Uptake, physiological responses and technical application of the aquatic fungus *Mucor hiemalis* EH5 for the removal of cyanobacterial toxins

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Auch eine Reise von tausend Meilen
beginnt mit dem ersten Schritt

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LIST OF MANUSCRIPTS

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

I)

Toxin resistance in aquatic fungi poses environmentally friendly remediation possibilities: A study on the growth responses and biosorption potential of *Mucor hiemalis* EH5 against cyanobacterial toxins.

Evelyn Balsano, Maranda Esterhuizen-Londt, Enamul Hoque, and Stephan Pflugmacher

International Journal of Water and Wastewater Treatment, 2015, 1(1), 1-9
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II)

Fungal pellets as potential tools to control water pollution: Strategic approach for the pelletization and subsequent microcystin-LR uptake by *Mucor hiemalis*.

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III)

Responses of the antioxidative and biotransformation enzymes in the aquatic fungus *Mucor hiemalis* exposed to cyanotoxins.

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LIST OF CONFERENCES

Toxin resistance in aquatic fungi poses environmentally friendly remediation possibilities: A study on the growth responses and biosorption potential of *Mucor hiemalis* EH5 against MC-LR, BMAA, and CYN.

Evelyn Balsano, Maranda Esterhuizen-Londt, Enamul Hoque, and Stephan Pflugmacher

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ABSTRACT

Many cyanobacteria produce various harmful secondary metabolites, a.k.a. cyanotoxins, which cause global concerns because of their hazardousness to ecosystems and human health. Due to their diversity in origin and structure, cyanotoxins express different biological activities commonly including the generation of oxidative stress. This work focuses on the three most frequently occurring cyanotoxins, the hepatotoxin microcystin-LR (MC-LR), the cytotoxin cylindrospermopsin (CYN) and the neurotoxin β-*N*-methylamino-L-alanine (BMAA) at environmentally relevant concentrations as a representative study, with special emphasis on MC-LR. The removal of cyanotoxins is essential to ensure water quality and safety and therefore mycoremediation of these toxins using *Mucor hiemalis* EH5 in pellet morphology was considered. *M. hiemalis* has been shown to resist environmental perturbations and still sporulates at groundwater temperatures of 5 °C. Most importantly, it expresses high glutathione *S*-transferase activity, which was shown to be involved in the enzymatic degradation of the herbicide isoproturon in combination with the lignolytic model white-rot fungus *Phanerochaete chrysosporium*. The aim of the present study was therefore to investigate cyanotoxin uptake, physiological responses, and the degradation ability of *M. hiemalis* with regards to assess its applicability for future technical applications for the removal of cyanotoxins from surface waters.

First (Paper I), mycelial *M. hiemalis* grown on agar petri dishes in the presence of cyanotoxins was investigated in terms of sensitivity using an adaptation of the Kirby-Bauer disk diffusion assay, and fungal growth and biomass production *via* radial extension and dry weight measurements, respectively. Although cyanotoxins are considered antimicrobial, *M. hiemalis* exhibited unchanged radial expansion and biomass production. Quantification of internalized cyanotoxin concentration was achieved *via* liquid chromatography tandem mass spectrometry of the fungal extracts to show the biosorption capability of *M. hiemalis*. The second part (Paper II) investigated the influence of different factors, media type, temperature, agitation rate, inoculum size, pH, additives, flask shape, and volume on pellet formation probability of *M. hiemalis* in submerged cultivation. Considering the advantages of using fungal pellets in bioreactors, an optimized method for the pellet production of the aquatic fungus was established. Fungal pellets were, in a third part (Paper III), exposed to MC-LR, CYN, and BMAA to investigate antioxidative and biotransformation enzyme responses, including the biomarkers, catalase, glutathione reductase, glutathione peroxidase, and glutathione *S*-transferase. It was thereby confirmed that *M. hiemalis* does not or only slightly

suffer from oxidative stress in the presence of MC-LR and BMAA as well as that it can handle CYN-induced oxidative stress. These findings are important when considering a fungus for mycoremediation application as it indicates the fungus's ability to cope with exposure to the cyanotoxins in question. The degradation ability of *M. hiemalis* was estimated, using MC-LR as an example, by monitoring the reduction in cyanotoxin concentration from medium over time (Chapter 5). Removal efficiency was compared in nutrient-rich and limiting medium and *versus* the degradation ability of *P. chrysosporium* in single as well as in co-culture. Results indicate an involvement of internal/external MC-LR degradation processes by *M. hiemalis*, however, at the same time, the need of further optimization in order to consider mycoremediation applications.

In conclusion, this work demonstrated the resistance and overall growth fitness of *M. hiemalis* towards a broad range of exposure concentrations of the three structurally diverse cyanotoxins MC-LR, CYN, and BMAA and thus proves its survival, viability, and longevity in their presence. *M. hiemalis* was easily stimulated and maintained in growing as pellets, which facilitates the handling and cultivation. Oxidative stress tolerance of the fungal pellets towards the three cyanotoxins was demonstrated, which indicates its ability to cope with the exposure on a physiological level. Cyanotoxin removal capacity of *M. hiemalis* was demonstrated, however, efficiency needs to be further optimized. Future studies should therefore include the influence of different factors, such as co-cultivation, pH optimum establishment, temperature variations, medium composition, *etc*. in order to enhance fungal degradation efficiency. The results from the present study do, however, present *M. hiemalis* as an attractive candidate for future applications in the removal of cyanotoxins.

ZUSAMMENFASSUNG

Viele Cyanobakterienarten produzieren verschiedene giftige Sekundärmetabolite, so genannte Cyanotoxine, die weltweit eine Bedrohung für das Ökosystem und die menschliche Gesundheit darstellen. Aufgrund ihrer diversen Herkunft und Struktur, unterscheiden sich Cyanotoxine in ihrer Wirkungsweise, oftmals mit der gemeinsamen Eigenschaft, oxidativen Stress zu generieren. Diese Arbeit befasst sich mit drei meist vorkommenden Cyanotoxinen, dem Hepatotoxin microcystin-LR (MC-LR), dem Cytotoxin cylindrospermopsin (CYN) und dem Neurotoxin β-*N*-methylamino-L-alanine (BMAA) repräsentativ bei umweltrelevanten Konzentrationen, mit speziellem Fokus auf MC-LR. Die Entfernung von Cyanotoxinen ist essentiell, um die Wasserqualität und -sicherheit zu gewährleisten und zu diesem Zweck wurde die Mykoremediation mittels *Mucor hiemalis* EH5 in Pelletmorphologie in Betracht gezogen. *M. hiemalis* hat sich als beständig gegen störende Umwelteinflüsse erwiesen, sporuliert auch noch bei Grundwassertemperaturen von 5 °C und besitzt vor allem eine hohe Glutathion *S*-Transferaseaktivität. Letzteres spielte eine entscheidende Rolle beim Abbau des Herbizids Isoproturon in Verbindung mit *Phanerochaete chrysosporium*, dem bekanntesten Vertreter der lignolytischen Weißfäulepilze. Das Ziel der vorliegenden Arbeit bestand deshalb darin, die Cyanotoxinaufnahme, physiologischen Antworten und das Degradationspotential von *M. hiemalis* zu untersuchen mit Berücksichtigung auf seine Anwendbarkeit zur technischen Umsetzung für die Cyanotoxinentfernung aus Oberflächengewässern. Als erstes wurde das Myzel von *M. hiemalis* auf Agarplatten auf seine Empfindlichkeit mittels einer Adaptation des Kirby-Bauer Diffusionsassays, als auch sein Wachstum und die Biomasseproduktion mittels radialer Wachstumsrate und Trockengewichtmessungen bestimmt. Trotz der antimikrobiellen Wirkung von Cyanotoxinen wurde kein Einfluss auf die radiale Ausbreitung und Biomasseproduktion von *M. hiemalis* beobachtet. Mittels Flüssigkeitschromatographie-Tandem-Massenspektrometrie der Pilzextrakte wurde die aufgenommene Cyanotoxinmenge quantifiziert und die Fähigkeit von *M. hiemalis* zur Biosorption gezeigt. Der zweite Teil erforschte den Einfluss verschiedener Faktoren (Art des Nährmediums, Temperatur, Schüttelfrequenz, Konzentration des Inokulums, pH-Wert, Zusätze, Kolbenform und Volumen) auf die Wahrscheinlichkeit der Pelletbildung von *M. hiemalis* in der Flüssigkultur. Da Pilzpellets in Bioreaktoren viele Vorteile mit sich bringen, wurde eine optimale Methode zur Pelletbildung des aquatischen Pilzes etabliert. Im dritten Teil wurden die gebildeten Pilzpellets mit MC-LR, CYN und BMAA exponiert, um die enzymatischen Antworten des antioxidativen Systems und der Biotransformation zu

untersuchen, was die Biomarker Katalase, Glutathionreduktase, Glutathionperoxidase und Glutathion *S*-Transferase miteinschließt. Dabei wurde ersichtlich, dass *M. hiemalis* keinen, bzw. nur geringen oxidativen Stress in Gegenwart von MC-LR und BMAA erleidet, sowie CYN generierten oxidativen Stress tolerieren kann. Diese Erkenntnisse sind wichtig, um einen Pilz zur Mykoremediation umzusetzen, da sie seine Fähigkeit zeigen, die Exposition mit Cyanotoxinen auszuhalten. Die Degradationsfähigkeit von *M. hiemalis* wurde am Beispiel von MC-LR ermittelt, wobei die Abnahme der Cyanotoxinkonzentration im Medium über die Zeit gemessen wurde. Die Eliminierungsleistung von *M. hiemalis* wurde in nährstoffreichem und nährstoffarmem Medium verglichen und dem Degradationspotential von *P. chrysosporium* in alleiniger, bzw. Co-Kultur gegenübergestellt. Die Ergebnisse deuten auf interne/externe MC-LR Degradationsprozesse durch *M. hiemalis* hin, machen aber gleichzeitig deutlich, dass es weiterer Optimierung bedarf, um tatsächlich eine Mykoremediation als Anwendung in Erwägung ziehen zu können.

Zusammenfassend zeigt diese Arbeit die Resistenz und allgemeine Wachstumsfitness von *M. hiemalis* gegen drei strukturell unterschiedliche Cyanotoxine MC-LR, CYN und BMAA in einem breiten Konzentrationsbereich und beweist deshalb sein Überleben, Überlebensfähigkeit und Langlebigkeit in deren Gegenwart.

Pellets von *M. hiemalis* konnten leicht induziert und auch beibehalten werden, was das Handling und die Kultivierung erleichtert. Es konnte eine oxidative Stresstoleranz der Pilzpellets gegenüber der drei Cyanotoxinen bewiesen werden, was die Fähigkeit zur Expositionsbewältigung auf der physiologischen Ebene beweist. Auch wenn die Cyanotoxinentfernung durch *M. hiemalis* verdeutlicht werden konnte, muss die Effizienz weiterhin verbessert werden. Zukünftige Studien sollten deshalb den Einfluss verschiedener Faktoren, wie z.B. Co-Kultivierung, optimale pH-Wert Bestimmung, Temperaturänderungen, Mediumzusammensetzung, usw. umfassen, um die Abbauleistung der Pilze zu steigern. Nichtsdestotrotz präsentieren die Ergebnisse der vorliegenden Arbeit *M. hiemalis* als attraktiven Kandidaten für künftige technische Anwendungen zur Entfernung von Cyanotoxinen.

ABBREVIATIONS

ANOVA	analysis of variance
ATP	adenosine-5'-triphosphate
BMAA	β-*N*-methylamino-L-alanine
CAT	catalase
CDNB	1-chloro-2,4-dinitrobenzene
CYN	cylindrospermopsin
d	day(s)
DSMZ	German Collection of Microorganisms and Cell Cultures
DTE	1,4-dithioerythriol
DW	dry weight
EDTA	ethylendiamintetraacetic acid
e.g.	for example
EtOH	ethanol
FW	fresh weight
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GST	glutathione *S*-transferase
GSSG	glutathione disulfide
H_2O	water
i.a.	inter alia (amongst others)
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
max.	maximum
min.	minimum
min	minutes
MC-LR	microcystin-LR
MeOH	methanol
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
PBS	phosphate buffer saline

pH	pondus hydrogenii
rpm	rounds per minute
s	second(s)
SAB	Sabouraud dextrose broth
SE	experimental standard error of the mean
SD	experimental standard deviation of the mean
sp.	species
SPSS	statistical package for social sciences
UV-Vis	ultraviolet-visible spectroscopy
v/v	volume per volume
VOC	volatile organic compound
°C	degree Celsius
g	gram
g/L	gram per liter
L	liter
mL	milliliter
mM	millimolar
mm	millimeter
m/z	mass to charge ratio
MW	molecular weight
U	Unit, i.e., enzyme activity (µmol substrate per min)
C	carbon
Fe	iron
N	nitrogen
O	oxygen
S	sulfur

1 INTRODUCTION

Cyanobacteria, a.k.a. blue-green algae, occur worldwide and are ubiquitous in marine, brackish or freshwaters, and terrestrial environments. Taxonomically, they belong to the phylum Bacteria and are gram-negative photosynthesizing prokaryotes. As organisms that have survived for about three billion years, they are amongst the earliest on Earth and the pioneers of oxygen production (Whitton, 2012). Many of them have the capability to assimilate atmospheric carbon dioxide (CO_2) and nitrogen (N_2) and therefore they significantly contribute to the nitrogen cycle as well as to the primary production of ecosystems by providing fixed N_2 to their hosts. Even though their evolutionary and ecological importance remains uncontroversial, they are now a growing environmental and public health concern. Increased nutrient inputs, especially nitrogen and phosphorous, due to anthropogenic activities (urban, agricultural and industrial expansion, fertilizer run-off, discharge of untreated sewage and inadequate management of watersheds) favor the development of microalgae and cyanobacteria (Scholz et al., 2017). Such eutrophication of water bodies in combination with climate change and high light intensity became a driving force for massive proliferations (Carmichael, 1997; Pearl and Otten, 2013). Many species of cyanobacteria contain gas-filled inclusions, known as gas vesicles that reduce the mass density of the cells and thus enable buoyancy regulation and the adjustment of their vertical position in the water column (Walsby, 1994). The advantageous effects that result from their active movement are reduction in loss by sedimentation, improvement in the supply of light by accumulation on the water surface (Walsby, 1997), and the possibility to balance the supply of limiting resources and to overcome the vertical separation in light and nutrient availability that develops in waters where turbulence is reduced by thermal stratification. Consequently, cyanobacteria find optimized positions for survival and growth and become increasingly dominant forming so-called cyanobacterial blooms that affect the ecology of freshwaters as well as human health, especially when the water is used for recreation and as a drinking water supply (de Figueiredo et al., 2004; Merel et al., 2013; Saqrane et al., 2007; Wiegand and Pflugmacher, 2005). The depletion of oxygen in the water as a consequence of dense blooms constitutes a serious risk for the ecological balance and the functioning of ecosystems and affects plants, fish, and other life forms. Most importantly, many cyanobacterial species are able to produce cyanotoxins as secondary metabolites, which can be taken up and accumulated in aquatic biota and animals (Chen et al., 2009; Esterhuizen-Londt et al., 2015a), thereby entering the food chain, which leads to further biomagnification

(Cox et al., 2003). Humans may therefore be exposed to cyanotoxins through several routes, with oral exposure being the most important, including consumption of contaminated drinking water or food or ingestion of water during recreational activities. However, transdermal or inhalation exposure routes are also possible. Depending on the concentration, cyanotoxins can cause direct toxicity to aquatic animals, livestock, and humans (Azevedo et al., 2002; Fleming et al., 2002; Trevino-Garrison et al., 2015), leading to illness and death and are therefore an important group of natural toxins from an ecotoxicological and toxicological perspective.

1.1 CYANOTOXINS

Many of the bloom-forming cyanobacteria are known to produce a variety of bioactive secondary metabolites designated as cyanotoxins. In most cases, cyanotoxins exist intracellularly in the cytoplasm and are enclosed within the cell. However, during senescence and cell lysis, substantial amounts are released into the surrounding water leading to increased extracellular toxin concentrations (Saker and Griffiths, 2000; Sivonen and Jones, 1999). Their diversity in origin and structure entails that they are a diverse group of compounds, both from the chemical and the toxicological points of view. Structurally, they can be divided into three groups: cyclic peptides, heterocyclic compounds (alkaloids) or lipopolysaccharides (endotoxins). Lipopolysaccharides are integral components of cell walls of all cyanobacteria responsible for irritation and affecting any exposed tissue (Codd, 1995; Sivonen and Jones, 1999). According to their biological activity, cyanotoxins fall into five groups: hepatotoxins, neurotoxins, dermatotoxins, cytotoxins, and irritant toxins (Codd et al., 2005; Wiegand and Pflugmacher, 2005). The classification in terms of their toxicological target is the most frequently used. Fig. 1-1 shows three ecologically important cyanotoxins representing the various cyanotoxin classifications and differing in structure and biological activity.

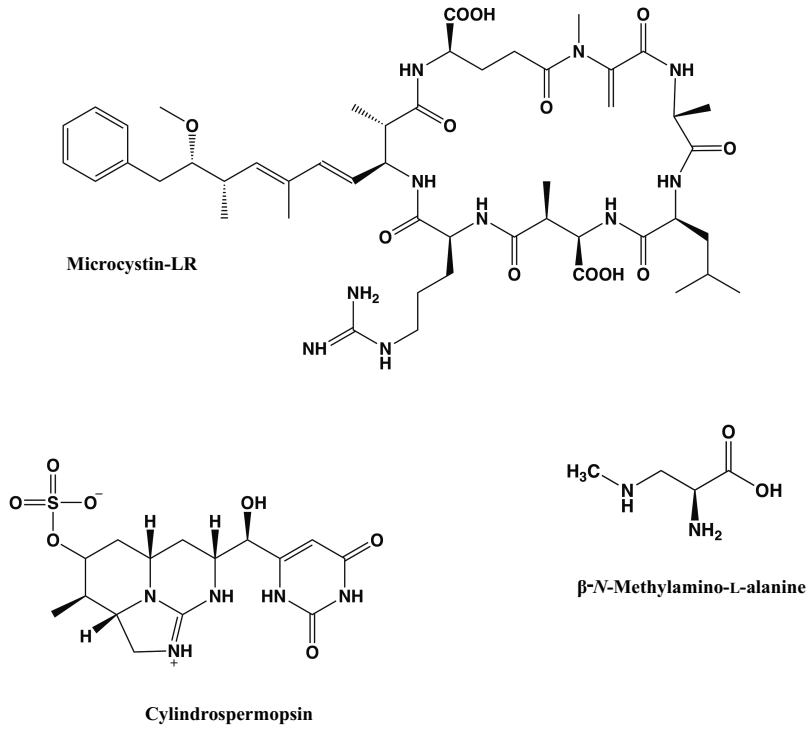

Fig. 1-1: Molecular structures of the cyanotoxins MC-LR, CYN, and BMAA**.**

The present work is based on these three selected cyanotoxins, microcystin-LR (MC-LR), cylindrospermopsin (CYN), and β-*N*-methylamino-L-alanine (BMAA) with special focus on MC-LR.

Microcystins

Microcystins (MCs) are the most studied and the most abundant cyanotoxins throughout the world. Commonly, they are produced by *Microcystis*, but also by the cyanobacterial genera *Anabaena*, *Planktothrix* (*Oscillatoria*), *Anabaenopsis*, *Nostoc* and *Hapalosiphon* (Lanaras and Cook, 1994; Luukkainen et al., 1993; Namikoshi et al., 1992; Prinsep et al., 1992; Sivonen et al., 1992). MCs are cyclic heptapeptides composed of different amino acids, with two variable sides, which make the differentiation between isoforms of MCs. Characteristic for all MC structures is the unique β-amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda), which is also responsible for the toxicity and critical to MC activity, as well as the presence of *N*-methyl dehydroalanine (Mdha). Among the more than 200 MCs identified to date (Stirling and Miles, 2016), only a few occur frequently and in high concentrations. MC-LR, containing the amino acids leucine (L) and arginine (R) at the variable positions, is among the most frequent and most toxic MC congeners (Imanishi et al.,

2005). The primary target organ for MC toxicity in mammals is the liver, as MC-LR crosses cell membrane chiefly through the bile acid transporter actively into liver cells *via* the ATP (adenosine-5'-triphosphate) dependent OATP (organic anion transporting polypeptide) (Feurstein et al., 2009; Fischer et al., 2005). It accumulates in the liver where it can lead to necrosis and death (Ho et al., 2012), being a potent hepatotoxin with genotoxic and carcinogenic properties. MC-LR acts as a specific inhibitor of eukaryotic serine/threonine protein phosphatases (PPs) such as PP1 and PP2A (MacKintosh et al., 1990) through the binding to these enzymes. Excessive phosphorylation of proteins may occur and lead to alterations in cytoskeleton, loss of cell shape with subsequent destruction of liver cells causing intra-hepatic hemorrhage, or hepatic insufficiency (Yoshizawa et al., 1990). On the other hand, MCs have been shown to interact with mitochondria of cells triggering oxidative stress (Campos and Vasconcelos, 2010; Ding et al., 2000; Žegura et al., 2003). This pathway together with the inhibition of PPs is probably the main mechanism of action through which a cascade of events responsible for cell necrosis or apoptosis takes place (Campos and Vasconcelos, 2010).

Cylindrospermopsin

CYN is a polycyclic uracil derivative containing guanidine and sulfate groups and according to its structure it is classified as an alkaloid. Because of its zwitterionic character, it is highly water-soluble. Its cyanobacterial producers are the genera *Cylindrospermopsis*, *Aphanizomenon*, *Umezakia*, *Raphidiopsis*, and *Anabaena* (Harada et al., 1994; Li et al., 2001; Ohtani et al., 1992; Preußel et al., 2006; Vasas et al., 2002). The toxic structure in the molecule is attributed to the presence of the hydroxyl-group on the uracil bridge or the keto-enol status of the uracil moiety (Banker et al., 2001). CYN is cytotoxic and genotoxic; its mode of action includes inhibition of protein synthesis (Froscio et al., 2008; Metcalf et al., 2004), as well as inhibition of glutathione synthesis (Gutiérrez-Praena et al., 2011a; Gutiérrez-Praena et al., 2011b; Norris et al., 2002), metabolic activation (Bazin et al., 2010), and the promotion of oxidative stress (Guzmán-Guillén et al., 2013).

β-*N*-Methylamino-L-alanine

BMAA is an environmentally ubiquitous small non-protein amino acid, biosynthesized by most cyanobacteria (Cox et al., 2005; Esterhuizen and Downing, 2008) under nitrogen limiting conditions (Downing et al., 2011; Scott et al., 2014). Interestingly, BMAA closely resembling glutamate in its molecular structure binds directly to *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate

receptors and thus acts as a glutamate receptor agonist (Ross et al., 1987), causing neurotoxicity and being involved in the development of neurodegenerative diseases (Lobner et al., 2007). Moreover, BMAA acts on the cysteine/glutamate antiporter (system Xc^-) and hence inhibits cysteine uptake, causing depletion of cellular GSH and increased oxidative stress (Liu et al., 2009; Lobner et al., 2007).

1.2 OXIDATIVE STRESS AND ANTIOXIDANT DEFENSIVE SYSTEM

Amongst the broad range of adverse biological activities of cyanotoxins, the production of oxidative stress has been proposed as an essential factor in their toxicity leading to mitochondrial damage (Ding et al., 2000), oxidative DNA damage (Žegura et al., 2003), apoptosis induction (Ding et al., 2000), and neurodegenerative diseases (Liu et al., 2009; Lobner et al., 2007).

Reactive oxygen species (ROS) are highly reactive molecules, e.g. peroxides, superoxide, hydroxyl radical, and singlet oxygen. Under normal conditions, ROS play an important role in the normal physiological functions of the reproductive system (Sena and Chandel, 2012); however, when its level increases dramatically, ROS can damage essential cell structures such as DNA, proteins, and lipids thereby altering vital cellular functions. Aerobic organisms have evolved various defense mechanisms, including the antioxidative enzymes superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and in parts glutathione *S*-transferase (GST), in order to eliminate ROS and avoid oxidative destruction. Fig. 1-2 shows the cycling of excessively formed H_2O_2 in the cell with respective enzymatically catalyzed pathways to defend organisms from oxidative stress and keep the balance between free radicals and antioxidant defenses within the cell.

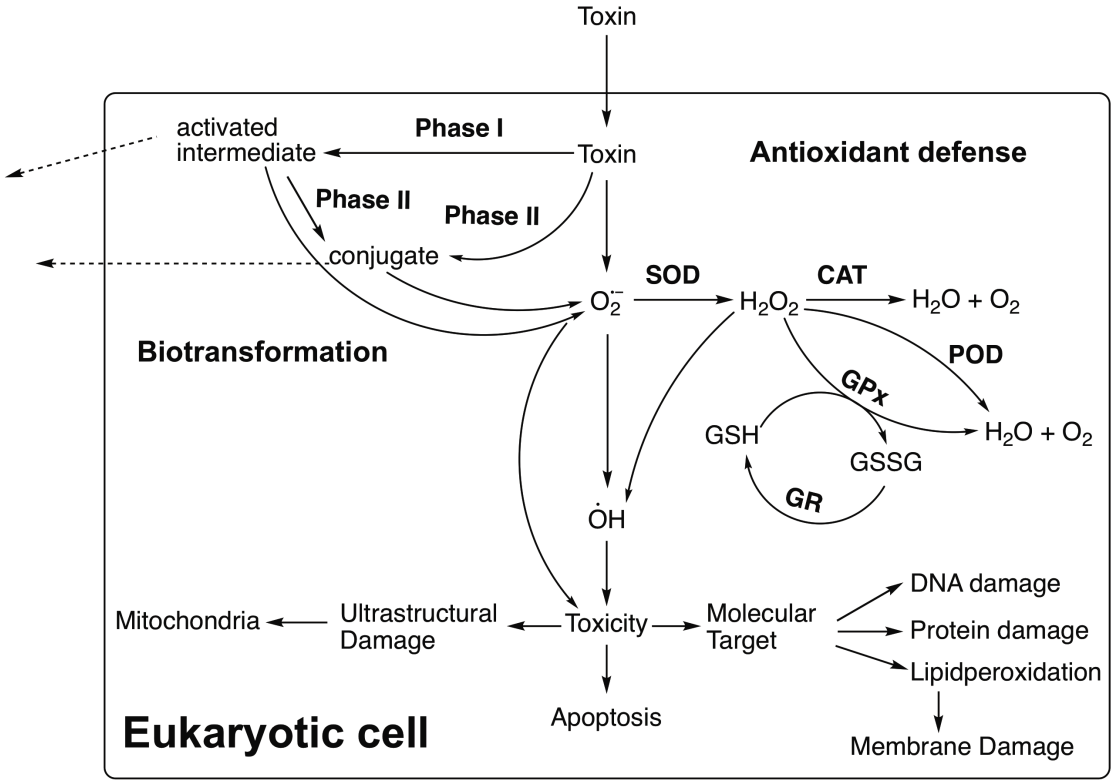

Fig. 1-2: Antioxidative defense mechanisms, adapted and generalized from Jiang et al. (2011) (Superoxide $O_2^{\bullet-}$, hydroxylradical $OH^{\bullet}$)

Antioxidant enzymes may respond with alterations in activity after exposure to a toxin and thus signal responses to oxidative stress produced by the toxin; selected antioxidative enzymes can therefore serve as biomarkers for toxin-induced oxidative stress (Contardo-Jara et al., 2009). The effect of cyanotoxins on the enzyme responses in many aquatic plants and animals has been widely examined and is further described below.

Toxicity of MC-LR related to the induction of oxidative stress was shown in the submerged macrophyte *Vallisneria natans* (Lour.) Hara with alterations in mesophyll cells (Jiang et al., 2011). Phytotoxic effects and oxidative stress induction due to MC-LR accumulation was shown in the aquatic plant *Lemna gibba* (Saqrane et al., 2007). POD and SOD activities in rape (*Brassica napus*) seedlings increased and decreased, respectively, with MC-LR increasing concentration, whereas SOD activity in rice (*Oryza sativa*) seedlings strongly increased with lower MC-LR concentrations and only slightly increased at higher exposure concentrations (Chen et al., 2004). Uptake of MC-LR in aquatic animals has also been shown to enhance the production of ROS, leading to an increase in lipid peroxidation, DNA damage,

protein crosslinks, mitochondrial damage, and alteration of the antioxidant defense system (Amado and Monserrat, 2010).

Enzyme activity enhancements with CYN exposure were observed in the free-floating macrophyte *Lemna minor* L, with CAT showing the most representative antioxidant response after 24 h (2.5 and 25 µg/L) and remaining elevated throughout the whole experimental time (168 h) (Flores-Rojas et al., 2015). In the same study, GST and GR responded at an exposure concentration of 25 µg/L after 24 h and as well at a concentration of 2.5 µg/L but only after a longer exposure period (168 h) (Flores-Rojas et al., 2015). CYN led to elevated oxidative stress (increased GST and GPx activities) in rice plants (*Oryza sativa*) after short-term exposure to toxic *Aphanizomenon ovalisporium* cyanobacterial extracts containing 0.13 µg CYN/L. Moreover, longer exposure periods led to tissue necrosis concomitant with oxidative stress (Prieto et al., 2011a). Some studies showed the involvement of oxidative stress as a mechanism of action of CYN in aquatic animals, e.g. in tilapia fish. Enhancement of GST in liver and kidney samples and inhibition of GPx in the kidneys have been reported in Nile tilapia (*Oreochromis niloticus*) acutely exposed to pure CYN (200 and 400 µg/L) by gavage (Puerto et al., 2011). Further changes in oxidative stress biomarkers of the same species were shown by oral and intraperitoneal routes (Gutiérrez-Praena et al., 2011a; Gutiérrez-Praena et al., 2012; Gutiérrez-Praena et al., 2011b), and it has been proven that oxidative stress plays a role in the pathogenicity induced by CYN in *O. niloticus* at concentrations of 10 and 100 µg/L after 7 and 14 d of exposure (Guzmán-Guillén et al., 2013).

In the aquatic macrophyte *C. demersum* after 24 h of exposure, BMAA had an inhibitory effect on all the antioxidant enzymes (CAT, GPx, POD, GR, SOD, and GST) tested with the exposure concentrations ranging from 0.5 to 100 µg/L. However, enzymes not related to oxidative stress response were not affected by BMAA in this study (Esterhuizen-Londt et al., 2011) showing a clear selective response of the antioxidant system. Inhibition of several oxidative stress biomarkers (SOD, CAT, and sGST) was also shown in the animal model *Daphnia magna* as a consequence of oxidative stress response due to BMAA exposure (Esterhuizen-Londt et al., 2015b). BMAA was taken up by the daphnia and subsequently led to an inhibition of the oxidative stress defense and biotransformation system of the aquatic animals within 24 h of exposure to BMAA (100, 500 and 1,000 µg/L) (Esterhuizen-Londt et al., 2015b). Unlike the clear inhibition of enzymatic defense responses in the mentioned aquatic plant and animal, BMAA exposure (10, 100 and 500 µg/L) to freshwater mussels provoked changes in selected oxidative stress enzymes (CAT, GR, GPx, and GST), which

resulted in the enhancement of GST and GR activity after 24 h and the inhibition of CAT and GPx activity after 48 h in *Corbicula javanicus*, and in the enhancement of CAT and GR activity after 24 h and a decrease of GPx activity after a longer exposure for four or seven days in *Unio tumidus* (Contardo-Jara et al., 2014a).

Although oxidative stress induced by cyanotoxins has been extensively examined for many aquatic organisms including plants and animals, the effect of cyanotoxins in aquatic fungi is yet unknown. However, it is an important factor, especially when considering the use of fungi in bioremediation as it imposes significant effects on fungal cultures in terms of morphology, growth rate, metabolism, and protein secretion (Li et al., 2011). Additionally, it can lead to growth and reproduction inhibition and/or mortality of the organism. Hence, elucidation of the enzymatic responses upon exposure to cyanotoxins provides insight on the applicability and longevity of the fungal organism.

1.3 CYANOTOXIN METABOLISM AND DETOXIFICATION IN LIVING ORGANISMS

The metabolism of a foreign chemical (drug/xenobiotic/natural toxin) in a living organism routinely involves three phases, activation, conjugation, and excretion in order to detoxify and remove the toxin from the cells. In activation phase I, the compound is chemically activated by the introduction of functional reactive groups, such as hydroxyl-, carboxyl- and amino-groups through hydroxylation, oxidation and reduction reactions that are catalyzed by different phase I enzymes, mostly by cytochrome P450 monooxygenase (hydroxylase), NADPH-cytochrome P450 reductase. After chemical transformation, the toxic compound can either be directly excreted if water-soluble enough or is prepared to enter conjugation phase II. Consequently, covalent attachment of the activated reactive metabolite to specific endogenous polar molecules, glucuronic acid, sulfate, acetate, amino acids, and the tripeptide glutathione occurs. The generated metabolic conjugates possess increased hydrophilicity and therefore decreased toxicity when compared with the original compound. Consequently, they are then more easily excreted by the organism in phase III. Similar pathways for xenobiotic metabolism and enzymes involved in the biotransformation phase I and II are found in microbial, mammalian, and plant systems but different behavior in phase III, excretion by microbial and mammalian systems, compartmentalization in plants within vacuoles, or the apoplast (Coleman et al., 1997).

Proposed mechanism for cyanotoxin metabolism

The mechanism of biotransformation depends in part, on the chemistry of the compound being degraded, and whether or not it is a substrate for one of the various phase I or II enzymes. Fig 1-3 shows the proposed mechanism of detoxification of MC-LR, the glutathione metabolic pathway. The tripeptide γ-L-glutamyl-L-cysteinyl-glycine known as glutathione (GSH), is the most important low molecular weight antioxidant synthesized in cells. The thiol group (-SH) of the cysteine (Cys) is involved in the reduction and conjugation reactions that are usually considered as the most important functions of GSH, for the removal of peroxides and many xenobiotic compounds. The GSH adduct (MC-LR-GSH), formed by the nucleophilic addition of GSH to the electrophilic α,β-unsaturated carbonyl of the Mdha moiety, chemically known as a Michael addition, has been shown to occur spontaneously and enzymatically, catalyzed by an important group of biotransformation phase II enzymes, the glutathione *S*-transferases (GST).

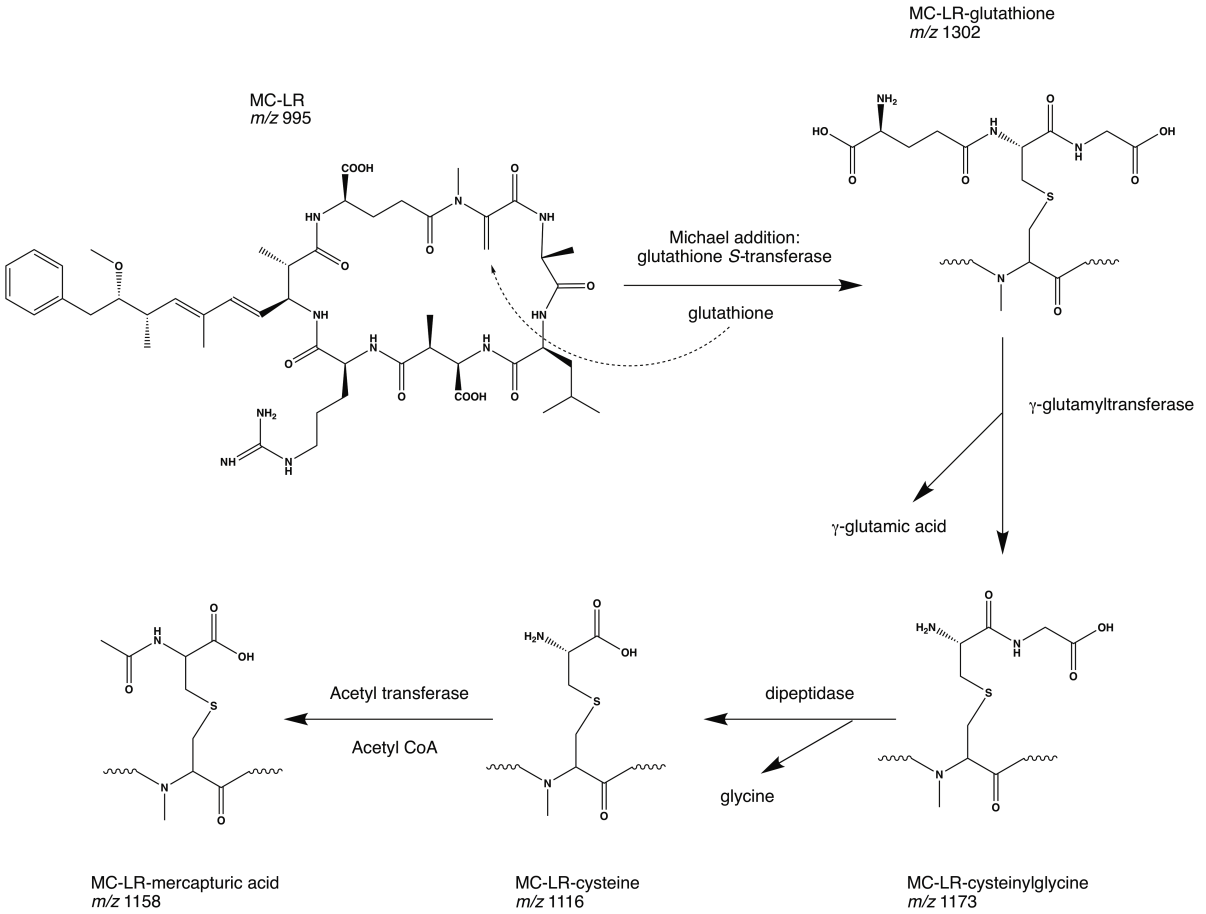

Fig. 1-3: Metabolic detoxification of MC-LR *via* conjugation to GSH, adapted from Schmidt et al. (2014).

