Interspecies interplay between cyanobacteria and green algae - the first step to know why cyanobacteria produce what we call "Toxins"

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LIST OF MANUSCRIPTS

This doctoral thesis is presented in a cumulative form and is based on the following three manuscripts, referred to in the text by their corresponding Roman numerals. The three manuscripts, which form separate chapters in the thesis, have been published in international peer-reviewed journals.

I

Still challenging: the ecological function of the cyanobacterial toxin microcystin – What we know so far.

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Interspecies interactions between *Microcystis aeruginosa* PCC 7806 and *Desmodesmus subspicatus* SAG 86.81 in a co-cultivation system at various growth phases.

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III

***Desmodesmus subspicatus* co-cultured with microcystin producing (PCC 7806) and the non-producing (PCC 7005) strains of *Microcystis aeruginosa*.**

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LIST OF CONFERENCES

I

Allelopathic interactions between *Microcystis aeruginosa* 7806 and *Desmodesmus subspicatus* in a co-culture experimental model.

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Co-cultivation of *Microcystis aeruginosa* PCC 7806 and *Desmodesmus subspicatus* SAG 86.81 new insights into the ecological implication of cyanobacterial toxin "Microcystin."

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ABSTRACT

Over the past decades, cyanobacteria have attracted the researcher's attention due to their ability to produce cyanotoxins. Among cyanotoxins, microcystins (MCs) are the most commonly studied. However, their significance for the producing species beyond their toxicity is still mysterious (was reviewed in Paper I). In the present study, the interactions between a common bloom-forming cyanobacterium "*Microcystis aeruginosa*" and a green alga "*Desmodesmus subspicatus*" were studied to provide more insights into the ecological importance of the algal secondary metabolites concerning MCs. To achieve this, a dialysis co-cultivation system was established. First, *M. aeruginosa* PCC 7806 and *D. subspicatus* were co-cultured (1:1) at different phases of growth (Paper II). Second, *D. subspicatus* was co-cultured with *M. aeruginosa* PCC 7806 and PCC 7005 (toxic and non-toxic strains, respectively) that were grown to the stationary phase of growth (Paper III). As a result, the concentration of intracellular and extracellular (inner and outer membrane) MC-LR was quantified using liquid chromatography-tandem mass spectrometry and the cell density was monitored using a haemocytometer under the light microscopy. The growth of *M. aeruginosa* PCC 7806 and *D. subspicatus* in the culture filtrates of each other and the effects of toxic *M. aeruginosa* crude extracts on the growth of *D. subspicatus* were studied as well (Paper II). The results provided clues to the involvement of the algal secondary metabolites in the interspecies interplay. The growth of the two species was inhibited in the culture filtrates of one another (> 25% exudates). The MC-containing crude extracts (> 140 µg/L) inhibited the growth of green alga. Moreover, the co-cultured species influenced the growth of each other negatively depending on the stages of the growth. During the exponential phase of growth, the green alga inhibited the growth and MC production but has not influenced the MC release. While, at the stationary phase of growth, MC production and release were enhanced, coinciding with the inhibition of the growth of green alga (Paper II). Moreover, MC-producing strain excluded the green alga greater and earlier compared to non-toxic strain. The MC-producing *M. aeruginosa* benefited from MC, in addition to or rather than the other secondary metabolites, to improve its fitness and the greater exclude of the green alga. However, inhibition of the growth of green alga at the concentrations of MC-LR above the environmental levels may explain the frequent co-existence of the green algae with cyanobacterial blooms. Moreover, the results indicated that the diffusion rate of extracellular MC-LR across the dialysis membrane was positively related to the time and negatively to the concentration of extracellular MC-LR.

ZUSAMMENFASSUNG

In den letzten Jahrzehnten haben Cyanobakterien viel Aufmerksamkeit auf sich gezogen, weil sie Cyanotoxine produzieren können. Die am häufigsten untersuchten Cyanotoxine sind die Microcystine (MCs). Neuere Studien haben die Rolle der MCs in den ökologischen und physiologischen Prozessen herausgestellt (Publikation I). In der vorliegenden Studie wurden die Wechselbeziehungen zwischen dem Cyanobakterium *Microcystis aeruginosa* und der Grünalge *Desmodesmus subspicatus* untersucht, um die ökologische Rolle der Algen-Sekundärmetaboliten, insbesondere der MCs, zu klären. Daher wurde ein Dialyse-Co-Kultursystem entworfen. Zuerst wurden die MC-produzierende *M. aeruginosa* PCC 7806 und *D. subspicatus* in verschiedenen Wachstumsstadien co-kultiviert (Publikation II). Zusätzlich wurde *D. subspicatus* mit den toxischen und nichttoxischen Stämmen von *M. aeruginosa* (PCC 7806 und PCC 7005), die bis zur stationären Wachstumsphase gezüchtet wurden, co-kultiviert (Publikation III). Die Konzentration des intrazellulären und extrazellulären (innere und äußere Membranen) MC-LR wurde mit der LC-MS/MS quantifiziert. Die Zelldichte der Arten wurde mit der Zählkammer und der Lichtmikroskopie gezählt. Anschließend wurde das Algenwachstum in den Kulturfiltraten gemessen und die Auswirkungen des Rohextrakts von toxischem *M. aeruginosa* auf das Wachstum der Grünalge bestimmt. Die Ergebnisse liefern Hinweise auf die Beteiligung der Algen-Sekundärmetaboliten an den interspezifischen Wechselwirkungen. Das Algenwachstum wurde in den Kulturfiltraten bei erhöhtem Anteil der Filtrate gehemmt. Das MC-haltige Rohextrakt von *M. aeruginosa* hemmte auch das Wachstum der Grünalge. Die co-kultivierten Arten wurden in Abhängigkeit von den Wachstumsphasen negativ beeinflusst. Während der exponentiellen Wachstumsphase inhibierte die Grünalge das Wachstum und die MC-Produktion von *M. aeruginosa*. In der stationären Wachstumsphase wurde die MC-Produktion und die Freisetzung von *Microcystis* gesteigert und das Wachstum der Grünalgen gehemmt. Die MC-produzierende Stamm von *M. aeruginosa* hemmte das Wachstum der Grünalge früher und in höherem Umfang als der MC-defiziente Stamm. Die MC-produzierende *Microcystis* profitierte vom MC in der Kombination mit oder anstelle der anderen Sekundärmetaboliten, mit dem Effekt, ihre Fitness zu verbessern und die Grünalge auszukonkurrieren. Die Hemmung des Grünalgenwachstum bei Konzentrationen über dem Umweltniveau der MC-LR kann jedoch die Koexistenz der Grünalgen und der Cyanobakterien erklären. Andererseits zeigten die Ergebnisse, dass die Diffusionsrate des MC-LR positiv abhängig von der Zeit und negativ von der Konzentration des extrazellulären MC-LR war.

ABBREVIATIONS

ACN	acetonitrile
ANOVA	analysis of variance
Chl	chlorophyll
d	day (s)
DIC	dissolved inorganic carbon
EDTA	ethylenediaminetetraacetic acid
EPS	extracellular polysaccharides
h	hour
kDa	kilodalton
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
lm	lumen
MC	microcystin
MC-LR	microcystin-LR
MeOH	methanol
min	minute (s)
MWCO	molecular weight cut-off
m/z	mass to charge ratio
NADPH	nicotinamide adenine dinucleotide phosphate
OATP	organic anion transporting polypeptide
O/I	outer to inner
pH	pondus hydrogenii
rpm	rounds per minute
SD	experimental standard deviation of the mean
SE	experimental standard error of the mean
S/N	signal to noise
sp.	species
SPSS	statistical package for social sciences
TFA	trifluoroacetic acid
°C	degree Celsius
cm	centimeter

fg	femtogram
µg	microgram
mg	milligram
ng	nanogram
µM	micromolar
mM	millimolar
L	litre
µl	microlitre
mL	millilitre
C	carbon
Fe	iron
N	nitrogen
O	oxygen
P	phosphorus

1 INTRODUCTION

1.1 Cyanobacteria - general characteristics and causes of bloom formation

Cyanobacteria, also known as blue-green algae, are gram-negative photosynthetic prokaryotes resembling both bacteria and algae. Similar to bacteria, they contain a double-stranded circular chromosome and a cell wall composed of peptidoglycan and lipopolysaccharide layers and lack internal organelles (Castenholz, 2015). In common with eukaryotic algae, they have a photosynthetic system and carry out oxygenic photosynthesis (Skulberg, 1993; Hoiczyk and Hansel, 2000; Mulkidjanian et al., 2006). They are the first photosynthetic organisms that have been involved in the increased oxygen level in the atmosphere 2.32 - 2.45 billion years ago (Rasmussen et al., 2008; Schirrmeister et al., 2011). In addition to chlorophyll a and carotenoids, cyanobacteria possess specific accessory photosynthetic pigments known as phycobilins containing phycoerythrin, phycocyanin, and allophycocyanin, which are organized into phycobilisomes (Maccoll, 1998; Adir, 2005). Some species of cyanobacteria have the ability of atmospheric nitrogen fixation as well, then significantly contribute to the nitrogen cycle (Stal, 2001; Berman-Frank et al., 2003).

Morphologically, cyanobacteria are highly variable from unicellular and colonial forms to filamentous and branched filaments (Whitton, 2012). They do not have flagella, but some filamentous species, such as *Oscillatoria* sp., can move actively over the surfaces by gliding (Hoiczyk, 2000; Mcbride, 2001). Many species contain gas vesicles, enabling them to regulate the buoyancy, and changing their vertical position in the water column to get the higher irradiance of light supply and the limited resources and reduced the sedimentation loss (Walsby et al., 1997).

Cyanobacteria are found in a wide range of environments on Earth such as the ice fields in polar and alpine regions, fresh and marine waters, thermal springs and deserts (Codd, 1997; Ward et al., 1998; Wynn-Williams, 2000; Krienitz et al., 2005; Sompong et al., 2005; Singh and Elster, 2007; Bhaya et al., 2012). Under favorable growth conditions such as high levels of nutrients, especially nitrogen (N) and phosphorus (P), abundant sunlight, and warm water temperature (> 25°C), they dominate the phytoplankton community and develop into massive blooms (Paerl and Otten, 2013). Eutrophication of water bodies that have resulted from the

increased nutrient inputs by anthropogenic activities such as fertilizer run-off, discharge of untreated sewage and inadequate management of watersheds, in combination with the global warming and high light intensity became a driving force for heavy cyanobacterial blooms formation (Paerl and Otten, 2013; Wells et al., 2015; Scholz et al., 2017). Cyanobacterial blooms contain a mixture of toxin and non-toxin producing species. Global warming and eutrophication caused a noticeable shift towards more toxic blooms (Davis et al., 2009). The occurrence of toxic cyanobacterial blooms with undesirable effects on aquatic animals, livestock, and humans, have been frequently reported in many countries around the world (Zanchett and Oliveira-Filho, 2013; Lévesque et al., 2014; Svirčev et al., 2017).

1.2 Cyanobacterial secondary metabolites - cyanotoxins

Cyanobacteria are good sources of a wide range of toxic secondary metabolites designated as cyanotoxins which can be enclosed within the cells (intracellular toxins) or released into the surrounding waterbody (extracellular toxins), depending on the nature of toxin, due to the leakage of the intracellular toxin at various stages of the growth or by cell lysis (Sivonen and Jones, 1999; Cordeiro-Araújo and Bittencourt-Oliveira, 2013). Cyanotoxins include a diverse group of organic compounds from the chemical and toxicological points of view. Structurally, they fall into three main groups: cyclic peptides, heterocyclic compounds (alkaloids), and lipopolysaccharides (Sivonen and Jones, 1999; Kaebernick and Neilan, 2001). According to their biological activity, they can be classified into five groups: hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and irritant toxins (Codd et al., 2005; Wiegand and Pflugmacher, 2005; Smith et al., 2008). Table 1.1 shows the important cyanotoxins representing the various cyanotoxin types, their biological activity, and the most common producing genera.

Table 1.1: Cyanotoxins, their biological modes of action, and representative producers.

Toxin classification	Toxins	Most common producing genera	Biological modes of action	References
Hepatotoxins	Microcystins Nodularins	*Microcystis* *Planktothrix* *Anabaena* *Nodularia* *Nostoc* *Hapalosiphon*	Inhibition of protein phosphatase 1 and 2A, severe liver disruption	Mankiewicz et al. (2003) and Bláha et al. (2009)

Neurotoxins	Anatoxin-a Anatoxin a-(s) Saxitoxin	*Anabaena* *Aphanizomenon* *Nostoc* *Oscillatoria*	Interfere with the function of neuromuscular systems	Funari and Testai (2008)
Cytotoxins	Cilyndrospermopsins	*Cylindrospermopsis* *Umezakia* *Aphanizomenon* *Raphidiopsis* *Anabaena*	Inhibition of protein synthesis, damages in liver, kidneys, spleen, thymus, and heart, DNA damage	Mankiewicz et al. (2003), Valério et al. (2010), and De La Cruz et al. (2013)
Dermatotoxins	Aplysiatoxins Debromoaply Siatoxins Lyngbyatoxins	*Oscillatoria* *Lyngbya* *Schizothrix*	Protein kinase C activators, dermatitis as inflammatory agents	Mankiewicz et al. (2003)
Endotoxins (Irritants)	Lipopolysaccharides of the outer membrane	Cyanobacteria in general	Allergy, skin and respiratory disease, gastrointestinal inflammation	Bláha et al. (2009) and Durai et al. (2015)

Of the cyanobacterial genera that include toxin-forming and -deficient species, the ones of concern when mass populations occur include *Microcystis* sp. that can produce microcystins (MCs), which are the most abundant and consequently the most prevalently studied of the cyanobacterial toxins.

1.3 MCs - structure and their biological modes of action

MCs were first isolated from *Microcystis aeruginosa*, the organism from which MCs take their names (Carmichael et al., 1988). Other *Microcystis* species such as *M. viridis*, *M. wesenbergii*, and several cyanobacterial genera produce MCs as well (Table 1.1). However, *M. aeruginosa* is the species most commonly identified with freshwater cyanobacterial toxic blooms (Carmichael, 2001).

MCs are a group of cyclic heptapeptides composed of seven different amino acids, five nonprotein, and two protein amino acids, with the general structure of cyclo (D- Ala^1 – X^2 – D-$MeAsp^3$ – Z^4 – $Adda^5$ – D-Glu^6 – $Mdha^7$), in which variable L-amino acid residues are found at the X and Z positions which make the differentiation between isoforms of MCs, D-MeAsp is D-erythro methylaspartic acid, Adda is a unique β -amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid and Mdha is N-methyl dehydroalanine (Fig. 1.1). Using sensitive mass spectrometric techniques, the number of known MCs congeners is

increasing rapidly. In recent years, more than 200 MC variants have been identified from cyanobacterial blooms and cultures (Stirling and Miles, 2016). The most common MCs are MC-LR, MC-RR and MC-YR, containing L-amino acids leucine (L), arginine (R) or tyrosine (Y), respectively, at the X position (Fig. 1.1).

Among the MCs congeners, MC-LR which contains the amino acids leucine (L) and arginine (R) at the variable X and Z positions, is the most frequently reported and one of the most hepatotoxic congeners (Harada, 1996; Chorus and Bartram, 1999; Pearson et al., 2010; Ufelmann et al., 2012; Yu et al., 2014).

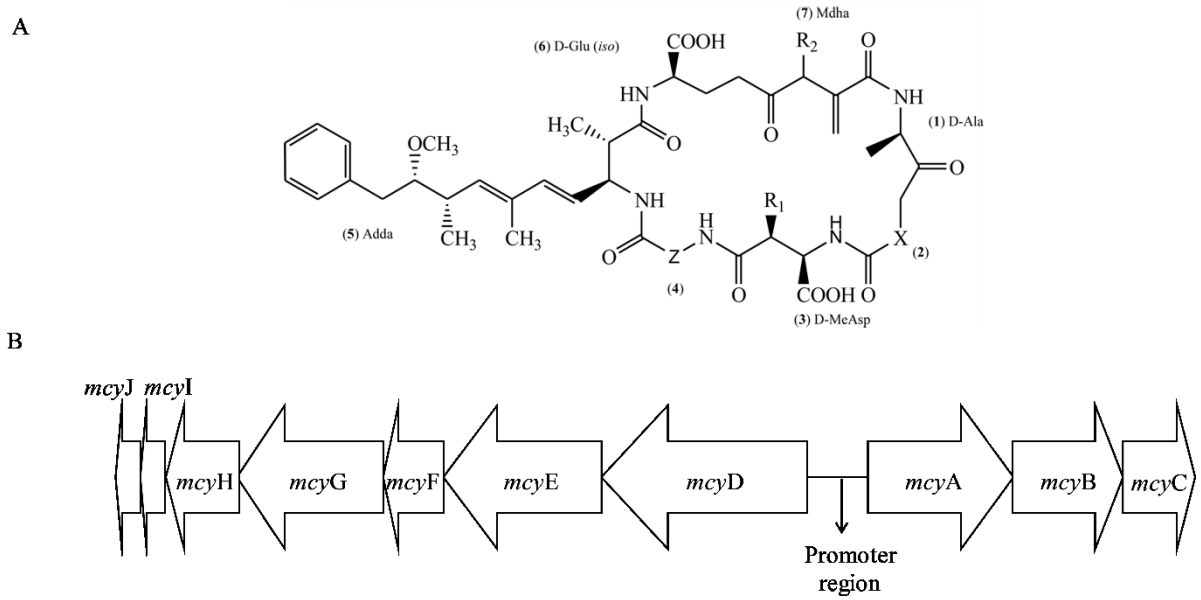

Fig. 1.1: Chemical structure of MCs (A), and MC synthesis gene cluster (*mcy*) (B).

In mammals, MCs pass through the membrane of hepatocytes *via* the ATP dependent membrane transporters (organic anion transporting polypeptide, OATP) (Fischer et al., 2005; Feurstein et al., 2009) and inhibit the eukaryotic serine/threonine protein phosphatase 1 and 2A through the strong covalent binding of their Mdha residue to these enzymes (Honkanen et al., 1990; Mackintosh et al., 1990). Consequently, higher phosphorylation of target proteins occurs that leads to the hyperphosphorylation of the intermediate filaments of the cytoskeleton and destruction of liver cells that results in the intra-hepatic hemorrhage, or hepatic insufficiency (Yoshizawa et al., 1990; Falconer and Yeung, 1992). Besides, MCs interact with mitochondria in animal cells, which can result in the induction of oxidative stress and cell apoptosis (Ding et al., 2000; Žegura et al., 2003; Campos and Vasconcelos, 2010).

1.4 Significance of MCs for the producing organisms

Over the past decades, a lot of efforts have been actively made to conduct toxicological studies concerning the effects of MCs on humans, animals, and aquatic biota. However, regarding the evolutionary time scales, cyanobacteria have evolved years before the advent of eukaryotic organisms. On the other hand, MCs are synthesized non-ribosomally *via* a giant multifunctional complex enzyme (MC synthetase) (Tillett et al., 2000); then, MC-producing species pay a high price for MC production.

Nowadays, understanding of the natural functions of MCs rather than their toxicity has received growing attention. Recent studies revealed the physiological and ecological significance of MCs for the producing species (was reviewed by Omidi et al. (2017), Paper I). A schematic view of the proposed intra- and extracellular functions of MCs is shown in Fig. 1.2.

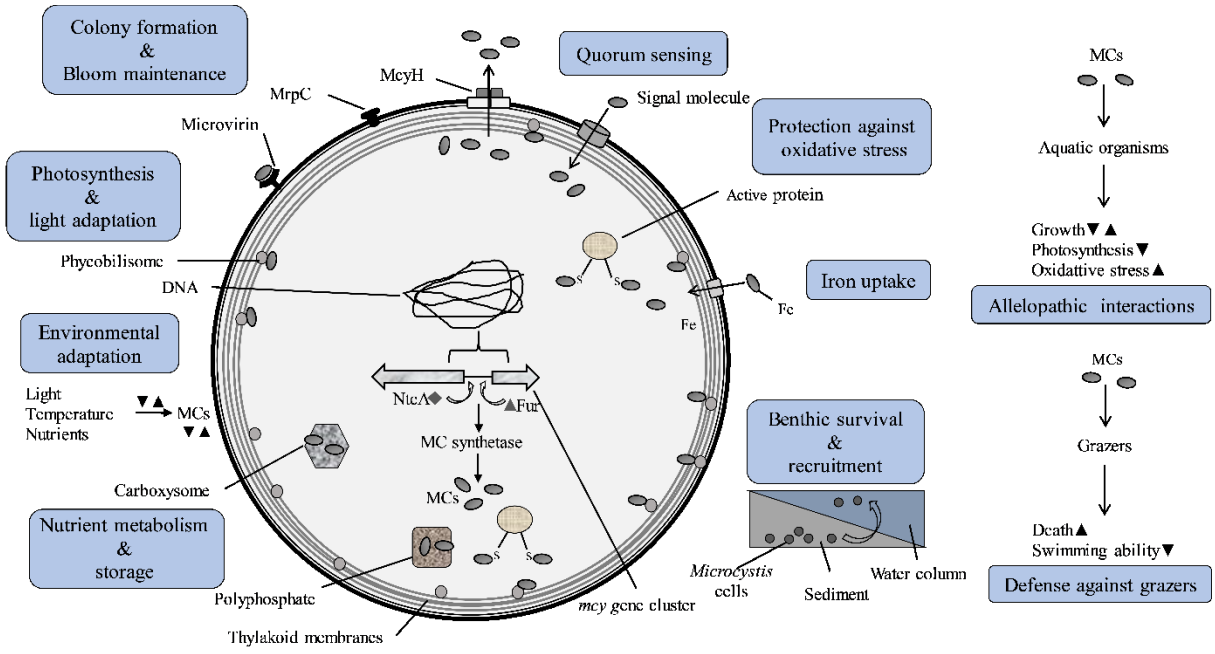

Fig. 1.2: A schematic illustration of the functions of MCs in *Microcystis aeruginosa.*

MCs were mostly found attached to the thylakoids membranes (Shi et al., 1995; Young et al., 2005). Moreover, MC production was regulated by the light quantity (Utkilen and Gjølme, 1992; Wiedner et al., 2003). Under high light irradiancy, the toxic strain of *M. aeruginosa* PCC 7806 was more resistant (Phelan and Downing, 2011) and had greater photosynthesis compared to the mutant (Meissner et al., 2015), suggesting the role of MCs in photosynthesis and light adaptation.

The elevated water temperature stimulated the formation of *Microcystis* blooms towards more toxic blooms (Davis et al., 2009; Liu et al., 2011). Further studies showed that the increased temperature and high light intensity induced oxidative stress in *M. aeruginosa*, coinciding with the raised MC content and MC-protein bindings that nominated MCs as protein-modulating factors or radical scavengers under the stress conditions (Dziallas and Grossart, 2011; Zilliges et al., 2011).

Localization of MCs in polyphosphate bodies (Gerbersdorf, 2006; Young et al., 2008) and formation of MC-metal (zinc and copper) complexes (Humble et al., 1997) supported the hypothesis of MCs involvement in metal detoxification or storage.

MC synthesis was regulated by nutrient availability (Harke and Gobler, 2013). The binding site of "NtcA" (a global nitrogen regulator in cyanobacteria) and Fur (ferric uptake regulator proteins) was found in the promoter region of *mcy* gene cassette (Martin-Luna et al., 2006; Ginn et al., 2010). Under iron deficiency, toxic *M. aeruginosa* PCC7806 survived longer compared to the mutant (Alexova et al., 2011). However, the low affinity of MCs to ferric iron candidates MCs as iron shuttles (Klein et al., 2013).

The similarity between the sequence of *mcy* gene cluster in *M. aeruginosa* and quorum sensing genes in *Rhizobium leguminosarum,* as well as the light-regulated proteins of *M. aeruginosa* PCC7806 and signal mediator proteins of *R. leguminosarum* (Dittmann et al., 2001), nominated MCs as infochemicals in intracellular communications.

Survival of *Microcystis* sp. capable of MC synthesis in depth of the lake sediments (Ihle et al., 2005; Misson et al., 2012) and the greater recruitment of MC-producing *Microcystis* compared to non-toxic strain (Schöne et al., 2010) suggested the role of MCs in benthic survival and recruitments.

Binding of MCs to lectin microvirin (a surface-exposed protein) (Kehr et al., 2006) and their interferences with the stability of MrpC (a surface-exposed MC-related protein) (Zilliges et al., 2008), supported the hypothesis of MCs involvement in cellular aggregations.

The induction of colony formation and the increased MC production were reported as inducible defense strategies in several strains of *M. aeruginosa,* which were exposed to zooplankton and flagellate *Ochromonas* sp. (Jang et al., 2003; Yang and Kong, 2012).

In the end, MCs were known as allelochemicals due to the induction of oxidative stress and inhibition of the growth and photosynthesis of the phytoplankton species and aquatic plants (Pflugmacher, 2002, 2004; Leflaive and Ten-Hage, 2007; Granéli et al., 2008).

1.5 Dynamics of phytoplankton community composition

1.5.1 Factors influencing the structure of the phytoplankton community

Cyanobacteria are important members of the phytoplankton community, which contains other members such as diatoms, dinoflagellates, and a diverse group of algae as well (Reynolds, 2006). Concerning the increased occurrence of toxic cyanobacterial blooms, understanding the factors that induce a noticeable shift in the phytoplankton composition towards predominating of toxic bloom-forming cyanobacteria, is of growing interest.

The structure of phytoplankton community changes in seasonal cycles, a phenomenon called seasonal successions (Reynolds, 1980), where the species dominate the community in successive waves, according to the seasonal pattern. For instance, towards the end of winter and spring, diatoms; during the summer, green algae; and in the late summer and fall, cyanobacteria may become predominant (Sommer, 2012).

Previous studies indicated that the species composition of planktonic community changes depending on the seasonal variations of the environmental factors such as light and temperature as well as the nutrient availability, where the species with a greater competitive ability gain this advantage over other members of the community to dominate the phytoplankton populations (Passarge et al., 2006; Zhang et al., 2013; Sourisseau et al., 2017; Sommer, 2012). In response to the favorable environmental conditions such as high nutrient availability, abundant sunlight, and high temperature in freshwater and marine environments, cyanobacteria can successfully proliferate and form massive blooms (Huisman et al., 2018). The cyanobacterial biomass cycles, bloom formation and disappearance, caused changes in the environmental conditions that influenced the co-occurrence of the eukaryotic plankton, such as the green algae, which dominate the phytoplankton communities when the cyanobacteria were at low numbers (Liu et al., 2019).

Recent studies revealed that the structure of the phytoplankton community is influenced not only by the abiotic factors but also by the interspecies interactions through the bioactive compounds, called allelopathy (Legrand et al., 2003; Leflaive and Ten-Hage, 2007; Leão et al., 2009). As a result, the species capable of producing the bioactive compounds benefit from allelopathy to outcompete the other members of the phytoplankton community (Rengefors and Legrand, 2001; Legrand et al., 2003; Granéli et al., 2008).

Scientists believe that the algal succession is originated as a consequence of a complex interaction between the ecological processes and the interspecies interactions related to the

secondary metabolites that are produced for other purposes and act as allelochemicals (Legrand et al., 2003; Figueredo et al., 2007; Zhang et al., 2015).

In 1996, the International Allelopathy Society defined allelopathy as follows: "The science that studies any process involving secondary metabolites produced by plants, microorganisms, viruses, and fungi that influence the growth and development of agricultural and biological systems (International Allelopathy Society, 1996). Secondary metabolites that are not directly involved in the basic metabolism of the producers influence the growth of the target species as allelochemicals. Consequently, the target species may be inhibited, stimulated, eliminated, or be resistant to the allelochemicals of the emitter. Based on this definition, the interspecies interplay between microalga, including photoautotrophic micro-eukaryotes and cyanobacteria, is included in this category.

1.5.2 Microalgal interspecies interactions through the secondary metabolites

Microalgae produce a variety of bioactive compounds such as alkaloids, esters, fatty acids, and peptides (Watson, 2003; Leflaive and Ten-Hage, 2007; De Morais et al., 2015). Some of these compounds have been isolated and identified with allelopathic activity, while the biological or physiological activity of the vast majority has not been explored yet (Gantar et al., 2008; Borowitzka, 2016; Liu et al., 2018). Allelopathic compounds which are released into the environment under various environmental conditions during different stages of the growth or by cell lysis can affect the target species through different mechanisms such as inhibition of the growth, photosynthesis, enzymes activities, nucleic acid synthesis, induction of oxidative stress and cell paralysis (Leflaive and Ten-Hage, 2007).

Previous studies showed that the interactions between cyanobacteria, other members of the phytoplankton community, and aquatic plants could be taken into account as allelopathy (Leflaive and Ten-Hage, 2007; Żak and Kosakowska, 2016). Keating (1978) proposed that cyanobacteria overcome diatoms in eutrophic Linsley Pond, USA, through allelopathy. The allelopathic interactions among cyanobacteria and phytoplankton species have been reported in other investigations as well (Vardi et al., 2002; Gantar et al., 2008; Zhang et al., 2013; Bittencourt-Oliveira et al., 2015).

Moreover, previous studies considered the phytoplankton toxins, such as cyanotoxins as allelochemicals (Rengefors and Legrand, 2001; Pflugmacher, 2002, 2004; Pflugmacher et al., 2006; Berry et al., 2008; Granéli et al., 2008; Jaiswal et al., 2008). MC-LR at the

environmentally relevant concentration (5 μg/L) promoted oxidative stress and inhibited the growth and photosynthesis of the aquatic macrophytes, *Ceratophyllum demersum* and *Myriophyllum spicatum* (Pflugmacher, 2002, 2004). Cyanotoxins, anatoxin-a and MCs, induced oxidative stress and inhibited the growth and photosynthesis of the strains of aquatic plant model, duckweed, *Lemna minor* and *Wolffia arrhizal* (Weiss et al., 2000; Mitrovic et al., 2004, 2005).

