Diversity, impact and fate of cyanobacterial toxins in freshwater ecosystems

Dissertation submitted for the degree of Doctor of Natural Sciences (Dr. rer. nat.)

Presented by

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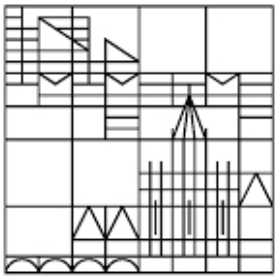

Faculty of Sciences

Department of Biology

Konstanz, 2015

“Nothing great in the world has been accomplished without passion.”

– Georg Wilhelm Friedrich Hegel

I. Publications and Honours

A. Publications

Cerasino, L., **Shams, S**., Boscaini A., Salmaso, N., 2015. Inter-annual variability of the microcystins pool in the oligo-mesotrophic Lake Garda (Italy). ***In Preparation***.

Shams, **S**., Capelli, C., Cerasino, L., Ballot, A., Dietrich, D.R., Sivonen, K., Salmaso, N., 2015. Anatoxin-a producing *Tychonema* (cyanobacteria) in European water bodies. ***Water Research*** 69, 68-79.

Shams, **S**., Cerasino, L., Salmaso, N., Dietrich, D.R., 2014. Experimental models of microcystin accumulation in *Daphnia magna* grazing on *Planktothrix rubescens*: Implications for water management. ***Aquatic Toxicology*** 148, 9-15.

Salmaso, N., Copetti, D., Cerasino, L., **Shams**, **S**., Capelli, C., Boscaini, A., Valsecchi, L., Pozzoni, F., Guzzell, L., 2014.Variability of microcystin cell quota in metapopulations of *Planktothrix rubescens*: causes and implications for water management**.** ***Toxicon*** 90, 82-96.

Jiang, L., Eriksson, J., Lage, S., Jonasson, S., **Shams**, **S**., Mehine, M., Ilag, L. Rasmussen, U., 2014. Diatoms: A Novel Source for the Neurotoxin BMAA in Aquatic Environments. ***PLoS One*** 9(1), e84578.

Salmaso, N., Boscaini, A., **Shams**, **S**., Cerasino, L., 2013. Strict coupling between the development of *Planktothrix rubescens* and microcystin content in two nearby lakes south of the Alps (lakes Garda and Ledro). ***Annales de Limnologie - International Journal of Limnology*** 49 (4), 309-318.

B. Conference talks

July 1-5, 2013, **Münster, Germany**

Shams, S., Cerasino, L., Salmaso, N., Dietrich, D.R. Experimental models of microcystin accumulation in *Daphnia magna* grazing on *Planktothrix rubescens*: potential for microcystin transfer through the food web. SEFS 8 (8th Symposium for European Freshwater Science).

October 29 - November 2, 2012, **Changwon**, **South Korea**

Shams, S., Cerasino, L., Salmaso, N., Dietrich, D.R. Diversity and Seasonality Of cyanotoxins In Lake Garda (Italy): Potential for Hepatotoxic Microcystins Transfer through the Food Web. 15th International Conference on Harmful Algae, Changwon, Republic of Korea.

November 28-30, 2011, **Vienna, Austria**

Shams, S., Cerasino, L., Salmaso, N. Diversity and seasonality of cyanotoxins in Lake Garda. 4th EULAKE meeting and science day, University of Natural Resources and Life Sciences.

August 21-28, 2011, **Trento, Italy**

Shams, S, Milan, M., Ranjan, J., Tolotti, M., Cerasino, L., Boscaini, A., Salmaso, N. Ecological changes of Central European Lakes within the EU Project EULAKES: nuisance cyanobacteria and cyanotoxins, and their impact on aquatic ecosystems and water quality at different temporal scales. 16th Workshop of the International Association of Phytoplankton Taxonomy and Ecology (IAP) held in San Michele all'Adige, Trento, Italy.

C. Posters

October 8-12, 2012, **Konstanz, Germany**

Cerasino, L., **Shams, S**., Salmaso, N., Dietrich, D.R. Toxic potential of cyanobacteria in oligo-mesotrophic lakes: the case of Lake Garda (Italy). 3rd European Large Lakes Symposium University of Konstanz.

June 26-July 1st, 2011, **Girona, Spain**

Shams, S., Cerasino, L., Ranjan, J., Salmaso, N. Cyanotoxins and their impact on water quality in Central Europe lakes. The 7th Symposium of the European Freshwater Science, Girona, Spain.

D. Other conference contributions

February 22-27, 2015, Granada, Spain

Capelli, C., Shams, S., Cerasino, L., Cavalieri, D., Salmaso, N. Toxic cyanobacteria in the deep lakes south of the alps: A molecular assessment on cyanotoxin producing genotypes. Aquatic Sciences Meeting (ASLO).

February 22-27, 2015, Granada, Spain

Cerasino, L., Shams S., Boscaini A., Salmaso, N. Evolution of the toxin diversity in the oligo-mesotrophic environment of Lake Garda (Italy). Aquatic Sciences Meeting (ASLO).

February 22-27, 2015, Granada, Spain

Salmaso, N., Capelli, C., Shams, C., Boscaini, A., Tolotti, M., Cerasino, L. Invasion of *Dolichospermum lemmermannii* (cyanobacteria) to the deep lakes south of the alps: an unusual colonization from north to south? Aquatic Sciences Meeting (ASLO).

September 1-5, 2014, Perugia, Italy

Capelli, C., Shams, S., Cerasino, L., Cavalieri, D., Salmaso, N. Toxic cyanobacteria in lake Garda: a molecular assessment on cyanotoxin producing genotypes. 15th World Lake Conference (WLC15).

September 1-5, 2014, Perugia, Italy

Cerasino, L., Shams, S., Boscaini, A., Salmaso, N. Toxin dynamics inside the resident cyanobacterial community of Lake Garda (Italy). 15th World Lake Conference (WLC15).

September 1-5, 2014, Perugia, Italy

Salmaso, N., Tolotti, M., Milan, M., Shams, S., Capelli, C., Boscaini, A., Pareeth, S., Neteler, M., Cerasino, L. Impact of anthropogenic pressures and long-term climate changes on the trophic state of Lake Garda (Northern Italy): a multidisciplinary assessment. 15th World Lake Conference (WLC15).

September 15-17, 2014, Ferrara, Italy

Salmaso, N., Shams, S., Capelli, C., Boscaini, A., and Cerasino, L. From species to strains: production of toxins in populations of cyanobacteria, and implications for water management. Dipartimento di Scienze della Vita e Biotecnologie (SVeB), Università degli Studi di Ferrara, Italy.

May 30, 2013, Gardone Riviera, Brescia, Italy

Salmaso, N., Shams, S., Cerasino, L. Development of cyanobacteria and production of cyanotoxins in Lake Garda: statistical models and predictive power. Workshop of Quality and sustainable use of water resources in Lake Garda and in other large water bodies in Europe:

experiences within the project EULAKES.

May 30, 2013, Gardone Riviera, Brescia, Italy

Cerasino, L., Shams, S., Salmaso, N. Cyanotoxins in Lake Garda? The critical evaluation of chemodiversity. Workshop of Quality and sustainable use of water resources in Lake Garda and in other large water bodies in Europe: experiences within the project EULAKES.

April 7-10, 2014, Palermo, Italy

Capelli, C., Shams, S., Cerasino, L., Cavalieri, D., Papini, A., Salmaso, N. Toxin-producing cyanobacteria in the large lakes south of the Alps: detection of new producers and molecular identification methods. 10th PhD students meeting in Ecology and Aquatic Systems.

April 7-12, 2013, Vienna, Austria

Cerasino, L., Shams, S., Salmaso, N., Dietrich, D.R. The impact of toxic cyanobacteria on the water quality in the Deep Subalpine Lakes (DSL). European Geosciences Union General Assembly.

June 26-July 1, 2011, Girona, Spain

Ranjan, J., Shams, S., Cerasino, L., Salmaso, N. Ecological changes of central European lakes within the EU project EULAKES: nuisance cyanobacteria and impact on the aquatic ecosystems. The 7th Symposium of the European Freshwater Science.

E. Research awards and scholarship

March-May 2013

COST action award, Short Term Scientific Missions (STSMs) for learning new molecular methods at the laboratory of Prof. Kaarina Sivonen, Microbiology department, Helsinki University, Finland.

October 29-November 2, 2012

Student Travel award for participation in 15th International Conference on Harmful Algae held in Changwon, Republic of Korea.

January 2011-January 2015

PhD scholarship from Fondazione Edmund Mach, San Michele All'adige Trento, Italy.

II. Deusche Zusammenfassung

Massive Blaualgen (Cyanobakterien)-blüten werden weltweit und zunehmend in Oberflächengewässern registriert. Diese Cyanobacterienblüten sind vielfach toxisch aufgrund der Präsenz von hepato- und neurotoxinen. Mehrere Vergiftungsfälle mit Menschen und Nutztieren aber auch Wildtieren sind auf diese toxischen Cyanobakterienblüten zurückzuführen. Entsprechend müssen Oberflächengewässer routinemässig auf das Vorkommen von Cyanobakterienblüten und möglicher Toxien untersucht werden.

Im ersten Teil dieser Doktorarbeit wurden LC-MS Methoden zur Identifizierung und Quantifizierung von Cyanobakterientoxinen im Lago di Garda etabliert und optimiert. Anatoxin-a (ATX) und Microcystine (MCs) konnten im Lago di Garda regelmässig nachgewiesen werden, jedoch zu unterschiedlichen Jahreszeiten. ATX dominierte primär im Frühsommer während MCs typischerweise erst im Spätsommer/Spätherbst in höheren Konzentrationen nachzuweisen war. Obwohl 5 verschiedene MCs nachgewiesen werden konnten, dominierte das MC-RRdm Kongener in allen analysierten Proben.

In einem weiteren Kapitel dieser Doktorarbeit wurden die kinetischen Aspekte des trophischen Transfers von MC in *Planktothrix rubescens* zum Wasserfloh *Daphnia magna* untersucht. Modelierungen der MC Akkumulation in der Wasserfloh *Daphnia magna* untersucht zeigetn, dass die MC Akkumulation prinzipiell von der ursprünglichen MC Konzentration und der Expositionsdauer der Daphnien abhängt. Innerhalb der ersten 24 h der Exposition ist die MC Akkumulation in der Wasserfloh, *Daphnia magna*, nahezu linear, unabhängig davon wie hoch die Dichte der *Planktothrix rubescens* und die MC Konzentration war. Nach 48h Exposition, verlief die MC Akkumulation in der Wasserfloh *Daphnia magna* exponentiell.

Im letzten Teil dieser Doktorarbeit wurde ein polyphasischer Ansatz gewählt um die Oscillatoriales zu identifizieren, welche für die Synthese von ATX im Lago di Garda verantwortlich ist. Dieser Ansatz beinhaltete mikroskopische, molkelar-biologische und analytische Methoden. Aufgrund dieser Analysen konnte *Tychonema bourrellyi* als neuer ATX Produzent und auch die entsprechenden Synthesegene identifiziert werden.

III. Summary

Massive proliferations of cyanobacteria (bloom) are common in aquatic environments worldwide. These blooms are often toxic due to the presence of hepatotoxins or neurotoxins and have become a worldwide environmental problem. Various incidents of animal and human poisonings have been attributed to these toxins. Therefore, monitoring of potentially toxic cyanobacteria and the associated toxins need to be investigated routinely in each water body.

In the first part of present study, LC-MS methods were applied for identifying and quantifying cyanotoxins diversity in Lake Garda. Anatoxin-a (ATX) and microcystins (MC) were always present in this lake with a different seasonal pattern. ATX represented an early summer peak, while MC showed a typical late summer-early autumn peak.The results of toxin analysis also revealed the presence of 5 variants of MC in this lake, but the variants MC-RRdm was always dominant over the others.

In another chapter of this thesis the kinetic aspects of MC transfer from *Planktothrix rubescens* to *Daphnia magna* was investigated. Models of MC accumulation obtained from this part of study differed largely as a result of the duration of exposure and initial MC concentrations used. Within the first 24 h of exposure, MC accumulation in *D. magna* was linear, irrespective of the initial densities of toxic *P. rubescens* and MC concentrations. After 48h of exposure, MC accumulation in *D. magna* showed an exponential pattern.

In the last part of this study, the taxonomic identification of new Oscillatoriales was carried out adopting a polyphasic approach and new potential ATX producers were screened through chemical characterization and identification of specific toxins encoding genes. The analyses were made on several strains isolated from environmental samples collected in Lake Garda.

The results allowed identifying a new ATX producer, *Tychonema bourrellyi*. This is the first discovery of a planktonic genus belonging to the Oscillatoriales able to produce ATX.

IV. Acknowledgement

This doctoral program would be impossible to accomplish alone, therefore, I would like to express my deepest gratitude to those who provided me help in many ways during this study.

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Konstanz University, 2015

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VI. Abbreviations

ATX	Anatoxin
CyanoHABs	Cyanobacterial harmful blooms
DNA	Deoxyribonucleic acid
DIN	Dissolved inorganic nitrogen
DIP	Dissolved inorganic phosphorus
DW	Dry Weight
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
i.p	intraperitoneal
ITS	Internal transcribed spacer region
LC-MS	Liquid chromatography-mass spectrometry
LD_{50}	Lethal dose, 50%
LOQ	Limit of quantitation
MC	Microcystin
mcy	Microcystin synthetase gene cluster
mcyD	Microcystin synthetase gene D
mcyE	Microcystin synthetase gene E
m/z	Ratio of molecular mass and ion charge
NOD	Nodularin
PCR	Polymerase chain reaction
PKS	Polyketide synthase
PPIA	Protein phosphatase inhibition assay
qPCR	Quantitative polymerase chain reaction
sp.	Species
TN	Total nitrogen
TP	Total phosphorus
UHCC	University of Helsinki culture collection
WHO	World Health Organisation

Chapter 1

1. INTRODUCTION

1.1 Cyanobacteria

Cyanobacteria or blue green algae are considered as the oldest organisms on Earth with the fossil dating back to 3.5 billion years ago.They are believed as the earliest form of life and responsible for creating oxygenic atmosphere when the planet was without oxygen and void of life (Schopf, 2000).

Cyanobacteria can thrive in wide variety of habitats both terrestrial and aquatic. They can grow as the dominant phytoplankton in freshwater, brackish and marine ecosystems (Chorus and Bartram, 1999; Mur *et al*., 1999).Moreover, they have also been reported from extreme ecosystems such as deserts (Friedmann and Ocampo-Friedmann, 1984), tropical acidic soils (Lukešová, 2001), Antarctic lakes (Taton *et al*., 2003) and thermal springs (Sompong *et al*., 2005).

Their ability to grow in such a diverse range of habitats can be explained because cyanobacteria have high adaptive capacity. Some of their abilities include: tolerance to a wide range of temperatures; different strategies to optimize light harvesting; buoyancy; ability to fix atmospheric-N; high tolerance to salinity,pH and UV; the capacity to form akinetes as dormant stage under harsh conditions (Carey *et al*., 2012).

The traditional classification system of cyanobacteria was mainly based on morphology and type of division. According to Anagnostidis and Komárek (1999), the polarity of cells, the position of cells in a colony and the structure of a colony were also important markers.Therefore, cyanobacteria were classified in two groups; non-filamentous (Chroococcales, Pleurocapsales) and filamentous (Oscillatoriales, Nostocales and Stigonematales).

Since phenotype and morphology of cyanobacteria can change under different environmental or culture conditions, the morphology analysis alone can lead to misidentification (Lyra *et al*., 2001). The further adoption of different and complementary criteria to classify cyanobacteria allowed to deeply revise the taxonomic classification of cyanobacteria. At present, the use of the classical morphological criteria, coupled with the genetic and autecological characterization of species, strongly increased the accuracy in the identification and classification of cyanobacteria. The frequently used approach for the phylogenetic classification of cyanobacteria has been the analysis of genes encoding the small subunit ribosomal RNA, the 16S rRNA, because culture or growth conditions do not have any effect on the sequence of this gene. Moreover, this gene is universal and conserved and this makes it suitable for many phylogenetic studies (Nübel *et al*., 1997).

1.2 Cyanobacterial harmful blooms (CyanoHABs)

Toxic blooms of cyanobacteria are a growing problem throughout the world and frequent episodes have been reported very frequently from many aquatic ecosystems in all continents (Fig. 1). Most planktonic cyanobacteria contain gas vesicles which enable them to regulate their buoyancy, so during the summer months, in warm, slow moving and nutrient rich water bodies, they can proliferate at surface water and form a scum which is defined “bloom”.

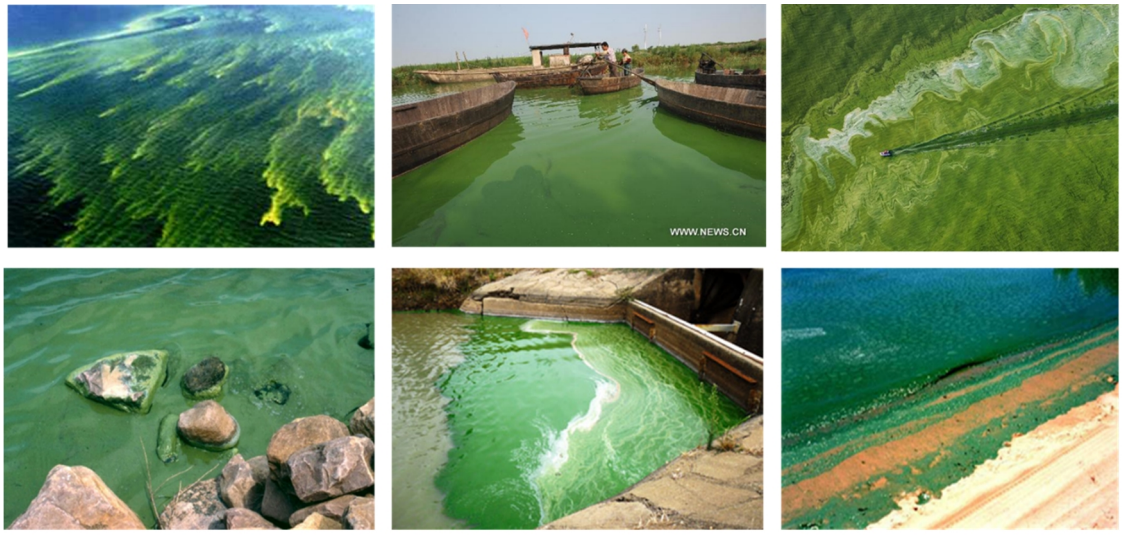


Figure1. Examples of water bodies around the world that have experienced cyanobacterial harmful algal bloom. Up left: Baltic Sea-Gulf of Finland. Up middle: Chaohu Lake in east China. Up right: Lake Erie near Toledo, Ohio, US. Bottom left: Umgeni River, South Africa. Bottom middle: Northern end of Lake Albert, New South Wales, Australia. Bottom right:Matilda Bay, Swan-Canning Estuary, Western Australia (See chapter 7, Section 7.3. Figure captions).

Bloom forming cyanobacteria are divided in 3 categories, I) those capable for N_2 fixation and buoyancy (e.g, *Nostocales*), II) those capable of buoyancy but lacking the ability for N_2 fixation (e.g, *Chroococcales*), III) those not able of neither buoyancy nor N_2 fixation (e.g, many *Oscillatoriales*) (Anagnostidis and Komárek, 1985). Figure 2 reports a few examples among the most frequent cyanobacterial bloom forming genera throughout the world.

According to Mur *et al*. (1999) the most common bloom forming genera such as *Microcystis, Anabaena (Dolichospermum)* and *Aphanizomenon* form blooms in the epilimnion of eutrophic lakes. However, some genera, such as *Planktothrix*, require low irradiance and temperature to grow, and thus they can bloom in the metalimnetic zone. Moreover, at this layer there is limited competition for available nutrient with other photosynthetic organisms (Feuillade *et al*., 1992).

The blooms of cyanobacteria are also defined "harmful" because, apart from their negative effect on water quality by producing taste-and-odor compounds, they are able to produce toxins, posing a serious risk for human health and also for aquatic organisms leading to diversity losses in aquatic food webs (Graham *et al*., 2010).

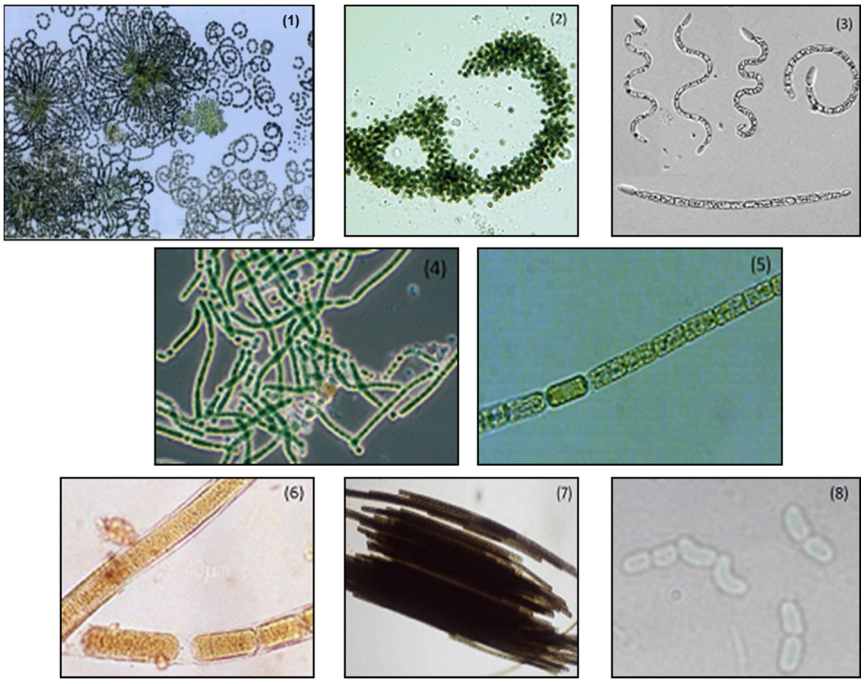


Figure2. Major bloom forming cyanobacteria genera. Top: freshwater: (1) *Dolichospermum,* (2) *Microcystis* (3) *Cylindrospermopsis*); Middle: estuarine: (4) *Nodularia* (5) *Aphanizomenon*; Bottom: marine environments: (6) *Lyngbya* (7) *Trichodesmium* and (8) *Synechococcus*. (Revised form O'Neil *et al*., 2012; See chapter 7, Section 7.3. Figure captions).

1.3 Role of eutrophication and climate change in CyanoHAB

CyanoHAB are not caused by a single environmental factor, but there is consensus that a complex of factors occurring simultaneously triggers the proliferation of cyanobacteria (Heisler *et al*., 2008). Among all environmental drivers responsible for harmful algal bloom, nutrient pollution and eutrophication have received more attention. By increasing human population and anthropogenic

activities, the water bodies have loaded by nutrients, especially nitrogen and phosphorous, which can shift the phytoplankton community towards dominance by cyanobacteria (Paerl and Huisman, 2009).

Moreover, the increase in surface water temperatures due to climate change has been also mentioned as an important factor in the global expansion of harmful algal bloom worldwide (Paul, 2008). By rising temperature and exceeding 20°C, the growth rate of many freshwater eukaryotic phytoplankton decreases while many cyanobacteria keep growing and this is regarded as a competitive advantage for them (Peperzak, 2003).

However, in a recent study by Lürling *et al*. (2013), cyanobacteria and chlorophytes showed a similar mean optimum growth temperature (around 29.2 °C). They concluded that if global warming will probably lead to mass occurrence and intensification of cyanobacterial blooms, it can not be due to a higher growth rate of cyanobacteria compared with their chlorophyte competitors. What gives cyanobacteria a competitive advantage over chlorophytes is their ability to migrate through water coloumn and prevent their sedimentation in warm and stratified water.

Beside the direct effects of temperature on cyanobacterial growth rates, under the effect of climate change many of the physical characteristics of aquatic environments will also change favouring the cyanobacteria dominance. The increase of thermal stratification and the consequent reduction of the vertical turbulent mixing and the widening of the stratification period strengthen and extend the period of optimal growth of many cyanobacterial species. Moreover, higher temperatures will lead to decline in viscosity with promotion of the sedimentation of larger, non motile phytoplankton with weak regulation mechanisms (such as diatoms), giving further advantage to cyanobacteria with the ability to regulate the buoyancy (Wagner and Adrian, 2009).

In stratified ecosystems, less nutrients are available at the surface and since cyanobacteria are able to regulate their buoyancy, they will obtain nutrients from deeper layers of water (Paerl and Huisman, 2009).

1.4 Cyanobacterial toxins

Many cyanobacteria are able to produce a wide range of secondary metabolites, most with unclear or unknown physiological functions and ecological role. Some of these compounds have proven to be toxic for mammals and have been found responsible in many human or animal poisoning episodes. They are called cyanotoxins (van Apeldoorn, 2007).

Cyanotoxins are categorized in different groups based on their "target organ" including: hepatotoxins (e.g. microcystins, nodularins), neurotoxins (e.g. anatoxin-a, homoanatoxin-a, anatoxin-a(s), BMAA), cytotoxins (e.g. cylindrospermopsins), dermatotoxins (e.g. lipopolysaccharides, lyngbyatoxin-a, and aplysiatoxins), and irritant toxins (e.g. lipopolysaccharides) (Wiegand and Pflugmacher, 2005). Each bloom may contain either one dominant species, or several genera of cyanobacteria. In both cases, the existence of more than one type of cyanotoxin is possible (Oehrle, 2010) (Table 1).

Table1. Cyanobacteria known to produce the major classes of cyanotoxins (Revised and updated from Metcalf and Codd, 2012)

Toxin	Producers
Microcystins	**Chroococcales**: *Microcystis* spp., *M. aeruginosa, M. viridis* **Oscillatoriales**: *Planktothrix agardhii, Leptolyngbya boryana , Phormidium corium, Phormidium splendidum, Arthrospira fusiformis* **Nostocales**: *Anabaena* sp., *Dolichospermum flosaquae, A. cylindrica, Trichormus variabilis, Nostoc* sp., *Nostoc carneum. Anabaenopsis* sp., *Gloeotrichia echinulata, Rivularia biasolettiana, R. haematites. Tolypothrix distorta* **Stigonematales**: *Hapalosiphon* sp.
Nodularins	**Nostocales**: *Nodularia spumigena*
Anatoxin-a and homoanatoxin-a	**Oscillatoriales**: *Arthrospira fusiformis, Phormidium formosum, Phormidium* sp., *Oscillatoria* sp. **Nostocales**: *Anabaena* spp., *Aphanizomenon* sp., *Dolichospermum flosaquae, Anabaena planctonica, Cylindrospermum* sp., *Raphidiopsis mediterranea*
Anatoxin-a(S)	**Nostocales**: *Dolichospermum flosaquae, Dolichospermum lemmermannii*
Saxitoxins	**Oscillatoriales**: *Lyngbya wollei, Planktothrix* sp. **Nostocales**: *Aphanizomenon flosaquae, Dolichospermum sigmoideum, Cylindrospermopsis raciborskii*
Cylindrospermopsins	**Nostocales**: *Cylindrospermopsis raciborskii*, *Chrysosporum ovalisporum, Anabaena* sp., *Anabaena lapponica, Raphidiopsis curvata Stigonematales, Umezakia natans*

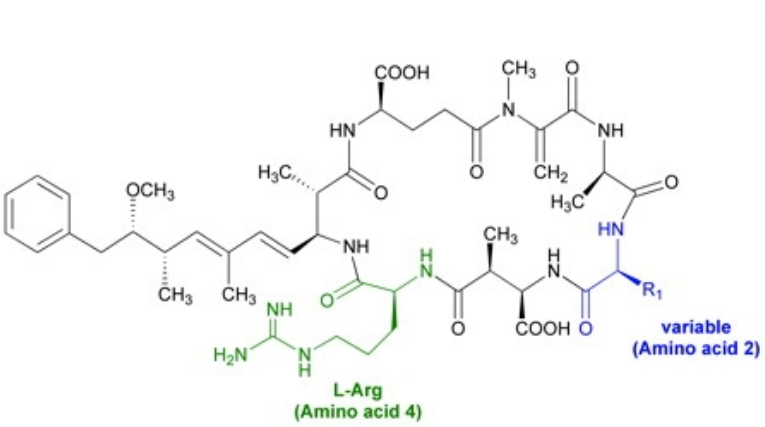
The major routes of exposure to these toxins in humans are represented by the ingestion of contaminated drinking water or contaminated sea food (such as fish and shellfish) and by dermal contact when doing recreational activities (e.g, swimming, bathing, wind and jet skiing) in contaminated water (Drobac *et al*., 2013).

Besides the sanitary consequences, CyanoHAB are also considered a major threat for freshwater ecosystems health. The presence of high biomass and toxins can both have an adverse effect on aquatic organisms. This can cause a considerable change in food web and consequently changes in ecosystem function. For instance, it can lead to modifications of trophic links among organisms, changing the biodiversity, causing oxygen depletion and decreasing the light penetration especially during the bloom episodes (Christoffersen, 1996; Bláha, 2009).

