Cyanobacterial Toxin (Microcystins):

Occurrence, Accumulation and Effects on Freshwater Clam

A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Biological Sciences

(Doctoral Program in Bioindustrial Sciences)

Pham Thanh Luu

Abstract

Increasing of human population and the consequent intensification of agricultural and industrial activities along with deficient water management have led to enhancement of eutrophication and cyanobacterial blooms (CYBs) in freshwater bodies. Occurrence of toxic CYBs in freshwater environments is a global problem and public health concern due to produce secondary metabolites with toxic properties, named cyanotoxins. Among various cyanotoxins known, microcystins (MCs) are the most widespread in freshwaters. While MCs have been associated with many aquatic animal deaths as well as chronic effects in humans, bioaccumulation and adverse effects of sub-chronic exposure at environmentally relevant concentrations are not yet fully understood. Consuming water or food contaminated with MCs poses risks to human health. To minimize these risks, the World Health Organization (WHO) recommends a value of 1 $\mu g\ L^{-1}$ for total MC for drinking water guideline and a chronic tolerable daily intake (TDI) of 0.04 $\mu g\ kg^{-1}$ body weight per day for human consumption.

Episodes of toxic CYBs have recently been well documented in subtropical and temperate regions. However, information on toxic cyanobacteria and their toxins from tropical regions like Southeast Asia is limited. Dau Tieng Reservoir provides drinking water for millions of people in southern Vietnam. Although CYBs have recently reported, cyanobacterial toxins are not considered as an important parameter of water quality in Vietnam. Therefore, during excessive proliferation of cyanobacteria, local people may be facing health risks caused by cyanotoxins.

This work focused on (1) isolated cyanobacteria and their toxins in Dau Tieng Reservoir. (2) Investigated the accumulation and elimination of free MC and covalently bound MC (Co-MC) in the edible clam *Corbicula leana* under laboratory conditions. (3) Evaluated oxidative stress and toxic effects caused by various cyanobacterial biomass including toxic, non-toxic *Microcystis* intact cells and a natural cyanobacterial crude extract (CCE) containing MCs in

the clam tissues. And, (4) calculated potential health risks associated with consumption of the clam contaminated with MCs.

A total of 68 cyanobacterial strains belonging to 5 genera: *Microcystis* (59 strains), *Dolichospermum* (4 strains), *Arthrospira* (2 strains), *Pseudanabaena* (2 strains) and *Cylindrospermopsis* (1 strain) have been characterized from Dau Tieng Reservoir. Investigation these strains revealed the co-existence of toxic and non-toxic genotypes. In which *Microcystis aeruginosa* was bloom-forming species and toxin producer. The MC concentrations from bloom samples and cultures were as high as 669 and 2,129 µg g^{-1} dry weight (DW), respectively.

In the aquatic environment, MCs present not only in ambient water but also contaminate in aquatic animals via bioaccumulation through food web. Aquatic animals may take up and accumulate MCs via the two main routes: uptake dissolved toxins via a transdermal route or intake of toxic cyanobacterial cells via ingestion. In this study, the accumulation and depuration of MC in clam *C. leana* were investigated in the two main routes by using both toxic cells (toxic *Microcystis*, for via ingestion) and CCE containing MCs (for via uptake from dissolved fraction). Results showed that clam *C. leana* was able to accumulate a considerable amount of MCs via both ways, ingest toxic cyanobacteria and/or via uptake from dissolved fraction in water column. The clam *C. leana* accumulated up to 12.7 ± 2.5 µg g^{-1} DW of free and 4.2 ± 0.6 µg g^{-1} DW of Co-MC when feed with toxic cells, and 3.4 ± 0.63 µg g^{-1} DW of free and 0.3 ± 0.013 µg g^{-1} DW of Co-MC when exposed to CCE containing MCs. Results also show that although free MC was quickly took up and rapidly excreted after 1 – 3 days of exposure and depuration, the Co-MC was still detectable after 10 days of depuration.

Both toxic, non-toxic cyanobacteria cells and/or CCE containing MCs caused many toxic effects on clam tissues. They induced the detoxification and antioxidant enzymes in various organs of *C. leana*. The activity of superoxide dismutase (SOD), catalase (CAT), and

glutathione *S*-transferases (GST) in the gills and mantle increased. However, CAT and GST activity was significantly inhibited in the foot and only slightly changed in the remaining tissues when treated clams with CCE. In contrast, GST activity inhibited significantly in the gills, foot and remaining tissues while CAT and SOD activities elevated significantly in the gills and mantle whereas they were almost no change in foot and other tissues when exposed the clams to toxic and non-toxic *Microcystis*.

These results suggested that both toxic, non-toxic cyanobacteria and CCE caused oxidative stress in the clam tissues, but did not kill the clam. At the same time, the animal would both improve their antioxidant abilities and metabolize the toxin to combat these adverse effects. However, toxic effects are only temporary in both cases and that prolonged exposure may lead to the adaptations to cope with deleterious effects. Consequently, in toxin-free water, both of the antioxidant enzymes and detoxification enzyme showed adaptive responses at several time points whereby enzyme activity was induced and then returned to control levels. In other words, clams are temporarily affected by toxic and non-toxic cyanobacteria but later show signs of resistant to cyanotoxins.

Consumption of food contaminated with MCs could be potentially cancer promotion. My results showed that the total MC contents in clams exceed the TDI of 0.04 μg kg^{-1} of body mass per day. It suggested that *C. leana* represents a health risk to consumers. Consumption of this clam should be limited when there are high concentrations of toxic cyanobacteria present in the water from which it is harvested. They need detoxification process before marketing.

Contents

List of abbreviations

Abbreviations and definitions

4–PB	4-phenylbutyric acid
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
ANOVA	one-way analysis of variance
BW	body weight
CAT	catalase
CCE	cyanobacterial crude extract
Co-MC	covalently bound microcystin
CR	clearance rate
CTAB	cetyltrimethyl ammonium bromide
CYB(s)	cyanobacterial bloom(s)
DAD	diode array detector
D-MeAsp	D-erythro-b-methylas-partic acid
DNA	deoxyribonucleic acid
DTE	dithioerythritol
DW	dry weight
EDI	estimated daily intake
EDTA	ethylenediaminetetraacetic acid
FW	fresh weight
GC/MS	gas chromatography–mass spectrometry
GSH	glutathione
GST(s)	glutathione *S*-transferase(s)
HPLC	high-performance liquid chromatography
LD_{50}	lethal dose, 50%
MC(s)	microcystin(s)
MC-LR	microcystin which has leucine and arginine in positions R_1 and R_2, respectively (see Figure 1–2)
MC-LY	microcystin which has tyrosine and arginine in positions R_1 and R_2, respectively (see Figure 1–2)
MC-RR	microcystin which has arginine and arginine in positions R_1 and R_2, respectively (see Figure 1–2)
mcy	microcystin synthetase gene cluster

Mdha	N-methyldehydroalanine
me4-PB	methyl 4-phenylbutyric acid
meMMPB	methyl 2-methyl-3-methoxy-4-phenylbutyric acid
MeOH	methanol
MMPB	2-methyl-3-methoxy-4-phenylbutyric acid
MXR	multixenobiotic resistance
NIES	National Institute for Environmental Studies
NRPS	non-ribosomal peptide synthesis
OATP(s)	organic anion transporting polypeptide(s)
PCR	polymerase chain reaction
PDA	photodiode array detector
PKS	polyketide synthesis
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PPFD	photosynthetic photon flux density
PPs	protein phosphatase
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RSD	relative standard deviations
SD	standard deviations
SEM	soil extract medium
SIM	selected ion monitoring
SOD	superoxide dismutase
TDI	tolerable daily intake
TE	tris-EDTA buffer solution
TN	total nitrogen
TP	total phosphorus
UV	ultraviolet
WHO	World Health Organization
WW	wet weight

Chapter 1

Introduction

1.1. Background

1.1.1. Cyanobacteria

Cyanobacteria (also known as blue-green algae) belong to the kingdom Monera (Prokaryota), division Eubacteria, class Cyanobacteria (Stanier and van Niel, 1941). The class includes about 150 genera and approximately 2,000 species (Hoek *et al.*, 1995; Duy *et al.*, 2000). Cyanobacteria are photosynthetic prokaryotes and common in limnic systems where they can exist as solitary, free-living cells or as colonies or filaments (Catherine *et al.*, 2013). They also can be found in marine environments, terrestrial habitats or even surviving in extreme environments of Antarctica or hot springs (Zanchett and Oliveira-Filho, 2013). Unlike photosynthetic bacteria, however cyanobacteria possess chlorophyll-a in common with photosynthetic eukaryotes, and they liberate oxygen during photosynthesis. Cyanobacteria are considered as an important contributor to the formation of the Earth's atmosphere and nitrogen fixation (Hoek *et al*., 1995).

The cyanobacteria were traditionally classified by morphology into five sections, referred to by the numerals I – V: Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales and Stigonematales (Hoek *et al*., 1995). In which, group I (Chroococcales) comprises solitary and colonical unicellular cyanobacteria, for example, *Microcystis*, *Synechococcus* and *Gloeocapsa*. Group II (Pleurocapsales) consists of unicellular to pseudo-filamentous, thallus-forming cyanobacteria, with cells capable of multiple, as well as binary, fission, for example, *Pleurocapsa*. In Pleurocapsales, the cells have the ability to form internal spores (baeocytes). Group III (Oscillatoriales) comprises filamentous cyanobacteria without cell differentiation, for example, *Oscillatoria*, *Lyngbya* and *Phormidium* whereas Group IV (Nostocales) consists of filaments marked by cell differentiation to produce akinetes and heterocysts, for example,

Anabaena, *Nostoc* and *Cylindrospermopsis*. And group V (Stigonematales) comprises cell-differentiating cyanobacteria with more complex multicellular organization and includes species with truly branched trichomes, for example, *Mastigocladus* and *Stigonema* (Knoll, 2008).

1.1.2. Bloom-forming cyanobacteria and causes

Increasing of human population and the consequent intensification of agricultural and industrial activities has led to enhancement of eutrophication creating conditions favored cyanobacteria blooms (CYBs) in freshwater bodies (Figueiredo *et al.*, 2004).

There is no international definition for what a CYB is, however, this phenomenon is generally considered as an excessive production of cyanobacteria biomass over a short period of time (several weeks). In fact, blooms of cyanobacteria are often mono-specific (or nearly so) (Merel *et al.*, 2013). During the bloom, generally, a green layer or scum of cyanobacteria will form on surface water visible to the un-aided eye (Figure 1–1). Bloom-forming cyanobacteria often contain gas vesicles and are capable of floating up, enabling them to form a dense surface blooms where they can take advantage of high levels of irradiance to optimize photosynthesis (Paerl and Paul, 2012). Most of cyanobacteria prefer living in warm aquatic habitat, which therefore resulting in blooms mostly happening in summer (Havens, 2008).

Generally, the formation of CYBs is regulated by a combination of three primary environmental factors (Merel *et al.*, 2013). The first one is water temperature, many species of cyanobacteria preferring warmer conditions (20°C or above). Consequently, climate change and global warming may increase the frequency and magnitude of CYBs by favoring cyanobacteria among other phytoplankton species (Arheimer *et al.*, 2005; Wiedner *et al.*, 2007; Heisler *et al.*, 2008; Paerl and Huisman, 2009; El-Shehawy *et al.*, 2012; Kosten *et al.*, 2012; O'Neil *et al.*, 2012; Paerl and Paul, 2012; Reichwaldt and Ghadouani, 2012). The second environmental factor influencing CYBs is light intensity. Although several species of cyanobacteria can be considered hetero- or chemo-trophic, most species need a minimum of

light availability for photosynthesis (Merel *et al.*, 2013). Many cyanobacteria cannot survive high light intensities over longer periods. This may limit their distribution to more turbid and eutrophic habitats. However, some species are extremely flexible in their response to light exposure because buoyancy regulation enables them to find light conditions that are optimal for their growth (Chorus and Bartram, 1999). And the third factor leading to bloom formation is the trophic status of the aquatic system. Nitrogen and phosphorus are two nutrients that are most essential for the growth and survival of cyanobacteria. Thus, CYBs mainly occur in eutrophic water bodies and well correlate with the concentration of TN and TP (Downing *et al.*, 2001; Heisler *et al.*, 2008; El-Shehawy *et al.*, 2012).

1.1.3. Cyanobacterial hepatotoxin: Microcystins

CYBs are often toxic because they produce a variety of secondary metabolites, known as cyanotoxins. Among these, hepatotoxin microcystins (MCs) form the main family since they are the most frequently studied and the most widespread. MCs are produced by various species within the genera *Dolichospermum* (planktic *Anabaena*), *Microcystis*, *Oscillatoria*, *Planktothrix*, *Nostoc*, *Aphanizomenon*, *Arthrospira*, *Lyngbya*, *Pseudanabaena* and *Anabaenopsis* in freshwaters (Chorus and Bartram, 1999).

MCs contain seven peptide-linked amino acids, with the two terminal amino acids of the linear peptide being condensed (joined) to form a cyclic compound. The general structure is: cyclo-(D-alanine-R1-D-MeAsp-R2-Adda-D-glutamate-Mdha) in which R1 and R2 are variable L amino acids, D-MeAsp is D-erythro-b-methylas-partic acid, and Mdha is N-methyldehydroalanine (Duy *et al.*, 2000). The R1, R2 variable amino acids for MC-LR, MC-RR and MC-YR are leucine (L), arginine (R) and tyrosine (Y) (Figure 1–2). The amino acid Adda, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, is the most unusual structure in this group of cyclic peptide toxins. There are over 90 structure variants have so far been reported and the three most common are MC-LR, -RR and -YR (Pearson *et al.*, 2010).

1.1.4. Microcystin synthetases

MCs are synthesized by the thiotemplate mechanism characteristic for non-ribosomal peptide synthesis (NRPS), polyketide synthesis (PKS) and fatty acid synthesis (Kurmayer and Christiansen, 2009). The MC biosynthesis gene cluster (*mcy*) contains peptide synthetases, polyketide synthases and tailoring enzymes that are encoded by ten (in *Microcystis*, *Anabaena*) or nine (in *Planktothrix*) genes (Figure 1–3, Nishizawa *et al.*, 1999; Tillett *et al.*, 2000; Christiansen *et al.*, 2003; Rouhiainen *et al.*, 2004; Kurmayer and Christiansen, 2009).

The *mcy* gene was the first complex metabolite gene cluster to be fully sequenced from a cyanobacterium. In *M. aeruginosa* PCC7806, the *mcy* gene spans 55 kb and comprises 10 genes arranged in two divergently transcribed operons (*mcyA–C* and *mcyD–J*). The larger of the two operons, *mcyD–J*, encodes a modular PKS (*mcyD*), two hybrid enzymes comprising NRPS and PKS modules (*mcyE* and *mcyG*), and enzymes putatively involved in the tailoring (*mcyJ*, *F*, and *I*) and transport (*mcyH*) of the toxin. The smaller operon, *mcyA–C* encodes three NRPS (*mcyA–C*). Interestingly, the arrangement in the *mcy* cluster was found to be different in the organisms *Microcystis*, *Anabaena* and *Planktothrix* (Figure 1–3, Kurmayer and Christiansen, 2009; Pearson *et al.*, 2010). These fundamental studies have afforded insight into the evolution of cyanotoxin biosynthesis, and have additionally provided much of the groundwork for current PCR-based cyanobacterial detection methods (Neilan *et al.*, 2008).

1.1.5. Occurrence of hepatotoxic blooms

In recent centuries toxic CYBs have been reported worldwide and intensively reviewed on literature (Paerl *et al.*, 2001; Figueiredo *et al.*, 2004; Havens, 2008; Blaha *et al.*, 2009; Cheung *et al.*, 2013; Merel *et al.*, 2013). The most dominant bloom forming cyanobacterial genera in freshwaters are: *Microcystis*, *Dolichospermum*, *Aphanizomenon*, *Oscillatoria*, *Cylindrospermopsis*, *Phormidium* and *Nostoc* (Figueiredo *et al.*, 2004).

CYBs have been reported from many lakes and reservoirs in Europe, such as Lale Vela Portuguese, Lake Tuusulanjärvi in Finland, Lake Grand-Lieu, Saint-Caprais reservoir in

France, El Atazar reservoir in Spanish, ponds Liège in Belgium, lakes in southwestern Germany, lakes of northern Poland, all used for recreational or drinking purposes. They have been found to have hepatotoxic blooms, mainly associated with the dominance of *M. aeruginosa* and in some cases *Aphanizomenon*, *Dolichospermum* and *Oscillatoria* (Vasconcelos *et al.*, 1996; Vezie *et al.*, 1998; Wirsing *et al.*, 1998; Hummert *et al.*, 2001b; Vasconcelos *et al.*, 2001; Frank, 2002; Barco *et al.*, 2004; Mankiewicz *et al.*, 2005).

Many lakes used for drinking water from America, Canada and United States have also suffered with *M. aeruginosa* blooms that sometime have MC contents higher than the WHO guideline (Campos *et al.*, 1999; Amé *et al.*, 2003; Rinta-Kanto *et al.*, 2009; Ruiz *et al.*, 2013) and related to human exposed and animal deaths (Puschner *et al.*, 1998; Nasri *et al.*, 2008).

In Africa, occurrence of *Microcystis* and *Oscillatoria* blooms with production of MCs has been recorded in River Nile from Egypt, in Lake Victoria from Uganda, Lake Lalla Takerkoust from Morocco, Lake Oubeira in Algeria, Dam Lebna in Tunisia, dam Hartbeespoort and Roodeplaat in South African (Oudra *et al.*, 2002; Sabour *et al.*, 2002; Haande *et al.*, 2007; El-Herry *et al.*, 2008; Nasri *et al.*, 2008; Nyakairu *et al.*, 2010; Okello *et al.*, 2010; Conradie and Barnard, 2012).

The occurrence of severe *Microcystis*, *Dolichospermum* and *Cylindrospermopsis* blooms associated with MCs and other cyanobacterial toxins seems to be occur very frequent in Australia (Figueiredo *et al.*, 2004; Kemp and John, 2006; Mitrovic *et al.*, 2011; Al-Tebrineh *et al.*, 2012).

In Asian countries, intensive blooms of cyanobacteria (mainly *Microcystis*, *Dolichospermum* and *Planktothrix*) have been reported from Lake Taihu, Lake Chaohu, Lake Dianchi, Guanting Reservoir in China (Chen and Xie, 2005a; Dai *et al.*, 2008; Xu *et al.*, 2008; Chen *et al.*, 2009; Krüger *et al.*, 2010; Ma *et al.*, 2014), from Lake Kasumigaura, Lake Biwa, Lake Sagami, Isahaya Bay in Japan (Park *et al.*, 1993a; Park *et al.*, 1993b; Park *et al.*, 1998a; Tsujimura *et al.*, 2000; Ozawa *et al.*, 2005; Umehara *et al.*, 2012). CYBs associated with MCs

have also been found from eutrophic aquaculture ponds and water reservoirs in Taiwan (Lee *et al.*, 1998), many lakes from Korea (Park *et al.*, 1998b), from Laguna de Bay, the largest freshwater lake in the Philippines (Cuvin-Aralar *et al.*, 2002), as well as Kranji Reservoir in Singapore (Te and Gin, 2011), Lake Thanh Cong, Tri An, Dau Tieng and Nui Coc Reservoirs in Vietnam (Hummert *et al.*, 2001a; Dao *et al.*, 2010; Duong *et al.*, 2014).

CYBs are now global environmental problems and public concerns. Therefore, it is necessary to make more efforts to minimize the impacts of CYBs and their toxins in aquatic ecosystems.

1.2. Previous studies on accumulation and effects of cyanotoxins

1.2.1. Accumulation

The ability of MCs to accumulate in a wide range of aquatic animals, including zooplankton, bivalves, gastropods, fish and macrocrustaceans in both laboratory and field studies is well known from literature (for reviews: Ibelings and Chorus, 2007; Smith *et al.*, 2008; Martins and Vasconcelos, 2009; Ferrão-Filho and Kozlowsky-Suzuki, 2011).

Zooplankton is reported the best bioaccumulator of MCs, reaching value over 1,000 µg g^{-1} dry weight (DW). This leads to transport of MCs through the food chains because zooplankton community is one of the most important links between the primary producers and higher aquatic consumers such as fish (Ferrão-Filho *et al.*, 2002; Sotton *et al.*, 2014).

Gastropods and bivalves may be among the most threatened groups of aquatic organisms in the presence of toxic cyanobacteria blooms because they are sessile filter-feeders (Martins and Vasconcelos, 2009). Freshwater as well as marine gastropods and bivalves are able to accumulate cyanobacteria toxins via ingestion of toxic cyanobacteria or uptake MCs dissolved in water column (Ibelings and Chorus, 2007). The highest concentrations of MCs in gastropods and bivalves were 436 and 630 µg g^{-1} DW in hepatopancreases, respectively (Ozawa *et al.*, 2003; Yokoyama and Park, 2003).

In contrast, larger crustaceans (*i.e., Macrobrachium nipponensis*) seem to be the less efficient accumulator of MCs with a maximum concentration of only 12 μg g^{-1} DW (Chen and Xie, 2005b).

Fish may be accumulated MCs via ingestion of toxic cyanobacteria or contaminated food and to a lesser extent via uptake of dissolved toxins (Sotton *et al*., 2014). MCs were found to accumulate in different organs of freshwater fish and the highest concentrations (up to 874 μg g^{-1} DW) often found in the liver (Ibelings *et al.*, 2005).

The MCs concentrations accumulated in aquatic organisms depends on species, exposure routes, target organs as well as the MCs concentration from surrounding water. This bioaccumulation of MCs in food chains is a public concern for human health. However, most previous studies have reported only the concentration of the accumulation of free MC and did not adequately consider the content of MCs covalently bound to protein phosphatases (PPs), which may represent a considerably part of the MCs burden (Lance *et al.*, 2010; 2014).

1.2.2. Toxicity and effect of microcystins

MCs are cyclic heptapeptides because they target liver cells, and its cellular uptake requires organic anion transporter polypeptides (OATPs) activity (Fischer *et al.*, 2005). Once in hepatocytes, they are potent inhibitors of eukaryotic protein phosphatases PP1 and PP2A by interacting with the catalytic subunits of these enzymes (MacKintosh *et al.*, 1990; Duy *et al.*, 2000). While PPs serve as a regulator to maintain homeostasis in the cell, inhibition of PPs leads to hyperphosphorylation, causing severe cell damage (Eriksson *et al.*, 1990; Dawson, 1998). This is a major post-transitional modification which can result in excessive signaling and may lead towards cell proliferation, cell transformation and tumor promotion (MacKintosh *et al.*, 1990; Apeldoorn *et al.*, 2007; Amado and Monserrat, 2010).

Besides inhibition of PPs, oxidative stress, produced by reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO•) may play an important role in the pathogenesis of MCs toxicity in various organisms (Pašková *et*

al., 2008; Gonçalves-Soares *et al.*, 2012; Paskerová *et al.*, 2012; Pavagadhi *et al.*, 2012; Galanti *et al.*, 2013).

As a preventive step to protect the public health from adverse effects, WHO recommends a provisional guideline value of 1 μg L^{-1} for MC-LR concentration in drinking water in 1998, and a chronic tolerable daily intake (TDI) of 0.04 μg kg^{-1} body mass per day for human consumption (Chorus and Bartram, 1999).

Although MCs are present in surface water bodies worldwide, there is still little study on the toxicity of complex cyanobacterial crude extract (CCE) containing MCs or intact toxic-cells. Water from CYBs contain not only MCs but a complex hazardous compounds such as microviridins, micropeptins, oscillapeptins, or lipopolysaccharsides that can evoke more pronounced toxic effects than MCs or other well-recognized cyanotoxins (Burýšková *et al.*, 2006; Falconer, 2007; Palíková *et al.*, 2007; Smutná *et al.*, 2014). Therefore, it is necessary to consider and evaluate the effects of complex cyanobacterial biomasses or intact toxic-cells, especially at the environmentally relevant concentration on aquatic organisms.

1.3. Aims of this study

To reduce the impacts of CYBs on public health, it is important to characterize toxins in waters sources and mitigate their effects on aquatic animals. Testing toxicity of different MCs sources by using animal models is an important step toward understanding how they will interact in human health. Therefore, the aims of this study are:

1) Identify MCs producers and characterize MCs production/profiles of CYB samples from Dau Tieng Reservoir, which has been supplying drinking water for millions of people in southern Vietnam.

2) Investigate accumulation/depuration of free MC, Co-MC and adverse effects from CCE containing MCs on the edible clam *Corbicula leana*.

3) Investigate accumulation/depuration of free MC, Co-MC and adverse effects of toxic and non-toxic cyanobacteria *Microcystis* on the clam *C. leana.* And, determination human health risks associated with consumption of the animal contaminated with MCs.

1.4. Research contents

In order to understand occurrence, accumulation/depuration and effects of MCs, a series of experiments both in field and laboratory have been conducted in this study. The contents for this thesis consist 5 chapters and are summarized as follows:

Chapter 1: Introduction of research background, previous studies and objectives.

Chapter 2: Reported the identification and characterization a total of 68 cyanobacterial strains isolated from Dau Tieng Reservoir, determined their toxin profiles, toxic abilities and detected the MC concentrations of CYBs collected from this reservoir.

Chapter 3: In order to understand the toxicity of cyanobacterial cell lysis containing MCs (often occur at the end of a bloom), I collected toxic cyanobacterial biomass from water blooms in Kasumigaura Lake. The samples were then used to expose to the clam *C. leana*. The uptake and depuration of free- and Co-MC were investigated. And adverse effects on various tissues caused by this biomass in *C. leana* were evaluated through changes of antioxidant and detoxification enzymes.

Chapter 4: In order to understand the toxicity of toxic *Microcystis* (one of the most cyanobacteria frequently causes bloom throughout the world), I cultured the toxic *M. aeruginosa* (strain NIES-1086) and non-toxic *M. aeruginosa* (strain NIES-101); both were then used to expose to clam *C. leana*. And again, the uptake, depuration of free- and Co-MC as well as adverse effects on various tissues in clam *C. leana* were investigated by comparison with the green alga *Chlorella vulgaris* (strain NIES-2170).

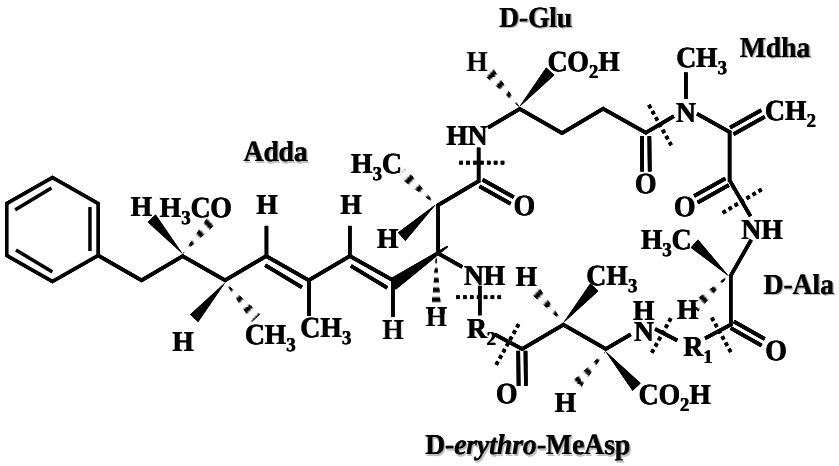
Chapter 5: General conclusions and recommendations.

Lake Kasumigaura, Japan; August 2013

Dau Tieng Reservoir, Vietnam; July 2011

Lake Chao Hu, China; June 2008 (photo from www.researchgate.net)

Figure 1–1. Blooms of cyanobacteria.



	R_1	R_2
microcystin-RR	arginine	arginine
microcystin-LR	leucine	arginine
microcystin-YR	tyrosine	arginine

Figure 1–2. Chemical structure of microcystin-LR, -RR, and -YR.

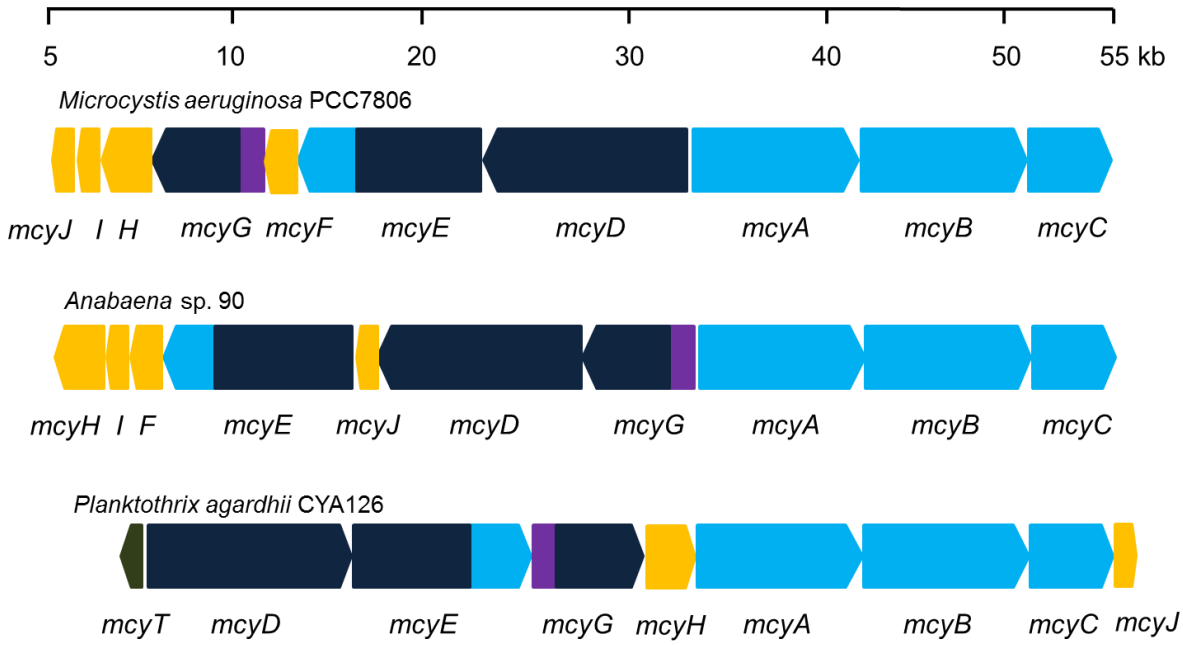


Figure 1–3. Structural organization of the *mcy* gene clusters in *Microcystis*, *Anabaena* and *Planktothrix* (modified from Pearson *et al*., 2010).

Chapter 2

Occurrence of toxic cyanobacterial blooms and their toxins in Dau Tieng Reservoir, Vietnam

2.1. Introduction

Cyanobacteria are notorious for their ability to produce a variety of toxic secondary metabolites called cyanotoxins, which can be especially problematic during CYBs (Sivonen, 1996). The common occurrence of high concentrations of cyanotoxins associated with CYBs in drinking, recreational, and irrigation water bodies poses a serious hazard for wild and domestic animals as well as humans (Pouria *et al.*, 1998; Carmichael *et al.*, 2001; Stewart *et al.*, 2008).

Among cyanotoxins, MCs are the most prominent cyanobacterial hepatotoxins in freshwater (Chorus and Bartram, 1999). Morphological methods have traditionally been used to monitor and characterize cyanobacteria. However, blooms of cyanobacteria usually consist of toxic and non-toxic strains (Neumann *et al.*, 2000). Morphological characteristics cannot be used as the basis for differentiation of toxic and non-toxic strains because many strains of cyanobacteria appear to be identical under the microscope. The presence or absence of the *mcy* gene cluster has been widely used as a means for distinguishing the two genotypes and has recently been used to reveal the presence of MC-producing cyanobacteria in both environmental samples and axenic cultures (Nishizawa *et al.*, 2000; Tillett *et al.*, 2000; Bittencourt-Oliveira, 2003; Hisbergues *et al.*, 2003; Kurmayer and Kutzenberger, 2003; Hotto *et al.*, 2005; Pedro *et al.*, 2011). However, because the conclusions of most of these studies have been based on only one gene/operon, the results have sometimes been unreliable in cases of homology and mutation on the *mcy* gene. The amplification of several genes responsible for synthesis of MC in both the NRPS and PKS modules may be a more appropriate way to detect potentially toxic cyanobacteria.

Episodes of toxic CYBs have recently been rather well documented in subtropical and temperate regions (Te and Gin, 2011). However, information on toxic cyanobacteria and cyanotoxins from tropical regions like Southeast Asia is limited. In Vietnam, few investigations of toxic cyanobacteria and their toxins have been undertaken within the last decade. The most commonly cited potentially toxic cyanobacterial species has been *M. aeruginosa*, which has been reported from Lake Thanh Cong (Hummert *et al.*, 2001a), the Huong River (Nguyen *et al.*, 2007b), and Tri An Reservoir (Dao *et al.*, 2010). Other potentially toxic species such as *M. botrys* and *M. wesenbergii* have also been reported (Nguyen *et al.*, 2007b). Recently, Nguyen *et al.* (2012) collected, morphologically characterized, and classified *Microcystis* strains in the middle and north of Vietnam, but provided no information on MC concentrations. In those studies, a small number of strains was isolated and characterized for MC production. There is still little understanding about the potential toxin production of other common species belonging to the genera *Anabaena* (*Dolichospermum*), *Arthrospira*, *Cylindrospermopsis*, and *Pseudanabaena*.

