Universidade de Aveiro Departamento de Biologia

Ano 2012

Carlos Filipe Carvalho Pinheiro

TOXICIDADE DE MISTURAS DE CIANOTOXINAS E COMPOSTOS QUÍMICOS

JOINT TOXICITY EFFECTS OF CYANOTOXINS AND CHEMICAL COMPOUNDS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada – Ramo de Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro (Investigadora Auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro) e co-orientação do Doutor Vítor Vasconcelos (Professor Catedrático do Departamento de Biologia da Faculdade de Ciências da Universidade do Porto e Investigador do LEGE, CIIMAR)

Dedico este trabalho à minha mãe pelo seu incansável apoio, sempre a relembrar-me que todos os dias é preciso lutar pelos meus objetivos pessoais e profissionais.

"A ciência é uma aventura de toda a raça humana para aprender a viver e talvez a amar o Universo onde se encontra. Ser uma parte dele é compreender, é conhecer-se a si próprio, é começar a sentir que existe dentro do Homem uma capacidade muito superior a que ele pensava ter e uma quantidade infinita de possibilidades humanas".

Isidor Isaac Rabi
(1898-1988)

o júri

presidente

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palavras-chave

Toxicidade de misturas, acção independente, cianotoxinas, metais, pesticidas, microcistina-LR, cilindrospermosina, cadmio, terbutilazina, microalgas, *Chlorella vulgaris*.

resumo

Os organismos aquáticos podem estar constantemente expostos a cianotoxinas e contaminantes antropogénicos provenientes das florescências de cianobactérias e das atividades humanas, respetivamente. A microcistina-LR (MC-LR) e a cilindrospermopsina (CYN) são as cianotoxinas mais frequentemente detetadas nas florescências de cianobactérias e têm sido encontradas simultaneamente na água. Os metais e pesticidas são contaminantes antropogénicos normalmente encontrados no ambiente aquático como resultado da intensificação das atividades agrícolas e industriais. O cádmio (Cd) e a terbutilazina (TBA) foram selecionados como exemplos de metais e pesticidas que podem co-ocorrer com cianotoxinas no ambiente. No entanto, o risco ecotoxicológico combinado de cianotoxinas e/ou contaminantes antropogénicos existentes no ambiente aquático é ainda pouco conhecido. O presente trabalho teve como objetivo identificar alguns padrões e comportamentos biológicos relativamente a este tipo de combinações. Os efeitos de misturas binárias de MC-LR, CYN, Cd e TBA foram avaliados nas taxas de crescimento da alga *Chlorella vulgaris* após 4 e 7 dias de exposição, usando o modelo de referência de ação independente (AI). A ferramenta MIXTOX foi usada para avaliar possíveis desvios ao modelo de referência (devido a interações entre compostos), tais como sinergismo/antagonismo, dependência da dose ou do rácio da mistura. Os resultados demonstraram vários padrões de resposta, dependendo da mistura binária testada. Foi detetado sinergismo na mistura de MC-LR e CYN em ambos os períodos de exposição. Na mistura de MC-LR e TBA, houve um desvio dependente do nível da dose entre os componentes para ambos os períodos de exposição, onde se observou antagonismo e sinergismo para concentrações baixas e elevadas de ambos os compostos, respetivamente. Na mistura de MC-LR e Cd, registou-se antagonismo após 4 dias de exposição e um desvio dependente do nível da dose entre os componentes após 7 dias de exposição, observando-se sinergismo e antagonismo para concentrações baixas e elevadas de ambos os compostos, respetivamente. Embora na mistura de CYN e TBA se tenha observado um desvio dependente do rácio entre os componentes, com um padrão de antagonismo perante a dominância da CYN, na mistura de CYN e Cd observou-se antagonismo após 4 dias de exposição. Após 7 dias de exposição foi observado um padrão semelhante de resposta em ambas as misturas contendo CYN, ou seja, um desvio dependente do nível da dose entre os componentes na qual se observou sinergismo para as concentrações baixas e antagonismo para as concentrações elevadas testadas de ambos os compostos.

Para a mistura de TBA e Cd, registou-se antagonismo após 4 dias de exposição e um desvio dependente do nível das doses entre os componentes (antagonismo para concentrações baixas e sinergismo para concentrações elevadas de ambos os componentes) após 7 dias de exposição. Devido à diversidade de efeitos e comportamentos que podem resultar da combinação de tóxicos bastante comuns, este estudo mostra a importância de avaliar os efeitos combinados de cianotoxinas e/ou contaminantes antropogénicos.

keywords

Mixture toxicity, independent action, cyanotoxins, metals, pesticides, microcystin-LR, cylindrospermopsin, cadmium, terbuthylazine, microalgae, *Chlorella vulgaris*.

abstract

Aquatic organisms may be exposed to cyanotoxins and anthropogenic contaminants originated from harmful cyanobacterial blooms and human activities, respectively. Microcystin-LR (MC-LR) and cylindrospermopsin (CYN) are the most frequently detected cyanotoxins in harmful cyanobacterial blooms and have been simultaneously reported in the water. Metals and pesticides are anthropogenic contaminants commonly found in the aquatic environment as a result of the intensification of agricultural and industrial activities. Cadmium (Cd) and terbuthylazine (TBA) were chosen as an example of the possible metals and pesticides that can co-occurr with cyanotoxins in the environment. However, the ecotoxicological risk of combinations of cyanotoxins and/or anthropogenic contaminants in the aquatic environment needs more studies. The present work aimed to elucidate some biological behaviours and patterns regarding these combinations. The effects of binary mixtures of MC-LR, CYN, Cd and TBA on the growth rate of the freshwater algae *Chlorella vulgaris* were assessed after 4 and 7 days of exposure using the reference model of independent action (IA). The MIXTOX tool was used to detect possible deviations (due to the interaction between compounds) from the reference model such as synergism/antagonism, dose ratio and dose level dependency. The results demonstrated that several patterns of response were obtained depending on the binary mixture. Synergism was detected in the mixture of MC-LR and CYN for the two exposure periods. In the MC-LR and TBA mixture, a dose-level deviation was observed for the two exposure periods indicating antagonism at low dose levels and synergism at high dose levels. In the MC-LR and Cd mixture, deviations for antagonism were found for a 4-day exposure period while a dose-level deviation was observed for a 7-day exposure period showing synergism at low dose levels and antagonism at high dose levels. A dose-ratio deviation was observed in the CYN and TBA mixture, with a pattern for antagonism when CYN was the compound dominant, while deviations for antagonism were observed in the CYN and Cd mixture for a 4-day exposure period. Similar patterns of response were obtained for both mixtures involving CYN after 7 days of exposure, namely dose-level deviation indicating synergism at low dose levels and antagonism at high dose levels. For TBA and Cd mixture, antagonism was found for a 4-day exposure period and a dose-level deviation (antagonism at low dose levels and synergism at high dose levels) was observed for a 7-day exposure period. Due to the diversity of effects and behaviours that can result from the combination of very common toxicants, this study shows the importance of evaluating the combined effects of cyanotoxins and/or anthropogenic contaminants.

Contents

List of Figures

Chapter I

Chapter II

Chapter III

List of Tables

Chapter I

Chapter II

Chapter III

Abbreviations

Listed alphabetically

A_r	Relative atomic mass
Ala	Alanine
Adda	(2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
ANOVA	One-way analysis of variance
ATP	Adenosine triphosphate
BBB	Blood brain barrier
CA	Concentration addition
CCE	Cyanobacterial crude extract
CYN	Cylindrospermopsin
CYP450	Cytochrome P450
D-MeAsp	D-erythro-β-methylaspartic acid
DL	Dose level dependency
DNA	Deoxyribonucleic acid
DR	Dose ratio dependency
EC_x	Effect concentration of the chemical *i* that provoke *x*% of the effect
EU	European Union
GJIC	Gap junctional intercellular communication
Glu	Glutamate
GPx	Glutathione peroxidase
GR	Glutathione redutase
GSH	Glutathione
GST	Glutathione *S*-transferase
HAB	Harmful cyanobacterial bloom
HPLC	High performance liquid chromatography
IA	Independent action
IARC	International Agency for Research on Cancer

LOEC	Lowest observed effect concentration
LPS	Lipopolysaccharide
MCs	Microcystins
Mdha	*N*-methyldehydroalanine
MoA	Mode of action
MW	Molecular weight
NOEC	No observed effect concentration
OATP	Organic anion transport protein
OECD	Organization for Economic Co-operation and Development
OD	Optical density
PDA	Photo diode array
POD	Peroxidase
PP	Protein phosphatases
PPP	Plant protection products
PSII	Photosystem II
PVC	Polyvinyl chloride
ROS	Reactive oxygen species
SOD	Superoxidase dismutase
SR	Synergistic ratio
TBA	Terbuthylazine
TDI	Tolerated daily intake
TFA	Trifluoracetic acid
TU	Toxic unit
U.S.EPA	US Environmental Protection Agency
WHO	World Health Organization

Chapter I

1. General introduction

1.1. Cyanobacteria

Cyanobacteria are ubiquitous prokaryotic photoautotrophs that used to be referred to as blue-green algae. Their geographic distribution ranges from polar to tropical regions in northern and southern hemispheres, where they are able to dominate planktonic and benthic primary production (Paerl & Paul 2012). They may be found in the most diverse environments, including terrestrial, marine, brackish and freshwater, occupying all kinds of habitats, including extreme habitats such as Antarctic lakes, thermal springs, arid deserts and tropical acidic soils (Kaebernick & Neilan 2001). The global distribution of cyanobacteria is related to their morphological, physiological and chemical diversity (Wiegand & Pflugmacher 2005).

Cyanobacteria are the Earth's oldest known oxygen-producing life forms (Kaebernick & Neilan 2001; Paerl & Paul 2012). Stromatolites provide proof of cyanobacterial presence in our planet dating back to ~3.5 billion years ago (Lazcano & Miller 1994). As cyanobacteria were the first organisms to carry out the oxygenic photosynthesis, cyanobacterial proliferation during the Precambrian period was largely responsible for the reductive to oxidative shift in our atmosphere and subsequent evolution of eukaryotic life (Kaebernick & Neilan 2001; Paerl & Paul 2012; Schopf 1994). Their long evolutionary history on Earth has enabled them to develop diverse and highly effective ecophysiological adaptations and strategies for ensuring survival and dominance in different environments undergoing natural and anthropogenic pressures and tolerance under a wide range of ecological conditions (Paerl & Paul 2012; Schopf 1994). Some adaptations and strategies are as follows (Kaebernick & Neilan 2001; Paerl & Paul 2012):

- Highly efficient nutrient (N, P, Fe and trace metal) uptake and storage capabilities;
- Light harvesting pigments (chlorophyll and phycocyanin);
- Nitrogen fixation in specialized cells (heterocysts);

- Formation of stress resistant cells (akinetes);
- Ability to regulate buoyancy through gas vesicles (vertical migration);
- Ability to form symbiotic (as endosymbionts) and mutualistic associations with several organisms (diatoms, fungi, protists, sponges, corals, lichens, plants).

At present, cyanobacteria display a great morphological diversity. They may present unicellular forms and more complex colonial and filamentous forms, with sizes ranging from less than 2 µm to 40 µm in diameter (Kaebernick & Neilan 2001). Additionally, they may live as free-living organisms or in symbiotic associations with a broad spectrum of other living organisms, such as plants, fungi, protists and sponges (Smith & Doan 1999). However, the most intriguing characteristic of cyanobacteria is their ability to produce cyanotoxins (Carmichael 1992).

1.2. Cyanotoxins

Cyanobacteria are able to produce a great variety of chemically unique secondary metabolites, many of which with powerful bioactivities (e.g. cytotoxicity towards tumor cells) (Carmichael 1992; Namikoshi & Rinehart 1996). The discovery of these biological and biochemical activities has led to a growing awareness on the biotechnological potential of these organisms (Abed et al. 2009; Tan 2007; Tan 2010). Of these secondary metabolites, cyanotoxins or cyanobacterial toxins are the most prominent group and the most studied (Namikoshi & Rinehart 1996).

The cyanotoxins are a diverse group of compounds, both from the chemical and the toxicological points of view. According to their chemical structure, cyanotoxins may be classified as cyclic peptides, alkaloids and lipopolysaccharides (Kaebernick & Neilan 2001). However, they are more commonly divided in terms of their mechanism of action on terrestrial vertebrates, especially mammals, as hepatotoxins, neurotoxins, cytotoxins and dermatotoxins and irritant toxins (Codd et al. 2005; Kaebernick & Neilan 2001; Prasanna et al. 2010). Table 1. shows the main cyanotoxins produced by cyanobacteria and their classification based on the previous criteria as well as the main producing genera,

their mechanisms of action and main detoxication mechanisms involved in the biotransformation of these compounds. The hepatotoxin microcystins and the cytotoxin cylindrospermopsin are the most frequently detected toxins in cyanobacterial blooms in fresh and brackish waters (Zurawell et al. 2005).

Table 1 – Cyanotoxins and their classifications based on the chemical structure and the mechanism of action on vertebrates including a brief description of their representative producers, the molecule mode of action, the causative residue of the toxin molecule and the detoxification pathways. GST is glutathione *S*-transferase, CYP450 is cytochrome P450. Adapted from Wiegand & Pflugmacher (2005).

Cyanotoxins	Type of toxin	Chemical structure	Producer (genera)	Toxic structure in the molecule	Mechanism of action	Detoxification
Microcystins	Hepatotoxins	Cyclic peptides	*Microcystis* *Anabaena* *Anabaenopsis* *Plankthotrix* *Nostoc* *Radiocystis* *Hapalosiphon*	Adda moiety	Inhibition of protein phosphatases (PP1 and PP2A)	GST
Nodularins	Hepatotoxins	Cyclic peptides	*Nodularia*	Adda moiety	Inhibition of protein phosphatases (PP1 and PP2A)	GST
Saxitoxins	Neurotoxins	Carbamate alkaloids	*Anabaena* *Aphanizomenon* *Cylindrospermopsis* *Lyngbya*		Binding and blocking the sodium channels in neural cells	GST
Anatoxin-a	Neurotoxin	Secondary amine alkaloid	*Anabaena* *Aphanizomenon* *Cylindrospermopsis* *Plankthotrix* *Oscillatoria* *Microcystis*		Binding irreversibly to the nicotinic acetylcholine receptorss	CYP450 GST
Anatoxin-a(s)	Neurotoxin	Guanidine methyl phosphate ester (alkaloid)	*Anabaena*		Inhibition of Ach-esterase activity	CYP450 GST
Cylindrospermopsin	Cytotoxin	Guanidine alkaloid	*Cylindrospermopsis* *Aphanizomenon* *Umezakia* *Raphidiopsis* *Anabaena* *Trichodesmium*	The presence of the hydroxyl on the uracil bridge or the keto-enol status of the uracil moiety	Inhibitior of protein biosynthesis Cytogenetic damage on DNA	CYP450
Lipopolysaccharides	Dermatotoxins and irritant toxins	Lipopolysaccharides	Cyanobacteria in general	Fatty acid component	Irritant; cause inflammation in exposed tissues	Deacylation via lysossomal pathway

1.2.1. Microcystins

The hepatotoxic microcystins (MCs), produced by members of several cyanobacterial genera (Table 1), are the largest and the most structurally diverse group of cyanotoxins, consisting of over 75 congeners (Amado & Monserrat 2010; Rinehart et al. 1994). Collectively, MCs (Figure 1) are described as small produced non-ribosomally monocyclic heptapeptides (MW between 900 and 1100 Daltons) and share the general chemical structure:

cyclo-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha

where X and Z are variable L-amino acids, Ala and Glu are alanine and glutamate respectively, D-MeAsp is D-erythro-β-methylaspartic acid, Adda is the unusual amino acid (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid and Mdha is *N*-methyldehydroalanine (van Apeldoorn et al. 2007; Zurawell et al. 2005). The most common and the best studied congener among MCs is MC-LR (994 Daltons, Figure 1), characterized by the presence of leucine (L) and arginine (R) as the variable L-amino acids (Amado & Monserrat 2010; Zurawell et al. 2005). Other variants that also occur frequently are MC-RR, MC-YR and MC-LA (de Figueiredo et al. 2004). However, all of these structurally related compounds differ in toxicity under different experimental conditions, being the MC-LR variant the most potent cyanotoxin followed by MC-YR and MC-RR (Gupta et al. 2003; Luukkainen et al. 1993).

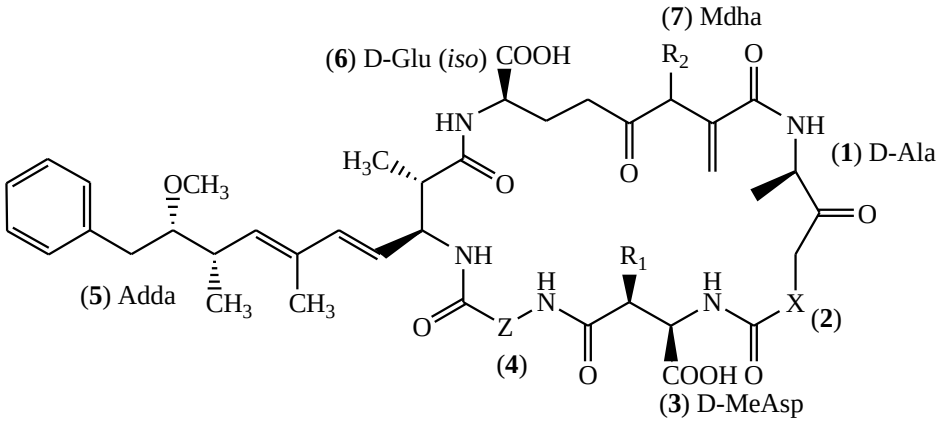

Figure 1 – General chemical structure of microcystis. In microcystin-LR, X represents L-leucine, Z L-arginine and R_1 and R_2 CH_3.

The hydrophobic amino acid Adda (Table 1), unique to cyanobacteria, is thought to be responsible for the toxic properties of MCs (Rudolph-Böhner et al. 1994; Vasconcelos 2001; Zurawell et al. 2005). Cleavage of the Adda amino acid from the cyclic peptide seems to render MCs inactive with regard to their mechanism of toxicity (Harada et al. 1990a; Harada et al. 1990b). Nevertheless, the Adda amino acid by itself shows no inhibitory activity even at high concentrations of 10 µM and no toxicity to mice at intraperitoneal doses of up to 10 $mg.kg^{-1}$ body weight (Harada et al. 2004). The cyclic structure of MCs appears to be also linked with their biological activity, since linear peptides which have the same amino acids as the cyclic peptides did not show apparently toxicity to mice (Choi et al. 1993; Rinehart et al. 1994).

MCs have been involved in many cases of intoxications of wild and domestic animals and humans (Azevedo et al. 2002; Carmichael et al. 2001; Codd 1995; Miller et al. 2010; Oberholster et al. 2009; Wood et al. 2010; Zurawell et al. 2005). The most famous fatal human intoxication occurred in 1996, when an acute poisoning caused the death of 52 people in Caruaru, Brazil, as a result of the usage of MC contaminated water from a nearby water reservoir without treatment in a haemodialysis centre (Azevedo et al. 2002).

The majority of MC-related research has extensively focused on mammalian toxicity. The degree of severity of MC-induced toxicity depends on the level and duration of exposure, determined by the balance between MC absorption, detoxification and excretion (Funari & Testai 2008). MCs are water soluble and hydrophilic and therefore most of them are incapable of penetrating the lipid membranes of animals and plants by passive transport (Campos & Vasconcelos 2010; Funari & Testai 2008; van Apeldoorn et al. 2007). In animals and humans, MCs are actively transported into cells by the organic anion transport proteins (Oatps; OATPs in humans) (Campos & Vasconcelos 2010; van Apeldoorn et al. 2007). These active transporters are expressed in organs such as intestines, kidneys, heart, lungs, spleen, pancreas, brain and blood brain barrier (BBB) (Campos & Vasconcelos 2010; Funari & Testai 2008; van Apeldoorn et al. 2007). Thus, the toxicity of MCs is only restricted to organs expressing Oatps/OATPs on their cell membranes. In plant cell, there is no active MC transport system identified so far (Yin et al. 2005). MCs are conjugated with reduced glutathione (GSH) in aquatic organisms (Pflugmacher et al.

1999; Pflugmacher et al. 1998) and mammals (Kondo et al. 1992), process mediated by soluble glutathione *S*-transferase (GST). The MC-LR-GSH conjugate appears to be the first step in the detoxification of a cyanotoxin in aquatic organisms (Pflugmacher et al. 1998). The reaction of conjugation occurs through the methyl group of Mhda (Figure 1) (Kondo et al. 1992). The binding to GSH enhances the water solubility of MCs, facilitating their excretion from the cells (Wiegand & Pflugmacher 2005). Although it is known that the detoxification process occurs in many organisms, the underlying mechanism of MC biotransformation/excretion remains unknown (Campos & Vasconcelos 2010).

Acute toxicity of MCs may result not only in severe liver damage (main target organ) but also in injury to other organs such as heart, kidney, intestines and brain (Campos & Vasconcelos 2010; van Apeldoorn et al. 2007). The main mechanism of toxicity is the irreversible inhibition of eukaryotic protein phosphatases types 1 and 2A (PP1 and PP2A, respectively) both *in vitro* and *in vivo* from both animals and higher plants (Mackintosh et al. 1990). PP1 and PP2A are key regulatory enzymes responsible for catalyzing the dephosphorylation of serine and threonine residues of phosphoproteins (e.g. structural proteins, enzymes, regulators) (Bláha et al. 2009). These enzymes are involved in a number of important molecular and physiological processes to maintain the cell's homeostasis such as gene expression, protein synthesis, cell growth and differentiation and carbon and nitrogen metabolism (Babica et al. 2006). Inhibition of protein phosphatases is followed by loss of cytoskeletal integrity, cytolysis or apoptosis (primarily of hepatocytes) and ultimately death by intrahepatic haemorrhage and hypovolaemic shock and heart failure (Bláha et al. 2009; van Apeldoorn et al. 2007; Wiegand & Pflugmacher 2005).

Chronic exposure to low concentrations of MCs may also be a serious problem to the public health, since MC-LR may act as a tumour promoter (Nishiwakimatsushima et al. 1992). Due to their cyclic structure, MCs are chemically stable and may persist in the environment for extend periods of time leading to long-term exposures for humans (Tsuji et al. 1994; van Apeldoorn et al. 2007). The danger of tumour promotion by chronic exposure of MC-LR led World Health Organization (WHO) in 1998 to establish a provisional guideline value of 1 $\mu g.L^{-1}$ for MC-LR in drinking water based on tolerated daily intake (TDI) of 0.04 $mg.kg^{-1}$ body weight (Dietrich & Hoeger 2005; Žegura et al.

2011). The International Agency for Research on Cancer (IARC) recently reviewed available data on MC-LR carcinogenicity and classified MC-LR as possibly carcinogenic to humans (Group 2B), whereas there is not enough available information for the classification of other MCs (IARC 2010). Moreover, U.S. Environmental Protection Agency (U.S.EPA) classified MC on the list of compounds with high priority for hazard characterization (U.S.EPA 2010).

Oxidative stress seems to be another important biochemical mechanism of MC toxicity (Amado & Monserrat 2010; Bláha et al. 2009). MCs have been shown to induce formation of reactive oxygen species (ROS) in different terrestrial and aquatic organisms that might cause serious cellular damage such as lipid peroxidation, genotoxicity, mitochondrial damage, alterations in the antioxidant defence system and modulation of apoptosis (Amado & Monserrat 2010; Bláha et al. 2009; Ding & Ong 2003). The formation of ROS is the most likely the mechanism responsible for oxidative damage of DNA, genotoxic and clastogenic effects of MCs (Humpage & Falconer 1999; Humpage et al. 2000). However, the underlying mechanism of oxidative stress induced by MCs is still not known (Bláha et al. 2009; Campos & Vasconcelos 2010).

1.2.2. Cylindrospermopsin

The cytotoxic CYN (MW 415 Daltons; Figure 2), produced by a larger number of cyanobacterial species (Table 1), consists of an uracil combined with a tricyclic guanidine group through a hydroxyl bridge, both critical to its biological activity (Banker et al. 2001; Ohtani et al. 1992). Only two natural structural variants, 7-epiCYN (Banker et al. 2000) and 7-deoxyCYN (Li et al. 2001; Norris et al. 1999; Seifert et al. 2007), have been identified so far with variations occurring at the hydroxyl group (Figure 2). The first variant appears to have similar toxicity as CYN (Banker et al. 2000), whereas the second variant is devoid of toxicity (Norris et al. 2002). However, 7-deoxyCYN was recently reported to have toxic potency similar to CYN, suggesting that the toxicological studies on this structural variant are controversial and the potential risks should be clarified

(Neumann et al. 2007). CYN is zwitterionic and highly water soluble because of the negatively charged sulphate group and the positively charged guanidine group (Falconer & Humpage 2006). It is chemically stable when exposed to sunlight, high temperature and a wide range of pH conditions (Chiswell et al. 1999). Due to its stability, CYN may persist in the environment for extend periods of time.

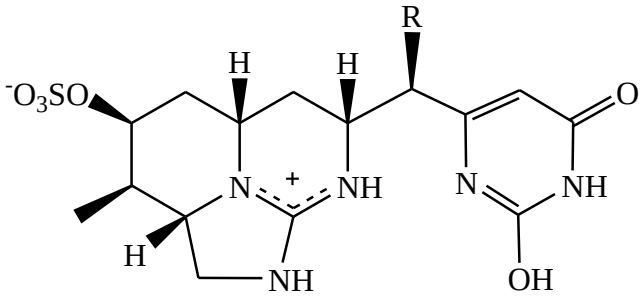

Figure 2 – Chemical structure of cylindrospermopsin. In cylindrospermopsin, R represents OH group while in 7-epicylindrospermopsin R represents OH epimer group. In the other structural variant, 7-deoxycylindrospermopsin, R is H.

CYN has been implicated in a considerable number of animal and human poisonings (Griffiths & Saker 2003; Shaw et al. 1999). One of the most known and serious episodes of human intoxication occurred in 1979 at Palm Island, Queensland, Australia, where over 100 children of Aboriginal families had to be hospitalized with several symptoms of gastroenteritis as a consequence of the usage of CYN contaminated water from a water reservoir (Solomon Dam) treated with copper sulphate to control the CYN-producing *Cylindrospermopsis raciborskii* bloom (Griffiths & Saker 2003).

In comparison with MCs, substantially less attention has been given to CYN, especially from the ecotoxicological point of view. Nevertheless, CYN seems to be gaining some importance taking in consideration the increased number of published reports about its occurrence in Europe (Bláhová et al. 2009; Bogialli et al. 2006; Fastner et al. 2003; Mankiewicz-Boczek et al. 2012; Preussel et al. 2006; Rücker et al. 2007; Spoof et al. 2006).

As for MCs, the toxic effects of CYN have been studied in mammals. More recent CYN-related studies have expanded their focus to other organisms such as invertebrates, fish and plants (Berry et al. 2009; Beyer et al. 2009; Kinnear et al. 2007; Kinnear et al. 2008; Nogueira et al. 2006; Silva & Vasconcelos 2010; Soares et al. 2009; White et al. 2007). However, this expansion is still not enough and the need to more closely study the toxic effects of CYN on a wide range of organisms including microalgae is clearly evident. In mammals, CYN has been discovered to interfere with several metabolic pathways (Kinnear 2010). The main target of CYN toxicity is the liver, although other organs may be damaged following exposure to the cytotoxin such as kidneys, lungs, thymus, spleen, heart, adrenal glands, stomach, intestinal tract and immune system (Falconer & Humpage 2006; Terao et al. 1994). This widespread CYN-induced toxicity depends on the levels and duration of exposure, balanced by CYN absorption, detoxification and excretion. There is evidence that CYN is more toxic in the short term repeated dosing exposure (1-2 weeks) than in the long-term exposure (Shaw et al. 2000). The pathway of CYN uptake is still poorly understood and further investigations with respect to this issue are necessary (Kinnear 2010). CYN is highly hydrophilic and cannot cross the cell membranes, requiring therefore active transport systems to its absorption as MCs (Funari & Testai 2008; Kinnear 2010). Nevertheless, the small size and weight of the molecule (415 Daltons) makes passive diffusion, albeit limited, a real possibility (Funari & Testai 2008; Kinnear 2010).

