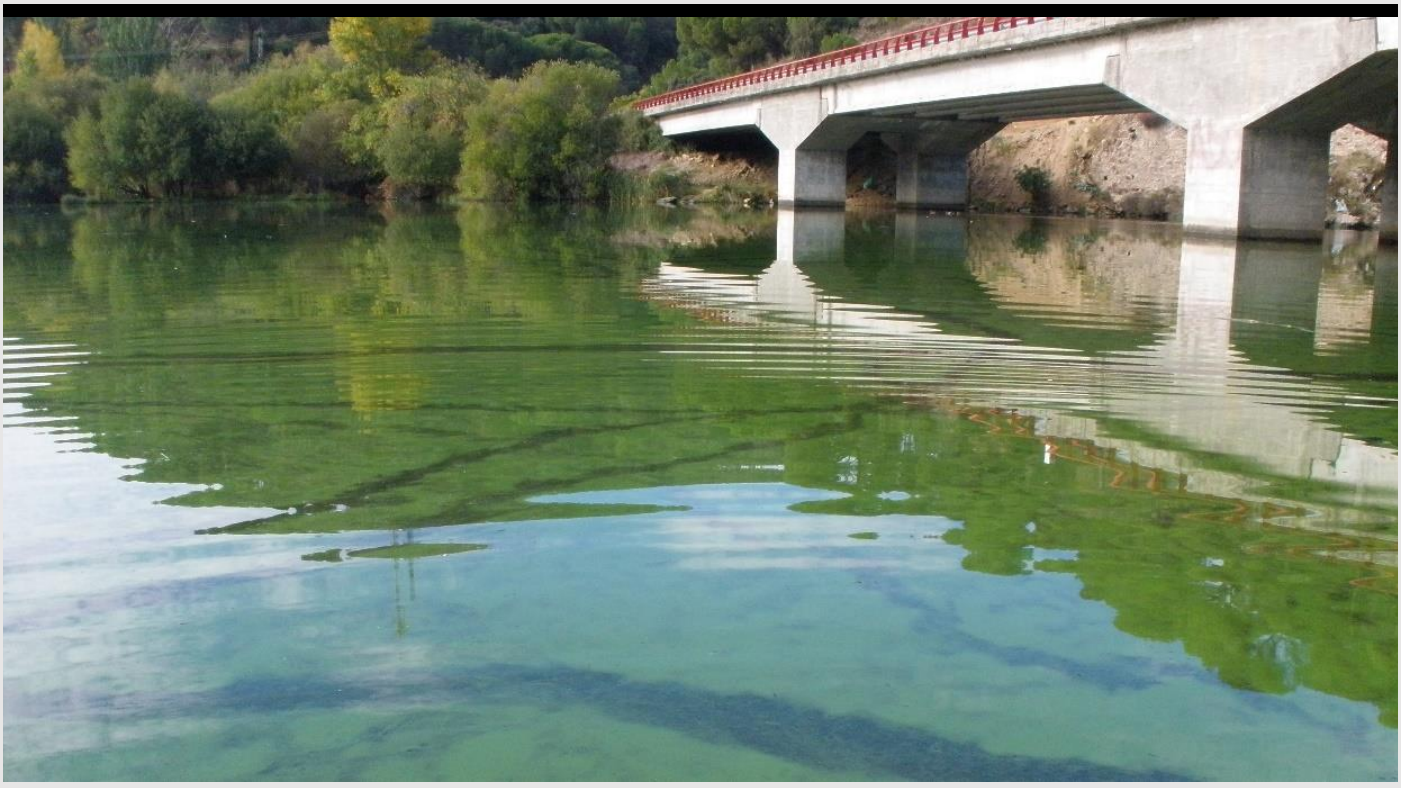
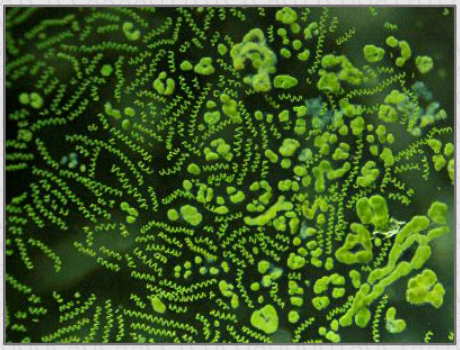
UNIVERSIDAD AUTONOMA DE MADRID

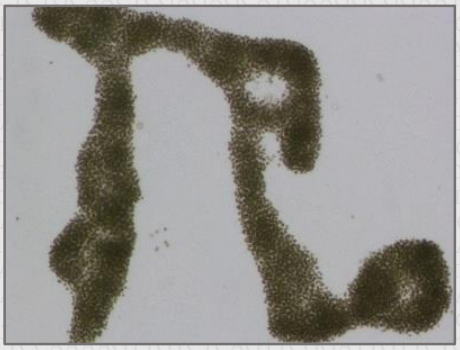
Doctoral Dissertation

Biodegradation of microcystins by freshwater bacteria:

New genetic and ecological perspectives







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Departamento de Biología

Biodegradation of microcystins by freshwater bacteria: new genetic and ecological perspectives

Biodegradación de microcistinas por bacterias de aguas dulces: nuevas perspectivas genéticas y ecológicas

Doctoral Dissertation
Tesis Doctoral

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A mi familia

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Abstract

Freshwater cyanobacterial blooms are frequent phenomena increasing worldwide, and a matter of concern due to the associate negative effects on the ecosystem and water uses. Some bloom-forming cyanobacterial strains also produce toxins (cyanotoxins) that adversely affect humans and other animals. Among the array of toxins produced by these massive cyanobacterial proliferations, microcystins (a group of potent hepatotoxins) are the most frequent and widespread cyanotoxins in freshwater systems. The microcystins are structurally stable molecules resistant to physical and chemical processes naturally occurring in the environment and also to those applied in conventional water treatment technologies. Therefore, the study of cyanobacterial blooms and microcystins allow to improve water management strategies and water treatment processes for cyanobacterial and toxin removal.

Cyanobacterial massive proliferations coexist and exert different interactions with other microorganisms present in the aquatic environment. A group of bacteria with the ability to degrade efficiently microcystins is frequently associated with toxic cyanobacterial blooms. Despite their relevance for the removal of microcystins in the environment, this bacterial community has been scarcely studied worldwide and, specially, in the Mediterranean region. Therefore, the studies comprised in this thesis onsets on the assessment of the biological microcystin degradation capacity in a reservoir from central Spain, and further dives into the genetic and ecological characteristics, as well as the bacterial diversity underlying this biodegradation process. The thesis approaches the freshwater bacteria associated with cyanobacterial blooms from different angles, using various analytical methods and including a three-year field study.

Once a microcystin biodegradation activity was detected in the reservoir of study, the following sequence of events was developed: optimization of an efficient method for the isolation of microcystin-degrading bacteria, isolation and taxonomic identification of the bacterial strains and, then, detection of the genes involved in the microcystin degradation process (*mlr* gene cluster). Two different bacterial genotypes possessing and lacking *mlr* genes (mlr^+ and mlr^-, respectively) were identified within the microcystin-degrading bacterial community. Afterwards, an evaluation of the microcystin degradation efficiencies between each mlr^+ and mlr^- genotype under the presence of alternative carbon and nitrogen compounds was performed. The identification of a bacterial isolate that exhibited high microcystin degradation efficiency in presence of a wide range of alternative carbon and

nitrogen concentrations lead to the development and optimization of a method for an efficient use of this strain as a biological tool for the removal of microcystins in water.

The identification of two bacterial genotypes (*mlr*$^{+}$ and *mlr*$^{-}$) coexisting within the microcystin-degrading bacterial community in the environment lead to the development of a diversity study on the bacterial community associated with toxic cyanobacterial blooms. Finally, a seasonal dynamic study on the two microcystin-producing and microcystin-degrading bacterial communities, as well as the influence of the most relevant physicochemical factors on their temporal shifts, was carried out to make significant inroads into the interaction they have in the environment. The development of successful water management strategies to prevent, halt and reduce cyanobacterial bloom episodes and their toxins necessary requires a good knowledge on both the temporal dynamics of these blooms and the associated bacterial community able to degrade the microcystins.

Resumen

Las proliferaciones (o *blooms*) de cianobacterias en aguas dulces son un fenómeno frecuente que está aumentando a escala global y un tema de preocupación debido a los efectos nocivos que acarrean sobre el ecosistema y los usos del agua. Algunas cepas de cianobacterias formadoras de *blooms* también producen toxinas (cianotoxinas) que afectan negativamente a los humanos y otros animales. Entre estas cianotoxinas, las microcistinas (un grupo de potentes hepatotoxinas) son las más frecuentes y las más ampliamente distribuidas en los ecosistemas de agua dulce. Las microcistinas son moléculas estructuralmente estables y resistentes a procesos físicos y químicos, tanto a los que tienen lugar de manera natural en el medio ambiente como a los aplicados en tecnologías convencionales de tratamiento del agua. Por tanto, el estudio de las cianobacterias y cianotoxinas permite mejorar las estrategias de gestión del agua y los procesos para su eliminación en plantas de tratamiento.

Las proliferaciones masivas de cianobacterias coexisten e interaccionan con otros microorganismos presentes en el medio acuático. Un grupo de bacterias específicas capaces de degradar de manera eficiente microcistinas está frecuentemente asociado a las proliferaciones masivas de cianobacterias. A pesar de su relevancia en la eliminación de dichas toxinas del medio acuático, esta comunidad bacteriana está escasamente estudiada a nivel global y, muy especialmente, en la región mediterránea. Esta tesis evalúa, en primer lugar, la capacidad de biodegradación de microcistinas en un embalse situado en el centro de España y, posteriormente, profundiza en las características genéticas y ecológicas, así como en la diversidad bacteriana que está detrás de este proceso de biodegradación. Esta tesis se enfoca sobre las bacterias de agua dulce asociadas a los *blooms* de cianobacterias desde diferentes ángulos, utilizando varios métodos analíticos e incluyendo un estudio de tres años de muestreos en campo.

Tras la detección de actividad de biodegradación de microcistinas en el embalse de estudio, se desarrolló la siguiente secuencia de eventos: optimización de un método eficiente de aislamiento de bacterias degradadoras de microcistinas, aislamiento e identificación taxonómica de las cepas degradadoras y, posteriormente, detección de los genes responsables del proceso de degradación de microcistinas (genes *mlr*). Se identificaron, dentro de la comunidad bacteriana degradadora, dos genotipos diferentes de bacterias en base a la presencia o ausencia de los genes *mlr* (mlr^+ y mlr^-, respectivamente). Posteriormente se evaluaron las eficiencias de degradación de microcistinas de cada uno de

los genotipos en presencia de otros compuestos de carbono y nitrógeno en el medio. La detección de una alta eficiencia de degradación de microcistinas, en presencia de un amplio rango de concentraciones de otros compuestos de carbono y nitrógeno en una de las bacterias aisladas, llevó al desarrollo y optimización de un método para un uso eficiente de dicha bacteria como herramienta biológica en la eliminación de microcistinas del agua.

La identificación de los dos genotipos (*mlr*$^{+}$ y *mlr*$^{-}$) de bacterias degradadoras de microcistinas coexistiendo en el medio acuático condujo al desarrollo de un estudio de diversidad sobre la comunidad bacteriana asociada a las proliferaciones de cianobacterias. Por último, se realizó un estudio de las dinámicas estacionales de las comunidades de cianobacterias productoras y bacterias degradadoras de microcistinas, así como la influencia de los factores fisicoquímicos más relevantes del medio acuático sobre ambas dinámicas estacionales. El desarrollo de estrategias adecuadas de gestión del agua para prevenir, frenar y reducir los episodios de *blooms* de cianobacterias y sus toxinas requiere, necesariamente, de un buen conocimiento de las dinámicas temporales de dichos *blooms* y de la comunidad bacteriana asociada capaz de degradar las microcistinas.

Abbreviations / Abreviaturas

¼ R2A: 4-fold diluted R2A medium

BLASTN: Basic local alignment search tool for nucleotide

bp: Base pairs

CE: Cell-free extract

Chl-*a*: Chlorophyll *a*

Cl.: Class

DNA: Deoxyribonucleic acid

dNTPs: deoxynucleotides triphosphate

DOC: Dissolved organic carbon

ESI: Electrospray ionization

HPLC: High performance liquid chromatography

HPLC-TOF: High performance liquid chromatography coupled to a time-of-flight mass spectrometry

ICBN: International code of botanic nomenclature

ICNP: International code of nomenclature of prokaryotes

LD_{50}: Lethal dose 50

m/z: Mass / charge ratio

MC: Microcystins

ML: Maximum likelihood

mlr^{-}: Absence of the complete *mlr* gene cluster

mlr^{+}: Presence of the complete *mlr* gene cluster

*mlr*A^{-}: Absence of the *mlr*A gene

*mlr*A^{+}: Presence of the *mlr*A gene

MSM: Mineral salts medium

NCBI: National center for biotechnology information

Or.: Order

PCA: Principal component analysis

PCR: Polymerase chain reaction

PKS: Polyketide synthase

PS: Peptide synthetase

qPCR: Quantitative polymerase chain reaction

rRNA: Ribosomal ribonucleic acid

TN: Total nitrogen

TOC: Total organic carbon

TOF: Time of fight

WHO: World health organization

λ_{max}: Maximum absorption wavelength

Abbreviations other than those listed here are defined in their first mention in the text.

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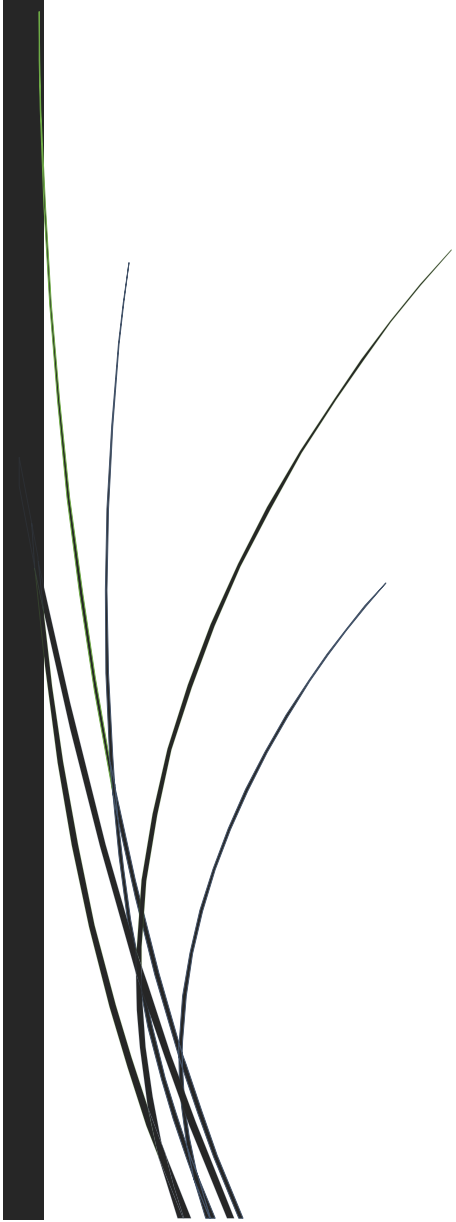


1. General introduction

1.1. Cyanobacteria and mass proliferations

1.1.1. General features of cyanobacteria

The cyanobacteria is a group of photoautrophic bacteria able to produce oxygenic photosynthesis associated with photosystems I and II (Castenholz and Waterbury, 1989) and comprises a wide morphological variety of prokaryotic microorganisms, ranging from unicellular to multicellular. In the oxygenic photosynthesis, cyanobacteria use light as energy source, water as an electron donor and produces oxygen as by-product. Because of that, they are considered the first organisms contributing with oxygen to the anoxic original atmosphere (Madigan et al., 2003).

The cyanobacterial cell size is typically less than 10 µm of diameter and, as prokaryotes, they have simple internal structure with lacking-membrane organelles (Bellinger and Sigee, 2010). Their genome is a circular, large and condensed double-stranded DNA molecule that constitutes the nucleoid, which is dispersed in the cytoplasm (Figure 1.1), and they are Gram negative bacteria (Madigan et al., 2003). The cyanobacteria may have a mucilage layer outside the cell wall, allowing the formation of colonies. Some may also contain gas vesicles (vacuoles) in their cytoplasm, allowing them to control their buoyancy in the water column for an optimal nutrient and/or light position for the photosynthesis (Walsby, 1987). Cyanobacteria possess a photosynthetic light-harvesting system located in the thylakoids that lie free in the cytoplasm close to the cell periphery. The photosynthetic system is mainly composed by chlorophyll *a* and other accessory pigments such as phycobilins. Phycobilins and their associated proteins (phycobiliproteins) are assembled into a macromolecular aggregate called phycobilisome, located at the surface of the thylakoids. The cyanobacteria may possess four types of phycobiliproteins that enable them to harvest a wide range of light spectra: phycocyanin (maximum absorption wavelength (λ_{max}) at 620 nm) and allophycocyanin (λ_{max}: 650 nm) are always present in cyanobacteria, and phycoerythrin (λ_{max}: 565 nm) and phycoerythrocyanin (λ_{max}: 568 nm) are present only in some species providing red colour to their cells (Edward, 2008). The presence of phycocyanin, together with the chlorophyll *a*, is the responsible for the cyanobacterial blue-green colour.

Some cyanobacteria have polymer inclusions in the cytoplasm, such as glycogen or cyanophycin granules (carbon and nitrogen reservoirs), and carboxysomes (compartments containing enzymes involved in the carbon fixation).

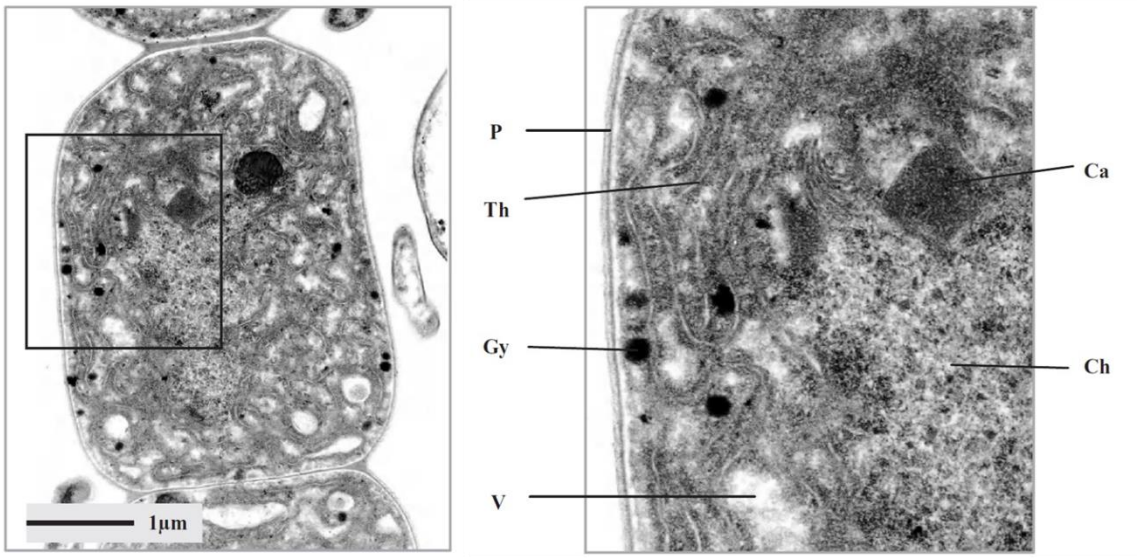


Figure 1.1. Transmission electron micrograph of a vegetative cell of Anabaena sp. (left) and the details of the structures (right). Ch: central region of chromatin (no membrane), Ca: carboxysome (polyhedral body), Gy: glycogen granule, Th: peripheral thylakoid, V: vacuole, P: thin peptidoglycan cell wall (Bellinger and Sigee, 2010)

Apart from the above mentioned characteristics, some cyanobacterial species form specialized cells, such as heterocysts and akinetes. The heterocysts are cells responsible for nitrogen fixation when the nitrogen concentrations in the environment are below their requirements. The reduction from the N_2 to the NH_4^+ is performed under anoxic conditions via the enzyme-complex nitrogenase. Thus, heterocysts present a thickened cell wall and lack the photosystem II, to avoid oxygen diffusion and oxygen production during photosynthesis. Since this photosystem is also responsible for the reducing power of the H_2O, the heterocysts communicate with vegetative cells to obtain fixed carbon compounds to act as electron donor for the N_2 reduction (Madigan et al., 2003). The akinetes are present in some filamentous cyanobacteria and consist in resistant cells containing reserve materials that are surrounded by a thickened cell wall that protect the organism under unfavourable environmental conditions (dryness, darkness, nutrient limiting conditions, etc.) (Sukenik et al., 2013). Some examples of the above mentioned cyanobacterial features are present in Figure 1.2.

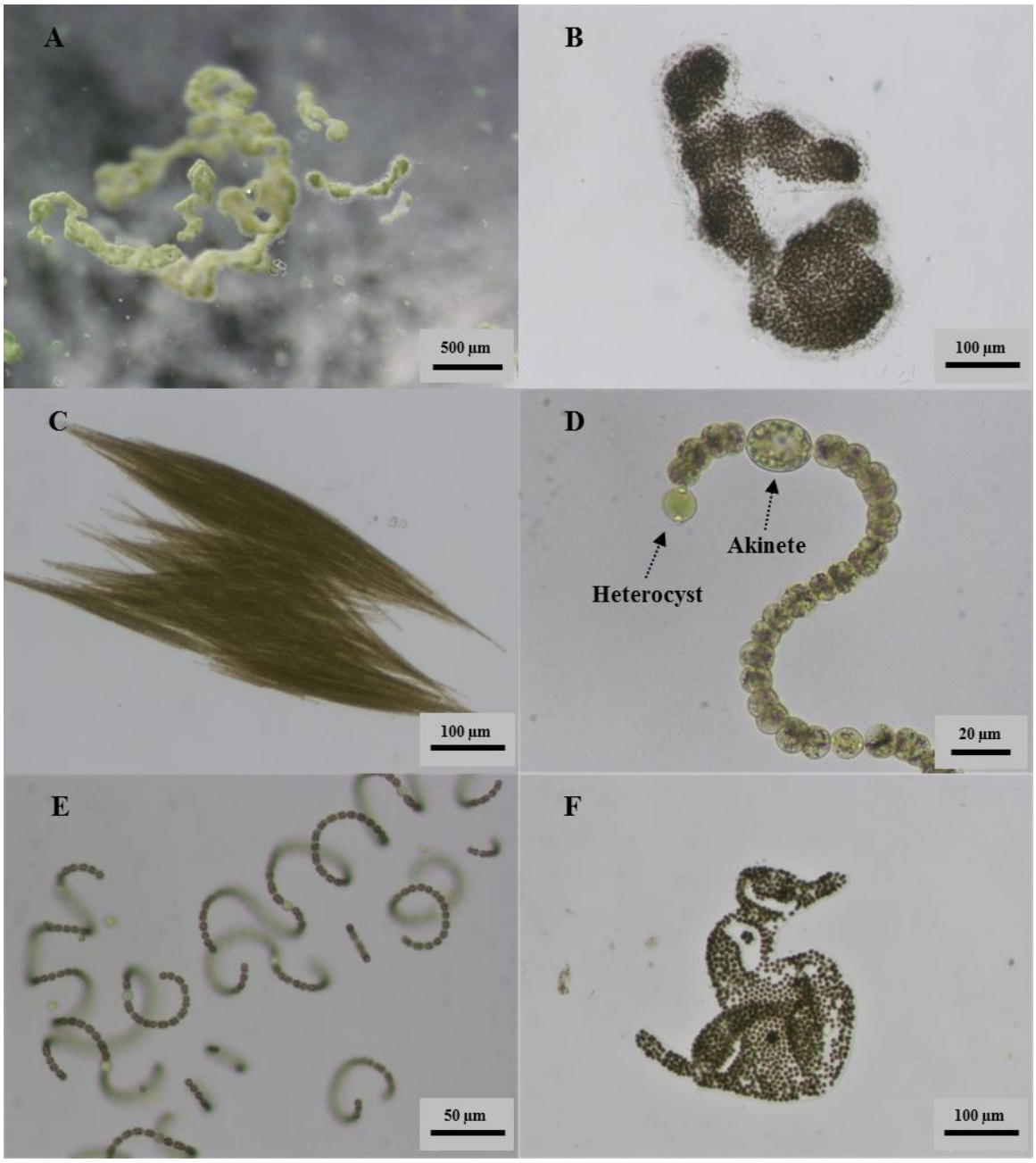


Figure 1.2. Microphotograhs of planktonic cyanobacteria collected in San Juan reservoir (Madrid, Spain) in 2013 and 2014. From the top left to the bottom right: A) Colonies of *Microcystis aeruginosa* and *Dolichospermum flos-aquae* observed under the binoculars; B) colony of *Microcystis aeruginosa* with a thick mucilage containing filaments of *Pseudanabaena mucicola*; C) colony formed by filaments of *Aphanizomenon flos-aquae* grouped in fascicles; D) filament of *Dolichospermum crassum* with an akinet and a heterocyst; E) coiled *Dolichospermum* sp. filament containing heterocysts, and F) colony of *Microcystis wessenbergii* with a thin mucilage layer.

Other structures that cyanobacteria may form are the hormogonia and the baeocytes, related to their asexual reproduction. The hormogonia are gliding motile filaments generated by fragmentation from the parent filament, and the baeocytes are endospores produced by successive divisions of the mother vegetative cells (Kunkel, 1984).

As a result of their wide variety of structures, cyanobacteria are versatile organisms that colonize a wide range of ecosystems, both terrestrial and aquatic habitats, including fresh, brackish and saline waters (Whitton and Potts, 2012). Despite of their ubiquity, freshwater systems with diverse trophic states are their prominent habitats, further discussed in the section "1.1.3. Ecology of cyanobacteria".

1.1.2. Taxonomy of cyanobacteria

Given the pigment composition and the capacity to perform the photosynthesis, cyanobacteria have been considered as a group of microalgae for a long time. As consequence, cyanobacteria have been also called as "blue-green algae" (still in use). The difficulty to stablish an appropriate boundary between the prokaryotic cyanobacteria and the eukaryotic microalgae has led to a great theoretical discussion and has hampered the study of the cyanobacteria by the coexistence of two classifications: the botanical and the bacteriological.

The botanical classification, mainly based on the morphological features of cyanobacteria (cell size, colony form, presence of sheath, etc.) (Kómarek and Anagnostidis, 1999), follows the International Code of Botanic Nomenclature (ICBN) (McNeill et al., 2006) and have classified about 2000 species within four different orders (*Chroococcales*, *Oscillatoriales*, *Nostocales* and *Stigonematales*). The increasing information of the 16S rRNA gene, electron microscopy and biochemical analysis, have led to a new revision of the classification. In this polyphasic approach, main changes are found at the order and family levels. Thus, eight orders, instead of four, are newly considered: *Gloeobacterales*, *Synechococcales*, *Spirulinales*, *Chroococcales*, *Pleurocapsales*, *Oscillatoriales*, *Chroococcidiopsidales* and *Nostocales* (Komárek et al., 2014).

On the other side, the bacteriological approach classify the cyanobacteria according to the International Code of Nomenclature of Prokaryotes (ICNP), based not only on the morphological features, but also on the physiological, cytological and biochemical characteristics of axenic cyanobacterial cultures (Rippka et al., 1979). This approach also incorporates the information provided by the 16S rRNA gene and was revised several times until the current nomenclature. So far, this classification stablishes 5 subsections of cyanobacteria comprising 57 different genera and about 82 different species (Garrity et al., 2004).

The main difference between both classifications is the lower number of cyanobacterial species considered within the bacteriological approach compared to those within the botanical code. For example, the genus *Microcystis* is only represented by the species *Microcystis aeruginosa* using the bacteriological approach, while it is comprised by 11 different species under the botanical code. As consequence, the coexistence of the dual nomenclature system causes confusion. Even though a consensus that is acceptable by both bacteriologists and botanists has been requested (Oren, 2004), the use of the most suitable system is still under debate (Oren and Ventura, 2017). Thus far, efforts are being directed towards an improvement of the botanical code based on polyphasic approaches, as commented previously (Komárek et al., 2014), and followed by studies combining both morphological and genetic analysis.

In this thesis, cyanobacteria is classified under the botanical approach and the information provided by the 16S rRNA gene.

1.1.3. Ecology of cyanobacteria

The physiological characteristics of the cyanobacteria confer them certain ecological advantages to succeed in the environment and dominate the phytoplankton communities. The cyanobacteria are ancient microorganisms, as reported by the presence of cyanobacterial fossil deposits (stromatolites and oncolites) that date about 2,700 million years ago (Brasier et al., 2002). Thanks to these studies we assume that the oxygen concentrations present in the current atmosphere were mainly driven by the cyanobacterial metabolism (Madigan et al., 2003).

1.1.3.1. Occurrence in nature: cyanobacterial blooms

Despite the ubiquity of the cyanobacteria in the biosphere, their predominant habitats are the aquatic environments, both freshwater and marine. Cyanobacteria are present in lotic (flowing waters) and lentic aquatic systems (standing waters; e.g., oceans, lakes and reservoirs), in which the high residence time of the water masses favours the concentration of nutrients for the primary production (nitrogen and phosphorus) and offers stability for growth (Merel et al., 2013).

In certain circumstances, the physiological characteristics of the cyanobacteria allow them to overcome unfavourable conditions and to outcompete other phytoplankton species, allowing them to dominate the aquatic systems. This dominance, together with the increasing nutrient concentrations due to the industrial development and agricultural practices, have led

to an increasing frequency of massive cyanobacterial proliferations or blooms in the aquatic systems. Although no international definition exists for what a cyanobacterial bloom is, it is commonly accepted that it refers to a significant production of biomass over a short period of time, correlated with a diminution in the phytoplankton diversity (Merel et al., 2013). Due to the different interpretations that can be made of this definition, alternative explanations that consider experimental data establish that chlorophyll *a* concentrations above 10 µg L^{-1} (with cyanobacterial dominance) or cyanobacterial cells above 20,000 per mL may be considered as blooms (Oliver and Ganf, 2000). These massive cyanobacterial proliferations may form a dense layer of cells at the surface of the water column visible to the naked eye, also called scum (Figure 1.3). In other cases, these cyanobacterial blooms may even develop in deeper water layers that may go unnoticed, as occur with species more sensitive to light intensities (e.g., *Planktothrix rubescens*) (Chorus and Bartram, 1999).

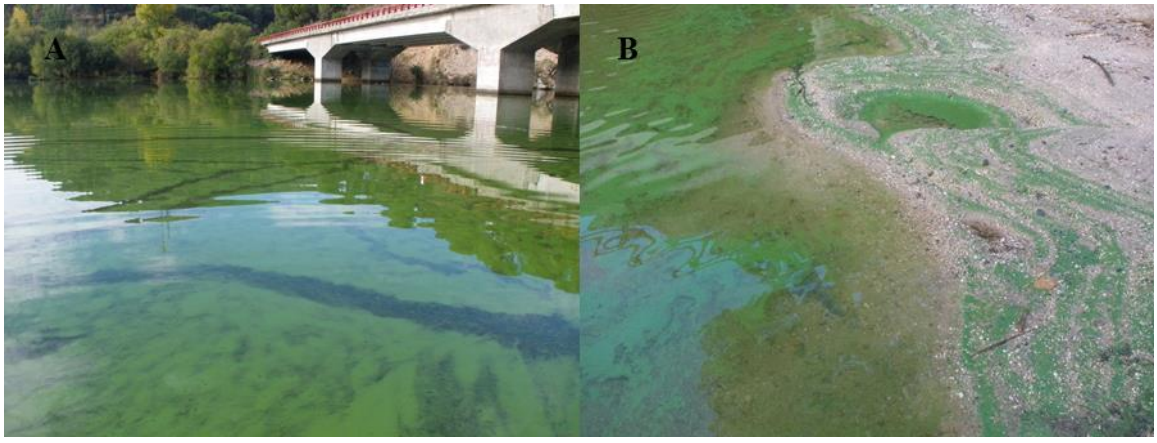


Figure 1.3. Cyanobacterial blooms dominated by *Microcystis aeruginosa* in a permanent pond close to the San Juan reservoir dam (Madrid, Spain). A) Cyanobacterial scum present in the surface water layer and B), cyanobacterial scum accumulated at the shoreline.

The presence of freshwater cyanobacterial blooms are common natural phenomena in the environment, but their increasing frequency worldwide and their associated adverse effects are a matter of concern for the population and the water authorities. The cyanobacterial massive proliferations increase the water turbidity, hampering the light penetration for macrophyte growth and thus affecting the habitat of macroinvertebrates and fishes. As a result, the biodiversity of the aquatic system is reduced. Moreover, the decomposition of the cyanobacterial biomass generates hypoxia conditions, leading to water chemistry changes and influencing the survival of fish and other aquatic organisms. The presence of cyanobacterial blooms also affects the aesthetic properties of the water masses by the release of unpleasant odours (geosmin and 2-methylisoborneol (2-MIB)) that impact the

organoleptic properties and, therefore, the water uses (Merel et al., 2013). Apart from these effects, the most problematic issue of the cyanobacterial massive proliferations is the production of cyanobacterial secondary metabolites, some of them considered as potent toxins (hereafter cyanotoxins) (Sivonen and Jones, 1999). The synthesis of these cyanotoxins represents a significant health problem to humans and other animals (e.g., some invertebrates) (Codd et al., 2005a, 2005b). One of the most frequent and widely-distributed toxin is the microcystin (MC), a potent hepatotoxin described in detail in the section "1.1.4. Cyanotoxin production: microcystins" and the object of study of this thesis.

1.1.3.2. Factors controlling cyanobacterial blooms

The success and dominance of cyanobacteria in the aquatic environment has been shown to depend on the optimization in the capture of resources, their efficient use and the reduction of losses (Oliver and Ganf, 2000). As a result, cyanobacterial blooms are regulated by a combination of bottom-up control factors, such as the physicochemical properties of the environment (water temperature, light exposure, nutrient concentrations) and top-down factors, such as the interaction with other co-existing organisms (grazing, parasitism, etc.) (Steffen et al., 2015; Van Wichelen et al., 2016). The relevance and contribution of each factor for the development of the cyanobacterial blooms and the production of toxins is still under debate. During the last decades, the nitrogen and phosphorous concentrations, the N:P ratio, the light intensity and the water temperature (leading thermal stratification of the water column) were shown to be important driving factors for the onset and the decline of the cyanobacterial blooms, as well as for the production of toxins (Monchamp et al., 2014; Sivonen, 1990) and the expression of the responsible toxic genes (Kaebernick et al., 2000). However, in the recent times, an increasing number of studies are focused on the relationships between the cyanobacteria and other organisms (bacteria, fungi, protists) to explain the cyanobacterial community dynamics in the environment (Gerphagnon et al., 2015; Van Wichelen et al., 2016) and the variations in the toxicity during bloom development (Agha et al., 2014; Sønstebø and Rohrlack, 2011).

In temperate lakes and reservoirs, cyanobacterial blooms occur during the summer season, commonly after a temporal succession of diatoms in spring and green-algae at the early stratification of the water column (Bellinger and Sigee, 2010). Considering the predictive models of climate change, the expected warmer conditions are supposed to favour the eco-physiological adaptations from cyanobacteria (Carey et al., 2012). The increase in temperatures would extend the periods of thermal stratification, thus lengthening the

growing season of primary producers and uncoupling the trophic relationships (De Senerpont Domis et al., 2013). This, together with the increasing nutrient concentrations, toxic cyanobacterial blooms are expected to be more frequent, start earlier and last longer.

1.1.4. Cyanotoxin production: microcystins

Cyanobacteria are described to produce secondary metabolites, that is, molecules synthetized by metabolic pathways not directly involved in their growth, development or reproduction and, thus, whose presence is not strictly essential for their survival. The rising interest in these compounds not only stems in the harmful effects produced on animals (Sivonen and Jones, 1999), but also in their pharmacological potential and exploitation as antibiotics, antivirals and anticancer drugs, among others (Rastogi and Sinha, 2009). The biological role of these metabolites still remains unclear although some hypothesis have been proposed, such as grazing protection (Ger et al., 2016; Rohrlack et al., 2004), *quorum sensing* signalling (Pereira and Giani, 2014; Schatz et al., 2007), allelopatic effect (Pflugmacher, 2002; Sukenik et al., 2002) or enhancing the fitness under oxidative stress conditions (Zilliges et al., 2011).

Some of these cyanobacterial metabolites are considered as potent toxins (cyanotoxins) due to their effects on animals, while others are considered merely as bioactive compounds. The distinction between both types is somewhat artificial and anthropocentric, since some cyanobacterial metabolites are known to produce effects on viruses and other bacteria (Sivonen and Börner, 2008). These cyanotoxins are classified into four groups based on their effects on vertebrates: hepatotoxins, neurotoxins, cytotoxins and dermatotoxins (Table 1.1).

Table 1.1. Main groups of cyanobacterial toxins, acute toxicities, structures, action mechanisms and producers (adapted from Codd et al., (2005a, 2005b))

Toxins (LD_{50})	Number of variants	Structure and Activity	Toxigenic genera[1]
Hepatotoxins			
MCs (25 to ≈ 1000)	>90	Cyclic heptapeptides; Hepatotoxic, protein phosphatase-inhibition, membrane integrity and conductance disruption, tumour promoters	*Microcystis*, *Dolichospermum*[4], *Nostoc*, *Anabaenopsis*, *Planktothrix*, *Oscillatoria* *Hapalosiphon*
Nodularins (30 to 50)	9	Cyclic pentapeptides; Hepatotoxic, protein phosphatase-inhibition, membrane integrity and conductance disruption, tumour promoters, carcinogenic	*Nodularia*, *Theonella* (sponge-containing cyanobacterial symbionts)
Cylindrospermopsins (200-2100)	3	Guanidine alkaloids; Necrotic injury to liver (also kidneys, spleen, lungs, intestine), protein synthesis-inhibitor, genotoxic	*Cylindrospermopsis*, *Aphanizomenon*, *Umezakia* *Dolichospermum*[4], *Raphidiopsis*
Neurotoxins			
Anatoxin-a (including homoanatoxin-a) (250)	5	Alkaloids; Postsynaptic, depolarising neuromuscular blockers	*Dolichospermum*[4], *Oscillatoria*, *Phormidium*, *Aphanizomenon*, *Raphidiopsis*
Anatoxin-a (S) (40)	1	Guanidine methyl phosphate ester; Acetylcholinesterase inhibitor	*Dolichospermum*
Saxitoxins (10-30)	20	Carbamate alkaloids; Sodium channel blockers	*Aphanizomenon*, *Dolichospermum*[4], *Lyngbya*, *Cylindrospermopsis*, *Planktothrix*
BMAA[2] (β-N-methylamino-L-alanine)	2	Non-protein aminoacid; Change protein conformation, linked to theamyotrophic lateral sclerosis and Parkinson-dementia complex (ALS/PDC)	*Nostoc*, *All?*[3]
Dermatotoxins and cytotoxins			
Lyngbyatoxin-a	1	Alkaloid; Inflammatory agent, protein kinase C activator	*Lyngbya*, *Schizotrix*, *Oscillatoria*
Aplysiatoxin	2	Alkaloids; Inflammatory agents, protein kinase C activators	*Lyngbya*, *Schizotrix*, *Oscillatoria*
Lipopolysaccharides	Many	Lipopolysaccharides; inflammatory agents, gastrointestinal irritants	All cyanobacteria

LD_{50} refers to the dose that kills the 50 per cent of the mice exposed to a single cyanotoxin through an intraperitoneal injection. It is expressed as µg/kg body weight. [1]Not all species within a genus and not all strains within a species produce the particular toxin. [2]Two stereoisomers have been identified, the L-isomer-(BMAA) and the D-isomer (DAB). [3](Cox et al., 2005). [4]Former *Anabaena.*

1.1.4.1. MC structure and toxicity

The most frequently found cyanotoxin in freshwater blooms are the MCs, a group of potent heptatotoxins that produce toxicity to the liver cells. They have been reported in several countries of Europe, Asia, Africa and America (Codd et al., 2005b) and are produced by the genera *Microcystis*, *Dolichospermum*, *Planktothrix*, *Oscillatoria*, *Nostoc*, *Anabaenopsis* and *Hapalosiphon* (terrestrial) (Sivonen and Jones, 1999). Recent studies reported also the presence of MCs in other genera, such as *Gleitlerinema*, *Leptolyngbya*, *Pseudanabaena*, *Synechococcus*, *Spirulina*, *Phormidium* and *Radiocystis* ((Buratti et al. (2017) and references therein).

The MCs are cyclic heptapeptides consisting of seven amino acids with the following structure: cyclo-(D-Ala^1-X^2-D-MeAsp^3-Z^4-Adda^5-D-glu^6-Mdha^7) (Figure 1.4). The amino acids X and Z are variable L-amino acids, D-MeAsp is D-*erythro*-β-methylaspartic acid, Mdha is N-methyldehydroalanine and Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid).

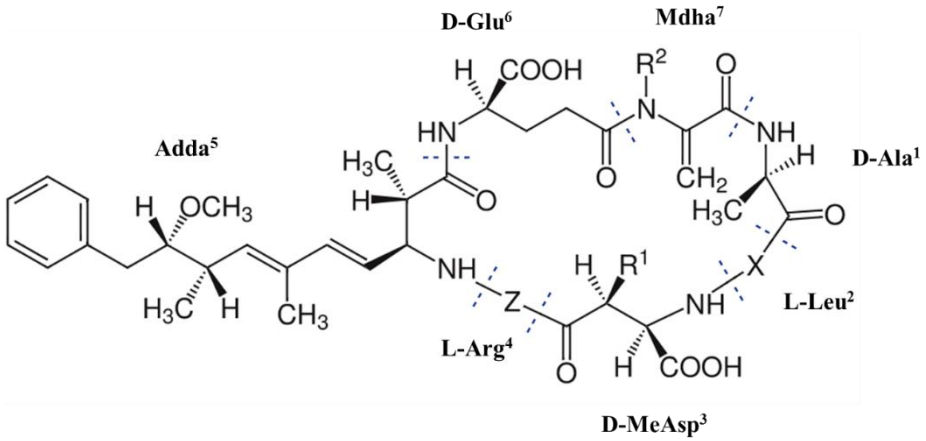


Figure 1.4. Structure of the microcystin-LR (MC-LR). The variable X and Z amino acids are L-Leucine and L-Arginine, respectively (Sivonen and Jones, 1999).

The MCs comprise a high number of chemical variants, so far more than 90 (Merel et al., 2013), in which main variations occur at positions 2 and 4. For example, MC-LR contains L-Leucine at position 2 and L-Arginine at position 4. Although other variations may also occur in other positons, they are less frequent (e.g., demethylations at positions 3 and 7). These variations in the amino acid composition, as well as in the presence of methyl groups, causes differences in the toxicity. For example, the three most common MCs in the aquatic systems (MC-LR, MC-RR and MC-YR) show dissimilar LD_{50} in mouse bioassays (intraperitoneal injection): 50 µg kg^{-1} for MC-LR, 600 µg kg^{-1} for MC-RR and 70 µg kg^{-1}

for MC-YR (Sivonen and Jones, 1999). Comparing the LD_{50} of the MCs with other contaminant substances present in the aquatic systems, such as the insecticide parathion (banned in several countries) and the herbicide atrazine (banned in the European Union), the toxicity of the MC-LR is in range or even higher (170-fold), respectively, showing the high toxicity of the MCs (Falconer, 2004).

The MCs are usually contained within the cyanobacterial cells and are abundantly released to the environment during cell lysis. These toxins are soluble in water and unable to penetrate directly into the lipid membranes of animals, plants or bacterial cells, being necessary a membrane transporter for the cell uptake (Sivonen and Jones, 1999). As water-soluble molecules, they are easily ingested by animals (the major exposure route), in which they cause disruption of the cytoskeletal components of hepatocytes leading to cell liver failure (Falconer and Yeung, 1992). The mechanism of toxicity is based on the inhibition of the protein phosphatases 1 (PP1) and 2A (PP2A) of eukaryotic cells, enzymes that catalyse the dephosphorylation (removal of a phosphate group) of the serine/threonine amino acids from proteins and are important for many signal transduction pathways in the cell (cytoskeletal rearrangement, cell movement, apoptosis, etc.). The different amount of phosphatases and kinases (enzymes that incorporates a phosphate group to the proteins) in the cell may seriously affect the cellular structure and function by even causing uncontrolled cell proliferation and cancer development (Buratti et al., 2017; Cohen and Cohen, 1989). As a result, MCs have been also described as possible tumour promoters (MacKintosh et al., 1990; Zhou et al., 2002).

1.1.4.2. MC biosynthesis

The MCs are synthetized by non-ribosomal pathways, as suggested by their particular structure and presence of the unusual amino acid Adda (Welker and von Döhren, 2006). In this synthesis, multifunctional modular enzyme complexes, named as non-ribosomal peptide synthases (NRPS), are the responsible for the activation, modification and condensation of individual amino acids, serving as template for the biosynthesis of peptides (Figure 1.5). Together with the NRPS, polyketide synthases (PKS), another multienzyme complexes, are involved in the synthesis of the Adda amino acid (Börner and Dittmann, 2005).

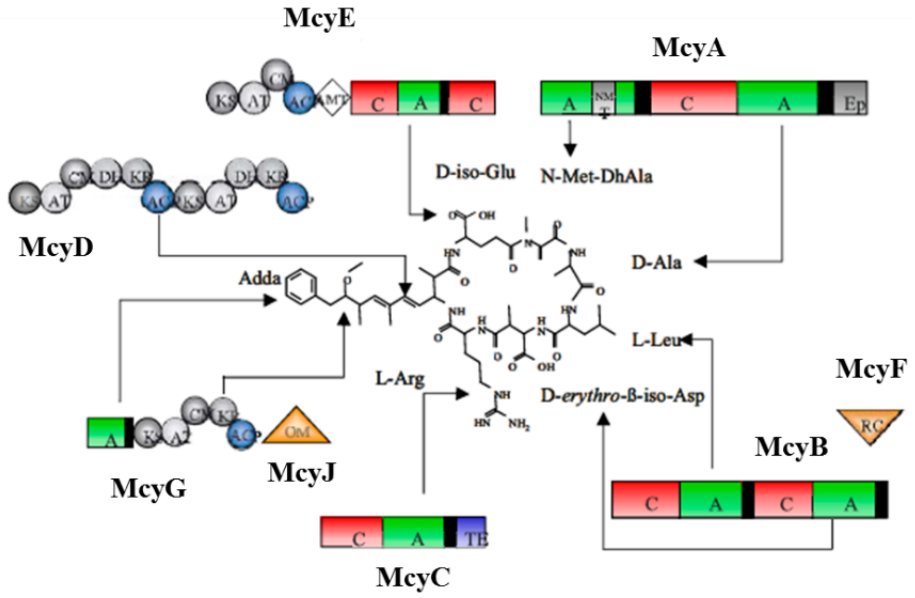


Figure 1.5. The role of the components of the MC synthetase complex (Mcy proteins). Arrows indicate the assignment of individual proteins to the MC molecule. PKS domains: AT: acyltransferase; ACP: acyl carrier protein; KS: ß-ketoacyl synthase, KR: ketoacyl reductase; DH: dehydratase; CM: C-methyltransferase; AMT: aminotransferase; NRPS domains: A:aminoacyl adenylation; C: condensation; NMT: N-methyltransferase; Ep: epimerase, TE:thioesterase; McyF: racemase, OM (McyJ): O-methyltransferase. Black bars represent thethiolation motif of NRPS modules (Börner and Dittmann, 2005)

Both NRPS and NRPS/PKS hybrids are encoded in the *mcy* gene cluster, that comprises 10 genes (*mcy*A-J) embedded in two bidirectionally transcribed operons (Figure 1.6) (Tillett et al., 2000). The gene cluster was firstly elucidated in *Microcystis*, and then in *Planktothrix* and *Anabaena*. Although the multienzyme components encoded by the individual gene clusters are similar and produce the same MC molecule in the three genera, the arrangement of certain genes is different. Since the evolutionary aspects of the *mcy* gene cluster is out of the scope of this thesis, see Börner and Dittmann (2005).

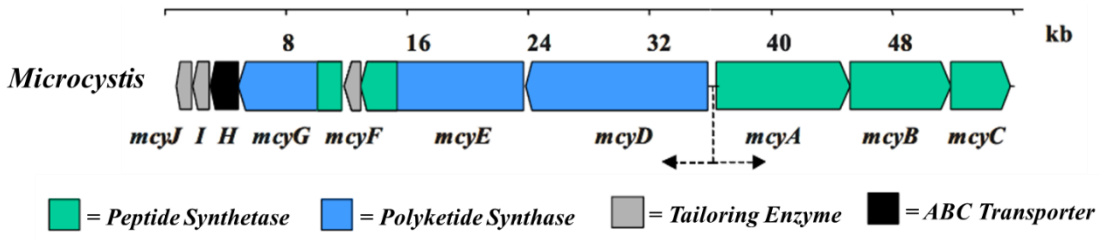


Figure 1.6. The *mcy* gene cluster for the biosynthesis of MCs in *Microcystis*. Arrows indicate the starting site for transcription (Börner and Dittmann, 2005).

1.2. Degradation of MCs in the environment

1.2.1. Physical and chemical degradation

The MCs are structurally stable molecules resistant to physical and chemical processes such as high temperatures and extreme pH. The half-life of MC-LR at 40 ºC and pH 1 was found in 3 weeks, and at pH 9 (commonly found during the overgrowth of photosynthetic organisms) in 10 weeks (Harada et al., 1996). The stability of MCs was also corroborated after boiling water with MCs and after boiling carp muscles contaminated with MCs, since no complete destruction of the toxins was observed (Chen et al., 1998; D. Zhang et al., 2010).

In full sunlight, MCs show slow photochemical breakdown and isomerization. However, increasing degradation rates were found in presence of cell pigments acting as photosensitizers, but a minimum of two weeks were required for the decomposition of more than the 90% of the MCs (Tsuji et al., 1994). The dependence of photosensitizers for the degradation of MCs in the natural waters was also confirmed by Welker and Steinberg (2000, 1999), who observed that no degradation of MCs occurred in Milli-Q water without humic substances. These humic substances indirectly mediate the breakdown of the toxins through the formation of highly reactive molecules during their decomposition with the sunlight (hydroxyl-radicals, singlet oxygen or hydrogen peroxide, among others) (Cooper et al., 1989). Welker and Steinberg (2000) also observed that the half-life of the MC-LR (concentrations from 5 to 55 μg L^{-1}) with a sunlight intensity comparable to the mid-European values were estimated in 90-120 days per meter depth of the water column. A more rapid MC breakdown was observed by Wörmer et al. (2010), although the authors confirmed the higher MCs half-life times as depth increases. In this work, about 4.5 μg L^{-1} of MCs were photodegraded between 6.3 and 11.5 days at 2 m., and between 21.5 and 52 days at 10 m. These results show photodegradation as an important mechanism for the MC removal in only few limited situations, such as shallow systems with high residence times. In addition, these experiments were performed with concentrations sometimes below to those found during the occurrence of cyanobacterial blooms (Carrasco et al., 2006; Orihel et al., 2012), especially when dense scums develop (Sivonen and Jones, 1999). Therefore, the contribution of the natural photodegradation for the MCs breakdown represents a small percentage of the overall processes of MCs elimination in the environment.

The MCs can also be degraded under intense ultraviolet (UV) light (Tsuji et al., 1995), which is highly relevant for water treatment technologies, but with less relevance for the understanding of the degradation processes occurring in nature. In the same line, strong

oxidation processes by chlorine, permanganate or ozone, among others (Westrick et al., 2010), are interesting for water treatment, but do not represent the naturally occurring degradation in the environment (see section "1.3. MC removal in drinking-water treatment").

Apart from the molecular breakdown of the MCs, it is important to mention that other processes, such as adsorption to particles and dilution, may also contribute to reduce MC concentrations in the water. MCs appear to be retained weakly to the natural suspended solids, usually below the 20 % of the total MC concentrations present in the water (Li et al., 2016; Sivonen and Jones, 1999). Another fraction of the pelagic MCs may settle down within the living cells, whose sedimentation rate during a bloom episode has been described around the 4.5 % of the total MCs present in the water (Wörmer et al., 2011). However, both adsorption-to-particles and sedimentation processes only shuttle MCs out of the water column but does not eliminate them from the system because MCs are afterwards detached and/or released back to the water column after cell lysis of the sediment cells.

1.2.2. Biological degradation

The cyanobacterial massive proliferations have been described to coexist with other microorganisms living free or embedded in the mucilage of cyanobacteria, whether they are bacteria (Parveen et al., 2013), virus (Van Hannen et al., 1999) or fungi (Sønstebø and Rohrlack, 2011), among others. In spite of the chemical stability of the MCs, specific bacteria that coexist with cyanobacteria are able to degrade these toxins in the aquatic environment. Only few studies reported MC degradation capacities by other microorganisms, such as fungi (Jia et al., 2012) or ochrophyta (*Poterioochromonas* sp.) (Zhang et al., 2008), thus conferring to bacteria the major biological MC degradation in nature.

1.2.2.1. Diversity of MC-degrading bacteria

The first evidence for the MC degradation by bacteria in a freshwater system was observed by Jones and Orr (1994) after an algicide treatment applied to a *Microcystis aeruginosa* bloom. The authors identified a rapid decrease in the MC concentrations after 9 days of lag-phase. In a further study the authors isolated the bacterial strain *Sphingomonas* MJ-PV (further changed to ACM-3962), initially identified as *Pseudomonas* sp. (Jones et al., 1994). Since then, the collection of MC-degrading bacteria has been increasing worldwide reaching up to 45 strains, approximately (Table 1.2). Most of the bacteria belong to the phylum *Proteobacteria*, and it was not until 2007 that other strains from the phyla *Firmicutes*

(Nybom et al., 2007) and *Actinobacteria* (Manage et al., 2009a) were also confirmed as MC degraders. Since then, biological MC degradation was mainly associated to the order *Sphingomonadales* (phylum *Proteobacteria*).

Although the study of the MC biodegradation dates back 1994, which is relatively new compared with other fields, the number of the MC-degrading strains isolated around the world is still scarce, hindering the study of the biological MC degradation process and the comparison of results. This is due to the general difficulties that involve bacterial isolation from environmental samples and the lack of a standardized procedure describing the steps and conditions for the isolation of MC-degrading bacteria. The number of steps, the type of MCs added (pure or mixture), the concentration of MCs used (Manage et al., 2009a; M. Zhang et al., 2010), as well as the type of the culture media used (with absence or presence of additional carbon and/or nitrogen sources) (Lemes et al., 2008; Valeria et al., 2006; M. Zhang et al., 2010) are some examples of these differences. Thus, a standardization of an efficient method for the isolation of MC-degrading bacteria is required for increasing the number of strains, make data comparison possible among laboratories and facilitate the studies on the MC degradation processes (e.g., efficiencies of degradation and characterization of degradation pathways).

