Universidad Autónoma de Madrid

Facultad de Ciencias

Departamento de Biología

Chemotypical subpopulations in planktonic cyanobacteria. Ecology and Application in advanced monitoring tools

Subpoblaciones quimiotípicas en cianobacterias planctónicas. Ecología y aplicación en herramientas avanzadas de monitorización

Doctoral Dissertation

Tesis Doctoral

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A mis padres

To my parents

Table of Contents / Índice

Abbreviations /Abreviaturas

Adda : 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid

Adha: 3-amino-(2-hydroxy) decenoid acid

AHC: Ascending Hierarchical Classification

Ahp: 3-amino-6-hydroxy-2-piperidone

ALS/PDC: amyotrophic lateral sclerosis and Parkinson-dementia complex

ANOVA: Analysis of Variance

ATX-a: Anatoxin-a

BMAA: β-Methylamino-L-alanine

Chl-a: Chlorophyll-a

Choi: 2-carboxy-6-hydroxyoctahydroindole

CT: Chemotype

CYN: Cylindrospermopsin

DAB: α-γ -diaminobutyric acid

DGGE: Denaturing Gradient Gel Electrophoresis

DIN: Dissolved Inorganic Nitrogen

DNA: Desoxyribonucleic acid

DOM: Dissolved Organic Matter

EPS: Exopolysaccharides

ESA: European Space Agency

ESI: Electrospray Ionization

HCCA: α-cyano-4-hydroxycinnamic acid

HPLC: High Performance Liquid Chromatography

IDF: Interreplicate Detection Frequency

Ka: Non-synonymous substitution

Ks: Synonymous substitution

LC: Liquid Chromatography

LC-MS/MS: Liquid Chromatography tandem Mass Spectrometry

LD_{50}: Lethal Dose 50

LH: High Light

LL: Low Light

LM: Medium Light

LT-SEM: Low Temperature – Scanning Electron Microscopy

m/z: mass / charge ratio

MALDI-TOF MS: Matrix- Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry

MC: Microcystin

MERIS: MEdium Resolution Imaging Spectrometer

NC: Nutrient Control

NGR: Net Growth Rate

NIR: Near-Infrared Region

NMR: Nuclear Magnetic Resonance

NN: Nitrogen-poor treatment

NP: Phosphorous-poor treatment

NRPS: Non-Ribosomal Peptide Synthase

PAM Flurometry : Pulse Amplitude Modulated Flurometry

PC: Phycocyanin

PCA: Principal Component Analysis

PDA: Photodiode Array

PKS: Polyketide Synthase

PSTs: Paralytic Shellfish Toxins

PVC: Polyvinyl Chloride

q-PCR: quantitative Polymerase Chain Reaction

RIT: Relative Intensity Threshold

RMSEs: Root Mean Squared Errors

RNA: Ribonucleic acid

rRNA: Ribosomal Ribonucleic acid

SAR: Structure-Activity Relation

SR: Sedimentation Rate

SRP: Soluble Reactive Phosphorous

TFA: Trifluoracetic acid

TOF: Time Of Flight

WFD: Water Framework Directive

WHO: World Health Organization

*Abbreviations not listed here are defined in the text when mentioned for the first time.

Figure Index / Índice de Figuras

Table Index / Índice de Tablas

“Though this be madness, yet there is a method in ’t”

Polonius in “Hamlet”, Act II, Scene 2

William Shakespeare

1. Introduction

1.1. Cyanobacteria: general features

Cyanobacteria are photoautotrophic prokaryotes, capable of performing oxygenic photosynthesis by photosystems I and II, using water as electron source and releasing oxygen as by-product. Cyanobacteria are considered the first organisms producing oxygen as a photosynthetic product and are in fact considered responsible for the transformation of the atmosphere to oxygenic conditions (Schopf 2001). Endosymbiosis between a protist and a cyanobacterium led to the formation of a membrane-bound photosynthetic organelle, the chloroplast (Bhattacharya et al. 2003). Oxygenic photosynthesis by modern green and red algae, as well as plants, is hence of cyanobacterial origin.

Cyanobacteria harvest light thanks to photosynthetic pigments chlorophyll *a* (Chl-a) and phycocyanin, which provide their characteristic blue-green pigmentation that gave rise to the term “blue-green algae”, still used in some publications. Other accessory pigments may also be present, such as phycoerythrine and carotenoids, which allow the absorption of light at a wider range of wavelengths (Fig. 1.1). The photosynthetic machinery is located in the thylakoids. In cyanobacteria, thylakoid membranes are not differentiated into granum and stroma regions as it happens in plants. Instead, they form stacks of parallel sheets close to the cytoplasmic membrane (van de Meene et al. 2006). While Chl-a protein complexes, photosynthetic reaction centers and electron transfer system are located within the thylakoids, phycobilisomes are attached to the surface of the thylakoids. Phycobilisomes contain the major light harvesting pigments in cyanobacteria, the phycobilins. Phycobilisomes are formed by a series of rods that are connected to the phycobilisome core. The core contains allophycocyanin (A_{max}=650 nm), whereas the rods contain phycocyanin (A_{max}=620 nm) and sometimes phycoerythrine (A_{max}=560 nm), among other pigments. Phycobilisomes harvest energy from light, which flows unidirectionally from the rods to the core and then to the photosynthetic reaction centers in the thylakoid.

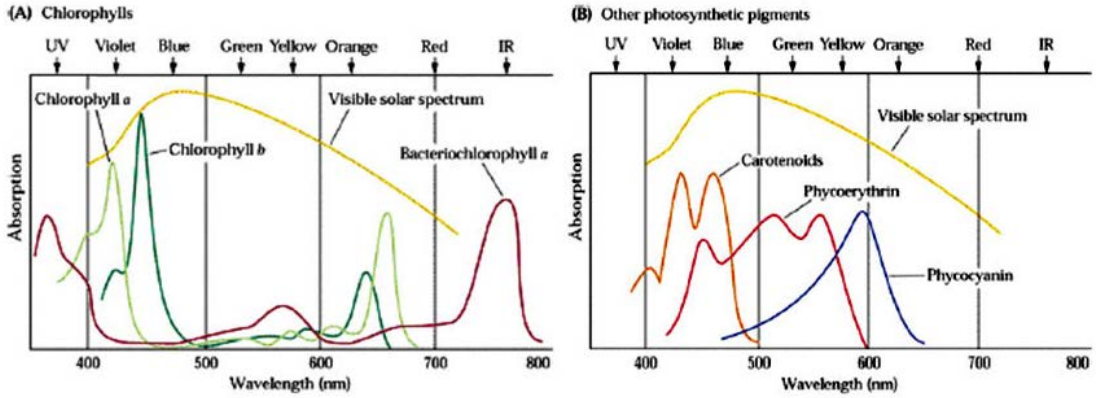


Figure 1.1. Absorption spectra of the main photosynthetic pigments. (A) Chlorophylls; (B) Other pigments. Source: Creative Commons

Although traditionally considered algae, cyanobacteria present similar intracellular organization (lack of nucleus and membrane-bound organelles), cell wall structure (peptidoglycan layer) and protein synthesis machinery to those of other prokaryotes, and are therefore now classified as bacteria, namely Gram negative bacteria.

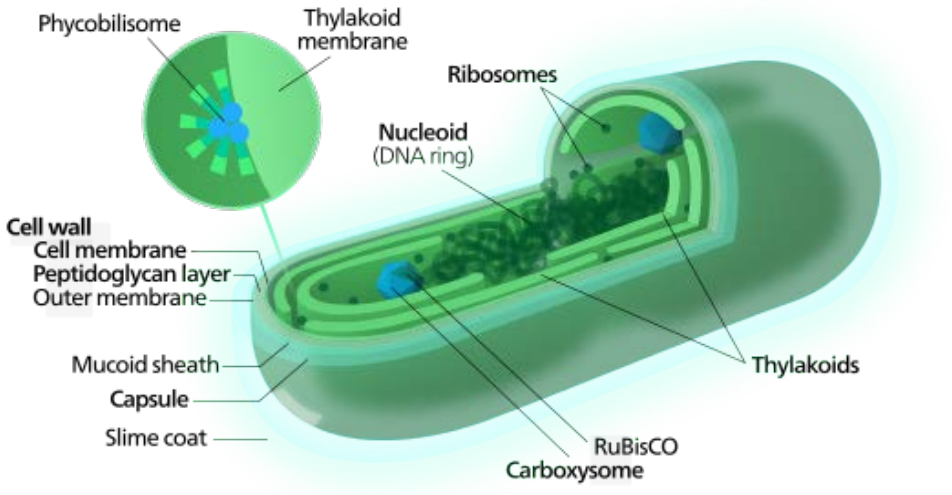


Figure 1.2. Diagram of a typical cyanobacterial cell. Source: Creative Commons

Cyanobacteria lack nucleus and genetic material appears as a circular double-stranded DNA chromosome which is not linked to hystones and is therefore dispersed within the cytoplasm. Their cell wall consists of a few layers of peptidoglycan surrounded by an outer membrane containing lipopolysaccharides and lipoproteins. The cell protoplast presents

inclusions, such as carboxysomes (i.e. compartments containing enzymes involved in carbon fixation) or granules of polymers (e.g. glycogen, polyphosphate, cyanophycin) that serve as energy and nutrient storage. Many planktonic cyanobacteria also present gas vesicles, proteinacious protoplastic inclusions filled with gas, which allow the regulation of the overall cell density and enable some aquatic cyanobacteria to control their buoyancy and thereby their position in the water column.

Some cyanobacteria present distinctive features with regard to their ability to form specialized cells. For instance, heterocysts – specialized cells with thickened walls and lacking thylakoids that are responsible for atmospheric N_2 fixation. These cells provide an anoxic microenvironment that allows the enzymatic reduction of atmospheric N_2 via nitrogenase to ammonia (NH_4^+) and its further assimilation. Nitrogen fixation by cyanobacteria represents in fact an important input of nitrogen into oceanic and terrestrial ecosystems (Vitousek et al. 2002, Kitajima et al. 2009). Another type of specialized cells present in some filamentous genera are akinetes, spore-like cells with a different morphology than vegetative cells that contain energy-rich polymers such as cyanophycin and glycogen. Akinetes serve as resting forms after filament death and germinate under favorable environmental conditions, regenerating a new filament of vegetative cells. Akinetes are not always present and their differentiation is triggered under nutrient or energy limiting conditions (Sili et al. 1994, Sukenik et al. 2013). Other examples of specialized cells in cyanobacteria are hormogonia, reproductive structures in form of motile filaments that disperse in the environment, and baeocytes, groups of small endospores that can become motile when in contact with a solid surface.

1.2. Cyanobacterial taxonomy

Given the ecological features shared with eukaryotic algae, cyanobacteria were considered in the first place a special group of microalgae. For this reason and due to their particular pigment composition, cyanobacteria were called “blue-green algae”, although nowadays this term is not much in use. Similarly to the taxonomy of other non-sexually-reproducing organisms, the concept of species in cyanobacteria has a diffuse boundary. The controversy on what are the distinctive features (if any at all) that delimit a species in the prokaryotic world is partially reflected in the taxonomy of cyanobacteria by the dual use of botanical and bacteriological criteria for taxa definition, and is therefore a matter of scientific discussion yet to be solved.

The botanical approach (e.g. Komárek and Anagnostidis 1999) is based mainly on morphological features of cyanobacteria, such as cell morphology, presence of envelopes or

sheaths, type of cell division, etc. This approach is included under the International Code of Botanic Nomenclature (Greuter et al. 2000), which results in definition of over 2000 cyanobacterial species, grouped in 4 different orders (Table 1.1).

Order	Main features	Families	Examples of freshwater planktonic genera within order
Chroococcales	Unicellular, some genera grouped as colonies surrounded by mucilage; cell division in one, two or three planes.	Chroococcaceae Synechoccaceae Microcystaceae Merismopediaceae Entophysalidaceae Hydrococcaceae Chamaesiphonaceae Dermocarpellaceae Xenococcaceae Hyellaceae	*Aphanocapsa, Chroococcus, Merismopedia, Microcystis, Synechocystis, Synechococcus, Radiocystis, Snowella, Woronichinia*
Oscillatoriales	Filamentous. Lacking heterocysts or akinetes. Sheath appears commonly.	Pseudanbaenaceae Schizotrichaceae Phormidiaceae Oscillatoriaceae Borziaceae Gomontiellaceae	*Planktothrix, Pseudanabaena, Arthrospira, Limnothrix*
Nostocales	Filamentous; with heterocysts and akinetes; may form hormogonia; some taxa show false branching	Scytonemaceae Microchaetaceae Rivulariaceae Nostocaceae	*Anabaena, Aphanizomenon, Cylindropermopsis, Nodularia, Raphidiopsis,*
Stigonematales	Filamentous; with heterocysts and akinetes; show true branching	Chlorogloeopsaceae Capsosiraceae Stigonomataceae Fischerellaceae Bourzinemataceae Loriellaceae Nostochopsaceae Mastigocladaceae	*Umezakia**

Table 2.1. Cyanobacterial taxonomic classification after Komárek and Anagnostidis (1989, 1999, 2005)
*Genus *Umezakia* was traditionally classified under order Stigonematales, but recent phylogenetic analysis motivated its inclusion into Family Nostocaceae (Niiyama et al. 2011).

Alternatively to the botanical approach, cyanobacteria are also classified after bacteriological systematics, according to the International Code for Nomenclature of Bacteria. This approach is not limited to morphological features, but also considers physiological, cytological and biochemical properties of axenic cyanobacterial cultures. These aspects include criteria like pigment composition, fatty acid and nucleotide compositions, genome length and 16S rRNA based phylogeny (i.e. phylogeny based on the sequences codifying ribosomic RNA), which were gradually incorporated to the bacteriological classification, leading to several revisions since the late 70's (Rippka et al. 1979, Boone & Castenholz 1989, 2001) until the

current classification (table 1.2) which establishes 5 subsections. These subsections can be assimilated to the cyanobacterial orders proposed by the botanical approach.

Class	Subsection	Part	Examples of freshwater planktonic genera
Cyanobacteria	I (Chroococcales)	I	*Aphanocapsa, Chroococcus, Merismopedia, Microcystis, Radiocystis, Synechococcus, Synechocystis, Snowella, Woronichinia*
	II (Pleurocapsales)[1]	I	-
		II	-
	III (Oscillatoriales)	I	*Pseudanabaena, Arthrospira, Limnothrix, Planktothrix*
	IV (Nostocales)	I	*Anabaena, Anabaenopsis, Aphanizomenon, Cylindrospermopsis, Nodularia,*
		II	*Gleotrichia*
	V (Stigonematales) [1]	I	*Umezakia*[2]

Table 1.2. Cyanobacterial classification according to Bergey's Manual of Systematic Bacteriology (Boone and Castenholz, 2001). Names in parenthesis correspond to the denomination according to botanical classification. [1] Only benthic genera described. [2] This genus has recently been classified under subsection IV (Niiyama et al. 2011).

The microbiological approach delimits however a significantly lower number of species than the botanical approach. An illustrative example of this is the Chroococcacean genus *Microcystis,* for which a single species was assumed by the Bergey's Manual of Systematic Bacteriology, and is in contrast split in 11 different species under the botanical classification (Komárek & Komárkova 2002), taking into consideration their morphological differences. This controversy has been temporally resolved by the use of the term "morphospecies" for other *Microcystis* species other than *M. aeruginosa* (i.e. *M. flos-aquae, M. novaceckii, M. wesenbergii, M. botrys, M. smithii* and others).

The prevailing systematic view points to an integrative perspective, taking into account phenotypic, genotypic and phylogenetic features, and integrate them into the so-called polyphasic taxonomy (Vandamme et al. 1996). However, (cyano)bacterial taxonomy is still far from achieving a general consensus, as evidenced by the recurrent revisions in taxonomy and nomenclature. From 2005 onwards, several changes at the family, genus and species level have been proposed. These revisions are based chiefly on botanical criteria, and to a lesser extent, on 16S rRNA phylogeny and some biochemical properties (Zapomelova et al. 2009). In the context of this thesis, the most important changes proposed relate to genus *Anabaena,* (typically comprising both planktonic and benthic taxa), which is proposed to be maintained only for mat forming species, whereas planktonic species traditionally under genus *Anabaena* are now comprised within the genus *Dolichospermum* (Komárek & Zapomelova 2007).

Moreover, *Aphanizomenon issatschenkoii* is substituted by the new genus *Cuspidothrix* (Rajaniemi et al. 2005) Lastly, species *Aphanizomenon aphanizomenoides* and *Anabaena kisseleviana* are substituted by the genus *Sphaerospermum* (Zapomelova et al. 2009). However, as these recent modifications have not achieved a full scientific consensus, the present work will stick to the previous nomenclature after Komárek and Anagnostidis (1989, 1999, 2005).

1.3. Ecology of cyanobacteria

Cyanobacteria are ancient organisms, with fossil evidences of their existence dating from the earliest records of life on Earth. The most studied of these fossil deposits refers to the Aphex Chert fossils, located in Western Australia (Schopf 1993). These fossils were generated from sand, silt and mud by pressure and heat over long periods of geological time and are dated from around 3.5 billion years. They revealed fossil evidence of a wide range of spherical and filamentous organisms which are similar in size and shape to the current cyanobacteria. Another important fossil deposit is the Gunflint Chert located in Lake Erie (United States) dating from approximately 2.09 billion years. The presence of fine ferric oxide bands in geologically adjacent rocks indicates the presence of oxygen and evidences photosynthetic activity in an otherwise anaerobic environment (Klein & Beukes 1992).

According to fossil records, cyanobacteria were strongly present over most of the history of Life, mainly as stromatolites. In the present however, stromatolites are scarce, with the most representative redoubts located in Western Australia, close to the Aphex Chert deposits, and at Shark Bay in the Caribbean Sea. Instead, cyanobacteria are extremely frequent in other forms and have colonized almost every habitat on Earth, including extreme environments such as the polar regions, desert areas, as well as brackish, saline and thermal waters (Ward & Castenholz 2000, Quesada & Vincent 2012) and have also established symbiotic relationships with diverse organisms including plants, fungi and animals (Whitton & Potts 2000).

Cyanobacteria are common inhabitants of aquatic environments, including oceans, seas, lakes and reservoirs worldwide. In these aquatic systems, human-induced environmental changes, most notably nutrient enrichment associated to urban, agricultural and industrial development, have led to increased rates of primary production and the dominance and periodic massive proliferations of cyanobacteria or blooms.

Cyanobacterial blooms typically refer to massive proliferations of cyanobacteria, with Chl-a concentrations above 10 μgL^{-1} and/or 20,000 cyanobacterial cells per milliliter (Oliver & Ganf 2000). Blooms often cause cyanobacterial biomass to accumulate in the surface, leading to the formation of scums or paint-like stains floating on the water (fig. 1.3). Under these circumstances, Chl-a concentrations may exceed 1 mgL^{-1} and 10^6 cells mL^{-1}. Cyanobacterial blooms are relevant phenomena causing a variety of adverse effects from different perspectives. Massive cyanobacterial proliferations increase the turbidity of the water, often suppressing the growth of macrophytes and thereby affecting the habitat of many macroinvertebrates and fish species. Furthermore, they tend to induce hypoxia during stratification, sometimes causing massive fish mortality. In addition, blooms cause aesthetic problems and unpleasant odors due to the production of geosmin and other chemical compounds, which affect recreational activities and impact the organoleptic properties of drinking waters. However, the most relevant threat associated to cyanobacterial blooms is related to the ability of some genera to produce diverse toxic compounds, which will be covered in detail later on in this introduction.

Figure 1.3. Surface scums at Alcántara Reservoir, Central Spain.

In aquatic ecosystems, planktonic cyanobacterial populations are co-modulated by both top-down and bottom-up mechanisms, which jointly comprise a complex matrix of interactions with abiotic (physicochemical, hydrological, etc.) and biotic factors (grazing, pathogens, parasites etc., fig 1.4). In this sense, cyanobacterial dominance over their competitors has been associated to their ability to harvest and utilize the existing resources more efficiently, as well as to minimize population losses.

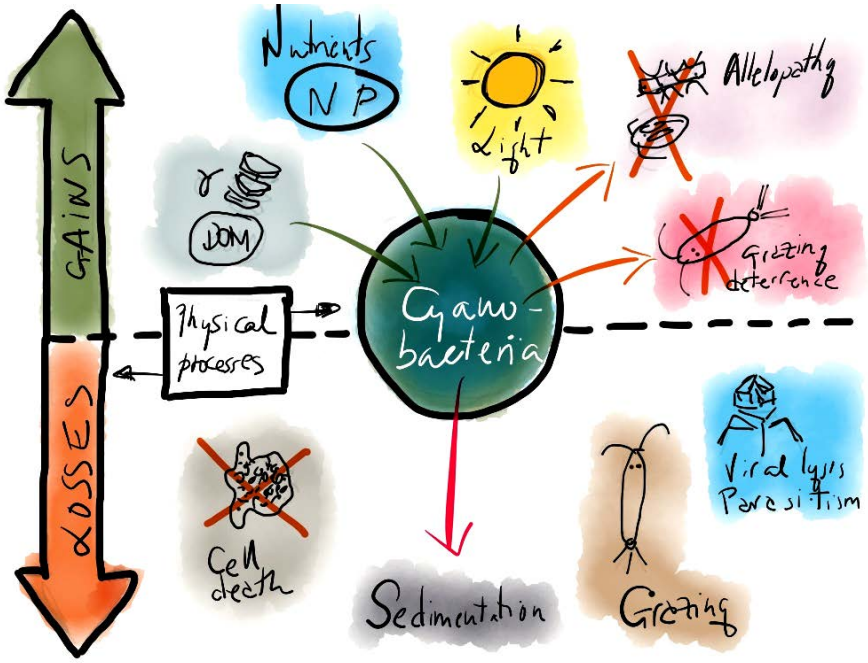


Figure 1.4. Factors affecting cyanobacterial gains and losses. In the upper part, cyanobacterial gains are influenced by their ability to utilize dissolved organic matter (DOM), nutrient and light uptake and, the release of allelochemicals under adverse conditions that affect other algal competitors or grazers. In the lower part, cyanobacterial loss processes include cell death, sedimentation, grazing, viral lysis and diverse forms of parasitism. Adapted from Granèli and Turner (2006).

In temperate freshwater lakes and reservoirs, cyanobacterial blooms tend to occur during the summer season. In deep lentic systems, a typical phytoplanktonic succession pattern is often observed, consisting in an initial growth of diatoms during spring, followed by a sharp increase in green algae, coincident with nutrient rich conditions. Toward the end of the summer, with maximal water temperatures and gradual nutrient depletion, cyanobacteria become dominant in the planktonic community, often as massive blooms (Reynolds et al. 1984, Oliver & Ganf 2000). The dominance of cyanobacteria compared to other algal groups is inconsistent with their lower growth rates compared eukaryotic algae. Instead, cyanobacteria display ecophysiological adaptations to outcompete their competitors.

Cyanobacteria take advantage of the thermal stratification of waters. The high stability of a stratified water column allows bloom-forming cyanobacteria to optimally control their position in the water column by regulating their buoyancy and thereby find advantageous depths for optimal light and nutrient harvesting. Some species (e.g. *Microcystis aeruginosa*) can accumulate in the surface as a result of overbuoyancy, leading to the formation of scums during wind calm summer periods.

Besides, the array of accessory pigments in cyanobacteria constitutes a competitive advantage. The spectral composition of the incident light in the water column is strongly influenced by depth and turbidity. Consequently, changes in depth as a result of buoyancy control imply changes in the qualitative (and also quantitative) composition of light, which cyanobacteria have to cope with. Accessory pigments allow cyanobacteria to harvest energy of virtually the whole photosynthetically active spectrum. In the presence or dominance of green algae, for instance, chlorophytes absorb an important fraction of the incoming irradiance, although wavelengths in the orange-green spectra region cannot be utilized by them (Kirk 1983). Instead, cyanobacteria, endowed with a wider range of photosynthetic pigments, are capable of gathering energy from this residual irradiance. Conversely, under conditions of high turbidity or presence of surface scums, light is rapidly attenuated in the first centimeters depth, generating an underlying water column with very low or even non-existent light availability. Under low light conditions, cyanobacteria indeed show to grow faster than other algal groups, as a result of their lower basal energy usage or more efficient electron transfer (Mur 1983, Bartram & Chorus 1999).

Nitrogen and phosphorus have typically been considered the main nutrients enhancing phytoplankton growth (Reynolds & Walsby 1975), although the relative importance of each nutrient in the development of cyanobacterial blooms is still a matter of discussion (e.g. Downing et al. 2001 and references therein). In this context, cyanobacteria display ecophysiological adaptations to enhance nutrient harvesting. Cyanobacteria cope with P limitation with two mechanisms; first, by the synthesis of phosphatases, enzymes that hydrolyze phosphate from organic solutes and allow its uptake, and second, by intracellular accumulation of excess P under replete conditions (Healey 1982, Kromkamp et al. 1989). This constitutes an important competitive advantage under P limitation, as cyanobacteria can divide up to three or four times without further P uptake, using only intracellular reserves (Mur et al. 1999). In the case of nitrogen, which can often be a limiting nutrient, cyanobacteria also present strategies to outcompete other algal groups. As is the case for other phytoplankton groups, nitrate and ammonia are the preferred forms of nitrogen assimilated by cyanobacteria. However, many cyanobacterial taxa are also capable of fixing atmospheric nitrogen when other preferred forms are strongly depleted. Although N_2 fixation is energetically expensive, chiefly due to the need both of breaking the triple bond in N_2 and providing anoxic conditions inside the heterocysts to prevent nitrogenase inhibition, this energetic investment is compensated by a significant competitive advantage under nitrogen limiting conditions. Some non-heterocystous taxa are also capable of fixing N_2, for example by

separating oxygen-producing photosynthesis during the day and nitrogenase activity at night (e.g. *Lyngbya* spp.) or, alternatively via the development of diazocytes (Stal et al. 2010, Sandh et al. 2012).

The relative importance of nitrogen and phosphorus for the dominance and proliferation of cyanobacteria in freshwater ecosystems has been studied for decades and is still a matter of debate. Smith (1983) suggested that low N:P molar ratios (<16) favor cyanobacteria, while higher ratios (>20) enhance eukaryotic algae. This rule of thumb seems to be valid in many tropical, subtropical and temperate systems that present summer stratification and long residence times (Downing et al. 2001). However, other authors have highlighted that in many occasions this may not apply, for instance in systems with high loads of both N and P, when none of these nutrients are limiting and other factors modulate cyanobacterial growth (Oliver & Ganf 2000, Paerl & Huisman 2008). However, it is widely accepted that N and P are the main nutrients affecting phytoplankton dynamics, and there is a general consensus when associating the gradual nutrient enrichment derived from human activities and the occurrence of cyanobacterial blooms.

1.4. Genus *Microcystis*

The scope of this thesis focuses on genus *Microcystis*. Therefore, some brief introductory remarks on this genus seem appropriate. *Microcystis* is a unicellular and coccoid cyanobacterium that aggregates into colonies with a more or less homogeneous mucilaginous envelope. The previously discussed discrepancies between botanical and bacteriological taxonomic approaches in *Microcystis* species delimitation (see Section 1.2) led to the use of the term *morphospecies* to refer to species other than *M. aeruginosa*. The term morphospecies refers to clearly distinguishable morphotypes occurring in nature that may undergo morphological changes under culture conditions.

Cyanobacteria of the genus *Microcystis* are among the most successful and cosmopolitan organisms in freshwater phytoplankton communities. In fact, *Microcystis* is considered the most frequent bloom-forming freshwater cyanobacterial genus in Mediterranean latitudes (Cook et al. 2004). The typical annual life-cycle of genus *Microcystis* is schematized in figure 1.5. During the summer season, under favorable conditions, *Microcystis* develops in the pelagial, sometimes causing massive blooms. Towards autumn, coincident with decreasing water temperatures, pelagic *Microcystis* settles to the bottom as a result of a

gradual loss of buoyancy, entering the benthic stage. Once on the sediments, colonies reduce metabolic activities to a minimum and spend the winter in the first centimeters of the sediment. This phase is commonly known as benthic overwintering. Toward spring, some colonies regain buoyancy and re-colonize the water column. This so-called re-invasion or recruitment phase constitutes the inoculum for pelagic growth back into the summer season.

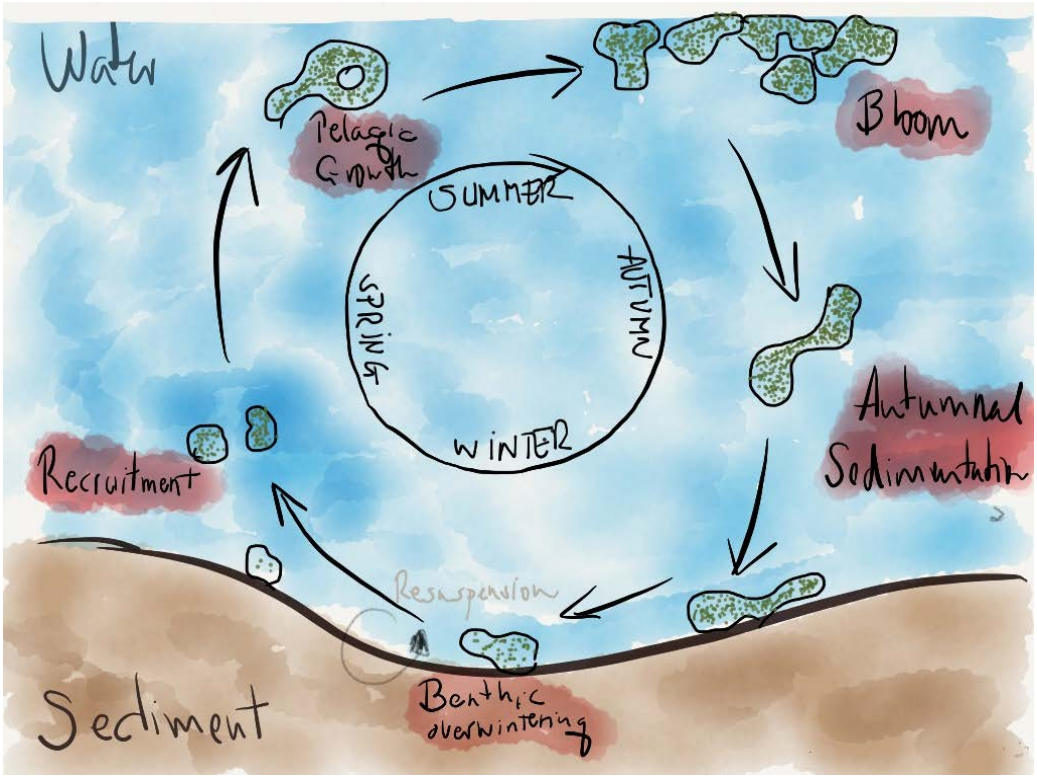


Figure 1.5. Schematic representation of the lifecycle of genus *Microcystis.*

1.5. Cyanobacterial toxins and other secondary metabolites

Bacterial secondary metabolites are extremely diverse and their distribution among species and strains is remarkably heterogeneous. The secondary metabolism refers to metabolic pathways that are not directly involved in the growth, development or reproduction of the organism. Much like other bacteria, cyanobacteria are endowed with a very active secondary metabolism and produce a wide range of secondary metabolites. Some of them are toxic to human and other eukaryotes (therefore considered cyanotoxins), whereas other "non-toxic" metabolites present other bioactive properties, whose main interest is driven by their unique biological activities and potential pharmacological applications. The separation of these metabolites into toxins and other bioactive compounds is somewhat artificial and results from a rather anthropocentric view, having compounds causing adverse effects on human or

livestock traditionally considered cyanotoxins. However, the truth is that the biological function of secondary metabolites remains unclear at present and hence, from a strictly biological standpoint, a distinction between cyanotoxins and other bioactive compounds is arguable. However, given the importance of cyanotoxins in the context of water management and human health, this thesis will adhere to the usual terminology and discern between cyanotoxins and other secondary metabolites.

1.5.1. Cyanotoxins

Cyanotoxins comprise a diverse group of compounds that can be classified according to their chemical structure in peptides, alkaloids and lipopolysaccharides (Sivonen & Jones 1999). According to their toxic effects on eukaryotes, cyanotoxins can be grouped as hepatotoxins, neurotoxins, cytotoxins and dermatotoxins (Table 1.3).

Microcystins

The most prevalent and best studied cyanotoxins are microcystins, a group of heptatotoxic cyclic heptapeptides. The general structure of microcystins is cyclo(-D-Ala-L-X-D-erythro-methylAsp-L-Z-Adda-D-Glu-N-methyldehydro-Ala), where Adda stands for the unique amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid (Fig 1.6).

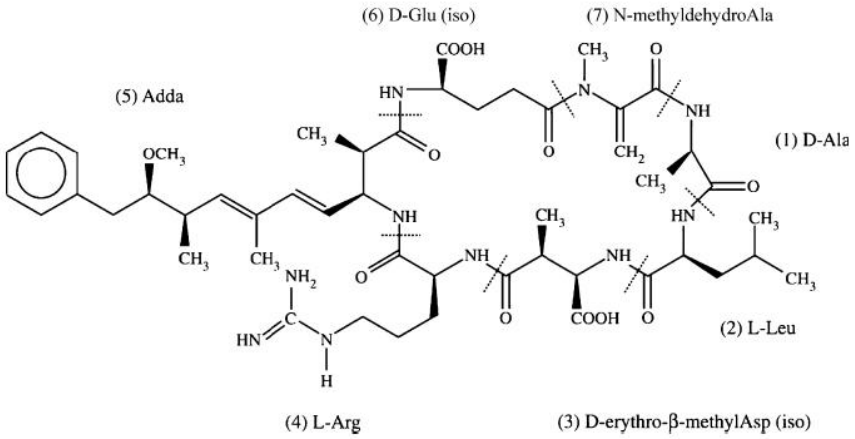


Figure 1.6. Chemical structure of microcystin LR, characterized by presenting leucine (L) and arginine (R) as L-amino acids in variable positions 2 and 4.

Microcystins comprise a numerous set of chemical variants, with over 90 variants as per Fastner et al. (2001). The main structural variations consist in changes in the L-amino acid residues at positions 2 and 4, although variability in all other residues is possible, usually consisting in demethylations in the respective residues. Microcystins are relatively polar molecules, due to the presence of carboxylic acids in positions 3 and 6, as well as the common presence of arginine in positions 2 and 4. The Adda residue confers a partial hydrophobicity to the molecule.

Cyanotoxin	Number of structural variants	Structure and activity	Examples of toxigenic genera
Hepatotoxins			
Microcystins	>90	Cyclic heptapetides; hepatotoxic; protein phosphatase inhibition, membrane integrity and conductance disruption, tumour promoters	*Microcystis, Anabaena, Anabaenopsis, Planktothrix, Oscillatoria, Hapalosiphon, Nostoc*
Nodularins	9	Cyclic pentapeptides; Hepatotoxic; protein phosphatase inhibition, membrane integrity and conductance disruption, tumour promoters	*Nodularia, Theonella* (sponge endosymbiont)
Neurotoxins			
Anatoxin-a	5	Alkaloids; postsynaptic, depolarizing neuromuscular blockers	*Anabaena, Oscillatoria, Phormidium, Aphanizomenon, Raphidiopsis*
Anatoxin-a (S)	1	Guanidine methyl phosphate ester; acetylcholinesterase inhibitor	*Anabaena*
Saxitoxins / PSP toxins (Paralytic Shellfish Poisoning)	20	Carbamate alkaloids; Na-channel blockers	*Aphanizomenon, Anabaena, Lyngbya, Cylindrospermopsis, Planktothrix,*
BMAA β-methylamino-L-alanine)	2 [1]	Non-proteinic amino acids; incorporate to proteins, affecting their structure; linked to amyotrophic lateral sclerosis and Parkinson-dementia complex (ALS/PDC) disease.	*Nostoc*; all? [2]
Cytotoxins and dermatotoxins			
Cylindrospermopsins	3	Guanidine alkaloids; necrotic injury to liver, kidneys, spleen, lungs and intestine; protein synthesis inhibition, genotoxic	*Cylindrospermopsis, Aphanizomenon, Anabaena, Umekazia, Raphidiopsis, Lyngbya*
Lyngbyatoxin-a	1	Alkaloid; inflammatory agent, protein kinase C activator	*Lyngbya, Schizothrix, Oscillatoria*
Aplysiatoxins	2	Alkaloids; inflammatory agents, protein kinase C activators	*Lyngbya, Schizothrix, Oscillatoria*
Lipopolysaccharides	Many	Lipopolysaccharides; inflammatory agents, gastrointestinal irritants	All

Table 1.3. Main groups of cyanotoxins, including their mechanisms of action and potentially producing genera. Adapted from Codd et al. (2005). [1] Two different isomers exist: the L-isomer (BMAA) and the D-isomer (DAB). [2] after Cox et al. (2005).

Mechanisms of toxicity of microcystins are based on the inhibition of enzymes, namely protein phosphatases. Protein phosphorilation commonly modulates enzyme activities and therefore plays an important role in the metabolism of eukaryotic cells (Barford 1996). Disruption of phosphatases activity severely affects cellular structure and functioning and has been associated with the proliferation of cancer cells (Cohen & Cohen 1989). In particular, microcystins inhibit some groups of protein phosphatases, namely serine/threonine-specific phosphates (1 and 2A), while other phosphates remain unaffected. Similarly, bacterial and cyanobacterial phosphatases are unaffected by microcystins (Mackintosh et al. 1990). The unusual amino acid Adda has shown to be responsible for their inhibitory effect, as a result of its covalent binding with the cystein residues present in the catalytic centre of phosphatases (Mackintosh et al. 1995, Runnegar et al. 1995). In fact, the elimination or modification of the Adda amino acid results in a strong reduction or complete loss of toxicity (Abdelrahman et al. 1993).

Microcystins are considered hepatotoxins, as the most evident and firstly observed toxic effects consisted in the deformation of hepatic cell lines. In fact, liver cells are particularly vulnerable to microcystins due to the fact that their entry into hepatocytes is facilitated by the polypeptidic organic anion membrane transporters, allowing their absorption through the intestinal barrier (Fischer et al. 2010). Once in the cytoplasm, microcystins exert their inhibitory effect, although they can also access the nucleus and trigger programmed cell death cascades. In fact, sublethal chronic exposures to microcystins have shown to promote skin cancer (Falconer & Buckley 1989, Shen et al. 2003), colon cancer (Humpage et al. 2000) and liver cancer (Nishiwaki-Matsushima et al. 1992) in rodents. In humans, several epidemiological studies have been carried out to associate the incidence of cancer and microcystin intake. In China, a positive correlation between the concentration of microcystin in drinking waters and the incidence of colon cancer was found (Zhou et al. 2002). Similarly, microcystin in drinking waters was pointed out as a risk factor to explain the high occurrence of liver cancer in the Serbian population (Svircev et al. 2009). Acute microcystin intoxications may result in lethal consequences (e.g. Lürling & Faassen 2013). The comparison of microcystin lethal doses (LD_{50}) with those of frequent drinking water contaminants evidences that microcystins are remarkably potent toxins, displaying LD_{50}s two orders of magnitude lower than atrazine, an insecticide banned in the European Union in 2004 (Table 1.4).

Compound	Oral LD_{50} (mg kg^{-1})
Atrazine	850
Copper	400
Acryl amide	100-270
Chlorpyrifos	60
Parathion	5
Microcystin LR	5
Cylindrospermopsin	6 (in 7 days)
Saxitoxin	0.12

Table 1.4. Acute toxicity of different possible drinking water contaminants in mice, expressed as LD_{50} (Oral dose causing mortality in 50% of the individuals in 24 hrs). After Falconer (2005).

Producing organisms - Microcystins can be produced by different cyanobacterial species of the genera *Microcystis*, *Anabaena*, *Planktothrix* (formerly known as *Oscillatoria*), *Nostoc* and *Nodularia*. Microcystins were also found in *Radiocystis fernandoii* (Lombardo et al. 2006) and marine *Synechococcus* (Carmichael & Li 2006). However, within potential microcystin-producing taxa, both toxigenic and non-toxigenic strains exist, as a result of the uneven distribution of the genes encoding the synthesis of microcystins among strains.

Biosynthesis and evolution of microcystin synthesis - The first attempts to unravel the synthetic pathways of microcystins employed ^{13}C labeled substrates. Cultures of *Microcystis aeruginosa* were grown in an array of possible precursors, and the incorporation of the labeled atoms into the final structure of microcystin was evaluated by means of Nuclear Magnetic Resonance (NMR) spectroscopy (Moore et al. 1991). This allowed the identification of precursor molecules involved in the synthetic pathways. These studies also showed that the constituent amino acids, despite consisting mainly of D-amino acids, were synthesized by pathways typical of L-amino acids, evidencing that the racemization from L- to D- amino acids occurred during the synthesis of the peptidic ring. The biosynthesis of other compounds in other bacteria and fungi, like peptide antibiotics that also present D-amino acids and cyclic structures, follows non-ribosomal pathways (Kleinkauf & Von Döhren 1990), in which large multienzyme complexes are involved. By comparing conserved regions encoding both fungal and bacterial non-ribosomal pathways, it was possible to identify similar sequences in the genome of *M. aeruginosa* (Borchert et al. 1992), which were absent in non-microcystin-producing strains and were otherwise only found in toxic strains. Later studies identified the first modular regions corresponding to non-ribosomal peptide synthases, namely *mcy*A, *mcy*B and *mcy*C (Meissner et al. 1996). Further research revealed the existence of seven additional modules within the *mcy* gene cluster (*mcy*D, *mcy*E, *mcy*F, *mcy*G, *mcy*H, *mcy*I and *mcy*J) and clarified their individual functions (Nishizawa et al. 1999, Tillett et al. 2000). These genes

account for a 55kb operon, which is responsible for the synthesis of microcystins by encoding non-ribosomal peptide synthases (NRPS), polyketide synthases (PKS), mixed NRPS/PKS and tailoring enzymes (Nishizawa et al. 1999, Tillett et al. 2000, Christiansen et al. 2003). Microcystin synthase gene cluster, known as *mcy* gene cluster, consist of different modules, each one involved in the activation and incorporation of individual amino acids into the growing peptide chain. Its elucidation in genus *Microcystis* constitutes the first complex metabolite cluster fully described in cyanobacteria (Tillett et al. 2000).

Similar approaches rapidly led to the characterization of the *mcy* gene cluster in other genera (Christiansen et al. 2003, Dittmann & Borner 2005). Interestingly, a detailed analysis of the *mcy* operon in genera *Planktothrix* and *Anabaena* revealed differences in sequences match and gene organization within the cluster (Fig. 1.7). These syntenic differences suggest divergence or independent origin of these genes (Rouhiainen et al. 2004).

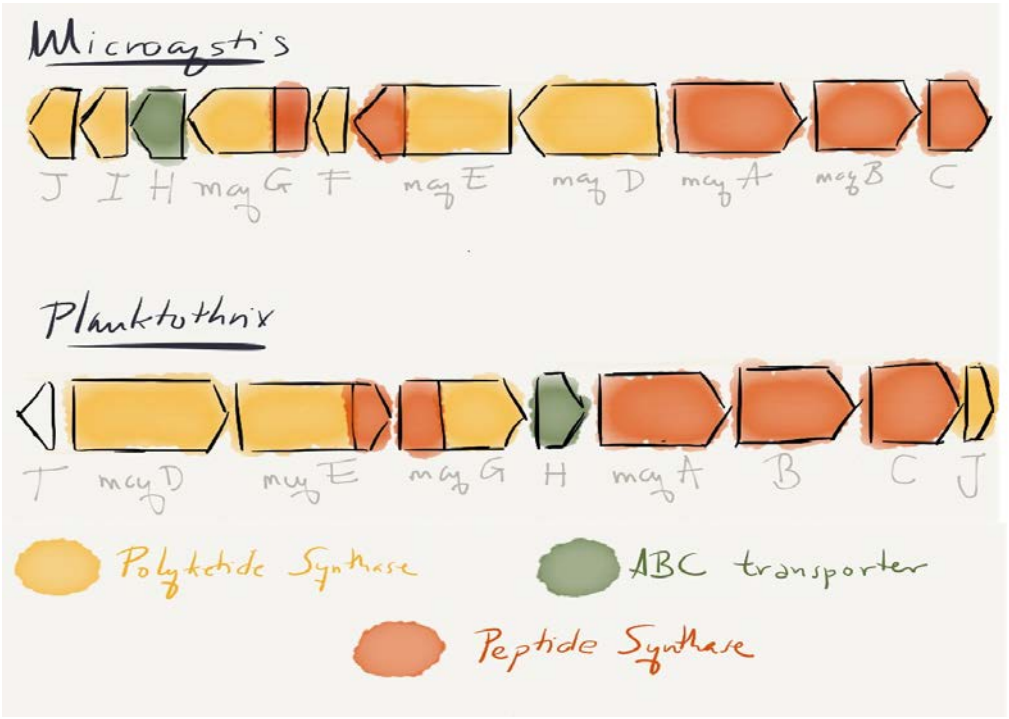


Fig 1.7. Schematic syntenic comparison of the mcy gene cluster in genera Microcystis and Planktothrix exemplifying modular reorganization among genera. Yellow and red areas denote sequences encoding for polyketide synthases (PKS) and peptide synthases (NRPS). Green color stands for regions encoding a putative ABC transporter. Adapted from Christiansen et al. (2003) and Dittmann and Börner (2005).

