Vakgroep Biologie
Laboratorium voor Protistologie en Aquatische ecologie

Population structure of the cyanobacterium *Microcystis* mediated by history, grazing and interstrain interactions

Populatiestructuur van de cyanobacterie *Microcystis* gemedieerd door historische factoren, begrazing en interacties tussen stammen

Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de Wetenschappen: Biologie

Publieke verdediging: 06/03/2009

Ineke van Gremberghe
Academiejaar 2008-2009

Promotor: Prof. Dr. Wim Vyverman (Universiteit Gent)
Co-promotor: Prof. Dr. Luc De Meester (Katholieke Universiteit Leuven)

Leden van de leescommissie:
Prof. Dr. Wim Vyverman (Universiteit Gent)
Prof. Dr. Luc De Meester (Katholieke Universiteit Leuven)
Prof. Dr. Lucien Hoffmann (Centre de Recherche Public - Gabriel Lippmann)
Dr. Petra Visser (Universiteit van Amsterdam)

Overige leden van de examencommissie:
Prof. Dr. Dominique Adriaens (Universiteit Gent)
Prof. Dr. Anne Willems (Universiteit Gent)
Dr. Sofie Derycke (Universiteit Gent)
Dr. Pieter Vanormelingen (Universiteit Gent)

The research reported in this thesis was performed in the Laboratory of Protistology and Aquatic Ecology, Biology Department, Ghent University, Krijgslaan 281-S8, B-9000 Ghent, Belgium. http://www.pae.ugent.be/

Dankwoord

De voorbije vier jaar zou ik kunnen samenvatten als een periode van hard werken, zeer veel bijleren op korte tijd en ook veel fijne momenten in het labo voor Protistologie en Aquatische ecologie. Ik heb de kans gekregen om zeer afwisselende dingen te doen tijdens mijn doctoraat. Dit ging van meewerken met projectaanvragen, moleculair werk, cultuurwerk, experimenten, staalnames, statistische analyses tot het schrijven van artikels, en mocht hierbij deel uit maken van verschillende projecten. Ik ben dan ook zeer dankbaar dat ik deze ervaring heb mogen meemaken en hoop dat ik nog een tijdje in het onderzoekswereldje kan blijven.

Mijn doctoraatsthesis zou nooit tot stand zijn gekomen zonder de hulp van vele mensen die ik hierbij dan ook allemaal wil bedankten. Moest ik toch iemand vergeten zijn, is dit uiteraard niet met opzet.

Mijn dank gaat allereerst uit naar mijn promotor Prof. Wim Vyverman die mij de kans heeft gegeven een doctoraat te mogen doen in het labo voor Protistologie en Aquatische ecologie en daarnaast ook geholpen heeft met de concrete invulling van dit doctoraat.

Mijn dank gaat ook uit naar mijn co-promotor Prof. Luc De Meester voor de zeer concrete opmerkingen en suggesties bij mijn verschillende hoofdstukken.

Katleen Van der Gucht heeft mij vooral in het begin van mijn doctoraat goed op weg geholpen, wat er mede voor heeft gezorgd dat er niet te veel tijd verloren ging en ik wil haar hiervoor bedanken.

Pieter Vanormelingen heeft mij veel geholpen tijdens het schijven van de verschillende hoofdstukken (tot op het laatst tijdens de kerstvakantie net voor het indienen in januari) waarvoor mijn oprechte dank.

Frederik Leliaert en Joachim Mergeay hebben zeer veel bijgedragen tot hoofdstuk 2, tijdens een ook voor hen drukke periode waarvoor ik hen wil bedanken.

Tsehaye Asmelash, Tadesse Dejenie, Luc De Meester, Steven Declerck en alle andere mensen van het Aquatic Ecology project in Tigray (Ethiopië) wil ik bedanken voor de staalnames voor hoofdstuk 3.

Sofie D'hondt wil ik bedankten voor de 'DGGE hulp', en natuurlijk ook voor de fijne momenten in het Centrum voor Moleculaire Fylogenie en Evolutie waar ik de eerste drie jaar van mijn doctoraat werkte.

Sofie D'hondt en Antoniya Mancheva dank ik ook voor de praktische hulp bij het experiment dat beschreven werd in hoofdstuk 4 en Caroline Souffreau en Pieter Vanormelingen voor de praktische hulp bij het 'grote' experiment dat leidde tot hoofdstukken 5 en 6.

Bart Vanelslander wil ik bedanken voor de ideeën en concrete hulp bij het schrijven van hoofdstuk 5.

Jeroen Van Wichelen wil ik bedanken voor de staalnames van Appendix A en de samenwerking tijdens B-Blooms en B-Blooms2.

Annick Wilmotte en Petra Visser dank ik voor algemene tips i.v.m. het werken met Microcystis.

Ook de studenten waarmee ik gewerkt heb wil ik bedanken voor hun inzet: Kevin De Keyser, Niels Halewyck, Mike Maveau, Evelien Van de Vyver, Jonas D'Hondt en Ann-Eline Debeer. Ann-Eline wil ik dan ook nog bedankten voor de hulp bij hoofdstuk 2.

Daarnaast gaat mijn dank ook uit naar de mensen die misschien minder rechstreeks meewerken aan de verschillende hoofdstukken van mijn doctoraat maar toch bijdraagden met praktische tips of gewoon gezellige momenten tijdens het werk. Hierbij denk ik aan mijn collega's in het Centrum voor Moleculaire Fylogenie en Evolutie: Sylvie Cousin, Fabian Van Haelst, Tine Verstraete, en mijn collega's op de Sterre: Caroline Souffreau, Katrijn Vannerum en Maureen Fagot (collega's uit het 'vrouwenkot', onze bureau in de S8), en aan Griet Casteleyn en Mieke Sterken voor het samen 'afzien' op het einde van het doctoraat, en alle andere mensen van de PAE.

Natuurlijk zijn er ook nog mijn vrienden en familie die ik ook wil bedankten voor hun steun en hulp. Mijn ouders, broer en zus wil ik bedanken voor hun hulp en interesse in mijn doctoraat. Mijn vrienden ('De bende van de bosklappers': Raf Roelant, Filip De Bock, Karen Migneaux en Venessa Van Eeckhoven, allemaal met vriend/vriendin ondertussen) waarmee ik drie fantantische reizen meemaakte en waarmee lief en leed werd gedeeld op café. Raf wou ik ook nog eens extra bedanken voor de hulp met LaTeX toen Benny en ik ogen te kort kwamen de laatste nacht voor het indienen in januari.

En tot slot wil ik mijn vriend Benny bedanken voor zijn geduld en steun in de afgelopen vier jaar (en de jaren daarvoor...) die toch ook wel stressvol waren, en alle concrete IT-hulp (o.a. met LaTeX en Manteltester). Ik hoop na alle drukte van het doctoraat voor ons nog een goede verhuis en fijn huwelijk dit jaar!

Ineke
maart 2009

Acknowledgement of financial support

The research was carried out in the framework of different projects:

- B-Blooms ('Algal blooms: emerging problem for health and sustainable use of surface waters'), nationally supported by BELSPO (Belgian Science Policy)
- B-Blooms2 ('Cyanobacterial blooms: toxicity, diversity, modelling and management'), nationally supported by BELSPO
- BIOPOOL ('Connectivity, dispersal and priority effects as drivers of biodiversity and ecosystem function in pond and pool communities'), ESF (European Science Foundation) EURODIVERSITY project, nationally supported by BELSPO and FWO (Fonds Wetenschappelijk Onderzoek Vlaanderen)
- Aquatic Ecology project in the VLIR IUS-MEKELLE programme ('Aquatic Ecology and sustainable management of inland water bodies in Tigray, North Ethiopia'), supported by VLIR (Vlaamse Interuniversitaire Raad)
- BIOMAN ('Biodiversity and human impact in shallow lakes'), supported by the European Union
- SALGA ('South American Lake Gradient Analysis'), supported by NWO/WOTRO and other financiers[1]
- 'Thermal adaptation and its interplay with biotic interactions across a latitude gradient', research project (G.0419.08) of FWO

[1] www.projectenaew.wur.nl/salga/

Contents

Chapter 1

General Introduction

1.1 The bloom forming cyanobacterium *Microcystis*

Cyanobacteria form a diverse group of autotrophic Bacteria and are found in aquatic (fresh water as well as marine) and terrestrial environments. They arose probably more than 3 billion years ago (Knoll 2008) and have played a major role in the evolution of life on earth because they were responsible for the rise in atmospheric oxygen over 2 billion years ago (Van den Hoek *et al.* 1995). Cyanobacteria play a key role in the functioning of inland and marine aquatic ecosystems, as they are a major constituent of phytoplankton communities. Massive growth of cyanobacterial phytoplankton however, generally in response to eutrophication, can lead to dense, often almost monospecific water blooms. The most important factors stimulating the development of these blooms are a high temperature, a relatively low light intensity, a stable water column (stratified lakes), a low CO_2 concentration, a high phosphorus concentration and a low N/P ratio (Chorus and Bartram 1999; Huisman *et al.* 2005). Once established, a cyanobacterial bloom may itself influence (a)biotic conditions in such a way that positive feedback loops (internal phosphorus loading, light conditions, temperature, CO_2 depletion) are established, which stabilize cyanobacterial dominance (Scheffer *et al.* 1997; Bicudo *et al.* 2007), making it difficult to disentangle cause and consequence. Furthermore, it is important to know the species identity of the bloom forming cyanobacterium because several cyanobacteria produce hepato-, neuro- and cytotoxins, irritants and gastrointestinal toxins (e.g. *Microcystis*, *Planktothrix*, *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*) that are highly toxic to eukaryotes (Codd *et al.* 2005), and cause health problems in recreational waters and drinking water reservoirs. Due to both global warming and eutrophication of standing waters, the frequency of these blooms has increased worldwide in the last decade, and this increase will probably continue (Huisman *et al.* 2005; Jöhnk *et al.* 2008).

One of the most widespread bloom forming cyanobacteria is *Microcystis* (order Chroococcales), which has, as a result, received much scientific attention (Huisman *et al.* 2005). *Microcystis* is easily recognizable morphologically based on the coccal shape of the cells, the presence of gas vesicles which give the cells their characteristic

granulous view and the formation of large colonies of diverse shapes surrounded by mucilaginous sheets (Fig. 1.1). *Microcystis* forms a well-supported clade on the basis of 16S ribosomal DNA (rDNA) sequences, with little intraclade variation (Otsuka *et al.* 1998).

Figure 1.1: Photographs of *Microcystis* blooms with dense scums in the nature reserves Leeuwenhof (Ghent, Belgium) taken by Jeroen Van Wichelen (A) and Renaat Dasseville (B) and Tiens Broek (Tienen, Belgium) taken by Pieter Vanormelingen (C), and microscopic photograph of *Microcystis* colony isolated from Leeuwenhof taken by Ineke van Gremberghe. Scale bar = 100 µm.

Blooms of *Microcystis* (Fig. 1.1) often occur during summer in temperate regions and year-round in (sub)tropical regions in ponds and lakes with a high nutrient load and stable water column (Reynolds *et al.* 1981; Chorus and Bartram 1999; Visser *et al.* 2005, Asmelash *et al.* in preparation). During colder periods, *Microcystis* forms resting stages in the sediment and is recruited from the sediment in spring (Verspagen *et al.* 2004). Early in the season, when *Microcystis* biomass is still low, unicellular or small colony forming *Microcystis* can be found, but at the peak of the bloom the colonies can be very large and individual colonies can be visible by eye. The ability to form colonies entails advantages such as protection against predation (Fulton and Paerl 1987), the ability to float, a higher photosynthetic efficiency and enhanced stress tolerance compared to unicellular strains[1] (Zhang *et al.* 2007; Wu and Song 2008). The gas vesicles inside the cells make the colonies buoyant, and a dense floating layer of colonies may be present at the water surface under calm weather conditions, which may accumulate on one side of the water body due to wind action. When carbohydrates accumulate in the cell due to photosynthetic activity under high light conditions, the *Microcystis* colonies sink to the bottom.

One of the most remarkable characteristics of *Microcystis* is its ability to produce the hepatotoxic microcystins, which is the best-studied group of toxins produced by cyanobacteria (Carmichael 1994; Codd *et al.* 2005). Microcystin is a cyclic heptapeptide,

[1] *Microcystis* strain: *Microcystis* cell or colony in nature or monoclonal *Microcystis* culture

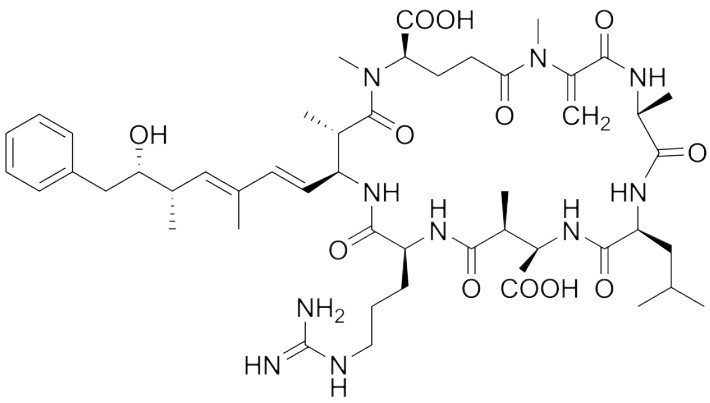


Figure 1.2: Molecular structure of microcystin-LR.

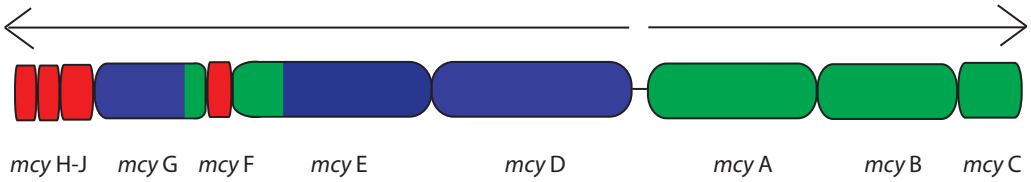


Figure 1.3: Microcystin synthetase gene cluster in *Microcystis aeruginosa* PCC 7806. Green = genes encoding peptide synthetase modules, blue = genes encoding polyketide synthase modules, red = genes encoding modifying enzymes. Arrows indicate the direction of transcription.

non-ribosomally synthesized by a multifunctional enzyme complex (Börner and Dittmann, 2005). The heptapeptide consists of five non-protein amino acids and two variable protein amino acids. These two amino acids distinguish several microcystin variants, of which more than 70 are described. The best-known microcystin is microcystin-LR consisting of leucine (L) and arginine (R) (Fig. 1.2). The gene cluster coding for the microcystin synthetase complex consists of ten *mcy*-genes (Tillett *et al.* 2000) and has a total length of 55 kb. The genes are transcribed as two polycistronic operons, *mcy*ABC en *mcy*DEFGHIJ, from a central bidirectional promoter (Fig. 1.3). Not all *Microcystis* strains possess a complete microcystin synthetase gene cluster, and these strains probably lost their ability to produce microcystins in the course of evolution (Rantala *et al.* 2004). Toxicity is mediated through the active transport of microcystin into hepatocytes by the bile acid organic anion transport system, followed by inhibition of eukaryotic serine/threonine protein phosphatases 1 and 2A (Runnegar *et al.* 1995) and causing oxidative stress (Ding *et al.* 2000). Poisoning by microcystin causes gastro-intestinal problems in humans and cattle and can lead to casualties (Codd *et al.* 2005). It also causes mortality of zooplankton and algae (Falconer 1999; Carmichael *et al.* 2001; Huisman *et al.* 2005). The toxicity of microcystins can be compared with cobra poison (intravenous LD50 for mice for microcystin-LR: 50 $\mu g\, kg^{-1}$ body weight). Accordingly, the World Health Organization has set the concentration of microcystin-LR that can be present in drinking water to be lower than 1 $\mu g\, l^{-1}$ (WHO 1998). Microcystins are mainly present within the cells. There is a limited active

excretion of microcystins (Pearson *et al.* 2004), but they are usually released into the water column when the cells die and lyse. Moreover, microcystins can remain in the water column for several days or weeks even when the bloom has disappeared (Chorus and Bartram 1999). The toxicity of a bloom is variable and depends on the biomass of the bloom, its genetic composition, the cellular microcystin production and the variants of microcystin produced (Kardinaal and Visser 2005). Microcystin production is influenced by external growth stimuli such as light, temperature and concentrations of iron, nitrogen and phosphorus (Lukac and Aegerter 1993; Utkilen and Gjolme 1995; Rapala *et al.* 1997; Kaebernick *et al.* 2000), as well as by the presence of zooplankton (Jang *et al.* 2003). The function of microcystin is still heavily debated. Some studies suggest a function of microcystin in the regulation of light harvesting (Kaebernick *et al.* 2000), in iron uptake (Utkilen and Gjolme 1995) or intercellular communication (Dittmann *et al.* 2001). Microcystin might also play an allelopathic role and suppress the growth of other phytoplankton species (Babica *et al.* 2006). Several studies also suggest an important role of microcystin as a defense mechanism against predation by zooplankton (DeMott 1999; Rohrlack *et al.* 1999a, 2001; Lotocka 2001).

1.2 Taxonomy, genetic diversity and population structure of *Microcystis*

Cyanobacteria were originally classified as blue green algae by Linnaeus (1753). This classification was further developed by botanists using morphological characteristics under the rules of the Botanical Code. Using new technologies like electron microscopy and biochemical analyses, one discovered that cyanobacteria were in fact prokaryotes. Stanier *et al.* (1978) and Rippka *et al.* (1979) proposed to develop the taxonomy of cyanobacteria under the rules of the Nomenclature of Bacteria based on morphological characteristics and cellular division of axenic cultures, and this taxomomy was revised in 'Bergey's Manual of Systematic Bacteriology' (Castenholz and Waterbury 1989; Castenholz 2001). Several studies have also shown the usefulness of 16S rDNA as molecular marker in taxonomic studies of cyanobacteria (Giovannoni *et al.* 1988; Wilmotte and Golubic 1991) as in other prokaryotes.

In the genus *Microcystis*, differences in the size and shape of cells and colonies have led traditional taxonomists to distinguish several morphospecies (Komárek and Anagnostidis 1986, 1999). However, the delineation of morphospecies does not correspond to genetic lineages based on molecular markers such as 16S rDNA, 16S-23S rDNA ITS (internal transcribed spacer) and *cpc*BA IGS (intergenic spacer) (Otsuka *et al.* 1998, 1999; Tillett *et al.* 2001; Janse *et al.* 2004; Yoshida *et al.* 2005). DNA-DNA hybridizations of strains belonging to the morphospecies *Microcystis aeruginosa*, *M. ichthyoblabe*, *M. novacekii*, *M. viridis* and *M. wesenbergii* showed a similarity of more than 70 % , indicating they belong in fact to one species based on criteria for prokaryote species delimitation (Otsuka *et al.* 2001). Several morphological characteristics, including colony shape and size, appear to be very plastic and may even disappear when strains are grown under laboratory conditions (Otsuka *et al.*

2000), indicating that the taxonomy based on only morphological characteristics is not consistent. Today, several genotypic (e.g. AFLP, RFLP, REP-PCR, ERIC-PCR, HIP-PCR, sequencing of rRNA-genes and phycocyanin genes) and chemotypic (MALDI-TOF mass spectrometry) techniques are used to distinguish different genotypes[2] of *Microcystis* regardless of their morphology (Bittencourt-Oliveira *et al.* 2001; Lyra *et al.* 2001; Humbert *et al.* 2005; Janse *et al.* 2004; Oberholster *et al.* 2005; Wilson *et al.* 2005; Wu *et al.* 2007 Welker *et al.* 2004, 2007). While all these techniques are useful in studying the genetic composition of *Microcystis* populations, the most widely used marker at present is rDNA ITS. This marker is effective in phylogeographic and population genetic studies because it is variable enough and is present in the genome as a single or several identical rDNA operons (Otsuka *et al.* 1999; Boyer *et al.* 2001; Humbert *et al.* 2005), and can be directly used to determine the composition of natural *Microcystis* populations using Denaturing Gradient Gel Electrophoresis (DGGE) (Kardinaal *et al.* 2007a). Moreover, Otsuka *et al.* (1999) and Janse *et al.* (2004) indicated that *Microcystis* strains with identical rDNA ITS sequences are homogeneous for microcystin production (i.e. they are toxic or non-toxic). Yoshida *et al.* (2008a) showed a relationship between ITS type and phenotype for at least some *Microcystis* strains.

Studies based on several molecular markers (see before) have shown that naturally occurring *Microcystis* populations are generally composed of several genotypes, that temporal turnover in genetic composition can be high and that neighbouring lakes may harbour different genotypes (e.g. Bittencourt-Oliveira *et al.* 2001; Humbert *et al.* 2005; Janse *et al.* 2004; Wilson *et al.* 2005; Kardinaal *et al.* 2007a). Strains isolated from a single bloom population often also differ in ecological and physiological traits (Wilson *et al.* 2006; Bañares-España *et al.* 2006; Yoshida *et al.* 2008a). In particular, the toxicity of a *Microcystis* bloom is to a large extent determined by its strain composition (Janse *et al.* 2004; Kardinaal *et al.* 2007a) and several studies have shown that the microcystin concentration per unit of *Microcystis* biomass is highest at the onset of a bloom, probably because the percentage of toxic strains is highest at that moment (Kardinaal and Visser 2005; Kardinaal *et al.* 2007a). However, little is known about the mechanisms generating local and regional diversity of *Microcystis* bloom populations nor about the factors affecting population structure. This is an important gap in our knowledge because a better understanding of these mechanisms can be useful in predicting bloom formation and bloom toxicity, and may eventually help preventing bloom occurrence.

Generally, community and population structure are determined by an interplay of historical and environmental factors (Slatkin 1985, 1987; Ricklefs 1987; Leibold *et al.* 2004), including biotic interactions, that can also interact with each other. Below, we discuss the potential effects of (1) historical factors (dispersal limitation and priority effects), (2) local environmental factors (especially grazing by zooplankton) and (3) interstrain interactions on the population structure of *Microcystis*.

[2] *Microcystis* genotype: genetic features of a *Microcystis* strain

1.3 Influence of historical factors

Historical factors may influence the genotypic composition of natural *Microcystis* populations by two mechanisms: (1) through contemporary dispersal limitation leading to differences in both the local and regional genotype pool, and (2) by long-lasting effects of immigration history on local population structure, as differences in the arrival order of genotypes from the regional genotype pool in a new habitat may have lasting consequences for community or population structure, resulting in so-called priority effects (Morin 1984; Drake 1991). None of these two processes has been studied in detail, however.

The role of dispersal limitation for micro-organisms is controversial. The Baas Becking hypothesis (Baas Becking 1934), 'everything is everywhere, but, the environment selects', stating that dispersal limitation is not an issue for micro-organisms, is currently hotly debated (e.g. Finlay and Fenchel 2004; Martiny *et al.* 2006), which has resulted in a renewed interest in the biogeography of micro-organisms. The introduction of new molecular techniques for studying natural microbial diversity has made it possible to test this hypothesis more rigorously. The general picture emerging from these and other studies is that different groups of micro-organisms differ in their dispersal capacities and susceptibility to environmental conditions, reflected in cosmopolitan to geographically confined distribution patterns (e.g. Finlay and Clarke 1999; Finlay 2002; Whitaker *et al.* 2003; Green *et al.* 2004; Horner-Devine *et al.* 2004; Vyverman *et al.* 2007; Van der Gucht *et al.* 2007). However, inconsistencies in the inferred role of historical and environmental factors may also be due to differences in sampling scale (Dolan 2005, 2006; Martiny *et al.* 2006) and phylogenetic resolution. Also with regard to cyanobacteria, a variety of geographical distributions have been documented, ranging from cosmopolitan to limited (Hoffmann 1996). rDNA ITS lineages of the marine planktonic cyanobacteria *Synechococcus* and *Prochlorococcus* do not have distinct geographical distribution patterns (Rocap *et al.* 2002), while allopatric divergence has been observed in hot spring (Papke *et al.* 2003) and symbiotic *Synechococcus* (Erwin and Thacker 2008). Also, rDNA ITS sequences of the freshwater cyanobacterium *Cylindrospermopsis* cluster according to continent of origin (Haande *et al.* 2008), whereas the absence of geographically distributed ITS lineages was reported in the (sub)tropical freshwater cyanobacterium *Arthrospira* (Scheldeman *et al.* 1999; Baurain *et al.* 2002). While *Microcystis* is a cosmopolitan genus, it is unclear whether lineages within *Microcystis* are geographically restricted to a single continent or cosmopolitan. Studies based on rDNA ITS and *cpc*BA IGS suggest a cosmopolitan distribution of at least several *Microcystis* genotypes (Bittencourt-Oliveira *et al.* 2001; Janse *et al.* 2004; Haande *et al.* 2007). In contrast, Wu *et al.* (2007) and Oberholster *et al.* (2005) found evidence for a geographical signal in the distribution of *Microcystis* genotypes using whole-cell polymerase chain reaction of *cpc*BA IGS and AFLP (amplified fragment length polymorphism) respectively, although it is not clear if this geographical pattern results from dispersal limitation or environmental selection.

Even when dispersal is a common phenomenon, the arrival order of genotypes,

which is determined by regional abundance, dispersal ability and chance, may still be an important factor in determining contemporary community and population structure, and may result in strong priority effects (Drake 1991; Beisner *et al.* 2003; Schröder *et al.* 2005). Two mutually non-exclusive mechanisms may be responsible for such priority effects. The first is a purely numerical effect as later colonists have a numerical disadvantage compared to the first colonists. Secondly, early colonists can also alter the environment in a favourable or detrimental way for later colonists. For example, shading by growing plants can facilitate the growth of seedlings by protection against drought in semi-arid environments (Callaway and Walker 1997; Maestre *et al.* 2001, 2003; Brooker *et al.* 2008) and unpalatable plants can protect seedlings from herbivory (Brooker *et al.* 2008). While the importance of priority effects for community composition has convincingly been shown for a variety of organisms (e.g. Alford and Wilbur 1985; Robinson and Dickerson 1987; Blaustein and Margalit 1996; Louette and De Meester 2007; Zhang and Zhang 2007; Körner *et al.* 2008), the same mechanisms may also hold at the population level, to the extent that genotypes differ in ecologically important traits, with a strength and direction depending on the local environment. Priority effects in *Microcystis* populations may be important during population assembly in new habitats, as well as in determining strain composition in seasonal blooms that build up each year when conditions become favourable in spring or summer (Verspagen *et al.* 2004), but nothing is known about the importance of this additional historical factor.

1.4 Influence of environmental factors

When *Microcystis* strains have reached a particular habitat, they have to cope with the contemporary climatic conditions and local abiotic and biotic conditions in order to establish themselves (Leibold *et al.* 2004; Ricklefs 2004). Since *Microcystis* strains are physiologically very diverse (Wilson *et al.* 2006; Bañares-España *et al.* 2006), different strains probably react in a strain-specific manner to local environmental conditions. *Microcystis* is a successful competitor for light compared to other phytoplankton species in lakes with a stable water column thanks to its buoyancy regulation (Huisman *et al.* 2004) and has a competitive advantage at high temperatures (Reynolds 1997). At high pH and low CO_2 concentrations *Microcystis* can profit of its capacity to take up bicarbonate (Shapiro 1997). After phosphorus concentrations decreased in the water column, *Microcystis* is able to survive for a certain period because of its efficient phosphorus storage (Kromkamp *et al.* 1989). The factors responsible for the dominance of *Microcystis* in lakes might be important for the dominance of particular *Microcystis* genotypes too. For instance, non-toxic strains seem to be better competitors for light than toxic strains (Kadinaal *et al.* 2007b) and high levels of nutrients may favour the growth of toxic strains over non-toxic strains (Vézie *et al.* 2002; Yoshida *et al.* 2007). Other studies suggested the importance of temperature (Ohkubo *et al.* 1991) and CO_2 concentration (Bañares-España *et al.* 2006) for strain-specific growth of *Microcystis*. Furthermore, recent studies showed the importance of cyanophages for *Microcystis* population dynamics by host-specific infections of *Microcystis* strains (Yoshida *et al.* 2006, 2008b).

Another important aspect that may explain the dominance of *Microcystis* is the toxicity of particular strains and their capacity to form large colonies which provides resistance to zooplankton grazing (DeMott 1999; Rohrlack *et al.* 1999ab). As shown by van Gremberghe *et al.* (2008), zooplankton grazing can be very important in structuring the cyanobacterial community in a mesotrophic lake (see appendix). Zooplankton grazing might also influence *Microcystis* population structure through different mechanisms. Grazers may preferentially consume certain strains (e.g. unicellular strains rather than colony forming strains, non-toxic above toxic strains). Copepods, for example, can select cyanobacteria based on size and chemical characteristics like toxicity and nutritional value (DeMott and Moxter 1991). Infochemicals produced by zooplankton can also lead to changes in functional traits of *Microcystis* strains (Jang *et al.* 2003; Ha *et al.* 2004), which might change interstrain interactions and hence population structure. Finally, zooplankton species do not recycle nitrogen and phosphorus at the same relative rates which can influence nutrient concentrations in the water column (Vanni and Layne 1997; Elser and Urabe 1999; Vanni 2002), and may result into changes in *Microcystis* population structure (Yoshida *et al.* 2007).

Studying the interactions between zooplankton grazers and *Microcystis* is also important considering the possible role for zooplankton in preventing bloom formation. There is conflicting evidence on the extent to which grazing by zooplankton can control the development of cyanobacterial blooms. It is well-known that the presence of cyanobacteria can influence zooplankton abundance and composition, because some cyanobacteria are toxic, have a low nutritional value or form large colonies and filaments which can negatively affect several zooplankton species (Ghadouani *et al.* 2003; Murrel and Lores 2004; Ruokolainen *et al.* 2006). *Daphnia* (Cladocera) is a key grazer in standing waters (Fig. 1.4), but its capacity to suppress *Microcystis* is heavily debated (Ghadouani *et al.* 2003). The fact that *Daphnia* cannot distinguish between toxic and non-toxic *Microcystis* strains and is poisoned by microcystins has been extensively studied (e.g. DeMott 1999; Rohrlack *et al.* 1999a, 2001; Lotocka 2001). Other negative effects of *Microcystis* on *Daphnia* are reduction of feeding activity (Rohrlack *et al.* 1999b) and fatal molting disruption by the toxin microviridin J (Rohrlack *et al.* 2004). Overall, *Microcystis* seems to produce several metabolites harmful for *Daphnia* (Czarnecki *et al.* 2006). However, despite the negative effects of *Microcystis* on *Daphnia*, some studies have provided evidence that *Daphnia* may suppress developing *Microcystis* blooms depending on initial conditions and history (Christoffersen *et al.* 1993; Matveev *et al.* 1994; Sarnelle 2007). *Daphnia* is the most efficient grazer on phytoplankton and efficient grazing at low *Microcystis* biomass might prevent bloom formation, but not suppress existing blooms. Moreover, *Daphnia* can increase its tolerance to *Microcystis* toxins through maternal effects (Gustafsson *et al.* 2005) and microevolution (Hairston *et al.* 1999). Understanding *Daphnia*-*Microcystis* interactions is important to evaluate possibilities of suppressing bloom development by grazing.

Another intriguing question is whether zooplankton influences the degree to which *Microcystis* blooms are toxic. If toxin production is a defense against zooplankton grazing, one may expect that dense zooplankton populations may enhance the blooms

Figure 1.4: Photograph of *Daphnia magna* taken by Joachim Mergeay. Scale bar = 1 mm.

to become toxic. On the other hand, zooplankton grazing might also prevent the development of toxic blooms. Insight in this matter may be crucial to reduce the occurrence of toxic *Microcystis* blooms.

From the above it is clear that there is a need for more studies on the importance of historical and environmental factors for *Microcystis* population structure and diversity. Large-scale surveys of the occurrence and distribution of particular lineages or genotypes may, next to assessing their geographical distribution, also reveal environmental preferences, e.g. for certain climatic conditions. Assessments of genetic structure and diversity in metapopulations may determine which environmental gradients are important determinants of local population structure and determine their role relative to spatial structure (e.g. Van der Gucht *et al.* 2007). These field studies should be complemented by competition experiments using a large number of well-characterized *Microcystis* strains to verify the role of specific environmental and historical factors, such as priority effects, in determining population structure.

1.5 Interstrain interactions

Since *Microcystis* strains within populations can differ considerably in functional traits, strong and complex interactions between these strains are very likely to occur. Moreover, the high diversity of *Microcystis* within and between blooms may in part be maintained by these interactions. Generally, interactions between species or strains

can be negative (resource competition and allelopathy) or positive (facilitation and niche complementarity) and depend on particular functional traits of the organisms (Callaway and Walker 1997; Hulot *et al.* 2001; Suikkanen *et al.* 2004; Agawin *et al.* 2007; Jiang 2007). Several studies have documented allelopathic effects of *Microcystis* against other cyanobacteria, green algae and dinoflagellates (Ikawa *et al.* 1996; Singh *et al.* 2001; Sukenik *et al.* 2002). However, interstrain interactions between cyanobacteria can also occur, as shown recently by strain-specific allelopathic interactions between strains of the filamentous cyanobacterium *Planktothrix* (Oberhaus *et al.* 2008). Interstrain interactions in *Microcystis* are poorly studied and focused mainly on toxic versus non-toxic strains. For instance, non-toxic strains may have a competitive advantage over toxic strains, as suggested by Kardinaal *et al.* (2007b), and experiments by Schatz *et al.* (2005) suggest the occurrence of allelopathic interactions between toxic and non-toxic *Microcystis* strains. Several functional traits (absolute growth rate in monoculture, critical light intensity for growth, colony formation, production of secondary metabolites including microcystins...), may be important for the sign and strength of interstrain interactions in *Microcystis*. Ideally, experiments with a large number of strains characterized by many functional traits should be carried out to disentangle competitive relationships.

In plant communities, interactions among members of the same guild can be strongly influenced by local abiotic and biotic conditions. Increased environmental severity (abiotic stress or herbivory) tends to increase the importance of positive interactions relative to negative interactions (Bertness and Callaway 1994; Callaway and Walker 1997; Brooker *et al.* 2008). For instance, while in the absence of herbivores strong competitive interactions can occur between palatable and unpalatable plants, unpalatable plants can have a strong indirect positive effect on palatable plants in the presence of herbivores by acting as a grazer deterrent (Callaway *et al.* 2005; Graff *et al.* 2007). The same mechanism might work for non-toxic unicellular *Microcystis* strains which might be protected against predation by a non-selective zooplankton grazer such as *Daphnia* by toxic and/or colonial *Microcystis* strains.

1.6 Thesis outline

The aim of this thesis is to obtain information on the local and regional diversity of *Microcystis* populations and the factors influencing diversity and population structure. We paid attention to the importance of history (dispersal limitation and priority effects), environmental factors (especially zooplankton grazing) and interstrain interactions on *Microcystis* population structure, and the interactions between all these factors (Fig. 1.5). In a first part of this thesis (**chapter 2** and **3**), we studied dispersal, diversity and genotypic structure of natural *Microcystis* populations at different spatial scales, including the influence of abiotic and biotic factors on diversity and population structure. In a second part of this thesis (**chapter 4**, **5** and **6**), we studied functional traits and interactions between *Microcystis* strains, and the influence of priority effects and *Daphnia* grazing on population structure by laboratory experiments.

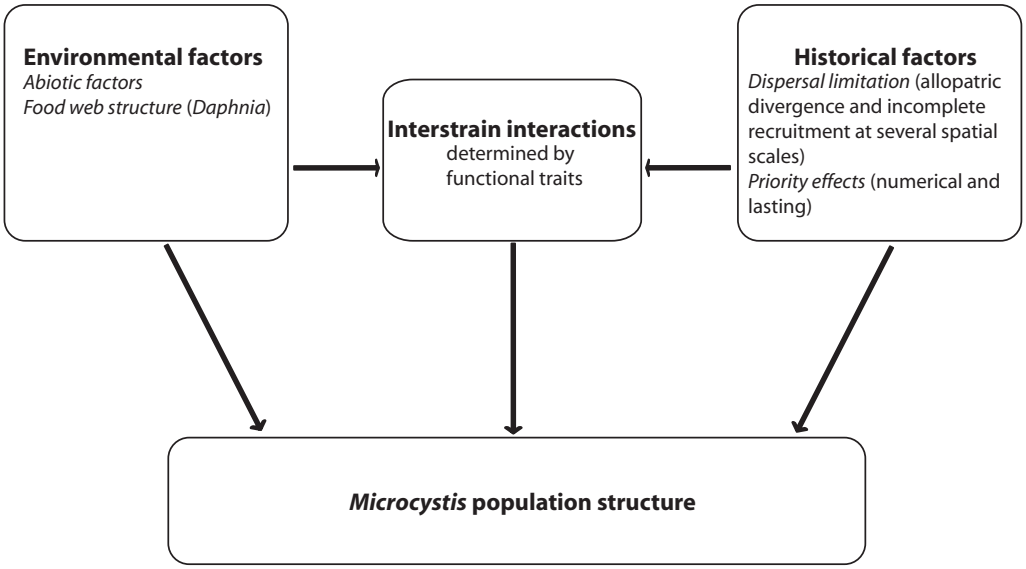


Figure 1.5: Diagram of the factors potentially determining *Microcystis* population structure. Historical factors (dispersal limitation: chapter 2 and 3, priority effects: chapter 6) and environmental factors (abiotic factors: chapter 2 and 3, food web structure: chapter 3 and 5) can directly influence *Microcystis* population structure, or indirectly by influencing interstrain interactions (chapter 4, 5 and 6). Interstrain interactions can also influence *Microcystis* population structure independently of historical and environmental influences (chapter 5).

In **chapter 2**, we studied the rDNA ITS phylogeography of *Microcystis* on a global scale to reveal the degree of geographical structuring of ITS types according to continents, which would reflect allopatric divergence and/or incomplete dispersal from the global ITS type pool. In addition, we tested structuring of ITS types according to climatic conditions.

In **chapter 3**, we investigated the local and regional diversity and genotypic composition of natural *Microcystis* populations in man-made reservoirs in Tigray (Northern Ethiopia) by DGGE of rDNA ITS, and related population structure to spatial configuration of the reservoirs and environmental gradients, including the abundance and species composition of zooplankton. This study was part of the Aquatic Ecology project in the VLIR IUS-MEKELLE programme studying the ecology of cyanobacterial blooms in man-made reservoirs in Tigray forming a direct threat to the local people. In a supplementary study, we also compared local and regional ITS diversity of *Microcystis* between different regions. This included the ITS diversity in Flanders, which was done in the framework of a national project studying cyanobacterial blooms in Belgium (B-Blooms), and the ITS diversity in Europe and South America using samples collected in the framework of projects on biodiversity of shallow lakes along a longitudinal gradient (BIOMAN and SALGA). See acknowledgement of financial support for details of the respective projects.

In **chapter 4**, we determined key functional traits (growth rate, microcystin production, colony formation and cell size) and their dependence on *Daphnia* infochemicals of eight *Microcystis* strains isolated from two bloom populations in Flemish water bodies.

In **chapter 5**, we studied interactions between four of the above-mentioned *Microcystis* strains, which were isolated from a single bloom population and differed in important functional traits. Experiments were carried out in the presence and absence of *Daphnia magna*, and growth rate of the strains in mixed populations was compared to growth rate in monoculture. This way, we could determine the sign (competition or facilitation) and strength of interstrain interactions, how they depended on the functional traits of the strains, and whether they were altered by the presence of a grazer.

In **chapter 6**, we investigated the influence of arrival order on the development and structure of *Microcystis* populations in the presence and absence of *Daphnia magna*. Using competition experiments with time lags we determined the final population structure and measured growth rate of the different strains in function of arrival order. This allowed quantification of priority effects in determining population structure, and to assess whether priority effects were altered by zooplankton grazing.

The latter three studies were done in the framework of an European project studying the importance of connectivity, dispersal and priority effects as drivers of biodiversity and ecosystem function in pond and pool communities (BIOPOOL).

In **chapter 7**, the main conclusions are summarized and discussed and perspectives for future research are given.

References

Agawin, N. S. R., Rabouille, S., Veldhuis, M. J. W., Servatius, L., Hol, S., van Overzee, H. M. J. and Huisman, J. 2007. Competition and facilitation between unicellular nitrogen-fixing cyanobacteria and non-nitrogen-fixing phytoplankton species. Limnology and Oceanography 52: 2233-2248.

Alford, R. A. and Wilbur, H. M. 1985. Priority effects in experimental pond communities - competition between *Bufo* and *Rana*. Ecology 66: 1097-1105.

Baas Becking, L. G. M. 1934. Geobiologie of Inleiding tot de Milieukunde. Van Stockum and Zoon, The Hague, The Netherlands.

Babica, P., Blaha, L. and Marsalek, B. 2006. Exploring the natural role of microcystins - A review of effects on photoautotrophic organisms. Journal of Phycology 42: 9-20.

Bañares-España, E., Lopez-Rodas, V., Salgado, C., Costas, E. and Flores-Moya, A. 2006. Inter-strain variability in the photosynthetic use of inorganic carbon, exemplified by the pH compensation point, in the cyanobacterium *Microcystis aeruginosa*. Aquatic Botany 85: 159-162.

Baurain, D., Renquin, L., Grubisic, S., Scheldeman, P., Belay, A. and Wilmotte, A. 2002. Remarkable conservation of internally transcribed spacer sequences of *Arthrospira* ('*Spirulina*') (Cyanophyceae, Cyanobacteria) strains from four continents and of recent and 30-year-old dried samples from Africa. Journal of Phycology 38: 384-393.

Beisner, B. E., Haydon, D. T. and Cuddington, K. 2003. Alternative stable states in ecology. Frontiers in Ecology and the Environment 1: 376-382.

Bertness, M. D. and Callaway, R. 1994. Positive Interactions in Communities. Trends in Ecology & Evolution 9: 191-193.

Bicudo, D. D. C., Fonseca, B. M., Bini, L. M., Crossetti, L. O., Bicudo, C. E. D. M. and Araujo-Jesus, T. 2007. Undesirable side-effects of water hyacinth control in a shallow tropical reservoir. Freshwater Biology 52: 1120-1133.

Bittencourt-Oliveira, M. D., de Oliveira, M. C. and Bolch, C. J. S. 2001. Genetic variability of Brazilian strains of the *Microcystis aeruginosa* complex (Cyanobacteria/Cyanophyceae) using the phycocyanin intergenic spacer and flanking regions (*cpc*BA). Journal of Phycology 37: 810-818.

Blaustein, L. and Margalit, J. 1996. Priority effects in temporary pools: Nature and outcome of mosquito larva toad tadpole interactions depend on order of entrance. Journal of Animal Ecology 65: 77-84.

Börner, T. and Dittmann, E. 2005. Molecular biology of cyanobacterial toxins. Genetic basis of microcystin production. In Huisman, J., Matthijs, H. C. P., and Visser, P. M. (eds). Harmful Cyanobacteria. Springer, Dordrecht, The Netherlands. pp. 25-40.

Boyer, S. L., Flechtner, V. R. and Johansen, J. R. 2001. Is the 16S-23S rRNA internal transcribed spacer region a good tool for use in molecular systematics and population genetics? A case study in cyanobacteria. Molecular Biology and Evolution 18: 1057-1069.

Brooker, R. W., Maestre, F. T., Callaway, R. M., Lortie, C. L., Cavieres, L. A., Kunstler, G., Liancourt, P., Tielborger, K. *et al.* 2008. Facilitation in plant communities: the past, the present, and the future. Journal of Ecology 96: 18-34.

Callaway, R. M., Kikodze, D., Chiboshvili, M. and Khetsuriani, L. 2005. Unpalatable plants protect neighbors from grazing and increase plant community diversity. Ecology 86: 1856-1862.

Callaway, R. M. and Walker, L. R. 1997. Competition and facilitation: A synthetic approach to interactions in plant communities. Ecology 78: 1958-1965.

Carmichael, W. W. 1994. Toxins of Cyanobacteria. Scientific American 270: 78-86.

Carmichael, W. W., Azevedo, S. M. F. O., An, J. S., Molica, R. J. R., Jochimsen, E. M., Lau, S., Rinehart, K. L., Shaw, G. R. and Eaglesham, G. K. 2001. Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. Environmental Health Perspectives 109: 663-668.

Castenholz, R. W. 2001. Oxygenic photosynthetic bacteria Phylum BX. Cyanobacteria. In: Boone D. R. and Castenholz, R. W. (eds). Bergey's Manual of Systematic Bacteriology. 2nd Edition. Volume One: The Archaea and the deeply branching and phototrophic Bacteria. Springer, New-York. pp. 473-600.

Castenholz, R. W. and Waterbury, J. B. 1989. Group I. Cyanobacteria, Preface. In J. T. Staley and M. P. Bryant (eds). Bergey's Manual of systematic Bacteriology. Volume Three. Springer, New-York, pp. 1710-1727.

Chorus, I. and Bartram, J. (eds). WHO. 1999. Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. E & FN Spon. London, United Kingdom.

Christoffersen, K., Riemann, B., Klysner, A. and Sondergaard, M. 1993. Potential role of fish predation and natural populations of zooplankton in structuring a plankton community in eutrophic lake water. Limnology and Oceanography 38: 561-573.

Codd, G. A., Lindsay, J., Young, F. M., Morrison, L. F. and Metcalf, J. S. 2005. Harmful cyanobacteria. From mass mortalities to management measures. In: Huisman, J., Matthijs, H. C. P. and Visser, P. M. (eds). Harmful Cyanobacteria. Springer. Dordrecht, The Netherlands. pp 1-24.

Czarnecki, O., Henning, M., Lippert, I. and Welker, M. 2006. Identification of peptide metabolites of *Microcystis* (Cyanobacteria) that inhibit trypsin-like activity in planktonic herbivorous *Daphnia* (Cladocera). Environmental Microbiology 8: 77-87.

DeMott, W. R. 1999. Foraging strategies and growth inhibition in five daphnids feeding on mixtures of a toxic cyanobacterium and a green alga. Freshwater Biology 42: 263-274.

DeMott, W. R. and Moxter, F. 1991. Foraging on cyanobacteria by copepods - Responses to chemical defenses and resource abundance. Ecology 72: 1820-1834.

Ding, W. X., Shen, H. N. and Ong, C. N. 2000. Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. Hepatology 32: 547-555.
Dittmann, E., Erhard, M., Kaebernick, M., Scheler, C., Neilan, B. A., von Dohren, H. and Borner, T. 2001. Altered expression of two light-dependent genes in a microcystin-lacking mutant of *Microcystis aeruginosa* PCC 7806. Microbiology-Sgm 147: 3113-3119.
Dolan, J. R. 2005. An introduction to the biogeography of aquatic microbes. Aquatic Microbial Ecology 41: 39-48.
Dolan, J. R. 2006. Microbial biogeography? Journal of Biogeography 33: 199-200.
Drake, J. A. 1991. Community-assembly mechanics and the structure of an experimental species ensemble. American Naturalist 137: 1-26.
Elser, J. J. and Urabe, J. 1999. The stoichiometry of consumer-driven nutrient recycling: Theory, observations, and consequences. Ecology 80: 735-751.
Erwin, P. M. and Thacker, R. W. 2008. Cryptic diversity of the symbiotic cyanobacterium *Synechococcus spongiarum* among sponge hosts. Molecular Ecology 17: 2937-2947.
Falconer, I. R. 1999. An overview of problems caused by toxic blue-green algae (cyanobacteria) in drinking and recreational waters. Environmental Toxicology 14: 5-12.
Finlay, B. J. 2002. Global dispersal of free-living microbial eukaryote species. Science 296: 1061-1063.
Finlay, B. J. and Clarke, K. J. 1999. Ubiquitous dispersal of microbial species. Nature 400: 828-828.
Finlay, B. J. and Fenchel, T. 2004. Cosmopolitan metapopulations of free-living microbial eukaryotes. Protist 155: 237-244.
Fulton, R. S. and Paerl, H. W. 1987. Effects of colonial morphology on zooplankton utilization of algal resources during blue-green algal (*Microcystis aeruginosa*) blooms. Limnology and Oceanography 32: 634-644.
Ghadouani, A., Pinel-Alloul, B. and Prepas, E. E. 2003. Effects of experimentally induced cyanobacterial blooms on crustacean zooplankton communities. Freshwater Biology 48: 363-381.
Giovannoni, S. J., Turner, S., Olsen, G. J., Barns, S., Lane, D. J. and Pace, N. R. 1988. Evolutionary relationships among cyanobacteria and green chloroplasts. Journal of Bacteriology 170: 3584-3592.
Graff, P., Aguiar, M. R. and Chaneton, E. J. 2007. Shifts in positive and negative plant interactions along a grazing intensity gradient. Ecology 88: 188-199.
Green, J. L., Holmes, A. J., Westoby, M., Oliver, I., Briscoe, D., Dangerfield, M., Gillings, M. and Beattie, A. J. 2004. Spatial scaling of microbial eukaryote diversity. Nature 432: 747-750.
Gustafsson, S., Rengefors, K. and Hansson, L. A. 2005. Increased consumer fitness following transfer of toxin tolerance to offspring via maternal effects. Ecology 86: 2561-2567.
Ha, K., Jang, M. H. and Takamura, N. 2004. Colony formation in planktonic algae induced by zooplankton culture media filtrate. Journal of Freshwater Ecology 19: 9-16.
Haande, S., Ballot, A., Rohrlack, T., Fastner, J., Wiedner, C. and Edvardsen, B. 2007. Diversity of *Microcystis aeruginosa* isolates (Chroococcales, Cyanobacteria) from East-African water bodies. Archives of Microbiology 188: 15-25.
Haande, S., Rohrlack, T., Ballot, A., Roberg, K., Skulberg, R., Beck, M. and Wiedner, C. 2008. Genetic characterisation of *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria) isolates from Africa and Europe. Harmful Algae 7: 692-701.
Hairston, N. G., Lampert, W., Caceres, C. E., Holtmeier, C. L., Weider, L. J., Gaedke, U., Fischer, J. M., Fox, J. A. and Post, D. M. 1999. Lake ecosystems - Rapid evolution revealed by dormant eggs. Nature 401: 446-446.
Hoffmann, L. 1996. Geographic distribution of freshwater blue-green algae. Hydrobiologia 336: 33-40.
Horner-Devine, M. C., Lage, M., Hughes, J. B. and Bohannan, B. J. M. 2004. A taxa-area relationship for bacteria. Nature 432: 750-753.
Huisman, J., Matthijs, H. C. P. and Visser, P. M. 2005. Harmful cyanobacteria. Springer. Dordrecht, The Netherlands. 243 pp.
Huisman, J., Sharples, J., Stroom, J. M., Visser, P. M., Kardinaal, W. E. A., Verspagen, J. M. H. and Sommeijer, B. 2004. Changes in turbulent mixing shift competition for light between phytoplankton species. Ecology 85: 2960-2970.
Hulot, F. D., Morin, P. J. and Loreau, M. 2001. Interactions between algae and the microbial loop in experimental microcosms. Oikos 95: 231-238.
Humbert, J. F., Duris-Latour, D., Le Berre, B., Giraudet, H. and Salencon, M. J. 2005. Genetic diversity in *Microcystis* populations of a French storage reservoir assessed by sequencing of the 16S-23S rRNA intergenic spacer. Microbial Ecology 49: 308-314.
Ikawa, M., Haney, J. F. and Sasner, J. J. 1996. Inhibition of *Chlorella* growth by the lipids of cyanobacterium *Microcystis aeruginosa*. Hydrobiologia 331: 167-170.
Jang, M. H., Ha, K., Joo, G. J. and Takamura, N. 2003. Toxin production of cyanobacteria is increased by exposure to zooplankton. Freshwater Biology 48: 1540-1550.
Janse, I., Kardinaal, W. E. A., Meima, M., Fastner, J., Visser, P. M. and Zwart, G. 2004. Toxic and nontoxic *Microcystis* colonies in natural populations can be differentiated on the basis of rRNA gene internal transcribed spacer diversity. Applied and Environmental Microbiology 70: 3979-3987.
Jiang, L. 2007. Negative selection effects suppress relationships between bacterial diversity and ecosystem functioning. Ecology 88: 1075-1085.
Jöhnk, K. D., Huisman, J., Sharples, J., Sommeijer, B., Visser, P. M. and Stroom, J. M. 2008. Summer heatwaves promote blooms of harmful cyanobacteria. Global Change Biology 14: 495-512.
Kaebernick, M., Neilan, B. A., Borner, T. and Dittmann, E. 2000. Light and the transcriptional response of the microcystin biosynthesis gene cluster. Applied and Environmental Microbiology 66: 3387-3392.

Kardinaal, W. E. A., Janse, I., Kamst-van Agterveld, M., Meima, M., Snoek, J., Mur, L. R., Huisman, J., Zwart, G. and Visser, P. M. 2007a. *Microcystis* genotype succession in relation to microcystin concentrations in freshwater lakes. Aquatic Microbial Ecology 48: 1-12.

Kardinaal, W. E. A., Tonk, L., Janse, I., Hol, S., Slot, P., Huisman, J. and Visser, P. M. 2007b. Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*. Applied and Environmental Microbiology 73: 2939-2946.

Kardinaal, W. E. A. and Visser, P. M. 2005. Dynamics of cyanobacterial toxins. Sources of variability in microcystin concentrations. In: Huisman, J., Matthijs, H. C. P. and Visser, P. M. (eds). Harmful Cyanobacteria. Springer. Dordrecht, The Netherlands. pp. 41-64.

Knoll, A. H. 2008. Cyanobacteria and earth history. In: Herrero, A. and Flores, E. (eds). The Cyanobacteria: Molecular Biology, genetics and evolution. Horizon Scientific Press. pp 1-19.

Komárek, J. and Anagnostidis, K. 1986. Modern approach to the classification system of cyanophytes 2 Ű Chroococcales. Archiv für Hydrobiologie Supplement 83: 377-401.

Komárek, J. and Anagnostidis, K. 1999. Cyanoprokaryota 1. Teil: Chroococcales, In: Ettl, H., Gärtner, G., Heynig, H. and Mollenhauer, D.(eds). Süsswasserflora von Mitteleuropa, band 19/1, Gustav Fisher. Berlin, pp. 1-548.

Körner, C., Stocklin, J., Reuther-Thiebaud, L. and Pelaez-Riedl, S. 2008. Small differences in arrival time influence composition and productivity of plant communities. New Phytologist 177: 698-705.

Kromkamp, J., Vandenheuvel, A. and Mur, L. R. 1989. Phosphorus uptake and photosynthesis by phosphate-limited cultures of the cyanobacterium *Microcystis aeruginosa*. British Phycological Journal 24: 347-355.

Leibold, M. A., Holyoak, M., Mouquet, N., Amarasekare, P., Chase, J. M., Hoopes, M. F., Holt, R. D., Shurin, J. B. *et al.* 2004. The metacommunity concept: a framework for multi-scale community ecology. Ecology Letters 7: 601-613.

Linnaeus, C. 1753. Species plantarum.

Lotocka, M. 2001. Toxic effect of cyanobacterial blooms on the grazing activity of *Daphnia magna* Straus. Oceanologia 43: 441-453.

Louette, G. and De Meester, L. 2007. Predation and priority effects in experimental zooplankton communities. Oikos 116: 419-426.

Lukac, M. and Aegerter, R. 1993. Influence of trace-metals on growth and toxin production of *Microcystis aeruginosa*. Toxicon 31: 293-305.

Lyra, C., Suomalainen, S., Gugger, M., Vezie, C., Sundman, P., Paulin, L. and Sivonen, K. 2001. Molecular characterization of planktic cyanobacteria of *Anabaena*, *Aphanizomenon*, *Microcystis* and *Planktothrix* genera. International Journal of Systematic and Evolutionary Microbiology 51: 513-526.

Maestre, F. T., Bautista, S. and Cortina, J. 2003. Positive, negative, and net effects in grass-shrub interactions in mediterranean semiarid grasslands. Ecology 84: 3186-3197.

Maestre, F. T., Bautista, S., Cortina, J. and Bellot, J. 2001. Potential for using facilitation by grasses to establish shrubs on a semiarid degraded steppe. Ecological Applications 11: 1641-1655.

Martiny, J. B. H., Bohannan, B. J. M., Brown, J. H., Colwell, R. K., Fuhrman, J. A., Green, J. L., Horner-Devine, M. C., Kane, M. *et al.* 2006. Microbial biogeography: putting microorganisms on the map. Nature Reviews Microbiology 4: 102-112.

Matveev, V., Matveeva, L. and Jones, G. J. 1994. Study of the ability of *Daphnia carinata* King to control phytoplankton and resist cyanobacterial toxicity - implications for biomanipulation in Australia. Australian Journal of Marine and Freshwater Research 45: 889-904.

Morin, P. J. 1984. Odonate guild composition - experiments with colonization history and fish predation. Ecology 65: 1866-1873.

Murrel, M. C. and Lores, E. M. 2004. Phytoplankton and zooplankton seasonal dynamics in a subtropical estuary: importance of cyanobacteria. Journal of Plankton Research 26: 371-382.

Oberhaus, L., Briand, J. F. and Humbert, J. F. 2008. Allelopathic growth inhibition by the toxic, bloom-forming cyanobacterium *Planktothrix rubescens*. FEMS Microbiology Ecology 66: 243-249.

Oberholster, P. J., Botha, A. M., Muller, K. and Cloete, T. E. 2005. Assessment of the genetic diversity of geographically unrelated *Microcystis aeruginosa* strains using amplified fragment length polymorphisms (AFLPs). African Journal of Biotechnology 4: 389-399.

Ohkubo, N., Yagi, O. and Okada, M. 1991. Effects of temperature and illumination on the growth of blue-green alga *Microcystis viridis*. Japanese Journal of Limnology 52: 255-261.

Otsuka, S., Suda, S., Li, R. H., Matsumoto, S. and Watanabe, M. M. 2000. Morphological variability of colonies of *Microcystis* morphospecies in culture. Journal of General and Applied Microbiology 46: 39-50.

Otsuka, S., Suda, S., Li, R. H., Watanabe, M., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 1998. 16S rDNA sequences and phylogenetic analyses of *Microcystis* strains with and without phycoerythrin. FEMS Microbiology Letters 164: 119-124.

Otsuka, S., Suda, S., Li, R. H., Watanabe, M., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 1999. Phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis* based on 16S to 23S internal transcribed spacer sequence. FEMS Microbiology Letters 172: 15-21.

Otsuka, S., Suda, S., Shibata, S., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 2001. A proposal for the unification of five species of the cyanobacterial genus *Microcystis* Kutzing ex Lemmermann 1907 under the Rules of the Bacteriological Code. International Journal of Systematic and Evolutionary Microbiology 51: 873-879.

Papke, R. T., Ramsing, N. B., Bateson, M. M. and Ward, D. M. 2003. Geographical isolation in hot spring cyanobacteria. Environmental Microbiology 5: 650-659.

Pearson, L. A., Hisbergues, M., Borner, T., Dittmann, E. and Neilan, B. A. 2004. Inactivation of an ABC transporter gene, *mcy*H, results in loss of microcystin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. Applied and Environmental Microbiology 70: 6370-6378.

Rantala, A., Fewer, D. P., Hisbergues, M., Rouhiainen, L., Vaitomaa, J., Borner, T. and Sivonen, K. 2004. Phylogenetic evidence for the early evolution of microcystin synthesis. Proceedings of the National Academy of Sciences of the United States of America 101: 568-573.

Rapala, J., Sivonen, K., Lyra, C. and Niemela, S. I. 1997. Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena spp.* as a function of growth stimuli. Applied and Environmental Microbiology 63: 2206-2212.

Reynolds, C. S. 1997. Vegetation processes in the pelagic: a model for ecosystem theory. Excellence in Ecology 9. Ecology Institute. Oldendorf, Germany.

Reynolds, C. S., Jaworski, G. M. H., Cmiech, H. A. and Leedale, G. F. 1981. On the annual cycle of the blue-green algae *Microcystis aeruginosa* Kütz. Emend. Elenkin. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 293: 419-477.

Ricklefs, R. E. 1987. Community diversity: relative roles of local and regional processes. Science 235: 167-171.

Ricklefs, R. E. 2004. A comprehensive framework for global patterns in biodiversity. Ecology Letters 7: 1-15.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. and Stanier, R. Y. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. Journal of General Microbiology 111: 1-61.

Robinson, J. V. and Dickerson, J. E. 1987. Does invasion sequence affect community structure? Ecology 68: 587-595.

Rocap, G., Distel, D. L., Waterbury, J. B. and Chisholm, S. W. 2002. Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S-23S ribosomal DNA internal transcribed spacer sequences. Applied and Environmental Microbiology 68: 1180-1191.

Rohrlack, T., Christoffersen, K., Kaebernick, M. and Neilan, B. A. 2004. Cyanobacterial protease inhibitor microviridin J causes a lethal molting disruption in *Daphnia pulicaria*. Applied and Environmental Microbiology 70: 5047-5050.

Rohrlack, T., Dittmann, E., Borner, T. and Christoffersen, K. 2001. Effects of cell-bound microcystins on survival and feeding of *Daphnia spp.* Applied and Environmental Microbiology 67: 3523-3529.

Rohrlack, T., Dittmann, E., Henning, M., Borner, T. and Kohl, J. G. 1999a. Role of microcystins in poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 65: 737-739.

Rohrlack, T., Henning, M. and Kohl, J. G. 1999b. Mechanisms of the inhibitory effect of the cyanobacterium *Microcystis aeruginosa* on *Daphnia galeata*'s ingestion rate. Journal of Plankton Research 21: 1489-1500.

Runnegar, M., Berndt, N., Kong, S.-M., Lee, E. Y. C. and Zhang, L. 1995. In vivo and in vitro binding of microcystin to protein phosphatases 1 and 2A. Biochemical and Biophysical Research Communications 216: 162-169.

Ruokolainen, L., Lilley, T. M., Tammi, M. and Vuorinen, P. 2006. Zooplankton in relation to cyanobacteria across a geographic gradient in Archipelago Sea, northern Baltic. Boreal Environment Research 11: 1-11.

Sarnelle, O. 2007. Initial conditions mediate the interaction between *Daphnia* and bloom-forming cyanobacteria. Limnology and Oceanography 52: 2120-2127.

Schatz, D., Keren, Y., Hadas, O., Carmeli, S., Sukenik, A. and Kaplan, A. 2005. Ecological implications of the emergence of non-toxic subcultures from toxic *Microcystis* strains. Environmental Microbiology 7: 798-805.

Scheffer, M., Rinaldi, S., Gragnani, A., Mur, L. R. and van Nes, E. H. 1997. On the dominance of filamentous cyanobacteria in shallow, turbid lakes. Ecology 78: 272-282.

Scheldeman, P., Baurain, D., Bouhy, R., Scott, M., Muhling, M., Whitton, B. A., Belay, A. and Wilmotte, A. 1999. *Arthrospira* ('*Spirulina*') strains from four continents are resolved into only two clusters, based on amplified ribosomal DNA restriction analysis of the internally transcribed spacer. FEMS Microbiology Letters 172: 213-222.

Schröder, A., Persson, L. and De Roos, A. M. 2005. Direct experimental evidence for alternative stable states: a review. Oikos 110: 3-19.

Shapiro, J. 1997. The role of carbon dioxide in the initiation and maintenance of blue-green dominance in lakes. Freshwater Biology 37: 307-323.

Singh, D. P., Tyagi, M. B., Kumar, A. and Thakur, J. K. 2001. Antialgal activity of a hepatotoxin-producing cyanobacterium, *Microcystis aeruginosa*. World Journal of Microbiology & Biotechnology 17: 15-22.

Slatkin, M. 1985. Gene flow in natural populations. Annual Review of Ecology and Systematics 16: 393-430.

Slatkin, M. 1987. Gene flow and the geographic structure of natural populations. Science 236: 787-792.

Stanier, R. Y., Sistrom, W. R., Hansen, T. A., Whitton, B. A., Castenholz, R. W., Pfennig, N., Gorlenko, V. N., Kondratieva, E. N. *et al.* 1978. Proposal to place nomenclature of Cyanobacteria (blue-green-algae) under rules of international code of nomenclature of Bacteria. International Journal of Systematic Bacteriology 28: 335-336.

Suikkanen, S., Fistarol, G. O. and Graneli, E. 2004. Allelopathic effects of the Baltic cyanobacteria *Nodularia spumigena*, *Aphanizomenon flos-aquae* and *Anabaena lemmermannii* on algal monocultures. Journal of Experimental Marine Biology and Ecology 308: 85-101.

Sukenik, A., Eshkol, R., Livne, A., Hadas, O., Rom, M., Tchernov, D., Vardi, A. and Kaplan, A. 2002. Inhibition of growth and photosynthesis of the dinoflagellate *Peridinium gatunense* by *Microcystis sp.* (cyanobacteria): A novel allelopathic mechanism. Limnology and Oceanography 47: 1656-1663.