The new technologies on molecular genetics (e.g. massive sequencing) have allowed us to obtain information of the bacterial diversity directly from the environment, surpassing the constraints of the isolation in the laboratory. A high diversity of bacteria have been reported to be associated with toxic cyanobacterial blooms, among which, *Proteobacteria* (classes α, β, γ and δ), *Bacteroidetes* and *Actinobacteria* have been the most represented phyla (Li et al., 2016; K. Shao et al., 2014). However, little is known about the implication of these bacteria in the degradation of MCs in the environment. A microcosm study with field water amended with MCs observed a bacterial community shift in which other orders different to the *Sphingomonadales* appeared to be more responsive to the MC addition (e.g. *Methylophilales* and *Burkholderiales*) and, thus, suggested as more important for the toxin removal (Mou et al., 2013). However, this remains to be verified in the environment, since a lack of information still exists on the diversity and the seasonal dynamics of the MC-degrading bacterial community associated to toxic cyanobacterial blooms. This information is crucial to find out the bacteria that is engaged in the MC degradation processes during toxic cyanobacterial blooms and to make significant inroads into the relationship and interaction between the MC-producing and MC-degrading bacterial communities.

Table 1.2. MC-degrading bacterial isolates and whether they possess or not the *mlr* genes. "n.d." means not detected.

Phylum	Class	Order	Family	Genera/species	Strain	Accession number	*mlr*A presence/absence	References
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae*	*Microbacterium* sp.	DC8*	-	unknown	(Ramani et al., 2012)
			Micrococcaceae	*Arthrobacter* sp.	C6	FN392690	n.d.	(Manage et al., 2009a)
					F10	FN392691	n.d.	
					F7	FN392693	n.d.	
					R1	FN392694	n.d.	
					R4	FN392695	n.d.	
					R6	FN392696	n.d.	
					R9	FN392697	n.d.	
			Nocardiaceae	*Rhodococcus* sp.	C1	FN392688	n.d.	
					C3	FN392689	n.d.	
			Brevibacteriaceae	*Brevibacterium* sp.	F3	FN392692	n.d.	
		Bifidobacteriales	*Bifidobacteriaceae*	*Bifidobacterium lactis*	420	-	unknown	(Nybom et al., 2008, 2007)
					Bb12	-	unknown	
				Bifidobacterium longum	46	-	unknown	
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus* sp.	AMRI-03	GU294753	*mlr*A	(Alamri, 2010)
					EMB	FJ526332	*mlr*A	(Hu et al., 2012)
				Bacillus nanhaiencis	JZ-2013	KF841622	unknown	(Zhang et al., 2015)
		Lactobacillales	*Lactobacillaceae*	*Lactobacillus rhamnosus*	GG	AY370682	unknown	(Nybom et al., 2008, 2007)
					LC-705	-	unknown	
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiaceae*	*Rhizobium gallicum*	DC7*	AY972457	unknown	(Ramani et al., 2012)
		Sphingomonadales	*Sphingomonadaceae*	*Novosphingobium* sp.	THN1	HQ664117	*mlr*A, *mlr*B, *mlr*C, *mlr*D	(Jiang et al., 2011)
				Sphingomonas sp.	7CY	AB076083	unknown	(Ishii et al., 2004)
					ACM-3962	AF411072	*mlr*A, *mlr*B, *mlr*C, *mlr*D	(Bourne et al., 2001, 1996; Jones et al., 1994)
					B9	AB159609	unknown	(Imanishi et al., 2005)
					CBA4	AY920497	unknown	(Valeria et al., 2006)
					MD-1	AB110635	*mlr*A	(Saito et al., 2003)
					MDB2	AB219940	unknown	(Maruyama et al., 2006)
					MDB3	AB219941	unknown	
					NV3	-	*mlr*A, *mlr*B, *mlr*C, *mlr*D	(Somdee et al., 2013)
				Sphingomonas stygia	-	-	unknown	(Saitou et al., 2003)
				Sphingopyxis sp.	C-1	AB161684	*mlr*A, *mlr*B, *mlr*C, *mlr*D	(Okano et al., 2009)
					LH21	DQ112242	*mlr*A, *mlr*B, *mlr*C, *mlr*D	(Ho et al., 2007)
					TT25	JQ398614	*mlr*A	(Ho et al., 2012b)
					USTB-05	EF607053	*mlr*A	(Wang et al., 2010; M. Zhang et al., 2010)
				Sphingosinicella microcystinivorans	Y2	AB084247	*mlr*A, *mlr*B, *mlr*C, *mlr*D	(Lezcano et al., 2016; Maruyama et al., 2006; Saito et al., 2003)
	Betaproteobacteria	*Burkholderiales*	*Alcaligenaceae*	*Bordetella* sp.	MC-LTH1	KC734882.1	*mlr*A	(Yang et al., 2014a)
			Burkholderiaceae	*Burkholderia* sp.	UPC-BI05	DQ459360	unknown	(Lemes et al., 2008)
				Paucibacter toxinivorans	2C20	NR_042941	unknown	(Rapala et al., 2005)
				Ralstonia solanacearum	-	-	unknown	(Yan et al., 2004; Zhang et al., 2011)
		Methylophilales	*Methylophilaceae*	*Methylobacillus sp.*	J10	FJ418599	unknown	(Hu et al., 2009)
	Gammaproteobacteria	*Enterobacteriales*	*Enterobacteriaceae*	*Morganella morganii*	-	-	unknown	(Eleuterio and Batista, 2010)
		Aeromonadales	*Aeromonadaceae*	*Aeromonas* sp.	-	-	n.d.	(Mankiewicz-Boczek et al., 2015)
		Pseudomonadales	*Pseudomonadaceae*	*Pseudomonas aeruginosa*	-	-	unknown	(Takenaka and Watanabe, 1997)
		Xanthomonadales	*Xanthomonadaceae*	*Stenotrophomonas acidaminiphila*	MC-LTH2	-	n.d.	(Yang et al., 2014b)
				Stenotrophomonas sp.	EMS	FJ712028	*mlr*A	(J. Chen et al., 2010)

* *Microbacterium* sp. and *Rhizobium gallicum* only degrade MCs when they are together in a consortia.

1.2.2.2. MC degradation genes and pathways

The first evidences of a gene cluster involved in the degradation of MCs was reported by Bourne et al. (2001) in the isolated strain *Sphingomonas* sp. ACM-3962 (Bourne et al., 1996). The gene cluster was named *mlr* and comprises four genes (*mlr*A-D) with two transcription directions (Figure 1.7).

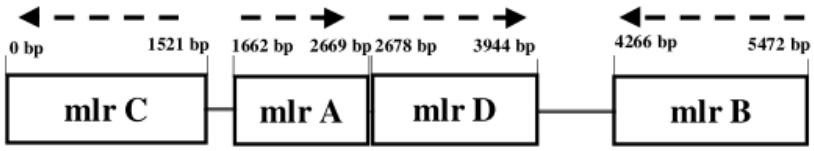


Figure 1.7. The *mlr* gene cluster for the synthesis of the enzymes involved in the MC-LR degradation. Dotted lines indicate the direction of transcription of each individual gene (Bourne et al., 2001).

The *mlr* gene cluster encodes three hydrolytic enzymes involved in the MC degradation pathway (encoded in the *mlr*A-C genes), and a putative oligopeptide transporter (encoded in the *mlr*D gene) (Bourne et al., 2001). The enzymes MlrA and MlrC are recognized as metalloproteases and the MlrB as a serine protease. The degradation pathway begins with the linearization of the cyclic MC by the action of the MlrA enzyme (also named as microcystinase) that cleaves the Adda-Arg peptide bond. After linearization, the linear MC is then hydrolyzed by the MlrB at the Ala-Leu peptide bond, resulting in a tetrapeptide molecule. Finally, the tetrapeptide is further decomposed by the MlrC, giving rise to the Adda amino acid and smaller peptides and/or amino acids that have not been yet fully identified. Further studies confirmed the same MC-LR degradation pathway in other strains (Harada et al., 2004; Imanishi et al., 2005), and recent studies have shown that the MlrC enzyme has a double cleavage activity also involved in the direct degradation of the linear MC into smaller peptides, amino acids (Dziga et al., 2012; Shimizu et al., 2012), as well as into an hexapeptide (Dziga et al., 2016). Further degradation of the hexapeptide has been confirmed not to be mediated by the MlrC enzyme and thus the following degradation steps and enzymes involved still remains unknown (Figure 1.8).

Although this degradation pathway was firstly identified for the degradation of MC-LR, some studies have further confirmed that the same *mlr* gene cluster is involved in the degradation of other MC variants (Imanishi et al., 2005; Xu et al., 2015). However, it was found that the efficiency of degradation of the different MC variants may vary due to the dissimilar affinity of the Mlr enzymes to the specific peptide bonds. Thus, the MlrA, that

cleaves the Adda-Arg peptide bond (e.g., in MC-LR or MC-RR), hydrolyzes more slowly the MC-LF, that possess the Adda-Phe peptide bond (Imanishi et al., 2005).

These enzymatic activities are predicted to take place in the periplasmic space of the membranes. Due to the close distance between the *mlr*A and *mlr*D genes, it was also speculated that the MlrD may be responsible for the uptake of the digested peptides into the cell (Bourne et al., 2001) although never experimentally proven. Another suggested function for MlrD is the uptake of MCs from outside the cell into the periplasmic space for the subsequent enzymatic degradation.

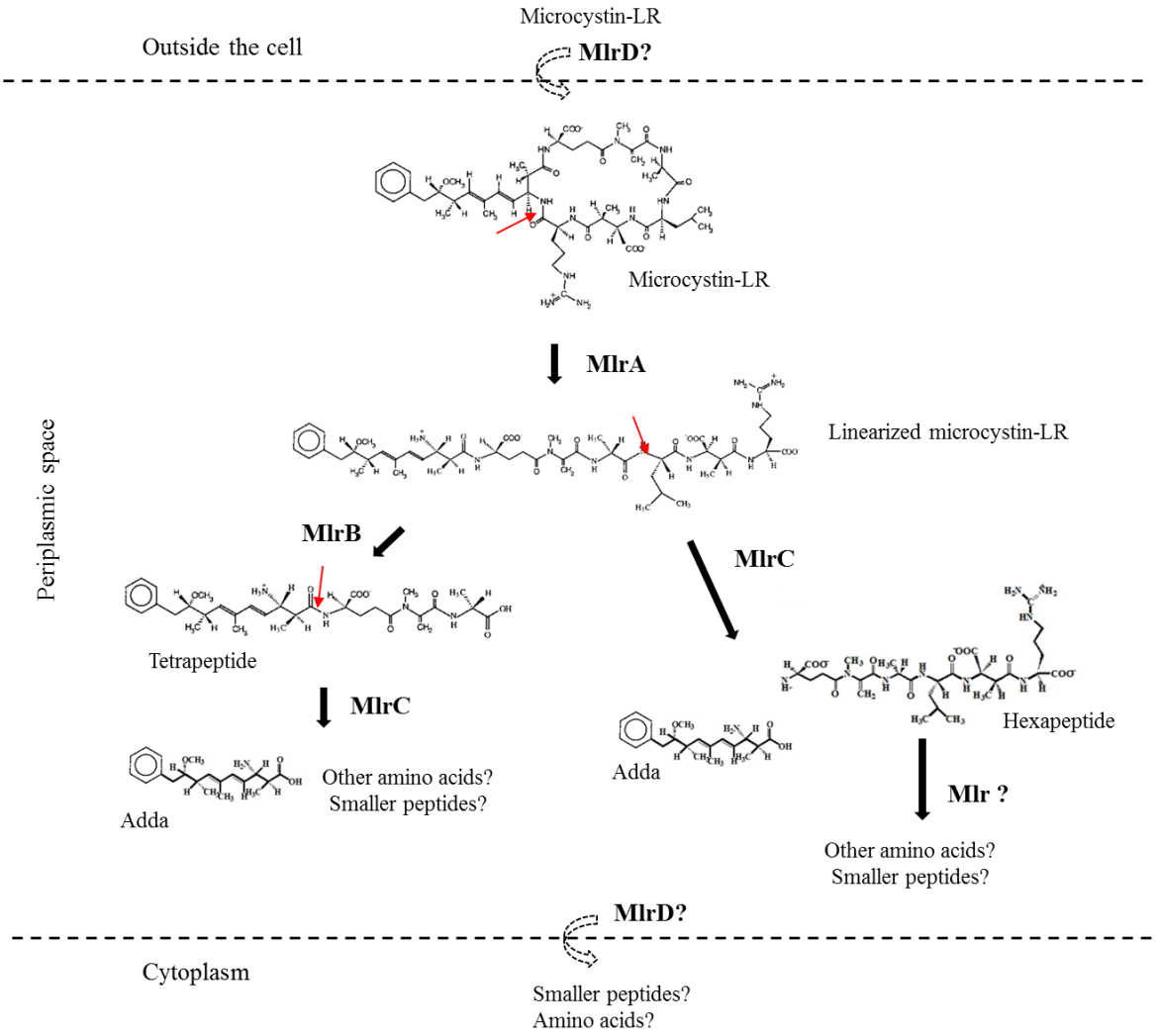


Figure 1.8. MC-LR degradation pathway in *Sphingomonas* sp. strain ACM-3962. Dotted arrows indicate possible functions of the MlrD enzyme and red arrows indicate sites of peptide hydrolysis. Adapted from Bourne et al. (1996) and Li et al., (2017) with information from the studies of Dziga et al. (2016, 2012).

The importance of the MC degradation relies on the significantly less toxicity of the intermediate products compared to the parent cyclic MC. The concentration of the linear MC-LR and the tetrapeptide molecules that cause the 50% of the phosphatase inhibition are 95 nM and 12 nM, which means about 160 and 20 times less toxic than the cyclic MC (0.6 nM), respectively (Bourne et al., 1996). As a result, the *mlr*A gene has been considered as an important gene marker for the detection of MC-degrading bacteria. The identification of inactivation mutations (stop codons) in the *mlr*B gene in some MC-degrading strains (Jiang et al., 2011) also supports the use of the *mlr*A as the best gene marker for the identification of MC-degrading bacteria (Saito et al., 2003). The *mlr* gene cluster has been reported as constitutive (Alamri, 2010; Bourne et al., 2001; Ishii et al., 2004), thus showing a continuous expression, although upregulated with the MC concentrations. The study conducted by Jiang et al. (2011) observed that the higher the exposure to the MC concentrations, the more rapid and higher transcriptional response of the *mlr*A gene.

Apart from the *mlr* genes, other isolated bacterial strains have shown to degrade MCs without the detection of the *mlr* gene cluster (Table 1.2), pointing towards alternative MC degradation pathways operating in nature (Manage et al., 2009a). This has aroused interest within the scientific community, since the use of the *mlr* genes alone may underestimate the real contribution of the total bacterial community for the degradation of MCs in the environment. However, the absence of gene markers, the unknown MC degradation pathways and the few isolated MC-degrading bacterial strains lacking *mlr* genes have hindered the study of these *mlr*-lacking bacterial community. While all studies have been focused on bacteria with *mlr* genes and the factors that influence their MC degradation efficiencies under laboratory conditions (see section "1.3.2.2. Factors controlling the MC degradation efficiency"), no information exists regarding the influence of these factors on the MC-degrading bacteria lacking *mlr* genes. Moreover, it is still unknown the taxonomical affiliation of these *mlr*-lacking bacteria, their contribution in the environment for the total MC removal and the characterization of the alternative degradation pathways. Only a recent metagenomics study developed in a microcosm experiment amended with MCs showed that the genes for the xenobiotic metabolism were more represented than the *mlr* genes, suggesting their possible involvement in the MC degradation in nature (Mou et al., 2013). However, neither the identification of the MC-degrading bacteria lacking *mlr* genes nor their seasonal dynamics have been studied in environmental samples associated to naturally occurring toxic cyanobacterial blooms.

Henceforth, both MC-degrading bacterial communities with and without the *mlr* genes are named as *mlr*$^{+}$ and *mlr*$^{-}$ bacteria throughout the thesis.

1.2.2.3. Factors controlling the MC degradation efficiency

Several studies have focused on the factors that influence the MC degradation efficiency. These studies have been performed in *in vitro* assays and can be divided in those with pure isolated bacteria and those with total bacterial communities from biological treatment facilities (biofilms), lake water or sediments. The most studied parameters are the carbon and nitrogen compounds alternative to MCs (e.g., glucose, peptone, acetate, nitrate and ammonium), the phosphate and the temperature. Most of these studies coincide that the MC degradation efficiency increases at higher temperatures up to 30-35 ºC, approximately (X. Chen et al., 2010; Park et al., 2001; Yang et al., 2014b). However, inconclusive results were obtained in relation with the effect of nutrients in the MC degradation efficiency. While in some studies the presence of alternative carbon and nitrogen substrates enhanced the MC degradation rates in pure bacterial isolates (Surono et al., 2008; Zhang et al., 2015), in others impaired the degradation process (Eleuterio and Batista, 2010) or did not show any significant influence (Ishii et al., 2004). This controversy increases even more when comparing the previous results with those performed with whole bacterial communities. In most these studies, the presence of alternative carbon sources showed an inhibitory effect on the MC degradation efficiency (Eleuterio and Batista, 2010; J. Li et al., 2012, 2011), while in others did not show any effect (Christoffersen et al., 2002). Moreover, while the ammonium and the phosphate caused an inhibitory effect, the presence of nitrate enhanced the MC degradation efficiency (J. Li et al., 2012, 2011). Besides the aerobic degradation, the degradation of MC has been also studied in lake sediments and in drinking water sludge under anoxic conditions. In this case, the results are also contradictory. In some studies the presence of glucose impaired the MC degradation rates (Ma et al., 2016), while in others glucose and ammonium had no effect, and only showed inhibition under the presence of nitrate (X. Chen et al., 2010).

The reported negative effects in the MC degradation efficiency of bacteria in presence of alternative carbon and nitrogen compounds under both aerobic and anaerobic conditions has been related to the primary use of these compounds instead of the MCs (Eleuterio and Batista, 2010; Ma et al., 2016). However, other experiments pointed to the use of MC as a primary substrate (J. Li et al., 2012) and associate both inhibitory and enhanced effects to

the dissimilar expression of the *mlr* genes under different environmental factors (J. Li et al., 2011; Li et al., 2014).

As observed, substrate competition with other organic and inorganic compounds appear to play a crucial role in the MC degradation efficiency, impacting ultimately on the MC lifetimes in the water column. As mentioned above, all these studies have been focused on bacteria with *mlr* genes or on bacterial communities ignoring the possible presence of those without *mlr* genes. Considering that both *mlr*$^{+}$ and *mlr*$^{-}$ bacterial genotypes may have different MC degradation pathways, it would also be necessary to study the influence of alternative carbon and nitrogen sources on the MC degradation efficiency in *mlr*$^{-}$ bacteria.

1.2.2.4. MC degradation rates

As pointed out in the previous section, the MC degradation rates depend on the influence of several physicochemical factors. The absence of a standardized method to perform the *in vitro* MC degradation assays hinders the comparison between the degradation efficiencies of the strains. The use of diverse media (Lemes et al., 2008; Maruyama et al., 2006; Takenaka and Watanabe, 1997), the different initial MC concentrations (Manage et al., 2009b; M. Zhang et al., 2010), the possible application of a starvation period, the different initial bacterial cell concentrations and the use of cell-free (CE) extracts instead of living cells (Xiao et al., 2010) are some examples of the different conditions used. In general, the MC degradation rates of bacteria have been reported to range from less than 1 μg L^{-1} day^{-1} (Ho et al., 2007) to 16.7 mg L^{-1} day^{-1} (M. Zhang et al., 2010), approximately. In some cases, the recorded low MC degradation rates in cultures have been associated to extensive bacterial lag phases (Ho et al., 2007; J. Li et al., 2011), and it has been also reported to occur in the environment (X. Chen et al., 2010; Jones and Orr, 1994).

1.3. MC removal in drinking-water treatment

The first step for cyanotoxin control is the prevention of cyanobacterial massive proliferations by controlling the factors that triggers bloom occurrence, such as eutrophication. However, once blooms are developed in water intended for consumption, three different management strategies may undertake to minimize health risks: change of the water supply, adjust the water intake depth and, in the last instance, implement the water treatment.

The removal of MCs in drinking-water treatment plants (and for the rest of cyanotoxins) comprises the physical removal, chemical inactivation and biological inactivation (Westrick

et al., 2010). The foremost consideration prior treatment is to maximize the MC removal by drawing intact cells, thus avoiding further release of the intracellular toxins into the water. Therefore, the use of pre-oxidants, such as chlorine, are questioned and only those that avoid cell damage may be suitable (e.g., potassium permanganate ($KMnO_4$) (Chen and Yeh, 2005)). So far, the conventional treatment options, such as coagulation, sand filtration, and chlorination, among others, have shown limited effect on their removal, especially when there are high levels of organic matter during cyanobacterial blooms (Chow et al., 1998; Himberg et al., 1989; Keijola et al., 1988). On the contrary, advanced oxidation treatments based on the production of highly active and non-selective oxidizing radical species have demonstrated effective removal of MCs. Within these advanced oxidation treatments are found the ozonation, photochemical degradation, titanium dioxide (TiO_2), photocatalysis, Fe (III) and Fe (IV) photocatalysis, Fenton ($Fe_2^+ + H_2O_2$), Photo-Fenton and ultrasonication (Pantelic et al., 2013). However these sophisticated processes are expensive, they are not always available in small treatment facilities and they are usually optimized for other contaminants present in higher concentrations than the MCs. In this sense, the bacterial degradation of MC arises as a promising water treatment option considering the available number of strains with high MC degradation efficiency, as well as it is characterized as a high cost-effective and an environmental-friendly process that does not involve the use of harmful chemicals. As consequence, these advantages are motivating the scientific community to adapt available systems present in water treatment plants (e.g., sand filtration and granular activated carbon, among others) for the removal of MCs using efficient MC-degrading bacteria (Bourne et al., 2006; Ho et al., 2006; Wang et al., 2007).

1.4. Legislation

The awareness about the human and animal health risks related to the occurrence of toxic cyanobacterial blooms has increased in the last years, as observed by the incorporation of non-mandatory guidelines and recommendations of cyanobacterial cell densities and cyanotoxin concentrations in monitoring programs and in national legislations. As an example, the World Health Organization (WHO) incorporated the cyanotoxins into the *Guidelines for drinking-water quality* (Gorchev and Ozolins, 2011) and in the *Guidelines for safe recreational water environments: Coastal and freshwaters* (WHO, 2003). Regarding drinking water, the WHO stablishes a guideline value of 1 µg L^{-1} for MCs (WHO, 1998), adopted in several European and non-European legislations (Chorus, 2012) and incorporated in the Spanish Royal Decree 140/2003 *(R.D. 140/2003 by which health criteria for the*

quality of water intended for human consumption are established). In this Royal Decree, MCs should be only analysed at the exit of the drinking water treatment plant when eutrophication in the water uptake is observed.

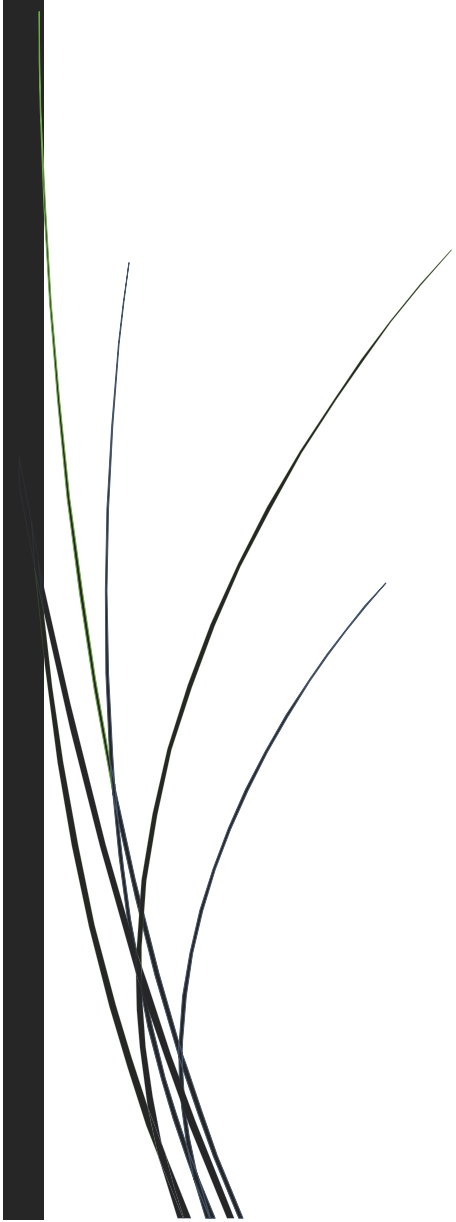
In relation with the recreational waters, the European Bathing Water Directive (2006/7/EC) stablishes as mandatory the analysis of cyanobacteria for the development of recreational water profiles in each country and a risk assessment for bathing sites with frequent toxic cyanobacterial blooms. The purpose of this Directive is to preserve, protect and improve the quality of the environment and to protect human health by complementing the Water Framework Directive (2000/60/EC). The Bathing Water Directive was further incorporated in the Spanish Royal Decree 1341/2007 (*R.D. 1341/2007 regarding the quality management of bathing waters*).

1.5. Objectives of the thesis

The general objective of this thesis is to investigate the genetic and ecological aspects underlying the biological degradation of MCs mediated by freshwater bacteria.

1. Assess the MC degradation capacity of the bacterial community in a Spanish water reservoir, isolate the responsible MC-degrading bacteria, classify them into different genotypes (*mlr*$^{+}$ and *mlr*$^{-}$) and compare their MC biodegradation efficiencies under the presence of alternative carbon and nitrogen sources.
2. Investigate the diversity and the temporal shifts of the *mlr*$^{+}$ and *mlr*$^{-}$ fractions of the MC-degrading bacterial community associated with a toxic cyanobacterial bloom.
3. Investigate the relationship between the MC-producing cyanobacteria and the fraction of the MC-degrading bacterial community with *mlr* genes in the environment.
4. Optimize a method for:
    i. an efficient isolation of MC-degrading bacteria from water bodies with episodes of toxic cyanobacterial blooms.
    ii. the use of an efficient MC-degrading strain as a biological tool for the degradation of MCs in aqueous medium with a wide range of organic carbon concentrations.

Chapter 2



2. Presence or absence of *mlr* genes and nutrient concentrations co-determine the microcystin biodegradation efficiency of a natural bacterial community

María Ángeles Lezcano[1,2], Jesús Morón-López[1], Ramsy Agha[3], Isabel López-Heras[1], Leonor Nozal[1], Antonio Quesada[2] and Rehab El-Shehawy[1]

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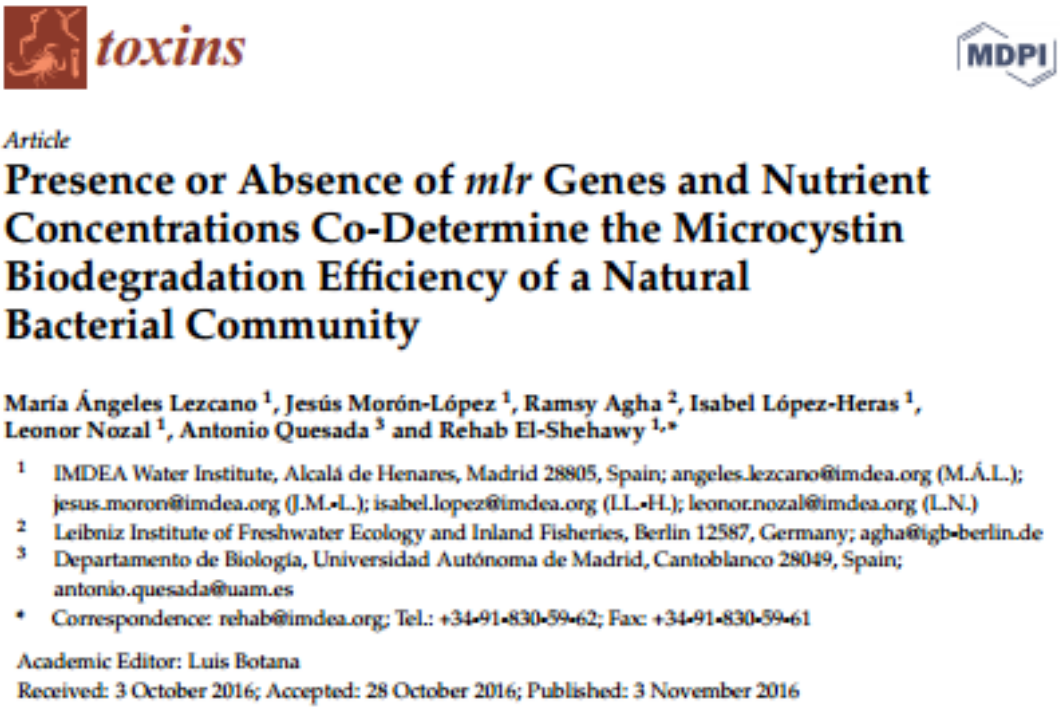
Part of the content of this research was used for the application of a patent with international protection (PCT). The information regarding the patent is found in Chapter 5 and in the Appendix.



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Presence or Absence of *mlr* Genes and Nutrient Concentrations Co-Determine the Microcystin Biodegradation Efficiency of a Natural Bacterial Community

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2.1. Abstract

The microcystin biodegradation potential of a natural bacterial community coexisting with a toxic cyanobacterial bloom was investigated in a water reservoir from central Spain. The biodegradation capacity was confirmed in all samples during the bloom and an increase of *mlr*A gene copies was found with increasing microcystin concentrations. Among the 24 microcystin degrading strains isolated from the bacterial community, only 28% showed presence of *mlr*A gene, strongly supporting the existence and abundance of alternative microcystin degradation pathways in nature. *In vitro* biodegradation assays with both *mlr*$^{+}$ and *mlr*$^{-}$ bacterial genotypes (with presence and absence of the complete *mlr* gene cluster, respectively) were performed with four isolated strains (*Sphingopyxis* sp. IM-1, IM-2 and IM-3; *Paucibacter toxinivorans* IM-4) and two bacterial degraders from the culture collection (*Sphingosinicella microcystinivorans* Y2; *Paucibacter toxinivorans* 2C20). Differences in microcystin degradation efficiencies between genotypes were found under different total organic carbon and total nitrogen concentrations. While *mlr*$^{+}$ strains significantly improved microcystin degradation rates when exposed to other carbon and nitrogen sources, *mlr*$^{-}$ strains showed lower degradation efficiencies. This suggests that the presence of alternative carbon and nitrogen sources possibly competes with microcystins and impairs putative non-*mlr* microcystin degradation pathways. Considering the abundance of the *mlr*$^{-}$ bacterial community and the increasing frequency of eutrophic conditions in aquatic systems, further research on the diversity of this community and the characterization and conditions affecting non-*mlr* degradation pathways deserves special attention.

2.2. Introduction

Mass developments of cyanobacteria in freshwater systems are increasing worldwide due to anthropogenic eutrophication and global warming (El-Shehawy et al., 2012; Paerl et al., 2011; Paerl and Huisman, 2008). Some cyanobacterial genera are known to synthesize toxic secondary metabolites, so-called cyanotoxins, which cause human health problems and pose ecological risks (Campos and Vasconcelos, 2010; Codd, 1995; Codd et al., 2005a; Sivonen and Börner, 2008). The most widespread and frequently found cyanotoxins are microcystins (hereafter MCs), a group of potent hepatotoxins produced by the genera *Microcystis*, *Dolichospermum*, *Planktothrix*, *Nostoc*, and *Anabaenopsis* (Cirés and Quesada, 2011; Sivonen and Jones, 1999). MCs act as an inhibitor of protein phosphatases 1 and 2A and are, therefore, toxic to eukaryotic cells (MacKintosh et al., 1990; Spoof, 2005). MCs are heptapeptides synthesized non-ribosomally by large multi-enzyme complexes encoded by the *mcy*A-J gene cluster (Börner and Dittmann, 2005), which serves for the biosynthesis of the complete MC molecule by joining each amino acid. Due to variable incorporation of amino acids, MCs presents over 90 different variants (Pearson et al., 2010; Sivonen and Börner, 2008).

MCs are structurally stable and resistant against physical and chemical processes such as high temperatures, extreme pH, sunlight, general hydrolytic enzymes, etc. (Rastogi et al., 2014; Wörmer et al., 2010). However, some naturally occurring bacterial populations are reported to effectively degrade MCs (Dziga et al., 2013; Edwards et al., 2008; Kormas and Lymperopoulou, 2013). A MC-LR biodegradation pathway has been characterized in some bacterial strains that involve the action of three specific peptidases and a putative oligopeptide transporter encoded in the *mlr*A-D gene cluster (Bourne et al., 2001). The first enzyme encoded by the *mlr*A gene cleaves the Adda-Arg peptide bond from the MC cyclic structure, resulting in a linearized molecule that is 160 times less toxic (Bourne et al., 1996). Therefore, the *mlr*A gene has been considered to be an important marker for the detection of bacterial populations with MC degradation capacity (Hoefel et al., 2009; Saito et al., 2003). However, the absence of *mlr* genes (or no PCR amplification) has been reported in some MC degraders, suggesting the existence of alternative degradation pathways (Manage et al., 2009b; Mou et al., 2013). A large array of MC-degrading bacteria, both containing and lacking *mlr* genes, have been isolated from both water and sediment samples. Most of these strains belong to the phyla *Proteobacteria* (especially classes α- and β-*Proteobacteria*) (Ho et al., 2007; Valeria et al., 2006; Xiao et al., 2010), although strains from the phyla

Actinobacteria and *Firmicutes* have also been reported (Manage et al., 2009b; Nybom et al., 2007).

The study of MC degradation kinetics of indigenous bacteria can increase understanding of the fate and lifetimes of MCs in the water column. Although substrate competition with other organic and inorganic compounds in natural waters appears to play a crucial role in the MC degradation process (J. Li et al., 2012), no studies have been performed to study the effect of nutrient availability on the MC biodegradation efficiency between *mlr*$^+$ and *mlr*$^-$ bacterial genotypes (with presence and absence of the *mlr* gene cluster, respectively). Few studies have focused on comparing MC degradation rates in bacterial strains under different nutrient conditions, and those they have been carried out report contradictory results. Studies performed directly on biofilm communities showed lower MC-LR degradation removal rates in media spiked with acetate (Eleuterio and Batista, 2010), glucose and peptone (J. Li et al., 2011), but enhanced rates with the addition of nitrate (J. Li et al., 2011). On the other hand, studies developed with individual strains found that the presence of exogenous carbon and/or nitrogen sources enhanced MC-LR removal rates in some cases (Surono et al., 2008; Zhang et al., 2015) and impaired them in others (Eleuterio and Batista, 2010). Unraveling the effect of nutrients in MC biodegradation is especially relevant after a bloom collapse and sestonic MCs are released, since cell debris and other nutrients from the water column may serve as alternative C and N sources. In this sense, *mlr*$^+$ and *mlr*$^-$ genotypes from the natural MCs-degrading bacterial community may respond differently in terms of degradation efficiency. To address this, field and experimental approaches with a natural bacterial community and isolates from said community were used.

This study aims to analyze the diversity among strains of a natural bacterial community in relation to the presence/absence of *mlr* genes and their MC degradation efficiencies. In particular, the objectives of the present work are: (i) to evaluate the MC biodegradation capacity of a natural bacterial community from a water body in the Iberian Peninsula (Mediterranean region) and isolate the responsible bacterial strains; and (ii) to study the MCs biodegradation efficiency of both *mlr*$^+$ and *mlr*$^-$ bacterial genotypes under variable total organic carbon and total nitrogen concentrations.

2.3. Materials and Methods

2.3.1. Sampling

A permanent pond in Alberche river located near the exit of the San Juan dam (Madrid) (40°22′3.43″ N and 4°18′12.28″ W) was sampled from 7 August 2012 to 29 October 2012, comprising a cyanobacterial bloom episode. Samples were taken on a monthly basis from 7 August to 4 September and then collected every two weeks until the end of the sampling period. At every sampling event, a total of 4 L of sub-surface water samples were collected with sterile polyethylene bottles and transported in the dark at 4 °C. One liter of water was sequentially filtered through fiberglass filters (2.7-µm approx., Millipore, Darmstadt, Germany) to remove algae and large cyanobacteria and then through 0.22-µm polycarbonate filters (Millipore) to collect the bacterioplankton. Polycarbonate filters were stored at −20 °C until DNA extraction. Another 1 L was passed through fiberglass filters (0.7-µm approx., Millipore) to collect seston and stored at −20 °C until analysis. Some water aliquots were used immediately after collection to screen for MC-degrading bacteria. Finally, 1 L of water was used for taxonomic identification of buoyant cyanobacteria under microscope (Olympus CX41, Tokyo, Japan) after 24 h of flotation (Kómarek and Anagnostidis, 1999).

2.3.2. MCs extraction

A crude microcystin extract for experiments was extracted from a scum collected in San Juan reservoir. The scum was extracted with 100% methanol, vortexed for 1 min, sonicated for 10 min in an ultrasonication bath (P-Selecta Ultrasons, Barcelona, Spain) and stored at 4 °C for one hour for extraction. The extracted sample was then centrifuged (Sorvall RC-5C, GMI, Ramsey, MN, USA) for 15 min at 4000× g and supernatant was stored at −20 °C. The whole extraction process was repeated three times. The MC crude extract was vacuum dried at 40 °C in a rotavapor (Rotavapor-R, Büchi Labortechnik AG, Flawil, Switzerland), resuspended in 10% methanol and bonded to 5 g C18 cartridges (Extrabond C18, 5 g, 20 mL, Sharlab, Barcelona, Spain) for partial purification. Cartridges were activated with 100% methanol, followed by Milli-Q water and equilibrated with 10% methanol. After that, samples were passed through and washed by Milli-Q water and 30% methanol. MCs were eluted in 20 mL of 90% methanol and dried down under vacuum in a multiple evaporator (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) at 40 °C. Finally, MCs were resuspended in Milli-Q water, passed through sterile syringe 0.22-µm filters (25 mm, Pall Corporation, Port Washington, NY, USA) for sterilization and stored at −20 °C.

Sestonic MCs from fiberglass filters were extracted twice by sonication with 90% aqueous methanol and evaporated at 40 °C under vacuum in the multiple evaporator. Final extracts were stored at −20 °C until analysis.

2.3.3. Reservoir's MC biodegradation capacity

The determination of the reservoir's MC biodegradation capacity was performed in duplicates in 20 mL of raw water enriched with 1.2 mg equivalent MC-LR·L^{-1} of crude MC extract. Flasks were incubated for 15 days at 27 °C with 120 rpm shaking in the dark to prevent photosynthetic growth. Negative controls consisting of autoclaved reservoir water were included. Samples for quantifying MCs (MC-LR, -RR and -YR) were collected at the start and after 15 days of incubation. Additional samples were filtered through 0.22-µm polycarbonate membranes and filters containing retained microorganisms were stored at −20 °C for DNA extraction.

2.3.4. Screening and isolation of MC-degrading bacteria

Samples showing biodegradation activity in the previous experiment were 10-fold serially diluted and transferred to R2A agar (Sigma-Aldrich, St. Louis, MO, USA) plates. Plates were incubated for 7 days at 27 °C in the dark. A total of 90 colonies with different morphologies were selected and purified by streaking on plates. Each isolated colony was transferred individually into R2A liquid medium and incubated under same conditions until late exponential phase. From each bacterial culture, an aliquot was washed and resuspended in 100 µL of 4-fold R2A liquid medium with 1 mg equivalent MC-LR·L^{-1} of crude MC extract to get a final optical density of 0.45 (maximum error ± 0.15) at 600 nm. Aliquots were transferred to sterile 96-well plates containing 150 µL of 4-fold R2A liquid medium with 1 mg equivalent MC-LR·L^{-1} of raw MCs extract in each well. Negative controls (no bacterial inoculation) and positive controls inoculated with strains Y2 (Park et al., 2001) and 2C20 (Rapala et al., 2005) were also included. Plates were covered with a sterile plastic film and incubated at 27 °C in the dark at 120 rpm for 6 days. Total MC concentrations were analysed at start and after 6 days of incubation. Classification of bacterial phenotypes according to MC degradation efficiencies was performed using an ascending hierarchical classification analysis with XLSTAT software (Addinsoft, 2016) with a minimum and maximum variability intra- and inter-cluster, respectively.

2.3.5. MC degradation under different TOC and TN concentrations

Four new MC-degrading isolates were incubated in R2A medium at 27 °C in the dark at 120 rpm for 24 h with initial absorbance of 0.05 measured at 600 nm. Cells in the late exponential phase were centrifuged at 5.000 rpm for 5 min, washed in Mineral Salts Medium (MSM) (Valeria et al., 2006) and incubated in MSM for 14 h at the same conditions to induce nutrient starvation. Cells were then resuspended at a final absorbance of 0.05 at 600 nm (equivalent to 10^8 CFU mL^{-1}, approximately) in various media (MSM, reservoir water and 4-fold R2A liquid medium) with different total organic carbon (TOC) and total nitrogen (TN) concentrations, containing 1 mg equivalent MC-LR·L^{-1} of crude MC extract. Experiments were performed in duplicate. TOC and TN concentrations from the three media were analysed in triplicates in a Total Organic Carbon analyzer (TOC-V CSH, Shimadzu, Kyoto, Japan) and Total Nitrogen kit (range 0.5–15.0 mg L^{-1}, Spectroquant, Merck Millipore, Dramstadt, Germany), respectively. Incubation was carried out under previously described conditions for 120 h and samples were collected at different time intervals (0, 3, 6, 9, 24, 48 and 120 h) for quantification of MCs and bacterial biomass. A standard curve of optical density vs. biomass (dry weight) was performed to calculate the bacterial biomass at each time interval and, thus, the average growth rate.

2.3.6. Analysis of MCs

All samples for MC analysis were filtered through 0.22-µm syringe filters (Acrodisc GHP, Pall Corporation, Port Washington, NY, USA) before analysis and commercial MC-LR, MC-RR and MC-YR pure standards (Sigma-Aldrich, St. Louis, MO, USA) were used for calibration curves.

Sestonic MC concentrations from water samples and MCs from the kinetic experiment were measured on a high-performance liquid chromatography (HPLC) system (Agilent series 1100, Agilent Technologies, Santa Clara, CA, USA) coupled to a time-of-flight (TOF) mass spectrometer (Agilent 6230 accurate mass TOF Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation of MC-LR, MC-RR and MC-YR was performed using a Pursuit C18 150 mm × 2 mm column with 3 µm of particle size (Agilent Technologies, Santa Clara, CA, USA) and thermostated at 40 °C. The mobile phase consisted of 0.1% of acetic acid in water (A) and 0.1% of acetic acid in acetonitrile (B) with a flow rate of 0.3 mL min^{-1}. Gradient profile started at 30% B, increased to 60% B over 9 min and changed to 100% B over 1 min for cleaning. For re-equilibration of the column, B was reduced to 30% over 1 min and was held for further 6 min. Fifty microliters of each sample was injected.

MCs concentrations for the rest of experiments were measured on a Varian 500 Ion Trap Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) supported by two Varian 212 LC chromatographic pumps (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation of MC-LR, MC-RR and MC-YR was performed using a Pursuit C18 150 mm × 2 mm column with 3 µm of particle size (Agilent Technologies, Santa Clara, CA, USA) following conditions from Agha et al. (2012).

2.3.7. Genomic DNA extraction

Genomic DNA extraction from filters and bacterial pellets was performed using DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following manufacturer's instructions with several modifications: Filters and pellets were introduced in 2 mL microcentrifuge tubes with addition of the first buffer (Buffer AP1). For fiberglass filters, they were previously cut into two pieces, each acting as a single sample, to avoid excess of fibre filter residues that may interfere with DNA extraction. Immediately, tubes were introduced for 30 s in liquid N_2 and thawed at room temperature, repeating the process twice. After thawing, cells were disrupted using sterile glass beads (212–300 µm, acid-washed, Sigma Aldrich, St. Louis, MO, USA) and a homogenizer (Precellys, Bertin Technologies, Montigny-le-bretonneux, France) at 5000 r.p.m. for 40 s. RNAse was added to tubes, incubated at 65 °C for 10 min and then centrifuged at 20,000× g for 2 min. Supernatants were transferred to new microcentrifuge tubes avoiding filter residues. At this point, manufacturer's instructions were followed and both tubes belonging to the same glass fibre filter were combined to the same DNeasy Mini spin column. Genomic DNA was dissolved in sterile Milli-Q water and stored at −20 °C until analysis.

2.3.8. mlrA-D genes detection

For the identification of the *mlr* gene cluster, a PCR using the specific primer sets MF/MR (Saito et al., 2003), mlrBf1/mlrBr1, mlrCf1/mlrCr1 and mlrDf1/mlrDr1 (Ho et al., 2007) was carried out. Amplifications were performed in 50 µL of total volume containing final concentration of 0.25 µM of each primer, 1× PCR buffer (HotStarTaq Master Mix kit, QIAGEN, Hilden, Germany) and 1 µL of DNA. Amplification of the *mlr*A gene in a PCR thermal cycler (Techne TC-5000, Bibby Scientific, Staffordshire, UK) was performed under the following conditions: initial activation step at 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 40 s and 72 °C for 40 s. Final extension step at 72 °C for 10 min. Amplification of *mlr*B, *mlr*C and *mlr*D genes were performed with an initial activation step at 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 40 s and 72 °C for

40 s. Final extension step was set at 72 °C for 10 min. PCR products were separated on 1.5% agarose gel electrophoresis and visualized on a AlphaImager HP (Alpha Innotech, San Leandro, CA, USA). The obtained *mlr*A-D sequences were deposited in the GeneBank under the following accession numbers: *mlr*A (KY002139.1-KY002141.1), *mlr*B (KY002142.1-KY002145.1), *mlr*C (KY002146.1-KY002149.1) and *mlr*D (KY002150.1-KY002153.1).

2.3.9. mcyE gene detection

Genomic DNA extracted from fiberglass filters were used for identification of the *mcy*E gene as a marker for the presence of potentially MC-producing cyanobacteria. Specific primer set HEPF and HEPR (Jungblut and Neilan, 2006) was used for the PCR. Amplification was performed in 10 µL of total volume, containing final concentration of 0.25 µM of each primer, 1× PCR buffer (HotStarTaq Master Mix kit, QIAGEN) and 1 µL of DNA. PCR amplification was performed under the following conditions: initial activation step at 95 °C for 15 min, followed by 35 cycles of 94 °C for 20 s, 52 °C for 30 s and 72 °C for 1 min. Final extension step at 72 °C for 10 min. PCR products of 472 bp were separated by 1.5% agarose gel electrophoresis and visualized on the AlphaImager HP (Alpha Innotech, San Leandro, CA, USA).

2.3.10. Quantification of mlrA gene: Real-Time PCR

Serial dilutions of purified PCR product of *mlr*A gene (807 bp) from strain IM-2 were used to generate a standard curve for *mlr*A detection. A specific primer set qmlrAf and qmlrAr, and the TaqMan probe qmlrA-tm with a variation in the quencher (Hoefel et al., 2009), were used for quantification on a Real-Time PCR (AB7300, Applied Biosystems, Foster City, CA, USA). Reactions resulted in an amplification of 120 bp product and were carried out in duplicate in 25 µL of volume containing 1× of QuantiTech Probe PCR Master Mix (QIAGEN), 0.4 µM of each primer, 0.2 µM of qmlrA-tm probe and 1 µL of either a DNA standard or sample. Thermal cycling conditions were performed with an initial activation step of 95 °C for 15 min, followed by 45 cycles of denaturation at 94 °C for 15 s and annealing/extension at 62 °C for 1 min. Data were collected at the end of the annealing/extension step. Gene copies per sample were calculated using a standard curve of the target gene copy number vs. threshold cycle (Ct) with a correlation coefficient (R^2) of 0.999 and an efficiency of 85% in a linear range of 3.16×10^3 to 3.16×10^9 *mlr*A gene copy number per sample. Quantification of *mlr*A gene copies per litre were calculated considering the volume filtered.

2.3.11. Identification of bacterial isolates using 16S rRNA gene analysis

Genomic DNA of the new isolated MC-degrading bacteria was used for 16S rRNA gene analysis. A PCR was performed using two universal primer sets: (1) 27F and 907R; and (2) 533F and 1492R_l (Lane, 1991; Turner et al., 1999; Weisburg et al., 1991). Amplification was performed in 50 µL of total volume, containing final concentration of 0.25 µM of each primer, 1× PCR buffer (HotStarTaq Master Mix kit, QIAGEN) and 1 µL of DNA. Amplification for both primer sets was performed under the following conditions: initial activation step at 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min. Final extension step at 72 °C for 10 min. PCR products were separated by 1.5% agarose gel electrophoresis with bands visualized on the AlphaImager HP. PCR products were purified with QIAquick PCR purification kit (QIAGEN) following manufacturer's instructions and sent for sequencing to the Molecular Biology Centre of the University of Alcalá (Madrid). Nucleotide sequences obtained from both primer sets were combined (1344–1420 bp) and compared with sequence information available in the NCBI using BLASTN. Multiple alignment of the sequences was performed using CLUSTAL W (Thompson et al., 1994) from the current BioEdit software (version 7.2.5, Ibis Biosciences, Carlsbad, CA, USA) (Hall, 1999). The construction of the phylogenetic tree was performed with MEGA6 software (Tamura et al., 2013) and a tree based on the 16S rRNA gene was constructed using Maximum Likelihood method with bootstrap analysis of 1000 replicates. The obtained 16S rRNA sequences were deposited in the GeneBank under the following accession numbers: KX085478, KX085479, KX085480 and KX085481.

2.4. Results

2.4.1. Cyanobacterial bloom and biodegradation capacity of the natural bacterial community

The presence of potentially MC-producing cyanobacteria along the bloom episode was recorded in all samples by microscopic identification (Kómarek and Anagnostidis, 1999). *Microcystis aeruginosa* was the dominant species along the bloom period, but *Microcystis flos-aquae*, *Microcystis wessenbergii* (non-toxic), *Dolichospermum crassum*, *Woronichinia naegeliana* and *Aphanizomenon flos-aquae* were also identified. MC-producing cyanobacteria were also confirmed by the presence of *mcy*E gene in all samples, used in this study as a marker for potentially MC-producing cyanobacteria. Total sestonic MCs ranged from 0.54 to 49.52 µg L^{-1}, peaking on 24 September (Table 2.1). MC-LR was the most abundant variant in all samples, representing approximately the 59% (±8%) from total MCs,

followed by MC-RR, with 29% (±5%) and MC-YR, with 12% (±3%). In order to assess the MC degradation capacity of the natural bacterial community at different bloom stages, water samples were spiked with 1.2 mg equivalent MC-LR·L^{-1} from crude MC extract. After 15 days of incubation all samples showed complete removal of MCs (Table 2.2), evidencing an endogenous MC degradation capacity during the bloom episode.

Table 2.1. Sestonic MC concentrations during cyanobacterial bloom. (+) refers to the presence of *mcy*E gene. Errors represent standard error of two replicates.

Date	Sestonic MCs (µg L^{-1})				*mcy*E
	MC-LR	MC-RR	MC-YR	Total MCs	
7-Aug-2012	0.31 ± 0.20	0.16 ± 0.02	0.07 ± 0.00	0.54 ± 0.22	+
4-Sep-2012	10.17 ± 1.59	3.14 ± 0.18	0.99 ± 0.07	14.31 ±1.84	+
24-Sep-2012	25.75 ± 6.10	16.82 ± 3.82	6.95 ± 1.45	49.52 ± 11.37	+
8-Oct-2012	13.64 ± 1.43	5.89 ± 0.74	2.42 ± 0.26	21.95 ± 0.43	+
29-Oct-2012	0.85 ± 0.26	0.52 ± 0.14	0.23 ± 0.06	1.60 ± 0.06	+

Table 2.2. Total MC concentrations in the water from different sampling dates enriched with 1.2 mg equivalent MC-LR·L^{-1} from crude MC extract before and after 15 days of incubation. Controls represent autoclaved water. Errors represent standard errors of two replicates. "n.d." means "not detected".

Date	Control (µg L^{-1})		Reservoir water (µg L^{-1})	
	Day 0	Day 15	Day 0	Day 15
7-Aug-2012	1315 ± 32	1239 ± 26	1196 ± 80	n.d.
4-Sept-2012	1238 ± 35	1219 ± 95	1206 ± 22	n.d.
24-Sep-2012	1303 ± 15	1321 ± 52	1200 ± 30	n.d.
8-Oct-2012	1145 ± 69	1179 ± 125	1173 ± 65	n.d.
29-Oct-2012	1183 ± 45	1284 ± 22	1211 ± 44	n.d.

2.4.2. Isolation of MC-degrading bacteria from the bacterial community

In order to characterize the natural MC-degrading bacterial community, 90 bacterial strains were isolated from the MC enrichment assays. Using six-day *in vitro* degradation assays in 4-fold R2A medium enriched with 1 mg equivalent MC-LR·L^{-1} from crude MC extract, individual isolates were classified into four phenotypes according to their MC degradation efficiencies (Figure 2.1). Phenotype A comprises the most efficient MC-degrading group of bacteria with a degradation efficiency ranging between 80% and 100%, followed by

phenotype B and C, with efficiencies ranging between 60% and 79%, and 40% and 59%, respectively. Phenotype D comprises non-MC-degraders and represents the largest group, with the 73.33% of the total bacterial isolates (66 strains), followed by phenotype A, C and B, represented by the 17.78% (16 strains), 5.56% (5 strains) and 3.33% (3 strains), respectively. Among the 24 MC-degrading bacterial isolates belonging to phenotypes A, B and C (mostly isolated on 4 September), only the 19 strains from the most efficient phenotypes (A and B) were screened for the presence of *mlr*A gene, revealing that *mlr*A was only present in a relatively small fraction of the MC-degrading strains investigated (27.78%).

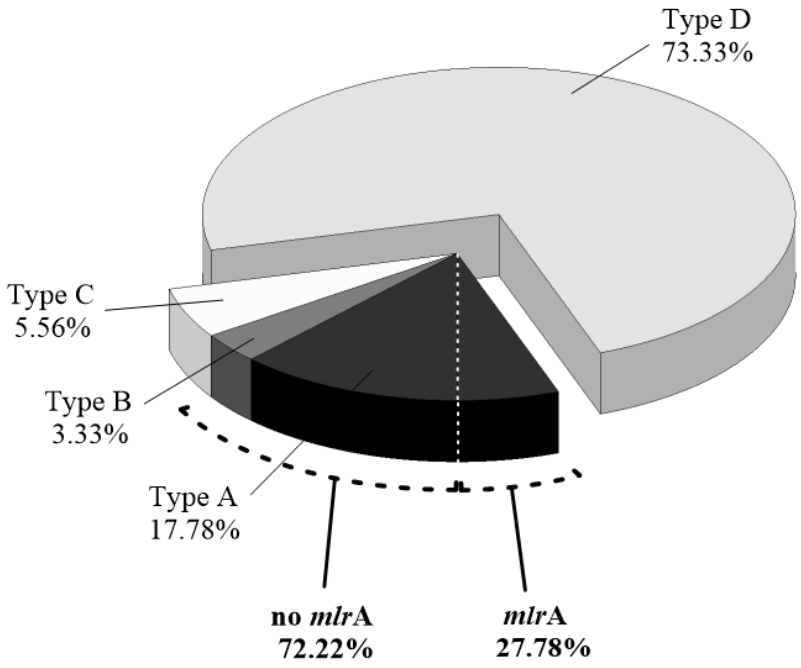


Figure 2.1. Proportion of the different cultivable MC-degrading bacterial phenotypes found in the water samples. Classification was performed according to MC degradation efficiencies. Type A represents bacteria with a MC degradation efficiency between 80% and 100%, type B between 60% and 79% and type C between 40% and 59%. Non-MC-degrading bacteria are represented in type D. The proportion of the most efficient MC-degrading bacteria (phenotypes A and B) with presence and absence of *mlr*A gene are also represented.

