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Effects of the allelopathically active macrophyte *Myriophyllum spicatum* on the potentially toxic cyanobacterium *Microcystis aeruginosa*

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Contents

List of Figures

List of Tables

List of Abbreviations

Chl *a*	**Chl**orophyll ***a***
DIN	**D**issolved **I**norganic **N**itrogen
dw	**d**ry **w**eight
EC_{50}	half maximal **E**ffective **C**oncentration
EDTA	**E**thylene**d**iamine**t**etraacetic **a**cid
EET	**E**astern **E**uropean **T**ime
fw	**f**resh **w**eight
GLS	**G**eneralized **L**east **S**quares model
GPP	**G**ross **P**rimary **P**roduction
HAB	**H**armful **A**lgae **B**loom
HUB	**H**umboldt **U**niversity of **B**erlin
ISO	**I**nternational **S**tandards **O**rganization
LAND	Lithuanian Environmental Protection Normative Document (lit.**L**ietuvos **A**plinkos Apsaugos **N**ormatyvinis **D**okumentas)
LD_{50}	Median **L**ethal **D**ose
LM	General **L**inear **M**odel
LME	**L**inear **M**ixed **E**ffects models
MC	**Mic**rocystin
mcyB	**m**icro**cy**stin-synthetase gene **B**
NIES	**N**ational **I**nstitute for **E**nvironmental **S**tudies
NIVA	**N**orwegian **I**nstitute for **W**ater **R**esearch
NO_X	NO_2^- + NO_3^-
PC	**P**hyco**c**yanin operon

PCC	**P**asteur **C**ulture **C**ollection of Cyanobacteria
PCR	**P**olymerase **C**hain **R**eaction
PPM	**P**earson **P**roduct-**M**oment correlation analysis
PSU	**P**ractical **S**alinity **U**nits
qPCR	**q**uantitative **P**olymerase **C**hain **R**eaction
RU	**R**elative **U**nit
SAG	The Culture Collection of Algae at the University of Göttingen
SD	**S**tandard **D**eviation
SE	**S**tandard **E**rror of the mean
SRP	**S**oluble **R**eactive **P**hosphorus
TA	**T**annic **A**cid
TN	**T**otal **N**itrogen
TP	**T**otal **P**hosphorus
TPC	**T**otal **P**henolic **C**ontent
Tris	**Tris**(hydroxymethyl) Aminomethane
UTEX	**U**niversity of **Tex**as at Austin
UWO	**U**niversity of **W**isconsin Culture Collection at **O**shkosh
WGS	**W**orld **G**eodetic **S**ystem
WHO	**W**orld **H**ealth **O**rganisation

mano seneliui, mokslininkui be laipsnių

1
Introduction

1.1 Scope of the study

Cyanobacteria are an important component in aquatic ecosystems. However, the massive growth of cyanobacteria (the so-called blooms) is a public health and environmental concern (Francis, 1878; Chorus and Bartram, 1999; de Figueiredo et al., 2004). One of the most widespread harmful algae bloom forming cyanobacteria, species *Microcystis aeruginosa* (Kützing) Lemmerman (order *Chroococcales*), is being increasingly reported in freshwater ecosystems around the world (Hallegraeff, 1993; Paerl et al., 2011). *M. aeruginosa* blooms are primarily caused by anthropogenic eutrophication (Heisler et al., 2008; Smith and Schindler, 2009) and are further facilitated by increasing water temperatures (Liu et al., 2011), stability of stratification (Bonnet and Poulin, 2002; Chung et al., 2014) and levels of atmospheric CO_2 (Sandrini et al., 2015). Due to *M. aeruginosa* ability to produce the hepatotoxic microcystins (MCs, Carmichael, 1992), it may contaminate drinking and recreational waters, cause a wide range of acute or chronic illnesses to human and livestock, and, in the overall, threaten the ecological integrity of many water bodies worldwide (reviewed in Paerl and Otten, 2013). One of the measures assumed to potentially help control *M. aeruginosa* blooms is the use of allelopathically active macro-

phytes[1] (e.g. Nam et al., 2008; Zhang et al., 2009; Shao et al., 2013). Eurasian watermilfoil *Myriophyllum spicatum* L. is one of the aquatic plants with the strongest allelopathic effects on phytoplankton based on its high content of polyphenolic allelochemicals (Gross et al., 1996; Gross, 2003a). Under *in situ* conditions, effects of macrophytes and their allelochechemicals should be primarily observed in shallow water bodies characterised by the so-called "crashing" phase in which cyanobacteria blooms are present in summer, despite the occurrence of macrophytes in spring (Sayer et al., 2010a,b). Due to the reoligotrophication of water bodies in Europe and North America (Jeppesen et al., 2005; Søndergaard et al., 2007) and consequent reoccurrence of macrophytes (Hilt et al., 2013), relevance of the impact of macrophytes and their allelochemicals in aquatic ecosystem should increase in the future. However, the relevance of the allelopathic effect of macrophytes under *in situ* conditions is poorly understood. Although the toxicity of *M. aeruginosa* blooms is significantly determined by the ratio of MC-producing to non-MC-producing genotypes (Kurmayer et al., 2003; Zurawell et al., 2005), it is not known how these genotype ratios are affected by macrophytes and their excreted allelochemicals. A better understanding of the effects of macrophytes and their excreted allelochemicals on potentially toxic *M. aeruginosa* cyanobacterium would increase the understanding of the processes that affect the occurrence and severity of harmful cyanobacteria blooms.

1.2 Aim and objectives of the study

The aim of the study was to evaluate the effect of the allelopathically active macrophyte *M. spicatum* on *M. aeruginosa* and its toxicity.

[1] aquatic plants

The objectives of the study were:

1. To evaluate if the presence of *M. spicatum* reduces the biomass of cyanobacteria, influences the phytoplankton community composition and abundances of zooplankton taxa under *in situ* like conditions, and to estimate the effect of *M. spicatum* allelochemicals.

2. To estimate the effect of polyphenolic allelochemicals on MC-producing and non-MC-producing *M. aeruginosa* strains.

3. To evaluate the role of the production of MCs in *M. aeruginosa* in its resistance against polyphenolic allelochemicals.

4. To evaluate if the coexistence of MC-producing and non-MC-producing *M. aeruginosa* strains influences the effects of allelochemicals on *M. aeruginosa*.

1.3 Novelty of the study

This dissertation evaluates how allelopathically active macrophyte *M. spicatum* affects MC-producing and non-MC-producing *M. aeruginosa* genotypes. The study evaluates the protective role of allelopathy of *M. spicatum* under *in situ* like conditions. This study provides evidence that the coexistence of MC-producing and non-MC-producing *M. aeruginosa* strains influence the effects of allelochemicals of *M. spicatum* on *M. aeruginosa* strains. The study evaluates the role of MCs in *M. aeruginosa* against allelochemicals of *M. spicatum* and gives indications that biochemical characteristics of phytoplankton to produce certain metabolites could be one of the reasons of differential sensitivities of phytoplankton organisms to allelochemicals of macrophytes.

1.4 Scientific and practical significance of the results

This dissertation broadens the understanding of how macrophytes and their allelochemicals may affect the occurrence and severity of toxic cyanobacterial blooms. We argue that more mesocosm experiments with complex natural phytoplankton communities are needed to unravel the ecological relevance of macrophyte allelopathy. This study suggests that interactions between *M. aeruginosa* genotypes need to be taken into consideration in order to assess the potential of macrophyte allelochemicals to suppress *M. aeruginosa* blooms. The results of this study question the potential use of allelochemicals as artificial treatments to control *M. aeruginosa* blooms, as an increased toxicity of *M. aeruginosa* blooms may follow artificial treatments. This study implies that macrophyte-derived allelochemicals are one of the biotic factors that can significantly affect MC- and non-MC-producing *M. aeruginosa* genoptype ratios and deserve further attention.

1.5 Defensive statements

1. The presence of *M. spicatum* reduces biomass of cyanobactieria and influences the phytoplankton community composition and abundance of certain zooplankton taxa under *in situ* like conditions.

2. Polyphenolic allelochemicals suppress MC-producing less than non-MC-producing strains of *M. aeruginosa*.

3. The lower suppression of MC-producing than non-MC-producing *M. aeruginosa* strains by polyphenolic allelochemicals is not related to the production of MCs *per se*.

4. The coexistence of MC-producing and non-MC-produ-

cing *M. aeruginosa* strains can influence the effects of allelochemicals of *M. spicatum* on *M. aeruginosa*.

1.6 Scientific approval

The dissertation is based on 2 publications, 1 submitted manuscript and 2 manuscripts in preparation:

- Tuomainen, M., Ahonen, V., Kärenlampi, S.O., Schat, H., Paasela, T., Švanys, A., Tuohimetsä, S., Peräniemi, S., Tervahauta, A., 2011. Characterization of the glyoxalase 1 gene *TcGLX1* in the metal hyperaccumulator plant *Thlaspi caerulescens*. Planta 233(6), 1173-1184.

- Švanys, A., Paškauskas, R., Hilt, S., 2014. Effects of the allelopathically active macrophyte *Myriophyllum spicatum* on a natural phytoplankton community: a mesocosm study. Hydrobiologia 737(1), 57-66.

- Švanys, A., Eigemann, F., Grossart, H.P. and Hilt, S. Microcystin is not responsible for lower sensitivities of microcystin- versus non-microcystin-producing *Microcystis aeruginosa* strains to polyphenolic allelochemicals. (in preparation).

- Švanys, A., Eigemann, F., Grossart, H.P. and Hilt, S. Effects of mixtures of microcystin- and non-microcystin-producing *Microcystis aeruginosa* strains on their allelopathic inhibition by allelochemicals (in preparation).

- Švanys, A., Anne, O. et al. Effects of environmental parameters on microcystin-producing *Microcystis aeruginosa* genotypes (in preparation).

The results of this study were presented at 13 international conferences and seminars:

- Open lecture at Klaipėda University. Klaipėda, Lithuania, May 2011.
- SUBMARINER Partner Meeting, Trelleborg, Sweden, May 2011.
- SUBMARINER Cooperation Event "Present and Potential Uses of Algae", Trelleborg, Sweden, September 2011.
- Public meeting "Maudyklų vandens kokybės ir kitos aplinkos sveikatinimo bei neinfekcinių ligų profilaktikos aktualijos" Trakai, Lithuania, October 2011.
- International congress: The 6th World Congress on Allelopathy, Guangzhou, China, December 2011.
- International conference: International Symposium on Aquatic Plants, Poznan, Poland, August 2012.
- International conference: Estuarine Coastal and Shelf Science 51st symposium, Klaipėda, Lithuania, September 2012.
- International conference: 28th annual meeting of International Society of Chemical Ecology, Vilnius, Lithuania, July 2012.
- National conference: Marine and coastal research, Klaipėda, Lithuania, April 2013.
- International Conference: 2nd International Conference on Biodiversity and the UN Millennium Development Goals, Berlin, Germany, April 2013.

- National conference: Intern-ships of doctorate students at scientific centers abroad 2012-2013. Vilnius, Lithuania, October 2013.
- National conference: Marine and coastal research, Klaipėda, Lithuania, April 2014.
- International congress: The 7th World Congress on Allelopathy, Vigo, Spain, July 2014.

1.7 Structure of the dissertation

The dissertation is presented in the following chapters: Introduction, Literature Review, Materials and Methods, Results, Discussion, Conclusions and Bibliography. Bibliography has 201 sources. The dissertation contains 5 tables and 23 figures. The size of the dissertation is 140 pages. Dissertation is written in English with extended summary in Lithuanian and English.

2

Literature review

2.1 Harmful cyanobacterial blooms

Cyanobacteria[1] are a phylum of prokaryotic photosynthetic bacteria. They obtain light energy through photosynthesis, use carbon dioxide, essential nutrients and water to grow and produce oxygen as a byproduct. Cyanobacteria are one of the Earth's oldest life forms. Evidence of their existence on earth, derive from fossil records, that are the oldest ($\sim$ 3.5 billion years old) known fossils in the world (Schopf, 2000). Cyanobacteria play an important role in the functioning of all freshwater, estuarine and marine ecosystems worldwide, as they are one of the major constituent in phytoplankton communities. Thus, cyanobacteria contribute significantly to ecosystem productivity, however, their massive growth can lead to a dense mono-species specific nuisance water blooms (Fig. 2.1).

Harmful cyanobacterial blooms have become a worldwide troublesome phenomenon (de Figueiredo et al., 2004) that are mainly caused due to excessive anthropogenic nutrient loadings and are further stimulated by high temperature and pH, low turbulence (e.g. Paerl et al., 2001). These blooms are undesirable, not only because of their smell and lack of appeal for recreational purposes, but also because some cya-

[1] gr. $\kappa\upsilon\alpha\nu\sigma\varsigma$ (cyano) - blue, dark blue.

(A) Villerest reservoir, France (B) Lake Müggelsee, Germany

FIGURE 2.1: Cyanobacterial bloom. Photos A and B were taken by Algirdas Švanys and Sebastian Panwitz, respectively.

nobacteria produce toxic substances that have adverse effects on aquatic ecosystems, terrestrial animals and even humans (reviewed by Chorus and Bartram, 1999; Paerl et al., 2001; Paerl and Otten, 2013). The first adverse effects of cyanobacterial toxins were reported by Georg Francis (1878) as cattle stock deaths in Australia, after consumption of cyanobacteria-contaminated drinking water. Since then many human illnesses associated with exposure to cyanobacterial toxins have also been reported (Carmichael, 1992; Chorus and Bartram, 1999; de Figueiredo et al., 2004). One of them, most tragic incidents caused by cyanotoxins, was the death of 52 dialysis patients in Brazil, caused by MCs (Jochimsen et al., 1998; Fig. 2.2).

As a consequence to the increasing spread of HABs, in 1998, the World Health Organization (WHO) introduced provisional guideline values for the concentration of MC-LR[2],

[2]L and R are abbreviations of the amino acids leucine and arginine in MC, respectively. The subsection 2.2.1 reviews structural variants of

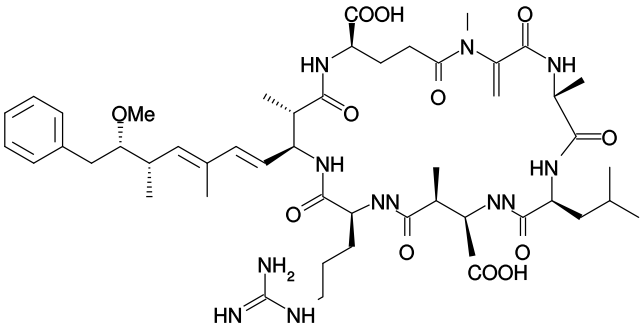


FIGURE 2.2: Molecular structure of microcystin-LR

with maximum values of 1.0 μg MC-LR L^{-1} as a provisional guideline value for drinking-water quality (WHO, 2004) and 20 μg MC-LR L^{-1} as a provisional guideline value for recreational waters (WHO, 2003). A number of countries have also developed regulations or guidelines for cyanobacteria derived toxins and cyanobacteria in drinking, recreational and agricultural waters (Burch, 2008).

2.2 Bloom forming *Microcystis aeruginosa*

Among the most frequent toxin-producing cyanobacteria genera *Microcystis*, *Anabaena* and *Planktothrix*, *Microcystis aeruginosa* is one of the most widespread harmful algae bloom forming cyanobacteria species (Hallegraeff, 1993; Paerl et al., 2011). *M. aeruginosa* is a successful competitor for light compared to other phytoplankton species due to its capability to regulate its buoyancy (Visser et al., 1997), has a competitive advantage at high temperatures (Fujimoto et al., 1997), due to its capability to uptake bicarbonate (Sandrini et al., 2014), *M. aeruginosa* wins competition at high pH and low carbon

MCs into more detail.

dioxide concentrations. When phosphorus concentrations decease, *M. aeruginosa* is able to survive certain periods due to its efficient phosphorus storage (Kromkamp et al., 1989).

The troublesome characteristics of *M. aeruginosa* is its ability to produce the hepatotoxic MCs (Carmichael, 1992; Chorus and Bartram, 1999) although both MC-producing and non-MC-producing *M. aeruginosa* genotypes co-exist in the field (Fastner et al., 2001). MC-producing *M. aeruginosa* dominated blooms are primarily caused by anthropogenic eutrophication (Heisler et al., 2008; Smith and Schindler, 2009) and are suggested to increase in their severity due to the climate change (Dziallas and Grossart, 2011; Paerl et al., 2011).

2.2.1 Synthesis and function of microcystins

MCs are a family of cyclic heptapeptides produced by many cyanobacterial genera (de Figueiredo et al., 2004). MCs consist of five non-protein amino acids and two variable protein amino acids (Tillett et al., 2000). The general structure of MCs is cyclo(Adda-D-Glu-Mdha-D-Ala-L-X-D-MeAsp-L-Z-), where X and Z are variable L-amino acids, Adda is 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-4,6-decadienoic acid, D-MeAsp is 3-methylaspartic acid, and Mdha is N-methyldehydroalanine (Fig. 2.2). More than 100 MC variants have been described (Welker and Von Döhren, 2006; Puddick, 2013), mostly differing in amino acid in position X and Z. The most common MC is MC-LR, which includes leucine (L) and arginine (R) in the places of X and Z amino acids. A significant part of this structural variability is achieved by relaxation of substrate specificities, resulting in variable incorporation of amino acids and thereby leading to the existence of multiple chemical variants synthesized by the same enzyme complex. MCs are produced by a hybrid polyketide synthetase and non-

ribosomal peptide synthetase called microcystin synthetase. Microcystin synthetase gene cluster consist of ten mcy-genes (non-ribosomal peptide synthetase and polyketide synthase genes) and has total length of 55kb (Fig. 2.3). The mcy-genes are organized in two bi-directional operons (mcyABC and mcyDEFGHIJ) separated by a bi-directional promoter (Tillett et al., 2000).

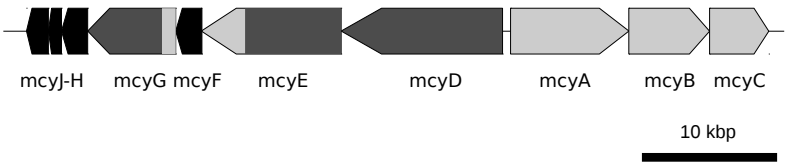


FIGURE 2.3: Organisation of the cluster of microcystin synthetase genes in PCC 7806 strain. The light and dark grey modules represent peptide synthetase and polyketide synthase gene regions, respectively, and black modules represent genes coding modifying enzymes.

The larger operon (mcyDEFGHIJ) encodes modules responsible for the formation of Adda and its linkage to D-glutamate. The smaller operon (mcyABC) encodes modules responsible for the extension of this intermediate to form a heptapeptide and subsequent cyclisation. The mcyB gene mutated strain used in this study lacks mcyB enzyme (Dittmann et al., 1997), which incorporate L-leucine and D-MeAsp into the peptide chain. As this peptide incorporation step is eliminated, the whole MC synthesis is disrupted in the PCC 7806 $\Delta mcyB$ mutated strain (Dittmann et al., 1997).

