Cyanotoxins in agricultural watersheds and their quantification in the soil-plant system

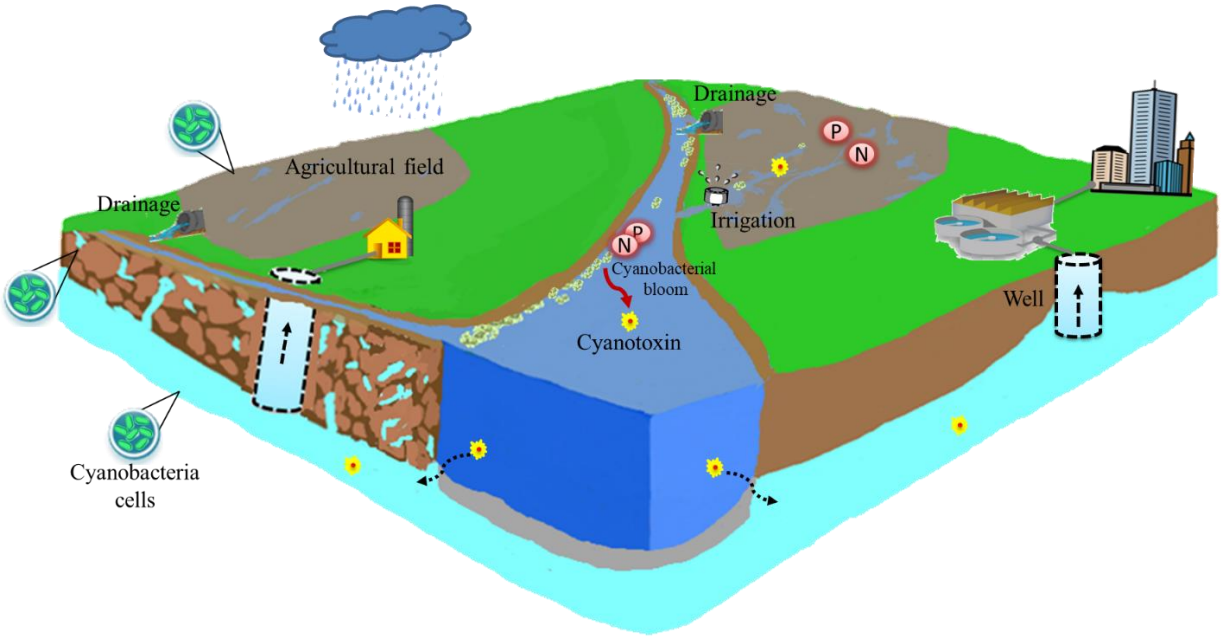
Yanyan Zhang

Department of Natural Resource Sciences

Faculty of Agricultural and Environmental Sciences

McGill University, Montréal

November 2020



A thesis submitted to McGill University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

山重水复疑无路，柳暗花明又一村

(After endless mountains and rivers that leave doubt whether there is a path out, suddenly one encounters the shade of a willow, bright flowers and a lovely village)

— *Song Poem in the ancient China*

TABLE OF CONTENTS

ABSTRACT

Cyanobacteria and their cyanotoxins are present in many regions and ecosystems worldwide. Eutrophic freshwater lakes that receive regular inputs of nutrient-rich runoff from agricultural land are an ideal environment for cyanobacteria blooms, which are associated with cyanotoxin production, but cyanotoxins could also be present in, or originate from, the soils, groundwater and the vadose zone of agricultural watersheds. Humans will be exposed to these toxins when they consume cyanobacteria-contaminated groundwater and crops grown in contaminated soils. However, we do not understand fully how agricultural nutrient loading may trigger cyanotoxin production and the occurrence of cyanotoxins in ecosystem compartments besides eutrophic waterbodies. One of the major challenges is to develop quantitative methods that can detect the biologically significant cyanotoxins at low concentrations, and another challenge is to sensitize the agricultural community to the risk of cyanotoxins to agricultural plants, which has consequences for the health of animals and humans that consume those plants. Therefore, my thesis aimed to address these knowledge gaps.

First, I completed a critical review, proposing that nutrient loading from agricultural runoff may trigger beta-N-methylamino-L-alanine production in cyanobacterial blooms, based on evidence from Lake Winnipeg, Canada. I provide evidence that allochthonous agricultural nutrients may trigger beta-N-methylamino-L-alanine production by influencing the composition of potentially toxic cyanobacteria species and the nitrogen availability. Then, I confirmed that cyanotoxins are present in ecosystem compartments beside eutrophic waterbodies, based on the detection of microcystins in agricultural soils and subsurface water (drainage water, well water and municipal drinking water) in agricultural watersheds in Quebec, Canada. However, the semi-quantitative method in this study was not conclusive and led me to develop a method to extract

and quantify the cyanotoxins in soil. My new method can efficiently detect 15 cyanotoxins in soils. Finally, I evaluated the phytotoxicity of microcystins in soil on agricultural plants and determined the human health risk from consuming microcystins-contaminated plants. Based on my meta-analysis, microcystins are potentially most phytotoxic to potato but leafy vegetables such as dill, parsley and cabbage could bioconcentrate ~3 times more microcystins than other agricultural plants. Consuming leafy vegetables containing microcystins could be risky to adults and children because the estimated daily intake values (> 0.2 μg $kg^{-1}d^{-1}$) exceed the WHO guidelines (0.04 μg $kg^{-1}d^{-1}$). In conclusion, my research showed that cyanotoxins are detectable in terrestrial and subterranean environments where they are seldom studied, and reveals a public health risk associated with microcystins in the edible components of agricultural plants. My findings support the One Health approach to manage the risk of public exposure to toxic substances, and indicate that cyanotoxins warrant investigation in a greater number of environments than are monitored at present.

RÉSUMÉ

Les cyanobactéries et leurs cyanotoxines sont présentes dans de nombreuses régions et écosystèmes du monde entier. Les lacs d'eau douce eutrophes qui reçoivent des apports réguliers de ruissellement riches en nutriments provenant des terres agricoles sont un environnement idéal pour les proliférations de cyanobactéries, qui sont associées à la production de cyanotoxines, mais les cyanotoxines pourraient également être présentes ou provenir des sols, des eaux souterraines et de la zone vadose des bassins versants agricoles. Les humains seront exposés à ces toxines lorsqu'ils consommeront des eaux souterraines contaminées par des cyanobactéries et des cultures cultivées dans des sols contaminés. Cependant, nous ne comprenons pas pleinement comment la charge de nutriments agricoles peut déclencher la production de cyanotoxines et la présence de cyanotoxines dans les compartiments de l'écosystème en plus des plans d'eau eutrophiques. L'un des défis majeurs est de développer des méthodes quantitatives capables de détecter les cyanotoxines biologiquement significatives à de faibles concentrations, et un autre défi est de sensibiliser la communauté agricole au risque de cyanotoxines pour les plantes agricoles, ce qui a des conséquences pour la santé des animaux et des humains qui consomment ces plantes. Par conséquent, ma thèse visait à combler ces lacunes dans les connaissances.

Tout d'abord, j'ai terminé un examen critique, proposant que la charge en nutriments provenant du ruissellement agricole puisse déclencher la production de bêta-N-méthylamino-L-alanine dans les efflorescences cyanobactériennes, sur la base de preuves provenant du lac Winnipeg, Canada. Je démontre que les nutriments agricoles allochtones peuvent déclencher la production de bêta-N-méthylamino-L-alanine en influençant la composition des espèces de cyanobactéries potentiellement toxiques et la disponibilité en azote. Ensuite, j'ai confirmé que les cyanotoxines sont présentes dans les compartiments de l'écosystème à côté des plans d'eau

eutrophiques, sur la base de la détection de microcystines dans les sols agricoles et les eaux souterraines (eaux de drainage, eau de puits et eau potable municipale) dans les bassins versants agricoles du Québec, Canada. Cependant, la méthode semi-quantitative de cette étude n'a pas été concluante et m'a conduit à développer une méthode pour extraire et quantifier les cyanotoxines dans le sol. Ma nouvelle méthode peut détecter efficacement 15 cyanotoxines dans les sols. Enfin, j'ai évalué la phytotoxicité des microcystines dans le sol des plantes agricoles et déterminé le risque pour la santé humaine de consommer des plantes contaminées par des microcystines. Selon ma méta-analyse, les microcystines sont les plus phytotoxiques pour la pomme de terre, mais les légumes à feuilles tels que l'aneth, le persil et le chou pourraient bioconcentrer ~ 3 fois plus de microcystines que les autres plantes agricoles. La consommation de légumes à feuilles contenant des microcystines pouurait donc être risquée pour les adultes et les enfants, car les valeurs journalières estimées (> 0,2 µg $kg^{-1}d^{-1}$) dépassent les recommandations de l'OMS (0,04 µg $kg^{-1}d^{-1}$). En conclusion, mes recherches ont montré que les cyanotoxines sont détectables dans les environnements terrestres et souterrains où elles sont rarement étudiées, et révèlent un risque pour la santé publique associé aux microcystines dans les composants comestibles des plantes agricoles. Mes résultats soutiennent l'approche *One Health* pour gérer le risque d'exposition du public aux substances toxiques et indiquent que les cyanotoxines méritent d'être étudiées dans un plus grand nombre d'environnements que ce qui est actuellement surveillé.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my profound gratitude to my primary supervisor, Dr. Joann Whalen, for giving me the opportunity to undertake this project under her tutelage. Having been working with Joann is one of the great learning experiences in my life. Give a man a fish and you feed him for a day; teach a man to fish and you feed him for a lifetime. Joann is the one who "teaches me to fish". She leads me to appreciate the beauty of science so that I gradually fall in love with science and the passion of being a good researcher is triggered. I believe things l learned from her will lead me to the success in my future academic career. My next profound gratitude goes to my co-supervisor Dr. Sébastien Sauvé, whose support laid the foundation of completing this project. I am grateful to Sébastien for his expertise, guidance and constructive critiques of my approach and manuscripts, and for providing me unlimited access to his laboratory to use the consumable materials and liquid chromatography-mass spectrometry instrument. I would like to also thank Dr. Geoffrey Sunahara for providing me with constructive comments on my experimental design and manuscript writing, and Dr. Alistair Boxall for hosting me as a visiting student at the University of York, UK.

I am equally grateful to Barry Husk for assisting Joann to give me the opportunity to conduct this project and supporting this project all the time. I would like to also express my thanks to Hicham Benslim for his support and encouragement, and his assistance in field sampling. I am also thankful to all the former and current members of the Soil Ecology Research Group at McGill University and Analytical Chemistry Lab at Université de Montréal for their help and support during my Ph.D. program. Very special thanks to Dr. Chen Cai for his encouragement and guidance when I felt lost in the first year of my Ph.D. study, and Dr. Sung

Vo Duy for guiding and supporting my lab work. My thanks are extended to Hélène Lalande for her technical support, and to all the staff of the Department of Natural Resource Sciences.

I am also thankful to China Scholarship Council for its financial support. I am thankful to my master's supervisor, Dr. Zejun Tang, who gave me freedom and financial support so that I got the opportunity to pursue my scientific career in McGill University.

During my time in Canada, I was lucky to meet Dr. Najmeh Mazloom as my first friend in Canada. Her niceness and optimism were like a light on me, driving away my trepidation when I first came to McGill. To my like-minded friends in China, I owe great deal of thanks for their endless support, especially to Yu Zhang, Yang Yang and Miaoqian Teng.

Words fail me when I try to express my heartfelt gratitude to my parents and my grandmother. My grandmother accompanied my whole childhood, and my parents brought me in this world and raise me up. They have given me the best they can give without any strings attached. My great thanks also go to my two brothers, sister-in-law, two clever nephews for their unchanging love, support and encouragement. Very special thanks to my boyfriend, who gave me a harbor to rest in—let my heart from now on no longer stray and feel lonely in a foreign country.

This thesis is dedicated to my memory of my grandfathers who did not have the opportunity to see their lovely granddaughter's dream coming true. I also dedicate this thesis to my parents and grandmas, who are strong backing on the way to my dream. Thank you all for your unconditional love!

CONTRIBUTION TO KNOWLEDGE

The research conducted in this thesis provides several unique contributions to knowledge:

(1). I was the first to propose the ecophysiological functions of beta-N-methylamino-L-alanine for cyanobacteria populations. It was the first study that explained how the nitrogen loading from agricultural runoff triggers the production of beta-N-methylamino-L-alanine by increasing the biomass of toxic cyanobacteria and altering the nitrogen availability in the freshwater environment.

(2). I was the first to confirm that cyanotoxins are present in the vadose zone (soil and drainage water from the soil profile) and in groundwater collected from the aquifer of agricultural watersheds. I had qualitative confirmation (DNA-based analysis) of cyanobacteria with potential to produce cyanotoxins, as well as semi-quantitative and quantitative detection of two cyanotoxins, anatoxin-a and microcystins, in agricultural soils of Quebec, Canada. Furthermore, I detected microcystins in drinking water sourced from an artesian well and in the municipal drinking water supply of rural communities in south-central Quebec, Canada. This confirms that cyanotoxins occur in many environments, either because they are produced there or because they are transferred between ecosystem compartments (e.g., from the vadose zone to subsurface drainage and groundwater receptors).

(3). I developed a new method that is capable of extracting and quantifying 17 common cyanotoxins with diverse chemistry from soil. Purification was achieved quickly by coupling the pre-concentration process for a large volume of analyte to the analytical system with an on-line SPE-UHPLC-MS/MS. My extraction method with methanol + 200 mM ammonium acetate and rigorous quantification approach provide suitable accuracy and precision, within acceptance criteria, to quantify 15 distinct cyanotoxins in soil.

(4). This was first meta-analysis study to evaluate the microcystins phytotoxicity and bioconcentration in agricultural plants. Furthermore, it is the first time that the public health risk of microcystins in the edible parts of agricultural plants is reported.

CONTRIBUTION OF AUTHORS

This thesis is composed of five chapters, preceded by a general introduction, and ends with general conclusions and suggestions for future research. The contributions to knowledge from this thesis are listed, according to the guidelines of the Graduate and Postdoctoral Studies Office, McGill University. The first chapter is a literature review that summarizes the previous work and knowledge gaps about cyanotoxins detected in agricultural watersheds, as well as methods to quantify cyanotoxins in the soil-plant system. Chapters two to five are my original research on cyanotoxins from laboratory and field experiments, which are written in manuscript format according the guidelines of the Graduate and Postdoctoral Studies Office, McGill University.

The candidate is the first author of all manuscripts. Co-authors include Joann K. Whalen, Sébastien Sauvé, Barry R. Husk, Hicham Benslim, Sung Vo Duy, Gabriel Munoz, Quoc Tuc Dinhc and Juan Sebastian Sanchez. The candidate reviewed the literature and formulated the research goals, developed the methodological approach, performed the experiments, collected data, verified the overall replication/reproducibility of results/experiments, used statistical and mathematical techniques to analyze the data, synthesized information, interpreted findings, and wrote the all manuscripts. Dr. Whalen provided financial support, advice about research goals and experimental work, and editorial assistance with the manuscripts. Dr. Sauvé provided study materials, access to state-of-the-art analytical instruments, computing resources, and data analysis tools, coordinated responsibility for the planning and execution of research activities, and edited manuscripts of Chapters 3, 4 and 5. Mr. Husk assisted with field sampling and edited the manuscripts of Chapters 3 and 4. Dr. Sung helped to develop the analytical method, sample analysis and interpretation of data, as well as editing the manuscripts of Chapters 3 and 4. Dr. Munoz checked the data interpretation and edited the manuscript of Chapter 4. Mr. Dinhc and

Mr. Sanchez assisted with field sampling and provided advice on manuscripts (Chapters 3). The thesis research was funded by China Scholarship Council (CSC), NSERC Discovery grant and the ATRAPP grant funded by Genome Quebec and Génome Canada. The manuscript-based chapters are presented in the following order:

Chapter 2. Zhang, Y., Whalen, J.K., 2020. Production of the neurotoxin beta-N-methylamino-L-alanine may be triggered by agricultural nutrients: An emerging public health issue. Water Research 170: 115335. https://doi.org/10.1016/j.watres.2019.115335.

Chapter 3. Zhang, Y., Husk, B.R., Duy, S.V., Dinh, Q.T., Sanchez, J.S., Sauvé, S, Whalen, J.K., 2020. Quantitative screening for cyanotoxins in soil and groundwater of agricultural watersheds in Quebec, Canada. (Major Revision, Chemosphere)

Chapter 4. Zhang, Y., Whalen, J.K., Duy, S.V., Munoz, G., Husk, B.R., Sauvé, S., 2020. Improved extraction of multiclass cyanotoxins from soil and sensitive quantification with on-line purification liquid chromatography tandem mass spectrometry. Talanta 120923. https://doi.org/10.1016/j.talanta.2020.120923.

Chapter 5. Zhang, Y., Whalen, J.K. and Sauvé, S., 2020. Phytotoxicity and bioconcentration of microcystins in agricultural plants: Meta-analysis and risk assessment. Environmental Pollution 115966. https://doi.org/10.1016/j.envpol.2020.115966.

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

Cyanobacteria are ubiquitous in aquatic and terrestrial environments, and the largest populations are present at the surface where there is ample light to support photosynthesis, the main source of energy and carbon for these autotrophs. Aquatic cyanobacteria can number as many as 200 million cells per cm^3 in the surface water, whereas soil can support 5.2 million to 51 million cyanobacteria cells per cm^2 in agroecosystems of humid temperate regions (Whitman et al., 1998). Still, cyanobacteria are known to be bioactive in subsurface soil, aquifers and groundwater, because they can consume hydrogen gas and produce energy in the dark, a trait developed by their non-photosynthetic ancestors (Puente-Sánchez et al., 2018).

Since they are the survivors of more than 3.0 billion years of evolution and architects of the Great Oxygenation Event, cyanobacteria evolved many survival strategies. One of these strategies is to produce secondary metabolites – cyanotoxins – in response to ecophysiological cues related to nitrogen and carbon metabolism, photosynthesis regulation, quorum sensing and competition with other organisms (Zhang and Whalen, 2019). Cyanotoxin production is usually associated with harmful cyanobacterial blooms in eutrophic surface water. Cyanotoxins can be lethal, but they also bioaccumulate in the cells of microorganisms, plants and animals, which can lead to bioconcentration of cyanotoxins in secondary consumers, including humans. The human health risk from cyanotoxins must not be underestimated because the hepatotoxic peptides of microcystins and cylindrospermopsins are associated with acute and chronic liver damage, while the chronic exposure to neurotoxic anatoxin-a, saxitoxins and beta-N-methylamino-L-alanine increase the risk of human neurodegenerative diseases (Huisman et al., 2018).

Since cyanotoxins biosynthesis is energetically and nutritionally costly for cyanobacteria, there must be an evolutionary advantage for cyanobacteria to synthesize these compounds.

Cyanotoxins are stored intracellularly (intracellular cyanotoxin) or released to the surrounding environment (extracellular cyanotoxins). Except for cylindrospermopsin, which is secreted into the environment when it is produced, the other cyanotoxins are retained in living cyanobacteria and only release into the surrounding environment when cyanobacterial cells lyse. These molecules may ensure the organism's functional niche (ecological advantage) or improve their fitness (physiological efficiency).

Cyanotoxins present in the environment are dangerous to human health, and the risk to public health depends upon the dose and duration of exposure. Humans are most likely to be exposed to cyanotoxins from harmful cyanobacterial bloom because of the global distribution and frequent occurrence of these toxic blooms. However, biologically significant amount of cyanotoxins also exist in soil crusts, in soils irrigated with cyanobacteria-contaminated water, and in plants that grow in these soil environments. As we move towards a One Health approach to manage the risk of public exposure to cyanotoxins, we must also include soil as a source of these emerging biological contaminants. Eco-epidemiological surveys indicate that human health is at risk from exposure to soil-borne cyanotoxins, since cyanotoxins are inhaled along with dust (wind-eroded soil particles) and ingested by consuming plants that contain cyanotoxins in their tissues or by accidental ingestion of contaminated soil and water (Funari and Testai, 2008; Serrano et al., 2015). Environmental exposure levels must be quantified to understand risks and improve public health outcomes, consistent with One Health goals (Hilborn and Beasley, 2015).

The overarching goal of my thesis is to explain how agricultural nutrients may trigger cyanotoxin production, to understand the occurrence of cyanotoxins in agricultural watersheds and to quantify the human health risks of cyanotoxins in soil-plant system. The specific objectives were: 1): to explain how nutrient loading from agricultural runoff trigger beta-N-

methylamino-L-alanine production in cyanobacterial blooms, focusing on the ecophysiological reasons why nitrogen availability controls beta-N-methylamino-L-alanine production and supporting this with evidence from in-lake studies. 2): to investigate the occurrence of cyanotoxins outside of eutrophic waterbodies by detecting cyanotoxins in agricultural soils and subsurface water (drainage water, well water and municipal drinking water) in agricultural watersheds in Quebec, Canada. 3): to develop a new method to extract and quantify 17 diverse cyanotoxins from soil. 4): to evaluate the phytotoxicity of microcystins in soil to agricultural plants and determine the human health risk from consuming microcystins-contaminated plants.

CHAPTER 1

Literature review

1.1. The global distribution of cyanobacteria

Cyanobacteria are the oldest phototrophic prokaryotes that appeared on Earth about 3.2 to 3.5 billion years ago (De, 2000). They may be unicellular, colonial, or filamentous, with cell sizes varying from < 2 um to 40 μm in diameter. Cyanobacteria are classified in three orders: *Chroooccales,* which include coccoid unicellular or colony-forming cells, *Nostocales* are the filamentous forms that can fix atmospheric nitrogen in their heterocysts, and *Oscillatoriales*, which are the filamentous types lacking heterocysts (Komárek et al., 2014). Cyanobacteria have a cosmopolitan distribution and are common in the benthos and water column of freshwater and marine environments, in soil biological crusts, and as symbionts with plants and fungi. Large cyanobacteria populations are present at the surface of aquatic and terrestrial environments where there is ample light to support photosynthesis, the main source of energy and carbon for these autotrophs (Bar-On et al., 2018). Surface water can contain as many as 200 million cyanobacteria cells per cm^3, while soil cyanobacteria populations are estimated to be between 5.2 and 51 million cells per cm^2 in agroecosystems of humid temperate regions (Whitman et al., 1998). Cyanobacteria are also detected in subsurface soil, aquifers and groundwater, probably because they can consume hydrogen gas and produce energy in the dark, a survival strategy that might trace back to their non-photosynthetic ancestors (Puente-Sánchez et al., 2018).

1.2. Cyanotoxins, the secondary metabolites of cyanobacteria

1.2.1. History of the emergence of cyanotoxins as pollutants

Cyanobacteria are dangerous to humans, and this was known for thousands of years. One of the earliest narrative reports of cyanobacteria poisoning occurred during the Han dynasty in China, about 2,000 years ago. General Zhu Ge-ling, while on a military campaign in southern China, reported losing troops from poisoning whilst crossing a river. He reported that the river was green in color and his troops drank from the green water (Bartram and Chorus, 1999). The first detailed scientific inquiry of the toxic action of a cyanobacterial bloom was published in Nature in 1878 (Francis, 1878). George Francis, a local chemist, was commissioned to investigate the cause of mass mortalities of farm livestock along the Murray River and the shores of Lake Alexandrina in South Australia. He observed that a dense bloom of the cyanobacterium *Nodularia spumigena* floated to the surface during calm weather, forming a scum at the lee shores of the lake. Livestock along the shores ingested this scum while drinking from the lake and died a few hours later. Francis reproduced the characteristic signs and timing of illness, mortalities and gross organ pathology by dosing healthy sheep with fresh scum material. He correctly inferred that *Nodularia spumigena* contained a toxin responsible for the sheep deaths, which is now known as "cyanotoxin". More than 40 toxin-producing cyanobacterial genera have since been identified (Sivonen and Jones, 1999; Svirčev et al., 2019) and there are an unknown number of "potentially" toxic species. More than nine different types and hundreds of variants of cyanotoxins have been detected from 60% CyanoHABs samples all over the world (Hipsher et al., 2020; Organization, 2003; Rastogi et al., 2014) (Figure 1.1).