This pathway, mediated by GST, was suggested to play a primary role in the metabolic detoxification of MCs in various aquatic organisms ranging from plants (*C. demersum*), invertebrates (*Dreissena polymorpha*, *D. magna*) up to fish eggs, fish (*Danio rerio*, *O. niloticus*), and rat, stating that the pathway for xenobiotic metabolism expressed in all major groups of organisms is involved in the detoxification of MC (Pflugmacher et al., 1998b; Takenaka, 2001; Wang et al., 2006). Moreover, the MC-LR conjugation with GSH catalyzed by five recombinant human GSTs has been characterized (Buratti et al., 2011). GSH-conjugation is often the first in a series of metabolic alterations to a xenobiotic, ultimately producing a mercapturic acid conjugate that is quickly excreted by the organism (Manahan, 2003). The γ-glutamic acid group is removed by γ-glutamyltransferase, yielding the MC-LR-cysteinylglycine conjugate. The glycine group of the MC-LR-cysteinylglycine conjugate is removed by dipeptidase, forming the MC-LR-Cys conjugate. On the basis of the LD_{50} values obtained after intravenous injection to mice, GSH and Cys conjugates showed a significant reduction in toxicity compared with MC-LR (Kondo et al., 1992). The MC-LR-Cys conjugate is finally oxidized through acetyl transferase, forming the MC-LR-mercapturic acid conjugate.

Investigation of GST activity as the biotransformation enzyme responsible for the first step in this metabolic pathway can elucidate the involvement of biotransformation phase II *via* conjugation to GSH as a possible route for MC-LR metabolism in exposed organisms.

The detoxification pathways for CYN and BMAA are yet unknown. However, GSH seems to be required to inactivate CYN, but concurrently in its presence, GSH synthesis is severely inhibited, which is most likely an important factor for the cytotoxicity of CYN (Runnegar et al., 1995). GSH depletion may hinder biotransformation phase II conjugation catalyzed *via* GST to be a possible mechanism for CYN detoxification. The involvement of cytochrome P450 is suggested to play a part in the biotransformation of CYN, however with generation of metabolically activated intermediates that may be more toxic and/or potent inhibitors of GSH synthesis than the parent CYN (Froscio et al., 2003; Humpage et al., 2005).

BMAA bioaccumulates and biomagnifies within the environment. It either associates with proteins and can be found protein-bound in plants and animals (Contardo-Jara et al., 2014b; Contardo-Jara et al., 2013; Esterhuizen et al., 2011; Esterhuizen-Londt et al., 2015b; Karlsson et al., 2014) or is covalently modified as shown in selected freshwater mussels (Downing et al., 2014) and the aquatic plant *C. demersum* (Downing et al., 2015). Other possible models suggest acetylation (Reece and Nunn, 1988), oxidation followed by hydrolysis and decarboxylation of subsequent metabolites (Hashmi and Anders, 1991) and demethylation by

formation of methylamine in rat liver and kidney homogenates exposed to BMAA (Nunn and Ponnusamy, 2009). However, the exact mechanism of BMAA metabolism in a range of environmentally relevant organisms, including fungi, and the possible involvement of phase I and II enzymes in the detoxification is yet unknown.

1.4 REMOVAL OF CYANOTOXINS – BIODEGRADATION

The World Health Organization (WHO) has established provisional drinking water guidelines of 1 µg/L for MC-LR and a proposed guideline of 1 µg/L for CYN, and a recreational exposure guideline value of 10 µg/L for total MC-LR (free plus cell-bound) (WHO, 2011). Since 2003, the Federal Environmental Agency recommends bathing prohibition at concentrations exceeding 100 µg/L and warning declarations at concentrations exceeding 10 µg/L (UBA, 2015). Efficient treatment strategies are needed to prevent cyanotoxins from reaching consumers. The removal of cyanotoxins from water bodies is therefore an important environmental issue to improve the supply of drinking water and to ensure water quality and safety of bathing waters. The presence of cyanotoxins is of particular concern in drinking water supplies where conventional water treatment, such as flocculation/filtration and chlorination is only restrictedly applicable and often fails to eliminate them (Himberg et al., 1989; Hoffman, 1976; Keijola et al., 1988). Tsuji et al. (1997) nevertheless suggested chlorination at an adequate chlorine dose as an effective water treatment for the removal of MCs, however, preoxidation of the cell itself with chlorine must be avoided, because of additional toxin release and trihalomethane production. Ozonation and reverse osmosis treatment are effective techniques in water treatment to remove MCs (Chang et al., 2014; Liu et al., 2010). Advanced and special methods (activated carbon either as powder or as granulates, membrane filtration) (Himberg et al., 1989; Keijola et al., 1988) result in high costs and physicochemical methods may partly be ineffective because of the relative stability of cyanotoxins under a wide range of physicochemical conditions (Chiswell et al., 1999; Harada, 1996; Metcalf and Codd, 2000). In fact, there is evidence supporting bioremediation as being the main route for natural cyanotoxin removal from the environment (Edwards and Lawton, 2009). Therefore, the exploitation of using microbes to remove cyanotoxins from contaminated waters poses a suitable and effective remediation strategy, known as biodegradation. Biodegradation is a process with low technology expenditure, long-term sustainability including low maintenance and operating costs, and, most importantly, with the advantage of a natural and sustainable treatment strategy (Ho et al., 2012).

Many studies have focused on screening and isolating cyanotoxin-, especially MC-degrading microbes under which mostly bacteria have been identified belonging to the *Proteobacteria* (Kato et al., 2007), especially to the *Sphingomonadaceae* family (Kormas and Lymperopoulou, 2013). The bacterial degradation pathway of MC-LR has been elucidated and its mechanisms thereby include the involvement of an *mlr* gene cluster, which is responsible for sequential enzymatic hydrolyses of the peptide bonds (Fig. 1-4).

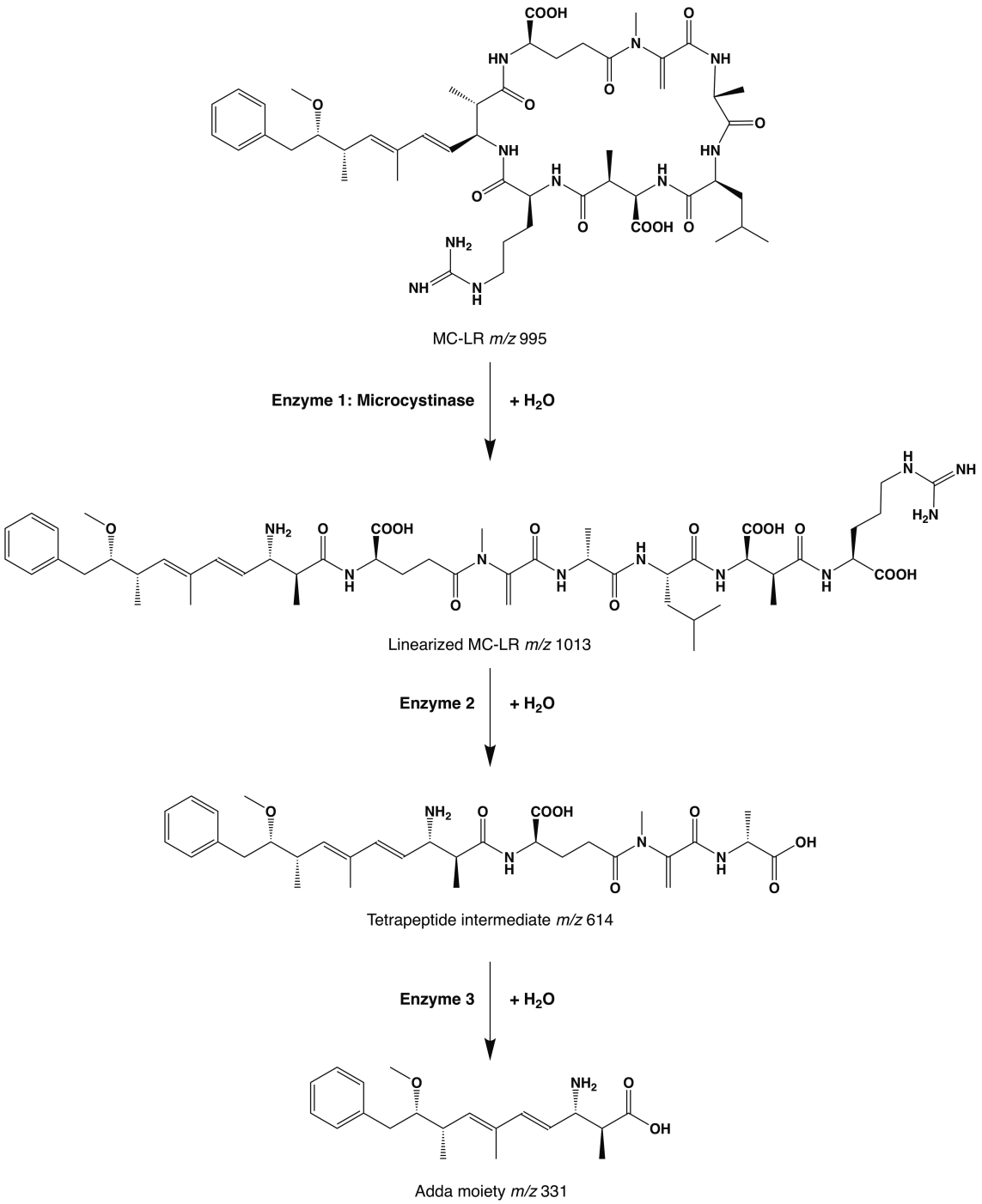

Fig. 1-4: Proposed bacterial degradation pathway of MC-LR, adapted from Bourne et al. (1996).

There are four genes that encode intracellular enzymes able to break MC bonds, namely *mlrA*, *mlrB*, *mlrC* und *mlrD* (Bourne et al., 2001), of which the first *mlrA* microcystinase plays the most important role, because it encodes the enzyme that breaks the Adda-arginine peptide bond and thus opens the cyclic structure of MC (Bourne et al., 2001; Ho et al., 2012) resulting in a linearized product with 160-fold decreased toxicity (Somdee et al., 2013). Subsequently, further enzymatic catalyzed peptide hydrolyses occur, yielding a tetrapeptide intermediate and the Adda moiety as well as other smaller peptides and amino acids, which were found to be nontoxic (Harada et al., 2004; Imanishi et al., 2005). Currently, this is the only bacterial degradation mechanism that has been characterized, but other ways are suggested, as bacteria without the characteristic *mlr* gene cluster are still able to degrade MCs (Ho et al., 2012; Hu et al., 2009; Manage et al., 2009). Moreover, periphyton, a biofilm in surface water with floral and faunal microorganisms has been reported to adsorb and degrade MCs and to be applicable as an environmentally friendly remediation system for their removal (Wu et al., 2010). Although, fungi represent another important degrading microbial group, they have been studied only to a very limited extent with regards to cyanotoxin degradation. The white-rot fungus *Trichaptum abietinum* has been reported to degrade MC-LR in a *Microcystis* culture and to lower the toxicity of the degraded culture, as proven by the micronucleus test (Jia et al., 2012). Another fungus, *Trichoderma citrinoviride* has been described to inhibit *Microcystis* growth and to degrade its microcystin toxin (Mohamed et al., 2014). Biodegradation related to the use of fungi is called mycoremediation and involves organisms with a broad substrate specificity and high adaptation ability to extreme environmental conditions and perturbations and could open the way to a new cyanotoxin remediation strategy.

Mycoremediation

Fungi play a crucial role in the balance of ecosystems. White-rot fungi, mostly basidiomycetes such as *Phanerochaete chrysosporium, Trametes versicolor, and Pleurotus ostreatus* are important decomposers and recyclers of decaying organic matter and are therefore indispensable for the carbon cycle of the biosphere and responsible for resupplying elements such as nitrogen and phosphorous to the environment. The exploitation of the natural ecosystem functions of fungal species, to remove organic and inorganic pollutants from the environment, has been proven as a good alternative to traditional treatment technologies. The advantage of eukaryotic fungi is their very low substrate specificity as they excrete H_2O_2 as well as non-specific extracellular enzymes. Among them are, most importantly, the lignin-modifying enzymes, lignin peroxidases (LiP), manganese-dependent peroxidases (MnP), and laccases (Barr and Aust, 1994; Bumpus and Aust, 1987; Kersten,

1990; Kersten and Kirk, 1987). White-rot fungi are able to break down many persistent complex organic substances, such as lignin and cellulose (Tien, 1987; Ulmer et al., 1983), and are therefore responsible for wood decay. This natural degradation ability of the non-specificity of the fungal enzyme machinery is used for industrial applications to degrade, mineralize, and remove other natural or chemical organic pollutants as well (Novotný et al., 2004). The first fungal xenobiotic degradation application was reported in 1985 with the complete removal of free and lignin bound chloranilines from the environment (Arjmand and Sandermann Jr, 1985) and mineralization of polychlorinated biphenyls (Eaton, 1985), using the white-rot model fungus *P. chrysosporium*. Besides the lignolytic enzymes, white-rot fungi additionally possess intracellular oxidative biotransformation phase I enzymes including cytochrome P450s, which have been demonstrated to play further central roles in the metabolism of various exogenous compounds *via* chemical transformation and to be attractive for a series of practical degradation applications. The degradation of the endocrine-disrupting chemical nonylphenol in nutrient-limited (lignolytic conditions) and –sufficient (non-lignolytic conditions) cultures of *P. chrysosporium* evidences the involvement of P450 enzymes in the degradation of the xenobiotic (Subramanian and Yadav, 2009). Cytochrome P450 monooxygenases were further involved in the degradation of various PAHs, diuron and benzoic acid by *P. chrysosporium* (Coelho-Moreira et al., 2013; Ning et al., 2010; Subramanian and Yadav, 2009; Syed and Yadav, 2012), trichlorobenzenes by *T. versicolor* (Marco-Urrea et al., 2009), antibiotics such as norfloxacin and ciprofloxacin by *T. versicolor* (Prieto et al., 2011b), the pesticide *trans*-chlordane by *Phlebia* spp. (Xiao et al., 2011), hydroxylation of toluenes by *P. chrysosporium* (Teramoto et al., 2004), and phenanthrene degradation by *P. ostreatus* (Bezalel et al., 1997).

The white-rot fungus *P. chrysosporium*, which is partly used within this study, has become a model fungus of the white-rot class and is successfully applied in the degradation of various persistent compounds. However, although *P. chrysosporium* and other white-rot fungi are clearly effective degraders with the ability to mineralize the original compound to CO_2, H_2O, and nutrients, there are some disadvantages encountered, such as temperature limitation due to high optimal working temperatures (30-39 °C for *P. chrysosporium*) and high oxygen demand (Couto et al., 2006; Kirk et al., 1978). Both factors do not correspond with realistic environmental conditions and would lead to high technological costs and energy expenditure in reactor technology. To overcome these disadvantages, fungi with high GST biotransformation phase II enzymes became of special interest and include the filamentous fungi, *Mucor* spp. This genus of zygomycetes has been successfully employed in the

remediation of several pentachlorophenol (Carvalho et al., 2011), PAHs (Jia et al., 2016; Su et al., 2006), insecticides such as carbofuran (Seo et al., 2007) and endosulfan (Shetty et al., 2000), oil hydrocarbons (Marchut-Mikolajczyk et al., 2015; Mirbagheri et al., 2016), slime extracellular polymeric substances, and polysaccharides, contributing to the improvement of sludge dewaterability (Wang et al., 2015). Functional groups on the fungal cell wall surface enable adsorption of heavy metals, such as chromium (VI) (Tewari et al., 2005), nickel (Shroff and Vaidya, 2011), and intracellular mercury accumulation (Hoque and Fritscher, 2016). *Mucor hiemalis* EH5 is an aquatic fungus that is reported to express high intracellular GST activity (Hoque et al., 2007) and has been used in the remediation of isoproturon involving phase II conjugation as a pathway for degradation in co-culture with *P. chrysosporium* (Hoque, 2003). *M. hiemalis* therefore represents an ideal candidate for mycoremediation attempts of cyanotoxin contaminated freshwaters, especially MC-LR that is known to be primarily detoxified *via* this specific route in plants, animals, and humans (Buratti et al., 2011; Pflugmacher et al., 1998b; Takenaka, 2001). Most importantly, the fungus still sporulates at ground water temperatures of 5 °C enabling low energy expenditure in biotechnological applications; and is resistant to high hydrogen sulfide concentrations (Hoque, 2003) enabling its use also under extreme environmental perturbations. *M. hiemalis* has been used in the biotransformation of steroids and flavonoids, for synthetically challenging hydroxylation, and glycosylation reactions (Kolet et al., 2014; Xiao and Lee, 2016) indicating the variety of fungal biotransformation ability of different substrates.

The co-cultivation of two different fungi, *P. chrysosporium*, a basidiomycete with lignolytic and high levels of phase I enzymes and *M. hiemalis*, a zygomycete with high levels of phase II GST enzyme activity, could result in the generation of complementary systems, i.e. the mutual elicitation of enzyme activities and therefore enhanced biotransformation and degradation efficiency. This was illustrated with the successful mycoremediation of isoproturon, where only after additional treatment with *M. hiemalis*, complete remediation of the herbicide by *P. chrysosporium* was achieved (Hoque, 2003). This technique may also be translated on the remediation of cyanotoxins and other natural or synthetic toxins.

Technical applications of fungi are realized in bioreactors. One is the utilization of fungi as biofactories for the synthesis of valuable bioproducts of commercial importance, such as organic acids, antibiotics, enzymes, mycotoxins, proteins (native and heterologous), vitamins, and other native fungal products (El-Enshasy, 2007; Richter et al., 2014). Another is the implementation of fungi in water and wastewater treatment. The decolorization of wastewaters from textile, printing, and paper industry is achieved by fungal degradation of

synthetic dyes, inorganic and organic pollutants. Wastewaters containing phenols from pharmaceuticals, pesticides, solvents, paper and pulp industries, and pharmaceutical compounds (i.e., anti-inflammatory agents, analgesics, antibiotics, psychiatric and anticancer drugs) are also often purified *via* fungal remediation. Moreover, fungi are used to treat water by biosorption of heavy metals and metalloids (Espinosa-Ortiz et al., 2016).

Filamentous fungi are morphologically diverse including the growth as filaments, clumps, or pellets in submerged fermentation. Fungal pellets are well settling aggregates formed by self-immobilization in liquid shaking cultures under species specific conditions. Their use in bioreactors is promising as it avoids the practical and technical difficulties usually encountered with dispersed mycelium and offers advantages, such as improvement of harvesting, good settling ability and quick separation of biomass, improvement in culture rheology (low-medium viscosity, Newtonian flow behavior), lower power consumption for sufficient bulk heat and mass transfer, biomass reuse and continuous operation of the process, better mass exchange of oxygen and nutrients due to the decrease surface to volume ratio, low clogging effect, ease of scale up, high cell loading and volumetric productivities, and no adherence to any part of the bioreactors (Espinosa-Ortiz et al., 2016; Kim et al., 1983; Olsvik and Kristiansen, 1994).

Factors influencing fungal morphology in submerged cultivation are many that have to be considered and include medium composition, type and concentration of the carbon source, levels of nitrogen and phosphate, presence of solid particles, trace metals, culture pH, dissolved oxygen and carbon dioxide, temperature, the type of the inoculum (vegetative or spores), inoculum size and a large number of physical factors such as agitation, rheology, fermenter geometry, as well as the mode of culture itself, e.g. batch, fed-batch or continuous culture (Papagianni, 2004). The role of the factors affecting fungal morphology and the mechanism of pelletization is still not fully understood and therefore brings some challenges to the development of a thorough understanding and methodology for efficient pellet formation of the fungus of interest. Moreover, morphological behavior has shown to be not only genus, but also species specific, thus pellet formation of the species in question is only achievable after an experimental adaptation of the culture parameters. At present, pellet formation of *Mucor* species is reported to a very limited extent (Xia et al., 2011, and the present study).

1.5 AIMS AND OBJECTIVES

The objectives of this study were to investigate the concept of cyanotoxin mycoremediation using the filamentous aquatic fungus *M. hiemalis*, to provide information on cyanotoxin uptake, physiological responses and degradation ability of *M. hiemalis* with comparison to the degradation efficiency of the lignolytic white-rot fungus *P. chrysosporium* in single- and co-culture, with regards to evaluate the possible technical applications of *M. hiemalis* in bioreactor technology.

The main question within this study was:

Is *M. hiemalis* a suitable candidate for mycoremediation of cyanotoxins in regard for future bioreactor applications?

The matters of interest concerning the main question suggested are shown below.

- Effect of cyanotoxins on sensitivity, growth and biomass production of *M. hiemalis*
- Uptake of cyanotoxins by the aquatic fungus *M. hiemalis*
- Morphological investigations in submerged cultivation with factor variations and establishment of a cultivation method for the pelletization of *M. hiemalis*
- Production of oxidative stress by cyanotoxins and antioxidant enzyme responses in *M. hiemalis*
- Influence on phase II biotransformation enzyme GST in *M. hiemalis*
- Possibility of using *M. hiemalis* to remove cyanotoxins from water bodies, implementation of the mycoremediation concept with first degradation experiments on the example of MC-LR
- Comparison of the degradation ability towards *P. chrysosporium* in single- and co-culture

Therefore, the following hypotheses were set for this study and were tested by one or more specific objectives stated to each of them. These hypotheses were explored in three separate publications (Paper I-III in Chapter 2-4) and one manuscript in preparation (Chapter 5).

1) **Fungi are highly resistant microorganisms that can develop under extreme environments and in polluted areas (Paper I and III)**

   → Study effect of cyanotoxins on aquatic fungus *M. hiemalis*, including radial inhibition, biomass production, and the influence at a physiological level, i.e. oxidative stress

2) **Cyanotoxins are taken up and degraded intra- and extracellularly by aquatic fungi (Paper I, II and Chapter 5)**

   → Assess cyanotoxin uptake by *M. hiemalis* in a biomass- and time-dependent manner and monitor remaining media concentration over time

3) **Filamentous fungi are morphologically diverse and can be manipulated such as growing in pellets under submerged cultivation and optimal conditions**

   → Investigate various factors influencing fungal morphology and develop an optimized strategy for the pelletization of *M. hiemalis*

4) **Exposure to cyanotoxins does not lead to oxidative stress and negative morphological and growth effects in aquatic fungi (Paper I and III)**

   → Examine changes in the activity of antioxidative enzymes in connection with ROS formation in *M. hiemalis* exposed to cyanotoxins related to growth behavior

5) **Aquatic fungi can biotransform cyanotoxins and this process may be associated with an increase of GST activity (Paper III)**

   → Determine alterations in enzyme activities of the biotransformation system in *M. hiemalis* in response to cyanotoxin type- and concentration-dependent exposure

6) **Aquatic fungi have good potential for being used as remediating agents for cyanotoxins in aquatic environments**

   → Assess the removal ability of *M. hiemalis* on the example of MC-LR and compare towards the removal ability of the white-rot model fungus *P. chrysosporium* (Chapter 5)

7) **Degradation efficiency may be enhanced in co-cultivation and temperature limitation of *P. chrysosporium* may be overcome**

   → Compare the removal ability of single- and co-culture under the level of the optimal working temperature

In this study, the aquatic fungus *M. hiemalis* was chosen because of its known high GST activity and temperature-resistance over a broad range with known metabolic activity even at ground water temperatures of 5 °C and because of its ability to co-pelletize with the lignolytic white-rot fungus *P. chrysosporium*. Three diverse cyanotoxins were chosen to compare in between structure and biological activity, however, with special focus on MC-LR throughout the whole work, as it is known that MC-LR is primarily metabolized through GSH conjugation thereby posing a possible metabolic process catalyzed by fungal GST conjugation reaction.

2 TOXIN RESISTANCE IN AQUATIC FUNGI POSES ENVIRONMENTALLY FRIENDLY REMEDIATION POSSIBILITIES: A STUDY ON THE GROWTH RESPONSES AND BIOSORPTION POTENTIAL OF *MUCOR HIEMALIS* EH5 AGAINST CYANOBACTERIAL TOXINS

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Own contribution:

Literature review

Design of experiments and laboratory studies

Fungal culture and exposure settings

Toxin extraction and sample preparation

Performing all required laboratory works

Statistical analysis and interpretation of the experimental results

Preparation of the manuscript, which includes all texts and figures

Revision of the manuscript after receiving the reviewer's comments

ABSTRACT

Cyanobacteria produce various harmful secondary metabolites, which pose a serious global threat to aquatic ecosystems and human health. Biodegradation is an important topic of water purification research and offers especially an environmentally friendly remediation strategy. Here, we present a water fungus of the genus *Mucor* that shows considerable promise to be applied as a mycoremediation agent for the removal of cyanobacterial toxins from aquatic environments. In the present study, we investigated the effect of three different cyanobacterial toxins, namely the hepatotoxin microcystin-LR, the neurotoxin β-*N*-methylamino-L-alanine and the cytotoxin cylindrospermopsin, on the sensitivity of *Mucor hiemalis* EH5 using an adaptation of the Kirby-Bauer disk diffusion assay, and the influence of the toxins on fungal growth and biomass production *via* radial extension and dry weight measurements. Additionally, we established an optimized strategy for the individual cyanobacterial toxin extraction from the vegetative part of *Mucor hiemalis* EH5 and analyzed its biosorption potential *via* LC-MS/MS measurements. The fungal microorganism showed a fast adaptation behavior and strong resistance towards the toxins. No significant differences in terms of growth were perceived when comparing the exposed fungi to an untreated control. This indicates that the cyanobacterial toxins are not lethal to the fungus and that the organism can grow and develop undisturbed in their presence. Toxin uptake was quantified by LC-MS/MS detection with recoveries for the established extraction methods of > 60-85 %. After exposing the fungi to each of the toxins respectively for 24 and 48 hours, we found a significant uptake ($p < 0.05$) in the range of 0.1 to 1.7 mg of the applied toxin per gram mycelial biomass (dw). Our results suggest that *Mucor hiemalis* EH5 is an ideal organism to be tested in further studies as a biodegrading system for the remediation of cyanobacterial toxins from contaminated waters.

Keywords: cyanobacterial toxins, biodegradation, *Mucor hiemalis* EH5, growth response, biosorption, toxin uptake.

2.1 INTRODUCTION

Cyanobacteria are ubiquitous organisms, mostly found in aquatic environments. They are amongst the earliest organisms on Earth and the pioneers of oxygen production. Even though their evolutionary and ecological importance remains uncontroversial [1], they are now a growing environmental and public health concern because of their ability to form various bioactive secondary metabolites [2,3]. Eutrophication of water bodies and climate change factors promote the development of microalgae and cyanobacteria yielding to an explosive formation of massive blooms [4,5]. The produced cyanobacterial toxins are mainly retained within the cyanobacterial cells but are especially released during senescence and cell lysis [6,2]. These secondary metabolites display diverse modes of action thereby manifesting adverse effects on the aquatic flora and fauna [7-10] and human health [11]. According to their biological effects, they can be divided into five different groups: hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and lipopolysaccharides [2]. Their structural diversity is clearly illustrated in Fig. 2-1, which shows three commonly occurring cyanotoxins.

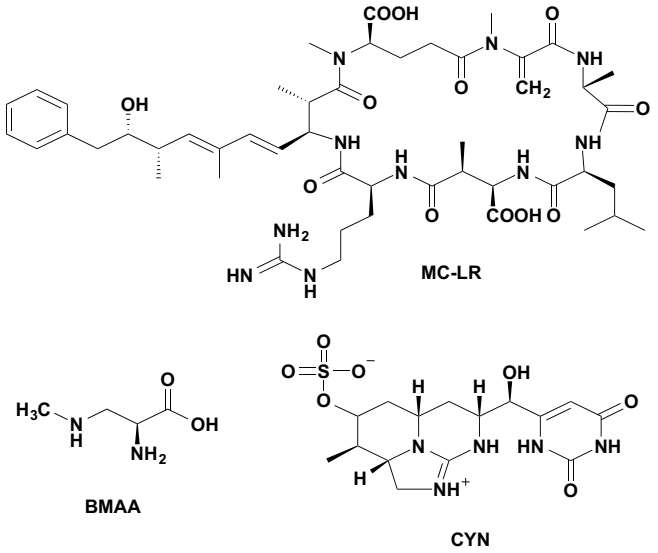

Fig. 2-1: Molecular structures of the cyanotoxins microcystin-LR (MC-LR), β-*N*-methylamino-L-alanine (BMAA) and cylindrospermopsin (CYN).

Microcystins, cyclic heptapeptides, are the most widespread toxins and are therefore the best studied. Microcystin-LR (MC-LR, Fig. 2-1) is considered the most toxic compound of this family [12]. β-*N*-Methylamino-L-alanine (BMAA, Fig. 2-1) is a highly reactive non-protein amino acid likely synthesized by all cyanobacteria [13-15] and can occur either free or protein-bound [16]. BMAA is assumed to cause various neurodegenerative diseases such as ALS-PDC and Alzheimer's disease [16] and recently has been shown to induce neural damage at very low concentrations [17,18]. Cylindrospermopsin (CYN, Fig. 2-1) has caused

human poisonings in Australia and Brazil [19] and is accountable for the death of animals [20].

Cyanobacterial toxin removal from water bodies is of critical importance [21] and an emerging research area of increasing interest in order to improve water quality and safety. Biosorption and biotransformation proves to be the most appropriate method for the efficient elimination of cyanobacterial toxins from water bodies in environmental conditions [22] and offers particularly the advantage of a natural and sustainable strategy [23].

Fungal biosorption is achieving prime attention in effluent treatment processes. Extensive research exists on metal biosorption by terrestrial and aquatic fungi as an alternative treatment for heavy metal bearing wastewaters. The sorption of heavy metals and radionuclides by fungi such as *Aspergillus* spp., *Mucor* spp., *Rhizopus* spp. and *Penicillium* spp., and yeast (*Saccharomyces* spp.) has been observed to varying extents [24-27].

Furthermore, fungal uptake and biodegradation of different xenobiotics is known. Four selected ectomycorrhizal fungi have been shown to almost completely remediate 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) from the media within 15 days and only 40-50 % was found being accumulated in mycelia, whereas the remaining DDT was degraded to metabolites, which were identified by GC-MS [28].

The most successful application of white rot fungi lies in the purification of effluents of textile industries, paper and pulp industries, because of their high decolorization capacity. Fungal mycelium of *Trametes versicolor* showed initial adsorption of synthetic dyes in the first hour of contact of the dye with the mycelium [29]. An efficient decolorization of 17 disperse dyes due to sorption of dyes to fungal cells was also observed in *Cunninghamella polymorpha* cultures [30]. In general, dye molecule biosorption onto cell surface appears to be quick and often completes in a few hours. Amino, carboxyl, thiol, and phosphate groups, present in the fungal cell wall, are responsible for binding dye molecules [31]. Fu and Viraraghavan [32] studied biosorption of four dyes using *Aspergillus niger* biomass. *Phanerochaete chrysosporium* (*P. chrysosporium*) is used in the MyCoR (mycelial color removal) reactor [33] or immobilized on alginate beds it serves for the removal of chlorophenols [34].

To date, despite the important role of fungi in water treatment, very limited information exists on fungal biosorption and degradation of cyanotoxins. Research has primarily focused on screening bacteria for their ability to control and degrade harmful cyanobacteria and their produced toxins in aquatic environments. Little attention has been paid to fungi, including studies on white rot fungi, which can inhibit the growth of cyanobacteria species [35-37].

Moreover, the ability of the white rot fungus *Trichaptum abietinum* 1302BG to degrade MC-LR in a culture of *Microcystis aeruginosa PCC7806* has been reported. After 12 hours, complete degradation of extracellular MC-LR was observed. More recently, the strain *Trichoderma citrinoviride* has been identified to selectively inhibit the growth of the cyanobacterium *Microcystis aeruginosa* and effectively degrade its microcystin toxins. After 72 hours, complete elimination of the toxin was achieved [38]. These results show that fungal strains can degrade microcystins more rapidly (12-72 hours) than bacterial strains (6-25 days) [39,40]. Thus it is necessary to screen more fungal species, especially aquatic fungi that may be used as efficient bioagents against cyanobacterial toxins.

Eukaryotic fungi exceed bacteria in their degradation ability due to their very low substrate specificity [41,42]. While bacteria work through genetic specific degradation mechanisms, fungi act with unspecific extracellular oxidation enzymes, such as the peroxidase systems [43,44] and the glutathione-*S*-transferase enzymes [45]. Glutathione-*S*-transferases are key enzymes in the detoxification metabolism and protect the cell against toxicants by catalyzing the conjugation of xenobiotics to glutathione. Fungal species with glutathione-*S*-transferase activity, like the genera *Basidiomycotina*, *Deuteromycotina* or *Zygomycotina*, e.g. *Cephalosporium*, *Penicillium*, *Trichoderma* and *Mucor* spp. can be utilized for the purification of sulfidic waters [46]. *Mucor hiemalis* (*M. hiemalis*) *f. irnsingii* (DSM 14200, alias EH5), which has been isolated from such waters, possesses a distinctive high glutathione-*S*-transferase activity and shows a high tolerance against H_2S [47]. Moreover, the fungus has functional groups on the cell wall enabling biosorption of heavy metals, such as chrome VI [48] and nickel [49], and is also known for its fast and complete remission potential of the herbicide isoproturon, if it is used in combination with *P. chrysosporium* [47]. However, *P. chrysosporium* requires an optimal growing temperature of 39 °C leading to high temperature expenditure in biotechnological applications. In contrast, *M. hiemalis* EH5 is temperature-independent, and still sporulates at temperatures lower than groundwater temperature (e.g. 5 °C). This paves the way for a season-independent application of the fungal organism and enables pollutant removal in deep sediment layers as well as under extreme environmental conditions. *M. hiemalis* can be applied in facultative aerobic/anaerobic, reducing or H_2S polluted water; moreover, it resists metal contamination over a wide pH range (3-11) and can be used in ground and surface water, sewage, wastewater, and industrial and mine waters [50].

Because of the promising characteristics of *M. hiemalis* EH5 to possibly degrade and remove cyanobacterial toxins from contaminated water bodies, the goal of the present work was to

obtain an insight into the effect of MC-LR, BMAA and CYN on the sensitivity and growth responses of *M. hiemalis* EH5 and its biosorption ability towards the cyanotoxins.

2.2 MATERIALS AND METHODS

2.2.1 FUNGAL STRAIN

M. hiemalis EH5 (DSM 14200), previously isolated as an aquatic H_2S-resistant strain from the sulfidic-sulfurous Irnsing spring water biofilms in Bavaria, Germany [45], was obtained from the culture collection of the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures.

2.2.2 CYANOBACTERIAL TOXINS

The cyanobacterial toxins microcystin-LR (MC-LR), β-*N*-methylamino-L-alanine hydrochloride (BMAA) and cylindrospermopsin (CYN) were purchased from commercial suppliers (Enzo Life Sciences, Alexis Biochemicals ALX-350-012-M001; Sigma-Aldrich, Munich, Germany CAS Number: 16676-91-8; Enzo Life Sciences, Alexis Biochemicals ALX-350-149-M001). Pre-stock solutions with a concentration of 1 mg/mL were prepared in pure methanol and stored at -20 °C. Serial dilutions (5, 50, 100, 250, 500 and 1000 µg/L) were prepared in sterilized double distilled water for the experiments.

2.2.3 CULTURE CONDITIONS

Short-term conservation of the pure culture in the laboratory was achieved by periodical inoculation of malt extract agar plates once per month.

For strain maintenance and propagation, cultures were grown on a solid malt extract agar substrate. The nutrient medium consisted of 30 g malt extract broth (Sigma-Aldrich, Fluka 70146), 15 g agar bacteriological (Oxoid no 1, LP0011) in 1 L double distilled water (Roth, T172.3) supplemented with 0.82 mM sodium thiosulfate and 100 ppm streptomycin sulfate (Roth, HP66.1) [45]. The medium was autoclaved for 20 min at 121 °C. After cooling to 60 °C, 10 mL of the agar medium were aseptically poured into petri dishes. The plates were sealed with parafilm and stored in the dark at 4 °C for further experiments.

2.2.4 DISK DIFFUSION ASSAY: INOCULATION AND EXPOSURE

Mycelial growth experiments were assessed in the dark at 25 °C, which has been reported to be the optimum growing temperature for *M. hiemalis* EH5 [51]. A mycelial mat containing sporangiospores of a 3-4 week old colony was gently removed with a sterile pair of tweezers from a 1 cm^2 agar surface and positioned at 1 cm distance to the petri dish wall.

The procedure was adapted according to a modification of the Kirby-Bauer disc diffusion test [52]. Small filter paper disks, with an approximately diameter of 5 mm, were immersed in a highly concentrated toxin solution (100 µg/mL) of MC-LR, BMAA and CYN respectively. Different disks were used per toxin and placed onto separate agar plates for the inhibition zone assay studies. Water was used as a negative control, and antimicotic (±)-miconazole nitrate salt as a positive control. Zone diameters were measured from edge to edge across the zone of inhibition over the center of the disc. The zone of inhibition of fungal growth is used as a measure of susceptibility. Large zones of inhibition indicate that the organism is susceptible (S), while small or no zone of inhibition indicate resistance (R). Three independent replicates were performed.

2.2.5 MEASUREMENT OF RADIAL GROWTH RATE AND BIOMASS PRODUCTION

Agar plates separately coated with 1 mL of MC-LR, BMAA and CYN toxin solution at different concentrations (5, 100, 250, 500 and 1000 µg/L) on malt extract agar were prepared. Five mm diameter mycelial disks were taken from a 3-4 week old grown culture and transferred onto the plates. The plates were incubated at 25 °C in the dark.

Radial extension was marked at intervals of 24 hours for a seven day period or until the maximum extension was reached. Colony diameters were measured starting from the center of the inoculum. Distance values were expressed as the average of three measurement points of the plate (one middle axis and the left and right axes at an angle of 45 °). The radial growth rate was calculated by linear regression of the colony radius versus time.

For biomass production evaluation, the dry weights were determined. The fungal colony was lifted out of the plate and transferred with the agar gel into 50 mL tubes filled with distilled water. The tubes were heated up to 110 °C for 30 min in the autoclave in order to melt the agar, the content was then immediately poured through a strainer with a mesh size of 0.5 mm and rinsed with distilled water. Intact mycelia were collected on pre-weighted filter papers (Whatman no.1 pore size), dried in the oven at 80 °C until a constant mass was reached (about 20 hours) and cooled to room temperature in a desiccator. Dry weights were noted after seven days of incubation [53].