Dau Tieng Reservoir has been supplying drinking water for millions of people in southern Vietnam. Nutrient enrichment, especially phosphorus and nitrogen, has led to conditions that favor cyanobacterial growth in the reservoir. However, there is still lack of data relevant to polymorphism of *mcy* genes, toxic and non-toxic genotypes, and toxin profiles from toxin producers. This study aimed to collect cyanobacterial assemblages, including samples from blooms, and to isolate numerous strains for investigation. Identification of cyanobacterial species was based on microscopic observations. MC concentrations in both isolated strains and bloom samples were quantified by high performance liquid chromatography (HPLC). The ability of isolated strains to produce MCs was elucidated with *mcyA*, *mcyB*, and *mcyD* molecular markers.

2.2. Materials and methods

2.2.1. Sites and sampling

Dau Tieng Reservoir is located in southern Vietnam, about 85 km northwest of Ho Chi Minh City. The reservoir is located within a quadrat bounded by 11°12'–12°00'N, 106°10'–106°30'E. Its maximum depth, mean depth, surface area, and volume are 24 m, 3 m, 264 km^2, and 1.08 × 10^8 m^3, respectively (Figure 2–1). Dau Tieng Reservoir serves multiple purposes. It is a source of drinking and irrigation water, a recreational resource, a means of flood control, and an attraction to tourists (Vietnam-MOSTE, 2001). The reservoir falls into the eutrophic category according to the classification scheme described by Ye *et al.* (2009) that associates eutrophy with total phosphorus concentration of 25–100 µg L^{-1} and total nitrogen concentration of 600–1500 µg L^{-1} (unpublished data).

I used a conical plankton net with a mesh size of 25 µm to collect cyanobacterial samples monthly during July–October 2011 and March–May 2012 at both shallow- (DT3 and DT5) and deep- (DT1, DT2 and DT4) water sites (Figure 2–1). The sites included one location commonly used for recreation (DT1); two locations used intensively for fish and duck farming (DT2 and DT4); and two sites near stream mouths (DT3 and DT5). The characteristics of the water at the sampling sites were determined in situ during sampling and included pH (model 744 pH meter, Metrohm, Herisau, Switzerland), temperature (WTW Oxi197i, Metrohm, Weilheim, Germany), and water transparency (Secchi disk). The values of these parameters were used as metrics of the abiotic characteristics of the sites where the cyanobacteria were collected. Samples for isolation of cyanobacteria were maintained under viable conditions, and samples for identification were fixed with Lugol's solution in the field (Sournia, 1978). Samples of the scum from blooms were collected in July and August 2011 at the lake shore, filtered onto GF/C glass fiber filters (Whatman, Kent, England), dried at 45°C overnight, and stored at –80°C prior to MCs analysis.

2.2.2. Morphological characterization

Cyanobacteria were observed at 800 × magnification under a microscope Olympus BX51 equipped with a digital camera and DP71 software (Olympus, Tokyo, Japan). Taxonomic classification was based on the system of (Komárek and Anagnostidis, 1989; Komárek and Anagnostidis, 1999; Komárek and Anagnostidis, 2005). Descriptions of cyanobacteria were based on observations of both preserved and cultured samples.

Cyanobacterial isolation and cultivation: Cyanobacteria were isolated by micropipetting and washing. A single cyanobacterial trichome or colony was isolated by micropipetting, washed, and transferred into cyanobacterial growth medium (Belcher and Swale, 1988). Cyanobacteria were grown in MA medium (Ichimura, 1979). All cultures were grown on a 12:12 light:dark cycle at a temperature of 28°C and light intensity of 20 µmol photons m^{-2} s^{-1}. Biomasses of cyanobacterial cultures were harvested onto GF/C glass fiber filters (Whatman, Kent, England) and processed as described above.

2.2.3. DNA extraction

DNA was extracted by the method of Hisbergues *et al.* (2003) with minor modifications. Briefly, 2 mL of fresh culture was harvested by centrifugation (10,000 × *g*, 15 min, 4°C). Cell pellets were suspended in 567 µL TE buffer (50 mM Tris/HCl, 40 mM EDTA, pH 8.0). An aliquot of 30 µL of 10% SDS (sodium dodecyl sulfate) and proteinase K (final concentration: 100 µg mL^{-1} in 0.5% SDS) was then added and incubated for 60 min at 37°C. Then 5 M NaCl (100 µL) and CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) (80 µL) were added, and the samples were incubated for 10 min at 65°C. DNA was then extracted twice with 780 µL phenol:chloroform:isoamyl alcohol (25:24:1 v/v). After centrifugation for 5 min at 6,000 × *g* at 4°C, the supernatant was collected and transferred to a fresh tube. DNA was then precipitated by the addition of a volume of isopropanol equal to 0.6 times the volume of the supernatant, followed by centrifugation for 20 min at 20,000 × *g* at 4°C. The precipitated

DNA was rinsed with 1 mL of 70% ethanol and dried under vacuum. The final DNA sample was rehydrated in 20 µL of 1 × TE buffer (10 mM Tris and 1 mM EDTA [pH 8.0]). Quantitation was done by absorbance (GeneQuant pro, Cambridge, England) at 260 nm using a Sub-Micro quartz cuvette (NSG, New York, USA).

2.2.4. PCR amplification

Two peptide synthetase gene fragments, *mcyA* and *mcyB*, (297 bp and 78 bp, respectively) were amplified using primer pairs *mcyA*-Cd1F/*mcyA*-Cd1R (Hisbergues *et al.*, 2003) and *mcyB*-30F/*mcyB*-108R (Kurmayer and Kutzenberger, 2003). The fragment of the *mcyA* gene has previously proven to be suitable for detection of MC-producing cells of the genera *Dolichospermum*, *Microcystis*, and *Planktothrix* (Hisbergues *et al.*, 2003). The polyketide synthase fragment (*mcyD*, 297 bp) was amplified using primer pair *mcyD*-F2/*mcyD*-R2 (Kaebernick *et al.*, 2000). To detect the presence of cyanobacterial DNA, the CYA primer pair (Urbach *et al.*, 1992) was used to amplify a 1200 bp fragment of the 16s rRNA gene common to all cyanobacteria. For each sample, four separate PCRs were conducted. DNA of *M. aeruginosa* NIES-102 and *M. aeruginosa* NIES-1086, both of which produce MCs (Yasuno *et al.*, 1998), were used for positive controls. As a negative control, I used DNA extracted from a non-toxic strain, *M. aeruginosa* NIES-101 (Baeka *et al.*, 2009)**.** All PCR reactions were prepared in a volume of 20 µL containing 2 µL of 10 × Ex-Taq Buffer, 200 µM of each dNTP, 0.5 µL of each primer (10 µM), 0.5 U of Ex-Taq polymerase (Takara Bio Inc., Shiga, Japan), and 20–25 ng of template DNA. If any strain was positive for one *mcy* marker but not for others, each template DNA was diluted and used for PCR again, but with 10, 25, 50, and 100 ng DNA to confirm the results. Amplification was performed in a Veriti 200 Thermal Cycler (Applied Biosystems, Foster City, California, USA) according to the protocols summarized in Table 2–1. PCR products were examined on 1.5% or 2.5% (w/v) agarose gels stained with ethidium bromide. The images were obtained under ultraviolet (UV)

transillumination and digitalized with a Canon Digital EOS Kis X2 system (Canon Inc., Tokyo, Japan).

2.2.5. Microcystins extraction and analysis

The filters containing cells (about 20 mg of dried cells) were cut into small pieces (1–2 mm^2) with scissors. Extraction of MCs was conducted as described by Barco *et al.* (2005) with minor modifications. Briefly, MCs were first extracted in 5 mL of 100% aqueous methanol (MeOH) by shaking for 60 min followed by two 60-min extractions in 3 mL of 75% (vol/vol) aqueous MeOH. Each extraction step was followed by sonication (1 min, 150W) and centrifugation (1,800 × *g*, 4°C, 30 min). The supernatants of all extractions from each sample were pooled, dried at room temperature, re-dissolved in 0.5 mL MeOH (100%) and centrifuged at 6,000 × *g* at 4°C for 5 minutes. The supernatant was passed through a Minisart RC4 filter membrane (0.2 µm pore size, Sartorius Stedim Biotech, Göttingen, Germany), and kept at –20°C prior to reversed-phase HPLC analysis.

A reverse-phase HPLC with UV-visible photodiode array (PDA) detector (Shimadzu 10A series, Kyoto, Japan) was equipped with a silica–based, reverse-phase C_{18} column (Waters SunFire^{TM} 5 µm, 3 × 250 mm, Milford, Massachusetts, USA) and maintained at 40°C. The samples were separated with a mobile phase consisting of methanol: 0.05 M phosphate buffer (pH 2.5; 50:50 v/v) at a flow rate of 0.58 mL min^{-1}. MC congeners were detected by UV detection at 238 nm and identified on the basis of both their retention time and characteristic UV spectra. Microcystins (MC-LR, MC-RR, and MC-YR) purchased from Wako Pure Chemical Industries, Ltd. (Chuoku, Osaka, Japan), were used as standards. The HPLC system had a detection limit of 0.1 µg L^{-1}.

2.3. Results

2.3.1. Isolation and morphological characteristics

Microscopic examination of the July and August 2011 CYB samples revealed the dominance of *Microcystis* (mainly *M. aeruginosa*) and the less frequent occurrence of other genera *Dolichospermum* (*Anabaena*), *Arthrospira*, *Planktothrix*, *Pseudanabaena*, and *Cylindrospermopsis*. During the blooms, which lasted for several weeks, the pH and water temperature of all sites were in the ranges of 8.3–9.1 and 29.0–31.0°C, respectively. Identification of individual *Microcystis* colonies revealed the occurrence of three species: *M. aeruginosa*, *M. botrys*, and *M. wesenbergii*. I successfully isolated 68 cyanobacterial strains from the cyanobacterial samples (collected from July–October 2011 and March–May 2012) and maintained them in cultures. Those 68 strains were representatives of eight species that belonging to three orders of cyanobacteria, Chroococcales (59), Nostocales (5) and Oscillatoriales (4) (Table 2–2). A taxonomic breakdown of the eight isolated species is as follows:

Order CHROOCOCCALES

Family MICROCYSTACEAE

Microcystis aeruginosa (Kützing) Kützing 1846

Colonies of this species were embedded in a colourless mucilage that varied in shape from more or less spherical to strongly irregular assemblages of sub-colonies (Figures 2–2a, b). The individual cells were more or less spherical, with aerotopes present. The cells were 4–6 µm in diameter and separated from each other, even in dense colonies. The colonial form of this species was not maintained, and the colonies were mostly separated as single and/or double cells in culture.

Microcystis botrys Teiling 1942

The colonies of this species were composed of more or less spherical sub-colonies, with densely packed cells in the centre of colonies (Figure 2–2c). Sub-colonies were 50–150 µm in diameter. The colonial form of this species was maintained in cultures during its exponential

phase. The mucilage was colourless and surrounded clusters of cells. Cells were dark in colour, 5.5–8.0 µm in diameter, and had many aerotopes.

Microcystis wesenbergii (Komárek) Komárek in Kondrateva 1968

This species formed elongate, often lobate, clathrate, and sometimes spherical colonies that were commonly composed of sub-colonies with a distinct refractive mucilage edge (Figure 2–2d). In nature, a sub-colony of this species was 15–100 µm in diameter and contained from a few (2–3) to many (> 150) cells, depending on the age of the colony. Cells were more or less evenly spread throughout the colony, 5.5–8.5 µm in diameter, with many spherical aerotopes.

Order NOSTOCALES

Family NOSTOCACEAE

Dolichospermum nygaardii (Cronberg & Komárek) Wacklin, L.Hoffmann & Komárek 2009

Trichomes were solitary, planktic, with regular coils 30–40 µm in width. The distance between coils was 10–25 µm. Cells were spherical, 5.5–6.5 µm in diameter with aerotopes (Figures 2–3 a–c). Heterocytes were spherical, slightly bigger than the vegetative cell, and 6–6.5 µm in diameter. Akinetes were oval or somewhat kidney-shaped, solitary, distant from heterocytes, with dimensions of 7–10 × 11.5–19 µm. The akinetes were only observed in the cultures. Usually only one heterocyte or akinete was present on each trichome.

***Dolichospermum* sp.**

Trichomes were solitary, planktic, with coils of 30–35 µm in width. The distance between coils was 7.5–10 µm. (Figures 2–3 d–g). Cells were oval, 6.5–8.5 µm broad, 7–10 µm long, with aerotopes. Heterocytes were spherical, somewhat larger than the vegetative cells, and 7.5–10 µm in diameter. Akinetes were not observed in either field samples or cultures.

Cylindrospermopsis raciborskii (Woloszynska) Seenayya & Subba Raju in Desikachary 1972

Trichomes were solitary, straight, or slightly curved. Cells were cylindrical, 4–5.5 µm wide, and 7–13 µm long (Figures 2–3 h–j). The terminal heterocytes were long and conical, 3–6 µm wide, and 6–14 µm long. The akinetes was one or two cells distant from terminal heterocytes and sometimes occurred in pairs. They were long ovals, 5–8 µm wide and 13–19.5 µm long. Akinetes were observed in both field samples and cultures.

Order OSCILLATORIALES

Family PHORMIDIACEAE

Arthrospira massartii Kufferath 1914

Trichomes were greyish-blue-green, planktic, solitary, spiral, with indistinct cross walls. They were not constricted at cross walls and were tapered slightly at the ends (Figures 2–3 k–n). The distance between coils of spiral trichomes was 20–40 µm, and the trichomes had a coiled diameter of 35–45 µm. Cells were 2.5–3 µm long and 4.5–6 µm wide, with aerotopes. Apical cells were tapered and rounded.

Family PSEUDANABAENACEAE

Pseudanabaena limnetica (Lemm.) Komárek 1974

Trichomes were solitary, planktic, straight or slightly curved, and not tapering toward the ends (Figure 2–4). Cells were pale blue-green, cylindrical, without aerotopes, 1.5–2 µm wide and 3–6.5 µm long, and constricted at the cross wall. Apical cells were bluntly rounded.

2.3.2. Microcystin producers

The ability to produce MCs of all strains was determined by using three molecular markers: *mcyA*, *mcyB*, and *mcyD*. Results of the *mcy* gene analysis MC-producing ability, as determined by *mcyA* showed that among 59 strains of *Microcystis*, all three toxin genes *mcyA*, *mcyB*, and *mcyD* were present in 25 strains (42% of the *Microcystis* strains). Twelve *Microcystis* strains were positive for *mcyA* and *mcyB* but negative for *mcyD*; the 22 remaining *Microcystis* strains (37% of the *Microcystis* strains) were negative for all *mcyA*, *mcyB*, and

mcyD genes. None of the strains of *Dolichospermum*, *Arthrospira*, *Pseudanabaena* and *Cylindrospermopsis* were positive for an *mcyA*, *mcyB*, or *mcyD* amplicon. All strains were positive with 16S rRNA (Figure 2–5).

Within the genus *Microcystis*, relationships between morphospecies and MC genotypes as well as between morphospecies and MC production were apparent. Colonies of *M. aeruginosa* had the greatest potential for MC production. In contrast, no strains assigned to *M. botrys* and *M. wesenbergii* yielded fragments of *mcy* genes in the PCR amplification products, the indication being that these strains were non-toxic.

2.3.3. Microcystin production

Results of HPLC analysis indicated that 25 cultured strains (37% of all isolates) contained MCs. The total concentration of MCs from toxic isolated strains ranged from 39 to 2,129 µg g^{-1} DW. The MCs content of these strains was quite variable, the highest concentration being found in strain M.ae–42 and the lowest concentration in strain M.ae–25. The predominant MC variant in these strains was MC-RR. It was present in most of the toxic strains, followed by MC-LR and MC-YR (Figure 2–6).

Two bloom samples collected in July and August contained three MCs, MC-LR, MC-RR and MC-YR (Figure 2–7). The concentration of MCs in those samples ranged from 521 to 669 µg g^{-1} DW. The MC-RR concentration was high in the first sample but much lower in the second one. In contrast, the concentrations of MC-YR and MC-LR were low in the first sample (8% and 6%, respectively) but much higher (22% and 23%, respectively) in the second. In both bloom samples, MC-RR was the dominant congener (Table 2–3).

2.4. Discussion

There is still little known about the tropical cyanobacterial microflora (Cronberg and Komárek, 2004); this is especially true in the case of Vietnam. Like many other cyanobacterial species, *Microcystis* spp. are distributed throughout the world and can be

found in habitats ranging from freshwater to brackish water (Komárek and Anagnostidis, 1999; Cronberg and Annadotter, 2006). The species *M. aeruginosa* and *M. botrys* are common in Vietnam (Pham, 1969; Phung *et al.*, 1992; Duong, 1996; Nguyen *et al.*, 2007b; Dao *et al.*, 2010). In my study, *M. aeruginosa* was dominant in bloom samples from the Dau Tieng Reservoir; an observation that is consistent with previous records from Vietnam (Nguyen *et al.*, 2007b; Dao *et al.*, 2010). In general, cell sizes of *Microcystis* spp. were slightly larger in the Dau Tieng Reservoir than in some other freshwater environments in Vietnam (Nguyen *et al.*, 2007b; Dao *et al.*, 2010).

The genus *Dolichospermum* (formerly known as planktic species of *Anabaena*) comprises more than 40 species that are distributed worldwide (Cronberg and Annadotter, 2006; Wacklin *et al.*, 2009). *Dolichospermum nygaardii* is a tropical cyanobacterial species that has previously been recorded only in Africa (Cronberg and Komárek, 2004). Morphologically, it was more or less similar to *D. flos-aquae.* However, the coiling characteristics and akinete shapes of cultivated strains were more similar to those of *D. nygaardii* (Komárek and Zapomelova, 2007). Therefore, I preferred to identify it as *D. nygaardii*, but I recommend further molecular investigations of this strain, such as 16S rRNA gene sequencing, for confirmation of the identity of this species. I also suggest molecular characterization for species identification of the *Dolichospermum* sp. because of the absence of information about its akinetes from the field samples and cultures. My investigation recorded *D. nygaardii* for the first time in Vietnam.

About ten species of *Cylindrospermopsis* have been described (Cronberg and Annadotter, 2006), and *C. raciborskii* is the most commonly recorded and investigated (Falconer and Humpage, 2005). In Vietnam, this species was initially identified as *Anabaenopsis raciborskii* Woloszynska in some freshwater bodies in northern Vietnam (Duong, 1996) but subsequently updated with the current name by Nguyen *et al.* (2007b). This species has also been reported from the Tri An Reservoir in southern Vietnam (Dao *et al.*,

2010). However, the occurrence of *C. raciborskii* in this water body reflects in part the wide distribution of this harmful species in Vietnamese waters, especially drinking water supplies. The cell size and especially the cell length of *C. raciborskii* isolated from the Dau Tieng Reservoir are greater than the size and length of cells from the Tri An Reservoir or Hue localities (Nguyen *et al.*, 2007b; Dao *et al.*, 2010).

The genus *Arthrospira* comprises about ten species that occur mainly in tropical and semi-tropical regions (Cronberg and Annadotter, 2006). In Vietnam, I have found four *morphotypes* of this genus, including *A. gigantea, A. platensis*, *A. jenneri* and *A. massartii* (Pham, 1969; Phung *et al.*, 1992; Duong, 1996; Nguyen *et al.*, 2007b). The dimensions of trichomes and cells of this species in my study and in a previous investigation (Nguyen *et al.*, 2007b) are comparable. The genus *Pseudanabaena* consists of about 30 species (Komárek and Anagnostidis, 2005). However, few species of this genus have been described from Vietnam. *Pseudanabaena limnetica* was first identified as *Oscillatoria limnetica* Lemm. from the Mekong Delta in Vietnam (Pham, 1969); it has also been recorded from the Tri An Reservoir (Dao *et al.*, 2010) and now (my study) from the Dau Tieng Reservoir. Both *A. massartii* and *P. limnetica* are properly characterized as cosmopolitan species (Komárek and Anagnostidis, 2005).

The cyanobacterial biota from the Dau Tieng Reservoir consists of several potentially toxic species, including species of *Microcystis*, *Dolichospermum*, *Planktothrix*, *Pseudanabaena* and *Cylindrospermopsis*. My results are consistent with those of Joung *et al.* (2011) and Neumann *et al.* (2000) that cyanobacterial population exists as a mixture of toxic and non-toxic genotypes. *Arthrospira* and *Pseudanabaena* are not well known in terms of toxin production, but some reports of their toxicity have been published (Iwasa *et al.*, 2002; Ballot *et al.*, 2004; Nguyen *et al.*, 2007b). *Cylindrospermopsis* isolated from water bodies in the cities of Hue and Pleiku in the middle and central highlands of Vietnam have been reported to produce cylindrospermopsin (Nguyen *et al.*, 2010). Because I found no *mcy* gene

fragments and detected no production of MCs in my samples of *Arthrospira*, *Dolichospermum, Pseudanabaena*, and *Cylindrospermopsis* strains, I concluded that *Microcystis* was the major MCs producer in the Dau Tieng Reservoir. Nevertheless, there is no information for these strains concerning the presence of other toxins such as cylindrospermopsin, saxitoxins, and anatoxin-a; their potential presence warrants further investigation.

PCR-based detection of genes involved in the synthesis of MCs (*mcyA-J*) is the cornerstone of rapid detection techniques and has been successfully applied for early detection of the mass occurrence of potentially toxic cyanobacterial (Bittencourt-Oliveira, 2003; Rantala *et al.*, 2006; Martins *et al.*, 2009; Gkelis and Zaoutsos, 2014). The use of *mcyA*, *mcyB*, and *mcyD* for assessment of MC toxigenicity has previously been described (Hisbergues *et al.*, 2003; Hotto *et al.*, 2005; Rinta-Kanto *et al.*, 2005; Vasconcelos *et al.*, 2010; Kumar *et al.*, 2011). Microscopic and PCR results revealed that the different morphospecies varied considerably with respect to the proportion of genotypes that produced MCs. In general, non-toxic strains do not contain *mcy* genes. However, some strains may have fragments or mutant forms of *mcy* genes. Consequently, the *mcy* genes of these strains can be amplified with *mcy* primers, although the strains are not able to produce toxins (Kaebernick *et al.*, 2000; Dittmann *et al.*, 2001; Tillett *et al.*, 2001; Martins *et al.*, 2009; Vasconcelos *et al.*, 2010). Homology or down regulation of the *mcy* gene region or the presence of adenylation domains with other loci or even the presence of a cluster of *mcy* genes in some non-toxic *Microcystis* strains may also lead to such discrepancies (Janse *et al.*, 2004). In this study, I used three *mcy* genes from both NRPS and PKS domains for screening toxigenicity. Strains showing positive results for all three *mcyA*, *mcyB*, and *mcyD* genes generated at least one variant of MC, including MC-RR. However, several non-toxic strains were positive for the two molecular markers *mcyA* and *mcyB*. The same observations have been reported in cyanobacterial samples collected from Portuguese water bodies (Martins *et al.*, 2009) and

Central Mexico water bodies (Vasconcelos *et al.*, 2010). In contrast, Gkelis and Zaoutsos (2014) detected MCs in water samples from Greece, where the *mcyA* gene region was not amplified. Therefore, it is evident that *mcy* genes differ with respect to their ability to predict MC toxigenicity. My results indicate that *mcyD*, which encodes for parts of both the β-ketoacyl synthase and the acyltransferase domains (Rantala *et al.*, 2004), is one of the best molecular markers for determination of potential toxicity of cyanobacteria. The *mcyA* gene has been reported from *Microcystis* strains isolated from localities near Hue in Vietnam (Nguyen *et al.*, 2007a). However, my study is the first to apply *mcyB* and *mcyD* for the analysis of the toxicity of cyanobacterial strains from Vietnam. The combination of HPLC and PCR provides a robust tool for reliably characterizing lakes in terms of MCs. The HPLC assay measures the actual level of toxin production, whereas PCR provides an estimate of the potential for toxin biosynthesis.

The presence of MCs from cultures was revealed with the three variants MC-RR, MC-LR, and MC-YR, among which MC-RR was the dominant congener. MC production and yield varied considerably among strains. The most highly toxic strains were strains of *M. aeruginosa*. The cyanobacterial population of the Dau Tieng Reservoir is thus a mosaic of genotypes, the various strains being genetically different, with distinct toxicity potential and toxic ability. From the Dau Tieng Reservoir, MCs have been reported in a mixed *Microcystis* culture, the highest concentration being 128 µg L^{-1} (only MC-LR present) (unpublished data). Together with the MC-LR reported previously, I also identified MC-RR and MC-YR, but the most common variant in this reservoir was MC-RR. The MC concentrations in the bloom samples (521–669 µg g^{-1} DW) were within the range of concentrations reported for the Tri An Reservoir (450–640 µg g^{-1} DW) by Dao *et al.* (2010).

MCs are highly water soluble and more than 90% of the total amount is contained within the cells in healthy cyanobacterial populations. However, dying and decaying cyanobacteria may release MCs into the water (Chorus and Bartram, 1999). The lower the

concentration of intracellular MC at the end of blooms might indicate that release of MC from cells into their environment occurred during the senescence and the decomposition periods of *Microcystis* cells. My data agreement with those of (Park *et al.*, 1998a) that high concentrations of intracellular MC were found during the exponential growth phase of the blooms.

Risks associated with exposure to MCs present in surface waters and reservoirs worldwide are a serious concern from the standpoint of human health because of the potent hepatotoxicity of MCs and their tendency to promote tumors (Chorus and Bartram, 1999). The Dau Tieng Reservoir is a eutrophic body of water that receives nutrients from a large drainage area as well as the runoff from fish farms, duck farms, cattle ranches and other agricultural activities, especially during the dry season (unpublished data). MCs have been reported in the raw water (unpublished data), although the occurrence of blooms has not been observed previously. A succession of blooms was first observed along the southern shore of this reservoir during the summer of 2011 (Figure 2–1). I strongly recommend further investigations to elucidate the cause of these blooms.

Local authorities have not yet addressed the public health risks associated with the presence of cyanobacterial toxins in drinking water in Vietnam. There are no facilities for removal of MCs from drinking water, nor is monitoring being conducted to detect MCs in drinking water. Dau Tieng Reservoir supplies drinking water for million people in southern Vietnam. Both raw and tap water from this reservoir were found to contain MCs. MC concentration in raw water was sometimes higher than the WHO guideline value of 1.0 $\mu g\ L^{-1}$ (unpublished data). The concentrations of MCs in tap water corresponded to the concentrations in the reservoir (unpublished data). Hence, during periods of high MCs concentrations in the reservoir, local residents may suffer from hepatotoxic effects via their daily consumption of MC-contaminated drinking water. Monitoring of toxic cyanobacterial abundance and the concentrations of their toxins as well as further investigations of the

toxicity of the Dau Tieng Reservoir water are strongly recommended to reduce impacts on humans and ecosystems.

2.5. Summary

In this chapter, cyanobacterial assemblages, isolated strains, and bloom samples from the Dau Tieng Reservoir were used for species identification, MC synthetase gene analysis, and MC determination. Microcystin concentrations in both isolated strains and bloom samples were quantified by high performance liquid chromatography, whereas toxic and nontoxic strains were distinguished by three molecular markers: *mcyA*, *mcyB*, and *mcyD*.

I isolated and characterized a total of 68 cyanobacterial strains belonging to 5 genera: *Microcystis* (59 strains), *Dolichospermum* (4 strains), *Arthrospira* (2 strains), *Pseudanabaena* (2 strains) and *Cylindrospermopsis* (1 strain). Cyanobacterial populations from the reservoir consisted of toxic and nontoxic genotypes. *Dolichospermum nygaardii* was morphologically identified and described for the first time in Vietnamese waters. The results also showed that *mcyD* is the best molecular marker for determination of toxicogenic strains. *Microcystis* included toxic and nontoxic genotypes. The MC concentrations from bloom samples and cultures were as high as 669 and 2,129 μg g^{-1} DW, respectively. These results argue strongly for the implementation of a monitoring program for cyanobacteria and their toxins in the water to minimize potential health risks to animal and human populations.

Table 2–1. Primers used for PCR amplification.

Target	Primer set	Sequence (5'–3')	Reference
Cyanobacteria	CYA-F	ACGGGTGAGTAACRCGTRA	Urbach *et al*. 1992
	CYA-R	CTTCAYGYAGGCGAGTTGCAGC	
Microcystis, *Anabaena* and *Planktothrix*	*mcyA*-Cd1F	AAAATTAAAAGCCGTATCAAA	Hisbergues *et al*. 2003
	mcyA-Cd1R	AAAAGTGTTTTATTAGCGGCTCAT	
Microcystis	*mcyB*-30F	CCTACCGAGCGCTTGGG	Kurmayer and Kutzenberger 2003
	mcyB-108R	GAAAATCCCCTAAAGATTCCTGAGT	
Microcystis	*mcyD*-F2	GGTTCGCCTGGTCAAAGTAA	Kaebernick *et al*. 2000
	mcyD-R2	CCTCGCTAAAGAAGGGTTGA	

Table 2–2. Cyanobacterial strains examined in this study.

Orders, genera, and species	Number of isolated strains
Chroococcales	
Microcystis aeruginosa	52
Microcystis botrys	5
Microcystis wesenbergii	2
Nostocales	
Dolichospermum nygaardii	2
Dolichospermum sp.	2
Cylindrospermopsis raciborskii	1
Oscillatoriales	
Arthrospira massartii	2
Pseudanabaena limnetica	2

Table 2–3. Microcystin concentrations (mean ± SD, n = 4) in two bloom samples from Dau Tieng Reservoir.