Tillett, D., Dittmann, E., Erhard, M. von Dohren, H., Börner, T. and Neilan, B. A. 2000. Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase

system. Chemistry & Biology 7:753-764.
Tillett, D., Parker, D. L. and Neilan, B. A. 2001. Detection of toxigenicity by a probe for the microcystin synthetase A gene (*mcy*A) of the cyanobacterial genus *Microcystis*, comparison of toxicities with 16S rRNA and phycocyanin operon (phycocyanin intergenic spacer) phylogenies. Applied and Environmental Microbiology 67: 2810-2818.
Utkilen, H. and Gjolme, N. 1995. Iron-stimulated toxin production in *Microcystis aeruginosa*. Applied and Environmental Microbiology 61: 797-800.
Van den Hoek, C., Mann, D. G. and Jahns, H. M. (eds). 1995. Algae: an introduction to phycology. Cambridge University Press. Cambridge, United Kingdom.
Van der Gucht, K., Cottenie, K., Muylaert, K., Vloemans, N., Cousin, S., Declerck, S., Jeppesen, E., Conde-Porcuna, J. M. *et al.* 2007. The power of species sorting: Local factors drive bacterial community composition over a wide range of spatial scales. Proceedings of the National Academy of Sciences of the United States of America 104: 20404-20409.
van Gremberghe, I., Van Wichelen, J., Van der Gucht, K., Vanormelingen, P., D'Hondt, S., Boutte, C., Wilmotte, A. and Vyverman, W. 2008. Covariation between zooplankton community composition and cyanobacterial community dynamics in Lake Blaarmeersen (Belgium). FEMS Microbiology Ecology 63: 222-237.
Vanni, M. J. 2002. Nutrient cycling by animals in freshwater ecosystems. Annual Review of Ecology and Systematics 33: 341-370.
Vanni, M. J. and Layne, C. D. 1997. Nutrient recycling and herbivory as mechanisms in the "top-down" effect of fish on algae in lakes. Ecology 78: 21-40.
Verspagen, J. M. H., Snelder, E., Visser, P. M., Huisman, J., Mur, L. R. and Ibelings, B. W. 2004. Recruitment of benthic *Microcystis* (Cyanophyceae) to the water column: Internal buoyancy changes or resuspension? Journal of Phycology 40: 260-270.
Vézie, C., Rapala, J., Vaitomaa, J., Seitsonen, J. and Sivonen, K. 2002. Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intracellular microcystin concentrations. Microbial Ecology 43: 443-454.
Visser, P. M., Ibelings, B. W., Mur, L. R. and Walsby, A. E. 2005. The ecophysiology of the harmful cyanobacterium *Microcystis*. Features explaining its success and measures for its control. In: Huisman, J., Matthijs, H. C. P. and Visser, P. M. (eds). Harmful Cyanobacteria. Springer. Dordrecht, The Netherlands. pp. 109-142.
Vyverman, W., Verleyen, E., Sabbe, K., Vanhoutte, K., Sterken, M., Hodgson, D. A., Mann, D. G., Juggins, S. *et al.* 2007. Historical processes constrain patterns in global diatom diversity. Ecology 88: 1924-1931.
Welker, M., Brunke, M., Preussel, K., Lippert, I. and von Dohren, H. 2004. Diversity and distribution of *Microcystis* (Cyanobacteria) oligopeptide chemotypes from natural communities studied by single-colony mass spectrometry. Microbiology-Sgm 150: 1785-1796.
Welker, M., Sejnohova, L., Nemethova, D., von Dohren, H., Jarkovsky, J. and Marsalek, B. 2007. Seasonal shifts in chemotype composition of *Microcystis sp.* communities in the pelagial and the sediment of a shallow reservoir. Limnology and Oceanography 52: 609-619.
Whitaker, R. J., Grogan, D. W. and Taylor, J. W. 2003. Geographic barriers isolate endemic populations of hyperthermophilic Archaea. Science 301: 976-978.
W. H. O. 1998. Cyanobacterial toxins: Microcystin-LR. Guidelines for Drinking-Water Quality. World Health Organization, Geneva.
Wilmotte, A. and Golubic, S. 1991. Morphological and genetic criteria in the taxonomy of Cyanophyta Cyanobacteria. Archiv für Hydrobiologie Supplement 92: 1-24.
Wilson, A. E., Sarnelle, O., Neilan, B. A., Salmon, T. P., Gehringer, M. M. and Hay, M. E. 2005. Genetic variation of the bloom-forming cyanobacterium *Microcystis aeruginosa* within and among lakes: Implications for harmful algal blooms. Applied and Environmental Microbiology 71: 6126-6133.
Wilson, A. E., Wilson, W. A. and Hay, M. E. 2006. Intraspecific variation in growth and morphology of the bloom-forming cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 72: 7386-7389.
Wu, Z. X., Gan, N. Q. and Song, L. R. 2007. Genetic diversity: Geographical distribution and toxin profiles of *Microcystis* strains (Cyanobacteria) in China. Journal of Integrative Plant Biology 49: 262-269.
Wu, Z. X. and Song, L. R. 2008. Physiological comparison between colonial and unicellular forms of *Microcystis aeruginosa* Kutz. (Cyanobacteria). Phycologia 47: 98-104.
Yoshida, T., Takashima, Y., Tomaru, Y., Shirai, Y., Takao, Y., Hiroishi, S. and Nagasaki, K. 2006. Isolation and characterization of a cyanophage infecting the toxic cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 72: 1239-1247.
Yoshida, M., Yoshida, T., Satomi, M., Takashima, Y., Hosoda, N. and Hiroishi, S. 2008a. Intra-specific phenotypic and genotypic variation in toxic cyanobacterial *Microcystis* strains. Journal of Applied Microbiology 105: 407-415.
Yoshida, M., Yoshida, T., Kashima, A., Takashima, Y., Hosoda, N., Nagasaki, K. and Hiroishi, S. 2008b. Ecological dynamics of the toxic bloom-forming cyanobacterium *Microcystis aeruginosa* and its cyanophages in freshwater. Applied and Environmental Microbiology 74: 3269-3273.
Yoshida, M., Yoshida, T., Takashima, Y., Hosoda, N. and Hiroishi, S. 2007. Dynamics of microcystin-producing and non-microcystin-producing *Microcystis* populations is correlated with nitrate concentration in a Japanese lake. FEMS Microbiology Letters 266: 49-53.
Yoshida, M., Yoshida, T., Takashima, Y., Kondo, R. and Hiroishi, S. 2005. Genetic diversity of the toxic cyanobacterium *Microcystis* in Lake Mikata. Environmental Toxicology 20: 229-234.

Zhang, M., Kong, F. X., Tan, X., Yang, Z., Cao, H. S. and Xing, P. 2007. Biochemical, morphological and genetic variations in *Microcystis aeruginosa* due to colony disaggregation. World Journal of Microbiology & Biotechnology 23: 663-670.

Zhang, Q. G. and Zhang, D. Y. 2007. Colonization sequence influences selection and complementarity effects on biomass production in experimental algal microcosms. Oikos 116: 1748-1758.

Chapter 2

Global dispersal of *Microcystis* inferred from rDNA Internal Transcribed Spacer (ITS) sequences

Ineke van Gremberghe[1], Frederik Leliaert[2], Joachim Mergeay[3], Katleen Van der Gucht[1], Pieter Vanormelingen[1], Ann-Eline Debeer[1], Luc De Meester[3] and Wim Vyverman[1]

Unpublished manuscript

Contribution to this chapter by IVG: culturing *Microcystis* strains, part of the molecular work, statistical analyses and writing

[1]Laboratory of Protistology and Aquatic Ecology, Ghent University, Krijgslaan 281 - S8, 9000 Ghent, Belgium

[2]Phycology Research Group, Ghent University, Krijgslaan 281 - S8, 9000 Ghent, Belgium

[3]Laboratory of Aquatic Ecology and Evolutionary Biology, Katholieke Universiteit Leuven, Charles Debériotstraat 32, 3000 Leuven, Belgium

Abstract

The geographical distribution of closely related cyanobacterial lineages is to a large extent unknown and the few phylogeographic studies show contrasting results. We determined the global phylogeography of the freshwater cyanobacterium *Microcystis* based on an extensive dataset of 302 16S-23S rDNA ITS sequences sampled from six continents. Phylogenetic analyses revealed largely unresolved and non-diverging relationships among ITS types, indicating the absence of independently evolving lineages in *Microcystis*. Our analyses also revealed the presence of short, hypervariable regions in the ITS region showing incongruent phylogenetic signals, possibly as a result of DNA transfer between different *Microcystis* strains or recombination within the genome itself. Overall, our data revealed a high diversity of *Microcystis* ITS types in all continents and the presence of several identical ITS types on more than one continent. Analysis of variance showed a lack of overall genetic differentiation between continents. Our results suggest high dispersal potential of *Microcystis* ITS types on a global scale. To what extent environmental selection is important for the distribution of ITS types is largely unclear, but climatic conditions apparently do not have a considerable impact.

2.1 Introduction

Phylogeography is an integrative field of science that uses genetic information to study the geographical distribution of genealogical lineages, especially those found within species (Avise 2000). Geographic isolation, past range restrictions and expansions, drift processes, and founder effects associated with rare long-distance dispersal events may all leave their signature in the lineage composition of contemporary populations. The importance of such historical factors for prokaryote populations is contentious however, given the view that the huge population sizes of microbial species drive their ubiquitous dispersal (Baas Becking 1934; Finlay 2002). However, allopatric divergence has been observed in Archaea and Bacteria (including Cyanobacteria) inhabiting isolated habitats such as hot springs and hydrothermal vent mussels (Whitaker *et al.* 2003; Papke *et al.* 2003; DeChaine *et al.* 2006). In less sparsely distributed habitats, some at first sight conflicting results were obtained. On one hand, based on sequences of rDNA ITS1, PC-IGS, *nif*H and *rpo*C1, Haande *et al.* (2008) showed that strains of the freshwater planktonic cyanobacterium *Cylindrospermopsis raciborskii* from the same continent were more closely related than strains originating from different continents. On the other hand, little evidence was found for dispersal limitation of 16S rDNA lineages of freshwater bacterioplankton from a local to continental scale (Van der Gucht *et al.* 2007) and soil bacteria on a global scale (Fierer and Jackson 2006). In addition, Rocap *et al.* (2002) reported the absence of geographically distributed lineages of the planktonic marine cyanobacteria *Synechococcus* and *Prochlorococcus* based on rDNA ITS sequence data, and Scheldeman *et al.* (1999) and Baurain *et al.* (2002) showed the absence of geographically distributed ITS lineages in the (sub)tropical freshwater cyanobacterium *Arthrospira*. These differences may be attributable to differences in resolution of the markers used, the degree of habitat isolation, or differences in sampling scale (Dolan 2005, 2006; Martiny *et al.* 2006). In any case, it is clear that more studies are needed to determine the role of evolutionary history in shaping the lineage composition of prokaryote populations.

Microcystis is a planktonic cyanobacterium often displaying mass developments at the surface of freshwater bodies, causing economical and ecological problems worldwide (Huisman *et al.* 2005). Such nuisance blooms of *Microcystis* are composed of genetically and ecologically different strains, with most strains producing the hepatotoxin microcystin (Chorus and Bartram 1999; Janse *et al.* 2004). Differences in the size and shape of cells and colonies have led traditional taxonomists to distinguish several morphospecies in *Microcystis* (Komárek and Anagnostidis 1986, 1999). However, the delineation of morphospecies does not correspond to genetic lineages based on molecular markers such as 16S rDNA, rDNA ITS and *cpc*BA IGS (Otsuka *et al.* 1998, 1999; Tillett *et al.* 2001; Janse *et al.* 2004). Phylogenetic studies based on 16S rDNA sequences and DNA-DNA hybridization suggest that different *Microcystis* strains are closely related to each other (Otsuka *et al.* 1998, 2001).

Microcystis is distributed worldwide from tropical to cold-temperate regions (Chorus and Bartram 1999). Evidence for discrete geographical distribution patterns of *Microcystis* genotypes was provided by Wu *et al.* (2007) based on whole-cell PCR of

*cpc*BA IGS and Oberholster *et al.* (2005) using AFLP. On the other hand, studies based on rDNA ITS and *cpc*BA IGS suggest a cosmopolitan distribution of these genotypes (Bittencourt-Oliveira *et al.* 2001; Janse *et al.* 2004; Haande *et al.* 2007). However, as these studies were relatively limited in scope, they did not imply that all or most *Microcystis* genotypes are ubiquitous. A comprehensive study is still lacking to gain insight into the worldwide dispersal of *Microcystis* genotypes and the influence of environmental factors on lineage distribution. The rDNA ITS region has proven to be variable enough for phylogeographic studies of *Microcystis* and is present in the genome as a single or several identical rDNA operons (Otsuka *et al.* 1999; Boyer *et al.* 2001; Humbert *et al.* 2005). Several studies have suggested a link between ITS type and phenotypic and chemotypic traits in *Microcystis* (Otsuka *et al.* 1999; Janse *et al.* 2004; Yoshida *et al.* 2008; Cadel-Six *et al.* 2008). The present study was designed to obtain a better understanding of biogeographical patterns of *Microcystis* based on a global dataset of rDNA ITS sequences. In particular, we were interested to find out (1) if similar *Microcystis* ITS types are distributed worldwide, or if distinct geographical distribution patterns could be detected, and (2) if the distribution of *Microcystis* ITS types is influenced by climatic conditions.

2.2 Materials and methods

2.2.1 Dataset construction

Our dataset consisted of 302 rDNA ITS sequences of *Microcystis* sampled from six continents: Europe (199), Africa (39), Asia (45), North America (1), South America (12) and New Zealand (6). All sequences from Belgium (52), South-America (12) and Ethiopia (29) were newly obtained in this study in addition with some sequences from Denmark (5), The Netherlands (7) and Spain (2) (Table 2.1, Fig. 2.1). See acknowledgements for the different projects in which these sequences were obtained and chapter 3 for more information about the sampled lakes. New sequences were generated using three methods. Most were obtained from sequencing bands of Denaturing Gradient Gel Electrophoresis (DGGE) of natural water samples (57), several from cloning of mixed PCR products from natural water samples (38), and some from direct sequencing of isolated *Microcystis* strains (12) (see also below). These were completed with sequences from GenBank (all 195 sequences of *Microcystis* including the full ITS sequence available on the 14th of June 2008 were considered), which were also obtained from cultivated isolates, sequenced DGGE bands or cloned PCR fragments. For accession numbers of sequences included from GenBank see figure 2.4. Because no quantitative sequence data were available for most geographical areas, only a single sequence of each ITS type was included for each country in the dataset.

2.2.2 Sampling, strain isolation and culture conditions

Water samples for DNA extractions from lakes and ponds in Europe, South America and Ethiopia were filtered over a 25 mm 0.2 µm GSWP filter (Millipore) and frozen

Figure 2.1: Global map with indication of the regions from which *Microcystis* ITS sequences were used to build a global phylogeography.

as soon as possible at -20 °C. Individual *Microcystis* colonies of samples from Flanders (Belgium) and Tigray (Ethiopia) were picked out using sterile glass Pasteur pipettes under a stereo microscope. The strains were grown in WC medium (Guillard and Lorenzen 1972) at 19 °C at an irradiance of approximately 30 µmol m^{-2} s^{-1} and a 12:12 h light:dark cycle. In total, ten ITS sequences were included from isolated strains from Belgium and two from Ethiopia.

2.2.3 DNA extraction and PCR amplification

DNA from the water samples and isolated strains was extracted as described by Zwart *et al.* (1998), including bead beating with phenol extraction and ethanol precipitation. After extraction, the DNA was purified on a Wizard column (Promega). 16S-23S rDNA ITS sequences of the isolated *Microcystis* strains were amplified using the protocol described by Janse *et al.* (2003). In addition, fragments of the *mcy* A and *mcy* E genes were amplified using the protocols from respectively Hisbergues *et al.* (2003) and Vaitomaa *et al.* (2003) to examine if the isolated strains are (potentially) toxic. For the DNA from the water samples, a specific nested-PCR protocol based on Janse *et al.* (2003) was developed to amplify only *Microcystis* ITS sequences. In a first PCR, a specific 16S rDNA primer for *Microcystis* (CH) described by Rudi *et al.* (1997) was used as forward primer and a universal 23S rDNA primer (ULR) as reverse primer (Janse *et al.* 2003). The PCR was performed using the following reaction mixtures: 1 µl of template DNA, 0.5 µM of each primer, 200 µM of each deoxynucleoside triphosphate, 2.5 units of Taq DNA polymerase (Ampli Taq), 10x PCR buffer [Tris/HCl: 100 mM, pH: 8.3; KCl: 500 mM; MgCl2: 15 mM; Gelatine: 0.01 % (w/v)] and 400 ng of

bovine serum albumin. The mix was adjusted to a final volume of 50 µl with sterile water. The PCR program started with a denaturation step of five minutes at 94 °C. A touch down procedure was performed consisting of 20 cycles in which the annealing temperature decreased by 1 °C every second cycle from 62 to 52 °C. In the next 10 cycles, the annealing temperature was 52 °C. Cycle step times were one minute each for denaturation (94 °C), annealing and extension (72 °C). A final extension step was performed for 30 minutes at 72 °C. The resulting PCR product was purified using a QiaQuick PCR purification kit (QiaGen), diluted 10X, and used as template (2 µl in a total volume of 50 µl) for a second PCR with the cyanobacterium-specific 16S rDNA primer (GC)-CSIF (with GC-clamp for DGGE-analysis, without GC-clamp for cloning) in combination with the universal primer ULR (Janse *et al.* 2003). The composition of the reaction mix was the same as for the first PCR. The PCR program started with a denaturation step of five minutes at 94 °C. After pre-incubation, 30 cycles were performed. Cycle step times were one minute each for denaturation (94 °C), annealing (65 °C) and extension (72 °C). A final extension step was performed for 30 minutes at 72 °C.

2.2.4 DGGE profiling

DGGE was essentially performed as described by Muyzer *et al.* (1993). Equal amounts of PCR products were loaded onto 8 % (w/v) polyacrylamide gels (1 mm thick, in 1X TAE [20 mM Tris acetate (pH 7.4), 10 mM acetate, 0.5 mM disodium EDTA]). The denaturing gradient contained 35-40 % denaturant [100 % denaturant corresponded to 7 M urea and 40 % (v/v) formamide]. Electrophoresis was performed for 16 h at 75 V and the temperature was set at 60 °C. Finally, the gels were stained with ethidium bromide and photographed on a UV transillumination table with a CCD camera. Next, a small piece of gel from the middle of the target band was excised from the DGGE gel and incubated in 50 µl of sterile TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA) for 24 h at 4 °C. The eluent was then reamplified and purified on DGGE one or two times. The resulting PCR products were purified using a QiaQuick PCR purification kit (QiaGen).

2.2.5 Cloning

Microcystis-specific ITS sequences obtained from samples from Belgium and Ethiopia were ligated into pGEM®-T Easy Vectors (Promega), and transformed into competent *Escherichia coli* JM109 cells. The transformed cells were plated on Luria-Bertani (LB) plates containing 20 µg l^{-1} ampicillin, 20 µg l^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 5 µg l^{-1} IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated overnight at 37 °C. White recombinants were picked out and grown overnight in ampicillin-supplemented liquid medium (Luria-Bertani-Broth). The clones were screened for inserts using primers CSIF and ULR (see before). 40-70 clones per sample were screened by DGGE analysis (see before) to identify groups of clones containing (presumably) the same inserts. One or more representatives of each group were then

chosen for sequencing. In total, 34 ITS sequences were included from Belgium and four from Ethiopia obtained by cloning (see also chapter 3).

2.2.6 Sequencing

Sequencing was performed with the ABI-Prism sequencing kit and the resulting sequencing reaction products were analysed on an automatic sequencer (ABI-Prism 3100).

2.2.7 Microcystin production

Microcystin production of two strains of each ITS type was checked by ELISA (Enzyme-Linked Immunosorbent Assay). For the extraction of microcystins, the cells were put in 1.5 ml tubes and placed in a boiling water bath during 20 minutes (van der Oost 2007). Next, the cultures were centrifuged for 10 minutes at 14 000 rpm and the supernatant was used for ELISA. The ELISA-test was done according to the manufacturer's instructions (SDI - EnviroGard Microcystin ELISA plate kit). The toxicity of ITS types obtained from GenBank was also included based on the detection of microcystins when this information was available in published studies.

2.2.8 Phylogenetic analysis

The 302 ITS sequences were aligned using MUSCLE (Edgar 2004)[1], and manually adjusted. The alignment was straightforward and included a limited number of insertions / deletions (indels). This full sequence alignment suggested the presence of multiple hypervariable regions in the ITS region (fig 2.2). Detailed analyses of DNA site polymorphism using DnaSP 4.50.3 (Rozas *et al.* 2003) by means of the sliding window option (window size = 2, step size = 1) revealed eight hypervariable regions that displayed extremely high nucleotide divergence (between 0.55 and 0.30, positions 18, 44, 81-87, 114-116, 217, 226-248, 269-284, 306-307) compared to the average (0.03). We suspected that these hypervariable regions were localized artefacts of unknown origin. Sequencing artefacts could be excluded based on the observation that similar or identical mutations were observed for all regions in all sequences. These regions are only phylogenetically informative if their different genetic variants were autapomorphies, and if they were only transferred vertically and did not suffer from recombination. Under this model of inheritance, we expected particular variants of these mutation-prone regions to be highly correlated ($R > 0.90$) to each other due to complete physical linkage. In the opposite case, macromutations were expected to behave as more independent units, meaning that associations with other macromutation variants at other loci would be broken up. This lack of linkage would increase the level of noise in the phylogenetic signal. Linkage was tested in the software program Genetix v. 4.5 (Belkhir *et al.* 2004) by encoding each variant of a particular macromutation locus as a distinct allele and testing the Black and Krafsur (1985) correlation coefficient for linkage disequilibrium.

[1] http://www.ebi.ac.uk/Tools/muscle/

For this test to run, haploid data were considered as homozygous diploid data as suggested by Goudet (2001). Usually this test is used to test for independence of inheritance of supposedly physically unlinked genetic markers. We used the same test statistic, however, to test the degree of correlation of proximate loci (< 300 base pairs apart). All loci showed significant but weak linkage ($R = 0.7 - 0.22$; $p < 0.05$), showing that these regions display incongruent phylogenetic signals in the large majority of cases, justifying their removal from the dataset. This sequence alignment is further referred to as stripped ITS sequence alignment.

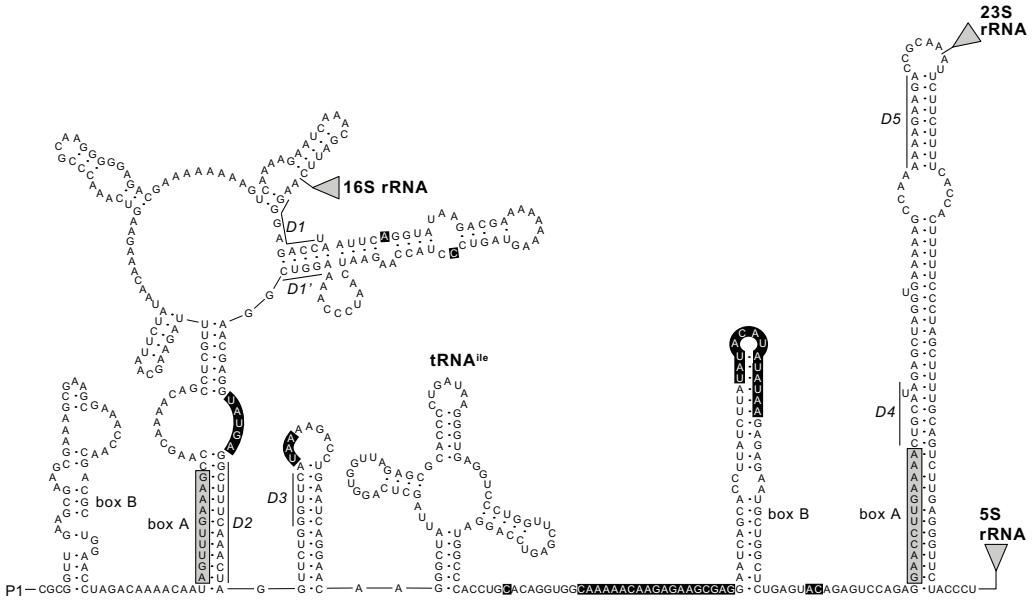


Figure 2.2: Predicted secondary structure of the spacer region of the rrn operon transcript (i.e. the 5' leader sequence upstream from the 16S rRNA, the 16S-23S rRNA ITS and 23S-5S rRNA spacer) of *Microcystis* strain NIES843 (EMBL accession number AP009552: ITS sequence identical to BG08 of the present study). P1 indicates the position of the promotor. Locations of the 16S rRNA, 23S rRNA and 5S rRNA are represented by triangles. The antiterminator box B stems and box A sequences are indicated in the leader and spacer domains, in accordance with Rocap *et al.* (2002). The conserved motifs (D1-D5, defined by Iteman *et al.* 2000) are marked. Positions of the hypervariable regions, identified in the present study, are indicated by a black background.

The amount of phylogenetic signal versus noise in the full and stripped ITS alignments was assessed by three different approaches. First, the Iss statistic, a measure of substitution saturation in molecular phylogenetic data sets, was calculated with DAMBE (Xia and Xie 2001). Second, the rDNA data were tested for substitutional saturation by plotting the uncorrected distances against the corrected distances as determined with the model of sequence evolution yielding the best fit to the data (estimated with PAUP/Modeltest 3.6) (Swofford 2002; Posada and Crandall 1998). Third, the measure of skewness [g1-vallue calculated by using 10,000 randomly selected trees in PAUP* 4.0b10 (Swofford 2002)] was compared with the empirical threshold values in Hillis and Huelsenbeck (1992) to verify for non-random structuring of the data.

Phylogenetic reconstruction using the full or stripped alignment was performed with the Bayesian inference (BI) and maximum likelihood (ML) methods, using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) and PhyML v2.4.4 (Guindon and Gascuel 2003), respectively. The full alignment was analysed under a general time-reversible model with proportion of invariable sites and gamma distribution split into four categories (GTR+I+G4), as determined by the Akaike Information Criterion in PAUP / Modeltest 3.6. In the stripped dataset, no separate rate class for invariable sites was accounted for (GTR+G4). BI analyses consisted of two parallel runs of four incrementally heated chains, and 5 million generations with sampling every 1000 generations. Summary statistics and trees were generated using the last 2 million generations, well beyond the point of convergence between the two runs. For the ML trees, the reliability of each internal branch was evaluated based on 1000 bootstrap replicates.

The production of PCR artefacts (e.g. chimeras and heteroduplexes) is a potential risk when mixed templates of related sequences are amplified by PCR (Wang and Wang 1996; Thompson *et al.* 2002). This leads to an overestimation of the genetic variation through the amplification of non-existent sequences (von Wintzingerode *et al.* 1997; Hugenholtz and Huber 2003). When performing DGGE and cloning using PCR-amplified DNA obtained from water samples to obtain *Microcystis*-specific ITS sequences, there is a risk of chimeric DNA molecules. Therefore we also performed our phylogenetic analyses using sequences from isolated strains only.

Statistical parsimony networks of the full and stripped datasets (Templeton *et al.* 1992) were constructed with TCS 1.21 (Clement *et al.* 2000), with calculated maximum connection steps at 95 % and alignment gaps treated as missing data.

2.2.9 Biogeographic and climatic structure analysis

The sequence dataset was divided into distinct pre-defined populations, each population consisting of sequences from a single country (Table 2.1). For each ITS sequence, the climate of the region of origin was indicated according to Köppen-Geiger (Kottek *et al.* 2006). For some larger countries spanning different climate zones, more than one population was considered. In total, twelve distinct climates were distinguished (Table 2.1). Patterns of genetic structuring based on the stripped ITS sequence alignment among geographical localities (continents) and climates were estimated by analysis of molecular variance (AMOVA) using Arlequin 3.1 (Excoffier *et al.* 2005). For intercontinental comparisons, using the total dataset can give a biased view as more populations were sampled in Europe compared to the other continents. To obtain equal sample sizes, samples were selected based on the number of lakes sampled in a population (2-5 lakes per population, Table 2.1). In addition, we divided the selected dataset into two 'super continents': Afro-Eurasia (selection of four populations: Greece, Denmark, Ethiopia and Japan) and South America. Three climate groupings were considered. First, a grouping based on the climate according to Köppen-Geiger resulting in twelve different climates. Second, only climates represented by more than ten ITS types were considered (Aw, BSh, Cfb, Csa, Cfa). Third, the dataset was split into cold-temperate

(Cfb, Dfc, Cfc, Csb, Dfb, ET) and (sub)tropical climate (Af, As, Aw, Cfa, Csa, BSh), based on the average year temperature. To perform AMOVA, a distance matrix was calculated using Tamura and Nei distances (Tamura and Nei, 1993) and significance levels were determined with 1000 permutations. Additionally, nucleotide divergence (mean percentage of pairwise differences, Tamura and Nei correction) and its standard deviation based on the stripped ITS sequence alignment were calculated for each population using Arlequin 3.1. One-way analysis of variance (ANOVA) was performed using SPSS 15 (SPSS Inc. Chicago, USA) to test for significant differences in average nucleotide divergence between the continents (excluding New Zealand, as only one population was sampled there) using the restricted dataset correcting for differences sample size (Table 2.1).

Table 2.1: Origin of the ITS sequences used to infer the global phylogeography of *Microcystis*. Only the populations indicated with an asterisk were used in the restricted dataset correcting for differences in sample size (asterisk in the column 'number of sampled lakes' indicates the number of lakes selected in the restricted dataset). Climates were indicated according to Köppen-Geiger. Main climates: A = equatorial, B = arid, C = warm temperate, D = snow, E = polar. Precipitation: S = steppe, f = fully humid, s = summer dry, w = winter dry. Temperature: a = hot summer, b = warm summer, c = cool summer, h = hot arid. T = polar tundra.

Continent	Population	Climate	No. of sampled lakes	No. of distinct sequences
Europe	Belgium	Cfb	37	52
	The Netherlands	Cfb	10	38
	Germany*	Cfb	3	8
	Italy	Csa	1	3
	Spain	Csa	3	11
	Portugal-Csa	Csa	1	1
	Portugal-Csb	Csb	1	1
	Greece*	Csa	2	26
	Romania	Dfb	1?	6
	France	Cfb	1	37
	Scotland*	Cfb	3	9
	Czech Republic	Cfb	1	1
	Denmark*	Cfb	5	6
Africa	Uganda-Af	Af	1	4
	Uganda-Aw*	Aw	2	3
	Kenya-Af*	Af	2	2
	Kenya-Aw	Aw	1	1
	Ethiopia*	BSh	29 (*: 5)	29
Asia	China*	Cfa	2	7
	Japan-Cfa*	Cfa	8 (*: 5)	26
	Japan-Dfb	Dfb	2	3
	Thailand*	Aw	3	7
	Israel	Csa	1	2
North America	Canada	Dfc	1	1
South America	Brazil-Aw*	Aw	5	5
	Brazil-As*	As	2	3
	Argentina-ET*	ET	3	3
	Argentina-Cfc	Cfc	1	1
Oceania	New Zealand*	Cfb	5	6

2.3 Results

2.3.1 Phylogenetic analysis

Specifications of the full and stripped ITS sequence alignments and evolutionary models applied are given in Table 2.2. The saturation plot (Fig. 2.3) of the full dataset was found to level off with increasing genetic distance, indicating mild saturation in the sequence alignment. The stripped dataset showed a nearly linear correlation between uncorrected and corrected distances, indicating that this dataset did not suffer from saturation. The Iss statistic did not reveal significant saturation in any of the alignments. The measure of skewness of the full alignment equalled the empirical threshold values in Hillis and Huelsenbeck (1992), indicating that the length distributions of random trees of all data sets were only slightly left-skewed, and that the alignment was only slightly more structured than random data. Removal of the hypervariable and putative recombinant regions resulted in an alignment that was significantly more structured than random data.

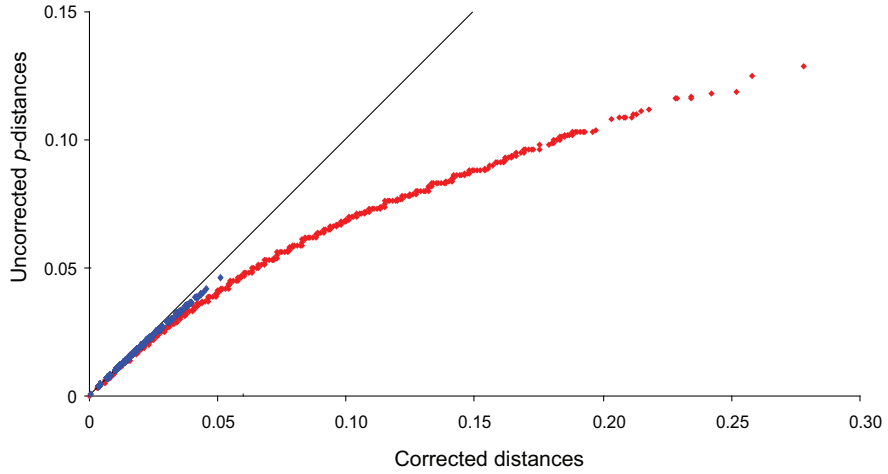


Figure 2.3: Analysis of saturation of the full (red dots) and stripped (blue dots) ITS sequence alignments by plotting corrected distances versus uncorrected p-distances. Corrected distances are calculated using models estimated by PAUP/Modeltest for each specific data partition (see Table 2.2)

BI and ML analyses of the full and stripped ITS sequence alignments yielded trees which were largely unresolved, except for a small number of well-supported clades. Only the BI 50 % majority rule consensus tree inferred from the stripped alignment is shown in figure 2.4. In this tree, a large unresolved group of ITS sequences is notable, with several weak to more strongly supported clades emerging from this polytomy (indicated as clades A to G). The unresolved group included a mixture of ITS types from all continents.

Table 2.2: Specification of the full and stripped ITS sequence alignment and summary of models and model parameters obtained.

	ITS sequence alignment	
	Full	Stripped
Alignment length / variable sites / parsimony informative sites	374 / 139 / 66	317 / 99 / 33
Number of distinct ITS types (gaps treated as missing / 5th state)	234 / 249	119 / 142
Uncorrected pairwise sequence divergence (max / average %)	13 / 4	5/ 1
Measure of skewness (g1-value)	-0.129	-0.378
Iss statistic (Iss / Iss.c, p-value of 32 taxon data subsets)	0.100 / 0.654, $p < 0.001$	0.076 / 0.681, $p < 0.001$
Model estimated by the Akaike information criterion (AIC)	GTR + I + G	GTR + G
Base frequencies (A/C/G/T)	0.34 / 0.18 / 0.30 / 0.18	0.31 / 0.22 / 0.29 / 0.18
Substitution rates (AC / AG / AT / CG / CT / GT)	2.09 / 2.81 / 1.16 / 1.33 / 4.11 / 1.00	1.67 / 6.83 / 2.17 / 0.49 / 5.24 / 1.00
Among-site rate variation: proportion of invariable sites (I) / gamma distribution shape parameter (G)	0.42 / 0.35	0 / 0.60

Most clades consisted of ITS genotypes from several continents (C, E, F and G), while some others were exclusively restricted to a single continent (A, B and D). Toxic *Microcystis* strains from different continents are distributed in different clades along with non-toxic strains, and strains identical in ITS sequence are not always similar in the capacity to produce microcystins. When considering the full ITS sequence alignment, six ITS types were represented by toxic strains only, eight by non-toxic strains, and three by both toxic and non-toxic strains. For the stripped ITS sequence alignment five ITS types were represented by toxic strains only, four by non-toxic strains, and seven by toxic and non-toxic strains. BI and ML analysis using only isolated strains resulted in comparable, poorly resolved phylogenetic trees (not shown), indicating that PCR artefacts due to sequences obtained by DGGE and cloning were not the main cause of the low support values.

The statistical parsimony analysis based on the full ITS sequence alignment resulted in a single, highly interconnected network based on a maximum connection limit of 9 steps (Fig. 2.5). In total, twelve ITS types were found at two continents, three ITS types were found at three continents and one ITS type was found at four continents. The parsimony network derived from the stripped ITS sequence alignment was to a large extent still unresolved, with a high degree of connection uncertainties (Fig. 2.6). Nevertheless, one particular ITS type accounted for 15 % of all sequences. This ITS type was also considered as the most likely root of the network by TCS, following the general trend that ancestral types tend to be centrally placed, are abundant and have many closely related derivates (cfr. the star-shaped pattern, Templeton 1998). This central type was moreover distributed over five out of six sampled continents. The sixth continent (Oceania) was represented twice by ITS types that were only one mutational step away from this central type. Four smaller clusters (numbered 1 - 4 in figure 2.6) could be discriminated, connected to this central group by one to five mutational steps, each of which containing sequences from three to five continents. The subcluster 1 is most distantly related to the ancestral ITS type and corresponds to clade X in the BI tree (Fig. 2.4). The sequences from South America and Oceania were mostly grouped around the ancestral ITS type, but few sequences were available from these continents. The overall pattern suggests little or no relation between geographic origin and position in the network. From the 119 distinct stripped ITS types found, sixteen occur on more than one continent, eight occur at two continents, six at three continents, one at four continents and one at five continents. Eight ITS types occur in more than one country within a single continent. One stripped ITS type was detected twice in Brazil and one was detected three times in The Netherlands. The unique ITS types were generally connected with more frequently observed ITS types by only a single mutational step. 22 ITS types were found in different climate regimes, of which 18 were detected in (sub)tropical as well as cold-temperate climate regimes (Fig. 2.4 and 2.6).

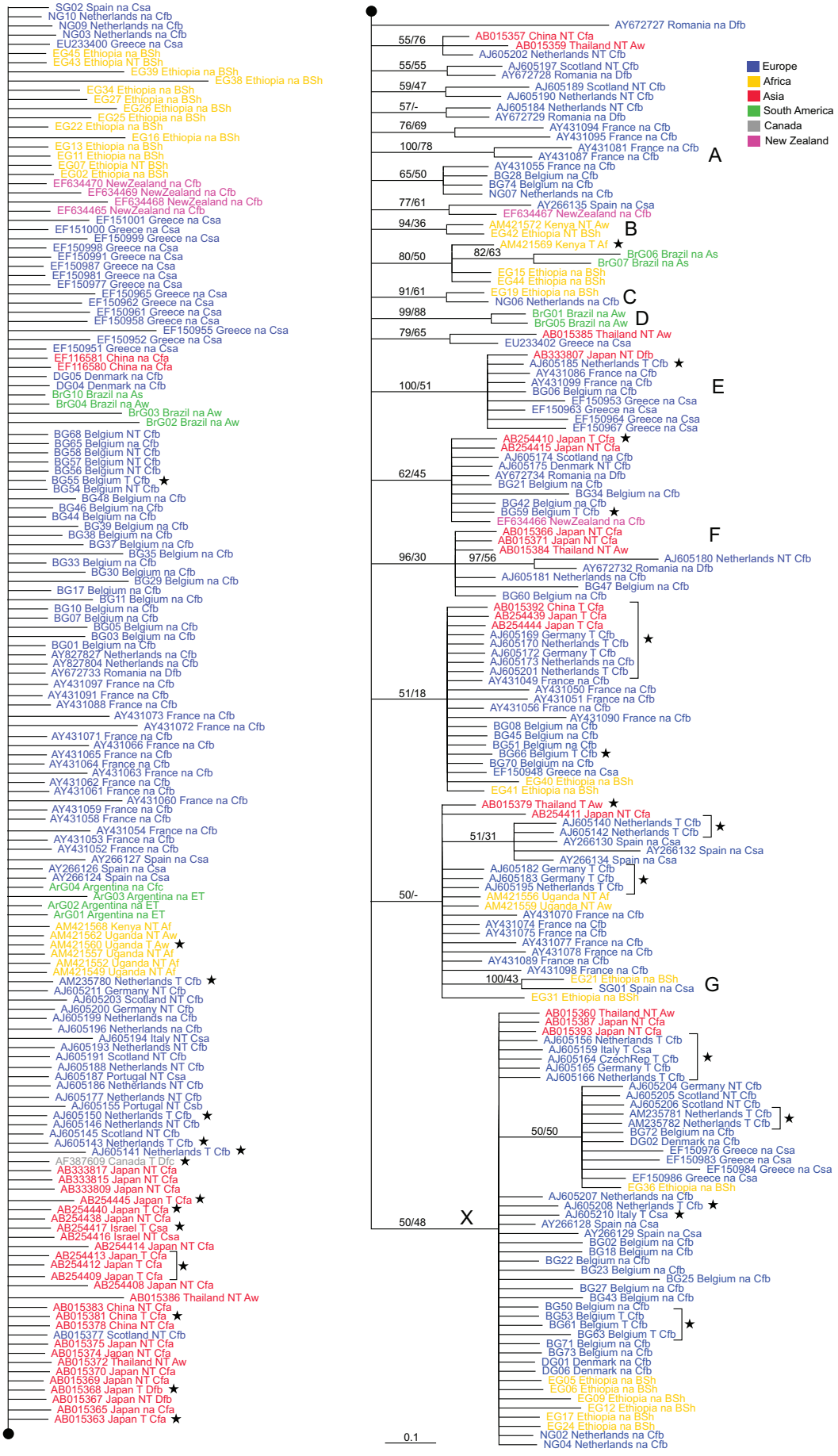

SG02 Spain na Csa
NG10 Netherlands na Cfb
NG09 Netherlands na Cfb
NG03 Netherlands na Cfb
EU233400 Greece na Csa
EG45 Ethiopia na BSh
EG43 Ethiopia NT BSh
EG39 Ethiopia na BSh
EG38 Ethiopia na BSh
EG34 Ethiopia na BSh
EG27 Ethiopia na BSh
EG26 Ethiopia na BSh
EG25 Ethiopia na BSh
EG22 Ethiopia na BSh
EG16 Ethiopia na BSh
EG13 Ethiopia na BSh
EG11 Ethiopia na BSh
EG07 Ethiopia NT BSh
EG02 Ethiopia na BSh
EF634470 NewZealand na Cfb
EF634469 NewZealand na Cfb
EF634468 NewZealand na Cfb
EF634465 NewZealand na Cfb
EF151001 Greece na Csa
EF151000 Greece na Csa
EF150999 Greece na Csa
EF150998 Greece na Csa
EF150991 Greece na Csa
EF150987 Greece na Csa
EF150981 Greece na Csa
EF150977 Greece na Csa
EF150965 Greece na Csa
EF150962 Greece na Csa
EF150961 Greece na Csa
EF150958 Greece na Csa
EF150955 Greece na Csa
EF150952 Greece na Csa
EF150951 Greece na Csa
EF116581 China na Cfa
EF116580 China na Cfa
DG05 Denmark na Cfb
DG04 Denmark na Cfb
BrG10 Brazil na As
BrG04 Brazil na Aw
BrG03 Brazil na Aw
BrG02 Brazil na Aw
BG68 Belgium NT Cfb
BG65 Belgium na Cfb
BG58 Belgium NT Cfb
BG57 Belgium NT Cfb
BG56 Belgium NT Cfb
BG55 Belgium T Cfb ★
BG54 Belgium NT Cfb
BG48 Belgium na Cfb
BG46 Belgium na Cfb
BG44 Belgium na Cfb
BG39 Belgium na Cfb
BG38 Belgium na Cfb
BG37 Belgium na Cfb
BG35 Belgium na Cfb
BG33 Belgium na Cfb
BG30 Belgium na Cfb
BG29 Belgium na Cfb
BG17 Belgium na Cfb
BG11 Belgium na Cfb
BG10 Belgium na Cfb
BG07 Belgium na Cfb
BG05 Belgium na Cfb
BG03 Belgium na Cfb
BG01 Belgium na Cfb
AY827827 Netherlands na Cfb
AY827804 Netherlands na Cfb
AY672733 Romania na Dfb
AY431097 France na Cfb
AY431091 France na Cfb
AY431088 France na Cfb
AY431073 France na Cfb
AY431072 France na Cfb
AY431071 France na Cfb
AY431066 France na Cfb
AY431065 France na Cfb
AY431064 France na Cfb
AY431063 France na Cfb
AY431062 France na Cfb
AY431061 France na Cfb
AY431060 France na Cfb
AY431059 France na Cfb
AY431058 France na Cfb
AY431054 France na Cfb
AY431053 France na Cfb
AY431052 France na Cfb
AY266127 Spain na Csa
AY266126 Spain na Csa
AY266124 Spain na Csa
ArG04 Argentina na Cfc
ArG03 Argentina na ET
ArG02 Argentina na ET
ArG01 Argentina na ET
AM421568 Kenya NT Af
AM421562 Uganda NT Aw
AM421560 Uganda T Aw ★
AM421557 Uganda NT Af
AM421552 Uganda NT Af
AM421549 Uganda NT Af
AM235780 Netherlands T Cfb ★
AJ605211 Germany NT Cfb
AJ605203 Scotland NT Cfb
AJ605200 Germany NT Cfb
AJ605199 Netherlands na Cfb
AJ605196 Netherlands na Cfb
AJ605194 Italy NT Csa
AJ605193 Netherlands NT Cfb
AJ605191 Scotland NT Cfb
AJ605188 Netherlands NT Cfb
AJ605187 Portugal NT Csa
AJ605186 Netherlands NT Cfb
AJ605177 Netherlands NT Cfb
AJ605155 Portugal NT Csb
AJ605150 Netherlands T Cfb ★
AJ605146 Netherlands NT Cfb
AJ605145 Scotland NT Cfb
AJ605143 Netherlands T Cfb ★
AJ605141 Netherlands T Cfb ★
AF387609 Canada T Dfc ★
AB333817 Japan NT Cfa
AB333815 Japan NT Cfa
AB333809 Japan NT Cfa
AB254445 Japan T Cfa ★
AB254440 Japan T Cfa ★
AB254438 Japan NT Cfa
AB254417 Israel T Csa ★
AB254416 Israel NT Csa
AB254414 Japan NT Cfa
AB254413 Japan T Cfa
AB254412 Japan T Cfa ★
AB254409 Japan T Cfa
AB254408 Japan NT Cfa
AB015386 Thailand NT Aw
AB015383 China NT Cfa
AB015381 China T Cfa ★
AB015378 China NT Cfa
AB015377 Scotland NT Cfb
AB015375 Japan NT Cfa
AB015374 Japan NT Cfa
AB015372 Thailand NT Aw
AB015370 Japan NT Cfa
AB015369 Japan NT Cfa
AB015368 Japan T Dfb ★
AB015367 Japan NT Dfb
AB015365 Japan na Cfa
AB015363 Japan T Cfa ★
AY672727 Romania na Dfb
55/76
AB015357 China NT Cfa
AB015359 Thailand NT Aw
AJ605202 Netherlands NT Cfb
55/55
AJ605197 Scotland NT Cfb
AY672728 Romania na Dfb
59/47
AJ605189 Scotland NT Cfb
AJ605190 Netherlands NT Cfb
57/-
AJ605184 Netherlands NT Cfb
AY672729 Romania na Dfb
76/69
AY431094 France na Cfb
AY431095 France na Cfb
100/78
AY431081 France na Cfb
AY431087 France na Cfb
A
65/50
AY431055 France na Cfb
BG28 Belgium na Cfb
BG74 Belgium na Cfb
NG07 Netherlands na Cfb
77/61
AY266135 Spain na Csa
EF634467 NewZealand na Cfb
94/36
AM421572 Kenya NT Aw
EG42 Ethiopia NT BSh
B
AM421569 Kenya T Af ★
80/50
82/63
BrG06 Brazil na As
BrG07 Brazil na As
EG15 Ethiopia na BSh
EG44 Ethiopia na BSh
91/61
EG19 Ethiopia na BSh
NG06 Netherlands na Cfb
C
99/88
BrG01 Brazil na Aw
BrG05 Brazil na Aw
D
79/65
AB015385 Thailand NT Aw
EU233402 Greece na Csa
AB333807 Japan NT Dfb
AJ605185 Netherlands T Cfb ★
AY431086 France na Cfb
100/51
AY431099 France na Cfb
BG06 Belgium na Cfb
E
EF150953 Greece na Csa
EF150963 Greece na Csa
EF150964 Greece na Csa
EF150967 Greece na Csa
AB254410 Japan T Cfa ★
AB254415 Japan NT Cfa
AJ605174 Scotland na Cfb
AJ605175 Denmark NT Cfb
62/45
AY672734 Romania na Dfb
BG21 Belgium na Cfb
BG34 Belgium na Cfb
BG42 Belgium na Cfb
BG59 Belgium T Cfb ★
EF634466 NewZealand na Cfb
AB015366 Japan NT Cfa
AB015371 Japan NT Cfa
AB015384 Thailand NT Aw
F
96/30
97/56
AJ605180 Netherlands NT Cfb
AY672732 Romania na Dfb
AJ605181 Netherlands na Cfb
BG47 Belgium na Cfb
BG60 Belgium na Cfb
AB015392 China T Cfa
AB254439 Japan T Cfa
AB254444 Japan T Cfa
AJ605169 Germany T Cfb
AJ605170 Netherlands T Cfb
AJ605172 Germany T Cfb
AJ605173 Netherlands na Cfb
AJ605201 Netherlands T Cfb
AY431049 France na Cfb
★
51/18
AY431050 France na Cfb
AY431051 France na Cfb
AY431056 France na Cfb
AY431090 France na Cfb
BG08 Belgium na Cfb
BG45 Belgium na Cfb
BG51 Belgium na Cfb
BG66 Belgium T Cfb ★
BG70 Belgium na Cfb
EF150948 Greece na Csa
EG40 Ethiopia na BSh
EG41 Ethiopia na BSh
AB015379 Thailand T Aw ★
AB254411 Japan NT Cfa
AJ605140 Netherlands T Cfb ★
AJ605142 Netherlands T Cfb ★
51/31
AY266130 Spain na Csa
AY266132 Spain na Csa
AY266134 Spain na Csa
AJ605182 Germany T Cfb
AJ605183 Germany T Cfb
AJ605195 Netherlands T Cfb
★
50/-
AM421556 Uganda NT Af
AM421559 Uganda NT Aw
AY431070 France na Cfb
AY431074 France na Cfb
AY431075 France na Cfb
AY431077 France na Cfb
AY431078 France na Cfb
AY431089 France na Cfb
AY431098 France na Cfb
100/43
EG21 Ethiopia na BSh
SG01 Spain na Csa
G
EG31 Ethiopia na BSh
AB015360 Thailand NT Aw
AB015387 Japan NT Cfa
AB015393 Japan NT Cfa
AJ605156 Netherlands T Cfb
AJ605159 Italy T Csa
AJ605164 CzechRep T Cfb
AJ605165 Germany T Cfb
AJ605166 Netherlands T Cfb
★
AJ605204 Germany NT Cfb
AJ605205 Scotland NT Cfb
AJ605206 Scotland NT Cfb
AM235781 Netherlands T Cfb
AM235782 Netherlands T Cfb
★
50/50
BG72 Belgium na Cfb
DG02 Denmark na Cfb
EF150976 Greece na Csa
EF150983 Greece na Csa
EF150984 Greece na Csa
EF150986 Greece na Csa
EG36 Ethiopia na BSh
AJ605207 Netherlands na Cfb
AJ605208 Netherlands T Cfb ★
AJ605210 Italy T Csa ★
AY266128 Spain na Csa
AY266129 Spain na Csa
BG02 Belgium na Cfb
BG18 Belgium na Cfb
BG22 Belgium na Cfb
BG23 Belgium na Cfb
BG25 Belgium na Cfb
BG27 Belgium na Cfb
BG43 Belgium na Cfb
BG50 Belgium na Cfb
BG53 Belgium T Cfb
BG61 Belgium T Cfb
BG63 Belgium T Cfb
★
50/48
X
BG71 Belgium na Cfb
BG73 Belgium na Cfb
DG01 Denmark na Cfb
DG06 Denmark na Cfb
EG05 Ethiopia na BSh
EG06 Ethiopia na BSh
EG09 Ethiopia na BSh
EG12 Ethiopia na BSh
EG17 Ethiopia na BSh
EG24 Ethiopia na BSh
NG02 Netherlands na Cfb
NG04 Netherlands na Cfb
0.1
Europe
Africa
Asia
South America
Canada
New Zealand

Figure 2.4: Bayesian 50 % majority-rule consensus tree based on the stripped ITS sequence alignment of *Microcystis*. The tree has been split at the solid black circles to facilitate visualisation. Values at branches indicate BI posterior probabilities and ML bootstrap values. Clade letters (A-G) are indicated for clusters that received posterior probability support of 90 % or more. Clade X shows a clade corresponding to subcluster 1 in the parsimony network (Fig. 2.6). T = toxic strain (also indicated with an asterisk), NT = non-toxic strain, na = toxicity not available. Climates according to Köppen-Geiger are also indicated.

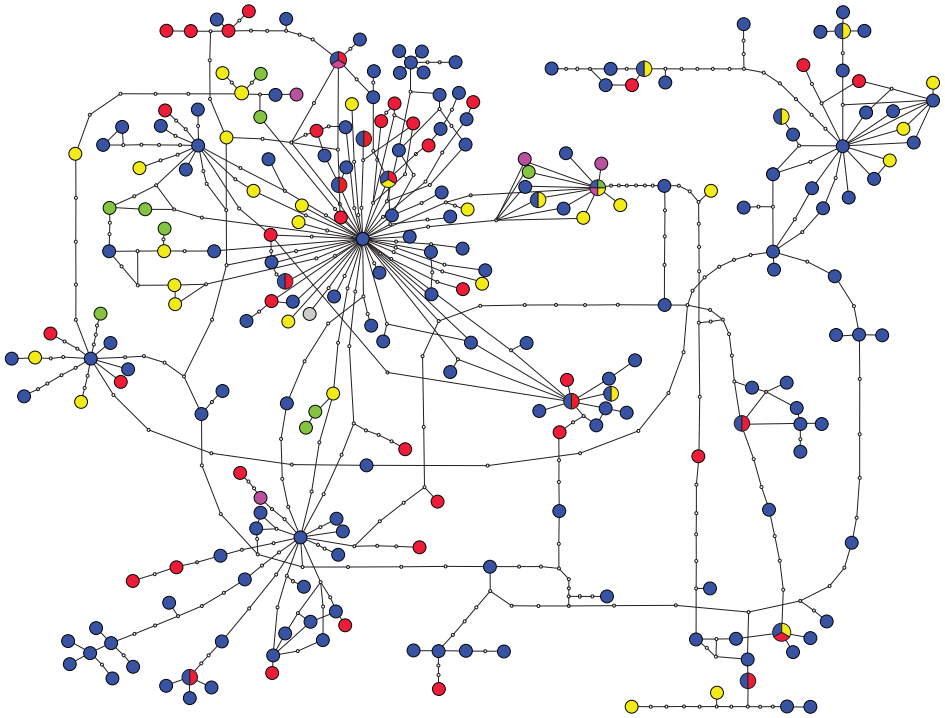


Figure 2.5: 95 % probability parsimony network of *Microcystis* rDNA ITS sequences based on the full ITS sequence alignment. Colours indicate region of origin (blue: Europe, yellow: Africa, red: Azia, green: South America, grey: Canada, pink: New Zealand). A line between ITS types represents one mutational step, open circles represent ITS types not sampled.

2.3.2 Biogeographic and climatic structure analysis

Analysis of molecular variance based on the stripped ITS sequence alignment showed no genetic structuring according to the continents (Table 2.3). The dominant component of genetic variation was found within (96.54 %) instead of between continents (3.46 %). A limited, but significant, genetic structuring was seen based on the super continents Afro-Eurasia and South America ($F_{CT} = 0.092$; $p < 0.05$), and the dominant component of genetic variation was again found within (90.82 %) instead of between these super continents (9.18 %). No significant genetic structuring was found among all twelve climates, among the five climates represented by more than ten ITS types, or between cold-temperate and (sub)tropical climates (Table 2.3).

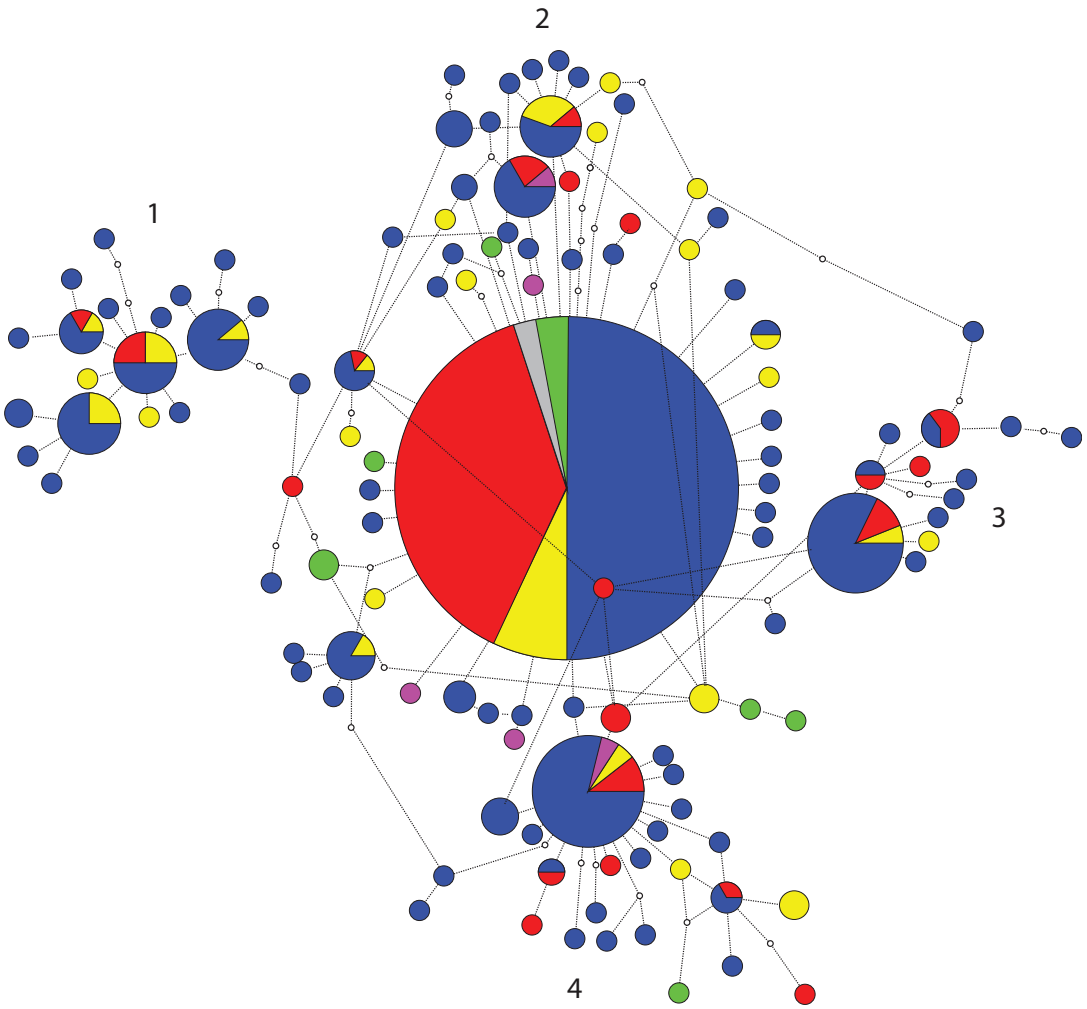


Figure 2.6: 95 % probability parsimony network of *Microcystis* rDNA ITS sequences based on the stripped ITS sequence alignment. Colours indicate region of origin (blue: Europe, yellow: Africa, red: Azia, green: South America, grey: Canada, pink: New Zealand). A line between ITS types represents one mutational step, open circles represent ITS types not sampled. Radius of the circles represents abundance, numbers 1-4 indicate subclusters.

Table 2.3: Results of AMOVA based on the stripped ITS sequence alignment. Significant p-values are shown in bold.

	F_{CT}	p
Continent	0.035	0.062
Afro-Eurasia/South America	0.092	**0.034**
Climate (all 12)	- 0.003	0.587
Climate (5 most important)	- 0.009	0.658
Cold-temperate/(sub)tropical	- 0.003	0.628

Table 2.4 shows the nucleotide divergence based on the stripped ITS sequence alignment of all sampled populations. It is difficult to compare the nucleotide divergence directly between all populations, as several populations differed in the number of sampled lakes and/or the sampling area. However, the number of sampled lakes and total sampled area of the populations from Belgium and Ethiopia are very similar (chapter 3) and the present study shows that the nucleotide divergence is similar too (1.31 % and 1.38 % respectively). In both New Zealand and Denmark six distinct ITS sequences were obtained from five lakes and in Thailand seven distinct sequences were obtained from three lakes. Although the sampling area was larger in New Zealand compared to Denmark and Thailand, the nucleotide divergence was lower in New Zealand compared to Denmark and Thailand (0.76 %, 1.43 % and 1.56 % respectively). No significant differences in nucleotide divergence between the continents were found by analysis of variance ($F = 1.334$; $p > 0.05$) (Fig. 2.7).

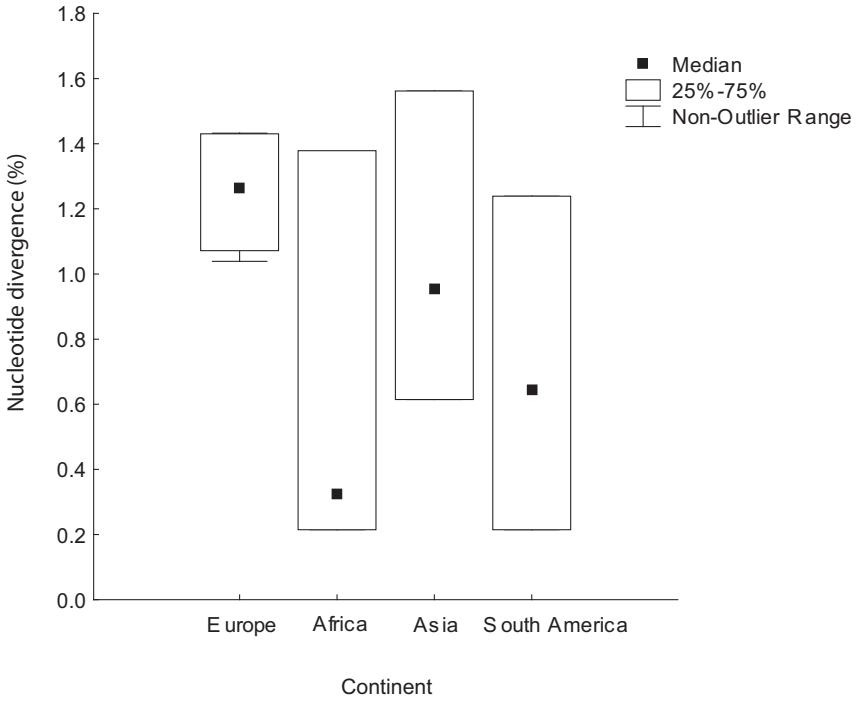


Figure 2.7: Box and Whisker plot of the nucleotide divergence (mean percentage of pairwise differences, Tamura and Nei correction) of the *Microcystis* ITS types from Europe, Africa, Asia and South America based on the restricted dataset correcting for differences in sample size.

Table 2.4: The nucleotide divergence (mean percentage of pairwise differences, Tamura and Nei correction) based on the stripped ITS sequence alignment and standard deviation of the sampled populations.

	Nucleotide divergence (%)	Standard deviation of nucleotide divergence
Belgium	1.31	0.74
The Netherlands	1.17	0.67
Germany	1.10	0.73
Italy	1.74	1.44
Spain	1.33	0.81
Greece	1.43	0.81
Romania	0.78	0.57
France	1.11	0.64
Scotland	1.04	0.67
Denmark	1.43	0.94
New Zealand	0.76	0.55
Uganda-Af	0.48	0.43
Uganda-Aw	0.21	0.27
Kenya-Af	0.32	0.46
Ethiopia	1.38	0.78
China	0.61	0.45
Japan-Cfa	0.95	0.59
Japan-Dfb	0.49	0.50
Thailand	1.56	0.99
Brazil-Aw	1.24	0.87
Brazil-As	0.64	0.61
Argentina-ET	0.21	0.27

2.4 Discussion

Our study shows a high diversity of *Microcystis* ITS types in the studied continents. Pairwise sequence divergence was relatively low (maximum divergence of 13 % for the full and 5 % for the stripped ITS sequence alignment). The phylogenetic analyses revealed largely unresolved and non-diverging relationships among ITS types. The lack of distinct phylogenetic structure in our ITS data indicates the absence of independently evolving lineages within this cyanobacterial genus, suggestive of a single bacterial species. Our findings are in contrast to phylogenetic studies of other bacterial genera based on rDNA ITS sequence data, where divergent evolutionary entities have been distinguished. For example, in the alphaproteobacterial genus *Ochrobactrum* distinct, well supported ITS genotypic clusters were revealed, corresponding to described species (Lebuhn 2006). Similarly, in the marine cyanobacterial genera *Synechococcus*, *Prochlorococcus* and *Cyanobium*, discrete genetic entities have been distinguished (Rocap *et al.* 2002; Chen *et al.* 2006).

The observation of several hypervariable regions in the ITS alignment, which display a lack of genetic correlation, could be the result of DNA transfer between different *Microcystis* strains or recombination within the genome itself. Several studies revealed exchange of genetic information through horizontal gene transfer and recombination in cyanobacteria (Rudi *et al.* 1998, 2002; Lodders *et al.* 2005; Rounge *et al.* 2008). Recently, Tanabe *et al.* (2007) suggested that recombination is an important evolutionary force for the generation and maintenance of the genetic diversity of *Microcystis*. The vectors involved in genetic exchange among *Microcystis* strains have yet to be identified, but plasmids (Takahashi *et al.* 1996; Wallace *et al.* 2002) and cyanophages (Tucker and Pollard 2005; Yoshida *et al.* 2006) have been isolated from natural populations of *Microcystis*, and may be responsible. In addition, Kaneko *et al.* (2007) and Frangeul *et al.* (2008) showed very recently that the genome of two *Microcystis aeruginosa* strains is very plastic and characterized by high transposon activity. Genome plasticity in prokaryotes is often considered to be an adaptive strategy allowing microorganisms to promote diversification (Aras *et al.* 2003). Through recombination events, *Microcystis* strains might be able to enhance adaptation to different ecological niches.