2.2.6 BIOSORPTION: EXPOSURE SCENARIO AND SAMPLE COLLECTION

Ten days old cultures of *M. hiemalis* EH5 grown on Sabouraud Dextrose Broth (Sigma-Aldrich, Fluka S3306) agar medium in petri dishes at 25 °C in the dark, were exposed to 1 mL of a 1000 µg/L concentrated MC-LR, BMAA and CYN solution separately for 24 and 48 hours under optimal growth conditions. Five replicates were performed and pure

water was used for the untreated controls. Before harvesting, fungal mycelia were washed thoroughly three times with five mL water, followed by one washing step with five mL methanol and repetitively three times with five mL water to completely remove all toxin residues from the plate and the mycelial mat surface. Then, mycelia were collected with a pair of tweezers, snap frozen in liquid nitrogen and lyophilized overnight (-48.3 °C, 0.1163 mbar).

2.2.7 TOXIN EXTRACTION

Disruption and homogenization of the vegetative part of *M. hiemalis* EH5 was achieved by Ultra-Turrax treatment (25,000 rpm, for max. 30 s) followed by glass potter grinding of the lyophilized mycelia (20-50 mg dw) in 1-1.5 mL of the respective disruption/extraction solvent described for each toxin below in this section. As sporangiospores were resistant to the mechanical disruption method used, only the toxin content in the mycelia of the fungi could be determined.

MC-LR was extracted with 0.1 % trifluoroacetic acid (TFA) in 70 % methanol. Each mycelial homogenate was sonicated for two hours in a water bath, shaken for 45 min and centrifuged (4000 x *g* for 10 min). The supernatant was collected and the pellet re-suspended in 500 µL of the extraction solvent. The shaking and centrifuging cycle was performed three times in total. The combined extracts were evaporated to dryness at 30 °C in a vacuum concentrator and the obtained dried fractions were re-dissolved in 250 µL methanol 100 % (MS grade) and centrifuged before insertion to the HPLC-MS/MS system.

BMAA was extracted following the protocol applied for BMAA extraction from cyanobacterial isolates [54] by sonication with 0.1 M trichloroacetic acid (TCA) for one hour in a water bath. Free BMAA was obtained in the supernatant after centrifugation at 15,800 x *g* for 3 min at 4 °C to precipitate proteins. The pellet was washed twice with 250 µL of 0.1 M TCA, and all supernatants were combined. In order to release protein-bound BMAA, the pellet was suspended in 1 mL 6 M hydrochloric acid and hydrolyzed overnight. BMAA was derivatized prior to HPLC-MS/MS analysis using the Phenomonex EZ:faast^{TM} kit following the manufacturers specifications. The derivatization involves a concentration step on a sorbent tip, washing, elution from and removal of the stationary phase, and derivatization with a proprietary chloroformate derivative as well as sample clean-up *via* liquid-liquid extraction and evaporation of the organic solvent under a gentle stream of nitrogen. The remaining dried amino acid derivatives were re-dissolved in a mixture of LC mobile phase components (water/methanol, 3.2:6.8).

A modification of the method published by Welker et al. [55] has been applied for CYN extraction and pure water has proven to efficiently extract CYN from mycelial material. The

homogenized mycelial water suspension was sonicated for 15 min in a water bath, shaken for one hour, sonicated again and centrifuged for 15 min at 4000 x *g*. The combined supernatants were dried in a vacuum concentrator and the dried fractions re-suspended in a mixture of acetonitrile and water (95:5). All steps were performed in the dark, as CYN is light sensitive.

2.2.8 LC-MS/MS

The chromatographic separation of MC-LR was accomplished with a Kintex C18 column (2.6 µm, 2.1 x 50 mm) on an Agilent 1200 Infinity Series liquid chromatography system coupled to an Agilent Technologies 6460 Triple Q^{TM}. The column oven temperature was set to 40 °C and the injection volume used was 10 µL. A flow rate of 0.2 mL/min was used during the analysis, using a solvent gradient with 0.1 % TFA in H_2O (MS grade, mobile phase A) and 0.1 % TFA in acetonitrile (MS grade, mobile phase B) for separation. At the start of the run, mobile phase B was increased from 0 to 35 % over 3 min followed by an increase to 65 % until 3.75 min, abidance at this condition was held for 5 min, concluding with a post time of 3 minutes. The retention time of MC-LR was 6.15 min. For the subsequent MS–MS detection, the MRM mode (positive mode) was used with a mass transfer of 995.5 (Q1) and 135, 213 and 379 (Q3) for MC-LR. Method calibrations were linear ($R^2 = 0.999$) between 0.01 and 100 µg/L, with a lower limit of quantification being 2 pg on column.

Derivatized BMAA and internal standards were chromatographically separated on a Phenomenex AAA-MS amino acid analysis column (2.0 x 250 mm, included in the kit) (on the same equipment) at a column oven temperature of 35 °C. The sample injection volume was 1 µL at a flow rate of 0.25 mL/min using the following solvent gradient with 10 mM ammonium formate in H_2O (MS grade, mobile phase A) and 10 mM ammonium formate in MeOH (MS grade, mobile phase B): mobile phase B was increased from 68 to 83 % within 13 min followed by an immediate decrease to 68 % mobile phase B and an abidance at this condition until 17 min. The retention time of the derivatized BMAA was determined as 8.2 min. For the subsequent MS detection the MRM mode (positive mode) will be used with *m/z* of 333 (Q1, derivatized BMAA) measuring the transition to product ions *m/z* 273 and 245. Calibration was linear ($R^2 = 0.999$) between 1 and 100 µg/L. Limit of detection (LOD) was 1 ng/mL derivatized BMAA. Quantification of derivatized BMAA was conducted with the internal standard homo-arginine (included in the kit) allowing the consideration of derivatization efficiency [54,10].

Chromatographic separation of CYN was achieved with the Kinetex HILIC column (2.6 µm, 2.1 x 100 mm) on the same equipment as for the MC-LR. The column oven temperature was set to 35 °C and the injection volume used was 20 µL at a flow rate of 0.5 mL/min. A gradient

elusion was used starting at 95 % acetonitrile (MS grade) for 5 minutes which was then decreased to 50 % over 3 minutes with a post time of 2 minutes, resulting in a retention time of 4.2 min for CYN. For the subsequent MS–MS detection the MRM mode (positive mode) will be used with a mass transfer of 416 (Q1) and 176 and 194 (Q3) for CYN. Calibrations for this method were linear ($R^2 = 0.998$) between 0.01 and 100 µg/L with a LOD of 2 pg on column.

2.2.9 STATISTICAL ANALYSIS

The effect of MC-LR, BMAA and CYN on the growth and biosorption potential of *M. hiemalis* EH5 was analyzed using the SPSS Statistics software ($\alpha = 0.05$, 95 % CI). A Shapiro-Wilk's test ($p > 0.05$) showed that the values (radial growth extension, dry weight and uptake) were with some exceptions (explained below) normally distributed, and ANOVA tables of different responses (radial growth extension, dry weight and uptake) were used to evaluate the factors ($p > 0.05$). Data, which did not follow a normal distribution (growth at day seven and biomass determined *via* dry weight of CYN 250 µg/L), was analyzed with non-parametric tests, such as the Kruskal-Wallis and Mann-Whitney-U-test ($p > 0.05$). Uptake was tested with the t-test ($p < 0.05$).

2.3 RESULTS AND DISCUSSION

The removal of cyanobacterial toxins from water bodies is fundamental to maintain human and ecosystem health. This research focuses on three cyanotoxins, which occur worldwide and differ in their mode of action: the hepatotoxin MC-LR, the neurotoxin BMAA and the cytotoxin CYN. As a first step, inhibition zone assays were conducted to study the sensitivity of *M. hiemalis* EH5 towards MC-LR, BMAA and CYN. Data of three independent replicates and photos taken after three days of incubation are shown in Table 2-1 and Fig. 2-2 (A-C show 100 µg/L MC-LR, BMAA, and CYN, respectively). *M. hiemalis* EH5 appears to be resistant to all of the cyanotoxins tested, which is underlined by the lack of inhibition zone formation (Fig. 2-2 A-C) compared to the positive control antimicotic (±)-miconazole nitrate salt (Fig. 2-2 D). In A-C the fungus shows fast adaptation and clearly grows over the toxic impregnated filter zones without expressing any sensitivity towards MC-LR, BMAA, or CYN. D shows an example of the positive control, where a clear zone formation of 14.3 ± 0.6 mm was observed.

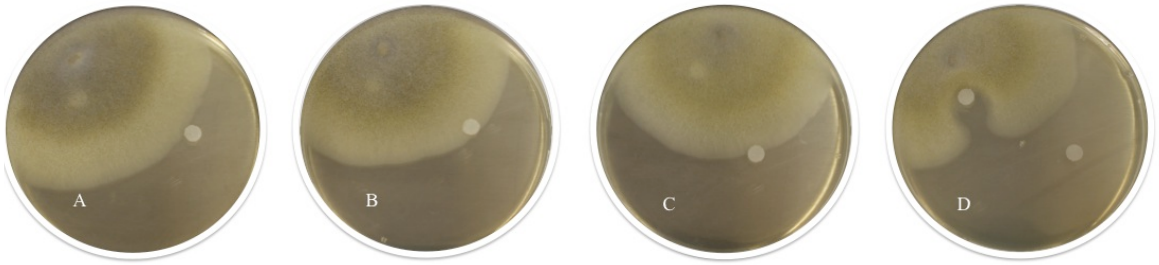

Fig. 2-2: Inhibition zone assay as an adaptation of the Kirby-Bauer disk diffusion test of the cyanobacterial toxins MC-LR (A), BMAA (B), and CYN (C) compared to the control (±)-miconazole nitrate salt (C).

Table 2-1: Zone diameter of the inhibition zone assays and characterization of susceptibility. Data are means ± SE. Water was used as a negative control and (±)-miconazole nitrate salt as a positive control (R = resistant, S = susceptible).

	Zone diameter [mm]	**Susceptibility**
MC-LR	0	R
BMAA	0	R
CYN	0	R
Negative control	0	R
Positive control	14.3±0.6	S

This behavior gives evidence that fungal growth is not affected and that *M. hiemalis* EH5 is able to grow in the presence of the tested cyanobacterial toxins.

Growth characteristics were examined in more detail in petri dishes, where the agar surface was coated homogenously and separately with the pure MC-LR, BMAA and CYN toxin solution in order to elucidate the effect on the growth rate constants of *M. hiemalis* EH5 in the presence of MC-LR, BMAA and CYN at various ecologically relevant concentrations [56]. The maximum concentration was chosen according to the highest cyanotoxin concentration reported in the blooms in Lake Chaohu, China [57] and the lower concentration related to the lower doses occurring in German fresh waters (up to 119 µg/L of MC-LR in Berlin water bodies) [56].

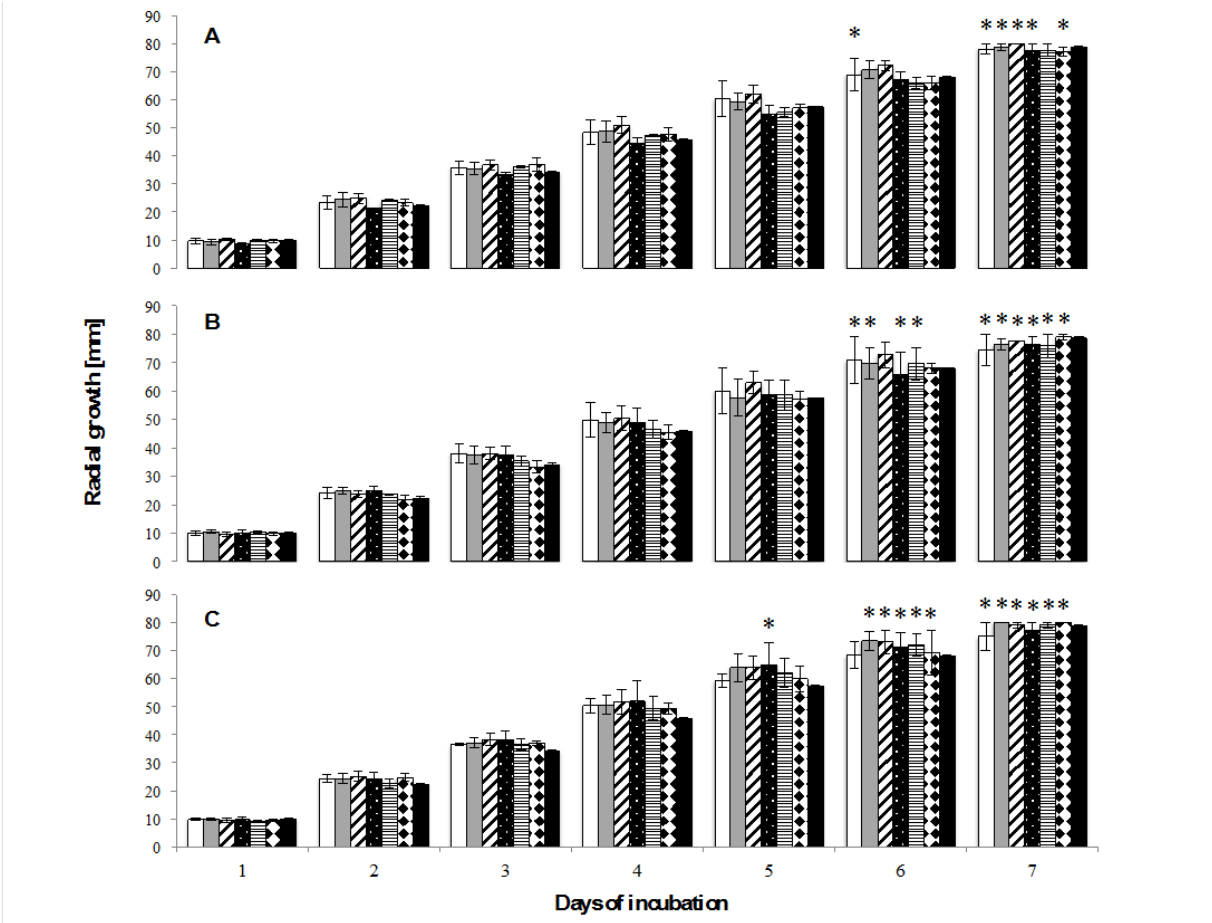

Fig. 2-3: Concentration-dependent growth kinetic of *M. hiemalis* EH5 in the presence of the cyanobacterial toxins MC-LR (A), BMAA (B) and CYN (C) at different concentrations (bars are white 5 µg/L, grey 50 µg/L, diagonally striped 100 µg/L, dotted 250 µg/L, horizontally striped 500 µg/L, squared 1000 µg/L and black control). Data are means ± SE of three independent replicates. * indicate maximal periphery, where the maximum distance of 80 mm was reached, and this value was used for calculations and graphical illustration. No statistical differences were observed when comparing concentration/toxin to the control at each time point ($p > 0.05$).

The radial extension of the culture was monitored daily and plotted versus time. White-grey colonies of *M. hiemalis* EH5 were expanding circularly at constant rates of 11.2 ± 0.5 mm per day. The linear growth profiles are shown in Fig. 2-3A-C.

Data, expressed as the distance in mm, were analyzed at all time points separately for each concentration of the individual toxin compared to the control (without addition of cyanobacterial toxins). Within the whole concentration range (5 µg/L - 1000 µg/L), no significant difference was observed (ANOVA, $p > 0.05$) at each time point, showing that the growth of *M. hiemalis* EH5 was not negatively affected by exposure of the fungus to increasing concentrations of MC-LR, BMAA, and CYN. Even at maximum exposure concentration, the fungus continued to grow and no toxic impact was observed. Where maximum extension was reached before the end of the experiment, the distance value from the inoculum to the plate wall (80 mm) was taken for calculations and graphical illustration; the bars are marked with stars in Fig. 2-3A-C.

To reveal more precise information about the effect of cyanobacterial toxins on mycelial development and productivity, aerial growth was considered as well. Additionally to lateral growth, the biomass production was determined *via* dry gravimetric analysis.

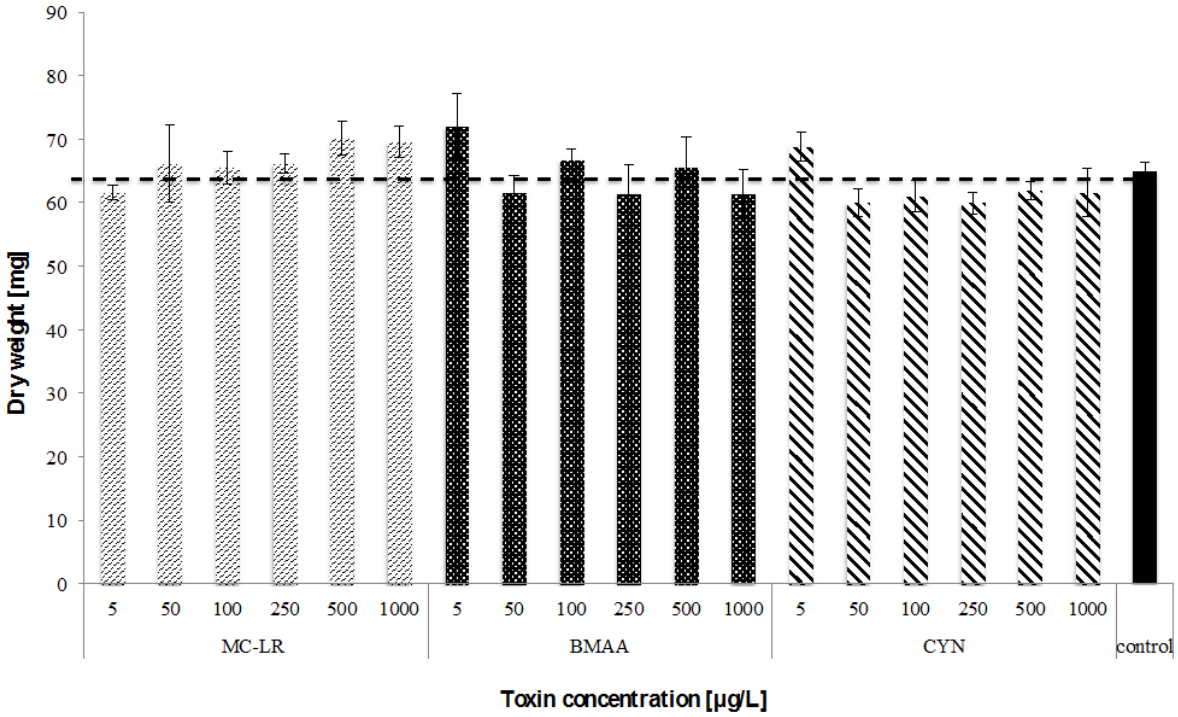

Fig. 2-4: Biomass production expressed as dry weights after one week of exposure. Data are means ± SE of three independent replicates. No statistical differences were observed when comparing concentration/toxin to the control ($p > 0.05$).

After seven days, when the fungal colonies had reached diameters of between 70 and 80 mm, dry weights were recorded. Statistical Shapiro tests showed normal distribution of the dry weight values, with the exception of CYN at 250 µg/L. The obtained data set was analyzed with ANOVA, and the evaluated toxin and concentration dependent responses did not show any significant deviations compared to the control ($p > 0.05$). For CYN at 250 µg/L, the non-paramagnetic Mann-Whitney-U-test evidenced the same hypothesis ($p > 0.05$) when comparing the data set to the control.

The cyanobacterial toxins neither showed to affect biomass production of *M. hiemalis* EH5 nor to negatively influence mycelial growth after a seven day exposure (Fig. 2-4).

Concentration-depending dry weight results were tested for all the toxins independently proving that there is no significant deviation from the control within the whole concentration range (ANOVA, $p > 0.05$ and Kruskal-Wallis/Mann-Whitney-U-test, $p > 0.05$).

These results indicate high tolerance to MC-LR, BMAA and CYN of the aquatic fungus, possibly depending on the capacity to degrade the toxins by specific enzymes.

Uptake experiments were conducted to examine biosorption capacity and possible fungal-toxin interactions. The extraction methods, which were applied to isolate the cyanobacterial toxins from the fungal cells, yielded adequate recoveries, with recoveries for MC-LR being above 60 % and CYN above 85 %. BMAA extraction and derivatization was adapted from Esterhuizen and Downing [14] using the EZfaast^{TM} amino acid analysis kit for LC/MS (Phenomenex).

An appropriate amount of mycelial biomass was obtained upon growing cultures of *M. hiemalis* EH5 for ten days under optimal growth conditions. *M. hiemalis* EH5 controls not exposed to MC-LR, BMAA and CYN contained no cyanobacterial toxin after a period of 24 and 48 h (Fig. 2-5). The highest used concentration from the growth experiment (1000 µg/L) was applied as the exposure dosage with an exposure time of 24 and 48 h in five replicates for each toxin separately. All the three cyanobacterial toxins tested were taken up by *M. hiemalis* EH5 (Fig. 2-5) showing good biosorption capacity. After 24 h exposure time, toxin levels were observed in the milligram per gram range and were still detected after 48 h in the mycelium of *M. hiemalis* EH5. However, no time-dependent uptake was observed, and therefore no statement on the rate of uptake/efflux and possible bioaccumulation can be made. After 24 h exposure, a maximum amount of 0.58 mg MC-LR per gram mycelial biomass (dw) was taken up in the vegetative part of *M. hiemalis* EH5, the BMAA and CYN level detected was 0.22 and 0.13 mg toxin/g dw respectively. After a prolonged exposure of 48 h, no significant alterations in the biosorption pattern were observed, when the data for each single

toxin was compared at the different exposure times ($p > 0.05$). However, mycelial biomass exposed to MC-LR was found to contain significantly greater toxin concentrations than mycelia exposed to BMAA and CYN respectively ($p < 0.05$), whereas between the BMAA and CYN uptake extent, no significant difference was observed ($p > 0.05$). The uptake requires that the toxin penetrates the fungal cell and its efficiency is attributed to the specific structure of the cell wall with chitin and chitosan as main constituents in the cell walls of fungal organisms. Depending on their chemical properties, molecules may be absorbed by either passive diffusion or active transport. The hydrophobicity of MC-LR may in part be a possible explanation for the enhanced toxin uptake in *M. hiemalis* EH5 mycelial cells, as the chitin cell wall is highly hydrophobic, and hydrophobic molecules may therefore have a facilitated entrance into the cells as they may interact more easily with the lipophilic cell wall. BMAA is highly hydrophilic, but as a small molecule consisting of only one amino acid, it may be taken up by simple diffusion or alternatively be transported into the cell by one of the amino acid carriers; the similarity of BMAA to glutamate connected with its agonistic activity on the glutamate receptor has been reported and may explain a possible uptake route for the toxin in fungal mycelia. As BMAA exists in plasma in several forms (neutral, zwitterion, tripolar cation, and α- and β-carbamate), it is possible that each of the species may express affinity for one or more carriers. In contrast to (aquatic) plant and animal uptake mechanisms [16], no mechanism of association of BMAA with proteins was observed in the aquatic fungus, suggesting a different mode of uptake possibly accompanied by a lower toxicity on the fungal organism, as BMAA will not accumulate and be incorporated into cellular proteins. BMAA protein incorporation in mycelia might be prevented by activated defense mechanisms of *M. hiemalis* EH5, however, this needs to be further investigated, because the assumption of an insufficient exposure time should not be rejected either. It is possible that longer turnaround time may be needed. Despite the hydrophilic nature of CYN, making the molecule unlikely to cross cell walls, its small molecular weight makes passive diffusion nevertheless a considerable probability [58].

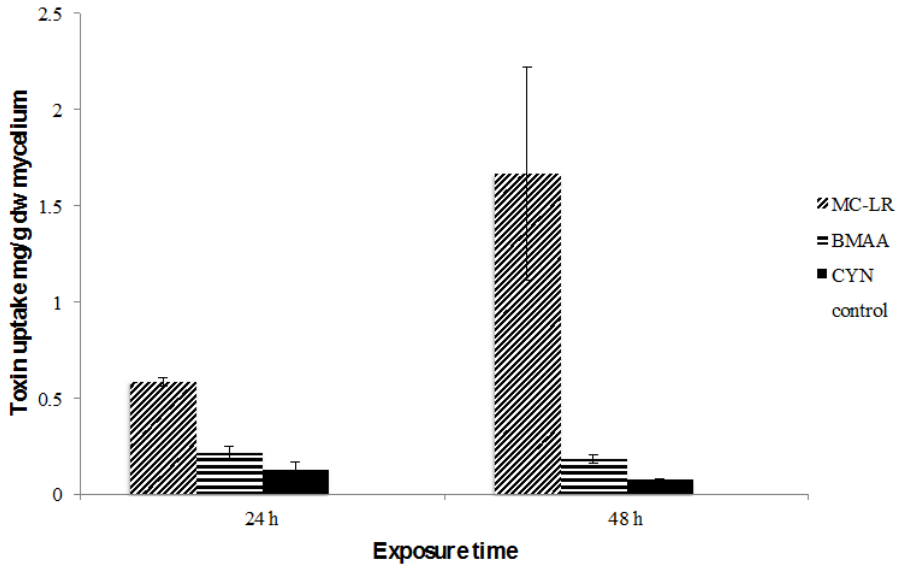

Fig. 2-5: Total toxin uptake by *M. hiemalis* EH5 expressed as milligram toxin per gram dry weight of lyophilized mycelial biomass after 24 and 48 h exposure to 1000 µg/L MC-LR, BMAA and CYN. Data are means ± SE (n=5).

Because of the resistance of the fungal sporangiospores to the mechanical disruption methods used in this experiment, the amounts of the cyanotoxins quantified by LC-MS/MS are only attributed to the uptake of MC-LR, BMAA and CYN in the vegetative mycelial part of the aquatic fungi (Table 2-2). This is the first report of an uptake study of cyanobacterial toxins by aquatic fungi. Its results are very promising as the biosorption capability of *M. hiemalis* EH5 lies in the range and in some cases exceeds the uptake potential of several aquatic plants, which have been previously reported to effectively accumulate significant amounts of cyanobacterial toxins. An uptake of between 0.6 % and 1.75 % of the applied radiolabeled MC-LR has been shown in the three rooted aquatic plants *C. demersum*, *Elodea canadensis*, and *Vesicularia dubyana* [59]. In comparison, a higher uptake percentage was observed in *M. hiemalis* EH5, which showed an uptake of between 1.95 to 2.9 % of the applied MC-LR. Another study demonstrated MC-LR uptake in *Lemna minor* (*L. minor*) and *Chladophora fracta* (*C. fracta*) [60]. *L. minor* took up 0.288 ± 0.009 mg/g ww after a 5 d exposure to MC-LR but with a 20 fold higher treatment dosage used. *C. fracta* reached a maximum uptake of 0.042 ± 0.015 mg/g ww but when a six fold treatment dosage was applied. When comparison is restricted to the results obtained with the lower concentration, which was still three fold higher than the concentration applied to *M. hiemalis* EH5 in the present study, MC-LR uptake levels detected were 0.046 ± 0.007 mg/g ww in *L. minor* and 0.041 ± 0.016 mg/g ww in *C. fracta*, which under these relative comparable concentration conditions are both substantially lower than the MC-LR content taken up by

M. hiemalis EH5. The submerged macrophyte *Vallisneria natans* took up a maximum of 0.013.63 ± 0.00342 mg/g dw when exposed for 14 d to 25 µg/L MC-LR.

BMAA uptake by the aquatic fungus *M. hiemalis* EH5 was distinctly higher than by the model aquatic plant *C. demersum*. Interestingly, as stated above no protein-associated form was detected in *M. hiemalis* EH5, thereby showing a difference between fungal and (aquatic) plant or animal biosorption characteristics of BMAA. Both free and protein-bound BMAA was found in aquatic plants, e.g. *C. demersum* [54], *Fontinalis antipyretica*, *Riccia fluitans*, and *Lomariopsis lineata* [10], in wheat *Triticum aestivum* [61] and in animals, e.g. freshwater mussels [62] and rats [63].

Cyanobacterial toxins are effectively taken up by *M. hiemalis* EH5 and are so no longer available to food chain. More particularly, the toxins could be broken down in situ or ex situ and by this means eliminated completely by *M. hiemalis* EH5, and lasting removed from contaminated waters.

Table 2-2: Mycelial concentration (mg per g dw ± SE) of MC-LR, BMAA (free and protein-bound) and CYN after exposure of *M. hiemalis* EH5 to 1000 µg/L for 24 and 48 hours. Values are means ± SE. Significant differences (ANOVA, t-test, $p < 0.05$) between treatment and control of both sampling dates with no time-dependency ($p > 0.05$) were observed. *ND – not detected.

	Exposure time/concentration	24 h	48 h
MC-LR	1 µg/mL	0.58 ± 0.02	1.67 ± 0.55
BMAA free	1 µg/mL	0.22 ± 0.03	0.18 ± 0.02
BMAA protein-bound		0	0
CYN	1 µg/mL	0.13 ± 0.04	0.075 ± 0.01
Untreated control	0 µg/mL	ND*	ND*

In summary, this study showed that the three tested cyanobacterial toxins have no toxic impact on the aquatic fungus *M. hiemalis* EH5 and that the organism can easily grow in their presence. No decrease in growth and biomass production was observed in *M. hiemalis* EH5 cultures exposed to MC-LR, BMAA and CYN for up to seven days at concentrations ranging from 5-1000 µg/L. Moreover, rapid and significant MC-LR, BMAA and CYN uptake by *M. hiemalis* EH5 was demonstrated. These results constitute the first report of uptake of cyanobacterial toxins by an aquatic fungus. The ability to adapt to the toxic perturbation upon biosorption indicates a strong resistance of the water fungus *M. hiemalis* EH5, which is a prerequisite for its use as a bioremediation organism. Previous studies have shown the ability

of *M. hiemalis* EH5 to breakdown the herbicide isoproturon. Possible sites to be attacked were C-C and C-N bonds [47], which are also present in MC-LR, BMAA and CYN (structures are shown in Fig. 2-1). Therefore, it is possible, that *M. hiemalis* EH5 might be able to breakdown the structures of the cyanobacterial toxins as well and use the metabolites as a carbon source, which makes *M. hiemalis* EH5 an ideal organism to be tested for mycoremediation purposes. The utilization of the water fungus, which acts based on simple ecosystem functions in its natural habitat, might be a breakthrough in the field of biodegradation and removal of cyanobacterial toxins from contaminated water bodies and could offer an environmentally friendly and sustainable elimination process. The aquatic fungus has shown to be resistant to high toxin concentrations; this together with its known effectiveness even under extreme environmental conditions and low temperatures [47] enables a universal and season independent application. Future studies are suggested to examine metabolism and degradation ability of *M. hiemalis* EH5 towards cyanobacterial toxins.

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REFERENCES

1. Whitton BA (2012) Ecology of Cyanobacteria II. XV, Springer: 760.
2. Wiegand C, Pflugmacher S (2005) Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. Toxicol Appl Pharmacol 203: 201-218.
3. Saqrane S, Ghazali IE, Ouahid Y, Hassni ME, Hadrami IE, et al. (2007) Phytotoxic effects of cyanobacteria extract on the aquatic plant *Lemna gibba*: Microcystin accumulation, detoxication and oxidative stress induction. Aquatic Toxicol 83: 284-294.
4. de Figueiredo DR, Azeiteiro UM, Esteves SM, Gonçalves FJM, Pereira MJ (2004) Microcystin-producing blooms—a serious global public health issue. Ecotoxicol Environ Saf 59: 151-163.
5. Merel S, Walker D, Chicana R, Snyder S, Baurès E, et al. (2013) State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environ Int 59: 303-327.

6. Saker ML, Griffiths DJ (2000) The effect of temperature on growth and cylindrospermopsin content of seven isolates of *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) from water bodies in northern Australia. Phycologia 39: 349-354.
7. Pflugmacher S (2002) Possible allelopathic effects of cyanotoxins, with reference to microcystin-LR, in aquatic ecosystems. Environ Toxicol 17: 407-413.
8. Nogueira ICG, Saker ML, Pflugmacher S, Wiegand C, Vasconcelos VM (2004) Toxicity of the cyanobacterium *Cylindrospermopsis raciborskii* to *Daphnia magna*. Environ Toxicol 19: 453-459.
9. Esterhuizen-Londt M, Pflugmacher S, Downing TG (2011) The effect of β-*N*-methylamino-L-alanine (BMAA) on oxidative stress response enzymes of the macrophyte *Ceratophyllum demersum*. Toxicon 57: 803-810.
10. Contardo-Jara V, Sebastian Funke M, Peuthert A, Pflugmacher S (2013) β-*N*-Methylamino-L-alanine exposure alters defense against oxidative stress in aquatic plants *Lomariopsis lineata*, *Fontinalis antipyretica*, *Riccia fluitans* and *Taxiphyllum barbieri*. Ecotoxicol Environ Saf 88: 72-78.
11. Funari E, Testai E (2008) Human Health Risk Assessment Related to Cyanotoxins Exposure. Crit Rev Toxicol 38: 97-125.
12. Imanishi S, Kato H, Mizuno M, Tsuji K, Harada Ki (2005) Bacterial Degradation of Microcystins and Nodularin. Chem Res Toxicol 18: 591-598.
13. Cox PA, Banack SA, Murch SJ, Rasmussen U, Tien G, et al. (2005) Diverse taxa of cyanobacteria produce β-*N*-methylamino-L-alanine, a neurotoxic amino acid. Proc Natl Acad Sci USA 102: 5074-5078.
14. Esterhuizen M, Downing TG (2008) β-*N*-Methylamino-L-alanine (BMAA) in novel South African cyanobacterial isolates. Ecotoxicol Environ Saf 71: 309-313.
15. Metcalf JS, Banack SA, Lindsay J, Morrison LF, Cox PA (2008) Co-occurrence of β-*N*-methylamino-L-alanine, a neurotoxic amino acid with other cyanobacterial toxins in British waterbodies, 1990–2004. Environ Microbiol 10: 702-708.
16. Murch SJ, Cox PA, Banack SA (2004) A mechanism for slow release of biomagnified cyanobacterial neurotoxins and neurodegenerative disease in Guam. Proc Natl Acad Scie USA 101: 12228-12231.
17. Lobner D, Piana PMT, Salous AK, Peoples RW (2007) β-*N*-Methylamino-L-alanine enhances neurotoxicity through multiple mechanisms. Neurobiol Dis 25: 360-366.

18. Liu X, Rush T, Zapata J, Lobner D (2009) β-*N*-Methylamino-L-alanine induces oxidative stress and glutamate release through action on system Xc−. Exp Neurol 217: 429-433.
19. Humpage AR, Fontaine F, Froscio S, Burcham P, Falconer IR (2005) Cylindrospermopsin Genotoxicity and Cytotoxicity: Role Of Cytochrome P-450 and Oxidative Stress. J Toxicol Environ Health A 68: 739-753.
20. Saker ML, Thomas AD, Norton JH (1999) Cattle mortality attributed to the toxic cyanobacterium *Cylindrospermopsis raciborskii* in an outback region of North Queensland. Environ Toxicol 14: 179-182.
21. Codd GA (1995) Cyanobacterial toxins: Occurrence, properties and biological significance. Water Science and Technology 32: 149-156.
22. Edwards C, Lawton LA (2009) Chapter 4 Bioremediation of Cyanotoxins. Vol. 67, Allen I. Laskin, S.S., Geoffrey, M.G., Eds. In: Advances in Applied Microbiology, Academic Press 109-129.
23. Ho L, Sawade E, Newcombe G (2012) Biological treatment options for cyanobacteria metabolite removal - A review. Water Res 46: 1536-1548.
24. Mullen MD, Wolf DC, Beveridge TJ, Bailey GW (1992) Sorption of heavy metals by the soil fungi *Aspergillus niger* and *Mucor rouxii*. Soil Biol Biochem 24: 129-135.
25. Yan G, Viraraghavan T (2003) Heavy-metal removal from aqueous solution by fungus *Mucor rouxii*. Water Res 37: 4486-4496.
26. Wang J, Chen C (2006) Biosorption of heavy metals by *Saccharomyces cerevisiae*: A review. Biotechnol Adv 24: 427-451.
27. Luo Jm, Xiao X, Luo sl (2010) Biosorption of cadmium(II) from aqueous solutions by industrial fungus *Rhizopus cohnii*. Transactions of Nonferrous Metals Society of China 20: 1104-1111.
28. Huang Y, Zhao X, Luan S (2007) Uptake and biodegradation of DDT by 4 ectomycorrhizal fungi. Sci Total Environ 385: 235-241.
29. Wang Y, Yu J (1998) Adsorption and degreadation of synthetic dyes on the mycelium of *Trametes versicolor*. Water Sci Technol 38: 233-238.
30. Sugimori D, Banzawa R, Kurozumi M, Okura I (1999) Removal of disperse dyes by the fungus *Cunninghamella polymorpha*. J Biosci Bioeng 87: 252-254.
31. Kaushik P, Malik A (2009) Fungal dye decolourization: Recent advances and future potential. Environ Int 35: 127-141.
32. Fu Y, Viraraghavan T (2002) Dye biosorption sites in *Aspergillus niger*. Bioresource Technology 82: 139-145.