M. aeruginosa was shown to severely inhibited the growth and photosynthesis of dinoflagellate *Peridinium gatunense* through inhibition of carbonic anhydrase activity, which led to the induction of oxidative stress and programmed cell death (Sukenik et al., 2002; Vardi et al., 2002). On the other hand, the study by Sukenik et al. (2002) showed that MC only at a high concentration has slightly affected the photosynthesis and growth of dinoflagellate. While a heat-stable relatively hydrophobic compound with a molecular weight of < 5 kDa was involved in the growth and photosynthetic restriction of dinoflagellate. The filtrates of dinoflagellate *Peridinium gatunense* induced cell lysis in *Microcystis* sp. and raised the transcript levels of *mcy*B as well (Vardi et al., 2002).

The green alga, *Scenedesmus obliquus*, could produce allelochemicals which inhibited the growth and photosynthesis of *M. aeruginosa* (Jia et al., 2008). *Chlamydomonas reinhardtii*, a green alga, inhibited the MC production in cyanobacterium *Anabaena flos-aquae* through the production of allelochemicals (Kearns and Hunter, 2000). The study by Zhang et al. (2014) showed that the exudates of mono- and co-cultures inhibited the growth of *M. aeruginosa* while stimulated the growth of *Anabaena flos-aquae*. They found that several allelochemicals such as sulfur compounds, naphthalene derivatives, cedrene derivatives, quinones, phenol derivatives, diphenyl derivatives, anthracene derivatives, and phthalate ester were involved in the interspecies interactions.

The investigation of the interspecies interferences between cyanobacteria and the other members of the phytoplankton community may clarify how some species dominate the whole algal community and which factors control the formation and disappearance of blooms. Moreover, the toxin-related studies can provide more insight into the significance of toxin to the producing cyanobacterium, and the conditions influencing the toxin production and release. Besides, it may open the ways for the biocontrol of harmful algal blooms through the allelochemicals produced by the co-occurring organisms.

1.5.3 Investigation of the microalgal chemically - mediated interactions

The interspecies interactions are a major topic of the current research, which includes both direct and indirect interactions. Several laboratory approaches have been developed to detect the microalgal interspecies interferences (Borowitzka, 2016). Cross-culturing is one of the most common classical methods in which the target species was grown in a medium enriched with the culture filtrates of the donor species to study the effects of the released metabolites of the emitter on the target species.

The interspecies interactions can be further considered through the exposure of target species to the crude extracts or the purified metabolites of the species whose allelopathic activity is studied. As a result, the interactions between species would be approved by any changes in the growth, photosynthesis, and biochemical status of the target organism. The conventional approaches have been widely used to consider the possible involvement of the algal secondary metabolites in the microalgal interactions. However, they focused on the single or repeated exposure of the interacting organism to the metabolites of the emitter while do not cover the natural phenomena where the organisms exist in diverse microbial communities, and intact cells communicate with each other. In axenic cultures in the absence of the diverse microbial compounds, many biosynthetic genes remain silent or expressed differently.

The interspecies interplay can be studied in mixed cultures where the species are grown together. Unlike the classical routine methods such as cross culturing, the co-cultivation procedures study the effects of the intact cells of the co-growing species on each other (Goers et al., 2014). Moreover, the co-cultivation techniques give the opportunity of the investigation of interspecies interactions under a constitutive production of allelochemicals, a situation similar to the natural ecosystems.

Commonly, in co-cultivation methods, the species are mixed in a culture container (Wang and Tang, 2008), which covers the probable effects of the physical cell-cell contacts in addition to the chemically mediated interactions. The dialysis co-cultivation system is a mixed culture in which the species are separated physically using a dialysis membrane, but still communicate through their diffusible extracellular metabolites (Yamasaki et al., 2007; Paul et al., 2009). The dialysis semi-permeable membrane contains pores of a specified size range. The intact cells retain inside and outside of the tubing while the chemicals and extracellular products which are smaller than the pores, diffuse freely across the membrane and affect the target organism.

At the next step, the allelochemicals can be isolated and identified by the metabolomic profiling-based approaches using analytical technologies (Prince and Pohnert, 2010; Weston et al., 2015).

1.6 Model species - *Microcystis aeruginosa* and *Desmodesmus subspicatus*

The model organisms studied in this work were the cyanobacterium *Microcystis aeruginosa*, MC-producing strain of *M. aeruginosa* PCC 7806 and the naturally occurring MC-deficient strain of *M. aeruginosa* PCC 7005, and a green alga *Desmodesmus subspicatus*.
M. aeruginosa is the most common bloom-forming cyanobacterium in eutrophic freshwaters all over the world (Harke et al., 2016). *M. aeruginosa* exists in spherical unicells (3-7 µm in diameter) in laboratory culture conditions (Fig. 1.3) but forms colonies under natural conditions (Komárek, 2002; Xiao et al., 2018). *M. aeruginosa* consists of toxin-producing strains that can produce hepatotoxin MCs, and non-toxic strains, which lost their ability of MC production due to the partial or total lack of the *mcy* gene cluster (Meissner et al., 1996; Christiansen et al., 2008).

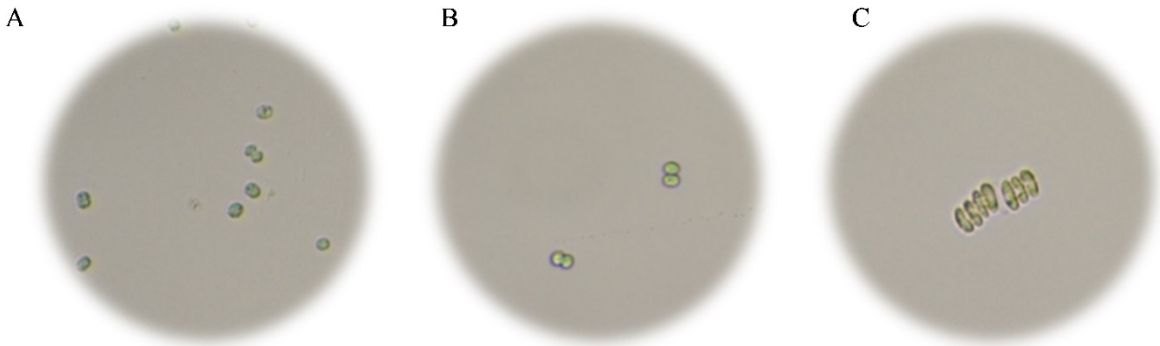

Fig. 1.3: Microscopic images of *Microcystis aeruginosa* PCC 7806 (A)*, Microcystis aeruginosa* PCC 7005 (B), and *Desmodesmus subspicatus* SAG 86.81(C).

The genus of *Desmodesmus* (formerly known as Scenedesmus) is globally distributed green algae in freshwater habitats (Hegewald, 2000). They are characterized by their ornamented cell wall formed by the outer layer with warty (*D. subspicatus*), net-like (*D. quadricauda*), or star-like (*D. serratus*) morphology. *D. subspicatus* cells are elliptically shaped (length 7 - 10 µm and width 3 - 4 µm), spiny unicells, or linearly arranged in 2 - 8 celled colonies (Fig. 1.3) (Shubert et al., 2014; Hegewald and Braband, 2017). *D. subspicatus* responds rapidly to the

changes in ecosystems, often provides one of the first signals of the ecosystem variations; then is highly recommended as a useful test organism in ecotoxicological studies (Rojíčková and Maršálek, 1999; Berthet, 2015). Moreover, it was reported that *Desmodesmus* sp. could produce bioactive compounds with the potential of allelopathic/antimicrobial activity (Leflaive et al., 2008; El Semary, 2011).

In the present study, the species, *M. aeruginosa* and *D. subspicatus*, were grown in mono- and co-cultures in the dialysis co-cultivation system (Fig. 1.4).

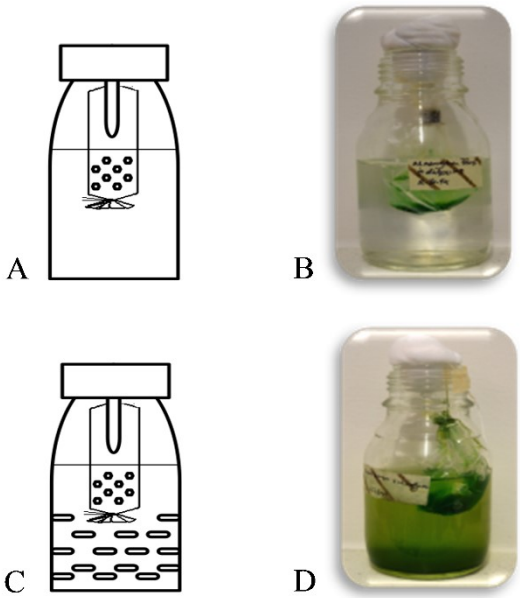

Fig. 1.4: A schematic view of the microalgal monoculture in dialysis tubing (A), a monoculture of *M. aeruginosa* PCC 7806 inside of the dialysis tubing (B), a schematic view of a dialysis co-cultivation system (C), and co-cultivation of *M. aeruginosa* inside and *D. subspicatus* outside of the dialysis tubing (D)

1.7 Main objectives

Among cyanotoxins, MCs are the most commonly studied. Over the past decades, numerous efforts have been made to conduct toxicological studies of the risks of the exposure of humans, animals, aquatic biota, and higher plants to MCs. Nowadays, there is a growing interest in understanding the probable natural physiological and ecological importance of MCs for the producing organisms (was reviewed in Paper I).

In lakes, cyanobacterial blooms are frequently associated with the green algae, another member of the phytoplankton community, which dominate the phytoplankton community in successive waves. Recent studies have disclosed that the phytoplankton seasonal fluctuations are influenced not only by the abiotic environmental factors but also by the interspecies interferences. In the present study, the interactions between two freshwater microalgae, the most common cause of toxic cyanobacterial blooms "*Microcystis aeruginosa*" and a green alga "*Desmodesmus subspicatus*," were considered to provide more insights into the probable ecological importance of the algal secondary metabolites concerning MCs in the variation of phytoplankton community structure. Moreover, the probable ability of the green algae to influence the bloom-forming *M. aeruginosa* was studied.

The primary concern of the present study was:

What advantages do secondary metabolites such as MCs lend to the producers?

Therefore, the interspecies interplay between *M. aeruginosa* and *D. subspicatus* was studied as follows:

- To study whether the species produced metabolite(s) capable of altering the growth of each other:
  - The two species, "*M. aeruginosa* PCC 7806" and green alga "*D. subspicatus*," were grown in BG 11 medium enriched with different percentages of the culture filtrates of each other (Paper II).
  - The green alga "*D. subspicatus*" was exposed to the crude extracts of MC-producing *M. aeruginosa* PCC 7806 containing different concentrations of MC-LR (Paper II).

As a result, the growth of species was monitored by cell counting using a haemocytometer under the light microscopy.

- To study how the species influenced each other in mixed cultures:

A co-cultivation system was designed using a dialysis tubing where two microalgal populations were grown together in mixed cultures to communicate with each other through their probable diffusible extracellular products beyond their physical associations. The co-cultivation experiments were performed based on two experimental designs:

- Co-cultivation of the toxic strain of *M. aeruginosa* PCC 7806 with the green alga "*D. subspicatus*" for one month at various phases of growth to consider whether the physiological status of the co-cultured species affected the growth of species as well as MC production and release in *M. aeruginosa* (Paper II).
- Co-cultivation of the green alga "*D. subspicatus*" with MC-producing and -deficient strains of *M. aeruginosa*, PCC 7806 and PCC 7005, respectively, which were grown to the stationary phase of growth to consider whether MCs gave any advantages to the MC-producing species over the non-toxic populations to influence the growth of co-existing green alga at the collapse phase of growth (Paper III).

As a result, the growth of species was monitored using haemocytometer and light microscopy. The concentration of intracellular and extracellular MC-LR was assessed using LC-MS/MS as well. Additionally, the potential of the dialysis membrane in the interspecies communications was considered through the measurement of the concentration of extracellular MC-LR at both sides of the dialysis tubing (Paper II and III).

2 PAPER I

Still challenging: the ecological function of the cyanobacterial toxin microcystin – What we know so far

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Own contribution:

- Literature review of the suggested roles for MCs
- Preparation of the manuscript which includes all texts, tables, and the figure
- Revision of the manuscript after receiving the reviewer's comments

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Abstract

Microcystins (MCs) are the most commonly studied cyanotoxins. While these past studies have mainly focused on the toxicity of MCs, the evolutionary history of life has shown that toxicity can be considered as an assigned role to MCs. Nowadays, there is a growing interest in understanding the importance of cyanotoxins in any of the physiological processes or beyond at the ecological level. This review evaluates the variously proposed intracellular and extracellular functions of MCs and how they benefit the producing cyanobacterium. However, the strain-specific and divergent laboratory and field results obtained to date has made it difficult to generalize.

Keywords: Cyanobacteria, Cyanotoxins, Microcystins, physiological function

2.1 Introduction

Cyanobacteria, commonly known as blue green algae, are the first photosynthetic organisms that released oxygen to the atmosphere 2.32 - 2.45 billion years ago during the great oxygenation event (Büdel, 2011; Rasmussen et al., 2008; Schirrmeister et al., 2011). Cyanobacteria are found almost in every imaginable habitat on earth from the ice fields such as the polar and alpine regions to hot springs and deserts (Bhaya et al., 2012; Büdel, 2011; Castenholz, 2015; Sompong et al., 2005; Singh & Elster, 2007; Ward et al., 1998; Whitton, 2012; Zakhia et al., 2008). Under favorable conditions, cyanobacteria form highly toxic blooms which have undesirable effects on humans, animals, and aquatic life (Catherine et al., 2013; Falconer, 2008; Falconer & Humpage, 2005; Lévesque et al., 2014; Ressom et al., 1994; Smith et al., 2008; Stewart et al., 2008; Zanchett & Oliveira-Filho, 2013). These toxic blooms, often but not always occurring in eutrophic lakes, rivers, estuarine and coastal waters, have been reported to occur frequently in many countries throughout the world (Paerl et al., 2011; Sivonen & Jones, 1999; Stewart et al., 2008). Cyanobacterial toxicity is caused by a variety of secondary metabolites termed cyanotoxins which include a diverse group of organic compounds both from the chemical and toxicological points of view (Wiegand & Pflugmacher, 2005). According to their chemical structures, they fall into three main groups including cyclic peptides, (microcystin and nodularin), heterocyclic compounds like alkaloids (anatoxin-a, anatoxin-a(s), saxitoxin, cylindrospermopsin, aplysiatoxins, lyngbyatoxin-a), and lipopolysaccharides (Kaebernick & Neilan, 2001). Based on the biological effects on targets,

they are classified into five groups; 1) the hepatotoxins, which include the microcystins (MCs) and nodularins, cause severe liver disruption by inhibition of protein phosphatase 1 and 2A in hepatocytes, 2) the neurotoxins, composed of anatoxin-a, anatoxin a-(s), and saxitoxin, interfere with the functioning of neuromuscular systems, 3) the cytotoxins include cylindrospermopsins and alkaloids, cause damages in the liver, kidneys, spleen, thymus, and heart by inhibition of protein synthesis, 4) the dermatotoxins, such as aplysiatoxins, debromoaplysiatoxins, and lyngbyatoxins cause dermatitis as inflammatory agents and protein kinase C activators, and lastly 5) the irritant toxins, such as endotoxins, which are lipopolysaccharides associated with outer membranes of cyanobacteria that elicit irritation and allergic responses in human and animal tissues and also cause gastroenteritis and inflammation (Bláha et al., 2009; Carmichael, 2001; Codd, 1994; Codd et al., 2005; Falconer, 2008; Leflaive & Ten-Hage, 2007; Pearson et al., 2010; Smith et al., 2008; Wiegand & Pflugmacher, 2005; Zanchett & Oliveira-Filho, 2013).

Among the cyanotoxins, MCs are the most commonly studied and was first isolated from *Microcystis aeruginosa*, the organism from which their name is derived. Although other cyanobacterial species such as *Anabaena*, *Planktothrix*, *Nostoc*, and some species of benthic *Oscillatoria* produce MCs, *M. aeruginosa* is the species most often identified in freshwater cyanobacterial harmful blooms (Carmichael, 1992, 2001; Leflaive & Ten-Hage, 2007; Pearson et al., 2010; Wiegand & Pflugmacher, 2005; Zurawell et al., 2005).

MCs are cyclic hepatopeptides composed of seven amino acids with the general structure of cyclo D-Ala^{1} – X^{2} – D-MeAsp^{3} – Z^{4} – Adda^{5} – D-Glu^{6} – Mdha^{7}, in which X and Z are variable L-amino acids, D-MeAsp is D-erythro methylaspartic acid, Adda is a unique 3-amino-9-methoxy-2, 6,8-trimethyl-10-phenyl-deca-4,6-dienoicacid, and Mdha is N-methyldehydroalanine (Fig. 2.1A) (Van Apeldoorn et al., 2007). Currently, more than 100 different congeners of MCs have been identified (Niedermeyer, 2014). The variants typically vary at the X and Z-amino acids positions, the presence or absence of methyl group on D-MeAsp and / or Mdha and the substations of other moieties (Namikoshi et al., 1998; Rinehart et al., 1994; Sivonen, 1996). The most common MC congeners are MC-LR, MC-RR, and MC-YR, containing L-amino acids leucine (L), arginine (R) or tyrosine (Y) at the X position, respectively (Harada, 1996). Among the MCs congeners, MC-LR is the best studied variant because of its abundance and toxicity (Harada, 1996; Pearson et al., 2010; Ufelmann et al., 2012; Yu et al., 2014). MC-LR is the most toxic variant followed by MC-YR and MC-RR (Yu et al., 2014). However, the limited information for the other congeners, possibly due to the focus on MC-LR or lacking analytical methods, might have resulted in the underestimation of

the toxicity of these MC variants and consequently, accepting MC-LR as the most toxic of the congeners (Fischer et al., 2010; Pacheco et al., 2016).

MCs are synthesized non-ribosomally *via* a complex multifunctional enzyme, MC synthetase, which includes non-ribosomal peptide synthetases (NRPS), polyketide synthetase (PKS), tailoring enzymes and an ABC transporter (Kaplan et al., 2012; Nishizawa et al., 2000; Pearson et al., 2010; Welker & Von Döhren, 2006). MC synthetase is encoded by a 55-kb *mcy* gene cluster composed of 10 bidirectional genes, called *mcy*A-J, with a bidirectional promoter region of 732-bp between *mcy*A and *mcy*D (Fig. 2.1B) (Kaplan et al., 2012; Pearson et al., 2010; Tillett et al., 2000).

Studies revealed that non-toxic strains had lost a partial or total part of the MC synthetase genes resulting in a sporadic distribution of the *mcy* gene cluster (Christiansen et al., 2008; Rantala et al., 2004). On the other hand, MC-deficient mutants were generated by insertional inactivation of genes such as *mcy*A or *mcy*B and partial deletion of *mcy*H in the *mcy* gene cassette (Dittmann et al., 1997; Pearson et al., 2004). As a result, MC-lacking mutants are able to produce other non-ribosomal peptides but not the MC variants which nominate them as useful tools to study the functions of MCs. For instance, competition studies between toxic (MC-producing) and non-toxic (MC-deficient) strains of *M. aeruginosa* under various conditions have provided more information regarding the importance of MCs to the producing cyanobacterium.

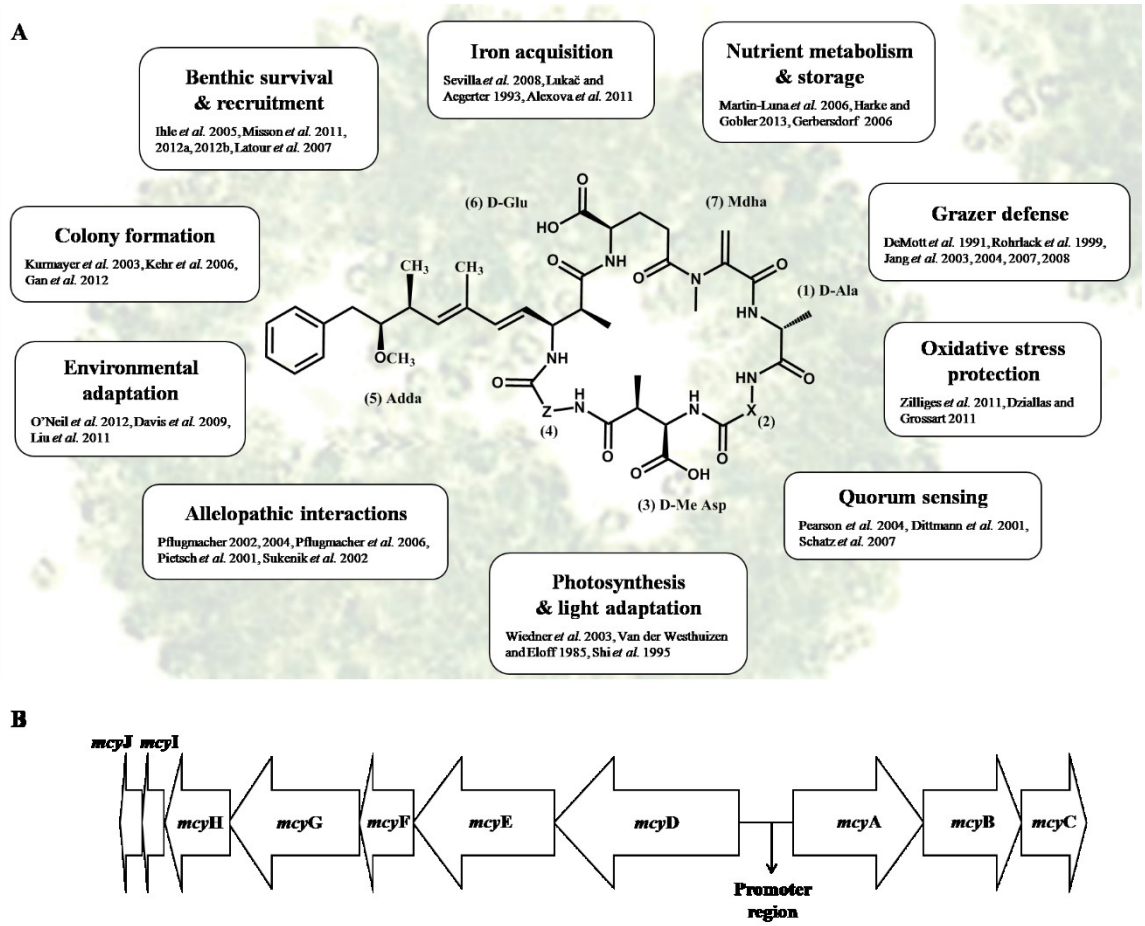

Fig. 2.1: (A) The chemical structure of MCs and their proposed intracellular and extracellular functions and (B) MC synthesis gene cluster (*mcy*).

From a toxicological point of view, MCs are known as eukaryotic protein phosphatase inhibitors. They inhibit eukaryotic serine/threonine protein phosphatases types 1 and 2A by irreversible covalent binding to the thiol group of cysteines of the catalytic domain which leads to a hyperphosphorylation of cytoskeleton proteins resulting in severe liver disruption and death by hypovolemic shock (Pearson et al., 2010; Runnegar et al., 1995). Therefore, a vast amount of research has been focused on the harmful effects of MCs on humans, animals, and aquatic organisms, while their natural physiological and / or ecological functions remain unclear (Merel et al., 2013ab). There are so many open questions which need to be answered: Why do cyanobacteria produce toxins in the first place? What is the advantage of toxin production for the producer? MCs are N-rich and high-cost products; since producers pay a high energetic price for MC synthesis, they might be an effective factor in the cellular metabolism beyond their toxicity. In the present review, the suggested roles of MCs, including intracellular and extracellular functions with the focus on *M. aeruginosa,* will be discussed to

clarify the significance of MCs to the producing organism (Table 2.1, Table 2.2, and Fig. 2.1 A).

2.2 MCs intracellular functions

2.2.1 Photosynthesis and light adaptation

The involvement of MCs in photosynthesis and light adaptation were proposed as a result of studies which found that the MC production rate depended on the photon irradiancy (Utkilen & Gjølme, 1992; Wiedner et al., 2003). In contrast, light quality did not have a significant effect on MC production (Utkilen & Gjølme, 1992). It has been reported that a very low light intensity decreased the production MCs, however, so did high light intensity (Van der Westhuizen & Eloff, 1985). Under natural conditions, the toxicity at the bloom's surface was higher and decreased with an increase in depth, suggesting a link to the light penetration depth (Utkilen & Gjølme, 1992). Under laboratory conditions, an increase in light intensity to the saturated level at 2960-3700 lm m^{-2}, resulted in increased MC production under continuous culture. However, with a further increase in light intensity the MC concentration was decreased without any changes in the growth rate (Kaebernick & Neilan, 2001; Utkilen & Gjølme, 1992; Wiedner et al., 2003).

On the other hand, genetic studies indicated that the transcription of the *mcy* gene cluster and therefore MC production is regulated by light quantity (Renaud et al., 2011). Studies showed that a three-fold higher concentration of MC-LR was produced during the light period of light–dark cycle (do Carmo Bittencourt-Oliveira et al., 2005), suggesting that MC production is regulated *via* circadian photosynthetic rhythm. From this, it can be inferred that MCs plausibly participate in the central metabolism although they are known as secondary metabolites (Straub et al., 2011).

Research investigating the relation between MC production rate and chlorophyll a content raised more evidence in support of the hypothesis that MCs participate in photosynthesis activity and affected the chromatic pigment contents (Lyck, 2004). It was also established that MCs occurred in a constant ratio to Chl a (1:2 mol: mol) (Long et al., 2001). The MC content in cyanobacteria was directly related to the chlorophyll a quota but was reversely related to the growth rate (Deblois & Juneau, 2010; Van der Westhuizen & Eloff, 1985). Under light limited conditions, the *M. aeruginosa mcy*B-lacking mutant contained less photosynthetic pigments, β-carotene, zeaxanthin, echinenone, and chlorophyll a, but a greater ratio of phycocyanine to

chlorophyll a compared to the wild type. On the other hand, the growth of the wild type and the mutant strain was similar, suggesting that MCs might play a role in the light harvesting processes (Hesse et al., 2001). Although, other study obtained conflicting results with the *mcy*-knockout mutant strain at different light intensities, no changes in the photosynthesis efficiency were observed, however, differences in the thylakoids membrane and irregular gas vacuoles were demonstrated (Dittmann et al., 1997).

The hypothesized involvement of MCs in photosynthesis was further supported by studies using an immunogold-labeling technique that disclosed that the thylakoids membrane is the most MC-occupied cell site followed by the nucleoplasmic area. Physically, more than two-thirds of MCs were attached to the thylakoids membranes (Shi et al., 1995, 2008; Young et al., 2005). Although, further study using the cryofixation / cryosectioning technique demonstrated that most of the MCs were localized in the nucleoplasmic area and intracellular inclusions such as carboxysomes and polyphosphate bodies, rather than thylakoids membranes and the cell wall (Gerbersdorf, 2006). Under high light irradiation, the ratio of MCs in outer to inner cellular parts increased, and a higher percentage of MCs were found close to the thylakoids membrane, suggesting the probable role of MCs in light adaptation (Gerbersdorf, 2006). Moreover, the *M. aeruginosa mcy*A-knockout mutant has been found to be dominant under low light, with the toxic genotype *M. aeruginosa* PCC 7806 showing a greater fitness to high light, suggesting that MCs play a role in protection against photooxidation (Phelan & Downing, 2011). In contrast, another study showed under both low and high light irradiation (1480 and 5920 lm m^{-2}, respectively) that a mixed culture was dominated by the MC-producing strain *M. aeruginosa* UTCC 300 which further emphasized the importance of MCs in light adaptation (Renaud et al., 2011). Comparative proteomic studies also revealed two NADPH-dependent reductases, phycobiliproteins and RuBisCo, which is a Calvin cycle enzyme, were expressed differently in the wild type and *mcy*B^- mutant of *M. aeruginosa* PCC 7806. Furthermore, MC-protein binding was significantly enhanced under high light (51800 lm m^{-2}) which was assumed to increase the protein stability and avoid redox changes (Zilliges et al., 2011). Therefore, the potential role of MCs in photooxidative protection under high light is an advantage for the organism (Gerbersdorf, 2006; Phelan & Downing, 2011). In another study with *M. aeruginosa* PCC7806 and its MC-deficient mutant, differences in metabolic responses between strains upon exposing to high light intensity (18500 lm m^{-2}) were observed. Trehalose and sucrose, two general stress markers, accumulated more in the mutant while carbon reserves such as glycolate accumulated faster in the wild type. Additionally, the photosynthesis rate and high molecular weight carbohydrate contents were greater in the wild type (Meissner et al., 2015).

2.2.2 Environmental adaptations

In addition to light, the links between MC quota and a variety of other environmental factors such as water temperature, pH, and nutrient availability (specifically nitrogen and phosphorus) were observed (Dai et al., 2016).