In agreement with previous studies, no apparent correlation was retrieved between toxicity and phylogeny (Otsuka *et al.* 1999). However, Otsuka *et al.* (1999) and Janse *et al.* (2004) indicated that *Microcystis* strains with identical rDNA ITS sequences are homogeneous for microcystin production (i.e. they are toxic or non-toxic) using 125 ITS sequences from Europe, Asia and Morocco. While our results point at some association between ITS type and toxicity, some strains identical in ITS sequence were found to differ in toxicity, indicating that one should be careful when linking biochemical characteristics to ITS sequence on a global scale. Possibly, the lack of consistent relationships between biochemical traits and ITS phylogeny may be partly attributed to recombination events within *Microcystis*. For instance, Yoshida *et al.* (2008) showed a relationship between ITS type and phenotype for three ITS lineage groups, but several strains had high phenotypic and genotypic diversity and

did not show distinct lineages. Cadel-Six *et al.* (2008) showed that the distribution of peptide synthetase gene clusters, coding for nonribosomal peptide synthetases that synthesize oligopeptides, was in relative agreement with their ITS phylogeny, whereas the distribution of the associated halogenase genes appears to be sporadic, suggesting horizontal gene transfer. Accordingly, recombination between cyanobacterial peptide synthetase gene modules, involving exchanges of substrate-specific domains, has been shown for the microcystin synthetase genes (Mikalsen *et al.* 2003).

Our phylogenetic analyses revealed no clear phylogeographic structure and similar ITS types are distributed globally on several continents. In total, 62 % of the ITS types detected more than once in our dataset occur on more than one continent. This was further confirmed by the lack of overall genetic differentiation between continents shown by analysis of molecular variance, and the finding that the dominant component of genetic variation was found within, instead of between continents. This finding indicates that the dispersal rate of new ITS types is higher than the rate of evolution. While a clear geographical structuring based on the different continents was absent, we found a subtle geographical structuring between Afro-Eurasia and South America, and ITS types from South America were absent in some of the subclusters in the ITS network. Although sampling in South America occurred along a broad longitudinal gradient in eleven lakes (as opposed to Africa, where sampling was geographically more restricted), one may argue that the lower number of samples from South America compared to Europe, Asia and Africa might be responsible for the geographical structuring. While the total dataset was corrected for sample size when performing biogeographic structure analyses, more sampling is needed in America to confirm our results. In addition, ITS types from New Zealand seem to have a lower nucleotide divergence than those from other regions, but samples came from a limited set of five lakes on the North Island, necessitating additional sampling.

Contrary to the classical view that aquatic microbial organisms are globally distributed due to their huge population sizes and high dispersal potential (also known as the 'everything is everywhere' or EisE hypothesis, Baas-Becking 1934; Finlay and Clarke 1999), several recent studies have shown at least some degree of phylogeographic structuring in aquatic micro-organisms (Whitaker *et al.* 2003; Cho *et al.* 2000; Fontaneto *et al.* 2008; Erwin and Thacker 2008). Our data, although limited in genetic resolution, support the idea that a large number of *Microcystis* ITS types are ubiquitous and that global dispersal of these ITS types is not limited. This is remarkable given the fact that globally, freshwater bodies are small and highly isolated habitat patches in a vast marine and terrestrial matrix. Moreover, geographical structuring of ITS lineages according to continents was indeed shown for another bloom forming freshwater cyanobacterium, *Cylindrospermopsis* (Haande *et al.* 2008). Possible reasons for the lack of phylogeographic structure in *Microcystis* may be a high dispersal capacity of all or most genotypes. Unfortunately, there is little information on the dispersal ability and mechanism of transport of *Microcystis* strains (Round 1981; Hoffmann 1996). The probability of successful long-distance passive dispersal depends strongly on the effectiveness of the carrier (animal or airborne dispersal), and the ability of *Microcystis* to tolerate the transport conditions (Kristiansen 1996ab). An effective way for

Microcystis to disperse might be by bird migrations (Baurain *et al.* 2002). Birds often swim in or drink from lakes containing a *Microcystis* bloom, which can form a dense scum on the water surface, and can take colonies of *Microcystis* with them during migration. In addition, wind dispersal of *Microcystis* strains might also occur (Kellogg and Griffin 2006). As *Microcystis* cells have thick resistant cell walls, are grouped into colonies surrounded by a protective mucilage layer and form resting stages (Brunberg and Blomqvist, 2002; Oberholster *et al.* 2005), they might resist drought or intestinal conditions for a certain period, which could explain their global dispersal at least on the time scale of ITS evolution (Hoffmann 1996; Marshall and Chalmers 1997).

The average nucleotide divergence of ITS types of the sampled populations did not differ between continents. On population level, the nucleotide divergence was very similar in Belgium and Ethiopia, whereas the local (within lakes) and regional (among lakes) ITS diversity (number of distinct ITS types) was lower in Ethiopia compared to Belgium, possibly because the Ethiopian ponds are much younger than the Belgian ones (chapter 3). As dispersal does not seem to be limited, it is possible that with time the local and regional diversity will converge in these regions. Interestingly, in Ethiopia one ITS type was very dominant and this was the dominant and ancestral ITS type seen in the network. This seems to be a very successful ITS type with good dispersal capacities and/or tolerance to different conditions.

The importance of local environmental selection for ITS type distribution (Cottenie 2005; Van der Gucht *et al.* 2007) could not be studied as we could not obtain local environmental parameters for all lakes. It is possible that adaptation to local environmental factors resulted in the evolution of different ecotypes, corresponding with the subclusters detected in the ITS network. However, no climatic structuring was detected (including factors such as temperature and drought), indicating that *Microcystis* ITS types tolerate different climatic conditions. Moreover, 82 % of the ITS types detected in more than one climate regime, were found under (sub)tropical as well as cold-temperate climate regimes. Generally, the importance of environmental selection is difficult to falsify because there could be unmeasured aspects of the environment which can also differ among regions (Foissner 2006).

In conclusion, our study suggests that at least several *Microcystis* ITS types are very widespread, supporting the 'everything is everywhere' part of the Baas Becking hypothesis. To what extent 'the environment selects' (Baas Becking 1934) on *Microcystis* ITS types is less clear, although the climatic conditions apparently do not have a considerable impact on the distribution of ITS types. In addition, our study shows a complex phylogenetic structure of *Microcystis* rDNA ITS calling for a cautious use and interpretation of *Microcystis* phylogenetic analyses based on this molecular marker.

Acknowledgements

Sampling of lakes containing *Microcystis* was done in the framework of different projects: B-Blooms and B-Blooms2, nationally supported by BELSPO (Belgian Science Policy), BIOMAN, supported by the European Union, Aquatic Ecology project in the VLIR IUS-MEKELLE programme, supported by VLIR (Vlaamse Interuniversitaire

Raad) and SALGA, supported by NWO/WOTRO and other financiers[2]. We thank all people who sampled lakes in the framework of the different projects. Phylogenetic analyses were carried out on the KERMIT computing cluster (Ghent University) and the Computational Biology Service Unit (Cornell University and Microsoft Corporation). FL and JM are indebted to the Research Foundation, Flanders (FWO) for a postdoctoral fellowship grant.

[2]www.projectenaew.wur.nl/salga/

References

Aras, R. A., Kang, J., Tschumi, A. I., Harasaki, Y. and Blaser, M. J. 2003. Extensive repetitive DNA facilitates prokaryotic genome plasticity. Proceedings of the National Academy of Sciences of the United States of America 100: 13579-13584.

Avise, J. C. 2000. Phylogeography: The History and Formation of Species. Harvard University Press, Cambridge, Massachusetts.

Baas Becking, L. G. M. 1934. Geobiologie of Inleiding tot de Milieukunde. Van Stockum and Zoon, The Hague, The Netherlands.

Baurain, D., Renquin, L., Grubisic, S., Scheldeman, P., Belay, A. and Wilmotte, A. 2002. Remarkable conservation of internally transcribed spacer sequences of *Arthrospira* ('*Spirulina*') (Cyanophyceae, Cyanobacteria) strains from four continents and of recent and 30-year-old dried samples from Africa. Journal of Phycology 38: 384-393.

Belkhir, K., Borsa, P., Goudet, J. and Bonhomme, F. 2004. GENETIX 4.05, logiciel sous WindowsTM pour la génétique des populations. Montpellier Laboratoire Génome, Populations, Interactions CNRS UMR 5000, Université de Montpellier II, Montpellier (France).

Bittencourt-Oliveira, M. D., de Oliveira, M. C. and Bolch, C. J. S. 2001. Genetic variability of Brazilian strains of the *Microcystis aeruginosa* complex (Cyanobacteria/Cyanophyceae) using the phycocyanin intergenic spacer and flanking regions (*cpc*BA). Journal of Phycology 37: 810-818.

Black, W. C. IV and Krafsur, E. S. 1985. A FORTRAN program for the calculation and analysis of two-locus linkage disequilibrium coefficients. Theoretical and Applied Genetics 70: 491-496.

Boyer, S. L., Flechtner, V. R. and Johansen, J. R. 2001. Is the 16S-23S rRNA internal transcribed spacer region a good tool for use in molecular systematics and population genetics? A case study in cyanobacteria. Molecular Biology and Evolution 18: 1057-1069.

Brunberg, A. K. and Blomqvist, P. 2002. Benthic overwintering of *Microcystis* colonies under different environmental conditions. Journal of Plankton Research 24: 1247-1252.

Cadel-Six, S., Dauga, C., Castets, A. M., Rippka, R., Bouchier, C., de Marsac, N. T. and Welker, M. 2008. Halogenase genes in nonribosomal peptide synthetase gene clusters of *Microcystis* (Cyanobacteria): Sporadic distribution and evolution. Molecular Biology and Evolution 25: 2031-2041.

Chen, F., Wang, K., Kan, J., Suzuki, M. and Wommack, E. 2006. Diverse and unique picocyanobacteria found in the Chesapeake Bay. Applied and Environmental Microbiology 72: 2239-2243.

Cho, J. C. and Tiedje, J. M. 2000 Biogeography and degree of endemicity of fluorescent *Pseudomonas* strains in Soil. Applied and Environmental Microbiology 66: 5448-5456.

Chorus, I. and Bartram, J. WHO. 1999. Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. E and FN Spon. London, United Kingdom.

Clement, M., Posada, D. and Crandall, K. 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology 9: 1657-1660

Cottenie, K. 2005. Integrating environmental and spatial processes in ecological community dynamics. Ecology Letters 8: 1175-1182.

DeChaine, E. G., Bates, A. E., Shank, T. M. and Cavanaugh, C. M. 2006. Off-axis symbiosis found: characterization and biogeography of bacterial symbionts of Bathymodiolus mussels from Lost City hydrothermal vents. Environmental Microbiology 8: 1902-1912.

Dolan, J. R. 2005. An introduction to the biogeography of aquatic microbes. Aquatic Microbial Ecology 41: 39-48.

Dolan, J. R. 2006. Microbial biogeography? Journal of Biogeography 33: 199-200.

Erwin, P. M. and Thacker, R. W. 2008. Cryptic diversity of the symbiotic cyanobacterium *Synechococcus spongiarum* among sponge hosts. Molecular Ecology 17: 2937-2947.

Edgar, R. C. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5: 113.

Excoffier, L., Laval, G. and Schneider, S. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1: 47-50.

Fierer, N. and Jackson, R. B. 2006. The diversity and biogeography of soil bacterial communities. Proceedings of the National Academy of Sciences of the United States of America 103: 626-631.

Finlay, B. J. and Clarke, K. J. 1999. Ubiquitous dispersal of microbial species. Nature 400: 828-828.

Finlay, B. J. 2002. Global dispersal of free-living microbial eukaryote species. Science 296: 1061-1063.

Foissner, W. 2006. Biogeography and dispersal of micro-organisms: A review emphasizing protists. Acta Protozoologica 45: 111-136.

Fontaneto, D., Barraclough, T. G., Chen, K., Ricci, C. and Herniou, E. A. 2008. Molecular evidence for broad-scale distributions in bdelloid rotifers: everything is not everywhere but most things are very widespread. Molecular Ecology 17: 3136-3146.

Frangeul, L., Quillardet, P., Castets, A. M., Humbert, J. F., Matthijs, H. C. P., Cortez, D. *et al.* 2008. Highly plastic genome of *Microcystis aeruginosa* PCC 7806, a ubiquitous toxic freshwater cyanobacterium. Bmc Genomics 9.

Goudet, J. 2001. Fstat version 2.9.3. Institute of Ecology, Biology Building, UNIL, CH 1015, Lausanne, Switzerland.

Guillard, R. R. and Lorenzen, C. J. 1972. Yellow-green algae with chlorophyllide c. Journal of Phycology 8: 10-14.

Guindon, S. and Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52: 696-704.

Haande, S., Ballot, A., Rohrlack, T., Fastner, J., Wiedner, C. and Edvardsen, B. 2007. Diversity of *Microcystis*

aeruginosa isolates (Chroococcales, Cyanobacteria) from East-African water bodies. Archives of Microbiology 188: 15-25.

Haande, S., Rohrlack, T., Ballot, A., Roberg, K., Skulberg, R., Beck, M. and Wiedner, C. 2008. Genetic characterisation of *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria) isolates from Africa and Europe. Harmful Algae 7: 692-701.

Hillis, D. M. and Huelsenbeck, J. P. 1992. Signal, noise, and reliability in molecular phylogenetic analyses. Journal of Heredity 83: 189-195.

Hisbergues, M., Christiansen, G., Rouhiainen, L., Sivonen, K. and Borner, T. 2003. PCR-based identification of microcystin-producing genotypes of different cyanobacterial genera. Archives of Microbiology 180: 402-410.

Hoffmann, L. 1996. Geographic distribution of freshwater blue-green algae. Hydrobiologia 336: 33-40.

Hugenholtz, P. and Huber, T. 2003. Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. International Journal of Systematic and Evolutionary Microbiology 53: 289-293.

Huisman, J., Matthijs, H. C. P. and Visser, P. M. 2005. Harmful cyanobacteria. Springer, Dordrecht, The Netherlands. 243 pp.

Humbert, J. F., Duris-Latour, D., Le Berre, B., Giraudet, H. and Salencon, M. J. 2005. Genetic diversity in *Microcystis* populations of a French storage reservoir assessed by sequencing of the 16S-23S rRNA intergenic spacer. Microbial Ecology 49: 308-314.

Iteman, I., Rippka, R., de Marsac, N. T. and Herdman, M. 2000. Comparison of conserved structural and regulatory domains within divergent 16S rRNA-23S rRNA spacer sequences of cyanobacteria. Microbiology-Sgm 146: 1275-1286.

Janse, I., Meima, M., Kardinaal, W. E. A. and Zwart, G. 2003. High-resolution differentiation of cyanobacteria by using rRNA-internal transcribed spacer denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69: 6634-6643.

Janse, I., Kardinaal, W. E. A., Meima, M., Fastner, J., Visser, P. M. and Zwart, G. 2004. Toxic and nontoxic *Microcystis* colonies in natural populations can be differentiated on the basis of rRNA gene internal transcribed spacer diversity. Applied and Environmental Microbiology 70: 3979-3987.

Kaneko, T., Nakajima, N., Okamoto, S., Suzuki, I., Tanabe, Y., Tamaoki, M. *et al.* 2007. Complete genomic structure of the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* NIES-843. DNA Research 14: 247-256.

Kellogg, C. A. and Griffin, D. W. 2006. Aerobiology and the global transport of desert dust. Trends in Ecology & Evolution 21: 638-644.

Komárek, J. and Anagnostidis, K. 1986. Modern approach to the classification system of cyanophytes 2 - Chroococcales. Archiv für Hydrobiologie Supplement 83: 377-401.

Komárek, J. and Anagnostidis, K. 1999. Cyanoprokaryota 1. Teil: Chroococcales. In: Ettl, H., Gärtner, G., Heynig, H. and Mollenhauer, D. (eds). Süsswasserflora von Mitteleuropa, band 19/1, Gustav Fisher, Berlin, pp. 1-548.

Kottek, M., Grieser, J., Beck, C., Rudolf, B. and Rubel, F. 2006. World map of the Köppen-Geiger climate classification updated. Meteorologische Zeitschrift 15: 259-263.

Kristiansen, J. 1996a. Biogeography of Freshwater Algae. Developments in Hydrobiology 118, Kluwer Academic Publishers. Dordrecht, The Netherlands. pp. 1-161.

Kristiansen, J. 1996b. Dispersal of freshwater algae - a review. Hydrobiologia 336: 151-157.

Lebuhn, M., Bathe, S., Achouak, W., Hartmann, A., Heulin T. and Schloter, M. 2006. Comparative sequence analysis of the internal transcribed spacer 1 of *Ochrobactrum* species . Systematic and Applied Microbiology 29: 265-275.

Lodders, N., Stackebrandt, E. and Nubel, U. 2005. Frequent genetic recombination in natural populations of the marine cyanobacterium *Microcoleus chthonoplastes*. Environmental Microbiology 7: 434-442.

Marshall, W. A. and Chalmers, M. O. 1997. Airborne dispersal of antarctic terrestrial algae and cyanobacteria. Ecography 20: 585-594.

Martiny, J. B. H., Bohannan, B. J. M., Brown, J. H., Colwell, R. K., Fuhrman, J. A., Green, J. L. *et al.* 2006. Microbial biogeography: putting microorganisms on the map. Nature Reviews Microbiology 4: 102-112.

Mikalsen, B., Boison, G., Skulberg, O. M., Fastner, J., Davies, W., Gabrielsen, T. M. *et al.* 2003. Natural variation in the microcystin synthetase operon *mcy*ABC and impact on microcystin production in *Microcystis* strains. Journal of Bacteriology 185: 2774-2785.

Muyzer, G., Dewaal, E. C. and Uitterlinden, A. G. 1993. Profiling of complex microbial populations by Denaturing Gradient Gel Electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S ribosomal RNA. Applied and Environmental Microbiology 59: 695-700.

Oberholster, P. J., Botha, A. M., Muller, K. and Cloete, T. E. 2005. Assessment of the genetic diversity of geographically unrelated *Microcystis aeruginosa* strains using amplified fragment length polymorphisms (AFLPs). African Journal of Biotechnology 4: 389-399.

Otsuka, S., Suda, S., Li, R. H., Watanabe, M., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 1998. 16S rDNA sequences and phylogenetic analyses of *Microcystis* strains with and without phycoerythrin. FEMS Microbiology Letters 164: 119-124.

Otsuka, S., Suda, S., Li, R. H., Watanabe, M., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 1999. Phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis* based on 16S to 23S internal transcribed spacer sequence. FEMS Microbiology Letters 172: 15-21.

Otsuka, S., Suda, S., Shibata, S., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 2001. A proposal for the unification of five species of the cyanobacterial genus *Microcystis* Kützing ex Lemmermann 1907 under the Rules of the Bacteriological Code. International Journal of Systematic and Evolutionary Microbiology 51:

873-879.
Papke, R. T., Ramsing, N. B., Bateson, M. M. and Ward, D. M. 2003. Geographical isolation in hot spring cyanobacteria. Environmental Microbiology 5: 650-659.
Posada, D. and Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817-818.
Rocap, G., Distel, D. L., Waterbury, J. B. and Chisholm, S. W. 2002. Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S-23S ribosomal DNA internal transcribed spacer sequences. Applied and Environmental Microbiology 68: 1180-1191.
Ronquist, F. and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-74
Round, F. E. 1981. The Ecology of Algae. Cambridge University Press. Cambridge, pp. 653.
Rounge, T. B., Rohrlack, T., Kristensen, T. and Jakobsen, K. S. 2008. Recombination and selectional forces in cyanopeptolin NRPS operons from highly similar, but geographically remote *Planktothrix* strains. Bmc Microbiology 8: 141.
Rozas, J., Sánchez-DelBarrio, J. C., Messeguer, X. and Rozas, R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496-2497.
Rudi, K., Skulberg, O. M., Larsen, F. and Jakobsen, K. S. 1997. Strain characterization and classification of oxyphotobacteria in clone cultures on the basis of 165 rRNA sequences from the variable regions V6, V7, and V8. Applied and Environmental Microbiology 63: 2593-2599.
Rudi, K., Skulberg, O. M. and Jakobsen, K. S. 1998. Evolution of cyanobacteria by exchange of genetic material among phyletically related strains. Journal of Bacteriology 180: 3453-3461.
Rudi, K., Fossheim, T. and Jakobsen, K. S. 2002. Nested evolution of a tRNA(Leu)(UAA) group I intron by both horizontal intron transfer and recombination of the entire tRNA locus. Journal of Bacteriology 184: 666-671.
Scheldeman, P., Baurain, D., Bouhy, R., Scott, M., Muhling, M., Whitton, B. A., Belay, A. and Wilmotte, A. 1999. *Arthrospira* ('*Spirulina*') strains from four continents are resolved into only two clusters, based on amplified ribosomal DNA restriction analysis of the internally transcribed spacer. FEMS Microbiology Letters 172: 213-222.
Swofford, D. L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates. Sunderland, Massachusetts.
Takahashi, I., Hayano, D., Asayama, M., Masahiro, F., Watahiki, M. and Shirai, M. 1996. Restriction barrier composed of an extracellular nuclease and restriction endonuclease in the unicellular cyanobacterium *Microcystis sp*. FEMS Microbiology Letters 145: 107-111.
Tamura, K. and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial-DNA in humans and chimpanzees. Molecular Biology and Evolution 10: 512-526.
Tanabe, Y., Kasai, F. and Watanabe, M. M. 2007. Multilocus sequence typing (MLST) reveals high genetic diversity and clonal population structure of the toxic cyanobacterium *Microcystis aeruginosa*. Microbiology-Sgm 153: 3695-3703.
Templeton, A. R., Crandall, K. A. and Sing, C. F. 1992. Acladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and sequencing data. III. Cladogram estimation. Genetics 132: 619-633.
Templeton, A. R. 1998. Nested clade analysis of phylogenetic data: testing hypotheses about gene flow and population history. Molecular Ecology 7: 381-397.
Thompson, J. R., Marcelino, L. A. and Polz, M. F. 2002. Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. Nucleic Acids Research 30: 2083-2088.
Tillett, D., Parker, D. L. and Neilan, B. A. 2001. Detection of toxigenicity by a probe for the microcystin synthetase A gene (*mcy*A) of the cyanobacterial genus *Microcystis*, comparison of toxicities with 16S rRNA and phycocyanin operon (phycocyanin intergenic spacer) phylogenies. Applied and Environmental Microbiology 67: 2810-2818.
Tucker, S. and Pollard, P. 2005. Identification of cyanophage Ma-LBP and infection of the cyanobacterium *Microcystis aeruginosa* from an Australian subtropical lake by the virus. Applied and Environmental Microbiology 71: 629-635.
Vaitomaa, J., Rantala, A., Halinen, K., Rouhiainen, L., Tallberg, P., Mokelke, L. and Sivonen, K. 2003. Quantitative real-time PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. Applied and Environmental Microbiology 69: 7289-7297.
Van der Gucht, K., Cottenie, K., Muylaert, K., Vloemans, N., Cousin, S., Declerck, S. *et al.* 2007. The power of species sorting: Local factors drive bacterial community composition over a wide range of spatial scales. Proceedings of the National Academy of Sciences of the United States of America 104: 20404-20409.
van der Oost, R. 2007. Cyanotoxine monitoring: standaardisering en validatie van methoden voor de Nederlandse waterkwaliteitsbeheerders. Rapport STOWA onderzoek 2005 and 2006. Waternet/Waterproef. Amsterdam.
von Wintzingerode, F., Göbel, U. B. and Stackebrandt, E. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiology Reviews 21: 213-229.
Wallace, M. M., Miller, D. W. and Raps, S. 2002. Characterization of pMa025, a plasmid from the cyanobacterium *Microcystis aeruginosa* UV025. Archives of Microbiology 177: 332-338.
Wang, G. C. Y. and Wang, Y. 1996. The frequency of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species. Microbiology 142: 1107-1114.
Whitaker, R. J., Grogan, D. W. and Taylor, J. W. 2003. Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science 301: 976-978.

Wu, Z. X., Gan, N. Q. and Song, L. R. 2007. Genetic diversity: geographical distribution and toxin profiles of *Microcystis* strains (Cyanobacteria) in China. Journal of Integrative Plant Biology 49: 262-269.

Xia, X. and Xie, Z. 2001. DAMBE: data analysis in molecular biology and evolution. Journal of Heredity 92: 371-373.

Yoshida, T., Takashima, Y., Tomaru, Y., Shirai, Y., Takao, Y., Hiroishi, S. and Nagasaki, K. 2006. Isolation and characterization of a cyanophage infecting the toxic cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 72: 1239-1247.

Zwart, G., Huismans, R., van Agterveld, M. P., Van de Peer, Y., De Rijk, P., Eenhoorn, H. *et al.* 1998. Divergent members of the bacterial division *Verrucomicrobiales* in a temperate freshwater lake. FEMS Microbiology Ecology 25: 159-169.

Chapter 3

rDNA ITS diversity and population structure of *Microcystis* in a tropical region (Tigray, Northern Ethiopia)

Ineke van Gremberghe[1], Katleen Van der Gucht[1], Pieter Vanormelingen[1], Tsehaye Asmelash[2], Tadesse Dejenie[2], Sofie D'hondt[1], Steven Declerck[3], Luc De Meester[3] and Wim Vyverman[1]

Unpublished manuscript

Contribution to this chapter by IVG: culturing *Microcystis* strains, part of the molecular work, statistical analyses and writing

[1]Laboratory of Protistology and Aquatic Ecology, Ghent University, Krijgslaan 281 - S8, 9000 Gent, Belgium

[2]Mekelle University, P.O. Box 231, Mekelle, Ethiopia

[3]Laboratory of Aquatic Ecology and Evolutionary Biology, Katholieke Universiteit Leuven, Charles Debériotstraat 32, 3000 Leuven, Belgium

Abstract

The cyanobacterium *Microcystis* is notorious for forming extensive and potentially toxic blooms in nutrient-rich freshwater bodies. Despite the importance of population structure for bloom toxicity, little is known about the factors underlying diversity and metapopulation structure of natural *Microcystis* blooms. We studied genetic diversity and structure of *Microcystis* populations in 30 small man-made reservoirs in Tigray (Northern Ethiopia) using Denaturing Gradient Gel Electrophoresis of the 16S-23S rDNA ITS, and assessed the importance of local environmental conditions and geographic position of the reservoirs for the observed patterns. The analyses showed that both the regional and local *Microcystis* ITS diversity in Tigray was low compared to other regions, as several dense blooms contained only a single ITS type. While a possible role of climate cannot be excluded, a plausible explanation for the relatively low ITS diversity is the recent construction of the reservoirs in a semi-arid region with a low historic availability of suitable *Microcystis* habitat. Especially one non-toxic ITS type dominated many *Microcystis* blooms and was spatially distributed. Taken together, this suggests that long-distance dispersal is infrequent enough to (still) limit local and regional ITS diversity and to cause the geographically restricted distribution of the dominant ITS type. In addition to the effects of dispersal limitation, the significant correlations of *Microcystis* ITS population structure with abiotic variables (water clarity, pH) and zooplankton (especially *Daphnia* biomass) suggest an important additional influence of these factors on *Microcystis* population structure in the reservoirs of Tigray.

3.1 Introduction

The cyanobacterium *Microcystis*, which produces the hepatotoxin microcystin, is one of the most common bloom formers of freshwater phytoplankton (Huisman *et al.* 2005). *Microcystis* blooms are generally limited to summer in temperate regions but may occur year-round in (sub)tropical regions. High densities of this cyanobacterium can intoxicate a variety of eukaryotes (Rohrlack *et al.* 1999; Ferrao-Filho *et al.* 2000; Pflugmacher 2004; Codd *et al.* 2005). During the last years, there has been considerable interest in the strain composition of these blooms, as not all strains of *Microcystis* produce microcystins and the toxicity of a bloom depends to a high extent on the genotypic composition (Via-Ordorika *et al.* 2004; Janse *et al.* 2004; Kardinaal *et al.* 2007a). Furthermore, strains can also differ in the production of other potentially toxic secondary metabolites, which could play a role in the development of a bloom (Welker *et al.* 2004, 2007), and can differ in other functional traits like growth rate and colony formation (Wilson *et al.* 2006). Therefore, information about the genetic diversity of *Microcystis* blooms is a prerequisite for understanding bloom dynamics and toxicity. The genotypic diversity of *Microcystis* has been studied in temperate as well as tropical regions (e.g. Bittencourt-Oliveira *et al.* 2001; Humbert *et al.* 2005; Janse *et al.* 2004; Wilson *et al.* 2005). These studies show a high diversity of *Microcystis* populations within and among lakes by genetically characterizing cultures using fingerprint techniques, or rDNA or phycocyanin gene sequences. This way, a limited number of strains from a few lakes are generally characterized. By applying Denaturing Gradient Gel Electrophoresis (DGGE) on natural samples, the population structure of a large number of *Microcystis* blooms can directly be determined and their temporal dynamics can be followed (Janse *et al.* 2004; Kardinaal *et al.* 2007a).

Little is known yet about the historical and environmental factors that influence the population structure and diversity of natural *Microcystis* blooms (Yoshida *et al.* 2007), despite the fact that this knowledge is essential to understand the build-up of these blooms. Since *Microcystis* strains have a diverse physiology (Wilson *et al.* 2006; Bañares-España *et al.* 2006), different strains probably react strain-specific on local environmental conditions. For instance, non-toxic strains seem to be better competitors for light than toxic strains (Kardinaal *et al.* 2007b) and high levels of nitrate may favour the growth of toxic over non-toxic strains (Vézie *et al.* 2002; Yoshida *et al.* 2007). This implies that local environmental conditions will influence strain composition. Moreover, in prokaryotes, a lack of recombination and a resulting clonal population composition may cause a correlation between functional traits and neutral molecular markers, such as ITS sequences, although recombination may also be frequent enough to prevent this (Lodders *et al.* 2005, chapter 2). This implies that one has to take into account the influence of local environmental factors on the genotypic structure of a population, even when analyzing a selectively neutral molecular marker such as ITS rDNA.

Although *Microcystis* blooms are assumed to occur very often in Africa due to the (sub)tropical climate, the genetic diversity of *Microcystis* is hardly studied on this continent (Haande *et al.* 2007). Information about toxic cyanobacterial blooms in

African regions is important because of the direct threat to local people who often make intensive use of existing water bodies. Recent surveys showed that *Microcystis* blooms are very frequent in man-made reservoirs in the semi-arid highlands of Tigray, Northern Ethiopia (Asmelash *et al.* unpublished). In the present study we determined the genetic diversity and population structure of *Microcystis* populations in these reservoirs using the 16-23S rDNA Internal Transcribed Spacer (ITS). Janse *et al.* (2003) have developed a cyanobacteria-specific PCR to amplify ITS sequences and visualised the community structure of cyanobacteria by DGGE. We used this protocol in a nested PCR with a specific *Microcystis* primer in the first PCR to selectively amplify only rDNA ITS of *Microcystis*. Our specific goals were to (1) determine the diversity and population structure of the *Microcystis* populations present and (2) to evaluate the influence of the geographical position of the reservoirs and local environmental factors on the *Microcystis* population structure.

3.2 Materials and methods

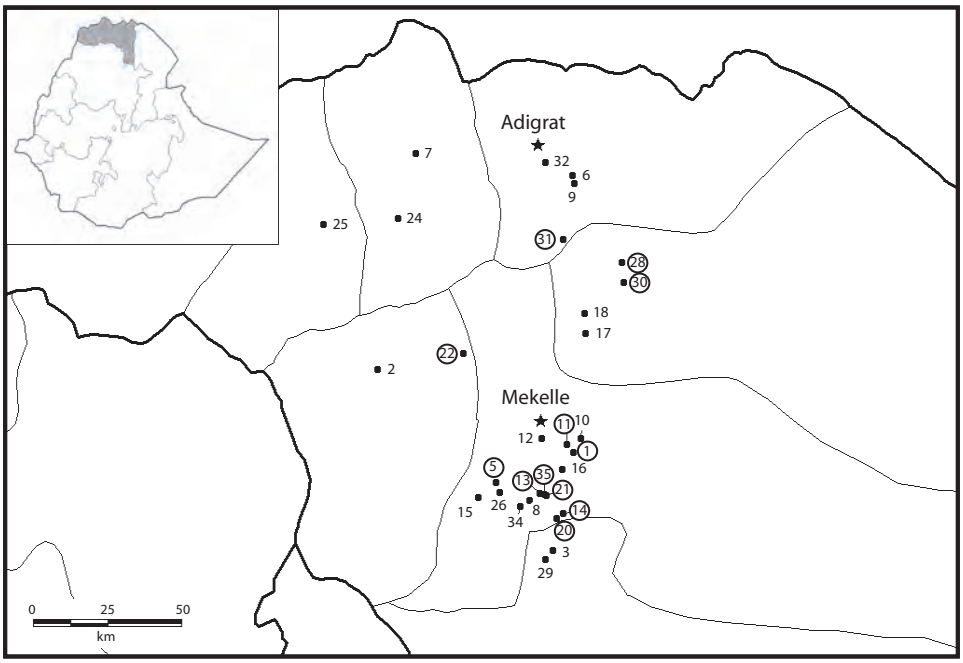
3.2.1 Study area

The highlands of Tigray are generally characterized as tropical semi-arid. The annual rainfall ranges between 546 to 879 mm, with an average annual rainfall of ca. 700 mm. Most authors classify the period from October to May as dry and June to September as rainy. Most of the reservoirs have a surface area between 2 and 20 ha and were constructed after 1994 to provide the local people with water for irrigation. A detailed description of the limnology and aquatic food web structure of these reservoirs is given in Dejenie *et al.* (2008).

A map of the sampled reservoirs (30 in total) is given in figure 3.1. The total sampling area is approximately 1.5*10^6 ha. The mean distance between two sampled reservoirs is 60 km, and ranges from 0.75 to 135 km. Fifteen reservoirs were sampled twice, the first time in September 2004 at the end of the wet season, the second time in April 2005 near the end of the dry season. Fourteen reservoirs were sampled once, six of those in the wet season sampling campaign and eight in the dry season. Several reservoirs contained dense *Microcystis* blooms at the time of sampling (bloom visible by eye from a concentration of 0.5*10^6 pg C ml^{-1}). In addition, temporal variation in genotypic composition of *Microcystis* populations was assessed by a monthly sampling of eight reservoirs (including one reservoir that was not sampled in the wet and dry season of 2004 and 2005 respectively) during the course of one year (September 2005 - September 2006).

3.2.2 Environmental and spatial variables

Environmental variables and phytoplankton and zooplankton communities in the reservoirs at the time of sampling have been described elsewhere and we made a selection of these variables to include in our analyses (Dejenie *et al.* 2008, Dejenie *et al.* unpublished, Asmelash *et al.* unpublished). Water samples were taken with a Heart



Reservoir	Code	Reservoir	Code	Reservoir	Code	Reservoir	Code
Adi Amharay	1	Dur Anbesa	8	Hizaeti Wedi Cheber	16	Mai Seye	25
Adi Asme'e	2	Enda Gabriel	9	Korir	17	Meala	26
Adi Gela	3	Era Quihila	10	Laelay Wukro	18	Ruba Feleg	28
Adi Kenafiz	34	Gereb Awso	11	Mai Delle	20	Shilanat IV	29
Betequa	5	Gereb Beati	12	Mai Gassa I	21	Tegh'ane	30
Bokoro	6	Gereb Mihiz	13	Mai Gassa II	35	Tsinkanet	31
Dibdibo	7	Gum Selasa	14	Mai Leba	22		
Dibla	32	Haiba	15	Mai Sessella	24		

Figure 3.1: Map of Tigray including the sampled reservoirs with indication (circle around number of reservoir) of a very successful ITS type (EG07), which was geographically distributed (see also table 3.1, figure 3.2 and 3.3). The table under the figure shows the names of the reservoirs sampled in the wet season of 2004 and/or dry season of 2005 and the codes indicated on the map. Underlined reservoirs were also sampled monthly from September 2005 to September 2006 (Mai Leba was not sampled in the wet season of 2004 and dry season 2005). Position of Tigray in Ethiopia is shown in the upper left corner.

valve sampler at five sampling points and from three depths (surface, middle and near-bottom) and pooled. From this pooled sample, subsamples were taken for the analysis of nutrient concentrations, and phytoplankton and rotifer biomass. Water samples for DNA extraction were filtered over a 25 mm 0.2 µm GSWP filter (Millipore) and frozen as soon as possible at -20 °C. Abiotic parameters (temperature, oxygen concentration, pH and conductivity) of the water were measured with electrodes. Water transparency was measured using a cylindrical Snell's tube. Total nitrogen and total phosphorus concentrations were determined with a Technicon autoanalyser II following to the manual (Technicon Instruments Corporation, New York, USA).

Macrozooplankton was sampled with a Schindler-Patalas trap (12 l content, 64 µm mesh size) at three depths and pooled. Phytoplankton samples were fixed with acid lugol and zooplankton samples were fixed with sugar-saturated formalin. Phytoplankton, including *Microcystis*, was counted using an inverted microscope (Asmelash *et al.* unpublished). *Microcystis* colony density was converted into biovolume by using colony dimensions and a visually estimated fraction of the colonies occupied by the cells, and into biomass using a general conversion factor (Menden-Deuer and Lessard 2000). Zooplankton from the reservoirs in Tigray was identified and counted as individuals per litre and abundance was converted to biomass using published length-weight regressions (Bottrell *et al.* 1976). Copepods were identified and counted as calanoids and cyclopoids. Cladocerans and rotifers were identified up to species level. For more information about the zooplankton in the reservoirs see Dejenie *et al.* (unpublished). Geographical UTM coordinates were determined by GPS recordings for each of the sampled reservoirs.

3.2.3 *Microcystis* cultures

Individual colonies of *Microcystis* from the reservoir Mai Gassa I, sampled on the 11th of July 2006, were picked up with sterile glass Pasteur pipettes under a binocular microscope. The strains were grown in WC medium at 18 °C at an irradiance of approximately 30 µmol $\mathrm{m}^{-2}\,\mathrm{s}^{-1}$ (12:12 h light:dark cycle). All 54 strains were screened by Denaturing Gradient Gel Electrophoresis (see below) to identify groups of strains with (presumably) identical rDNA ITS sequences and three or more representatives of each group were then chosen for sequencing.

3.2.4 DNA extraction and PCR amplification

DNA from the isolated strains and DNA from the GSWP filters from the natural water samples was extracted as described by Zwart *et al.* (1998). 16S-23S rDNA ITS sequences of the isolated *Microcystis* strains were amplified by PCR using the protocol described by Janse *et al.* (2003). In addition, fragments of the *mcy* A and *mcy* E genes were amplified from all isolated strains using the protocols from respectively Hisbergues *et al.* (2003) and Vaitomaa *et al.* (2003) to examine if the strains are (potentially) toxic. A nested-PCR protocol based on the protocol described by Janse *et al.* (2003) was used to specifically amplify only *Microcystis* ITS sequences from the

water samples (see chapter 2).

3.2.5 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was essentially performed as described by Muyzer *et al.* (1993) and described in detail in chapter 2. From every DGGE gel, individual bands were cut out and sequenced (see below). On every gel, three standard lanes were run in parallel with the samples. These standard lanes are composed of several bands with a distinct position on the gel and used to align the different gels. Digitalized DGGE images were analysed using the software package BioNumerics 4.5 (Applied Maths BVBA). The program detects the bands and, based on their position in the gel, groups the bands into band classes which normally represent single ITS types. This was verified manually using sequence information obtained from the bands. Next, one matrix was compiled with the relative abundances of the different ITS types in the samples based upon relative band intensity in a sample and a second matrix with presence/absence data.

3.2.6 Cloning

To obtain additional information about the ITS diversity in Mai Gassa I and II, *Microcystis*-specific ITS sequences of two natural samples from Mai Gassa I (September 2004 and April 2006) and two natural samples from Mai Gassa II (April 2005 and January 2006) were cloned using the pGEM®-T vector system II (Promega). For a detailed description of the method, we refer to chapter 2. In total, 73 clones were screened by Denaturing Gradient Gel Electrophoresis to identify groups with (presumably) identical rDNA ITS sequences and one or more representatives of each group were then chosen for sequencing.

3.2.7 Sequencing

Sequencing was performed with the ABI-Prism sequencing kit and the resulting sequencing reaction products were analysed on an automatic sequencer (ABI-Prism 3100). The rDNA ITS sequences were screened against GenBank using the BLAST search algorithm (Altschul *et al.* 1990) to obtain more information about the geographical distribution of these ITS types. One sequence of each ITS type was already used for a phylogeographic analysis (chapter 2).

3.2.8 Microcystin production

Microcystin production of two strains of each of the three ITS types was checked with an Enzyme-Linked Immunosorbent Assay (ELISA) using the SDI - EnviroGard Microcystin ELISA plate kit (see chapter 2 for more details). The toxicity of identical ITS types from GenBank was also included based on the detection of microcystins when this information was available in published studies.

3.2.9 Statistical analysis

Variables included in the statistical analyses were *Microcystis* local diversity (number of distinct ITS types in the DGGE profile per sample) and *Microcystis* population structure (presence/absence and relative abundances of ITS types in the DGGE profile per sample), temperature, oxygen concentration, pH, conductivity, water transparency (Snell depth), total nitrogen and phosphorus concentrations, total biomass of phytoplankton, zooplankton, *Daphnia* and *Microcystis*, phytoplankton and zooplankton community composition based on absolute abundances, and geographical distances. Before statistical analyses, all variables (except pH) were $\log(x+1)$ transformed.

A Mann-Whitney U test was performed to test for significant differences in local ITS diversity between the wet and dry season for the fifteen reservoirs sampled in both the wet and dry season of 2004 and 2005 respectively. Correlations between the local *Microcystis* ITS diversity of the wet and dry season separately and biomass of *Microcystis* and local environmental variables (not including zooplankton and phytoplankton communities or geographical distances), and between the relative abundance of one particular abundant *Microcystis* ITS type and *Daphnia* biomass were determined by calculating Spearman rank correlation coefficients in SPSS 15 (SPSS Inc. Chicago, USA).

Possible differences in *Microcystis* population structure between the wet and dry season were tested with Analysis of similarity (ANOSIM) using 999 permutations for the 15 reservoirs sampled in both seasons (Primer 5, Clarke and Gorley 2001). ANOSIM generates a test statistic R, and the magnitude of R is indicative of the degree of separation between groups, with a score of 1 indicating complete separation and 0 indicating no separation. Monte-Carlo randomization of the group labels was used to generate null distributions in order to test the hypothesis that within-group similarities are higher than would be expected if sample DGGE profiles were grouped at random.

Mantel tests (Mantel 1967; Mantel and Valand 1970) were used to investigate the relation between *Microcystis* ITS diversity or population structure and environmental variables or geographical distance. Distance matrices for the mantel tests were constructed using Bray-Curtis similarities for the *Microcystis* DGGE profiles, phytoplankton, and zooplankton community composition (Primer 5, Clarke and Gorley 2001). Euclidean distances were used to construct distance matrices for each separate abiotic variable, *Daphnia* biomass (the most important large cladoceran in Tigray, see Dejenie *et al.* unpublished), total phytoplankton biomass, *Microcystis* biomass and diversity. A distance matrix representing geographic distance was constructed by calculating the distance in meter between each pair of reservoirs. Separate analyses were performed for the samples from the wet and dry season and for the samples from the eight reservoirs monitored during one year. For the samples from the reservoirs monitored during one year an extra distance matrix was constructed based on the dummy variable 'reservoir' to test for effects independent of differences between reservoirs. In the Mantel test, the null hypothesis is that the pair-wise distances between samples in distance matrix A are independent of the distances in another distance matrix B. The null hypothesis is tested using a randomization procedure in which the original value of the statistic is compared with the distribution found by randomly reallocating the order of the elements in one

of the matrices. The statistic used for the measure of the correlation between the matrices is the Pearson correlation coefficient. Simple Mantel tests evaluate whether the association between two independent similarity matrices, describing the same set of entities, is stronger than one would expect from chance. Partial Mantel tests are used to determine the relationship between two matrices while controlling the effect of a third matrix. Simple and partial Mantel tests were carried out by the Zt software tool (Bonnet and Van de Peer 2002). The program Manteltester[1] was used to automate the Mantel tests. As the correlations found using relative abundances of ITS types were similar to the correlations found based on the presence or absence of ITS types, we only show the correlations with relative abundances of ITS types.

3.3 Results

3.3.1 Local and regional ITS diversity

The total biomass and ITS population structure of *Microcystis* in the reservoirs sampled in the wet and dry season is given in figure 3.2. *Microcystis* was present in all reservoirs sampled, although biomass was sometimes very low. In both the wet and the dry season, four reservoirs contained dense *Microcystis* blooms at the time of sampling (Fig. 3.2). Based on the sequencing of the different DGGE bands, the clone library and strain sequences, a total of 25 different ITS types could be found in the region (Table 3.1). Reservoirs often differed completely in their ITS population structure, as the average number of ITS types per sample was only 1.8 ($\pm$ 1.0). Half of the populations contained a single ITS type (= one band) (Fig. 3.2). No significant differences were found between the local ITS diversity (number of ITS types per sample) in the wet and dry season ($U = 84;\ p > 0.05$). A comparison with the local ITS diversity in other areas is given as supplementary study. No significant correlations were found between local ITS diversity and total biomass of *Microcystis*, environmental variables or geographical distances (all $p > 0.05$) in the wet and dry season (Table 3.2).

The total biomass and ITS population dynamics of *Microcystis* in the eight reservoirs followed during the course of one year is given in figure 3.3. In Gum Selasa, Mai Gassa I and Mai Gassa II *Microcystis* dominated the phytoplankton during (almost) the whole sampling period, and biomass was often high, although there were also strong fluctuations (Asmelash *et al.* unpublished). These three reservoirs were dominated by a single ITS type, termed EG07, during the whole sampling period. This ITS type was also dominant in five and seven reservoirs sampled in the wet and dry season respectively (Fig. 3.2). EG07 appears to be a very abundant ITS type in the Tigray reservoirs as it dominated in twelve out of the total of 30 sampled reservoirs during at least one sampling date (Fig. 3.1). This result was confirmed by cloning and sequencing of cultures (Table 3.1). In Gereb Awso, Ruba Feleg, Mai Leba, Tegh'ane and Tsinkanet *Microcystis* generally did not dominate the phytoplankton and biomass was often low to below the counting detection limit of the inverted microscope (Asmelash

[1] http://manteltester.berlios.de

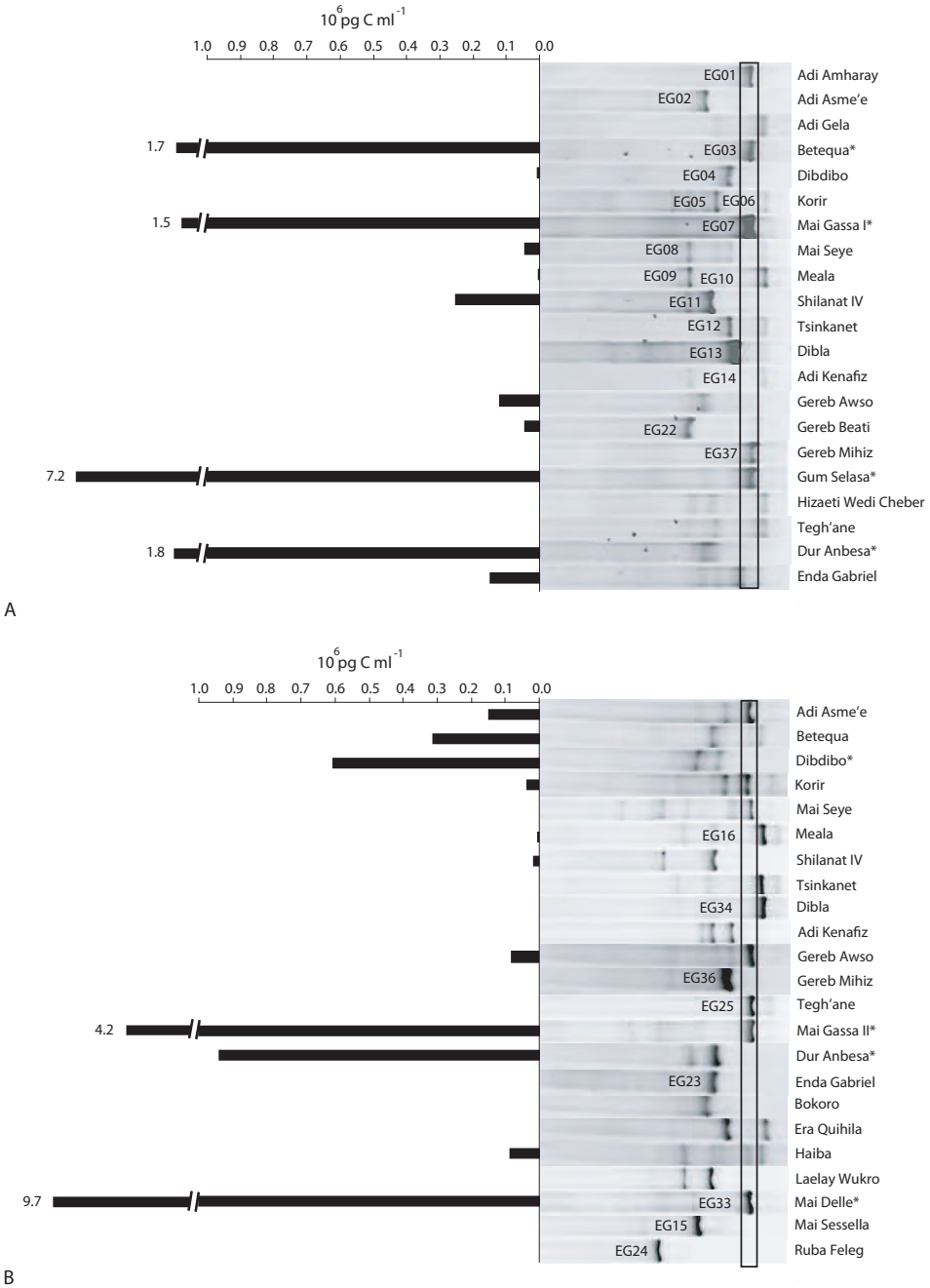


Figure 3.2: Photograph of the DGGE gels obtained by *Microcystis*-specific PCR amplification of DNA from samples taken in 21 reservoirs in the wet season (September) of 2004 (A) and 23 reservoirs in the dry season (April) of 2005 (B) in Tigray. Sequenced bands are numbered, bar includes bands representing one dominant ITS type (EG07, see also table 3.1). Reservoir names are indicated at the right and asterisks indicate the presence of a *Microcystis* bloom. Total biomass of *Microcystis* in the samples is shown at the left.

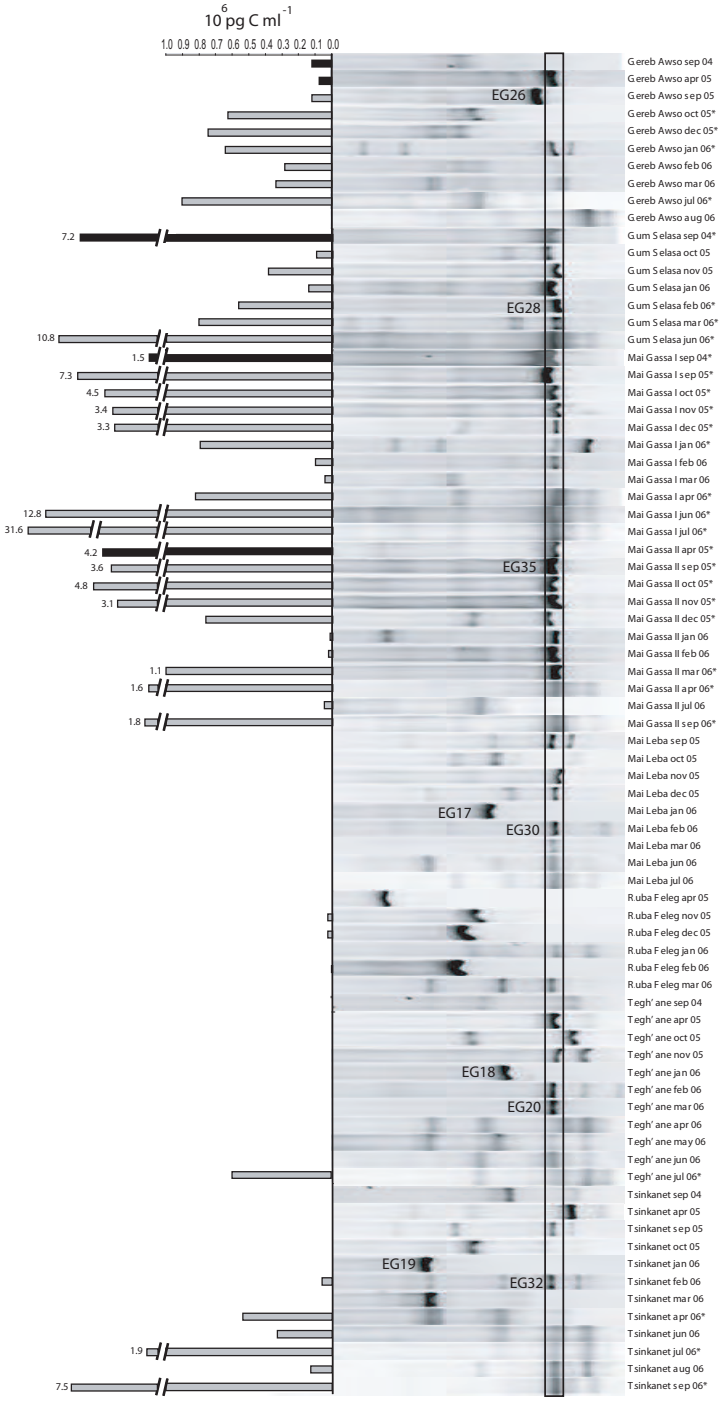


Figure 3.3: Photograph of the DGGE gels obtained by *Microcystis*-specific PCR amplification of DNA from samples taken in eight reservoirs in September 2004 and April 2005 and from September 2005 to September 2006. Reservoir names and sampling periods are indicated at the right and asterisks indicate the presence of a *Microcystis* bloom. Sequenced bands are numbered, bar includes bands representing one dominant ITS type (EG07, see table 3.1). Total biomass of *Microcystis* in the samples is shown at the left (black bars: September 2004 and April 2005, grey bars: September 2005 to September 2006).

et al. unpublished). *Microcystis* biomass was temporarily very high in Tsinkanet (July and September). The ITS diversity was often higher in these reservoirs and the ITS population structure varied more strongly in time. Mantel tests showed that the ITS diversity was correlated with *Microcystis* biomass (a higher diversity was associated with a lower biomass, $r = 0.097$; $p < 0.01$, controlled for reservoir), total nitrogen concentration (a higher diversity was associated with a lower nitrogen concentration, $r = -0.064$; $p < 0.05$, controlled for reservoir) and zooplankton community composition ($r = -0.118$; $p < 0.01$, controlled for reservoir) (Table 3.2). These correlations were independent of each other as they remained significant when performing a partial mantel test controlling for the effect of the other factor.

Table 3.1: Information about rDNA ITS sequences of *Microcystis* obtained in this study (see also figure 3.2 and 3.3): method used (DGGE, cloning or culture), toxicity of the ITS types (NT = non-toxic, T = toxic, na = not available) and occurrence in other countries based on the ITS sequences present in Genbank. *: toxicity determined from isolated strain with the same ITS sequence as DGGE bands. Sequences obtained from DGGE bands are grouped per band class. For cloning and cultures, only sequences that differ from DGGE bands are included in the table. For cloning, five distinct ITS types were detected: identical sequence as EG07 represented by 32 clones in Mai Gassa I and 34 clones in Mai Gassa II, EG38 represented by one clone in Mai Gassa I, EG39 and EG40 represented by one clone and EG41 represented by four genotypes in Mai gassa II. For cultures, three distinct ITS types were detected: identical sequence as EG07 represented by 46 cultures, EG42 represented by five cultures and EG43 represented by three cultures.

	Method	Toxicity	Occurrence of identical sequence in other countries	Accession number of identical sequence
EG02	DGGE		-	
EG04	DGGE		Greece (na)	EF150950
EG18				
EG05	DGGE		Belgium (na)	
EG06	DGGE		-	
EG10				
EG14				
EG01	DGGE	NT*	Belgium (na)	
EG03			The Netherlands (NT)	AJ605146
EG07			Denmark (na)	
EG20			Scotland (NT)	AJ605145
EG25			Greece (na)	EF150951
EG28			Argentina (na)	
EG30			Brazil (na)	
EG32			New Zealand (na)	EF634470
EG33				
EG35				
EG37				
EG08	DGGE		Belgium (NT)	
EG22			The Netherlands (na)	AY827804
			Israel (T)	AB254417
			Japan (T)	AB015368
EG09	DGGE		-	

	Method	Toxicity	Occurrence of identical sequence in other countries	Accession number of identical sequence
EG11	DGGE		Kenya (NT) Uganda (NT) Brazil (na)	AM421568 AM421552
EG12	DGGE		-	
EG13	DGGE		-	
EG15	DGGE		Kenya (T)	AM421569
EG16	DGGE		-	
EG17	DGGE		Denmark (na)	
EG19	DGGE		The Netherlands (na)	
EG23	DGGE		-	
EG24	DGGE		Belgium (T) Germany (T) Czech Republic (T) The Netherlands (T) Italy (T) Denmark (na)	 AJ605165 AJ605164 AJ605156 AJ605159
EG26	DGGE		-	
EG34	DGGE		-	
EG36	DGGE		Germany (NT) Scotland (NT) Denmark (na)	AJ605204 AJ605205
EG38	Cloning		-	
EG39	Cloning		-	
EG40	Cloning		-	
EG41	Cloning		Belgium (na) China (T) Germany (T) France (na) The Netherlands (T) Japan (T)	 AB015392 AJ605169 AY431049 AJ605170 AB254444
EG42	Culture	NT	Kenya (NT)	AM421572
EG43	Culture	NT	-	

3.3.2 Determinants of *Microcystis* ITS population structure

Analysis of similarity revealed that there were no significant differences ($p > 0.05$) in ITS population structure (presence/absence and relative abundance of the ITS types) between the wet and dry season. *Microcystis* population structure was correlated with *Microcystis* biomass in the dry season ($r = -0.122$; $p < 0.05$), but not in the wet season. Mantel tests showed that the *Microcystis* ITS population structure in the wet season is correlated with the distance between the different reservoirs ($r = -0.221$; $p < 0.01$), the zooplankton community composition ($r = 0.195$;

Table 3.2: Correlations of *Microcystis* diversity and population structure with environmental and spatial variables as determined by Mantel tests and Spearman rank order correlations. Correlations are presented as Pearson correlation coefficients (when *Microcystis* population, zooplankton and phytoplankton community or geographical distances are included and for the correlations obtained from the monthly sampled reservoirs) or Spearman rank correlation coefficients. Significant correlations are in bold, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$; ****: $p < 0.0001$, nd: not determined. 1: controlled for geographical distances, 2: controlled for reservoir, 3: controlled for zooplankton community composition.

	Wet season		Dry season		Monthly sampling	
	Microcystis diversity	*Microcystis* population structure	*Microcystis* diversity	*Microcystis* population structure	*Microcystis* diversity[2]	*Microcystis* population dynamics[2]
Microcystis biomass	−0.110	−0.097	−0.003	**−0.122***	**0.097****	0.029[3]
Temperature	−0.241	0.061	0.233	0.085	0.014	−0.068
Oxygen concentration	0.021	−0.125	−0.018	0.083	−0.052	−0.059
pH	0.040	**−0.195***	−0.255	0.077	−0.001	0.045
Conductivity	0.261	−0.049	−0.025	0.053	0.016	−0.082[3]
Water transparency (Snell depth)	0.229	−0.049	−0.047	0.001	0.042	**−0.150***
Total nitrogen concentration	−0.027	−0.089	−0.274	−0.071	**−0.064***	−0.055
Total phosphorus concentration	−0.176	−0.077	−0.190	−0.084	−0.049	−0.072
Phytoplankton community	−0.066	0.064	−0.078	0.046	0.004	−0.001
Phytoplankton biomass	−0.068	−0.066	−0.141	−0.078	−0.046	−0.048
Zooplankton community	−0.028	0.086[1]	0.001	0.003	**−0.118****	**0.263****
Zooplankton biomass	0.124	0.024	0.038	0.020	0.023	−0.091
Daphnia biomass	0.253	0.057	0.060	**−0.213*****	0.074	**−0.240*****
Geographical distances	−0.041	**−0.221****	0.016	**−0.195****	nd	nd

$p < 0.05$) and the pH ($r = -0.195$; $p < 0.05$). However, the correlation found with the zooplankton community composition was lost when controlling for the geographical distances ($r = 0.086$; $p > 0.05$), because both the zooplankton communities and the *Microcystis* ITS population were geographically distributed (Table 3.2). In the dry season, the *Microcystis* ITS population structure was again correlated with the geographical distances ($r = -0.195$; $p < 0.01$) and the total biomass of *Daphnia* ($r = -0.213$, $p < 0.001$). These correlations were independent of each other as they remained significant when performing a partial mantel test controlling for the effect of the other factor. There was a trend for *Daphnia* to be geographically distributed, although not significant ($r = 0.121$; $p = 0.06$). The correlation with *Daphnia* biomass was mainly due to the dominant ITS type EG07, as there was no correlation between the *Microcystis* population structure without EG07 and *Daphnia* biomass ($r = -0.032$; $p > 0.05$). Spearman rank correlation coefficients revealed a significant negative correlation between the relative abundance of ITS type EG07 and *Daphnia* biomass ($\mathrm{Spearman\ R} = -0.607$, $p < 0.01$) in the dry season (Fig. 3.4). Also, the correlation of *Microcystis* population structure with geographical distance in the wet and dry season was due to the dominant ITS type EG07 (Fig. 3.1). When correlating the population without this ITS type with the geographical distances, no correlation was found anymore (wet season: $r = -0.091$; $p > 0.05$, dry season: $r = -0.001$; $p > 0.05$).

For the eight reservoirs monitored during one year, Mantel tests showed that the *Microcystis* ITS population dynamics is correlated with water transparency ($r = -0.150$; $p < 0.05$, controlled for reservoir), conductivity ($r = -0.151$; $p < 0.05$, controlled for reservoir) and the zooplankton community composition ($r = 0.263$; $p < 0.0001$, controlled for reservoir). However, the correlation with conductivity is lost when controlling for the zooplankton community composition ($r = -0.082$; $p > 0.05$). In addition, total *Daphnia* biomass was strongly correlated with *Microcystis* population dynamics ($r = -0.240$; $p < 0.001$, controlled for reservoir). Spearman rank correlation coefficients revealed a significant negative correlation between the relative abundance of ITS type EG07 and *Daphnia* biomass ($\mathrm{Spearman\ R} = -0.474$; $p < 0.0001$). When ITS type EG07 was present, the biomass of *Daphnia* was zero or low in the reservoir (Fig. 3.4). Not only the *Microcystis* population dynamics was correlated with zooplankton community composition, the total *Microcystis* biomass was also strongly correlated with the zooplankton community composition ($r = -0.435$; $p < 0.0001$, controlled for reservoir). Moreover, the correlation between *Microcystis* biomass and *Microcystis* population dynamics ($r = -0.111$; $p < 0.01$) was lost when controlling for zooplankton community composition ($r = 0.029$; $p > 0.05$) (Table 3.2).