Date collected	MC-RR	MC-YR	MC-LR	Total µg g^{-1} DW
20/07/2011	582.9 ± 37.5	51.2 ± 15.4	39.3 ± 14.9	668.9 ± 24.8
06/08/2011	286.0 ± 14.8	115.7 ± 5.3	118.8 ± 13.5	520.5 ± 22.5

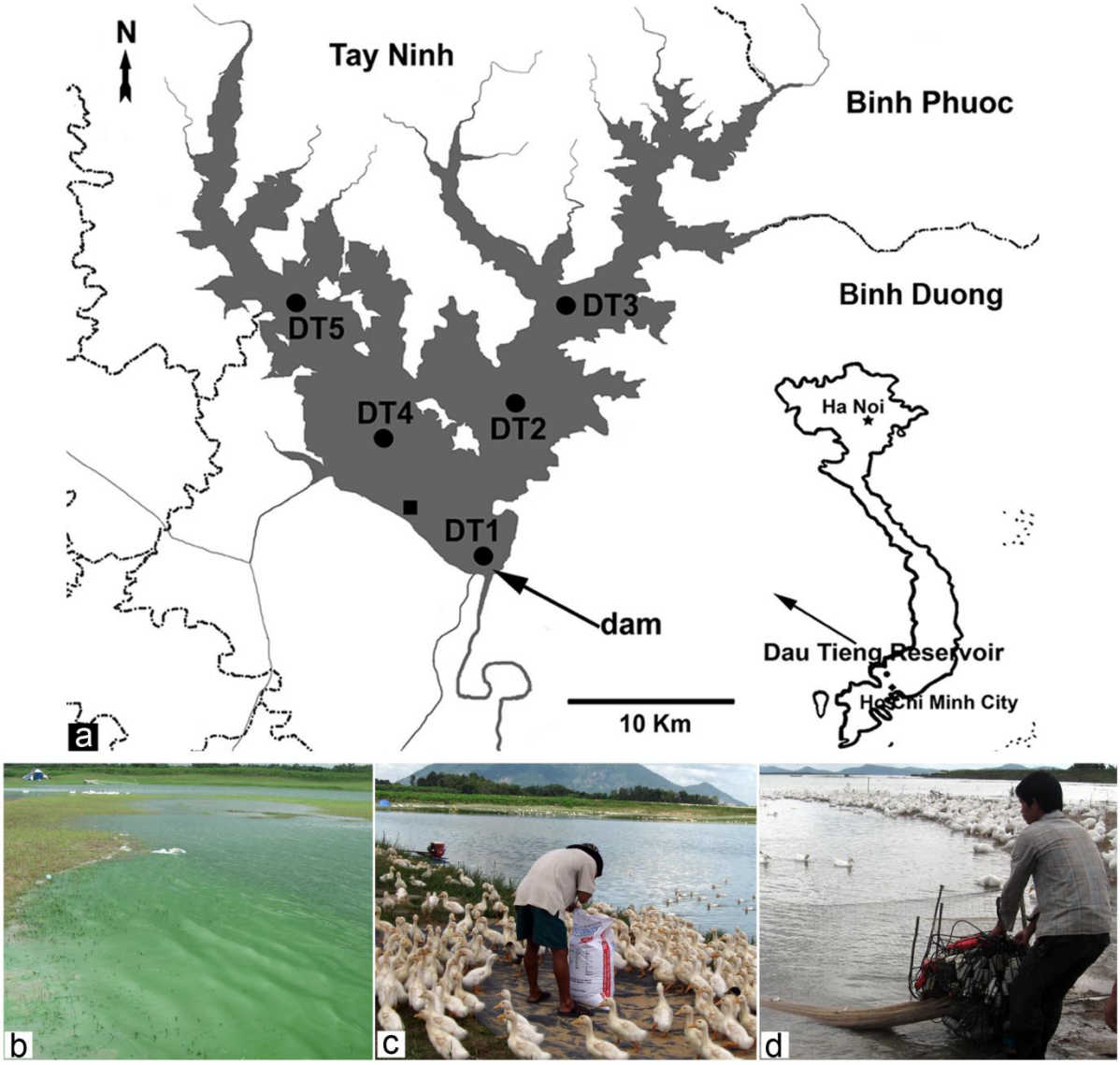
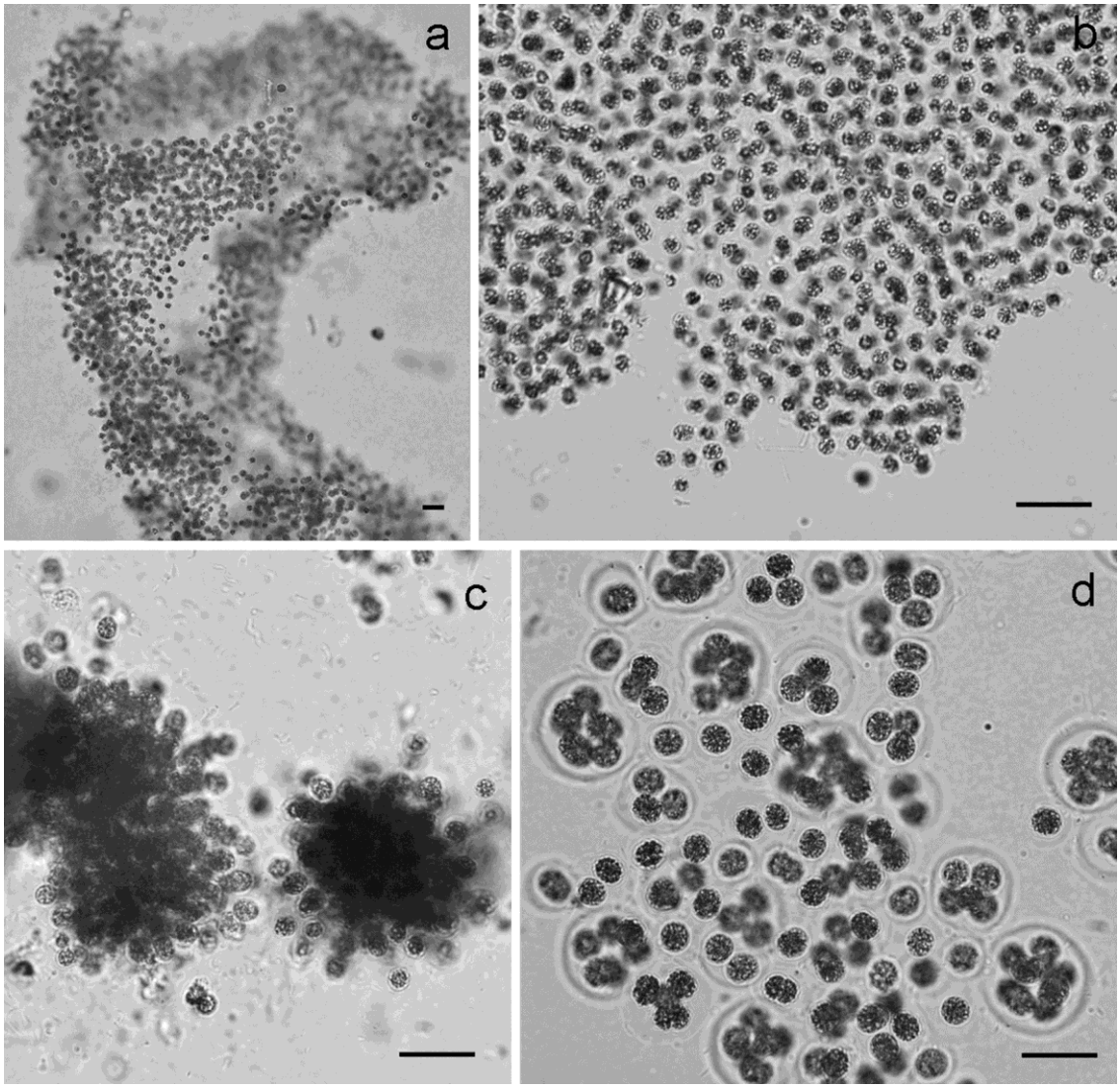


Figure 2–1. Map of the Dau Tieng Reservoir (a). The round black dots indicate locations where cyanobacteria were sampled for culture work. DT3 (11°27’24”N; 106°22’37”E) and DT5 (11°25’48”N; 106°15’52”E) represented shallow-water sites; DT1 (11°19’34”N; 106°20’36”E), DT2 (11°23’38”N; 106°21’20”E) and DT4 (11°22’21”N; 106°17’11”E) represented deep-water sites. Black square indicate location where bloom occurred (b). Duck farming on the reservoir (c). And (d) a fisherman is catching fish with his fishing net.



Figures 2–2. Photographs (a, b) *Microcystis aeruginosa*; (c) *Microcystis botrys*; (d) *Microcystis wesenbergii.* Photos were taken from preserved samples. Scale bars = 20 µm.

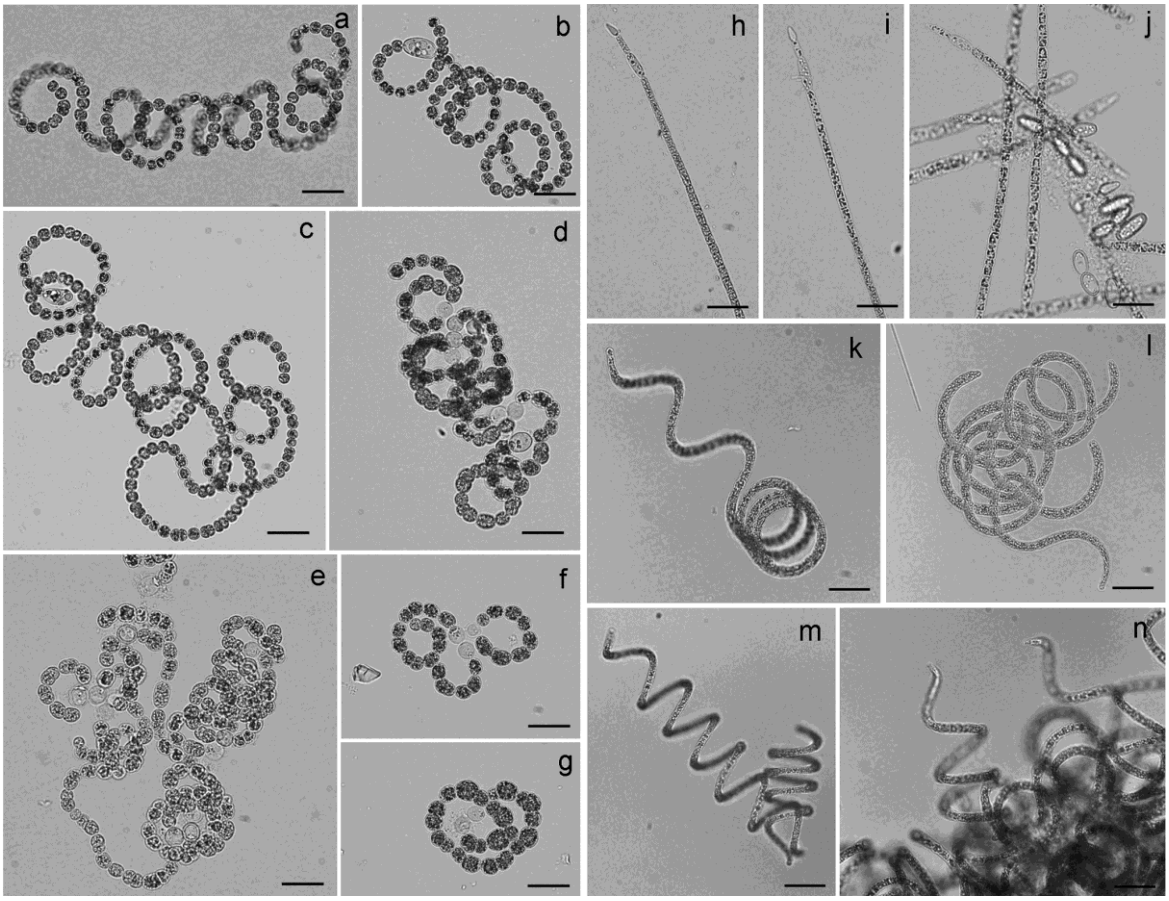


Figure 2–3. Photographs of (a–c) *Dolichospermum nygaardii*; (d–g) *Dolichospermum* sp.; (h–j) *Cylindrospermopsis raciborskii*; (k–n) *Arthrospira massartii*. Photos were taken from cultured samples. Scale bars = 20 µm.

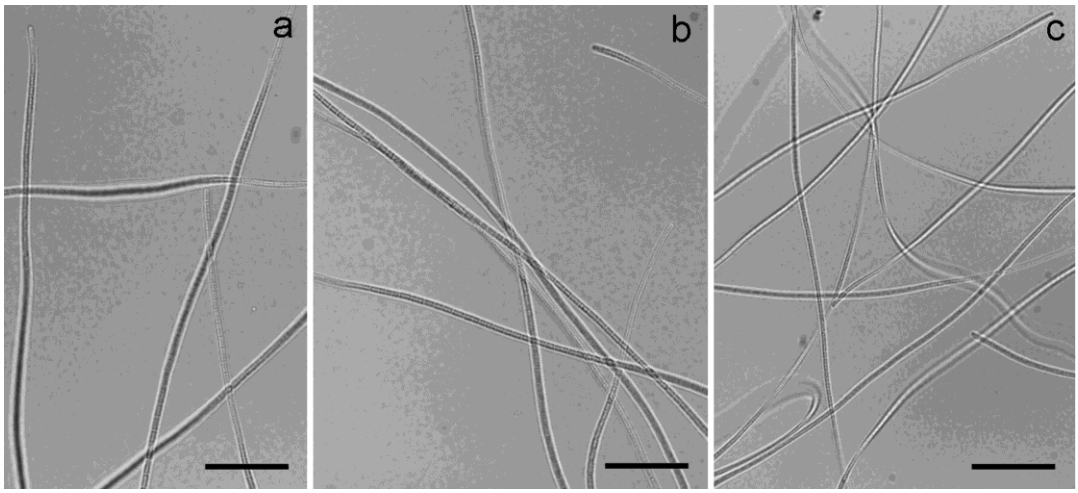


Figure 2–4. Photographs of (a–c) *Pseudanabaena limnetica*. Photos were taken from cultured samples. Scale bar = 20 μm.

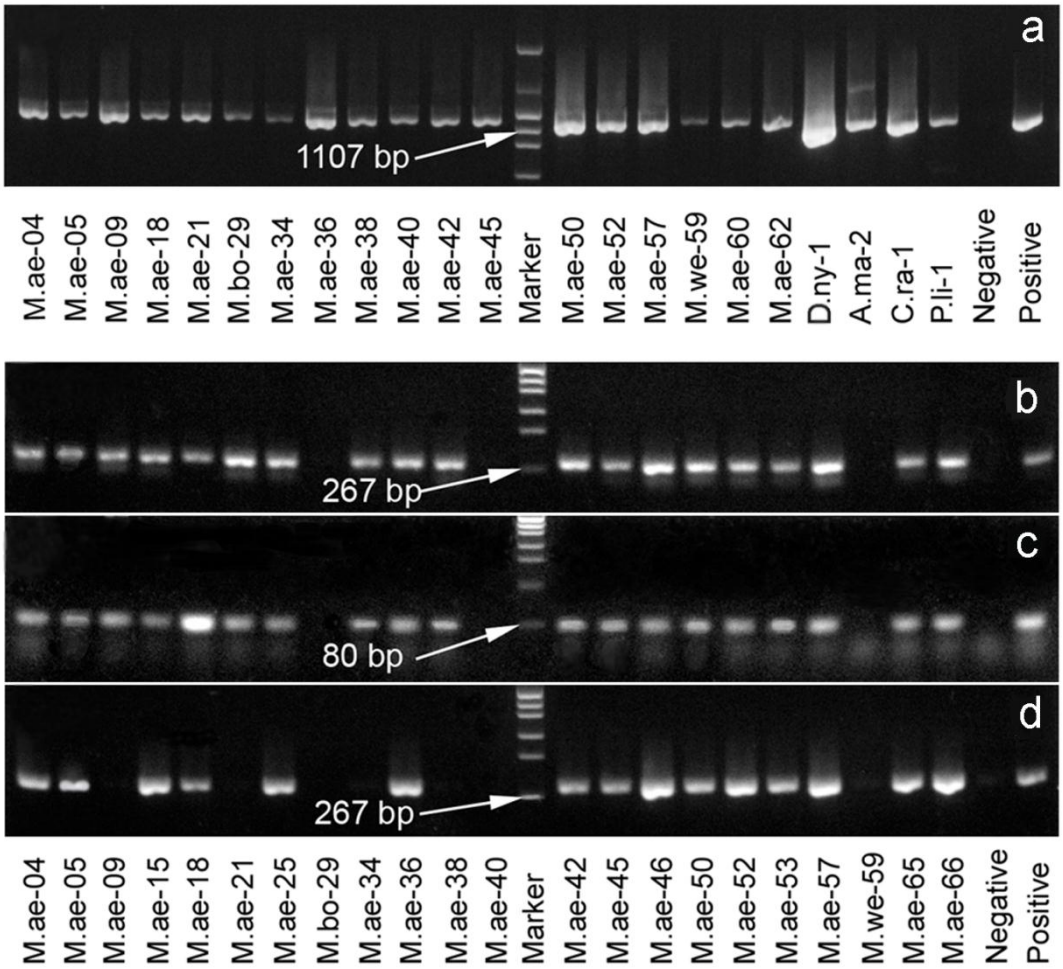


Figure 2–5. Photographs of 1.5% agarose gels (*mcyA* 297 bp, *mcyD* 297 bp, and 16S rRNA 1200 bp) and 2.5% agarose gel (*mcyB* 78 bp) stained with ethidium bromide showing the PCR products amplified by using (a) 16S rRNA, (b) *mcyA*, (c) *mcyB*, and (d) *mcyD* primer pairs. Molecular marker: PHY marker; negative control: NIES-101; positive control: NIES-1086. For 16S rRNA amplification, a sample containing no DNA template was used as the negative control. M.ae: *Microcystis aeruginosa*; M.bo: *Microcystis botrys*; M.we: *Microcystis wesenbergii*; D.ny: *Dolichospermum nygaardii*; A.ma: *Arthrospira massartii*; C.ra: *Cylindrospermopsis raciborskii* and P.li: *Pseudanabaena limnetica*. D.ny, A.ma, C.ra and P.li are not showed in *mcy* production figures (b, c and d) because they are non-toxic species and all negative with *mcy* gene.

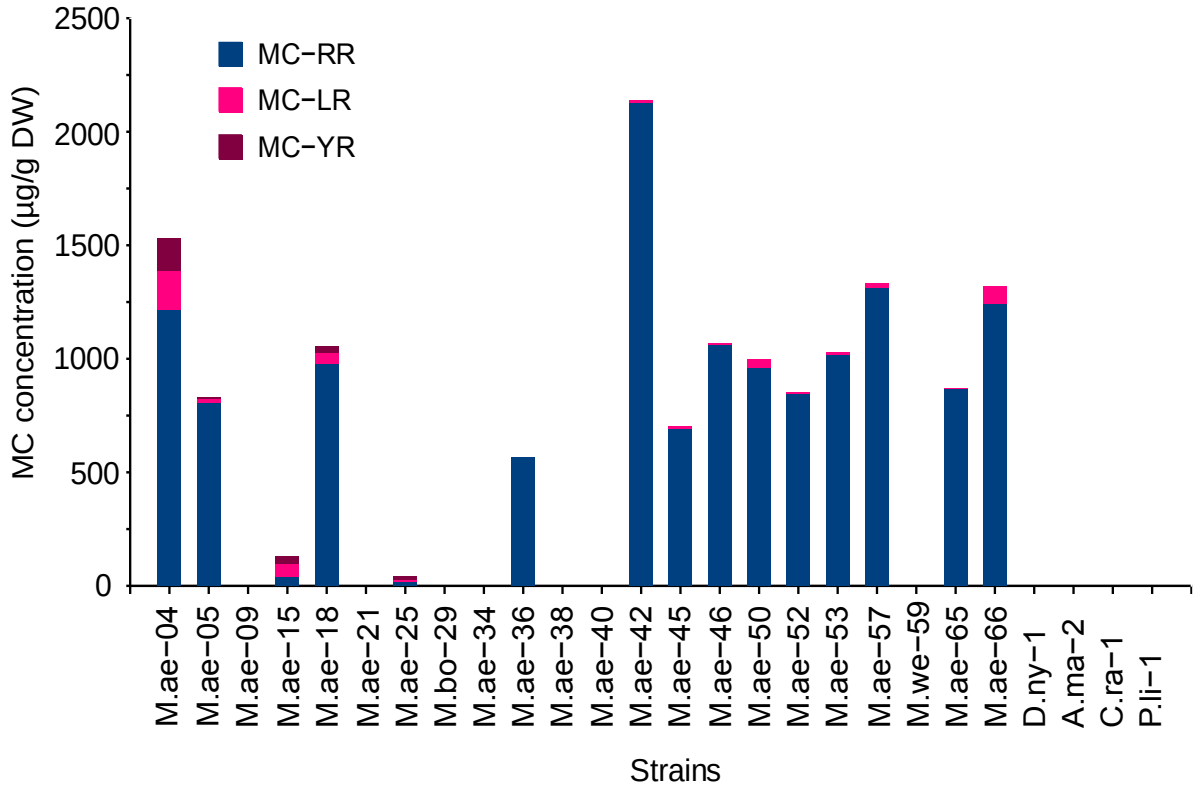


Figure 2–6. Microcystin profiles detected from isolated strains. M.ae: *Microcystis aeruginosa*; M.bo: *Microcystis botrys*; M.we: *Microcystis wesenbergii*; D.ny: *Dolichospermum nygaardii*; A.ma: *Arthrospira massartii*; C.ra: *Cylindrospermopsis raciborskii*; P.li: *Pseudanabaena limnetica.*

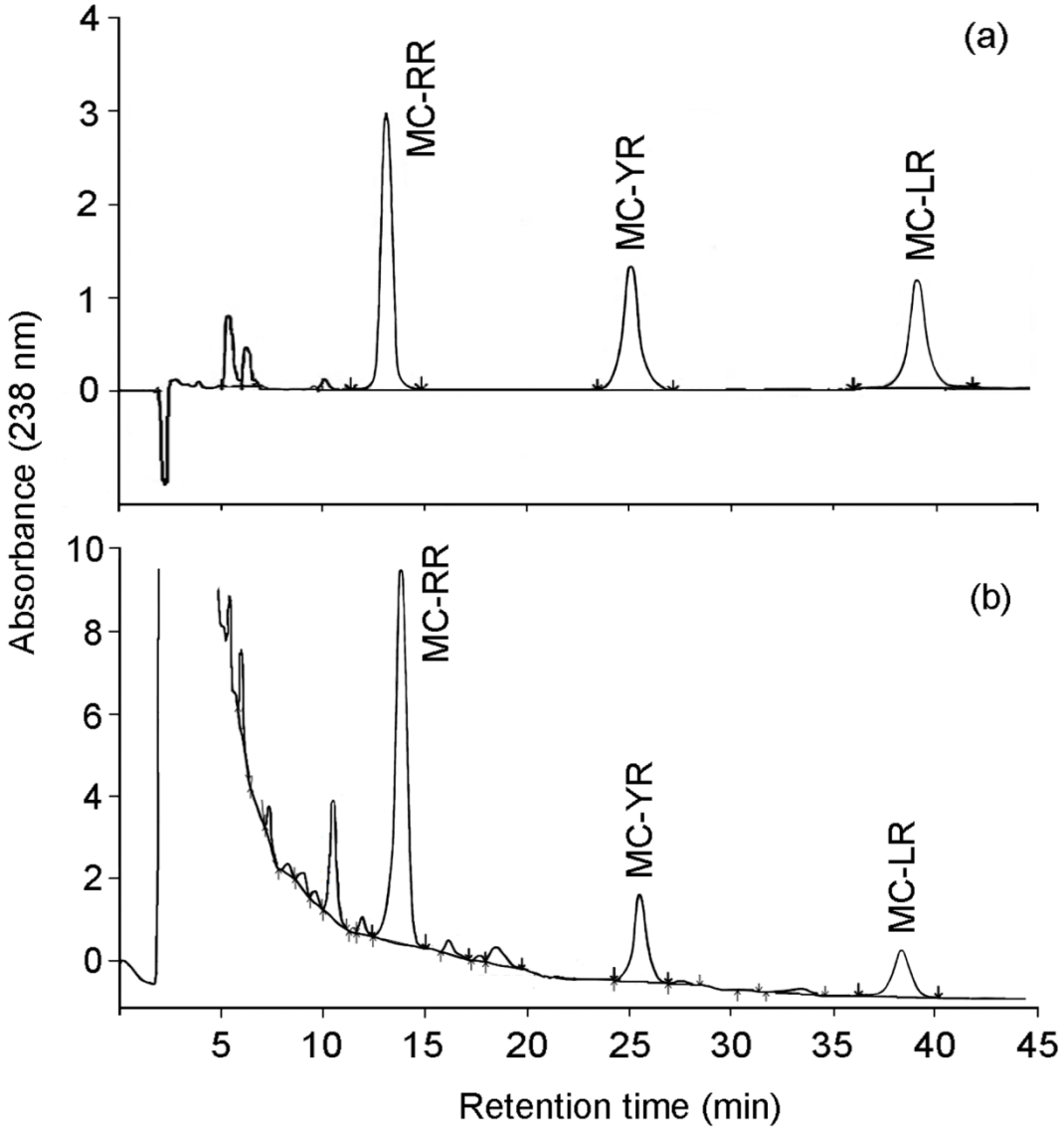


Figure 2–7. HPLC-chromatograms of (a) microcystin standards, and (b) bloom sample from Dau Tieng reservoir.

Chapter 3

Accumulation and effects of cyanobacterial crude extract containing microcystins on the edible clam *Corbicula leana*

3.1. Introduction

The occurrence of CYBs in eutrophic lakes, reservoirs and recreational waters has become a global environmental and public health concerns. These blooms produce a wide range of toxic secondary metabolites, especially MCs, the most widespread and occur in up to 75% of CYB incidents (Chorus and Bartram, 1999). MCs are intracellular but may rapidly and massively be released by cell lyses due to natural senescence, herbicides or physical stress (Issam *et al.*, 2010). Concentrations of dissolved MC from traces up to 1,800 µg L^{-1} have been reported in many inland waters worldwide (Chorus and Bartram, 1999; Te and Gin, 2011). Therefore, aquatic animals may be subchronic exposed to high concentration of dissolved MCs for a period of time (Ibelings and Chorus, 2007). These dissolved toxins can be responsible for toxic effects observed in both animals and humans and are actually associated with massive deaths of aquatic animals (Chorus and Bartram, 1999).

MCs target liver cells, and their cellular uptake requires the activity of OATPs (Fischer *et al.*, 2005). Once in the cell, they can accumulate as free form of MC or specifically interact with protein phosphatases (PP1 and PP2A) in a two-step mechanism involving a rapid and reversible binding potentially followed several hours later by covalent binding; they can thus accumulate as covalently bound MC (Co-MC) with hyperphosphorylation and tumour-promoting abilities (MacKintosh *et al.*, 1990; Amado and Monserrat, 2010; Lance *et al.*, 2010). Moreover, MCs induced the production of ROS in various tissues of aquatic organisms (Pinho *et al.*, 2005; Burmester *et al.*, 2012; Gonçalves-Soares *et al.*, 2012; Turja *et al.*, 2014) leading to oxidative stress.

The cellular system of defense against MC toxicity comprises enzymatic antioxidant and detoxification enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione *S*-transferases (GSTs). SOD and CAT are antioxidant enzymes. SOD catalyzes the dismutation of superoxide anion ($O_2^{\bullet -}$) into oxygen and H_2O_2, whereas CAT catalyzes the conversion of two molecules of H_2O_2 into two molecules of water and one of oxygen (Lushchak, 2011). The mechanism of MC detoxification in aquatic organisms involves GSTs, members of the phase II detoxification enzyme family that catalyze conjugation of MCs with glutathione (GSH) (Pflugmacher *et al.*, 1998). This conjugation is generally considered the primarily route of MC detoxification in aquatic organisms; it results in the formation of compounds that are more polar and thus more easily excreted (Kondo *et al.*, 1992; Pflugmacher *et al.*, 1998; Wiegand *et al.*, 1999; Beattie *et al.*, 2003; Campos and Vasconcelos, 2010). Furthermore, many aquatic species are able to survive in polluted environments by increasing their use of a multixenobiotic resistance (MXR) mechanism (Bard, 2000). The overexpressed membrane and intracellular transporters, such as P-glycoprotein, that pump unmetabolized and metabolized toxins directly out of the cell have been found in various organisms (Bard, 2000; Amé *et al.*, 2009; Faria *et al.*, 2011). This MXR mechanism is responsible for resistance to cyanotoxin bioaccumulation or insensitivity to natural toxins in mussels (Contardo-Jara *et al.*, 2008; Amé *et al.*, 2009; Campos *et al.*, 2014).

Nevertheless, toxic cyanobacteria and cyanotoxins, especially MCs, can have negative impacts on aquatic organisms. Bivalves usually insert themselves into sediments on the beds or shores of lakes or rivers and filter small particles via their gills. These sessile filter feeders are therefore seriously affected by the presence of toxic cyanobacterial colonies during CYBs or after the blooms have begun to decay. Increased attention is being paid to the accumulation and effects of MCs in bivalves, because humans consume these organisms (Ibelings and Chorus, 2007). Unlike the case in fish and mammals, there have been relatively few studies of the biological effects of cyanotoxins in bivalve mollusks (Gérard *et al.*, 2009; Sabatini *et al.*,

2011). MC accumulation in bivalves has been reported both in the field and in the laboratory studies (for a review see Martins and Vasconcelos, 2009). Previous studies, however, have not adequately considered MCs that are covalently bound to PPs, which may represent a considerable part of the MC burden (Lance *et al.*, 2010; 2014).

The toxicology and ecotoxicology of MCs have been investigated in detail (Duy *et al.*, 2000; Wiegand and Pflugmacher, 2005). However, toxicologists have focused only on isolating MCs (Beattie *et al.*, 2003; Li *et al.*, 2003; Kist *et al.*, 2012) or using purified MCs (Cazenave *et al.*, 2006; Contardo-Jara *et al.*, 2008; Pavagadhi *et al.*, 2012; Sun *et al.*, 2012) in toxicity studies; the toxicity of the complex CCE has not been evaluated to the same extent. Several recent findings indicate that water from CYBs contain not only MCs but a mixture of hazardous substances that can evoke more pronounced toxic effects than can MCs or other well-recognized cyanotoxins alone (Pietsch *et al.*, 2001; Burýšková *et al.*, 2006; Falconer, 2007; Palíková *et al.*, 2007; Smutná *et al.*, 2014). It would therefore be value to evaluate the effects of these complex cyanobacterial biomasses on aquatic organisms.

In this chapter, I examined the effects of a crude extract of CYBs containing MCs on the freshwater edible clam *C. leana*, as well as the accumulation and depuration of MCs by this species. My aims were to increase food safety and to reveal the clam's system of defense against MCs via the capacity of the antioxidant or detoxification enzymes CAT, SOD and GST in various organs (gills, foot, mantle and remaining tissues). Finally, evidences for the clam's resistance to cyanotoxins were also discussed.

3.2. Materials and methods

3.2.1. Rearing the organisms

Freshwater clam *C. leana* P. (Figure 3–1) was collected at a freshwater fisheries experimental station in Oita Prefecture, Japan, and transported alive to the laboratory. The clams were introduced into sufficient aerated 50-L aquatic aquariums containing

dechlorinated tap water and 5-cm sand layer as the substrate. Before the experiments, clams were kept at a density of below 100 individual per 50 L and acclimatized for 1 month at a photosynthetic photon flux density (PPFD) of 20 μmol photons m^{-2} s^{-1} under a 12:12 light:dark photoperiod. The water temperature was 22 ± 1°C, the pH 7.5 ± 0.3, and the dissolved oxygen concentration 7.9 ± 0.6 mg L^{-1}. All of the incubation water was renewed every 3 days. The clams were fed daily with the green alga *C. vulgaris* at a concentration of 2 × 10^3 cell mL^{-1} cultivated in soil extract medium (SEM) (Kong *et al.*, 2012). The wet weight (WW) of individual clams was 5.14 ± 0.72 g and the shell length was 2.12 ± 0.53 cm.

3.2.2. Preparation of cyanobacterial crude extract

CCE was prepared as previously reported by Pietsch *et al.* (2001) with minor modifications. Briefly, 4 kg wet weight of bloom material (mainly *Microcystis* spp., collected from Lake Kasumigaura, Japan by using plankton net) was frozen at −30°C for two days and then thawed at room temperature. After the material had thawed completely, it was ice-cooled and sonicated for 1 min (Taitec Ultrasonic 150W, Saitama, Japan). This freeze–thaw–sonicate cycle was repeated four times. The samples were then centrifuged at 3,000 × *g* at 4°C for 30 min to remove cell debris. The CCE supernatant was collected and kept at −30°C until use.

Subsamples of CCE were used for MCs analysis. Briefly, the CCE was centrifuged at 6,000 × *g* at 4°C for 15 min. The supernatant was collected, dried completely, and redissolved in 500 μL of 100% MeOH. The samples were analyzed by HPLC for MCs quantification. MC-RR, MC-LR and MC-YR (Wako, Osaka, Japan) were used as standards. The HPLC analysis showed that the CCE contained three MC congeners, namely MC-RR (53%) and MC-LR (45%), and the minor congener MC-YR (2%), at a total concentration of 7892 μg L^{-1}.

3.2.3. Experimental set up

The clams were placed in eight aquariums (30 clams per aquarium) containing 2 L distilled water and a 2-cm sand layer as a substrate. Clams were allocated randomly to an

exposure and a control group. Light intensity and water conditions were the same as previously described.

In the exposure group, CCE containing MCs was added to each aquarium to a final concentration of 400 µg MC-LR_{eq} L^{-1} on day 0. The water and MC were completely replaced on day 5 of the 10-day exposure period. The clams were then collected and relocated into aquariums containing distilled water, toxin-free water; they stayed in these aquariums for 5 days of depuration. The experiment therefore lasted a total of 15 days (10 days of MCs exposure following by 5 days of toxin depuration). In the control group only the water was replaced on day 5. No food was provided during the uptake and depuration periods. Dead clams were removed and counted daily.

Six hours after the start of exposure and again on days 1, 3, 5, and 10 of the exposure period and days 11, 13, and 15 of the depuration period, 12 – 15 clams were sampled. For MC quantification the shells of 8 – 10 clams were immediately removed; 10 clams were collected only before the experiment from control group for MC control. The clams were then freeze-dried for 48 h and keep at –30°C until MC analysis. For measurement of enzyme activity, the clams (in both groups) were rinsed gently under dechlorinated tap water. The gills, mantle, and foot of three to five clams (pooled) and the remaining tissues (kept individually) were dissected on ice. The samples were then immediately frozen in liquid nitrogen and stored at –80°C until enzyme extraction.

3.2.4. Extraction and analysis of MCs in incubation water

I measured MC concentrations in the incubation water immediately after the start of exposure and then at the same sampling times as listed above. The incubation water (about 10 to 100 mL) was collected and filtered through GF/C filter. The filtrate was then passed through the Presep C18 (ODS) cartridge (Wako Pure Chemical Industries Ltd., Osaka, Japan) that had been preconditioned with 5 mL MeOH 100% and 10 mL ultrapure water; it was then

subjected to final elution with 3 mL MeOH 100% and dried completely. The MC fraction was then redissolved in 500 µL MeOH 100% and kept at –30°C until HPLC analysis. MCs (-RR, -LR, -YR) were analyzed with an reversed-phase HPLC system equipped with a UV detector (Shimadzu 10A series, Shimadzu Corporation, Kyoto, Japan) by using the method as described in the 2.2.5 section.

3.2.5. Extraction and analysis of free MC in clam

Free MC was extracted as previously reported by Xie and Park (2007), with minor modifications. Briefly, freeze-dried tissues (about 100 to 150 mg per sample) were homogenized in 3 mL of BuOH:MeOH:H_2O (5:20:75, v/v/v) by using a homogenizer (Polytron, Kinematica AG, Littau-Luzern, Switzerland) and extracted three times with 5 mL of the same solution, each time for 24 h with shaking in darkness. After sonication for 1 min (Taitec Ultrasonic 150W, Saitama, Japan), the samples were centrifuged at 2,000 × *g* at 4°C for 30 min. The supernatants were then combined, evaporated to 10 mL, diluted three times with ultrapure water and applied to an Oasis HLB cartridge (60 mg, Waters Corp., Milford, MA, USA) that had been preconditioned with 3 mL MeOH 100% and 10 mL ultrapure water. The column was first washed with 3 mL MeOH 20%, and then eluted with 3 mL MeOH 100%. This elution fraction was evaporated to dryness under reduced pressure at below 40°C. MCs were suspended in 500 µL MeOH 100%; they were then kept at –20°C prior to reversed-phase HPLC analysis as described above. Duplicate samples with duplicate analysis were used in this determination (n = 4).

