough toxin to suppress toxin-sensitive strains. Our experiments were run with very high cell densities (up to 25 million cells ml<sup>-1</sup>) typical of dense cyanobacterial blooms. Moreover, in one of our competition experiments, the toxic strain was given a much higher initial abundance than the non-toxic strain. Yet, in the end, in all competition experiments the non-toxic strain became dominant. Furthermore the outcome of the competition experiment with strains V145 and V163 followed the prediction on the basis of their growth in monocultures, i.e. the strain with the lowest critical light intensity, V145, won the competition. Any effect of microcystins or other allelopathic substances would have counteracted this result. Hence, our findings do not support the suggestion that microcystins play an ecologically important role as allelopathic compounds in *Microcystis* population dynamics.

One explanation for the absence of allelopathic effects might be that the non-microcystin producing strains used in our study were resistant to microcystins. An alternative explanation might be that the extracellular microcystin concentrations in our experiments never exceeded 20 µg l<sup>-1</sup>, which is only 5 % of the cell-bound microcystin in the experiments. Despite high *Microcystis* densities, the extracellular microcystin concentrations in our experiments may still have been too low to have a significant negative effect on the growth of non-microcystin producing *Microcystis* strains. Similarly, Babica et al. (2006) recently concluded that ecologically relevant microcystin concentrations, as commonly found in *Microcystis* blooms, are generally too low to cause allelopathic effects on other photoautotrophic organisms.

*Seasonal dynamics of toxic and non-toxic strains.* Controlled laboratory experiments provide very simple environments in comparison to the full complexity of real aquatic ecosystems. For instance, in our laboratory experiments we found competitive exclusion, resulting in the dominance of a single strain. Under field conditions, however, coexistence of several *Microcystis* genotypes is often found (Kurmayer et al. 2002, Sanchis et al. 2005, Wilson et al. 2005, Ouelette et al. 2006). It might be that ecologically relevant aspects not investigated in our experiments, like zooplankton grazing or nutrient limitation, promote the coexistence of multiple strains in natural waters. Furthermore, differences in pigment composition may enable strains containing phycoerythrin, like strain V163, to use another part of the light spectrum, which may prolong their coexistence with strains containing phycocyanin (Stomp et al. 2004).

Yet, despite the simplified environments in laboratory experiments, there are striking similarities between the population dynamics in our competition experiments and the seasonal dynamics of toxic and non-toxic *Microcystis* strains in natural waters. In eutrophic lakes, the increase in total *Microcystis* biomass during the growing season is often accompanied by a decrease of the average microcystin concentration per cell (Fig. 1; see also Welker et al. 2003, Kardinaal and Visser 2005). We hypothesize that this seasonal pattern reflects a competitive replacement from toxic to non-toxic *Microcystis* strains within *Microcystis* blooms. The population dynamics in our competition experiments indicate that the strain composition within *Microcystis* populations determines the overall microcystin concentration. Moreover, the toxic strains were weaker competitors for light than the non-toxic strains, resulting in gradually declining microcystin concentrations during the competition experiments (Fig. 4, Fig. 6). Hence, our laboratory experiments demonstrate that, in principle, competition for light can drive a seasonal succession from toxic to non-toxic strains in dense *Microcystis* blooms.

*Acknowledgements* We thank G. Zwart for critical reading of the manuscript. The research of WEAK and IJ was funded by the Technology Foundation (STW, project ACH 4874). LT, SH, JH and PMV were supported by the Earth and Life Sciences Foundation (ALW), which is subsidized by the Netherlands Organization for Scientific Research (NWO). LT and PMV were additionally supported by a European Union grant within the program PEPCY.

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# Chapter 7

## SYNTHESIS



## 7.1 INTRODUCTION

Harmful cyanobacterial blooms are a globally occurring problem. The toxins produced by these cyanobacteria form health risks to both animals and humans, and can make lakes and reservoirs temporally unsuitable for use as drinking water or for recreational purposes. This thesis presents a series of studies aiming to improve our knowledge on cyanopeptide production. Microcystins are the most intensively studied group of cyanopeptides. Yet, until now, many aspects of microcystin production still remain unclear. For example, the physiological and/or ecological function of microcystins are not yet understood, the mechanisms underlying microcystin synthesis have not been fully elucidated, and the adaptive response of microcystin synthesis to environmental factors still holds several surprises. In particular, the large variation in microcystin concentrations observed in lakes and other aquatic ecosystems deserves further attention. Other bioactive oligopeptides produced by harmful cyanobacteria, in addition to microcystin, have scarcely been studied at all.

Although little information exists on the toxicity of cyanobacterial oligopeptides, other than the major cyanotoxin classes, it is clear that they represent a vast diversity of bioactive substances. The use of the term toxicity can be confusing since it generally refers to tests performed with mice or human cell lines. However, the effect of cyanopeptides on aquatic organisms living in the same ecosystem may be adverse. Vice versa, cyanopeptides that do not appear to be toxic to humans may have a lethal effect on aquatic organisms. Indeed, while the bioactivity of cyanopeptides may range from enzyme inhibitors to cytotoxicity, preliminary results based on short-term experiments with non-microcystin cyanobacterial peptides showed no direct cytotoxic effects on human cell lines (Hoeger and Dietrich 2004). However, we have no clue about the long-term effects of exposure to peptide concentrations in drinking or recreational water. The immense peptide diversity makes it impossible to investigate all potentially toxic peptides. A recent effort to classify cyanobacterial oligopeptides according to their chemical properties (Welker et al. 2006) may allow further study of peptide production using a systematic approach.

In this thesis, I studied several aspects of cyanobacterial oligopeptides, to increase our understanding of the oligopeptide production and regulation by harmful cyanobacteria. More precisely, we investigated the effects of salinity, light, nitrogen, and amino acid availability on the microcystin production and composition of the cyanobacteria *Microcystis aeruginosa* and *Planktothrix agardhii* (Chapters 2-4). Also, we investigated the effects of different environmental conditions on the production of other oligopeptides (like cyanopeptolins, anabaenopeptilides, and anabaenopeptins) by *Anabaena* sp. and *M. aeruginosa* (Chapter 5). Finally, we investigated the population dynamics of microcystin producing and non-microcystin producing *Microcystis* strains competing for light (Chapter 6).

In this chapter, our findings on the production and regulation of cyanobacterial oligopeptides will be discussed. In addition, the potential ecological implications of our findings will be presented.