Further phylogenetic analysis across MC-producing genera by Rantala and coworkers (2004) ruled out the existence of horizontal gene transfer of the *mcy* gene cluster between genera. Instead, genes responsible for microcystin synthesis showed to have coevolved with housekeeping genes (i.e. genes involved in the primary metabolism) during the entire

evolutionary history of the toxin, strongly suggesting that microcystin genes were present in the common ancestor of contemporary cyanobacteria (Rantala et al. 2004). These findings are supported by the chromosomal location of the *mcyABC* gene, which is conserved in all toxigenic *Microcystis* strains, strengthening the idea of a common ancestral origin of microcystins (Tillett et al. 2000). This implies that the heterogeneous distribution of the *mcy* gene cluster among strains was caused by repeated gene losses in non-producing strains, which is consistent with reports of strains possessing *mcy* genes but lacking microcystin production (Mikalsen et al. 2003, Kurmayer et al. 2004, Mbedi et al. 2005, Christiansen et al. 2006).

Cylindrospermopsin

Cylindrospermopsin is a small cytotoxic alkaloid. It presents a tricyclic ring structure of a guanidine derivative and a hidroxymethyl-uracil (Fig. 1.8). Cylindrospermopsin is highly soluble in water due to presenting a double charge, both positive and negative (Ohtani et al. 1992). Two further structural variants of cylindrospermopsin exist, namely deoxycylindrospermopsin (Norris et al. 1999) and 7-epicilindrospermopsin (Banker et al. 2000). They differ in the absence of one oxygen atom at C-7, and the position of the hydroxyl group at C-7, respectively.

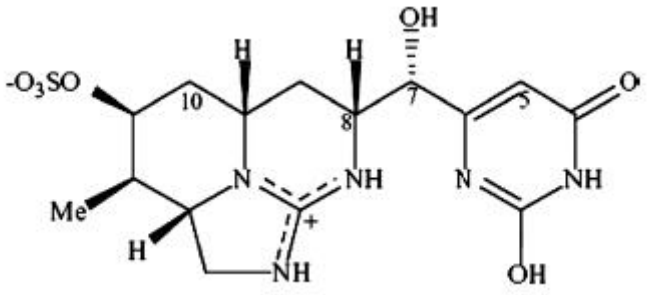


Figure 1.8. Chemical structure of cylindrospermopsin. Source: Creative Commons

As a result of its polarity, cylindrospermopsin is able to penetrate the membranes of a wide range of cells. Unlike microcystins, cylindrospermopsin toxicity is not limited to liver, but also affects kidneys, thymus, heart and other organs (Chong et al. 2002). Once in the cytoplasm, cylindrospermopsin acts as an irreversible inhibitor of protein synthesis and causes a variety of negative effects (Froscio et al. 2003). Besides, the uracil group in its structure interacts with adenine present in DNA and RNA by intercalation between nucleotides in the double helix, causing genotoxic and carcinogenic effects (Falconer 2005, Straser et al. 2011, Zegura et al. 2011, Straser et al. 2013). Notably, experiments on the carcinogenic potential of

cylindrospermopsin showed that about 10% of the rodents exposed to sublethal doses of cylindrospermopsin during 30 weeks developed tumors, while no tumors were found in the control group, unexposed to the toxin (Falconer & Humpage 2001).

Producing organisms

Cylindrospermopsin was detected for the first time in Australia and associated to the presence of the cyanobacterium *Cylindrospermopsis racirborskii*. Later, however, cylindrospermopsin was detected in the Sea of Galilee (Israel), the most important water supply in the region (Banker et al. 1997) and associated with the presence of *Aphanizomenon ovalisporum*. This species was found to be responsible for cylindrospermopsin production in Australia as well a few years later (Shaw et al. 1999). Together, these two species are the most important cylindrospermopsin producers, although other toxigenic species have also been reported, including *Aphanizomenon flos-aquae* (Preussel et al. 2006), *Umezakia natans* (Harada et al. 1994), *Anabaena lapponica* (Spoof et al. 2006), *Raphidopsis curvata* (Li et al. 2001) and *Raphidiopsis mediterranea* (McGregor et al. 2011). Some of these taxa have recently expanded their geographical distribution to more temperate regions (Briand et al. 2004), putatively due to an expansion of their ecological niches, as a result of global warming.

Biosynthesis of cylindrospermopsin

As in the case of microcystins, investigations on the biosynthetic pathways of cylindrospermopsin were initially undertaken by radiolabeling possible precursor substrates (Burgoyne et al. 2000) and the search for possible genetic regions that might be responsible for its synthesis in *Cylindrospermopsis racirborskii* and *Anabaena bergii* (Schembri et al. 2001). These studies were based on the assumption that cylindrospermopsin was synthesized by non-ribosomal peptide synthases and polyketide synthases. In fact, the presence of NRPS and PKS genes was directly correlated with the ability to produce cylindrospermopsin. Later studies focused on the genetic exploration of the polyketide synthase. Shalev-Alon et al. (2002) sequenced this set of genes in *Aphanizomenon ovalisporum,* and identified 3 genes responsible for a part of the cylindrospermopsin synthetic pathway (*aoa*A, *aoa*B and *aoa*C). The first of these genes codifies an amidinotransferase (AoaA), responsible for the synthesis of the guanidinoacetic acid, the first step in the synthesis of cylindrospermopsin. The guanidinoacetic acid is then recognized by AoaB (a NRPS/PKS hybrid) that, together with AoaC (a PKS), are responsible for the polymerization of the polyketide chain. These three genes were found in cylindrospermopsin-producing strains of *Cylindrospermopsis racirborskii, Aphanizomenon*

ovalisporum, Umezakia natans and Aphanizomenon flos-aquae (Schembri et al. 2001, Shalev-Alon et al. 2002, Kellmann et al. 2006, Preussel et al. 2006). The complete synthetic pathway was finally described by Mihali et al. (2008). The first three steps, previously described for *Aph. ovalisporum*, were confirmed for *Cylindrospermopsis racirborskii* by the presence of three homologous genes (*cyr*A, *cyr*B, *cyr*C). The complete cylindrospermopsin biosynthetic pathway is comprised by fifteen synthases. Interestingly, one of them, CyrK, shows similarities to proteins involved in the cellular export of type-NorM toxic compounds, which suggests that this enzyme may be involved in the excretion of cylindrospermopsin to the medium.

Neurotoxins: Anatoxin-a and Paralytic Shellfish Toxins (PSTs)

The occurrence of neurotoxic alkaloids produced by cyanobacteria has been reported in Europe, North America, and Australia. Neurotoxins can cause death by respiratory arrest in short periods of time. Three main families of cyanobacterial neurotoxin exist: Anatoxin-a (including homoanatoxin-a), Anatoxin-a(S) and saxitoxins (or PSPs).

Anatoxin-a is a small alkaloid with the structure 2-acetyl-9-azabicyclo(4-2-1)non-2-ene (Fig 1.9). Anatoxin-a acts as a nicotinic antagonist and a postsynaptic depolarizing muscular blocking agent. LD_{50}s for this toxin are estimated between 200 and 250 μg kg^{-1} body weight in rodents (intraperitoneal; Carmichael 1997). Anatoxin-a is produced by several species from the genera *Anabaena, Oscillatoria, Planktothrix, Aphanizomenon* and *Raphidiopsis*. Homoanatoxin-a is an homologue of anatoxin-a firstly isolated from a *Planktothrix formosum* laboratory culture (Skulberg et al. 1992) that presents a propionyl group at C2, instead of the acetyl group in anatoxin-a (Fig. 1.9).

Anatoxin-a(S) is a phosphate ester of a cyclic N-hydroxyguanine (Fig. 1.9), isolated from strains of *Anabaena flos-aquae* (Matsunaga et al. 1989) and *Anabaena lemmermannii* (Henriksen et al. 1997). LD_{50} of anatoxin-a(S) is 20 μg kg^{-1} body weight by intraperitoneal injection in mice.

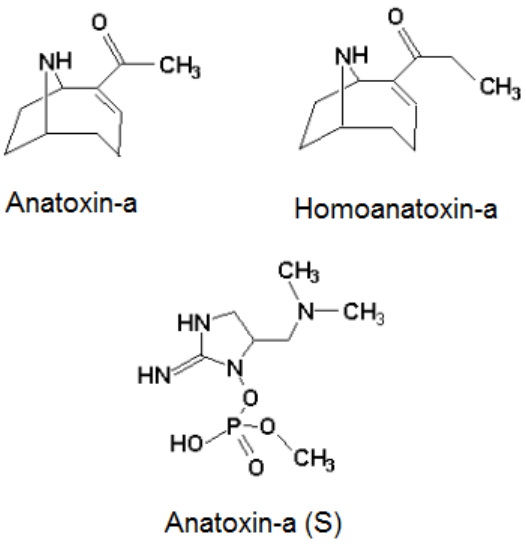


Figure 1.9. Chemical structure of Anatoxin-a, Homoanatoxin-a and Anatoxin-a (S).

Saxitoxins comprise a diverse group of small neurotoxic alkaloids produced by some marine dinoflagellates and some cyanobacteria of the genera *Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Planktothrix* and *Raphidiopsis* (Araoz et al. 2010 and references therein). Saxitoxins are also known as paralytic shellfish toxins (PSTs), as they were firstly isolated from shellfish, where they were concentrated from marine dinoflagellates. PSTs block nerve-cell sodium channels and cause severe intoxication and even death upon ingestion (e.g. García et al. 2004). Chemically, saxitoxins comprise a diverse group of carbamide alkaloids with different structural configurations. The general structure of PSTs is detailed in figure 1.10. While intensively studied in marine ecosystems, data on the occurrence of PSTs in freshwaters is still limited. For instance, the first detection of PSTs in Spanish freshwaters was reported only recently (Wörmer et al. 2011a).

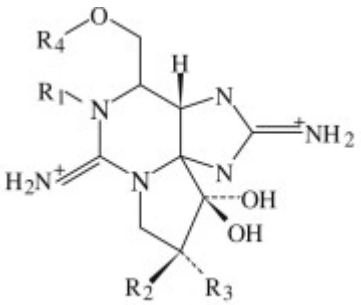
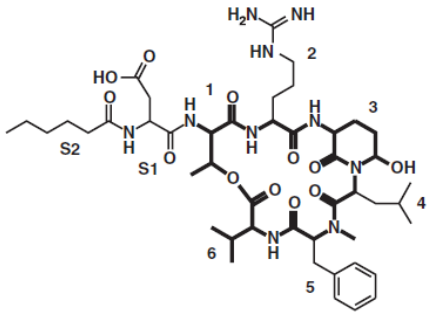
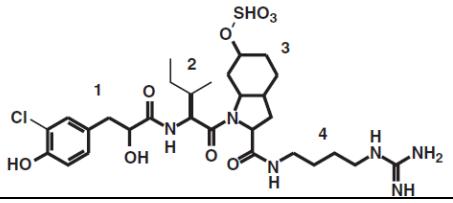


Figure 1.10. General structure of saxitoxins (PSP toxins). R1, R2 R3 and R4 correspond to variable residues that give rise to sixteen different structural variants.

1.5.2. Cyanobacterial oligopeptides

Like microcystins, a major portion of the numerous secondary metabolites produced by cyanobacteria can be classified as peptides or possess peptidic structures. These are commonly known as oligopeptides. Thanks to the recent advances in chemical structure elucidation techniques, together with the growing interest toward cyanobacterial peptides with antimicrobial, anticancer and other unique bioactive properties, new oligopeptides are being steadily discovered and characterized. According to their structural similarities, oligopeptides can be grouped in different classes: Microcystins, cyanopeptolins, aeruginosins, anabaenopeptins, microginins, cyclamides and microviridins (Table 1.5). Whereas this classification, proposed by Welker and von Döhren (2006), does not fully represent the vast diversity of oligopeptides, it includes a major portion of them, which cyanobacterial oligopeptide-based chemotyping is generally based on. Similarly to microcystins, oligopeptides are synthesized by NRPS or NRPS/PKS hybrid pathways, with the exception of cylamides and microviridins, whose synthesis follow ribosomal pathways (Ziemert et al. 2008, Hemscheidt 2012). NRPSs and PKS are huge multienzyme complexes that represent some of the largest proteins known. A single enzyme-complex can be responsible for dozens of individual biochemical reactions (Hutchinson 2003). NRPS and PKS are comprised by different modules, each one containing a set of active sites catalyzing the condensation of individual aminoacids and elongation of the peptide/polyketide chain.

Oligopeptide class	**Representative structural variant**	**Characteristic features and bioactive properties (when described)**	**Nr. of variants**
Cyanopeptolins		Depsipeptides with a unique residue Ahp (3-amino-6-hydroxy-2-piperidone. **Serine protease inhibitors**.	82
Aeruginosins		Linear peptides with the unique amino acid Choi (2-carboxy-6-hydroxyoctahydroindole). **Serine protease inhibitors**	27

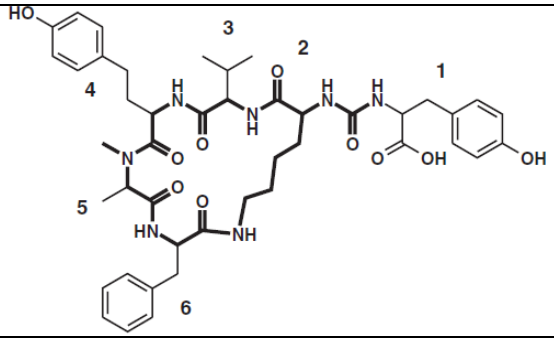
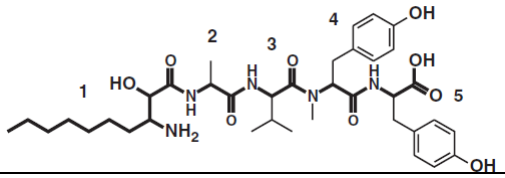
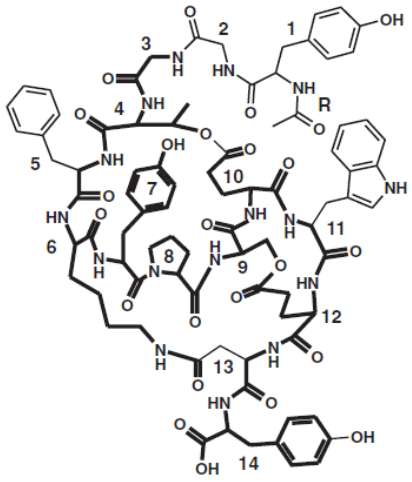
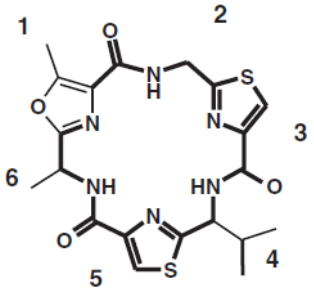
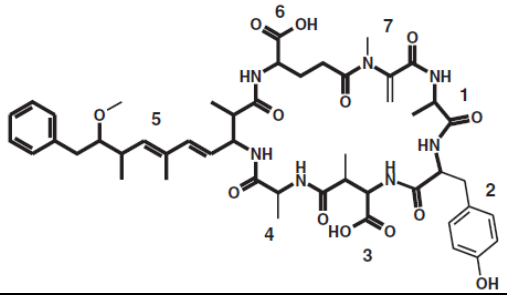
Anabaenopeptins		Hexapeptides containing D-lysine and a unique ureido bond. **Protease inhibitors.**	32
Microginins		Linear peptides containing the amino acid Adha (3-amino-(2-hydroxy) decenoid acid).	38
Microviridins		Depsipeptides displaying a tricyclic ring system. Not toxic in mouse bioassay, but some variants **may have significant effects on grazers** (Rohrlack et al. 2004). Ribosomal synthesis and posttranslational modifications (Hemscheidt 2012)	10
Cyclamides		Cyclic peptides with characteristic thiazole and oxazone moieties. Ribosomal biosynthesis.	21
Microcystins		See section 1.5.1.1. for details	89

Table 1.5. Main oligopeptide classes after the structural classification proposed by Welker and Von Dohren (2005). Bold lines in the chemical structures represent conserved parts of the molecule, while thin lines represent variable parts. The number of variants represents described chemical congeners in 2006, after Welker and von Döhren (2006).

Oligopeptide Analysis by MALDI-TOF MS

Oligopeptides can be rapidly detected in a qualitative way using Matrix Assisted Laser Desorption/Ionization Time Of Flight Mass Spectrometry (MALDI-TOF MS). This mass spectrometric technique consists in the ionization, separation and detection of analytes (fig. 1.11). Samples (e.g. *Microcystis* colonies) are mixed with a low molecular weight co-crystalizing matrix, which enhances ionization. By focusing a high energy laser beam on the sample, a cloud of ions is produced. Ionized molecules are then accelerated in an electromagnetic field. Ions then enter a field-free drift area where they move at different velocities depending on their respective mass/charge ratios (m/z). Thereby, analyte separation is achieved. Accelerated molecules move until colliding with the detector, which provides a time-resolved plot.

While other analytical techniques such as High Performance Liquid Chromatography (HPLC) or Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) may also be suitable for the analysis of oligopeptides, in the context of oligopeptide chemotyping, MALDI-TOF MS presents the advantage that only a small amount of sample is necessary to obtain reliable signals and no extraction or purification steps are needed. This allows a rapid screening of a wide array of oligopeptides without complex sample preparation.

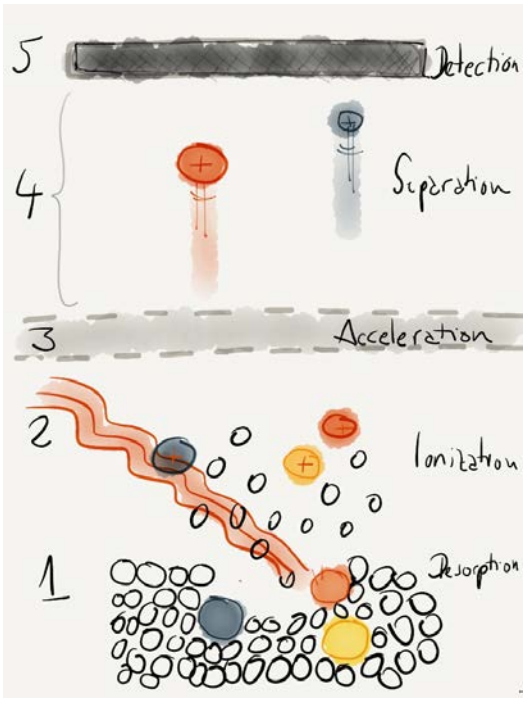


Figure 1.11. Schematic representation of the principles of MALDI-TOF MS. (1) Co-crystalized matrix (blank circles) and analyte molecules (colored). (2) Laser beam pulses on the sample, desorbing and ionizing molecules. (3) Grid electrode accelerates ions. (4) Ions move at different velocities in a field-free drift area and are thereby separated. (5) Analytes reach the ion detector. Adapted from Welker et al. (2002).

Efforts on identifying the bioactive properties of oligopeptides have been traditionally driven by their pharmacological interest, while their role in the physiology and ecology of cyanobacteria remains at present poorly understood. Hypotheses on the ecological function of oligopeptides include grazing protection (Ghadouani et al. 2004, Rohrlack et al. 2004, Czarnecki et al. 2006), competition with eukariotic algae (von Elert & Juttner 1997, Schagerl et al. 2001, Sukenik et al. 2002, Suikkanen et al. 2004) *quorum sensing* signaling (Dittmann et al. 2001, Schatz et al. 2007) or bloom termination (Sedmak et al. 2008). In the context of the putative role of oligopeptides in cyanobacteria, two important evolutionary aspects are worth considering: The first aspect refers to the huge chemical variability within oligopeptidic classes. Each peptide class comprises a multitude of structural variants caused, first, by variable amino acids moieties in particular positions in the molecule and, second, by modifications like methylations, glycosylation, sulfatation or halogenations (Ishida et al. 1997, Rouhiainen et al. 2000, von Elert et al. 2005, Ishida et al. 2007, Cadel-Six et al. 2008). However, the different structural variants within individual peptide classes are synthesized by a single enzyme complex. The existence of multiple chemical congeners results from relaxed substrate specificities during biosynthesis (Mikalsen et al. 2003). It is important to note, however, that certain positions in the general oligopeptide structures have been conserved and are therefore likely indispensable to conserve their bioactive properties. It is somewhat notorious that during their evolutionary history, the vast pool of peptide congeners has not been reduced to a few efficient ones, and on the contrary, substrate promiscuity in oligopeptide synthases seems to be positively selected (e.g. Tooming-Klunderud et al. 2008), leading to a huge range of individual variants with peptide classes. A second evolutionary aspect in oligopeptide production refers to the distribution of oligopeptide production abilities among cyanobacterial strains, which is discussed in the next section.

1.6. Oligopeptide-based chemotypes

Oligopeptide production is widespread in at least four of the five cyanobacterial orders (Welker & von Dohren 2006). The ability to produce individual oligopeptides is determined by the presence or absence of the corresponding gene cluster. However, the distribution of individual clusters among cyanobacterial strains is extremely patchy, which is attributed to frequent horizontal gene transfer among strains, recombination and gene loss events (Welker & von Dohren 2006, Ishida et al. 2009, Rouhiainen et al. 2010, Rounge et al. 2010). As a result, natural cyanobacterial populations are comprised by a number of strains, each one producing a different set of oligopeptides. Such differences in oligopeptide production among strains

allow the delimitation of subspecific subpopulations with a characteristic oligopeptide fingerprint, which conform oligopeptide-based chemotypes (onwards termed chemotypes). Chemotypes have been found to coexist in natural systems (Fastner et al. 2001, Welker et al. 2004) and are subject to strong seasonal dynamics (Welker et al. 2007, Rohrlack et al. 2008). However, the factors shaping chemotype composition and succession remain unknown.

Although microcystins have traditionally been in the spotlight of cyanobacterial research, cyanotoxins only represent a minor portion of the wide array of cyanobacterial oligopeptides. In light of the diversity and complexity of cyanobacterial metabolites, the study of cyanobacterial populations as communities of oligopeptide-based chemotypes, represents an appealing approach to increase our insights into their ecological role and into the factors governing community dynamics under bloom conditions. The high diversity of chemotypical subpopulations reported in natural systems (Welker et al. 2007) suggests that the production of a wide array of peptides is more beneficial to cyanobacteria compared to a few efficient ones. In contrast, the ability to produce individual peptides in cyanobacterial laboratory isolates does not seem to affect the fitness of strains. Even in a narrow geographical range (e.g. same lake) chemotype diversity shows to be strikingly high (e.g. Welker et al. 2007), implying that chemotypes coexist in the environment without displaying any clear competitive advantage of one over the other. In this context, addressing the question on whether cyanobacterial chemotypes represent ecologically relevant units seems a promising way to gather new insights into the biological functions of cyanobacterial metabolites, including microcystins. Furthermore, understanding the mechanisms that control the dynamics and compositions of chemotypical subpopulations may provide answers to unresolved questions about the origin, evolution and diversification of cyanobacterial secondary metabolites and chemotypical subpopulations.

While many studies dealing on cyanobacterial populations below the species level have focused on the dynamics of subpopulations with regard to *mcy+* and *mcy-* genotypes (i.e. toxigenic and non-toxigenic genotypes) (Kardinaal et al. 2007, Yepremian et al. 2007), the delimitation of subpopulations based on oligopeptide fingerprints constitutes a rather new perspective to approach the interaction of cyanobacteria with their environment. At present, however, the concept of what delimits a chemotype has not been clearly defined. As a result, the criteria used for the delimitation of chemotypes in the literature are not homogeneous, often hampering comparisons among studies, for example in terms of the existing diversity or

the seasonal dynamics of chemotypes: While some studies use the presence or absence of general oligopeptide classes (cyanopeptolins, anabaenopetolins, etc.) for the delineation of chemotypes (Martins et al. 2009), other studies consider the composition of individual oligopeptides, including structural variants, as chemotype markers, sometimes applied in a strict (Welker et al. 2007) or rather permissive way (Rohrlack et al. 2008). As the role of the oligopeptides remains unclear at present (for example, the unknown ecological or physiological significance of the existence of multiple oligopeptide structural variants), defining how precise an ideal chemotype marker should be, in order to delineate ecologically meaningful chemotypes, seems currently a challenging task. However, a consensus on the definition of cyanobacterial oligopeptide chemotypes is definitely needed. More importantly, the integrity of the oligopeptide signatures as chemotype marker has not been assessed, in spite of the reported physiological variations in oligopeptide production (e.g. Repka et al. 2004, Rohrlack & Utkilen 2007, Halstvedt et al. 2008). Whereas peptide signatures are assumed to be a stable marker, given that their synthesis is constitutive (Repka et al. 2004) and that cyanobacterial isolates have been observed to produce the same oligopeptides for years (Tillett et al. 2000, Fastner et al. 2001, Bister et al. 2004), the physiologically induced variations in the peptide fingerprints that define chemotypes have not been assessed and, if exist, may have major implications when interpreting their dynamics in natural systems.

1.6. Cyanobacterial blooms and water management

The issues related to the presence of cyanotoxins in drinking waters have been substantially reduced in the last years as a result of the use of modern water treatment techniques such as ozonation or active carbon filtration (Newcombe & Nicholson 2004, Westrick et al. 2010), which efficiently eliminate cyanotoxins before its distribution to end-users. However, these sophisticated techniques are usually not available in small drinking water distribution systems, like those of small towns. In these cases, chlorination is typically the technique of choice, which eliminates a fraction of the dissolved toxins, but dramatically enhances the release of cell bound toxins to the water. Besides, recreational waters pose an additional risk of exposure, as they imply direct contact with untreated water, often near shorelines, where scums tend to accumulate.

The awareness of the risks to human and animal health involving the occurrence of cyanobacterial blooms by public administrations and water managers has increased in the last years, as reflected by the development of non-mandatory guidelines and recommendations,

including guideline values for cyanobacterial cell densities and cyanotoxin concentrations, monitoring programs and risk management measures. A good example of this are the "*Guidelines for safe recreational water environments"* published by the World Health Organization (WHO 2003). Furthermore, different countries are gradually incorporating cyanotoxins into the respective national legislations (Chorus 2012). Moreover, the European Bathing Water Directive (2006/7/EC) requires for the first time the analysis of cyanobacteria as a mandatory item for the elaboration of recreational water profiles in each country and an adequate "risk assessment" for bathing sites with a tendency for cyanobacterial proliferation. In particular, the implementation of the European Bathing Waters Directive in Spain, including the analysis and assessment of the risks for cyanobacterial proliferation of all Spanish official bathing sites, is covered in the first chapter of this thesis.

Current monitoring strategies addressing the occurrence of cyanobacterial blooms are chiefly based on routine conventional samplings. However, cyanobacterial blooms display inherent sources of variability which often hamper efficient monitoring actions. First, blooms are prone to display wide variations in biomass distribution in space and time. In this context, conventional sampling techniques lack the necessary resolution to report changes in phytoplankton abundance and distribution, which can occur within short periods of time, especially under bloom conditions. Emerging techniques like remote sensing display the potential to address such variability and overcome these limitations (Kutser et al. 2006). The use of air or space borne sensors can be used to detect cyanobacterial biomass over wide geographical areas, taking advantage of the characteristic absorbance of the photosynthetic pigments chlorophyll-a and cyanobacterial phycocyanin and thus yields improved spatial coverage that cannot be achieved by conventional water sampling (Kutser 2009). Secondly, cyanobacterial blooms display marked fluctuations in toxin contents of up to several orders of magnitude (Carrasco et al. 2006). As microcystin production at the individual level is constitutively regulated and varies within a narrow range (Orr & Jones 1998), the observed differences in toxin loads cannot be explained by physiological changes in microcystin biosynthesis. Instead, shifts in the proportion of coexisting toxigenic and non-toxigenic strains in natural populations are arguably a critical factor modulating microcystin concentrations in cyanobacterial blooms. While the use of molecular techniques are suitable to investigate variations in the proportion of toxin and non-toxic genotypes (e.g. Kurmayer & Kutzenberger 2003, Janse et al. 2005), these require laborious and time-consuming sample preparation. An emerging alternative approach is the analysis of chemotypical subpopulations by MALDI-TOF

MS, which allows a rapid identification of toxigenic and non-toxigenic individuals in the population that can potentially explain the variations in microcystin concentration and thereby allow rapid water management decision-making.

In light of the existing sources of uncertainty related to the heterogeneous spatiotemporal distribution of cyanobacterial biomass and the fluctuations in toxin concentrations, new monitoring strategies are needed to address these sources of variability and enable a more accurate assessment of the risks associated to bloom events. With this aim, we designed and tested a monitoring approach combining conventional water sampling, remote sensing techniques and the analysis of cyanobacterial chemotypes, which is covered in the sixth Chapter of this dissertation.

2. Objectives / Objetivos

The objectives of this thesis are:

1) Investigate the occurrence of freshwater planktonic cyanobacteria in the 212 official Spanish bathing sites and assess their risk with regard to the proliferation of toxic cyanobacteria in the context of the European Bathing Waters Directive 2006/7/EC.

2) Assess the stability of oligopeptide patterns as a consistent marker of cyanobacterial subpopulations under different nutrient and light conditions.

3) Evaluate whether chemotypical subpopulations present different ecological traits in natural ecosystems and constitute ecologically relevant units below the species level.

4) Incorporate the analysis of cyanobacterial chemotypical subpopulations in the design and validation of new-generation strategies for the monitoring of cyanobacterial blooms, with an emphasis on the reduction of the main sources of uncertainty affecting the assessment of the risks associated to bloom events.

Los objetivos de esta tesis son:

1) Explorar la incidencia de afloramientos de cianobacterias planctónicas en las 212 zonas de baño oficiales y evaluar el riesgo de dichas zonas en relación a su tendencia a la proliferación de cianobacterias en el marco de aplicación de la Directiva de Aguas de Baño EC/2007/CE

2) Evaluar la estabilidad de los patrones oligopeptídicos como marcadores de subpoblaciones quimiotípicas bajo distintas condiciones de nutrientes y luz.

3) Evaluar si las poblaciones quimiotípicas presentan rasgos ecológicos distintivos en sistemas naturales y por tanto, si representan unidades subespecíficas con relevancia ecológica.

4) Incorporar el análisis de subpoblaciones quimiotípicas en el diseño y validación de estrategias de monitorización de nueva generación, con especial énfasis en la reducción de las fuentes de incertidumbre que impactan el análisis de riesgos asociados a episodios de blooms cianobacterianos.

3. Cyanobacteria and cyanotoxin occurrence in Spanish recreational inland waters

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3.1. Abstract

Cyanobacterial proliferation is a public health concern due to the ability of some genera to produce potent toxins. In the particular case of recreational waters, risks of exposure are substantially increased due to users direct contact with untreated water. The European Bathing Waters Directive 2006/07/CE required the elaboration of bathing water profiles for every official bathing site. One of the parameters required in the profiles refers to the tendency of harmful cyanobacteria proliferation. To comply with such regulations, all Spanish official inland bathing sites (n=212) were sampled during the bathing seasons of 2008 and 2009 and analyzed for cyanobacteria and cyanotoxin occurrence. Cyanobacteria showed generally low abundance, although they were observed to strongly dominate planktonic communities under specific circumstances. *Microcystis* was the most commonly occurring genus, together with *Anabaena* and *Aphanizomenon*, including several potential cyanotoxin-producing species. Microcystins and cylindrospermopsin were found in 6.1% and 2.4% of the bathing sites, respectively. Anatoxin-a was only detected in one bathing site. According to the designed risk assessment, only 29.5% of the bathing sites presented a low risk, while moderate and high risks of toxic cyanobacteria blooms were found in 50.2% and 19.8% of the surveyed bathing sites, respectively, denoting that Spanish bathing sites are prone to host harmful cyanobacterial blooms and stressing the need of implementing efficient monitoring strategies.

3.2. Introduction

Surface inland waters are of strategic importance in Spain, as they constitute the main source of drinking resources and are intensively used for recreational activities. Given the dry summers inherent to Mediterranean climate, with virtually non-existing precipitations in some regions, most of this water is stored in reservoirs (> 1000 in Spain). High temperatures (Robarts & Zohary 1987) and high levels of eutrophication (Heisler et al. 2008) have been suggested as important factors enhancing the proliferation of cyanobacteria. In fact, the review of previous studies focusing on Spanish freshwaters (Margalef et al. 1976, Sabater & Nolla 1991, de Hoyos et al. 2004) evidences a substantial increase in the number and occurrence of cyanobacterial species, which can putatively be associated to the increasing eutrophication in the Spanish waters during the last decades, but also to the gradual temperature increase as a consequence of climate change (Paerl & Huisman 2008). Cyanobacteria are an increasing concern for public health authorities, due to the ability of some genera to produce a wide range of toxic metabolites, commonly termed cyanotoxins. Cyanobacteria and cyanotoxins have been associated with the death of wild animals, livestock and fish (Carmichael 1992), as well as human illness (Kuiper-Goodman et al. 1999) and even death due to cyanotoxin exposure via haemodialysis (Jochimsen et al. 1998). Besides, sublethal chronic exposures to microcystins in rodent experiments have shown to promote cancer (e.g. Shaw et al. 1999, Humpage et al. 2000), whereas epidemiological studies in humans showed a positive correlation between the concentration of microcystin in drinking waters and the incidence of colon cancer (Zhou et al. 2002). Given the common occurrence of massive cyanobacterial proliferations or blooms and their widespread distribution, the World Health Organization (WHO) established a preliminary guideline concentration for the cyanotoxin microcystin-LR of 1 μg L^{-1} in drinking waters. This guideline was assimilated in the Spanish legislation in 2007 – being microcystins (MCs) the only legislated cyanotoxin in Spain - establishing a mandatory limit for microcystins of 1 μg L^{-1} after drinking water treatment.

Cyanotoxins comprise a diverse group of bioactive compounds. MCs, the most prominent group of cyanotoxins, are cyclic heptapeptides which act as potent hepatotoxins, inhibiting serine/threonine protein phosphatases 1 and 2A. Tumor-promoting activity, gastroenteritic and hepatic diseases have been associated with their presence (Falconer 1991). Microcystins are produced by different species of the genera *Microcystis, Anabaena* and *Planktothrix,* among others (Sivonen & Jones 1999), although within potentially MC-producing taxa, both producing and non-producing strains coexist. Another cyanotoxin, cylindrospermopsin (CYN), is a small alkaloid that displays protein inhibition properties, as well

as genotoxic and carcinogenic activity (Humpage & Falconer 2003). Given its ability to penetrate a wider range of cells, its toxic effects are not limited to the liver and causes extensive damage in a variety of organs, including heart, kidneys and others. Reported planktonic CYN producers are *Aphanizomenon ovalisporum* (Banker et al. 1997, Shaw et al. 1999) *Aphanizomenon flos-aquae* (Preussel et al. 2006), *Cylindrospermopsis raciborskii* (Ohtani et al. 1992), *Umekazia natans* (Harada et al. 1994), *Anabaena bergii* (Schembri et al. 2001), *Anabaena lapponica* (Spoof et al. 2006), *Raphidopsis curvata* (Li et al. 2001) and *Raphidiopsis mediterranea* (McGregor et al. 2011). CYN has received less attention compared to microcystins due to its apparently limited geographical distribution. Main interest in CYN was until recently localized in Australia, Israel and other tropical and subtropical regions, such as Florida (USA) and Brazil. However, during the last years, CYN has gained importance also in Europe. During the summer of 2004 the first massive bloom of CYN-producing cyanobacteria in Europe was reported in Spain (Quesada et al. 2006), followed by later reports of CYN occurrence in European freshwaters (Blahova et al. 2009, Brient et al. 2009, Kokocinski et al. 2009). In fact, the expansion of CYN producers *Aph. ovalisporum* and *Cylindrospermopsis raciborskii* to more temperate regions has been suggested by Mehnert et al. (2010) and Sukenik et al. (2012). Anatoxin-a (ATX-a) is a potent neurotoxin, which acts as a neuromuscular blocking agent through the nicotinic acetylcholine receptor and can cause death via asphyxia from respiratory arrest (Carmichael 1994). ATX-a is produced by some planktonic cyanobacterial genera including *Anabaena, Aphanizomenon*, *Oscillatoria, Raphidiopsis* and *Planktothrix*, although the production of ATX-a seems to be more frequent in benthic cyanobacteria (Hamill 2001, Gugger et al. 2005).

The risk of human exposure to cyanotoxins has been substantially reduced with the development and implementation of modern water treatment techniques, such as ozonation and activated carbon filtration, which are capable of virtually removing cyanotoxins from drinking waters (Lawton & Robertson 1999). However, recreational activities involving direct contact with untreated water may result in increased effect risks, due to accidental or incidental ingestion of water, inhalation of aerosols or direct skin contact with waters containing cyanobacteria (Stewart et al. 2006). As a result of this concern, the European Parliament and Council developed in 2006 the Directive 2006/7/CE related to the management of the water quality in water bodies for recreational use. This Directive aims to improve water quality and protect public health as a complement to the EU Water Frame Directive (WFD) 2000/60/CE. To achieve this objective, the Directive stresses the need of elaborating individual bathing water profiles for the involved water bodies according to its annex 3. One of the

elements stipulated in the characterization of these water bodies (point c) is the assessment of the risk of toxic cyanobacteria proliferation. The Spanish Decree RD 1341/2007 is the result of the transposition of the European Directive to the Spanish regulation. According to this text, 212 official sampling points were defined throughout Spain that are subject of such characterization.

In this work, we present the results of the monitoring campaigns carried out in 212 Spanish official inland bathing sites in the context of their characterization of the tendency for toxic cyanobacteria proliferation. This includes water sampling and its physicochemical characterization, cyanobacterial quantification and taxa identification, cyanotoxin analysis, as well as the subsequent assessment of the risks for toxic cyanobacteria proliferation.

3.3. Materials and methods

Spanish official bathing sites (n=212) were sampled during the summer periods (July-October) of 2008 and 2009 (fig. 3.1). Each designated sampling point was sampled thrice, once during 2008 and twice during 2009. Sampling consisted in the collection of an integrated water sample from 5 different shore points (2L per point) within the first meter of depth, covering the whole bathing area. Water samples were analyzed in situ for NH_4^+, NO_3^- and reactive soluble phosphorous (SRP) by colorimetric methods using a DREL-2010 Portable Laboratory (Hach Company, USA) following APHA (1992). The remaining water samples were then transported cool (4˚C) to the laboratory for further analysis, consisting of the quantification of total and cyanobacterial chlorophyll *a* (Chl-a) concentrations, cyanobacterial taxa identification and cyanotoxin (MC, CYN, ATX-a) extraction and analysis.

Total Chl-a, and cyanobacterial Chl-a concentrations were determined using a benchtop BBE-Moldaenke Algae Analyser Fluorimeter, capable of discriminating among algal groups (green algae, diatoms, cryptophytes and cyanobacteria) within a sample. Cyanobacterial taxa identification of each sample was carried out microscopically using an Olympus BH2 microscope equipped with a Leica DF300 FX camera (Leica Microsystems, Germany). Species identification was based on diagnostic morphological traits according to Geitler (1932) and Komárek and Anagnostidis (1989, 1999, 2005).

Water samples were filtered by GF/F glass fiber filters (Whatman, UK) and stored at -20˚C until toxin extraction. Each sample was analyzed for microcystins (LR, RR and YR variants) and cylindrospermopsin in both sestonic and dissolved fractions. Additionally, samples were also analyzed for sestonic anatoxin-a.

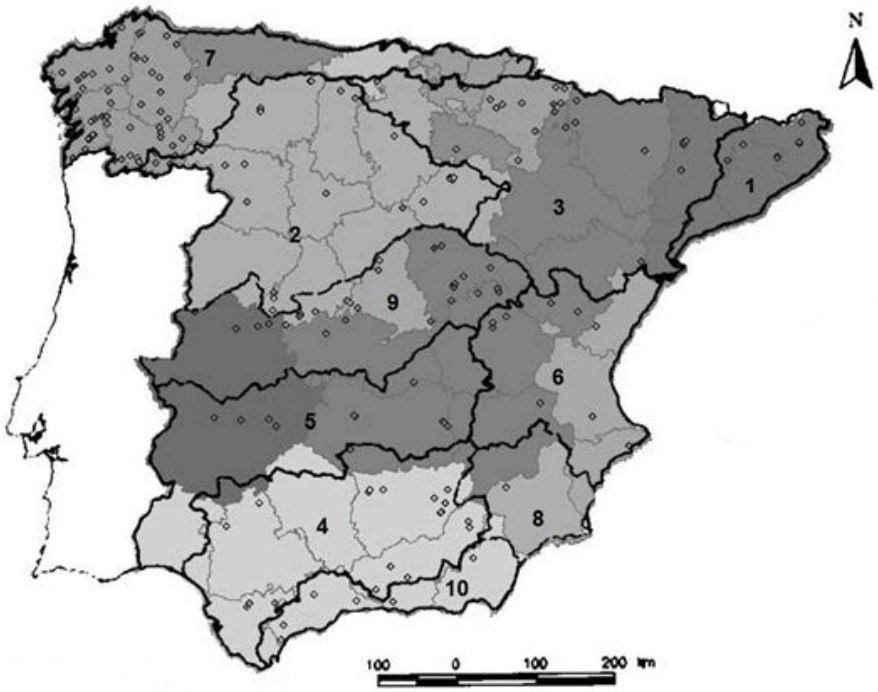

Figure 3.1. Map of Spanish official bathing sites. Greyscale represents Spanish autonomous regions. Bold lines delimit administrative watersheds. (1:CIC ; 2:Duero; 3:Ebro; 4:Guadalquivir; 5:Guadiana; 6:Segura; 7:Norte; 8:Júcar; 9:Tajo; 10:Sur)

MCs were extracted twice by sonication into methanol 90% after Carrasco et al. (2006). CYN was extracted twice into Milli-Q water after Torokne et al. (2004). ATX-a was extracted twice into methanol 100% after Carrasco et al. (2007). All extracts were concentrated under vacuum using a Heidolph Synthesis multiple evaporator (Heidolph Instruments GmbH, Germany) and prepared for analysis. For the analysis of MCs and CYN in the dissolved fraction, one liter of filtered water of each sampling point was concentrated by solid phase extraction: Filtered samples were pumped through a pre-activated C18 cartridge (MegaBondElut, Varian, Inc.) for MCs, and through a graphite carbon cartridge (LiChrolut RP-18, Merck) for CYN samples after Wörmer et al. (2009). Retained toxins were eluted by washing the cartridges with methanol 90% (for MCs) and a 1:4 dichloromethane: methanol + 5% formic acid solution (for CYN). Eluents were then concentrated under vacuum and prepared for mass spectrometric analysis.

MCs and CYN were identified and quantified using a Varian 500MS Ion Trap Mass Spectrometer coupled to two Varian 212 LC chromatographic pumps and a 410 autosampler. Chromatographic separation of MC-LR, MC-RR, MC-YR and CYN was achieved using a Pursuit

C18 3µm 2x150mm column and mobile phases MilliQ water (A) and methanol (B) both acidified with 0.2% formic acid and buffered with 2mM ammonium formate. For MCs, a chromatographic gradient (%A/%B) 60/40 to 0/100 in 18 minutes was applied. CYN was analyzed separately using a gradient 95/5 to 50/50 in 10 minutes. ATX-a was analyzed by High Performance Liquid Chromatography (HPLC) on a Waters Alliance 2695 HLPC System equipped with a 996 PDA (Photo Diode Array) detector and a Luna C18 3µm 4.6 x 100mm column (Phenomenex Inc., USA). MilliQ water (A) and acetonitrile (B), both acidified with 0.05% trifluoracetic acid (TFA) were used as mobile phases, applying the chromatographic gradient detailed in Edwards et al. (1992). All quantifications were made by injecting commercial standards (MC: DHI, Denmark; CYN: NRC, Canada; ATX-a: Abraxis, USA) and plotting calibration curves.