3.2.6. Extraction of total MC in clam

Total MC (free and Co-MC) was extracted as previously reported by Neffling *et al.* (2010) and Cadel-Six *et al.* (2014), with minor modifications. Briefly, freeze-dried tissues were homogenized and trypsinated with 3 mL of 500 µg mL^{-1} of trypsin in Sorensen's phosphate buffer (pH 7.5) at 37°C for 3 h; this was followed by oxidation with 0.1 M $KMnO_4$

and 0.1 M $NaIO_3$ (pH 9.0) for 3 h at room temperature. The reaction was quenched with sodium bisulphite solution (40% w/v) until colorless at pH 2 with 10% sulphuric acid. After sample centrifugation (2,000 × *g*, 4°C, 30 min), the supernatant was collected, diluted five times with ultrapure water, and then applied to an Oasis HLB cartridge (60 mg, Waters Corp.) that had been preconditioned with 3 mL MeOH 100% and 10 mL ultrapure water. The column was first washed with 3 mL MeOH 20%, and then 2-methyl-3-methoxy-4-phenylbutanoic acid (MMPB) fraction, which is the product of MC oxidation, was eluted with 3 mL MeOH 80%. The elution fraction was evaporated to dryness and redissolved in 500 µL MeOH 100%. The MMPB was converted to its methyl ester (meMMPB) by using a 10% BF_3–methanol kit (Sigma-Aldrich, Tokyo, Japan) (Figure 3–2). The derivatized samples were dissolved in *n*-hexane and kept at –20°C prior to GC-MS analysis. The Co-MC content was thus estimated by subtracting the fee MC from the total MC content. 4-phenylbutylric acid (4–PB) was used as an internal standard (Sano *et al.*, 1992; Tsuji *et al*., 2001). MMPB-d_3 and MC-LR purchased from Wako Pure Chemical Industries (Osaka, Japan) were used as external standards.

3.2.7. GC-MS analysis

GC-MS analyses were performed on a DSQ II mass spectrometer linked to a Trace GC Ultra gas chromatograph system (Thermo Scientific, Waltham, MA, USA), equipped with an Rxi-5ms column (30 m × 0.25 mm ID, phase thickness 0.25 mm; Restek, Bellefonte, PA, USA). Helium was used as the carrier gas at a flow rate of 1.5 mL min^{-1} (splitless mode). The program used for the analysis was 80°C for 1 min followed by an increase to 280°C at 8°C min^{-1}. The other conditions were as follows: ion source temperature 200°C, injection port temperature 230°C, detector temperature 250°C, and interface temperature 280°C. Methylated 4–PB (me4–PB) and meMMPB were detected by using selected ion monitoring (SIM) mode. Ions at 91 and 104 m/z were selected for me4–PB; and those at 75, 78, 91, 131, and 134 m/z

for meMMPB (Tsuji *et al*., 2001; Suchy and Berry, 2012). Xcalibur software was used for quantitative analysis of these analytes. Duplicate samples with duplicate analysis were used in this determination (n = 4).

3.2.8. Enzyme extraction and measurement

Enzymes were extracted as previously reported by Wiegand *et al.* (2000) with minor modifications. Briefly, samples (gill, foot, mantle, remaining soft tissues) were homogenized in 0.1 M sodium phosphate buffer (pH 6.5) (1:5 w/v) containing 20% (v/v) glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1.4 mM dithioerythritol (DTE), as a reducing agent and a protease inhibitor, in ice. The homogenate was centrifuged at 10,000 × *g* at 4°C for 15 min to eliminate cell debris and the other fragments. The supernatant was used for enzyme activity measurements. I used a Fluoroskan Ascent fluorometer (Thermo Electron Corp., Milford, MA, USA) to detect the activities of GST (EC 2.5.1.18), SOD (EC 1.15.1.1), and CAT (EC 1.11.1.6) at wavelengths of 340, 460, and 540 nm, respectively, with GST, SOD, and CAT assay kits purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All enzyme activities were calculated in terms of the protein content, as measured with a Quick Start Bradford protein assay kit purchased from Bio-Rad Laboratories **(**Hercules, CA, USA). Each enzymatic assay was performed in triplicate.

3.2.9. Recovery experiment

In order to determine the recovery ratio of MC contents extracted from clam tissues, the recovery experiment was conducted by spiking 100 mg lyophilised clam tissues with a CCE that contained of 1 μg of MCs (MC-LR_{eq}). Samples were prepared in quadruplicate. MC contents were then extracted and analysed as previous described. The recovery and relative standard deviation of the analytical method were calculated. The mean recovery for the clam tissues (n = 4) was 95.8% (relative standard deviations RSD = 4.9%) (data not shown).

3.2.10. Statistical analyses

Data on CAT, SOD, GST and MCs are presented as mean ± SD. Differences between the exposure and control groups were tested for significance by using one-way analysis of variance (ANOVA). When the ANOVAs were significant, I used pairwise comparison by using Tukey's HSD Post-hoc test to detect significant differences between the exposure concentrations and the control. *P*-values less than 0.05 were considered statistically significant.

3.3. Results

3.3.1. Microcystins concentration in incubation water

MC concentrations in the control incubation water were under the detection limit (data not shown). I monitored MC concentrations in the incubation water during the first 5 days of uptake and during the depuration period. In the uptake experiment, MC was immediately and continuously cleared from the incubation water. After only 6 h the MC concentration in the water had decreased to 326.3 ± 13.5 μg L^{-1}; after 1 day it was 262.2 ± 12.9 μg L^{-1}, after 3 days 185.8 ± 10.7 μg L^{-1}, and after 5 days 121.1 ± 3.1 μg L^{-1} (Figure 3–3). There was no release of the unmetabolized parent compound into toxin-free water during depuration period.

3.3.2. Uptake and depuration of free- and Co-MC

There were no deaths in either of the groups of animals during the experiments. The control samples contained no MCs at detectable concentrations (data not shown).

Extractable free MC accumulated in the clams during the uptake and depuration periods was presented in Figure 3–3. Typically, the free MC concentration in the whole clams increased rapidly after the start of exposure and peaked (at 3.4 ± 0.63 μg g^{-1} DW) after about 1 day. It then gradually declined over the rest of the exposure period. The free MC content was well correlated with the concentration of MCs in the incubation water (R = 0.65, P <

0.01). The Co-MC concentration increased slowly during the uptake and depuration periods, peaking (at 0.31 ± 0.013 μg g^{-1} DW) on day 11. It gradually declined thereafter.

During the depuration period, free MC was quickly eliminated from the clam tissues and below the limit of detection by HPLC. In contrast, the Co-MC concentration was enhanced on the first day of depuration and then gradually declined, although Co-MC was still detectable at the end of the depuration period (Figure 3–3).

3.3.3. Biotransformation enzyme

I measured GST activity in various tissues of both the exposure and the control groups (Figure 3–4). GST activity in the gills was significantly greater in the exposure group than in the control group, but only on days 0.25, 1, 3, and 11. Significant elevation of GST was also observed on days 10 and 11 in mantle. In contrast, GST activity in the foot was significantly lower in the exposure group than in the control group on days 3, 5, 10, and 13, although it had returned to the control level by the end of the experiment. GST activity in the remaining tissues did not differ significantly over time between the two groups.

3.3.4. Antioxidant enzyme activities

I examined the effects of CCE containing MCs on SOD activity in the various clam tissues (Figure 3–5). SOD activity in the gills was significantly greater in the exposure group than in the control group on all measurement days in the exposure period except day 10. In the mantle this was also true for all measurement days in the exposure period except day 3. In contrast, in the foot there were no significant differences in SOD activity between the two groups at any time. In the remaining tissues SOD activity was significantly greater in the exposure group than in the control group, but only on days 0.25 and 5. Unlike the case with GST, during the depuration period there were no differences in SOD activity between the two groups in any of the tissues.

I then examined changes in CAT activity (Figure 3–6). CAT activity in the gills was significantly greater in the exposure group than in the control group, but only on days 0.25, 1,

and 3. In contrast, CAT activity in the foot was significantly lower in the exposure group than in the control group on days 5, 10, and 11 but thereafter returned to normal. There were no detectable trends in CAT activity in the mantle and the remaining tissues. CAT activity in the mantle was significantly greater in the exposure group than in the control group on days 0.25, 1, 10, and 11 but significantly lower at the end of the experiment, on day 15. In the remaining tissues, CAT activity was significantly greater in the exposure group than in the control group on day 1 but significantly lower than in the controls on day 13.

3.4. Discussion

In natural environment and under experimental conditions, concentration of dissolved MCs in water can be expected to be decreased by such processes as adsorption onto particulate materials, attachment to subtracts, UV or photolysis degradation, and degradation by intracellular organic matter and bacteria (Harada and Tsuji, 1998; Grützmacher *et al.*, 2009; Ding et al., 2010; Wörmer *et al.*, 2010; Shimizu *et al.*, 2011; Ma *et al.*, 2012; Shimizu *et al.*, 2012). In my experiment, the concentration of MCs in the incubation water had decreased by about 69% after 5 days of incubation. This result agreed well with the finding in another study that after 3 days of incubation the concentration of dissolved MC-LR had decreased by more than 50% (Contardo-Jara *et al.*, 2008). However, the natural degradation of MCs in complex CYB extracts is still unknown. My results may suggest that the degradation of MCs in CCE is a result of the combined effects of physical, chemical, and biological factors, including uptake by aquatic animals. However, the main contributors to toxin degradation remain unknown and need further investigation.

The extraction solvents used for extraction of MCs from animal tissues vary greatly in the literature (Msagati *et al.*, 2006; Smith and Boyer, 2009). The solvent selected in this study was successfully used also in bivalve *Anodonta woodiana*, *Cristaria plicata* and *Unio douglasiae* (Yokoyama and Park, 2002), fish *Silurus glanis*, *Carassius auratus* and *Cyprinus*

carpio (Xie *et al.*, 2007), gadtropod *Sinotaia histrica*, *Lymnaea stagnalis* (Xie *et al.*, 2007; Lance *et al.*, 2010), with similar percentages of recovery.

I revealed here that toxin uptake by *C. leana* was lower than that by most other mussels and snails. The maximum levels of free MC measured in *C. leana* (3.4 ± 0.63 µg g^{-1} DW) were similar to the MC content in the mussel *Anodonta* sp. collected from Lake Kastoria, in Greece (Gkelis *et al.*, 2006), but they were much lower than those in other bivalve species e.g 16 µg g^{-1} DW in *Mytilus galloprovincialis* (Amorim and Vasconcelos, 1999), 16.3 µg g^{-1} DW and 21 µg g^{-1} WW in *Dreissena polymorpha* (Pires *et al.*, 2004; Contardo-Jara *et al.*, 2008) and 70 µg g^{-1} DW in *Anodonta cygnea* (Eriksson *et al.*, 1989), during laboratory exposure. Different species no doubt have different capacities for toxin accumulation, uptake, and tolerance. The decline in extractable MC content in *C. leana* after day 1 of exposure could have been the result of either enhanced biliary excretion of MCs and their metabolites (Sahin *et al.*, 1996) or slow covalent addition of MCs to the catalytic subunit of PPs and other thiol-containing cellular proteins (MacKintosh *et al.*, 1995) such as GSH (Kondo *et al.*, 1992). Whereas the former mechanism (biliary excretion) would progressively reduce the amount of free MC in the animal, the covalent binding of MCs to PPs would prevent the MCs from being excreted. This interpretation is consistent with my results showing that the Co-MC concentration gradually increased with time.

Because MCs covalently bind to PPs and cannot be extracted from the covalent complex by using organic solvents, detection of MCs in animal tissues has been limited to free MC (for reviews see Ibelings and Chorus, 2007; Smith *et al.*, 2008; Martins and Vasconcelos, 2009). By using an oxidation procedure adapted from previously developed methods (Sano *et al.*, 1992; Harada *et al.*, 1996; Tsuji *et al.*, 2001; Lance *et al.*, 2010; Neffling *et al.*, 2010; Suchy and Berry, 2012), I provided evidence for the existence and accumulation of Co-MC in *C. leana* tissues (Figure 3–3). On average, 0.5% of total MC was bound in *C. leana* during the 15-day experiment (data not shown). However, the clam rapidly

eliminated the MC when cultured in toxin-free water. Williams *et al.* (1997b) reported that the total MC content in the mussels *Mytilus edulis* transferred to untreated saltwater dropped from 337 μg to 11 μg g^{-1} FW in 4 days, after which time it was undetectable. Prepas *et al.* (1997) have also shown that MC concentrations significantly decrease within 6 days of depuration in the clam *Anodonta grandis simpsoniana*. Also, immediate uptake and rapid release of MC have been observed in *D. polymorpha* (Pires *et al.*, 2004; Contardo-Jara *et al.*, 2008), and *M. galloprovincialis* (Amorim and Vasconcelos, 1999). I found here that free MC rapidly began to be released when the clam was transferred to toxin-free water, but the percentage of bound MC increased (and reached 100% of the total MC content) during the depuration period (Figure 3–3). This increase may have occurred because the enhancement of the free MC binding to PPs. At the end of the 5-day depuration period, *C. leana* tissues still contained 0.15 ± 0.01 μg g^{-1} DW of Co-MC. Although depuration is commonly judged to be rapid, it is equally clear that depuration is incomplete, even after a considerable period of time (Wiegand *et al.*, 1999; Ibelings and Chorus, 2007). Therefore, Co-MC level should be considered in predictions of risk to higher trophic organisms and humans.

The current study revealed the toxin uptake by *C. leana* from dissolved MCs is possible. Despite these relatively low levels, however, my results raise concern about chronic toxicity from a human health perspective, because human may be consuming clams contaminated with MCs, and consumption of food contaminated with MCs could promote liver cancer (Duy *et al.*, 2000). I used a coefficient of 100 to convert dry weight to wet weight in the case of this clam; my results showed that the total MC content in clams exceeded the TDI of 0.04 μg kg^{-1} of body mass per day (Figure 3–7). My results therefore suggest that *C. leana* represents a health risk to consumers when aquatic MC concentrations are high. They need detoxification process before marketing.

In this study, I investigated the defense system against MC toxicity in the clam that focused on GST activity. It is well known that the family of GST enzymes is the most

important group for MCs detoxification (Li *et al.*, 2008; Burmester *et al.*, 2012; Galanti *et al.*, 2013; Pereira *et al.*, 2013). I found an elevation in GST activity in the gills during the first 3 days of exposure, suggesting that there was an immediate response by the tissue to the CCE. This response can be due either to an increase in MC conjugation with GSH or to the detoxification of endogenous molecules such as membrane peroxides (Pinho *et al.*, 2005). The higher GST activity in the exposure group suggested that there was an increase in MC conjugation capability in the gills of these animals. The same defense system against MC toxicity has been reported in the gut and gills of *M. galloprovincialis* mussel (Vasconcelos *et al.*, 2007), in the gills of crabs exposed to an aqueous extract of the toxic cyanobacterium *M. aeruginosa* (Vinagre *et al.*, 2003; Pinho *et al.*, 2005), and in the gills and intestine of the catfish *Corydoras paleatus* exposed to high concentration of MC-RR (Cazenave *et al.*, 2006). The further increase in GST activity in the gills on day 11 in *C. leana* may have been the result of excessive GST synthesis, which was thereafter regulated in response to the lack of MCs in the depuration period. Higher GST activity in the gills, digestive glands and muscle during depuration period has been reported in mussel *M. galloprovincialis* after the termination of exposure to toxic *M. aeruginosa* (Fernandes *et al.*, 2009). A similar pattern has been reported in the freshwater snail *Bellamya aeruginosa* after the end of exposed to *M. aeruginosa* (Zhu *et al.*, 2011). Contrastingly to the gills, in the foot, mantle, and remaining tissues GST expression was inhibited or did not change after exposure. Decreased GST activity in these tissues may be related to GSH depletion in response to MC toxicity (Amado *et al.*, 2011; Sabatini *et al.*, 2011); it may also result in altered biochemical effects in organisms exposed to MCs (Malbrouck *et al.*, 2003). In contrast with my results Gélinas *et al.* (2012) reported no increase of GST activity as well as lipid peroxidation in both liver and brain tissues of rainbow trout (*Oncorhynchus mykiss*) after exposure to cyanobacterial extracts.

CCE containing MCs induces ROS production, resulting in oxidative stress to organisms (Jos *et al.*, 2005; Burýšková *et al.*, 2006; Palíková *et al.*, 2007). These ROS

activate the expression of several antioxidant enzymes, including SOD and CAT, which constitute the major defensive system against ROS (Amado and Monserrat, 2010; Lushchak, 2011; Paskerová *et al.*, 2012). Exposure of the freshwater clam *Diplodon chilensis patagonicus* to toxic *Microcystis* leads to an increase in oxidative stress, as indicated by enhanced CAT and SOD activities (Sabatini *et al.*, 2011). Similarly, exposure of the mussel *M. edulis* to an extract of the cyanobacterial toxin nodularin leads to an increase in CAT activity (Kankaanpää *et al.*, 2007). Here, I found significant changes in both CAT and SOD enzyme activities in various tissues of *C. leana*. These findings indicate that there was an activation of the antioxidant defensive system as a direct or indirect respond to ROS generation after exposure to CCE containing MCs. More specifically, the alterations that I found in antioxidant enzyme activity were likely caused mainly by the presence of MCs and partly by the presence of other compounds in the CCE (Dao *et al.*, 2013). Also, my results are consistent with the observations of Burmester *et al.* (2012), who found that SOD activity in two bivalves, *D. polymorpha* and *U. tumidus*, was elevated in various tissues after exposure with purified MC-LR or CCE.

Elevation of CAT activity and other antioxidant enzymes have been observed in the crab hepatopancreas after 48 h of exposure to MCs from CCE (Pinho *et al.*, 2005) or in shrimp (*Litopenaeus vannamei*) injected with MCs (Gonçalves-Soares *et al.*, 2012). In contrast, CAT activity was significantly reduced, and SOD activity unchanged, in the crab hepatopancreas after 7 day's exposure to a high-dose *M. aeruginosa* aqueous extract (Pinho *et al.*, 2005). Likewise, CAT activity in larvae of the bighead carp *Hypophthalmythys nobilis* is significantly reduced upon MC-LR exposure, suggesting that CAT activity is inhibited by MC-LR (Sun *et al.*, 2013). In my clam, CAT activity in the mantle was significantly lower in the exposure group than in the control group at the end of the experiment, possibly because at that point the mantle was less efficient than gills and foot at neutralizing the impact of oxidative stresses. In contrast, the reduction in CAT activity in the foot toward the end of the

exposure period could have been due to the generation of superoxide radicals during oxidative stress; these molecules have been reported to inhibit CAT activity (Kono and Fridovich, 1982). Therefore, toxicity effects depend not only on the dose and kind of toxins, the routes of exposure, and the duration time of exposure, but also on the target organs, the state of the organisms, specific species as well as method of detection (Malbrouck and Kestemont, 2006; Pavagadhi *et al.*, 2012; Sun *et al.*, 2013).

Contrastingly, MXR in the freshwater mussel *D. polymorpha* is evidence of the insensitivity of bivalves to purified cyanobacterial toxins (Contardo-Jara et al., 2008). My results also correspond to those of Fischer and Dietrich (2000), who observed no deaths, malformations, or growth inhibition in *Xenopus laevis* embryos exposed to purified MCs at up to 2,000 μg L^{-1} for 96 h. Similarly, no developmental toxicity of MCs (at up to 20,000 μg L^{-1}) has been observed in the toad *Bufo arenarum* (Chernoff *et al.*, 2002). Antioxidant enzyme levels may be elevated in response to cellular oxidative stress in animal cells (Dias *et al.*, 2009; Turja *et al.*, 2014), and the increased rate of synthesis of these antioxidant enzymes could be a plausible explanation for the insensitivity following MC exposure in some experimental groups (Pavagadhi *et al.*, 2012). My results demonstrate that biochemical toxic effects in *C. leana* are only temporary and that prolonged exposure can lead to adaptations to cope with deleterious effects. The significant change in GST, SOD, and CAT activities that I found in *C. leana* probably reflect adaptation to oxidative conditions. However, in toxin-free water, both of the antioxidant enzymes and detoxification enzyme showed adaptive responses at several time points whereby enzyme activity was induced and then returned to control levels. The responses of antioxidant and detoxification enzymes might thus contribute to the MC and cyanotoxins tolerance of *C. leana*.

Many aquatic organisms live and reproduce in contaminated waters, suggesting that they have ways to resist or tolerate contaminants (xenobiotics) in their environment (Cornwall *et al.*, 1995). Exposure to toxins can trigger the MXR mechanism, which serves as a first line

of defense against a broad spectrum of natural and man-made toxicants in the cells (Bard 2000; Eufemia and Epel, 2000; Faria *et al*., 2011). Contardo-Jara *et al.* (2008) pointed out that the interactions of various defense mechanisms against MC toxicity in the freshwater mussel *D. polymorpha* are due to high constitutive level of P-gp and the reaction of MXR mechanisms; this explains the clams' survival success, even when they are exposed to high MC concentrations. Further studies are needed to give us an integrated view of toxin insensitivity and the MXR mechanism in the clam *C. leana*. To my knowledge, this is the first report of MC uptake by, and the biochemical responses of, this edible clam in the context of safe food production.

3.5. Summary

In this chapter the accumulation and effects of CCE containing MC on the edible clam *C. leana* were investigated. Toxic effects were evaluated through the activity of antioxidant and detoxification enzymes: CAT, SOD, and GSTs from gills, foot, mantle and remaining soft tissues. Clams were exposed to CCE containing 400 µg MC-LR_{eq} L^{-1} for 10 days and were then kept in toxin-free water for 5 days. Clam accumulated MCs (up to 3.41 ± 0.63 µg g^{-1} DW of free- and 0.31 ± 0.013 µg g^{-1} DW of Co-MC). The activity of SOD, CAT, and GST in the gills and mantle increased in MC-treated animals. In contrast, CAT and GST activity was significantly inhibited in the foot and mostly only slightly changed in the remaining tissues.

My findings in this chapter provide insights into the uptake of CCE containing MCs at high concentrations by *C. leana* and the consequent biochemical responses of the clam under laboratory conditions. Results highlight the involvement of antioxidant and biotransformation systems in detoxification of MCs. It explains the possible tolerance of *C. leana* continuously exposed to high level of MCs. In addition, it reveals that MCs accumulated by the clam via the uptake of dissolved MCs in water bodies is possible. My findings should also improve our

understanding of the impacts of MC-containing cyanobacteria dissolved in the water column on aquatic life under natural conditions. The results also showed that the total MC content in clams exceeded the TDI of 0.04 μg kg^{-1} of body mass per day. My results therefore suggest that *C. leana* represents a health risk to human when aquatic MC concentrations are high. They need detoxification process before marketing. Consumption of this clam should be limited when there are high concentrations of dissolved MC present in the water (especially after cells lyse at the end of the blooms). However, further research is required to deepen our understanding of the fate and transfer of MCs and the toxicity of other hazardous substances from CCE.

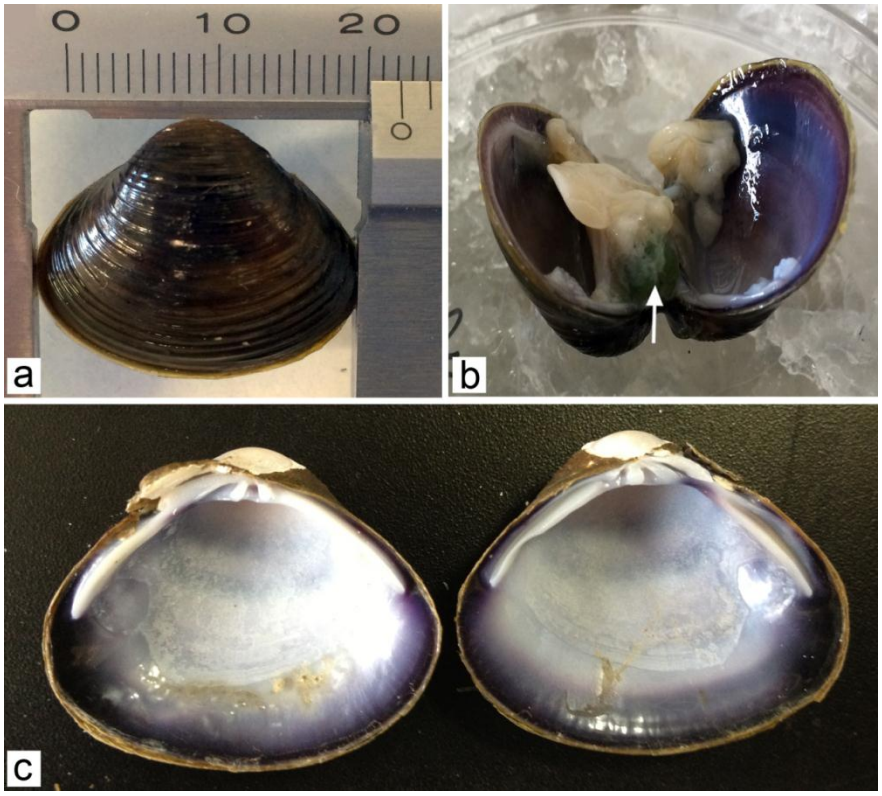


Figure 3–1. Photos of the clam *Corbicula leana*. (a) Whole external shell; (b) Soft tissues, arrow indicates cyanobacterial cells accumulated in soft tissues; and (c) the inner shell-side.

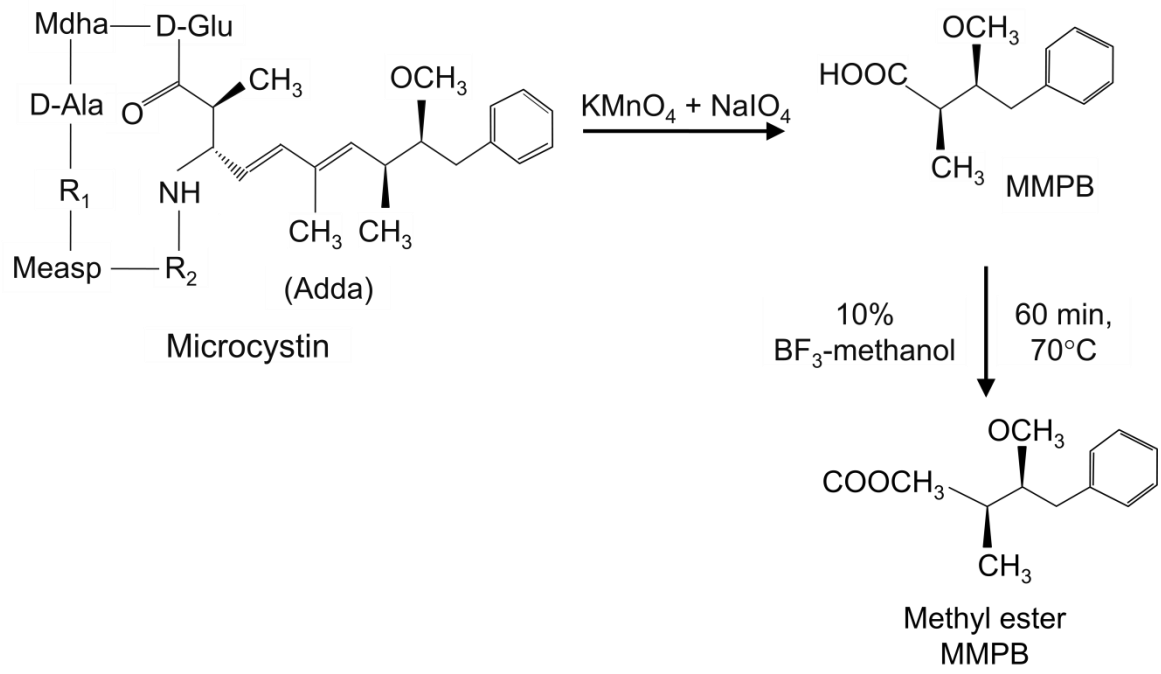


Figure 3–2. The oxidation of 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) in microcystins to the carboxylic acid 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) and its methyl ester (modified from Tsuji *et al*., 2001).

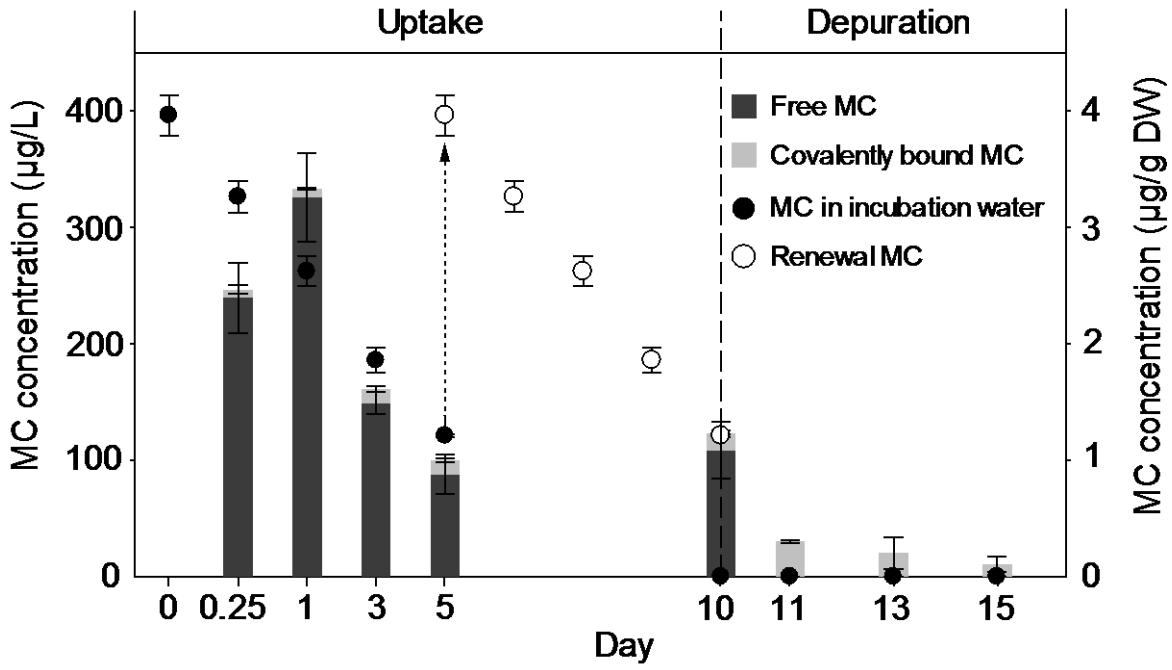


Figure 3–3. Concentrations of free microcystin (MC), covalently bound MC in *Corbicula leana*, and of MC in incubation water, during the uptake and depuration periods. Arrow indicates the time of renewal of the MC concentration and the water during the uptake period.

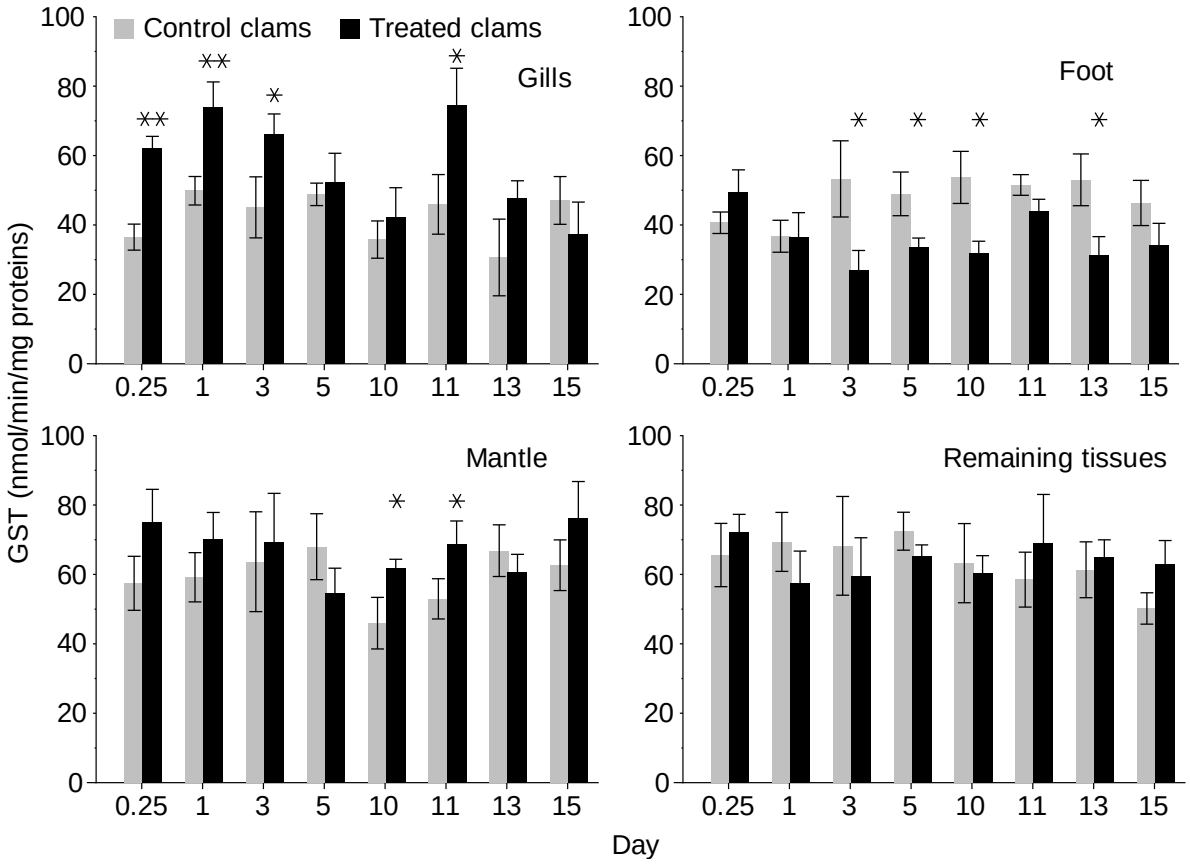


Figure 3–4. Production of glutathione *S*-transferase (GST) (nmol min^{-1} mg^{-1} proteins) in various tissues of *Corbicula leana* exposed to toxic cyanobacterial bloom crude extract. Asterisks indicate significant differences compared with controls at the respective time points (*$P < 0.05$, **$P < 0.01$).