## 7.2. PEPTIDE PRODUCTION AND REGULATION

7.2.1. *Bioactive oligopeptide production.* The study described in Chapter 5 is a first attempt to obtain an overview of the effects of different environmental factors on various bioactive oligopeptides, produced by two cyanobacterial species. In addition to microcystin, we also focused on the production of cyanopeptolins, anabaenopeptins and anabaenopeptilides in *M. aeruginosa* PCC 7806 and *Anabaena* 90. Our results show that the production of these oligopeptides resembles the constitutive production of microcystins (reviewed by Kardinaal and Visser, 2005). Microcystin and cyanopeptolin contents varied by a factor 2 to 3 across different experimental conditions, whereas the anabaenopeptins and anabaenopeptilides of *Anabaena* 90 varied more strongly, by a factor 6 to 10 (Chapter 5).

Little is known about the regulation of cyanobacterial peptide production. Unlike many bacterial secondary metabolites, which are mainly produced when growth ceases (Madigan et al. 2003), microcystins are mainly produced during the growth phase. Regulation may take place at the level of transcription, transcript stability, translation, enzyme activity and peptide turnover rate (Börner and Dittmann 2005). Regulatory mechanisms may be triggered by basic cellular processes and/or a variety of environmental stimuli. Indeed, a wide range of environmental factors is known to influence microcystin production (Chorus and Bartram 1999). An early review of these studies indicated that conditions most favorable for cyanobacterial growth also seemed to result in highest microcystin production rates (Sivonen and Jones 1999). The growth rate of cyanobacteria has even been proposed as a master factor steering microcystin production, regardless of the environmental conditions (Orr and Jones 1998). However, this growth-rate hypothesis has not been supported by subsequent studies. For instance, different patterns were found in a study on microcystin production by *M. aeruginosa* PCC 7806 as a function of photon irradiance (Wiedner et al. 2003), and by a study on the direct effect of light on the transcription and/or transcript stability of microcystin synthetase genes in the same strain (Kaebernick et al. 2000). Likewise, our data show a more complex response of peptide production to different environmental conditions. In fact, peptide production decreased with specific growth rate in many cases (Chapter 5). We therefore reject the growth-rate hypothesis, and suggest instead that different environmental conditions affect peptide production in different ways, depending on the peptides concerned, the pathways for peptide production, and the physiology of the organisms producing these peptides.

7.2.2. *Peptide variants and compensatory dynamics.* Many cyanobacteria produce different peptide variants simultaneously. These variants may differ in their toxicity. Nevertheless, few studies have investigated how the production of different variants is regulated (Oh et al. 2000, Rapala et al. 1997). The regulation of peptide variants is particularly interesting. While the core molecule of an entire peptide class is gene encoded, the variation within peptide classes does not necessarily have to be linked to a particular gene sequence. This may give cyanobacteria quite some flexibility in the production of different variants,

as illustrated by the microcystin variants [Asp<sup>3</sup>]microcystin-RR and [Asp<sup>3</sup>]microcystin-LR produced by *P. agardhii* 126/3 (Tonk et al. 2005, Chapter 3). While no clear variation was seen in the total microcystin content of *P. agardhii* exposed to a range of different light conditions, investigation of both microcystin variants separately revealed distinct patterns. [Asp<sup>3</sup>]Microcystin-RR content decreased twofold with increasing photon irradiance, whereas [Asp<sup>3</sup>]microcystin-LR content increased threefold. In other words, the total microcystin content of *P. agardhii* did not respond to photon irradiance, because the decrease in one variant was compensated by an increase of the other variant. Similar compensatory changes were observed for cyanopeptolin variants in *Microcystis aeruginosa* PCC 7806 (Chapter 5). Decreased cyanopeptolin A and C contents were accompanied by increased cyanopeptolin 970 contents. Variation in environmental conditions poses multiple challenges in cellular metabolism. We therefore suggest that the production of a range of peptide variants with similar functions might provide cyanobacteria with a better chance to sustain the production rate of the peptide class, and thus uphold their yet unknown function in a variable environment. Compensatory changes may not necessarily be restricted to peptide variants within a peptide class, but may also be observed between different peptide classes with comparable protease inhibitory effects. In *Anabaena* 90, we found that decreased anabaenopeptin A and C contents were accompanied by an increase in anabaenopeptilide 90B contents (Chapter 5).

*7.2.3 Regulation of peptide variants by amino acid availability.* In Chapter 3, we propose a regulatory mechanism for the compensatory dynamics of different microcystin variants synthesized by *P. agardhii* 126/3. Our hypothesis is based on differences in amino acid availability. [Asp<sup>3</sup>]Microcystin-RR and [Asp<sup>3</sup>]microcystin-LR differ from each other by one amino acid. [Asp<sup>3</sup>]Microcystin-RR contains arginine (R) at the exact position where the more toxic [Asp<sup>3</sup>]microcystin-LR contains leucine (L). Addition of leucine increased the LR/RR ratio of [Asp<sup>3</sup>]microcystin in a chemostat culture of *Planktothrix*, while addition of arginine decreased the LR/RR ratio (Chapter 4). This shows that the relative availability of amino acids indeed plays a direct role in the production of microcystin variants. In theory, amino acid availability could also be of importance for the synthesis of different variants within other peptide classes. To test whether regulation of peptide variants by amino acid availability is a common phenomenon, the experiments should be repeated with other strains producing other sets of oligopeptide variants.

Now that we know that amino acid availability can steer oligopeptide composition, a next step would be to understand how environmental changes may render differences in the intracellular availability of amino acids. This is by no means simple, because many processes in the cellular metabolism of cyanobacteria have an effect on amino acid availability. For instance, in addition to the formation of new amino acids through nitrogen assimilation, amino acids may also become available through the breakdown of cyanophycin or the degradation of phycocyanin pigments. During nitrogen excess, cyanobacteria can store their nitrogen surplus in cyanophycin, a polypeptide consisting of arginine (R) and aspartic acid (D) (Oppermann-Sanio and Steinbüchel 2002).

In rapidly growing cyanobacteria, cyanophycin is present only in small amounts (Allen 1988). During periods of nitrogen deficiency, however, arginine stored in cyanophycin can play an important role in the balance between the carbon and nitrogen metabolism of cyanobacteria (Maheswaran et al. 2006). Thus, nitrogen storage in cyanophycin might provide a buffer against changes in external nitrogen availability. To be more precise, under conditions of nitrogen excess there is a high demand for carbon-skeleton molecules, typically pyruvate, that are used for the synthesis of arginine through glutamine synthesis, and also for the synthesis of aspartic acid through oxaloacetate (Fig. 7.1). This drain on carbon-skeletons may deplete the availability of pyruvate for leucine synthesis. Thus, excess nitrogen may reduce the intracellular concentration of leucine. Conversely, if nitrogen is limited, a transient imbalance may increase the leucine level in cells relative to the levels of arginine and aspartic acid (Fig. 7.1). These processes, in turn, are likely to affect the production of arginine-based or leucine-based microcystin variants. Interacting effects of nitrogen and light on the production of different microcystin variants and the potential role of cyanophycin as a major store of arginine, deserve further investigation.