The principal mechanism of CYN toxicity is the irreversible inhibition of protein synthesis; process most likely mediated by cytochrome P450 (CYP450)-generated metabolites (Froscio et al. 2001; Froscio et al. 2008; Humpage et al. 2005; Terao et al. 1994). Evidence is given that activation of CYN by CYP450 is an important factor for its cytotoxicity but not for its protein synthesis (Froscio et al. 2003; Norris et al. 2002; Runnegar et al. 1995). Thus there appear to be two toxic responses. The first one – acute toxicity – seems to be mediated by CYP450-generated metabolites (probably more toxic than the parent compound) while the second one – longer-term toxicity – is due to protein synthesis inhibition (Froscio et al. 2003; Humpage et al. 2005; Norris et al. 2002; Runnegar et al. 1995). CYN also inhibits GSH synthesis (Humpage et al. 2005; Runnegar et al. 1995; Runnegar et al. 2002).

Moreover, CYN has demonstrated genotoxic effects in mammals both *in vitro* and *in vivo* (Humpage et al. 2005; Shen et al. 2002). The depletion of GSH could lead to an increase in oxidative stress that could contribute to its genotoxicity. Nevertheless, oxidative stress and ROS formation were found not to be likely involved in its genotoxicity, as no changes in lipid peroxidation were observed after exposure to CYN (Humpage et al. 2005). Genotoxicity seems to be caused by the ability of CYN itself or CYP450-generated metabolites to induce DNA strand breaks and chromosome loss (aneuploidy) (Humpage et al. 2000; Shen et al. 2002).

In addition, there are still preliminary evidences indicating that CYN may act as a carcinogen *in vivo* (Falconer & Humpage 2001). The uracil group could possibly interact with adenine groups in RNA and DNA, interfere with DNA synthesis, induce mutations and consequently cause carcinogenicity (Žegura et al. 2011). Studies related to CYN-mediated carcinogenesis are scarce and the underlying mechanisms of carcinogenicity are not well understood (Žegura et al. 2011). Although there are not enough data available to assess the potential of CYN for carcinogenicity, U.S.EPA has classified it on the list of compounds with high priority for hazard characterization (U.S.EPA 2010).

Taken into account the CYN-related toxicity and the evidence for carcinogenicity, Humpage & Falconer (2003) proposed a provisional guideline value of 1 $\mu g.L^{-1}$ for CYN in drinking water. Despite the available literature on the CYN toxicity, WHO believes its adequacy for toxicological assessment is questionable and therefore do not propose any guideline value.

1.3. Cyanobacterial blooms

Cyanobacterial blooms in freshwater, brackish and coastal marine ecosystems have become a major environmental problem worldwide (Bláha et al. 2009). Since the 60's, the number of publications and reports about cyanobacterial blooms has globally increased, primarily in freshwater and estuarine environments, in part due to the increased monitoring efforts (Carey et al. 2012).

Blooms of cyanobacteria have been generally associated with eutrophication processes (i.e. increased input of nutrients, especially phosphorous but also nitrogen). The increase of human population and the consequent intensification of urban, agricultural and industrial activities have promoted the enhancement of eutrophication in aquatic ecosystems. Anthropogenic induced eutrophication in many freshwater and brackish ecosystems along with specific and favorable environmental conditions such as high temperatures, elevated light intensity and low turbulence, may favor periodic proliferation and dominance of cyanobacteria, both in planktonic and benthic environments (Figure 3). Mass development of cyanobacteria often gives rise to harmful cyanobacterial blooms (HABs) which may create significant water quality and aesthetics problems and severe impacts on aquatic communities (Bláha et al. 2009; Lindholm et al. 1989; Naselli-Flores et al. 2007; Sukenik et al. 1998; Zurawell et al. 2005). Although the massive proliferation of cyanobacteria might be a natural event, the notable nutrient over-enrichment associated with the increasing levels of human activity has been leading to higher HAB frequencies. Climatic changes, especially the predicted rise of global temperatures and the changes in the global hydrological cycle (expressed as changes in precipitation and drought) are thought to benefit such HAB occurrence in fresh and brackish water systems by increasing not only its frequency and intensity but also its dominance, duration, persistence and geographic distribution (El-Shehawy et al. 2012; Elliott 2012; Paerl & Huisman 2008; Paerl & Huisman 2009; Paerl & Paul 2012). Some studies suggest that eutrophication and global warming may act synergistically in promoting proliferation, dominance and persistence of HABs (El-Shehawy et al. 2012; Elliott 2010; Elliott 2012; Paerl & Paul 2012; Wagner & Adrian 2009). However, the relative importance of global warming versus eutrophication in freshwater was questioned, since decreasing phosphorus and nitrogen inputs can counteract the direct positive effect of warming temperatures on bloom proliferation (Brookes & Carey 2011).

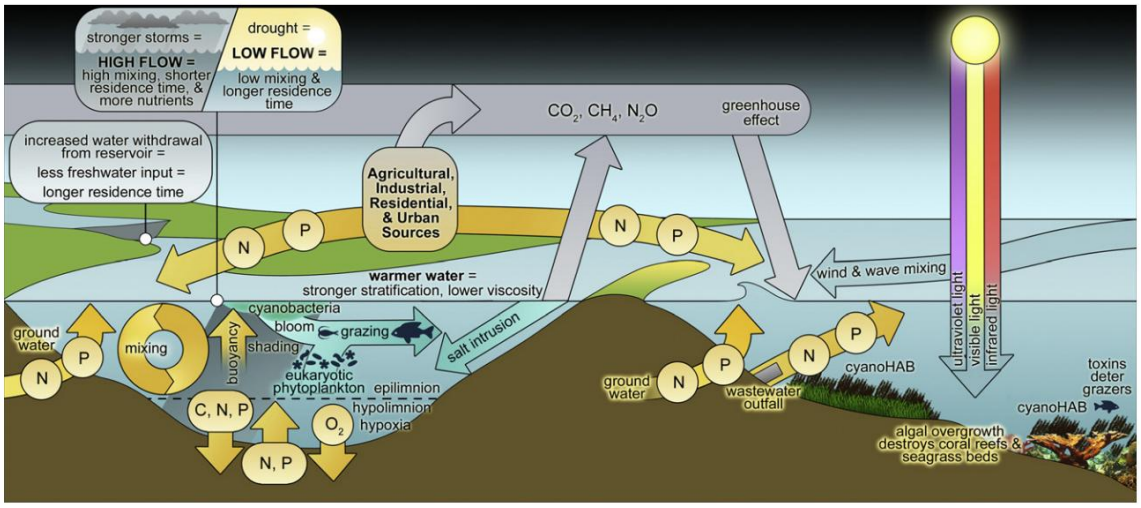

Figure 3 – Conceptual diagram, illustrating the multiple interacting environmental factors controlling harmful cyanobacterial bloom formation and proliferation along the freshwater-marine continuum. Key factors controlling cyanobacterial growth and dominance such as nutrient inputs/availability, water column transparency, mixing conditions, water residence times, temperature and grazing are shown. From Paerl & Paul (2012).

HABs may have numerous consequences. The first consequence of HAB occurrence is the water quality reduction, which may lead to negative economical, ecological and public health implications (Lindholm et al. 1989; Naselli-Flores et al. 2007; Sukenik et al. 1998). From an ecological point of view, HABs may impact ecosystem functioning by disturbing the relationships among organisms, declining biodiversity at all trophic levels and deteriorating habitats (Bláha et al. 2009; de Figueiredo et al. 2006). As high densities of cyanobacteria may concentrate at the water surface forming scums, HBAs increase the turbidity and hence block light penetration in the water column in impacted ecosystems. This, in turn, reduces the phytoplankton growth and suppresses the establishment and growth of aquatic macrophytes and benthic microalgae, negatively affecting planktonic and benthic flora and fauna (Paerl & Huisman 2009; Paerl & Paul 2012; Vasconcelos 1995). On the other hand, HABs cause nighttime oxygen depletion and production of substances such as ammonia resulting from respiration and bacterial decomposition of dense blooms. The anoxic conditions and ammonia release may lead to the death of many fish populations and loss of benthic infauna and flora (Paerl & Paul 2012; Vasconcelos 1995). In addition, HABs may also cause aesthetic problems as well as changes in organoleptic properties of the water by the production of noxious substances that gives an unpleasant and intense taste and a characteristic earthy-musty odour such as

geosmin and 2-methylisoborneol (Vasconcelos 1995). Lastly, HABs may become dangerous due to the ability of many cyanobacteria species to produce potent cyanotoxins, which are a major threat for several organisms, mainly aquatic organism, and human drinking and irrigation water supplies, fisheries and recreational resources (Paerl & Huisman 2009; Paerl & Paul 2012).

During HAB development, cyanotoxins such as MCs and nodularins are largely retained within the cyanobacterial cells (Wiegand & Pflugmacher 2005). Cyanotoxins such as CYN may be continuously liberated to the water due to its hydrophilic nature and apparent membrane permeability (Shaw et al. 1999; Wiegand & Pflugmacher 2005; Wormer et al. 2008). However, high concentrations of cyanotoxins are released into the surrounding medium upon senescence and/or cell lyses. Concentration of dissolved MCs between 10-50 $\mu g.L^{-1}$ and 350 $\mu g.L^{-1}$ have been reported, but much higher levels (up to 25000 $\mu g.L^{-1}$) can occur in the water after collapse of HABs (Fastner et al. 1999; Kemp & John 2006; Máthé et al. 2007; Nagata et al. 1997). On the contrary, CYN may be found at concentrations between 11.8 and 800 $\mu g.L^{-1}$ (Bogialli et al. 2006; Gallo et al. 2009; Griffiths & Saker 2003; Rücker et al. 2007). Nevertheless, these high levels are usually not long-lasting due to strong dilution in the water body, wind mixing, adsorption to the sediment and (bio)degradation (Funari & Testai 2008).

Once released in the water, cyanotoxins may persist in the aquatic environment depending on local environmental conditions and endemic bacterial population (Jones & Orr 1994). MCs may persist in water for relatively long time, ranging between 21 days and 2-3 months (Funari & Testai 2008; Jones & Orr 1994), while CYN may persist until 40 days with no degradation by co-occurring natural bacterial communities (Wormer et al. 2008). In surface waters, a half-life of 11-15 days was reported for CYN (Chiswell et al. 1999). Considering the stability and persistence of cyanotoxins, a wide range of aquatic organisms including microalgae and aquatic plants may be directly exposed to cyanotoxins via food and/or dissolved in the water during long periods of time, which may cause diverse toxic effects. Some toxic effects of MCs on aquatic microalgae are shown in Table 2. Ingestion and accumulation of these cyanotoxins may subsequently be transferred possibility along the food chain and cause serious animal and human health concerns.

Table 2 – Review on the studies investigating the effects of MCs on aquatic microalgae from literature.

Species	Investigated variant of MC	Exposure concentrations ($mg.L^{-1}$)	Exposure duration	Observed effects	Reference
OCHROPHYTA					
Poterioochromonas sp.	MC-LR, -RR	0.1-4	16 days	Growth stimulation (≥ 1 $mg.L^{-1}$)	Ou et al. (2005)
Poterioochromonas sp.	MC-LR	0.5	3 days	Increase in SOD activity, enhanced lipid peroxidation, non-significant changes in GSH pool	Ou et al. (2005)
Poterioochromonas sp.	MC-LR	1	15 days	Changes in cell ultrastructure (vacuolization, chloroplast swelling), low cellular viability (similar to programmed cell death)	Ou et al. (2005)
Poterioochromonas sp.	MC-LR	1.05-2.5	5-10 days	Biodegradation of MC-LR (most probably due to activity of extracellular substances), no accumulation of MC-LR in the cells observed	Ou et al. (2005)
CRYPTOPHYTA					
Cryptomonas erosa	MC-RR	0.519	12 days	Growth stimulation (after 6 days) followed by the growth inhibition (after 10 days) accompained by the cell degradation in low-light conditions	Sedmak & Kosi (1998)
Cryptomonas ovata	Pure MC-LR	0.02-1.06	14 days	Growth inhibition (at least in the first 4-6 days)	B-Béres et al. (2012)
Cryptomonas ovata	CCEs containing MCs	0.02-1.06	14 days	Growth stimulation (at 0.09 and 0.53 $mg.L^{-1}$), growth inhibition (at 1.06 $mg.L^{-1}$)	B-Béres et al. (2012)
Cryptomonas ovata	MC-LR	2.5-50	3 days	Growth inhibition	B-Béres et al. (2012)
DINOFLAGELLATA					
Peridinium galunense	MC-LR	0.05	24h	Increased formation of ROS (within 24h), effects on activity of protein kinases (0.5-22h), no inhibition of photosynthesis	Sukenik et al. (2002), Vardi et al. (2002)
CHLOROPHYTA					
Chlamydomonas reinhardtii	CCEs containing semi-purified MCs	0.01	12 days	Growth inhibition	Kearns & Hunter (2000)
Chlamydomonas reinhardtii	MC-LR	0.01		Inhibition of motility (12 days of exposure); enhanced settling rate (1h exposure)	Kearns & Hunter (2001)
Chlamydomonas reinhardtii	Pure MC-LR, -RR	0.001-25	11 days	Growth inhibition (at 25 $mg.L^{-1}$)	Babica et al. (2007)
Chlorella kesslerii	Pure MC-LR, -RR	0.001-25	11 days	Growth inhibition (at 25 $mg.L^{-1}$)	Babica et al. (2007)

Table 2 (continued)

Species	Investigated variant of MC	Exposure concentrations ($mg.L^{-1}$)	Exposure duration	Observed effects	Reference
Chlorella vulgaris	Pure MC-LR, CCE containing MC-LR	0.001-0.1	14 days	Growth inhibition (only at 3rd day), decrease of pigment content (chlorophyll a and b and carotenoids), accumulation of MC-LR in the cells observed (only at the 3rd day), overproduction of intracellular polysaccharides, great amounts of extracellular polysaccharides in the medium, enhanced lipid peroxidation (only at 3rd day), decrease of GSH content (only at 3rd day), increase in GST and GPx activity (only at 3rd day).	Mohamed (2008)
Coelastrum microporum	MC-RR	0.104 and 0.519	14 days	Growth stimulation (after 8/10 and 10 days at lower and higher concentrations) followed by the growth inhibition (after 12 days) under low-light conditions	Sedmak & Kosi (1998)
Dunaliella tertiolecta	MC-LR	0.0001-0.1	24h	Inhibition of chlorophyll a accumulation under low-light conditions, no growth inhibition	Escoubas et al. (1995)
Monoraphidium contortum	MC-RR	0.104 and 0.519	16 days	Growth stimulation (after 10 days) under low-light conditions	Sedmak & Kosi (1998)
Nephroselmis olivacea	MC-LR	0.157	10 day	Weak growth inhibition	Christoffersen (1996)
Pediastrum duplex	Pure MC-LR, -RR	0.001-25	11 days	Growth inhibition (≥ 5 $mg.L^{-1}$ for MC-LR, at 25 $mg.L^{-1}$ for MC-RR)	Babica et al. (2007)
Pseudokirchneriella subcapitata	Pure MC-LR, -RR	0.001-25	11 days	Growth inhibition (≥ 1 $mg.L^{-1}$)	Babica et al. (2007)
Pseudokirchneriella subcapitata	Pure MC-LR, -RR	0.3	10 days	No growth alteration (10 days of exposure), increase in GR activity after 3 and 24h (168h exposure), non-significant changes in GSH pool and GST and GPx activity (168h exposure)	Bartova et al. (2011)
Pseudokirchneriella subcapitata	CCE containing MCs	0.3	10 days	No growth alteration (10 days of exposure), non-significant changes in GSH pool and GR, GST and GPx activity (168h exposure)	Bartova et al. (2011)
Scenedesmus armatu	Pure MC-LR, -RR; CCE	0.00025	1h	Pure MCs increased POD and sGST activities and had no effect on photosynthesis; exposure to CCE elevated POD activity but inhibited sGST and photosynthesis	Pietsch et al. (2001)
Scenedesmus quadricauda	MC-RR	0.104 and 0.519	16 days	Growth stimulation (after 10 days) under low-light conditions	Sedmak & Kosi (1998)
Scenedesmus quadricauda	MC-LR, -RR, -YR	0.5	14 days	Induction of cell aggregation, increase of cell volumes and overproduction of chlorophyll a	Sedmak & Elersek (2006)
Scenedesmus quadricauda	Pure MC-LR, -RR	0.001-25	11 days	Growth inhibition (at 25 $mg.L^{-1}$)	Babica et al. (2007)

Table 2 (continued)

Species	Investigated variant of MC	Exposure concentrations ($mg.L^{-1}$)	Exposure duration	Observed effects	Reference
Scenedesmus quadricauda	Pure MC-LR, CCE containing MC-LR	0.001-0.1	14 days	Growth inhibition (only at the 3rd day), decrease of pigment content (chlorophyll a and b and carotenoids), accumulation of MC-LR in the cells observed (only at the 3rd day), overproduction of intracellular polysaccharides, great amounts of extracellular polysaccharides in the medium, enhanced lipid peroxidation (only at 3rd day), decrease of GSH content (only at 3rd day), increase in GST and GPx activity (only at 3rd day)	Mohamed (2008)
CHAROPHYTA					
Klebsormidium sp.	Pure MC-LR, -RR, -YR	2.5-50	14 days	No growth alteration	Valdor & Aboal (2007)
Klebsormidium sp.	CCEs	1.33-30.78 (total MCs)	14 days	No growth alteration, cell deformation, thylakoidal disaggregation	Valdor & Aboal (2007)

MC, microcystin; L, leucine; R, arginine; Y, tyrosine; CCE, cyanobacterial crude extract; SOD, superoxide dismutase; GSH, glutathione; ROS, reactive oxygen species; sGST, soluble glutathione *S*-transferase; GPx, glutathione peroxidase; GR, glutathione reductase; POD, peroxidase.

1.4. Toxic metals and pesticides in aquatic environments

Pollution by metals and pesticides is another major problem in aquatic ecosystems. The intensive use of pesticides in agricultural practices may lead to the contamination of surface and ground waters by drift, runoff, drainage and leaching. In recent years, the pollution of water by pesticides has been of great concern due to the increasing number of pesticides used and detected in water as well as to their persistence, mobility and toxicity as well as their metabolites. On the other hand, the development of human activities and industrialization has led to an increased accumulation of metals in the aquatic environment over the last century. Unlike pesticides, metal compounds cannot be degraded or destroyed; instead, they can be bioaccumulated by aquatic organisms and also take part in the process of biomagnification. Metal compounds are also one of the most persistent pollutants in the aquatic environment, thus constituting a worldwide problem. Some of the most common toxic metals and pesticides that can be found in the environment and therefore be used as chemical substance models in ecotoxicology are the cadmium and the terbuthylazine, respectively.

1.4.1. Cadmium

Cadmium (Cd) is a metallic element (A_r 112.4) which is widely distributed in the Earth's crust in very small amounts (IHCP 2007; OECD 1994). In nature, this metal always occurs as a minor constituent in most of zinc (Zn), lead (Pb) and copper (Cu) ores, the main deposits of Cd, and hence is closely linked to the production of these non-ferrous metals (OECD 1994; OSPAR 2002). Consequently, Cd is obtained mainly as a by-product from mining, smelting and refining sulphide ores of Zn and, to a lesser degree, Pb and Cu (OECD 1994; OSPAR 2002).

Cd exhibits excellent resistance to corrosion processes to low melting temperatures and it has both high electrical and thermal conductivity (Marcano et al. 2009). Due to this

great variety of unique properties, this element is used in the manufacture of pigments, stabilizers of PVC, alloys, metallic coatings, electronic components and rechargeable nickel-cadmium (Ni-Cd) batteries. Ni-Cd batteries are by far the most significant application of Cd (OECD 1994). Direct releases of Cd to the environment or significant exposures of populations to Cd are unlikely from the use of these products. The manufacture and disposal of these products, however, have the potential for releasing Cd to the environment (OECD 1994). During the 1990s, Cd usage has decreased considerably in European countries, mainly due to the gradual phase-out of Cd products other than Ni-Cd batteries and the implementation of more stringent environmental legislation in the EU (Jarup 2003). However, Cd production, consumption and emissions to the environment have increased worldwide dramatically during the 20th century, since Cd containing products are rarely recycled but frequently dumped together with household waste, thereby contaminating the environment (Jarup 2003).

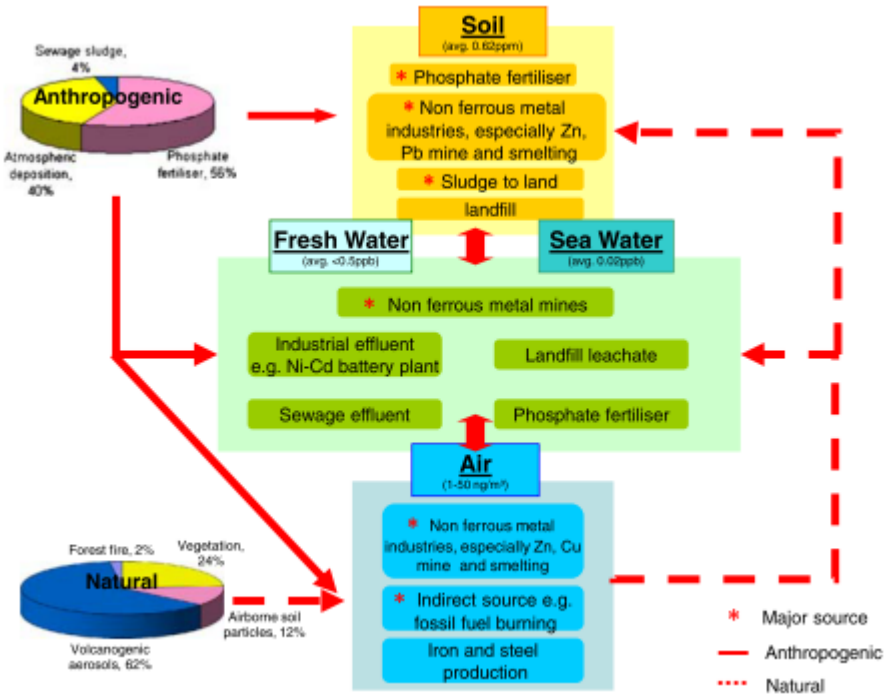

Figure 4 – Source-pathway model for general cadmium flow. From Pan et al. (2010).

Cd is released by several natural and anthropogenic sources to the atmosphere, aquatic environments (fresh and salt water environments) and terrestrial environments,

thereby existing fluxes between these compartments (Figure 4). The most significant releases to the aquatic environment arise from the volcanic activity and weathering of rocks – *natural sources* – and production of non-ferrous metals (Zn, Pb and Cu), production of iron and steel, atmospheric deposition, direct discharges from industrial operations, domestic wastewaters, leakage from landfills and contaminated sites, production and use of phosphate fertilizers, application of manure and sewage sludge in agriculture – *anthropogenic sources* (OECD 1994). Anthropogenic sources account for more than 90% of Cd in the surface environments (Pan et al. 2010).

Once released to the aquatic environment, much of Cd is rapidly adsorbed by particulate matter, which may remain suspended or settle down depending on natural or man-induced physic-chemical conditions (e.g. pH, water hardness, suspended matter levels, redox potential, salinity) and on man-made interventions (e.g. dredging) (OECD 1994; OSPAR 2002). Levels of dissolved Cd may hence be low even in rivers receiving and transporting large quantities of the metal (Pan et al. 2010).

Generally, Cd may be present in aquatic environmental as the hydrated ion ($Cd^{2+}.6H_20$), as inorganic complexes with CO_3^{2-}, OH^-, Cl^- or SO_4^{2-}, or as organic complexes with humic acids (OSPAR 2002). The free metal ion Cd^{2+} is considered the most toxic species (IHCP 2007). The bioavailability and potential for toxic effects depends to a great extent on the form of Cd. For example, $CdSO_4$ (cadmium sulphate) and $CdCl_2$ (cadmium chloride) are quite soluble in water, whereas $CdCO_3$ (cadmium carbonate) is practically insoluble in water (OECD 1994; OSPAR 2002). Cd is persistent (it cannot be broken down into less toxic substances) in the environment (Robards & Worsfold 1991) and tend to bioaccumulate throughout the food chain (McLaughlin et al. 1999).

Cd is considered to be a non-essential metal for animal or plant life, even though Cd has showed nutritional beneficial effects to the growth-inhibited marine diatom *Thalassiosira weissflogii* under conditions of Zn limitation (Lee et al. 1995; Stohs & Bagchi 1995). In this case, growth recovery resulted from formation of a specific Cd-carbonic anhydrase, a zinc-dependent enzyme involved in carbon acquisition (Cullen et al. 1999; Lee et al. 1995).

Cd in the environment has been a concern since 1960s, when a painful bone disease (a combination of osteomalacia and osteoroporis) called “itai-itai” (ouch-ouch) was reported to have been caused by Cd-contaminated water used for irrigation of local rice fields in an area in Japan (Jarup 2003; Pan et al. 2010). Since then, an increasing number of studies have reported adverse health effects of Cd exposure in general population, even at much lower levels than previously anticipated (Jarup & Åkesson 2009).

Cd is one of the most toxic metals to aquatic organisms, even at low concentrations, and may cause long-term adverse effects in the aquatic environment (Deckert 2005; OECD 1994; Simon et al. 2008). According to the European commission, some Cd compounds were classified as dangerous for the environment (R50-53) (Annex 1 of Directive 67/548/EEE, 2004). The toxicity of Cd to aquatic organisms depends on the exposure duration, species and life-stage, being the early-stages and the reproductive system the most vulnerable (OECD 1994). In aquatic systems, Cd is most readily absorbed by organisms directly from the water in its free ionic form (OSPAR 2002) and hence high concentrations may bioaccumulate in aquatic vertebrates and invertebrates and algae. The rate of uptake and the toxic impact of Cd on aquatic organisms vary considerably even between closely related species, and are greatly related to physic-chemical factors of the water such as free ionic concentration of the metal, temperature, pH, hardness and organic matter content (OECD 1994). Cd interacts with calcium metabolism of aquatic organisms. In fish, Cd may cause hypocalcaemia (low calcium levels), probably by inhibiting calcium uptake from the water (OECD 1994). Effects of long-term exposure may include mortality and temporary reduction in growth (OSPAR 2002).

Effects of Cd on algae have been extensively studied over the last years at the level of growth processes (Bišová et al. 2003; Huang et al. 2009; Lam et al. 1999; Marcano et al. 2009; Monteiro et al. 2011; Pereira et al. 2005; Qian et al. 2009; Visviki & Rachlin 1994a), photosynthesis (Awasthi & Das 2005), chlorophyll content (Marcano et al. 2009; Qian et al. 2009; Vymazal 1987) and ultrastructural changes (Torres et al. 2000; Visviki & Rachlin 1992; Visviki & Rachlin 1994b). However, the molecular mechanism underlying Cd toxicity are still not completely understood (Faller et al. 2005). Cd can interfere with a great number of metabolic processes, altering enzymatic activities by nonspecific binding

to functional groups necessary for catalytic activity such as sulphydryl (-SH) and carboxyl (-COOH) or by replacing some essential metallic elements with similar molecular size and charge such as Ca and Zn that play a key role in active sites of enzymes, thus resulting in inhibition of photosynthesis, respiration, biosynthesis of photosynthetic pigments and cell growth (Aravind & Prasad 2004; Báscik-Remisiewicz et al. 2011; di Toppi & Gabbrielli 1999; Prasad et al. 1998). It has been demonstrated that Cd may arrest the photosynthetic electron flow (Voigt & Nagel 2002), inhibit the water-splitting complex of the oxidizing site of photosystem II (PSII) (Mallick & Mohn 2003) or competitively bind to the essential Ca^{2+} site in PSII during photoactivation (Faller et al. 2005).