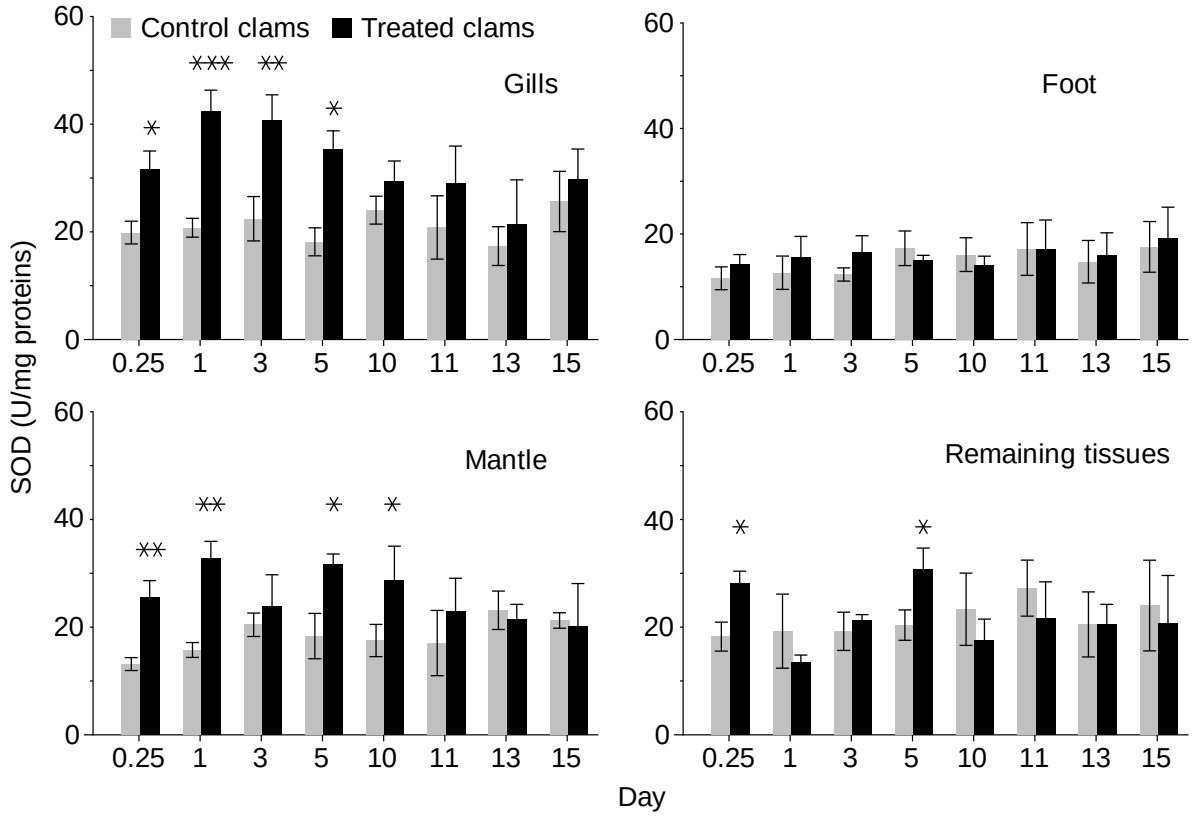


Figure 3–5. Production of the antioxidant enzyme superoxide dismutase (SOD) (U mg^{-1} proteins) in various tissues of *Corbicula leana* exposed to toxic cyanobacterial bloom crude extract. Asterisks indicate significant differences compared with controls at the respective time points (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

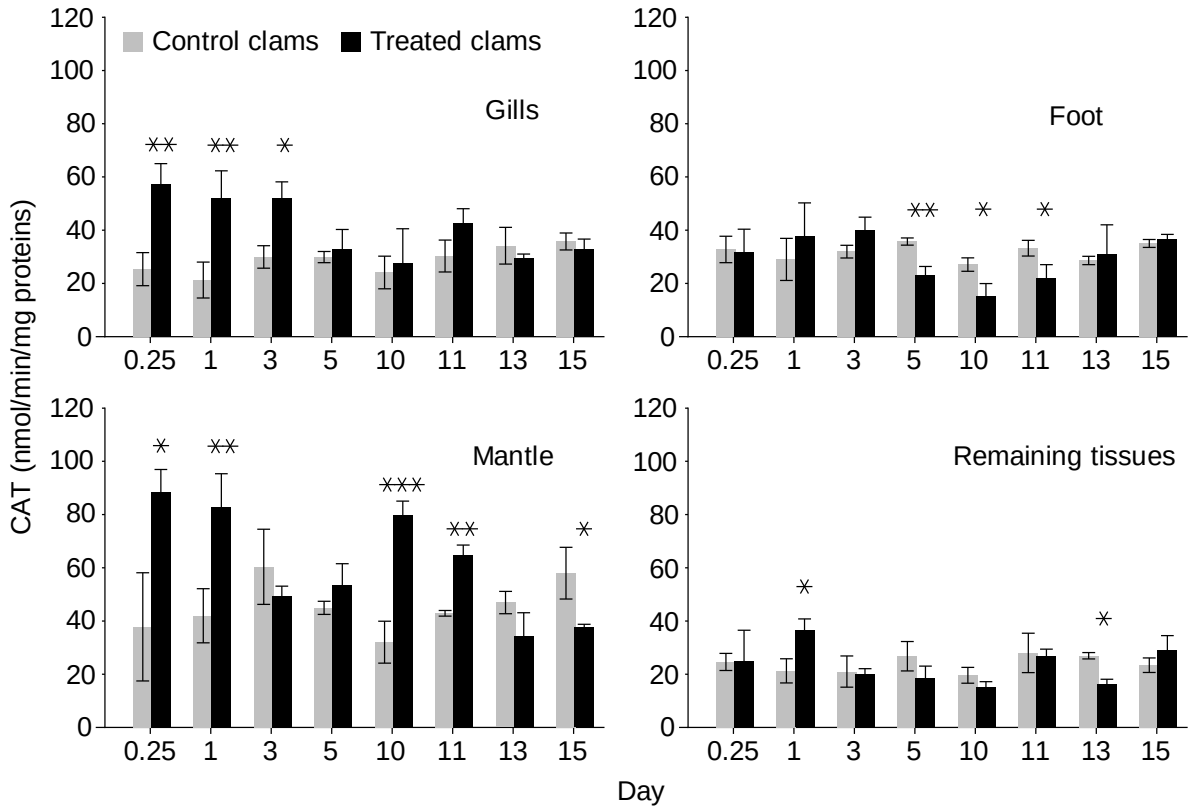


Figure 3–6. Production of the antioxidant enzyme catalase (CAT) (nmol min^{-1} mg^{-1} proteins) in various tissues of *Corbicula leana* exposed to toxic cyanobacterial bloom crude extract. Asterisks indicate significant differences compared with controls at the respective time points (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

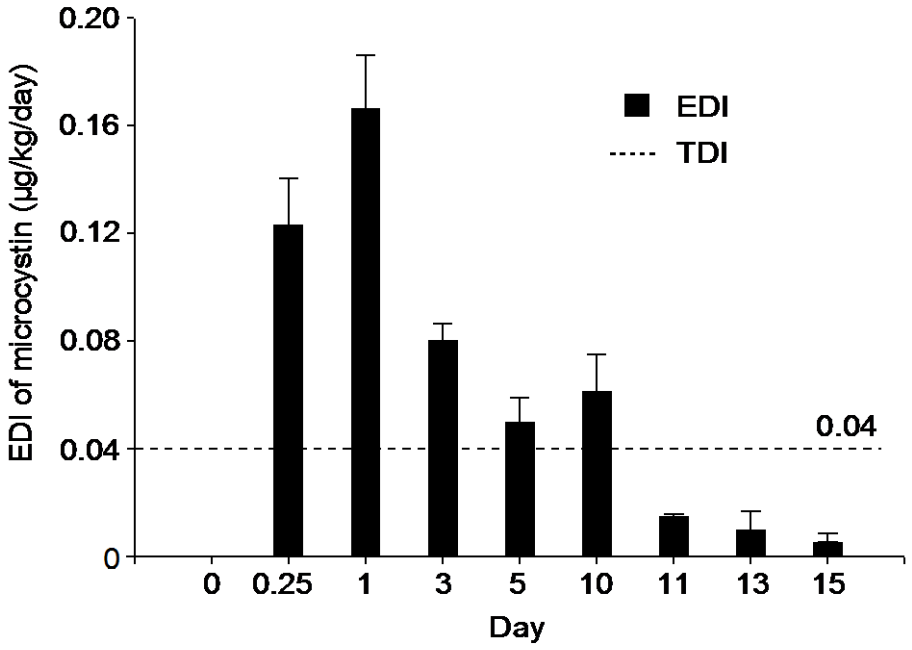


Figure 3–7. Estimated daily intake (EDI) of microcystin by a person (60 kg) consuming 300 g wet weight of *Corbicula leana* (Ibelings and Chorus, 2007). Horizontal line indicates the maximum tolerable daily intake (TDI) for humans (0.04 µg $kg^{-1}day^{-1}$), as proposed by the World Health Organization (WHO) (Chorus and Bartram, 1999).

Chapter 4

Accumulation and effects of toxic and non-toxic cyanobacteria on the edible clam *Corbicula leana*

4.1. Introduction

Toxic cyanobacteria as well as MCs pronounce negative effects on aquatic animals, including zooplankton, fish, gastropods and bivalves (Amado and Monserrat, 2010; Paskerová *et al.*, 2012; Pavagadhi and Balasubramanian, 2013). In aquatic environment, animals may take up and accumulate cyanotoxins via the two main routes: From dissolved toxins via a transdermal route or ingestion via the in-take of toxic cyanobacterial cells (Ibelings and Chorus, 2007). High concentrations of cyanotoxins in water are likely to be short-lived due to a lysis of surface blooms. Thus, aquatic animal may be exposed to high dissolved MCs for short time periods. However, filter feeding organisms like mussels may directly graze on toxic cyanobacteria (Pires *et al.*, 2005). Previous studies have concluded that bivalves uptake of MCs via the food (ingestion) is the main route for accumulation (Yokoyama and Park, 2003).

In previous chapter, I reported the accumulation/depuration of free- and Co-MC, and potential adverse effects in *C. leana* via absorption/uptake caused by MC dissolved in ambient water column. In this chapter, I investigated the accumulation and elimination of free- and Co-MC in the same animal under laboratory experiments, but exposed to toxic and non-toxic cyanobacterial cells. An oral route of exposure was employed, in which clam (*C. leana*) was given a single oral dose of approximately 100 μg L^{-1} of MC-LR_{eq} via food supply (toxic *Microcystis*) for ten days, and then the clam was exposed to a non-toxic *Microcystis* for another 10 days. Moreover, I also performed a clearance rate (CR) experiment to test the hypothesis that whether *C. leana* filtered toxic and non-toxic *M. aeruginosa* and the green alga *C. vulgaris* at different rates. The MC concentrations (MC-RR, -YR and -LR) in the whole body of mussels and in incubation water were monitored during the experiment. In

addition, the changes of antioxidant enzymes CAT, SOD, and detoxification enzyme GST in various clam tissues (gills, foot, mantle and remaining tissues) were also examined. Finally, evidences for the clam's resistance to cyanotoxins were also discussed.

4.2. Materials and methods

4.2.1. Culture of toxic, non-toxic *Microcystis* and green alga

The toxic cyanobacterium *M. aeruginosa* (strain NIES-1086), non-toxic *M. aeruginosa* (strain NIES-101), and green alga *C. vulgaris* (strain NIES-2170) were ordered from the National Institute for Environmental Studies (NIES) collection (Tsukuba, Ibaraki, Japan). The toxic strain NIES-1086 produced MCs at a concentration of 15.21 fg $cell^{-1}$ as determined by HPLC. Toxic strain was cultured in MA medium and non-toxic strain in CB medium (Ichimura, 1979; Shirai *et al.*, 1989). The green alga was grown in SEM medium (Kong *et al.*, 2012). All culture was maintained at 24°C under a 12:12 light:dark photoperiod with white fluorescent lights at a PPFD of 30 µmol m^{-2} s^{-1}. When the culture had reached the stationary phase, cells were harvested by centrifugation at 4,200 × *g* at 4°C for 30 min (Avanti HP-26XP, Beckman Coulter, Brea, CA, USA). Both cyanobacteria and green alga were cultured as single cells.

4.2.2. Collection and maintenance of clams

As described in the 3.2.1 section.

4.2.3. Clearance rate

The clearance rate (CR) experiment was performed to determine whether *C. leana* filtered toxic and non-toxic *M. aeruginosa* and the green alga *C. vulgaris* at different rates. Nine clams of the same shell size were gently scrubbed under tap water and allocated randomly to three groups of three. They were then incubated individually in 500-mL beakers with 300 mL of dechlorinated tap water, with constant aeration at a temperature of 22 ± 1°C

(Figure 4–1). Bakers with phytoplankton but no clam were used as controls. After 12 h of acclimation with no food, when each individual had its valves open, toxic *M. aeruginosa*, non-toxic *M. aeruginosa*, or *C. vulgaris* was added to the water to a final concentration of 2×10^6 cells mL^{-1}. This density is often found in water bodies (Davis *et al.*, 2009; Okello *et al.*, 2010; Sabart *et al.*, 2010). Water samples were taken at 1-h interval for 6 h. Cell density was estimated directly by using a Speirs-Levy Eosinophil counting slide under an Olympus light microscope, as described by Andersen (2005). CR, defined as the volume of water (mL) cleared with suspended particles per unit time (h) by each individual, was calculated according to the method of Coughlan (1969) as follows:

$$\mathrm{CR} = \frac{V}{wt}\left(\ln\frac{C_o}{C_t} - \ln\frac{C'_o}{C'_t}\right)$$

In which *CR* is the clearance rate expressed in mL g^{-1} wet weight (WW) h^{-1}. *V* is the water volume (300 mL), *w* is the whole body WW of the clam in gram, *t* is the duration course of the experiment in *h*, C_o is the cell density (cell L^{-1}) at $t = 0$ or one time step before *t* and C_t is the cell density at time *t*. C'_o is the cell density in the control baker at $t = 0$ and C'_t is the cell density at time *t*. The overall *CR* was calculated as the mean of the six consecutive measurements.

4.2.4. Feeding experiment

After acclimation, clams were placed in 8 aquariums (35 clams in each) containing 2 L of dechlorinated tap water and a 2-cm sand layer as a substrate, with constant aeration. Clams were allocated randomly to an exposure group and control group. Light intensity and water temperature were the same as previously described.

For MCs uptake, toxic *M. aeruginosa* cells were added to the aquarium to a final concentration of 100 µg MC-LR_{eq} L^{-1} on days 0, 3, 5, and 7 of the uptake period which lasted for 10 days. This concentration of MC is often found in natural environment during blooms (Chorus and Bartram, 1999; Znachor *et al.*, 2006; Davis *et al.*, 2009). The clams were then

collected and relocated into aquariums containing dechlorinated tap water as a toxin-free water. They were kept in these aquariums for another 10 days as a depuration period. During the depuration, the toxic *M. aeruginosa* was completely replaced by non-toxic *M. aeruginosa* at a concentration of 6×10^6 cells mL^{-1}. The non-toxic *Microcystis* cells were completely renewed on days 0, 3, 5 and 7 of the depuration period, which corresponded to days 11, 13, 15, 17 and 20 of the experiment. The water was also completely replaced at the same times. The entire experiment therefore lasted for 20 days (10 days of exposure following by 10 days of depuration). The control group was incubated in dechlorinated tap water and fed three times a week with *C. vulgaris* at a concentration of 6×10^6 cells mL^{-1}. Dead clams were removed and counted daily.

On days 1, 3, 5, 7, and 10 (in the uptake period) and days 11, 13, 15, 17 and 20 (in the depuration period) I sampled randomly about 12 – 15 clams in assigned aquariums. For MC quantification 8 – 10 clams were rinsed gently under dechlorinated tap water. The shell was removed immediately and the remaining tissues were freeze-dried completely and kept at –30°C until MC extraction. Ten clams collected before the start of the experiment were used as controls. To measure enzyme activity, in both groups I first dissected the gills, foot, and mantle of three to five clams (pooled) and the remaining tissues (kept individually) on ice. The samples were immediately frozen in liquid nitrogen and stored at –80°C until enzyme extraction.

4.2.5. Extraction and analysis of MCs in incubation water

MCs (intracellular content) in the incubation water were monitored every day during uptake and on days 11, 13, 15, 17 and 20 in the depuration period. The incubation water (10 to 100 mL) was filtered through GF/C filters. The filters were then dried completely at 45°C and kept at –30°C. MCs extraction and analysis were performed as described in 2.2.5 section. The preliminary experiment showed that more than 99% of MCs concentration was

intracellular (data not shown). Therefore I ignored the extracellular MCs in the incubation water.

4.2.6. Extraction and analysis of free MC in clam

As previously described in the 3.2.5 section.

4.2.7. Extraction of total MC in clam

As previously described in the 3.2.6

4.2.8. GC-MS analysis

GC-MS analyses were performed on a GCMS-QP2010 Plus mass spectrometer linked to a GC-2010 gas chromatography system (Shimadzu, Kyoto, Japan) equipped with an Rxi-5ms column (30 m × 0.25 mm ID, phase thickness 0.25 mm; Restek, Bellefonte, PA, USA). Helium was used as the carrier gas, at a flow rate of 1.5 mL min^{-1} in splitless mode. The program used for the analysis was 80°C for 1 min, followed by an increase to 280°C at 8°C min^{-1}. The other conditions were as follows: ion source temperature 200°C, injection port temperature 230°C, detector temperature 250°C and interface temperature 280°C. Methyl 4-PB (me4-PB) and meMMPB were detected by using SIM mode. Ions at 91 and 104 m/z were selected for me4-PB, and those at 75, 78, 91, 131, and 134 m/z were selected for meMMPB (Tsuji *et al*., 2001; Suchy and Berry, 2012). GC-MS solution software was used for the quantitative calculation. Duplicate samples with duplicate analysis (yielded 4 measurements, n = 4) were used in this determination.

4.2.9. Enzyme extraction and measurement

As previously described in the 3.2.8 section.

4.2.10. Statistical analyses

As previously described in the 3.2.10 section.

4.3. Results

4.3.1. Clearance rate

In the control bakers the mean cell densities of all strains was almost constant over the course of the experiment. The CR of 2×10^6 cells mL^{-1} of toxic *Microcystis* by *C. leana* was 16.0 ± 3.3 mL g^{-1} WW h^{-1}; that of non-toxic *Microcystis* was 15.3 ± 3.2 mL g^{-1} WW h^{-1}, and that of *C. vulgaris* was 7.0 ± 1.1 mL g^{-1} WW h^{-1} (Figure 4–2).

One-way ANOVA showed that the *Microcystis* CRs were significantly higher than that of green alga ($P < 0.03$). There was no significant different in CR between the toxic and non-toxic *Microcystis*. I observed that larger amount of pseudo-faeces were expelled in the bakers containing the toxic or non-toxic *Microcystis* than in the *C. vulgaris* beakers.

4.3.2. Microcystin concentrations in incubation water

Concentrations of MC in the control incubation water were under the detection limit (data not shown).

At the starting and renewal points, the concentrations of MC in the incubation water were 105 ± 4.7 µg MC-LR_{eq} L^{-1}. The MC concentrations in the incubation water slowly and continuously declined after each renewal. After 1 day the MC level had decreased to 96 ± 3.8 µg L^{-1}, after 2 days to 89 ± 4.1 µg L^{-1}, and after 3 days to 83 ± 1.5 µg L^{-1} (Figure 4–3).

4.3.3. Uptake and depuration of free- and Co-MC

There were no deaths in the exposure or control groups during the experiments. The control samples contained no detectable MCs, whereas exposed clams showed substantially enhanced toxin accumulation. I monitored the changes in MC accumulation by the clams during the uptake and depuration periods (Figure 4–3). Typically, free MC concentrations rose rapidly after the start of exposure and peaked (at 12.7 ± 2.5 µg g^{-1} DW) after about 3 days. The free MC content was relatively constant at the different time point during the uptake period. In contrast, the Co-MC concentration slowly increased during uptake period,

peaking at 4.2 ± 0.6 µg g^{-1} DW on day 10. Free MC was quickly eliminated from the clam tissues in the depuration period and declined from 12.7 ± 2.5 to 5.7 ± 0.3 µg L^{-1} after 24 h (day 11), then to 1.3 ± 0.3 µg L^{-1} after 72 h (day 13). It was under the detection limit after 7 days of depuration, which corresponded to day 17 of the experiment. In contrast, Co-MC level was almost unchanged during the first 5 days of depuration. It then gradually declined but was still detectable at the end of the depuration period (Figure 4–3).

4.3.4. Biotransformation enzyme

I examined the effects of toxic and non-toxic *Microcystis* on GST activity in different organs in the exposure and control groups (Figure 4–4). Inhibition of GST activity in the gills was observed after exposure of the clam to toxic *Microcystis* for 5 days, and there was significant difference compared with the controls after 7 days and at the end of the experiment. A decrease in GST activity was also observed on days 10 and 15 in the foot and days 10 and 20 in the remaining tissues. In contrast, in the mantle, GST activity was significantly higher than in the controls on day 11, but there were no significant differences at other time points.

4.3.5. Antioxidant enzyme activities

I examined the effects of toxic and non-toxic cyanobacteria on SOD activity in the different clam tissues (Figure 4–5). In the gills, SOD activity was significant higher in the exposed clams than in the controls on days 5 and 10 (exposure period) and on days 11 and 15 (depuration period). There was also a significant elevation of SOD activity on days 1 and 5 in the foot and on days 10 and 17 in the mantle. However, SOD activity in the gills, foot and mantle eventually returned to control levels during exposure to non-toxic *Microcystis* at the the end of the experiment (day 20). In the remaining tissues, SOD activity did not differ significantly from that in the controls, with the exception of a significantly inhibition at the end of the experiment (day 20).

I examined changes in CAT activity in the different tissues (Figure 4–6). There were no definable trends. In the gills, CAT activity in the exposure group was significantly higher than in the controls on days 5, 7, and 15 but was significantly lower on day 20. Significant elevation of CAT activity in the exposure group was observed on day 13 in the foot and on days 5 and 11 in the mantle. In the remaining tissues there were no differences in CAT activity between the control and exposure groups over the course of the experiment.

4.4. Discussion

This study is the first to describe uptake and depuration in the edible clam *C. leana* grazing on toxic and non-toxic cyanobacteria *Microcystis* and on the green alga *C. vulgaris*. Although aquatic animals have been shown to select and ingest only non-toxic food in laboratory experiments (Zurawell *et al.*, 2005; Juhel *et al.*, 2006b), my results rejected the hypothesis that the clams would graze less efficiently on toxic strains than on non-toxic ones, because no significant difference in CRs was observed. My observations agreed well with the results of Pires and Donk (2002), Pires *et al.* (2005) and Gazulha *et al.* (2012) who reported no significant differences in CR between mussels grazing on toxic and on non-toxic *Microcystis*. The second hypothesis that clams would graze less efficiently on toxic strains than on green algae was rejected as well. In contrast, the clams had higher CRs on toxic *Microcystis* than on the green alga (Figure 4–2). I observed more pseudo-faeces were expelled by clams fed cyanobacteria than by those fed the green alga (data not shown); clams are able to sort captured material, ingesting the nutritious particles (*C. vulgaris*) and rejecting the unpalatable ones (*Microcystis*) as pseudo-faeces. Their pumping rates may therefore be enhanced when there is a relative abundance of unpalatable food. Similar observations have been reported by Pires *et al.* (2004) namely that mussels filter toxic *Microcystis* faster than the green algae *Nannochloropsis* and *Scenedesmus*. Efficient expulsion of toxic cells via pseudo-feces has been described as a primary defense mechanism in zebra mussel *D. polymorpha*

when exposed to highly toxic cyanobacteria (Juhel *et al.*, 2006a). However, my observations contradict those of Pires *et al*. (2007) and and Liu et al. (2009), in which the CR did not change significantly in the three mussels *Anodonta anatina*, *Dreissena polymorpha* and *Unio douglasiae* when they were provided with varying concentrations of cyanobacteria and green algae. Differently, Gazulha *et al.* (2012) reported that highest filtration rates of the golden mussel *Limnoperna fortunei* were registered when mussels fed on diatom *Nitzschia* instead of *Microcystis*. Possibly, CR may depend not only on the food type (including the MC content) and the mussel species but also the food concentration and physical parameters such as temperature, salinity, pH, and season (Riisgård *et al.*, 2003; Bontés *et al.*, 2007; Naddafi *et al.*, 2007; Pestana *et al.*, 2009; Hwang *et al.*, 2010; Riisgård *et al.*, 2011). My results supported that the CR is even higher in case of bloom occurred with one species of *Microcystis*. This characteristic indicated that mussels are capable of removing cyanobacteria from water, regardless of whether the cyanobacteria are toxic or not. Hence, mussels may be used as a biofilter for mitigating harmful CYBs in eutrophic shallow lakes.

Field and experimental studies have reported the accumulation and distribution of MCs in different bivalve species fed on toxic cyanobacteria cells or exposed to purified MCs. Commonly, the highest MC content is found in the hepatopancreas (Prepas *et al.*, 1997; Yokoyama and Park, 2003; Pires *et al.*, 2004; Chen and Xie, 2005a; Chen *et al.*, 2005; Chen and Xie, 2007; Contardo-Jara *et al.*, 2009; Fernandes *et al.*, 2009; Sabatini *et al.*, 2011). Despite intensive studies, the accumulation and distribution of free MC and Co-MC in bivalve gavaged with toxic cyanobacteria at environmentally relevant concentrations are not yet fully understood. My laboratory experiments revealed that free MC was accumulated by *C. leana* by 1 day after the start of exposure. The peak levels of free MC measured in *C. leana* (12.7 $\mu g\ g^{-1}$ DW) were similar to the MC concentration measured in the zebra mussel *D. polymorpha* (11 $\mu g\ g^{-1}$ DW; Pires *et al.*, 2004), and the freshwater mussel *Mytilus galloprovincialis* (10.5 $\mu g\ g^{-1}$ DW; Vasconcelos, 1995) during laboratory exposure. However,

even when data on accumulation in other bivalves are available (Watanabe *et al.*, 1997; Yokoyama and Park, 2003; Chen and Xie, 2005a; Vareli *et al.*, 2012) they are not suitable for comparison with mine, because most were obtained from measurement in individual tissues and not the whole body. In general, MC accumulation in aquatic animals is likely to be affected by a number of factors, such as the exposure route, exposure duration and exposure dose, target tissues as well as by the mussel species (Galanti *et al.*, 2013). Thus, laboratory studies, using different approaches, can contribute to understand the dynamic of cyanotoxins accumulation.

Because of the difficulties of extracting covalently linked MC, analysis of MCs in animal tissues has until now been limited to quantification of the free MC content (for reviews see Ibelings and Chorus, 2007; Martins and Vasconcelos, 2009; Ferrão-Filho and Kozlowsky-Suzuki, 2011). By using an oxidation procedure adapted from previously developed methods (Tsuji *et al*., 2001; Neffling *et al.*, 2010; Suchy and Berry, 2012; Cadel-Six *et al.*, 2014) I were able to detect Co-MC in *C. leana* tissues (Figure 4–3). On average, 3.2% (the number was calculated from mean percentages of the Co-MC contents from the MC concentrations in incubation water on days 1, 3, 5, 7 and 10) of total MC was bound in *C. leana* during the 10-day exposure period (data not shown). The existence of high content of Co-MC has been reported in several organisms such as fish (Williams *et al.*, 1997a; Suchy and Berry, 2012; Cadel-Six *et al.*, 2014), gastropods (Lance *et al.*, 2010; 2014) and mussels (Williams *et al.*, 1997b; Pires *et al.*, 2004).

Fortunately, my observations showed that clams rapidly eliminated the free MC content within several days when culture in toxin-free water, this has been reported elsewhere (Prepas *et al.*, 1997; Williams *et al.*, 1997b). Immediate uptake and rapid release of MCs were also observed in *D. polymorpha* (Pires *et al.*, 2004; Contardo-Jara *et al.*, 2008), and in *M. galloprovincialis* (Amorim and Vasconcelos, 1999). In contrast, the rate of elimination of Co-MC in *C. leana* was lower than that of free MC, and the clam's tissues still contained a

detectable amount of Co-MC after 10 days in toxin-free water. Therefore, to accurately assess the risks to humans, Co-MC in edible aquatic animals need to be quantified. Meanwhile, their toxicity and potential transfer to higher trophic levels remain unknown.

Freshwater mussels are among the most numerous macro-invertebrates in the world's streams, rivers, ponds, and lakes (Byrne *et al.*, 2000), where cyanobacteria frequently bloom. Consumption of bivalve mollusks is a potential route of human exposure to hepatotoxic MCs, especially in the case of mollusks harvested from lakes and reservoirs that routinely experience toxic CYBs (Chen and Xie, 2007; Song *et al.*, 2007; Funari and Testai, 2008; Dyble *et al.*, 2011; Mulvenna *et al.*, 2012; Cheung *et al.*, 2013). In this study, I used a linear equation $y = 0.0116x$ ($r^2 = 0.94$, $n = 30$), to convert the clam's whole-body wet weight to dry weight. Assuming that a 60-kg person consumes 100 g WW (~1.16 g DW) of the clam per day (Ibelings and Chorus, 2007), the maximum EDI during the accumulation period would be more than seven times the TDI guideline value (Figure 4–7), suggesting that *C. leana* poses high risks to human consumers. Consumption of this clam should be limited when there are high concentrations of toxic cyanobacteria present in the water from which it is harvested. My estimation was made for a healthy adult, and the risks for children, the elderly and sensitive individuals are likely to be higher.

Detoxification of MCs by aquatic animals occurs first via conjugation to GSH, a reaction catalyzed by GSTs (Kondo *et al.*, 1992; Pflugmacher *et al.*, 1998; Zhang *et al.*, 2012; Li *et al.*, 2014). Nevertheless, the responses of GST activity to MCs in mussels and other aquatic animals are variable (Vasconcelos *et al.*, 2007; Amado and Monserrat, 2010; Gélinas *et al.*, 2012; Paskerová *et al.*, 2012). It appears that the exposure time, route, composition of MCs, and target tissues all influence the GST response after exposure to MCs or toxic cyanobacteria. An increase in the activity of these enzymes can arise from an activation of existing enzymes or from de novo enzyme synthesis, whereas a decreased activity can be sign of saturation due to substrate inhibition caused by the presence of high toxin concentration

(Cazenave *et al.*, 2006; Hao *et al.*, 2008; Burmester *et al.*, 2012). Inhibition of GST activity has been observed in the mussel *U. tumidus* exposed to MC-LR or CCE (Burmester *et al*., 2012). Similar results have been reported in the gills of the freshwater mussel *D. polymorpha* after exposure to MC-LR at 100 μg L^{-1} for 1 h; the highest MC content in whole mussel tissue was detected at this time point (Contardo-Jara *et al*., 2008). In this study, I found significant inhibition of GST activity after 7 days of exposure in the gills and at two time points in the foot, but there was no inhibition in the mantle. This response of the detoxification system was synchronous with the increase in the rate of MC accumulation by the clam (Figures 4–3 and 4–4). Depression of GST activity may result from inhibition of GST synthesis as a result of the high levels of accumulation of MCs during the exposure period (Hao *et al.*, 2008; Li *et al*., 2008). In contrast, Vasconcelos *et al*. (2007) and Fernandes *et al*. (2009) reported increase of GST activity in gills of the blue mussel (*M. galloprovincialis*) after exposure to toxic *Microcystis* for 24 h or 2 weeks. Increase of GST activity was also observed in the freshwater clam *D. chilensis patagonicus* after exposure to toxic *Microcystis* for 6 weeks (Sabatini *et al*., 2011). Out of mussel group, Gavrilović *et al*. (2014) reported that GST activity in the gills of three cyprinids fish, *Rutilus rutilus* (roach), *Blicca bjoerkna* (white bream), and *Carassius gibelio* (Prussian carp), was inhibited during a CYB with high concentration of MCs. The same observations have been reported in the gills of zebrafish (*Danio rerio*) upon sub-chronic exposure to MC-LR (Chen *et al*., 2012) and in the liver, gills, intestine, and brain of the catfish *Corydoras paleatus* exposed to dissolved MC-RR at concentrations from 0.5 to 10 μg L^{-1} for 24 h (Cazenave *et al*., 2006). In another study (Cazenave *et al.*, 2008) observed an increase of GST activity in liver and brain of one-sided livebearer (*Jenynsia multidentata*) exposed to MC-LR at 1 μg g^{-1} but decreased when exposed to MC-LR at 0.1 μg g^{-1} for 24 h. On the contrary, the GST in zebra fish (*Danio rerio*) and tilapia fish (*Oreochromis* sp.) remained stable after exposure (Best *et al.*, 2002; Atencio *et al.*, 2009). Probably, CYBs or MCs, or both, can inhibit GST activity in a range of aquatic animals.