2.4.3. Abundance of mlrA gene during the bloom episode

Quantitative analysis of the *mlr*A gene by Real-Time PCR during the bloom episode ranged from unquantifiable (but detected) gene copies L^{-1} on 7 August and 4 September, to 4.72×10^4 gene copies L^{-1} on 24 September, 1.76×10^4 gene copies L^{-1} on 8 October and 1.37×10^4 gene copies L^{-1} on 29 October (Figure 2.2). These results, together with the high correlation recorded between *mlr*A gene abundance and sestonic MCs, show that the *mlr*A^+ bacterial community (with presence of *mlr*A gene) follows a similar trend with total sestonic MC concentrations, peaking on 24 September, when maximum MC concentrations were also

recorded. Quantitative analysis of the *mlr*A gene in reservoir water spiked with 1.2 mg equivalent MC-LR·L^{-1} showed an increase of gene abundance near two orders of magnitude in all samples, indicating a positive response of *mlr*A^+ bacterial community to MC concentrations in the water.

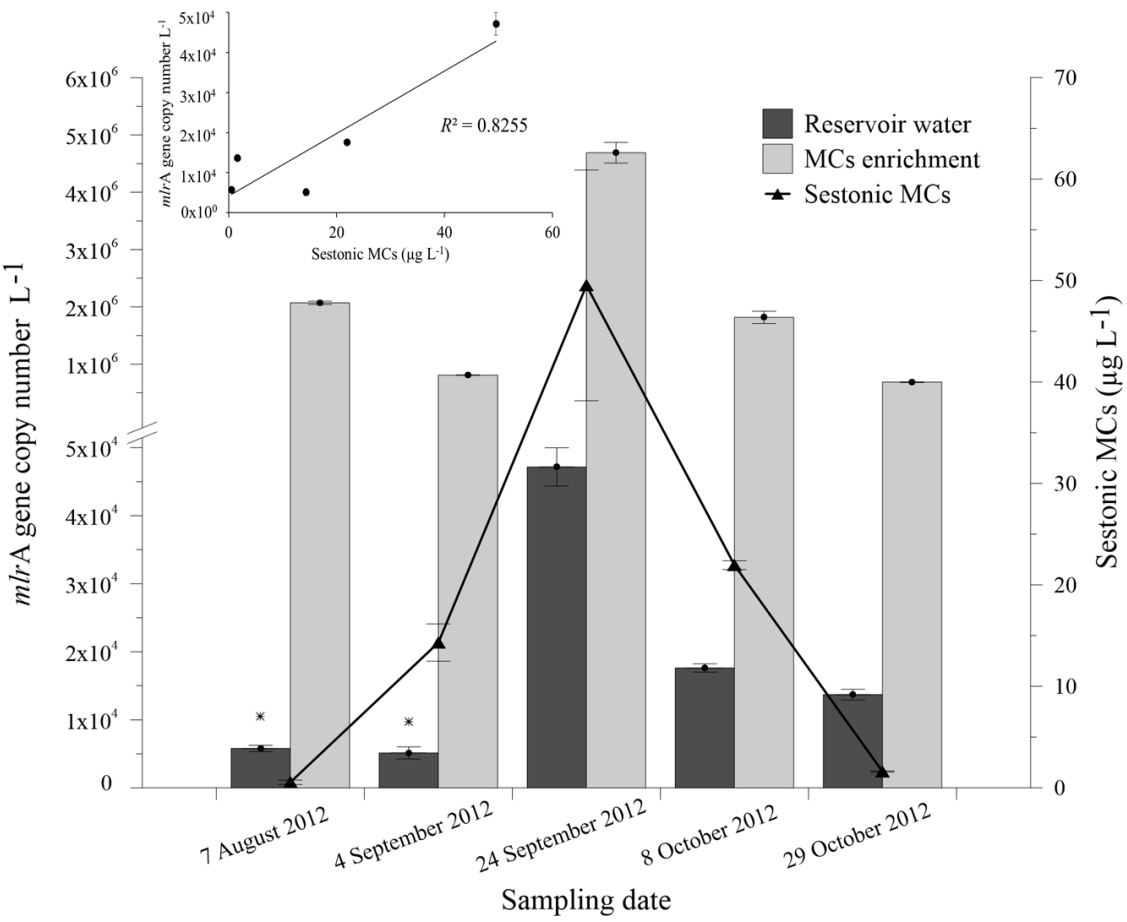


Figure 2.2. Abundance of *mlr*A gene copies L^{-1} in reservoir water samples and 15 days after MCs enrichment. Line represents total sestonic MCs and the small inserted plot represents the correlation between *mlr*A gene copies L^{-1} and total sestonic MCs. Asterisk indicates data below quantification limit and error bars represent standard errors of two replicates.

2.4.4. In vitro MC biodegradation assays and kinetics

In order to evaluate whether the presence or absence of *mlr* genes determine MC degradation efficiencies under alternative carbon and nitrogen sources, four new isolates and two MC-degrading bacteria from culture collection (*Sphingosinicella microcystinivorans*, strain Y2 and *Paucibacter toxinivorans*, strain 2C20) were selected. Results from the identification of the complete *mlr* gene cluster showed that the new isolates IM-1, IM-2 and IM-3, identified as *Sphingopyxis* sp. based on 16S rRNA analysis (Figure 2.3), contain the whole *mlr* cluster (*mlr*A-D) (*mlr*$^+$ genotype) while strain IM-4, identified as *Paucibacter toxinivorans*, lack the

complete *mlr* gene cluster (*mlr*$^{-}$ genotype). Bacterial strains Y2 and 2C20 were identified as *mlr*$^{+}$ and *mlr*$^{-}$ genotypes, respectively.

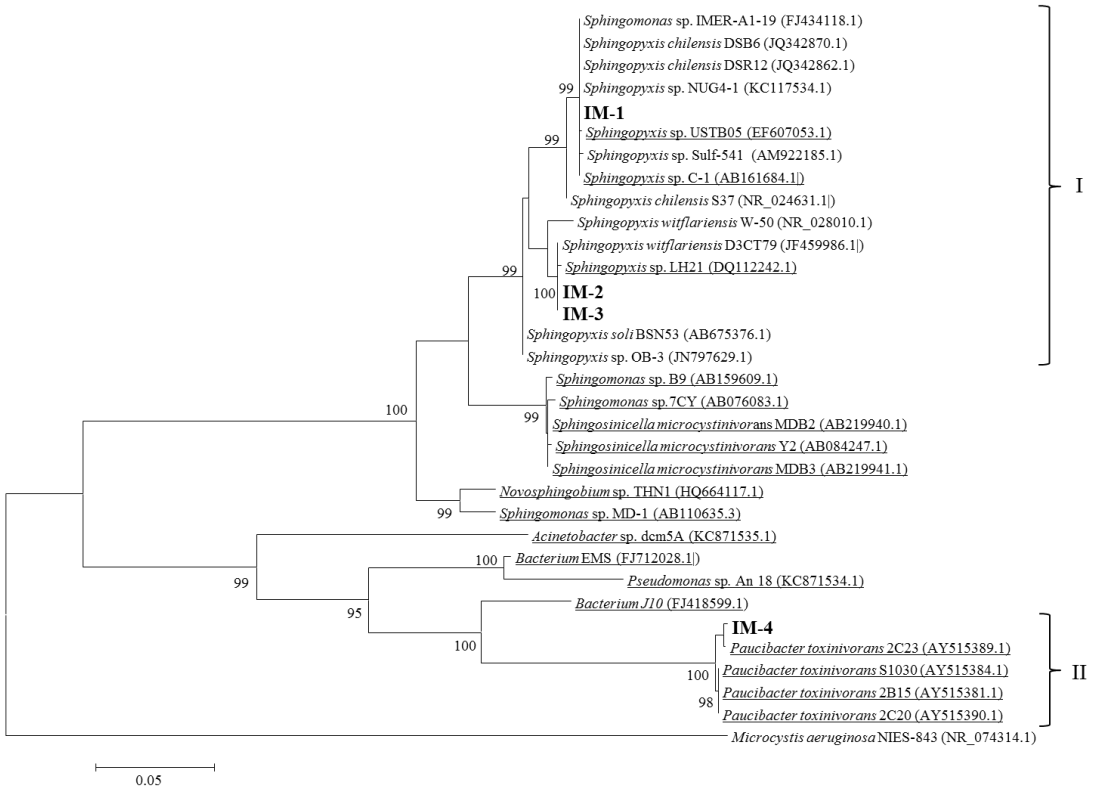


Figure 2.3. Maximum likelihood tree based on the 16S rRNA gene (1344–1420 bp) showing, in bold, the position of the sequences obtained in the present study. The numbers near nodes indicate bootstrap values greater than or equal to 95, as a percentage of 1000 replicates resulting from the analysis. Underlined sequences indicate already known MC-degrading bacteria. Bar, 0.05 substitutions per nucleotide position. Cluster I represents *Sphingopyxis* sp. and cluster II, *Paucibacter toxinivorans*.

MC degradation kinetics of strains were assessed under different concentrations of alternative carbon and nitrogen sources by using Minimal Salts Medium (Valeria et al., 2006) (absence of carbon and nitrogen compounds) and 4-fold R2A medium (258.37 ± 0.06 mg L^{-1} of TOC and 1.9 mg L^{-1} of TN). An experiment in reservoir water (3.83 ± 0.02 mg L^{-1} of TOC and <0.05 mg L^{-1} of TN) was also performed to evaluate degradation efficiencies similar to real life scenarios. Experiments were performed with 1 mg equivalent MC-LR·L^{-1} from the crude MC extract containing 92.37% (±0.86%) of MC-LR, 3.91% (±0.82%) of MC-RR and 3.71% (±0.50%) of MC-YR. Figure 2.4 shows MC degradation curves of selected strains over time. In all cases, no losses of MCs were found in negative controls, indicating that removal of MCs was biologically-mediated. *mlr*$^{+}$ bacterial genotype (strains IM-1, IM-2, IM-3 and Y2) considerably improved MC degradation and reduced lag phases

under high carbon and nitrogen concentrations (4-fold R2A medium) compared to MSM and reservoir water. Contrarily, *mlr*$^{-}$ genotype (strains 2C20 and IM-4) showed longer lag phases and required 120 h or more to completely degrade the toxins in presence of carbon and nitrogen sources. Only strain IM-1 (*mlr*$^{+}$) was equally efficient degrading 90% of total MCs in 6 h regardless of the different TOC and TN concentrations. MC degradation rates in 4-fold R2A medium comparing to MSM was enhanced about 1.5, 3.8 and 3.0 times in Y2, IM-2 and IM-3, respectively (*mlr*$^{+}$), and was depressed about 1.6 and 4.7 times in 2C20 and IM-4, respectively (*mlr*$^{-}$) (Table 2.3). Regardless of the genotype, no bacterial growth was observed either in MSM medium or in reservoir water, where no or few carbon and nitrogen sources apart from MCs (and other natural components of the MC raw extract) were present (Table S 2.1 from Supplementary Materials).

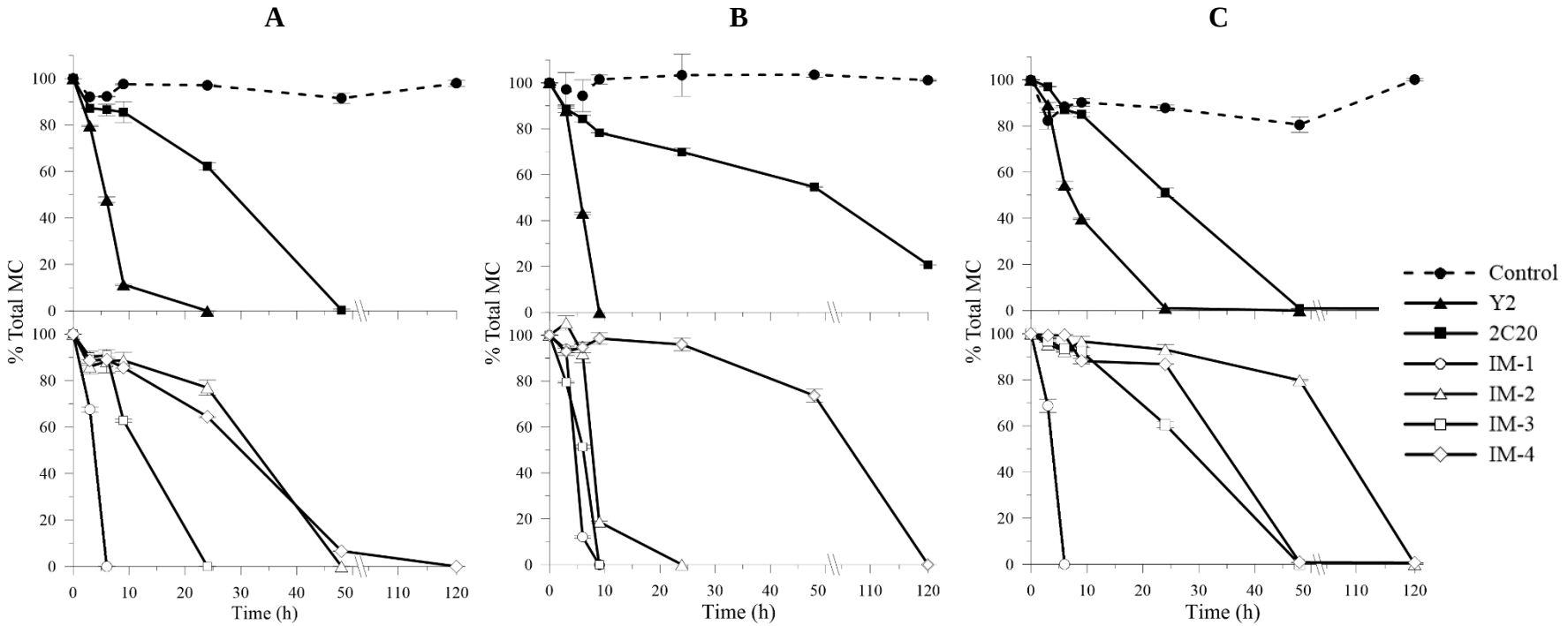


Figure 2.4. Biodegradation of total MCs by bacterial genotypes *mlr*$^{+}$ (strains Y2, IM-1, IM2 and IM-3) and *mlr*$^{-}$ (strains 2C20 and IM-4) incubated in: (A) MSM, (B) 4-fold R2A medium, and (C) reservoir water for 120 h. Upper plots represent bacteria from culture collection and bottom plots represent new isolated strains. A negative control without bacteria was included. Error bars represent standard errors of two technical replicates.

Table 2.3. MC degradation rates under different media with variable total organic carbon and total nitrogen concentrations. Ratios correspond to assay period until 90% of total MC degradation was achieved. (+) and (−) mean presence and absence of *mlr* genes, respectively. Errors represent standard errors of two replicates.

Bacterial strains	*mlr*A-D genes	Degradation rates (µg MC L^{-1} h^{-1})		
		MSM	¼ R2A medium	Reservoir water
Y2	+	73.24 ± 6.09	113.36 ± 22.33	48.2 ± 16.80
2C20	-	12.75 ± 2.93	8.04 ± 4.94	20.62 ± 4.00
IM-1	+	171.15 ± 15.34	146.92 ± 23.79	144.9 ± 54.02
IM-2	+	25.35 ± 6.04	97.03 ± 40.47	11.39 ± 5.28
IM-3	+	37.10 ± 7.20	112.14 ± 21.36	16.30 ± 0.71
IM-4	-	19.42 ± 1.12	4.12 ± 1.37	21.26 ± 3.36

Individual analysis of MC-LR and MC-RR biodegradation in selected bacterial strains showed no apparent differences in degradation rates under above-mentioned testing conditions (Figure S 2.1 – S 2.3 from Supplementary Materials). Data from MC-YR were excluded from this analysis because of discrepancies among technical replicates due to its low sensitivity and low initial concentration.

2.5. Discussion

In the current study, all samples, except those collected on 7 August, exceeded the provisional drinking water guideline value of 1 µg L^{-1} for MCs (WHO, 1998). Although no guideline value for MCs is reported for recreational waters, the WHO establishes a maximum of 20,000 cyanobacterial cells mL^{-1} or 10 µg L^{-1} of chlorophyll *a* for low health effects, where about 2–4 µg L^{-1} of MCs is expected (WHO, 2003). Accordingly, samples collected on 4 September, 24 September and 8 October exceeded these values. These MC concentrations were within the range or higher than those reported in previous studies that were conducted on water samples taken in Spain (Agha et al., 2012; Carrasco et al., 2006; Moreno et al., 2005), Europe (Gkelis et al., 2014; Jančula et al., 2014; Messineo et al., 2009) and other countries (Orihel et al., 2012; Su et al., 2015), which questions the capacity of the ecosystem to tackle high concentrations of MCs and, thus, prevent their potential harmful effects on humans, animals and other living beings. The depletion of MCs in spiked reservoir water samples collected during the bloom episode revealed the MC biodegradation capacity of the indigenous bacterial community to overcome a release of intracellular MCs during bloom decay. This capacity may also explain, together with photodegradation, adsorption

and/or dilution factors, why the extracellular fraction is always several orders of magnitude below the sestonic (Sivonen and Jones, 1999). Considering predictive models of climate change, temperate lakes are expected to extend periods of thermal stratification under warmer conditions, lengthening growing season of primary producers and uncoupling trophic relationships (De Senerpont Domis et al., 2013; Paerl et al., 2011). This, in addition to expected decreases in precipitation in the Mediterranean area, will lead to longer water residence times resulting in higher total cyanobacterial biomass and increased cyanotoxin concentrations in water (Romo et al., 2013). In such projected scenarios, the endogenous MC biodegradation process in Mediterranean reservoirs and lakes will become an important mechanism for restoration of initial conditions and water uses. Thus, the study of said mechanism and the factors controlling the efficiency of the process in nature deserves special attention.

Coexisting MC-degrading strains within the bacterial community were classified according to their different degradation efficiencies into three phenotypes (A–C). Thus far, most studies have been mainly focused on isolation and simple identification of new MCs-degrading strains. However, the screening and classification of coexisting bacteria according to their degradation efficiencies has allowed understanding that the composition of these phenotypes within the indigenous bacterial community may modulate and ultimately be responsible for the final MC concentrations in nature when a cyanobacterial bloom develops. Previous studies on cultivable heterotrophic bacteria have demonstrated the coexistence of different bacterial genera associated with cyanobacterial blooms (Berg et al., 2009), and identified qualitative and quantitative family composition changes in relation to the amount of cyanobacterial biomass (H. Li et al., 2012). These results support our observations of the existence of diverse MCs-degrading bacteria with different degradation efficiencies, and that the occurrence, proportion, and spatio-temporal distribution of these MCs-degrading phenotypes contribute to the regulation of the final concentration of MCs in the water. The presence of two different genotypes, *mlr*A^{+} and *mlr*A^{-}, within the MCs-degrading bacterial community support previous studies that suggest the existence of alternative pathways for the MC degradation process to that described by Bourne et al. (2001) (Manage et al., 2009b; Mou et al., 2013). Another possibility is that, contrary to the general consensus in the field, *mlr*A gene is not highly conserved among the genera capable of degrading MCs. This, in addition to the lack of *mlr*A gene analysis of some novel MC-degrading bacteria (Kormas and Lymperopoulou, 2013), makes it difficult to establish a firm conclusion about the

suitability of the *mlr*A as an unequivocal gene marker for MC-degrading capacity. According to our results, *in vitro* MC biodegradation assays currently represent the most reliable test to assess the MC-degrading ability of a strain. The design of universal primers or the elucidation of new enzymatic pathways may reveal new insights into the MC degradation process and the biological role these pathways play in the removal of MCs in nature.

The observed temporal variations in *mlr*A gene abundance during bloom development, together with the positive response of *mlr*A to enriched water samples with crude MCs, revealed that the *mlr*A$^+$ bacterial community responded positively to a release of MCs in water. Similar results were observed by Zhu et al. (2014), who found that seasonal variations of extracellular MCs in water were related to variations in MC-degrading bacterial abundance based on *mlr*A gene analysis. Our results, together with other studies, suggest that these *mlr*A$^+$ bacteria make an important contribution to the biodegradation of MCs in water (Li et al., 2015; Zhu et al., 2014). However, the real contribution of the total biodegradation of MCs in nature can only be understood by considering the whole MC-degrading bacterial community which, according to our results, goes beyond the analysis of the *mlr*A gene. Therefore, although correlations between *mlr*A gene copy number and MC-LR biodegradation rate identified by Li et al. (2015) exhibited a similar changing trend, the low correlation (R^2 = 0.54) actually indicates that biodegradation rate of MCs is only partially explained by *mlr*A-possessing MC-degrading bacteria. This is consistent with the relatively higher abundance of *mlr*A$^-$ over *mlr*A$^+$ MC-degrading bacteria found in our study and in Mou et al. (2013), which supports our observation that the MCs-degrading bacterial community that lacks *mlr* genes may be an important contribution to the MC biodegradation process in nature. In an effort to better understand the importance and advantage of *mlr* genes for the MC-degrading bacterial community, we studied the degradation efficiencies of both bacterial genotypes (*mlr*$^+$ and *mlr*$^-$) in presence of various TOC and TN concentrations.

Our results show dissimilar MC degradation efficiencies between *mlr*$^+$ and *mlr*$^-$ bacterial genotypes in presence of alternative C and N sources. The marked improvement of MC removal rates by genotype *mlr*$^+$ under alternative C and N compounds compared to bacteria in absence of *mlr* genes (Figure 2.4) suggests a different MC substrate affinity between *mlr* and alternative-*mlr* degradation pathways. According to our results, the presence of alternative organic carbon and nitrogen compounds (in this study provided by the 4-fold R2A medium) stimulate both growth (Table S 2.1 from Supplementary Materials) and MCs

degradation in *mlr*$^+$ strains, indicating that MC removal rates seem to depend more on the abundance of bacterial biomass, as reported in other studies (Ho et al., 2010; Hoefel et al., 2009) than on the stimulation or depression of specific nutrients to *mlr* genes expression (Li et al., 2014). Our results are also in accordance with Zhang et al. (2015), where removal percentage of MC-LR increased by increasing bacterial growth under addition of glucose and ammonium chloride. In contrast, despite showing growth of *mlr*$^-$ strains in presence of alternative C and N sources, MC degradation was not enhanced, indicating a possible competition between MCs and alternative substrates. Accordingly, the absence of *mlr* genes and, consequently, the presence of alternative-*mlr* MC degradation pathways (Manage et al., 2009b; Mou et al., 2013), drives lower degradation rates in presence of additional C and N sources compared to those containing *mlr* genes. These differences in the degradation efficiency among natural MC-degrading bacteria raise concern about its impact in the aquatic ecosystem, where alternative C and N sources are always present in the water column. The low C and N concentrations found in the reservoir water we used in this study explain why MC degradation rates are similar to those obtained in absence of alternative C and N sources (MSM). However, the increasing eutrophication of aquatic ecosystems raises doubts about the MC degradation efficiency of the natural bacterial community. It appears to us that eutrophication may not only drive more incidences of toxic cyanobacterial blooms, but may also drive lower overall elimination of MCs. Despite the enhanced MC removal of *mlr*$^+$ bacteria when other C and N sources are present in the water, the degradation capacity of the abundant *mlr*$^-$ bacterial community is negatively affected under increasing C and N concentrations. This, in turn, may result in prolonged toxicity of the bloom episode.

The equal degradation of both MC-LR and MC-RR by our isolated strains in the presence of variable C and N concentrations (Figures S1–S3) indicates that both *mlr* and alternative-*mlr* pathways do not appear to exhibit preference among MC variants, which is consistent with other studies where no differences were found either in reservoir water samples (Ho et al., 2012b) or in mineral salts medium (Li et al., 2015). Therefore, regardless of common shifts in the relative abundance of MC variants along the bloom episode, MC degraders from the bacterial community will face these changes with similar efficiency, as they do not show any preference for one MC variant over the other.

2.6. Conclusions

Our results demonstrate that the MC degradation activity found in an aquatic system during a cyanobacterial bloom is mediated by a MC-degrading bacterial community and contributes

to the natural capacity of the ecosystem to cope with high MC concentrations. The presence or absence of the *mlr* gene cluster in the MC-degrading bacterial community has been proven to be an important factor for the MCs degradation efficiency under the presence of alternative carbon and nitrogen sources. Bacteria with *mlr* genes showed better MC substrate affinity and higher degradation rates than MC-degrading bacteria without *mlr* genes. Accordingly, the MC removal rates in nature are not only determined by the availability of alternative C and N sources, but also the relative abundance of mlr^+ over mlr^- genotypes within a MC-degrading bacterial community. Our findings further highlight the need to increase our knowledge on the diversity, abundance and function of the MC-degrading bacterial community under eutrophication and global warming scenarios in order to predict their impacts on the ecosystem.

2.7. Acknowledgements

The authors acknowledge funding from Marie Curie-Cofund (AMAROUT Europe) to Rehab El-Shehawy.

2.8. Supplementary materials

Table S 2.1. Average bacterial growth rates (mg L^{-1} h^{-1}) during MC degradation in MSM, reservoir water and 4-fold R2A medium enriched with 1 mg equivalent MC-LR ·L^{-1} from MC crude extract. Errors represent standard errors of two replicates. "n.d." means not detected.

Average bacterial growth rates (mg L^{-1} h^{-1})			
Bacterial strains	**MSM**	**Water reservoir**	**¼ R2A medium**
Control	n.d.	n.d.	n.d.
Y2	n.d.	n.d.	0.25 ± 0.01
2C20	n.d.	n.d.	0.28 ± 0.00
IM-1	n.d.	n.d.	0.47 ± 0.00
IM-2	n.d.	n.d.	0.87 ± 0.06
IM-3	n.d.	n.d.	0.73 ± 0.04
IM-4	n.d.	n.d.	0.21 ± 0.01

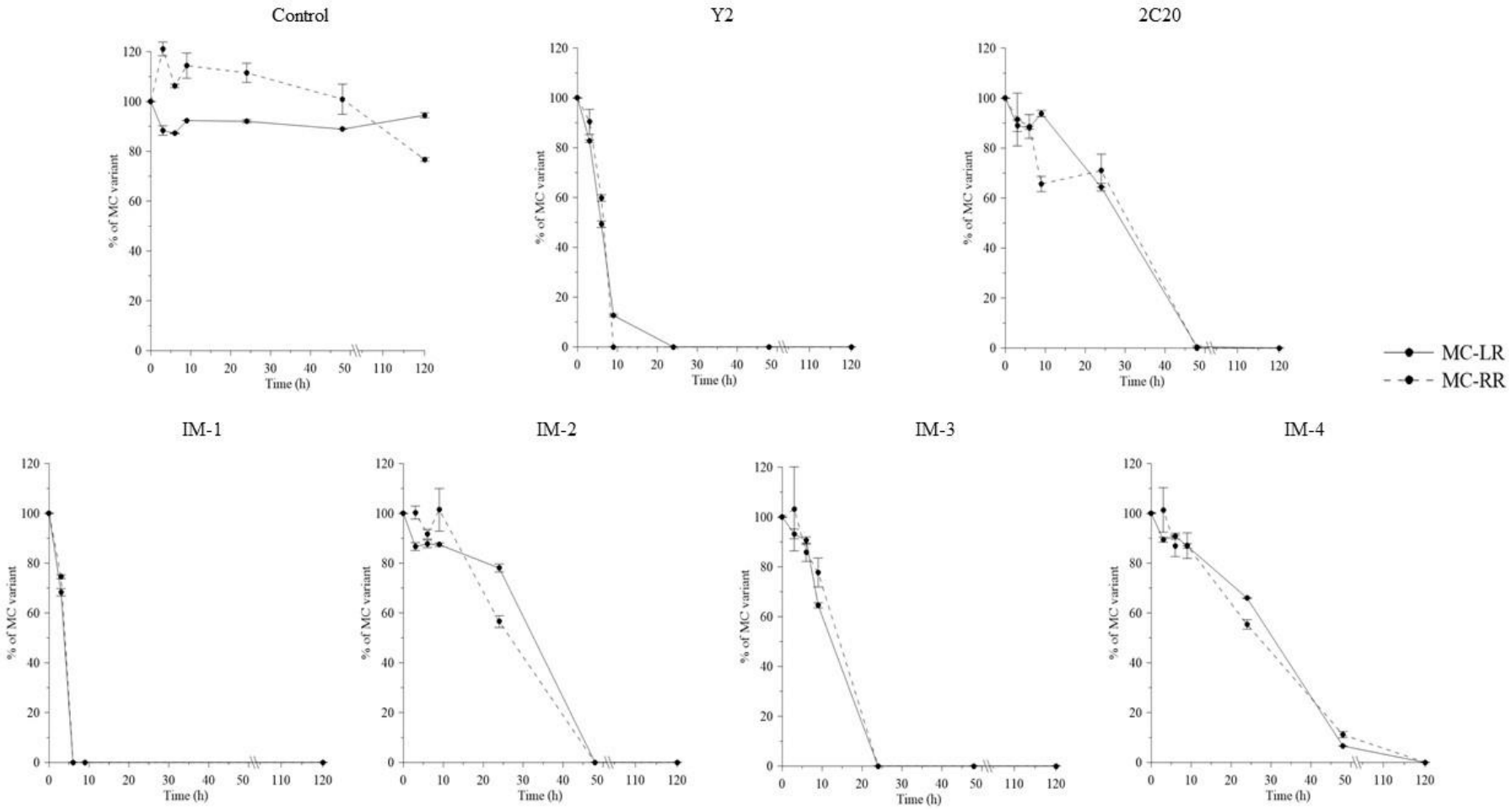


Figure S 2.1. Biodegradation of MC-LR and MC-RR variants by bacterial genotypes *mlr*$^{+}$ (strains Y2, IM-1, IM2 and IM-3) and *mlr*$^{-}$ (strains 2C20 and IM-4) incubated in MSM for 120 h. A negative control without bacteria was included. Error bars represent standard errors of two replicates.

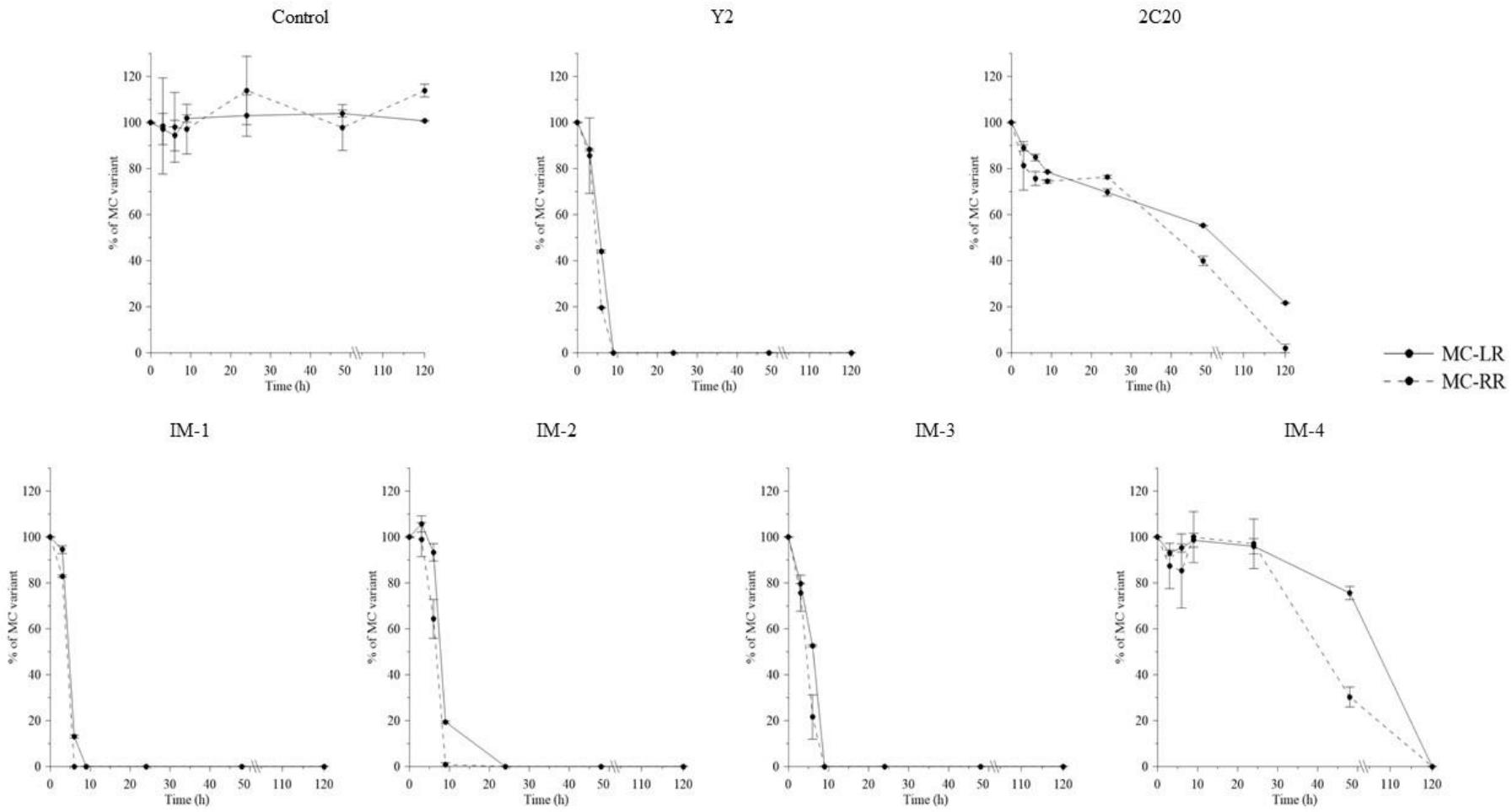


Figure S 2.2. Biodegradation of MC-LR and MC-RR variants by bacterial genotypes *mlr*$^+$ (strains Y2, IM-1, IM2 and IM-3) and *mlr*$^-$ (strains 2C20 and IM-4) incubated in 4-fold R2A medium for 120 h. A negative control without bacteria was included. Error bars represent standard errors of two replicates.

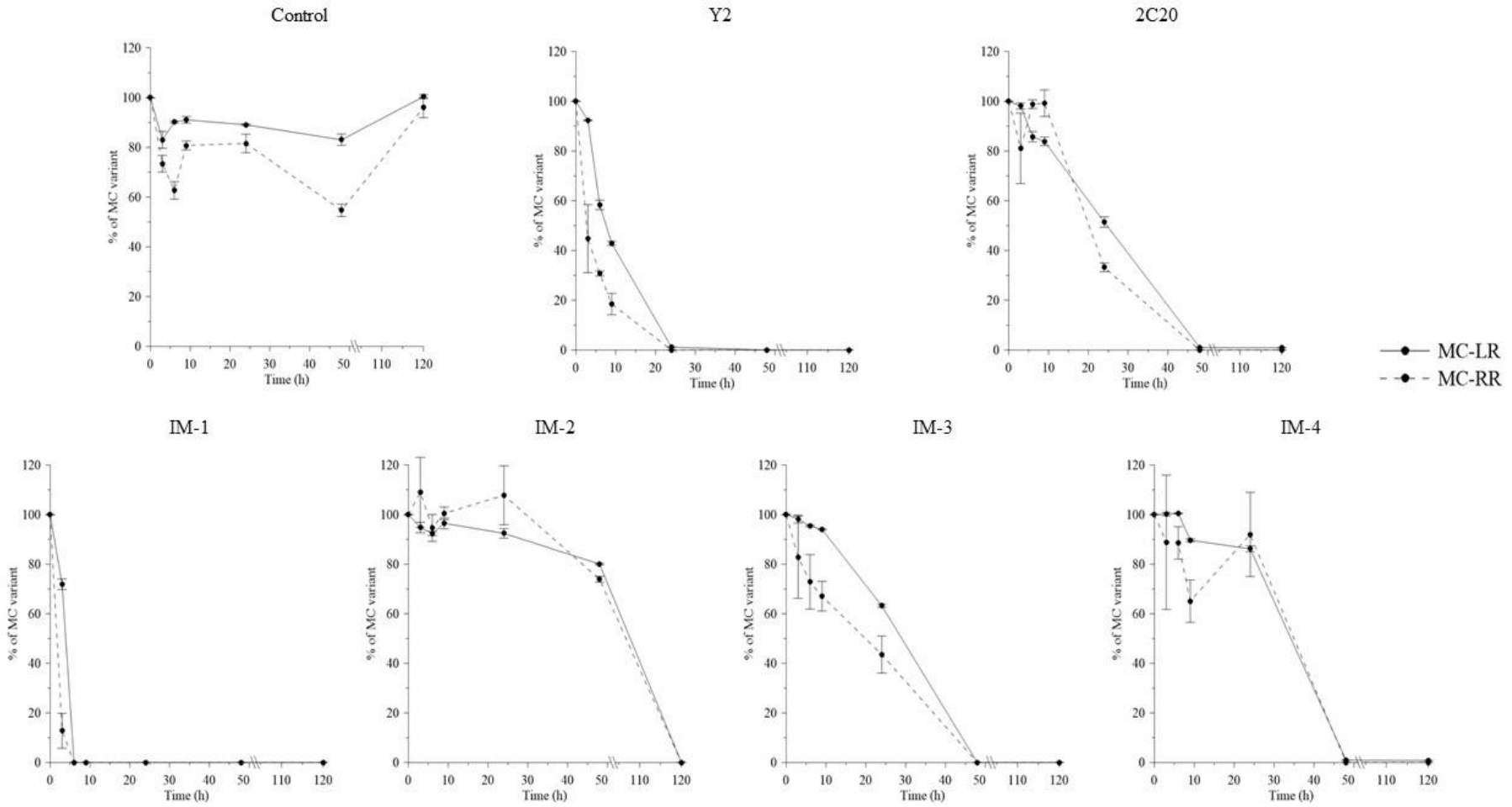
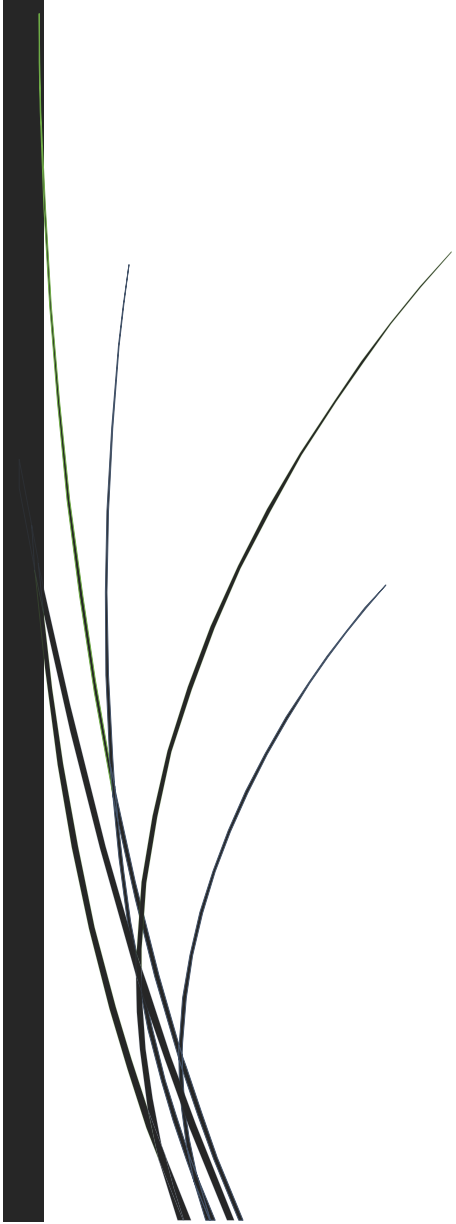


Figure S 2.3. Biodegradation of MC-LR and MC-RR variants by bacterial genotypes *mlr*$^{+}$ (strains Y2, IM-1, IM2 and IM-3) and *mlr*$^{-}$ (strains 2C20 and IM-4) incubated in reservoir water for 120 h. A negative control without bacteria was included. Error bars represent standard errors of two replicates.

Chapter 3



3. Diversity and temporal shifts of the bacterial community associated with a toxic cyanobacterial bloom: an interplay between microcystin producers and degraders

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3.1. Abstract

The biodegradation of microcystins (MCs) by bacteria constitutes an important process in freshwater ecosystems to prevent the accumulation of toxins. However, little is known about the diversity and the seasonal dynamics of the bacterial community composition (BCC), as well as the potential MC degradation pathways involved in nature. To explore these BCC shifts, high-throughput sequencing was used to analyse the 16S rRNA, *mcy*E and *mlr*A genes during a year in a freshwater reservoir with a toxic cyanobacterial bloom episode. The variability within the *mcy*E and *mlr*A genes from water samples revealed the coexistence of different MC-producing and MC-degrading genotypes, respectively. The patchy temporal distribution of the *mlr*A genotypes (from the families *Sphingomonadaceae* and *Xanthomonadaceae*) suggests their dissimilar response to environmental conditions and the influence of other factors besides the MCs that control their presence and relative abundance. During the maximum toxic cyanobacterial biomass and cell lysis, other bacterial taxa that lack *mlr* genes increased their relative abundance. Among these bacteria, those with a recognized role in the degradation of xenobiotic compounds (e.g., orders *Myxococcales*, *Ellin6067*, *Spirobacillales* and *Cytophagales*) were the most representative.

3.2. Introduction

Freshwater cyanobacterial blooms are common natural phenomena, but their increasing frequency and intensity worldwide are considered undesirable consequences of the eutrophication and global change on aquatic systems (De Senerpont Domis et al., 2013; Paerl et al., 2011). As part of the phytoplankton community, the cyanobacteria are essential to biogeochemical cycles and constitute the basis of aquatic food webs (Cho and Azam, 1990). However, their excessive growth entails significant disruption to ecosystem function (Codd et al., 2005a), especially when the toxin-producing cyanobacteria dominate blooms (Moustaka-Gouni et al., 2006). The most widespread and frequent toxins are the microcystins (MCs), which are synthesized non-ribosomally by large multienzyme complexes encoded by the *mcy*A-J gene cluster (Börner and Dittmann, 2005). The MCs are potent inhibitors of protein phosphatases, which cause liver damage, promote tumour activity (MacKintosh et al., 1990; Zhou et al., 2002) and pose serious harm to a broad group of organisms from the ecosystem (Bláha et al., 2009; Sivonen and Jones, 1999).

In the region surrounding cyanobacteria, the so-called phycosphere (Bell and Mitchell, 1972), other bacterioplankton communities play an important role in the mineralization and turnover of the organic matter. Some of these bacteria, especially those from the family *Sphingomonadaceae* (phylum *Proteobacteria*), are able to degrade the MCs (Ho et al., 2007; Valeria et al., 2006) using an enzymatic pathway encoded by the *mlr*A-D gene cluster (hereafter, *mlr*$^+$ genotype) (Bourne et al., 2001). However, bacterial isolates lacking *mlr* genes (hereafter, *mlr*$^-$ genotype) from the families *Burkholderiaceae, Micrococcaceae*, *Nocardiaceae*, *Brevibacteriaceae, Xanthomonadaceae* and *Aeromonadaceae*, have also been reported as MC degraders (Lezcano et al., 2016; Manage et al., 2009b; Mankiewicz-Boczek et al., 2015; Rapala et al., 2005; Yang et al., 2014b). The different MC-degrading bacterial families isolated from field samples (both *mlr*$^+$ and *mlr*$^-$ genotypes), together with the diverse array of bacterial taxa associated with cyanobacterial blooms (H. Li et al., 2012) and responsive to MCs in microcosm experiments (Mou et al., 2013), point towards the existence of alternative-*mlr* degradation pathway(s) and reveals the largely undetermined MC-degrading bacterial diversity in nature.

Despite the well-studied multiple interactions between cyanobacteria and the associated bacterial assemblage (Ramanan et al., 2016), there is a gap of knowledge about the composition and the seasonal dynamics of the specialized MC-degrading bacterial community, considering both *mlr*$^+$ and *mlr*$^-$ genotypes, under environmental conditions. To

unravel this diversity and the seasonal patterns, a high-throughput sequencing experiment of a water reservoir with periodic toxic cyanobacterial blooms was designed. Therefore, in the present work, we studied the following: i) the composition and the seasonal dynamics of the MC-producing cyanobacteria and the fraction of the MC-degrading bacteria with *mlr* genes by analysing the *mcy*E and *mlr*A genes, respectively, and ii) the composition and the seasonal dynamics of the bacteria lacking *mlr* genes that are responsive to the release of MCs during the cyanobacterial bloom decay. This study is the first to analyse the diversity and the seasonal dynamics of both the mlr^+ and mlr^- fractions of the MC-degrading bacterial community in a water reservoir with a toxic cyanobacterial bloom episode.

3.3. Materials and methods

3.3.1. Sampling site and sample collection

The San Juan reservoir is a water body located in the Iberian Central Plateau (Madrid, Spain) with a granitic catchment and a history of persistent toxic cyanobacterial blooms. It was periodically sampled at the shoreline (40° 22' 44.10" N and 4° 19' 40.95" W) from 29 January 2014 to 2 December 2014. Water samples were collected every two months during winter and spring, which was changed to a monthly basis from August to November and to a fortnightly basis throughout the massive cyanobacterial bloom episode until December. Each sample was comprised of a total of 2 L of subsurface water collected with sterilized polyethylene bottles that were rinsed three times on site with reservoir water and stored in dark and cold (4°C) conditions during transport (less than 2 hours) to the laboratory. Samples were split in two aliquots, and each aliquot was treated with a different protocol: one litre of water was sequentially filtered through 2-µm (25 mm, Whatman, Maidstone, United Kingdom) and 0.22-µm pore size (25 mm, Millipore, Darmstadt, Germany) polycarbonate filters to collect the fractions containing the larger and smaller cyanobacteria and other aquatic bacteria (both those attached to the cyanobacterial mucilage and those living free). Filter membranes were stored at -20 °C for further analysis. The remaining one litre of water was filtered through fibreglass filters (0.7 µm approx., Millipore) to collect the sestonic fraction and then stored at -20°C for subsequent MC and chl-*a* analysis. The flow-through was also preserved at -20°C for quantification of the dissolved MCs.

3.3.2. Bacterial community DNA extraction

Genomic DNA from 2-µm and 0.22-µm polycarbonate filters was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions with several modifications in the cell disruption step, as described in Lezcano et

al. (2016). The extracted DNA was dissolved in sterilized Milli-Q water and quantified using an Epoch spectrophotometer (BioTek Instruments, Winooski, VT, USA). Equal genomic DNA concentrations from both filtered fractions and the sampling events were used for the subsequent high-throughput sequencing analysis.

3.3.3. Amplicon sequencing and analysis

The bacterial community composition (BCC) was examined using a bar-coded 16S rRNA amplicon sequencing strategy with primers 341F (5´-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3´) and 805R (5´-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3´) (Klindworth et al., 2013) which include the V3-V4 hypervariable regions of the 16S rRNA gene with specific linking sequences and sequencing adaptors. To study the potentially MC-producing cyanobacterial community and the fraction of the MC-degrading bacterial community possessing the *mlr* gene cluster (Bourne et al., 2001), *mcy*E and *mlr*A were used as the targeted genes due to their essential function in the synthesis (Jungblut and Neilan, 2006) and degradation of MCs (Saito et al., 2003), respectively. Moreover, both *mcy*E and *mlr*A, although functional genes, have been reported to be conserved between different genera and enough variable to detect differences (J. Chen et al., 2010; Jungblut and Neilan, 2006), which allow us to approach to the taxonomic identification of both MC-producing and MC-degrading communities. The primer pairs used for the *mcy*E gene were HEPF and HEPR (Jungblut and Neilan, 2006), due to their wide application on the different MC-producing genera; for the *mlr*A gene, the primer pairs were MF and MR (Saito et al., 2003), which are the most widely used. The quantity and quality of the resulting PCR products were verified using the Epoch spectrophotometer and a 1.5% agarose gel. Equal concentrations of each PCR product were pooled, and the resulting amplicon pool was pair-end sequenced (2x250 bp) on an Illumina MiSeq platform (Foundation for the Promotion of Health and Biomedical Research, FISABIO, Valencia, Spain).

Denoising, filtration of low-quality reads and the removal of chimaeras were performed using QIIME software v.1.8.0. (Caporaso et al., 2010), following the 16S Profiling Data Analysis Pipeline from the Brazilian Microbiome Project (Pylro et al., 2014). The sequences were paired-end aligned using the Trimmomatic tool (Bolger et al., 2014). The sequencing reads were also quality filtered to minimize the effects of random sequencing errors. Singletons were discarded to avoid overestimations and clustering bias using UPARSE

(Edgar, 2013). The reads were clustered into OTUs with 97% sequence similarity using the UPARSE algorithm. The OTU sequences of the 16S rRNA, *mcy*E and *mlr*A genes were aligned in-house with sequences obtained from the NCBI GenBank (Altschul et al., 1990: accession 05/07/2016) and used as seed sequences in the MUSCLE algorithm. The final alignment lengths were 460 bp for 16S rRNA, 470 bp for *mcy*E and 807 bp for *mlr*A. Using this method, a total of 2,680 bacterial OTUs were obtained (2,642 for 16S rRNA genes, 19 for *mcy*E genes and 19 for *mlr*A genes). The taxonomic assignations of OTUs from the 16S rRNA gene were performed with 97% similarity. A profile of OTU similarities from 90 to 99% for *mcy*E and *mlr*A genes was generated to study the match differences among samples. Finally, the taxonomic assignations were performed with 97% similarity for *mcy*E genes and 95% similarity for *mlr*A genes, since both values were the highest percentages that accounted for the highest number of different OTUs. Sequences from the 16S rRNA gene that were assigned to non-bacterial entities (Archaea and chloroplasts from Eukaryota) were removed (Logue et al., 2016). Since the analysis of the 16S rRNA gene is focused on the exploration of the specific MC-degrading bacterial community that lack *mlr* genes, OTUs represented by fewer than 15 sequences among all the water samples were discarded to avoid biased conclusions due to underrepresented bacteria from the ecosystem. Thus, the threshold considered OTUs with a minimum of 0.001% of representation in the total bacterial community and discarded 0.55% of the total sequences.

3.3.4. MCs and chl-a extraction

Duplicates of sestonic MCs and chl-*a* from fibreglass filters were double-extracted by sonication (P-Selecta Ultrasons, Barcelona, Spain) with 90% aqueous methanol and evaporated at 40 °C under vacuum in a multiple evaporator (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany). Chl-*a* was quantified before evaporation by spectrophotometric measurement at 665 nm (Shimadzu Multispec-1501, Kyoto, Japan) and the final extracts were stored at -20 °C for MC quantification. Dissolved MCs from 600 mL of filtered water was extracted by solid phase extraction using C18 cartridges (200 mg, 6 cc) (OASIS HLB, Waters, Milford, MA, USA) and equilibrated with 10% methanol. The elution of the MCs was performed in 90% methanol. Both sestonic and dissolved MC extractions were filtered through 0.22-µm syringe filters (Acrodisc GHP, Pall Corporation, Port Washington, NY, USA) before analysis. Quantification of the MCs was performed on an HPLC system (Agilent series 1100, Agilent Technologies, Santa Clara, CA, USA) coupled to a time-of-flight (TOF) mass spectrometer (Agilent 6230 accurate mass TOF Agilent

Technologies, Santa Clara, CA, USA) by plotting calibration curves from commercial MC-LR, MC-RR and MC-YR pure standards (Sigma-Aldrich, St. Louis, MO, USA). The chromatographic separation and the gradient profile of the mobile phase were described in Lezcano et al. (2016).

3.3.5. Statistical analysis

A Pearson correlation analysis was performed with STATGRAPHICS Centurion XV v.15.1.02 software (Statpoint Technologies, Inc. Warrenton, VA, USA) using the whole phylum, order and family datasets to study the relationship between the log-transformed total MC concentrations and the log-transformed relative abundances of each bacterial taxon. Significant positive or negative correlations were assumed when the calculated *p*-value was lower than 0.05. Additionally, higher *p*-values up to 0.15 were also considered in this study for the exploration of moderate correlations. Moreover, a principal component analysis (PCA) was also performed using CANOCO5 v.5.04 software (Microcomputer Power, Ithaca, NY, USA) to explore the relationship between the log-transformed total MC concentrations and the log-transformed relative abundances of the whole bacterial phylum, order and family datasets over the period of study. Shannon-Wiener and Chao1 diversity and evenness indices were calculated as α-diversity estimators for the BCC for all sampling dates.

3.4. Results

3.4.1. MCs and chl-a concentrations

The chl-*a* concentrations from water samples ranged from the 1.93 ± 0.53 µg L^{-1} detected in December to the 349.82 ± 54.78 µg L^{-1} detected in autumn (19 November), coincident with a *Microcystis* spp. bloom episode (Figure 3.1). Sestonic MC concentrations (the sum of the most frequent variants: MC-LR, MC-RR and MC-YR) varied considerably from undetectable levels (detection limit: 0.2 µg L^{-1}) during the first semester of 2014 to 9,857.90 ± 1051.36 µg L^{-1} on 19 November. The dissolved fraction of the MCs was detected in all water samples, with concentrations below 1 µg L^{-1} in the first semester of 2014 and reaching 35.25 ± 2.14 µg L^{-1} at the cyanobacterial bloom peak (19 November). Both sestonic and dissolved MC concentrations decreased approximately 2,230-fold and 90-fold, respectively, after the bloom collapsed (2 December), suggesting an important *in situ* MC biodegradation activity by the natural bacterial community. The toxicity of the cyanobacterial bloom increased over time, as reported by the increasing MC:Chl-*a* ratios (from 1.52 the 9 September to 28.51 the 19 November, Table S 3.1 in the supplementary materials).

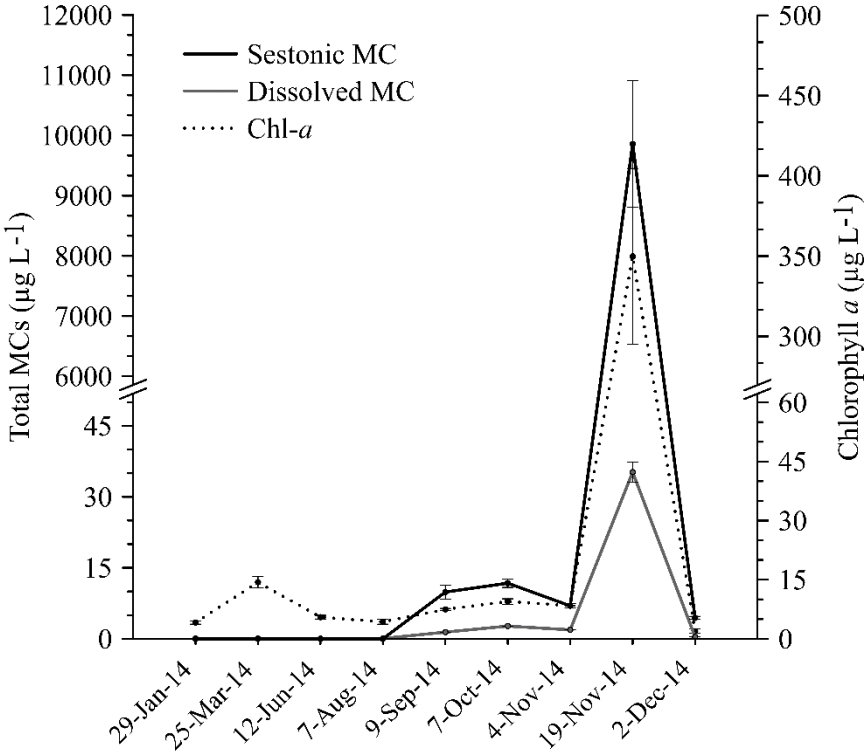


Figure 3.1. Temporal changes of chl-*a* (dotted line) and both sestonic (black line) and dissolved MC (grey line) concentrations in the San Juan reservoir. The error bars for the sestonic MC and chl-*a* concentrations represents the standard errors of two replicates and from the dissolved MC concentrations represent the standard errors of two technical replicates.