3.4 Discussion

The DGGE profiles gave a clear view of the rDNA ITS diversity of *Microcystis* populations in Tigray and revealed that the population structure was rather stable, as temporal changes in population structure were limited. The wet and dry season had a similar population structure and in three of the eight reservoirs monitored during one year,

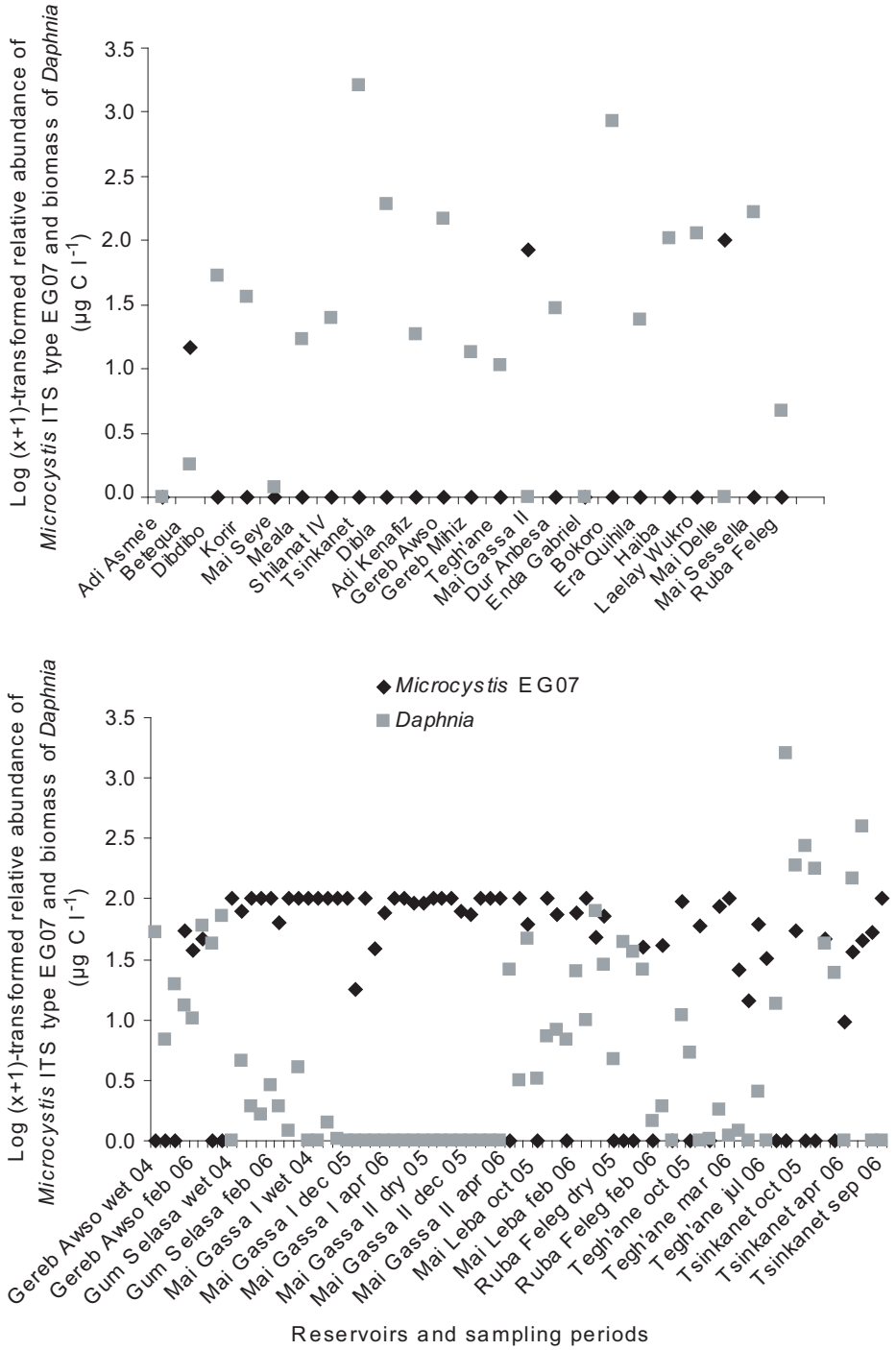


Figure 3.4: Negative correlation between relative abundance of *Microcystis* ITS type EG07 and *Daphnia* biomass in the dry season of 2005 (upper graph) and in the eight reservoirs sampled monthly during one year (lower graph). For the reservoirs sampled monthly, not all sample names are indicated, see figure 3.3 for all sample names.

almost no changes were observed. However, in some reservoirs temporal dynamics were higher suggesting variation among the reservoirs. Several blooms apparently contained only one ITS type, indicating that no high diversity is needed to obtain a high biomass. Moreover, in the monthly sampled reservoirs, a high biomass was associated with a low diversity, although the reason for this was that blooms were dominated by one particular ITS type.

Previous studies revealed a high local *Microcystis* ITS diversity. Kardinaal *et al.* (2007a) found 5-7 types per lake by ITS-DGGE in three Dutch lakes sampled twice a month in the growth season and once a month in winter for one year. Humbert *et al.* (2005) detected 37 ITS types using clone libraries in a French storage reservoir sampled at two stations in winter and in summer. Other studies showed the presence of several ITS types in a single water body by isolating strains, for example in Asia (Otsuka *et al.* 1999), Europe (Janse *et al.* 2004) and Uganda (Haande *et al.* 2007). According to these studies, the local ITS diversity in Tigray was very low, with on average only 1.8 ITS types per sample. The regional diversity in Tigray seems low too as a total of only 25 ITS types were encountered in the 23 reservoirs sampled. However, apart from the fact that not a single other study attempted to recover the full regional ITS diversity, one should be careful in comparing local and regional diversity among studies because of differences in region size, and water body area and density. To avoid these problems we compared the ITS diversity in Tigray with a similar unpublished study in 37 water bodies in Flanders, a region of similar size with shallow water bodies with a comparable area, but completely different in climate, and history and density of water bodies (see supplementary study). This comparison confirmed the low local and regional ITS diversity in Tigray. A plausible explanation for the low ITS diversity in *Microcystis* populations in Tigray, as compared to other regions, is that the reservoirs were recently constructed (after 1994) in an area with a history of severe droughts and a low density of water bodies (SAERT 1994; De Wit unpublished). This is in contrast to the historically abundant and well-connected water bodies in Flanders, where dispersal can lead to the accumulation and persistence of more ITS types. However, contemporary factors may also play a role. The higher temporal variability in temperature in a temperate climate might prevent the dominance of a single ITS type whereas in (sub)tropical climates strains which can grow very fast at high temperatures win the competition from other strains. As shown in the supplementary study, the relatively low local ITS diversity in Brazil suggests climate factors may play a role, although in Argentina (temperate to polar climate) a similar low diversity was seen. In addition, Haande *et al.* (2007) showed that at least four different *Microcystis* ITS types were present in Murchison Bay (Lake Victoria) in Uganda. Definitely, more comparative studies in temperate and (sub)tropical regions are needed to determine whether there is a consistent difference in local and regional ITS diversity between cold-temperate and (sub)tropical areas and what is the cause of the difference. Additionally, priority effects might also play a role in maintaining the low ITS diversity (chapter 6).

One ITS type was especially successful in Tigray and occurred often in dense blooms where it was generally the only ITS type present. On the other hand, its distribution seems to be geographically limited. A similar geographical pattern in

bacterial community composition on a relatively small scale was reported by Reche *et al.* (2005) who studied the bacterial composition in eleven high-mountain lakes in Sierra Nevada (Spain) and found that lakes that were closer together had more similar bacterial fingerprints. The geographical distribution of the *Microcystis* ITS type in Tigray was not caused by any of the environmental factors studied, suggesting the observed pattern is caused by dispersal limitation, possibly in combination with ongoing effects of immigration history (chapter 6). On the other hand, several ITS types recorded in Tigray, including the successful one mentioned above, have been recorded previously from other continents, implying that long-distance dispersal occurs from time to time. Overall, the emerging picture is that long-distance dispersal is frequent enough for ITS types to colonize the newly created habitats in Tigray, but infrequent enough to (still) limit local and regional ITS diversity and to cause the geographically restricted distribution of the dominant ITS type.

As we could isolate the dominant ITS type from Mai Gassa I, we determined that it was non-toxic. The two other strains from Mai Gassa I did not produce microcystins either, although detection of microcystin synthetase genes by PCR suggests that toxic strains frequently occur in the reservoirs (Asmelash *et al.* unpublished). More research about the toxicity of these blooms is definitely needed. A relatively high occurrence of toxic strains has been reported in Europe (Janse *et al.* 2004), China (Wu *et al.* 2007) and USA (Wilson *et al.* 2005). On the other hand, Haande *et al.* (2007) detected only three toxic ITS types from a total of ten in water bodies from Uganda and Kenya.

Abiotic environmental influences on ITS population structure seem to be relatively limited in Tigray, as only a correlation with pH was found in the wet season, probably reflecting primary production as pH is high throughout the region (not lower than 7.5, see Dejenie *et al.* 2008). *Microcystis* strains differ in capacity to take up bicarbonate, which could also be the cause of the correlation between *Microcystis* population structure and pH (Bañares-España *et al.* 2006). For the monthly sampled reservoirs, a correlation was found between *Microcystis* population dynamics and water transparency, possibly reflecting competition for light between *Microcystis* ITS types (Kardinaal *et al.* 2007b).

One of the most striking results from this study was the strong relationship between zooplankton community composition and *Microcystis* population dynamics. This was mainly due to the negative correlation between the relative abundance of the dominant ITS type EG07 and *Daphnia* biomass. The influence of zooplankton community composition on cyanobacterial community dynamics was reported before (van Gremberghe *et al.* 2008, see appendix). However, based on our field study it is difficult to know if the zooplankton community composition influenced the *Microcystis* population dynamics, or the other way around as the biomass of *Microcystis* was often very high and *Microcystis* strains can differ considerably in harmful effects on zooplankton. However, as shown by Dejenie *et al.* (unpublished), the most important large-bodied grazers in Tigray were *Daphnia* species (*D. barbata*, *D. carinata* and *D. magna*) and total *Daphnia* biomass was strongly correlated with *Microcystis* population structure. Generally, the effect of *Daphnia* on *Microcystis* biomass is debated, as *Microcystis* may strongly suppress growth of *Daphnia* (Ghadouani *et al.* 2003), while other studies have provided

evidence that *Daphnia* may suppress developing *Microcystis* blooms depending on initial conditions and history (Christoffersen *et al.* 1993; Sarnelle 2007). Dejenie *et al.* (unpublished) suggested a top-down effect of *Daphnia* grazing on *Microcystis* in the reservoirs of Tigray by an enclosure experiment. In fact, biomass of *Daphnia* was low or zero when the dominant ITS type EG07 was present. As this strain did not produce microcystins, it is more reasonable that *Daphnia* controlled growth of this strain by grazing. In addition, a strong influence of *Daphnia* on population structure of *Microcystis* was also confirmed by laboratory experiments using four *Microcystis* strains from Belgium (chapter 5). These aspects indicate that a top-down effect of zooplankton grazing on *Microcystis* population structure in Tigray is more likely than suppression of zooplankton species by particular (toxic) *Microcystis* strains, although more experiments are needed to confirm this.

In summary, we observed that the local and regional *Microcystis* ITS diversity was low in man-made reservoirs in Tigray compared to other regions, probably due to their low density and recent construction, and especially one non-toxic ITS type was very abundant and geographically restricted in its distribution. In addition to the effects of dispersal limitation, the correlations of *Microcystis* ITS population structure with abiotic variables (water clarity, pH) and *Daphnia* biomass suggest an important additional influence of these factors on *Microcystis* population structure in the Tigray highland reservoirs.

3.5 Supplementary study: Comparison of local *Microcystis* ITS diversity between regions

Because of a lack of available datasets in the literature on local and regional *Microcystis* ITS diversity, we compared the local (and regional) ITS diversity between different regions using unpublished studies from Europe and South America (van Gremberghe *et al.* unpublished) in addition to our dataset of Tigray (Ethiopia). Using these datasets, we also aimed to test several hypotheses which might explain differences in local ITS diversity. These included (1) climatic conditions, (2) turbidity, (3) lake size, (4) lake depth, (5) *Microcystis* biomass, (6) latitude, (7) habitat availability, (8) lake age and (9) dissolved nutrient concentrations. Shallow lakes, moderately to highly productive and differing in size, nutrient load and structural diversity, were sampled in summer in Europe (98 lakes in total) in 2000 and 2001, and in South America (83 lakes in total) in 2004-2006 along a latitudinal gradient, and only the lakes in which *Microcystis* was detected were used in this study. In Europe, nine lakes were included from Denmark, four and two from Belgium and The Netherlands respectively, and nine from Spain. In South America, fourteen lakes were included from Brazil and six from Argentina. A global map with indication of these sampled regions is shown in chapter 2. Additional samples were obtained from 37 ponds in Flanders (Belgium), which were sampled in the summer periods of 2003-2005, of which 24 contained a *Microcystis* bloom. The sampled ponds in Flanders are eutrophic fishing and urban ponds and most of them were made decades to centuries ago. The sampled region covers an area

of 1.3*10^6 ha, with a mean, minimum and maximum distance of 49 km, 0.12 km and 144 km respectively between ponds, which is similar to Tigray. Denaturing Gradient Gel Electrophoresis (DGGE) was used to assess the *Microcystis* ITS diversity in each of the sampled lakes. The total number of bands in the DGGE profile obtained for a sample was counted as the local ITS diversity. In Flanders, additional cloning and sequencing of cultures was performed in a similar way as the samples from Tigray (see chapter 2 for more information). Statistical analyses included non-parametric tests to test for significant differences in local diversity between regions (Mann-Whitney U test and Kruskal Wallis test) and Spearman rank order correlations (R = Spearman rank correlation coefficient) to test for correlations between the local diversity and local conditions in the lakes. Variables were $log(x+1)$ transformed and the statistical analyses were performed in SPSS 15 (SPSS Inc. Chicago, USA).

Table 3.3: Average local ITS diversity of *Microcystis* (number of ITS types per sample obtained by DGGE) and standard deviation of the different regions.

	average	standard deviation
Tigray	1.8	1.0
Flanders	3.6	1.8
Europe	4.0	2.5
South America	2.2	1.5
Denmark	4.3	2.0
Belgium/The Netherlands	3.6	1.9
Spain	3.8	2.6
Brazil	2.1	1.3
Argentina	2.3	2.2

Firstly, we compared the local and regional ITS diversity of *Microcystis* in Tigray with the ITS diversity in Flanders. A Mann-Whitney U test revealed that the local ITS diversity is significantly higher in Flanders than in Tigray ($U = 220$; $p < 0.0001$) (Fig. 3.5). The average number of ITS types per sample is 3.6 and 1.8 for Flanders and Tigray, respectively (Table 3.3). This difference also resulted in a large difference in total regional diversity. In Flanders, a total of 47 different ITS sequences was detected in seven ponds (sampled only once). In contrast, in Tigray 25 ITS types were obtained from 23 reservoirs (of which several were sampled more than once). In Flanders, cloning revealed that the diversity was in reality higher than determined by DGGE, suggesting that DGGE underestimates *Microcystis* ITS diversity when the diversity is high, as bands of different ITS types may be situated very close to each other in that case. In Tigray, cloning revealed no considerable higher diversity as compared to DGGE.

Secondly, we compared the local ITS diversity of *Microcystis* between Europe and South America. A Mann-Whitney U test revealed that the local ITS diversity is significantly higher in Europe than in South America ($U = 111$; $p = 0.01$) (Fig. 3.6). The average number of ITS types per sample is 4.0 and 2.2 for Europe and South America, respectively (Table 3.3). Over the latitudinal gradients in Europe and South America, there were apparently no differences in local ITS diversity as we did not find significant differences in local diversity between Denmark, Belgium/The Netherlands

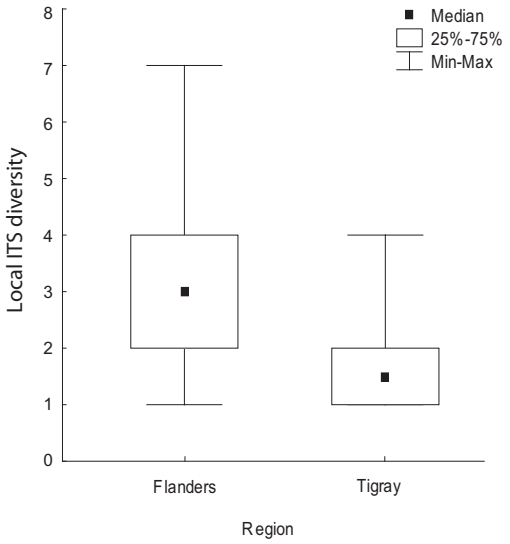


Figure 3.5: Box and Whisker plots of the local ITS diversity of *Microcystis* (number of ITS types per sample) in Flanders (Belgium) and Tigray (Ethiopia).

and Spain (Kruskal Wallis: $\chi^2 = 2.28$; $p > 0.05$), and between Brazil and Argentina (Mann-Whitney U test: $U = 40.5$; $p > 0.05$) (Fig. 3.7).

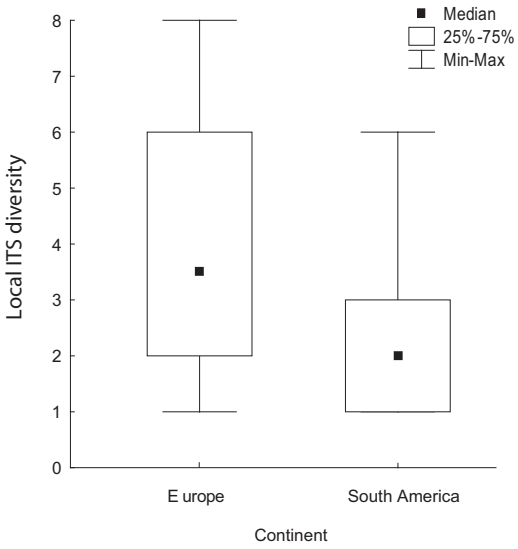


Figure 3.6: Box and Whisker plots of the local ITS diversity of *Microcystis* (number of ITS types per sample) in Europe and South America.

The above analyses clearly show that regions strongly differ in their local *Microcystis* ITS diversity. Finally, we tested several hypotheses which might explain these differences using the available datasets. Firstly, we stated that climatic conditions may cause differences in local diversity through their effects on the degree of temporal environmental variability, as tropical regions as Tigray and Brazil had a low diversity whereas in Europe a higher diversity was detected. However, this factor may not be the main cause of differences in diversity, as there were no significant differences in

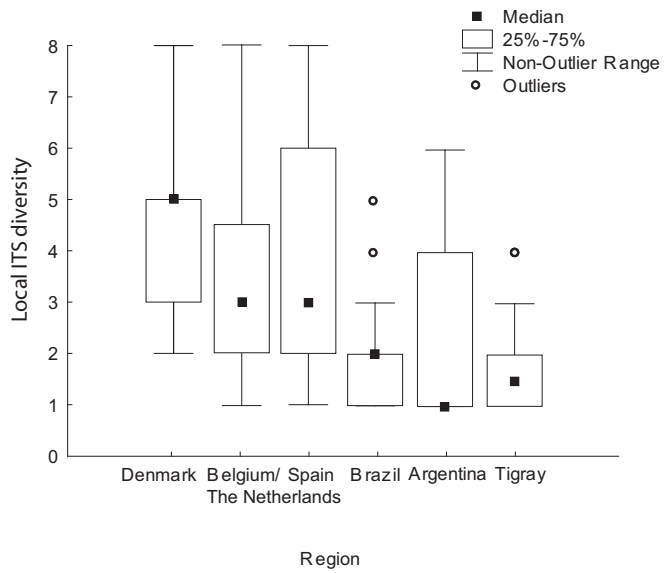


Figure 3.7: Box and Whisker plots of the local ITS diversity of *Microcystis* (number of ITS types per sample) in the different sampled regions (local diversity in 'Belgium / The Netherlands' included both the local diversity of the sampled ponds in Flanders and the shallow lakes in Belgium and The Netherlands).

local diversity between Brazil (tropical climate) and Argentina (temperate and polar climate).

Secondly, lake turbidity may be an important factor causing differences in diversity, as more light availability may result in a higher diversity. However, no significant correlation between local diversity and turbidity ($R = -0.297$; $p > 0.05$) was found for the European lakes, which included turbid as well as clear water lakes (determined by the percentage of the lake covered by submerged macrophytes).

Thirdly, lake size may be an important factor causing differences in diversity, as large lakes contain often more ecological niches than small lakes and are colonized more easily resulting in a higher diversity. In Europe, lakes ranging in area from 0.5 to 170 ha were sampled, but we did not find a correlation between local diversity and lake size ($R = -0.149$; $p > 0.05$).

Fourthly, lake depth may cause differences in diversity due to the large limnological differences between stratified and unstratified lakes. In South America, lakes ranging in depth from 1 to 10 m were sampled, but no correlation was found between local diversity and lake depth ($R = -0.133$; $p > 0.05$).

Fifthly, differences in diversity might be attributed to differences in *Microcystis* biomass, as a higher diversity might result in a higher biomass. However, in Tigray, blooms were found containing only one ITS type whereas in Flanders blooms were found containing more than five ITS types, and neither in Tigray or Flanders, correlations were found between local diversity and biomass of *Microcystis* ($R = -0.134$; $p > 0.05$ and $R = 0.038$; $p > 0.05$, respectively).

Sixthly, the latitudinal position of the lakes may cause differences in diversity, as climatic conditions and water body density vary strongly with latitude. In South

America, samples were taken in Brazil at the equator and in the south of Argentina, but there was no correlation between local diversity and Y-coordinates ($R = -0.087$; $p > 0.05$).

Seventhly, water body density in a certain region may cause differences in diversity, as dispersal will be more frequent in regions with a higher density of water bodies. However, no significant correlation between local diversity and percentage of water in a radius of 10 km around the lake was found in South America ($R = 0.165$; $p > 0.05$).

Eightly, we stated that the age of a water body is an important factor that may cause differences in diversity, as older lakes may have accumulated more ITS types. In South America, lakes of different age classes (10 years, 100 years and 1000 years old) were sampled. However, no significant differences were seen in local diversity between these age classes (Kruskal Wallis: $\chi^2 = 1.326$; $p > 0.05$), indicating that in this range of water body histories, differences in migration history do not cause differences in local diversity. In Tigray, most of the water bodies are younger than 15 years, but no correlation was found between the local diversity and age of the water bodies, probably because all lakes are relatively young ($R = -0.086$; $p > 0.05$).

Finally, concentrations of dissolved nutrients may cause differences in diversity as they play an important role in the development of *Microcystis* blooms. However, no correlation was found between local diversity and dissolved phosphorus ($R = -0.163$; $p > 0.05$) and nitrogen ($R = 0.044$; $p > 0.05$) concentrations in Europe.

In conclusion, none of our hypotheses was directly confirmed by the statistical analyses indicating that none of the factors tested (climatic conditions, turbidity, lake size and depth, biomass of *Microcystis*, geographical positioning, water body density and history, and dissolved nutrient concentrations) is the main cause of the observed differences in local diversity. Probably, more than one factor is important in determining local diversity or different factors are important for different regions. Unmeasured factors might also play a role. The extreme low diversity in Tigay is probably caused by several factors, including the fact that all reservoirs were relatively young and constructed in an area with a history of severe droughts and a low density of water bodies. Historical factors as dispersal limitation and priority effects (chapter 6) might be important in determining local diversity, and are largely determined by chance which could also explain the lack of correlations between local diversity and local environmental factors. In our datasets, sampling was done according to different projects and we need a more standardized sampling to reveal the causes of differences in local *Microcystis* diversity between individual lakes and between regions.

Acknowledgements

This research was financially supported by Aquatic Ecology project in the VLIR IUS-MEKELLE programme ('Aquatic Ecology and sustainable management of inland water bodies in Tigray, North Ethiopia'), supported by VLIR (Vlaamse Interuniversitaire Raad). We also thank Ann-Eline Debeer for helping with DGGE analyses.

The comparison of local ITS diversity (supplementary study) was possible using

samples obtained from different projects: B-Blooms ('Algal blooms: emerging problem for health and sustainable use of surface waters'), nationally supported by BELSPO (Belgian Science Policy), SALGA ('South American Lake Gradient Analysis'), supported by NWO/WOTRO and other financiers[2] and BIOMAN ('Biodiversity and human impact in shallow lakes') supported by European Union.

[2] www.projectenaew.wur.nl/salga/

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. 1990. Basic local alignment search tool. Journal of Molecular Biology 215: 403-410.

Bañares-España, E., Lopez-Rodas, V., Salgado, C., Costas, E. and Flores-Moya, A. 2006. Inter-strain variability in the photosynthetic use of inorganic carbon, exemplified by the pH compensation point, in the cyanobacterium *Microcystis aeruginosa*. Aquatic Botany 85: 159-162.

Bittencourt-Oliveira, M. D., de Oliveira, M. C. and Bolch, C. J. S. 2001. Genetic variability of Brazilian strains of the *Microcystis aeruginosa* complex (Cyanobacteria/Cyanophyceae) using the phycocyanin intergenic spacer and flanking regions (*cpc*BA). Journal of Phycology 37: 810-818.

Bonnet, E. and Van de Peer, Y. 2002. Zt: a software tool for simple and partial Mantel tests. Journal of Statistical software 7: 1-12.

Bottrell, H. H., Duncan, A., Gliwicz, Z. M., Grygierek, E., Herzig, A., Hillbrichtilkowska, A. *et al.* 1976. Review of some problems in zooplankton production studies. Norwegian Journal of Zoology 24: 419-456.

Christoffersen, K., Riemann, B., Klysner, A. and Sondergaard, M. 1993. Potential role of fish predation and natural populations of zooplankton in structuring a plankton community in eutrophic lake water. Limnology and Oceanography 38: 561-573.

Clarke, K. R. and Gorley, R. N. 2001. PRIMER 5 for Windows. PRIMER-E Ltd. Plymouth, UK.

Codd, G. A., Morrison, L. F. and Metcalf, J. S. 2005. Cyanobacterial toxins: risk management for health protection. Toxicology and Applied Pharmacology 203: 264-272.

Dejenie, T., Asmelash, T., De Meester, L., Mulugeta, A., Gebrekidan, A., Risch, S. *et al.* 2008. Limnological and ecological characteristics of tropical highland reservoirs in Tigray, Northern Ethiopia. Hydrobiologia 610: 193-209.

Ferrao-Filho, A. S., Azevedo, S. and DeMott, W. R. 2000. Effects of toxic and non-toxic cyanobacteria on the life history of tropical and temperate cladocerans. Freshwater Biology 45: 1-19.

Ghadouani, A., Pinel-Alloul, B. and Prepas, E. E. 2003. Effects of experimentally induced cyanobacterial blooms on crustacean zooplankton communities. Freshwater Biology 48: 363-381.

Haande, S., Ballot, A., Rohrlack, T., Fastner, J., Wiedner, C. and Edvardsen, B. 2007. Diversity of *Microcystis aeruginosa* isolates (Chroococcales, Cyanobacteria) from East-African water bodies. Archives of Microbiology 188: 15-25.

Hisbergues, M., Christiansen, G., Rouhiainen, L., Sivonen, K. and Borner, T. 2003. PCR-based identification of microcystin-producing genotypes of different cyanobacterial genera. Archives of Microbiology 180: 402-410.

Huisman, J., Matthijs, H. C. P. and Visser, P. M. 2005. Harmful cyanobacteria. Springer, Dordrecht, The Netherlands. 243 pp.

Humbert, J. F., Duris-Latour, D., Le Berre, B., Giraudet, H. and Salencon, M. J. 2005. Genetic diversity in *Microcystis* populations of a French storage reservoir assessed by sequencing of the 16S-23S rRNA intergenic spacer. Microbial Ecology 49: 308-314.

Janse, I., Meima, M., Kardinaal, W. E. A. and Zwart, G. 2003. High-resolution differentiation of cyanobacteria by using rRNA-internal transcribed spacer Denaturing Gradient Gel Electrophoresis. Applied and Environmental Microbiology 69: 6634-6643.

Janse, I., Kardinaal, W. E. A., Meima, M., Fastner, J., Visser, P. M. and Zwart, G. 2004. Toxic and nontoxic *Microcystis* colonies in natural populations can be differentiated on the basis of rRNA gene internal transcribed spacer diversity. Applied and Environmental Microbiology 70: 3979-3987.

Kardinaal, W. E. A., Janse, I., Kamst-van Agterveld, M., Meima, M., Snoek, J., Mur, L. R. *et al.* 2007a. *Microcystis* genotype succession in relation to microcystin concentrations in freshwater lakes. Aquatic Microbial Ecology 48: 1-12.

Kardinaal, W. E. A., Tonk, L., Janse, I., Hol, S., Slot, P., Huisman, J. and Visser, P. M. 2007b. Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*. Applied and Environmental Microbiology 73: 2939-2946.

Lodders, N., Stackebrandt, E. and Nubel, U. 2005. Frequent genetic recombination in natural populations of the marine cyanobacterium *Microcoleus chthonoplastes*. Environmental Microbiology 7: 434-442.

Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Research 27:209-220.

Mantel, N. and Valand, R. S. 1970. A technique of nonparametric multivariate analysis. Biometrics 26:547-558.

Menden-Deuer, S. and Lessard, E. J. 2000. Carbon to volume relationships for dinoflagellates, diatoms, and other protist plankton. Limnology and Oceanography 45: 569-579.

Muyzer, G., Dewaal, E. C. and Uitterlinden, A. G. 1993. Profiling of complex microbial populations by Denaturing Gradient Gel Electrophoresis analysis of Polymerase Chain Reaction amplified genes coding for 16S ribosomal RNA. Applied and Environmental Microbiology 59: 695-700.

Otsuka, S., Suda, S., Li, R. H., Watanabe, M., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 1999. Phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis* based on 16S to 23S internal transcribed spacer sequence. FEMS Microbiology Letters 172: 15-21.

Pflugmacher, S. 2004. Promotion of oxidative stress in the aquatic macrophyte *Ceratophyllum demersum* during biotransformation of the cyanobacterial toxin microcystin-LR. Aquatic Toxicology 70: 169-178.

Reche, I., Pulido-Villena, E., Morales-Baquero, R. and Casamayor, E. O. 2005. Does ecosystem size determine aquatic bacterial richness? Ecology 86: 1715-1722.

Rohrlack, T., Dittmann, E., Henning, M., Borner, T. and Kohl, J. G. 1999. Role of microcystins in poisoning and

food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 65: 737-739.

SAERT (Sustainable Agricultural and Environmental Rehabilitation in Tigray). 1994. Sustainable agriculture in Tigray working document. Unpublished, Mekelle, Ethiopia.

Sarnelle, O. 2007. Initial conditions mediate the interaction between *Daphnia* and bloom-forming cyanobacteria. Limnology and Oceanography 52: 2120-2127.

Vaitomaa, J., Rantala, A., Halinen, K., Rouhiainen, L., Tallberg, P., Mokelke, L. and Sivonen, K. 2003. Quantitative real-time PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. Applied and Environmental Microbiology 69: 7289-7297.

Vézie, C., Rapala, J., Vaitomaa, J., Seitsonen, J. and Sivonen, K. 2002. Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intracellular microcystin concentrations. Microbial Ecology 43: 443-454.

Via-Ordorika, L., Fastner, J., Kurmayer, R., Hisbergues, M., Dittmann, E., Komarek, J. *et al.* 2004. Distribution of microcystin-producing and non-microcystin-producing *Microcystis sp.* in European freshwater bodies: Detection of microcystins and microcystin genes in individual colonies. Systematic and Applied Microbiology 27: 592-602.

Welker, M., Brunke, M., Preussel, K., Lippert, I. and von Dohren, H. 2004. Diversity and distribution of *Microcystis* (Cyanobacteria) oligopeptide chemotypes from natural communities studied by single-colony mass spectrometry. Microbiology-Sgm 150: 1785-1796.

Welker, M., Sejnohova, L., Nemethova, D., von Dohren, H., Jarkovsky, J. and Marsalek, B. 2007. Seasonal shifts in chemotype composition of *Microcystis* sp communities in the pelagial and the sediment of a shallow reservoir. Limnology and Oceanography 52: 609-619.

Wilson, A. E., Sarnelle, O., Neilan, B. A., Salmon, T. P., Gehringer, M. M. and Hay, M. E. 2005. Genetic variation of the bloom-forming cyanobacterium *Microcystis aeruginosa* within and among lakes: Implications for harmful algal blooms. Applied and Environmental Microbiology 71: 6126-6133.

Wilson, A. E., Wilson, W. A. and Hay, M. E. 2006. Intraspecific variation in growth and morphology of the bloom-forming cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 72: 7386-7389.

Wu, Z. X., Gan, N. Q. and Song, L. R. 2007. Genetic diversity: Geographical distribution and toxin profiles of *Microcystis* strains (Cyanobacteria) in China. Journal of Integrative Plant Biology 49: 262-269.

Yoshida, M., Yoshida, T., Takashima, Y., Hosoda, N. and Hiroishi, S. 2007. Dynamics of microcystin-producing and non-microcystin-producing *Microcystis* populations is correlated with nitrate concentration in a Japanese lake. FEMS Microbiology Letters 266: 49-53.

Zwart, G., Huismans, R., van Agterveld, M. P., Van de Peer, Y., De Rijk, P., Eenhoorn, H. *et al.* 1998. Divergent members of the bacterial division *Verrucomicrobiales* in a temperate freshwater lake. FEMS Microbiology Ecology 25: 159-169.

Chapter 4

Influence of *Daphnia* infochemicals on functional traits of *Microcystis*

Ineke van Gremberghe[1], Pieter Vanormelingen[1], Katleen Van der Gucht[1], Antoniya Mancheva[1], Sofie D'hondt[1], Luc De Meester[2] and Wim Vyverman[1]

Submitted to Hydrobiologia

Contribution to this chapter by IVG: experimental set-up, sample analyses, statistical analyses and writing

[1]Laboratory of Protistology and Aquatic Ecology, Ghent University, Krijgslaan 281 - S8, 9000 Ghent, Belgium

[2]Laboratory of Aquatic Ecology and Evolutionary Biology, Katholieke Universiteit Leuven, Charles Debériotstraat 32, 3000 Leuven, Belgium

Abstract

We conducted a laboratory experiment to investigate the influence of *Daphnia* infochemicals on growth rate, microcystin production, colony formation and cell size of eight *Microcystis* strains isolated from two lakes. The strains were characterized genetically by their 16S-23S rDNA ITS sequence. The experiment was composed of four treatments: 1) a control using WC medium, 2) addition of *Scenedesmus obliquus* culture medium filtrate, 3) addition of *Daphnia magna* culture medium filtrate and 4) addition of sodium octyl sulphate, a commercially available *Daphnia* infochemical. Our results showed that sympatric strains differed strongly for the measured functional traits, while no correlations between traits were found. Between-strain differences in growth rate, microcystin production, colony formation and cell size were generally larger than the differences in phenotypes observed between treatments. Despite this, several strains reacted to the infochemicals by changing functional trait values. *Daphnia* culture medium filtrate and, to a lesser extent, sodium octyl sulphate had a negative influence on the growth of half of the strains and stimulated microcystin production in one strain, but this last effect was not *Daphnia*-specific as *Scenedesmus* culture medium filtrate had the same effect. *Daphnia* culture medium filtrate also induced colony formation in one strain. In conclusion, our data suggest that *Daphnia* infochemicals generally have a weak influence on growth rate, microcystin production and colony formation of *Microcystis* strains as compared to the inter-strain variability, while existing inducible effects are often highly strain-specific.

4.1 Introduction

Phytoplankton has evolved various morphological, biochemical and behavioral defense mechanisms against grazing of zooplankton. The fact that herbivorous zooplankton can induce these defense mechanisms in phytoplankton by releasing infochemicals is well known (van Donk 2007). In particular, the influence of zooplankton infochemicals on the morphology of the closely related green algae *Scenedesmus* and *Desmodesmus* has been intensively studied (e.g. Hessen and van Donk 1993; Lürling and van Donk 1997; Verschoor *et al.* 2004). The presence of infochemicals from grazers has been shown to induce changes in cell size, colony formation and spine formation in a range of species from both genera. Other chlorococcal green algae may show a similar response (van Donk *et al.* 1999; Luo *et al.* 2006). Also, several flagellated algal species regulate their recruitment rate from the sediment depending on the presence or absence of grazers in the water column (Hansson 1996, 2000). These adaptive changes reduce phytoplankton mortality due to herbivory, while the energetic cost of the response is lowered by the fact that the defense can be attuned depending on the presence of predators (van Donk 2007).

Among phytoplankton, cyanobacteria are very widespread and may form nuisance blooms (Huisman *et al.* 2005). *Microcystis* is a common genus that forms toxic blooms, especially in eutrophic water bodies at high temperatures (Chorus and Bartram 1999; Visser *et al.* 2005). There is conflicting evidence in literature on the extent to which grazing by zooplankton can control the development of cyanobacterial blooms. For instance, the effect of *Daphnia*, a key grazer in standing waters, on *Microcystis* biomass is debated, as *Microcystis* may strongly suppress growth of *Daphnia* (Ghadouani *et al.* 2003), while other studies have provided evidence that *Daphnia* may suppress developing *Microcystis* blooms depending on initial conditions and history (Christoffersen *et al.* 1993; Sarnelle 2007). Understanding the mechanism of defense of *Microcystis* against predation is important to evaluate possibilities of suppressing bloom development by grazing. Previous research has shown that *Microcystis* blooms are typically composed of several genotypes that can differ in functional traits such as growth rate, microcystin production, colony formation and cell size (Wilson *et al.* 2005, 2006; Carrillo *et al.* 2003; Janse *et al.* 2004; Kardinaal *et al.* 2007). With respect to the extent to which the presence of grazers may induce phenotypic shifts in these traits, the data of different studies lead to different conclusions. Zooplankton infochemicals seem to have no (Yang *et al.* 2006a, Yang and Li 2007) or a slightly positive impact (Jang *et al.* 2007, 2008) on the growth rate of *Microcystis*. Jang *et al.* (2003, 2007, 2008) reported that grazing or culture medium filtrate of *Daphnia magna* and *Moina macrocopa* increased microcystin production in several *Microcystis* strains. In addition, Jang *et al.* (2003) and Ha *et al.* (2004) showed that grazing or culture medium filtrate of *Daphnia magna* and *Moina macrocopa* can induce colony formation in *Microcystis* strains. However, other studies failed to find changes in this trait induced by *Daphnia magna* (Hessen and van Donk 1993; Yang *et al.* 2006b). Most studies were carried out on one or a few strains. Given the high inter-strain variability observed in field studies and in laboratory experiments, there is a need for

studies that include several strains.

In this study, we carried out a laboratory experiment to examine the diversity in growth rate, microcystin production, colony formation and cell size in eight *Microcystis* strains isolated from two bloom forming populations, and the influence of *Daphnia* infochemicals on these traits.

4.2 Materials and methods

4.2.1 Isolation and genotypic characterization of *Microcystis* strains

Monoclonal *Microcystis* cultures were obtained by picking up single colonies with a micropipette from a bloom sample from a pond in the nature reserve Leeuwenhof (Ghent, Belgium) on 07/09/04 and from a pond in the nature reserve Tiens Broek (Tienen, Belgium) on 10/08/05. The colonies were grown in WC medium (Guillard and Lorenzen 1972) at a temperature of 18 °C and a light intensity of 30 µmol photons m^{-2} s^{-1} (Light Dark cycle: 12h:12h). As the taxonomy of *Microcystis* is still largely based on morphologic features and does not correspond to genetic features (Otsuka *et al.* 2001), we used the 16S-23S rDNA ITS sequence as a genetic marker to discriminate among different *Microcystis* genotypes irrespective of morphospecies identification (Janse *et al.* 2004). We detected four ITS types in Leeuwenhof and seven ITS types in Tiens Broek. Five ITS types were identified as microcystin producing genotypes (two toxic genotypes from Leeuwenhof named T1 and T2, and three toxic genotypes from Tiens Broek of which we randomly selected two genotypes named T3 and T4) and six as non-microcystin producing genotypes (two non-toxic genotypes form Leeuwenhof named NT1 and NT2, and four non-toxic genotypes from Tiens Broek of which we randomly selected two genotypes named NT3 and NT4), both by detecting the presence of *mcy* genes A (Hisbergues *et al.* 2003) and E (Vaitomaa *et al.* 2003) as well as by directly measuring microcystin concentrations in cultures by ELISA (Enzyme-Linked Immunosorbent Assay) with SDI - EnviroGard Microcystin ELISA plate kits (see below for more details). A rDNA ITS distance tree (Neighbor Joining with Jukes and Cantor correction) was constructed using the program BioNumerics 4.5 (Applied Maths BVBA).

4.2.2 Experimental design

All eight genotypes were grown under standardized conditions, in WC medium at a temperature of 23 °C and a light intensity of 120 µmol photons m^{-2} s^{-1} (Light Dark cycle: 12h:12h) for several generations. Exponentially growing cells were used as inoculum for the experiment. The experimental cultures were grown during eight days in 50 ml flasks at an initial density of 200 000 cells ml^{-1}. Four treatments were imposed: 1) a control with addition of pure filtered WC medium, 2) addition of filtered medium from a *Scenedesmus obliquus* culture containing 250 000 cells ml^{-1} ('*Scenedesmus*'

treatment), 3) addition of *Daphnia* infochemicals obtained by filtering medium from a culture in which 300 *Daphnia magna* individuals per liter grazed for 24 hours on approximately 50 000 cells ml^{-1} of *Scenedesmus obliquus* ('*Daphnia*' treatment), and 4) addition of filtered WC medium containing sodium octyl sulphate (final culture concentration of 1000 µg l^{-1}), a commercially available *Daphnia* infochemical which can induce colony formation in *Scenedesmus gutwinskii* as described by Yasumoto *et al.* (2005) ('SOS' treatment). Sterile Nalgene filter units with CN membrane and pore size of 0.2 µm were used for filtration. Each culture flask contained 90 % standard WC medium and 10 % culture medium filtrate. New culture medium filtrate was added every second day for all four treatments because, as the cultures were not axenic, infochemicals could be degraded by micro-organisms (Lürling and van Donk 1997). There were three replicate cultures for each strain x treatment combination, which resulted in 96 flasks that were gently shaken by hand daily. The position of the cultures was randomized daily.

4.2.3 Sample analyses

The optical density (OD) of the cultures was measured with a spectrophotometer ($\lambda = 750$ nm) at the start of the experiment and after two, five and eight days. The growth rate of each strain was determined by calculating the slope of the regression line between time and Ln(OD). Samples for microcystin analysis were taken after 21 hours and after eight days of growth. The total microcystin concentration (µg ml^{-1}) in the samples was determined by ELISA. For the extraction of microcystins, the cells were boiled during 20 minutes (van der Oost 2007). Next, the cultures were centrifuged during 10 minutes at 14 000 rpm and the supernatant was used for ELISA. The ELISA-test was done according to the manufacturer's instructions using SDI - EnviroGard Microcystin ELISA plate kits. To calculate microcystin concentration per unit biomass, cell densities were converted into biomass (pg C µl^{-1}) using the formula pg C cell^{-1} = 0.216*volume cell$^{0.939}$ (Menden-Deuer and Lessard 2000). Cells were counted and cell biovolumes were estimated by measuring the average cell diameter of each strain using the program CellC (Selinummi *et al.* 2005) (see below). Microscopical pictures of the cells and colonies were taken at the start and at the end of the experiment. After eight days of growth, 10 ml from each culture was fixed with formalin (end concentration 2 %). Colony formation was determined quantitatively using the fixed samples by counting the unicellular cells with a counting chamber and recounting the cells after boiling the samples for 6 minutes to make the colonies fall apart (Joung *et al.* 2006). From these data, the percentage of cells in unicellular form and in colony form could be calculated. We also screened the samples for colonies and measured the size (largest diameter) of a limited number of arbitrary chosen colonies for each culture to have an idea of the size range of the colonies. In addition, 5 ml of the fixed sample was filtered on black polycarbonate filters to measure cell size by fluorescence microscopy using the program CellC.

4.2.4 Statistical analyses

Correlations between the studied traits were investigated by simple and partial Mantel tests using the Zt software tool (Bonnet and Van de Peer 2002). This was done with Euclidean distance dissimilarity matrices for the four functional traits based on the mean values of the control treatment and a similarity matrix based on sequence data (% similar nucleotides in rDNA ITS sequence). To test whether there are significant differences in functional traits between strains and treatments, we carried out two-way analyses of variance (ANOVA; fixed factors: strain and treatment) followed by Post Hoc tests for growth rate, microcystin production and cell size, and the non-parametric Kruskal Wallis test for colony formation. Statistical analyses were performed by SPSS 15 (SPSS Inc. Chicago, U.S.).

4.3 Results

Figure 4.1 shows the rDNA ITS distance tree of the eight *Microcystis* ITS types, in which it is clear that strain T2 is more distantly related to the other strains and that there is no clustering per lake or toxicity.

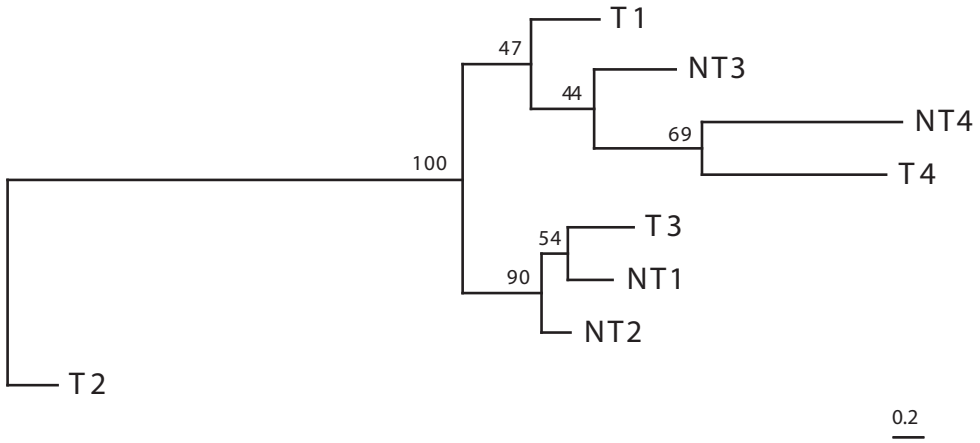


Figure 4.1: Neighbor Joining tree of the eight studied *Microcystis* rDNA ITS types (correction: Jukes and Cantor). Bootstrap values are indicated. T = toxic strains and NT = non-toxic strains, strains numbered 1 and 2 are isolated from Leeuwenhof and strains numbered 3 and 4 are isolated form Tiens Broek.

Simple Mantel tests showed no significant correlations between traits, except for cell size, which was correlated with genetic distance ($r = -0.439$; $p = 0.031$) and growth rate ($r = 0.417$; $p = 0.043$). However, partial Mantel tests showed that the correlation between cell size and growth rate was lost when controlling for the genetic distances ($r = 0.350$; $p > 0.05$). When correlations were recalculated without T2 (this strain differs more than average from the other strains), no significant correlations between the traits were found at all ($p > 0.05$).

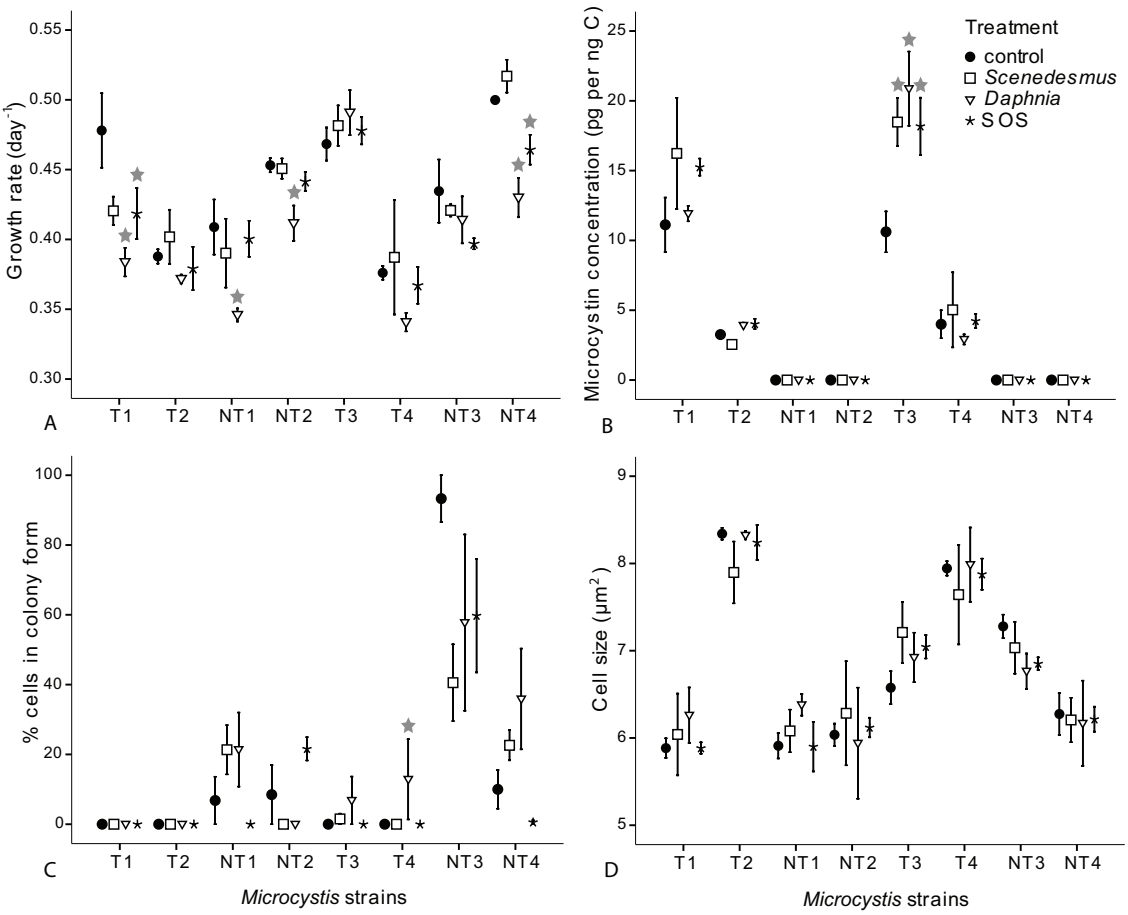


Figure 4.2: Growth rate (A), microcystin concentration (B), colony formation (C) and cell size (D) after eight days of growth of eight *Microcystis* strains in four treatments: control, *Scenedesmus* treatment, *Daphnia* treatment and sodium octyl sulphate treatment (SOS). Error bars indicate the standard error of the mean. Grey asterisks indicate treatments that differ significantly ($p < 0.05$) in the studied trait from the control for the respective strain.

Results of two-way ANOVA revealed significant differences in growth rate between strains ($F = 30.94$; $p < 0.001$) and between treatments ($F = 11.31$; $p < 0.001$), but no strain x treatment interaction effect ($F = 1.56$; $p > 0.05$). Post Hoc Tukey tests ($p < 0.05$) revealed four groups of strains: T4-T2-NT1, T2-NT1-NT3, NT3-T1-NT2 and NT4-T3 (from slow to fast growth, no significant differences in growth rate within each group). Post Hoc LSD tests showed that the *Daphnia* treatment had a significantly lower growth rate than the control for four of the eight strains ($0.001 < p < 0.01$ for T1 and NT4; $0.01 < p < 0.05$ for NT1 and NT2) and that the SOS treatment had a significantly lower growth rate than the control for two strains (T1 and NT4; $0.01 < p < 0.05$) (Fig. 4.2A). The four strains that did not produce microcystins at the start of the experiment (NT1-4) similarly did not produce microcystins in any of the treatments during the experiment (Fig. 4.2B). For each strain, the microcystin concentration after 21h and after eight days of growth was very similar, but the differences between the treatments were slightly larger at the end of

the experiment (data not shown), so we focused on the data collected after eight days. Two-way ANOVA revealed significant differences in microcystin production between the four toxic strains ($F = 65.73; p < 0.001$) and between treatments ($F = 3.39; p = 0.030$), and a marginal not significant strain x treatment interaction effect ($F = 2.06; p = 0.065$) after eight days of growth. Post Hoc Tukey tests of the strains showed that T2 and T4 were characterized by a significantly lower microcystin concentration than T1 and T3 ($p < 0.001$) and that T3 produced a significantly higher microcystin concentration than T1 ($0.01 < p < 0.05$). Post Hoc LSD tests revealed that the microcystin concentration was significantly higher in the *Scenedesmus*, *Daphnia* and SOS treatments compared to the control for T3 ($0.01 < p < 0.05$ for *Scenedesmus* and SOS; $0.001 < p < 0.01$ for *Daphnia*) (Fig. 4.2B). At the start of the experiment, all strains were composed of single cells and only a few small colonies were observed. After eight days of growth in the control treatment, only NT3 formed large colonies (size ranging from a few cells to approximately 300 µm) and four strains (T1, T2, T3 and T4) were exclusively composed of single cells. T1 and T2 also failed to produce colonies in the other treatments. Kruskal-Wallis tests for each strain separately revealed that T4 had a significantly higher percentage of cells in colonies in the *Daphnia* treatment compared to the other treatments ($\chi^2 = 10.74;\ p = 0.013$) indicating that colony formation was induced by *Daphnia* infochemicals in this strain. For NT1, T3 and NT4 a trend for increased colony formation in the presence of *Daphnia* infochemicals was observed, but the pattern was not significant ($0.05 < p < 0.1$) (Fig. 4.2C). Two-way ANOVA revealed significant differences in cell size between strains (F = 33.35; $p < 0.001$), but not between treatments ($F = 0.10;\ p > 0.05$), and no strain x treatment interaction effect ($F = 0.47;\ p > 0.05$). Post Hoc Tukey tests showed that the strains could be divided into three significantly different groups ($p < 0.05$) according to their cell size: small (T1, NT1, NT2 and NT4), medium (T3 and NT3) and large cell size (T2 and T4) (Fig. 4.2D).

4.4 Discussion

Overall, our results confirm the large variability in functional traits among *Microcystis* strains isolated from the same population (Carrillo *et al.* 2003; Bañares-España *et al.* 2006, 2007; Wilson *et al.* 2006). Apart from a correlation between the genetic distances and cell size, which was due to the presence of one highly divergent ITS type, no significant correlations in variation among the studied traits were found across strains. This is in accordance to Wilson *et al.* (2006), who did not find a correlation between growth rate and microcystin production using 32 *Microcystis* strains. They also found a similar growth rate for unicellular and colony forming strains. Alltogether, this indicates that microcystin production and colony formation are not costly in terms of growth rate.

The differences in growth rate, microcystin production, colony formation and cell size between the studied strains were generally larger than the inducible differences between the treatments for each strain. Despite this, several strains reacted to the

infochemicals by changing functional trait values. In contrast to previous studies (Yang *et al.* 2006a, Yang and Li 2007; Jang *et al.* 2007, 2008), *Daphnia* infochemicals had a specific negative influence on the growth rate of four of the eight studied strains, while sodium octyl sulphate had a similar but reduced effect on two of the strains. This growth cost may be due to investment of the strains in a defense against grazing other than colony formation and microcystin production. Possible candidates are an increase in other metabolites harmful for *Daphnia* (Czarnecki *et al.* 2006), for instance microviridin J (Rohrlack *et al.* 2004). This aspect should be investigated further. *Daphnia* culture medium filtrate induced colony formation in one strain, as reported in literature (Jang *et al.* 2003; Ha *et al.* 2004). Apparently, the infochemical involved was not sodium octyl sulphate as this substance was not effective, although the response appears to be *Daphnia*-specific because colony formation was not induced by *Scenedesmus* culture medium filtrate. In another strain, microcystin production increased in the presence of *Daphnia* culture medium filtrate, similar to Jang *et al.* (2003). However, the same effect was found for the *Scenedesmus* treatment indicating that the effect was not *Daphnia*-specific. Yet, sodium octyl sulfate also had a significant positive influence on microcystin production. Probably, more than one substance enhances microcystin production.

Our results clearly show that the response of *Microcystis* to zooplankton infochemicals is strongly strain-dependent, which may explain the often conflicting results reported by different studies. Inter-strain variability in growth rate and colony formation as reaction to zooplankton infochemicals was also reported in *Scenedesmus* (Lürling 1999; Verschoor *et al.* 2004). Moreover, although this was not the focus of our study, the influence of zooplankton infochemicals on traits of a particular *Microcystis* strain might also vary with culture conditions, as it has been shown that several environmental factors influence growth rate and microcystin production (Lukac and Aegerter 1993; Sivonen and Jones 1999; Wiedner *et al.* 2003). As seen in another experiment we conducted (chapter 5), the functional traits of the *Microcystis* strains studied in this experiment can vary under different circumstances. In addition, Lürling and van Donk (1999) showed that temperature influenced colony induction by zooplankton infochemicals in *Scenedesmus*, and Hessen and van Donk (1993) and Lürling *et al.* (1997) reported on a *Scenedesmus* strain that formed colonies upon exposure to *Daphnia* infochemicals in one experiment and not in another experiment conducted under the same conditions, suggesting an influence of growth phase or physiological state of the algae.

Environmental conditions thus add another dimension to a complicated picture and merit further investigation in *Microcystis*. However, they do not affect the main findings of our study, being (1) the large differences in functional traits between different, even sympatric, *Microcystis* strains, (2) a lack of correlation between functional traits, and (3) the fact that by *Daphnia* infochemicals induced changes in these traits are generally smaller than differences among strains. Our data therefore also indicate that, for future research on the role of particular infochemicals on functional traits of *Microcystis*, it will be important to take inter-strain variability into account.

Acknowledgements

We thank Jeroen Van Wichelen and Renaat Dasseville for sampling lake Leeuwenhof, Victor Chepurnov for isolating the *Microcystis* strains and Caroline Souffreau for helping with the sampling during the experiment. This research was financially supported by ESF EURODIVERSITY project BIOPOOL (nationally supported by BELSPO - Belgian Science Policy and FWO - Fonds Wetenschappelijk Onderzoek Vlaanderen) and Belgian project B-BLOOMS2 (nationally supported by BELSPO).

References

Bañares-España, E., Lopez-Rodas, V., Salgado, C., Costas, E. and Flores-Moya, A. 2006. Inter-strain variability in the photosynthetic use of inorganic carbon, exemplified by the pH compensation point, in the cyanobacterium *Microcystis aeruginosa*. Aquatic Botany 85: 159-162.

Bañares-España, E., Lopez-Rodas, V., Costas, E., Salgado, C. and Flores-Moya, A. 2007. Genetic variability associated with photosynthetic pigment concentration, and photochemical and nonphotochemical quenching, in strains of the cyanobacterium *Microcystis aeruginosa*. FEMS Microbiology Ecology 60: 449-455.

Bonnet, E. and Van de Peer, Y. 2002. Zt: a software tool for simple and partial Mantel tests. Journal of Statistical software 7: 1-12.

Carrillo, E., Ferrero, L. M., Alonso-Andicoberry, C., Basanta, A., Martin, A., Lopez-Rodas, V. and Costas, E. 2003. Interstrain variability in toxin production in populations of the cyanobacterium *Microcystis aeruginosa* from water-supply reservoirs of Andalusia and lagoons of Doñana National Park (southern Spain). Phycologia 42: 269-274.

Chorus, I. and Bartram, J. WHO. 1999. Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. E and FN Spon. London, United Kingdom.

Christoffersen, K., Riemann, B., Klysner, A. and Sondergaard, M. 1993. Potential role of fish predation and natural populations of zooplankton in structuring a plankton community in eutrophic lake water. Limnology and Oceanography 38: 561-573.

Czarnecki, O., Henning, M., Lippert, I. and Welker, M. 2006. Identification of peptide metabolites of *Microcystis* (Cyanobacteria) that inhibit trypsin-like activity in planktonic herbivorous *Daphnia* (Cladocera). Environmental Microbiology 8: 77-87.

Ghadouani, A., Pinel-Alloul, B. and Prepas, E. E. 2003. Effects of experimentally induced cyanobacterial blooms on crustacean zooplankton communities. Freshwater Biology 48: 363-381.

Guillard, R. R. and Lorenzen, C. J. 1972. Yellow-green algae with chlorophyllide c. Journal of Phycology 8: 10-14.

Ha, K., Jang, M. H. and Takamura, N. 2004. Colony formation in planktonic algae induced by zooplankton culture media filtrate. Journal of Freshwater Ecology 19: 9-16.

Hansson, L. A. 1996. Behavioural response in plants: Adjustment in algal recruitment induced by herbivores. Proceedings of the Royal Society of London Series B-Biological Sciences 263: 1241-1244.

Hansson, L. A. 2000. Synergistic effects of food chain dynamics and induced behavioral responses in aquatic ecosystems. Ecology 81: 842-851.

Hessen, D. O. and van Donk, E. 1993. Morphological changes in *Scenedesmus* induced by substances released from *Daphnia*. Archiv für Hydrobiologie 127: 129-140.

Hisbergues, M., Christiansen, G., Rouhiainen, L., Sivonen, K. and Borner, T. 2003. PCR-based identification of microcystin-producing genotypes of different cyanobacterial genera. Archives of Microbiology 180: 402-410.

Huisman, J., Matthijs, H. C. P. and Visser, P. M. 2005. Harmful cyanobacteria. Springer. Dordrecht, The Netherlands. 243 pp.

Jang, M. H., Ha, K., Joo, G. J. and Takamura, N. 2003. Toxin production of cyanobacteria is increased by exposure to zooplankton. Freshwater Biology 48: 1540-1550.

Jang, M. H., Jung, J. M. and Takamura, N. 2007. Changes in microcystin production in cyanobacteria exposed to zooplankton at different population densities and infochemical concentrations. Limnology and Oceanography 52: 1454-1466.

Jang, M. H., Ha, K. and Takamura, N. 2008. Microcystin production by *Microcystis aeruginosa* exposed to different stages of herbivorous zooplankton. Toxicon 51: 882-889.

Janse, I., Kardinaal, W. E. A., Meima, M., Fastner, J., Visser, P. M. and Zwart, G. 2004. Toxic and nontoxic *Microcystis* colonies in natural populations can be differentiated on the basis of rRNA gene internal transcribed spacer diversity. Applied and Environmental Microbiology 70: 3979-3987.

Joung, S. H., Kim, C. J., Ahn, C. Y., Jang, K. Y., Boo, S. M. and Oh, H. M. 2006. Simple method for a cell count of the colonial cyanobacterium, *Microcystis sp.* Journal of Microbiology 44: 562-565.

Kardinaal, W. E. A., Janse, I., Kamst-Van Agterveld, M., Meima, M., Snoek, J., Mur, L. R., Huisman, J., Zwart, G. and Visser, P. M. 2007. *Microcystis* genotype succession in relation to microcystin concentrations in freshwater lakes. Aquatic Microbial Ecology 48: 1-12.

Lukac, M. and Aegerter, R. 1993. Influence of trace-metals on growth and toxin production of *Microcystis aeruginosa*. Toxicon 31: 293-305.

Luo, W., Pflugmacher, S., Proschold, T., Walz, N. and Krienitz, L. 2006. Genotype versus phenotype variability in *Chlorella* and *Micractinium* (Chlorophyta, Trebouxiophyceae). Protist 157: 315-333.

Lürling, M., De Lange, H. J. and van Donk, E. 1997. Changes in food quality of the green alga *Scenedesmus* induced by *Daphnia* infochemicals: biochemical composition and morphology. Freshwater Biology 38: 619-628.

Lürling, M. and van Donk, E. 1997. Morphological changes in *Scenedesmus* induced by infochemicals released in situ from zooplankton grazers. Limnology and Oceanography 42: 783-788.

Lürling, M. 1999. Grazer-induced coenobial formation in clonal cultures of *Scenedesmus obliquus* (Chlorococcales, Chlorophyceae). Journal of Phycology 35: 19-23.

Lürling, M. and van Donk, E. 1999. Grazer-induced colony formation in *Scenedesmus* acutus (Chlorophyceae): Ecomorph expression at different temperatures. Journal of Phycology 35: 1120-1126.

Menden-Deuer, S. and Lessard, E. J. 2000. Carbon to volume relationships for dinoflagellates, diatoms, and other protist plankton. Limnology and Oceanography 45: 569-579.

Otsuka, S., Suda, S., Shibata, S., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 2001. A proposal for the unification of five species of the cyanobacterial genus *Microcystis* Kutzing ex Lemmermann 1907 under the Rules of the Bacteriological Code. International Journal of Systematic and Evolutionary Microbiology 51: 873-879.

Rohrlack, T., Christoffersen, K., Kaebernick, M. and Neilan, B. A. 2004. Cyanobacterial protease inhibitor microviridin J causes a lethal molting disruption in *Daphnia pulicaria*. Applied and Environmental Microbiology 70: 5047-5050.

Sarnelle, O. 2007. Initial conditions mediate the interaction between *Daphnia* and bloom-forming cyanobacteria. Limnology and Oceanography 52: 2120-2127.

Selinummi, J., Seppala, J., Yli-Harja, O. and Puhakka, J. A. 2005. Software for quantification of labeled bacteria from digital microscope images by automated image analysis. Biotechniques 39: 859-863.

Sivonen, K. and Jones, G. 1999. Cyanobacterial toxins. In: Chorus, I. and Bartram, J. (eds). Toxic cyanobacteria in water - a guide to their public health consequences, monitoring and management. E and FN Spon. London, England. pp. 41-111.

Vaitomaa, J., Rantala, A., Halinen, K., Rouhiainen, L., Tallberg, P., Mokelke, L. and Sivonen, K. 2003. Quantitative real-time PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. Applied and Environmental Microbiology 69: 7289-7297.

van der Oost, R. 2007. Cyanotoxine monitoring: standaardisering en validatie van methoden voor de Nederlandse Waterkwaliteitsbeheerders. Rapport STOWA onderzoek 2005 and 2006. Waternet / Waterproef. Amsterdam.

van Donk, E., Lürling, M. and Lampert, W. 1999. Consumer-induced changes in phytoplankton: inducibility, costs, benefits, and the impact on grazers. In: Tollrian R., Harvell C.D. (eds). The ecology and evolution of inducible defenses. Princeton University Press, Princeton, New Jersey.

van Donk, E. 2007. Chemical information transfer in freshwater plankton. Ecological Informatics 2: 112-120.

Verschoor, A. M., Van Der Stap, I., Helmsing, N. R., Lürling, M. and van Donk, E. 2004. Inducible colony formation within the *Scenedesmaceae*: Adaptive responses to infochemicals from two different herbivore taxa. Journal of Phycology 40: 808-814.

Visser, P. M., Ibelings, B. W., Mur, L. R. and Walsby, A. E. 2005. The ecophysiology of the harmful cyanobacterium *Microcystis*. Features explaining its success and measures for its control. In: Huisman, J., Matthijs, H. C. P. and Visser, P. M. (eds). Harmful cyanobacteria. Springer. Dordrecht, The Netherlands. pp. 109-142.

Wiedner, C., Visser, P. M., Fastner, J., Metcalf, J. S., Codd, G. A. and Mur, L. R. 2003. Effects of light on the microcystin content of *Microcystis* strain PCC 7806. Applied and Environmental Microbiology 69: 1475-1481.

Wilson, A. E., Sarnelle, O., Neilan, B. A., Salmon, T. P., Gehringer, M. M. and Hay, M. E. 2005. Genetic variation of the bloom-forming cyanobacterium *Microcystis aeruginosa* within and among lakes: Implications for harmful algal blooms. Applied and Environmental Microbiology 71: 6126-6133.