Among different kind of cyanotoxins, microcystins and anatoxins are generally the most frequent toxins identified as responsible of many human and animal casualties caused by cyanoHABs.

1.4.1 Microcystin (MC) - Structure and properties

Microcystins are cyclic heptapeptides. The structure is very variable, as structural variations have been reported in all seven amino acids, although the most frequent variations involve amino acids at position 2 and 4. For instance, in figure 3, a list of MC differing for the nature of the amino acid in position 2 is reported: microcystins-LR (MC-LR) contains Leucine (L), MC-RR contains Arginine (R), MC-YR contains Tyrosine (Y) in position 2 (Fig.3) (Chorus and Bartram, 1999; Van Apeldoorn, 2007). So far, more than 110 different variants of MC have been reported (Dietrich and Hoeger, 2005).



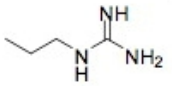
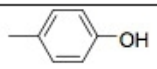
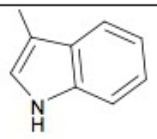
Compound	R_1
MC-LR	
MC-RR	
MC-YR	
MC-WR	

Figure3. Structural variants of Microcystin.

Microcystins were first isolated from the cyanobacterium *Microcystis aeruginosa* (Carmichael, 1988) but many other genera of cyanobacteria were reported as MC producers, e.g, *Anabaena, Nostoc*, *Planktothrix*, *Anabaenopsis* and *Hapalosiphon* (Sivonen and Jones, 1999). The most toxic microcystin variants, microcystin-LR, has an intraperitoneal (i.p.) LD_{50} value of 50 µg kg^{-1} body weight in mice (Dow and Swoboda, 2000). The World Health Organization (WHO) has established a guideline of 1 µg/l as a maximum concentration of microcystin-LR in drinking water (WHO, 2003). Microcystins are water soluble, therefore they cannot penetrate lipid membranes of humans, animals and plants, but can be actively transported through the bile acid-type transporters and then concentrated in liver due to active uptake by hepatocystes (Gorham and Carmichael, 1988). By inhibiting protein phosphatases 1 (PP1) and 2A (PP2A), two important enzymes involved in tumor suppression, MC can cause severe liver damage and promote liver cancer (Ito *et al*., 1997; Zurawell

et al., 2005). Microcystin poisoning is usually characterized by symptom such as anorexia, respiratory problems, vomiting, diarrhoea (Codd, 2000). Depending on the species, it can also follow by liver necrosis, hemorrhagic shock and death after some hours or days (Gorham and Carmichael, 1988).

1.4.1.1 Negative effects of MCs on aquatic organisms

MC is a very stable toxin and its toxicity and negative effect on aquatic organism have been shown by many studies. The motile green alga *Chlamydomonas reinhardtii*, was paralyzed at presence of MC-LR and this led to its settlement and creating a lake zone free of competitors for microcystin-producing cyanobacteria (Kearns and Hunter, 2001). The sensitivity of brine shrimp, *Artemia salina*, to MC-LR was also demonstrated by Delaney and Wilkins (1995). Acute exposure to toxic cyanobacterial cells containing microcystins caused oxidative stress in tilapia fish (*Oreochromis niloticus*) (Prieto *et al*., 2007). MC causes other negative effects such as mortality and delayed hatching in fish embryos of carp (*Cyprinus carpio*) and affecting feeding behaviour (Malbrouck and Kestemont, 2006; Palíková *et al*., 2007). In some laboratory studies, the negative effect of MC on survival, growth and reproduction rates of zooplankton have been demonstrated (DeMott *et al*. 1991; Ferrão-Filho *et al*., 2000; Ghadouani *et al*., 2004). In a recent study by Dao *et al*. (2010), the direct negative effect of microcystin on zooplankton was also demonstrated when the growth and reproduction of parent daphnids were slightly affected by microcystin-LR (see references in the paper presented in CHAPTER 2 for more studies about the negative effect of MC on aquatic organisms).

1.4.1.2 Accumulation of MC in zooplankton

There has been a great attention towards the effects of MC on zooplankton and specially on the larger cladocerans such as *Daphnia* because these organisms play an important role in the aquatic food web (Benndorf *et al*., 2002; Reichwaldt *et al*., 2013). They feed on primary producers and are regarded as major food source for juvenile fish; consequently, they can act as important vectors to transfer cyanobacterial toxins to higher consumers such as fish along the food web (Rohrlack *et al*., 2005). Unlike copepods, which are able to differentiate between toxic and non-toxic cells (DeMott and Moxter, 1991), daphnids are regarded as non-selective filter feeders and are not able to discriminate food particles with different quality (DeMott, 1986).

Ferrão-Filho *et al*. (2002) demonstrated that zooplankton are an efficient accumulator of microcystins. They showed that microcystins accumulated in zooplankton individuals ranged from 0.3 to 16.4 µg/g DW, while in phytoplankton MC were around 0.3-3.9 µg/g DW.

Most of the field studies have indicated the potential of MC accumulation in the entire zooplankton community (Table 2), even if with different accumulation pattern among different species (see CHAPTER 4).

The main route of MC accumulation is through ingestion of cell-bound MC. This route of ingestion has been recently investigated by Shams *et al.* (2014) (see CHAPTER 4).

MC uptake from aqueous extracts is considered as another route of exposure. MC uptake from aqueous extracts obtained from a natural bloom sample has been investigated very recently by Ferrão-Filho *et al.* (2014) in 3 different cladocerans. They demonstrated that *Moina micrura* and *Daphnia laevis* presented the highest MC concentrations in their tissues, while *Daphnia similis* showed the lowest.

Table 2. Microcystin accumulation in zooplankton by different studies (Revised from Ferrão-Filho *et al*., 2002).

Zooplankton	Habitat	MC-LR eq. units ($\mu g g^{-1}$DW or WW)	Detection	Reference
Entire community	Freshwater (Kasumigaura, Lake, Japan)	75.0-1387 (DW)	I-EC	Watanabe *et al*. (1992)
Entire community	Freshwater (four lakes Central Alberta, Canada)	Up to 67.0 (WW)	HPLC PPase	Kotak *et al*. (1996)
Daphnia magna	Freshwater	Up to 24.5	ELISA	Thostrup and Christoffersen (1999)
Entire community	Brackish (Jacarepagua, Lagoon)	0.3-16.4 (DW)	ELISA	Ferrão-Filho *et al*., (2002)

MCYST-LR Eq. Units, microcystin-LR equivalent units; I-EC, Ion-Exchange Chromatography; Ppase, protein phosphatase bioassay; ELISA, Enzyme-linked immunosorbent assay. DW, Dry Weight; WW, Wet Weight.

Grazing behavior of Cladocerans can be affected by the size and shape of cyanobacteria (Lampert 1987). For instance, the mechanical interference of feeding activity in *Daphnia* grazing on filamentous cyanobacteria have been reported by Infante and Abella (1985) and Burns *et al*. (1968),

while some other studies showed that even high densities of filaments did not inhibit the grazing and consumption of cyanobacteria (Holm *et al*., 1983).

Kurmayer (2001) reported that mechanical interference was not important when *Daphnia galeata* was ingesting the filamentous cyanobacterium *Aphanizomenon flexuosum*. Finally, Oberhaus *et al*., (2007) reported that *D. pulicaria* could efficiently control *Planktothrix* blooms in their early stages by grazing on short filaments of *P.rubescens* and *P.agardhii.*

The contradictory results reported in different investigations suggest that the ingestion of filamentous cyanobacteria by *Daphnia* is highly dependent on both grazer and cyanobacterial species.

In spite of the widespread occurrence of toxic filamentous cyanobacterium *Planktothrix* in European lakes (Salmaso *et al*., 2003; Ernst *et al*., 2009), *Daphnia* grazing over *Planktothrix* has been investigated by only a limited number of studies (see e.g. Kurmayer and Jüttner, 1999; Oberhaus *et al*., 2007; Pires *et al*., 2007; Reichwaldt and Abrusan, 2007).The accumulation kinetics of MC in large cladocerans such as *Daphnia* has been poorly investigated so far and more studies are needed to fill this gap of knowledge. (The paper presented in CHAPTER 4 was prepared based on a laboratory experiment to further elucidate this aspect).

1.4.1.3 Microcystin Biosynthesis and Genetic

Microcystin is synthesized non-ribosomally by enzyme complex which encoded by the 55kb microcystin synthetase gene cluster. It includes genes for nonribosomal peptide synthetase (NRPS), polyketide synthase (PKS) and tailorig enzymes (Tillett *et al*., 2000).

The gene clusters encoding microcystin synthetase were sequenced and characterized from *Anabaena* (Rouhiainen *et al*., 2004), the unicellular *Microcystis aeruginosa* (Tillett *et al*., 2000; Nishizawa *et al*., 2000) and from the filamentous *Planktothrix agardhii* (Christiansen *et al*., 2003). The comparison of the gene cluster of these species from 3 genera indicated that the genes mcyA, mcyB, mcyC, mcyD, mcyE, mcyG, and mcyJ that are involved in MC synthesis are always present (Tillett *et al*, 2000, Rouhiainen *et al*., 2004) (Fig.4).

Both toxic and nontoxic strains occur in the same species. The difference between microcystin-producing (toxic) and nonproducing (nontoxic) strains of cyanobacteria is primarily due to the presence or absence of microcystin synthetase gene cluster or inactivation of single genes (Christiansen *et al*., 2008; Ostermaier *et al*., 2012).

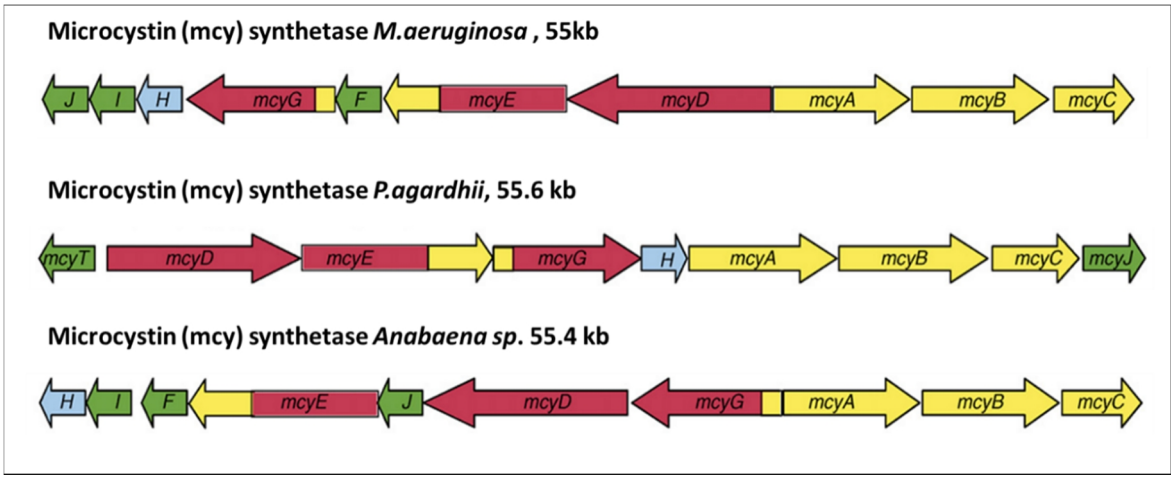


Figure4. Comparison of microcystin gene clusters in three microcystin-producing species; *Microcystis* (up), and *Planktothrix* (middle), *Anabaena* (bottom). Genes encoding polyketide synthases (red), non-ribosomal peptide synthetases (yellow), tailoring enzymes (green) and ABC transporters (blue) (Pearson and Neilan, 2008).

1.4.1.4 Regulation of microcystin synthetase gene expression

It have been shown that different physical and environmental variables, including nitrogen, phosphorous, temperature, light, pH and trace metals can affect the MC production in cyanobacteria (Song *et al*.,1998; Neilan *et al*., 2013).

Description and sequencing the MC gene cluster by Tillett and colleagues (2000) revealed that there is a bidirectional promoter between mcyA and mcyD which is responsible for the transcription of mcy genes in *M. aeruginosa*. In this central regulatory part of the mcy cluster there are sequence motifs for Fur (ferric uptake regulator) and NtcA (global nitrogen regulator) DNA binding proteins. These finding could demonstrate the role of iron and nitrogen in controlling microcystin biosynthesis.

Many studies have investigated the effect of iron as one of the most frequently studied metal stressor on cyanotoxin production. For instance, iron deprivation, in *Microcystis* spp. has been shown to be responsible for both increase and decrease of microcystin production (Li *et al*., 2009; Neilan *et al*., 2013).

In a study by Tonk *et al*. (2005), an increase in mcy gene transcription and toxin production rates in *Planktothrix agardhii* cultures was observed under high light conditions up to 60 mmol photons m^{-2} s^{-1}). Interestingly, as a response to the differing light intensities, the produced microcystin variants (dmMC-LR and dmMC-RR) varied while the cellular microcystin content remained constant. Kaebernick *et al*. (2000) also showed that maximum microcystin transcription rates were observed at high light intensities and under red light. In contrast, the blue light decreased the transcription rate. Temperature has also shown to influence the type of toxin. In selected strains (*Anabaena* 90 and *Anabaena* 202A1), high temperature (>25°C) was shown to enhance MC-RR production, while MC-LR correlated with temperatures below 25°C (Rapala and Sivonen, 1998).

Many laboratory studies have shown the effect of environmental conditions on cyanotoxin production, but it is still not clear how these affects can be regulated at molecular level and how this can translate to actual responses in the environment. Therefore, more studies are needed to investigate more in detail the promoters and transcription factor binding sites of the toxin biosynthesis clusters in order to fill these knowledge gaps (Neilan *et al*., 2013).

1.4.2 Anatoxin-a (ATX) - Structure and properties

Anatoxin-a (ATX) is an alkaloid neurotoxin with a semi-rigid bicyclic secondary amine structure and a molecular weight of 165 Dalton (Devlin *et al*., 1977) (Fig.5). It is produced by different genera of Nostocales (*Anabaena, Aphanizomenon, Cylindrospermum*) and benthic Oscillatoriales (*Phormidium* and *Oscillatoria*) (Sivonen *et al*.,1989, Bumke-Vogt *et al*.,1999; Namikoshi *et al*., 2003; Ballot *et al*., 2005; Gugger *et al*., 2005; Aráoz *et al*., 2005). Recently, ATX production in pelagic Oscillatoriales was demonstrated by (Shams *et al*., 2015; CHAPTER 5).

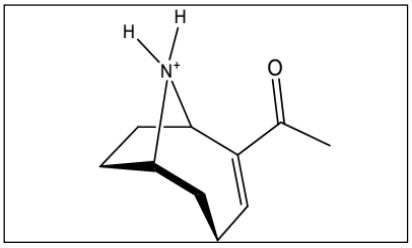


Figure5. Chemical Structure of Anatoxin-a.

ATX can lead to death by respiratory arrest through binding irreversibly to nicotinic acetylcholine receptors and trigger the receptor's channel to stay excited (Devlin *et al*., 1977). It has been also

shown that ATX can negatively affect the heart rate, gas exchange and cause hypoxia and respiratory arrest and led to animal death (Adeymo and Siren, 1992). After 2 min with intraperitoneal (i.p.) LD_{50} value of 375 μg/kg $^{-1}$ in mice, or after 10 min with intraperitoneal (i.p.) LD_{50} value of 250μg/kg^{-1} in mice, death can occur because of muscle spasm, respiratory arrest and paralysis (Rogers *et al*., 2005).

Many cases of fatal intoxications of dogs and livestock due to ATX have been reported from different countries, e.g. Canada (Carmichael and Gorham, 1978), Scotland (Edwards *et al*., 1992), Japan (Park *et al*., 1993) Kenya (Krienitz *et al*., 2003; Ballot *et al*., 2005), France (Cadel-Six *et al*., 2007), Netherland (Faassen *et al*., 2012).

1.4.2.1 Effect/ accumulation on aquatic organisms

ATX is not a stable toxin and can be degraded easily into non-toxic derivatives such as dehydroanatoxin-a and epoxyanatoxin-a. Under strong sunlight and high pH, it has a half-life of several hours or days (Smith and Sutton., 1993). Due to its low persistency in aquatic ecosystem, it has attracted little scientific attention so far to investigate the fate and impact of this toxin in aquatic ecosystems.

Only few studies as Osswald *et al*. (2007, 2008) have investigated the negative effect of ATX in aquatic organisms such as fish and mussels. For instance, it was shown that all the juvenile fishes Cyprinus carpio died between 26-29h after exposure to higher cell density of toxic strain of the cyanobacterium *Anabaena* sp. (Osswald *et al*., 2007).

Moreover, in a very recent study by Ha *et al*. (2014) oxidative stress in aquatic plant *Ceratophyllum demersum* was induced following ATX uptake. A rapid toxin uptake was observed during the 24h, afterward it was followed by a constant accumulation.

Thus, considering the accelerated eutrophication and climate change which can facilitate massive proliferation of cyanobacteria and can be followed by increased amount of cyanotoxins, more studies needed to elucidate the ATX uptake and metabolism in different aquatic organisms (e.g. zooplankton, fishes and molluscs) (Carmichael, 2008).

1.4.2.2 Anatoxin-a Biosynthesis and Genetic

According to study by Cadel-Six *et al*. (2009), the polyketide synthase (PKS) which is specific for anatoxin-a, or homoanatoxin-a producing strains was identified in benthic *Oscillatoria* strain PCC 6506. Only recently, by partial genome sequencing of this strain the identification of the putative gene cluster responsible for anatoxin-a and homoanatoxin-a production was revealed (Méjean *et al*., 2009; 2010). These findings opened the way to design a genetic marker for the detection of gene coding for anatoxin-a in other genera, such as *Oscillatoria, Phormidium, Aphanizomenon* and *Anabaena* (Cadel-Six *et al*., 2009; Ballot *et al*., 2010; Wood *et al*., 2010; Rantala-Ylinen *et al*., 2011).

On the basis of comparison of the anatoxin-a biosynthesis gene of *Oscillatoria* sp. PCC 6506, Rantala-Ylinen *et al*. (2011) could identify the Anatoxin-a synthetase gene cluster for the strain *Anabaena flos-aquae* 37. The anatoxin genes of *Anabaena flos-aquae* 37 are very similar with those of *Oscillatoria* sp. PCC 6506 but the cluster is slightly rearranged (Fig. 6). In *Anabaena flos-aquae* 37, the three genes anaI, anaJ, and anaA are downstream of the anaG gene. There are several genes between anaG and anaI that are very likely not involved in the biosynthesis of anatoxin-a (Rantala-Ylinen *et al*., 2011). The sequences of the ana genes in these strains are very similar (at least 70% identity in nucleotide sequence), and according to Méjean *et al*. (2014) these clusters might have evolved from a common ancestor. As seen in Figure 6, there are transposase genes shown in hatched and these genes might have responsible for putative horizontal transfer of these clusters within cyanobacteria.

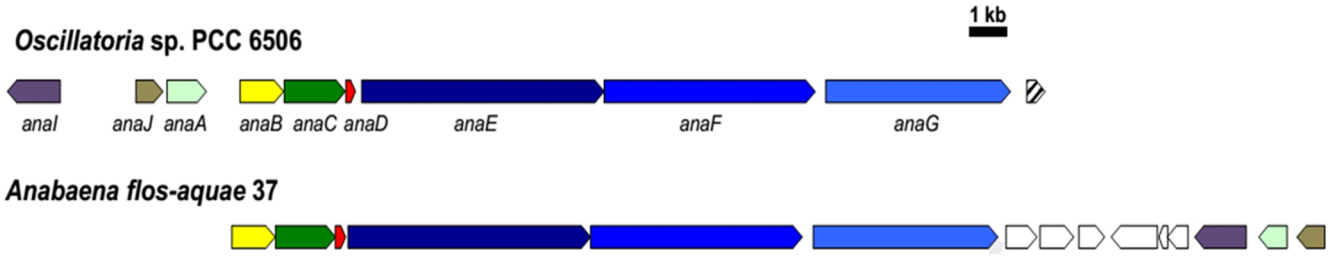


Figure6. Anatoxin-a biosynthetic gene clusters in (up) *Oscillatoria sp*. strain PCC 6506, (bottom) *Anabaena flos-aquae* strain 37. The clusters starting from anaB (yellow), Transposase genes (hatched), genes that have no function in the biosynthesis of anatoxins *in Anabaena flos-aquae* 37(white) (Méjean *et al*., 2014).

1.4.2.3 Regulation of anatoxin-a synthetase gene expression

It has been shown that metal stressors can affect the cyanobacterial growth and cyanotoxin production (Neilan *et al*., 2013). Maldonado *et al*. (2002) demonstrated that increasing in copper or reduction in iron can activate the neurotoxic domoic acid production in the diatom *Pseudo-nitzschia* spp. Harland *et al*. (2013) investigated the effect of iron and copper on the growth and anatoxin-a production by the benthic mat-forming species *Phormidium autumnale.* They showed within the first two weeks of growth, ATX concentrations varied between 0.49 and 0.55 pg $cell^{-1}$ and growth rates were significantly affected by copper and iron.

The role of other environmental factor on regulating ATX production is not still clear and more studies are needed to elucidate this issue as it can provide useful information for predicting periods when anatoxin-a producers proliferate and the highest health risk is inevitable.

1.5 Monitoring of cyanobacteria and cyanotoxin

Considering the negative effects and threats posed by cyanobacterial toxins to water supplies, animals and human health, efficent monitoring programs play a fundamental role for predicting bloom event and for water management.

1.5.1 Chemical analysis of cyanobacterial toxins

As stated earlier, a huge chemical variability exists among cyanotoxins. Different chemical structure means different physical and chemical properties and, importantly, different toxicity. The toxicity of a given bloom is rarely determined by a single toxin; more often a mixture of toxins is present. Therefore, the full knowledge of the chemical diversity in a sample is mandatory for a complete and robust investigation. Many analytical methods are available for the characterization of toxins. They differ for sensitivity (measured as LOQ, limit of quantitation), selectivity (low in case of methods which give the total amount of toxins; high in case of methods that quantify the single congeners), and resources needed (costs of equipment and training).

ELISA (enzyme-linked immunosorbent assay) is widely used for the analysis of hepatotoxins (MC and NOD) and saxitoxins; PPIA (protein phosphatase inhibition assay) is sometimes used for hepatotoxins. However, these techniques have very low selectivity, as they are not able to distinguish among the different toxin variants. The techniques based upon High Performance Liquid

chromatography combined with Mass Spectrometry (LC-MS) represent the best performing techniques in terms of sensitivity and selectivity. They allow rapid, sensitive and reliable identification and quantification of different toxin variants.

1.5.2 Microscopic analysis of cyanobacteria

Microscopic identification and cell counting have traditionally been used as basic methods for monitoring cyanobacteria. However, it is very time-consuming, and it requires a skilled person for species identification. Moreover, identification and quantification of cyanobacteria based on microscopic approach do not provide information regarding the potentially toxin producers because even though different strains from same species present similar morphologies, they could possess different toxigenicity (Sivonen and Jones, 1999).

1.5.3 Genetic analysis of cyanobacteria

Chemical analysis of cyanobacterial toxins does not indicate which cyanobacteria produce the toxins, since different genera of cyanobacteria may produce similar toxins. Moreover, the classical morphological taxonomy was not able to differentiate the toxin and nontoxic cyanobacterial strains. Therefore, researchers were driven for a complementary method which, along with chemical and taxonomical analysis, could solve the above drawbacks.

Polymerase chain reaction (PCR), quantitative real-time PCR (qPCR) are now widely used as a routine in many laboratories and are considered as very rapid and sensitive tools for detecting the potentially toxic cyanobacteria in water supplies even when the quantities of toxic genes are low (Sivonen, 2008).

Nübel *et al*. (1997) designed three 16S rRNA cyanobacterial specific primers: CYA359F (forward), CYA781R (a) and CYA781R (b) (reverse) which can amplify ca. 420 bp 16S rRNA gene sequence. CYA781R (a) primer targets filamentous cyanobacteria whereas the CYA781R (b) targets unicellular cyanobacteria. An equimolar mixture of these three primers allows an optimum investigation of the diversity of the cyanobacterial community.

Larger fragments of 16S rRNA gene (1432–1439 bp) can be also analysed by applying primers such as pA and B23S (Rajaniemi *et al*., 2005). Amplification of the rRNA operon containing the ITS region (internal transcribed spacer region) has been also used to study the cyanobacterial diversity of environmental samples (Taton *et al*., 2003; Iteman *et al*., 2000).

Primers targeting housekeeping genes (e.g. rbcLX and rpoC1 genes) along with 16S rRNA and ITS region are also regarded as very useful molecular markers to analyse the genetic diversity of cyanobacteria and to discriminate between cyanobacterial species and strains.

Discovering the gene cluster of cyanotoxin synthesis in some cyanobacteria has resulted in an explosion of molecular detection methods. Designing general and/or genus-specific primers and probes to detect and characterize the toxin producing cyanobacteria in environmental samples have made these molecular methods as early warning tools in monitoring systems and protecting water bodies.

To detect and differentiate the potential microcystin-producing species in natural bloom communities in Finland the general forward primer, mcyE-F2, was applied in combination with genus-specific reverse primers, mcyE-plaR3, mcyE-12R and mcyE-R8, to detect hepatotoxic *Planktothrix*, *Anabaena* and *Microcystis* species, respectively (Vaitomaa *et al*., 2003; Rantala *et al*., 2006). To discover the potential anatoxin producers, general and genus-specific primers were designed to amplify regions of the anatoxin-a synthetase gene in two different genus (e.g, *Anabaena* and *Oscillatoria*) (Rantala-Ylinen *et al*., 2011).However, more research is needed to discover the gene cluster of other cyanobacteria and also the genes responsible for cyanotoxin synthesis which can lead to designing more genus specific primers targeting specific toxic genes and will provide very fast and valuable information for monitoring a water body.

Quantitative PCR (qPCR or real-time PCR) has been widely used to quantify gene copy numbers present in environmental samples and this is regarded as its advantages over traditional PCR which can only detect the presence of a specific gene without reflecting its actual abundance. Therefore, it is a robust and highly sensitive method to quantify genes and to monitor their temporal and spatial dynamic.

The quantitative data generated by qPCR can be used to relate variation in gene copy number and toxic concentration or to find out the relation between environmental factors and gene abundance to elucidate for instance under which environmental condition the highest gene copy number will be observed. Thus, this method can be used as very useful tools to predict toxic cyanobacterial blooms.

In qPCR, the amplicon numbers are measured in real-time during the PCR through detection of a fluorescent reporter which indicates accumulation of amplicon in each cycle. Two different reporter systems are commonly used: SYBR green assay and the TaqMan probe system. SYBR green is easy to use and least costly approach than TaqMan probe. It is nonspecific and binds to all double-stranded

DNA. After binding to DNA, a fluorescent signal is released following light excitation. Since SYBER green binds to all dsDNA, it is important to use specific primer pairs which target specific genes to avoid producing the nonspecific products that can result in overestimation of the target.

1.6 Aim of the study

It is nowadays well recognized that cyanobacteria may produce wide range of toxins with harmful effects on humans and animals. Furthermore, they are regarded as an increasing public concern in freshwater ecosystems. Many investigations have been conducted so far, however, further research in this area is still needed to obtain up-to-date information on cyanobacteria and the associated toxins, and to forecast cyanobacterial bloom occurrence, reducing the risks to human and aquatic ecosystems.

This PhD project was structured into 3 research lines focused on toxic cyanobacteria and cyanotoxins in a freshwater ecosystem in Italy, Lake Garda. This lake is the largest Italian lake and, since the 1960s, it has become a well-known mass tourist destination, with a yearly visitor presence approaching 20 million during the last years. It was also included in the LTER (Long-Term Ecological Research) network since 2006.

The main objectives addressed by the PhD thesis were:

1) To elucidate the nature, quantity and seasonality of toxins produced by cyanobacteria in Lake Garda (**CHAPTER 3).**

2) To investigate the potential accumulation of toxins produced by the dominant cyanobacterial species of Lake Garda (*Planktothrix rubescens*) in the main zooplankton grazers (*Daphnia*). As mentioned earlier (section 1.4.1.2), zooplankton are considered as an important vectors for cyanotoxins transfer to higher trophic level and still little is known for accumulation kinetics of MC in large cladocerans such as *Daphnia* when grazing on a most widespread MC-producing filamentous cyanobacteria, *Planktothrix rubescens* **(CHAPTER 4).**

3) To identify the main producers of ATX in Lake Garda. Cyanotoxins analyses (confirmed in CHAPTER 3) showed a significant production of ATX in Lake Garda, with concentrations higher than MC. Until a short time ago, the producers were unknown, and their clear identification was not possible, because biological analyses on isolated strains were not available. This discrepancy has found solution during the completion of this PhD thesis, through the discovery of a pelagic Oscillatoriales able to synthetise ATX (i.e. *Tychonema bourrellyi*). The discovery was possible by applying a polyphasic approach based on morphology, genetics and chemistry analysis carried out on cultures of strains isolated from environmental samples **(CHAPTER 5).**

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Chapter 2

2. SAMPLING AND METHODOLOGY

2.1 Lake Garda – Study site

Lake Garda is one of the largest freshwater bodies in Europe and the most important freshwater resource in Italy. Waters supplied by this lake are intensively used in agriculture and industry, becoming a life-sustaining element for the economy of the most densely populated and productive area in Italy. In addition, these water bodies are one of the key elements for the tourist economy of the Alpine region (Salmaso and Mosello, 2010). Lake Garda is located at the southern border of the north eastern Italian Alps, at 65 m a.s.l; it has a volume of more than 49×10^9 m^3, a maximum depth of 350 m and a surface of 368 km^2. The lake was originally oligotrophic (average values of total phosphorus <10 $\mu g\ l^{-1}$ on the whole water column). Lake Garda was included in the LTER (Long-Term Ecological Research) network since 2006. Lake Garda hosts two main potential toxic cyanobacterial species: *Planktothrix rubescens* and *Dolichospermum lemmermannii*. The first species is known to produce MC and the latter is known to produce both MC and ATX (Sivonen *et al*., 1992; Landsberg, 2002) (Fig. 1).