MCs may constitute up to 2% cell mass in *M. aeruginosa* (Ballot et al., 2003), suggesting a significant investment in energy and possible ecophysiological role, however, natural function of MCs in *M. aeruginosa* is still debated. Many functional roles have been proposed for MCs, including pro-

tection against grazing (DeMott and Moxter, 1991), chelation of trace elements (Utkilen and Gjølme, 1995), gene regulation (Dittmann et al., 2001), allelopathic effects (Sukenik et al., 2002), colony formation (Kehr et al., 2006) and intraspecific signalling (Schatz et al., 2007). MCs may also covalently bind to cysteines of proteins and in this way increase the fitness of *M. aeruginosa* under oxidative stress (Zilliges et al., 2011; Meissner et al., 2013; Leunert et al., 2014).

2.3 Environmental triggers of *Microcystis aeruginosa* toxicity

The toxicity of *M. aeruginosa* is variable from one bloom to another and during the course of a single bloom (Briand et al., 2009), and depends on *M. aeruginosa* biomass, its genetic composition, cellular MC production and the variants of MC produced (reviewed by Neilan et al., 2013). MC content within *M. aeruginosa* typically shows 2-fold to 3-fold variation, and was shown to be influenced by light, temperature and concentration of phosphorus (Neilan et al., 2013). The median lethal dose (LD_{50}) of the structural variants of MC in general range from 50 to 300 μg kg^{-1} (reviewed in Chorus and Bartram, 1999, Table 3.2). Numerous studies suggest that MC dynamics in lakes mainly result from the succession of a large number of MC-producing genotypes (Zurawell et al., 2005). The ratio between MC-producing and non-MC-producing genotypes was shown to be influenced by light intensity, temperature, concentration of nutrients and CO_2. Non-MC-producing *M. aeruginosa* seem to be a better competitor for low light (Kardinaal et al., 2007) and low nutrients (Vézie et al., 2002; Yoshida et al., 2007), but are weaker to withstand oxidative stress (Dziallas and Grossart, 2011) and low CO_2 (Van de Waal et al., 2011) than MC-producing strains. A

potential importance of cyanophages to affect the MC-producing and non-MC-producing *M. aeruginosa* dynamics in lakes has also been recently suggested (Yoshida et al., 2008a).

2.4 Effects of macrophytes on *Microcystis aeruginosa* toxicity

Remesotrophication and successful mitigation of *M. aeruginosa* and other harmful phytoplankton blooms require reduction of nutrient loadings (Hallegraeff, 1993; Smith and Schindler, 2009; Heisler et al., 2008), e.g. industrial, agricultural and sewage effluents. However, efficient reductions of nutrient loadings are of considerable cost, require long-term nutrient control and management strategy and may take a decade or more for an observable result (de Figueiredo et al., 2004). Regardless to the managements of nutrient loading, a number of more rapid approaches have been developed in order to help controlling harmful algae proliferations (e.g. Paerl and Otten, 2013). These approaches include chemical, physical and biological techniques. Chemical approaches can effectively and rapidly remove algal blooms, however, most chemicals such as $CuSO_4$ and herbicides cause secondary pollution and are not selective. Physical approaches are less polluting (e.g. filtration), but are limited to an application in a large scale. Biological approaches are increasingly suggested to offer an environmentally friendly and efficient way for controlling toxic cyanobacteria and HABs.

One of the biological approaches that may potentially help to control *M. aeruginosa* blooms is the use of allelopathically-active aquatic plants or macrophytes (e.g. Nam et al., 2008; Zhang et al., 2009; Shao et al., 2013). Macrophytes significantly contribute to the suppression on phytoplankton proliferations and stabilize clear water conditions especially in

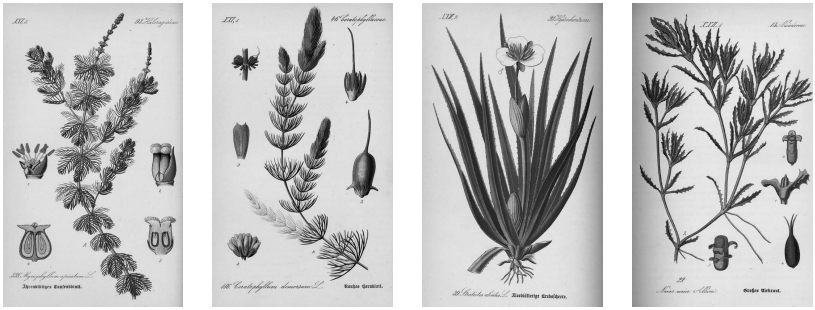
shallow lakes (Scheffer et al., 1993). Several mechanisms contribute to the impact of macrophytes on phytoplankton. These include competition for nutrients and light, the provision of a spatial refuge against predators for zooplankton and habitat for piscivorous fish, the reduction of sediment resuspension and increased sedimentation (Søndergaard and Moss, 1998). In addition, the excretion of allelopathic[3] substances by submerged macrophytes has been suggested as a potentially important mechanism suppressing phytoplankton growth (Wium-Andersen et al., 1982; Gross et al., 1996).

2.4.1 Allelopathic macrophyte species

A number of macrophyte species exude allelopathic substances with algicidal and bactericidal effects (Hilt and Gross, 2008), and thus were suggested to be used to control harmful cyanobacterial blooms (Shao et al., 2013). Allelopathic effects of submerged macrophytes on phytoplankton have been shown for at least 37 species (Mulderij, 2006). For instance, *M. aeruginosa* has been found to be inhibited by at least 10 different submerged macrophyte species when tested in single-species cultures (Chang et al., 2012, and literature quoted therein). Although studies directly comparing the activity of submerged macrophytes against phytoplankton are scarce, Hilt and Gross (2008) have ranked macrophytes according to their allelopathic activity. There are a number of very active species in temperate regions such as Eurasian watermilfoil

[3]Allelopathy (*gr. allelon* - mutual, *pathos* - affection, harm) in its original definition by the Viennese botanist Hans Molisch (1937) describes any inhibitory or stimulatory effect of one plant on another plant, mediated by the release of some chemical factor. International Allelopathy Society (IAS) defines allelopathy as "any process involving secondary metabolites produced by plants, algae, bacteria and fungi that influences the growth and development of agriculture and biological system"

(*Myriophyllum spicatum*) and Rigid hornwort (*Ceratophyllum demersum* L.), species with a medium activity (*Najas*, *Elodea*, *Stratiotes* and *Chara* species), and species that have little or no allelopathic activity such as most pondweeds, e.g. *Potamogeton* spp. (Hilt and Gross, 2008, Fig. 2.4). Other species of *Myriophyllum* (e.g. *Myriophyllum verticilaltum* (Haloragaceae)) are also allelopathically active (Hilt et al., 2006; Chang et al., 2012).



(A) (B) (C) (D)

FIGURE 2.4: Allelopathically active macrophyte species: A. *Myriophyllum spicatum*, B. *Ceratophyllum demersum*, C. *Stratiotes alloides*, D. *Najas marina*. The pictures are derivatives of drawings from Thomé (1903).

2.4.2 Allelochemicals of macrophytes

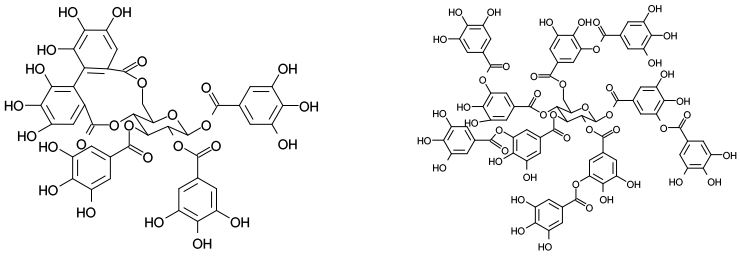
Aquatic allelochemicals present a variety of chemical structures as they have in terrestrial ecosystems. Chemicals responsible for allelopathic interactions in aquatic ecosystems can be divided broadly into seven categories according to their chemical nature: (1) phenolic compounds, (2) saturated and unsaturated fatty acids, (3) terpenoids, (4) oligopeptides, (5) polyethers, (6) polysaccharides and (7) miscellaneous com-

pounds (Macías et al., 2008).

Phenolic compounds are the type of compounds most often reported as algicidal from aquatic organisms. *M. spicatum*, one of the best investigated allelopathically active species, contains polyphenolic compounds such as gallotannins and ellagitannins (Nakai et al., 2000, 2001) of which tellimagrandin II (Fig. 2.5A) may reach up to 6% of biomass in growing apical meristems (Gross et al., 1996). Polyphenols of *M. spicatum* cause oxidative stress (Wang et al., 2011) and inhibit the activities of the alkaline phosphatase (Gross et al., 1996), esterase (Eigemann et al., 2013a) and photosystem II (Körner and Nicklisch, 2002; Leu et al., 2002) of different phytoplankton species. Besides polyphenolic compounds, *M. spicatum* releases algicidal fatty acids such as nonanoic and octadecenoic acids (Nakai et al., 2005). Fatty acid ethyl 2-methylacetoacetate from *Phragmites australis* (Cav.) Trin. ex Steud. (syn. *Phragmites communis*) was shown to be involved in changes in plasma membrane integrity and leakage of ions in the protoplast (Li and Hu, 2005). Diterpenes reported as allelochemicals include entlabdanes from several *Potomageton* spp. (Macías et al., 2008). Although a large number of allelopathically active substances of macrophytes are known (Bauer, 2011), most of them are still to be identified (Gross et al., 2012).

2.4.3 Factors influencing the allelopathic effect of macrophytes

The strength of allelopathic effects of a donor organism (e.g. macrophytes) on an acceptor organism (e.g. phytoplankton species) is modulated by a variety of factors. A number of biotic and abiotic factors may influence the sensitivity of the acceptor organism to allelochemicals of macrophytes (Bauer,



(A) Tellimagrandin II (B) Tannic acid

FIGURE 2.5: Chemical structures of major inhibiting compounds of *M. spicatum* tellimagrandin II (A) and its substitute tannic acid (B) used in this study.

2011). For instance, solar radiation, oxygen and changing redox conditions may influence the stability of allelochemicals (Gross, 2003a). The content of allelochemicals in macrophytes may also vary with season (Hilt et al., 2006; Bauer et al., 2009).

Biotic parameters, such as microbial capacity to degrade of allelochemicals (Müller et al., 2007), species-specific bacteria interactions, coexisting alga species may further affect the allelopathic effect (Eigemann, 2013). For instance, Chang et al. (2012) showed that interactions with green algae turned the inhibiting effect of macrophyte-released allelochemicals on *M. aeruginosa* into an enhancement resulting in increased growth rates. They, therefore, suggested that future studies searching for the potential of natural allelochemicals to inhibit toxic cyanobacteria blooms should consider interactions of cyanobacteria with other species present in respective phytoplankton communities. However, in most field studies, it was not possible to exclude competition for resources (nutrients and light) between coexisting species as a potentially confounding

factor (Mjelde and Faafeng, 1997; Van den Berg et al., 1998; Blindow et al., 2002; Lombardo, 2005). *In situ* evidences of allelopathy is difficult to achieve so that all requisites (inhibition, production, release, transport, uptake, exclusion of other factors) stated by Willis (1985) are fulfilled (Gross et al., 2007). Mesocosm studies may offer a reasonable compromise allowing for a higher degree of control over a number of potentially confounding effects (Vanderstukken et al., 2011).

Target organisms themselves exhibit specific traits that may influence their sensitivities to allelochemicals. Sensitivities of algae and cyanobacteria to allelochemicals of macrophytes differ significantly at the phylum (Hilt and Gross, 2008), species (Körner and Nicklisch, 2002) and strain levels (Eigemann et al., 2013b). The reasons for these differences remain largely unknown. *M. aeruginosa* strains possess high genomic (Humbert et al., 2013) and proteomic (Alexova et al., 2011) plasticity and diversity in morphological (Rico et al., 2006), physiological (Bañares-España et al., 2013) and biochemical (Agha et al., 2014) characteristics. We propose that the sensitivity of *M. aeruginosa* to allelochemicals could be affected by their ability to produce certain metabolites. Of these metabolites, MCs are likely to be of particular importance.

Few studies checked sensitivities of both MC-producing and non-MC-producing *M. aeruginosa* strains to extracts, exudates and allelochemicals of macrophytes (Mulderij et al., 2005; Liu et al., 2007; Wu et al., 2009), but provided contrasting results. Liu et al. (2007) found that the chlorophyll *a* (Chl *a*) and carotenoid concentrations of non-MC-producing *M. aeruginosa* strain FACHB-942 decreased more quickly than in MC-producing *M. aeruginosa* strain FACHB-469 in treatments with the allelochemical pyrogallol. In contrast,

Mulderij et al. (2005) showed that exudates from *S. aloides* suppres MC-producing more than non-MC-producing strains of *M. aeruginosa*. Similarly, Wu et al. (2009) found that extracts from three macrophytes (*Elodea nuttallii* Planch, *Hydrilla verticillata* Royle and *Vallisneria spiralis* L.) inhibit MC-producing more than non-MC-producing strains of *M. aeruginosa*. However, a broader study is needed to unravel the potential role of MCs against allelochemicals of macrophytes in *M. aeruginosa* cyanobacteria.

3
Materials and Methods

3.1 Overview

To test the potential effects of the allelopathically active macrophyte *M. spicatum* on *M. aeruginosa* and its toxicity of and to fulfil the objectives of the study (Fig. 3.1), several field, mesocosm and laboratory studies were combined.

Firstly, a field study was conducted to identify environmental parameters that affect the proportion of toxic *M. aeruginosa* genotypes in a highly eutrophic Villerest reservoir in France (Fig. 3.2). Secondly, a 13 day lasting study with 8 mesocosms (4 controls and 4 treatments with *M. spicatum* sets) was performed to evaluate the impact of *M. spicatum* on natural mixed phytoplankton communities sampled from the Curonian Lagoon during a cyanobacteria bloom that included different *M. aeruginosa* genotypes. Third, the effects of allelochemicals on MC- and non-MC-producing *M. aeruginosa* were studied by employing TA bioassays on single *M. aeruginosa* strains and tests with single and mixed MC- and non-MC-producing *M. aeruginosa* strains either treated with allelochemical TA or placed in dialysis bags among *M. spicatum* shoots.

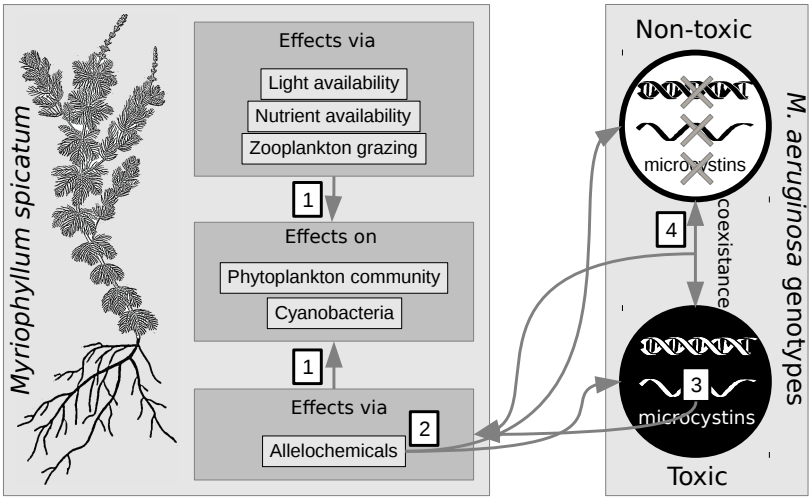


FIGURE 3.1: Layout of the objectives investigated in this study. Factors investigated are marked with Roman numerals and interactions between factors are indicated with arrows. The drawing of *Myriophyllum spicatum* is a derivative of drawings from Thomé (1903) and Kops et al. (1830).

3.2 Field study

3.2.1 Study area and sampling

The influence of different environmental factors on the development MC- and non-MC- producing *M. aeruginosa* was studied in Villerest reservoir (Fig. 3.2), which is one the most eutrophicated water bodies in France with frequent developments of *M. aeruginosa* dominated blooms (Aleya et al., 1994; Bonnet and Poulin, 2002). Few general characteristics of the Villerest reservoir: area - 7.12 km^2, length - 34 km, maximum depth - 45 m, volume - 130 $\times$ 10^6 m^3, mean annual total phosphorus inflow - 0.08 mg L^{-1}, Chl *a* concentration (μg L^{-1}) annual - 20.5, maximum - 62.9, retention time – 3

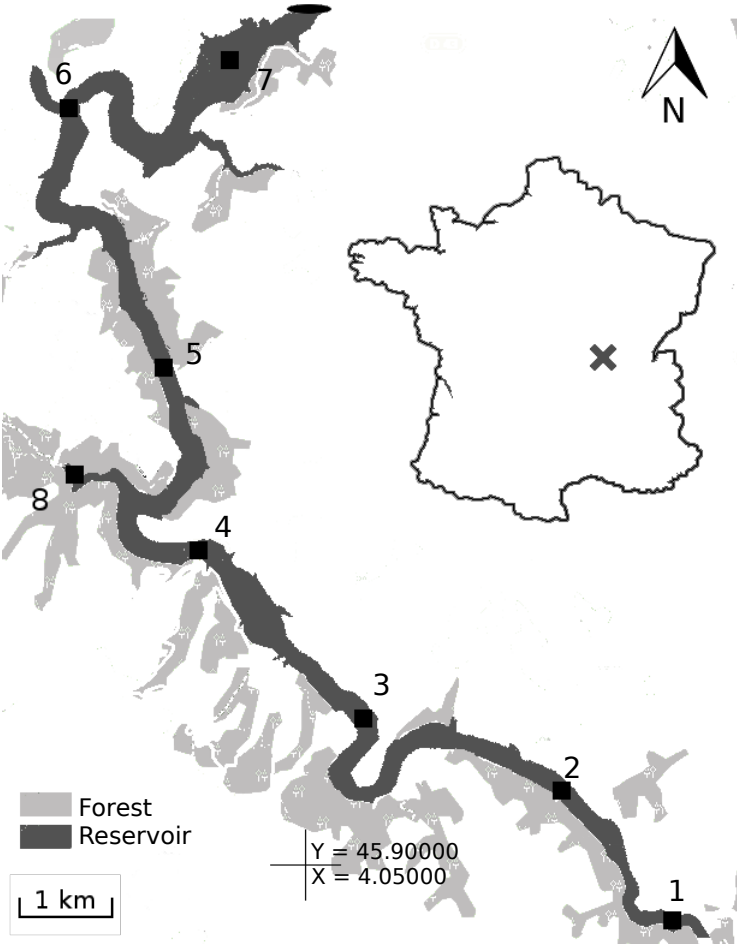


FIGURE 3.2: Geographic locations of the Villerest reservoir in France (marked with cross) and the sampling sites in the Villerest reservoir (no. 1-8). The map data available under the Open Database Licence is sourced from the Direction Générale des Impôts and ©OpenStreetMap contributors.

month.