33. Chang HM, Joyce TW, Kirk TK (1987) Process of treating effluent from a pulp or papermaking operation. US 4655926 A.
34. Lewandowski GA, Armenante PM, Pak D (1990) Reactor design for hazardous waste treatment using a white rot fungus. Wat. Res. 24: 75-82.
35. Jia, Y., Han, G., Wang, C., Guo, P., Jiang, W., Li, X., Tian, X., 2010. The efficacy and mechanisms of fungal suppression of freshwater harmful algal bloom species. Journal of Hazardous Materials 183 (1–3), 176-181.
36. Wang, Q., Su, M., Zhu, W., Li, X., Jia, Y., Guo, P., Chen, Z., Jiang, W., Tian, X., 2010. Growth inhibition of *Microcystis aeruginosa* by white-rot fungus *Lopharia spadicea*. Water Science & Technology 62 (2), 317-323.
37. Jia Y, Wang C, Zhao G, Guo P, Tian X (2011) The possibility of using cyanobacterial bloom materials as a medium for white rot fungi. Lett Appl Microbiol 54: 96-101.
38. Mohamed ZA, Hashem M, Alamri SA (2014) Growth inhibition of the cyanobacterium *Microcystis aeruginosa* and degradation of its microcystin toxins by the fungus *Trichoderma citrinoviride*. Toxicon 86: 51-58.
39. Cousins IT, Bealing DJ, James HA, Sutton A (1996) Biodegradation of microcystin-LR by indigenous mixed bacterial populations. Water Research 30: 481-485.
40. Christoffersen K, Lyck S, Winding A (2002) Microbial activity and bacterial community structure during degradation of microcystins. Aquat Micro Ecol 27: 125-136.
41. Pointing SB (2001) Feasibility of bioremediation by white-rot fungi. Appl Microbiol Biotechnol 57: 20-33.
42. Mansur M, Arias ME, Copa-Patiño JL, Flärdh M, González AE (2003) The white-rot fungus *Pleurotus ostreatus* secretes laccase isozymes with different substrate specificities. Mycologia 95: 1013-1020.
43. Bumpus JA, Aust SD (1987) Biodegradation of environmental pollutants by the white-rot fungus *Phanerochaete chrysosporium*: Involvement of the lignin degrading system. BioEssays 6: 166-170.
44. Barr DP, Aust SD (1994) Mechanisms white rot fungi use to degrade pollutants. Environ Sci Technol 28: 78A-87A.
45. Hoque E, Pflugmacher S, Fritscher J, Wolf M (2007) Induction of Glutathione *S*-Transferase in Biofilms and Germinating Spores of *Mucor hiemalis* Strain EH5 from Cold Sulfidic Spring Waters. Appl Environ Microbiol 73: 2697-2707.
46. Phae CG, Shoda M (1991) A new fungus which degrades hydrogen sulfide, methanethiol, dimethyl sulfide and dimethyl disulfide. Biotech Lett 13: 375-380.

47. Hoque E (2003) Process for the degradation of xenobiotics by fungi with monooxygenase/dioxygenase activity in the presence of fungi with glutathione *S*-transferase
48. Tewari N, Vasudevan P, Guha BK (2005) Study on biosorption of Cr(VI) by *Mucor hiemalis*. Biochem Eng J 23: 185-192.
49. Shroff KA, Vaidya VK (2011) Kinetics and equilibrium studies on biosorption of nickel from aqueous solution by dead fungal biomass of *Mucor hiemalis*. Chem Eng J 171: 1234-1245.
50. Fritscher J, Hoque E, Stöckl M (2004) Verfahren zur Beseitigung von Schwermetallen aus wässrigen Medien durch kompatible *Mucor hiemalis* Stämme. DE102004020836.
51. Hoque E (2002) Microbial decomposition of xenobiotics, useful especially for degrading isoproturon, uses mixture of fungi with oxygenase and glutathione *S*-transferase activities. DE10125365A1.
52. Bauer AW, Kirby WM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45(4): 493-6.
53. Reeslev M, Kjøller A (1995) Comparison of biomass dry weights and radial growth rates of fungal colonies on media solidified with different gelling compounds. Appl Environ Microbiol. 61: 4236–4239.
54. Esterhuizen M, Pflugmacher S, Downing TG (2011) β-*N*-Methylamino-L-alanine (BMAA) uptake by the aquatic macrophyte *Ceratophyllum demersum*. Ecotoxicol Environ Saf 74: 74-77.
55. Welker M, Bickel H, Fastner J (2002) HPLC-PDA detection of cylindrospermopsin-opportunities and limits. Water Res 36: 4659-4663.
56. Corbel S, Mougin C, Bouaïcha N (2014) Cyanobacterial toxins: Modes of actions, fate in aquatic and soil ecosystems, phytotoxicity and bioaccumulation in agricultural crops. Chemosphere 96: 1-15.
57. Krüger T, Wiegand C, Kun L, Luckas B, Pflugmacher S (2010) More and more toxins around–analysis of cyanobacterial strains isolated from Lake Chao (Anhui Province, China). Toxicon 56: 1520-1524.
58. Chong MW, Wong BS, Lam PK, Shaw GR, Seawright AA (2002) Toxicity and uptake mechanism of cylindrospermopsin and lophyrotomin in primary rat hepatocytes. Toxicon 40: 205-211.

59. Pflugmacher S, Wiegand C, Beattie KA, Codd GA, Steinberg C (1998) Uptake of the cyanobacterial hepatotoxin microcystin-LR by aquatic macrophytes. J Appl Botany 72: 228-232.
60. Mitrovic SM, Allis O, Furey A, James KJ (2005) Bioaccumulation and harmful effects of microcystin-LR in the aquatic plants *Lemna minor* and *Wolffia arrhiza* and the filamentous alga *Chladophora fracta*. Ecotoxicol Environ Saf 61: 345-352.
61. Contardo-Jara V, Schwanemann T, Pflugmacher S (2014) Uptake of a cyanotoxin, β-*N*-methylamino-L-alanine, by wheat (*Triticum aestivum*). Ecotoxicol Environ Saf 104: 127-131.
62. Downing S, Contardo-Jara V, Pflugmacher S, Downing TG (2014) The fate of the cyanobacterial toxin β-*N*-methylamino-L-alanine in freshwater mussels. Ecotoxicol Environ Saf 101: 51-58.
63. Karlsson O, Jiang L, Andersson M, Ilag LL, Brittebo EB (2014) Protein association of the neurotoxin and non-protein amino acid BMAA (β-*N*-methylamino-L-alanine) in the liver and brain following neonatal administration in rats. Toxicol Lett 226: 1-5.

3 FUNGAL PELLETS AS POTENTIAL TOOLS TO CONTROL WATER POLLUTION: STRATEGIC APPROACH FOR THE PELLETIZATION AND SUBSEQUENT MICROCYSTIN-LR UPTAKE BY *MUCOR HIEMALIS*

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Own contribution:

Literature review on fungal morphology and factors inducing pelletization of various filamentous fungi

Design of experiments and laboratory studies

Fungal culture from petri dish, spore harvest to submerged cultivation

Performing all required laboratory works

Exposure, toxin extraction and sample preparation

Statistical analysis (model suggested from M. E-L) and interpretation of the experimental results

Preparation of the manuscript, which includes all texts and figures

Revision of the manuscript after receiving the reviewer's comments

ABSTRACT

Microcystin-LR is one of the most prevalent and toxic secondary metabolites produced by cyanobacteria worldwide, causing global concerns because of its hazardousness to ecosystems and human health. Green Liver Systems® have been developed to purify contaminated water, however, system capacities need to be extended to allow season- and location independent applications. Therefore, mycoremediation using temperature resistant *Mucor hiemalis* in pellet morphology was considered. In submerged liquid cultures, fungal morphology is species specific and strongly depends on the cultivation environment. One main focus of the present study was the investigation of diverse factors influencing pelletization. Moreover, we translated the pellet product into an immediate application and studied its biosorption ability towards microcystin-LR. Our results showed that pH was a key factor stimulating pellet formation of *M. hiemalis* and that inoculum size played an essential role as well. Final pellet size was limited by the available space in the flask and is therefore directly related to inoculum size. Microcystin-LR was found to be taken up by pelletized *M. hiemalis* as quantified *via* LC-MS/MS measurements. Our results report for the first time optimized pelletization of *M. hiemalis* and cyanotoxin uptake by these fungal pellets in liquid cultures.

Keywords: *Mucor hiemalis*, pelletization, mycoremediation, microcystin-LR, uptake

3.1 INTRODUCTION

In the last decades, concern regarding the negative effects of cyanobacterial toxins on aquatic ecosystems as well as human health has grown worldwide. Microcystins are heptapeptides, which attracted attention not only due to their high acute and chronic toxicities but also due to their global abundance. Microcystin-LR (MC-LR) in particular, is considered the most toxic candidate of this family [1] and is produced by cyanobacterial species belonging to the genera *Anabaena*, *Microcystis*, *Nostoc*, and *Anabaenopsis* [2-5]. It is toxic to the liver, promoting liver tumors in humans [6-10], and showed to be acutely toxic in mice [11]. In the aquatic environment, it can have significantly negative effects on the survival of zooplankton including species of *Daphnia* [12]. Investigations of the effect in different early life stages of zebrafish (*Danio rerio*) showed uptake of the toxin resulting in growth reduction and malformations [13]. Phytotoxic effects were studied in various aquatic plants such as *Lemna gibba* [14, 15] and *Ceratophyllum demersum* [16]; a clear dose-dependent inhibition of macrophyte growth with exposure to low concentrations of MC-LR has been shown. Reduction in growth, rate of photosynthesis and changes in plant pigment composition were observed [17]. The stable cyclic structure of MCs makes conventional water treatment (such as chlorination, chlorine dioxide, and ozonation) and physicochemical techniques a challenge, as the removal capability remains limited [18]. More advanced methods (such as activated carbon in granular or powder form and membrane filtration) result in high costs and therefore, the investigation and development of more effective and low-cost water treatment technologies is of great importance in order to ensure the removal of cyanotoxins from aquatic ecosystems. Green Liver Systems® present a possible way for the purification of cyanotoxin-contaminated water bodies in a cost-effective, sustainable and environmentally friendly manner. The system uses the phytoremediation potential of aquatic submerged plants [19], i.e. the capability to take up contaminants from the water, biotransform them enzymatically and lastly, store them in cell wall fractions, the apoplast or in the vacuoles. Successful results in respect to cyanobacterial toxin removal were obtained in simulating Green Liver Systems® in the laboratory and as well in a small pilot plant in Hefei (Anhui Region, PR China) [20] and Itacuruba (Nordeste Region, Brazil) [19]. Despite the promising remediation capacities of Green Liver Systems®, their application is limited to warm seasons and climates with temperature ranges suitable for plant growth, development and survival. Hence, there is a demanding need to search for alternative bioremediating agents that could be applied season-independently and worldwide for effective purification of cyanotoxin-contaminated water bodies. It is therefore of high scientific interest to investigate the

applicability of further organisms as an alternative to aquatic macrophytes currently used in Green Liver Systems®. Microorganisms that play a major role in decomposition and degradation in the ecosystem, based on natural functions are the fungi. Mycoremediation, as a process of using fungi to degrade contaminants in the environment, offers a wide application area and has raised international interest throughout the last decades. *Via* non-specific extracellular enzymes (peroxidase, manganese peroxidase, laccase), fungi are able to breakdown many persistent complex organic substances, such as lignin and cellulose [21, 22], and are therefore responsible for wood decay. This natural degradation ability of the non-specificity of the fungal enzyme machinery is used for industrial applications to degrade, mineralize and remove other natural or chemical organic pollutants as well [23]. Many white-rot fungi are known to degrade major environmental pollutants such as munitions waste, pesticides, organochlorines, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), synthetic dyes, wood preservatives and synthetic polymers [24]. *Phanerochaete* (*P.*) *chrysosporium* is extensively used as a model for white-rot fungal biodegradation through the involvement of the lignin degrading system. Mineralization of chloraniline/lignin conjugates, free chloraniline and their produced metabolites has been successfully characterized [25, 26]. The fungus proved to remediate atrazine-contaminated soils [27, 28], various PAHs [29], PCBs [30], TNT [31], pentachlorophenol [32], 2,4,5-trichlorophenol [33] and many more. A recent high-tech application is the use of *P. chrysosporium* as a tool in different fungal bioreactor systems for the bleaching in Kraft plant effluents [34], the removal of phenolic compounds from coking wastewater [35], or the decolorization of textile dyes [36, 37].

Overall, the use of fungi may be a promising alternative to plant organisms, however, similar drawbacks are encountered in regard to temperature limitation of white-rot fungi. *P. chrysosporium* for example, has a growth optimum of 30-39 °C [38], therefore, cultivation would lead to energy expenditure in biotechnological processes and would still not be the solution for a universal and season-independent bioremediating system. In order to overcome temperature limitations, the search for an appropriate fungal organism has to be extended. Instead of using fungi with peroxidase enzymes, one can focus on fungi with glutathione *S*-transferase activity. *Mucor hiemalis* is an aquatic fungus that is known to express high levels of extracellular glutathione *S*-transferases [39], which are a class of detoxification enzymes. Its degradation potential has been highlighted in regard to the herbicide isoproturon [40]. Moreover, it displays functional groups on the cell wall surface that enable absorption of heavy metals and is therefore used for the removal of nickel [41], chrome [42], mercury, *etc*.

from ground- and surface water, purification plants, waste water and industrial water [43]. Most importantly, the fungus still sporulates even at ground water temperatures of 5 °C and is resistant to high hydrogen sulfide concentrations [39]. Our previous study showed its resistance to three globally occurring and structural diverse cyanobacterial toxins [44]. Growth and development were not affected in the presence of these toxins and uptake could be detected in the mycelium after exposure. Xenobiotic resistance and good biosorption ability are prerequisites for the use of the fungus as a bioremediating agent. These promising results sustain further research to answer the question: Is *M. hiemalis* a possible alternative for the remediation of cyanotoxins from contaminated waters? Hence, investigations on uptake in submerged liquid cultures have to be undertaken in preliminary laboratory experiments. As the present fundamental research will serve for the development of future fungal bioreactors, it is important to establish systems that are operating with the ideal morphological growth form. Concerning biotechnological applications, it may be useful to develop devices that work with pelletized fungal biomass. The filamentous mycelial growth causes problems in bioreactors as it could interfere with bioreactor components leading to decreased productivity, reduced growth and negatively affecting the potential for bioremediation [45]. Hence, using compact pelletized fungal biomass in bioreactor technology provides many advantages, including decreased broth viscosity, easier separation, improved aeration, stirring, heat transfer, and a larger surface area which reduces the mass transfer limitations and enhances uptake [46, 47]. Many filamentous fungi have the ability to grow in the form of small spherical pellets of intertwined hyphae, however, this morphology has been shown to be species specific and to strongly depend on cultural conditions. Pellet formation has been extensively studied for different filamentous fungi, and many approaches for the pellet formation of *Rhizopus* (*R.*) spp. [48-51] and different species of the genus *Aspergillus* (*A.*) [52-54] have been found. The factors affecting pellet formation are not only genus but also species specific. To the authors' knowledge, pellet formation of only one *Mucor* species has yet been reported [55]. The aim of this work was therefore to investigate the optimal condition for the pelletization of *M. hiemalis*, as to date no information exists on pellet formation of this species and pelletized fungal biomass offers many advantages in biotechnological applications. Investigations of various factors that have been reported to influence fungal morphology, such as temperature, medium composition, pH, inoculum size, additives, agitation rate, volume, or flask shape are presented. Finally, we demonstrate the ability of *M. hiemalis* to incorporate MC-LR into the produced fungal pellets to show uptake ability, which is required for the remediation of toxins from water.

3.2 MATERIALS AND METHODS

3.2.1 MICROORGANISM AND INOCULUM PREPARATION

The fungal strain *M. hiemalis* EH5 (DSM 14200) was previously isolated as an aquatic H_2S-resistant strain from the sulfidic-sulfurous Irnsing spring water biofilms in Bavaria, Germany [56]. *M. hiemalis* cultures were grown on solid malt extract agar substrate as previously reported [44]. Spores were harvested from four-week-old colonies by washing the mycelial surface with sterile distilled water. Suspended sporangiospores were collected in Falcon tubes, centrifuged (5 min, 4000 x *g*) and washed three times with sterile distilled water. The spore concentration was determined using a Neubauer hemocytometer and various concentrations (10^3-10^8 spores/mL) were prepared in sterile distilled water for further inoculation procedures described in section 3.2.3.

3.2.2 CULTURE MEDIUM

Pelletization was tested in three different culture media; (1) nitrogen (N)-limiting medium, adapted from Kirk et al. [57], (2) Sabouraud dextrose broth (SAB) (Sigma-Aldrich) and (3) SAB containing the same vitamin composition as described by Kirk et al. [57]. The vitamin solution consisted of biotin (5 mg/L), folic acid (5 mg/L), thiamine hydrochloride (12.5 mg/L), pyridoxine hydrochloride (12.5 mg/L), cyanocobalamin (2.5 mg/L), nicotinic acid (12.5 mg/L), DL-calcium pantothenate (12.5 mg/L), *p*-aminobenzoic acid (12.5 mg/L) and thioctic acid (12.5 mg/L) (Sigma-Aldrich) [57]; of which 0.5 mL was added to one set of SAB medium per liter. The influence of solid particles was investigated by adding $CaCO_3$ (9 g/L, Sigma-Aldrich) and CaO_2 (0.025%, Sigma-Aldrich), into the culture medium respectively as described in section 3.2.3; and trace metals used consisted of $MgSO_4 \cdot 7\ H_2O$ (25 ppm, Merck), $ZnSO_4 \cdot 7\ H_2O$ (4 ppm, Roth), $FeSO_4 \cdot 7\ H_2O$ (250 ppb, Roth). HCl, NaOH and phosphate-buffered saline (PBS, 10 mM) were used to correct the pH of the media. PBS was adapted as a 1 x solution following the Cold Spring Harbor Protocols instructions [58].

3.2.3 CULTIVATION METHOD

Submerged cultivation of *M. hiemalis* was carried out in Erlenmeyer flasks (100 mL medium) on a rotary shaker (Orbital thermoregulated bath 9006, HT INFORS AG) for a maximum of 21 days in the dark. Spore stocks (10^3-10^8 spores per milliliter) were prepared by dilution and stored in distilled water at 4 °C in the dark until inoculation. The inoculation volume was kept constant (1 mL) resulting in various inoculum sizes (Table 3-1). Triplicates per test experiment were performed to observe the influence of the cultivation conditions on fungal morphology. Parameters are specified and summarized in Table 3-1. First, fungal growth and

morphology using two different inoculum sizes (10^2 and 10^4 spores/mL) were observed in different media types in order to choose the most appropriate medium for further experiments. Temperature, agitation rate and the addition of calcium carbonate, calcium peroxide and trace metals respectively, were tested in a matrix to observe morphological characteristics. A pH range from 4 to 8 and inoculum sizes ranging from 10^1 to 10^6 spores/mL were tested on the effect of morphological growth. Optimal pH was defined, and the effect on the morphology after adjustment with sodium hydroxide, calcium carbonate and PBS was compared. Normal and baffled shaped flasks, and a change in culture volume (100 and 200 mL) were tested to evaluate the influence on pellet formation.

Table 3-1: Parameters and ranges tested that influence the probability of pellet formation of *M. hiemalis* in liquid submerged cultivation.

Parameter tested	Unit	Range
Media type		N-limiting, SAB+V, SAB
Temperature	°C	20, 25, 30, 35, 40
Agitation rate	rpm	110, 125, 130, 180
pH		4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8
Inoculum size	mL^{-1}	10^1, 10^2, 10^3, 10^4, 10^5, 10^6
Additives		$CaCO_3$, CaO_2, trace metals, HCl, NaOH, PBS
Flask shape		normal, baffled
Volume	mL	100, 200

Size variation of pellets was observed by inoculating Erlenmeyer flasks with different amounts of seven-day old pellets (5, 10, 20 and 50 pellets per flask). Flasks containing 100 mL of SAB medium and the specified amounts of pellets, were incubated at 25 °C and constantly shaken at 130 rpm in the dark. Diameters were measured after 24 and 48 hours of incubation.

3.2.4 ANALYTICAL METHODS

The pH of the media was measured using a digital pH meter. The morphological growth form of *M. hiemalis* was observed visually after seven to fourteen days of incubation and was classified into one of four characterized groups: pellets (uniform spherical shaped balls), clumps (non-uniform irregular large aggregates), filaments (freely dispersed mycelia, non-aggregated fungal material), or no growth. Pellet-forming cultures from each flask were dispensed separately in sterile Petri dishes and counted with a sterile pair of tweezers. Biomass was determined as dry weight (dw) after lyophilization (-50.2 °C, 0.1043 mbar for 24 hours in a Lio 5P Freeze-drier, Kambič Laboratorijska oprema d.o.o.).

3.2.5 MC-LR EXTRACTION METHOD

For the determination of extraction recovery from the pellets, a defined aliquot of MC-LR (100 ng) was directly added to 50 mg pelletized fungal biomass (dw). Extraction recovery was investigated in triplicate and the established extraction method was then applied for toxin extraction from exposed fungi. Lyophilized samples were snap-frozen and disrupted mechanically using a TissueLyzer (5 mm beads, 5 min treatment, 50/s) until a fine powder was obtained. Then, 0.1% trifluoroacetic acid in 70% methanol was added (5-10 µL per mg dw) and the suspension was sonicated for 60 minutes in an ultrasonic water bath (Allpax, Gmbh & Co. KG). This was followed by vigorous vortexing for three minutes, followed by centrifugation (15 min, 8000 x *g*). The supernatant was transferred and the remaining pellet re-suspended in half of the volume used at first extraction cycle, repeated twice in total. The remaining pellets were discarded and supernatants were combined and re-centrifuged before analysis on the liquid chromatography tandem mass spectrometer (LC-MS/MS) system. Measurement and quantification of the toxin was performed as previously reported [44].

3.2.6 MC-LR EXPOSURE

In order to examine the biosorption ability of pelletized *M. hiemalis*, seven-day old pellets (with a diameter of 5 mm) were transferred into Erlenmeyer flasks containing 50 mL of SAB medium. Varying pellet numbers (3, 5, 10 and 25 pellets per flask) were exposed to 100 ng/mL MC-LR. The control contained three pellets and no toxin to ensure the absence of MC-LR in the fungal strain. The experiment was conducted in triplicate. Sample collection was performed after 48 hours of incubation on a rotary shaker at 130 rpm, at 25 °C in the dark. Pellets were sieved, washed thoroughly with distilled water, snap-frozen and freeze-dried (-50.2 °C, 0.1043 mbar for 24 hours in a Lio 5P Freeze drier, Kambič Laboratorijska oprema d.o.o.). MC-LR extraction was conducted as described in section 3.2.5. LC-MS/MS quantification was performed as per Balsano et al. [44].

3.2.7 STATISTICAL ANALYSIS

SPSS Statistic software was used for statistical analysis ($\alpha = 0.05$, 95% CI). Multi-variate analysis was performed as an overall multiple linear regression to investigate the effect of all the factors listed in Table 3-1 on the morphological form of *M. hiemalis*. Sectional multiple linear regression was additionally applied in order to investigate especially the effect of pH and inoculum size on the probability of pellet formation. Post-Hoc and t-tests were conducted to analyze uptake and growth results after fungal exposure to MC-LR.

3.3 RESULTS AND DISCUSSION

Morphological characteristics of filamentous fungi grown in liquid culture strongly depend on the culture parameters. The pelletized form of *M. hiemalis* offers many advantages in industrial purposes and may be useful for application in bioreactor technology for the removal of cyanobacterial toxins from contaminated water. Therefore, different factors that may enhance the probability for pellet formation have been studied. A matrix table was established, which correlates all the tested factors with each other to summarize under which conditions pellet formation of *M. hiemalis* was observed (Table 3-2). We show a strategic approach by a stepwise optimization of different factors that influence fungal morphology and subsequently analyze the influence of all the factors on the likelihood to promote pellet formation by an overall multivariate linear regression.

Table 3-2: Matrix table with factor correlation.

		Temperature [°C]					Agitation rate [rpm]				pH									Inoculum size 10^5/mL						Additives*						Flask shape		V [mL]	
		20	25	30	35	40	110	125	130	180	4	4.5	5	5.5	6	6.5	7	7.5	8	1	2	3	4	5	6	A	B	C	D	E	F	n	b	100	200
Temperature [°C]	20																																		
	25																																		
	30																																		
	35																																		
	40																																		
Agitation rate [rpm]	110	✔	✔	✔	×	×																													
	125	✔	✔	✔	×	×																													
	130	✔	✔	✔	×	×																													
	180	✔	✔	✔	×	×																													
pH	4	×	×	×	×	×	×	×	×	nd																									
	4.5	×	×	×	×	×	×	×	×	nd																									
	5	×	×	×	×	×	×	×	×	nd																									
	5.5	×	×	×	×	×	×	×	×	×																									
	6	×	×	×	×	×	×	×	×	nd																									
	6.5	×	×	×	×	×	×	×	×	nd																									
	7	×	×	×	×	×	×	×	×	nd																									
	7.5	✔	✔	✔	×	×	✔	✔	✔	✔																									
	8	✔	✔	×	×	×	×	×	×	nd																									
Inoculum size 10^5/mL	1	×	×	×	×	×	nd	×	×	nd	×	×	×	×	×	×	×	×	×																
	2	×	×	×	×	×	nd	×	×	nd	×	×	×	×	×	×	×	×	×																
	3	✔	✔	✔	✔	✔	✔	✔	✔	✔	×	×	×	×	×	×	×	✔	×																
	4	×	×	×	×	×	×	×	×	nd	×	×	×	×	×	×	×	×	×																
	5	×	×	×	×	×	×	×	×	nd	×	×	×	×	×	×	×	×	×																
	6	×	×	×	×	×	×	×	×	nd	×	×	×	×	×	×	×	×	×																
Additives*	A	×	×	×	×	×	×	×	×	nd	nd	nd	nd	nd	nd	nd	nd	×	nd	×	×	×	×	×	×										
	B	×	×	×	×	×	×	×	×	nd	nd	nd	nd	nd	nd	×	nd	nd	nd	×	×	×	×	×	×										
	C	×	×	×	×	×	×	×	×	nd	nd	nd	nd	×	nd	nd	nd	nd	nd	×	×	×	×	×	×										
	D	×	×	×	×	×	×	×	×	nd	×	×	×	nd	nd	nd	nd	nd	nd	×	×	×	×	×	×										
	E	✔	✔	✔	×	×	✔	✔	✔	✔	nd	nd	nd	nd	×	×	×	✔	×	×	×	✔	×	×	×										
	F	×	×	×	×	×	×	×	×	nd	nd	nd	nd	nd	nd	nd	nd	×	nd	×	×	×	×	×	×										
Flask shape	n	✔	✔	✔	×	×	✔	✔	✔	✔	×	×	×	×	×	×	×	✔	×	×	×	✔	×	×	×	×	×	×	×	✔	×				
	b	✔	✔	✔	×	×	✔	✔	✔	✔	×	×	×	×	×	×	×	✔	×	×	×	✔	×	×	×	×	×	×	×	✔	×				
V [mL]	100	✔	✔	✔	×	×	✔	✔	✔	✔	×	×	×	×	×	×	×	✔	×	×	×	✔	×	×	×	×	×	×	×	✔	×	✔	✔		
	200	nd	×	×	×	×	nd	nd	×	nd	nd	nd	nd	×	nd	nd	nd	×	nd	nd	nd	×	×	×	×	nd	nd	×	nd	×	nd	×	×		

*Additives are $CaCO_3$ (A), CaO_2 (B), trace metals (C), HCl (D), NaOH (E), and PBS (F). Flask shape is normal (n) or baffled (b). Tick indicates pellet formation, and cross no pellet formation; nd stands for not determined.

The presented overall multiple linear regression model (Equation 1) analyzed the effect of all tested factors on pellet formation (results were characterized as "pellet formation yes/no", Table 3-2) and predicted that flask shape exhibited significant effects on fungal morphology (p = 0.011), however, medium composition (p = 0.995), temperature (p = 0.116), agitation rate (p = 0.633), inoculum size (p = 0.683) and additives (p = 0.993) did not have a statistically significant influence on pellet formation if the complete data set was considered. The results on fungal morphology are highlighted in Equation 1.

$$\textit{Morphology} = 0.002 - (0.002 \times \textit{media type}) + (0.062 \times \textit{temperature}) - (0.012 \times \textit{agitation rate}) + (0.280 \times \textit{pH}) - (0.051 \times \textit{inoculum size}) - (0.001 \times \textit{additives}) + (1.338 \times \textit{flask shape}) - (0.011 \times \textit{volume}) \quad \text{Eq. (1)}$$

The equation was developed using SPSS and multiple linear regression. Dependent variable (morphology) and independent variables (factors affecting morphology) were modeled using linear regression analysis and the result was expressed as a linear function of all variables. The unstandardized coefficients calculated by the model have been inserted into a linear function (Equation 1). Accordingly, the low factor values of the additives, media type, volume, and agitation rate, listed in ascending order do not act significantly on fungal morphology. Flask shape and pH demonstrate strongest effects on fungal morphology, followed by temperature and inoculum size.

3.3.1 THE INFLUENCE OF MEDIA TYPE

Three different basic types of media were compared to investigate growth behavior and morphological forms of *M. hiemalis* in liquid submerged cultures: The N-limiting variation of the medium developed by Kirk et al. [57], SAB medium with the addition of vitamins (SAB+V) and SAB medium without (SAB). Biomass production was characterized as dry weight (lyophilized biomass) after three weeks of cultivation on a continuously shaking rotator. Dry weights were 0.6 ± 0.03 g and 0.6 ± 0.01 g for SAB+V and SAB respectively, which shows that there is no statistical difference if media were supplemented with vitamins or not (p = 0.991). However, growth was found being three fold higher in both SAB media if compared to the fungal growth in the N-limiting medium, where dry weights were only 0.2 ± 0.03 g ($p < 0.001$) (Fig. 3-1).

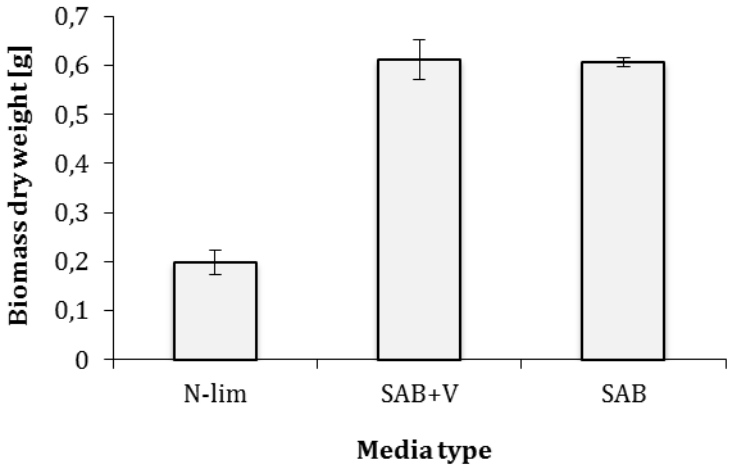

Fig. 3-1: Biomass production of *M. hiemalis* expressed as dry weights after three weeks of cultivation in different media types (Nitrogen-limiting, Sabouraud dextrose broth with vitamins and Sabouraud dextrose broth without the addition of vitamins) on a rotary shaker. Data represent means ± standard errors (n = 3).

Even though the fungal growth was influenced by the media type, morphology was not. In all three media, with an initial inoculum size of 10^2 sporangiospores per milliliter, the fungus grew in the form of filaments, whereas if the spore concentration was raised to 10^4 spores per milliliter, clumps were prevalent. Opposite results were found for *P. chrysosporium*, where an increased inoculum level led to the transition of clumps to more filamentous morphologies [59] and similar results were found for *A. niger*, however, much higher inoculum sizes were used in both cases, i.e. 10^4 to 10^6, 10^9 spores per milliliter, respectively [52]. However, in this study, media type did only influence the growth extent but not the morphological growth form of *M. hiemalis*. Because of the simpler formulation of the medium, the enhanced growth and the same morphological profile of *M. hiemalis* in SAB without the addition of vitamins, this medium was chosen for further experiments. On the basis of SAB, additional factors were studied to evaluate the effect on fungal morphology. Table 3-1 shows all the parameters that have been varied to investigate the effect on the morphology of *M. hiemalis*. Additives ($CaCO_3$, CaO_2, and trace metals) that led to the pelletization of other filamentous fungi [49, 55, 60, 61] were tested for the pellet formation of *M. hiemalis* as discussed in section 3.3.6. HCl, NaOH and PBS buffer were used to adjust the pH of the medium and the effect is discussed in section 3.3.2.

3.3.2 THE INFLUENCE OF PH

The pH and culture media volume both did not have a statistically significant effect when analyzed by a general multiple regression model, however, p-values were lower compared to the other factors (p = 0.070 and 0.075, respectively). Therefore, data was analyzed separately in more detail by sectional multiple linear regression in order to elucidate these effects only

after optimization of cultural conditions. The effects of the different sources that contributed to an alkaline pH shift in cultural media were compared. Focusing on the effects caused after addition of sodium hydroxide, the resulting pH had a statistically significant effect on pellet formation (p = 0.032). The pH of the culture medium is a measure of the concentration of H^+ ions present in a solution and contributes to surface phenomena, which might explain its role in relation to pellet formation. The negatively charged functional groups existing on the surface of the fungal cell wall, phosphates, proteins, and carboxylate groups, may undergo protonation at low pH, leading to an increase in the positive charge density on the cell surface. The magnitude of net charge influenced by pH is species dependent [62]. Here, development of dispersed mycelial filaments of *M. hiemalis* in the pH range from 4 to 7 were observed, likely due to mutual repulsion. At an acidic to neutral pH, charged cell wall surfaces are predominant in the fungus and hyphae with net charges of the same sign may repel each other and push themselves to grow apart. The exertion of repulsive forces resulted in the growth of dispersed filaments lacking the affinity of fungal cells to aggregate. With the decrease of repulsive electrostatic interactions in correlation with the increase of the pH toward the cell isoelectrical point, the surface charge decreases, and cells were more likely to aggregate and the fungus grew in the form of intertwined hyphae. Similar observations were reported for *A. nidulans*, where pellet formation was mostly attributed to the pH dependent electrical charge and hydrophobicity of the conidiospores [53]. Favorable hyphal attraction of *M. hiemalis* was expressed in the range of 7 to 8, where coagulation of hyphae resulted in the formation of homogenously compact pellets. Within this pH range after adjustment with sodium hydroxide, filamentous growth did not occur. However, clumpy growth was observed, when pH reached 7.5 after addition of calcium carbonate or adjustment with phosphate buffer, showing that morphology not only depended on dissociated H^+ protons but also on the counter ions present in the media, which may interfere with fungal cell surfaces inducing agglomeration. In comparison, *M. circinelloides* formed small pellets with little growth at an initial pH of 3. When the pH was adjusted to 5.3 after 18 hours, cell growth was stimulated, showing compact, spherical, smooth pellets in the presence of $CaCO_3$ and loosely packed, fluffy pellets in the presence of NaOH [55]. In contrast, *M. hiemalis* did not pelletize if pH was lower than 7 and we concluded, that initial pH adjustment with NaOH was necessary however, only needed to stimulate pellet formation, and no further pH control and adjustment was necessary. Once the pellet was formed, the fungus kept growing in the form of a pellet, even if the pH was not controlled constantly or has dropped due to fungal metabolism. Fig. 3-2 shows the spherical growth of transferred pellets in original SAB medium. This is in

contrast with pellet studies on *M. circinelloides*, where pH was monitored and kept constant at 5.3 in order to enable pellet formation and spherical growth [55].

3.3.3 GROWTH OF PELLETS

Different amounts of pellets were transferred to new flasks and growth was monitored after 24 and 48 hours of incubation. Pellets kept growing spherically ($p < 0.05$) and reached maximum sizes that were limited by the available space in the flask (Fig. 3-2).

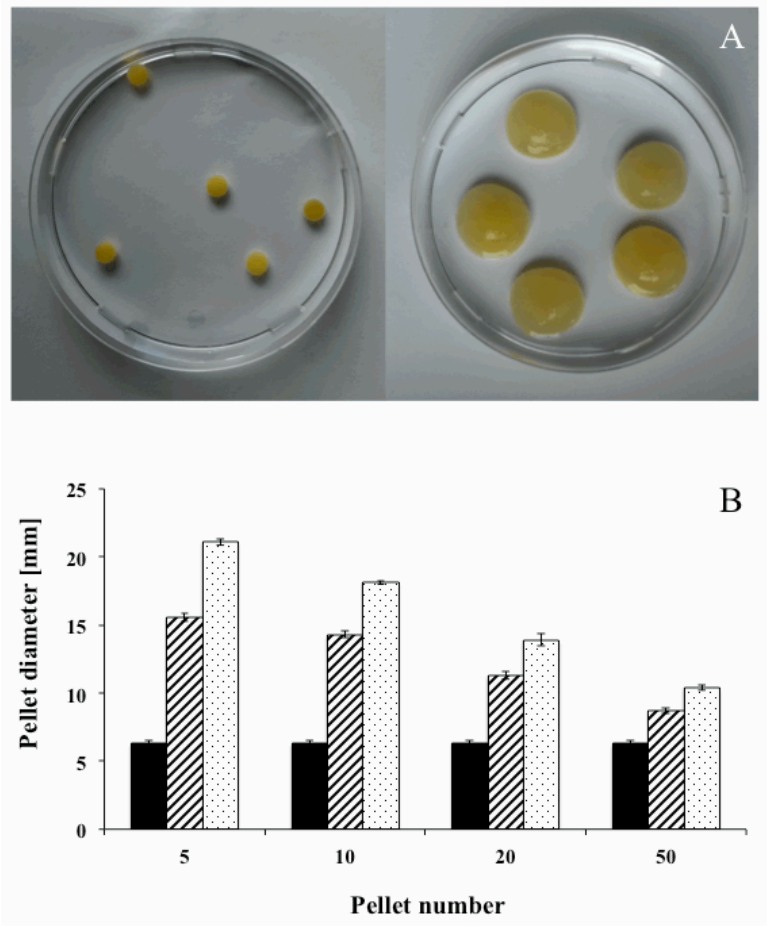

Fig. 3-2: Growth of pellets. A) Aliquot of seven-day old *M. hiemalis* pellets (left) and "transferred" pellets after 48 hours, re-incubation in original SAB medium (right). B) Spherical growth of *M. hiemalis* (measured in millimeters) in original SAB medium dependent on the incubation pellet number. Data are means ± standard errors of five replicates measuring five pellets per replicate. Bars represent measurement points at time point 0, 24 and 48 hours (in the order black, striped and dotted).

Fig. 3-2 shows how maximum pellet sizes depended on the inoculation pellet number. Fig. 3-2A represents the typical diameter (5 mm) of the fungal pellets and the larger diameter (20 mm) achieved when re-inoculated and allowed to grow with more space (Fig. 3-2B). The pellet diameter increased with the decrease in pellet number ($p < 0.05$) monitored after 24 and 48 hours ($p < 0.05$). This shows that sufficient available space is needed for pellets to expand

in size and may in part explain why pellet formation was not observed at all spore concentrations within the fixed pH optimum as explained in section 3.3.4.