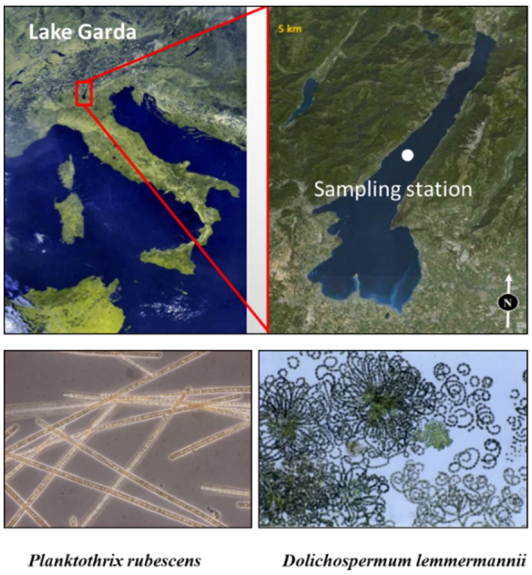

Figure1. Lake Garda, Italy **(top)**, two toxic cyanobacteria species in this lake **(bottom)**.

2.2 Collection of water samples

Since April 2010, a monitoring campaign was conducted to assess the presence of cyanotoxins in Lake Garda. The chemical diversity and the abundance of cyanotoxins were investigated by advanced analytical techniques (LC-MS) with a monthly frequency.

Along the chemical analyses, samples were also collected for genetic analysis since January 2013 form Lake Garda. Moreover, samples for preparing isolated cultures were taken since February 2014.

Water samples for chemical and genetic analyses were taken from 4 depths (0, 10, 20 and 60m), brought to laboratory and filtered on GF/C filters (Whatman - GE Healthcare Life Sciences). The filters were frozen and stored at -20°C until further processing (See fig. 2). For filament isolation, phytoplanktons were collected by single vertical tows from the depth 10-15 m to the surface using a 80 µm mesh (25 cm diameter).

Figure2. Schematic representing the sampling collection form Lake Garda and preparing samples for different analysis.

2.3 Methodology

2.3.1 *Part I - Chemical analysis of cyanobacterial toxins*

Objectives and experimental set up

In the first part of this PhD study we investigated in depth the cyanobacterial toxin diversity in Lake Garda. Specific objectives included:

I) Temporal dynamic of MC variants

II) Temporal dynamic of total MC and ATX

Toxin extraction

For chemical analysis, water samples taken from 4 different depths were filtered on GF/C filters (Fig. 2). The filters were processed for toxin extraction by adding 7 ml of extraction solvent (70% methanol containing 0.1% formic acid) and homogenizing for 5 min. After centrifugation (10000 rpm, 10 min; 9850 G), the supernatant was transferred and the pellet was extracted again with 7 ml of extraction solvent. The two supernatants were finally put together. A 2 ml aliquot of the extract was filtered on Phenex-RC syringe filter (0.2 µm pore size, Phenomenex) and used for the determination of the MC content. The remaining solution was concentrated under vacuum to get rid of the organic solvent. The resulting water solution was filtered on RC syringe filter and used for the determination of alkaloids (ATX, CYN) using the LC-MS instrument.

LC-MS analysis

LC-MS analyses were carried out on a Waters Acquity UPLC system directly coupled to an AB SCIEX 4000 QTRAP mass spectrometer equipped with a turbo ion spray interface. Standard injection volume was 2µL. For more detailed information on LC-MS analysis (see Shams *et al*., 2015).
Microcystins (RR, [D-Asp^3]-RR, YR, LR, WR, LA, LY, LW, LF), NOD-R and CYN analytical standards were purchased fromVinci Biochem, and ATX from Tocris Cookson Ltd. All solvents and reagents used in this procedure were LC-MS grade. The limits of quantitation (LOQ) were between 30 and 500 ng L^{-1}(different MCs congeners), 140 ng L^{-1} (NOD-R), 30 ng L^{-1}(ATX), and 8 ng L^{-1}(CYN).
See CHAPTER 3 for the results obtained after analysing samples.

2.3.2 *Part II- Cyanotoxin transfer through foodweb*

Objectives and experimental set up

For the second part of this PhD study, we were interested to investigate the transfer of one of the most common cyanotoxins, microcystin, through the food web.

We wanted to elucidate better the microcystin accumulation patterns in *D. magna* after exposure to populations of *P. rubescens* and to clarify the following objectives:

I) To verify if *D. magna* can take up the filaments of MC-producing *Planktothrix rubescens* (the dominant cyanobacterium in Lake Garda) and to investigate whether the density of filaments will decrease over time.

II) Assuming that MC accumulation occurs in *D.magna*, which accumulation pattern are expected (linear or exponential) to explain the relationships between MC accumulation in *Daphnia*, ambient MC concentrations, and both initial MC exposure and time after initial exposure.

To fulfill the objectives, a short-term laboratory experiment was conducted. Four different MC exposure (A, B, C and D) glass beakers (1L) filled with *P.rubescens* cultures were considered for this experiment (Fig.3). In order to achieve the different MC concentrations, different proportions of toxic and non-toxic strains of *P. rubescens* were mixed and diluted with BG11 medium to 1L. A control group was used containing a comparable density of the chlorophyte *Scenedesmus* sp.

All the analyses (MC content and algal densities) were made on three independent replicates. The experiments were started after adding 100 adults of *D.magna* in each 1L glass beakers.The chemical and biological analyses were carried out on samples collected after a time exposure of 0, 6, 24, 48, 72, and 144 h Fig. 4).

See CHAPTER4 for the results obtained after analysing samples.

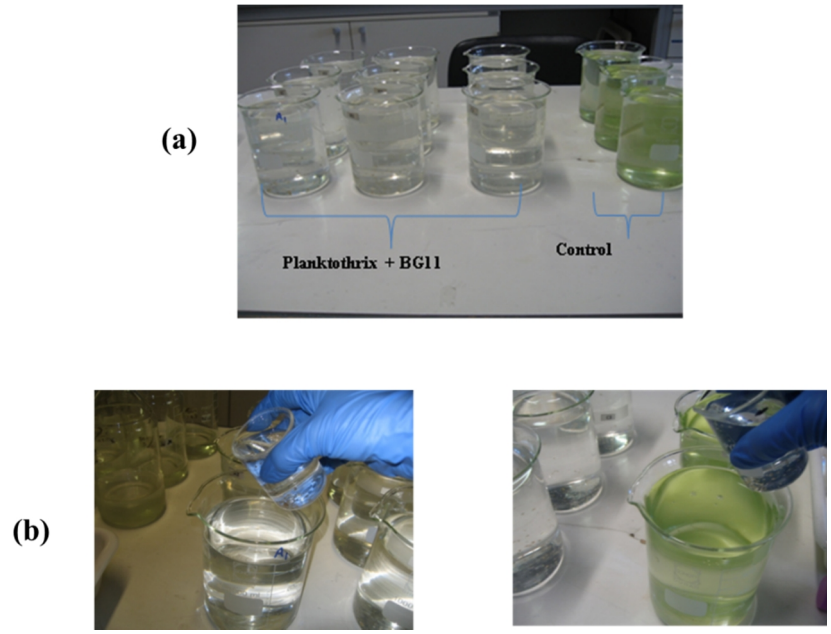

Figure3. Experimental set up to investigate the toxin transfer through food web. (a) Different exposures and the control group that was used in this experiment. (b) Adding daphnids to each exposure and starting the experiment.

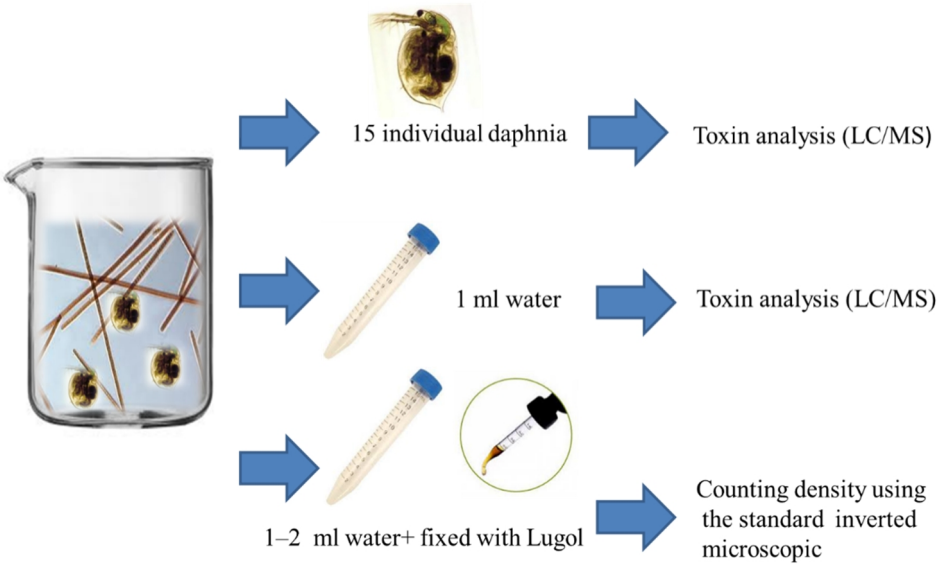

Figure4. Sample collected at certain time exposure for different analysis

2.3.3 *Part III- Genetic analysis*

Objectives and Experimental set up

For the last part of this study, we were interested to investigate the presence of different cyanotoxins producing genotypes in Lake Garda. Specific objectives included:

I) To check the presence and seasonality of the two main potential toxic cyanobacteria analysed in environmental samples collected in Lake Garda (*Planktothrix rubescens* and *Dolichospermum lemmermannii*), by applying genus specific primers targeting house keeping genes.
II) To screen the environmental samples for seasonal dynamic of cyanotoxin producing genotypes, specifically MC and ATX genes, by using general/specific primers.
III) Chemical (toxins) and genetic (taxonomic and toxins encoding genes) characterization of cultures of cyanobacterial strains isolated from environmental samples
IV) Investigating temporal dynamic of ATX copy numbers by appling qPCR to see if there is any correlation between ATX concentration and ATX copy numbers.

Cyanobacteria isolation, culture conditions and microscopy analysis

After diluting the net-phytoplankton samples, single filaments of cyanobacteria were isolated under a stereomicroscope using a glass micropipette. The single filaments were washed 3 times in Z8 medium to get rid of other algae, and then placed in microwell plates containing 3 ml Z8 medium. After 1-2 week when they grew enough, the cultures were checked for the presence of other contaminating algae, if they did not contain any contaminants then the first subset culture obtained from a single filaments were inoculated to 30ml flask containing Z8 medium, and after around 10 days when they grew enough they were transferred to 150 ml flask with Z8 medium. All the flasks were maintained at 20 °C under continuous light conditions (25 μmol m^{-2} s^{-1}).
At the end of the exponential growth stage, after careful homogenization each single culture went through 3 different analyses: 15 ml were preserved with Lugol's solution for phytoplankton analysis, whereas 250 ml were filtered through 0.45 μm GF/C filters (Whatman – GE Healthcare Life Sciences) for both cyanotoxins and genetic analyses.
Morphological features were analyzed using an inverted microscope (Zeiss Axiovert 135). Single specimens were identified following morphometric and morphological criteria described in Komárek and Albertano (1994) and Komárek and Anagnostidis (2007).

DNA extraction, PCR amplification

DNA was extracted from all the filters, environmental samples and isolate cultures, using the Mo Bio PowerWater® DNA Isolation Kit (Mo Bio Laboratories, Inc., CA, USA) following manufacturer's instructions. The quantity and quality of DNA was measured by spectrophotometry with a NanoDrop ND-8000 (Thermo Fisher Scientific Inc., MA, USA).

The DNA extracts were then examined for different cyanotoxin producing genotypes using general or genus-specific primers which were classified in different categories as follows (see also CHAPTER 5, Section 5.9. Supplementary Tables, for the complete list of primers used in this study):

Category I- Primers specific for cyanobacteria and house keeping genes

◆*16S rRNA*: Samples were checked for amplification of a short fragment (ca. 420 bp) of the 16S rRNA gene specific for cyanobacteria to confirm the presence of cyanobacterial DNA using the forward primer CYA359F and an equimolar mixture of the reverse primers CYA781Ra and CYA781Rb (Nübel *et al*., 1997).

◆*House-keeping genes (rbcLX and rpoC1 genes) specific in Planktothrix*

To confirm the presence of *P. rubesens* in environmental samples collected in 2013 and in isolated strains, two sets of primers were used: (rbcLX- Prbc, F/R) and (rpoC1-RPO, F/R); the former primer pair amplifies a 824-bp rbcLX gene, while the latter amplifies a 608-bp rpoC1 gene in *Planktothrix* (Lin *et al*., 2010).

◆*House-keeping genes (rpoB genes) specific in Dolichospermum*

The presence of *Dolichospermum lemmermannii* in environmental samples of 2013 was checked using a house keeping gens specific for genus *Anabaena* (revised to *Dolichospermum*) (rpoBana-F/R) (Rajaniemi *et al*., 2005).

Category II- Primers targeting the mcy genes

The production of microcystin by *P.rubescens* and *D.lemmermannii* in environmental samples of 2013 and isolated strains in 2014 were checked with genus-specific primers to target mcyE genes in *Planktothrix* and *Dolichospermum*. Moreover, another primer pair was also used to target the mcyB gene in *Planktothrix*).

Planktothrix agardhii strain 126/3 and *Anabaena* 90 were used as a positive control (Pla 126/3;

UHCC), (Ana-90; UHCC).

- *mcyE gene in Planktothrix*

The mcyE-F2/ mcyE-plaR3 primer pair was used to amplify a 249-bp mcyE gene specific for *Planktothrix* (Rantala *et al*., 2006).

- *mcyE gene in Anabaena (revised to Dolichospermum)*

The mcyE-F2/ AnamcyE-12R primer pair was used to amplify a 247-bp mcyE gene specific for *Anabaena* (Vaitomaa *et al*., 2003).

- *mcyB in Planktothrix*

The (mcyBA1tot R/F) primer pair was used to amplify a 1692-bp fragment of the mcyB gene in *Planktothrix* (Kurmayer *et al*., 2005).

Category III- Primers targeting the ATX gene

The presence of anatoxin gene in samples was checked first with general primer (anaC-gen) which amplifies a 366-bp anaC gene in anatoxin producers (Rantala-Ylinen *et al*., 2011). Moreover, samples were further analysed using genus-specific primers to target anaC gene in *Oscillatoria* and *Dolichospermum*. *Oscillatoria* 193 (Osc-193, UHCC) and *Anabaena* 37 (Ana-37, UHCC) were used as positive controls.

- *anaC gene in Anabaena(revised to Dolichospermum)*

The primer pair (anaC-anab F/R) was applied to amplify a 263-bp anaC gene specific for genus *Dolichospermum* (Rantala-Ylinen *et al*., 2011).

- *anaC gene in Planktothrix*

Since still there is no primer to target anaC gene in *Planktothrix*, therefore, to investigate the possible presence of this gene, samples were checked with primer pair (anaC-oscF/ R) which is a gene-specific primer for *Oscillatoria* and amplifies a 216-bp fragment of anaC gene in *Oscillatoria* (Rantala-Ylinen *et al*., 2011).

All PCR protocols and thermal cycling for each primer can be found on (CHAPTER 5- Section 5.9. Supplementary Tables). Thermal cycling was carried out using an Eppendorf Mastercycler ep (Eppendorf AG, Hamburg, Germany).

A volume of 10-15µl of PCR product was run on a 1.5% agarose gel. The gels were stained with ethidium bromide and photographed under UV transillumination.

After analysing the isolated strains for cyanotoxin producing genotypes, particularly ATX and MC, the approach led to the discovery and characterization of a new unexpected filamentous cyanobacterial producer of ATX (see CHAPTER 5).

Quantitative PCR (qPCR) analysis

Environmental samples collected in 2013 were quantified using qPCR to determine the abundance of cells carrying anaC-osc genes using a genomic DNA standard (Osc-193 UHCC).
Standard curves were set up using the genomic DNA of anatoxin producing *Oscillatoria* Osc-193 (UHCC) with 6 serial dilutions. The first dilution was made from DNA of *Oscillatoria* Osc-193 which was aslready diluted to 1:10. Afterward, other five dilutions were made consecutively (Fig. 5).

For each standard, the concentration (number of copies) of diluted DNA was plotted against the mean C_p (crossing point, determined in triplicate) value (Rodríguez-Lázaro and Hernández, 2013), and the slope of the standard curve was calculated by performing a linear regression analysis following instrument protocols (Roche Diagnostics GmbH, 2008).

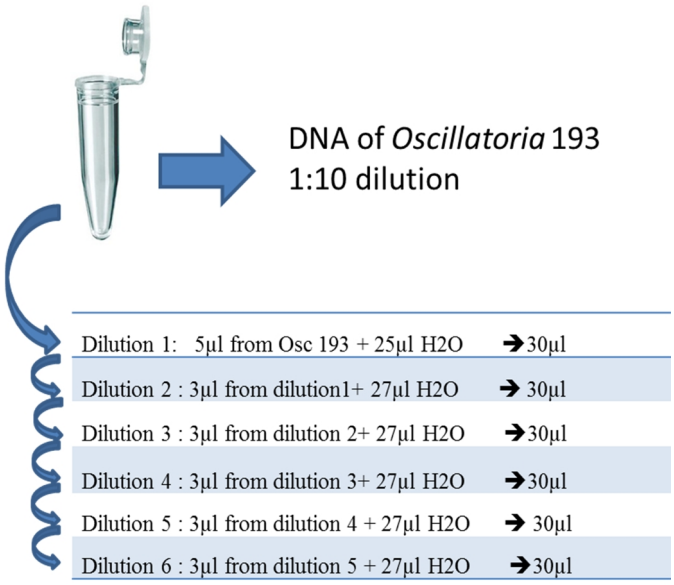

Figure5. Preparing serial dilution for standard curve.

The extracted DNA from environmental samples of 2013 and positive control were diluted with distilled water at a ratio of 1:100. All the reactions were prepared with 30µl volumes in 96-well qPCR plates. The reaction mix contained 12.5 µl iTaq SYBR Green super mix (BIO RAD), 1.25µl of primer

anaC-osc -F/R, 10 µl of distilled water, and 5µl of the DNA template. Besides environmental samples, analyses were made also on negative controls without DNA, and positive controls comprising the genomic DNA of the strain *Oscillatoria* 193 (Osc-193, UHCC). Each sample was prepared in triplicate. Table1 shows the thermal cycle used for qPCR analysis in this study.

The results of qPCR analysis are reported in Chapter 5- Section 5.10.3. Additional data.

Table1. qPCR protocol and thermal cycle applied in this study.

Step	Program Name	Temp.	Time	Cycle	Ram rate (°C/s)	Acquisitions (per °C)
1	Activation	95 °C	3 min	1	4.4	------
2	qPCR	95 °C	15 Sec	35	4.4	------
		60°C	45 Sec		2.2	------
3	HRM	95°C	15 Sec	1	4.4	------
		40°C	1 min		2.2	------
		58°C	1 Sec		1.1	------
		95°C	------		0.02	------
4	Cooling	37°C	00:00:00	1		25

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Chapter 3

3. Inter-annual variability of the microcystins pool in the oligo-mesotrophic Lake Garda (Italy)

Manuscript: **(*In preparation*)**

Inter-annual variability of the microcystins pool in the oligo-mesotrophic Lake Garda (Italy)

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ABSTRACT

Cyanobacteria have the ability to produce an extraordinary variety of secondary metabolites, some of which are toxic for humans. Massive developments (blooms) of these microorganisms represent a major concern in many natural and artificial water bodies, because of the high levels of toxins which can be potentially released in the water and eventually uptaken by humans (mainly by ingestion). Microcystins, potent hepatotoxins, represent the most frequent toxins produced by cyanobacteria. They show a big variability in chemical structure and, in fact, more than 90 variants have been reported. The chemical diversity has an impact on the toxicity, which differs substantially from variant to variant. The factors triggering the toxin production in cyanobacteria as well as the reasons of the wide chemical variability are still matter of debate. In order to elucidate the dynamics of toxin production inside a population of cyanobacteria, we have investigated the temporal and spatial variability of the microcystin diversity in the cyanobacterial populations of Lake Garda. The lake, which represents the biggest water basin in Italy, was sampled on a monthly basis from 2008 till 2013. Toxin analysis, based on LC-MS/MS technique, was performed on phytoplankton samples taken at discrete depths in the trophogenic layer. The investigation showed a seasonal pattern of toxin production (with typical late summer-early autumn peaks) and a relatively constant toxin diversity (with five variants accounting for almost the totality of the microcystin content) with one variant (MC-RRdm) always dominating over the others.

Keywords

Lake Garda, Cyanobacteria, Microcystins

3.1. Introduction

Toxic cyanobacteria blooms represent a serious threat for freshwater environments. Cyanobacteria prefer stable and stratified freshwaters, where they grow in higher abundances. Factors that favor their growth are nutrient availability and warm water. Eutrophication and global warming are therefore the stressors that can trigger cyanobacteria blooms (Paerl and Huisman, 2009).

Besides the ecological problems related to the development of massive biomasses, cyanobacteria blooms have sanitary implications; some species, in fact, have the ability to produce toxic substances (cyanotoxins) which can poison animals and humans. Cyanobacteria produce a wide range of toxic compounds: peptides (microcystins, microginines, microviridines, etc.), alkaloids (cylindrospermopsins, anatoxins, saxitoxins). Microcystins are the most frequent compounds, more than 110 variants have been described so far (Dietrich and Hoeger, 2005).

MCs have been subject to toxicological studies which have demonstrated that they can have serious acute and also chronic effects; they have been classified by IARC as potential carcinogenic for humans. It is worthy underlying that most of the toxicological studies have been performed on the most common MC-LR variant, but few studies demonstrated that a huge variability exists among variants.

Microcystins are cyclic eptapetides synthetized by cellular non ribosomal protein synthetase (NRPS) system. In Fig. 1 the chemical structure of MC-RR is reported, having two Arginine (R) residues in the variable positions 2 and 4. The same picture shows also other sites of common modifications, i.e. aminoacids 3 and 7. The brief table (Table 1) reports the MC analogs with variations on these two aminoacids. Although more than 110 MC variants have been described, it is no point out that cyanobacteria usually produce one or few variants in high amounts, and others at trace levels. From the available literature we have found that the dominant MC variants in the environment are LR, RR, YR, and their respective closest demethylated derivatives. Since in water samples complex mixture of MC variants may occur, to provide an accurate measure of the toxin concentration, the toxicity equivalent factor (TEF) is used (van Apeldoorn *et al*. 2007) and the TEF for microcystin-LR was set as 1.0 as the most toxic MC variant (see Table 3).

In the present paper, we report the results of a 5 years investigation carried out in Lake Garda aimed at describing the toxin dynamics in a oligo-mesotrophic lake hosting a population of *Planktothrix rubescens*. The toxin analysis was performed with the same protocol thus providing a reliable and

intravalidated database. We set up an analytical protocol for the detection of 40 MC analogs starting from 8 commercial standards and enlarging the panel to their close related demethylated and bis demetylated analogs. As illustrated in Fig. 1, where the case of MC-RR is shown, for each reference toxin we added three demethylated (dm) analogs, and one bisdemethylated (ddm) analog.

MC producing cyanobacteria in Lake Garda are represented mainly by *P. rubescens*, with sporadic and limited (in time and space) presence of *D. lemmermannii*. Conversely, populations of *Tychonema bourrellyi*, which were recently discovered in Lake Garda, did not show any MC production (Shams *et al*., 2015). Therefore Lake Garda represents a good study site where investigate the dynamics of MC production by *P. rubescens* in natural conditions, thus offering the possibility of spotting changes in toxin profile. Importantly, Lake Garda is an LTER site where a detailed measure of physical, chemical and biological variables are measured, thus providing the opportunity of linking the eventual variation in toxic profile to specific environmental drivers.

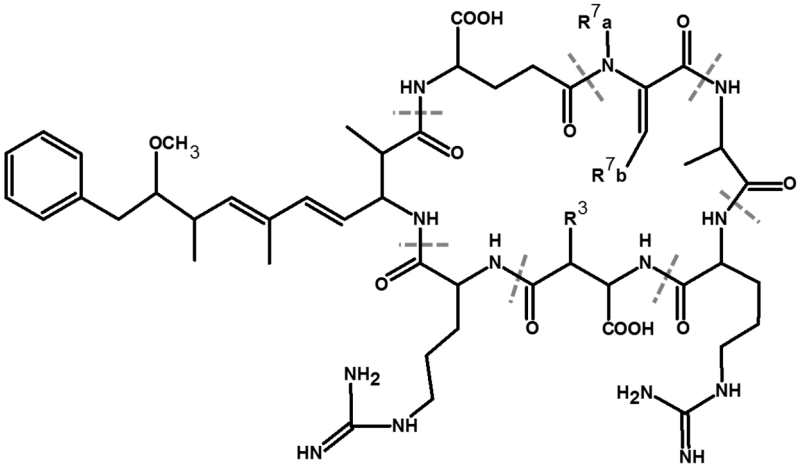

Figure1. Chemical structure of MC-RR showing variability positions at aminoacids 3 and 7.

Table1. MC analogs with variations on aminoacids 3 and 7.

Toxin analog	abbreviation	Molecular weight	R^3	R^7a	R^7b
MC-RR		1037	CH3	CH3	H
[D-Asp3] MC-RR	RRdm	1023	H	CH3	H
[Dha7] MC-RR	RRdm	1023	CH3	H	H
[DAsp3, Dhb7] MC-RR	RRdm	1023	H	H	CH3
[D-Asp3, Dha7] MC-RR	RRddm	1009	H	H	H

3.2. Methods

3.2.1 Study site and sample collection

Lake Garda is the largest Italian lake. It is located at the southern border of the north eastern Italian Alps. Sampling was conducted in the LTER station (Brenzone) which is located in the deepest part of the lake (45.69 N, 10.72 E). Sampling was conducted with a monthly frequency from October 2008 till October 2013. Water sample (2 liters) for chemical analysis was collected at 4 dephts: 0, 10, 20 and 60 m. The water was filtered on a 0.45 μm GF/C filter to collect the phytoplankton. The filter was stored at -20°C until toxin extraction.

3.2.2 Cyanotoxins analyses

3.2.2.1 Toxin extraction

Extraction was performed as follows: each freeze-thawed filter was homogenated for 5 minutes with 8 ml of extracting solvent (water: methanol 30:70, 0.1% formic acid). The suspension was centrifuged at 10000g for 5 minutes; the supernatant was transferred and the pellet was re-extracted with 8 ml of the extraction solvent. The two supernatants were combined. A 2 ml aliquot of the extract was filtered on Phenex-RC syringe filter (0.2 μm pore size, Phenomenex) and analyzed by LC-MS

3.2.2.2 LC-MS analysis

The LC-MS analyses was carried out on a Waters Acquity UPLC system directly coupled to an AB SCIEX 4000 QTRAP mass spectrometer equipped with a turbo ion spray interface. Standard injection volume was 2 μL. MC analysis was carried out using a Phenomenex Kinetex XB-C18 column (1.7 μm particle size, 2.1 × 50 mm) at 40 °C. The mobile phase consisted of water (A) and acetonitrile (B), both containing 0.1% formic acid. A linear gradient scheme was employed: the starting eluent was 80% A, decreased to 30% A at 4.5 min, and finally restored at 80% A at 6.5 min (hold 0.5 min). The total run time was 7 min with a flow rate of 0.25 ml min-1. The mass detector was operated in positive Electro Spray mode (ESI+) using the Multiple Reaction Monitoring (MRM) scanning mode. General settings were as follows: ion spray voltage 5000 V, entrance potential 10 V, cell exit potential 10 V, interface heater temperature 300 °C. For each target compound, two transitions were monitored. The quantification of the toxins was performed with the external standard procedure, in which, for each compound, a calibration curve was obtained using the most intense transitions. For demethylated (dm) and bisdemethylated (ddm) variants, quantification was carried out using the calibration curve

of the parent methylated variant, assuming a similar instrumental response factor. Compound-specific settings of the mass detector and performance data of the methods are listed in Table 2. In the same table, a full list of monitored variants is reported. Microcystins analytical standards (RR, YR, LR, WR, LA, LY, LW, LF) were purchased from Vinci Biochem. All solvents and reagents used in this procedure were LC-MS grade. The limits of quantitation (LOQ) were between 30 and 500 ng L-1 (different MC congeners).