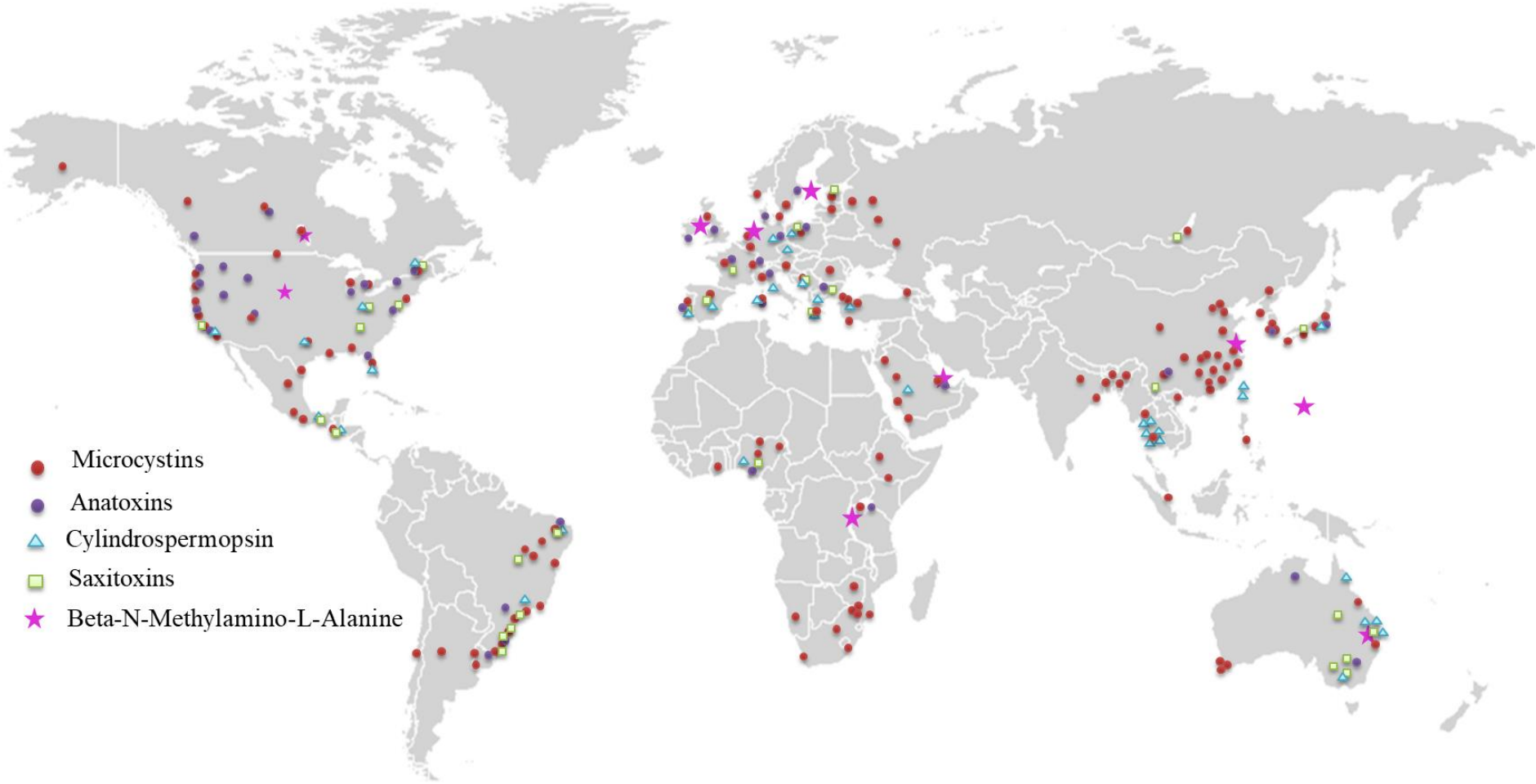


Figure 1.1: The worldwide distribution of commonly studied cyanotoxins (adapted from Svirčev et al., 2019).

Cyanotoxins can be classified into three groups based on their chemical composition: (1) cyclic peptides (microcystins and nodularin), (2) alkaloids (anatoxin-a, anatoxin-a(s), saxitoxins, cylindrospermopsin, aplysiatoxin, lyngbiatoxin-a) and (3) lipopolysaccharides (Rastogi et al., 2014). The toxicology, environmental fate, analytical methods and removal technologies for microcystins have received much more attention than other cyanotoxins, e.g. cylindrospermopin (~7 times less), beta-N-methylamino-L-alanine (~10 times less), anatoxins (~5 times less) and saxitoxins (~3 times less) (Figure 1.2). This is probably due to the widespread and frequent occurrence of microcystins in cyanobacterial blooms, persistence in the environment and well-developed analytical techniques than the other cyanotoxins, which are more active and less common in nature with trace amount.

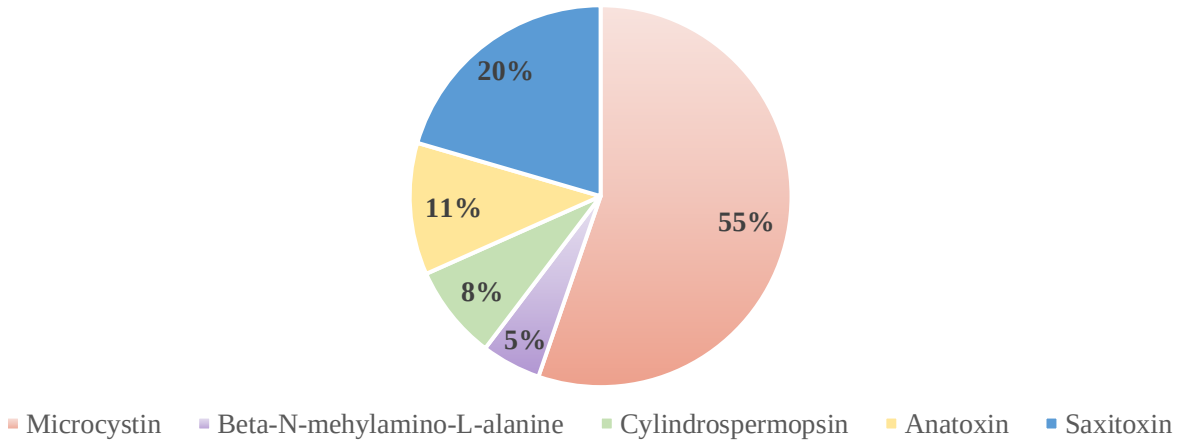


Figure 1.2: The number of studies on different cyanotoxins published by 2020 (Web of Science). The data was obtained by searching keywords (TITLE-ABS-KEY (Cyanobacterial bloom OR Cyanotoxin)) *AND (Microcystin* Cylindrospermopsin OR Anatoxin* OR Saxitoxin* OR PSP OR BMAA OR heptato*OR peptide toxin* OR neuroto* OR alkali* OR cyano*) AND (*accumulation* OR *take OR assimila* OR expos*) AND (*toxicity OR inhibit* OR negative*(influen* OR effect OR impact* OR grow* OR health*) AND (human OR animal OR plant OR soil OR water)*.

1.2.2. General introduction to cyanotoxins

Chemically, microcystins are monocyclic heptapeptides with a molecular weight of 800–1,100 Da. The general structure of microcystins shown in Figure 1.3 is cyclo-(-D-Ala^{1}-L-X^{2}-DisoMeAsp^{3}-L-Z^{4}-Adda^{5}-D-isoGlu^{6}-Mdha^{7}), where D-MeAsp^{3} is D-erythro-b-methylaspartic acid, Mdha^{7} is N-methyldehydroalanine and Adda^{5} is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4, 6-dienoic acid. X^{2} and Z^{4} are substituted amino acids that vary according to the biosynthesis process, resulting in more than 240 different variants of microcystins (Bouaïcha et al., 2019; Sivonen and Jones, 1999). Microcystins are potent and specific inhibitors of protein phosphatase1 and 2A, which accounts for their extreme toxicity in plant and animal cells (Corbel et al., 2014).

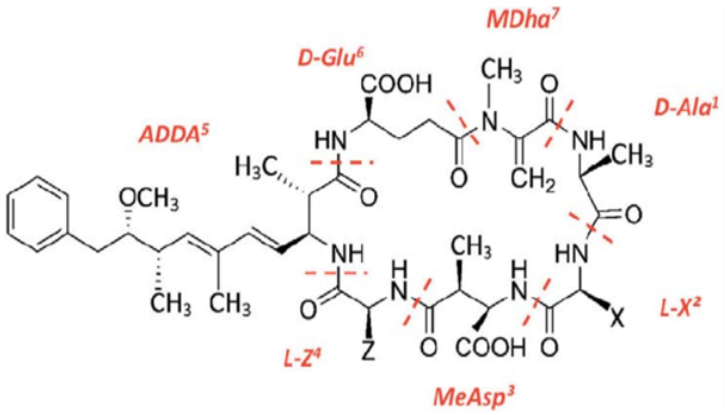


Figure 1.3: General structure of microcystins.

Cylindrospermopsin is a highly water-soluble polyketide-derived alkaloid with a central functional guanidino moiety combined with hydroxymethyluracil attached to its tricyclic carbon skeleton (Figure 1.4; (Ohtani et al., 1992). Natural analogs of 7-deoxy-Cylindrospermopsin, which lack the hydroxyl group on the tricyclic skeleton, and 7-epi-Cylindrospermopsin that has an epimer at the hydroxyl bridge (Armah et al., 2013). The uracil moiety and the hydroxyl group are responsible for cylindrospermopsin toxicity (Banker et al., 2001). Cylindrospermopsin could

irreversibly inhibit protein synthesis and glutathione synthesis in plant and animal cells (Froscio et al., 2003; Machado et al., 2017; Runnegar et al., 1994).

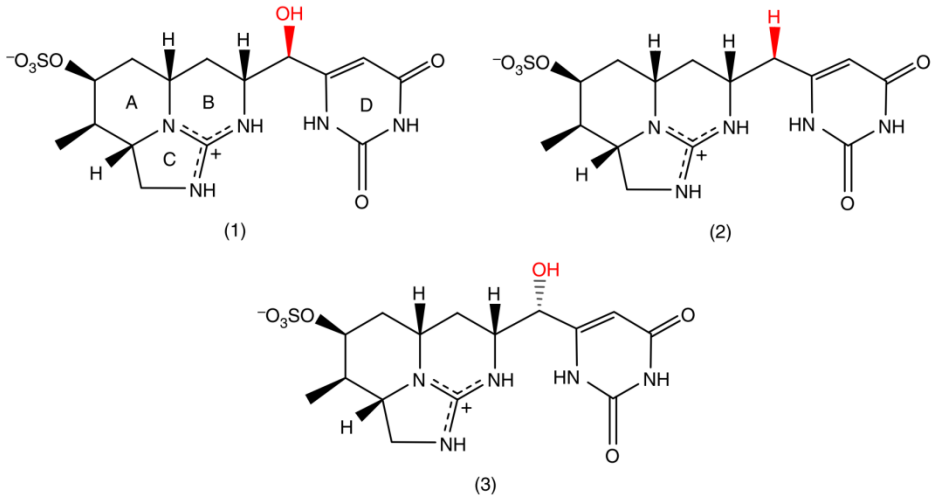


Figure 1.4: The molecular structure of cylindrospermopsin (1) and its analogs 7-deoxy-Cylindrospermopsin (2) and 7-epiCylindrospermopsin (3) (Moreira et al., 2013).

Anatoxin-a is a secondary amine bicyclic alkaloid with a molecular weight of 165. It has a structural similarity to cocaine, although it does not have the pharmatological function. Homoanatoxin-a (molecular weight 179) is structurally related to anatoxin-a, but it differs due to the presence of a propionyl group instead of the acetyl group (Figure 1.5). Anatoxin-a is a potent pre- and post-synaptic depolarizing agent, which competes efficiently with acetylcholine (being 100 times more selective) for nicotinic receptors in neuromuscular junctions and the central nervous system (Mahmood and Carmichael, 1986). Homoanatoxin-a has nearly identical toxicological properties and the same mode of action as anatoxin-a (Lilleheil et al., 1997).

Figure 1.5: Chemical structure of anatoxin-a (left) and homoanatoxin-a (right).

Saxitoxin is the parent molecule in a class of neurotoxic alkaloids. Since its initial discovery, 57 naturally occurring saxitoxin analogs have been identified in several organisms, collectively referred to as paralytic shellfish toxins (Wiese et al., 2010). The basic structure of saxitoxin is a trialkyl tetrahydropurine, with positions 2 and 8 of the purine ring containing the NH_2 groups, which form the two permanent guanidinium moieties (Figure 1.6; (Cusick and Sayler, 2013). Saxitoxin is one of the world's most potent natural toxin; for instances, its LD_{50} (10 μg kg^{-1} bw after intraperitoneal injection in mice) was more than 5 times higher than microcystin-LR (50 μg kg^{-1} bw). Saxitoxin is a neurotoxin that acts as a selective sodium channel blocker (Thottumkara et al., 2014). It acts on the voltage-gated sodium channels of neurons, preventing normal cellular function and leading to paralysis (O'Neill et al., 2016).

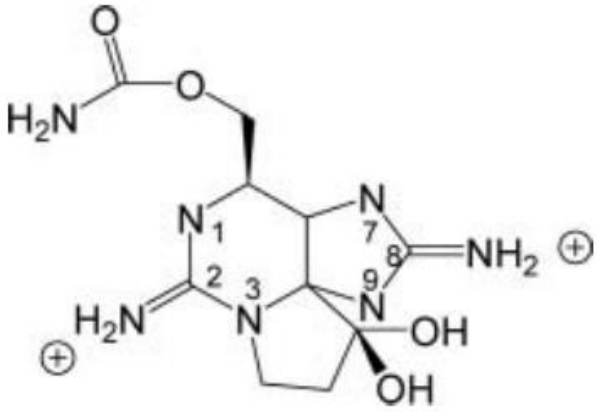


Figure 1.6: Basic structure of saxitoxin.

Beta-N-methylamino-L-alanine (BMAA) is a non-lipophilic, hydrophilic, and highly polar non-protein amino acid (Figure 1.7) that is produced by diverse taxa of cyanobacteria (nearly 95% of cyanobacteria) (Cox et al., 2005b), including dinoflagellates and diatoms. It was first isolated from the seeds of *Cycad micronesia* serendipitously, a cycad indigenous to Guam that is eaten by the native residents (Vega and Bell, 1967). Beta-N-methylamino-L-alanine is a slow-acting toxin that is linked to the amyotrophic lateral sclerosis/parkinsonism-dementia complex syndrome of Guam (Borenstein et al. 2007), and is suspected to contribute to chronic

neurotoxicology in disorders like Alzheimer's disease (Cox et al., 2003a) and amyotrophic lateral sclerosis (Cox et al. 2009).

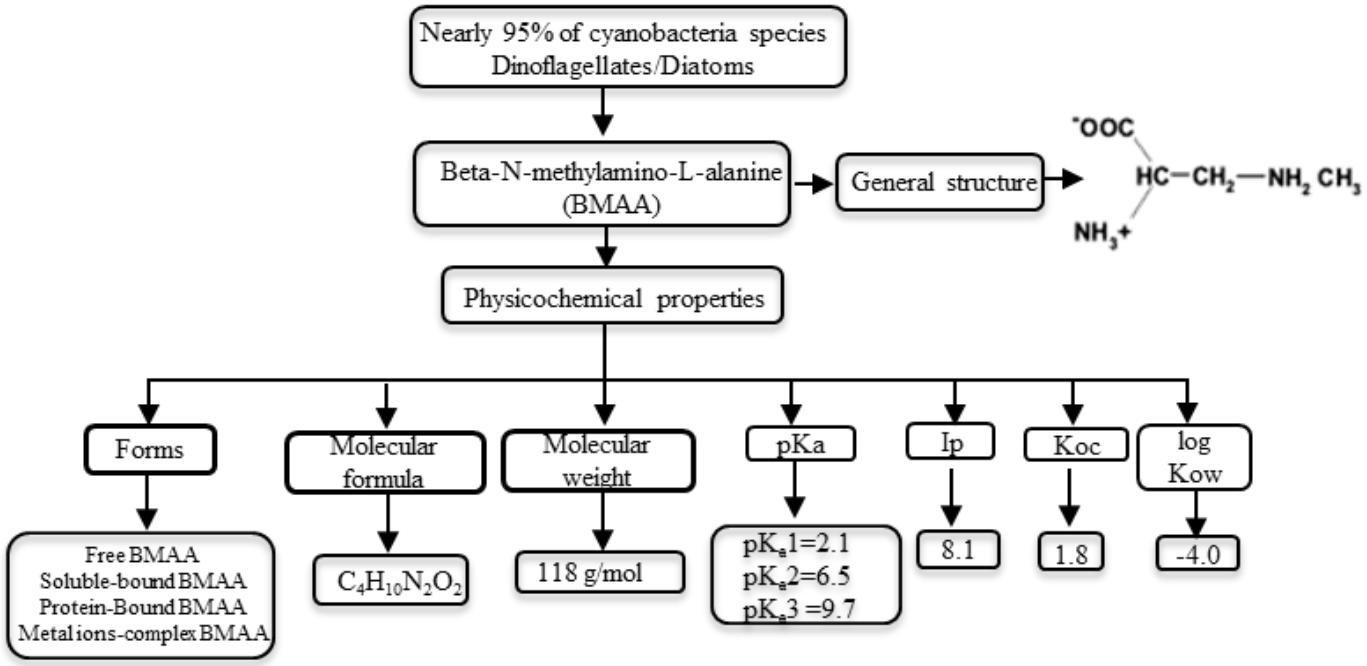


Figure 1.7: General structure and physicochemical properties of beta-N-methylamino-L-alanine.

1.2.3. Environmental occurrence and concentration of cyanotoxins

Cyanotoxins are often produced by CyanoHABs in eutrophic surface water, but this is not the only environment where cyanotoxins are detected. The cyanobacteria and cyanotoxins in surface water could be transferred from aquatic systems to subsurface water through hydrologic processes and to agricultural lands through irrigation. Furthermore, metabolically active cyanobacteria live in the vadose zone, the aquifer and soil biological crusts. These cyanobacteria could produce the cyanotoxins found in the subsurface water (groundwater) and soils. Therefore, we expect to find cyanotoxins in eutrophic surface water where CyanoHABs occur and in adjacent environments.

The presence and concentration of cyanotoxins, as well as the putative cyanobacteria that could produce these toxins, varies among environments, as summarized in Table 1.1.

Microcystins are the most common cyanotoxins in surface water, groundwater, agricultural soils and biological soil crust. Plants, animals and humans are more likely to be exposed to microcystins than other cyanotoxins. In animals and human, organic anion transporting polypeptides in the cell membrane are responsible for transporting microcystins into the cells of affected organisms (Campos and Vasconcelos, 2010). However, plants do not have a specific microcystins transporter (Redouane et al., 2019); they might passively adsorb and diffuse microcystins throughout their organs, or they could take up biotransformed microcystins conjugates. In plants, less than 10% of microcystin will be conjugated non-enzymatically to glutathione (Pflugmacher, 2002). The remaining microcystins can bind to protein phosphatases and other cellular proteins, or they are absorbed by chloroplasts. Microcystins can cause excessive formation of reactive oxygen species in plant, which places an enormous burden on the antioxidant system, damaging to DNA, proteins, carbohydrates and lipids cells (Machado et al., 2017; Pham and Utsumi, 2018). Specifically, microcystins can pose negative effects on histology, cytology and morphology of plant including (1) tissue development; (2) activation of enzymes involved in CO_2 fixation; (3) starch storage; (4) gene expression; (5) regulation of ionic channels; (6) carbon and nitrogen metabolism; (7) hormone transport and (8) photosynthesis.

Table 1.1: Environmental occurrence, range of concentration and health guideline of cyanotoxins and their producers. The data were obtained from field investigation, while laboratory-controlled experiments are excluded.

Environment	Cyanotoxins	Concentration	Producers species/strains	Guidelines	Reference
Surface water (μg L^{-1})	Microcystins	0.1–29,000	*Anabaena; Geitlerinema; Leptolyngbya; Pseudanabaena; Synechococcus; Spirulina; Phormidium; Microcystis sp.; Microcystis aeruginosa; Microcystis flos-aquae; Nostoc sp.; Oscillatoria agardhii; Planktothrix agardhii; Radiocystis fernandoii*	Recreational water: WHO/Ohio/Illinois/ Washington: 10 California: 0.8 Indiana/Kansas: 4 Lowa/Nebraska/Oklahoma/Texas: 20 Massachusetts/Rhode Island: 14	(Billam et al., 2006; Cao et al., 2018a; Flores et al., 2018; Liu et al., 2011; Merel et al., 2013; Nasri et al., 2008; Spoof et al., 2003)
	Cylindrospermopsin	0.07–202	*Anabaena bergii; Anabaena planctonica; Anabaena ovalisporum; Oscillatoria sp.; Cylindrospermopsis raciborskii; Raphidiopsis curvata; Raphidiopsis mediterranea; Aphanizomenon ovalisporum; Umezakia natans; Lyngbya wollei*	Recreational water: Washington: 4.5 Ohio: 5 Oregon: 6	(Berry and Lind, 2010; Bláhová et al., 2008; Blahova et al., 2009; Hawkins et al., 1997; Jiang et al., 2014b; Li et al., 2001; Mankiewicz-Boczek et al., 2012; Rzymski and Poniedziałek, 2014; Shaw et al., 1999; Vasas et al., 2010)
	Anatoxin-a	0.01–444	*Arthrospira fusiformis; Anabaena spp.; Anabaena lemmermannii; Aphazinomenon spp; Aphanizomenon issatschenkoi; Aphazinomenon flos-aquae; Cylindrospermum sp.; Microcystis aeruginosa; Phormidium autumnale;*	Recreational water: Washington: 1 Vermont: 10 Oregon: 20 Ohio: 80	(Ballot et al., 2010; Bumke‐Vogt et al., 1999; James et al., 1997; Testai et al., 2016)
	Saxitoxin	3–1,000	*Anabaena circinalis; Anabaena perturbata; Anabaena spiroides; Aphanizomenon flos-aquae; Aphanizomenon gracile; Cylindrospermopsis*	Recreational water: Washington: 75 Ohio: 0.8	(Ledreux et al., 2010; Molica et al., 2005; Orr et al., 2004; Rapala et al., 2005)

			raciborskii;Plankthotrix FP1; *Lyngbya wollei*		
	Beta-N-methylamino-L-alanine	1–231	*Microcystis; Prochlorococcus marinus; Synechocystis; Chroococcidiopsis indica; Myxosarcina burmensis; Lyngbya majuscula; Planktothrix agardhii; Plectonema; Phormidium; Symploca; Trichodesmium; Anabaena; Aphanizomenon flos-aquae; Cylindrospermopsis raciborskii; Nodularia; Nostoc; Calothrix; Fischerella; Scytonema*	N/A	(Al-Sammak et al., 2014; Metcalf et al., 2008; Pip et al., 2016)
Groundwater ($\mu g\ L^{-1}$)	Microcystins	0.2–2.5	*Oscillatoria limnetica; Chroococcus minor; Gleocapsa sp.; Oscillatoria limnetica; Spirulina sp.*	Drinking water: WHO/Ohio:1 Health Canada: 1.5 Minnesota: 0.04	(Chen et al., 2006b; Eynard et al., 2000; Mohamed and Al Shehri, 2009; Tian et al., 2013; Yang et al., 2016)
	Cylindrospermopsin	–	–	Drinking water： New Zealand/Ohio:1	–
	Anatoxin-a	–	–	Drinking water: New Zealand: 6 Ohio: 20	–
	Saxitoxin	–	–	Drinking water: New Zealand: 3 Ohio: 0.2	–
	Beta-N-methylamino-L-alanine	–	–	N/A	–
Agricultural soil ($\mu g\ kg^{-1}$)	Microcystins	2.1–1,690		[a] 13.7	(Chen et al., 2012; Xiang et al., 2019)

	Cylindrospermopsin	Detectable	*Hormoscilla pringsheimii;*	N/A	(Bohunická et al., 2015; Rzymski and Poniedzialek, 2015)
	Anatoxin-a	–	–	N/A	–
	Saxitoxin	–	–	N/A	–
	Beta-N-methylamino-L-alanine	758,000	*Chlorogloeopsis* PCC 6912	N/A	(Cox et al., 2005b)
Soil biological crust ($\mu g\ kg^{-1}$)	Microcystins	1.5–11,058	*Hapalosiphon hibernicus; Microcoleus; Nostoc sp.; Nostoc paludosum; Nostoc paludosum; Chroococcus sp; Oscillatoria sp., and Phormidium sp.*	[b] 0.001–2.25	(Chen et al., 2006a; Chrapusta et al., 2015; Metcalf et al., 2012; Prinsep et al., 1992)
	Cylindrospermopsin	[c] Detectable	*Hormoscilla pringsheimii;*	N/A	(Bohunická et al., 2015; Rzymski and Poniedzialek, 2015)
	Anatoxin-a	322–633	*Oscillatoria; Phormidium autumnale; Phormidium favosum*	N/A	(Chrapusta et al., 2015)
	Saxitoxin	–	–	N/A	–
	Beta-N-methylamino-L-alanine	[c] Detectable		N/A	(Cox et al., 2005a; Metcalf et al., 2015a; Richer et al., 2015)

[a] Predicted No-effect Concentration (Xiang et al., 2019); [b] Tolerate Daily Inhale ($\mu g\ kg^{-1}\ day^{-1}$) (Metcalf et al., 2012); [c] Specific value is not available.