3.4.2. Diversity and seasonal dynamics of potentially MC-producing cyanobacteria and MC-degrading bacteria with mlr genes

To identify the cyanobacterial genera/species potentially producing the MCs, a high-throughput sequencing analysis of the *mcy*E gene was performed. A total of 602,703 sequences were recovered from the gene amplicons after quality filtering and the removal of artificial reads. The sequencing effort varied between samples, ranging from 13,810 (25 March) to 109,102 (4 November) reads (Table 3.1).

Table 3.1. Number of filtered sequences (S), number of OTUs, Shannon-Wiener index (H') and Chao1 index from the high-throughput sequencing data of the bacterial 16S rRNA, *mcy*E and *mlr*A genes from the water reservoir. The mark "n.d." means "no data" (absence of doubletons).

	16SrRNA gene				*mcy*E gene				*mlr*A gene			
Date	**S**	**OTUs**	**H'**	**Chao1**	**S**	**OTUs**	**H'**	**Chao1**	**S**	**OTUs**	**H'**	**Chao1**
29-Jan-14	149831	1006	5.07	1091	35783	17	0.76	18.00	3253	14	0.88	20.25
25-Mar-14	134377	715	4.41	805	13810	11	0.18	11.13	1393	12	1.09	14.25
12-Jun-14	178872	626	4.55	698	69145	16	0.65	16.00	182	4	0.68	n.d.
7-Aug-14	144443	803	4.37	920	60244	17	0.46	n.d.	2818	13	1.22	n.d.
9-Sep-14	166150	761	4.53	886	96185	17	0.62	19.00	936	12	1.02	13.50
7-Oct-14	160511	926	4.66	1042	100781	17	0.68	17.00	8127	13	0.87	15.00
4-Nov-14	199534	1075	4.97	1144	109102	19	0.74	20.00	5441	14	0.99	14.25
19-Nov-14	166009	1065	4.67	1123	85942	19	0.58	19.67	2766	12	0.91	20.00
2-Dec-14	68535	1023	5.14	1135	31712	16	0.51	16.17	1861	10	0.88	12.00

A total of 19 different OTUs of the *mcy*E gene were identified in the water samples over the period of study; 7 of them belonged to the species *Microcystis aeruginosa*, 1 to *Microcystis viridis* and 11 to the genus *Microcystis* (Figure 3.2). The analysis showed the dominance of two OTUs in all water samples, which were assigned to *Microcystis* sp., both accounting for more than 98.9% of the relative abundance on all sampling dates, with variations from 16 to 96% (OTU 1) and from 3 to 83% (OTU 2) over the period of study. The rest of the cyanobacterial OTUs were present in significantly less relative abundance (maximum of 0.4%). Some of the OTUs reached their maximum relative abundance during the cyanobacterial bloom episode (e.g., OTUs 11, 12, 14, 15, 18 and 19), while others thrived outside the period of the massive growth (the remainder of the OTUs). The Shannon-Weiner (H') and the Chao1 indices showed variations in the diversity among sampling dates, 25 March being the less diverse (H'=0.18 and Chao1=11.13), and both 29 January (H'=0.76 and Chao1=18.00) and 4 November (H'=0.74 and Chao1=20.00) being the most diverse.

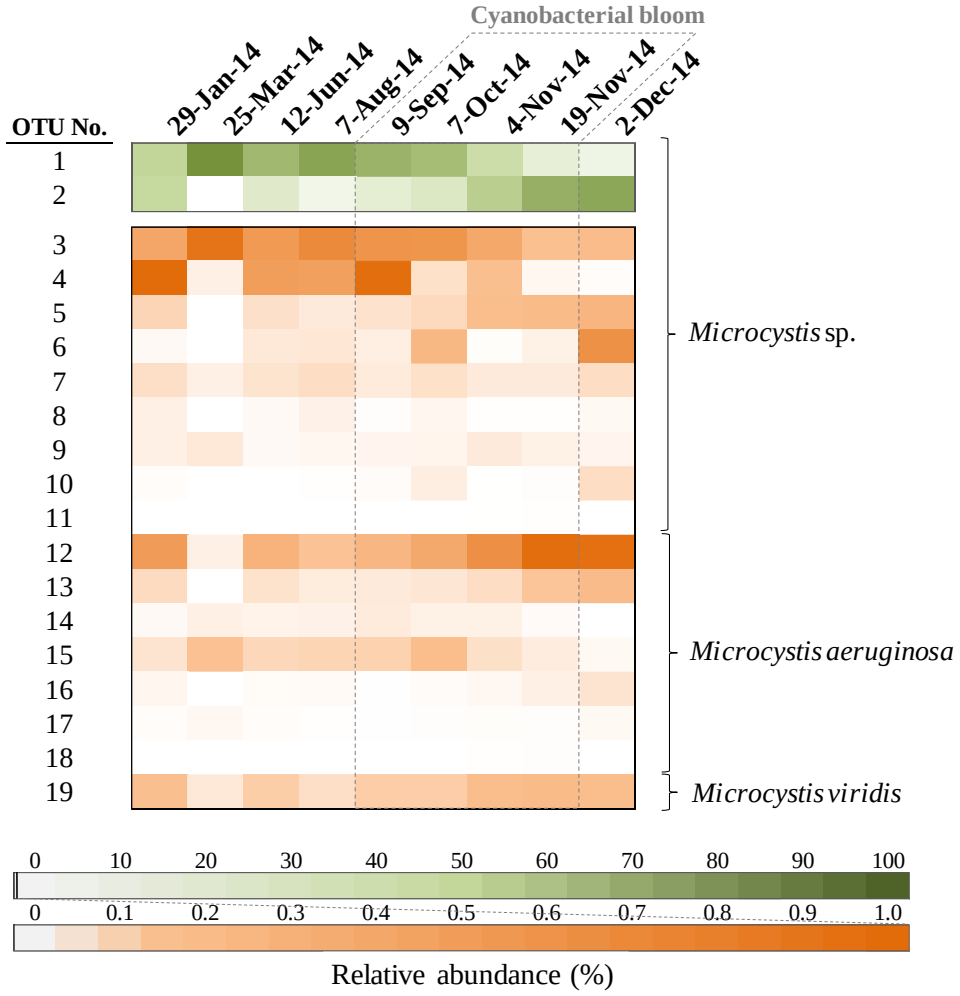


Figure 3.2. Temporal shifts of potentially toxic cyanobacterial OTUs during the period of study. The colours indicate the relative abundance of each OTU over the total MC-producing cyanobacterial community at each sampling date. Two colour scales are displayed for an enhanced view of the higher and lower relative abundances. The taxonomic assignations were performed with gene similarities equal or above 97%.

To identify the potentially MC-degrading bacteria encoding the already described enzymatic pathway for the degradation of MCs, the *mlr*A gene was analysed. This analysis showed a total of 26,777 sequences after quality filtering. The number of reads varied among samples, ranging from 182 (12 June) to 8,127 (7 October) (Table 3.1). A total of 19 OTUs from the genera *Stenotrophomonas* sp., *Novosphingobium* sp., *Sphingomonas* sp., *Sphingopyxis* sp. and uncultured bacteria were identified over the period of study (Figure 3.3). The results showed that most of the OTUs (2-11) belonged to the family *Sphingomonadaceae*, followed by OTUs 12-19, which were assigned to uncultured bacterium, and OTU 1, which belonged to the family *Xanthomonadaceae*. The three most abundant OTUs (1-3) accounted for more than 89% of the relative abundance in all water samples, followed by OTUs 4 and 5, which

accounted for between 0.32 and 8.54%. Except for the four most abundant OTUs (1-4), which were present in all samples, the rest (5-19) showed a patchy temporal distribution over the period of study; some of them showed their maximum relative abundance during the cyanobacterial bloom episode (e.g., OTUs 1, 9-13, 15, 16) while others peaked outside the period of the massive growth (rest of OTUs). Regardless of the temporal evolution of the cyanobacterial bloom and the variations in the MC concentrations over the period of study, on most of the sampling dates, a similar number of OTUs (between 10 and 14) coexisted. 12 June was an exception, in that only the 4 most abundant OTUs were present (1-4). The Shannon-Weiner and the Chao1 indices confirm the observed slight variations in the diversity, considering samples before and after 12 June (H'= 0.98 ± 0.12 and Chao1=15.61 ± 3.22).

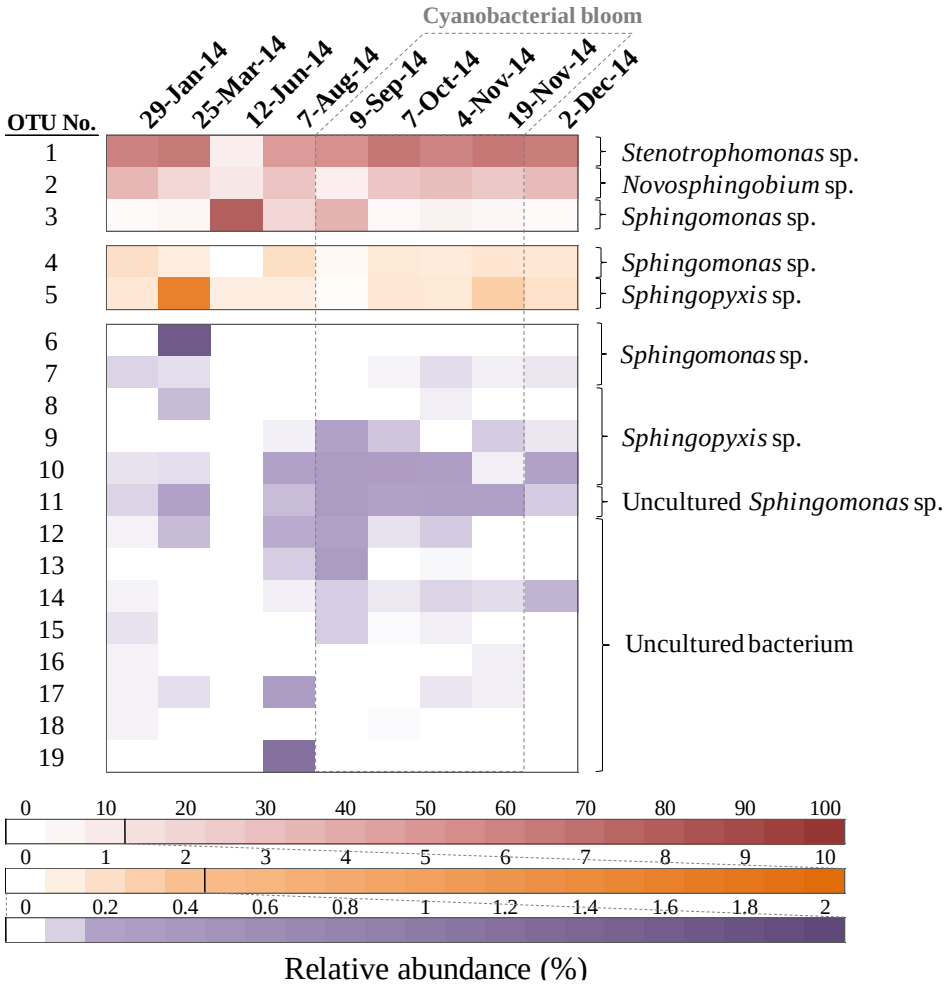


Figure 3.3. Temporal shifts of potentially MC-degrading bacterial OTUs with *mlr*A genes during the period of study. The colours indicate the relative abundance of each OTU over the total MC-degrading bacterial community at each sampling date. The three scales are displayed for an enhanced view of the higher and lower relative abundances. The taxonomic assignations were performed with gene similarities equal or above 95%.

3.4.3. Shifts in the BCC over the period of study

A total of 1,368,262 sequences were recovered from the 16S rRNA gene amplicons from all water samples after quality filtering. The sequencing effort was highly similar in all samples, from 134,377 to 199,534 reads, except for the water sample collected the 2 December (68,535 sequences) (Table 3.1). A total of 1,332 different bacterial OTUs, classified in 27 phyla, were observed among all samples (Figure 3.4), and only 15 of them showed relative abundances higher than 0.25% in at least one sample. *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* dominated the sampling profiles with slight variations and accounted for more than 60% of the relative abundance in all water samples. Phyla *Verrucomicrobia* and *Cyanobacteria* were the next most abundant, with temporal shifts in their relative abundances ranging from 3.54 to 19.81% and from 0.31 to 17.87%, respectively. The 22 remaining phyla represented between 6 and 12% of the total BCC in all water samples.

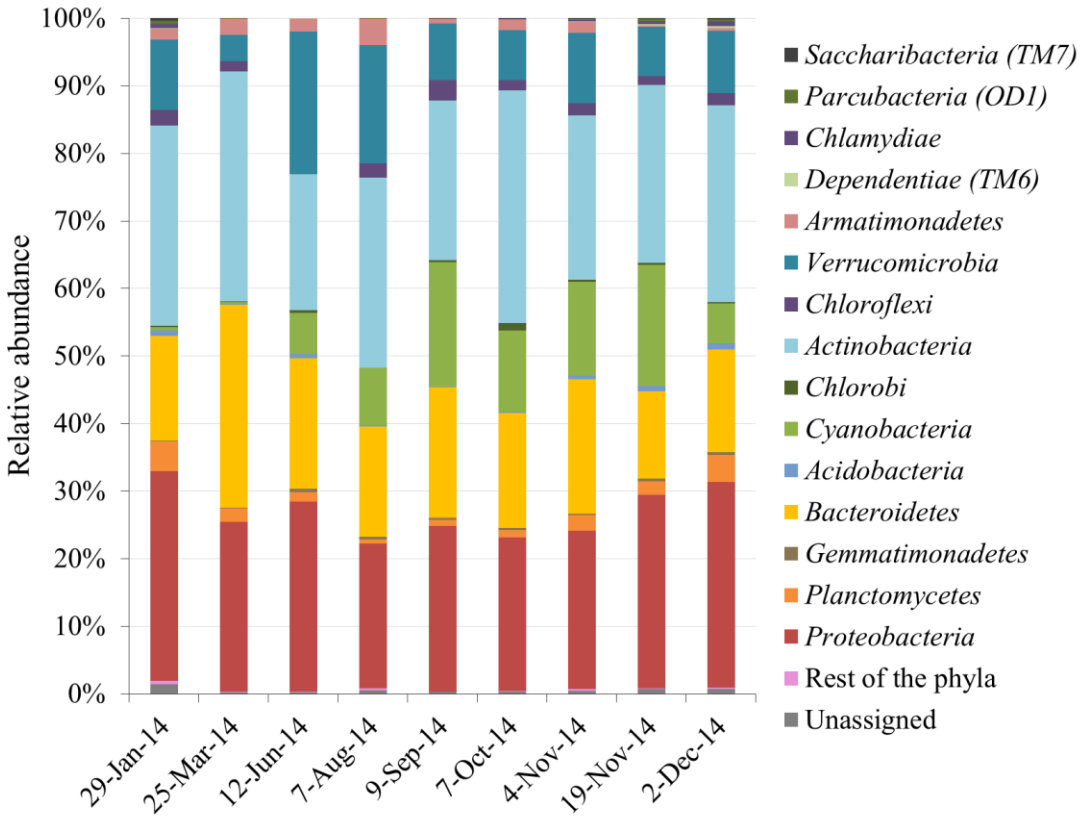
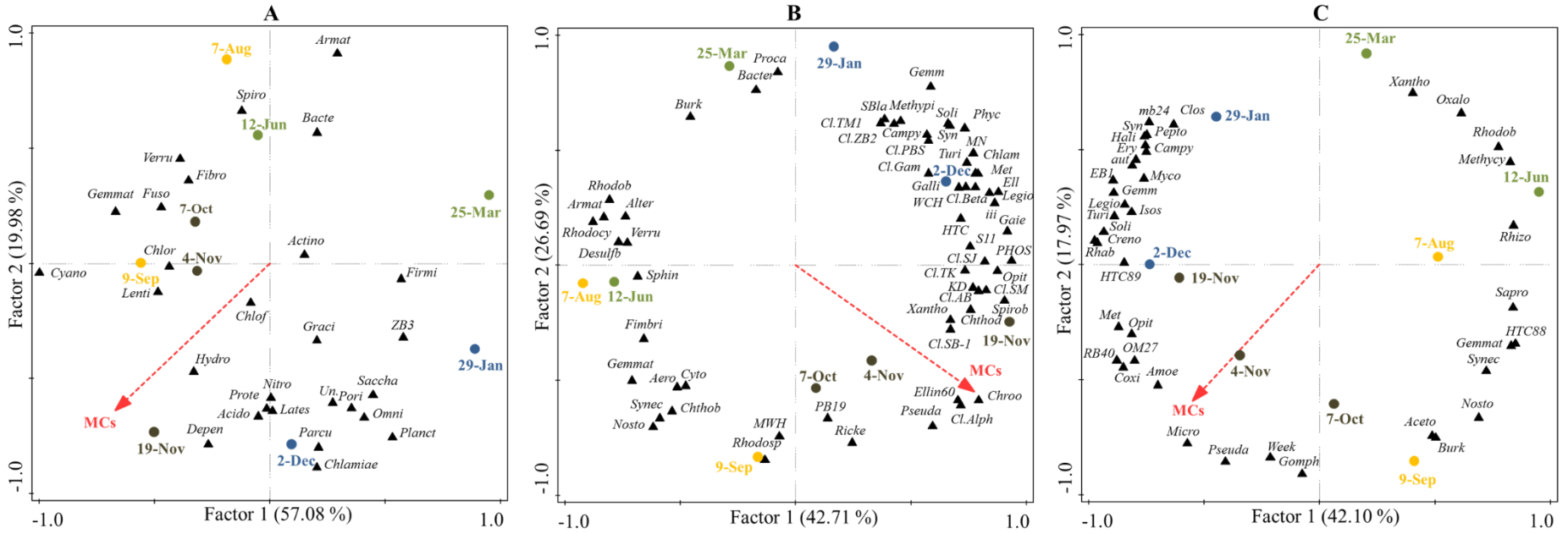


Figure 3.4. Temporal shifts of the total bacterial community composition at the phylum level in terms of relative abundance. The phyla with relative abundances higher than 0.25% on at least in one sampling date are represented in the graph. The remaining phyla and the unassigned sequences are included the "rest of the phyla" and the "unassigned" groups, respectively.

3.4.4. Relationships between the BCC and the MC concentrations

To identify possible relationships between the BCC and the toxic cyanobacterial bloom, a Pearson correlation analysis (Table S 3.2 in supplementary materials) and a set of PCA plots at the phylum, order and family levels (Figure 3.5) were performed, considering the temporal shifts of the MC concentrations and the relative abundances of each bacterial taxon.

All PCA plots showed a seasonal cyclic trend of the BCC, as shown with the clearly separated sampling dates at all the studied taxonomic levels. For example, winter and summer bacterial assemblages were well differentiated from each other since their sampling dates were opposed in all PCA plots. Despite of the cyclic trend of the BCC as a whole, some specific phyla such as *Actinobacteria*, *Gracilibacteria* and *Chloroflexi* barely showed seasonal variations, based on their central location in the PCA plot (Figure 3.5A). Descending to lower taxonomic levels, a higher number of bacterial orders and families were found to be related to the maximum and subsequent decay of the cyanobacterial bloom (late autumn) compared to those present before (spring) or at the early stage (summer) of the massive cyanobacterial growth (Figure 3.5B, C). The Shannon-Wiener and Chao1 diversity indices confirmed this increasing diversity during the cyanobacterial bloom episode, from its start on 9 September (H'= 4.53, Chao1= 886) to its decay and the release of toxins on 2 December (H'=5.14, Chao1=1135). The toxicity of the bloom appeared to be mainly caused by the cyanobacterial family *Microcystaceae*, as demonstrated a high and significant correlation with the MC concentrations (r = 0.9581, p < 0.05) and was consistent with the identification of the genus *Microcystis* in the analysis of the *mcy*E gene.



AB: ABY1; Aceto: Acetobacteraceae; Acido: Acidobacteria; Actino: Actinobacteria; Aero: Aeromonadales; Alph: Alphaproteobacteria; Alter: Alteromonadales; Amoe: Amoebophilaceae; Armat: Armatimonadetes/Armatimonadales; Aut: auto67-4W; Bacte: Bacteroidetes; Bacter: Bacteroidales; Beta: Betaproteobacteria; Burk: Burkholderiales/Burkholderiaceae; Campy: Campylobacterales; Chlam: Chlamydiales; Chlamiae: Chlamydiae; Chlof: Chloroflexi; Chlor: Chlorobi; Chroo: Chroococcales; Chthob: Chthoniobacterales; Clos: Clostridiaceae; Coxi: Coxiellaceae; Creno: Crenotrichaceae; Cyano: Cyanobacteria; Cyto: Cytophagales; Depen: Dependentiae; Desulfb: Desulfobacterales; EB1: EB1017; Ell: Ellin329; Ellin60: Ellin6067; Ery: Erysipelotrichaceae; Fibro: Fibrobacteres; Fimbri: Fimbriimonadales; Firmi: Firmicutes; Fuso: Fusobacteria; Gaie: Gaiellales; Galli: Gallionellales; Gemm: Gemmatales/Gemmataceae; Gemmat: Gemmatimonadetes/Gemmatimonadales/Gemmatimonadaceae; Gomph: Gomphosphaeriaceae; Graci: Gracilibacteria; Hali: Haliangiaceae; HTC: HTCC2188; HTC88: HTCC2188; HTC89: HTCC2089; Hydro: Hydrogenedentes; iii: iii1-15; Isos: Isosphaeraceae; KD: KD8-87; Lates: Latescibacteria; Lenti: Lentisphaerae; Legio: Legionellales/Legionellaceae; mb24: mb2424; Met: Methylococcales/Methylococcaceae; Methycy: Methylocystaceae; Methypi: Methylophilales; Micro: Microcystaceae; MN:MND1; MWH: MWH-UniP1; Myco: Mycobacteriaceae; Nitro: Nitrospirae; Nosto: Nostocales/Nostocaceae; Omni: Omnitrophica; Opit: Opitutales/Opitutaceae; Oxalo: Oxalobacteraceae; Parcu: Parcubacteria; PBS: PBS-25; Pepto: Peptostreptococcaceae; PHOS: PHOS-HD29; Phyc: Phycisphaerales; Planct: Planctomycetes; Pori: Poribacteria; Proca: Procabacteriales; Prote: Proteobacteria; Pseuda: Pseudanabaenales/Pseudanabaenaceae; Rhab: Rhabdochlamydiaceae; Rhizo: Rhizobiaceae; Rhodob: Rhodobacterales/Rhodobacteraceae; Rhodocy: Rhodocyclales; Rhodosp: Rhodospirillales; Ricke: Rickettsiales; S11: S1198; Saccha: Saccharibacteria; Sapro: Saprospiraceae; SBla: SBla14; SJ: SJA-4; SM: SM2F11; Soli: Solibacterales/Solibacteraceae; Sphin: Sphingomonadales; Spiro: Spirochaetes; Spirob: Spirobacillales; Syn: Syntrophobacterales/Syntrophaceae; Synec: Synechococcales/Synechococcaceae; TK: TK17; TM1: TM7-1; Turi: Turicibacterales;Turicibacteraceae; Un.: Undetermined; Verru: Verrucomicrobia/Verrucomicrobiales; WCH: WCHB1-50; Week: Weeksellaceae; Xantho: Xanthomonadales/Xanthomonadaceae

Figure 3.5. PCA ordination triplots of data obtained from the water reservoir over a year. The data included the bacterial community composition (16S rRNA gene) at the phylum (A) order (B) and family (C) levels (triangles), the total MC concentrations (red vectors) and the sampling dates (coloured points indicating winter (blue), spring (green), summer (yellow) and autumn (brown)). Plot A shows all the bacterial phyla and, for an enhanced view, plots B and C only show the 60 orders and the 40 families (in absence, the upper taxonomic level) with the greatest relationships to the PCA factors.

To determine the bacterial families (if absent, their orders (Or.) or classes (Cl.)) closely related with the toxic *Microcystis* bloom episode, the Pearson correlation analysis allowed the classification of bacteria based on their degree of relationship with the temporal shifts in the MC concentrations. Group I represents the core bacteria with a significant and positive correlation with the toxin concentrations ($r > 0.5$; $p < 0.05$) (Figure 3.6A), and group II represents the bacteria trended with the temporal variations in the MC concentrations ($r > 0.5$; $0.05 < p < 0.15$) (Figure 3.6B). The bacterial group I is represented by 6 families (apart from *Microcystaceae*), 4 orders and 3 classes from 7 different phyla, among which, the relative abundance of 9 increased more than 1.5 times during the cyanobacterial bloom peak (asterisks in Figure 3.6A). These bacteria that are highly responsive to the release of MCs are comprised in the phyla *Proteobacteria* (*Coxiellaceae*, 0319-6G20, Or. *Ellin6067*, Or. *PHOS-HD29*, Or. *Spirobacillales*), *Bacteroidetes* (*Amoebophilaceae* and Cl. SM1A07), *Acidobacteria* (RB40) and *Gemmatimonadetes* (Or. KD8-87), which are potential candidates for the degradation of MCs during the bloom collapse and depict an important interplay with the toxic cyanobacterial bloom. The bacterial Group II was comprised of 5 families, 4 orders and 6 classes from 10 different phyla, among which, the relative abundance of only 7 bacterial taxa from the phyla *Proteobacteria* (*Sinobacteriaceae*, *Legionellaceae*, Or. *Legionellales*), *Dependentiae* (Cl. SJA-4 and Or. S1198), *Actinobacteria* (*Gaiellaceae*) and *Chlamydiae* (*Parachlamydiaceae*) increased more than 1.5 times during the cyanobacterial bloom peak (asterisks in Figure 3.6B). The members of this bacterial group are trended with the toxic cyanobacterial dynamics and, thus, are also potential candidates to have interactions with cyanobacteria and/or their MCs.

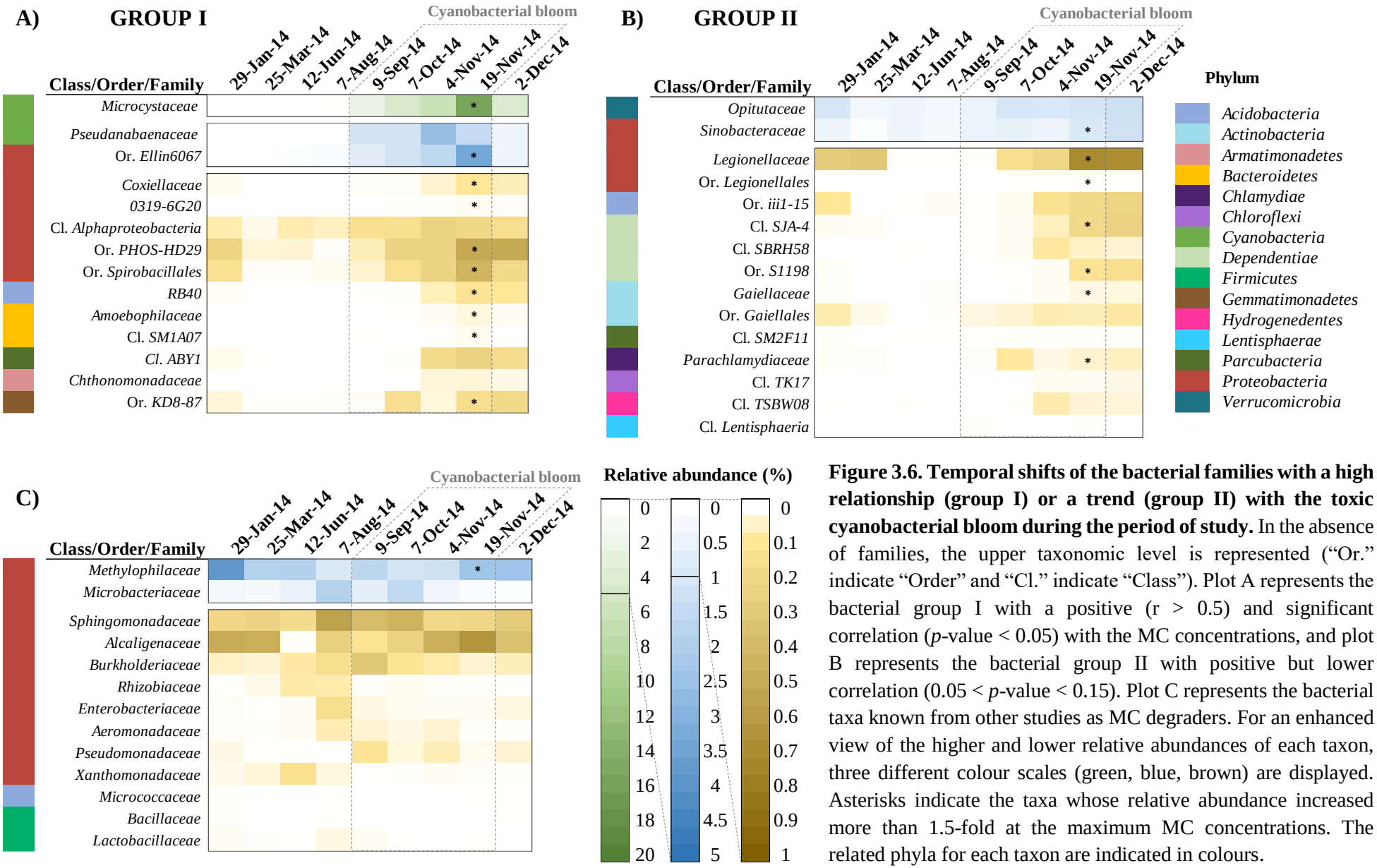


Figure 3.6. Temporal shifts of the bacterial families with a high relationship (group I) or a trend (group II) with the toxic cyanobacterial bloom during the period of study. In the absence of families, the upper taxonomic level is represented ("Or." indicate "Order" and "Cl." indicate "Class"). Plot A represents the bacterial group I with a positive ($r > 0.5$) and significant correlation (p-value < 0.05) with the MC concentrations, and plot B represents the bacterial group II with positive but lower correlation ($0.05 < p$-value < 0.15). Plot C represents the bacterial taxa known from other studies as MC degraders. For an enhanced view of the higher and lower relative abundances of each taxon, three different colour scales (green, blue, brown) are displayed. Asterisks indicate the taxa whose relative abundance increased more than 1.5-fold at the maximum MC concentrations. The related phyla for each taxon are indicated in colours.

In addition to the classification of bacteria in groups I and II, the families previously described in other studies as MC degraders (Table S 3.3 in supplementary materials) were also included in this analysis, so that their seasonal dynamics could be followed (Figure 3.6C). These MC-degrading families were present in our water samples, with the exceptions of *Nocardiaceae*, *Brevibacteriaceae* and *Bifidobacteriaceae*. Within this group, the relative abundance of only *Methylophilaceae* increased more than 1.5 times at the cyanobacterial bloom peak. However, its higher occurrence in winter led to a low correlation with the MC concentrations ($r = 0.0089$, $p > 0.15$). In the same line, the rest of the families also showed low positive or negative correlation with the MCs concentrations ($r < 0.40$).

3.5. Discussion

In the present study, extremely high sestonic and dissolved MC concentrations were recorded in the water reservoir due to a toxic cyanobacterial bloom dominated by *Microcystis* spp. that formed thick scum on 19 November 2014. Total MC concentrations (the sum of sestonic and dissolved MCs) from the start of the bloom until its decline were above the WHO guideline value of 1 µg L^{-1} for drinking water (WHO, 1998). The large decrease of the sestonic and dissolved MC concentrations after the bloom collapse (2,230-fold and 90-fold, respectively) may suggest an important *in situ* MC degradation activity by the aquatic natural bacterial community. The lower MCs degradation rates by photodegradation (Welker and Steinberg, 2000; Wörmer et al., 2010) as compared to biological processes (Dziga et al., 2013; Ho et al., 2007; Lezcano et al., 2016), the lower light intensity in autumn and the few variations in the water volume of the San Juan reservoir over the period of study (Table S 3.4 in supplementary materials) support biodegradation as an important process that contributes for the decrease in MC concentrations.

Considering the coexistence of MC-producing and MC-degrading activities in the water reservoir, a high-throughput sequencing analysis was performed to explore the diversity and the seasonal dynamics of both bacterial communities over a year. A season-specific BCC was found at all the studied phylogenetic levels (phylum, order and family), according to the PCA analysis. This seasonality is consistent with other studies (Allgaier and Grossart, 2006; Dai et al., 2015) and supports the existence of variable temporal ecological niches (Jaspers et al., 2001), indicating certain environmental factors (e.g., phytoplankton succession, grazing, virus lysis, temperature) may trigger the BCC shifts.

The occurrence of the toxic cyanobacterial bloom in the late summer and autumn seasons likely offered new niche opportunities and may result in the observed BCC changes. The higher bacterial diversity at the order and family levels during the development and decay of the toxic cyanobacterial bloom, compared to samples collected before or during the early state of the massive cyanobacterial growth, supports this idea. Moreover, previous studies have shown that the overgrowth and the subsequent decay of phytoplankton cause changes in the dissolved organic matter (DOM) concentrations (Søndergaard et al., 2000) and, thus, induce shifts in the BCC (Dinasquet et al., 2013; H. Li et al., 2012; K. Shao et al., 2014), favouring growth of those bacterial taxa able to degrade not only the amount, but also the quality of the dissolved organic matter (low- or high-molecular-weight carbon) (Logue et al., 2016). Therefore, the breakdown of the toxic cyanobacterial bloom described here may lead to a release of labile and commonly biodegradable molecules from the cell as well as complex compounds, such as toxins with high molecular weights (>600 Da), that cannot be readily taken up by microorganisms across the membrane (Weiss et al., 1991) and require specialized bacterial communities that used specific enzymatic cleavages (Logue et al., 2016). Thus, the maximum diversity observed during and after the toxic cyanobacterial bloom episode is most likely the answer of a specialized bacterial community to the increase of complex organic matter in the ecosystem. Among these bacteria, those with a role on MC degradation may represent an important fraction, considering the fast decrease in MC concentrations that was detected after the bloom peak. A previous study in a microcosm experiment also reported shifts in the BCC in response to the addition of MCs (Mou et al., 2013), supporting the idea that, despite the multiple factors governing the *in situ* environmental samples, the MCs released during the cyanobacterial bloom constitute an important factor driving BCC changes.

The study of the potentially MC-producing cyanobacteria disclosed the coexistence of different *mcy*E genotypes. Their different seasonal dynamics and the increase of bloom toxicity over time reveal the existence of *Microcystis* subpopulations that exhibit *mcy* gene heterogeneity (genotypes) and produce different MC variants and content (chemotypes). This genotype and chemotype diversity has been reported previously for other *mcy* genes (Kurmayer et al., 2002; Milkalsen et al., 2003) and in different cyanobacterial genera (Rohrlack et al., 2008; Welker et al., 2007), suggesting dissimilar ecological functions and different responses to the environmental conditions (Agha et al., 2014).

The identical analysis performed on the potentially MC-degrading bacterial community possessing the *mlr* gene cluster also revealed an *mlr*A gene variability. The coexistence, the patchy temporal distribution and the variations in the relative abundance of the observed *mlr*A genotypes in the period of study, mostly assigned to the genera *Sphingomonas* and *Sphingopyxis*, may reveal the formation of subpopulations that respond differently to the environmental conditions. This ubiquitous temporal distribution point toward the existence of other environmental factors, apart from the concentrations of MCs, that determine their presence and provide evidence for other relevant roles alternatives to the degradation of MCs that they may play in the ecosystem. The recognized ability of the families *Sphingomonadaceae* and *Xanthomonadaceae* to degrade other complex organic compounds, including xenobiotics (Bosso and Cristinzio, 2014; Leng et al., 2016), supports this idea and suggests that only a fraction of these families were the responsible for the MC degradation after the bloom decay.

In addition to the two families *Sphingomonadaceae* and *Xanthomonadaceae*, that represented the fraction of the MC-degrading bacteria using specific Mlr enzymes, higher diversity of other bacterial taxa identified from the 16S rRNA gene analysis responded markedly to the toxic cyanobacterial bloom (Figure 3.6A, B). The different magnitudes of relationships between the bacterial groups (I and II) and the MC concentrations actually reveal the degree of influence that toxic cyanobacteria and/or their exudates have on the role that each bacterial group plays in the ecosystem. Thus, bacterial group I seems to be strongly influenced by the cyanobacteria and their toxins, while interactions with bacterial group II are more uncertain and other factors are likely to contribute to their temporal distribution and relative abundances.

So far, bacteria have been reported to have different types of interaction with cyanobacteria, such as parasitism (Kim et al., 2008; Rashidan and Bird, 2001), commensalism (Briand et al., 2016; H. Li et al., 2011) and mutualism (Ramanan et al., 2016). Here, both direct and indirect relationships were observed within the bacterial community associated with the toxic cyanobacterial bloom. The candidate phylum *Dependentiae* (Group II), the phylum *Chlamydiae* (from group II) and the candidate phylum *Parcubacteria* (Groups I and II) have been described as ectosymbionts or parasites of other organisms (Lagkouvardos et al., 2014; Nelson and Stegen, 2015; Yeoh et al., 2015), such as the free-living amoeba that coexist and feed on toxic cyanobacterial blooms (Urrutia-Cordero et al., 2013). This also occurred with the order *Legionellales*, a

potential pathogen represented in this study by the families *Coxiellaceae* (Group I) and *Legionellaceae* (Group II). The relationship between *Legionellales* and the toxic cyanobacterial blooms has been previously reported (H. Li et al., 2011) and highlights massive cyanobacterial growths as reservoirs for opportunistic pathogens and waterborne diseases (Mediannikov et al., 2010; Tison et al., 1980). On the other hand, cyanobacteria also establish a direct relationship that provides protection to other bacteria. For example, the cyanobacterial family *Pseudanabaenaceae*, highly represented in this study (Group I), is one example of bacteria that is usually associated with the *Microcystis* mucilage (Vasconcelos and Pereira, 2001), which benefits from the protection it offers.

Apart from the shelter protection, the release of the high amount of cyanobacterial exudates to the environment during the cyanobacterial growth and lysis may offer nourishment and encourage the association of bacterial scavengers in the phycosphere, which plays a key role in the turnover of the organic matter. The physical and chemical stability of the MCs (Tsuji et al., 1995, 1994) due to their cyclic structure (De la Cruz et al., 2011) requires specialized enzymes and thus suggests the existence of specific enzymatic pathways for the degradation of MCs. A high enzymatic activity of the *mlr*$^{+}$ bacterial genotype could explain the degradation of the MCs in the water reservoir. However, the greater relationship of the *mlr*-lacking bacteria (Groups I and II) to the toxic cyanobacterial bloom compared with the relationship of the bacterial families with *mlr* genes (*Sphingomonadaceae* and *Xanthomonadaceae*) with the toxic bloom, strongly suggests the joint contribution of both *mlr*$^{+}$ and *mlr*$^{-}$ genotypes for the degradation of MCs. In fact, most of the bacteria from group I, especially from the phyla *Proteobacteria* and *Bacteroidetes*, reported abilities to degrade complex organic compounds (Muangchinda et al., 2014). The uptake of nitrogen-rich peptides and amino acids have also been described as the top functions of their ABC membrane transporters (Penn et al., 2014), indicating that the peptides are important sources of organic carbon and supporting the possible intake and catabolism of MCs in both phyla. Furthermore, the order *Myxococcales* (the family 0319-6G20 belongs to this order) is able to produce several degradative enzymes and to decompose a number of biomacromolecules (Reichenbach, 2015). The order *Ellin6067* has been reported to be ammonia-oxidizing bacteria (Xia et al., 2005), and the classes *Alphaproteobacteria* and *Betaproteobacteria* (the order *Spirobacillales* belongs to this class) have been described as degraders of complex organic compounds (Debroas et al., 2009). Although little is known about the class TA18

(represented by the order PHOS-DH29 in this study) from the phylum *Proteobacteria*, this class was found in oxygenic sediments enriched with genes associated with xenobiotic metabolism (Robinson et al., 2016). Moreover, the capability of the order *Cytophagales* (represented by the family *Amoebophilaceae* in this study) to degrade large complex organic molecules (Van Hannen et al., 1999), the capacity of the family *Chthnomonadaceae* (phylum *Armatimonadetes*) to transport and use a wide range of carbohydrates (Lee et al., 2014) and the metabolically flexible members of the phylum *Acidobacteria* (represented by the family RB40 in this study) suggest the contribution of these taxa to the successfully removal of the MCs. The lack of *mlr* genes and the ability of these bacteria to degrade complex and recalcitrant compounds support the possibility of alternative-*mlr* pathway(s) that are likely associated with the xenobiotic metabolism for the degradation of MCs. Consistent with these results were those found in the study conducted by Mou et al. (2013), in which an overrepresentation of genes from xenobiotic metabolism and the higher abundance of bacteria from orders other than *Sphingomonadales* were observed in a microcosm experiment amended with MCs. Thus, a diverse array of bacterial taxa from the freshwater bacterial community may present alternative-*mlr* MC-degradation pathway(s) for successfully removing the MCs released after a cyanobacterial bloom collapse.

3.6. Conclusions

Beyond the fraction of the MC-degrading bacterial community with *mlr* genes, represented here by the families *Sphingomonadaceae* and *Xanthomonadaceae*, other bacteria with xenobiotic degradation ability were closely related with the toxic cyanobacterial bloom. The decrease in the MC concentrations during and after bloom collapse suggest, not only an important role played by the *mlr*$^{+}$ bacteria, but also by other bacteria operating the alternative-*mlr* degradation pathway(s), as judge from the correlation of bacteria lacking *mlr* genes with the MC concentrations. The degree to which both fractions contribute to the overall removal of MCs in the environment is still unknown. Therefore, the quantification of each bacterial fraction, the influence of the environmental factors on their seasonal dynamics and MC degradation activity, as well as the characterization of the alternative-*mlr* degradation pathway(s) deserve further in-depth study.

Accession numbers: All the genetic data generated in this study have been submitted to the GenBank. Accession numbers of the BioSamples are: SAMN06826931,

SAMN06826932, SAMN06826933, SAMN06826934, SAMN06826935, SAMN06826936, SAMN06826937, SAMN06826938, SAMN06826939.

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3.8. Supplementary materials

Table S 3.1. Total MC (sum of sestonic and dissolved fractions) and chl-*a* concentrations from the water reservoir, and the ratio between both variables during the period of study.

Date	Total MCs (µg L^{-1})	Chl-*a* (µg L^{-1})	Total MC : Chl-*a*
29-Jan-2014	0.079 ± 0.006	4.139 ± 0.416	0.019 ± 0.002
25-Mar-2014	0.022 ± 0.001	14.345 ± 1.424	0.002 ± 0.000
12-Jun-2014	0.036 ± 0.000	5.497 ± 0.460	0.007 ± 0.001
7-Aug-2014	0.065 ± 0.004	4.314 ± 0.591	0.015 ± 0.002
9-Sep-2014	11.283 ± 1.458	7.468 ± 0.241	1.519 ± 0.243
7-Oct-2014	14.439 ± 0.920	9.461 ± 0.701	1.528 ± 0.018
4-Nov-2014	8.852 ± 0.490	8.410 ± 0.000	1.053 ± 0.056
19-Nov-2014	9893.138 ± 1053.495	349.816 ± 54.779	28.509 ± 1.459
2-Dec-2014	4.805 ± 0.336	1.927 ± 0.526	2.744 ± 0.920

Table S 3.2. Pearson correlations between the bacterial phyla, orders and families, and the total MC concentrations. Numbers in bold indicate positive or negative significant correlations ("*" indicate *p*-value <0.05 and "**" indicate 0.05 < *p*-value <0.15).

Phylum /Order/Family	Short name	Total MCs
Acidobacteria	*Acido*	0.3595
Solibacterales	*Soli*	0.1399
Solibacteraceae	*Soli*	0.4029
RB41	*RB4*	-0.4763
Ellin6075	*Ell6075*	-0.4763
Holophagales	*Holo*	-0.2071
Holophagaceae	*Holo*	-0.2071
Cl. RB25	*Cl.RB*	-0.2610
CCU21	*CCU*	-0.1768
iii1-15	*iii*	**0.5369****
mb2424	*mb24*	0.1791
RB40	*RB40*	**0.7644***
Actinobacteria	*Actino*	0.1105
Solirubrobacterales	*Solir*	0.1949
Conexibacteraceae	*Cone*	0.0391
Gaiellales	*Gaie*	**0.5713****
Gaiellaceae	*Gaie*	**0.5603****
Acidimicrobiales	*Acidi*	0.0596
Microthrixaceae	*Microth*	0.1141
EB1017	*EB1*	0.1650
C111	*C111*	0.0264
Actinomycetales	*Actino*	0.1526
Mycobacteriaceae	*Myco*	0.1520
Micrococcaceae	*Microc*	-0.2901
Microbacteriaceae	*Microb*	-0.2278
ACK-M1	*ACK*	0.1932
Armatimonadetes	*Armat*	**-0.7583***
Chthonomonadales	*Chthod*	**0.7086***
Chthonomonadaceae	*Chthod*	**0.7086***
Armatimonadales	*Armat*	**-0.7721***
Armatimonadaceae	*Armat*	**-0.7721***
Fimbriimonadales	*Fimbri*	-0.3097
Fimbriimonadaceae	*Fimbri*	-0.3097
Bacteroidetes	*Bacte*	-0.4258
Cl. VC2-1-Bac22	*Cl. VC*	0.2211
Cl. SM1A07	*Cl. SM1*	**0.8259***
Sphingobacteriales	*Sphinb*	-0.0263
Sphingobacteriaceae	*Sphinb*	-0.5012
Flavobacteriales	*Flavo*	**-0.5727****
Flavobacteriaceae	*Flavo*	-0.1950
Cytophagaceae	*Cyto*	0.1532
Cryomorphaceae	*Cryo*	**-0.7447***
Weeksellaceae	*Week*	0.1854
Cytophagales	*Cyto*	-0.0415
Flammeovirgaceae	*Flam*	**-0.5591****
Cyclobacteriaceae	*Cyclo*	-0.4528
Amoebophilaceae	*Amoe*	**0.8553***
Saprospirales	*Sapro*	0.3707
Saprospiraceae	*Sapro*	-0.4556
Chitinophagaceae	*Chit*	0.4775
Bacteroidales	*Bacter*	**-0.5412****
SB-1	*SB-1*	-0.4231
Rikenellaceae	*Rike*	-0.3850
Porphyromonadaceae	*Porphy*	-0.2507

Bacteroidaceae	*Bacter*	-0.0131
Chlamydiae	*Chlamiae*	0.3771
Chlamydiales	*Chlam*	0.3771
Parachlamydiaceae	*Para*	**0.5899****
Rhabdochlamydiaceae	*Rhab*	0.2897
Chlorobi	*Chlor*	0.3643
Cl. OPB56	*Cl. OP*	0.3643
Chloroflexi	*Chlof*	0.1911
Cl. TK17	*Cl. TK*	**0.5258****
Cl. SL56	*Cl. SL*	0.1461
envOPS12	*envO*	-0.3945
Roseiflexales	*Rose*	0.1949
Cyanobacteria	*Cyano*	**0.7052***
YS2	*YS*	-0.3289
ML635J-21	*ML*	-0.2683
Nostocales	*Nosto*	-0.1046
Nostocaceae	*Nosto*	-0.1046
Chroococcales	*Chroo*	**0.9601***
Gomphosphaeriaceae	*Gomph*	0.3692
Microcystaceae	*Micro*	**0.9581***
Pseudanabaenales	*Pseuda*	**0.7939***
Pseudanabaenaceae	*Pseuda*	**0.7939***
Synechococcales	*Synec*	-0.1882
Synechococcaceae	*Synec*	-0.1895
Acaryochloridaceae	*Acar*	0.2442
SM1D11	*SM1D*	0.2038
Dependentiae (TM6)	*Depen*	**0.6812***
Cl. SBRH58	*Cl. SB*	**0.6215****
Cl. SJA-4	*Cl. SJ*	**0.6067****
S1198	*S11*	**0.6149****
Fibrobacteres	*Fibro*	0.1057
Fibrobacterales	*Fibro*	0.1057
Firmicutes	*Firmi*	-0.4061
Turicibacterales	*Turi*	0.3416
Turicibacteraceae	*Turi*	0.3416
Lactobacillales	*Lacto*	-0.4618
Streptococcaceae	*Strep*	-0.2968
Lactobacillaceae	*Lacto*	-0.3755
Enterococcaceae	*Entco*	-0.3603
Erysipelotrichales	*Ery*	-0.4798
Erysipelotrichaceae	*Ery*	-0.4798
Clostridiales	*Clos*	-0.2330
Peptostreptococcaceae	*Pepto*	0.0107
Lachnospiraceae	*Lach*	-0.1051
Clostridiaceae	*Clos*	-0.1469
Christensenellaceae	*Chris*	-0.2880
Mogibacteriaceae	*Mogi*	-0.4050
Bacillales	*Baci*	-0.2709
Planococcaceae	*Plano*	**-0.5704****
Paenibacillaceae	*Paeni*	0.1393
Bacillaceae	*Baci*	-0.2222
Fusobacteria	*Fuso*	0.0343
Fusobacteriales	*Fuso*	0.0343
Fusobacteriaceae	*Fuso*	0.0343
Gemmatimonadetes	*Gemmat*	0.2009
Cl. Gemm-1	*Cl. Ge*	0.2254
KD8-87	*KD*	**0.6744***
Gemmatimonadales	*Gemmat*	-0.2221
Gemmatimonadaceae	*Gemmat*	-0.2395
A1-B1	*A1*	0.0461

Hydrogenedentes (NKB19)	*Hydro*	**0.5258****
Cl. TSBW08	*Cl. TS*	**0.5258****
Latescibacteria (WS3)	*Lates*	0.2200
Sediment-1	*Sed*	0.2200
PRR-10	*PRR*	0.2200
Lentisphaerae	*Lenti*	0.4340
Victivallales	*Victi*	-0.0113
Victivallaceae	*Victi*	-0.0113
Cl. Lentisphaeria	*Cl. Len*	**0.5354****
Nitrospirae	*Nitro*	0.3001
Nitrospirales	*Nitro*	0.3001
Nitrospiraceae	*Nitro*	0.3001
Omnitrophica (OP3)	*Omni*	0.1736
Cl. PBS-25	*Cl. PBS*	0.1736
Planctomycetes	*Planct*	0.0525
WD2101	*WS*	-0.4396
CL500-15	*CL500*	-0.0147
Phycisphaerales	*Phyc*	0.2105
Phycisphaeraceae	*Phyc*	0.4821
Gemmatales	*Gemm*	-0.0418
Isosphaeraceae	*Isos*	-0.1978
Gemmataceae	*Gemm*	0.1333
Pirellulales	*Pire*	-0.0740
Pirellulaceae	*Pire*	-0.0740
Planctomycetales	*Planct*	-0.0458
Planctomycetaceae	*Planct*	-0.0458
DH61	*DH61*	-0.0859
Poribacteria (BRC1)	*Pori*	-0.0547
Cl. PRR-11	*Cl.PR*	-0.0547
Proteobacteria	*Prote*	0.3772
PB19	*PB19*	0.4073
Thiotrichales	*Thio*	-0.1967
Piscirickettsiaceae	*Pisci*	-0.1967
SC-I-84	*SC*	-0.2324
Oceanospirillales	*Ocea*	-0.1817
Halomonadaceae	*Halo*	-0.1817
GMD14H09	*GMD*	-0.1647
Nitrosomonadales	*Nitrom*	0.2663
Nitrosomonadaceae	*Nitros*	0.2663
Desulfobacterales	*Desulfb*	**-0.5448****
Desulfobulbaceae	*Desulbu*	-0.5127
Desulfobacteraceae	*Desulfb*	-0.3767
Desulfuromonadales	*Desulfm*	-0.4678
Pelobacteraceae	*Pelo*	-0.4827
Geobacteraceae	*Geob*	-0.3959
Campylobacterales	*Campy*	0.1840
Helicobacteraceae	*Heli*	-0.0303
Campylobacteraceae	*Campy*	0.3552
Aeromonadales	*Aero*	-0.1204
Aeromonadaceae	*Aero*	-0.1204
MIZ46	*MIZ*	0.3549
34P16	*34P*	0.0225
BD7-3	*BD7-3*	0.0897
Caulobacterales	*Caulo*	-0.4825
Caulobacteraceae	*Caulo*	-0.4825
Ellin329	*Ell*	0.4784
Rhizobiales	*Rhizo*	0.4852
Rhizobiaceae	*Rhizo*	**-0.5320****
Methylocystaceae	*Methycy*	**-0.5961****
Hyphomicrobiaceae	*Hypho*	-0.2199

Beijerinckiaceae	*Beij*	0.1963
Bradyrhizobiaceae	*Brad*	-0.3112
Rhodobacterales	*Rhodob*	**-0.7113***
Hyphomonadaceae	*Hypho*	-0.4462
Rhodobacteraceae	*Rhodob*	**-0.6990***
Rhodospirillales	*Rhodosp*	0.4183
Acetobacteraceae	*Aceto*	0.2621
Rhodospirillaceae	*Rhodosp*	0.4353
Rickettsiales	*Ricke*	0.4586
Pelagibacteraceae	*Pela*	0.3743
Rickettsiaceae	*Ricke*	0.0305
Sphingomonadales	*Sphin*	-0.4652
Erythrobacteraceae	*Ery*	0.2324
Sphingomonadaceae	*Sphin*	-0.0503
Cl. Alphaproteobacteria	*Cl. Alph*	**0.8340***
Burkholderiales	*Burk*	**-0.6905***
Alcaligenaceae	*Alca*	0.3839
Burkholderiaceae	*Burk*	0.0550
Comamonadaceae	*Coma*	-0.5135
Oxalobacteraceae	*Oxalo*	**-0.7931***
Ellin6067	*Ellin60*	**0.9556***
Methylophilales	*Methypi*	0.0201
Methylophilaceae	*Methypi*	0.0089
MND1	*MN*	0.3359
MWH-UniP1	*MWH*	0.2934
Neisseriales	*Neiss*	-0.3584
Neisseriaceae	*Neiss*	-0.3584
Nitrosomonadales	*Nitro*	0.2663
Procabacteriales	*Proca*	**-0.5332****
Procabacteriaceae	*Proca*	**-0.5332****
Gallionellales	*Galli*	0.4350
Gallionellaceae	*Galli*	0.4350
Rhodocyclales	*Rhodocy*	**-0.6982***
Rhodocyclaceae	*Rhodocy*	**-0.6982***
SBla14	*SBla*	-0.1181
Cl. Betaproteobacteria	*Cl. Beta*	0.3540
Bdellovibrionales	*Bdell*	0.3035
Bdellovibrionaceae	*Bdell*	-0.0537
Bacteriovoracaceae	*Bacvor*	0.2746
Myxococcales	*Myxo*	0.2190
OM27	*OM27*	0.2686
Myxococcaceae	*Myxo*	-0.1409
Haliangiaceae	*Hali*	-0.0841
0319-6G20	*031*	**0.7947***
Spirobacillales	*Spirob*	**0.8614***
Syntrophobacterales	*Syn*	0.1703
Syntrophaceae	*Syn*	-0.1663
Syntrophobacteraceae	*Synb*	0.2404
Cl. Deltaproteobacteria	*Cl. Delt*	0.0985
Alteromonadales	*Alter*	**-0.6797***
HTCC2188	*HTC88*	-0.3478
Chromatiaceae	*Chroma*	-0.5154
125ds10	*125ds*	-0.1065
211ds20	*211ds*	-0.5013
Alteromonadaceae	*Alter*	0.0394
HTCC2188	*HTC*	0.4011
HTCC2089	*HTC89*	0.4011
OM60	*OM60*	**-0.5394****
Shewanellaceae	*Shewa*	0.0642
Chromatiales	*Chrom*	0.4423

Enterobacteriales	*Entero*	-0.2261
Enterobacteriaceae	*Entero*	-0.2261
Legionellales	*Legio*	**0.5698****
Coxiellaceae	*Coxi*	**0.8124***
Legionellaceae	*Legio*	**0.5307****
Methylococcales	*Met*	0.4588
Crenotrichaceae	*Creno*	0.4533
Methylococcaceae	*Met*	0.4906
Pseudomonadales	*Pseudo*	-0.2117
Moraxellaceae	*Morax*	-0.4445
Pseudomonadaceae	*Pseudo*	0.3670
Xanthomonadales	*Xantho*	**0.6302****
Sinobacteraceae	*Sino*	**0.6843***
Xanthomonadaceae	*Xantho*	**-0.5465****
Marinicellales	*Marin*	-0.3115
Marinicellaceae	*Marin*	-0.3115
PHOS-HD29	*PHOS*	**0.7279***
Cl. Gammaproteobacteria	*Cl. Gam*	0.3148
Saccharibacteria (TM7)	*Saccha*	-0.0262
Cl. TM7-3	*Cl. TM3*	-0.2963
Cl. SC3	*Cl. SC*	0.2042
Cl. TM7-1	*Cl. TM1*	-0.0537
Spirochaetes	*Spiro*	-0.3816
Spirochaetales	*Spiro*	-0.3843
Spirochaetaceae	*Spiro*	-0.3843
Leptospirales	*Lepto*	-0.3744
Leptospiraceae	*Lepto*	-0.3744
Verrucomicrobia	*Verru*	-0.2247
WCHB1-50	*WCH*	0.4296
Verrucomicrobiales	*Verru*	**-0.5772****
Verrucomicrobiaceae	*Verru*	**-0.5772****
S-BQ2-57	*S-BQ*	-0.2372
Opitutales	*Opit*	**0.6206****
Opitutaceae	*Opit*	**0.6206****
Methylacidiphilales	*Methyla*	0.3237
LD19	*LD*	0.3237
Cerasicoccales	*Cera*	-0.1971
Cerasicoccaceae	*Cera*	-0.1971
Chthoniobacterales	*Chthob*	-0.0514
Chthoniobacteraceae	*Chthob*	-0.0514
Pedosphaerales	*Pedos*	0.2755
R4-41B	*R4*	0.1325
Ellin515	*Ell51*	-0.3413
auto67-4W	*aut*	-0.1437
ZB3	*ZB3*	-0.2154
Cl. BS119	*Cl. BS1*	-0.2154
Gracilibacteria (GN02)	*Graci*	0.0357
Cl. BD1-5	*Cl. BD1*	0.0357
Parcubacteria (OD1)	*Parcu*	0.3923
Cl. ZB2	*Cl. ZB2*	-0.0483
Cl. SM2F11	*Cl. SM*	**0.6457****
Cl. ABY1	*Cl. AB*	**0.7298***
Unassigned	*Un.*	0.1871

Table S 3.3. MC-degrading bacterial isolates and whether they possess or do not possess the *mlr* genes. "n.d." means not detected.