Wilson, A. E., Wilson, W. A. and Hay, M. E. 2006. Intraspecific variation in growth and morphology of the bloom-forming cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 72: 7386-7389.

Yang, Z., Kong, F. X., Shi, X. L. and Cao, H. S. 2006a. Differences in response to rotifer *Brachionus urceus* culture media filtrate between *Scenedesmus obliquus* and *Microcystis aeruginosa*. Journal of Freshwater Ecology 21: 209-214.

Yang, Z., Kong, F. X., Shi, X. L. and Cao, H. S. 2006b. Morphological response of *Microcystis aeruginosa* to grazing by different sorts of zooplankton. Hydrobiologia 563: 225-230.

Yang, Z. and Li, J. J. 2007. Effects of *Daphnia*-associated infochemicals on the morphology and growth of *Scenedesmus obliquus* and *Microcystis aeruginosa*. Journal of Freshwater Ecology 22: 249-253.

Yasumoto, K., Nishigami, A., Yasumoto, M., Kasai, F., Okada, Y., Kusumi, T. and Ooi, T. 2005. Aliphatic sulfates released from *Daphnia* induce morphological defense of phytoplankton: isolation and synthesis of kairomones. Tetrahedron Letters 46: 4765-4767.

Chapter 5

Genotype dependent interactions among ecologically different *Microcystis* strains mediated by *Daphnia* grazing

Ineke van Gremberghe[1], Pieter Vanormelingen[1], Bart Vanelslander[1], Katleen Van der Gucht[1], Sofie D'hondt[1], Luc De Meester[2] and Wim Vyverman[1]

Accepted in Oikos pending revision

Contribution to this chapter by IVG: experimental set-up, sample analyses, statistical analyses and writing

[1]Laboratory of Protistology and Aquatic Ecology, Ghent University, Krijgslaan 281 - S8, 9000 Ghent, Belgium

[2]Laboratory of Aquatic Ecology and Evolutionary Biology, Katholieke Universiteit Leuven, Charles Debériotstraat 32, 3000 Leuven, Belgium

Abstract

Natural populations of the bloom forming cyanobacterium *Microcystis* are typically composed of several distinct genotypes. Using *Microcystis* strains that differ in growth rate, microcystin production and colony formation, we conducted a laboratory experiment in the presence and absence of a grazer, the water flea *Daphnia*, to investigate whether interactions among strains can be predicted from functional traits, and whether the outcome of competition between strains is influenced by a grazer. Two toxic and two non-toxic *Microcystis* strains, isolated from a single lake, were grown during four weeks as single strains, in all possible combinations of two strains and all together, in the presence and absence of *Daphnia magna*. The relative abundance of strains in the populations was assessed using Denaturing Gradient Gel Electrophoresis, and the growth rate of each strain in mixed populations was compared to its growth rate in monoculture to determine interactions between strains. The observed interactions were strain-specific, and the relative abundances of strains in mixed populations could be partially explained by taking toxicity and colony formation into account. Importantly, some of the interactions were strongly altered by the presence of *Daphnia*. *Daphnia* induced colony formation in one strain, which then became a better competitor. *Daphnia* grazing also caused a higher evenness in the populations, both through a weakening of competitive interactions as well as by facilitation effects. Strong facilitation effects were due to non-toxic strains benefiting from the protection offered by toxic strains in the presence of predation.

Overall, our results emphasize the presence of strong competitive interactions between *Microcystis* genotypes in the absence of grazing, whereas indirect positive interactions are prevalent in the presence of a generalist grazer. Our results suggest that differences in functional traits and grazer-mediated facilitation effects may enhance coexistence of *Microcystis* strains, including toxic and non-toxic strains.

5.1 Introduction

Ecological communities are characterized by complex interactions between species. Interactions among members of the same guild can be negative (resource competition and allelopathy) or positive (facilitation and niche complementarity) and depend on particular physiological traits of the species (Callaway and Walker 1997; Suikkanen *et al.* 2004; Agawin *et al.* 2007). Additionally, the strength and sign of the interactions depend upon the ecological context, as the presence of predators or competitors can alter a species' behaviour or morphology and this can influence interspecific interactions (i.e. trait-mediated interactions) (Abrams 1995; Relyea and Yurewicz 2002). In studies on plant community structure, negative effects are mainly caused by resource competition, whereas positive effects can be directly caused by habitat amelioration or indirectly by predator-mediated facilitation. Increased environmental severity (abiotic stress or herbivory) tends to increase the importance of positive interactions relative to negative interactions (Bertness and Callaway 1994; Callaway and Walker 1997; Brooker *et al.* 2008). For instance, Callaway *et al.* (2005) and Graff *et al.* (2007) showed that palatable plants could be protected against herbivory when growing near unpalatable plants. While in the absence of herbivores the two plant species showed strong competitive interactions, the unpalatable plant species had a strong indirect positive effect on the palatable plant species in the presence of herbivores by acting as a grazer deterrent. While these sometimes complex interactions are well known in plant communities (e.g. Hambäck *et al.* 2000; Rousset and Lepart 2000; Milchunas and Noy-Meir 2002; Baraza *et al.* 2006), much less is known about interactions in communities of micro-organisms, especially in combination with predation (Hulot *et al.* 2001; Fox 2004; Jiang 2007).

Bacteria are genetically and biochemically very diverse and complex interactions may occur among strains within morphospecies. This is indicated by recent research on the cyanobacterium *Microcystis*, a cosmopolitan micro-organism that forms toxic blooms under warm weather conditions in eutrophic water bodies (Chorus and Bartram 1999; Visser *et al.* 2005). Naturally occurring *Microcystis* populations are typically composed of several genotypes with different ecophysiological traits (Wilson *et al.* 2005, 2006; Bañares-España *et al.* 2006; Kardinaal *et al.* 2007a), but little is known about the mechanisms through which this diversity is maintained. The toxicity of a *Microcystis* bloom is mainly determined by its strain composition, as not all strains possess the microcystin synthetase genes (Janse *et al.* 2004; Kardinaal *et al.* 2007a). Therefore, it is expected that the structure of *Microcystis* populations and the interactions between strains can have a strong impact on other components of the food web (Hansson *et al.* 2007). Ecologically relevant traits like growth rate, microcystin production and colony formation may be important for the sign and strength of the interactions among strains as well as with other species. For instance, non-toxic strains may be better competitors for light than toxic strains, as suggested by Kardinaal *et al.* (2007b). The ability to form colonies entails advantages such as protection against predation (Fulton and Paerl 1987), the ability to float and shade unicellular strains, a higher photosynthetic efficiency, and enhanced stress tolerance compared to unicellular

strains (Zhang *et al.* 2007; Wu and Song 2008). Previous studies have shown that microcystin production and colony formation appear to have no direct growth rate costs (Wilson *et al.* 2006) and can both be influenced by zooplankton (Jang *et al.* 2003; Ha *et al.* 2004).

In this study, we investigated interactions between four naturally co-occurring *Microcystis* strains that differ in functional traits (growth rate, microcystin production and colony formation) in experimental populations with and without a key grazer, the large-bodied cladoceran *Daphnia magna*. We tested the hypothesis that interactions are strain-specific and influenced by functional traits such as toxin production and colony formation. In addition, we hypothesized that the presence of a grazer changes among-strain interactions substantially. More specifically, we predicted less strong negative interactions among strains, and a facilitation effect of toxic strains on non-toxic strains in the presence of *Daphnia*. If interactions are strain-dependent and involve both competition and facilitation as predicted, then this provides a strong mechanism for the maintenance of strain polymorphism in natural populations of *Microcystis*, including the coexistence of toxic and non-toxic strains.

5.2 Materials and methods

5.2.1 Isolation and genotypic characterization of *Microcystis* strains

Lake Leeuwenhof (a lake of 7 ha, part of a nature reserve in Ghent, Belgium, 51°04'01" N, 3°39'23" E) was sampled on the 6th of September 2004 when a very dense *Microcystis* bloom was present. 25 monoclonal *Microcystis* cultures were obtained by picking up single colonies with a micropipette and growing them in WC medium (Guillard and Lorenzen 1972, but without pH adjustment) at a temperature of 18 °C and a light intensity of 30 µmol photons $\mathrm{m}^{-2}\ \mathrm{s}^{-1}$ (Light Dark cycle: 12h:12h). As the taxonomy of *Microcystis* is still largely based on morphology and does not correspond to genetic features (Otsuka *et al.* 2001), it is preferable to use genetic markers such as the 16S-23S rDNA ITS sequence to distinguish different *Microcystis* genotypes from each other irrespective of morphospecies identification (Janse *et al.* 2004). We determined the 16S-23S rDNA ITS sequence of each strain. Four ITS genotypes could be distinguished and one strain of each ITS type was used in the experiment. Two ITS genotypes were identified as microcystin producing genotypes (toxic genotypes, named T1 and T2) based on the presence of *mcy* genes A (Hisbergues *et al.* 2003) and E (Vaitomaa *et al.* 2003) and through direct measurement of microcystin concentrations by ELISA (Enzyme-Linked Immunosorbent Assay) with SDI - EnviroGard Microcystin ELISA plate kits (see below for more details). Two strains were identified to be non-microcystin producing genotypes (non-toxic genotypes, named NT1 and NT2).

5.2.2 Experimental design

Before the start of the experiment, the four *Microcystis* strains were grown in WC medium at a temperature of 23 °C and a light intensity of 120 µmol photons m^{-2} s^{-1} (Light Dark cycle: 12h:12h). Culture conditions were the same throughout the study. Exponentially growing cells were used as inocula for the experiment. The four *Microcystis* strains were grown in triplicate in monoculture, in all possible combinations of two strains, and in a four strain mixed culture in 200 ml Erlenmeyer flasks during four weeks. All population treatments were grown with and without addition of four individuals of the large-bodied zooplankton species *Daphnia magna*. *Daphnia* is a key grazer in standing waters. Its effect on *Microcystis* biomass is debated, as *Microcystis* may strongly suppress growth of *Daphnia* (Ghadouani *et al.* 2003), while other studies have provided evidence that *Daphnia* may suppress developing *Microcystis* blooms depending on initial conditions and history (Christoffersen *et al.* 1993; Sarnelle 2007). Our design resulted in a total of 11 populations x 2 grazing treatments x 3 replicate observations = 66 experimental units. All cultures were shaken by hand and their position randomized daily. Cultures without *Daphnia magna* ('no predation', 'NP') were inoculated at a total density of 50 000 cells per ml, whereas the cultures with *Daphnia magna* ('predation', 'P') were inoculated at a total density of 500 000 cells per ml. We used two different inoculation densities in the two predation treatments because a pilot experiment indicated that this was the best compromise to obtain comparable densities towards the end of the experiment (3-4 weeks), when most samples were taken. This reflects the strong impact of *Daphnia* grazing on these cultures, resulting in a collapse of *Microcystis* populations unless inoculated at relatively high densities. Within each mixed population, all strains had the same initial relative abundance. The *Daphnia magna* individuals were obtained from a multiclonal population grown in the laboratory and fed *Scenedesmus obliquus*. Before adding *Daphnia* to the *Microcystis* cultures, they were starved for at least three hours. During the experiment, the *Daphnia magna* individuals were systematically replaced by new individuals every 1-3 days in order to keep densities the same across cultures. *Daphnia* survival was low in some of the cultures, especially from the third week of the experiment onwards. During the third week, *Daphnia* survival was monitored in all cultures as the number of individuals alive 24 hours after inoculation of four fresh *Daphnia* individuals.

To determine *Microcystis* cell density and microcystin concentrations, a weekly subsample (1 ml) was taken from each culture. After optical density measurement at 750 nm with a spectrophotometer (Shimadzu UV-1601), the sample was stored at -20°C until measurement of microcystin concentrations (see below). Once a week, colony formation was checked microscopically, and pictures of the cells and colonies were taken at the start of the experiment, after two and after four weeks. For single cell and colony counting, 6 ml of each culture was fixed at the end of the experiment with formalin (end concentration 2 %) and stored at 4°C. Samples for DNA extraction (2 ml) were taken after two, three and four weeks and stored at -20°C.

5.2.3 Sample analyses

To test to what extent a particular relative amount of cells corresponds to an equal relative band intensity as determined by Denaturing Gradient Gel Electrophoresis (DGGE), a pilot experiment was carried out in which, for all possible two-strain combinations, the two strains were mixed in a gradient of relative cell densities (10:1, 5:1, 2:1, 1:1, 1:2, 1:5 and 1:10). The relative abundance of each strain in the mixtures was determined by DGGE using the protocol of Janse *et al.* (2003, 2004). DNA from the samples was extracted as described by Zwart *et al.* (1998), which includes bead beating with phenol extraction and ethanol precipitation. The rDNA ITS sequence was amplified using the primers (GC)-CSIF and ULR, and a denaturing gradient of 34-40 % denaturant was used. Digitalized DGGE images were analysed using the software package BioNumerics 4.5 (Applied Maths BVBA). A matrix was compiled based on band intensities, and absolute values were converted into relative values. The results clearly showed that DGGE worked well to determine the relative abundance of *Microcystis* ITS genotypes (average R^2 of the correlations between the known relative abundance and relative abundance determined by DGGE in the mixtures is 0.96, data not shown), which is in agreement with the observations of Kardinaal *et al.* (2007b). We used the same method as described above to study the relative abundance of the strains in the mixed populations during the experiment. The population structure after two and three weeks of growth was comparable with the population structure at the end of the experiment, although evenness gradually declined in all mixed populations. We only present the data after four weeks of growth. The relative abundances of the strains in the populations at the end of the experiment were used to determine which strains are superior and which strains inferior competitors in particular mixtures. *Microcystis* cell density of the monocultures was estimated by converting the optical density into cell density (cells ml^{-1}), which was determined by counting cells by the program CellC (chapter 4), using two standard curves made in a pilot experiment. One curve was fitted for strains T1, NT1 and NT2 ($R^2 = 0.996$), and one for strain T2 ($R^2 = 0.998$) as this strain is characterized by slightly larger cells than the other ones. *Microcystis* cell density of each strain in the mixtures was estimated by converting the optical density into cell density using the standard curves as described above and combining this with the relative abundance of each strain as determined by DGGE. Pielou's evenness (Pielou 1966) was calculated using Primer 5 (Clarke and Gorley 2001) based on cell densities of the strains in the mixtures at the end of the experiment. Following Loreau (1998), we defined interactions between strains to be present when the growth rate of a particular strain in a mixture departs from the growth rate in monoculture, implying that the presence of other strain(s) influences the growth rate of that particular strain positively or negatively. Therefore, we determined the growth rate of each strain in monoculture and in the mixtures using the formula [Ln(cells ml^{-1})t2 - Ln(cells ml^{-1})t1]/(t2-t1) with t1 = start of the experiment and t2 = after four weeks of growth. For the cultures with *Daphnia* only the nett growth rate was actually known (= cell growth rate minus grazing rate). The total microcystin concentration (μg ml^{-1}) in the samples was determined by ELISA. For the extraction of microcystins, the cells were boiled for 20 minutes (van der Oost 2007). Next, the cultures were centrifuged for 10 minutes at 14

000 rpm and the supernatant was used for ELISA. The ELISA-test was done according to the manufacturer's instructions (SDI - EnviroGard Microcystin ELISA plate kit). To calculate the microcystin concentration per biomass, the cell density was converted into biomass (pg C μl^{-1}) using the formula pg C cell^{-1} = 0.216*volume cell$^{0.939}$ (Menden-Deuer and Lessard 2000). Cell biovolumes were estimated by measuring the average cell diameter of each strain.

Colony formation in the monocultures was determined quantitatively using the fixed samples by counting the cells in unicellular form with a counting chamber and recounting the cells after boiling the samples for 6 minutes to make the colonies fall apart (Joung *et al.* 2006). This way, the percentage of cells as single cells and colonies could be calculated. In addition, we screened the samples for colonies and measured the size (largest diameter) of a limited number of arbitrary chosen colonies for each culture to have an idea of the size range of the colonies rather than to perform a detailed quantitative analysis of colony size.

5.2.4 Statistical analyses

As our design involved a difference in inoculation densities among predation treatments, a direct comparison of total cell densities and growth rates among predator treatments cannot be interpreted as a biological effect. Within predator treatments, however, total cell densities and growth rates can be compared among monocultures and mixtures. Moreover, we carried out two-way ANOVA (fixed factors: population and predation) on total densities in the cultures as a methodological test to determine whether final densities (fourth week) in the predation treatments differed. As there was no main effect of predation treatment on total final cell densities ($F = 0.449$; $p = 0.506$), we conclude that we can meaningfully compare predation treatments for variables that were measured during the fourth week of our experiment.

One-way ANOVA (fixed factor: strain) was used to test for significant differences in monoculture growth rate between the four strains for each predation treatment separately. Post Hoc Tukey tests were performed to identify which strains differed significantly in growth rate in monoculture. Two-way ANOVA (fixed factors: strain and predation) was used to test for significant differences in microcystin production between the two toxic strains and between predation treatments after four weeks of growth. To know whether the presence of a non-toxic strain influenced microcystin production of a toxic strain, two-way ANOVA (fixed factors: presence non-toxic strain and predation) was also performed on the microcystin concentrations of T1 en T2 separately in monoculture and with NT1 and NT2 after four weeks of growth. One-way ANOVA (fixed factor: predation) was used to test for an effect of predation on colony formation (percentage cells in colony form) in T2 after four weeks of growth.

Concerning the mixed populations, one-way ANOVA (fixed factor: population) for each predation treatment separately was used to determine significant differences between total cell densities after four weeks of growth. One-tailed t-tests with Bonferroni correction were used to determine whether the total cell density of the different mixtures after four weeks of growth was significantly higher than the total

cell density of the respective monoculture with the highest yield (referred to as 'overyielding', Loreau 1998). Two-way ANOVA (fixed factors: mixed population and predation) and Post Hoc LSD tests were performed to test for significant differences in evenness in the mixed populations between the predation treatments after four weeks of growth. Only the results of Post Hoc tests were shown. One-way ANOVA (fixed factor: competing strain) and Post Hoc LSD tests were performed for each strain separately to test for significant differences between the monoculture growth rate and the growth rate in each of the mixed populations for each predation treatment separately. Only the results of Post Hoc tests were shown.

All statistical analyses were performed in SPSS 15 (SPSS Inc. Chicago, U.S.).

5.3 Results

5.3.1 Strain characteristics in the absence and presence of *Daphnia* grazing

A summary of the traits of the four strains grown in monoculture in the absence and presence of predation is given in table 5.1. The growth rate differed significantly between the four strains (Table 5.1 and 5.2) in both predation treatments. In the absence of predation, T2 had a lower growth rate than the other three strains, and T1 and NT1 grew slower than NT2. In the presence of predation, NT1 had a lower growth rate (around zero) than the other three strains (Fig. 5.1), and T2 and NT2 grew slower than T1 (Table 5.1 and 5.2).

Table 5.1: Summary of the mean value and standard error (between parentheses) of growth rate, microcystin production and colony formation of monocultures of the four *Microcystis* strains in the absence (NP) and presence (P) of predation by *Daphnia*. Growth rate was measured over a period of four weeks, whereas the reported values of microcystin concentration and colony formation were quantified after four weeks of growth.

	T1		T2		NT1		NT2	
	NP	P	NP	P	NP	P	NP	P
Growth rate (day^{-1})	0.212 (± 0.001)	0.143 (± 0.002)	0.187 (± 0.003)	0.108 (± 0.006)	0.213 (± 0.003)	0.000 (± 0.001)	0.227 (± 0.002)	0.105 (± 0.017)
Microcystin concentration (pg per ng C)	17.9 (± 4.5)	14.0 (± 2.3)	2.8 (± 0.7)	3.9 (± 0.8)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)
Colony formation (% cells in colony)	0 (± 0)	0 (± 0)	37.5 (± 10.9)	39.7 (± 7.6)	0 (± 0)	0 (± 0)	0 (± 0)	90 (± 9.9)

The different *Microcystis* populations differentially affected mortality in the grazing *Daphnia*. When grazing on non-toxic strains, the *Daphnia* individuals were in good condition and were found to reproduce asexually. When grazing on toxic strains (or toxic strains together with non-toxic strains), the *Daphnia* showed high mortality. In the last week of the experiment they often died within 24 hrs, i.e. before they were replaced, and the *Microcystis* strains could reach high densities. Figure 5.2 shows that *Daphnia* exhibited high survival rates when grazing on only non-toxic strains, low

survival rates in cultures containing T2 but without T1, and very high mortality in cultures containing T1. The overall mean of *Daphnia* individuals alive after 24 hrs was 3.22, 1.56 and 0.27, respectively.

Table 5.2: ANOVA table on the differences in growth rate of monocultures of the four *Microcystis* strains in the absence (NP) and presence of *Daphnia* (P). Results of Tukey Post Hoc tests are given (p-values) for all strain combinations (significant p-values are in bold).

Effect	df	MS	F	p	Post Hoc	NP	P
strain (NP)	3	0.001	44.45	$<$ **0.001**	T1/T2	**0.001**	**0.003**
strain (P)	3	0.012	187.77	$<$ **0.001**	T1/NT1	0.972	$<$ **0.001**
					T1/NT2	**0.009**	**0.039**
					T2/NT1	$<$ **0.001**	$<$ **0.001**
					T2/NT2	$<$ **0.001**	0.274
					NT1/NT2	**0.017**	$<$ **0.001**

As expected, no microcystin was detected in the populations with only non-toxic strains. There was no effect of grazing on microcystin production of these two strains, but T1 had a significantly higher microcystin production than T2 after four weeks of growth (Table 5.1 and 5.3). Microcystin concentrations of T1 and T2 decreased towards the end of the experiment, especially for T1 (size range of mean microcystin concentrations: for T1, NP = 25.7 - 17.9, P = 27.3 - 14.0 and for T2, NP = 5.4 - 2.8, P = 7.1 - 3.9 pg per ng C). There were no significant differences in microcystin production for T1 and T2 between monocultures and in presence of NT1 or NT2 (all $p > 0.05$).

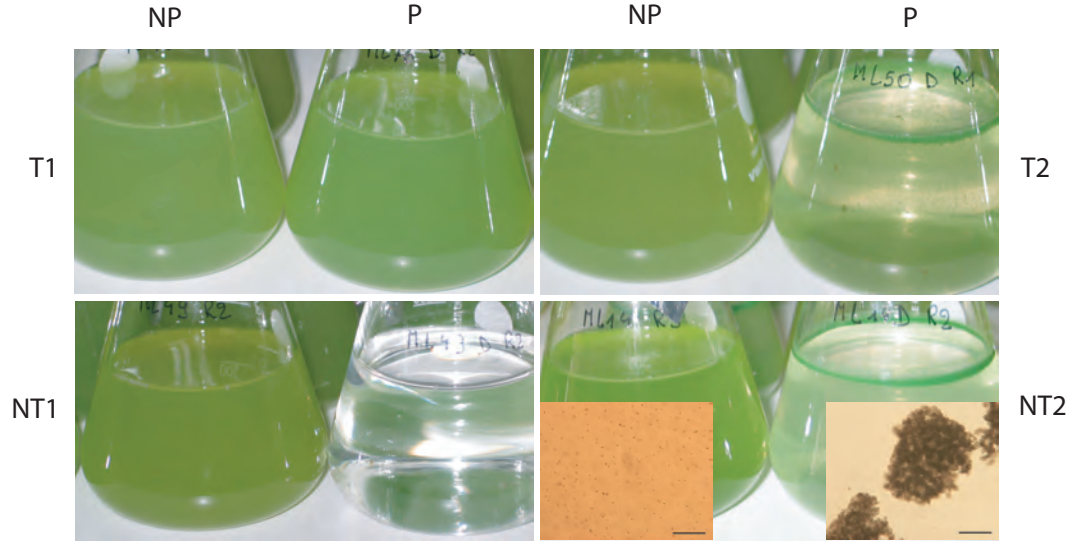


Figure 5.1: Photographs of the four *Microcystis* strains in monoculture at the end of the experiment, and for strain NT2 microscopic photographs of the cells and colonies showing colony induction in the presence of *Daphnia magna* (scale bar = 100 µm). NP = no predation, P = predation.

No colony formation was detected in T1 and NT1 during the experiment. T2 formed large, loose colonies (size ranging from a few cells to large colonies of

approximately 1 mm diameter) in both the presence and absence of predation. There were no significant differences in the percentage of cells in colony form in the presence and absence of predation ($p > 0.05$) after four weeks of growth for this strain (Table 5.1). NT2 formed large, dense colonies after two weeks of growth in the presence of predation (size ranging from a few cells to large colonies of approximately 1 mm diameter) and kept its colony form until the end of the experiment. In the absence of predation, only single cells were seen in cultures of this strain (Fig. 5.1 and Table 5.1).

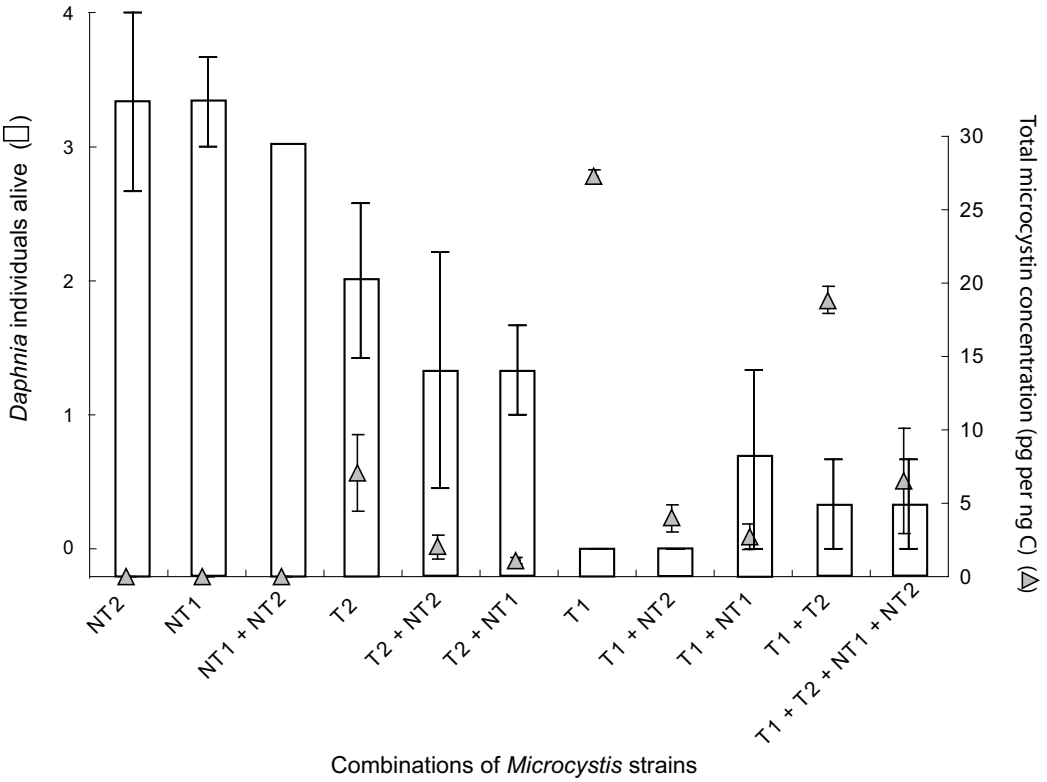


Figure 5.2: *Daphnia* individuals alive after 24hrs grazing on *Microcystis* cultures grown for three weeks and total microcystin concentration in the *Microcystis* cultures at the third week. Error bars indicate the standard error of the mean.

Table 5.3: ANOVA table on the effects of strain and predation on microcystin production of monocultures of T1 and T2 after four weeks of growth (significant p-values are in bold).

Effect	df	MS	F	p
strain	1	4.794	24.332	**0.001**
predation	1	0.056	0.283	0.609
strain*predation	1	0.180	0.912	0.367

5.3.2 Interactions between *Microcystis* strains in the absence and presence of *Daphnia* grazing

Figure 5.3 shows the total cell density of the monocultures and mixed populations as well as the relative abundances of the strains in the mixed populations after four weeks of growth. In both predation treatments, the populations differed significantly in total cell density (NP: $F = 24.91$; $p < 0.001$, P: $F = 11.61$; $p < 0.001$). In the absence of predation, none of the mixed populations had a total cell density that was significantly higher than the highest respective monoculture cell density ($p > 0.05$), indicating the absence of overyielding. However, in the presence of predation, total cell densities of the mixtures T1 + NT2 and T2 + NT1 were significantly higher than the monoculture cell densities of T1 and T2, respectively ($p < 0.05$), resulting in significant overyielding in these combinations of toxic and non-toxic strains.

In many of the mixed populations, one of the strains was clearly dominant, whereas in other mixtures, the different strains were co-dominant. Predation by *Daphnia* tended to reduce dominance of specific strains in the mixed populations, resulting in a higher evenness (Fig. 5.3). A significant higher evenness in the presence than in the absence of *Daphnia* was seen for mixtures T1 + NT2, T2 + NT2 and the four-strain mixtures ($p < 0.05$). A significant lower evenness in the presence than in the absence of *Daphnia* was seen in the mixture involving the two non-toxic strains NT1 + NT2 ($p < 0.05$), where *Daphnia* grazing induced dominance of NT2.

The growth rate of strains was often influenced by the presence of other strains. As a result, the dominance of a strain in a mixture could not be explained by differences in monoculture growth rate alone. Overall, we detected complex and strain-dependent patterns of suppression and facilitation of growth in the mixed populations (see figure 5.4 for significant results). For instance, T1 never suppressed another strain but was suppressed by all other strains in the absence of *Daphnia* (very strongly by NT2), and moderately suppressed by T2 in the presence of *Daphnia*. T2 strongly suppressed NT2 in the absence of predation. NT1 weakly suppressed T1 and NT2 in the absence of grazing and was never suppressed by another strain, except in the four-strain cultures in the absence of grazing. T1 was never facilitated by any other strain, but it facilitated all other strains in both grazed and non-grazed populations. It very strongly facilitated both non-toxic strains in the presence of predation. T2 was facilitated by all other strains, except by NT1 in the absence of grazing, and NT1 was facilitated by both toxic strains in the presence of *Daphnia* and by NT2 in the absence of *Daphnia*. Generally, strong negative interactions among strains were detected in the absence of predation, whereas strong positive interactions were especially seen in the presence of predation (Fig. 5.4).

In Figure 5.4, suppression and facilitation are quantified relative to growth rates in monocultures. For an assessment of the fitness of strains and a prediction of their occurrence in nature, absolute growth rates in the presence of other strains are also important (Fig. 5.4 and resulting population densities in Fig. 5.3). Taking both absolute growth rates as well as competition/facilitation effects into account, it is clear that strain T1 is well-adapted to grow in isolation in both the presence and absence

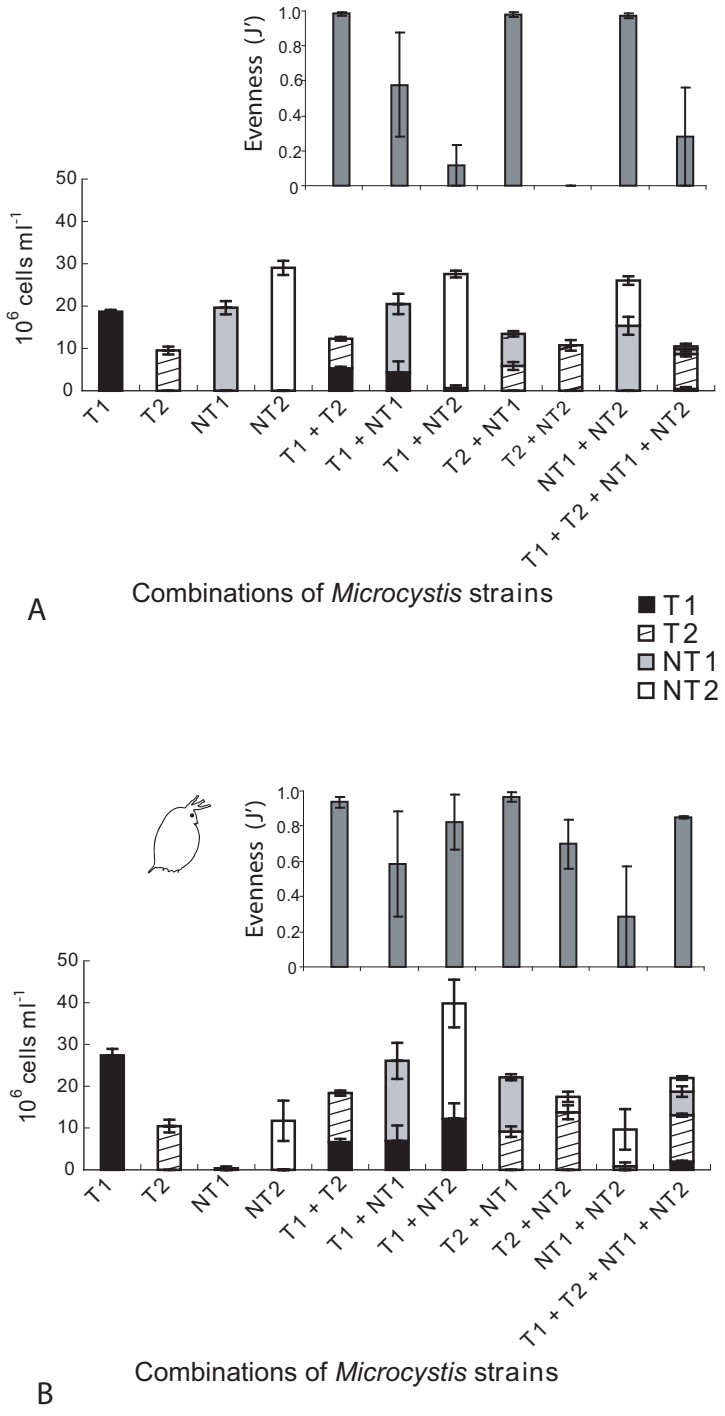


Figure 5.3: Total cell density of *Microcystis* monocultures and mixed populations of the four strains after four weeks of growth, with indication of the relative contribution of each strain in the mixed populations (lower graphs) and Pielou's evenness (J') of the mixed populations after four weeks of growth (upper graphs), A: in the absence of predation, B: in the presence of predation. Error bars indicate the standard error of the mean.

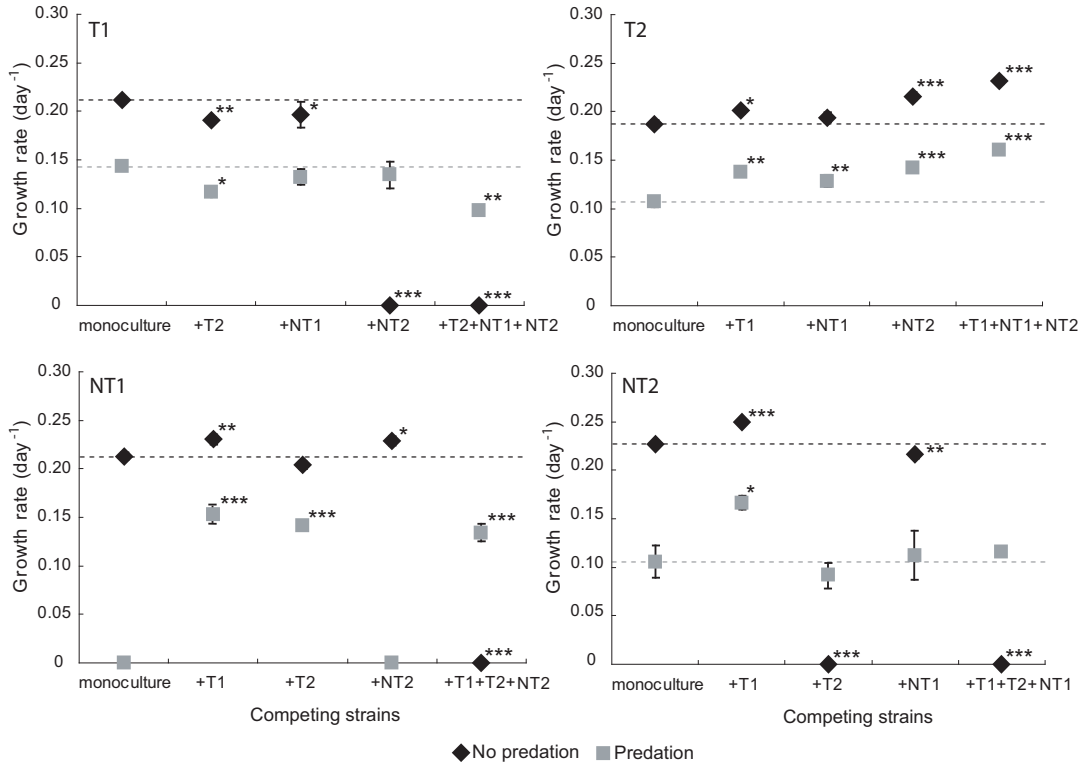


Figure 5.4: Growth rate of the four *Microcystis* strains in monoculture and in mixed populations in the absence (No predation) and presence (Predation) of predation. Hatched lines indicate the growth rate in monoculture. Zero-values for the growth rate mean that the growth rate was zero or negative. Asterisks indicate significant differences (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) between the growth rate of the strain in the mixed population and the growth rate in monoculture. Error bars indicate the standard error of the mean (non-visible error bars are smaller than symbols).

of grazers. In the absence of grazers, it did not grow faster than the non-toxic strains, but in the presence of grazers, it was by far the best growing strain. This strain seems, however, sensitive to competition, and especially to competition from strain NT2. T2, which had a lower monoculture growth rate than the other three strains in the absence of predation, was co-dominant or dominant in all mixed populations. This strain seems very well adapted to co-occur with other strains. In the four-strain cultures, this strain completely outcompeted all other strains in the absence of grazers. Both non-toxic strains performed well in the absence of grazers, but were sensitive to strain-specific interactions: NT1 was very sensitive to the combined interaction with the other three strains, whereas NT2 was very sensitive to T2. In the presence of grazers, NT1 performed very poorly when grown in isolation but was able to grow in mixed cultures with the toxic strains and then became even co-dominant or dominant. NT2 could grow in the presence of grazers, and gained additional advantage from co-occurrence with T1.

5.4 Discussion

Our experiment revealed strong negative as well as positive interactions between *Microcystis* strains in mixed cultures, and these interactions were modulated by the presence of *Daphnia magna*. Allelopathic interactions as well as resource competition may lead to negative interactions, and additional experiments are needed to distinguish between these mechanisms. In the filamentous cyanobacterium *Planktothrix*, it was recently shown that growth inhibition in mixed cultures was at least partly caused by allelopathic interactions among strains, and that these interactions are strain-specific (Oberhaus *et al.* 2008). The interactions observed in our experiment are highly strain-specific too, and several other functional traits than the ones we quantified are probably involved. Yet, many of the interactions observed are in line with expectations based on among-strain variation in toxin production and colony formation and their association with competitive strength. Strain T1 confirmed the lower competitive strength of toxic strains compared to non-toxic strains when light becomes limited (Kardinaal *et al.* 2007b) and probably grows well at low cell densities early in the growing season or under pioneer conditions. Its relatively fast growth in the presence of predation may be due to its high microcystin production, however the toxicity is mainly determined by the microcystin variants produced and these were not measured. Strain T2 exhibited a striking dominance in competition. This may be related to the capacity of T2 to form colonies, which may convey a strong advantage in competition because of shading effects of the floating colonies, thus reducing light availability for competing strains. Alternatively, strain T2 may exhibit traits that we did not quantify and that strongly interact with other strains in a strain-specific manner. The latter is not unlikely, given that T2 did not suppress strain NT1 to the same degree as it did strain NT2. Colony formation may also explain other instances of competitive dominance in our experiment, such as the dominance of T2 over T1 in both presence and absence of grazing, and the dominance of NT2 over NT1 in the presence of grazing.

We observed striking facilitation and/or niche complementarity in the mixed *Micro-*

cystis populations, as growth rate of some of the strains was stimulated by the presence of other strains. These interactions too were strain-specific. We observed a tendency for more facilitation in the presence than in the absence of predation, which is largely due to very strong facilitation of non-toxic strains by toxic strains in the presence (three cases) but not in the absence of predation (two cases of mild facilitation).

One of the key results of our experiment was indeed the strong impact of grazing by *Daphnia magna* on the interactions among *Microcystis* strains. When taking absolute growth rates into account, it appears that non-toxic strains have an equally high or higher growth rate than toxic strains in the absence of grazing, but are less buffered against grazing when growing in isolation. Additionally, colony formation conveys an advantage in the presence of grazing. Admittedly, these conclusions are based on observations on just a few strains, but are largely consistent with expectations from literature (e.g. Fulton and Paerl 1987; Kardinaal *et al.* 2007b; Zhang *et al.* 2007; Wu and Song 2008). Predators can diminish strong competitive interactions within a prey community directly by grazing more on the dominant species, or indirectly by the recycling of limiting nutrients and the secretion of growth-promoting substances (Vanni and Layne 1997; Elser and Urabe 1999; Vanni 2002). Although in our experiment the effect of *Daphnia* on the population structure of *Microcystis* can be caused by grazing and/or through the excretion of nutrients that are taken up by the *Microcystis* strains, the strain-specific effects we observed rather point to specific mechanisms and facilitation. First, *Daphnia* induced colony formation in one non-toxic strain (NT2), and this resulted in a protection against predation, which gave the strain a competitive advantage over the non-toxic unicellular strain NT1. The occurrence of colony induction in *Microcystis* by *Daphnia* has been reported before (Jang *et al.* 2003; Ha *et al.* 2004), although other studies did not observe colony induction (Hessen and van Donk 1993; Yang *et al.* 2006). Our results confirm that induction of colony formation is strain-specific as only one strain responded to the presence of *Daphnia* by colony formation. Colonies of *Microcystis* can reduce the filtering rate of cladocerans by mechanical interference with the filtering system (Fulton and Paerl 1987), and *Daphnia* may avoid ingestion of large colonies by narrowing the carapace gape (Lampert 1987). Second, grazing by *Daphnia* caused very strong indirect facilitation effects (Callaway *et al.* 2005; Graff *et al.* 2007) in some of the mixed populations. More specifically, non-toxic strains benefited from the presence of toxic strains because *Daphnia* suffered severely from the microcystins. This protection offered by toxic strains is parallel to herbivory protection as reported in plants (Callaway *et al.* 2005; Graff *et al.* 2007). Once protected, it is conceivable that the non-toxic strains are even stimulated in their growth by the nutrients that *Daphnia* excrete (Vanni 2002). Our results do not allow, however, testing this idea. The fact that *Daphnia* cannot distinguish between toxic and non-toxic strains and is poisoned by microcystins has been extensively studied (e.g. DeMott 1999; Rohrlack *et al.* 1999; 2001; Lotocka 2001). As expected, the microcystin concentration seemed to influence the survival rate of *Daphnia*. Contrary to Jang *et al.* (2003), however, we did not find an increased microcystin production in the presence of *Daphnia magna*. One may argue that we had to replace the *Daphnia* in our grazing treatment, thereby artificially increasing

the impact of grazing by zooplankton compared to the natural situation. We replaced the grazers, as we wanted to investigate what the impact of continued grazing is on mixed populations that involve both toxic and non-toxic strains of *Microcystis*. *Daphnia* is often co-occurring with *Microcystis* in nature, and *Daphnia* grazing can even control an upcoming *Microcystis* bloom (Christoffersen *et al.* 1993; Sarnelle 2007). Moreover, *Daphnia* can increase its tolerance to *Microcystis* toxins through maternal effects (Gustafsson *et al.* 2005) and microevolution (Hairston *et al.* 1999).

We did not find direct evidence for populations with a higher diversity to have a generally higher productivity as reported in several studies that have evaluated the relationship between biodiversity and ecosystem functioning (e.g. Loreau and Hector 2001; Reusch *et al.* 2005; Hooper *et al.* 2005). However, non-toxic unicellular strains cannot grow in the presence of grazers, and while toxic strains decreasing grazing pressure are needed to obtain a high total biomass of *Microcystis* in the presence of *Daphnia*, it is the combination of defended toxic strains and fast growing non-toxic strains that yields the highest total biomass under grazing. In natural circumstances, grazers are usually present, and a higher genotypic diversity in *Microcystis* populations will increase the likelihood that toxic strains and/or strains forming large colonies are present, resulting in a higher probability of *Microcystis* blooms. In a meta-analysis of the impact of grazers on periphyton biomass, Hillebrand and Cardinale (2004) also found evidence that a higher diversity of prey decreases consumer effects. We also followed the population dynamics and total density of the populations in time, as it is known that interactions and diversity effects on productivity can change over time (Fox 2004; Weis *et al.* 2007). However, we did not find considerable changes in the structure or relative total density of the different populations after two, three and four weeks of growth (data not shown). This indicates that strain interactions do not seem to be strongly influenced by the cell densities used in our experiment.

Despite the fact that our study is based on only four strains and that more research involving a broader set of genotypes and environmental conditions would be most welcome to elucidate how interactions among strains structure *Microcystis* populations in nature, our study allows some general conclusions. First, our results show that complex strain-specific interactions occur among *Microcystis* strains, and that the nature and strength of these interactions depends on the presence of grazers. Also, it is difficult to predict the responses entirely on the growth characteristics of the strains in isolation. Knowledge on toxicity and colony formation appears very useful to predict competitive dominance in mixed cultures, but does not lead to perfect predictions. Our observations suggest that strain polymorphism in populations of *Microcystis* can be maintained by strain-specific interactions that are modulated by differences in key functional traits, and by interactions with environmental variation. In the absence of grazing, polymorphism is mediated by specific interactions that partly involve trade-offs between growth characteristics in isolation and in mixtures. In the presence of grazing, polymorphism is enhanced by facilitation effects. Our results suggest that complex strain-specific interactions and predator-mediated facilitation effects may contribute to our understanding of the so-called 'paradox of plankton' (Hutchinson 1961; Huisman *et al.* 2001; Scheffer *et al.* 2003). Another notable observation is that toxic and

non-toxic strains can coexist both in the absence and presence of intense *Daphnia* grazing. From our observations on pure cultures, one would expect that *Daphnia* grazing would promote toxic strains over non-toxic strains, whereas non-toxic strains would be expected to predominate in the absence of grazing. Yet, all but two of the two-strain mixtures that involved toxic and non-toxic strains showed an appreciable fraction of the population to consist of the toxic strain in the absence of *Daphnia*, whereas all mixed populations had relatively high abundances of the non-toxic strain in the presence of *Daphnia*. Especially the latter observation is striking, and reflects strong facilitation effects of non-toxic strains being protected by the presence of toxic strains.

The development and characteristics of *Microcystis* populations should be viewed in the context of both environmental conditions and strain composition, and account for environment-strain interactions. Whether the relationship between strain composition and environmental conditions is predictable in terms of lineage sorting (Baas Becking 1934; Finlay and Clarke 1999; Finlay 2002; Van der Gucht *et al.* 2007) or impacted by colonization effects and dispersal limitation requires further study. Our results, however, indicate that lineage sorting, if prevalent, may not operate in a simple manner, but on combined traits of strains in relation to both environmental variation and strain composition of the resident population. It might be a fruitful approach to view *Microcystis* populations as communities of strains and analyze their ecology (e.g. occurrence, blooms and toxicity) from that perspective.

Acknowledgements

We thank Jeroen Van Wichelen and Renaat Dasseville for sampling lake Leeuwenhof, Victor Chepurnov for isolating the *Microcystis* strains, Caroline Souffreau for helping with the sampling during the experiment and Antoniya Mancheva for microscopical counting of cells and colonies. This research was financially supported by ESF EURODIVERSITY project BIOPOOL (nationally supported by BELSPO - Belgian Science Policy and FWO - Fonds Wetenschappelijk Onderzoek Vlaanderen) and Belgian project B-BLOOMS2 (nationally supported by BELSPO).

References

Abrams, P. A. 1995. Implications of dynamically variable traits for identifying, classifying, and measuring direct and indirect effects in ecological communities. American Naturalist 146: 112-134.

Agawin, N. S. R., Rabouille, S., Veldhuis, M. J. W., Servatius, L., Hol, S., van Overzee, H. M. J. and Huisman, J. 2007. Competition and facilitation between unicellular nitrogen-fixing cyanobacteria and non-nitrogen-fixing phytoplankton species. Limnology and Oceanography 52: 2233-2248.

Baas Becking, L. G. M. 1934. Geobiologie of Inleiding tot de Milieukunde. Van Stockum and Zoon, The Hague, The Netherlands.

Bañares-España, E., Lopez-Rodas, V., Salgado, C., Costas, E. and Flores-Moya, A. 2006. Inter-strain variability in the photosynthetic use of inorganic carbon, exemplified by the pH compensation point, in the cyanobacterium *Microcystis aeruginosa*. Aquatic Botany 85: 159-162.

Baraza, E., Zamora, R. and Hodar, J. A. 2006. Conditional outcomes in plant-herbivore interactions: neighbours matter. Oikos 113: 148-156.

Bertness, M. D. and Callaway, R. 1994. Positive interactions in communities. Trends in Ecology & Evolution 9: 191-193.

Brooker, R. W., Maestre, F. T., Callaway, R. M., Lortie, C. L., Cavieres, L. A., Kunstler, G., Liancourt, P. *et al.* 2008. Facilitation in plant communities: the past, the present, and the future. Journal of Ecology 96: 18-34.

Callaway, R. M. and Walker, L. R. 1997. Competition and facilitation: A synthetic approach to interactions in plant communities. Ecology 78: 1958-1965.

Callaway, R. M., Kikodze, D., Chiboshvili, M. and Khetsuriani, L. 2005. Unpalatable plants protect neighbors from grazing and increase plant community diversity. Ecology 86: 1856-1862.

Chorus, I. and Bartram, J. WHO. 1999. Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. E and FN Spon. London, United Kingdom.

Christoffersen, K., Riemann, B., Klysner, A. and Sondergaard, M. 1993. Potential role of fish predation and natural populations of zooplankton in structuring a plankton community in eutrophic lake water. Limnology and Oceanography 38: 561-573.

Clarke, K. R. and Gorley, R. N. 2001. PRIMER 5 for Windows. PRIMER-E Ltd. Plymouth, UK.

Demott, W. R. 1999. Foraging strategies and growth inhibition in five daphnids feeding on mixtures of a toxic cyanobacterium and a green alga. Freshwater Biology 42: 263-274.

Elser, J. J. and Urabe, J. 1999. The stoichiometry of consumer-driven nutrient recycling: Theory, observations, and consequences. Ecology 80: 735-751.

Finlay, B. J. and Clarke, K. J. 1999. Ubiquitous dispersal of microbial species. Nature 400: 828-828.

Finlay, B. J. 2002. Global dispersal of free-living microbial eukaryote species. Science 296: 1061-1063.

Fox, J. W. 2004. Effects of algal and herbivore diversity on the partitioning of biomass within and among trophic levels. Ecology 85: 549-559.

Fulton, R. S. and Paerl, H. W. 1987. Effects of colonial morphology on zooplankton utilization of algal resources during blue-green-algal (*Microcystis aeruginosa*) blooms. Limnology and Oceanography 32: 634-644.

Ghadouani, A., Pinel-Alloul, B. and Prepas, E. E. 2003. Effects of experimentally induced cyanobacterial blooms on crustacean zooplankton communities. Freshwater Biology 48: 363-381.

Graff, P., Aguiar, M. R. and Chaneton, E. J. 2007. Shifts in positive and negative plant interactions along a grazing intensity gradient. Ecology 88: 188-199.

Guillard, R. R. and Lorenzen, C. J. 1972. Yellow-green algae with chlorophyllide c. Journal of Phycology 8: 10-14.

Gustafsson, S., Rengefors, K. and Hansson, L. A. 2005. Increased consumer fitness following transfer of toxin tolerance to offspring via maternal effects. Ecology 86: 2561-2567.

Ha, K., Jang, M. H. and Takamura, N. 2004. Colony formation in planktonic algae induced by zooplankton culture media filtrate. Journal of Freshwater Ecology 19: 9-16.

Hairston, N. G., Lampert, W., Caceres, C. E., Holtmeier, C. L., Weider, L. J., Gaedke, U., Fischer, J. M., Fox, J. A. and Post, D. M. 1999. Lake ecosystems - Rapid evolution revealed by dormant eggs. Nature 401: 446-446.

Hamback, P. A., Agren, J. and Ericson, L. 2000. Associational resistance: Insect damage to purple loosestrife reduced in thickets of sweet gale. Ecology 81: 1784-1794.

Hansson, L. A., Gustafsson, S., Rengefors, K. and Bomark, L. 2007. Cyanobacterial chemical warfare affects zooplankton community composition. Freshwater Biology 52: 1290-1301.

Hessen, D. O. and van Donk, E. 1993. Morphological changes in *Scenedesmus* induced by substances released from *Daphnia*. Archiv für Hydrobiologie 127: 129-140.

Hillebrand, H. and Cardinale, B. J. 2004. Consumer effects decline with prey diversity. Ecology Letters 7: 192-201.

Hisbergues, M., Christiansen, G., Rouhiainen, L., Sivonen, K. and Borner, T. 2003. PCR-based identification of microcystin-producing genotypes of different cyanobacterial genera. Archives of Microbiology 180: 402-410.

Hooper, D. U., Chapin, F. S., Ewel, J. J., Hector, A., Inchausti, P., Lavorel, S., Lawton, J. H., Lodge, D. M. *et al.* 2005. Effects of biodiversity on ecosystem functioning: A consensus of current knowledge. Ecological Monographs 75: 3-35.

Huisman, J., Johansson, A. M., Folmer, E. O. and Weissing, F. J. 2001. Towards a solution of the plankton paradox: the importance of physiology and life history. Ecology Letters 4: 408-411.

Hulot, F. D., Morin, P. J. and Loreau, M. 2001. Interactions between algae and the microbial loop in experimental microcosms. Oikos 95: 231-238.

Hutchinson, G. E. 1961. The Paradox of the Plankton. American Naturalist 95: 137-145.

Jang, M. H., Ha, K., Joo, G. J. and Takamura, N. 2003. Toxin production of cyanobacteria is increased by exposure

to zooplankton. Freshwater Biology 48: 1540-1550.
Janse, I., Kardinaal, W. E. A., Meima, M., Fastner, J., Visser, P. M. and Zwart, G. 2004. Toxic and nontoxic *Microcystis* colonies in natural populations can be differentiated on the basis of rRNA gene internal transcribed spacer diversity. Applied and Environmental Microbiology 70: 3979-3987.
Janse, I., Meima, M., Kardinaal, W. E. A. and Zwart, G. 2003. High-resolution differentiation of cyanobacteria by using rRNA-internal transcribed spacer denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69: 6634-6643.
Jiang, L. 2007. Negative selection effects suppress relationships between bacterial diversity and ecosystem functioning. Ecology 88: 1075-1085.
Joung, S. H., Kim, C. J., Ahn, C. Y., Jang, K. Y., Boo, S. M. and Oh, H. M. 2006. Simple method for a cell count of the colonial cyanobacterium, *Microcystis sp.* Journal of Microbiology 44: 562-565.
Kardinaal, W. E. A., Janse, I., Kamst-van Agterveld, M., Meima, M., Snoek, J., Mur, L. R., Huisman, J., Zwart, G. and Visser, P. M. 2007a. *Microcystis* genotype succession in relation to microcystin concentrations in freshwater lakes. Aquatic Microbial Ecology 48: 1-12.
Kardinaal, W. E. A., Tonk, L., Janse, I., Hol, S., Slot, P., Huisman, J. and Visser, P. M. 2007b. Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*. Applied and Environmental Microbiology 73: 2939-2946.
Lampert, W. 1987. Laboratory studies on zooplankton-cyanobacteria Interactions. New Zealand Journal of Marine and Freshwater Research 21: 483-490.
Loreau, M. 1998. Separating sampling and other effects in biodiversity experiments. Oikos 82: 600-602.
Loreau, M. and Hector, A. 2001. Partitioning selection and complementarity in biodiversity experiments (vol 412, pg 72, 2001). Nature 413: 548-548.
Lotocka, M. 2001. Toxic effect of cyanobacterial blooms on the grazing activity of *Daphnia magna* Straus. Oceanologia 43: 441-453.
Menden-Deuer, S. and Lessard, E. J. 2000. Carbon to volume relationships for dinoflagellates, diatoms, and other protist plankton. Limnology and Oceanography 45: 569-579.
Milchunas, D. G. and Noy-Meir, I. 2002. Grazing refuges, external avoidance of herbivory and plant diversity. Oikos 99: 113-130.
Oberhaus, L., Briand, J. F. and Humbert, J. F. 2008. Allelopathic growth inhibition by the toxic bloom-forming cyanobacterium *Planktothrix rubescens*. FEMS Microbiology Ecology 66: 243-249.
Otsuka, S., Suda, S., Shibata, S., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 2001. A proposal for the unification of five species of the cyanobacterial genus *Microcystis* Kutzing ex Lemmermann 1907 under the Rules of the Bacteriological Code. International Journal of Systematic and Evolutionary Microbiology 51: 873-879.
Pielou, E. C. 1966. The measurement of diversity in different types of biological collections. Journal of Theoretical Biology 13: 131-144.
Relyea, R. A. and Yurewicz, K. L. 2002. Predicting community outcomes from pairwise interactions: integrating density- and trait-mediated effects. Oecologia 131: 569-579.
Reusch, T. B. H., Ehlers, A., Hammerli, A. and Worm, B. 2005. Ecosystem recovery after climatic extremes enhanced by genotypic diversity. Proceedings of the National Academy of Sciences of the United States of America 102: 2826-2831.
Rohrlack, T., Dittmann, E., Borner, T. and Christoffersen, K. 2001. Effects of cell-bound microcystins on survival and feeding of *Daphnia spp.* Applied and Environmental Microbiology 67: 3523-3529.
Rohrlack, T., Dittmann, E., Henning, M., Borner, T. and Kohl, J. G. 1999. Role of microcystins in poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 65: 737-739.
Rousset, O. and Lepart, J. 2000. Positive and negative interactions at different life stages of a colonizing species *Quercus humilis*. Journal of Ecology 88: 401-412.
Sarnelle, O. 2007. Initial conditions mediate the interaction between *Daphnia* and bloom-forming cyanobacteria. Limnology and Oceanography 52: 2120-2127.
Scheffer, M., Rinaldi, S., Huisman, J. and Weissing, F. J. 2003. Why plankton communities have no equilibrium: solutions to the paradox. Hydrobiologia 491: 9-18.
Suikkanen, S., Fistarol, G. O. and Graneli, E. 2004. Allelopathic effects of the Baltic cyanobacteria *Nodularia spumigena*, *Aphanizomenon flos-aquae* and *Anabaena lemmermannii* on algal monocultures. Journal of Experimental Marine Biology and Ecology 308: 85-101.
Vaitomaa, J., Rantala, A., Halinen, K., Rouhiainen, L., Tallberg, P., Mokelke, L. and Sivonen, K. 2003. Quantitative real-time PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. Applied and Environmental Microbiology 69: 7289-7297.
Van der Gucht, K., Cottenie, K., Muylaert, K., Vloemans, N., Cousin, S., Declerck, S., Jeppesen, E., Conde-Porcuna, J. M. *et al.* 2007. The power of species sorting: Local factors drive bacterial community composition over a wide range of spatial scales. Proceedings of the National Academy of Sciences of the United States of America 104: 20404-20409.
van der Oost, R. 2007. Cyanotoxine monitoring: standaardisering en validatie van methoden voor de Nederlandse waterkwaliteitsbeheerders. Rapport STOWA onderzoek 2005 and 2006. Waternet/Waterproef. Amsterdam.
Vanni, M. J. 2002. Nutrient cycling by animals in freshwater ecosystems. Annual Review of Ecology and Systematics 33: 341-370.
Vanni, M. J. and Layne, C. D. 1997. Nutrient recycling and herbivory as mechanisms in the "top-down" effect of

fish on algae in lakes. Ecology 78: 21-40.
Visser, P. M., Ibelings, B. W., Mur, L. R. and Walsby, A. E. 2005. The ecophysiology of the harmful cyanobacterium *Microcystis*. Features explaining its success and measures for its control. In: Huisman, J., Matthijs, H. C. P. and Visser, P. M. (eds). Harmful cyanobacteria. Springer, Dordrecht, The Netherlands. pp. 109-142.
Weis, J. J., Cardinale, B. J., Forshay, K. J. and Ives, A. R. 2007. Effects of species diversity on community biomass production change over the course of succession. Ecology 88: 929-939.
Wilson, A. E., Sarnelle, O., Neilan, B. A., Salmon, T. P., Gehringer, M. M. and Hay, M. E. 2005. Genetic variation of the bloom-forming cyanobacterium *Microcystis aeruginosa* within and among lakes: Implications for harmful algal blooms. Applied and Environmental Microbiology 71: 6126-6133.
Wilson, A. E., Wilson, W. A. and Hay, M. E. 2006. Intraspecific variation in growth and morphology of the bloom-forming cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 72: 7386-7389.
Wu, Z. X. and Song, L. R. 2008. Physiological comparison between colonial and unicellular forms of *Microcystis aeruginosa* Kutz. (Cyanobacteria). Phycologia 47: 98-104.
Yang, Z., Kong, F. X., Shi, X. L. and Cao, H. S. 2006. Morphological response of *Microcystis aeruginosa* to grazing by different sorts of zooplankton. Hydrobiologia 563: 225-230.
Zhang, M., Kong, F. X., Tan, X., Yang, Z., Cao, H. S. and Xing, P. 2007. Biochemical, morphological, and genetic variations in *Microcystis aeruginosa* due to colony disaggregation. World Journal of Microbiology & Biotechnology 23: 663-670.
Zwart, G., Huismans, R., van Agterveld, M. P., Van de Peer, Y., De Rijk, P., Eenhoorn, H., Muyzer, G., van Hannen, E. J., Gons, H. J. and Laanbroek, H. J. 1998. Divergent members of the bacterial division *Verrucomicrobiales* in a temperate freshwater lake. FEMS Microbiology Ecology 25: 159-169.

Chapter 6

Priority effects in experimental populations of *Microcystis*

Ineke van Gremberghe[1], Pieter Vanormelingen[1], Katleen Van der Gucht[1], Caroline Souffreau[1], Wim Vyverman[1] and Luc De Meester[2]

Submitted to Environmental Microbiology

Contribution to this chapter by IVG: experimental set-up, sample analyses, statistical analyses and writing

[1]Laboratory of Protistology and Aquatic Ecology, Ghent University, Krijgslaan 281 - S8, 9000 Ghent, Belgium

[2]Laboratory of Aquatic Ecology and Evolutionary Biology, Katholieke Universiteit Leuven, Charles Debériotstraat 32, 3000 Leuven, Belgium

Abstract

The arrival order of colonists in developing populations can have a lasting influence on community and population structure, with potential impact on ecosystem functioning. To explore whether priority effects are important in determining strain composition of the nuisance cyanobacterium *Microcystis*, four *Microcystis* strains isolated from a single lake were grown during four weeks in the laboratory in all possible pairwise combinations, with the two strains either inoculated at the same time or with a time lag of one week, in the presence and absence of grazing *Daphnia magna*. Changes in the relative abundance of the strains in the mixed populations were monitored using Denaturing Gradient Gel Electrophoresis, and the growth rate of each strain in the mixtures was determined for the last two weeks of the experiment. We observed strong effects of inoculation order on the final population structure, which were influenced by grazing *Daphnia*. The priority effects were strain-specific and occurred in two directions, with strains either growing faster or slower when inoculated second than when inoculated first. The observed interactions and priority effects were in line with expectations based on variation in toxicity and colony formation among the strains and their consequences for competitive strength and grazing protection.

6.1 Introduction

Community assembly is determined by historical (dispersal) and local environmental factors, including biotic interactions. To become a dominant member of a community, species have to first colonize the habitat and establish themselves, which requires that they are sufficiently well adapted to the local environmental conditions and can deal with the resident community (Leibold *et al.* 2004; Ricklefs 2004). Not all species reach a particular habitat at the same time and the order of arrival is determined by both chance and dispersal rate of the different species of the regional pool, the latter being influenced by dispersal ability and regional abundance of the species. Priority effects occur when the order of arrival of colonists has a lasting influence on community structure (Drake 1991; Beisner *et al.* 2003; Schröder *et al.* 2005). Two mutually non-exclusive mechanisms may be responsible for such priority effects. The first is a purely numerical effect as later colonists have a disadvantage in numbers compared to the first colonists. Carrying capacity may be reached as a consequence of rapid population growth before later immigrants can become abundant, as is exemplified by initial soil exploration and root biomass formation in plants (Körner *et al.* 2008). Secondly, early colonists can also alter the environment in a favourable or detrimental way for later colonists. For instance, shading by growing plants can inhibit germination of seeds (Harper 1961), but can also facilitate the growth of seedlings in semi-arid environments by protection against drought, which is often indicated as the 'nurse plant syndrome' (Callaway and Walker 1997; Maestre *et al.* 2001; 2003; Brooker *et al.* 2008). Additionally, unpalatable plants can protect seedlings from herbivory (Brooker *et al.* 2008).

The occurrence of priority effects, which can be very strong, has been observed in a variety of taxa ranging from protists to plants, insects and amphibians (Morin 1984; Alford and Wilbur 1985; Robinson and Dickerson 1987; Drake 1991; Blaustein and Margalit 1996; Sunahara and Mogi 2002; Louette and De Meester 2007; Zhang and Zhang 2007; Körner *et al.* 2008). In addition, the strength and direction of priority effects has been shown to interact with the local conditions as these may influence competitive interactions among species. For instance, in aquatic communities, the presence of predators has a large influence on community composition and has been shown to alter priority effects (Morin 1984, 1999; Louette and De Meester 2007).