Toxic cyanobacteria and MCs could alter antioxidant systems and induce ROS production, resulting in the oxidative stress that has been well documented in aquatic species (Prieto *et al.*, 2007; Leão *et al.*, 2008; Amado and Monserrat, 2010; Chen *et al.*, 2012; Jiang *et al.*, 2012; Paskerová *et al.*, 2012; Sun *et al.*, 2013; Gavrilović *et al.*, 2014). SOD, CAT, and other molecules such as lipoic and dihydrolipoic acid are an essential components of antioxidative defense systems (Lushchak, 2011; Amado and Monserrat, 2010), and increase SOD and CAT activities in animal tissues plays an important role in eliminating excessive ROS (Li *et al.*, 2003; Jos *et al.*, 2005; Zhang *et al.*, 2013). In a recent study, Burmester *et al.* (2012) reported that SOD activity in *D. polymorpha* was elevated in most mussel tissues after exposure to MC-LR or CCE, or both, but CAT activity was barely affected. Increased SOD and CAT activities have been observed in the freshwater clam *D. chilensis patagonicus*, but only at 5 and 6 weeks after exposure to toxic *Microcystis* (Sabatini *et al.*, 2011). I found here that SOD and CAT activities in *C. leana* were elevated only at some points and in some tissues during the exposure and depuration periods (Figures 4–5 and 4–6). My results were partly in line with those of Burmester *et al*. (2012), who observed that SOD in *U. tumidus* was slightly induced during 7 days exposure to MC-LR or CCE, or both. Similar response of SOD activity was reported in *U. tumidus* after exposure to aquatic contaminations in the Fensch River for 15 and 30 days (Cossu *et al.*, 1997). In contrast, studies performed on crustaceans such as shrimp (*Palaemonetes argentinus*), on tilapia fish (*Oreochromis* sp.), on bighead carp (*Aristichthys nobilis*), and on loach (*Misgurnus mizolepis*) given intraperitoneal MC-LR injections or exposed to toxic cyanobacteria have shown significant elevations of antioxidant enzymes, especially in the liver (Li *et al.*, 2005; Prieto *et al.*, 2006; Li *et al.*, 2010; Galanti *et al.*, 2013). Probably, SOD and CAT play key roles in scavenging free radicals and ROS in fishes but play minor roles in mussels.

Although no MCs were present in the incubation water during the depuration period, SOD, CAT, and GST activities were still induced by the non-toxic *Microcystis*. This is

evidence that both toxic and non-toxic *Microcystis* cause oxidative stress in the clam and that MCs are not the only, or even the main, toxic compounds in cyanobacteria. Probably, other components of the complex cyanobacteria biomass contribute to this oxidative stress. However, these adverse effects are only temporary in the case of both toxic and non-toxic *Microcystis,* and prolonged exposure may lead to adaptation. I found here that although enzyme activity was induced it returned to control levels at later time points. In other words, clams are temporarily affected by toxic and non-toxic cyanobacteria. This resistance may occur because of an ability to efficiently expel toxic cyanobacteria cells without metabolising them (Fernandes *et al*., 2009; Burmester *et al*., 2012). In addition, recent evidence indicates that MXR mechanisms represent a general biological defense of many marine and freshwater mussels against environmental toxicants (Eufemia and Epel, 2000; Achard *et al.*, 2004; Contardo-Jara *et al.*, 2008; Faria *et al.*, 2011). Animal cells can trigger an MXR mechanism that serves as an efflux transporter against a broad spectrum of natural and man-made toxicants under stress conditions. This mechanism needs further investigation in *C. leana*.

4.5. Summary

In this chapter the accumulation and toxicity of toxic and non-toxic *Microcystis* in the edible clam *C. leana* were investigated. Treated clams were exposed to toxic *Microcystis* at 100 µg of MC (microcystin)-LR_{eq} L^{-1} for 10 days. The experimental organism was then placed in toxin-free water and fed on non-toxic *Microcystis* for the following 10 days for depuration. CRs by *C. leana* of toxic and non-toxic *Microcystis* and of the green alga *Chlorella vulgaris* as a control were estimated. Toxic effects were evaluated though the activity of CAT, SOD and GST. Clam accumulated MCs (up to 12.7 ± 2.5 µg g^{-1} DW of free- and 4.2 ± 0.6 µg g^{-1} DW of Co-MC). My results suggest that although both toxic and non-toxic cyanobacteria caused oxidative stress by inducing the detoxification and antioxidant

defense system, the clam was quite resistant to cyanotoxins. The estimated MC concentration in *C. leana* was far beyond the WHO's provisional TDI (0.04 μg kg^{-1} day^{-1}), suggesting that consuming clams harvested during cyanobacterial blooms carries a high health risk.

My experiments confirmed the transient bioaccumulation of free MC and Co-MC in *C. leana* after exposure to toxic cyanobacteria. Although free-MC was partially eliminated from the clams after transferring to MC-free water, the Co-MC would have still been contaminated in their tissues. Both toxic and non-toxic *Microcystis* caused oxidative stress, as shown by the induction of antioxidant and detoxification systems. However, my laboratory experiments suggested that GST activity did not play a major role in the elimination of MC. The clam may be able to efficiently expel toxic cyanobacteria cells or eliminate cyanotoxins without metabolising them. Alternative mechanisms, such as MXR, could be responsible for MC removal and resistance during exposure. These mechanisms need to be studied further.

Figure 4–1. Clearance rate experiment.

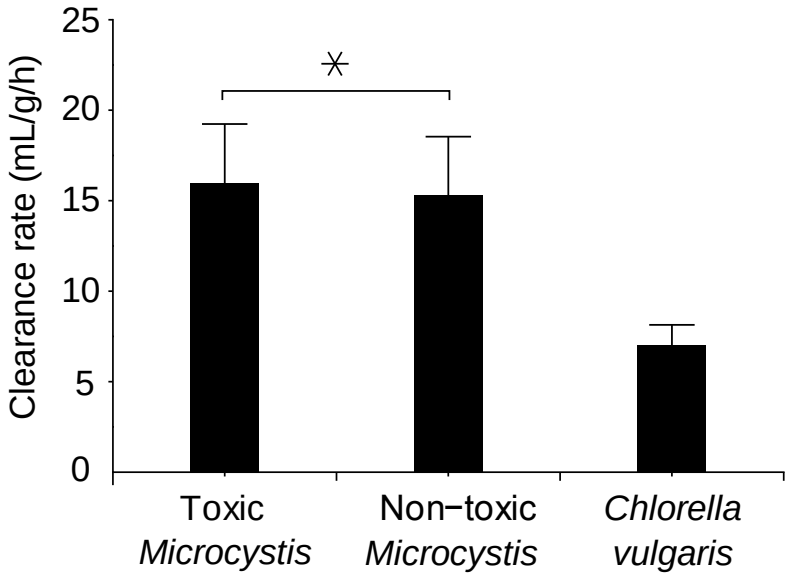


Figure 4–2. Mean (±SD) clearance rates (CRs) (in mL g^{-1} wet weight h^{-1} over 6 h) after feeding of *Corbicula leana* with toxic *Microcystis* (NIES-1086), non-toxic *Microcystis* (NIES-101), or the green alga *Chlorella vulgaris* (NIES-2170). Asterisk indicates significant difference ($P < 0.05$).

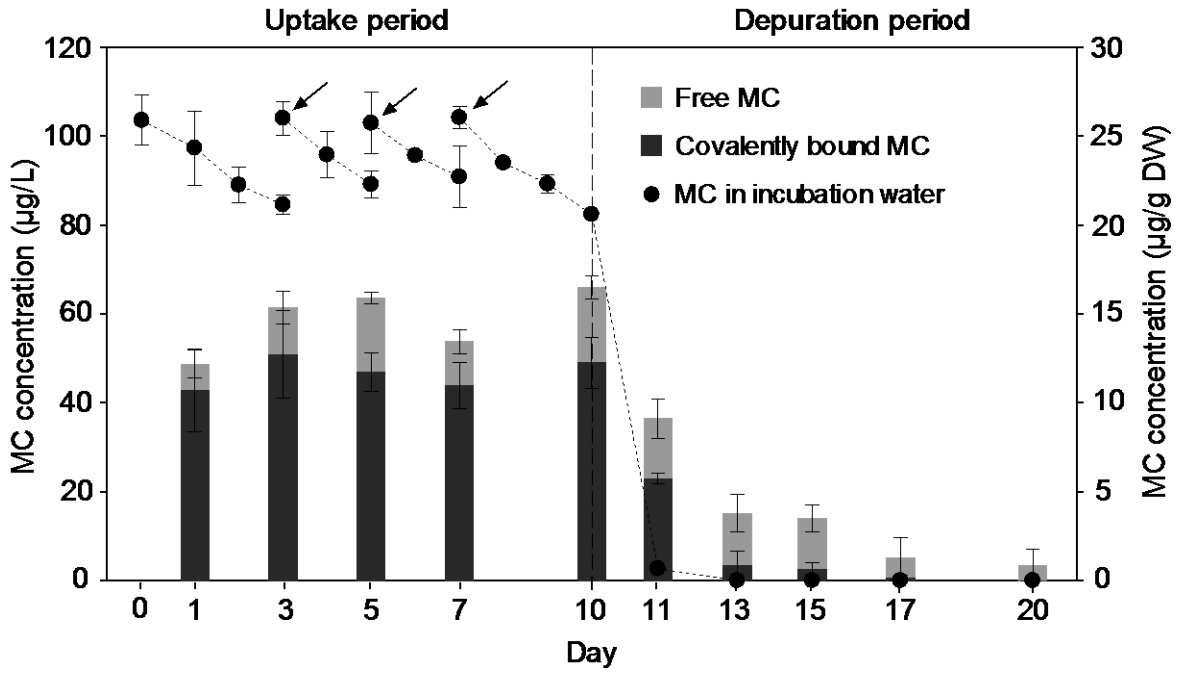


Figure 4–3. Concentrations of microcystin (MC) in incubation water, of free MC, and covalently bound MC accumulated in clams during the uptake and depuration periods. Arrows indicate points at which the MC and the water were renewed during the uptake period.

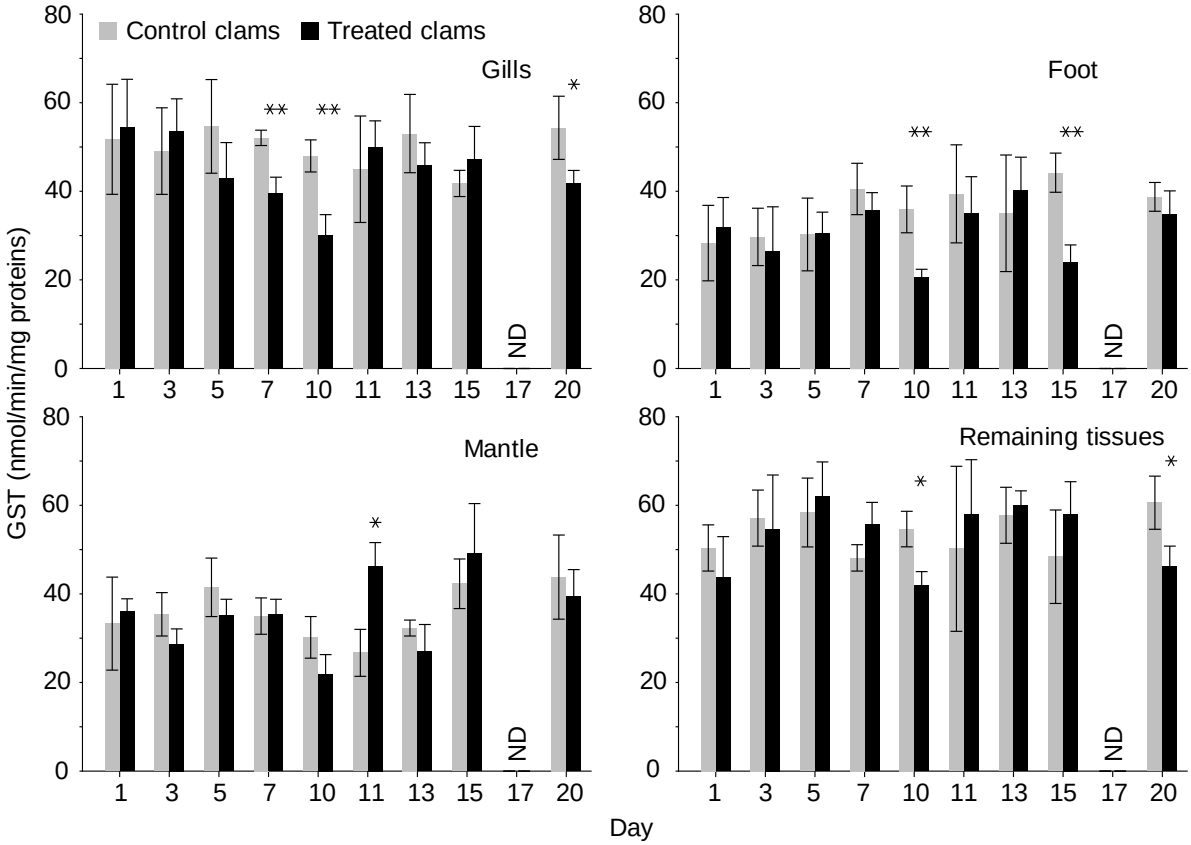


Figure 4–4. Changes in glutathione *S*-transferase (GST) activity (nmol min^{-1} mg^{-1} protein) in various tissues of clams fed with toxic *Microcystis* (days 0 to 10) or non-toxic *Microcystis* (days 11 to 20). Asterisks indicate significant differences compared with controls (fed with *Chlorella vulgaris*) at the respective time points (*$P < 0.05$, **$P < 0.01$). ND: not detected.

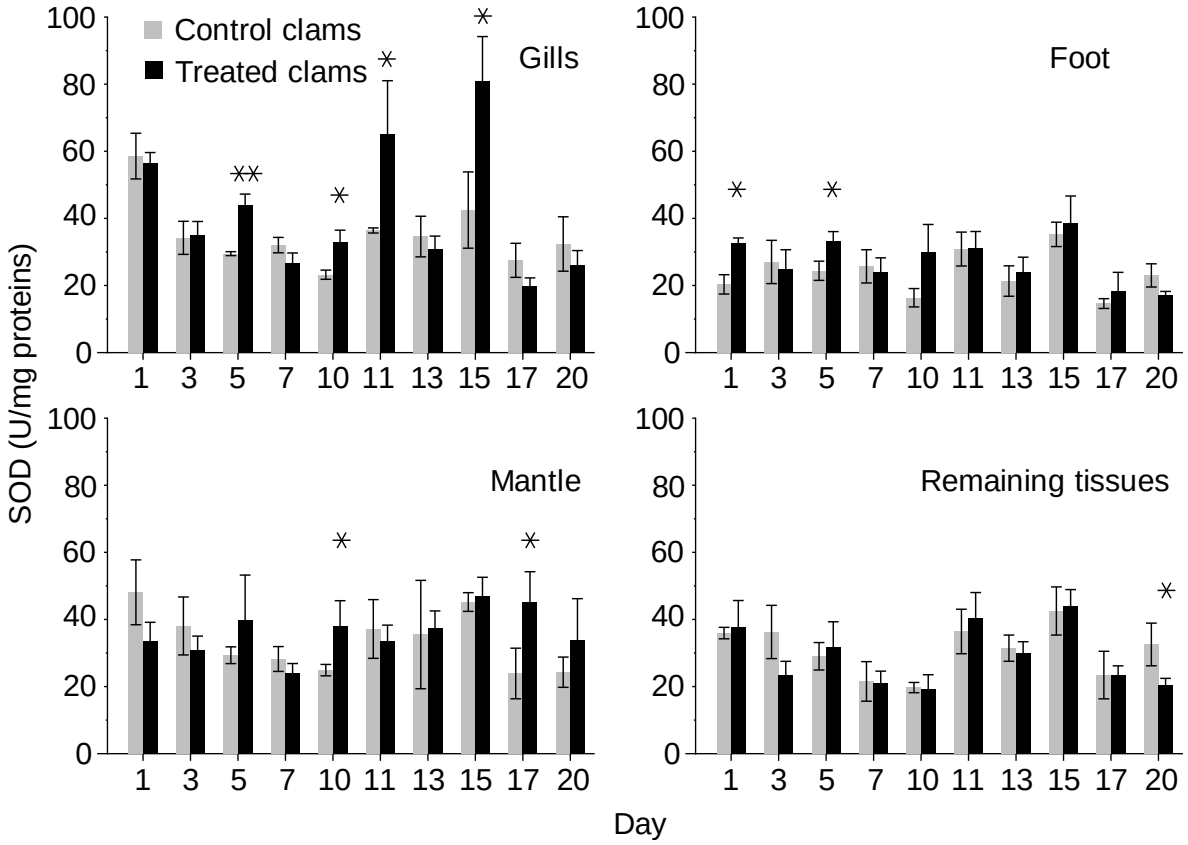


Figure 4–5. Changes in superoxide dismutase (SOD) activity (U mg^{-1} proteins) in various tissues of clams fed with toxic *Microcystis* (days 0 to 10) or non-toxic *Microcystis* (days 11 to 20). Asterisks indicate significant differences compared with controls (fed with *Chlorella vulgaris*) at the respective time points (*$P < 0.05$, **$P < 0.01$).

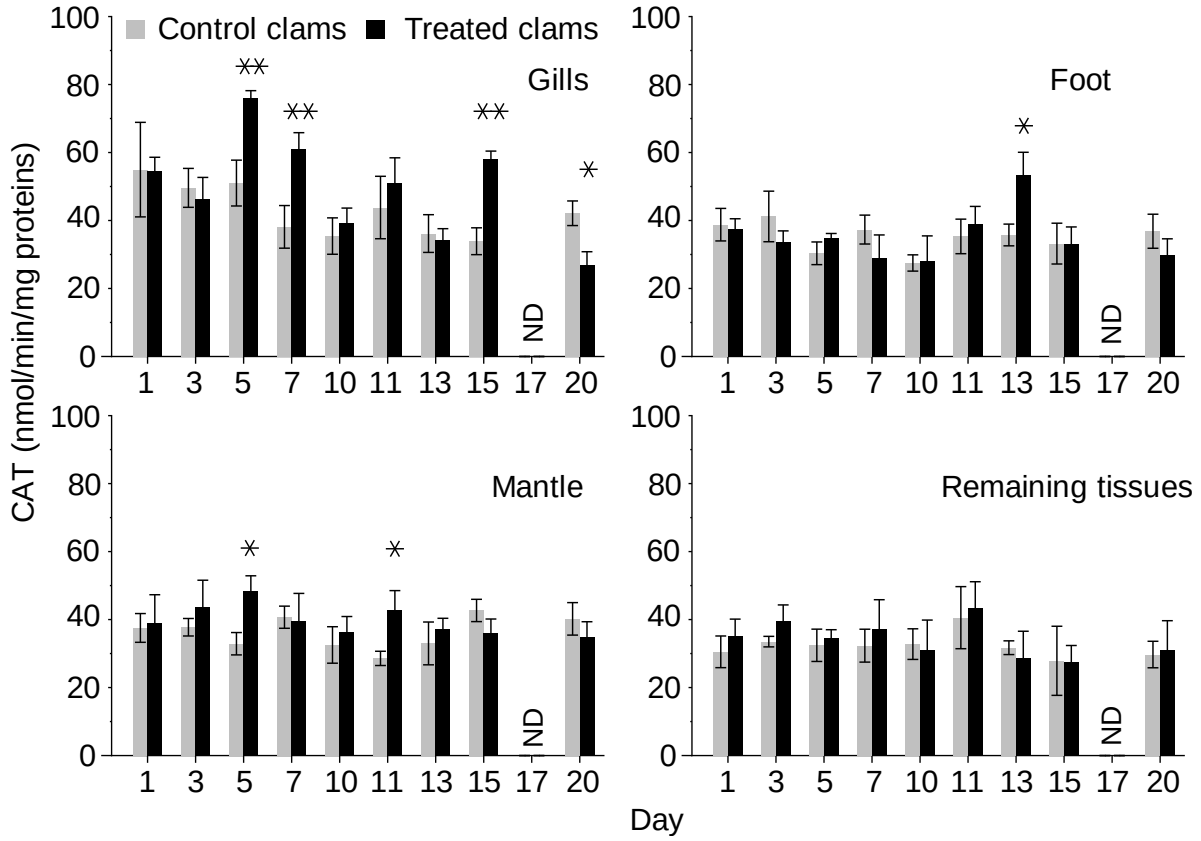


Figure 4–6. Changes in catalase (CAT) activity (nmol min^{-1} mg^{-1} protein) in various tissues of clams fed with toxic *Microcystis* (days 0 to 10) or non-toxic *Microcystis* (days 11 to 20). Asterisks indicate significant differences compared with controls (fed with *Chlorella vulgaris*) at the respective time points (*$P < 0.05$, **$P < 0.01$). ND: not detected.

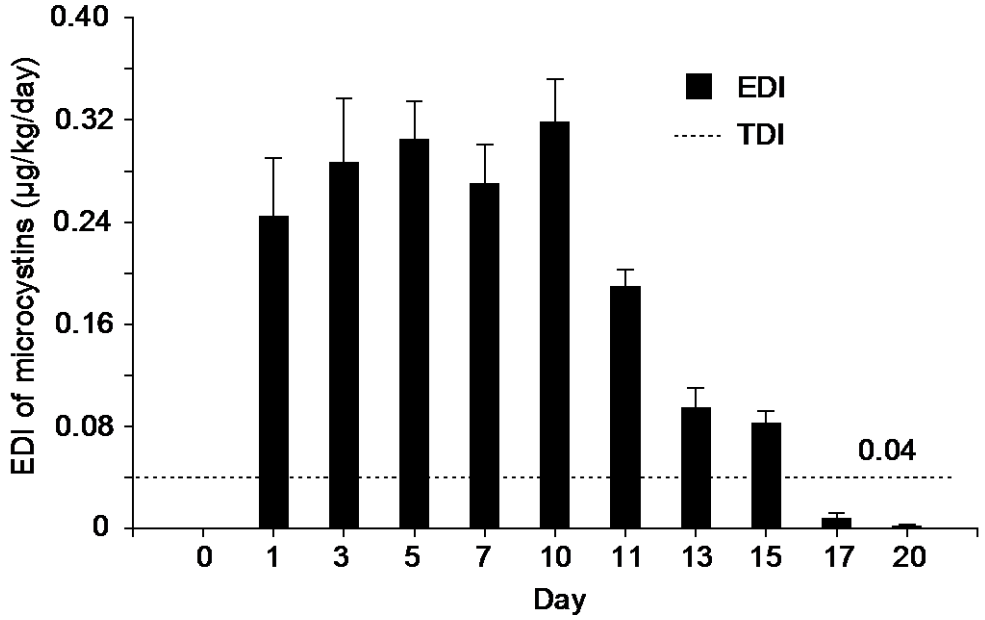


Figure 4–7. Estimated daily intake (EDI) of microcystins by a person (60 kg) consuming 100 g of clam wet weight per day (Ibelings and Chorus, 2007). Horizontal line indicates the maximum tolerable daily intake (TDI) of 0.04 µg kg^{-1} day^{-1}, as proposed by the World Health Organization (WHO) (Chorus and Bartram, 1999).

Chapter 5

General conclusions and recommendations

5.1. Conclusions

Toxic CYBs and their toxins are a global threat to human health and aquatic biota. MCs are the most common cyanotoxins and can be produced wherever blooms of cyanobacteria occur in surface water. The worldwide occurrence of MC-producing blooms mainly dominated by *M. aeruginosa* strains is well established, although other species of *Microcystis* and of other genera such as *Dolichospermum, Arthrospira* and *Cylindrospermopsis* may co-dominate with *Microcystis.* Blooms of *Microcystis* are also the most common recorded in Vietnamese waters.

Cyanotoxins cause adverse effects on aquatic animals. Freshwater mussels are a fascinating group of animals that reside in the bottom of streams, rivers, and lakes. They play an important role in natural ecosystems by taking up phytoplankton, filtering organic matter and suspended particles from the water column and providing food for many types of fish and aquatic mammals. These filter feeders inhabit the littoral areas where cyanobacteria frequently form scums. They therefore are among the most threatened group of aquatic animals during CYBs or after blooms decay. This study reveals that MCs can be accumulated in the edible clam *C. leana* via both ingestion of toxic cells and/or via uptake of MCs dissolved in water column. MCs can be accumulated in the animal up to thousands ng g^{-1} DW but the actual concentration of MC accumulated may depend on the levels of MC dissolved in water column or in food seston, routs and time of exposure as well as target organs and species. It may be possible to correlate the toxin level in mussels with levels in the water column. MCs not only accumulated under unbound forms (free forms) but also covalently bound to PPs. However, the clam could metabolize effectively and/or eliminate MCs. Free MC was completely eliminated within a few days, whereas the elimination of Co-MC last for

weeks. Results on the presence of Co-MC in bivalve tissues illustrate the necessity to consider the total of MC content (free- and Co-MC) when assess the toxicity in aquatic animals and human health.

Toxic, non-toxic cyanobacteria as well as CCE containing MCs stimulated the detoxification and antioxidant enzymes in the mussel. CCE induced these systems to a greater extent but less bioaccumulation than the intact cells. Exposure of the mussel to natural CCE containing MCs caused significant changes in levels and activities of these enzymes, while exposure to toxic intact cells caused minor changes of these enzymes. These changes could potentially lead to chronic toxic effects on the clam. In addition, both toxic and non-toxic cyanobacterium caused oxidative stress in bivalves and that MCs are not the only, or even the main, toxic compounds in cyanobacteria. Probably, other components of the complex cyanobacteria biomass contribute to this oxidative stress. However, toxic effects are only temporary in all cases and that prolonged exposure may lead to the adaptations to cope with deleterious effects. Effectively eliminate free MC and adaptation in detoxification and antioxidant systems are evidences that there is an adaptive response in the detoxification system and possible tolerance of *C. leana* with continuous exposure to high levels of toxic cyanobacteria or to aqueous extract containing MCs.

Results of this study suggested that toxic, non-toxic cyanobacteria as well as CCE cause increasing of ROS contents in clam tissues leading oxidative stress, at the same time, the animal would both improve their antioxidant abilities and metabolize the toxin to overcome these adverse effects. It is likely that GST is not the only one involved in the elimination of MCs in clam. Other alternative mechanisms, like MXR could be responsible for partly MCs removal in clam tissues.

Humans are potentially exposed to harmful algal blooms from contact with toxic algal blooms via recreational activities, via oral uptake by drinking contaminated water or via ingestion food contaminated with cyanotoxins. This study reveals MCs from water or toxic

Microcystis cells, or both, can accumulate in edible clam *C. leana*. Moreover, my findings clearly demonstrate that consumption of the clam contaminated with MC poses a serious health risk for consumers. Cyanotoxins concentrations accumulated in bivalve were often highest during bloom period, which often occurs during summer or after cells lyse at the end of the blooms. Therefore, consumption risks may be reduced or avoided if the local government adjusts harvesting time. Additionally, bioaccumulation of cyanotoxins is expected to be higher in the tropics and may be transported to higher trophic levels; humans might be at high risks, especially in undeveloped countries where the control of toxins in food and in water supply is not well regulated.

5.2. Recommendations

In order to reduce the risk in humans and animals exposure to cyanobacterial toxins, it is necessary to have on-going monitoring of both CYBs and their toxins in surface water bodies. More studies on the impact of CYBs and their toxins in tropical regions should be carried out, since the incidence and persistence of these blooms are greater than that in temperate regions. Blooms of cyanobacteria in surface waters need to be regularly monitored due to their potential toxicity. Water treatment for removing cyanotoxins in drinking water is also important to mitigate effects from CYBs. Hence, more efficient and affordable on monitoring strategies and drinking water treatment should be developed to address these concerns.

It is important to predict the presence and occurrence of potential CYBs in water bodies. Although cyanotoxins and their toxicity have been well documented, predicting the occurrence of CYBs remains challenging. Climate change has been promoting the frequency and magnifying of blooms and altering toxin distribution to latitudes outside of their current range. Hence, future research should address on prediction the impact of climate change on bloom occurrence and challenge to mitigate the effects from CYBs.

The majority of studies in freshwater systems have focused only on MCs and other cyanotoxins has not been investigated to the same extent. It is likely that other cyanotoxins (e.g. anatoxins, saxitoxins, cylindropermopsin) have been presented in CYB waters, and these toxins should also be characterized in both water and aquatic animals. Also, special attention should be paid to other metabolites of cyanobacteria such as microviridins, micropeptins, oscillapeptins, lipopolysaccharides and β-Methylamino-L-alanine (BMAA).

Cyanotoxin concentrations should be characterized in all levels of the food web in order to gain further insight into the trophic transfer of cyanotoxins (particularly through the analysis of non-fish food web components). In addition, attention to the covalent complex MC bound to PPs, its toxicity and bioavailability for the next trophic level still require to be elucidated.

In particular, it remains unknown whether the resistance of bivalves to MCs may be a result of a higher efficiency in detoxification systems or to a decrease in uptake of MCs by the efflux systems, or both. Additional research is needed with respect to the insensitivity of bivalves toward MCs in natural aquatic environments. Studies are needed to investigate the MXR mechanism in bivalves that serves as a driver against a broad spectrum of natural and man-made toxicants in the cells. In aquatic ecosystems, it is likely that aquatic organisms are simultaneously exposed to multiple stresses not only natural toxins but also anthropogenic pollutants. Characterization of single and combine effects of these toxicants on aquatic animals under relevant condition are also necessary in future research.

Acknowledgements

Most of all I am profoundly grateful to my supervisor Professor Motoo Utsumi for guiding me through my study. His critical discussion, valuable comments, suggestion, encouragement and great support at all times have brought me to such an academic achievement. I greatly appreciated for all his help throughout my study at the University of Tsukuba, Japan.

I grateful thank Professor Norio Sugiura, Professor Kouji Nakamura, Professor Toshiaki Nakajima, Professor Yingnan Yang, Professor Do-Hong Lan-Chi for many advices, invaluable guidance and comments for the thesis content. I would like to express my thanks to Assistant Professor Kazuya Shimizu of Toyo University for assistance on GC/MS analysis, paper reviewing, and sharing valuable experiences in laboratory works. I deeply thank Dr. Utsumi Kunihiro for collecting the clams used in this research.

I wish to express my thanks to Dr. Jieming Li, Dr. Chie Amano, Dr. Yu Gao, Dr. Xiaowei Tian, Dr. Xin Wang, Ms. Ayako Kanazawa, Ms. Mariko Hirokawa, Mr. Akiyama, Mr. Hailong Cui, Mr. Yoshiaki Hamatani for their assistance throughout my study. I thank all lab members for providing numerous assistance, nice working environment and helpfulness during my study time in Tsukuba. Special thanks also go to Dr. Dao Thanh Son from Ho Chi Minh City University of Technology for his kindly help solving cyanobacterial taxonomic problems, paper reviewing, and giving me valuable discussion and comments.

I am greatly indebted Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) for giving me this chance to study in Doctoral Program of Graduate School of Life and Environmental Science, University of Tsukuba, Japan by supporting scholarship.

Thanks to my wife, Nguyen Bich Thuy for her love, sympathies, sacrifice and beside me all time. I am grateful my parents, sisters and brothers for encouragement and mental support. Thanks all my friends for spending time with me. Your friendship and companion are the treasures in my life.

References

Achard, M., Baudrimont, M., Boudou, A., and Bourdineaud, J.P. (2004) Induction of a multixenobiotic resistance protein (MXR) in the Asiatic clam *Corbicula fluminea* after heavy metals exposure. *Aquat.Toxicol.* 67: 347–357.

Al-Tebrineh, J., Merrick, C., Ryan, D., Humpage, A., Bowling, L., and Neilan, B.A. (2012) Community composition, toxigenicity, and environmental conditions during a cyanobacterial bloom occurring along 1,100 kilometers of the Murray River. *Appl. Environ. Microbiol.* 78: 263–272.

Amado, L.L., and Monserrat, J.M. (2010) Oxidative stress generation by microcystins in aquatic animals: Why and how. *Environ. Int.* 36: 226–235.

Amado, L.L., Garcia, M., Ramos, P., Yunes, J., and Monserrat, J. (2011) Influence of a toxic *Microcystis aeruginosa* strain on glutathione synthesis and glutathione-S-transferase activity in common carp *Cyprinus carpio* (Teleostei: cyprinidae). *Arch. Environ. Contam. Toxicol.* 60: 319–326.

Amé, M.V., Díaz, M.D.P., and Wunderlin, D.A. (2003) Occurrence of toxic cyanobacterial blooms in San Roque Reservoir (Córdoba, Argentina): A field and chemometric study. *Environ. Toxicol.* 18: 192–201.

Amé, M.V., Baroni, M.V., Galanti, L.N., Bocco, J.L., and Wunderlin, D.A. (2009) Effects of microcystin–LR on the expression of P-glycoprotein in *Jenynsia multidentata*. *Chemosphere* 74: 1179–1186.

Amorim, Á., and Vasconcelos, V.T. (1999) Dynamics of microcystins in the mussel *Mytilus galloprovincialis*. *Toxicon* 37: 1041–1052.

Andersen, R.A. (2005) Algal culturing techniques. Academic Press, New York. 589 pp.