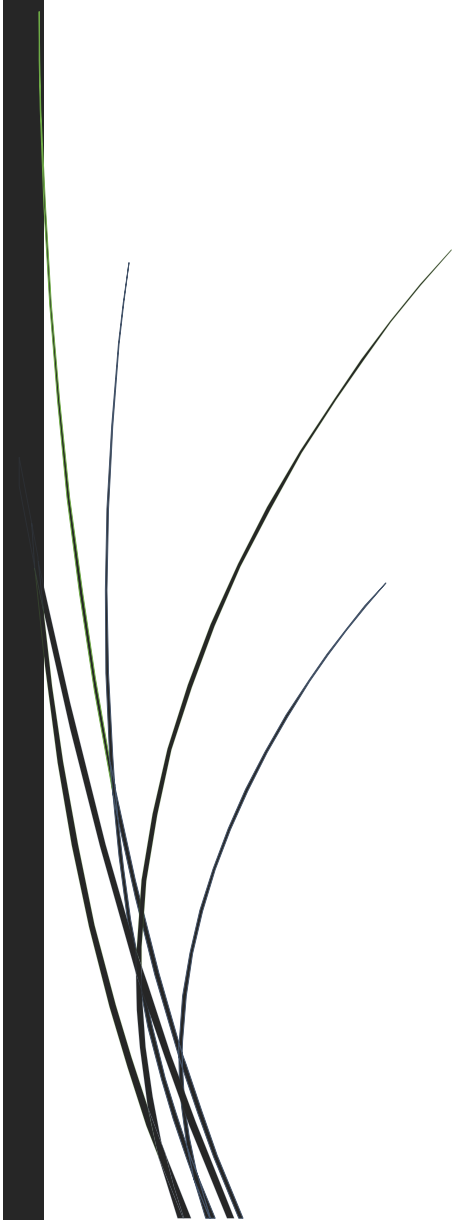
Phylum	Class	Order	Family	Genera/species	Strain	Accession number	*mlr*A presence/absence	References
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae*	*Microbacterium* sp.	DC8*	-	unknown	(Ramani et al., 2012)
			Micrococcaceae	*Arthrobacter* sp.	C6	FN392690	n.d.	(Manage et al., 2009a)
					F10	FN392691	n.d.	
					F7	FN392693	n.d.	
					R1	FN392694	n.d.	
					R4	FN392695	n.d.	
					R6	FN392696	n.d.	
					R9	FN392697	n.d.	
			Nocardiaceae	*Rhodococcus* sp.	C1	FN392688	n.d.	
					C3	FN392689	n.d.	
			Brevibacteriaceae	*Brevibacterium* sp.	F3	FN392692	n.d.	
		Bifidobacteriales	*Bifidobacteriaceae*	*Bifidobacterium lactis*	420	-	unknown	(Nybom et al., 2008, 2007)
					Bb12	-	unknown	
				Bifidobacterium longum	46	-	unknown	
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus* sp.	AMRI-03	GU294753	*mlr*A	(Alamri, 2010)
					EMB	FJ526332	*mlr*A	(Hu et al., 2012)
				Bacillus nanhaiencis	JZ-2013	KF841622	unknown	(Zhang et al., 2015)
		Lactobacillales	*Lactobacillaceae*	*Lactobacillus rhamnosus*	GG	AY370682	unknown	(Nybom et al., 2008, 2007)
					LC-705	-	unknown	
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiaceae*	*Rhizobium gallicum*	DC7*	AY972457	unknown	(Ramani et al., 2012)
		Sphingomonadales	*Sphingomonadaceae*	*Novosphingobium* sp.	THN1	HQ664117	*mlr*A, *mlr*B, *mlr*C, *mlr*D	(Jiang et al., 2011)
				Sphingomonas sp.	7CY	AB076083	unknown	(Ishii et al., 2004)
					ACM-3962	AF411072	*mlr*A, *mlr*B, *mlr*C, *mlr*D	(Bourne et al., 2001, 1996)
					B9	AB159609	unknown	(Imanishi et al., 2005)
					CBA4	AY920497	unknown	(Valeria et al., 2006)
					MD-1	AB110635	*mlr*A	(Saito et al., 2003)
					MDB2	AB219940	unknown	(Maruyama et al., 2006)
					MDB3	AB219941	unknown	
					NV3	-	*mlr*A, *mlr*B, *mlr*C, *mlr*D	(Somdee et al., 2013)
				Sphingomonas stygia	-	-	unknown	(Saitou et al., 2003)
				Sphingopyxis sp.	C-1	AB161684	*mlr*A, *mlr*B, *mlr*C, *mlr*D	(Okano et al., 2009)
					IM-1	KX085478	*mlr*A, *mlr*B, *mlr*C, *mlr*D	(Lezcano et al., 2016)
					IM-2	KX085479	*mlr*A, *mlr*B, *mlr*C, *mlr*D	
					IM-3	KX085480	*mlr*A, *mlr*B, *mlr*C, *mlr*D	
					LH21	DQ112242	*mlr*A, *mlr*B, *mlr*C, *mlr*D	(Ho et al., 2007)
					TT25	JQ398614	*mlr*A	(Ho et al., 2012b)
					USTB-05	EF607053	*mlr*A	(Wang et al., 2010; M. Zhang et al., 2010)
				Sphingosinicella microcystinivorans	Y2	AB084247	*mlr*A, *mlr*B, *mlr*C, *mlr*D	(Lezcano et al., 2016; Maruyama et al., 2006; Saito et al., 2003)
	Betaproteobacteria	*Burkholderiales*	*Alcaligenaceae*	*Bordetella* sp.	MC-LTH1	KC734882.1	*mlr*A	(Yang et al., 2014a)
			Burkholderiaceae	*Burkholderia* sp.	UPC-BI05	DQ459360	unknown	(Lemes et al., 2008)
				Paucibacter toxinivorans	2C20	NR_042941	n.d.	(Lezcano et al., 2016; Rapala et al., 2005)
					IM-4	KX085481	n.d.	(Lezcano et al., 2016)
				Ralstonia solanacearum	-	-	unknown	(Yan et al., 2004; Zhang et al., 2011)
		Methylophilales	*Methylophilaceae*	*Methylobacillus sp.*	J10	FJ418599	unknown	(Hu et al., 2009)
	Gammaproteobacteria	*Enterobacteriales*	*Enterobacteriaceae*	*Morganella morganii*	-	-	unknown	(Eleuterio and Batista, 2010)
		Aeromonadales	*Aeromonadaceae*	*Aeromonas* sp.	-	-	n.d.	(Mankiewicz-Boczek et al., 2015)
		Pseudomonadales	*Pseudomonadaceae*	*Pseudomonas aeruginosa*	-	-	unknown	(Takenaka and Watanabe, 1997)
		Xanthomonadales	*Xanthomonadaceae*	*Stenotrophomonas acidaminiphila*	MC-LTH2	-	n.d.	(Yang et al., 2014b)
				Stenotrophomonas sp.	EMS	FJ712028	*mlr*A	(J. Chen et al., 2010)

* *Microbacterium* sp. and *Rhizobium gallicum* only degrade microcystins when they are together in a consortia.

Table S 3.4. Water volumes from San Juan reservoir in dates closest to the samplings. Data are obtained from the Hydrographic Confederation of the Tagus River.

Date	Volume (Hm^3)	Variation rate	
		Hm3	%
27-Jan-2014	109		
24-Mar-2014	116	7	6
9-Jun-2014	119	3	3
4-Aug-2014	98	-21	-18
8-Sep-2014	83	-15	-15
6-Oct-2014	80	-3	-4
3-Nov-2014	87	7	8
17-Nov-2014	92	5	6
1-Dec-2014	106	14	16
8-Dec-2014	104	-2	-2

Chapter 4



4. Seasonal dynamics of microcystin-degrading bacteria and toxic cyanobacterial blooms: interaction and influence of abiotic factors

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4.1. Abstract

Massive proliferations of cyanobacteria coexist and have different interactions with other microorganisms, including microcystin-degrading bacteria. Despite their relevance in the environment for the removal of microcystins (MCs), this community has been scarcely studied. We investigated the influence of physicochemical factors and the seasonal dynamics of toxic cyanobacteria on the relative abundance and seasonal dynamics of the MC-degrading bacterial community with *mlr* genes (*mlr*$^+$) during a two-year study at a water reservoir in central Spain. The capacity of the total bacterial community on the degradation of MCs during the whole period of study was also evaluated. The results showed that *mlr*$^+$ bacteria started to increase in relative abundance after an increase in the relative abundance of toxic cyanobacteria, indicating a related seasonal dynamic between the two communities. The correspondence of a high abundance of *mlr*$^+$ bacteria with a decrease in toxic cyanobacteria and vice versa suggests an important interaction between the two communities in the environment and a possible antagonistic relationship that deserves an in-depth study. The lack of a significant relationship observed between the seasonal dynamics of both toxic cyanobacteria and *mlr*$^+$ bacterial communities with the physicochemical factors also supports their interaction as an important driver of their seasonal dynamics in nature. The MC biodegradation capacity of the total bacterial community was only observed during the toxic cyanobacterial bloom episodes, highlighting the importance of pre-exposure to MCs for triggering the MC biodegradation process.

4.2. Introduction

The occurrence of massive cyanobacterial growth due to eutrophication is becoming a prevalent situation worldwide, causing water quality problems for recreational and domestic uses. Moreover, climate change predictions point towards an elongation of the growing season, the uncoupling of trophic relationships and the disruption of ecosystem functions (De Senerpont Domis et al., 2013). The dominance of microcystin-producing cyanobacteria and the associated development of hepatic diseases, irritant reactions and tumour-promoting activities (Falconer and Yeung, 1992; Ueno et al., 1996) have increased the concern of water authorities and health organizations. Microcystins (MCs) are the most frequent and widespread toxins produced by several freshwater cyanobacterial genera, including *Microcystis*, *Dolichospermum* and *Planktothrix*, (Sivonen and Jones, 1999) and are synthesized by multienzyme complexes encoded by the *mcy* gene cluster (Börner and Dittmann, 2005). MCs consist of seven amino acids formed in a cyclic structure that is stable in water and resistant to some physicochemical processes (Chen et al., 1998; Harada et al., 1996; Tsuji et al., 1994). However, some freshwater bacteria are able to degrade MCs efficiently under environmental conditions (Jones and Orr, 1994; Kormas and Lymperopoulou, 2013). An enzymatic pathway encoded by the *mlr* gene cluster has been found responsible for the breakdown of MCs (Bourne et al., 2001). The *mlr* gene cluster has been reported to be constitutive (Alamri, 2010; Bourne et al., 2001; Ishii et al., 2004) although upregulated with the MC concentrations, thus showing higher transcriptional responses with increased MC concentrations (Jiang et al., 2011). However, recent studies have shown evidence for alternative-*mlr* MC degradation pathways operating in nature. The absence of *mlr* genes in some isolated MC-degrading bacteria (*mlr*$^{-}$) (Lezcano et al., 2016; Manage et al., 2009b), and the relationships observed between *mlr*-lacking bacteria associated to toxic cyanobacterial blooms and the xenobiotic metabolism (Mou et al., 2013a; Chapter 3), support the contribution of alternative-*mlr* MC degradation pathways to the degradation of MCs in the environment.

The dominance of cyanobacteria within the phytoplankton community, as well as the temporal shifts in the cyanobacterial composition, has been traditionally believed to be driven by bottom-up control factors (abiotic). Light, nutrients and temperature have been reported to influence the growth of cyanobacteria, the production of MCs (Monchamp et al., 2014; Orr and Jones, 1998; Sivonen, 1990; Vézie et al., 2002) and the expression of the *mcy* gene cluster (Kaebernick et al., 2000; Sevilla et al., 2008). However, top-down control

factors, such as interactions with other organisms (virus, fungi, bacteria, and zooplankton), are receiving special attention to understand the success of the cyanobacterial blooms and the variations in their toxicity (Sønstebø and Rohrlack, 2011; Van Wichelen et al., 2016; Wilken et al., 2014). Although the heterotrophic bacteria associated with toxic cyanobacterial blooms have been reported to have different types of interactions with cyanobacteria, such as parasitic, predatory or mutualistic (Rashidan and Bird, 2001; Van Wichelen et al., 2016), the ecological significance of the specific MC-degrading bacterial community has hardly been studied and, hence, is poorly understood in nature.

As far as we know, only one previous study performed by Zhu and co-authors (2014) studied the seasonal dynamics of the potentially MC-degrading bacteria with *mlr* genes (*mlr*$^+$) in the environment. The study showed an increase in the *mlr*A gene abundance after an increase in the MC concentrations during a toxic cyanobacterial bloom, pointing towards a close interaction between the two communities. However, the scarce number of studies performed on the seasonal dynamics of *mlr*$^+$ bacteria, as well as the currently unknown influence of the environmental factors on their temporal shifts, are hindering the understanding of the relationship between the *mlr*$^+$ bacteria and the toxic cyanobacterial blooms in nature. Moreover, several studies have reported a link between the MC biodegradation rates and the pre-exposure of the bacterial communities or uni-bacterial cultures to the MC *in vitro* (Ho et al., 2007; Morón-López et al., 2017). However, the MC degradation capacity and efficiency of the bacterial community in the environment before, during and after a naturally occurring toxic cyanobacterial bloom is still poorly understood and deserve in-depth study.

Therefore, the present study has the following aims: i) to determine the influence of the physicochemical factors and the toxic cyanobacterial dynamics on the relative abundance and seasonal dynamics of the *mlr*$^+$ bacterial community and ii) to assess the MC degradation capacity and efficiency of the aquatic bacterial community before, during and after toxic cyanobacterial bloom episodes. To achieve this, we monitored over two years the most relevant physicochemical factors (temperature, pH, dissolved oxygen and nutrients) and the relative abundance of both potentially MC-producing (*mcy*E gene as a marker) and MC-degrading (*mlr*A gene as a marker) bacterial communities in a reservoir in central Spain. To assess the MC degradation capacity of the aquatic bacterial community, we performed *in vitro* MC-degradation assays with the collected water samples.

4.3. Materials and methods

4.3.1. Sampling setup

The study was conducted in the San Juan reservoir, a waterbody located in central Spain (Madrid) with a history of persistent toxic cyanobacterial blooms. Water samples were periodically collected at the shoreline (40° 22' 44.10" N and 4° 19' 40.95" W) from 20 August 2013 to 28 January 2015. From the start of the cyanobacterial bloom in late summer until the collapse of the bloom in late autumn, samples were collected fortnightly. During the cold period, samples were collected bimonthly and then monthly during the summer before the onset of the cyanobacterial bloom. The measurements of the physical and chemical parameters were performed *in situ* using portable individual probes for dissolved oxygen (Crison OXI 45 P, Hach Lange, Spain), pH (Crison PH 25 Hach Lange, Spain) and temperature. Each sample was comprised of 4 L of subsurface water samples collected with sterilized polyethylene bottles and stored in dark and cold (4 °C) conditions during transport (less than 2 hours) to the laboratory. The samples were homogenized and split into four aliquots and treated with different protocols. One litre of water was sequentially filtered through 2-µm (25 mm, Whatman, Maidstone, United Kingdom) and 0.22-µm (25 mm, Millipore, Darmstadt, Germany) pore size polycarbonate filters to collect the larger and smaller fractions of the cyanobacteria and other aquatic bacteria (both those attached to the cyanobacterial mucilage and those free-living), respectively. The filter membranes were stored at -20 °C for further DNA analysis. The second litre of water was filtered through fibreglass filters (approximately 0.7 µm, Millipore) to collect seston and then stored at -20 °C for subsequent MCs and chl-*a* quantification. The flow-through was also collected and preserved at -20 °C for the analysis of dissolved MCs and nutrient concentrations (Dissolved Organic Carbon (DOC), nitrate and soluble reactive phosphorus). The third litre of water was immediately used to test the *in vitro* MC degradation capacity and the efficiency of the aquatic bacterial community. The fourth litre was used for the identification of cyanobacteria under a microscope (Olympus CX41, Tokyo, Japan) after 24 hours of flotation (Cirés and Quesada, 2011).

4.3.2. Nutrient analysis

The water samples that were filtered through fibreglass filters (approximately 0.7 µm) for the analysis of DOC, nitrogen from nitrate ($N\text{-}NO_3^-$) and phosphorous from soluble phosphate ($P\text{-}PO_4^{3-}$) were filtered again through 0.45-µm nylon syringe filters (25 mm, LLG, Meckenheim, Germany) before analysis. The analysis of DOC was performed in a Total

Organic Carbon analyser (TOC-V CSH, Shimadzu, Japan) after previous digestion of the samples by burning at 680 °C and subtracting the inorganic carbon fraction. The analysis of nitrate was performed in an 861 Advanced Compact IC system (Metrohm, Switzerland) with an autosampler 838 Advanced Sample Processor (Metrohm, Switzerland). Chromatographic separation was performed using a Metrosep A Supp 7-250 column (Metrohm, Switzerland) thermostated at 45 °C. The mobile phase consisted of 3.6 mM Na_2CO_3 with a flow rate of 0.7 mL min^{-1}. The analysis of soluble phosphate was performed following the ascorbic acid method described in the American Public Health Association – American Water Works Association Standard Methods (AWWA, 1992).

4.3.3. MCs and chl-a extraction from water samples

Sestonic MCs and chl-*a* from fibreglass filters were extracted in duplicate and double-extracted by sonication (P-Selecta Ultrasons, Barcelona, Spain) with 90% aqueous methanol. Chl-*a* was immediately quantified using spectrophotometric measurement at 665 nm (Shimadzu Multispec-1501, Kyoto, Japan). The final extracts were evaporated at 40 °C under a vacuum in a multiple evaporator (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany), and the volume was stored at -20 °C until quantification of sestonic MCs. The dissolved fraction of the MCs was extracted using solid-phase extraction from 600 mL of filtered water using C18 cartridges (200 mg, 6 cc) (OASIS HLB, Waters, Milford, MA, USA) activated with 100% methanol and then distilled water and finally equilibrated with 10% methanol. The flow rate was 1 mL min^{-1}, and the final elution of MCs was performed in 90% methanol. Both sestonic and dissolved MC extractions were filtered through 0.22-µm syringe filters (Acrodisc GHP, Pall Corporation, Port Washington, NY, USA) before analysis. The quantification of MCs was performed on an HPLC system (Agilent series 1100, Agilent Technologies, Santa Clara, CA, USA) coupled with a time-of-flight (TOF) mass spectrometer (Agilent 6230 accurate mass TOF Agilent Technologies, Santa Clara, CA, USA) by plotting the calibration curves from commercial MC-LR, MC-RR and MC-YR pure standards (Sigma-Aldrich, St. Louis, MO, USA). The chromatographic separation and the gradient profile used for the mobile phase are described in Lezcano et al. (2016).

4.3.4. MCs extraction from a toxic cyanobacterial scum

A toxic cyanobacterial scum collected from San Juan in 2012 (Lezcano et al., 2016) was extracted and used for the MC biodegradation assays performed on the collected water samples. For the MCs extraction, approximately 600 mL of the scum was extracted, partially

purified by solid phase extraction on 5 g C18 cartridges (Extrabond C18, 5 g, 20 mL, Sharlab, Barcelona, Spain) and quantified in the HPLC-TOF system, following the same procedure as previously described in Lezcano et al. (2016).

4.3.5. MC degradation capacity and efficiency of the bacterial community

To determine the MC degradation capacity and the efficiency of the aquatic bacterial community inhabiting the water reservoir, experiments were performed in duplicates with 20 mL of water samples enriched with 0.5 mg L^{-1} of MCs (sum of MC-LR, MC-RR and MC-YR) previously extracted from the scum. The flasks were incubated for 15 days at 27 °C with 120 rpm shaking in the dark to prevent photosynthetic growth. Negative controls consisting of autoclaved reservoir water were included. The samples used for MCs quantification (MC-LR, MC-RR and MC-YR) were collected at the start and after 15 days of incubation. The quantification of MCs was performed in the HPLC-TOC system as previously described.

4.3.6. DNA extraction from water samples

Genomic DNA from 2-µm and 0.22-µm polycarbonate filters was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions with several modifications to the cell disruption step, as described in Lezcano et al. (2016). The extracted DNA was dissolved in sterilized Milli-Q water and quantified using an Epoch spectrophotometer (BioTek Instruments, Winooski, VT, USA). Equal genomic DNA concentrations from both filtered fractions were combined to ensure a representative DNA sample from each water sample event.

4.3.7. Quantification of 16S rRNA, mcyE and mlrA genes by real-time PCR

Serial dilutions of purified 16S rRNA (959 bp) and *mlr*A (807 bp) gene PCR products from *Sphingopyxis* sp. strain IM-2 were used to generate a standard curve for the quantification of both genes in the water samples. For the quantification of the *mcy*E gene, serial dilutions of genomic DNA from *Microcystis aeruginosa* strain UAM 265 were used. The primer sets used for the 16S rRNA gene were 533F and 1492R_l (Turner et al., 1999; Weisburg et al., 1991), which are universal for bacteria. The primer sets used for the *mlr*A gene were MF and MR (Saito et al., 2003), which are specific to the MC-degrading bacteria possessing *mlr* genes. The conditions used for the PCR were previously described in Lezcano et al. (2016).

For the real-time PCR analysis, specific primer sets and TaqMan probes were used. All were previously designed, with the exception of the quencher TAMRA label, which was used

instead of BHQ1 in both the *mlr*A and *mcy*E TaqMan probes. For quantification of the 16S rRNA gene, we used the primer set BACT1369F and PROK1492R (123 bp) and the probe TM1389F (Suzuki et al., 2000), which are designed for universal bacteria and suggested for environmental samples. For *mlr*A quantification, the primer set included qmlrAF and qmlrAR (120 bp), and the probe was mlrA-tm (Hoefel et al., 2009); this primer and probe combination is currently the most widely used. Finally, for *mcy*E quantification, we used the primer set mcyF and mcyR and the probe mcyP (Al-Tebrineh et al., 2012), which amplify a gene region that encodes key enzymes for the MC biosynthesis and, thus, are unlikely to produce false negatives.

Prior to gene quantification, the DNA from each water sample was diluted to equal concentrations (10 ng μL^{-1}). Thus, the 16S rRNA gene analysis allowed us to verify that we used the same amount of DNA and further allowed us to quantify the relative abundance (%) of each specific gene (*mcy*E and *mlr*A) over the total bacterial community. All reactions were performed in duplicate using a Real-Time PCR machine (AB7300, Applied Biosystems, Foster City, CA, USA) in 25 µL of volume containing 1X of QuantiTech Probe PCR Master Mix (QIAGEN), 0.4 µM of each primer, 0.2 µM of each probe and 1 µL of either a DNA standard or sample. Thermal cycling conditions for 16S rRNA gene amplification were performed with an initial activation step at 95 °C for 15 min, followed by 45 cycles of denaturation at 94 °C for 15 s and annealing/extension at 56 °C for 1 min. Data were collected at the end of the annealing/extension step. The same thermal cycling conditions were used for *mlr*A and *mcy*E gene amplifications with the temperature in the annealing/extension step changed to 62 °C for *mlr*A and 60°C for *mcy*E. The number of gene copies per sample was calculated using the standard curve of the target gene copy number vs. the threshold cycle (Ct). The standard curve for the 16S rRNA gene was linear between $1.63x10^4$ and $1.63x10^9$ gene copies with a correlation coefficient (R^2) of 0.999 and an efficiency of 97%. The standard curve for the *mlr*A gene was linear between $3.14x10^2$ and $3.14x10^9$ gene copies with a R^2 of 0.999 and an efficiency of 89%, and the standard curve for the *mcy*E gene was linear between $1.28x10^2$ and $1.28x10^7$ gene copies with a R^2 of 0.999 and an efficiency of 84%.

4.3.8. Statistical analysis

A Pearson correlation analysis was performed with STATGRAPHICS Centurion XV v.15.1.02 software (Statpoint Technologies, Inc. Warrenton, VA, USA) using the data on the relative abundances of the *mcy*E and *mlr*A genes (MCs producers and degraders,

respectively) and the environmental factors (physicochemical parameters) previously square root transformed for normality. Statistically significant correlations were defined as those with *p*-values less than 0.05. Additionally, a principal component analysis (PCA) was performed in CANOCO5 v.5.04 software (Microcomputer Power, Ithaca, NY, USA) using the previously transformed data to explore the relationships between the relative abundance of both MCs producer and degrader communities with the environmental factors.

4.4. Results

4.4.1. Environmental parameters

Two cyanobacterial blooms occurred in the San Juan reservoir during the late-summer and autumn periods in 2013 (20 August to 5 November) and 2014 (20 August to 19 November). During both bloom episodes, chl-*a* increased to concentrations above 10 µg L^{-1} and reached 17.04 ± 0.83 µg L^{-1} in 2013 (5 November) and 349.82 ± 54.78 µg L^{-1} in 2014 (19 November) (Table 4.1). A chl-*a* peak (14.34 ± 1.42 µg L^{-1}) was also recorded in spring 2014 (25 March), although not related to MC-producing cyanobacteria. Total MC concentrations (sum of sestonic and dissolved) above 1 µg L^{-1} were found during and after the decay of both cyanobacterial blooms. The maximum MC concentrations were 140.91 ± 8.00 µg L^{-1} during the bloom of 2013 (5 November) and 9,893.14 ± 1053.49 µg L^{-1} during the bloom of 2014 (19 November), which coincided with the maximum chl-*a* peaks. The toxicity of both cyanobacterial episodes varied and progressed over time, with total MCs concentration per biomass ratios (MC:chl-*a*) ranging from 1.26 ± 0.09 to 8.31 ± 0.88 during the 2013 bloom and from 1.05 ± 0.06 to 28.51 ± 1.46 during the 2014 bloom. The DOC concentrations varied slightly during the two-year samplings (3.48 ± 0.54 mg L^{-1}). The nitrogen ($N\text{-}NO_3^-$) and soluble phosphorus ($P\text{-}PO_4^{3-}$) concentrations were lower during cyanobacterial overgrowths, increased during bloom peaks and peaked from bloom decay to spring. The maximum recorded concentrations of $N\text{-}NO_3^-$ and soluble $P\text{-}PO_4^{3-}$ were 451.81 ± 0.00 µg L^{-1} and 23.32 µg L^{-1} during the winter of 2015 (28 January), respectively.

Table 4.1. Physicochemical parameters from San Juan reservoir and the ratio of MCs to chl-*a* concentrations.

Date	Water chemistry								Ratio
	Total MCs ($\mu g\ L^{-1}$)	Chl-*a* ($\mu g\ L^{-1}$)	DOC ($mg\ L^{-1}$)	N-NO_3^- ($\mu g\ L^{-1}$)	P-PO_4^{3-} ($\mu g\ L^{-1}$)	T (ºC)	pH	Dissolved O_2 ($mg\ L^{-1}$)	MCs : Chl-*a*
20-Aug-2013	45.40 ± 2.70	10.29 ± 0.22	4.14 ± 0.00	96.57 ± 0.56	2.61	25.60	9.48	9.67	4.42 ± 0.36
4-Sep-2013	13.90 ± 3.66	5.04 ± 0.22	4.13 ± 0.11	92.73 ± 0.56	1.30	23.30	9.15	9.01	2.73 ± 0.61
18-Sep-2013	7.43 ± 0.63	4.42 ± 0.48	3.95 ± 0.01	90.25 ± 0.34	2.61	21.70	7.86	8.41	1.72 ± 0.33
1-Oct-2013	7.08 ± 0.97	5.61 ± 0.35	3.72 ± 0.09	156.10 ± 0.45	2.94	20.80	7.57	8.60	1.26 ± 0.09
15-Oct-2013	20.62 ± 0.46	12.68 ± 0.64	3.55 ± 0.09	85.05 ± 0.34	2.28	19.10	7.85	8.70	1.63 ± 0.12
5-Nov-2013	140.91 ± 8.00	17.04 ± 0.83	3.32 ± 0.08	208.17 ± 1.24	5.54	15.90	7.60	7.82	8.31 ± 0.88
26-Nov-2013	1.34 ± 0.32	5.21 ± 0.39	2.94 ± 0.02	250.19 ± 0.79	16.96	11.70	7.94	8.60	0.25 ± 0.04
11-Dec-2013	0.72 ± 0.15	4.64 ± 0.26	2.81 ± 0.00	301.02 ± 0.34	19.89	9.60	7.70	8.54	0.16 ± 0.04
29-Jan-2014	0.08 ± 0.01	4.14 ± 0.42	2.83 ± 0.02	397.14 ± 0.90	21.82	7.90	7.85	11.09	0.02 ± 0.00
25-Mar-2014	0.02 ± 0.00	14.34 ± 1.42	3.47 ± 0.37	306.10 ± 1.13	15.92	10.40	8.19	12.05	0.00 ± 0.00
12-Jun-2014	0.04 ± 0.00	5.50 ± 0.46	4.02 ± 0.06	39.53 ± 1.13	2.15	22.10	9.80	9.95	0.01 ± 0.00
8-Jul-2014	0.30 ± 0.30	9.64 ± 0.88	3.98 ± 0.08	31.63 ± 0.00	2.45	22.30	9.46	11.95	0.03 ± 0.03
7-Aug-2014	0.07 ± 0.00	4.31 ± 0.59	3.52 ± 0.18	31.63 ± 0.00	3.06	24.70	8.38	8.82	0.02 ± 0.00
20-Aug-2014	4.49 ± 0.46	4.27 ± 0.07	3.10 ± 0.01	29.37 ± 0.00	29.97	23.60	8.08	8.55	1.05 ± 0.09
9-Sep-2014	11.28 ± 1.46	7.47 ± 0.24	3.04 ± 0.00	28.24 ±1.13	2.00	23.40	7.73	8.83	1.52 ± 0.24
24-Sep-2014	17.22 ± 0.46	8.10 ± 0.66	2.79 ± 0.06	27.11 ± 0.00	1.85	21.60	7.90	9.20	2.13 ± 0.12
7-Oct-2014	14.44 ± 092	9.46 ± 0.70	2.75 ± 0.25	27.11 ± 0.00	3.06	20.30	8.16	9.64	1.53 ± 0.02
22-Oct-2014	27.57 ± 0.10	12.79 ± 0.88	3.72 ± 0.13	57.61 ± 1.13	2.15	18.80	7.94	9.58	2.17 ± 0.14
4-Nov-2014	8.85 ± 0.49	8.41 ± 0.00	4.60 ± 0.00	101.66 ± 4.52	5.02	16.90	7.56	8.86	1.05 ± 0.06
19-Nov-2014	9893.14 ± 1053.49	349.82 ± 54.78	4.07 ± 0.11	282.38 ± 2.26	8.35	14.50	7.30	7.5	28.51 ± 1.46
2-Dec-2014	4.81 ± 0.34	1.93 ± 0.53	3.15 ± 0.04	306.10 ± 5.65	19.41	13.00	7.51	8.69	2.74 ± 0.92
28-Jan-2015	0.09 ± 0.00	1.40 ± 0.70	3.04 ± 0.11	451.81 ± 0.00	23.32	6.80	7.56	11.4	0.09 ± 0.04

4.4.2. Cyanobacterial community composition

Cyanobacteria were microscopically identified in all water samples except for samples from the winter (29 January 2013 and 28 January 2014) and one from the summer (8 July 2014) (

Table **4.2**). A strong hailstorm followed by heavy rains occurred three days before 8 July 2014, which may have mixed the water column and may explain the absence of cyanobacteria. Both cyanobacterial blooms were dominated by *Microcystis aeruginosa* with a large and correlative presence of *Microcystis flos-aquae* and *Pseudanabaena mucicola*. *Dolichospermum flos-aquae* and *Aphanizomenon flos-aquae* appeared at the end of the cyanobacterial bloom in 2013 and the onset of the cyanobacterial bloom in 2014. The non-toxic cyanobacterium *Microcystis wesenbergii* was identified in a few samples during both cyanobacterial blooms, and *Woronichinia naegeliana* was identified in all seasons except winter.

Table 4.2. Taxonomic identification of cyanobacterial morphospecies under the microscope after 24 hours of flotation. An asterisk indicates the estimated dominant morphospecies.

Cyanobacterial taxa	20-Aug-13	4-Sep-13	18-Sep-13	1-Oct-13	15-Oct-13	5-Nov-13	26-Nov-13	11-Dec-13	29-Jan-14	25-Mar-14	12-Jun-14	8-Jul-14	7-Aug-14	20-Aug-14	9-Sep-14	24-Sep-14	7-Oct-14	22-Oct-14	4-Nov-14	19-Nov-14	2-Dec-14	28-Jan-15
Dolichospermum crassum																						
Dolichospermum flos-aquae								*			*		*	*	*							
Aphanizomenon flos-aquae	*																					
Microcystis aeruginosa			*	*	*	*	*	*						*	*	*	*	*	*	*		
Microcystis flos-aquae																						
Microcystis viridis																						
Microcystis wesenbergii																						
Pseudanabaena mucicola																						
Woronichinia naegeliana		*																				

4.4.3. Seasonal shifts of potentially MC-producing cyanobacteria and MC-degrading bacteria

The seasonal dynamics of the potentially MC-producing cyanobacteria were monitored over the period of the study and were expressed as the ratio of the *mcy*E gene over the 16S rRNA gene abundance of the total bacterial community (Figure 4.1A). Relative abundances above 1% were found in summer and autumn water samples during both cyanobacterial blooms of 2013 and 2014. The highest relative abundance of *mcy*E genes in 2013 was observed in summer at the onset of the cyanobacterial bloom episode (5.53%, 4 September), and remained in similar proportions until the end of the cyanobacterial overgrowth (2.28% ± 1.38%). However, a greater difference in the relative abundance of *mcy*E genes was observed during the 2014 bloom, which reached a maximum of 31.18% at the end of the cyanobacterial growing season (19 November). The relative abundances of the *mcy*E genes in the winter and spring water samples ranged below 0.5%.

The seasonal dynamics of the fraction of potentially MC-degrading bacteria with *mlr* genes were monitored over the period of the study and were expressed as the ratio of the *mlr*A gene over the 16S rRNA gene abundance of the total bacterial community (Figure 4.1B). The increase in the relative abundances of the *mlr*A genes occurred towards the middle-end of both cyanobacterial bloom episodes, reaching 0.19% in 2013 (26 November) and 0.15% in 2014 (4 November). The *mlr*A peak in 2013 appeared after the recorded maximum MCs concentrations, and the *mlr*A peak in 2014 appeared after a continuous presence of MCs but just before the cyanobacterial bloom peak and the maximum MC concentrations. A drop in *mlr*A genes was observed during the maximum cyanobacterial biomass of 2014, followed by a small rebound after the bloom lysis. Additionally, another small peak in *mlr*A genes was observed during the summer of 2014 (7 August), before the development of the toxic cyanobacterial bloom and when total MC concentrations were below 0.5 µg L^{-1}.

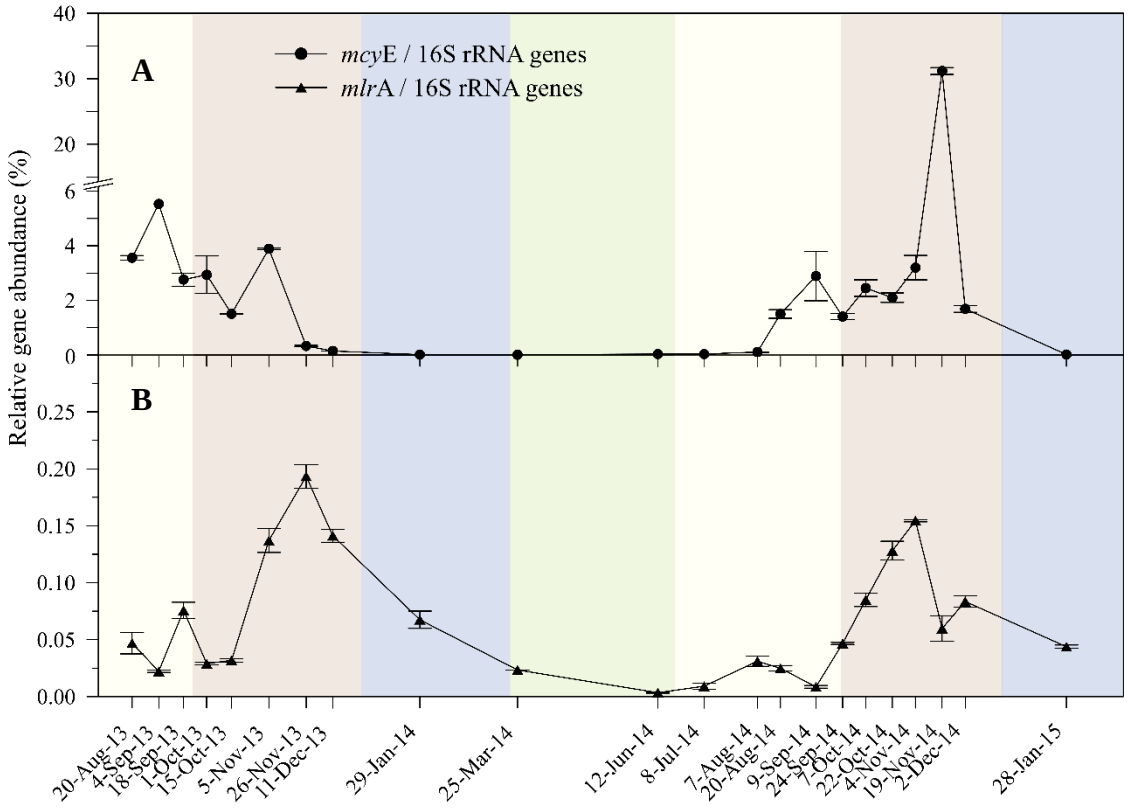


Figure 4.1. Seasonal dynamics of the relative abundances (%) of *mcy*E gene (A) and *mlr*A gene (B) over the total bacterial community (16S rRNA gene) in San Juan reservoir. The coloured sections indicate seasons: summer in yellow, autumn in brown, winter in blue and spring in green. Error bars indicate standard errors of duplicates.

4.4.4. Seasonal shifts of the sestonic and dissolved MC concentrations

The seasonal dynamics of the sestonic and dissolved MC concentrations followed a similar trend as the temporal shifts of the *mcy*E gene relative abundances (Figure 4.2A). The sestonic MCs represented the highest fraction in all samples (between 76% and 100% of the total MC concentrations), except for those from the winter, spring and early summer, when only dissolved MCs were detected (below 0.1 µg L^{-1}). Despite the similar trends observed between the *mcy*E and both the sestonic and dissolved MC concentrations, they did not increase in the same proportions. The ratio between the total MC concentration (sum of sestonic and dissolved) and the *mcy*E genes ranged between 1 and 14 over the whole study period and reached 37 during the cyanobacterial bloom peak in 2013 and 317 during the peak in 2014 (data not shown).

When comparing the sestonic and dissolved MC concentrations with the relative abundances of the *mlr*A genes, their increases were also not proportional. While the sestonic and dissolved MCs concentrations were 70-fold and 97-fold higher during the cyanobacterial

bloom peak of 2014 compared to those of 2013, the relative abundance of the *mlr*A genes in 2014 was 2.3-fold lower. Despite this difference, both the sestonic and dissolved fractions of the MCs decreased in concentration by nearly 100% after the cyanobacterial bloom lysis in 2013 and 2014, pointing towards an important MCs degradation capacity of the aquatic bacterial community in the environment.

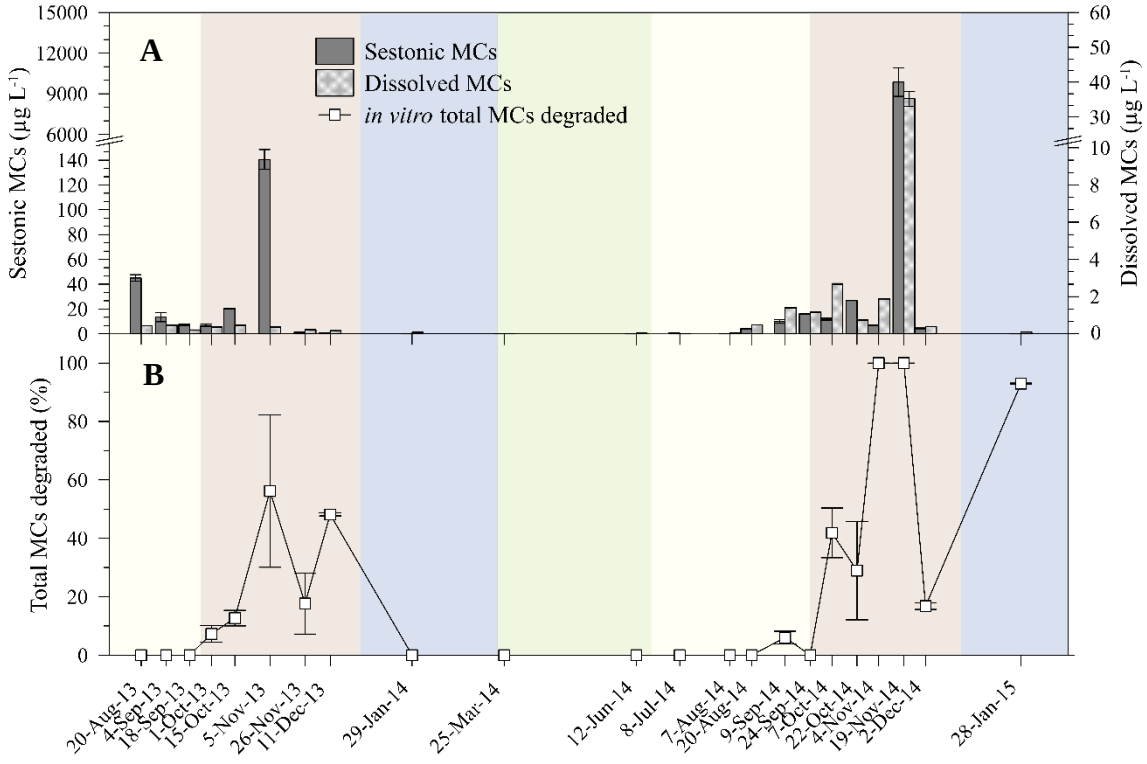


Figure 4.2. Seasonal dynamics of both sestonic and dissolved MCs concentrations in the reservoir (upper plot) and the percentage of degraded MCs after 15 days of incubation of the total bacterial community present in the reservoir water samples enriched with 0.5 mg L^{-1} of MCs (lower plot). The coloured sections indicate seasons: summer in yellow, autumn in brown, winter in blue and spring in green. Error bars indicate standard errors of duplicates.

4.4.5. Seasonal shifts of the in vitro MC degradation capacity and efficiency of the bacterial community

The *in vitro* MC degradation experiments in the water samples collected from the reservoir and amended with 0.5 mg L^{-1} of MCs demonstrated that the autumn bacterial communities were mainly responsible for the degradation of MCs (Figure 4.2B). This capacity occurred after the pre-exposure of the bacterial community to MCs from the reservoir, and maximum degradation efficiencies were reached during the cyanobacterial bloom peaks and higher MC concentrations.

The *in vitro* MC degradation capacity of the bacterial communities from autumn water samples (and from 9 September 2014 and 28 January 2015) coincided with the presence of *mlr*A genes. However, the presence of *mlr*A genes in the bacterial communities from the other seasons did not exhibit an MC degradation capacity under our experimental conditions. When considering only those water samples showing MC biodegradation, the efficiency of the biodegradation of MCs did not show any significant relationship with the relative abundance of the *mlr*A genes (r=0.1929, p-value > 0.05) (Figure 4.3). This low relationship is partially explained by the drop in the relative abundance of *mlr*A genes on 19 November 2014 (61% compared to the previous date) when the *in vitro* MCs degradation efficiency remained 100%.

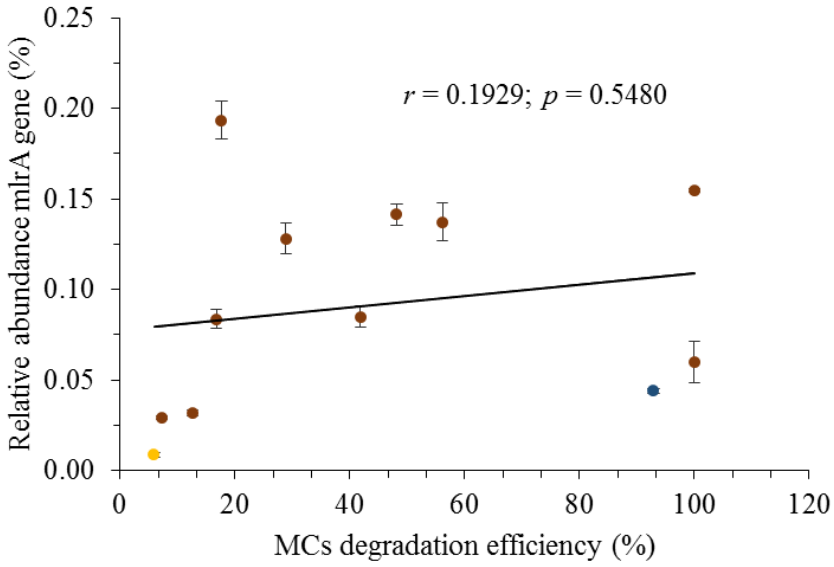


Figure 4.3. Correlation between the relative abundance of *mlr*A genes and the *in vitro* MC degradation efficiencies of the aquatic bacterial community. Only water samples that showed the removal of MCs were considered for the plot. The coloured points indicate seasons: summer in yellow, autumn in brown and winter in blue. Error bars indicate standard errors of duplicates. "*r*" refers to the Pearson correlation coefficient and "*p*" refers to the *p*-value.

4.4.6. Relationships between the relative abundance of MC producers, MC degraders and the environmental parameters

To explore the influence of the environmental factors and the seasonal dynamics of the toxic cyanobacteria on the seasonal dynamics of the *mlr*$^+$ bacteria, a Pearson correlation analysis (Table 4.3) and a PCA plot (Figure 4.4) were constructed. In the analysis, we considered the relative abundances of the *mcy*E and *mlr*A genes, the concentrations of chl-*a*, MCs (sestonic and dissolved) and nutrients (DOC, P-PO_4^{3-} and N-NO_3^-), and the *in situ* physical and chemical parameters (T, pH and dissolved oxygen).

Table 4.3. Pearson correlations between physical, chemical and biological parameters from San Juan reservoir. The numbers in bold indicate a statistically significant positive or negative correlation ($r > 0.5$, p-value < 0.05).

	***mlr*A genes**	***mcy*E genes**	**Sestonic MCs**	**Dissolved MCs**	**Chl-*a***	**DOC**	**N-NO_3^-**	**P-PO_4^{3-}**	**T**	**Dissolved O_2**	**pH**
***mlr*A genes**											
***mcy*E genes**	0.1197										
Sestonic MCs	0.0596	**0.8498**									
Dissolved MCs	0.1165	**0.8855**	**0.9511**								
Chl-*a*	0.0350	**0.8144**	**0.9856**	**0.9358**							
DOC	-0.1869	0.3762	0.2542	0.1882	0.2815						
N-NO_3^-	0.3877	-0.0142	0.2059	0.0858	0.1625	-0.2137					
P-PO_4^{3-}	0.2737	-0.2545	-0.0107	-0.0632	-0.0587	**-0.4980**	**0.6782**				
T	-0.4363	0.2285	-0.0669	-0.0039	-0.0514	0.4188	**-0.9071**	**-0.7383**			
Dissolved O_2	-0.3705	**-0.6330**	-0.4073	-0.4583	-0.3355	-0.0663	0.1193	0.1420	-0.2794		
pH	**-0.5256**	-0.2833	-0.2780	0.3570	-0.2340	0.3824	-0.4618	-0.3908	**0.4973**	0.4196	

The *mcy*E genes, chl-*a* and the sestonic and dissolved MCs concentrations in the water were grouped together in the PCA plot and showed a positive and significant correlation. The *mcy*E genes presented a significant and negative relationship with the dissolved oxygen in the PCA plot, which is consistent with the correlation analysis (r=-0.6330; $p < 0.05$). The PCA plot and the correlation analysis showed a lack of significant relationship between the *mlr*A and *mcy*E genes due to the temporal displacement of both seasonal patterns (Figure 4.1), and between these genes and the nutrient concentrations (N, P and DOC). The analysis also showed a lack of significant relationship between the relative abundance of the *mlr*A genes and both the sestonic and dissolved MC concentrations. Only the pH appeared to be significantly and negatively correlated with the *mlr*A genes, but the correlation was weak (r=-0.5256, $p < 0.05$). Nitrogen and phosphorus appeared to be significantly and negatively related to low temperatures, which was consistent with the detection of higher concentrations in the late autumn, winter and spring water samples.

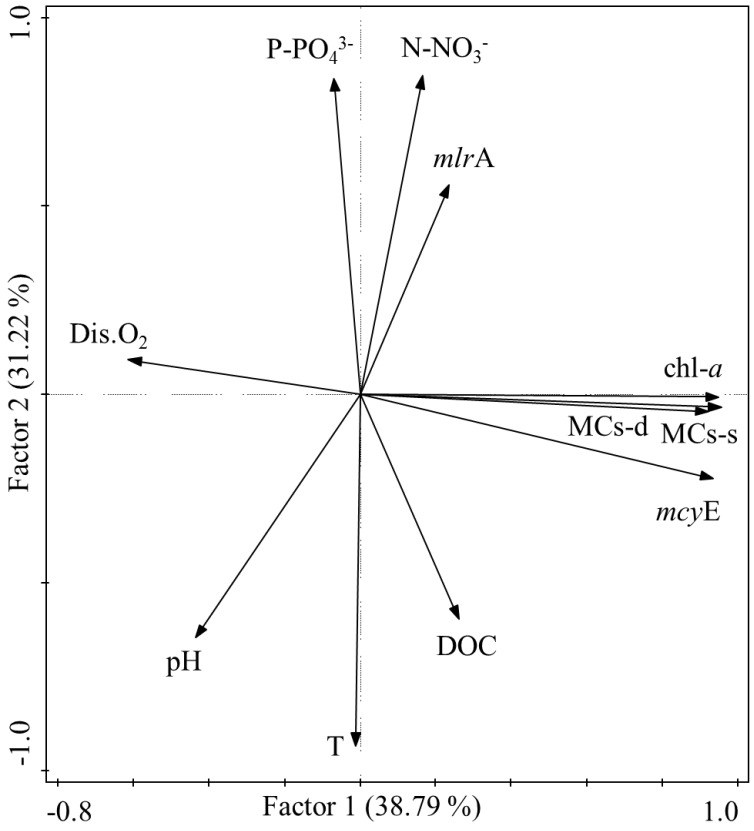


Figure 4.4. PCA ordination biplot of data obtained from the reservoir from 20 August 2013 to 28 January 2015. The physical and chemical parameters, quantification of *mlr*A and *mcy*E genes, concentration of nutrients ($P\text{-}PO_4^{3-}$, $N\text{-}NO_3^-$, DOC) and both sestonic and dissolved MCs concentrations (MCs-s and MCs-d, respectively) are represented by arrows.