Table2. Compound-specific parameters in LC-MS analysis of toxins. Only data for the eight standards are shown. For the demethylated (dm) and bis demethylated (ddm) variants the MRM transitions are modified according to their molecular weights. All other parameters are kept constant.

RT = chromatographic retention time; DP = declustering potential; CE = collision energy.

Toxin variant	**RT (min)**	**MRM transitions (m/z)**	**DP (V)**	**CE (V)**
RR	1.43	520.1/135	85	44
		520.1/213	85	50
YR	2.63	523.6/135	45	20
		523.6/911	45	20
LR	2.78	498.6/135	40	19
		498.6/213	40	43
WR	3.06	535.0/135	40	18
		535.0/213	40	42
LA	4.30	911.6/135	85	90
		911.6/213	85	65
LY	4.40	1002.6/135	106	96
		1002.6/213	106	75
LW	4.80	1025.6/135	111	100
		1025.6/213	111	80
LF	4.93	986.6/135	96	95
		986.6/213	96	73

3.2.2.3 Estimation of cyanotoxins diversity

The diversity of cyanotoxins was estimated by applying two diversity indices of general use, i.e. the inverse of the Simpson index, 1/D,

$$D = \sum_{i=1}^{n} p_i^2$$

and the Shannon index, H',

$$H' = -\sum_{i=1}^{n} p_i \ln p_i$$

where p_i is the proportional abundance of the congener *i*.

3.3. Results and discussion

Monthly data about the toxin profile of Lake Garda have been collected from September 2008 to October 2013 with few exceptions (Dec 2008, Dec 2009, Dec 2011, Apr and Aug 2012, Jan 2013), and a total of nearly 230 samples have been analyzed. In Fig. 2, the total MC content (sum of the different variants) measured in the five years survey is shown. There is a clear seasonality in the MCtot, with lower values in winter and spring seasons and higher in summer and autumn. In particular, values were below 40 ng l^{-1} from November to June in all years. From July to October, values were higher. A great variability is present from year to year: the summer peak showed a progressive increase from 2009 until 2011 (95 ngl^{-1} in 2009, around 200 ng l^{-1} in 2010 and 2011) and afterwards a progressive decrease (73 ngl^{-1} in 2012 and 33 ngl^{-1} in 2013). In the graph, we can also note that the summer peak occurred in Aug-Sept period with the only exception for 2009 when the peak occurred one month later (Sept-Oct).

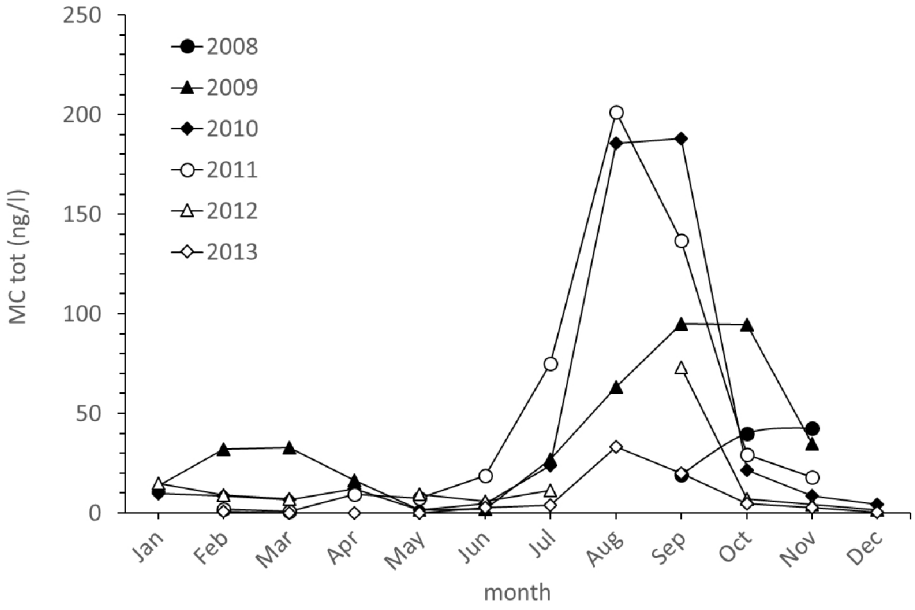

Figure2. Montly 0-20 m mean values of MC tot plotted per year.

In Fig. 3 the statistical distribution of the MCtot among the four considered depths is shown. It is evident that MC is basically distributed in the 0 – 20 m water layer with very similar concentrations. The median values were 7.5, 8.2, 12.4 ngl^{-1} (at 0, 10 and 20 m, respectively). The MC present at 60 m are much lower (median =3.8 ngl^{-1}).The boxplot in Fig. 3 also shows that the highest concentrations of toxins (in coincidence of the MC summer peaks) occur at 20 m depth, where 5-95th percentile bars are about threefold higher than 0 and 10 m. The highest value found in the survey was recorded at 20 m depth (540 ngl^{-1}), while highest values at 10 and 0 m were, respectively, 290 and 260 ngl^{-1}.

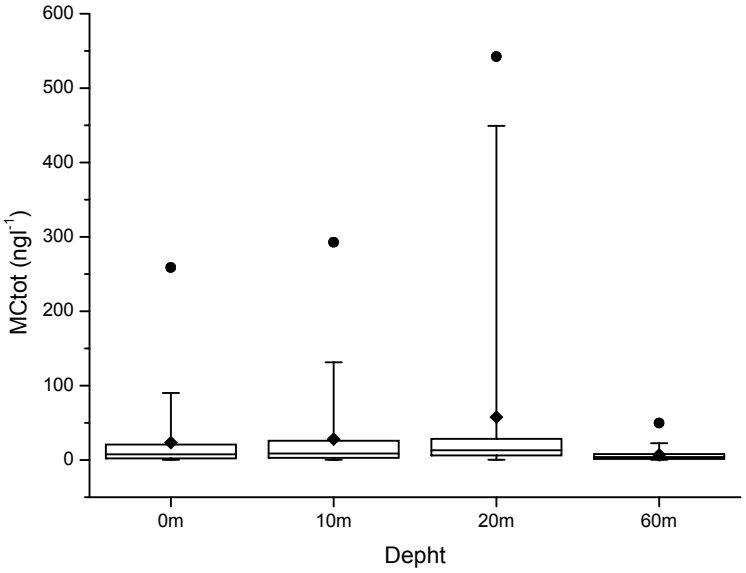

Figure3. Distribution of the MC content among layers.
Legend: ● = max, ♦ = mean, bars = 5 – 95th percentile, box = 25 – 75th percentile with median.

As far as the MC diversity is concerned, we could detect five different variants out of the 40 (Table 3).Two demethylated variants (RRdm and LRdm) represented almost the totality of the MC pool, with the former being the dominant one. The other MC variants were MC YR, LR and RR. In Table 3 a synoptic view of the MC diversity and distribution in the four depths is reported. The MC diversity is very constant in the water column, with RRdm around 93% on average, LRdm around 4%, YR around 1.7%, LR around 0.4% and finally RR around 0.6%.

Table3. Relative contribution of different MC congeners at different depths. Mean % values and (in brackets) the min and max values are reported. Relative toxicity values (compared to MC-LR=1) indicate that MC-RRdm (the dominant congener) has a toxicity four times lower than MC-LR.

depht	RRdm	LRdm	YR	LR	RR
0 m	93.0 (79.4 – 100)	4.2 (0 – 20.6)	1.7 (0 – 10.2)	0.6 (0 – 5.3)	0.5 (0 – 16.1)
10 m	93.0 (70.1 - 100.0)	3.7 (0 - 22.2)	1.7 (0 – 12.6)	0.4 (0 - 4.5)	1.2 (0 - 23.9)
20 m	92.0 (70 - 100.0)	4.4 (0 - 21.3)	2.3 (0 – 19.2)	0.7 (0 – 14.2)	0.5 (0 - 10.4)
60 m	94.5 (80.1 - 100.0)	4.0 (0 - 18.2)	1.2 (0 - 14.1)	0.02 (0 - 1.1)	0.3 (0 - 4.5)
Relative toxicity	0.23	0.33	0.29	1	0.1

In order to better follow the temporal dynamics of the MC diversity, in Fig. 4 we reported the temporal behavior of the five variants. It is evident a certain degree of fluctuation in the MC composition. In

particular, considering the two major variants (MC RRdm and LR dm), reported in panel b of figure 4, they seem to be inversely correlated: when MC RRdm declines, LRdm grows, and *vice-versa*. RRdm showed an increasing trend from Oct 2008, when it was approx. 80%, to Oct 2010, when it reached 100%; then a steady state followed (4-5 months long), before a decreasing trend started lasting approximately one year in which the RRdm dropped down to 78% (May 2012). Afterwards, RRdm againg went gradually up againg to 100% (Nov 2012), when a new steady state occurred (3-4 months), before a new decreasing trend took place in the last 6 months of observations. All these changes in the RRdm relative abundance were paralleled by a counter-changes in the LR dm abundances (Fig. 4b), which reached highest value (ca. 21%) in May 2102. The other 3 MC variants (YR, RR and LR) also showed a fluctuation (Fig.4c). More appropriately, for these variants an annual oscillation can be detected. In fact, for MC YR and LR the highest percentages were registered during the summer-autumn time of each year (in coincidence with the maxima in MC concentrations). MC RR showed a similar pattern in 2011 and 2012; but showed a different pattern in the other years, when this toxin exhibited annual oscillations with maxima a couple of months ahead of the MC peak: in May 2009, June 2010, and June 2013.

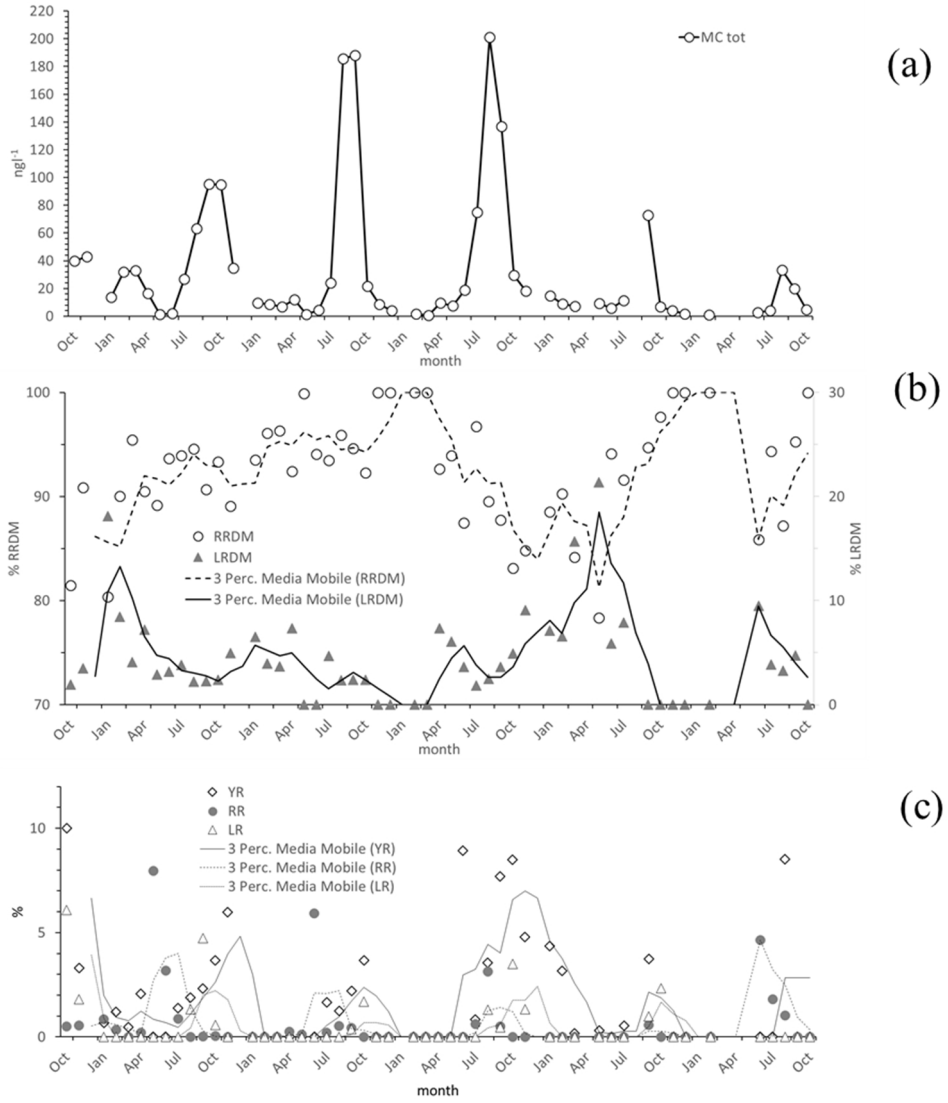

Figure4. Temporal behavior of the five MCvariants; mean values are considered. (a) Temporal dynamic of total MC, (b) Temporal dynamic of two major variants (MC RRdm and LR dm), (c) Temporal dynamic of other 3 MC variants (YR, RR and LR).

To check whether the observed MC diversity could be influenced by the MC concentration, as the higher the concentration the better the resolution in the LC-MS experiments and in turn the better the quantification of minority variants, we performed a series of statistical calculations (Fig. 5) which demonstrated that actually the concentration did not influence the diversity data, thus valorizing what we argumented before.

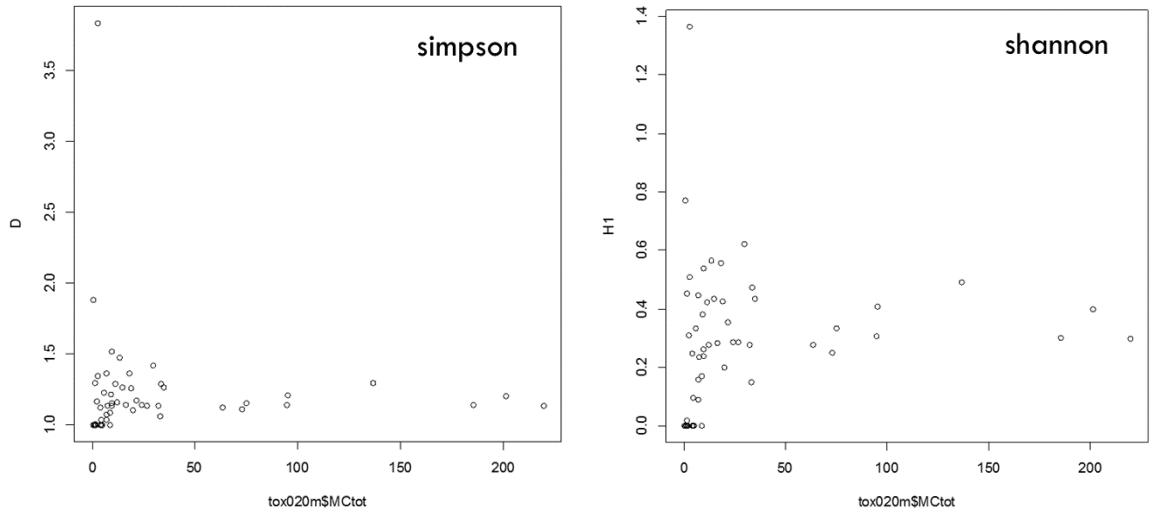

Figure5. Statistical approach for correlating MC diversity and abundances.

We applied a PCA study on our data (fig. 6) which clearly showed a seasonality pattern in MC production: between July and October all five variants increased, with LR showing a typical preference for later periods. The figure also shows the annual cycle in MC production. Another information that can be obtained by the PCA analysis is the relationship between variants. Together with correlation chart (Table 4) they show that four MC variants are highly correlated, which means they form a "cluster" and they are produced at the same time. On the contrary, MC-LR does not belong to that cluster but appears to be produced independently.

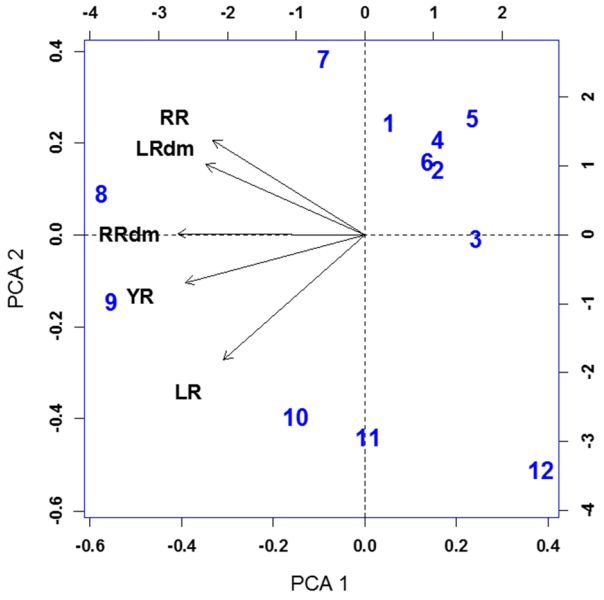

Figure6. PCA showing the seasonality in MC production and inter-variant correlations.

Table4. Correlations among MC variants. Correlations with MC-LR (which appears to be produced independently from the other congeners) are highlighted in yellow.

	RR	RRdm	YR	LR	LRdm
RR	1.00	0.85	0.77	0.03	0.80
RRdm	0.85	1.00	0.87	0.28	0.92
YR	0.77	0.87	1.00	0.24	0.85
LR	0.03	0.28	0.24	1.00	0.18
LRdm	0.80	0.92	0.85	0.18	1.00

n= 54

P

	RR	RRdm	YR	LR	LRdm
RR		0.0000	0.0000	0.8521	0.0000
RRdm	0.0000		0.0000	0.0423	0.0000
YR	0.0000	0.0000		0.0870	0.0000
LR	0.8521	0.0423	0.0870		0.1957
LRdm	0.0000	0.0000	0.0000	0.1957	

Implications for water management. As reported in table 3, the toxicities of the five variants are very different. In the case of Lake Garda, the health risks are basically due to the presence of the MCRRdm variant, which represent typically more than 90 % of the total. This variant is less toxic than the reference LR variant (by a factor of 4). This toxic profile is quite constant in the lake also in the summer season when higher cyanobacteria biomasses develop. Guidelines referring to MC content based on the MC-LR will therefore greatly overestimate the risks.

NOTE: The manuscript is in preparation, other parts will be completed as future work.

3.4. ADDITIONAL DATA

Beside the above mentioned results which are going to be included in manuscript 1 presented in CHAPTER 3, some results on ATX concentration have been also obtained which may use for future publications.

3.4.1 Temporal dynamic of ATX

Chemical analyses of samples taken from Lake Garda on 2010 till 2013, demonstrated that ATX is always present thtoughout a year with the maximum value recorded for the summer month; June and July (Fig. 7)

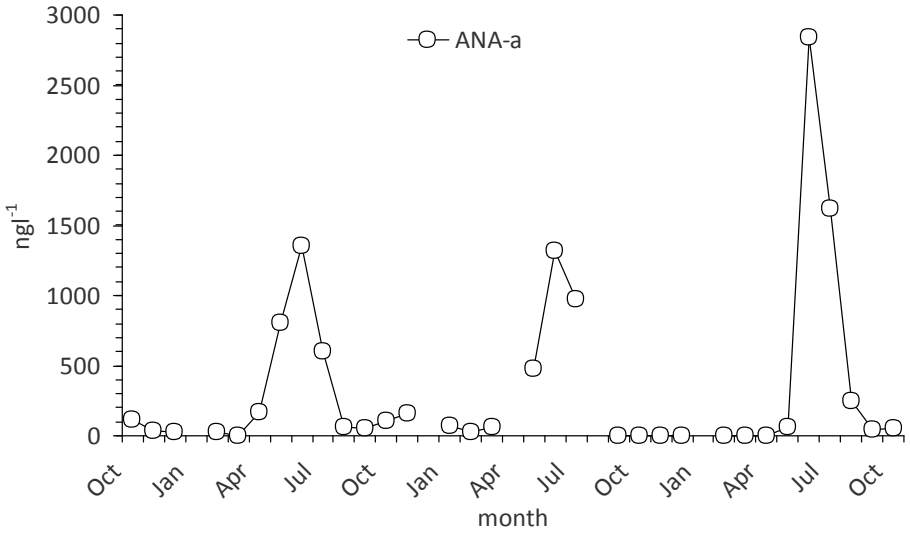

Figure7. ATX concentrations from Oct 2010 till Oct 2013 (0 – 20 m mean values).

3.4.2 Temporal dynamic of ATX and total MC

As shown in (Fig. 8), the ATX value has been always higher than MCtot value. The investigation showed a seasonal pattern for ATX production with early summer peak specially on June-July and for MCtot with typical late summer-early autumn peaks.

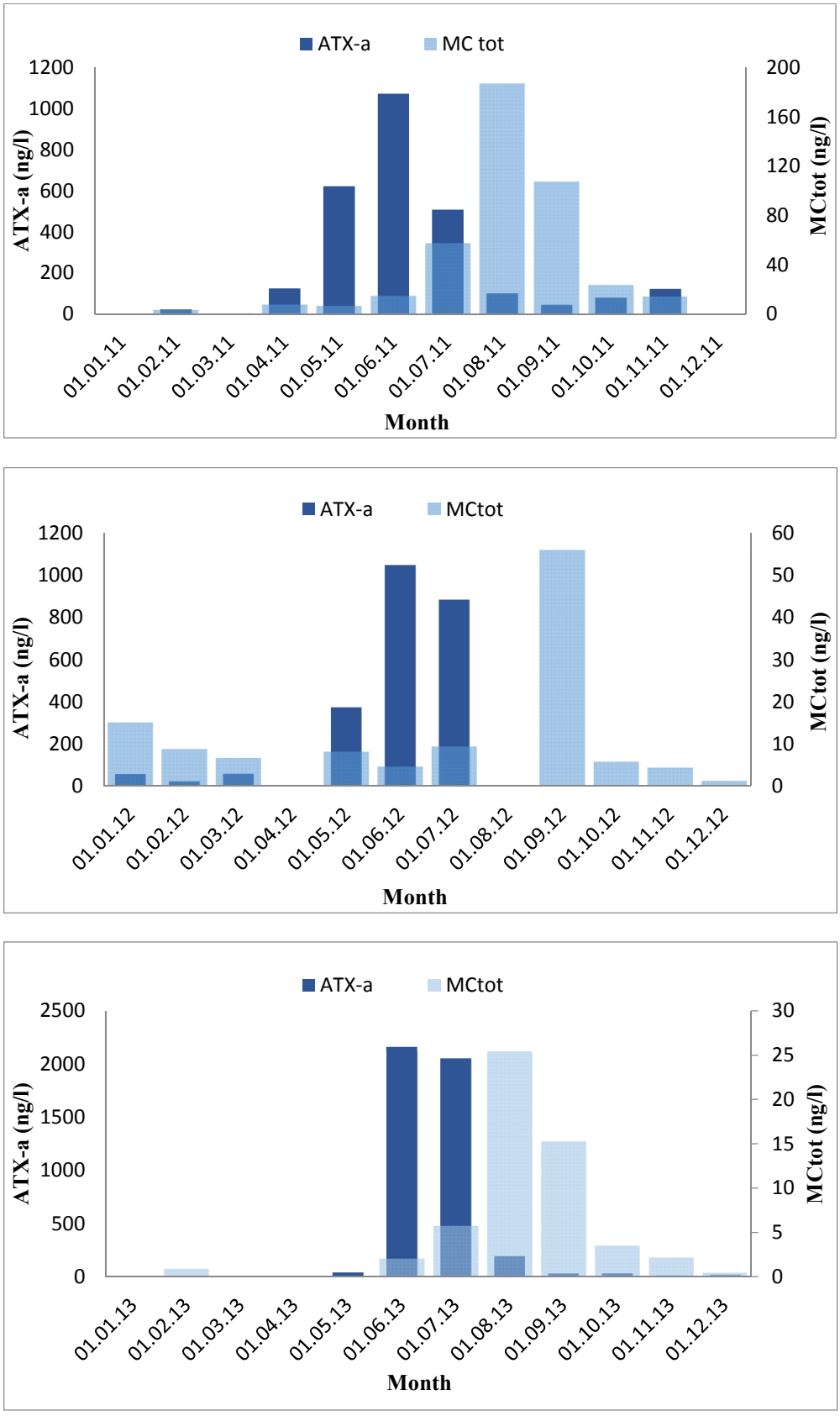

Figure8. Temporal dynamic of ATX and MCtot in Lake Garda from 2011 till 2013.

Chapter 4

4. Experimental models of microcystin accumulation in *Daphnia magna* grazing on *Planktothrix rubescens*: Implications for water management

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Experimental models of microcystin accumulation in *Daphnia magna* grazing on *Planktothrix rubescens*: Implications for water management

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ABSTRACT

In this study, we investigated the kinetic aspects of the microcystin (MC) transfer from *Planktothrix rubescens* to *Daphnia magna* by carrying out exposure experiments in small simple mesocosms. We hypothesized that higher fractions of toxic cyanobacteria in the diet of grazers would shift the balance towards a greater than linear, i.e. non-linear accumulation of MC in *D. magna*. This hypothesis was tested by exposing *D. magna* to varying initial densities of MC-producing *P. rubescens*. The evolving models of MC accumulation differed largely as a result of the duration of exposure and initial MC concentrations used. Within the first 24 h of exposure, MC accumulation in *D. magna* was linear, irrespective of the initial densities of toxic *P. rubescens* and thus MC concentrations. After 48h of exposure, MC accumulation in *D. magna* showed an exponential pattern, possibly due to a delayed digestion of *P. rubescens* and/or decreased MC detoxification capabilities when compared with higher ambient concentrations of MC.After 72h toxin concentrations in *Daphnia* drop in all experiments as a consequence of the reduced cyanobacterial cells in the medium and the detoxification of MC within *Daphnia*. The results obtained suggest that in lakes with higher MC content and longer cyanobacterial bloom period MC accumulation in *D. magna* should be more pronounced than in mesotrophic lakes with lower MC content. The latter interpretation, however, should be verified investigating accumulation of MC both in larger mesocosms and in situ, in lakes of different trophic status.

Keywords

Planktothrix rubescens; Cyanobacteria; *Daphnia magna*; Microcystins; Bioaccumulation; Lake Garda

4.1. Introduction

Cyanobacterial blooms have become a growing global concern due to their increased occurrence as well as to the massive increase of freshwater utilization throughout the world (Paerl *et al.,* 2011). Many cyanobacterial species are able to produce a wide range of toxins (Sivonen and Jones, 1999; Sivonen and Börner, 2008). One of the more intensely studied toxin groups is represented by microcystins (MCs) (Metcalf and Codd, 2012). So far, more than 110 different variants of MCs have been reported (Dietrich and Hoeger, 2005; Spoof, 2005; Puddick, 2013), primarily produced by freshwater cyanobacteria, e.g. *Microcystis*, *Planktothrix*, *Anabaena*, and occasionally *Nostoc* (Sivonen and Jones, 1999; Salmaso *et al*., 2013).MCs are cyclic heptapeptides that consist of 5 D- and 2 variable L- amino acids. They are characterized by high chemical stability. The degradation of MCs in water occurs very slowly, and is primarily the result of microbial breakdown and to a lesser extent the result of photolytic and hydrolytic processes (Tsuji *et al*., 1994). Due to their stability, MCs have the capability of being accumulated in a variety of aquatic organisms including bivalves, crustaceans, zooplankton and fish (Ferrão-Filho *et al*., 2002; Zhang *et al*., 2009; Ernst *et al*.,2007, 2009; Lemaire *et al*., 2012; Wojtal-Frankiewicz *et al*., 2013).There has been growing attention towards the effects of MCs on zooplankton and especially on the larger cladoceran Daphnia because of the key role that these organisms have in the aquatic food web (Elser, 1999; Benndorf *et al*., 2002; Reichwaldt *et al*.,2013). They feed on primary producers and represent a major source of food for juvenile fish, consequently they can act as transfer vectors of toxins to higher trophic levels (Rohrlack *et al*., 2005) Unlike copepods, which appear to be able to discriminate between toxic and non-toxic cells (DeMott and Moxter, 1991), daphnids are non-selective filter feeders and are a priori not able to select food particles that differ in quality (DeMott, 1986). However, *Daphnia pulicaria* was demonstrated to be able to discriminate between toxic and non toxic *Microcystis aeruginosa* cells, whereby MC content was obviously not the determining factor for reducing filter feeding when *D. pulicaria* was confronted with toxic *M. aeruginosa* (Jungmann *et al*., 1991; Jungmann, 1995). Several investigations have shown that filamentous cyanobacteria have a negative effect on *Daphnia* because of the interference of filaments with grazing on other available food sources (Porter and McDonugh, 1984; Hawkins and Lampert, 1989; Kurmayer and Jüttner, 1999). Kurmayer (2001) reported that *Daphnia galeata* was able to ingest the filamentous cyanobacterium *Aphanizomenon flexuosum* and that mechanical interference was not important. Nadin-Hurley and Duncan (1976) have argued that the width of filaments is more important than length in limiting ingestion by daphnids, while DeMott (1995) suggested that filament size together with filament hardness are important for Daphnia feeding on large particles. Oberhaus *et al*. (2007) finally reported that *D. pulicaria* preferred to

graze on short filaments of *Planktothri*x *rubescens* and *P. agardhii* and could efficiently control *Planktothrix* blooms in their early stages. Above results highlight the complexity of feeding mechanisms underlying grazing of filamentous cyanobacteria by *Daphnia*, whereby the specific feeding preferences appear to be largely dependent on the *Daphnia* and cyanobacteria species involved (Hulot *et al*., 2012). Nevertheless, in spite of the widespread occurrence of toxic *Planktothrix* in European lakes (Davis *et al*., 2003; Salmaso *et al*., 2003; Ernst *et al*., 2009), *Daphnia* grazing on filamentous cyanobacteria has been investigated so far by only a limited number of studies (see e.g.Kurmayer and Jüttner, 1999; Oberhaus *et al*., 2007; Pires *et al*., 2007; Reichwaldt and Abrusan, 2007).