Samples were collected in eight study sites (Fig. 3.2), from 4th of June to 28th of August, 2009 in seven sampling campaigns performed approximately once in two week interval. Sites 1-7 were characteristic to a deep open water body (depth > 4 m), whereas the 8th sampling site was in a narrow semi-enclosed shallow bay (depth ~ 1 m) with a low density and sporadic presence of macrophytes (macrophyte densities and species were not identified). This site was surrounded by dense terrestrial vegetation and received water from a 2.2 km long densely vegetated rivulet La Goutte de Plaigne. Coordinates of the sampling sites were: 1. 45.89587, 4.10198; 2. 45.90833, 4.08500; 3. 45.91500, 4.05666; 4. 45.93274, 4.03085; 5. 45.95056, 4.02778; 6. 45.97639, 4.01389; 7. 45.98167, 4.03667; 8. 45.94028, 4.01389 (WGS).

At each sampling site, phytoplankton biomass and temperature vertical profiles were made by submersible fluorescence probe (bbe Moldaenke, Kiel, Germany) which enabled the quantitative differentiation of different phytoplankton groups (green algae, cyanobacteria, diatoms, cryptophyta) according to their different fluorescence excitations (Beutler et al., 2002). MC- and non-MC-producing *M. aeruginosa* quantification were performed from 15 L water samples collected from the surface layer. Sampled water was filtered through 20 μm mesh after which *M. aeruginosa* colonies were concentrated on polycarbonate filter (pore size - 1 μm; Nucleopore) and stored at -20 °C till further molecular and MCs analysis (samples for MCs analyses were taken on the sites 1, 4 and 7). Data of day averaged total solar irradiation on horizontal plane from HelioClim-3 Database (Blanc et al., 2011) were kindly provided by MINES ParisTech – Armines.

3.2.2 Molecular analyses

DNA was extracted according to the protocol of Humbert et al. (2005). Briefly, after filtering the sample, pieces of filter were placed in a tube and subjected to ultrasonication in 2–4 ml cell lysis buffer (0.05 M Tris-HCl, 0.004 M EDTA, 0.75 M sacharoze at pH 8, and with a final concentration of 10 mg mL^{-1} of lysozyme). The tubes were incubated at 37 °C for 45 min. Proteinase K (0.5 mg mL^{-1}) and 1% sodium dodecyl sulfate were then added, and the tubes were placed in a water bath at 55 °C for 90 min. After a phenol – chloroform extraction, and ethanol precipitation, the extracted DNA was stored at -20 °C until used.

Table 3.1: List of primers and probes

Name	Nucleotide sequence
mcyB-F	CCTACCGAGCGCTTGGG
mcyB-R	GAAAATCCCCTAAAGATTCCTGAGT
mcyB probe	FAM-ACCAAAGAAACACCCGAATCTGAGAGG-TAMRA
PC-F	GCTACTTCGACCGCGCC
PC-R	TCCTACGGTTTAATTGAGACTAGCC
PC probe	CYA-CCGCTGCTGTCGCCTAGTCCCTG-BHQ-2

FAM - 6-carboxyfluorescein; TAMRA - 6-carboxytetramethylrhodmine; CYA - 5-indocarboxycyanine; BHQ-2 - black hole quencher-2.

The proportion of toxic *M. aeruginosa* genotypes were determined by means of qPCR similarly as described by Briand et al. (2009). Regardless to quantification of micro organisms in ecological studies the qPCR technique have a wide applicability in detection and quantification of nucleic acids (both DNA and RNA) in molecular biology (e.g. Tuomainen et al.,

2011; Mackay, 2007), clinical quantifications and genotyping of infectious diseases, cancer and genetic abnormalities (reviewed by Kaltenboeck and Wang, 2005), food safety, food spoilage, fermentation analyses (e.g. Postollec et al., 2011), detection of genetically modified organisms (e.g. Ahmed, 2002).

Two target gene regions were used: the intergenic spacer region within the phycocyanin (PC) operon and the *mcyB* gene region, which carries out one step in MC biosynthesis (Tillett et al., 2000). The primers and probes listed in Table 3.1 were specific for *Microcystis* (Kurmayer and Kutzenberger, 2003). The sizes of the amplicons were 66- and 78-bp for the *PC* and *mcyB* genes, respectively. Fluorescent reporter and quencher dyes were used to perform mutiplex qPCR and to discriminate between the two amplifications in a same run. qPCR was carried out using an Mx3005P thermal cycler (Stratagene, the Netherlands). All the reactions were performed with 20 μL volumes in 96-well plates (Stratagene). The reaction mix contained 10 μL of 2x PCR QuantiTec Probe Mix Kit (Qiagen), a 900 nM concentration of each primer, a 250 nM concentration of the TaqMan probes and 2 μL of template containing various amounts of genomic DNA. The mix was filled to 20 μL with sterile water. Each sample was prepared in triplicates of 1, 10 and 100 fold diluted template DNA. Negative controls without DNA were included for each qPCR run. For each run of samples, serial dilutions containing duplicates 1.1×10^{1}, 1.1, 1.1×10^{-1}, 1.1×10^{-2}, 1.1×10^{-3}, 1.1×10^{-4} and 1.1×10^{-4} ng of the genomic DNA μL^{-1} from the DNA extract of an PCC 7806 strain was included to serve as external standard. The temperature cycle consisted of an initial preheating step of 15 min at 95 °C, followed by 40 cycles of 30 s at 95 °C (denaturing), 1 min at 60 °C (annealing) and 30 s at 72 °C (extension). For data anal-

ysis, the threshold for the fluorescence of all the samples was set manually to 132 (relative fluorescence level) for PC gene amplification, and to 665 for *mcyB* amplification to obtain the best qPCR efficiency using linear–log calibration curves.

The change in threshold cycle (ΔC_t ; Briand et al. 2008) was used to estimate the percentage of the MC-producing *M. aeruginosa* genotypes in the whole *M. aeruginosa* population by a single multiplex qPCR. We had tested the reproducibility of the method by applying it to several MC-producing *M. aeruginosa* strains. Standard curves for the *PC* and *mcyB* genes were constructed using the genomic DNA of eight MC-producing *M. aeruginosa* strains PCC 7806, PCC 7813, PCC 7820, PCC 7941, PCC 9354, PCC 9355, PCC 9443 and PCC 9808. The genomic DNA of these strains was kindly provided by Marion Sabart. For each strain, serial dilutions containing duplicates 1.1×10^{1}, 1.1, 1.1×10^{-1}, 1.1×10^{-2}, 1.1×10^{-3}, 1.1×10^{-4} and 1.1×10^{-4} ng of the genomic DNA μL^{-1} were prepared. The standard curves were established by relating the known quantity of DNA to the threshold cycle (C_t) number (the cycle number at which the fluorescence exceeds the threshold) for each diluted sample (Fig. 3.3A). The regression equations, and the resulting ΔC_t between the two genes are shown in the Table 3.2.

We found that there was a narrow variation in ΔC_t values (mean = 0.056 ± 0.28 SD) for all eight MC-producing *M. aeruginosa* strains. As the C_t decreases by 1 when the quantity of DNA decreases by half, theoretical ΔC_t equation was deduced which was used to calculate the percentage of MC-producing strains in sampled water: $y = 1.44ln(x) + 6.59$, where y is the ΔC_t, and x is the percentage of the MC-producing *M. aeruginosa* genotypes (Fig. 3.3B). This method of the deduction of the ΔC_t equation was validated using var-

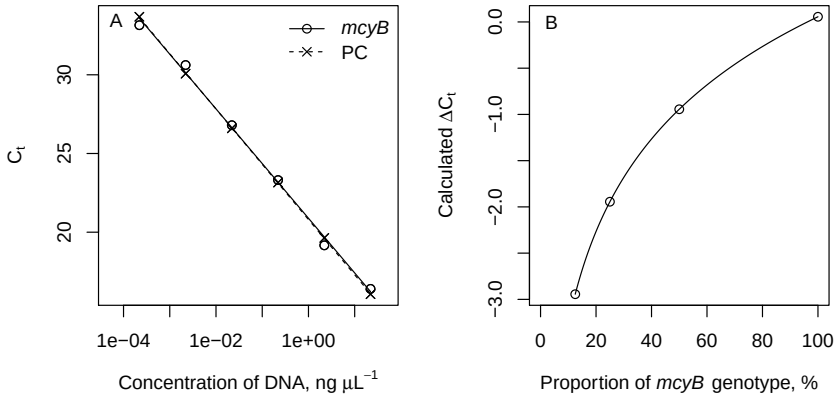


FIGURE 3.3: qPCR calibration curves: A - an example of a calibration curve to estimate ΔC_t using the MC-producing *Microcystis aeruginosa* strain PCC 7806; B - theorethically deduced ΔC_t curve to calculate the percentage of MC-producing *Microcystis aeruginosa* strains.

ious mixtures of the MC- and non-MC-producing strains by Briand et al. (2009).

3.2.3 Microcystin analysis

Intracellular MC-LR and MC-RR extraction and quantitative determination were performed identically as described by Sotton et al. (2011). In brief, each filter was placed in a haemolysis tube and extracted with 75% methanol. Then, the extracts were quantified by high performance liquid chromatography with photodiode array (Waters).

3.3 Mesocosm study

3.3.1 Experimental design

The mesocosm experiment was performed in eight 85 L non-transparent white plastic cone-shaped tanks (top diame-

TABLE 3.2: Regression equations of the calibration curves for the tested MC-producing *Microcystis aeruginosa* strains for *PC* and *mcyB* amplicons and estimated ΔC_t values.

Strain	Equations	ΔC_t
PCC 9443	*PC*, $y = -3.395ln(x) + 20.48$, Eff. $= 97.0\%$ *mcyB*, $y = -3.427ln(x) + 20.04$, Eff. $= 95.8\%$	-0.44
PCC 7813	*PC*, $y = -3.304ln(x) + 20.61$, Eff. $= 100.8\%$ *mcyB*, $y = -3.430ln(x) + 20.52$, Eff. $= 95.7\%$	-0.09
PCC 7820	*PC*, $y = -3.383ln(x) + 20.03$, Eff. $= 97.5\%$ *mcyB*, $y = -3.418ln(x) + 19.97$, Eff. $= 96.1\%$	-0.06
PCC 7808	*PC*, $y = -3.305ln(x) + 22.12$, Eff. $= 100.7\%$ *mcyB*, $y = -3.333ln(x) + 22.26$, Eff. $= 99.5\%$	0.14
PCC 9354	*PC*, $y = -3.307ln(x) + 20.22$, Eff. $= 100.6\%$ *mcyB*, $y = -3.474ln(x) + 20.22$, Eff. $= 94.0\%$	0
PCC 9355	*PC*, $y = -3.260ln(x) + 19.75$, Eff. $= 102.7\%$ *mcyB*, $y = -3.324ln(x) + 20.08$, Eff. $= 99.9\%$	0.33
PCC 7806	*PC*, $y = -3.511ln(x) + 20.80$, Eff. $= 92.7\%$ *mcyB*, $y = -3.474ln(x) + 20.89$, Eff. $= 94.0\%$	0.09
PCC 7941	*PC*, $y = -3.219ln(x) + 18.07$, Eff. $= 104.5\%$ *mcyB*, $y = -3.221ln(x) + 18.55$, Eff. $= 104.4\%$	0.48

ter×bottom diameter×height: $53 \times 46 \times 43$ cm) with a 1 mm diameter mesh cover on the top to exclude insects and aligned along a straight west-east axis in the vicinity of Klaipėda city in Lithuania.

M. spicatum individuals were obtained from the Curonian Lagoon at 55.494481, 21.243083 (WGS) in early April, 2011. Macrophytes were rinsed to remove invertebrate grazers and fish eggs, planted in 0.5 L plastic pots with sediments taken from the same location, placed in an aquarium with water from a nearby pond and grown outdoors for 3 months prior to experimentation. On the 2nd of July, 2011, all eight meso-

cosms were filled with fresh (salinity = 0 PSU) water taken from the Curonian Lagoon at 55.354967, 21.202329 (WGS) during a cyanobacteria bloom. This site was chosen to avoid brackish water that intruded from the Baltic Sea at the macrophyte sampling point. Water was cross mixed between mesocosms to ensure homogeneity. Ten to twelve *M. spicatum* sets potted into sediment were placed into each of four mesocosms whereas the four control mesocosms received potted sediments without plants. 1.25 mg P L^{-1} as KH_2PO_4 and 5.95 mg N L^{-1} as $NaNO_3$ were added into each of the mesocosms on day 0. The experiment terminated after 13 days. The final *M. spicatum* aboveground biomass (determined after drying at 105 °C) varied from 0.38 to 0.53 g dw L^{-1} (32 to 45 g dw per mesocosm or 164 to 229 g dw m^{-2}), which is within the range of maximum *M. spicatum* biomass *in situ* reported by Grace and Wetzel (1978). Water temperature (measured daily in the mesocosms at 7 AM, 2 PM and 9 PM (EET) varied from 15 to 28 °C throughout the experimental period with a mean of 23 °C at 2 PM (EET). The mesocosms received direct sun light throughout the day (length of daytime was 17 ± 0.2 hours) and 41 mm of rain fall compensated for water losses due to evaporation and sampling. Meteorological data were kindly provided by Lithuanian Hydrometeorological Service under the Ministry of Environment of the Republic of Lithuania.

3.3.2 Sampling and sample analysis

Conductivity, nutrient concentrations, pH, Chl *a* content, zooplankton abundance, nitrogenase activity and gross primary production (GPP) were measured in water sampled from the Curonian Lagoon on day 0 and in the mesocosms on days 3, 6, 10 and 13, whereas water samples for quantification of *M. aeruginosa* subpopulations were taken on days

0 and 13. Water was mixed prior to sampling and sampled once and consistently for all analyses from 8 to 11 AM (EET) on each sampling day.

Concentrations of NH_4^+, NO_3^- + NO_2^- (NO_x), total nitrogen (TN), soluble reactive phosphorus (SRP) and total phosphorus (TP) were determined in 100 mL water sample (stored frozen prior to analyses) following standard procedures (LAND 38-2000, ISO 13395-2000, ISO 11905-1:1997 and ISO 15681-1:2005, respectively).

Biomasses of different phytoplankton groups were determined with a cuvette-equipped submersible fluorescence probe (bbe Moldaenke, Kiel, Germany) which enabled the quantitative differentiation of different phytoplankton groups (green algae, cyanobacteria, diatoms, cryptophyta) according to their different fluorescence excitations (Beutler et al., 2002). Dominant phytoplankton species were identified in the formalin-fixed sample taken on day 0 with a light microscope (600×) following Starmach (1989), Komárek and Anagnostidis (1999) and John et al. (2002).

Zooplankton counting was performed in 60 mL formalin-fixed water samples using Bogorov's counting chamber under a binocular microscope (600×). Zooplankton taxa were identified to genus or species level using Kutikova (1970) and Rybak and Błędzki (2010) and their biomass was calculated according to the allometric body length-weight relationships (Salazkin et al., 1984; Jorgensen et al., 1995). Potential grazing rates were calculated for the functional groups known to feed on phytoplankton. We assumed that the daily grazing rates of cladocerans are equivalent to their biomass, and that the daily consumption rates of copepods are equivalent to 50% of their biomass (Vanderstukken et al., 2011).

Nitrogenase activity was estimated by acetylene reduction

to ethylene (Capone, 1993) using a gas chromatograph (Focus, Thermo Scientific) with flame ionization detector. Fifty mL of sampled water were placed in 62 mL volume crimp-topped vials, 5 mL of freshly prepared acetylene from CaC_2 were injected at 9 AM (EET) on the sampling day after which vials were immersed in a water bath to ensure similar conditions to those in the mesocosms. Ethylene production was terminated after four hours by injecting 0.5 mL of 0.25 M $HgCl_2$.

The gross primary production of phytoplankton was determined from in vitro changes in dissolved oxygen after 24 h light and dark bottle incubations. Water samples were incubated in 3 clear and 3 opaque 50 mL bottles immersed in a water bath to ensure similar conditions to those in the mesocosms. Dissolved oxygen concentration was determined by Winkler's titration technique (Grasshoff, 1983).

3.3.3 Quantification of *Microcystis aeruginosa* genotypes

In order to quantify the proportion of MC-producing *M. aeruginosa* genotypes we filtered 1.5 L of water on days 0 and 13 using 0.2 μm pore-size cellulose acetate membrane filters. Filters were frozen and remained frozen until DNA extraction. MC-producing and total *M. aeruginosa* genotypes were quantified by means of real-time PCR (Kurmayer and Kutzenberger, 2003). Genomic DNA from the *M. aeruginosa* PCC 7806 strain (kindly provided by Hans-Peter Grossart) and water samples from the mesocosms were extracted with phenol-chloroform (Zhou et al., 1996). Similarly as described above two target gene regions were used: PC operon, to quantify the total *M. aeruginosa* genotypes, and the *mcyB* gene, to quantify MC-producing *M. aeruginosa* genotypes. The ucleotide sequences of primers (*mcyB*-F, *mcyB*-R and *PC*-F, *PC*-R)

were the same as those described in the Table 3.1. qPCR analyses were carried out using the Maxima SYBR Green qPCR Master Mix (Fermentas GmbH) on the Bio-Rad CFX96 Real-Time System with a C1000 Thermal Cycler for both primer pairs in separate wells of the same amplification run. The reaction mix contained 10 μL of Maxima SYBR Green qPCR Master Mix (2×), 900 nM concentration of each primer and 60 ng of template DNA. Each sample was prepared in triplicate and negative controls without DNA were included. PC operon and *mcyB* genes were quantified from the standard curves which were established performing amplifications on 10-fold serial DNA dilutions of genomic DNA of the *M. aeruginosa* PCC 7806 strain. The temperature cycle consisted of an initial preheating step of 10 min at 95 °C, followed by 40 cycles each consisting of 15 s at 95 °C (denaturing) and 60 s at 60 °C (annealing/extension). For data analysis, the fluorescence threshold of all of the samples and standard curves was set manually to 180. Highly significant standard curves were obtained for both genes: PC: Eff. = 101%; $R^2 = 0.99$; $P < 0.001$; slope = 3.29; *mcyB*: Eff. = 100%; $R^2 = 0.99$; $P < 0.001$; slope = 3.322.

3.4 Laboratory experiments

3.4.1 Origin and growth of *Microcystis aeruginosa* strains

In total, 13 strains of *M. aeruginosa* were tested for their sensitivity to allelochemicals (Table 3.3). Strains were purchased from the culture collections of the National Institute for Environmental Studies, Japan (NIES), the Norwegian Institute for Water Research (NIVA), the University of Göttingen, Germany (SAG), the University of Texas at Austin, USA (UTEX) and the Pasteur Culture Collection of Cyanobacte-

ria, France (PCC). Strains from the University of Wisconsin Culture Collection at Oshkosh (UWO) were kindly provided by Elke Dittmann and strains from the Humboldt University of Berlin (HUB) were kindly provided by Manfred Henning. Two sympatric pairs of MC- and non-MC-producing strains HUB 524 and HUB P461 originate from Lake Pehlitz (Germany), and strains SAG 14.85 and UTEX LB2386 originate from Lake Little Rideau (Canada). To omit intrinsic inter-strain variability we employed the PCC 7806 $\Delta mcyB$ strain, which is genetically identical to the PCC 7806 strain, but lacks the ability to produce MCs (due to an insertion of a chloramphenicol resistance cassette into the *mcyB* gene; Dittmann et al., 1997). The UWO MRC strain (genetically identical to UWO MRD; Kaebernick et al., 2001) is unstable in its production of MCs (Alexova et al., 2011).