4.5. Discussion

The presence of chl-*a* concentrations above 8-10 µg L^{-1} revealed an increase in the phytoplankton biomass during the spring, summer and autumn, reaching an overgrowth (or bloom) on 19 November 2014, when 350 µg L^{-1} of chl-*a* was detected in the water (Bellinger and Sigee, 2010). According to these data and microscopic inspection, two summer-autumn blooms dominated by *Microcystis aeruginosa* and one spring bloom with non-cyanobacterial dominance occurred over the two-year samplings. The relationships between the chl-*a* concentrations and both the relative abundance of the potentially toxic cyanobacteria (monitored with the *mcy*E gene) and the MC concentrations indicated that the two summer-autumn blooms were toxic. The concentrations of total MCs during these cyanobacterial blooms were above the WHO guideline value of 1 µg L^{-1} for drinking water (WHO, 1998). Moreover, approximately 43-63% of the water samples during the growing seasons showed chl-*a* concentrations above 10 µg L^{-1}, which exceeds the threshold established for low health risk in recreational waters (WHO, 2003).

The greater increase in the total MCs concentrations (sestonic and dissolved) compared to the increase in the relative abundance of the *mcy*E genes over the period of study (MC:*mcy*E ratios from 1 to 317) raises questions about the suitability of the *mcy*E gene as an indicator of MC concentrations in water, as has also been reported in other studies (Beversdorf et al., 2015; Vaitomaa et al., 2003). Previous studies investigating the influence of several physicochemical factors (temperature, light, pH, and others) on *mcy* expression under laboratory conditions only reported variations in the MC content by a maximum factor of five (Rapala et al., 1997; Sivonen, 1990) and thus were unable to explain the large oscillations in the MC concentrations observed in the field. Conversely, some studies have ascribed these variations in the MC concentrations to the temporal shifts in the relative abundance of *Microcystis* and *Planktothrix* subpopulations with different toxin profiles (chemotypes) and content (Agha et al., 2014; Rohrlack et al., 2008). Thus, one explanation for the greater increase in the MC concentrations compared to the increase in the relative abundance of the *mcyE* genes is the presence of cyanobacterial strains with higher MC contents. Another possibility is the increase of bacterial strains with higher 16S rRNA gene copy numbers, which ultimately translates into lower *mcy*E relative abundances (*mcy*E:16S RNA genes) and overestimates the MC:*mcy*E proportion. In this study, we do not refer to genome copies per cell because the environmental water samples have a complex bacterial community composition that contains very different genome copy numbers (Acinas et al.,

2004; Kembel et al., 2012). However, it is estimated that *Microcystis aeruginosa* possesses, on average, 3 copies of the 16S rRNA gene and 1 copy of the *mcy*E gene per cell (Al-Tebrineh et al., 2012). Therefore, this may also explain the increasing MC:*mcy*E ratios during *Microcystis* spp. bloom development. Then, monitoring the relative abundance of the *mcy*E gene to predict MC concentrations in the water should be considered with caution.

The general increase in the relative abundance of the *mlr*A gene just after the increase in the relative abundance of the *mcy*E gene and the MC concentrations point towards a bacterial community shift in which the presence of cyanobacteria and toxins likely stimulate, at least, the growth of those potential degraders of MCs with *mlr* genes. Thus, the pelagic growth and overwintering periods described in *Microcystis* sp. (Cirés et al., 2013; Reynolds et al., 1981) may be followed by, at least, the *mlr*$^+$ bacterial community. These results are also consistent with those reported in the study conducted by Zhu et al., (2014), in which the increase in the abundance of the *mlr*A gene occurred after an increase in the toxic *Microcystis* spp. and MC concentrations in the water.

Despite the likely stimulation of the *mlr*$^+$ bacterial growth under the presence of MCs in the environment, we did not observe a significant relationship between the relative abundance of the *mlr*A gene and either the sestonic or dissolved MC concentrations in our study. This lack of significant relationship can be partly explained by the decline in the *mlr*A gene during the highest peak of toxic cyanobacteria in the water (19 November 2014). While we expected a significant increase in the relative abundance of the *mlr*A gene with regard to the 1,118-fold increase in the total MC concentrations (sestonic and dissolved), the *mlr*A gene decreased 2.6–fold. On the one hand, it may be possible that the high cyanobacterial biomass at that point could mask the increase in the abundance of the *mlr*$^+$ bacteria; thus, it would not be reflected when considering relative abundances. On the other hand, since the toxic cyanobacterial bloom represents approximately 31% of the total bacterial community, there is still a remaining 69% that is likely represented by other bacteria, which raises questions about the factors that influence the dynamics of the *mlr*$^+$ bacteria and the type of interaction with the toxic cyanobacteria. In this sense, the correspondence of the increase in the relative abundance of the *mlr*$^+$ bacteria with the decrease in the relative abundance of the toxic cyanobacteria and vice versa, during the period of study, may outline an antagonist interaction between the two communities. On the one hand, a resource competition may be possible, in which the available nutrients favour the growth of cyanobacteria due to their ecophysiological characteristics (Visser et al., 2005), and a substantial growth of *mlr*$^+$

bacteria is only observed when the cyanobacterial bloom declines and cell lysis has occurred. Thus, the *mlr*$^+$ bacteria may act as decomposers (Zhang et al., 2012). Another possibility is that the *mlr*$^+$ bacteria could be implicated in the bloom collapse and cell lysis and afterwards in the degradation of the released MCs. In fact, previous studies have ascribed the collapse of cyanobacterial blooms to the growth of microbial antagonists, such as cyanolytic bacteria that produce algicidal compounds (Gerphagnon et al., 2015; Hee-Jin et al., 2005; Rashidan and Bird, 2001; J. Shao et al., 2014; Van Wichelen et al., 2016). Previous studies describing the seasonal dynamics of the lytic bacteria showed a peak in their abundance coincident or closely followed by a decline in the cyanobacterial bloom (Manage, 2009; Manage et al., 2001; Rashidan and Bird, 2001). The lack of a significant relationship observed in our study between the physicochemical factors and the relative abundances of *mlr*A and *mcy*E genes also supports the notion that biotic factors are the main drivers of the seasonal dynamics of both toxic cyanobacteria and *mlr*$^+$ bacterial communities (top-down mechanisms).

Considering the aforementioned, the decline in *mlr*$^+$ bacteria at the highest toxic cyanobacterial peak suggests an impairment in *mlr*$^+$ bacterial growth due to the development of the cyanobacterial bloom, possibly by either preventing access to the cyanobacterial cells or competing with the nutrients. Cyanobacterial cell aggregation has been described to cause opposite responses in the associated bacteria. In some cases, bacteria seem to be unaffected by colony formation and even induce cyanobacterial cell aggregation as an efficient way to concentrate food (Nakamura et al., 2003). In other cases, the thick mucilage matrix of the cyanobacteria acts as a physical barrier by preventing cell lysis and limiting the proliferation of heterotrophic bacterial populations (Liu et al., 2013; Wang et al., 2013; Xu et al., 2012). A diversity study on the *mlr*$^+$ bacterial community would be required to explore this hypothesis, since other microorganisms present in the environment may also influence the seasonal dynamics of both MC-producing and MC-degrading communities.

The *in vitro* biodegradation of MCs mainly found during the toxic cyanobacterial bloom episodes (autumn) and the absence of such capacity during the inter-bloom period despite the detection of *mlr*A genes suggest that previous exposure of the bacterial communities to MCs in the natural environment is an important factor for the MC biodegradation process. This pre-exposure to MCs may cause an activation of the *mlr* genes (Ho et al., 2007; Shimizu et al., 2011) or a bacterial community shift (K. Shao et al., 2014) by increasing the abundance of *mlr*$^+$ bacteria. Despite the constitutive expression of the *mlr* genes (Bourne et al., 2001; Jiang et al., 2011), it may also be possible that the total amount of *mlr*$^+$ bacteria present

during the inter-bloom period was insufficient to detect MC degradation activity. Thus, the MC biodegradation capacity of the bacterial community seems to be related to the toxic cyanobacterial bloom episode, and the detection of the *mlr*A gene only indicates a presence of potentially MC-degrading bacteria, but not an occurring MC degradation activity.

Attending solely to the efficiency of the bacterial communities that exhibited an *in vitro* degradation of MCs, the lack of a significant relationship with the relative abundance of the *mlr*A gene supports previous studies that have highlighted the contribution of bacteria operating *mlr*-alternative MC degradation pathways for the overall degradation of MCs in the environment. The use of the same conditions for the *in vitro* MC biodegradation experiments allows for the better association of the different MC degradation efficiencies to changes in the bacterial community composition than to dissimilar *mlr* gene expression, thus supporting the possible presence of *mlr*$^{-}$ bacteria and alternative-*mlr* degradation pathways.

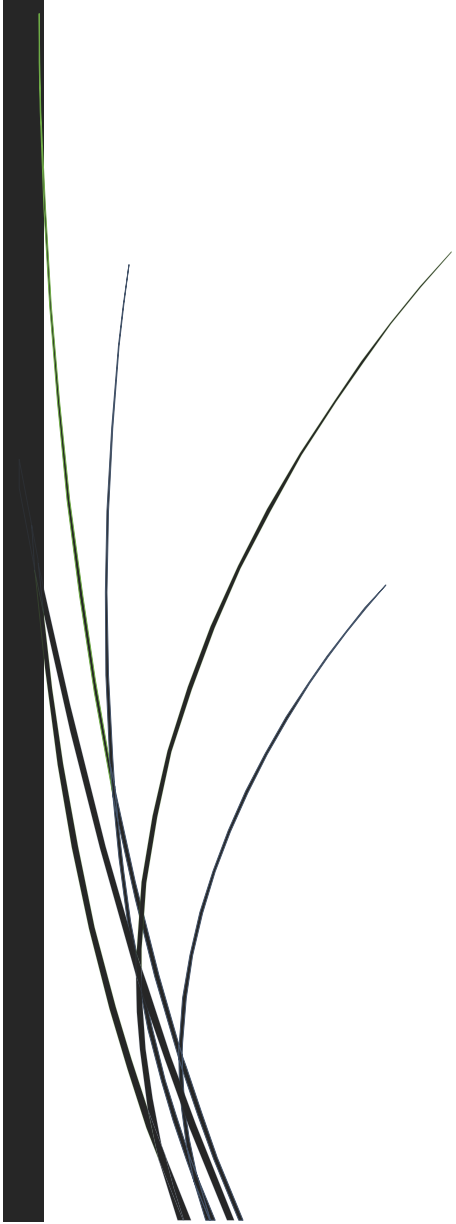
4.6. Conclusions

The closely related seasonal dynamics between the toxic cyanobacteria and the *mlr*$^{+}$ bacteria, as well as the absence of a significant relationship with the physicochemical factors, suggest an important interaction between both communities. The increase in the relative abundance of the *mlr*$^{+}$ bacteria coincident with the decrease in the relative abundance of the toxic cyanobacteria indicates a possible antagonistic relationship between the two communities that needs further research. The observation of biodegradation of MCs only in water samples during the toxic cyanobacterial bloom episodes indicates that the biodegradation of MCs by the bacterial community is related to the previous presence of cyanobacteria and MCs in the environment. Thus, the pre-exposure of the bacterial community to MCs in nature may play an important role in both triggering a bacterial community shift, including both *mlr*$^{+}$ and *mlr*$^{-}$ bacteria, and/or activating the *mlr* genes. Moreover, the lack of a significant correlation between the efficiency of MC biodegradation and the relative abundance of *mlr*$^{+}$ bacteria suggests an important contribution of alternative-*mlr* MC degradation pathways for the efficient biodegradation of MCs in nature during the toxic cyanobacterial bloom episodes.

4.7. Acknowledgements

We would like to thank María Isabel López Heras for the analysis of MCs and to extend the thanks to Carolina Guillén and Patricia García for the analysis of nitrate, phosphate and DOC.

Chapter 5



5. Method for degrading MCs in an aqueous medium (PCT/ES2016/070026)

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Previous considerations:

A patent application with international protection (PCT) was considered after developing the following:

1. A method for the efficient isolation of MC-degrading bacteria in water samples collected from a reservoir with frequent toxic cyanobacterial bloom episodes.
2. An efficient MC degradation method in aqueous media with different nutrient concentrations using the bacterial isolate *Sphingopyxis* sp, strain CECT 8967. This strain was able to degrade the 90% of 1 mg L^{-1} of total MCs (MC-LR, MC-RR and MC-YR) in 6 hours regardless of the different total organic carbon (TOC) concentrations (from 0 to 258 mg L^{-1}, approx.) present in the aqueous media.

The complete information of the invention is found in the PCT application located in Appendix. The methods developed in this patent were used in the study published in Lezcano et al. (2016) (Chapter 2). Therefore, the information explained in this section only refers strictly to the development of the methods and the aspects that have not been previously discussed in Chapter 2.

5.1. Introduction

Cyanobacterial blooms are frequent phenomena in eutrophic water masses from lentic systems like ponds, lakes and reservoirs worldwide (Merel et al., 2013). They are considered a potential hazard to human health and to ecosystems due to the production of cyanotoxins (Codd, 1995). The microcystins (MCs) are the most abundant cyanotoxins in freshwater systems and more than 90 variants have been identified so far (Merel et al., 2013; Sivonen and Börner, 2008). The MC-LR, MC-RR and MC-YR are the most common variants and a provisional guideline value of 1 μg L^{-1} of MC-LR is stablished for drinking water (WHO, 1998).

MCs are a group of potent hepatotoxins with cyclic structure and very stable to traditional water treatment technologies where conventional processes such as adsorption, coagulation, chlorination, among others, have limited effect on their removal (Himberg et al., 1989; Keijola et al., 1988). However, the MCs can be degraded by aquatic heterotrophic bacteria present in water and sediments from lakes and reservoirs (Ishii et al., 2004; Rapala et al., 2005), conferring a promising method for toxin removal from water (Ho et al., 2012a, 2006; Wang et al., 2007). So far, several MC-degrading bacteria have been isolated around the world, but the number of strains is scarce (Dziga et al., 2013) due to the difficulties in the isolation process. Some different methods for the isolation of MC-degrading bacteria have been described in the scientific literature, but they showed differences in the steps of isolation, the type of MC added (pure or mixture), the concentrations of MCs used (Manage et al., 2009b; M. Zhang et al., 2010) and in the type of the culture media employed during the process (with presence or absence of alternative carbon and/or nitrogen sources) (Lemes et al., 2008; Valeria et al., 2006; M. Zhang et al., 2010), among others. Thus, a standardization of an efficient isolation method is required.

The isolation of MC-degrading bacteria allows, in one hand, to facilitate and improve the studies of the biological degradation of MCs occurring in the environment, but also has a biotechnological potential for water treatment plants as a biological tool for removing MCs from the water. In this sense, it must be considered the influence of other organic carbon substrates present in the water, since alternative carbon sources may compete with the MCs and impair the MC degradation process (J. Li et al., 2012, 2011). Despite the importance of such aspect, few MC-degrading bacterial isolates have been cheked individually for the MC degradation efficiency in media with a wide range of TOC concentrations. As far as we know, our isolated bacterial strain *Sphingopyxis* sp. CECT8967 is the only one described

able to degrade efficiently high MC concentrations under the presence of different organic carbon loads. Therefore, it is required the development of method for the use of this bacterial strain as a biotechnology-based solution for the removal of MCs in water with different organic carbon concentrations.

5.2. Method I: A method for the efficient isolation of MC-degrading bacteria from water samples

5.2.1. Objective of the invention

The invention provides a method for the efficient isolation of MC-degrading bacteria from water bodies with episodes of harmful cyanobacterial blooms.

5.2.2. Method

The method of isolation is comprised of four different steps: sampling, enrichment assay, bacterial isolation and biodegradation assay. In order to facilitate the understanding of the method, described in detail in the patent application (Appendix), a flowchart is represented in the Figure 5.1. The lines below are a brief explanation of the steps.

To increase the success of the isolation of MC-degrading bacteria, water samples were collected from a water reservoir with a toxic cyanobacterial bloom episode (*sampling* step). For a positive selection of the MC-degrading bacteria over the total bacterial community, the water samples were enriched with MCs extracted from a toxic cyanobacterial bloom (e.g., addition of 1 mg L^{-1} of total MCs). The water samples were then incubated (e.g., at 27 ºC) for 15 days (*enrichment assay* step) and, after incubation, the MC degradation activity of the aquatic bacterial community was analysed by quantifying the remaining MCs present in the water. Those water samples with MCs concentrations below a pre-selected threshold value were transferred to a solid medium (e.g., R2A medium) and were immediately incubated (e.g., at 27 ºC) for 7 days. Bacterial colonies that were grown on the solid medium were isolated according to their morphology (*bacterial isolation* step) and were further checked for their MC degradation capacity. In this step, pure and individual colonies were incubated simultaneously in a 96-well plate with a diluted-rich medium (e.g., 4-fold diluted R2A liquid medium) with MCs (e.g., 1 mg L^{-1}) (*biodegradation assay* step). The use of a diluted-rich medium offers an input of nutrients which enhances bacterial growth and allows the metabolically active cells to degrade the MCs. Compared to previously described methods, this method allow the isolation of freshwater bacteria with different MC-degrading efficiencies.

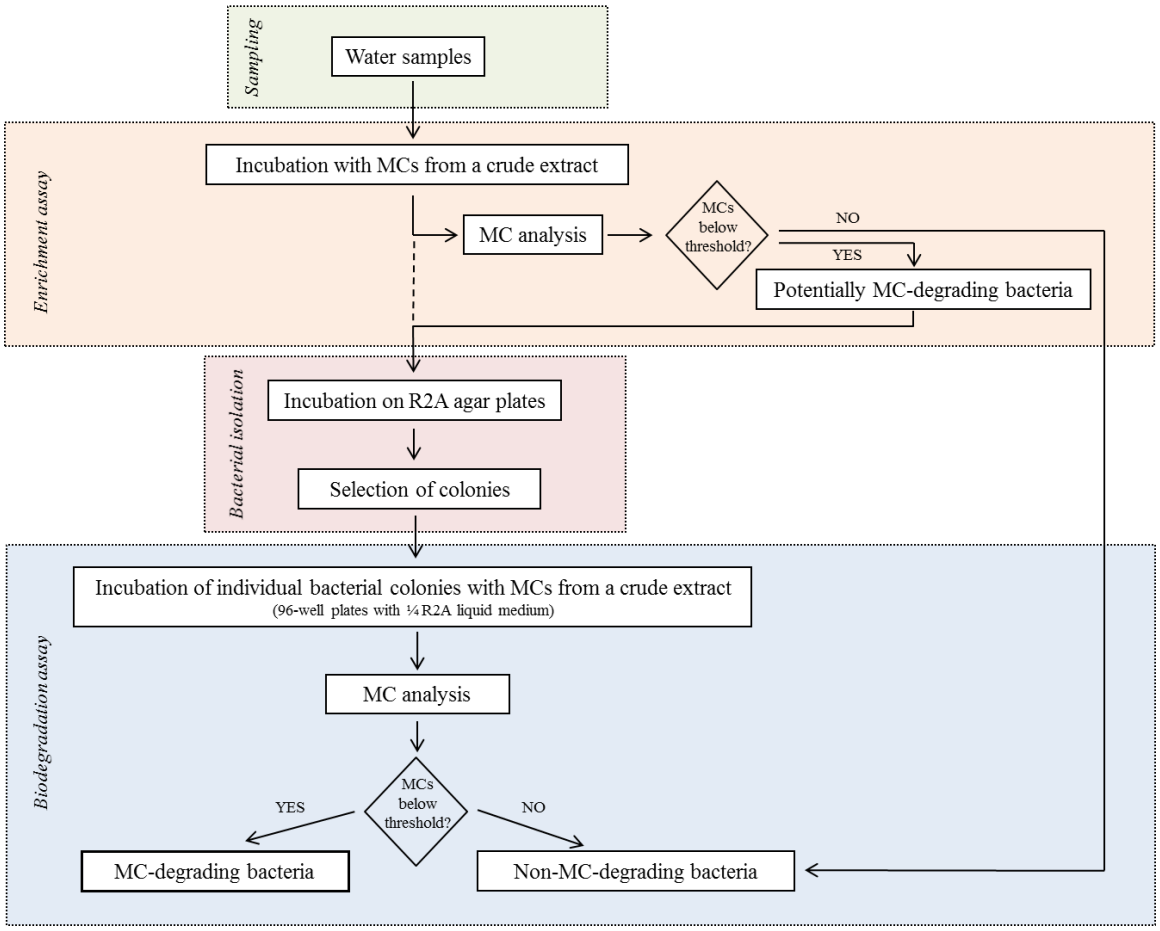


Figure 5.1. Flowchart for the screening and identification of MC-degrading bacteria isolated from water samples.

5.2.3. Results and discussion

The toxic cyanobacterial blooms and the release of toxins in freshwater systems have captured the attention and the concern of the water authorities and the scientific community, driving efforts to the removal of MCs from the water. During the last years, several studies have shown the isolation of the MC-degrading bacteria from freshwater systems and their exploitation as a low-cost and environmental-friendly solution for the MC removal (Ho et al., 2012a; Merel et al., 2013). However, the already described isolation methods vary greatly in their steps and have shown low success rates. Thus, a standardized and optimized method was required. The present invention provides an isolation method that considers the most successful steps previously described and the incorporation of new ones in order to isolate bacteria with different degradation efficiencies. As a result, a total of 24 MC-degrading bacterial strains were isolated from a reservoir during a toxic cyanobacterial bloom episode (Lezcano et al., 2016).

A general consensus is stablished for the sampling step to be performed in water bodies with a history of toxic cyanobacterial blooms. However, some differences have been found in the rest of the steps. In our method, we consider important the incubation of the water samples with an addition of high MC concentrations (*enrichment assay* step) as a pre-requisite for the successful isolation of MC-degrading bacteria. Some studies considered that this MC enrichment assay is a non-necessary step (Alamri, 2010; Ho et al., 2007; Ishii et al., 2004; Jiang et al., 2011; Maruyama et al., 2006; Park et al., 2001; Takenaka and Watanabe, 1997). However, we verified that the incubation of the water samples with MCs causes a positive selection of, at least, the fraction of the MC-degrading bacterial community with *mlr* genes, which resulted in two orders of magnitude higher compared to the initial abundance (Lezcano et al., 2016). Then, this positive selection further enhances the success of isolation of MC-degrading bacteria. Our method also consider the development of the enrichment step in the original water sample to allow the bacteria to grow without exposing them to further stress, as may occur when they are transferred to artificial media (J. Chen et al., 2010; Eleuterio and Batista, 2010; Hu et al., 2009; Okano et al., 2009; Ramani et al., 2012; Somdee et al., 2013; Valeria et al., 2006; Zhang et al., 2011). Moreover, some studies reported several additions of MCs to the water samples instead of one, in order to enhance the relative abundance of MC-degrading bacteria over the total bacterial community. However, it may cause unnecessary extension of the incubation period and higher MC consumption, thus increasing overall costs, especially when pure MCs are used (J. Chen et al., 2010; Hu et al., 2009; Lemes et al., 2008; Okano et al., 2009; Ramani et al., 2012; Somdee et al., 2013; Valeria et al., 2006; Yang et al., 2014a; M. Zhang et al., 2010; Zhang et al., 2011).

Regarding the isolation step, we consider that the transfer of an aliquot from the enrichment step to a solid rich medium is enough to find MC-degrading bacterial strains among the colonies that have grown on the plate. Some authors reported this transfer to a diluted-rich medium or a minimal medium containing only MCs to enhance the growth of those able to degrade only the toxins (J. Chen et al., 2010; Hu et al., 2009; Valeria et al., 2006; Yang et al., 2014a; Zhang et al., 2011). We think that the addition of toxins to the solid medium is not strictly necessary if a proper concentration of MCs and incubation time have been performed during the enrichment step. The bacterial transfer between liquid and solid media is an additional stress factor that may be minimized providing the necessary requirements for the bacterial growth. Moreover, nutrient shortages may result in avoiding the growth of those able to degrade the MCs in presence of additional nutrients.

Although the MC enrichment step increases the success of isolation of MC-degrading bacteria, the isolated strains may be not able to degrade the MCs and, thus, a MC degradation assay is required. Several strategies have been performed to identify these MC-degrading bacteria. In one hand, some authors incubate the potentially MC-degrading bacteria on minimal medium containing MCs (J. Chen et al., 2010; Ho et al., 2012b; Hu et al., 2009; Lemes et al., 2008; Manage et al., 2009b; Ramani et al., 2012; Somdee et al., 2013; Valeria et al., 2006; Yang et al., 2014a; M. Zhang et al., 2010), thus avoiding the growth of those unable to use MCs as a sole carbon or nitrogen source. On the other side, other authors incubate the bacteria on nutrient rich media with MCs (Alamri, 2010; Eleuterio and Batista, 2010; Ishii et al., 2004; Maruyama et al., 2006; Park et al., 2001), which is not convenient due to the possible lengthening of the lag phases that may lead to false negatives. As a result, in our method we use a diluted-rich medium (e.g., 4-fold diluted R2A medium) that provide a suitable input of nutrients to enhance the bacterial growth without disturbing the MC degradation activity and without lengthening the lag phase. Moreover, the previously described methods use large reagent volumes in the biodegradation assay step, which increases the costs and the time to perform the assay. As consequence, this step may be time-consuming when there is a large number of potentially MC-degrading isolates to check. Therefore, in our method we use a 96-well plate that allow to test simultaneously a high number of individual bacteria, reducing time and costs. A BiologMT2, a 96-well microplate used for the screening of several bacterial strains that degrades MCs as a sole carbon source, has been also described (Manage et al., 2009b). Since it is based on the colour change produced in the media when the substrate is metabolized (MC in this case), additional carbon sources cannot be used. Therefore, MC-degrading bacteria that may need additional nutrients for their optimal growth and, thus, consumption of MCs, may be considered as a false negative. To minimize the risk of losing any potentially MC-degrading bacteria, the present method consider the incubation of the isolated strains in 96-well plates containing diluted-rich medium with addition of MCs.

Besides the divergences in the methodological steps, no consensus exists in the concentration and/or type of MCs used during the enrichment, isolation and biodegradation assays. In general, the MC concentrations used in other studies ranged from 0.1 to 50,200 μg L^{-1} (Manage et al., 2009b; M. Zhang et al., 2010), being most of them pure MCs (Alamri, 2010; Eleuterio and Batista, 2010; Ho et al., 2012b; Lemes et al., 2008; Manage et al., 2009b; Maruyama et al., 2006; Park et al., 2001; Ramani et al., 2012; Somdee et al., 2013; Takenaka

and Watanabe, 1997; Valeria et al., 2006; Yang et al., 2014a; M. Zhang et al., 2010; Zhang et al., 2011) instead of a mixture of MCs extracted from a cyanobacterial bloom (J. Chen et al., 2010; Hu et al., 2009; Ishii et al., 2004). We consider that a mixture of MCs extracted from a cyanobacterial bloom is recommended to determine the ability of the bacterial strain to degrade different MC variants in a matrix similar to that found in the environment. Therefore, a MC crude extract was used along the described method, which, in addition, reduces costs compared to those that use pure toxins.

5.2.4. Conclusions

The present invention allows the isolation of a wide range of MC-degrading bacteria with different degradation efficiencies using a method that saves time and cost by reducing steps, reagents and encouraging the use of crude MCs, instead of pure MC variants, for the assays.

5.3. Method II: An efficient MC degradation method using *Sphingopyxis* sp. strain CECT 8967 in aqueous medium with different organic carbon concentrations

5.3.1. Objective of the invention

The invention provides a method for the biological treatment of MCs from an aqueous medium with a wide range of organic carbon concentrations. This method uses the bacterial isolate *Sphingopyxis* sp. strain CECT8967 able to degrade efficiently high MC concentrations, constituting a low-cost and potential tool for toxin removal.

5.3.2. Method

A bacterial strain with the ability to degrade MCs (at least, MC-LR, MC-RR and MC-YR) was isolated with the previously described method. The strain was identified as *Sphingopyxis* sp. and was further deposited in the Spanish Type Culture Collection (CECT) under the strain number CECT8967. This bacteria was equally efficient degrading the 90% of the total MCs (1 mg L^{-1} of MC-LR, MC-RR and MC-YR) in less than 6 hours regardless of the different TOC concentrations (from 0 to 258 mg L^{-1}, approx.) present in the aqueous medium. Therefore, it was considered as a good candidate for the biological elimination of MCs in water with presence of different organic carbon concentrations.

In order to facilitate the understanding of the method for the biological degradation of MCs using the bacteria *Sphingopyxis* sp. strain CECT8967 (described in detail in the patent application (Appendix)), a flowchart of the steps is represented in the Figure 5.2. An example of the time needed for the bacterial strain CECT8967 to degrade the 90% of the MC

concentrations present in different media with variable TOC concentrations is also included in the figure, as well as the time needed for the control strains *Sphingosinicella microcystinivorans* Y2 (Park et al., 2001) and *Paucibacter toxinivorans* 2C20 (Rapala et al., 2005). The lines below are a brief explanation of the steps.

The bacterial strain CECT8967 is incubated in a rich-nutrient medium (e.g., R2A medium) with an initial absorbance of 0.05 (measured at 600 nm) until the exponential phase. Cells were then centrifuged, resuspended and incubated at initial absorbance of 0.05 (600 nm) for 14 hours in a medium without organic compounds to induce nutrient starvation (e.g. MSM). At this step, bacteria is prepared to efficiently degrade high concentrations of MCs (reduced lag phases) in aqueous media with a wide range of TOC concentrations (tested conditions from 0 to 258 mg L^{-1}), which are below, in range and far above the concentrations present in water bodies intended for drinking water. The media used with the variable TOC concentrations were MSM, reservoir water and 4-fold diluted R2A medium. The use of this bacterial strain CECT8967 following the previously described steps allows to efficiently degrade MCs in water bodies intended for consumption, or for other uses that either involve an aqueous media with a wide variation of organic carbon concentrations.

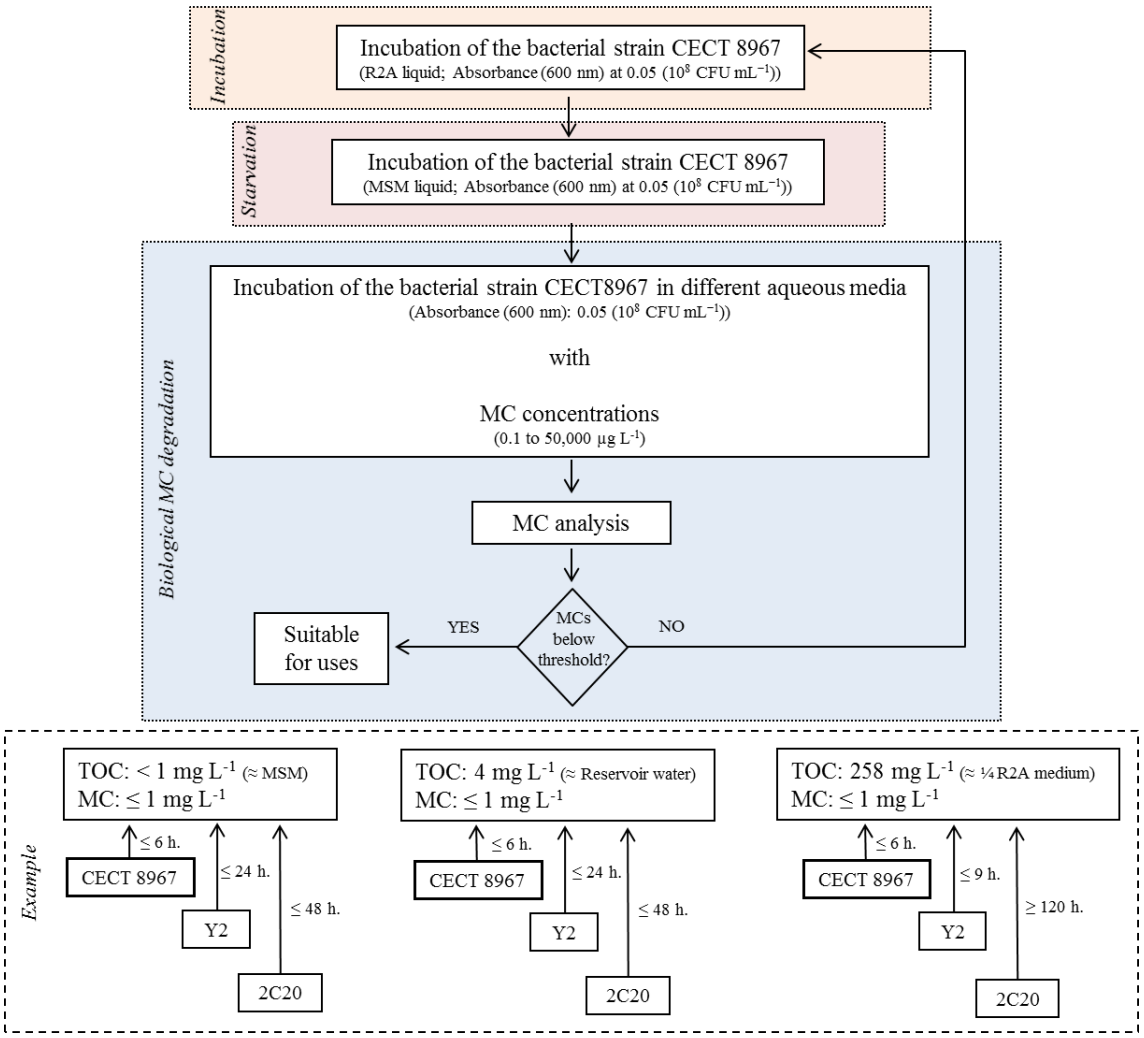


Figure 5.2. Flowchart of steps for the biological degradation of MCs present in an aqueous medium with different TOC concentrations. An example is also included to compare the time needed for the bacterial strain *Sphingopyxis* sp. CECT 8967 to degrade, at least, the 90% of the MCs concentrations (1 mg L^{-1}) present in the aqueous medium, with the time need for the controls *Sphingosinicella microcystinivorans* Y2 and *Paucibacter toxinivorans* 2C20. A detail of the results is found in Lezcano et al. (2016).

5.3.3. Results and discussion

The isolated strain *Sphingopyxis* sp. CECT 8967 reported a high potential for the degradation of MCs in an aqueous medium with different TOC concentrations. While our strain was able to degrade the 90 % of the total MC concentrations (1 mg L^{-1} of MC-LR, MC-RR and MC-YR) in less than 6 hours in all tested media, that is, a degradation rate of 166.7 µg L^{-1} h^{-1}, the control strains Y2 and 2C20 required, at least, from 9 to more than 120 hours to degrade the same concentration. Thus, the strain CECT 8967 recorded the highest MC degradation rates compared to those obtained from the control strains, and these degradation rates were similar despite the different organic carbon concentrations present in the water. A detail of

these results are found in the Table 2 from the Appendix (no replicates; n=1) and in Lezcano et al. (2016) (two replicates; n=2).

Several bacterial isolates have been checked for their MC degradation efficiencies in other studies (a compilation of MC degradation rates can be found in Dziga et al. (2013)), but the absence of a standardized method for the MC biodegradation assay is currently hindering the comparison of the biodegradation efficiencies between the isolates. The use of various media (Lemes et al., 2008; Maruyama et al., 2006; Takenaka and Watanabe, 1997), different initial MC concentrations (Manage et al., 2009b; M. Zhang et al., 2010), the application or not of a starvation period and different initial cell concentrations are examples of various conditions that may lead to confusion when comparing the MC degradation efficiencies. As an example, few studies documented the initial cell abundance used in the experiments (Eleuterio and Batista, 2010; Manage et al., 2009b; Takenaka and Watanabe, 1997), which constitutes a crucial information since the type of relationship (linear, logarithmic, etc.) that exists between the bacterial cell density and the MC concentrations, as well as the bacterial cell density and the efficiency of MCs degradation in the water, is still unknown. Thus, the addition of the double of the bacterial inoculum in the assay may extend or maintain the same lag phase but accelerate, afterwards, the MC biodegradation rates.

The partial 16S rRNA gene analysis of the strain CECT 8967 showed 100% similarity with the MC-degrading strains *Sphingopyxis* sp. C-1 (Okano et al., 2009) and *Sphingopyxis* sp. USTB05 (M. Zhang et al., 2010). The strain C-1 was reported to remove 1 mg L^{-1} of MC-LR in 3 hours, that is, a degradation rate of 333.33 µg L^{-1} h^{-1}. This efficiency is higher than the obtained by the strain CECT 8967. However, a closer look to the conditions used reveals that the strain C-1 was incubated in a 0.1 phosphate buffer (pH=6.72), was only tested to degrade pure MC-LR and the initial bacterial absorbance was 0.5 (measured at 600 nm). This means that the absorbance of the starting inoculum was 10-fold higher than the absorbance we used and the degradation assay in a buffer with a sole and pure MC-LR is not related to real life scenarios, where other nutrients and MC variants coexist in the aquatic environment during the toxic cyanobacterial bloom (Sivonen and Börner, 2008). Therefore, we suggest the use of raw MCs extracted from toxic cyanobacterial blooms rather than the use of pure toxins when testing the capacity and the efficiency of a strain for the degradation of MC in environmental water samples.

Similarly, the strain USTB05 was able to remove 50.2 mg L^{-1} of pure MC-RR in 3 days in a 50 mM potassium phosphate buffer, that is, a MC-RR degradation rate of 697.22 µg L^{-1} h^{-1} (M. Zhang et al., 2010). In addition to the above mentioned drawbacks with the use of buffers and pure MCs, the experiments were performed with cell-free extracts (CE) instead of living cells and, thus, results cannot be comparable. In fact, an experiment performed with the same strain (USTB-05) and pure MC-LR to evaluate the differences between the use of living cells and the CE showed that the CE was able to degrade the MC concentrations faster than the living cells. About 28.8 mg L^{-1} of MC-LR were degraded in 3 hours by the CE, and about 4 mg L^{-1} in 4 days by the living cells, that is, a degradation rate of 41.67 µg L^{-1} h^{-1} (Xiao et al., 2010). Although the use of living cells appeared to be less efficient, it has the advantage to work initially with low cell concentrations that increase over time when incubation conditions are favourable, allowing saving costs and time comparing with the preparation of CE. It is also worth noting that both 50.2 and 28.8 mg L^{-1} of MCs would be exceptional situations in real life scenarios, where concentrations usually ranges from less than 1 µg L^{-1} to several few mg L^{-1} (Sivonen and Jones, 1999). Experimental setup with MC concentrations near or slightly higher to what is commonly found during and after toxic cyanobacterial blooms provide better information for a MC degradation efficiency to overcome a real problem of toxicity.

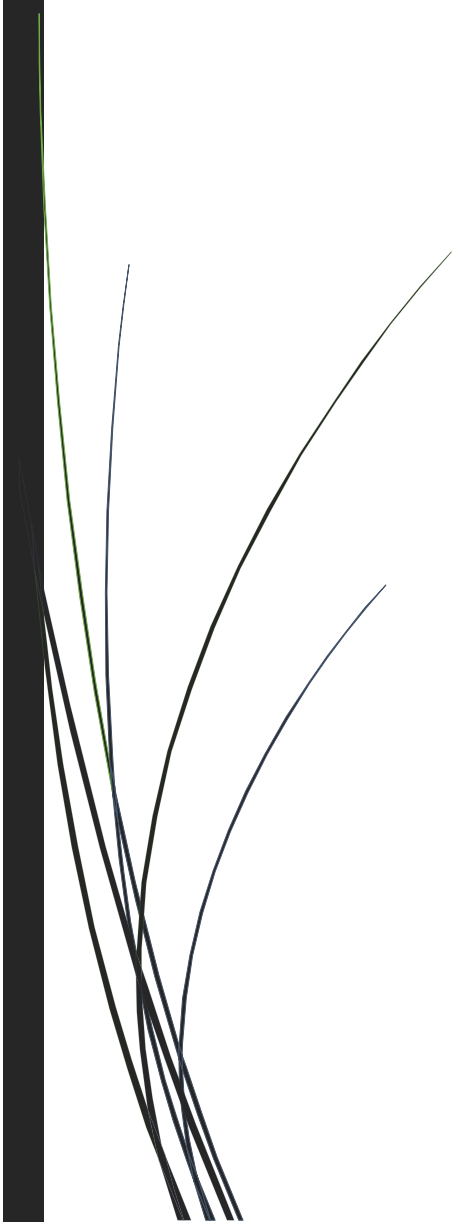
5.3.4. Conclusions

In the biological treatment of MCs, the presence of alternative carbon sources in the water may slow down or totally impair the MC degradation process by bacteria due to their different affinity to the MCs. The method described here and the use of the *Sphingopyxis* sp. strain CECT 8967, which is able to degrade high concentration of MCs regardless of a wide range of TOC concentrations present in the aqueous media, overcomes this situation. Therefore, the high capacity of the strain CECT 8967 to degrade different MCs variants from a cyanobacterial crude extract makes this strain as a promising technology-based solution to remove MCs from the aquatic environment during a toxic cyanobacterial bloom. Finally, the description of a method for the removal of MCs enables the direct exploitation of the bacterial strain and the easy comparison with other bacterial isolates from other laboratories.

5.4. Acknowledgements

We would like to thank Antonio Quesada for his valuable comments and assistance.

Chapter 6



6. General discussion

This thesis comprises a set of studies that goes from the assessment of a biological MC degradation process in a reservoir from central Spain, to deepen into the genetic characteristics, diversity of the responsible bacteria, interaction between MC producers and degraders and the ecological significance of this process in the environment. The thesis also includes an enhanced method for the isolation of MC-degrading bacteria to encourage and facilitate research in this field considering the scarce number of existing isolates around the world. Moreover, and regarding the increasing frequency of toxic cyanobacterial blooms in water bodies intended for drinking-water, this thesis also aims to bring a method for an efficient degradation of MCs using the bacterial isolate CECT8967 as an environmental-friendly and cost-efficient tool for the removal of MCs in the water.

Thus, in this section, all findings are synthetized and discussed to explore the scope of this thesis, as well as a discussion of some new observations glimpsed from the results.

6.1. MC-degrading capacity and efficiency of freshwater bacterial communities

The increasing frequency of toxic cyanobacterial proliferations due to eutrophication (Paerl et al., 2011), and the consequent harmful effects on humans and other living beings, seem to be reduced by the capacity of a MC-degrading bacterial community that degrade efficiently the MC concentrations present in the water. This MC-degrading bacterial community is comprised by strains with presence and absence of *mlr* genes, and appeared to contribute together to the overall MC degradation in the environment. The isolation of MC-degrading bacteria lacking *mlr* genes (Chapter 2) and the lack of relationship between the abundance of *mlr*A gene and the efficiency of *in vitro* MC degradation in reservoir water samples (Chapter 4), supports the significant contribution of the *mlr*$^{-}$ bacterial community to the degradation of MCs in the environment.

During cyanobacterial blooms, MCs are accompanied by high concentrations of other organic carbon and nitrogen compounds (Søndergaard et al., 2000). Previous studies performed on the influence of these compounds over the MC degradation efficiency were only focused on *mlr*$^{+}$ bacterial isolates (Ishii et al., 2004; Surono et al., 2008) or on the whole bacterial community from water or biofilms (Christoffersen et al., 2002; J. Li et al., 2011), ignoring the possible presence of *mlr*$^{-}$ bacteria. Our results showed that both genotypes exhibit dissimilar MC degradation efficiencies and, thus, the proportion of each genotype

within the total bacterial community may determine the overall MC degradation rates and also explain the different results observed between studies performed on different water, sediment or biofilm samples (Christoffersen et al., 2002; Eleuterio and Batista, 2010; J. Li et al., 2011). While *mlr*$^{+}$ bacteria enhances the efficiency of MC degradation in presence of alternative carbon and nitrogen compounds, by either increasing their abundance (Zhang et al., 2015) and/or by increasing the expression of the *mlr* gene cluster (Li et al., 2014), the *mlr*$^{-}$ bacteria showed lower efficiency. This may point towards a possible substrate competition with the MCs through the alternative-*mlr* MC degradation pathway(s) (Chapter 2).

Despite the reported constitutive expression of the *mlr* gene cluster (Bourne et al., 2001; Jiang et al., 2011) and the high MC degradation efficiency observed in *mlr*$^{+}$ bacteria under laboratory conditions, the detection of *mlr* genes in the environment do not ensure an occurring MC degradation activity. The preceding presence of toxic cyanobacteria and MCs concentrations in the field seem to be also an important factor triggering the bacterial MC degradation process by either activating the responsible genes (Jiang et al., 2011) and/or by changing the bacterial community composition so that the abundance of the MC-degrading bacteria (*mlr*$^{+}$ or *mlr*$^{-}$) increases. Thus, the capacity of the bacterial community to degrade de MCs appear to be dependent and closely linked with the toxic cyanobacterial bloom period (Chapter 4).

6.2. Integrating the bacterial MC degradation capacity in water monitoring strategies

As observed with all the aforementioned, the variations in the MC concentrations reported in the water during the cyanobacterial bloom episode are not only explained by the MC-producing cyanobacteria and the seasonal dynamics of their chemotypic subpopulations (Agha et al., 2014; Rohrlack et al., 2008), but also on the MC-degrading bacterial community and its efficiency of degradation. Therefore, the relative abundance of each MC-degrading genotype (*mlr*$^{+}$ or *mlr*$^{-}$) within the bacterial community, the concentration of alternative carbon and nitrogen compounds, as well as the MC pre-exposure in the environment are all important factors modulating the half-life of MC concentrations in nature. Therefore, it would be interesting to incorporate the assessment of the MC degradation capacity and efficiency of the aquatic bacterial community associated to toxic cyanobacterial blooms to water management plans.

So far in Spain, current monitoring programs for official bathing sites are only focused on cyanobacteria and cyanotoxins, ignoring the contribution of the bacterial communities to degrade the MCs. Regarding water bodies intended for consumption, MCs are only analysed at the exit of the drinking-water treatment plants if eutrophication is observed at the water uptake. In both cases, water management strategies only consider the MC producers and their toxins, passing over the biological degradation losses and, thus, being able to fall in an over or underestimation of the problem. As consequence, decision-making may lead to inefficient and cost-intensive management measures. An example for a monitoring strategy incorporating the analysis of the MC degradation capacity and efficiency of the aquatic bacterial community is explained in Figure 6.1. Further study for their validation and suitability would be required.

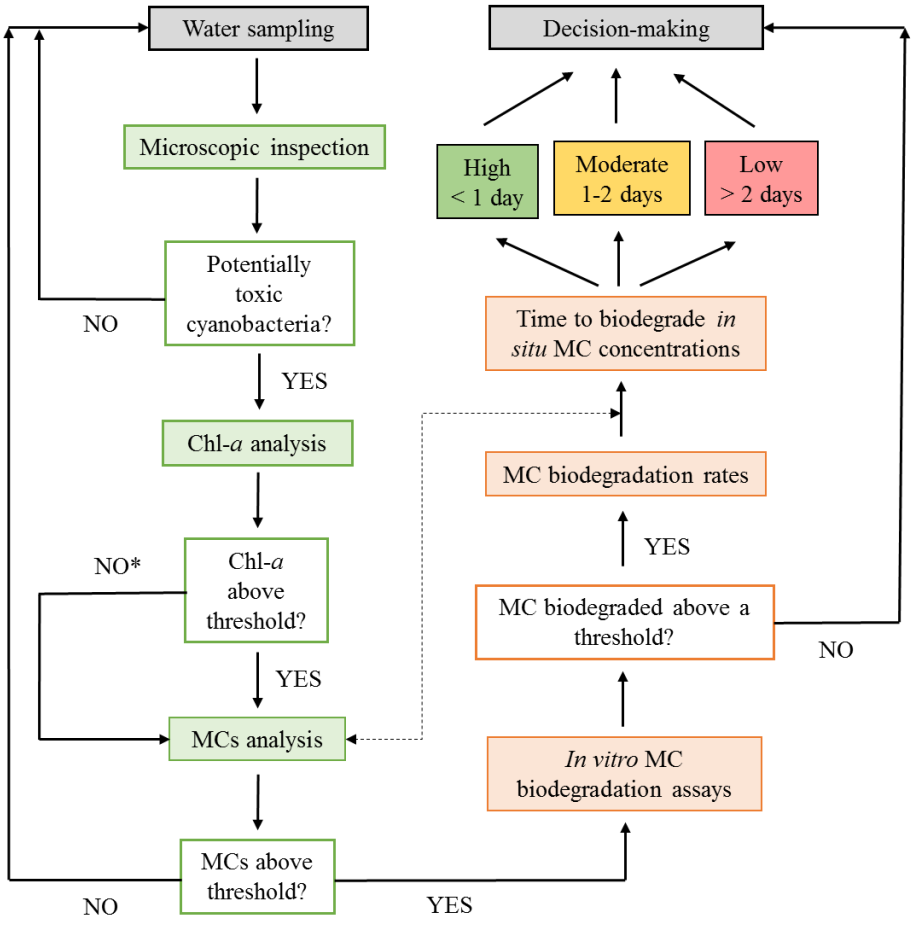


Figure 6.1. Flowchart representing a proposal for a monitoring program considering both MC-producing (green) and MC-degrading (orange) communities for a proper decision-making in water management plans. The decision-making is based on three categories that represents high, moderate and low MC degradation capacities of the aquatic bacterial community. The asterisk indicate that a MC analysis is strongly recommended despite chl-*a* concentrations are below the threshold. This decision is based on the reported variations in the MC content during bloom development and, thus, on the ununiformed MC:Chl-*a* ratios (Chapter 4).

In the proposed monitoring program, the presence or absence of potentially toxic cyanobacteria in water samples is firstly identified under the microscope (Komárek et al., 2014; Kómarek and Anagnostidis, 1999). Then, chl-*a* analysis are performed in those water samples potentially toxic. Irrespective of whether the chl-*a* concentrations exceed or not a specific threshold value (stablished according to the national or local guidelines), analysis of MCs are recommended due to the common variations reported in the MC content during bloom development and, thus, on the ununiformed MC:Chl-*a* ratios (Agha et al., 2014; Sønstebø and Rohrlack, 2011; Chapter 4). After the MC analysis, samples above a certain threshold value (e.g. 1 μg L^{-1} of MCs for drinking-water in most European countries (Chorus, 2012)) are recommended to pass by an *in vitro* MC biodegradation assay to check the capacity of the aquatic bacterial community to degrade the MC concentrations present in the water. The length and the conditions used for the bioassays are crucial for further decision-making process and, accordingly, should be well-established. In this monitoring program, a two-day assay is proposed to be more conservative and avoid lengthening the decision over time. Dark conditions (to avoid growth of photosynthetic organisms) and the use of the mean temperature from the water reservoir at the sampling season are also encouraged. Then, a determination of the MC biodegradation rates would be useful to calculate the time needed for the bacterial community to degrade the MC concentrations detected in the field. Three categories of biological MC degradation capacity are established: "high" (< 1 day to degrade the MC concentrations), "moderate" (1-2 days) and "low" (> 2 days). These categories still need to be established according to more *in situ* studies on MC life-times in the environment.

Although the incorporation of the MC biodegradation capacity to monitoring programs may be interesting for the better understanding of the aquatic system and to take better decisions, the proposed monitoring program has also some limitations. Even though the biological degradation is a relevant process in the environment, physical and chemical degradation (Wörmer et al., 2011, 2010) also contribute to the elimination of MCs in nature. Moreover, the assessment of the MC biodegradation capacity gives information about the potential degradation of the bacterial community in an immediate future, but not at the moment of the sampling, as occur with the information of chl-*a* and MC concentrations. Indeed, the MC biodegradation rates are calculated under laboratory conditions and, thus, may be far from the reality if physicochemical conditions are not well established. Troubleshooting such limitations would require a good knowledge of the characteristics of the aquatic system and both the biotic and abiotic interactions occurring in nature.

Notwithstanding, and despite the aforementioned drawbacks, the information of the MC biodegradation capacity of the aquatic bacterial community may complement the information obtained from the cyanobacteria and their toxins and, thus, lead to better water management decisions.

6.3. MC-degrading bacterial diversity: possible origin and evolution of the MC biodegradation

Toxic cyanobacterial proliferations coexist and exert different interactions with a wide array of bacteria in the aquatic environment. Among these bacteria, those able to degrade the MCs possess an important role, at least from an anthropocentric point of view, of detoxifying MCs in the natural environment. Thus far, several diversity studies were performed on the bacterial assemblage associated to toxic cyanobacterial blooms (Dinasquet et al., 2013; H. Li et al., 2012; K. Shao et al., 2014) and on the potentially MC degraders that responded to an addition of MCs in laboratory experiments (Mou et al., 2013). However, a lack of information existed regarding the diversity of both fractions of the MC-degrading bacterial community (*mlr*$^+$ and *mlr*$^-$) during a naturally occurring toxic cyanobacterial bloom. This thesis showed that the diversity within the *mlr*$^+$ bacterial community associated to a toxic cyanobacterial bloom was mainly represented by the families *Sphingomonadaceae* (genera *Novosphingobium* sp., *Sphingomonas* sp. and *Sphingopyxis* sp.) and *Xanthomonadaceae* (genera *Stenotrophomonas* sp.), which appeared to coexist even when there is no development of cyanobacterial blooms in the water body (Chapter 3). On the contrary, other bacteria lacking *mlr* genes with a recognized ability of degrading complex and/or xenobiotic compounds appeared more related to toxic cyanobacterial blooms and to MCs concentrations than the *Sphingomonadaceae* and *Xanthomonadaceae* families (Chapter 3). This supports the hypothesis of non-*mlr* MC degradation pathways operating in nature (Chapter 2; Manage et al., 2009) and suggests the possible implication of genes for the xenobiotic catabolism in the degradation of MCs (Chapter 3; Mou et al., 2013). Further studies on the characterization of the alternative-*mlr* degradation pathway(s) would be necessary to confirm this hypothesis.

The possible presence of more than one pathway for the degradation of MCs, and the presence of MC degradation capacity in bacteria from different phyla, raises questions about the origin and evolution of the MC degradation process. Although no specific studies have been developed in this sense, the results from this thesis allow to make some speculations. In one hand, the information gathered about the MC-degrading bacterial strains isolated around the world (Table 1.2) and the high-throughput sequencing study performed on the

*mlr*A gene (Chapter 3) showed that the *mlr* gene cluster is not restricted to the order *Sphingomonadales*, as previously thought, but spread on different families, orders and even phyla (*Proteobacteria* and *Firmicutes*). On the other hand, if we attend solely to the bacterial MC degradation capacity regardless of the presence of the *mlr* genes, the bacterial diversity with MC degradation capacity is even higher, including the phylum *Actinobacteria*, and suspected to include other bacterial phyla that are both associated with toxic cyanobacterial blooms and related to the xenobiotic degradation (Mou et al. (2013), Chapter 3). The phylogenetic tree constructed with the isolated MC-degrading strains IM-1 - IM-4, and other bacteria with and without the ability to degrade MCs (Figure 2.3), confirmed that this capacity is present in phylogenetically distinct bacteria. Thus, the acquisition of the genes responsible for the degradation of MCs (at least the *mlr* gene cluster) through horizontal genetic transfer appeared as the most plausible explanation, instead of a vertical transmission of genes from the "parent" to the "offspring". In fact., the horizontal genetic transfer has been reported as an important mechanism for the dispersion of antibiotic resistance (Touchon et al., 2017) and the degradation of xenobiotic compounds (Nojiri et al., 2004) as a rapid adaptation to the changing environmental stimuli. The coexistence of multitude of microorganisms during the cyanobacterial proliferations, including the presence of virus (Van Wichelen et al., 2016), which have been described as important gene transfer agents, supports the horizontal genetic transfer as a possible mechanism to distribute the genes responsible for the degradation of MCs between bacteria in the aquatic environment.