Oxidative stress seems to be another molecular mechanism of Cd toxicity (Leonard et al. 2004; Pinto et al. 2003; Valko et al. 2005). In contrast with other metallic elements such as Cu and Cr, Cd is not a redox-active metal and therefore cannot itself generate directly ROS through the Fenton type-reaction (Benavides et al. 2005; Bertin & Averbeck 2006; Pinto et al. 2003; Valko et al. 2005). However, Cd may indirectly produce ROS by affecting the cellular antioxidant capacity and general enzyme function through binding to thiols such as GSH (Pinto et al. 2003). This, in turn, results in DNA damage, lipid peroxidation, depletion of GSH, change in calcium and sulphydryl homeostasis, membrane depolarization and acidification of the cytoplasm (Badisa et al. 2007; Conner & Schmid 2003; Valko et al. 2005) that may generate cell death.

As detoxification mechanisms, documented responses to Cd exposure for a great number of organisms involve, beside the production of oxidative stress responses proteins, the synthesis of molecular chaperones (heat shock proteins) to cope with the damaged and misfolded proteins, the activation of membrane transporters that export Cd from the cell or transport it into vacuoles, the production of enzymes in sulphate assimilation, the biosynthesis of GSH, metallothioneins and phytochelatins (Bertin & Averbeck 2006; Clemens 2001; Deckert 2005; Perales-Vela et al. 2006; Robinson 1989).

Cd is also a potent carcinogen and has been associated with cancers of the lung, prostate, kidney and probably with liver, pancreas and stomach, but this evidence is controversial (Bertin & Averbeck 2006; Jarup 2003; Jarup & Åkesson 2009; Valko et al.

2005). Because of its carcinogenic properties, Cd has been classified as a #1 category human carcinogen by the IARC of USA (1997), while the European Commission has classified some Cd compounds as possibly carcinogenic (Carcinogen category 2) (Annex 1 of Directive 67/548/EEE, 2004). Cd has also recently been listed as an endocrine-disrupting substance because of its ability to bind to cellular estrogen receptors and to hence mimic the actions of estrogens (act as an agonist) (Darbre 2006). Furthermore, Cd has been identified as a priority hazardous substance in the field of water quality and therefore extensively included in environmental monitoring programmes (European Commission 2001) as well as a metal of primary interest in the framework for metals risk assessment (U.S.EPA 2007). Cd was also included in 1998 on the OSPAR List of Chemicals for Priority Action (OSPAR 2011) as well as on the HELCOM Recommendation 19/5 List of Substances for Immediate Priority Action (HELCOM 1998). In addition, Cd was chosen by OECD as one of the five chemical to be included in the risk reduction programme (OECD 1994).

1.4.2. Terbuthylazine

Terbuthylazine (TBA) is a relatively widespread triazine selective systemic herbicide with increasing agricultural significance, since it has gradually replaced the better know and longer studied atrazine (another triazine herbicide) which has been banned in the European Union (Dezfuli et al. 2006; Gikas et al. 2012; Steinberg et al. 1994). Chemically, TBA belongs to the cloro-*s*-triazine family with the following structure: N^2-*tert*-butyl-6-chloro-N^4-ethyl-1,3,5-triazine-2,4-diamine (Figure 5).

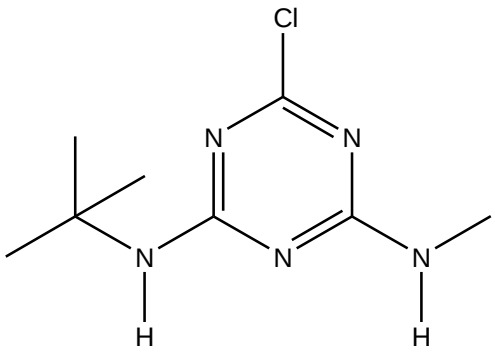

Figure 5 – Chemical structure of terbuthylazine.

TBA has been used as a broad spectrum herbicide for the control of both grasses and broadleaf weeds in agricultural crops such as maize, wheat, sorghum, potatoes, citrus, vines, coffee, olives and forestry, being particularly effective against annual dicotyledons (Gikas et al. 2012; Singh et al. 2001; WHO 2003). It is taken up through the roots and leaves and is distributed throughout the plant after the uptake, which enables it to be used in both pre- and post-emergence treatment (WHO 2003).

The triazine TBA is one of the most used plant protection products (PPPs) in Europe territory in agricultural fields, especially maize (Eurostat 2007). PPPs are defined by plant protection products directive as "active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to: a) protect plants or plants products against all harmful organisms or prevent the action of such organisms (…); b) influence the life processes of plants, other than as a nutrient (e.g. growth regulators); c) prevent plant products (…); d) destroy undesired plants and e) destroy parts of plants, check or prevent undesired growth of plants" (article 2 of 91/414/ECC). In 2010, TBA was the second most sold herbicide in Portugal (DGADR 2011).

s-Triazine herbicides including TBA may be directly or indirectly released into the aquatic environment through effluents discharges from manufacturing facilities and through runoff and accidental spillage during and/or after their application in both agriculture and forestry (Navarro et al. 2004b; Tchounwou et al. 2000). Among *s*-triazines,

TBA is considered to be one of the most persistent in water (Carafa et al. 2007; Guzzella et al. 2006), representing a potential risk to aquatic ecosystems; in riverwater and groundwater, its half-life is 196 and 263-366 days, respectively (Navarro et al. 2004a; Navarro et al. 2004b). Monitoring studies carried out in Europe have been shown that TBA is frequently detected in surface water (Azevedo et al. 2001; de Almeida Azevedo et al. 2000; Lacorte et al. 1998; Loos et al. 2010b; Loos et al. 2007; Palma et al. 2009; Pérez et al. 2010) and groundwater (Guzzella et al. 2006; Hernández et al. 2008; Hildebrandt et al. 2008; Loos et al. 2010a; Mansilha et al. 2011).

Degradation of TBA in aquatic environment depends on bacterial activity and on abiotic factors such as organic matter content, pH and, above all, temperature that directly or indirectly influence the degradation rate (Barra Caracciolo et al. 2010). The main degradation pathways of TBA are dechlorination with concominant hydroxylation and/or dealkylation of amine groups (Gikas et al. 2012) and are summarized in Figure 6. Hydroxy-TBA is the main degradation product in water from an abiotic process while deethyl-TBA and deisopropyl-atrazine result from biotic mechanisms (Gikas et al. 2012). The combination of the two mechanisms (dealkylation and subsequent hydroxylation) gives rise to deethyl-hydroxy-TBA and deisopropyl-hydroxy-TBA (Figure 5). Another major degradation product of TBA, namely N^2-*tert*-buthyl-N^4-ethyl-6-methoxy-1,3,5-triazine-2,4-diamine, was recently identified (Gikas et al. 2012). However, little is known about the toxicological effects of TBA degradation products and how their toxicity compares to that of the parent compound.

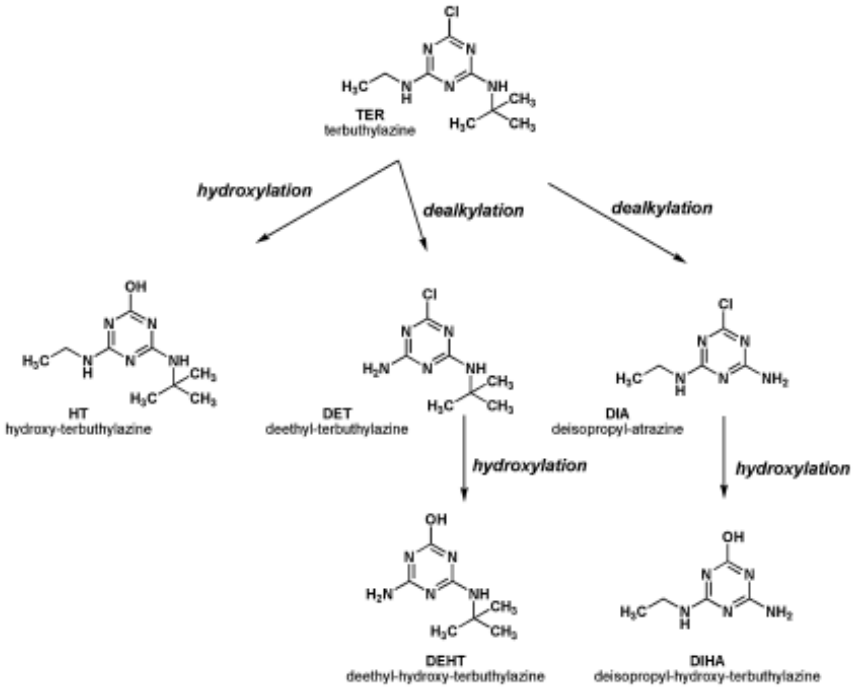

Figure 6 – Major degradation pathways (biotic and abiotic) of terbuthylazine. From Gikas et al. (2012).

The toxic effects of TBA have been primarily studied in terrestrial animals. Once ingested, TBA is quickly absorbed, metabolized and excreted via urine and faeces with a half-life of 16-17h (WHO 2003). Several CYP450 enzymes are involved in the hepatic oxidative phase-I metabolism of TBA in exposed humans and animals (Hodgson 2003; Lang et al. 1996; Lang et al. 1997). However, CYP450 1A2 enzyme alone is probably the most important in the metabolism of TBA, since it is active at low substrate concentrations whereas the other enzymes (e.g. CYP450 3A4 and 2C19) appear to be active at high substrate concentration only (Hodgson 2003; Lang et al. 1997).

Toxicity data involving aquatic organisms, specially algae, and TBA are still limited (Cedergreen & Streibig 2005; Faust et al. 2001; Munkegaard et al. 2008; Pérez et al. 2011). TBA is a specific inhibitor of the photosynthetic electron transport. Its mode of action is through the competitive and reversible binding to the domain of the D1 protein of the PSII reaction center, thus displacing the electron acceptor plastoquinone Q_B from this site (Faust et al. 2001). The interruption of photosynthetic electron transport leads to the concomitant inhibition of ATP production and carbon fixation (van Rensen et al. 1999).

Due to this specific mechanism of action and taken into account that *s*-triazines are designed to kill unwanted plants, aquatic plants and algae are expected to be the most sensitive group of aquatic non-target organisms.

1.5. Models used in mixture toxicity assessment

Aquatic organisms are constantly exposed to a cocktail of different environmental contaminants. However, most of the knowledge and comprehension of chemical contamination effects in aquatic organisms is based upon the effects of single toxicant exposure, instead of exposure to complex environmental mixtures. Exposure to chemical mixtures is a reality and, fortunately, the interest of scientists and regulatory policies in the toxicology and potential risk of combined exposures is growing. As a consequence of this awareness, it is now widely recognized that the adverse effects caused by exposure to chemical mixtures must be an integral part of environmental and human health risk assessment (Groten 2000).

In order to describe and assess the chemical mixture toxicity, two theoretical reference models based on the mode of action (MoA) of single chemicals have been widely used: concentration addition (CA) and independent action (IA). Both models assume no interaction between toxicants in the mixture (meaning that each component does not influence the biological action of the other component present in the mixture) and allow the calculation of expected mixture toxicity on the basis of known individual toxicities of the mixture components.

The CA model assumes that individual toxicants have the same MoA and hence act upon the same molecular target inside the organism (Konemann & Pieters 1996; Loewe & Muischnek 1926). This reference model is defined as a summation of the relative toxicities of each individual component in the mixture (Groten 2000). Mathematically, the CA model may be expressed as:

$$\sum_{i=1}^{n} \frac{c_i}{\mathrm{EC}x_i} = 1$$

where c_i is the concentration of the chemical i in the mixture and $\mathrm{EC}x_i$ is the effect concentration of chemical i that provoked x% if applied singly. The quotient $c_i/\mathrm{EC}x_i$ is also referred as the dimensionless toxic units (TU), which quantifies the contribution of the toxicity of chemical i in the mixture of n chemicals (Jonker et al. 2005). TU gives the contribution of concentration of a component in the mixture scaled for its relative toxicity. This means that given an example where EC_{50} value for a chemical X is 0.5 mg.L^{-1} 1 TU will correspond to the concentration of 0.5 mg X.L^{-1} and 2 and 0.5 TU will correspond to the concentration of 1 mg X.L^{-1} and 0.25 mg X.L^{-1}, respectively. If the sum of the TUs of the mixture components equals 1 at a mixture concentration provoking x% effect, CA holds. Consequently, CA stipulates that one mixture component can be substituted by another chemical with the same mechanism of action, without changing the overall mixture toxicity, as long as its concentration expressed in TU remains the same (van Gestel et al. 2006). The effect concentration (ECx) can be chosen, albeit the level where 50% of effect is observed (EC_{50}) is normally the most relevant, since it is in the middle of the dose response curve and thus less prone to variability.

The CA reference model has predicted successfully the effects of mixtures of similar acting compounds in a large number of toxicological and ecotoxicological studies (Altenburger et al. 2000; Cleuvers 2004; Faust et al. 2001; Junghans et al. 2003; Munkegaard et al. 2008; Pérez et al. 2011; Porsbring et al. 2010). CA has been also proposed as a reasonable model for the worst mixture toxicity assessment scenarios, due to its predictability power even in mixtures with dissimilar compounds (Backhaus et al. 2004; Boedeker et al. 1993; Lock & Janssen 2002).

Alternatively, the IA model assumes that individual toxicants have different MoA and hence acts upon different molecular targets in the organisms (Bliss 1939; Konemann & Pieters 1996). It is based on chemicals working independently, meaning that the relative effect of a toxicant remains unchanged in the presence of another chemical. This reference model calculates the joint effects by multiplying the probabilities of responses of

individual mixture components. Contrary to the CA model, under the IA concept only the components that cause an effect are considered, meaning that components present at doses or concentrations below effect threshold (i.e. EC_0) will not contribute to the toxicity of the mixture and if this condition is fulfilled for all components there will be no combination effect (van Gestel et al. 2006). Mathematically, the IA model may be formulated as:

$$Y = u_{\max} \prod_{i=1}^{n} q_i(c_i)$$

where Y denotes the biological response, c_i is the concentration of chemical i in the mixture, $q_i(c_i)$ the probability of non-response, $u_{\max}$ the control response for endpoints and $\prod$ the multiplication function (Jonker et al. 2005).

The IA model has been able to predict the effects of mixtures of dissimilarly acting chemicals in a large number of toxicological and ecotoxicological studies (Backhaus et al. 2000; Faust et al. 2003; Jonker et al. 2004; Pérez et al. 2011; Syberg et al. 2008; VanGestel & Hensbergen 1997; Walter et al. 2002). IA has been also reported to predict the effects of similar acting compounds (Cedergreen et al. 2008; Syberg et al. 2009).

However, the application of the theoretical reference models is not always linear. Chemical mixtures may also be composed by chemicals whose MoA is unknown or ambiguous. In such cases, both CA and IA models may be used for toxicity prediction and then the one showing the best fit on the data is chosen over the other. MoA ambiguity may be explained by the toxicodynamic properties, i.e., individual toxicants in a mixture may act in the same targets (e.g. enzyme, cell or organ), but they may impair different physiological processes (Loureiro et al. 2010).

Deviations from the reference models may also occur when chemicals affect the bioavailability of one another (related to the environmental conditions), their MoA and their behavior after uptake (e.g. bioaccumulation) (Loureiro et al. 2010). In a mixture, some compounds may interact synergistically or antagonistically becoming more or lower toxic than expected, respectively, by the CA and IA models from the toxicity of single

compounds. More complex interactions between different compounds may also take place depending on the mixture dose level (DL, deviations from the reference models are different at low and high dose levels) or on the dose ratio (DR, deviations from the reference models is dependent of the mixture composition, i.e., which chemical is mainly responsible for toxicity) (Jonker et al. 2005).

1.6. Rationale and aims

The idea for the present work came from the following:

- **i.** Ecotoxicological risk of chemical mixtures of cyanotoxins in aquatic environments is still unknown;
- **ii.** Ecotoxicological risk of chemical mixtures of cyanotoxins with anthropogenic environmental contaminants in aquatic environments is still poorly investigated.

Taken into consideration the topics pointed out above, the present work was undertaken with three main objectives:

1. Evaluation of the toxicity of the cyclic peptide MC-LR and the alkaloid CYN and their respective combination for the growth rate of the freshwater algae *Chlorella vulgaris*;
2. Evaluation of the joint effects of MC-LR and CYN with anthropogenic environmental contaminants representatively present in aquatic systems on the growth rate of the freshwater algae *C. vulgaris*. The anthropogenic environmental contaminants selected were: pesticides, in this case *s*-triazine herbicide TBA, and metals, in this case Cd;
3. Evaluation of the effects of the *s*-triazine TBA and the metal Cd in both single and combined exposures on the growth rate of *C. vulgaris*.

1.7. Relevance of the study

The growing massive proliferations of cyanobacteria (HABs) in freshwater, brackish and coastal marine waters pose an environment and public health risk because of the ability of some cyanobacterial species to produce cyanotoxins. Among these cyanotoxins, CYN is of increasing concern due to its potent cytotoxic effects and the growing number of worldwide locations in which CYN has been reported during the last years. However, not much is known about the toxic effects of CYN on microalgae, an ecologically important group which plays an essential role as primary producers in aquatic food chains.

Since massive proliferations of cyanobacteria are quite often characterized by the presence of a mixture of cyanotoxins, aquatic organism may likely be exposed to combinations of different cyanotoxins. Nevertheless, this issue has not received much scientific attention so far. To our knowledge, there is only one paper published on the study of the effects of simultaneous exposure to cyanobacterial extracts containing CYN and MC-LR on the aquatic plant *Oryza sativa* (Prieto et al. 2011).

Cyanotoxins may also occur simultaneously with other stressors in a cyanobacterial bloom, e.g., anthropogenic environmental toxicant such as metals and pesticides. However, the interaction between cyanotoxins and anthropogenic environmental contaminants has not been sufficiently investigated until now.

Given the number of studies which have been reported the occurrence of TBA in surface waters, it is also important to understand the toxic effects of TBA on non-target aquatic organisms, especially aquatic plants and algae. However, few studies have investigated the TBA effects on aquatic plants and algae (Faust et al. 2001; Munkegaard et al. 2008; Nitschke et al. 1999; Pérez et al. 2011). So far, TBA toxicity on the freshwater algae *Chlorella vulgaris* has not been investigated.

1.8. Dissertation organization

The present dissertation was organized in four chapters:

- **Chapter 1:** current "General Introduction" to the themes of cyanobacterial ecology and secondary metabolites, cyanobacterial blooms, pollution of aquatic environment by pesticides and metals as well as mixture toxicity assessment;
- **Chapter 2:** "The interactive effects of microcystin-LR and cylindrospermopsin on the growth rate of the freshwater algae *Chlorella vulgaris*", where the effects of MC-LR and CYN on the growth rate of *C. vulgaris* after 4 and 7 days of exposure were assessed in both single and binary combinations using Z8 medium as exposure medium. For the assessment of cyanotoxins' combination, the MIXTOX tool was used to evaluate if the reference model IA described the data obtained or if deviations (interactions) between cyanotoxins occurred in the mixture.
- **Chapter 3:** "Effects of binary mixtures of cyanotoxins and xenobiotics on the growth rate of *Chlorella vulgaris*", where the growth response of *C. vulgaris* exposed to four single compounds (MC-LR, CYN, TBA and Cd) and five binary mixtures (MC-LR + TBA; MC-LR + Cd; CYN + TBA; CYN + Cd; TBA + Cd) was investigated at 4^{th} and 7^{th} days using Z8 medium as exposure medium. Again, for the evaluation of the joint effects, the MIXTOX tool was used in order to see if the reference model IA described the data or if deviations (interactions) between the compounds occurred in the binary mixtures.
- **Chapter 4:** "General discussion and final considerations", where the main results were discussed in terms of biological mechanisms underlying the combined effects of the several mixtures performed. In conclusion, an encompassing perspective of the suitability of assessing chemical mixtures instead of the single exposure approach usually followed in ecotoxicological studies was accomplished.

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Chapter II

2. The interactive effects of microcystin-LR and cylindrospermopsin on the growth rate of the freshwater algae *Chlorella vulgaris*

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Abtract

Microcystin-LR (MC-LR) and cylindrospermopsin (CYN) are the most representative cyanobacterial toxins. They have been simultaneously detected in water, but their combined ecotoxicological risk to aquatic organisms, especially microalgae, is unknown. In the present study, we examined the effects of these cyanotoxins individually and as a binary mixture on the growth rate of the freshwater algae *Chlorella vulgaris*. Using the MIXTOX tool, the reference model independent action (IA) was selected to evaluate the combined effects of MC-LR and CYN on the growth of the freshwater algae due to their dissimilar modes of action. Deviations from the IA model such as synergism/antagonism, dose ratio and dose level dependency were also assessed. In single exposures, our results demonstrated that MC-LR and CYN had different impacts on the growth rate of *C. vulgaris* at the highest tested concentrations, being CYN the most toxic. In mixture exposure trial, MC-LR and CYN showed a synergistic deviation from the conceptual model IA as the best descriptive model. From these results, the combined exposure of MC-LR and CYN should be considered for risk assessment as this study has shown that more severe effects than expected by single cyanotoxin exposure might be observed. This study also represents an important step to understand the interactions among MC-LR and CYN detected previously in aquatic systems.

Keywords: Cyanotoxin, Microcystin-LR, Cylindrospermopsin, *Chlorella vulgaris*, Combined toxicity, Independent action

2.1. Introduction

Cyanobacterial blooms in eutrophic water bodies have become a serious environmental problem worldwide, as many genera of bloom-forming cyanobacteria are able to produce potent cyanotoxins that are released in significantly high concentrations into the aquatic environment when cell ruptures (Bláha et al. 2009; Codd 1995; Codd et al. 2005; Prasanna et al. 2010; Wiegand & Pflugmacher 2005). These cyanotoxins include hepatotoxins, neurotoxins, cytotoxins and dermatotoxins and irritant toxins, representing a major health hazard for animals and humans (Codd et al. 1999; Falconer 1999; van Apeldoorn et al. 2007). Furthermore, there is a prediction that such cyanobacterial blooms are likely to increase in prevalence and magnitude in the future with climate changes, especially with the predicted rise of global temperatures (Paerl & Huisman 2008; Paerl & Huisman 2009; Paerl & Paul 2012). With this predicted rising of cyanobacterial bloom occurrence, the release of high cyanotoxin concentrations could considerably become more common in the aquatic environment, and therefore instigating the evaluation of a wide range of concentrations which include extremely high concentrations.

The majority of cyanotoxin poisoning reports have been directly related to two toxin groups, microcystins (MCs) and cylindrospermopsin (CYN) (Falconer & Humpage 2005). Both cyanotoxins are produced by a larger number of cyanobacterial species around the world (Prasanna et al. 2010; Wiegand & Pflugmacher 2005). MCs are the most common and ubiquitous cyanotoxin in brackish and freshwater blooms (Zurawell et al. 2005) and to date more than 80 MC variants have been isolated and identified, being MC-LR the most common and toxic variant (Dittmann & Wiegand 2006; Hoeger et al. 2005). They are stable cyclic heptapeptides (Duy et al. 2000; Tsuji et al. 1994; van Apeldoorn et al. 2007), whose mechanism of toxicity is mainly based on the induction of oxidative stress (Amado & Monserrat 2010) and inhibition of protein phosphatases 1 and 2A (Gulledge et al. 2002; Mackintosh et al. 1990; Mezhoud et al. 2008; Runnegar et al. 1995a) in aquatic animals and higher plants. CYN is a widespread (Falconer & Humpage 2006; Fastner et al. 2007; Quesada et al. 2006; Spoof et al. 2006) and stable (Chiswell et al. 1999; Wormer et al. 2008) tricyclic alkaloid. Only two CYN variants have been reported: 7-epiCYN, with

similar toxicity to CYN (Banker et al. 2000), and 7-deoxyCYN, whose toxicity is well recognized by some authors (Neumann et al. 2007), but was questioned by others (Norris et al. 1999; Norris et al. 2002). It is established that CYN may act through the glutathione and protein synthesis inhibition in mammals (Froscio et al. 2001; Froscio et al. 2008; Runnegar et al. 1995b; Terao et al. 1994), a process likely mediated by cytochrome P450 (CYP450)-generated metabolites (Humpage et al. 2005).

Individual blooms may contain multiple cyanobacterial species in the same water body and many cyanobacterial strains may produce more than one type of cyanotoxin as well as different congeners of the same type of cyanotoxin (e.g. MC congeners). Some studies have reported the concurrent presence of MC-LR and CYN in water (Bogialli et al. 2006; Brient et al. 2009; Oehrle et al. 2010) as well as the coexistence of potentially MC-LR and CYN-producing cyanobacteria (Bláhová et al. 2009; Kokociński et al. 2009; Vasas et al. 2004). Therefore, it might be expected that when MC-LR and CYN occur simultaneously in the water, and persist for days in the region as blooms (Eaglesham et al. 1999; Lahti et al. 1997), they have serious combined impacts on aquatic organisms, including microalgae. Although the single toxic effects of MC-LR and CYN on aquatic invertebrates, fish, plants and algae have been widely investigated as pure cyanotoxins (Babica et al. 2007; Beyer et al. 2009; Chen et al. 2005; Lindsay et al. 2006; Máthé et al. 2009; Metcalf et al. 2002; Ortiz-Rodríguez & Wiegand 2010; Wiegand et al. 2002) and/or MC-LR- and CYN-containing crude extracts (Dao et al. 2010; Kinnear et al. 2008; Oberemm et al. 1997; Pflugmacher et al. 1999; Prieto et al. 2011), information available on their combined effects is still scarce. To date, there is only one study on plants, showing that the exposure of the rice plant *Oryza sativa* to a mixture of MC-LR and CYN-containing crude extracts induced a synergistic effect on the anti-oxidative enzyme activities (Prieto et al. 2011). Given the co-occurrence, stability and persistence of MC-LR and CYN, it is important to assess the extent to which synergism is a concern.

The aim of this study was thus to examine the effects of MC-LR and CYN, independently and in combination, on the growth of the freshwater *Chlorella vulgaris*. To predict the combined effects of both cyanotoxins on *C. vulgaris*, the non-interaction conceptual model of independent action (IA) was used in this study (Jonker et al. 2005).

The theory behind the IA model is that the components in a mixture of chemicals have dissimilar modes of action (MoA) and do not interfere with each other during exposure, uptake and toxic action (Olmstead & LeBlanc 2005). Hence the IA model is usually used if the question asked is whether the probability of toxicity to one chemical is independent from the probability of toxicity exposure to another chemical (Garcia et al. 2010; Jonker et al. 2004; Jonker et al. 2005). However, deviations from the IA model may occur and therefore a different behaviour may be expected. These deviations are those where a given mixture causes a more severe (synergism) or less severe (antagonism) effect than the predicted by the IA model. These deviations can be constant throughout the concentrations used or vary and follow a dose-level dependency (i.e. different effects at high and low concentrations) and a dose-ratio dependency (i.e. effects differ depending on the mixture composition) (Jonker et al. 2005).

Specifically, we tested the following hypothesis: (1) dissolved CYN and MC-LR will have a similar impact on the growth response of the freshwater microalgae and (2) there will be an additivity of effects between these two cyanotoxins regarding the IA model. We tested these hypotheses by determining the growth rate of freshwater microalgae *C. vulgaris* after 4 and 7 days of exposure over a range of high pure toxin levels.