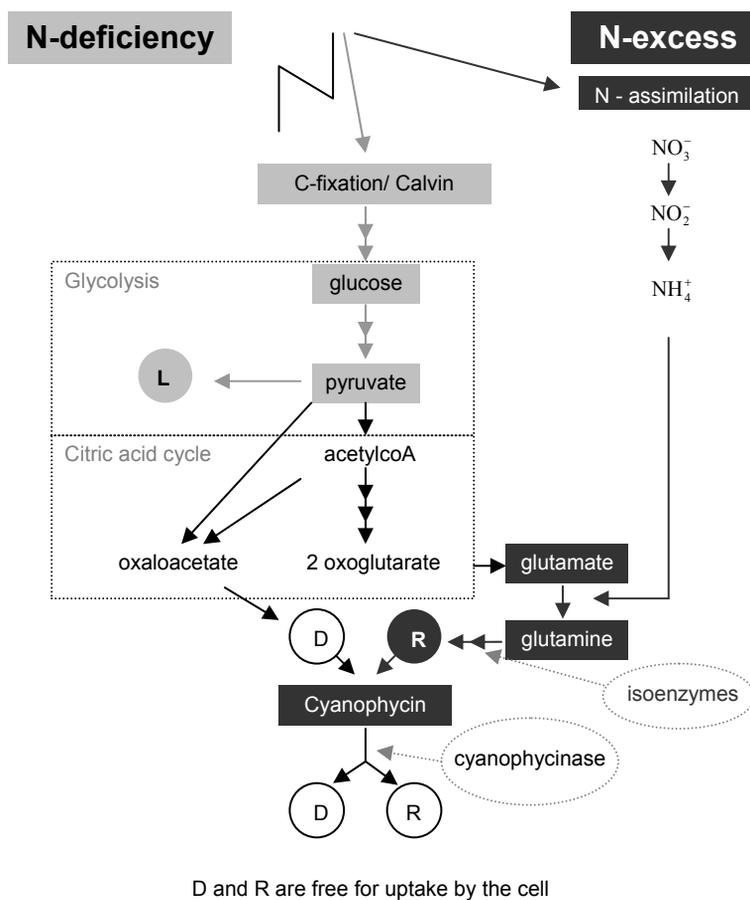


Figure 7.1: The pathway of cyanophycin (multi-L-arginine (R) poly-L-aspartic acid (D)) synthesis through the Calvin cycle (light-grey boxes) and N-assimilation (dark-grey boxes). Leucine (L) is synthesized earlier in the glycolysis from pyruvate. Arginine (R) is synthesized through glutamine from the citric acid cycle in the R biosynthetic pathway. Aspartic acid (D) is synthesized from oxaloacetate through pyruvate and acetylCoA. Cyanophycin synthesis is regulated by isoenzymes from the R biosynthetic pathway, which differentiate between R supply for either protein or cyanophycin synthesis (Leganés et al. 1998). Cyanophycinase degrades cyanophycin.

### 7.3. ECOLOGICAL IMPLICATIONS

In terms of ecological impacts and risk assessment it is now clear that a high level of differentiation is required to estimate the effects of cyanobacterial blooms. For a comprehensive overview one should not only investigate the different phytoplankton groups, the cyanobacterial species and strains, but also the peptide classes and variants produced by each strain. In fact, the dynamics of cyanopeptide concentrations in lakes are structured like a layered onion, in which one layer is embedded within the other:

- 1) Total phytoplankton biomass. At the highest level, there is variation in total phytoplankton biomass, for instance driven by nutrient or light conditions.
- 2) Harmful species. The total phytoplankton community consists of many species. Only some of these species belong to the harmful cyanobacteria. The relative proportion of harmful species in the total phytoplankton community changes in response to processes like competition and predation.
- 3) Toxic strains. Cyanobacterial species, in turn, consist of several different strains. Some of these strains are toxic, while others are not toxic.
- 4) Peptide classes. Toxic strains produce a variety of different peptide classes, like the microcystins, cyanopeptolins, and anabaenopeptilides.
- 5) Peptide variants. Each peptide class consists of several variants. These variants can differ in their toxicity.

The same environmental factors can have different, and sometimes even opposite, effects at different levels within this layered structure. For instance, light-limited conditions may favor the dominance of cyanobacteria, while within the cyanobacterial species the non-microcystin producing strains might be favored (Chapter 6), and the peptide composition within the microcystin producing strains may shift towards a less toxic peptide variant (Chapter 3). Hence, the actual changes in cyanopeptide concentrations in a given lake depend on the balance of different processes at different levels. With this knowledge, we are now ready to discuss how environmental conditions affect different levels of this layered structure:

*7.3.1 Peptide variants.* Environmental conditions in lakes may change on several time scales. Light conditions, for instance, vary due to changes in cloudiness or wind-induced resuspension of sediments. In Chapter 3, we showed that such changes in light conditions may profoundly affect the microcystin composition and thereby the toxicity of *P. agardhii* (Tonk et al. 2005), a widespread cyanobacterium of shallow, turbid lakes. As a result, monitoring the total abundance or the strain composition of *P. agardhii* is not sufficient to predict the toxicity of *P. agardhii* blooms. The harmful cyanobacterium *P. agardhii* produces a more toxic variant during periods of sunny weather, when recreational activities in lakes are most attractive, than during cloudy days.

Similar changes in cyanopeptide composition induced by environmental factors have been observed among the cyanopeptolins and between anabaenopeptins and anabaenopeptilides (Chapter 5). While the toxic effects (tested on human cell lines) of other cyanobacterial peptides seem to be lower than the toxicity of microcystins, some of these peptides, such as the anabaenopeptins and anabaenopeptilides, may compensate this lower toxicity by a higher variation in their content across different experimental conditions (Chapter 5). However, in order to explain differences in variability among different peptide groups in terms of compensatory dynamics, further knowledge on the toxicity of these different peptide groups will be desirable.

*7.3.2 Cyanobacterial strains.* Lakes dominated by *Microcystis* and *Planktothrix* may show considerable seasonal variability in total microcystin concentrations (Fastner et al. 1999, Kardinaal and Visser 2005). This striking seasonal variability exceeds the physiological variability in cellular microcystin contents reported for isolated *Microcystis* and *Planktothrix* strains in laboratory experiments (Oh et al. 2000, Hesse and Kohl 2001, Wiedner et al. 2003, Tonk et al. 2005). It is therefore likely that the variability in total microcystin concentration commonly observed in *Planktothrix*-dominated and *Microcystis*-dominated lakes results from a succession of closely related microcystin producing and non-microcystin producing strains. This is consistent with recent findings on the coexistence of microcystin-producing and non-microcystin-producing strains in *Planktothrix* and *Microcystis* dominated lakes (Fastner et al. 2001, Kurmayer et al. 2004). However, which factors determine the population dynamics of microcystin producing versus non-microcystin producing strains?