3.3.4 THE INFLUENCE OF INOCULUM SIZE

Separate statistical analysis was used to reveal the influence of inoculum size on fungal growth behavior by means of a sectional multiple linear regression model. All data at concentrations 10^1, 10^2, 10^3, 10^4 spores/mL and initial pellet inducing pH control were analyzed in respect to the effect of inoculum size on pellet formation. Our results showed a statistically significant effect of inoculum size on the probability of pellet formation ($p = 0.027$). The optimal spore concentration for pelletization was found to be 10^3 spores per milliliter. Cultures with lower spore concentrations (10^1-10^2 spores/mL) resulted in low or not macroscopically visible growth. Cultures with higher spore concentrations (10^4-10^6 spores/mL) yielded large clumps. Takahashi and Yamada distinguish two mechanisms of pellet formation, the coagulating and non-coagulating type and it is assumed that the genus *Mucor* is classified into the non-coagulative type [63]. In this case, spores germinate and young hyphae form small clumps that grow to form pellets. The extreme case of a non-coagulative behavior occurs if inoculum size is small enough that the distance between spores remains large enough to enable each single spore to grow and form one pellet. This explains not only that pellet growth was restricted to space (Fig. 3-2) but also that there must be a critical inoculum size in non-coagulative fungi, which nonetheless depends on the agitation level [64]. Keeping the agitation rate constant at 130 rpm for the cultivation of *M. hiemalis*, biomass was too large if inoculum size was higher than 10^3 spores per milliliter. Exceeding this inoculum size (i.e. 10^4-10^6 spores/mL) resulted in clumping of all hyphae and the formation of larger irregular clumps, whereby pellet formation was prevented. At high concentrations the hyphae interact with each other in the early stages of growth, thereby preventing the development of pellets. Therefore, the spore concentration of 10^3 per milliliter has been characterized as the critical inoculum size for *M. hiemalis*, which was two magnitudes higher than for *M. circinelloides*, but much lower in comparison to other pellet-forming fungi. *P. chrysosporium* has been reported to pelletize at a spore concentration of 10^5/mL or higher [65, 66]. The filamentous fungus *R. oryzae* showed pellet formation when 10^9 spores per milliliter were incubated [67]. Inoculum size appeared to be a critical factor in pellet formation; however, pH changes still provoked the strongest effects on fungal morphology of *M. hiemalis*. Refinement of the initial pH of the growth medium was crucial for promoting pellet formation, and adjustment of spore concentration was essential in order

to provide appropriate space to grow. Interplay of both factors has to be considered for successful pelletization of *M. hiemalis*.

3.3.5 EFFECT OF SHEAR FORCES

Additionally, in the present study factors connected to impact on mechanical forces have been investigated. Among them, volume had substantial effects on pellet formation. Pellets were only formed when 100 mL media were used, whereas in 200 mL no pellet formation was observed. A change in volume creates a different force and resulting fluid dynamics. It is possible that a larger volume requires higher agitation speed to enable a comparable liquid movement required for pelletization. However, this was not tested here. Moreover, too much volume reduces oxygen transfer, whereby a smaller volume maximizes the surface area exposed to the available air and produces sufficient supply of oxygen and removal of carbon dioxide [68]. Flask shape had negligible effects on the morphology under optimized medium composition conditions, however smoother pellets were obtained if baffled flasks were used rather than normal shaped flasks, showing that additional shear forces slightly influenced pellet characteristics. Agitation was important for good mixing to keep spore-to-spore distance large enough to prevent clumpy growth, and moreover it created shear forces, which influenced fungal morphology. The agitation rate (110-180 rpm), however, did not have a statistical significant role in pellet formation ($p = 0.633$).

3.3.6 EFFECT OF ADDITIVES

The supplementation of additives ($CaCO_3$, CaO_2, or trace metals) did not exhibit positive effects on pellet formation in contrast to what was reported for other filamentous fungi [49, 55, 60, 61]. Trace metals in the cultivation medium significantly promoted the formation of pellets of *R. oryzae* ATCC 20344 [49, 60], whereas in the present study trace element addition did not promote pelletization. Addition of solid particles, such as calcium carbonate, which was barely soluble in the medium, showed a clear negative effect on the probability of pelletization of *M. hiemalis*. Conversely, numerous studies showed that the addition of solid particles enhanced the probability of pelletization of different fungi, e.g. the addition of rice promoted pellet formation of *R. oryzae* [67] and titanate ($TiSiO_4$) microparticles resulted in the pelletization of *A. niger* [69]. Moreover, the addition of calcium carbonate not only favored pellet formation in *R. oryzae* [70] but also induced pelletization of the species *circinelloides* of the genus *Mucor* [55]. Generally, the addition of calcium ions (in the form of $CaCO_3$ or CaO_2) both did not promote pellet formation of *M. hiemalis*, however in *R. oryzae*

and *A. niger*, the addition of Ca^{2+} ions in the form of $CaCl_2$ resulted in the production of pellets for both fungi [50, 71].

3.3.7 EFFECT OF TEMPERATURE

In regards to cultural temperature, *M. hiemalis* grew from 23 °C to 30 °C. Variation of the temperature within that range did not have an influence on pellet probability, but fungal growth was inhibited if the cultivation temperature reached 40 °C. Future studies are required to investigate growth and biosorption potential of pelletized *M. hiemalis* at lower temperatures.

By modifying the cultivation conditions, the filamentous fungus *M. hiemalis* was successfully manipulated into growing as pellets. In summary, a total of eight different factors and their synergistic effects were considered. The factors included media type, temperature, agitation rate, inoculum size, pH, additives, flask shape, and volume. The different combinations resulted in growth, ranging from large irregular shaped clumps or spherical pellets to completely dispersed mycelia. When comparing morphological results to other fungi tested in literature, it clearly shows that influence not only varies from genus to genus, but also between species. A multifactorial experimental design developed and applied to *Rhizopus* sp. showed that temperature not only led to a faster development but also increased the probability, and agitation rate, calcium ion concentration, pH and solid cellulose particles each had significant effects on pelletization of the fungus, whereas inoculum size and liquid volume, both factors that showed to influence *M. hiemalis* pellet formation, were not found to have a significant effect on *Rhizopus* sp. [50]. While pH adjustment during cell growth *via* addition of calcium carbonate was used to induce pellet formation of *M. circinelloides* [55], the addition of calcium carbonate suppressed pellet formation of the *Mucor* species in question and constant pH control was not necessary for pellet growth. Obviously, the factors influencing fungal morphology are many that have to be considered, and only an experimental adaptation of the culture parameters allows pellet formation for the fungus in question.

3.3.8 MC-LR UPTAKE BY FUNGAL PELLETS OF *M. HIEMALIS*

Uptake experiments were performed to examine the biosorption capacity of *M. hiemalis* at varying pellet numbers per exposure flask. The extraction method used to isolate MC-LR from fungal pellets yielded excellent recoveries (100 ± 2%) as a total of 97.8 ± 2.5 ng MC-LR (n = 3) were quantified *via* LC-MS/MS analysis in the test samples containing 50 mg untreated pelleted fungal biomass (dw) and 100 ng MC-LR. The same extraction procedure

was applied to toxin treated samples and extracted toxin was analyzed and quantified *via* LC-MS/MS. The fungus showed uptake between 0.2-0.4 micrograms MC-LR per gram pelletized biomass (dw) ($p < 0.05$) for all pellet numbers tested. No statistical difference of uptake could be observed when comparing 3, 5 or 10 pellets ($p > 0.05$). However, a statistically higher concentration of MC-LR was detected when 25 pellets per flasks were used ($p < 0.05$). Fig. 3-3 graphically shows the total uptake in nanograms that was achieved by using pelletized *M. hiemalis*. Growth expressed as milligram dry weight after 48 hours of exposure was plotted on a secondary axis. The ratio between total uptake and biomass lies in the same range for all the pellet numbers ($p > 0.05$), which explains why a statistically higher amount of toxin has been biosorbed within the pellets when 25 pellets were used, as a much higher amount of biomass was obtained after 48 hours of incubation and exposure ($p < 0.05$). Comparing biomass of three pellets exposed to the toxin to three pellets of untreated controls, we observed that no statistically significant growth variation occurred ($p = 0.365$). This explains that growth behavior of the fungus was not negatively affected in the presence of cyanotoxin, which confirms the results obtained in a previous study [44].

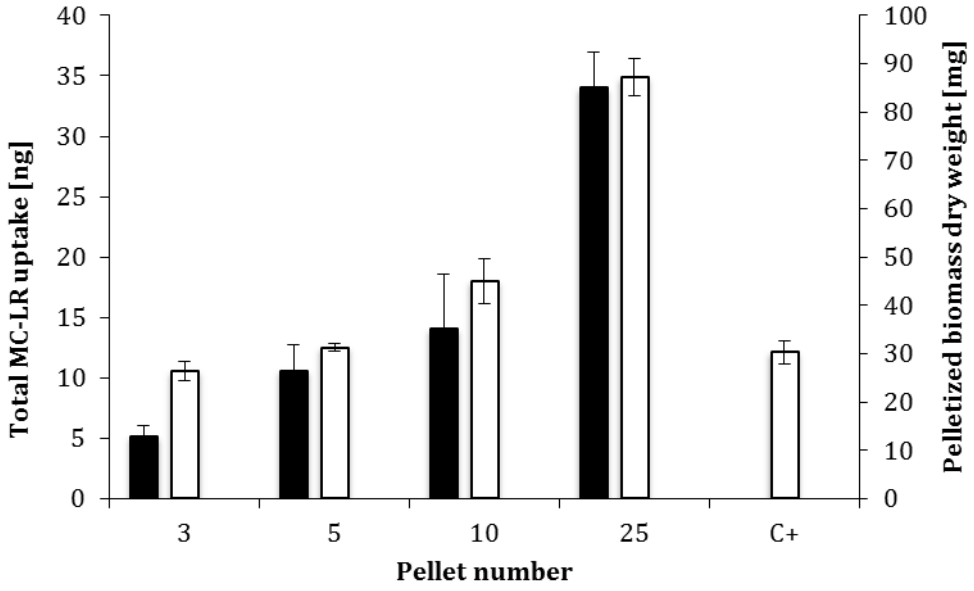

Fig. 3-3: Microcystin-LR uptake in fungal pellets of *M. hiemalis*. Total toxin uptake [ng] plotted on the primary axis (black bars) and fungal biomass expressed as lyophilized dry weight [mg] plotted on the secondary axis (white bars) after 48 hours of incubation and exposure to 100 ng/mL of the cyanotoxin versus different amounts of fungal pellets. The positive control (C+) in the absence of toxin contained three pellets. Data represent means ± standard errors ($n = 3$).

To the authors' knowledge, this is the first report to introduce cyanobacterial toxin uptake in pelletized filamentous fungi. Further studies are suggested to enhance biosorption ability using larger fungal biomass and a longer exposure range, as well as examine the optimal

concentration range for best toxin removal. It can be concluded that *M. hiemalis* is an attractive organism to be further studied for the capacity expansion of the approved Green Liver Systems® due to the similar biosorption potential of the fungus towards MC-LR compared to several aquatic plants. In *M. hiemalis* pellets, 0.7% of the total applied MC-LR was internalized when using 25 pellets. This is similar compared to the uptake reported in *Ceratophyllum demersum*, *Elodea canadensis* and *Vesicularia dubyana*, where 0.6-1.75% of the total toxin exposure concentration could be detected intracellularly [72]. *Lemna minor* and *Chladophora fracta* were exposed to a concentration that was 100-200 times higher than what was applied to *M. hiemalis* cultures in the present study, nonetheless, similar intracellular toxin concentrations were detected and this only after a prolonged exposure of five days [73]. The present study focuses only on fungal uptake of MC-LR, but further studies are suggested to investigate possible degradation of the toxin *via* extracellular or intracellular enzymatic pathways in the fungus. Toxin removal from the water could be achieved by using *M. hiemalis* pellets in bioreactor technology, which inspires future work on pelletized *M. hiemalis* as a mycoremediation tool in bioreactors for the removal of cyanobacterial as well as other hazardous toxins from contaminated water.

3.4 CONCLUSIONS

The present study is the first report of a cultivation method for the pellet production of *M. hiemalis* in liquid submerged cultures. When comparing factors that influence fungal morphology, there is a clear difference not only between genera, but also between species of the same genus. Initial sodium hydroxide based pH adjustment of Sabouraud dextrose broth medium was crucial for the pellet induction in *M. hiemalis* cultures with inoculum size of 10^3 spores per milliliter in baffled (or normal shaped) Erlenmeyer flasks with a volume of 100 mL medium. The study combines the application of the obtained fungal pellets with biosorption experiments towards the cyanotoxin microcystin-LR. Toxin uptake into fungal pellets was demonstrated, which is an essential prerequisite to the applicability of an organism in water remediation. The results motivate further work in order to establish fungal bioreactors that may be used for efficient cyanobacterial as well as other toxin removal from contaminated water.

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REFERENCES

1. Imanishi S, Kato H, Mizuno M, Tsuji K, Harada KI. Bacterial degradation of microcystins and nodularin. Chemical Research in Toxicology. 2005; 18(3):591-598.
2. Namikoshi M, Sivonen K, Evans WR, Sun F, Carmichael WW, Rinehart KL. Isolation and structures of microcystins from a cyanobacterial water bloom (Finland). Toxicon. 1992; 30(11):1473-1479.
3. Luukkainen R, Namikoshi M, Sivonen K, Rinehart KL, Niemelä SI. Isolation and identification of 12 microcystins from four strains and two bloom samples of *Microcystis* spp.: Structure of a new hepatotoxin. Toxicon. 1994; 32(1):133-139.
4. Sivonen K, Namikoshi M, Evans WR, Färdig M, Carmichael WW, Rinehart KL. Three new microcystins, cyclic heptapeptide hepatotoxins, from *Nostoc* sp. strain 152. Chemical Research in Toxicology. 1992; 5(4):464-469.
5. Lanaras T, Cook CM. Toxin extraction from an *Anabaenopsis milleri* — dominated bloom. Science of The Total Environment. 1994; 142(3):163-169.
6. Azevedo SMFO, Carmichael WW, Jochimsen EM, Rinehart KL, Lau S, Shaw GR, Eaglesham GK. Human intoxication by microcystins during renal dialysis treatment in Caruaru—Brazil. Toxicology. 2002; 181–182(0):441-446.
7. Jochimsen EM, Carmichael WW, An J, Cardo DM, Cookson ST, Holmes CEM, Antunes MB, de Melo Filho DA, Lyra TM, Barreto VST, Azevedo SMFO, Jarvis WR. Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. New England Journal of Medicine. 1998; 338(13):873-878.
8. Falconer IR, Humpage AR. Tumour promotion by cyanobacterial toxins. Phycologia. 1996; 35(6S):74-79.
9. Falconer IR. Tumor promotion and liver injury caused by oral consumption of cyanobacteria. Environmental Toxicology and Water Quality: An International Journal. 1991; 6(2):177-184.

10. Nishiwaki-Matsushima R, Ohta T, Nishiwaki S, Suganuma M, Kohyama K, Ishikawa T, Carmichael WW, Fujiki H. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. Journal of Cancer Research and Clinical Oncology. 1992; 118(6):420-424.
11. Yoshida T, Makita Y, Nagata S, Tsutsumi T, Yoshida F, Sekijma M, Tamura S, Ueno Y. Acute oral toxicity of microcystin-LR, a cyanobacterial hepatotoxin, in mice. Natural Toxins. 1997; 5(3):91-95.
12. DeMott WR, Zhang QX, Carmichael WW. Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of *Daphnia*. Limnology and Oceanography. 1991; 36(7):1346-1357.
13. Ghazali IE, Saqrane S, Carvalho AP, Ouahid Y, Oudra B, del Campo FF, Vasconcelos V. Compensatory growth induced in zebrafish larvae after pre-exposure to a *Microcystis aeruginosa* natural bloom extract containing microcystins. International Journal of Molecular Sciences. 2009; 10(1):133-146.
14. Saqrane S, Ghazali IE, Ouahid Y, Hassni ME, Hadrami IE, Bouarab L, del Campo FF, Oudra B, Vasconcelos V. Phytotoxic effects of cyanobacteria extract on the aquatic plant *Lemna gibba*: Microcystin accumulation, detoxication and oxidative stress induction. Aquatic Toxicology. 2007; 83(4):284-294.
15. LeBlanc S, Pick FR, Aranda-Rodriguez R. Allelopathic effects of the toxic cyanobacterium *Microcystis aeruginosa* on duckweed, *Lemna gibba* L. Environmental Toxicology. 2005; 20(1):67-73.
16. Pflugmacher S, Codd GA, Steinberg CEW. Effects of the cyanobacterial toxin microcystin-LR on detoxication enzymes in aquatic plants. Environmental Toxicology. 1999; 14(1):111-115.
17. Pflugmacher S. Possible allelopathic effects of cyanotoxins, with reference to microcystin-LR, in aquatic ecosystems. Environmental Toxicology. 2002; 17(4):407-413.
18. Kormas KA, Lymperopoulou DS. Cyanobacterial toxin degrading bacteria: Who are they? BioMed Research International. 2013; 2013(0):1-12.
19. Pflugmacher S, Kühn S, Lee SH, Choi JW, Baik S, Kwon KS, Contardo-Jara V. Green liver systems® for water purification – using the phytoremediation potential of aquatic macrophytes for the removal of different cyanobacterial toxins from water. American Journal of Plant Sciences. 2015; 6(9):1607-1618.

20. Nimptsch J, Wiegand C, Pflugmacher S. Cyanobacterial toxin elimination *via* bioaccumulation of MC-LR in aquatic macrophytes: An application of the "Green Liver Concept". Environmental Science & Technology. 2008; 42(22):8552-8557.
21. Tien M. Properties of ligninase from *Phanerochaete chrysosporium* and their possible applications. Critical Reviews in Microbiology. 1987; 15(2):141-168.
22. Ulmer D, Leisola M, Puhakka J, Fiechter A. *Phanerochaete chrysosporium*: Growth pattern and lignin degradation. European Journal of Applied Microbiology and Biotechnology. 1983; 18(3):153-157.
23. Novotný Č, Svobodová K, Erbanová P, Cajthaml T, Kasinath A, Lang E, Šašek V. Ligninolytic fungi in bioremediation: Extracellular enzyme production and degradation rate. Soil Biology and Biochemistry. 2004; 36(10):1545-1551.
24. Reddy CA. The potential for white-rot fungi in the treatment of pollutants. Current Opinion in Biotechnology. 1995; 6(3):320-328.
25. Arjmand M, Sandermann Jr H. *N*-(Chlorophenyl)-succinimides: A novel metabolite class isolated from *Phanerochaete chrysosporium*. Pesticide Biochemistry and Physiology. 1987; 27(2):173-181.
26. Arjmand M, Sandermann Jr H. Mineralization of chloraniline/lignin conjugates and of free chloranilines by the white-rot fungus *Phanerochaete chrysosporium*. Journal of Agricultural and Food Chemistry. 1985; 33(6):1055-1060.
27. Hickey WJ, Fuster DJ, Lamar RT. Transformation of atrazine in soil by *Phanerochaete chrysosporium*. Soil Biology and Biochemistry. 1994; 26(12):1665-1671.
28. Mougin C, Laugero C, Asther M, Dubroca J, Frasse P, Asther M. Biotransformation of the herbicide atrazine by the white-rot fungus *Phanerochaete chrysosporium*. Applied and Environmental Microbiology. 1994; 60(2):705-708.
29. Bumpus JA. Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Applied and Environmental Microbiology. 1989; 55(1):154-158.
30. Eaton DC. Mineralization of polychlorinated biphenyls by *Phanerochaete chrysosporium*: A ligninolytic fungus. Enzyme and Microbial Technology. 1985; 7(5):194-196.
31. Fernando T, Bumpus JA, Aust SD. Biodegradation of TNT (2,4,6-trinitrotoluene) by *Phanerochaete chrysosporium*. Applied and Environmental Microbiology. 1990; 56(6):1666-1671.

32. Mileski G, Bumpus JA, Jurek MA, Aust SD. Biodegradation of pentachlorophenol by the white-rot fungus *Phanerochaete chrysosporium*. Applied and Environmental Microbiology. 1988; 54(12):2885-2889.

33. Joshi DK, Gold MH. Degradation of 2,4,5-trichlorophenol by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Applied and Environmental Microbiology. 1993; 59(6):1779-1785.

34. Cammarota MC, Sant'Anna Jr GL. Decolorization of kraft bleach plant E_1 stage effluent in a fungal bioreactor. Environmental Technology. 1992; 13(1):65-71.

35. Lu Y, Yan L, Wang Y, Zhou S, Fu J, Zhang J. Biodegradation of phenolic compounds from coking wastewater by immobilized white rot fungus *Phanerochaete chrysosporium*. Journal of Hazardous Materials. 2009; 165(1–3):1091-1097.

36. Miranda Rde C, Gomes Ede B, Pereira Jr N, Marin-Morales MA, Machado KM, Gusmão NB. Biotreatment of textile effluent in static bioreactor by *Curvularia lunata* URM 6179 and *Phanerochaete chrysosporium* URM 6181. Bioresource Technology. 2013; 142(0):361-367.

37. Mielgo I, Moreira MT, Feijoo G, Lema JM. Biodegradation of a polymeric dye in a pulsed bed bioreactor by immobilised *Phanerochaete chrysosporium*. Water Research. 2002; 36(7):1896-1901.

38. Lamar RT, Larsen MJ, Kirk TK, Glaser JA. Growth of the white-rot fungus *Phanerochaete chrysosporium* in soil. In: Barkley NP, Martin JF, editors. Land disposal, remedial action, incineration and treatment of hazardous waste: Proceedings of the 13th annual research symposium, U.S. EPA, Cincinnati, OH: Hazardous waste and engineering research laboratory, Office of research and development; 1987, p. 419-424.

39. Hoque E, inventor; PatentDe, assignee. Verfahren zum Abbau von Xenobiotika durch Pilzarten mit Monooxygenase-/Dioxygenase-Aktivität in Gegenwart von Pilzen mit Glutathion-*S*-Transferase-Aktivität. Deutsches Patentdokument DE10125365C2. 2003 June 5.

40. Rønhede S, Jensen B, Rosendahl S, Kragelund BB, Juhler RK, Aamand J. Hydroxylation of the herbicide isoproturon by fungi isolated from agricultural soil. Applied and Environmental Microbiology. 2005; 71(12):7927-7932.

41. Shroff KA, Vaidya VK. Kinetics and equilibrium studies on biosorption of nickel from aqueous solution by dead fungal biomass of *Mucor hiemalis*. Chemical Engineering Journal. 2011; 171(3):1234-1245.

42. Tewari N, Vasudevan P, Guha BK. Study on biosorption of Cr(VI) by *Mucor hiemalis*. Biochemical Engineering Journal. 2005; 23(2):185-192.

43. Fritscher J, Hoque E, Stöckl M, inventor; PatentDe, assignee. Fungus *Mucor hiemalis*, useful for the removal of heavy metals e.g. mercury, chromium, uranium and aluminum in ground- and surface water, purification plants, waste water and industrial water is new. Deutsches Patentdokument DE102004020837B4. 2006 Feb 23.

44. Balsano E, Esterhuizen-Londt M, Hoque E, Pflugmacher S. Toxin resistance in aquatic fungi poses environmentally friendly remediation possibilities: A study on the growth responses and biosorption potential of *Mucor hiemalis* EH5 against cyanobacterial toxins. International Journal of Water and Wastewater Treatment. 2015; 1(1):1-9.

45. Gibbs PA, Seviour RJ, Schmid F. Growth of filamentous fungi in submerged culture: Problems and possible solutions. Critical Reviews in Biotechnology. 2000; 20(1):17-48.

46. Kim JH, Lebeault JM, Reuss M. Comparative study on rheological properties of mycelial broth in filamentous and pelleted forms. European Journal of Applied Microbiology and Biotechnology. 1983; 18(1):11-16.

47. Olsvik E, Kristiansen B. Rheology of filamentous fermentations. Biotechnology Advances. 1994; 12(1):1-39.

48. Liao W, Liu Y, Chen S. Studying pellet formation of a filamentous fungus *Rhizopus oryzae* to enhance organic acid production. In: Mielenz JR, Klasson KT, Adney WS, McMillan JD, editors. Applied Biochemistry and Biotechnology, Biotechnology for Fuels and Chemicals, The Twenty-Eight Symposium, New York: Humana Press; 2007, p. 689-701.

49. Liao W, Liu Y, Frear C, Chen S. A new approach of pellet formation of a filamentous fungus – *Rhizopus oryzae*. Bioresource Technology. 2007; 98(18):3415-3423.

50. Nyman J, Lacintra MG, Westman JO, Berglin M, Lundin M, Lennartsson PR, Taherzadeh MJ. Pellet formation of zygomycetes and immobilization of yeast. New Biotechnology. 2013; 30(5):516-522.

51. Žnidaršič P, Komel R, Pavko A. Studies of a pelleted growth form of *Rhizopus nigricans* as a biocatalyst for progesterone 11α-hydroxylation. Journal of Biotechnology. 1998; 60(3):207-216.

52. Papagianni M, Mattey M. Morphological development of *Aspergillus niger* in submerged citric acid fermentation as a function of the spore inoculum level.

Application of neural network and cluster analysis for characterization of mycelial morphology. Microbial Cell Factories. 2006; 5(3):1-12.

53. Dynesen J, Nielsen J. Surface hydrophobicity of *Aspergillus nidulans* conidiospores and its role in pellet formation. Biotechnology Progress. 2003; 19(3):1049-1052.

54. Carlsen M, Spohr AB, Nielsen J, Villadsen J. Morphology and physiology of an α-amylase producing strain of *Aspergillus oryzae* during batch cultivations. Biotechnology and Bioengineering. 1996; 49(3):266-276.

55. Xia C, Zhang J, Zhang W, Hu B. A new cultivation method for microbial oil production: Cell pelletization and lipid accumulation by *Mucor circinelloides*. Biotechnology for Biofuels. 2011; 4(15):1-10.

56. Hoque E, Pflugmacher S, Fritscher J, Wolf M. Induction of glutathione *S*-transferase in biofilms and germinating spores of *Mucor hiemalis* strain EH5 from cold sulfidic spring waters. Applied and Environmental Microbiology. 2007; 73(8):2697-2707.

57. Kirk TK, Schultz E, Connors WJ, Lorenz LF, Zeikus JG. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Archives of Microbiology. 1978; 117(3):277-285.

58. Sambrook J, Russel T. Preparation of reagents and buffers used in molecular cloning, phosphate-buffered saline (PBS). In: Argentine J, Irwin N, Janssen KA, Curtis S, Zierler M, McInerny N, Brown D, Schaefer S, editors. Molecular cloning: a laboratory manual, New York: Cold Spring Harbor Laboratory Press; 2001, p. A1.7.

59. Tucker KG, Thomas CR. Mycelial morphology: The effect of spore inoculum level. Biotechnology Letters. 1992; 14(11):1071-1074.

60. Zhou Y, Du J, Tsao GT. Mycelial pellet formation by *Rhizopus oryzae* ATCC 20344. Applied Biochemistry and Biotechnology. 2000; 84(1):779-789.

61 Sladdin M, Lynch JM. Antimicrobial properties of calcium peroxide in relation to its potential use as a seed dressing. Journal of General Microbiology. 1983; 129(7):2307-2314.

62. Douglas HW, Collins AE, Parkinson D. Electric charge and other surface properties of some fungal spores. Biochimica et Biophysica Acta. 1959; 33(2):535-538.

63. Takahashi J, Yamada H. Studies on the effect of some physical conditions on the submerged mold culture. Part II. On the two types of pellet formation in the shaking culture. Journal of Agricultural Chemical Society. 1959; 33(8):707-710.

64. Van Suijdam JC, Kossen NWF, Paul PG. An inoculum technique for the production of fungal pellets. European Journal of Applied Microbiology and Biotechnology. 1980; 10(3):211-221.

65. Gerin PA, Dufrene Y, Bellon-Fontaine MN, Asther M, Rouxhet PG. Surface properties of the conidiospores of *Phanerochaete chrysosporium* and their relevance to pellet formation. Journal of Bacteriology. 1993; 175(16):5135-5144.

66. Wu J, Li QB. Biosorption of lead by *Phanerochaete chrysosporium* in the form of pellets. Journal of Environmental Sciences. 2002; 14(1):108-114.

67. Liu Y, Liao W, Chen S. Study of pellet formation of filamentous fungi *Rhizopus oryzae* using a multiple logistic regression model. Biotechnology and Bioengineering. 2008; 99(1):117-128.

68. Henzler HJ, Schedel M. Suitability of the shaking flask for oxygen supply to microbiological cultures. Bioprocess Engineering. 1991; 7(3):123-131.

69. Driouch H, Hänsch R, Wucherpfennig T, Krull R, Wittmann C. Improved enzyme production by bio-pellets of *Aspergillus niger*: Targeted morphology engineering using titanate microparticles. Biotechnology and Bioengineering. 2012; 109(2):462-471.

70. Liu Y, Liao W, Chen S. Co-production of lactic acid and chitin using a pelletized filamentous fungus *Rhizopus oryzae* cultured on cull potatoes and glucose. Journal of Applied Microbiology. 2008; 105(5):1521-1528.

71. Pera LM, Callieri DA. Influence of calcium on fungal growth, hyphal morphology and citric acid production in *Aspergillus niger*. Folia Microbiologica. 1997; 42(6):551-556.

72. Pflugmacher S, Wiegand C, Beattie KA, Codd GA, Steinberg C. Uptake of the cyanobacterial hepatotoxin microcystin-LR by aquatic macrophytes. Journal of Applied Botany. 1998; 72(5-6):228-232.

73. Mitrovic SM, Allis O, Furey A, James KJ. Bioaccumulation and harmful effects of microcystin-LR in the aquatic plants *Lemna minor* and *Wolffia arrhiza* and the filamentous alga *Chladophora fracta*. Ecotoxicology and Environmental Safety. 2005; 61(3):345-352.

4 RESPONSES OF THE ANTIOXIDATIVE AND BIOTRANSFORMATION ENZYMES IN THE AQUATIC FUNGUS *MUCOR HIEMALIS* EXPOSED TO CYANOTOXINS

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Own contribution:

Literature review on antioxidative and biotransformation enzymes and responses in aquatic organisms, plants and animals upon cyanotoxin exposure

Design of experiments and laboratory studies

Fungal culture and exposure to toxins

Optimization and measurement of enzyme assays

Performing all required laboratory works

Statistical analysis and interpretation of the experimental results

Preparation of the manuscript, which includes all texts and figures

Revision of the manuscript after receiving the reviewer's comments

ABSTRACT

Objectives

To investigate antioxidative and biotransformation enzyme responses in *Mucor hiemalis* towards cyanotoxins considering its use in mycoremediation applications.

Results

Catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx) in *M. hiemalis* maintained their activities at all tested microcystin-LR (MC-LR) exposure concentrations. Cytosolic glutathione *S*-transferase (GST) activity decreased with exposure to 100 μg MC-LR l^{-1} while microsomal GST remained constant. Cylindrospermopsin (CYN) exposure led to CAT activity increase and GR inhibition at 100 μg l^{-1}, as well as to a concentration-dependent GPx inhibition. Microsomal GST was clearly inhibited at all concentrations tested. β-*N*-methylamino-L-alanine (BMAA) inhibited GR activity in a concentration-dependent manner, however, CAT, GPx, and GST remained unaffected.

Conclusions

M. hiemalis showed enhanced oxidative stress tolerance and intact biotransformation enzyme activity towards MC-LR and BMAA in comparison to CYN, concluding its applicability in bioreactor technology in terms of viability and survival in their presence.

Keywords: Biotransformation, Cyanotoxin, Degradation, Microcystin-LR, *Mucor hiemalis*, Oxidative stress.

4.1 INTRODUCTION

Cyanotoxins, produced by many cyanobacteria as harmful secondary metabolites, in the aquatic environment constitute a serious risk for the ecological balance and the functioning of ecosystems (Corbel et al. 2014). The generation of oxidative stress plays an important role in their toxicity and the response of antioxidative enzymes has been extensively examined for many aquatic organisms exposed to cyanotoxins including plants (Flores-Rojas et al. 2015; Jiang et al. 2011; Pflugmacher 2004) and animals (Esterhuizen-Londt et al. 2016; Puerto et al. 2011) as an indicator of their ability to cope with the exposure. The conjugation of glutathione with microcystins was suggested to play a primary role in the metabolic pathway leading to detoxification of microcystins mediated by glutathione *S*-transferase (GST) in various aquatic organisms ranging from plants and invertebrates up to fish eggs, fish, and rat; demonstrating that the phase II conjugation pathway for xenobiotic metabolism expressed in all major groups of organisms is involved in the detoxification of microcystins (Pflugmacher et al. 1998; Wang et al. 2006). At present, the effect of cyanotoxins on the antioxidative and biotransformation enzymes in aquatic fungi is unknown and is especially of interest when considering the use of the fungal species for mycoremediation.

Mucor spp. have been successfully employed in the remediation of several pentachlorophenol *via* phase II conjugation and oxidation-reduction reactions (Carvalho et al. 2011) and the insecticide endosulfan involving oxidative and hydrolytic pathways (Shetty et al. 2000). The aquatic fungus *Mucor hiemalis* possesses functional groups that enable adsorption and hence removal of heavy metals, such as mercury (Hoque and Fritscher 2016) and expresses high intracellular GST activity (Hoque et al. 2007) that enable the remediation of the herbicide isoproturon involving phase II conjugation as a pathway for degradation in co-culture with *Phanerochaete chrysosporium* (Hoque 2003). It has been proven as an ideal candidate for mycoremediation attempts of cyanotoxins (Balsano et al., 2015, 2016) and it was therefore deemed necessary to investigate the antioxidative responses in *M. hiemalis* associated with cyanotoxin exposure to gauge its capability to deal with the toxins on a physiological level. The aim of the present study was therefore to evaluate the antioxidative and biotransformation enzyme responses in *M. hiemalis* with exposure to the three most common cyanotoxins: microcystin-LR (MC-LR), cylindrospermopsin (CYN) and β-*N*-methylamino-L-alanine (BMAA), representing the various cyanotoxin classifications at environmentally reported concentrations.

4.2 MATERIAL AND METHODS

4.2.1 CHEMICALS AND REAGENTS

All chemicals were analytical grade and purchased from Sigma-Aldrich unless specified otherwise. From MC-LR, CYN, and BMAA (Enzo Life Sciences, Alexis Biochemicals ALX-350-012-M001; Sigma-Aldrich, CAS Number: 16676-91-8; Enzo Life Sciences, Alexis Biochemicals ALX-350-149-M001) stock solutions (1 mg ml^{-1}) were prepared in MS grade methanol and stored at -20 °C. Necessary dilutions were prepared in sterile deionized water for exposure experiments.

4.2.2 FUNGAL STRAIN *MUCOR HIEMALIS* EH5 CULTIVATION

M. hiemalis EH5 (DSM 14200) was obtained from the culture collection of the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures. Cultivation and pelletization was performed as previously described by Balsano et al. (2016). Pellets were grown in Sabouraud dextrose broth pH 7.5 by incubating 2.5 x 10^3 spores per milliliter for seven days on a rotary shaker (130 rpm, 23 °C) in the dark.

4.2.3 EXPOSURE

Twenty pellets (with an average diameter of 5 ± 1 mm) were aseptically transferred into 100 ml Erlenmeyer flasks containing 50 ml of Sabouraud dextrose broth medium and were exposed to environmentally relevant concentrations (1, 10, and 100 µg l^{-1}) of the cyanobacterial toxins MC-LR, CYN, and BMAA, respectively. The control contained only the fungal strain in the absence of toxins. The cultures were incubated on a rotary shaker (130 rpm, r.t.) in the dark. The experiment was conducted in replicates of five for each toxin and concentration individually. Sample collection was performed after 24 h of exposure. Fungal pellets were removed from the exposure media, washed thoroughly with distilled water and surface-dried. Consequently, the pellet samples were snap-frozen, immediately ground to a fine powder in liquid nitrogen using a mortar and pestle before enzyme extraction.

4.2.4 EXPOSURE MEDIA CONFIRMATION

Exposure media concentrations were confirmed by liquid chromatography tandem mass spectrometry (LC-MS/MS) at the inception of the experiments. For MC-LR, the method as described by Balsano et al. (2015) was used. CYN was quantified according to Esterhuizen-Londt et al. (2015) and BMAA as per Esterhuizen-Londt et al. (2011b).

4.2.5 ENZYME EXTRACTION

The enzymes were extracted as described by Pflugmacher (2004). Frozen samples were homogenized in 0.1 M sodium phosphate buffer (pH 6.5) containing 20 % (v/v) glycerol, 1 mM ethylene-diamine-tetraacetic acid, and 1.4 mM dithioerythriol with a tissue grinder (Ultra-Turrax). Suspensions were then stirred on ice for 30 min, followed by centrifugation (10 min at 5,400 x *g* at 4 °C) to remove cell debris. For the collection of the microsomal fraction, the supernatant was again centrifuged (60 min at 86,900 x *g*) and the resulting pellet was re-suspended in 0.5 ml 20 mM sodium phosphate buffer (pH 7.0) containing 20 % (v/v) glycerol. Soluble proteins were concentrated and purified by ammonium sulfate precipitation, collecting the 35-80 % (w/v) saturation fraction after centrifugation (30 min at 48,900 x *g*). The resulting pellet was suspended in 20 mM sodium phosphate buffer (pH 7.0) and desalted by gel filtration using NAP-5 columns (Pharmacia, Uppsala, Sweden). Enzyme extracts were snap-frozen and stored at -80 °C.

4.2.6 ENZYME ACTIVITY ASSAYS

Enzyme activity was related to the protein content of the samples. Microsomal and cytosolic GST (mGST and cGST, EC 2.5.1.18) activity was determined according to Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene as substrate, and following the formation of its glutathione conjugate with absorbance maximum at 340 nm over a 5-min period. Catalase (CAT, EC 1.11.1.6) activity was assayed in the decrease of hydrogen peroxide at 240 nm for 5 min (Claiborne 1985). Glutathione reductase (GR, EC 1.8.1.7) activity was determined according to Schaedle and Bassham (1977). The glutathione disulfide dependent NADPH oxidation was followed kinetically at 340 nm over a 3-min period. Glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined according to Livingstone et al. (1992) by measuring the decrease in the co-factor NADPH at 340 nm for 5 min, using hydrogen peroxide as substrate.