6.4. Ecological relationship between MC producers and degraders

The success and dominance of cyanobacterial blooms have been related to a combination of bottom-up (e.g., temperature, nutrients, etc.) and top-down control factors (grazing, parasitism, etc.) (Steffen et al., 2015). Since cyanobacterial blooms have been considered as relatively poor food sources due to the lack of essential compounds for zooplankton nutrition, such as sterols and polyunsaturared fatty acids (Gulati and Demott, 1997; Müller-Navarra et al., 2000), most of the attention over the factors that may control cyanobacterial blooms and their toxicity have been largely focused on the physicochemical properties of the aquatic environment (Kaebernick et al., 2000; Monchamp et al., 2014; Sivonen, 1990). On the other hand, the interest on the interaction of cyanobacteria with other microorganisms is gaining importance and the results are giving to the biotic factors a crucial role in the cyanobacterial success and bloom collapse (Gerphagnon et al., 2015; Van Wichelen et al., 2016). The results obtained in this thesis also point towards this direction (Chapter 4). The

physicochemical factors measured did not influence significantly to neither the seasonal dynamics of the toxic cyanobacteria nor the *mlr*$^+$ bacterial community. However, both seasonal dynamics appeared closely linked, with an increase in the relative abundance of the *mlr*$^+$ bacteria just after the increase in the relative abundance of the toxic cyanobacteria and the MC concentrations. This suggests that the presence of cyanobacteria and MCs favours the growth of the *mlr*$^+$ MC-degrading bacteria, as also reported in Zhu et al., (2014), and the observed decline of toxic cyanobacteria when *mlr*$^+$ bacteria reached its maximum, and vice versa, may as well point towards an antagonist interaction between both communities that requires further research (Chapter 4).

In the diversity study developed in the reservoir during the year 2014, between the 48-67% (except June 12, with a 9%) of the total *mlr*$^+$ bacterial community was represented by an *mlr*A-homolog that is, at least, 95% similar to the strain *Stenotrophomonas* sp. EMS (Chapter 3). Due to its highest dominance, this *mlr*A-homolog was the main responsible for the seasonal dynamic of the total *mlr*$^+$ bacterial community (Figure 6.2). The diversity study within the 16S rRNA genes also confirmed the presence of the family *Xanthomonadaceae*, in which the genus *Stenotrophomonas* is classified, supporting the possible presence of this genus within the MC-degrading bacterial community (Chapter 3).

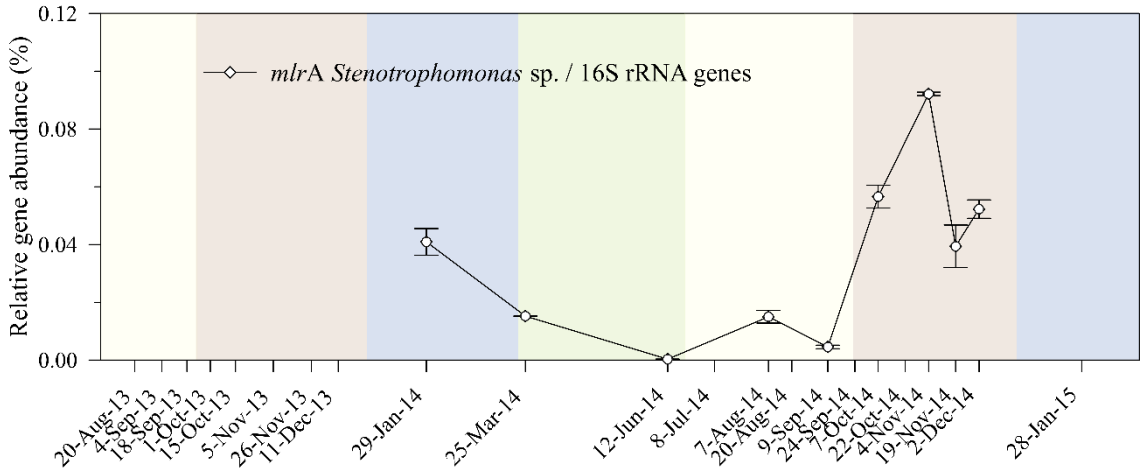


Figure 6.2. Seasonal dynamics of the relative abundance (%) of the *mlr*A genes from the most abundant OTU during the sampling year of 2014 (at least, 95 % similarity with *Stenotrophomonas* sp. strain EMS) over the total bacterial community (16S rRNA genes). This relative abundance is calculated considering the percentage that represents the specific *Stenotrophomonas* sp. EMS over the *mlr*$^+$ bacterial community (Chapter 3) and the percentage that represents the *mlr*$^+$ bacterial community over the total bacterial community (based on general 16S rRNA genes) (Chapter 4).

The genus *Stenotrophomonas* sp. has been reported in a wide range of habitats, including animals and plant hosts (Hayward et al., 2010), as well as associated to toxic cyanobacterial blooms (Mou et al., 2013). Some isolated *Stenotrophomonas* strains have been also reported as degraders of MCs (J. Chen et al., 2010; Yang et al., 2014b) and producers of fungicidal (Dunne et al., 2000) and algicidal compounds that cause lytic activity in both marine and freshwater cyanobacteria (Lin et al., 2016; Osman et al., 2017). Therefore, considering the dominance of the *mlr*A-homolog to *Stenotrophomonas* sp. within the *mlr*$^+$ bacterial community, and the apparent antagonistic relationship exhibited between the *mlr*$^+$ bacteria and the toxic cyanobacterial communities in the environment (Chapter 4), it may point towards a possible algicide activity of this *mlr*$^+$ bacteria on the toxic cyanobacteria.

The algicides produced by the cyanolytic bacteria entail structural and functional protein degradation, increase the permeability of the membranes, induce the production of intracellular reactive oxygenic species (ROS) and decrease the antioxidase activity of the cells (Cai et al., 2016; Guo et al., 2015), thus leading to suppression and even termination of cyanobacterial blooms (Osman et al., 2017). In this possible scenario, the *mlr*$^+$ bacteria would play an active role on the bloom lysis and subsequent degradation of MCs (micropredation), rather than to exhibit a comensalistic interaction in which only decompose the released MCs by exogenous causes. Previous studies performed with cyanolytic bacteria from the family *Cytophagaceae* and the cyanobacteria *Anabaena* sp. and *Synechococcus* sp. showed a maximum lytic bacterial abundance coincident with a decline in the abundance of cyanobacteria, suggesting a key role in their bloom decline (Rashidan and Bird, 2001). The lysis of cyanobacterial cells by the lytic bacteria have been usually attributed to nutrient shortage in the environment to cause the release of nutrients that may be further used by the bacteria for their own growth (Rashidan and Bird, 2001), which supports the possible cyanolytic activity and the subsequent degradation of MCs by the *mlr*$^+$ bacteria.

Considering all this, some *mlr*$^+$ MC-degrading bacteria may be newly added to the so far long catalogue of microorganisms potentially terminators of cyanobacterial blooms (Gerphagnon et al., 2015; Van Wichelen et al., 2016). Notwithstanding, further studies would be required to explore the feasibility of this hypothesis since other microorganisms coexist and interact with the cyanobacteria in the environment. Thus far, it remains as a possible scenario that may explain a more sophisticated ecological interaction among MC producers and degraders in freshwater environments.

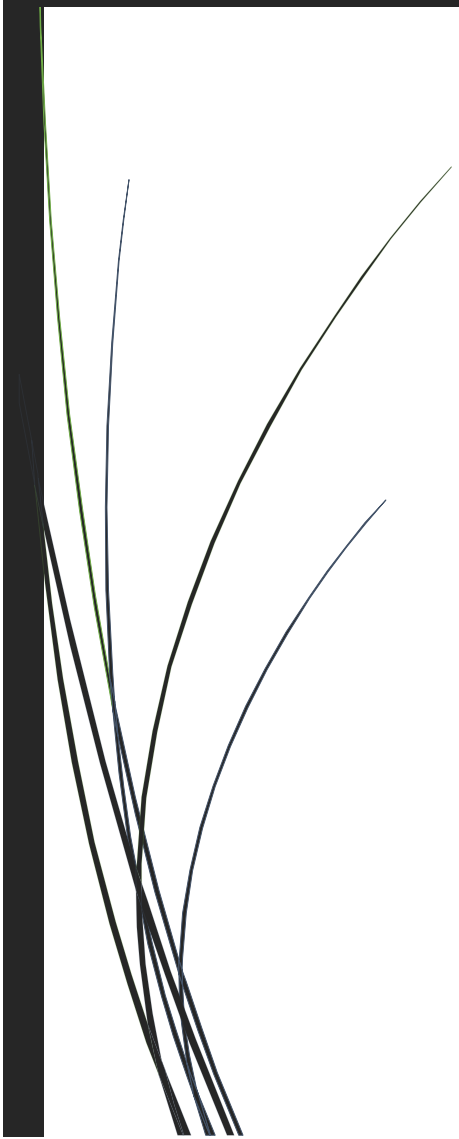
6.5. Isolation of MC-degrading bacteria and their potential in water treatment technologies

Most of the aforementioned results and discussion of this thesis would not have been possible without a previous optimization of a method for the isolation of MC-degrading bacteria from the aquatic environment (Chapter 5). Since the isolation of the first MC-degrading bacteria in 1994 (Jones and Orr, 1994), a relative small number of strains (about 45) have been isolated around the world (Table 1.2). This low number has hampered the studies of the MC biodegradation processes and their further application as a biological tool for the removal of MCs in the water. The lack of a standardized procedure with optimized conditions for the isolation of this group of bacteria was the main constraint. Previous isolation methods differed in the procedure: used different culture media (with or without additional carbon and/or nitrogen sources) (Lemes et al., 2008; Valeria et al., 2006; M. Zhang et al., 2010), a wide range of MC concentrations (from 0.1 to 50,200 µg L^{-1}) (Manage et al., 2009a; M. Zhang et al., 2010) and different type of MCs (pure or extract) (Ho et al., 2012b; Ishii et al., 2004; Lemes et al., 2008), among other differences, thus leading to different success rates, duration and costs of the isolation process. The method described in this thesis enhances the isolation of MC-degrading bacteria by performing an enrichment step in which the addition of 1 mg L^{-1} of MCs (crude cyanobacterial extract) to the water samples increased in two orders of magnitude the initial abundance of this specific bacterial community (Chapter 2 and 5). This enrichment step lead to a positive selection and increased, ultimately, the isolation success. Moreover, the screening of the selected colonies for their capacity to degrade MCs was performed by doing multiple MC biodegradation assays simultaneously on a 96-well plate amended with MCs (crude extracts), which allow to save up time and costs. This method allowed the isolation of 24 MC-degrading bacterial strains from the water reservoir with different MC degradation efficiencies (Chapter 2). In comparison with previous methods, this one allowed a higher success rate (Table 1.2 and references therein).

The high MC degradation efficiency exhibited by the isolate *Sphingopyxis* sp. CECT8967, regardless of the different concentration of total organic carbon present in the medium (tested conditions from 0 to 258 mg L^{-1}), makes this strain interesting from a biotechnological point of view (Chapter 5). Conventional water treatment technologies (e.g. chlorination, flocculation, sand filtration, etc.) have shown limited effect on the removal of MCs (Chow et al., 1998; Himberg et al., 1989; Keijola et al., 1988). On the other side, advanced oxidation

treatments (e.g. ozonation, titanium dioxide, etc.) are highly efficient (Pantelic et al., 2013 and references therein), but more expensive and not always present in water treatment facilities. Therefore, the use of the biodegradation capacity of the bacterial strain CECT8967 emerges as a cost-efficient and a more environmental-friendly option for water treatment technologies for the removal of MCs. Previous isolated strains, such as *Sphingopyxis* sp. USTB05 and C-1, were also reported as high efficient for the degradation of MCs, but they were not tested under the presence of alternative carbon compounds in the water. Moreover, the conditions used for the degradation of MCs were not related to real-life scenarios. As an example, incubations were performed in phosphate or potassium phosphate buffers, with pure and single MC variants and, in the case of the strain USTB05, using cell-free extracts instead of living cells (Okano et al., 2009; M. Zhang et al., 2010) (discussed in Chapter 5). Even though the absence of a standardized method makes the comparison of MC biodegradation efficiencies between strains more difficult, the *Sphingopyxis* sp. CECT8967 represents a suitable and efficient bacterial strain to be used in the water treatment technologies for the removal of MCs. The adaptation of available technology from water treatment plants to the use of efficient MC-degrading bacteria (Bourne et al., 2006; Ho et al., 2006; Wang et al., 2007) would be as an alternative option to both the use of chemicals and more expensive treatments to fulfil requirements from national legislations.

Chapter 7



7. Conclusions

1. The bacterial communities associated with the toxic cyanobacterial blooms in San Juan reservoir showed MC biodegradation capacity. The optimization of a method for the screening and isolation of MC-degrading bacterial strains allowed to improve the isolation process. Twenty four bacterial strains with dissimilar degradation efficiencies and two different genotypes (with and without *mlr* genes; *mlr*$^+$ and *mlr*$^-$ genotypes, respectively) were isolated from the reservoir.

2. The presence or absence of *mlr* genes in the isolates appeared as an important factor for their MC biodegradation efficiency in presence of alternative carbon and nitrogen compounds in the medium. In such conditions, the *mlr*$^+$ bacteria showed higher MC degradation rates than the *mlr*$^-$ bacteria. Then, the MC biodegradation efficiency of the aquatic bacterial community and, ultimately, the MC lifetimes in the water bodies depend, at least, in the relative abundance of each *mlr*$^+$ and *mlr*$^-$ bacterial genotype and the nutrient concentrations present in the aquatic environment.

3. The high MC biodegradation efficiency of the bacterial isolate *Sphingopyxis* sp. CECT8967, regardless of the total organic carbon concentrations present in the medium (from 0 to 258 mg L^{-1}), makes this strain suitable as a biological tool for the removal of MCs from water. A method was optimized for the efficient use of this strain for the removal of MCs.

4. The diversity study within the *mlr*$^+$ bacterial community present in the reservoir showed an *mlr*A gene variability and a representation of the families *Sphingomonadaceae* and *Xanthomonadaceae*. Apart from them, other bacterial taxa lacking *mlr* genes, with a described ability in the degradation of xenobiotic compounds, were more related to the toxic cyanobacterial bloom dynamics and MC concentrations than the *mlr*$^+$ bacterial community. This suggests a possible contribution of alternative-*mlr* MC degradation pathways in nature.

5. The seasonal dynamic study performed on the MC-producing cyanobacteria and the *mlr*$^+$ MC-degrading bacterial communities in the reservoir highlighted an important interaction between each other. The lack of significant relationship between the seasonal dynamics of both communities and the most relevant physicochemical factors from the aquatic environment also supports this observation. Moreover, the highest relative abundance of the *mlr*$^+$ bacteria coincident with the decrease in the

relative abundance of the toxic cyanobacteria suggest a possible antagonistic relationship between the two communities that needs further research.

6. The dominance of an *mlr*A gene homolog, at least 95% similar, with a strain of the genus *Stenotrophomonas* sp. within the mlr^+ bacterial community, and the described algicidal activity of several strains of this genus, support the possible antagonistic relationship observed between the MC-producing cyanobacteria and the mlr^+ bacterial communities in the environment. This would outline a possible involvement of some mlr^+ bacteria in the bloom lysis and subsequent degradation of MCs rather than only the decomposition of the released MCs.

7. The MC degradation capacity of the bacterial community was only detected during the toxic cyanobacterial bloom episodes. This highlights the importance of the pre-exposure to MCs in nature by triggering a bacterial community shift (increasing the mlr^+, mlr^- or both MC-degrading bacterial fractions) and/or by activating the *mlr* genes. The identification of the *mlr*A gene during the inter-bloom period and the absence of MC biodegradation activity indicate that the gene *mlr*A should only be considered as a marker to detect potentially MC-degrading bacteria.

8. Given the capacity of the aquatic bacterial community to degrade the MCs present in the environment, the oscillations observed in the MC concentrations are not only explained by the relative abundance and seasonal dynamics of the MC-producing cyanobacterial strains, but also by the seasonal dynamics and efficiency of the MC-degrading bacterial community. Monitoring strategies that incorporates the assessment of the MC degradation capacity and efficiency of the aquatic bacterial community could represent a new criterion to improve water management decision-making.

Conclusiones

1. La comunidad bacteriana asociada a las proliferaciones de cianobacterias tóxicas del embalse de San Juan mostró capacidad de degradación de MC. La optimización de un método para la búsqueda y aislamiento de dichas bacterias degradadoras permitió mejorar el proceso de aislamiento. Se aislaron un total de veinticuatro cepas bacterianas con diferentes tasas de degradación y con dos genotipos diferentes (con presencia y ausencia de los genes *mlr*; genotipos *mlr*$^{+}$ y *mlr*$^{-}$, respectivamente).

2. La presencia o ausencia de los genes *mlr* en las bacterias aisladas aparece como un factor importante en la eficiencia de degradación de MC cuando otros compuestos de carbono y nitrógeno están presentes en el medio. En dichas condiciones, las bacterias del genotipo *mlr*$^{+}$ mostraron mayores tasas de degradación de MC que las bacterias del genotipo *mlr*$^{-}$. Por tanto, la eficiencia de degradación de MC de la comunidad bacteriana y, en última instancia, la vida media de dichas toxinas en la masa de agua depende, al menos, de la abundancia relativa de las bacterias con los genotipos *mlr*$^{+}$ y *mlr*$^{-}$, así como de las concentraciones de nutrientes presentes en el medio acuático.

3. La alta eficiencia de degradación de MC de la bacteria aislada *Sphingopyxis* sp. CECT8967, independientemente de la concentración de carbono orgánico total presente en el medio (desde 0 a 258 mg L^{-1}), la convierte en una herramienta biológica adecuada para la eliminación de MC del agua. Se optimizó un método para el uso de esta cepa en la eliminación de dichas toxinas del agua.

4. En el estudio de diversidad de la comunidad bacteriana con el genotipo *mlr*$^{+}$ presente en el embalse se observó una variabilidad del gen *mlr*A y se identifcaron las familias *Sphingomonadaceae* y *Xanthomonadaceae*. Además de estas familias, otros taxones bacterianos con ausencia de los genes *mlr* (genotipo *mlr*$^{-}$) y con una habilidad descrita para degradar compuestos xenobióticos, mostraron mayor relación con la dinámica temporal de las cianobacterias tóxicas y las concentraciones de MC que la comunidad bacteriana con el genotipo *mlr*$^{+}$. Esto sugiere una posible contribución de otras rutas de degradación de MC diferentes a las vinculadas con los genes *mlr*.

5. El estudio de la dinámica estacional de las comunidades de cianobacterias productoras de MC y de las bacterias del genotipo *mlr*$^{+}$ degradadoras de dichas

toxinas presentes en el embalse, mostró una importante interacción entre ambas comunidades. La ausencia de relaciones significativas entre las dinámicas temporales de ambas comunidades y los factores fisicoquímicos más relevantes del medio acuático apoya también esta observación. Además, la coincidencia en los picos de abundancias relativas de la comunidad bacteriana con el genotipo *mlr*$^{+}$ y la disminución en las abundancias relativas de la comunidad de cianobacterias tóxicas sugiere la posibilidad de una relación de antagonismo entre ambas comunidades que requiere mayor investigación.

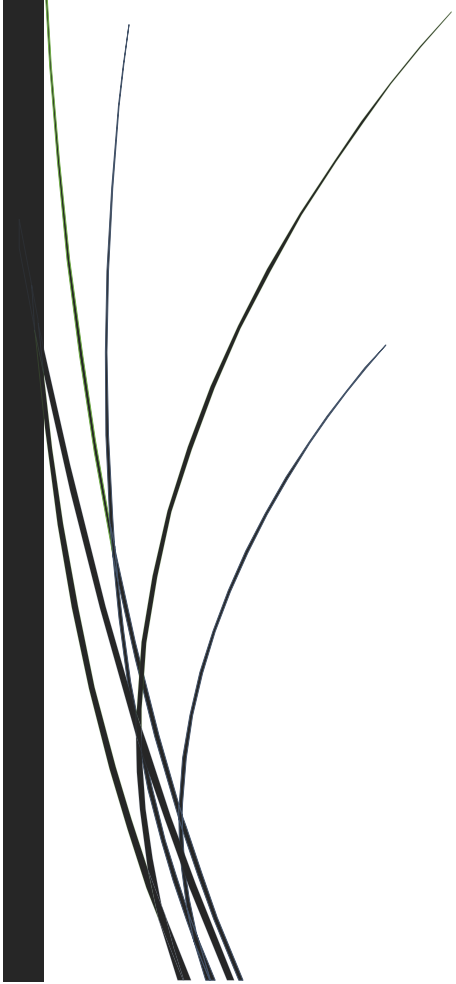
6. La representación mayoritaria de un gen *mlr*A homólogo (al menos en un 95% de similitud) con el de una cepa del género *Stenotrophomonas* dentro de la comunidad bacteriana *mlr*$^{+}$, y la actividad algicida descrita en algunas cepas de este mismo género, apoya la posibilidad de una relación antagonista entre las comunidades de cianobacterias productoras y bacterias degradadoras de MC en el medio ambiente. Este hecho podría describir un posible papel de terminación de proliferaciones de cianobacterias y posterior degradación de MC en algunas bacterias degradadoras con el genotipo *mlr*$^{+}$, en lugar de únicamente una actividad de descomposición de MC.

7. La capacidad de degradación de MC de la comunidad bacteriana sólo fue detectada durante los episodios de proliferaciones de cianobacterias tóxicas. Esto indica la necesidad de una preexposición a las MC en el medio ambiente para desencadenar un cambio en la comunidad bacteriana (aumentando las bacterias degradadoras con el genotipo *mlr*$^{+}$, *mlr*$^{-}$ o ambas) y/o en la activación los genes *mlr*. La identificación del gen *mlr*A también en periodos en ausencia de proliferaciones masivas de cianobacterias y sin detección de actividad biodegradadora de MC, indica que el gen *mlr*A sólo debe ser considerado como marcador genético de comunidades bacterianas potencialmente degradadoras de MC.

8. Dada la capacidad de la comunidad bacteriana para degradar MC presentes en el medio acuático, las variaciones observadas en la concentración de MC en el embalse no sólo están explicadas por las abundancias relativas y la dinámica estacional de las cepas de cianobacterias productoras de MC, sino también por las de la comunidad bacteriana degradadora de dichas toxinas. Por tanto, la incorporación en las estrategias de monitorización de una evaluación de la capacidad y eficiencia de

degradación de MC de la comunidad bacteriana podrían ser consideradas un criterio más útil para la toma de decisiones en la gestión del agua.

Chapter 8



8. Further work

In this section, other experiments and preliminary results initiated during the thesis and still ongoing are described. The study presented here started during my stay at the Department of Biosciences in Åbo Akademi University (Turku, Finland) under the supervision of Dr. Sonja Nybom and Dr. Jussi Meriluoto. This stay was possible thanks to a grant from the Short Term Scientific Missions (STSM) program from the CYANOCOST project (COST ES 1105).

8.1. Background

In a previous study, bacteria with *mlr*$^+$ genotype showed higher MC degradation rates than those lacking *mlr* genes in presence of increasing concentrations of alternative carbon and nitrogen compounds (Chapter 2). In a following study based on the diversity of the bacterial community composition associated with toxic cyanobacterial blooms, several bacterial taxa without *mlr* genes and with reported ability to degrade xenobiotic compounds were highly related to the cyanobacterial bloom seasonal dynamics and the MC concentrations (Chapter 3). As consequence of the aforementioned results and other studies that reported the presence of MC-degrading bacteria lacking *mlr* genes in aquatic systems (Manage et al., 2009b; Mou et al., 2013), at least two different MC degradation pathways seem to operate in the environment. Bourne and co-authors (1996) described the MC-LR pathway encoded by the *mlr* genes (Figure 1.8) and was further studied in other MC variants (Dziga et al., 2016; Yan et al., 2012) and nodularin (Imanishi et al., 2005). However, the MC degradation pathway lead by the MC-degrading bacteria lacking *mlr* genes is still unknown.

We hypothesize that *mlr*$^-$ bacteria may have a different MC degradation pathway that could result in a dissimilar breakdown of the MC-LR molecule from that described in *mlr*$^+$ bacteria. Therefore, we performed a comparative study of the MC-LR degradation pathways between six MC-degrading strains with both *mlr*$^+$ and *mlr*$^-$ genotypes.

8.2. Materials and methods

8.2.1. Bacterial strains and chemicals

The MC-degrading bacteria selected for this study comprises four strains with the *mlr*$^+$ genotype (*Sphingopyxis* sp. strains IM-1, IM-2, IM-3 and *Sphingosinicella microcystinivorans*, Y2) and two strains with the *mlr*$^-$ genotype (*Paucibacter toxinivorans* IM-4 and 2C20). The strains IM-1, IM-2, IM-3 and IM-4 were isolated in a previous study (Lezcano et al., 2016) and the strains Y2 (Park et al., 2001) and 2C20 (Rapala et al., 2005)

were obtained from the DSMZ culture collection. The analysis and identification of the complete *mlr*A-D gene cluster from the bacterial strains were performed in Lezcano et al. (2016) and the obtained gene sequences were deposited in the GenBank under the accession numbers: *mlr*A (KY002139.1-KY002141.1), *mlr*B (KY002142.1-KY002145.1), *mlr*C (KY002146.1-KY002149.1) and *mlr*D (KY002150.1-KY002153.1).

Stocks of dried and pure MC-LR to perform the MC biodegradation assays were kindly provided by the Professor Jussi Meriluoto. Prior the experiments, the stock of MC-LR was dissolved in sterile Milli-Q water, filtered through sterile syringe 0.22-µm filters (25 mm, Pall Corporation, PortWashington, NY, USA) and stored at -20 ºC until use.

8.2.2. MC-LR biodegradation assays

Each MC-degrading bacterial strain was incubated in R2A medium with an initial bacterial absorbance of 0.05 (measured at 600 nm) at 27 ºC in the dark at 120 rpm for 24 hours. Cells in the late exponential phase were centrifuged at 5.000 rpm for 5 min., washed in 4-fold diluted R2A liquid medium and then suspended in the same media with 0.7 mg L^{-1} of pure MC-LR. Final bacterial absorbance was 0.025 ± 0.008 (measured at 600 nm), equivalent to 10^4 CFU mL^{-1} approximately, and experiments were performed in duplicates. A control of 4-fold diluted R2A liquid medium without bacteria was also included. Incubation was performed under previously described conditions for 120 hours and samples were collected at different time intervals: 0, 6, 12, 24, 48 and 120 hours. Samples were immediately centrifuged at 5000 rpm for 5 minutes and the supernatant was bound to C18 cartridges (30 mg, 1 cc) (OASIS HLB, Waters, Milford, MA, USA). Cartridges were activated with 100% methanol and then Milli-Q water. After that, samples were passed through and washed by Milli-Q water and final elution of MC-LR was performed in 100% methanol. Then, samples were dried at 40 ºC with N_2 gas, resuspended in 50:50 methanol:water and filtered through syringe 0.2-µm filters (Acrodisc GHP, Pall Corporation, PortWashington, NY, USA) before analysis.

8.2.3. Analysis of MC-LR in HPLC-UV

Analysis of MC-LR was performed in a HPLC system (Agilent 1100 Series HPLC system, Waldbronn, Germany) with diode-array detector operated at 238 nm with a column oven regulated at 40 ºC. The chromatographic separation for MC-LR was performed using a Purospher STAR RP C18 55 mm x 4 mm with 3 µm particle size (Merck, Darmstadt, Germany). The mobile phase consisted of 0.05 % (v/v) TFA in water (A) and 0.05 % (v/v)

TFA in acetonitrile (B) with a flow rate of 1 mL min^{-1}. Gradient profile started at 25% B, increased to 70% B over 5 minutes followed by 1 minute at 70% B, changed to 25% B over 10 seconds and held at 25% B for 3 minutes for column re-equilibration. The injection volume was 10 µL and the MC-LR eluted at 3.4 min., approximately. Two external reference standards of MC-LR, NIES-107 and Ma-7820, were diluted in 75% methanol to appropriate concentrations and included in the analysis.

8.2.4. Analysis of putative MC-LR biotransformation products in HPLC-TOF

A preliminary analysis to identify tentative MC-LR biotransformation products were performed with two samples from the bacterial strains IM-2 (*mlr*$^+$) and 2C20 (*mlr*$^-$) at 12 and 48 hours of incubation, respectively. The samples were measured on a high-performance liquid chromatography (HPLC) system (Agilent series 1100, Agilent Technologies, Santa Clara, CA, USA) coupled to a time-of-flight (TOF) mass spectrometer (Agilent 6230 accurate mass TOF Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation of MC-LR was performed using a Pursuit C18 150 mm × 2 mm column with 3 µm of particle size (Agilent Technologies, Santa Clara, CA, USA) and thermostated at 40 ºC. The mobile phase consisted of 0.1% of acetic acid in water (A) and 0.1% of acetic acid in acetonitrile (B) with a flow rate of 0.3 mL min^{-1}. Gradient profile started at 5% B, increased to 100% B over 20 min and continued to 100% B over 5 min for cleaning. For re-equilibration of the column, B was reduced to 5% over 1 min and was held for further 6 min. Fifty microliters of each sample was injected. Mass spectra were acquired in the positive ion mode in the scan range of m/z 100-3000. The ESI source conditions were set as follows: a capillary voltage of 3500 V, drying gas flow rate of 9 L min^{-1}, drying gas temperature at 350 ºC and nebulizer at 35 psi. Ion recording used in this analysis were m/z 507.2829 for the acyclo MC-LR and 615.3388 for the tetrapeptide.

8.3. Results and discussion

8.3.1. MC-LR biodegradation assay and analysis in HPLC-UV

The analysis of MC-LR concentrations during the bioassay experiments showed higher MC degradation efficiencies in bacterial strains possessing *mlr* genes than those lacking the *mlr* gene cluster. As observed in Figure 8.1, the strains IM-1 (*mlr*$^+$) and Y2 (*mlr*$^+$) depleted the MC-LR concentrations in 12 hours, followed by strains IM-3 (*mlr*$^+$) and IM-2 (*mlr*$^+$), which required 24 hours to deplete the MC-LR concentrations. On the contrary, strains 2C20 (*mlr*$^-$) and IM-4 (*mlr*$^-$) exhibited lower degradation efficiencies, requiring 48 and 120 hours to completely deplete the initial MC-LR concentrations, respectively. Thus, these results

confirmed previous findings of dissimilar MC degradation efficiencies observed between *mlr*$^{+}$ and *mlr*$^{-}$ bacterial genotypes when they are exposed to identical MC concentrations and presence of alternative sources of carbon and nitrogen compounds (in this study, provided by the 4-fold diluted R2A medium).

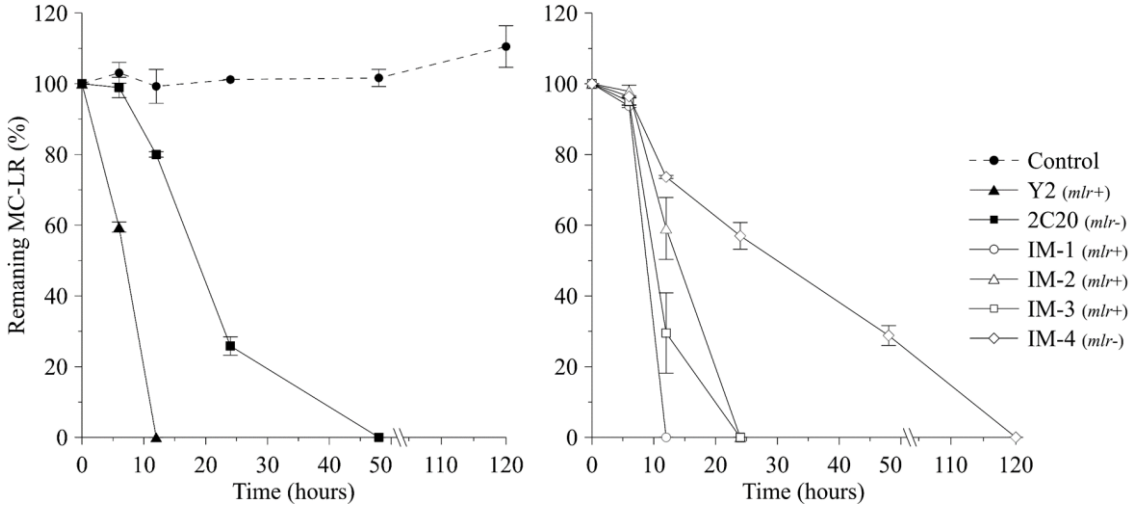


Figure 8.1. Degradation of pure MC-LR by the bacterial genotypes *mlr*$^{+}$ (strains Y2, IM-1, IM2 and IM-3) and *mlr*$^{-}$ (strains 2C20 and IM-4) over time in a 4-fold diluted R2A medium with 0.7 mg L^{-1} of MC-LR. Error bars represent standard errors of two replicates.

The analysis of HPLC chromatograms showed several potentially MC-LR biodegradation compounds that eluted at different times as compared to the MC-LR molecule (Retention time (RT): 3.41 min.) and increased in absorbance intensity as MC-LR decreases. The **Figure 8.2** shows an example of an HPLC chromatogram at 238 nm with two possible biodegradation products of MC-LR that eluted at 2.83 and 3.27 min. after 48 hours of incubation of the strain IM-4 (*mlr*$^{-}$).

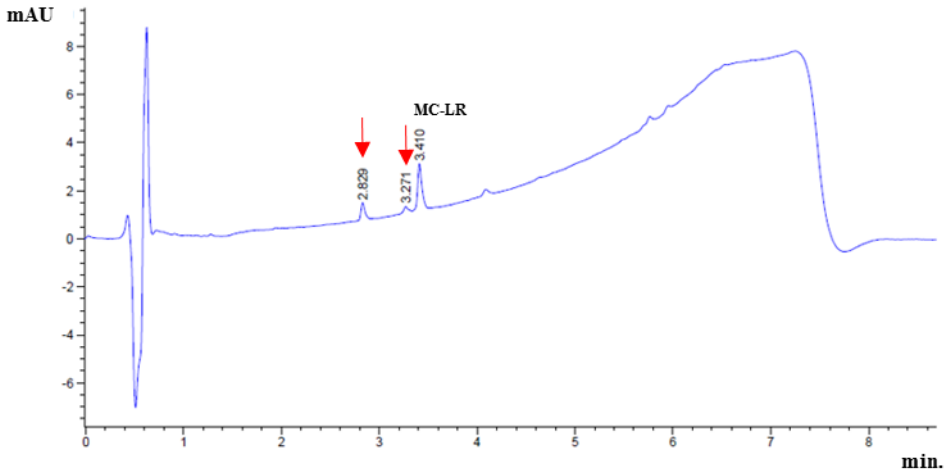


Figure 8.2. HPLC chromatogram of the MC-LR molecule and two possible biodegradation products (red arrows) after 48 hours of incubation of strain IM-4 (*mlr*$^{-}$) in 4-fold R2A medium with initial 0.7 mg L^{-1} of MC-LR. The chromatogram correspond to 238 nm following conditions described in "8.2.3. Analysis of MC-LR in HPLC-UV".

The comparison of the HPLC chromatograms from the MC-LR biodegradation assays lead to a total of four different peaks potentially biodegradation products, summarized in Table 8.1. Strains Y2 (*mlr*$^{+}$) and IM-1 (*mlr*$^{+}$) did not show any additional peak apart from the MC-LR, possibly due to their high MC-degradation efficiencies. Strain IM-2 (*mlr*$^{+}$) showed two peaks at the RT of 2.83 min. (peak A) and 3.06 min (peak B), and strain IM-3 (*mlr*$^{+}$) only showed peak B. If we compare the chromatograms from *mlr*$^{+}$ strains with those from *mlr*$^{-}$ strains, only coincide in peak A, and two new peaks with different RT appeared; one with RT of 3.28 min. (peak C) and one with RT of 3.38 min (peak D). These results may suggest two possibilities. In one hand, it may be possible that *mlr*$^{+}$ bacteria also degraded the MC-LR molecule into peaks C and D, but these molecules were rapidly degraded due to the higher MC degradation efficiencies compared to the *mlr*$^{-}$ strains. On the other hand, it may be possible that peaks C and D were only MC-LR degradation products present in the *mlr*$^{-}$ bacterial assays and, thus, suggesting a different MC-degradation pathway from that described in *mlr*$^{+}$ bacteria. To shed some light, an analysis of mass spectrometry to search for the acyclo MC-LR and the tetrapeptide degradation products described in the *mlr*$^{+}$ pathway were performed on two bioassay samples from the two *mlr*$^{+}$ and *mlr*$^{-}$ bacterial genotypes.

Table 8.1. Tentative identification of putative MC-LR transformation products detected in the HPLC chromatograms resulted from the biodegradation of MC-LR over time by bacterial genotypes *mlr*$^+$ (strains Y2, IM-1, IM2 and IM-3) and *mlr*$^-$ (strains 2C20 and IM-4). Retention times of the possible MC-LR biotransformation products were 2.83 min for A, 3.06 min for B, 3.28 min. for C and 3.38 min. for D. Maximum retention time deviation between analyses did not exceed 0.01 min.

Bacterial strains	Genotype	Tentative MC-LR transformation products					
		0 h.	6 h.	12 h.	24 h.	48 h.	120 h.
Sphingosinicella microcystinivorans Y2	*mlr*$^+$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sphingopyxis sp. IM-1	*mlr*$^+$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sphingopyxis sp. IM-2	*mlr*$^+$	n.d.	n.d.	**A** **B**	n.d.	n.d.	n.d.
Sphingopyxis sp. IM-3	*mlr*$^+$	n.d.	n.d.	**B**	n.d.	n.d.	n.d.
Paucibacter toxinivorans 2C20	*mlr*$^-$	n.d.	n.d.	**A**	**A** **C**	**A** **C** **D**	**C** **D**
Paucibacter toxinivorans IM-4	*mlr*$^-$	n.d.	n.d.	**A**	**A** **C**	**A** **C**	**A** **C**

8.3.2. Mass spectrometry analysis for the identification of potentially MC-LR biodegradation products with HPLC-TOF

One strategy for the identification of new or unknown molecules is the use of Mass Spectrometry (MS) with enough spectral resolution to measure "exact masses". By increasing the mass accuracy, the number of possible elemental compositions considerably reduces. In this study we used a LC-MS with an electrospray source (ESI) and time-of-flight (TOF) analyser in full scan. Firstly, we look for the identification of already known compounds, such as the linearized MC-LR and the tetrapeptide (the two most relevant MC-LR degradation products) (Bourne et al., 1996), to further search for unknown molecules.

Considering previous results of possible dissimilar MC-LR degradation pathways in the two *mlr*$^+$ and *mlr*$^-$ bacterial genotypes, a preliminary study was performed with two bioassay samples from strains IM-2 (*mlr*$^+$) and 2C20 (*mlr*$^-$) at 12 hours and 48 hours, respectively. Ion extractions of the linearized MC-LR performed on the chromatograms showed the presence of this compound in the two bacterial genotypes (Figure 8.3A,B).

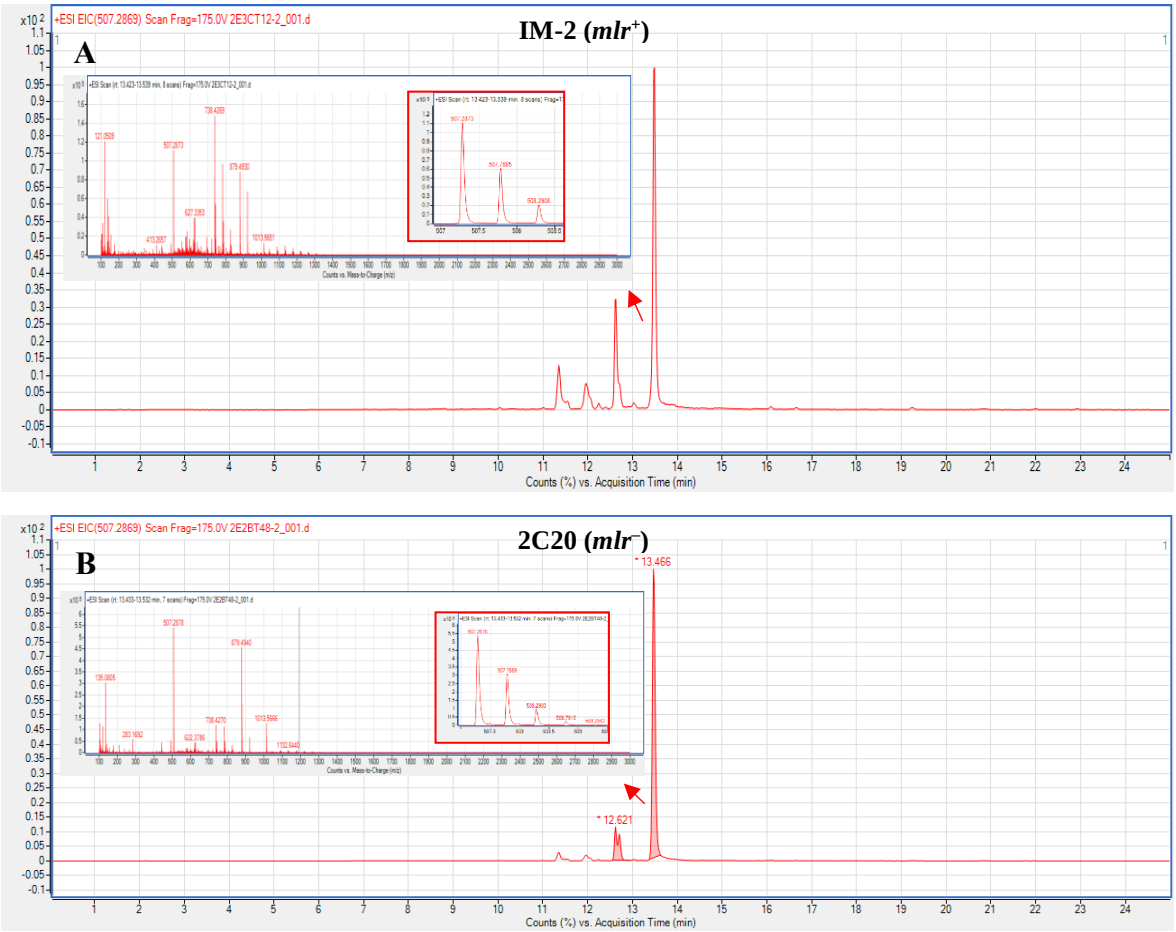


Figure 8.3. Extracted ion chromatograms of the linearized MC-LR (507.2829 m/z) molecule and their respective mass spectra for verification. Chromatogram A) refers to the MC-LR biodegradation assay performed with strain IM-2 (*mlr*$^+$). The linearized MC-LR molecule was identified in the four peaks although only the mass spectra of the most abundant peak is shown. Chromatogram B) refers to the MC-LR biodegradation assay performed with strain 2C20 (*mlr*$^–$). The linearized MC-LR molecule was identified in the two peaks although only the mass spectra of the highest peak is shown.

When looking for the tetrapeptide molecule, two different peaks were observed in the chromatogram of the *mlr*$^+$ bacteria, and only one in the chromatogram of the *mlr*$^–$ strain (

Figure **8.4**A, B). The analysis of the mass spectra allowed to discard the peak eluted at 11.766 min. and to confirm the tetrapeptide in the peak eluted at 12.776 min. Bacteria with *mlr*$^–$ genotype lacked the peak at 12.776 min and the mass spectra analysis allowed to confirm the absence of the tetrapeptide. These results are in accordance with those observed in the HPLC-UV analysis, in which peak A was observed in both *mlr*$^+$ and *mlr*$^–$ strains and peak B was only found in *mlr*$^+$ bacteria. This coincidence may point to the relation between peak A and the linearized MC-LR molecule, and the peak B and the tetrapeptide.

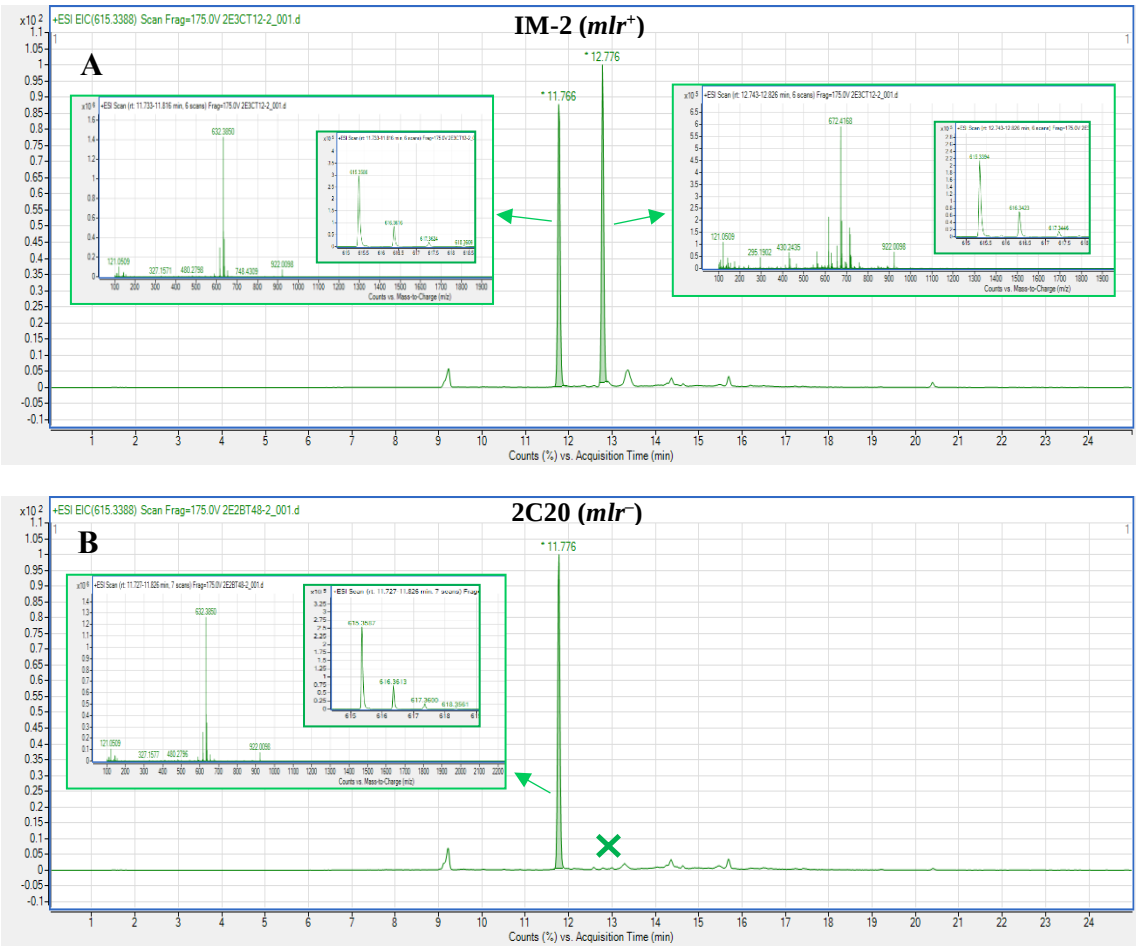


Figure 8.4. Extracted ion chromatograms of the tetrapeptide (615.3388 m/z) molecule and their respective mass spectra for verification. Chromatogram A) refers to the MC-LR biodegradation assay performed with strain IM-2 (*mlr*$^+$) and B) with strain 2C20 (*mlr*$^–$). The tetrapeptide molecule was identified in the peak with retention time at 12.776 min.

Although all these results are preliminary, it suggests that MC-degrading bacteria lacking *mlr* genes may have different MC degradation pathway(s) from the pathway described in *mlr*$^+$ bacteria. This would support previous hypothesis of the coexistence of more than one pathway operating in nature for the degradation of MCs. To confirm this, the next step would require the identification of possible new MC-LR biodegradation compounds in the *mlr*$^–$ bioassays, as tentatively identified in the HPLC-UV chromatograms.

Even though more experiments and analysis are still required, so far it remains as an interesting line to continue with. The confirmation of a possible new MC-degradation pathway(s) would open a new research line that would require the characterization of the genes involved in this putative alternative-*mlr* degradation pathway(s) and the design of new primers for their use as markers of the MC-degrading bacteria lacking *mlr* genes. In this scenario, new questions will arise regarding the specificity of the enzymes involved for the

degradation of MCs, the importance of the existence of two different MC degradation pathways in nature, the role each bacterial fraction (*mlr*$^{+}$ and *mlr*$^{-}$ genotypes) plays in the environment and the contribution of each bacterial fraction for the overall degradation of MCs in the environment.

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Appendix

MINISTERIO
DE INDUSTRIA, ENERGÍA
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Oficina Española
de Patentes y Marcas

TRATADO DE COOPERACIÓN EN MATERIA DE PATENTES NOTIFICACIÓN DE LA RECEPCIÓN DE LOS DOCUMENTOS QUE CONSTITUYEN SUPUESTAMENTE UNA SOLICITUD INTERNACIONAL PRESENTADA DE FORMA ELECTRÓNICA.

(Instrucciones Administrativas del PCT, Parte Séptima)

1.-Se notifica al solicitante que la Oficina Receptora ha recibido en la fecha de recepción indicada más abajo, los documentos que supuestamente constituyen una solicitud internacional.

2.-Se llama la atención del solicitante sobre el hecho de que la Oficina Receptora no ha comprobado aún si estos documentos satisfacen las condiciones del art. 11.1, es decir, si cumple los requisitos para que le sea atribuida una fecha de presentación internacional. En cuanto la Oficina Receptora haya comprobado los documentos, avisará al solicitante.

3.-El número de la supuesta solicitud internacional indicado más abajo ha sido otorgado automáticamente a estos documentos. Se invita al solicitante a mencionar este número en toda la correspondencia con la Oficina Receptora.

Número de presentación	300182559
Solicitud Número PCT	PCT/ES2016/070026
Fecha de recepción	20 enero 2016
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Referencia del expediente del solicitante o mandatario	P11812PC00
Solicitante	FUNDACIÓN IMDEA AGUA
Número de solicitantes	1
País	ES
Título de la invención	MÉTODO PARA DEGRADAR MICROCISTINAS EN UN MEDIO ACUOSO

Documentos presentados	eolf-pkda.xml	eolf-requ.xml
	eolf-appb.xml	eolf-tapp-000001.pdf (34 p.)
	eolf-fees.xml	eolf-biod-000001.xml
	eolf-biod-000002.pdf (1 p.)	eolf-vlog.xml
	eolf-othd-000001.pdf (40 p.)	eolf-abst.txt
	eolf-appb-P000001.pdf (3 p.)	

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METHOD FOR DEGRADING MICROCYSTINS IN AN AQUEOUS MEDIUM

FIELD OF THE INVENTION

The invention relates to the field of biological control of microcystins produced by cyanobacterial blooms. In particular, the invention relates to a microorganism from the species *Sphingopyxis* sp. which is able to degrade microcystins in an aqueous medium, to the uses of said microorganism in methods for degrading or preventing the accumulation of microcystins water polluted by cyanobacteria and to a method for isolating microcystin-degrading microorganisms.

BACKGROUND OF THE INVENTION

When closed-water areas such lakes, reservoirs, ponds and inland seas are eutrophicated due to inflow of sewage or the like, a large amount of cyanobacteria, for example, cyanobacteria belonging to the genus *Microcystis,* proliferate to generate the so-called water-bloom. Said cyanobacteria produce microcystins, also known as called toxic water-bloom, and thus the closed-water areas are polluted with microcystins.

Microcystins are cyclic peptides consisting of seven amino acids. Microcystin LR, microcystin RR, and microcystin YR are typical microcystins from closed-water areas. These compounds may poison human or livestock when orally ingested. There are various reports on poisoning by microcystins in several countries.

Conventionally, various methods for treating microcystin polluted water have been reported. In order to prevent poisoning by microcystins, a method for suppressing the generation of water-bloom is generally used. This can be carried out by adding an inhibitor of algae proliferation such as lysine to a closed-water area. In addition, it is also possible to spray an algaecidal copper ion compound over a closed-water area with generated water-bloom. However, said methods of adding an algae proliferation inhibitor causes organic substance pollution, since the algae proliferation inhibitor itself is an organic substance such as lysine. Moreover, algaecidal cause lysis of cyanobacterial cells and release of intracellular microcystins.

Another method for degrading microcystins is based on transmitting ultrasonic waves to polluted water prepared by evacuating a part of a closed-water area with water-bloom generated, thereby crushing cells of water-bloom so as to destroy the algae. However, said method has drawbacks in that oscillation of ultrasonic waves requires a massive amount of energy, and the residual crushed cells cause secondary pollution such as eutrophication. Moreover, the produced microcystins are accumulated in the cells of the cyanobacteria and even if the water-bloom is removed by the above-described method, a large amount of microcystins are released from the crushed cyanobacteria. Therefore, there is a problem that the closed-water area is increasingly polluted with microcystins.

In order to solve this problem, methods based on the microcystin treatment with ozone or with chlorine have been developed. However, in such methods, since ozone or chlorine react with a large amount of contaminants in a closed-water area, there is problem in that microcystins cannot be degraded efficiently. In addition, there is also a problem in that toxic by-products are generated due to the strong oxidation ability of ozone or chlorine.

Thus, an efficient method for degrading microcystins from polluted water is required.

BRIEF DESCRIPTION OF THE INVENTION

In a first aspect, the invention relates to a microorganism from the species *Sphingopyxis sp*. deposited in the Spanish Type Culture Collection (CECT) with accession number CECT 8967 or a mutant thereof which substantially maintains the ability of degrading microcystins.

In a second aspect, the invention relates to a biologically pure culture of the microorganism of the first aspect of the invention.

In a third aspect, the invention relates to a biomass comprising the microorganism of the first aspect of the invention.

In a fourth aspect, the invention relates to a bacterial consortium comprising the microorganism of the first aspect of the invention.

In a fifth aspect, the invention relates to a cell extract of the microorganism of the first aspect of the invention

In a sixth aspect, the invention relates to a method for obtaining a biomass of the microorganism of the first aspect of the invention comprising culturing said microorganism under conditions suitable for growth.

In a seventh aspect, the invention relates to a method for preventing the accumulation or for reducing the content of microcystins present in an aqueous medium which comprises adding, to said medium, a microorganism according to the first aspect of the invention or a biologically pure culture according to the second aspect of the invention, a biomass according to the third aspect of the invention, a bacterial consortium according to the fourth aspect of the invention or a cell extract according to the fifth aspect of the invention and maintaining the culture under conditions suitable for the degradation of said microcystins.

In an eight aspect, the invention relates to the use of a microorganism according to the first aspect of the invention, a biologically pure culture according to the second aspect of the invention, a biomass according to the third aspect of the invention, a bacterial consortium according to the fourth aspect of the invention or a cell extract according to the fifth aspect of the invention for preventing the accumulation or for reducing the content of microcystins present in an aqueous medium.