Apeldoorn, M.E.V., Egmond, H.P.V., Speijers, G.J., and Bakker, G.J. (2007) Toxins of cyanobacteria. *Mol. Nutr. Food Res.* 51: 7–60.

Arheimer, B., Andreasson, J., Fogelberg, S., Johnsson, H., Pers, C.B., and Persson, K. (2005) Climate change impact on water quality: Model results from southern Sweden. *Ambio* 34: 559–566.

Atencio, L., Moreno, I., Jos, Á., Prieto, A.I., Moyano, R., Blanco, A., and Cameán, A.M. (2009) Effects of dietary selenium on the oxidative stress and pathological changes in tilapia (*Oreochromis niloticus*) exposed to a microcystin-producing cyanobacterial water bloom. *Toxicon* 53: 269–282.

Baeka, S.H., Hong, S.-S., Song, S.-Y., Lee, H.-O., Nakano, S.-i., and Han, M.-S. (2009) Grazing effects on toxic and non-toxic *Microcystis aeruginosa* by the mixotrophic flagellate *Ochromonas* sp. *J. Freshw. Ecol.* 24: 367–373.

Ballot, A., Krienitz, L., Kotut, K., Wiegand, C., Metcalf, J.S., Codd, G.A., and Pflugmacher, S. (2004) Cyanobacteria and cyanobacterial toxins in three alkaline Rift Valley lakes of Kenya – Lakes Bogoria, Nakuru and Elmenteita. *J. Plankton Res.* 26: 925–935.

Barco, M., Flores, C., Rivera, J., and Caixach, J. (2004) Determination of microcystin variants and related peptides present in a water bloom of *Planktothrix* (*Oscillatoria*) *rubescens* in a Spanish drinking water reservoir by LC/ESI-MS. *Toxicon* 44: 881–886.

Barco, M., Lawton, L.A., Rivera, J., and Caixach, J. (2005) Optimization of intracellular microcystin extraction for their subsequent analysis by high-performance liquid chromatography. *J. Chromatogr. A* 1074: 23–30.

Bard, S.M. (2000) Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. *Aquat. Toxicol.* 48: 357–389.

Beattie, K.A., Ressler, J., Wiegand, C., Krause, E., Codd, G.A., Steinberg, C.E.W., and Pflugmacher, S. (2003) Comparative effects and metabolism of two microcystins and nodularin in the brine shrimp *Artemia salina*. *Aquat. Toxicol.* 62: 219–226.

Belcher, H., and Swale, E. (1988) Culturing algae: A guide for schools and colleges. The Ferry House, UK, 25 pp.

Best, J.H., Pflugmacher, S., Wiegand, C., Eddy, F.B., Metcalf, J.S., and Codd, G.A. (2002) Effects of enteric bacterial and cyanobacterial lipopolysaccharides, and of microcystin-LR, on glutathione S-transferase activities in zebra fish (*Danio rerio*). *Aquat. Toxicol.* 60: 223–231.

Bittencourt-Oliveira, M.d.C. (2003) Detection of potential microcystin-producing cyanobacteria in Brazilian reservoirs with a *mcyB* molecular marker. *Harmful Algae* 2: 51–60.

Blaha, L., Babica, P., and Marsalek, B. (2009) Toxins produced in cyanobacterial water blooms - toxicity and risks. *Interdiscip. Toxicol.* 2: 36–41.

Bontés, B., Verschoor, A., Dionisio Pires, L.M., Donk, E., and Ibelings, B. (2007) Functional response of *Anodonta anatina* feeding on a green alga and four strains of cyanobacteria, differing in shape, size and toxicity. In Gulati, R., Lammens, E., Pauw, N., and Donk, E. (eds.), Shallow lakes in a changing world. 196: 191–204.

Burmester, V., Nimptsch, J., and Wiegand, C. (2012) Adaptation of freshwater mussels to cyanobacterial toxins: response of the biotransformation and antioxidant enzymes. *Ecotox. Environ. Saf.* 78: 296–309.

Burýšková, B., Hilscherová, K., Babica, P., Vršková, D., Maršálek, B., and Bláha, L. (2006) Toxicity of complex cyanobacterial samples and their fractions in *Xenopus laevis* embryos and the role of microcystins. *Aquat. Toxicol.* 80: 346–354.

Byrne, M., Phelps, H., Church, T., Adair, V., Selvakumaraswamy, P., and Potts, J. (2000) Reproduction and development of the freshwater clam *Corbicula australis* in southeast Australia. *Hydrobiologia* 418: 185–197.

Cadel-Six, S., Moyenga, D., Magny, S., Trotereau, S., Edery, M., and Krys, S. (2014) Detection of free and covalently bound microcystins in different tissues (liver, intestines, gills, and muscles) of rainbow trout (*Oncorhynchus mykiss*) by liquid chromatography–tandem mass spectrometry: Method characterization. *Environ. Pollut.* 185: 333–339.

Campos, A., and Vasconcelos, V. (2010) Molecular mechanisms of microcystin toxicity in animal cells. *Int. J. Mol. Sci.* 11: 268–287.

Campos, B., Altenburger, R., Gómez, C., Lacorte, S., Piña, B., Barata, C., and Luckenbach, T. (2014) First evidence for toxic defense based on the multixenobiotic resistance (MXR) mechanism in *Daphnia magna*. *Aquat. Toxicol.* 148: 139–151.

Campos, V., Cantarero, S., Urrutia, H., Heinze, R., Wirsing, B., Neumann, U., and Weckesser, J. (1999) Microcystin in cyanobacterial blooms in a Chilean Lake. *Syst. Appl. Microbiol.* 22: 169–173.

Carmichael, W.W., Azevedo, S.M., An, J.S., Molica, R.J., Jochimsen, E.M., Lau, S., Rinehart, K.L., Shaw, G.R., and Eaglesham, G.K. (2001) Human fatalities from cyanobacteria: Chemical and biological evidence for cyanotoxins. *Environ. Health Perspect.* 109: 663–668.

Catherine, Q., Susanna, W., Isidora, E.-S., Mark, H., Aurélie, V., and Jean-François, H. (2013) A review of current knowledge on toxic benthic freshwater cyanobacteria – ecology, toxin production and risk management. *Water Res.* 47: 5464–5479.

Cazenave, J., Bistoni, M.d.l.A., Pesce, S.F., and Wunderlin, D.A. (2006) Differential detoxification and antioxidant response in diverse organs of *Corydoras paleatus* experimentally exposed to microcystin-RR. *Aquat. Toxicol.* 76: 1–12.

Cazenave, J., Nores, M.L., Miceli, M., Díaz, M.P., Wunderlin, D.A., and Bistoni, M.A. (2008) Changes in the swimming activity and the glutathione S-transferase activity of *Jenynsia multidentata* fed with microcystin-RR. *Water Res.* 42: 1299–1307.

Chen, J., Xie, P., Guo, L., Zheng, L., and Ni, L. (2005) Tissue distributions and seasonal dynamics of the hepatotoxic microcystins-LR and -RR in a freshwater snail (*Bellamya aeruginosa*) from a large shallow, eutrophic lake of the subtropical China. *Environ. Pollut.* 134: 423–430.

Chen, J., and Xie, P. (2005a) Seasonal dynamics of the hepatotoxic microcystins in various organs of four freshwater bivalves from the large eutrophic lake Taihu of subtropical China and the risk to human consumption. *Environ. Toxicol.* 20: 572–584.

Chen, J., and Xie, P. (2005b) Tissue distributions and seasonal dynamics of the hepatotoxic microcystins-LR and -RR in two freshwater shrimps, *Palaemon modestus* and *Macrobrachium nipponensis*, from a large shallow, eutrophic lake of the subtropical China. *Toxicon* 45: 615–625.

Chen, J., and Xie, P. (2007) Microcystin accumulation in freshwater bivalves from Lake Taihu, China, and the potential risk to human consumption. *Environ. Toxicol. Chem.* 26: 1066–1073.

Chen, J., Zhang, D., Xie, P., Wang, Q., and Ma, Z. (2009) Simultaneous determination of microcystin contaminations in various vertebrates (fish, turtle, duck and water bird) from a large eutrophic Chinese lake, Lake Taihu, with toxic *Microcystis* blooms. *Sci. Total Environ.* 407: 3317–3322.

Chen, Y., Zeng, S.-F., and Cao, Y.-F. (2012) Oxidative stress response in zebrafish (*Danio rerio*) gill experimentally exposed to subchronic microcystin-LR. *Environ. Monit. Assess*. 184: 6775–6787.

Chernoff, N., Hunter, E.S., Hall, L.L., Rosen, M.B., Brownie, C.F., Malarkey, D., Marr, M., and Herkovits, J. (2002) Lack of teratogenicity of microcystin-LR in the mouse and toad. *J. Appl. Toxicol.* 22: 13–17.

Cheung, M., Liang, S., and Lee, J. (2013) Toxin-producing cyanobacteria in freshwater: A review of the problems, impact on drinking water safety, and efforts for protecting public health. *J. Microbiol.* 51: 1–10.

Chorus, I., and Bartram, J. (1999) Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. Published on behalf of WHO, Spon Press, London, 416 pp.

Christiansen, G., Fastner, J., Erhard, M., Borner, T., and Dittmann, E. (2003) Microcystin biosynthesis in *Planktothrix*: Genes, evolution, and manipulation. *J. Bacteriol.* 185: 564–572.

Conradie, K. R., and Barnard, S. (2012) The dynamics of toxic *Microcystis* strains and microcystin production in two hypertrofic South African reservoirs. *Harmful Algae* 20: 1–10.

Contardo-Jara, V., Pflugmacher, S., and Wiegand, C. (2008) Multi-xenobiotic-resistance a possible explanation for the insensitivity of bivalves towards cyanobacterial toxins. *Toxicon* 52: 936–943.

Contardo-Jara, V., Klingelmann, E., and Wiegand, C. (2009) Bioaccumulation of glyphosate and its formulation Roundup Ultra in *Lumbriculus variegatus* and its effects on biotransformation and antioxidant enzymes. *Environ. Pollut.* 157: 57–63.

Cornwall, R., Toomey, B.H., Bard, S., Bacon, C., Jarman, W.M., and Epel, D. (1995) Characterization of multixenobiotic/multidrug transport in the gills of the mussel *Mytilus californianus* and identification of environmental substrates. *Aquat. Toxicol.* 31: 277–296.

Cossu, C., Doyotte, A., Jacquin, M.C., Babut, M., Exinger, A., and Vasseur, P. (1997) Glutathione reductase, selenium-dependent glutathione peroxidase, glutathione levels, and lipid peroxidation in freshwater bivalves,*Unio tumidus*, as biomarkers of aquatic contamination in field studies. *Ecotox. Environ. Saf.* 38: 122–131.

Coughlan, J. (1969) The estimation of filtering rate from the clearance of suspensions. *Marine Biol.* 2: 356–358.

Cronberg, G., and Komárek, J. (2004) Some nostocalean cyanoprokaryotes from lentic habitats of Eastern and Southern Africa. *Nova Hedwigia* 78: 71–106.

Cronberg, G., and Annadotter, H. (2006) Manual on aquatic cyanobacteria: A photo guide and a synopsis of their toxicology. *Kerteminde Tryk A/S,* 106 pp.

Cuvin-Aralar, M.L., Fastner, J., Focken, U., Becker, K., and Aralar, E.V. (2002) Microcystins in natural blooms and laboratory cultured *Microcystis aeruginosa* from Laguna de Bay, Philippines. *Syst. Appl. Microbiol.* 25: 179–182.

Dai, R., Liu, H., Qu, J., Ru, J., and Hou, Y. (2008) Cyanobacteria and their toxins in Guanting Reservoir of Beijing, China. *J. Hazard. Mater.* 153: 470–477.

Dao, T.S., Cronberg, G., Nimptsch, J., Do-Hong, L.-C., and Wiegand, C. (2010) Toxic cyanobacteria from Tri An Reservoir, Vietnam. *Nova Hedwigia* 90: 433–448.

Dao, T.-S., Ortiz-Rodríguez, R., Do-Hong, L.-C., and Wiegand, C. (2013) Non-microcystin and non-cylindrospermopsin producing cyanobacteria affect the biochemical responses and behavior of *Daphnia magna*. *Int. Rev. Hydrobiol*. 98: 235–244.

Davis, T.W., Berry, D.L., Boyer, G.L., and Gobler, C.J. (2009) The effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms. *Harmful Algae* 8: 715–725.

Dawson, R.M. (1998) The toxicology of microcystins. *Toxicon* 36: 953–962.

Dias, E., Andrade, M., Alverca, E., Pereira, P., Batoreu, M.C., Jordan, P., and Silva, M.J. (2009) Comparative study of the cytotoxic effect of microcistin-LR and purified extracts from *Microcystis aeruginosa* on a kidney cell line. *Toxicon* 53: 487–495.

Ding, J., Shi, H., Timmons, T., and Adams, C. (2010) Release and removal of microcystins from *Microcystis* during oxidative-, physical-, and UV-based disinfection. *J. Environ. Eng.* 136: 2–11.

Dittmann, E., Erhard, M., Kaebernick, M., Scheler, C., Neilan, B.A., von Dohren, H., and Borner, T. (2001) Altered expression of two light-dependent genes in a microcystin-lacking mutant of *Microcystis aeruginosa* PCC 7806. *Microbiology* 147: 3113–3119.

Downing, J.A., Watson, S.B., and McCauley, E. (2001) Predicting cyanobacteria dominance in lakes. *Can. J. Fish Aquat. Sci.* 58: 1905–1908.

Duong, D.T. (1996) Taxonomy of cyanobacteria of Vietnam. Agricultural publishing house, Ha Noi, 220 pp (In Vietnamese).

Duong, T., Jähnichen, S., Le, T., Ho, C., Hoang, T., Nguyen, T., Vu, T., and Dang, D. (2014) The occurrence of cyanobacteria and microcystins in the Hoan Kiem Lake and the Nui Coc reservoir (North Vietnam). *Environ. Earth Sci.* 71: 2419–2427.

Duy, T., Lam, P.S., Shaw, G., and Connell, D. (2000) Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water. *Rev. Environ. Contam. T.* 163: 113–185.

Dyble, J., Gossiaux, D., Landrum, P., Kashian, D.R., and Pothoven, S. (2011) A kinetic study of accumulation and elimination of microcystin-LR in yellow perch (*Perca flavescens*) tissue and implications for human fish consumption. *Marine Drugs* 9: 2553–2571.

El-Shehawy, R., Gorokhova, E., Fernández-Piñas, F., and del Campo, F.F. (2012) Global warming and hepatotoxin production by cyanobacteria: What can we learn from experiments? *Water Res.* 46: 1420–1429.

El-Herry, S., Fathalli, A., Rejeb, A.J.-B., and Bouaïcha, N. (2008) Seasonal occurrence and toxicity of *Microcystis* spp. and *Oscillatoria tenuis* in the Lebna Dam, Tunisia. *Water Res.* 42: 1263–1273.

Eriksson, J., Meriluoto, J., and Lindholm, T. (1989) Accumulation of a peptide toxin from the cyanobacterium *Oscillatoria agardhii* in the freshwater mussel *Anadonta cygnea*. *Hydrobiologia* 183: 211–216.

Eriksson, J.E., Toivola, D., Meriluoto, J.A.O., Karaki, H., Han, Y.G., and Hartshorne, D. (1990) Hepatocyte deformation induced by cyanobacterial toxins reflects inhibition of protein phosphatases. *Biochem. Biophys. Res. Commun.* 173: 1347–1353.

Eufemia, N.A., and Epel, D. (2000) Induction of the multixenobiotic defense mechanism (MXR), P-glycoprotein, in the mussel *Mytilus californianus* as a general cellular response to environmental stresses. *Aquat. Toxicol.* 49: 89–100.

Falconer, I.R., and Humpage, A.R. (2005) Health risk assessment of cyanobacterial (blue-green algal) toxins in drinking water. *Int. J. Environ. Res. Publ. Health*. 2: 43–50.

Falconer, I.R. (2007) Cyanobacterial toxins present in *Microcystis aeruginosa* extracts – more than microcystins. *Toxicon* 50: 585–588.

Faria, M., Navarro, A., Luckenbach, T., Piña, B., and Barata, C. (2011) Characterization of the multixenobiotic resistance (MXR) mechanism in embryos and larvae of the zebra mussel (*Dreissena polymorpha*) and studies on its role in tolerance to single and mixture combinations of toxicants. *Aquat. Toxicol.* 101: 78–87.

Fernandes, S., Welker, M., and Vasconcelos, V.M. (2009) Changes in the GST activity of the mussel *Mytilus galloprovincialis* during exposure and depuration of microcystins. *J. Exp. Zool. Part A.* 311A: 226–230.

Ferrão-Filho, A.D.S., Kozlowsky-Suzuki, B., and Azevedo, S.M.F.O. (2002) Accumulation of microcystins by a tropical zooplankton community. *Aquat. Toxicol.* 59: 201–208.

Ferrão-Filho, A.D.S., and Kozlowsky-Suzuki, B. (2011) Cyanotoxins: Bioaccumulation and effects on aquatic animals. *Marine Drugs* 9: 2729–2772.

Figueiredo, D.R., Azeiteiro, U.M., Esteves, S.M., Goncalves, F.J., and Pereira, M.J. (2004) Microcystin-producing blooms – A serious global public health issue. *Ecotox. Environ. Saf.* 59: 151–163.

Fischer, W.J., and Dietrich, D.R. (2000) Toxicity of the cyanobacterial cyclic heptapeptide toxins microcystin-LR and -RR in early life-stages of the African clawed frog (*Xenopus laevis*). *Aquat. Toxicol.* 49: 189–198.

Fischer, W.J., Altheimer, S., Cattori, V., Meier, P.J., Dietrich, D.R., and Hagenbuch, B. (2005) Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. *Toxicol. Appl. Pharmacol.* 203: 257–263.

Frank, C.A. (2002) Microcystin-producing cyanobacteria in recreational waters in southwestern Germany. *Environ. Toxicol.* 17: 361–366.

Funari, E., and Testai, E. (2008) Human health risk assessment related to cyanotoxins exposure. *Crit. Rev. Toxicol.* 38: 97–125.

Galanti, L.N., Amé, M.V., and Wunderlin, D.A. (2013) Accumulation and detoxification dynamic of cyanotoxins in the freshwater shrimp *Palaemonetes argentinus*. *Harmful Algae* 27: 88–97.

Gavrilović, B.R., Despotović, S.G., Gavrić, J.P., Borković-Mitić, S.S., Ognjanović, B.I., Pavlović, S.Z., and Saičić, Z.S. (2014) Changes in antioxidant enzyme activities in the livers and gills of three cyprinids after exposure to a cyanobacterial bloom in the Gruža Reservoir, Serbia. *Ecol. Indic.* 38: 141–148.

Gazulha, V., Mansur, M.C., Cybis, L.F., and Azevedo, S.M. (2012) Feeding behavior of the invasive bivalve *Limnoperna fortunei* (Dunker, 1857) under exposure to toxic cyanobacteria *Microcystis aeruginosa*. *Braz. J. Biol.* 72: 41–49.

Gélinas, M., Juneau, P., and Gagné, F. (2012) Early biochemical effects of *Microcystis aeruginosa* extracts on juvenile rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* 161: 261–267.

Gérard, C., Poullain, V., Lance, E., Acou, A., Brient, L., and Carpentier, A. (2009) Influence of toxic cyanobacteria on community structure and microcystin accumulation of freshwater molluscs. *Environ. Pollut.* 157: 609–617.

Gkelis, S., Lanaras, T., and Sivonen, K. (2006) The presence of microcystins and other cyanobacterial bioactive peptides in aquatic fauna collected from Greek freshwaters. *Aquat. Toxicol.* 78: 32–41.

Gkelis, S., and Zaoutsos, N. (2014) Cyanotoxin occurrence and potentially toxin producing cyanobacteria in freshwaters of Greece: A multi-disciplinary approach. *Toxicon* 78: 1–9.

Gonçalves-Soares, D., Zanette, J., Yunes, J.S., Yepiz-Plascencia, G.M., and Bainy, A.C.D. (2012) Expression and activity of glutathione S-transferases and catalase in the shrimp *Litopenaeus vannamei* inoculated with a toxic *Microcystis aeruginosa* strain. *Mar. Environ. Res.* 75: 54–61.

Grützmacher, G., Wessel, G., Klitzke, S., and Chorus, I. (2009) Microcystin elimination during sediment contact. *Environ. Sci. Technol.* 44: 657–662.

Haande, S., Ballot, A., Rohrlack, T., Fastner, J., Wiedner, C., and Edvardsen, B. (2007) Diversity of *Microcystis aeruginosa* isolates (Chroococcales, cyanobacteria) from East-African water bodies. *Arch. Microbiol.* 188: 15–25.

Hao, L., Xie, P., Fu, J., Li, G., Xiong, Q., and Li, H. (2008) The effect of cyanobacterial crude extract on the transcription of GST mu, GST kappa and GST rho in different organs of goldfish (*Carassius auratus*). *Aquat. Toxicol.* 90: 1–7.

Harada, K.-I., Murata, H., Qiang, Z., Suzuki, M., and Kondo, F. (1996) Mass spectrometric screening method for microcystins in cyanobacteria. *Toxicon* 34: 701–710.

Harada, K.-I., and Tsuji, K. (1998) Persistence and decomposition of hepatotoxic microcystins produced by cyanobacteria in natural environment. *Toxin Reviews* 17: 385–403.

Havens, K. (2008) Cyanobacteria blooms: Effects on aquatic ecosystems. In Hudnell, H.K. (ed.), Cyanobacterial harmful algal blooms: State of the science and research needs. Springer New York, 619: 733–747.

Heisler, J., Glibert, P.M., Burkholder, J.M., Anderson, D.M., Cochlan, W., Dennison, W.C., Dortch, Q., Gobler, C.J., Heil, C.A., Humphries, E., Lewitus, A., Magnien, R., Marshall, H.G., Sellner, K., Stockwell, D.A., Stoecker, D.K., and Suddleson, M. (2008)

Eutrophication and harmful algal blooms: A scientific consensus. *Harmful Algae* 8: 3–13.

Hisbergues, M., Christiansen, G., Rouhiainen, L., Sivonen, K., and Börner, T. (2003) PCR-based identification of microcystin-producing genotypes of different cyanobacterial genera. *Arch. Microbiol.* 180: 402–410.

Hoek, C. V. D., Mann, D.G., and Jahns, H.M. (1995) Algae: An introduction to phycology. Cambridge University Press, Cambridge, 623 pp.

Hotto, A., Satchwell, M., and Boyer, G. (2005) Seasonal production and molecular characterization of microcystins in Oneida Lake, New York, USA. *Environ. Toxicol.* 20: 243–248.

Hummert, C., Dahlmann, J., Reinhardt, K., Dang, H., Dang, D., and Luckas, B. (2001a) Liquid chromatography-mass spectrometry identification of microcystins in *Microcystis aeruginosa* strain from lake Thanh Cong, Hanoi, Vietnam. *Chromatographia* 54: 569–575.

Hummert, C., Reichelt, M., Weiß, J., Liebert, H.-P., and Luckas, B. (2001b) Identification of microcystins in cyanobacteria from the Bleiloch former drinking-water reservoir (Thuringia, Germany). *Chemosphere* 44: 1581–1588.

Hwang, S.J., Kim, H.S., Park, J.H., and Kim, B.H. (2010) Effects of cyanobacterium *Microcystis aeruginosa* on the filtration rate and mortality of the freshwater bivalve *Corbicula leana*. *J. Environ. Biol.* 31: 483–488.

Ibelings, B.W., Bruning, K., de Jonge, J., Wolfstein, K., Pires, L.M.D., Postma, J., and Burger, T. (2005) Distribution of microcystins in a lake foodweb: No evidence for biomagnification. *Microb. Ecol.* 49: 487–500.

Ibelings, B.W., and Chorus, I. (2007) Accumulation of cyanobacterial toxins in freshwater "seafood" and its consequences for public health: A review. *Environ. Pollut.* 150: 177–192.

Ichimura, T. (1979) Methods for isolating and culturing freshwater algae. In: Nishizawa K, Chihara M. (eds), Research methods of algae, Kyoritsu, Tokyo. 294–305.

Issam, E.G., Saqrane, S., Carvalho, A.P., Ouahid, Y., Del Campo, F.F., Oudra, B., and Vasconcelos, V. (2010) Effect of different microcystin profiles on toxin bioaccumulation in common carp (*Cyprinus carpio*) larvae via *Artemia nauplii*. *Ecotox. Environ. Saf.* 73: 762–770.

Iwasa, M., Yamamoto, M., Tanaka, Y., Kaito, M., and Adachi, Y. (2002) Spirulina-associated hepatotoxicity. *Am. J. Gastroenterol.* 97: 3212–3213.

Janse, I., Kardinaal, W.E., Meima, M., Fastner, J., Visser, P.M., and Zwart, G. (2004) Toxic and non-toxic *Microcystis* colonies in natural populations can be differentiated on the basis of rRNA gene internal transcribed spacer diversity. *Appl. Environ. Microbiol.* 70: 3979–3987.

Jiang, J., Shi, Y., Shan, Z., Yang, L., Wang, X., and Shi, L. (2012) Bioaccumulation, oxidative stress and HSP70 expression in *Cyprinus carpio* L. exposed to microcystin-LR under laboratory conditions. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* 155: 483–490.

Jos, Á., Pichardo, S., Prieto, A.I., Repetto, G., Vázquez, C.M., Moreno, I., and Cameán, A.M. (2005) Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis* sp.) under laboratory conditions. *Aquat. Toxicol.* 72: 261–271.

Joung, S.-H., Oh, H.-M., Ko, S.-R., and Ahn, C.-Y. (2011) Correlations between environmental factors and toxic and non-toxic *Microcystis* dynamics during bloom in Daechung Reservoir, Korea. *Harmful Algae* 10: 188–193.

Juhel, G., Davenport, J., O'Halloran, J., Culloty, S., Ramsay, R., James, K., Furey, A., and Allis, O. (2006a) Pseudodiarrhoea in zebra mussels *Dreissena polymorpha* (Pallas) exposed to microcystins. *J. Exp. Mar. Biol.* 209: 810–816.

Juhel, G., Davenport, J., O'Halloran, J., Culloty, S.C., O'Riordan, R.M., James, K.F., Furey, A., and Allis, O. (2006b) Impacts of microcystins on the feeding behaviour and energy balance of zebra mussels, *Dreissena polymorpha*: A bioenergetics approach. *Aquat. Toxicol.* 79: 391–400.

Kaebernick, M., Neilan, B.A., Borner, T., and Dittmann, E. (2000) Light and the transcriptional response of the microcystin biosynthesis gene cluster. *Appl. Environ. Microbiol.* 66: 3387–3392.

Kankaanpää, H., Leiniö, S., Olin, M., Sjövall, O., Meriluoto, J., and Lehtonen, K.K. (2007) Accumulation and depuration of cyanobacterial toxin nodularin and biomarker responses in the mussel *Mytilus edulis*. *Chemosphere* 68: 1210–1217.

Kemp, A., and John, J. (2006) Microcystins associated with *Microcystis* dominated blooms in the southwest wetlands, western Australia. *Environ. Toxicol.* 21: 125–130.

Kist, L.W., Rosemberg, D.B., Pereira, T.C., de Azevedo, M.B., Richetti, S.K., de Castro Leao, J., Yunes, J.S., Bonan, C.D., and Bogo, M.R. (2012) Microcystin-LR acute exposure increases AChE activity via transcriptional ache activation in zebrafish (*Danio rerio*) brain. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 155: 247–252.

Knoll, A.H. (2008) Cyanobacteria and earth history. In Herrero A, F.E. (ed.), The cyanobacteria: Molecular biology, genomics and evolution. Caister Academic, Norfolk, UK, 484 pp.

Komárek, J., and Anagnostidis, K. (1989) Modern approach to the classification system of Cyanophytes. 4 - Nostocales. *Arch. Hydrobiol. Suppl.* 82: 247–345.

Komárek, J., and Anagnostidis, K. (1999) Cyanoprokaryota 1. Teil: Chroococcales. 548 pp.

Komárek, J., and Anagnostidis, K. (2005) Cyanoprokaryota 1. Teil: Oscillatoriales. 759 pp.

Komárek, J., and Zapomelova, E. (2007) Planktic morphospecies of the cyanobacterial genus *Anabaena* = subg. *Dolichospermum* – 1. part: Coiled types. *Fottea* 7: 1–31.

Kondo, F., Ikai, Y., Oka, H., Okumura, M., Ishikawa, N., Harada, K., Matsuura, K., Murata, H., and Suzuki, M. (1992) Formation, characterization, and toxicity of the glutathione and cysteine conjugates of toxic heptapeptide microcystins. *Chem. Res. Toxicol.* 5: 591–596.

Kong, W.-B., Hua, S.-F., Cao, H., Mu, Y.-W., Yang, H., Song, H., and Xia, C.-G. (2012) Optimization of mixotrophic medium components for biomass production and biochemical composition biosynthesis by *Chlorella vulgaris* using response surface methodology. *J. Taiwan Inst. Chem. E.* 43: 360–367.

Kono, Y., and Fridovich, I. (1982) Superoxide radical inhibits catalase. *J. Biol. Chem.* 257: 5751–5754.

Kosten, S., Huszar, V.L.M., Bécares, E., Costa, L.S., van Donk, E., Hansson, L.-A., Jeppesen, E., Kruk, C., Lacerot, G., Mazzeo, N., De Meester, L., Moss, B., Lürling, M., Nõges, T., Romo, S., and Scheffer, M. (2012) Warmer climates boost cyanobacterial dominance in shallow lakes. *Glob. Change Biol.* 18: 118–126.

Krüger, T., Wiegand, C., Kun, L., Luckas, B., and Pflugmacher, S. (2010) More and more toxins around–analysis of cyanobacterial strains isolated from Lake Chao (Anhui Province, China). *Toxicon* 56: 1520–1524.

Kumar, A., Kumar, A., Rai, A.K., and Tyagi, M.B. (2011) PCR-based detection of *mcy* genes in blooms of *Microcystis* and extracellular DNA of pond water. *Afr. J. Microbiol. Res.* 5: 374–381.

Kurmayer, R., and Kutzenberger, T. (2003) Application of real-time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium *Microcystis* sp. *Appl. Environ. Microbiol.* 69: 6723–6730.

Kurmayer, R., and Christiansen, G. (2009) The genetic basis of toxin production in cyanobacteria. *Freshwater Reviews* 2: 31–50.

Lance, E., Neffling, M.-R., Gérard, C., Meriluoto, J., and Bormans, M. (2010) Accumulation of free and covalently bound microcystins in tissues of *Lymnaea stagnalis* (Gastropoda) following toxic cyanobacteria or dissolved microcystin-LR exposure. *Environ. Pollut.* 158: 674–680.

Lance, E., Petit, A., Sanchez, W., Paty, C., Gérard, C., and Bormans, M. (2014) Evidence of trophic transfer of microcystins from the gastropod *Lymnaea stagnalis* to the fish *Gasterosteus aculeatus*. *Harmful Algae* 31: 9–17.

Leão, J.C., Geracitano, L.A., Monserrat, J.M., Amado, L.L., and Yunes, J.S. (2008) Microcystin-induced oxidative stress in *Laeonereis acuta* (Polychaeta, Nereididae). *Mar. Environ. Res.* 66: 92–94.

Lee, T.-H., Chen, Y.-M., and Chou, H.-N. (1998) First report of microcystins in Taiwan. *Toxicon* 36: 247–255.

Li, G., Xie, P., Fu, J., Hao, L., Xiong, Q., and Li, H. (2008) Microcystin-induced variations in transcription of GSTs in an omnivorous freshwater fish, goldfish. *Aquat. Toxicol.* 88: 75–80.

Li, L., Xie, P., and Guo, L. (2010) Antioxidant response in liver of the phytoplanktivorous bighead carp (*Aristichthys nobilis*) intraperitoneally-injected with extracted microcystins. *Fish Physiol. Biochem.* 36: 165–172.

Li, W., Chen, J., Xie, P., He, J., Guo, X., Tuo, X., Zhang, W., and Wu, L. (2014) Rapid conversion and reversible conjugation of glutathione detoxification of microcystins in bighead carp (*Aristichthys nobilis*). *Aquat. Toxicol.* 147: 18–25.

Li, X., Liu, Y., Song, L., and Liu, J. (2003) Responses of antioxidant systems in the hepatocytes of common carp (*Cyprinus carpio* L.) to the toxicity of microcystin-LR. *Toxicon* 42: 85–89.

Li, X.-Y., Chung, I.-K., Kim, J.-I., and Lee, J.-A. (2005) Oral exposure to *Microcystis* increases activity-augmented antioxidant enzymes in the liver of loach (*Misgurnus*

mizolepis) and has no effect on lipid peroxidation. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* 141: 292–296.

Liu, Y., Xie, P., and Wu, X.-P. (2009) Grazing on toxic and non-toxic *Microcystis aeruginosa* PCC7820 by *Unio douglasiae* and *Corbicula fluminea*. *Limnology* 10: 1–5.

Lushchak, V.I. (2011) Environmentally induced oxidative stress in aquatic animals. *Aquat. Toxicol.* 101: 13–30.

Ma, J., Brookes, J.D., Qin, B., Paerl, H.W., Gao, G., Wu, P., Zhang, W., Deng, J., Zhu, G., Zhang, Y., Xu, H., and Niu, H. (2014) Environmental factors controlling colony formation in blooms of the cyanobacteria *Microcystis* spp. in Lake Taihu, China. *Harmful Algae* 31: 136–142.