1.2.4. The hotspots of cyanotoxins production—hot and nutritious bloom

As shown in Table 1.1, the concentration of cyanotoxins in a particular environment can vary by hundreds to thousands of orders of magnitude. The largest concentrations are recorded during times of the year when cyanobacteria populations are growing rapidly or reach their peak growth. Warm temperature, proper sunlight, long water residence time, low disturbance and abundant nutrients favor the growth of cyanobacteria (Figure 1.8). Cyanobacteria were present at bloom levels (≥20,000 cyanobacterial cells ml^{-1} or 10 μg L^{-1} chlorophyll a) across a wide range of temperatures (3–27 °C) (Bartram and Chorus, 1999). As a group, cyanobacteria exhibit optimal growth rates at relatively high temperatures, usually over 25 °C (Paerl and Huisman, 2009). The Intergovernmental Panel on Climate Change predicts increases in the global average surface temperature of 1.4 to 5.8 °C by the year 2100, which will favor cyanobacterial growth (i.e., they are better competitors at higher temperatures) and will increase the frequency of CyanoHABs in the future. (Change, 2007; Mooij et al., 2005).

Light is often considered to be one of the limiting factors of phytoplankton growth because solar radiation is the energy source of the aquatic ecosystem (Tilzer, 1987). Cyanobacteria contain chlorophyll a as a major pigment for harvesting light and conducting photosynthesis. They also contain other pigments such as the phycobiliproteins which include allophycocyanin (blue), phycocyanin (blue) and sometimes phycoerythrine (red) (Whitton and Potts, 2012). These pigments harvest light in the green, yellow and orange part of the spectrum (500-650 nm) which is hardly used by other phytoplankton species. The ability of cyanobacteria to grow at low light intensity, and to use radiative energy from more wavelengths for photosynthesis, enables them to grow in the "shadow" of other phytoplankton and eventually out-compete them.

Nitrogen (N) is an essential macronutrient for cyanobacteria (Flores and Herrero, 1994). Cyanobacteria prefer assimilable ammonium to nitrate and nitrite for their metabolic requirements. Some cyanobacteria can also use urea or organic nitrogen such as amino acids as a source of nitrogen. These nitrogen sources are taken up through permeases and metabolized to ammonium, which is incorporated into carbon skeletons through the glutamine synthetase-glutamate synthase pathway. Nitrogen is then biosynthesized from glutamine or glutamate into other amino acids, proteins and organic nitrogen compounds (Flores and Herrero, 2005). However, ionic forms of nitrogen and soluble amino acids are often in short supply because most of nitrogen exists as N_2 gas in the Earth's atmosphere. Consequently, some cyanobacteria possess heterocysts, specialized cells that convert N_2 into soluble ammonia that is transformed by glutamine synthetase into glutamine. Cyanobacteria are typically distinguished as N-fixing cyanobacteria and non-N-fixing cyanobacteria.

Cyanobacteria also require appreciable quantities of phosphorus (P) to maintain a stoichiometric homeostasis in their C:N:P chemistry, and for vital metabolic functions (structural integrity, energy relations and as an information carrier) (Codd et al., 2005). Cyanobacteria can absorb orthophosphate ($H_2PO_4^-$, HPO_4^{2-}, or PO_4^{3-}) and some small organic phosphate esters through specific transporter proteins in their cellular membrane (Raven, 2010). These compounds are usually present in minute amounts in freshwater environments, since the majority of the total phosphorus in aquatic systems are dissolved organic phosphates with high molecular weight or associated with colloidal material (Bentzen et al., 1992). Cyanobacteria can access some of this organic phosphorus by secreting alkaline phosphatases that hydrolyze esters of organic phosphorus into bioavailable inorganic phosphorus (Raven, 2010).

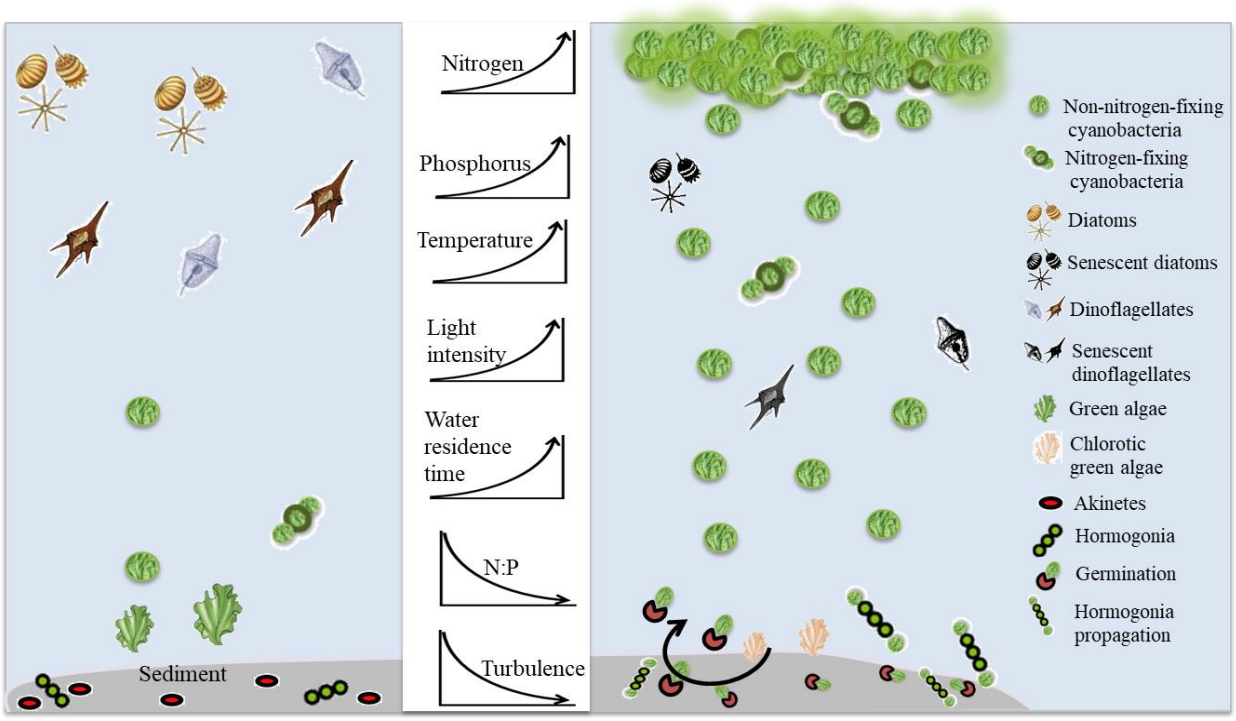


Figure 1.8: Environmental factors controlling cyanobacterial harmful algal bloom (CyanoHABs) abundance.

1.3. Why do cyanobacteria produce cyanotoxins?

Cyanotoxins are secondary metabolites of cyanobacteria. Since cyanotoxin biosynthesis is energetically and nutritionally costly for cyanobacteria, there must be an evolutionary advantage for cyanobacteria that retained the capacity to synthesize these compounds. Cyanotoxins are stored intracellularly (intracellular cyanotoxin) or released to the surrounding environment (extracellular cyanotoxins). With the exception of cylindrospermopsin, which is secreted into the environment when it is produced, the other cyanotoxins are retained in living cyanobacteria and only release into the surrounding environment when cyanobacterial cells lyse. These molecules may ensure the organism's functional niche (ecological advantage) or improve their fitness (physiological efficiency). Based on the literature, I synthesized and summarized the 6 known

ecological roles (Figure 1.9) and 5 physiological functions (Figure 1.10) of cyanotoxins for cyanobacteria.

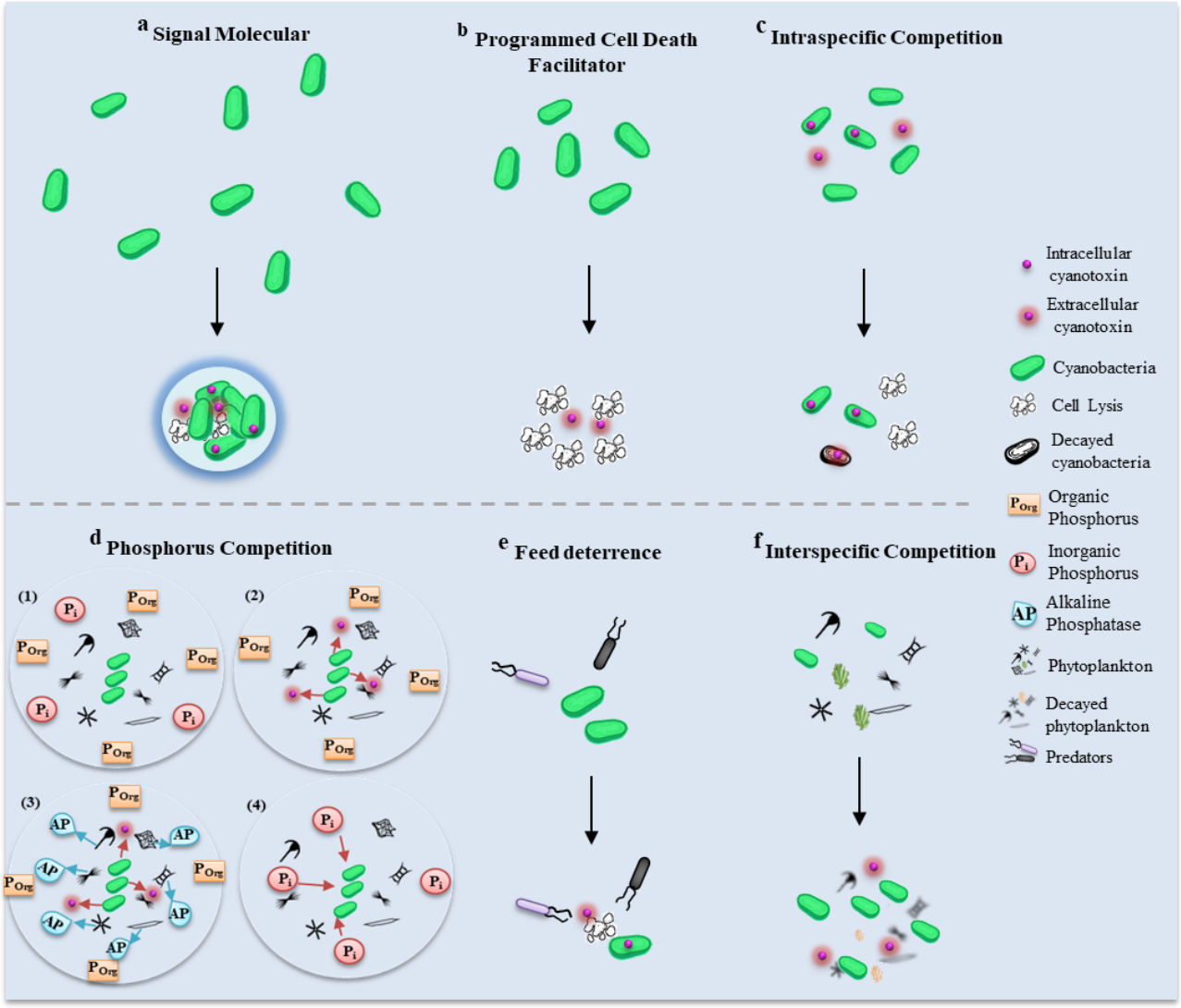


Figure 1.9: The reported ecological roles of cyanotoxins in cyanobacteria to date.

[a] Signal Molecule (Microcystins, Beta-N-methylamino-L-alanine); [b] Programmed Cell Death Participator (Microcystins, Beta-N-methylamino-L-alanine); [c] Interspecific Competition (Microcystins, Cylindrospermopsin, Beta-N-methylamino-L-alanine, Anatoxin, Saxitoxin); [d] Phosphorus Competition (Cylindrospermopsin); [e] Feed Deterrence (Microcystins,

Cylindrospermopsin, Anatoxin-a, Saxitoxin); [f] Intraspecific Competition (Microcystins, Cylindrospermopsin, Anatoxin, Saxitoxin).

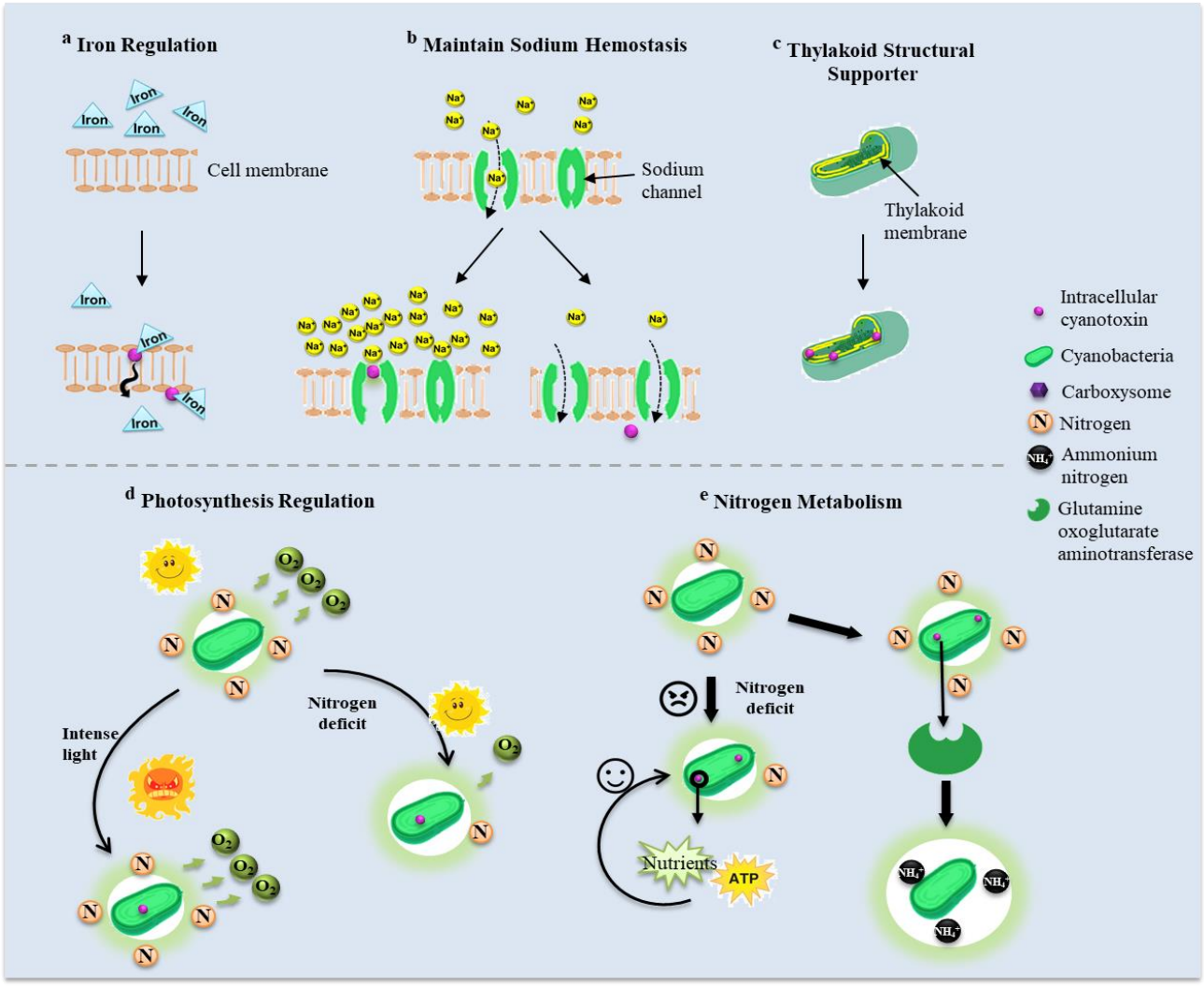


Figure 1.10: The reported physiological roles of cyanotoxins in cyanobacteria to date. [a] Iron Regulation (Microcystins); [b] Maintain Sodium Hemostasis (Anatoxin, Saxitoxin); [c] Thylakoid Structural Supporter (Microcystins); [d] Photosynthesis Regulation (Microcystins, Beta-N-methylamino-L-alanine); [e] Nitrogen Metabolism (Microcystins).

1.3.1. Ecological roles of cyanotoxins

1.3.1.1. Signal Molecule

Some cyanotoxins, especially microcystins and beta-N-methylamino-L-alanine, are potential signal molecules. When low concentrations of microcystins are released to the surrounding environment, they act as info chemicals, leading to phenotypic changes in colony formation from the lowest proportion of microcystins-producing genotypes (colonies < 50 μm) to the highest proportion of microcystins-producing cells (colonies > 100 μm) (Gan et al., 2012; Kurmayer et al., 2003). Additionally, the remaining cyanobacterial cells can sense the microcystins released from lysed cyanobacteria and respond by synthesizing more microcystins, thereby enhancing their fitness in their ecological niche (Schatz et al., 2007). Beta-N-methylamino-L-alanine has chemical characteristics that are analogous to other signal molecules produced by prokaryotes. These include: high hydrophibility (LogKow = -4.00), low molecular weight of 118 Da and rapid diffusion coefficient of 0.78 (Zhang and Whalen, 2019). Most cyanobacteria taxa seem to produce beta-N-methylamino-L-alanine, and it is postulated to be a signal molecule responsible for communication among cyanobacteria species (Cox et al., 2005b). Other non-protein amino acids that are chemically similar to beta-N-methylamino-L-alanine, such as gamma-Aminobutyric acid, accumulate rapidly in plants under stress and initiate defense pathways against drought and insect herbivory (Bown and Shelp, 2016). If beta-N-methylamino-L-alanine is a gamma-Aminobutyric acid analog, it may regulate the cyanobacteria metabolism in response to stress or be an adaptive response to mitigate stress, but this remains to be determined.

1.3.1.2. Programmed Cell Death Participator

Microcystins and beta-N-methylamino-L-alanine may be involved in initiating programmed cell death of cyanobacteria when the cyanobacterial bloom reaches the decline and collapse phases (Hu and Rzymski, 2019; Zhang and Whalen, 2019). Like other prokaryotes, cyanobacteria initiate programmed cell death under stressful environmental conditions. The

genetic basis for programmed cell death is the metacaspases, which initiate targeted protein degradation in the apoptotic programmed cell death pathways with high catalytic efficiencies and strict substrate specificities (Asplund-Samuelsson et al., 2016). Phylogenetic analysis of cyanobacteria with 16S RNA analysis revealed metacaspase orthologues, which suggests that cyanobacteria initiate programmed cell death when they encounter stressful conditions (Bar-Zeev et al., 2013; Franklin, 2014; He et al., 2016). The increase in exogenous microcystins and beta-N-methylamino-L-alanine concentration could induce the expression of metacaspase genes and initiate programmed cell death as a negative feedback to control the size of cyanobacteria populations (Hu and Rzymski, 2019; Sigee et al., 2007; Zhang and Whalen, 2019).

1.3.1.3. Intraspecific Competition

Cyanotoxins are "weapons" that allow cyanobacteria to compete for limited resources. Microcystins could act as allelochemicals, which provide a selective and competitive advantage of microcystin-producing strains to access limited resources (do Carmo Bittencourt-Oliveira et al., 2015; Kaebernick and Neilan, 2001). For instance, the inhibitory compounds released by microcystins-producing strains could inhibit non-microcystins-producing organisms, allowing the microcystins-producting strains to acquire more soluble nutrients or occupy more space (Briand et al., 2008; Van de Waal et al., 2011). The released microcystins can be taken up and localized in the thylakoid membranes of non-microcystins-producing strains, where photosynthesis II activity was inhibited (Phelan and Downing, 2014). The target of cylindrospermopsin is cyanobacteria species that produce other cyanotoxins. Cylindrospermopsin can inhibit the production of microcystins-producing cyanobacteria species, thereby reducing the fitness of the microcystins-producing cyanobacteria population (Rzymski et al., 2014). Microcystins and anatoxin-a govern the allelopathic interactions between microcystin-

producing (*Microcystis*) and anatoxin-producing cyanobacteria strains (*Anabaena*). This allelopathic interaction is linked to nutrient availability in the environment. Microcystins and anatoxin-a production alter the dominant cyanobacteria species under different nutrient conditions, enhancing the competitive advantage of their producers for nutrient acquisition (Chia et al., 2018). Nitrogen availability plays a critical role in the production of beta-N-methylamino-L-alanine by cyanobacteria (Downing et al., 2011; Monteiro et al., 2016; Scott et al., 2014). Under nitrogen starvation, non-N-fixing cyanobacteria are the dominant beta-N-methylamino-L-alanine producers. The beta-N-methylamino-L-alanine produced by non-N-fixing cyanobacteria under nitrogen starvation could be taken up by N_2-fixing cyanobacteria and inhibit their nitrogenase activity, thus altering the competitive interactions among non-N-fixing and N-fixing cyanobacteria. Beta-N-methylamino-L-alanine production kills some of the N-fixing cyanobacteria and nutrients released from their lysed cells can be recycled by the non-N-fixing cyanobacteria population. Besides, beta-N-methylamino-L-alanine blocks nitrogenase activity and constrains the growth of N-fixing cyanobacteria, thereby reducing their population size and liberating resources such as space and oxygen needed by the non-N-fixing cyanobacteria population (Zhang and Whalen, 2019). Saxitoxin is less well studied in this regard, although saxitoxin can kill the non-saxitoxin producing cyanobacteria (Holland and Kinnear, 2013). There is a possibility that the saxitoxin-producing cyanobacteria use this toxin to gain a competitive advantage over non-saxitoxin-producing cyanobacteria in environments with high pH or salt stress (Holland and Kinnear, 2013).

1.3.1.4. Phosphorus Competition

Cylindrospermopsin is reported to be involved in phosphate biogeochemical cycling, making cylindrospermopsin-producing cyanobacteria the best competitors for inorganic phosphorus.

When inorganic phosphorus is limited, cyanobacteria release cylindrospermopsin into the surrounding environment and induce metabolic changes in phytoplankton. This stimulates production and secretion of alkaline phosphatase into the extracellular environment. The alkaline phosphatase transforms organic phosphorus compounds into bioavailable inorganic phosphorus, which is rapidly uptaken by cylindrospermopsin-producing cyanobacteria.

1.3.1.5. Feed deterrence

Adaptations in morphology and biochemical make-up (toxicity) are two main grazer-induced defenses in cyanobacteria (Ger et al., 2016b). For instance, microcystins, cylindrospermopsin anatoxin-a and saxitoxin can reduce zooplankton fitness in laboratory studies (Chislock et al., 2013; DeMott et al., 1991; Ger et al., 2016b; Sadler and von Elert, 2014). The role of microcystins as an anti-grazer is better studied than cylindrospermopsin, anatoxin-a and saxitoxin. Microcystins could assemble cyanobacteria into large colonies that slow or probihit zooplanton ingestion (Ger et al., 2016a; Yang and Kong, 2012). Another trait, i.e. toxicity of microcystins may be also involved in grazing deterrents. Microcystins inhibit eukaryotic phosphatase enzyme activity and drive declines of growth and survival rate of zooplanktons such as daphnia and copepod (Ger et al., 2016a; Tillmanns et al., 2008; Wilson et al., 2006).

1.3.1.5. Interspecific Competition

There are concurrent allelopathic interactions between cyanobacteria, phytoplankton and macrophytes (Mohamed, 2017). Cyanobacteria that release microcystins, cylindrospermopsin and anatoxin-a into the environment can inhibit the growth of other algal species and macrophytes, thereby accessing the limited resources (Kearns and Hunter, 2001; Keating, 1978; Kinnear et al., 2007; Máthé and Vasas, 2013).

1.3.2. Physiological roles of cyanotoxins

1.3.2.1. Iron Regulation

Microcystins interact with trace metal ions as trace metal-complexing ligands and iron chelators (siderophores), and by detoxifying metals in cells (Facey et al., 2019). Given the inconsistent interactions between microcystins and trace metals from one study to another, the current belief is that microcystins act primarily as transporters, assisting iron transport through the cell membrane of cyanobacteria (Facey et al., 2019; Saito et al., 2008; Wang et al., 2012).