The above arguments on the importance of priority effects for community composition may also hold at the population level. To the extent that genotypes differ in ecologically important traits, priority effects consisting of both numerical and ecological mechanisms may play a role in populations too, with the strength and direction depending on the local environment. This would add an additional historical component to population structure. Populations of prokaryotes are often characterized by the presence of (functionally) highly divergent strains and differentiation in lineage composition between populations has been attributed to environmental factors (Bronikowski *et al.* 2001; Bhaya *et al.* 2007) or geographic isolation (DeChaine *et al.* 2006). Here, we experimentally investigated whether, in addition to these factors, colonization order

might determine the resulting strain composition of prokaryote populations as this has hardly been investigated (Fukami *et al.* 2007).

Microcystis is a common and cosmopolitan cyanobacterium that forms toxic blooms during warm weather conditions in eutrophic water bodies (Chorus and Bartram 1999; Visser *et al.* 2005). Naturally occurring *Microcystis* bloom populations are generally composed of several genotypes with different functional traits including growth rate, toxicity and colony morphology (Wilson *et al.* 2005, 2006; Bañares-España *et al.* 2006; Kardinaal *et al.* 2007a). Also, different *Microcystis* blooms often strongly differ in rDNA ITS genotype composition (Janse *et al.* 2004; Kardinaal *et al.* 2007a). Little is known about the mechanisms through which this diversity, both within and between lakes, is maintained, although competitive interactions and grazer-mediated facilitation are likely involved (chapter 5). Strain composition of a *Microcystis* bloom is expected to have important consequences for other components of the food web through its effect on bloom toxicity (Hansson *et al.* 2007), as not all strains possess microcystin synthetase genes (Janse *et al.* 2004; Kardinaal *et al.* 2007a). Priority effects may be important during population assembly in new habitats as well as in seasonal bloom populations that build up each year when conditions become favourable in spring or summer (Verspagen *et al.* 2004).

The aim of the present study was to experimentally test the hypothesis that priority effects among *Microcystis* strains that differ in functional traits, including the degree to which they produce microcystins and form colonies, can have a significant influence on strain composition in *Microcystis* populations. We also tested whether the presence of a grazer changes the strength and direction of priority effects by including a treatment with the addition of the large-bodied zooplankton species *Daphnia magna*. Finally, we verified whether the strength and direction of priority effects is in concordance with predictions based on trait differences.

Our predictions are (1) that colony formation makes priority effects in mixtures with a non-colony forming *Microcystis* strain stronger in case the colony forming *Microcystis* strain is inoculated first and weaker when this strain is inoculated second (asymmetric priority effects), because of the added competitive advantage offered by colony formation, (2) for microcystin production, the opposite pattern is expected, as non-toxic strains seemed better competitors for light than toxic strains (Kardinaal *et al.* 2007b; chapter 5), (3) in the presence of grazing pressure, the cost of being inoculated second might be lower for toxic or colony forming strains than in the absence of grazing when they are combined with strains that are more vulnerable to grazing (non-toxic, unicellular strains), and (4) non-toxic strains may benefit from being inoculated simultaneously or second to a toxic strain compared to when inoculated first in the presence of grazers, as they are facilitated by the protection offered by the toxins produced by their competitors (Rohrlack *et al.* 1999a), and a similar mechanism might work for the unicellular versus colonial strains, as colonies hinder *Daphnia* grazing (Rohrlack *et al.* 1999b).

6.2 Material and methods

6.2.1 Isolation and genotypic characterization of *Microcystis* strains

As the taxonomy of *Microcystis* is still largely based on morphologic features and does not correspond to genetic relationships (Otsuka *et al.* 2001), we used the 16S-23S rDNA ITS sequence to distinguish *Microcystis* genotypes from each other irrespective of morphospecies identification (Janse *et al.* 2004). Four *Microcystis* rDNA ITS genotypes, isolated from lake Leeuwenhof (Ghent, Belgium) and differing in ecologically important characteristics (growth rate, microcystin production and colony formation; chapter 5) were used in the experiment. Strains T1 and T2 are toxic (microcystin concentration in monoculture in absence of grazing in pg per ng C: 17.9 for T1 and 2.8 for T2, chapter 5) while NT1 and NT2 are non-toxic. T2 has a lower monoculture growth rate than the other three strains and forms colonies in both the absence and presence of grazing *Daphnia*. T1 and NT1 have a lower monoculture growth rate than NT2 (growth rates of strains in monoculture in absence of grazing in day^{-1}: 0.212 for T1, 0.187 for T2, 0.213 for NT1 and 0.227 for NT2, chapter 5). The latter strain forms only colonies in the presence of grazing while T1 and NT1 never form colonies. The two toxic strains T1 and T2 have a strongly negative influence on the survival of *Daphnia magna* individuals, and the *Daphnia* individuals seem to graze more easily on unicellular than on colonial strains (chapter 5).

6.2.2 Experimental design

Before the start of the experiment, the four *Microcystis* strains were grown in WC medium at a temperature of 23 °C and a light intensity of 120 µmol photons m^{-2} s^{-1} (Light Dark cycle: 12h:12h). Culture conditions were the same throughout the study. Exponentially growing cells were used as inocula for the experiment. The four *Microcystis* strains were grown in triplicate in all possible combinations of two strains, either inoculated at the same time or with a time lag of one week (both directions) in 200 ml Erlenmeyer flasks during four weeks. All treatments were grown with and without addition of four individuals of *Daphnia magna*. This design resulted in a total of 6 different mixtures x 3 inoculation sequences x 2 grazing treatments x 3 replicate observations = 108 experimental units. All cultures were shaken by hand and their position randomized daily. Cultures without *Daphnia magna* ('no predation', 'NP') were inoculated at a total density of 50 000 cells per ml, whereas the cultures with *Daphnia magna* ('predation', 'P') were inoculated at a total density of 500 000 cells per ml. We used two different inoculation densities because a pilot experiment indicated that this resulted in comparable densities towards the end of the experiment (third and fourth week) when the interactions are expected to be the strongest and most samples were taken (chapter 5). Inoculation at low and similar densities in both predation treatments in the pilot experiment resulted in the near-extermination of the *Microcystis* populations in the grazing treatment and thus widely different densities at the

end of the experiment, reflecting the strong potential of *Daphnia* to prevent *Microcystis* population development. This procedure implies that the total cell density at the end of the experiment cannot be compared between predator treatments. Yet, to the extent that densities in the final period of the experiment are similar across predation treatments, we can directly compare growth rates during that period (see below). Within each mixed population, all strains had the same initial relative abundance (two times 25 000 cells per ml for cultures without *Daphnia* and two times 250 000 cells per ml for cultures with *Daphnia*). The *Daphnia* individuals were obtained from a multiclonal population grown in the laboratory and fed *Scenedesmus obliquus*. Before adding *Daphnia* to the *Microcystis* cultures, they were starved for at least three hours. During the experiment, the *Daphnia* individuals were systematically replaced by new individuals every 1-3 days in order to keep densities the same across cultures. *Daphnia* survival was low in some of the cultures, especially from the third week of the experiment onwards (chapter 5). To determine *Microcystis* cell density, a weekly subsample (1 ml) was taken from each culture and the optical density was measured immediately at 750 nm with a spectrophotometer (Shimadzu UV-1601). Samples for DNA extraction (2 ml) were taken after two and four weeks and stored at -20°C.

6.2.3 Sample analyses

The methods used for DNA extraction, PCR and Denaturing Gradient Gel Electrophoresis (DGGE), are described in chapter 5 and based on the protocol of Janse *et al.* (2003, 2004). DGGE band intensity was used to monitor the relative abundance of the *Microcystis* strains as it was shown previously to accurately reflect relative strain abundance (chapter 5). The relative abundances of the strains in the populations were determined after two and four weeks of growth of the first strain. Cell densities of the different strains in the mixtures were estimated by converting optical density into cell density using standard curves in combination with the relative abundance of each strain as determined by DGGE (chapter 5). Based on these data, the growth rate during the last two weeks of the experiment was determined for each strain in the different mixtures using the formula: [Ln(cells ml^{-1})t2 - Ln(cells ml^{-1})t1]/(t2 - t1) with t1 = day 14 and t2 = day 28. For the cultures with *Daphnia* only the nett growth rate was actually known (= cell growth rate minus grazing rate).

6.2.4 Statistical analyses

For each strain and predation treatment separately, two-way ANOVA was used to test for significant differences in relative abundance (arcsine of square root transformed relative abundances) at the end of the experiment among mixtures that differ in inoculation order and competing strain. Post Hoc LSD tests adjusted by Bonferroni correction were performed for each competing strain separately to test for significant differences in relative abundances of strains inoculated at the same time and inoculated with a time lag, and between first inoculation and second inoculation. Only the results of the Post Hoc tests are shown.

Prior to further statistical analysis, we tested whether population densities on day 14 were similar across predation treatments for the samples in which strains were inoculated with a time lag using three-way ANOVA (factors: predation, population and inoculation). As no significant main effect of predation on population density on day 14 was observed ($F = 0.592$; $p = 0.445$), we included predation as a factor in our analyses on growth rate. Removing predation from the analyses outlined below and performing separate ANOVA on the two predation treatments separately does not change our conclusions on the strength and direction of priority effects. For each strain separately, three-way ANOVA was used to test for significant differences in growth rate (day 14 to day 28) among mixtures that differ in inoculation order, competing strain and predation treatment. Post Hoc LSD tests were performed to test for significant differences in growth rate in relation to inoculation order for each competing strain separately, in the presence and absence of predation. We conclude that (long-lasting) priority effects occur when there is a significant inoculation order effect on growth rate, indicating that early colonists influence the establishment of later arrivals (Louette and De Meester 2007). By using growth rate data of the last two weeks of the experiment, we assure that differences in growth rates are not merely the result of the first colonist being inoculated in an empty habitat and thus having a better scope for growth. At that time, all strains were present in the populations for at least one week, and population densities were similar across treatments.

All statistical analyses were performed in SPSS 15 (SPSS Inc., Chicago, USA).

6.3 Results

Figure 6.1 shows the relative abundances of the strains after four weeks of growth. Pronounced differences in strain composition in relation to inoculation order were often present with strains generally having a higher relative abundance when inoculated first in the absence of grazing, but this positive effect was often reduced by the presence of *Daphnia*. When inoculated at the same time, strain T1 was generally a weaker competitor than the other strains. However, when T1 was inoculated first, it became dominant or co-dominant at the end of the experiment in both predation treatments. When inoculated second in the absence of grazers, T1 was almost outcompeted in most mixtures but, in contrast, in the presence of grazers it became dominant when inoculated after NT1 and co-dominant when inoculated after NT2 (Fig. 6.1). T2 was generally a good competitor when inoculated at the same time with a competing strain, and when inoculated first, it strongly dominated the final population. Even when inoculated after T1 or NT2, T2 was dominant to co-dominant at the end of the experiment. However, when inoculated after NT1, T2 was only dominant in the presence of predation (Fig. 6.1). NT1 also benefited from being inoculated first in the absence of predation in combination with NT2, as it dominated the final population in that case, while it was largely outcompeted when inoculated after NT2. In contrast, in the presence of predation, NT1 was outcompeted by NT2 in all inoculation orders (Fig. 6.1).

As the relative abundances of the strains at the end of the experiment partly

Table 6.1: ANOVA table on the effects of inoculation order, competing strain and predation treatment on the growth rate (day 14 to day 28) of the four studied *Microcystis* strains in mixed populations. Post Hoc LSD tests for inoculation order are presented (p-values) separately for each competing strain in the absence of predation (NP) and the presence of predation (P). Significant p-values are in bold.

	df	MS	F	p	Post Hoc inoculation	NP	P
Effect T1							
Inoculation order (I)	1	0.248	141.670	**< 0.001**	T2	0.435	**0.033**
Competing strain (C)	2	0.237	135.481	**< 0.001**	NT1	**< 0.001**	**0.028**
Predation (P)	1	0.357	204.343	**< 0.001**	NT2	**0.023**	**0.047**
I*C	2	0.278	159.120	**< 0.001**			
I*P	1	0.527	301.669	**< 0.001**			
C*P	2	0.307	175.889	**< 0.001**			
I*C*P	2	0.284	162.231	**< 0.001**			
Effect T2							
Inoculation order (I)	1	0.007	6.360	**0.019**	T1	0.188	**0.021**
Competing strain (C)	2	0.002	1.380	0.271	NT1	0.403	0.136
Predation (P)	1	0.007	6.575	**0.017**	NT2	0.465	0.495
I*C	2	0.002	1.928	0.167			
I*P	1	0.002	1.759	0.197			
C*P	2	0.001	1.096	0.350			
I*C*P	2	7.66E-5	0.070	0.932			
Effect NT1							
Inoculation order (I)	1	0.329	377.165	**< 0.001**	T1	0.224	0.069
Competing strain (C)	2	0.981	1.122.758	**< 0.001**	T2	**0.023**	**< 0.001**
Predation (P)	1	0.174	198.729	**< 0.001**	NT2	**< 0.001**	**< 0.001**
I*C	2	1.765	2.020.700	**< 0.001**			
I*P	1	0.265	303.738	**< 0.001**			
C*P	2	0.196	223.865	**< 0.001**			
I*C*P	2	0.157	179.440	**< 0.001**			
Effect NT2							
Inoculation order (I)	1	0.033	15.407	**0.001**	T1	0.827	0.115
Competing strain (C)	2	0.209	97.905	**< 0.001**	T2	0.890	**< 0.001**
Predation (P)	1	0.023	10.850	**0.003**	NT1	0.051	**< 0.001**
I*C	2	0.130	60.793	**< 0.001**			
I*P	1	0.008	3.813	0.063			
C*P	2	0.105	49.289	**< 0.001**			
I*C*P	2	0.177	82.960	**< 0.001**			

reflected differences in the amount of time the strains could grow, we also calculated the growth rate for each strain in the mixed populations from day 14 to day 28 to detect priority effects (Table 6.1, Fig. 6.2). For T1, a significant effect of inoculation order, competing strain and predation treatment on the growth rate was detected as well as significant interaction effects between these factors (Table 6.1). The growth rate of T1 was significantly lower when inoculated after NT1 or NT2 compared to when inoculated before these strains in the absence of predation to such an extent that the growth rate of T1 was negative when inoculated after NT1, and zero when inoculated after NT2. In the presence of predation, the growth rate of T1 was higher when inoculated second compared to when inoculated first in all three combinations (Table 6.1, Fig. 6.2). For T2, there was a significant effect of inoculation order and predation treatment on growth rate (Table 6.1). The growth rate of T2 was higher when inoculated after T1 compared to when inoculated before T1 in the presence of predation (Table 6.1, Fig. 6.2). For NT1, a significant effect of inoculation order, competing strain and predation treatment on growth rate was found as well as significant interaction effects between these factors (Table 6.1). The growth rate of NT1 was higher when inoculated after T2 compared to when inoculated before T2, which was especially clear in the presence of grazing where the growth rate of NT1 was negative when inoculated before T2. When inoculated after NT2, the growth rate of NT1 was lower (even negative) compared to when inoculated before NT2 in both the absence and presence of grazing (Table 6.1, Fig. 6.2). Also for NT2, a significant effect of inoculation order, competing strain and predation treatment on growth rate was detected as well as significant interaction effects between the factors, except for the interaction between inoculation order and predation treatment (Table 6.1). In the presence of predation, the growth rate of NT2 was lower (even negative) when inoculated after T2 compared to when inoculated before T2, whereas the growth rate of NT2 was higher when inoculated after NT1 compared to when inoculated before NT1 (Table 6.1, Fig. 6.2).

Generally, the pairwise comparisons showed that inoculation order was important in determining growth rate of T1 during the final part of our experiment in five out of six mixtures, growth rate of T2 in one out of six mixtures, growth rate of NT1 in four out of six mixtures (+ one borderline non-significant), and growth rate of NT2 in two out of six mixtures (+ one borderline non-significant). Our experiment thus revealed a significant priority effect in 12 out of 24 tests. In some cases, the priority effect was very strong, from a clear positive to a clear negative growth (T1 in combination with NT1 and NT1 in combination with NT2 in the absence of grazing; NT1 in combination with NT2 and NT2 in combination with T2 in the presence of grazing) or vice versa (NT1 in combination with T2 in the presence of grazing). In most of these cases, arriving first or second made the difference between dominating the final population or being outcompeted. Importantly, we observed negative as well as positive priority effects in the mixed populations. Significant negative effects of the first colonizers on the later arrivals were detected in five out of 24 tests (three in the absence of predation and two in the presence of predation), whereas positive effects of the first colonizers on second colonists were detected in seven cases. Most of these facilitation effects (six out of seven cases) were detected in the treatment with grazers.

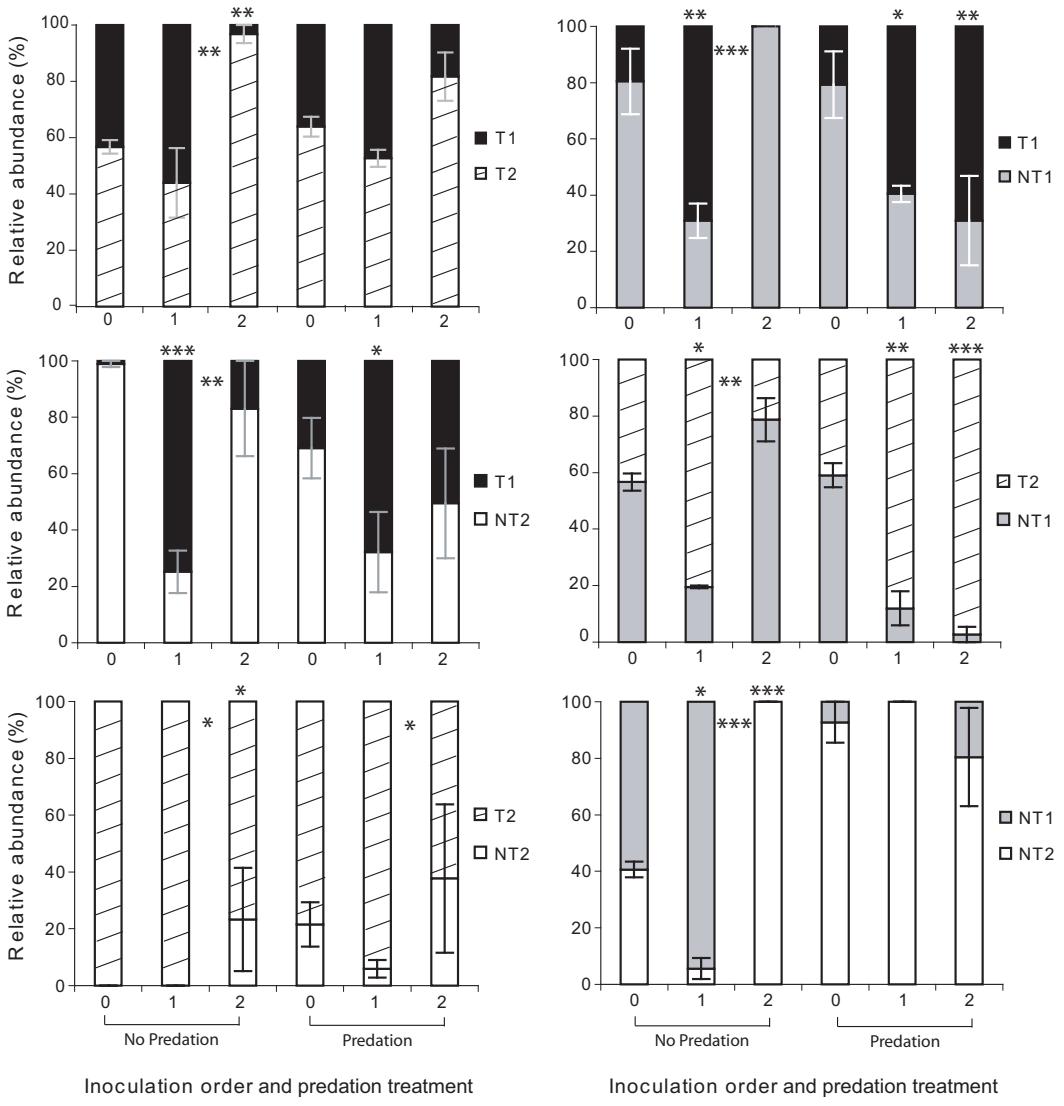


Figure 6.1: The relative abundances of the four *Microcystis* strains in the mixed populations as determined by DGGE after four weeks of growth. (0) the two strains inoculated at the same time, (1): the upper strain inoculated first and (2): the upper strain inoculated second in the absence and the presence of predation. Error bars indicate the standard error of the mean. Asterisks above the bars of the (1) or (2) treatment indicate significant differences in relative abundance compared to the (0) treatment, and asterisks between bars indicate significant differences between the relative abundances of the (1) and (2) treatments (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

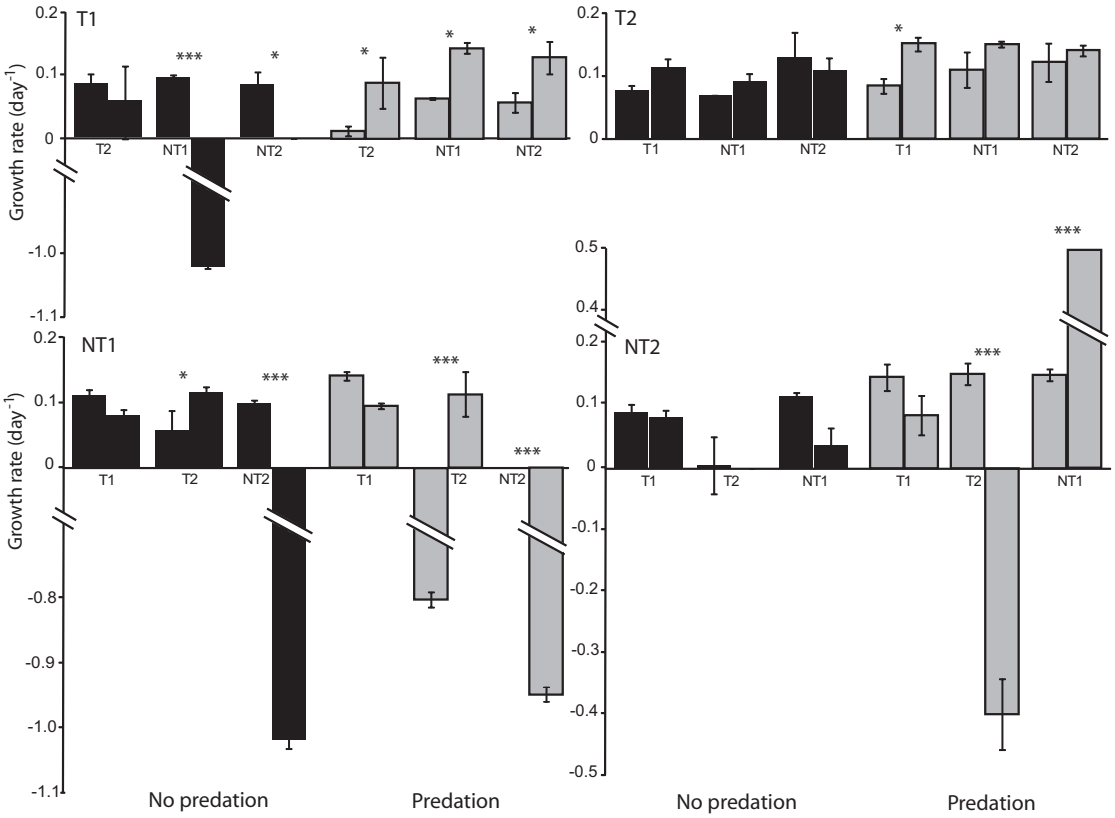


Figure 6.2: The growth rate of the four *Microcystis* strains from day 14 to day 28 in the mixed populations. The competing strain is indicated on the x-as, the first bar indicates the growth rate when the strain is inoculated before the competing strain, the second bar indicates the growth rate when the strain is inoculated after the competing strain. Growth rates in the absence of predation are in black and growth rates in the presence of predation in grey. Error bars indicate the standard error of the mean. Asterisks indicate a significant effect of inoculation order on the growth rate (*: $p < 0.05$; ***: $p < 0.001$).

6.4 Discussion

Our results highlight the importance of priority effects in determining strain composition of *Microcystis* populations. Inoculation order was found to be important in determining growth rate in half of the mixtures. A time lag of just one week had pronounced qualitative implications. The observation that growth rates are negative when inoculated second (four cases) or first (one case) clearly indicates that the observed priority effects have long-lasting consequences and the dominance patterns of the competing strains in these mixtures are not a temporary phenomenon. These observations are in concordance with a growing number of studies that detect important priority effects in a wide range of taxa (e.g. Drake 1991; Shorrocks and Bingley 1994; Louette and De Meester 2007; Fukami *et al.* 2007; Körner *et al.* 2008). Strikingly, we observed as many positive as negative priority effects. In agreement with the observations of Kardinaal *et al.* (2007b), this implies that a numerical advantage of a *Microcystis* strain does not systematically result in the final dominance of that strain. Overall, negative effects were very strong, involving a shift from positive to negative or zero growth in all cases, whereas positive effects were weaker except in one case. Thus, positive effects of arriving after a time lag of one week are common, but tend to have less impact than negative effects associated with being second. Most priority effects reported in literature are negative (e.g. Drake 1991; Shorrocks and Bingley 1994; Louette and De Meester 2007; Körner *et al.* 2008). However, some studies on communities of amphibians (Alford and Wilbur 1985) and coral reef fish (Almany 2003) also detected a facilitative effect of first colonizing taxa on later colonizing taxa. In plant communities, resident plants can also protect seedlings from drought (Maestre *et al.* 2001; 2003; Brooker *et al.* 2008) and taxa arriving first may protect others arriving later against herbivory (Brooker *et al.* 2008).

The priority effects observed in our experiment are largely in line with a priori predictions based on information on toxin production and colony formation combined with their impact on competitive strength. When the two toxic strains are combined, T2 dominates when inoculated first and T1 takes little advantage of being inoculated before T2, which is likely the result of T2 producing colonies giving it an additional competitive advantage over T1 when it is inoculated first. When the two non-toxic strains are combined in the absence of predation, a similar asymmetry is seen: when inoculated together NT1 is a better competitor than NT2, but NT2 takes more advantage of being first. In the presence of predation, NT2 always dominates because it is better protected against grazing thanks to colony formation. Here we see a striking facilitation effect: NT1 is not outcompeted when inoculated after NT2 compared to when inoculated before NT2. In the presence of grazing, it is disadvantageous for NT1 to arrive before NT2 compared to arriving together or after NT2, because it is readily eaten in the absence of NT2 and can take profit of the protection offered by colonies of NT2 hindering grazing. In the presence of predation, it seems that it is better for non-toxic strains to be inoculated simultaneously with or second to toxic strains than being the first colonizer. There is a negative effect of being first compared to the control for non-toxic strains in three (two significant for NT1 and one

borderline non-significant for NT2) out of four combinations, and the effect is stronger for NT1 than for NT2. The latter can be interpreted as being the result of NT2 being somewhat protected against grazing because it forms colonies. Strain NT1 does not produce toxins nor produces colonies, and performs very poorly when inoculated first in the presence of grazers. In the case of T2 and NT2, there is a strong bias towards dominance of T2 in the presence and absence of predation, a pattern that we cannot explain from our knowledge on trait variation among these strains, and possibly reflects interactions through additional functional traits of these strains, such as the production of allelopathic substances (Oberhaus *et al.* 2008). We can conclude that priority effects between toxic and non-toxic strains and between a colony forming and non-colony forming strains are asymmetric and differ between predation treatments. It is better for non-toxic strains to be inoculated simultaneously or second to a toxic strain and it is better for unicellular strains to be inoculated simultaneously or second to a colony forming strain, than being the first colonizer in the presence but not in the absence of grazers. This is because the non-toxic and unicellular strains are facilitated by the protection offered by microcystin production and colony formation of their competitors, respectively. Admittedly, we worked with only a few strains that all differ in combinations of traits, and further experiments are needed to investigate whether the observed patterns can be generalized. Also, we do not claim to have information on all traits that may be relevant to the observed interactions. It is, however, striking that the observed patterns are strongly in line with a priori predictions based on the two key traits considered.

Our study showed that priority effects can differ profoundly in the absence and presence of the large-bodied cladoceran *D. magna*. The mechanism by which predation impacts priority effects in our experiment is twofold. First, by reducing competitive interactions, a tendency for negative priority effects is reduced by the presence of grazers. Similarly, Morin (1984) showed that negative effects of early odonate breeders on late breeders occurred only in the absence and not in the presence of fish. Predators can eliminate competitive priority effects by reducing the abundance of preceding species and increasing resource levels for late arrivals and/or reducing the abundance of late species and lowering the demand for remaining resources (Morin 1987). Louette and De Meester (2007) also showed the importance of the presence of a predator on the occurrence of priority effects in experiments with three large cladoceran zooplankton species. Priority effects were reduced or alternative priority effects occurred due to selective predation that influenced competitive interactions between the cladoceran species. The second mechanism by which grazing *Daphnia* in our experiment caused strong inoculation order effects is by their strong impact on strains that are not protected against grazing when they are inoculated first. The fact that unprotected *Microcystis* strains perform better when inoculated simultaneously with protected strains than when inoculated first, is similar to the protection against herbivory of palatable plants by unpalatable plants (Callaway *et al.* 2005; Graff *et al.* 2007; Brooker *et al.* 2008) and results in strain-specific responses to inoculation order.

In conclusion, our experiment shows pronounced and long-lasting priority effects on the strain composition in *Microcystis* populations, and illustrates that these are strongly

strain-specific and profoundly influenced by the presence of grazers. In addition, priority effects occurred in both directions, with strains either growing faster or slower when inoculated second than when inoculated first. Overall, the observed priority effects are in broad agreement with predictions based on key traits such as microcystin production and colony formation. Arriving first can be advantageous for inferior competitors while arriving simultaneously or second seemed to be mainly advantageous in the presence of grazers, depending on the toxicity and colony formation of the competing strains. The priority effects observed are so strong that they may make the difference between a toxic and non-toxic *Microcystis* population. Especially in the absence of grazers, inoculation order in combinations of toxic and non-toxic strains has a pervasive effect on the degree to which toxic strains dominate in the population. Strikingly, in the presence of grazers, simultaneous colonization of toxic and non-toxic strains leads to the lowest relative abundances of toxic strains. Whereas the interactions among strains seem to be predictable from their ecology, priority effects will introduce a strong element of stochasticity in strain composition of natural *Microcystis* populations.

Acknowledgements

We thank Jeroen Van Wichelen and Renaat Dasseville for sampling lake Leeuwenhof, Victor Chepurnov for isolating the *Microcystis* strains and Sofie D'hondt for the DGGE analyses. This research was financially supported by ESF EURODIVERSITY project BIOPOOL (nationally supported by BELSPO - Belgian Science Policy and FWO - Fonds Wetenschappelijk Onderzoek Vlaanderen) and Belgian project B-BLOOMS2 (nationally supported by BELSPO).

References

Alford, R. A. and Wilbur, H. M. 1985. Priority effects in experimental pond communities: competition between *Bufo* and *Rana*. Ecology 66: 1097-1105.

Almany, G. R. 2003. Priority effects in coral reef fish communities. Ecology 84: 1920-1935.

Bañares-España, E., López-Rodas, V., Salgado, C., Costas, E. and Flores-Moya, A. 2006. Inter-strain variability in the photosynthetic use of inorganic carbon, exemplified by the pH compensation point, in the cyanobacterium *Microcystis aeruginosa*. Aquatic Botany 85: 159-162.

Beisner, B. E., Haydon, D. T. and Cuddington, K. 2003. Alternative stable states in ecology. Frontiers in Ecology and the Environment 1: 376-382.

Bhaya, D., Grossman, A. R., Steunou, A.-S., Khuri, N., Cohan, F. M., Hamamura, N., Melendrez, M. C., Bateson, M. M., Ward, D. M. and Heidelberg, J. F. 2007. Population level functional diversity in a microbial community revealed by comparative genomic and metagenomic analyses. The ISME Journal 1: 703-713.

Blaustein, L. and Margalit, J. 1996. Priority effects in temporary pools: Nature and outcome of mosquito larva toad tadpole interactions depend on order of entrance. Journal of Animal Ecology 65: 77-84.

Bronikowski, A. M., Bennett, A. F. and Lenski, R. E. 2001. Evolutionary adaptation to temperature. VIII. Effects of temperature on growth rate in natural isolates of *Escherichia coli* and *Salmonella enterica* from different thermal environments. Evolution 55: 33-40.

Brooker, R. W., Maestre, F. T., Callaway, R. M., Lortie, C. L., Cavieres, L. A., Kunstler, G., Liancourt, P. *et al.* 2008. Facilitation in plant communities: the past, the present, and the future. Journal of Ecology 96: 18-34.

Callaway, R. M., Kikodze, D., Chiboshvili, M. and Khetsuriani, L. 2005. Unpalatable plants protect neighbors from grazing and increase plant community diversity. Ecology 86: 1856-1862.

Callaway, R. M. and Walker, L. R. 1997. Competition and facilitation: A synthetic approach to interactions in plant communities. Ecology 78: 1958-1965.

Chorus, I. and Bartram, J. (eds). 1999. Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. WHO. London, E and FN Spon.

DeChaine, E. G., Bates, A. E., Shank, T. M. and Cavanaugh, C. M. 2006. Off-axis symbiosis found: characterisation and biogeography of bacterial symbionts of *Bathymodiolus* mussels from Lost City hydrothermal vents. Environmental Microbiology 8: 1902-1912.

Drake, J. A. 1991. Community-assembly mechanisms and the structure of an experimental species ensemble. American Naturalist 137: 1-26.

Fukami, T., Beaumont, H. J. E., Zhang, X. X. and Rainey, P. B. 2007. Immigration history controls diversification in experimental adaptive radiation. Nature 446: 436-439.

Graff, P., Aguiar, M. R. and Chaneton, E. J. 2007. Shifts in positive and negative plant interactions along a grazing intensity gradient. Ecology 88: 188-199.

Hansson, L. A., Gustafsson, S., Rengefors, K. and Bomark, L. 2007. Cyanobacterial chemical warfare affects zooplankton community composition. Freshwater Biology 52: 1290-1301.

Harper, J. L. 1961. Approaches to the study of plant competition. In: Mechanisms in biological competition. Milthorpe, F.L. (ed.), pp. 1-39. Cambridge, University Press.

Janse, I., Kardinaal, W. E. A., Meima, M., Fastner, J., Visser, P. M. and Zwart, G. 2004. Toxic and nontoxic *Microcystis* colonies in natural populations can be differentiated on the basis of rRNA gene internal transcribed spacer diversity. Applied and Environmental Microbiology 70: 3979-3987.

Janse, I., Meima, M., Kardinaal, W. E. A. and Zwart, G. 2003. High-resolution differentiation of cyanobacteria by using rRNA-internal transcribed spacer denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69: 6634-6643.

Kardinaal, W. E. A., Janse, I., Kamst-van Agterveld, M., Meima, M., Snoek, J., Mur, L. R., Huisman, J., Zwart, G. and Visser, P. M. 2007a. *Microcystis* genotype succession in relation to microcystin concentrations in freshwater lakes. Aquatic Microbial Ecology 48: 1-12.

Kardinaal, W. E. A., Tonk, L., Janse, I., Hol, S., Slot, P., Huisman, J. and Visser, P. M. 2007b. Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*. Applied and Environmental Microbiology 73: 2939-2946.

Körner, C., Stocklin, J., Reuther-Thiebaud, L. and Pelaez-Riedl, S. 2008. Small differences in arrival time influence composition and productivity of plant communities. New Phytologist 177: 698-705.

Leibold, M. A., Holyoak, M., Mouquet, N., Amarasekare, P., Chase, J. M., Hoopes, M. F., Holt, R. D., Shurin, J. B. *et al.* 2004. The metacommunity concept: a framework for multi-scale community ecology. Ecology Letters 7: 601-613.

Louette, G. and De Meester, L. 2007. Predation and priority effects in experimental zooplankton communities. Oikos 116: 419-426.

Maestre, F. T., Bautista, S., Cortina, J. and Bellot, J. 2001. Potential for using facilitation by grasses to establish shrubs on a semiarid degraded steppe. Ecological Applications 11: 1641-1655.

Maestre, F. T., Bautista, S. and Cortina, J. 2003. Positive, negative, and net effects in grass-shrub interactions in mediterranean semiarid grasslands. Ecology 84: 3186-3197.

Morin, P. J. 1984. Odonate guild composition: experiments with colonization history and fish predation. Ecology 65: 1866-1873.

Morin, P. J. 1987. Predation, breeding asynchrony, and the outcome of competition among treefrog tadpoles. Ecology 68: 675-683.

Morin, P. J. 1999. Community Ecology. Blackwell Science.

Oberhaus, L., Briand, J. F. and Humbert, J. F. 2008. Allelopathic growth inhibition by the toxic bloom-forming cyanobacterium *Planktothrix rubescens*. FEMS Microbiology Ecology 66: 243-249.

Otsuka, S., Suda, S., Shibata, S., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 2001. A proposal for the unification of five species of the cyanobacterial genus *Microcystis* Kutzing ex Lemmermann 1907 under the Rules of the Bacteriological Code. International Journal of Systematic and Evolutionary Microbiology 51: 873-879.

Ricklefs, R. E. 2004. A comprehensive framework for global patterns in biodiversity. Ecology Letters 7: 1-15.

Robinson, J. V. and Dickerson, J. E. Jr. 1987. Does invasion sequence affect community structure? Ecology 68: 587-595.

Rohrlack, T., Dittmann, E., Henning, M., Borner, T. and Kohl, J. G. 1999a. Role of microcystins in poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 65: 737-739.

Rohrlack, T., Henning, M. and Kohl, J. G. 1999b. Mechanisms of the inhibitory effect of the cyanobacterium *Microcystis aeruginosa* on *Daphnia galeata*'s ingestion rate. Journal of Plankton Research 21: 1489-1500.

Schröder, A., Persson, L. and De Roos, A. M. 2005. Direct experimental evidence for alternative stable states: a review. Oikos 110: 3-19.

Shorrocks, B. and Bingley, M. 1994. Priority effects and species coexistence - Experiments with fungal-breeding *Drosophila*. Journal of Animal Ecology 63: 799-806.

Sunahara, T. and Mogi, M. 2002. Priority effects of bamboo-stump mosquito larvae: influences of water exchange and leaf litter input. Ecological Entomology 27: 346-354.

Verspagen, J. M. H., Snelder, E., Visser, P. M., Huisman, J., Mur, L. R. and Ibelings, B. W. 2004. Recruitment of benthic *Microcystis* (Cyanophyceae) to the water column: Internal buoyancy changes or resuspension? Journal of Phycology 40: 260-270.

Visser, P. M., Ibelings, B. W., Mur, L. R. and Walsby, A. E. 2005. The ecophysiology of the harmful cyanobacterium *Microcystis*. Features explaining its success and measures for its control. In: Huisman, J., Matthijs, H. C. P. and Visser, P. M. (eds). Harmful cyanobacteria. Springer, Dordrecht, The Netherlands. pp. 109-142.

Wilson, A. E., Sarnelle, O., Neilan, B. A., Salmon, T. P., Gehringer, M. M. and Hay, M. E. 2005. Genetic variation of the bloom-forming cyanobacterium *Microcystis aeruginosa* within and among lakes: Implications for harmful algal blooms. Applied and Environmental Microbiology 71: 6126-6133.

Wilson, A. E., Wilson, W. A. and Hay, M. E. 2006. Intraspecific variation in growth and morphology of the bloom-forming cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 72: 7386-7389.

Zhang, Q. G. and Zhang, D. Y. 2007. Colonization sequence influences selection and complementarity effects on biomass production in experimental algal microcosms. Oikos 116: 1748-1758.

Chapter 7

General discussion

7.1 Introduction

Blooms of the toxic cyanobacterium *Microcystis* are a common phenomenon worldwide and, as a result, have been intensively studied. Attention has focused on the factors influencing bloom formation and the toxic effects of the hepatotoxic microcystins on eukaryotes (e.g. Falconer 1999; Chorus and Bartram 1999; Carmichael 1994, 2001; Huisman *et al.* 2005; Codd *et al.* 2005). More recently, some attention was paid on the characteristics of individual *Microcystis* strains and the genetic composition of *Microcystis* blooms as this may have large consequences for bloom dynamics and toxicity (e.g. Kardinaal *et al.* 2007ab; Wilson *et al.* 2005, 2006; Yoshida *et al.* 2007, 2008). To date, however, little is known about the processes influencing the genotypic composition and diversity of *Microcystis* populations. More specifically, the relative roles of dispersal, priority effects and zooplankton grazing in maintaining local and regional diversity of *Microcystis* bloom populations are not yet understood. In this thesis, we investigated the diversity and distribution patterns of *Microcystis* genotypes from a local to global scale, to assess the roles of historical and environmental processes in structuring genetic diversity. We used the 16S-23S rDNA Internal Transcribed Spacer region (ITS) as a marker to study the genetic diversity of *Microcystis* because it is variable enough for phylogeographic analysis and can be used to directly study natural *Microcystis* populations using Denaturing Gradient Gel Electrophoresis (DGGE) (Otsuka *et al.* 1999; Boyer *et al.* 2001; Kardinaal *et al.* 2007a). In these natural populations, we focused on the following questions: (1) Is there a geographical structuring of *Microcystis* lineages on a global scale? and (2) on a smaller spatial scale, how do local and regional factors influence diversity and population structure in nature? As *Microcystis* strains differ highly in functional traits, we also focused on interactions between *Microcystis* strains as these may be important determinants of population structure. Moreover, we paid attention to interactions between these interstrain interactions, zooplankton grazing and colonisation history in determining *Microcystis* population structure.

7.2 Distribution of *Microcystis* ITS types at different spatial scales

7.2.1 Global scale distribution patterns

In **chapter 2**, a global dataset consisting of 302 rDNA ITS sequences from six continents was used to explore geographical distribution patterns of *Microcystis* lineages. The general lack of phylogenetic structure in our ITS data indicates the absence of independently evolving lineages within this cyanobacterium. Furthermore, the pairwise sequence divergence was low (maximum divergence of 13 % for the full ITS sequence). These findings point at a very close phylogenetic relationship of *Microcystis* strains confirming earlier less extensive studies based on 16S rDNA and DNA-DNA hybridizations (Otsuka *et al.* 1998, 2001), and suggesting these strains belong to a single bacterial species. Taking into account differences in sampling intensity, we found a high continental-scale diversity of *Microcystis* ITS types in Europe, Asia, Africa and South America and no clear global phylogeographic structure, confirming earlier studies (Janse *et al.* 2004). After removing short, hypervariable regions showing incongruent phylogenetic signals from the ITS sequence (see below), no significant differences in average nucleotide divergence of the sampled populations were found between the continents. In total, 62 % of the ITS types detected more than once in our dataset occur on more than one continent, and the ITS types detected only once were often connected with more frequently observed ITS types by only one mutational step. It thus appears that global dispersal of *Microcystis* ITS types is not or little limited at the time scale of ITS evolution. There was no genetic structuring by climatic conditions (temperature, drought) and 82 % of the ITS types detected in more than one climate regime, were found in (sub)tropical as well as cold-temperate climate regimes. However, this does not preclude the possibility that at least some genotypes may be differentially adapted to climatic conditions, and, for example, have a more restricted temperature range than others (Ohkubo *et al.* 1991). Generally, in prokaryotic populations (including cyanobacteria), ubiquitous dispersal as well as geographically restricted distribution patterns attributable to limited dispersal and allopatric divergence have been reported (Rocap *et al.* 2002; Erwin and Thacker 2008; Haande *et al.* 2008; Papke *et al.* 2003; DeChaine *et al.* 2006).This difference may be partly attributable to differences in the resolution of the markers used, in the degree of habitat isolation, or in sampling scale (Dolan 2005, 2006; Martiny at al. 2006). However, in another bloom forming freshwater cyanobacterium, *Cylindrospermopsis*, ITS lineages show geographically restricted distributions (Haande *et al.* 2008), in stark contrast with the situation in *Microcystis*. Although additional phylogeographic studies are needed, this shows that, using comparable molecular markers, different species of freshwater cyanobacteria do differ in the importance of geography in determining lineage distribution. Future studies should be directed at explaining this difference, which might for instance be caused by differential dispersal abilities.

7.2.2 Regional and local scale distribution patterns

A case study of how *Microcystis* ITS population structure and diversity (number of ITS types per sample) vary over small spatiotemporal scales involved a survey of 30 man-made reservoirs in Tigray (Northern Ethiopia) in the framework of a VLIR-IUS project (**chapter 3**). This study revealed that in these recent, man-made reservoirs, half of the *Microcystis* populations contained a single ITS type. Several of these populations formed blooms, indicating that a high diversity is not needed to obtain a high biomass. The average number of ITS types per sample was only 1.8, with a total of 25 different ITS types found in the region. The low local ITS diversity in Tigray seems to be exceptional since other studies revealed several co-dominant ITS types in *Microcystis* populations (Otsuka *et al.* 1999; Janse *et al.* 2004; Humbert *et al.* 2005; Kardinaal *et al.* 2007a; Haande *et al.* 2007). This difference was confirmed by a comparison with the ITS diversity in Flanders (Belgium), a region comparable in size with Tigray and sampled in a similar way (**chapter 3**, supplementary study). We also included data of *Microcystis* ITS diversity in three additional regions in Europe (Denmark, Belgium/The Netherlands and South Spain) and two regions in South America (Brazil and North to South Argentina), and found that, at a larger geographical scale, *Microcystis* populations have on average a higher local diversity in Europe compared to South America. Furthermore, it seems that there are no significant trends in local ITS diversity over latitudinal gradients in Europe and South America. As our datasets showed that regions can strongly differ in their local ITS diversity, we tested the influence of climatic conditions, latitude, habitat availability, lake age, lake characteristics (turbidity, dissolved nutrient concentrations, lake depth and size), as well as *Microcystis* biomass on local ITS diversity. However, none of the factors seems to be the main cause of the observed differences in local ITS diversity. Probably, several factors interact in determining local diversity or different combinations of factors are important for different regions. Unmeasured factors may also play a role. The extreme low diversity in Tigay is probably caused by several factors, including dispersal limitation, as all reservoirs are relatively young and constructed in an area with a history of severe droughts and a low density of water bodies. Additionally, priority effects (**chapter 6**) might be important in determining local diversity. The importance of historical factors in determining local ITS diversity might explain the observed lack of correlations between local diversity and environmental factors, as these are largely determined by chance. A more standardized sampling approach would be useful to reveal the causes of differences in local diversity between individual lakes and between regions.

In Tigray, the relatively low ITS diversity and the availability of biotic and abiotic environmental parameters allowed us to check for the influence of environmental conditions on *Microcystis* population structure and dynamics (**chapter 3**). This is much more difficult when diversity is as high as in Flanders, due to saturation of the DGGE gels. With the exception of pH during the wet season and water transparency in the reservoirs followed during one year, no abiotic factors were found to be correlated with *Microcystis* ITS population structure or dynamics. Temporal changes in population structure were also limited. The wet and dry season had a similar population

structure, and in the eight reservoirs monitored during one year, relatively few changes were observed at a monthly timescale. One ITS type was very dominant in Tigray, and this ITS type had a geographical distribution not correlated to any of the environmental factors studied, suggesting that the observed pattern is caused by dispersal limitation, possibly in combination with priority effects (see below). On the other hand, this successful ITS type occurs on all continents studied (**chapter 2**), implying that long-distance dispersal occurs from time to time. Overall, the emerging picture is that long-distance dispersal allows ITS types to colonize the recently created habitats in Tigray, but is strain-specific and occurs infrequent enough to still limit local and regional ITS diversity in these recently constructed reservoirs.

7.2.3 The usefulness of ITS for inference of phylogenetic relationships and population structure

Our phylogenetic analysis of *Microcystis* ITS sequences obtained from sampling in six continents revealed the presence of short, hypervariable regions showing incongruent phylogenetic signals, possibly as a result of DNA transfer between different *Microcystis* strains or recombination within the genome itself. Recently, Tanabe *et al.* (2007) suggested that recombination is an important evolutionary force for the generation and maintenance of the genetic diversity of *Microcystis*. The vectors involved in genetic exchange among *Microcystis* strains have yet to be identified, but plasmids (Takahashi *et al.* 1996; Wallace *et al.* 2002) and cyanophages (Tucker and Pollard 2005; Yoshida *et al.* 2006) have been isolated from natural populations of *Microcystis* and may be responsible. Kaneko *et al.* (2007) and Frangeul *et al.* (2008) also showed that the genome of two *Microcystis aeruginosa* strains is very plastic and has a high transposon activity. Overall, our results call for a cautious use and interpretation of ITS phylogeny within *Microcystis* and support the plasticity of the *Microcystis* genome.

Otsuka *et al.* (1999) and Janse *et al.* (2004) indicated that *Microcystis* strains with identical rDNA ITS sequences are homogeneous for microcystin production (i.e. they are toxic or non-toxic) using 125 ITS sequences from Europe, Asia and Morocco. Our global ITS phylogeny showed that identical ITS sequences can differ in microcystin production. 17 % and 44 % of the ITS types, for the full ITS and ITS without hypervariable regions respectively, represented by more than one strain of which the capacity to produce microcystins was known, differed in toxin production. These findings suggest that caution is needed when making assumptions about biochemical characteristics based on ITS sequences, at least on a global scale. Possibly, the lack of a consistent relationship between biochemical traits and ITS phylogeny may also be attributed to horizontal gene transfer and recombination (Mikalsen *et al.* 2003; Yoshida *et al.* 2008; Cadel-Six *et al.* 2008). This may also explain the lack of correlation between different functional traits such as colony formation, microcystin production, cell size, and growth rate in strains in culture (**chapter 4**).

As few correlations were found between *Microcystis* ITS population structure and environmental conditions, there also seems to be no clear relationship between ITS type and ecotype. This is in contrast to the cyanobacteria *Prochlorococcus* and *Synecho-*

coccus (Rocap *et al.* 2002; Ferris *et al.* 2003), in which a close relationship between ITS type and ecotype was found. In these genera, several distinct ITS lineages exist, probably without much DNA transfer between them, allowing for a correlation between ITS type and ecotype. One ITS type may represent more than one ecotype in *Microcystis*. If recombination within *Microcystis* occurs frequently, it might be difficult to impossible to find a neutral marker that has a consistent relationship with ecological traits of strains. By recombination, *Microcystis* strains might also be able to adapt to different ecological niches.

7.3 Functional traits and resulting interstrain interactions in *Microcystis*

7.3.1 Functional traits

In **chapter 4**, we showed a high functional diversity of sympatric *Microcystis* strains, which is in agreement with previous studies (Wilson *et al.* 2006; Bañares-España *et al.* 2006; Yoshida *et al.* 2008). We studied the growth rate, microcystin production, colony formation and cell size of eight *Microcystis* strains, isolated from two bloom populations, and detected that these strains differed considerably in these traits and that there were no correlations between the traits. These strains had a minimum and maximum pairwise ITS sequence divergence of only 1.0 and 10.1 % respectively, and after removing the hypervariable regions (**chapter 2**), two groups of strains (NT1-NT2 and T1-NT3-NT4) had identical ITS sequences, indicating that strains with (almost) identical ITS sequences can be functionally very different.

Functional traits can be modulated by environmental variation, for instance by predation (i.e. trait-mediated interactions) (Abrams 1995; Relyea and Yurewicz 2002), which is illustrated in our experiments by *Daphnia* grazing and infochemicals (see also below). However, the differences in functional traits between *Microcystis* strains were more important than the influence of *Daphnia* on these traits, and inducible effects were strongly strain-specific, which may explain the often conflicting results considering the influence of grazing and infochemicals on colony formation in *Microcystis* reported by different studies (Hessen and van Donk 1993; Jang *et al.* 2003; Ha *et al.* 2004; Yang *et al.* 2006) (**chapter 4** and **5**). It has been shown previously that the influence of environmental variation on microcystin production is lower than the interstrain variability (Kardinaal and Visser 2005). In the experiments described in **chapter 4** and **5**, the same *Microcystis* strains were used, however, growth rate and colony formation of particular strains differed between these experiments indicating that some functional traits can vary with slight differences in culture conditions (e.g. culture flask size and material, duration of the experiment, direct or indirect exposure to *Daphnia*) or physiological state of the cultures. Altogether, as *Microcystis* strains are functionally very diverse, it might be worthwhile to study *Microcystis* populations as communities of functionally different strains and study biogeography and influences of environmental factors on *Microcystis* population structure directly based on these functional traits

instead of using molecular markers (Green *et al.* 2008).

7.3.2 Interstrain interactions

Community and population structure are often determined by complex interactions between species and strains (e.g. Callaway and Walker 1997; Hulot *et al.* 2001; Jiang 2007). An important result from this thesis was the detection of strong strain-specific interactions between four *Microcystis* strains differing in functional traits (monoculture growth rate, toxicity and colony formation) (**chapter 5**), suggesting that strain polymorphism in populations of *Microcystis* can be maintained by these complex interactions. In agreement with Kardinaal *et al.* (2007b) we detected strong competitive interactions between *Microcystis* strains, sometimes leading to competitive exclusion of strains. Both allelopathic interactions as well as resource competition may lead to these negative interactions, but more experiments are needed to distinguish between these two possibilities. We also found evidence for the occurrence of positive interactions between *Microcystis* strains, especially in the presence of *Daphnia* grazing, where strong facilitation effects of non-toxic strains being protected by the presence of toxic strains were seen (see below). How toxic and non-toxic strains interact with each other is an intriguing question as the toxicity of a *Microcystis* bloom depends to a large extent on the abundance of toxic strains in the population (Kardinaal *et al.* 2007a).

We showed that interstrain interactions depend on the combination of several functional traits of the strains. More specifically, many of the interactions were in line with expectations based on among-strain variation in growth rate, microcystin production and colony formation and their association with competitive strength. Our experiment generally confirmed the lower competitive strength of toxic strains compared to non-toxic strains when competing for light (Kardinaal *et al.* 2007b), and the competitive advantage of colony formation because of shading by the floating colonies on competing strains and the protection against predation offered by the large colony size (Fulton and Paerl 1987; Zhang *et al.* 2007; Wu and Song 2008).

7.4 Priority effects in *Microcystis* populations

Differences in arrival time of taxa or genotypes present in the regional species or genotype pool can have lasting influence on community and population structure (Drake 1991; Beisner *et al.* 2003; Schröder *et al.* 2005, Fukami *et al.* 2007). Arrival order is determined by regional abundance, dispersal ability and chance. Our experimental study demonstrated the potential for strong strain-specific priority effects (**chapter 6**). In our experiment, we used a time lag of only one week resulting in strong differences in population structure after four weeks of growth. Strikingly, priority effects were found in two directions, with strains either growing faster (positive effects) or slower (negative effects) when inoculated second than when inoculated first. In accordance with previous studies (e.g. Drake 1991; Shorrocks and Bingley 1994; Louette and De Meester 2007), negative effects were very strong, involving a shift from

positive to zero or negative growth, whereas positive effects were generally weaker and especially seen in the presence of grazers (see below). The observed priority effects can be so strong that they may make the difference between a toxic and non-toxic population. In the absence of grazers, first arrival of a non-toxic strain might result in a non-toxic bloom, while first arrival of a toxic strain might result in a toxic bloom. In the presence of grazers, simultaneous colonization of toxic and non-toxic strains leads to the lowest relative abundances of toxic strains. Priority effects will introduce a strong element of stochasticity in the genotypic composition of natural *Microcystis* populations. Functional traits are very important for the occurrence of priority effects too. Arriving first can be advantageous for inferior competitors while arriving simultaneously or second seemed to be mainly advantageous in the presence of grazers, depending on the toxicity and colony formation of the competing strains.

The observation of clear differences in growth rate when inoculated first or second suggests that these priority effects can have long-lasting influences on population structure in natural populations. In regions like Tigray (**chapter 3**), where *Microcystis* populations are relatively young and the regional pool of ITS types is limited, priority effects might be involved in structuring these populations. We found one dominant ITS type in Tigray, successful worldwide, and this ITS type might be one of the first colonists in Tigray, still dominating in many of the reservoirs. Priory effects in nature are expected to be heavily influenced by environmental variation, however, more studies are needed on the occurrence of priority effects in a natural setting.

7.5 Influence of zooplankton grazing on *Microcystis* population structure

7.5.1 *Daphnia* grazing

Zooplankton grazing has a large impact on phytoplankton and cyanobacterial communities (van Gremberghe *et al.* 2008, see appendix; Degans *et al.* 2002; Muylaert *et al.* 2002), and these interactions between zooplankton grazing and community composition depend to a large extent on morphological and biochemical traits of species (Sarnelle 2005). As *Microcystis* populations are composed of strains differing in morphology and production of toxic metabolites, we predicted that zooplankton grazing also influences strain composition of *Microcystis* populations. During the field survey in Tigray, Dejenie *et al.* (unpublished) detected that large *Daphnia* species were the most important grazers in the reservoirs. We found that the relative abundance of the dominant *Microcystis* ITS type was negatively correlated with *Daphnia* biomass, suggesting that *Daphnia* negatively influenced this ITS type relative to other ITS types (**chapter 3**).

The fact that *Daphnia* can influence population structure of *Microcystis* was confirmed by laboratory experiments (**chapter 4**, **5** and **6**). These experiments indicated that *Daphnia* influences *Microcystis* population structure through two mechanisms. Firstly, as shown in **chapter 4** and **5**, *Daphnia* grazing and infochemicals can reduce

growth rate, stimulate microcystin production and induce colony formation in a strain-specific way, the latter two in agreement with earlier studies (Jang *et al.* 2003; Ha *et al.* 2004). The induction of colony formation in a particular strain was advantageous in competition with a unicellular strain (**chapter 5**). Influences of zooplankton infochemicals on growth rate and microcystin production of particular *Microcystis* strains may also change interstrain interactions, but this was not directly investigated in our experiments. Secondly, *Daphnia* grazing can also cause a higher evenness in *Microcystis* populations, both through a weakening of competitive interactions as well as by indirect facilitation effects. Competitive interactions can be weakened directly by grazing more on the dominant species, or indirectly by the recycling of limiting nutrients and the secretion of growth-promoting substances (Vanni and Layne 1997; Elser and Urabe 1999; Vanni 2002). As shown in **chapter 5**, *Daphnia* clearly grazed more easily on unicellular non-toxic strains than on colony forming non-toxic strains in monoculture. This way, *Daphnia* may influence *Microcystis* population structure directly by grazing more on unicellular strains (**chapter 5**), although the latter should be proven quantitatively by experiments including a large number of mixed populations of unicellular and colony forming strains in which the ingestion rate of *Daphnia* on the different strains is determined. Strong facilitation effects were due to non-toxic strains benefiting from the protection offered by toxic strains in the presence of predation. This grazer-mediated facilitation enhanced the coexistence of toxic and non-toxic *Microcystis* strains and is similar to herbivory protection of palatable plants by unpalatable plants (Callaway *et al.* 2005; Graff *et al.* 2007). Similarly, in **chapter 6** we showed that grazing diminished the frequency of negative priority effects, and that in the presence of *Daphnia*, unicellular or non-toxic *Microcystis* strains perform better when inoculated simultaneously or after colony forming or toxic strains than when inoculated first.

As stated in **chapter 1**, the effect of *Daphnia* on *Microcystis* biomass is debated (Christoffersen *et al.* 1993; Matveev *et al.* 1994; Ghadouani *et al.* 2003; Sarnelle 2007). However, based on the results from our laboratory experiments (**chapter 5** and **6**) and the field study in Tigray (**chapter 3**, Dejenie *et al.* unpublished), we postulate that *Daphnia* is indeed able to control the development of *Microcystis* blooms, but that this ability probably depends strongly on the functional traits of *Microcystis* strains (**chapter 5**) and on the initial conditions (initial *Daphnia* and *Microcystis* density), which is shown in the pilot experiment described in **chapter 5**, and is in agreement with Sarnelle (2007). In particular, a bloom with only non-toxic unicellular strains can probably only develop in the absence of grazers, as our experiments showed that, although they have an equally high growth rate as toxic strains in the absence of grazing, non-toxic unicellular strains can only reach high cell densities in the presence of *Daphnia* when growing together with toxic strains.

7.5.2 Selective predation

While we focused on the importance of *Daphnia* grazing in this thesis, other more selective predators might be as important as *Daphnia* in structuring *Microcystis* po-

pulations. Copepods for example can select cyanobacteria based on size and chemical characteristics like toxicity and nutritional value (DeMott and Moxter 1991). The cladoceran *Bosmina* also grazes selectively (DeMott and Kerfoot 1982) and has been shown to structure the cyanobacterial community in the mesotrophic lake Blaarmeersen in Ghent, Belgium (van Gremberghe *et al.* 2008, see appendix). Several selective predators might be able to select between individual *Microcystis* strains based on their toxicity or morphology. Furthermore, the first results of a high-resolution two-year study of *Microcystis* bloom dynamics in a small urban pond (Westveldpark, Ghent - see cover picture), including an assessment of population structure with microscopy and DGGE of ITS, showed that in 2007 two *Microcystis* morphotypes could be distinguished, which corresponded to the two dominant ITS types present (Van Wichelen *et al.* unpublished results). The *aeruginosa* type strongly dominated in June, but was almost completely replaced in a single week by the *viridis* type, that dominated the rest of the growth season. This shift coincided with a temporary bloom biomass decrease. Apparently amoebae were responsible for this dramatic shift and they reached a pronounced peak in abundance at the time of the shift and infected a high percentage of the *aeruginosa* colonies. From the first results of 2008, a similar pattern emerges. These data indicate that amoebae may graze selectively on *Microcystis* colonies, thereby strongly influencing population structure.

7.6 Influence of history, grazing and interstrain interactions on *Microcystis* population structure: synthesis

This thesis evidenced the high genotypic and functional diversity of *Microcystis* at local, regional and global scale, and provided information about the importance of history (dispersal limitation and priority effects), grazing and interstrain interactions on diversity and population structure. We can conclude that all these factors may influence the composition of *Microcystis* populations in a highly strain-specific way. At global scale and over long time spans, dispersal limitation seems not very important for *Microcystis*, though in regions with a low density of young water bodies, differences in dispersal rate and, possibly, priority effects among *Microcystis* genotypes can be important in structuring local populations and limiting regional and local diversity. Importantly, the toxicity of a bloom may also be influenced by the arrival order of strains. Interstrain interactions, mediated by functional traits, are probably one of the major factors structuring *Microcystis* populations given the highly complex positive and negative interactions between sympatric strains. These interactions also determine the sign and strength of priority effects. Furthermore, grazing of zooplankton can influence the population structure and temporal dynamics of *Microcystis* populations. The interaction between *Daphnia* and *Microcystis* seems to depend to a large extent on toxicity and colony formation of *Microcystis* strains. *Daphnia* may also influence, although relatively weak compared to interstrain differences, the functional traits themselves of *Microcystis* strains. In addition, grazing can change the strength and direction

of interstrain interactions and priority effects, as illustrated by predator-mediated facilitation in the presence of *Daphnia*.

Furthermore, this thesis also suggests the occurrence of recombination within the rDNA ITS region of *Microcystis* possibly resulting in the absence a clear relationship between ITS type and ecotype.

Concluding, history, grazing and interstrain interactions influence *Microcystis* population structure in a strongly strain-specific way depending on the functional traits of the strains, and it might be a fruitful approach to view *Microcystis* populations as communities of functionally different strains and analyze their ecology (e.g. occurrence, blooms and toxicity) from that perspective.

7.7 Perspectives

Although this thesis elucidates several important aspects about the ecology of *Microcystis* populations, more research is definitely needed for a better understanding of the structure and functioning of *Microcystis* bloom populations. Below we discuss some of the questions resulting from our work which should be answered in the future.

The local diversity of *Microcystis* populations differs between regions, but it is not clear yet which factors influence these differences. A more intensive and standardized sampling of additional regions, in cold-temperate as well as (sub)tropical climates, is needed to understand the effects of various environmental and historical factors influencing *Microcystis* diversity. While we did not find a correlation between temperature and *Microcystis* ITS diversity, some strains may still have a more restricted temperature optimum than others and strains may be adapted to local climatic conditions. Bearing global warming in mind, it would be interesting to compare the growth rate of *Microcystis* strains, isolated from different climates, at different temperatures to see whether these strains are adapted to the local temperature regime.

Our study does not show a clear relationship between ITS type and ecotype. The presence of hypervariable regions in the ITS sequence suggests that regular horizontal gene transfer and recombination might be the reason for the lack of a consistent relationship. Future research has to reveal if distinct *Microcystis* ecotypes (isolated from the same lake and from different lakes occurring in different geographical localities) can be discriminated by molecular markers. In addition, fingerprint techniques (e.g. AFLP, RFLP, RAPD) should be tested for their power to discriminate between different ecotypes.

We found complex positive as well as negative interactions between *Microcystis* strains. However, more experiments are needed to understand the mechanisms leading to these interactions. For instance, are negative interactions the result of allelopathic interactions or resource competition and how important are direct facilitative interactions and niche complementarity? Experiments in which strains are grown directly

together in batch or continuous culture, and experiments in which filtered culture medium from a strain is added to another strain would be very informative. A large part of the interactions in our experiments could be predicted based on microcystin production and colony formation, however, experiments including a larger number of *Microcystis* strains, and characterized by more functional traits (including metabolic diversity) would be useful to better understand these interactions.