3.4. Results

Total Chl-a concentrations in the analyzed samples were generally low, with concentrations below 5 µg L^{-1} in 87% of the samples (Fig. 3.2a). Cyanobacterial presence was accordingly low (Fig. 3.2b) with 97% of the analyzed samples presenting cyanobacterial Chl-a concentrations below 5 µg L^{-1}. Regarding the contribution of cyanobacteria to the phytoplanktonic composition (Fig. 3.2c), around half of the samples (51%) showed cyanobacterial presence below 5%. In order to further analyze the data, results were grouped by administrative watershed. In line with the frequently low total and cyanobacterial Chl-a concentrations in the samples, all watersheds showed similar medians below 5 µg L^{-1} (Figs. 3.3a and 3.3b, respectively). However, Tajo, Norte and Guadalquivir watersheds were characterized by exhibiting several outliers corresponding to sampling points where high cyanobacterial presence was evident (fig. 3.3b). In terms of cyanobacterial dominance (Fig. 3.3c), these differences were less notorious. Although medians remained low for all watersheds, the occurrence of outliers showing a clear dominance of cyanobacteria in almost all watersheds indicates that cyanobacteria can strongly dominate the phytoplanktonic community under specific circumstances.

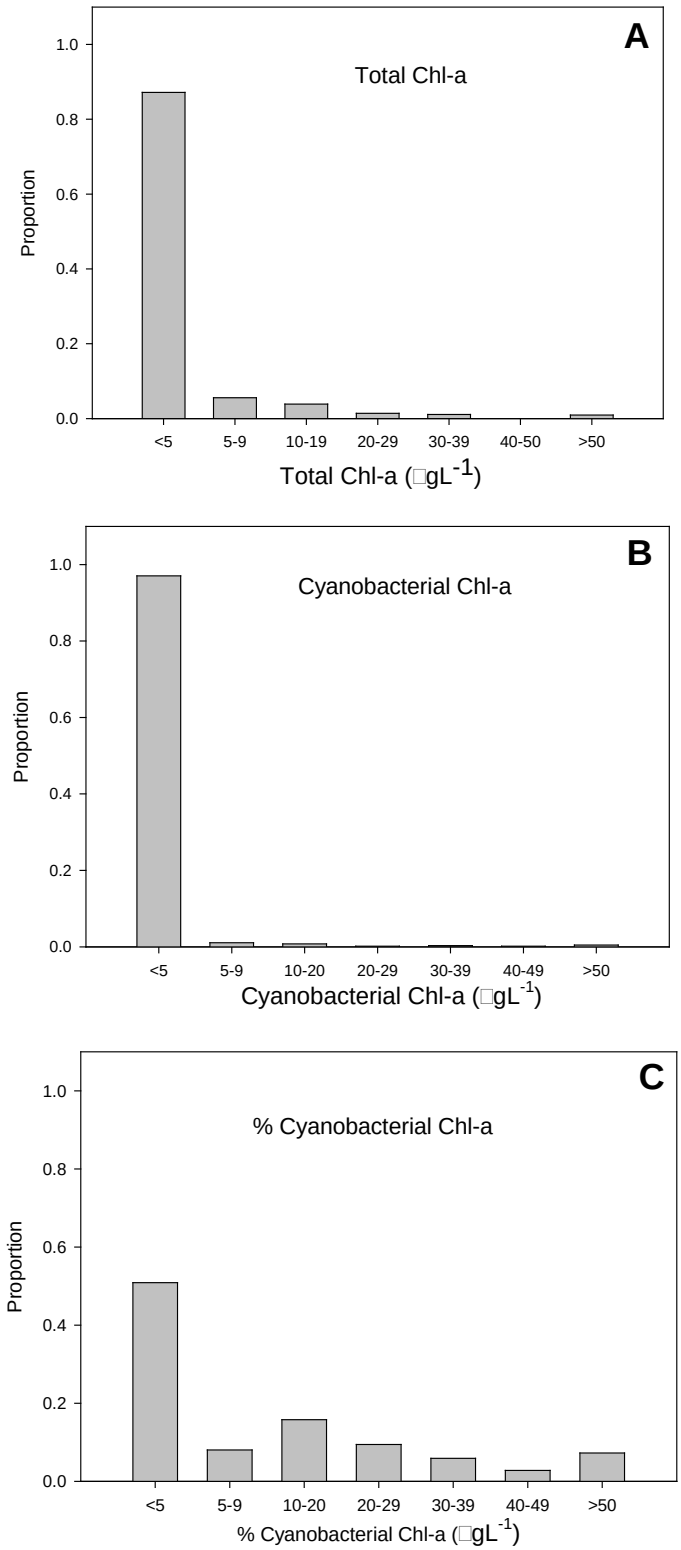

Figure 3.2. Proportion of the samples presenting different ranges of (A) Total chlorophyll-a concentrations, (B) cyanobacterial chlorophyll-a and (C) contribution of cyanobacterial chlorophyll-a to total chlorophyll-a concentrations.

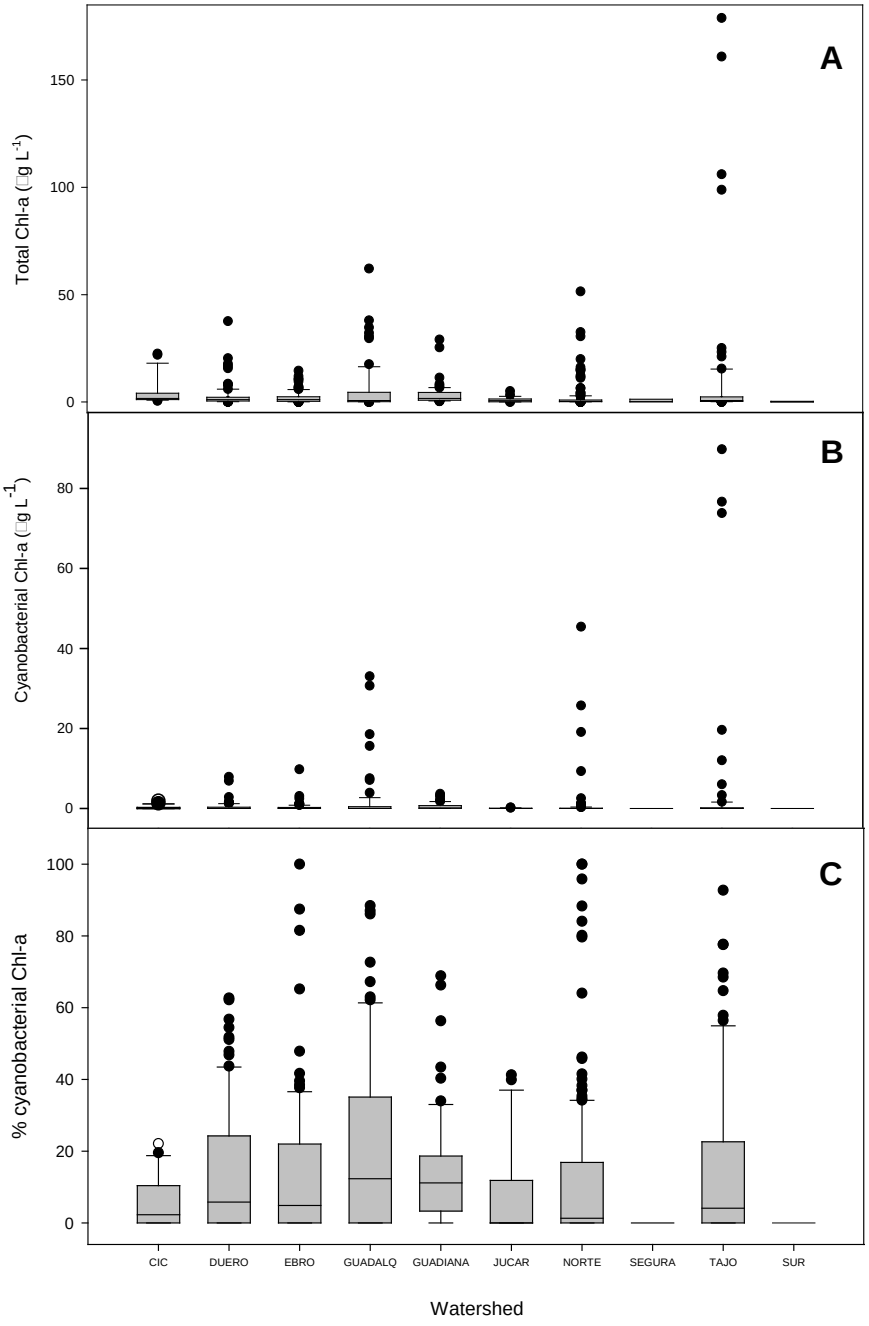

Figure 3.3. Box-plots representing (A) total chlorophyll-a concentrations, (B) cyanobacterial chlorophyll-a concentrations and (C) contribution of cyanobacterial chlorophyll-a to total chlorophyll-a concentrations of individual samples grouped by administrative watersheds

The occurrence of cyanobacterial taxa in the analyzed samples is summarized in Table 3.1. The most frequently occurring cyanobacterial order was Chroococcales (60.9%), mainly represented by genus *Microcystis* (51.7%). Within this genus, *M. aeruginosa*, *M. flos-aque* (potential MC-producers) and M*. wesenbergii* were the most frequent species. Cyanobacteria of the order Nostocales were also found in almost half (49.4%) of the samples where any cyanobacterial presence was detected. Within this order, genus *Anabaena* (34.5%),

represented by several potential cyanotoxin-producing species, and genus *Aphanizomenon* (24.1%), including the invasive CYN-producer *Aph. ovalisporum* (2.3%) were the most frequently occurring. *Cylindrospermopsis raciborskii* was found in 8% of the samples presenting cyanobacteria. Regarding order Oscillatoriales, *Planktothrix agardhii* was the most frequently occurring species (16.1%).

Cyanotoxins MCs, CYN and ATX-a were analyzed by LC-MS/MS or HPLC-PDA. Table 3.2 shows the number of analyzed samples and bathing sites, together with the number of positive detections in each case. MCs were found in 6.1% of the analyzed bathing sites, CYN in 2.4% of the surveyed bathing sites, and ATX-a was only found at a single bathing site (0.5%). Figure 3.4 shows the toxin concentrations found in samples with positive cyanotoxin detections. Total MC concentrations were widely variable, ranging from trace concentrations (minimum 0.6 ng L^{-1}) to maximum concentrations of 123.4 µg L^{-1}. In the case of CYN, positive samples showed total concentrations ranging from 18 ng L^{-1} to 3.38 µg L^{-1}. ATX-a was detected in only one sample at low concentrations (0.16 µg L^{-1}).

When considering the contribution of sestonic and dissolved cyanotoxin concentrations (Fig. 3.5), differences between MC and CYN were evident. MC was mainly present in the intracellular fraction, with detectable dissolved concentrations only in 3 out of 20 positive samples. In these cases, the contribution of dissolved MCs to total toxin loads was low (0.2-12.5%). In contrast, CYN was typically present in both cell bound and dissolved fractions at significant concentrations. When both sestonic and dissolved CYN were detectable, the latter accounted for 40-92% of total CYN concentrations. Exceptionally, in two occasions CYN could only be detected in the dissolved fraction at very low concentrations. The relation between total Chl-a and total cyanotoxin (MC and CYN) concentrations as well as the relation between cyanobacterial Chl-a and cyanotoxin concentrations (Figs. 3.6a and 3.6b, respectively), did not reveal any evident correlation.

Order	Genus	Species	Frequency
Chroococcales			60.9%
	Chroococcus	*Chroococcus sp.*	1.1%
	Microcystis		51.7%
		M. aeruginosa [m]	27.6%
		M. ichtyoblabe [m]	6.9%
		M. viridis [m]	1.1%
		M. smithii [m]	3.4%
		M. flos-aquae [m]	20.7%
		M. novaceckii [m]	2.3%
		M. wesenbergii	17.2%
		Microcystis sp.[m]	8.0%
	Woronichinia		34.5%
		W. naegeliana	26.4%
		Woronichinia sp.	9.2%
Nostocales			49.4%
	Anabaena		34.5%
		A. circinalis	1.1%
		A. crassa	6.9%
		A. flos-aquae [m,a]	10.3%
		A. heterospora	2.3%
		A. lemmermannii [a]	2.3%
		A. planctonica [a]	3.4%
		A. viqueri	1.1%
		Anabaena sp.[m]	10.3%
	Aphanizomenon		24.1%
		Aph. aphanizomenoides	2.3%
		Aph. flos-aquae	5.7%
		Aph. gracile	3.4%
		Aph. issatchenkoii	1.1%
		Aph. ovalisporum [c]	2.3%
		Aphanizomenon sp.	15.9%
	Cylindrospermopsis	*C. raciborskii*	8.0%
Oscillatoriales			26.4%
	Limnothrix		10.3%
		Limnothrix redekeii	6.9%
		Limnothrix sp.	3.4%
	Planktothrix	*P. agardhii* [m]	16.1%
	Pseudanabaena	*Pseudanabaena sp.*	2.3%
	Raphidiopsis	*Raphidiopsis*	2.3%

Table 3.1. Occurrence of cyanobacterial taxa in the analyzed samples. Frequencies are expressed as the percentage of samples where the respective taxa were identified, out of the samples with any cyanobacterial presence. m: potential microcystin producer; c: potential cylindrospermopsin producer; a: potential anatoxin-a producer

Cyanotoxin	Samples		Sampling sites	
	Analyzed	Positive	Analyzed	Positive
Microcystin	647	20	212	13
Cylindrospermopsin	647	8	212	7
Anatoxin - a	647	1	212	1

Table 3.2. Samples and bathing sites analyzed for cyanotoxin presence and number of positive detections by LC-MS/MS.

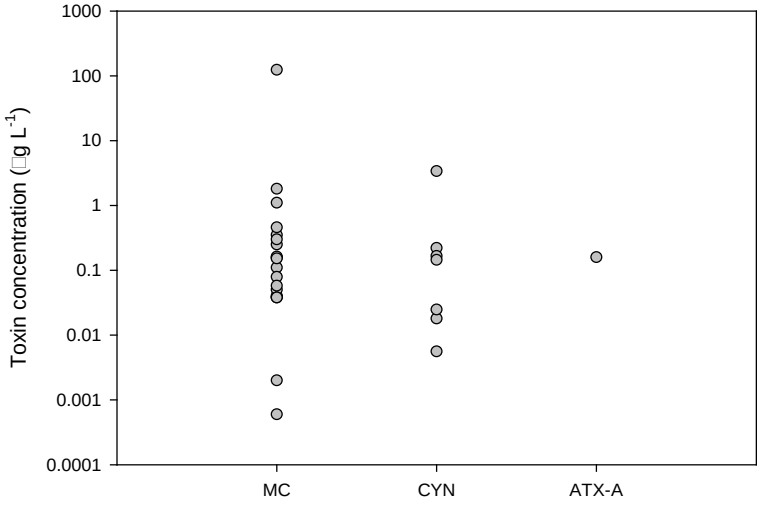

Figure 3.4. Toxin concentrations in positive water samples. Concentrations are in logarithmic scale.

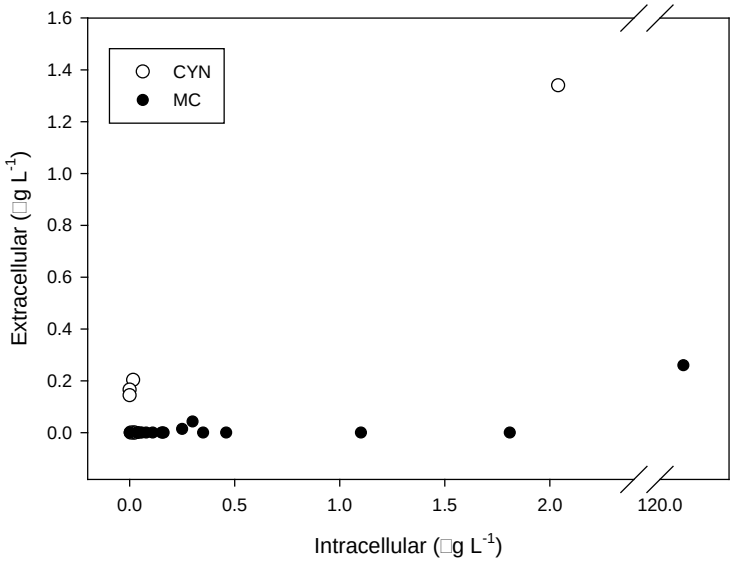

Figure 3.5. Contribution of intra- and extracellular fractions to total cyanotoxin concentrations. Black points stand for microcystin, whereas white circles stand for cylindrospermopsin.

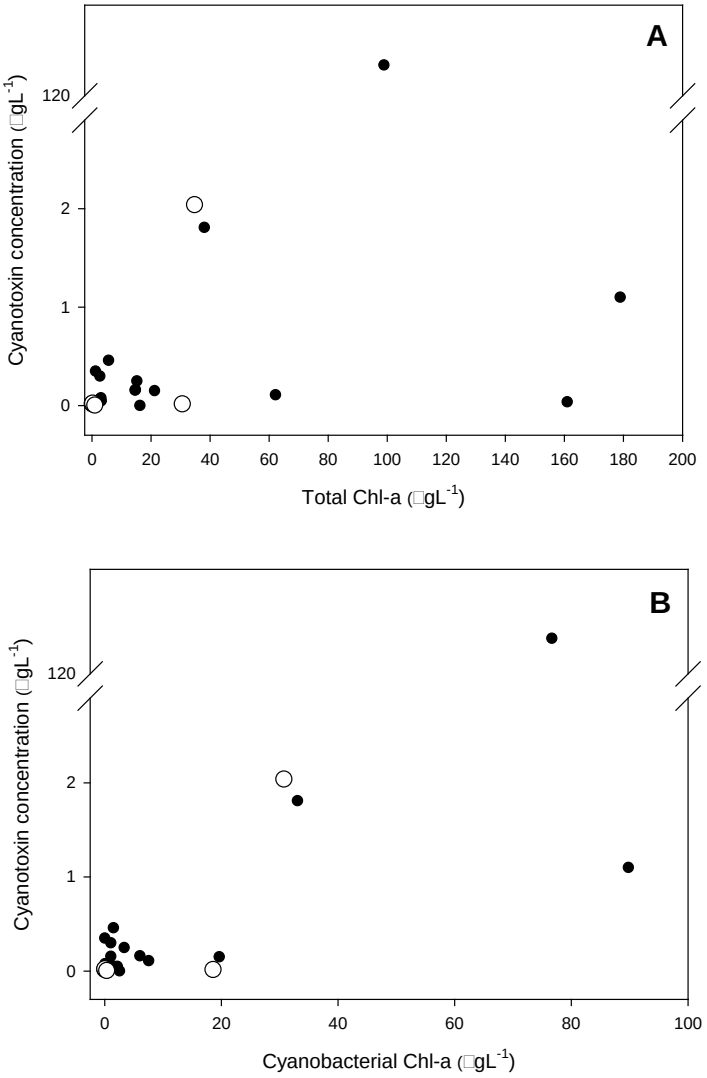

Figure 3.6. Relation between cyanotoxin concentrations and total chlorophyll-a concentrations (A), and cyanobacterial chlorophyll-a concentrations (B). Black points stand for microcystin, whereas white circles stand for cylindrospermopsin.

In order to assess the tendency of toxic cyanobacteria proliferation, we employed a stepwise decision tree schematized in fig. 3.7. Such decision tree was designed *ad hoc* for Spanish water bodies, setting concentration thresholds that enable a realistic assessment of the situation of Spanish waters in terms of harmful cyanobacteria proliferation, based on our current results, historical data and previous experience. Such thresholds should however be adapted to local/national guidelines if applied elsewhere. The proposed assessment includes a set of preliminary verifications (level 0; fig. 3.7): the presence of visual scums, as well as the presence of CYN or CYN producers, lead to directly classify the risk as high. In case these preliminary verifications are negative, a first level of assessment is carried out by verifying direct and indirect trophic parameters using current and historical data (level 1), namely total

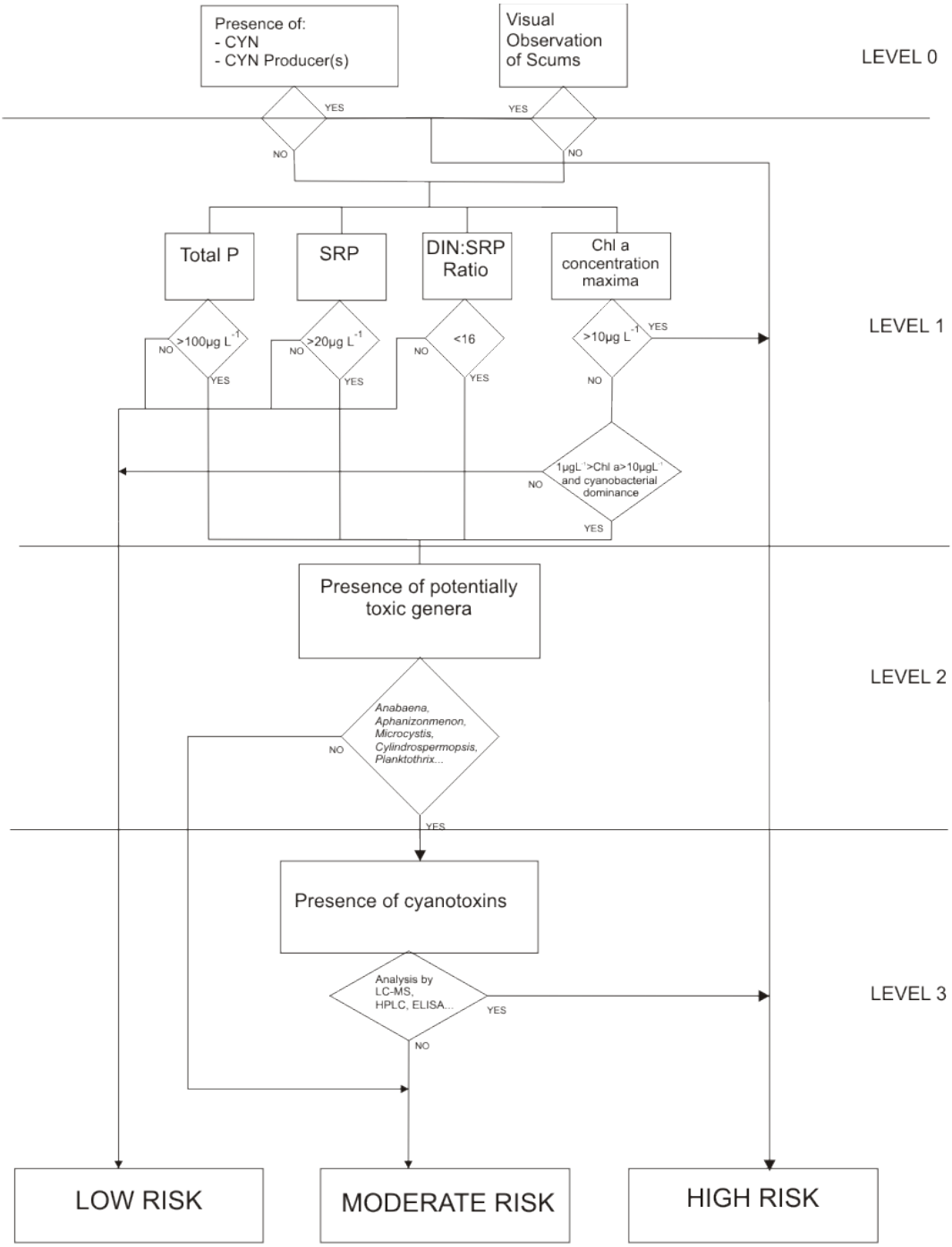

Figure 3.7. Flow chart representing the proposed assessment of the risk for toxic cyanobacteria proliferation. The decision tree is organized in 4 levels of decision (see text).

P and reactive soluble P (SRP) concentrations, DIN:SRP ratios and Chl-a concentration maxima according to the specified thresholds. Exceptionally, maximal Chl-a concentrations above 10 µg L^{-1} trigger the evaluation of the risk of toxic cyanobacteria proliferation as high without further verifications. If none of these trophic parameters exceeds threshold values, the risk of proliferation can be considered low. Otherwise, it is assumed that the corresponding water body possesses the trophic potential to host cyanobacterial blooms, and the presence of

potentially toxic cyanobacterial genera should hence be evaluated (level 2). The absence of potentially toxic genera results in a moderate risk of toxic cyanobacteria proliferation. As the presence of potentially toxic genera does not necessarily imply the presence of cyanotoxin producers, cyanotoxin analysis (level 3) should be carried out as a final verification to classify the risk as high. The results of the assessment on the official Spanish bathing water sites are summarized in table 3.3. Half (50.2%) of the analyzed bathing sites showed a moderate risk of toxic cyanobacteria proliferation, while high risks accounted for 19.8% of the bathing sites. Only 29.5% of the analyzed bathing sites presented a low risk of cyanobacterial proliferation.

Risk	Official Bathing Sites	%
High Risk	43	19.8
Moderate Risk	109	50.2
Low Risk	64	29.5
Insufficient data	1	0.5

Table 3.3. Results of the assessment of the risks for toxic cyanobacterial proliferation

3.5. Discussion

Cyanobacteria and their toxins have been considered a menace in Mediterranean water bodies in the last decades (Vasconcelos 1994, Vezie et al. 1997, Cook et al. 2004, Messineo et al. 2009). Local and national scale studies carried out in Spain have demonstrated that Spain is not an exception (de Hoyos et al. 2004, Quesada 2004, Aboal & Puig 2005, Carrasco et al. 2006). The present work constitutes the most extensive study carried out in Spanish freshwaters concerning harmful cyanobacteria. It has to be noted however that the obtained results may somewhat be biased towards reduced cyanobacterial presence, chiefly due to the limited temporal resolution of the samplings (one or two samplings per season) and also due to the fact that sampled sites may not be fully representative, as sampling was restricted to officially designated bathing sites. As such designation was done by the individual regional authorities, some regions reported a high number of official bathing sites and were hence extensively sampled (e.g. northwest Spain, Norte watershed), while others (e.g. east and southeast regions, Segura watershed; Fig. 3.1) were poorly represented. Similarly, many bathing sites corresponded to fluvial systems, where planktonic communities are often unable to develop. This fact partially explains the high frequency of samples with low (< 5 μgL^{-1}) total and cyanobacterial Chl-a concentrations. However, these sampling points also demand special attention from a public health standpoint, as a remarkable fraction of the lentic freshwater ecosystems in Spain are reservoirs, derived from dammed rivers (de Hoyos et al. 2004). Fluvial

sampling sites are thus often affected by upstream reservoirs discharges in terms of cyanobacteria and cyanotoxins. In fact, 2 out of the 6 samples with positive CYN detections, although presenting low concentrations, corresponded to river systems located downstream reservoirs with CYN presence, highlighting the importance of monitoring recreational areas with evident influence of upstream reservoirs.

In line with previous studies in Mediterranean freshwaters (Cook et al. 2004), *Microcystis* was the most commonly found cyanobacterial genus. *Anabaena* and *Aphanizomenon* were also frequent genera in Spanish bathing waters, including the species *Aph. ovalisporum*, whose recent appearance in temperate latitudes is considered an expansion of its natural distribution in tropical and subtropical regions (Mehnert et al. 2010). Such expansion is matter of growing concern, as the presence of *Aph. ovalisporum* likely implies the production of CYN (Stuken et al. 2009). A similar geographical expansion was highlighted in by de Hoyos et al. (2004) regarding *Cylindospermopsis raciborskii*, a typically tropical species that, as our data confirm, can currently be considered firmly established in Spanish freshwaters.

In terms of cyanotoxins, MCs were clearly the most abundant cyanotoxins in Spanish freshwaters, present in 13 of the 212 (6.1%) sampled sites. The dominance of MCs among cyanotoxins has previously been described in reservoirs in Central Spain (Carrasco et al. 2006) and also worldwide (Sivonen & Jones 1999). However, MCs were detected in low concentrations, usually below the WHO guideline value of 1 μg L^{-1}. These low MC concentrations can be partially explained by the large fraction of analyzed bathing sites presenting low cyanobacterial presence. However, the toxicity of the biomass (expressed as MC : cyanobacterial Chl-a ratio) was high in most cases, with 52% of the samples showing ratios above 0.1 (data not shown). No clear relationship could be identified between MC concentrations and total or cyanobacterial Chl-a, denoting that biomass indicators (both total and cyanobacterial Chl-a concentrations) are poor predictors of toxin concentrations, chiefly due to the existence of toxin-producing and non-producing species in the community, together with the coexistence of both toxic and non-toxic strains below the species level and their temporal dynamics.

CYN was detected in 6 bathing sites (2.8% of the surveyed bathing sites), generally at low concentrations below 1 μg L^{-1}. Until recently, CYN was typically a concern in tropical and subtropical latitudes, while remained absent from European waters. During the last few years, however, CYN detections have been increasingly reported in several countries in Europe, including Germany (Fastner et al. 2003), Italy (Messineo et al. 2009), Spain (Quesada et al.

2006), Finland (Spoof et al. 2006), Poland (Kokocinski et al. 2009), Czech Republic (Blahova et al. 2009) and France (Brient et al. 2009). CYN dynamics in the water greatly differ from those of microcystins: MCs mostly remain in the producing cells and are majorly released only after cell lysis. Consequently, the contribution of the extracellular fraction to the total toxin pool is usually small, with concentrations one or more orders of magnitude below those of the intracellular fraction (e.g. Oh et al. 2001, Poon et al. 2001). In contrast, CYN seems to be exported by the producing organisms and is therefore largely present in the extracellular fraction, with similar or even higher concentrations as the cell bound fraction. In addition, the degradation of CYN under natural conditions has shown to be extremely limited (Wörmer et al. 2008). Both toxin export and lack of degradation allow the accumulation of CYN in the dissolved fraction, which implies a significant risk of exposure, even in the absence of cyanobacteria.

ATX-a was only detected in a single bathing site during the survey performed. Our results support the low incidence of ATX-a, previously reported in Spanish freshwaters (Carrasco et al. 2007). Together with additional studies carried out in Italy (Messineo et al. 2009), our results seem to confirm that ATX-a is an infrequent cyanotoxin in Mediterranean latitudes. In contrast, ATX-a seems to be more frequent in colder latitudes, e.g. in Finland (Rapala et al. 1993), Germany (Bumke-Vogt et al. 1999, Ballot et al. 2010) and even subalpine Italy (Cerasino & Salmaso 2012).

For the assessment of the risk of toxic cyanobacteria proliferation, a stepwise decision tree was employed. We established a preliminary level of decision (level 0) to contemplate the circumstances of massive bloom occurrence and CYN presence. The presence of cyanobacterial blooms evidently implies a high risk of proliferation without further verifications. Additionally, given the particular problematic of CYN (lack of degradation and accumulation in the dissolved fraction, see above), its presence in the water also implied a high risk of exposure. Although the Directive refers to an assessment of “the risks for cyanobacteria proliferation” and not the risk to cyanotoxin exposure, the detection of CYN implies a recent presence of toxic cyanobacterial populations. Therefore, we believe that considering the presence of CYN as a trigger for evaluating the risk as high, pursues the aim of the Directive of protecting users from the harmful effects of cyanobacteria and was therefore contemplated in the assessment decision tree.

N:P ratios (Smith 1983), total P and algal biomass (Trimbee & Prepas 1987, Downing et al. 2001) have been proposed as the best linear correlates with cyanobacterial dominance.

Although the identification of the best trophic parameter predicting cyanobacterial proliferation in surface waters has been surrounded by controversy for decades, there is a general consensus about the overwhelming evidence for the importance of increased nutrient loads for the dominance of cyanobacteria in the planktonic community and bloom occurrence. The defined threshold values employed in our study may seem unrestrictive to be applied elsewhere, but they aim to enable a realistic assessment, according to the trophic state of Spanish freshwaters after the analysis of historical data gathered during the last 2 decades. In fact, 65% of the sampling sites exceeded any of the trophic thresholds, denoting that the proposed trophic values do not act as a permissive limit in the context of the Spanish freshwaters. As a result of this assessment, around half (50.2%) of the surveyed bathing sites presented a moderate risk of toxic cyanobacteria proliferation, while 20% of the official bathing sites were highly prone to host cyanobacterial blooms. This data evidences widespread susceptibility of Spanish inland waters to be affected by cyanobacterial blooms, which may be subject in the near future, not only by a putative intensification in bloom occurrence, but also by the invasion of new harmful species in the upcoming global warming scenario. The high fraction of official bathing sites with moderate or high risks of bloom occurrence stresses the need of implementing efficient monitoring strategies to ensure users safety during recreational activities, as well as extending such monitoring to other non-official, yet heavily-used bathing sites. Similarly, monitoring efforts should also address the occurrence of additional cyanotoxins, including the neurotoxic PSP toxins, whose presence has recently been reported in Spain (Wörmer et al. 2011a). This study constitutes however one of the most extensive monitoring efforts carried out in Spain, in which national Administrations have been actively involved and represents a significant step in building full awareness of water authorities and users about the issues related to the occurrence of cyanobacteria and their toxins.

3.6 Acknowledgements

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4. Limited stability of microcystins in oligopeptide compositions of *Microcystis aeruginosa* (Cyanobacteria): Implications in the definition of chemotypes

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Article

Limited Stability of Microcystins in Oligopeptide Compositions of *Microcystis aeruginosa* (Cyanobacteria): Implications in the Definition of Chemotypes

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Abstract: The occurrence of diverse oligopeptides in cyanobacteria, including the cyanotoxins microcystins, has been recently used to classify individual clones into sub-specific oligopeptide chemotypes, whose composition and dynamics modulate microcystin concentrations in cyanobacterial blooms. Cyanobacterial chemotyping allows the study of the ecology of chemotypical subpopulations, which have been shown to possess dissimilar ecological traits. However, the stability of chemotypes under changing abiotic conditions is usually assumed and has not been assessed in detail. We monitored oligopeptide patterns of three strains of *Microcystis aeruginosa* under different nutrient and light conditions. MALDI-TOF MS revealed alterations in the microcystins signatures under N and P poor conditions and high light intensities (150 and 400 µmol photons $m^{-2}s^{-1}$). Variations in the general oligopeptide composition were caused by a gradual disappearance of microcystins with low relative intensity signals from the fingerprint. The extent of such variations seems to be closely related to physiological stress caused by treatments. Under identical clonal compositions, alterations in the oligopeptide fingerprint may be misinterpreted as apparent shifts in chemotype succession. We discuss the nature of such variations, as well as the consequent implications in the use of cyanobacterial chemotyping in studies at the subpopulation level and propose new guidance for the definition of chemotypes as a consistent subpopulation marker.

4.1. Abstract

The occurrence of diverse oligopeptides in cyanobacteria, including the cyanotoxins microcystins, has been recently used to classify individual clones into sub-specific oligopeptide chemotypes, whose composition and dynamics modulate microcystin concentrations in cyanobacterial blooms. Cyanobacterial chemotyping allows the study of the ecology of chemotypical subpopulations, which have been shown to possess dissimilar ecological traits. However, the stability of chemotypes under changing abiotic conditions is usually assumed and has not been assessed in detail. We monitored oligopeptide patterns of three strains of *Microcystis aeruginosa* under different nutrient and light conditions. MALDI-TOF MS revealed alterations in the microcystins signatures under N and P poor conditions and high light intensities (150 and 400 µmol photons $m^{-2}s^{-1}$). Variations in the general oligopeptide composition were caused by a gradual disappearance of microcystins with low relative intensity signals from the fingerprint. The extent of such variations seems to be closely related to physiological stress caused by treatments. Under identical clonal compositions, alterations in the oligopeptide fingerprint may be misinterpreted as apparent shifts in chemotype succession. We discuss the nature of such variations, as well as the consequent implications in the use of cyanobacterial chemotyping in studies at the subpopulation level and propose new guidance for the definition of chemotypes as a consistent subpopulation marker.

4.2. Introduction

Freshwater cyanobacteria are well known for their occasional massive proliferation in temperate water bodies during late summer periods. Cyanobacterial blooms are of public health concern, due to the ability of many genera to produce a wide range of toxins and other bioactive compounds (Codd et al. 2005). An important group of these compounds are non-ribosomal peptides, termed onwards oligopeptides. Microcystins (MCs), a group of potent hepatotoxins, are one of these compounds.

The traditional cyanobacterial taxonomy following botanical criteria is based mainly on morphological features. However, the existence of transitional populations with wide morphological variations (Komárek & Kling 1991), together with the observed loss of original morphological features under culture conditions (Laamanen et al. 2001), implies a remarkable polymorphism, which has raised discussions about the validity of taxonomic classification with morphological criteria alone. Indeed, from a toxicological standpoint, it is well known that morphological characteristics do not correlate well with toxin profiles, which have been observed to vary widely (Sivonen & Jones 1999).

Oligopeptides are produced by non-ribosomal peptide synthases (NRPS) (Neilan et al. 1999), with the exception of cyclamides and microviridins, which are synthesized by ribosomic pathways. More than 600 structural variants of oligopeptides have been described, which have been classified in seven classes: aeruginosins, cyanopeptolins, microcystins, microviridins, microginins, anabaenopeptins and cyclamides (Welker & von Dohren 2006). The ability of cyanobacteria to produce oligopeptides is determined by the presence or absence of NRPS-encoding gene-clusters (Dittmann et al. 1997). Cyanobacterial cells can present any combination of gene clusters, generating a specific pattern of oligopeptide production, which can be used as a characterizing oligopeptide fingerprint. The coexistence of individuals with distinct oligopeptide compositions within a population has been reported (Fastner et al. 2001). This finding allowed the use of distinct oligopeptide fingerprints as subspecific cyanobacterial markers to divide populations into oligopeptide-based subpopulations or chemotypes (Welker et al. 2007), which have been observed to possess different ecological traits (Rohrlack et al. 2008). Besides, MC concentrations in the course of the bloom often display marked temporal variations of up to several orders of magnitude (e.g. Carrasco et al. 2006) that represent one of the main hindrances to evaluate the risks associated with cyanobacterial blooms. Since MC is

produced constitutively, its synthesis varies only about three-fold and is related to growth or cell division rates (Orr & Jones 1998). These variations cannot be explained by physiological changes at the individual level. Conversely, the relative abundance of dissimilar chemotypes in natural communities, as well as the shifts in chemotypical subpopulations, have been shown to be responsible for the observed varying MC concentrations (Agha et al. 2012) and, consequently, have to be considered critical factors modulating bloom toxicity.

Although changes in the synthesis of individual oligopeptides under changing abiotic conditions have been examined (Repka et al. 2004, Rohrlack et al. 2007, Halsvedt et al. 2008) these differences have not been assimilated in the context of the conservation of oligopeptide fingerprints and its evaluations as a consistent chemotype marker. Understanding potential variations in the peptide composition of clonal strains is crucial for tracking the succession of chemotypical subpopulations in natural systems and, thereby, investigate their ecology. To evaluate their stability, we tracked oligopeptide compositions of three clonal strains of *Microcystis aeruginosa* producing a wide range of microcystin variants and cultured under different scenarios. We focused on *M. aeruginosa*, a species that has been pointed out as the most important microcystin producer in Mediterranean latitudes (Cook et al. 2004, Carrasco et al. 2006). Selected strains were exposed to N and P reductions and different light intensities. Their oligopeptide contents were analyzed by Matrix Assisted Laser Desorption/Ionization Time Of Flight Mass Spectrometry (MALDI-TOF MS) to determine whether physiological stress causes alterations in oligopeptide fingerprints, which may have major implications when delimiting chemotypes and interpreting their dynamics under natural conditions.

4.3. Materials and methods

4.3.1. *Microcystis aeruginosa* strains

Three strains of *M. aeruginosa* were used, namely UAM254 (non-colonial morphotype), UAM264 and UAM265 (colonial morphotypes), which were isolated between 1998 and 2000 from Santillana Reservoir, a eutrophic and typically monomictic water body located in Madrid, central Spain. Limnological features of the reservoir are described elsewhere (Sanchis et al. 2002). After isolation, strains were maintained as clonal cultures in a BG11 medium (Sanchis et al. 2004) under constant conditions at 28 °C and 10 µmol photons m^{-2} s^{-1} illumination provided by continuous cool white fluorescent light.

4.3.2. Experimental setup

Nutrient treatments were cultured under continuous light irradiance of 70 µmol photons $m^{-2}s^{-1}$. All experiments were conducted after 2 weeks acclimation period to assayed conditions. Nutrient control (NC) conditions used the original BG11 medium ([P] = 175 µM; [N] = 2 mM). N-poor (NN) treatment consisted in the use of a modified BG11 medium containing only 10% of the original $NaNO_3$ concentration ([N] = 200 µM), while P-poor treatment (NP) consisted in a modified BG11 medium containing only 10% of the original K_2HPO_4 concentration ([P] = 17.5 µM). Glassware containing P-poor medium was washed previously with HCl (10%) to eliminate P traces. Light experiments were carried out using the original BG11 medium under different continuous light intensities: low light (LL) conditions consisted in a 10 µmol photons $m^{-2}s^{-1}$ irradiance and were used as control for the comparison of oligopeptide compositions, while medium (LM) and high light (LH) conditions provided 150 and 400 µmol photons $m^{-2}s^{-1}$, respectively. Sterile glassware with treatment-specific nutrient medium was inoculated with 1 mL aliquots of the original cultures in the exponential phase and placed under the appropriate light conditions. The resulting cultures were maintained under the same temperature, and their growth was monitored by spectrophotometric readings at 750 nm. Culture growth rates were calculated for each treatment during the exponential phase [$\mu = \ln(Nt/No)/\Delta t$].

4.3.3. MALDI-TOF MS analysis

During the active growth phase and until the stationary growth phase was achieved, individual colonies or free cells were collected for the determination of their oligopeptide compositions through MALDI-TOF MS. Twenty replicates for each strain-treatment-time combination were analyzed. Colony biovolume (for colonial strains) and optical density (for unicellular cultures) were correlated to cell numbers for each strain (data not shown), and were used to indirectly ensure sufficient biomass in the samples (>6000 cells) and, thereby, obtain unambiguous mass spectra. Single colonies of at least 0.03 mm^3 or 5 µL of free cell suspension were collected with disposable glass capillaries and placed in 0.2 mL Eppendorf tubes, which were allowed to dry at room temperature during 2 h. Ten microliters of an acetonitrile, ethanol and water (1:1:1) extractant solution, acidified with 0.03% (*v*/*v*) trifluoroacetic acid (TFA), were then added to each sample. Samples were introduced into liquid nitrogen for 2 h to induce cell lysis and stored at −80°C until analyzed. For MS analysis, 0.5 µL of individual samples analyte solution were placed onto the matrix spots of a Prespotted AnchorChip (PAC 284/96 HCCA, Bruker Daltonics, Bremen, Germany), which contain α-cyano-

4-hydroxycinnamic acid (HCCA) as co-crystallizing matrix. Spots were allowed to dry at room temperature and were washed with ammonium phosphate (10 mM) for 5 s before MALDI-TOF analysis. Mass spectrometric analysis was performed on a Bruker Reflex MALDI mass spectrometer equipped with a time of flight (TOF) mass analyzer on positive ion detection mode and reflector mode. Acceleration voltage was set to 25 kV and mirror voltage to 26.3 kV. Mass spectra were accumulated from 1000 laser pulses scanning the entire sample spot for a mass range of 500–2000 Da.

4.3.4. Data processing

The resulting mass spectra were processed using Bruker FlexAnalysis software (version 3.0). The SNAP algorithm (Bruker Daltonics, Bremen, Germany) was used for mass determination using a signal to noise threshold of 6. Putative oligopeptides were identified according to their mass/charge ratio, compared with previously described oligopeptides (Welket et al. 2006). The occurrence of Na and K adducts in the ionization was also examined, and their assignment was corrected to the appropriate protonated oligopeptide. Mass signals that could not be assigned to any previously described oligopeptide were excluded from the analysis. Relative intensities were calculated based on the absolute signal intensity of the highest identified oligopeptide peak in the mass spectrum. Obtained data were used to construct a matrix for each strain, illustrating the presence or absence of oligopeptides along the different treatments and sampling times. To evaluate the differences between the oligopeptide fingerprints recorded under different treatments, an ascending hierarchical clustering (AHC) was conducted: for each strain, multiple datasets consisting of each treatment and its respective control were constructed. The resulting individual datasets were examined by AHC, which analyzed dissimilarities using Euclidean distances and applying Ward's agglomeration method. The number of clusters was predefined (k = 2), so that data variability within the dataset was separated into two clusters. The resulting clusters contained samples with similar (or identical) peptide compositions.