1.3.2.2. Maintain Sodium Hemostasis.

Saxitoxin acting as a selective sodium channel blocker and is linked to Na^+ homeostasis in cyanobacteria (Holland and Kinnear, 2013; Pomati et al., 2003). Similar to saxitoxin, anatoxin-a is also able to bind to the nicotinic receptor on sodium channels, which opens the channels and allows sodium ions to flow in (Bownik, 2010). Several genera of cyanobacteria (*Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Planktothrix*) produce both saxitoxin and anatoxin-a (Cirés and Ballot, 2016; Méjean et al., 2014; Pearson et al., 2010), which could help to maintain sodium homeostasis at different environmental scales (high Na^+ (saxitoxin), low Na^+ (anatoxin-a)) (Holland and Kinnear, 2013).

1.3.2.3. Thylakoid Structural Supporter

In cyanobacterial cells, two-thirds of microcystins are localised in thylakoid area, which can alter the thylakoid ultrastructure slightly (Shi et al., 1995; Young et al., 2005).

1.3.2.4. Photosynthesis Regulation

Cyanobacteria must protect their photosynthetic apparatus from damage by photooxidation, and the protective effect could be enhanced by microcystins and beta-N-methylamino-L-alanine. Microcystins bioaccumulate preferentially in the thylakoid area, where photosynthetic pigments

and enzymes are found. Furthermore, there are approximately 20% more photosynthetic pigments in the cells of microcystins-producing cyanobacteria (*Microcystis*) than non-microcystins producing cyanobacteria (Hesse et al., 2001; Young et al., 2005). Light intensity can induce transcription of microcystins biosynthesis gene (*mcyD*) in cyanobacteria, which suggests that microcystins may help to regulate photosynthesis (Deblois and Juneau, 2010; Sevilla et al., 2012).

Studies with biological soil crust from arid environments also suggest that microcystins and beta-N-methylamino-L-alanine prevent cyanobacteria damage by high light intensity, but their contribution depends upon the nitrogen availability to cyanobacteria cells (Downing et al., 2015). In biological soil crusts with high nitrogen availability, microcystins buffer the oxygen evolution capacity under high light intensity, thereby preventing excessive oxygen radical formation and associated light-induced reaction center damage. In nitrogen limited conditions, beta-N-methylamino-L-alanine appears to regulate oxygenic photosynthesis by blocking light signal transduction to protect cyanobacteria from oxidative damage under high light intensity (Zhang and Whalen, 2019). Beta-N-methylamino-L-alanine might also regulate chlorophyll *a* activity to avoid its photooxidation. Since beta-N-methylamino-L-alanine is produced under nitrogen starvation conditions that induce chlorosis and the degradation of photosynthetic pigments, it would be logical for beta-N-methylamino-L-alanine to protect pigments in the xanthophyll cycle to dissipate light radiation before it damages the chloroplast, or to inhibit the activity of chlorophyll *a*, as proposed by Downing et al. (2012) and Berntzon et al. (2013). Thus, microcystins offers protection from sudden onset high light stress whereas beta-N-methylamino-L-alanine helps the cyanobacteria to avoid and repair oxidative damage. Physiologically, this would prepare the cyanobacteria to rapidly respond to variations in nitrogen availability and high

light intensity. With the assistance of these two cyanotoxins, the cyanobacteria can simultaneously maintain the integrity of its photosynthetic apparatus while taking advantage of available nutrients for cellular metabolism, growth and reproduction (Downing et al., 2015; Metcalf et al., 2015b; Richer et al., 2015).

1.3.2.3. Nitrogen Metabolism

As described above, beta-N-methylamino-L-alanine biosynthesis is associated with cellular nitrogen stress (Downing et al., 2011; Holtcamp, 2012; Popova et al., 2018). Cultures grown under nitrogen starvation conditions (< 10 μM) may produce beta-N-methylamino-L-alanine to initiate the ionotropic glutamate receptors (iGLuRs) and thus activate glutamate receptor cation (Ca^{2+}) channels, which control Ca^{2+} movement and maintain the integrity of cellular membranes (Berntzon et al., 2013; Brenner et al., 2003). Another possible role for beta-N-methylamino-L-alanine is to initiate the incorporation of Ca^{2+} into the carbon skeleton of 2-oxoglutarate after enzymatic reduction (Weiland et al., 2016). Although it seems counter-intuitive to use limited N resources to synthesize a non-protein amino acid-like beta-N-methylamino-L-alanine, there is evidence that the primary amino-N group in beta-N-methylamino-L-alanine can be redistributed to glutamate and glutamine via transamination of the enzyme glutamine synthetase-glutamine oxoglutarate aminotransferase (GOGAT) (Downing et al., 2011; Martín-Figueroa et al., 2000). Therefore, beta-N-methylamino-L-alanine has important physiological functions that maintain the integrity of cyanobacteria cells under N starvation conditions, and mechanisms to reassimilate the amino-N portions of the molecule once the protective glutamate pathways are initiated.

Cyanobacteria are the survivors of more than 3.0 billion years of evolution and architects of the Great Oxygenation Event, which occurred around 2.3– 2.5 billion years ago. The genes

encoding for cyanotoxins were selected from billions of possible permutations that arose over millennia. Although their ecophysiological effects are not completely understood, there is no doubt that cyanotoxins fulfill multiple functions for cyanobacteria. Most of the current studies on the ecophysiological roles of cyanotoxins focus on microcystins. Given that cyanobacteria produce many cyanotoxins besides microcystins, it seems advisable to consider multiple cyanotoxins, as well as their individual and synergistic effects, to understand the importance of these bioactive molecules for cyanobacteria (Huisman et al., 2018; Kaplan et al., 2012).

1.4. Detecting cyanotoxins in soil and water samples

1.4.1. Soil samples preparation prior to cyanotoxins analysis

Cyanotoxins are generally associated with harmful algal blooms in aquatic ecosystems, but also exist in soil crusts and in soils irrigated with cyanobacteria-contaminated water. Biologically significant amounts of cyanotoxins may be present in the soil, and humans will be exposed to these toxins when they inhale dust particles or consume cyanobacteria-contaminated groundwater and crops grown in contaminated areas. For example, two dogs died after consuming rainwater covering neurotoxin-producing cyanobacterial crust in 2013-2014 in the Qatar desert (Chatziefthimiou et al., 2014). During an average dust storm, an individual can breathe 6.6 g of dust and particles of <10 μm in size penetrate the lungs, representing a human exposure pathway for cyanotoxins (Griffin, 2007). Researchers suspect that "Gulf War Syndrome" is associated with inhaling neurotoxins in airborne dust particles (PM 2.5) when vehicular disturbance and military activities generated large amounts of dust (Cox et al., 2009; Metcalf et al., 2015a; Metcalf et al., 2012). However, it remains challenging to assess the safe exposure limits to cyanotoxins in soil, and the problem mainly stems from inefficient extraction procedures and insensitive analytical methods. Clay and organic matter in the soil matrix bind

with cyanotoxins, which prevents their recovery from soil, while molecules with similar structure or functional groups can react with antibodies and decline the sensitivity and detectability of the methods used, thus creating challenges to accurately quantify cyanotoxins in soils (Manubolu et al., 2018; Zhang et al., 2020).

The first step to improve cyanotoxins quantification is to recover them from the soil matrix and reduce the interference from matrix components. Solid phase extraction (SPE) is a separation and pre-concentration procedure that isolates cyanotoxins from the complex soil matrix by sorption onto a solid sorbent, which achieves matrix separation, reduces matrix effects, and improves the detection limits of analytical techniques (Mashile and Nomngongo, 2017). Solid phase extraction can be done offline (the sample preparation is done with separate equipment) or in an online (the sample preparation is coupled to the analytical instrument) mode (Zhang et al., 2020). Procedures for solid phase extraction for separation and pre-concentration of cyanotoxins in soil samples are summarized in Table 1.2. Solid phase extraction columns were first developed to extract microcystins from soil samples, and there are fewer options for other cyanotoxins like cylindrospermopsin, anatoxin-a, saxitoxin and beta-N-methylamino-l-alanine. Therefore, quantifying cyanotoxins in soil samples is still challenging. Research is needed to test various solvents and extraction methods to eliminate the matrix interferences and improve the extraction efficiency for cylindrospermopsin, anatoxin-a, saxitoxin and beta-N-methylamino-l-alanine.

Table 1.2: Reported solid phase extraction (SPE) methods of extracting cyanotoxins from soils prior to analysis.

Cyanotoxin	Solvent	Supernatant	SPE column (*Condition*)	Rinse	Elution	Concentrated	Reconstitute	Reference
Microcystins	75% methanol/water acidified with 0.1 M acetic acid	Ultrasonication /Centrifugation	Strata X column *(Methanol/Water)*	5% Methanol	90% acetonitrile	Dryness (N_2 gas)	Deionized water	Abraxis Inc.
	Na_4P_2O and 0.1 M ethylenediamine tetraacetic acid	Ultrasonication /Centrifugation (Modify pH 3 with trifluoroacetic acid)	C18 cartridge *(Methanol/MilliQ water)*	–	Methanol acidified with 0.1% trifluoroacetic acid	Dryness (N_2 gas)	Aqueous 50% acetonitrile (v/v)	(Corbel et al. 2014)
	Na_4P_2O and 0.1 M ethylenediamine tetraacetic acid	Ultrasonication /Centrifugation (Modify pH 3 with trifluoroacetic acid)	Sep-pak ODS cartridge	20% (v/v) aqueous methanol,	90% aqueous methanol	Dryness (N_2 gas)	0.05 M acetic acid	(Chen et al., 2006) (Cao et al., 2017)
	Na_4P_2O and 0.1 M thylenediaminetetraacetic acid	Ultrasonication /Centrifugation	Hydrophilic-Lipophilic Balance/Sep-pak C18 *(Methanol followed by ultrapure water)*	20% methanol	80% methanol	Dryness (N_2 gas)	80% Methanol	(Manubolu et al., 2018)
	Acetic Acid and 0.01 M ethylenediamine tetraacetic acid	Ultrasonication /Centrifugation	Hydrophilic-Lipophilic Balance/Sep-pak C18	20% methanol	80% methanol	Dryness (N_2 gas)	80% Methanol	(Manubolu et al., 2018)

			(Methanol followed by ultrapure water)					
	Methanol with 200 mM ammonium acetate	Ultrasonication /Centrifugation	–	–	–	Dryness (N_2 gas)	High performance liquid chromatography water	(Zhang et al., 2020)
Cylindrospermopsin	Methanol with 200 mM ammonium acetate	Ultrasonication /Centrifugation	–	–	–	Dryness (N_2 gas)	High performance liquid chromatography water	(Zhang et al., 2020)
Anatoxin-a	Methanol with 200 mM ammonium acetate	Ultrasonication /Centrifugation	–	–	–	Dryness (N_2 gas)	High performance liquid chromatography water	(Zhang et al., 2020)
Saxitoxin	–	–	–	–	–	–	–	–
Free Beta-N-methylamino-L-alanine	6 M hydrochloric acid	–	–	–	–	–	20 mM hydrochloric acid	(Richer et al., 2015)
	0.1 M trichloroacetic acid	Ultrasonication /Centrifugation	–	–	–	Dryness (N_2 gas)	20 mM hydrochloric acid	(Li et al., 2019)
Protein-bound Beta-N-methylamino-L-alanine	6 M hydrochloric acid	hydrolyzed in 6 M hydrochloric acid at 110 °C for 16 h	–	–	–	–	20 mM hydrochloric acid	(Richer et al., 2015)
	0.1 M trichloroacetic acid	Ultrasonication /Centrifugation hydrolyzed in 6 M hydrochloric acid at 110 °C for 24 h	–	–	–	Dryness (N_2 gas)	20 mM hydrochloric acid	(Li et al., 2019)

1.4.1. Analytical methods for analyzing cyanotoxins

Biological and physical-chemical methods were developed to detect cyanotoxins in water, and they should be appropriate to analyze cyanotoxins in soil extracts. Biological approaches include mouse bioassays, enzyme-linked immunosorbent assays (ELISA), and phosphatase inhibition assays (e.g., PP2A). Physicochemical approaches include gas chromatography coupled with mass spectrometry, high-performance liquid chromatography (HPLC) coupled with photodiode array detection, and HPLC coupled with mass spectrometry or tandem mass spectrometry (LC/MS or LC/MS/MS) (Kaushik and Balasubramanian, 2013; Moreira et al., 2014). The ELISA and LC-MS/MS methods are the most common.

The ELISA test is based on coupling a specific binding antibody or an antigen to a sensitive assay enzyme. The precision of the assay is enhanced by coating the plate with high-affinity antibodies. There are several formats used for ELISAs. These fall into either direct, indirect, or sandwich capture and detection methods. Currently, microcystins are detected with the indirect competition ELISA kits while direct competition ELISA kits are available to determine cylindropermopsin, anatoxin-a, saxitoxin and beta-N-methylamino-L-alanine. The indirect competitive ELISA involves two binding processes with a primary antibody and a labeled secondary antibody (Figure 1.11). Specifically, it is based on the recognition of microcystins and their congeners by specific biotinylated antibodies. Toxin, when present in a sample, and a microcystins-protein analogue immobilized on the plate compete for the binding sites of the biotinylated anti-microcystins antibodies in solution. The plate is then washed and streptavidin-horseradish peroxidase is added. After a second washing step and the addition of the substrate solution, the color signal is generated. The direct competition ELISA for cylindropsermopsin, anatoxin-a, saxitoxin and beta-N-methylamino-L-alanine only involves in one binding process,

since these cyanotoxins bind to specific antibodies (Figure 1.12). Cyanotoxins, when present in a sample, and a cyanotoxin-labeled analog compete for binding sites on anti-cyanotoxin antibodies in solution. The anti-cyanotoxin antibodies are then bound by a second antibody immobilized on the wells of the microtiter plate. After a washing step and addition of the substrate solution, a color signal is generated. In all ELISA methods, the intensity of the blue color in each well (absorbance measured at 450 nm) is inversely proportional to the concentration of cyanotoxins present in the sample. The cyanotoxins concentrations are determined by interpolation from the standard curve constructed with each ELISA plate (generally a 96-well microtiter plate).

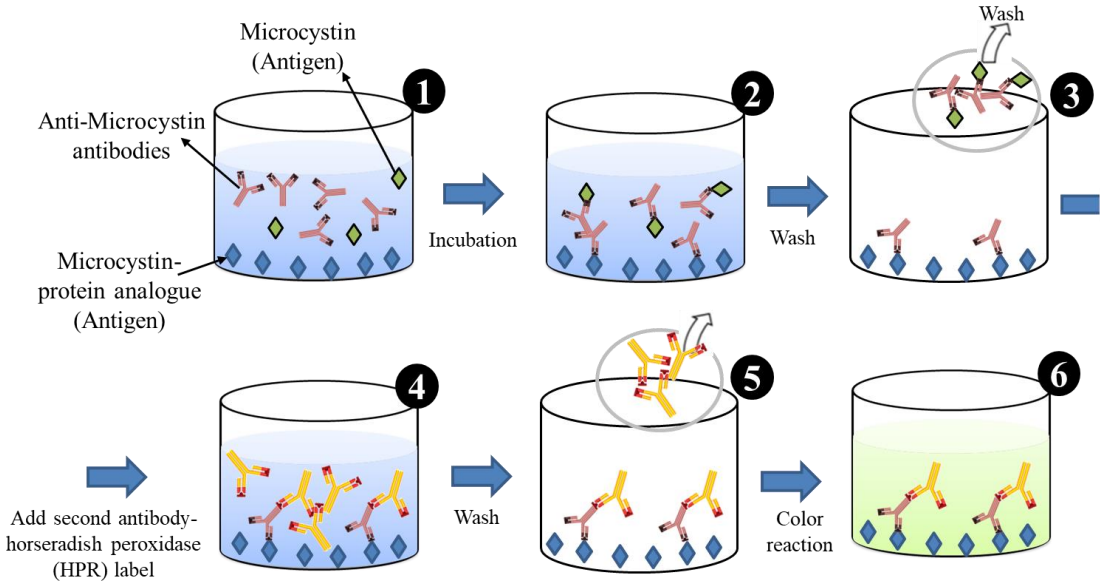


Figure 1.11: Steps involved in the indirect competition ELISA procedure for determining microcystins concentration in solution.

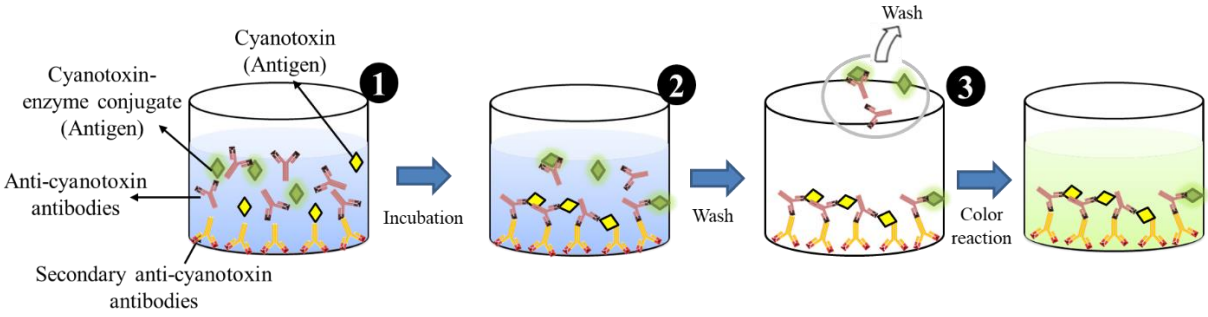


Figure 1.12: Principles of the direct ELISA method for determining cyanotoxins like cylindrospermosin in solution.

The ELISA method, which detects multiple cyanotoxins variants, is a good semi-quantitative screening tool to evaluate the presence of cyanotoxins in environmental samples. The most common uses of ELISA are for monitoring the toxicity associated with a dynamic cyanobacterial bloom, tracking relative changes in toxin concentrations, and taking actions to control cyanobacterial blooms in source waters. However, the method cannot provide a quantitative assessment of the cyanotoxins in water samples. It has a high limit of detection (0.04–4 µg/L), and the nonlinear calibration curve means that small differences in measured absorbance can translate into large concentration differences, thus the ELISA method can overestimate the actual concentration by two to three times. Furthermore, molecules with similar chemistry and functionality as cyanotoxins can produced false positives in both direct and indirect competition ELISA methods. In direct competition ELISA, the error occurs when foreign proteins in the sample bind to the non-specific fixed antigen. In indirect competition ELISA for microcystins, the secondary antibody is prone to generate nonspecific signals through cross-reaction with antigen.

The other common method for cyanotoxins detection is liquid chromatography coupled to mass spectrometry (LC-MS). The LC-MS is a precise quantitative method for determining cyanotoxins in water samples without false positives, and its lower limit of detection is around two orders of magnitude smaller than the ELISA assay (Duy et al., 2019; Munoz et al., 2017; Roy-Lachapelle et al., 2019). Mass spectrometry is combined with chromatographic separation to achieve simultaneous separation and identification of cyanotoxins in a mixture, based on the characteristic ions in their mass spectra. The application of MS/MS detection greatly enhances

the identification of unknown toxins by the fragmentation pattern (Teta et al., 2015). Fragment ions generated by collision-induced dissociation provide a sensitive and reliable confirmation of the identity of microcystin variants in the environmental samples (Roy-Lachapelle et al., 2019; Yen et al., 2011). The LC-MS system is further enhanced by coupling it with an on-line SPE system. On-line SPE automates all steps of the sample preparation (conditioning, sample enrichment, wash and elution), which would be done manually in the traditional off-line method (Fayad et al., 2015). The off-line SPE technique is the time consuming and laborious (~1 hour/sample), while on-line SPE approach is quicker, allows for smaller sample size, less handling and preparation, improved reproducibility, higher sample throughput as well as less waste and solvent consumption. On-line SPE coupled with LC-MS/MS allowed simultaneous analysis of multiple cyanotoxins in < 8 min per sample from environmental matrices, with low detection limits (0.005–0.8 μg L^{-1}, depending on the cyanotoxin; Zhang et al., 2020).

1.5. Risk assessment and public health concerns related to cyanotoxins: case studies

Cyanotoxins present in the environment are dangerous to human health, but the risk to public health depends upon the dose and duration of exposure. Humans can be exposed to cyanotoxins through three major pathways: ingesting cyanotoxin-contaminated food and water, dermal contact and the inhalation of particles (sediment, dust or soil) containing cyanotoxins (Figure 1.13). Humans are most likely to be exposed to cyanotoxins from CyanoHABs because of the global distribution and frequent occurrence of these toxic blooms. Another important exposure pathway is from ingesting agricultural plants that bioaccumulated cyanotoxins because they were irrigated with cyanobacteria-contaminated water. For this section, we focused on the risk and public health concerns for beta-N-methylamino-L-alanine associated with CyanoHABs and microcystins present in agricultural plants as two distinct case studies.

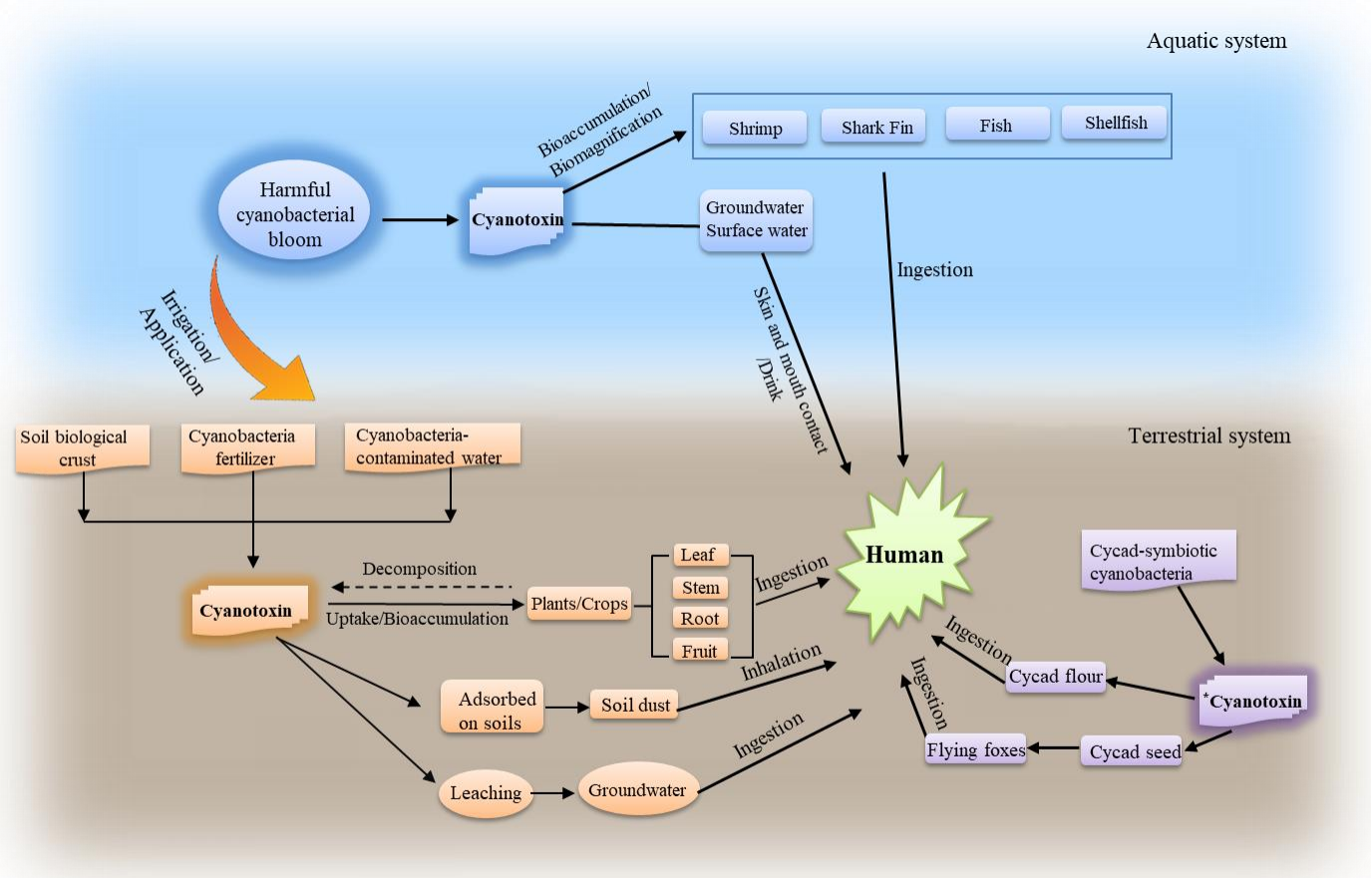


Figure 1.13: A hypothetical human exposure scenario to cyanotoxins. *Cyanotoxin: only applicable to beta-N-methylamino-L-alanine.