The strains were maintained and experiments were carried out in a modified MIIIKS medium ($CaSO_4$ 0.5 mM, $CaCl_2$ 0.5 mM, $MgSO_4$ 0.25 mM, NaN_3 0.5 mM, KH_2PO_4 0.05 mM, KCl 0.1 mM, Na_2SiO_3 0.4 mM, HCl 0.75 mM, $NaHCO_3$ 2 mM, $FeCl_3$ 0.010 mM, Na_2EDTA 0.020 mM, H_3BO_3 4 μM, $MnSO_4$ 0.8 μM, $ZnSO_4$ 0.08 μM, Na_2MoO_4 0.04 μM, $CuSO_4$ 0.04 μM, $AlK(SO_4)_2$ 0.08 μM, $CoCl_2$ 0.04 μM, $NiSO_4$ 0.04 μM, KBr 0.08 μM, KJ 0.04 μM, and H_2SeO_3 0.06 μM;Nicklisch 1992) with a pH of 8.3 $\pm$ 0.1, at 22 $\pm$ 1 °C in a conditioning cabinet. Light was supplied in a 12:12 h light:dark cycle by fluorescent tubes (Osram L36W/965 Biolux T8, Germany) that provided an emission spectrum similar to daylight with a light intensity of 200 μmol photons m^{-2} s^{-1}. Cultures were shaken gently at ca. 80 rpm. Prior to experimentation the cultures were diluted once every three to four days and Chl *a* concentrations were maintained within the range of 15 - 100 μg L^{-1} in order to sustain non-limited exponential growth.

TABLE 3.3: List of *Microcystis aeruginosa* strains used and their characteristics.

Strain	MC	Isolate location	Isolate date	Reference
HUB 018	−	Federal State of Brandenburg, Germany	1977	Schwabe et al. (1988)
HUB 524	+	Lake Pehlitz, Germany	1978	Schwabe et al. (1988)
HUB P461	−	Lake Pehlitz, Germany	1995	Dziallas and Grossart (2011)
NIES 101	−	Lake Suwa, Japan	1982	Yoshida et al. (2008b)
NIES 102	+	Lake Kasumigaura, Japan	1982	Yoshida et al. (2008b)
NIVA CYA 143	−	Lake Akersvatnet, Norway	1984	Rudi et al. (1997)
PCC 7806	+	Braakman reservoir, The Netherlands	1972	Kappers (1984)
PCC 7806 Δ*mcyB*	−	Braakman reservoir, The Netherlands	1972	Dittmann et al. (1997)
SAG 14.85	+	Little Rideau Lake, Canada	1954	Hughes et al. (1958)
SAG 14.50	+	Lake Mendota, USA	1946	Gerloff et al. (1950)
UTEX LB2386	−	Little Rideau Lake, Canada	1956	Allen and Gorham (1981)
UWO MRC	±*	Malpas Dam, Australia	1973	Kaebernick et al. (2001)
UWO MRD	+	Malpas Dam, Australia	1973	Kaebernick et al. (2001)

Signs + and − indicate MC- and non-MC-producing strains, respectively; * − strain was shown to be unstable in MC production (Alexova et al., 2011).

3.4.2 Sensitivity of single strains to allelochemicals

To test the sensitivity of *M. aeruginosa* strains to allelochemicals, quadruplicated bioassays with three concentrations (5, 10 and 20 mg L^{-1}) of the TA (filling code: 403955/1 64400; Fluka, Germany) were performed. TA is a major active compound in submerged macrophytes of the genus *Myriophyllum* (Gross et al., 1996; Gross, 2003b; Hilt, 2006; Bauer et al., 2009). Experiments were run for 3 days in 50 mL conical flasks that contained 20 mL of the strain culture with the initial Chl *a* concentrations of 20 μg L^{-1}. As few strains slowed down their growth after a transfer to a new flask, respective cultures were grown for three days to pre-adjust the flasks after which pre-cultures were discarded and new respective cultures were added to pre-adjusted flasks to initiate the experiment. A freshly prepared TA stock solution (1 g L^{-1}) was added to the respective treatment to reach final concentrations. TA did not change the pH value in the buffered MIIIKS medium (Eigemann et al., 2013b).

3.4.3 Sensitivity of mixed strains to allelochemicals

Two types of tests (lasting 3 days each) with mixed cultures of three sympatric MC- and non-MC-producing strains pairs (SAG 14.85 and UTEX LB2386, HUB 524 and HUB P461, PCC 7806 and PCC 7806 Δ*mcyB*; Table 3.3) were performed: 1) bioassay tests with multiple addition of an allelochemical tannic acid (TA) and 2) *M. spicatum* - *M. aeruginosa* coexistence tests. These tests were carried out at 22 $\pm$ 0.5 °C in a climate controlled chamber with 600 μmol photons m^{-2} s^{-1} light intensity. For both tests *M. aeruginosa* monoculture tests were paralleled by tests with mixtures of two sympatric MC- and non-MC-producing *M. aeruginosa* strains. Both, monocultures and mixtures included experi-

mental controls and allelopathic treatments. For all tests the initial concentration of strains was set to 20 μg L^{-1} Chl *a*. For mixtures, strains were combined at a ratio 1:1 based on initial Chl *a* concentration.

Bioassay tests were performed in quintuplicates in 50 mL conical flasks that contained 20 mL of the strain culture. As some strains slowed down in their growth after a transfer to a new flask, pre-cultures were grown for three days after which these were discarded and new culture solutions were added to initiate the experiment. Optimum amounts of freshly prepared TA stock solution (1 g L^{-1}) was added once at the initiation of the experiment and then once in every subsequent 24 hour in order to mimic continue release of allelochemicals from macrophytes. The each time added TA was set to reach 7 mg L^{-1} final concentration in SAG 14.85/UTEX LB2386 and 14 mg L^{-1} final concentration in HUB 524/HUB P461 and PCC 7806/PCC 7806 Δ*mcyB* cultures. The added TA was shown not to change the pH value in the buffered MIIIKS medium (Eigemann et al., 2013a).

For *M. spicatum* – *M. aeruginosa* coexistence tests dialysis bag approach was used (Körner and Nicklisch, 2002). *M. spicatum* was harvested from Lake Flakensee (coordinates: 52.434855, 13.764779 (WGS); Germany) and was maintained rooted in sandy sediments in plastic tanks at outdoor conditions. The apical tips (length $\sim$ 5 cm) of *M. spicatum*, as they contain highest amounts of allelochemicals (Bauer et al., 2009), were harvested 3 days in advanced and kept at experimental conditions to prevent allelochemical and nutrient leaching from the wounds. At the day of the start of experiment, apical parts were rinsed carefully with distilled water to remove epiphyton. Sterile sausage skin dialyse bags from regenerated cellulose Wienie-Pak Skinless Sausage Casings (De-

vro Teepak, Scarborough, Ontario, Canada), with a molecular cut-off weight of 7000 (ca. 30 cm long), were pulled over 40 mL bottomless Schott-bottles, fixed with rubber clamps and filled with 50 mL of algal culture. These dialysis bags were placed in 500 mL Erlenmeyer flasks containing 450 mL of modified MIIIKS solution. Experimental treatments received 0.90 ± 0.11 g fw apical shoots of *M. spicatum* resulting in macrophyte densities 0.08 ± 0.02 g dw L^{-1} that are comparable with those occurring in lakes (Grace and Wetzel, 1978). The experimental controls received plastic plants of similar shape and density in order to provide similar light shading.

For all laboratory experiments positions of flasks were randomized every day to ensure equal light conditions. At the end of the experiments Chl *a* concentrations and maximum quantum yields of the photosystem II (from now on termed photosynthetic yield) were determined in 20 min dark adapted samples with a PHYTO-PAM fluorometer (Walz, Germany), as described in Körner and Nicklisch (2002). In order to suppress the background fluorescence of the nutrient solution, Chl *a* concentrations were determined after off-setting the fluorescence of the culture filtrates (filtered through 0.2 μm pore size filters). All measurements were performed at the same time of a given photoperiod to ensure similar physiological states between cultures.

3.4.4 Determination of total phenolic content

Total phenolic content (TPC) was determined in culture filtrates at the end of TA bioassays with single PCC 7806, PCC 7806 Δ*mcyB*, SAG 14.85 and UTEX LB2386 strains. TPC was also determined in culture filtrates after the experiment with PCC 7806 and PCC 7806 Δ*mcyB* strains and their mixture treated by daily additions of TA. In order to

test if the allelopathic activities of *M. spicatum* shoots were not different between *M. spicatum* treatments in MC-producing, non-MC-producing strain and their mixture tests, TPC was determined at the end of all experiments with dialysis bags approach in *M. aerugiosa* culture filtrate. The cultures for TPC analyses were filtered via 1 μm pore size filter (Nucleopore).

The determination of TPC of was performed with the Folin-Ciocalteau assay (Folin and Ciocalteu, 1927) as described in Ainsworth and Gillespie (2007). The Folin-Ciocalteau assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic / phosphotungstic acid complexes, which are determined spectroscopically at 765 nm. Calibration of the method was done with TA. Total phenolic content were calculated and given as TA equivalents.

3.5 Data analysis

3.5.1 Field study

For statistical analyses of the data of Villarest reservoir, solar radiation for all sampling dates was obtained by averaging daily solar radiations of 14 days before to the respective sampling date. To omit diurnal variation, temperature data were averaged for the depth 1 - 2 m. Chl *a* concentrations were averaged for 0 - 2 m water layer. Site clustering was performed using the Ward's Minimum Variance Clustering method based on dissimilarities calculated using the Bray–Curtis index (Bray and Curtis, 1957) obtained from data of the percentage of MC-producing *M. aeruginosa* genotypes, total phytoplankton, green algae, cyanobacteria and diatoms Chl *a* concentrations. Differences in total phytoplankton, green algae, cyanobacteria and diatoms Chl *a* concentrations between sites 1, 2, 3, 4 *versus* sites 5, 6, 7 were compared

by fitting a two-factorial general linear model (LM) with site and sampling date as fixed factors. Data for total Chl *a* were log transformed to obtain normal and homoscedastic distributions of residuals. Multiple linear regression (MLR) was used to create a model to predict the percentage of MC-producing *M. aeruginosa* by biotic (total phytoplankton, cyanobacteria, green algae, diatom concentrations) and abiotic (temperature and solar radiation) environmental variables. Non-normally distributed data were log transformed prior to analysis. A backward stepwise model selection based on Akaike Information Criterion was employed selecting for the independent variables that makes the highest contribution to the prediction of the data. Total phytoplankton and green algae were excluded from the model due to co-linearity with cyanobacteria. The relative importance of the predictor variables (lmg; Chevan and Sutherland 1991) were calculated by calc.relimp function from relaimpo package in R (Grömping, 2006). Site clustering and backward stepwise MLR model selection were performed with vegan and MASS packages in R (Oksanen et al., 2015; Venables and Ripley, 2002).

3.5.2 Mesocosm study

Effects of the presence of *M. spicatum* on the different parameters throughout the whole experiment and short-term effect during the first 6 days were tested using linear generalised least squares (GLS) and linear mixed effects (LME) models, respectively, following the procedures in Zuur et al. (2009). Shapiro-Wilk test and homogeneity of variance was tested by Brown-Forsythe test. The following variables were transformed in order to meet assumptions of normality and homogeneity of variance: TP – square root; pH and DIN – exponential; SRP and copepoda biomass – rank; GPP, total zoo-

plankton biomass and potential grazing rate – Tukey's transformation (Tukey, 1962). Normality was not reached for diatoms, cryptophyta, cladocera, ciliate biomass, conductivity and nitrogenase activity and thus treatments *versus* control pairwise comparisons for each day were performed by Mann-Whitney U test with Bonferroni adjustment ($\alpha = 0.0125$). The GLS model with a proper auto-correlation structure was applied using treatment (i.e. macrophyte *versus* control) as a factor variable, time as a continuous variable and treatment × time interaction, whereas the latter was excluded in case of non-significance. The LME model was applied using treatments and day as a categorical variables and number of mesocosm as a random factor. *M. aeruginosa* populations were compared by Student's t-test (t-test) after inspection of normality and homogeneity of variance. The GLS and LME models were applied using the nlme package in R (Jose et al., 2014).

3.5.3 Laboratory experiments

Growth rates of *M. aeruginosa* strains were calculated by the formula:

$$\mu = \frac{ln\frac{X_t}{X_0}}{\Delta t} \tag{3.1}$$

where μ is growth rate, X_t and X_0 are Chl *a* concentrations at day 3 and 0, respectively, and Δt is the duration of the experiments in days.

Changes (i.e. reductions or increases) in Chl *a* and photosynthetic yields were calculated by the formula:

$$Changes = \frac{V_C - V_T}{V_C} \times 100 \tag{3.2}$$

where V_C is the mean value of the controls, and V_T is the value obtained from the treatment.

For the single strain experiments differences between changes in Chl *a* concentrations and photosynthetic yields within strains were tested by Kruskal-Wallis test (KWT) and subsequent Nemenyi post-hoc test. The changes in Chl *a* and photosynthetic yield for all TA treatments between MC-producing *versus* non-MC-producing strains and between sympatric MC-producing *versus* non-MC-producing strains in strain pairs were compared by fitting a two-factorial general linear model (LM) with MC production and TA concentrations as fixed factors. Data on the changes in Chl *a* concentrations for comparisons between PCC 7806 *versus* PCC 7806 $\Delta mcyB$ strains were square root transformed and data on the changes in photosynthetic yields for SAG 14.85 *versus* UTEX LB2386 strains were rank transformed to obtain normal and homoscedastic distributions of residuals. The concentrations at which TA reduced Chl *a* concentrations and photosynthetic yields by 50% i.e. half maximal effective TA concentrations (EC_{50-C} and EC_{50-Y}, respectively) were calculated by simple linear regression. Chl *a* concentration, photosynthetic yield mean values in control treatments and EC_{50-C} values between MC-producing and non-MC-producing strains were compared by Student's t-test (t-test). EC_{50-C} values of different origins were compared by the inspection of 95% confidence intervals. Associations between variables were sought with Pearson product-moment correlation analysis (PPM). For parametric tests, assumptions of normality and homoscedasticity were checked by Shapiro-Wilk and Brown-Forsythe tests, respectively. Final comparisons between MC-producing and non-MC-producing strains excluded data from the MRC strain due to its known unstable MC-production as well as the PCC 7806 $\Delta mcyB$

strain due to its genetic transformation.

For the mixed strain experiments, the Chl *a* and photosynthetic yield and their changes in mixtures were compared to respective theoretical values by one sample Student's t-test if normality assumptions (tested by Shapiro–Wilk test) were met. Theoretic concentrations of Chl *a* and Chl *a* weighted photosynthetic yield values were calculated by the following formulas, respectively:

$$PY_{theoretic} = \frac{X_{0_T} e^{\mu_T \Delta t} \times PY_T + X_{0_N} e^{\mu_N \Delta t} \times PY_N}{X_0 e^{\mu_T \Delta t} + X_0 e^{\mu_N \Delta t}} \quad (3.3)$$

$$Chl\, a_{theoretic} = X_0 e^{\mu_T \Delta t} + X_0 e^{\mu_N \Delta t} \quad (3.4)$$

where X_{0_T}, X_{0_N} are initial concentrations of Chl *a* of MC- and non-MC-producing strains, respectively, in mixed strain tests, μ_T and μ_N are growth rates of MC- and non-MC-producing strains, respectively, from the paralleled monoculture strain tests and PY_T and PY_N are photosynthetic yields of MC- and non-MC-producing strains, respectively, from the paralleled monoculture strain tests. Differences between changes in Chl *a* were tested Student's t-test.

The differences in TPC between MC- and non-MC-producing *M. aeruginosa* single strains and their mixtures in *M. spicatum* - *M. aeruginosa* coexistence tests were tested by KWT and with the general linear model (LM) with strain and TA concentrations as fixed factors for TA bioassays. TPC in *M. spicatum* - *M. aeruginosa* coexistence tests with HUB 524 / HUB P461 strains was different between treatments and thus was excluded from further analyses.

All statistical analyses were performed in the R Statistical Environment (R Core Team, 2014); for graphical data explo-

ration and figure graphing lattice and ggplot2 packages were employed (Sarkar, 2008; Wickham, 2009).

4

Results

4.1 *Microcystis aeruginosa* toxicity *in situ*

The Villerest reservoir experienced sporadic dense phytoplankton proliferations (Fig. 4.1) reaching total phytoplankton Chl *a* concentrations more than 100 μg Chl *a* L^{-1}, but on average Chl *a* concentrations kept below 10 μg Chl *a* L^{-1} of different phytoplankton groups (Table 4.1).

Green algae biomass were low throughout the summer (Table 4.1; Fig. 4.1A). Cyanobacteria peaked in the middle of summer, but their dense proliferation were only observed sporadically at different sampling sites, dates (Fig. 4.1B) and depths (Appendix A). Total phytoplankton biomasses were highest at the beginning of the summer mainly due dominating diatoms in the phytoplankton community (Fig. 4.1C and D). The variability of green algae, cyanobacteria and diatoms biomasses at different depths are detailed in the Appendix A.

Percentage of MC-producing *M. aeruginosa* genotypes were high till the end of July (mean = 72.9 % ± 13.1 SD, min. = 39.6 %, max. = 95.4 %) and declined in the middle of August (mean = 17.7 % ± 7.5 SD, min. = 9.9 %, max. = 34.1 %; Fig. 4.2). The lowest averaged percentage of MC-producing *M. aeruginosa* genotypes were in the site number 8 (mean = 52.3 % ± 25.3 SD; Fig. 4.2). MC-LR and MC-RR concentrations kept below 0.5 μg L^{-1} and were observed in only 10 out of 21 samples. Solar radiation kept high at the early sum-

TABLE 4.1: Descriptive statistics of environmental parameters measured in the Villerest reservoir.

Parameter	Unit	Statistics			
		Min.	Median	Mean	Max.
Biomasses of:					
- total phytoplankton	μg Chl *a* L^{-1}	1.2	14.8	18.8	102.9
- green algae	μg Chl *a* L^{-1}	0	2.4	3.2	23.2
- cyanobacteria	μg Chl *a* L^{-1}	0.1	3.8	7.4	46.4
- diatoms	μg Chl *a* L^{-1}	0.2	6.0	8.2	89.1
Percentage of MC-producing *M. aeruginosa* genotypes	%	9.9	69.5	60.2	95.3
Sum concentration of MC-LR and MC-RR	μg L^{-1}	LOD	LOD	0.03	0.41
Solar radiation	Wh m^{-2}	1399	6498	6047	8290
Water temperature	°C	16.4	23.8	23.5	27.9

LOD - lower limit of detection.

mer and declined thereafter following shorter days (data not shown). The surface water temperature peaked in the middle of the summer and then declined steadily (data not shown).