Adverse effects of MC on *Daphnia* have been reported by many studies, e.g. laboratory experiments by DeMott (1999) investigated the effects of *M. aeruginosa* on five different *Daphnia* species. Out of the five species tested, *D. pulicaria* showed the lowest growth inhibition and *D. pulex* the highest. *D. galeata*, instead, exhibited symptoms of exhaustion that finally led to death (Rohrlack *et al*., 2005). Recently, Dao *et al*. (2010) provided evidence that offspring produced by *Daphnia magna* pre-exposed to MC-LR or cyanobacteria crude extract, not only showed delayed maturation but also increased mortalities.

A few other studies explored the accumulation of MCs in large cladocerans. Thostrup and Christoffersen (1999) in a laboratory experiment documented that *D.magna* grazing on *M. aeruginosa* could lead to an accumulation of MCs up to 24.5 µg g^{-1}dry weight. Similarly, Oberhaus *et al*. (2007) demonstrated that *D. pulicaria* was able to accumulate MCs up to 1099 µg g^{-1}dry weight when grazing on filaments of *Planktothrix*. Nevertheless, the accumulation kinetics of MC in large cladocerans was poorly investigated. At the same time, no information was available regarding the type of relationships governing MC accumulation as a function of exposure time and ambient MC concentrations.

Following the observed feeding behaviours of *D. pulicaria* towards toxic and non-toxic small-celled *M. aeruginosa*, the question was raised whether feeding on different proportions of toxic and non-toxic filaments would result in different MC accumulation patterns in exposed daphnids.

On the basis of above considerations we decided to investigate the MC accumulation patterns in *D. magna* after exposure to populations of *Planktothrix rubescens* with different proportions of toxic and non toxic strains. Assuming that accumulation occurs when a positive net balance exists between MC uptake and concomitant MC loss resulting from excretion and detoxification (e.g. oxidation

and/or conjugation), we hypothesized that higher concentrations of toxic filaments would result in a proportionally higher and non-linear accumulation of MC in *Daphnia*. More specifically, we analyzed the suitability of linear and exponential models for explaining the relationships between MC accumulation in *Daphnia*, ambient MC concentrations, and both initial MC exposure and time after initial exposure.The results will be discussed also taking into considerations the implications for water management in lakes of different trophic status and abundance of *Planktothrix*.

4.2. Materials and methods

4.2.1. Chemicals and analytical equipment

Solvents and reagents used for toxin extraction and analysis were LC-MS purity grade. MC (including [d-Asp^3] MC-RR) analytical standards were purchased from Sigma–Aldrich.The ultrasonic homogenizer was an Omni Sonic Ruptor 4000 equipped with a processing tip of 4 mm in diameter. The LC–MS system consisted of a Waters Acquity UPLC® directly coupled to an AB Sciex 4000 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Cerasino and Salmaso, 2012).

4.2.2. *P. rubescens* and *D. magna* cultures

Single filaments of toxic and non-toxic *P. rubescens* were isolated at the Long Term Ecological Research (LTER) station of Lake Garda (Lat 45 °41"N, Long 10°43' 15") and cultured in flasks containing BG11 medium in a temperature-controlled chamber at 15 °C with 8:12 h light:dark cycle and a light intensity of 30 mmol photon $m^{-2}s^{-1}$. Cultures were periodically analysed for density and MC content. Toxic cultures contained only one toxin, namely [dAsp ^{3}] MC-RR.

D.magna batches were provided by Eschematteo srl, Italy. *Daphnia* were cultured in a glass aquarium filled with dechlorinated tap water in a temperature-controlled chamber at 15°C with 8:12 h light:dark cycle and a light intensity of 30 µmol photon $m^{-2}s^{-1}$. *Daphnia* were fed with cultures of green algae (*Scenedesmus sp*.) and baking yeast (*Saccharomyces cerevisiae*). The yeast was resuspended in water before feeding.

4.2.3. Experimental setup

To investigate MC accumulation, 100 adult *D. magna* were put into 1 L glass beakers filled with *P. rubescens* cultures with MC concentrations of 9.0, 3.8, 2.4 and 0.6 μg $^{l-1}$, hereafter referred as exposures A, B, Cand D. In order to achieve the different MC concentrations, different proportions of toxic and non-toxic strains of *P. rubescens* were mixed and diluted with BG11 medium to 1L. A control group was used containing a comparable density of the chlorophyte *Scenedesmus sp*.

Exposures B–D contained the same density of *P.rubescens* (approx. 54,000 cells m^{l-1}), while exposure A contained approx.22,000 cells ml−1. All beakers were placed in a temperature controlled climatic chamber at 15°C with 8:12 h light: dark cycle and a light intensity of 30 μmol photons $m^{-2}s^{-1}$.Neither exposure medium (BG11) nor *P.rubescens* (exposures)and *Scenedesmus* (controls) were exchanged or replenished during the exposures. *P.rubescens* and *Scenedesmus* density as well as total MC concentrations in the exposure medium and in exposed and control daphnids were determined at 0, 6, 24, 48, 72, and 144 h of exposure. The fate of different densities of *P. rubescens* over time without concurrent grazing pressure was examined in parallel experiments in which *P. rubescens* cultures were diluted at the required densities with the same BG11 medium and kept in the same conditions of the *Daphnia*-containing treatments. All the analyses (MC content and algal densities) were made on three independent replicates, with the exclusion of the densities of *Planktothrix* in the experiments with *Daphnia* (cf. Fig. 1).

4.2.4. Algal density estimation

Densities of *P. rubescens* were determined by counting of Lugol's fixed water sub-samples (1–2 ml) taken from each exposure at the sampling times described above. After dilution, Lugol's fixed samples were counted using the standard inverted microscopic method using 10 ml, 2.5 mm-diameter sedimentation chambers (Lawton *et al*., 1999). *Planktothrix* densities were estimated by determining the length of filaments in 5 equidistant transects at 200× (width of the optical field, 1mm) located on the bottom of the sedimentation chamber, and then dividing the total length of filaments by the length of one cell (5 μm). The procedure and the reliability of this type of density estimation for filamentous species were previously reported by Rott *et al*. (2007).

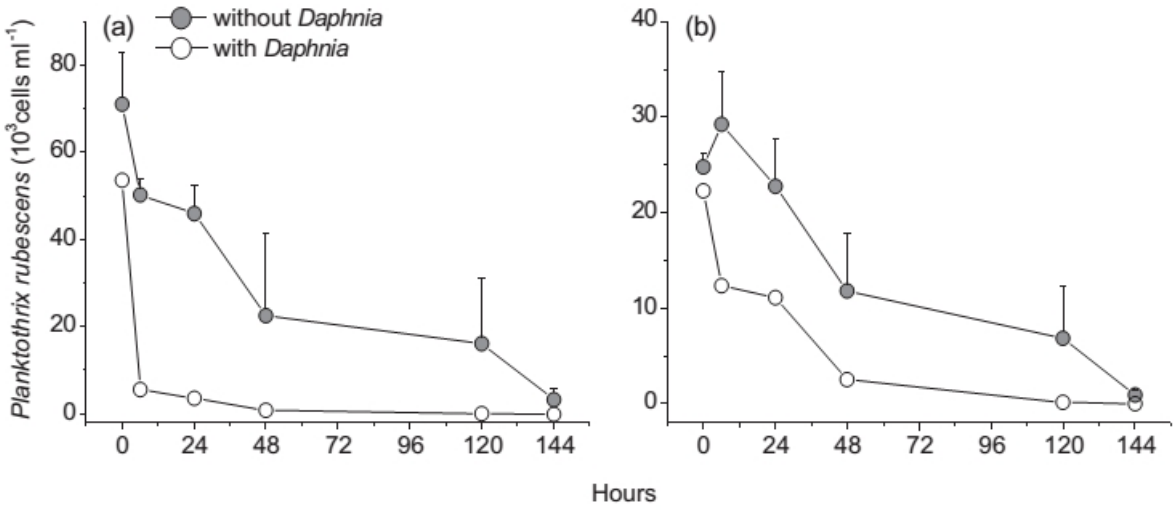

Figure1.Decrease of *P. rubescens* over time in experiments with and without *D.magna* at two different *P. rubescens* densities. (Starting densities) (a): 71,025 cells ml^{-1} without *Daphnia* and 53,630 cells $ml^{-1}$with *Daphnia* present; (b): 24,771 cells ml^{-1} without *Daphnia* and 22,280 cells $ml^{-1}$with *Daphnia* present.) Values "without *Daphnia*" are mean ± SEM (standard errors of the means) of n =3 replicate experiments. Note the different scales of the y-axis in (a) and (b).

4.2.5. Toxin extraction and analysis

The content of MC in both *D. magna* and water was analysed via LC-MS/MS. For MC extraction from *D. magna*, 15 *Daphnia* were randomly collected from the exposure beakers using a pipette with a large tip, gently rinsed twice with distilled water to remove any algae attached to the daphnids, transferred to Eppendorf-tubes with 1ml of water and then subjected to freeze-thawing. For MC extraction from water, 1ml water sample taken from each exposure was freeze-thawed. MC-extracts of *Daphnia* and water samples were prepared by adding 1ml methanol to 1ml sample volume.

This mix was probe-sonicated for 8 min at 120 W in pulsed mode and then filtered through 0.2 µm filter.The filtrate was analysed via LC-MS within 24h of preparation. A more detailed description of the analytical procedures is provided in Guzzella *et al*. (2010) and Cerasino and Salmaso (2012). This method should allow estimation of the overall MC content within *Daphnia*, i.e. including both the metabolized fraction and the fraction contained inside the filaments trapped in the carapace and phyllopods. For experiment A, MC concentrations at time = 0 were not measured, therefore initial MC concentrations for this experiment were inferred from a model relating MC measured after 0 and 6 h in experiments B and C ($r^2 = 0.93$).

4.2.6. Data analysis

The comparison of the decrease of the densities of *Planktothrix* in the experiments with and without *Daphnia* was evaluated computing paired t-tests on the original data (Sokal and Rohlf, 1995). The linear rate of decrease of the densities of *Planktothrix* in the exposures A–D was evaluated using linear regression analysis. Since the decrease of *Planktothrix* densities reflected an exponential pattern the data were log transformed before statistical analyses. The slopes of the regression lines were compared by an analysis of covariance (ANCOVA), with the 4 exposures representing the levels.

The increase of MC in *Daphnia* as a function of initial MC concentrations was evaluated computing both a linear (1) and exponential (2) model,

$$MCD = a + b \times MCi \quad (1)$$

$$MCD = a \times \exp(b \times MC_i) \quad (2)$$

where MC_i and MCD are the MC concentrations in the exposures A–D at the beginning of the experiment and inside *Daphnia* individuals at a given exposure time, respectively.The models were computed and tested for 3 exposure times, i.e. 24, 48 and 72 h (see also Fig. 2). In the above analyses, ANCOVA and regression models were compared based on the Akaike information criterion (AIC) and ANOVA tests. Statistical analyses were calculated in R 3.0.0 (R Core Team, 2013).

4.3. Results

4.3.1. Density of *P. rubescens*

The density of *P. rubescens* in both experimental groups, with and without *Daphnia*, showed an exponential decrease over time. Fig. 1 shows the decrease of *P. rubescens* in two experiments with different cell densities. In experiments with a high cell density (Fig. 1a) and presence of *Daphnia* the average densities decreased considerably, from 53,630 to 5590 cells ml−1 , already after 6 h, while in *Daphnia*-free experiments, the decrease was less steep, i.e. From 71,025 cells ml^{-1} to 50,230 cells ml^{-1} (Fig. 1a). In experiments with low cell densities, the densities decreased less dramatically at the 6 h time-point, from 22,280 cells ml^{-1} to 12,365 cells ml^{-1} in presence

of *Daphnia*. In *Daphnia*-free experiments *Planktothrix* showed a slight increase within the first 6 h (from 24,771 cells ml^{-1} to 29,289 cells ml^{-1}), showing thereafter a gradual decrease (Fig. 1b). In experiments lasting ≥24 h, filaments started to break to smaller sizes. In both cases, the decrease of *Planktothrix* in the grazing experiments with *Daphnia* was significantly larger compared with the experiments without Daphnia (paired t-test, as for Fig. 1a and b, $p = 0.01$ and $p < 0.05$, respectively). In all of the 6-days grazing experiments with *Daphnia* (cf. Fig. 2; Section 3.3), the densities of *Planktothrix* decreased at the same rate, i.e. following similar patterns of an exponential decrease in the 4 experiments A–D (ANCOVA, $p = 0.53$). This demonstrated a comparable grazing effect of *Daphnia* on *P. rubescens*, apparently irrespective of the MCs concentrations/densities of MC containing *P. rubescens* used in the 4 experiments.

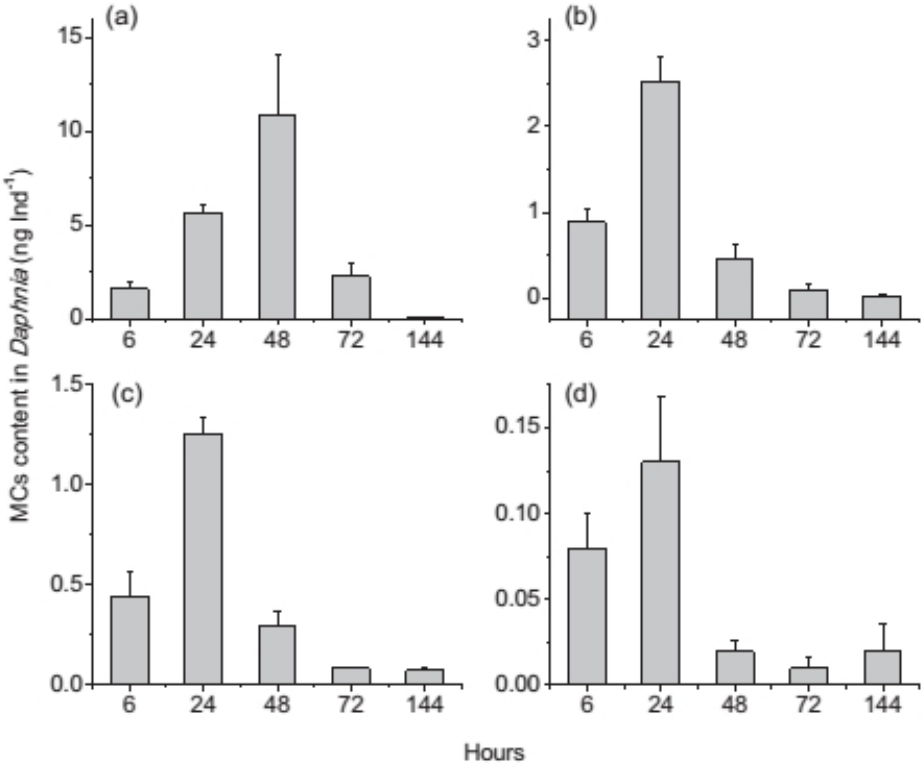

Figure2. MC accumulation in individuals of *D.magna* grazing on *Planktothrix rubescens* in four different exposures of decreasing toxic *P. rubescens* densities and consequently MC concentrations (a = approx. 9, b = 3.8, c = 2.4 and d = 0.6 $\mu g\, l^{-1}$) over 6 days of exposure time. Values are mean + SEM (standard errors of the means) of n = 3 replicate experiments. Note the different scales of the y-axis in (a)–(d).

4.3.2. Total MC concentration

Total MC content (in filaments and water) in all beakers slightly decreased in the first 6 h but then remained approximately stable until 72h, with averages (mean ± SD) of 8.2 ±

0.54, 3.4 ± 0.27, 2.4 ± 0.21 and 0.52 ± 0.07 μg l^{-1} for experiments A, B, C, and D, respectively (data not shown).The dissolved: cell bound toxin ratio increased with time as *P.rubescens* population decreased and cell lysis induced liberation of internal MC added to the MC concentration in the ambient water.

4.3.3. MC accumulation in *Daphnia*

MC was detected in *D. magna* in all experiments containing toxic *P. rubescens* (Fig. 2).The highest MC concentrations in individual *Daphnia* were observed in experiment A (Fig. 2a), and the lowest in experiment D (Fig. 2d) and thus in accordance with the corresponding MC concentrations initially used in the experiments. In experiment A, the highest content of MC in *Daphnia* was reported at 48 h (about 11 ng ind−1), while for experiments B, C and D it was observed at 24 h (about 2.5, 1.2, and 0.1 ng ind^{-1}, respectively). After these concentration peaks, the MC content in the body of *Daphnia* declined, and after 6 days (144 h), MC concentrations were very low: 0.07, 0.03, 0.07, and 0.02 ng ind^{-1} in experiments A, B, C, and D, respectively.

4.3.4. Modelling the accumulation of MC in *Daphnia*

Models of toxin accumulation demonstrated that during the first 24 h, the toxin accumulation was linear, irrespective of the initial concentrations of MC in the experiments (Fig. 3a). However, after an additional 24h toxin accumulation presented an exponential pattern (Fig. 3b), albeit with a considerable drop in the MC accumulation. This pattern was also clearly apparent at 72h (Fig. 3c).

Both the linear (Fig. 3a) and the exponential (Fig. 3b and c) models of MC accumulation were highly significant (Table 1). Owing to the almost constant content of MC in the experiments (Section 4.3.2), very similar results were obtained when comparing the MC accumulation in *Daphnia* with the actual concentrations of MC measured at 24 h, 48 h and 72 h. Despite numerical comparability in the results, we did not continue with modelling of the MC concentrations in the *Daphnia* via ambient MC concentrations, as the internalized MC concentration within the *Daphnia* is the result of MC accumulation, metabolism and excretion and is not governed by a mere concentration diffusion model. The latter considerations are supported by the observed continuous accumulation of MCs in *Daphnia* and the increase in the dissolved:cell bound toxin ratio during the exposure experiments (Section 4.3.2).

Table1. Parameter estimates of the models fitted to the data in Fig. 3a and b. a and b are the parameters in Eq. (1) or (2). RSE, residual standard error (root mean square error, RMSE) on 10 degrees of freedom.

Time exposure	Model	*a*	*b*	RSE	*p*-Value
24 h (Fig. 3a)	Linear (1)	−0.15850	0.64746	0.502	<0.001
48 h (Fig. 3b)	Exponential (2)	0.00122	1.00441	0.808	<0.001
72 h (Fig. 3c)	Exponential (2)	0.06710	0.38610	0.593	<0.001

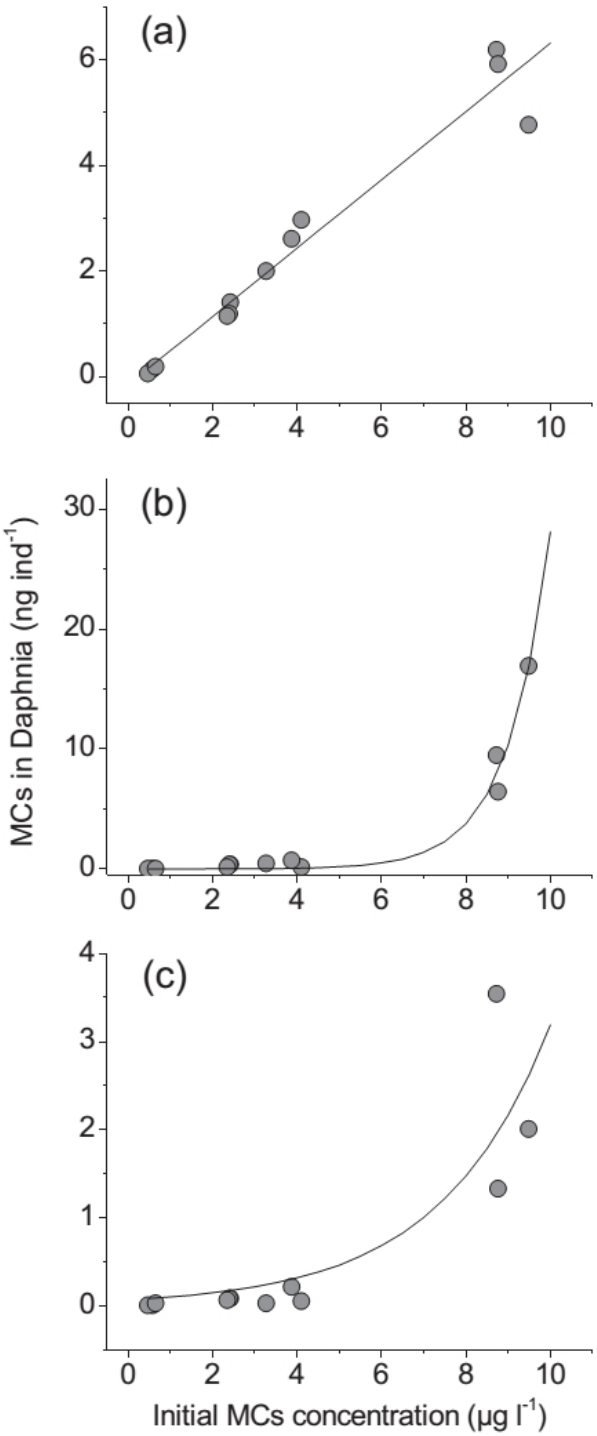

Figure3. Accumulation of MC in *D. magna* as a function of different initial concentrations of toxins, and after (a) 24 h, (b) 48 h and (c) 72h. Data of three independent replicates per initial MC concentrations (MC containing *Planktothrix rubescens* densities) are shown. Note the different scales of the y-axis in (a)–(c).

4.4. Discussion

This study demonstrated the effective and significant grazing of *D. magna* over *P. rubescens* under controlled conditions in a microcosm experiment. In both experimental groups, with and without grazers, *Planktothrix* filaments declined over time, but a more remarkable decrease was observed in the presence of grazers. The disappearance of filaments from water in the experiments without *Daphnia* can be explained by disintegration of filaments and bacterial degradation and/or parasitic infection. In contrast, the decrease of filaments in experiments with *Daphnia* appears to result from the specific grazing activity of *Daphnia*, whereby the ingestion of shorter filaments that are more easily ingested could be favoured, as observed to occur after 24 h. Parasitic chytrid fungi can cause mortality on most cyanobacteria. Nevertheless, Rohrlack *et al*. (2013) demonstrated that the production of microcystins, microviridins and anabaenopeptins, as the most common oligopeptides produced by most cyanobacteria, can reduce the virulence of chytrids to Planktothrix, thereby increasing the host's chance of survival. Therefore, the decrease of filaments in grazing experiments, as observed in the experiments presented here, is most likely the result of a combination of three factors, presence of grazers, disintegration of filaments and bacterial degradation, and/or possible interaction with parasitic chytrid fungi.

No mortality of *Daphnia* was observed during this study. However, the adverse effects of MCs on *Daphnia* such as reduced growth, survival and reproduction, have been investigated in many studies (DeMott and Moxter, 1991; Rohrlack *et al*., 2005; Trubetskova and Haney, 2006; Dao *et al*., 2010). Therefore, we cannot exclude toxic effects of MCs on the physiology of *Daphnia* during our experiments. However, the exponential MC accumulation due to active feeding in *Daphnia* after 48 h can be interpreted as an indirect indication of viability of daphnids in this experiment.

Accumulation of MCs in *Daphnia* was observed in all experiments containing toxic *P. rubescens*. However, in experiment A, with the highest MC concentration at the beginning of the experiment (9 $\mu g\, l^{-1}$), the maximum peak of MC accumulation in *Daphnia* was reached later (48 h) compared with experiments B–D, which had lower initial MC concentrations. Indeed, in experiments B–D the peak for MC content was recorded already at 24 h.

The MC content in *Daphnia* decreased after 72 h in all of the experiments, most likely due to reduced uptake resulting from the degradation of *P.rubescens* filaments as well as due to increased metabolism and excretion of internalized MC. Indeed the number of *P.rubescens* filaments was decisively reduced after 72 h. In conjunction with lowered MC uptake via filament ingestion, the rate of metabolism, e.g.

conjugation to more hydrophilic moieties, and concomitant excretion would, in the sum of the uptake, metabolism and excretion kinetics, decrease the overall MC concentration within *Daphnia*. In view of the potentially higher dissolved MC concentrations in the ambient water post 72h, this also suggests that dissolved MCs in the ambient water are most likely not readily taken up by *Daphnia*.

To characterize the kinetics of MC accumulation in *D. magna*, we used models to estimate how much of the available MC in different diets (here *P. rubescens* filaments) would accumulate in the body of *Daphnia* over time.We found that the degree and pattern of MC accumulation in *D. magna* was directly related to the initial MC and to the exposure time. Within the first 24 h of exposure, a linear relationship was observed between initial ambient MC concentrations in the food and the MC concentrations detected per individual daphnid (Fig.3a). Subsequent to the first 24 h the toxin accumulation followed an exponential pattern, with proportionally higher MC concentrations at higher initial ambient MC concentrations in the food. The exponential pattern at 48 h (Fig. 3b) resulted not only from the larger accumulation of MC at higher initial ambient MC concentrations in the food, but also from concomitantly decreasing MC containing food in the experiments with lower initial MC concentrations (see Fig. 2). As accumulation kinetics were evaluated considering the initial MC concentrations in the food, the actual accumulation kinetics may have been underestimated, thus suggesting that MC retained within *Daphnia* could be not only dependent on MC availability in food and on the corresponding grazing activity. Indeed, partial or full inhibition of digestion and detoxification pathways, resulting from high internalized MC concentrations could have evolved. Rohrlack *et al*. (2001) previously suggested that MC detoxification pathways in cladocerans could be more efficient at low MC concentrations. Correspondingly, Chen *et al*. (2005) showed that while low concentrations of dissolved MC-LR had no harmful consequences for *D. magna*, high concentrations and long-term exposure resulted in a reduction of antioxidant enzyme activities, most likely resulting from an overburdening of the detoxification system by MC metabolism. Indeed, at low MC concentrations the crucial protein phosphatases were not entirely inhibited, thereby allowing at least partial functioning of signal transduction, i.e. enzyme activation/deactivation control pathways. Overall, the latter observations fit with the hypothesis that higher proportions of toxic filaments are able to shift the balance between accumulation and excretion/detoxification towards a greater accumulation of MC in grazers, with a corresponding exponential increase of MCD as a function of MC_i.

The models that we elucidated with these experiments could have important implications for the transfer of toxins along the trophic webs. Nevertheless, our study showed that with the existence of

non-linear patterns of MC accumulation, trophic transfer of MC to higher trophic levels would be strongly dependent on the trophic status of water bodies and the degree of toxicity of cyanobacterial strains characterized by different toxin per cell quota. The presence of MC at different trophic levels has been reported by many studies (Ibelings *et al*., 2005; Lehman *et al*., 2010). Sotton *et al*. (2014) analyzed the accumulation of MC in the whitefish (*Coregonus*), which is one of the most important commercial fish in the peri-alpine region. The whitefish was found below the thermocline, where metalimnetic blooms of *P. rubescens* also occurred. Though an unintentional ingestion of filaments was expected from earlier experiments by Ernst et al. (2007, 2009), after analysing the whitefish gut only a few or no filaments were observed. Instead, zooplanktonic herbivores were clearly demonstrated as the vectors of MCs to whitefish by encapsulating grazed cyanobacteria through their diel vertical migration. Actually, 75% and 21% of the total MCs in the white fish came from Chaoborus larvae and *Daphnia*, respectively.