1.5.1. Beta-N-methylamino-L-alanine and harmful cyanobacterial bloom

The beta-N-methylamino-L-alanine concentrations (~1–231 µg L^{-1}) in freshwater lakes and seafood harvested from CyanoHAB-affected aquatic systems represent an important human exposure pathway (Craighead et al., 2009; Esterhuizen and Downing, 2008). Spatial analysis of amyotrophic lateral sclerosis cases and lakes with a history of cyanobacterial blooms in New Hampshire revealed that people living within 1 km of cyanobacteria-contaminated lakes had a 2.3 times greater risk of developing amyotrophic lateral sclerosis, and inhabitants of Lake Mascoma had up to 25 times greater risk of amyotrophic lateral sclerosis than the general population (Caller et al., 2009; Zhang and Whalen, 2019). From 1990-2004, beta-N-

methylamino-L-alanine was found in the untreated/unprocessed water taken from British waterbodies and used for human drinking water, highlighting the dangers of beta-N-methylamino-L-alanine to human health (Metcalf et al., 2008). Additionally, high beta-N-methylamino-L-alanine levels, ranging from 0–7000 μg kg^{-1}, were found in muscle and brain of bottom-dwelling fishes, shrimp, crab and shark fin, which are consumed by people (Brand et al., 2010; Jonasson et al., 2010). Through the food web, the concentration of beta-N-methylamino-L-alanine could be biomagnified ~8 times from phytoplankton to zooplankton (Jiang et al., 2014a; Mondo et al., 2012).

Relatively low exposure to beta-N-methylamino-L-alanine is damaging, based on in vitro studies showing that beta-N-methylamino-L-alanine causes selective motor neuron demise at concentrations as low as 3,540 μg L^{-1} and damages motor neurons at concentrations of 1,180 μg L^{-1} (Lobner et al., 2007; Rao et al., 2006). The biomagnification of beta-N-methylamino-L-alanine and its ability to bind with proteins in the brain mean that people can accumulate this endogenous neurotoxin throughout their lifespan, and it might slowly release as free beta-N-methylamino-L-alanine into the brain tissue during aging (Rodgers et al., 2018). Consequently, long-term exposure to beta-N-methylamino-L-alanine is a huge threat to public health that will disproportionately affect the health and well-being of senior citizens (Holtcamp, 2012).

1.5.2. Microcystins accumulation by agricultural plants

Microcystins are another group of toxic substance of concern to humans. Its presence in agricultural fields can be from irrigating with cyanobacteria-contaminated water or applying cyanobacteria as an organic fertilizer. These cyanobacteria can be harvested from lakes and applied directly to the field, or after they are treated in activated sludge systems (Figure 1.13)

(Bouaïcha and Corbel, 2016). For instance, irrigated agricultural soils in South China had up to 1,690 μg microcystins kg^{-1} soil (Chen et al., 2012; Xiang et al., 2019).

Microcystins are amphiphilic molecules with hydrophobic groups in combination with polar groups of hydrophilic character (ionizable carboxyl groups and amino group). In neutral soil environment (pH 6.0~8.5), microcystins are neutral or anionic (microcsytins minus). Natural formation of micelles and ion-pairs with negatively charged microcsytins means that they do not absorb strongly to the negatively charged surfaces of soil minerals and organo-minerals, so they remain in soil solution (McCord et al., 2018; Wu et al., 2011). The half-life of microcystins in soils is from ~5 days to ~2 months, indicating that the microcystins could be bioavailable to plants that covers the agricultural fields (Bouaïcha and Corbel, 2016). Microcystins can pass through the root membrane barrier, translocate within plant tissues and accumulate into different organs, including edible parts, which is a great concern for food safety (Cao et al., 2018a). Due to the bioaccumulation potential of microcystins in edible plants, two guideline thresholds are proposed, the tolerable daily intake (TDI, WHO) and chronic reference dose (RfD, United States Environmental Protection Agency). For microcystin-LR, the TDI should not exceed 0.04 μg kg^{-1} d^{-1} and RfD should not exceed 0.003 μg kg^{-1} d^{-1} (Edition, 2011; EPA:, 2015).

The human health risk of ingesting microcystins is determined by calculating the estimated daily intake (EDI) of microcysitn-LR equivalent. The EDI of microcystins via consumption of the microcystins-polluted agricultural plants is calculated as follows:

$$\text{Estimated Daily Intake (EDI)} = \frac{C_{MC} \text{ X } D_{intake}}{bw}$$

where C_{MC} is an average microcystin-LR equivalent concentrations in agricultural plants (μg g^{-1} wet weight); D_{intake} is the daily consumption of the edible part of an agricultural plant (g d^{-1}),

based on standards published by the United States Food and Drug Administration (US FDA 2015) and bw is the average body weight (adult: 60 kg, child: 25 kg).

Recent studies have reported EDI values (0.06–0.2 μg microcystins kg^{-1} d^{-1}) that exceed the recommended WHO guideline of 0.04 μg microcystins kg^{-1} d^{-1} for edible plants (Cao et al., 2018b; Lee et al., 2017; Xiang et al., 2019; Zhu et al., 2018). However, the likelihood that humans will ingest microcystins from agricultural plants has not been thoroughly investigated, especially under field conditions. Most of previous studies were conducted by greenhouse pot experiments or hydroponic experiments at non-environmentally relevant concentrations, marginally representing real bioaccumulation of microcystins in field soils, or exaggerating the bioavailability and accumulation of microcystins in agricultural crops (Cao et al., 2018a). When field data are available, they are often obtained without adequate monitoring plans, or refer to plants that are not commonly eaten by humans, so that they do not represent the actual human exposure (Funari and Testai, 2008). Therefore, it is urgent to understand the actual concentrations of microcystins in agricultural fields and to assess the bioaccumulation and corresponding human health risks of various microcystins variants in actual soil-crop systems in agricultural fields. Meanwhile, we can make some effort to educate farmers to avoid using irrigation water that may contain microcystins and increasing the awareness of farmers and the general public about the health risk of microcystins in agricultural plants.

FORWARD TO CHAPTER 2

The literature review highlighted the important role of nutrients to stimulate cyanotoxins production by CyanoHABs. Agricultural nutrient loadings to freshwater environments may create a preferential environment for cyanobacteria producing cyanotoxins. In chapter 2, I present my critical review paper that demonstrates a connection between agricultural nutrient inputs and cyanotoxins production. I explain how the nitrogen loading from agricultural runoff may trigger beta-N-methylamino-L-alanine production in CyanoHABs in freshwater

environments, focusing on the ecophysiological reasons why nitrogen availability controls beta-N-methylamino-L-alanine production and supporting this with evidence from in-lake studies.

CHAPTER 2

Production of the neurotoxin beta-N-methylamino-L-alanine may be triggered by agricultural nutrients: An emerging public health issue

Yanyan Zhang[a], Joann K. Whalen[a]

a. McGill University, Department of Natural Resource Sciences, Macdonald Campus, 21,111 Lakeshore Road, Ste-Anne-de Bellevue, Quebec, H9X 3V9, Canada

2.1. Abstract

Diverse taxa of cyanobacteria, dinoflagellates and diatoms produce β-N-methylamino-L-alanine (BMAA), a non-lipophilic, non-protein amino acid. BMAA is a neurotoxin in mammals. Its ingestion may be linked to human neurodegenerative diseases, namely the Amyotrophic lateral

sclerosis/Parkinsonism dementia complex, based on epidemiological evidence from regions where cyanobacterial harmful algal blooms occur frequently. In controlled environments, cyanobacteria produce BMAA in response to ecophysiological cues such as nutrient availability, which may explain the elevated BMAA concentrations in freshwater environments that receive nutrient-rich agricultural runoff. This critical review paper summarizes what is known about how BMAA supports ecophysiological functions like nitrogen metabolism, photosyntheis and provides a competitive advantage to cyanobacteria in controlled and natural environments. We explain how BMAA production affected competitive interactions among the N_2-fixing and non-N_2-fixing populations in a freshwater cyanobacterial bloom that was stimulated by nutrient loading from the surrounding agricultural landscape. Better control of nutrients in agricultural fields is an excellent strategy to avoid the negative environmental consequences and public health concerns related to BMAA production.

Keywords: Neurotoxin; β-N-methylamino-L-alanine production; CyanoHABs; Ecophysiological role; Agricultural nutrients; Best management practices.

2.2. Introduction

The neurotoxin β-N-methylamino-L-alanine (BMAA), a non-protein amino acid that is a suspected causative agent of human neurodegenerative diseases, is produced by diverse cyanobacteria, dinoflagellates and diatoms (Cox et al., 2005; Lage et al., 2014; Réveillon et al., 2014). Evidence of the connection between BMAA and the Amyotrophic lateral sclerosis/Parkinsonism dementia complex was first documented in Guam, where local residents who consumed cycad flour and flying foxes containing BMAA had 100 times greater incidence of these neurological disorders than the global baseline (Cox and Sacks, 2002; Cox et al., 2016). Humans are exposed to BMAA through ingestion and dermal contact with contaminated water

and food, or inhalation of dust containing BMAA, but the greatest environmental health risk is from cyanobacterial harmful algal blooms (CyanoHABs) because of their global occurrence (Huisman et al., 2018; Merel et al., 2013). This perspective is supported by eco-epidemiological modelling showing that sporadic Amyotrophic lateral sclerosis is associated with BMAA produced by CyanoHABs (Snyder et al., 2009; Torbick et al., 2014).

Most cyanobacteria can produce BMAA, whether they are plant symbionts, in mats growing in ponds and on sediments, or in biological soil crusts and surface soil (0-2 cm) (Cox et al., 2005; Jungblut et al., 2018; Metcalf et al., 2015; Richer et al, 2015). However, detection of BMAA production in CyanoHABs is alarming due to the increasing frequency, magnitude and duration of CyanoHABs in fresh water systems worldwide, especially in watersheds where nutrient loading occurs due to urban and rural activities (Huisman et al., 2018). In controlled studies, cyanobacteria produce BMAA in response to ecophysiological cues, including nutrient availability. We postulate that nutrients in agricultural runoff triggers BMAA production in CyanoHABs, but this requires nutrient loading to occur in the right proportions, at the right time and possibly in combination with other factors that induce BMAA production by cyanobacteria.

The objective of this critical review paper is to present evidence of the connection between nutrient loading from agricultural runoff and BMAA production in CyanoHABs in freshwater environments. We begin by discussing the metabolic and ecological functions of BMAA for cyanobacteria populations and the factors that trigger BMAA production. Then, we describe the known and hypothesized connections between nutrients in agricultural runoff and BMAA production by freshwater CyanoHABs. Finally, we discuss the opportunities and limitations of agricultural best management practices to reduce the environmental and public health risks associated with the cyanotoxin BMAA.

2.3. BMAA production and its ecophysiological role in cyanobacteria

Understanding BMAA synthesis and functions in cyanobacteria remains challenging, since it may exist as a free amino acid, be associated with or incorporated into protein, or complexed with metal ions (Cao et al. 2019). This section summarizes the factors influencing BMAA production and its ecophysiological role in cyanobacteria.

2.3.1. Factors contributing to BMAA production

Cyanobacteria produce several forms of BMAA through distinct biosynthesis pathways. Cyanobacteria could biosynthesize the free amino acid form (keto acid β-N-methylaminopyruvate) directly by methylation of free 2,3-Diaminopropionic acid (DAP) or by enzymatic hydrolysis of BMAA associated with proteins (Downing et al., 2011; Tripathi and Gottesman, 2016). Alternatively, the cyanobacteria *Synechocystis* PCC6803 may biosynthesize BMAA through hydrolysis of a cyclic urea compound (Downing and Downing, 2016). The genetic basis for BMAA biosynthesis in the cycad-cyanobacteria symbiosis is associated with glutamate receptor-like genes (Brenner et al., 2003). Ongoing research into BMAA stereochemistry and molecular transcriptomics should improve understanding of BMAA biosynthesis pathways in cyanobacteria (Diaz-Parga et al., 2018; Popova et al., 2018).

Like other cyanotoxins, the free amino acid form of BMAA probably exists in the nucleoplasmic region, thylakoid area, cell wall and sheath of cyanobacteria cells (Vega and Bell, 1967). It also associates with membrane, bound peptides or proteins in the cytoplasm (Faassen et al., 2016). After cyanobacteria biosynthesize BMAA, the BMAA molecule remains in the cell during its lifespan. Dead or damaged cyanobacteria cells lyse and release BMAA into the environment. Therefore, BMAA appearing in the environment originated from (a) *de novo*

BMAA biosynthesis in the cell, and (b) BMAA released into the environment through cellular lysis (Pernil et al., 2015; Main et al., 2018).

Under nitrogen (N)-deficient conditions, both non-N_2-fixing and N_2-fixing cyanobacteria can synthesize BMAA (Monteiro et al., 2017). However, the non-N_2-fixing cyanobacteria are expected to produce more BMAA than N_2-fixing cyanobacteria in N-limited environments (Scott et al., 2014, Downing et al., 2011; Downing et al., 2012; Monteiro et al., 2017; Popova et al., 2018). N concentrations of 0-40 μM in the form of NH_4 and NO_3 triggers BMAA production in the non-N_2-fixing cyanobacteria when they growing in a bloom and laboratory cultures (Table 2.1). In addition to *de novo* BMAA biosynthesis, cyanobacteria can absorb exgeneous BMAA from the environment (Berntzon et al., 2013; Downing et al., 2011). Absorbed BMAA could be biodegraded within the cell, or it could be re-released into the environment after cell death and lysis. In laboratory cultures, cyanobacteria populations are viable for about 30 days (Nizan and Shilo, 1986), but the generation time could be as short as 7 days in CyanoHABs during summer months (Bidle and Falkowski, 2004) or as long as 90 days in biological soil crusts (Mugnai et al., 2018). Apoptosis, necrosis and senescence are the main forms of cell death (Bidle and Falkowski, 2004), followed by lysis of the plasma membrane by viral, enzymatic or osmotic mechanisms. Abiotic stressors that induce cellular lysis include nutrient stress due to excess / deficiency of phosphorus or iron (Bidle 2016; Berman et al, 2004; Popova et al., 2018), salt stress (Ning et al., 2002), high light stress (Berman et al., 2007) and oxidative stress (Ross et al., 2006). In addition, biotic stress due to viral and bacteriophage infections, as well as predation from animal-like protists, flagellates and other microbiota, cause cellular lysis (Haraldsson et al., 2018).

Table 2.1: Production of β-N-methylamino-L-alanine (BMAA) by non-N_2-fixing cyanobacteria in laboratory cultures as influenced by nitrogen.

Species	Nitrogen source	Nitrogen exposure (μM)	BMAA concentration (ng/g cells (dry weight))	Reference
Synechocystis J341	$NaNO_3$	$1.8x10^3$ μM $NaNO_3$	ND[a]	(Scott et al., 2014) (Downing et al., 2011)
		0	[b] The peak area of LC/MS detection of BMAA is 1.5×10^6	
Microcystis PCC7806	-	0	[b] The peak area of LC/MS detection of BMAA is 1.2×10^5	
	NH_4Cl	1000 μM NH_4Cl	ND[a]	
	$NaNO_3$	1000 μM NaNO3	ND[a]	
Microcystis aeruginosa	$NaNO_3$	$1.76x10^3$ μM $NaNO_3$	ND[a]	(Fan et al., 2015)
		$17.6x10^3$ μM $NaNO_3$	ND[a]	
		$52.8x10^3$ μM $NaNO_3$	ND[a]	
Microcystis aeruginosa bloom	-	~0 μM Combined nitrogen	252	(Scott et al., 2014)
	Nitrate	40 μM Combined nitrogen	34	
	Nitrite Ammonia	>40 μM Combined nitrogen	ND[a]	

ND[a] = not detectable; [b] Peak area, as BMAA concentration was not quantified by the authors.

2.3.2. Ecophysiological role of BMAA

Since there is an energetic cost to synthesize all cyanotoxins, including BMAA, there must be an evolutionary advantage for cyanobacteria that retained BMAA biosynthesis capacity. This molecule may ensure the organism's functional niche (ecological advantage) or improve its fitness (physiological efficiency). Based on the literature, we suggest three ecological roles and two physiological roles of BMAA for cyanobacteria (Figure 2.1). Other ecophysiological functions of BMAA may be discovered in the future.

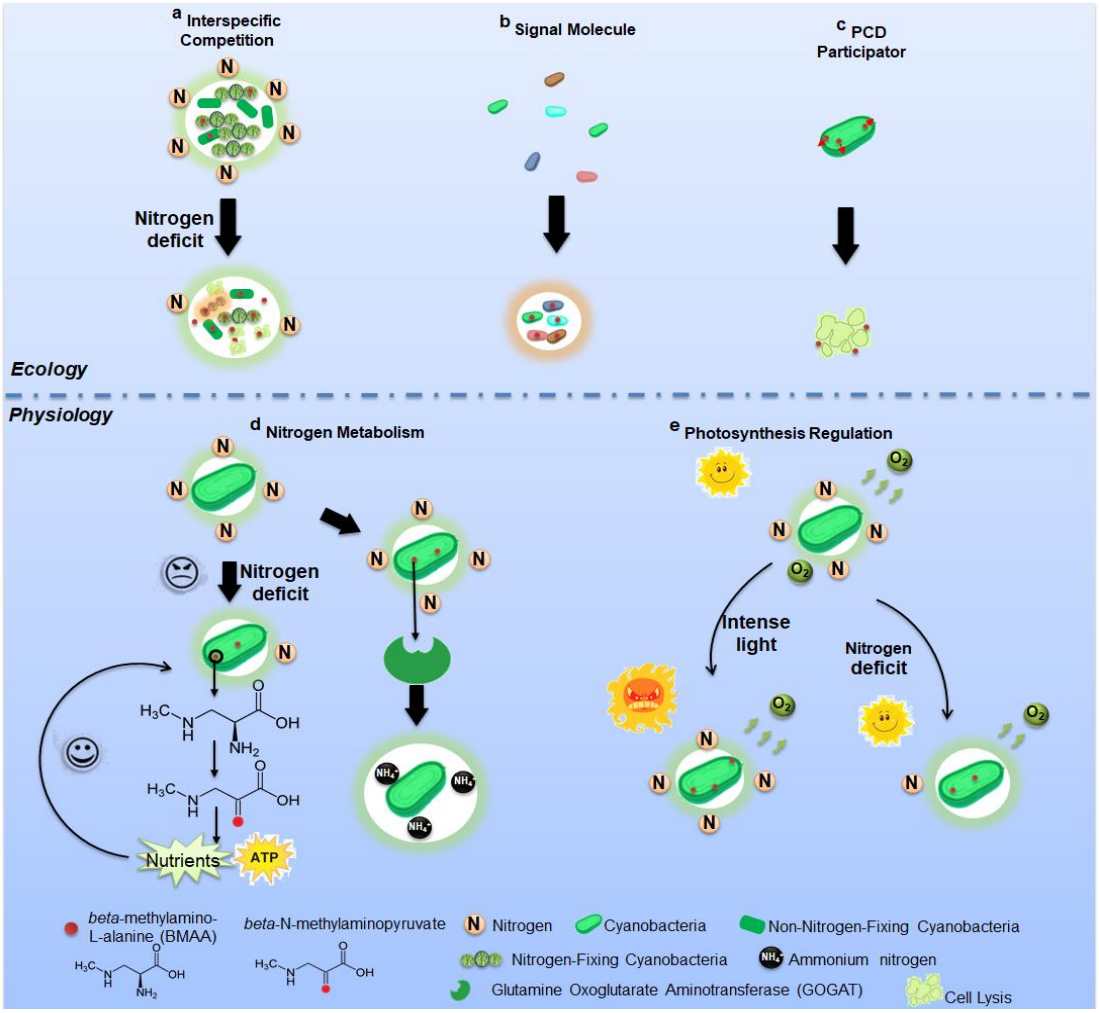


Figure 2.1: Proposed ecological and physiological roles of b-N-methylamino-L-alanine (BMAA) in cyanobacteria.

Interspecific Competition. N availabilty plays a critical role in the production of BMAA by cyanobacteria (Downing et al., 2011; Monteiro et al., 2017). When the available nitrate concentration falls below 0.3 to 0.4 µM, N_2-fixing cyanobacteria initiate the N_2 fixing reaction, which is energetically costly, requiring 18 molecules of ATP and 8 low-potential electrons per molecule of N_2 fixed (Canfield, Glazer et al. 2010). This metabolic process requires P for energy and several elements (Fe, Mo, S, O) to build the electron-donating iron-molybdenum cofactor in nitrogenase, thereby depleting these essential nutrients from the environment.

Under N-deficient conditions, both non-N_2-fixing and N_2-fixing cyanobacteria can synthesize BMAA (Cox et al., 2005; Main et al., 2018; Monteiro et al., 2018; Rodgers et al., 2018; Violi et al., 2019). However, the non-N_2-fixing cyanobacteria are expected to produce more BMAA than N_2-fixing cyanobacteria in N-limited environments. This statement is consistent with the observation that the non-N_2-fixing *Microcystis aeruginosa* had >7 times higher BMAA concentration in a reservoir with ~1 µM NO_3 than when the environment contained 40 µM NO_3 (Scott et al., 2014). Furthermore, N deprivation induced higher BMAA production in *Synechocystis* and *Microcystis* (non-N_2-fixing cyanobacteria) in laboratory experiments (Downing et al., 2011, Downing et al., 2012, Popova al., 2018). In contrast, there was 4-fold less BMAA produced by *Nostoc* sp. (N_2-fixing cyanobacteria) in a N-free cultures than in N-containing medium (the actual N concentration in the culture media is not reported in the paper) (Monteiro et al., 2017). Together, these reports suggest that non-N_2-fixing cyanobacteria are the dominant BMAA producers in N-deficient conditions.

The BMAA produced by non-N_2-fixing cyanobacteria under N starvation could be uptaken by N_2-fixing cyanobacteria and inhibit their nitrogenase activity, thus altering the competition interactions among non-N_2-fixing and N_2-fixing cyanobacteria (Berntzon et al., 2013; Popova et al., 2018). For example, non-N_2-fixing *Microcystis* outcompeted the N_2-fixing *Aphanizomenon* when the soluble N concentration was 0.5 to 2 mg N /L in the freshwater Lake Mendota, located in Wisconsin, USA (Beversdorf et al., 2013). The competitive advantage permitted the non-N_2-fixing *Microcystis* population to increase from 10^3 cells /mL to 10^6 cells /mL, while the population of N_2-fixing *Aphanizomenon* declined from 10^6 cells /mL to 10^5 cells /mL from July to September (Beversdorf et al., 2013). Although these authors did not evaluate BMAA production by the *Microcystis* population, we posit that if the non-N_2-fixing cyanobacteria

produced BMAA, it could have two benefits for their population. First, BMAA production kills some of the N_2-fixing cyanobacteria and nutrients released from their lysed cells can be recycled by the non-N_2-fixing cyanobacteria population. Second, BMAA blocks nitrogenase activity and constrains the growth of N_2-fixing cyanobacteria, thereby reducing their population size and liberating resources such as space and oxygen needed by the non-N_2-fixing cyanobacteria population.

We also know that N_2-fixing cyanobacteria produce BMAA and can have a high BMAA concentration in laboratory cultures and CyanoHABs (Cox et al., 2005; Main et al., 2018). However, we do not know whether the high BMAA concentration in N_2-fixing cyanobacteria is due to *in vivo* synthesis of the molecule, or whether the BMAA was originally produced by another organism and assimilated by the N_2-fixing cyanobacteria population. How environmental conditions, particularly N availability, affect BMAA production by N_2-fixing cyanobacteria, still remains to be understood. We believe that cyanobacteria have physiological adaptations that allow them to bioaccumulate and sequester BMAA of which concentration is under threshold level (no documented specific threshold value) in its organelles, to avoid toxic effects. This possibility offers hope for the development of genetic and biochemical therapies to treat humans and mammals exposed to BMAA. It could also explain the co-existance of non-N_2-fixing and N_2-fixing cyanobacteria in freshwater environments, despite the ability of both groups to produce lethal BMAA. Doubtless, each group has adapted to the selective pressure of the BMAA molecule and developed strategies to maintain their functional niche after millenia of co-evolution.

Signal Molecule. BMAA has chemical characteristics that are analagous to other signal molecules produced by prokaryotes such as high hydrophibility ($LogK_{ow}$ =-4.00), molecular

weight of 118 Da and diffusion coefficient of 0.78 (Defra 2008 and Diaz-Parga et al. 2018). Most cyanobacteria taxa seem to produce BMAA, and it is postulated to be a signal molecule responsible for communication among cyanobacteria species (Cox et al., 2005). Other non-protein amino acids that are chemically similar to BMAA, such as γ-Aminobutyric acid (GABA), accumulate rapidly in plants under stress and initiate defense pathways against drought and insect herbivory (Bown and Shelp, 2016). If BMAA is a GABA analogue, it may regulate the cyanobacteria metabolism in response to stress or be an adaptive response to mitigate stress, but this still remains to be determined.