Finally, in a ninth aspect, the invention relates to a method for isolating microcystin-degrading microorganism from an aqueous medium comprising:

i) adding microcystins to a sample obtained from said aqueous medium at an appropriate concentration and incubating the sample under conditions suitable for the growth of microcystin-degrading microorganisms present in the sample, thereby enriching said sample in microcystins degrading microorganisms.

ii) culturing the microorganisms of the sample of step i) on a solid culture medium comprising at least one carbon source and at least one nitrogen source during an appropriate incubation time until colonies are obtained,

iii) culturing the microorganism from the colonies obtained of step ii) on a culture medium comprising at least one carbon source and at least one nitrogen source wherein said at least one carbon source and at least one nitrogen source are present in a concentration which is about or lower than the concentration which results after a dilution which is at least a two-fold dilution of the concentration of said components in the culture media of step ii) in the presence of microcystins; and

iv) selecting microcystin-degrading microorganisms as the microorganism which grow after the culture step iii).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Total MC degradation by bacteria CECT 8967, Y2 and 2C20 in a) MSM medium, b) reservoir's water and c) 4-fold diluted R2A medium in 48 hours of incubation. Control refers to medium without bacteria. Error bars relate to technical replica.

Figure 2. Estimated total MC degradation by isolated bacterial strains with different degradation rates.

DETAILED DESCRIPTION OF THE INVENTION

The inventors of the present invention have surprisingly discovered a bacterial strain of species *Sphingopyxis sp*. which degrades microcystins in solution with higher efficiency compared to other bacterial strains and wherein said degradation capacity is not inhibited by high levels of total organic carbon present in the solution. Moreover, the inventors have developed a method for an efficient isolation of microcystin-degrading bacteria from water bodies suffering episodes of harmful cyanobacterial blooms.

Based on these findings, the inventors have developed the methods of the present invention in their different embodiments that will be described now in detail.

MICROORGANISM OF THE INVENTION

In a first aspect, the invention relates to a microorganism from the species *Sphingopyxis sp*, hereinafter "the microorganism of the invention", deposited in the Spanish Type Culture Collection (CECT) with accession number 8967 which degrades microcystins or to a mutant thereof which substantially maintains the ability of degrading microcystins.

Said strain was deposited before the date of filing the present patent application in the Spanish Type Culture Collection (CECT) at c/ Catedrático Agustín Escardino, 9, 46980, Paterna, Valencia (Spain), as a legally recognized depositary institution for that purpose in accordance with the Budapest Treaty of April 28, 1977, on international recognition of the deposit of microorganisms for the purposes of patent.

The depositor was Fundación IMDEA Agua with registered office at Av. Punto Com, 2, Parque Científico y Tecnológico de la Universidad de Alcalá, 28805 Alcalá de Henares, Madrid, Spain.

As it is used herein, the term "microorganism" or "microbe" refers to a single-celled or multicellular microscopic organism with the capacity of degrading microcystins. Particularly, the microorganism of the invention is a bacterium of the genus *Sphingopyxis*

sp, specifically the strain CECT 8967 or a mutant strain thereof which substantially maintains the ability of degrading microcystins.

As it is used herein, the term "strain" refers to a genetic variant or subtype of a certain organism.

As it is used herein, the term "mutant" or "mutant strain", refers to any microorganism resulting from a mutation or change in the DNA of one or several genes of the *Sphingopyxis sp* strain CECT 8967 maintaining substantially the properties of degradating microcystins.

In a particular embodiment, the mutant of *Sphingopyxis sp* strain CECT 8967 has a genome having a sequence identity of at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99% or higher with the genome of *Sphingopyxis sp* strain CECT 8967. The sequence identity between the genomes of two microorganisms can be determined by using algorithms implemented in a computer and methods which are widely known by the persons skilled in the art. The identity between two nucleotide sequences is preferably determined using the BLASTN algorithm (BLAST Manual, Altschul, S. et al., NCBI NLM NIH Bethesda, Md. 20894, Altschul, S., et al., J., 1990, Mol. Biol. 215:403-410).

The mutant can be produced naturally or intentionally by mutagenesis methods known in the state of the art, such as, for example but not being limited to, growing the original microorganism in the presence of mutagenic or stress-causing agents, or by means of genetic engineering aimed at modifying specific genes.

Variants may or may not have the same identifying biological characteristics of the specific strains exemplified herein, provided they share similar advantageous properties in terms of their cholesterol-absorbing capacity of the reference strain. For example, the 16S rRNA genes of a "variant" strain as contemplated herein may share about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a strain disclosed herein.

In another embodiment, a variant of the strains according to the present invention refer to any strain the genome of which hybridizes under stringent conditions with the genome of any of the CECT 8605 L. reuteri V3401 strain or of the CECT 8606 B. breve BT820 strain. In general, a low stringency hybridization reaction is carried out at about 40 degrees centigrade in 10xSSC or a solution of equivalent ionic strength/temperature. A moderate stringency hybridization is typically performed at about 50 degrees centigrade in 6xSSC, and a high stringency hybridization reaction is generally performed at about 60 degrees centigrade in 1xSSC.

In another embodiment, the degree of relatedness between the variant and the parent strains is determined as the average nucleotide identity (ANI), which detects the DNA conservation of the core genome (Konstantinidis K and Tiedje JM, 2005, Proc. Natl. Acad. Sci. USA 102: 2567–2592). In some embodiments, the ANI between the variant and the parent strain is of about 95%, about, 96%, about 97%, of about 98%, of about 99%, of about 99.1%, of about 99.5%, of about 99.6%, of about 99.7%, of about 99.8%, of about 99.9%, of about 99.99%, of about 99.999%, of about 99.9999%, of about 99.99999%, of about 99.999999% or more but less than 100%.

In another embodiment, the degree of relatedness between the variant and the parent strains is determined as the Tetranucleotide Signature Frequency Correlation Coefficient, which is based on oligonucleotide frequencies (Bohlin J. et al. 2008, BMC Genomics, 9:104). In some embodiments, the Tetranucleotide Signature Frequency Correlation coefficient between the variant and the parent strain is of about 0.99, 0.999, 0.9999, 0.99999, 0.999999, 0.999999 or more but less than 1.

In another embodiment, the degree of relatedness between the variant and the parent strains is determined as the degree of similarity obtained when analysing the genomes of the parent and of the variant strain by Pulsed-field gel electrophoresis (PFGE) using one or more restriction endonucleases. The degree of similarity obtained by PFGE can be measured by the Dice similarity coefficient. In some embodiments, the Dice similarity coefficient between the variant and the parent strain is of about 95%, about, 96%, about 97%, of about 98%, of about 99%, of about 99.1%, of about 99.5%, of about 99.6%, of about 99.7%, of about 99.8%, of about 99.9%, of about 99.99%, of about 99.999%, of about 99.9999%, of about 99.99999%, of about 99.999999% or more but less than 100%.

In another embodiment, a strain is considered a variant of a given parent strain when both strains have the same ribotype, as obtained using any of the methods known in the art an described, for instance, by Bouchet et al. (Clin. Microbiol. Rev., 2008, 21:262–273).

In another embodiment, the degree of relatedness between the variant and the parent strains is the Pearson correlation coefficient obtained by comparing the genetic profiles of both strains obtained by repetitive extragenic palindromic element-based PCR (REP-PCR) (see e.g. Chou and Wang, Int J Food Microbiol. 2006, 110:135-48). In some embodiments, the Pearson correlation coefficient obtained by comparing the REP-PCR profiles of the variant and the parent strain is of about 0.99, 0.999, 0.9999, 0.99999, 0.999999, 0.999999 or more but less than 1.

In another embodiment, the degree of relatedness between the variant and the parent

strains is the linkage distance obtained by comparing the genetic profiles of both strains obtained by Multilocus sequence typing (MLST) (see e.g. Maiden, M. C., 1998, Proc. Natl. Acad. Sci. USA 95:3140-3145). In some embodiments, the linkage distance obtained by MLST of the variant and the parent strain is of about 0.99, 0.999, 0.9999, 0.99999, 0.999999, 0.999999 or more but less than 1.

In a preferred embodiment, the variant and the parent strain are of the same genus. In a still more preferred embodiment, the variant and the parent strain are of the same species or subspecies.

The term "microcystins", "MC", or "cyanoginosins", as used herein, refers to a class of toxins produced by certain freshwater cyanobacteria; primarily *Microcystis aeruginosa* but also other *Microcystis* species, as well as members of the *Planktothrix, Anabaena, Anabaenopsis, Oscillatoria* and *Nostoc* genera. Chemically, microcystins are cyclic heptapeptides synthetized nonribosomally by peptide synthases, with a general structure of *cyclo*-(D-alanine^{1}-X^{2}-D-MeAsp^{3}-Z^{4}-Adda^{5}-D-glutamate^{6}-Mdha^{7}), in which X and Z are variable L-amino acids and Adda refers to a (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, an unusual amino acid principally responsible of the toxicity of eukaryotic cells. Among 90 MC variants have been characterized but the main isoforms of MC are MC-RR, MC-LR and MC-YR. Variations occur in all amino acids, but most frequently at positions 2 and 4 with variable L-amino acids and at positions 3 and 7 with methylations and demethylations on the D-erythro-methylaspartic acid and/or N-methyldehydroalanine. For instance, MC-LR comes from leucine at position 2 and arginine at position 4.

As used herein, the expression "degrading microcystins" refers to the ability of the microorganism of the invention of metabolizing microcystins by using said molecules as a source of carbon and/or nitrogen in presence of additional carbon and/or nitrogen sources. The determination of the ability of metabolizing microcystins by the microorganism of the invention may be carried out by any suitable technique which allows the detection and/or quantification of the microcystins-degradation intermediates, such as the linearized (acyclo-) MC-LR (NH2-Adda-Glu-Mdha-Ala-Leu-MeAsp-Arg-OH), the tetrapeptide (NH2-Adda-Glu-Mdha-Ala-OH) and the Adda moiety among others. Illustrative non-limitative techniques which allow the detection of said metabolites include high performance liquid chromatography (HPLC) or liquid chromatography/mass spectrometry (LC/MS). Alternatively, the determination of MC-degrading capacity may be carried out by analyzing the initial and final concentration of microcystins in a given sample after incubation with the

microorganism of the invention by HPLC, LC/MS, enzyme-linked inmunoabsorbent assay (ELISA) and/or protein phosphatase inhibition assay. Therefore, as the person in the art will understand, calculations of the degradation rate (i.e. concentration of microcystins/time unit) not only allows the determination of the ability of the microorganism of the invention to degrade microcystins, but also the efficient removal of the microcystins from an aqueous solution.

The microorganism of the invention also refers to a mutant strain thereof which substantially maintains the capacity of degrading microcystins. As it is used herein, the expression "substantially maintains the capacity of degrading microcystins" of the mutant strain according to the invention means that said microorganism maintains at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% the ability of degrading microcystins when compared with *Sphingopyxis sp.* strain CECT 8967.

In another aspect, the invention relates to a biologically pure culture, hereinafter "the biologically pure culture of the invention" comprising the microorganism of the invention. As it is used herein, the expression "biologically pure culture" refers to a culture in which the microorganism of the invention can be found in a proportion of 95% or higher, for example 96% or higher, 97% or higher, 98% or higher, 99% or higher, or 100%, compared with other organisms present in the culture. As it is used herein, the term "culture" refers to a population of the microorganisms of the invention. A culture can comprise other elements in addition to the microorganism of the invention, such as the culture medium or any other substance that can be added to the culture medium that is beneficial for growth or maintenance. The term "culture medium" or "medium" is recognized in the art and generally refers to any substance or preparation that is used for the culture of live cells. As it is used in reference to a cell culture, the term "medium" includes the components of the environment surrounding the cells. The medium can be solid medium, liquid medium, gaseous medium, or a mixture of the phases and materials. The growth media include liquid culture media as well as liquid media that do not support cell growth. The medium may also include gelatinous media such as agar, agarose, gelatin and collagen matrices. Exemplary gaseous media include the gas phase to which the cells growing in a Petri dish or another solid or semisolid support are exposed. The term "medium" also refers to a material that must be used in a cell culture, even if it has still not been contacted with the cells. In other words, a liquid rich in nutrients prepared for bacterial culture is a medium. Likewise, a powder

mixture which when mixed with water or with another liquid becomes suitable for the cell culture can be called a "powder medium". "Defined medium" refers to the media made up of components having a defined chemical constitution (generally purified). The defined media do not contain biological extracts that are not completely characterized, such as meat broth and yeast extract. "Rich medium" includes media designed to support growth of most or all viable forms of a particular species. Enriched media often include complex biological extracts. Any conventional culture medium suitable for *Sphingopyxis sp* known in the art can be used in the present invention, such as, for example, nutrient broth made up of yeast extract (0.5 g/L), proteose peptone (0.5 g/L), casamino acids (0.5 g/L), glucose (0.5 g/L), starch (0.5 g/L), Na-pyruvate (0.3 g/L), K_2HPO_4 (0.3 g/L) and $MgSO4 \cdot 7H_2O$ (0.05 g/L), pH 7.2 or a culture medium comprising $MgSO_4 \cdot H_2O$ (1.12 mg/L), KH_2PO_4 (340 mg/L), $ZnSO_4 \cdot H_2O$ (5 mg/L), $CaCl_2$(14 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (2.5 mg/L), $Na_2HPO_4 \cdot 7H_2O_2$ (670 mg/L), $FeCl_3$(0.13 mg/L), pH 7.

In a further aspect, the invention relates to a biomass comprising the microorganism of the invention. As it is used herein, the term "biomass" refers to the biological material of living organisms, particularly of the microorganism of the invention.

In another aspect, the invention relates to a bacterial consortium comprising the microorganism of the invention. The term "bacterial consortium", as used herein, refers to a natural association of two or more bacterial populations belonging to different species, which act together as a community, in a complex system where everyone benefits from the activities of others. By way of illustration, the bacterial consortium comprising the microorganism of the invention may also comprise genera S*phingopyxis sp*., *Sphingomonas sp*. or *Paucibacter sp*.

In a fifth aspect, the invention relates to a cell extract, hereinafter "the cell extract or the extract of the invention", of a microorganism of the invention. The term "cell extract", as used herein, refers to a cellular homogenate, particularly to a cellular homogenate of the microorganism of the invention, which can be in different grades of cell lysis depending on the treatment of the cells. The lysis of the cells of the microorganism of the invention may be done by any suitable method known in the art, such as heat lysis, lysis in basic medium, lysis in acidic medium, enzymatic lysis using enzymes such as proteases or polysaccharide-degrading enzymes (amylases), lysis by means of ultrasounds, mechanical lysis or lysis by means of osmotic shock. These methods can be carried out in a separate or combined manner, and in the case of combined use, they can be carried out simultaneously or sequentially. The degree of cell rupture can be determined by means of microscopic analysis.

As the person skilled in the art will understand, the cell extract of the invention is characterized in that it maintains or substantially maintains the ability of degrading microcystins.

The term "microorganism of the invention" has been previously defined in the context of the first aspect of the invention and equally applies to the second, third, fourth and fifth aspect of the invention.

METHOD FOR OBTAINING A BIOMASS

In a further aspect the invention relates to a method for obtaining a biomass of the microorganism of the invention, hereinafter "the first method of the invention", comprising culturing said microorganism under conditions suitable for growth.

The term "microorganism of the invention" has been defined in the first aspect of the invention and equally applies to the first method of the invention.

Conditions suitable for growth of the microorganism of the invention will be those conditions which allow microorganism maintenance and multiplication. In a particular embodiment, said conditions comprise culturing the microorganism of the invention in the presence of a culture medium or substrate containing one or several carbon sources, one or several nitrogen sources and inorganic and organic salts at concentrations suitable for obtaining maximum biomass yields. Said medium or substrate can be solid or liquid. The carbon sources consist of monosaccharides, polysaccharides, cereals or plant extracts. The nitrogen sources comprise plant protein hydrolysates, peptones or free, pure or mixed amino acids. The salts are sulfates or phosphates of elements such as Na, Ca, Mg, Fe, or K. In a particular embodiment, the culture medium or substrate contains between 1 and 5 carbon sources, between 1 and 5 nitrogen sources and between 1 and 10 salts. Suitable culture media which can be used for obtaining a biomass of the microorganism of the invention have been mentioned above in the context of the microorganism of the invention.

In a preferred embodiment, the conditions suitable for growth of the microorganism of the invention comprise culturing the microorganism in the culture medium under constant temperature, pH and aeration conditions. Preferably, the temperature is comprised between 15ºC and 35ºC, preferably 27ºC. Typically, the pH is comprised between 6.0 and 8.0, preferably 7.0. Normally, aeration is achieved by means of stirring at speeds between 50 and 150 rpm. The time during which the microorganism must be kept under conditions suitable for growth is the time needed for the microorganism to reach a concentration corresponding to a minimum substrate to biomass conversion of 80%. Typically, said time of growth for

reaching these yields is between 1 and 120 hours.

Once the microorganism growth step has ended, the obtained biomass can be recovered from the used up substrate by applying one or several unit operations which can comprise centrifugation, decantation, filtration or a combination of several of these operations. Therefore, if desired, the first method of the invention additionally comprises a step for separating the biomass from the substrate by means of one or more steps of centrifugation, decantation or filtration, or a combination thereof.

METHOD FOR TREATING MICROCYSTIN-POLLUTED WATER OR FOR PREVENTING THE ACCUMULATION OF MICROCYSTINS IN WATER

As mentioned above, microcystins are accumulated in aqueous medium due to the proliferation of cyanobacteria. The microorganism of the invention is useful for treating said polluted water or for preventing the accumulation of microcystins in said media. Thus, in a seventh aspect, the invention relates to a method for preventing the accumulation or for reducing the content of microcystins present in an aqueous medium, hereinafter, "the second method of the invention", which comprises adding to said medium a microorganism according to the first aspect of the invention, a biologically pure culture according to the second aspect of the invention, a biomass according to the third aspect of the invention, a bacterial consortium according to the fourth aspect of the invention or a cell extract according to the fifth aspect of the invention and maintaining the culture under conditions suitable for the degradation of said microcystins.

The term "preventing the accumulation of microcystins present in an aqueous medium", as used herein refers to the maintenance of a concentration of microcystins in the aqueous medium which is considered as tolerable concentration according to the World Health Organization. The term "tolerable concentration", as used herein refers to the amount of microcystins which can be ingested without causing health damage. In a particular embodiment of the first method of the invention, the concentration of microcystins is maintained below 1.5 µg/L, preferably below 1 µg/L.

The term "reducing the content of microcystins present in an aqueous medium" as used herein, include not only the elimination of the almost total of microcystins present in said aqueous medium but also the elimination of at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of the microcystins present in said aqueous medium. The initial concentration of microcystins in an aqueous medium may vary within a range. However, in

a particular embodiment, said aqueous medium has microcystins concentration about < 1 µg/L to 500 µg/L or even higher. Conventional methods can be used for determining the concentration of microcystins in said aqueous medium. Illustrative examples of said methods are mentioned in the context of the first aspect of the invention.

The term "aqueous medium" as used herein, refers to any liquid phase comprising water. In a particular and preferred embodiment of the invention said aqueous medium refers to inland water such lakes, reservoirs, ponds or streams.

The term "microcystins" have been previously defined in the context of the first aspect of the invention. In a particular embodiment of the second method of the invention, said microcystins are selected from the group consisting of microcystin-RR, microcystin-LR, microcystin-YR and combinations thereof.

Conditions suitable for degradation of microcystins in an aqueous medium according to the second method of the invention include said conditions which allow the development and growth of the microorganism of the invention, the biologically pure culture of the invention, the biomass according to the invention or the bacterial consortium. Said conditions also include those that do not inhibit the microcystin-degrading activity of the cell extract. Such conditions include operation under aerobic conditions; a selection of an appropriate temperature; a selection of an appropriate time of incubation of the microorganism, the biologically pure culture, the biomass, the bacterial consortium or the cell extract of the invention with the aqueous medium to be treated; as well as the addition or the omission of nutrients in order that microorganisms can develop their microcystins-degrading activity.

If necessary, the aqueous medium may be supplemented with a carbon source and/or nitrogen source and/or essential nutrients, in order to facilitate the survival of the microorganism of the invention, the biologically pure culture of the invention, the biomass according to the invention or the bacterial consortium of the invention. By way of illustration, in order to optimize the process of the invention, suitable amounts of micronutrient solution, along with magnesium, cobalt, molybdenum and other essential metals, typically in the micromolar order (µM) may be added. In any case, the choice and amount of nutrients and/or micronutrients to be added to the polluted aqueous medium will depend on the composition of said aqueous medium to be treated and on the microbiological demand in each particular case. The person skilled in the art can take appropriate steps to achieve such suitable conditions.

In a particular embodiment, said conditions suitable for degrading microcystins comprise:

- temperature between 15ºC and 35ºC
- aerobic conditions
- pH between 6 and 8 and/or
- incubation time between 1 and 120 hours

In a preferred embodiment, said conditions comprise incubating said sample at 27ºC, under aerobic conditions and during 24 hours. Said incubation may be carried out in absence of light.

In a particular and preferred embodiment, the second method of the invention comprises a previous step wherein the microorganism, the biomass, the biologically pure culture or the bacterial consortium of the invention has been expanded in a culture medium comprising at least one carbon source and at least one nitrogen source in appropriate conditions for growth. Suitable conditions which can be used for growing the microorganism of the invention have been detailed in the context of the first method of the invention. In a preferred embodiment, said step comprises expanding the microorganism of the invention during 24 hours and at 27ºC in a culture medium which comprises yeast extract (0.5 g/L) proteose peptone (0.5 g/L), casamino acids (0.5 g/L), glucose (0.5 g/L), starch (0.5 g/L), Na-pyruvate (0.3 g/L), K_2HPO_4 (0.3 g/L) and $MgSO4 \cdot 7H_2O$ (0.05 g/L), and at pH 7.2.

In a more particular and preferred embodiment, the second method of the invention comprises a previous step wherein the microorganism, the biomass, the biologically pure culture or the bacterial consortium of the invention has been maintained prior to addition to the aqueous medium containing microcystins under nutrient starvation conditions. The term "nutrient starvation conditions", as used herein, refers to growing cells in nutrient deprivation media. Normally, under said conditions, cells do not duplicate. Said step allows depletion of residual carbon and nitrogen sources in order to achieve the maximum velocity of degradation of microcystins by the microorganism of the invention. Typically, nutrient starvation conditions include supplementing the culture medium with micronutrients but without adding carbon or nitrogen sources. Illustratively, said step comprises culturing the microorganism of the invention in a minimum salt medium (MSM) comprising 112 mg/L $MgSO_4 \cdot H_2O$, 5 mg/L $ZnSO_4 \cdot H_2O$, 2.5 mg/L $Na_2MoO_4 \cdot 2H_2O$, 340 mg/L KH_2PO_4 670 mg/L $Na_2HPO_4 \cdot 7H_2O$, 14 mg/L $CaCl_2$, 0.13 mg/L $FeCl_3$ at pH 7.2. The person skilled in the art may use any method known from the state of the art for determining that cells have reached starvation mode such as any suitable method for detecting cell division.

As the person skilled in the art will understand said step should be carried out during an appropriate time which allows cells reaching starvation period but without dying due to the absence of carbon and nitrogen sources. Preferably, said step is carried out during 8 hours, during 9 hours, during 10 hours, during 11 hours, during 12 hours, during 13 hours, during 14 hours, during 15 hours, during 16 hours, during 17 hours, during 18 hours, during 19 hours or during 20 hours. More preferably, said step is carried out during 12-15 hours.

The microorganism, the biomass, the biologically pure culture, the cell extract or the bacterial consortium according to the invention is characterized in that it can degrade microcystins in presence of additional carbon sources. Thus, in another particular embodiment of the second method of the invention, the concentration of total organic carbon in the aqueous medium is from <1 to 300 mg/L.

In another particular embodiment of the second method of the invention, said aqueous medium comprises microcystins in a concentration from 0.1 to 50000 µg/L.

METHOD FOR THE ISOLATION OF MICROCYSTIN-DEGRADING MICROORGANISMS

The authors of the present invention have developed an efficient method which allows isolating microcystin-degrading microorganisms from an aqueous medium. Thus, in another aspect, the invention relates to a method for isolating microcystin-degrading microorganism from an aqueous medium, hereinafter "the third method of the invention", comprising:

i) adding a microcystin to a sample obtained from said aqueous medium at an appropriate concentration and incubating the sample under conditions suitable for the growth of microcystin-degrading microorganisms present in the sample, thereby enriching said sample in microcystins degrading microorganisms;

ii) culturing the microorganisms of the sample of step i) on a solid culture medium comprising at least one carbon source and at least one nitrogen source during an appropriate incubation time until colonies are obtained,

iii) culturing the microorganism from the colonies obtained of step ii) on a culture medium comprising at least one carbon source and at least one nitrogen source wherein said at least one carbon source and at least one nitrogen source are present in a concentration which is about or lower than the concentration which results after a dilution which is at least a two-fold

dilution of the concentration of said components in the culture media of step ii) in the presence of microcystins; and

iv) selecting microcystin-degrading microorganisms as the microorganism which grow after the culture step iii)

The term "microorganism", "microcystins" and "aqueous medium" have been previously defined.

According to the third method of the invention, the step i) comprises adding microcystins to a sample obtained from said aqueous medium at an appropriate concentration and incubating the sample under conditions suitable for the growth of microcystin-degrading microorganisms present in the sample. This step allows enriching the microcystins-degrading population over the rest of microorganisms present in the sample. The term "enriched" as used herein, refers to a sample which comprises microcystin-degrading microorganisms in a proportion of more than 50%, more than 60%, more than 70% more than 80%, more than 90%, more than 91%, more than 92%, more than 93%, more than 94%, more than 95%, more than 96%, more than 97% more than 98% or more than 99% with respect to the total of the microorganisms of said sample.

The sample used in step i) can be any sample of water suspected of containing microorganisms with microcystin-degrading capacity, for example a water polluted sample due to a cyanobacteria bloom or a non-polluted freshwater isolated from inland waters such as ponds, lakes, reservoirs or streams. In a particular embodiment, said aqueous sample is freshwater. As it will be understand by the person skilled in the art, special care will be taken in order to ensure the survival of the microorganism population of said sample.

A carbon source comprising microcystins susceptible of being degraded by said microorganism is added to said sample and the resulting medium is incubated under conditions allowing the growth of said microorganism. Illustrative conditions which can be used in this step include operation under aerobic conditions; a selection of an appropriate temperature; a selection of an appropriate time of incubation of the microorganism with the aqueous medium. In a particular embodiment, said conditions comprise:

- temperature between 15ºC and 35ºC
- aerobic conditions and/or
- incubation time between 1 and 20 days

In a preferred embodiment, said conditions comprise incubating said sample at 27ºC, under aerobic conditions and during 15 days. Said incubation may be carried out in absence of light if desired.

The term "appropriate concentration of microcystins", as used herein refers to a concentration of microcystins which favors growth of microcystins-degrading microorganism. Suitable concentration of microcystins which can be used in this step is from about 0.1 mg/L to 10 mg/L. In a particular embodiment, said concentration of microcystins is 1 mg/L. Any type of microcystins can be used in this step. However it is particularly preferred using an extract comprising microcystins. Said extract can be obtained from cyanobacteria. Illustratively, microcystins extraction can be carried out following methanol extraction method, purified through solid phase extraction method (SPE), dried and finally eluted in sterile Milli-Q water.

According to step ii), the third method of the invention comprises culturing the microorganism of the sample of step i) on a solid culture medium comprising at least one carbon source and at least one nitrogen source during an appropriate incubation time until colonies are obtained. Suitable solid culture media which can be used in step ii) include any rich culture medium known by the person skilled in the art. In a particular embodiment, said solid medium comprises yeast extract (0.5 g/L) proteose peptone (0.5 g/L), casamino acids (0.5 g/L), glucose (0.5 g/L), starch (0.5 g/L), Na-pyruvate (0.3 g/L), K_2HPO_4 (0.3 g/L),$MgSO4 \cdot 7H_2O$ (0.05 g/L) and agar (15 g/L) at pH 7.2. As the person skilled in the art will understand, the incubation comprises a selection of appropriate temperature. Preferably, the temperature is comprised between 15ºC and 35ºC, preferably 27ºC. Said incubation may be carried out in absence of light, if desired.

The incubation is carried out until colonies are obtained. The term "colony" as used herein, refers to individual organisms of the same species living closely together, usually for mutual benefit and which growth on the surface of or within a solid medium, presumably cultured from a single cell. Typically, the incubation time until colonies are obtained is from 1 to 10 days. In a preferred embodiment the incubation time is 7 days. Colonies are selected and, optionally, purified using standard purification methods such as streak plate method.

The third method of the invention comprises in step iii) culturing the microorganisms from the colonies obtained of step ii) on a culture medium, preferably on a liquid culture medium, comprising at least one carbon source and at least one nitrogen source, wherein said at least one carbon source and at least one nitrogen source are present in a concentration which is about or lower than the concentration which results after a dilution which is at least a two-fold dilution of the concentration of said components in the culture media of step ii) in the presence of microcystins. This step provides an advantage for the isolation of microcystins-degrading microorganisms with highly different metabolism since the diluted

media used in this step offers an input of nutrients which enhances growing while still allows metabolically active cells to degrade microcystins. In a particular and preferred embodiment of the invention, this step is carried out on a 96-well plate since it allows to perform high number of MC-degrading assays simultaneously, reducing time and costs of reagents.

The medium used in this step for growing cells is a diluted rich medium. Particularly, the medium used in step iii) which has been at least 2-fold diluted, which means that the concentration of the nitrogen and carbon sources in the medium used in step ii) of the present method is at least double than the concentration of said nitrogen and carbon sources in the medium of step iii). Suitable diluted media which may be used in this step include, but are not limited to, the culture medium of step ii) 3-fold diluted, 4-fold diluted, 5-fold diluted, 6-fold diluted, 7-fold diluted, 8-fold diluted, 9-fold diluted, 10 fold-diluted, 20 fold-diluted or more. In a particular and preferred embodiment of the invention the medium which is used in this step results after a fourth-fold dilution of the medium used in step ii). Any suitable diluent which does not interfere with both growing and microcystins-degrading ability of the microorganisms which are being selected can be used. In a preferred embodiment, the diluent is sterile Milli-Q water.

Said step is carried out in presence of microcystins in order to ensure that the microorganisms which are being selected possess the degrading-microcystins ability. Suitable concentrations that can be used in this step are from about 20 µg/L to 50 mg/L. In a particular and preferred embodiment of the invention the concentration of microcystins in step ii) is 0.5 mg/L.

As the person skilled in the art will understand, step iii) is carried out under conditions suitable for growth of the microorganisms degrading microcystins to be isolated according to the invention and which allows the development of their microcystins-degrading ability. Said conditions comprise culturing the microorganism in the culture medium under constant temperature, pH, and aeration conditions during an appropriate time. Preferably, the temperature is comprised between 15ºC and 35ºC, more preferably 27ºC. Typically, the pH is comprised between 6.0 and 8.0, preferably 7.0. Normally, aeration is achieved by means of stirring at speeds between 50 and 150 rpm. Preferably, the incubation time of said step is from 1 to 10 days, more preferably 6 days.

Finally, the third method of the invention comprises in step iv) selecting microcystins-degrading microorganisms as the microorganisms which grow after the culture step iii).

USES OF THE INVENTION

The invention also relates to the use of the microorganism of the invention for preventing the accumulation or for reducing the content of microcystins present in an aqueous medium.

The invention also relates to the use of the biologically pure culture of the invention for preventing the accumulation or for reducing the content of microcystins present in an aqueous medium.

The invention also relates to the use of the biomass of the invention for preventing the accumulation or for reducing the content of microcystins present in an aqueous medium.

The invention also relates to the use of the bacterial consortium of the invention for preventing the accumulation or for reducing the content of microcystins present in an aqueous medium.

The invention also relates to the use of the cell extract of the invention for preventing the accumulation or for reducing the content of microcystins present in an aqueous medium.

The terms “microorganism”, “biologically pure culture”, “biomass”, “bacterial consortium”, “cell extract”, “microcystins”, “preventing the accumulation of microcystins present in an aqueous medium”, “reducing the content of microcystins present in an aqueous medium” , “aqueous medium” and the particulars thereof have been previously defined.

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The following examples are provided as merely illustrative and are not to be construed as limiting the scope of the invention.

EXAMPLE 1: Efficient removal of MC by *Sphingopyxis sp*. strain CECT 8967 at different total organic carbon concentrations in water

Removal of MCs from water with different organic carbon loads by using isolate *Sphingopyxis sp*. strain CECT 8967:

1. Isolated cells from *Sphingopyxis sp*. strain CECT 8967 were incubated in R2A medium [(0.5 g/L) proteose peptone (0.5 g/L), casamino acids (0.5 g/L), glucose (0.5 g/L), starch (0.5 g/L), Na-pyruvate (0.3 g/L), K_2HPO_4 (0.3 g/L), and $MgSO4 \cdot 7H_2O$ (0.05 g/L) at pH 7.2] at 27ºC, in the dark and at120 rpm for 24 hour with initial absorbance of 0.05 measured at 600 nm.
2. To induce nutrients starvation, cells were collected at 5.000 rpm, washed with MSM (Valeria et al., 2006) and resuspended in MSM medium (112 mg/L $MgSO_4 \cdot H_2O$ 5 mg/L $ZnSO_4 \cdot H_2O$, 2.5 mg/L, $Na_2MoO_4 \cdot 2H_2O$, 340 mg/L KH_2PO_4 670 mg/L

$Na_2HPO_4 \cdot 7H_2O$, 14 mg/L $CaCl_2$ and 0.13 mg/L $FeCl_3$ at pH 7.2) at 0.05 measured at 600 nm. Cells were then incubated for 14 hours at the same incubation conditions.

3. Following the starvation period, cells were resuspended in various media (MSM, reservoir's water and 4-fold diluted R2A liquid medium) containing 1 mg/L of MC extract at 0.05 measured at 600 nm (10^8 CFU · mL^{-1}). See TOC concentration from different mediums at Table 1.
4. Incubation was carried on under previously described conditions for 120 hours. Samples were collected at different time intervals for MC quantification by using Liquid Chromatography/Mass Spectrometry (LC/MS).

Table 1. Total organic carbon (TOC) concentrations in different mediums. Errors refers to standard deviation (n=3)

MSM medium	Reservoir water	¼ R2A medium
< 1.00	3.83 ± 0.02	258.37 ± 0.06

The isolated bacteria *Sphingopyxis sp*. strain CECT 8967 was able to degrade efficiently 1 ppm of MC extract containing MC-LR, MC-RR and MC-YR in MSM, reservoir's water and 4-fold diluted R2A liquid medium. This strain removed the 90% of MC between 6 and 9 h of incubation regardless of the media used, while *Sphingosinicella microcystinivorans* Y2 (Park et al., 2001, Environ. Toxicol, 16:337-43) and *Paucibacter toxinivorans* 2C20 (Lathi et al., 1997, Water Res., 31: 1005-1012; Rapala et al., 2005, Int. J. Syst. Evol. Microbiol, 55: 1563-1568) (purchased from Leibniz-Institut DSMZ GmbH, and used here as controls for comparison of degradation rates) needed between, at least, 12 and up to 120h. These results demonstrate the high capacity of our isolate for MC removal.

The average degradation rate of *Sphingopyxis sp*. strain CECT 8967 was 155.81, 198.92 and 123.13 μg · L^{-1} · h in MSM, reservoir's water and 4-fold diluted R2A liquid medium, respectively (Table 2), calculated by averaging the rates at each time interval until the 90% of MC concentration was degraded. The strain Y2 showed an average degradation rate of 79.32, 65.00 and 91.03 μg · L^{-1} · h^{-1} in MSM, reservoir's water and 4-fold diluted R2A liquid medium, respectively, whilst 2C20 displayed an average rate of 15.68, 24.61 and 12.98 μg · L^{-1} · h^{-1} in the same media. It is important to underline the low efficiency of Y2 and 2C20 strains for MC degradation in reservoir's water, being 3-folds and 8-folds lower than IM-1, respectively.

The obtained data demonstrate the high degradation capacity of *Sphingopyxis sp*. strain CECT 8967 regardless of the concentration of total organic carbon present in the solution.

Table 2. Microcystins-degradation capacity by bacteria CECT 8967, Y2 and 2C20. Data is represented by ne value (n=1)

Degradation rate (µg MCs · L^{-1} · h^{-1})			
Strain	**MSM**	**Reservoir water**	**¼ R2A medium**
CECT 8967	155.81	198.92	123.13
Y2	79.32	65.00	91.03
2C20	15.68	24.61	12.68

Genetic analysis of the 16S rRNA partial gene of *Sphingopyxis sp*. strain CECT 8967 shows high similarity with MC-degrading strains *Sphingopyxis sp.* C-1 (Okano et al., 2009, J. Toxicol., 954291) and *Sphingopyxis sp.* USTB05 (Zhang et al., 2010, J. Envirom. Sci., 22: 168-175), as well as with non-reported MC-degrading strains *Sphingopyxis sp.* NUG4-1, *Sphingopyxis chilensis* DSR12 and *Sphingomonas sp.*IMER-A1-19, among others). The strain C-1 is a pH-dependent MC degrader, able to remove in three hours 1 mg/L of MC-LR in 0.1 phosphate buffer (pH=6.72) with 0.5 of initial O.D. at 600 nm. Apparently, this degradation rate of 333.33 µg · L^{-1} ·h^{-1} is greater than what we report for *Sphingopyxis sp*. strain CECT 8967, however a closer look to the results shows that the former strain was only tested with pure MC-LR and only in buffer; conditions that do not correspond to real life scenarios.

In the same way, strain USTB05 was demonstrated to be able to remove 50.2 mg/L of pure MC-RR in 3 days in a 50 mM potassium phosphate buffer and using intracellular enzyme extract instead of living cells. It is also worth noting that experiments done using that high concentration of MC hugely exceed the limits of reality, where in average common concentrations during a cyanobacterial bloom can be 1-500 µg/L. Experimental setup with MC concentrations near or slightly higher to what is commonly found during and after a cyanobacterial bloom provide better information for a MC degradation process to overcome a real problem of toxicity. Moreover, cyanobacterial breakdown at the end of the bloom period always causes the release of a mixture of different MC variants. In this sense, experiments performed with pure toxins do not represent the reality for an issue of water-borne toxins and their removal. Accordingly, we tested and we suggest the use of MC-extract

rather than pure MC variants when testing strain capacity for MC degradation as a method for MC removal during water treatment.

In the biological treatment for the removal of MC in water, the presence of other organic carbon can slow down or totally impair MC degradation due to inherited different affinity of the biodegraders to carbon sources present in water. The use of *Sphingopyxis sp*. strain CECT 8967 overcomes this situation through its metabolic versatility, since it is able to degrade MC with high efficiency regardless of the different total organic carbon concentrations present in the aqueous water. Moreover, the high potential of *Sphingopyxis sp*. strain CECT 8967 for degrading MC in reservoir's water with a natural MC-extract, and its ability to decrease the MC concentration under the guideline threshold value (WHO, 1998), raises the strain as a promising biological tool to solve global problems with MC toxicity in water.

EXAMPLE 2: Isolation of MC-degrading bacteria using an optimized method

Sampling

1. Surface water samples (1 L) were collected from a local reservoir during a cyanobacterial bloom period with sterile polyethylene bottles. Samples were stored in dark at 4ºC during transport.

Enrichment assay

2. Duplicate water samples (20 ml) were enriched with 1 mg/L of MC extracted from a cyanobacterial bloom. MC extraction was carried out following methanol extraction method, purified through Solid Phase Extraction method (SPE), dried and finally eluted in sterile Milli-Q water. Samples were then incubated at 27 ºC in the dark at 120 rpm for 15 days. Negative controls were prepared in duplicate using autoclaved reservoir´s water.
3. MC concentration at 0 and 15 days of incubation was quantified as by LC-MS. Only samples showing degradation activity were selected.

Bacterial isolation

4. After 15 days of incubation, ten-fold serial dilutions of each sample showing degradation activity were prepared and 100 µL from each dilution were plated on R2A medium (Sigma). Plates were incubated at 27ºC in the dark for 7 days.

5. After 7 days of incubation, colonies with different morphology and color were selected and purified using standard purification method, such as streak plate method.

Biodegradation assay

6. Each isolated colony and positive controls were transferred into R2A liquid medium and incubated under same incubation conditions till late exponential phase. From each liquid culture 100 µL was washed and resuspended in 4-fold diluted R2A liquid medium containing 1 mg/L of MC extract to get a final O.D of 0.2-0.6 at 600 nm.
7. A sterile 96-well plate was prepared with 150 µL of 4-fold diluted R2A liquid medium with 1 mg/L of MC extract and the previous bacterial solution from point 6 was added. A negative control was included.
8. Plates were covered with a sterile film and incubated at 27ºC in the dark at 120 rpm for 6 days.
9. MC concentration was quantified at time 0 and after 6 days of incubation as previously described in step 3.

Our developed method possesses different essential steps for the isolation of indigenous MC-degrading bacteria with highly different metabolisms. This process comprises: sampling, enrichment, bacterial isolation and biodegradation assay.

Sampling was performed in a waterbody with historical bloom occurrences to increase the success of finding bacteria with the ability of using MC as a carbon and/or nitrogen source. Once in the laboratory, the *enrichment step* with the addition of a MC crude extract causes a positive selection of MC-degrading bacteria from the large bacterial population inhabiting water samples. After 15 days, degradation activity was analyzed and *isolation* of potentially MC-degrading bacteria was performed on solid medium according to their morphology. In the *biodegradation assay*, single bacterial colonies were checked simultaneously in a 96-well plate for MC degrading capacity in a diluted-rich medium (4-fold diluted R2A liquid medium) with MC extract. This step provides an advantage for the isolation of MC-degrading bacteria with highly different metabolism with regard to the methods already described. The diluted-rich medium offers an input of nutrients which enhances bacterial growth while still allowing metabolically active cells to degrade microcystins. The R2A medium was chosen for the isolation method because of its features, which allows growth of bacteria after a stress period.

Most of the studies agree with sampling in places where the MC occurrence is known, but great differences are found for the rest of the steps of the isolation method. Some studies consider the MC enrichment as a non-necessary step. However, it is important for positively selecting the MC-degrading population over the rest of the bacterial populations in the water samples. Moreover, MC enrichment in the own natural water sample allows indigenous bacteria to grow without exposing them to further stress compared to enrichments carried on in artificial media. Performing several enrichment subcultures is widely used as a technique for increasing MC-degrading populations in the sample. However, it causes unnecessarily extension of the incubation periods and higher microcystin consumption, thus increasing the overall cost (especially when pure MC is used).

In the isolation process, some authors prepare plates with diluted-rich media or minimal media containing MC to enhance the isolation of MC-degrading bacteria, but the addition of toxin is not strictly necessary if the previous enrichment step is performed with proper concentration of MC and incubation time. Besides, bacterial transference between liquid and solid mediums causes stress conditions that could lead to limited diversity.

Several strategies have been performed to identify MC-degrading bacteria during the isolation process. Most of these experiments were done on minimal media containing MC, which could avoid the growth of some MC- degrading bacteria if they are not capable of using MC as their sole C or N source. Furthermore, nutrient rich media are also not convenient because they may lead to an extended lag phase periods and providing false negatives. To avoid these problems, diluted-rich mediums (4-fold diluted R2A) add a suitable input of nutrients to enhance bacterial growth without disturbing MC degradation activity and shortening the lag phase period. In general, reported biodegradation assays are limited by the use of large volumes, which increases costs of reagents and time to perform the assay on a large number of potentially MC-degrading isolates. In this sense, simultaneous assays on 96-well plates show a great advantage because it allows the simultaneous testing of higher number of bacteria at the same time, reducing time and costs. In the literature, only one simultaneous assay has been described to check for MC degradation (Manage et al., 2009, Appl. Env. Microbiol., 75:6924-6928). It was performed in BiologMT(a 96-well microplate based on a colorimetric measurement) with MC as a single carbon source in a matrix with minimal medium, which may lead to the consequences explained above. However, in our invention we bring a new approach using diluted-rich medium in 96-well plates, reducing time and costs of the assay and minimizing the risk of losing any potentially MC-degrading bacteria.

In the literature, there is no consensus on the concentration of MC used during the enrichment, isolation or during the biodegradation assay. The concentration used ranges from 0.1 to 50,200 µg/L. Moreover, there is no consensus on the use of purified or crude-extract of MC. The use of MC extract from a toxic cyanobacterial bloom mimic the natural conditions at laboratory scale, as well as reduces costs. Most of the experiments have been performed with pure MC-LR since it is the most common and toxic variant described. However, other isoforms are also found in the environment. Our invention leads to the isolation and identification of bacteria capable of degrading the main MC variants (MC-LR, -RR and –YR), and even other MC that may be present in the water, at low cost.

The present invention allows the isolation of a wide range of MC-degrading bacteria with different degradation rates. The low percentage of MC-degrading population from the total bacterial community in the waterbody supposes a challenge for the isolation process. Our described method allowed us to identify 25 strains with different MC degradation ability (Figure 2) whilst in most of the studies just a single bacterium was reported. Compared with other isolation methods, we achieved to isolate the highest number of degrading bacteria reported with various biodegradation ability that could be used not only as an efficient and environmental-friendly method for MC-removal but also for studying the metabolic pathway(s) of MC degradation allowing us to better understand and sustainably preserve such an important environmental process.

CLAIMS

1. A microorganism from the species *Sphingopyxis sp*. deposited in the Spanish Type Culture Collection (CECT) with accession number CECT 8967 or a mutant thereof which substantially maintains the ability of degrading microcystins.

2. A biologically pure culture of a microorganism according to claim 1, a biomass comprising the microorganism according to claim 1, a bacterial consortium comprising the microorganism of claim 1 or a cell extract of a microorganism according to claim 1.

3. A method for obtaining a biomass of the microorganism of claim 1 comprising culturing said microorganism under conditions suitable for growth.

4. A method for preventing the accumulation or for reducing the content of microcystins present in an aqueous medium which comprises adding to said medium a microorganism according to claim 1 or a biologically pure culture, a biomass, a bacterial consortium or an extract according to claim 2 and maintaining the culture under conditions suitable for the degradation of said microcystins.

5. The method according to claim 4 wherein the microorganism or the biologically pure culture, the biomass, or the bacterial consortium has been expanded in a culture medium comprising at least one carbon source and at least one nitrogen source in appropriate conditions suitable for growth.

6. The method according to claims 4 or 5 wherein the microorganism, the biologically pure culture, the biomass, or the bacterial consortium has been maintained prior to the addition to the aqueous medium containing microcystins under nutrient starvation conditions.

7. The method according to any of claims 4 to 6, wherein the concentration of organic carbon in the aqueous medium is from <1 to 300 mg/L.

8. The method according to any of claims 4 to 7 wherein said aqueous medium comprises microcystins in a concentration from 0.1 µg/L to 50,000 µg/L.

9. The method according to any of claims 4 to 8, wherein said microcystins are selected from the group consisting of microcystin-RR, microcystin-LR, microcystin-YR and combinations thereof.

10. The method according to any of claims 4 to 9, wherein the conditions suitable for degrading microcystins comprise:
    - temperature in range between 15ºC and 35ºC
    - aerobic conditions
    - pH between 6 and 8 and/or
    - incubation time between 1 and 120 hours.

11. Use of the microorganism according to claim 1, or a biologically pure culture, a biomass, an extract or a bacterial consortium according to claim 2 for preventing the accumulation or for reducing the content of microcystins present in an aqueous medium.

12. A method for isolating a microcystin-degrading microorganism from an aqueous medium comprising:
    i) adding microcystins to said aqueous medium at an appropriate concentration and incubating the sample under conditions suitable for the growth of microcystin-degrading microorganisms present in the sample, thereby enriching said sample in microcystins degrading microorganisms;
    ii) culturing the microorganism of the sample of step i) on a solid culture medium comprising at least one carbon source and at least one nitrogen source during an appropriate incubation time until colonies are obtained,
    iii) culturing the microorganisms from the colonies obtained on step ii) on a culture medium comprising at least one carbon source and at least one nitrogen source wherein said at least one carbon source and at least one nitrogen source are present in a concentration which is about or lower than the concentration which results after dilution which is at least two-fold dilution of the concentration of said components in the culture media of step ii) in the presence of microcystins; and
    iv) selecting microcystin-degrading microorganisms as the microorganisms which grow after the culture step iii)

13. The method according to claim 12, wherein said aqueous sample is freshwater.

14. The method according to any of claims 12 or 13 wherein the appropriate concentration of microcystins of step i) is 0.5 mg/L and wherein the appropriate concentration of microcystins of step iv) is from 20 µg/L to 50 mg/L.

15. The method according to any of claims 12 to 14, wherein the appropriate conditions suitable for the growth of microcystin-degrading microorganisms in step i) comprise:

- temperature in range between 15ºC and 35ºC
- aerobic conditions and/or
- incubation time between 1 and 20 days.

16. The method according to any of claims 12 to 15, wherein the culturing step iii) is carried out between 4 and 10 days.

ABSTRACT

The invention relates to a bacterial strain capable of degrading microcystins in an aqueous medium. A method for reducing the content or for preventing the accumulation of microcystins in an aqueous medium is also disclosed. Finally, the present invention relates to an efficient method for isolating microcystin-degrading microorganisms.

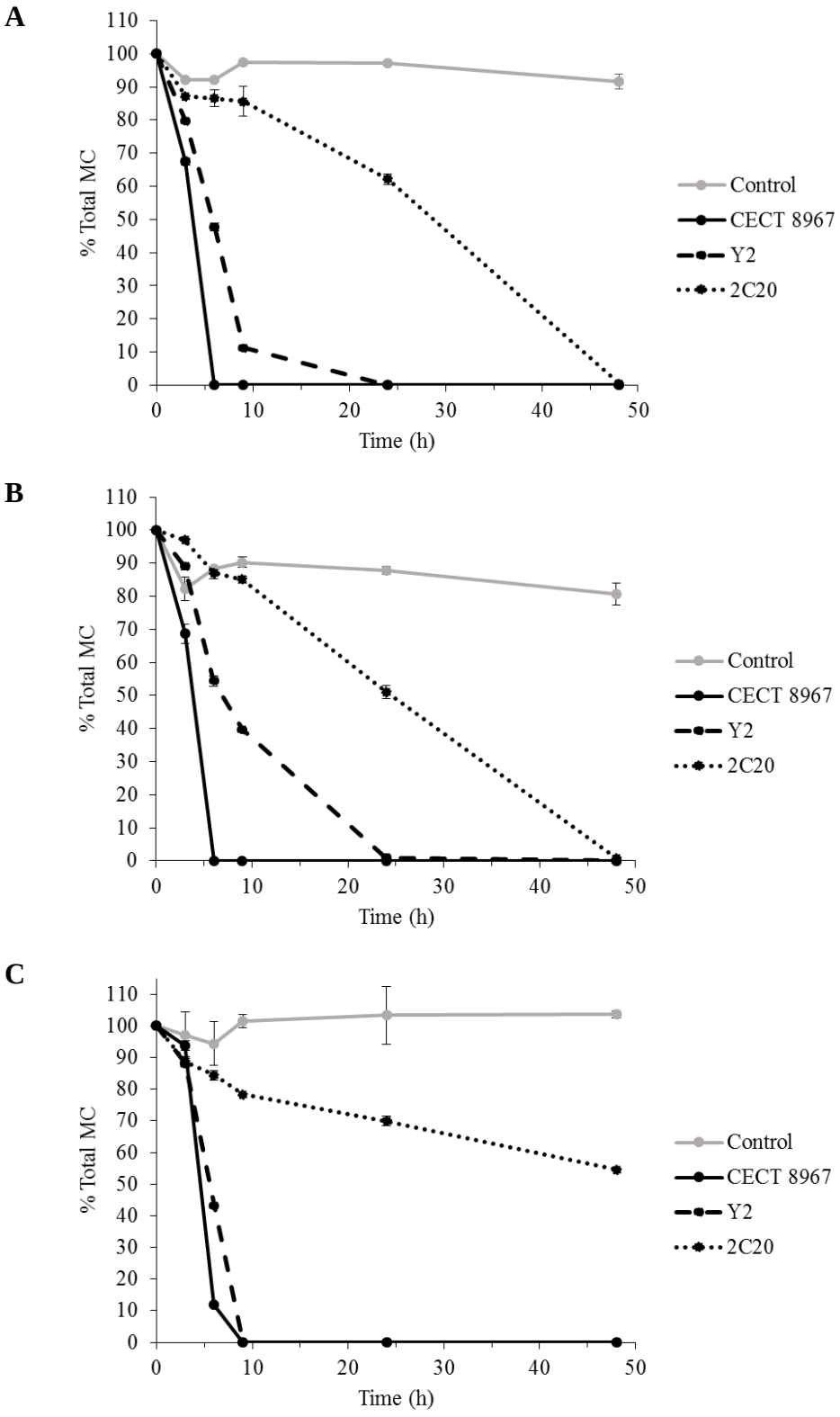

FIGURE 1

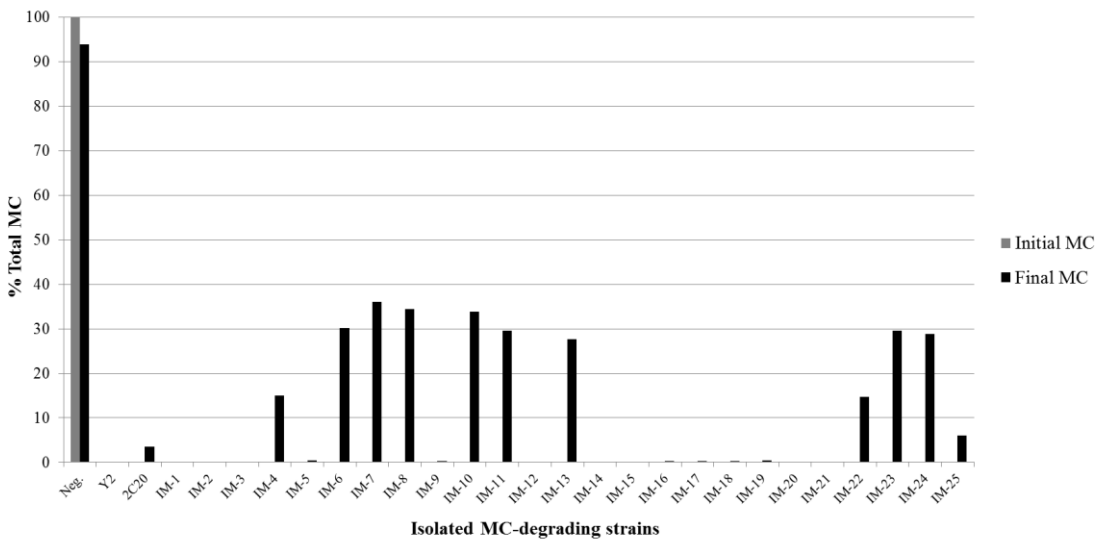
100
90
80
70
60
50
40
30
20
10
0
% Total MC
Neg.
Y2
2C20
IM-1
IM-2
IM-3
IM-4
IM-5
IM-6
IM-7
IM-8
IM-9
IM-10
IM-11
IM-12
IM-13
IM-14
IM-15
IM-16
IM-17
IM-18
IM-19
IM-20
IM-21
IM-22
IM-23
IM-24
IM-25
Isolated MC-degrading strains
Initial MC
Final MC

FIGURE 2