Competition for light is an important selective factor in phytoplankton communities of eutrophic waters (Mur and Schreurs 1995, Huisman et al. 1999, 2004), and plays a key role during the development of *Microcystis* blooms. The gradual increase in *Microcystis* biomass during bloom development will cause substantial shading, and thereby limits the light available for growth. The phytoplankton species (or strain) which can maintain its growth rate at the lowest critical light intensity will be the best competitor for light (Huisman et al. 1999). Our competition experiments for light between microcystin producing and non-microcystin producing *Microcystis* strains indicate that non-microcystin producing *Microcystis* strains are better competitors for light (Chapter 6; Kardinaal et al. 2007). Although the generality of this finding needs further testing with more microcystin producing and non-microcystin producing *Microcystis* strains, the competitive replacement of microcystin producing by non-microcystin producing strains offers a plausible explanation for the gradual decrease in average toxicity per cell commonly observed during the development of dense *Microcystis* blooms. In addition to competition for light, microcystin producing and non-microcystin producing strains may also compete for other resources, such as nutrients or inorganic carbon. For a more complete understanding of the population dynamics of microcystin producing and non-microcystin producing strains, it would therefore be interesting to run competition experiments for nutrients and inorganic carbon as well.

**7.3.3 Harmful cyanobacterial blooms.** Phytoplankton communities consist of many different taxonomic groups, including diatoms, dinoflagellates, green algae, and cyanobacteria. What determines the dominance of harmful cyanobacteria in many eutrophic waters? Many harmful cyanobacteria have lower maximal growth rates than other phytoplankton, such as diatoms or green algae (Van Liere and Walsby 1982, Huisman et al. 2004). The competitive ability of harmful cyanobacteria often lies in their unique characteristics, such as the possession of gas vesicles to adjust their vertical position in the water column (Walsby et al. 1997, Huisman et al. 2004), their ability to store essential nutrients and metabolites (Fig. 7.1), their nitrogen fixation capabilities (Smith 1983, De Nobel et al. 1997), and their ability to thrive under suboptimal environmental conditions such as low photon irradiance (Van Liere and Mur 1978). Although not all harmful cyanobacterial species share all these characteristics, many species often possess at least two or three. These distinctive characteristics of cyanobacteria contribute to their dominance over other phytoplankton groups, especially in eutrophic waters.

Increasing salinities in freshwater ecosystems, caused by agricultural practices, droughts, or sea level rise, are also likely to affect the phytoplankton composition. A number of harmful freshwater cyanobacteria, such as *Anabaena* and *Aphanizomenon*, are known to tolerate considerable increases in salt concentration. Chapter 2 shows that *Microcystis* PCC 7806 also has a rather high salt tolerance compared to most other freshwater phytoplankton species (Tonk et al. 2007). This indicates that in freshwater ecosystems exposed to increasing salinity, *Microcystis* may gain a competitive advantage over freshwater phytoplankton species. Conversely, when estuaries face a sudden input of freshwater, *Microcystis* may gain an advantage over resident marine phytoplankton species (Robson and Hamilton 2003). Thus, increasing salinity of freshwater ecosystems is another factor that might favor the dominance of harmful cyanobacteria.

On a global scale, water temperature and salinity are expected to increase during the next decades. *Microcystis aeruginosa* has a high temperature optimum and salt tolerance. Therefore, *Microcystis* blooms in particular are very likely to increase in frequency and abundance. Current debates in The Netherlands consider whether several Dutch freshwater lakes dominated by *Microcystis* should be converted into brackish waters (e.g., Lake Volkerak; Verspagen et al. 2006). Our results show that temporary exposure to salinities between 10 and 20 g liter<sup>-1</sup> not only allows survival of *Microcystis* populations, but may also lead to an increase of the extracellular microcystin concentrations. This implies that salinity fluctuations in brackish waters may not only favor *Microcystis* over other freshwater phytoplankton species, but may also enhance the exposure of many aquatic organisms to elevated microcystin concentrations.

Despite extensive studies of phytoplankton species, there are still many gaps in our scientific knowledge on the optimum ranges of environmental factors of phytoplankton species, including many harmful cyanobacteria. More data on the effects of environmental factors on phytoplankton growth rates and peptide production, and knowledge of the temperature and salinity ranges of the main phytoplankton species will be essential for models to predict the population dynamics in phytoplankton communities, and thus to predict harmful algal blooms. Improved knowledge of the growth optima and cyanopeptide production of harmful cyanobacteria is especially relevant in the context of global change.

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SAMENVATTING

&

SUMMARY



## SAMENVATTING

De impact van omgevingsfactoren op de productie van toxines en bioactieve peptiden door schadelijke cyanobacteriën.

Cyanobacteriën vormen een rijke bron van verschillende bioactieve stoffen. Van een aantal van deze stoffen is bekend dat zij een schadelijk effect op hun omgeving kunnen hebben. Microcystine (MC) is een voorbeeld van een door cyanobacteriën geproduceerd toxine dat regelmatig voorkomt in Nederlandse meren. Het is toxisch vanwege haar schadelijke effect op de lever. Met name in de zomer, wanneer de omgevingsfactoren gunstig zijn voor de groei van cyanobacteriën, zorgen deze voor veel overlast door in een dikke laag te accumuleren aan de wateroppervlakte. De concentratie van bioactieve stoffen kan tijdens een bloei zo hoog oplopen dat zij schadelijk is voor de dieren en planten in het betreffende ecosysteem. Toxineproductie veroorzaakt wereldwijd problemen in zoet water. Meren en zoetwaterbronnen met te hoge cyanotoxinegehalten worden tijdelijk ongeschikt verklaard voor gebruik als drinkwater en zwemwater. Microcystine is het meest onderzochte cyanotoxine. Andere bioactieve cyanopeptiden zijn tot dusver niet of nauwelijks onderzocht. In dit proefschrift heb ik een aantal aspecten van de productie van microcystines en andere bioactieve peptiden onderzocht, met als doel ons begrip van oligopeptide-productie en -regulatie door schadelijke cyanobacteriën te vergroten.