2.2. Materials and methods

2.2.1. Test organism, cyanobacterial strains and culture conditions

C. vulgaris is normally used for algal toxicity tests and being from freshwater environments may co-occur with MC-LR and CYN-producing cyanobacteria. Therefore it is a relevant species to provide further insights on the effects of cyanotoxin mixtures in freshwater phytoplankton. *C. vulgaris* LEGE Z-001 was cultivated non-axenically in Z8 medium (Kotai 1972), incubated at 25 ± 2ºC under a cool-white light intensity of 10 $\mu mol.m^{-2}.s^{-1}$ photon irradiance with a photoperiod of 14h light and 10h dark. For the

maintenance of the laboratorial cultures, and the start of new cultures, an aliquot of *C. vulgaris* culture in exponential growth rate were harvested every 7-8 days and inoculated in fresh culture medium. Cultures were aerated with ambient air filtered through 0.22 µm.

Microcystis aeruginosa LEGE 91094 and *Aphanizomenon ovalisporum* LEGE X-001 were grown in non-axenic cultures in Z8 medium (Kotai 1972) with the same conditions described above for *C. vulgaris*. After 3-4 weeks of growth, biomass was collected and concentrated by centrifugation (4495 *g*, 15 min) in case of *M. aeruginosa* culture and by filtration (20 µm pore plankton net) of *A. ovalisporum* culture. The concentrated biomass was frozen at -80ºC and freeze-dried. Lyophilized material was stored at room temperature and in the dark until cyanotoxin extraction and purification procedure. The strain *M. aeruginasa* LEGE 91094 was reported to produce mainly the MC-LR variant, accounting for approximately 95% of the total intracellular MCs (Pereira et al. 2009). The strain *A. ovalisporum* LEGE X-001 was verified by high performance liquid chromatography (HPLC) Water Alliance e2695 coupled with a photo diode array (PDA) 2998 to produce only CYN (data of our lab).

2.2.2. MC-LR extraction, purification and quantification

MC-LR was extracted according to the method described by Ramanan et al. (2000), with some modifications. Briefly, the lyophilized *M. aeruginosa* biomass (0.5 g) was mixed with 15 mL of MeOH 75% (v/v) by continuous stirring at room temperature. After 20 min, the sample was sonicated in a bath for 15 min and then ultrasonicated on ice at 60 Hz with 5 cycles of 1 min (VibraCell 50 sonics & Material Inc. Danbury, CT, USA). The homogenate was centrifuged at 10000 *g* for 15 min to remove cell debris. The extraction procedure was repeated with the same volume of MeOH 75% (v/v). The supernatants resulting from both extraction steps were then pooled together and applied to a solid-phase extraction with a Water Sep-Pak® Vac 6 mL C_{18} cartridge at 1 $mL.min^{-1}$, which had been preconditioned with MeOH 100% and distilled water. The loaded column was washed with MeOH 20% (v/v) and then the MC-LR was eluted using MeOH 80% (v/v). The MC-LR

fraction was evaporated by rotary evaporation at 35ºC to remove the entire MeOH portion. The concentrated MC-LR fraction was thereafter purified and quantified by HPLC-PDA following modified versions (Ramanan et al. 2000; Xie & Park 2007). The limit of detection of this cyanotoxin in the HPLC-PDA system is 0.2 $mg.L^{-1}$. A reversed phase column (Phenomenex Luna RP-18, 25 cm × 10 mm, 10 µm) kept at 40ºC were used for MC-LR purification. The gradient elution used MeOH and water both acidified with 0.1% trifluoracetic acid (TFA) with a flow rate of 2.5 $mL.min^{-1}$. The injected volume was 500 µL. Peak purity and percentage of purified MC-LR was calculated at 214 nm and 238 nm. The fractions containing purified MC-LR were then combined and evaporated with air nitrogen for 1 day until removing all the solvent. The residue was resuspendend in culture medium to the desired concentration. For purified MC-LR quantification, a reversed phase column (Merck Lichrospher RP-18 endcapped, 25 cm × 4.6 mm, 5 µm) equipped with a guard column (Merck Lichrospher RP-18 endcapped, 4 × 4 mm, 5 µm) both kept at 45ºC were used. The gradient elution consisted of (A) MeOH + TFA 0.1% and (B) H_2O + TFA 0.1% (55% A and 45% B at 0 min, 65% A and 35% B at 5 min, 80% A and 20% B at 10 min, 100% A at 15 min, 55% A and 45% B at 15.1 and 20 min) with a flow rate of 0.9 $mL.min^{-1}$. The injected volume was 20 µL. The PDA range was 210-400 nm, with a fixed wavelength at 238 nm. The linearity method was achieved between 0.5 and 20 $mg.L^{-1}$. The MC-LR was identified by comparison of spectra and retention time with a standard of MC-LR (≥ 95% purity, Sigma-Aldrich). All HPLC solvents were filtered (Pall GH Polypro 47 mm, 0.2 µm) and degassed by ultrasound bath.

After analysis, the final concentration of the MC-LR stock solution was 308.5 $mg.L^{-1}$ and its chromatographic purity was 97%. The purified MC-LR was then diluted in Z8 medium to the concentration range used in experiments.

2.2.3. CYN extraction, purification and quantification

CYN was extracted from *A. ovalisporum* following a modified version of the method described by Welker et al. (2002). Briefly, freeze-dried cells (0.7 g) were mixed with 5 mL of distilled water acidified with TFA 0.1% (v/v) by continuous stirring for 1h at

room temperature. The homogenate was then sonicated in bath for 15 min followed by 5 cycles of 1 min of ultrasonication on ice. After the extraction step, the homogenate was centrifuged (20000 g, 20 min) and the supernatant collected. A second extraction step was performed to the pellet. The supernatants were pooled together and stored at -20ºC.

CYN was thereafter purified by the same HPLC system using a Gemini C_{18} column (250 mm × 10 mm, 5 µm) from Phenomenex, kept at 40ºC. The isocratic elution was with MeOH 5% (v/v) containing 2 mM of sodium 1-heptanesulfonate monohydrate (99%) with a flow rate of 3 $mL.min^{-1}$. The injection volume was 500 µL. Peak purity and percentage of purified CYN was calculated at 262 nm. The fractions containing purified CYN were combined and then evaporated by speed-vac at 30ºC. The residue was resuspended in culture medium to the desired concentration. The solution containing the purified CYN was also quantified in the same HPLC system on an Atlantis® HILLIC phase column (250 mm × 10 mm, 5 µm) from Waters kept at 40ºC. The isocratic elution was a solution of MeOH 5% (v/v) containing 2 mM of sodium 1-heptanesulfonate monohydrate (99%) with a flow rate of 0.9 $mL.min^{-1}$ and a injected volume of 10 µL. the PDA range was 210-400 nm with a fixed wavelength of 262 nm. The linearity method was achieved between 0.3 and 25 $mg.L^{-1}$. The CYN was identified by comparison of spectra and retention time with a standard of CYN (100% purity, Cork University, Ireland). The limit of detection of this cyanotoxin in the HPLC-PDA system is 0.3 $mg.L^{-1}$. All HPLC solvents were filtered (Pall GH Polypro 47 mm, 0.2 µm) and degassed by ultrasound bath.

After analysis, the final concentration of CYN stock solution was 325.1 $mg.L^{-1}$ and its chromatographic purity was 98%. The purified CYN was diluted in Z8 medium to the concentration range used in experiments.

2.2.4. Experimental design

An experimental design which includes simultaneously single exposures of each cyanotoxin and a set of 25 binary combinations was chosen for the mixture testing. A ray design was chosen to assess the mixtures of MC-LR and CYN (Figure 1).

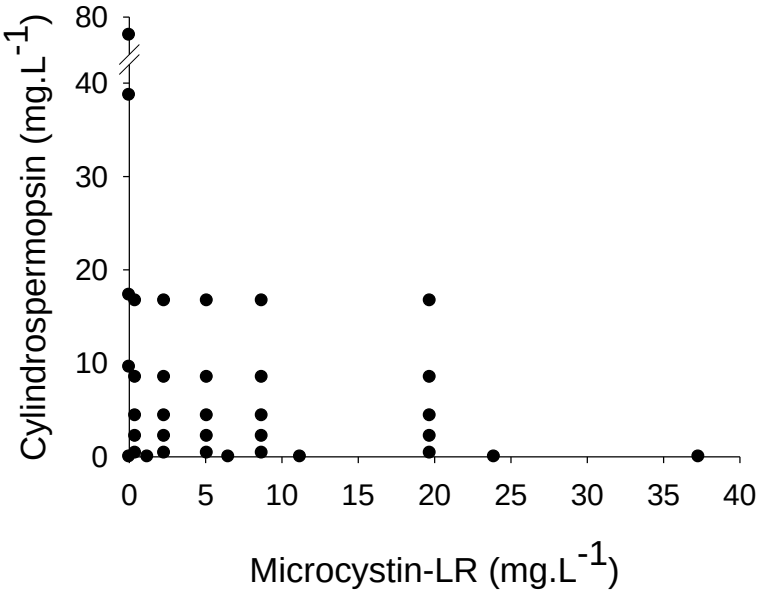

Figure 1 – A schematic ray design of the combinations used for the microcystin-LR and cylindrospermopsin mixture.

Nominal concentrations of MC-LR used in single exposures were 1, 5, 10, 20 and 40 $mg.L^{-1}$ and in combined exposures were 0.5, 2.5, 5, 10 and 20 $mg.L^{-1}$. For CYN nominal concentrations were 10, 20, 40 and 80 $mg.L^{-1}$ in single exposures and 0.5, 2.5, 5, 10 and 20 $mg.L^{-1}$ in combined exposures. This concentration range was selected based on our previous work (Pinheiro et al. in press). As the toxicity prediction of binary mixture exposures is based on the dose-response curve of each of the cyanotoxins independently and the previous single exposures to high concentrations of MC-LR and CYN did not cause 50% response in the growth rate of *C. vulgaris*, a high concentration range of CYN was used for the single exposures in the mixture experiment in order to obtain a EC_{50} value. For MC-LR, concentrations higher than 40 $mg.L^{-1}$ were not used. Each concentration in single and combined experiments was tested in three replicates. In addition, a negative control with Z8 medium was also tested in triplicate. The single and mixture exposures were carried out at the same time so that differences in organisms responses, due to the sensitivity variations, could be controlled and not invalidate the analysis.

All the samples of the MC-LR and CYN-containing exposure medium were quantified by HPLC-PDA. The stability of MC-LR and CYN was also monitored during the exposure period by HPLC-PDA using samples with the same concentrations in the same conditions used for the experiments.

Validity of the experiments was controlled using the reference substance potassium dichromate in three concentrations (5, 10 and 20 $mg.L^{-1}$) with 5 replicates each.

2.2.5. Growth inhibition test with *C. vulgaris*

The growth inhibition test with the freshwater algae *C. vulgaris* was performed in 96-well polystyrene microplates based on the method described by Gantar et al. (2008) due to the experimental design used and the amount of cyanotoxins necessary for the concentration range selected for the experiments. Each well consisted of 200 µL of test solution (with or without cyanotoxin). The log-phase growing microalgae was exposed for 7 days to each cyanotoxin singly and in mixture and the algae growth was determined in accordance with the OECD 201 Guideline (2006). The Z8 medium was used as control. The pH values was recorded in the beginning (range, 7.3-7.4) and at the end (range, 9.1-9.4) of the experiments.

Microplates were sealed with perforated parafilm (to reduce evaporation and allow gas exchanges) and incubated for 7 days under the conditions described above for *C. vulgaris* cultures. The initial cell concentration of *C. vulgaris* was of approximately 5×10^5 $cell.mL^{-1}$. At the beginning of the experiments and after 4 and 7 days the algae concentration was measured by optical density (OD) using microplate reader (PowerWave, Biotek, Vermont, USA) at a wavelength of 750 nm. Before the measurement of OD (on day 4 and 7), the well content was ressuspended with a pipette. The OD values were converted in $cell.mL^{-1}$ using the equation:

$$C = 3.00 \times 10^7 \times Abs + 2.17 \times 10^5 \ (R^2 = 0.99)$$

where C is the algae concentration (cell.mL^{-1}) and Abs is the absorbance obtained at 750 nm.

The average specific growth rate was calculated as the logarithmic increase in cell concentration for the period of 4 and 7 days from the equation:

$$\mu_{i-j} = \frac{\ln B_j - \ln B_i}{t_j - t_i}$$

where μ_{i-j} is the average specific growth rate from time i to j; t_i is the time for the start of the exposure period; t_j is the time for the end of the exposure period; B_i is the cell concentration at time i and B_j is the cell concentration at time j.

2.2.6. Data analysis

Significant differences in the growth rate of *C. vulgaris* between the control and the treatments were analyzed using a one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test. Results were considered significant at $P < 0.05$. The EC_{50} values for *C. vulgaris* single exposures to cyanotoxins at 4 and 7 days were, when possible, calculated through a three-parameter logistic regression curve, the same dose-response regression curve used within the MIXTOX tool. When the dose-response curve could not be obtained, the analysis in the MIXTOX tool were conducted with fixed EC_{50} and slope parameters according to Loureiro et al. (2006).

In addition, estimation of EC_{50} values for CYN was also obtained for each MC-LR concentration used in the mixture experiment using, where feasible, the same three-parameter logistic regression curve. Synergistic ratios (SRs) were calculated by dividing the EC_{50} value for CYN (without MC-LR) by the EC_{50} value for each of the MC-LR and CYN treatments. SR values was used to give an indication of how strong was the

synergistic pattern on the growth rate of *C. vulgaris* exerted by the cyanotoxin binary mixture. SRs of 1.0 indicate no effects of the MC-LR on CYN toxicity (or an additive response), whereas values of > 1.0 and < 1.0 indicate greater and less effects than expected, respectively.

The mixture data were analyzed using the MIXTOX tool described by Jonker et al. (2005) which allowed comparing the observed combined toxic effect and the expected combined effect calculated from the single cyanotoxin exposures. Growth rates from exposure to mixtures with the cyanotoxins were firstly fit to the IA model as cyanotoxins have dissimilar MoA. In a second step of the data analysis, IA model was extended to test the interactions between the two cyanotoxins with deviation functions describing synergism/antagonism (S/A), dose ratio dependent deviation (DR) and dose level dependent deviations (DL).

The S/A deviations are extensions of the IA model and the DR and DL deviations are further extensions of the S/A function (see details in Jonker et al. (2005). These deviations are obtained with the addition of the parameters *a* and *b* forming a nested framework. The extra parameter *a* in the S/A deviations model can become negative or positive for IA. If the value of parameter *a* is positive, this means that a smaller effect than expected (antagonism) was observed; if the referred parameter *a* is negative, thus it expresses a higher effect than expected (synergism). When the value of parameter *a* is zero, the S/A model reduces to the IA reference model. For DR dependency, a second parameter b_{DR} is included in addition to *a*, to generate the DR deviation model. In this deviation function, the parameter b_{DR} allows the deviation from IA model to depend on the composition of the mixture. If the b_{DR} value is positive, antagonism may be observed where the toxicity of the mixture is caused mainly by one of the toxicants; if the b_{DR} value is negative, synergism may be observed where the toxicity of the mixture is caused mainly by the other one. To describe deviations of DL dependency, again a second parameter b_{DL} is included in addition to *a*. DL describes synergism/antagonism depending on the doses of each toxicant in the mixture. In this case *a* value allows to observe whether synergism occurs at low doses and antagonism at high doses (parameter *a* smaller than zero) or whether antagonism occurs at low doses and synergism at high doses (parameter *a* higher

than zero). The parameter b_{DL} indicates at what dose level the change between the two deviations occurs (i.e. from antagonism to synergism or vice versa); e.g., at the EC_{50}, below the EC_{50} or above the EC_{50} level. The biological interpretations of the additional parameters are described in more detail in Table 1.

The IA model and their deviations were fitted to the data using the method of maximum likelihood and statistically compared through likelihood testing. The best fit was chosen at the significance level of 0.05 using the Chi-square test which implies a decrease in the residuals of the sum of squares (SS) and an increase in the description of the variation of the data (R^2). When a deviation from IA model was obtained, the effects pattern was deduced directly from the parameter values as described in Table 1.

Table 1 – Interpretation of additional parameters (*a* and *b*) that define the functional form of deviation patterns from independent action. EC_{50} is the median effect concentration.

Deviation pattern	**Independent action**	
	Parameter *a*	**Parameter *b***
Synergism/Antagonism (S/A)	$\boldsymbol{a > 0}$**:** antagonism $\boldsymbol{a < 0}$**:** synergism	
Dose ratio dependent (DR)	$\boldsymbol{a > 0}$**:** antagonism except for those mixture ratios where negative b value indicate synergism	$\boldsymbol{b_i > 0}$**:** antagonism where the toxicity of the mixture is caused mainly by toxicant *i*
	$\boldsymbol{a < 0}$**:** synergism except for those mixture ratios where positive b value indicate antagonism	$\boldsymbol{b_i < 0}$**:** synergism where the toxicity of the mixture is caused mainly by toxicant *i*
Dose level dependent (DL)	$\boldsymbol{a > 0}$**:** antagonism low dose level and synergism high dose level	$\boldsymbol{b_{DL} > 2}$**:** change at lower EC_{50} level $\boldsymbol{b_{DL} = 2}$**:** change at EC_{50} level
	$\boldsymbol{a < 0}$**:** synergism low dose level and antagonism high dose level	$\boldsymbol{1 < b_{DL} < 2}$**:** change at higher EC_{50} level $\boldsymbol{b_{DL} < 1}$**:** no change, but the magnitude of S/A is effect level dependent

Adapted from Jonker et al. (2005)

2.3. Results

2.3.1. Chemical analysis

To assess contamination accuracy, MC-LR and CYN analyzes were made by HPLC-PDA and the results showed that some measured concentrations varied generally more than 20% from the nominal concentrations. So, all calculations were based on effective concentrations.

Stability analyzes for MC-LR and CYN were also made by HPLC-PDA and the results showed no toxin degradation throughout the 7 days of exposure. No significant changes in the pH were observed during the exposure period, indicating that this parameter was not interfering with *C. vulgaris* response to the cyanotoxin treatments.

2.3.2. Single exposures

The growth of *C. vulgaris* was assessed after 4 and 7 days of exposure to each cyanotoxin alone in the mixture experiment according to the OECD 201 Guideline (2006). The growth was expressed as the logarithmic increase in cell concentration after exposure.

C. vulgaris growth rates were slightly increased at the highest tested concentrations of pure MC-LR after 7 days of exposure (Figure 2, one-way ANOVA, $F_{5,12}$=4.901, P=0.011); the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) values of 1.2 and 6.5 $mg.L^{-1}$ were obtained for pure MC-LR, respectively (Figure 2). No significant differences compared to control were observed in *C. vulgaris* growth rates after 4 days of exposure to pure MC-LR (Figure 2, one-way ANOVA, $F_{5,12}$=2.511, P=0.089).

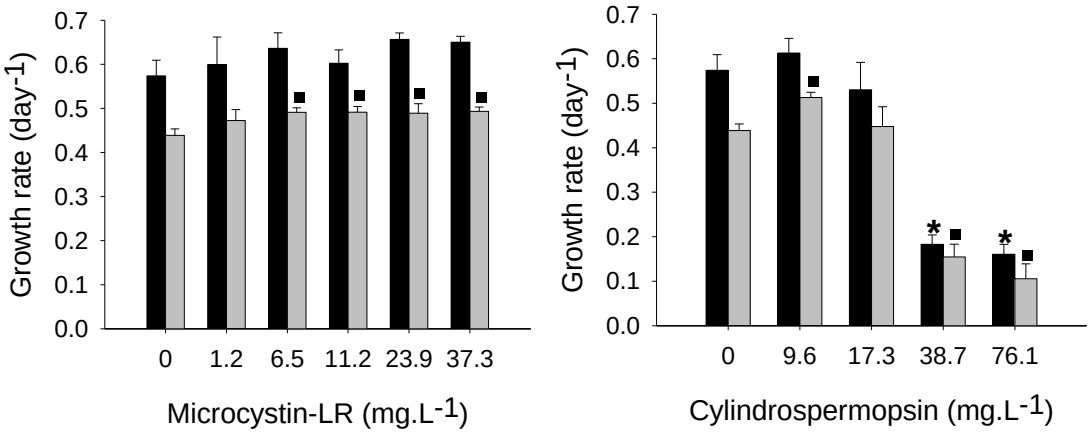

Figure 2 – Growth rate of the microalgae *Chlorella vulgaris* after 4 (black bars) and 7 (grey bars) days of exposure to pure MC-LR (left side) and CYN (right side) in the single exposures of the mixture experiment. Results are expressed as average ± standard error. (*) and (■) Denotes data significantly different from control at the fourth and seventh day of exposure (Dunnett's method, $P < 0.05$).

The growth response of *C. vulgaris* over the 4 and 7 days' exposure to pure CYN followed a dose-response relationship as the growth rates decreased significantly with increasing pure CYN concentrations (Figure 2). The effects became more pronounced within prolonged exposure time. Significant differences compared to control were found at the highest concentrations (38.7 and 76.1 $mg.L^{-1}$) for both exposure periods (one-way ANOVA, $F_{4,10}$=104.362, $P \leq 0.001$ for 4th day; one-way ANOVA, $F_{4,10}$=123.527, $P \leq 0.001$ for 7th day). At the concentrations of 38.7 and 76.1 $mg.L^{-1}$ of pure CYN, the growth rate values were about 3.14- and 3.58-fold lower than the control after 4 days of exposure, and 2.84- and 4.15-fold lower than the control after 7 days of exposure, respectively. A significant increase in the growth rate of *C. vulgaris* was observed at 9.6 $mg.L^{-1}$ of pure CYN after 7 days of exposure (Figure 2, Dunnett's method, $P<0.05$) but it was only 0.86-fold higher than the value found for the control. The NOEC and LOEC values of 17.3 and 38.7 $mg.L^{-1}$ were obtained for pure CYN on the 4th day of exposure, respectively. For the 7th day of exposure, the LOEC value for pure CYN was 9.6 $mg.L^{-1}$.

The EC_{50} values obtained when *C. vulgaris* was exposed to pure CYN were 32.66 $mg.L^{-1}$ (SE = 3.91, R^2 = 0.90) and 33.24 $mg.L^{-1}$ (SE = 3.47, R^2 = 0.91) for the 4th and 7th days of exposure, respectively. For MC-LR exposure, it was impossible to calculate a valid

EC_{50} value for *C. vulgaris* growth rate and further analysis in the MIXTOX tool had to be undertaken with fixed EC_{50} and slope parameters.

2.3.3. Mixture exposures

The toxicity of the binary mixture of pure MC-LR and CYN on the 4th and 7th days of exposure was predicted by using the MIXTOX tool to fit the data set and generate the best description of the biological response of *C. vulgaris* to the combination of these cyanotoxins. For that, the IA model was basically the only conceptual model used to fit our data set, considering that MC-LR and CYN have different MoA on the organism. All parameters and significance test results obtained from data fitted with the MIXTOX tool are presented in Table 2 and 3.

Table 2 – Summary of the analysis done for the effects on the growth rate of *C. vulgaris* exposed for 4 days to the binary mixture of MC-LR and CYN.

	Independent action			
	Reference	**S/A**	**DR**	**DL**
Max	0.63	0.63	0.63	0.62
$\beta_{MC\text{-}LR}$	*30*	*30*	*30*	*30*
β_{CYN}	2.07	2.37	2.43	2.30
$EC_{50\ MC\text{-}LR}$	*83*	*83*	*83*	*83*
$EC_{50\ CYN}$	27.84	28.83	29.35	30.54
a	-	-1.76	-3.20	-0.01
$b_{DR/DL}$	-	-	4.34	-1220.30
SS	0.26	0.24	0.24	0.22
R^2	0.79	0.80	0.81	0.82
$p(\chi^2)$	-	5.75×10^{-03}	0.26	3.90×10^{-03}

S/A is synergism/antagonism, DR is dose ratio deviation and DL is dose level deviation from the reference model; Max is the maximum response value obtained for the given endpoint; β is the slope of the individual dose-response curve; EC_{50} is the median effect concentration value; a and $b_{DR/DL}$ are parameters of the deviation functions; SS is the sum of squared residuals; R^2 is the coefficient of determination; χ^2 is the Chi-squared test and $p(\chi^2)$ indicates the outcome of the likelihood ratio test (significance level $p < 0.05$). Fixed EC_{50} and β parameters are indicated in italics.

On the 4th day, the fit of the IA model to the binary mixture data of pure MC-LR and CYN exposure yielded a SS value of 0.26, explaining 79% of our data. After adding parameter *a* to the IA model in order to describe synergism or antagonism, the SS value decreased slightly to 0.24 ($p(\chi^2) < 0.001$, Table 2), explaining 80% of the data. Parameter *a* had a value of -1.76, which indicates synergism (Table 1 and 2). Continuing in testing for deviations for DR dependency, no significant improvement was obtained on the data fit ($p(\chi^2) = 0.26$). However, when adding parameter *a* and b_{DL} to the DL deviation, the SS value decreased significantly to 0.22 ($p(\chi^2) < 0.001$) explaining 82% of the data. Parameter *a* had a value of -0.01, which indicates synergism at low dose levels and antagonism at high dose levels, and the parameter b_{DL} had a value of -1220.30, which indicates that the magnitude of synergism/antagonism became effect level dependent, i.e, suggesting that antagonism would be observed at concentrations much higher than the concentrations tested in the experimental design (see Figure 1, Table 1 and 2). Although a DL dependency was observed for the binary mixture data, nothing can be concluded due to the inaccurate value obtained for the parameter b_{DL}. Therefore, a synergism deviation from IA model was shown to be the best description for our data set (SS = 0.24, $R^2 = 0.80$ and $p(\chi^2) < 0.001$, Figure 3).

Table 3 – Summary of the analysis done for the effects on the growth rate of *C. vulgaris* exposed for 7 days to the binary mixture of MC-LR and CYN.

	Independent action			
	Reference	**S/A**	**DR**	**DL**
Max	0.48	0.48	0.48	0.48
$\beta_{MC\text{-}LR}$	*35*	*35*	*35*	*35*
β_{CYN}	2.26	2.66	2.76	2.64
$EC_{50\ MC\text{-}LR}$	*85*	*85*	*85*	*85*
$EC_{50\ CYN}$	30.27	31.44	31.84	32.54
a	-	-2.32	-4.03	-0.02
$b_{DR/DL}$	-	-	4.94	-956.82
SS	0.14	0.13	0.13	0.12
R^2	0.80	0.82	0.83	0.84
$p(\chi^2)$	-	6.23×10^{-04}	0.19	7.54×10^{-03}

S/A is synergism/antagonism, DR is dose ratio deviation and DL is dose level deviation from the reference model; Max is the maximum response value obtained for the given endpoint; β is the slope of the individual dose-response curve; EC_{50} is the median effect concentration value; *a* and $b_{DR/DL}$ are parameters of the deviation functions; SS is the sum of squared residuals; R^2 is the coefficient of determination; χ^2 is the Chi-squared test and $p(\chi^2)$ indicates the outcome of the likelihood ratio test (significance level $p < 0.05$). Fixed EC_{50} and β parameters are indicated in italics.

For data on the 7th day of exposure, a significant fit was observed, explaining 80% of the data (SS = 0.14, $p < 0.05$). After adding parameter *a* to the IA equation the SS value decreased slightly to 0.13 and the R^2 increased significantly to 0.82 and a synergistic pattern was suggested for the binary mixture of cyanotoxins (a = -2.32, $p(\chi^2) < 0.001$, Table 1 and 3). No significance decrease to the SS value was observed when adding parameter *a* and b_{DR} to the DR deviation (SS = 0.13, $p(\chi^2) = 0.19$, Table 3). However, when adding parameter *a* and b_{DL} to the DL deviation, the SS value decreased slightly to 0.12 ($p(\chi^2) < 0.001$). The DL deviation fit explained 84% of the data. Parameter *a* had a value of -0.02, which indicates synergism at low dose levels and antagonism at high dose levels, and the parameter b_{DL} had a value of -956.82, which indicates that the magnitude of synergism/antagonism became effect level dependent (Table 1 and 2). Again a DL dependency was observed for the binary mixture data, but nothing can be concluded due to the inaccurate value obtained for the parameter b_{DL}. Therefore, a synergism deviation from IA model, which was achieved by adding parameter *a* to the equation, was shown to be the best description for our data set (SS = 0.13, $R^2 = 0.82$ and $p(\chi^2) < 0.001$, Figure 3).