To evaluate whether applied treatments caused changes in the peptide composition compared to controls, the percentage of treatment and control replicates assigned to each cluster was examined. When treatments induced variations in the peptide fingerprint, control replicates were majorly and selectively concentrated in cluster A, while most of the treatment replicates were grouped in cluster B. Conversely, when assayed conditions did not cause changes in the peptide composition, non-selective assignment occurred, having samples

corresponding to both control and treatment majorly assigned to the same cluster. Given that chemotypical subpopulations are delimited by their respective peptide compositions, which are usually defined by the presence of individual oligopeptides, the gain or loss of one or more peptides would imply a change in the chemotype-specific fingerprint. Conserved oligopeptide patterns were, hence, considered those which conserved their complete oligopeptide combination regardless of treatments and time. Therefore, stability was analyzed as the consistency in detection of individual peptides. Detection frequencies were expressed as a percentage of positive detections among same-treatment replicates (termed onwards IDF, interreplicate detection frequency) and aim to provide a metric of the variations in detection of individual peptides between replicates. Oligopeptides presenting IDFs below 60% were regarded as unstable signals and consequently considered to account for changes in the oligopeptide fingerprint. This value aimed to provide a conservative limit to discern between stable and unstable peptides. To express the extent of the fingerprint instability, a relative intensity threshold (RIT) was calculated, which represents the maximal mean relative intensity among unstable oligopeptides (i.e., those showing IDFs below 60%). RIT values represent the delimitation, in terms of mass spectral relative intensity, between stable and unstable peptides for each treatment and strain.

4.4. Results

4.4.1. Oligopeptide compositions

A total of 1401 samples (colonies or groups of free cells) were analyzed by MALDI-TOF MS for their qualitative oligopeptide composition, generating a pool of more than 15,500 mass signals corresponding to, among others, 10 previously described oligopeptides (Welker et al. 2006). Obtained data were used to construct an oligopeptide presence/absence matrix for each strain and condition (Figures 1 and 2). The selected strains produced mass spectra containing a wide range of putative MCs congeners and one cyanopeptolin variant (Table 4.1). Strain UAM254 showed a peptide composition consisting of a range of nine MC-variants. Strain UAM264 and UAM265 provided identical peptide patterns, with nine MC variants and one cyanopeptolin (cyanopeptolin 1006A, 1007.5 Da). The most intense signals corresponded to MC-LR, MC-RR and MC-YR, independently of the analyzed strain.

Peptide Name	Protonated m/z $[M+H]^+$ (Da)	UAM254	UAM264	UAM265
Desmethyl-MC-LR	981.5	x	x	x
MC-LR	995.6	x	x	x
Cyanopeptolin 1006A	1007.5		x	x
Desmethyl-MC-RR	1024.6	x	x	x
MC-FR	1029.5	x	x	x
Desmethyl-MC-YR	1031.5	x	x	x
MC-RR	1038.6	x	x	x
MC-YR	1045.5	x	x	x
MC-H4YR	1049.6	x	x	x
MC-WR	1068.6	x	x	x

Table 4.1. Detected oligopeptides by MALDI-TOF MS for each analyzed strain and their repective protonated m/z ratios

4.4.2. Growth rates

We employed reductions in growth rates *versus* controls as a proxy of the fitness of the cultures under assayed conditions and, thereby, evaluate the effectiveness of treatments. N-poor treatment caused significant decreases in growth rates in two of the three analyzed strains, while P-poor only caused significant reductions in the growth rates of one strain (Table 4.2). UAM254 showed similar growth rates under control and P-poor treatments, significantly higher than those observed in the N-poor culture. Strain UAM264 did not show differences in growth rates between control and both N- and P-poor conditions. Nutrient treatments also induced reductions in growth rates in strain UAM265. In the light intensity experiment, non-colonial strain UAM254 exhibited under medium light (LM) and high light (LH) treatments significantly lower growth rates than control (low light; LL). Strain UAM264 also showed lower growth rates when compared to control, although differences were only statistically significant under LM treatment. Strain UAM265 did not present positive growth rates under high light conditions (LH) and started to decline a few days after inoculation. Although some colonies of this culture were analyzed, they presented poor pigmentation and produced only mass spectra with extremely low signals and were hence excluded from the analysis. Cultures under LM treatment presented significantly lower growth rates than under low light conditions (LL).

Strain		Growth rates (d^{-1})	Relative Intensity Threshold [(a)]
Strain UAM254	NC	0.146	0.04
	NN	0.108 (*)	0.34
	NP	0.181	0.04
	LL	0.135	0.08
	LM	0.060 (*)	0.25
	LH	0.070 (*)	0.25
Strain UAM264	NC	0.274	0 (stable)
	NN	0.205	0 (stable)
	NP	0.275	0 (stable)
	LL	0.206	0 (stable)
	LM	0.117 (*)	0.03
	LH	0.163 (*)	0.03
Strain UAM265	NC	0.383	0 (stable)
	NN	0.172 (*)	0.07
	NP	0.179 (*)	0.06
	LL	0.190	0 (stable)
	LM	0.103 (*)	0.03
	LH	-----	-----

Table 4.2. Growth rates and relative intensity threshold for each strain and treatment. (a) Relative intensity threshold refers to the highest mean relative intensity among peptides, which presented interreplicate detection frequencies (IDFs) below 60% (i.e. unstable). NC: nutrient control; NN: N-poor treatment; NP; P-poor treatment; LL: low light (Control), 10 µmol photons $m^{-2}s^{-1}$; LM: medium light, 150 µmol photons $m^{-2}s^{-1}$; LH: high light, 400 µmol photons $m^{-2}s^{-1}$.
(*) Value significantly different to respective control treatments (ANOVA Holm-Sidak test; $p < 0.05$).

4.4.3. Stability under N- and P-reduction

N-poor conditions did not cause changes in the oligopeptide pattern in strain UAM264. However, in the remaining strains, ascending hierarchical classification (AHC) did reveal variations under N-poor conditions (UAM254 and UAM265). Differences in the peptide patterns under the P-poor treatment could only be detected in one strain (UAM265).

Until the last days before achieving the stationary phase, strain UAM254 provided, under both control and P-poor conditions, spectra containing mass signals corresponding to all the range of MCs, except for MC-FR, which could not always be detected (Figure 4.1). However, during the last days before achieving the stationary growth phase (t = 15 days), oligopeptide detection under control conditions (nutrient control; NC) was dramatically reduced to signals corresponding to MC-LR, MC-YR and MC-RR. Under N-poor conditions, spectra lacked many of the mass peaks (Figure 4.1), which corresponded to low intensity oligopeptides, while only signals that exhibited high mean relative intensities (>0.34; Table 4.3) were conserved. In fact, oligopeptide compositions recorded in the N-poor replicates were clearly separated in different clusters from those recorded in the control (except control at t =

15 days; Table S1A). AHC did not evidence significantly different oligopeptide profiles in P-poor cultures and control treatment (except control at *t* = 15 days), as most of the replicates were grouped in the same cluster.

Strain UAM264 presented a conserved oligopeptide pattern under all treatments (Figure 4.1), as confirmed by AHC, which did not show separation between oligopeptide fingerprints in any of the nutrient treatments, when compared to control conditions, having most of the samples consistently assigned to the same cluster (Table S1B).

Strain UAM265 showed differences between peptide patterns under control conditions and those obtained in both N- and P- poor treatments (Table S1C). Under P-poor conditions, interreplicate detection frequencies (IDFs) corresponding to oligopeptides with the lowest relative intensity signals (Desmethyl-MC-YR and MC-FR) falling below 60%, leading to instability of the peptide fingerprint. N-poor treatment showed mass peaks corresponding to Desmethyl-MC-YR, MC-FR and also Desmethyl-MC-RR as non-stable signals, with IDFs below 30% (Table 4.3).

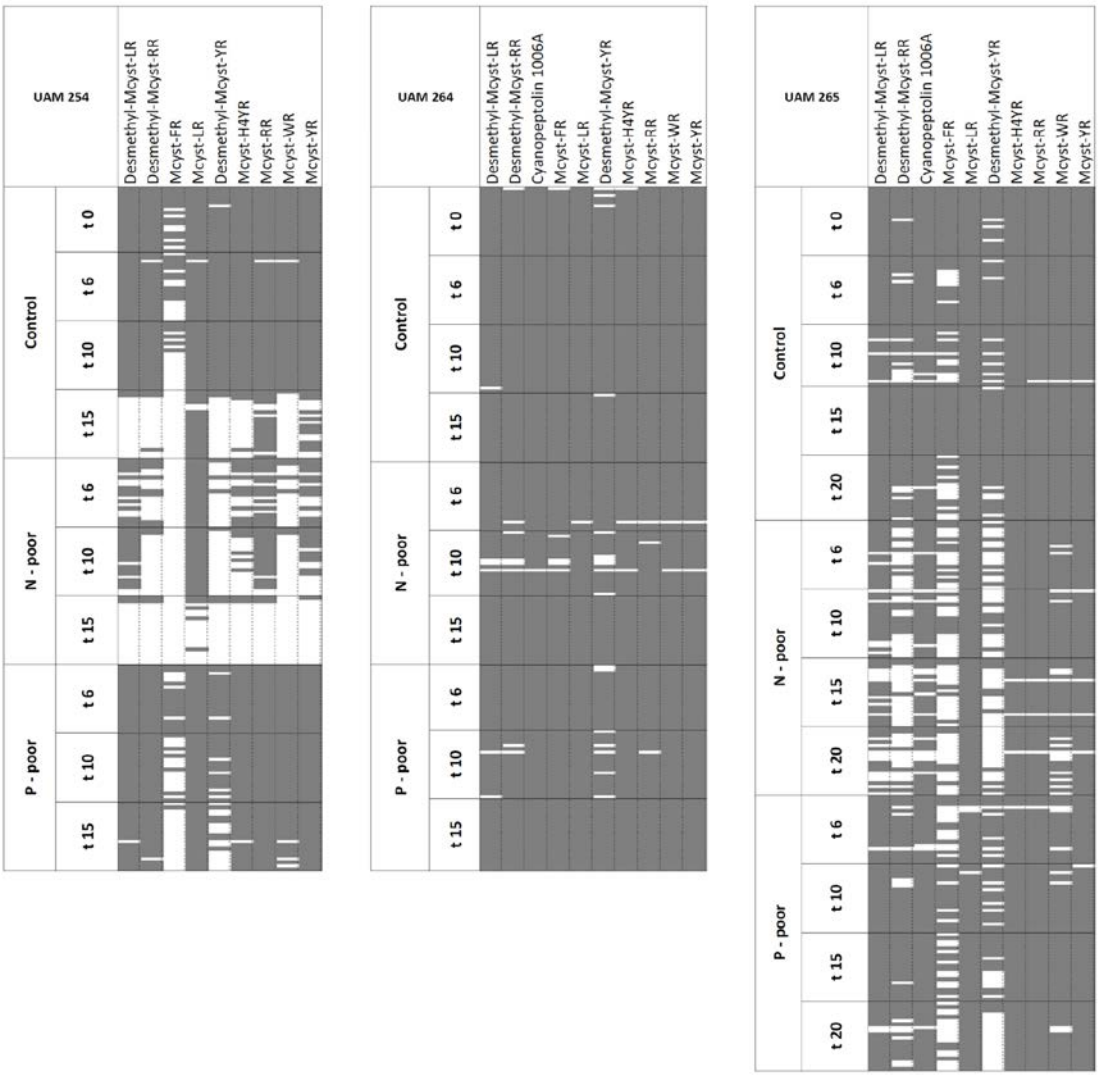

Figure 4.1. Presence/absence matrices of oligopeptides for strains UAM 254, UAM264 and UAM265 under N- and P-poor treatments. Columns show previously described oligopeptides, whereas rows represent the analyzed samples, sorted by nutrient treatment applied and time of sampling (days). Filled/colored cells correspond to presence; blank cells stand for absence.

4.4.4. Stability under high light intensities

Increased light irradiation (both LM and LH) caused variations in the peptide compositions in all strains. Strain UAM254 provided spectra with incomplete oligopeptide fingerprints (Figure 4.2), even under control conditions (10 µmol $m^{-2}s^{-1}$), lacking microcystins that presented average low relative intensity signals (≤0.08; Table 4.3). Peptide compositions of all light treatments were similar during the first days: AHC grouped replicates of LL and both

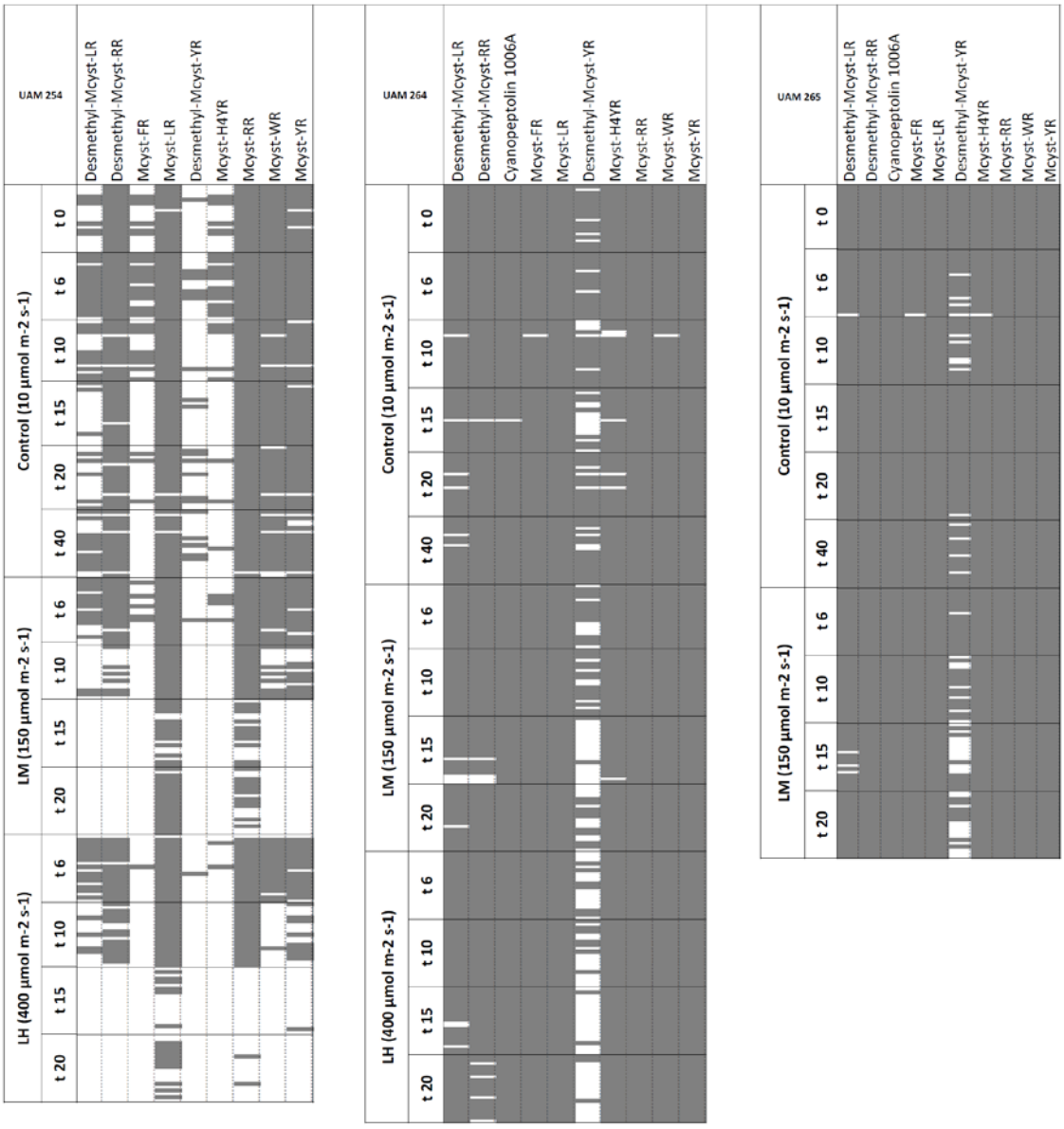

Figure 4.2. Presence/absence matrices of oligopeptides for strains UAM 254, UAM264 and UAM265 under different light intensities. Columns show previously described oligopeptides, whereas rows represent the analyzed samples, sorted by nutrient treatment applied and time of sampling (days). Filled/colored cells correspond to presence; blank cells stand for absence.

higher light intensities in the same cluster for the initial days of growth (t = 6 days). However, in peptide patterns obtained in later days (t = 15, 20 days), some peptides were missing, and replicates were therefore grouped in a different cluster by AHC, thereby evidencing variations in the oligopeptidic fingerprint (Table S2A). Under increased light conditions, mass signals corresponding to unstable oligopeptides showed always average relative intensities below 0.25 (Table 4.3). Oligopeptide compositions of strains UAM264 and UAM265 were consistent along treatments (Figure 4.2), with the exception of signal corresponding to Desmethyl-MC-YR, which showed the lowest relative intensities and could only be detected with IDFs below 60%

under higher light intensities (Table 4.3). Based on its absence, AHC evidenced differences in peptide fingerprints under LM (both strains) and LH treatments (UAM264), when respectively compared to LL treatment (Table S2B,C).

4.4.5. Extent of the peptide fingerprint stability

In general, a significant reduction in growth rates caused by treatments was followed by a gradual disappearance of microcystins mass signals with increasing relative intensities in oligopeptide profiles. The extent of the instability in the oligopeptidic fingerprint could hence be expressed as a relative intensity threshold, above which oligopeptides could be consistently detected. This threshold served as a metric of the extent of the instability, as well as a comprehensive delimitation between stable and unstable oligopeptides, according to the IDF criteria set. Strain UAM264 did not exhibit significant reductions in growth rates under nutrient limiting conditions and presented a stable oligopeptide fingerprint. In the light treatments though, where reduction in growth rates was significant (Table 4.2), differences in the fingerprint were caused by Desmethyl-MC-YR, which was detected with the lowest mean relative intensity (0.03). Instability in the peptide fingerprint of strain UAM265 was caused by the absence of more peptides, whose maximal mean relative intensity (i.e., relative intensity threshold) ascended to 0.07 (Table 4.3). Non-colonial strain UAM254, showing the lowest growth rates, provided incomplete peptide compositions, even under control conditions, presenting even higher relative intensity thresholds (0.34 and 0.24 in nutrient and light experiments, respectively; Table 4.3).

4.5. Discussion

MALDI-TOF MS has been pointed out as a useful and fast screening technique for the detection of cyanobacterial peptides, toxins and other bioactive compounds (Erhard et al. 1997, Fastner et al. 2001, Welker et al. 2002). Recent studies dealt with the description of oligopeptide-based subspecific units or chemotypes, their ecology, dynamics and diversity (Welker et al. 2007, Rohrlack et al. 2008, Fewer et al. 2009, Martins et al. 2009). However, this approach was based on the assumption that qualitative cyanobacterial oligopeptide composition was a stable feature, unaffected by abiotic conditions. Although there is abundant bibliography about the factors affecting synthesis of single oligopeptides (Repka et al. 2004, Rohrlack & Utkilen 2007, Halstvedt et al. 2008) and cyanobacterial cultures have been observed to produce oligopeptides cyanopeptolin and microcystin for years under constant laboratory conditions (Tillett et al. 2000, Bister et al. 2004), to our knowledge, no specific

studies have related the observed differences in oligopeptide production under changing conditions in the context of the definition of chemotypes. Evaluating the consistency and conservation of oligopeptide fingerprints under varying environmental conditions, as well as understanding to what extent these are reliable in the frame of chemotype delimitation, is central for the correct interpretation of the dynamics of chemotypical subpopulations under field conditions.

In our study, the different extent of the reduction in growth rates under different treatments evidences that strains show disparate susceptibility to nutrient deficiency. Intraspecific variations in growth rates under identical conditions have previously been described (Kosol et al. 2009). While strain UAM264 grew with similar rates under all nutrient treatments during the studied period, UAM265 suffered both N- and P-deficiency (Table 4.2). Strain UAM254 exhibited overall lower growth rates, which may be attributed to its non-colonial morphology (Wu & Song 2008). All in all, our observations evidence that strains may show different growth optima, even when belonging to the same chemotype (UAM264 and UAM265). In line with the varying susceptibility to changing nutrient and light conditions, we also found a different response among strains in terms of stability of their oligopeptide profiles. While peptide composition changed in strains UAM254 and UAM265 when nutrient and/or light conditions were altered, strain UAM264 conserved its original peptide pattern. Interestingly, variations in microcystin compositions were only observable when treatments caused significant reductions in growth rates. Strains unaffected by treatments (i.e., no statistically significant reductions in growth rates) provided highly conserved peptidic profiles. Indeed, missing oligopeptides always provided the lowest average relative intensities in the mass spectra (Table 4.3). The gradual disappearance of low intensity producing peptides enabled the use of a relative intensity threshold as an indicator of the extent of the oligopeptide fingerprint stability. Microcystins with average relative intensities below the relative intensity threshold (RIT) value could not be consistently detected. In other words, RITs can potentially be used as a threshold to discern between stable and unstable peptides. Together with the number of unstable peptides, RIT values were also higher when greater reductions in growth rates were observed (Table 4.2), denoting that the fitness of the culture, and the degree of conservation of microcystin signatures are closely linked.

	IDF					Average relative intensities			
Strain UAM254	Total	NC	NN	NP		Total	NC	NN	NP
MC-LR	**91%**	96%	75%	100%	STABLE	**1.00**	1.00	1.00	1.00
MC-RR	**84%**	94%	60%	100%	STABLE	**0.63**	0.73	0.39	0.65
MC-YR	**78%**	89%	**42%**	100%	**UNSTABLE**	**0.34**	0.39	0.24	0.31
MC-H4YR	**71%**	80%	**33%**	98%	**UNSTABLE**	**0.18**	0.20	0.17	0.16
Desmethyl-MC-LR	**76%**	77%	**52%**	98%	**UNSTABLE**	**0.18**	0.15	0.31	0.13
Desmethyl-MC-RR	**66%**	77%	**20%**	98%	**UNSTABLE**	**0.14**	0.15	0.15	0.13
MC-WR	**61%**	75%	**10%**	95%	**UNSTABLE**	**0.11**	0.13	0.08	0.10
Desmethyl-MC-YR	**53%**	76%	**10%**	67%	**UNSTABLE**	**0.08**	0.09	0.11	0.06
MC-FR	**26%**	**37%**	**0%**	**37%**	**UNSTABLE**	**0.04**	0.04	ND	0.04
Strain UAM264	Total	NC	NN	NP		Total	NC	NN	NP
MC-LR	**99%**	100%	97%	100%	STABLE	**0.99**	0.97	0.99	1.00
MC-RR	**98%**	100%	95%	98%	STABLE	**0.81**	0.85	0.79	0.77
MC-YR	**99%**	100%	95%	100%	STABLE	**0.47**	0.53	0.39	0.47
MC-H4YR	**98%**	99%	95%	100%	STABLE	**0.34**	0.32	0.26	0.43
Cyanopeptolin 1006A	**99%**	100%	97%	100%	STABLE	**0.30**	0.26	0.37	0.30
MC-WR	**99%**	100%	95%	100%	STABLE	**0.23**	0.27	0.19	0.22
MC-FR	**97%**	99%	92%	100%	STABLE	**0.10**	0.10	0.09	0.11
Desmethyl-MC-RR	**96%**	99%	90%	97%	STABLE	**0.09**	0.09	0.10	0.09
Desmethyl-MC-LR	**97%**	99%	93%	97%	STABLE	**0.09**	0.08	0.11	0.09
Desmethyl-MC-YR	**91%**	95%	88%	88%	STABLE	**0.05**	0.05	0.05	0.05

Strain UAM265	Total	NC	NN	NP		Total	NC	NN	NP
MC-LR	**99%**	100%	100%	96%	STABLE	**1.00**	1.00	1.00	1.00
MC-H4YR	**98%**	100%	96%	99%	STABLE	**0.39**	0.32	0.28	0.58
MC-RR	**98%**	99%	96%	99%	STABLE	**0.33**	0.38	0.26	0.34
MC-YR	**98%**	99%	95%	99%	STABLE	**0.33**	0.38	0.28	0.32
Cyanopeptolin 1006A	**92%**	96%	82%	96%	STABLE	**0.17**	0.15	0.13	0.23
MC-WR	**90%**	99%	78%	91%	STABLE	**0.14**	0.16	0.13	0.13
Desmethyl-MC-LR	**88%**	97%	69%	96%	STABLE	**0.13**	0.12	0.09	0.17
Desmethyl-MC-RR	**67%**	86%	**27%**	84%	**UNSTABLE**	**0.07**	0.06	0.04	0.08
Desmethyl-MC-YR	**58%**	85%	**26%**	**56%**	**UNSTABLE**	**0.06**	0.06	0.04	0.07
MC-FR	**53%**	74%	**29%**	**50%**	**UNSTABLE**	**0.05**	0.05	0.04	0.05

	IDF					Average relative intensities			
Strain UAM254	Total	LL	LM	LH		Total	LL	LM	LH
MC-RR	**79%**	99%	76%	61%	STABLE	**0.97**	1.00	0.89	0.98
MC-LR	**87%**	97%	91%	70%	STABLE	**0.61**	0.29	0.87	0.91
MC-YR	**59%**	91%	**37%**	**34%**	**UNSTABLE**	**0.25**	0.24	0.23	0.28
Desmethyl-MC-RR	**61%**	93%	**33%**	**41%**	**UNSTABLE**	**0.24**	0.25	0.15	0.29
MC-WR	**56%**	93%	**34%**	**24%**	**UNSTABLE**	**0.16**	0.17	0.15	0.14
Desmethyl-MC-LR	**32%**	**47%**	**20%**	**23%**	**UNSTABLE**	**0.08**	0.05	0.08	0.15
Desmethyl-MC-YR	**8%**	**18%**	**1%**	**1%**	**UNSTABLE**	**0.06**	0.06	0.04	0.07
MC-FR	**14%**	**28%**	**7%**	**1%**	**UNSTABLE**	**0.03**	0.03	0.03	0.04
MC-H4YR	**13%**	**25%**	**5%**	**3%**	**UNSTABLE**	**0.03**	0.03	0.04	0.05
Strain UAM264	Total	LL	LM	LH		Total	LL	LM	LH
MC-RR	**100%**	100%	100%	100%	STABLE	**0.80**	1.00	0.71	0.60
MC-LR	**100%**	100%	100%	100%	STABLE	**0.79**	0.51	0.99	0.99
Cyanopeptolin 1006A	**100%**	99%	100%	100%	STABLE	**0.33**	0.15	0.36	0.56
MC-YR	**100%**	100%	100%	100%	STABLE	**0.33**	0.29	0.38	0.33
MC-WR	**100%**	99%	100%	100%	STABLE	**0.20**	0.17	0.25	0.18
MC-FR	**100%**	99%	100%	100%	STABLE	**0.16**	0.10	0.17	0.23
MC-H4YR	**98%**	96%	99%	100%	STABLE	**0.12**	0.06	0.17	0.13
Desmethyl-MC-RR	**97%**	99%	95%	95%	STABLE	**0.11**	0.14	0.09	0.08
Desmethyl-MC-LR	**95%**	95%	94%	96%	STABLE	**0.06**	0.04	0.06	0.07
Desmethyl-MC-YR	**52%**	75%	**47%**	**23%**	**UNSTABLE**	**0.03**	0.03	0.03	0.04

Strain UAM265	Total	LL	LM	LH		Total	LL	LM	LH
MC-RR	**100%**	100%	100%		STABLE	**0.88**	1.00	0.71	
MC-LR	**100%**	100%	100%		STABLE	**0.74**	0.57	0.99	
MC-YR	**100%**	100%	100%		STABLE	**0.42**	0.38	0.48	
MC-WR	**100%**	100%	100%		STABLE	**0.23**	0.20	0.27	
Cyanopeptolin 1006A	**100%**	100%	100%		STABLE	**0.23**	0.13	0.36	
MC-H4YR	**99%**	99%	100%		STABLE	**0.15**	0.09	0.23	
Desmethyl-MC-RR	**100%**	100%	100%		STABLE	**0.12**	0.14	0.10	
MC-FR	**99%**	99%	100%		STABLE	**0.10**	0.08	0.14	
Desmethyl-MC-LR	**98%**	99%	96%		STABLE	**0.06**	0.04	0.08	
Desmethyl-MC-YR	**77%**	88%	**60%**		**UNSTABLE**	**0.03**	0.03	0.05	

Table 4.3. Interreplicate detection frequencies (IDFs) and average relative intensities of individual peptides. Peptides are classified as stable or unstable, according to their observed IDFs. Peptide with IDFs ≤ 60% are considered unstable

In light of these observations, we hypothesize that physiological effects caused by intense light irradiation and/or nutrient limitation, especially N-deficiency, causes an overall reduction in microcystins cellular concentrations, leading to alterations in the oligopeptide profiles employed for chemotype delimitation. Under high light conditions, free microcystin concentration in the cell may be reduced as a result of covalent binding of microcystin to the cysteine residues of proteins involved in photosynthesis, as recently demonstrated by Zilliges et al. (2011). This binding was observed to be dramatically enhanced under high light conditions, suggesting that microcystins may play a role as a protein-modulating metabolite under oxidative stress conditions. Alternatively, a decrease in the NPRS/PKS enzyme complex activity as a result of physiological stress would explain an overall reduction in MC synthesis: The coexistence of multiple MC variants partially results from the NPRS/PKS multi-enzyme complex substrate inspecificity in the incorporation of amino acids in positions 2 and 4 (Mikalsen et al. 2003). A reduction in the activity of the NRPS/PKS enzyme complex would therefore reduce the synthesis of all coexisting MC congeners homogeneously, causing minority MC variants (i.e., those providing the lowest intensity signals) to fall below detection limits. Reduced NPRS activities under physiological stress are consistent with previous findings demonstrating that maximal oligopeptide concentrations are found under optimal growth conditions (Repka et al. 2004, Rohrlack & Utkilen 2007) and that MC production is directly and linearly correlated with growth rates (Orr & Jones 1998). A further plausible explanation refers to reductions in the availability of amino acids involved in oligopeptide synthesis as a result of N-limitation. Similarly, increased photosynthesis under high light irradiation would have an equivalent effect, raising the C/N cellular ratio and ultimately leading to N-deficiency. Nitrogen availability modulates the cellular amino acid composition, reducing nitrogen-rich amino acids, such as arginine, under limiting conditions (Van de Waal et al. 2010). All detected microcystin variants in our study contained one arginine in their structure, so that overall microcystin production would be homogenously reduced. Exceptionally, variants MC-RR and Desmethyl-MC-RR contain arginine in both positions 2 and 4. Interestingly, we observed reductions in the relative intensity of signals corresponding to MC-RR in favor of MC-LR (the co-dominant variant in the mass spectra) under both high light and N-poor conditions (Table 4.3). Reductions in MC-RR production among other variants under N-limiting conditions and high light intensities have been reported previously (Tonk et al. 2008, Van de Waal et al. 2010), evidencing that its synthesis is closely related to arginine availability. Desmethyl-MC-RR is also probably affected in this way, but changes in signals with initially low absolute intensities remain negligible in

terms of relative intensity. Previous studies reported that MALDI-TOF MS shows preferential ionization of peptides containing arginine in their structure (Krause et al. 1999). One could therefore concede that MALDI-TOF results may not represent the complete cellular oligopeptide composition, having non-arginine-containing peptides as underestimated signals close to or even below detection limits. As all MC variants detected in our study contained arginine, the presence of further oligopeptides cannot be discarded. However, variations in a peptide pattern with respect to controls (presence/absence or changes in relative intensities) have to be regarded as actual variations in the oligopeptide net production that transcend the mere alteration of the peptide fingerprint, raising important implications regarding the definition of the chemotype itself. For instance, UAM264 and UAM265 presented identical peptide compositions under control conditions and have therefore to be considered the same chemotype. However, in light of the observed variations in the general oligopeptide pattern under N and P limitation, the dilemma is evident: the same chemotype under nutrient rich conditions or two distinct chemotypes under limiting conditions? The oligopeptide profiles of the selected strains in our study only represent a portion of the complete array of oligopeptides typically used for chemotype delimitation, and the observed fingerprint instability was caused by the loss of microcystin signatures. Although most oligopeptides are produced via non-ribosomal biosynthetic pathways, a generalization of our findings to other oligopeptide classes has to be done with caution. Further investigations should, hence, focus on addressing a wider range of bioactive compounds to assess whether our findings are applicable to other oligopeptide classes. Still, the distortion of oligopeptidic fingerprints, although consisting solely of microcystins, constitutes an unreported issue in the context of chemotypes definition. Assessing the instability of oligopeptide compositions is crucial for an unbiased interpretation of the dynamics of chemotypical subpopulations in natural systems. However, homogeneous criteria for chemotype delimitation are currently not available. In fact, studies dealing with the dynamics of chemotypical subpopulations show huge differences in terms of chemotype diversity. For instance, Rohrlack et al. (2008) identified four *Planktothrix* chemotypes in Lake Steinsfjorden throughout a period of 33 years. In contrast, Welker et al. (2007) found 37 different *Microcystis* chemotypes in Brno reservoir in a study period of five months. Although these studies focus on different genera and may not be comparable, chemotype delimitation was performed in each case using different methodologies. On the one hand, the inclusion of strains with similar, but not identical oligopeptide patterns in the same chemotype (Lake Steinsfjorden) lead to the definition of a low number of

subpopulations. On the other hand, data on Lake Brno was analyzed on the basis that changes in individual oligopeptides implied a different chemotypical unit. Our study shows that such variations may occur within clonal subpopulations, suggesting that the number of chemotypes in Lake Brno could partially be overestimated. Sudden changes in abiotic conditions (e.g., nutrient inputs), together with the differences in physiological conditions among individuals within the same chemotype, may trigger changes in the recorded oligopeptide profiles, which may be misinterpreted as shifts in the chemotypical subpopulations, while the clonal composition of the population actually remains unchanged. Therefore, we believe that chemotypical delimitation needs to be done on the basis of the presence of high intensity oligopeptides, as the detection of low intensity producing microcystins has been shown to be highly dependent on the physiological condition of the subpopulation and is, therefore, not always possible.

Thus, setting a relative intensity threshold to identify reliable (*i.e.*, stable) oligopeptides for chemotype definition seems a plausible option to circumvent this problem. However, a definition of a relative intensity threshold to generally discern between "stable" and "potentially unstable" oligopeptides is still needed, given the differences in susceptibility to stressing conditions among strains and the variability in detection limits in the MALDI-TOF technique from laboratory to laboratory. Further research should, hence, focus on describing the relationship between the degree of instability in the oligopeptidic patterns (e.g., via RIT) and specific physiological indicators, which can normalize the observed intraspecific variations in growth optima. Still, our findings provide unreported evidence for variability in the detection of microcystin signatures, which may lead to a biased delimitation of oligopeptide-based chemotypes. While restricted to microcystins, such variability affects oligopeptide signatures as a whole and needs to be addressed to allow the definition of consistent chemotypical subpopulations that can be unequivocally identified under the complete range of natural conditions.

4.6. Conclusions

The stability of oligopeptide fingerprints used for the definition of cyanobacterial chemotypes has so far not been assessed. Our results evidence that oligopeptide fingerprints may be subject to distortion under suboptimal physiological conditions, consisting in the gradual loss of low intensity signals corresponding to minor microcystin variants. Changes in the oligopeptide patterns may be misinterpreted as shifts in the composition of chemotypes

and may lead to overestimation of their diversity in natural populations. Therefore, criteria for the definition of oligopeptide chemotypes should be based on the detection of high intensity producing peptides, which can be consistently detected over the complete range of environmental conditions, allowing an accurate analysis of the dynamics of chemotypes in natural ecosystems.

4.7. Acknowledgements

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5. Seasonal dynamics and differential sedimentation losses of oligopeptide-based Microcystis chemotypes reveal subpopulations with different ecological traits

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5.1. Abstract

The synthesis of a range of oligopeptides by cyanobacteria has been recently used to subdivide individual clones into oligopeptide-based chemotypes. In order to evaluate the ecological significance of such subpopulations, we tracked seasonal dynamics and sedimentation losses of *Microcystis* chemotypes in eutrophic Valmayor reservoir (Central Spain). A total of 53 distinct chemotypes could be identified throughout the season, 6 of them only present as benthic colonies. The seasonal succession of chemotypes in the water column, which successfully explained temporal fluctuations in microcystin-contents of the bloom, was driven by differential sedimentation fluxes among dominant chemotypes and occurred synchronically in pelagic and littoral habitats and at different depths. While differential sedimentation among chemotypes was in general the most important loss process shaping their succession, we also observed the existence of alternative processes (excluding sedimentation) selectively inflicting massive losses to individual chemotypes. Together, the different sedimentation patterns and pelagic net growth rates among chemotypes, their segregation among pelagic and benthic habitats, as well as the existence of chemotype-selective loss processes, evidence that *Microcystis* oligopeptide chemotypes interact differently with their environment and represent commonly ignored ecologically functional subpopulations.

5.2. Introduction

Cyanobacterial blooms in freshwaters used for drinking and recreational uses are an increasingly concerning issue due to the ability of some genera to produce potent toxins. Cyanobacterial toxins, commonly termed cyanotoxins, pose a hazard to animal and human health (Codd et al. 2005). The most common cyanotoxins are microcystins (MCs), a group of cyclic heptapeptides with two variable amino acid residues and the unusual amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda). MCs display inhibitory effects on eukaryotic protein phosphatases and have been associated with gastroenteritic and hepatic diseases (Falconer 2005). Among the potentially toxic cyanobacteria, *Microcystis* represents the most frequent bloom-forming genus in Mediterranean latitudes (Cook et al. 2004, Carrasco et al. 2006) whose massive proliferations impact the ecology of lakes and reservoirs by disrupting the food web and inducing hypoxia in lentic systems.

In addition to microcystins, cyanobacteria are able to synthesize a wide array of non-ribosomal oligopeptides with diverse bioactive properties. More than 600 chemical variants have been described so far (Welker & von Döhren 2006) and reports of new peptide variants steadily enlarge this number. Cyanobacterial oligopeptide biosynthesis is constitutively regulated, with up to 5-fold variations in cellular concentrations under different conditions (Repka et al. 2004, Rohrlack & Utkilen 2007, Halstvedt et al. 2008). Oligopeptide production, including microcystins, is generally performed via non-ribosomal pathways by large multienzyme complexes (frequently non-ribosomal peptide synthases, NRPS or combined NRPS/polyketide synthases). These peptides are encoded in large gene clusters, whose transcription and translation imply a significant demand of cellular resources in order to produce small secondary metabolites. Arguably, such energy burden has to be counterbalanced by an adaptive value of oligopeptides. In natural cyanobacterial populations, the distribution of individual oligopeptide-encoding gene clusters is highly heterogeneous, due to frequent horizontal gene transfer, recombination and gene loss events (Christiansen et al. 2003, Mikalsen et al. 2003, Kurmayer & Gumpenberger 2006). As a result, conspecific strains display distinctive oligopeptide patterns, which can be used for the delimitation of oligopeptide-based subpopulations or chemotypes. While ecological studies on cyanobacteria typically focus on the species as the lowest taxonomic level, the coexistence of intraspecific lineages and their ecological significance are often overlooked. However, evidence for population subdivision in picoplanktonic *Prochlorococcus* and *Synechococcus* has been

observed, resulting in niche partitioning among distinct ecotypes (Becker et al. 2002, Casamayor et al. 2002, Rocap et al. 2002, Becker et al. 2007, Huang et al. 2012). Ecotype differentiation would confer these genera increased ecological versatility under different environmental conditions and was suggested as one of the reasons for their worldwide distribution (Johnson et al. 2006). With the aim of identifying similar ecologically relevant subpopulations in more complex cyanobacteria, previous studies revealed the coexistence of numerous oligopeptide chemotypes in natural populations (Fastner et al. 2001), whose composition showed to be highly dynamic along the season (Welker et al. 2007). Although such dynamics lead to propose that chemotypical subpopulations possess dissimilar ecological traits (Rohrlack et al. 2008), additional quantitative field data seem needed regarding their differential interaction with their environment including loss processes, which would corroborate their ecological significance.

Understanding the dynamics of cyanobacterial chemotypes is particularly important, first, from an ecological perspective. Differences among chemotypes in terms of growth, susceptibility to grazers and pathogens, as well as sedimentation losses, can in principle shape the composition and succession of strains and thereby dynamically affect the properties of the whole-population. Furthermore, the study of cyanobacterial populations focusing on the differential interactions of chemotypes with their environment provides important insights into the yet unclear biological function(s) of oligopeptides, the selective forces driving chemotype diversification and into how such a vast metabolic diversity evolved, considering the high energetic cost linked to their biosynthesis. Secondly, in the context of water management and public health, the phenology of chemotypes in the water column arguably constitutes an important factor exerting a direct effect on the relative toxicity of cyanobacterial populations. Cyanobacterial blooms often display variations in microcystin concentrations of several orders of magnitude in both spatial (Agha et al. 2012) and temporal scales (Sanchis et al. 2002). As fluctuations in bloom toxicity cannot be explained by the rather constant microcystin synthesis at the individual level (Orr & Jones 1998), shifts in the composition of toxigenic and non-toxigenic chemotypes in the population are a critical factor modulating bloom toxicity.

Here, we study the dynamics of *Microcystis* chemotypes in Valmayor reservoir, a typical Mediterranean reservoir located in central Spain, paying special attention to their participation in sedimentation fluxes and differences in pelagic net growth. Previous studies in

Valmayor reservoir showed a high diversity of MC variants and temporal fluctuations in MC contents of settled seston during the season, which suggested differential sedimentation of chemotypes (Wörmer et al. 2011b, Cires et al. 2013). In this study we evaluate whether: (1) the distribution and dynamics of *Microcystis* chemotypes are habitat-dependent, i.e. display differences in littoral and pelagic areas, (2) shifts in the composition of *Microcystis* chemotypes are responsible for temporal fluctuations in microcystin-contents (i.e. relative toxicity) of the bloom, (3) individual chemotypes display differences in pelagic net growth rates, (4) individual chemotypes differ in their temporal sedimentation patterns and (5) chemotypes can be selectively affected by alternative loss processes other than sedimentation.

5.3. Material & Methods

5.3.1. Sampling setup

The study was performed in the eutrophic Valmayor reservoir (40°31'39''N 04°03'19''W), a typical monomictic Mediterranean reservoir located in Central Spain used for drinking water supply and recreational activities. Limnological features of the reservoir are described in detail elsewhere (Wörmer et al. 2011b, Cires et al. 2013). Valmayor reservoir was sampled during summer and fall 2010, namely from July 7th until November 24th (table 5.1). Two sampling points were defined: A pelagic sampling point located in the central part of the reservoir (z_{max} =34m) and a second point in the littoral area (z_{max} =4m). Vertical profiles of temperature, dissolved oxygen and chlorophyll a (Chl-a) were obtained using an YSI 6920 multiparameter probe at each sampling date. Light irradiance along the water column was measured using a LiCor 2π PAR sensor attached to a LiCor data logger (LI-1000). Thermal structure of the water column was also monitored by installing a line of 6 TidbiT temperature data loggers (Onset Computer Corporation, USA) at the pelagic sampling point at depths 0.5m, 3m, 6m, 12m and 18m, which recorded water temperature at 30min intervals throughout the whole study period. Additionally, three sediment cores were collected at the beginning of the study period (July 7th) at both pelagic and littoral sampling points using a gravity corer (Uwitec, Austria) in order to analyze the composition of chemotypes in the non-recruited *Microcystis* population. After a prolonged clear phase (August 24th – September 29th) and a subsequent significant appearance of *Microcystis* colonies in the water column, sediment traps were installed in the pelagic sampling point to study *Microcystis* sedimentation until the end of the study period.

Date	Discrete depths	Integrated net samples		Sediment
	Pelagic	Pelagic	Littoral	20m
07 July	X			
14 July	X			
20 July	X			
27 July	X	X	X	
11 August	X	X	X	
24 August	X			
09 September	X			
28 September	X			
15 October	X	X	X	D
19 October	X			
25 October	X	X	X	X
03 November	X	X	X	X
11 November	X	X	X	X
24 November	X	X	X	X

Table 5.1. List of samples collected at Valmayor reservoir at each sampling date. D: deployment of the sediment traps

5.3.2. Water sampling

Two types of water samples were collected during the study period (table 5.1). Firstly, discrete depth water samples were collected at the pelagic sampling point at 0.5m, 3m, 5m, 10m and 20m using a 5L water sampler (Uwitec, Austria). These samples were used for (1) the determination of total Chl-a and algal group composition, (2) the identification of present cyanobacterial taxa, (3) *Microcystis* cell density calculations and biovolume estimations, (4) quantification of sestonic MCs and (5) *Microcystis* chemotyping at individual depths (chemotyping was only performed when sufficient colonies were present; see table 5.2 for dates). At these sampling days, integrated net samples were also collected with a 20µm mesh size plankton net (Uwitec, Austria) at both pelagic (0-20m depth) and littoral (0-3m depth) sampling points and were used for chemotyping of single *Microcystis* colonies.