A correlation between temperature and MC production has been reported. It was found that increasing temperature to the optimal growth temperature enhanced toxicity. In contrast, temperatures exceeding 28°C led to a declined MCs quantity (Van der Westhuizen & Eloff, 1985). A recent study by Mowe *et al.*(2015) showed that elevated temperatures of 33 °C led to an increase in total MC cell quota of tropical *Microcystis* species while the further increase in temperature to 36 °C resulted in a decrease in total cellular MC content. Increased temperature stimulated *Microcystis* blooms formation during warm months in summer (Liu et al., 2011; O'Neil et al., 2012). Recent studies revealed that increased temperature promoted the frequency of *Microcystis* toxic strains over non-toxic strains as well (Davis et al., 2009; Dziallas & Grossart, 2011; Lehman et al., 2013; Yu et al., 2014). Consequently, global warming raised not only the possibility of cyanobacterial blooms formation but also caused more toxic blooms. Dziallas and Grossart (2011) reported that in Boulder Lake (USA), Hamburg Innenalster (Germany), and Lake Taihu (China) the ratio of toxic to non-toxic populations was raised with increased water temperature. A field study of four lakes in the northeast US showed that temperatures elevated by 4°C caused a noticeable shift toward more toxic blooms (Davis et al., 2009). A laboratory study also showed that increasing the temperature from 20 to 32°C led to a significant rise in the ratio of toxic vs. non-toxic strains of *M. aeruginosa* and the transcript levels of *mcy*D as well. Additionally, the presence of heterotrophic bacteria affected both quantity and quality of MCs in *M. aeruginosa* HUB W333 depending on the temperature. Raised temperature enhanced the proportion of MC-LR to MC-YR and led to a greater toxicity in a mixed culture (Dziallas & Grossart, 2011). Raised temperature to 36 °C also resulted in an increase in the ratio of MC-LR, more MC toxic variant, to MC-RR of *M. ichthyoblabe* (Mowe et al., 2015). Moreover, the toxicity of *M. aeruginosa* was increased both at a pH higher or lower than the optimal growth pH value which was pH 9 (Van der Westhuizen & Eloff, 1983).

Recent studies demonstrated a correlation between dissolved inorganic carbon (DIC) and the growth and MC production of *M. aeruginosa*. In a competitive study, the effect of low and high DIC (0.365 and 7.658 mmol l^{-1} $KHCO_3$) on *M. aeroginosa* toxic and non-toxic strains, FACHB 912 and FACHB 469, co-cultured with green algae *Chlamydomonas microsphaera* were investigated. The growth of *M. aeruginosa* toxic and non-toxic strains was negatively

affected by DIC without any significant changes in the chlorophyll content; however, the photosynthesis efficiency and chlorophyll content of green algae decreased. The results proposed that *M. aeruginosa* might be more adapted to low DIC condition (Zhang et al., 2012). Increased dissolved inorganic carbon had an adverse effect on the frequency of toxic *Microcystis* and MCs concentration in Lake Chaohu, China as well (Yu et al., 2014). Deficiency of intracellular inorganic carbon resulted in an increase in MC production of *M. aeruginosa* PCC 7806. Moreover, the toxic wild type contained greater chlorophyll a content and consequently displayed higher photosynthetic efficiency compared to the *mcy*B$^-$ mutant, suggesting a role of MCs in environmental adaptation (Jähnichen et al., 2007).
In another study, the toxic *Microcystis* sp. KLL strain MG-K isolated from Lake Kinneret, Israel, severely inhibited the growth of its non-toxic laboratory emerged successor (strain MG-J). Additionally, during the *Microcystis* bloom season, an MC-producing strain placed in dialysis membrane in Lake Kinneret survived while the non-toxic spontaneous mutant lysed within 24 h. These results suggested that *Microcystis* toxic strains have an advantage under natural conditions (Schatz et al., 2005).

2.2.3 Protection against oxidative stress

Under stress-induced conditions which trigger reactive oxygen species generation, toxigenic *Microcystis* survive longer (Dziallas & Grossart, 2011). In a comparative study investigating the effect of hydrogen peroxide exposure on both the toxic and non-toxic *M. aeruginosa* strains, the toxic strain showed a lesser decrease in its chlorophyll a content. Moreover, by increasing the temperature both strains' MC content and reactive oxygen species were elevated (Dziallas & Grossart, 2011). Another study with two toxic *Microcystis* spp. isolated from Brazilian water bodies, disclosed that limiting the nutrients (nitrogen and phosphorus) as a form of stress-induction led to increased MC production and *mcy*D expression (Pimentel & Giani, 2014). A recent proteomic study using continuous cultures of *M. aeruginosa* PCC 7806 showed that under iron depletion physiological changes such as reduction in chlorophyll a content besides enhancement in MCs production. Increased intracellular and extracellular MCs in continuous culture of *M. aeruginosa* PCC 7806 under iron depletion provided more evidence of the assistance role of MCs in the better fitness of *Microcystis* under oxidative stress conditions (Yeung et al., 2016). In addition, *mcy* gene transcripts were enhanced under oxidative stress in

various quantities i.e. 20% for *mcy*B, 270% for *mcy*A, 330% for *mcy*H, and 370% for *mcy*D (Straub et al., 2011).

Proteomics studies revealed the potential role of MCs in protection against oxidative stress as MC bound covalently to the cysteine residues of certain proteins *via* its N-methyl-dehydroalanine moiety (Dziallas & Grossart, 2011; Kaplan et al., 2012; Zilliges et al., 2011). These proteins include phycobiliproteins, CpcB and ApcA, RuBisCo, glutathione reductase, and various hypothetical proteins that were expressed differentially in the wild type and mutant strain (Zilliges et al., 2011). Under oxidative stress due to the iron depletion, MCs showed a greater tendency to the binding sites in thioredoxin-regulated proteins (Alexova et al., 2016). In *M. aeruginosa* PCC 7806 wild type grown under high light and iron deficiency or exposed to 10 µM hydrogen peroxide, MC-protein formation was stimulated. On the other hand, in cultures treated with a protease such as subtilisin under high light (51800 lm m^{-2}), the large subunit of RuBisCo was more stable in the wild type. It was assumed that MC attachment to proteins avoid the dimerization of cysteines and consequently caused a delay in conformational changes of proteins and enzymes inactivation (Zilliges et al., 2011). Thus, the increased protein stability led to more adaptation to the various stresses (Kaplan et al., 2012; Zilliges et al., 2011). Moreover, under high light, the decreased oxygenase function of RuBisCo protected the cells against photorespiration (Gerbersdorf, 2006). On the other hand, current findings indicated MCs as additional radical scavengers which might protect the cells against oxidative stress damage (Martin-Luna et al., 2006a; Zilliges et al., 2011). The ability of MCs to bind to metals such as zinc and cadmium also point to the possible role of the toxin in metal detoxification in metal-induced oxidative stresses (Dziallas & Grossart, 2011).

2.2.4 Nutrient metabolism and storage

It has previously been shown that changes in the concentration of nutrients such as nitrogen, carbon, and phosphorus led to a change in the MC production rate indicating a possible relation between toxicity and primary cell metabolism (Oh et al., 2000).

Increased nitrogen concentration promoted MC production to the highest level, whereas nitrogen depletion led to a decrease in MCs synthesis (Holland & Kinnear, 2013). These findings confirm a high dependency of the MC production rate on the nitrogen supply, also as it is such an N-rich molecule (Harke & Gobler, 2013). Moreover, the binding site of NtcA, a global nitrogen regulator in cyanobacteria, was found in the promoter region of the *mcy* gene

cassette include bidirectional *mcy*DA promoter, *mcy*E, *mcy*H, *mcy*G, and *mcy*J promoters (Kuniyoshi et al., 2011). Under nitrogen starvation, *ntc*A transcripts increased, and reversely *mcy* gene expressions decreased (Harke & Gobler, 2013). Therefore, NtcA might regulate *Microcystis* toxicity (Harke & Gobler, 2013; Pimentel & Giani, 2014). It was shown that under nitrogen depletion, 2-oxoglutarates, a signal of the carbon/nitrogen balance, increased NtcA binding to the *mcy*A promoter leading to a decrease in *mcy* gene expression (Kuniyoshi et al., 2011). As a result, based on the carbon-nitrogen status, MCs synthesis can be regulated by NtcA as a repressor (Harke & Gobler, 2013; Kuniyoshi et al., 2011).

On one hand, in a comparative proteomics study, different isoforms of the same proteins were found in *M. aeruginosa* toxic and non-toxic strains. Moreover, proteins involved in carbon-nitrogen metabolism and redox balance were expressed in various quantities. Comparisons between toxic and non-toxic strains also showed Calvin cycle enzymes and proteins involved in glycolysis and respiration expressed in higher quantity in *M. aeruginosa* PCC 7820, a known toxic strain (Tonietto et al., 2012). Under nitrogen depletion, proteins involved in carbon and nitrogen metabolism such as chaperones and proteases were accumulated more in the non-toxic *M. aeruginosa* strains, PCC 7005 and *mcy*H$^-$ mutant (Alexova et al., 2016).

On the other hand, a study using immunogold labeling revealed MCs are generally localized within carboxysomes suggesting a possible relation between MCs synthesis and carbon fixation (Gerbersdorf, 2006). Studies with *M. aeruginosa* PCC 7806 wild type and its *mcy*B$^-$ mutant cultured under inorganic carbon deficiency showed that the intracellular and extracellular quota of MCs had been elevated. Moreover, in the wild type, the concentration of chlorophyll a was higher. Consequently, *M. aeruginosa* PCC 7806 could adapt to the C-limited conditions better than the non-toxic mutant strain (Jähnichen et al., 2007). It was elucidated that MCs act as a RuBisCo inhibitor to better adapt to carbon fluctuations (Dziallas & Grossart, 2011; Gerbersdorf, 2006; Jähnichen et al., 2007). Under inorganic carbon limitation, which occurs in dense blooms by inhibition of the RuBisCo synthesis, the oxygenase function of RuBisCo decreased leading to lower CO_2 consumption, decreased carbon leakage by photorespiration and, consequently retaining the CO_2/O_2 ratio at a higher level (2007, Jähnichen et al., 2001).

MCs were also found in the vicinity of polyphosphate inclusion bodies which are known as phosphate storage granules and metal trapping area (Gerbersdorf, 2006). Enhancing the concentration of phosphorus led to an increase in the ratio of toxic to the non-toxic population (Davis et al., 2009; Yu et al., 2014). Increased MCs beside a lower C-fixation rate in *M. aeruginosa* UTEX 2388 under P-limited conditions were observed (Oh et al., 2000). Under

severe P-limitation, the ratio of MC-LR to MC-RR was increased leading to shifting toward the more toxic variant MC-LR. The MC content was greater while the carbon fixation rate and as a result growth was lower (Oh et al., 2000).

Concerning MC's tendency to form complexes with metals such as zinc and cadmium, a possible role of MCs in detoxification or metal storage has been proposed (Young et al., 2008). Whether the intracellular function of MCs is multifarious and multifaceted or just not fully elucidated yet remains unknown, however, of all currently proposed roles (Table 2.1) each holds some merit in its own right.

Table 2.1: MCs possible intracellular functions.

Role	Evidences	References
Photosynthesis and Light adaptation	Regulation of MC production rate and transcript levels of *mcy*D by the light intensity	Utkilen & Gjølme (1992), Wiedner et al. (2003) and Renaud et al. (2011)
	Higher MC production during the light phase of light – dark cycle	do Carmo Bittencourt-Oliveira et al. (2005)
	Direct relation of MCs content to chlorophyll a quota	Van der Westhuizen & Eloff (1985), Long et al.(2001) and Deblois & Juneau (2010)
	Light limitation • Lower photosynthetic pigments in MC-deficient mutant • Higher ratio of phycocyanin to Chlorophyll a	Hesse et al. (2001)
	Higher photosynthesis rate in *Microcystis* PCC 7806	Meissner et al. (2015)
	Localization of MCs in thylakoids membrane	Shi et al. (1995), Young et al. (2005, 2008)
	Different expression of two NADPH-dependent reductases, phycobiliproteins and RuBisCo in *Microcystis* wild type and *mcy*B$^-$ mutant	Zilliges et al. (2011)
	High light irradiation • Dominance of toxic strain in a mixed culture of toxic-non-toxic strains	Renaud et al. (2011)
	Higher percentage of MCs close to the thylakoids membrane	Gerbersdorf (2006)
	• Enhancement in MC attachment to certain proteins	Zilliges et al. (2011)
	• Faster accumulation of carbon reserves • Greater contents of high molecular weight carbohydrate in the wild type of *M. aeruginosa*	Meissner et al. (2015)
	Temperature	

Environmental adaptations	Enhanced MCs by increased temperature to the optimal growth temperature and decreased MCs quantity at exceeded temperatures	Van der Westhuizen & Eloff (1985)
	Increased temperature	
	• Stimulation of Microcystis blooms formation in summer	Liu et al. (2011) and O'Neil et al. (2012)
	• Greater proportion of toxic population in the lakes	Dziallas & Grossart (2011)
	• Higher frequency of *Microcystis* toxic blooms	Davis et al. (2009)
	• Higher ratio of toxic vs. non-toxic strains of *M. aeruginosa*	Dziallas & Grossart (2011)
	• Higher *mcy*D transcript levels	
	• Greater proportion of MC-LR to MC-YR in a mixed culture with heterotrophic bacteria	
	pH higher or lower than the pH 9	Van der Westhuizen & Eloff (1983)
	• Increased toxicity	
	Toxic *Microcystis* sp. (KLL strain MG-K) outcompete the non-toxic laboratory emerged successor (strain MG-J)	Schatz et al. (2005)
	Dissolved inorganic carbon (DIC)	
	Low and high concentrations of DIC	Zhang et al. (2012)
	• Greater growth at lower DIC and no change in the chlorophyll content of *M. aeroginosa*	
	• Lower photosynthesis efficiency and chlorophyll content of *Chlamydomonas microsphaera*	
	Increased DIC (Lake Chaohu, China)	Yu et al. (2014)
	• Lower abundance of toxic Microcystis and MCs quota	
	Decreased DIC	Jähnichen et al. (2007)
	• Increased MCs production	
	• Greater chlorophyll a content of *M. aeruginosa* PCC 7806 wild type	
	• Growth inhibition of non-toxic laboratory emerged successor by the toxic *Microcystis* sp	Schatz et al. (2005)
	• Survival of toxic strain and lysis of non-toxic spontaneous mutant during the bloom season	
Protection against oxidative stress	Exposure to hydrogen peroxide	Dziallas & Grossart (2011)
	• Longer survival of toxigenic *Microcystis*	
	• Lesser decrease in its chlorophyll a content	
	Increased temperature	Dziallas & Grossart (2011)
	• Higher increase in MCs content and reactive oxygen species	
	Nitrogen and phosphorus limitation	Pimentel & Giani (2014)
	• Increased MC production and *mcy*D transcript levels	
	Iron depletion	Yeung et al. (2016)
	• Increased intracellular MCs in *M. aeruginosa* PCC 7806	

	• Reduction in the cell size and chlorophyll a content	
	High light irradiation	Meissner et al. (2015)
	• Increased stress markers in *M. aeruginosa* *mcy*B$^-$ mutant	
	Oxidative stress	Straub et al. (2011)
	• Enhancement in *mcy* gene cluster transcripts	
	Different expression of proteins phycobiliproteins, CpcB and ApcA, RuBisCo, glutathione reductase and some hypothetical proteins in the wild type and mutant strain	Zilliges et al. (2011)
	High light, iron deficiency and exposure to hydrogen peroxide	Zilliges et al. (2011)
	• Stimulation of MC-protein binding in *M. aeruginosa* PCC 7806 wild type	
	Iron depletion	Alexova et al. (2016)
	• Greater tendency of MC to the binding sites of thioredoxin-regulated proteins	
	High light (51800 lm m^{-2})	Zilliges et al. (2011)
	• More stability of the large subunit of RuBisCo in the wild type	
	High light	Gerbersdorf (2006)
	• Decrease in oxygenase function of RuBisCo, Protection against photorespiration	
	C-limitation	Jähnichen et al. (2007)
	• Higher intracellular and extracellular MCs quota	
	• Higher concentration of chlorophyll a	
	MC binding to metals such as zinc and cadmium	Dziallas & Grossart (2011)
	MC as additional radical scavengers	Zilliges et al. (2011)
Nutrient metabolism and storage	Dependency of MC production on the nitrogen concentration	Harke & Gobler (2013) and Holland & Kinnear (2013)
	Binding site of NtcA to the promoter region of *mcy* gene cluster	Kuniyoshi et al. (2011)
	Nitrogen starvation	Kuniyoshi et al. (2011), Harke & Gobler (2013) and Pimentel & Giani (2014)
	• Increased *ntc*A transcripts	
	• Decreased *mcy* gene cluster expression	
	2-oxoglutarates addition	Kuniyoshi et al. (2011)
	• Increased binding of NtcA to the *mcy*A promoter	
	• Decreased *mcy* gene expression.	
	Toxic and non-toxic strains are different in	Tonietto et al. (2012)
	• The isoforms of the same proteins	
	• Quantities of proteins involved in carbon-nitrogen metabolism	
	Nitrogen depletion	Alexova et al. (2016)

• Greater accumulation of proteins involved in carbon and nitrogen metabolism in non-toxic *M. aeruginosa* PCC 7005 and *mcy*H^{-} mutant	
Localization of MCs within carboxysomes	Gerbersdorf (2006)
MCs in the vicinity of polyphosphate inclusion bodies	Gerbersdorf (2006)
C-limitation	
• Better adaptation of *M. aeruginosa* PCC 7806 wild type	Jähnichen et al. (2007)
• Higher intracellular and extracellular MCs quota	
• Higher concentration of chlorophyll a	
• Inhibition of oxygenase function of RuBisCo	Jähnichen et al. (2001, 2007)
Higher phosphorus concentration	Davis et al.(2009) and
• Greater ratio of toxic to the non-toxic population	Yu et al. (2014)
Phosphorus limitation	Oh et al. (2000)
• Increased MCs content	
• Higher ratio of MC-LR to MC-RR	
• Lower carbon fixation rate	

2.3 MCs extracellular functions

2.3.1 Quorum sensing (cell - cell communication)

Many bacterial species produce signal molecules such as acylated homoserine lactones and oligo-peptides to synchronize cellular activities in response to the environmental changes, a process called “quorum sensing" (Miller & Bassler, 2001). Do MCs play any role as infochemicals in intercellular communications? To have this extracellular function, first MCs must be secreted from the cells to the environment. MCs are produced continuously from the early logarithmic phase to the late stationary phase (Lyck, 2004). During growth and under different environmental conditions, MCs are exported partly to the surrounding media as an extracellular toxin. Therefore, MCs are released into the extracellular environment which is a known character of a signaling molecule (Hotto, 2007). Increased extracellular MCs under iron limitation in continuous cultures of *M. aeruginosa* PCC 7806 proposed the probable role of MCs as infochemical to enhance toxin production and consequently increase the fitness of *Microcystis* blooms (Yeung et al., 2016).

The hypothesis of MCs involvement in signaling processes was also supported by the discovery of a protein with homology to the ABC transporter that is encoded by a part of MCs synthesis gene cluster, *mcy*H. Thus, McyH might be involved in MCs export (Pearson et al., 2004).

However, by inactivation of *mcy*H gene in the Δ*mcy*H mutant of *M. aeruginosa*, MC synthetase has not been detected, and consequently, MC synthesis was completely blocked. From this, it was proven that MC export is linked to MC production (Pearson et al., 2004). Another hypothesis proposed that McyH might play a role in the stability of MC synthetase or act as a sensor of MCs in *Microcystis* cell membranes; however, this remains unclear (Kaplan et al., 2012; Pearson et al., 2004).

Since quorum sensing is a cell density-dependent mechanism, it is essential to know the effect of cell density on MC production rate and if a certain cell density is required to trigger toxin production. The study with different initial cell numbers of *M. aeruginosa* inoculated to the Bold's medium and unfiltered water from Lake Sinclair (Georgia, USA) showed that the final cell density was positively related to the initial cell numbers. Therefore, *M. aeruginosa* proliferated in a cell density-dependent manner (Dunn & Manoylov, 2016). In a mesocosm study, *Microcystis* cells collected in proximity were added to the mesocosm. Consequently, increase in the cell density from 5×10^5 cells ml^{-1} to 7×10^6 cells ml^{-1} resulted in a considerable increase in MC production from 0.1 to 1.38 pg $cell^{-1}$ over a period of 6 h (Wood et al., 2012). Though, the increase in cell numbers might be considered as a stress factor and therefore could trigger an increase in toxin production induced under oxygen deficiency or nutrient limitations. Besides, extracellular MC was not increased which might be due to the time required to start toxin export (Wood et al., 2012). A field study at the Lake Rotorua (South Island, New Zealand) with a dense bloom of toxic *Microcystis* demonstrated that an increase in the cell concentrations from 7×10^4 to 4×10^6 cells ml^{-1} coincided with significantly increased *mcy*E transcripts, and total and extracellular MC quota (Wood et al., 2011). As a result, MC synthesis depended on the *Microcystis* cell density (Wood et al., 2011, 2012). However, cultures started with different inoculum sizes, regardless of the number of initial cells, had the same MC production rate in the end (Orr & Jones, 1998).

A further study clarified that addition of pure MC-LR or crude extracts caused an enhancement in the toxicity of resting cells. In *Microcystis* cultures exposed to the *Microcystis* crude extracts for 24 h, the *mcy*B transcripts enhanced 12-fold (Schatz et al., 2007). Moreover, MCs released after cell lysis due to mechanical treatments or environmental stresses caused an increase in toxin production in resting cells, a known character of an intercellular signal (Schatz et al., 2007). Genetic studies also provided more pieces of evidences to confirm this hypothesis. It was revealed that the sequence of the *mcy* gene cluster is similar to the quorum sensing genes of *Rhizobium leguminosarum* (Dittmann et al., 2001). Additionally, two light regulated proteins, MrpA and B, were found in *M. aeruginosa* PCC 7806 with the similarities to Rhi A

and B proteins of *R. leguminosarum* (Dittmann et al., 2001). Rhi A and B are known as signal mediators and induced directly by N-acyl-homoserine lactones (AHLs) in a cell density-dependent manner (Rodelas et al., 1999). MrpA was expressed strongly in the wild type but not detected in a *mcy*B^{-} mutant (Dittmann et al., 2001). MC addition to pure cultures enhanced the transcription levels of these proteins, especially in the wild type. Since the MC quota is also regulated by light irradiancy, it was assumed that there is an indirect relation between these light-regulated proteins and MCs as an intercellular signaling molecule (Dittmann et al., 2001). However, these light regulated proteins were not found in the toxic *M. aeruginosa* NIES-843 strain indicating that they are strain-specific proteins and not necessarily linked to the toxicity (Frangeul et al., 2008).

2.3.2 Benthic survival and recruitment processes

Microcystis has a biphasic life cycle, pelagic growth that proliferates as a planktonic form in the summer and, benthic sedimentation that sinks and joins the sediments in the winter which is called overwintering (Reynolds et al., 1981; Takamura et al., 1984). The benthic population contains a mixture of toxic and non-toxic genotypes (Catherine et al., 2013). Recent studies showed that in sediments, toxic *Microcystis* cells could survive and preserve their toxic ability for an extended period up to several years (Catherine et al., 2013; Ihle et al., 2005; Misson et al., 2012 a,b;Torres & Adámek, 2013). The study of the sediments taken from Lake Grangent (Loire, France) showed that *Microcystis* cells were present at the surface and the depth of sediments (Latour et al., 2007; Misson et al., 2012a,b). Latour et al. (2007) studied *Microcystis* cells in the sediments of Grangent reservoir (Lior, France), at the surface (0-2 cm), depths of 25-35 cm (1.5-year- old colonies) and 70 cm (14-year-old colonies). The highest biomass of *Microcystis* cells was found in the 25-35 cm layer (2300 colonies ml sediments $^{-1}$ compared to 250 colonies ml sediments $^{-1}$ at the surface and 600 colonies ml sediments $^{-1}$ in 70 cm layer). Then, it was assumed that *Microcystis* cells could be accumulated and preserved in sediments. Although depth and the age of the sediments caused physiological changes as well as decreased colony number and cellular metabolites, MCs were detected at higher concentrations, e.g. 1 pg MC-LR cell^{-1} at the 30 cm sediment layer compared to 0.3 pg MC-LR cell^{-1} at the surface of the sediment (Latour et al., 2007). Then it was assumed that MCs did not use as a nitrogen source. It might play a role in sustaining of *Microcystis* colonies. Moreover, the fermentation of endogenous glucose could provide the required energy to maintain the vegetative cells in

deep sediments (Moezelaar & Stal, 1997). Further studies demonstrated that MCs could be synthesized in the cells that were deeply buried in the benthic sediments even after several years of sedimentations (Lake Grangent, Loire, France) (Misson et al., 2012b). Moreover, the cellular MC quota varied with the age of sediments (Misson et al., 2012a,b). MC content decreased as the duration of the benthic life stage increased (Misson et al., 2012b). It was reported that MC quota had been changed sharply in the first few months of sedimentation, first significantly increasing followed by a significant decrease, whereas it was reduced gradually in old sediments and stayed almost stable at a low level for up to 6 years (Misson et al., 2012a). The decline in MC quotas in long term sedimentations could result from a decrease in MCs synthesis, MCs release from the old damaged cells, or MCs consumption as a nitrogen source (Jähnichen et al., 2008; Latour et al., 2007; Misson et al., 2012b). However, MCs were not used as cellular nitrogen supply (Latour et al., 2007).

The synthesis of MCs is a high energy cost process, and whether the toxic strains gain an advantage through MC production over non-toxic subpopulations is still in doubt. The continued presence and stability of MCs in the benthic phase in spite of the extreme environmental conditions of sediments such as low temperature, oxygen deficiency, and darkness, propose the hypothesis of MC's involvement in benthic survival (Misson et al., 2012b). It was suggested that MCs play a role in the maintenance of vegetative cells during the benthic phase under the oxidative stress caused by unfavorable conditions of sediments (Ihle et al., 2005; Latour et al., 2007; Misson et al., 2012a; Torres & Adámek, 2013; Zilliges et al., 2011). It has been shown that MCs could be attached to the proteins under stress conditions to keep them active and preserve their functional structure (Misson et al., 2012a ; Zilliges et al., 2011). Therefore, there might be a link between the ecological importance and the physiological functions of MCs.

In the life cycle of *Microcystis*, overwintering followed by the recruitment of cells from sediments to the water column in the spring is an important process to the annual succession of *Microcystis* (Catherine et al., 2013; Misson et al., 2011; Reynolds et al., 1981; Takamura et al., 1984). Although the recruitment is initiated in response to changing environmental factors, mainly increase in temperature and light penetration of water, the implication of MCs in benthic recruitment was proposed in recent laboratory and field studies (Ihle et al., 2005; Misson et al., 2012b; Schöne et al., 2010). Laboratory studies showed that reinvasion was profoundly affected by light and temperature (Misson et al., 2011; Schöne et al., 2010). By increased temperature (from 4 to 8°C) under the light, different subpopulation left the sediment and reentered the water column (Misson et al., 2011). Increased temperature and low light intensity

that penetrates to the bottom of sediments promote the resumption of metabolic activity of the benthic *Microcystis* cells, resulting in regaining the buoyancy of benthic colonies to migrate back into the water column (Reynolds et al., 1981; Schöne et al., 2010; Tan et al., 2008; Tsujimura et al., 2000).

The study of the sediments of Lake Quitzdorf (Germany) showed a preferred recruitment of MC-producing *Microcystis* to non-toxic strains under light (Schöne et al., 2010). During the recruitment process, the disappearance of *Microcystis viridis* (*mcy*B^{+} genotype) from the sediments was greater than *Microcystis wesenbergii* (*mcy*B^{-} genotype). Moreover, up to 70% of the toxic genotype of *M. aeruginosa* were also re-entered from sediments to the water column (Schöne et al., 2010). Furthermore, studies of sediments from Lake Villerest (France) showed there was a preferential reinvasion selection related to the MC content and the colony size in the toxic subpopulations of *Microcystis* (Misson et al., 2011; Schöne et al., 2010). The smaller colonies (> 160 μm) with higher MC content (0.021 ± 0.004 pg eq MC-LR cell^{-1}) displayed a greater recruitment rate. Furthermore, the recruited *Microcystis* contained higher cellular quotas of MCs compared to the benthic cells. Regarding the unfavorable experimental conditions for MC production at the beginning of pelagic phase (darkness at 8°C), the preferential selection of more toxic population was proposed (Misson et al., 2011).

Moreover, during spring an annual decrease in *Microcystis* population in sediments was observed (Ihle et al., 2005). Field studies showed only a low number of cells (3-4%) were recruited successfully from sediments to the water column (Ihle et al., 2005; Schöne et al., 2010). During the recruitment process, cell lysis or programmed cell death was observed with an increase in extracellular MCs (Ihle et al., 2005). The addition of purified MC-LR (in the μg L^{-1} range) to the sediments led to a decrease in recruitment rates of both toxic (*mcy*B genotype) and non-toxic subpopulations that in the toxic subpopulation (25.7% and 6.4% reduction, respectively), with a greater decrease seen in the toxic subpopulation. Then, it was proposed that MCs play a role as an extracellular messenger (signal) in the regulation of reinvasion processes (Misson et al., 2012b).