*Hoofdstuk 2* behandelt de zouttolerantie van *Microcystis aeruginosa* PCC 7806. Dit is een uitermate relevant onderwerp aangezien zoetwater ecosystemen wereldwijd geconfronteerd worden met toenemende zoutgehalten. Voor deze studie werd *Microcystis aeruginosa* PCC 7806 in semi-continu culturen gekweekt om het effect van geleidelijk toenemende zoutgehalten (tot  $20 \text{ g L}^{-1}$  totaal opgeloste zouten) op de groeisnelheid, het intra- en extracellulaire MC-gehalte en de MC-productie te onderzoeken. Daarnaast werd een experiment uitgevoerd om het effect van een zoutshock op de groei en microcystine-productie van *M. aeruginosa* te testen. De specifieke groeisnelheid, het intracellulaire MC-gehalte en de MC-productiesnelheid bleven min of meer gelijk bij zoutgehalten tot  $10 \text{ g L}^{-1}$ . De specifieke groeisnelheid nam dramatisch af bij een hoger zoutgehalte dan  $10 \text{ g L}^{-1}$  gedurende enige weken. De zoutshockexperimenten onthulden dat *Microcystis* tijdelijke verhogingen van het zoutgehalte tot zelfs  $17,5 \text{ g L}^{-1}$  kan overleven. Deze resultaten geven aan dat *Microcystis*, voor een zoetwater soort, een hoge zouttolerantie heeft. Stijgende zoutgehalten in zoetwaterecosystemen kunnen de blootstelling van aquatische organismen aan verhoogde extracellulaire microcystineconcentraties doen toenemen.

In *Hoofdstuk 3* hebben we het effect van de lichtintensiteit op de productie van twee microcystinevarianten van *Planktothrix agardhii* onderzocht. Dit is gedaan door het meten van het *mcyA* transcript, de totale microcystineproductie en de gehalten van de microcystinevarianten van *P. agardhii*, die gekweekt werd in lichtgelimiteerde turbidostataten.

Zowel het mRNAtranscriptgehalte van het *mcyA*-gen als de totale microcystine-productiesnelheid namen toe tot een lichtintensiteit van  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ , om vervolgens af te nemen bij een lichtintensiteit boven de  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Het totale microcystinegehalte van de celinhoud van *P. agardhii* filamenten bleef constant bij toenemende lichtintensiteit. Van de twee microcystinevarianten geproduceerd door *P. agardhii* nam het  $[\text{Asp}^3]\text{MC-RR}$  gehalte met een factor twee af met de lichtintensiteit, terwijl het  $[\text{Asp}^3]\text{MC-LR}$  gehalte met een factor drie toenam met de lichtintensiteit. Het is niet bekend wat ervoor zorgt welke microcystinevariant geproduceerd wordt.

In *Hoofdstuk 4* hebben we de invloed van de hoeveelheid beschikbare aminozuren op de synthese van verschillende microcystinevarianten door *P. agardhii* onderzocht. In dit hoofdstukponeren we de hypothese dat verschillen in de hoeveelheid beschikbare aminozuren eventueel een verklaring zouden kunnen zijn voor het feit dat *Planktothrix* minder arginine (R)- en meer leucine (L) bevattende microcystines (MC-RR respectievelijk MC-LR) produceert bij een hogere lichtintensiteit zoals werd gevonden in hoofdstuk 2. Om dit te testen hebben we leucine en arginine toegevoegd aan culturen, gekweekt bij lage en hoge lichtintensiteit ( $20$  and  $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). De toename van de  $[\text{Asp}^3]\text{MC-LR} / [\text{Asp}^3]\text{MC-RR}$  ratio in leucine verrijkt medium en de afname van deze ratio in arginine verrijkt medium impliceren dat de hoeveelheid beschikbare aminozuren een directe rol spelen bij de productie van specifieke microcystinevarianten.

Veranderingen in omgevingsfactoren hebben effect op het celmetabolisme en kunnen tot verschillen in de intracellulaire beschikbaarheid van leucine en arginine leiden. Een verschil in intracellulaire beschikbaarheid van aminozuren kan vervolgens de productie van  $[\text{Asp}^3]\text{MC-LR}$  en/of  $[\text{Asp}^3]\text{MC-RR}$  beïnvloeden. Naar aanleiding van het feit dat leucine één stikstofatoom bevat terwijl arginine vier stikstofatomen bevat hebben we de hypothese opgesteld dat een tekort aan stikstof (N) het relatieve aandeel van leucine in de aminozuur samenstelling ten gunste kan komen. Om dit te testen hebben we een continu cultuur van *P. agardhii* bemonsterd tijdens de overgang van een N verzadigende situatie naar een N limiterende situatie. De cellulaire concentraties van leucine en arginine namen af maar er vond geen verschuiving van de L/R ratio in het voordeel van leucine plaats. We vonden ook geen verandering in de  $[\text{Asp}^3]\text{MC-LR} / [\text{Asp}^3]\text{MC-RR}$  ratio onder invloed van N limitatie. De gecombineerde effecten van stikstof limitatie en licht limitatie op de productie van microcystine varianten en de mogelijke rol die cyanophycine daarbij speelt als opslag voor reserve arginine, behoeven verder onderzoek.

Gedurende de laatste jaren zijn er veel cyanobacteriële bioactieve peptiden ontdekt. Om ons inzicht in de drijfveren van peptide productie te vergroten en een zo juist mogelijke risico-inschatting te kunnen maken, is er ook informatie over de productie van deze “niet-microcystine” peptiden nodig. Mede daarom zijn in *Hoofdstuk 5* de effecten van veranderingen in lichtintensiteit, temperatuur en fosfaatbeschikbaarheid op de microcystine, cyanopeptoline- en anabaenopeptilide-productie van *Microcystis* PCC 7806 en *Anabaena* 90 onderzocht. *Microcystis* PCC 7806 produceerde twee microcystinevarianten en

drie cyanopeptolines, terwijl *Anabaena* 90 vier microcystinevarianten, drie anabaenopeptines en twee anabaenopeptilides produceerde. De microcystine- en cyanopeptolinegehalten varieerden met een factor 2 tot 3, terwijl de anabaenopeptines en de anabaenopeptilides een grotere variatie hadden. Evenals microcystine werden de overige peptiden onder alle opgelegde omstandigheden aangetroffen. Tijdens fosfaatlimitatie stegen de productiesnelheden van de peptiden evenredig met de groeisnelheid. Lichtintensiteit en temperatuur hadden een meer complex effect op de productiesnelheid en in veel gevallen nam deze juist af met een toenemende groeisnelheid. De stelling dat groeisnelheid de belangrijkste sturende factor is in peptide productie wordt dan ook niet ondersteund door onze studie. Onze hypothese luidt dat verschillende omgevingsfactoren de peptideproductie op verschillende manieren beïnvloedt, afhankelijk van het peptide in kwestie, het productietraject en de fysiologie van het organisme dat deze peptiden produceert. Tevens hebben we voor verschillende varianten waargenomen dat er compensatie plaatsvond. Terwijl de concentratie van bijvoorbeeld cyanopeptoline 970 toenam, nam de concentratie van cyanopeptolines A en C af. Op eenzelfde manier werd een afname in anabaenopeptine A en B gecompenseerd door een toename in anabaenopeptilide 90B. Wij stellen daarom dat het produceren van een reeks peptidenvarianten met vergelijkbare functie de kans vergroot dat een cyanobacterie een bepaalde peptideklasse in voldoende mate produceert en daarmee zijn eventuele functie onder variërende omstandigheden in stand kan houden. Oftewel, compensatie dynamiek in peptideproductie zou cyanobacteriën kunnen helpen een stabiel peptideniveau te handhaven in een variabele omgeving.