4.2.7 STATISTICAL ANALYSIS

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software (version 21, SPSS, Inc, Chicago, IL, USA; $\alpha = 0.05$, 95 % CI). Data were tested for normality and homogeneity of variance using Shapiro-Wilk test and Levene test, respectively. Variables were log-transformed ($\log_{10}$) if data were non-normally distributed. The Dunnett test and Student's t-test were used to assess the differences between means, if variables were homogeneous or heterogeneous, respectively. Data were displayed as means ± standard errors (SE, $n = 5$). P value < 0.05 was set for statistical significance.

4.3 RESULTS

Oxidative stress tolerance is important when considering a fungus for mycoremediation application as it indicates the fungus's ability to cope with exposure to the xenobiotics in question. We therefore exposed *M. hiemalis* to three structurally diverse cyanotoxins, namely MC-LR, CYN, and BMAA, and evaluated the effects on the antioxidative enzymes CAT, GR, and GPx, as well as the biotransformation enzyme GST.

4.3.1 EXPOSURE CONCENTRATION CONFIRMATION

Table 4-1: Nominal and mean ± SD (n = 5) of the actual toxin exposure concentrations [μg l^{-1}] during the 24 h exposure (measured by LC-MS/MS).

Toxin / nominal concentration	MC-LR	CYN	BMAA
Control (0 μg l^{-1})	ND	ND	ND
1 μg l^{-1}	0.94 ± 0.02	1.40 ± 0.16	1.15 ± 0.21
10 μg l^{-1}	11.3 ± 2.9	10.8 ± 0.66	10.3 ± 0.57
100 μg l^{-1}	102.1 ± 6.7	103.4 ± 5.8	109.1 ± 7.9

ND = not detected

Table 4-1 shows mean values of quantified exposure media concentrations measured by LC-MS/MS of five replicates versus selected nominal concentrations ranging from 1 to 100 μg l^{-1} for the three cyanotoxins individually.

4.3.2 RESPONSE OF THE ANTIOXIDATIVE ENZYME SYSTEM

The enzymes CAT, GR, and GPx were selected as markers for oxidative stress and the effect on the antioxidative defense system in *M. hiemalis* upon exposure to MC-LR (Fig. 4-1), CYN (Fig. 4-2), and BMAA (Fig. 4-3) was evaluated independently by comparing different toxin concentrations to a control group, respectively.

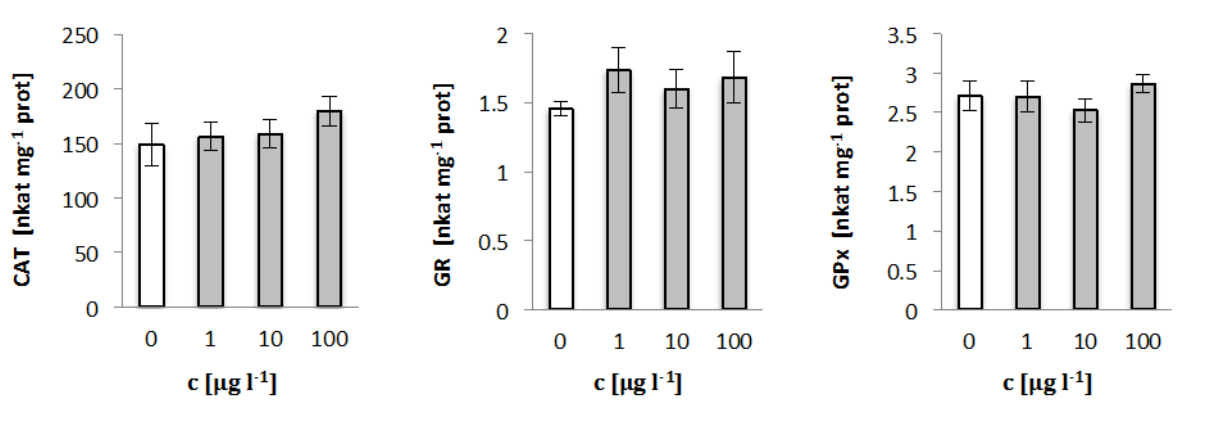

Fig. 4-1: Antioxidative enzyme activities of CAT, GR, and GPx [nkat mg^{-1} protein] in *M. hiemalis* after 24 h exposure to various concentrations of MC-LR. Quantified actual concentrations are given in Table 4-1. Data represents mean ± SE ($n = 5$).

The activities of all three antioxidative enzymes tested remained constant with exposure to MC-LR ranging in concentration from 1 to 100 µg l^{-1} and were similar to enzyme responses in the control ($p > 0.05$) (Fig. 4-1), showing that the toxin does not induce oxidative stress in *M. hiemalis*. In contrast, enzyme activities changed in the presence of CYN (Fig. 4-2). Of the used cyanotoxins, CYN was the only toxin that affected all three enzymes tested and thus caused oxidative stress. CAT activity visibly increased with increasing CYN exposure concentration, which was significant at 100 µg l^{-1} with 31 % increase compared to the control ($p < 0.001$). GR and GPx activities were inhibited in the presence of CYN in a concentration-dependent manner. GR activity decreased by 25 % at 100 µg l^{-1} ($p < 0.001$). GPx activity decreased by 18 % at 10 µg l^{-1} ($p = 0.01$) and by 33 % at 100 µg l^{-1} ($p < 0.001$).

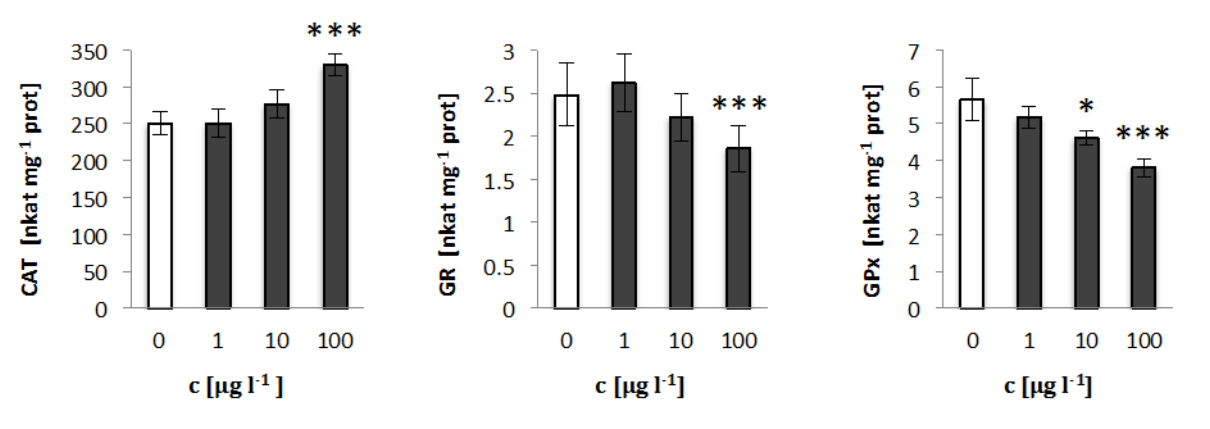

Fig. 4-2: Antioxidative enzyme activities of CAT, GR, and GPx [nkat mg^{-1} protein] in *M. hiemalis* after 24 h exposure to various concentrations of CYN. Quantified actual concentrations are given in Table 4-1. Data represents mean ± SE ($n = 5$). Asterisks indicate significant statistical differences compared to the control with * $p < 0.05$, *** $p < 0.001$.

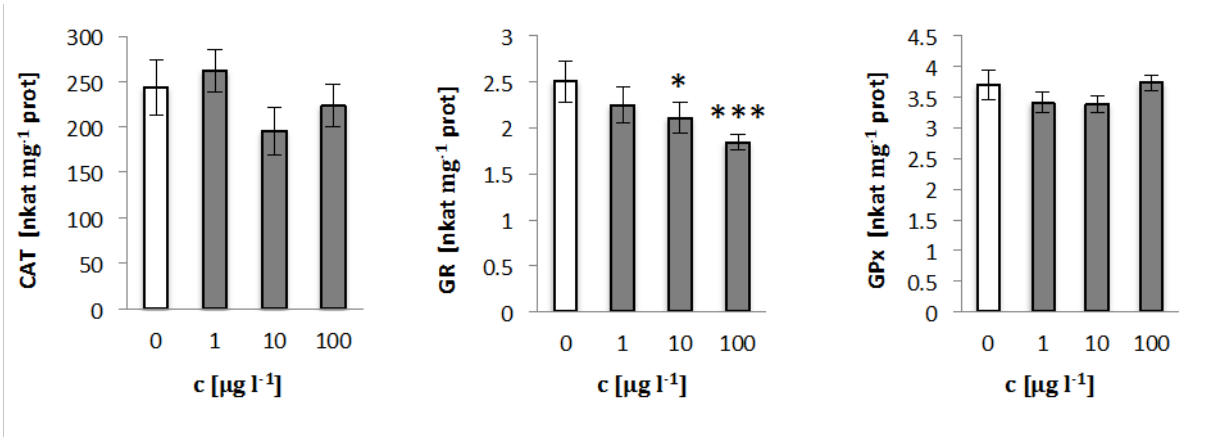

Fig. 4-3: Antioxidative enzyme activities of CAT, GR, and GPx [nkat mg^{-1} protein] in *M. hiemalis* after 24 h exposure to various concentrations of BMAA. Quantified actual concentrations are given in Table 4-1. Data represents mean ± SE (n = 5). Asterisks indicate significant statistical differences compared to the control with * $p < 0.05$, *** $p < 0.001$.

Exposure to BMAA (Fig. 4-3) provoked exclusively alterations in GR activity, a decrease at 10 ($p = 0.01$) and 100 µg l^{-1} ($p < 0.001$) by 16 and 26 % compared to the control, respectively. However, CAT and GPx activity remained unchanged compared to the control within the whole concentration range ($p > 0.05$).

4.3.3 RESPONSE OF THE BIOTRANSFORMATION ENZYME SYSTEM

The responses of the biotransformation enzyme GST after exposure of *M. hiemalis* to MC-LR, CYN, and BMAA are shown in Fig. 4-4 to 4-6.

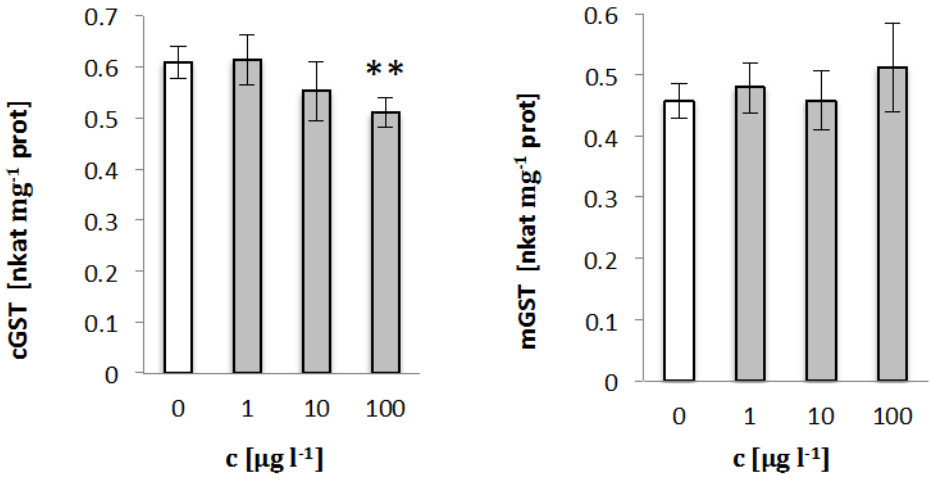

Fig. 4-4: Enzymatic responses of GST activities from the soluble cytosolic (cGST) and membrane-bound microsomal (mGST) [nkat mg^{-1} protein] protein fractions in *M. hiemalis* after 24 h exposure to various concentrations of MC-LR. Quantified actual concentrations are given in Table 4-1. Data are means ± SE (n = 5). Asterisks indicate significant statistical differences compared to the control with ** $p < 0.01$.

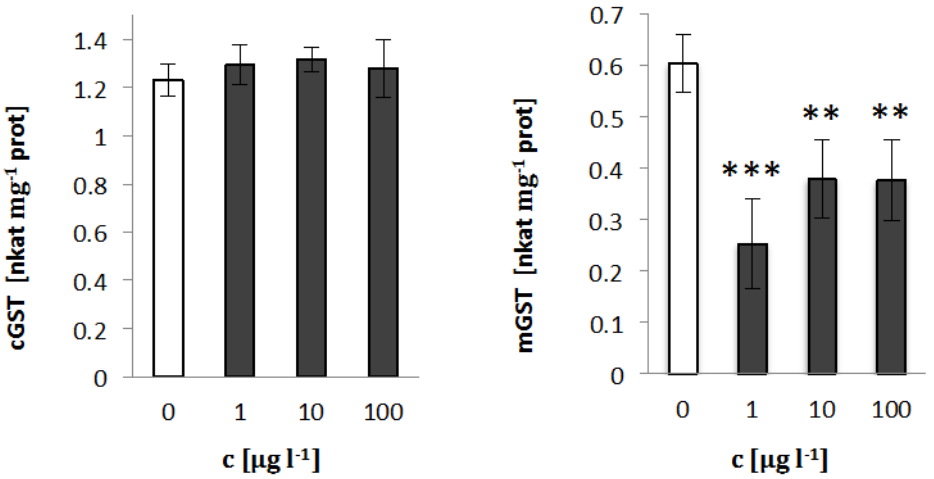

Fig. 4-5: Enzymatic responses of GST activities from the soluble cytosolic (cGST) and membrane-bound microsomal (mGST) [nkat mg^{-1} protein] protein fractions in *M. hiemalis* after 24 h exposure to various concentrations of CYN. Quantified actual concentrations are given in Table 4-1. Data are means ± SE (n = 5). Asterisks indicate significant statistical differences compared to the control with ** $p < 0.01$, *** $p < 0.001$.

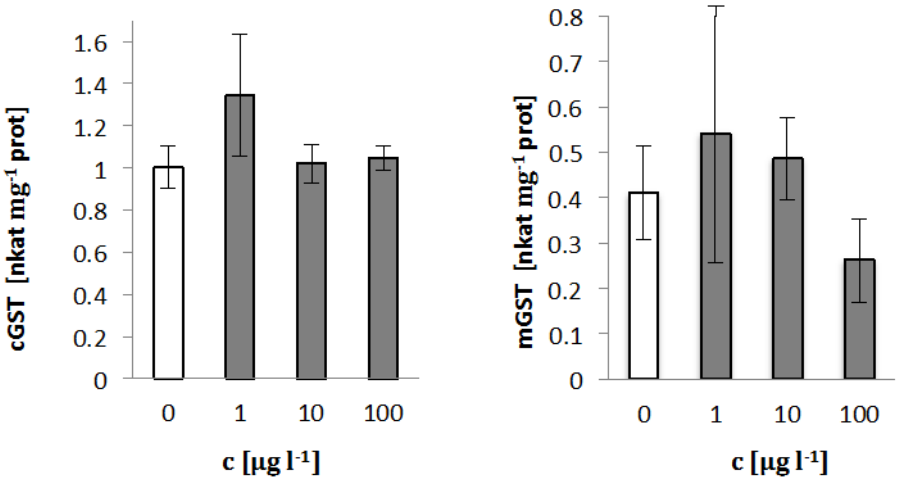

Fig. 4-6: Enzymatic responses of GST activities from the soluble cytosolic (cGST) and membrane-bound microsomal (mGST) [nkat mg^{-1} protein] protein fractions in *M. hiemalis* after 24 h exposure to various concentrations of BMAA. Quantified actual concentrations are given in Table 4-1. Data are means ± SE (n = 5).

After 24 h of exposure, cGST decreased by 16 % with exposure to 100 µg MC-LR l^{-1} ($p = 0.002$), whereas mGST remained unaffected for all MC-LR exposure concentrations (Fig. 4-4). In contrast, significant decreases of mGST activities were observed with CYN exposure at all concentrations assayed in comparison to the control group (Fig. 4-5); with 1 µg CYN l^{-1} the enzyme activity was inhibited by 58 % ($p < 0.001$), and with exposure to both, 10 and 100 µg l^{-1} mGST decreased by 37 % ($p < 0.01$). However, cGST activity

remained unchanged. BMAA did not have any effect on either cGST or mGST activity ($p > 0.05$), and for all concentrations used in the experiment (Fig. 4-6).

4.4 DISCUSSION

Oxidative stress is an important biomarker to assay when considering the mycoremediation potential of a fungus as it imposes significant effects on fungal cultures in terms of morphology, growth rate, metabolism, and protein secretion (Li et al. 2011) and can lead to growth and reproduction inhibition and/or mortality of the organism. Hence, elucidation of the enzymatic responses upon exposure to cyanotoxins provides insight on the applicability and longevity of the fungal strain in question. Oxidative stress-related mechanisms are associated with the toxicity exerted by cyanotoxins and has been clearly reported in many organisms. However, to our knowledge, information on enzymatic responses, including defense mechanisms in fungi upon cyanotoxin exposure is lacking. The metabolism of a toxin in a living organism can include phase II biotransformation. These reactions involve covalent attachment of small polar endogenous molecules such as glutathione to form hydrophilic compounds that are less toxic compared to the parent compound (Kondo et al. 1992) and more easily excreted by the organism. The biotransformation enzyme GST catalyzes such conjugation reactions. Hence, GST activity investigation may elucidate possible conjugation pathway as a detoxification mechanism of cyanotoxins by the fungus *M. hiemalis*, which is known to express high levels of intracellular GST (Hoque et al. 2007).

Although MC-LR is considered one of the most toxic cyanotoxins worldwide with negative effects on the enzyme expressions in aquatic plants (Jiang et al. 2011; Pflugmacher 2004) and aquatic animals (Amado and Monserrat 2010), our results showed that it had no impact on the antioxidative system in *M. hiemalis*. The conjugation of MC-LR with glutathione catalyzed by GST is considered the primary detoxification route in many organisms (Pflugmacher et al. 1998; Wang et al. 2006). Activity of GST was not considerably affected; MC-LR provoked inhibition of cGST, however only at the highest concentration used, whereas mGST maintained its activity within the whole concentration range. It is possible that *M. hiemalis* detoxifies MC-LR *via* biotransformation phase II and hence, exposure to MC-LR does not result in oxidative stress for the fungal organism. However, this needs further investigation, with a longer exposure time and identification of metabolic products.

In contrast, CYN affected all three enzymes of the antioxidative stress system tested and clearly showed to have effects on the enzymatic level of *M. hiemalis*. The most pronounced effect was observed by the selective inhibition of the glutathione cycle enzymes. Both

enzymes of the glutathione pathway, GR and GPx, responded to CYN in a concentration-dependent decrease of the enzyme activities. CYN depletes glutathione (Norris et al. 2002) and in turn depletion of glutathione might lead to fundamental disturbances in the defense against oxidative stress; this could be the explanation for the decrease in the activity of all enzymes that use glutathione as a co-factor for their catalytic activity, including GST. In the present study, mGST was clearly inhibited at all CYN concentrations tested. Glutathione serves as a co-substrate in the biotransformation of xenobiotics and lipid peroxidation products. Therefore, depletion of glutathione and partial inactivation of GST in *M. hiemalis* due to CYN exposure might disturb essential cell functions, result in the defense inability against toxins, and lipid peroxidation intermediates *via* the biotransformation phase II and so lead to enhanced oxidative stress and elevated susceptibility of the aquatic fungus. Comparison of the modes of action of CYN between fungal and plant or animal enzyme responses reveals difference in the pattern of enzyme expressions. While all glutathione involved enzymes were inhibited in *M. hiemalis*, all enzymes tested in the aquatic plant *L. minor* L, such as CAT, GST, and GR were enhanced upon CYN exposure (Flores-Rojas et al. 2015). Enhanced GST, but reduced GPx were shown in tilapia fish (Puerto et al. 2011). This clearly shows the induction of oxidative stress by CYN in aquatic plants, animals, and fungi (as shown in the present study), however, the defense systems of diverse organisms reply with different patterns of oxidative stress responses.

BMAA caused minimal effects on the antioxidative enzymes of *M. hiemalis*, the enzymes CAT and GPx remained unchanged, and only GR was inhibited. BMAA has a high affinity for chelating transition metals, such as nickel (II), copper (II) and zinc (II) (Nunn et al. 1989) and could directly interact with the catalytic metal centers of enzymes, leading to conformational change and biological activity loss as suggested as a reason for inhibition in the aquatic macrophyte *C. demersum* (Esterhuizen-Londt et al. 2011a). Interestingly, these findings could not be translated on the effect that BMAA had on the enzymatic responses in the aquatic fungus *M. hiemalis*, where solely the enzyme GR, not containing a catalytic metal center, was inhibited and metal-containing oxidative stress response enzymes, CAT and GPx, maintained their activity, showing that chelation is unlikely and/or does not trigger oxidative stress in *M. hiemalis*. As BMAA is a small and hydrophilic amino acid it is suggested that GST is not involved in the detoxification process. Moreover, the constant pattern throughout the concentration range predicts that the defense system through phase II conjugation in *M. hiemalis* is not affected in the presence of BMAA and therefore together with the

negligible impact on GR activity suggested that BMAA did not have considerable negative impacts on the antioxidative enzymes' activities in *M. hiemalis*.

This is the first report of enzymatic responses in fungi upon cyanotoxin exposure and shows toxin- and concentration-dependent effects. Alterations in antioxidative enzyme activities as a response to oxidative stress was not observed for all cyanotoxins tested, which seemed to depend on molecular structure and function. Based on the results, we inferred that no or negligible oxidative stress was observed in the presence of MC-LR and BMAA, and that the fungus could be suitable in terms of viability and longevity regarding these two toxins. In contrast, antioxidative enzymes and in parts the biotransformation enzyme GST were inhibited in the presence of CYN, which can lead to an accumulation of reactive oxygen species from normal cell function over to cell damage and cell death, and can cause the fungus to be more susceptible to other xenobiotics and environmental stress. But even though we show that the antioxidative enzyme activities were affected in *M. hiemalis* as a biochemical feature of CYN, the aquatic fungus may either overcome or cope with the possible resulting oxidative stress, as an overall growth fitness of *M. hiemalis* with no significant decrease in biomass production compared to the control was previously reported in the presence of up to 1,000 µg CYN l^{-1} after one-week exposure period (Balsano et al. 2015). Therefore, studies over a longer time period are necessary to see if oxidative stress can be overcome.

4.5 CONCLUSION

Based on the data, we can confirm the tolerance of *M. hiemalis* against MC-LR and BMAA on the enzymatic level and therefore suggest its longevity in their presence. Moreover, *M. hiemalis* may overcome the inhibited antioxidative enzyme activities due to CYN exposure, as previously evaluated overall growth fitness has been demonstrated. We therefore further confirm the applicability of the aquatic fungus *M. hiemalis* for water purification in terms of toxin tolerance and its ability of toxin uptake (Balsano et al. 2015). However, in order to discuss its effectiveness as a cyanotoxin remediating microorganism, especially for MC-LR that did not produce oxidative stress and could possibly be biotransformed *via* glutathione conjugation, further investigations are needed to examine the degradation and removal of toxins from media by *M. hiemalis* over a longer exposure period and to identify possible degradation products.

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REFERENCES

Amado LL, Monserrat JM (2010) Oxidative stress generation by microcystins in aquatic animals: Why and how. Environ Int 36:226-235. doi: 10.1016/j.envint.2009.10.010

Balsano E, Esterhuizen-Londt M, Hoque E, Pflugmacher S (2015) Toxin resistance in aquatic fungi poses environmentally friendly remediation possibilities: A study on the growth responses and biosorption potential of *Mucor hiemalis* EH5 against cyanobacterial toxins. Int J Water Wastewater Treat 1:1-9. doi: 10.16966/ijwwwt.101

Balsano E, Esterhuizen-Londt M, Hoque E, Pflugmacher S (2016) Fungal pellets as potential tools to control water pollution: Strategic approach for the pelletization and subsequent microcystin-LR uptake by *Mucor hiemalis*. J App Biol Biotech 4:031-041. doi: 10.7324/JABB.2016.40403

Carvalho MB, Tavares S, Medeiros J, Núñez O, Gallart-Ayala H, Leitão MC, Galceran MT, Hursthouse A, Silva Pereira C (2011) Degradation pathway of pentachlorophenol by *Mucor plumbeus* involves phase II conjugation and oxidation–reduction reactions. J Haz Mat 198:133-142. doi: 10.1016/j.jhazmat.2011.10.021

Claiborne A (1985) Catalase activity. In: Greenwald RA (ed) Handbook of methods for oxygen radical research, CRC Press, Boca Raton, FL, pp 283-284

Corbel S, Mougin C, Bouaïcha N (2014) Cyanobacterial toxins: Modes of actions, fate in aquatic and soil ecosystems, phytotoxicity and bioaccumulation in agricultural crops. Chemosphere 96:1-15. doi: 10.1016/j.chemosphere.2013.07.056

Esterhuizen-Londt M, Kühn S, Pflugmacher S (2015) Development and validation of an in-house quantitative analysis method for cylindrospermopsin using hydrophilic interaction liquid chromatography–tandem mass spectrometry: Quantification demonstrated in 4 aquatic organisms. Environ Toxicol Chem 34:2878-2883. doi: 10.1002/etc.3138

Esterhuizen-Londt M, Downing S, Downing TG (2011) Improved sensitivity using liquid chromatography mass spectrometry (LC-MS) for detection of propyl chloroformate derivatised β-*N*-methylamino-L-alanine (BMAA) in cyanobacteria. WaterSA 37:133-138.

Esterhuizen-Londt M, Pflugmacher S, Downing TG (2011) The effect of β-*N*-methylamino-L-alanine (BMAA) on oxidative stress response enzymes of the macrophyte *Ceratophyllum demersum*. Toxicon 57:803-810. doi: 10.1016/j.toxicon.2011.02.015

Esterhuizen-Londt M, von Schnehen M, Kühn S, Pflugmacher S (2016) Oxidative stress responses in the animal model, *Daphnia pulex* exposed to a natural bloom extract versus artificial cyanotoxin mixtures. Aquat Toxicol 179:151-157. doi: 10.1016/j.aquatox.2016.09.003

Flores-Rojas NC, Esterhuizen-Londt M, Pflugmacher S (2015) Antioxidative stress responses in the floating macrophyte *Lemna minor* L. with cylindrospermopsin exposure. Aquat Toxicol 169:188-195. doi: 10.1016/j.aquatox.2015.11.002

Guo X, Xie P, Chen J, Tuo X, Deng X, Li S, Yu D, Zeng C (2014) Simultaneous quantitative determination of microcystin-LR and its glutathione metabolites in rat liver by liquid chromatography–tandem mass spectrometry. J Chromatogr B 963:54-61. doi: 10.1016/j.jchromb.2014.05.046

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione *S*-transferase: the first step in mercapturic acid formation. J Biol Chem 249:7130-7129

Hoque E (2003) Verfahren zum Abbau von Xenobiotika durch Pilzarten mit Monooxygenase-/Dioxygenase-Aktivität in Gegenwart von Pilzen mit Glutathion-*S*-Transferase-Aktivität, Patentnr.: DE10125365 C2

Hoque E, Fritscher J (2016) A new mercury-accumulating *Mucor hiemalis* strain EH8 from cold sulfidic spring water biofilms. MicrobiologyOpen 5:763-781. doi: 10.1002/mbo3.368

Hoque E, Pflugmacher S, Fritscher J, Wolf M (2007) Induction of glutathione *S*-transferase in biofilms and germinating spores of *Mucor hiemalis* strain EH5 from cold sulfidic spring waters. Appl Environ Microbiol 2697-2707. doi: 10.1128/AEM.02786-06

Jiang J, Gu X, Song R, Wang X, Yang L (2011) Microcystin-LR induced oxidative stress and ultrastructural alterations in mesophyll cells of submerged macrophyte *Vallisneria natans* (Lour.) Hara. J Haz Mat 190:188-196. doi: 10.1016/j.jhazmat.2011.03.023

Kondo F, Ikai Y, Oka H, Okumura M, Ishikawa N, Harada K-i, Matsuura K, Murata H, Suzuki M (1992) Formation, characterization, and toxicity of the glutathione and cysteine conjugates of toxic heptapeptide microcystins. Chem Res Toxicol 5:591-596. doi: 10.1021/tx00029a002

Li Q, Bai Z, O'Donnell A, Harvey LM, Hoskisson PA, McNeil B (2011) Oxidative stress in fungal fermentation processes: the roles of alternative respiration. Biotechnol Lett 33:457-467. doi: 10.1007/s10529-010-0471-x

Livingstone DR, Lips F, Martinez PG, Pipe RK (1992) Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*. Mar Biol 112:265-276. doi: 10.1007/bf00702471

Norris RLG, Seawright AA, Shaw GR, Senogles P, Eaglesham GK, Smith MJ, Chiswell RK, Moore MR (2002) Hepatic xenobiotic metabolism of cylindrospermopsin *in vivo* in the mouse. Toxicon 40:471-476. doi: 10.1016/S0041-0101(01)00243-4

Nunn PB, O'Brien P, Pettit LD, Pyburn SI (1989) Complexes of zinc, copper, and nickel with the nonprotein amino acid L-α-amino-β-methylaminopropionic acid: A naturally occurring neurotoxin. J Inorg Biochem 37:175-183. doi: 10.1016/0162-0134(89)80040-6

Pflugmacher S (2004) Promotion of oxidative stress in the aquatic macrophyte *Ceratophyllum demersum* during biotransformation of the cyanobacterial toxin microcystin-LR. Aquat Toxicol 70:169-178. doi: 10.1016/j.aquatox.2004.06.010

Pflugmacher S, Wiegand C, Oberemm A, Beattie KA, Krause E, Codd GA, Steinberg C (1998) Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. Biochim Biophys 1425:527-533. doi: 10.1016/S0304-4165(98)00107-X

Puerto M, Jos A, Pichardo S, Gutiérrez-Praena D, Cameán A (2011) Acute effects of pure cylindrospermopsin on the activity and transcription of antioxidant enzymes in tilapia (*Oreochromis niloticus*) exposed by gavage. Ecotoxicology 20:1852-1860. doi: 10.1007/s10646-011-0723-0

Schaedle M, Bassham JA (1977) Chloroplast glutathione reductase. Plant Physiol 59:1011-1012. doi: 10.1104/pp.59.5.1011

Shetty PK, Mitra J, Murthy NBK, Namitha KK, Savitha KN, Raghu K (2000) Biodegradation of cyclodiene insecticide endosulfan by *Mucor thermo-hyalospora* MTCC 1384. Curr Sci 79:1381-1383

Wang L, Liang X-F, Liao W-Q, Lei L-M, Han B-P (2006) Structural and functional characterization of microcystin detoxification-related liver genes in a phytoplanktivorous fish, Nile tilapia (*Oreochromis niloticus*). Comp Biochem Physiol C Toxicol Pharmacol 144:216-227. doi: 10.1016/j.cbpc.2006.08.009

5 FUNGAL DEGRADATION OF MICROCYSTIN-LR

The chapter represents unpublished results (manuscript in preparation) of the first MC-LR removal attempts using *M. hiemalis* and *P. chrysosporium* in single and co-culture.

5.1 INTRODUCTION

Microcystin-LR (MC-LR) is a toxic secondary metabolite produced by many cyanobacteria worldwide. During cyanobacterial cell lysis, thus toxin release, concentrations in surface waters can reach values that are dangerous to aquatic life forms and humans (DeMott et al., 1991; Falconer, 1991). Studies regarding mycoremediation of cyanotoxins are to date very limited (Mohamed et al., 2014), but could, however, represent a promising strategy for their removal. The white-rot fungus *P. chrysosporium* is reported to degrade many persistent compounds with the aid of its lignolytic system, extracellular laccases and peroxidases, as well as intracellular biotransformation enzymes. Its degradation ability finds technical application in bioreactors, e.g. for the treatment of hazardous waste (Shim and Kawamoto, 2011), the decolorization of textile dyes from industrial effluents (Pakshirajan and Singh, 2010), as well as the removal of pharmaceuticals (Rodarte-Morales et al., 2012). *M. hiemalis* is used in several industrial processes as it produces lipases and proteases (Alves et al., 2002; Hiol et al., 1999). Moreover, it exhibits high glutathione *S*-transferase activity against fluoridifen and high tolerance against hydrogen sulfide, as well as temperature independency with ability to sporulate still at 5 °C (Hoque et al., 2003, 2007). Functional groups on the fungal surface enable biosorption and removal of heavy metals (Hoque and Fritscher, 2016; Shroff and Vaidya, 2011; Tewari et al., 2005). In co-culture with *P. chrysosporium*, fast and complete degradation of the herbicide isoproturon was achieved (Hoque, 2003; Rønhede, 2005). The aim of the present work was to investigate the degradation ability of *M. hiemalis* and *P. chrysosporium* in pellet morphology in single- as well as co-culture towards the highly abundant cyanotoxin MC-LR and present mycoremediation as a sustainable and potential technique for the removal of cyanotoxins.

5.2 METHODS

5.2.1 FUNGAL STRAINS

M. hiemalis EH5 (DSM 14200) was purchased from the culture collection of the Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures, and *P. chrysosporium* ATCC® 24725™ from ATCC, the American Type Culture Collection, Manassas, VA 20108 USA.

5.2.2 REVIVING FUNGAL CULTURE

M. hiemalis was obtained as an activated agar slant and was directly inoculated to petri dishes (section 5.2.3). *P. chrysosporium* was revived from a freeze-dried culture according to the ATCC instructional guide for Reviving Freeze-Dried Microorganisms, as follows. Double-vial was opened with a flame, insulation and inner vial, and cotton were gently removed with forceps, 1 mL sterile distilled water was directly added to the pellet, and stirred suspension was aseptically transferred to a falcon tube and filled to 5 mL total volume. The culture was rehydrated over night at 25 °C to obtain increased viability. Suspension was mixed well, and several drops were used to inoculate agar plates.

5.2.3 CULTIVATION AND PELLETIZATION OF FUNGI

Cultivation and pelletization of *M. hiemalis* were performed as per Balsano et al. (2015, 2016).

Cultivation of *P. chrysosporium* (ATCC® 24725™) was carried out following the ATCC company's product sheet on solid agar medium containing 15 g/L Sabouraud dextrose broth (SAB), 3 g/L malt extract, 20 g/L agar bact oxoid No 1 in deionized H_2O, at 30 °C under typical aerobic conditions and in the dark. Basidiospores were released after ultrasonic treatment of 3-4 weeks grown mycelial mat in water bath for 60 min and sieved through a strainer with mesh size 0.5 mm. Spore suspensions were centrifuged (15 min, 4,000 x *g*) and washed three times with sterile distilled water. The fungal spore stock of 10^7 spores / mL was determined by Neubauer hemocytometer count. The growth medium (100 mL SAB, pH 5.6 ± 0.2) was inoculated with 1.0 % (v/v) of the spore stock and then incubated on an orbital shaker in the dark at 30 °C and 150 rpm.

Co-pellets were obtained accordingly in SAB medium pH 7.5, with 10^3 *M. hiemalis* spores / mL and 10^5 *P. chrysosporium* spores / mL, agitation rates of 130 and 150 rpm, as well as temperatures of 20 and 30 °C to stimulate predominant *M. hiemalis* and *P. chrysosporium* growth, respectively.

5.2.4 EXPOSURE OF FUNGI TO MC-LR

After seven days of growth, single- and co-pellets were washed with sterile deionized water and approximately 100 mg (dw) thereof were separately transferred to 100 mL Erlenmeyer flasks in replicates of five and were exposed to 100 µg/L MC-LR at 20 and/or 30 °C. The control contained only 100 µg/L MC-LR, in order to determine the natural degradation rate. One set was conducted in replicates of five for each time point in order to harvest fungal biomass for uptake studies. Removal efficiency was compared in nutrient-rich (SAB) and limiting medium. The limiting medium consisted of 0.2 g/L calcium chloride, 0.103 g/L sodium bicarbonate, 0.1 g/L sea salt, 5 g/L glucose, and 0.05 g/L ammonium chloride.

5.2.5 SAMPLE PREPARATION AND LC-MS/MS MEASUREMENT

Samples (1 mL) were collected at the inception of the experiment and after 24 h, 96 h, and 7 d for all exposures, as well as 14 d, however, only for *M. hiemalis* in SAB and related uptake studies. MC-LR extraction from pellets and subsequent quantification of internalized toxin, as well as media concentration over time was performed as previously described (Balsano et al., 2015, 2016).

5.3 RESULTS AND DISCUSSION

The degradation efficiency of *M. hiemalis* was estimated by measuring the reduction of MC-LR in media over time *via* LC-MS/MS with comparison to the control, which was run under the same conditions but in the absence of fungal pellets (Fig. 5-1).

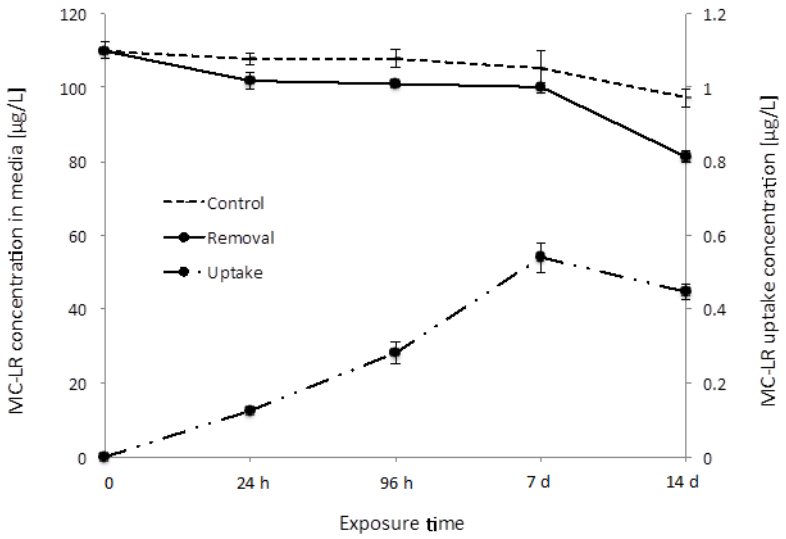

Fig. 5-1: MC-LR uptake and removal from medium by *M. hiemalis* in single-culture in SAB medium at 20 °C over time, expressed as micrograms per liter. Data represent mean ± SE (n = 5).