2.3.3 Colony formation and bloom maintenance

Microcystis occurs in large colonies in nature but as unicellular forms in laboratory-grown cultures (Reynolds et al., 1981; Sun et al., 2015; Zhang et al., 2007). This colony formation characteristic assists *Microcystis* to form large blooms in nature, allowing it to dominate over

other phytoplankton species, and migrate easier vertically in the water column to obtain the available nutrients (Bonnet & Poulin, 2002; Gan et al., 2012; Yang & Kong, 2012). Moreover, *Microcystis* in colonial form is more tolerant to the stress conditions (Wu et al., 2007). From this, the question as to whether MCs play a role in cellular aggregation arises. Continuous predator pressure by *Ochromonas* sp. as well as culturing with the filtered cultures of the flagellate fed with *M. aeruginosa* induced colony formation in unicellular *M. aeruginosa* PCC 7806 (Yang & Kong, 2012; Yang et al., 2009).

The addition of MC-RR to *Microcystis* cultures caused a significant increase in extracellular polysaccharides (EPS) that subsequently caused larger colonies to form. Therefore, extracellular MCs might be considered as an important factor in the maintenance of *Microcystis* blooms (Gan et al., 2012). A field study of *Microcystis* blooms in Lake Wannsee (Berlin, Germany) showed larger colonies (>100 mm) contained a greater proportion of toxic strains to non-toxic and also higher toxicity. Moreover, by increasing the colony size from the ratio of toxic to non-toxic genotypes, the concentration of MCs per cell was enhanced (Kurmayer et al., 2003).

On one hand, further studies regarding cellular surface exposed proteins involved in cellular interactions provided more evidence of the probable role of MCs in colony formation (Kehr et al., 2006; Zilliges et al., 2008). MCs were shown to bind covalently to a lectin microvirin protein which has a known role in cell to cell attachments. It is important to note that non-toxic strains contain different kinds of microvirin and that the microvirin deficient mutant produced lower MC quantities (Kehr et al., 2006). Thus, there might be a relation between microvirin, toxin production rate, and colony formation (Kehr et al., 2006).

On the other hand, a recent study revealed a relation between MCs and MrpC, a novel surface-exposed MC-related protein. MrpC is a strain-specific glycoprotein of *M. aeruginosa* 7806 that has a role in cellular aggregation (Zilliges et al., 2008). In the MC-deficient mutant *mcy*B$^-$, the MrpC quantity has been remarkably increased, and mutant cells showed a greater cellular aggregation compared to the wild type (Zilliges et al., 2008). Since the expression level of MrpC in the mutant was the same as wild type, MCs may affect the general stability of MrpC by interfering with the post-transitional modifications such as glycosylation status, or the expression level of other binding surface partners such as sugar binding proteins or lipopolysaccharides (Zilliges et al., 2008). The absence of glycosylation may lead to a protein inactivation or degradation that has been shown in other pathogenic bacteria like *Haemophilus influenza* (Grass et al., 2003). Cell surface-associated proteins, lectin microvirin and MrpC, were accumulated differentially in the wild type and MC-deficient mutant (Kehr et al., 2006,

Zilliges et al., 2008). However, no evidence concerning the direct interactions of MCs with these proteins has been found.

2.3.4 Defense against zooplankton

Interactions between cyanobacteria and zooplankton are considered as aquatic predator–prey relations. Colony formation, lower long chain saturated fatty acids production, and increased toxicity have been reported in *Microcystis* cultures exposed to grazers (Jang et al., 2003; Müller-Navarra et al., 2000; Watson, 2003; Yang & Kong, 2012; Yang et al., 2006).
Direct and indirect exposure of toxigenic *M. aeruginosa* strains (NIES 44, 87 and 88) to the zooplankton *Moina macrocopa*, *Daphnia magna,* and *Daphnia pulex* resulted in a significant increase and then decrease in MC production during six-day exposure experiments (Jang et al., 2003). The rate of increase has depended on the growth stages, population densities, and concentrations of cellular exudates of the zooplankton. Compared to the juveniles and neonates, the adult zooplankton produced higher infochemicals that resulted in a greater increase in MC production (Jang et al., 2008). Additionally, higher cell density and a higher concentration of culture media filtrate of zooplankton led to a significant increase in both intracellular and extracellular toxin production by *Microcystis*. Results suggested that MC synthesis was triggered by infochemicals released from herbivorous zooplankton (Jang et al., 2007b). Moreover, colony formation was observed in *M. aeruginosa* exposed to the flagellate *Ochromonas* sp. as well (Yang & Kong, 2012; Yang et al., 2006). Then, the increased MC production and colony formation were considered as efficient inducible defensive responses of *M. aeruginosa* to the grazing force of herbivorous zooplankton as exposure caused death in zooplankton (Jang et al., 2003, 2007b; Rohrlack et al., 1999b). Another study reported that *Daphnia galeata* fed with the wild type of *M. aeruginosa* PCC7806 died rapidly compared to the cultures exposed to the non-toxic mutant strain (Rohrlack et al., 1999b). Additionally, the toxic strain caused a decrease in the mobility of the daphnids. Thus, the toxigenic *Microcystis* can use the benefits of toxicity as an anti-predator defense mechanism. However, the ingestion rate of the wild type versus mutant strains was the same, meaning that the daphnids were not deterred by toxin production (Rohrlack et al., 1999b). In fact, filtering and feeding behavior of *Daphnia* spp. was not influenced by the presence or absence of MCs (Rohrlack et al., 1999a,b). The inhibitory effects were related to the mechanical inhibition of maxillule movement and swallowing rate caused by the morphology of *Microcystis* such as the high viscosity of

mucilaginous envelope and colony size of colony forming *Microcystis* that exceeded the size limit for food intake by the daphnids (Rohrlack et al., 1999a). Regarding the refuse of MC effects on *Daphnia* sp.'s ingestion rate, the significant inhibition of food intake reported by unicellular toxic strain may be related to other factors which require further investigation (Müller-Navarra et al., 2000; Rohrlack et al., 1999a).

The diversity and distribution of *Daphnia* spp. were negatively correlated to the *Microcystis* blooms biomass (Reichwaldt et al., 2013). In a small eutrophic lake, the higher diversity and larger zooplankton biomass were observed at the sites of lower *Microcystis* bloom densities (Reichwaldt et al., 2013). Increased MC production in response to the direct and indirect exposures of *M. aeruginosa* toxic strains NIES 44, 88 and 99 to phytoplanktivorous (*Hypophthalmichthys molitrix*) and omnivorous fish (*Carassius gibelio langsdorfi*) provided more evidence in support of the inducible defensive role of MC which are triggered by the infochemicals, known as kairomones, released from fish (Ha et al., 2009; Jang et al., 2004). The increased concentrations of infochemicals induced increased MC production. Infochemicals produced by fish influenced MC production even though physical contact was not necessary (Ha et al., 2009).

Above all, cyanobacteria evolved a long time before the metazoans, and therefore defense against predators as the primary role of MCs is doubted, however; perhaps toxicity was introduced as a recent evolutionary role of MCs (Kaplan et al., 2012). Furthermore, the role of MCs as anti-herbivore chemical defenses is ambiguous. Further field studies showed that even high concentration of toxin is not enough to stop daphnid proliferation so that it might be used as a biological control of blooms (Chislock et al., 2013). Although the co-existence of zooplankton and toxic *Microcystis* in eutrophic habitats might be due to the reciprocal defenses that contain the induced toxin increase in *Microcystis* and induced tolerance in zooplankton (Jang et al., 2007b). Feeding inhibition of toxic *Microcystis* by chemosensory means (Copepods) or inhibitory consumption of large colonies (some small Cladocerans) and physiological resistance to MCs (*Brachionus calyciflorusor* and *Daphnia pulicaria*) were introduced as defense mechanisms in zooplankton (DeMott et al., 1991; Fulton & Paerl, 1987).

2.3.5 Iron acquisition

Iron is a vital growth factor involved in photosynthesis, respiration, nitrogen fixation, and detoxification of oxygen radicals (Sunda, 2001). MC production rate has been reported to

change with different concentrations of iron (Sevilla et al., 2008; Zakhia et al., 2008). Under low iron level (below 2.5 μmol L^{-1}), the cellular growth was slower, but the toxicity was 20 – 40% higher (Lukač & Aegerter, 1993). Increased MCs content under iron depletion (10 nmol l^{-1}) together with a greater accumulation of phycobilisome proteins and FutA, the ferric iron transporter, were also observed in toxic *M. aeruginosa* PCC 7806 strain, but not in non-toxic *mcy*H^{-} mutant and PCC 7005 strains (Alexova et al., 2011, 2016). Moreover, at severe and long-term iron limitations, MC-producing strain survived longer and had a greater iron uptake compared to non-toxic strains, and the toxin production rate was found to be higher (Alexova et al., 2011; Martin-Luna et al., 2006b). Therefore, it was proposed that MCs perhaps serve as a siderophore especially during extended iron starvation or act as an iron storage component (Alexova et al., 2011; Martin-Luna et al., 2006b). However, increased toxicity under iron depletion might be considered as a response to this environmental stress as well.

It was shown that under low concentration of Fe (III), *M. aeruginosa* 7806 released superoxide to the extracellular environment that converts Fe (III) to Fe (II) which was later taken up through a siderophore-mediated system (Fujii et al., 2010). However, regarding low affinity of MCs to ferric iron which is inconsistent with the properties of siderophore, it was suggested that MCs might act as a shuttle to transfer iron through the cellular membrane (Klein et al., 2013).

Supplementary evidence of the relation between toxicity and iron uptake was obtained by finding the binding site of Fur in the promoter region of *mcy* gene cassette between *mcy*A and *mcy*D (Kaplan et al., 2012; Martin-Luna et al., 2006a). Fur, a ferric uptake regulator, is known as an iron availability and oxidative stress antenna in prokaryotes (Whitton, 2012). It is involved in iron homeostasis, regulation of the genes responsible for oxidative stress responses, and cellular metabolism (Martin-Luna et al., 2006b). At sufficient concentration of iron; binding of Fur to the regulator region of DNA suppresses the expression of these genes (Whitton, 2012). Under iron starvation, Fur was expressed 2-fold higher alongside an increase in the MC production rate (Martin-Luna et al., 2006a,b; Whitton, 2012). Thus, Fur is involved in the regulation of iron uptake and MC biosynthesis (Alexova et al., 2011; Dittmann et al., 1997; Martin-Luna et al., 2006a; Whitton, 2012). On the other hand, the iron uptake capacity of cells at high light has been shown to be enhanced (Kaebernick & Neilan, 2001). Light intensity controls both iron uptake and MC biosynthesis. Moreover, reduction of Fe (III) resulting from photoreduction or by superoxide dismutase made iron more available for the organism, which was later taken up by an iron-transfer system (Fujii et al., 2010). Thus, the possibility of a light regulating system was proposed (Whitton, 2012).

2.3.6 Allelopathic interspecies interactions

Cyanobacteria are important members of the phytoplankton community which also contains other organisms such as diatoms, dinoflagellates, as well as green, red and brown algae. In aquatic habitats, cyanobacteria dominate the algal communities at the different seasonal cycle in a successive wave (El Herry et al., 2008). Previous publications showed that not only the environmental physical factors but also the interspecies biological interactions known as allelopathy, influence the algal succession and bloom formation (Figueredo et al., 2007; Legrand et al., 2003; Zhang et al., 2015). The production of allelopathic compounds has been observed in a wide variety of phytoplankton species (Gantar et al., 2008; Legrand et al., 2003; Lewis Jr, 1986). In several studies, it has been shown that toxins can be considered as allelochemical (Granéli et al., 2008; Jaiswal et al., 2008; Rengefors & Legrand, 2001). The producer can use this benefit to outcompete the other algal species in aquatic ecosystems (Granéli et al., 2008; Legrand et al., 2003; Rengefors & Legrand, 2001). The interactions between *M. aeruginosa* and other cyanobacteria, other members of phytoplankton community, and aquatic plants can be taken into account as allelopathy. MCs affect the target species by photosynthesis inhibition, growth inhibition, and oxidative stress induction (Gantar et al., 2008; Legrand et al., 2003).

Allelopathy was proposed as an essential tool of cyanobacteria to outcompete diatomic population in a eutrophic lake (Keating, 1978). The results were obtained by measuring the growth rate in mono and mixed cultures (Leão et al., 2009). The allelopathic function of MCs was further observed as the growth inhibition of various algal species, such as *Chlamydomonas, Haematococcus, Navicula and Cryptomonas*, and cyanobacteria with exposure to *Microcystis* and MC-LR (Babica et al., 2006; Kaebernick & Neilan, 2001; Leão et al., 2009; Singh et al., 2001). However, the growth and photosynthesis of the dinoflagellate *Peridinium gatunense* grown in the cell-free filtrate of *Microcystis* sp. (KLL strain MB) were severely inhibited; exposure to pure MC-LR only resulted in slight effects. It was later proposed that cellular extracts include some metabolites that enhance the effects of produced toxins (Sukenik et al., 2002). In another study, exposure of different aquatic organisms to the crude extract of a cyanobacterial bloom isolated from Lake Müggelsee (Berlin, Germany) caused an elevation in the activity of the detoxification enzymes and, moreover, led to photosynthesis inhibition in *Scenedesmus armatus* and *Ceratophyllum demersum*. However, the toxicity of the crude extract was remarkably higher than the concentration of pure MC used (Pietsch et al., 2001). The inhibitory effects of MC-LR on the growth and photosynthesis of aquatic macrophytes *C.*

demersum and *Myriophyllum spicatum* were observed at the environmentally relevant concentration of 5 µg L^{-1} (Pflugmacher, 2002). The photosynthetic pigment composition changed and the chlorophyll contents reduced (higher ratio of Chl b to Chl a) (Pflugmacher, 2002). Additionally, the antioxidative enzymes of *C. demersum*, superoxide dismutase, glutathione peroxidase, ascorbate peroxidase, and dehydroascorbate reductase, were induced indicating oxidative stress as a result of MC exposure (Pflugmacher, 2004). In another study, *Medicago sativa* was exposed to 5.0 µg $L^{-1}$of MC-LR and cyanobacterial bloom extract that led to the oxidative stress induction, lipid peroxidation and elevation of antioxidative enzymes, germination inhibition, and a decrease in the root length of alfalfa seedlings (Pflugmacher et al., 2006). Short time exposure of *Cyprinus carpio L* to MC-LR-induced oxidative stress mediated hydroxyl radicals in the carp (Jiang et al., 2013). The allelopathic interaction between duckweed *(Lemna japonica Landolt*) and toxic *M. aeruginosa* strains resulted in MC concentration increase and growth inhibition in the cyanobacterium and decrease in the growth of the aquatic plant (Jang et al., 2007a). By increasing the initial density of *Microcystis* cells in a co - cultivation of *M. aeruginosa* and *Chlorella* sp., the growth of green algae was changed from stimulation to the growth inhibition (Hong et al., 2010).

Concerning allelopathic studies, most investigations include two main procedures; exposing the target species to the pure MC as well as to *Microcystis* crude extract and culturing of target species with the culture media filtrates of *Microcystis* strains. Besides the cellular exudates and pure toxin, studies of co - cultivation systems and under continues toxin exposure to mimic the natural ecosystems can provide more practical information. The interspecies interactions of two MC-producing species *M. aeruginosa* CPCC 299 and *Planktothrix agardhii* NIVA-CYA 126 revealed that the presence of competing cyanobacteria affected the growth, *mcyE* gene copies, and McyE transcripts of both species negatively in mixed cultures compared to monocultures (Ngwa et al., 2014).

The presence of a diverse group of heterotrophic microorganisms associated with cyanobacterial blooms was reported in the field studies (Eiler & Bertilsson, 2004; Kolmonen et al., 2004). Interspecies interactions with heterotrophic bacteria influenced MC production (Dziallas & Grossart, 2011). Exposure of toxic strain of *M. aeruginosa* PCC 7806 and its mutant to hydrogen peroxide led to a decrease in MCs content. This decline was higher in axenic cultures compared to the xenic cultures containing heterotrophic bacteria added from a nutrient-poor Lake Stechlin (Germany) or the nutrient-rich Lake Dagow (Germany) indicating the contribution of heterotrophic bacteria to radical scavenging. Moreover, with increased

temperature the MC production in the xenic cultures was significantly greater than the axenic culture (Dziallas & Grossart, 2011).

Table 2.2: MCs possible extracellular functions.

Role	Evidences	References
Quorum sensing	Detection of extracellular MCs	Hotto (2007)
	Homology of McyH to the ABC transporter	Pearson et al. (2004)
	No detection of MCs in the Δ*mcy*H mutant of *M. aeruginosa*	Pearson et al. (2004) and Kaplan et al. (2012)
	Increased cell density	
	• Increased MC production rate	Wood et al.(2012)
	• Higher *mcy*E transcripts	Wood et al. (2011, 2012)
	• Increased total and extracellular MC quota	
	Exposure to pure MC-LR or crude extract	Schatz et al. (2007)
	• Increased *mcy*B transcript levels	
	Iron limitation	Yeung et al. (2016)
	• Increased extracellular MCs	
	Similarity between the sequence of the *mcy* gene cluster of *M. aeruginosa* and quorum sensing genes of *R. leguminosarum*	Dittmann et al. (2001)
	Similarity between light regulated proteins MrpA & B of *M. aeruginosa* PCC7806 and RhiA & B, signal mediator proteins of *R. leguminosarum*	Dittmann et al. (2001)
	• Strong expression of MrpA in the wild type • No detection of MrpA in a *mcy*B$^-$ mutant. • Enhancement in the transcription levels of MrpA & B proteins in response to MC addition	Dittmann et al. (2001)
Benthic survival and recruitment	Survival of *Microcystis* cells at the surface and depth of sediments	Ihle et al. (2005), Latour et al. (2007), Misson et al. (2012a,b), Catherine et al. (2013) and Torres & Adámek (2013)
	MCs detection in benthic sediments	Latour et al (2007)
	Preservation of MCs synthesize after several years of sedimentations (lake Grangent, France)	Misson et al. (2012a,b)
	Decrease in cellular MCs quota by increase in the age of sediments	Misson et al. (2012a,b)
	preferred recruitment of toxic *Microcystis* to non-toxic subpopulation (Lake Quitzdorf, Germany)	Schöne et al. (2010)
	Greater recruitment rate of smaller colonies (> 160 µm) with higher MCs content (Lake Villerest (France))	Misson et al. (2011)
	Annual decrease in *Microcystis* population in sediments during spring	Ihle et al. (2005)

	MC-LR addition • Greater decrease in recruitment of toxic *Microcystis*	Misson et al. (2012a)
Colony formation	Exposure to *Ochromonas* spp. • Induction of colony formation in *M. aeruginosa* PCC 7806	Yang et al. (2009) and Yang & Kong (2012)
	MC-RR addition • Increased extracellular polysaccharides (EPS) and larger colony formation	Gan et al. (2012)
	Larger colonies (>100 mm) (Lake Wannsee Germany) contained • Greater ratio of toxic to non-toxic genotypes	Kurmayer et al. (2003)
	• Covalently binding of MCs to lectin microvirin protein • Lower MC production in microvirin-deficient mutant Different kinds of microvirin detected in non-toxic strains	Kehr et al. (2006)
	MC-deficient mutants • Higher accumulation of MrpC • Greater aggregation tendency	Kehr et al. (2006) and Zilliges et al. (2008)
Defense against grazers	Exposure to grazers • Colony formation, lower long chain saturated fatty acids production and increased toxicity in *Microcystis*	Müller-Navarra et al. (2000), Jang et al. (2003), Watson (2003), and Yang & Kong (2012)
	Exposure to zooplanktons M. macrocopa, D. magna and D. Pulex • Increase in MC production depended on the growth stage, population densities and concentrations of cellular exudates	Jang et al. (2003)
	MC synthesis triggered by infochemicals released from herbivorous zooplankton	Jang et al. (2007a, 2008)
	Repaid death and decrease in the mobility of *D. galeata* fed with the *M. aeruginosa* PCC7806 wild type	Rohrlack et al. (1999b)
	Negative correlation between distribution and density of *Daphnia* to *Microcystis* blooms	Reichwaldt et al. (2013)
	Exposure to phytoplanktivorous and omnivorous fish • MCs increased in response to released infochemcals	Jang et al. (2004) and Ha et al. (2009,
Iron uptake or transfer	Dependency of MC production rate to iron concentration	Zakhia et al. (2008)
	Higher toxicity under low iron concentration	Lukač & Aegerter (1993)
	Severe and long-term iron limitations • Longer survival of MC-producing strain with a greater iron uptake and higher MCs production	Martin-Luna et al. (2006a) and Alexova et al. (2011)
	Iron depletion	Alexova et al. (2016)

	• Increased MCs • Greater accumulation of phycobilisome proteins and FutA in toxic *M. aeruginosa* PCC 7806	
	Low affinity of MCs to ferric iron	Klein et al. (2013)
	Binding site of Fur in the promoter region of *mcy* gene cassette	Martin-Luna et al. (2006b), Kaplan et al. (2012)
	Iron depletion • Higher expression of Fur • Increased MC production rate	Martin-Luna et al. (2006a,b)
	High light • Increase in the iron uptake	Kaebernick & Neilan (2001)
Allelopathic interactions	Exposure to MC-LR • Growth inhibition of various algal species	Kaebernick & Neilan (2001), Singh et al. (2001), Babica et al. (2006), and Leão et al. (2009)
	• Growth inhibition • Photosynthesis inhibition in *Ceratophyllum demersum* and *Myriophyllum spicatum*	Pflugmacher (2002)
	Increase in antioxidative enzymes activity in *Ceratophyllum demersum*	Pflugmacher (2004)
	Exposure of alfalfa (*Medicago sativa*) seedlings to MC-LR • Inhibition Germination and root development • Oxidative stress induction	Pflugmacher et al. (2006)
	• Promotion of oxidative stress in *Cyprinus carpio L*	Jiang et al. (2013)
	Cell-free filtrate of *Microcystis* sp. (KLL strain MB) • Growth and photosynthesis inhibition of dinoflagellate *Peridinium gatunense*	Sukenik et al. (2002)
	Exposure of *S. armatus* and *C. demersum* to the crude extract of a cyanobacterial bloom • Oxidative stress induction • Photosynthesis inhibition	Pietsch et al. (2001)
	Direct exposure of *Lemna japonica Landolt* to *M. aeruginosa* • MCs increase and growth inhibition of *M. aeruginosa* • Growth inhibition of *L. japonica*	Jang et al. (2007b)
	Co - cultivation of *M. aeruginosa* and *Chlorella* sp. • Growth stimulation and then inhibition by increase in the initial density of *Microcystis*	Hong et al. (2010)
	Co - cultivation of *M. aeruginosa* CPCC 299 and *P. agardhii* NIVA-CYA 126 • Decline in the biomass, *mcyE* gene copies and McyE transcripts of both species	Ngwa et al. (2014)

Exposure to hydrogen peroxide or increased temperature • Greater MCs content in xenic cultures of *M. aeruginosa* PCC 7806 containing heterotrophic bacteria	Dziallas & Grossart (2011)
Indirect exposure to phytoplanktivorous and omnivorous fish • Greater MC production in response to the released infochemcals	Jang et al. (2004) and Ha et al. (2009)

Collectively, the proposed extracellular functions (Table 2.2) might clarify the ecological importance of MCs in the dominance of toxic bloom-forming *Microcystis*. MCs might be considered as an assistant factor that in combination with other environmental factors helps *Microcystis* to outcompete the other co-existing organisms and form blooms in a successive wave.

2.4 Future outlook and concluding remark

M. aeruginosa, the most common freshwater bloom-forming cyanobacterium, possess a rich reservoir of strain-specific flexible genes that support its evolutionary ecological adaptations and success in bloom formation (Humbert et al., 2013). A large number of genes encode secondary metabolites containing MCs (Humbert et al., 2013). MCs are the most commonly studied cyanotoxins in the environment. Although as research to date mainly focusing on the toxicity of MC, the possible primary functions of MCs have not yet been clarified. However, there is a growing interest in understanding the advantages of MCs as a product that proved its worth for the producer. Recent investigations, ranging from field studies to laboratory experiments provided some insights into the different potential intra- and extracellular functions of MCs.

First, MCs are known as cyanobacterial secondary metabolites; however, the evidence obtained by current studies indicated they are produced continuously from the early logarithmic phase to the late stationary phase, making this definition unlikely (Gantar et al., 2008; Lyck, 2004, Orr & Jones, 1998; Tonietto et al., 2012). Moreover, current studies clarified the probable metabolic roles of MCs as an essential cellular compound (Gantar et al., 2008). Therefore, there might be a close connection between toxin production and the primary metabolism of toxigenic cyanobacteria (Holland & Kinnear, 2013; Lyck, 2004; Zilliges et al., 2011).

Second, MCs are known as endotoxins, but they can be released into the surrounding environments at different growth stages or under diverse environmental conditions (Hotto, 2007). Moreover, the discovery of an ABC transporter encoded by a part of the *mcy* gene cassette strengthens the theory of possible MC export (Dittmann et al., 2001). However, the extracellular toxin fraction constitutes about 10% of the total produced MCs; it's possible physiological and / or ecological importance especially in dense blooms might be considerable (Dittmann & Börner, 2005).

On the other hand, the necessity of MCs in the central metabolic pathway is still doubtable due to the existence of non-toxic strains that contain the *mcy* gene cluster but do not produce any detectable MCs (Christiansen et al., 2008). However, proteomic studies revealed that different quantity and forms of the same proteins were expressed in toxic and non-toxic strains (Tonietto et al., 2012). Moreover, in cyanobacterial blooms toxic and non-toxic strains co-existed. How can we explain this occurrence? Perhaps non-MC-producing strains exploit the available extracellular toxins produced by the toxic genotypes in blooms, or they might produce other metabolites with the similar chemical structure but which are not toxic such as cyanopeptolines or microginins (Leflaive & Ten-Hage, 2007; Namikoshi & Rinehart, 1996).

Cyanobacterial blooms contain a mixture of toxic and non-toxic strains in various proportions. The population dynamics of cyanobacterial blooms also change with time during bloom formation. Under favorable growth conditions, non-toxic strain might outcompete toxic populations. In contrast, under various stress conditions induced by biotic and abiotic factors such as nutritional limitations, which often accompany increased blooms density, the toxic strains can survive longer by virtue of their toxicity (Briand et al., 2012; Kardinaal et al., 2007; Renaud et al., 2011). Indeed, the strains which are equipped with the stress adaptation systems can dominate the competition with others in natural ecosystems.

Stress-induced conditions such as high light intensity, iron deficiency, and exposure to hydrogen peroxide promote MCs attachment to certain proteins (Zilliges et al., 2011). Covalent binding of MCs to proteins that are involved in carbon and nitrogen metabolism might increase the stability of these proteins against oxidative stress-induced damage and redox changes. As a result, the proteins maintain their natural conformation or activity under various stresses. Moreover, containing a higher quantity of proteins involved in cellular metabolism by toxigenic strains might lend the advantage of providing a larger cellular metabolic reservoir to endure longer under limited nutritional conditions or to produce more ATP which makes the cost of toxin production meaningful (Tonietto et al., 2012). Under oxidative stress, more MCs are released to the surrounding environment and therefore might play a role as a signal

molecule to synchronize the cell responses to the stress-inducing agents. Increased intracellular MC concentration in response to oxidative stress conditions such as iron limitation support the hypothesis of MCs involvement in protection against oxidative stress, while the enhancement in extracellular MCs suggests the possible role of MCs as a signal molecule to make a better environmental adaptation (Yeung et al., 2016). Additionally, *in vitro* MC-metals complex formation, besides MC localization in polyphosphate bodies, supports the possibility of MCs involvement in metal detoxification or storage within the producer organism.

In contrast, some current findings highlight the involvement of MCs in central metabolism. Differences in the proteins related to the carbon and nitrogen metabolism between MC-producing and non-toxic strains provides new insights into the possibility of MCs involvement in the cellular processes. The proximity of MCs to thylakoid membranes and carboxysomes, aside from the evidence of regulation of both toxicity and photosynthesis efficiency by light, suggests a possible role of MCs in photosynthesis and carbon fixation. Identification of binding sites of the nitrogen regulator NtcA in the *mcy* gene cluster promoter, as well as the dependency of MC synthesis on nitrogen supplies and the carbon/nitrogen status, suggest a possible link between toxicity and primary cellular metabolism.

Light-regulated proteins similar to the proteins involved in signal transduction and the regulatory effect of light on MC synthesis propose that *Microcystis* might have a light sensing mechanism with the features of a quorum sensing system in which light intensity can be considered as a controlling agent (Dittmann et al., 2001). Moreover, finding of a Fur binding sequence in the promoter region of the *mcy* gene cluster and increased iron uptake under high light reinforces the possible role of MCs in iron acquisition regulated through a light signaling cascade.

From an ecological point of view, the importance of MCs has been more elucidated by studies of interactions between *Microcystis* and other aquatic organisms. MC's role in the maintenance of bloom formation and as a feeding deterrent offers defense mechanisms against grazers linked to MC production (Gan et al., 2012). Furthermore, toxin related interspecies interactions, allelopathy, resulted in the suppression of growth and photosynthesis together with induction of oxidative stress in other aquatic species, especially the members of the phytoplankton community. This may improve success leading to cyanobacterial dominance, a critical function of MCs for the producer to dominate over other phytoplankton community members. MCs play a role in the benthic survival of *Microcystis* vegetative cells and as a signal molecule in the regulation of *Microcystis* recruitment, an important process for the annual succession of *Microcystis* blooms.