In veel meren neemt het gemiddelde microcystine gehalte per cel geleidelijk af: van hoge gehalten aan het begin van een *Microcystis* bloei tot lage gehalten gedurende de piek van een bloei. Deze seizoensdynamica zou een gevolg kunnen zijn van de successie van toxische en niet-toxische stammen, die doorgaans samen in *Microcystis* gedomineerde meren voorkomen. Om deze hypothese te onderzoeken, hebben we in *Hoofdstuk 6* competitie-experimenten uitgevoerd met twee toxische en twee niet-toxische *Microcystis* stammen, waarbij we gebruik hebben gemaakt van lichtgelimiteerde chemostaten. De populatiedynamica van deze twee stammen is gevolgd met behulp van karakteristieke veranderingen in lichtabsorptiespectra en PCR-amplificatie van de rRNA ITS regio in combinatie met Denaturatie-Gradiënt Gel Electroforese (DGGE), welke een identificatie en semi-quantificatie van de stammen bewerkstelligde. In alle experimenten hebben de toxische stammen de competitie om licht verloren van de niet-toxische stammen. Met als gevolg dat de totale microcystineconcentratie in de competitie-experimenten geleidelijk afnam. We hebben geen bewijs voor allelopathische interacties van toxische op niet-toxische stammen gevonden, aangezien de niet-toxische stammen zelfs domineerden wanneer de toxische stam een extreem hogere concentratie had bij aanvang van het experiment. Deze bevindingen tonen aan dat, in onze experimenten, de niet-toxische stammen van *Microcystis* sterkere 'strijders om het licht' zijn dan de toxische stammen. De vervanging van niet-toxische voor toxische stammen als gevolg van competitie

om licht vormt een plausibele verklaring voor de afname in toxineconcentratie per cel gedurende de ontwikkeling van een *Microcystis* bloei.

Wat betreft ecologische impact en risico-analyse is het duidelijk dat er een hoge mate van differentiatie nodig is om de effecten van een cyanobacteriële bloei goed in te kunnen schatten. Om een goed beeld te kunnen vormen van het risico van cyanotoxines in een meer zou je niet alleen de fytoplanktonsamenstelling, de verschillende cyanobacterie-soorten en -stammen moeten bepalen, maar ook de toxine-klassen en -varianten geproduceerd door elke stam. Dit wordt nog duidelijker nu blijkt dat omgevingsfactoren verschillende, en soms zelfs tegengestelde effecten hebben op de verschillende niveaus zoals hierboven beschreven. Bijvoorbeeld: lichtgelimiteerde condities kunnen cyanobacteriebloeï bevorderen, terwijl binnen de verschillende soorten de niet-toxische stammen voordeel hebben bij lichtlimitatie en de peptidesamenstelling naar de minder toxische variant verschuift. In de toekomst zullen met name verhoogde zoutgehaltes en temperatuur de fytoplankton samenstelling met name gaan beïnvloeden. Ondanks het vele onderzoek aan fytoplankton ontbreekt er nog veel kennis omtrent de optimum ranges van omgevingsfactoren van veel fytoplanktonsoorten, de schadelijke cyanobacteriën inbegrepen. Meer data over het effect van omgevingsfactoren op fytoplanktongroei en toxineproductie en kennis omtrent de temperatuuroptima en zouranges van fytoplankton zijn nodig om modellen te creëren om populatiedynamica in fytoplanktonsamenstelling en dus ook cyanobacteriebloeï beter te kunnen voorspellen.

## SUMMARY

Cyanobacteria produce a wide variety of bioactive compounds. Some of these compounds are known to have a toxic effect on their environment. Microcystin (MC) is an example of a cyanobacterial toxin that is frequently detected in Dutch lakes. Microcystin is toxic because of its harmful effect on the liver. During warm summers, when weather conditions are stable and photon irradiance and temperature are most favorable, cyanobacterial blooms often develop in freshwater lakes and brackish waters. During such a bloom the concentration of bioactive compounds can increase to heights that may be harmful for the animals and plants living in the ecosystem. Harmful cyanobacterial blooms are a globally occurring problem. The toxins can make lakes and reservoirs temporally unsuitable for use as drinking water or for recreational purposes. Microcystins are the most intensively studied group of cyanopeptides. Other bioactive oligopeptides produced by harmful cyanobacteria have scarcely been studied at all. In this thesis, I studied several aspects of cyanobacterial oligopeptides, to increase our understanding of the oligopeptide production and regulation by harmful cyanobacteria.

*Chapter 2* deals with the salt tolerance of the harmful cyanobacterium *Microcystis aeruginosa*. This is a pressing issue since freshwater ecosystems are confronted with increasing salinities worldwide. In this study, *Microcystis* strain PCC 7806 was grown in semi-continuous cultures to investigate the effect of a gradual salinity increase (up to 20 g L<sup>-1</sup> total dissolved salts) on the growth rate and toxin production in *M. aeruginosa*. Additionally, an experiment was performed to test the effect of a salinity shock on the growth rate and microcystin production of *Microcystis* PCC 7806. Specific growth rate, intracellular microcystin content and microcystin production rate remained more or less unaffected by salinity levels up to 10 g L<sup>-1</sup>. Specific growth rate collapsed when salinity was increased beyond 10 g L<sup>-1</sup> for several weeks. Salt-shock experiments revealed that *Microcystis* can temporarily endure salinities as high as 17.5 g L<sup>-1</sup>. These results indicate that *Microcystis* has a high salt tolerance for a freshwater species. Rising salinities in freshwater ecosystems may enhance the exposure of aquatic organisms to elevated concentrations of extracellular microcystins.