The results obtained in this study require to be interpreted with care. The statistical parameters representing the bioaccumulation models are valid only in this particular experimental system and with the cyanobacterial cell density curves reported. However, the experiments showed quite clearly how the relationship between the accumulation of MC in *Daphnia* and the initial concentrations of toxic cyanobacteria and toxins (i.e. the variables mostly related to eutrophication) can be described with general linear and exponential models, depending on the exposure time. Needless to state that in order to decisively improve the bioaccumulation model, the corresponding kinetic parameters for MC metabolism and excretion in *Daphnia* would be required. The latter kinetic parameters would also allow elucidation of whether at higher internal MC concentrations, metabolism and excretion of MCs can be overwhelmed or even inhibited, thus resulting in the observed overall accumulation of MCs. The experimental setup we used does not adequately represent the natural environment. Indeed, the grazing activity by *Daphnia* was influenced by the availability of shorter filaments in the algal cultures. Moreover, the experiments could be biased by the tendency of *Planktothrix* to degrade (microbially) in these small mesocosms. However, considering that the rate of decrease (and therefore food consumption) in the abundances of *P. rubescens* was similar in the 4 (A–D) grazing experiments, the results seems to further confirm that a proportionally larger accumulation of MC can be observed in presence of more toxic variants of cyanobacteria. Considering the above weaknesses in the experimental setup, the results should be verified in larger mesocosms, with durable and long-living populations of *Planktothrix*.

The possibility to further generalize the results of these experiments should also take into consideration the characteristics of *D. magna*. This species in one of the large grazers, i.e. individuals used in this work were around 3 mm, and thus much larger than other common *Daphnia* inhabiting lentic waters. For example, in Lake Garda, and in many other large subalpine lakes, waters are populated by smaller individuals of *Daphnia hyalina*/*galeata*, several of them with dimensions of 1 mm and therefore with a smaller ability to graze on long filamentous algae (Salmaso and Naselli Flores, 1999). Moreover, differences in hydrophobicity among MC variants must also be taken into account to interpret the bioaccumulation patterns (Ward and Codd, 1999). In this experiment [d-Asp^3] MCRR produced by *Planktothrix* is more hydrophilic than MC-LR, the experimentally most employed variant of MC. In the presence of more hydrophobic MC, e.g. MC-LA, -LF or -LW, even lower depuration rates and thus higher accumulation rates would be expected in daphnids, thus emphasizing that not only the cyanobacterial species and their anatomical descriptors, but also the specific MC produced may be key factors governing the accumulation, depuration and thus residual time of MC within daphnids. The latter will be key determinants for the potential trophic transfer of MC within a given surface water system.

4.5. Author contributions

The experiment was planned by all the authors. S. Shams conducted the experiment. L.Cerasino performed the LC–MS/MS analysis. The models were prepared by N. Salmaso. The manuscript was written by S. Shams and revised by L.Cerasino, N. Salmaso and D. R. Dietrich.

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Chapter 5

5. Anatoxin-a producing *Tychonema* (cyanobacteria) in European waterbodies

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Anatoxin-a producing *Tychonema* (cyanobacteria) in European waterbodies

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ABSTRACT

In order to identify the cyanobacterial species responsible of anatoxin-a (ATX) production in Lake Garda (Northern Italy), an intensive isolation and culturing of filamentous cyanobacteria were established since 2014 from environmental samples. In this work, we report a detailed account of the strategy adopted, which led to the discovery of a new unexpected producer of ATX, *Tychonema bourrellyi*. So far, this species is the first documented example of cultured Oscillatoriales able to produce ATX isolated from pelagic freshwater ecosystems. The isolated filaments were identified adopting a polyphasic approach, which included microscopic species identification, genetic characterisation and phylogenetic analyses based on 16S rRNA genes. The taxonomic identification was further confirmed by the high (>99%) rbcLX sequence similarities of the *T. bourrellyi* strains of Lake Garda with those deposited in DNA sequence databases. More than half of the isolates were shown to produce a significant amount of ATX, with cell quota ranging between 0.1 and 2.6 µg mm^3, and 0.01 and 0.35 pg $cell^{-1}$. The toxic isolates were tested positive for anaC of the anatoxin-a synthetase (ana) gene cluster. These findings were confirmed with the discovery of one ATX producing *T. bourrellyi* strain isolated in Norway. This strain and a further non-ATX producing Norwegian *T. bornetii* strain tested positive for the presence of the anaF gene of the ana gene cluster. Conversely, none of the Italian and Norwegian *Tychonema* strains were positive for microcystins (MCs), which was also confirmed by the absence of mcyE PCR products in all the samples analysed. This work suggests that the only reliable strategy to identify cyanotoxins producers should be based on the isolation of strains and their identification with a polyphasic approach associated to a concurrent metabolomic profiling.

Keywords

anatoxin-a; *Tychonema*; cell quota; polyphasic approach; phylogenetic analysis; European waterbodies

5.1. Introduction

The long evolutionary history is the basis of the high competitive ability that characterizes cyanobacteria. They are distributed in most aquatic and terrestrial habitats, including extreme environments (Paerl *et al*., 2003; Boyer and Zimba, 2007; Kleinteich *et al*., 2012). In water bodies characterized by high concentrations of nutrients, limited water exchange and high temperatures and thermal stability, cyanobacteria can develop with high biomasses, giving rise to the formation of blooms at the surface, euphotic zone or in the metalimnic layers, largely depending on the respective species (Paerl and Paul, 2012). Cyanobacteria represent one of the major causes of ecosystem degradation and impairment of the economical value of freshwater resources. Specific strains produce a wide range of powerful toxins, with important implications for health risks associated with the human exploitation of recreational and drinking waters (Meriluoto and Codd, 2005; Mankiewicz-Boczek *et al*., 2011; Zamyadi *et al*., 2012). The principal classes of cyanotoxins are microcystins, nodularins, anatoxin-a and homoanatoxin-a, anatoxin-a(S), saxitoxins and cylindrospermopsins (Metcalf and Codd, 2012; Méjean *et al*., 2014).Compared with microcystin (MC) producers, only a few anatoxin-a (ATX) producing taxa have been distinctly isolated and characterized (Table 1). Other reports, based on analyses carried out on bulk environmental samples, suggest the existence of a wide spectrum of potential cyanobacterial taxa able to produce ATX (see, among the others, Carrasco *et al*., 2007; van Apeldoorn *et al*., 2007; Aráoz *et al*., 2010; Metcalf and Codd, 2012; Quiblier *et al*., 2013). Many reports, however, were not confirmed by analyses made on isolated strains. Toxic species can be detected using direct analytical chemical approaches (Meriluoto and Codd, 2005; Humpage *et al*., 2012; Metcalf *et al*., 2012) as well as molecular methods able to detect the presence of toxin biosynthetic genes (Pearson and Neilan, 2008; Sivonen, 2008; Rantala-Ylinen *et al*., 2011a). Nevertheless, until a few years ago, a genetic molecular approach to identify ATX encoding genes was not feasible because of the unknown biosynthetic pathway leading to the production of anatoxin. Biosynthetic genes coding for ATX have been characterized only recently in a benthic Oscillatoria PCC 6506 (Méjean *et al*., 2009; 2010) and planktonic Anabaena sp. strain 37 (Rantala- Ylinen *et al*., 2011b), opening the way to the design and use of primers for the detection of genes coding ATX in *Oscillatoria*, *Phormidium*, *Aphanizomenon* and *Anabaena* strains (Cadel-Six *et al*., 2009; Ballot *et al*., 2010; Wood *et al*., 2010; Rantala-Ylinen *et al*., 2011b). In a recent work, Cerasino and Salmaso (2012) documented a widespread presence of ATX in the Lake District south of the Alps. Based on analyses carried out on environmental samples collected during the warmer months, detectable concentrations of ATX ranging between 0.1 and 0.6 μg L^{-1}were found in the lakes Garda, Iseo, Como and Maggiore, i.e. the

largest lakes that experienced a recent colonization and summer surface blooms of *Dolichospermum lemmermannii* (Salmaso *et al*., 2012). However, a clear identification of producers in the different seasons was not possible because biological analyses on isolated strains were not available. Based on the hypothesis that filamentous cyanobacteria could possibly be amongst the ATX producers, cultures of Oscillatoriales were established from environmental samples collected since 2014 in Lake Garda with the aim to isolate potential new producers. Owing to the very low abundance of cyanobacteria usually recorded in the winter months (Salmaso, 2011), samples were collected using plankton nets and initial cultures established. The isolated cyanobacteria were then examined and identified following a polyphasic approach (Vandamme *et al*., 1996; Lee *et al*., 2014), which included microscopic species identification, genetic and phylogenetic analyses. Culture strains were further screened for cyanotoxins, particularly ATX and MCs, and tested for the presence of ATX and MCs biosynthesis encoding genes. Above approach led to the discovery and characterization of a new unexpected filamentous cyanobacterial producer of ATX.

Table1. Cyanobacterial anatoxin-a producers. The list, at the genus level, includes only the results obtained from analyses carried out on isolated strains in culture conditions.

	Genus	Selected references
Heterocytous genera	*Dolichospermum*/*Anabaena*	Sivonen et al. (1989); Lakshmana Rao et al.(2002); Rantala-Ylinen et al. (2011b)
	Aphanizomenon	Sivonen et al. (1989); Osswald et al. (2009)
	Cuspidothrix (*Aphanizomenon*)	Wood et al. (2007a); Ballot et al. (2010); Hodoki et al. (2013)
	Cylindrospermum	Sivonen et al. (1989)
Oscillatoriales	*Oscillatoria*[(a)]	Sivonen et al. (1989); Edwards et al. (1992); Aráoz et al. (2005); Rantala-Ylinen et al. (2011b)
	Phormidium[(b)]	Wood et al. (2012); Harland et al. (2013; 2014)
	Tychonema	This work

[(a)] Including *O. limnetica* (*Pseudanabaena limnetica*). [(b)] Populations of *Phormidium* producing ATX were observed for the first time in benthic river mats (Wood et al., 2007b).

5.2. Methods

5.2.1. Study site

Lake Garda is located at the southern border of the north eastern Italian Alps, at 65 m a.s.l.. With a volume of more than $49\times10^9 m^3$, a maximum depth of 350 m and a surface of 368 km^2, Lake Garda is one of the largest freshwater bodies in Europe. From the 1970s to the 1990s the average concentrations of total phosphorus in the whole water column doubled, from 10 to 20 µg $L^{-1}$and beyond. Present concentrations are decreasing and stabilising around 18 µg L^{-1}. Information on the lake and previous investigations were reported in Salmaso and Mosello (2010).

5.2.2. Collection of samples and environmental variables

The sampling station was located at the deepest point of the lake (350 m), between the villages of Brenzone and Gargnano (45.69 N, 10.72 E). Field measurements and collection of samples were made between February and April 2014 (Table 2).

Table2. Chemical and physical characteristics at the surface and around the upper boundary of the euphotic layer (Zeu, 20 m) in the three sampling dates. DIN, dissolved inorganic nitrogen; SRP, soluble reactive phosphorus; TP, total phosphorus; Kd, vertical light attenuation coefficient.

Variable		11 Feb		11 March		8 April	
Depth	m	0	20	0	20	0	20
Temperature	°C	9.1	9.1	10.0	9.2	12.4	9.9
pH		7.6	7.9	7.7	8	8.4	8.2
Conductivity	µS cm^{-1} at 20°C	211	214	212	213	211	214
NO_3-N	µg N L^{-1}	273	256	337	341	227	268
DIN	µg N L^{-1}	280	261	342	346	232	275
SRP	µg P L^{-1}	9	8	3	4	2	<2
TP	µg P L^{-1}	14	11	10	16	15	12
K_d	m^{-1}	0.17		0.21		0.19	
Z_{eu}	m	27		22		24	

Owing to the very low abundance, in February and March 2014 phytoplankton was collected by single vertical tows from 10-15 m to the surface with a 25 cm diameter 80 µm mesh plankton net, which resulted in 0.5-0.7 m^3 of filtered water. In April 2014, filamentous cyanobacteria were collected with plankton nets and Niskin bottles. Vertical profiles of water temperature were carried out with a multi-parameter probe (Idronaut Ocean Seven 316). Secchi disk readings were carried out with the aid of a bathyscope, while light attenuation coefficients (Kd) were measured with a submersible irradiance sensor, LiCor 192SA.

The euphotic depth was computed as Z_{eu}= ln (100)×Kd-1(Kirk, 1994). Concentrations of dissolved inorganic nitrogen (DIN), soluble reactive phosphorus (SRP) and total phosphorus (TP) were carried out using standard methods (APHA *et al*., 2000; Cerasino and Salmaso, 2012).

5.2.3. Isolation of strains, culture conditions and morphological characterization

Single filaments of cyanobacteria were isolated from diluted net-phytoplankton samples under a stereomicroscope (Leica M125) and a macroscope (WILD M420) using a micropipette. The single filaments were washed 3 times and placed in microwell plates containing 3 mL Z8 medium (Kotai, 1972). After initial growth, as assessed by visual inspection and the macroscope, single strains were first transferred to 30 mL Z8 medium and, upon successful growth, to 150 mL medium Z8 CELLSTAR (Greiner Bio-One GmbH) cell culture flasks. The flasks were maintained at 20 °C under continuous light conditions (25µmol $m^{-2}s^{-1}$).From each algae culture, after careful homogenization, 15mL were preserved with Lugol's solution for biovolume determinations, whereas 250 mL were filtered with a 0.45 µm GF/C filter (Whatman – GE Healthcare Life Sciences) for subsequent cyanotoxin and genetic analyses.Depending on abundances, cell densities and biovolumes were estimated by measuring cell sizes and length of filaments from 1 to 3 transects at 200 magnification in10 mL sedimentation chambers of 25 mm diameter. Morphological features were analysed using an inverted microscope (Zeiss Axiovert 135).Single specimens were identified following morphometric and morphological criteria described in Komárek and Albertano (1994) and Komárek and Anagnostidis (2007). Over 65 single filament cultures were analysed microscopically, while 24 randomly selected cultures were analysed for the presence of cyanotoxins and for preliminary sequencing of 16S rRNA genes with reverse primer . Complete analyses of toxins, cyanotoxins encoding genes, and sequencing of 16S rRNA and rbcLX genes (with forward and reverse primers) were carried out in 4 single filament cultures selected randomly from each of the 3 sampling time points (12 isolates).

5.2.4 Cyanotoxins analyses

5.2.4.1 Toxin extraction

To measure intracellular concentrations of toxins, 250 mL of cyanobacterial cultures were filtered with a 0.45 µm GF/C filter. The filter was frozen and stored at -20 °C until further processing (within one week of filtration). For toxin extraction, the filter was homogenized for 5 min after addition of 7 mL of extraction solvent (70% methanol containing 0.1% formic acid) in a homogenization tube. After centrifugation (9850 G), the supernatant was transferred and the pellet was re-extracted again

with an additional 7mL of extraction solvent. The evolving supernatants were combined. A 2 mL aliquot of the filter-extract was filtered on Phenex-RC syringe filter (0.2 µm pore size, Phenomenex) and used for the determination of MCs and nodularin (NOD- R) content via liquid chromatography–mass spectrometry (LC-MS). The remaining filter-extract was reduced under vacuum and filtered with a 0.2 µm pore size RC syringe filter and the filtrate subjected to alkaloid (ATX, and cylindrospermopsin, CYN) LC-MS analytics.

5.2.4.2 LC-MS analysis

LC-MS analyses were carried out on a Waters Acquity UPLC system directly coupled to an AB SCIEX 4000 QTRAP mass spectrometer equipped with a turbo ion spray interface. Standard injection volume was 2 µL. The analysis of MC and nodularin-R was carried out using a Phenomenex Kinetex XB-C18 column (1.7 µm particle size, 2.1 × 50 mm) at 40°C. The mobile phase consisted of water (A) and acetonitrile (B), both containing 0.1% formic acid. A linear gradient scheme was employed: the starting eluent was 80% A, decreased to 30% A at 4.5 min, and finally restored at 80% A at 6.5 min (hold 0.5 min). The total run time was 7 min with a flow rate of 0.25 mL min-1. The analysis of CYN and ATX was carried out using a Phenomenex Kinetex HILIC column (1.7 µm particle size, 2.1 × 50 mm) at 30°C. The mobile phase consisted of water with 1% acetonitrile (A), containing ammonium acetate (10 mM) and acetic acid (10 mM), and acetonitrile (B). A linear gradient scheme was employed: the starting eluent was 10% A (hold 0.5 min), raised to 25% A at 1 min (hold 1 min), raised to 60% A at 3.5 min (hold 2 min), and finally returned to 10% A at 8 min (hold 2 min). The total run time was 10 min with a flow rate of 0.25 mL min-1. The mass detector was operated in positive Electro Spray mode (ESI+) using the Multiple Reaction Monitoring (MRM) scanning mode. General settings were as follows: ion spray voltage 5000 V, entrance potential 10 V, cell exit potential 10V, interface heater temperature 300°C. For each target compound, two transitions were monitored. Toxin identification was achieved by comparing the chromatographic retention time and relative intensity of the two transitions with those of the commercial standards. Toxin quantification was performed with the external standard procedure, in which, for each compound, a calibration curve was obtained using the most intense transitions. Compound-specific settings of the mass detector and performance data of the methods are listed in Table 3.

Table3. Compound-specific parameters in LC-MS analysis of toxins. All toxins are reported in the same table for clarity, but MC/NOD and CYN/ATX are analysed in different chromatographic conditions. RT = chromatographic retention time; DP = declustering potential; CE = collision energy.

		MS acquisition parameters			
Toxin variant	RT (min)	MRM transitions[(a)] (m/z)	ratio[(b)]	DP (V)	CE (V)
[D-Asp3]RR	1.20	512.8/135	17	85	44
		512.8/213		85	50
RR	1.43	520.1/135	15	85	44
		520.1/213		85	50
NOD-R	2.20	825.6/135	1	140	83
		825.6/70		90	100
YR	2.63	523.6/135	6	45	20
		523.6/911		45	20
LR	2.78	498.6/135	17	40	19
		498.6/213		40	43
WR	3.06	535.0/135	18	40	18
		535.0/213		40	42
LA	4.30	911.6/135	2	85	90
		911.6/213		85	65
LY	4.40	1002.6/135	2	106	96
		1002.6/213		106	75
LW	4.80	1025.6/135	2	111	100
		1025.6/213		111	80
LF	4.93	986.6/135	2.5	96	95
		986.6/213		96	73
CYN	1.90 (HILIC)	416.3/194	1.5	80	53
		416.3/336		80	53
ATX	4.10 (HILIC)	166.1/149	1.6	60	21
		166.1/131		60	24

[(a)] For all compounds the most intense transition is reported in the first line, the less intense in the second line. [(b)] The relative ratios between the intensities of the two MRM transitions are reported in this column.

Homoanatoxin-a and other degradation products e.g. dihydro- and epoxy-homoanatoxin-a were not quantified because analytical protocols were still under evaluation. Microcystins (RR, [D-Asp^3]-RR, YR, LR, WR, LA, LY, LW, LF), NOD-R and CYN analytical standards were purchased from Vinci Biochem, ATX from Tocris Cookson Ltd. All solvents and reagents used in this procedure were LC-MS grade. The limits of quantitation (LOQ) were between 30 and 500 ng L^{-1}(different MCs congeners), 140 ng L^{-1}(NOD-R), 30 ng L^{-1}(ATX), and 8 ng L^{-1}(CYN).

5.2.5 DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from the filters using the Mo Bio PowerWater® DNA Isolation Kit (Mo Bio Laboratories, Inc., CA, USA) following manufacturer's instructions. The quantity and quality of DNA was measured by spectrophotometry with a NanoDrop ND-8000 (Thermo Fisher Scientific Inc., MA,USA).Taxonomic identification and phylogenetic analyses of filamentous cyanobacteria were carried out by the amplification of a short fragment (ca. 420 bp) of the 16S rRNA gene using the forward primer CYA359F and an equimolar mixture of the reverse primers CYA781Ra and CYA781Rb (Nübel *et al*., 1997) synthesized commercially (Sigma-Aldrich Co. LLC). The PCRs were carried out on an Eppendorf Mastercycler ep (Eppendorf AG, Hamburg, Germany). The reaction mix, with a final volume of 25 µl, contained 1X Optimized DyNAzyme PCR Buffer (Thermo Scientific), 0.2 mM dNTPs mix (Thermo Scientific), 0.1 µM forward primer, 0.05 µM 200 each of the two reverse primers, 1U of DyNAzyme II DNA Polymerase (Thermo Scientific), and 1 µl of DNA templates. Genomic DNA concentrations were in the range 4.9–20.3 ng µL-1. The cycling protocol consisted of a first denaturation step at 94°C for 3 minutes followed by 35 cycles of DNA denaturation at 94° C for 30 s, primer annealing at 53° C for 30 s, strand elongation at 72° C for 1 minute, and a final elongation step at 72° C for 5 min. PCR products were checked and separated by 1.5% agarose gel electrophoresis stained with ethidium bromide. Sizing of DNA fragments were first evaluated with a commercial DNA ladder (GeneRuler Express, Fermentas).

Besides 16S rRNA phylogenetic analyses, taxonomic identification of isolates presented in Table 4 was further checked using the rbcLX gene region. rbcLX products (878 bp) were amplified using the primers CW and CX, following the protocols in Rudi *et al*. (1998), with the exception of the number of cycles in the second cycle step (35 instead of 38).

PCR products (16S rRNA and rbcLX genes) were cleaned with Exonuclease plus Shrimp Alcaline Phosphatase (ExoSAP). The same primers as in the PCR (CYA359F and CYA781Ra for 16S rRNA) were used with the BigDye Terminator Cycle Sequencing technology (Applied Biosystems), according to the manufacturers' protocols. After purification in automation using the Agencourt CleanSEQ® Kit (Beckman), products were run on an Automated Capillary Electrophoresis Sequencer 3730XL DNA Analyzer (Applied Biosystems). In order to trim the low quality ends, sequences were checked with Chromatogram Explorer 3.3.0 (Heracle Biosoft). Forward and reverse chromatograms were further evaluated and assembled using the BioEdit 7.2.5 sequence alignment editor (Hall, 1999). Sequences were deposited to the European Nucleotide Archive (ENA) and analysed with Megablast (NCBI) against 16S rRNA and rbcLX gene sequences.

In addition to the toxins analyses (section 2.4), the isolated strains were analysed for the presence of MCs and ATX encoding genes. The presence of mcyE genes was evaluated according to the PCR protocols of Rantala *et al*. (2006) using general primers (mcyE-F2/R4) and Anabaena 90 as a positive control (Ana-90; UHCC). The presence of anatoxin-a synthetase gene (anaC) was determined using the primer pairs anaC-osc, Osc-193 (UHCC) as positive control, and the methods described in Rantala-Ylinen *et al*. (2011b).

Table4. (a) Codes of Tychonema bourrellyi strains isolated in Lake Garda (Northern Italy) between February and April 2014, and corresponding ENA accession numbers of 16S rRNA and rbcLXgenes.(b) Polymerase chain reaction amplification of ATX and MCs biosynthesis encoding genes (anaC and mcyE), and concentrations of anatoxin-a (ATX) and total microcystins (MCs); "+" and "-"indicate the presence and absence of expected amplicons using agarose gel electrophoresis. "nd", not detectable.

(a)					(b)			
Date	Isolate number	Isolate code	Accession number		*anaC*	ATX	*mcyE*	MCs
			16S rRNA	*rbcLX*		µg L^{-1}		µg L^{-1}
11 Feb	1	FEM_GaT0214-3	LM997416	LM997428	+	6.44	-	*nd*
	2	FEM_GaT0214-12	LM997417	LM997429	+	10.46	-	*nd*
	3	FEM_GaT0214-16	LM997418	LM997430	+	11.32	-	*nd*
	4	FEM_GaT0214-21	LM997419	LM997431	+	5.76	-	*nd*
11 Mar	5	FEM_GaT0314-2	LM997420	LM997432	+	2.92	-	*nd*
	6	FEM_GaT0314-4	LM997421	LM997433	-	*nd*	-	*nd*
	7	FEM_GaT0314-13	LM997422	LM997434	-	*nd*	-	*nd*
	8	FEM_GaT0314-17	LM997423	LM997435	+	2.91	-	*nd*
08 Apr	9	FEM_GaT0414-14	LM997424	LM997436	+	1.48	-	*nd*
	10	FEM_GaT0414-16	LM997425	LM997437	+	2.02	-	*nd*
	11	FEM_GaT0414-26	LM997426	LM997438	+	1.55	-	*nd*
	12	FEM_GaT0414-27	LM997427	LM997439	+	0.48	-	*nd*

5.2.6 Comparison with Norwegian Tychonema strains

Eight Tychonema *bornetii* and *Tychonema bourrellyi* strains isolated in Norway between 1976 and 1982 from phytoplankton samples collected in the River Glåma and Lake Mjøsa were included in the study. The eight Norwegian *Tychonema* strains are cultivated in the culture collection of algae of the Norwegian Institute for Water Research (NIVA-cca). DNA extraction was conducted according to Ballot *et al*. (2014), PCR and sequencing of the 16S rRNA gene was conducted u sing the methods described in Ballot *et al*. (2008). The Norwegian *Tychonema* sp. strains were investigated for the production of MC and ATX using the Abraxis Microcystins/Nodularins (ADDA), ELISA Kit and the Abraxis Anatoxin-a Receptor-Binding Assay (Biosense, Bergen, Norway) respectively. The same strains were tested for the anaF encoding gene using the primers atxoaf and atxoar and the protocol according to Ballot *et al*. (2010) and Ballot *et al*. (2014). The presence of mcyE genes was evaluated according to the PCR protocols of Rantala *et al*. (2006) using general primers (mcyE-F2/R4). Sequences were submitted to ENA.

5.2.7 Phylogenetic analysis

The 16S rRNA genes of the 20 *Tychonema* strains listed in tables 4 and 5 were analysed using molecular sequence assembly software Seqassem version 04/2008 (SequentiX-Digital DNA processing, Klein Raden, Germany).The Align(version 03/2007) MS Windows-based manual sequence alignment editor (SequentiX -Digital DNA processing, Klein Raden, Germany) was used to obtain DNA sequence alignments, which were then corrected manually. Segments with highly variable and ambiguous regions and gaps making proper alignment impossible were excluded from the analyses. A 16S rRNA gene set containing 405 bp was used. *Gloeobacter violaceus* (AF132790) was employed as outgroup in the 16S rRNA tree. Thirty-three additional Oscillatoriales from GenBank were included in the 16S rRNA gene sequence analysis.

A phylogenetic tree for 16S rRNA gene sequences was constructed using the maximum likelihood (ML) algorithm with 1000 bootstrap replicates. In the ML analysis, evolutionary substitution model was evaluated in MEGA version 6 (Tamura *et al*., 2013) and K2+G+I was found to be the best-fitting evolutionary model for the 16S rRNA gene.

Table5. (a) Codes of Tychonema bourrellyi strains isolated in Lake Mjøsa and River Glåma (Norway), and corresponding ENA accession numbers of 16S rRNA genes. (b) Polymerase chain reaction amplification of anaF and mcyE genes, and positive or negative detection of anatoxin-a (ATX) and total microcystins (MCs) measured using ELISA Kit and Anatoxin-a ReceptorBinding Assay. "+" and "-"indicate the presence and absence of expected amplicons using agarose gel electrophoresis (anaF and mcyE), and the presence and absence of corresponding toxins (ATX, MCs), respectively.

(a)					(b)			
Code	Year of isolation	Species	Origin	Accession number 16S rRNA	*anaF*	ATX	*mcyE*	MCs
NIVA-CYA 33/1	1976	*T. bourrellyi*	Lake Mjøsa	LM651410	-	-	-	-
NIVA-CYA 33/3	1976	*T. bourrellyi*	Lake Mjøsa	LM651411	-	-	-	-
NIVA-CYA 33/4	1976	*T. bourrellyi*	Lake Mjøsa	LM651412	-	-	-	-
NIVA-CYA 33/5	1976	*T. bourrellyi*	Lake Mjøsa	LM651413	-	-	-	-
NIVA-CYA 60	1978	*T. bornetii*	Lake Mjøsa	LM651414	+	-	-	-
NIVA-CYA 95	1982	*T. bornetii*	River Glåma	LM651415	-	-	-	-
NIVA-CYA 96/1	1982	*T. bourrellyi*	Lake Mjøsa	LM651416	-	-	-	-
NIVA-CYA 96/3	1982	*T. bourrellyi*	Lake Mjøsa	LM651417	+	+	-	-

5.3. Results

5.3.1. Environmental samples

In the first 20 m, water temperatures in the three sampling dates ranged between 9.1 and 12.4°C (Table 2). An incipient stratification was apparent beginning in April. DIN and TP concentrations were in the range 232-342 µg N $L^{-1}$and 10-16 µg P L^{-1}. The euphotic depth was between 22 and 27m.

5.3.2. Microscopic examinations

Over 65 cultures were obtained from the isolation of single filaments collected in February, March and April. In the original samples, the filaments were solitary and free floating, pale red, sometimes longer than 2 mm. All the specimens looked quite different from the filaments of *Planktothrix rubescens*,the predominant cyanobacterium in Lake Garda, and more similar to other Phormidioideae, namely *Tychonema* spp. (Komárek and Albertan, 1994; Kotare and Anagnostidis, 2007). A first preliminary round of PCR and sequencing with the reverse primer CYA781Ra on 24 cultures matched well to various *Tychonema* species (similarity between 99% and 100%), as well as to sheathed Oscillatoriales (*Phormidium, Microcoleus*) (see section 5.3.3).