However, conflicting results in different studies, possibly due to the lack of standardized experimental designs and analysis using different culturing methods and growth conditions, have limited a generalized definition of the role of MCs and applying it to MC-producing species other than *M. aeruginosa* (Kardinaal et al., 2007; Neilan et al., 2013). For instance, studies of the effects of various light intensities on MC production showed controversial results. Some studies indicated that MC production was elevated at high light intensity while the other studies revealed that it was increased under low light intensity (Kaebernick et al., 2000; Rapala et al., 1997; Sivonen, 1990; Utkilen & Gjølme, 1992; Van der Westhuizen & Eloff, 1985). Strain-specific toxin production rate has further hindered the comparison of studies and interpretation of tested parameters. For instance, the presence of some proteins such as MrpC are highly strain specific and may be associated with distinct colony types of *Microcystis* (Gan et al., 2012; Zilliges et al., 2008). On the contrary, controlled laboratory conditions might be different from the natural conditions where a variety of complex biotic and abiotic factors influence cyanobacterial proliferation and toxin production. It is further necessary to realize that laboratory experiments do not provide a holistic view of the function of MCs as *Microcystis* behaves differently under laboratory conditions compared to the field conditions as is evident with colony formation in nature versus unicellular growth when cultured (Sun et al., 2015; Zhang et al., 2007). In addition to the morphological shifts, more changes have been reported in *Microcystis* strains isolated from the field and cultured in the laboratory such as loss of MC production ability or changes in the toxicity and MCs variants (Cuvin-Aralar et al., 2002; Schatz et al., 2005). On the other hand, previously known non-toxic strains might retain the genes for MCs synthesis (Rantala et al., 2004). These changes have made it difficult to obtain a clear understanding of what happens in nature. For instance, the different defense mechanism against grazers has been reported for the unicellular laboratory cultures and colonial field samples. For the unicellular cultures, toxicity, and in the case of the field samples, large colony formation, have been explained as defense mechanisms.

Consequently, despite the vast amount of research on MCs, a general function has not been elucidated, but it is possible that MC can have multiple functions. There are still many raised questions that need to be answered. Further studies are required to clarify the natural role(s) of MCs.

In the end, evidence suggests that global warming and eutrophication will increase the occurrence of cyanobacterial blooms with a shift toward more toxic populations in the future. Therefore, deeper studies need to get more insights into the success of *Microcystis* in making blooms worldwide. Improvement of the current knowledge concerning the ecological or

physiological significance of MCs and the conditions influencing MC production might open the way for the control of harmful cyanobacterial blooms. For instance, allelochemicals produced by co-occurring organisms might be useable as eco-friendly biocontrol agents of *Microcystis* blooms. Therefore, further understanding of allelopathy can aid in biological bloom control in the future.

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3 PAPER II

Interspecies interactions between *Microcystis aeruginosa* PCC 7806 and *Desmodesmus subspicatus* SAG 86.81 in a co-cultivation system at various growth phases

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Own contribution:

- Literature review
- Design of experiments and laboratory studies
- Setting up the Algal monocultures and co-cultivation system
- Performing all required laboratory works
- MC analysis and growth measurements
- Statistical analysis and interpretation of the experimental results
- Preparation of the manuscript, which includes all texts and figures
- Revision of the manuscript after receiving the reviewer's comments

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Highlights

- The species affected each other depending on the physiological growth status.
- Green alga excluded *M. aeruginosa* at the exponential phase of growth.
- *Microcystis* might benefit from its other metabolites rather than or along with MC.
- In the presence of the green alga, MC production and release was changed.

Abstract

In lakes, cyanobacterial blooms are frequently associated with green algae and dominate the phytoplankton community in successive waves. In the present study, the interactions between *Microcystis aeruginosa* PCC 7806 and *Desmodesmus subspicatus* were studied to clarify the probable ecological significance of algal secondary metabolites; focusing on the role of cyanotoxin 'microcystin-LR' (MC-LR). A dialysis co-cultivation technique was applied where *M. aeruginosa* was grown inside and *D. subspicatus* was cultured outside of the dialysis tubing. The concentration of the intra- and extracellular MC-LR and the growth of two species were measured at different time points over a period of one month. Additionally, the growth of the two species in the culture filtrate of one another and the effect of the purified MC-LR on the growth of the green alga were studied. The results indicated that the co-existing species could affect each other depending on the growth phases. Despite the early dominance of *D. subspicatus* during the logarithmic phase, *M. aeruginosa* suppressed the growth of the green alga at the stationary phase, which coincided with increased MC production and release. However, the inhibitory effects of *Microcystis* might be related to its other extracellular metabolites rather than, or possibly in addition to, MC.

Keywords: Interspecies interactions, Co-cultivation, *M. aeruginosa*, *D. subspicatus*

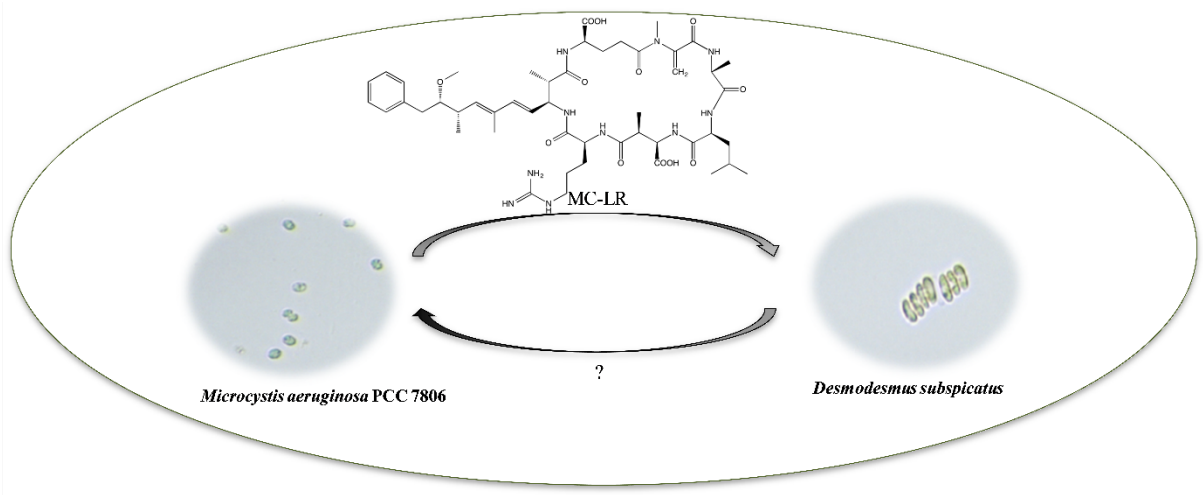

Fig. 3.1: Graphical abstract.

Abbreviations: Microcystins (MCs), Methanol (MeOH), Trifluoroacetic acid (TFA), Acetonitrile (ACN)

3.1 Introduction

Monitoring the composition of the phytoplankton populations has shown that algal species undergo a sequence of dominance; a phenomenon called seasonal succession (Reynolds, 1980). According to the seasonal pattern, diatoms (Diatomophyceae) dominate the phytoplankton community during the winter and spring, whereas during the summer green algae (Chlorophyceae) prevail, and in the late summer and autumn cyanobacteria outcompete their predecessors (Sommer, 1989). However, under environmental parameters favouring algal growth such as high nutrient availability (primarily nitrogen and phosphorous) in eutrophic waters, abundant sunlight, warm water temperature (> 25°C), and stagnant water, some species of cyanobacteria grow explosively and form large blooms outside of their typical season (Paerl and Otten, 2013; Rastogi et al., 2015; Scholz et al., 2017). Additionally, eutrophication of lakes and climate change influence the algal seasonal pattern, favouring the formation of harmful cyanobacterial blooms. The occurrence of toxic cyanobacterial blooms, which have undesirable effects on humans, animals, and aquatic biota, has been reported in many countries throughout the world (Zanchett and Oliveira-Filho, 2013; Svirčev et al., 2017).

Understanding the factors that induce the shift in the phytoplankton composition to the domination of a toxic bloom holds many advantages, especially concerning water quality, treatment, and governance. Recent studies reported that the seasonal fluctuation of

phytoplankton species is influenced not only by the environmental factors (Chen et al., 2003; Karadžić et al., 2013; Yang et al., 2018) but also by the interspecies interactions (Sukenik et al., 2002; Vardi et al., 2002; Legrand et al., 2003; Leão et al., 2009; Chia et al., 2018). In freshwater ecosystems, cyanobacterial blooms influence the composition of microbial communities and the co-occurrence patterns of eukaryotic plankton (Xue et al., 2018; L. Liu et al., 2019; M. Liu et al., 2019).

Microalgae, including cyanobacteria and eukaryotic algae, are known to produce and release a great variety of secondary metabolites during their life cycles or by cell lysis (Leflaive and Ten-Hage, 2007) as a result of natural senescence or chemical treatment. Cyanotoxins, produced by cyanobacteria, are a broad range of toxic secondary metabolites (Sivonen, 2009). Among them, microcystins (MCs), which are synthesised non-ribosomally via a large multifunctional enzyme MC synthetase, are the most widely studied cyanotoxins (Nishizawa et al., 2000; Sivonen, 2009). The past studies have mainly focused on the toxicity of MCs while their probable natural functions are not well understood. It is also not yet clear why cyanobacteria pay such a high energetic price for MC synthesis and what advantages the toxin lends to the producer.

A large volume of significant work has already invested in understanding the physiological and ecological roles of MCs (as reviewed by Omidi et al. (2017)). It has been found that MCs could interfere in the photosynthesis and nutrient metabolism, iron uptake, quorum sensing, benthic survival and recruitment process, bloom maintenance, protection against the oxidative stress, defence against the grazers, and the interspecies interactions. In this regard, the ecological significance of MCs in aquatic ecosystems has been more elucidated by the studies of the toxin-related interactions between *Microcystis* spp. and other aquatic co-existing species (Vardi et al., 2002; Li and Li, 2012; Yang et al., 2014; Bittencourt-Oliveira et al., 2015).

Cyanobacterial blooms are frequently associated with green algae and together dominate the aquatic environments in successive waves (Sedmak and Kosi, 1998; Chen et al., 2003; Harel et al., 2013). Therefore, a study of the interactions between these two environmentally co-existing organisms, cyanobacteria and green algae, might provide more insights into the seasonal variation dynamics of phytoplankton populations.

In the present study, the interactions between the most common species to cause toxic cyanobacterial blooms, *Microcystis aeruginosa* PCC 7806, and a common freshwater green alga, *Desmodesmus subspicatus* SAG 86.81 were studied. To achieve this, we designed a co-cultivation system where two populations were growing together in close proximity but separated physically using dialysis tubing to study the effects of the co-growing species on

each other in a continuous exposure mode through diffusible secondary metabolites beyond their physical cell-cell contacts. As a result, the changes in the growth of both species and MC-LR contents of *M. aeruginosa* were assessed. Moreover, the growth of the two species in the cell-free spent medium of each other was monitored. The green alga was exposed to different concentrations of the extracted MC-LR as well. Based on the results, the possible ecological significance of cyanobacterial secondary metabolites, with particular reference to MC-LR, in interspecies interactions, as well as the possibility of the production of secondary metabolites by green algae to influence the toxic cyanobacteria species, were considered.

3.2 Theory

Microcystins (MCs) are the most commonly studied cyanotoxins. Recently, the significance of MCs to the producing organisms beyond their toxicity gained particular attention. In an attempt to improve this knowledge, we applied a co-cultivation system in the present study, i.e. using a dialysis membrane to study the effects of the probable diffusible secondary metabolites of these two organisms on one another, concurrently excluding the effects of mixed-cultivation. To our knowledge, this is the first study to consider the efficiency of dialysis tubing in the interspecies interaction studies through the measurements of MC-LR on both sides of the tubing. This method may provide a more practical approach and information for future laboratory and field studies of microbial interactions.

3.3 Materials and methods

3.3.1 Algal monocultures and culture condition

The cyanobacterium, *Microcystis aeruginosa* PCC 7806, was provided by the Pasteur Culture Collection of Cyanobacteria (PCC) (Paris, France) and the green alga, *Desmodesmus subspicatus* SAG 86.81 (formerly *Scenedesmus subspicatus*), was obtained from the SAG Culture Collection of Algae (Sammlung von Algenkulturen) (University of Göttingen, Germany). The species were cultivated sterile BG 11 medium (Rippka et al., 1979), which was adjusted to pH of 8 using 1 N HCl and 1 N NaOH, respectively. The cultures were incubated at 24 ± 1°C under 12:12 light: dark cycle, with a light intensity of 2220 lm m^{-2} provided by cool white fluorescent irradiation. The cultures were grown for ten days to mid-exponential growth phase to use as inoculum in the co-cultivation experiments.

3.3.2 Setting up the co-cultivation system

The dialysis membranes (molecular weight cut-off (MWCO) 12 - 14 kDa; diameter of 29 mm; Spectra/ Por®, Spectrum Laboratories, USA) were cut into 30 cm lengths before being soaked in distilled water for 15 min, incubated in 10 mM sodium bicarbonate ($NaHCO_3$) for 30 min at 80°C, and then soaked in 10 mM Na_2EDTA for 30 min. The membranes were then placed in distilled water and autoclaved for 10 min at 121°C. The prepared sterilised dialysis bags with an open top and a closed bottom end were each positioned aseptically in a 250 mL Schott bottle containing 180 mL fresh BG 11 medium.

M. aeruginosa PCC 7806 was transferred into the dialysis tubing containing 20 mL BG 11 medium and therefore the cell density at the start of the experiment was 1×10^7 cells/mL. *D. subspicatus* was inoculated into the bottle containing the dialysis tubing but outside of the tubing, which contained 180 mL of BG 11. The starting cell density of the green alga then amounting to 1×10^6 cells/mL (Fig. 3.2). Two controls were set up, i.e. one where *M. aeruginosa* was cultured in a dialysis tube without *D. subspicatus* on the outside (control 1, monoculture of *M. aeruginosa*) and the second where the green alga was cultured in a glass bottle medium containing a dialysis tube filled only with BG 11 medium without *M. aeruginosa* (control 2, monoculture of *D. subspicatus*) (Fig. 3.2). The co-cultivation experiments were evaluated at three phases in the life cycle of the microalgae, i.e. after the first, second, and fourth weeks representing the early logarithmic phase, the logarithmic phase, and the late logarithmic and stationary phases of growth, respectively. In other words, the treatments were prepared in triplicate for three incubation periods, i.e. one, two, and four weeks, after which time samples were collected for cell counting and MC-LR analysis. The prepared cultures were incubated under the same conditions as described for the initial cultivation on a rotary shaker at 75 rpm at 23 ± 2°C. Every five days, 5 mL of fresh, sterilised medium was added on the outside of the membranes under aseptic conditions to eliminate the possibility of competition for nutrients. At the end of the first, second and fourth weeks, respectively, 1 mL was sampled from the inside and outside of dialysis tubing from each replicate. Taking into account, a dilution factor of 10 was applied to the reported biomass of *Microcystis,* regarding the ratio of the volume of the culture medium inside of the dialysis tubing to the whole medium (20: 200).

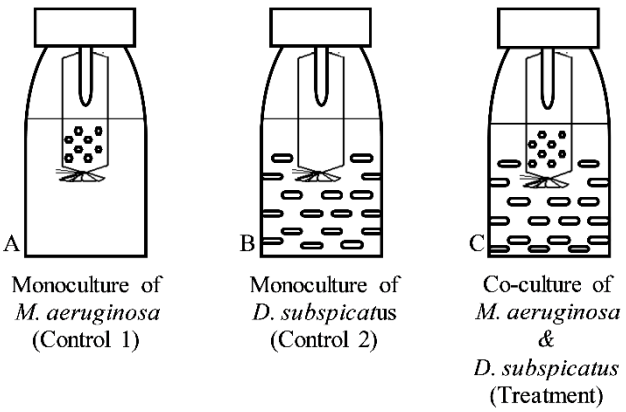

Fig. 3.2: A schematic view of the experimental design: A) monoculture of *M. aeruginosa* PCC 7806 in the dialysis tubing (control 1), B) monoculture of *D. subspicatus* SAG 86.81 out of the dialysis tubing (control 2), and C) a co-cultivation system containing *M. aeruginosa* PCC 7806 inside and *D. subspicatus* SAG 86.81 outside of the dialysis bag (treatment).

3.3.3 Growth of algal species in cell-free spent medium

Cell-free spent medium of each species was obtained from the algal monocultures at logarithmic phase of growth after centrifugation (4000 × g, 30 min, 4°C). Then, 100 mL of culture media containing different percentages of spent medium (25, 50, 75, and 100) was prepared using fresh BG 11 medium. The target species each at the initial density of 5×10^5 cells/mL was inoculated, and the prepared cultures (five replicates) were incubated under the described culture conditions of the algal monocultures for one, two and three weeks. Finally, 1 mL of sample was collected at the end of the incubation periods for cell counting.

3.3.4 Exposure of the green alga to the extracted MC-LR

MC-LR was extracted from 25 mg of the lyophilised *Microcystis* biomass as described below in section 3.3.6. The toxin concentration was determined using LC-MS/MS, as described section 3.3.8. Then, 100 mL of culture media containing different concentrations of the extracted MC-LR (70, 140, 270, 410 and 520 µg/L) was prepared in 300 mL Erlenmeyer flasks. *D. subspicatus* at an initial density of 1×10^5 cells/mL (five replicates) were grown under the previously described monoculture conditions. The experiment was run for two weeks, and every 2 or 3 days 1 mL sample was taken for the growth measurements.

3.3.5 Growth measurement

The cell densities of *M. aeruginosa* and *D. subspicatus* were monitored by counting the number of cells using a haemocytometer (Neubauer) and light microscopy (Olympus CH-2).

3.3.6 Intracellular MC-LR extraction

Microcystis cells were harvested by centrifugation (4000 × g, 30 min, 4°C) of 5 mL sample, which was taken from the inside of the dialysis tubing. The resulting pellet was lyophilised using an LIO-5P (5 Pascal, Italy) freeze dryer. The cells of 3 mg freeze-dried biomass were disrupted in an ultrasonic bath (Allpax, Germany) for 15 min in 3 cycles of 5 min. Then, 1 mL of 70% methanol (MeOH) acidified with 0.1% trifluoroacetic acid (TFA) was added, and the mixture was continuously shaken (Intelli-mixer, neoLab®) for one hour. The resulting slurry was centrifuged (10 000 × g, 10 min, 4°C), whereafter the supernatant was collected, and the pellet re-extracted following the same method. The procedure was repeated four times. Then, the resulting supernatants were combined and dried at 30°C using a Concentrator Plus (Eppendorf, Germany). Finally, the residue was dissolved in 1 mL of 100% MeOH and centrifuged (20 800 × g, 15 min, and 4°C). The supernatant was stored at -20°C until quantification with liquid chromatography-tandem mass spectroscopy (LC-MS/MS).

3.3.7 Extracellular MC-LR preparation

The concentration of extracellular MC-LR was measured both inside and outside of the dialysis tubing at the end of the first, second, and fourth incubation weeks in control 1 (monoculture of *M. aeruginosa*) and treatments. From 5 mL of sample collected from the inside of dialysis tubing, *Microcystis* cells were harvested by centrifugation (4000 × g, 30 min, 4°C), and the supernatant was filtered through a 0.22 µm Whatman filters (Sigma-Aldrich). From 30 mL sample collected from the outside of the dialysis membrane, the supernatant was collected after centrifugation (4000 × g, 15 min, 4°C) and then was filtered through a 0.22 µm Whatman filters (Sigma-Aldrich). The cell-free supernatants which have been collected from the inner and outer sides of the dialysis membrane were applied to a solid phase extraction column (Sep-Pak tC18 6 cc Vac Cartridge, 500 mg Sorbent per Cartridge, pore size 125 Å, particle size 37-55 µm, Waters). All columns were pre-conditioned with 10 mL of 100% MeOH, followed by 10 mL

of distilled water both at a flow rate of approximately 5 mL/min. Sample loading and elution with 10 mL MeOH 100% were conducted at a flow rate of 1 mL/min. Extracts were evaporated in a Concentrator Plus (Eppendorf, Germany) at 30°C. The resulting precipitates were solubilised in 1 mL of MeOH 100% and stored at -20°C until quantification. The outer membrane MC content was divided by the inner membrane MC-LR concentration (O / I: Outer / Inner) to obtain the diffusion rate.

3.3.8 MC-LR quantification

Determination and quantification of MC-LR were carried out on an Alliance 2695 UHPLC coupled to a Micromass Quattro micro™, (Waters, Milford, MA, USA). The chromatographic separation of the samples was carried out on a reverse phase column using a Kinetex™ C18 column (2.1 × 50 mm, 2.6 µm pore size, Phenomenex). The column temperature was thermostated at 40°C. The mobile phase consisted of solution A (MS-grade water containing 0.1% trifluoroacetic acid (TFA) and 5% acetonitrile (ACN)) and solution B (ACN containing 0.1% TFA) at a flow rate of 0.2 mL/min. The linear gradient conditions of solutions were as follows: 0 min 100% A; 3.75–7 min 35% A, and 7.8–12 min 100% A. The injection volume was 20 µL. The elution peak of MC-LR was observed at 7.95 min.

For the tandem mass spectroscopy, the electrospray ionisation (ESI) conditions were set as follows: capillary voltage of 3 kV, source temperature of 120°C, desolvation temperature of 500°C, and cone gas flow-rate of 100 L/h. The collision energy was 65 V, and the cone voltage was 60 V. Desolvation gas flow-rate was 1000 L/h. Nitrogen was used as trigger gas and Argon as the collision gas. Parent compound and its fragment ions, respectively, were scanned at the following mass-to-charge ratio (m/z) 995.5 → 135.1 for MC-LR. Limit of MC-LR detection was set at 1 ng/mL and limit of quantification at 5 ng/mL (signal to noise S/N > 5). Standard solution of purified solid MC-LR (Enzo, Germany) in MeOH 100% was used to set the calibration curve.

3.3.9 Statistical analysis

Statistical analyses were performed for the cell numbers and MC contents using SPSS, version 24. Shapiro-Wilk test and Levene test were used to verify the normality of the data and homogeneity of variance, respectively. The observed data were assessed statistically by one-

way analyses of variance (ANOVA) for differences between the mono- and co-cultures ($p \leq 0.05$). The Turkey HSD analysis and Student's t-test were used to assess the differences between means, if variables were homogeneous or heterogeneous, respectively. Data, which did not follow a normal distribution (the intracellular MC-LR in co-cultures at the end of the second week and the diffusion rate of extracellular MC-LR in co-cultures at the end of the fourth week) was analysed with non-parametric tests, such as the Kruskal-Wallis and Mann-Whitney-U-test. All data were presented as mean ± standard deviation (n=3). The Dunnett test and Student's t-test was used to assess the differences between means of algal biomass in cell-free spent media and the growth of green alga which was exposed to the extracted MC-LR, if variables were homogeneous or heterogeneous, respectively. Data were displayed as means ± standard errors (n=5). P value < 0.05 was set for statistical significance.

3.4 Results

3.4.1 MC concentration in co-cultivation experiments

After the first week of co-cultivation, i.e. during the early logarithmic phase of growth, neither intracellular nor extracellular MC-LR was detected in either monoculture (control 1) or simultaneous co-cultures (treatment). Over time, towards the end of the second week, i.e. during the logarithmic phase of growth, less total MC, which is the sum of intracellular and extracellular MC-LR, was detected in the co-cultivated (Fig. 3.3C and D, $p < 0.05$). In co-cultures, both the extracellular and intracellular MC-LR were significantly lower compared to monocultures ($p < 0.05$, Fig. 3.3A and B, respectively). However, the difference between intracellular MC-LR achieved with mono- and co-cultivated was greater than that seen with the extracellular MC-LR ($p < 0.05$, Fig. 3.3A and B). In co-cultures, the concentration of extracellular MC-LR (µg/L) was three-fold less than the monocultures while the content of the intracellular MC-LR (µg/mg dry weight) was four-fold less than the monoculture ($p < 0.05$, Fig. 3.3A and B, respectively). The results indicated that the MC release from cells was constant ($p > 0.05$) while the amount of intracellular MC-LR per cell was 4-fold decreased in co-cultures compared to monocultures (Fig. 3.3C, $p < 0.05$).

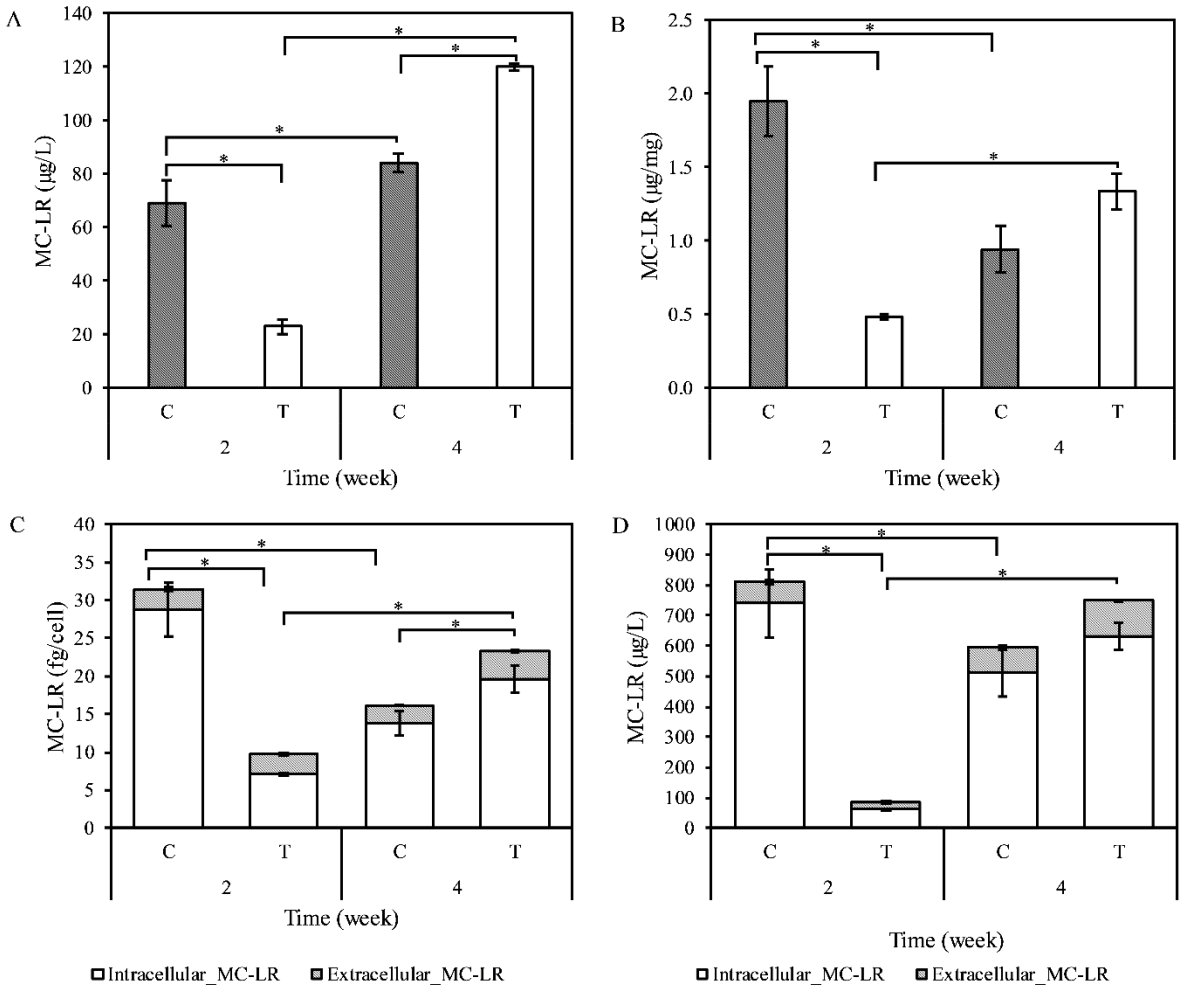

Fig. 3.3: The concentration of A) extracellular MC-LR (µg/L), B) intracellular MC-LR (µg/mg dry weight), C) MC-LR (intra- and extracellular) concentration per cell (fg/cell), and D) total MC-LR (intra- and extracellular) content (µg/L) in mono- and co-cultures at the end of the 2nd and the 4th week (C: control (monoculture); T: treatment (co-culture)). Data represent mean values ± standard deviation (n = 3). Significant differences observed at $p \leq 0.05$ (*).

After the fourth week, which was during the late logarithmic and stationary phases of growth, in the treatments, total MC content (the sum of intracellular and extracellular MC-LR (µg/L)) had not changed compared to the simultaneously conducted monoculture ($p > 0.05$, Fig. 3.3D). Thus, the amount of extracellular MC-LR was elevated ($p < 0.05$, Fig. 3.3A) while the concentration of intracellular toxin was at the same level as the monocultures ($p > 0.05$, Fig. 3.3B). However, the total MC content per cell was higher compared to the simultaneous monoculture. The results indicated a significant elevation in the concentration of both intracellular and extracellular MC-LR per cell ($p < 0.05$, Fig. 3.3C).

The results showed that the dynamic variation of MC content was different in mono- and simultaneous co-cultures. In the presence of the green alga, with time, the increased release of MC into the surrounding media was higher ($p < 0.05$, Fig. 3.3A). From the second to the fourth

week, in the monocultures, the extracellular MC-LR continuously increased from 69.3 µg/L after the second week to 84.3 µg/L after the fourth week. While, in the mixed cultures at the end of the second week, the extracellular MC-LR concentration was one-third of that measured with the monoculture (22.9 µg/L) which reached the highest quantified level (119.95 µg/L) ($p < 0.05$, Fig. 3.3A). Conversely, from the second to the fourth week, the concentration of intracellular MC-LR was significantly decreased with monoculture ($p < 0.05$) while it was increased in co-cultures ($p < 0.05$, Fig. 3.3B). The highest level of intracellular MC-LR (2.0 µg/mg dry weight) was quantified in the monocultures after the second week; then over time from the second to the fourth week, it was reduced by half ($p < 0.05$, Fig. 3.3B). In contrast, the lowest level of intracellular MC-LR (0.5 µg/mg dry weight) was quantified in co-cultures after the second week, which had been raised approximately three-fold over time to the fourth week ($p < 0.05$, Fig. 3.3B).