In *Chapter 3* we investigated the effect of photon irradiance on the production and composition of the two microcystin variants produced by *Planktothrix agardhii*. This was accomplished by measuring the *mcyA* transcript, the total microcystin production and the production rates of [Asp<sup>3</sup>]MC-RR and [Asp<sup>3</sup>]MC-LR separately in light-limited turbidostats. Both the amount of mRNA transcript of the *mcyA* gene and the total microcystin production rate increased with photon irradiance up to 60 μmol m<sup>-2</sup> s<sup>-1</sup>, but started to decrease with irradiance beyond 100 μmol m<sup>-2</sup> s<sup>-1</sup>. The cellular content of total microcystin remained constant, independent of irradiance. However, of the two main microcystin variants detected in *P. agardhii*, the [Asp<sup>3</sup>]MC-RR content decreased twofold with photon irradiance, whereas the [Asp<sup>3</sup>]MC-LR content increased threefold. [Asp<sup>3</sup>]MC-RR contains arginine at the position where the more toxic [Asp<sup>3</sup>]MC-LR incorporates leucine. It is not known what exactly causes the synthesis of one variant opposed to another.

In *Chapter 4* we investigated the importance of the amino acid availability on the composition of microcystin variants in *Planktothrix agardhii*. We hypothesized that differences in amino acid availability might explain why *Planktothrix* produced relatively less [Asp<sup>3</sup>]MC-RR and more [Asp<sup>3</sup>]MC-LR at higher light intensities. In order to test this hypothesis leucine (L) and arginine (R) were added to cultures grown at low and high photon irradiance (20 and 80  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). The increase of the [Asp<sup>3</sup>]MC-LR/[Asp<sup>3</sup>]MC-RR ratio in leucine enriched medium and the decrease of this ratio in arginine enriched medium indicates that the amount of available amino acids play a direct role in the production of the specific microcystin variants.

Environmental changes affecting cell metabolism may render differences in the intracellular availability of L and R, which can affect the production of [Asp<sup>3</sup>]MC-LR and/or RR. Since L contains one N atom while R contains four N atoms, we hypothesized that low nitrogen availability might shift the amino acid composition in favor of L. However, when a continuous culture of *P. agardhii* was shifted from nitrogen-saturated to nitrogen-limited mineral medium, the cellular concentrations of both L and R decreased, but the L/R ratio did not change in favor of L. Accordingly, while the total microcystin concentration of the cells decreased, we did not observe changes in the [Asp<sup>3</sup>]MC-LR/[Asp<sup>3</sup>]MC-RR ratio in response to nitrogen limitation. The interactive effects of nitrogen limitation and light limitation on the production of different microcystin variants and the potential role of cyanophycin as a major store of R deserve further investigation.

During recent years, various new bioactive oligopeptides have been discovered in cyanobacteria, and more information on their production rates is required to improve our understanding of what drives peptide production and to guide cyanotoxin risk assessment. In *Chapter 5*, we investigated effects of light intensity, temperature, and phosphorus limitation on the peptide production of the cyanobacteria *Microcystis* PCC 7806 and *Anabaena* 90. *Microcystis* PCC 7806 produced two microcystin variants and three cyanopeptolins, whereas *Anabaena* 90 produced four microcystin variants, three anabaenopeptins, and two anabaenopeptilides. Microcystin and cyanopeptolin contents varied by a factor 2 to 3, whereas the anabaenopeptins and anabaenopeptilides of *Anabaena* varied more strongly. Under phosphorus limitation, peptide production rates increased with the specific growth rate. The response of peptide production to light intensity and temperature was more complex, and in many cases peptide production decreased with specific growth rate. We observed compensatory changes of different peptide variants. For instance, decreased cyanopeptolin A and C contents were accompanied by increased cyanopeptolin 970 contents. Likewise, decreased anabaenopeptin A and C contents were accompanied by increased anabaenopeptilide 90B contents. Compensatory dynamics in peptide production may enable cyanobacteria to sustain stable peptide levels in a variable environment.

In several lakes the average microcystin content per cell gradually declines from high levels at the onset of *Microcystis* blooms towards low levels at the height of the bloom. Such seasonal dynamics might result from a succession of toxic to non-toxic strains. In *Chapter 6* we ran competition experiments with two toxic and two non-toxic *Microcystis* strains using

light-limited chemostats to investigate this hypothesis. The population dynamics of these closely related strains were monitored by means of characteristic changes in light absorbance spectra and by PCR amplification of the rRNA ITS region in combination with denaturing gradient gel electrophoresis (DGGE), which allowed identification and semi-quantification of the competing strains. In all experiments the toxic strains lost competition for light from non-toxic strains. As a consequence, the total microcystin concentrations in the competition experiments gradually declined. We did not find evidence for allelopathic interactions, as non-toxic strains became dominant even when toxic strains were given a major initial advantage. These findings show that, in our experiments, non-toxic strains of *Microcystis* were better competitors for light than toxic strains. The generality of this finding deserves further investigation with other *Microcystis* strains. The competitive replacement of toxic by non-toxic strains offers a plausible explanation for the gradual decrease in average toxicity per cell during the development of dense *Microcystis* blooms.

In terms of ecological impacts and risk assessment it is now clear that a high level of differentiation is required to estimate the effects of cyanobacterial blooms. For a comprehensive overview one should not only investigate the different phytoplankton groups, the cyanobacterial species and strains, but also the peptide classes and variants produced by each strain. This becomes even more clear since we found that the same environmental factors can have different, and sometimes even opposite, effects at different levels within this layered structure. For instance, light-limited conditions may favor the dominance of cyanobacteria, while within the cyanobacterial species the non-microcystin producing strains might be favored, and the peptide composition within the microcystin producing strains may shift towards a less toxic peptide variant. In the future, increasing salinity levels and temperature are likely to affect phytoplankton composition. Despite extensive studies of phytoplankton species, there are still many gaps in our scientific knowledge on the optimum ranges of environmental factors of phytoplankton species, including many harmful cyanobacteria. More data on the effects of environmental factors on phytoplankton growth rates and peptide production, and knowledge of the temperature and salinity ranges of the main phytoplankton species will be essential for models to predict the population dynamics in phytoplankton communities, and thus to predict harmful algal blooms. Improved knowledge of the growth optima and cyanopeptide production of harmful cyanobacteria is especially relevant in the context of global change.



# DANKWOORD

Zoals ik met nieuwjaar bij binnenkomst in een volle kamer ook het liefste riep: “Gelukkig nieuwjaar allemaal, ik ga niet zoenen!”, wat trouwens niet altijd opging, zou ik nu ook willen zeggen: “Mensen bedankt!”. Maar ja zo werkt het natuurlijk niet, dus hier volgt mijn dank.

Ten eerste wil ik mijn promotoren bedanken die dit proefschrift mede mogelijk gemaakt hebben. Jef wil ik graag bedanken voor z’n enorme toewijding en geduld, met name in de eindfase van de hoofdstukken en Petra voor het langeafstandswerk en de fijne samenwerking, die goede sfeer heeft in hoge mate bijgedragen aan een relatief relaxte promotietijd. Erg waardevol was voor mij ook de prettige samenwerking met Hans Matthijs, met wie ik vele interessante en plezierige discussies heb mogen voeren.