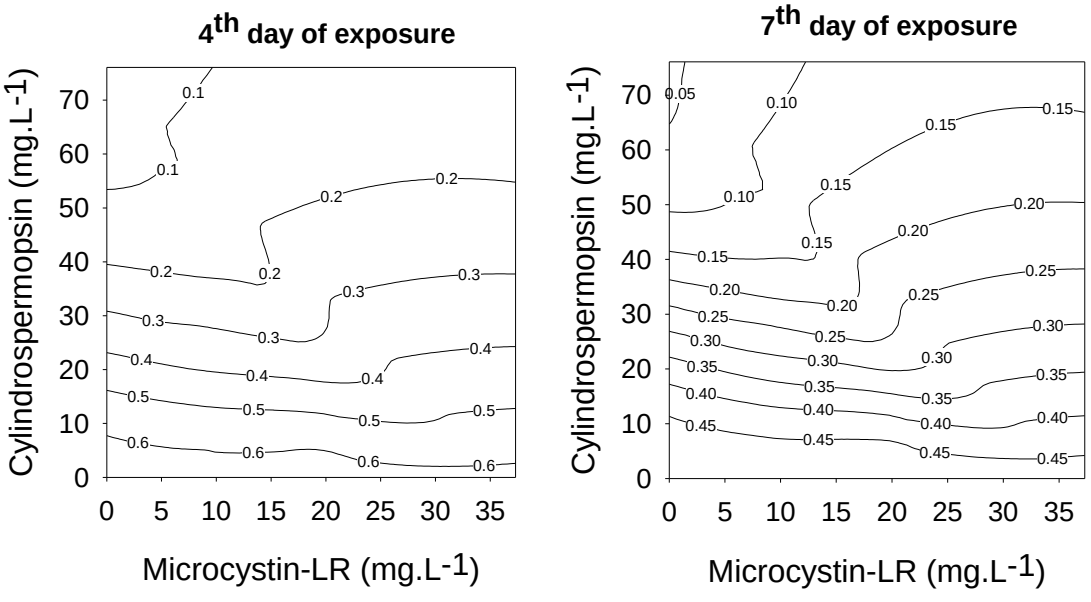

Figure 3 – Concentration-response relationship for the binary mixture of microcystin-LR and cylindrospermopsin showing a synergist pattern after IA model for the growth rate of *C. vulgaris* on the 4th and 7th day of exposure (2D isobolic surface). Concentrations of cyanotoxins reported as effective values.

For both exposure periods, a significant synergistic interaction was found between MC-LR and CYN in the mixture. Although MC-LR has not caused any inhibitory effect on the growth rate of *C. vulgaris* at high concentrations when tested individually either on the 4th or 7th day of exposure, the presence of MC-LR at levels lower than 20 mg.L^{-1} in the mixture significantly increased the toxicity of CYN. In order to provide information on the magnitude of the synergistic effect on the growth rate of *C. vulgaris*, the EC_{50} values for CYN in all MC-LR levels were estimated and the SRs calculated. The EC_{50} values and standard errors are provided in Table 4 with the corresponding SRs. From Table 4, it is clear that MC-LR had an effect on CYN toxicity with SRs of almost 2 at concentrations between 0.4 and 19.7 mg.L^{-1}. This means that independently of MC-LR concentration present in the mixture, CYN toxicity was increased by about a factor of 2 when combined with MC-LR.

Table 4 – EC_{50} values (with the standard errors, in mg.L^{-1}) and synergistic ratios (SRs) estimated and calculated, respectively, for cylindrospermopsin when co-occurring with each concentration of microcystin-LR in the mixture experiments.

Exposure time (days)		**Microcystin-LR (mg.L^{-1})**					
		0	**0.4**	**2.3**	**5.1**	**8.7**	**19.7**
4	EC_{50} (± SE)	32.66 (± 3.91)	19.64 (± 5.41)	17.53 (n.d.)	17.22 (n.d.)	17.26 (n.d.)	18.27 (± 0.99)
	SR[a]	-	1.66	1.86	1.90	1.89	1.87
7	EC_{50} (± SE)	33.24 (± 3.47)	17.40 (n.d.)	17.38 (n.d.)	17.08 (± 0.15)	17.28 (± 0.28)	16.94 (± 0.13)
	SR[a]	-	1.91	1.91	1.95	1.92	1.96

SE is the standard error; n.d. is not determined

[a] SR = $EC_{50\ CYN\ without\ MC\text{-}LR}$ / $EC_{50\ CYN\ and\ MC\text{-}LR\ treatments}$

2.4. Discussion

2.4.1. Single exposures

In this study, the log-phase growing freshwater algae *C. vulgaris* was exposed to extremely high concentrations of pure MC-LR and CYN for 7 days aiming to estimate the

EC_{50} values for each of the cyanotoxins and then predict their combined toxicity in the MIXTOX tool. Our experiments demonstrate that pure CYN at higher concentrations (≥ 38.7 $mg.L^{-1}$) could strongly affect the growth of *C. vulgaris*, inhibiting their growth rates by a factor higher or similar to 3 after 4 and 7 days of exposure. In our previous work, pure CYN slightly inhibited the growth rate of *C. vulgaris* (< 10% of inhibition) after 4 days of exposure at concentrations of 8.5 and 16.7 $mg.L^{-1}$, but no growth rate inhibition was observed on the 7th day of exposure (Pinheiro et al. in press). However, concentrations corresponding to 9.6 and 17.3 $mg.L^{-1}$ in our experiments were found to be either completely ineffective or causing a weak growth stimulation (0.86-fold higher than control) in *C. vulgaris* during the exposure period. Moreover, our experiments also demonstrated that pure MC-LR at higher concentrations (≥ 6.5 $mg.L^{-1}$) could stimulate the growth rates of *C. vulgaris* only after 7 days of exposure. Growth rate of *C. vulgaris* was also previously found to be increased after the same prolonged exposure time, but only at pure MC-LR concentration of 37.3 $mg.L^{-1}$ (Pinheiro et al. in press). Similarly to our results, a few studies have reported significant stimulations on microalgae growth when exposed to MC concentrations lower or similar to 4 $mg.L^{-1}$. Ou et al. (2005) showed that the growth of the grazing chrysophyte *Posterioochromonas* sp. increased remarkably with the presence of MC-LR and MC-RR at concentrations between 0.1 and 4 $mg.L^{-1}$ within 17 days of exposure. Sedmak & Kosi (1998) observed an early growth stimulation of the green alga *Coelastrum microporum* when exposed to pure MC-RR concentrations of 0.1 and 0.5 $mg.L^{-1}$ in the first 10 days and a subsequent growth inhibition in the last 4 days. In a similar experiment with pure MC-RR and a different exposure time (16 days of exposure), the authors also observed an increase on the growth of the green algae *Monoraphidium contortum* at a concentration of 0.104 $mg.L^{-1}$ (Sedmak & Kosi 1998). In addition, we can notice a slight difference between the growth rates assessed on the 4th and 7th days of exposure which, in the case of pure CYN, tend to disappear with increasing concentrations. Since no cyanotoxin degradation was observed and *C. vulgaris* was in log-phase at the end of 7 days, the expressive decreasing of growth rates between the 4th and 7th days may possibly be a result of nutrient depletion.

Previous works have shown that pure MCs may have detrimental effects on the microalgae growth at concentrations as high as those used in the present work. For

example, Babica et al. (2007) demonstrated that the growth of five planktonic microalgae representatives of Chlorophyta (*Chlamydomonas reinhardtii, Chlorella kesslerii*, *Pediastrum duplex, Pseudokirchneriella subcapitata* and *Scenedesmus quadricauda*) was strongly inhibited by pure MC-LR and MC-RR at a concentration of 25 $mg.L^{-1}$ after 11 days of exposure. *P. subcapitata* showed to be the most sensitive microalgae, being highly affected by both MC variants at even low concentrations (1 and 5 $mg.L^{-1}$). In our study, we did not observe any growth inhibition at *C. vulgaris* exposed to pure MC-LR at concentrations below 25 $mg.L^{-1}$. It is possible that green algae species display differential susceptibility to MCs. Moreover, the responses of microalgae to MCs seem to be also influenced by physic-chemical and environment factors. Some of these features are reported by Sedmak & Kosi (1998) that found that MC-RR at 0.104 and 0.519 $mg.L^{-1}$ inhibited the growth of *C. microporum*, but induced the growth of *M. contortum* and *S. quadricauda* under low light conditions.

Data obtained in the present study for single exposures to cyanotoxin in the mixture experiment do not corroborate our hypothesis that dissolved CYN and MC-LR have a similar impact on the growth response of *C. vulgaris*, since at approximately equivalent concentrations pure CYN inhibited the growth rates of the freshwater algae while pure MC-LR did not have any negative effect on their growth rates. In the present study, concentrations of 32.66 and 33.24 $mg.L^{-1}$ CYN decreased the growth rate of *C. vulgaris* by 50% in a 4- and 7-day exposure period, respectively. However, CYN toxicity is considered not ecologically relevant because toxic effects on microalgae growth in aquatic systems are likely caused by high CYN concentrations (again, such high concentrations were used to estimate the EC_{50} values for CYN in order to further analysis in MIXTOX tool), and probably not found in the environment. In most water bodies, the presence of CYN is characterized by a high dissolved fraction (Wormer et al. 2009). In Europe, concentrations of dissolved CYN associated with cyanobacterial blooms range from 0.08 to 18.4 $\mu g.L^{-1}$ (Bogialli et al. 2006; Gallo et al. 2009; Messineo et al. 2010; Quesada et al. 2006; Rücker et al. 2007), and up to 0.8 $mg.L^{-1}$ in Australia (Griffiths & Saker 2003; Shaw et al. 1999). Although it was agreed that CYN toxicity only occurs at high concentrations (here EC_{50} = 33 $mg.L^{-1}$ on the 4^{th} and 7^{th} days of exposure), toxicity at low concentrations of CYN may not be excluded in even longer-term exposures. Unlike MCs, dissolved CYN tends to

accumulate in the aquatic systems because of elevated extracellular release by CYN-producing cyanobacteria (Preussel et al. 2009) and, considering its limited photodegradation (Wörmer et al. 2010) and biodegradation (Wormer et al. 2008), CYN may impair the environment and aquatic organisms.

For pure MC-LR, it was not possible to observe a dose-response curve for the growth rate in *C. vulgaris* and estimate the EC_{50} value, thus limiting the toxicity prediction for this endpoint in the binary mixture of MC-LR and CYN. Although high pure MC-LR concentrations were not toxic to *C. vulgaris*, the fact cannot be disregarded that low concentrations of MC-LR may affect aquatic organism, especially microalgae, in longer-term exposures. There are some studies that have reported toxic effects of MC-LR on microalgae at concentrations below 1.1 $mg.L^{-1}$ in a 12- and 14-day exposure period (B-Béres et al. 2012; Kearns & Hunter 2000; Kearns & Hunter 2001; Sedmak & Eleršek 2006), suggesting that for longer exposures periods, low concentrations may have a negative impact. Concentrations of dissolved MCs in natural waters are generally reported below 10 $\mu g.L^{-1}$ because they are mainly retained within healthy cyanobacterial cells (Babica et al. 2006; Babica et al. 2007; Lahti et al. 1997; Wiegand & Pflugmacher 2005). However, concentrations of MCs above 10 $\mu g.L^{-1}$ in the environment can occur immediately after the collapse of a cyanobacterial bloom or the application of algicides. Jones and Orr (1994) measured 1.3-1.8 $mg.L^{-1}$ MCs following algicide treatment of a *M. aeruginosa* bloom in a recreational lake. Furthermore, in a few cases, accumulation of cyanobacterial cells in surface scum may raise MC concentrations to levels higher than 1.8 $mg.L^{-1}$. In addition, concentrations of total MCs up to 8.4-25 $mg.L^{-1}$ have been reported in natural bloom samples (Fastner et al. 1999; Kemp & John 2006; Máthé et al. 2007; Nagata et al. 1997). High concentrations of MC-LR in addition to longer exposure periods may also cause severe impact on aquatic ecosystems. Evidence supporting this is shown in the work of Sedmak & Kosi (1998). These authors studied the relationship between the species diversity and the development of toxic cyanobacterial blooms and MC content in natural water bodies in which it was found a negative correlation between high cyanobacterial cell densities and high MC values (> 0.01 $mg.L^{-1}$) and the number of phytoplankton species present.

2.4.2. Mixture exposures

Considering the growth response of *C. vulgaris* submitted to the combined exposure of pure MC-LR and CYN, the MIXTOX analysis showed a synergistic deviation from the conceptual model of IA for the growth rate endpoint in both exposure periods. The choice of IA to fit the data set from this binary mixture was mainly based on the assumption of different MoA of MC-LR and CYN, which means that they act in different target sites on the biological systems and/or follow different pathways to cause any observed effects. At the molecular level, MC-LR mainly inhibits protein phosphatases 1 and 2A in mammals and higher plants (Mackintosh et al. 1990), which cause intracellular problems with cell growth, differentiation and osmoregulation (Gulledge et al. 2002; Monserrat et al. 2003; Runnegar et al. 1995a). MC-LR can also cause oxidative stress in aquatic animals, plants and algae, leading to an increase in lipid peroxidation, DNA damage, mitochondrial damage and alteration of the antioxidant defense system (Amado & Monserrat 2010; Bártová et al. 2011; Mohamed 2008; Pflugmacher 2004). CYN, on the other hand, inhibits glutathione and protein synthesis in mammals (Froscio et al. 2001; Froscio et al. 2008; Runnegar et al. 1995b; Terao et al. 1994) interfering with several metabolic pathways. Studies performed with dissimilar acting chemicals generally show that the IA model demonstrates a good ability in predicting the combined toxicity of those chemicals, but there have been cases where deviations from this model have occurred when those chemical were tested (Ferreira et al. 2008; Loureiro et al. 2010; Munkegaard et al. 2008).

Interpretation of modeled data for the endpoint of growth rate in both exposure periods suggested a synergistic interaction of pure MC-LR and CYN on *C. vulgaris*. It indicates that observed growth rates of *C. vulgaris* were impaired by the MC-LR and CYN mixture exposure, with toxic effects being higher than those predicted by IA model. Therefore, IA model underestimated the mixture toxicity and the synergistic pattern was established for that endpoint. This result does not corroborate our hypothesis that pure MC-LR and CYN could have an additive response on the microalgae growth. As far as we know, until now there is no published data on response patterns of *C. vulgaris* to the same

cyanotoxin mixture, but there are data from other species, *O. sativa*, where the joint effects of MC-LR- and CYN-containing crude extracts were evaluated (Prieto et al. 2011). In a 48h-experiment, Prieto et al. (2011) showed higher activity of GST in the roots and leaves of the rice plant *O. sativa* exposed to the mixture of MC-LR and CYN-containing cyanobacterial extracts compared to that of the plant exposure to the individual cyanobacterial extracts, suggesting a synergistic interaction between both cyanotoxins. It should be noted that both cyanobacterial extracts are complex mixtures of cyanobacterial metabolites (including cyanotoxins) and potential interactions among the components of these complex mixtures cannot be neglected. As an example, Nováková et al. (2012) studied the combined effects of crude extracts from two non-producing cyanobacteria, *Aphanizomenon gracile* and *Cylindrospermopsis raciborskii*, on gap junctional intercellular communication (GJIC) and showed that both extracts (without cyanotoxins) when mixed caused an additive response on GJIC, suggesting that unknown metabolites are responsible for the inhibitory activity of both combined extracts on GJIC. Recent studies also demonstrated synergistic effects between cyanobacterial metabolites such as portoamide A and B (Leão et al. 2010), lobocyclamides A and B (MacMillan et al. 2002) or laxaphycins A and B (Bonnard et al. 2007). Therefore, the effect of mixtures of cyanobacterial extracts containing cyanotoxins should be analyzed with more reflection and prudency.

In the current study, high concentrations of MC-LR (0.4-19.7 $mg.L^{-1}$) and CYN (0.4-16.7 $mg.L^{-1}$) were used in order to assess their combined effects on the growth rate of *C. vulgaris*. Pure MC-LR at 0.4-19.7 $mg.L^{-1}$ increased the toxicity of pure CYN by about a factor of 2. This is a substantial increase when considering that levels of 2-10 times this amount of pure MC-LR did not cause toxicity by itself. The most likely explanation for this increased toxicity is that CYN inhibits the GSH synthesis and prevent the GST catalyzed conjugation of MC-LR to GSH, which represents the first step in detoxification of MCs (Pflugmacher et al. 1998), prolonging thereby the residence time of MC-LR in *C. vulgaris* and resulting in a much higher toxicity. Best et al. (2002), who investigated the combined effect of MC-LR and lipopolysaccharides (LPS) on the activity of microsomal and soluble GST of zebra fish *Danio rerio*, also reported that LPS (0.5 $\mu g.L^{-1}$) may exert a synergistic effects on MC-LR-induced toxicity (0.5 $\mu g.L^{-1}$), since LPS seem to potentially

decrease the GST activity (microsomal and soluble), thus reducing MC detoxification. In contrast to the previous study, Lindsay et al. (2006) showed antagonistic effects between MC-LR or CYN and LPS in invertebrates (*Artemia salina*, *Daphnia magna* and *Daphnia galeata*); pre-exposure with a sublethal level of LPS (2 μg.L^{-1}) protect the invertebrates against the toxicity of MC-LR (1000 ng.L^{-1}-20 mg.L^{-1}) and CYN (1000 ng.L^{-1}-20 mg.L^{-1}). This protective effect was also found for the co-exposure of LPS and MC-LR, but was less pronounced than the conferred by pre-exposure with LPS. The mechanism involved in the antagonistic effects is not fully understood yet, but appears to involve effects of LPS on detoxification pathways other than GST, including suppression of the invertebrates CYP450 system (Lindsay et al. 2006). On the other hand, Pires et al. (2011) did not found clear evidences for synergistic effects of MCs and LPS, but the interaction between *Microcystis* strain type (non-producing and MC-producing), concentration of MC-producing cells and LPS (absence or presence) was significant, indicating composition dependent effects of MCs and LPS, i.e., dose ratio dependency. The variety of responses produced by cyanotoxin mixtures indicates that the observed toxic effects are likely dependent on the species, cyanotoxin mixture, exposure type and levels of cyanotoxins used.

The combined toxicity of pure MC-LR and CYN has not been reported and our experiments confirmed a synergistic MC-LR-CYN interaction on *C. vulgaris* growth rate. Although the synergistic interaction between MC-LR and CYN occurred at high concentrations, negative impacts of these cyanotoxins in combination on microalgae community should not be ignored. The impact to the microalgae community can be increased if we consider that MC-LR and CYN are relatively stable compounds (Chiswell et al. 1999; Duy et al. 2000; Tsuji et al. 1994; Wormer et al. 2008; Wörmer et al. 2010), persisting in the water after cyanobacterial senescence and/or cell lyses (Eaglesham et al. 1999). Given predictions that cyanobacterial blooms will increase in frequency and magnitude in the future (Paerl & Huisman 2008; Paerl & Huisman 2009; Paerl & Paul 2012), we might anticipate that synergistic effects on growth responses of microalgae, which will influence exponential population growth, may have pronounced effects on populations and communities of zooplankton.

Given the importance of microalgae such as *C. vulgaris* in the food webs and the growing concerns regarding cyanobacterial blooms, we suggest that there is a need to carefully assess the mechanism behind this synergistic effect. We also emphasize that similar experiments with other freshwater microalgae and even with environmentally relevant concentrations should be undertaken, as differential susceptibilities will undoubtedly occur between genera and species. Revealing such sensitivities in the microalgae growth response to MC-LR and CYN mixtures may indicate the consequences to the microalgae communities and in turn to the zooplankton communities.

2.5. Conclusions

In the present study, *C. vulgaris* was chosen as a test species to discuss the toxic effects of individual and mixture exposure of pure MC-LR and CYN. Our results showed that the interaction between MC-LR and CYN was synergistic and MC-LR enhanced the CYN toxicity on the growth rate of *C. vulgaris*. Although the synergistic interaction between MC-LR and CYN occurred at high concentrations, the possibility of interaction between these two cyanotoxins in aquatic environment cannot be excluded. Thus, when aquatic organisms, especially microalgae, are simultaneously exposed to both cyanotoxins, the increased environmental risks should not be ignored. To the best of our knowledge, this is the first study concerning combined toxicity of MC-LR and CYN as pure cyanotoxins. Considering the predicted expansion of cyanobacterial blooms on a global scale, this report is also an important contribution to our understanding of an increasing potential environmental risk between MC-LR and CYN and of how both cyanotoxins interact with each other in microalgae. Moreover, our results demonstrated that high concentrations of pure CYN applied as single cyanotoxin caused impairments on growth rates of *C. vulgaris* while pure MC-LR can lead generally to an increase of the growth rates.

As a final conclusion, this work emphasize that more research needs to be done regarding the effects of binary mixtures of cyanotoxins on several microalgae species at different combinations of concentrations, including environmentally relevant concentrations.

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Chapter III

3. Effects of binary mixtures of cyanotoxins and xenobiotics on the growth rate of the freshwater algae *Chlorella vulgaris*

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Abstract

Aquatic organisms are constantly exposed to several natural and anthropogenic contaminants such as cyanotoxins, metals and pesticides in eutrophic brackish and freshwaters mainly due to toxic cyanobacterial blooms and human activities. However, the ecotoxicological risk of their combinations in the aquatic environment is unknown. The aim of the present study was to investigate the growth responses of *Chlorella vulgaris* exposed to four single chemical compounds (microcystin-LR, cylindrospermopsin, terbuthylazine and cadmium) and five binary mixtures. In the growth inhibition tests, terbuthylazine was the most toxic to *C. vulgaris*, followed by cadmium, cylindrospermopsin and microcystin-LR, which was the less toxic. The MIXTOX tool was used to evaluate mixture toxicity. Observed data was compared with the expected mixture effects predicted by independent action model; deviations for synergistic/antagonistic interactions, dose ratio and dose level dependency were also assessed. In the mixture toxicity assessment, several patterns of response were obtained depending on the mixture. Antagonism was the prevailing type of interaction between the chemical compounds in a 4-day exposure period while dose level dependency was the only deviation obtained in a 7-day exposure period. In the case of dose level dependency, synergism at low doses of each chemical compound involved in the mixture was observed in three of the five binary mixtures. This synergistic effect represents a significant risk for aquatic organisms co-exposed to cyanotoxins and anthropogenic contaminants in the environment. Toxicokinetic and toxicodynamic studies should be made in the future as a way to understand the toxicological mechanisms involved in complex mixture exposures.

Keywords: Cyanotoxin, Microcystin-LR, Cylindrospermopsin, Terbuthylazine, Cadmium, *Chlorella vulgaris*, Mixture toxicity, Independent action

3.1. Introduction

The global intensification of agricultural and industrial activities has enhanced eutrophication in brackish and freshwaters, which has led to the increasing worldwide frequency of harmful cyanobacterial blooms (HABs) (Paerl et al. 2001). Climate changes, specially the predicted rise of global temperatures and the changes in the global hydrological cycle (precipitation and drought), are thought to favour the HAB occurrence in the future not only in frequency and intensity but also in dominance, duration, persistence and geographic distribution (El-Shehawy et al. 2012; Elliott 2012; Paerl & Huisman 2008; Paerl & Huisman 2009; Paerl & Paul 2012). Such blooms are of great ecological and human health concern mainly because of the ability of many bloom-forming cyanobacterial species to produce potent cyanotoxins (Codd et al. 1999; Falconer 1999; van Apeldoorn et al. 2007; Zurawell et al. 2005). Among these cyanotoxins, the hepatotoxin microcystins (MCs) and the cytotoxin cylindrospermopsin (CYN) are the most frequently detected in HABs in brackish and freshwaters (Codd et al. 2005; Prasanna et al. 2010; van Apeldoorn et al. 2007).

During HAB development, MCs are mainly retained within the cyanobacterial cells while CYN may be continuously released to the water due to its hydrophilic properties and apparent membrane permeability (Shaw et al. 1999; Wiegand & Pflugmacher 2005; Wormer et al. 2008). After senescence and/or lysis of cyanobacterial cells, high concentrations of both cyanotoxins may be released into the aquatic environment during the collapse of HABs. Concentrations of dissolved MCs may reach levels as high as 25 $mg.L^{-1}$ (Kemp & John 2006; Máthé et al. 2007; Nagata et al. 1997) while concentrations of dissolved CYN may attain 0.8 $mg.L^{-1}$ (Griffiths & Saker 2003). These high levels, which may become more common with the HAB increase in prevalence and magnitude (Paerl & Huisman 2008; Paerl & Huisman 2009; Paerl & Paul 2012), inspired us to examine a range of concentrations that include extremely high concentrations. As MCs and CYN are highly stable and persist for days under conditions found in most natural water bodies (Chiswell et al. 1999; Duy et al. 2000; Eaglesham et al. 1999; Lahti et al. 1997; Tsuji et al. 1994; Wormer et al. 2008), the released cyanotoxins might come into contact direct with a range

of aquatic organisms, including microalgae, and cause adverse effects on them (Babica et al. 2007; Berry et al. 2009; Beyer et al. 2009; Pflugmacher 2004; Pflugmacher et al. 1999).

MCs and CYN may seriously affect important cellular processes such as growth (B-Béres et al. 2012; Babica et al. 2007; Beyer et al. 2009), photosynthesis (Pietsch et al. 2001; Weiss et al. 2000; Wiegand et al. 2002), detoxification pathways (Mohamed 2008; Wiegand et al. 2002) and reproduction (Kinnear et al. 2007). MCs act as potent inhibitors of protein phosphates 1 and 2A in mammals and higher plants (Gulledge et al. 2002; Mackintosh et al. 1990; Pereira et al. 2011; Runnegar et al. 1995a). Moreover, many studies have indicated that oxidative stress may play a significant role in the toxicity mechanism of MCs in aquatic animals, plants and algae (Amado & Monserrat 2010; Bártová et al. 2011; Mohamed 2008; Pflugmacher 2004). CYN inhibits glutathione and protein synthesis (Froscio et al. 2001; Froscio et al. 2008; Runnegar et al. 1995b; Terao et al. 1994) and potentially interferes with DNA structure (Shaw et al. 2000) in mammals. Its toxicity mechanism is likely mediated by cytochrome P450-generated metabolites (Humpage et al. 2005).

Concomitant with the eutrophication of brackish and freshwaters and massive proliferation of cyanobacteria, the development of agriculture and industrialization has also led to the release of high amounts of persistent anthropogenic contaminants such as metals and pesticides to the aquatic systems. Such high levels may be toxic to a range of aquatic organisms, including microalgae (Bišová et al. 2003; Chalifour & Juneau 2011; Fairchild et al. 1997; Huang et al. 2009; Pérez et al. 2011; Visviki & Rachlin 1994; Weiner et al. 2007).

Aquatic organisms may therefore be simultaneously exposed to several natural and anthropogenic environmental contaminants originating from different sources that may have more deleterious effects on aquatic communities than those induced by the contaminants individually. The combined effects of cyanotoxins (especially MC-LR) and anthropogenic contaminants have been reported on invertebrates (Cerbin et al. 2010; Yang et al. 2012; Yang et al. 2011), fish (Notch et al. 2011; Sun et al. 2012), birds (Wang et al. 2012) and plants (Ge et al. 2012; Wang et al. 2012). However, such combined effects on

freshwater microalgae are unknown so far. Although there is an increasing concern about the combined exposure to both natural and anthropogenic contaminants on aquatic organisms, the ecotoxicological risks of their combinations on aquatic ecosystems is still limited.