PCD (Programmed Cell Death) Participator. Like other prokaryotes, cyanobacteria initiate PCD under stressful environmental conditions. The genetic basis for PCD are the metacaspases, which initiate targeted protein degradation in the apoptotic PCD pathways with high catalytic efficiencies and strict substrate specificities (Asplund et al., 2016). Phylogenetic analysis of N_2-fixing cyanobacteria with 16S RNA analysis revealed metacaspase orthologues, which suggests that cyanobacteria initiate PCD when they encounter stressful conditions (Bidle and Falkowski, 2004; Bar-Zeev et al., 2013; He et al., 2016; Ross et al., 2006). The presence of exogenous BMAA is an indication that cyanobacteria cells are dying and being lysed by abiotic and biotic stressors, described in section 2.1 (Bishop et al., 2018; Monteiro et al., 2016). Thus, an increase in exogenous BMAA concentration could induce the expression of metacaspase genes and initiate PCD as a negative feedback to control the size of cyanobacteria populations.

Nitrogen Metabolism. As described previously, BMAA biosynthesis is associated with cellular N stress (Holtcamp 2012; Popova et al., 2018; Downing et al., 2011). Cultures grown under N starvation conditions (< 10 μM) may produce BMAA to initiate the ionotropic glutamate receptors (iGLuRs) and thus activate glutamate receptor cation (Ca^{2+}) channels, which

controls Ca^{2+} movement and maintains the integrity of cellular membranes (Berntzon et al., 2013; Brenner et al., 2000). Another possible role for BMAA is to initiate the incorporation of Ca^{2+} into the carbon skeleton of 2-oxoglutarate after enzymatic reduction (Chang et al., 2015). Although it seems counter-intuitive to use limited N resources to synthesize a non-protein amino acid like BMAA, there is evidence that the primary amino-N group in BMAA can be redistributed to glumate and glutamine via transamination of the enzyme glutamine synthetase-glutamine oxoglutarate aminotransferase (GOGAT) (Martín et al., 2000; Downing et al., 2011). Therefore, BMAA has important physiological functions that maintain the integrity of cyanobacteria cells under N starvation conditions, and mechanisms to reassimilate the amino-N portions of the molecule once the protective glutamate pathways are initiated.

Photosynthesis Regulation. Cyanobacteria are photosynthetic organisms that must protect their photosynthetic apparatus from damage by photooxidation, and this could be assisted by the BMAA molecule. First, BMAA acts as a glutamate receptor agonist that alters the cell membrane potential of the model plant *Arabidopsis*, thus affecting the light signal transduction (Brenner et al., 2000). The glutamate receptor of *Arabidopsis* is highly homologous with the same receptor in the cyanobacteria *Synechocystis* sp. (Chen et al., 1999). It is thought that BMAA regulates oxygenic photosynthesis by blocking light signal transduction to protect cyanobacteria from oxidative damage when photosynthesis occurs under high light intensity and limited N availability (Downing et al., 2015). Second, BMAA might regulate chlorophyll *a* activity to avoid its photooxidation. Since BMAA is produced under N starvation conditions that induce chlorosis and the degradation of photosynthetic pigments, it would be logical for BMAA to protect pigments used in the xanthophyll cycle to dissipate light radiation before it damages the

chloroplast, or to inhibit the activity of chlorophyll *a*, as proposed by Downing et al. (2012) and Berntzon et al. (2013).

2.4. Contribution of agricultural nutrients to BMAA production in freshwater environments

Virtually every CyanoHABs in a freshwater environment is associated with elevated nutrient concentrations in the waterbody. The exponential growth that is characteristic of an algal bloom will not occur if the cyanobacteria are lacking in phosphorus (< 0.2 mg total P/L) and N (< 0.8 mg total N/L) (Paerl et al., 2011; Xu, H., et al., 2014; Schindler et al., 2008). Nitrogen may originate from biogeochemical cycling within the aquatic system, but also enters as runoff from upstream terrestrial environments, intrusion of N-rich groundwater and via atmospheric deposition (Gruber and Galloway, 2008; Elser and Bennett, 2011). As an example, we critically review the specific case of the eleventh-largest freshwater lake (by area) in the world, Lake Winnipeg in Manitoba, Canada. In Lake Winnipeg, the total N budget is 96, 000 ton/yr, where 29% of N comes from in-lake processes and the remaining 71% is derived from sources outside the aquatic system (Board, 2006). At least 49% of the exogeneous N is delivered in runoff from the surrounding agricultural land (Rattan et al., 2017). Agricultural runoff is responsible for N levels of up to 18.5 mg total N/L and phophorous levels as high as 4 mg total P/L, which simulate and sustain the high-biomass bloom in excess of 20 µg cells /L in Lake Winnipeg (Rattan et al., 2017; Paerl et al., 2011; Hamilton et al., 2016; Michalak et al., 2013). Agricultural activities contribute to nutrient loading because fertilizer, manure and other nutrient-rich materials applied for crop production release dissolved and particulate-associated nutrients that are susceptible to transport from farm fields (Li et al., 2018; Castellano et al., 2019; Whalen et al., 2019). However, the N transfer from agroecosystems to downstream rivers and lakes is

seasonally variable because it is influenced by climatic conditions, hydrologic processes within the watershed and agricultural management. Dissolved and particulate nutrients are readily transported during snowmelt in spring, and after rainfall events in the early growing season that have enough intensity and/or duration to generate overland flow from fertilized agroecosystems to waterbodies, and these nutrient sources are associated with the emergence and exponential growth phases in CyanoHABs (Schneider et al., 2019; Zhang et al., 2012a; Smith et al., 2007). Nutrient transfers decline during late summer to late fall because of rainfall is more sporatic at this time of year, so agricultural fields are drier and receiving little or no fertilizer as crops mature and are harvested. The late summer to late fall period is a time when CyanoHABs go into decline and collapse (Reichwaldt and Ghadouani, 2012).

The dynamics of CyanoHABs depend on many factors, including N loading from agroecosystems, but we hypothesize that relationships exist between the CyanoHABs growth phase, BMAA concentration and N loading in Lake Winnipeg. It is known that BMAA was produced throughout the CyanoHABs forming season. From emergence to the bloom phase, the BMAA concentration ranged from undetectable to low, and this increased approximately 20-fold to as much as 306 µg BMAA/L during the decline and collapse phases, in late fall (Pip et al., 2016; Bishop et al., 2018; Main et al., 2018; Zguna et al., 2019). We posit that agricultural runoff triggers BMAA production because it influences the N entering the lake at different phases of bloom. We can evaluate these relationships in Lake Winnipeg because data exists on the temporal variation in BMAA production and CyanoHABs, as well as the origin of N loading into this lake Winnipeg. The next sections discuss the known and hypothesized connections between the recurrent CyanoHABs in Lake Winnipeg and agricultural activities in its 982,900 km^2

watershed, which includes three US states (North Dakota, South Dakota and Minnesota) and four Canadian provinces (Alberta, Saskatchewan, Ontario and Manitoba).

2.4.1. Lake Winnipeg, a freshwater lake where BMAA is produced by CyanoHABs

Detection of BMAA production in CyanoHABs was reported in British freshwater lakes and brackish waterbodies (Metcalf et al., 2008), the Baltic Sea (Jonasson et al., 2010), several freshwater environments in South Floria (Brand et al., 2010), Lake Taihu, China (Jiao et al., 2014), Lake Houston, the US (Holtcamp, 2012), Thau lagoon, French Mediterranean Sea (Reveillon et al., 2015) and Lake Winnipeg, Canada (Bishop et al., 2018; Pip et al., 2016). The specific example of Lake Winnipeg, Canada is compelling because this freshwater environment has experienced regular CyanoHABs since the late 1990s due to anthropogenic forces, primarily agricultural activities.

Lake Winnipeg is located in the cold humid temperate region of Manitoba, Canada and covers an area of 24,514 km^2, with two distinct basins, the South basin and the North basin (Wassenaar and Rao, 2012; Zhang et al., 2012b). The CyanoHABs in this lake often cover thousands of square kilometers (Benoy et al., 2016). From 2002-2011, extensive blooms covered as much as 93% of the lake surface (Zhang et al., 2012b; Binding, 2018). This represents a 5-fold increase in nuisance blooms of heterocystous cyanobacteria from the 1990s (Wassenaar and Rao., 2012).

In 2016, BMAA was present in about 25% of CyanoHABs in Lake Winnipeg (Bishop et al., 2018; Pip et al., 2016). Temporally, the BMAA production occurrred during the cyanobacterial bloom forming-season from early summer to late fall. We do not know what cyanobacteria are responsible for BMAA production in Lake Winnipeg, but BMAA could be associated with the toxic non-N_2-fixing cyanobacteria, such as *Planktothrix* and *Microcystis*. In the past decade,

these genera have emerged as co-dominant populations together with the N_2-fixing cyanobacteria *Aphanizomenon* and *Dolichospermum*, which historically dominated the CyanoHABs. This widespread community-level change in CyanoHABs is attributed to the elevated N and phosphorus concentrations in lakes (Kling et al., 2011; McKindles et al., 2019). Furthermore, phosphorus-rich lakes that receive exogenous N inputs can see a 500% increase in cyanobacteria production and toxicity (Leavitt et al., 2006; Donald et al., 2011; Vogt et al., 2017). Therefore, temporal variation in BMAA production may be due to the emergence of toxic non-N_2-fixing cyanobacteria within the CyanoHABs (Pip et al., 2016), but a simpler explanation may be that BMAA production in this lake is responding to N availability, a major driver of BMAA production in controlled studies.

2.4.2. Lake Winnipeg, a freshwater lake where BMAA production is associated with agricultural nutrient loading

Lake Winnipeg's biggest problem stems from its large watershed, the high drainage to surface area ratio about 40 and the change in surrounding land use (Board, 2006; Benoy et al., 2016). Agriculture expansion occurred throughout the 20th century, particularly in the southern region of the Lake Winnipeg watershed. From 1990 to 2010, there was an intensification of hog production, more annual crop production and overall increase in livestock density (Bunting et al., 2011; Liu et al., 2014). At the same time, much of the post-glacial terrain was systematically drained through the creation of surface ditches and channels to accelerate drainage on agricultural land in southern Manitoba (Rattan et al., 2017). This also involved filling or draining wetlands to expand agricultural production (Schindler et al., 2012). The SPAtially Referenced Regressions on Watershed attributes (SPARROW) model indicates that about 65% of the total phosphorus load and approximately 75% of the total N load entering Lake Winnipeg originated

from agricultural sources (Benoy et al., 2016). Approximately 7900 t phosphorus /year and 96 000 t N /year enter the watershed, primarily during snowmelt and spring runoff events (McCullough et al., 2012; Schindler et al., 2012).

The N concentration in Lake Winnipeg varies seasonally and corresponds to the loading pattern of agricultural nutrients (Schindler et al., 2012; Bunting et al., 2016). The highest N concentrations are recorded in late spring and summer months (Mayer and Wassenaar, 2012; Soto et al., 2019). From late summer to fall, nutrient loading from agricultural land is limited because fertilizer is not applied at the pre/post-harvest stage and there are few rainfall events that could induce N loss from the agroecosystem. During development of the cyanobactrial bloom, the in-lake N concentration declines to <0.1 mg total N /L in late autumn (Mayer and Wassenaar, 2012; Pip et al., 2016). Furthermore, there is spatial variability in the NO_3 concentrations of Lake Winnipeg. The South basin receives about 69% more of the N load than the North basin in April and May, prior to the emergence and bloom phases of CyanoHABs (Environment and Climate Change Canada (2016)). By early July, the South basin becomes dominated by several non-N_2-fixing *Microcystis* and *Planktothrix* (Kling et al., 2011; McKindles et al., 2019). In an average flow year, nutrients in South basin reach North basin after two or three months, in time to support the bloom that peak in summer in both basins (Zhang et al., 2012b; Binding et al., 2016). Consequently, the non-N_2-fixing cyanobacteria begin to experience N starvation during late summer and autumn in both basins (Bishop et al., 2018; Orihel et al., 2012). In-lake N dynamics are negatively related to BMAA concentration (Pip et al., 2016; Pick 2016; Vo Duy et al., 2019), and is expected to be lowest in the early summer (bloom emergence) and increasing from summer to autumn, with the peak BMAA concentration occurring just before bloom collapse (Figure 2.2). Overall, the allochthonous agricultural nutrients may trigger BMAA production by

influencing the composition of potentially toxic cyanobacteria species and nitrogen level in Lake Winnipeg.

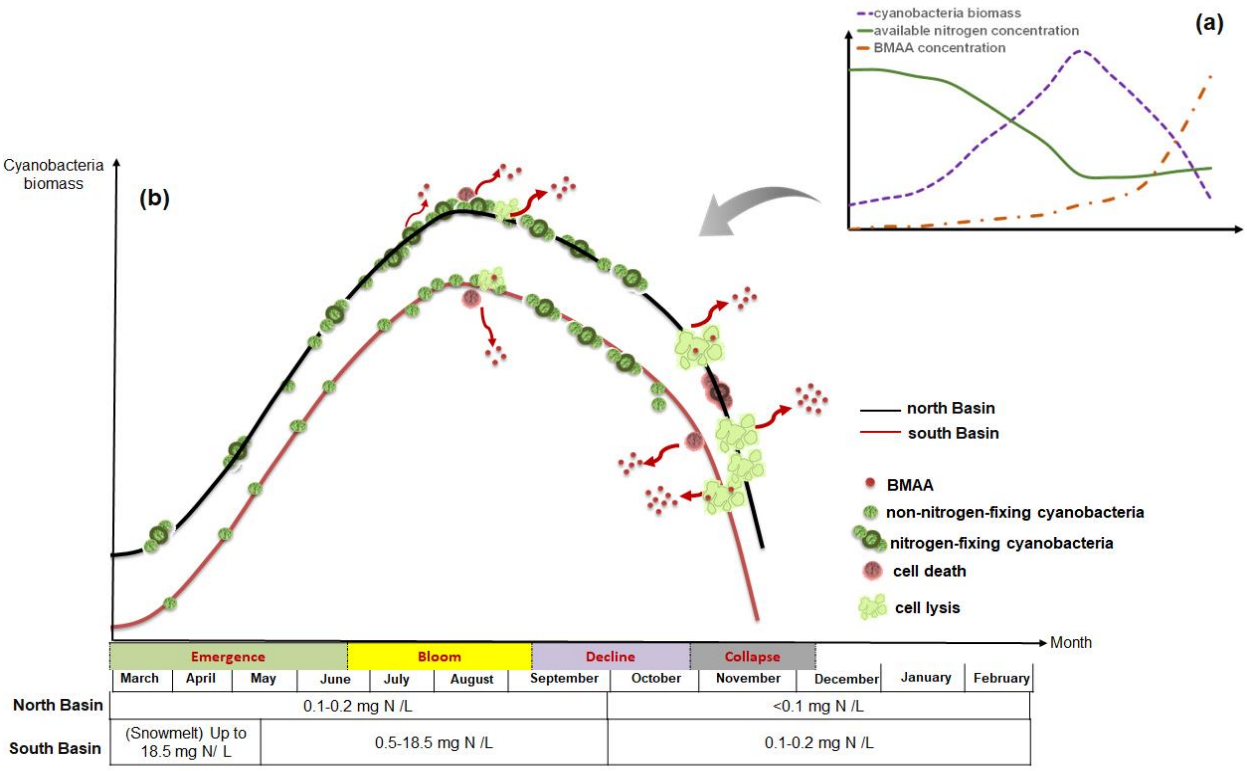


Figure 2.2: Hypothesized relationship among available nitrogen concentration, cyanobacteria biomass and b-N-methylamino-L-alanine (BMAA) production (a) and relationship between BMAA production and development of cyanobacterial harmful algal blooms composed of the non-N2-fixing cyanobacteria and N2-fixing cyanobacteria in the North basin and South basin of Lake Winnipeg, Canada (based on CyanoHABs dynamics reported by Manitoba Sustainable Development and Environment and Climate Change Canada (2012, 2016) (b).

2.5. Controlling nutrients in agricultural fields to reduce human health risks from BMAA production in CyanoHABs

Detection of BMAA in freshwater lakes, many of which are drinking water sources, is an emerging public health issue (Craighead et al., 2009; Esterhuizen and Downing et al., 2008).

Compared to other environmental sources, the BMAA concentration in freshwater lakes is high and represents an important exposure pathway (Table 2.2). Spatial analysis of Amyotrophic lateral sclerosis cases and lakes with a history of cyanobacterial blooms in New Hampshire revealed that people living within 1 km of cyanobacteria-contaminated lakes had a 2.3 times greater risk of developing Amyotrophic lateral sclerosis, and inhabitants of Lake Mascoma had up to 25 times greater risk of Amyotrophic lateral sclerosis than the general population (Caller et al., 2009). Relatively low exposure to BMAA is damaging, based on *in vitro* studies showing that BMAA causes selective motor neuron demise at concentrations as low as 30 μM (Rao et al., 2006) and damages motor neurons at concentrations of 10 μM (Lobner et al., 2007). Still, BMAA needs to be understood as a chronic neurotoxin because it binds with proteins and has a long latency period before the proteins degrade and slowly release free BMAA into human brain tissues (Holtcamp, 2012). The public health risk from long-term exposure to BMAA in freshwater environments should not be underestimated.

Table 2.2: Human exposure pathways to environmental sources of β-N-methylamino-L-alanine (BMAA).

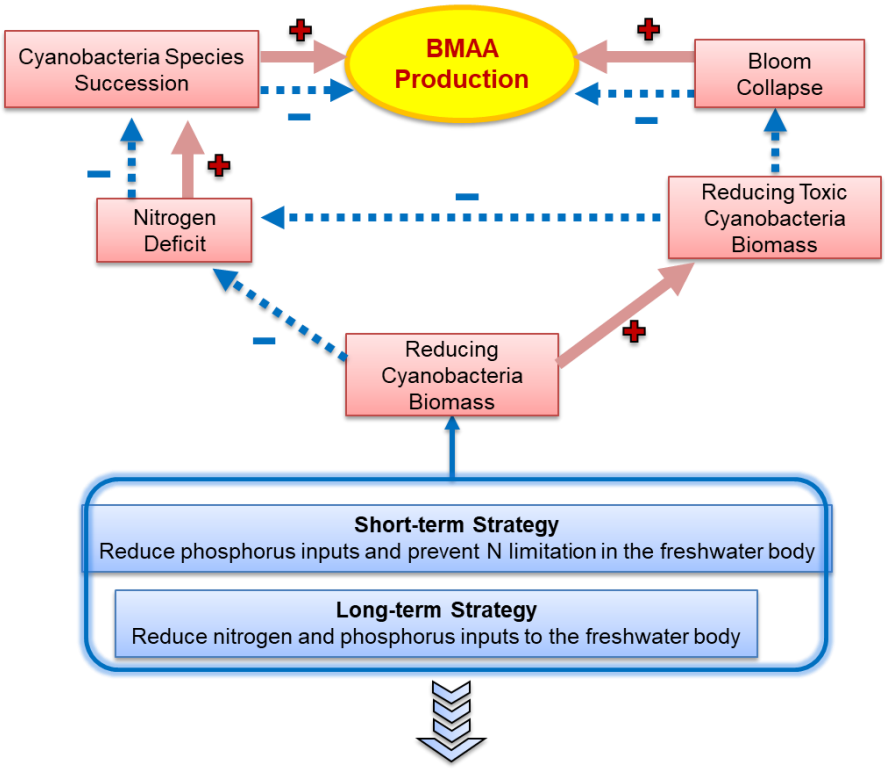
Exposure pathway	Environmental source	Sampling location	Concentration (μg/g DW)	Concentration (μg/ L water)	Reference
Ingestion	Cycad flour	Guam, USA	40-169	-	(Cheng and Banack, 2009)
	Shark fin	South Florida	144 -1836	-	(Mondo et al., 2012)
	Shark cartilage dietary supplements	South Florida	86-265	-	(Mondo et al., 2012)
	Fa cai Nostoc soup	China	[a]ND-0.7	-	(Roney et al., 2009)
	Algae dietary supplements	Pacific/Hawaii/ Klamath Lake	Detectable	-	(Roy-Lachapelle et al., 2017)
	Spirulina natural health products	Health food store in Canada	0.01-0.74	-	(Faassen et al., 2009)
	Spirulina powder from commercial raw ingredient producers	N/A	0.01-0.07	-	(Baker et al., 2018)
	Aphanizomenon flosaquae dietary supplements	Klamath Lake	~0.04	-	(Roy-Lachapelle et al., 2017)
Inhalation	Biological crust	Gulf region/Qatar	Detectable		(Cox et al., 2009; Richer et al., 2015; Metcalf et al., 2015)
Oral/dermal contact	Water	Nebraska lakes	-	1.8 ~ 24.5	(Al-Sammak et al., 2014)
		Lake Winnipeg Canada	-	~1.0	(Pip et al., 2016)
	Cyanobacteria	Lake Winnipeg Canada	4.1 ~ 22.5	-	(Bishop et al., 2018)
		Baltic Sea	0.001 ~ 0.015	-	(Jonasson et al., 2010)
		Freshwater impoundments (Southern Africa)	0 ~ 2757	-	(Esterhuizen and Downing, 2008) (Esterhuizen and Downing, 2008)
		Lake Taihu, China	2.0 ~ 7.1	-	(Jiao et al., 2014)
		Portuguese estuaries	0.04 ~ 63	-	(Cervantes et al., 2012)
		Multiple water bodies (Eastern Australia)	[a]ND~ 47.26	-	(Main et al., 2018) (Main et al., 2018)
	Urban waters (cyanobacteria bloom)	Dutch	Maximum 42	-	(Faassen et al., 2009)
	Untreated water (Drinking water reservior)	British waterbodies	Maximum 48	Maximum 231	(Metcalf et al., 2008)

[a]ND = not detectable; LD_{50}=10 μg /L (fish larvae Danio rerio); 1500~5000 μg /L (brine shrimps Artemia salina); 5000 μg /L (the ciliate Nassula Sorex); ED_{50}= 1430 to 1604 μM (human neurons).

2.5.1. Managing agricultural nutrients to prevent CyanoHABs and the associated BMAA production

Since BMAA production occurs during the CyanoHABs collapse phase, and is fueled by N availability during the entire bloom, the most efficient way to eliminate BMAA production in freshwater environments is to prevent CyanoHABs. This will involve preventative measures

from multiple anthropogenic actors that contribute to N loading into vulnerable watersheds, due to the influence of N availability on cyanobacteria species succession and CyanoHABs collapse (Figure 2.3). Agricultural best management practices that control the diffuse sources of nitrogen and phosphorus will be critical, and these focus on controlling the transport processes and sources of excess nutrients. As illustrated in Figure 2.3, interventions that enhance natural attenuation processes in water and solute transport pathways, as well as those that constrain the rate and time that N and phosphorus are applied to agricultural fields, are two critical ways to reduce N loading to freshwater bodies (Hamilton et al., 2016). End-of-field treatments with buffer strips, bioreactors and wetland restoration should be implemented to slow hydrologic processes and capture nutrients before they enter the freshwater system. Precision agriculture approaches that combine remote sensing with judicious fertilizer use should prove effective in delivering N, phosphorus and other nutrients to agricultural fields in the quantities needed by crops during the growing season. Fall fertilization should be strongly discouraged since crop nutrient demand is low in autumn and the residual nutrients are susceptible to loss during winter and early spring, when most of the agricultural runoff occurs in cold humid temperate regions of the world. In the short-term, we cautiously suggest that one strategy to reduce BMAA production could be to avoid N starvation of the non-N_2-fixing cyanobacteria. This would be accomplished by inhibiting the growth and biomass accumulation of cyanobacteria by controlling the P input, and adding N to prevent the *de novo* BMAA production by the N limited non-N_2-fixing cyanobacteria (Figure 2.3). This possibility needs to be verified experimentally before attempting any intervention in freshwater bodies, as it is probably too simplistic to assume that N concentrations in the lake are the sole trigger of BMAA production in CyanoHABs.