Ma, M., Liu, R., Liu, H., and Qu, J. (2012) Chlorination of *Microcystis aeruginosa* suspension: Cell lysis, toxin release and degradation. *J. Hazard. Mater.* 217–218: 279–285.

MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P., and Codd, G.A. (1990) Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Letters* 264: 187–192.

MacKintosh, R.W., Dalby, K.N., Campbell, D.G., Cohen, P.T.W., Cohen, P., and MacKintosh, C. (1995) The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1. *FEBS Letters* 371: 236–240.

Malbrouck, C., Trausch, G., Devos, P., and Kestemont, P. (2003) Hepatic accumulation and effects of microcystin-LR on juvenile goldfish *Carassius auratus* L. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 135: 39–48.

Malbrouck, C., and Kestemont, P. (2006) Effects of microcystins on fish. *Environ. Toxicol. Chem.* 25: 72–86.

Mankiewicz, J., Komárková, J., Izydorczyk, K., Jurczak, T., Tarczynska, M., and Zalewski, M. (2005) Hepatotoxic cyanobacterial blooms in the lakes of northern Poland. *Environ. Toxicol.* 20: 499–506.

Martins, J., Saker, M., Moreira, C., Welker, M., Fastner, J., and Vasconcelos, V. (2009) Peptide diversity in strains of the cyanobacterium *Microcystis aeruginosa* isolated from Portuguese water supplies. *Appl. Microbiol. Biotechnol.* 82: 951–961.

Martins, J.C., and Vasconcelos, V.M. (2009) Microcystin dynamics in aquatic organisms. *J. Toxicol. Environ. Health Part B, Critical Reviews* 12: 65–82.

Merel, S., Walker, D., Chicana, R., Snyder, S., Baurès, E., and Thomas, O. (2013) State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. *Environ. Int.* 59: 303–327.

Mitrovic, S.M., Hardwick, L., and Dorani, F. (2011) Use of flow management to mitigate cyanobacterial blooms in the lower Darling River, Australia. *J. Plankton Res.* 33: 229–241.

Msagati, T.A.M., Siame, B.A., and Shushu, D.D. (2006) Evaluation of methods for the isolation, detection and quantification of cyanobacterial hepatotoxins. *Aquat. Toxicol.* 78: 382–397.

Mulvenna, V., Dale, K., Priestly, B., Mueller, U., Humpage, A., Shaw, G., Allinson, G., and Falconer, I. (2012) Health risk assessment for cyanobacterial toxins in seafood. *Int. J. Environ. Res. Publ. Health* 9: 807–820.

Naddafi, R., Pettersson, K., and Eklöv, P. (2007) The effect of seasonal variation in selective feeding by zebra mussels (*Dreissena polymorpha*) on phytoplankton community composition. *Freshwater Biol.* 52: 823–842.

Nasri, H., El-Herry, S., and Bouaïcha, N. (2008) First reported case of turtle deaths during a toxic *Microcystis* spp. bloom in Lake Oubeira, Algeria. *Ecotox. Environ. Saf.* 71: 535–544.

Neffling, M.R., Lance, E., and Meriluoto, J. (2010) Detection of free and covalently bound microcystins in animal tissues by liquid chromatography-tandem mass spectrometry. *Environ. Pollut.* 158: 948–952.

Neilan, B.A., Pearson, L.A., Moffitt, M.C., Mihali, K.T., Kaebernick, M., Kellmann, R., and Pomati, F. (2008) The genetics and genomics of cyanobacterial toxicity. *Adv. Exp. Med. Biol.* 61: 417–452.

Neumann, U., Campos, V., Cantarero, S., Urrutia, H., Heinze, R., Weckesser, J., and Erhard, M. (2000) Co-occurrence of non-toxic (cyanopeptolin) and toxic (microcystin) peptides in a bloom of *Microcystis* sp. from a Chilean Lake. *Syst. Appl. Microbiol.* 23: 191–197.

Nguyen, L.A.V., Tanabe, Y., Matsuura, H., Kaya, K., and Watanabe, M.M. (2012) Morphological, biochemical and phylogenetic assessments of water-bloom-forming tropical morphospecies of *Microcystis* (Chroococcales, cyanobacteria). *Phycol. Res.* 60: 208–222.

Nguyen, T.T.L., Cronberg, G., Annadotter, H., and Larsen, J. (2007a) Planktic cyanobacteria from freshwater localities in Thuathien-Hue province, Vietnam II. Algal biomass and microcystin production. *Nova Hedwigia* 85: 35–49.

Nguyen, T.T.L., Cronberg, G., Larsen, J., and Moestrup, O. (2007b) Planktic cyanobacteria from freshwater localities in Thuathien-Hue province, Vietnam I. Morphology and distribution. *Nova Hedwigia* 85: 1–34.

Nguyen, T.T.L., Nguyen, T.C., and Le, T.T.N. (2010) Morphology and cylindrospermopsin - producing ability of cyanobacteria strains isolated from some lakes and ponds in vietnam. *Vietnam J. Biotech.* 8: 103–108.

Nishizawa, T., Asayama, M., Fujii, K., Harada, K., and Shirai, M. (1999) Genetic analysis of the peptide synthetase genes for a cyclic heptapeptide microcystin in *Microcystis* spp. *J. Biochem.* 126: 520–529.

Nishizawa, T., Ueda, A., Asayama, M., Fujii, K., Harada, K., Ochi, K., and Shirai, M. (2000) Polyketide synthase gene coupled to the peptide synthetase module involved in the biosynthesis of the cyclic heptapeptide microcystin. *J. Biochem.* 127: 779–789.

Nyakairu, G.W.A., Nagawa, C.B., and Mbabazi, J. (2010) Assessment of cyanobacteria toxins in freshwater fish: A case study of Murchison Bay (Lake Victoria) and Lake Mburo, Uganda. *Toxicon* 55: 939–946.

O'Neil, J.M., Davis, T.W., Burford, M.A., and Gobler, C.J. (2012) The rise of harmful cyanobacteria blooms: The potential roles of eutrophication and climate change. *Harmful Algae* 14: 313–334.

Okello, W., Portmann, C., Erhard, M., Gademann, K., and Kurmayer, R. (2010) Occurrence of microcystin-producing cyanobacteria in Ugandan freshwater habitats. *Environ. Toxicol.* 25: 367–380.

Oudra, B., Loudiki, M., Vasconcelos, V., Sabour, B., Sbiyyaa, B., Oufdou, K., and Mezrioul, N. (2002) Detection and quantification of microcystins from cyanobacteria strains isolated from reservoirs and ponds in Morocco. *Environ. Toxicol.* 17: 32–39.

Ozawa, K., Yokoyama, A., Ishikawa, K., Kumagai, M., Watanabe, M.F., and Park, H.-D. (2003) Accumulation and depuration of microcystin produced by the cyanobacterium *Microcystis* in a freshwater snail. *Limnology* 4: 131–138.

Ozawa, K., Fujioka, H., Muranaka, M., Yokoyama, A., Katagami, Y., Homma, T., Ishikawa, K., Tsujimura, S., Kumagai, M., Watanabe, M.F., and Park, H.-D. (2005) Spatial distribution and temporal variation of *Microcystis* species composition and microcystin concentration in Lake Biwa. *Environ. Toxicol.* 20: 270–276.

Paerl, H.W., Fulton, R.S., Moisander, P.H., and Dyble, J. (2001) Harmful freshwater algal blooms, with an emphasis on cyanobacteria. *Scientific World Journal* 1: 76–113.

Paerl, H.W., and Huisman, J. (2009) Climate change: A catalyst for global expansion of harmful cyanobacterial blooms. *Environ. Microbiol. Rep.* 1: 27–37.

Paerl, H.W., and Paul, V.J. (2012) Climate change: Links to global expansion of harmful cyanobacteria. *Water Res*. 46: 1349–1363.

Palíková, M., Krejčí, R., Hilscherová, K., Babica, P., Navrátil, S., Kopp, R., and Bláha, L. (2007) Effect of different cyanobacterial biomasses and their fractions with variable microcystin content on embryonal development of carp (*Cyprinus carpio* L.). *Aquat. Toxicol.* 81: 312–318.

Park, H.D., Watanabe, M.F., Harada, K.-I., Suzuki, M., Hayashi, H., and Okino, T. (1993a) Seasonal variations of *Microcystis* species and toxic heptapeptide microcystins in Lake Suwa. *Environ. Toxicol. Water Qual.* 8: 425–435.

Park, H.D., Watanabe, M.F., Harda, K., Nagai, H., Suzuki, M., Watanabe, M., and Hayashi, H. (1993b) Hepatotoxin (microcystin) and neurotoxin (anatoxin-a) contained in natural blooms and strains of cyanobacteria from Japanese freshwaters. *Nat. Toxins* 1: 353–360.

Park, H.D., Iwami, C., Watanabe, M.F., Harada, K.-I., Okino, T., and Hayashi, H. (1998a) Temporal variabilities of the concentrations of intra- and extracellular microcystin and toxic *Microcystis* species in a hypertrophic lake, Lake Suwa, Japan (1991–1994). *Environ. Toxicol.Water Qual.* 13: 61–72.

Park, H.D., Kim, B., Kim, E., and Okino, T. (1998b) Hepatotoxic microcystins and neurotoxic anatoxin-a in cyanobacterial blooms from Korean lakes. *Environ. Toxicol. Water Qual.* 13: 225–234.

Paskerová, H., Hilscherová, K., and Bláha, L. (2012) Oxidative stress and detoxification biomarker responses in aquatic freshwater vertebrates exposed to microcystins and cyanobacterial biomass. *Environ. Sci. Pollut. Res.* 19: 2024–2037.

Pašková, V., Adamovský, O., Pikula, J., Skočovská, B., Band'ouchová, H., Horáková, J., Babica, P., Maršálek, B., and Hilscherová, K. (2008) Detoxification and oxidative stress responses along with microcystins accumulation in Japanese quail exposed to cyanobacterial biomass. *Sci. Total Environ.* 398: 34–47.

Pavagadhi, S., Gong, Z., Hande, M.P., Dionysiou, D.D., de la Cruz, A.A., and Balasubramanian, R. (2012) Biochemical response of diverse organs in adult *Danio rerio* (zebrafish) exposed to sub-lethal concentrations of microcystin-LR and microcystin-RR: A balneation study. *Aquat. Toxicol.* 109: 1–10.

Pavagadhi, S., and Balasubramanian, R. (2013) Toxicological evaluation of microcystins in aquatic fish species: Current knowledge and future directions. *Aquat. Toxicol.* 142–143: 1–16.

Pearson, L., Mihali, T., Moffitt, M., Kellmann, R., and Neilan, B. (2010) On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. *Marine Drugs* 8: 1650–1680.

Pedro, O., Correia, D., Lie, E., Skåre, J.U., Leão, J., Neves, L., Sandvik, M., and Berdal, K.G. (2011) Polymerase chain reaction (PCR) detection of the predominant microcystin–producing genotype of cyanobacteria in Mozambican lakes. *Afr. J. Bio.* 10: 19299–19308.

Pereira, S.R., Vasconcelos, V.M., and Antunes, A. (2013) Computational study of the covalent bonding of microcystins to cysteine residues – a reaction involved in the inhibition of the PPP family of protein phosphatases. *FEBS Journal* 280: 674–680.

Pestana, D., Ostrensky, A., Boeger, W.A.P., and Pie, M.R. (2009) The effect of temperature and body size on filtration rates of *Limnoperna fortunei* (Bivalvia, Mytilidae) under laboratory conditions. *Braz. Arch. Biol. Technol.* 52: 135–144.

Pflugmacher, S., Wiegand, C., Oberemm, A., Beattie, K.A., Krause, E., Codd, G.A., and Steinberg, C.E.W. (1998) Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: The first step of detoxication. *Biochim. Biophys. Acta.* 1425: 527–533.

Pham, H.H. (1969) Quelques algues d'eau douce de la région de Cantho. Annals of the university of Cantho. *Sci. Agri.* 39–59 (in French).

Phung, T.N.H., Couté, A., and Bourrelly, P. (1992) Cyanophyceae in Mekong Delta (Vietnam). *Nova Hedwigia* 54: 403–446 (in French).

Pietsch, C., Wiegand, C., Amé, M.V., Nicklisch, A., Wunderlin, D., and Pflugmacher, S. (2001) The effects of a cyanobacterial crude extract on different aquatic organisms: Evidence for cyanobacterial toxin modulating factors. *Enviro. Toxicol.* 16: 535–542.

Pinho, G.L.L., Moura da Rosa, C., Maciel, F.E., Bianchini, A., Yunes, J.S., Proença, L.A.O., and Monserrat, J.M. (2005) Antioxidant responses after microcystin exposure in gills of an estuarine crab species pre-treated with vitamin E. *Ecotox. Environ. Saf.* 61: 361–365.

Pires, L.M.D., and Donk, E.V. (2002) Comparing grazing by *Dreissena polymorpha* on phytoplankton in the presence of toxic and non-toxic cyanobacteria. *Freshwater Biol.* 47: 1855–1865.

Pires, L.M.D., Karlsson, K.M., Meriluoto, J.A.O., Kardinaal, E., Visser, P.M., Siewertsen, K., Donk, E.V., and Ibelings, B.W. (2004) Assimilation and depuration of microcystin–LR by the zebra mussel, *Dreissena polymorpha*. *Aquat. Toxicol.* 69: 385–396.

Pires, L.M.D., Bontes, B.M., Van Donk, E., and Ibelings, B.W. (2005) Grazing on colonial and filamentous, toxic and non-toxic cyanobacteria by the zebra mussel *Dreissena polymorpha*. *J. Plankton Res.* 27: 331–339.

Pires, L.M.D., Bontes, B., Samchyshyna, L., Jong, J., Donk, E., and Ibelings, B. (2007) Grazing on microcystin-producing and microcystin-free phytoplankters by different filter-feeders: implications for lake restoration. *Aquat. Sci.* 69: 534–543.

Pouria, S., de Andrade, A., Barbosa, J., Cavalcanti, R.L., Barreto, V.T.S., Ward, C.J., Preiser, W., Poon, G.K., Neild, G.H., and Codd, G.A. (1998) Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. *The Lancet* 352: 21–26.

Prepas, E.E., Kotak, B.G., Campbell, L.M., Evans, J.C., Hrudey, S.E., and Holmes, C.F. (1997) Accumulation and elimination of cyanobacterial hepatotoxins by the freshwater clam *Anodonta grandis simpsoniana*. *Can. J. Fish. Aquat. Sci.* 54: 41–46.

Prieto, A.I., Jos, Á., Pichardo, S., Moreno, I., and Cameán, A.M. (2006) Differential oxidative stress responses to microcystins LR and RR in intraperitoneally exposed tilapia fish (*Oreochromis* sp.). *Aquat. Toxicol.* 77: 314–321.

Prieto, A.I., Pichardo, S., Jos, Á., Moreno, I., and Cameán, A.M. (2007) Time-dependent oxidative stress responses after acute exposure to toxic cyanobacterial cells containing microcystins in tilapia fish (*Oreochromis niloticus*) under laboratory conditions. *Aquat. Toxicol.* 84: 337–345.

Puschner, B., Galey, F.D., Johnson, B., Dickie, C.W., Vondy, M., Francis, T., and Holstege, D.M. (1998) Blue-green algae toxicosis in cattle. *J. Am. Vet. Med. Assoc.* 213: 1605–1607.

Rantala, A., Fewer, D.P., Hisbergues, M., Rouhiainen, L., Vaitomaa, J., Borner, T., and Sivonen, K. (2004) Phylogenetic evidence for the early evolution of microcystin synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 101: 568–573.

Rantala, A., Rajaniemi-Wacklin, P., Lyra, C., Lepisto, L., Rintala, J., Mankiewicz-Boczek, J., and Sivonen, K. (2006) Detection of microcystin-producing cyanobacteria in Finnish lakes with genus-specific microcystin synthetase gene E (*mcyE*) PCR and associations with environmental factors. *Appl. Environ. Microbiol.* 72: 6101–6110.

Reichwaldt, E.S., and Ghadouani, A. (2012) Effects of rainfall patterns on toxic cyanobacterial blooms in a changing climate: Between simplistic scenarios and complex dynamics. *Water Res.* 46: 1372–1393.

Riisgård, H.U., Kittner, C., and Seerup, D.F. (2003) Regulation of opening state and filtration rate in filter-feeding bivalves (*Cardium edule*, *Mytilus edulis*, *Mya arenaria*) in response to low algal concentration. *J. Exp. Mar. Biol. Ecol.* 284: 105–127.

Riisgård, H.U., Egede, P.P., and Barreiro S.I. (2011) Feeding behaviour of the mussel, *Mytilus edulis*: New observations, with a minireview of current knowledge. *J. Mar. Biol.* 2011: 1–13.

Rinta-Kanto, J.M., Ouellette, A.J., Boyer, G.L., Twiss, M.R., Bridgeman, T.B., and Wilhelm, S.W. (2005) Quantification of toxic *Microcystis* spp. during the 2003 and 2004 blooms in western Lake Erie using quantitative real-time PCR. *Environ. Sci. Technol.* 39: 4198–4205.

Rinta-Kanto, J.M., Konopko, E.A., DeBruyn, J.M., Bourbonniere, R.A., Boyer, G.L., and Wilhelm, S.W. (2009) Lake Erie *Microcystis*: Relationship between microcystin production, dynamics of genotypes and environmental parameters in a large lake. *Harmful Algae* 8: 665–673.

Rouhiainen, L., Vakkilainen, T., Siemer, B.L., Buikema, W., Haselkorn, R., and Sivonen, K. (2004) Genes coding for hepatotoxic heptapeptides (microcystins) in the cyanobacterium *Anabaena* strain 90. *Appl. Environ. Microbiol.* 70: 686–692.

Ruiz, M., Galanti, L., Ruibal, A., Rodriguez, M., Wunderlin, D., and Amé, M. (2013) First report of microcystins and anatoxin-a co-occurrence in San Roque Reservoir (Córdoba, Argentina). *Water Air Soil Pollut.* 224: 1–17.

Sabart, M., Pobel, D., Briand, E., Combourieu, B., Salencon, M.J., Humbert, J.F., and Latour, D. (2010) Spatiotemporal variations in microcystin concentrations and in the proportions of microcystin-producing cells in several *Microcystis aeruginosa* populations. *Appl. Environ. Microbiol.* 76: 4750–4759.

Sabatini, S.E., Brena, B.M., Luquet, C.M., San Julián, M., Pirez, M., and Carmen Ríos de Molina, M.d. (2011) Microcystin accumulation and antioxidant responses in the freshwater clam *Diplodon chilensis patagonicus* upon subchronic exposure to toxic *Microcystis aeruginosa*. *Ecotox. Environ. Saf.* 74: 1188–1194.

Sabour, B., Loudiki, M., Oudra, B., Vasconcelos, V., Martins, R., Oubraim, S., and Fawzi, B. (2002) Toxicology of a *Microcystis ichthyoblabe* waterbloom from Lake Oued Mellah (Morocco). *Environ. Toxicol.* 17: 24–31.

Sahin, A., Tencalla, F.G., Dietrich, D.R., and Naegeli, H. (1996) Biliary excretion of biochemically active cyanobacteria (blue-green algae) hepatotoxins in fish. *Toxicology* 106: 123–130.

Sano, T., Nohara, K., Shiraishi, F., and Kaya, K. (1992) A method for micro-determination of total microcystin content in waterblooms of cyanobacteria (blue-green algae). *Int. J. Environ. Anal. Chem.* 49: 163–170.

Shimizu, K., Maseda, H., Okano, K., Itayama, T., Kawauchi, Y., Chen, R., Utsumi, M., Zhang, Z., and Sugiura, N. (2011) How microcystin-degrading bacteria express microcystin degradation activity. *Lake Reserv. Res. Manage.* 16: 169–178.

Shimizu, K., Maseda, H., Okano, K., Kurashima, T., Kawauchi, Y., Xue, Q., Utsumi, M., Zhang, Z., and Sugiura, N. (2012) Enzymatic pathway for biodegrading microcystin LR in *Sphingopyxis* sp. C-1. *J. Biosci. Bioeng.* 114: 630–634.

Shirai, M., Matumaru, K., Ohotake, A., Takamura, Y., Aida, T., and Nakano, M. (1989) Development of a solid medium for growth and isolation of axenic microcystis strains (cyanobacteria). *Appl. Environ. Microbiol.* 55: 2569–2571.

Sivonen, K. (1996) Cyanobacterial toxins and toxin production. *Phycologia* 35: 12–24.

Smith, J.L., Boyer, G.L., and Zimba, P.V. (2008) A review of cyanobacterial odorous and bioactive metabolites: impacts and management alternatives in aquaculture. *Aquaculture* 280: 5–20.

Smith, J.L., and Boyer, G.L. (2009) Standardization of microcystin extraction from fish tissues: A novel internal standard as a surrogate for polar and non-polar variants. *Toxicon* 53: 238–245.

Smutná, M., Babica, P., Jarque, S., Hilscherová, K., Maršálek, B., Haeba, M., and Bláha, L. (2014) Acute, chronic and reproductive toxicity of complex cyanobacterial blooms in *Daphnia magna* and the role of microcystins. *Toxicon* 79: 11–18.

Song, L., Chen, W., Peng, L., Wan, N., Gan, N., and Zhang, X. (2007) Distribution and bioaccumulation of microcystins in water columns: A systematic investigation into the environmental fate and the risks associated with microcystins in Meiliang Bay, Lake Taihu. *Water Res.* 41: 2853–2864.

Sotton, B., Guillard, J., Anneville, O., Maréchal, M., Savichtcheva, O., and Domaizon, I. (2014) Trophic transfer of microcystins through the lake pelagic food web: evidence for the role of zooplankton as a vector in fish contamination. *Sci. Total Environ.* 466-467: 152–163.

Sournia, A. (1978) Phytoplankton manual. UNESCO, UK, 337 pp.

Stanier, R.Y., and van Niel, C.B. (1941) The main outlines of bacterial classification. *J. Bacteriol.* 42: 437–466.

Stewart, I., Seawright, A.A., and Shaw, G.R. (2008) Cyanobacterial poisoning in livestock, wild mammals and birds-an overview. *Adv. Exp. Med. Biol.* 619: 613–637.

Suchy, P., and Berry, J. (2012) Detection of total microcystin in fish tissues based on lemieux oxidation, and recovery of 2-methyl-3-methoxy-4-phenylbutanoic acid (MMPB) by solid-phase microextraction gas chromatography-mass spectrometry (SPME-GC/MS). *Int. J. Environ. Anal. Chem.* 92: 1443–1456.

Sun, H., Lü, K., Minter, E.J.A., Chen, Y., Yang, Z., and Montagnes, D.J.S. (2012) Combined effects of ammonia and microcystin on survival, growth, antioxidant responses, and lipid peroxidation of bighead carp *Hypophthalmythys nobilis* larvae. *J. Hazard. Mater.* 221–222: 213–219.

Sun, H., Wang, W., Geng, L., Chen, Y., and Yang, Z. (2013) In situ studies on growth, oxidative stress responses, and gene expression of juvenile bighead carp (*Hypophthalmichthys nobilis*) to eutrophic lake water dominated by cyanobacterial blooms. *Chemosphere* 93: 421–427.

Te, S.H., and Gin, K.Y.-H. (2011) The dynamics of cyanobacteria and microcystin production in a tropical reservoir of Singapore. *Harmful Algae* 10: 319–329.

Tillett, D., Dittmann, E., Erhard, M., von Dohren, H., Borner, T., and Neilan, B.A. (2000) Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. *Chem. Biol.* 7: 753–764.

Tillett, D., Parker, D.L., and Neilan, B.A. (2001) Detection of toxigenicity by a probe for the microcystin synthetase a gene (*mcyA*) of the cyanobacterial genus *Microcystis*: Comparison of toxicities with 16S rRNA and phycocyanin operon (phycocyanin intergenic spacer) phylogenies. *Appl. Environ. Microbiol.* 67: 2810–2818.

Tsuji, K., Masui, H., Uemura, H., Mori, Y. and Harada, K.-I. (2001) Analysis of microcystins in sediments using MMPB method. *Toxicon* 39: 687–692.

Tsujimura, S., Tsukada, H., Nakahara, H., Nakajima, T., and Nishino, M. (2000) Seasonal variations of *Microcystis* populations in sediments of Lake Biwa, Japan. *Hydrobiologia* 434: 183–192.

Turja, R., Guimarães, L., Nevala, A., Kankaanpää, H., Korpinen, S., and Lehtonen, K.K. (2014) Cumulative effects of exposure to cyanobacteria bloom extracts and benzo[a]pyrene on antioxidant defence biomarkers in *Gammarus oceanicus* (Crustacea: Amphipoda). *Toxicon* 78: 68–77.

Umehara, A., Tsutsumi, H., and Takahashi, T. (2012) Blooming of *Microcystis aeruginosa* in the reservoir of the reclaimed land and discharge of microcystins to Isahaya Bay (Japan). *Environ. Sci. Pollut. Res.* 19: 3257–3267.

Urbach, E., Robertson, D.L., and Chisholm, S.W. (1992) Multiple evolutionary origins of prochlorophytes within the cyanobacterial radiation. *Nature* 355: 267–270.

URL: http://www.researchgate.net [30 January 2015].

Vareli, K., Zarali, E., Zacharioudakis, G.S.A., Vagenas, G., Varelis, V., Pilidis, G., Briasoulis, E., and Sainis, I. (2012) Microcystin producing cyanobacterial communities

in Amvrakikos Gulf (Mediterranean Sea, NW Greece) and toxin accumulation in mussels (*Mytilus galloprovincialis*). *Harmful Algae* 15: 109–118.

Vasconcelos, V.M. (1995) Uptake and depuration of the heptapeptide toxin microcystin-LR in *Mytilus galloprovincialis*. *Aquat. Toxicol.* 32: 227–237.

Vasconcelos, V.M., Sivonen, K., Evans, W.R., Carmichael, W.W., and Namikoshi, M. (1996) Hepatotoxic microcystin diversity in cyanobacterial blooms collected in portuguese freshwaters. *Water Res.* 30: 2377–2384.

Vasconcelos, V.M, Oliveira, S., and Teles, F.O. (2001) Impact of a toxic and a non-toxic strain of *Microcystis aeruginosa* on the crayfish *Procambarus clarkii*. *Toxicon* 39: 1461–1470.

Vasconcelos, V.M., Wiegand, C., and Pflugmacher, S. (2007) Dynamics of glutathione-S-transferases in *Mytilus galloprovincialis* exposed to toxic *Microcystis aeruginosa* cells, extracts and pure toxins. *Toxicon* 50: 740–745.

Vasconcelos, V., Martins, A., Vale, M., Antunes, A., Azevedo, J., Welker, M., Lopez, O., and Montejano, G. (2010) First report on the occurrence of microcystins in planktonic cyanobacteria from central Mexico. *Toxicon* 56: 425–431.

Vezie, C., Brient, L., Sivonen, K., Bertru, G., Lefeuvre, J.C., and Salkinoja-Salonen, M. (1998) Variation of microcystin content of cyanobacterial blooms and isolated strains in lake Grand-Lieu (France). *Microb. Ecol.* 35: 126–135.

Vietnam-MOSTE. (2001) The valuable biodiversity and environment wetlands in Vietnam. Agricultural Publishing House, Hanoi, 192 pp (In Vietnamese).

Vinagre, T.M., Alciati, J.C., Regoli, F., Bocchetti, R., Yunes, J.S., Bianchini, A., and Monserrat, J.M. (2003) Effect of microcystin on ion regulation and antioxidant system in gills of the estuarine crab *Chasmagnathus granulatus* (Decapoda, Grapsidae). *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 135: 67–75.

Wacklin, P., Hoffmann, L., and Komárek, J. (2009) Nomenclatural validation of the genetically revised cyanobacterial genus *Dolichospermum* (RALFS ex BORNET et FLAHAULT) comb. nova. *Fottea* 9: 59–64.

Watanabe, M.F., Park, H.D., Kondo, F., Harada, K., Hayashi, H., and Okino, T. (1997) Identification and estimation of microcystins in freshwater mussels. *Natural Toxins* 5: 31–35.

Wiedner, C., Rücker, J., Brüggemann, R., and Nixdorf, B. (2007) Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. *Oecologia* 152: 473–484.

Wiegand, C., Pflugmacher, S., Oberemm, A., Meems, N., Beattie, K.A., Steinberg, C.E.W., and Codd, G.A. (1999) Uptake and effects of microcystin-LR on detoxication enzymes of early life stages of the zebra fish (*Danio rerio*). *Environ. Toxicol.* 14: 89–95.

Wiegand, C., Pflugmacher, S., Oberemm, A., and Steinberg, C. (2000) Activity development of selected detoxification enzymes during the ontogenesis of zebrafish *Danio rerio*. *Int. Rev. Hydrobiol.* 854: 413–422.

Wiegand, C., and Pflugmacher, S. (2005) Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. *Toxicol. Appl. Pharmacol.* 203: 201–218.

Williams, D.E., Craig, M., McCready, T.L., Dawe, S.C., Kent, M.L., Holmes, C.F., and Andersen, R.J. (1997a) Evidence for a covalently bound form of microcystin-LR in salmon liver and *Dungeness* crab larvae. *Chem. Res. Toxicol.* 10: 463–469.

Williams, D.E., Dawe, S.C., Kent, M.L., Andersen, R.J., Craig, M., and Holmes, C.F.B. (1997b) Bioaccumulation and clearance of microcystins from salt water mussels, *Mytilus edulis*, and *in vivo* evidence for covalently bound microcystins in mussel tissues. *Toxicon* 35: 1617–1625.

Wirsing, B., Hoffmann, L., Heinze, R., Klein, D., Daloze, D., Braekman, J.-C., and Weckesser, J. (1998) First report on the identification of microcystin in a water bloom collected in Belgium. *Syst. Appl. Microbiol.* 21: 23–27.

Wörmer, L., Huerta-Fontela, M., Cirés, S., Carrasco, D., and Quesada, A. (2010) Natural photodegradation of the cyanobacterial toxins microcystin and cylindrospermopsin. *Environ. Sci. Technol.* 44: 3002–3007.

Xie, L., and Park, H.D. (2007) Determination of microcystins in fish tissues using HPLC with a rapid and efficient solid phase extraction. *Aquaculture* 271: 530–536.

Xie, L., Yokoyama, A., Nakamura, K., and Park, H.D. (2007) Accumulation of microcystins in various organs of the freshwater snail *Sinotaia histrica* and three fishes in a temperate lake, the eutrophic Lake Suwa, Japan. *Toxicon* 49: 646–652.

Xu, Q., Chen, W., and Gao, G. (2008) Seasonal variations in microcystin concentrations in Lake Taihu, China. *Environ. Monit. Assess.* 145: 75–79.

Yasuno, M., Sugaya, Y., Kaya, K., and Watanabe, M.M. (1998) Variations in the toxicity of *Microcystis* species to *Moina macrocopa*. *Phycol. Res.* 46: 31–36.

Ye, W., Liu, X., Tan, J., Li, D., and Yang, H. (2009) Diversity and dynamics of microcystin producing cyanobacteria in China's third largest lake, Lake Taihu. *Harmful Algae* 8: 637–644.

Yokoyama, A., and Park, H.-D. (2002) Mechanism and prediction for contamination of freshwater bivalves (Unionidae) with the cyanobacterial toxin microcystin in hypereutrophic Lake Suwa, Japan. *Environ. Toxicol.* 17: 424–433.

Yokoyama, A., and Park, H.D. (2003) Depuration kinetics and persistence of the cyanobacterial toxin microcystin-LR in the freshwater bivalve *Unio douglasiae*. *Environ. Toxicol.* 18: 61–67.

Zanchett, G., and Oliveira-Filho, E. (2013) Cyanobacteria and cyanotoxins: From impacts on aquatic ecosystems and human health to anticarcinogenic effects. *Toxins* 5: 1896–1917.

Zhang, D., Yang, Q., Xie, P., Deng, X., Chen, J., and Dai, M. (2012) The role of cysteine conjugation in the detoxification of microcystin-LR in liver of bighead carp (*Aristichthys nobilis*): A field and laboratory study. *Ecotoxicology* 21: 244–252.

Zhang, D.L., Hu, C.X., Li, D.H., and Liu, Y.D. (2013) Lipid peroxidation and antioxidant responses in zebrafish brain induced by *Aphanizomenon flos-aquae* DC-1 aphantoxins. *Aquat. Toxicol.* 144–145: 250–256.

Zhu, J., Lu, K., Zhang, C., Liang, J., and Hu, Z. (2011) Biochemical and ultrastructural changes in the hepatopancreas of *Bellamya aeruginosa* (Gastropoda) fed with toxic cyanobacteria. *Scientific World Journal* 11: 2091–2105.

Znachor, P., Jurczak, T., Komarkova, J., Jezberova, J., Mankiewicz, J., Kastovska, K., and Zapomelova, E. (2006) Summer changes in cyanobacterial bloom composition and microcystin concentration in eutrophic Czech reservoirs. *Environ. Toxicol.* 21: 236–243.

Zurawell, R.W., Chen, H., Burke, J.M., and Prepas, E.E. (2005) Hepatotoxic cyanobacteria: A review of the biological importance of microcystins in freshwater environments. *J. Toxicol. Environ. Health. Part B, Critical Reviews* 8: 1–37.