In the present study, the growth responses of the freshwater unicellular algae *Chlorella vulgaris* exposed to several chemical substances, singly and in binary mixture, were investigated. For that, the following four chemicals with dissimilar modes of action (MoA) were chosen: 1) cyclic heptapeptide MC-LR as the most common and toxic variant of MCs (Dittmann & Wiegand 2006; Hoeger et al. 2005); 2) tricyclic alkaloid CYN due to its worldwide occurrence (Falconer & Humpage 2006; Fastner et al. 2007; Quesada et al. 2006; Spoof et al. 2006); 3) triazine-ring herbicide terbuthylazine (TBA) which acts at the photosystem II (PSII) of photoautotroph organisms inhibiting the photosynthetic electron transport (Faust et al. 2001) and is frequently detected in surface waters (Azevedo et al. 2001; de Almeida Azevedo et al. 2000; Lacorte et al. 1998; Loos et al. 2010; Loos et al. 2007; Palma et al. 2009; Pérez et al. 2010); and 4) the non-essential metal cadmium (Cd), commonly found in the aquatic environment due to a variety of anthropogenic and natural sources, is known as an oxidative stress inductor, causing lipid peroxidation (Badisa et al. 2007; Leonard et al. 2004; Pinto et al. 2003; Valko et al. 2005), and a competitor by the essential Ca^{2+} site in PSII during photoactivation (Faller et al. 2005). We hypothesized that growth responses of *C. vulgaris* will not only be adversely affected by these four chemical substances independently, but also interactive effects between them will occur.

3.2. Material and methods

3.2.1. Test chemicals

Chemical substances used in this study were cadmium chloride anhydrous ($CdCl_2$, 99% purity, FLUKA, Sigma-Aldrich), TBA (formula: $C_9H_{16}ClN_5$, molecular weight: 229.7

Daltons) tested as a commercial formulation (SAPEC with 500 g active ingredient.L^{-1}), pure MC-LR (formula: $C_{49}H_{74}N_{10}O_{12}$, molecular weight: 994 Daltons, 97% purity) and pure CYN (formula: $C_{15}H_{21}N_5O_7S$, molecular weight: 415 Daltons, 98.7% purity). The cyanotoxins were purified by high performance liquid chromatography (HPLC) Water Alliance e2695 coupled with a photo diode array (PDA) 2998. All the TBA concentrations in the experiments are given as active ingredient per L Z8 medium.

Exposure medium contamination was controlled by chemical analysis by HPLC-PDA for MC-LR and CYN. To control medium contamination, chemical analysis were performed to two samples of the stock solutions and 32 and 29 samples of both single and combined exposures for MC-LR and CYN, respectively. For Cd and TBA, actual concentrations were not checked. The stability of MC-LR and CYN was also monitored during the exposure period by HPLC-PDA using samples with the same concentrations in the same conditions used for the experiments.

3.2.2. Test organism and cyanobacterial strains

All experiments were carried out with the freshwater unicellular algae *C. vulgaris* LEGE-Z001 as test organism. Non-axenic cultures of *C. vulgaris* were maintained in Z8 medium (Kotai 1972) and incubated at 25 ± 2ºC under a cool-white light intensity of 10 µmol.m^{-2}.s^{-1} photon irradiance with a photoperiod of 14h light and 10h dark. Cultures were aerated with ambient air sterilized by passing through 0.22 µm filter. For the maintenance of the laboratory cultures, and start of new cultures, *C. vulgaris* were harvested while still in the exponential growth phase (7-8 days old) and inoculated in fresh Z8 medium.

The MC-LR and CYN-producing cyanobacterial strains used in this study were *Microcystis aeruginosa* LEGE 91094 and *Aphanizomenon ovalisporum* LEGE X-001. In the strain LEGE 91094, the most produced MC variant MC-LR was reported to account for approximately 95% of the total intracellular MCs (Pereira et al. 2009b). The strain LEGE X-001 produces only CYN as assessed by HPLC-PDA (data not shown). Non-axenic

cultures of both cyanobacterial species were maintained in Z8 medium (Kotai 1972) at the same conditions described above for *C. vulgaris* culture. After 3-4 weeks of growth, *M. aeruginosa* biomass was harvested by centrifugation (4495 *g*, 15 min) and *A. ovalisporum* biomass was collected by filtration through 20 µm pore plankton net. The harvested biomass was frozen at -80ºC and freeze-dried. Lyophilized material was stored at room temperature and in the dark until cyanotoxin extraction and purification procedures.

3.2.3. MC-LR extraction, purification and quantification

MC-LR was extracted from lyophilized *M. aeruginosa* biomass based on the method described by Ramanan et al. (2000) with some modifications. Briefly, the freeze-dried cells (0.5 g) were mixed with 15 mL of MeOH 75% (v/v). The homogenate was continuously stirred for 20 min at room temperature, sonicated in a bath for 15 min and then ultrasonicated on ice at 60 Hz with 5 cycles of 1 min (VibraCell 50 sonics & Material Inc. Danbury, CT, USA). Cell debris was separated by centrifugation (10000 *g*, 15 min) after the extraction step. The pellet was thereafter re-extracted with the same volume of MeOH 75% (v/v). The supernatants resulting from both extraction steps were combined and passed through a Water Sep-Pak® Vac 6 mL C_{18} cartridge (solid-phase extraction), preconditioned with MeOH 100% and distilled water before. The flow rate was of 1 $mL.min^{-1}$. MeOH 20% (v/v) was used to wash the loaded cartridge and MeOH 80% (v/v) was used for MC-LR elution. After elution, the MC-LR containing fraction was evaporated by rotary evaporation at 35ºC to completely remove MeOH. The concentrated MC-LR fraction was subsequently purified and quantified by HPLC-PDA following modified versions (Ramanan et al. 2000; Xie & Park 2007). The limit of detection of this compound in the HPLC-PDA system is 0.2 $mg.L^{-1}$. A reversed phase column (Phenomenex Luna RP-18, 25 cm × 10 mm, 10 µm) kept at 40ºC were used for MC-LR purification. The gradient elution utilized MeOH and water both acidified with 0.1% trifluoracetic acid (TFA) with a flow rate of 2.5 $mL.min^{-1}$. The injected volume was 500 µL. Peak purity and percentage of purified MC-LR was calculated at 214 and 238 nm. The fractions containing purified MC-LR were then combined, evaporated to dryness with air nitrogen for 1 day and redissolved

in culture medium. For purified MC-LR quantification, a reversed phase column (Merck Lichrospher RP-18 endcapped, 25 cm × 4.6 mm, 5 µm) equipped with a guard column (Merck Lichrospher RP-18 endcapped, 4 × 4 mm, 5 µm) both kept at 45ºC were used. The binary gradient of elution consisted of (A) MeOH + TFA 0.1% and (B) H_2O + TFA 0.1% (55% A and 45% B at 0 min, 65% A and 35% B at 5 min, 80% A and 20% B at 10 min, 100% A at 15 min, 55% A and 45% B at 15.1 and 20 min) with a flow rate of 0.9 $mL.min^{-1}$. The injected volume was 20 µL. The PDA range was 210-400 nm, with a fixed wavelength at 238 nm. The linearity method was achieved between 0.5 and 20 $mg.L^{-1}$. MC-LR was identified by comparison of spectra and retention time with a standard of MC-LR (≥ 95% purity, Sigma-Aldrich). All HPLC solvents were filtered (Pall GH Polypro 47 mm, 0.2 µm) and degassed by ultrasound bath.

After analysis, the final concentration of the MC-LR stock solution was 673.3 $mg.L^{-1}$ and its purity was 97% according to HPLC chromatograms. Purified MC-LR was then diluted in Z8 medium to the concentration range used in experiments.

3.2.4. CYN extraction, purification and quantification

CYN was extracted from lyophilized *A. ovalisporum* biomass according to the method described by Welker et al. (2002), with some modifications. Briefly, freeze-dried cells (0.7 g) were mixed with 5 mL of distilled water acidified with TFA 0.1% (v/v). The homogenate was then stirred for 1h at room temperature, sonicated in bath for 15 min and ultrasonicated on ice at 60 Hz with 5 cycles of 1 min (VibraCell 50 sonics & Material Inc. Danbury, CT, USA). Cell debris was separated by centrifugation (20000 *g*, 20 min) after the extraction step. The pellet was thereafter re-extracted with the same volume of distilled water acidified with TFA. The supernatants resulting from both extraction steps were pooled together and stored at -20ºC.

The same HPLC system was used to purify and quantify the CYN content in the extract. The limit of detection of this compound in the HPLC-PDA system is 0.3 $mg.L^{-1}$. A reversed phase column (Phenomenex Gemini C_{18}, 250 cm × 10 mm, 5 µm) kept at 40ºC

were used for CYN purification. The isocratic elution utilized MeOH 5% (v/v) containing 2 mM of sodium 1-heptanesulfonate monohydrate (99%) with a flow rate of 3 $mL.min^{-1}$. The injection volume was 500 µL. Peak purity and percentage of purified CYN was calculated at 262 nm. The fractions containing purified CYN were then combined, evaporated by speed-vac at 30ºC and redissolved in culture medium to the desired concentration. For purified CYN quantification, a reversed phase column (Waters Atlantis® HILLIC, 250 mm × 10 mm, 5 µm) kept at 40ºC were used. The isocratic elution utilized MeOH 5% (v/v) containing 2 mM of sodium 1-heptanesulfonate monohydrate (99%) with a flow rate of 0.9 $mL.min^{-1}$ and an injected volume of 10 µL. The PDA range was 210-400 nm with a fixed wavelength of 262 nm. The linearity method was achieved between 0.3 and 25 $mg.L^{-1}$. The CYN was identified by comparison of spectra and retention time with a standard of CYN (100% purity, Cork University, Ireland). All HPLC solvents were filtered (Pall GH Polypro 47 mm, 0.2 µm) and degassed by ultrasound bath.

After analysis, the final concentration of CYN stock solution was 608.7 $mg.L^{-1}$ and its purity exceeded 98% according to HPLC chromatograms. Purified CYN was diluted in Z8 medium to the concentration range used in experiments.

3.2.5. Experimental design

3.2.5.1. Single-chemical exposures

The growth inhibition tests with the freshwater algae *C. vulgaris* were performed in 96-well polystyrene microplates based on the method described by Gantar et al. (2008) due to the experimental design used and the amount of cyanotoxins necessary for the concentration range selected for the experiments.

The log-phase growing microalgae at a cell concentration of approximately 5×10^5 $cell.mL^{-1}$ was exposed for 7 days to the chemical compounds and the algae growth was determined based on the OECD 201 Guideline (2006). Nine concentrations of Cd and TBA

plus a negative control (Z8 medium only) with 5 replicates each were used for the experimental setup. Nominal concentrations for Cd ranged from 0.25 to 64 mg.L^{-1} and for TBA from 0.001 to 0.5 mg.L^{-1}. Five effective concentrations of pure MC-LR (range, 1.2-37.3 mg.L^{-1}) and pure CYN (range, 0.4-16.7 mg.L^{-1}) with 3 replicates each were previously used in our study (Pinheiro et al. in press). Each well consisted of 200 µL of test solution (with or without chemical) prepared in fresh culture medium. The pH values was recorded in the beginning and at the end of the experiments.

Microplates were sealed with perforated parafilm (to reduce evaporation and allow gas exchanges) and incubated for 7 days at the same temperature and luminosity conditions mentioned above for microalgae culture. Algal growth was measured indirectly by optical density (OD) using microplate reader (PowerWave, Biotek, Vermont, USA) at a wavelength of 750 nm. The first reading was taken at the beginning of each experiment and subsequent readings were taken 4 and 7 days later. Before the measurement of OD (on day 4 and 7), the well content was ressuspended with a pipette. All the OD values were converted in cell.mL^{-1} using the equation:

$$C = 3.00 \times 10^7 \times Abs + 2.17 \times 10^5 \ (R^2 = 0.99)$$

where C is the algae concentration (cell.mL^{-1}) and Abs is the absorbance obtained at 750 nm.

The average specific growth rate was calculated as the logarithmic increase in cell concentration for the period of 4 and 7 days from the equation:

$$\mu_{i-j} = \frac{\ln B_j - \ln B_i}{t_j - t_i}$$

where μ_{i-j} is the average specific growth rate from time i to j; t_i is the time for the start of the exposure period; t_j is the time for the end of the exposure period; B_i is the cell concentration at time i and B_j is the cell concentration at time j.

In order to meet the validity of the experiments, the reference substance potassium dichromate was used in three concentrations (5, 10 and 20 $mg.L^{-1}$) with 5 replicates each.

3.2.5.2. Binary mixture exposures

The growth inhibition test with the freshwater algae *C. vulgaris* were conducted using the same methodology as the single-chemical tests.

The following binary combinations were tested: MC-LR and TBA, MC-LR and Cd, CYN and TBA, CYN and Cd and TBA and Cd. A ray experimental design was used with the exception of the mixture of Cd and TBA where a full factorial experimental design was applied.

For the MC-LR and TBA and MC-LR and Cd combinations, the experimental design consisted of single exposures to 7 concentrations of MC-LR and 5 concentrations of TBA and Cd and to 25 combinations of substances for each case (Figure 1). The nominal concentrations that were used for MC-LR single exposures ranged from 0.5 to 80 $mg.L^{-1}$ and for combined exposures from 0.5 to 20 $mg.L^{-1}$. As the toxicity prediction of binary mixture exposures is based on the dose-response curve of each of the chemicals independently, a high concentration range of MC-LR was used in each single exposure in order to calculate the EC_{50} value since in our previous study no growth inhibition was observed for the growth rate of *C. vulgaris* at the tested concentrations, thereby preventing the calculation of the IC_{50} value (Pinheiro et al. in press). For TBA and Cd nominal concentrations ranged from 0.03 to 0.48 and 0.05 to 5 $mg.L^{-1}$ in single and combined exposures, respectively.

For the CYN and TBA and CYN and Cd combinations, the experimental design consisted of single exposures to 4 concentrations of CYN and 5 concentration of TBA and Cd and to 25 combinations of substances for each case (Figure 1). The nominal concentrations that were used for CYN single exposures ranged from 10 to 80 $mg.L^{-1}$ and for combined exposures from 0.5 to 20 $mg.L^{-1}$. Again, for the same reason, a high concentration range of CYN was used in each single exposure in order to get reliable EC_{50}

estimates, because in our previous study only weak growth inhibitions were registered for the growth rate of *C. vulgaris* after 4 days of exposure to CYN, overestimating the calculation of the IC_{50} value (Pinheiro et al. in press). For TBA and Cd nominal concentrations ranged from 0.03 to 0.48 and 0.05 to 5 $mg.L^{-1}$ in single and combined exposures, respectively.

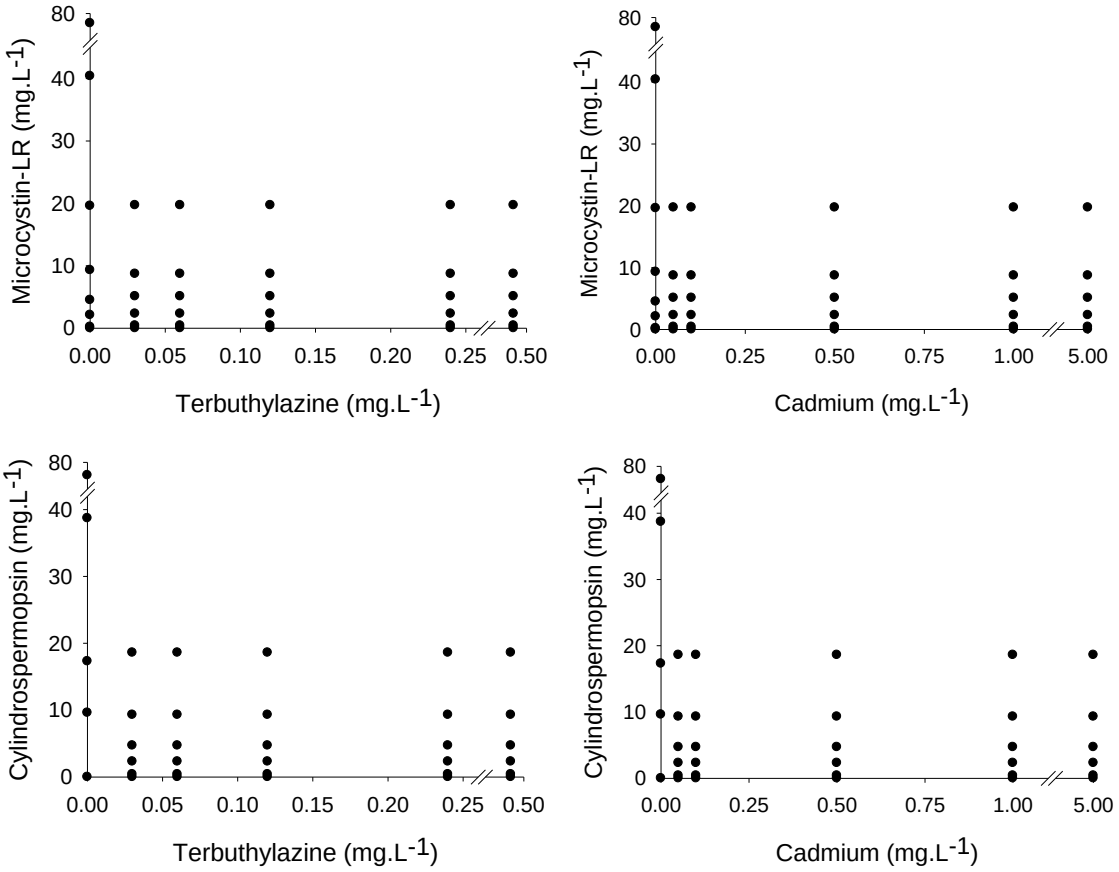

Figure 1 – A schematic ray design of the combinations used for the microcystin-LR and terbuthylazine, microcystin-LR and cadmium, cylindrospermopsin and terbuthylazine, cylindrospermopsin and cadmium.

For TBA and Cd combination, the experimental design consisted of single exposures to 6 concentrations of each chemical and to 36 combinations of both chemicals (Figure 2). Nominal concentration of TBA and Cd ranged from 0.03 to 0.48 and 0.05 to 5 $mg.L^{-1}$ in single and combined exposures, respectively.

In the binary mixture experiments, individual and mixture exposures were carried out simultaneously so that differences in organisms' responses, due to the sensitivity variations, could be controlled and not invalidate the analysis. Three replicates were used for single and mixture experiments.

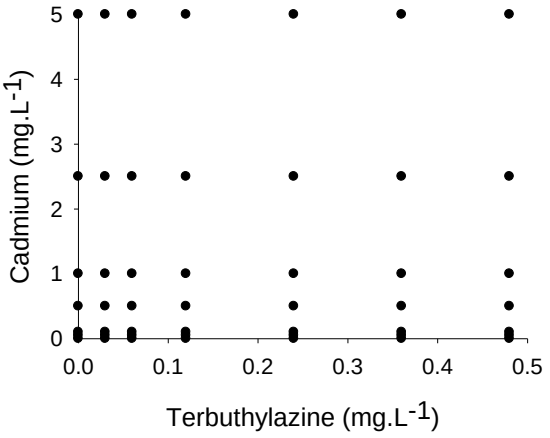

Figure 2 – A schematic full factorial design of the combinations used for the terbuthylazine and cadmium mixtures.

3.2.6. Data analysis

In order to determine the no observed effect concentration (NOEC, i.e. the highest concentration to cause no significant effect on algal growth) and the lowest observed effect concentration (LOEC, i.e. the lowest concentration to cause a significant effect on algal growth), a one-way analysis of variance (ANOVA) was performed using the SigmaPlot software (SPSS, 2002), followed by the multiple comparisons Dunnett's method to detect differences between the data that followed a normal distribution and homogeneity of variances. When the normality test and data transformation procedures failed, a non-parametric Kruskal-Wallis one-way ANOVA test was used and the multiple comparisons Dunn's method conducted. All significant differences were established at $P \leq 0.05$.

The EC_{50} values for each chemical compound in the single exposures were, when possible, calculated using the following three parameter logistic curve (the same dose-response curve used within the MIXTOX tool):

$$Y_{c_i} = \frac{Y_{max}}{1 + \left(\frac{c_i}{EC_{50}}\right)^{\beta}}$$

where Y_{ci} is the response of a given parameter at a concentration c_i of a chemical, Y_{max} is the maximum response value for the parameter, EC_{50} is the effect concentration that provoke 50% of the response and β is the slope. When it was not possible to fit this curve to get reliable EC_{50} estimates, the analysis in the MIXTOX tool were conducted with fixed EC_{50} and slope parameters according to Loureiro et al. (2010).

Whenever it was not possible to observe a full dose response curve for a particular component of a binary mixture, synergistic ratios (SR) were calculated to estimate how strong was the synergistic inhibition of the growth rates of *C. vulgaris* exerted by that binary mixture. For this, the EC_{50} values were estimated for the component of the binary mixture where a full dose response curve was observed (e.g. Chem1), in the presence of each concentration of the other component where a full dose response curve was not obtained (e.g. Chem2). The estimation of EC_{50} values was obtained using, when feasible, the same three parameter logistic curve described above. After that, SRs were calculated by dividing the EC_{50} value for the Chem1 (without the presence of the Chem2) by EC_{50} value for each of Chem1 and Chem2 treatments. SRs of 1.0 indicate no effects of the Chem2 on Chem1 toxicity (or an additive response), whereas values of > 1.0 and < 1.0 indicate greater and less effects than expected, respectively.

To predict the toxic effects in the binary mixture experiments, the observed effect was compared to the expected effect of mixtures calculated from the single chemical exposure, using the conceptual model of independent action (IA) described by Jonker et al. (2005). This conceptual model assumes that both components in the mixture have dissimilar modes of action, acting at different target sites, which is the case of the selected chemical substances. However, the toxic behaviour of a mixture may deviate from this

conceptual model, thereby being more or less severe than expected. Deviations from the IA model such as synergism/antagonism (S/A), dose ratio (DR) dependency and dose level (DL) dependency were obtained by the addition of two parameter (*a* and *b*) to the mathematical model that describe IA [see in more detail in Jonker et al. (2005)]. The biological interpretation of these additional deviation parameters are summarized in Table 1 and may be found in more detail in Jonker et al. (2005). The tool used to analyze and compare the data in the present study was the MIXTOX already described by Jonker et al. (2005), which allowed to fit the IA model to the data and also to extend it to test for deviations. The data fitted to the IA model or their deviations was compared using the method of maximum likelihood and the best fit chosen using the Chi-square test at the significance level of 0.05. When a deviation from IA model was obtained, the effects pattern was deduced directly from the parameter values described in Table 1.

Table 1 – Interpretation of additional parameters (*a* and *b*) that define the functional form of deviation patterns from independent action. EC_{50} is the median effect concentration.

Deviation pattern	**Independent action**	
	Parameter *a*	**Parameter *b***
Synergism/Antagonism (S/A)	$a > 0$: antagonism $a < 0$: synergism	
Dose ratio dependent (DR)	$a > 0$: antagonism except for those mixture ratios where negative b value indicate synergism	$b_i > 0$: antagonism where the toxicity of the mixture is caused mainly by toxicant *i*
	$a < 0$: synergism except for those mixture ratios where positive b value indicate antagonism	$b_i < 0$: synergism where the toxicity of the mixture is caused mainly by toxicant *i*
Dose level dependent (DL)	$a > 0$: antagonism low dose level and synergism high dose level	$b_{DL} > 2$: change at lower EC_{50} level $b_{DL} = 2$: change at EC_{50} level
	$a < 0$: synergism low dose level and antagonism high dose level	$1 < b_{DL} < 2$: change at higher EC_{50} level $b_{DL} < 1$: no change, but the magnitude of S/A is effect level dependent

Adapted from Jonker et al. (2005)

3.3. Results

3.3.1. Chemical analysis

In all tests performed, pH ranged between 7.3-7.6 and 9.1-9.5 for MC-LR and CYN, and between 7.8-8.2 and 9.6-10.5 for Cd and TBA in the beginning and at the end of the experiments. In the highest tested Cd concentration, pH measured at the end of the experiments ranged between 7.3-7.5.

In order to assess contamination accuracy, MC-LR and CYN analyzes were made by HPLC-PDA and the results showed that measured concentrations varied generally more than 20% from the nominal concentrations. So, all calculations were based on effective concentrations. Stability analyzes for MC-LR and CYN were also made by HPLC-PDA and the results showed no toxin degradation throughout the 7 days of exposure.

3.3.2. Single chemical exposures

The NOEC, LOEC and EC_{50} values obtained from each of the four single chemical exposures are reported in Table 2, along with the data obtained in the single-chemical exposures from the binary mixture experiments. After 4 days of exposure, it is clear that TBA was the most toxic to *C. vulgaris*, being approximately 5 times more toxic than Cd, which in turn is approximately 28 times more toxic than CYN. After 7 days of exposure, the toxicity difference between TBA and Cd is much smaller, with TBA being only 2.5 times more toxic than Cd. On the other hand, the toxicity difference between Cd and CYN is much higher, being CYN approximately 46 times less toxic than Cd. MC-LR was clearly the less toxic to *C. vulgaris*, differing approximately 2 times from CYN toxicity on the 4^{th} and 7^{th} days of exposure.

In general, the NOEC and LOEC for pure MC-LR after 4 and 7 days of exposure was 40.4 and 78.9 $mg.L^{-1}$ (run in the binary mixture set up), respectively. In mixture experimental set ups, growth rate of *C. vulgaris* exposed to single pure MC-LR showed a maximum reduction of 23% and 44% on the 4^{th} and 7^{th} days of exposure, respectively, when compared to the control. It was therefore not possible to calculate a valid EC_{50} value for pure MC-LR in both single chemical exposures, and further analysis in the MIXTOX tool had to be undertaken with fixed EC_{50} and slope parameters. The same situation was also observed to single TBA exposure from the binary mixture with CYN. In spite of the LOEC value for TBA was 0.24 $mg.L^{-1}$ for 4 days of exposure and 0.12 $mg.L^{-1}$ for 7 days of exposure, a maximum inhibition of 37% and 47% was registered for the growth rate of *C. vulgaris* exposed to single TBA at 0.48 $mg.L^{-1}$. Again, it was not possible to obtain a valid EC_{50} value for TBA, and further analysis in the MIXTOX tool were made by fixing the EC_{50} and slope parameters.

The EC_{50} values between the experiments were similar, showing reproducibility on the methodology, with the best belonging to TBA and the most variable belonging to Cd (Table 2).

Table 2 – NOEC, LOEC and EC_{50} values (with corresponding standard errors), in $mg.L^{-1}$, for the effect of selected chemical substances on the growth rate of *Chlorella vulgaris* exposed for 4 and 7 days in Z8 medium. NOEC is the no observed effects concentration, LOEC the lowest observed effect concentration and EC_{50} the median effect concentration.