Increased uptake into pellets with time was shown over a 7 d period with decreased uptake at day 14. Greatest removal from medium, however, was observed after 14 d, which evidences additional mechanisms potentially involved in the removal process, and suggests enzymatic breakdown to be more likely than intracellular storage and bioaccumulation. Of the 26 % of the initial applied MC-LR that has been removed from medium after 14 d exposure and fungal treatment, 11 % can be attributed to photodegradation, adsorbance on glass walls, binding to medium components *etc*. as it has been also observed in the control. Only 0.4 % of the parent compound has been found internalized within the fungal pellets; the remaining 14.6 % that were eliminated from medium but were not detected in the fungus might have in parts been adsorbed on the fungal pellet surface and washed off after sample collection, but most importantly might have been removed by internal and/or external fungal degradation.

These results suggest indeed MC-LR degradation by *M. hiemalis*, however the resulting degradation efficiency is not sufficient for mycoremediation purposes and needs to be improved. Therefore, degradation by *M. hiemalis* was compared in nutrient-rich *vs* limiting media and was successfully improved when the fungus was exposed under starving conditions in limiting media (Fig. 5-2).

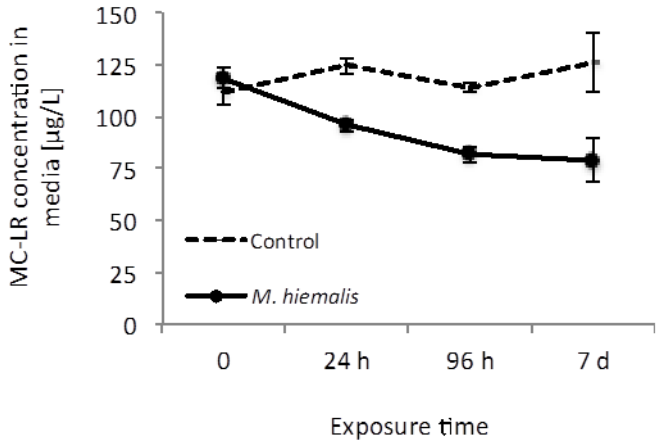

Fig. 5-2: MC-LR removal from medium by *M. hiemalis* in single culture in limiting medium at 20 °C over time, expressed as micrograms per liter. Data represent mean ± SE (n = 5).

Degradation efficiency, expressed as percentage removal after seven days from initial MC-LR concentration, was 5 % in nutrient-rich medium (Fig. 5-1) and reached 30 % in limiting medium. In addition, the degradation rate was augmented and allowed shortening exposure time; a statistically confirmed removal compared to the control was measured after 14 d exposure in nutrient-rich medium (Fig. 5-1) whereas in limiting medium, there was a noticeable statistical difference already after 24 h fungal treatment (Fig. 5-2).

Mycoremediation of MC-LR was compared treating the toxin with *M. hiemalis* and *P. chrysosporium*, respectively in single-culture in order to compare two fungi with different

metabolic characteristics. The white-rot fungus is known to release extracellular lignin-modifying enzymes with low substrate-specificity and intracellular cytochrome P450 monooxygenases posing two possible enzymatic pathways that have been reported responsible for the degradation of various persistent exogenous compounds (Syed and Yadav, 2012). Subramanian and Yadav (2009) evidenced that P450 enzymes are involved in the degradation of nonylphenol in nutrient-rich conditions, but are not responsible for the degradation of the compound in limiting conditions. It is highly probable that these P450 enzymes are predominantly involved in the degradation of M-LR by *P. chrysosporium* as well, because its degradation seemed to be more efficient in nutrient-rich (Fig. 5-3) rather than limiting medium (Fig. 5-4). As MC-LR is faster degraded in non-lignolytic conditions, meaning nutrient-sufficient cultures, where MnP and LiP are not likely produced, it is suggested, that these lignolytic enzymes are not essential for the degradation of MC-LR, but other biotransformation enzymes may be more important.

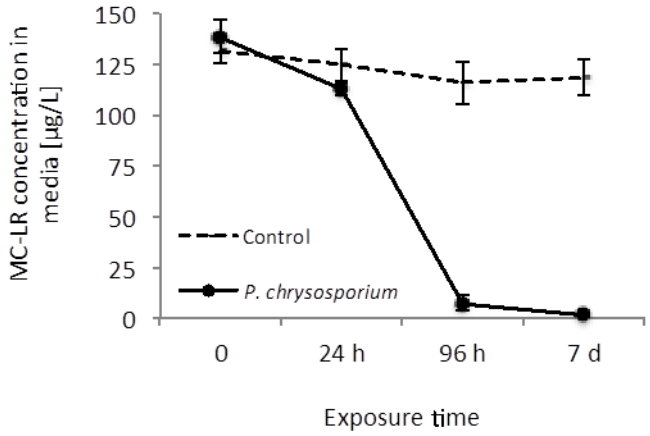

Fig. 5-3: MC-LR removal from medium by *P. chrysosporium* in single culture in nutrient-rich SAB medium at 30 °C over time, expressed as micrograms per liter. Data represent mean ± SE (n = 5).

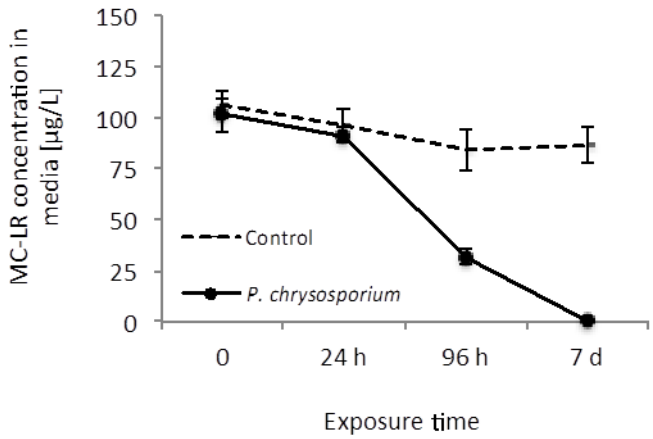

Fig. 5-4: MC-LR removal from medium by *P. chrysosporium* in single culture in limiting medium at 30 °C over time, expressed as micrograms per liter. Data represent mean ± SE (n = 5).

In relation to the model white-rot fungus *P. chrysosporium,* which efficiently degraded MC-LR in non-lignolytic as well as in lignolytic conditions and completely removed the cyanotoxin within seven days from medium (Fig. 5-3 and 5-4), degradation by *M. hiemalis* was small and complete degradation from medium was not achievable (Fig. 5-1 and 5-2). However, the disadvantage of *P. chrysosporium* is the need of a temperature setting and external heating, here 30 °C, in order to ensure growth, metabolism, and enzyme production as well as activity; this would lead to high operational expenditure in bioreactor technology and hinder the applicability under environmental realistic conditions. *M. hiemalis* on the other hand sporulates even at groundwater temperatures of 5 °C and is temperature-independent over a broad range. The next step of optimization attempt was therefore to test the degradation efficiency of *M. hiemalis* in co-culture with *P. chrysosporium* at r.t. (20 °C), thereby providing complementary systems: a temperature-resistant fungus with high GST biotransformation II enzyme activity and a white-rot fungus with lignin-modifying and monooxygenases enzymes (Fig. 5-5).

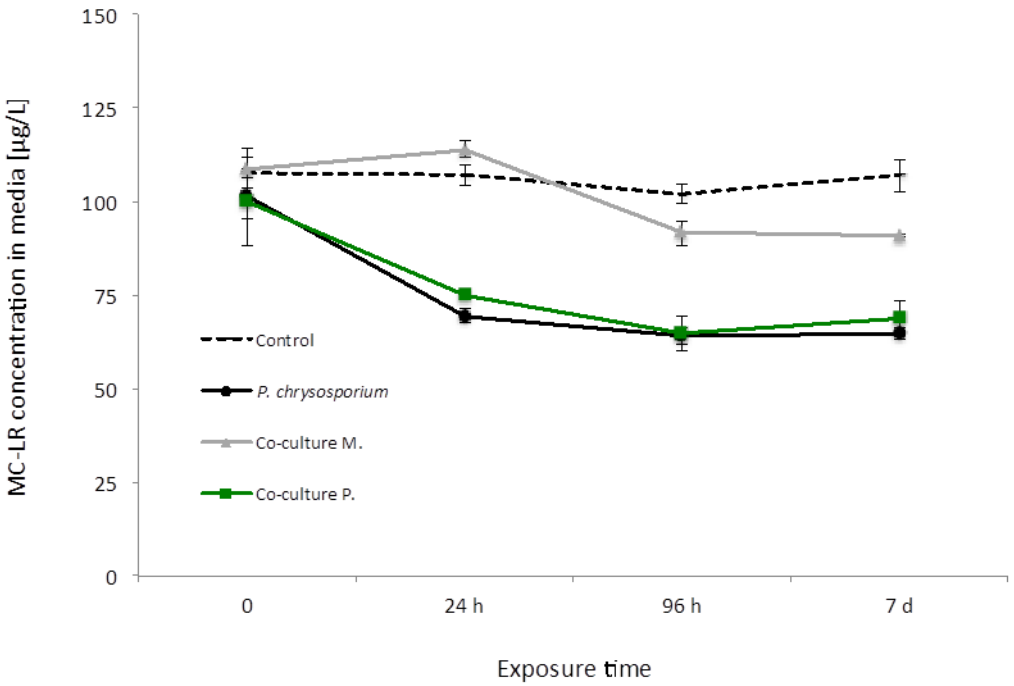

Fig. 5-5: MC-LR removal from medium by *P. chrysosporium* in single culture (black line) and in co-culture with *M. hiemalis* with M. (*M. hiemalis* predominant, grey line) and P. (*P. chrysosporium* predominant, green line) in SAB medium at 20 °C exposure temperature over time, expressed as micrograms per liter. Data represent mean ± SE (n = 5).

The co-culture reached similar degradation efficiency as the lignolytic fungus *P. chrysosporium* in single-culture at 20 °C, and enhanced degradation ability than *M. hiemalis* alone stating that this could be a better alternative than using *M. hiemalis* alone.

5.4 CONCLUSION

Fungal degradation was shown to depend on the fungal strain, its temperature optimum and medium composition. Although *M. hiemalis* was not efficient enough to degrade MC-LR completely, its temperature resistance makes it an attractive candidate to be further investigated. Future studies should therefore include the investigation of co-cultures, especially in combination with *P. chrysosporium*, which was proven to degrade MC-LR within 7 d. Moreover, optimal degradation conditions need to be established, which include the examination of the addition of enzyme inducers, pH optimum, variation of temperature, and carbon/nitrogen sources as well as their ratios. This study shows the application of fungi to degrade cyanotoxins from aquatic media and simultaneously describes a new potential treatment strategy of surface waters containing these natural pollutants.

REFERENCES

Alves, M.H., Campos-Takaki, G.M., Porto, A.L.F., Milanez, A.I., 2002. Screening of *Mucor* spp. for the production of amylase, lipase, polygalacturonase and protease. Brazilian Journal of Microbiology 33, 325-330.

Balsano, E., Esterhuizen-Londt, M., Hoque, E., Pflugmacher, S., 2015. Toxin resistance in aquatic fungi poses environmentally friendly remediation possibilities: A study on the growth responses and biosorption potential of *Mucor hiemalis* EH5 against cyanobacterial toxins. International Journal of Water and Wastewater Treatment 1, 1-9.

Balsano, E., Esterhuizen-Londt, M., Hoque, E., Pflugmacher, S., 2016. Fungal pellets as potential tools to control water pollution: Strategic approach for the pelletization and subsequent microcystin-LR uptake by *Mucor hiemalis*. Journal of Applied Biology and Biotechnology 4, 031-041.

DeMott W.R., Zhang Q.X., Carmichael, W.W., 1991. Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of *Daphnia*. Limnology and Oceanography. 36, 1346-1357.

Falconer I.R., 1991. Tumor promotion and liver injury caused by oral consumption of cyanobacteria. Environmental Toxicology and Water Quality: An International Journal 6, 177-184.

Hiol, A., Jonzo, M.D., Druet, D., Comeau, L., 1999. Production, purification and characterization of an extracellular lipase from *Mucor hiemalis f. hiemalis*. Enzyme and Microbial Technology 25, 80-87.

Hoque, E., 2003. Verfahren zum Abbau von Xenobiotika durch Pilzarten mit Monooxygenase-/Dioxygenase-Aktivität in Gegenwart von Pilzen mit Glutathion-*S*-Transferase-Aktivität, Patentnr: DE10125365 C2.

Hoque, E., Fritscher, J., 2016. A new mercury-accumulating *Mucor hiemalis* strain EH8 from cold sulfidic spring water biofilms. MicrobiologyOpen 5, 763-781.

Hoque, E., Pflugmacher, S., Fritscher, J., Wolf, M., 2007. Induction of glutathione *S*-transferase in biofilms and germinating spores of *Mucor hiemalis* strain EH5 from cold sulfidic spring waters. Applied and Environmental Microbiology, 2697-2707.

Mohamed, Z.A., Hashem, M., Alamri, S.A., 2014. Growth inhibition of the cyanobacterium *Microcystis aeruginosa* and degradation of its microcystin toxins by the fungus *Trichoderma citrinoviride*. Toxicon 86, 51-58.

Rønhede, S., Jensen, B., Rosendahl, S., Kragelund, B.B., Juhler, R.K., J. Aamand. 2005. Hydroxylation of the herbicide isoproturon by fungi isolated from agricultural soil. Applied Environmental Microbiology 71, 7927-7932.

Pakshirajan, K., Singh, S., 2010. Decolorization of synthetic wastewater containing azo dyes in a batch-operated rotating biological contactor reactor with the immobilized fungus *Phanerochaete chrysosporium*. Industrial & Engineering Chemistry Research 49, 7484-7487.

Rodarte-Morales, A.I., Feijoo, G., Moreira, M.T., Lema, J.M., 2012. Operation of stirred tank reactors (STRs) and fixed-bed reactors (FBRs) with free and immobilized *Phanerochaete chrysosporium* for the continuous removal of pharmaceutical compounds. Biochem. Eng. J. 66, 38-45.

Shim, S-S., Kawamoto, K., 2011. Enzyme production activity of *Phanerochaete chrysosporium* and degradation of pentachlorophenol in a bioreactor. Water Research 33, 4445-4454.

Shroff, K.A., Vaidya, V.K., 2011. Kinetics and equilibrium studies on biosorption of nickel from aqueous solution by dead fungal biomass of *Mucor hiemalis*. Chemical Engineering Journal 171, 1234-1245.

Subramanian, V., Yadav, J.S., 2009. Role of P450 monooxygenases in the degradation of the endocrine-disrupting chemical nonylphenol by the white rot fungus *Phanerochaete chrysosporium*. Applied Environmental Microbiology 75, 5570-5580.

Syed, K., Yadav, J.S., 2012. P450 monooxygenases (P450ome) of the model white rot fungus *Phanerochaete chrysosporium*. Critical Reviews in Microbiology 38, 339-363.

Tewari, N., Vasudevan, P., Guha, B.K., 2005. Study on biosorption of Cr (VI) by *Mucor hiemalis*. Biochemical Engineering Journal 23, 185-192.

6 SYNTHESIS AND CONCLUSION

Eutrophic environments and high water temperatures are conducive to the appearance of cyanobacterial blooms that not only affect water quality, but also produce highly toxic secondary metabolites known as cyanotoxins. These pose a serious risk to ecosystems and their livestock, as well as to human health, especially when the water is used for recreational purposes, irrigation, or as a drinking water supply. The removal of cyanotoxins from contaminated waters is essential to ensure the quality and safety of water. Mycoremediation, the intentional application of fungi to degrade and so reduce or eliminate chemical and biological contaminants from polluted systems, offers an environmentally friendly water treatment strategy. *Mucor* spp. have been successfully employed in the remediation of several organic and inorganic compounds, and heavy metals (Carvalho et al., 2011; Hoque and Fritscher, 2016; Jia et al., 2016; Marchut-Mikolajczyk et al., 2015; Mirbagheri et al., 2016; Seo et al., 2007; Shetty et al., 2000; Shroff and Vaidya, 2011; Su et al., 2006; Tewari et al., 2005; Wang et al., 2015). With the focus of this thesis on *M. hiemalis* EH5, this aquatic fungus was used as it is known to express high GST activity and has been applied for the remediation of the herbicide isoproturon involving phase II conjugation as a pathway for degradation in co-culture with *P. chrysosporium* (Hoque, 2003). The conjugation of GSH catalyzed *via* the biotransformation enzyme GST has been found to be the primary detoxification route of many cyanotoxins in plants, animals, and humans (Kondo et al., 1996; Pflugmacher et al., 1998b; Takenaka, 2001; Wang et al., 2006). Therefore, mycoremediation of cyanotoxins from water using temperature and toxin resistant *M. hiemalis* in pellet morphology was considered.

The presented work is composed of six chapters. The first is an introductory and problem stating chapter, the three consecutive chapters are articles published in peer-reviewed journals, the fifth chapter represents a manuscript in preparation in parts, and the last embodies general concluding remarks. All are concerned with the utilization of the aquatic fungus, *M. hiemalis* EH5, related to its interaction with cyanotoxins, i.e. growth, uptake, antioxidative and biotransformation enzyme responses and degradation ability. The work aimed to introduce the concept of mycoremediation of cyanotoxins and to discuss the applicability of *M. hiemalis* for future environmental technical devices, especially fungal pellet bioreactors.

6.1 GROWTH AND BIOSORPTION

As cyanotoxins are known for their antimicrobial nature (Frankmölle et al., 1992; Ramos et al., 2015; Zanchett and Oliveira-Filho, 2013), it was necessary to preliminary investigate whether they could express antifungal activity towards the fungus in question and/or if *M. hiemalis* could tolerate cyanotoxins different in their structure and mechanistic action at a broad range of concentration. Paper I represented the first report of fungal growth responses and uptake upon cyanotoxin exposure; three cyanotoxins were considered important for their effect studies: MC-LR, CYN, and BMAA, representing the various cyanotoxin classifications. The first experiments were performed in order to elucidate the influence on the development of *M. hiemalis*. The results of an adaptation of the Kirby-Bauer disk diffusion assay showed the resistance of *M. hiemalis* towards high concentrations (100 mg/L) of the toxins. Mycelia continuously grew over the toxin containing filter paper disks radially without forming any inhibition zones around the contaminated areas, thereby showing that the fungus was able to grow in the presence of cyanobacterial toxins. Additionally, measuring radial growth rate on petri dishes helped to understand if the fungal growth rate was affected upon cyanotoxin exposure at concentrations ranging from 5 – 1,000 µg/L. *M. hiemalis* expanded radially at constant rates of 11.2 ± 0.5 mm per day over a 7 d period with statistically confirmed growth rate similarities between toxin-treated fungi within the whole concentration range tested and untreated control fungi. *M. hiemalis* grew branched in lateral and aerial expansion. In order to include aerial growth, biomass production was determined in the presence and absence of toxins after 7 d incubation to estimate its overall growth and developmental fitness. Results showed that cyanotoxins did not affect biomass production of *M. hiemalis,* as no statistical difference was observed between treated and untreated controls.
Although cyanotoxins have been shown to exhibit antimicrobial properties (Frankmölle et al., 1992; Ramos et al., 2015; Zanchett and Oliveira-Filho, 2013) and can act as growth inhibitors (Máthé et al., 2007; Pflugmacher, 2002), they did neither affect fungal growth nor biomass production of *M. hiemalis*, which proved the fungus's overall growth fitness and undisturbed development in the presence of cyanotoxins and that the toxins are not lethal to the fungus. This outcome stimulated further investigation on toxin uptake ability. Mycelia that were exposed for 24 and 48 h to 1,000 µg cyanotoxin/L, showed biosorption capacity with detection of 1.95 to 2.9 % of the total applied MC-LR amount internalized within the fungal hyphae. In relation to three aquatic macrophytes, which reached uptake between 0.6 and 1.75 % from the total initial MC-LR content after 24 h exposure (Pflugmacher et al., 1998a), uptake by the aquatic fungus was nearly by a factor of two higher. Comparable uptake was

demonstrated as well for BMAA and CYN in the present study. However, for all cyanotoxins, no time-dependent uptake was observed with statistically confirmed similarities between the two time points; thus possible bioaccumulation cannot be concluded, but a longer exposure period is necessary in order to discuss the rate of uptake/efflux and possible bioaccumulation. Most importantly, the concept of cyanotoxin uptake by fungi was hereby introduced and together with the demonstrated adaptation ability of *M. hiemalis* towards cyanotoxins, the aquatic fungus seems to be a promising candidate to be further investigated for the implementation in mycoremediation. The experiments to follow involved *M. hiemalis* grown as fungal pellets, as this growth morphology is advantageous in bioreactor applications (Kim et al., 1983; Olsvik and Kristiansen, 1994).

6.2 PELLET FORMATION

The results in Paper I assured the ability of *M. hiemalis* to grow and develop in the presence of cyanotoxins and internalize the toxins into fungal mycelia, fulfilling essential requirements for the consideration as a mycoremediating agent. An important aspect was to discuss the applicability of the fungal species with focus on bioreactor technology. Dispersed mycelia are only significant in static cultures. In stirred-tank bioreactors or low-shear stress bioreactors (airlift, bubble column), the excessive growth of filamentous mycelia provokes operational problems, interference with bioreactor components, decrease in the productivity due to viscosity increase, and mass transfer limitations, hence negatively affecting the bioremediation capability and efficiency (Allen and Robinson, 1989; Gibbs et al., 2000). For this purpose, the morphological form of *M. hiemalis* growing as compact pellets was chosen in order to facilitate rheology, bioreactor operation and handling by providing decreased broth viscosity, easier separation, improved aeration, stirring, heat transfer, and a larger surface area to reduce mass transfer limitations (Kim et al., 1983; Olsvik and Kristiansen, 1994).

The growth morphologies of filamentous fungi in liquid cultures range from dispersed mycelia to compact pellets and are strongly dependent on the cultivation environment. A large and variable amount of factors play important roles in the influence of fungal growth morphology specific not only for the fungal genus but also for species. The objective of paper II was to analyze the effect of parameter modification on the growth morphology of the filamentous fungus *M. hiemalis*. Media type, temperature, agitation rate, inoculum size, pH, additives, flask shape, and volume are all factors previously reported in literature to influence fungal morphology and have been varied to enhance pellet formation probability of *M. hiemalis*. Paper II evidences the complexity and specificity of the morphological behavior

and discusses similarities and differences in morphological characteristics between *M. hiemalis* and other species as well as genera. Initial pH adjustment was a crucial factor for stimulating pellet formation, but pH was not necessarily further controlled or maintained constant, which is in contrast to the pellet behavior of *M. circinelloides* (Xia et al., 2011) and facilitates the handling of *M. hiemalis*. Inoculum size played an important role as well and should not exceed the critical spore inoculum defined as 10^3 spores/mL. In comparison to other pellet-forming fungi, such as *P. chrysosporium* and *R. oryzae*, which pelletized at inoculum sizes of 10^5 (or more) and 10^9 spores/mL (Gerin et al., 1993; Juan and Qing-biao, 2002; Liu et al., 2008), the inoculum size of *M. hiemalis* was 2 to 6 magnitudes lower, respectively, thus entailing a substantially lower yield requirement of spore harvest to form *M. hiemalis* pellets. Moreover, additive addition needed for other filamentous fungi to grow as pellets, such as the addition of calcium carbonate, calcium peroxide, or trace metals (Liao et al., 2007; Sladdin and Lynch, 1983; Xia et al., 2011; Zhou et al., 2000), did not induce pellet formation in *M. hiemalis*, thereby offering the possibility of a simplified medium formulation without the need of supplements. Temperature did not affect the probability of *M. hiemalis* to grow as pellets, in contrast to *Rhizopus* sp., where temperature enhanced the development and probability of pellet formation (Nyman et al., 2013). In summary, the method established in Paper II describes a reproducible, simple, and high yield pellet production report for *M. hiemalis* and is the first for this *Mucor* species. As pH stability and temperature regulation is not required, low inoculum size and no additives are needed, *M. hiemalis* seems to be a robust candidate for pellet bioreactors and practical and technical difficulties encountered in culturing fungi might be overcome. Consequently, the control and regulation of hyphal extension and pellet size, which is of great importance for the potential application of fungi in continuous operation, are likely to be easily fulfilled.

6.3 ANTIOXIDATIVE AND BIOTRANSFORMATION ENZYME RESPONSES

The production of oxidative stress provoked by exposure to cyanotoxins was reported in many aquatic organisms including plants (Flores-Rojas et al., 2015; Jiang et al., 2011; Pflugmacher, 2004; Saqrane et al., 2007) and animals (Esterhuizen-Londt et al., 2016; Gutiérrez-Praena et al., 2011a; Guzmán-Guillén et al., 2013; Puerto et al., 2011); however, knowledge on the effect of cyanotoxins regarding fungal antioxidative and biotransformation enzyme responses was still lacking. Moreover, fungal oxidative stress tolerance towards cyanotoxins is of special interest when considering the microorganism for mycoremediation purposes, to ensure viability and longevity of the fungal strain. In bioreactors, oxidative stress

can cause significant effects on culture morphology, growth rate, metabolism, and protein secretion (Li et al., 2011) and can lead to growth and reproduction inhibition and/or mortality of the organism. High intracellular GST production by *M. hiemalis* (Hoque et al., 2007) as a well-known process of fungal cell protection could possibly be used in biotechnological applications for the detoxification of cyanotoxins *via* the biotransformation phase II conjugation. The conjugation of GSH with MCs to yield more hydrophilic and less toxic compounds has been found to be the primary detoxification mechanism in plants and animals (Pflugmacher et al., 1998b; Takenaka, 2001; Wang et al., 2006). The purpose of Paper III was to study the effect of cyanotoxins on selected antioxidative and biotransformation enzyme responses of *M. hiemalis*, namely those of CAT, GR, GPx, and GST.

Oxidative stress as a result of cyanotoxin exposure was not observed for all cyanotoxins tested, but depended on toxin structure and concentration. Although MC-LR is considered one of the most toxic cyanotoxins worldwide with negative effects on the enzyme expressions in aquatic plants (Jiang et al., 2011; Pflugmacher, 2004; Saqrane et al., 2007), terrestrial plants such as rape and rice (Chen et al., 2004), and aquatic animals (Amado and Monserrat, 2010), the toxin did not alter antioxidative enzyme responses in *M. hiemalis* showing the unique resistance of fungal organisms towards contaminants and environmental stress. Additionally, nearly all enzymes in *M. hiemalis* maintained their activity despite the exposure of the fungus to BMAA, exclusively GR was inhibited at 10 and 100 µg BMAA/L, however, CAT and GPx remained unaffected for all exposure concentrations. *M. hiemalis* did not substantially suffer from oxidative stress when exposed to BMAA. Correspondingly, mGST and cGST activities remained constant at all levels of BMAA concentration, showing that the biotransformation phase II cycle for the cell protection in *M. hiemalis* remained intact in the presence of BMAA enabling further detoxification mechanisms. The only toxin that clearly showed an impact on antioxidative and biotransformation enzyme activities in *M. hiemalis*, was CYN with an enhancement of CAT activity and an inhibitory effect on both GSH cycling antioxidative enzymes GR and GPx, as well as on mGST. CYN has been reported to deplete GSH (Norris et al., 2002); this was proposed to be a reason for the inhibition on all enzymes that use GSH as a co-factor in their reaction they catalyze. *M. hiemalis* responded with alterations in enzyme activities, however, no difference in growth and biomass production was previously observed even at higher concentrations (1,000 µg/L) and up to a longer exposure period of 7 d (Paper I). *M. hiemalis* might either cope with oxidative stress or overcome it over time. Therefore, investigations of enzyme activities over a longer time period are needed to better understand antioxidative defense mechanisms upon CYN exposure.

Generally, the results in Paper III demonstrate the tolerance of *M. hiemalis* towards MC-LR and in parts BMAA on a physiological level and the ability of the fungus to handle oxidative stress produced by CYN with consideration of an overall growth fitness proven in Paper I.
Although the involvement of GST in the biotransformation of MC-LR *via* phase II GSH conjugation could not be proven within this work, it remains still likely to occur and important for further clarification. Thus, GST activity in response to cyanotoxin exposure needs to be recorded over time and possible biotransformation products such as MC-LR-GSH, –Cys and mercapturic acid adducts (Guo et al., 2014) need to be identified.

6.4 FUNGAL DEGRADATION OF MICROCYSTIN-LR

Papers I, II, and III evidenced the suitability of *M. hiemalis* in terms of an overall growth fitness, toxin uptake, and tolerance on an enzymatic level. Moreover, the stable pelletized fungal growth morphology further supports the applicability of the fungal strain in pellet bioreactor technology. Chapter 5 subsequently shows an approach of implementing *M. hiemalis* pellets in the remediation of cyanotoxins on the example of MC-LR, the toxin that generated smallest alterations on fungal enzyme responses without the involvement of oxidative stress compared to BMAA and CYN (Paper III).
The degradation ability of *M. hiemalis* was shown by measuring the reduction of MC-LR in media over time *via* LC-MS/MS with comparison to the control, which was run under the same conditions but in the absence of fungal pellets. Only 0.4 % of the initial MC-LR concentration was taken up by *M. hiemalis* pellets after 14 d exposure. However, 14.6 % were eliminated by the fungal culture, which were neither taken up nor attributed to natural degradation. The results suggested fungal degradation, however, insufficient for mycoremediation purposes. Consequently, degradation efficiency was enhanced by means of medium composition substitution, nutrient-rich *vs* limiting medium. Degradation rates as well as efficiency were thereby both successfully improved, resulting in a noticeable reduction in toxin concentration after 24 h and reaching 30 % removal after seven days exposure.
Mycoremediation of MC-LR by *M. hiemalis* was compared towards *P. chrysosporium* in single- and co-culture, in order to include two fungi with different metabolic properties. In comparison to *P. chrysosporium*, which degraded MC-LR in non-lignolytic as well as in lignolytic conditions achieving complete elimination, removal by *M. hiemalis* was small and complete degradation from medium was not possible. However, the optimal removal working temperature of *P. chrysosporium* was 30 °C, and therefore operation in bioreactor would need external heating to ensure fungal activity. In contrast, *M. hiemalis* still sporulates at

groundwater temperatures. Co-pellets were produced with the aim to overcome the drawbacks of *P. chrysosporium*'s temperature limitation and *M. hiemalis*'s degradation weakness by providing complementary systems and mutual enzyme elicitations, with reference to a previous study on isoproturon (Hoque et al., 2007). The co-culture reached similar degradation efficiency as the lignolytic fungus *P. chrysosporium* in single-culture at 20 °C, and enhanced degradation ability than *M. hiemalis* in single-culture at 20 °C stating that this could be the better alternative than using *M. hiemalis* alone. Nonetheless, within this study, complete degradation was achieved neither with *M. hiemalis* in single nor in co-culture with *P. chrysosporium* within 7 d. Therefore, the hypothesis that *M. hiemalis* may degrade cyanotoxins sufficiently in order to implement the fungus in efficient mycoremediation cannot be confirmed within this work, but needs further investigation and improvement. Especially *in situ* treatment of water bodies with fungal pellets require a wide temperature range below 20 °C, which needs to be considered for the energy-saving degradation of cyanotoxins, preferably with co-cultures of aquatic *M. hiemalis* with terrestrial *P. chrysosporium*. In summary, results in this section show the versatility to influence fungal degradation efficiency and evidences the optimization potential of cyanotoxin mycoremediation as a promising strategy to clean up the water. Studies on fungal remediation of cyanotoxins, although promising, are until today, with some exceptions, lacking (Mohamed et al., 2014). This study enlarges the intentional application field of fungi to degrade contaminants in the environment including cyanotoxins as potential substrates and thereby presents a new approach to the treatment of cyanotoxins in water.

6.5 CONCLUSION

Cyanotoxin exposure of *M. hiemalis* towards MC-LR, CYN, and BMAA at concentrations from 5 to 1,000 µg/L did not influence fungal sensitivity, growth rate and biomass production. Therefore, it can be concluded that the aquatic fungus resists different types and high concentrations of cyanotoxins and thus its undisturbed growth and development in their presence is ensured.

The cyanotoxins MC-LR, CYN, and BMAA could be taken up by the mycelia of the aquatic fungus *M. hiemalis*, but were not accumulated over time within 48 h. Moreover, decreased MC-LR concentration was found internalized within the pellets of *M. hiemalis* after 14 d compared to 7 d exposure. Therefore, removal of cyanotoxins from water by the aquatic fungus including internal degradation rather than intracellular storage is suggested.

Pelletization of *M. hiemalis* was achieved in submerged cultivation on a rotary shaker. Conditions for pelletization were found being critical spore inoculum consideration, media volume, and initial pH adjustment. Pellet growth needed to be initiated by pH adjustment, but pH stability was not necessary to keep pellet morphology constant. Thus it can be concluded that pellet morphology is easily maintained without the need of continuous pH setting, which simplifies practical and technical reactor usage as well as continuous operation.

The components of fungal defense systems such as a variety of antioxidative enzymes, CAT, GR and GPx, as well as the biotransformation enzyme GST, seemed to be not or only partially affected in the presence of MC-LR and BMAA suggesting that the two cyanotoxins, although known to induce oxidative stress in many organisms, did not produce substantial oxidative stress in *M. hiemalis*. Moreover, antioxidative and biotransformation enzyme responses in *M. hiemalis* after CYN exposure showed that the aquatic fungus can cope with CYN induced oxidative stress as an overall growth fitness was previously confirmed even at higher concentrations and over a longer time period. This further proved the unique tolerance and adaptability of fungal organisms towards toxins and environmental perturbations. The postulation of the growth studies that fungi are resistant organisms, and can handle possible cyanotoxin-induced oxidative stress was hereby further reinforced on an enzymatic level. Consequently, the applicability of *M. hiemalis* in reactor technology is confirmed in terms of viability and longevity of the fungus in the presence of cyanotoxins.

The biotransformation enzyme mGST remained unaffected in the presence of MC-LR at all concentrations; cGST was inhibited at the highest concentration used in the experiment (100 µg/L). In the presence of BMAA, both mGST and cGST maintained their activity, whereas CYN inhibited mGST at all concentrations assayed, but left cGST untouched. Thus, the hypothesis that fungal GST of *M. hiemalis* may detoxify MC-LR and other cyanotoxins could not be answered within this work. However, the result, that MC-LR inhibits exclusively cGST at the highest toxin concentration used and the constant pattern of GST activities after BMAA exposure, states that GST mostly maintains its activity for further intact detoxification and cell protection. Measurements over a longer time period including metabolite identification are needed, in order to evaluate the role of fungal GST in the possible biotransformation of cyanotoxins.

MC-LR removal from medium by *M. hiemalis* has been observed, which was not detected internalized within the fungal pellets, suggesting in addition to internal also possible external degradation processes. Degradation efficiency of *M. hiemalis* was enhanced by substituting nutrient-rich with limiting medium as well as by implementing the aquatic fungus in co-culture with the lignolytic white-rot fungus *P. chrysosporium*. Thus, it has been shown that there are potential factors that may enable method development for efficient degradation and removal optimization.

The implementation of fungi in bioreactor technology for the removal of cyanotoxin polluted waters, could offer a promising solution to manage the toxins in an economically and sustainable way using natural ecosystem functions. *M. hiemalis* is an outstanding candidate in terms of cyanotoxin type and concentration tolerance, an overall developmental and growth fitness, uptake ability, pelletization and its inertness towards certain cyanotoxins on the enzymatic level as well as its ability to cope with cyanotoxin-induced oxidative stress. The improvement of its degradation efficiency is feasible by varying media composition and approaching co-cultivation albeit imperative for the realization of efficient mycoremediation. Thus, the technical application of *M. hiemalis* for cyanotoxin removal can only partially be confirmed within this work, but great potential is suggested if fungal degradation efficiency is further optimized. Future research should therefore include the investigation of co-cultures as complementary systems, studying temperature effects with the goal of lowering the working temperature in order to simulate realistic environmental conditions and avoid energy expenditure in reactor technology. Moreover, factors such as the use of inducers, pH optimum, temperature, oxygenation, and carbon/nitrogen sources and ratios, that could influence the elicitation of fungal degradation enzyme activities, which are important for cyanotoxin biotransformation and/or breakdown, need further clarification in order to stimulate optimal enzyme production and guarantee enzyme stability and thus be able to effectively degrade cyanotoxins and consider the possibility of mycoremediation with aquatic fungi. Consequently, identification of produced metabolites and their toxicity assessment after successful removal are required for generating evaluated purifying systems with confirmed detoxification ability yielding to reduced toxicity, and opening the way to a new field of cyanotoxin remediation.

7 REFERENCES

Allen, D.G., Robinson, C.W., 1989. Hydrodynamics and mass transfer in *Aspergillus niger* fermentations in bubble column and loop bioreactors. Biotechnology and Bioengineering 34, 731-740.

Amado, L.L., Monserrat, J.M., 2010. Oxidative stress generation by microcystins in aquatic animals: Why and how. Environment International 36, 226-235.

Arjmand, M., Sandermann Jr, H., 1985. Mineralization of chloraniline/lignin conjugates and of free chloranilines by the white-rot fungus *Phanerochaete chrysosporium*. Journal of Agricultura and Food Chemistry 33, 1055-1060.

Azevedo, S.M.F.O., Carmichael, W.W., Jochimsen, E.M., Rinehart, K.L., Lau, S., Shaw, G.R., Eaglesham, G.K., 2002. Human intoxication by microcystins during renal dialysis treatment in Caruaru-Brazil. Toxicology 181–182, 441-446.

Banker, R., Carmeli, S., Werman, M., Teltsch, B., Porat, R., Sukenik, A., 2001. Uracil Moiety is Required for Toxicity of the Cyanobacterial Hepatotoxin Cylindrospermopsin. Journal of Toxicology and Environmental Health 62, 281-288.

Barr, D.P., Aust, S.D., 1994. Mechanisms white rot fungi use to degrade pollutants. Environmental Science & Technology 28, 78A-87A.

Bazin, E., Mourot, A., Humpage, A.R., Fessard, V., 2010. Genotoxicity of a freshwater cyanotoxin, cylindrospermopsin, in two human cell lines: Caco-2 and HepaRG. Environmental and Molecular Mutagenesis 51, 251-259.

Bezalel, L., Hadar, Y., Cerniglia, C.E., 1997. Enzymatic mechanisms involved in phenanthrene degradation by the white rot fungus *Pleurotus ostreatus*. Applied and Environmental Microbiology 63, 2495-2501.