Chl *a* concentrations of total phytoplankton, cyanobacteria, diatoms and green algae was significantly higher at the riverine part of the reservoir (sites 1, 2, 3, 4) compared to the sites 5, 6, 7 that are closer to the dam (LM: $F_{2,42} > 15.0$, $P < 0.001$, for all parameters; Fig. 4.1) and was statistically equal for the percentage of MC-producing *M. aeruginosa* genotypes (LM: $F_{2,42} = 0.3$, $P = 0.867$; Fig. 4.2). The site 8 had on average highest concentrations of cyanobacteria, diatoms and total phytoplankton (means 10.6 ± 13.2 SD, 11.2 ± 8.6 SD, 24.7 ± 16.0 SD, respectively; all values are in μg Chl *a* L^{-1};

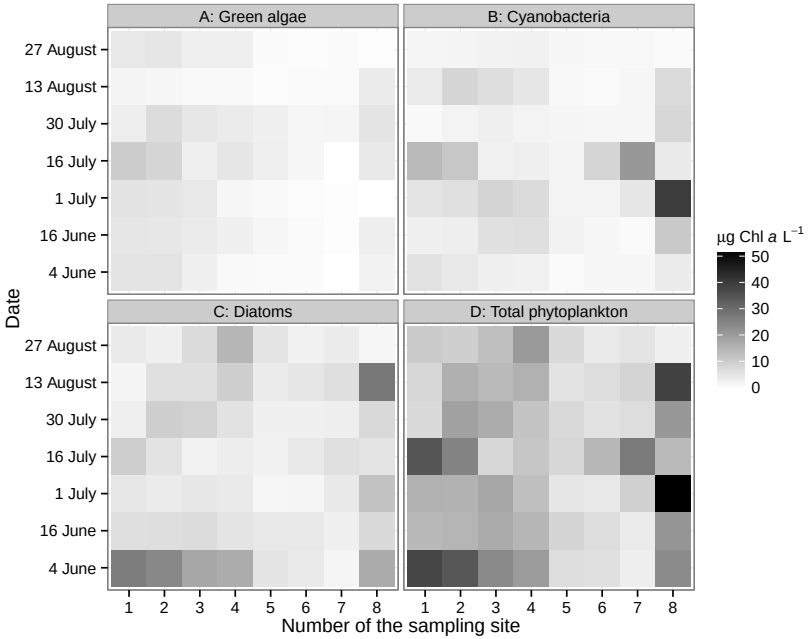


FIGURE 4.1: Spatiotemporal variation of biomass of the main phytoplankton groups (A - green algae; B - cyanobacteria; C - diatoms; D - total phytoplankton) in the Villerest reservoir. The data are averaged concentrations of the upper (to 2 m depth) water layer.

Fig. 4.1). Distinction between the site number 8 compared to other sites is graphically seen in the dendrogram of the sampling sites (Fig. 4.3A).

The proportion of MC-producing *M. aeruginosa* genotypes significantly positively correlated with the solar radiation (MLR: lmg = 0.75, $F_{6,46} = 15.5$ $P < 0.001$; Fig. 4.3B) and cyanobacteria (MLR: lmg = 0.09, $F_{1,46} = 51.5$ $P < 0.001$) and negatively correlated with water temperature (MLR: lmg = 0.16, $F_{1,46} = 47.2$ $P < 0.001$). The proportion of MC-producing *M. aeruginosa* genotypes did not correlate with diatoms (MLR:

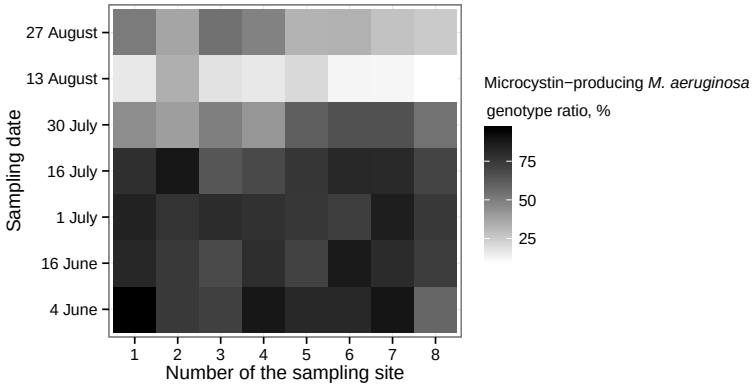


FIGURE 4.2: Spatiotemporal variation of the percentage of MC-producing *Microcystis aeruginosa* genotypes in the Villerest reservoir.

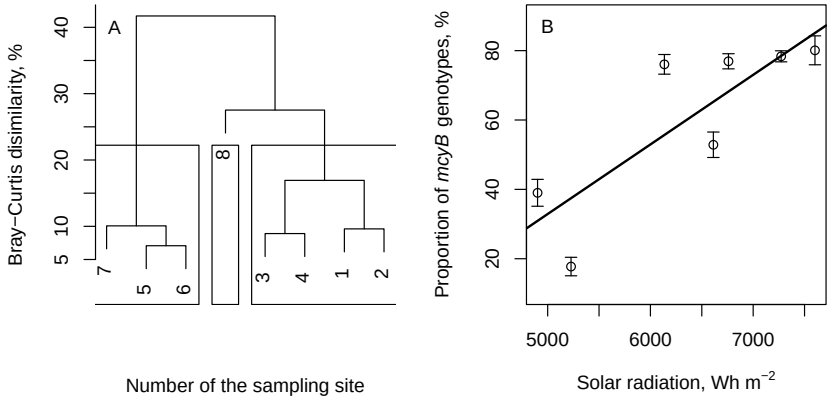


FIGURE 4.3: A - dendrogram based on Bray-Curtis dissimilarity matrix of biotic parameters measured in Villerest reservoir; B - relationship between the percentage of MC-producing *Microcystis aeruginosa* genotypes and daily solar radiation in the field study in the Villerest reservoir (error bars represent ± standard errors).

lmg = 0.01, $F_{1,46}$ = 0.08 P = 0.785). The concentrations of MCs did not correlate with the percentages of MC-producing *M. aeruginosa* genotypes (PPM: r = -0.43, P = 0.211).

4.2 Effects of *Myriophyllum spicatum* under *in situ* like conditions

4.2.1 Hydrochemical characteristics

For the mesocosm study, SRP concentrations decline significantly within the first 3 days in macrophyte treatments, and on average were more than 6 times lower than SRP concentrations in controls (Fig. 4.4A) whereas a less-pronounced effect was observed in dissolved inorganic nitrogen (DIN) concentrations (Fig. 4.4B). Both SRP and DIN were consistently and significantly lower in *M. spicatum* compared to control treatments throughout the experiment (GLS: $F_{1,29}$ = 122.9; $P < 0.001$ and $F_{1,29}$ = 6.1; P = 0.02, respectively; Fig. 4.4A and B). TP and TN concentrations were also lower in macrophyte treatments (GLS: $F_{1,29}$ = 91.2; $P < 0.001$ and $F_{1,29}$ = 8.6; P = 0.006), whereas concentrations of ammonium and conductivity did not differ between the treatments (GLS: $F_{1,29}$ = 0.9; P = 0.34, Mann-Whitney U, $P > 0.0125$ for all days, respectively). pH increased from 7.6 (day 0) to more than 10 within 6 days in all the mesocosms and kept high till the end of the experiment (Fig. 4.4C). pH was significantly higher in *M. spicatum* compared to control treatments (GLS: $F_{1,29}$ = 11.7; P = 0.002).

4.2.2 Zooplankton

Total zooplankton biomass and potential grazing rates did not differ between experimental treatments (GLS: $F_{1,29}$ = 0.37; P = 0.54 and $F_{1,29}$ = 0.18; P = 0.68, respectively; Fig. 4.4D and E). Only the biomass of copepods was significantly

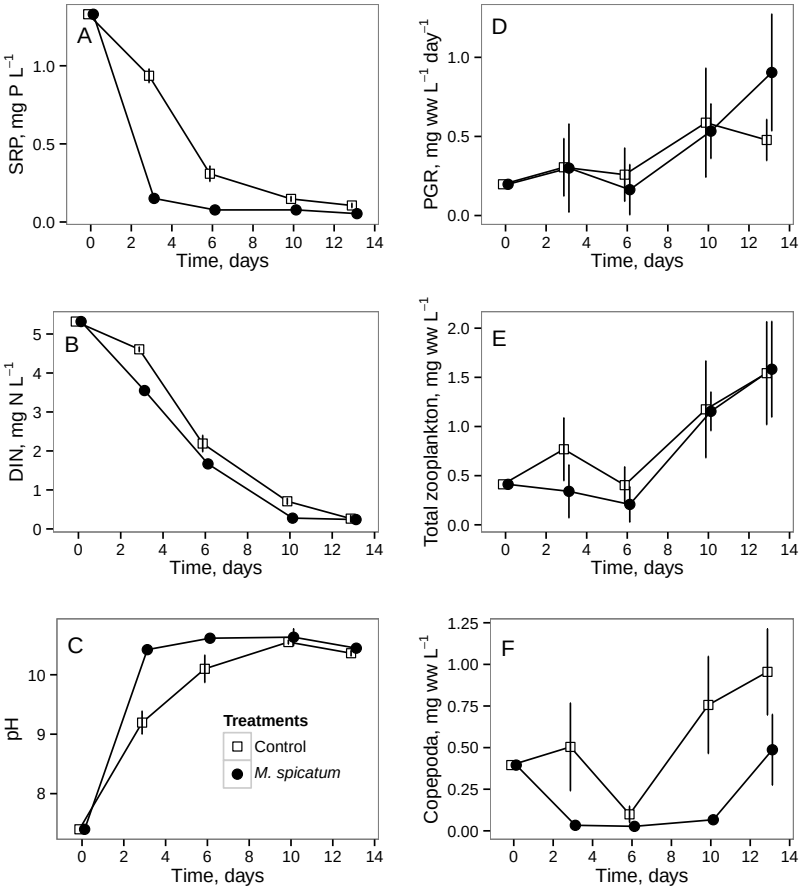


FIGURE 4.4: Changes of soluble reactive phosphorus (SRP) concentrations (A), dissolved inorganic nitrogen (DIN) concentrations (B), pH (C), potential grazing rate (PGR; D), biomass of total zooplankton (E), and copepods (F) in control mesocosms (solid circles) and in mesocosms with *M. spicatum* (open squares) for different days (n = 4, error bars represent ± standard errors).

lower in the presence of *M. spicatum* when all experimental dates were compared (GLS: $F_{1,29} = 9.8$; $P = 0.003$; Fig. 4.4F) whereas biomasses of rotifera, cladocera and ciliates did not differ between the treatments (GLS: $F_{1,29} = 0.007$; $P = 0.93$ (rotifera); Mann-Whitney U, $P > 0.0125$ for all days (cladocera and ciliates).

4.2.3 Phytoplankton community composition

The water taken from the Curonian Lagoon for the mesocosm study was initially dominated by cyanobacteria (27.7 μg Chl *a* L^{-1}) and green algae (12.6 μg Chl *a* L^{-1}). The dominant cyanobacteria species were *Aphanizomenon flos-aquae* Ralfs ex Bornet & Flahault, *Woronichinia compacta* (Lemmerm.) Komárek & Hindák and *Aphanocapsa holsatica* (Lemmerm.) Cronberg & Komárek and the dominant green algae were *Planctonema lauterbornii* Schmidle and *Mucidosphaerium pulchellum* (H. C. Wood) C. Bock, Pröschold & Krienitz. Diatoms and cryptophytes were observed in lower quantities (0.5 and 0.3 μg Chl *a* L^{-1}, respectively).

Total phytoplankton, green algae and cyanobacteria Chl *a* concentrations declined in both *M. spicatum* and control treatments within the first three days of the experiment (Fig. 4.5A, B and C). Concentrations began to increase again soon thereafter, but with rates differing between phytoplankton groups and treatments. The total phytoplankton Chl *a* peaked on day 6 and declined thereafter in the controls, while in *M. spicatum* treatments the total phytoplankton Chl *a* peaked only by the end of the experiment on days 10 and 13. Both total phytoplankton and green algae Chl *a* concentrations were significantly higher in controls compared to *M. spicatum* treatments till day 6 (LME: $F_{1,6} = 10.1$; $P = 0.02$ and $F_{1,6} = 16.2$; $P = 0.007$, respectively), but did not differ when

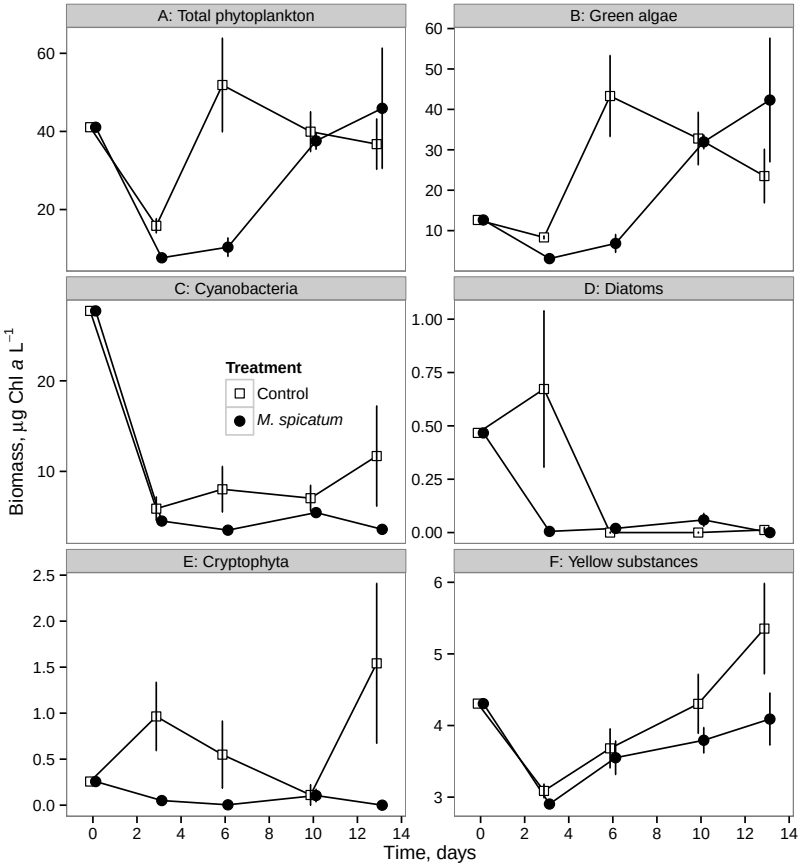


FIGURE 4.5: Changes in Chl *a* concentrations of the main phytoplankton groups i.e. total (A), green algae (B), cyanobacteria (C), diatoms (D), cryptophytes (E) and yellow substances (F) in control mesocosms (open squares) and in mesocosms with *M. spicatum* (solid circles) for different days (n = 4, error bars represent ± standard errors).

all days were included into the analyses (GLS: $F_{1,29} = 2.9$; $P = 0.1$ and $F_{1,29} = 1.02$; $P = 0.32$, respectively).

Cyanobacteria Chl *a* concentrations were consistently lower in macrophyte treatments throughout the experiment. Cyanobacteria biomass was on average 48 % lower in *M. spicatum* treatments than in control treatments (Fig. 4.5C), but the effect was only statistically significant at the $\alpha = 0.1$ level (GLS: $F_{1,29} = 3.2$; $P = 0.083$). Chl *a* concentrations of diatoms and cryptophytes were relatively low throughout the experiment and did not differ between the treatments (Fig. 4.5D and E). The phytoplankton community composition changed profoundly over the course of the experiment. Cyanobacteria were replaced by green algae, and the latter dominated both treatments from day 6 until the end of the experiment.

4.2.4 *Microcystis aeruginosa* genotypes

The initial percentage of the MC-producing *M. aeruginosa* genotypes with respect to the total *M. aeruginosa* population was 28 ± 5 %. At the end of the experiment, this proportion was 24 ± 3 % in control treatments and 35 ± 6 % in macrophyte treatments. The percentage of MC- and non-MC-producing genotypes in macrophyte treatments with respect to control treatments at the end of the experiment was not significantly different (t-test, n = 4; df = 6; $P = 0.14$; Fig. 4.6).

4.2.5 Gross primary production and nitrogenase activity

Gross primary production of phytoplankton ranged from 0.86 to 8.16 mg O_2 L^{-1} d^{-1}, and was significantly lower in mesocosms with *M. spicatum* (GLS: $F_{1,25} = 11.1$; $P = 0.003$). Ethylene production rates (nitrogenase activity) ranged from

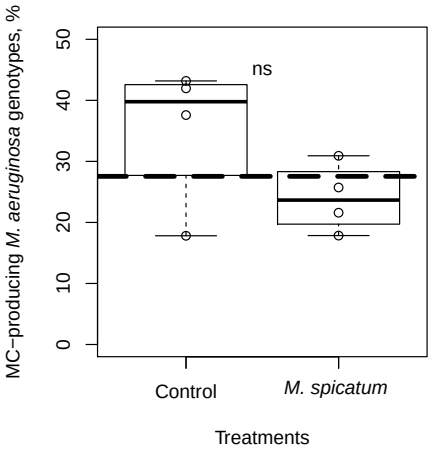


FIGURE 4.6: Percentage of the MC-producing *Microcystis aeruginosa* genotypes with respect to the total *Microcystis aeruginosa* population in control and *Myriophyllum spicatum* treatments at the end of the mesocosm experiment. Horizontal dashed line shows the initial percentage of the MC-producing *M. aeruginosa* genotypes at day 0. Open circles show percentages of the MC-producing *Microcystis aeruginosa* genotypes of each mesocoms at day 13.

0 to 1100 nmol C_2H_4 μg Chl a^{-1} h^{-1} were generally low and highly variable with mean ethylene production rates of 91 $\pm$ 271 SD and 133 $\pm$ 244 SD C_2H_4 μg Chl a^{-1} h^{-1} for macrophyte and control treatments, respectively. Nitrogenase activity did not differ significantly between macrophyte and control treatments (Mann-Whitney U, $P > 0.0125$ for all days).

4.3 Sensitivity of *Microcystis aeruginosa* strains to allelochemicals

4.3.1 Effects of tannic acid on single *Microcystis aeruginosa* strains

In the laboratory experiments with single strains, the growth rates calculated from Chl *a* of *M. aeruginosa* in the control treatments ranged from 0.18 d^{-1} up to 0.63 d^{-1} (Fig. 4.7A), and photosynthetic yields ranged from 0.42 to 0.55 (shown as relative units; Fig. 4.7B). Significantly negative as well as positive effects of TA on *M. aeruginosa* Chl *a* and photosynthetic yield were observed (Fig. 4.8A and B). On average the increasing concentration of TA decreased both Chl *a* concentrations and photosynthetic yields (PPM: slope = -3.11, R^2 = 0.535, $P < 0.001$ and slope = -0.91, $R^2 = 0.405$, $P = 0.002$, respectively; Fig. 4.9A and B).