Substances	NOEC		LOEC		EC_{50} (± SE)		Experiment
	Day 4	Day 7	Day 4	Day 7	Day 4	Day 7	
Microcystin-LR	> 37.3	23.9	> 37.3	37.3	> 37.30	> 37.30	Single exposure experiments[a]
(MC-LR)	40.4	40.4	78.9	78.9	> 78.9	> 78.9	Mixture experiment with TBA
	19.6	40.4	40.4	78.9	> 78.9	> 78.9	Mixture experiment with Cd
Cylindrospermopsin	4.4	> 16.7	8.5	> 16.7	> 16.70 (n.d.)	> 16.70 (n.d.)	Single exposure experiments[a]
(CYN)	17.3	9.6	38.7	17.3	49.21 (± 3.29)	47.67 (± 2.98)	Mixture experiment with TBA
	17.3	9.6	38.7	17.3	56.34 (± 2.73)	51.26 (± 1.72)	Mixture experiment with Cd
Terbuthylazine	0.01	0.025	0.025	0.05	0.39 (± 0.03)	0.39 (± 0.02)	Single exposure experiments
(TBA)	0.12	0.03	0.24	0.06	0.37 (± 0.03)	0.46 (± 0.03)	Mixture experiment with MC-LR
	0.12	0.06	0.24	0.12	> 0.48	> 0.48	Mixture experiment with CYN
	< 0.03	0.03	0.03	0.06	0.35 (± 0.01)	0.46 (± 0.02)	Mixture experiment with Cd
Cadmium	1	< 0.25	2	0.25	1.17 (± 0.19)	0.88 (± 0.08)	Single exposure experiments
(Cd)	0.1	0.1	0.5	0.5	0.92 (± 0.08)	0.76 (± 0.04)	Mixture experiment with MC-LR
	1	0,1	5	0.5	4.15 (n.d.)	1.80 (± 0.14)	Mixture experiment with CYN
	0.1	< 0.05	0.5	0.05	1.27 (± 0.08)	0.88 (± 0.03)	Mixture experiment with TBA

[a] Results for *C. vulgaris* from Pinheiro et al. (in press).

3.3.3. Binary mixture exposures

To understand growth responses of *C. vulgaris* to several binary mixture exposures, the reference model of IA was used to evaluate the combined effects of the chemical substances on the growth rate of this microalga in accordance with the mode of action of each chemical. The toxicity of each binary mixture was predicted through the MIXTOX tool. The best descriptive model and the parameters obtained from data fitted with the MIXTOX tool are shown in Table 3.

Comparing the effects of MC-LR and TBA mixture on the growth rates of *C. vulgaris*, after 4 days of exposure, to the IA model a SS value of 0.37 was obtained explaining 67% of the data set. However, adding parameters a and b_{DL} the SS value decreased significantly (SS = 0.15, r^2 = 0.85, $p(\chi^2) < 0.05$) and a DL dependent deviation from IA model was observed (Table 3). Parameter a was positive (a = 1.59) indicating antagonism at low dose levels and synergism at high dose levels (Figure 3A) and parameter b_{DL} was negative (b_{DL} = -5.87) indicating that the magnitude of antagonism/synergism became effect level dependent, which means that synergism is predicted to occur at concentrations much higher than the ones tested in the experimental design (see Figure 1, Table 1 and 3). The same behaviour was observed after 7 days of exposure to the MC-LR and TBA mixture (Table 3, Figure 3B).

Exposure to mixtures of MC-LR and Cd showed a different trend of toxicity on the growth rates of *C. vulgaris*. After 4 days of exposure, a significant antagonistic effect (SS = 0.83, r^2 = 0.63, $p(\chi^2) < 0.05$, a = 4.49) was detected when compared to the IA model (Table 3, Figure 3C). After 7 days of exposure, a significant DL dependent deviation from the IA model (SS = 0.49, r^2 = 0.77, $p(\chi^2) < 0.001$) was observed with the deviation parameters a (a = -7.30) indicating synergism at low dose levels and antagonism at high dose levels (Figure 3D). The switch between synergism and antagonism may be calculated from 1/ b_{DL} × EC_{50} (Jonker et al. 2005). Thus, the change occurred at (1/2.96 = 0.34 × EC_{50}) doses lower than the EC_{50} level (Table 1 and 3).

Table 3 – Summary of the analysis done for the effects on the growth rate of *C. vulgaris* exposed for 4 and 7 days to the binary mixture of microcystin-LR and terbuthylazine, microcystin-LR and cadmium, cylindrospermopsin and terbuthylazine, cylindrospermopsin and cadmium and finally terbuthylazine and cadmium (Chem 1 × Chem 2, respectively).

Mixture	Exposure time (days)	Fit method[a]	Independent action[b]							
			Reference		Deviation					
			SS	r^2	Type	$p(\chi 2)$	SS	r^2	a	b_{DR}/b_{DR}
Microcystin-LR and terbuthylazine	4	MC-LR drc fix	0.37	0.67	DL	3.46×10^{-03}	0.15	0.85	1.59	-5.87
	7	MC-LR drc fix	0.07	0.88	DL	4.62×10^{-03}	0.04	0.93	0.51	-8.52
Microcystin-LR and cadmium	4	MC-LR drc fix	0.91	0.59	A	1.25×10^{-03}	0.83	0.63	4.49	-
	7	MC-LR drc fix	0.66	0.69	DL	3.05×10^{-08}	0.49	0.77	-7.30	2.96
Cylindrospermopsin and terbuthylazine	4	TBA drc fix	0.26	0.66	DR	6.64×10^{-03}	0.15	0.81	0.98	4.99
	7	TBA drc fix	0.10	0.82	DL	7.36×10^{-07}	0.07	0.88	-0.998	5.45
Cylindrospermopsin and cadmium	4	Free	1.34	0.58	A	4.89×10^{-07}	1.06	0.67	15.75	-
	7	Free	0.73	0.62	DL	1.50×10^{-06}	0.57	0.70	-14.53	6.86
Terbuthylazine and cadmium	4	Free	0.24	0.93	A	9.20×10^{-04}	0.23	0.94	0.73	-
	7	Free	0.12	0.96	DL	1.69×10^{-03}	0.11	0.96	1.51	2.26

[a] The fit method indicates if any parameter had to be fixed during fitting due to the poor single chemical effect data, followed for statistics for the reference model of independent action and their best deviation function.
[b] The model parameters given are: a and b_{DR}/b_{DL} are the parameters obtained from the deviation functions; SS is the sum of squared residuals; r^2 is the coefficient of determination; χ^2 is the Chi-squared test and $p(\chi^2)$ indicates the outcome of the likelihood ratio test (significance level $p < 0.05$).
[c] A is antagonism; DR is dose ratio deviation and DL is dose level deviation.

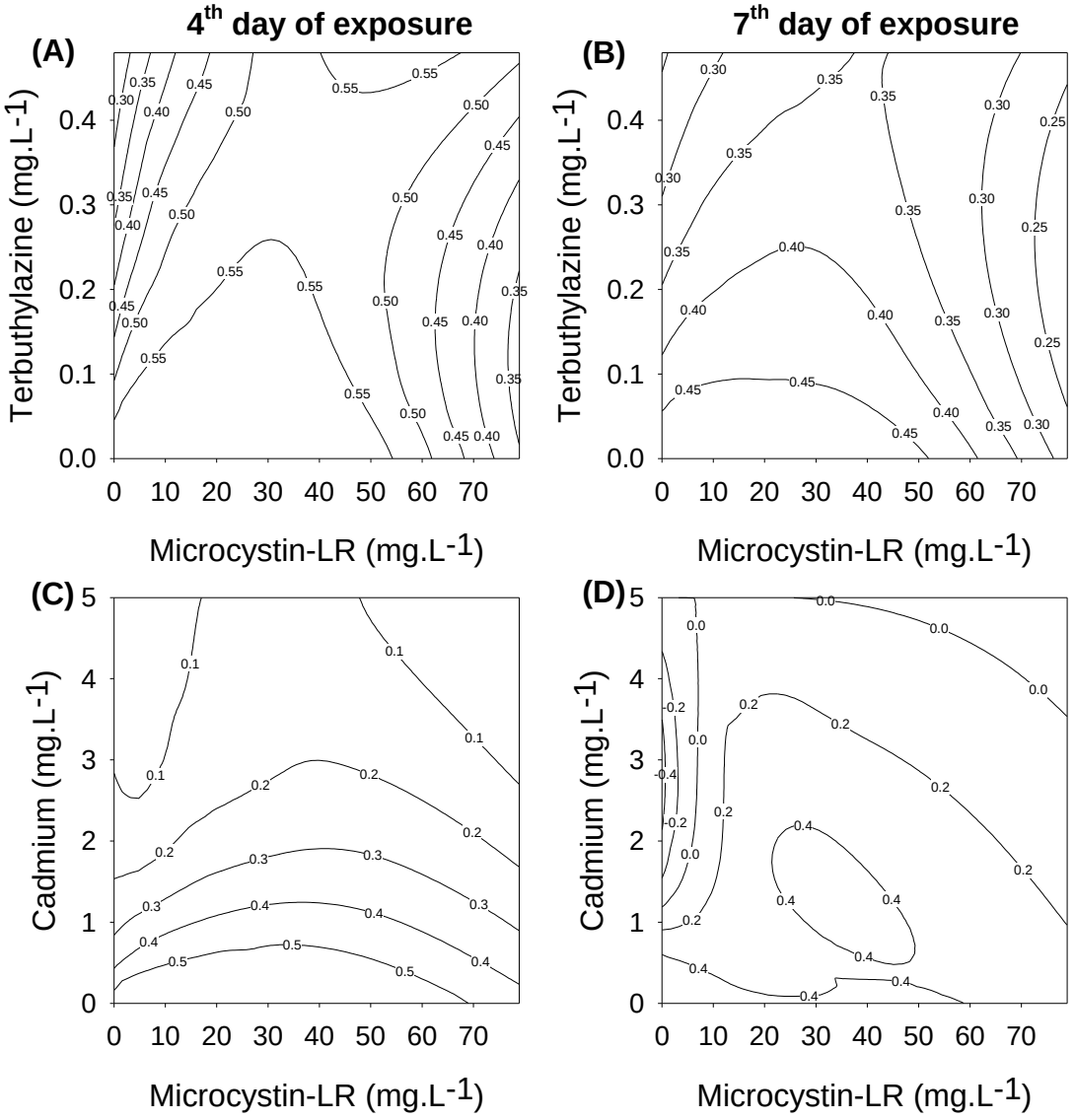

Figure 3 – Concentration-response relationship for the binary mixtures of microcystin-LR and terbuthylazine and microcystin-LR and cadmium tested on the growth rate of *C. vulgaris* after 4 and 7 days of exposure (2D isobolic surface). **A, B** and **D** show a dose level deviation from the IA model; **C** shows an antagonistic pattern after IA model fit. Concentrations of microcystin-LR reported as effective values and concentrations of terbuthylazine and cadmium reported as nominal concentrations.

Comparing the effects of CYN and TBA mixture on the growth rates of *C. vulgaris*, after 4 days of exposure, to the IA model a SS value of 0.26 was obtained explaining 66% of the data set. Nevertheless, adding the parameters a and b_{Dr} the SS value decreased significantly (SS = 0.15, r^2 = 0.81, $p(\chi^2) < 0.05$) and a DR dependent deviation from IA model was obtained (Table 3). In this case, an antagonistic effect was observed and explained mostly when CYN was the dominant chemical in the mixture (a = 0.98 and b_{DR}

= 4.99, Table 1 and 3, Figure 4A). On the 7th day of exposure, a significant DL dependent deviation (SS = 0.07, r^2 = 0.88, $p(\chi^2) < 0.001$) was detected as the deviation that explained better the data after fitting the data to the IA model, showing synergism at low dose levels and antagonism at high dose levels (a = -0.998, Table 1 and 3, Figure 4B). The change between synergism and antagonism occurred at (1/5.45 = 0.18 × EC_{50}) doses lower than the EC_{50} level (Table 1 and 3).

Relatively to the CYN and Cd mixture exposure, when data was modeled using IA a significant fit was observed and the SS value of 1.34 was obtained, explaining 58% of the data set ($p < 0.05$). Adding the parameter a to the IA model to achieve a S/A, the SS value decreased significantly (SS = 1.06, r^2 = 0.67, $p(\chi^2) < 0.001$, Table 3). Parameter a was positive (a = 15.75), indicating antagonism (Figure 4C). On the 7th days of exposure to the binary mixture, data fitted the IA model (SS = 0.73, r^2 = 0.62) but significantly better fit was obtained after adding parameter a and b_{DL} (SS = 0.57, r^2 = 0.70, $p(\chi^2) < 0.001$, Table 3), revealing a DL dependent deviation from the IA model. Parameter a had a negative value (a = -14.53) indicating synergism at low dose levels and antagonism at high dose levels (Figure 4D), and the parameter b_{DL} was higher than 2 (b_{DL} = 6.86) indicating that the change from synergism to antagonism occurred at (1/6.86 = 0.15 × EC_{50}) doses lower than the EC_{50} level (Table 1 and 3).

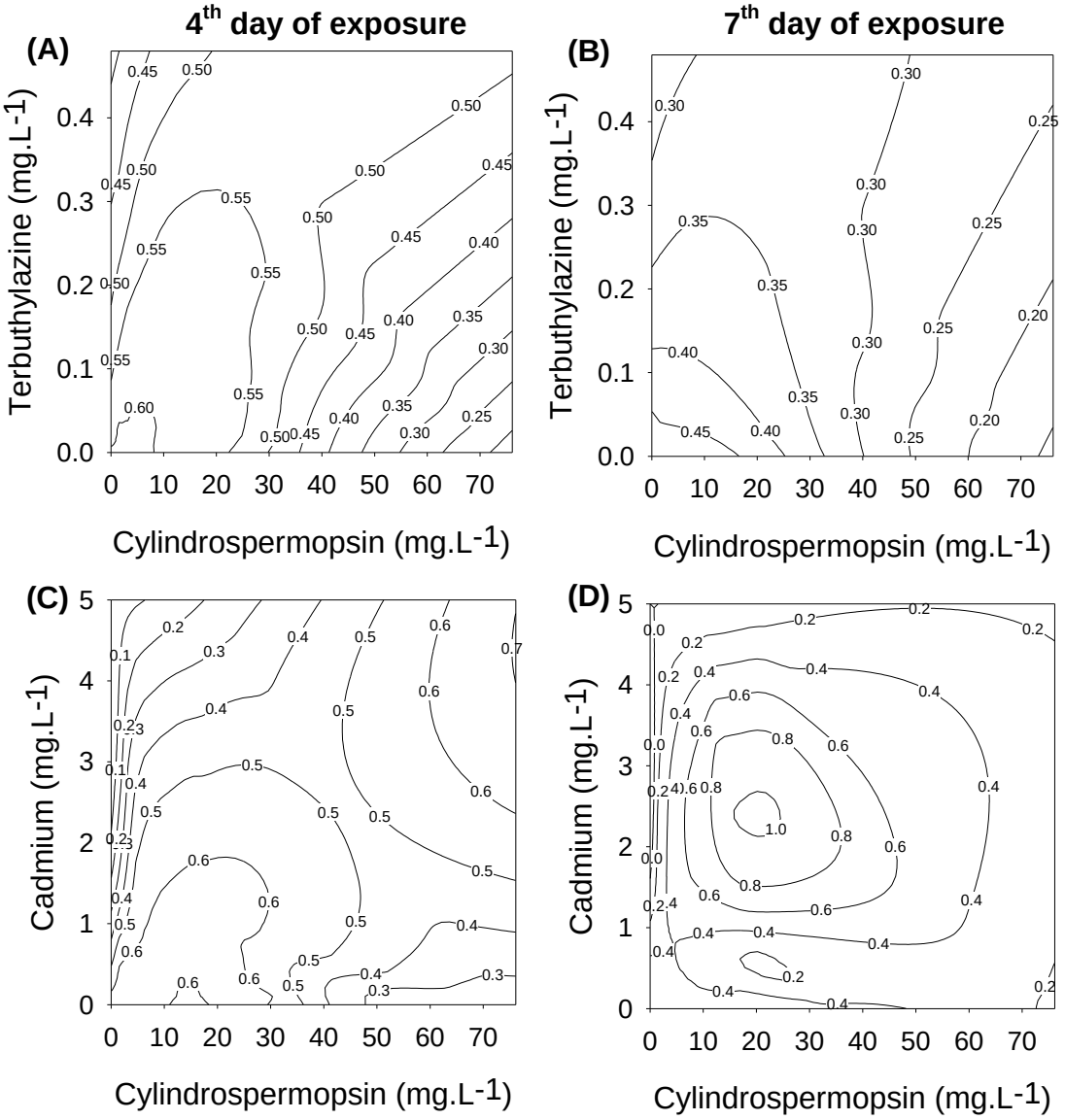

Figure 4 – Concentration-response relationship for the binary mixtures of cylindrospermopsin and terbuthylazine and cylindrospermopsin and cadmium tested on the growth rate of *C. vulgaris* after 4 and 7 days of exposure (2D isobolic surface). **A** shows a dose ratio deviation from the IA model; **B** and **D** show a dose level deviation from the IA model; and **C** shows an antagonistic pattern after IA model fit. Concentrations of cylindrospermopsin reported as effective values and concentrations of terbuthylazine and cadmium reported as nominal concentrations.

Results on the effects of TBA and Cd mixture on the growth rate of *C. vulgaris* after 4 days of exposure fitted significantly the IA model (SS = 0.24, $p < 0.05$), explaining 93% of the data set. However, while changing the function to assess S/A, a small decreased of the SS value was verified (SS = 0.24, $r^2 = 0.94$, $a = 0.73$, Table 3) with significant differences at $p(\chi^2) < 0.001$, indicating antagonism on growth response (Figure 5A). After

7 days of exposure, a significant DL dependent deviation from the IA model (SS = 0.11, r^2 = 0.96, $p(\chi^2) < 0.05$) was observed with the deviation parameters *a* (*a* = 1.51) indicating antagonism at low dose levels and synergism at high dose levels (Figure 5B). The change between antagonism and synergism occurred at (1/2.26 = 0.44 × EC_{50}) doses lower than the EC_{50} level (Table 1 and 3).

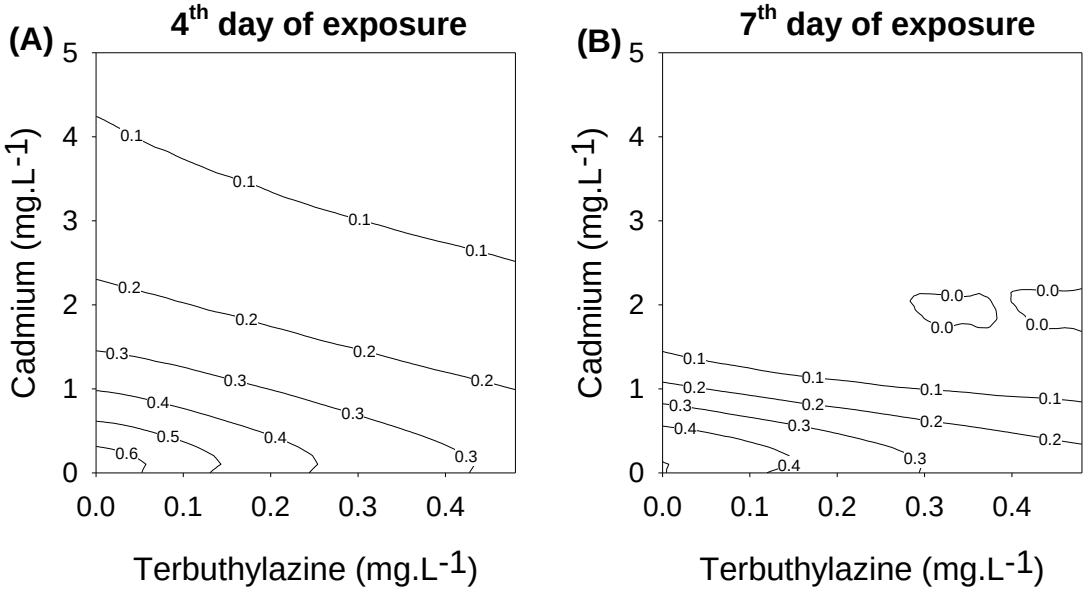

Figure 5 – Concentration-response relationship for the binary mixture of terbuthylazine and cadmium tested on the growth rate of *C. vulgaris* after 4 and 7 days of exposure (2D isobolic surface). **A** shows an antagonistic pattern after IA model fit and **B** show a dose level deviation from the IA model. Concentrations of terbuthylazine and cadmium reported as nominal concentrations.

The EC_{50} values and standard errors are provided in Table 4 with the corresponding SRs. After 4 days of exposure, MC-LR decreased the toxic effects of TBA with SRs of 0.46 at 8.7 $mg.L^{-1}$ and 0.33 at 19.7 $mg.L^{-1}$. The antagonistic effect of MC-LR and TBA mixture was less pronounced after 7 days of exposure with SRs of 0.62 at 8.7 $mg.L^{-1}$ and 0.51 at 19.7 $mg.L^{-1}$. MC-LR also decreased the Cd toxicity with SR of 0.46 at 19.7 $mg.L^{-1}$ on the 4th day of exposure. However, no obvious effect of MC-LR on the Cd toxicity was found after 7 days of exposure (Table 4), even though the MIXTOX tool fitting has revealed a significant synergism at low dose levels and a significant antagonism at high dose level with the change occurring at dose lower than the EC_{50} value (Table 3). In the

case of CYN and TBA mixture, it was not possible to calculate the SRs. Growth rates of *C. vulgaris* were significantly inhibited by CYN in the single exposure as the concentration were increased, following a dose-response relationship (see Table 2). But when co-occurring with TBA, the growth rate inhibition was completely eliminated and there was an increase of the growth rates at values compared with those of control or even a growth stimulus, justifying somehow the antagonism observed mainly when CYN was the dominant component in the mixture after 4 days of exposure. On the 7th day of exposure, there was a weak inhibition of the growth rates of *C. vulgaris* when CYN co-occurred with TBA at 0.03-0.12 mg.L^{-1}, contrasting with the increase of the growth rates to the level of control or even with the growth stimulation at concentration of TBA higher or similar to 0.24 mg.L^{-1}. This explains somehow the significant synergism at low dose levels and the significant antagonism at high dose level suggested by MIXTOX tool, with the change occurring at dose lower than the EC_{50} value (Table 3).

Table 4 – EC_{50} values (with the standard errors, in mg.L^{-1}) and synergistic ratios (SRs) estimated and calculated, respectively, for terbuthylazine (TBA) and cadmium (Cd) when co-occurring with each concentration of microcystin-LR in the mixture experiments.

Compound	Exposure time (days)		Microcystin-LR (mg.L^{-1})					
			0	**0.4**	**2.3**	**5.1**	**8.7**	**19.7**
TBA	4	EC_{50}	0.37	0.37	0.48	0.67	0.80	1.14
		(± SE)	(± 0.03)	(± 0.03)	(± 0.05)	(± 0.10)	(± 0.17)	(± 0.48)
		SR[a]	-	1.00	0.77	0.55	0.46	0.32
	7	EC_{50}	0.46	0.42	0.48	0.61	0.74	0.90
		(± SE)	(± 0.03)	(± 0.03)	(± 0.03)	(± 0.05)	(± 0.10)	(± 0.17)
		SR[a]	-	1.10	0.96	0.75	0.62	0.51
Cd	4	EC_{50}	0.92	0.75	1.76	0.96	0.97	2.02
		(± SE)	(± 0.08)	(± 0.04)	(± 0.15)	(n.d.)	(± 0.25)	(± 1.13)
		SR[a]	-	1.23	0.52	0.96	0.95	0.46
	7	EC_{50}	0.76	0.72	1.08	0.76	0.86	0.92
		(± SE)	(± 0.04)	(± 0.02)	(± 0.06)	(± 0.02)	(± 0.04)	(± 0.67)
		SR[a]	-	1.06	0.70	1.00	0.88	0.83

SE is the standard error; n.d. is not determined

[a] SR = $EC_{50\ TBA\ or\ Cd\ without\ MC\text{-}LR}$ / $EC_{50\ TBA\ or\ Cd\ and\ MC\text{-}LR\ treatments}$

3.4. Discussion

3.4.1. Single chemical exposures

The 7-day growth inhibition tests with the single cyanotoxins showed that pure MC-LR and CYN were toxic at the highest concentrations to the freshwater algae *C. vulgaris* (Table 2). In the present study, concentrations of 49.21 and 56.34 $mg.L^{-1}$ CYN decreased the growth rate of *C. vulgaris* by 50% in a 4-day exposure period. After 7 days of exposure, 50% of growth rate inhibition was observed at concentrations around 50 $mg.L^{-1}$, suggesting that CYN is more toxic with increasing exposure periods. The LOEC values found for MC-LR, in general, were 40.4 and 78.9 $mg.L^{-1}$ with a maximum growth rate inhibition of 23% and 44% on the 4^{th} and 7^{th} days of exposure, respectively. However, MC-LR and CYN toxicity to the *C. vulgaris* growth is considered not ecologically relevant because toxic effects on microalgae growth in aquatic environments are likely caused by high concentrations and probably not found in the water. Although, in a few cases, concentrations of dissolved MCs can reach 1.8 $mg.L^{-1}$ (Jones & Orr 1994) or higher (up to 25 $mg.L^{-1}$) (Fastner et al. 1999; Kemp & John 2006; Máthé et al. 2007; Nagata et al. 1997) in natural waters during the collapse of cyanobacterial blooms, they commnonly do not exceed 0.1 $mg.L^{-1}$ (Vasconcelos et al. 2011; Vasconcelos & Pereira 2001) and concentrations between 0.05 and 5 $\mu g.L^{-1}$ are considered to be typical for aquatic ecosystems with cyanobacterial massive development (Babica et al. 2006; Babica et al. 2007; Lahti et al. 1997). Relatively to CYN, cyanobacterial blooms are concurrent with 0.08-18 $\mu g.L^{-1}$ CYN in Europe (Bogialli et al. 2006; Gallo et al. 2009; Messineo et al. 2010; Quesada et al. 2006; Rücker et al. 2007) and up to 0.8 $mg.L^{-1}$ CYN in Australia (Griffiths & Saker 2003; Shaw et al. 1999). It is important to remember that such high concentrations of pure MC-LR and CYN were used to estimate the EC_{50} values for both cyanotoxins so that further analysis in MIXTOX tool were possible to perform.

For TBA (as a commercial formulation), there are no available toxicity data for microalgae. To our knowledge, this study provides for the first time evidence that a

commercial formulation containing TBA (SAPEC with 500 g a.i.L^{-1}) is highly toxic to *C. vulgaris*, reducing their growth rates by 50% at concentrations 0.35-0.39 mg.L^{-1} in a 4-day exposure period. On the 7th day of exposure, slightly higher EC_{50} values, 0.39-0.46 mg.L^{-1}, were registered in our study, indicating recovery of the growth rates. Although the toxicity of TBA as a commercial formulation has not received any scientific attention until now, information about the toxicity of TBA as a pure compound on microalgae is already known. Perez et al. (2011) studied the toxic effects of the *s*-triazine herbicide TBA on the growth rate of *Pseudokirchneriella subcapitata* and found 72-h EC_{50} value of 0.024 mg.L^{-1}. Okamura et al. (2000) found that *P. subcapitata* was also sensitive to TBA with 72-h EC_{50} value of 0.036 mg.L^{-1} which is a value comparable to that determined in the previous study. In a 48-h exposure period, Munkegaard et al. (2008) investigated whether interactions between organophosphorous insecticides and herbicides can take place in the algae *P. subcapitata* and a EC_{50} value of 0.60 mg.L^{-1} for TBA was found. Compared with previous studies, TBA used in our study as a commercial formulation seem to be less toxic to microalgae, even though different microalgae species (with different susceptibilities), exposure periods and exposure mediums have been used to observe toxic effects. This is not in accordance with some studies available in the literature that clearly demonstrated that commercial formulations exhibit higher toxicity to non-target organisms than the corresponding active ingredients (Cedergreen & Streibig 2005; Pereira et al. 2009a; Pereira et al. 2000). Commercial formulations are composed of the active ingredient and a number of other chemicals (generally called inert ingredients) that support its mixing, dilution, application and stability (Cox & Surgan 2006). The inert ingredients are not supposed to be toxic, but some authors have shown that they can contribute to the overall toxicity of the formulation, either by exerting toxic activity on their own, or by interacting with the active ingredient (Oakes & Pollak 2000; Solomon & Thompson 2003).