3.4.2 Growth measurements

3.4.2.1 Growth of algal species in the co-cultivation experiment

The growth curves of *M. aeruginosa* and *D. subspicatus* in single were studied and documented prior to the co-cultivation experiments (Fig. 3.4A). The results showed that the growth of the two species was different when growing together (Fig. 3.4B). At the end of the first week of co-cultivation, no changes in the growth of the green alga were observed ($p > 0.05$) while the growth of *Microcystis* was remarkably inhibited ($p < 0.05$). Over time to the end of the second week, the growth of *Microcystis* was reduced to one third which was at the same level as the treatment of the first week ($p > 0.05$) and significantly lower than its simultaneous monocultures ($p < 0.05$) whereas the growth of *D. subspicatus* was not altered ($p > 0.05$). Finally, despite the significant inhibition of the growth of *Microcystis* during the second week, the *Microcystis* biomass was increased from the second to the end of the fourth week of co-cultivation. However, at the end of the fourth week, it was still significantly lower than the monocultures ($p < 0.05$, Fig. 3.4B). In contrast, the growth of *D. subspicatus* has been notably inhibited at the end of the fourth week ($p < 0.05$, Fig. 3.4B).

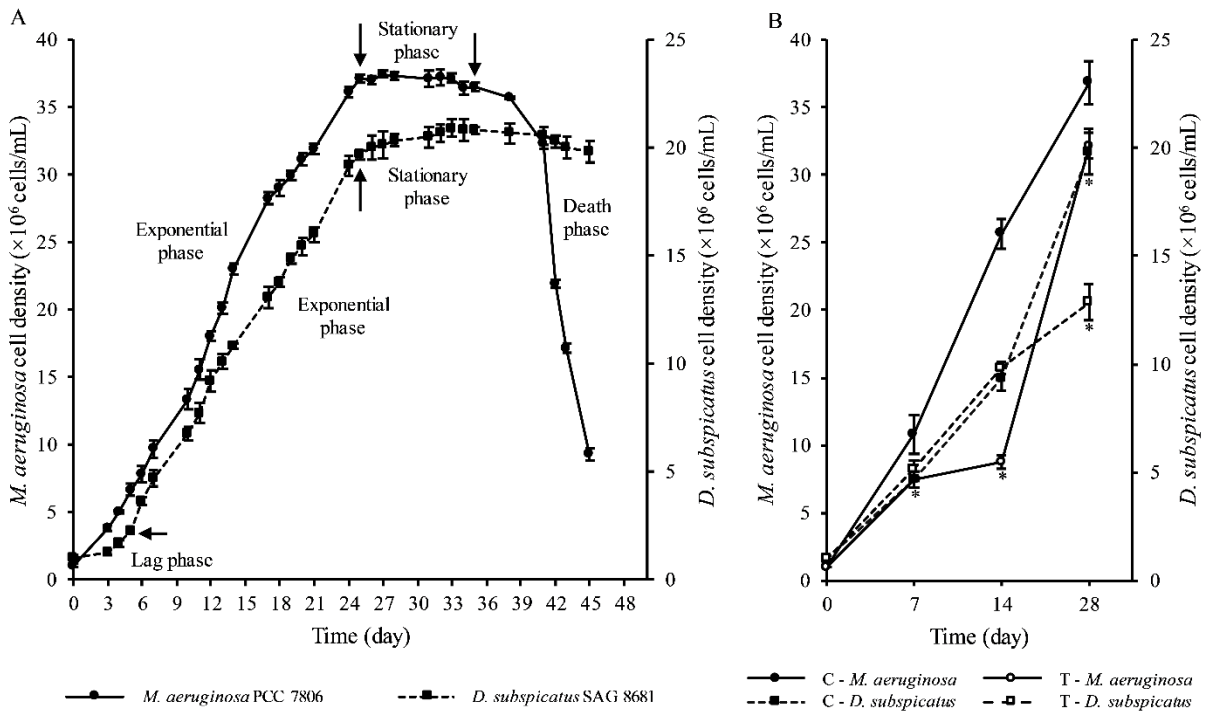

Fig. 3.4: Microalgal growth: A) the growth curves of *M. aeruginosa* PCC 7806 and *D. subspicatus*, and B) the growth of *M. aeruginosa* PCC 7806 and *D. subspicatus* in the co-cultivation system in mono- (controls) and co-cultures (treatments) (C: control, T: treatment). Data represent mean values ± standard deviation (n=3). Significant differences were accepted at a p-value of $p < 0.05$ (*).

3.4.2.2 Growth of algal species in cell-free spent medium

After the first week, no changes in the growth of *Microcystis* was observed at different percentages of the spent medium of the green alga. Afterwards, towards the end of the experiment, the biomass in 25% enriched medium was at the same level as the control ($p > 0.05$) while at the higher percentages of the filtrate, the biomass was significantly reduced ($p < 0.05$, Fig. 3.5A).

After the first week, the growth of the green alga at different percentages of the *Microcystis* filtrate stayed unchanged compared to the control ($p > 0.05$, Fig. 3.5B). After the second week, the growth was inhibited at 75% and 100% filtrate ($p < 0.05$) while at the lower percentages of the filtrate, the biomass was not altered ($p > 0.05$). Towards the end of the experiment, the biomass of the green alga was significantly reduced ($p < 0.05$) at the higher levels of the spent medium (50, 75, and 100%) while it was not changed at 25% filtrate, compared to the control ($p > 0.05$, Fig. 3.5B).

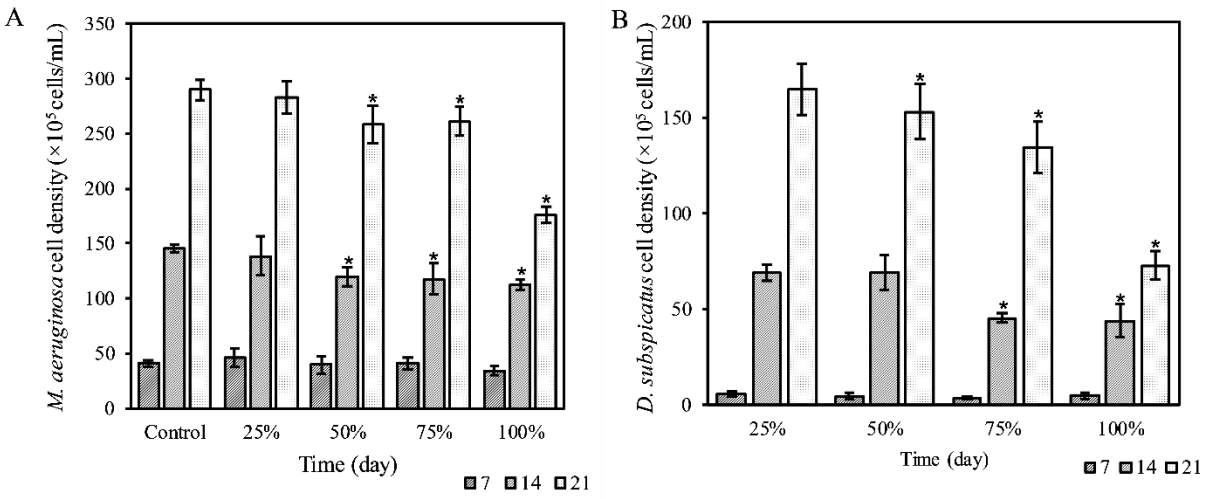

Fig. 3.5: The growth of A) *M. aeruginosa* in cell-free spent medium of *D. subspicatus* and B) *D. subspicatus* in cell-free spent medium of *M. aeruginosa*, at different percentages of the filtrates (25, 50, 70, and 100%). Data represent mean values ± standard deviation (n=5). Significant differences observed at $p < 0.05$ (*).

3.4.2.3 Growth of the green alga which was exposed to the extracted MC-LR

After ten days, the density of the green alga at the lower concentrations of MC-LR (70, 140, and 270 µg/L) was at the same level as the control ($p > 0.05$) while increased concentrations of MC-LR (410 and 520 µg/L) significantly inhibited the growth of green alga ($p < 0.05$, Fig. 3.6). Afterwards, on days 12 and 14, the density of *D. subspicatus* remained unchanged at the concentrations 70 and 140 µg/L ($p > 0.05$) while it was significantly reduced at the greater concentrations of MC-LR, compared to the control ($p < 0.05$, Fig. 3.6).

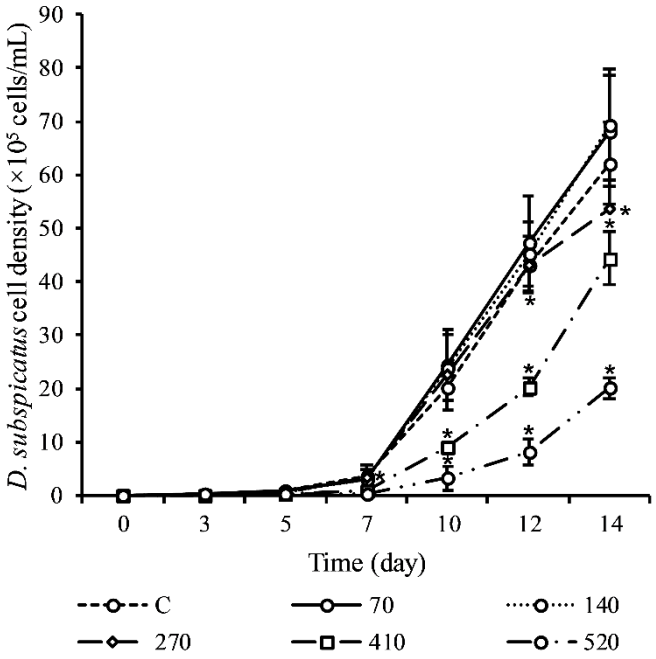

Fig. 3.6: The growth of *D. subspicatus*, which was exposed to the different concentrations of the extracted MC-LR from *M. aeruginosa* (70, 140, 270, 410, and 520 µg/L). Data represent mean values ± standard deviation (n=5). Significant differences observed at $p < 0.05$ (*).

3.4.3 The efficiency of the dialysis membrane in co-cultivation experiments

The efficiency of the dialysis membrane used in the co-cultivation system was considered by measuring the concentration of the extracellular MC-LR at both sides of the dialysis tubing for both the controls and treatments (Fig. 3.7). In all cultures, the concentration of extracellular MC-LR on the inside of dialysis tubing was significantly higher than the outside ($p < 0.05$, Fig. 3.7). The only exception was seen for the co-cultures during the second week where the extracellular MC-LR diffused through the membrane and was equally dispersed between the two sides, amounting to 52.29 ± 4.54% MC-LR on the inside and 47.71 ± 4.54% on the outside of the dialysis tubing ($p > 0.05$, Fig. 3.7 and Fig. 3.8A). For the monoculture sampled after the fourth week, the co-culture after the fourth week, and the monoculture after the second week, the diffusion rates were not equal, i.e. with the diffusion rates of 0.6, 0.5 and 0.2, respectively (Fig. 3.8B).

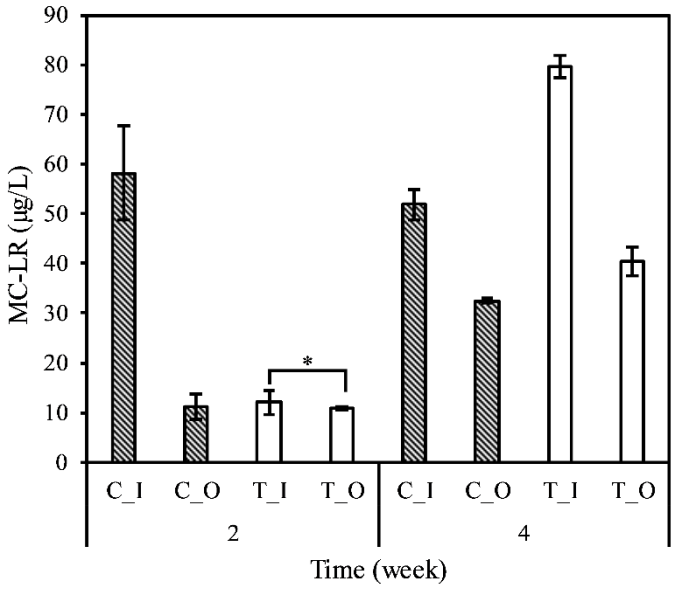

Fig. 3.7: The concentration of the extracellular MC-LR (µg/L) of *M. aeruginosa* at the inside and outside of the dialysis tubing in the controls and treatments (C: control, T: treatment, I: inner membrane, O: outer membrane). Data represent mean values ± standard deviation (n=3). An asterisk (*) indicates no significant difference ($p > 0.05$) observed between the inner and outer membrane MC-LR of control and treatment.

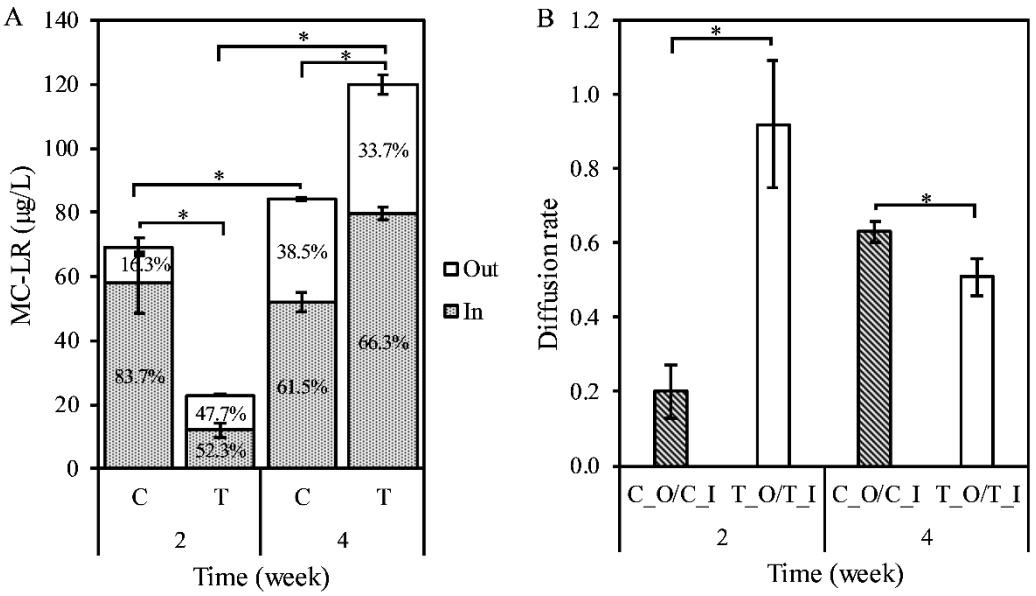

Fig. 3.8:The percentage (A) and the diffusion rate (B) of the extracellular MC-LR in mono and co-cultures at the 2nd and 4th week (C: control, T: treatment, I: inner membrane O: outer membrane). Data represent mean values ± standard deviation (n=3). Significant differences observed at $p < 0.05$ (*).

In the co-cultures, after the second week, where the minimum concentration of total extracellular MC-LR (22.9 µg/L) was quantified, the highest MC diffusion rate (0.9) was observed (Fig. 3.8B). After the fourth week, the smaller concentration of total extracellular

MC-LR in monocultures (84.3 µg/L) has been better equilibrated (Fig. 3.8A). On the other hand, the worst diffusion rate (0.2) has been observed in the monocultures of the second week, which contained a lower concentration of total extracellular MC-LR, compared to the control and treatments of the fourth week (Fig. 3.8B).

3.5 Discussion

In the present study, a co-cultivation system was designed which composed of two microalgae populations separated physically using a dialysis membrane, thereby still allowing the two to affect each other via their diffusible extracellular products beyond their physical associations. Over time, the inner membrane growing species, *M. aeruginosa*, released an increasing concentration of MC-LR, which diffused through the membrane and was detected at both sides of the tubing. The results indicated that at the lowest concentration of extracellular MC-LR, it could be equally distributed between the inner and outer sides of the dialysis membrane. Moreover, the diffusion rate of the released MC-LR was negatively related to the total concentration of extracellular MC-LR.

Regarding the experimental design, MC-producing *Microcystis* was placed inside of the dialysis tubing to avoid the reduction of the light intensity, which might have resulted from the shadow effects of the buoyant *Microcystis* cells in the mixture. Furthermore, the competition for nutrients was prevented according to the suggestion by Dunker et al. (2013) and Bittencourt-Oliveira et al. (2015). The species were cultivated in BG 11 medium which is a nutrient rich growth medium at the equal initial cell density (1:1), and fresh nutrient was added regularly to avoid giving one species the advantages of the higher initial biomass or the different ability in nutrient uptake to dominate the mixed culture. However, nutrient concentrations were not measured during the co-cultivation experiments for undoubted rejection of the possibility of resource competition between the co-cultured species.

The results indicated that in the co-cultivation system, both species affected one another depending on the growth stages. At the early logarithmic phase of growth, the presence of *Microcystis* did not influence the growth of green alga, while the green alga started to out-compete the co-cultured *Microcystis* which was further inhibited during the exponential phase of growth. The inhibitory effects of the green algae and some species of cyanobacteria on the growth, MC production and photosynthesis of *Microcystis* spp. was reported in other investigations through the reduction in chlorophyll a content (Jia et al., 2008), the production

of bioactive compounds capable of inhibition of MC production and increasing of the activity of alkaline phosphatase (ALP) (Rzymski et al., 2014), disturbing of the synthesis of proteins and polysaccharides and chlorophyll a content (Qiu et al., 2019), induction of cell lysis (Harel et al., 2013; Bittencourt-Oliveira et al., 2015), and the greater nutrient uptake ability of the green algae (Huan et al., 2006). Moreover, the results indicated that the reduction rate of MC release in co-cultures was at the same level as the decreased rate of the biomass of *Microcystis*. Therefore, the reduction of the extracellular MC-LR was due to the decreased *M. aeruginosa* cell density, and the decreased total MC content have resulted from the significant suppression of the MC synthesis. Since the MC production rate is positively related to the cell division rate, it can be assumed that the growth inhibition and suppression of MC synthesis might be related to the limiting factors such as light intensity and interferences in the nutrient uptake capability of *Microcystis* (Orr and Jones, 1998; Lyck, 2004; Downing et al., 2005; Deblois and Juneau, 2010; Chaffin et al., 2018; Kramer et al., 2018). MC production is highly depended on the nitrogen supply (Harke and Gobler, 2013; 2015). The binding site of NtcA, a global nitrogen regulator in cyanobacteria, was found in the promoter region of the *mcy* gene cassette (Ginn et al., 2010).

On the other hand, the study by Huan et al. (2006) demonstrated that in a mixed culture, the growth of *M. aeruginosa* was inhibited due to the greater ability of the green alga *Chlorella ellipsoidea* in the utilisation of nitrogen and phosphorous. Zhang et al. (2013) found that the green algae *Quadrigula chodatii* FACHB-1080 inhibited the growth of *M. aeruginosa* PCC7820 through the production of allelochemicals such as dibutyl phthalate and beta-sitosterol, and the interferences in the nitrogen uptake and utilisation by *Microcystis*. In the current study, the culture filtrate of the green alga negatively affected the growth of *Microcystis,* which might reinforce the probable interferences of the extracellular metabolites of the green alga in out-competing of the co-existing *Microcystis*. Moreover, the inhibition of the growth of *Microcystis* was observed earlier in co-cultures (after the first week) than compared to exposure with the *Desmodesmus* filtrate, which might be due to the interferences of the green alga in nutrient uptake capability of *Microcystis* in co-cultures. However, it is important to point out that the MC production rate is regulated by light intensity (Deblois and Juneau, 2010; Renaud et al., 2011). With the progression of the experiment, light limitation may have occurred due to the shading of the dense algal cultures, especially at the logarithmic phase of growth, which should be considered in future studies.

Over time, toward the stationary phase of growth, in co-cultures, MC production and release were increased, coinciding with the inhibition of the growth of the green alga. However, the

exposure of the green alga to the extracted MC-LR showed that the growth of green alga was inhibited only at MC concentrations that were greater than the outer membrane MC concentration (79.5 µg/L) of the co-culture experiments. Past studies have shown that the exposure of aquatic organisms to the *Microcystis* crude extracts caused greater activity in detoxification enzymes in the target species, compared to the purified MCs and the intact cells of *Microcystis* due to the presence of the other toxin modulating factors (Pietsch et al., 2001; Vasconcelos et al., 2007; Scoglio, 2018). However, the interspecies interference between intact cells in a consistent mode of microbial exposure might be more complicated. On the other hand, regarding the inhibitory effects of the *Microcystis* spent medium on the growth of green alga, it might be assumed that the other probable extracellular metabolites of *Microcystis* might be involved in the interspecies interplay. *Microcystis* is known to produce the other secondary metabolites such as micropeptin, microviridin, microgenin, as well as some unknown compounds that might be involved in the interferences of cyanobacteria with other phytoplankton species (Ikawa et al., 1996; Reshef and Carmeli, 2001; Ploutno et al., 2002). However, the probable synergistic effects between other metabolites and MC-LR to outcompete the co-existing species should be considered.

Previous studies have shown that in a mixed culture, *M. aeruginosa* severely inhibited the growth of the green algae *Chlorella pyrenoidosa* (Hong et al., 2010), and the growth and photosynthesis of the dinoflagellate *Peridinium gatunense* through abolishing carbonic anhydrase activity (Sukenik et al., 2002). Furthermore, the growth and photosynthesis of other aquatic organisms which were exposed to the purified MCs (25 - 50 µg/mL) or the crude extracts of *Microcystis*, were inhibited due to the reduction of CO_2 uptake, depletion of nitrogen fixation (Singh et al., 2001), and promotion of the oxidative stress (Pflugmacher, 2004; Paskerová et al., 2012). Therefore, at the exponential phase of growth, the growth of green alga was not altered, perhaps due to the repair systems of the green alga such as antioxidative enzymes (Cirulis et al., 2013). The study by Mohamed (2008) showed that MCs had been absorbed and biotransformed in the green algae, *Chlorella* and *Scenedesmus*. Additionally, it is speculated that the green alga could produce intra- and extracellular polysaccharides as an adaptive response to protect the cells against the oxidative stress (Mohamed, 2008; El-Sheekh et al., 2012). However, by entering the stationary phase of growth, the increased release of metabolites from *Microcystis* due to the increased lysis of *Microcystis* cells might have resulted in the inhibition of the growth of the green alga through the interferences in the photosynthesis process, inhibition of serine protease activity and the induction of oxidative stress (Smith et al., 2008).

On the other hand, the results of the co-cultivation experiments indicated that the rate of enhanced toxin release in co-cultures was significantly higher than the elevated rate of cell density. While in monocultures, they have enhanced approximately at the same level. Previous studies showed that the MC production and release could be induced under stress conditions such as the limited nutrient availability caused by the presence of the competitor species (Kaplan et al., 2012; Pimentel and Giani, 2014; Yeung et al., 2016). Additionally, the extracellular metabolites of green algae could induce MC production in *M. aeruginosa* (Bittencourt-Oliveira et al., 2015), and cause disruption of *Microcystis* cell membrane at the stationary phase of growth (Harel et al., 2013).

In summary, applying a co-cultivation system allowed investigating the interspecies interference between intact cells in a consistent mode of microbial exposure to simulate the natural ecosystems where microorganisms are co-existing within various microbial communities. However, the natural ecosystems are much more complicated than the laboratory-controlled conditions. A combination of the conventional exposure of target species to cellular exudates, purified metabolites such as MCs and co-cultivation studies might provide more insights into the probable mechanisms of the interspecies interplays, through the resource competitions, the interferences of the bio-compounds, or a combination of both. Moreover, the characterisation of the probable bioactive compounds of the green alga might open the ways for the control of harmful cyanobacterial blooms or minimise the harmful effects of the release of a high concentration of MCs into the natural ecosystem that come into contact with the aquatic species following toxic cyanobacterial bloom degradation. With this in mind, it should be considered whether exudates of green alga are non-toxic to non-target organisms.

3.6 Conclusions

This study highlights the potential of the dialysis membrane to determine the interspecific interactions between the intact cells of the co-growing species. It can be used in future laboratory or field studies by the positioning of the dialysis membrane containing the individual species or the mixed samples in the natural ecosystems of the occurring cyanobacterial blooms. It should be considered that the interspecies interactions through the bioactive compounds or due to the competition for the resources might influence the dynamics of the phytoplankton community. Both species might produce metabolites to influence the growth of each other negatively. The inhibition of the green alga at a concentration higher than the environmentally

relevant concentrations of MCs (1–10 µg/L) might explain the co-existence of the cyanobacteria and green algae in the phytoplankton community. MC might improve the fitness of *Microcysts* cells under the stress conditions induced by the presence of the green alga. However, the inhibitory effects of *Microcystis* might be related to other probable extracellular metabolites of *Microcystis* in addition to MC. The presence of MC may reinforce the inhibitory effects of the *Microcystis* on the growth of the co-existing green alga which should be considered in future studies.

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4 PAPER III

Desmodesmus subspicatus co-cultured with microcystin producing (PCC 7806) and the non-producing (PCC 7005) strains of *Microcystis aeruginosa*

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Own contribution:

- Literature review
- Design of experiments and laboratory studies
- Setting up the Algal monocultures and co-cultivation system
- Performing all required laboratory works
- MC analysis and growth measurements
- Statistical analysis and interpretation of the experimental results
- Preparation of the manuscript, which includes all texts and figures
- Revision of the manuscript after receiving the reviewer's comments

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Abstract

Although microcystins (MCs) are the most commonly studied cyanotoxins, their significance to the producing organisms remains unclear. MCs are known as endotoxins, but they can be found in the surrounding environment due to cell lysis, designated as extracellular MCs. In the present study, the interactions between MC producing and the non-producing strains of *Microcystis aeruginosa*, PCC 7806 and PCC 7005, respectively, and a green alga, *Desmodesmus subspicatus*, were studied to better understand the probable ecological importance of MCs at the collapse phase of cyanobacterial blooms. We applied a dialysis co-cultivation system where *M. aeruginosa* was grown inside dialysis tubing for one month. Then, *D. subspicatus* was added to the culture system on the outside of the membrane. Consequently, the growth of *D. subspicatus* and MC contents were measured over a 14-day co-exposure period. The results showed that *Microcystis* negatively affected the green alga as the growth of *D. subspicatus* was significantly inhibited in co-cultivation with both the MC-producing and -deficient strains. However, the inhibitory effect of the MC-producing strain was greater and observed earlier compared to the MC-deficient strain. Thus, MCs might be considered as an assistant factor that, in combination with other secondary metabolites of *Microcystis*, reinforce the ability to outcompete co-existing species.
Keywords: Co-cultivation; *D. subspicatus*; *M. aeruginosa*; MC-LR; interspecies interactions

4.1 Introduction

In recent decades, the increasing occurrence of cyanobacterial blooms in water bodies throughout the world has raised concerns (Buratti et al. 2017; Svirčev et al. 2017). Moreover, global warming and eutrophication have increased the occurrence of cyanobacterial blooms with a shift toward more toxic populations (Dziallas and Grossart 2011; Scholz et al. 2017). Cyanobacteria are known to produce toxic secondary metabolites designated as cyanotoxins which have undesirable effects on humans, animals, and aquatic organisms (Catherine et al. 2013; Zanchett and Oliveira-Filho 2013; Lévesque et al. 2014). Among the cyanotoxins, microcystins (MCs) are the most commonly studied. However, past studies have mainly focused on the toxicity of MCs while their importance to the producing organisms is still unknown. Recently, there has been a considerable growing interest in understanding the physiological and ecological importance of MCs to the producers beyond their toxicity.

MCs are produced by many species of cyanobacteria, however the most common bloom-forming species is *Microcystis aeruginosa* (Carmichael 1992). They are high-cost products which are synthesised non-ribosomally *via* a complex multifunctional enzyme, MC synthetase (Nishizawa et al. 2000; Welker and Von Döhren 2006). It is still uncertain why producers pay such a high energy price for the synthesis of MCs. A study by Christiansen et al. (2008) revealed that non-toxic strains could produce other non-ribosomal peptides, but not MC variants due to the partial or total lack of the MC synthetase genes, *mcy* gene cluster. MC-deficient strains, therefore, can be useful tools in competitive studies with toxic strains, to clarify the importance of MCs for the producing species. Previous studies demonstrated that MC-producing strains gain advantages from MC production over non-toxic subpopulations for better environmental adaptations under low or high light irradiation (Renaud et al. 2011), C-limited conditions (Jähnichen et al. 2007; Zhang et al. 2012), and elevated water temperature (Dziallas and Grossart 2011).

Recent studies have suggested some ecological and physiological functions for MCs; varying from the interference in photosynthesis and nutrient metabolism to quorum sensing, iron uptake, recruitment, defence against grazers, and allelopathic interactions (Omidi et al. 2017). The toxin-related interspecies interactions between *Microcystis* and other co-existing organisms such as the members of the phytoplankton community may further elucidate the ecological importance of MCs.