Growth rates in control treatments were higher for non-MC-producing compared to MC-producing strains (t-test: t = 2.28; $P = 0.048$), whereas no difference in photosynthetic yield was found (t-test: t = 0.15; $P = 0.882$). Both Chl *a* concentrations and photosynthetic yields were significantly diminished in non-MC-producing relative to MC-producing strains in all TA treatments (LM: $F_{1,36} = 21.2$; $P < 0.001$ and $F_{1,36} = 9.2$; $P = 0.004$, respectively, Fig. 4.9A and B). The reduction of Chl *a* was significantly higher for MC-producing PCC 7806 compared to its non-MC-producing $\Delta mcyB$ mutant, whereas reductions of photosynthetic yield did not differ (Table 4.2). When comparing sympatric MC-producing and non-MC-producing strain pairs, reductions of photosynthetic yield and Chl *a* concentrations under TA exposure were higher or equal for non-MC-producing compared to sympatric MC-producing strains in most cases (Table 4.2). The relation-

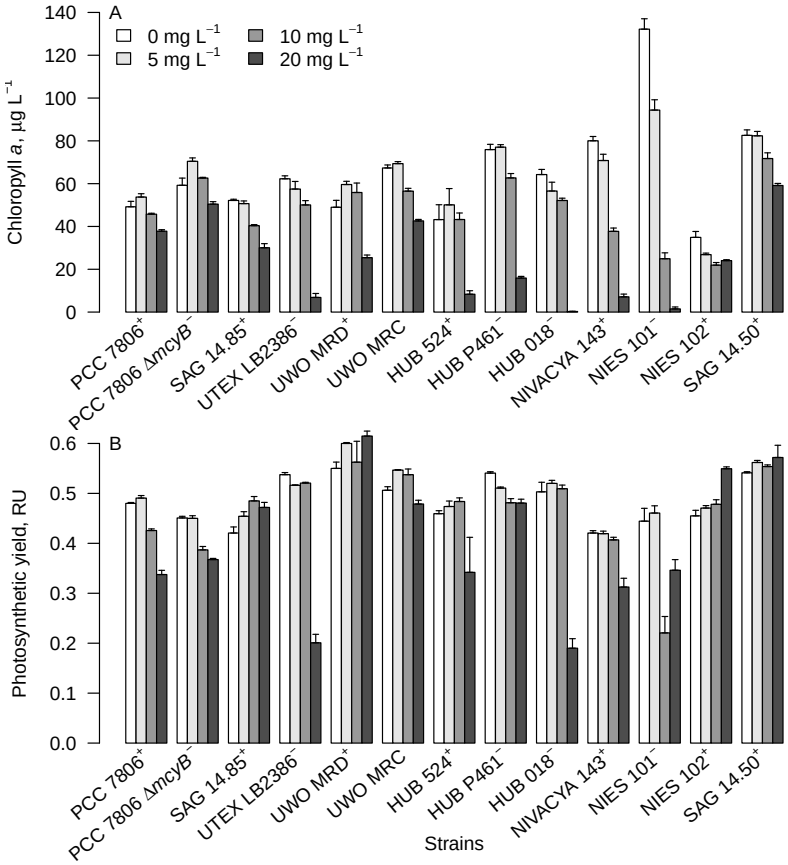


FIGURE 4.7: Mean final concentration of Chl *a* (A) and photosynthetic yield (B) for strains of *Microcystis aeruginosa* at different TA treatments. $^{+}$ and $^{-}$ signs at the ends of strain names indicate MC producing and non-MC-producing strains, respectively. Error bars represent ± standard errors.

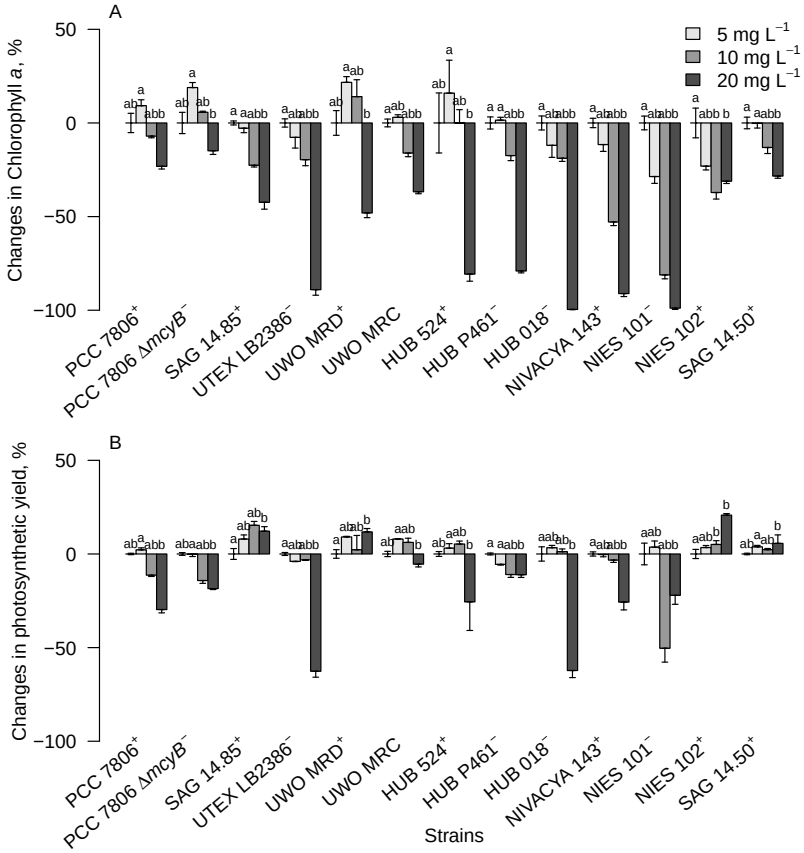


FIGURE 4.8: Changes in Chl *a* (A) and photosynthetic yield (B) in comparison to experimental controls for *Microcystis aeruginosa* strains at different TA treatments. Different letters indicate significant differences between TA treatments. $^{+}$ and $^{-}$ signs at the ends of strain names indicate MC producing and non-MC-producing strains, respectively. Error bars represent ± standard errors. The error bars at the beginnings of grouped bars represent variability of parameters in the experimental controls.

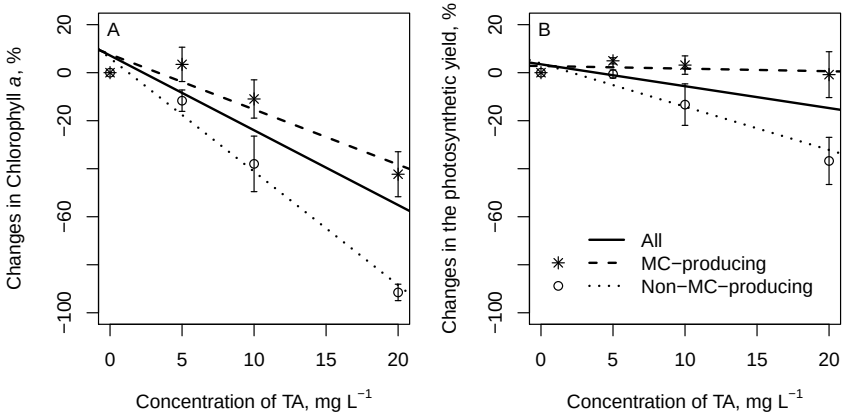


FIGURE 4.9: Relationships between concentrations of TA applied and changes in Chl *a* (A) and photosynthetic yield (B) in comparison to experimental controls for all, MC- and non-MC-producing *Microcystis aeruginosa* strains (error bars represent ± standard errors).

ships between reductions in Chl *a* and photosynthetic yield were positive, i.e. slopes were 0.03, 0.38 and 0.35 for 5, 10 and 20 mg L^{-1} TA treatments, respectively, whereas a significant relationship was only found for the 10 mg L^{-1} TA treatment (PPM: $R^2 = 0.272$, $P = 0.044$).

Half maximal effective TA concentration based on Chl *a* concentrations (EC_{50-C}) varied greatly between *M. aeruginosa* strains (Fig. 4.10) with the NIES 101 strain being the most sensitive and the PCC 7806 Δ*mcyB* being the most resistant (E_{50-C} values 8.2 and 58.4 mg L^{-1} TA, respectively). EC_{50-C} concentrations were significantly higher for MC-producing (mean 12.0 ± 1.1 SE) than non-MC-producing (mean 28.0 ± 3.5 SE) strains (t-test: $t = 3.96$, $P = 0.003$). EC_{50-C} concentrations were not related to the final Chl *a* concentrations of the control treatments of the respective strains (PPM: $r = 0.37$, $P = 0.218$; data not shown). The confidence

TABLE 4.2: Results of linear models for comparing changes of Chl *a* concentrations and photosynthetic yields of sympatric MC-producing and non-MC-producing *Microcystis aeruginosa* strains in single strain tannic acid bioassays.

Strain pair	Chl *a*			Photosynthetic yield		
	df	F	*P*	df	F	*P*
PCC 7806 / PCC 7806 Δ*mcyB*	2, 21	46.7	0.001^{T}	2, 21	0.97	0.335
HUB 524 / HUB P461	2, 20	2.22	0.151	2, 20	9.26	0.006^{N}
SAG 14.85 / UTEX LB2386	2, 21	6.41	0.020^{N}	2, 21	25.6	0.001^{N}

^{T} – MC-producing strain is more sensitive compared to non-MC-producing, ^{N} – non-MC-producing strain is more sensitive compared to MC-producing

intervals of EC_{50-C} values of strains from different continents highly overlapped (means ± confidence intervals for Asian 17.7 ± 27.8, Australian 26.5 ± 2.3, European 19.1 ± 11.1 and N. American 23.6 ± 14.5 strains, respectively). Half maximal effective TA concentration based on photosynthetic yield data (EC_{50-Y}) were not obtained for four MC-producing strains as photosynthetic yield was not on average enhanced within the range of applied TA concentrations. The obtained EC_{50-Y} values did not correlate with the EC_{50-C} values (PPM: r = 0.161, $P = 0.679$).

4.3.2 Effects of allelochemicals on mixed *Microcystis aeruginosa* strains

Similarly to the results of the experiments with single *M. aeruginosa* strain (Section 4.3), the MC-producing strain SAG 14.85 was less inhibited by TA than MC-producing strain

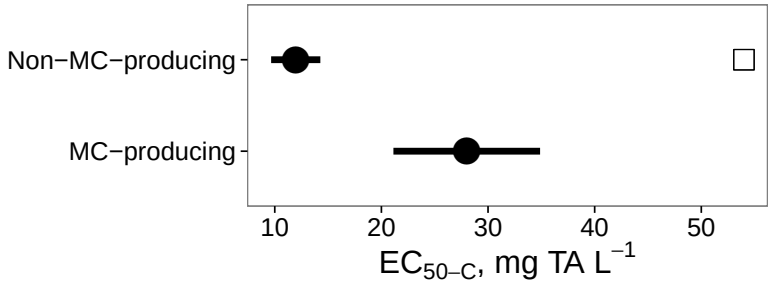


FIGURE 4.10: 95 % confidence intervals of half maximal effective TA concentrations based on Chl *a* (EC_{50-C}) for MC- and non-MC-producing *Microcystis aeruginosa* strains. The open square indicates EC_{50-C} for the PCC 7806 $\Delta mcyB$ mutant.

UTEX LB2386 (t-test: t = -2,86; $P = 0.021$) and MC-producing strain PCC 7806 was more inhibited by TA than *mcyB* gene mutated strain PCC 7806 $\Delta mcyB$ (t.test: t = 4.58; $P = 0.002$). In contrast to the latter, the PCC 7806 was less inhibited than *mcyB* gene mutated strain PCC 7806 $\Delta mcyB$ when co-cultured with *M. spicatum* (t-test: t = -3.58; $P = 0.021$) whereas the no difference in the effect on SAG 14.85 versus UTEX LB2386 was found (t-test: t = -2.61; $P = 0.076$).

Both Chl *a* and photosynthetic yield in control and TA treatments were different in mixed cultures of MC- and non-MC-producing *M. aeruginosa* strains (Fig. 4.11A and B) as compared to Chl *a* concentrations and photosynthetic yields calculated from the data of single strain cultures. The effect of mixture was positive and negative and varied between different strains pairs. Highest effects were for SAG 14.85 / UTEX LB2386 strain pair, that showed 29% and 53% higher Chl *a* concentrations in the mixtures in control and TA treatments, respectively, compared to calculated Chl *a* concentrations based on the data of single strains. The Chl *a* concentra-

tions of the mixed PCC 7806 and PCC 7806 Δ*mcyB* strains was enhanced in the control treatment (Fig. 4.11A) whereas the photosynthetic yield was diminished in both control and TA treatments (Fig. 4.11B) as compared to calculated Chl *a* and photosynthetic yield based on the data of single strains.

Chl *a* concentration in control treatment was different in mixed cultures of MC- and non-MC-producing *M. aeruginosa* strains as compared to their calculated Chl *a* concentrations based on the data of single strain cultures for SAG 14.85 and UTEX LB2386 strain pair (Fig. 4.12A). Whereas photosynthetic yield in control treatment was different in mixed cultures of MC- and non-MC-producing *M. aeruginosa* strains as compared to calculated photosynthetic yield based on the data of single strain cultures for SAG 14.85 and UTEX LB2386 strain pair (Fig. 4.12B). Changes of Chl *a* concentrations as compared to their experimental controls were different in mixed cultures of PCC 7806 / PCC 7806 Δ*mcyB* strain as compared to their calculated changes of Chl *a* concentrations (Fig. 4.12C).

4.3.3 Total phenolic content

Culture filtrates in bioassay test with 5, 10 and 20 mg TA L^{-1} on average remained 0.9 ± 0.1, 1.1 ± 0.2 and 1.6 ± 0.2 mg TA L^{-1}, respectively. The culture filtrates of the tests with mixed *M. aeruginosa* strains on average remained 2.2 ± 0.2 and 4.2 ± 0.5 mg TA L^{-1} in 7 and 14 mg L^{-1}, respectively, daily additions of TA treatment. Presence of different strains did not influence the TPC in all TA treatments (LM: $F_{2,25}$ = 0.54; P = 0.586). Culture filtrates of SAG 14.85 / UTEX LB2386 and PCC 7806 / PCC 7806 Δ*mcyB* cultures in *M. spicatum* treatments on average contained 1.0 ± 0.1 and 0.8 ± 0.1 mg TA L^{-1}, respectively. The presence of *M. spicatum*

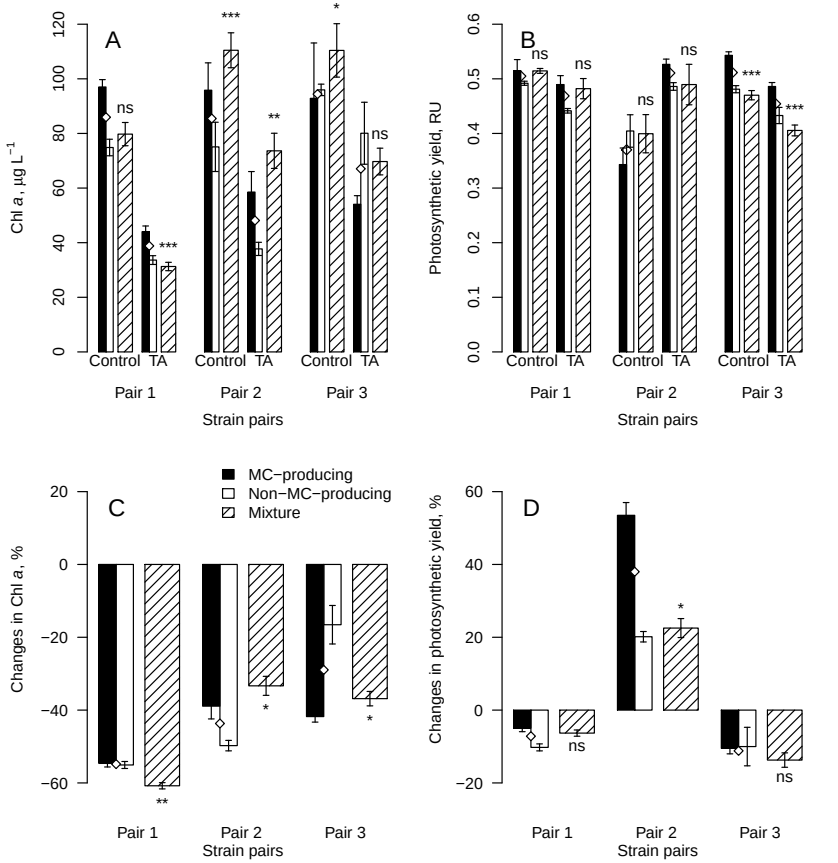


FIGURE 4.11: Chl *a* concentrations (A) and photosynthetic yields (B) and their changes as compared to their experimental controls (C and D, respectively) for MC-producing (black bars) and non-MC-producing (white bars) *Microcystis aeruginosa* single strains and their mixtures (striped bars) in control and TA treatments. Calculated Chl *a* concentrations and photosynthetic yields based on the data of single strains and changes of Chl *a* concentrations and photosynthetic yields as compared to their experimental controls are shown as open squares. Pair 1, 2 and 3 represent HUB 524 and HUB P461, SAG 14.85 and UTEX LB2386, PCC 7806 and PCC 7806 Δ*mcyB* strain pairs, respectively. Error bars represent ± standard errors. Levels of significance: ns - $P > 0.025$, * - $P < 0.025$, ** - $P < 0.005$, *** - $P < 0.0005$.

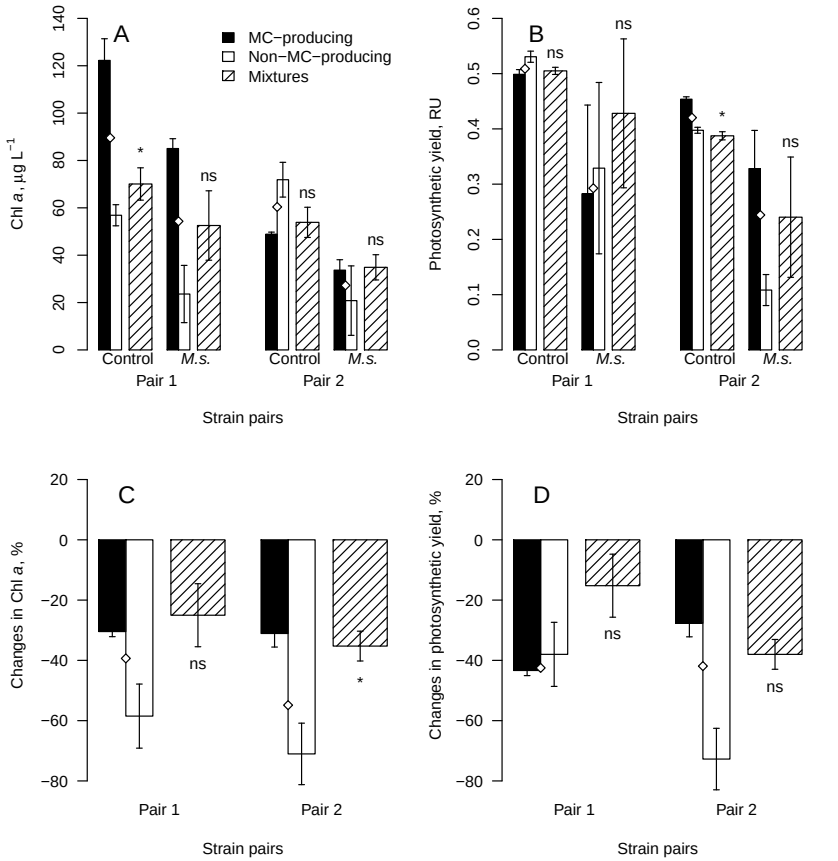


FIGURE 4.12: Chl *a* concentrations (A) and photosynthetic yields (B) and their changes as compared to their experimental controls (C and D, respectively) for MC-producing (black bars) and non-MC-producing (white bars) *Microcystis aeruginosa* single strains and their mixtures (striped bars) in control and *Myriophyllum spicatum* treatments. Calculated Chl *a* concentrations and photosynthetic yields based on the data of single strains and changes of Chl *a* concentrations and photosynthetic yields as compared to their experimental controls are shown as open squares. Pair 1 and 2 represent SAG 14.85 and UTEX LB2386, PCC 7806 and PCC 7806 Δ*mcyB* strain pairs, respectively. Error bars represent ± standard errors. Levels of significance: ns - $P > 0.025$, * - $P < 0.025$, ** - $P < 0.005$, *** - $P < 0.0005$.

in tests with dialysis bags yielded 6.5 ± 3 mg TA per 1 dw g of *M. spicatum* i.e. 0.8 ± 0.3 mg TA per 1 fw g of *M. spicatum*.