Management	Process controlled	Practices	Impact on nutrient loss		Reference
			N	P	
Transport management	Surface runoff Subsurface runoff Leaching	Buffer strips	↓	↓	(Schoumans et al., 2014)
		Wetland preservation, restoration or reconstruction	↓	↓	(Paerl et al., 2016)
		Bioreactors	↓	↓	(Husk et al., 2017; Husk et al., 2018; Rasouli et al., 2014)
		Subsurface drainage system	↑	↓	(Kopacek et al., 2013; Michaud et al., 2019)
Source management	Manure storage	Lagoon, impermeable storage, barnyard runoff control	↓	↓	(Sharpley et al., 2007)
	Manure volume	Compost, transport excess out of the watershed			(Sharpley et al., 2007)
	Manure treatment	Add alum to reduces NH_3 loss and P solubility Flyash, Fe oxide or gypsum to reduce P solubility	↓	↓	(Sharpley et al., 2007)
	Manure application rate	Rate chosen to meet, but not exceed, crop needs	↓	↓	(Sharpley et al., 2007)

Application timing	Growing season only, split application to synchronize N inputs with N-demanding growth stages	↓	↓	(Rasouli et al., 2014)
	Pre-sidedress soil NO_3-N test	↓	Neutral	(Rasouli et al., 2014)
Application method	Soil P testing	Neutral	↓	(Shigaki et al., 2006)
	Subsurface injection (reduce N and P loss in runoff)	↑	↓	
Crop management	Agroforestry systems, including short-rotation woody crops Biomass / bioenergy crops	↓	↓	(Rasouli et al., 2014; Wang et al., 2019)
	Crop rotation or intercropping	↓	↓	
	Keep soil surface covered by retaining crop residues / planting cover crop	↓	↓	

Figure 2.3: Agricultural best management practices with potential to reduce β-N-methylamino-L-alanine (BMAA) production by cyanobacterial harmful algal bloom.

2.6. Conclusion

This synthesis suggests reasons why cyanobacteria have evolved BMAA biosynthesis as a cellular function, and the environmental stimuli that induces BMAA production. For non-N_2-fixing cyanobacteria populations, BMAA production gives them a competitive strategy and guarantees their co-existence with N_2-fixing cyanobacteria. Seasonal nutrient loading from agricultural lands triggers and increases the production of BMAA by increasing the biomass of toxic cyanobacteria and altering the N availability in the freshwater environment. However, most of current understanding about BMAA production in cyanobacteria is still based on controlled laboratory work. Most field studies did not monitor the relationship between N availability and BMAA production, which makes it difficult to compare the lab and field work. Long-term monitoring is needed to confirm the relationship between agricultural nutrients, CyanoHABs and BMAA production. For instance, it is of utmost importance to identify the role of N availability

on BMAA production and release into freshwater environments. Understanding the patterns of N loading from agricultural land along with the change in BMAA concentration, cyanobacterial community dynamics and toxicity will eventually lead to more efficient agricultural management plans for preventing BMAA. Dual control on phosphorus and N inputs from agricultural land, as well as measures to limit the N recycling in freshwater could be the optimal strategies to inhibit the growth of toxic cyanobacterial strains and control BMAA production.

BMAA is associated with acute and chronic neurotoxicity in humans leading to disorders in the Amyotrophic lateral sclerosis/Parkinsonism dementia complex that are costly and burdensome for individuals, families and the health care profession. Reducing human health risk from BMAA production in CyanoHABs will require close collaboration between biochemists, hydrologists, ecologists, agronomists and policy makers to deal with this global problem. Public health officials need to be concerned about the fact that BMAA is produced in CyanoHABs, which are occurring more frequently, with longer duration and larger size, due to global warming and more anthropogenic nutrient loading in waterways around the world (Merel et al., 2013; Reichwaldt and Ghadouani, 2012). Part of the solution will be more aggressive strategies to stop agricultural nutrient loading in lakes. Policies that clearly state the responsibilities of various stakeholders are needed, as well as financial commitments from governments and citizens to manage eutrophic watersheds. We need to use our knowledge of cyanobacteria biology to control their populations and to prevent CyanoHABs that will normally result in BMAA production to preserve the freshwater environments that are critically connected to our long-term public health.

Acknowledgments

Financial support for this project was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) through Discovery Grant # RGPIN-2017-05391. YZ was supported by a postgraduate scholarship from the China Scholarship Council.

2.7. References

Agawin, N. S., Rabouille, S., Veldhuis, M. J., Servatius, L., Hol, S., van Overzee, H. M., Huisman, J., 2007. Competition and facilitation between unicellular nitrogen-fixing cyanobacteria and non-nitrogen-fixing phytoplankton species. Limnol Oceanogr. 52(5), 2233-2248. https://doi.org/10.4319/lo.2007.52.5.2233.

Asplund-Samuelsson, J., Sundh, J., Dupont, C. L., Allen, A. E., McCrow, J. P., Celepli, N. A., Ekman, M., 2016. Diversity and expression of bacterial betacaspases in an aquatic ecosystem. Front Microbiol. 7, 1043. https://doi.org/10.3389/fmicb.2016.01043.

Baker, T. C., Tymm, F. J. M., Murch, S. J., 2018. Assessing environmental exposure to beta- *N* -Methylamino-L-Alanine (BMAA) in complex sample matrices: a comparison of the three most popular LC-MS/MS methods. Neurotox Res. 33(1), 43-54. https://doi.org/10.1007/s12640-017-9764-3.

Bar-Zeev, E., Avishay, I., Bidle, K. D., Berman-Frank, I., 2013. Programmed cell death in the marine cyanobacterium *Trichodesmium* mediates carbon and nitrogen export. ISME J. 7(12), 2340-2348. https://doi.org/10.1038/ismej.2013.121.

Benoy, G. A., Jenkinson, R. W., Robertson, D. M., Saad, D. A., 2016. Nutrient delivery to Lake Winnipeg from the Red-Assiniboine River Basin-A binational application of the SPARROW model. Can Water Resour J. 41(3), 429-447. https://doi.org/10.1080/07011784.2016.1178601.

Berman-Frank, I., Bidle, K. D., Haramaty, L., Falkowski, P. G., 2004. The demise of the marine cyanobacterium, *Trichodesmium* spp., via an autocatalyzed cell death pathway. Limnol Oceanogr. 49(4), 997-1005. https://doi.org/10.4319/lo.2004.49.4.0997.

Berman-Frank, I., Rosenberg, G., Levitan, O., Haramaty, L., Mari, X., 2007. Coupling between autocatalytic cell death and transparent exopolymeric particle production in the marine cyanobacterium *Trichodesmium*. Environ Microbiol. 9(6), 1415-1422. https://doi.org/10.1111/j.1462-2920.2007.01257.x.

Berntzon, L., Erasmie, S., Celepli, N., Eriksson, J., Rasmussen, U., Bergman, B., 2013. BMAA inhibits nitrogen fixation in the cyanobacterium *Nostoc* sp. PCC 7120. Mar. Drugs. 11(8), 3091-3108. https://doi.org/10.3390/md11083091.

Beversdorf, L. J., Miller, T. R., McMahon, K. D., 2013. The role of nitrogen fixation in cyanobacterial bloom toxicity in a temperate, eutrophic lake. PLOS ONE 8(2), e56103. https://doi.org/10.1371/journal.pone.0056103.

Bidle, K. D., Falkowski, P. G., 2004. Cell death in planktonic, photosynthetic microorganisms. Nat Rev Microbiol. 2(8), 643-655. https://doi.org/10.1038/nrmicro956.

Bidle, K. D., 2016. Programmed cell death in unicellular phytoplankton. Curr. Biol. 26(13), R594-R607. https://doi.org/10.1016/j.cub.2016.05.056.

Bishop, S. L., Kerkovius, J. K., Menard, F., Murch, S. J., 2018. *N*-β-Methylamino-L-Alanine and its naturally occurring isomers in cyanobacterial blooms in Lake Winnipeg. Neurotox Res. 33(1), 133-142. https://doi.org/10.1007/s12640-017-9820-z.

Board, L. W. S., 2006. Reducing nutrient loading to Lake Winnipeg and its watershed: Our collective responsibility and commitment to action. Lake Winnipeg Stewardship Board. http://hdl.handle.net/1993/33947.

Bown, A. W., Shelp, B. J., 2016. Plant GABA: not just a metabolite. Trends Plant Sci. 21(10), 811-813. https://doi.org/10.1016/j.tplants.2016.08.001.

Brand, L. E., Pablo, J., Compton, A., Hammerschlag, N., Mash, D. C., 2010. Cyanobacterial blooms and the occurrence of the neurotoxin beta-N-methylamino-L-alanine (BMAA) in South Florida aquatic food webs. Harmful Algae 9(6), 620-635. https://doi.org/10.1016/j.hal.2010.05.002.

Braun, E., 2007. Reactive nitrogen in the environment: too much or too little of a good thing. UNEP/Earthprint.

Brenner, E. D., Martinez-Barboza, N., Clark, A. P., Liang, Q. S., Stevenson, D. W., Coruzzi, G. M., 2000. Arabidopsis mutants resistant to S (+)-β-methyl-α, β-diaminopropionic acid, a cycad-derived glutamate receptor agonist. Plant Physiol. 124(4), 1615-1624. https://doi.org/10.1104/pp.124.4.1615.

Brenner, E. D., Stevenson, D. W., McCombie, R. W., Katari, M. S., Rudd, S. A., Mayer, K. F., Dai, G., 2003. Expressed sequence tag analysis in *Cycas*, the most primitive living seed plant. Genome Biol. 4(12), R78. https://doi.org/10.1186/gb-2003-4-12-r78.

Bunting, L., Leavitt, P., Wissel, B., Laird, K., Cumming, B., St Amand, A., Engstrom, D., 2011. Sudden ecosystem state change in Lake Winnipeg, Canada, caused by eutrophication arising from crop and livestock production during the 20th century. Final report to Manitoba Water Stewardship, Winnipeg, Manitoba, Canada, 785.

Bunting, L., Leavitt, P. R., Simpson, G. L., Wissel, B., Laird, K. R., Cumming, B. F., Engstrom, D. R., 2016. Increased variability and sudden ecosystem state change in Lake Winnipeg, Canada, caused by 20th century agriculture. Limnol Oceanogr. 61(6), 2090-2107. https://doi.org/10.1002/lno.10355.Caller, T. A., Doolin, J. W., Haney, J. F., Murby, A. J., West, K. G., Farrar, H. E., Stommel, E. W., 2009. A cluster of amyotrophic lateral sclerosis in New Hampshire: a possible role for toxic cyanobacteria blooms. Amyotroph Lateral Scler. 10(sup2), 101-108. https://doi.org/10.3109/17482960903278485.

Canfield, D. E., Glazer, A. N., Falkowski, P. G., 2010. The evolution and future of Earth's nitrogen cycle. Science, 330(6001), 192-196. https://doi.org/10.1126/science.1186120.

Cao, Y., S. Hu, T. Gong, Q. Xian, B. Xu., 2019. Decomposition of β-N-methylamino-L-alanine (BMAA) and 2, 4-diaminobutyric acid (DAB) during chlorination and consequent disinfection byproducts formation. Water Res. 159: 365-374. https://doi.org/10.1016/j.watres.2019.05.007.

Castellano, M. J., Archontoulis, S. V., Helmers, M. J., Poffenbarger, H. J., Six, J., 2019. Sustainable intensification of agricultural drainage. Nat. Sustain. 2(10), 914-921. https://doi.org/10.1038/s41893-019-0393-0.

Chen, G.-Q., Cui, C., Mayer, M. L., Gouaux, E., 1999. Functional characterization of a potassium-selective prokaryotic glutamate receptor. Nature 402(6763), 817. https://doi.org/10.1038/45568.

Chen, X., Wo, F., Chen, C., Fang, K., 2010. Seasonal changes in the concentrations of nitrogen and phosphorus in farmland drainage and groundwater of the Taihu Lake region of China. Environ Monit Assess. 169(1), 159-168. https://doi.org/10.1007/s10661-009-1159-3.

Cox, P. A., Sacks, O. W., 2002. Cycad neurotoxins, consumption of flying foxes, and ALS-PDC disease in Guam. Neurology 58(6), 956-959. https://doi.org/10.1212/WNL.58.6.956.

Cox, P. A., Banack, S. A., Murch, S. J., Rasmussen, U., Tien, G., Bidigare, R. R., Bergman, B., 2005. Diverse taxa of cyanobacteria produce β-*N*-methylamino-L-alanine, a neurotoxic amino acid. Proc Natl Acad Sci U S A. 102(14), 5074-5078. https://doi.org/10.1073/pnas.0501526102.

Cox, P. A., Davis, D. A., Mash, D. C., Metcalf, J. S., Banack, S. A., 2016. Dietary exposure to an environmental toxin triggers neurofibrillary tangles and amyloid deposits in the brain. Paper presented at the Proc. R. Soc. B. https://doi.org/10.1098/rspb.2015.2397.

Craighead, D., Metcalf, J. S., Banack, S. A., Amgalan, L., Reynolds, H. V., Batmunkh, M., 2009. Presence of the neurotoxic amino acids β-*N*-methylamino-L-alanine (BMAA) and 2, 4-diamino-butyric acid (DAB) in shallow springs from the Gobi Desert. Amyotroph Lateral Scler. 10(sup2), 96-100. https://doi.org/10.3109/17482960903278469.

Diaz-parga, P., Goto, J. J., Krishnan, V., 2018. Chemistry and chemical equilibrium dynamics of BMAA and its carbamate adducts. Neurotoxicity research 33(1), 76-86. https://doi.org/10.1007/s12640-017-9801-2.

Donald, D. B., Bogard, M. J., Finlay, K., Leavitt, P. R., 2011. Comparative effects of urea, ammonium, and nitrate on phytoplankton abundance, community composition, and toxicity in hypereutrophic freshwaters. Limnol Oceanogr. 56(6), 2161-2175. https://doi.org/10.4319/lo.2011.56.6.2161.

Downing, S., Banack, S., Metcalf, J., Cox, P., Downing, T., 2011. Nitrogen starvation of cyanobacteria results in the production of β-*N*-methylamino-L-alanine. Toxicon 58(2), 187-194. https://doi.org/10.1016/j.toxicon.2011.05.017.

Downing, T. G., Phelan, R. R., Downing, S., 2015. A potential physiological role for cyanotoxins in cyanobacteria of arid environments. J. Arid Environ. 112, 147-151. https://doi.org/10.1016/j.jaridenv.2014.02.005.

Downing, S., Downing, T. G., 2016. The metabolism of the non-proteinogenic amino acid beta-*N*-methylamino-L-alanine (BMAA) in the cyanobacterium *Synechocystis* PCC6803. Toxicon 115, 41-48. https://doi.org/10.1016/j.toxicon.2016.03.005.

Elser, J., Bennett, E., 2011. Phosphorus cycle: a broken biogeochemical cycle. Nature, 478(7367), 29. https://doi.org/10.1038/478029a.

Environment and Climate Change Canada, 2016. Manitoba Environment. Manitoba sustainable development. Available from: http://www.manitoba.ca/sd/pubs/annual-reports/annual_report_2016_17.pdf.

Esterhuizen, M., Downing, T.G., 2008. *β-N*-methylamino-L-alanine (BMAA) in novel South African cyanobacterial isolates. Ecotoxicol Environ Saf. 71(2), 309-313. https://doi.org/10.1016/j.ecoenv.2008.04.010.

Faassen, E. J., Gillissen, F., Zweers, H. A., Lürling, M., 2009. Determination of the neurotoxins BMAA (β-*N-methylamino-L-alanine*) and DAB (α-, γ-diaminobutyric acid) by LC-MSMS in Dutch urban waters with cyanobacterial blooms. Amyotroph Lateral Scler. 10 Suppl 2, 79-84. https://doi.org/10.3109/17482960903272967.

Faassen, E. J., Antoniou, M. G., Beekman-Lukassen, W., Blahova, L., Chernova, E., Christophoridis, C., Harmsen, J., 2016. A collaborative evaluation of LC-MS/MS based methods for BMAA analysis: Soluble bound BMAA found to be an important fraction. Mar. Drugs. 14(3), 45. https://doi.org/10.3390/md14030045.

Ferber, L., Levine, S., Lini, A., Livingston, G., 2004. Do cyanobacteria dominate in eutrophic lakes because they fix atmospheric nitrogen? Freshwater Biol. 49(6), 690-708. https://doi.org/10.1111/j.1365-2427.2004.01218.x.

Gruber, N., Galloway, J. N., 2008. An Earth-system perspective of the global nitrogen cycle. Nature, 451(7176), 293. https://doi.org/10.1038/nature06592.

Hamilton, D. P., Salmaso, N., Paerl, H. W., 2016. Mitigating harmful cyanobacterial blooms: strategies for control of nitrogen and phosphorus loads. Aquat Ecol. 50(3), 351-366. https://doi.org/10.1007/s10452-016-9594-z.

Holtcamp, W., 2012. The emerging science of BMAA: do cyanobacteria contribute to neurodegenerative disease? Environ Health Perspect. 120(3), a110. https://doi.org/10.1289/ehp.120-a110.

Haraldsson, M., Gerphagnon, M., Bazin, P., Colombet, J., Tecchio, S., Sime-Ngando, T., Niquil, N., 2018. Microbial parasites make cyanobacteria blooms less of a trophic dead end than commonly assumed. ISME J. 12(4), 1008-1020. https://doi.org/10.1038/s41396-018-0045-9.

He, Y., Zhou, Q.H., Liu, B.Y., Cheng, L., Tian, Y., Zhang, Y.Y., Wu, Z.B., 2016. Programmed cell death in the cyanobacterium *Microcystis aeruginosa* induced by allelopathic effect of submerged macrophyte *Myriophyllum spicatum* in co-culture system. J Appl Phycol. 28(5), 2805-2814. https://doi.org/10.1007/s10811-016-0814-7.

Huisman, J., Codd, G. A., Paerl, H. W., Ibelings, B. W., Verspagen, J. M., Visser, P. M., 2018. Cyanobacterial blooms. Nat. Rev. Microbiol. 16, 471-483. https://doi.org/10.1038/s41579-018-0040-1.

Husk, B. R., Anderson, B. C., Whalen, J. K., Sanchez, J. S., 2017. Reducing nitrogen contamination from agricultural subsurface drainage with denitrification bioreactors and controlled drainage. Biosyst Eng. 153, 52-62. https://doi.org/10.1016/j.biosystemseng.2016.10.021.

Husk, B. R., Sanchez, J. S., Anderson, B. C., Whalen, J. K., Wootton, B. C., 2018. Removal of phosphorus from agricultural subsurface drainage water with woodchip and mixed-media bioreactors. J Soil Water Conserv. 73(3), 265-275. http://doi.org/10.2489/jswc.73.3.265.

Jiao, Y., Chen, Q., Chen, X., Wang, X., Liao, X., Jiang, L., Yang, L., 2014. Occurrence and transfer of a cyanobacterial neurotoxin beta-methylamino-L-alanine within the aquatic food webs of Gonghu Bay (Lake Taihu, China) to evaluate the potential human health risk. Sci Total Environ. 468, 457-463. https://doi.org/10.1016/j.scitotenv.2013.08.064.

Jonasson, S., Eriksson, J., Berntzon, L., Spáčil, Z., Ilag, L. L., Ronnevi, L. O., Bergman, B., 2010. Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure. Proc Natl Acad Sci U S A. 107(20), 9252-9257. https://doi.org/10.1073/pnas.0914417107.

Jungblut, A., Wilbraham, J., Banack, S., Metcalf, J., Codd, G., 2018. Microcystins, BMAA and BMAA isomers in 100-year-old Antarctic cyanobacterial mats collected during Captain RF Scott's Discovery Expedition. Eur J Phycol. 53(2), 115-121. https://doi.org/10.1080/09670262.2018.1442587.

Kling, H., Watson, S., McCullough, G., Stainton, M., 2011. Bloom development and phytoplankton succession in Lake Winnipeg: a comparison of historical records with recent data. Aquat Ecosyst Health Manag. 14(2), 219-224. https://doi.org/10.1080/14634988.2011.577722.

Kopacek, J., Hejzlar, J., Posch, M., 2013. Factors controlling the export of nitrogen from agricultural land in a large central European catchment during 1900-2010. Environ Sci Technol. 47(12), 6400-6407. https://doi.org/10.1021/es400181m.

Lage, S., Costa, P. R., Moita, T., Eriksson, J., Rasmussen, U., Rydberg, S. J., 2014. BMAA in shellfish from two Portuguese transitional water bodies suggests the marine dinoflagellate

Gymnodinium catenatum as a potential BMAA source. Aquat Toxicol. 152, 131-138. https://doi.org/10.1016/j.aquatox.2014.03.029.
Leavitt, P. R., Brock, C. S., Ebel, C., Patoine, A., 2006. Landscape-scale effects of urban nitrogen on a chain of freshwater lakes in central North America. Limnol Oceanogr. 51(5), 2262-2277. https://doi.org/10.4319/lo.2006.51.5.2262.
Liu, K., Elliott, J. A., Lobb, D. A., Flaten, D. N., Yarotski, J., 2014. Conversion of conservation tillage to rotational tillage to reduce phosphorus losses during snowmelt runoff in the Canadian prairies. J Environ Qual. 43(5), 1679-1689. http://doi.org/10.2134/jeq2013.09.0365.
Li, L., He, Z. L., Tfaily, M. M., Inglett, P., Stoffella, P. J., 2018. Spatial-temporal variations of dissolved organic nitrogen molecular composition in agricultural runoff water. Water Res. 137, 375-383. https://doi.org/10.1016/j.watres.2018.01.035.
Lobner, D., Piana, P. M. T., Salous, A. K., Peoples, R. W., 2007. β-*N*-methylamino-l-alanine enhances neurotoxicity through multiple mechanisms. Neurobiol. Dis. 25(2), 360-366. https://doi.org/10.1016/j.nbd.2006.10.002.
Main, B. J., Bowling, L. C., Padula, M. P., Bishop, D. P., Mitrovic, S. M., Guillemin, G. J., Rodgers, K. J., 2018. Detection of the suspected neurotoxin β-methylamino-l-alanine (BMAA) in cyanobacterial blooms from multiple water bodies in Eastern Australia. Harmful Algae 74, 10-18. https://doi.org/10.1016/j.hal.2018.03.004.
Martín-Figueroa, E., Navarro, F., Florencio, F. J., 2000. The GS-GOGAT pathway is not operative in the heterocysts. Cloning and expression of *glsF* gene from the cyanobacterium *Anabaena* sp. PCC 7120. FEBS Lett. 476(3), 282-286. https://doi.org/10.1016/S0014-5793(00)01722-1.
Mayer, B., Wassenaar, L. I., 2012. Isotopic characterization of nitrate sources and transformations in Lake Winnipeg and its contributing rivers, Manitoba, Canada. J Great Lakes Res. 38, 135-146. https://doi.org/10.1016/j.jglr.2012.02.004.
McCullough, G. K., Page, S. J., Hesslein, R. H., Stainton, M. P., Kling, H. J., Salki, A. G., Barber, D. G., 2012. Hydrological forcing of a recent trophic surge in Lake Winnipeg. J Great Lakes Res. 38, 95-105. https://doi.org/10.1016/j.jglr.2011.12.012.
McKindles, K. M., Zimba, P. V., Chiu, A. S., Watson, S. B., Gutierrez, D. B., Westrick, J., Davis, T. W., 2019. A multiplex analysis of potentially toxic cyanobacteria in Lake Winnipeg during the 2013 bloom season. Toxins, 11(10), 587.
Merel, S., Walker, D., Chicana, R., Snyder, S., Baurès, E., Thomas, O., 2013. State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environ. Int. 59, 303-327. https://doi.org/10.1016/j.envint.2013.06.013.
Metcalf, J. S., Banack, S. A., Lindsay, J., Morrison, L. F., Cox, P. A., Codd, G. A., 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(3), 702-708. https://doi.org/10.1111/j.1462-2920.2007.01492.x.
Metcalf, J. S., Banack, S. A., Richer, R., Cox, P. A., 2015. Neurotoxic amino acids and their isomers in desert environments. J. Arid Environ. 112, 140-144. https://doi.org/10.1016/j.jaridenv.2014.08.002.
Michalak, A. M., Anderson, E. J., Beletsky, D., Boland, S., Bosch, N. S., Bridgeman, T. B., Daloğlu, I., 2013. Record-setting algal bloom in Lake Erie caused by agricultural and meteorological trends consistent with expected future conditions. Proc Natl Acad Sci U S A. 110(16), 6448-6452. https://doi.org/10.1073/pnas.1216006110.

Michaud, A. R., Poirier, S. C., Whalen, J. K., 2019. Tile drainage as a hydrologic pathway for phosphorus export from an agricultural subwatershed. J Environ Qual. 48(1), 64-72. http://doi.org/10.2134/jeq2018.03.0104.

Monteiro, M., Costa, M., Moreira, C., Vasconcelos, V. M., Baptista, M. S., 2017. Screening of BMAA-producing cyanobacteria in cultured isolates and in in situ blooms. J Appl Phycol. 29(2), 879-888. https://doi.org/10.1007/s10811-016-1003-4.