After sampling, water samples were transported to the laboratory at 4°C and processed within 2 hours. Chl-a concentrations and algal group compositions were determined using an Algae Analyzer benchtop fluorometer (Moldaenke BBE, Germany), allowing the determination of the individual contributions of cyanobacteria and other algal groups to total Chl-a concentrations in the sample. A 100mL aliquot of discrete depth water samples was fixed in acid Lugol's solution for subsequent microscopic identification, cell counts and biovolume estimations of waterborne *Microcystis*. The remaining water was filtered through GF/F glass fiber filters (Whatman, UK) and stored at -20°C for MC analysis.

Date	Discrete depth samples					Net samples		Sediment traps
	Pelagial					Pelagial	Littoral	20m
	0.5m	3m	5m	10m	20m			
27 July	30	30	27	21		58	60	
11 August	30	30	26	27		59	60	
15 October	30	30	30	28	30	60	60	
25 October	30	30	30	30	30	60	60	60 (4)
3 November	30	30	30	30	30	60	60	60 (5)
11 November	30	30	30	30	26	60	60	60 (3)
24 November	30	29	27	19	22	60	54	60 (6)

Table 5.2. Number of *Microcystis* colonies collected for chemotyping for each sample type and sampling date. Numbers in brackets stand for colonies providing unknown oligopeptide compositions.

5.3.3. Sediment trap sampling

Sediment traps were designed and constructed by SegaInvex (Universidad Autónoma de Madrid). They consist of three identical black PVC cylinders (4.4 cm inner diameter) that prevent photosynthetic growth. The traps were attached to two buoys fixed by two anchors. A central weight allowed further stabilization. Traps were thereby freely suspended in the water column at a depth of 20m. After their deployment on Oct 15th, traps were collected simultaneously with water samples (table 5.1). Settled material was collected from the traps by carefully discarding the supernatant of each tube and collecting the 100mL remaining in the trap. After thorough homogenization of the settled material, a first aliquot was fixed in formaldehyde 4% (v/v) and stored dark at 4°C for microscopic species identification and quantification. A second aliquot was vacuum filtered through GF/F filters and stored at -20°C for MC extraction and analysis. The remaining volume was used to collect single colonies for chemotyping. All measurements were performed individually for each tube, and results were expressed as average values of three replicates.

5.3.4. Sediment cores sampling and separation of benthic *Microcystis*

In order to analyze the chemotype composition of the benthic *Microcystis* population, three profundal sediment cores and three littoral sediment cores were collected at each sampling point at the beginning of the study period using a gravity corer (Uwitec, Austria). Immediately after sampling, cores were transported undisturbed to the laboratory and sliced. *Microcystis* colonies were successfully separated from the sediment after centrifugation of 2g of homogenized fresh sediment (0-2cm slices) with 30% LUDOX TM-50 (Sigma-Aldrich) at 400 x

g during 20 minutes. After centrifugation, colonies accumulated in the supernatant and 60 colonies per sediment core were collected for chemotyping analysis.

5.3.5. Identification and quantification of *Microcystis* in water and sediment trap samples

Identification and quantification of cyanobacteria was performed in acid Lugol fixed samples following Utermöhl's technique (Ütermohl 1958). Morphospecies identification was performed under a Leica DM IL inverted microscope (Leica Microsystems, Germany) following Komárek and Anagnostidis (1999). In the case of sediment traps, formaldehyzed aliquots of the settled material of each tube of the trap were diluted 10-fold and 1mL of the resulting suspension was filtered through 0.2µm pore-size Anodisc membrane filters (Whatman, UK) under gentle vacuum to avoid colony disruption. The filters were mounted on microscope slides adding a drop of anti-fading mounting oil Aqua Poly/Mount (Polysciences Inc., USA) and examined under an Olympus BH2 microscope equipped with a BH2-RFCA epifluorescence system (Olympus, Japan), using the BP545 excitation filter, the DM570 dicroic mirror and the O590 emission filter. *Microcystis* colonies and cells were counted on the whole surface of the filter. Thereby total settled *Microcystis* colonies/cells were calculated for each sedimentation period.

5.3.6. Estimation of pelagic net growth rates and sedimentation rates

Cell counts in individual discrete-depth water samples allowed the estimation of *Microcystis* depth-time distribution along the first 20m during the sedimentation period. The vertical distribution of the four dominant chemotypes was estimated analogously, considering their relative abundances in each discrete-depth water sample. The calculated settling rates of the whole *Microcystis* population and individual chemotypes (cells m^{-2} d^{-1}), obtained from cell counts of the settled *Microcystis* and the relative abundances of chemotypes in the traps, could be related to total *Microcystis* (and individual chemotypes) cells in the overlying 20m water column above the traps. Thereby, settling rates could be assimilated to normalized sedimentation rates (d^{-1}) for both the whole *Microcystis* population and the individual chemotypes.

In order to quantify the contribution of natural sedimentation to the dynamics of chemotypes, a simple conceptual model was employed that can be described with the equation: $\Delta N = NGR - SR$, where ΔN (d^{-1}) is the recorded variation in the integrated chemotype cell numbers between sampling dates, SR is the calculated sedimentation rates (d^{-}

^{1}) and NGR stands for a net growth rate estimate (d^{-1}) which comprises chemotype intrinsic growth, as well as overall cellular losses (including grazing, pathogenic cell lysis, programmed cell death etc.), excluding sedimentation.

5.3.7. *Microcystis* chemotyping

When sufficiently abundant, individual *Microcystis* colonies were collected from individual discrete depth water samples, integrated net samples, sediment trap samples and sediment core slices under a dissecting microscope with disposable glass capillaries (Hirschmann Laborgeräte, Germany). The number of colonies collected from each type of sample and date is detailed in table 5.2. Once collected, individual colonies were placed in 0.2mL Eppendorf tubes and allowed to dry at room temperature for 2h. Ten µL of an acetonitrile, ethanol and water (1:1:1) extractant solution, acidified with 0.03% (v/v) trifluoracetic acid were added to each sample. Samples were introduced in liquid nitrogen to induce cell lysis and enhance oligopeptide extraction. Colonies were then stored at - 80°C until analyzed. The analysis of individual colonies was performed by Matrix Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) using a Bruker Reflex MALDI mass spectrometer equipped with a TOF (Time of Flight) detector. Subsequent oligopeptide identification and spectral data processing were performed using Bruker FlexAnalysis 3.0 software (Bruker Daltonics). MALDI-TOF MS data acquisition and spectral data processing are described in detail in Agha et al. (2012). Chemotype identification was carried out by clustering analysis of the mass spectral oligopeptide compositions by performing an Ascending Hierarchical Classification, using Euclidean distances and applying Ward's agglomeration method. Optimal classification was considered when intracluster variability was zero for all clusters. Chemotype composition of each sample was expressed as the relative abundance of each chemotype. To analyze the temporal dynamics of chemotypes in the reservoir, a principal component analysis (PCA) was performed. Thereby, dimensionality of the data was reduced. Each set of colonies from each individual sample was compared and plotted in a two-component plain. The diversity of chemotypes in the individual samples was expressed as the Shannon index of diversity.

5.3.8. Microcystin Extraction and LC/MS-MS Analysis

Sestonic microcystins in discrete depth water samples and sediment trap samples were extracted from the GF/F filters twice by sonication into methanol (90%). Extracts were

vacuum-concentrated and prepared for ESI LC-MS/MS analysis. Microcystin analysis was performed on a Varian 500 Ion Trap Mass Spectrometer equipped with two Varian 212 LC chromatographic pumps and a 410 autosampler. Chromatographic separation of MC-LR, MC-YR and MC-RR was achieved using a Pursuit C-18 2x150mm column as stationary phase and mobile phases MilliQ water (A) and methanol (B), both acidified with 0.2% formic acid and buffered with 2mM ammonium formate. Gradient (%A/%B) applied 60/40 to 0/100 in 18 minutes. Ionization conditions for each MC variant are detailed in table 5.3. Quantification of MC variants was made by injecting commercial standards (DHI, Denmark) to plot calibration curves.

	Nebulizing gas pressure	**Drying gas pressure (psi)**	**Drying gas pressure (°C)**	**Precursor ion (m/z)**	**Capillary Voltage (V)**	**Excitation Amplitude (V)**
MC-RR	50	30	300	519.7	60	0.9
MC-YR	50	30	300	1045.3	185	1.8
MC-LR	50	30	300	995.4	140	1.05

Table 5.3. Electrospray ionization conditions for the microcystin variants analyzed.

5.4. Results

5.4.1. Water column sampling

Cyanobacterial abundances during the study period were moderate and no massive cyanobacterial proliferation was observed along the season. Valmayor reservoir was thermally stratified at the beginning of the sampling period, with a thermocline located at 6m, which migrated deeper with the course of the season, reaching 16m on November 11th, when autumnal mixing of the water column occurred. During July and the first two weeks of August, green algae dominated the planktonic community, although diatoms and cyanobacteria (represented by *Microcystis* spp., *Woronichinia naegeliana*, and filamentous *Anabaena crassa* were also present in low abundances. Cyanobacterial Chl-a concentrations and *Microcystis* cell densities were accordingly low (Fig. 5.1). After a prolonged clear phase in late August and September, cyanobacterial presence in the water column increased, reaching cyanobacterial Chl-a concentration maxima around 6 μg L^{-1} on November 3rd. During this period and until the end of the study, *Microcystis aeruginosa*, *M. flos-aquae* and *M. novacekii* appeared as the dominant species in the cyanobacterial community, reaching subsurface cell densities of up to

700 cells mL^{-1}. Mixing of the water column occurred on November 11th and cyanobacterial abundance in the water column rapidly decreased.

5.4.2. Dynamics of *Microcystis* chemotypes

In the analysis of 2175 single *Microcystis* colonies, a wide range of oligopeptides were detected, belonging to different oligopeptide families. Microcystins were the most abundant class (present in 88% of the analyzed colonies) including multiple chemical variants (MC –LR, –RR, –YR, –H4YR, –WR, and other demethylated variants). Aeruginosins (101, 602 and 670a variants), cyanopeptolins (1063b, 1034Ac, 986A, 972A and 920B variants) and microginins (FR3, FR4, 478 and 755 variants) were also detected. Spectral data processing and subsequent clustering analysis enabled the identification of 53 different chemotypes throughout the study period, characterized by presenting a different combination of individual oligopeptides. Most chemotypes (41 out 53; 73%) never reached relative abundances above 5% in individual samples. In contrast, only 6 chemotypes (11%) jointly accounted for 40-90% of the *Microcystis* population in the individual water samples. The remaining 6 chemotypes could only be detected as benthic populations collected from sediment samples, but never as waterborne colonies. The different *Microcystis* morphospecies (*aeruginosa, flos-aquae and novacekii*) were represented by several chemotypes and no clear relationship could be found between chemotype affiliation and colony size (data not shown).

The comparison of chemotype compositions of each analyzed sampling point and date was expressed as a bi-factorial PCA plot (Fig. 5.2). The position of data points in the PCA space expresses the similarity between chemotype assemblies in each sample, i.e. closely positioned points represent sampling dates that display resembling chemotype compositions. Consequently, the shift of data points across the PCA plane can be interpreted as the dynamics of chemotypical subpopulations. Factor 1 was significantly and positively correlated with CT11 and negatively correlated with CT17, CT4 and CT22. Factor 2 was positively correlated with CT11 and CT17 and negatively correlated with CT1. Together, both factors accounted for 67.47% of the variance in the data. Chemotypes dynamics in Valmayor reservoir during the study period displayed a non-cyclic trend, with clearly dissimilar communities at the beginning

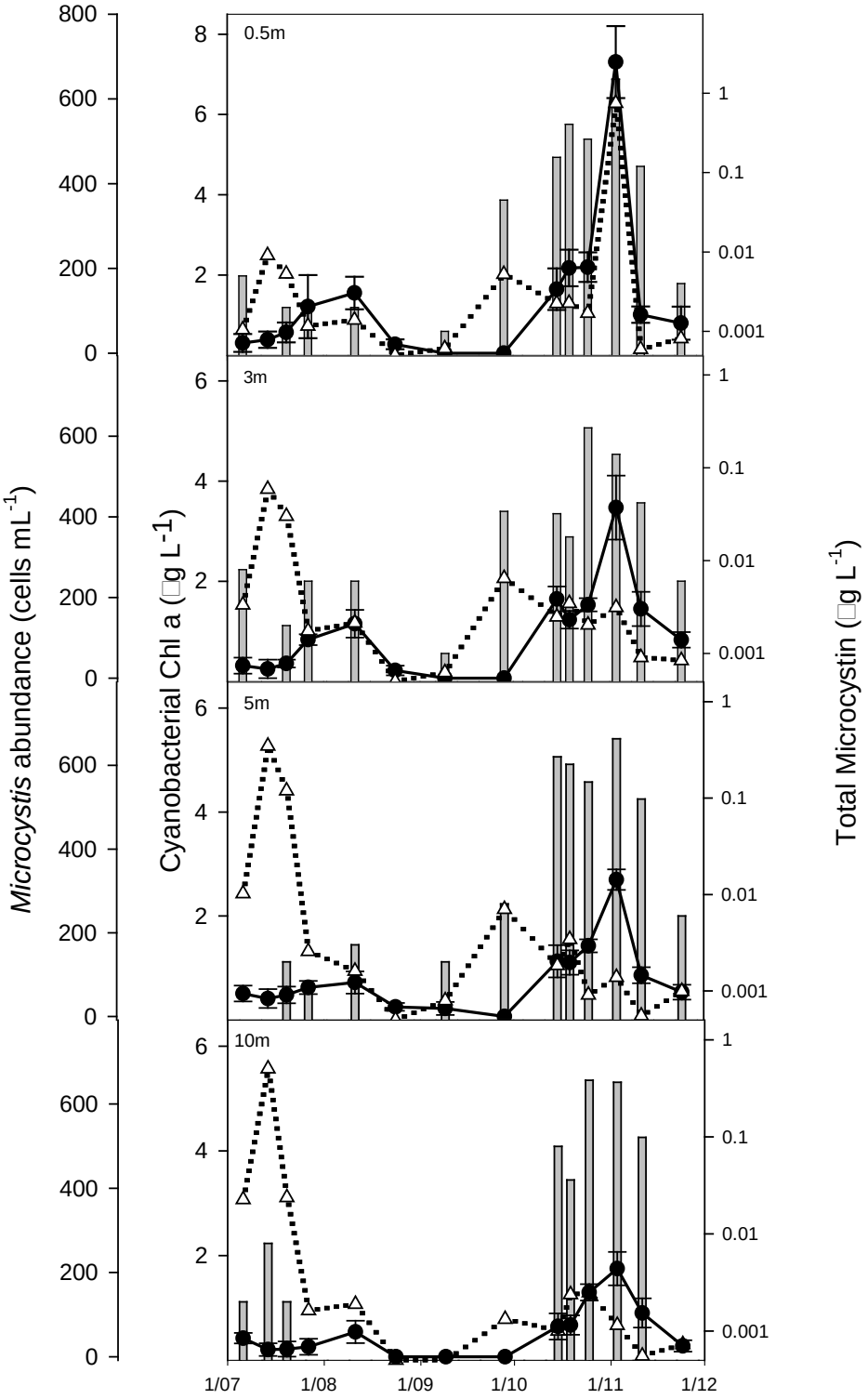

Figure 5.1. Temporal evolution of cyanobacterial chlorophyll-a (dotted lines), Microcystis cell densities (bold lines) and sestonic microcystin (bars) concentrations at the pelagic sampling point in Valmayor reservoir.

and end of the study. Points corresponding to the same sampling date grouped together, independently of their pelagic or littoral origin, indicating that the seasonal succession of chemotypes occurs homogeneously in both reservoir habitats. Interestingly, points corresponding to water samples were clearly separated from that of profundal sediment samples, evidencing marked differences between benthic and waterborne chemotype compositions during the blooming season. Surprisingly, no colonies could be found in the sediment cores collected in the littoral area. The reason for their absence remains unclear and might result from sediment focusing into deeper areas of the reservoir (Hilton 1985), or enhanced recruitment of colonies in shallow sediments with increased light availability (Schoene et al. 2010).

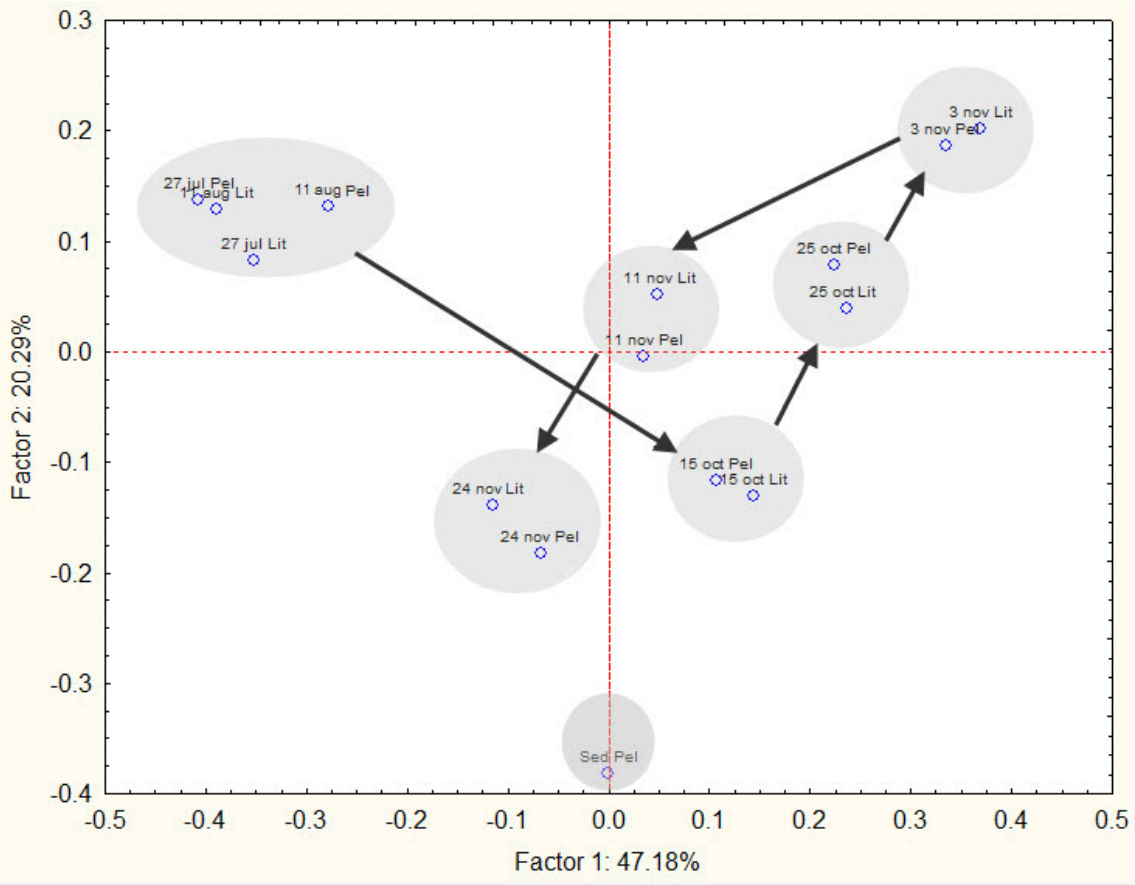

Figure 5.2. Bifactorial Principal Components Analysis (PCA) plot illustrating Microcystis chemotype compositions in Valmayor reservoir from July until November 2010. The corresponding sampling date and point (pelagic/littoral) are specified next to each data point. Arrows indicate the temporal evolution of the samples. *Sed Pel* stands for profundal sediment samples.

Chemotype diversity was high during the whole study period, although significant temporal variations in the Shannon index could be observed (Fig. 5.3). During the end of July and August (preceding the clear phase) chemotype diversity was lowest (Shannon diversity Index, H = 1.42 – 1.56) with chemotypes CT4, CT22 and CT17 dominating the population, jointly accounting for 73-94% of the colonies in the different samples during this period. After a clear phase of several weeks, *Microcystis* colonies reappeared in the water column Oct 15th comprising a significantly more diverse population than the previous period ($p<0.01$; H= 2.64 - 2.69). Chemotype diversity declined then steadily until Nov 3rd (H= 1.69 – 1.87) coincident with the seasonal cyanobacterial density maxima. In the next week (Nov 11th) diversity reached its maximum (H= 2.56 - 2.90) and remained constant until the end of the study period (no significant differences). Despite the marked succession of chemotypes, Spearman rank correlation tests did not reveal any significant ($p>0.01$) relationship between depth, light irradiance, macronutrients or water temperature and the relative abundance of the individual chemotypes along the study period (data not shown).

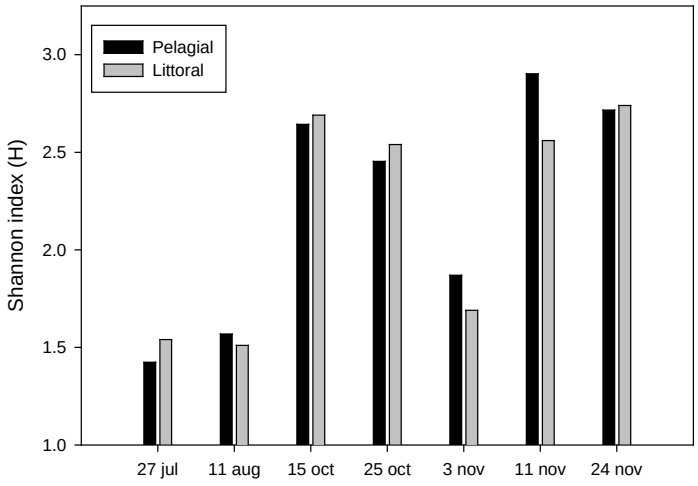

Figure 5.3. Shannon index of chemotype diversity at pelagic and littoral sampling points in Valmayor reservoir.

5.4.3. Microcystins and chemotype composition

In line with cyanobacterial abundances, total microcystin concentrations in Valmayor reservoir were in general low, varying from 0.03 to 1.49µgL^{-1}. Temporal fluctuations in sestonic toxin concentrations showed no clear agreement with cyanobacterial Chl-a concentrations or

Microcystis abundances in the water column (Fig. 5.1). Instead, the proportion of toxic chemotypes in the population recorded at each date, sampling point and depth showed a highly significant linear correlation with total microcystins : *Microcystis* biovolume ratios (r^2=0.912; p<0.01; Fig. 5.4), evidencing that sestonic microcystin contents in Valmayor reservoir are modulated by shifts in the composition of chemotypes.

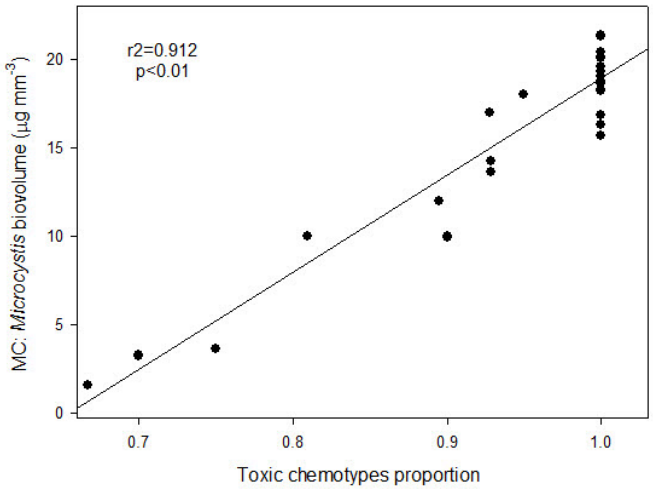

Figure 5.4. Correlation between the proportion of toxigenic chemotypes and microcystin:*Microcystis* biovolume ratios in Valmayor reservoir. Points correspond to discrete depth water samples

5.4.4. Sedimentation study

Together with the reappearance of *Microcystis* colonies after the clear phase, sedimentation traps were installed at the pelagic sampling point on October 15th to track and quantify the sedimentation losses of each *Microcystis* chemotype. Colonies collected from the sedimentation traps showed in general intense red autofluorescence when microscopically inspected and generally provided satisfactory mass spectra in the chemotyping analysis. A few colonies however, (n=18, table 5.2) provided putatively incomplete mass spectra, showing no coincidence with any of the peptide patterns observed in waterborne or benthic populations.

For the sedimentation study, we focused on the most abundant chemotypes during the last month before mixing, namely chemotypes CT5, CT11, CT14 and CT16. In particular, the phenology of these chemotypes (Fig. 5.5) was characterized by a marked dominance of CT11 during the first two weeks, accounting for up to 39% of the population, followed by a later dramatic decline and disappearance from the water column. The other chemotypes presented relative abundances between 3-10% that fluctuated without clear trends during this period

until Nov 24th, when cyanobacterial densities dramatically decreased and only CT5 could be found in the water column.

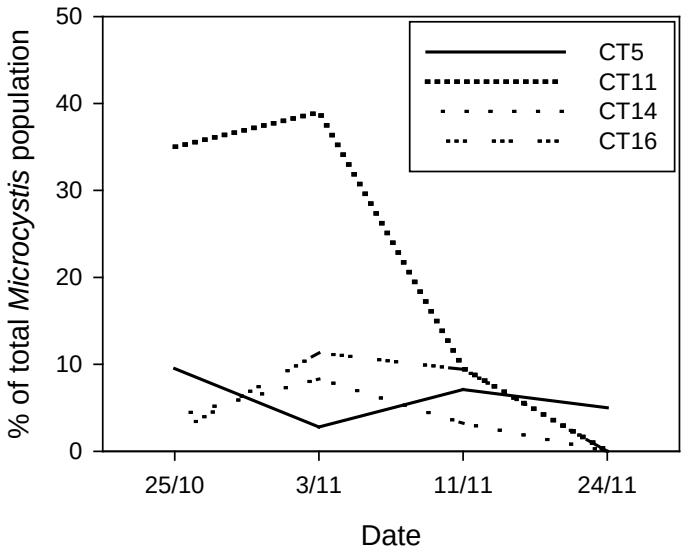

Figure 5.5. Relative abundances of the four major *Microcystis* chemotypes (CT5, CT11, CT14 and CT16) in the whole water column during the sedimentation study period.

Although the *Microcystis* whole-population showed moderate sedimentation rates (0.01 –0.12 d^{-1}), the dominant chemotypes displayed significantly higher sedimentation and net growth rates ($p<0.01$; one-way ANOVA, fig. 5.6). During the first sedimentation period, all major chemotypes displayed a rather similar behavior with marginal sedimentation rates and high net growth rates. In later sedimentation periods, CT5, CT14 and CT16 exhibited lower pelagic net growth rates and higher sedimentation rates (although significantly different among chemotypes, fig. 5.6), denoting that sedimentation was the main loss process in these subpopulations. Interestingly, CT11, the dominant chemotype at the beginning of the sedimentation study, showed a completely different behavior. After a period of 2 weeks dominating the community (accounting for 35% and 39% of the colonies in October 25th and November 3rd respectively; fig. 5.5), a sharp decrease in the abundances of this chemotype was evident. Strikingly, no colonies with the characteristic oligopeptidic signature of CT11 could be found in the sediment traps. To evaluate whether this observation was an analytical artifact, we explored the possibility of a loss in the oligopeptidic pattern caused by suboptimal physiological conditions (Agha et al. 2013) during sedimentation, which would mask the settling fluxes of CT11. However, we ruled out this possibility, as colonies providing unknown

peptide patterns in the traps, not consistent with any observed benthic or pelagic chemotype (see above), presented not only a lack of CT11-specific signals, but also one or more additional signals corresponding to oligopeptides not synthesized by CT11. As a result of the absence of sedimentation, net growth estimates corresponding to CT11 showed negative values, indicating that pelagic loss processes in the case of this chemotype were of a different nature, but not due to sedimentation.

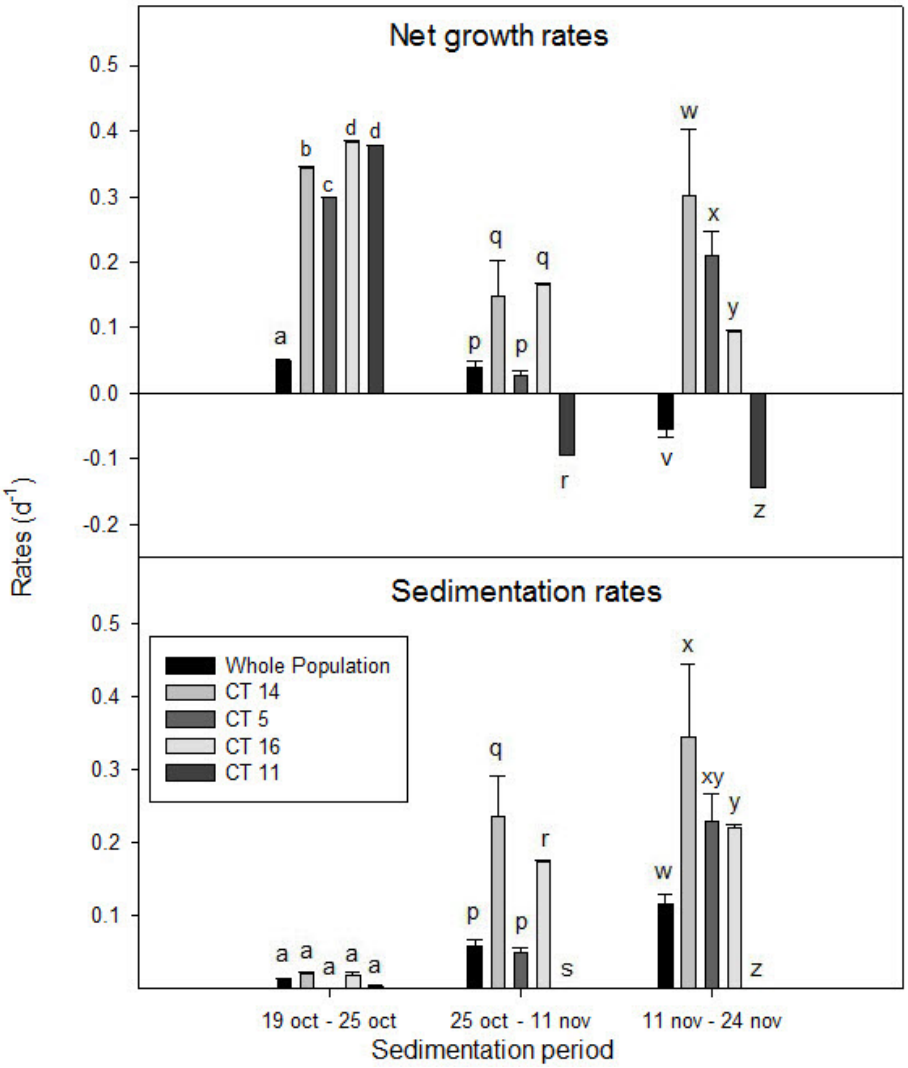

Figure 5.6. Net growth rates and sedimentation rates of the whole *Microcystis* population and the four major *Microcystis* chemotypes during the last weeks before mixing in Valmayor reservoir. Sedimentation rates were calculated based on the analysis of sedimentation traps. Net growth rates were estimated considering variations in chemotypes waterborne cell densities among sampling dates and their respective sedimentation rates during each period. Letters represent significant differences among chemotypes specific rates at the same date (one-way ANOVA, post-hoc Holm-Sidak test; $p<0.05$)

5.5. Discussion

The coexistence of conspecific lineages within a population is a common feature in many microorganisms. In cyanobacteria, population subdivision has been observed in picocyanobacteria *Prochlorococcus* and *Synechococcus* which show niche partitioning among ecotypes (Johnson et al. 2006, Becker et al. 2007). In more complex cyanobacteria, coexisting subpopulations have been recognized with regard to the production of non-ribosomal peptides in genera *Microcystis* (Welker et al. 2007), *Planktothrix* (Rohrlack et al. 2008) and *Nodularia* (Fewer et al. 2009). Classic botanical criteria used for the classification of cyanobacterial species are unsuitable to discriminate among chemically dissimilar strains within a population, as supported in this study by the lack of correspondence between chemotype affiliation and *Microcystis* morphotypes. Similarly, phylogenetic classification shows little or no agreement with the widely variable oligopeptide compositions (Neilan et al. 1997, Rounge et al. 2010). Instead, the ability of strains to produce different sets of peptides is determined, first, by the presence of the respective biosynthetic gene cluster and second, by relaxed substrate specificities of the NRPS enzymes that lead to the synthesis of different peptide variants. The erratic distribution of NRPS gene clusters among strains results in the coexistence of different chemotypes in the population.

This study reveals the existence of a highly diverse community of *Microcystis* chemotypes in Valmayor reservoir, which are subject to marked dynamic changes in their composition, leading to a continuous succession of chemotypes along the season. Coincident with previous studies in nutrient-rich water bodies (Welker et al. 2007), the high diversity of chemotypes in Valmayor reservoir at a given time point was characterized by the presence of a few abundant dominating subpopulations and a remarkable number of chemotypes coexisting at low cell densities. The disappearance of abundant chemotypes throughout the season gave rise to a sharp increase in other chemotypes. The resulting succession of chemotypes along the season showed to critically control microcystin contents in the population as a result of fluctuations in the proportion of toxigenic and non-toxigenic subpopulations. This is of special relevance when addressing uncertainties derived from the wide variations in toxin concentrations typically observed during bloom events. The inclusion of chemotypical approaches using MALDI-TOF MS in the development of monitoring strategies of cyanobacterial blooms has in fact been recently proposed (Agha et al. 2012) and constitutes a rapid and simple alternative to quantify the proportion of toxigenic clones within a population,

compared to genetic approaches based on the amplification of genes responsible for toxin production.

The temporal dynamics of *Microcystis* chemotypes showed to be synchronic in both horizontal and vertical gradients. No clear differences in chemotype compositions among pelagic and littoral areas were evident. Similarly, no correlation between depth and the distribution of individual *Microcystis* chemotypes in the water column could be found. The uniform vertical distribution of chemotypes is attributable to buoyancy control and consequent daily vertical migration of *Microcystis* colonies, but contrasts with the depth-dependent distribution of *Planktothrix* chemotypes observed by Rohrlack and coworkers (2008). In that case, however, oligopeptide chemotypes and gas vesicles genotypes (*sensu* Beard et al. 2000) showed a full agreement and it seems hence difficult to ascertain that oligopeptides are effectively involved in the vertical distribution of chemotypes. Despite the homogenous distribution of *Microcystis* chemotypes in both vertical and horizontal gradients, chemotype compositions in the water column and sediments were markedly different. Although studies on *Microcystis* recruitment showed that pelagic re-invasion from the sediment is triggered by physical and rather unspecific factors such as temperature (e.g. Reynolds et al. 1981, Latour et al. 2004), light (e.g. Rengefors et al. 2004, Schoene et al. 2010) and sediment resuspension and bioturbation (e.g. Stahl-Delbanco & Hansson 2002, Verspagen et al. 2005), positive selection of *mcy+ Microcystis* genotypes during recruitment has also been evidenced (Schoene et al. 2010, Misson et al. 2011). The latter indicates that benthic recruitment in genus *Microcystis,* instead of being an unspecific process solely mediated by physical factors*,* may act as a selective process influencing the composition of strains in pelagic summer populations. Hence, although a putative involvement of oligopeptides (not only MCs) in the successful re-invasion of the water column remains to be examined, the segregation of chemotypical subpopulations among benthic and pelagic habitats indicates that individual chemotypes go through shifts in their annual life-cycle with different outcomes and strongly suggest differences in their interaction with the environment.

The succession of chemotypes in Valmayor reservoir was generally well reflected by differential participation of chemotypes in sedimentation losses at each trap sampling date (Fig. 5.6). Recorded whole-population sedimentation rates are in the range of other studies (Reynolds & Rogers 1976, Fallon & Brock 1980, Verspagen et al. 2005), displaying sedimentation maxima toward the end of the season. However, sedimentation rates of the

dominant chemotypes greatly exceeded whole population rates, especially during the last two weeks before mixing. Strikingly, sedimentation rates of CT14 showed maximum values of 0.34±0.1 d^{-1}, corresponding to sinking velocities of 6.8±2m d^{-1}, which are, to our knowledge, the highest settling rates reported for *Microcystis*. However, while reported *Microcystis* sedimentation rates typically refer to whole-population rates, no studies specifically addressed the settling patterns of subspecific populations. The moderate rates recorded at the whole population level and the conspicuously high settling rates of some dominant chemotypes indicate that these subpopulations are the ones mostly contributing to *Microcystis* losses at a given time-point and suggest that chemotypes represent subpopulations that undergo sedimentation losses with dissimilar severity. Carbohydrate accumulation and reduced respiration under low temperatures (Visser et al. 1995), as well as the attachment of sediment particles to the mucilage (Verspagen et al. 2006) have been proposed as possible mechanisms resulting in loss of buoyancy and responsible for *Microcystis* sedimentation. Although such physical and rather unspecific processes may result in unspecific settling, for example during autumnal temperature decrease, these mechanisms alone seem insufficient to explain the differences in sedimentation patterns among chemotypes. Instead, the significant differences in net growth and sedimentation rates among subpopulations suggest that individual chemotypes present dissimilar physiological conditions at a given time point that result in an asynchronous proliferation of chemotypes along the season, leading to a continuous succession of subpopulations. Besides, whereas *Microcystis* losses were mostly caused by sedimentation, our data also indicate that individual chemotypes (in this study, CT11) may undergo massive chemotype-specific loss processes unrelated to sedimentation. Although the nonappearance of CT11 colonies in the traps evidences the absence of substantial sedimentation losses, the nature of such alternative loss process(es) remains unclear. However, the different participation in sedimentation losses among chemotypes, together with the existence of chemotype-specific loss processes, further support the idea that *Microcystis* chemotypes represent ecologically distinct subpopulations that interact in different ways with their environment.

Individual chemotypes in their environment can be controlled either by bottom-up or top-down mechanisms. Bottom-up control processes are mediated by resource competition among chemotypes (e.g. light, nutrients). However, no significant correlation between temperature, light availability and macronutrients and the prevalence of particular chemotypes could be found. Similar results were obtained elsewhere for genus *Planktothrix*

(Rohrlack et al. 2008), raising questions about the existence of bottom-up mechanisms effectively controlling chemotypical subpopulations. Conversely, top-down mechanisms shaping the dynamics of chemotypes constitute a more plausible explanation: Rohrlack and Sonstebø (2011) could demonstrate the existence of chemotype-selective chytrid infection in genus *Planktothrix* and proposed an evolutionary scenario in which *Planktothrix* chemotypes constitute functional evolutionary units that co-evolve with parasites by reciprocal adaptations. According to this model, *Planktothrix* populations are caught in a close relationship with parasites that results in an evolutionary arms race. As parasites typically display higher evolutionary rates, hosts would be strongly selected toward diversification (De Bruin et al. 2008), resulting in the subdivision of *Planktothrix* into different chemotypes. In fact, recently, Rohrlack and coworkers (2013) convincingly showed by knockout mutagenesis that oligopeptides microcystins, anabaenopeptins and microviridins could play a major role as antichytrid compounds in genus *Planktothrix*. Although formulated for genus *Planktothrix*, this hypothesis could in principle explain the metabolic diversification of *Microcystis* into oligopeptide-based subpopulations. Rather than maintaining an ideal genotype, *Microcystis* may profit from preserving a wide array of chemotypes and thereby hamper the ability of parasites to optimally exploit the whole population. In our study, the dramatic decline of the most abundant chemotype toward the end of the season (CT11, accounting for 35-39% of the total *Microcystis* population) and its subsequent absence in the sediment traps is compatible with the idea of chemotype-selective epidemics: According to the kill-the-winner concept (Thingstad & Lignell 1997), the most abundant host populations are the ones mostly affected by pathogenic losses, resulting in the maintenance of a highly diverse community despite the existence of competitive differences among chemotypes.

Existing research on naturally occurring oligopeptide chemotypes shows wide differences in terms of diversity. Welker and coworkers (2007) found 37 distinct *Microcystis* chemotypes in hypereutrophic Brno reservoir (Czech Rep.) in a period of 5 months, while we identified 53 *Microcystis* chemotypes in eutrophic Valmayor reservoir in the same period. In another study, 15 different *Planktothrix* chemotypes could be isolated from a single plankton net sample taken from lake Maxsee, Germany (Welker et al. 2004). This contrasts with the remarkably lower diversity of *Planktothrix* chemotypes in the Norwegian oligotrophic lake Steinsfjorden, where only 4 chemotypes were observed over more than 3 decades (Rohrlack et al. 2008). In the frame of the proposed evolutionary scenario, these marked differences in chemotype diversity, although observed for different genera, could be related to the trophic

state of the different ecosystems. In contrast to oligotrophic water bodies, where species co-existence and plankton diversity is promoted, highly eutrophic systems typically display lower specific diversity and are usually dominated by oligo- or monospecific cyanobacterial communities, represented by one or a few dominant taxa (Rasconi et al. 2012) that constitute rather homogeneous host communities that can be expected to be more readily exploited by the pool of co-existing parasites. We hence hypothesize that monospecific cyanobacterial populations, typically occurring in eutrophic systems, undergo intense selective pressure by parasites and pathogens and therefore, are more strongly selected toward diversification (De Bruin et al. 2008), resulting in populations comprised by a more diverse community of chemotypes. Future work will explore this hypothesis, which, if confirmed, would further consolidate the idea that population subdivision into oligopeptide chemotypes embodies a new dimension in cyanobacterial biodiversity.

5.6. Conclusion

In conclusion, this study revealed the co-existence of highly diverse *Microcystis* oligopeptide-based subpopulations in Valmayor reservoir, whose dynamics proved to be responsible for the fluctuations in the average toxicity of the population. We demonstrate that the succession of chemotypes results from temporal differences in chemotype-specific net growth and losses. Although such losses generally consist in sedimentation of *Microcystis* colonies, we provide evidence for alternative loss process other than sedimentation selectively affecting individual chemotypes. Although the nature of such processes remains unclear, the existence of extensive chemotype-selective loss processes, the temporal differences in terms of sedimentation during pelagic growth, as well as the partitioning of subpopulations in benthic and pelagic habitats, clearly demonstrate that *Microcystis* chemotypes interact differently with their environment, display dissimilar ecological traits and have therefore to be considered ecologically significant subspecific units.

5.7. Acknowledgements

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6. Multi-scale strategies for the monitoring of cyanobacteria: Reducing the sources of uncertainty

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Multi-scale strategies for the monitoring of freshwater cyanobacteria: Reducing the sources of uncertainty

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ABSTRACT

Cyanobacterial blooms are a frequent phenomenon in eutrophic freshwaters worldwide and are considered potential hazards to ecosystems and human health. Monitoring strategies based on conventional sampling often fail to cover the marked spatial and temporal variations in cyanobacterial distribution and fluctuating toxin concentrations inherent to cyanobacterial blooms. To deal with these problems, we employed a multi-scale approach for the study of a massive Microcystis bloom in Tajo River (Spain) utilizing 1) remote sensing techniques, 2) conventional water sampling and 3) analysis of chemotypical subpopulations. Tajo River at the study area is influenced by high temperatures waters diverted upstream from a nuclear power plant, the presence of a dam downstream and a high nutrient load, which provide optimal conditions for massive cyanobacterial proliferation. MERIS imagery revealed high Chl-a concentrations that rarely fell below 20 $\mu g\ L^{-1}$ and moderate spatio-temporal variations throughout the study period (March–November 2009). Although the phytoplanktonic community was generally dominated by Microcystis, sampling points highly differed in cyanobacterial abundance and community composition. Microcystin (MC) concentrations were highly heterogeneous, varying up to 3 orders of magnitude among sampling points, exceeding in some cases WHO guideline values for drinking and also for recreational waters. The analysis of single colonies by MALDI-TOF MS revealed differences in the proportion of MC-producing colonies among points. The proportion of toxic colonies showed a highly significant linear correlation with total MC biovolume ratio ($r^2 = 0.9$; $p < 0.001$), evidencing that the variability in toxin concentrations can be efficiently addressed by simple analysis of subpopulations. We propose implementing a multi-scale monitoring strategy that allows covering the spatiotemporal heterogeneities in both cyanobacterial distribution (remote sensing) and MC concentrations (subpopulation analysis) and thereby reduce the main sources of uncertainty in the assessment of the risks associated to bloom events.