Veel dank gaat uit naar de enthousiaste studenten die me vol overgave geholpen hebben tijdens hun stage, Oliver, Merijn en Kim, behalve kundig waren ze vooral ook erg leuk. Dan wil ik mijn kamergenoten bedanken, Edwin, Pedro en met name Jolanda omdat ze altijd bereid was uitgebreid op al m’n vragen in te gaan en zo gezellig is. Verder wil ik natuurlijk al m’n collega’s en ex-collega’s van aquatische microbiologie bedanken voor de goede samenwerking en de vriendelijke, collegiale sfeer die onze vakgroep ademt, best bijzonder. Met name wil ik Corrien, Dedmer, Hans Balke, Jutta, Klaus, Luuc, Maayke, Pascale, Pieter en Suzanne voor hun hulp bedanken. Ook wil ik even de zooxanthellen-bende (Robert, Pim en Pedro) bedanken, waar ik een beetje bij heb kunnen klussen bij wijze van hobby. Gabriel Zwart wil ik bedanken voor zijn hulp tijdens Petra’s verlof en Reshma en Marion voor hun hulp op het NIOO. De monsternemers van Rijnland wil ik graag bedanken, en dan vooral Kees en Marvin, met wie ik vaker mee de Zegerplas op ging en altijd behulpzaam en relaxed waren. In de weken dat Rijnland de Zegerplas niet bemonsterde kreeg ik de hulp van Gerard de Smits van windsurfvereniging Zegerplas, die dan met mij in het weekend op algenjacht ging, deze vriendelijkheid en gastvrijheid heb ik zeer gewaardeerd. Meneer van der Schilden wil ik daar ook graag voor bedanken. En als ik dan helemaal niemand meer kon vinden om op veldwerk te gaan, ging Vanessa mee! Eugenia wil ik ook nog bedanken voor het tellen van al die cellen.

Many thanks go out to the people of the CYANOTOX en PEPCY project. It has been wonderful to complete a PhD in such an esteemed scientific but also friendly environment! Special thanks go out to those I had the pleasure of cooperating more closely with, Claudia, Elke, Guntram, Rainer, Eva, Louise, Jutta, Ingrid and especially Martin, for guiding me through the mazes of peptide nomenclature and quantification and preventing me from introducing nonexistent ones.

Dan lijkt het me nu een mooi moment om mijn paranimfen Eveline en Edwin te bedanken. Ik heb ze niet voor niets uitgekozen om mij bij te staan in het hol van de leeuw. Eveline, geweldige vriendin en eindeloze positivist en Edwin, kameraad, buurman en copiloot, bij wie ik met al mijn vragen terecht kon, van taartvorm tot HPLC.

Berber kwam precies op het juiste moment met het aanbod de lay-out van dit proefschrift te verzorgen en dat heeft ze geweldig gedaan, superbedankt daarvoor!

M'n ouders wil ik bedanken voor hun onvoorwaardelijke liefde en steun waar ik al mijn hele leven van heb mogen genieten en omdat ze, vooral op het gebied van doorzettingsvermogen, mijn absolute voorbeeld zijn. Laura wil ik bedanken omdat ze een geweldige zus voor me is en ook omdat ze samen met Roger, Willem, Isabel en Rogier op de wereld heeft gezet en de liefste moeder is die zij zich kunnen wensen. Opa wil ik bedanken voor zijn interesse in mijn werk en zijn plezier in het leren van nieuwe dingen, waarvan ik ook het een en ander heb meegekregen.

Nu ik toch lekker bezig ben wil ik eigenlijk ook m'n vrienden bedanken voor hun steun, aanmoediging en gezelligheid, Weertenaren, Gruningers, Curaçao-gangers en Amsterdammers respectievelijk alaf, moi, hopi danki swahs en helemaal top! De mensen van opvanghuis Jan Steen, Eef, Jochem en Jillis, dank, dubbeldank voor steun, lekker eten, héérlijke wijntjes en veel champagne... en het is er wederom een mooie gelegenheid voor! Zonder andere sausers en niet-sausers te kort te doen wil ik ook nog even Annemarie en Mai-britt bedanken omdat elke activiteit in hun gezelschap op een knotsgekke gezellige gebeurtenis uitliep wat een enorme uitlaatklep voor me is geweest. Rogier en Marijke wil ik bedanken omdat ze hun vakantie naar Thailand speciaal voor deze gelegenheid hebben willen verzetten.

Als laatste wil ik Noam bedanken, vooral om de persoon en vriend die je bent maar ook om de woestijnen van bewegingsvrijheid en lucht die je me gegeven hebt. Ik had ze nodig en heb er nooit om hoeven vragen, respect chamoed. Zo en dan is het nu tijd voor een feestje! Want daar gaat per slot van rekening toch allemaal om...

### *Curriculum Vitae*

Linda Tonk werd geboren op 25 Januari 1976 te Weert. In 1993 haalde zij haar HAVO diploma aan de Philips van Horne in Weert, waar ze vervolgens in 1995 haar vwo diploma bemachtigde. Van 1995 tot 2000 studeerde zij Biologie aan de Rijksuniversiteit Groningen (RuG) waar ze afstudeerde in de richting Mariene Biologie. Haar stages betroffen “The genetic diversity in the coral genus *Madracis*”, bij de vakgroep Mariene Biologie aan de RuG en “The linkage of Caribbean *Clionid* sponges to seawater column bacteria by means of filterfeeding”, bij het Nederlands Instituut voor Onderzoek der Zee (NIOZ), uitgevoerd op Curaçao. Na haar studie kwam ze in 2001 terecht bij de vakgroep Aquatische Microbiologie aan de Universiteit van Amsterdam, waar ze op het Europese Unie project CYANOTOX aan toxische cyanobacteriën kwam te werken. Vervolgens werkte ze mee aan het Ecologie, Economie en Technologie (EET) project, waarna ze vanaf 2003 bij dezelfde vakgroep op het PEPCY project, wederom een project vanuit de Europese Unie, aan de peptiden productie van cyanobacteriën heeft gewerkt, wat tot dit proefschrift heeft geleid. Op het moment geniet zij van een typisch Nederlandse zomer waarna zij zich op een nog te verkrijgen post-doc baan zal richten, bij voorkeur op het gebied van Mariene Microbiologie. Voor een meer kleurrijke presentatie van mijn cv, zie mijn webpagina op [staff.science.uva.nl/~ltonk/](http://staff.science.uva.nl/~ltonk/).