Toxin production by *M. aeruginosa* has been suggested to be a continuous process that starts in the early logarithmic phase to the beginning of the stationary phase (Lyck 2004; Jähnichen et al. 2008). MCs can be enclosed within the cells (intracellular MCs) or released into surrounding water (extracellular MCs) (Sivonen and Jones 1999) at different growth stages and under various environmental or physiological conditions, by cell lysis or leakage of intracellular MCs (Rapala et al. 1997; Leflaive and Ten-Hage 2007). Varying levels of MCs have been detected in the water bodies ranging from < 3 $\mu g\ L^{-1}$ across Europe to 19 500 $\mu g\ L^{-1}$ in water samples from Japan (Turner et al. 2018). During senescence of scums or very dense cyanobacterial blooms, high concentrations of MC reaching up to 25 000 $\mu g\ L^{-1}$ have been reported (Sivonen and Jones 1999; World Health Organization 2003).

When blooms collapse due to artificial (mechanical or chemical) or natural processes, the cellular material containing high concentrations of toxins are released into the environments in a short period. In nature, the toxin concentrations do not stay at these high level indefinitely due to rapid dilution in the water column and degradation of MCs by light or certain species of

bacteria (Christoffersen et al. 2002; Gągała and Mankiewicz-Boczek 2012). However, before this happens, aquatic biota is constantly exposed to MCs for days (Jones and Orr 1994).
In aquatic habitats, the cyanobacterial blooms are frequently associated with green algae, another member of the phytoplankton community (Sedmak and Kosi 1998; Paerl et al. 2001). The phytoplankton community, contain other members such as diatoms, dinoflagellates, and a diverse group of algae as well (Reynolds 2006). The species composition of phytoplankton communities are dynamic and change in seasonal cycles, a phenomenon called seasonal successions where according to the seasonal pattern the species dominate the phytoplankton community in successive waves (Reynolds 1980; Sedmak and Kosi 1998; Chen et al. 2003; El Herry et al. 2008). Past studies have shown that not only the abiotic environmental conditions, but also the biological factors such as interspecies interferences, influenced the seasonal fluctuations of the algal populations in the phytoplankton community (Vardi et al. 2002; Chen et al. 2003; Legrand et al. 2003; Granéli and Hansen 2006; Leão et al. 2009; Zhang et al. 2015). Therefore, the present study aimed to understand the influence and possible advantage of toxin production better, i.e. if it provided a competitive advantage to the MC-producing strain of *M. aeruginosa*. A comparative study between toxic (MC-producing) and non-toxic (MC-deficient) strains of *M. aeruginosa*, and *Desmodesmus subspicatus*, a freshwater green alga, was performed in a co-exposure system to explore the probable importance of the cyanobacterial bioactive metabolites, especially MCs, when the bloom material starts to lyse, and a high concentration of toxin is released into the surrounding environment.

4.2 Materials and methods

4.2.1 Organisms and culture conditions

Axenic cultures of *M. aeruginosa* PCC 7806 and *M. aeruginosa* PCC 7005 were obtained from the Pasteur Culture Collection of Cyanobacteria (PCC), Paris, France. The green alga, *D. subspicatus* SAG 86.81 (formerly *Scenedesmus subspicatus*), was provided by the SAG Culture Collection of Algae (Sammlung von Algenkulturen), University of Göttingen, Germany. The species were grown in BG-11 liquid medium (Rippka et al. 1979) at 24 ± 1°C under an illumination intensity of 2220 lm m^{-2}, provided by cool white fluorescent irradiation, with a 12:12 light: dark interval. The growth phases of *M. aeruginosa* PCC 7806 and PCC 7005 were studied and documented prior to experimentation (Fig. 4.1c).

4.2.2 Co-cultivation experimental design

The dialysis membranes with a molecular weight cut-off of 12 - 14 kDa and diameter of 29 mm (Spectra/ Por®, Spectrum Laboratories, USA) were used to separate the cultures. The membranes were cut into 30 cm lengths and soaked in distilled water for 15 min. Then, they were incubated in 10 mM sodium bicarbonate ($NaHCO_3$) for 30 min at 80°C and soaked in 10 mM Na_2EDTA for 30 min. The wide-open end of a glass pasture pipette was used to hold the dialysis tube and to transfer the *Microcystis* inoculum into the tubing. One *end* of the membrane was tightly tied to the pasture pipette *with* a piece of *string*, as an open end to the latter injection of the cells into the dialysis tubing. Another end was tied closed by a knot, then fastened to the glass pipette with the string. The prepared tubings were autoclaved for 10 min at 121°C and placed aseptically in a 250 mL glass bottle containing 180 mL sterile BG-11 medium. The dialysis tubings were filled with 15 mL fresh sterilized BG-11 medium and *Microcystis* inoculum in 5 mL fresh BG-11 medium was injected into the dialysis tubing using a sterile syringe.

Monocultures of *M. aeruginosa* in the dialysis tubing, containing 20 mL fresh, sterilised BG-11 medium, nine replicates of *M. aeruginosa* PCC 7806 (MC-producing strain), and three replicates of *M. aeruginosa* PCC 7005 (MC-deficient strain), were prepared each at the initial cell density of 1×10^7 cells mL^{-1}. Then, the groups of toxic and non-toxic independent replicates were grown for one month before commencing with the co-cultivation experiments. Every 5 days, 5 mL of fresh sterilized BG-11 medium was added out of the membrane.

After one month, monocultures of the toxic strain (triplicate) was harvested as “initial” to measure the initial concentrations of the intracellular and extracellular MC-LR before the introduction of the green algae into the culture system.

The co-cultivation experiments then commenced, i.e. they were performed using one-month-old monocultures of toxic and non-toxic *Microcystis* (six and three replicates, respectively). *D. subspicatus* at the initial density of 1×10^4 cells mL^{-1} was inoculated on the outside of the membrane into the bottle containing the one-month-old *Microcystis* inside the dialysis membrane. As a control for the growth of the green alga, *D. subspicatus* was cultured in the bottle containing a dialysis tubing that was filled only with BG-11 medium without *M. aeruginosa*. The prepared cultures include one-month-old monoculture of toxic *Microcystis* (control 1: C 1), co-cultures of green alga with the toxic (treatment 1: T 1) and non-toxic (treatment 2: T 2) strains of *Microcystis*, and monoculture of green alga (control 2: C 2), were

kept on a shaker at 75 rpm under the same conditions as described for the unialgal cultures for 14 days.

4.2.3 Growth measurement of *D. subspicatus* and *M. aeruginosa* PCC 7806

Every 2 or 3 days, 1 mL of sample was taken from the outside of the dialysis tubing from each replicate of monocultures (C 2) and co-cultures of the green alga with toxic and non-toxic strains of *M. aeruginosa* (T 1 and T 2, respectively). Then, cells of *D. subspicatus* were counted using bright field microscopy (Olympus CH-2, Japan) and a Neubauer counting chamber (Roth, Karlsruhe, Germany).

The cell density of *M. aeruginosa* PCC 7806 was monitored in one-month-old monoculture (initial) and after two weeks co-cultivation from mono- (C 1) and co-cultures of toxic *Microcystis* with green alga (T 1). A 1 mL sample was collected from the inside of dialysis tubing from each replicate. The optical density of samples (OD_{750}) was measured using a spectrophotometer (UVIKON 922, France). Then, the cell numbers was calculated using the calibration curve from the OD_{750} vs cells mL^{-1}.

4.2.4 Extracellular MC-LR preparation

Samples from the inside (5 mL) and outside (40 mL) of the dialysis tubing were collected from the one-month-old monoculture of toxic *Microcystis* (initial) and after two weeks co-cultivation from mono- (C 1) and co-cultures of toxic *Microcystis* with green alga (T 1). *Microcystis* cells were harvested and the supernatant collected from the inner membrane after centrifugation at 4000 × g for 30 min (4°C). The supernatant was collected from the outer side of the membrane after centrifugation (4000 × g, 15 min, 4°C). The supernatants were filtered through a 0.22 µm Whatman filter (Millipore). Solid-phase extraction (SPE) was performed using C18 SPE cartridges (Sep-Pak tC18 6 cc Vac Cartridge, 500 mg Sorbent per Cartridge, pore size 125 Å, particle size 37-55 µm, hold up volume, Waters) which was conditioned with 10 mL of 100% methanol (MeOH) and subsequently washed with 10 mL of distilled water. The cell-free supernatants were applied to the pre-conditioned cartridge and eluted with 10 mL of 100% MeOH. The eluent was dried in a concentrator plus (Eppendorf, Germany) at 30°C, and the residue was resuspended in 1 mL of MeOH 100% and stored at -20°C until analysis.

4.2.5 Intracellular MC-LR extraction

Samples of one-month-old monoculture of toxic *Microcystis* (initial), and two weeks mono- (C 1) and co-cultures of toxic *Microcystis* with green alga (T 1) were collected from the inside of the dialysis tubing and were centrifuged (4000 × g, 30 min, 4°C). Then, the pellet was freeze-dried using an LIO-5P freeze dryer (5 pascals, Italy). The lyophilised cells (25 mg) were sonicated in an ultrasonic bath (Allpax, Germany) for 15 min in 3 cycles of 5 min. After that, intracellular MC-LR was extracted by addition of 1 mL of 70% methanol (MeOH) acidified with 0.1% trifluoroacetic acid (TFA) which was continuously shaken (Intelli-mixer, neoLab®) for one hour. The resulting supernatant was collected after centrifugation (10 000 × g, 10 min, 4°C), and the pellet was re-extracted with the same procedure. This procedure was repeated four times. Then, the combined supernatant was evaporated to dryness at 30°C using a concentrator plus (Eppendorf, Germany) and the dried material was dissolved in 1 mL of 100% MeOH and centrifuged (20 800 × g, 15 min, 4°C). The supernatant was stored at -20°C until analysis by liquid chromatography-tandem mass spectroscopy (LC-MS/MS).

4.2.6 MC-LR analysis

An Alliance 2695 UHPLC coupled to a Micromass Quattro micro™ (Waters, Milford, MA, USA) was used for determination and quantification of MC-LR. A reversed phase column Kinetex™ C18 column (2.1 × 50 mm, 2.6 µm pore size, Phenomenex) was used for chromatography. Solution A (MS-grade water containing 0.1% trifluoroacetic acid (TFA) and 5% acetonitrile (ACN)) and solution B (ACN containing 0.1% TFA) was used as the mobile phase at a flow rate of 0.2 mL min^{-1}. A linear gradient elution program was applied as follows: 0 min 100% A; 3.75 - 7 min35% A, and 7.8 - 12 min 100% A. The column oven temperature was set at 40°C. The injection volume was 20 µL. MC-LR in the samples was identified by the retention time at 7.95 min.

The tandem mass spectroscopy, using electrospray ionization (ESI), conditions were set as follows: the spray voltage was set at 3 kV, and the cone voltage at 60 V. The capillary temperature was set at 120°C, the desolvation gas temperature and cone gas flow-rate was set at 500°C and 1000 L h^{-1}, respectively. The collision energy was 65 and cone gas flow-rate of 100 L h^{-1}. The trigger gas and the collision gas were nitrogen and Argon, respectively. Parent compounds and its fragment ions, respectively, were analysed according to their alignment at the following mass-to-charge ratio (m/z) 995.5 → 135.1.

The detection limit of MC-LR was 1 ng mL^{-1} (signal to noise S/N > 3), and the limit of quantification was set at 5 ng mL^{-1} (signal to noise the S/N > 5). The toxin content was quantified by calibrating against the standard solution of purified solid MC-LR (Enzo, Germany) in MeOH 100%.

4.2.7 Statistical analysis

Statistical analyses were performed with SPSS (version 24). Data were tested for normality and homogeneity of variance using the Shapiro-Wilk test and Levene test, respectively. Differences between samples were determined using one-way analyses of variance (ANOVA) followed by Turkey HSD analysis and Student's t-test for the homogenous (the growth of *D. subspicatus* and *M. aeruginosa 7806*, intracellular MC-LR, and the diffusion rate of extracellular MC-LR) and heterogeneous variables (extracellular MC-LR), respectively ($p \leq 0.05$). Data, which did not follow a normal distribution (growth of *D. subspicatus* on the third day (treatments (T1 and T2), and the seventh day (treatment with *M. aeruginosa* PCC 7806 (T1)) was analysed with non-parametric tests, such as the Kruskal Wallis and Mann-Whitney-U-test.

4.3 Results

4.3.1 Inhibition of growth rate

After co-cultivation with the MC-producing *Microcystis* (treatment 1, T 1), the biomass of *D. subspicatus* was significantly decreased compared to the biomass concentration achieved in monoculture (C 2) and also co-culture with the MC-deficient strain (treatment 2, T 2) ($p < 0.05$, Fig. 4.1a). The results indicated a long lag phase, from day 3 to 10, in the growth of green alga co-cultivated with PCC 7806. After ten days, the density of green alga in co-cultivation with PCC 7806, sharply levelled off to 29×10^4 cells mL^{-1}, 6.3 and 5.1 times less than when grown as mono- or in co-culture with the MC-deficient strain, respectively ($p < 0.001$). Afterwards, the growth of the green alga slowly increased and reached $148 \times 10^4 \pm 12 \times 10^4$ cells mL^{-1} on day 14, which was 3.4 times less than in monoculture and 2.16 times lower than when co-cultured with the MC-deficient strain ($p < 0.001$).

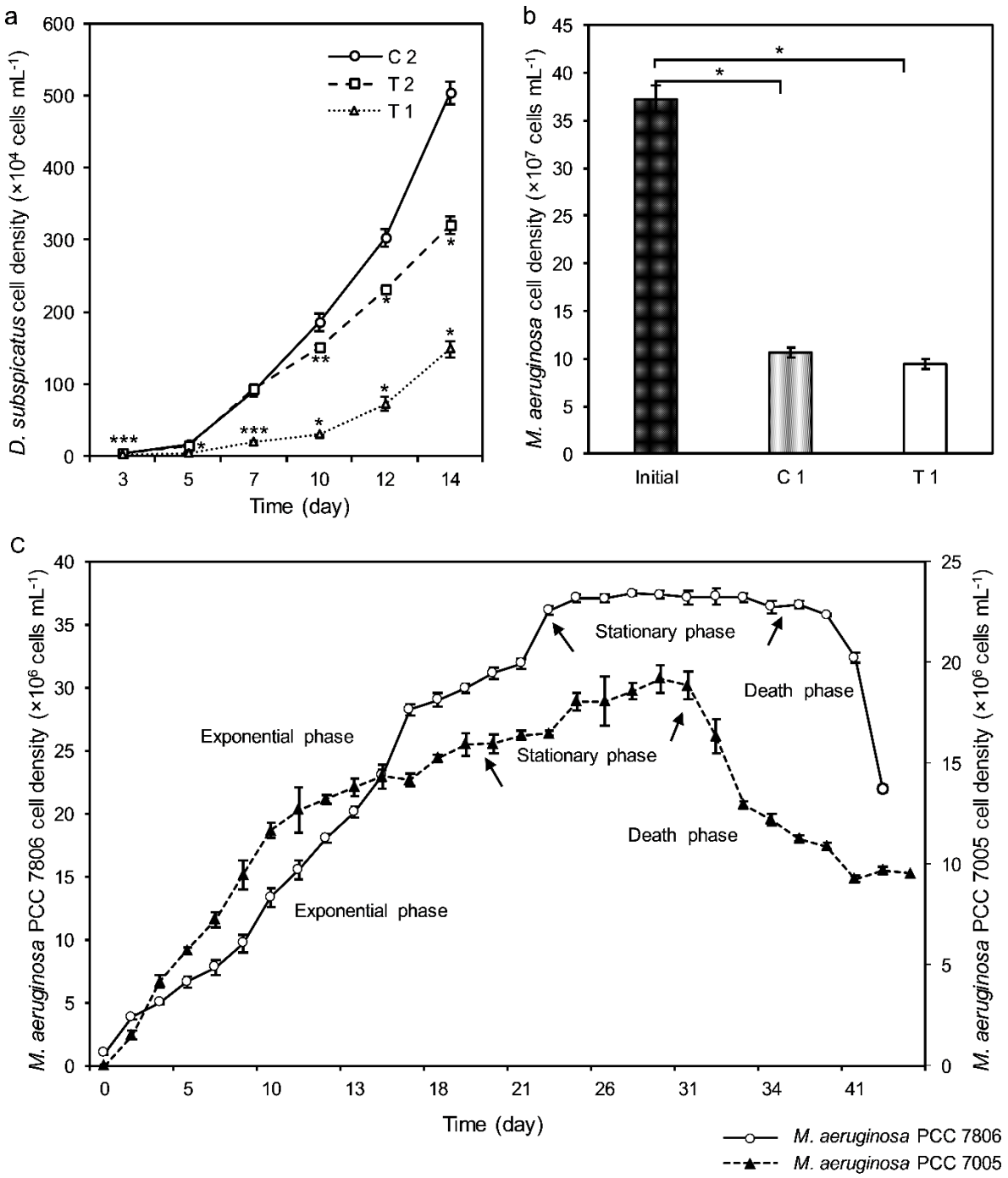

Fig. 4.1: The growth of *D. subspicatus* SAG 86.81 on the outside of the dialysis tubing in co-cultivation with *M. aeruginosa* (a), the density of *M. aeruginosa* 7806 inside of the dialysis tubing in co-cultivation with *D. subspicatus* (b), and the growth curves of *M. aeruginosa* PCC 7806 and PCC 7005 (c) (Initial, one-month-old monoculture of *M. aeruginosa* PCC 7806; C 1: control 1, 6 weeks old monoculture of *M. aeruginosa* PCC 7806; C 2: control 2, monoculture of *D. subspicatus*; T 1: treatment 1, co-culture of *M. aeruginosa* PCC 7806 with *D. subspicatus*; T 2: treatment 2, co-culture of *M. aeruginosa* PCC 7005 with D. subspicatus). Data represent mean values ± standard deviation (n = 3). Significant differences observed at p-values of $p < 0.001$ (*), $p \leq 0.01$ (**) and $p < 0.05$ (***)

In contrast, the density of the green alga, which was co-cultivated with MC-deficient strain, *M. aeruginosa* PCC 7005, was not significantly different to the control after the first seven days ($p > 0.05$). Afterwards, the biomass of green alga was decreased significantly and slowly fell to $320 \times 10^4 \pm 13 \times 10^4$ cells mL^{-1} at the end of the co-cultivation experiments on day 14 that was 1.6 times lower than the monoculture ($p < 0.001$, Fig. 4.1a).

The results showed that after 14 days of co-cultivation, the cell density of *M. aeruginosa* 7806 was significantly decreased in mono- (C 1) and co-cultures (T 1), compared to the one-month-old monoculture (initial) ($p < 0.001$, Fig. 4.1b). Moreover, the growth of *Microcystis* in co-culture (T 1) remained unchanged, compared to the simultaneously conducted monoculture (C 1) ($p > 0.05$, Fig. 4.1b).

4.3.2 Toxin concentration

At the inception of the experiment in one-month-old monoculture (initial), the total concentration of extracellular MC-LR (inside plus outside the dialysis membrane) was 1050.2 ± 51.7 µg L^{-1} (Fig. 4.2a) and the intracellular MC-LR concentration was 1214.5 ± 114.5 µg g^{-1} (Fig. 4.2b). After two weeks of co-cultivation, the concentration of intracellular MC-LR in mono- (C 1) and co-cultures (T 1) remained unchanged compared to the intracellular MC-LR concentration at the start of the experiment (initial) (Fig. 4.2b, $p > 0.05$). In contrast, the concentration of extracellular MC-LR was significantly raised both with mono- and co-cultivation ($p \leq 0.001$, Fig. 4.2a). However, after two weeks of co-cultivation, the increased MC release in co-culture (T 1) was at the same level as the simultaneous monoculture (C 1) ($p > 0.05$, Fig. 4.2a). Additionally, the amount of outer membrane extracellular MC-LR that was the actual concentration at which the green alga has been exposed was not significantly different relative to the control (C 1) ($p > 0.05$, Fig. 4.2).

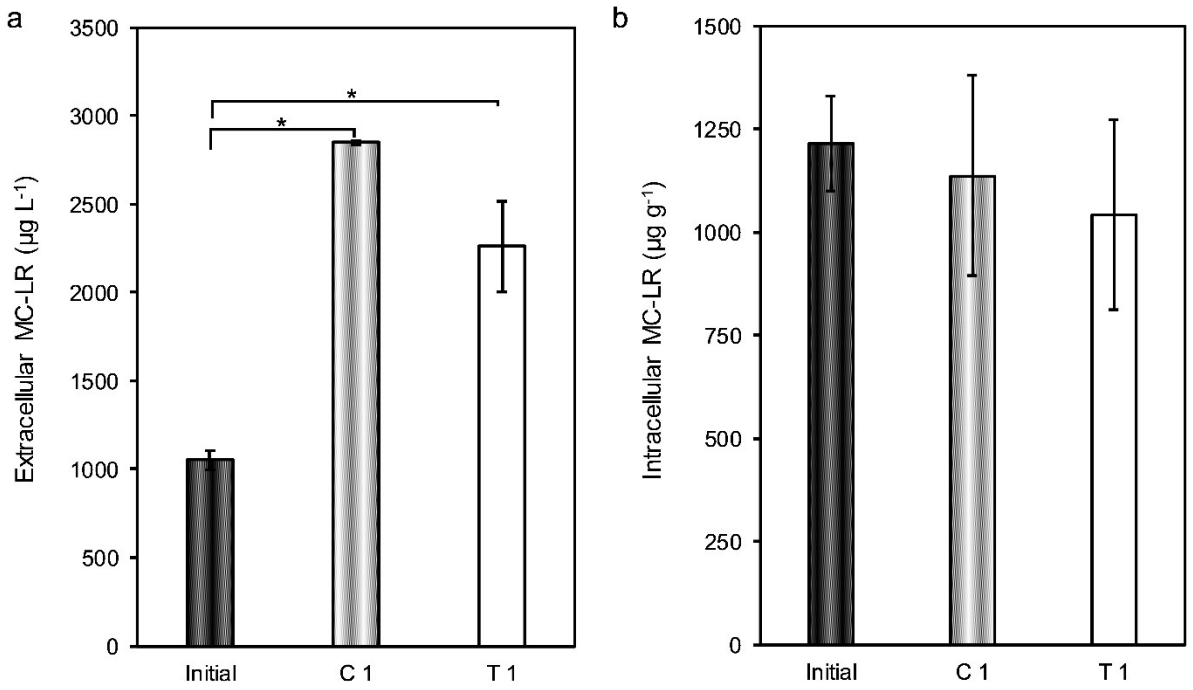

Fig. 4.2: The concentration of a) total extracellular MC-LR (µg L^{-1}) and b) intracellular MC-LR (µg g^{-1} dry weight) where initial is the monoculture of *M. aeruginosa* PCC 7806 sampled after 1 month, C 1 is the monoculture of *M. aeruginosa* PCC7806 sampled 14 days after inception of co-cultivation experiment (6 weeks), and T 1 is the co-culture of *M. aeruginosa* PCC 7806 with green alga-after 14 days. Data represent mean values plus minus standard deviation (n = 3). asterisk (*) indicates significant differences at a p-value of $p \leq 0.001$

The efficiency of employing the dialysis membrane in the co-cultivation system was assessed in terms of the diffusion rate of MC-LR. This was evaluated by measuring the concentration of extracellular MC-LR at both sides of the dialysis tubing (Fig. 4.3). The results showed that at the start of the co-cultivation experiment with *M. aeruginosa* PCC7806 monoculture (initial), as well as after 14 days of both mono- (C 1) and co-culture (T 1), the concentration of extracellular MC-LR inside the dialysis membrane was significantly higher than the outer of the membrane ($p < 0.01$, Fig. 4.3). However, in the one-month-old monoculture (initial) where the lowest concentration of extracellular MC-LR was measured, the highest diffusion rate of extracellular MC-LR (0.7) was observed which was at the same level as co-culture (T 1) and significantly greater than the monoculture of the co-cultivation experiment (C 1) ($p > 0.05$ and $p < 0.05$, respectively, Fig. 4.4). As *Microcystis* cells were entering the death phase, the release of MC-LR increased due to the increased cell lysis (Fig. 4.1b) after two weeks of co-cultivation. Therefore, the MC was less distributed between the inner and outer side of the dialysis membrane (Fig. 4.3 and Fig. 4.4).

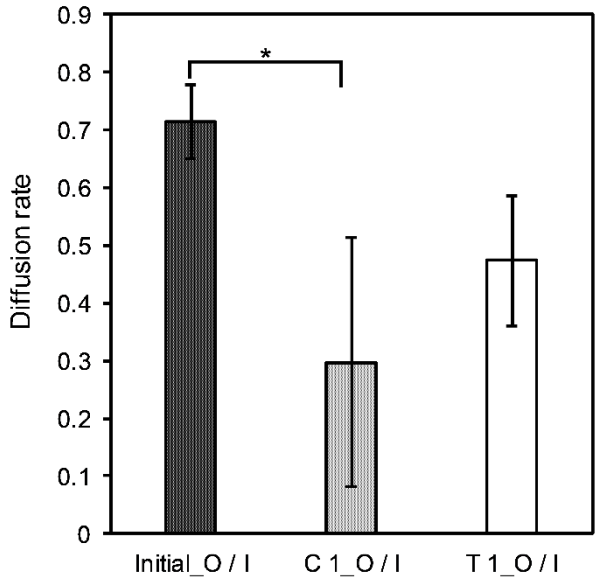

Fig. 4.3: The concentration of the extracellular MC-LR (µg L^{-1}) in the controls and treatment on the inside and outside of the dialysis tubing, measured after one month in monoculture (initial) and 6 weeks in monoculture (control 1) and co-culture (treatment 1) of toxic *M. aeruginosa* PCC 7806 (Initial: one-month-old monoculture, C 1: control 1, T 1: treatment 1, I: in the dialysis tubing, O: out of the dialysis tubing). Data represent means ± standard deviation (n = 3). Asterisk (*) indicates significant differences at a p-value of $p < 0.01$

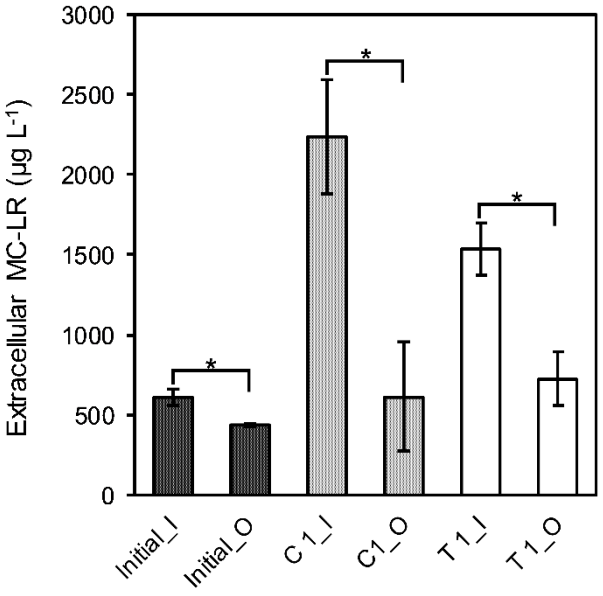

Fig. 4.4:The diffusion rate of extracellular MC-LR in controls and treatment (Initial: one-month-old monoculture of *M. aeruginosa* PCC 7806; C 1: control 1, 6 weeks old monoculture of *M. aeruginosa* PCC 7806; T 1: treatment 1, co-culture of *M. aeruginosa* PCC 7806 with *D. subspicatus*, I / O: in / out of dialysis tubing). Data represent means ± standard deviation (n = 3). Significant difference observed at a p-value of $p < 0.05$ (*)

4.4 Discussion

In the present study, a co-cultivation system was designed using a dialysis membrane as a separation technique, where *Microcystis* strains were grown inside of the dialysis tubing and the green alga was cultured outside of the tubing. The system allowed the species to influence each other through their diffusible extracellular products without any physical cell-cell contacts. Thus, the shading effects of the buoyant *Microcystis* cells which were grown to a dense culture could be restricted through confining the *Microcystis* cells inside of the dialysis membrane in the mixed culture. The results showed that over time the extracellular MC-LR diffused through the membrane. However, the diffusion rate of the released MC across the membrane was affected by a sudden large release of extracellular MC-LR, possibly delaying diffusion to achieve an equilibrium on both sides.

The results showed that during the co-cultivation experiment, coinciding with the decreased cell density of *M. aeruginosa* toxic strain in both the control and treatments, the concentration of intracellular MC-LR remained unchanged in mono- and co-cultures. However, the total extracellular MC-LR from both the inside and outside was significantly higher, which can be explained by the *Microcystis* cells entering into the stationary phase of growth (Lyck 2004; Jähnichen et al. 2008). This means that *D. subspicatus* which was growing to the exponential phase of growth was exposed to the contents of the *Microcystis* cells that passed into the collapse phase of growth. The growth of the green alga was inhibited in co-cultivation with both toxic and non-toxic strains of *Microcystis*. However, MC-producing strain inhibited the growth of green alga greater and in earlier days, compared to the MC-deficient strain. Then, it was assumed that the toxic strain might benefit from MC production through outcompeting of the co-existing green alga. Moreover, the green alga showed a longer lag phase in the presence of MC-producing strain compared to the control and MC-deficient *Microcystis*. The study by Mohamed (2008) indicated that the green algae, *Chlorella* and *Scenedesmus,* could absorb and biotransform MCs. The inhibitory effects of MCs on the growth and photosynthesis of the other members of phytoplankton community have been reported in other investigations as well (Singh et al. 2001; Yang et al. 2014). MCs could negatively affect the exposed species through the restriction of the carbonic anhydrase activity (Sukenik et al. 2002), the reduction of CO_2 uptake, depletion of nitrogen fixation (Singh et al. 2001), and the induction of oxidative stress that lead to programmed cell death (Pietsch et al. 2001; Pflugmacher 2004; Amado and Monserrat 2010). Therefore, it might be assumed that the green alga needed more time to fix

the damages which might have resulted from the presence of MC and/or the other secondary metabolites of *Microcystis*.