6.1. Abstract

Cyanobacterial blooms are a frequent phenomenon in eutrophic freshwaters worldwide and are considered potential hazards to ecosystems and human health. Monitoring strategies based on conventional sampling often fail to cover the marked spatial and temporal variations in cyanobacterial distribution and fluctuating toxin concentrations inherent to cyanobacterial blooms. To deal with these problems, we employed a multi-scale approach for the study of a massive Microcystis bloom in Tajo River (Spain) utilizing 1) remote sensing techniques, 2) conventional water sampling and 3) analysis of chemotypical subpopulations. Tajo River at the study area is influenced by high temperatures waters diverted upstream from a nuclear power plant, the presence of a dam downstream and a high nutrient load, which provide optimal conditions for massive cyanobacterial proliferation. MERIS imagery revealed high Chl-a concentrations that rarely fell below 20 mg L^{-1} and moderate spatiotemporal variations throughout the study period (March - November 2009). Although the phytoplanktonic community was generally dominated by Microcystis, sampling points highly differed in cyanobacterial abundance and community composition. Microcystin (MC) concentrations were highly heterogeneous, varying up to 3 orders of magnitude among sampling points, exceeding in some cases WHO guideline values for drinking and also for recreational waters. The analysis of single colonies by MALDI-TOF MS revealed differences in the proportion of MC-producing colonies among points. The proportion of toxic colonies showed a highly significant linear correlation with total MC: biovolume ratio ($r^2 = 0.9$; $p<0.001$), evidencing that the variability in toxin concentrations can be efficiently addressed by simple analysis of subpopulations. We propose implementing a multi-scale monitoring strategy that allows covering the spatiotemporal heterogeneities in both cyanobacterial distribution (remote sensing) and MC concentrations (subpopulation analysis) and thereby reduce the main sources of uncertainty in the assessment of the risks associated to bloom events.

6.2. Introduction

As a result of increasing eutrophication, cyanobacterial blooms are growing in frequency and intensity in many inland water bodies worldwide (Paerl et al. 2011). Such blooms are of increasing concern for environmental agencies, water authorities and health organizations, as they severely disrupt the ecosystem functioning and many cyanobacterial species are able to produce a variety of metabolites which are toxic to both human and animal. Among these toxic metabolites, microcystins (MCs) are the most frequent and widespread toxin produced by freshwater cyanobacteria (Chorus et al. 2000). MCs are potent inhibitors of protein phosphatases. Gastroenteritic and hepatic diseases, tumor promoting activity and irritant reactions have also been linked with their presence (Falconer, 2005). Among the potentially toxic cyanobacteria, Microcystis has been pointed out as the most frequent bloom-forming genus in Mediterranean latitudes (Cook et al., 2004).

Cyanobacterial distribution during bloom episodes displays marked spatial and temporal variations, which represent one of the main hindrances when monitoring the occurrence and extent of these phenomena. Conventional water sampling has so far been crucial for both basic and applied research on cyanobacteria. However, spatial and temporal resolution of conventional sampling is often insufficient to report changes in phytoplankton biomass, especially during bloom conditions, when the variability in phytoplankton density is particularly high. Remote sensing is an emerging technique suitable to overcome these limitations and improve surveillance on cyanobacterial proliferation by providing truly synoptic views of their heterogeneous spatial distribution (Simis et al. 2005, Kutser et al. 2006). Air- and space-borne sensors allow the monitoring of the distribution of cyanobacteria in freshwater ecosystems over wide geographical areas. This geographical coverage has been shown to be unapproachable by conventional sampling alone (e.g. Kutser, 2004).

The detection of cyanobacterial biomass by remote sensing is based on the absorption feature of the photosynthetic pigment phycocyanin (PC), present in cyanobacteria in considerable concentrations. Other spectral features are commonly used, such as Chl-a absorption at 440 and 675 nm, carotenoid absorption at 495 nm and scattering at the green and near-infrared (NIR) regions (Kutser 2009and references within). The MERIS sensor (MEdium Resolution Imaging Spectrometer) on board of the ENVISAT satellite allows the combined detection of PC and Chl-a, with an acceptable spatial resolution and a satisfactory

signal-to-noise ratio (Simis et al. 2005). It is the first sensor providing an adequate combination of narrow wavebands to target both Chl-a and PC. Algorithms based on ratios of reflectance in the red and NIR spectral region can be used to retrieve photosynthetic pigment concentrations in turbid inland waters (Simis et al. 2005, Guanter et al. 2009, Kutser et al. 2009).

In addition to cyanobacterial patchy spatiotemporal distribution, MC concentrations also display marked seasonal and spatial variations (Carrasco et al., 2006), which represent a second source of uncertainty that hinders an accurate assessment of the risks associated to cyanobacterial blooms. Variations in toxin concentrations cannot be explained by physiological regulations of MC synthesis at the individual level, as these are reported to range only within a factor of 3 (Orr & Jones, 1998). Instead, changes in MC concentrations are more likely the result of shifts in the composition of toxic (MC-producing) and non-toxic individuals in the population (Kardinaal et al. 2007). Several studies have employed genetic approaches to investigate the fluctuations in the proportion of toxic and non-toxic cells (Kurmayer & Kutzenberger 2003, Janse et al. 2005, Davis et al. 2009). An alternative emerging approach relies in the direct detection of secondary metabolites in single colonies/filaments by MALDI-TOF MS (Matrix- Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry). Besides MCs, cyanobacteria produce a wide range of secondary metabolites (usually oligopeptides) that give individual clones a characterizing peptide fingerprint. The analysis of oligopeptide compositions enables the discrimination between toxic and non-toxic colonies, as well as the identification of distinct oligopeptide chemotypes coexisting in the population (Fastner et al. 2001, Welker et al. 2004). The relative abundance of chemotypical subpopulations in natural systems can be highly dynamic (Rohrlack et al. 2008) and shifts in their composition may cause a high variability in the whole-population properties, including its toxicity.

Given the heterogeneity and variability in both cyanobacterial distribution and MC concentrations inherent to cyanobacterial blooms, a combined approach utilizing techniques at three different levels of detail may provide a potentially effective combination of strategies for the monitoring of freshwater ecosystems. The objectives of this study are 1) to use remote sensing signatures to monitor cyanobacterial uneven distribution in wide geographical areas and identify differences in the course of the bloom along the river-reservoir course, 2) to evaluate the analysis of chemotypical subpopulations as a proxy of the relative toxicity of the cyanobacterial population and 3) to design a combined monitoring strategy utilizing

conventional field sampling in combination with remote sensing and subpopulation analysis to address and reduce the main sources of uncertainty inherent to cyanobacterial blooms, i.e. the heterogeneity and variability in both cyanobacterial distribution and MC concentrations.

6.2. Materials and methods

6.2.1. Site description

Tajo River is the longest river in the Iberian Peninsula with the third biggest catchment area in Spain. It flows from east to west along 910 km. The study area is located in the mid-low course of the Tajo river, namely between Alcántara reservoir (one of the biggest water reservoirs in Spain, currently used for hydroelectric production, drinking and recreational uses) and about 40 km upstream from the reservoir (Fig. 6.1). Sampling was carried out in 4 locations. ALC1 corresponds to a point in the river course located within the Monfragüe National Park, 25 km downstream of the Almaraz nuclear power plant. ALC2 is located 8 km downstream the Monfragüe National Park and 15 km upstream the Alcántara reservoir. ALC3 and ALC4 correspond to different locations within the reservoir.

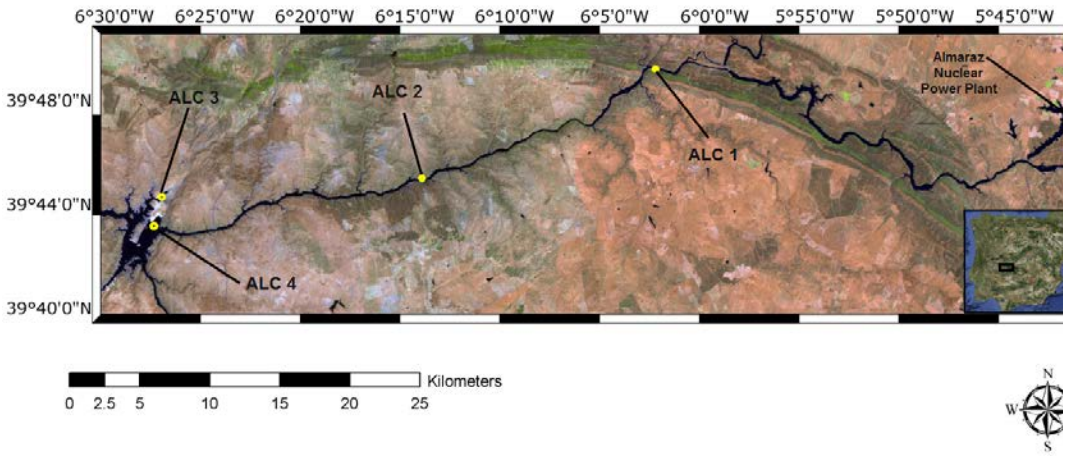

Figure 6.1. Map of the study area at Tajo river and Alcántara reservoir.

6.2.2. Remote sensing

Chl-a and PC concentrations were monitored by the MERIS (MEdium Resolution Imaging Spectrometer) sensor, on board the European Space Agency's ENVISAT. MERIS allows the acquisition of images every three days in 15 spectral bands, ideally positioned for water, land and atmospheric correction applications, with a spatial resolution of 300 m. Semianalytical algorithms were employed for Chl-a and PC retrieval (Dominguez et al. 2011).

These algorithms use normalized band-ratios of water-leaving reflectance: (R560 nm - R420 nm)/(R560 nm + R420 nm) for low Chl-a concentrations; (R705 nm - R665 nm)/(R705 nm + R665 nm) for high Chl-a and (R705 nm - R620 nm)/(R705 nm + R620 nm) for PC concentrations. Geometric correction of satellite images was based on ground control points, using the standard image of the study area with root mean squared errors (RMSEs) below 0.2 (Dominguez et al. 2009). Water/land pixels were discriminated as follows: 1) For river areas wider than 300m, a simple threshold in the near-infrared band was selected (Engman & Gurney 1991, Dominguez & Peña 1999), and 2) for river areas narrower than 300m, a spectral mixture analysis was used to estimate the proportion of each pixel covered by known cover types (water or vegetation). Thereby the spectral influence of vegetation in the water pixel could be erased.

6.2.3. Water sampling

Water sampling was carried out on July 15th, after a dramatic increase in pigment concentrations after MERIS signatures was recorded, which was also confirmed by water authorities by in situ visual inspection. Vertical profiles of temperature, Chl-a and dissolved oxygen were performed in the four designated sampling points using a YSI 6920 multiparametric probe. Water samples were collected at 3 depths with a 5 L watersampler (Uwitec). Microcystis colonies from the whole water column were collected with a 20 mm mesh plankton net (Uwitec) at each point. Due to access constraints, ALC1 samples consisted only of an integrated sample of epilimnetic water at 3 depths, namely 0.5 m, 3 m and 7 m. All samples were stored dark at 4°C during transport. Chl-a was quantified by spectrophotometric measurement at 665 nm (Shimadzu Multispec-1501) after methanolic extraction. Algal group composition was obtained as an average of three measurements on a benchtop fluorometer (Moldaenke BBE Algae Analyser). An aliquot of the sample was immediately fixed in acid Lugol's solution before taxonomic identification and quantification. Cell counting and biovolume calculations were carried out under a Leica DM IL inverted microscope (Leica Microsystems) after sedimentation following Utermöhl's method (1958). After GF/F filtration of another aliquot, sestonic MC was extracted twice by sonication into methanol (90%), the extracts were concentrated under vacuum and prepared for LC-MS/MS analysis.

6.2.4. MC analysis by ESI LC/MS-MS

MC analysis was performed on a Varian 500 Ion Trap Mass Spectrometer supported by two Varian 212 LC chromatographic pumps and a 410 autosampler. Chromatographic

separation of MC-LR, MC-YR and MC-RR was achieved using a Pursuit C-18 2 x 150 mm column and mobile phases MilliQ water (A) and methanol (B), both acidified with 0.2% formic acid and buffered with 2mM ammonium formate. Gradient (%A/%B) applied was 60/40 to 0/100 in 18 min. The quantification of MCs contained in the various samples was made by injecting commercial pure standard solutions (DHI, Denmark) to plot calibration curves.

6.2.5. Oligopeptide analysis and spectral data processing

Individual *Microcystis* colonies from both water (discrete depths) and net samples (integrated water column) were collected with disposable glass capillaries and placed in 0.2 ml Eppendorf tubes and allowed to dry at room temperature during 2 h. Ten mL of an acetonitrile, ethanol and water (1:1:1) extractant solution, acidified with 0.03% (v/v) trifluoroacetic acid (TFA) were then added to each sample. Samples were introduced into liquid nitrogen for 2 h to induce cell lysis and stored at -80°C until analyzed. For MS analysis, 0.5 mL of individual samples analyte solution were placed onto the matrix spots of a Prespotted AnchorChip (PAC 284/96 HCCA, Bruker Daltonics) containing α-cyano-4-hydroxycinnamic acid as co-crystallizing matrix. Spots were allowed to dry at room temperature and washed with ammonium phosphate (10 mM) during 5 s. MS analysis was performed on a Bruker Reflex MALDI mass spectrometer equipped with a Time of Flight (TOF) detector on positive ion detection mode and reflector mode. Acceleration and mirror voltage were set to 25 kV and 26.3 kV, respectively. Mass spectra were accumulated from 1000 laser pulses scanning the entire sample spot for a mass range of 500-2000 Da. Resulting mass spectra were processed using Bruker FlexAnalysis 3.0 software (Bruker Daltonics). SNAP algorithm (Bruker Daltonics) was used for mass determination. The identification of putative oligopeptides required matching of the mass/charge ratio (m/z) with previously described oligopeptides (Welker et al. 2006), the presence of predicted isotope patterns and a signal-to-noise ratio above 6. Chemotypes identification was performed by clustering analysis of the mass spectral oligopeptide compositions performing an Ascending Hierarchical Classification (AHC). AHC analyzed dissimilarities using Euclidean distances and applying Ward's agglomeration method. Optimal classification was considered when intracluster variability was zero for all clusters (i.e. all samples in each cluster presented identical oligopeptide compositions).

6.3. Results

6.3.1. Pigment concentrations

Chl-a and PC concentrations were monitored on a weekly basis (every 3 days during the first months) from March until November by remote sensing (see Video 1 in suppl. Material doi:10.1016/j.watres.2012.03.005). Chl-a concentrations above 20 mg L^{-1} could be observed throughout all the study period along the river course, indicating a persistent presence of an abundant phytoplanktonic community. River sampling points ALC1 and ALC2 showed marked fluctuations along time, especially in the summer period, in which both PC and Chl-a fluctuated jointly, denoting the dominance of cyanobacterial populations in these points (Fig. 5.2). ALC1 presented 3 peaks of Chl-a and PC concentrations. A first peak occurred at the beginning of summer (June), a second peak at midsummer (mid July, reaching maximal PC concentrations) and a last peak in August. Similar less pronounced peaks could be observed at ALC2 (downstream location) with a few days lag. Slighter fluctuations in PC concentration in points ALC3 and ALC4 (reservoir sampling points) were observed, but Chl-a concentrations remained rather constant, ranging from 20 to 30 mg L^{-1}. Exceptionally, ALC3 presented a dramatic increase in PC and Chla concentrations during May, and a further increase later in November, which could also be observed in ALC4. Supplementary video related to this article can be found at doi:10.1016/j.watres.2012.03.005.

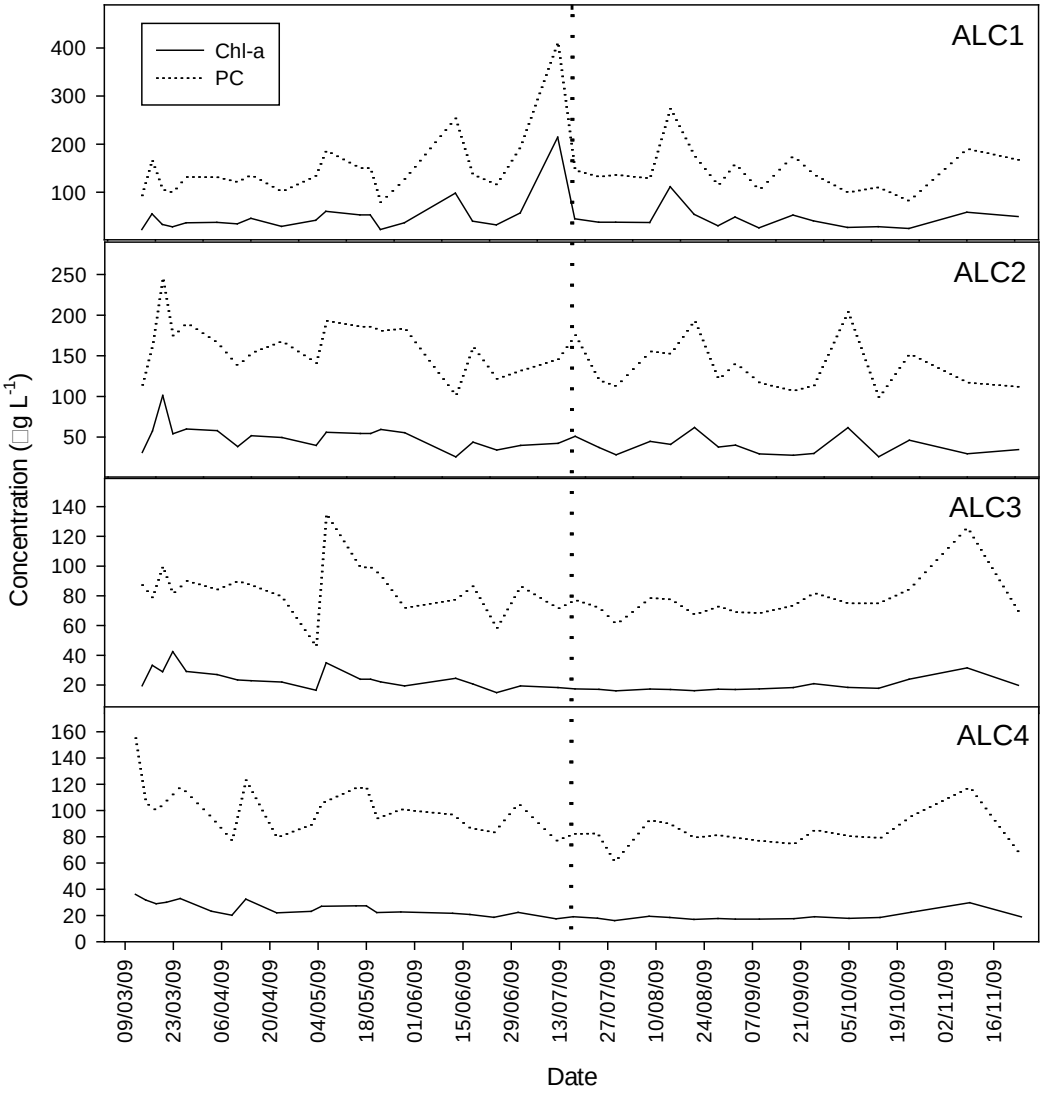

Figure 6.2 Temporal evolution of phycocyanin (PC) and chlorophyll-a (Chl-a) concentrations in each of the sampling points by MERIS sensor. Dashed line indicates the date of the field sampling. Note that locations show different scales.

6.3.2. Water sampling

Considering temperature and dissolved oxygen profiles, sampling points were thermally stratified at the moment of the water sampling (data not shown). Epilimnetic waters presented high temperatures (≈27 °C). Thermocline was found between 10 and 15 m depth in points ALC1, ALC2 and ALC4. Exceptionally ALC3, with a maximum depth of only 5 m, showed even higher water temperatures (≈29 °C) in the whole water column. Sampling points presented a high heterogeneity in terms of Chl-a concentration (Fig. 6.3) and species composition (Fig. 6.4).

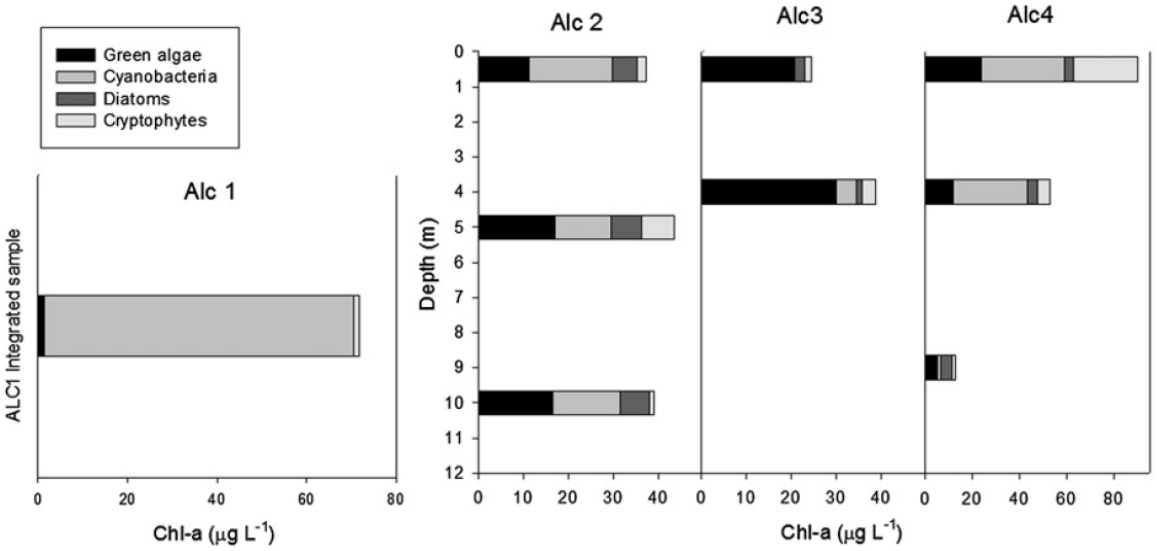

Figure 6.3. Chl-a concentrations attributable to the different algal groups by sampling point and depth.

ALC1 showed highest Chla concentrations in the water (71.83 mg L^{-1}) and visible scums starting to accumulate at the shore. The phytoplanktonic community was comprised exclusively by Microcystis aeruginosa and Microcystis flos aquae (6.7 x 10^4 cells mL^{-1}). ALC2 and ALC4 also presented high Chl-a concentrations ranging from 43.95 mg L^{-1} to 89.70 mg L^{-1} and a more diverse phytoplanktonic community, dominated by cyanobacteria, but with significant presence of green algae and diatoms. Cyanobacteria (3.0 x 10^4 and 4.7 x 10^4 cells mL^{-1} in ALC2 and ALC4, respectively) were dominated mainly by genus *Microcystis*, but also *Anabaena spiroides* and *Aphanizomenon gracile* were present in significant amounts in subsurface waters (Fig. 6.4). At these points, dense scums had accumulated at the shore. ALC3 presented dominance of green algae with a scarce and decaying cyanobacterial population, which presented only poor pigmentation. Although no scums were present at this point, wide green colored shore areas evidenced their prior presence.

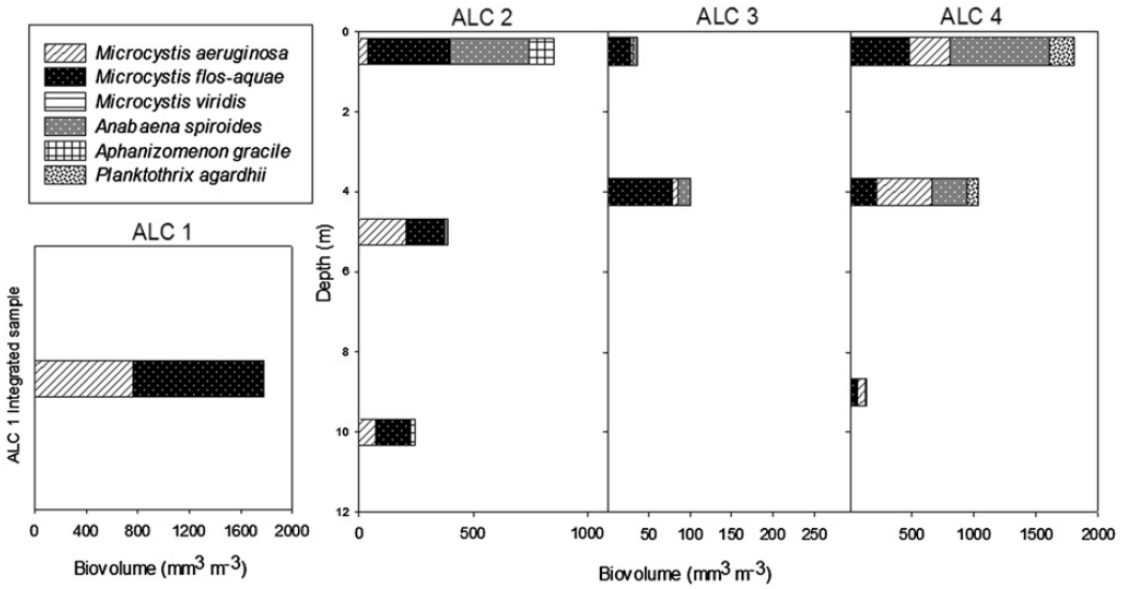

Figure 6.4. Biovolume of the identified cyanobacterial species in the studied sampling points and depths at Tajo river and Alcántara reservoir.

6.3.3. Microcystin concentrations

Along with the observed heterogeneity in Chl a concentrations and species composition along points, MC concentrations also presented a high spatial variability (Table 6.1). ALC1 presented the highest epilimnetic MC concentrations of all three analyzed MC variants (total 11.87 mg L^{-1}; integrated sample). ALC2 and ALC4, despite showing in some cases higher Chl-a concentrations, presented total MC concentrations one or two orders of magnitude below, consisting mainly of MC-RR and MC-LR and only traces of MC-YR. ALC3 showed only very low concentrations of MC-RR. Scums found at point ALC1 and ALC2 exhibited high MC concentrations (milligrams per liter), while scum at ALC 4 showed total MC concentration 2 orders of magnitude below and a slightly different MC variants shares (data not shown).

Sampling point	Depth (m)	MC-RR (μg L^{-1})	MC-YR (μg L^{-1})	MC-LR (μg L^{-1})	Total MC (μg L^{-1})
ALC1	0–8 (integrated)	8.6	1.68	1.59	11.87
ALC2	0.5	1.2	0.06	0.41	1.67
	5	0.5	Nd	0.12	0.62
	10	0.25	0.04	0.09	0.38
ALC3	0.5	0.09	Nd	Nd	0.09
	4	0.03	Nd	Nd	0.03
ALC4	0.5	1.47	0.06	1.45	2.98
	4	0.82	Nd	0.41	1.23
	9	0.12	Nd	0.01	0.13

Table 6.1. Sestonic microcystin concentrations by sampling point and depth.

6.3.4. Chemotype composition

The analysis of oligopeptide compositions of single colonies of Microcystis by MALDI-TOF MS revealed the presence of aeruginosin 101 ($[M + H]^+$ m/z = 643.3), cyanopeptolin 1006A ($[M + H]^+$ m/z = 1007.5) and microginin FR5 ($[M + H]^+$ m/z = 726.4), as well as several microcystin variants, namely MC-LR ($[M + H]^+$ m/z = 995.6), MC-YR ($[M + H]^+$ m/z = 1045.5) and MC-RR ($[M + H]^+$ m/z = 1038.6; Table 6.2). Ascending hierarchical classification of the obtained mass spectra resulted in the identification of 12 distinct Microcystis chemotypes. However, only a few of them presented high relative abundances in any sampling point and/or depth. The relative abundances of chemotypes along the integrated study points (i.e. net samples) are shown in Table 6.2. Points ALC1 and ALC2 were mainly dominated by chemotypes 1 and 2, the most frequent MC-producing chemotypes, while ALC 3 was dominated by chemotype 12, the most abundant non-MC-producing chemotype. Alternatively, ALC4 presented the highest diversity of oligopeptide-based subpopulations, with no clearly dominating chemotypes, besides chemotypes 2 (30%) and 12 (17%). To evaluate whether variations in MC concentrations could be explained by the composition of toxic and non-toxic subpopulation in the water column, chemotypes were grouped as toxic and non-toxic, according to their ability to produce MCs. Fig. 6.5a and b show the proportions of MC-producing and non-producing chemotypes for each point and depth. ALC1 was comprised exclusively by toxic chemotypes, while ALC2 and ALC4 presented significant proportions of non-toxic subpopulations, although toxin-producing chemotypes dominated the population. ALC3 exhibited toxic and non-toxic chemotypes in similar proportions. Accordingly, total MC concentrations were highest where toxic chemotypes dominated. In fact, scatter plots of the proportion of microcystin-producing colonies versus MC concentration: Microcystis biovolume ratio revealed a highly significant linear correlation (Fig. 6.6; $r^2 = 0.90$; $p < 0.001$).

Chemotype	Oligopeptide composition						Relative abundance[a]				Overall relative abundance[b] ($n = 250$)
	Aeruginosin 101 (643.3 Da)	Cyanopeptolin 1006A (1007.5 Da)	Microginin FR5 (726.4 Da)	MC-LR (995.6 Da)	MC-RR (1038.6 Da)	MC-YR (1045.5 Da)	ALC1 (%) $n = 28$	ALC2 (%) $n = 28$	ALC3 (%) $n = 17$	ALC4 (%) $n = 30$	
1		X		X	X	X	79	54	6	10	38.1%
2				X	X	X	7	32	–	30	19.6%
3	X	X		X	X	X	4	–	–	3	2.4%
4	X			X		X	–	–	24	–	3.6%
5	X			X	X	X	–	–	6	13	3.6%
6					X		3	–	–	3	4.4%
7				X		X	7	–	6		4.4%
8				X			–	–	12	10	5.6%
9				X	X		–	–	–	3	4.4%
10			X				–	–	–	3	7.2%
11	X		X				–	–	12	7	3.2%
12	X						–	15	35	17	10.8%

Table 6.2. Characterizing oligopeptide fingerprints of chemotypes and their relative abundances by sampling point. Note that chemotypes 10, 11 and 12 correspond to non-MC-producing chemotypes. [a] Relative abundances of colonies collected from integrated net sample in each sampling point. [b] Overall relative abundances refer to all analyzed colonies, including colonies collected at discrete depths and integrated net samples.

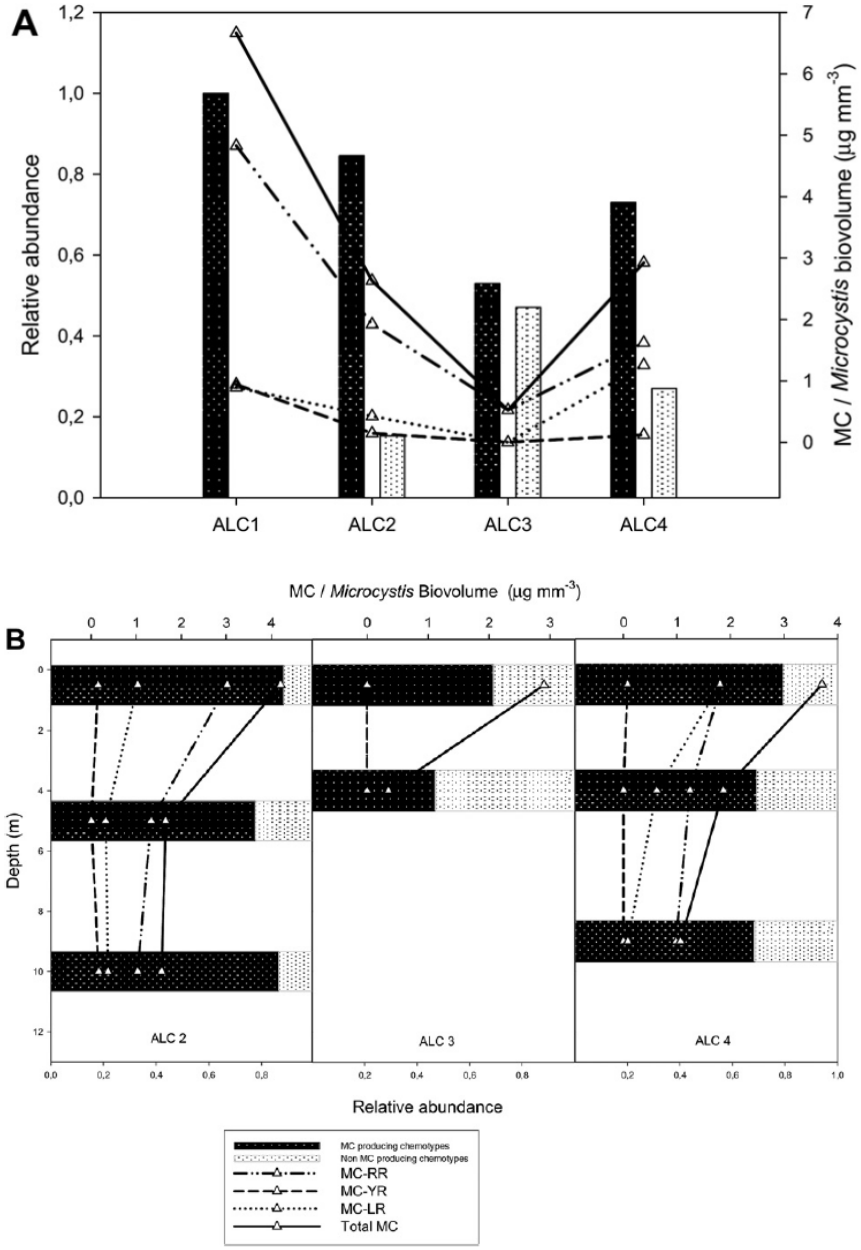

Figure 6.5. MC concentrations (lines) and proportions of MC-producing and non-producing colonies (bars) for each sampling point. A) Integrated samples; B) Discrete depth samples.

6.4. Discussion

In this work, we integrated different approaches at three different levels of detail (regional, local, subpopulational) for the study of cyanobacterial blooms. Besides conventional sampling, freshwater monitoring programs are starting to test new emerging techniques as an alternative to conventional sampling: Remote sensing, which has long been used in marine ecosystems (e.g. Roelfsema et al. 2006) due to its wide spatial coverage, is being increasingly incorporated in the monitoring of freshwaters (e.g. Matthews et al. 2010). However, this

technique presents several drawbacks in the context of routine monitoring: the need of high resolution sensors, the (still) high cost of the images and the strong dependence on meteorological conditions. An alternative approach, which intends to substitute conventional water sampling, is the use of unattended multiparametric probes in the water column that provide detailed real time data. Unattended sensors yield high temporal resolution, but they are not suitable to cope with spatial heterogeneity and their use is restricted by their high cost. Lastly, techniques at the subpopulation level focusing on the analysis of clones or groups of clones, such as oligopeptide-based chemotyping by MALDI-TOF MS (Welker et al. 2007), have shown to be useful to explain the variable toxicity of cyanobacterial populations, but at the moment they are mainly restricted to the field of basic research, while their application in routine monitoring programs is still marginal. We combined techniques at different levels in the study of an aquatic ecosystem with a remarkable tendency for massive and persistent cyanobacterial proliferation.

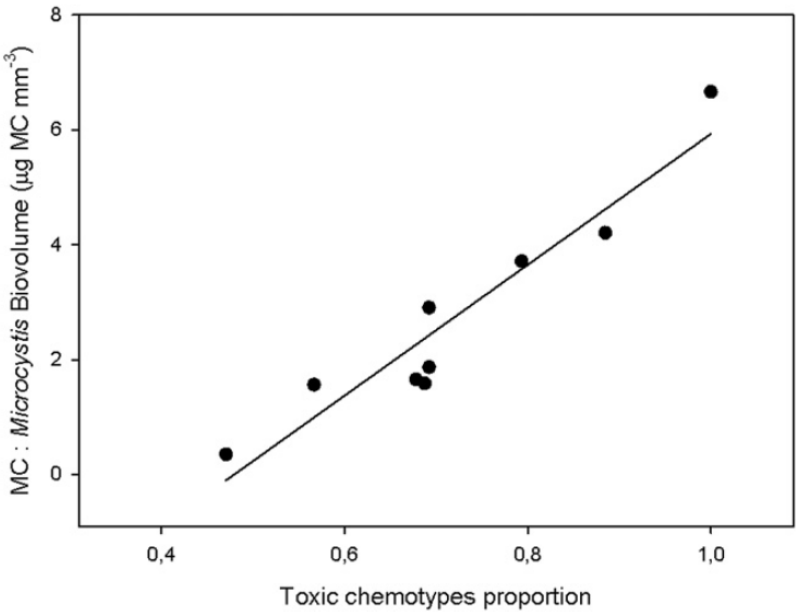

Figure 6.6. Relation between total MC concentrations and the proportion of toxin-producing Microcystis colonies. Total MC concentrations were determined by LC-MS/MS and are expressed as MC:Total Microcystis biovolume ratios ($r^2 = 0.90$; $p < 0.001$)

The study area in Tajo River is influenced by anthropogenic pressures which strongly modify the river natural conditions. High water temperatures favor the development of an abundant phytoplanktonic community, commonly dominated by cyanobacteria, which persisted over the whole study period. Seasonal variations are likely buffered by the constant input of high temperature water employed in the refrigeration of the nuclear power plant

reactors a few kilometers upstream. These waters may reach temperatures up to 40°C and are diverted into the river main course (CEDEX 1996). Consequently, epilimnetic waters in the studied area presented temperatures above 25°C, occasionally very close to 30°C. Additionally, the downstream dam at Alcántara reservoir exerts a critical effect in the hydrographic features of the river, minimizing the speed of the water flow and causing the river to behave as a lentic, rather than as a fluvial system. All this, together with the high nutrient concentrations observed in the studied sampling points (data not shown), provides ideal conditions for the massive proliferation of cyanobacterial blooms and its persistence over long periods of time, as evidenced by remote sensing data. Remote sensing was also capable of demonstrating an uneven distribution of the phytoplanktonic community along the river Tajo course and Alcántara reservoir. Horizontal distribution of cyanobacteria is known to be highly patchy (Welker et al. 2003) and represents one of the main issues when implementing monitoring strategies (e.g. Pobel et al. 2011). Therefore, monitoring of cyanobacterial blooms through MERIS imagery provides an effective solution to monitor large geographical areas and cope with such distributional heterogeneity. Despite its wide spatial and temporal coverage, MERIS presents some limitations in terms of spatiotemporal resolution. Wind, surface and subsurface currents, which have been pointed out as the most important factors influencing cyanobacterial distribution (Hotto et al. 2007, Moreno-Ostos et al. 2009, Qin et al. 2011), may dramatically affect the spatial distribution of cyanobacteria within a few hours. MERIS sensor - with a revisit time of 3 days - is unable to cope with such short term variability. Similarly, the effect of diurnal migration by buoyant cyanobacteria on their distribution cannot be addressed by routine remote sensing techniques (Hunter et al. 2008). MERIS spatial resolution is approximately 300 m, although suitable for reservoir sampling points (ALC3 and ALC4), is certainly low for fluvial systems (ALC1 and ALC2), limiting transversal resolution to one pixel in a wide river like Tajo River. However, water/land pixels can be discriminated using a simple threshold in the near-infrared (NIR) band. Terrestrial surface presents a high reflectance in the NIR spectral region that is rejected by water color algorithms, so that land signals are processed as errors (Kutser et al. 2009). Cyanobacterial scums are excluded in a similar way, as they look spectrally like terrestrial plants (Quibell 1992, Jupp et al. 1994). Currently, this represents an important limitation when monitoring cyanobacteria by remote sensing (Kutser 2004), although minor reflectance features of scums (local reflectance minima at 620 and 830 nm) may potentially be used to adapt pigment retrieval algorithms and discern scums from regular terrestrial signals (Dominguez, pers. comm.). Despite its limited spatial resolution,

MERIS Chl-a signatures at fluvial points (ALC1 and ALC2; interpolated from July 12th and July 17th images) are in good agreement with the Chl-a determinations carried out in the laboratory after sampling, with deviations from actual Chl-a concentrations of 11.8% and 7.8% in ALC1 and ALC2, respectively). Despite the discussed resolution issues, remote sensing is an extremely powerful tool in terms of both spatial and temporal coverage. Previous knowledge on the distribution of cyanobacterial biomass over long time periods can be easily obtained by remote sensing, enabling the definition of strategic sampling points, focusing on bloom "hotspots" and taking into account areas with increased risks of exposure (e.g. recreational areas). Thereby management decision-making can substantially be improved. Still, remote sensing alone fails to provide enough information (e.g. present cyanobacterial taxa, their vertical distribution and, naturally, toxin concentrations) for assessing the risk of exposure, making conventional water sampling indispensable.

Thanks to such conventional sampling, we observed a moderate heterogeneity in terms of community composition and cyanobacterial presence. Interestingly, sampling points showed features associated to different temporal stages of a cyanobacterial bloom: ALC1 can be associated to a point of initial proliferation, showing only sparse scums starting to accumulate at the shore and a single *Microcystis* population comprised almost exclusively by one toxic chemotype (chemotype 1). Accordingly, MC concentrations were highest at this point, which are usually observed at the onset of the bloom (Kardinaal et al. 2007; Welker et al. 2003). Alternatively, ALC2 and ALC4 represented further stages of the bloom: Scums had densely developed and accumulated at the shore, sometimes in an advanced stage of degradation (especially at ALC4). These sampling points hosted a more diverse community, still dominated by cyanobacteria, yet presenting significant amounts of other algal groups. Cyanobacterial community was comprised by other genera besides *Microcystis,* and chemotype diversity was higher at these points, with toxic chemotypes dominating the population, although showing a significant presence of non-toxic chemotypes. Lastly, ALC3 presented a senescent cyanobacterial population, exhibiting lowest cyanobacterial Chl-a concentrations and a community dominated by green algae. Toxic and non-toxic chemotypes comprised the scarce *Microcystis* population in similar proportions and only traces of MCs could be detected. These observations highlight the existence of marked spatial heterogeneities and suggest the presence of a gradient along the course of the river, that represents, not only different temporal stages of the bloom, but also different moments in the temporal succession of chemotypes, which exert critical variations in the whole-population

properties and, ultimately, its overall toxicity. Thus, the combined use of remote sensing with other monitoring approaches enables the detection of spatiotemporal variations in cyanobacterial distribution, which are typically a substantial source of uncertainty in the risk assessment.

In our study, WHO guideline value for microcystins in recreational waters of 10 mg L_{-1} (WHO, 2003) was exceeded at ALC1 (11.87 mg L^{-1}), while ALC2 and ALC4 (1.67 mg L^{-1} and 2.98 mg L^{-1}, respectively) presented total subsurface MC concentrations above the guideline value for drinking waters of 1 mg L^{-1} (WHO, 1998). Although the observed MC concentrations are within the range of other studies carried out in Spain (Barco et al. 2004, Aboal & Puig 2005), higher values have also been reported both in Spain (Carrasco et al. 2006) and elsewhere in Europe (Fastner et al. 1999, Cook et al. 2004). Microcystis blooms show typical average toxin contents of 0.2 pg per cell and 0.4 mg per mg Chl-a (Chorus et al. 2000). In our case, we observed maximum values for MC content per cell of 0.17 pg $cell^{-1}$and 0.18 mg mg^{-1} Chl-a at point ALC1, denoting a moderate toxicity of the cyanobacterial population at Tajo River. However, we observed differences of 2 orders of magnitude in toxin concentrations among sampling points. The existence of these dramatic differences stresses the need of implementing techniques capable of predicting toxin concentrations for the assessment of risks associated to cyanotoxin exposure. Current recommendations of the WHO, based on the monitoring of Chl-a concentrations and cyanobacterial cell counting of potentially toxic taxa seem insufficient to evaluate the toxicity of the bloom, as no obvious relation exists between biomass present (Chl-a concentration) and toxin concentration (Carrasco et al. 2006). Similarly, cyanobacterial cell densities observed in the studied points lead to underestimation of the exposure risk after WHO guidelines. This is not surprising, as almost all toxic cyanobacterial taxa present both toxic and non-toxic clones. Instead, we identified a highly significant linear relation between MC:*Microcystis* biovolume ratio and the proportion of MC-producing chemotypes, demonstrating that estimating the composition of toxic and non-toxic clones in the population by oligopeptide-based chemotyping is a good predictor of the fluctuations in MC concentrations. Total MC concentrations for a given proportion of toxic chemotypes are known to vary as a result of physiological or genetic differences in MC synthesis among chemotypes. However, in light of the excellent correlations found in this and other investigations (Welker et al. 2007), interstrain variations in MC synthesis seem to be negligible compared to those caused by shifts in the relative abundance of toxic chemotypes at the whole-population level. The use of chemotypical techniques at the subpopulation level allows

a rapid identification of chemotypes and enables the tracking of the dynamics in toxin concentrations, which is one of the main sources of uncertainty in the context of the assessment of the risks associated to cyanotoxin exposure.