In this thesis, evidence was found for the occurrence of priority effects in experimental *Microcystis* populations. However, field studies are needed to prove the occurrence of priority effects in nature, and to understand how priority effects are influenced by environmental variation. This should be done by monitoring the colonization of newly created ponds (as in Louette *et al.* 2007). In addition, in situ experiments in ponds in which the inoculation order of *Microcystis* strains is manipulated would allow testing hypotheses generated by laboratory experiments and field monitoring.

Microcystis produces a high number of secondary metabolites, several of them being toxic to *Daphnia* (Czarnecki *et al.* 2006; Welker *et al.* 2006). In this thesis we focused only on microcystins in predicting and understanding interactions between *Microcystis* strains and interactions with *Daphnia*, but it would be interesting to investigate the effect of other toxins such as microviridin J (Rohrlack *et al.* 2004) and the neurotoxic amino acid BMAA (β-N-methylamino-L-alanine) (Metcalf *et al.* 2008). In addition, chemotyping of *Microcystis* strains by MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) provides a fingerprint of the metabolites produced by different strains (Welker *et al.* 2004, 2006, 2007), and could be used as an alternative to molecular markers for characterizing *Microcystis* strains used in experiments.

In this thesis we focused mainly on the importance of *Daphnia* grazing on population structure of *Microcystis*. However, as we found in a recent study (Van Wichelen *et al.* unpublished), amoebae seem also capable of structuring *Microcystis* populations and this interaction has, as far as we know, not been studied yet. Laboratory experiments with amoebae grazing on different *Microcystis* strains and more field studies in which the population structure of *Microcystis* is related to amoebae infections are needed. Other zooplankton (e.g. copepods, cladocerans other than *Daphnia*) and parasites (chytrid fungi, viruses) should also be investigated for their potential to structure *Microcystis* populations.

References

Abrams, P. A. 1995. Implications of dynamically variable traits for identifying, classifying, and measuring direct and indirect effects in ecological communities. American Naturalist 146: 112-134.

Bañares-España, E., Lopez-Rodas, V., Salgado, C., Costas, E. and Flores-Moya, A. 2006. Inter-strain variability in the photosynthetic use of inorganic carbon, exemplified by the pH compensation point, in the cyanobacterium *Microcystis aeruginosa*. Aquatic Botany 85: 159-162.

Beisner, B. E., Haydon, D. T. and Cuddington, K. 2003. Alternative stable states in ecology. Frontiers in Ecology and the Environment 1: 376-382.

Boyer, S. L., Flechtner, V. R. and Johansen, J. R. 2001. Is the 16S-23S rRNA internal transcribed spacer region a good tool for use in molecular systematics and population genetics? A case study in cyanobacteria. Molecular Biology and Evolution 18: 1057-1069.

Cadel-Six, S., Dauga, C., Castets, A. M., Rippka, R., Bouchier, C., de Marsac, N. T. and Welker, M. 2008. Halogenase genes in nonribosomal peptide synthetase gene clusters of *Microcystis* (Cyanobacteria): Sporadic distribution and evolution. Molecular Biology and Evolution 25: 2031-2041.

Callaway, R. M., Kikodze, D., Chiboshvili, M. and Khetsuriani, L. 2005. Unpalatable plants protect neighbors from grazing and increase plant community diversity. Ecology 86: 1856-1862.

Callaway, R. M. and Walker, L. R. 1997. Competition and facilitation: A synthetic approach to interactions in plant communities. Ecology 78: 1958-1965.

Carmichael, W. W. 1994. Toxins of Cyanobacteria. Scientific American 270: 78-86.

Carmichael, W. W., Azevedo, S. M. F. O., An, J. S., Molica, R. J. R., Jochimsen, E. M., Lau, S., Rinehart, K. L., Shaw, G. R. and Eaglesham, G. K. 2001. Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. Environmental Health Perspectives 109: 663-668.

Chorus, I. and Bartram, J. (eds). WHO. 1999. Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. E & FN Spon. London, United Kingdom.

Christoffersen, K., Riemann, B., Klysner, A. and Sondergaard, M. 1993. Potential role of fish predation and natural populations of zooplankton in structuring a plankton community in eutrophic lake water. Limnology and Oceanography 38: 561-573.

Codd, G. A., Lindsay, J., Young, F. M., Morrison, L. F. and Metcalf, J. S. 2005. Harmful cyanobacteria: From mass mortalities to management measures. In: Huisman, J., Matthijs, H. C. P. and Visser, P. M. (eds). Harmful Cyanobacteria. Springer, Dordrecht, The Netherlands. pp 1-24.

Czarnecki, O., Henning, M., Lippert, I. and Welker, M. 2006. Identification of peptide metabolites of *Microcystis* (Cyanobacteria) that inhibit trypsin-like activity in planktonic herbivorous *Daphnia* (Cladocera). Environmental Microbiology 8: 77-87.

DeChaine, E. G., Bates, A. E., Shank, T. M. and Cavanaugh, C. M. 2006. Off-axis symbiosis found: characterization and biogeography of bacterial symbionts of *Bathymodiolus* mussels from Lost City hydrothermal vents. Environmental Microbiology 8: 1902-1912.

Degans, H., Zollner, E., Van der Gucht, K., De Meester, L. and Jurgens, K. 2002. Rapid *Daphnia*-mediated changes in microbial community structure: an experimental study. FEMS Microbiology Ecology 42: 137-149.

DeMott, W. R. and Kerfoot, W. C. 1982. Competition among Cladocerans - Nature of the Interaction between *Bosmina* and *Daphnia*. Ecology 63: 1949-1966.

DeMott, W. R. and Moxter, F. 1991. Foraging on cyanobacteria by copepods. Responses to chemical defenses and resource abundance. Ecology 72: 1820-1834.

Dolan, J. R. 2005. An introduction to the biogeography of aquatic microbes. Aquatic Microbial Ecology 41: 39-48.

Dolan, J. R. 2006. Microbial biogeography? Journal of Biogeography 33: 199-200.

Drake, J. A. 1991. Community-assembly mechanics and the structure of an experimental species ensemble. American Naturalist 137: 1-26.

Elser, J. J. and Urabe, J. 1999. The stoichiometry of consumer-driven nutrient recycling: Theory, observations, and consequences. Ecology 80: 735-751.

Erwin, P. M. and Thacker, R. W. 2008. Cryptic diversity of the symbiotic cyanobacterium *Synechococcus spongiarum* among sponge hosts. Molecular Ecology 17: 2937-2947.

Falconer, I. R. 1999. An overview of problems caused by toxic blue-green algae (cyanobacteria) in drinking and recreational waters. Environmental Toxicology 14: 5-12.

Ferris, M. J., Kuhl, M., Wieland, A. and Ward, D. M. 2003. Cyanobacterial ecotypes in different optical microenvironments of a 68 degrees C hot spring mat community revealed by 16S-23S rRNA internal transcribed spacer region variation. Applied and Environmental Microbiology 69: 2893-2898.

Frangeul, L., Quillardet, P., Castets, A. M., Humbert, J. F., Matthijs, H. C. P., Cortez, D., Tolonen, A., Zhang, C. C. *et al.* 2008. Highly plastic genome of *Microcystis aeruginosa* PCC 7806, a ubiquitous toxic freshwater cyanobacterium. Bmc Genomics 9.

Fukami, T., Beaumont, H. J. E., Zhang, X. X. and Rainey, P. B. 2007. Immigration history controls diversification in experimental adaptive radiation. Nature 446: 436-439.

Fulton, R. S. and Paerl, H. W. 1987. Effects of colonial morphology on zooplankton utilization of algal resources during blue green algal (*Microcystis aeruginosa*) blooms. Limnology and Oceanography 32: 634-644.

Ghadouani, A., Pinel-Alloul, B. and Prepas, E. E. 2003. Effects of experimentally induced cyanobacterial blooms on crustacean zooplankton communities. Freshwater Biology 48: 363-381.

Graff, P., Aguiar, M. R. and Chaneton, E. J. 2007. Shifts in positive and negative plant interactions along a grazing intensity gradient. Ecology 88: 188-199.

Green, J. L., Bohannan, B. J. M. and Whitaker, R. J. 2008. Microbial biogeography: From taxonomy to traits. Science 320: 1039-1043.

Ha, K., Jang, M. H. and Takamura, N. 2004. Colony formation in planktonic algae induced by zooplankton culture media filtrate. Journal of Freshwater Ecology 19: 9-16.

Haande, S., Ballot, A., Rohrlack, T., Fastner, J., Wiedner, C. and Edvardsen, B. 2007. Diversity of *Microcystis aeruginosa* isolates (Chroococcales, Cyanobacteria) from East-African water bodies. Archives of Microbiology 188: 15-25.

Haande, S., Rohrlack, T., Ballot, A., Roberg, K., Skulberg, R., Beck, M. and Wiedner, C. 2008. Genetic characterisation of *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria) isolates from Africa and Europe. Harmful Algae 7: 692-701.

Hessen, D. O. and van Donk, E. 1993. Morphological changes in *Scenedesmus* induced by substances released from *Daphnia*. Archiv Für Hydrobiologie 127: 129-140.

Huisman, J., Matthijs, H. C. P. and Visser, P. M. 2005. Harmful cyanobacteria. Springer, Dordrecht, The Netherlands. 243 pp.

Hulot, F. D., Morin, P. J. and Loreau, M. 2001. Interactions between algae and the microbial loop in experimental microcosms. Oikos 95: 231-238.

Humbert, J. F., Duris-Latour, D., Le Berre, B., Giraudet, H. and Salencon, M. J. 2005. Genetic diversity in *Microcystis* populations of a French storage reservoir assessed by sequencing of the 16S-23S rRNA intergenic spacer. Microbial Ecology 49: 308-314.

Jang, M. H., Ha, K., Joo, G. J. and Takamura, N. 2003. Toxin production of cyanobacteria is increased by exposure to zooplankton. Freshwater Biology 48: 1540-1550.

Janse, I., Kardinaal, W. E. A., Meima, M., Fastner, J., Visser, P. M. and Zwart, G. 2004. Toxic and nontoxic *Microcystis* colonies in natural populations can be differentiated on the basis of rRNA gene internal transcribed spacer diversity. Applied and Environmental Microbiology 70: 3979-3987.

Jiang, L. 2007. Negative selection effects suppress relationships between bacterial diversity and ecosystem functioning. Ecology 88: 1075-1085.

Kaneko, T., Nakajima, N., Okamoto, S., Suzuki, I., Tanabe, Y., Tamaoki, M., Nakamura, Y., Kasai, F. *et al.* 2007. Complete genomic structure of the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* NIES-843. DNA Research 14: 247-256.

Kardinaal, W. E. A., Janse, I., Kamst-van Agterveld, M., Meima, M., Snoek, J., Mur, L. R., Huisman, J., Zwart, G. and Visser, P. M. 2007a. *Microcystis* genotype succession in relation to microcystin concentrations in freshwater lakes. Aquatic Microbial Ecology 48: 1-12.

Kardinaal, W. E. A., Tonk, L., Janse, I., Hol, S., Slot, P., Huisman, J. and Visser, P. M. 2007b. Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*. Applied and Environmental Microbiology 73: 2939-2946.

Kardinaal, W. E. A. and Visser, P. M. 2005. Dynamics of cyanobacterial toxins. Sources of variability in microcystin concentrations. In: Huisman, J., Matthijs, H. C. P. and Visser, P. M. (eds). Harmful Cyanobacteria. Springer, Dordrecht, The Netherlands. pp. 41-64.

Louette, G. and De Meester, L. 2007. Predation and priority effects in experimental zooplankton communities. Oikos 116: 419-426.

Louette, G., Vanoverbeke, J., Ortells, R. and De Meester, L. 2007. The founding mothers: the genetic structure of newly established *Daphnia* populations. Oikos 116: 728-741.

Martiny, J. B. H., Bohannan, B. J. M., Brown, J. H., Colwell, R. K., Fuhrman, J. A., Green, J. L., Horner-Devine, M. C., Kane, M. *et al.* 2006. Microbial biogeography: putting microorganisms on the map. Nature Reviews Microbiology 4: 102-112.

Matveev, V., Matveeva, L. and Jones, G. J. 1994. Study of the ability of *Daphnia carinata* King to control phytoplankton and resist cyanobacterial toxicity - implications for biomanipulation in Australia. Australian Journal of Marine and Freshwater Research 45: 889-904.

Metcalf, J. S., Banack, S. A., Lindsay, J., Morrison, L. F., Cox, P. A. and Codd, G. A. 2008. Co-occurrence of beta-N-methylamino-L-alanine, a neurotoxic amino acid with other cyanobacterial toxins in British waterbodies, 1990-2004. Environmental Microbiology 10: 702-708.

Mikalsen, B., Boison, G., Skulberg, O. M., Fastner, J., Davies, W., Gabrielsen, T. M., Rudi, K. and Jakobsen, K. S. 2003. Natural variation in the microcystin synthetase operon *mcy*ABC and impact on microcystin production in *Microcystis* strains. Journal of Bacteriology 185: 2774-2785.

Muylaert, K., Van der Gucht, K., Vloemans, N., De Meester, L., Gillis, M. and Vyverman, W. 2002. Relationship between bacterial community composition and bottom-up versus top-down variables in four eutrophic shallow lakes. Applied and Environmental Microbiology 68: 4740-4750.

Ohkubo, N., Yagi, O. and Okada, M. 1991. Effects of temperature and illumination on the growth of blue-green alga *Microcystis viridis*. Japanese Journal of Limnology 52: 255-261.

Otsuka, S., Suda, S., Li, R. H., Watanabe, M., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 1998. 16S rDNA sequences and phylogenetic analyses of *Microcystis* strains with and without phycoerythrin. FEMS Microbiology Letters 164: 119-124.

Otsuka, S., Suda, S., Li, R. H., Watanabe, M., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 1999. Phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis* based on 16S to 23S internal transcribed spacer sequence. FEMS Microbiology Letters 172: 15-21.

Otsuka, S., Suda, S., Shibata, S., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 2001. A proposal for the unification of five species of the cyanobacterial genus *Microcystis* Kutzing ex Lemmermann 1907 under the

Rules of the Bacteriological Code. International Journal of Systematic and Evolutionary Microbiology 51: 873-879.
Papke, R. T., Ramsing, N. B., Bateson, M. M. and Ward, D. M. 2003. Geographical isolation in hot spring cyanobacteria. Environmental Microbiology 5: 650-659.
Relyea, R. A. and Yurewicz, K. L. 2002. Predicting community outcomes from pairwise interactions: integrating density- and trait-mediated effects. Oecologia 131: 569-579.
Rocap, G., Distel, D. L., Waterbury, J. B. and Chisholm, S. W. 2002. Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S-23S ribosomal DNA internal transcribed spacer sequences. Applied and Environmental Microbiology 68: 1180-1191.
Rohrlack, T., Christoffersen, K., Kaebernick, M. and Neilan, B. A. 2004. Cyanobacterial protease inhibitor microviridin J causes a lethal molting disruption in *Daphnia pulicaria*. Applied and Environmental Microbiology 70: 5047-5050.
Sarnelle, O. 2005. *Daphnia* as keystone predators: effects on phytoplankton diversity and grazing resistance. Journal of Plankton Research 27: 1229-1238.
Sarnelle, O. 2007. Initial conditions mediate the interaction between *Daphnia* and bloom-forming cyanobacteria. Limnology and Oceanography 52: 2120-2127.
Schröder, A., Persson, L. and De Roos, A. M. 2005. Direct experimental evidence for alternative stable states: a review. Oikos 110: 3-19.
Shorrocks, B. and Bingley, M. 1994. Priority effects and species coexistence. Experiments with fungal-breeding *Drosophila*. Journal of Animal Ecology 63: 799-806.
Takahashi, I., Hayano, D., Asayama, M., Masahiro, F., Watahiki, M. and Shirai, M. 1996. Restriction barrier composed of an extracellular nuclease and restriction endonuclease in the unicellular cyanobacterium *Microcystis sp.* FEMS Microbiology Letters 145: 107-111.
Tanabe, Y., Kasai, F. and Watanabe, M. M. 2007. Multilocus sequence typing (MLST) reveals high genetic diversity and clonal population structure of the toxic cyanobacterium *Microcystis aeruginosa*. Microbiology-Sgm 153: 3695-3703.
Tucker, S. and Pollard, P. 2005. Identification of cyanophage Ma-LBP and infection of the cyanobacterium *Microcystis aeruginosa* from an Australian subtropical lake by the virus. Applied and Environmental Microbiology 71: 629-635.
van Gremberghe, I., Van Wichelen, J., Van der Gucht, K., Vanormelingen, P., D'Hondt, S., Boutte, C., Wilmotte, A. and Vyverman, W. 2008. Covariation between zooplankton community composition and cyanobacterial community dynamics in Lake Blaarmeersen (Belgium). FEMS Microbiology Ecology 63: 222-237.
Vanni, M. J. 2002. Nutrient cycling by animals in freshwater ecosystems. Annual Review of Ecology and Systematics 33: 341-370.
Vanni, M. J. and Layne, C. D. 1997. Nutrient recycling and herbivory as mechanisms in the "top-down" effect of fish on algae in lakes. Ecology 78: 21-40.
Wallace, M. M., Miller, D. W. and Raps, S. 2002. Characterization of pMa025, a plasmid from the cyanobacterium *Microcystis aeruginosa* UV025. Archives of Microbiology 177: 332-338.
Welker, M., Brunke, M., Preussel, K., Lippert, I. and von Dohren, H. 2004. Diversity and distribution of *Microcystis* (Cyanobacteria) oligopeptide chemotypes from natural communities studied by single-colony mass spectrometry. Microbiology-Sgm 150: 1785-1796.
Welker, M., Marsalek, B., Sejnohova, L. and von Dohren, H. 2006. Detection and identification of oligopeptides in *Microcystis* (cyanobacteria) colonies: Toward an understanding of metabolic diversity. Peptides 27: 2090-2103.
Welker, M., Sejnohova, L., Nemethova, D., von Dohren, H., Jarkovsky, J. and Marsalek, B. 2007. Seasonal shifts in chemotype composition of *Microcystis sp.* communities in the pelagial and the sediment of a shallow reservoir. Limnology and Oceanography 52: 609-619.
Wilson, A. E., Sarnelle, O., Neilan, B. A., Salmon, T. P., Gehringer, M. M. and Hay, M. E. 2005. Genetic variation of the bloom-forming cyanobacterium *Microcystis aeruginosa* within and among lakes: Implications for harmful algal blooms. Applied and Environmental Microbiology 71: 6126-6133.
Wilson, A. E., Wilson, W. A. and Hay, M. E. 2006. Intraspecific variation in growth and morphology of the bloom-forming cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 72: 7386-7389.
Wu, Z. X. and Song, L. R. 2008. Physiological comparison between colonial and unicellular forms of *Microcystis aeruginosa* Kutz. (Cyanobacteria). Phycologia 47: 98-104.
Yang, Z., Kong, F. X., Shi, X. L. and Cao, H. S. 2006. Morphological response of *Microcystis aeruginosa* to grazing by different sorts of zooplankton. Hydrobiologia 563: 225-230.
Yoshida, M., Yoshida, T., Satomi, M., Takashima, Y., Hosoda, N. and Hiroishi, S. 2008. Intra-specific phenotypic and genotypic variation in toxic cyanobacterial *Microcystis* strains. Journal of Applied Microbiology 105: 407-415.
Yoshida, M., Yoshida, T., Takashima, Y., Hosoda, N. and Hiroishi, S. 2007. Dynamics of microcystin-producing and non-microcystin-producing *Microcystis* populations is correlated with nitrate concentration in a Japanese lake. FEMS Microbiology Letters 266: 49-53.
Yoshida, T., Takashima, Y., Tomaru, Y., Shirai, Y., Takao, Y., Hiroishi, S. and Nagasaki, K. 2006. Isolation and characterization of a cyanophage infecting the toxic cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 72: 1239-1247.
Zhang, M., Kong, F. X., Tan, X., Yang, Z., Cao, H. S. and Xing, P. 2007. Biochemical, morphological, and genetic

variations in *Microcystis aeruginosa* due to colony disaggregation. World Journal of Microbiology & Biotechnology 23: 663-670.

Chapter 8

Summary / Samenvatting

Summary

Cyanobacteria play a key role in the functioning of inland and marine aquatic ecosystems, as they are a major constituent of phytoplankton communities. Massive growth of cyanobacterial phytoplankton, generally in response to eutrophication, can lead to dense, often almost monospecific water blooms. One of the most widespread toxic bloom forming cyanobacteria is *Microcystis* (order Chroococcales), which has, as a result, received much scientific attention (Huisman *et al.* 2005). Blooms of *Microcystis* often occur during summer in temperate regions and year-round in (sub)tropical regions in ponds and lakes with a high nutrient load and stable water column. Studies based on several molecular markers have shown that naturally occurring *Microcystis* populations are generally composed of several genotypes, that temporal turnover in genetic composition can be high and that neighbouring lakes may harbour different genotypes (e.g. Janse *et al.* 2004; Wilson *et al.* 2005; Kardinaal *et al.* 2007). *Microcystis* strains isolated from a single bloom population often also differ in ecological and physiological traits (Wilson *et al.* 2006; Bañares-España *et al.* 2006; Yoshida *et al.* 2008). Particular genotypes of *Microcystis* produce the hepatotoxin microcystin, which is highly toxic to eukaryotes. Moreover, the toxicity of a *Microcystis* bloom is to a large extent determined by its strain composition (Kardinaal *et al.* 2007).

Although these blooms have been extensively studied, little is known about the mechanisms generating local and regional diversity of *Microcystis* bloom populations nor about the factors affecting population structure. This is an important gap in our knowledge because a better understanding of these mechanisms can be useful in predicting bloom formation and bloom toxicity, and may eventually help preventing bloom occurrence.

Generally, community and population structure are determined by an interplay of historical and environmental factors, including biotic interactions, that can also interact with each other. Historical factors may influence the genotypic composition of natural *Microcystis* populations by two mechanisms: (1) through contemporary dispersal limitation leading to differences in both the local and regional genotype pool, and (2) by long-lasting effects of migration history on local population structure, as

differences in the arrival order of genotypes from the regional genotype pool in a new habitat may have lasting consequences for community or population structure, resulting in so-called priority effects (Morin 1984; Drake 1991). When *Microcystis* strains have reached a particular habitat, they have to cope with the local abiotic and biotic conditions in order to establish themselves (Leibold *et al.* 2004; Ricklefs 2004). Since *Microcystis* strains are physiologically very diverse different strains probably react in a strain-specific manner to local environmental conditions.

In this thesis we investigated the diversity and distribution patterns of *Microcystis* ITS types (16S-23S rDNA Internal Transcribed Spacer) from a local to global scale, to assess the roles of historical and environmental processes in structuring genotypic diversity. We used the ITS as a marker to study the genetic diversity of *Microcystis* because it is variable enough for phylogeographic analysis and can be used to directly study natural *Microcystis* populations using Denaturing Gradient Gel Electrophoresis (DGGE) (Otsuka *et al.* 1999; Boyer *et al.* 2001; Kardinaal *et al.* 2007). As *Microcystis* strains differ highly in functional traits we also focused on interactions between *Microcystis* strains as these may be important determinants of population structure. Moreover, we paid attention to interactions between these interstrain interactions, zooplankton grazing and colonisation history in determining *Microcystis* population structure.

In a first part of this thesis (**chapter 2** and **3**), we studied dispersal, diversity and genotypic structure of natural *Microcystis* populations at different spatial scales, including the influence of abiotic and biotic factors on diversity and population structure. In a second part of this thesis (**chapter 4**, **5** and **6**), we studied functional traits and interactions between *Microcystis* strains, and the influence of priority effects and *Daphnia* grazing on population structure by laboratory experiments.

In **chapter 2**, we determined the global phylogeography of *Microcystis* based on an extensive dataset of 302 rDNA ITS sequences sampled from six continents. Phylogenetic analyses revealed largely unresolved and non-diverging relationships among ITS types, indicating the absence of independently evolving lineages in *Microcystis*. Our analyses also revealed the presence of short, hypervariable regions in the ITS showing incongruent phylogenetic signals, possibly as a result of DNA transfer between different *Microcystis* strains or recombination within the genome itself. Overall, our data revealed a high diversity of *Microcystis* ITS types in all continents and the presence of several identical ITS types on more than one continent. Analysis of variance showed a lack of overall genetic differentiation between continents. Our results suggest high dispersal potential of *Microcystis* ITS types on a global scale. To what extent environmental selection is important for the distribution of ITS types is largely unclear, but climatic conditions apparently do not have a considerable impact.

In **chapter 3**, we studied genetic diversity and structure of *Microcystis* populations in 30 small man-made reservoirs in Tigray (Northern Ethiopia) using denaturing gradient gel electrophoresis of the rDNA ITS, and assessed the importance of local environmental conditions and geographic position of the reservoirs for the observed

patterns. The analyses showed that both the regional and local *Microcystis* ITS diversity in Tigray was low compared to other regions, as several dense blooms contained only a single ITS type. While a possible role of climate cannot be excluded, a plausible explanation for the relatively low ITS diversity is the recent construction of the reservoirs in a semi-arid region with a low historic availability of suitable *Microcystis* habitat. Especially one non-toxic ITS type dominated many *Microcystis* blooms but appeared restricted in its geographical distribution. Taken together, this suggests that long-distance dispersal is infrequent enough to (still) limit local and regional ITS diversity and to cause the geographically restricted distribution of the dominant ITS type. In addition to the effects of dispersal limitation, the significant correlations of *Microcystis* ITS population structure with abiotic variables (water clarity, pH) and zooplankton (especially *Daphnia* biomass) suggest an important additional influence of these factors on *Microcystis* population structure in the reservoirs of Tigray.

In **chapter 4**, we conducted a laboratory experiment to investigate the influence of *Daphnia* infochemicals on growth rate, microcystin production, colony formation and cell size of eight *Microcystis* strains isolated from two lakes. The experiment was composed of four treatments: 1) a control using WC medium, 2) addition of *Scenedesmus obliquus* culture medium filtrate, 3) addition of *Daphnia magna* culture medium filtrate, 4) addition of sodium octyl sulphate, a commercially available *Daphnia* infochemical. Our results showed that sympatric strains differed strongly for the measured functional traits, while no correlations between traits were found. *Daphnia* infochemicals generally had a weak influence on growth rate, microcystin production and colony formation of *Microcystis* strains as compared to the inter-strain variability, while existing inducible effects were often highly strain-specific. *Daphnia* culture medium filtrate and, to a lesser extent, sodium octyl sulphate had a negative influence on the growth of half of the strains and stimulated microcystin production in one strain, but this last effect was not *Daphnia*-specific as *Scenedesmus* culture medium filtrate had the same effect. *Daphnia* culture medium filtrate also induced colony formation in one strain.

In **chapter 5**, we conducted a laboratory experiment to investigate whether interactions among *Microcystis* strains can be predicted from functional traits, and whether the outcome of competition between strains is influenced by a grazer. Two toxic and two non-toxic *Microcystis* strains, isolated from a single lake and studied in chapter 4, were grown during four weeks as single strains, in all possible combinations of two strains and all together, in the presence and absence of *Daphnia magna*. The relative abundance of strains in the populations was assessed using DGGE, and the growth rate of each strain in mixed populations was compared to its growth rate in monoculture to determine interactions between strains. The observed interactions were strain-specific, and the relative abundances of strains in mixed populations could be partially explained by taking toxicity and colony formation into account. Importantly, some of the interactions were strongly altered by the presence of *Daphnia*. *Daphnia* induced colony formation in one strain, which then became a better competitor. *Daphnia* grazing also caused a higher evenness in the populations, both through a weakening of competitive

interactions as well as by facilitation effects. Strong facilitation effects were due to non-toxic strains benefiting from the protection offered by toxic strains in the presence of predation. Overall, our results emphasize the presence of strong competitive interactions between *Microcystis* genotypes in the absence of grazing, whereas indirect positive interactions are prevalent in the presence of a generalist grazer. Our results suggest that differences in functional traits and grazer-mediated facilitation effects may enhance coexistence of *Microcystis* strains, including toxic and non-toxic strains.

In **chapter 6**, we conducted a laboratory experiment to explore whether priority effects are important in determining strain composition of *Microcystis*, and whether they are influenced by the presence of a grazer. The same four *Microcystis* strains as described in chapter 5 were grown during four weeks in the laboratory in all possible pairwise combinations, with the two strains either inoculated at the same time or with a time lag of one week, in the presence and absence of grazing *Daphnia magna*. Changes in the relative abundance of the strains in the mixed populations were monitored using DGGE, and the growth rate of each strain in the mixtures was determined for the last two weeks of the experiment. Effects of inoculation order on the growth rate of strains were considered evidence for long-lasting priority effects. We observed strong effects of inoculation order on the final population structure, which were influenced by grazing *Daphnia*. The priority effects were strain-specific and occurred in two directions, with strains either growing faster or slower when inoculated second than when inoculated first. The observed interactions and priority effects were in line with expectations based on variation in toxicity and colony formation among the strains and their consequences for competitive strength and grazing protection.

We can conclude from this thesis that history (dispersal limitation and priority effects), grazing and interstrain interactions influence *Microcystis* population structure in a strongly strain-specific way depending on the functional traits of the strains, and that these factors also interact with each other. At global scale and over long time spans, dispersal limitation seems not very important for *Microcystis*, though in regions with a low density of young water bodies, differences in dispersal rate and, possibly, priority effects among *Microcystis* genotypes can be important in structuring local populations and limiting regional and local diversity. Importantly, the toxicity of a bloom may also be influenced by the arrival order of strains. Interstrain interactions, mediated by functional traits, are probably one of the major factors structuring *Microcystis* populations given the highly complex positive and negative interactions between sympatric strains. These interactions also determine the sign and strength of priority effects. Furthermore, grazing of zooplankton can influence the population structure and temporal dynamics of *Microcystis* populations. The interaction between *Daphnia* and *Microcystis* seems to depend to a large extent on toxicity and colony formation of *Microcystis* strains. *Daphnia* may also influence, although relatively weak compared to interstrain differences, the functional traits themselves of *Microcystis* strains. In addition, grazing can change the strength and direction of interstrain interactions and priority effects, as illustrated by predator-mediated facilitation in the presence of *Daphnia*.

Samenvatting

Cyanobacteriën spelen een belangrijke rol in het functioneren van zoetwater en mariene aquatische ecosystemen, vermits zij een belangrijk deel uitmaken van de fytoplanktongemeenschap. Massale groei van cyanobacterieel fytoplankton, meestal als gevolg van eutrofiëring, kan aanleiding geven tot dense waterbloeien die vaak uit maar een cyanobacteriële soort bestaan. Een van de meest verspreide toxische bloeivormende cyanobacteriën is *Microcystis* (orde Chroococcales), die dan ook veel wetenschappelijke aandacht heeft gekregen (Huisman *et al.* 2005). Bloeien van *Microcystis* komen vooral voor tijdens de zomerperiode in gematigde gebieden en het ganse jaar door in (sub)tropische regio's in poelen en meren met hoge nutriëntengehaltes en een stabiele waterkolom. Studies gebaseerd op verschillende moleculaire merkers hebben aangetoond dat natuurlijk voorkomende *Microcystis* populaties bestaan uit verschillende genotypes, dat de temporele dynamiek van de genotype-samenstelling hoog kan zijn, en dat naast elkaar gelegen meren sterk kunnen verschillen in aanwezige genotypes (e.g. Janse *et al.* 2004; Wilson *et al.* 2005; Kardinaal *et al.* 2007). *Microcystis* stammen geïsoleerd uit eenzelfde bloeipopulatie kunnen vaak sterk verschillen in ecologische en fysiologische kenmerken (Wilson *et al.* 2006; Bañares-España *et al.* 2006; Yoshida *et al.* 2008). Sommige *Microcystis* genotypes produceren het hepatotoxine microcystine, wat zeer toxisch is voor eukaryote organismen. De toxiciteit van een *Microcystis* bloei wordt dan ook voor een groot deel bepaald door de genotype-samenstelling.

Ondanks het feit dat deze bloeien intensief werden bestudeerd is er nog weinig gekend over de mechanismen die de lokale en regionale diversiteit van *Microcystis* bloeipopulaties genereren, noch over de factoren die de populatiestructuur beïnvloeden. Dit is een belangrijk hiaat in de kennis over *Microcystis* bloeien omdat een beter begrijpen van deze mechanismen zeer nuttig kan zijn in het voorspellen van bloeivorming en bloeitoxiciteit en eventueel kan helpen in het vermijden van de ontwikkeling van deze bloeien.

Algemeen beschouwd wordt gemeenschaps- en populatiestructuur bepaald door een samenspel van historische- en omgevingsfactoren, met daarbij ook biotische interacties. Al deze factoren kunnen daarenboven ook interageren met elkaar. Historische factoren kunnen de genotype-samenstelling van natuurlijke *Microcystis* populaties beïnvloeden via twee mechanismen: (1) door dispersielimitatie, wat leidt tot verschillen in de lokale en regionale genotype-pool, en (2) door effecten van migratiegeschiedenis op de lokale populatiestructuur, vermits verschillen in kolonisatievolgorde van genotypes uit de regionale genotype-pool in een nieuw habitat langdurige consequenties kan hebben voor gemeenschaps- en populatiestructuur, wat kan resulteren in zogenoemde prioriteitseffecten (Morin 1984; Drake 1991). Wanneer *Microcystis* stammen een nieuw habitat koloniseren moeten ze voldoende aangepast zijn aan de lokale abiotische en biotische condities om een belangrijk deel uit te kunnen maken van de biotische gemeenschap in dit habitat (Leibold *et al.* 2004; Ricklefs 2004). Vermits individuele *Microcystis* stammen ook fysiologisch sterk kunnen verschillen (Wilson *et al.* 2006; Bañares-España *et al.* 2006), interageren ze wellicht op een stam-specifieke manier met lokale omgevingsfactoren.

In deze thesis werd de diversiteit en verspreidingspatronen van *Microcystis* ITS types (16S-23S rDNA Internal Transcribed Spacer) bestudeerd van lokale tot globale schaal om de invloed van historische en omgevingsfactoren in het structureren van de genotype-diversiteit te begrijpen. We gebruikten de ITS regio als moleculaire merker om de genetische diversiteit van *Microcystis* te bestuderen omdat deze variabel genoeg is voor fylogeografische analyse en ook kan gebruikt worden om natuurlijke *Microcystis* populaties te bestuderen via Denaturerende Gradiënt Gel Elektroforese (DGGE) (Otsuka *et al.* 1999; Boyer *et al.* 2001; Kardinaal *et al.* 2007). Vermits *Microcystis* stammen sterk verschillen in functionele kenmerken hebben we ons ook gefocust op interacties tussen *Microcystis* stammen omdat deze mogelijks een belangrijke invloed hebben op de populatiestructuur. Daarnaast hebben we ook aandacht besteed aan de wederzijdse beïnvloeding van interacties tussen *Microcystis* stammen, zoöplankton begrazing en kolonisatiegeschiedenis in het bepalen van de *Microcystis* populatiestructuur.

In een eerste deel van deze thesis (**hoofdstuk 2** en **3**), bestudeerden we verspreiding, diversiteit en genotype-structuur van natuurlijke *Microcystis* populaties op verschillende ruimtelijke schalen, met daarbij ook de invloed van abiotische en biotische factoren op diversiteit en populatiestructuur. In een tweede deel van deze thesis (**hoofdstuk 4**, **5** en **6**) bestudeerden we functionele kenmerken en interacties tussen *Microcystis* stammen, en de invloed van prioriteitseffecten en *Daphnia* begrazing op populatiestructuur aan de hand van laboratoriumexperimenten.

In **hoofdstuk 2** bestudeerden we de globale fylogeografie van *Microcystis* gebaseerd op een uitgebreide dataset van 302 rDNA ITS sequenties afkomstig uit zes continenten. Fylogenetische analyses resulteerden grotendeels in onopgeloste en niet-divergerende verwantschappen tussen ITS types, wat zou kunnen wijzen op de afwezigheid van afzonderlijke evolutielijnen binnen *Microcystis*. Onze analyses toonden ook de aanwezigheid aan van korte hypervariabele regio's in de ITS die incongruente fylogenetische signalen vertoonden, wellicht als gevolg van DNA-overdracht tussen verschillende *Microcystis* stammen of recombinatie in het genoom zelf. Onze data toonde een hoge diversiteit van *Microcystis* ITS types in alle continenten aan en de aanwezigheid van verscheidene identieke ITS types op meer dan één continent. Variantieanalyse toonde de afwezigheid van genetische differentiatie tussen continenten. Onze resultaten suggereren een hoog verspreidingspotentieel van *Microcystis* ITS types op globale schaal. In welke mate selectie door omgeving belangrijk is voor de verspreiding van ITS types is nog grotendeels onduidelijk, al lijken klimaatscondities weinig invloed te hebben.

In **hoofdstuk 3** bestudeerden we de genetische diversiteit en structuur van *Microcystis* populaties in 30 kleine kunstmatig aangelegde reservoirs in Tigray (Noord Ethiopië) via denaturerende gradiënt gel elektroforese van rDNA ITS en beoordeelden we het belang van lokale omgevingscondities en de geografische positie van de reservoirs voor de geobserveerde patronen. De analyses toonden aan dat zowel de lokale als regionale *Microcystis* ITS diversiteit in Tigray laag was in vergelijking met andere regio's en dat verscheidene dense bloeien zelfs maar een enkel ITS type bevatten. Terwijl een mogelijk rol van het klimaat niet kan worden uitgesloten, is een plausibele verklaring voor

de relatief lage ITS diversiteit de recente bouw van de reservoirs in een regio met veel droogte en een lage densiteit aan geschikt habitat voor *Microcystis*. Voornamelijk één niet-toxisch ITS type domineerde vele *Microcystis* bloeien maar bleek een beperkte geografische verspreiding te kennen. Dit suggereert dat langeafstandsdispersie infrequent genoeg is om lokale en regionale ITS diversiteit te limiteren en de beperkte geografische verspreiding van dit dominant ITS type te veroorzaken. Naast het belang van dispersielimitatie suggereren de correlaties tussen de *Microcystis* populatiestructuur en abiotische factoren (helderheid van de water kolom, pH) en zoöplankton (voornamelijk *Daphnia* biomassa) een belangrijke additionele invloed van deze factoren op de *Microcystis* populatiestructuur in de reservoirs van Tigray.

In **hoofdstuk 4** voerden we een laboratoriumexperiment uit met als doel de invloed te bepalen van *Daphnia* infochemicaliën (kairomonen) op de groeisnelheid, microcystineproductie, kolonievorming en celgrootte van acht *Microcystis* stammen, die geïsoleerd werden uit twee meren. Het experiment bestond uit vier behandelingen: 1) een controle met enkel WC medium, 2) toevoeging van *Scenedesmus obliquus* cultuurmediumfiltraat, 3) toevoeging van *Daphnia magna* cultuurmediumfiltraat, 4) toevoeging van natriumoctylsulfaat, een commercieel verkrijgbaar product dat ook een *Daphnia* kairomoon is. Onze resultaten toonden aan dat de *Microcystis* stammen uit eenzelfde meer sterk verschilden wat betreft de bestudeerde functionele kenmerken, terwijl er geen correlaties tussen deze kenmerken onderling waren. *Daphnia* kairomonen hadden over het algemeen een zwakke invloed op de groeisnelheid, microcystineproductie en kolonievorming van de *Microcystis* stammen in vergelijking met de variabiliteit tussen stammen onderling. De geïnduceerde effecten waren vaak ook sterk stam-specifiek. *Daphnia* cultuurmediumfiltraat en in mindere mate ook natriumoctylsulfaat hadden een negatieve invloed op de groei van de helft van de stammen en stimuleerden microcystineproductie in één stam, al was dit laatste effect niet *Daphnia*-specifiek want *Scenedesmus* cultuurmedium filtraat had hetzelfde effect. *Daphnia* cultuurmediumfiltraat induceerde ook kolonievorming in één stam.

In **hoofdstuk 5** gingen we via een laboratoriumexperiment na of de interacties tussen *Microcystis* stammen voorspeld kunnen worden gebaseerd op functionele kenmerken en of de uitkomst van competitie tussen stammen beïnvloed wordt door begrazing. Twee toxische en twee niet-toxische *Microcystis* stammen, die geïsoleerd werden uit eenzelfde meer en ook bestudeerd werden in hoofdstuk 4, werden (1) apart, (2) in alle mogelijke combinaties van twee stammen en (3) allemaal samen opgegroeid gedurende vier weken en dit zowel in aan- als afwezigheid van *Daphnia magna*. De relatieve abundantie van de stammen in de populaties werd in beeld gebracht via DGGE en de groeisnelheid van iedere stam in de mengpopulaties werd vergeleken met de groeisnelheid in monocultuur om interacties tussen stammen te bepalen. De geobserveerde interacties waren stam-specifiek en de relatieve abundantie van stammen in mengpopulaties kon voor een deel worden verklaard rekening houdend met toxiciteit en kolonievorming. Een belangrijke vaststelling was dat sommige interacties sterk werden beïnvloed door de aanwezigheid van *Daphnia*. *Daphnia* induceerde kolonievorming in één stam die dan een betere competitor werd. Begrazing door *Daphnia* veroorzaakte ook een groter evenwicht in de populaties, zowel door het verzwakken van competi-

tieve interacties als door facilitatie-effecten. Sterke facilitatie-effecten waren te wijten aan niet-toxische stammen die profiteerden van de bescherming geboden door toxische stammen in de aanwezigheid van predatie. Onze data toonden de aanwezigheid aan van sterke competitieve interacties tussen *Microcystis* stammen in de afwezigheid van begrazing, terwijl indirecte positieve interacties vooral voorkwamen in de aanwezigheid van *Daphnia*. Onze resultaten suggereren dat verschillen in functionele kenmerken en predator-gemedieerde facilitatie-effecten de coëxistentie van *Microcystis* genotypes bevorderen, hierbij ook toxische en niet-toxische stammen.

In **hoofdstuk 6** gingen we via een laboratoriumexperiment na in hoeverre prioriteitseffecten belangrijk zijn in het bepalen van de genotype-samenstelling van *Microcystis* en of deze beïnvloed worden door de aanwezigheid van een grazer. Dezelfde *Microcystis* stammen als beschreven in hoofdstuk 5 werden gedurende vier weken opgegroeid in het laboratorium in alle mogelijke paarsgewijze combinaties, met de twee stammen geïnoculeerd op hetzelfde tijdstip of met een tijdspanne van één week zowel in aan- als afwezigheid van grazende *Daphnia magna*. Veranderingen in de relatieve abundantie van de stammen in de mengpopulaties werden opgevolgd via DGGE en de groeisnelheid van iedere stam in de mengpopulaties in de laatste twee weken van het experiment werd bepaald. Effecten van inoculatievolgorde op de groeisnelheid van stammen werden beschouwd als aanwijzing voor langdurige prioriteitseffecten. We observeerden sterke effecten van inoculatievolgorde op de finale populatiestructuur die ook werden beïnvloed door grazende *Daphnia*. De prioriteitseffecten waren stamspecifiek en kwamen voor in twee richtingen: stammen groeiden zowel sneller als trager wanneer als tweede geïnoculeerd t.o.v. als eerste geïnoculeerd. De geobserveerde interacties en prioriteitseffecten kwamen grotendeels overeen met voorspellingen gebaseerd op variatie in toxiciteit en kolonievorming tussen stammen en hun consequenties voor competitieve sterkte en bescherming tegen predatie.

We kunnen uit deze thesis concluderen dat historische factoren (dispersielimitatie en prioriteitseffecten), begrazing en interacties tussen stammen de *Microcystis* populatiestructuur beïnvloeden op een sterk stam-specifieke manier afhankelijk van functionele kenmerken, en dat deze factoren ook interageren met elkaar. Op globale schaal en op lange termijn blijkt dispersielimitatie niet erg belangrijk te zijn voor *Microcystis*, maar in regio's met een lage densiteit aan jonge waterlichamen kunnen verschillen in dispersiesnelheid tussen *Microcystis* genotypes, en mogelijks ook prioriteitseffecten, belangrijk zijn in het structuren van lokale populaties en het limiteren van regionale en lokale diversiteit. De toxiciteit van een bloei blijkt ook beïnvloed te worden door de kolonisatievolgorde van stammen. Interacties tussen stammen, gemedieerd door functionele kenmerken, zijn wellicht een van de belangrijkste factoren in het structureren van *Microcystis* populaties, gezien de zeer complexe positieve en negatieve interacties tussen sympatrische stammen. Deze interacties bepalen ook de richting en de sterkte van prioriteitseffecten. Daarnaast beïnvloedt ook begrazing door zoöplankton de populatiestructuur en temporele dynamiek van *Microcystis* populaties. De interactie tussen *Daphnia* en *Microcystis* blijkt voor een groot deel af te hangen van de toxiciteit en kolonievorming van *Microcystis* stammen. *Daphnia* beïnvloedt ook,

alhoewel relatief weinig in vergelijking met verschillen tussen *Microcystis* stammen onderling, de functionele kenmerken van de stammen zelf. Daarnaast kan begrazing ook de sterkte en richting van interacties tussen stammen en prioriteitseffecten veranderen, zoals geïllustreerd werd door predator-gemedieerde facilitatie in de aanwezigheid van *Daphnia*.

References

Bañares-España, E., Lopez-Rodas, V., Salgado, C., Costas, E. and Flores-Moya, A. 2006. Inter-strain variability in the photosynthetic use of inorganic carbon, exemplified by the pH compensation point, in the cyanobacterium *Microcystis aeruginosa*. Aquatic Botany 85: 159-162.

Boyer, S. L., Flechtner, V. R. and Johansen, J. R. 2001. Is the 16S-23S rRNA internal transcribed spacer region a good tool for use in molecular systematics and population genetics? A case study in cyanobacteria. Molecular Biology and Evolution 18: 1057-1069.

Drake, J. A. 1991. Community-assembly mechanics and the structure of an experimental species ensemble. American Naturalist 137: 1-26.

Huisman, J., Matthijs, H. C. P. and Visser, P. M. 2005. Harmful cyanobacteria. Springer, Dordrecht, The Netherlands. 243 pp.

Janse, I., Kardinaal, W. E. A., Meima, M., Fastner, J., Visser, P. M. and Zwart, G. 2004. Toxic and nontoxic *Microcystis* colonies in natural populations can be differentiated on the basis of rRNA gene internal transcribed spacer diversity. Applied and Environmental Microbiology 70: 3979-3987.

Kardinaal, W. E. A., Janse, I., Kamst-van Agterveld, M., Meima, M., Snoek, J., Mur, L. R., Huisman, J., Zwart, G. and Visser, P. M. 2007. *Microcystis* genotype succession in relation to microcystin concentrations in freshwater lakes. Aquatic Microbial Ecology 48: 1-12.

Leibold, M. A., Holyoak, M., Mouquet, N., Amarasekare, P., Chase, J. M., Hoopes, M. F., Holt, R. D., Shurin, J. B. *et al.* 2004. The metacommunity concept: a framework for multi-scale community ecology. Ecology Letters 7: 601-613.

Morin, P. J. 1984. Odonate guild composition - experiments with colonization history and fish predation. Ecology 65: 1866-1873.

Otsuka, S., Suda, S., Li, R. H., Watanabe, M., Oyaizu, H., Matsumoto, S. and Watanabe, M. M. 1999. Phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis* based on 16S to 23S internal transcribed spacer sequence. FEMS Microbiology Letters 172: 15-21.

Ricklefs, R. E. 2004. A comprehensive framework for global patterns in biodiversity. Ecology Letters 7: 1-15.

Wilson, A. E., Sarnelle, O., Neilan, B. A., Salmon, T. P., Gehringer, M. M. and Hay, M. E. 2005. Genetic variation of the bloom-forming cyanobacterium *Microcystis aeruginosa* within and among lakes: Implications for harmful algal blooms. Applied and Environmental Microbiology 71: 6126-6133.

Wilson, A. E., Wilson, W. A. and Hay, M. E. 2006. Intraspecific variation in growth and morphology of the bloom-forming cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 72: 7386-7389.

Yoshida, M., Yoshida, T., Satomi, M., Takashima, Y., Hosoda, N. and Hiroishi, S. 2008. Intra-specific phenotypic and genotypic variation in toxic cyanobacterial *Microcystis* strains. Journal of Applied Microbiology 105: 407-415.

Appendix A

Covariation between zooplankton community composition and cyanobacterial community dynamics in Lake Blaarmeersen (Belgium)

Ineke van Gremberghe[1], Jeroen Van Wichelen[1], Katleen Van der Gucht[1], Pieter Vanormelingen[1], Sofie D'hondt[1], Christophe Boutte[2], Annick Wilmotte[2], Wim Vyverman[1]

Published in FEMS Microbiology Ecology 63 (2008) 222-237

Contribution to this chapter by IVG: molecular work, statistical analyses and writing

[1]Laboratory of Protistology and Aquatic Ecology, Ghent University, Krijgslaan 281 - S8, 9000 Ghent, Belgium

[2]Institute of Chemistry, University of Liège, Sart Tilman B6, 4000 Liège, Belgium

Abstract

The cyanobacterial community composition in the mesotrophic lake Blaarmeersen was determined by Denaturing Gradient Gel Electrophoresis (DGGE) of PCR-amplified 16S rDNA fragments during two consecutive years to asses the importance of different classes of explanatory variables (bottom-up and top-down factors, physical variables and phytoplankton) on cyanobacterial community dynamics. The most dominant cyanobacteria in Lake Blaarmeersen were *Synechococcus* (three genotypes), *Limnothrix redekei* and *Anabaena/Aphanizomenon*. ANOSIM (Analyses of Similarity) revealed that the cyanobacterial community in Lake Blaarmeersen differed significantly between the growing season and the winter season as well as between the epilimnion and hypolimnion during the stratified periods. Mantel tests revealed significant correlations between the DGGE data and bottom-up factors, physical variables, the phytoplankton community composition and, interestingly, the zooplankton community composition. In general, the zooplankton community composition (especially the cladoceran community) was more important in structuring the cyanobacterial community than the total zooplankton biomass. This study shows that grazing zooplankton communities can have a relatively strong impact on the cyanobacterial community dynamics and that this impact can be equally important as bottom-up processes regulated by nutrient concentrations and/or physical variables.

A.1 Introduction

Cyanobacteria have been attracting much attention in the last decade, as these autotrophic prokaryotes often dominate phytoplankton communities in nutrient rich freshwater lakes during warm weather conditions. Since many freshwater lakes worldwide have been affected by eutrophication, blooms of particular cyanobacterial species have become a common and recurrent phenomenon (Chorus and Bartram 1999; Huisman *et al.* 2005; Zurawell *et al.* 2005; Jayatissa *et al.* 2006).

Freshwater cyanobacterial communities are usually composed of biochemically and ecologically distinct species. For instance, different species and strains produce distinct secondary metabolites which can be toxic for eukaryotes (e.g. Milutinovic *et al.* 2003; Pflugmacher 2004; Viaggiu *et al.* 2004; Rohrlack *et al.* 2005), some cyanobacteria fix nitrogen while others do not (Ni *et al.* 1990; Downing *et al.* 2001; Fiore *et al.* 2005) and particular species exhibit different optima in relation to environmental parameters (Huisman and Hulot, 2005). Therefore, it is interesting to know the environmental conditions which tend to lead to the dominance of certain species. Whereas several studies revealed the influence of nutrients and physical variables (e.g. light, temperature, turbulence...) on the cyanobacterial community composition (e.g. Jacoby *et al.* 2000; Salmaso 2000; Pandey and Pandey 2002; Noges *et al.* 2003), the potential importance of top-down factors considering cyanobacterial community dynamics is poorly studied. This is probably due to the fact that cyanobacteria are thought to be less susceptible to grazing by zooplankton in comparison to other phytoplankton groups because they can form large colonies or filaments (Lampert 1987; Gliwicz and Lampert 1990; DeMott *et al.* 2001). Although some colonies and filaments are indeed too large to be consumed even by macrozooplankton, smaller cyanobacteria, being unicellular or consisting of small colonies or filaments, can easily be grazed. Several studies reported grazing by *Daphnia* (e.g. DeBernardi *et al.* 1981; Holm and Shapiro 1984; Rohrlack *et al.* 1999; Work and Havens 2003),*Bosmina* (Work and Havens 2003), copepods (Haney 1987; DeMott and Moxter 1991; Koski and Schmidt 2002; Work and Havens 2003), rotifers (Starkweather and Kellar 1983; Gilbert 1996), ciliates (Brabrand *et al.* 1983) and flagellates (Nishibe *et al.* 2002) on a range of cyanobacterial species. Some zooplankton species graze more efficiently on cyanobacteria than others do (Lampert 1987). Copepods for example can select cyanobacteria based on size and chemical characteristics like toxicity and nutritional value (DeMott and Moxter 1991; Engström *et al.* 2000). Therefore, in lakes with moderate to high nutrient concentrations where the cyanobacterial community is diverse, it is likely that both zooplankton abundance and composition play a role in cyanobacterial community dynamics. Previous studies indicate that grazing by zooplankton can influence the phytoplankton and the bacterioplankton community composition considerably (Bergquist *et al.* 1985; Degans *et al.* 2002; Muylaert *et al.* 2002; Sarnelle 2005). Although the effect of zooplankton grazing might be expected to be less pronounced concerning cyanobacteria (Sellner *et al.* 1993), more information is needed about the relationships between zooplankton and cyanobacterial community dynamics.

This study aims to evaluate the impact of top-down factors relative to other

important explanatory variables on seasonal changes in the cyanobacterial community in Lake Blaarmeersen. The cyanobacterial community in the lake was monitored for two years using Denaturing Gradient Gel Electrophoresis (DGGE) of PCR-amplified 16S rRNA-genes. Furthermore, the cyanobacterial community composition was related directly to top-down, bottom-up and other explanatory variables.

A.2 Materials and methods

A.2.1 Study site and sample collection

Lake Blaarmeersen (51°25' N, 3°41' E) is a temperate monomictic sand-pit lake with a high recreational value in the city of Ghent. Since 1982, the lake and the surrounding area has been opened to the public for recreation purposes. In the years following its construction in 1976, it was an oligotrophic, macrophyte-dominated lake but, like many other lakes in Belgium, eutrophication has resulted in a gradual increase of phytoplankton biomass, including cyanobacteria (Geysen and Coppejans 1985; several unpublished studies). Lake Blaarmeersen has a surface area of almost 20 ha and a maximal depth of 15 m. Samples from Lake Blaarmeersen were taken fortnightly in 2003 (but monthly in winter) and monthly in 2004 at a depth of 0.5 m and 7.5 m (some extra samples were taken at a depth of 1.5 m and 3.5 m) at a fixed position in the lake where the water depth is maximal (15 m).

Phytoplankton samples of Lake Blaarmeersen were taken with a vertical point water sampler. These samples were fixed with the alkalic lugol-formalin-sodiumthiosulfate method (Sherr and Sherr 1993). On each occasion, a depth integrated (0 m, 2.5 m, 5 m, 7.5 m, 10 m) zooplankton sample was gathered with a Schindler-Patalas sampler at the same locality. Zooplankton samples were fixed with sugar saturated formaldehyde solution (end concentration 4 %) (Haney and Hall 1973). Water samples for DNA extraction were filtered over a 25 mm 0.2 µm GSWP filter (Millipore) and frozen as soon as possible at - 20 °C. A depth profile for oxygen concentration, temperature, conductivity and pH was made with a CTD multi-meter (YSI 650 MDS). An underwater light profile was made with a Licor 1400 Data logger and two sensors (Aquamatic AOPL UV912). Water transparency was also measured with a Secchi-disk. For determination of dissolved nutrient concentrations (nitrate, ammonium and orthophosphate), water was filtered over a GF/F glass fibre filter and stored frozen until analysis using a Skalar auto-analyser (Koroleff 1976; Grasshoff 1976). Suspended matter was measured gravimetrically after filtration of a known volume of water on pre-weighed GF/F filters.

A.2.2 Microscopical determination and counting

Phytoplankton (including filamentous cyanobacteria) was identified according to Tikkanen and Willén (1992) and counted with an inverted microscope (Zeiss Axiovert), expressed as numbers per millilitre and converted into biomass (µg C l^{-1}) by determining the average biovolume (μm^3) of each taxon (Menden-Deuer and Lessard 2000). Phytoplankton was identified up to genus or species level. For the samples from

2004, an extra counting of picocyanobacteria was done using epifluorescence (Zeiss Axioplan microscope) with black polycarbonate filters. Zooplankton was identified and counted as individuals per litre with a binocular loupe and abundance was converted to biomass using published length-weight regressions (Bottrell *et al.* 1976). Copepods were identified and counted as calanoids, cyclopoids, copepodites and nauplii, cladocerans were identified up to genus level and rotifers were counted up to genus and species level.

A.2.3 DNA extraction

Genomic DNA from the GSWP filters containing water samples was isolated as described by Zwart *et al.* (1998), which includes the bead beating method with phenol extraction and ethanol precipitation. After extraction, the DNA was purified on a Wizard column (Promega).

A.2.4 PCR amplification for DGGE

16S rDNA fragments were amplified using the nested PCR protocol described by Zwart *et al.* (2005). The specific cyanobacterial primers cya-b-F371 and cya-R783 and the general primers F357 (GC) and R518 were used in the first en second PCR, respectively. In the first PCR a high specificity was obtained and in the second PCR the PCR fragment was shortened to obtain better profiles by DGGE. The first PCR was performed using the following reaction mixtures: 2 µl of template DNA, 0.5 µM of each primer, 200 µM of each deoxynucleoside triphosphate, 2.5 units of Taq DNA polymerase (Ampli Taq), 10X PCR buffer [Tris/HCl: 100 mM, pH: 8.3; KCl: 500 mM; MgCl2: 15 mM; Gelatine: 0.01 % (w/v)] and 400 ng of bovine serum albumin. The mix was adjusted to a final volume of 50 µl with sterile water. The PCR program started with a denaturation step of five minutes at 94 °C. A touch down procedure was performed consisting of 20 cycles in which the annealing temperature decreased by 1 °C every second cycle from 65 to 56 °C. Cycle step times were one minute each for denaturation (94 °C), annealing and extension (72 °C). A final extension step was performed for 10 minutes at 72 °C. After the cyanobacteria specific amplification and purification with a QiaQuick PCR purification kit (QiaGen), 1 µl of PCR product was transferred to a new 50 µl reaction mixture containing the general bacterial primers described above. The PCR program started with a denaturation step of five minutes at 94 °C. A touch down procedure was performed consisting of 20 cycles in which the annealing temperature decreased by 1 °C every second cycle from 65 to 56 °C (see before) and five additional cycles in which the annealing temperature was 55 °C. A final extension step was performed for 30 minutes at 72 °C. The presence of PCR products and their concentration was determined by analyzing 5 µl of each PCR product on 1.66 % agarose gels, staining with ethidium bromide, and comparing them to a molecular weight marker (SmartLadder; Eurogentec).

A.2.5 DGGE profiling

DGGE was essentially performed as described by Muyzer *et al.* (1993). Equal amounts of PCR products were loaded onto 8 % (w/v) polyacrylamide gels (1 mm thick, in 1X TAE [20 mM Tris acetate (pH 7.4), 10 mM acetate, 0.5 mM disodium EDTA]). The denaturing gradient contained 35-60 % denaturant [100 % denaturant corresponded to 7 M urea and 40 % (v/v) formamide]. Electrophoresis was performed for 16h at 75 V and the temperature was set at 60°C. Finally, the gels were stained with ethidium bromide and photographed on a UV transillumination table with a CCD camera. Furthermore, a small piece of gel from the middle of the target band was excised from the DGGE gel and incubated in 50 µl of sterile TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA) for 24h at 4 °C. The eluent was then reamplified and purified by DGGE one or two times. The resulting PCR products were purified using a QiaQuick PCR purification kit (QiaGen). Finally, sequencing was performed with the ABI-Prism sequencing kit and the resulting sequencing reaction products were analysed on an automatic sequencer (ABI-Prism 3100).

A.2.6 DGGE-profile analysis

On every gel, three standard lanes were analysed in parallel to the samples. These standard lanes were composed of several bands positioned distinctly in the gel. As these bands should always be formed at the same denaturant concentration in the gel, their position was used to compare the patterns formed in different gels. Digitalized DGGE images were analysed using the software package BioNumerics 4.5 (Applied Maths BVBA). The program detects the bands and groups the bands into band classes, based on their position in the gel. Sequence information of the bands was used to manually check the grouping of bands into band classes. Heteroduplex bands and very weak bands were removed. Further, a matrix was compiled, based upon the band intensity or the presence or absence of bands in band classes. The band intensities were then converted into relative intensities (the relative contribution of each band to the total band signal in the lane).

A.2.7 Cloning of ribosomal RNA genes

16S rDNA PCR products from the sample taken in Lake Blaarmeersen on 18/07/03 on a depth of 0.5 m were cloned and sequenced as described in Boutte *et al.* (2005). The DNA was amplified with the primers CYA359F (Nübel *et al.* 1997) and 23S30R (Taton *et al.* 2003). The PCR conditions and PCR programme were as described in Boutte *et al.* (2005). The TOPO TA Cloning Kit (Invitrogen) was used for cloning, following the manufacturer's instructions. Colonies were screened by a direct PCR with primers CYA359F and CYA784R (Nübel *et al.* 1997) using an annealing temperature of 60 °C instead of 54 °C to increase the specificity of the detection. Each positive clone was sequenced using primer 1092R. Sequences were obtained from Genome Express (Meylan). Sequencing reactions were performed using a ABI Prism system 3730 XL^{TM} (PE Applied Biosystems). The sequenced fragment has a length of 700-800 bp.

A.2.8 Sequence analysis and database deposits

The 16SrDNA sequences were screened against GenBank/EMBL using the algorithm BLAST (Altschul *et al.* 1990) to identify the cyanobacteria. The sequence data have been submitted to the EMBL database under accession numbers AM410055-AM410064 for the DGGE bands and AM411877-AM411904 for the clones.

A.2.9 Statistical analysis

Multivariate statistics were used to investigate the relation between cyanobacterial community composition and explanatory variables. The similarity of DGGE profiles (only the dominant cyanobacteria *Synechococcus*, *Limnothrix redekei* and *Anabaena/Aphanizomenon* were selected from the DGGE profile and their relative band intensities were used), cyanobacterial community obtained from counting (relative density), phytoplankton community composition (absolute biomass of taxa without cyanobacteria), zooplankton community composition (absolute biomass of taxa) and community composition of copepods, cladocerans and rotifers (absolute biomass of the three groups) was determined using the Bray-Curtis Index of similarity (Primer 5, Clarke and Gorley 2001). For each of these datasets a similarity matrix was prepared using $log(x+1)$ transformed data. The Bray Curtis similarity matrix of the DGGE profile was used to create a dendrogram, using weighted group average linkage in cluster analysis (Primer 5, Clarke and Gorley 2001). Dissimilarity matrices of environmental data were calculated using Euclidean distances (Primer 5, Clarke and Gorley 2001). Two matrices were constructed separately, one including the dissolved nutrients (nitrate, ammonium, total dissolved nitrogen and orthophosphate) and one including the physical variables (temperature, Secchi depth, oxygen level, pH and conductivity). Nutrient data were fourth root transformed and Secchi depth and conductivity were $log(x+1)$ transformed. Dissimilarity matrices of total zooplankton biomass, biomass of *Daphnia* and *Bosmina*, total cyanobacterial biomass and phytoplankton biomass were calculated using Euclidean distances (Primer 5, Clarke and Gorley 2001) on $log(x+1)$ transformed data.

Analyses of similarity (ANOSIM) using 999 permutations were run to test for statistically significant differences in cyanobacterial assemblage (Primer 5, Clarke and Gorley 2001). ANOSIM generates a test statistic R, and the magnitude of R is indicative of the degree of separation between groups, with a score of 1 indicating complete separation and 0 indicating no separation. Monte-Carlo randomization of the group labels was used to generate null distributions in order to test the hypothesis that within-group similarities are higher than would be expected if sample DGGE profiles were grouped at random.

Mantel tests (Mantel 1967; Mantel and Valand 1970) were used to investigate the relation between cyanobacterial community composition and abiotic/biotic variables. In the Mantel test, the null hypothesis is that distances in a matrix A are independent of the distances, for the same objects, in another matrix B. Testing of the null hypothesis is done by a randomization procedure in which the original value of the statistic is compared with the distribution found by randomly reallocating the order of the elements

in one of the matrices. The statistic used for the measure of the correlation between the matrices is the Pearson correlation coefficient. Simple Mantel tests evaluate whether the association between two independent similarity matrices, describing the same set of entities, is stronger than one would expect from chance. Partial Mantel tests are used to determine the relationship between two matrices while controlling the effect of a third matrix. Simple and partial Mantel tests were carried out by the Zt software tool (Bonnet and Van de Peer 2002) using Bray-Curtis similarity matrices for community data and Euclidean distance dissimilarity matrices for environmental data and total biomass (see before). The program Manteltester[1] was used to automate the Mantel tests.

Spearman rank correlation coefficients were calculated using Statistica 6.0 for Windows[2] to obtain extra information about positive or negative correlations among the different cyanobacterial genotypes (relative intensities of single DGGE band classes) and to obtain information about positive or negative correlations between each cyanobacterial genotype and biomass of *Daphnia* and *Bosmina*. In addition, Spearman rank correlation coefficients were calculated between the total cyanobacterial biomass and the biomass of *Daphnia* and *Bosmina*. All data were $log(x+1)$ transformed prior to the analyses.