Cd was also highly toxic towards *C. vulgaris* cells, with EC_{50} values ranging from 0.92 to 4.15 mg.L^{-1} for 4-day exposure period and 0.76 to 1.80 mg.L^{-1} for 7-day exposure period. Literature data suggest a great variability in the values of EC_{50} obtained for microalgae (Báscik-Remisiewicz et al. 2011; Lin et al. 2007; Magdaleno et al. 1997; Pereira et al. 2005; Yap et al. 2004). In a comparative study of single and mixture toxicities of Cd and Cu on the growth response of *C. vulgaris*, Lam et al. (1999) observed a 4-day

EC_{50} value for Cd of 4.01 $mg.L^{-1}$ from population density, which is in the same range of one EC_{50} value obtained in this study (Table 2). In contrast to our study, Rachin & Grosso (1993), who studied the growth response of the algae *C. vulgaris* to combined exposure of different metals (Cd, Cu and Co), obtained a lower EC_{50} value from population density, 0.10 $mg.L^{-1}$, for a 4-day exposure period, although close to our EC_{50} range. The differences in microalgae responses to Cd may result from chemical speciation of this metal in the culture medium. The chemical form of Cd in water strongly depends on the composition of this medium. It has been well documented that free Cd^{2+} ions are the most toxic form of Cd to organisms and that concentration of free metal ions can vary in the presence of complexing agents (Fernandezpinas et al. 1991).

3.4.2. Binary mixture exposures

According to the MIXTOX analysis, all the mixture exposures undertaken deviated from the non-interactive conceptual model of IA. Such deviations from the IA model may occur when compounds affect the bioavailability of one another, their modes of action and their behaviour after uptake (e.g. bioaccumulation) (Loureiro et al. 2010). In other words, interactions between compounds may be linked to toxicokinetic or toxicodymanic phases.

Fitting the IA model to the growth rate data from *C. vulgaris* species exposed to the MC-LR and TBA mixture resulted in a DL dependent deviation, indicating antagonism at low dose levels and synergism at doses above the concentrations tested in this experiment. The most likely explanation for the antagonistic response is that MC-LR affects *C. vulgaris* in a way that decreases the toxic action of TBA. Therefore, a specific mechanism is likely responsible for the decreased toxicity. TBA is a specific inhibitor of the photosynthetic electron transport in plants and algae (Faust et al. 2001), disturbing thereby the photosynthesis. It acts by the competitive and reversible binding to the domain of the D1 protein of the PSII reaction center, thus displacing the electron acceptor plastoquinone Q_B from this site (Faust et al. 2001; Giardi et al. 1995). MCs were also shown to affect photosynthesis in several photosynthetic organisms (Perron et al. 2012; Singh et al. 2001;

Weiss et al. 2000; Wiegand et al. 2002), but the detailed mode of action and pathways leading to the observed adverse effects in photosynthesis remain to be elucidated. It is well established that MCs induce the production of reactive oxygen species (ROS) leading to oxidative stress as well as the enhancement of antioxidant enzymes production (Amado & Monserrat 2010; Mohamed 2008; Pflugmacher 2004; Wiegand et al. 2002; Yin et al. 2005) in aquatic invertebrates, plants and algae. The intracellular increase of ROS, exceeding the cellular detoxification capability, may lead to the oxidation of biomolecules and induce preferential damage to PSII, with an irreversible oxidation of D1 protein causing its degradation (Drábková et al. 2007; Kim & Lee 2005; Krieger-Liszkay et al. 2008; Okada et al. 1996). TBA would be thus unable to bind to the domain of D1 protein and prevent the energy flow within PSII. Therefore, the antagonistic effects of MC-LR on the TBA toxicity are likely due to the oxidative changes in the PSII reaction center.

When the IA model was fitted to the growth rate data from *C. vulgaris* exposed to the MC-LR and Cd mixture for 4 days, an antagonism deviation was revealed probably due to a reduction on Cd toxicity caused by the induction of metallothioneins (MT) and phytochelatins (PCs), i.e., proteins of detoxification in algae (Perales-Vela et al. 2006; Robinson 1989), by cell's defensive mechanisms. The induction of PCs can also be linked to the presence of MC-LR in the mixture, since PCs are small metal-binding polypeptides enzymatically synthesized from glutathione (GSH) and Wang et al. (2012; 2011) found that combined exposure to the cyclic peptide MC-LR and linear alkylbenzene sulfonate increased the GSH content. Another plausible reason might be the fact that during the 4-day exposure period MC-LR-Cd complexes have been formed leading to smaller bioavailability of both compounds to the microalgae *C. vulgaris*, inside or outside the cells. Some studies have already documented the formation of these MC-metal complexes which could potentially arise from the coexistence of metals and cyanotoxins in aquatic systems (Humble et al. 1997; Saito et al. 2008; Yan et al. 2000). Humble et al. (1997) studied the interactions of Cu and Zn with three variants of MCs (MC-LR, -LW and -LF) at environmentally relevant pH values by differential pulse polarography and clearly demonstrated complexation between MC-LR and Cu in a pH dependent manner, with the extent of binding decreasing with decreasing pH values. Complex formation between MC-LR and Zn, however, was not influenced as strongly by pH (Humble et al. 1997).

Similarly, Yan et al. (2000) characterized the complexation of MC-LR with many metals (Hg, Pb, Cd, Cu and Zn) by using cyclic voltammetry and anodic stripping voltammetry. These authors demonstrated that MC-LR is able to form a stable complex with metal ions by accommodating them within its cavity region and complex formation was strongly dependent on the concentration of MCs. More recently, complexes of MC-LR and metals such as Fe (II), Zn, Cu and Mg were detected using cryospray ionization-Fourier transform ion cyclotron resonance mass spectrometry (Saito et al. 2008).

When the IA model was fitted to the growth rate data of the MC-LR and Cd mixture corresponding to the 7^{th} days of exposure, a DL dependent deviation was observed showing synergism at low dose levels and antagonism at high dose levels. Although the synergism/antagonism at different dose levels was not confirmed by the calculation of the EC_{50} values of Cd at different MC-LR concentrations in the mixture, the DL dependent deviation that resulted from the IA model was not rejected ($p(\chi^2) < 0.001$). The synergistic effect at low doses might be probably related to the production of ROS induced by both compounds culminating in oxidative stress (Amado & Monserrat 2010; Pinto et al. 2003) and consequently a reduction on cellular detoxification processes. Although it is not relevant at environmentally relevant concentrations, the antagonism observed at high dose levels may be associated with the interactions between MC-LR and Cd in the exposure medium or in the cytoplasmatic matrix or with the induction of extra detoxification proteins, which could be the explanation for the reduced toxicity in the MC-LR and Cd mixture.

The fit of the IA model to the binary mixture data of CYN and TBA exposure in the first four days resulted in a DR dependent deviation where a decrease of the mixture toxicity (antagonism) was observed and explained mainly when CYN was the dominant component in the mixture. One possible explanation for the antagonism detected in this mixture after 4 days of exposure is the activation of cellular detoxification mechanisms induced by CYN and TBA, causing a decrease of their toxicity and leading to the observed recovery of the *C. vulgaris* growth rates at levels close to those of control or even growth stimulation. It is well established that GST is an important enzyme responsible for the metabolism and detoxification of several classes of herbicides in plants, including members of the chloro-*s*-triazine group such as TBA and atrazine (Cummins et al. 2011;

Edwards & Dixon 2005; Edwards et al. 2000). The work of Tang et al. (1998) also provided evidence for the involvement of GST in atrazine metabolism in freshwater algae (*Chlamydomonas* sp., *Chlorella* sp., *Pediastrum* sp. and *Scenedesmus quadricauda*) based on the GSH-dependent formation of water soluble metabolites. It is therefore likely that the metabolism and detoxification of TBA follow similar paths in *C. vulgaris* as those expressed in atrazine metabolism in other microalgae species (Tang et al. 1998). Despite glutathione-CYN conjugate has not been identified so far, it is possible that the detoxification and biotransformation of CYN by microalgae occurs through the activity of GST as demonstrated for MC-LR (Kondo et al. 1992; Pflugmacher et al. 1999; Pflugmacher et al. 1998). An evidence supporting this issue is the fact that CYN at 2.5 and 12.5 $mg.L^{-1}$ increased the reduced GSH content by 50% and 80%, respectively, in the protest *Euglena gracilis* exposed to pure CYN for 7 days (Duval et al. 2005). Furthermore, CYP450 monooxygenases may also be involved in the detoxification of TBA in *C. vulgaris*, contributing thereby to the reduction of TBA toxicity in the mixture. There is evidence suggesting that CYP450 monooxygenases play a crucial role in the detoxification of herbicides in higher plants (Munkegaard et al. 2008). Similarly, scientists have concluded that the green algae *Chlorella fusca* had a wide range of CYP450 enzymes and that the degradation of metflurazone was CYP450 dependent (Thies et al. 1996). It is therefore likely that the degradation of TBA follow similar paths in algae as in higher plants.

When the IA model was fitted to the growth rate data of the same binary mixture but after 7 days of exposure, a different trend was observed. In this case a DL dependent deviation was obtained, showing synergism at low dose levels and antagonism at high dose levels. The increase of mixture toxicity (synergism) provided by low doses of CYN and TBA may be due to effects of CYN on detoxification enzyme pathways. CYN is able to directly affect TBA metabolism by inhibition of GSH synthesis (Humpage et al. 2005; Runnegar et al. 1995b; Runnegar et al. 2002). Thus, the intracellular decrease of the GSH content may lead to a limited conjugation of TBA to GSH by enzymatic activity of GST and subsequently to the increase of the residence time of TBA in *C. vulgaris*, resulting in a higher toxicity. The change in the response pattern in this mixture as a function of time is of great importance, suggesting that long-term exposures to CYN and TBA might cause an increase in toxicity. The decrease of mixture toxicity (antagonism) at higher doses, even

though not significant at environmentally relevant concentrations, may be explained by a possible increase of the CYP450 monooxygenases mediated degradation of TBA as well as the conjugation reaction of CYN and TBA with GSH.

In the case of the mixture of CYN and Cd, an antagonistic deviation from the IA model was observed after 4 days of exposure. As stressed before, a reduction on Cd toxicity could be due to the induction of MT and PCs by cell's defensive mechanisms. Along with the induction of MT and PCs caused by Cd, a possible increase of the enzymatic activity of GST in the detoxification of CYN could help to explain the reduction of mixture toxicity. In the present study, we cannot exclude the possibility of chemical interaction between these two compounds in the exposure medium and in the cytoplasmatic matrix similar to what happens with MC-LR and Cd. After 7 days of exposure, a DL dependent deviation from the IA model was obtained, showing synergism at low dose levels and antagonism at high dose levels. The synergistic pattern at lower exposure levels may be explained by the interference of Cd in several metabolic processes that leads to disruption of cellular homeostasis, such as oxidative stress (Leonard et al. 2004; Pinto et al. 2003; Valko et al. 2005), DNA damages (Badisa et al. 2007), membrane depolarization and acidification of the cytoplasm (Conner & Schmid 2003). Although there is a lack of information concerning the effects of CYN in freshwater algae, it act by inhibiting the protein synthesis (Froscio et al. 2001; Froscio et al. 2008; Metcalf et al. 2004; Terao et al. 1994) and potentially interfering with the microtubular organization (Beyer et al. 2009) and DNA structure (Shaw et al. 2000). Here it is important to note that CYN is only toxic after metabolic activation by the CYP450 system (Humpage et al. 2005; Runnegar et al. 1995b; Shaw et al. 2000). It is possible that CYN need to be activated by CYP450 system to act on its molecular target. In this mixture, it is also relevant to stress the temporal variation of the response pattern of *C. vulgaris*, indicating that long-term exposures to CYN and Cd might cause an increase in toxicity. The antagonistic effect at higher dose levels of CYN and Cd might be explained by a possible increase of the cellular detoxification processes in order to compensate the stress caused by both compounds, thus decreasing the mixture toxicity. However, the antagonism reported herein for higher exposure levels is likely unreal at concentrations of CYN and Cd found in the aquatic environment.

After fitting the IA model to the growth rates of *C. vulgaris* exposed to TBA and Cd mixture for 4 days, antagonism was the deviation that better explained the data, but when this model was fitted to the growth rate of *C. vulgaris* exposed to the same mixture for 7 days a DL dependent deviation indicating antagonism at low dose levels and synergism at high dose levels was observed. Again, the antagonistic patterns obtained in both exposure periods may be due to the reduction on TBA and Cd toxicity caused by the increase of MT and PC levels, in the case of Cd, and GSH and GST levels, in the case of TBA, by cell's defensive mechanisms together with the possible increase of the CYP450 monooxygenases mediated degradation of TBA. Antagonism was also detected in a work dealing with the combined effects of TBA and the chloroacetanilide herbicide metolachlor on the growth rate of the green alga *P. subcapitata* (Pérez et al. 2011). On the other hand, the synergistic effect observed at higher levels of TBA and Cd after 7 days of exposure may be related to the intracellular increase of ROS, exceeding the cellular detoxification capability, caused by Cd and the inhibition of the photosynthetic electron transport caused by TBA. Moreover, the increase on mixture toxicity could also be related to the ability of Cd to inhibit photoactivation (the last step in the assembly of PSII before it becomes functional) mostly by binding to the essential Ca^{2+} site (Faller et al. 2005; Vrettos et al. 2001). Faller et al. (2005) proposed that Cd binds competitively to the essential Ca^{2+} site in PSII during photoactivation and that this mechanism is likely to be important for Cd toxicity towards photosynthetic organisms. In contrast, Qian et al. (2009) demonstrated that Cd is able to inhibit the abundance of *psb*A in *C. vulgaris*, a gene that codes for an integral membrane protein D1 of PSII. The inhibition of *psb*A mRNA transcripts may decrease the activity of PSII and electron transfer rates in *C. vulgaris*.

In this study, antagonism was the common deviation from IA model for 4-day exposure period while DL dependency was the main deviation obtained when testing the IA model for 7-day exposure period. In the case of DL dependent deviation, a decrease of the mixture toxicity (antagonism) at low dose levels (environmentally relevant levels) was observed for the binary mixtures between MC-LR/TBA and TBA/Cd. For the binary mixtures between MC-LR/Cd and the binary mixtures involving CYN the opposite trend was obtained, i.e., an increase of the mixture toxicity (synergism) at low dose levels. As in our study, other authors have found synergistic effects between MC-LR or LPS and other

anthropogenic pollutants (Cerbin et al. 2010; Notch et al. 2011; Wang et al. 2012; Wang et al. 2011). In contrast to our study, no antagonistic response between MC-LR and other anthropogenic contaminants was reported to date. To our knowledge, our study is the first report observing antagonistic effects of MC-LR and TBA or Cd. The combined toxicity of CYN and other anthropogenic pollutants has not been reported, and our study confirmed an antagonistic effect between CYN and TBA or Cd on *C. vulgaris* growth rate for 4-day exposure period and a synergistic interaction at low dose levels of CYN and TBA or Cd for 7-day exposure period. Information about the combined effects of TBA and Cd is not also available as far as we know, although combined toxicity of TBA or Cd and other anthropogenic contaminants such as the *s*-triazine herbicides atrazine and simazine (Pérez et al. 2011), the organophosphorous insecticides malathion, endosulfan and chlorpyrifos (Munkegaard et al. 2008), the imidazole fungicide prochloraz (Cedergreen et al. 2006), and the metals Zn and Cu (Franklin et al. 2002; Lam et al. 1999; Qian et al. 2009; Rachlin & Grosso 1993) have been investigated.

The antagonism observed in all tested binary mixtures in shorter exposure period (4 days) demonstrate that the simultaneous presence of several toxics in the aquatic environment may lead to less toxic effects than expected by the single-chemical exposures and therefore cause more protective effects on ecosystems. On the other hand, the synergisms obtained in this study at low dose levels in almost all binary mixtures in longer exposure periods demonstrate that the simultaneous presence of several toxics in the aquatic environment may lead to more toxic effects than expected by the single-chemical exposures and therefore cause more devastating effects on ecosystems. As a result, more research is required in the area of interactions between different compounds in order to elucidate the response mechanisms involved.

The increasing anthropogenic eutrophication of the aquatic ecosystems (i.e., increased input of nutrients, especially phosphorous but also nitrogen) has created ideal conditions for the development of HABs (Bláha et al. 2009; Heisler et al. 2008). These aquatic ecosystems impacted by anthropogenic pollution frequently have increased concentrations of other contaminants, including metals such as Cd and pesticides such as TBA, making coexposure of cyanotoxins and anthropogenic contaminants likely to occur. Here, we show that the co-exposure of cyanotoxins and anthropogenic contaminants

resulted, in most of the cases, in a toxicity evolution over time (from antagonism to synergism). Given the predictions of the increase of HABs in frequency and magnitude in the future and the prevalence of co-exposure to cyanotoxins and anthropogenic contaminants, this toxicity evolution over time represents a real concern for aquatic organisms in contaminated habitats.

3.5. Conclusion

The first highlight that can be addressed from our results is that deviations from the IA model were found in all combinations studied (antagonism and also patterns that are dependent from the mixture composition and doses used). Antagonism was the prevailing type of interaction between dissimilar acting compounds in a 4-day exposure period while DL dependency was the only deviation obtained in a 7-day exposure period. The study of complex interactions between cyanotoxins and anthropogenic contaminants and how these compounds react simultaneously inside organisms needs further development in order to understand the underlying mechanisms and better predict mixture effects. Toxicokinetic and toxicodynamic studies can contribute significantly to the understanding of the main toxicological mechanisms behind a particular response of a complex mixture. The measure of antioxidative enzymes such as catalase, superodixade dismutase, glutathione peroxidase and GST and antioxidant molecules such as GSH, are examples of biomarkers that could help to better understand mixture exposure responses. To our knowledge, this is the first report concerning combined toxicity of CYN and other anthropogenic contaminants.

Another feature observed from analysis of our results was the switch from antagonism to synergism (at low dose levels) revealed by almost all binary mixture, respectively, from 4-day exposure period to 7-day exposure period. Concerning these results, synergistic patterns at low dose levels are a major concern for aquatic organisms exposed to combinations of cyanotoxins and anthropogenic contaminants in the environment.

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Chapter IV

4. General discussion and final considerations

The main purpose of this study was to assess the effects of cyanotoxins and anthropogenic environmental contaminants as binary mixtures on the growth rate of *Chlorella vulgaris*. The cyclic heptapeptide microcystin-LR (MC-LR) and the alkaloid cylindrospermopsin (CYN) were the cyanotoxins selected for this study because they are the most frequently detected cyanotoxins in harmful cyanobacterial blooms in brackish and freshwaters (van Apeldoorn et al. 2007). The non-essential metal cadmium (Cd) and the *s*-triazine herbicide terbuthylazine (TBA) were chosen as anthropogenic environmental contaminants representatively present in aquatic systems. Cd is commonly found in the aquatic environment due to a variety of anthropogenic and natural sources (Notch et al. 2011). TBA is frequently detected in surface waters (Loos et al. 2010; Loos et al. 2007; Palma et al. 2009; Pérez et al. 2010) because of its wide use as herbicide in agricultural fields. *C. vulgaris* was exposed to several binary mixtures for 4 and 7 days and then the results were analyzed using the reference model of independent action (IA) within the MIXTOX tool.

When *C. vulgaris* was exposed to MC-LR and CYN mixture, a synergistic deviation from IA model was obtained in both exposure periods, clearly demonstrating that the simultaneous presence of these cyanotoxins in the aquatic environment may lead to more toxic effects than expected by the single exposures to MC-LR and CYN. Best et al. (2002) also found synergistic effects of MC-LR and lipopolysaccharides (LPS) on the GST activity of *Danio rerio*. In contrast, Lindsay et al. (2006) found antagonistic effects when *Artemia salina, Daphnia magna* and *Daphnia galeata* were pre-exposed to LPS before MC-LR or CYN. This antagonism was also found for simultaneous exposure of LPS and MC-LR, but it was less pronounced than that conferred by the pre-exposure to LPS (Lindsay et al. 2006). The synergism observed in this study may be explained by the inhibition of the GSH synthesis caused by CYN (Runnegar et al. 1995) and subsequent absence of detoxification of MC-LR by GST activity (Pflugmacher et al. 1998), resulting in a much higher toxicity.

In the MC-LR and TBA mixture, a DL dependent deviation from the IA model was observed for 4- and 7-day exposure periods, indicating antagonism unless the doses of the two compounds were above the concentrations tested in this experiment where synergism would be expected to occur according to the MIXTOX tool. The antagonistic effect might be related to the production of reactive oxygen species (ROS) induced by MC-LR (Amado & Monserrat 2010) and the oxidation of D1 protein of the photosystem II (PSII) reaction center (Drábková et al. 2007; Kim & Lee 2005; Krieger-Liszkay et al. 2008; Okada et al. 1996), blocking the binding of TBA its target site.

In the case of MC-LR and Cd mixture, an antagonism deviation was observed for a 4-day exposure period while a DL dependent deviation indicating synergism at low dose levels and antagonism at high dose levels was obtained for a 7-day exposure period. The antagonism may be probably related to the induction of metallothioneins (MT) and phytochelatins (PCs) by cell's defensive mechanisms (Perales-Vela et al. 2006; Robinson 1989). The induction of PCs can also be linked to the presence of MC-LR in the mixture. Previous works demonstrated that the exposure of *Lemna minor* and *Lactuta sativa* to MC-LR and alkylbenzene sulfonate (LAS) mixtures, especially at high concentrations, increased GSH contents (Wang et al. 2012; Wang et al. 2011). As a precursor of PCs, the increase in the GSH pool may consequently lead to increased synthesis of PCs. Another possible explanation for the antagonism might be the reduction of the bioavailability of MC-LR and Cd inside or outside the cells by the formation of stable complexes as previously demonstrated for MCs and several metals, including Cd (Humble et al. 1997; Saito et al. 2008; Yan et al. 2000). The synergistic effect observed at low doses of MC-LR and Cd after 7 days of exposure may be possibly due to the increase of time of exposure and the production of ROS induced by both compounds (Amado & Monserrat 2010; Pinto et al. 2003) and the reduction on cellular detoxification processes. As in our study, synergistic interactions were also found between MC-LR/LAS (Wang et al. 2012; Wang et al. 2011), LPS/Cd (Notch et al. 2011), and carbaryl/MC-LR (as particulate MC-LR bound to *Microcystis aeruginosa*) (Cerbin et al. 2010), even though in the latter combination the synergism was not predicted from the effects of the single stressors.

In the binary mixtures involving CYN, the same pattern of response was obtained for a 7-day exposure period, i.e., DL dependent deviation showing synergism at low dose levels and antagonism at high dose levels. The synergism observed at low doses of CYN and TBA may be explained due to the inhibition of the GSH synthesis caused by CYN (Runnegar et al. 1995) and subsequent reduction of detoxification mechanism of TBA by GST activity, resulting in a much higher toxicity. Tang et al. (1998) provided evidence for the involvement of GST in atrazine metabolism in several microalgae species based on the GSH-dependent formation of water soluble metabolites. Therefore, it is likely that the metabolism and detoxification of TBA follow similar paths in *C. vulgaris*. In the case of CYN and Cd mixture, the synergistic pattern at lower exposure levels may be probably due to the cellular damage inflicted by the two compounds. Cd may cause oxidative stress (Pinto et al. 2003), DNA damage (Badisa et al. 2007), membrane depolarization and acidification of the cytoplasm (Conner & Schmid 2003). CYN, on the other hand, inhibit the protein synthesis (Froscio et al. 2001; Froscio et al. 2008; Metcalf et al. 2004; Terao et al. 1994) and may interfere with the microtubular organization (Beyer et al. 2009) and DNA structure (Shaw et al. 2000).

However, difference patterns of responses were registered after 4 day of exposure. For CYN and TBA mixture, a DR dependent deviation from the IA model was observed and the antagonism was mainly due to CYN. One possible explanation for this antagonism is the induction of cellular detoxification mechanisms generated by CYN and TBA, resulting in a reduction of the mixture toxicity. GST and cytochrome P450 monoxygenases are probably involved in the detoxification of TBA in *C. vulgaris* (Munkegaard et al. 2008; Tang et al. 1998; Thies et al. 1996). Duval et al. (2005) showed that CYN at 2.5 and 12.5 $mg.L^{-1}$ increased the reduced GSH content by 50% and 80%, respectively, in the protist *Euglena gracilis* after 7 days of exposure. Thus, it is likely that GST may also participate in the detoxification of CYN in *C. vulgaris*, even though no GSH-CYN conjugate has been identified so far. For CYN and Cd mixture, antagonism was the best descriptive deviation following IA model. A possible reason for this antagonism is the induction of MT and PCs, in the case of Cd, and possibly of GST activity, in the case of CYN, by cell's defensive mechanisms. In the present study, the possibility of chemical interaction between CYN and Cd in the exposure medium and/or in the cytoplasmatic matrix should not be neglected.

Relatively to the TBA and Cd mixture, antagonism deviation was observed for a 4-day exposure period while a DL deviation indicating antagonism at low dose levels and synergism at high dose levels was obtained for a 7-day exposure period. Again, the antagonistic patterns obtained for both exposure periods may be due to the increase of MT and PC levels, in the case of Cd, and GST levels, in the case of TBA, by cell's defensive mechanisms. As stated before, the increase of the CYP450 monooxygenases mediated degradation of TBA may also contribute to the reduction of TBA toxicity till a certain dose level. The synergistic effect observed at higher doses of TBA and Cd after 7 days of exposure may be associated to the oxidative stress caused by Cd (Pinto et al. 2003) and the inhibition of the photosynthetic electron transport caused by TBA (Faust et al. 2001). Moreover, the increase on mixture toxicity could also be related to the fact that Cd is able to inhibit photoactivation in algae and consequently decrease the activity of PSII (Faller et al. 2005; Qian et al. 2009; Vrettos et al. 2001).

Discussing the results of the combined toxicity of mixtures should be made alongside with the knowledge of the precise mode of toxic action of the cyanotoxins and the anthropogenic environmental contaminants inside the organisms, in order to make more assertive assumptions about the mechanisms involved in possible interactions between them. Due to the general lack of the systematic mechanisms underlying the specific toxic effects of cyanotoxins and anthropogenic contaminants, only some considerations could be made about the effects of the mixtures tested here.

An encompassing perspective should be made regarding the several binary mixtures tested with the two cyanotoxins and the two anthropogenic contaminants on the growth rate of *C. vulgaris*. All combinations tested deviated from the conceptual model of IA, suggesting that additive patterns are not always valid for binary mixtures. Indeed, this study demonstrate that a great variety of effects and behaviours can arise from the combination of toxicants commonly found in the aquatic environment, since all possible deviations from the IA model occurred (antagonism, synergism, DR and DL dependency). Antagonism was the interaction described in the majority of the binary mixtures in a 4-day exposure period; in 5 of the 6 binary mixtures (including the antagonism at low doses of MC-LR and TBA) the effects observed were below the effects predicted by the responses

observed in the single exposures. Synergism at lower exposure levels, on the other hand, was the prevailing type of interaction described in the majority of binary mixtures in a 7-day exposure period; in 4 of the 6 binary mixtures (including the synergism between MC-LR and CYN) the effects observed were much higher than expected from the single exposures. Synergism observed between MC-LR and CYN after 4 and 7 days of exposure is of particular interest since independently of concentration MC-LR increased the CYN toxicity by a factor of 2. From the analysis of our results, it is still possible to observe the switch from antagonism to synergism revealed by almost all binary mixture (with the exception of the binary mixture of MC-LR/CYN, MC-LR/TBA and TBA/Cd), respectively, from 4 to 7 days of exposure. Concerning these results, synergistic patterns at low dose levels are a major concern for aquatic organisms exposed to combinations of cyanotoxins and anthropogenic contaminants in the environment.

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