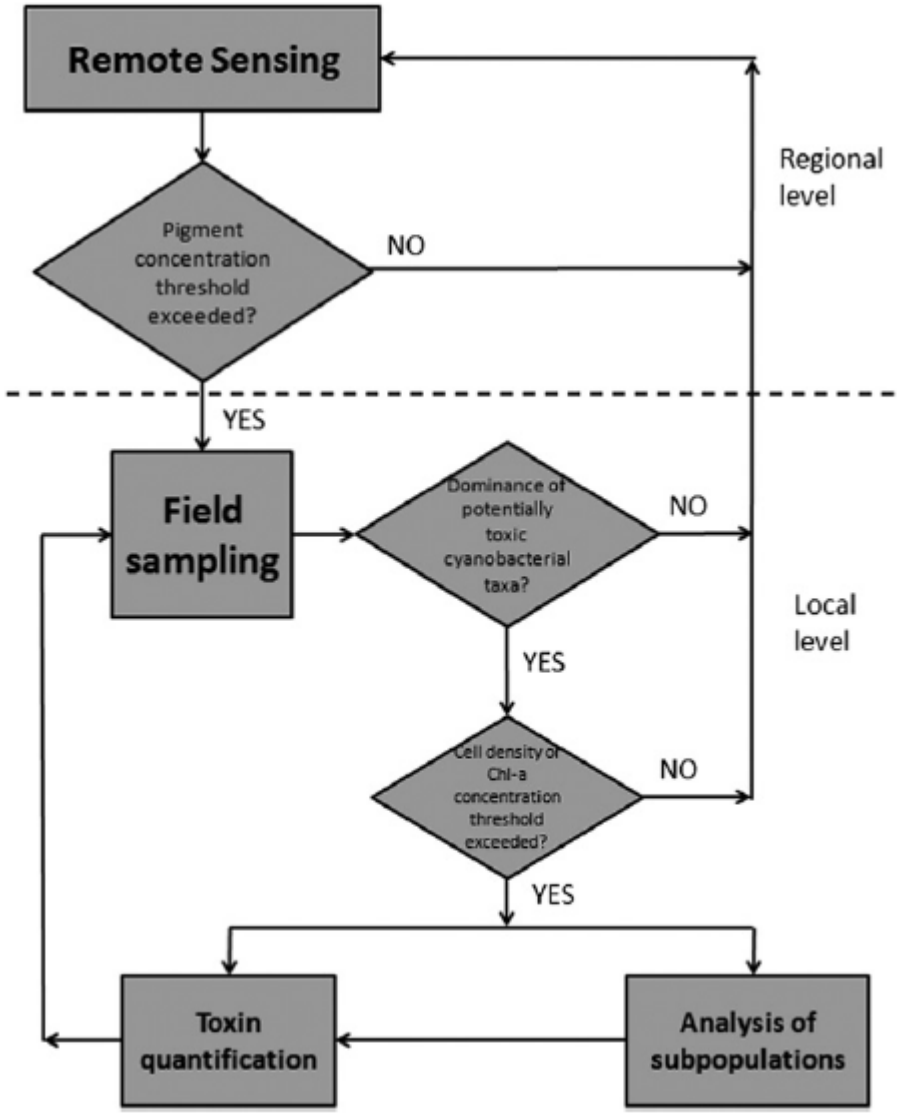

Figure 6.7. Flow-chart representing the proposed monitoring strategy. Regional level includes monitoring on a weekly basis of areas interest (e.g. bathing areas) and influencing surrounding areas by remote sensing. Pigment concentrations above a safety value trigger weekly field samplings at the pints of interest. Toxin quantification and subpopulations analysis should be carried out under conditions of dominance of potentially toxic taxa and high cyanobacterial cell densities. Field sampling, toxin and subpopulations analysis are maintained as long as these conditions prevail. Threshold values should ideally be adapted to national and/or local guidelines.

Efficient monitoring of cyanobacterial blooms has turned in the last years a growing need for water and health authorities, given the increasing occurrence of these phenomena and the recent discovery of new cyanotoxins (e.g. BMAA). Monitoring programs are usually

focused on single approaches, such as intensive water samplings or surveillance by remote sensing. These approaches alone seem insufficient to cope with marked heterogeneities in cyanobacterial distribution and dramatic fluctuations in MC concentrations inherent to bloom episodes. Instead, the joint use of techniques at different levels constitutes a potential solution to carry out efficient monitoring programs. For these reasons, we propose a multi-scale monitoring strategy based on different levels as schematized in Fig. 6.7. Routine monitoring by remote sensing on a weekly basis should provide information with wide spatial coverage about fluctuations in Chl-a concentrations in both points of interest (e.g. bathing areas) and potentially influencing surroundings (e.g. upstream waters, remote reservoir or lake areas, etc). In case total Chl-a concentrations surpasses a certain threshold, field sampling should be carried out in the area of interest (i.e. bathing area). These triggering thresholds need to be established according to national or local guidelines, aiming to provide a conservative limit to implement field sampling and thereby anticipate bloom formation. If limnological sampling confirmed the presence of potentially toxic cyanobacterial taxa, cell densities or total Chl-a concentration should be analyzed. Exceeding threshold values would prompt both the analysis of subpopulations and the analytical determination of uncertainty when assessing the risk associated to cyanotoxins exposure, mainly due to the highly patchy cyanobacterial distribution and the fluctuations in toxin concentrations. In this study we exemplified how the combination of conventional water samplings with remote sensing techniques providing wide spatial coverage, as well as approaches at the subpopulation level capable of explaining variations in toxin concentrations, allows the monitoring of the main sources of variability in cyanobacterial blooms. Multi-scale approaches as the one presented in this work, which intend to minimize the sources of uncertainty, are likely to constitute the backbone of the future monitoring programs and will substantially improve the assessment of the risks associated to cyanobacterial blooms.

6.5. Conclusion

The monitoring of cyanobacterial blooms has gained importance in the last years due to their increasing occurrence and the discovery of new harmful cyanotoxins. However, most monitoring programs are based on the sole use of conventional water sampling and cannot address important sources of uncertainty when assessing the risk associated to cyanotoxins exposure, mainly due to the highly patchy cyanobacterial distribution and the fluctuations in toxin concentrations. In this study we exemplified how the combination of conventional water samplings with remote sensing techniques providing wide spatial coverage, as well as

approaches at the subpopulation level capable of explaining variations in toxin concentrations, allows the monitoring of the main sources of variability in cyanobacterial blooms. Multi-scale approaches as the one presented in this work, which intend to minimize the sources of uncertainty, are likely to constitute the backbone of the future monitoring programs and will substantially improve the assessment of the risks associated to cyanobacterial blooms.

6.6. Acknowledgements

We would like to thank CHT (Confederación Hidrográfica del Tajo) and especially Mr. Pedro Soto for their collaboration in the field sampling, as well as Dr. Maite Alonso (SIdI-UAM) for her valuable assistance in the MALDI-TOF analysis. We also want to thank the ESA programme “Observing the Earth” for allowing the use of MERIS imagery in the context of the project “Development of an operational system for direct thematic mapping of photosynthetic pigments in lakes using MERIS application to the Spanish Reservoirs” (ID 594).

7. General discussion

This Dissertation covers two main topics in the context of cyanobacterial research, namely the monitoring of cyanobacterial blooms in freshwater ecosystems, using both conventional and advanced monitoring strategies, and the study of the dynamics and ecological significance of oligopeptide- based cyanobacterial subpopulations.

In particular, Chapter 3 covers the extensive survey of Spanish official bathing sites using conventional monitoring approaches, which provides a state of the art in the context of toxic cyanobacteria proliferation in Spanish inland waters. Next, in Chapter 4, we assessed the stability of oligopeptide fingerprints used for the delimitation of cyanobacterial chemotypes and thereby validate its use in further studies. In Chapter 5 we studied the dynamics of chemotypical subpopulations of genus *Microcystis* in their natural habitat, focusing on differential loss processes among subpopulations. Thereby, the ecological significance of chemotypes was explored. Lastly, in Chapter 6 we employed chemotyping approaches which, combined with remote sensing and conventional water sampling techniques, allowed the development of innovative monitoring strategies of cyanobacterial blooms that enable the reduction of sources of variability and uncertainty that typically hamper an accurate assessment of the risks associated to bloom events.

In the present Chapter we aim to synthesize and discuss the main findings on the various topics covered in this thesis and explore plausible scenarios on the driving forces behind the subdivision of chemotypical subpopulations and the biological role of oligopeptides in the ecology of cyanobacteria.

7.1. Cyanobacteria in Spanish recreational waters

The European Bathing Waters Directive constitutes beyond doubt a breaking through step in the inclusion of the cyanobacterial problematic in water management policies. For the first time, regulations at the European level explicitly include the assessment of the occurrence of cyanobacteria in freshwaters for recreational use. While much of the attention raised by cyanotoxins focused on their presence in drinking waters distribution systems, recreational waters are often overlooked. However, recreational activities represent an important potential route of exposure, especially in countries like Spain, where inland waters are traditionally heavily used during the summer season. Such activities imply a substantial risk of exposure due to the direct contact with untreated water.

Currently, Spanish legislations only establish mandatory limits for microcystins after drinking water treatment process. However, our results, together with a number of previous studies show that other cyanotoxins may be present in Spanish freshwaters (ATX-a, Carrasco et al. 2007; CYN, Quesada et al. 2006, PSTs, Wörmer et al. 2011), whose potential consequences are currently uncovered by the Spanish legislation. Of special relevance is cylindrospermopsin, whose occurrence in Spain is mainly attributed to the Nostocalean species *Aphanizomenon ovalisporum.* The distribution of this species has expanded in last years from tropical and subtropical areas to more temperate latitudes (Mehnert et al. 2010). In contrast to MCs, CYN is largely liberated to the dissolved fraction and is not bio- or photodegraded in the field (Wörmer et al. 2008, Wörmer et al. 2010), resulting in relevant CYN concentrations even in clear waters without cyanobacterial presence. Due to its increasing occurrence since it was first reported in Spain back in 2006 (Chapter 3, Quesada et al. 2006), CYN should beyond doubt be also included in the legislation on the topic. In our study, neurotoxic ATX-a could not be found in any of the surveyed bathing sites and supports previous reports of its infrequent occurrence in Spanish waters (Carrasco et al. 2007). Although PSTs were not analyzed in our study, their occurrence was recently reported for the first time in Spain (Wörmer et al. 2011). Moreover, recent studies undertaken in our research group showed that PSTs, much like CYN, can be significantly released to the dissolved fraction (Casero 2013). Therefore, future monitoring efforts should also address this emerging group of neurotoxins.

Accurately assessing the risks associated to cyanobacteria and their toxins is often a challenging task, subject to a delicate trade-off between analytical cost and uncertainties in the assessment. Obviously, the determination of cyanotoxin concentrations is the best risk indicator, but undertaking toxin analysis on a regular basis turns out prohibitive. Conversely, cyanobacterial presence does not necessarily imply the presence of cyanotoxins. Not even the presence of potentially toxic taxa grants an uncertainty-free risk assessment. The stepwise decision chart proposed in Chapter 3 aims to enable an evaluation of the tendency for toxic cyanobacterial proliferation, yielding a conservative assessment of the risk with the best possible trade-off between “analytical effort” and assessment accuracy. Therefore, the initial analysis in the risk assessment focus on easily determinable trophic parameters that constitute a prerequisite for the development of cyanobacteria. Only if these parameters indicate the trophic potential of the water body in question to host harmful cyanobacteria, more in depth and costly analyses are undertaken (e.g. taxonomic analysis, determination of toxin concentrations).

7.2. Advanced monitoring strategies

Moving further down the line, the assessment of exposure risks to cyanotoxins faces further sources of variability inherent to cyanobacterial blooms, which hamper an accurate assessment of the risks associated to cyanobacterial occurrence. In this work we covered two of these sources which arguably represent the most relevant uncertainties with regard to cyanobacterial blooms. First, the horizontal distribution of cyanobacterial biomass, caused by wind and water currents; second, fluctuations in toxin concentrations that result from the coexistence of toxigenic and non-toxigenic strains and their temporal dynamics. Conventional monitoring approaches are clearly insufficient to cope with these sources of variability. With the aim of addressing these issues, we developed multi-scale surveillance strategies as shown in Chapter 6.

Our study demonstrates that the highly heterogeneous spatial distribution of cyanobacterial biomass can be efficiently addressed by remote sensing techniques with wide spatial coverage. In Chapter 6, we employed MERIS signatures to quantify and chlorophyll-a and phycocyanin with remarkable accuracy. Unfortunately, ENVISAT satellite hosting the MERIS sensor is no longer operative. MERIS was the first sensor providing multiple spectral band readings and therefore, it was the first sensor allowing pigment concentrations data retrieval. MERIS main drawback was its limited spatial resolution (1 pixel ≈ 300m), which limits its use in small water bodies. However, new modern space borne sensors that provide both increased spatial and spectral resolution (i.e. more spectral bands) are now available. New algorithms are steadily being developed and validated, which do not only can track the distribution of cyanobacterial biomass, but could also potentially provide information about the presence of particular taxa with characteristic pigment compositions (Domínguez, pers. comm). Beyond doubt, remote sensing is experiencing a remarkable development and will further increase our ability to track cyanobacterial bloom developments over large geographical areas with much greater accuracy and resolution than we could show in our study with MERIS.

Bloom toxicity varies up to several orders of magnitude as a result of changes in the proportion of toxigenic genotypes in the population (e.g. Kardinaal et al. 2007) and the existence of toxic strains with different microcystin cell quota (Rohrlack et al. 2001, Carrillo et al. 2003, Welker et al. 2004). The use of molecular approaches for the detection *mcy*-genes containing individuals by q-PCR or DGGE yields reliable results to address this issue. However, these techniques are time-consuming and technically laborious and do not provide

information about the underlying diversity of microcystin variants produced. Furthermore, the presence of individual genes does not guarantee toxin production, in light of the presence of mutant genotypes, possessing one or more genes of the gene cluster, yet unable to produce the toxin (e.g. Kurmayer et al. 2004). Instead, we propose the analysis of *Microcystis* colonies by MALDI-TOF MS, which enables a high speed screening of microcystins on a single colony basis. The analysis of single colonies by MALDI-TOF MS takes no longer than 3 minutes, and data interpretation is straightforward, allowing for hundreds of colonies to be analyzed overnight and subsequent decision making by water managers within hours.

The proportion of toxigenic chemotypes in the population linearly correlated with the average cellular MC contents in Alcántara (Agha et al. 2012), Valmayor (Agha et al., under review; Chapter 5) and Brno (Welker et al. 2007) reservoirs. These findings evidence that differences among producing strains in terms of MC cell quota seem negligible and instead, the succession of toxic and non-toxic strains is the factor driving total microcystin contents of the bloom. Needless to say, understanding the factors that induce physiological and/or transcriptional changes in MC synthesis is important to gain insights into the largely debated cellular function of microcystins. However, in the context of water management, the dynamics of toxigenic and non-toxigenic strains shows to be the determining factor shaping toxin fluctuations; therefore, the improvement of current management strategies may profit from ecological studies on the dynamics of toxigenic and non-toxigenic subpopulations, rather than from physiological studies on MC synthesis at the individual level.

7.3. Oligopeptide fingerprints as chemotype markers

The analysis of taxon-specific metabolite patterns by mass spectrometric techniques in a number of organisms is being increasingly used for the rapid identification of infective or pathogenic species (e.g. Ho & Reddy 2011, Chen et al. 2012), as they yield simple, rapid and high-throughput measurements compared to molecular methods. In cyanobacteria, however, chemotypical approaches based on oligopeptide compositions are at their dawn. While much of the attention raised by planktonic cyanobacteria was traditionally driven by the occurrence of cyanotoxins, the progressive discovery of a vast diversity of secondary metabolites has promoted in the last few years a broader view of the secondary metabolism as an important feature closely related to their ecology. The patchy distribution of oligopeptide synthesis abilities among co-existing cyanobacterial strains prompted the use of oligopeptide compositions to delineate chemotypes in natural populations.

Cellular oligopeptide compositions can be rapidly screened by MALDI-TOF MS with minimal sample preparation, making it the technique of choice for cyanobacterial oligopeptide chemotyping (e.g. Welker et al. 2004, Martins et al. 2009), with LC-tandem mass spectrometry also being used to a lesser extent (e.g. Rohrlack et al. 2008). Peptides are suitable targets for MALDI-TOF MS, as free electron pairs in the amino groups are accessible for protonation and hence provide in general good ionization. Furthermore, MALDI ionization is less aggressive than other techniques (e.g. Electro-Spray Ionization) and produces singly charged ions, which are more easily identified. However, MALDI-TOF MS has to be regarded as a qualitative (or semi-quantitative) analytical method, as peptides differ in their ionization due to differences in their amino acid composition and hydrophobicity. Therefore mass signals do not directly reflect peptide concentrations, hampering a quantitative interpretation of the mass spectra.

The synthesis of cyanobacterial oligopeptides at the individual level is constitutively regulated (Repka et al. 2004) and oligopeptide cell quota fluctuates within a narrow range (Rohrlack & Utkilen 2007, Halstvedt et al. 2008, Pereira et al. 2012). Therefore, oligopeptide fingerprints of individual strains have been traditionally assumed to be consistent markers of chemotypes. However, so far, no specific studies have assessed the distorting effects of changing environmental conditions in peptide fingerprints. In Chapter 4, we evaluated the consistency in detection of individual oligopeptides by MALDI-TOF MS under changing nutrient conditions and light regimes. Our results revealed variations in peptide fingerprints, consisting in the misdetection of minor oligopeptides under suboptimal physiological conditions.

The difficulties in consistently detecting minor mass signals by MALDI-TOF MS have been recognized earlier (Czarnecki et al. 2006, Welker & Erhard 2007) but these usually correspond to minor peptides and are often accompanied by more intense signals corresponding to major peptides of the same class. However, these variations have not been assimilated in the frame of the definition of chemotypes and the study of their diversity and dynamics in natural systems. It is important to underline that, rather than questioning the integrity of chemotypes as subspecific entities, our findings highlight physiologically induced variations that call for caution when using MALDI-TOF MS retrieved oligopeptide compositions for the delimitation of chemotypes. Variability in the detection of minor peptides may lead to misinterpretations of the shifts in chemotype compositions and arguably to an overestimation of the coexisting chemotype diversity. In other words, the misdetection of low intensity peptides has to be regarded as a physiologically induced variation in the fingerprint and hence should not imply the adscription to a different chemotype.

In fact, existing studies analyzing naturally occurring chemotypes employ heterogeneous criteria for the delineation of chemotypical subpopulations. Chemotyping based on oligopeptide classes (e.g. Martins et al. 2010), "strict" individual-oligopeptide-based chemotyping (i.e. chemotypes display identical peptide compositions, Welker et al. 2007) or "lax" individual-oligopeptide chemotyping (i.e. chemotypes share resembling, but not always identical peptide compositions, Rohrlack et al. 2008) are used in the different studies on the topic and represent different approaches that hamper comparisons among studies in terms of chemotype diversity. Therefore, harmonized and integrative criteria for chemotype definition based on the synthesis of individual peptides, yet considering possible physiological variations in their synthesis and/or detection, are needed. Unfortunately, a problem to address this variability arises from the difficulties of assessing the physiological state of individual colonies collected from field samples, so that the assessment of a "complete" peptide fingerprint comes up problematic. Due to these problems, in Chapter 5, we decided to use chemotype delimitation based on the detection of individual peptides (*sensu* Welker et al. 2007), as this approach yields the delimitation of chemotypes with the highest degree of resolution, fingerprint variability notwithstanding, and allows for comparisons with existing data relative to *Microcystis* chemotype diversity in natural populations. In order to evaluate the potential impact of putative distortions in oligopeptide fingerprints from single colonies collected from the field, we assessed how many of the identified chemotypes in Valmayor reservoir could potentially constitute artifactual subpopulations (i.e. apparently genuine chemotypes presenting the same peptide composition as other coexisting chemotypes, except for the absence of one or more minor peptides, putatively resulting from oligopeptide fingerprint distortions). After pairwise comparisons of the oligopeptide compositions of each chemotypes, we could only find 9 candidates (out of a total of 53 chemotypes) that could potentially represent artifactual subpopulations. These chemotypes were minor components of the pelagic community that, when detected, always showed low relative abundances below 2%. This suggests that, if these subpopulations were not genuine, but artifactual chemotypes, the impact of physiologically induced fingerprint distortions in the interpretation of the dynamics and diversity of chemotypes in our study is limited. However, the implications of these inconsistencies in systems with a low number of coexisting chemotypes (e.g. Lake Steinsfjorden; 4 *Planktothrix* chemotypes, Rohrlack et al. 2008) might have a greater impact in data interpretation. One possible way to circumvent these issues and address fingerprint variability might consist in assessing the physiological conditions of single *Microcystis* colonies prior to MALDI-TOF analysis (e.g. by PAM fluorometry), although this seems unsuitable for

other non-colony-forming genera like *Planktothrix*. Alternatively, using criteria for chemotype delimitation with less resolution (e.g. presence of oligopeptide classes) may arguably be an alternative.

In this context, the appropriateness of a chemotype definition based on the synthesis of individual peptide variants (vs. chemotype definition based on general peptide classes) deserves to be discussed. The significance of the existence of oligopeptides with structural ambiguities has not been fully clarified. However, several studies undertaking structure-activity relationships (SAR) analysis of cyanobacterial peptides report changes in bioactivity resulting from structural variability. For example, cyanopeptolin structural variants display changes in protease inhibition capabilities (Czarckeni et al. 2006) and methylations in microcystin residues result in changes in chymotrypsin inhibition (Blom et al. 2001). Similarly, aeruginosin variants differ in their inhibition capabilities of different groups of serine proteases (Nagarajan et al. 2013). These findings strongly suggest that oligopeptide unique moieties, variable amino acid residues and general structural diversity affect the bioactive properties of oligopeptides and determine their degree of specificity and activity. Since structural variability seems to affect the bioactive properties of oligopeptides, delineating subpopulations based on the synthesis of individual peptide variants seems justified and may well define functionally dissimilar chemotypes.

However, chemotype delineation based on individual peptide variants embodies a picture of high complexity. The extent of the structural diversity throughout the different peptide classes could, at least in principle, be enormous. Microcystins are comprised by more than 90 described structural variants, while other oligopeptide classes could bear even more structural variability. Cyanopeptolins, for example, present variable positions at all residues, except two conserved positions, potentially leading to a much greater number of possible structures. One could argue that the potential diversity of oligopeptides in cyanobacteria, considering combinatorial incorporation of amino acids at reported variable positions across oligopeptide classes, can rise up to thousands of individual oligopeptides (Welker et al. 2007) and thus may lead to an almost endless number of different fingerprints and chemotypes. However, chemotypes in nature show unique ecological features (Chapter 5, Rohrlack et al. 2008) and therefore, the distribution of oligopeptide production abilities among cyanobacterial strains is questionably a random process. Instead, it likely constitutes an adaptive response to pressures exerted by their environment. Today, however, such pressures remain to be elucidated.

7.4. Unraveling the role of cyanobacterial oligopeptides

In this section we discuss recent findings that offer new insights into the biological role of oligopeptides and allow us to picture evolutionary scenarios that are capable of explaining the existing diversity of oligopeptide-based subpopulations, as well as the mechanisms behind the subdivision of cyanobacterial populations into different chemotypes.

7.4.1. Oligopeptide chemical diversity

NRPS or hybrid NRPS-PKS are pivotal enzymes in the bacterial secondary metabolism. NRPS genes may account for up to 5% of the cyanobacterial genome. However, end-products of NPRS pathways are usually small compounds, which are produced by huge enzymes. The transcription and translation of these enzyme complexes and their modular architecture imply an enormous demand of cellular resources. Notably, each NRPS catalytic unit (typically known as NRPS module) consists of roughly 1,000 to 1,500 amino acids and is responsible for the incorporation of a single monomer in the end-product peptide chain. This gives an idea of the size of NRPS "megaenzymes", which are one of the largest groups of enzymes known. However, these pathways have been conserved and exploited in distantly related lineages of microorganisms along their evolutionary history, in spite of the high energetic costs related to their synthesis. This apparent paradox can be explained by the modular architecture of non-ribosomal pathways, which allows microorganisms to take advantage of module reshuffling (e.g. module re-organization, duplication or skipping, recombination, point mutations, etc.), enabling the synthesis of a wide repertoire of metabolites with minimal genetic changes. A representative example of gene cluster re-organization resulting in brand new end products is the cyanotoxin nodularin, which evolved from the microcystin gene cluster through the deletion of two modules (Moffitt & Neilan 2004, Rantala et al. 2004).

In addition, single NRPS gene clusters are responsible for the synthesis of multiple chemical variants within a peptide class. A significant part of such structural diversity across oligopeptide classes is achieved by relaxation of substrate specificities in adenilation domains, leading to variable amino acids at some positions and thereby to multiple chemical variants. Substrate promiscuity in adenilation domains, together with further modifications like methylation, glycosylation, halogenation or sulfatation (Ishida et al. 1997, Rouhiainen et al. 2000, von Elert et al. 2005, Ishida et al. 2007) at different residues allow the existence of hundreds of congeners within the same peptide class, with differential bioactive properties (Czarckeni et al. 2006, Blom et al. 2001; Nagarajan et al. 2013). Both the modular architecture of encoding gen clusters and the high structural diversity of metabolites suggest that NPRS

pathways show high intrinsic versatility, which confers microorganisms an enormous metabolic plasticity and arguably counterbalances the high energetic costs associated with their synthesis.

7.4.2. Selective pressures driving oligopeptide diversification

The gradual identification of gene clusters responsible for the synthesis of different oligopeptide classes during the last years has increased our insights into their evolutionary history and the selective pressures acting on them. Sequence comparisons with regard to the occurrence of non-synonymous (i.e. substitutions resulting in changes in the encoded amino acid, Ka) versus synonymous nucleotide substitutions (i.e. substitutions causing no changes in the encoded amino acid, Ks) enable the identification of genomic regions under purifying ($Ka/Ks << 1$) and positive selection ($Ka/Ks > 1$) within a particular gene cluster. Studies focusing on different oligopeptide synthases encoding operons point out that an overwhelming majority of the encoding regions in the examined gene clusters are under strong purifying selection and are therefore highly conserved. However, small regions show remarkably higher Ka/Ks ratios, with values greater than unity, strongly suggesting positive selection acting on them. Interestingly, these regions are usually binding pockets within adenilation domains, which are involved in the recognition, activation and incorporation of different amino acids in the variable residues during oligopeptide biosynthesis and are hence responsible for the synthesis of multiple structural variants. These findings have been reported for the *mcy* gene cluster in genera *Microcystis, Planktothrix* and *Anabaena* (Mikalsen et al. 2003, Kurmayer & Gumpenberger 2006, Tooming-Klunderud et al. 2008), as well as for different oligopeptide classes, including anabaenopeptins (Christiansen et al. 2011) and cyanopeptolins (Rounge et al. 2008). Altogether, this evidences that the synthesis of new oligopeptide variants across cyanobacterial genera and oligopeptide classes is positively selected. This finding is highly insightful, as it strongly suggests that structural ambiguity does not exert a restraining effect on the function(s) of oligopeptides and that, instead, structural diversity seems crucial for the various chemotypes in their natural habitats.

7.4.3. The black box of the ecology of cyanobacteria

Identifying the factors that exert the above-mentioned selective pressures and hence drive oligopeptide chemical diversification and chemotype subdivision is needed to provide an unequivocal link to the role of oligopeptides, as well as to understand the significance of chemotypes in natural systems. The biological function of oligopeptides has been mainly investigated with regard to microcystins, the most prominently studied group of oligopeptides.

Over the last decades, different hypothetical functions of microcystins have been proposed, including nutrient scavenging (Utkilen & Gjolme 1995), protection against oxidative stress (Zilliges et al. 2011), grazing protection (Ghadouani et al. 2004, Rohrlack et al. 2004, Czarnecki et al. 2006), allelopathy (von Elert & Juttner 1997, Schagerl et al. 2001, Sukenik et al. 2002, Suikkanen et al. 2004, Leão et al. 2010) or quorum sensing (Kehr et al. 2006, Schatz et al. 2007). Although findings on which these hypothesis are based are deeply rooted in rigorous scientific work, the formulation of such hypothesis also contains elements of speculation, extrapolation and, naturally, controversy.

Similarly, an attempt to formulate a plausible scenario regarding the general role of oligopeptides and the ecological significance of cyanobacterial oligopeptide chemotypes as a corollary of this dissertation seems adequate. The formulation of this hypothesis is based, not only on the results derived from the studies compiled in this document, but also on the extensive new knowledge produced by the excellent scientific work undertaken during the last few years regarding various aspects of the synthesis, bioactive properties and evolutionary history of cyanobacterial oligopeptides. At some points during this discussion, we will inevitable step onto the grounds of speculation, although supportive evidence will be provided, with the intention of endowing our statements with a considerable degree of plausibility.

Much of the interest raised by cyanobacteria and their occurrence is directly or indirectly related to the adverse impacts of cyanotoxins in animal and human health. As mentioned above, elucidating the biological role of microcystins has been a matter of intense scientific debate during the last decades. At present, however, none of the proposed hypothesis on their biological function provides unequivocal evidence or seems fully conclusive. In this context, we have to acknowledge that the importance attributed to cyanotoxins results from a rather anthropocentric view and is undoubtedly the reason why microcystins, the most prominent of them, have been under the spotlight of cyanobacterial research. Still, from a strictly biological standpoint, microcystins constitute only a minor part of the wide repertoire of oligopeptides produced by cyanobacteria. Staying away from anthropocentric perspectives for a moment, one could argue (quite cynically indeed) that focusing our attention strictly on microcystins and disregarding other secondary metabolites, may have constituted a somewhat myopic approach to elucidate their biological function(s). Alternatively, the study of cyanobacterial populations from the chemotypical perspectives

represents an emerging, alternative, and certainly broader approach that offers new opportunities to increase our understanding on the biological role of oligopeptides as a whole.

As discussed above, NPRS and hybrid NRPS-PKS pathways, despite being highly energy demanding, constitute an extremely versatile and plastic machinery that endows cyanobacteria with a huge array of secondary metabolites. This versatility is manifested at the individual level by the existence of multiple chemical congeners encoded in the same gene clusters, but also at the population level, reflected by an extremely patchy distribution of oligopeptide production abilities among individuals, resulting in a highly diverse pool of co-existing chemotypes. Moreover, since the genetic regions responsible to a great extent for this structural variability have shown to be under positive selective pressure, this diversification has to be regarded as an on-going evolutionary process.

Attempts to correlate the occurrence and dynamics of chemotypical subpopulations with abiotic factors (and thereby attribute a putative biological function to oligopeptides) showed to be unfruitful. In Chapter 5, no relationship between abiotic components such as nutrient loads, light intensity or water temperature and the phenology and distribution of chemotypical subpopulations could be established. Similar findings were reported elsewhere (Rohrlack et al. 2008) and altogether, they speak against the importance of bottom-up mechanisms controlling chemotypical subpopulations. One could consider these findings somewhat unsurprising, as abiotic factors (which typically fluctuate on a more or less constant seasonal basis) unlikely support the existence of such a wide diversity of chemotypical subpopulations and individual oligopeptides and neither justify their on-going diversification.

In this context, the existence of top-down mechanisms controlling chemotypical subpopulations mediated by biotic ecosystem components seems a more plausible possibility: In contrast to abiotic factors, biotic components display an intrinsic adaptability, and as such, constitute a dynamic, responsive source of selective pressures that could in principle fit with the observed, on-going evolutionary diversification of oligopeptide repertoires in cyanobacterial populations. Moreover, most oligopeptide classes display a variety of bioactive properties that point toward an inhibitory and/or protective effect against other organisms: For instance, microcystins are potent inhibitors of serine/threonine-specific protein phosphatases (Mackintosh et al. 1990), while cyanopeptolins, aeruginosins and anabaenopeptins display serine proteases inhibitory effects (e.g. Matsuda et al. 1996, Namikoshi & Rinehart 1996, Fujii et al. 1997, Elkobi-Peer et al. 2013). The notion of a putative

protective and/or allelopathic role of oligopeptides is not new, although grazers and other phytoplankton have traditionally been regarded as the main target organisms. Two main considerations are typically raised against these possibilities though. A first argument refers to the evidence provided by Rantala and coworkers (2004). By comparing *mcy* genes from various cyanobacterial genera with housekeeping genes, the authors could clearly rule out the possibility of lateral gene transfer between cyanobacterial genera of the *mcy* gene cluster, strongly suggesting that the last common ancestor of present-day cyanobacteria already presented the ancestral *mcy* genes. Since this common ancestor predates the metazoan lineage, a primary defensive role against eukaryotic grazers comes up questionable, at best. A second consideration refers to the fact that oligopeptides remain within the producing cell (Young et al. 2005) and are only significantly released after cell lysis. Therefore an effective allelopathic and/or protective role of oligopeptides could only take place upon cell lysis or ingestion. Again, the benefits of such a "post-mortem" protection are questionable. In light of these considerations, it seems reasonable to explore other possible sources of selective forces driven by biotic components.

Parasitism

Parasitism is one of the earliest and most common ecological interactions in nature (Cavalier-Smith 1993). The best known phytoplankton pathogens are viruses, although bacterial pathogens and fungal parasites are also recognized as important algal pathogens. Although parasitism in aquatic ecosystems has received little attention, chiefly due to methodological constrains, parasites are increasingly being considered key players in the functioning of the ecosystem, with equal importance as that of predators (e.g. Montagnes et al. 2008). Parasitic interactions constitute a typically overlooked ecological dimension in aquatic ecosystems, that account for the majority of the food web links, enhance the recycling of organic matter, the transfer of energy and nutrients to higher trophic levels and the maintenance of diversity in the ecosystem (Lafferty et al. 2006, Amundsen et al. 2009). Parasitic interactions are recognized as important driving forces of ecological and evolutionary processes, including population dynamics, species succession, speciation and gene flows (Hudson et al. 2006). In particular, parasites impose a strong top-down control and, thereby, a strong selective pressure on host communities. Figure 7.1 illustrates to what extent overlooking the ecological role of parasites implies ignoring a significant part of the ecosystem complexity.

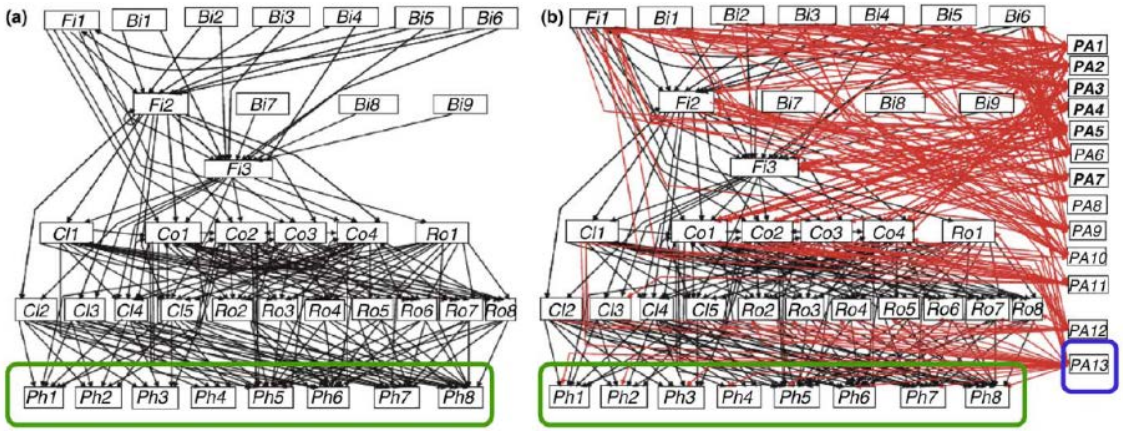

Figure 7.1. Schematic representation of the pelagic trophic web in Lake Takvatn (Norway) without parasite related links (a), and considering parasite-related connections (b). Phytoplankton species are marked in green. PA13, circled in purple, represent parasites of phytoplankton. Bi, birds; Cl, cladocerans; Co, copepods; Fi, fishes-, PA, parasites; Ph, phytoplankton; Ro, rotifers. Source: Sime-Ngando (2012), adapted from Gachon et al. (2010).

Like any other living organism, cyanobacteria are susceptible to parasites. Major parasites affecting cyanobacteria in aquatic ecosystems are fungi of the order Chytridiales, commonly referred to as chytrids. They are considered to be ubiquitous and therefore chytridiomycosis is an omnipresent phenomenon in aquatic ecosystems (Rasconi et al. 2012). Chytrids show absorptive nutrition: They encyst at the cell surface and form intracellular rhizoids to extract nutrients from the host cell. In this process, the secretion of proteases is a key mechanism involved in pathogenicity and is required for the digestion of the cellular constituents (Krarup et al. 1994, Rosenblum et al. 2008, Sun et al. 2011). In this situation, a putative defensive role of cyanobacterial oligopeptides, presumably mediated by their protease inhibitory properties, is compatible with the fact that oligopeptides remain largely within the producing cell.

Parasites and their hosts are usually caught in a close relationship, which results in an evolutionary arms race, according to the Red Queen hypothesis (Bell 1982). Parasites are characterized by displaying shorter life-cycles and generation times than their hosts and therefore present higher evolutionary rates (Hamilton et al. 1990) . For this reason, protective adaptations by hosts are rapidly countered by fast-evolving parasites. Under these circumstances of reciprocal adaptation, host populations are strongly selected toward diversification (Hamilton et al. 1990, De Bruin et al. 2008). Recent findings suggest that this could be the case for chytrids-cyanobacteria interactions. Rohrlack and Sonstebø (2011) showed that coexisting chytrid strains display chemotype-specific host preferences. The involvement of several oligopeptides in cyanobacterial resistance against chytrid infection was

in fact recently demonstrated by gene knockout mutagenesis in genus *Planktothrix* (Rohrlack et al. 2013). These findings lead to hypothesize that population subdivision into different chemotypes constitutes an adaptive response of cyanobacteria to resist parasites at the whole-population level. By strongly diversifying the repertoire of protease inhibitors, cyanobacteria would either: (a) face a pool of parasites with broad host ranges that unselectively exploit the population with suboptimal efficiency or, (b) force parasites themselves into diversification (i.e. parasite specialization) resulting in reciprocal, antagonistic coevolution with particular chemotypes. Both possibilities would favor cyanobacteria by hampering the ability of parasites to optimally exploit the whole population.

This hypothesis remains to be fully explored, but it provides a plausible scenario that could explain the population subdivision into chemotypes with dissimilar susceptibility to parasites and might constitute an adaptive strategy to resist parasites. While the recent lines of evidence point toward defensive mechanisms against fungal parasites, other sources of pathogenic diseases like viruses or pathogenic bacteria cannot be discarded. In this context, seasonal dynamics of subpopulations might be caused by chemotype specific pathogenic losses. Chemotype succession in Valmayor reservoir (Chapter 5) was caused mainly by sedimentation losses of individual *Microcystis* chemotypes. Unfortunately, we did not directly address the impact of pathogenic losses affecting individual subpopulations and therefore it is difficult to assess whether chemotype-specific sedimentation and pathogenic losses coexist at a given time point that may jointly cause the disappearance of chemotypes from the water column. In a number of algal species, rapid life-cycle shifts have been described as a strategy to escape parasite infection (Toth et al. 2004, Mayali et al. 2007, Frada et al. 2008). Although this has not been explicitly described for genus *Microcystis*, the outbreak of chemotype-specific epidemics in the water column might trigger the rapid sinking of susceptible colonies to avoid infection, thereby assuring a substantial pool of overwintering colonies in the sediments and consequently increasing the chances of successfully re-colonizing the water column in the following season. Although certainly speculative, this idea is compatible with the strikingly high sedimentation rates of some chemotypes observed in our study.

Differential synthesis of oligopeptides arguably implies competitive differences among coexisting chemotypes, most likely due to energetic burdens associated with their synthesis and/or antagonistic pleiotropy, i.e. interference of the inhibitory effects of oligopeptide(s) in the producer's own metabolism, for which evidence was found in genus *Microcystis* (Ghosh et al. 2008). Therefore, intraspecific competitive differences among chemotypes are likely to

exist. However, these do not seem to lead to a reduction of the diversity of coexisting oligopeptide subpopulations (via competitive exclusion) in the long term, as chemotype diversity shows to be maintained over decades (Rohrlack et al. 2008). Instead, if considering top-down control mechanisms mediated by parasites, the concept of "killing the winner" (Thingstad & Lignell 1997) provides a plausible explanation for the maintenance of the diversity of chemotypes in cyanobacterial populations. According to this concept, the most abundant subpopulation (i.e. superior competitor) is also most intensively infected by the pool of coexisting parasites. Thereby, the exclusion of otherwise less efficient subpopulations is avoided and diversity in the host population is maintained, despite competitive differences among chemotypes. This process seems to be compatible with our observations in natural systems. Chemotype compositions in the water column at given moment are characterized by the dominance of one or a few abundant chemotypes with other minor chemotypes coexisting in low abundances (Chapter 5, Welker et al. 2007), whereas successional shifts typically result from important losses of major chemotypes, followed by sharp increases of minor coexisting chemotypes.

Studies addressing the diversity and dynamics of cyanobacterial chemotypes in natural ecosystems are scarce. However, these studies show remarkable differences in chemotype diversity. Welker and coworkers (2007) found 37 distinct *Microcystis* chemotypes in hypereutrophic Brno reservoir (Czech Rep.) in a period of 5 months, while we identified 53 *Microcystis* chemotypes in eutrophic Valmayor reservoir in the same period (Chapter 5). In another study, 15 different *Planktothrix* chemotypes were isolated from a single plankton net sample taken from lake Maxsee, Germany (Welker et al. 2004). This contrasts with the remarkably lower diversity of *Planktothrix* chemotypes in the Norwegian oligotrophic lake Steinsfjorden, where only 4 chemotypes were observed over more than 3 decades (Rohrlack et al. 2008). Considering the trophic status of the different water bodies surveyed, a clear tendency showing a higher number of coexisting chemotypes with increasing lake productivity can be observed.

Oligotrophic conditions promote the coexistence of phytoplankton groups (chlorophytes, diatoms and cyanobacteria) which together constitute a wide range of hosts for the coexisting parasites; under this situations parasite diversity is accordingly higher and massive epidemics are rare events (e.g. Rasconi et al. 2012). Conversely, under eutrophic conditions, specific host range is for the most part much narrower, commonly consisting in monospecific cyanobacterial blooms. Under these circumstances, the control and selective pressures

imposed by parasites over cyanobacteria are arguably most intense. In the frame of the proposed evolutionary scenario, these conditions imply that cyanobacterial populations in eutrophic systems tend to be more strongly selected toward diversification (i.e. diversification of oligopeptide repertoires and chemotype subdivision), resulting in a higher number of coexisting chemotypes than in systems with less productivity (e.g. oligotrophic). Whereas this hypothesis remains to be tested in future studies, it provides a possible explanation for the marked differences in chemotype diversity that have been observed among studies.

This Discussion Chapter integrates various lines of evidence and sketches a plausible and coherent scenario on the ecological role of cyanobacterial oligopeptides and the subdivision of chemotypes. In summary, recent findings point towards a role of cyanobacterial oligopeptides as defensive compounds against parasites, putatively by inhibition of enzymes (proteases) involved in pathogenicity. The proposed scenario postulates an evolutionary arms race of reciprocal adaptations with parasites, resulting in population subdivision into different chemotypes with dissimilar susceptibility to pathogens as an adaptive strategy to resist rapidly evolving parasites. This hypothesis can in principle explain (i) the dynamics of chemotypes in natural systems, (ii) the maintenance of their diversity in time (by “killing the winner”) and (iii) the differences in chemotype diversity along the surveyed water bodies (higher diversifying pressure on narrow host ranges). While some aspects of this hypothesis remain to be experimentally demonstrated and consolidated, we believe that the coherence between the available pieces of evidence (both at the molecular and ecological levels) and the proposed evolutionary scenario is too striking to be overlooked. This hypothesis largely stimulates further experimentation and, in our opinion, constitutes a significant milestone to increase our understanding on the ecology of these fascinating organisms.