Bourne, D.G., Jones, G.J., Blakeley, R.L., Jones, A., Negri, A.P., Riddles, P., 1996. Enzymatic pathway for the bacterial degradation of the cyanobacterial cyclic peptide toxin microcystin-LR. Applied and Environmental Microbiology 62, 4086-4094.

Bourne, D.G., Riddles, P., Jones, G.J., Smith, W., Blakeley, R.L., 2001. Characterisation of a gene cluster involved in bacterial degradation of the cyanobacterial toxin microcystin-LR. Environmental Toxicology 16, 523-534.

Bumpus, J.A., Aust, S.D., 1987. Biodegradation of environmental pollutants by the white-rot fungus *Phanerochaete chrysosporium*: Involvement of the lignin degrading system. BioEssays 6, 166-170.

Buratti, F.M., Scardala, S., Funari, E., Testai, E., 2011. Human glutathione transferases catalyzing the conjugation of the hepatoxin microcystin-LR. Chemical Research in Toxicology 24, 926-933.

Campos, A., Vasconcelos, V., 2010. Molecular mechanisms of microcystin toxicity in animal cells. International Journal of Molecular Sciences 11, 268-287.

Carmichael, W.W., 1997. The cyanotoxins. In: Callow, JA (ed.), Advances in botanical research. Academic Press, London, UK.

Carvalho, M.B., Tavares, S., Medeiros, J., Núñez, O., Gallart-Ayala, H., Leitão, M.C., Galceran, M.T., Hursthouse, A., Silva Pereira, C., 2011. Degradation pathway of pentachlorophenol by *Mucor plumbeus* involves phase II conjugation and oxidation–reduction reactions. Journal of Hazardous Materials 198, 133-142.

Chang, J., Chen, Z.-l., Wang, Z., Shen, J.-m., Chen, Q., Kang, J., Yang, L., Liu, X.-w., Nie, C.-x., 2014. Ozonation degradation of microcystin-LR in aqueous solution: Intermediates, byproducts and pathways. Water Research 63, 52-61.

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

Chen, J.Z., Song, L.R., Dai, E., Gan, N.Q., Liu, Z.L., 2004. Effects of microcystins on the growth and the activity of superoxide dismutase and peroxidase of rape (*Brassica napus* L.) and rice (*Oryza sativa* L.). Toxicon 43, 393-400.

Chiswell, R.K., Shaw, G.R., Eaglesham, G., Smith, M.J., Norris, R.L., Seawright, A.A., Moore, M.R., 1999. Stability of cylindrospermopsin, the toxin from the cyanobacterium, *Cylindrospermopsis raciborskii*: Effect of pH, temperature, and sunlight on decomposition. Environmental Toxicology 14, 155-161.

Codd, G.A., 1995. Cyanobacterial toxins: Occurrence, properties and biological significance. Water Science and Technology 32, 149-156.

Codd, G.A., Morrison, L.F., Metcalf, J.S., 2005. Cyanobacterial toxins: risk management for health protection. Toxicology and Applied Pharmacology 203, 264-272.

Coelho-Moreira, J.S., Bracht, A., de Souza, A.C., Oliveira, R.F., de Sá-Nakanishi, A.B., de Souza, C.G., Peralta, R.M., 2013. Degradation of diuron by *Phanerochaete chrysosporium*: role of ligninolytic enzymes and cytochrome P450. BioMed Research International 2013.

Coleman, J.O.D., Blake-Kalff, M.M.A., Emyr Davies, T.G., 1997. Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. Trends in Plant Science 2, 144-151.

Contardo-Jara, V., Galanti, L.N., Amé, M.V., Monferrán, M.V., Wunderlin, D.A., Wiegand, C., 2009. Biotransformation and antioxidant enzymes of *Limnoperna fortunei* detect site impact in watercourses of Córdoba, Argentina. Ecotoxicology and Environmental Safety 72, 1871-1880.

Contardo-Jara, V., Otterstein, S.K.B., Downing, S., Downing, T.G., Pflugmacher, S., 2014a. Response of antioxidant and biotransformation systems of selected freshwater mussels (*Dreissena polymorpha*, *Anadonta cygnea*, *Unio tumidus*, and *Corbicula javanicus*) to the cyanobacterial neurotoxin β-*N*-methylamino-L-alanine. Toxicological and Environmental Chemistry 96, 451-465.

Contardo-Jara, V., Schwanemann, T., Pflugmacher, S., 2014b. Uptake of a cyanotoxin, β-*N*-methylamino-L-alanine, by wheat (*Triticum aestivum*). Ecotoxicology and Environmental Safety 104, 127-131.

Contardo-Jara, V., Sebastian Funke, M., Peuthert, A., Pflugmacher, S., 2013. β-*N*-Methylamino-L-alanine exposure alters defense against oxidative stress in aquatic plants *Lomariopsis lineata*, *Fontinalis antipyretica*, *Riccia fluitans* and *Taxiphyllum barbieri*. Ecotoxicology and Environmental Safety 88, 72-78.

Couto, S.R., Moldes, D., Sanromán, M.A., 2006. Optimum stability conditions of pH and temperature for ligninaseand manganese-dependent peroxidase from *Phanerochaete chrysosporium*. Application to *in vitro* decolorization of Poly R-478 by MnP. World Journal of Microbiology and Biotechnology 22, 607-612.

Cox, P.A., Banack, S.A., Murch, S.J., 2003. Biomagnification of cyanobacterial neurotoxins and neurodegenerative disease among the Chamorro people of Guam. Proceedings of the National Academy of Sciences of the United States of America 100, 13380-13383.

Cox, P.A., Banack, S.A., Murch, S.J., Rasmussen, U., Tien, G., Bidigare, R.R., Metcalf, J.S., Morrison, L.F., Codd, G.A., Bergman, B., 2005. Diverse taxa of cyanobacteria produce β-*N*-methylamino-L-alanine, a neurotoxic amino acid. Proceedings of the National Academy of Sciences of the United States of America 102, 5074-5078.

de Figueiredo, D.R., Azeiteiro, U.M., Esteves, S.M., Gonçalves, F.J.M., Pereira, M.J., 2004. Microcystin-producing blooms—a serious global public health issue. Ecotoxicology and Environmental Safety 59, 151-163.

Ding, W.X., Shen, H.M., Ong, C.N., 2000. Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. Hepatology 32, 547-555.

Downing, S., Banack, S.A., Metcalf, J.S., Cox, P.A., Downing, T.G., 2011. Nitrogen starvation of cyanobacteria results in the production of β-*N*-methylamino-L-alanine. Toxicon 58, 187-194.

Downing, S., Contardo-Jara, V., Pflugmacher, S., Downing, T.G., 2014. The fate of the cyanobacterial toxin β-*N*-methylamino-L-alanine in freshwater mussels. Ecotoxicology and Environmental Safety 101, 51-58.

Downing, S., Esterhuizen-Londt, M., Grant Downing, T., 2015. β-*N*-methylamino-L-alanine (BMAA) metabolism in the aquatic macrophyte *Ceratophyllum demersum*. Ecotoxicology and Environmental Safety 120, 88-92.

Eaton, D.C., 1985. Mineralization of polychlorinated biphenyls by *Phanerochaete chrysosporium*: A ligninolytic fungus. Enzyme and Microbial Technology 7, 194-196.

Edwards, C., Lawton, L.A., 2009. Chapter 4 Bioremediation of Cyanotoxins. In: Laskin, A.I., Sariaslani, S., Gadd, G.M. (eds.), Advances in Applied Microbiology. Academic Press, pp. 109-129.

El-Enshasy, H.A., 2007. Filamentous Fungal Cultures – Process Characteristics, Products, and Applications. In: Yang, S.-T. (ed.), Bioprocessing for Value-Added Products from Renewable Resources. Elsevier Press, Department of Chemical Engineering, Ohio State University, OH, USA, pp. 225-261.

Espinosa-Ortiz, E.J., Rene, E.R., Pakshirajan, K., van Hullebusch, E.D., Lens, P.N.L., 2016. Fungal pelleted reactors in wastewater treatment: Applications and perspectives. Chemical Engineering Journal 283, 553-571.

Esterhuizen, M., Downing, T.G., 2008. β-*N*-methylamino-L-alanine (BMAA) in novel South African cyanobacterial isolates. Ecotoxicology and Environmental Safety 71, 309-313.

Esterhuizen, M., Pflugmacher, S., Downing, T.G., 2011. β-*N*-Methylamino-L-alanine (BMAA) uptake by the aquatic macrophyte *Ceratophyllum demersum*. Ecotoxicology and Environmental Safety 74, 74-77.

Esterhuizen-Londt, M., Kühn, S., Pflugmacher, S., 2015a. Development and validation of an in-house quantitative analysis method for cylindrospermopsin using hydrophilic interaction liquid chromatography–tandem mass spectrometry: Quantification demonstrated in 4 aquatic organisms. Environmental Toxicology and Chemistry 34, 2878-2883.

Esterhuizen-Londt, M., Pflugmacher, S., Downing, T.G., 2011. The effect of β-*N*-methylamino-L-alanine (BMAA) on oxidative stress response enzymes of the macrophyte *Ceratophyllum demersum*. Toxicon 57, 803-810.

Esterhuizen-Londt, M., von Schnehen, M., Kühn, S., Pflugmacher, S., 2016. Oxidative stress responses in the animal model, *Daphnia pulex* exposed to a natural bloom extract versus artificial cyanotoxin mixtures. Aquatic Toxicology 179, 151-157.

Esterhuizen-Londt, M., Wiegand, C., Downing, T.G., 2015b. β-*N*-methylamino-L-alanine (BMAA) uptake by the animal model, *Daphnia magna* and subsequent oxidative stress. Toxicon 100, 20-26.

Feurstein, D., Holst, K., Fischer, A., Dietrich, D.R., 2009. Oatp-associated uptake and toxicity of microcystins in primary murine whole brain cells. Toxicology and Applied Pharmacology 234, 247-255.

Fischer, W.J., Altheimer, S., Cattori, V., Meier, P.J., Dietrich, D.R., Hagenbuch, B., 2005. Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. Toxicology and Applied Pharmacology 203, 257-263.

Fleming, L.E., Rivero, C., Burns, J., Williams, C., Bean, J.A., Shea, K.A., Stinn, J., 2002. Blue green algal (cyanobacterial) toxins, surface drinking water, and liver cancer in Florida. Harmful Algae 1, 157-168.

Flores-Rojas, N.C., Esterhuizen-Londt, M., Pflugmacher, S., 2015. Antioxidative stress responses in the floating macrophyte *Lemna minor* L. with cylindrospermopsin exposure. Aquatic Toxicology 169, 188-195.

Frankmölle, W.P., Larsen, L.K., Caplan, F.R., Patterson, G.M.L., Knübel, G., Levine, I.A., Moore, R.E., 1992. Antifungal cyclic peptides from the terrestrial blue-green alga *Anabaena laxa* - 1. Isolation and biological properties. The Journal of Antibiotics 45, 1451-1457.

Froscio, S.M., Humpage, A.R., Burcham, P.C., Falconer, I.R., 2003. Cylindrospermopsin-induced protein synthesis inhibition and its dissociation from acute toxicity in mouse hepatocytes. Environmental Toxicology 18, 243-251.

Froscio, S.M., Humpage, A.R., Wickramasinghe, W., Shaw, G., Falconer, I.R., 2008. Interaction of the cyanobacterial toxin cylindrospermopsin with the eukaryotic protein synthesis system. Toxicon 51, 191-198.

Gerin, P.A., Dufrene, Y., Bellon-Fontaine, M.N., Asther, M., Rouxhet, P.G., 1993. Surface properties of the conidiospores of *Phanerochaete chrysosporium* and their relevance to pellet formation. Journal of Bacteriology 175, 5135-5144.

Gibbs, P., Seviour, R., Schmid, F., 2000. Growth of filamentous fungi in submerged culture: problems and possible solutions. Critical Reviews in Biotechnology 20, 17 - 48.

Guo, X., Xie, P., Chen, J., Tuo, X., Deng, X., Li, S., Yu, D., Zeng, C., 2014. Simultaneous quantitative determination of microcystin-LR and its glutathione metabolites in rat liver by liquid chromatography–tandem mass spectrometry. Journal of Chromatography B 963, 54-61.

Gutiérrez-Praena, D., Jos, A., Pichardo, S., Cameán, A.M., 2011a. Oxidative stress responses in tilapia (*Oreochromis niloticus*) exposed to a single dose of pure cylindrospermopsin under laboratory conditions: Influence of exposure route and time of sacrifice. Aquatic Toxicology 105, 100-106.

Gutiérrez-Praena, D., Jos, Á., Pichardo, S., Moyano, R., Blanco, A., Monterde, J.G., Cameán, A.M., 2012. Time-dependent histopathological changes induced in Tilapia (*Oreochromis niloticus*) after acute exposure to pure cylindrospermopsin by oral and intraperitoneal route. Ecotoxicology and Environmental Safety 76, 102-113.

Gutiérrez-Praena, D., Pichardo, S., Jos, Á., María Cameán, A., 2011b. Toxicity and glutathione implication in the effects observed by exposure of the liver fish cell line PLHC-1 to pure cylindrospermopsin. Ecotoxicology and Environmental Safety 74, 1567-1572.

Guzmán-Guillén, R., Prieto, A.I., Vasconcelos, V.M., Cameán, A.M., 2013. Cyanobacterium producing cylindrospermopsin cause oxidative stress at environmentally relevant concentrations in sub-chronically exposed tilapia (*Oreochromis niloticus*). Chemosphere 90, 1184-1194.

Harada, K., 1996. Stability of microcystins from cyanobacteria—III. Effect of pH and temperature. Phycologia 35, 83-88.

Harada, K.-i., Imanishi, S., Kato, H., Mizuno, M., Ito, E., Tsuji, K., 2004. Isolation of Adda from microcystin-LR by microbial degradation. Toxicon 44, 107-109.

Harada, K.-i., Ohtani, I., Iwamoto, K., Suzuki, M., Watanabe, M.F., Watanabe, M., Terao, K., 1994. Isolation of cylindrospermopsin from a cyanobacterium *Umezakia natans* and its screening method. Toxicon 32, 73-84.

Hashmi, M., Anders, M.W., 1991. Enzymatic reaction of β-*N*-methylaminoalanine with L-amino acid oxidase. Biochimica et Biophysica Acta 1074, 36-39.

Himberg, K., Keijola, A.M., Hiisvirta, L., Pyysalo, H., Sivonen, K., 1989. The effect of water treatment processes on the removal of hepatotoxins from *Microcystis* and *Oscillatoria* cyanobacteria: A laboratory study. Water Research 23, 979-984.

Ho, L., Sawade, E., Newcombe, G., 2012. Biological treatment options for cyanobacteria metabolite removal - A review. Water Research 46, 1536-1548.

Hoffman, J.R.H., 1976. Removal of *Microcystis* toxins in water purification processes. Water SA 2, 58-60.

Hoque, E., 2003. Verfahren zum Abbau von Xenobiotika durch Pilzarten mit Monooxygenase-/Dioxygenase-Aktivität in Gegenwart von Pilzen mit Glutathion-*S*-Transferase-Aktivität, Patentnr: DE10125365 C2.

Hoque, E., Fritscher, J., 2016. A new mercury-accumulating *Mucor hiemalis* strain EH8 from cold sulfidic spring water biofilms. MicrobiologyOpen 5, 763-781.

Hoque, E., Pflugmacher, S., Fritscher, J., Wolf, M., 2007. Induction of glutathione *S*-transferase in biofilms and germinating spores of *Mucor hiemalis* strain EH5 from cold sulfidic spring waters. Applied and Environmental Microbiology, 2697-2707.

Hu, L.B., Yang, J.D., Zhou, W., Yin, Y.F., Chen, J., Shi, Z.Q., 2009. Isolation of a *Methylobacillus* sp. that degrades microcystin toxins associated with cyanobacteria. New Biotechnology 26, 205-211.

Humpage, A.R., Fontaine, F., Froscio, S., Burcham, P., Falconer, I.R., 2005. Cylindrospermopsin genotoxicity and cytotoxicity: Role of cytochrome P-450 and oxidative stress. Journal of Toxicology and Environmental Health, Part A 68, 739-753.

Imanishi, S., Kato, H., Mizuno, M., Tsuji, K., Harada, K.-i., 2005. Bacterial degradation of microcystins and nodularin. Chemical Research in Toxicology 18, 591-598.

Jia, C., Li, X., Allinson, G., Liu, C., Gong, Z., 2016. Composition and morphology characterization of exopolymeric substances produced by the PAH-degrading fungus of *Mucor mucedo*. Environmental Science and Pollution Research 23, 8421-8430.

Jia, Y., Du, J., Song, F., Zhao, G., Tian, X., 2012. A fungus capable of degrading microcystin-LR in the algal culture of *Microcystis aeruginosa* PCC7806. Applied Biochemistry and Biotechnology 166, 987-996.

Jiang, J., Gu, X., Song, R., Wang, X., Yang, L., 2011. Microcystin-LR induced oxidative stress and ultrastructural alterations in mesophyll cells of submerged macrophyte *Vallisneria natans* (Lour.) Hara. Journal of Hazardous Materials 190, 188-196.

Juan, W., Qing-biao, L., 2002. Biosorption of lead by *Phanerochaete chrysosporium* in the form of pellets. Journal of Environmental Sciences 14, 108-114.

Karlsson, O., Jiang, L., Andersson, M., Ilag, L.L., Brittebo, E.B., 2014. Protein association of the neurotoxin and non-protein amino acid BMAA (β-*N*-methylamino-L-alanine) in the liver and brain following neonatal administration in rats. Toxicology Letters 226, 1-5.

Kato, H., Imanishi, S.Y., Tsuji, K., Harada, K.-i., 2007. Microbial degradation of cyanobacterial cyclic peptides. Water Research 41, 1754-1762.

Keijola, A.M., Himberg, K., Esala, A.L., Sivonen, K., Hiisvirta, L., 1988. Removal of cyanobacterial toxins in water treatment process: Laboratory and pilot-scale experiments. Environmental Toxicology 3, 643-656.

Kersten, P.J., 1990. Glyoxal oxidase of *Phanerochaete chrysosporium*: Its characterization and activation by lignin peroxidase. Proceedings of the National Academy of Sciences of the United States of America USA 81, 2936-2940.

Kersten, P.J., Kirk, T.K., 1987. Involvement of a New Enzyme, Glyoxal Oxidase, in Extracellular H202 Production by *Phanerochaete chrysosporium*. Journal of Bacteriology 169, 2195-2201.

Kim, J., Lebeault, J.M., Reuss, M., 1983. Comparative study on rheological properties of mycelial broth in filamentous and pelleted forms. European Journal of Applied Microbiology and Biotechnology 18, 11-16.

Kirk, T.K., Schultz, E., Connors, W.J., Lorenz, L.F., Zeikus, J.G., 1978. Influence of Culture Parameters on Lignin Metabolism by *Phanerochaete chrysosporium* Archives of Microbiology 117, 277-285.

Kolet, S.P., Haldar, S., Niloferjahan, S., Thulasiram, H.V., 2014. *Mucor hiemalis* mediated 14α-hydroxylation on steroids: In vivo and in vitro investigations of 14α-hydroxylase activity. Steroids 85, 6-12.

Kondo, F., Ikai, Y., Oka, H., Okumura, M., Ishikawa, N., Harada, K.-i., Matsuura, K., Murata, H., Suzuki, M., 1992. Formation, characterization, and toxicity of the glutathione and cysteine conjugates of toxic heptapeptide microcystins. Chemical Research in Toxicology 5, 591-596.

Kondo, F., Matsumoto, H., Yamada, S., Ishikawa, N., Ito, E., Nagata, S., Ueno, Y., Makoto, S., Harada, K.-i., 1996. Detection and identification of metabolites of microcystins formed *in vivo* in mouse and rat livers. Chemical Research in Toxicology 9, 1355-1359.

Kormas, K.A., Lymperopoulou, D.S., 2013. Cyanobacterial toxin degrading bacteria: Who are they? BioMed Research International 2013, 12.

Lanaras, T., Cook, C.M., 1994. Toxin extraction from an *Anabaenopsis milleri* - dominated bloom. Science of The Total Environment 142, 163-169.

Li, Q., Bai, Z., O'Donnell, A., Harvey, L.M., Hoskisson, P.A., McNeil, B., 2011. Oxidative stress in fungal fermentation processes: the roles of alternative respiration. Biotechnology Letters 33, 457-467.

Li, R., Carmichael, W.W., Brittain, S., Eaglesham, G.K., Shaw, G.R., Liu, Y., Watanabe, M.M., 2001. First report of the cyanotoxins cylindrospermopsin and deoxycylindrospermopsin from *Raphidiopsis curvata* (cyanobacteria). Journal of Phycology 37, 1121-1126.

Liao, W., Liu, Y., Frear, C., Chen, S., 2007. A new approach of pellet formation of a filamentous fungus – *Rhizopus oryzae*. Bioresource Technology 98, 3415-3423.

Liu, X., Chen, Z., Zhou, N., Shen, J., Ye, M., 2010. Degradation and detoxification of microcystin-LR in drinking water by sequential use of UV and ozone. Journal of Environmental Sciences 22, 1897-1902.

Liu, X., Rush, T., Zapata, J., Lobner, D., 2009. β-*N*-methylamino-L-alanine induces oxidative stress and glutamate release through action on system Xc-. Experimental Neurology 217, 429-433.

Liu, Y., Liao, W., Chen, S., 2008. Study of pellet formation of filamentous fungi *Rhizopus oryzae* using a multiple logistic regression model. Biotechnology and Bioengineering 99, 117-128.

Lobner, D., Piana, P.M.T., Salous, A.K., Peoples, R.W., 2007. β-*N*-methylamino-L-alanine enhances neurotoxicity through multiple mechanisms. Neurobiology of Diseases 25, 360-366.

Luukkainen, R., Sivonen, K., Namikoshi, M., Färdig, M., Rinehart, K.L., Niemelä, S.I., 1993. Isolation and identification of eight microcystins from thirteen *Oscillatoria agardhii* strains and structure of a new microcystin. Applied and Environmental Microbiology 59, 2204-2209.

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

Manage, P.M., Edwards, C., Singh, B.K., Lawton, L.A., 2009. Isolation and identification of novel microcystin-degrading bacteria. Applied Environmental Microbiology 75, 6924–6928.

Manahan, S.E., 2003. Toxicological chemistry and biochemistry, 3rd ed. Lewis Publishers, Boca Raton, Florida.

Marchut-Mikolajczyk, O., Kwapisz, E., Wieczorek, D., Antczak, T., 2015. Biodegradation of diesel oil hydrocarbons enhanced with *Mucor circinelloides* enzyme preparation. International Biodeterioration & Biodegradation 104, 142-148.

Marco-Urrea, E., Pérez-Trujillo, M., Caminal, G., Vicent, T., 2009. Dechlorination of 1,2,3- and 1,2,4-trichlorobenzene by the white-rot fungus *Trametes versicolor*. Journal of Hazardous Materials 166, 1141-1147.

Máthé, C., M-Hamvas, M., Vasas, G., Surányi, G., Bácsi, I., Beyer, D., Tóth, S., Tímár, M., Borbély, G., 2007. Microcystin-LR, a cyanobacterial toxin, induces growth inhibition and histological alterations in common reed (*Phragmites australis*) plants regenerated from embryogenic calli. New Phytologist 176, 824-835.

Merel, S., Walker, D., Chicana, R., Snyder, S., Baurès, E., Thomas, O., 2013. State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environment International 59, 303-327.

Metcalf, J.S., Barakate, A., Codd, G.A., 2004. Inhibition of plant protein synthesis by the cyanobacterial hepatotoxin, cylindrospermopsin. FEMS Microbiology Letters 235, 125-129.

Metcalf, J.S., Codd, G.A., 2000. Microwave oven and boiling waterbath extraction of hepatotoxins from cyanobacterial cells. FEMS Microbiology Letters 184, 241-246.

Mirbagheri, M., Nahvi, I., Emamzadeh, R., Emtiazi, G., Shirani, E., 2016. Oil wastes management: medium optimization for the production of alpha-linolenic acid in *Mucor circinelloides*. International Journal of Environmental Science and Technology 13, 31-38.

Mohamed, Z.A., Hashem, M., Alamri, S.A., 2014. Growth inhibition of the cyanobacterium *Microcystis aeruginosa* and degradation of its microcystin toxins by the fungus *Trichoderma citrinoviride*. Toxicon 86, 51-58.

Namikoshi, M., Sivonen, K., Evans, W.R., Carmichael, W.W., Sun, F., Rouhiainen, L., Luukkainen, R., Rinehart, K.L., 1992. Two new l-serine variants of microcystins-LR and -RR from *Anabaena* sp. strains 202 A1 and 202 A2. Toxicon 30, 1457-1464.

Ning, D., Wang, H., Zhuang, Y., 2010. Induction of functional cytochrome P450 and its involvement in degradation of benzoic acid by *Phanerochaete chrysosporium*. Biodegradation 21, 297-308.

Norris, R.L.G., Seawright, A.A., Shaw, G.R., Senogles, P., Eaglesham, G.K., Smith, M.J., Chiswell, R.K., Moore, M.R., 2002. Hepatic xenobiotic metabolism of cylindrospermopsin *in vivo* in the mouse. Toxicon 40, 471-476.

Novotný, Č., Svobodová, K., Erbanová, P., Cajthaml, T., Kasinath, A., Lang, E., Šašek, V., 2004. Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. Soil Biology and Biochemistry 36, 1545-1551.

Nunn, P.B., Ponnusamy, M., 2009. Beta-*N*-methylaminoalanine (BMAA): metabolism and metabolic effects in model systems and in neural and other tissues of the rat *in vitro*. Toxicon 54, 85-94.

Nyman, J., Lacintra, M.G., Westman, J.O., Berglin, M., Lundin, M., Lennartsson, P.R., Taherzadeh, M.J., 2013. Pellet formation of zygomycetes and immobilization of yeast. New Biotechnology 30, 516-522.

Ohtani, I., Moore, R.E., Runnegar, M.T.C., 1992. Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. Journal of the American Chemical Society 114, 7941-7942.

Olsvik, E., Kristiansen, B., 1994. Rheology of filamentous fermentations. Biotechnology Advances 12, 1-39.

Papagianni, M., 2004. Fungal morphology and metabolite production in submerged mycelial processes. Biotechnology Advances 22, 189-259.

Pearl, H.W., Otten, T.G., 2013. Harmful cyanobacterial blooms: causes, consequences, and controls. Environmental Microbiology 65, 995-1010.

Pflugmacher, S., 2002. Possible allelopathic effects of cyanotoxins, with reference to microcystin-LR, in aquatic ecosystems. Environmental Toxicology 17, 407-413.

Pflugmacher, S., 2004. Promotion of oxidative stress in the aquatic macrophyte *Ceratophyllum demersum* during biotransformation of the cyanobacterial toxin microcystin-LR. Aquatic Toxicology 70, 169-178.

Pflugmacher, S., Wiegand, C., Beattie, K.A., Codd, G.A., Steinberg, C., 1998a. Uptake of the cyanobacterial hepatotoxin microcystin-LR by aquatic macrophytes. Journal of Applied Botany 72, 228-232.

Pflugmacher, S., Wiegand, C., Oberemm, A., Beattie, K.A., Krause, E., Codd, G.A., Steinberg, C., 1998b. Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. Biochimica et Biophysica Acta 1425, 527-533.

Preußel, K., Stüken, A., Wiedner, C., Chorus, I., Fastner, J., 2006. First report on cylindrospermopsin producing *Aphanizomenon flos-aquae* (Cyanobacteria) isolated from two German lakes. Toxicon 47, 156-162.

Prieto, A., Campos, A., Cameán, A., Vasconcelos, V., 2011a. Effects on growth and oxidative stress status of rice plants (*Oryza sativa*) exposed to two extracts of toxin-producing cyanobacteria (*Aphanizomenon ovalisporum* and *Microcystis aeruginosa*). Ecotoxicology and Environmental Safety 74, 1973-1980.

Prieto, A., Möder, M., Rodil, R., Adrian, L., Marco-Urrea, E., 2011b. Degradation of the antibiotics norfloxacin and ciprofloxacin by a white-rot fungus and identification of degradation products. Bioresource Technology 102, 10987-10995.

Prinsep, M.R., Caplan, F.R., Moore, R.E., Patterson, G.M.L., E. Honkanen, R., Boynton, A.L., 1992. Microcystin-LA from a blue-green alga belonging to the stigonematales. Phytochemistry 31, 1247-1248.

Puerto, M., Jos, A., Pichardo, S., Gutiérrez-Praena, D., Cameán, A., 2011. Acute effects of pure cylindrospermopsin on the activity and transcription of antioxidant enzymes in tilapia (*Oreochromis niloticus*) exposed by gavage. Ecotoxicology 20, 1852-1860.

Ramos, D.F., Matthiensen, A., Colvara, W., de Votto, A.P.S., Trindade, G.S., da Silva, P.E.A., Yunes, J.S., 2015. Antimycobacterial and cytotoxicity activity of microcystins. The Journal of Venomous Animals and Toxins Including Tropical Diseases 21, 9.

Reece, D.M., Nunn, P.B., 1988. Synthesis of ^{14}C-labelled L-α-amino-β-methylaminopropionic acid and its metabolism in the rat. Biochemical Society Transactions 17, 203-204.

Richter, L., Wanka, F., Boecker, S., Storm, D., Kurt, T., Vural, Ö., Süßmuth, R., Meyer, V., 2014. Engineering of *Aspergillus niger* for the production of secondary metabolites. Fungal Biology and Biotechnology 1, 1-13.

Ross, S.M., Seelig, M., Spencer, P.S., 1987. Specific antagonism of excitotoxic action of 'uncommon' amino acids assayed in organotypic mouse cortical cultures. Brain Research 425, 120-127.

Runnegar, M.T., Kong, S.-M., Zhong, Y.-Z., Lu, S.C., 1995. Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochemical Pharmacology 49, 219-225.

Saker, M.L., Griffiths, D.J., 2000. The effect of temperature on growth and cylindrospermopsin content of seven isolates of *Cylindrospermopsis raciborskii* (*Nostocales*, *Cyanophyceae*) from water bodies in northern Australia. Phycologia 39, 349-354.

Saqrane, S., El ghazali, I., Ouahid, Y., Hassni, M.E., El Hadrami, I., Bouarab, L., del Campo, F.F., Oudra, B., Vasconcelos, V., 2007. Phytotoxic effects of cyanobacteria extract on the aquatic plant *Lemna gibba*: Microcystin accumulation, detoxication and oxidative stress induction. Aquatic Toxicology 83, 284-294.

Schmidt, J.R., Wilhelm, S.W., Boyer, G.L., 2014. The fate of microcystins in the environment and challenges for monitoring. Toxins 6, 3354-3387.

Scholz, S.N., Esterhuizen-Londt, M., Pflugmacher, S., 2017. Rise of toxic cyanobacterial blooms in temperate freshwater lakes: causes, correlations and possible countermeasures. Toxicology and Environmental Chemistry 99, 543-577.

Scott, L.L., Downing, S., Phelan, R.R., Downing, T.G., 2014. Environmental modulation of microcystin and β-*N*-methylamino-L-alanine as a function of nitrogen availability. Toxicon 87, 1-5.

Sena, L.A., Chandel, N.S., 2012. Physiological roles of mitochondrial reactive oxygen species. Molecular Cell 48, 158-167.

Seo, J., Jeon, J., Kim, S.-D., Kang, S., Han, J., Hur, H.-G., 2007. Fungal biodegradation of carbofuran and carbofuran phenol by the fungus *Mucor ramannianus*: identification of metabolites. Water Science and Technology 55, 163.

Shetty, P.K., Mitra, J., Murthy, N.B.K., Namitha, K.K., Savitha, K.N., Raghu, K., 2000. Biodegradation of cyclodiene insecticide endosulfan by *Mucor thermo-hyalospora* MTCC 1384. Current Science 79, 1381-1383.

Shroff, K.A., Vaidya, V.K., 2011. Kinetics and equilibrium studies on biosorption of nickel from aqueous solution by dead fungal biomass of *Mucor hiemalis*. Chemical Engineering Journal 171, 1234-1245.

Sivonen, K., Jones, G.J., 1999. Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. E&FN Spon, London and New York.

Sivonen, K., Namikoshi, M., Evans, W.R., Färdig, M., Carmichael, W.W., Rinehart, K.L., 1992. Three new microcystins, cyclic heptapeptide hepatotoxins, from *Nostoc* sp. strain 152. Chemical Research in Toxicology 5, 464-469.

Sladdin, M., Lynch, M., 1983. Antimicrobial properties of calcium peroxide in relation to its potential use as a seed dressing. Journal of General Microbiology 129, 2307-2314.

Somdee, T., Thunders, M., Ruck, J., Lys, I., Allison, M., Page, R., 2013. Degradation of [Dha^7]MC-LR by a microcystin degrading bacterium isolated from lake rotoiti, New Zealand. ISRN Microbiology 2013, 596429.

Stirling, D.J., Miles, C.O., 2016. Marine algal toxin and cyanobacterial toxin mass lists. http://www.toxinology.no/Downloads.aspx. *Accessed* 08 March 2017.

Su, D., Li, P.-j., Frank, S., Xiong, X.-z., 2006. Biodegradation of benzo[a]pyrene in soil by *Mucor* sp. SF06 and *Bacillus* sp. SB02 co-immobilized on vermiculite. Journal of Environmental Sciences 18, 1204-1209.

Takenaka, S., 2001. Covalent glutathione conjugation to cyanobacterial hepatotoxin microcystin LR by F344 rat cytosolic and microsomal glutathione *S*-transferases. Environmental Toxicology and Pharmacology 9, 135-139.

Teramoto, H., Tanaka, H., Wariishi, H., 2004. Fungal cytochrome P450s catalyzing hydroxylation of substituted toluenes to form their hydroxymethyl derivatives. FEMS Microbiol Lett 234, 255-260.

Tewari, N., Vasudevan, P., Guha, B.K., 2005. Study on biosorption of Cr (VI) by *Mucor hiemalis*. Biochemical Engineering Journal 23, 185-192.

Tien, M., 1987. Properties of ligninase from *Phanerochaete chrysosporium* and their possible applications. CRC Critical Reviews in Microbiology 15, 141-168.

Trevino-Garrison, I., DeMent, J., Ahmed, F.S., Haines-Lieber, P., Langer, T., Ménager, H., Neff, J., van der Merwe, D., Carney, E., 2015. Human illnesses and animal deaths associated with freshwater harmful algal blooms—Kansas. Toxins 7, 353-366.

Tsuji, K., Watanuki, T., Kondo, F., Watanabe, M.F., Nakazawa, H., Suzuki, M., Uchida, H., Harada, K.-I., 1997. Stability of microcystins from cyanobacteria—IV. Effect of chlorination on decomposition. Toxicon 35, 1033-1041.

Umweltbundesamt, Bundesgesundheitsblatt, 2015. Empfehlung zum Schutz von Badenden vor Cyanobakterien-Toxinen. Springer-Verlag Berlin Heidelberg.

Ulmer, D., Leisola, M., Puhakka, J., Fiechter, A., 1983. *Phanerochaete chrysosporium*: Growth pattern and lignin degradation. European Journal of Applied Microbiology and Biotechnology 18, 153-157.

Vasas, G., Gáspár, A., Surányi, G., Batta, G., Gyémánt, G., M-Hamvas, M., Máthé, C., Grigorszky, I., Molnár, E., Borbély, G., 2002. Capillary electrophoretic assay and purification of cylindrospermopsin, a cyanobacterial toxin from *Aphanizomenon ovalisporum*, by plant test (blue-green *Sinapis* test). Analytical Biochemistry 302, 95-103.

Walsby, A.E., 1994. Gas vesicles. Microbiological Reviews 58, 94-144.

Walsby, A.E., 1997. The selective advantage of buoyancy provided by gas vesicles for planktonic cyanobacteria in the Baltic Sea. New Phytologist 136, 407-417.

Wang, L., Liang, X.-F., Liao, W.-Q., Lei, L.-M., Han, B.-P., 2006. Structural and functional characterization of microcystin detoxification-related liver genes in a phytoplanktivorous fish, Nile tilapia (*Oreochromis niloticus*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 144, 216-227.

Wang, Z., Zheng, G., Zhou, L., 2015. Degradation of slime extracellular polymeric substances and inhibited sludge flocs destruction contribute to sludge dewaterability enhancement

during fungal treatment of sludge using filamentous fungus *Mucor* sp. GY-1. Bioresource Technology 192, 514-521.

Whitton, B.A., 2012. Ecology of Cyanobacteria II. Springer.

World Health Organization, 2011. Guidelines for drinking-water quality, fourth ed. WHO Library Cataloguing-in-Publication Data, Geneva, CH.

Wiegand, C., Pflugmacher, S., 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. Toxicology and Applied Pharmacology 203, 201-218.

Wu, Y., He, J., Yang, L., 2010. Evaluating adsorption and biodegradation mechanisms during the removal of microcystin-RR by periphyton. Environmental Science & Technology 44, 6319–6324.

Xia, C., Zhang, J., Zhang, W., Hu, B., 2011. A new cultivation method for microbial oil production: cell pelletization and lipid accumulation by *Mucor circinelloides*. Biotechnology for Biofuels 4, 15.

Xiao, P., Mori, T., Kondo, R., 2011. Biotransformation of the organochlorine pesticide trans-chlordane by wood-rot fungi. New Biotechnology 29, 107-115.

Xiao, Y., Lee, I.-S., 2016. Microbial metabolism of prenylated apigenin derivatives by *Mucor hiemalis*. Phytochemistry Letters 16, 197-202.

Yoshizawa, S., Matsushima, R., Watanabe, M.F., Harada, K., Ichihara, A., Carmichael, W.W., Fujiki, H., 1990. Inhibition of protein phosphatases by microcystins and nodularin associated with hepatotoxicity. Journal of Cancer Research and Clinical Oncology 116, 609-614.

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

Žegura, B., Sedmak, B., Filipič, M., 2003. Microcystin-LR induces oxidative DNA damage in human hepatoma cell line HepG2. Toxicon 41, 41-48.

Zhou, Y., Du, J., Tsao, G., 2000. Mycelial Pellet Formation by *Rhizopus oryzae* ATCC 20344. Applied Biochemistry and Biotechnology 84, 779-789.