In the algal cultures, filaments were colourless or pale purplish/brown, rarely green, unbranched and without apparent firm sheaths. Cells usually were more or less isodiametric, with widths between 4.5 and 7 µm (Fig. 1). In some cultures, cells looked shorter (< 5 µm) than wide (e.g. Fig. 1d-e). Filaments looked immotile or slightly trembling, with rounded apical cells, without calyptra and with no or very slight attenuation at the ends. In some specimens, the width changed slightly along the filaments (e.g., Fig. 1a, c, d). Sometimes, the cells had clearly visible large holes, similar to "vacuoles" (quite apparent in Fig. 1a, c), which, actually, are widened thylakoids (Komárek and Albertano, 1994). The centripetal formation of the cross walls was often easily detectable (e.g., arrows in Fig. 1b).These characteristics were consistent with the diacritical features described for*T.bourrellyi*. However, taking into account that planktonic populations of *Tychonema tenue* could possibly be identical to *T. bourrellyi* (Komárek and Anagnostidis, 2007), the microscopic discrimination of these two species in pelagic environments is not straightforward.

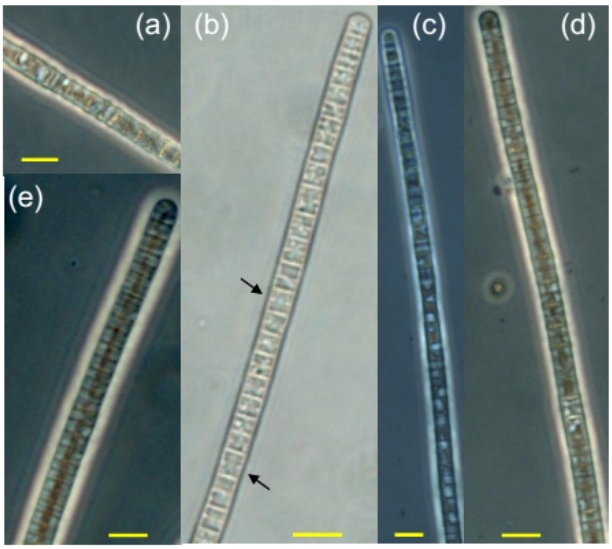

Figure1. Micrographs of *Tychonema* isolated from samples collected in Lake Garda in (a) February, (b, c) March and (d, e) April. Scale bars = 10 µm. From (a) to (e), filaments are 5.7, 5.8, 5.2, 5.3 and 6.8 µm wide, respectively. The arrows in (b) indicate the centripetal formation of the cross wall. Observations made at 400× and phase contrast.

5.3.3. Sequences and phylogenetic analyses

The morphological determination of the isolated strains was supported by phylogenetic analyses based on 16S rRNA performed on the 12 selected strains in Table 4. Phylogenetic relationships of the investigated strains are presented in the ML tree of the 16S rRNA region of Oscillatoriales strains (Fig. 2). All 16S rRNA sequences from the Italian and Norwegian *Tychonema* strains (Tables 4 and 5) were grouped in a distinct cluster together with *Tychonema* sequences derived from GenBank. The *Tychonema* cluster was very closely related to a *Phormidium autumnale* and a *Microcoleus antarcticus* strain (Fig. 2). The whole cluster was supported by a bootstrap value of 96%.

These findings were further confirmed by a BLAST (NCBI) homology search. Results showed that the rbcLX gene regions sequenced in the 12 selected strains (Table 4) were >99% similar to *T. bourrellyi* (7 strains) and *T. bornetii* (1 strain).

Figure2. Maximum likelihood tree determined on the basis of partial 16S rRNA gene sequences of 53 Oscillatoriales strains. Outgroup=Gloeobacter violaceaus (AF132790). Strains from this study are marked in bold. Bootstrap values above 50 are included. The scale bar indicates 2% sequence divergence.

5.3.4. Toxicity of the single strains

Of the 24 strains of *Tychonema* isolated from Lake Garda and submitted to LC-MS analyses, 14 produced ATX, although in some cases, at very low concentrations (3 isolates with ATX < 0.1 µg L^{-1}). A typical LC-MS chromatogram, showing the analyses of standards (ATX and CYN) and of a representative sample is reported in Fig. 3. The molecular analyses allowed amplifying the anaC encoding gene fragment of the anatoxin-a synthetase (ana) gene cluster. In the same group of 24 isolates, PCR products were identified in 11 strains. No anaC PCR products were identified in all the non-ATX producing strains, and in the 3 culture samples with very low concentrations of ATX (< 0.1 µg L^{-1}).

A representative picture of the anaC products amplified with anaC-osc primers in the 12 selected strains subjected to phylogenetic analysis and included in Table 4 is reported in Fig. 4. In these 12 selected strains, the cell quota of ATX on a biovolume basis were between 1.3 and 2.6 µg mm^{-3}in ebruary, 0.1 and 0.3 µg mm^{-3} in March, and 0.2 and 1.8 µg mm^{-3}April. On a cell basis, corresponding values were in the range .18-0.35 pg $cell^{-1}$(February), 0.01-0.04 pg $cell^{-1}$(March) and 0.02-0.20 pg $cell^{-1}$(April).

As for the strains isolated in the Norwegian freshwaters (Table 5), *Tychonema bourrellyi* strain NIVA-CYA 96/3 was confirmed as ATX producer using the Abraxis Anatoxin-a Receptor- Binding Assay, while the other seven strains from NIVAcca tested negative for ATX. *Tychonema bourrellyi* strain NIVA-CYA 96/3 and T. bornetii strain NIVA-CYA 60 tested positive for anaF of the ATX synthetase (ana) gene cluster (accession numbers LM651418 and LN555581, respectively).

None of the Italian and Norwegian *Tychonema* strains were positive for MC, which was also confirmed by the absence of mcyE PCR products in all the samples analysed (Tables 4 and 5).

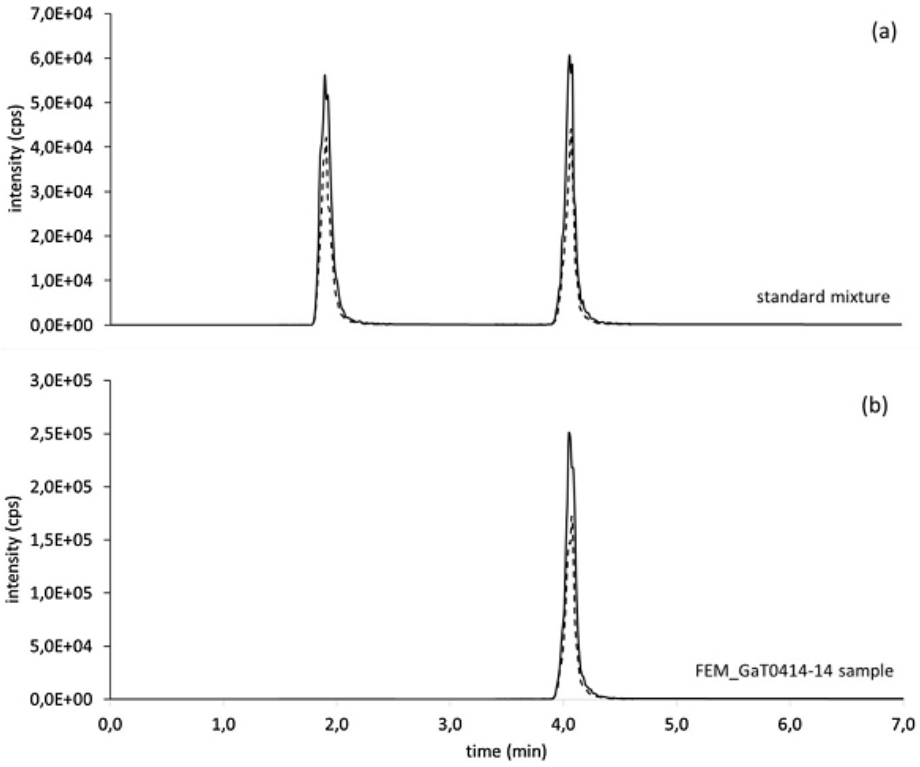

Figure3. Liquid chromatography–mass spectrometry chromatograms resulting from the injections of a mixture of pure standards of cylindrospermopsin (50 ng/mL) and anatoxin-a (165 ng mL^{-1}) (panel a) and of an extract of a *Tychonema* culture (panel b). For each toxin, the traces of the two monitored MRM transitions are shown (solid and dashed lines).

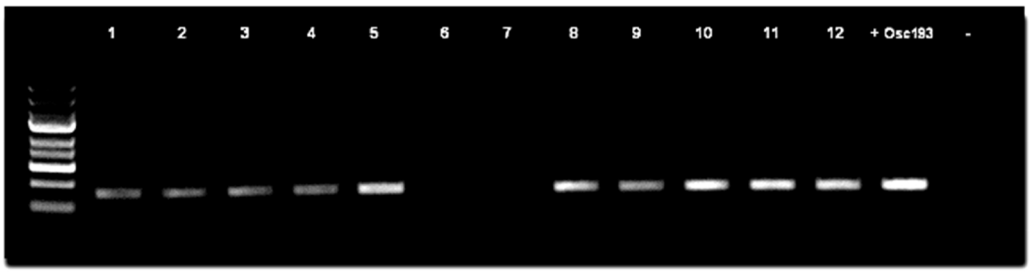

Figure4. PCR products amplified with anaC-osc primers (Rantala-Ylinen *et al*., 2011b). Samples from 1 to 12 are coded as in Table 4. "+" positive (Osc-193 UHCC strain) and "–"negative controls. Size of the ladder (in base pairs): 100, 300, 500, 750, 1000, 1500, 2000, 3000, 5000. The amplicon products are located between the bands 100-300 bp.

5.4. Discussion

After establishing several cultures of potentially toxic filamentous Cyanobacteria collected in Lake Garda, we discovered that *Tychonema* is able to produce ATX.This is the first discovery of a planktonic genus belonging to the Oscillatoriales able to produce ATX.These findings were confirmed with the discovery of ATX producing *Tychonema* strains isolated in Norway.

The discovery of *Tychonema* in Lake Garda was quite unexpected. The predominant Oscillatoriales in this lake and in the other large lakes south of the Alps (Iseo, Como, Lugano and Maggiore) is P. rubescens (Salmaso *et al*., 2012). Nevertheless, the high number of isolates of *P. rubescens* analysed so far by molecular methods (16S rRNA, rpoC1, rbcLX genes) in these lakes referred to samples mostly collected during the late spring and summer months, i.e. when the biomass development of this species was at its seasonal maximum (*P. rubescens* accession numbers are reported in D'Alelio *et al*., 2012; 2013). During winter, the abundances of *P. rubescens* and of the other Oscillatoriales (*Limnothrix sp*., *Planktolyngbya limnetica*) in Lake Garda are very low (Salmaso, 2011). In the work presented here, the isolation of filaments of *Tychonema* was possible on samples collected by means of plankton nets, and filtering 0.5-1 m^3 of lake water. Filaments in the samples collected with the Niskin bottles were rare and difficult to isolate. The presence of *Tychonema* appeared almost exclusive, because only a few filaments of *P. rubescens* were isolated from the net samples. On the other hand, the concurrent presence of *P. rubescens* was confirmed by molecular analysis carried out by checking the presence of rbcLX PCR products (see D'Alelio *et al*. 2013) on the environmental samples collected from February and April between the surface and 60 m (data not shown).

Tychonema is considered a cold-stenotherm genus of northern temperate regions (Komárek *et al*., 2003). Abundant populations of *T. bourrellyi* were documented particularly in northern Europe and Canada (Lund, 1955; Skulberg and Skulberg, 1985; Rudi *et al*., 1998; Komárek *et al*., 2003). In Lake Garda, the presence of this species in the colder months is consistent with these features. On the other side, the absence of gas vesicles, and therefore the inability to control buoyancy and vertical position, can represent a negative selective characteristic in deep and large lakes during the stratification months. The ecological role of the large "vacuoles", which are particularly apparent in the older cells, is still unclear.The large intracellular "holes" are due to the widened thylacoid membranes enveloping the enlarged intrathylacoidal spaces. Electron microscopy showed that these spaces were filled with electron dense fibrillar structures or globules similar to polyphosphate bodies (Komárek and Albertano, 1994). It is interesting to observe that another species – *T. sequanum* – was identified by microscopic methods in Lake Maggiore (Kamenir and Morabito, 2009). This taxon, however, has

smaller dimensions (2.5-5 μm width) compared to *T. bourrellyi*. Recently, *T. bourrellyi* was recorded also in Lake Erhai, in China. Comparative analyses of the 16S rDNA gene sequences determined from filaments isolated in this lake confirmed that the Chinese strains were grouped with *T. bourrellyi*/*T. tenue* (Wei *et al*., 2012).

The number of genera known to produce ATX is quite limited, especially when the list of producers is restricted to the only cases determined on species isolated and analysed in culture (Table 1). In Italy, reports of ATX are quite rare, referring exclusively to environmental samples (e.g., Cerasino and Salmaso, 2012). The production of ATX was associated with blooms or higher development of *Anabaena planctonica* (*Dolichospermum planctonicum*) (Bruno *et al*., 1994), Anabaena crassa (D. crassum) (Messineo *et al*., 2009) and "a peculiar *P.rubescens* population" (Viaggiu *et al*., 2004; Messineo *et al*., 2009). However, the ability of isolated populations of these species to produce ATX in culture conditions was not confirmed.

So far, the only Oscillatoriales proven to produce ATX in isolated populations belonged to the genera *Oscillatoria* and *Phormidium* (Table 1). These taxa are mostly detected in benthic or periphytic substrates. In contrast, *Tychonema bourrellyi* is known to develop pelagic populations (as in our study), opening new perspectives about the ability of pelagic Oscillatoriales to produce ATX. Taking into consideration the positive amplification of anaF genes in the strains of *T. bourrellyi* and *T. bornetii* isolated in Norway, the ability to produce ATX does not seem to be restricted to specific climatic regions or isolated populations. On the other hand, the high variability of the ATX quota in the isolates of *T. bourrellyi* grown under standard conditions suggests the existence of differences in the ability to produce ATX in strains isolated in different months. Overall, differences in the cell quota in the isolates of Lake Garda were between 0.01 and 0.35 pg cell^{-1}, i.e. within more than 1 order of magnitude, but well within the range estimated in cultures of *Phormidium autumnale* grown under different iron and copper stress conditions (between ca. 0 and 1.2 pg cell^{-1}; Harland *et al*., 2013), and within the variations observed in natural benthic *Phormidium* mats (100 fold differences in ATX quota; Wood *et al*., 2012).

The primer anaC-osc were specifically designed to amplify the anaC gene in the *Oscillatoria* genus (Rantala-Ylinen *et al*., 2011b). These primers proved to be useful also in the amplification of the anaC genes in *Tychonema*, suggesting the potential for a wider application of these specific protocols also to other Oscillatoriales. Similar considerations apply to the atxoaf-r primers, which were originally designed to detect *Aphanizomenon* species and other cyanobacteria (Ballot *et al*., 2010; 2014). In perspective, the comparison of the ana gene cluster encoding ATX in *Tychonema* and in the

other cyanobacteria will provide further insight for the design of protocols tailored for the detection of pelagic ATX producers (cf. Méjean *et al*., 2014).

Overall, our findings open new perspectives in the study of the ecology of phytoplankton and cyanotoxins producers in Lake Garda and in the deep alpine and subalpine lakes. The new discovery of *Tychonema* in Lake Garda will require to be studied in detail by evaluating the seasonal and spatial dynamics, distribution and diversity, as well as toxic potential assessed both in environmental and isolated strains. An open question that needs to be dealt with is the significance of this appearance and its potential evolution particularly in relation with the very recent oligotrophication of the lake and the decrease of *P. rubescens* populations (Salmaso and Cerasino, 2012).

5.5. Conclusions

In the work presented here, we identified a new pelagic cyanobacterium belonging to the Oscillatoriales able to synthesize ATX. This species –*Tychonema bourrellyi* – was isolated for the first time in the largest Italian lake (Lake Garda) during the winter and spring months.

- The identification of the species was carried out using a polyphasic approach, based on the microscopic identification of diacritical characters, molecular methods (16S rRNA and rbcLX genes) and phylogenetic analyses.

- Isolates of *Tychonema* were able to produce consistent amounts of ATX. The identification of this new ATX producer was also verified by the amplification of the anaC genes involved in the biosynthesis of ATX. These new findings were confirmed by the concurrent analyses of *Tychonema* strains isolated in Norway.

- The significance and impact of *Tychonema* producing ATX will require additional evaluation by studying the seasonal dynamics and toxic potential of populations in relation to the development of other toxic cyanobacteria as well as in a wider geographical context.

- This study further highlights how the number of cyanotoxins producers in the freshwater environments is possibly still underestimated. On the other hand, the way in which these results originated, suggests that the only reliable strategy to identify cyanotoxins producers should be based on the isolation of strains and their identification with a polyphasic approach associated to a concurrent metabolomic profiling performed with advanced analytical techniques.

5.6. Acknowledgements

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5.7. Author contributions

For samples of Lake Garda: S. Shams carried out all the experiments and wrote part of the manuscript; C. Capelli assisted in experimental procedures, strain isolation and maintenance cultures; L. Cerasino performed the LC-MS analysis; A. Ballot performed the whole analyses in the samples recorded in Norway, and conducted the phylogenetic analysis. D. Dietrich and K. Sivonen critically reviewed the whole mansuscript, discussing experiments and results. N.Salmaso was the supervisor of experiments and data analysis, and prepared the first draft of the manuscript, which was finalized by all the authors.

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5.9. Supplementary Tables:

Table1: List of primers used in this study

Target gene	Primer names	Amplicon length (bp)	Primer sequence (5'–3')	References
16S rRNA Cyanobacteria	CYA359F	~ 422	GGG GAA TYT TCC GCA ATG GG	Nübel etal., 1997
16S rRNA Cyanobacteria	CYA781R(a)	~ 422	GAC TAC TGG GGT ATC TAA TCC CAT T	Nübel etal., 1997
16S rRNA Cyanobacteria	CYA781R(b)	~ 422	GAC TAC AGG GGT ATC TAA TCC CTT T	Nübel etal., 1997
Housekeeping gene for *Planktothrix*	rbcLX-PrbcF	824	GGACATCCCTGGGGTAAT	Lin etal., 2010
Housekeeping gene for *Planktothrix*	rbcLX-PrbcR	824	TTGGACTTGCTTGACGAT	Lin etal., 2010
Housekeeping gene for *Planktothrix*	rpoC1-RPOF	608	TGGTCAAGTGGTTGGAGA	Lin etal., 2010
Housekeeping gene for *Planktothrix*	rpoC1-RPOR	608	GCCGTAAATCGGGAGGAA	Lin etal., 2010
Housekeeping gene for *Dolichospermum*	rpoBanaF	520-635	AGCMACMGGTGACGTTCC	Rajaniemi etal.,2005
Housekeeping gene for *Dolichospermum*	rpoBanaR	520-636	CNTCCCARGGCATATAGGC	Rajaniemi etal.,2005
mcyE in *Planktothrix*	mcyE-F2	249	GAA ATT TGT GTA GAA GGT GC	Vaitomaa etal., 2003
mcyE in *Planktothrix*	mcyE-plaR3	249	CTCAATCTGAGGATAACGAT	Rantala etal., 2006
mcyB in *Planktothrix*	mcyBA1tot	1692	CACCTAGTTGAAGAACAAGTTCT	Kurmayer etal., 2005
mcyB in *Planktothrix*	mcyBA1tot	1692	AGACTTGTTTAATAGCAAAGGC	Kurmayer etal., 2005
mcyE in *Dolichospermum*	mcyE-F2	247	GAA ATT TGT GTA GAA GGT GC	Vaitomaa etal., 2003
mcyE in *Dolichospermum*	AnamcyE-12R	247	CAA TCT CGG TAT AGC GGC	Vaitomaa etal., 2003
anaC	anaC-gen	366	TCTGGTATTCAGTCCCCTCTAT	Rantala etal., 2011
anaC	anaC-gen	366	CCCAATAGCCTGTCATCAA	Rantala etal., 2011
anaC gene in *Dolichospermum*	anaC-anab	263	GCCCGATATTGAAACAAGT	Rantala etal., 2011
anaC gene in *Dolichospermum*	anaC-anab	263	CACCCTCTGGAGATTGTTTA	Rantala etal., 2011
anaC gene in *Oscillatoria*	anaC-osc	216	CTCTATTCTCACAAGTTTGGTCT	Rantala etal., 2011
anaC gene in *Oscillatoria*	anaC-osc	216	GTTAGTTCAATATCAAGTGGTGGA	Rantala etal., 2011

NOTE. Detailed information about each primer and references used in the table can be found on Chapter 2, Part III- Genetic analysis.

Table2. All the PCR protocols and thermal cycles applied in this study.

16SrRNA gene in Cyanobacteria.

Component	Concentration
dd H2O	
10X PCR Buffer	1x
dNTP	0.2 mM
Primer 359 F	0.1 µM
Primer 781R a	0.05 µM
Primer 781R b	0.05 µM
Polymerase	1U
DNA Template = 1µl	
Final Volume= 25µl	

No	Step	Temp	Time	Cycle
1	Initial	94 °C	3 min	1x
2	Denaturation	94 °C	30 Sec	
3	Annealing	53°C	30 Sec	35x
4	Extension	72°C	1min	
	GO TO STEP 2			
5	Final extension	72°C	5 min	1x
6	Cooling	4°C	00:00:00	

House-keeping gene in Planktothrix (rbcLX).

Component	Concentration
dd H2O	
10X PCR Buffer	1x
dNTP	0.2 mM
Primer rbcLX-PrbcR	0.1 µM
Primer rbcLX-PrbcF	0.1 µM
Polymerase	1U
DNA Template= 2µl	
Final Volume= 25µl	

No	Step	Temp	Time	Cycle
1	Initial	94 °C	5 min	1x
2	Denaturation	94 °C	40 Sec	
3	Annealing	58°C	1 min	35x
4	Extension	72°C	2 min	
	GO TO STEP 2			
5	Final extension	72°C	8 min	1x
6	Cooling	4°C	00:00:00	

House-keeping gene in Planktothrix (rpoC1).

Component	Concentration
dd H2O	
10X PCR Buffer	1x
dNTP	0.2 mM
Primer rpoC1-PROF	0.1 µM
Primer rpoC1-PROF	0.1 µM
Polymerase	1U
DNA Template= 1µl	
Final Volume= 20µl	

No	Step	Temp	Time	Cycle
1	Initial	94 °C	5 min	1x
2	Denaturation	94 °C	40 Sec	
3	Annealing	58°C	50 Sec	40x
4	Extension	72°C	2 min	
	GO TO STEP 2			
5	Final extension	72°C	5 min	1x
6	Cooling	4°C	00:00:00	

House-keeping gene in Dolichospermum (rpoB).

Component	Concentration
dd H2O	
10X PCR Buffer	1x
dNTP	0.2 mM
Primer rpoBF	0.1 µM
Primer rpoBR	0.1 µM
Polymerase	1U
DNA Template= 2µl	
Final Volume= 20µl	

No	Step	Temp	Time	Cycle
1	Initial	94 °C	5min	1x
2	Denaturation	94 °C	1min	
3	Annealing	59°C	1min,30sec	30x
4	Extension	68°C	2 min	
	GO TO STEP 2			
5	Final extension	68°C	7 min	1x
6	Cooling	4°C	00:00:00	

mcyE gene in Planktothrix.

Component	Concentration
dd H2O	
10X PCR Buffer	1x
dNTP	0.2 mM
Primer mcyE-PlaR3	0.1 µM
Primer mcyE-F2	0.1 µM
Polymerase	1U
DNA Template= 2µl	
Final Volume= 20µl	

No	Step	Temp	Time	Cycle
1	Initial	95 °C	3 min	1x
2	Denaturation	94 °C	30 Sec	
3	Annealing	56°C	30 Sec	30x
4	Extension	75°C	30 Sec	
	GO TO STEP 2			
5	Final extension	72°C	5 min	1x
6	Cooling	4°C	00:00:00	

mcyB gene in Planktothrix.

Component	Concentration
dd H2O	
10X PCR Buffer	1x
dNTP	0.2 mM
Primer mcyBA1totfwd	0.1 µM
Primer mcyBA1totrev	0.1 µM
Polymerase	1U
DNA Template= 1µl	
Final Volume= 25µl	

No	Step	Temp	Time	Cycle
1	Initial	94 °C	5 min	1x
2	Denaturation	94 °C	40 Sec	
3	Annealing	58°C	1 min	35x
4	Extension	72°C	2 min	
	GO TO STEP 2			
5	Final extension	72°C	8 min	1x
6	Cooling	4°C	00:00:00	

mcyE gene in Dolichospermum.

Component	Concentration
dd H2O	
10X PCR Buffer	1x
dNTP	0.2 mM
Primer mcyE-F2	0.1 µM
Primer mcyE-12R	0.1 µM
Polymerase	1U
DNA Template= 1µl	
Final Volume= 25µl	

No	Step	Temp	Time	Cycle
1	Initial	95 °C	3 min	1x
2	Denaturation	95 °C	30 Sec	
3	Annealing	54°C	30Sec	30x
4	Extension	72°C	30 Sec	
	GO TO STEP 2			
5	Final extension	72°C	5 min	1x
6	Cooling	4°C	00:00:00	

anaC-gene (general primer).

Component	Concentration
dd H2O	
10X PCR Buffer	1x
dNTP	0.2 mM
Primer anaCf	0.1 µM
Primer anaCr	0.1 µM
Polymerase	1U
DNA Template= 2µl	
Final Volume= 20µl	

No	Step	Temp	Time	Cycle
1	Initial	94 °C	2 min	1x
2	Denaturation	94 °C	30 Sec	
3	Annealing	58°C	30 Sec	29x
4	Extension	72°C	30 Sec	
	GO TO STEP 2			
5	Final extension	72°C	5 min	1x
6	Cooling	4°C	00:00:00	

anaC gene in Oscillatoria.

Component	Concentration
dd H2O	
10XPCR Buffer	1x
dNTP	0.2 mM
Primer ana-oscR	0.1 µM
Primer ana-osc F	0.1 µM
Polymerase	1U
DNA Template= 2µl	
Final Volume= 20µl	

No	Step	Temp	Time	Cycle
1	Initial	94 °C	2 min	1x
2	Denaturation	94 °C	30 Sec	
3	Annealing	52°C	30 Sec	29x
4	Extension	72°C	30 Sec	
	GO TO STEP 2			
5	Final extension	72°C	5 min	1x
6	Cooling	4°C	00:00:00	

anaC gene in Dolichospermum.

Component	Concentration
dd H2O	
10X PCR Buffer	1x
dNTP	0.2 mM
Primer anaC-anabF	0.1 µM
Primer anaC-anabR	0.1 µM
Polymerase	1U
DNA Template= 2µl	
Final Volume= 20µl	

No	Step	Temp	Time	Cycle
1	Initial	94 °C	2 min	1x
2	Denaturation	94 °C	30 Sec	
3	Annealing	52°C	30 Sec	29x
4	Extension	72°C	30 Sec	
	GO TO STEP 2			
5	Final extension	72°C	5 min	1x
6	Cooling	4°C	00:00:00	

5.10. ADDITIONAL DATA

Beside the results published in manuscripts 3, this section reports a few additional results obtained with the genetic analysis of the environmental sample collected in 2013 with general and genus-specific primers. These data are preliminary, and will be used in future publications.

5.10.1 PCR anaylsis

When using the primer pair for 16S rRNA, all the samples collected from Lake Garda from January to December 2013, showed positive amplification which confirmed the presence of cyanobacterial DNA in the samples. PCR products corresponding to house-keeping genes (rbcLX and rpoC1) in *Planktothrix rubescens* showed strong amplification in the summer months and September, but faint amplification during winter months (Fig. 1a, 1b). However, while using housekeeping genes specific for *Dolichospermum,* strong products were observed in all the months, except January, February and March (Fig. 1c).