8. Conclusions / *Conclusiones*

1. The survey of the 212 Spanish official bathing sites highlights the importance of cyanobacteria as a key element in the assessment of the health risks associated to recreational activities in freshwaters. Half of the Spanish official bathing sites shows a moderate risk for toxic cyanobacteria proliferation, while almost 20% show a high risk to host toxic cyanobacteria.

1. *El análisis de los 212 puntos de baño oficiales españoles pone de manifiesto la importancia de la proliferación de cianobacterias como elemento clave en la evaluación de riesgos para la salud asociados a actividades recreativas en aguas continentales. La mitad de los puntos de baño oficiales muestran un riesgo moderado a la proliferación de cianobacterias tóxicas, mientras que en torno al 20% de ellos presentan un riesgo elevado.*

2. The Chroococalean genus *Microcystis* was confirmed as the most important MC producer in Spanish freshwaters, which was present in more than 50% of the Spanish official bathing sites hosting cyanobacteria. Besides, the recent invasion and increasing expansion of cyanobacterial species like *Aph. ovalisporum* is a matter of concern due to its ability to produce cylindrospermopsin, a so far uncommon cyanotoxin in Spanish freshwaters.

2. *El género* Microcystis *se confirma en España como el taxón productor de microcistinas más importante en aguas dulces, el cual se observó en más del 50% de los puntos de baño con presencia de cianobacterias. Además, la invasión y creciente expansión de especies cianobacterianas como* Aph. ovalisporum *demandan especial atención, dada su capacidad de producir cilindrospermopsina, una cianotoxina hasta el momento infrecuente en aguas españolas.*

3. Microcystins are the most commonly occurring cyanotoxin in Spanish waters, whose presence is closely associated to cyanobacteria of the genus *Microcystis.* Cylindrospermopsin was found to be less frequent, although its significant presence in the dissolved fraction and its strongly limited natural degradation demand the consideration of this cyanotoxin in the risk assessments and its inclusion in the legislation and water safety plans. ATX-a could not be detected in any of the Spanish official bathing sites and confirms the low incidence of this neurotoxin in Spanish freshwaters.

3. *Las microcistinas representan la cianotoxina más frecuente en aguas españolas y su incidencia está íntimamente relacionada con la presencia de cianobacterias del género* Microcystis*. La cilindrospermopsina resultó menos frecuente, aunque su significativa presencia en la fracción disuelta, así como su limitada degradación en condiciones naturales, demandan su consideración en los planes de gestión y legislación futuros. La anatoxina-a no fue detectada en ninguno de los puntos de baño analizados y confirma su escasa incidencia en aguas españolas.*

4. MALDI-TOF MS retrieved oligopeptide fingerprints used for the delimitation of cyanobacterial oligopeptide-based chemotypes may be subject to variations under nutrient poor conditions or high light irradiances. Variations consist in the gradual loss of low intensity producing peptides. The extent of the fingerprint distortion is closely related to suboptimal physiological conditions.

4. *Las huellas oligopeptídicas obtenidas mediante espectrometría de masas MALDI-TOF, empleadas en la delimitación de subpoblaciones quimiotípicas, son susceptibles de sufrir variaciones bajo condiciones pobres en nutrientes y alta luz. Dichas variaciones consisten en la desaparición gradual de péptidos con baja intensidad relativa. El alcance de la distorsión de la huella oligopeptídica está íntimamente relacionado con condiciones fisiológicas subóptimas.*

5. The proportion of MC-producing strains in the population can be rapidly determined by single colony MALDI-TOF MS analysis, compared to molecular methods. The composition of toxigenic and non-toxigenic chemotypes at any given moment linearly and significantly correlates with total MC:*Microcystis* biomass ratios, evidencing that physiological variations in MC synthesis at the individual level have a negligible effect on the whole population toxin contents and, instead, the dynamics of chemotypical subpopulations effectively control the average toxicity of the bloom.

5. *Comparado con métodos moleculares, el análisis mediante espectrometría de masas MALDI-TOF permite determinar rápidamente la proporción de cepas productoras de microcistinas en poblaciones naturales. La proporción de quimiotipos tóxicos en la población está linealmente correlacionada con el ratio Microcistinas totales/biomasa de* Microcystis*. Dicha correlación demuestra que las variaciones fisiológicas en la síntesis de microcistinas a nivel individual no tienen un efecto significativo en el contenido medio de*

toxina de la población y, por tanto, la dinámica de las subpoblaciones quimiotípicas es el factor que determina de modo efectivo la toxicidad relativa de los afloramientos.

6. *Microcystis* oligopeptide chemotypes in natural systems segregate among benthic and pelagic habitats. In the water column, chemotypes display a marked, non-cyclic seasonal dynamics, which result from asynchronous proliferation and sedimentation of individual chemotypes. Altogether, these findings demonstrate that *Microcystis* oligopeptide chemotypes interact differently with their environment and therefore present different ecological traits.

6. *Durante el periodo estival, las subpoblaciones quimiotípicas del género* Microcystis *en sistemas naturales se encuentran segregadas entre los hábitats béntico y pelágico. En la columna de agua, la composición de quimiotipos está sujeta a una fuerte dinámica no cíclica, causada por procesos de sedimentación y proliferación asincrónicos entre subpoblaciones. Conjuntamente, estos hallazgos demuestran que las subpoblaciones quimiotípicas del género* Microcystis *interaccionan de modo distinto con su entorno y, por tanto, muestran propiedades ecológicas distintas.*

7. The highly patchy cyanobacterial distribution during bloom events can be successfully addressed by remote sensing, while the spatiotemporal fluctuations in toxin concentrations can be explained by rapid analysis of subpopulations by MALDI-TOF MS. The combined use of remote sensing techniques, conventional water sampling and analysis of cyanobacterial chemotypes allows the efficient monitoring of cyanobacterial blooms and thereby reduces the most important sources of uncertainty in the assessment of the risks associated to bloom events and enables rapid decision making by water managers.

7. *El uso de técnicas de teledetección permite abordar la marcada heterogeneidad en la distribución de cianobacterias en afloramientos. Asimismo, el análisis de subpoblaciones quimiotípicas mediante espectrometría de masas MALDI-TOF permite explicar las fluctuaciones espaciotemporales en la concentración de toxina durante episodios de bloom. El uso combinado de técnicas de teledetección, junto con muestreos convencionales y análisis de subpoblaciones quimiotípicas permiten una monitorización eficiente de los afloramientos de cianobacterias, reduciendo las principales fuentes de incertidumbre asociadas a la evaluación de riesgos derivados de su proliferación y permite una rápida toma de decisiones por parte de las autoridades competentes.*

9. Further experiments

In addition to the results presented above, other experiments have been performed to explore hypotheses that arose from several observations during the various field samplings undertaken in the course of this Thesis. While these preliminary experiments somewhat fell outside the initial scope of this thesis, we believed that the underlying questions could be strongly related to the dynamics of *Microcystis* chemotypes and were hence worthwhile exploring. This chapter aims to synthesize and discuss the results obtained in these experiments, which constitute an initial approach to the topic and a basis for future investigations.

9.1. Background

With the aim of investigating the settling fluxes of the different *Microcystis* chemotypes (see Chapter 5), we installed a number of sediment traps at the pelagic sampling point in Valmayor reservoir. Microscopic examinations of the formaldehyzed samples gathered from these traps revealed the presence of *Microcystis* colonies that presented epiphytic growth of the cyanobacterium *Pseudanabaena mucicola*. Interestingly, not all *Microcystis* colonies present showed filaments of *Pseudanabaena* growing in their mucilage and, instead, both colonies with and without (or with marginal amounts of) epiphytic *Pseudanabaena* coexisted in the same samples (Fig. 9.1a). Such coexistence suggested that the colonization of *Microcystis* colonies by *Pseudanabaena* might occur on a selective basis. Moreover, in some cases, dead colonies (i.e. *Microcystis* free slimes) that were fully colonized by filaments of *Pseudanabaena* could be observed (Fig. 9.1b).

The occurrence of filaments of *Pseudanabaena* growing in the mucilage of *Microcystis* is a rather common phenomenon in natural systems (e.g. figs 9.1c and d). However, to our knowledge, literature addressing the interaction between *Microcystis* and *Pseudanabaena* is almost non-existing. In light of this gap of knowledge, we believe that exploring the nature of this interaction is worthwhile. In particular, the experiments presented herein, constitute an initial exploration of two main questions. First, we aim to confirm the selectivity of *Pseudanabaena mucicola* to colonize the mucilage of *Microcystis* and identify possible features that might determine this interaction to occur. Second, we aim to identify the effects of this ecological interaction on *Microcystis* in the water column and thereby assess whether this interaction (which putatively occurs with some degree of selectivity) could in principle contribute to the dynamics and succession of *Microcystis* strains in the water column.

To carry out the different laboratory experiments, we isolated the *Pseudanabaena* sp. strain UAM 700 from Valmayor reservoir and maintained it as a monoclonal culture with BG11 liquid medium.

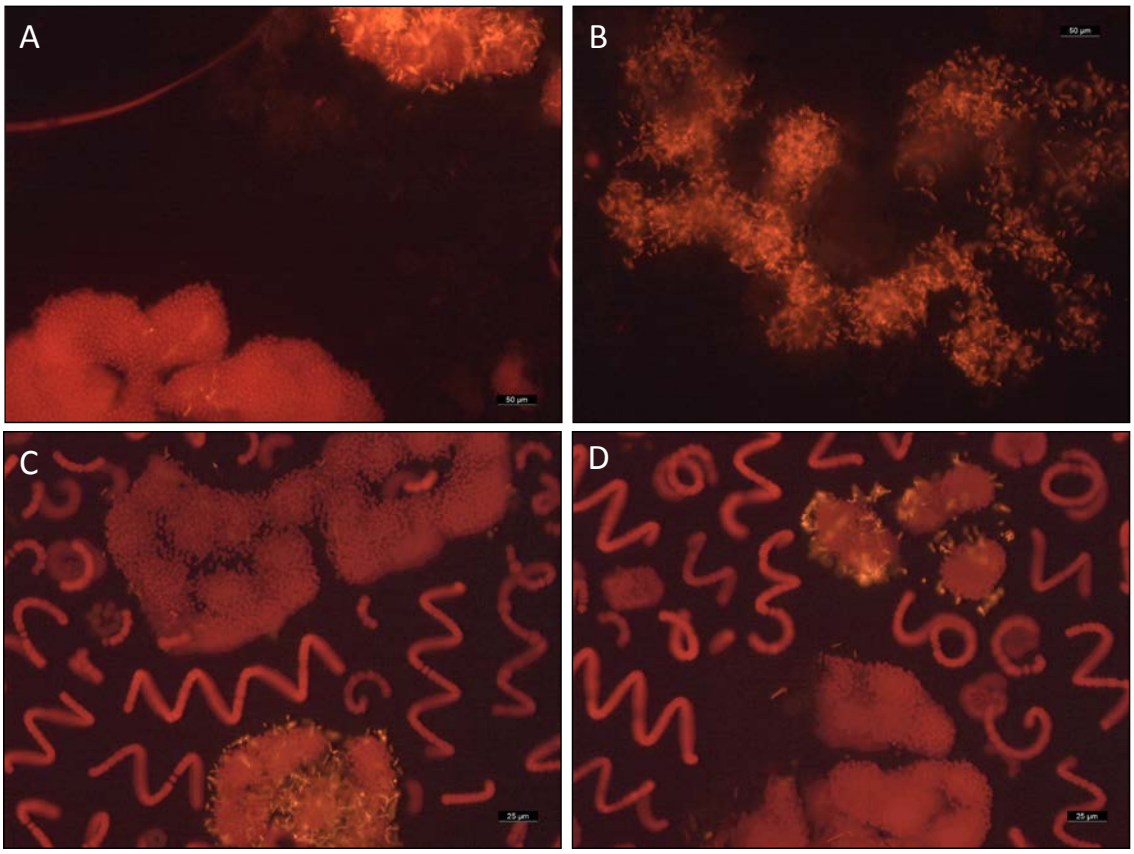

Figure 9.1. Epifluorescence micrographs of samples containing *Microcystis* colonies with epiphytic growth of *Pseudanabaena mucicola*. A) Co-existence of *Microcystis* colonies with and without epiphytic *Pseudanabaena* collected from sedimentation traps installed in Valmayor reservoir. B) "Empty" mucilage of a *Microcystis* colony fully occupied by filaments of *Pseudanabaena*. C) and D) Coexistence of *Microcystis* colonies with and without epiphytic *Pseudanabaena* collected from a natural subsurface water sample from San Juan reservoir (Central Spain).

9.2. Selectivity in the *Microcystis* - *Pseudanabaena* interaction

In order to evaluate the hypothesis regarding the existence of selectivity in the interaction between *Microcystis* and *Pseudanabaena*, three different colonial strains of the genus *Microcystis* (table 9.1) were inoculated with 10^5 filaments of the *Pseudanabaena sp.* strain UAM700. Experiments were carried out in 50mL Erlenmeyer flasks with BG11 liquid medium, kept at 29°C under continuous light fluorescent light of 50 µmol photons $m^{-2}s^{-1}$. The evolution of the interaction was monitored by regular microscopic inspection.

Strain name	Species	Colonial /Unicellular	Origin
UAM-KIN	*M. aeruginosa*	Colonial	See of Galilee (Israel)
UAM2C1B	*M. novacekii*	Colonial	Cazalegas reservoir (Central Spain)
UAM2C1F	*M. aeruginosa*	Colonial	Cazalegas reservoir (Central Spain)

Table 9.1. Microcystis strains used in the selectivity experiments.

Interestingly, microscopic inspection of the co-cultures at t_0, t_4 and t_8 revealed that the outcome of the interaction was dramatically different among *Microcystis* strains. *Pseudanabaena* filaments did not access the mucilage of the strain UAM2C1F and *Microcystis* colonies remained unaffected during the whole culture cycle (Fig. 9.2). In contrast, in the case of the *M. novacekii* strain UAM2C1B, *Pseudanabaena* filaments rapidly colonized the mucilage and proliferated significantly within the colony (fig. 9.4). However, microscopic inspection did not reveal any evident changes in the physiological state or growth of *Microcystis.* Strikingly, *M. aeruginosa* strain UAM-KIN (isolated from a bloom sample from the Sea of Galilee, kindly provided by Dr. Assaf Sukenik) showed dramatic effects after the inoculation of *Pseudanabaena.* In the case of this strain, filaments rapidly colonized the mucilage and strongly proliferated within it, while the density of *Microcystis* cells in the colonies rapidly declined with time. After a period of 8 days, the mucilage was massively occupied by *Pseudanabaena* and *Microcystis* cells in the colony were almost absent (Fig. 9.6).

The striking differences among strains confirmed the selective nature of the *Microcystis* - *Pseudanabaena* interaction. In particular, we could identify three situations: First, colonies (strain UAM2C1F) that grow normally in their presence and whose mucilage is not accessible to filaments of *Pseudanabaena* (onwards termed “resistant” strains). A second situation is exemplified by strain UAM2C1B, whose mucilage is readily accessible to *Pseudanabaena* filaments, where they strongly proliferate, but whose presence does not exert evident effects in the growth of *Microcystis* (onwards referred to as “indifferent” strains). Lastly a third kind of strain (UAM-KIN) could be observed, with colonies being rapidly and strongly colonized by *Pseudanabaena*, whose rapid growth leads to a dramatic decline in *Microcystis* cells in the colony and eventually to their disappearance (onwards termed “susceptible” strains).

These findings immediately led to wonder which factors could drive the observed selectivity. Our initial observations of naturally occurring *Pseudanabaena*-containing *Microcystis* colonies in the sediment traps at Valmayor reservoir, chronologically matched with

the pelagic disappearance of a particular chemotype from the water column, which could not be found later in the sediment traps (namely CT11, see Chapter 5). Therefore, the idea that the interaction between *Microcystis* and *Pseudanabaena* might occur on a chemotype-selective basis was appealing. A putative sedimentation of *Pseudanabaena*-containing colonies, would mask the oligopeptide signatures of *Microcystis* chemotypes, and might have been misinterpreted as a lack of sedimentation in our study (Chapter 5). To address this question, we analyzed the oligopeptide patterns of the three different *Microcystis* used by MALDI-TOF MS and compared them with that of *Pseudanabaena* UAM700. However, the comparison of oligopeptide compositions of the different strains used in the selectivity experiments did not reveal any differentiating oligopeptide that could explain or be involved in the differences in susceptibility to the interaction observed among *Microcystis* strains (table 9.2).

Alternatively, we analyzed the structure of the mucilage of the three different *Microcystis* strains by Low Temperature – Scanning Electron Microscopy (LT-SEM) to assess whether differences in the structure of the mucilage could determine the entry of *Pseudanabaena* to the mucilage and eventually, the subsequent effects on *Microcystis.* Interestingly, the inspection of single colonies by LT-SEM revealed marked differences in the mucilage structures among the studied *Microcystis* strains. Resistant strain UAM2C1F showed a massive, “concrete-like” mucilage, which densely surrounds individual cells (fig. 9.3). In contrast, susceptible *Microcystis* strain UAM-KIN displayed a mucilage with a diffuse structure, plenty of irregularities and cavities. In addition, we were able to distinguish *Pseudanabaena* filaments within the colony of strain KIN. Somewhat unexpectedly, filaments were not found in the cavities of the mucilage, but they are embedded within the mucilage (fig. 9.5). Lastly, colonies of the “indifferent” strain UAM2C1B displays in general a mucilage with a diffuse structure. However, cells are closely packed together and in these areas, mucilage with greater consistency densely surrounds the groups of cells (fig.9.7).

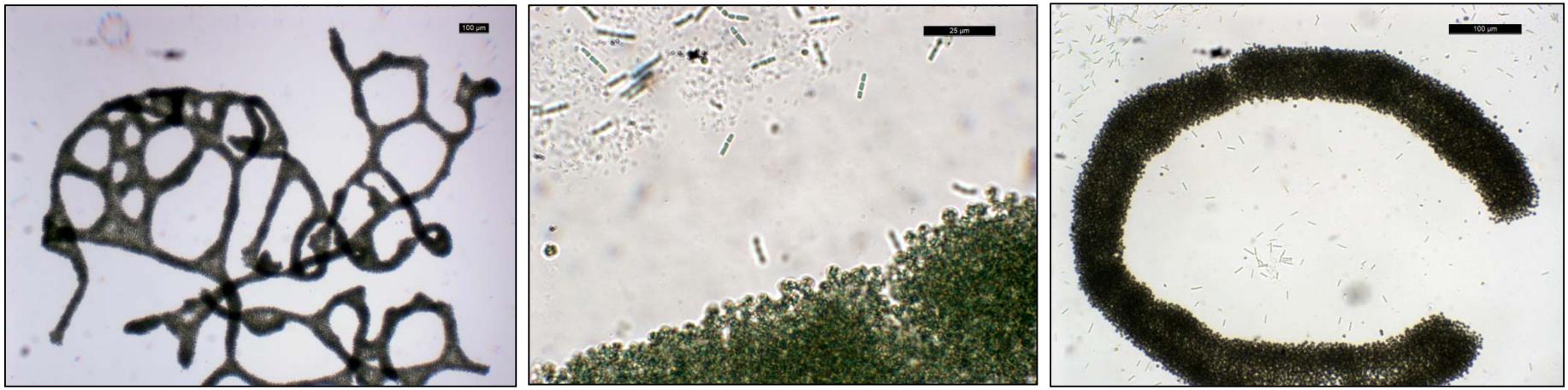

Figure 9.2. Micrographs of the co-culture of *Microcystis* strain UAM2C1F and *Pseudanabaena* sp. strain UAM700 at t=0 (left), t=4 d (center) and t=8 d (right). The mucilage was not accessible for filaments of *Pseudanabaena* and colonies remained unaffected.

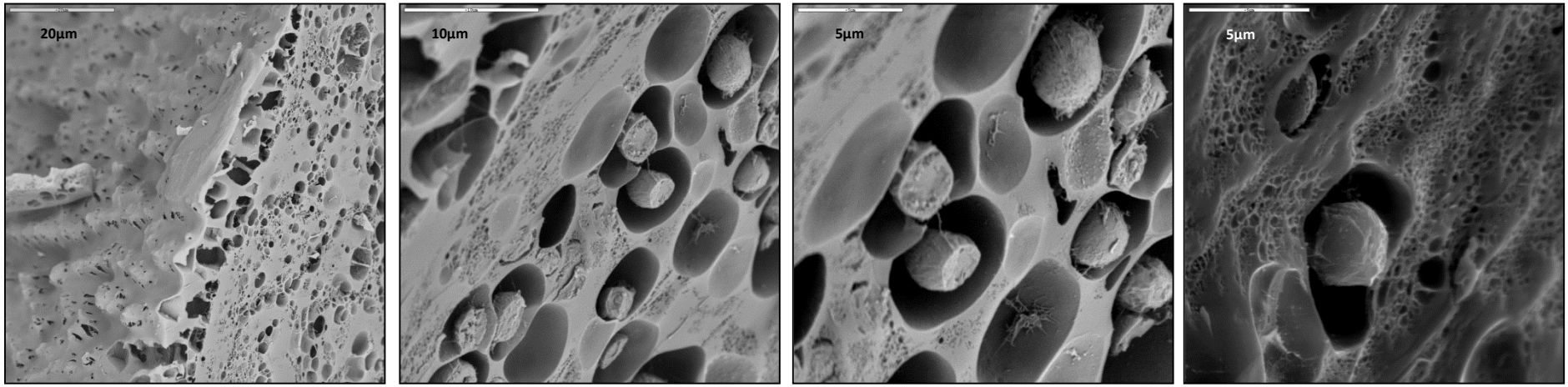

Figure 9.3. LT-SEM images of a colony of the *Microcystis* strain UAM2C1F. Colonies present a massive structure with a dense mucilage, where cells are embedded.

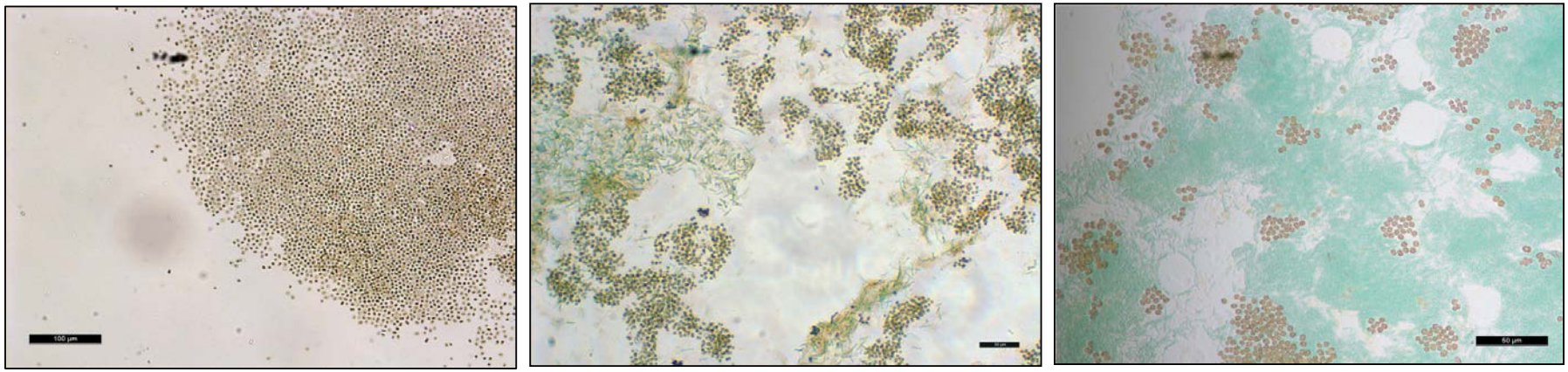

Figure 9.4. Micrographs of the co-culture of *Microcystis* strain UAM-KIN and *Pseudanabaena* sp. strain UAM700 at t=0 (left), t=4 d (center) and t=8 d (right). *Pseudanabaena* filaments rapidly accessed the slime and proliferated, while *Microcystis* cell density within the colony sharply declined. At day 8, *Pseudanabaena* filaments densely occupied most of the colony and only marginal amounts of *Microcystis* cells remained in the colony.

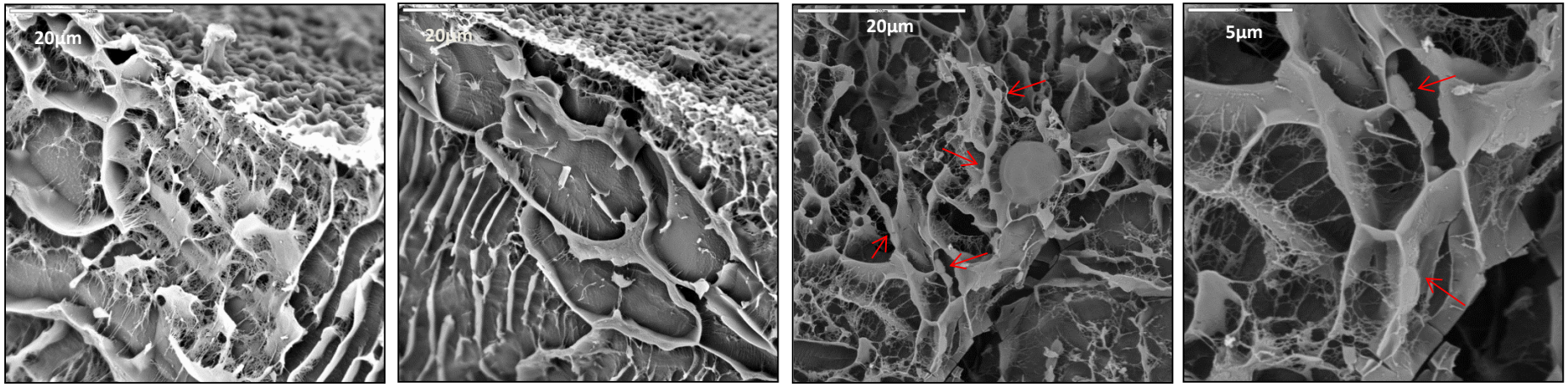

Figure 9.5. LT-SEM images of colonies of the *Microcystis* strain UAM-KIN. Colonies present a diffuse mucilage structure with numerous cavities. *Pseudanabaena* filaments are indicated by red arrows.

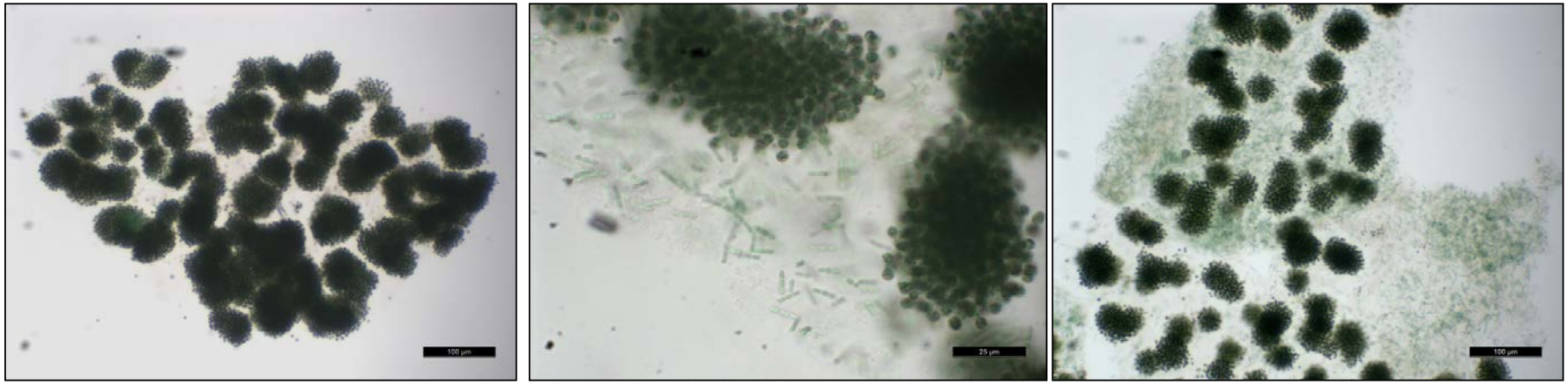

Figure 9.6. Micrographs of the co-culture of Microcystis strain UAM2C1B and Pseudanabaena sp. strain UAM700 at t=0 (left), t=4 d (center) and t=8 d (right). *Pseudanabaena* filaments rapidly colonized the mucilage of the strain UAM2C1B and proliferated, but no apparent effects on the cells of *Microcystis* were observable.

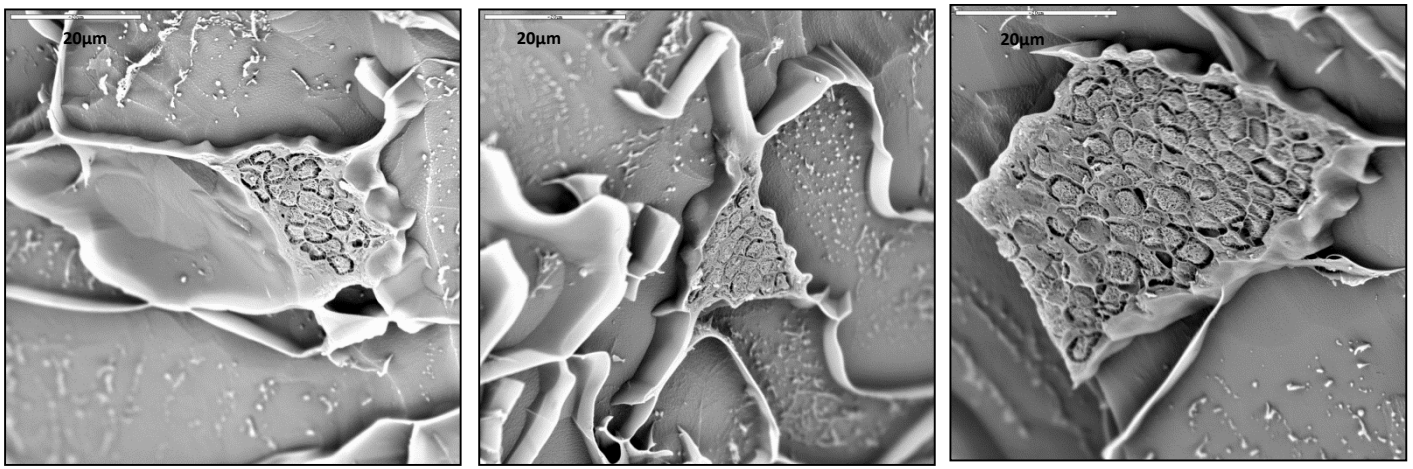

Figure 9.7. LT-SEM images of a colony of the *Microcystis* strain UAM2C1B. *Microcystis* cells are densely packed together in small subcolonies of dense mucilage, while the mucilage between subcolonies diplays a diffuse, loosely bound structure.

	Pseudanabaena sp. UAM700	*M. aeruginosa* UAM-KIN	*M. novacekii* UAM2C1B	*M. aeruginosa* UAM2C1F
Oligopeptide compositions	Aeruginosin 98Aa (609.32 Da)	Aeruginosin 98Aa (609.32 Da)	[Asp3]-Microcystin LR (981.5 Da)	Microcystin LR (995.6 Da)
	Aeruginosin 98Ba (575.4 Da)	Aeruginosin 89a (637.31 Da)	[Asp3]-Microcystin YR (1031.5 Da)	Microcystin YR (1045.5 Da)
	Microginin 764 (765.4 Da)	Microcystin LR (995.6 Da)	Microcystin LR (995.6 Da)	Microcystin RR (1038.6 Da)
	Microginin FR9 (751.4 Da)	Microcystin YR (1045.5 Da)	Cyanopeptolin 1014 (1015.6 Da)	
		Kasugamide C (639.3 Da)		

Table 9.2. MALDI-TOF MS-retrieved oligopeptide compositions of the cyanobacterial strains used in the selectivity experiments. Protonated mass/charge ratios (Da) are indicated in parenthesis.

9.3. Effects of the interaction

Alternatively to the exploration of the sources of selectivity, we were interested in characterizing the effects of the interaction. If the interaction selectively (and negatively?) affects particular strains in natural systems, it could in principle constitute a further ecological process that potentially modulates the composition and dynamics of co-existing *Microcystis* strains in natural systems. In particular, we evaluated whether the proliferation of *Pseudanabaena* in the mucilage of *Microcystis* causes an increase in the overall density of the colony, resulting in loss of buoyancy and thereby to selective settling of susceptible subpopulations.

To explore this question, we designed a simple experimental setup consisting of 20 cm high sterile tubes filled with BG11 medium. The walls of the lower 16cm of the tubes were covered with black opaque plastic to reduce light availability in the lower part of the tubes. By co-cultivating *Microcystis* and *Pseudanabaena* we could assess losses of buoyancy in a visual fashion, as well as easily collect settled and buoyant colonies separately for microscopic inspection. In our experiments, we evaluated a putative increase in the density of colonies resulting from *Pseudanabaena* association. We used the "indifferent" *Microcystis* strain UAM2C1B, whose mucilage was actively colonized by *Pseudanabaena* in the selectivity

experiments, but did not display adverse effects. *Pseudanabaena* sp. UAM700 (10^5 filaments) was added to 3 of the tubes, while the 3 remaining tubes served as a control.

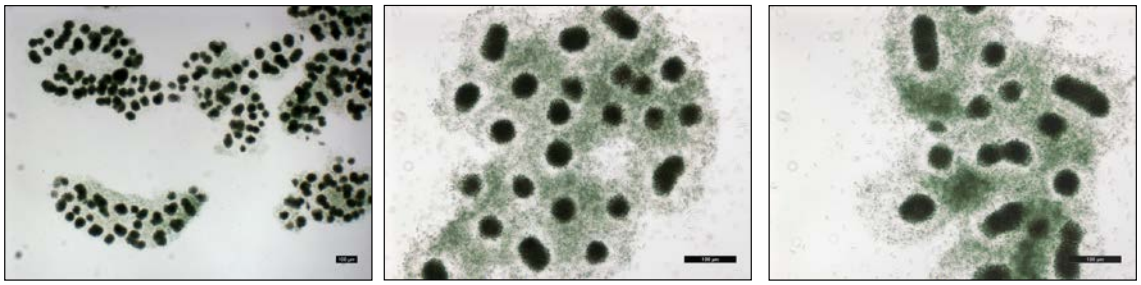

Figure 9.8. Micrographs of settled colonies of strain UAM2C1B three days after the inoculation of *Pseudanabaena* sp. UAM700.

After a period of 3 days, control colonies (i.e. no *Pseudanabaena* added) maintained positive buoyancy and remained in the surface of tubes, while those in presence of *Pseudanabaena* accumulated in the bottom of their respective tubes (fig. 9.8). To unequivocally demonstrate an increase in the overall density of the colonies in the presence of epiphytic *Pseudanabaena,* colonies from both control and treatment tubes were carefully collected and transferred to centrifuge tubes containing a previously generated Percoll (GE Healthcare Life Sciences) density gradient solution (1.23% Isotonic Percoll solution in NaCl 1.5M, generating an isopicnic layer in the middle of the centrifuge tubes of 1.008 g cm^{-3}). Once carefully loaded on the gradient solution, the tubes were centrifuged at 500g's during 15 minutes and colonies migrated to their respective isopicnic layer. After centrifugation, control colonies accumulated next to the surface, while colonies with associated *Pseudanabaena* accumulated in the bottom, evidencing a clear increase in the density of the colonies as a result of the epiphytic growth of *Pseudanabaena* (fig. 9.9).

Figure 9.9. Partitioning of colonies of *Microcystis* strain UAM2C1B after Percoll gradient centrifugation. Colonies without (left) and with (right) associated *Pseudanabaena.* (1.23% isotonic Percoll solution in NaCl 1.5M, generating an isopicnic layer in the middle of the tubes of 1.008 g cm^{-3})

9.4. Discussion

Although being a commonly observed phenomenon, epiphytic growth of bacteria and other organisms around phytoplankton in the so-called phycosphere (Cole 1982) has been largely overlooked as a potential driver for phytoplankton growth and dynamics (Paerl & Millie 1996, Salomon et al. 2003). Colonizing organisms of the phycosphere in general, and the mucilage of *Microcystis* colonies in particular, likely benefit from this microenvironment. The mucilage of *Microcystis* constitutes a rich source of nutrients and organic carbon. Besides, *Microcystis* colonies regulate their buoyancy and stay at depths that allow photosynthetic growth and likely provide shelter against grazing. Reported epibionts colonizing the mucilage of *Microcystis* include heterotrophic bacteria (most commonly α-proteobacteria), fungi, archea (Dziallas & Grossart 2011) and other cyanobacteria, as shown herein.

Epiphytic growth of these organisms may potentially have a number of effects on the host organism, both positive and negative. Potential positive effects of epibiota growth on the host include increased CO_2 availability due to respiration as well production of growth factors like vitamins by associated organisms (Kühl et al. 1996). However, epiphytic growth may also dramatically reduce light availability for the host and hinder the access of the host to nutrients in the water column by reduced diffusion (Wahl et al. 2010).

In particular, the interaction between *Microcystis* spp. and *Pseudanabaena mucicola* is a common phenomenon, although the nature, consequences and implications of such interaction are largely unknown. To our best knowledge, no peer-reviewed scientific works addressing this topic are available. Exceptionally, available data on this interaction could be found in a doctoral dissertation in German language by Dr. Tilo Ilhe (Ilhe 2008) after intensive search for data on the topic. In this work, the occurrence of *Microcystis* colonies with epiphytic *Pseudanabaena* in lake Quitzdorf (Germany) was recorded over a two years period. Ilhe identified that the occurrence of epiphytic *Pseudanabaena* was more frequently observed in morphospecies *M. aeruginosa* and *M. novacekii,* while its presence around colonies of *M. viridis and M. wesenbergii* was slightly less frequent. Somewhat unsurprisingly, no differences between toxigenic (*mcy+*) or non-toxigenic *(mcy-)* strains in terms of occurrence of epiphytic *Pseudanabaena* were evident. Similarly, MC cell quota among toxigenic did not correlate with the epiphytic association of *Pseudanabaena.*

In our experiments we show that epiphytic growth of *Pseudanabaena* occurs on a selective basis and may exert different effects on *Microcystis* colonies. We could identify "resistant" *Microcystis* strains that could not be colonized by *Pseudanabaena* and grew normally in co-culture. Alternatively, the mucilage of other "indifferent" strains was readily accessible for *Pseudanabaena* filaments, where they could grow massively, although no evident adverse effects on *Microcystis* could be observed. Lastly, we also identified "susceptible" *Microcystis* strains whose mucilage was rapidly colonized by *Pseudanabaena*. In these colonies, the rapid epiphytic growth of *Pseudanabaena* was followed by a dramatic decline in *Microcystis* cells in the colony, which eventually disappear. These findings indicate that the interaction, when taking place, seems to be detrimental for *Microcystis*, thus suggesting an antagonistic interaction between the two taxa.

In light of the marked differences observed among strains, we evaluated whether the selectivity in the interaction could be related to oligopeptide compositions of particular strains. While oligopeptides remain largely within the producing cells, the presence of oligopeptides (e.g. microcystins) has also been localized in the mucilage (Young et al. 2005) and could in principle modify its chemical environment. However, we could not find any distinctive oligopeptide among strains that might explain the observed *Pseudanabaena* preferences. Instead, the differences in the structure of the mucilage seems a more plausible possibility, as demonstrated by the inspection of colony mucilage structures by LT-SEM. Readily accessible mucilages for *Pseudanabaena* (strains UAM-KIN and UAM2C1B) showed a diffuse structure,

dominated by loosely bound EPS, presenting numerous cavities and irregularities. Instead, resistant strain UAM2C1F displays a massive, tightly bound mucilage, which was clearly inaccessible for *Pseudanabaena.* These findings suggest that the structure of the mucilaginous cover of *Microcystis* colonies may act as a first barrier for *Pseudanabaena* colony invasion. However, access to the mucilage by *Pseudanabaena* does not necessarily lead to detrimental effects for *Microcystis* cells, as observed for the strain UAM2C1B. Whether the dense mucilage surrounding groups of *Microcystis* cells (i.e. subcolonies, fig. 9.7) of strain UAM2C1B plays a role in the abolition of negative effects in this strain despite the strong presence of *Pseudanabaena* filaments in the mucilage, remains to be further explored. However, we could also observe that colonies with epiphytic growth of *Pseudanabaena* are subject to loss of buoyancy, likely due to an increase in the overall density of the colony. Consequently, epiphytic growth of *Pseudanabaena* in natural systems, even when not causing physiological decay of the colony, may result in the loss of buoyancy and eventual settling of colonies. In light of the observed selectivity and effects on buoyant colonies, epiphytic association of *Pseudanabaena* might represent an ecological process mediating the selective settling and/or decay of particular *Microcystis* strains and thereby might contribute to the dynamics of coexisting *Microcystis* strains in the water column.

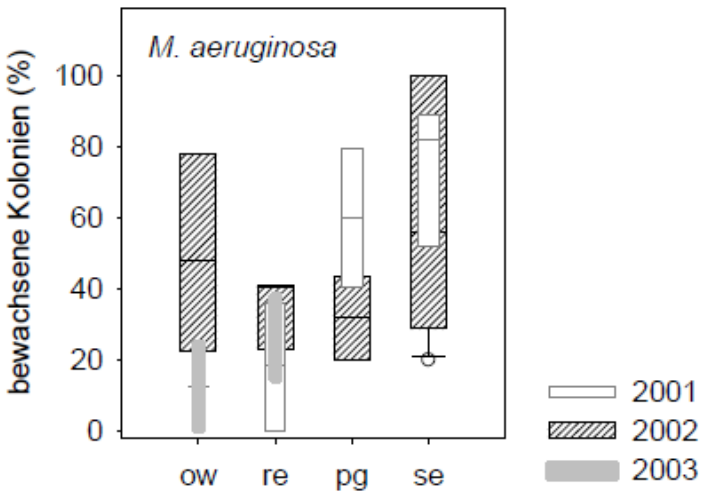

Figure 9.10. Proportion of *Microcystis aeruginosa* colonies with epiphytic *Pseudanabaena* during different phases of its lifecycle in Quitzdorf reservoir (Germany) during 2001-2003. Y-axis "bewachsene Kolonien (%)" stands for colonized colonies (%). ow: overwintering; re: recruitment; pg: pelagic growth; se: sedimentation. Reproduced from Ilhe (2008).

Cyanobacteria of the genus *Pseudanabaena* are typical inhabitants of shallow benthic habitats. Conversely, pelagic habitats do not seem suitable for *Pseudanabaena* spp., as the access to photic depths is strongly restricted due to their lack of gas vesicles. In this situation,

the attachment of *Pseudanabaena* to buoyant *Microcystis* colonies might provide adequate light conditions in an otherwise aphotic environment. Ilhe could observe that the occurrence of *M. aeruginosa* and *M novacekii* colonies displaying epiphytic P increased with time, being lowest during recruitment phases, steadily increasing during pelagic growth, and reaching maxima toward autumnal sedimentation periods (Fig, 9.10; Ilhe, 2008). In light of this data it is reasonable to propose that *Pseudanabaena mucicola* constitutes a differentiated ecotype that associates to *Microcystis* colonies as a strategy to colonize pelagic habitats. Much like in the case of *Microcystis,* the recruitment phase would constitute for *Pseudanabaena mucicola* the initial inoculum into the water column, followed by pelagic growth and reaching maximum epiphytic development toward autumnal sedimentation. Maximum proliferation toward the end of the season would ensure sufficient biomass to resist the overwintering phase on the sediments. Benthic overwintering under dark conditions likely represents a stage of latency associated to substantial population losses for *Pseudanabaena*, whose abundance in fact shows to be lowest when re-entering the water column in the recruitment phase.

While these results only represent an initial approximation to the study of this interaction, we could demonstrate that the interaction between *Pseudanabaena* and *Microcystis*, far from being unspecific, occurs on a selective basis that might be related to differences in the structural features of the mucilage of *Microcystis* colonies. Our preliminary observations indicate that the association of *Pseudanabaena* to the mucilage, when taking place, exerts negative effects on *Microcystis*, manifested either by the decay of *Microcystis* cells within the colony or the loss of buoyancy and eventual sedimentation of colonies due to an increase of their overall density. Thus, this interaction seems to be of antagonistic nature and may arguably constitute a further ecological process shaping the complex dynamics of *Microcystis* strains in the water column. Further work will aim to characterize the effects of the interaction and its nature, as well as to further elucidate the features that determine such interaction to occur.

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