5

Discussion

The aim of the study was to evaluate the effect of the allelopathically active macrophyte *M. spicatum* on *M. aeruginosa* and its toxicity. First, we discuss potential factors influencing *M. aeruginosa* toxicity *in situ*. Second, we estimate allelopathic effects of *M. spicatum* on cyanobacteria with particular interest on *M. aeruginosa* under *in situ* like conditions (section 5.2). Third, we estimate the effects of allelochemicals of *M. spicatum* on *M. aeruginosa* strains and the potential role of MCs against inhibitive effects of allelochemicals of *M. spicatum*. Fours, we discuss potential factors influencing inhibitory effects of macrophytes and their allelochemicals (section 5.4). Finally, we provide gaps of knowledge and future prospectives (section 5.5).

5.1 Factors influencing *Microcystis aeruginosa* toxicity *in situ*

Water in the site with vegetative surroundings (site no. 8) as compared to the deep open water sampling sites (no. 1-7) had the lowest percentage of MC-producing *M. aeruginosa* genotypes in the Villerest reservoir. This result indicates a higher suppression of MC-producing than non-MC-producing *M. aeruginosa* genotypes by vegetative conditions. However, besides the effect of the vegetative environment, many other environmental elements may have added many other interfering factors e.g. increased temperature, altered light

and turbulence conditions and nutient concentrations, effects of fishes, insects, birds. However, we lacked determination of most of those parameters. Nevertheless, this finding provides indication that MC-producing as compared to non-MC-producing *M. aeruginosa* genotypes may be more diminished by the close presence of terrestrial or aquatic vegetation, of which macrophytes and their allelochemicals may also have a role.

The found positive relationship between the percentage of MC-producing *M. aeruginosa* genotypes and solar radiation suggests a higher resistance of MC-producing as compared to non-MC-producing *M. aeruginosa* genotypes to high solar radiation. This result is in line with the study of (Ding et al., 2013) who found that MC-producing as compared to non-MC-producing *M. aeruginosa* strains are more affected by UVB radiation. These results may be explained by the ability of MCs to protect cells under oxidative environment (Zilliges et al., 2011; Dziallas and Grossart, 2011; Leunert et al., 2014), which is caused by increased light intensities. We discuss this aspect into more details in the subsection 5.3.1). Overall these findings indicate that increased solar radiation may influence *M. aeruginosa* genotype ratio of cyanobacterial blooms in favour of MC producers.

Negative relationship between the percentage of MC-producing *M. aeruginosa* genotypes and water temperatures were found in Villerest reservoir. Although, a similar seasonal pattern i.e. the decline of the percentage of MC-producing *M. aeruginosa* genotypes in the middle of summer was observed in an upstream Grangent reservoir (Briand et al., 2009), many other studies, however, report positive relationship between water temperature and the percentage of MC-producing *M. aeruginosa* genotypes (Davis et al., 2009; Joung et al., 2011;

Li et al., 2014). One possible explanation for these differences could be due reservoir specific characteristics that may have influenced the MC-producing *M. aeruginosa* genotypes.

5.2 Effects of *Myriophyllum spicatum* under *in situ* like conditions

The mesocosm study provided evidences that *M. spicatum* affects a number parameters in aquatic ecosystem. In particular, lower cyanobacteria concentrations, altered phytoplankton and zooplankton community compositions were observed in mesocosms containing *M. spicatum*. There are a number of potential mechanisms that may explain the observed results.

5.2.1 Effects on nutrients

Macrophytes and their epiphytes compete with phytoplankton for nutrients. *M. spicatum* can obtain most of its nutrients from sediments (Barko and James, 1998), but may also absorb as well as adsorb nutrients directly by its shoots (Best and Mantai, 1978; Zhang et al., 2011). The availability of nutrients for phytoplankton growth within macrophyte stands may also be diminished via indirect effects like uptake by epiphyton (Howard-Williams and Allanson, 1981), increased nitrification (Körner, 1999), denitrification (Weisner et al., 1994) or uptake by bacterioplankton (Huss and Wehr, 2004) due to increased dissolved organic carbon leached by macrophytes (Demarty and Prairie, 2009). As a result, SRP and DIN concentrations were significantly lower in mesocosms containing macrophytes. Furthermore, higher pH values in macrophyte treatments may indicate a depletion of dissolved CO_2 and lowered bicarbonate availability resulting in inorganic carbon limitation (e.g., Talling, 1976) and changing phytoplankton composition (Ibelings and Maberly, 1998). A potential com-

petition for nutrients and inorganic carbon between macrophytes and phytoplankton can not be excluded in this mesocosm study. However, phytoplankton growth was still most probably not nutrient-limited until day 10 as the detected SRP concentrations (mean in *M. spicatum* treatments: 0.08 mg P L^{-1}) were shown to be non-limiting for cyanobacteria and green algae growth in the Curonian Lagoon (Pilkaitytė and Razinkovas, 2007) and NO_x concentrations (mean in *M. spicatum* treatments: 0.29 mg N L^{-1}) were still a magnitude higher compared to the initial NO_x concentration in the water taken from the Curonian Lagoon.

5.2.2 Effects of the physical presence of macrophytes

The physical presence of submerged macrophytes may have reduced mixing of the water column (Barko and James, 1998) and may have influenced sinking losses and light climate for phytoplankton (Søndergaard and Moss, 1998). Declerck et al. (2007) detected a strong suppression of phytoplankton production by macrophytes in a mesocosm study at high nutrient levels that could not be explained by the mere structure of the plants as shown by using artificial plants. Similar results were obtained in a laboratory study showing strong inhibitory effects of *Chara globularis* Thuill., *Elodea canadensis* Rich. and *M. spicatum* on a green alga that were not detected when using plastic plants (Lürling et al., 2006). As cyanobacteria contain accessory pigments permitting net growth at a low irradiation (Scheffer et al., 1997) and buoyancy, allowing certain taxa to bloom at the water surface (Reynolds et al., 1987), increased sedimentation and potentially lower light availability in macrophyte treatments should have not explain the suppression of cyanobacteria. Apart from macrophyte shading

effects, phytoplankton self-shading could have occurred and may explain the decrease of total phytoplankton and green algae biomass in the control mesocosms after day 6.

5.2.3 Provision of a spatial refuge against predators

Macrophytes are inhabited by specific communities of plant-associated invertebrates (e.g., Gregg and Rose, 1985). The provision of a spatial refuge against predators for zooplankton (e.g., Timms and Moss, 1984) should not have played a role in the mesocosm experiment due to the lack of fish. The excretion of macrophyte chemicals that suppress zooplankton growth (Burks et al., 2000) may be a potential explanation for the lower biomass of copepods in the macrophyte mesocosms. In general, our results indicate that grazing by zooplankton did not explain the observed differences in phytoplankton communities between treatments. Similar conclusions were drawn in studies of Blindow et al. (2000) and Muylaert et al. (2006) showing that zooplankton grazing was not important for maintaining clear water conditions during the summertime in shallow vegetated temperate lakes.

5.2.4 Allelochemicals mediated interaction

The excretion of allelopathic substances by *M. spicatum*, could have also been involved in the observed effects despite lack of direct proof. This would have required measurements of concentrations of allelochemicals in the water and/or additional control treatments with artificial plants and additions of known allelochemicals, however, these were not feasible within the confines of the mesocosm study. Nevertheless, based on the determination of total phenolic content in dialysis bag experiments (subsection 4.3.3), theoretically the concentrations of total phenolics should have ranged from 2.5

to 3.4 mg L^{-1}. The involvement of allelopathic effect would support earlier findings of a high susceptibility of cyanobacteria towards allelochemicals excreted by submerged macrophytes (Hilt et al., 2006; Hilt and Gross, 2008) and explain lower cyanobacteria concentrations in the mesocosms containing *M. spicatum*. Jasser (1995) also measured a decline in biomass and percentage contribution of all cyanobacteria accompanied by an increase in biomass and percentage contribution of green algae in a natural lake water community in coexistence with the allelopathically-active macrophyte *Ceratophyllum demersum*. Our observed pattern of a slower reoccurrence of cyanobacteria in mesocosms containing *M. spicatum* after an initial decline in biomass in all treatments during the first three days (due to changes in growth conditions in the mesocosms compared to their natural environment) suggests an extension of the lag phase. This mechanism has been described by Mulderij et al. (2005) for the effects of allelochemicals excreted by *Stratiotes aloides* on a green alga and a cyanobacterium.

5.2.5 Effects of the presence of *Myriophyllum spicatum* on nitrogenase activities of phytoplankton

Although the initial phytoplankton community in mesocosms was dominated by the nitrogen-fixing cyanobacterium *A. flos-aquae*, the additionally measured nitrogenase activities were low, highly variable and not significantly different between treatments. Blooms of N-fixing species of cyanobacteria developing in environments with high levels of DIN with low levels of nitrogenase have been reported by Brownlee and Murphy (1983). Whether or not the nitrogenase activity could be potentially effected by macrophytes and their allelochemicals thus remains to be shown.

Taken together the latter findings, we conclude that the mesocosm study showed that *M. spicatum* reduces the biomass of cyanobacteria, influences phytoplankton community structure and abundance of certain zooplankton taxa at field like conditions and despite possible interference by resource limitation there were indications for a significant impact of *M. spicatum* excreted allelochemicals. Thus, we argue that more mesocosm experiments with complex natural phytoplankton communities are needed to unravel the ecological relevance of macrophyte allelopathy.

5.3 Effects of allelochemicals on *Microcystis aeruginosa* strains

The laboratory experiments with single *M. aeruginosa* strains treated with TA provided evidence that MC-producing strains of *M. aeruginosa* are less sensitive to polyphenolic allelochemicals than non-MC-producing strains, independently of their geographic origin. In contrast, the *mcyB* mutated, MC-deficient strain showed a better fitness to allelopathic stress compared to its wild-type, suggesting that MCs were not responsible for the lower sensitivities of MC-producing *M. aeruginosa* strains.

5.3.1 The role of microcystin production on *Microcystis aeruginosa* sensitivity to allelochemicals

Based on findings of previous studies MCs were expected to offer a trade-off between increased protection against oxygen radicals in the presence of allelochemicals (Zilliges et al., 2011; Dziallas and Grossart, 2011) and higher energetic costs related to MC synthesis (Kardinaal et al., 2007; Briand et al., 2012). The latter was confirmed in our study, as non-MC-producing strains grew faster in experimental controls (Fig. 4.7).

The observed general lower sensitivity of MC- *versus* non-MC-producing strains to allelochemicals suggested that the production of MCs in *M. aeruginosa* may contribute to defensive mechanisms against allelopathic stress which outweigh the energetic costs associated with MC production. On the one hand, this would be supported by the findings of several studies. Shao et al. (2009) showed that allelochemicals could up-regulate the expression of the *mcyB* gene, which is involved in the synthesis of MCs. Increased extracellular MC-LR concentrations were found under treatments with polyphenolic allelochemicals (Dziga et al., 2007). Zilliges et al. (2011) and Meissner et al. (2013) provided mechanistic insights into the intracellular protection of *M. aeruginosa* cells, whereby MCs stabilize proteins under oxidative stress, which can also be caused by allelochemicals (e.g., Nakai et al., 2001). The extracellular formation of H_2O_2 and subsequent oxidative stress may also be induced when allelochemicals are exposed to high light environments (Cooper and Zika, 1983; Leunert et al., 2014).

The expected defensive role of MCs and the trade-off of MCs production related costs were contradicted by our experiments with the MC-deficient mutant. The $\Delta mcyB$ mutant and its wild-type are nearly identical, except for MCs production. Thereby additional factors (genetic backgrounds, other metabolites etc.) that may provide protection against allelochemicals should have been eliminated, although the role of MC precursors cannot be fully neglected (Tillett et al., 2000). A number of arguments against a protective role of MCs to allelochemicals seem possible.

First, oxidative stress may be induced due to the formation of reactive oxygen species in the non-stress related processes of photosynthesis and metabolism, whereas the effect of

polyphenolic allelochemicals on *M. aeruginosa* is more complex and includes several modes of action such as the inhibition of alkaline phosphatase (Gross et al., 1996), esterase (Eigemann et al., 2013a) and photosystem II (Leu et al., 2002; Körner and Nicklisch, 2002). Thus the effect of allelochemicals cannot be restricted to oxidative stress induction *per se*.

Second, phylogenesis of photosynthetic cyanobacteria dates back at least 2.5 billion years ago (Kazmierczak and Altermann, 2002; Olson and Blankenship, 2004) whereas the oldest aquatic angiosperms evolved only 124.6 million years ago (Sun et al., 2002). Although the ancient evolution of MCs in cyanobacteria (Rantala et al., 2004) supports the defensive role of MCs against the oxidative environment that was present in the early years of cyanobacterial evolution, it is unlikely that the defensive role of MCs against allelochemicals from aquatic plants is an evolutionary response.

Third, MCs unrelated differences in genetic backgrounds (rRNA gene internal transcribed spacer sequences; Otsuka et al., 1999; Janse et al., 2004; Yang et al., 2013), proteomes (proteins that participate in carbon-nitrogen metabolism and redox balance maintenance; Otsuka et al., 1999; Tonietto et al., 2012) and profiles of secondary metabolites (Fastner et al., 2001; Welker and Von Döhren, 2006; Agha and Quesada, 2014) between MC- and non-MC-producing strains may also serve as underlying reasons for lower sensitivities to allelochemicals of MC- *versus* non-MC-producing strains.

Altogether, the latter findings indicate that although MC-producing strains of *M. aeruginosa* are less sensitive to polyphenolic allelochemicals than non-MC-producing strains, the resistance of *M. aeruginosa* against polyphenolic allelochemicals is not related to MCs. The latter suggests that other yet unknown protective mechanisms are present in MC-producing

M. aeruginosa strains.

5.3.2 Potential defensive mechanisms against polyphenolic allelochemicals in *Microcystis aeruginosa*

One speculative scenario is that MC-producing *M. aeruginosa* constitute similar groups of strains (or ecotypes) that rely on metabolites (MCs and most possibly many others) that protect against certain stressors (e.g. oxidative, light stress) in diverse ways. Therefore, this metabolic-rich strategy might collaterally provide MC-producing strains a greater protection against allelopathic stress, without direct mediation by MCs. One can argue that MC-producing ecotypes are "stress specialists" as, for instance, they tend to dominate at the beginning of algae blooms (e.g., Kardinaal et al., 2007) when exposed to high light stress. The found positive correlation between the percentage of MC-producing genotypes and solar radiation *in situ* in Villerest lake (Fig. 4.3) also supports the argument that MC-producing ecotypes are "stress (i.e. high light) specialists". It is therefore reasonable, that MC-producing ecotypes present other metabolites that provide higher protection also against allelopathic stress, whereas non-MC-producing strains arguably display a different set of metabolites, that provide lower protection. For instance, aeruginosins which occasionally accompany MCs in *M. aeruginosa* (Fastner et al., 2001; Humbert et al., 2013), or other secondary metabolites (Welker and Von Döhren, 2006, and references therein) play an important role in establishing defensive mechanisms against allelochemicals in MC-producing strains. A total of 13 gene clusters predicted to be involved in the biosynthesis of secondary metabolites have been identified among the *M. aeruginosa* genomes, of which six gene clusters encode enzymes for the biosynthesis of yet

unidentified metabolites (Humbert et al., 2013). The interrelation between MCs and other secondary metabolites has also been identified from transcriptomes of *M. aeruginosa*, suggesting hierarchical network between secondary metabolites (Makower et al., 2015). Meissner et al. (2014) showed that oxidative light stress increased the synthesis of general stress markers such as trehalose and sucrose in the Δ*mcyB* mutant. We may further hypothesize that the stress markers of allelopathic stress may induce other protective mechanisms such as the synthesis of antioxidants, enhanced protein replacement systems such as *de novo* protein synthesis, and photoprotective mechanisms (reviewed in Latifi et al., 2009), and that these may be more pronounced in MC-producing compared to non-MC-producing ecotypes. A MC-producing *M. aeruginosa* strain exposed to the allelochemical pyrogallol showed increased activities of superoxide dismutase and catalase (Shao et al., 2009). It also showed a greater increase in the expression of genes related to *de novo* fatty acid and photosynthesis-related protein synthesis than the increase in the expression of the Δ*mcyB* gene (Shao et al., 2009). However, the protective mechanisms of non-MC-producing strains against allelochemicals have not yet been studied. Taken together the latter findings, the presence of MCs *per se* unrelated protective mechanisms against polyphenolic allelochemicals in MC-producing ecotypes seems to have a reasonable theoretical background that needs to be further explored.

5.3.3 The role of microcystins against allelochemicals of *Myriophyllum spicatum*

However, in contrast to the findings of TA bioassays on Δ*mcyB* mutant and its wild type (Fig. 4.10), the Δ*mcyB* mutant was less inhibited by allelochemicals of *M. spicatum*

compared the wild type strain as shown in the experiment with dialysis bags (Fig. 4.12). This suggests that MCs may provide protection against the overall array of allelochemicals released from *M. spicatum*, but not against polyphenolic allelochemicals in particular. This is indirectly supported by the findings of Wu et al. (2009) who showed that pure extracts of macrophytes inhibited non-MC-producing strain more than the same extracts with removed phenolic compounds, whereas these extracts showed equal inhibition on MC-producing *M. aeruginosa* strain. Further studies are needed to unravel roles of MCs as protective compounds against different allelochemicals.