A.3 Results

A.3.1 Physico-chemical characteristics

Lake Blaarmeersen is a typical temperate, monomictic lake. The water layers are well mixed during winter and thermal stratification happens predominantly in summer. As a result, during the study period the water was well oxygenated during complete mixing but as soon as thermal stratification started, an oxycline developed first in deeper water and later more at the surface, dividing the water body in a well oxygenated epilimnion and a poorly oxygenated hypolimnion. In summer, severe oxygen depletion (with values less than 1 mg O_2 l^{-1}) was observed. During winter turnover, the euphotic depth was lower than the mixing depth and the primary producers could encounter light limitation when they were transported passively in the deeper darker zones. In late spring, a typical spring clear water phase was visible when the amount of suspended matter was lowest and Secchi depth and euphotic depth were maximal. This was probably induced by the high grazing pressure on phytoplankton, mainly by large cladocerans (*Daphnia*) at that time (see figure A.1). The pH values were higher in times when there was more phytoplankton production (mainly in summer at the surface of the water column). The total ion-concentration was somewhat higher at the bottom of the lake during the stratified period due to the presence of the sediment layer. Highest concentrations of dissolved nutrients (total dissolved nitrogen: up to 300 µg l^{-1}, orthophosphate: up to 30 µg l^{-1}) were observed in autumn and winter when organic matter became

[1] http://manteltester.berlios.de

[2] StatSoft. Tulsa, USA. http://www.statsoft.com

mineralised and the water column was mixed up to the bottom. During summer, dissolved nutrients became depleted in the epilimnion (see figure A.2).

A.3.2 Zooplankton community

In 2003 as well as in 2004, the zooplankton community was mainly dominated by predatorous as well as herbivorous rotifers, but the macrozooplankton community also reached high numbers. The herbivorous cladoceran *Daphnia* was encountered mainly in spring ('clear water phase') in both years and in winter 2003, but the highest density was found in October 2004 (up to 50 individuals per litre).*Bosmina* was very abundant in summer and autumn 2003 (up to 170 individuals per litre), a lower density was seen in summer 2004. Other cladocerans detected in Lake Blaarmeersen were *Ceriodaphnia* (low density in autumn 2003) and *Leptodora* (low density in summer and autumn 2004). Copepods were encountered mostly in spring and winter in both years but with maximum abundance in early spring 2004, just before the 'clear water phase' (up to 180 individuals per litre). Calanoid copepods were present in spring 2003 after which they were replaced by cyclopoid copepods. High numbers of rotifers were encountered in early summer 2003, just after the 'clear water phase' (more than 2000 individuals per litre). In August and September, rotifers were scarce, whereas in October, November and December they were abundant again. The same pattern was seen in 2004, although the total density was lower in comparison to 2003. The most abundant rotifers in Lake Blaarmeersen were *Polyarthra sp.*, *Keratella cochlearis*, *Keratella quadrata*, *Asplanchna priodonta*, *Synchaeta sp.*, *Kellicottia longispina* and *Pompholyx complanata*. To compare the grazing capacity of different zooplankton groups and species, biomass is more appropriate than numbers, therefore, the zooplankton community composition was studied using biomass data (see figure A.1).

A.3.3 Phytoplankton community

Similar values were obtained for the total biomass of phytoplankton at a depth of 0.5 m (epilimnion) and 7.5 m (hypolimnion), except in summer 2003 where the biomass was lower in the hypolimnion compared to the epilimnion. High biomass of phytoplankton (up to 1300 μg C l^{-1}) was especially seen in summer and autumn (see figure A.1). The phytoplankton group which dominated most frequently in Lake Blaarmeersen were cryptophytes, but dinophytes, chlorophytes, cyanobacteria and diatoms also reached high numbers in particular periods. In 2003, in early spring (end February), a bloom of small centricate diatoms (*Stephanodiscus sp.*) was detected, which was followed by a spring bloom (just before the 'clear water phase') of cryptophytes (*Cryptomonas sp.*). After the 'clear water phase', in June and July, high numbers of dinophytes (*Ceratium furcoides*, *Peridinium sp.*) were encountered, whereas in late summer filamentous phytoplankton (chlorophytes: *Mougeotia sp.* and cyanobacteria: *Anabaena sp.*, *Aphanizomenon flos-aquae* and *Limnothrix redekei*) was present in the lake. In autumn, there was again a bloom of cryptophytes. In 2004, the same pattern was observed, although there were some differences in the summer period because cyanobacteria,

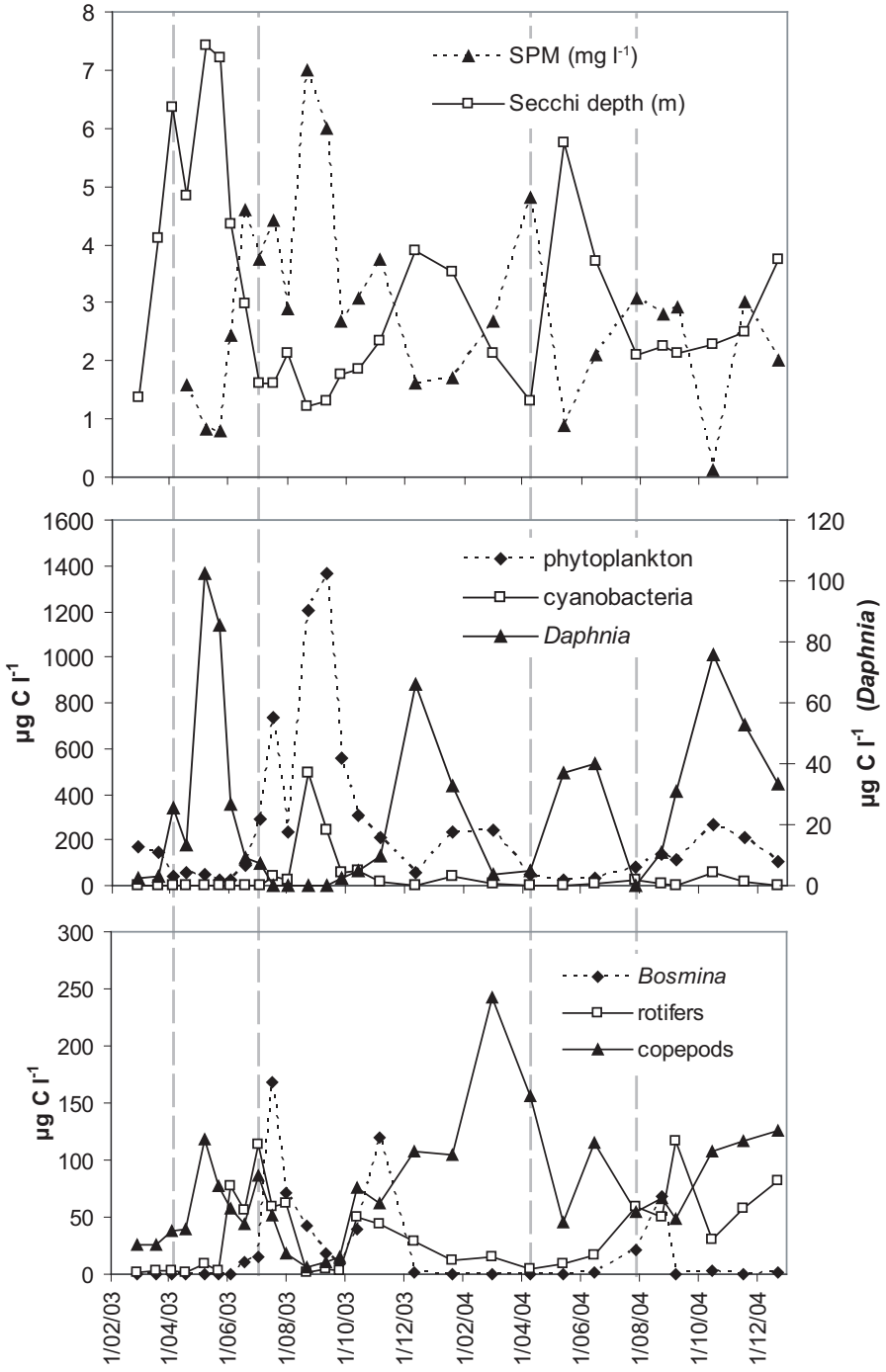


Figure A.1: Seasonal variation in water clarity expressed as the amount of suspended matter (SPM) or Secchi depth (top), total phytoplankton biomass (including cyanobacteria), cyanobacterial biomass and biomass of *Daphnia* (middle), and biomass of *Bosmina*, rotifers and copepods (bottom) in Lake Blaarmeersen in 2003 and 2004 [for SPM, phytoplankton and zooplankton biomass the data from the epilimnion (0.5 m) are used].

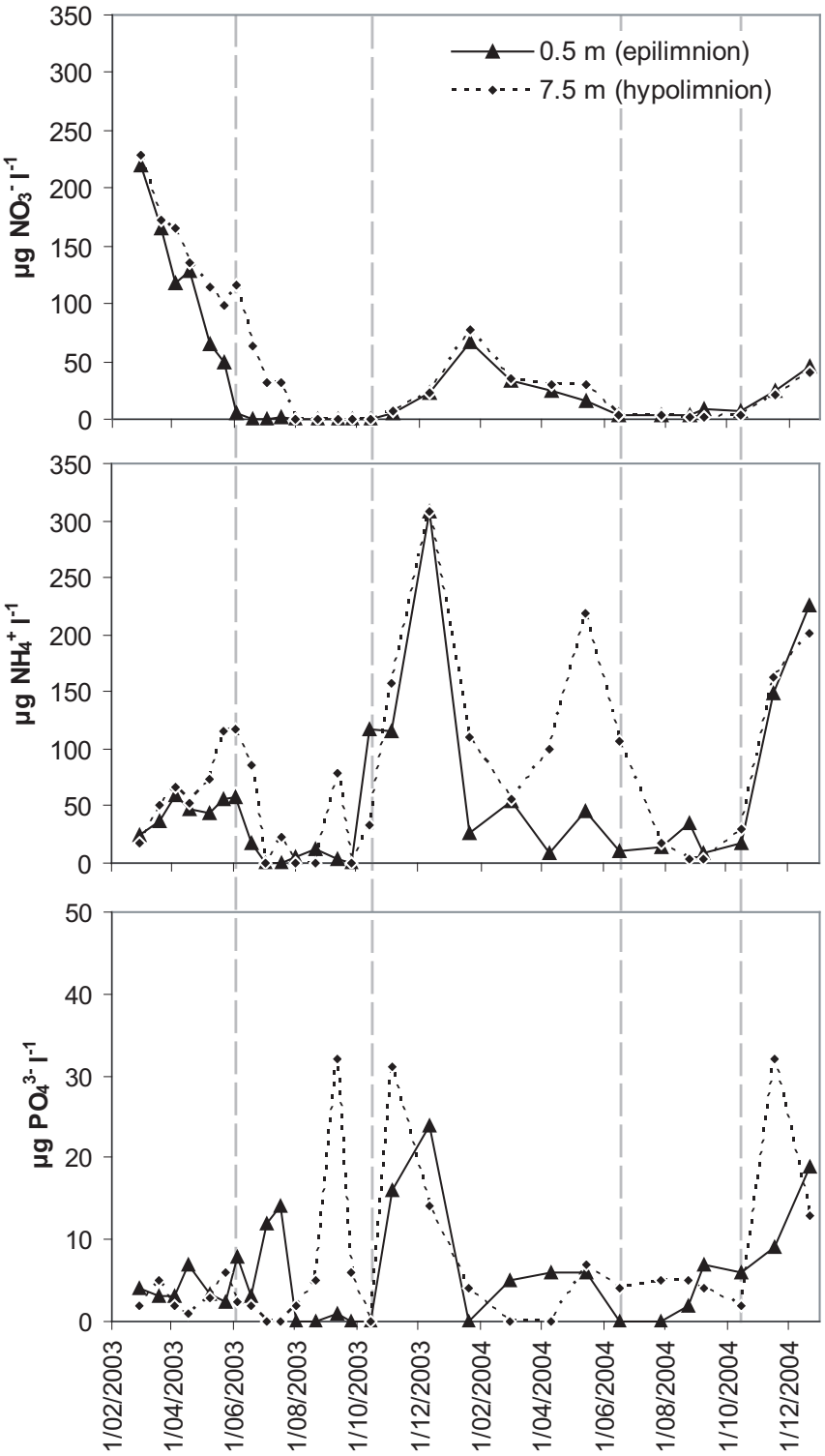


Figure A.2: Seasonal fluctuations in the concentration of dissolved nutrients in the water column of Lake Blaarmeersen in 2003 and 2004 in the epilimnion and hypolimnion.

diatoms, dinophytes and chlorophytes all reached high numbers at that time.

In general, cyanobacteria had a rather low contribution to the total phytoplankton biomass and reached a maximal contribution during summer and autumn (see figure A.1). During the sampling period, the biomass of cyanobacteria was similar or lower in the hypolimnion compared to the epilimnion, except in late summer and early autumn of 2004.

A.3.4 Cyanobacterial community

A significant correlation ($r = 0.145$; $p = 0.008$) was found by a simple Mantel test (Zt, Bonnet and Van de Peer 2002) between the cyanobacterial community composition data (relative contribution of the most dominant groups: *Synechococcus*, *Limnothrix redekei* and *Anabaena/Aphanizomenon*, see further) obtained by counting and obtained by DGGE for the growing season. The rather low correlation was probably mainly due to the fact that *Synechococcus* was underestimated in the counting data of 2003 because no extra counting with a fluorescence microscope was done for these samples. Furthermore, at several sampling dates during winter and spring no cyanobacteria were detected by the microscope in contrast to DGGE by which it was still possible to detect them. Considering these aspects, DGGE was used to study the cyanobacterial community composition.

Figure A.3 shows the DGGE profile obtained by cyanobacteria-specific PCR amplification of DNA from samples taken in Lake Blaarmeersen from april 2003 to december 2004 at a depth of 0.5 m and 7.5 m (and a few samples at a depth of 1.5 m and 3.5 m). Due to the fact that cyanobacteria were not dominant in the lake, many chloroplast sequences of other phytoplankton groups were also amplified (see table A.1). However, the sequence information of the bands was used to select the dominant cyanobacterial taxa and all the statistical analyses (see further) were done on the community composition of *Synechococcus* (band class 10, 14 and 18), *Limnothrix redekei* (band class 16) and *Anabaena/Aphanizomenon* (band class 19) (see figure A.3 and table A.1). The dominance of these cyanobacteria was confirmed by the counting data (these taxa reached the highest absolute biomass). Additionally, more 16S rDNA sequence information of these three cyanobacterial taxa was obtained by cloning to confirm and complement the DGGE data (see materials and methods section for accession numbers of the sequences).

The most abundant cyanobacterium in the DGGE profile is *Synechococcus*, which is represented by three genotypes (band classes 10, 14 and 18; see figure A.3 and table A.1). The genotype on position 10 was present in Lake Blaarmeersen during the whole sampling period on both depths, whereas the genotype on position 18 was only found in summer and autumn and the genotype on position 14 was only detected in the summer of 2004 in the hypolimnion. The cyanobacterium *Limnothrix redekei* (band class 16; see figure A.3 and table A.1) was dominant in 2003 in summer in the hypolimnion and in autumn on both depths. The nitrogen-fixing cyanobacterium *Anabaena/Aphanizomenon* was detected in late summer 2003 in the epilimnion (band class 19; see figure A.3 and table A.1). The relative contributions of *Syne-*

Table A.1: Closest relatives of sequenced bands from DGGE profile found in GenBank/EMBL (for numbers of bands and band classes see figure A.3, accession numbers of sequences in EMBL: AM410055-AM410064).

number band class	number band	sequence length (bp)	closest match	accession number of closest match	percentage similarity (bp/bp)
7	8	88	chloroplast (diatom)	AY858017	100 (87/87)
8	13, 14, 15, 17, 21	136	chloroplast	DQ363182	100 (129/129)
9	11	137	chloroplast (diatom)	AY678496	99 (132/133)
10	7, 9, 18	136	*Synechococcus*	AY436570	100 (118/118)
12	1	114	chloroplast (cryptophyte)	AY453067	100 (113/113)
14	16, 20	136	*Synechococcus*	AF448068	99 (129/130)
15	2, 3, 4	122	chloroplast (diatom)	AF418973	100 (121/121)
16	6, 12, 22	134	*Limnothrix redekei*	AJ544070	100 (128/128)
18	5, 19	120	*Synechococcus*	AY224199	100 (117/117)
19	10	121	*Aphanizomenon flos-aquae*	AY038035	100 (118/118)
			Aphanizomenon gracile	AJ293124	100 (118/118)
			Anabaena mendotae	AJ293107	100 (118/118)
			Anabaena affinis	AF247591	100 (118/118)

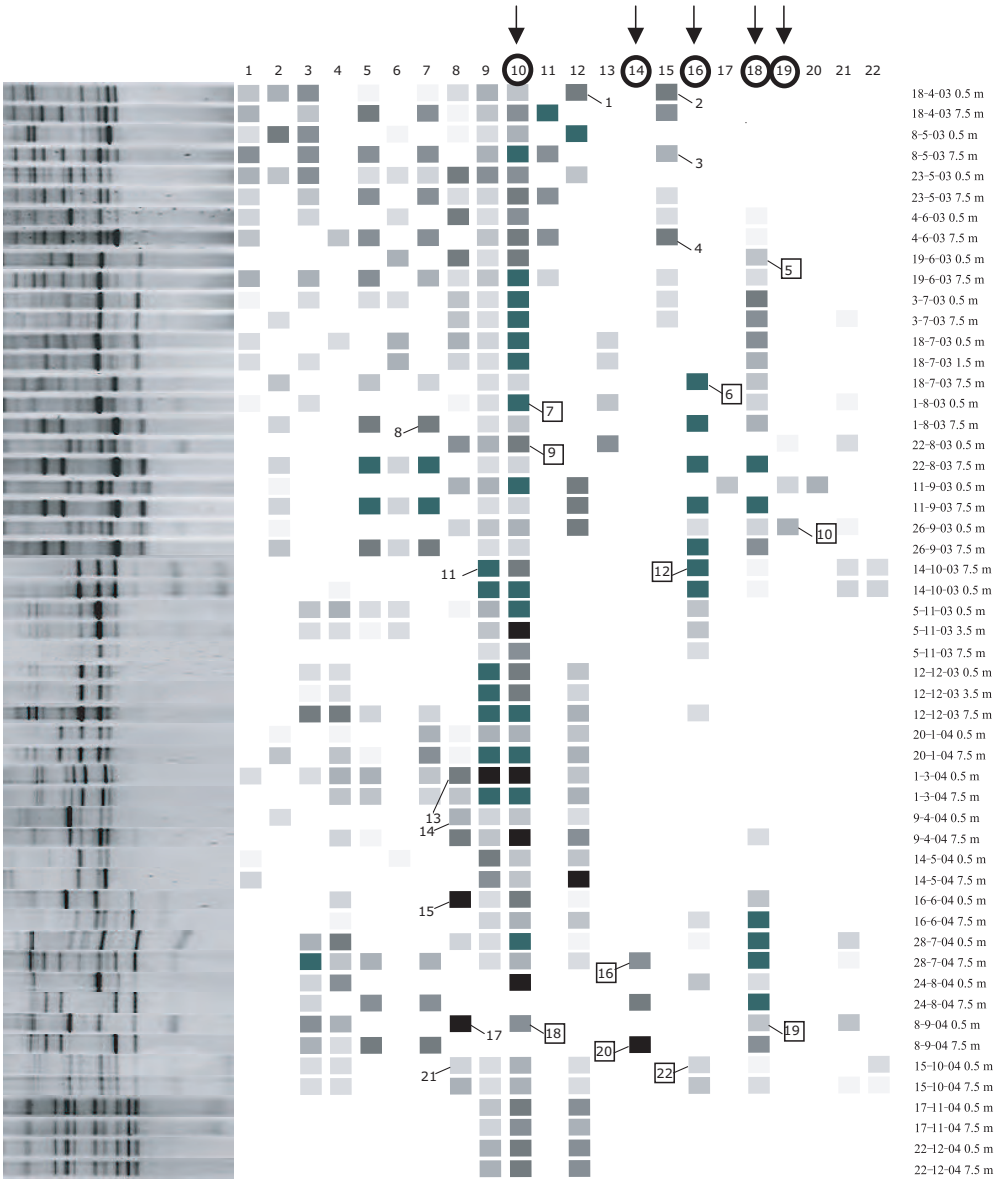


Figure A.3: Photograph of the original DGGE gels (left) and digitalized DGGE profile (right) obtained by cyanobacteria-specific PCR amplification of DNA from samples taken in Lake Blaarmeersen in 2003 and 2004. Sampling dates and depths are indicated at the completely right side of the figure. Bands are grouped into band classes (numbers of band classes are shown on top of the figure, see also table A.3). Individual bands which were sequenced are indicated and numbered (see also table A.3). Heteroduplex bands were removed from the band classes. Band classes representative for the most dominant cyanobacteria and used in the statistical analyses are indicated with arrows on top of the figure and circles around the band class number. Sequenced bands in these band classes have squares around their band number.

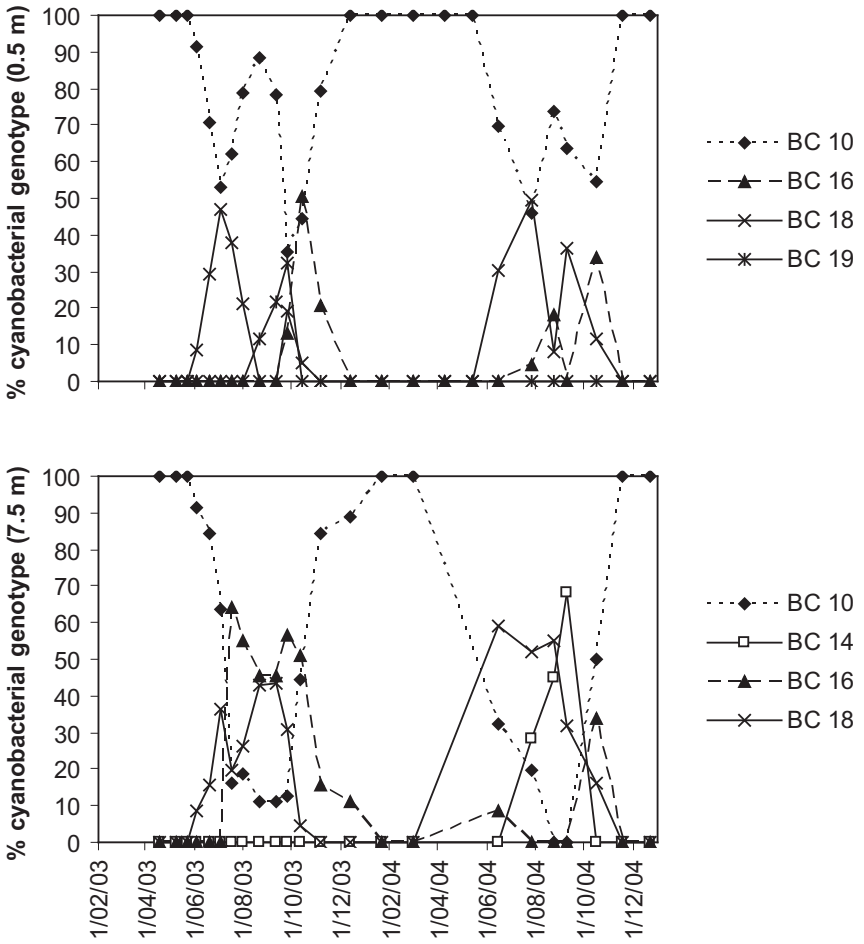


Figure A.4: Seasonal variation in the dominance of cyanobacterial genotypes (relative contribution) at a depth of 0.5 m (top) and 7.5 m (bottom) in Lake Blaarmeersen in 2003 and 2004 (BC10, BC 14 and BC18 = *Synechococcus*, BC16 = *Limnothrix redekei* and BC19 = *Anabaena/Aphanizomenon*; BC stands for band class, see also figure A.3 and table A.1).

chococcus genotypes, *Limnothrix redekei* and *Anabaena/Aphanizomenon* at a depth of 0.5 m and 7.5 m detected by DGGE are also visualized in figure A.4. Analyses of Similarity (ANOSIM, Primer 5, Clarke and Gorley 2001) revealed significant differences in the cyanobacterial community composition between the 'growing season' (late spring, summer and early autumn) and 'winter season' (late autumn, winter and early spring) ($R = 0.47$; $p = 0.001$) and significant differences in the cyanobacterial community composition between a depth of 0.5 m and 7.5 m ($R = 0.239$; $p = 0.001$) during the growing season, while no significant differences in the cyanobacterial community composition between a depth of 0.5 m and 7.5 m were found during the winter season. The Bray-Curtis cluster (Primer 5, Clarke and Gorley 2001) of the cyanobacterial community (see figure A.5) is composed of two sub clusters: one which groups the 'growing season samples' and one which groups the 'winter season samples'. Three samples taken in summer 2004 in the hypolimnion are grouped separately due to the presence of a *Synechococcus* genotype (band class 14). In the winter season there was little variation in the cyanobacterial community and especially one *Synechococcus* genotype (band class 10) was found.

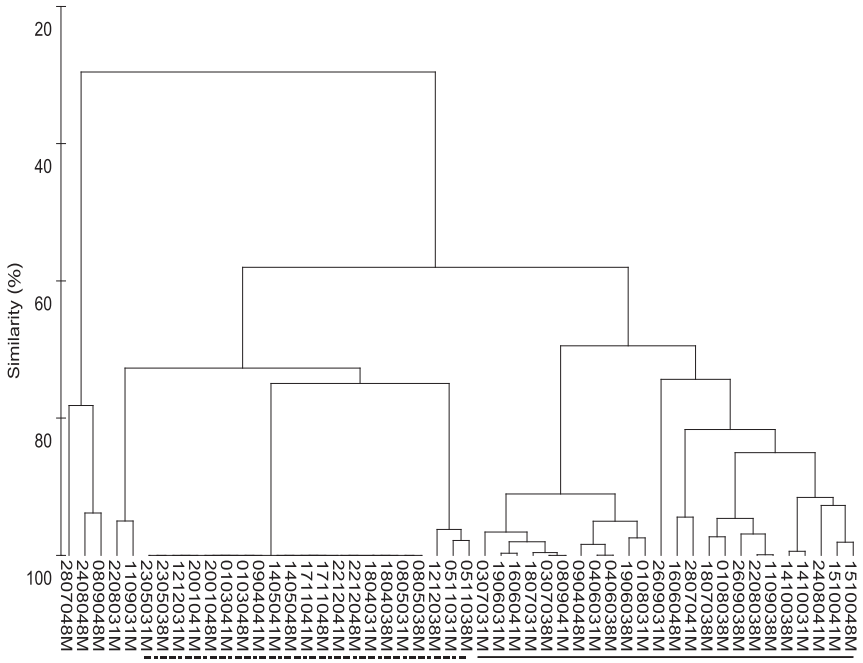


Figure A.5: Bray-Curtis cluster of the cyanobacterial community composition (band classes 10, 14, 16, 18 and 19, see figure A.3 and table A.1) of the samples taken in Lake Blaarmeersen in 2003 and 2004. Full line: samples taken in the 'growing season' (late spring, summer and early autumn), interrupted line: samples taken in the 'winter season' (late autumn, winter and early spring), 1M = 0.5 m, 8M = 7.5 m.

A.3.5 Influence of biotic and abiotic factors on the cyanobacterial community dynamics

Simple and partial Mantel tests (Zt, Bonnet and Van de Peer 2002) were done to investigate the relationship between the cyanobacterial community composition (relative contributions of *Synechococcus* genotypes, *Limnothrix redekei* and *Anabaena/Aphanizomenon*) as determined by DGGE and the community composition of zooplankton and phytoplankton (without cyanobacteria). In addition, the influence of dissolved nutrients and physical conditions (temperature, Secchi depth, oxygen level, pH and conductivity) was investigated (see table A.2). The simple Mantel tests revealed that the zooplankton and phytoplankton community composition as well as the dissolved nutrient concentrations and physical conditions of the lake are significantly related to the cyanobacterial community composition, while all these components are also related to each other. Partial Mantel tests revealed that each component independently influenced the cyanobacterial community dynamics. The highest correlations were found with dissolved nutrients, zooplankton community composition and physical variables and a slightly lower correlation was found with the phytoplankton community composition. For the Mantel tests described here, the band intensities in the DGGE profile were used (relative contributions of cyanobacterial taxa per sample). In addition, Mantel tests were also performed on the presence/absence DGGE data (presence or absence of cyanobacterial taxa per sample). In general, the correlations which were found were comparable with the correlations found when using band intensities, although they were slightly lower (not shown).

Simple Mantel tests also revealed that the zooplankton community composition influenced the total cyanobacterial biomass ($r = -0.346$; $p < 0.0001$) but the total biomass of zooplankton did not influence the cyanobacterial community dynamics ($r = -0.120$; $p > 0.05$). Furthermore, the zooplankton composition based on the absolute biomass of the three major groups (copepods, cladocerans and rotifers) has no significant correlation with the cyanobacterial community composition when controlling for nutrients ($r = 0.064$; $p > 0.05$) and physical variables ($r = 0.115$; $p > 0.05$). The different zooplankton groups were also correlated separately to the cyanobacterial community composition and total cyanobacterial biomass by simple and partial Mantel tests (see table A.3). The community composition of all three zooplankton groups has a significant correlation with the cyanobacterial community composition and biomass. However, when controlling for the phytoplankton community, only the cladoceran community has a significant correlation with the cyanobacterial community composition. The rotifer community has no significant correlation with the cyanobacterial biomass when controlling for phytoplankton biomass, cladoceran community and nutrients (see table A.3). *Bosmina* and *Daphnia* were the most abundant cladocerans in Lake Blaarmeersen (see figure A.1) and simple Mantel tests showed that the biomass of both species has a significant correlation with the cyanobacterial community composition and biomass. However, only *Bosmina* seemed to independently influence the cyanobacterial community dynamics (see table A.4).

Spearman rank correlation coefficients were also calculated between the relative

Table A.2: Results of Mantel tests relating the cyanobacterial community composition to the community of zooplankton and phytoplankton and abiotic variables. Results of simple Mantel tests are shown above the diagonal, results of partial Mantel tests below the diagonal (correlations are presented as Pearson correlation coefficients; *:$p < 0.05$; **:$p < 0.01$; ***:$p < 0.001$; ****:p<0.0001; n.s. = not significant).

	cyanobacterial community	zooplankton community	phytoplankton community	nutrients	physical variables
cyanobacterial community		0.306****	0.218***	−0.335****	−0.280****
zooplankton community	0.253*** controlled for phytoplankton community 0.237*** controlled for nutrients 0.282**** controlled for physical variables		0.341****	−0.272****	−0.135**
phyto-plankton community	0.127* controlled for zooplankton community 0.139* controlled for nutrients 0.167** controlled for physical variables	0.295**** controlled for cyanobacterial community 0.288**** controlled for nutrients 0.322**** controlled for physical variables		−0.275****	−0.220****
nutrients	−0.275*** controlled for zooplankton community −0.293*** controlled for phytoplankton community −0.274*** controlled for physical variables	−0.189** controlled for cyanobacterial community −0.197** controlled for phytoplankton community −0.245*** controlled for physical variables	−0.220*** controlled for cyanobacterial community −0.202** controlled for zooplankton community −0.224*** controlled for physical variables		0.302****
physical variables	−0.253**** controlled for zooplankton community −0.243**** controlled for phytoplankton community −0.199*** controlled for nutrients	−0.054 (n.s.) controlled for cyanobacterial community −0.065 (n.s.) controlled for phytoplankton community −0.057 (n.s.) controlled for nutrients	−0.169*** controlled for cyanobacterial community −0.186**** controlled for zooplankton community −0.149*** controlled for nutrients	0.230*** controlled for cyanobacterial community 0.278**** controlled for zooplankton community 0.258**** controlled for phytoplankton community	

Table A.3: Results of simple and partial Mantel tests relating the cyanobacterial community composition and total cyanobacterial biomass to the community of the different zooplankton groups (correlations are presented as Pearson correlation coefficients ; *:$p < 0.05$; **:$p < 0.01$; ***:$p < 0.001$; ****:$p < 0.0001$; n.s. = not significant).

	copepod community	cladoceran community	rotifer community
cyanobacterial community	0.186**	0.300****	0.151*
	0.118* controlled for cladoceran community	0.266**** controlled for copepod community	0.066 (n.s.) controlled for copepod community
	0.128* controlled for rotifer community	0.279**** controlled for rotifer community	0.096 (n.s.) controlled for cladoceran community
	0.128 (n.s.) controlled for phytoplankton community	0.250*** controlled for phytoplankton community	0.124 (n.s.) controlled for phytoplankton community
	0.139* controlled for nutrients	0.204*** controlled for nutrients	0.139* controlled for nutrients
	0.175* controlled for physical variables	0.261**** controlled for physical variables	0.147* controlled for physical variables
cyanobacterial biomass	−0.317****	−0.361****	−0.098*
	−0.250**** controlled for cladoceran community	−0.305**** controlled for copepod community	0.080* controlled for copepod community
	−0.313**** controlled for rotifer community	−0.350**** controlled for rotifer community	−0.027 (n.s.) controlled for cladoceran community
	−0.274**** controlled for phytoplankton biomass	−0.311**** controlled for phytoplankton biomass	−0.062 (n.s.) controlled for phytoplankton biomass
	−0.286**** controlled for nutrients	−0.293**** controlled for nutrients	−0.085 (n.s.) controlled for nutrients
	−0.317**** controlled for physical variables	−0.364**** controlled for physical variables	−0.097* controlled for physical variables

Table A.4: Results of simple and partial Mantel tests relating the cyanobacterial community composition and total cyanobacterial biomass to the biomass of *Daphnia* and *Bosmina* (correlations are presented as Pearson correlation coefficients; *:$p < 0.05$; **:$p < 0.01$; ***:$p < 0.001$; ****:$p < 0.0001$; n.s. = not significant).

	biomass of *Daphnia*	biomass of *Bosmina*
cyanobacterial community	−0.155**	−0.314****
	−0.006 (n.s.) controlled for *Bosmina* biomass	−0.276*** controlled for *Daphnia* biomass
	−0.113* controlled for phytoplankton community	−0.280*** controlled for phytoplankton community
	−0.053 (n.s.) controlled for nutrients	−0.240*** controlled for nutrients
	−0.127* controlled for physical variables	−0.289**** controlled for physical variables
cyanobacterial biomass	0.217***	0.311****
	0.081* controlled for *Bosmina* biomass	0.242*** controlled for *Daphnia* biomass
	0.149** controlled for phytoplankton biomass	0.280**** controlled for phytoplankton biomass
	0.141** controlled for nutrients	0.252**** controlled for nutrients
	0.216*** controlled for physical variables	0.311**** controlled for physical variables

Table A.5: Results of Spearman rank order correlations relating the relative abundance of single cyanobacterial genotypes (= relative intensity of respective DGGE bands) and biomass of *Daphnia* and *Bosmina* to each other (BC = band class, see figure A.3 and table A.1; correlations are presented as Spearman rank correlation coefficients; *:$p < 0.05$; **:$p < 0.01$; ***:$p < 0.001$; ****:$p < 0.0001$; n.s. = not significant).

	BC 10	BC 14	BC 16	BC 18	BC 19	*Daphnia*	*Bosmina*
BC 10 (*Synechococcus*)		−0.397**	−0.619****	−0.860****	−0.091 (n.s.)	0.517***	−0.705****
BC 14 (*Synechococcus*)	−0.397**		−0.173 (n.s.)	0.373**	−0.065 (n.s.)	−0.058 (n.s.)	0.131 (n.s.)
BC 16 (*Limnothrix redekei*)	−0.619****	−0.173 (n.s.)		0.296*	−0.023 (n.s.)	−0.293*	0.549****
BC 18 (*Synechococcus*)	−0.860****	0.373**	0.296*		−0.116 (n.s.)	−0.414**	0.512***
BC 19 (*Anabaena/ Aphanizomenon*)	−0.091 (n.s.)	−0.065 (n.s.)	−0.023 (n.s.)	−0.116 (n.s.)		−0.327*	0.169 (n.s.)
Daphnia	0.517***	−0.058 (n.s.)	−0.293*	−0.414**	−0.327*		−0.660****
Bosmina	−0.705****	0.131 (n.s.)	0.549****	0.512***	0.169 (n.s.)	−0.660****	

intensities of single DGGE band classes and biomass of *Daphnia* and *Bosmina* (Statistica 6.0) to know which species influenced each other in a positive or negative way. Table A.5 shows that band class 10 (*Synechococcus sp.*) has a negative correlation with band class 14 (*Synechococcus sp.*), 16 (*Limnothrix redekei*) and 18 (*Synechococcus sp.*) and band class 18 has a positive correlation with band class 14 and 16. Further, *Daphnia* is positively correlated to band class 10 and negatively to band class 16, 18 and 19 while *Bosmina* is negatively correlated to band class 10 and positively to band class 16 and 18. In addition, Spearman rank correlation coefficients show that the cyanobacterial biomass has a negative correlation with the biomass of *Daphnia* ($r = -0.457;\ p < 0.001$) and a positive correlation with the biomass of *Bosmina* ($r = 0.689;\ p < 0.0001$).

A.4 Discussion

Lake Blaarmeersen was characterized by a diverse cyanobacterial community dominated by the unicellular non-toxic cyanobacterium *Synechococcus*. The three *Synechococcus* genotypes probably represent different ecotypes because they were present in the lake during different periods and in different strata of the water column. It is unlikely that the three different band classes represent intragenomic sequence variation in 16S rRNA-genes of *Synechococcus* (Palenik *et al.* 2003) because the three genotypes did not occur in the same samples and negative correlations were found between the genotypes. In Lake Loosdrecht (The Netherlands), Zwart *et al.* (2005) also detected three different genotypes of *Synechococcus*, represented by three bands on different positions in the DGGE gradient. The relative intensities of these bands varied among spring, summer

and autumn, indicating that they may belong to different ecotypes. None of the *Synechococcus* genotypes found in Lake Blaarmeersen has 100 % sequence similarity with the genotypes from Lake Loosdrecht. The genus *Synechococcus* is polyphyletic (Honda *et al.* 1999; Robertson *et al.* 2001) and more sequence information is needed to determine the phylogenetic relationships between the different genotypes. The dominance of the filamentous non-toxic cyanobacterium *Limnothrix redekei* in the hypolimnion during the stratification period and in autumn agrees with other evidence that this species is better adapted to lower temperatures and light intensities than other cyanobacteria (e.g. Mur and Schreurs 1995; Havens *et al.* 1998). The nitrogen-fixing cyanobacteria *Anabaena* and *Aphanizomenon* were detected as a single band by DGGE. By sequencing a fragment of the 16S rDNA, it is not possible to distinguish between these two taxa (Kolmonen *et al.* 2004). Furthermore, phylogenetic studies have shown that *Anabaena* and *Aphanizomenon* probably belong to one genus (Castenholz 2001; Gugger *et al.* 2002). Microscopical determination confirmed that both cyanobacteria were present in Lake Blaarmeersen; *Anabaena* was mainly detected in August 2003 and *Aphanizomenon* was mainly detected in September 2003. *Anabaena* and *Aphanizomenon* are known to form surface blooms in summer when the water column is stable (Salmaso 2000). Some of the other bands in the DGGE profile were identified as chloroplast sequences. One should pay attention to the fact that primers specific for cyanobacterial 16S rRNA-genes also amplify most of the chloroplast 16S rRNA-genes (Kolmonen *et al.* 2004; Stiller and McClanahan 2005; Zwart *et al.* 2005; Ouellette *et al.* 2006). That this does not always happen is illustrated by the fact that several sequences in the databank Genbank/EMBL are indicated as belonging to cyanobacteria (especially clones) whereas in fact they represent chloroplasts. As a result, the DGGE profiles do not reflect only the cyanobacterial diversity but a combination of cyanobacteria and chloroplasts. Therefore, microscopical counts and cloning were additionally performed to provide extra information and complement the DGGE data presented here.

This study confirms the influence of several physical variables on the cyanobacterial community dynamics (e.g. Dokulil and Teubner 2000; Salmaso 2000; Pawlik-Skowronska *et al.* 2004), as well as the influence of dissolved nutrient concentrations (e.g. Pandey and Pandey 2002; Pawlik-Skowronska *et al.* 2004). On the other hand, two other classes of environmental variables, the zooplankton and phytoplankton community composition are also correlated to the cyanobacterial community composition. While the zooplankton and phytoplankton community are generally not independent of each other (Shapiro 1995; Sarnelle 2005; Abrantes *et al.* 2006), a significant and independent relationship was found with the cyanobacterial community composition, suggestive of direct effects. The rather low but significant correlation between the phytoplankton and the cyanobacterial community composition might reflect mechanisms of phytoplankton succession and competition (Huszar and Caraco 1998; Domingues *et al.* 2005; Huisman and Hulot 2005). The correlation between the zooplankton and cyanobacterial community composition is relatively high. When this relationship is analyzed further, it appears that the genus or species identity of zooplankton is more important than the major zooplankton group (copepods,

cladocerans and rotifers) to which they belong. The importance of the different zooplankton species present in the lake was confirmed by Mantel tests on the community composition of the copepods, cladocerans and rotifers respectively. Only the cladoceran community composition had a strong and unique influence on the cyanobacterial community dynamics. However, this finding needs to be interpreted with caution because of the lower taxonomic resolution of the copepod data (calanoids, cyclopoids, copepodites and nauplii) compared to those of cladocerans and rotifers (which were identified at the species or genus level), which may have obscured significant relationships. *Daphnia* and *Bosmina* were the most important grazing cladocerans in Lake Blaarmeersen and mainly *Bosmina* considerably influenced the cyanobacterial community dynamics. While *Daphnia* is a non-selective grazer,*Bosmina* grazes selectively (DeMott and Kerfoot 1982) and can consume small as well as large food particles (Bleiwas and Stokes 1985). Additionally,*Bosmina* might also indirectly influence the cyanobacterial community composition by grazing on heterotrophic nanoflagellates (DeMott and Kerfoot 1982) because heterotrophic nanoflagellates graze in turn on small cyanobacteria (Nishibe *et al.* 2002). In principle, it is also possible that the cyanobacterial community influences the zooplankton abundance and composition, because some cyanobacteria are toxic, have a low nutritional value or form large colonies and filaments which can negatively affect several zooplankton species (Ghadouani *et al.* 2003; Murrel and Lores 2004; Ruokolainen *et al.* 2006). However, the total biomass of cyanobacteria in Lake Blaarmeersen was low in comparison to other phytoplankton taxa and toxic cyanobacterial species were scarce, indicating that the influence of the cyanobacterial community on the zooplankton abundance and composition was limited. The positive correlation found between total cyanobacterial biomass and *Bosmina* biomass and the negative correlation between total cyanobacterial biomass and *Daphnia* biomass is in agreement with previous studies (Romo *et al.* 1996; DeMott *et al.* 2001; Abrantes *et al.* 2006) and might reflect a negative influence of filamentous cyanobacteria on *Daphnia*, however, it can also be the result of grazing of large *Daphnia* species on all cyanobacteria whereas *Bosmina* grazes more selectively.

The cyanobacterial community was composed of unicellular as well as filamentous cyanobacteria. Probably, the unicellular cyanobacteria are more easily grazed by different zooplankton species in comparison to the filamentous forms. Despite its low food quality, the picocyanobacterium *Synechococcus* is grazed by several zooplankton species (Martin-Creuzburg *et al.* 2005; Park *et al.* 2002). When grazed together with higher quality food, zooplankton is much less negatively affected (DeMott *et al.* 1998; Von Elert and Wolffrom 2001). Probably, the importance of zooplankton differs from lake to lake and is dependent of several biotic and abiotic characteristics. When the nutrient status is too high, the cyanobacterial community will become very dominant and will be composed by one or a few species. Under these circumstances, the influence of zooplankton on cyanobacteria will be very limited because zooplankton does not grow well (Ahlgren *et al.* 1990; Ghadouani *et al.* 2003; Müller-Navarra *et al.* 2004).

Several studies using a traditional microscopical approach studied the interactions

between phytoplankton (including cyanobacteria) and zooplankton community composition (e.g. van Donk *et al.* 1990; Schriver *et al.* 1995; Romo *et al.* 1996; Jeppesen *et al.* 1998; Romo *et al.* 2004) and showed that zooplankton can play a role in structuring the phytoplankton community. However, in these studies no direct correlations between the cyanobacterial and zooplankton community composition are calculated as cyanobacteria are seen as a part of the phytoplankton community. Therefore it is difficult to compare the zooplankton-cyanobacteria interactions found in this study to these traditional microscopical studies.

The goal of this study was to investigate the contribution of top-down variables relative to other explanatory variables in explaining cyanobacterial community dynamics in a natural setting. However, a significant relation between cyanobacterial community composition and community composition of potential predators may arise not only from a direct trophic interaction but also through indirect effects. Elser and Urabe (1999) showed that different zooplankton species differ in nutrient recycling which can influence the concentrations of dissolved nutrients which can in turn influence the cyanobacterial community dynamics (Callieri *et al.* 2004). However, this indirect effect of zooplankton was mainly excluded by calculating partial Mantel tests which allow controlling for confounding effects of co-varying variables. Despite these tests, this study cannot completely rule out the possibility that part of the correlation found is due to temporal changes in zooplankton and cyanobacterial community. Therefore, to confirm the importance of a direct relationship between zooplankton and cyanobacteria due to grazing, more field studies and experimental studies in which variables can be manipulated independently of each other are needed. The results from this study, however, strongly suggest that, while in general studies on the ecology of cyanobacteria rely mostly on the impact of bottom-up forces and physical conditions, one should also pay attention to top-down forces, which can be as important as dissolved nutrient concentrations and/or physical variables in structuring the cyanobacterial community.

Acknowledgements

The research presented here was carried out in the framework of the Belgian project B-BLOOMS (EV/13/34B) 'Algal blooms: emerging problem for health and sustainable use of surface waters' which was supported by the Federal Science Policy Office. We would like to thank Renaat Dasseville, Sam De Coster, Kevin De Keyser and Fanny De Ruysscher for helping with sampling and plankton analyses and Dirk Van Gansbeke for the nutrient analyses.

References

Abrantes, N., Antunes, S. C., Pereira, M. J. and Goncalves, F. 2006. Seasonal succession of cladocerans and phytoplankton and their interactions in a shallow eutrophic lake (Lake Vela, Portugal). Acta Oecologica-International Journal of Ecology 29: 54-64.

Ahlgren, G., Lundstedt, L., Brett, M. and Forsberg, C. 1990. Lipid-composition and food quality of some freshwater phytoplankton for cladoceran zooplankters. Journal of Plankton Research 12: 809-818.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. 1990. Basic local alignment search tool. Journal of Molecular Biology 215: 403-410.

Bergquist, A. M., Carpenter, S. R. and Latino, J. C. 1985. Shifts in phytoplankton size structure and community composition during grazing by contrasting zooplankton assemblages. Limnology and Oceanography 30: 1037-1045.

Bleiwas, A. H. and Stokes, P. M. 1985. Collection of large and small food particles by *Bosmina*. Limnology and Oceanography 30: 1090-1092.

Bonnet, E. and Van de Peer, Y. 2002. Zt: a software tool for simple and partial Mantel tests. Journal of Statistical software 7: 1-12.

Bottrell, H. H., Duncan, A. Gliwicz, Z. M., Grygierek, E., Herzig, A., Hillbrichtilkowska, A., Kurasawa, H., Larsson, P. and Weglenska, T. 1976. Review of some problems in zooplankton production studies. Norwegian Journal of Zoology 24: 419-456.

Boutte, C., Komárková, J., Grubisic, S., Znachor, P., Bataille, A., Zapomelová, E., Gullukaya, A., Jezberová, J. and Wilmotte, A. 2005. A polyphasic approach to assess the cyanobacterial diversity of summer samples from Czech reservoirs. Algological Studies 117: 177-195.

Brabrand, A., Faafeng, B. A., Kallqvist, T. and Nilssen, J. P. 1983. Biological control of undesirable cyanobacteria in culturally eutrophic lakes. Oecologia 60: 1-5.

Callieri, C., Balseiro, E., Bertoni, R. and Modenutti, B. 2004. Picocyanobacterial photosynthetic efficiency under *Daphnia* grazing pressure. Journal of Plankton Research 26: 1471-1477.

Castenholz, R. W. 2001. Phylum BX. Cyanobacteria. Oxygenic photosynthetic bacteria. In: The Archaea and the deeply branching and phototrophic Bacteria. Boone, D. R., Castenholz, R. W. and Garrity, G. M. (eds). Bergey's manual of systematic bacteriology, 2nd edition, vol. 1. Springer-Verlag, Berlin, Germany. pp. 473-599.

Chorus, I. and Bartram, J. (eds). WHO. 1999. Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. E and FN Spon. London, United Kingdom.

Clarke, K. R. and Gorley, R. N. 2001. PRIMER 5 for Windows. PRIMER-E Ltd, Plymouth, UK.

DeBernardi, R., Giussani, G. and Lasso Pedretti, E. 1981. The significance of blue-green algae as food for filter-feeding zooplankton: experimental studies on *Daphnia spp.* fed by *Microcystis aeruginosa*. Verhandlungen der internationalen Vereinigung für theoretische und angewandte Limnologie 21: 477-483.

Degans, H., Zollner, E., Van der Gucht, K., De Meester, L. and Jurgens, K. 2002. Rapid *Daphnia*-mediated changes in microbial community structure: an experimental study. FEMS Microbiology Ecology 42: 137-149.

DeMott, W. R. and Kerfoot, W. C. 1982. Competition among cladocerans - Nature of the interaction between *Bosmina* and *Daphnia*. Ecology 63: 1949-1966.

DeMott, W. R. and Moxter, F. 1991. Foraging on cyanobacteria by copepods - Responses to chemical defenses and resource abundance. Ecology 72: 1820-1834.

DeMott, W. R., Gulati, R. D. and Siewertsen, K. 1998. Effects of phosphorus-deficient diets on the carbon and phosphorus balance of *Daphnia magna*. Limnology and Oceanography 43: 1147-1161.

DeMott, W. R., Gulati, R. D. and van Donk, E. 2001. *Daphnia* food limitation in three hypereutrophic Dutch lakes: Evidence for exclusion of large-bodied species by interfering filaments of cyanobacteria. Limnology and Oceanography 46: 2054-2060.

Dokulil, M. T. and Teubner, K. 2000. Cyanobacterial dominance in lakes. Hydrobiologia 438: 1-12.

Domingues, R. B., Barbosa, A. and Galvao, H. 2005. Nutrients, light and phytoplankton succession in a temperate. estuary (the Guadiana, south-western Iberia). Estuarine Coastal and Shelf Science 64: 249-260.

Downing, J. A., Watson, S. B. and McCauley, E. 2001. Predicting cyanobacteria dominance in lakes. Canadian Journal of Fisheries and Aquatic Sciences 58: 1905-1908.

Elser, J. J. and Urabe, J. 1999. The stoichiometry of consumer-driven nutrient recycling: Theory, observations, and consequences. Ecology 80: 735-751.

Engström, J., Koski, M., Viitasalo, M., Reinikainen, M., Repka, S. and Sivonen, K. 2000. Feeding interactions of the copepods *Eurytemora affinis* and *Acartia bifilosa* with the cyanobacteria *Nodularia sp.* Journal of Plankton Research 22: 1403-1409.

Fiore, M. D., Neilan, B. A., Copp, J. N., Rodrigues, J. L. M., Tsai, S. M., Lee, H. and Trevors, J. T. 2005. Characterization of nitrogen-fixing cyanobacteria in the Brazilian Amazon floodplain. Water Research 39: 5017-5026.

Geysen, B. and Coppejans, E. 1985. Fytohydrobiologisch onderzoek van oppervlaktewaters in Vlaanderen, 2. De vijver in het sport- en recreatiedomein Blaarmeersen (Gent). Biologisch Jaarboek Dodonaea 53: 121-139.

Ghadouani, A., Pinel-Alloul, B. and Prepas, E. E. 2003. Effects of experimentally induced cyanobacterial blooms on crustacean zooplankton communities. Freshwater Biology 48: 363-381.

Gilbert, J. J. 1996. Effect of temperature on the response of planktonic rotifers to a toxic cyanobacterium. Ecology 77: 1174-1180.

Gliwicz, Z. M. and Lampert, W. 1990. Food thresholds in *Daphnia* species in the absence and presence of

blue-green filaments. Ecology 71: 691-702.
Grasshoff, K. 1976. Determination of nitrate and nitrite. In: Grasshoff, K. Methods of seawater analysis. Verslag Chemie, Weinheim, New York, 317 p.
Gugger, M., Lyra, C., Henriksen, P., Coute, A., Humbert, J. F. and Sivonen, K. 2002. Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. International Journal of Systematic and Evolutionary Microbiology 52: 1867-1880.
Haney, J. F. 1987. Field studies on zooplankton-cyanobacteria interactions. New Zealand Journal of Marine and Freshwater Research 21: 467-475.
Haney, J. F. and Hall, D. J. 1973. Sugar-coated *Daphnia*: A preservation technique for Cladocera. Limnology and Oceanography. 18: 331-333.
Havens, K. E., Phlips, E. J., Cichra, M. F. and Li, B. L. 1998. Light availability as a possible regulator of cyanobacteria species composition in a shallow subtropical lake. Freshwater Biology 39: 547-556.
Holm, N. P. and Shapiro, J. 1984. An examination of lipid reserves and the nutritional-status of *Daphnia pulex* fed *Aphanizomenon flos-aquae*. Limnology and Oceanography 29: 1137-1140.
Honda, D., Yokota, A. and Sugiyama, J. 1999. Detection of seven major evolutionary lineages in cyanobacteria based on the 16S rRNA gene sequence analysis with new sequences of five marine *Synechococcus* strains. Journal of Molecular Evolution 48: 723-739.
Huisman, J., Matthijs, H. C. P. and Visser, P. M. (eds). 2005. Harmful cyanobacteria. Springer, Dordrecht, The Netherlands. 243 pp.
Huisman, J. and Hulot, F. D. 2005. Population dynamics of harmful cyanobacteria. In: Harmful cyanobacteria. Huisman, J., Matthijs, H. C. P. and Visser, P. M. (eds). Springer, Dordrecht, The Netherlands. pp.143-176.
Huszar, V. L. D. and Caraco, N. F. 1998. The relationship between phytoplankton composition and physical-chemical variables: a comparison of taxonomic and morphological-functional descriptors in six temperate lakes. Freshwater Biology 40: 679-696.
Jacoby, J. M., Collier, D. C., Welch, E. B., Hardy, F. J. and Crayton, M. 2000. Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*. Canadian Journal of Fisheries and Aquatic Sciences 57: 231-240.
Jayatissa, L. P., Silva, E. I. L., McElhiney, J. and Lawton, L. A. 2006. Occurrence of toxigenic cyanobacterial blooms in freshwaters of Sri Lanka. Systematic and Applied Microbiology 29: 156-164.
Jeppesen, E., Sondergaard, M., Jensen, J. P., Mortensen, E., Hansen, A. M. and Jorgensen, T. 1998. Cascading trophic interactions from fish to bacteria and nutrients after reduced sewage loading: An 18-year study of a shallow hypertrophic lake. Ecosystems 1: 250-267.
Kolmonen, E., Sivonen, K., Rapala, J. and Haukka, K. 2004. Diversity of cyanobacteria and heterotrophic bacteria in cyanobacterial blooms in Lake Joutikas, Finland. Aquatic Microbial Ecology 36: 201-211.
Koroleff, F. 1976. Determination of ammonia, phosphorus and silicon. In: Grasshoff, K. Methods of seawater analysis. Verslag Chemie, Weinheim, New York. 317 p.
Koski, M., Schmidt, K., Engstrom-Ost, J., Viitasalo, M., Jonasdottir, S., Repka, S. and Sivonen, K. 2002. Calanoid copepods feed and produce eggs in the presence of toxic cyanobacteria *Nodularia spumigena*. Limnology and Oceanography 47: 878-885.
Lampert, W. 1987. Laboratory studies on zooplankton-cyanobacteria interactions. New Zealand Journal of Marine and Freshwater Research 21: 483-490.
Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Research 27: 209-220.
Mantel, N. and Valand, R. S. 1970. A technique of nonparametric multivariate analysis. Biometrics 26: 547-558.
Martin-Creuzburg, D., Wacker, A. and von Elert, E. 2005. Life history consequences of sterol availability in the aquatic keystone species *Daphnia*. Oecologia 144: 362-372.
Menden-Deuer, S. and Lessard, E. J. 2000. Carbon to volume relationships for dinoflagellates, diatoms and other protist plankton. Limnology and Oceanography 45: 569-579.
Milutinovic, A., Zivin, M., Zorc-Pleskovic, R., Sedmak, B. and Suput, D. 2003. Nephrotoxic effects of chronic administration of microcystins -LR and -YR. Toxicon 42: 281-288.
Müller-Navarra, D. C., Brett, M. T., Park, S., Chandra, S., Ballantyne, A. P., Zorita, E. and Goldman, C. R. 2004. Unsaturated fatty acid content in seston and tropho-dynamic coupling in lakes. Nature 427: 69-72.
Mur, L. R. and Schreurs, H. 1995. Light as a selective factor in the distribution of phytoplankton species. Water Science and Technology 32: 25-34.
Murrel, M. C. and Lores, E. M. 2004. Phytoplankton and zooplankton seasonal dynamics in a subtropical estuary: importance of cyanobacteria. Journal of Plankton Research 26: 371-382.
Muylaert, K., Van der Gucht, K., Vloemans, N., De Meester, L., Gillis, M. and Vyverman, W. 2002. Relationship between bacterial community composition and bottom-up versus top-down variables in four eutrophic shallow lakes. Applied and Environmental Microbiology 68: 4740-4750.
Muyzer, G., Dewaal, E. C. and Uitterlinden, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S ribosomal RNA. Applied and Environmental Microbiology 59: 695-700.
Ni, C. V., Yakunin, A. F. and Gogotov, I. N. 1990. Effect of bound forms of nitrogen on the growth and synthesis of different *Anabaena variabilis* nitrogenases. Microbiology 59: 260-263.
Nishibe, Y., Kawabata, Z. and Nakano, S. 2002. Grazing on *Microcystis aeruginosa* by the heterotrophic flagellate *Collodictyon triciliatum* in a hypertrophic pond. Aquatic Microbial Ecology 29: 173-179.
Noges, T., Noges, P. and Laugaste, R. 2003. Water level as the mediator between climate change and

phytoplankton composition in a large shallow temperate lake. Hydrobiologia 506: 257-263.
Nübel, U., Garcia-Pichel, F. and Muyzer, G. 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. Applied and Environmental Microbiology 63: 3327-3332.
Ouellette, A. J. A., Handy, S. M. and Wilhelm, S. W. 2006. Toxic *Microcystis* is widespread in Lake Erie: PCR detection of toxin genes and molecular characterization of associated cyanobacterial communities. Microbial Ecology 51: 154-165.
Palenik, B., Brahamsha, B., Larimer, F. W., Land, M., Hauser, L., Chain, P. *et al.* 2003. The genome of a motile marine *Synechococcus*. Nature 424: 1037-1042.
Pandey, J. and Pandey, U. 2002. Cyanobacterial flora and the physico-chemical environment of six tropical fresh water lakes of Udaipur, India. Journal of Environmental Sciences (China) 14: 54-62.
Park, S., Brett, M. T., Muller-Navarra, D. C. and Goldman, C. R. 2002. Essential fatty acid content and the phosphorus to carbon ratio in cultured algae as indicators of food quality for *Daphnia*. Freshwater Biology 47: 1377-1390.
Pawlik-Skowronska, B., Skowronski, T., Pirszel, J. and Adamczyk, A. 2004. Relationship between cyanobacterial bloom composition and anatoxin-a and microcystin occurrence in the eutrophic dam reservoir (SE Poland). Polish Journal of Ecology 52: 479-490.
Pflugmacher, S. 2004. Promotion of oxidative stress in the aquatic macrophyte *Ceratophyllum demersum* during biotransformation of the cyanobacterial toxin microcystin-LR. Aquatic Toxicology 70: 169-178.
Robertson, B. R., Tezuka, N. and Watanabe, M. M. 2001. Phylogenetic analyses of *Synechococcus* strains (cyanobacteria) using sequences of 16S rDNA and part of the phycocyanin operon reveal multiple evolutionary lines and reflect phycobilin content. International Journal of Systematic and Evolutionary Microbiology 51: 861-871.
Rohrlack, T., Dittmann, E., Henning, M., Borner, T. and Kohl, J. G. 1999. Role of microcystins in poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 65: 737-739.
Rohrlack, T., Christoffersen, K., Dittmann, E., Nogueira, I., Vasconcelos, V. and Borner, T. 2005. Ingestion of microcystins by *Daphnia*: intestinal uptake and toxic effects. Limnology and Oceanography 50: 440-448.
Romo, S., Miracle, M. R., Villena, M. J., Rueda, J., Ferriol, C. and Vicente, E. 2004. Mesocosm experiments on nutrient and fish effects on shallow lake food webs in a Mediterranean climate. Freshwater Biology 49: 1593-1607.
Romo, S., van Donk, E., Gylstra, R. and Gulati, R. 1996. A multivariate analysis of phytoplankton and food web changes in a shallow biomanipulated lake. Freshwater Biology 36: 683-696.
Ruokolainen, L., Lilley, T. M., Tammi, M. and Vuorinen, P. 2006. Zooplankton in relation to cyanobacteria across a geographic gradient in Archipelago Sea, northern Baltic. Boreal Environment Research 11: 1-11.
Salmaso, N. 2000. Factors affecting the seasonality and distribution of cyanobacteria and chlorophytes: a case study from the large lakes south of the Alps, with special reference to Lake Garda. Hydrobiologia 438: 43-63.
Sarnelle, O. 2005. *Daphnia* as keystone predators: effects on phytoplankton diversity and grazing resistance. Journal of Plankton Research 27: 1229-1238.
Schriver, P., Bogestrand, J., Jeppesen, E. and Sondergaard, M. 1995. Impact of submerged macrophytes on fish-zooplankton-phytoplankton interactions - Large-scale enclosure experiments in a shallow eutrophic lake. Freshwater Biology 33: 255-270.
Sellner, K. G., Brownlee, D. C., Bundy, M. H., Brownlee, S. G. and Braun, K. R. 1993. Zooplankton grazing in a Potomac river cyanobacteria Bloom. Estuaries 16: 859-872.
Shapiro, J. 1995. Lake restoration by biomanipulation-a personal view. Environmental Review 3: 83-93.
Sherr, E. B. and Sherr, B. F. 1993. Preservation and storage of samples for enumeration of heterotrophic protists. In: Handbook of methods in aquatic microbial ecology. Kemp, P. F., Sherr, B. F., Sherr, E. B. and Cole, J. J. (eds). CRC Press, Boca Raton: 207-212.
Starkweather, P. L. and Kellar, P. E. 1983. Utilization of cyanobacteria by *Brachionus calyciflorus* - *Anabaena flos-aquae* (Nrc-44-1) as a sole or complementary food source. Hydrobiologia 104: 373-377.
Stiller, J. W. and McClanahan, A. 2005. Phyto-specific 16S rDNA PCR primers for recovering algal and plant sequences from mixed samples. Molecular Ecology Notes 5: 1-3.
Taton, A., Grubisic, S., Brambilla, E., De Wit, R. and Wilmotte, A. 2003. Cyanobacterial diversity in natural and artificial microbial mats of lake Fryxell (McMurdo Dry Valleys, Antartica): a morphological and molecular approach. Applied and Environmental Microbiology 69: 5157-5169.
Tikkanenn, T. and Willénn, T. 1992. Växtplantonflora. Författarna Natur, Solna. 280 pp.
van Donk, E., Grimm, M. P., Gulati, R. D. and Klein Breteler, J. P. G. 1990. Whole-lake food-web manipulation as a means to study community interactions in a small ecosystem. Hydrobiologia 200/201: 275-289.
Viaggiu, E., Melchiorre, S., Volpi, F., Di Corcia, A., Mancini, R., Garibaldi, L. *et al.* 2004. Anatoxin-a toxin in the cyanobacterium *Planktothrix rubescens* from a fishing pond in northern Italy. Environmental Toxicology 19: 191-197.
Von Elert, E. and Wolffrom, T. 2001. Supplementation of cyanobacterial food with polyunsaturated fatty acids does not improve growth of *Daphnia*. Limnology and Oceanography 46: 1552-1558.
Work, K. A. and Havens, K. E. 2003. Zooplankton grazing on bacteria and cyanobacteria in a eutrophic lake. Journal of Plankton Research 25: 1301-1306.
Zurawell, R. W., Chen, H. R., Burke, J. M. and Prepas, E. E. 2005. Hepatotoxic cyanobacteria: a review of the biological importance of microcystins in freshwater environments. Journal of Toxicology and Environmental Health-Part B-Critical Reviews 8: 1-37.

Zwart, G., Huismans, R., van Agterveld, M. P., Van de Peer, Y., De Rijk, P., Eenhoorn, H. *et al.* 1998. Divergent members of the bacterial division *Verrucomicrobiales* in a temperate freshwater lake. FEMS Microbiology Ecology 25: 159-169.

Zwart, G., Kamst-van Agterveld, M. P., van der Werff-Staverman, I., Hagen, F., Hoogveld, H. L. and Gons, H. J. 2005. Molecular characterization of cyanobacterial diversity in a shallow eutrophic lake. Environmental Microbiology 7: 365-377.