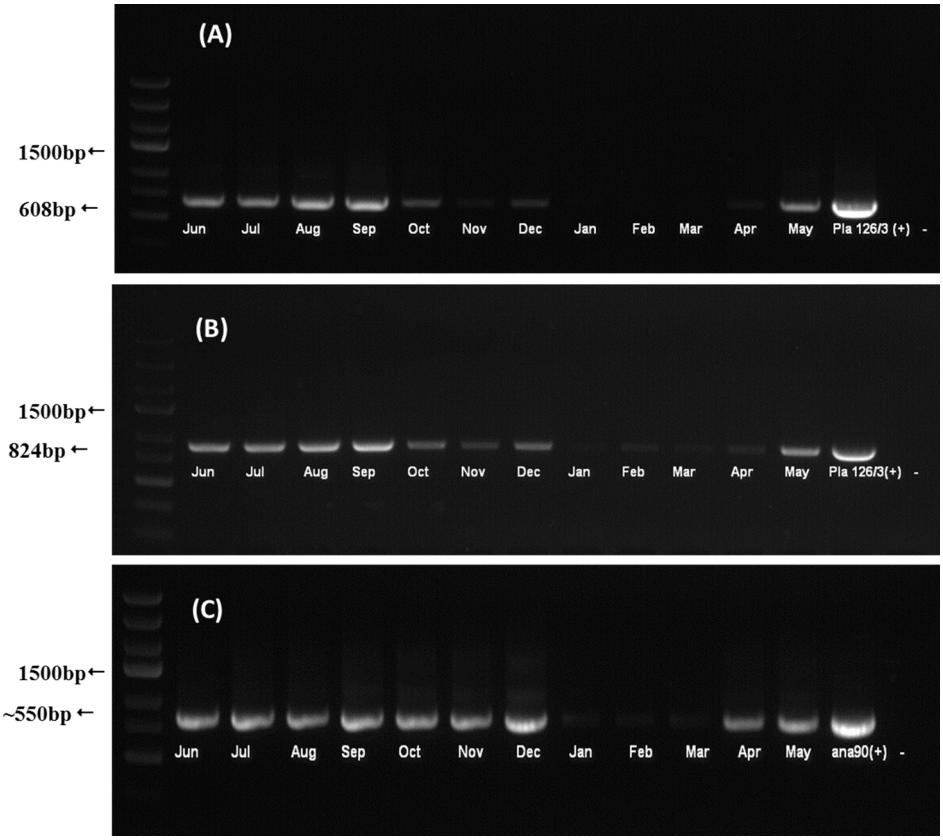

Figure1. (a) PCR products using rpoC primer pair specific for *Planktothrix*. (b) PCR products using rbcLX primer pair specific for *Planktothrix* (C) PCR products using rpoB primer pair specific for *Dolichospermum.*

The samples were also analysed for the presence of anaC gene (Fig. 2). When using the general primer for anaC gene, strong PCR products were detected in May and during the summer months (Fig. 2a). The same trend was observed when we used genus-specific primer to detect anaC gene in *Oscillatoria* (Fig. 2b). Interestingly, against our assumption that *Dolichospermum* can be a potential ATX producer, no PCR product was observed when using genus-specific primer to detect anaC gene in *Dolichospermum* (Fig. 2c).

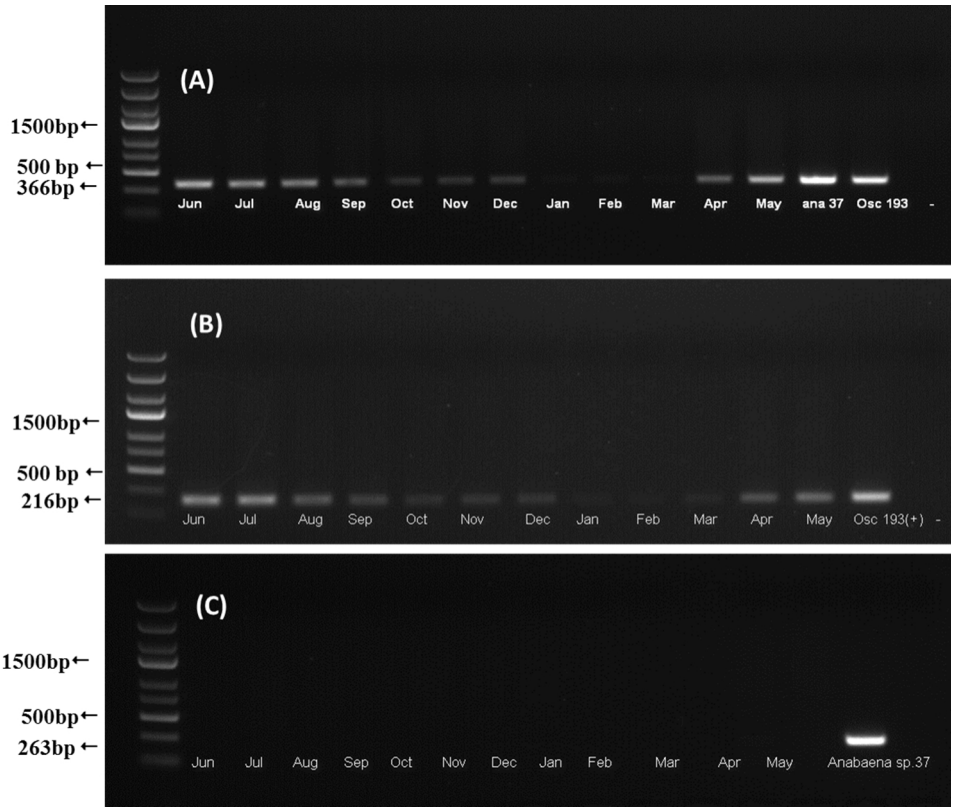

Figure2.(a) PCR products using general anaC primer pair to target anaC gene in all the possible ATX producers (b) PCR products using anaC-osc primer pair to target anaC gene in *Oscillatoria* (c) PCR products using anaC-anab primer pair to target anaC gene in *Dolichospermum.*

Eventually, in order to check the capability of the two known toxic cyanobacterial species in Lake Garda, *Planktothrix rubescens* and *Dolichospermum lemmermannii*, for producing MC, environmental samples were analysed with primers targeting mcyE and mcyB genes. We assumed

the both species are able to produce MC. No amplification was detected when using specific primers to target *Dolichospermum* specific mcyE genes (Fig. 3a). However very clear and strong PCR products were achieved during summer and early autumn when using specific primers to target mcyB and mcyE genes in *Planktothrix* (Fig.3 b,c). These results, confirming the conclusions in Shams et al. (2015), indicate that most (all) of the microcystins in the Lake are produced by *Planktothrix.*

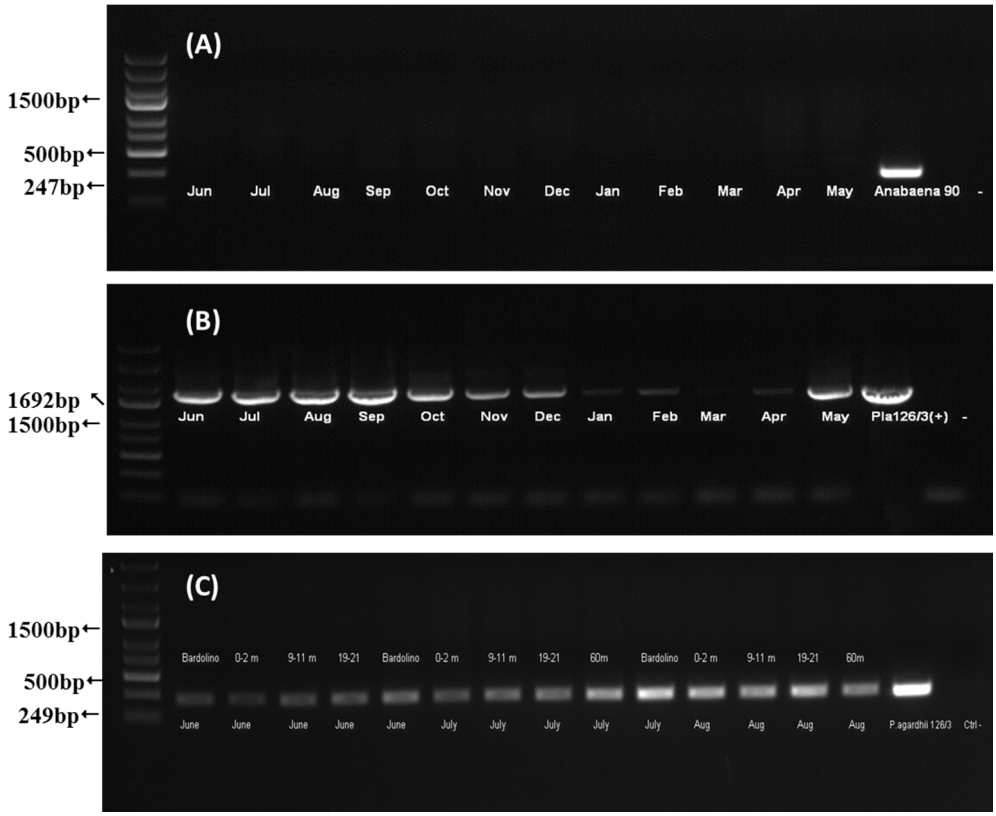

Figure3.(a) PCR products using mcyE-F2/ AnamcyE-12R primer pair to target mcyE gene in *Dolichospermum* (b) PCR products using mcyBA1tot primer pair to target mcyB gene in *Planktothrix* (c) PCR products using mcyE-F2/ mcyE-plaR3 primer to target mcyE gene in *Planktothrix*.

NOTE 1: Detailed information about positive controls used in each PCR analysis in this section can be found on (CHAPTER 2- *Part III: Genetic analysis)*.

NOTE 2: All the gel pictures represent samples starting from June to December 2013 and from January to May 2013 (left to right), except figure3-C, which represents the mcyE in *Planktorhrix* from June to August, at 4 different depths (0-2, 9-11, 19-21 and 60 m, left to right).

5.10.2 Phylogeny analysis

The taxonomic attribution of the Oscillatoriales identified in this research was confirmed adopting a polyphasic approach. The strategy behind this approach has been described in chapter 5 (Shams *et al*., 2015), which reports the description of the new *Tychonema* populations discovered in Lake Garda. The same approach has been adopted for the confirmation of the taxonomic identification of *Planktothrix rubescens*. The autecology and toxin production in this species have been widely described in previous works (Salmaso *et al*., 2014). The taxonomic identification of *P. rubescens* identified in the samples recorded in the summer 2013 (July-September) has been confirmed by phylogenetic characterization in (Fig.4). The individuals collected in Lake Garda in 2013 form a tight cluster along with other individuals collected in other European lakes (NIES, CCAP) or in previous sampling campaigns in Lake Garda (FEM DD).

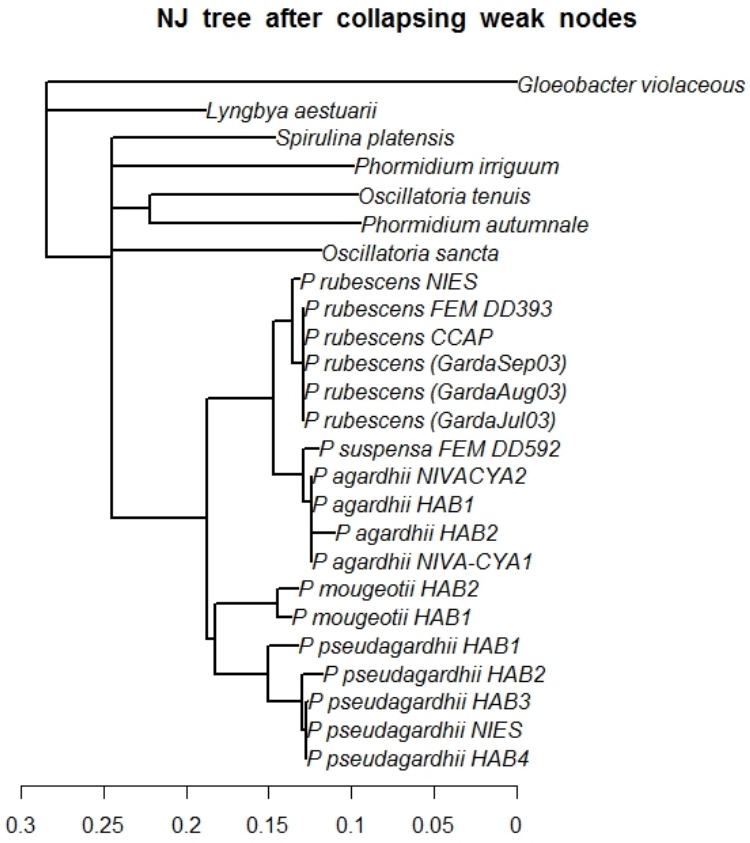

Figure4. Molecular phylogenetic characterization of *P. rubescens* (NJ, Tamura and Nei 1993 method, rooted) based on rpoC1 DNA sequences (DNA-dependent RNA polymerase gene) (>450 bp). The figure is obtained computing a Neighbour Joining tree after collapsing weak nodes (with bootstrapping values < 70%, based on 1000 replicates).

5.10.3 qPCR anaylsis

5.10.3.1 Seasonal and spatial dynamic of anaC-osc copy numbers

From June to November 2013, environmentals samples taken from 3 different depth (0, 10, 20m) were analysed with qPCR to quantify the ATX gene (anaC) copy numbers. The gene copies, which were detected at all depths, were particularly abundant between 10 and 20 m).

As shown in figure 5, the anaC-osc copy numbers were more abundant during the summer months, specifically on June and July, and the highest copy numbers were 38563 copies ml^{-1} recorded for the month July at the depth of 20 m.

From August onward, the copy numbers declined dramatically to less than 5000 copies ml^{-1}. The lowest value was recorded during the November with 79 copies ml^{-1}.

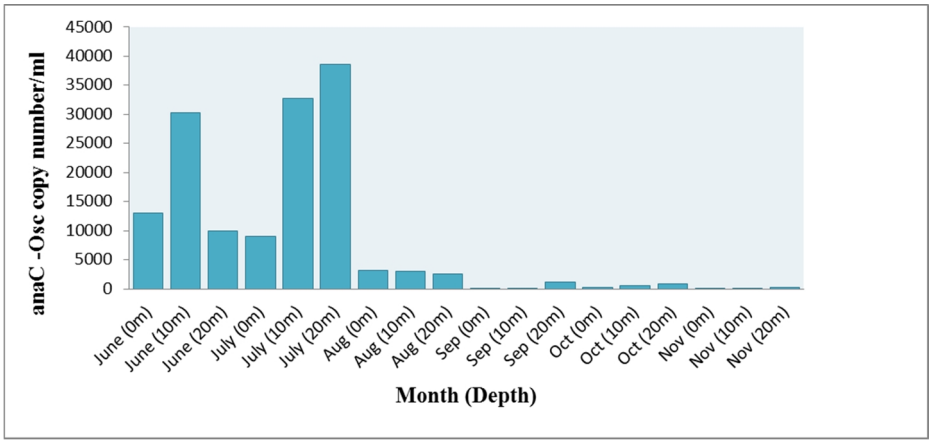

Figure5. Seasonal and spatial distribution of anaC-osc copy numbers during summer and autumn in Lake Garda.

5.10.3.2 Correlation between ATX conentration and anaC-osc copy numbers

As demonstrated in figure 6, the same trend was observed for ATX concentration. The highest values were recorded during summer specifically on June and July. However, towards the end of the summer

and autumn months, the ATX concentration decreased significantly. A strong, positive linear correlation between anaC copy numbers and ATX concentrations was found (Fig. 7).

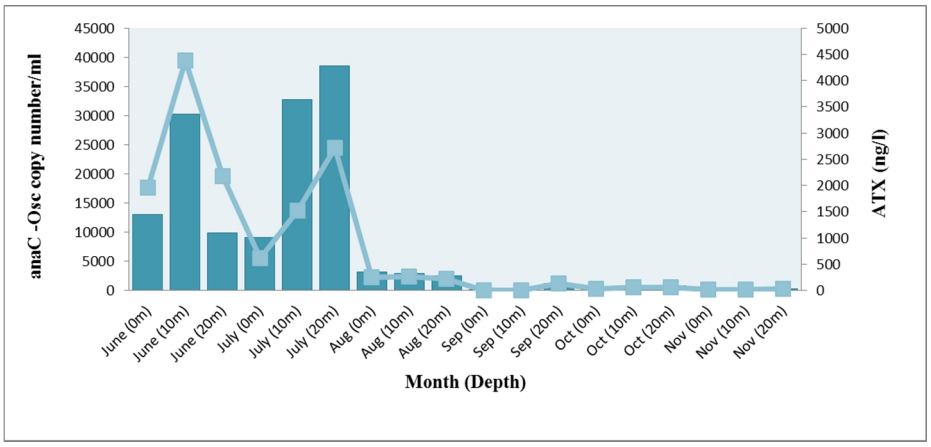

Figure6.The development of anaC copy numbers (measured with qPCR) and ATX concentrations (determined by LC–MS) in Lake Garda from June to November 2013.

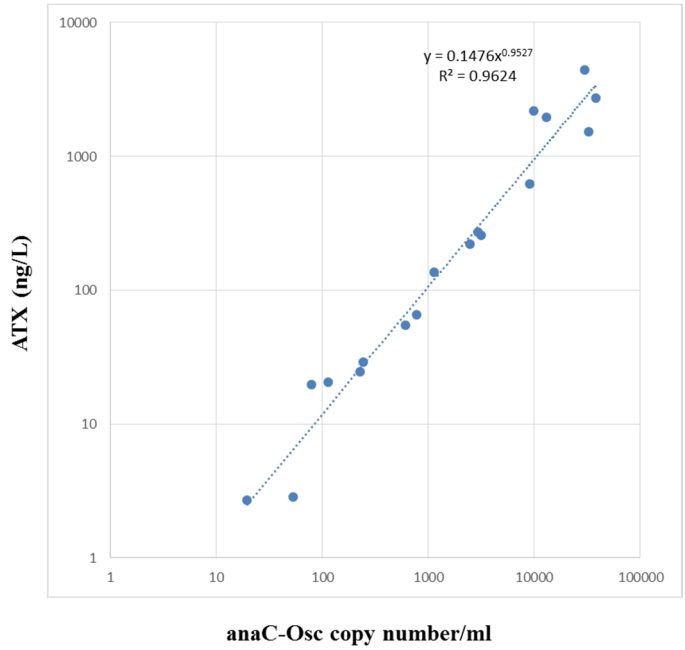

Figure7. Modeling the anaC-osc copy number and ATX concentration

5.11. References

Salmaso, N., Copetti, D., Cerasino, L., Shams, S., Capelli, C., Boscaini, A., Valsecchi, L., Pozzoni, F., Guzzell, L., 2014.Variability of microcystin cell quota in metapopulations of *Planktothrix rubescens*: causes and implications for water management. *Toxicon* 90, 82-96.

Shams, S., Capelli,C., Cerasino, L., Ballot, A., Dietrich, D.R., Sivonen, K., Salmaso, N., 2015. Anatoxin-a producing *Tychonema* (cyanobacteria) in European water bodies. Water Research 69, 68-79.

Chapter 6

6. DISCUSSION

6. Discussion

Planktonic cyanobacteria frequently form blooms which can pose a threat when water is used for drinking or recreational activities. Many of bloom forming cyanobacteria are able to produce a variety of toxins which are known as a global hazard to human and animal health. In this study, three aspects were addressed:

- **Cyanobacterial toxin diversity**. Many studies have investigated cyanotoxins diversity through different chemical analysis, among them ELISA it is a very popular screening tool. However, it provides limited quantitative information for specific groups of cyanotoxins and lack the ability to distinguish the different variants of microcystins. Therefore, in this study we applied the highly sensitive LC/MS methods to identify and quantify cyanotoxins in Lake Garda.

- **Toxin transfer through foodweb.** The adverse effect of cyanotoxin on aquatic animals has been documented by many papers. However, no investigations were madeon the cyanotoxin accumulation pattern in zooplankton. This aspect plays an important role in the transfer of cyanotoxins to higher trophic level. In this study, we studied accumulation pattern of MC in *Daphnia magna* while grazing on *Planktothrix rubescens*. The models obtained from this stuty could illustrate better the toxin accumulation pattern in the body of *D. magna*.

- **Genetic analysis.** Molecular detection methods have been reported as very fast and precise tools to study cyanotoxin genes in freshwater ecosystems. In this study, we investigated the presence of different toxin genotypes in environmental samples of Lake Garda and isolated strains by applying different general/genus specific primers.

Detailed information for each topic can be found in the respective CHAPTERS (3-5). In the following section, results of each part will be discussed:

6.1 Cyanobacterial toxin diversity

Cyanotoxins produced by freshwater cyanobacteria have been responsible of many intoxications in human and animals (Metcalf and Codd, 2012). Under certain circumstances, they can reach high concentrations in waters and cause health and ecological risks. The role of climate change (increased temperature, UV) has been discussed by many authors in connection with cyanobacterial growth and elevated cyanotoxin production (O'Neila *et al*., 2012 and references therein).

Microsystins are the most common hepatotoxins involved in animal poisoning events, including death of cattle, sheeps, dogs, horses (Briand *et al*., 2003). Their negative effect on aquatic biota (zooplankton, crustaceans, molluscs and fish) has been also investigated by many studies (Zurawell *et al*., 2005; Ferrão-Filho *et al*, 2002). Furthermore, several studies demonstrated that microcystins were associated with tumour incidence (Humpage *et al*., 2000; Dietrich and Hoeger, 2005; Svircev *et al*, 2009).

Anatoxin-a is a potent neurotoxin that has been responsible for fatal intoxications of dogs and livestock reported from different countries including; France (Cadel-Six *et al*., 2007), Netherland (Faassen *et al*., 2012), Scotland (Edwards *et al*., 1992), Japan (Park *et al*. 1993).

Thus, cyanotoxins are regarded as very important chemical compounds that can negatively affect human, aquatic animals and ecosystem functioning. Regular monitoring and up-to-date information regarding cyanotoxins in each water body play an important role for health authorities.

Cerasino and Salmaso (2012) reported data about the cyanobacterial toxin diversity in samples collected on 2009 from nine lakes located in the Italian subalpine region. They detected MCs in all lakes, and also ATX in four lakes (with concentrations varying from 45 to about 590 ng/l). They found in Lake Garda, the microcystin RRdm was always the most dominant variant of MC with more than 90% of the total MC with the highest concentration observed in late summer-early autumn. The ATX was also observed in 4 lakes with the highest amount recorded for Lake Garda in July.

The first part of the present work aimed to continue analysing the toxin diversity in Lake Garda and to elucidate the temporal and spatial distribution of MC variants and ATX in water sample collected during the period of 2011-2013.

Water samples were analysed with LC-MS and the investigation showed ATX and MC have been always present and it could better demonstrate that Lake Garda has a toxic potential. A seasonal pattern for both MC and ATX was found. MC observed with typical late summer-early autumn peaks,

however, ATX recorded with an early summer peak, specifically during June- July. Five different MC variants were identified and quantified, and the variant MC-RRdm was always dominant over the others. In CHAPTER 1, the temporal behavior of the five MC variants was furthermore demonstrated. Four MC variants (RRdm, LRdm, YR,RR) are highly correlated, which means they are produced at the same time. On the contrary, MC-LR appears to be produced independently.

6.2 Toxin transfer through food web

MC, the most common cyanotoxin, has been responsible of many negative effects in aquatic organisms and food webs throughout the world. The filamentous cyanobacterium *Planktothrix rubescens*, is the best known producer of MC. In spite of the widespread occurrence of toxic *Planktothrix* in European lakes, *Daphnia* grazing on filamentous cyanobacteria has been investigated so far by only a limited number of studies.Therefore, the second part of the study focused on the accumulation of MC in *Daphnia magna* grazing on *Planktothrix*. We demonstrated that *Planktothrix* filaments declined significantly over time in the presence of daphnia as a grazer.

This study was the first report demonstrating the kinetics of MC accumulation in *D. magna*, by applying models describing accumulation of MC (stored in *P. rubescens* cells) in the body of *Daphnia* over time. The experiments showed quite clearly how the relationship between the accumulation of MC in *Daphnia* and the initial concentrations of toxic cyanobacteria and toxins can be described with general linear and exponential models, depending on the exposure time.

The models obtained from this study could have important implications for the transfer of toxins along the trophic webs. The non-linear patterns of MC accumulation which was shown in this study could indicate that trophic transfer of MC to higher trophic levels would be strongly dependent on the trophic status of water bodies and the degree of toxicity of cyanobacterial strains characterized by different toxins cell quota.

6.3 Genetic analysis

In this study, the question about the potential ATX producer in Lake Garda was partially solved. After checking the environmental samples of 2013 with specific primers for anaC gene in *Dolichospermum*, a negative PCR amplification was observed, therefore our assumption for considering the *Dolichospermum* as a potential ATX producer was not confirmed.

Considering a few recent papers describing the presence of ATX encoding genes in several *Oscillatoriales* (Aráoz *et al*., 2005; Méjean *et al*., 2009), our first hypothesis was that production of

ATX in Lake Garda could be ascribed to *P. rubescens* (an *Oscillatoriales)*. Moreover, since there was no specific primer to target anaC gene in *Planktothrix*, the environmental samples were further checked with the anaC-osc primers, which target anaC gene in *Oscillatoria*. Interestingly, positive amplification was observed throughout a whole year of observations, confirming the presence of ATX genotypes in this lake. At this stage, however, the production could not be attributed to *P. rubescens*.

At the next step, cultures of single strains isolated from environmental samples were analysed adopting a polyphasic approach, which included cyanotoxins analysis, microscopic species identification, and genetic and phylogenetic analyses.

This approach led to the discovery of a new, unexpected Oscillatoriales producing ATX in Lake Garda, namely *Tychonema bourrellyi*. This is the first discovery of a planktonic genus belonging to the Oscillatoriales able to produce ATX.

The identification of this new ATX producer was also verified by the amplification of the anaC genes involved in the biosynthesis of ATX. The results were confirmed by the concurrent analyses of *Tychonema* strains isolated in Norway.

However, more study is needed to evaluate the seasonal and spatial dynamics of *Tychonema* in Lake Garda, as well as toxic potential assessed both in environmental and isolated strains.

Nevertheless, other questions still remain to solve. In particular the ability of *P.rubescens* and *D. lemmermannii* to produce ATX. Even thought environmental samples were negative for anaC gene in *Dolichospermum*, this does not necessarily mean that *D.lemmermannii* lacks this gene; further, its detection could be hampered by the low abundance of *Dolichospermum* and therefore lack of enough biomass for DNA extraction. The only reliable way is through investigating the toxic genotypes in isolated strains.

6.4 Outlook

This PhD work provided valuable information about cyanotoxin diversity and their seasonal patterns in Lake Garda. The accumulation of one of the most common cyanotoxins (MC) conveyed by *P. rubescens* in the body of *D.magna*, allowed to better investigate the toxic potential of the most abundant toxic cyanobacterium in Lake Garda. Finally, the discovery of a new ATX producer demonstrated the urgency to monitor extensively cyanobacterial toxins diversity and toxin genotypes in fresh water ecosystems. In fact, this new discovery further highlights how the number of cyanotoxins producers in freshwater environments is possibly still underestimated. On the other hand, the way in which these results originated in this study, suggests that the only reliable strategy to identify cyanotoxins producers should be based on the isolation of strains and their identification with a polyphasic approach associated to a concurrent metabolomic profiling performed with advanced analytical techniques.

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Up middle: *http://english.anhuinews.com/system/2011/07/25/004277730_02.shtml*

Up right: Photo by Peter Essick, National Geographic, *http://news.nationalgeographic.com/news/2014/08/140804-harmful-algal-bloom-lake-erie-climate-change-science/*

Bottom left: Photo from the South African River Health Program, *http://www.dwaf.gov.za/iwqs/rhp/state_of_rivers/state_of_umngeni_02/resource_units.html*

Bottom middle*: http://www.haloarchaea.com/resources/cyanobacterialBloom2013/*

Bottom right: Photo by Tom Rose (WA Waters and Rivers Commission) *http://www.ozcoasts.gov.au/indicators/econ_cons_algal_blooms.jsp*

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VII. DECLARATION OF SELF-CONTRIBUTION

This work was conducted without the help of unauthorized third parties, data and concepts from other literature sources discussed in this work were all referenced. This work or a similar was not yet presented to another examination authority in any country. This thesis was written solely by Shiva Shams and revised by Nico Salmaso, Daniel Dietrich and Leonardo Cerasino.

This PhD thesis was financed by EU Central Europe Programme (EULAKES Project, 2CE243P3) and the IASMA Research and Innovation Centre, Edmund Mach Foundation, and the PhD project was written by Nico Salmaso.
All the experiments were designed in consultation with Nico Salmaso, Daniel Dietrich and Leonardo Cerasino and Karl-Otto Rothhaupt. The individual contributions to the manuscripts are as stated below:

Chapter3- Inter-annual variability of the microcystins pool in the oligo-mesotrophic Lake Garda (Italy)

Shiva Shams conducted all the toxin extraction. Adriano Boscaini collected the samples. Leonardo Cerasino performed the LC-MS/MS analysis.The models were prepared by Nico Salmaso.The manuscript was written by Leonardo Cerasino.

Chapter4- Experimental models of microcystin accumulation in *Daphnia magna* grazing on *Planktothrix rubescens*: Implications for water management

The experiment was planned by all the authors. Shiva Shams conducted all the experiment; Leonardo Cerasino performed the LC-MS/MS analysis; and the models were prepared by Nico Salmaso. The manuscript was written by Shiva Shams and revised by Leonardo Cerasino, Nico Salmaso and Daniel Dietrich.

Chapter5- Anatoxin-a producing *Tychonema* (Cyanobacteria) in European waterbodies

For samples of Lake Garda: Shiva Shams carried out all the experiments and wrote part of the manuscript; Camilla Capelli assisted in experimental procedures, strain isolation and maintenance cultures; Leonardo Cerasino performed the LC-MS analysis; Andreas Ballot performed the whole analyses in the samples recorded in Norway, and conducted the phylogenetic analysis. Daniel Dietrich and Kaarina Sivonen critically edited and reviewed the whole manuscript, discussing experiments

and results. Nico Salmaso was the supervisor of experiments and data analysis, and prepared the first draft of the manuscript, which was finalized by all the authors.