5.3.4 Effect of geographic origin on strain sensitivity

The absence of any effect of geographic origin on strain sensitivity may reflect the cosmopolitan distribution and intercontinental dispersal of *M. aeruginosa*. No or marginal biogeographical genetic differentiations between continents, as well as a high continental variability, were also reported for strains of *M. aeruginosa* by Van Gremberghe et al. (2011) and Humbert et al. (2013). Biogeographically distinct sensitivities of *M. aeruginosa* to allelochemicals could have evolved as a result of local adaptations to allelopathically-active macrophytes. However, such differences were not observed in a previous study comparing the sensitivities of 23 green algae strains from lakes with and without allelopathically-active *Myriophyllum* (Eigemann et al., 2013b). However, most *Myriophyllum* species are distributed globally. Furthermore, the tested *M. aeruginosa* strains underwent a long (20+ years) in-culture adaptive evolution and may reveal other strain related biases (Lakeman et al., 2009).

5.3.5 Applied perspective

From the practical point of view, the lower sensitivities of MC-producing strains indicate that allelopathically-active macrophytes may not show the often suggested inhibitive effect on harmful cyanobacteria blooms (e.g., Shao et al., 2013). Instead, an increased toxicity, which is significantly determined by the ratio of MC- to non-MC-producing strains (Zurawell et al., 2005; Kurmayer et al., 2003), may follow artificial treatments with allelochemicals. Our study implies that macrophyte-derived allelochemicals are one of the biotic factors that can significantly affect the ratio between MC- and non-MC producing *M. aeruginosa* strains and deserve further attention.

The high resistance of the $\Delta mcyB$ mutant (EC_{50-C} = 58 mg L^{-1} TA) towards allelochemicals indicates its potential invasiveness in macrophyte-dominated shallow lakes. The $\Delta mcyB$ mutant was also shown to be an equal or higher competitor compared to its wild-type for low light and nutrients (Briand et al., 2012).

In conclusion, our results provided evidences that polyphenolic allelochemicals suppress MC-producing less than non-MC-producing strains of *M. aeruginosa* and this differential inhibition is not related to the production of MCs *per se*. The presence of MCs *per se* unrelated protective mechanisms against polyphenolic allelochemicals in MC-producing ecotypes seems to have a reasonable theoretical background that needs to be further explored.

5.4 Factors affecting the impact of macrophytes and allelochemicals

5.4.1 Coexistence of target organisms

The results with mixed MC- and non-MC-producing strains showed different allelopathic effects on single and mixed MC- and non-MC-producing *M. aeruginosa* strains. This indicates that the coexistence of MC- and non-MC-producing *M. aeruginosa* strains influences effect of allelochemicals of *M. spicatum* on *M. aeruginosa*.

In order to evaluate the effect of allelochemicals on co-cultured MC- and non-MC-producing *M. aeruginosa* strains, first the interactions in controls are discussed. Our results are in line with few other studies that also observed affected MC- and non-MC-producing strain growth in their mixture (Schatz et al., 2005; Kardinaal et al., 2007; Renaud et al., 2011). Van Gremberghe et al. (2011) further provided evidences that the effect of co-existence is strain specific and may affect both MC-producing and non-MC-producing strains.

Potential competition for light, nutrients and space could have caused the effect of strain mixture, but, as nutrient replete and high light conditions were used and high *M. aeruginosa* concentrations were not reached in our experimental design, the competition for resources between *M. aeruginosa* strains in their mixtures should be excluded.

Chemical interactions between MC- and non-MC-producing strains is another potential reason. For instance, Sedmak and Kosi (1998) provided evidences that non-MC-producing strain was stimulated to the addition of MC-RR. However, Schatz et al. (2005) showed that MC-producing strains inhibited non-MC-producing strains, but could not prove that the effect was caused by MCs and thus proposed an involve-

ment of other released chemicals. Chemical interactions via released substances may include other secondary metabolites and toxins (Welker and Von Döhren, 2006), allelochemicals (Legrand et al., 2003; Gross, 2003a), phytohormones (Dabas et al., 2014), quorum sensing substances (Zhai et al., 2012) present in *M. aeruginosa*. In contradiction, Briand et al. (2012) also found differences in growth between single PCC 7806 and its $\Delta mcyB$ mutant strains compared to co-culture of these two strains, and could not explain this difference neither by the addition of MCs nor exudates of *M. aeruginosa* strains.

One of the possible mechanisms is that the interaction between *M. aeruginosa* strains is mediated via membrane linked extracellular substances such as exoglycoproteins (Kehr et al., 2006; Kehr and Dittmann, 2015). Thus, physical interaction between *M. aeruginosa* strains might also be important and could explain the previous findings by Sedmak and Kosi (1998); Schatz et al. (2005); Briand et al. (2012). However, molecules that may be important in cell-to-cell interaction are to be identified and their potential function, e.g. as toxins, allelochemicals, infochemicals (Pohnert et al., 2007) and further molecular mechanism in the interaction between strains are to be elucidated.

Our results with mixed *M. aeruginosa* strains demonstrated that mixtures of MC-producing and non-MC-producing strains influenced the allelopathic effect of macrophyte *M. spicatum*. Similarly, Chang et al. (2012) found that interactions between phytoplankton species may alter the effects of allelochemicals by showing that interactions with green algae turned the inhibiting effect of macrophyte-released allelochemicals on *M. aeruginosa* into an enhancement. Although we did not measure MC-producing and non-MC-producing strains inhibition

in their mixtures, we calculated the overall positive or negative effects of mixtures on both strains. This result extends the list of environmental factors that influence the effect of allelochemicals of macrophytes and indicate that interactions between *M. aeruginosa* genotypes needs to be taken into consideration when investigating potential of macrophytes and their allelochemicals to suppress *M. aeruginosa* dominated cyanobacterial blooms.

5.4.2 Complexity of allelochemicals

The field study in Villerest reservoir, the mesocosm study and laboratory experiments provided different indications regarding the effect of macrophytes on *M. aeruginosa* toxicity (Fig. 5.1). There are a number of potential reasons that may explain the observed differential responses.

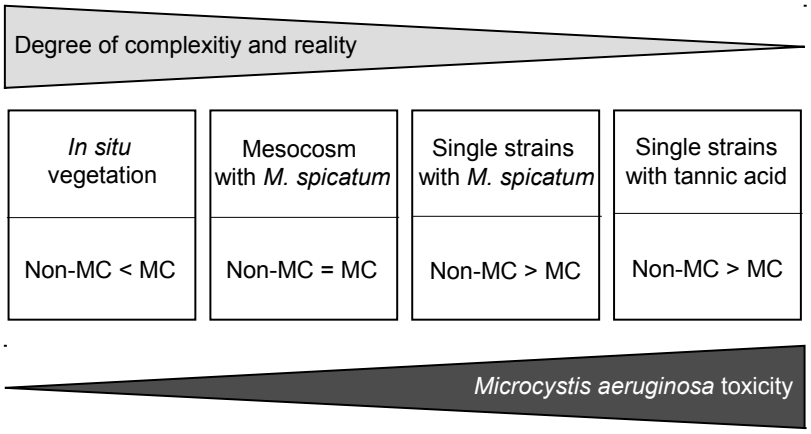


FIGURE 5.1: Inhibition of MC- and non-MC-producing *M. aeruginosa* strains in systems with a decreasing degree of complexity and reality.

Effects of different allelochemicals may explain some our and other reported different effects of macrophyte allelochem-

icals on MC-producing and non-MC-producing *M. aeruginosa* strains. TA bioassays indicated that non-MC-producing *M. aeruginosa* strains are more suppressed by allelochemicals than MC-producing strains. Single allelochemical use may partly explain differences to the previous studies that used macrophyte exudates or extracts. Our results are in line with Liu et al. (2007), who used polyphenol pyrogallol, but are in contrast to Mulderij et al. (2005); Wu et al. (2009), who found that the non-MC-producing strains are less sensitive to macrophyte exudates or extracts than MC-producing *M. aeruginosa* strains. More specifically, Liu et al. (2007) found that the Chl *a* and carotenoid concentrations of non-MC-producing *M. aeruginosa* strain FACHB-942 decreased more quickly than in MC-producing *M. aeruginosa* strain FACHB-469 in treatments with allelochemical pyrogallol, whereas Mulderij et al. (2005) showed that exudates from *S. aloides* to suppress MC-producing more than non-MC-producing strains of *M. aeruginosa*. Similarly, Wu et al. (2009) showed that extracts from macrophytes (*Elodea nuttallii* Planch, *Hydrilla verticillata* Royle and *Vallisneria spiralis* L.) suppressed MC-producing more than non-MC-producing strain of *M. aeruginosa*. Similarly, opposite sensitivities of $\Delta mcyB$ mutant *versus* its wild type strain to allelochemicals of *M. spicatum* via dialysis membrane and single allelochemical TA were found. Furthermore, Wu et al. (2009) reported different sensitivities *of M. aeruginosa* to pure extracts of macrophytes *versus* the same extracts with removed phenolic compounds.

Altogether the latter findings indicate that different allelochemicals may have different effects on MC- and non-MC-producing *M. aeruginosa* and, thus, differently affect *M. aeruginosa* toxicity.

5.4.3 *In situ* environmental complexity

The results of the mesocosm study did not show significantly different effects on MC-producing and non-MC-producing *M. aeruginosa*. This result is in contrast to the findings of TA bioassays (chapter 5.3) and the previous studies of Mulderij et al. (2005); Liu et al. (2007); Wu et al. (2009) that showed different (both lower and higher) sensitivities of MC-producing and non-MC-producing *M. aeruginosa* strains to allelochemicals of macrophytes. These results do not support the findings of Chang et al. (2012), either, who suggested that *M. aeruginosa* production may be promoted by *M. spicatum* when grown in coexistence with a green alga. These results may indicate that the effect of *M. spicatum* on a complex natural plankton community may differ substantially from those observed in laboratory experiments with single strains or mixtures of a few strains cultured at nutrient-replete, constant temperature and light regime.

Regardless to the effect of strain coexistence, other differences in environmental conditions, e.g. light, temperature, chemical parameters of medium, presence of grazing and interacting algae species could have had an effect. For instance, excretion of allelochemicals is light and nutrient dependent (Gross, 2003b), allelochemicals can be degraded/modified by UV-light and microbial activities (Bauer, 2011) and their stability is influenced by oxygen and redox conditions (Appel, 1993). Thus, the allelopathic effect of macrophytes on *M. aeruginosa* toxicity may be significantly affected by the complex manifold factors present at field like conditions.

On the other hand, *M. spicatum* may affect *M. aeruginosa* via many other mechanisms apart of allelopathy (subchapters 5.2.1, 5.2.2 and 5.2.3). Thus, the MC-producing *M. aeruginosa* genotype ratios may be affected via macrophyte

altered environmental parameters, i.e. light, phytoplankton, zooplankton or water chemistry. For instance, the non-MC-producing strain was a better competitor for low light compared to MC-producing strain (Briand et al., 2012). The percentage of MC-producing *M. aeruginosa* genotypes were positively correlated with nitrate concentrations in Japanese lake (Yoshida et al., 2007). The competitive ability of MC-producing strain was stronger than non-MC-producing when co-cultured with *Anabaena* sp. (Li and Li, 2012). do Carmo Bittencourt-Oliveira et al. (2014) showed increased MC production when MC-producing *M. aeruginosa* strain was cultivated in coexistence with green alga *Monoraphidium convolutum* (Corda). Zooplankton grazing mediated genotype-dependent interactions among MC-producing and non-MC-producing *M. aeruginosa* strains were demonstrated by Van Gremberghe et al. (2009). Thus, the latter findings indicate that the overall effects of *M. spicatum* (including effects on nutrients, light, bacteria, phytoplankton and zooplankton communities, and allelopathic affects) are likely to be different compared to sole allelopathic effects.

One speculative scenario is that the effect of macrophytes on MC-producing *versus* non-MC-producing *M. aeruginosa* genotypes is related to the degree of environmental complexity and reality (Fig. 5.1). However, testing this hypothesis is beyond the design of this study and needs further investigation.

5.5 Gaps of knowledge and future perspectives

The number of studies on the effects of macrophytes with a focus on the allelopathic effect on phytoplankton has considerably increased over the last 30 years (Mulderij, 2006; Bauer, 2011; Gross et al., 2012; Eigemann, 2013). This dissertation

contributes to the understanding of processes that affect the occurrence and severity of toxic cyanobacteria blooms. Still many knowledge gaps remain to be filled (Fig. 5.2).

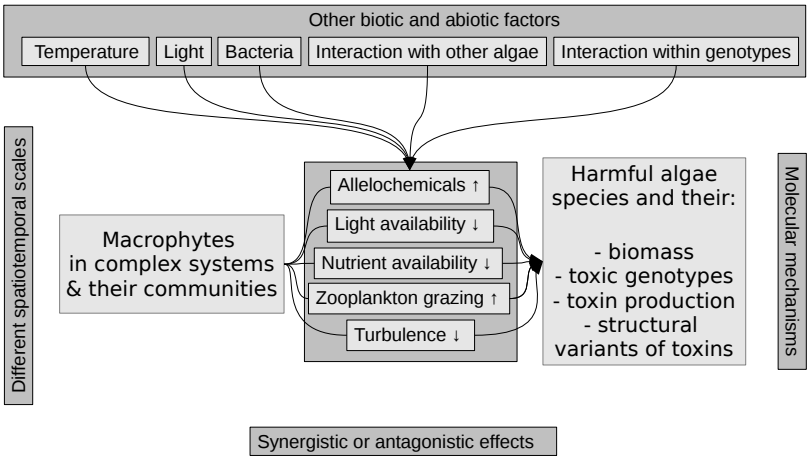


FIGURE 5.2: Mind map of parameters and their interactions to be considered in further investigations.

First, an assessment of the *in situ* influence of macrophytes on cyanobacteria and their toxicity requires more studies under *in situ* or *in situ* like conditions that include the complexity with respect to species diversity, both for macrophytes and cyanobacteria. There is a need to take into account other effects of macrophytes on *M. aeruginosa* such as competition for light and nutrients, provision of refuge for zooplankton against fish, etc. Synergisms (or antagonisms) between these effects are to explored.

Second, many of the macrophyte allelochemicals and their specific modes of action on *M. aeruginosa* remain to be identified. Most of the current knowledge is based on a few model systems mainly focussing on polyphenolic allelochemicals.

Third, MCs production rate and structural variants of MCs produced would provide a more comprehensive view regarding *M. aeruginosa* toxicity and should be considered in further studies.

Finally, the molecular understanding of the resistance mechanisms in phytoplankton against allelochemicals is superficially explored. We provided evidences that differences in the ability of *M. aeruginosa* to produce MCs is one of factors that may influence sensitivity of *M. aeruginosa* to allelochemicals. However, the latter needs further investigation on the molecular level in order to understand mechanisms that contribute in the protectiveness of *M. aeruginosa* and other phytoplankton species against allelochemicals of different macrophytes.

6
Conclusions

1. The presence of *M. spicatum* reduced the biomass of cyanobacteria, influenced phytoplankton community structure and abundance of certain zooplankton taxa under *in situ* like conditions in our mesocosm study. Despite the partial interference by resource limitation there were indications for an impact of *M. spicatum* excreted allelochemicals on phytoplankton. More mesocosm experiments with complex natural phytoplankton communities are needed to unravel the ecological relevance of macrophyte allelopathy.

2. TA bioassays on MC- and non-MC-producing *M. aeruginosa* strains showed lower inhibition of MC-producing (EC_{50-C} = 28 mg TA L^{-1}) than non-MC-producing (EC_{50-C} = 12 mg TA L^{-1}) *M. aeruginosa* strains. This indicates that polyphenolic allelochemicals suppress MC-producing less than non-MC-producing strains of *M. aeruginosa*. This suggests that the use of allelochemicals as artificial treatments to control *M. aeruginosa* blooms, may increase toxicity of *M. aeruginosa* blooms.

3. The lower sensitivity of MC synthesis impaired mutant (PCC 7806 $\Delta mcyB$) than its wild type *M. aeruginosa* strain (PCC 7806) to TA indicates that the lower sensi-

tivity of MC-producing than non-MC-producing *M. aeruginosa* strains to polyphenolic allelochemicals is not related to the production of MCs *per se*, but to other protective mechanisms present in MC-producing *M. aeruginosa* genotypes.

4. The different allelopathic effects of *M. spicatum* on single and mixed MC- and non-MC-producing *M. aeruginosa* strains indicate that the coexistence of MC-producing and non-MC-producing *M. aeruginosa* strains influences effects of allelochemicals of *M. spicatum* on *M. aeruginosa* strains. This suggests that interactions between *M. aeruginosa* genotypes need to be taken into consideration when investigating potential of macrophyte allelochemicals to suppress *M. aeruginosa* blooms.

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A Appendix

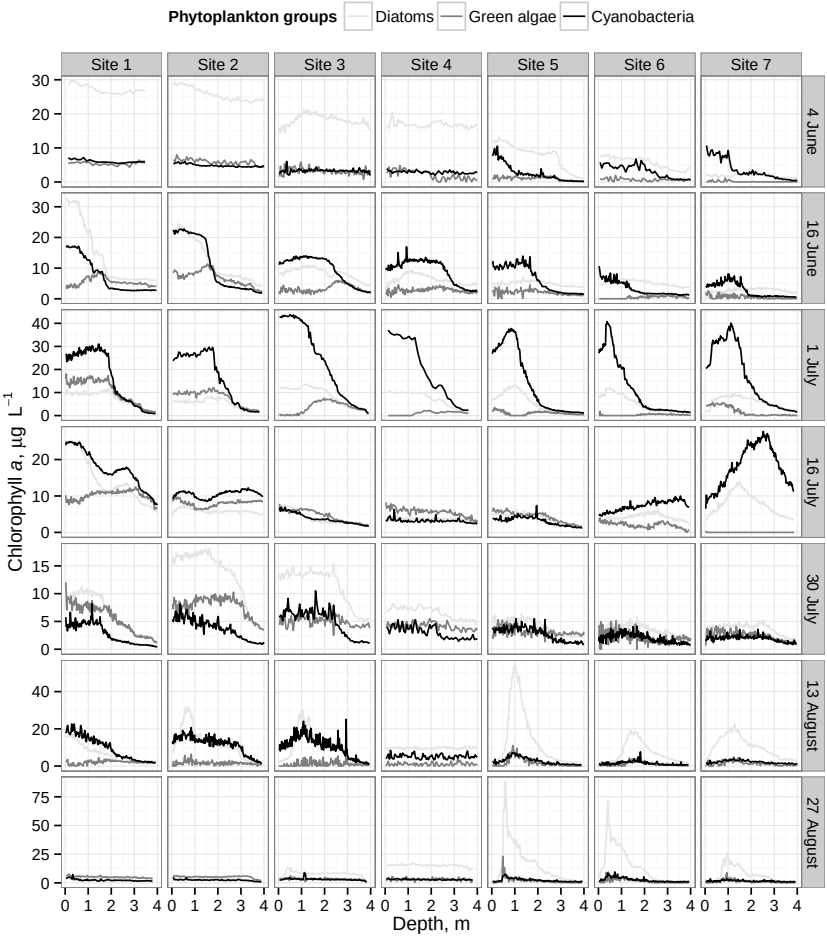

FIGURE A.1: Biomasses of the main phytotoplankton groups in the Villerest reservoir.

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