The results indicated that the naturally occurring non-toxic strain of *M. aeruginosa* PCC 7005 negatively influenced the growth of green alga as well. However, it took longer, and the inhibition was significantly smaller than the co-cultures with the wild-type MC-producing strain PCC 7806. Then, the inhibitory effects of *Microcystis* on the growth of green alga was not only related to MC but also the increased release of the other probable secondary metabolites over time were involved. Previous studies showed the other secondary metabolites of *Microcystis* such as micropeptin, microviridin, microgenin, as well as some unidentified compounds might interfere in the interspecies interactions (Banker and Carmeli 1999; Reshef and Carmeli 2001; Ploutno et al. 2002). However, the results of the current study showed that the presence of MC might reinforce the inhibitory effects of *Microcystis* on the growth of the co-existing green alga. More studies need to be done concerning the analysis of the secondary metabolic profile of both MC-producing and -deficient strains, for undoubted confirm or rejection of the probable involvement of the other cyanobacterial metabolites in combination with or rather than MC in the interspecies interactions.

On the other hand, the green alga did not affect the MC production and release from MC-producing strain that might be related to the population ratio and the physiological status of the co-existing species which influenced the pattern of the algal communications. The study by Bittencourt-Oliveira et al. (2015) showed that at the equal initial population ratio (1:1, 1×10^5 cells mL^{-1}), *Scenedesmus acuminatus* induced MC production in *M. aeruginosa*. The study by Harel et al. (2013) showed that the interspecies interactions between *Scenedesmus huji* and *M. aeruginosa* spp. at the same initial density of 1×10^5 cells mL^{-1}, was depending on the physiological status of the species. They showed that the metabolites derived from the stationary phase of the growth of green alga, *S. huji*, caused severe cell lysis in *Microcystis* spp. through the decrease of the integrity of the cell membrane. The study by Yang et al. (2018) showed *M. aeruginosa* and *Scenedesmus obliquus* at the similar algal initial abundance of 1×10^5 cells mL^{-1} have negatively affected one another's growth at 20 - 30°C, depending on their physiological status. As temperature increased from 20 to 30°C, the competitive advantages of the green alga was decreased where the green alga was superior for a shorter time at the initial phase of co-cultivation while towards the end of the co-cultivation period *Microcystis* resumed dominance in the mixed culture. Moreover, the results indicated that the growth of MC-producing *Microcystis* was not influenced in the presence of the co-cultured green alga. However, the growth of MC-deficient strain was not monitored. Therefore, due to the lack of

enough data it cannot be possible to assume whether the MC-producing strain benefited from MC over the MC-deficient strain to improve its fitness or not.

Taken together with the current study of the interspecies interplay between toxic and non-toxic strains of *M. aeruginosa* and the green alga, the probable importance of MC for the toxic *Microcystis* is evident. The results indicated that *Microcystis*, toxic and non-toxic strains, affected the growth of co-cultured green alga negatively. However, the growth inhibition in co-cultivation with the toxic *Microcystis* was significantly greater and occurred in earlier days. Then, in cyanobacterial blooms where toxic and non-toxic strains co-existed, the MC-producing strain may gain the advantage of MC production over non-toxic subpopulations to a greater exclusion of the co-existing species. MCs might play a role in the replacement of green algae with the cyanobacterial blooms at the end of summer.

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5 DISCUSSION

Cyanobacteria (blue-green algae) and green algae are members of the phytoplankton community. They are co-occurring in freshwater ecosystems and dominate at different seasonal cycles in successive waves. Previous studies indicated that the structure of the phytoplankton community was influenced not only by the environmental factors but also by the interspecies interactions.

In the present study, the interactions between a common bloom-forming cyanobacterium *"Microcystis aeruginosa"* and a green alga *"Desmodesmus subspicatus"* were studied in laboratory-designed experiments to get more insights into the probable ecological importance of algal secondary metabolites with a special focus on cyanotoxin "MCs." Therefore, a co-cultivation system was designed using a dialysis membrane to study the interspecies interferences, related to the algal extracellular metabolites beyond their physical associations. Moreover, *M. aeruginosa* PCC7806 (or *D. subspicatus*) was grown in the medium enriched with the cell-free filtrates of *D. subspicatus* (or *M. aeruginosa* PCC7806). The influence of the crude extracts of toxic *M. aeruginosa* PCC7806 containing different concentrations of MC-LR on the growth of green alga was studied as well.

5.1 Effects of *M. aeruginosa* on the growth of *D. subspicatus*

5.1.1 Inhibition of the growth of *D. subspicatus* in monoculture filtrates of *M. aeruginosa* PCC 7806 as well as co-cultures

The growth of *D. subspicatus* was monitored in BG11 medium supplemented with the cell-free spent medium of the toxic strain of *M. aeruginosa* PCC 7806, which was grown to the end of logarithmic phase of growth (Paper II). At the lowest concentration of the culture filtrates (25%), the growth of green alga has stayed at the same level as the control. While, the increased concentration of *Microcystis* exudates (50, 75, and 100%) inhibited the growth of green alga in a dose- and time-dependent manner where the higher concentrations of the filtrates inhibited the growth of green alga greater and earlier compared to the control. Therefore, it was suggested that inhibition of the growth of green alga was related to the extracellular metabolites containing MC-LR and / or the other bioactive compounds of *M. aeruginosa*.

On the other hand, in co-cultures, MC-producing *M. aeruginosa* PCC 7806 has negatively influenced the growth of the green alga, depending on the stages of the growth (Paper II). During the logarithmic phase of growth, in the absence or the presence of a lower concentration of MC-LR, the growth of *D. subspicatus* remained consistently unchanged, either in mono- or co-cultures. Then, towards the end of experiments, during the stationary phase of growth, the biomass of green alga was reduced, coinciding with the increased release of MC, which might support the hypothesis of MC involvement in the inhibition of the growth of green alga.

Previous studies have indicated that *M. aeruginosa* inhibited the growth of green algae. The exudates of *M. aeruginosa* from the exponential and stationary phases of growth have significantly inhibited the growth of the green algae, *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*, and a diatom *Cyclotella meneghiniana* (Wang et al. 2017). *M. aeruginosa* at the initial density of 1×10^6 cells/mL inhibited the growth of green alga *Chlorella* spp. (*C. pyrenoidosa C. vulgaris*, and *C. ellipsoidea*) in mixed cultures as well (Hong et al. 2010). Żak and Kosakowska (2014) have reported that the growth of green alga *Chlorella vulgaris* was inhibited in co-cultivation with the toxic strain of *Microcystis aeruginosa* PCC7820, and when exposed to the *Microcystis* filtrates.

It was reported that the green algae, *Scenedesmus* and *Chlorella*, could absorb and biotransform MCs (Mohamed, 2008). Therefore, it was assumed that at the exponential phase of growth, by the lower concentrations of the extracellular metabolites such as MC-LR, the growth of green alga has not been altered, perhaps due to the repair systems of the green algae such as antioxidative enzymes that have efficiently detoxified MC and / or the other secondary metabolites of *Microcystis* (Cirulis et al., 2013). Additionally, the green alga could produce intra- and extracellular polysaccharides as an adaptive response to protect the algal cells against the oxidative stress that was induced by MCs (Mohamed, 2008; El-Sheekh et al., 2012). By entering the stationary phase of growth, by the enhanced *Microcystis* cell lysis, the concentration of the released MC-LR and the other probable secondary metabolites of *Microcystis* were increased, which might have resulted in the inhibition of the growth of green alga.

On the other hand, regarding the results, the growth of *D. subspicatus* in *Microcystis*-filtrates was inhibited at the logarithmic phase of growth while in mixed cultures, it was suppressed later during the stationary phase of growth. Previous studies have shown a different pattern of the production of allelochemicals in mono and co-cultures. The study by Bittencourt-Oliveira et al. (2015) indicated that toxic and non-toxic strains of *M. aeruginosa* and *M. panniformis*, respectively, have negatively influenced the growth of green algae, *Scenedesmus acuminatus*

and *Monoraphidium convolutum*, in co-cultures. While the extracts of both MC-producing and -deficient strains have not affected the growth of green algae. The study by Mello et al. (2012) showed that the exudates of mixed cultures of *M. aeruginosa* and *Cylindrospermopsis raciborskii* with a high density of C. *raciborskii*, inhibited the growth of *Microcystis* while the monocultures filtrates of C. *raciborskii* has not influenced the growth of *Microcystis*. Therefore, the latter inhibition of the growth of green alga in co-cultivation with the MC-producing *Microcystis* might be related to the interspecies communications that have resulted in the different patterns of the production and / or release of the secondary metabolites in mixed cultures compared to monocultures.

5.1.2 Inhibition of the growth of *D. subspicatus* by the MC-containing crude extracts of *M. aeruginosa* PCC 7806

The exposure of green alga to the extracts from the MC-producing strain of *M. aeruginosa* PCC 7806 showed a negative concentration-dependent response of the green alga to the presence of MC-LR (Paper II). The results indicated that the growth of green alga was inhibited at the concentrations of MC-LR, which were greater than 140 µg/L. Previous studies showed that exposure to MCs, as well as the crude extracts of *Microcystis,* had promoted oxidative stress in aquatic organisms (Pietsch et al., 2001; Pflugmacher, 2004; Amado and Monserrat, 2010; Paskerová et al., 2012). The addition of 25 - 50 µg/mL of the purified MC inhibited the growth of green algae *Chlorella* and *Scenedesmus* sp. and cyanobacteria *Nostoc muscorum* and *Anabaena* BT1, due to the reduction in CO_2 uptake, and severe depletion of nitrogen fixation (Singh et al., 2001). The study by Zhang et al. (2014) demonstrated that the cellular exudates of *Anabaena flos-aquae,* which contained MC and anatoxin, reduced the growth of green alga *Chlamydomonas reinhardtii*. However, the addition of the purified toxins at the physiological concentration to the cultures of green alga *Chlamydomonas reinhardtii*, showed that MCs, but not anatoxin, might be partly responsible for the growth restriction.

The results of the current study went along with the study by Babica et al. (2007) where MCs influenced the growth of phytoplankton species such as different species of green algae at the concentrations of MC which were greater than the environmentally occurring concentrations (1-10 µg/L) that excluded MC from the common allelochemicals. Therefore, the results might reject the direct allelopathic role of MC-LR, at least in terms of the inhibition of the growth of

D. subspicatus. However, it may explain the common co-existence of the green algae with cyanobacterial blooms in freshwater ecosystems.

Moreover, the assistant role of MC in combination with the other secondary metabolites should be considered. It is important to mention that MCs are not the only products of *Microcystis*. A range of secondary metabolites has been isolated from *Microcystis* such as micropeptin, microviridin, microgenin, as well as some unknown compounds (Reshef and Carmeli, 2001; Ploutno et al., 2002; Beresovsky et al., 2006). It was shown that *Microcystis* sp. affected the growth of algal competitors negatively. The study by H. Ma et al. (2015) showed that the growth of cyanobacterium *Aphanizomenon flos-aquae* was inhibited in the monoculture filtrates of MC-producing strains of *Microcystis* as well as in co-cultures while the purified MC-LR (250 and 500 µg/L) has not influenced the growth of *A. flos-aquae*. Song et al. (2017) found that the toxic strain of *M. aeruginosa* FACHB-905 inhibited the growth of green alga *Chlorella vulgaris* through the release of linoleic acid. *Microcystis* sp. inhibited the growth and photosynthesis of dinoflagellate *Peridinium gatunense via* microcarbonin A (a carbonic anhydrase inhibitor) (Sukenik et al., 2002; Vardi et al., 2002). Beresovsky et al. (2006) identified different allelochemicals such as microcarbonin A (carbonic anhydrase inhibitor), micropeptin KT946, and anabaenopeptin KT864 (protease inhibitors) from exudates of MC-producing *Microcystis* sp. (MB-K) which were responsible for the retardation of photosynthesis and growth of *P. gatunense*.

On the other hand, inhibition of the growth of green alga at a lower concentration of MC-LR in co-cultures compared to the inhibitory concentrations of MC-LR in the crude extract (Paper II) might be related to the different pattern of production and release of the other secondary metabolites of *Microcystis* in the presence of green alga, influenced by the probable resource competition occurring at the stationary phase of growth or a combination of both which should be considered in future studies.

5.1.3 The advantage of MC for the MC-producing strain of *M. aeruginosa* over MC-deficient strain to a greater exclude of the co-cultured green alga

From the scums or very dense accumulations of cyanobacteria, the release of high concentrations of MCs as much as 25 000 µg/L were reported (Sivonen and Jones, 1999; World Health Organization, 2003). Moreover, organisms in close proximity to the MC-producers may experience higher concentrations of MCs in their microenvironment, especially in terms of the

microalgal interactions (Sedmak and Kosi, 1998; Hu et al., 2004). Therefore, the interactions between the green alga *D. subspicatus* and the toxic and non-toxic strains of *M. aeruginosa*, PCC 7806 and PCC 7005, respectively, were studied to investigate the effects of highly dense blooms of *Microcystis* sp. at the collapse phase on the growth of the co-existing green alga (Paper III).

The co-cultivation experiments were performed using MC-producing and -deficient strains of *M. aeruginosa,* which were grown inside of the dialysis tubings to the stationary phase of growth. Then, the green alga was grown out of the membrane for a 14-day co-cultivation period. Consequently, *D. subspicatus,* which was growing to the exponential phase of growth, was exposed to the increasing release of MC-LR and the other probable secondary metabolites from the toxic and non-toxic strains of *Microcystis* which entered the death phase of growth.

The results showed that the growth of green alga was inhibited in co-cultivation with both MC-producing and -deficient strains, which suggested the probable interferences of *Microcystis* secondary metabolites such as MC-LR in the interspecies interactions. However, the greater outcompeting of the green alga in the presence of MC, compared to the monocultures and the treatments with the non-toxic strain, supported the advantage of MC as an assistant factor that reinforced the inhibitory effects of the toxic *Microcystis* on the growth of green alga. Further studies are needed to identify the secondary metabolites profile of both MC-producing and -lacking strains, to clarify the probable interferences of the other secondary metabolites in the interspecies interplay, in addition to or rather than MC.

The study by Z. Ma et al. (2015) showed that both toxic and non-toxic strains of *M. aeruginosa*, FACHB-905 and FACHB-469, respectively, inhibited the growth of *Chlorella vulgaris*. However, the toxic *M. aeruginosa* caused more inhibition in the growth of green alga compared to the non-toxic strain. The study by Bittencourt-Oliveira et al. (2015) also indicated that both toxic and non-toxic strains of *Microcystis* sp. have negatively influenced the growth of green algae, *Scenedesmus acuminatus* and *Monoraphidium convolutum*, when grown together *via* the production of unknown allelochemicals. Moreover, in the presence of the toxic *Microcystis,* the growth of green algae was greater inhibited.

Additionally, the results indicated that in co-cultivation with the toxic strain of *Microcystis*, the growth was inhibited from the beginning of the co-cultivation period while in treatments with the non-toxic strain, it was inhibited after ten days. Exposure of the aquatic organisms to MCs or *Microcystis* crude extracts induced oxidative stress (Pietsch et al., 2001; Pflugmacher, 2004; Amado and Monserrat, 2010; Paskerová et al., 2012). Thus, the longer lag phase in the growth

of green alga in co-cultivation with the toxic strain might be related to the time that the green alga needed to fix the damages caused by MC.

5.2 Effects of *D. subspicatus* on the growth and MC production of *M. aeruginosa* PCC 7806

5.2.1 Inhibition of the growth of *M. aeruginosa* PCC 7806 in monoculture filtrates of *D. subspicatus* as well as co-cultures

The growth of the MC-producing strain of *M. aeruginosa* PCC 7806 was inhibited significantly in the filtered medium in which *D. subspicatus* was previously grown, suggesting that the green alga could produce metabolites to affect the growth of *M. aeruginosa* PCC 7806 negatively (Paper II). The results indicated that the culture filtrates from *D. subspicatus* at the levels higher than 25%, have significantly inhibited the growth of *M. aeruginosa* PCC 7806 in a concentration- and time-dependent manner where the increased concentrations of the cultural filtrates of the green alga inhibited the growth of *Microcystis* greater and earlier.

On the other hand, in the co-cultivation system, the growth of *M. aeruginosa* PCC 7806 was inhibited in the presence of the green alga as well (Paper II). At the early logarithmic phase of growth, the green alga has started to compete with the co-cultured *Microcystis* out. During the logarithmic phase of growth, the biomass of *M. aeruginosa* was reduced to one-third compared to the monocultures. Over time, during the stationary phase of growth, the growth of *Microcystis* was further inhibited in the presence of the green alga. However, from the logarithmic to the stationary phases of growth, the increased biomass of *Microcystis* was greater in co-cultures compared to the simultaneously conducted monocultures.

The inhibitory effects of the green algae on the growth and photosynthesis of *Microcystis* spp. have been reported in other investigations (Jia et al., 2008; Hong et al., 2010; Bittencourt-Oliveira et al., 2015). It was reported that cyanobacteria and green algae have different nutrient uptake capability (Levich, 1996; Huan et al., 2006; Wan et al., 2007). In eutrophic reservoirs and the mixed cultures, the decreased and increased ratio of N:P led to the dominance of the cyanobacteria and green algae, respectively (Levich, 1996; Wan et al., 2007). The study by Huan et al. (2006) demonstrated that in mixed cultures, the green alga *Chlorella ellipsoidea* inhibited the growth of *M. aeruginosa* due to its greater ability in the utilization of nitrogen and phosphorous. In the present study, to restrict the resource competition, the species were grown in BG-11 medium as a nutrient-rich growth medium, according to the suggestion by Dunker et

al. (2013) and Bittencourt-Oliveira et al. (2015). The fresh nutrient was added regularly during the co-cultivation periods as well. Additionally, the species were cultured at the equal initial cell density (1:1) to avoid giving one species the advantages of the higher initial biomass (Li and Li, 2012).

On the other hand, it was shown that the allelochemicals which were produced by the green alga could affect the growth of *Microcystis* sp. The green alga *Scenedesmus acuminatus* produced unknown allelochemicals that suppressed the growth of toxic and non-toxic strains of *Microcystis*, *M. aeruginosa* BCCUSP232 and *M. panniformis*, respectively (Bittencourt-Oliveira et al., 2015). *Scenedesmus obliquus* inhibited the growth and photosynthesis of *M. aeruginosa* through the production of allelochemicals that caused a reduction in the net photosynthesis and chlorophyll a content of *M. aeruginosa* (Jia et al., 2008). Zhang et al. (2013) reported that the growth of *M. aeruginosa* PCC 7820 was inhibited in the filtrates of the green alga *Quadrigula chodatii* FACHB-1080 as well as co-cultures through the suppression of the nitrogen utilization by *Microcystis*. Using gas chromatography-mass spectrometry (GC/MS) demonstrated the presence of allelochemicals such as dibutyl phthalate and beta-sitosterol in monocultures of the green alga as well as co-cultures (Zhang et al., 2013). Qiu et al. (2017) demonstrated that the growth of *Microcystis flos-aquae* was inhibited by the culture filtrates of the green algae *Scenedesmus* (*S. quadricauda* and *S. obliquus*) as well as in co-culture tests. They found that *S. quadricauda* inhibited the growth *M. flos-aquae via* the production of 4-tert-butylpyrocatechol (TBC), which disturbed the synthesis and secretion of proteins and carbohydrates, and damaged chlorophyll and the light-harvesting processes (Qiu et al., 2019).

5.2.2 Variation of MC content depending on the physiological status of *M. aeruginosa* and the presence of green alga

The results of the current study (Paper II) concurred with the previous studies which reported that MC synthesis is a continuous process which is varied over time, related to the status of the growth phase; starting at the early logarithmic phase, increased to reach the highest level at the exponential phase, and decreased as the growth passed into the stationary phase of growth (Lyck, 2004; Jähnichen et al., 2008). The dynamic variation of MC content in monocultures was changed depending on the various stages of the growth of *Microcystis*. During the first week of co-cultivation, the cultures were at the beginning of the logarithmic phase of growth,

where the cell numbers of *Microcystis* increased. However, neither intracellular nor extracellular MC-LR was detected, either in mono- or co-cultures. It was assumed that MC synthesis had not been initiated, or it may be started but did not achieve a concentration high enough to be detected by the quantification method used. After the second week, where *Microcystis* cells were in the exponential phase of growth, the highest level of intracellular MC-LR was measured in monocultures. Finally, after the fourth week *Microcystis* cells passed into the stationary phase of growth, the intracellular MC-LR of monocultures was reduced to the half level, and the released MC-LR has been increased significantly.

On the other hand, in the presence of the green alga in co-cultures, MC-production and release were variously regulated depending on the stages of the growth (Paper II). During the logarithmic phase of growth, coinciding with the inhibition of the growth of *M aeruginosa* PCC 7806, the concentration of both intracellular and extracellular MC-LR was less compared to the simultaneously conducted monocultures. The results showed that the decreased rate of extracellular MC-LR was at the same level as the reduction rate of *Microcystis* cell density. Besides, the reduction rate of the intracellular MC-LR was greater than the declined extracellular MC-LR. Therefore, MC production was suppressed, while MC release has not been influenced. It has been reported that the bioactive compounds of the phytoplankton species influenced the pattern of MC production and release. The monoculture filtrates of dinoflagellate *Peridinium gatunense* raised the transcript levels of *mcy*B in *Microcystis* sp. (Vardi et al., 2002). Kearns and Hunter (2000) found that the exudates of green alga *Chlamydomonas reinhardtii* induced anatoxin and reduced MC production in cyanobacterium *Anabaena flos-aquae* as well. They concluded that the toxin production was regulated by a combination of the physiological status, environmental factors, and the interspecies interplay.

Past studies have shown that MC production rate is positively related to the growth rate (Orr and Jones, 1998; Lyck, 2004). On the other hand, MCs are N-rich molecules. MC production is highly related to the nitrogen supply (Harke and Gobler, 2013). Therefore, it was assumed that during the exponential phase of growth, *D. subspicatus* might interfere with the nitrogen uptake or utilization by *Microcystis,* which has resulted in the suppression of MC production and inhibition of the growth of toxic *M. aeruginosa*.

Over time, during the stationary phase of growth, in the presence of the green alga, coinciding with the inhibition of the growth of *Microcystis*, MC production and release have increased. The study by Bittencourt-Oliveira et al. (2015) indicated that the green alga *Scenedesmus acuminatus* induced MC production, coinciding with the inhibition of the growth of the toxic strain of *M. aeruginosa* BCCUSP232, through the production of some unknown

allelochemicals. Harel et al. (2013) reported that the bioactive compounds from *Scenedesmus huji* (isolated from Lake Kinneret, Israel), which has grown to the stationary phase of growth, interfered with the integrity of the cellular membrane of *Microcystis* that caused severe cell lysis in *Microcystis* spp.

The study by Zilliges et al. (2011) showed that under the stress conditions, the attachment of MC to proteins increased that kept the proteins active and protected them against the oxidative stress, suggesting a protein-modulating role for MCs. The results of the current study (Paper II) indicated that in co-cultures, from the logarithmic to the stationary phase of growth, coinciding with the elevated MC content, the rate of increased biomass of *Microcystis* was greater than the monocultures that emphasized the concept of the probable involvement of MC in improving the fitness of *Microcystis* in the presence of the co-existing green alga.

Collectively, the growth of the toxic strain of *M. aeruginosa* PCC 7806 was inhibited in monoculture filtrates of *D. subspicatus* as well as the co-cultivation system, suggesting that the green alga produced metabolites to inhibit the growth of toxic *Microcystis*. The variation of MC content was influenced by the presence of the co-cultured green alga as well. Therefore, it was suggested that the green alga produced probable allelochemicals which affected the growth and MC production of *Microcystis* depending on the stages of the growth through different probable mechanisms, containing inhibition of the growth and MC production due to the restriction of nitrogen uptake at the logarithmic phase, and the stimulation of MC synthesis and release through the induction of oxidative stress at the stationary phase of growth. Moreover, the greater increase of the cell density of the co-cultured *Microcystis,* which was coincided with the enhanced MC content, suggesting the probable role of MC in improving the fitness of *Microcystis* cells under the stress conditions induced by the presence of the green alga. More studies need to be done to clarify which secondary metabolites from the green alga and how interfered with the interspecies interactions.

5.3 The efficiency of the dialysis membrane co-cultivation system

In the present study, a co-cultivation system was designed based on the separation properties of the dialysis membrane (Paper II and III). A semi-permeable dialysis membrane with the pores of a specified size range (12 - 14 kDa) was applied to separate two microalgal populations physically in a mixed culture while allowing a free pass of the chemicals and metabolites which were smaller than the pore size of the membrane.

Moreover, applying the dialysis membrane co-cultivation system gave the opportunity of the investigation of the interspecies interferences under a consistent mode of co-exposure to simulate the natural ecosystems where microorganisms are co-existing within various microbial communities. However, the natural ecosystems are much more complicated than the laboratory-controlled conditions.

Regarding the osmosis diffusion rules, a higher concentration of a metabolite at the inside of dialysis tubing is required to allow it to flow from the inside to the outside of the membrane, based on the concentration gradient. Therefore, the source of MC production, MC-producing strain of *M. aeruginosa* PCC 7806, was grown inside of the dialysis tubing.

Since MC synthesis is an ongoing process (Lyck, 2004; Jähnichen et al., 2008), over time, an increasing concentration of extracellular MC-LR was released, which could freely diffuse across the membrane to affect the green alga that was cultured out of the tubing. Besides, the extracellular metabolites of the green algae could come into the tubing and affect the inner membrane growing species, *M. aeruginosa*. On the other hand, positioning of the *Microcystis* cells, which contain gas vesicles at the inside of the tubes, restricted the reduction of the light intensity, which might have resulted from the shadow effects of the buoyant *Microcystis* cells in the mixture.

The results indicated that MC-LR could pass through the dialysis membrane. The extracellular MC-LR was detected at both sides of the tubing. However, the concentration of extracellular MC-LR inside of the dialysis tubing was significantly greater than the outer membrane MC. Since MC production is a continuous process and the MC-producing species was positioned inside of the tubing, detection of the greater concentrations of MC inside of the membrane was reasonable. The only exception was observed in the co-cultures after the second week in which the lowest quantified concentration of the extracellular MC-LR (22.94 µg/L) was equally distributed between the inner and outer membrane (Paper II).

Moreover, the diffusion rate of the extracellular MC-LR has been assessed to consider the efficiency of dialysis tubing in the co-cultivation system (Paper II and III). The results showed that the diffusion rate of MC-LR through the dialysis membrane was related to the concentration of extracellular MC-LR. Indeed, the smaller concentration of extracellular MC-LR either in the second or the fourth week of co-cultivation has been better equilibrated through the membrane (Paper II).

On the other hand, the worst diffusion rate (0.2) was observed in the monocultures after the second week where the concentration of the total extracellular MC-LR (69.29 µg/L) was significantly greater compared to the simultaneous co-culture and significantly lower compared

to the control and treatments of the fourth week (Paper II). Therefore, the rate of MC-LR diffusion through the dialysis membrane was positively related to the incubation time and negatively to the total concentration of extracellular MC-LR (Paper II). It was suggested that the increased release of MC LR needed more time to be distributed between the inside and outside of the tubing. However, a further increase in MC release has not influenced the diffusion rate of MC. After one month, the diffusion rate of the increased release of MC (Paper III) stayed at the same level as the co-cultures of the fourth week with a lower concentration of MC-LR (Paper II).

6 CONCLUSIONS

The present study showed that the studied species could communicate through their bioactive metabolites. The culture filtrates of each species, MC-producing *M. aeruginosa* and *D. subspicatus*, had generally inhibitory effects on the other one. Moreover, in the co-cultivation system, at the equal initial cell density, the species affected the growth of each other negatively depending on the stages of the growth. At the logarithmic phase of growth, the green alga has inhibited the growth of the co-cultured *Microcystis* and suppressed MC production. Over time, during the stationary phase of growth, the growth of both species "*D. subspicatus*" and "*M. aeruginosa* PCC 7806" was inhibited. However, MC production and release have increased, coinciding with the greater increased cell density of *Microcystis*, suggesting the probable involvement of MC in improving the fitness of *Microcystis* in the presence of the co-existing green alga.

Moreover, MC-producing strain of *M. aeruginosa* gained the advantages of MC production, in addition to or rather than the other secondary metabolites, over the non-toxic population to the greater exclude of the co-existing green alga. The MC-containing crude extracts have also negatively affected the growth of green alga. However, the inhibition of the growth of green alga at the concentrations of MC greater than the environmentally relevant levels may explain the co-existence of green algae with the cyanobacterial blooms in freshwater ecosystems.

On the other hand, the results indicated the potential of the dialysis co-cultivation system to study the MC-related interspecies communications, especially at the lower levels of MC-LR. Dialysis membranes holding the individual or mixed assemblages of species can be used in the laboratory conditions or future field studies during the occurrence of cyanobacterial blooms.

It should be noted that the focus of the present study was on MC. The data suggested that the presence of MC may reinforce the inhibitory effects of the *Microcystis* on the growth of the co-existing green alga. However, further studies are needed to unravel the compels interactions and involvements of other secondary metabolites. On the other hand, characterization of the bioactive compounds of the green alga and clarify their mode(s) of action and the mechanisms of the interspecies interactions may open the ways for the future control of harmful *Microcystis* blooms that is of growing global concern.

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