Mugnai, G., Rossi, F., Felde, V. J. M. N. L., Colesie, C., Büdel, B., Peth, S., De Philippis, R., 2018. Development of the polysaccharidic matrix in biocrusts induced by a cyanobacterium inoculated in sand microcosms. Biol Fert Soils. 54(1), 27-40. https://doi.org/10.1007/s00374-017-1234-9.

Ning, S. B., Guo, H. L., Wang, L., Song, Y. C., 2002. Salt stress induces programmed cell death in prokaryotic organism *Anabaena*. J. Appl. Microbiol. 93(1), 15-28. https://doi.org/10.1046/j.1365-2672.2002.01651.x.

Nizan, S., Dimentman, C., Shilo, M., 1986. Acute toxic effects of the cyanobacterium *Microcystis aeruginosa* on *Daphnia magna*. Limnol Oceanogr. 31(3), 497-502. https://doi.org/10.4319/lo.1986.31.3.0497.

Orihel, D. M., Bird, D. F., Brylinsky, M., Chen, H., Donald, D. B., Huang, D. Y., Kotak, B. G., 2012. High microcystin concentrations occur only at low nitrogen-to-phosphorus ratios in nutrient-rich Canadian lakes. Can. J. Fish. Aquat. Sci. 69(9), 1457-1462. https://doi.org/10.1139/f2012-088.

Paerl, H. W., Xu, H., McCarthy, M. J., Zhu, G., Qin, B., Li, Y., Gardner, W. S., 2011. Controlling harmful cyanobacterial blooms in a hyper-eutrophic lake (Lake Taihu, China): the need for a dual nutrient (N & P) management strategy. Water Res. 45(5), 1973-1983. https://doi.org/10.1016/j.watres.2010.09.018.

Paerl, H. W., Gardner, W. S., Havens, K. E., Joyner, A. R., McCarthy, M. J., Newell, S. E., Scott, J. T., 2016. Mitigating cyanobacterial harmful algal blooms in aquatic ecosystems impacted by climate change and anthropogenic nutrients. Harmful Algae 54, 213-222. https://doi.org/10.1016/j.hal.2015.09.009.

Pernil, R., Picossi, S., Herrero, A., Flores, E., Mariscal, V., 2015. Amino acid transporters and release of hydrophobic amino acids in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. Life (Basel) 5(2), 1282-1300. https://doi.org/10.3390/life5021282.

Pick, F. R., 2016. Blooming algae: a Canadian perspective on the rise of toxic cyanobacteria. Can. J. Fish. Aquat. Sci. 73(7), 1149-1158. https://doi.org/10.1139/cjfas-2015-0470.

Pip, E., Munford, K., Bowman, L., 2016. Seasonal nearshore occurrence of the neurotoxin β-N-methylamino-L-alanine (BMAA) in Lake Winnipeg, Canada. Environ Pollut. 5(1). http://doi.org/10.5539/ep.v5n1p110.

Popova, A. A., Rasmussen, U., Semashko, T. A., Govorun, V. M., Koksharova, O. A., 2018. Stress effects of cyanotoxin beta-methylamino-L-alanine (BMAA) on cyanobacterial heterocyst formation and functionality. Environ Microbiol Rep. 10(3), 369-377. https://doi.org/10.1111/1758-2229.12647.

Rao, S. D., Banack, S. A., Cox, P. A., Weiss, J. H., 2006. BMAA selectively injures motor neurons via AMPA/kainate receptor activation. Exp Neurol. 201(1), 244-252. doi:10.1016/j.expneurol.2006.04.017 https://doi.org/10.1016/j.expneurol.2006.04.017.

Rasouli, S., Whalen, J. K., Madramootoo, C. A., 2014. Review: Reducing residual soil nitrogen losses from agroecosystems for surface water protection in Quebec and Ontario, Canada:

Best management practices, policies and perspectives. Can. J. Soil Sci. 94(2), 109-127. https://doi.org/10.4141/cjss2013-015.
Rattan, K. J., Corriveau, J. C., Brua, R. B., Culp, J. M., Yates, A. G., Chambers, P. A., 2017. Quantifying seasonal variation in total phosphorus and nitrogen from prairie streams in the Red River Basin, Manitoba Canada. Sci Total Environ. 575, 649-659. https://doi.org/10.1016/j.scitotenv.2016.09.073.
Reichwaldt, E. S., Ghadouani, A., 2012. Effects of rainfall patterns on toxic cyanobacterial blooms in a changing climate: between simplistic scenarios and complex dynamics. Water Res. 46(5), 1372-1393. https://doi.org/10.1016/j.watres.2011.11.052.
Réveillon, D., Abadie, E., Séchet, V., Brient, L., Savar, V., Bardouil, M., Amzil, Z., 2014. Beta-*N*-methylamino-L-alanine: LC-MS/MS optimization, screening of cyanobacterial strains and occurrence in shellfish from Thau, a French Mediterranean lagoon. Mar. Drugs. 12(11), 5441-5467. https://doi.org/10.3390/md12115441.
Réveillon, D., Abadie, E., Séchet, V., Masseret, E., Hess, P., Amzil, Z., 2015. beta-N-methylamino-l-alanine (BMAA) and isomers: Distribution in different food web compartments of Thau lagoon, French Mediterranean Sea. Mar Environ Res. 110, 8-18. https://doi.org/10.1016/j.marenvres.2015.07.015.
Ross, C., Santiago-Vázquez, L., Paul, V., 2006. Toxin release in response to oxidative stress and programmed cell death in the cyanobacterium *Microcystis aeruginosa*. Aquat Toxicol. 78(1), 66-73. https://doi.org/10.1016/j.aquatox.2006.02.007.
Rattan, K. J., Corriveau, J. C., Brua, R. B., Culp, J. M., Yates, A. G., Chambers, P. A., 2017. Quantifying seasonal variation in total phosphorus and nitrogen from prairie streams in the Red River Basin, Manitoba Canada. Sci Total Environ. 575, 649-659. https://doi.org/10.1016/j.scitotenv.2016.09.073.
Richer, R., Banack, S. A., Metcalf, J. S., Cox, P. A., 2015. The persistence of cyanobacterial toxins in desert soils. J. Arid Environ. 112, 134-139. https://doi.org/10.1016/j.jaridenv.2014.01.023.
Rodgers, K. J., Main B. J., Samardzic K., 2018. Cyanobacterial neurotoxins: their occurrence and mechanisms of toxicity. Neurotox Res 33(1): 168-177. https://doi.org/10.1007/s12640-017-9757-2.
Roy-Lachapelle, A., Solliec, M., Bouchard, M. F., Sauvé, S., 2017. Detection of cyanotoxins in algae dietary supplements. Toxins (Basel) 9(3). https://doi.org/10.3390/toxins9030076.
Schindler, D. W., Hecky, R., Findlay, D., Stainton, M., Parker, B., Paterson, M., Kasian, S., 2008. Eutrophication of lakes cannot be controlled by reducing nitrogen input: results of a 37-year whole-ecosystem experiment. Proc Natl Acad Sci U S A. 105(32), 11254-11258. https://doi.org/10.1073/pnas.0805108105.
Schindler, D. W., Hecky, R. E., McCullough, G. K., 2012. The rapid eutrophication of Lake Winnipeg: Greening under global change. J Great Lakes Res. 38, 6-13. https://doi.org/10.1016/j.jglr.2012.04.003.
Schneider, K. D., McConkey, B. G., Thiagarajan, A., Elliott, J. A., Reid, D. K., 2019. Nutrient loss in snowmelt runoff: results from a long-term study in a dryland cropping system. J. Environ. Qual. https://doi.org /10.2134/jeq2018.12.0448.
Schoumans, O. F., Chardon, W. J., Bechmann, M. E., Gascuel-Odoux, C., Hofman, G., Kronvang, B., Dorioz, J. M., 2014. Mitigation options to reduce phosphorus losses from the agricultural sector and improve surface water quality: a review. Sci Total Environ. 468-469, 1255-1266. https://doi.org/10.1016/j.scitotenv.2013.08.061.

Scott, L., Downing, S., Phelan, R., Downing, T., 2014. Environmental modulation of microcystin and β-*N*-methylamino-l-alanine as a function of nitrogen availability. Toxicon 87, 1-5. https://doi.org/10.1016/j.toxicon.2014.05.001.

Sharpley, A. N., Kleinman, P., McDowell, R., 2007. Innovative management of agricultural phosphorus to protect soil and water resources. Commun Soil Sci Plan. 32(7-8), 1071-1100. https://doi.org/10.1081/CSS-100104104.

Shigaki, F., Sharpley, A., Prochnow, L. I., 2006. Animal-based agriculture, phosphorus management and water quality in Brazil: Options for the future. Sci Agr. 63(2), 194-209. http://dx.doi.org/10.1590/S0103-90162006000200013.

Smith, D., Owens, P., Leytem, A., Warnemuende, E., 2007. Nutrient losses from manure and fertilizer applications as impacted by time to first runoff event. Environ. Pollut. 147(1), 131-137. https://doi.org/10.1016/j.envpol.2006.08.021.

Snyder, L. R., Cruz-Aguado, R., Sadilek, M., Galasko, D., Shaw, C. A., Montine, T. J., 2009. Parkinson–dementia complex and development of a new stable isotope dilution assay for BMAA detection in tissue. Toxicol. Appl. Pharmacol. 240(2), 180-188. https://doi.org/10.1016/j.taap.2009.06.025.

Soto, D. X., Koehler, G., Wassenaar, L. I., Hobson, K. A., 2019. Spatio-temporal variation of nitrate sources to Lake Winnipeg using N and O isotope (δ^{15}N, δ^{18}O) analyses. Sci Total Environ. 647, 486-493. https://doi.org/10.1016/j.scitotenv.2018.07.346.

Torbick, N., Hession, S., Stommel, E., Caller, T., 2014. Mapping amyotrophic lateral sclerosis lake risk factors across northern New England. Int J Health Geogr. 13(1), 1. https://doi.org/10.1186/1476-072X-13-1.

Tripathi, A., Gottesman, S., 2016. Cell biology: Phosphate on, rubbish out. Nature 539(7627), 38. https://doi.org/10.1038/539038a.

Vega, A., Bell, E., 1967. α-Amino-β-methylaminopropionic acid, a new amino acid from seeds of *Cycas circinalis*. Phytochemistry 6(5), 759-762. https://doi.org/10.1016/S0031-9422(00)86018-5.

Violi, J. P., Mitrovic, S. M., Colville, A., Main, B. J., Rodgers, K. J., 2019. Prevalence of β-methylamino-L-alanine (BMAA) and its isomers in freshwater cyanobacteria isolated from eastern Australia. Ecotoxicol Environ Saf. 172, 72-81. https://doi.org/10.1016/j.ecoenv.2019.01.046.

Vogt, R. J., Sharma, S., Leavitt, P. R., 2017. Direct and interactive effects of climate, meteorology, river hydrology, and lake characteristics on water quality in productive lakes of the Canadian Prairies. Can J Fish Aquat Sci. 75(1), 47-59. https://doi.org/10.1139/cjfas-2016-0520.

Vo Duy, S., Munoz, G., Dinh, Q. T., Tien Do, D., Simon, D. F., Sauvé, S., 2019. Analysis of the neurotoxin β-N-methylamino-L-alanine (BMAA) and isomers in surface water by FMOC derivatization liquid chromatography high resolution mass spectrometry. PloS one, 14(8), e0220698. https://doi.org/10.1371/journal.pone.0220698.

Wang, M., Strokal, M., Burek, P., Kroeze, C., Ma, L., Janssen, A. B. G., 2019. Excess nutrient loads to Lake Taihu: Opportunities for nutrient reduction. Sci Total Environ. 664, 865-873. https://doi.org/10.1016/j.scitotenv.2019.02.051.

Wassenaar, L. I., Rao, Y. R., 2012. Lake Winnipeg: The forgotten great lake. J Great Lakes Res. 38, 1-5. https://doi.org/10.4319/lo.2007.52.5.2233.

Whalen, J. K., Thomas, B. W., Sharifi, M., 2019. Novel practices and smart technologies to maximize the nitrogen fertilizer value of manure for crop production in cold humid temperate regions. Adv. Agron. 153, 1-85. https://doi.org/10.1016/bs.agron.2018.09.002.

Xu, H., Paerl, H., Qin, B., Zhu, G., Hall, N., Wu, Y., 2014. Determining critical nutrient thresholds needed to control harmful cyanobacterial blooms in eutrophic Lake Taihu, China. Environ. Sci. Technol. 49(2), 1051-1059. https://doi.org/10.1021/es503744q.

Zguna, N., Karlson, A. M., Ilag, L. L., Garbaras, A., Gorokhova, E., 2019. Insufficient evidence for BMAA transfer in the pelagic and benthic food webs in the Baltic Sea. Sci. Rep. 9(1), 10406. https://doi.org/10.1038/s41598-019-46815-3.

Zhang, M., Duan, H., Shi, X., Yu, Y., Kong, F., 2012a. Contributions of meteorology to the phenology of cyanobacterial blooms: implications for future climate change. Water Res. 46(2), 442-452. https://doi.org/10.1016/j.watres.2011.11.013.

Zhang, W., Rao, Y. R., 2012b. Application of a eutrophication model for assessing water quality in Lake Winnipeg. J Great Lakes Res. 38, 158-173. https://doi.org/10.1016/j.jglr.2011.01.003.

FORWARD TO CHAPTER 3

In Chapter 2, I explained that cyanotoxin production in cyanobacterial blooms of agricultural watersheds responds to nutrient inputs and other environmental factors. However, I suspected that cyanobacterial blooms are not the only source of cyanotoxins in agricultural watersheds. Cyanotoxins in surface water could be transferred to groundwater through hydrologic processes. Furthermore, metabolically active cyanobacteria exist in the vadose zone and in aquifers, and are another potential source of cyanotoxins in groundwater. Therefore, I hypothesized that cyanotoxins will be presented in the vadose zone (soil and drainage water from the soil profile) and in groundwater collected from the aquifer in agricultural watersheds, which I discuss in Chapter 3.

CHAPTER 3

Quantitative screening for cyanotoxins in soil and groundwater of agricultural watersheds in Quebec, Canada

Yanyan Zhang[a], Barry R. Husk[b], Sung Vo Duy[c], Quoc Tuc Dinh[c], Juan Sebastian Sanchez[b], Hicham Benslim[a], Sébastien Sauvé[c], Joann K. Whalen[a]

a. McGill University, Department of Natural Resource Science, Macdonald Campus, 21,111 Lakeshore Road, Ste-Anne-de Bellevue, Quebec, Canada H9X 3V9

b. BlueLeaf Inc., 310 Chapleau Street, Drummondville, Quebec, Canada J2B 5E9

c. Université de Montréal, Department of Chemistry, c.p. 6128, Succursale Centre-Ville, Montréal, QC, Canada H3C 3J7

3.1. Abstract

Cyanotoxins, as secondary metabolites of cyanobacteria, are highly toxic to humans, animals and plants. The human health risk from cyanotoxins must not be underestimated. Among the cyanotoxins we investigated are hepatotoxic peptides of microcystins and cylindrospermopsins are associated with acute and chronic liver damage, while the chronic exposure to neurotoxic anatoxin-a, saxitoxins and beta-N-methylamino-L-alanine increase the risk of human neurodegenerative diseases. Cyanobacterial blooms are ‘hot spots’ for cyanotoxin production, but we hypothesize that cyanotoxins will be presented in multiple ecological compartments of agricultural watersheds. To test this hypothesis, we detect cyanotoxins in the vadose zone (soil and drainage water from the soil profile) and in groundwater used as drinking water of agriculutral watersheds. Cyanotoxins detection was confirmed with enzyme-linked immunosorbent assay kits and ultra-high liquid chromatography with tandem mass spectrometry. This work confirms that freshwater lakes are one of many ‘hot spots’ for cyanotoxin production. It is an alarmto find microcystins in the vadose zone and in drinking

water sourced from groundwater in agricultural watersheds. This suggests that cyanotoxins may be transferred from cyanobacterial blooms in lakes to groundwater through normal hydrologic processes. Public health authorities need to be alert to cyanotoxins in drinking water supplies, to implement proper monitoring and treatment protocols that protect citizens from this potential health hazard.

Keywords: Cyanotoxins; Microcystin; Anatoxin-a; Groundwater; ELISA and LC-MS/MS; Agricultural watersheds

3.2. Introducution

Cyanobacteria are ubiquitous in aquatic and terrestrial environments, and the largest populations are present at the surface where there is ample light to support photosynthesis, the main source of energy and carbon for these autotrophs. Aquatic cyanobacteria can number as many as 200 million cells per cm^3 in the surface water, whereas soil can support 5.2 million to 51 million cyanobacteria cells per cm^2 in agroecosystems of humid temperate regions (Whitman et al., 1998). Still, cyanobacteria are known to be bioactive in subsurface soil, aquifers and groundwater, because they can consume hydrogen gas and produce energy in the dark, a trait developed by their non-photosynthetic ancestors (Puente-Sánchez et al., 2018). Since their appearance on Earth about 3.2 to 3.5 billion years ago, cyanobacteria evolved many survival strategies. One of these strategies is to produce secondary metabolites – cyanotoxins – in response to ecophysiological cues related to nitrogen and carbon metabolism, photosynthesis regulation, quorum sensing and competition with other organisms (Zhang and Whalen 2020). Cyanotoxin production is usually associated with harmful cyanobacterial blooms in eutrophic surface water, and these compounds are toxic to plants, animals and humans. The human health risk from cyanotoxins must not be underestimated. These compounds include hepatotoxic

peptides of microcystins and cylindrospermopsins that are associated with acute and chronic liver damage, the neurotoxic anatoxin-a and saxitoxins, as well as beta-N-methylamino-L-alanine, to which chronic exposure can increase the risk of human neurodegenerative diseases (Huisman et al., 2018).

There is growing evidence that cyanotoxins are present in many environments, besides eutrophic waterbodies (Corbel et al., 2014). Cyanotoxins in surface water could be transferred from surface water to groundwater through hydrologic processes, resulting in well water contamination (Mohamed and Al Shehri 2009; Yang et al., 2016). Metabolically active cyanobacteria exist in the vadose zone and in aquifers (Puente-Sánchez et al., 2018), another potential source of cyanotoxins in groundwater. Therefore, we hypothesize that cyanotoxins will be presented in the vadose zone (soil and drainage water from the soil profile) and in groundwater collected from the aquifer of agricultural watersheds. To test this hypothesis, we evaluated the presence of cyanotoxins in agricultural watersheds. The following cyanotoxins were selected for evaluation: microcystins, cylindrospermopsin, anatoxin-a, saxitoxins and beta-N-methylamino-L-alanine. The sampling program included three ecosystem components: 1) agricultural soils, 2) subsurface drainage water from agricultural fields, and 3) drinking water sourced from groundwater wells in an agricultural watershed in south-central Quebec, Canada (Fig.1).

3.3. Materials and methods

3.3.1. Watershed characteristics for agricultural soil and drainage water samples

Petit-lac-St-François, located in St-François-Xavier-de-Brompton, Quebec, Canada (45.5°N, -72.1°W), is a hyper-eutrophic lake with total phosphorus concentration exceeding 0.3 mg L^{-1} (Pick 2016). This lake and its watershed are located in a cold humid temperate region where the

mean annual temperature is 5.6 ºC and annual precipitation is 1147 mm (Canada 2014). Land use in the watershed is predominately agricultural, and about 20% of the land is occupied by a mixed temperate deciduous-coniferous forest. Soils from the studied agricultural fields are of the Brompton stony loam series, a glacial till classified as a poorly drained Podzol soil. Soil, subsurface drainage water and well water collected for this study came from a commercial dairy farm that uses manure (dairy slurry) and supplemental inorganic fertilizers for crop nutrition, as well as conventional tillage and agrochemicals (primarily herbicides) for pest control (Husk et al. 2017). Due to the abundant precipitation in this region (>1000 mm per year), agricultural fields are not irrigated with groundwater or surface water.

3.3.2. Sampling methods

3.3.2.1. Soil samples

Soil samples (Soils M1-M3) for cyanobacteria metagenomic analysis were collected on 11 October 2016. The composite soil samples (5 cores from 3 randomly-selected locations) were taken at the 0–2 cm depth and at the 30 cm depth of the soil profile. We sampled three locations on the farm: S_{M1} was an agricultural field under corn (*Zea mays* L.), S_{M2} was an agricultural field under a mixed forage hay crop blend of 42% bromegrass (*Bromus* sp.), 37% alfalfa (*Medicago sativa*), 13% timothy-grass (*Phelum pratense*) and 8% fescue (*Festuca* sp.), and S_{M3} was a mixed temperate deciduous-coniferous forest adjacent to the agricultural fields. The DNA of fresh soil samples was extracted upon arrival at the lab and the DNA extract was then stored at -80℃ prior to metagenomic analysis.

Soil samples (Soils A-E) for cyanotoxins determination were collected on 13 May 2018 and 13 October 2018. Four soil samples (S_A - S_D) were taken from the 0–15 cm (mineral soil) layer of agricultural fields. S_A was from a soya (*Glycine max* L.))wheat (*Triticum* spp.) rotation, S_B

was a grass-based hayfield (dominated by *Phleum pratense* L.), S_C was a wheat (*Triticum* spp.) field and S_D was from a corn (*Zea mays* L.)-wheat (*Triticum* spp.) rotation. The fifth soil sample (S_E) was collected from the organic soil horizon ("O" layer, 0~15 cm deep) of a mixed temperate deciduous-coniferous forest adjacent to the agricultural fields. Soil samples were composites of 5 cores (~500 g soil) collected with a shovel, mixed homogenously and placed in a glass bottle wrapped with aluminum foil. Each composite soil represented one of three random locations (~1m^2) within each field, for a total of 15 soil samples on each sampling date.

3.3.2.2. Subsurface drainage and well water samples

Drainage water (Water A-D) and well water samples were collected on the same dates: 13 May 2018, 13 October 2018, and on 22 May 2019. Drainage water was collected from each of the agricultural fields by sequential sampling (3 times) of water flowing from the outlet of the subsurface tile drainage line in each field described above. Each replicate drainage water sample was placed in a glass container wrapped with aluminum foil. Well water (W_E) was collected from the drinking water source for this farm, a residential artesian well. Samples were transported in a cooler with ice. Within 2 h of collection, the samples were placed in a freezer at -20℃ in the dark to prevent cyanotoxin degradation. To prevent the breakage of glass bottles, we stored ~100 mL of water sample in a 250 mL bottle without tightening the cap and laid the sample on its side in the freezer.

3.3.2.3. Municipal drinking water sourced from groundwater

Groundwater-sourced municipal drinking water in 16 municipalities was sampled six times during a three-year period, during the late-autumn or early-winter months. The sampling dates were 22 October 2014, 7 November 2014, 22 November 2014, 28 October 2015, 5 November

2015 and 28 October 2016. All municipal drinking water samples were collected within a 6 h period on each sampling date. We returned to the same sampling site, a point of consumption within the drinking water distribution network, in each municipality on every sampling date. Similar sampling, transport and storage methods were used as described for drainage water samples in section 1.1. In the case of municipalities using chlorine treatment, 100 mg sodium thiosulfate /L was added to quench the chlorine.

3.3.3. DNA extraction and metagenomic analysis of soils

Prokaryotic DNA was extracted from S_{M1}–S_{M3} using the DNeasy PowerMax soil kit (QIAGEN Inc., Toronto, ON, Canada) according to manufacturer's instructions. DNA extracts were sequenced and prokaryotes were classified to the taxonomic order level by metagenomic analysis at the National Research Council Canada, Energy, Mining and Environment laboratory (Montreal, QC, Canada). Results were expressed by taxon and as the relative abundance of the total prokaryotic and cyanobacterial populations (Figure 3.1).

3.3.4. Extraction of cyanotoxins from soil

Cyanotoxins were extracted from fresh soil samples (S_A–S_E), in triplicate. We set up two groups, i.e., 1) field soil and 2) field soil spiked with cyanotoxins to test the recovery of cyanotoxins. The solid phase extraction (SPE) process is expected to purify the target compound from complex soil matrices, but the efficiency of SPE purification could vary with cyanotoxin and soil types. Therefore, two soils were chosen for comparison testing of SPE efficiency: an organic-poor soil (S1) and an organic-rich soil (S2).

In SPE efficiency test, microcystins were extracted using 75/25% methanol/water (*v/v*) acidified with 0.1 M acetic acid and then purified by SPE using a hydrophilic-lipophilic balance cartridge (6 mL, 200 mg, Phenomenex Inc., USA).The recovery of microcystins increased 27%

with SPE purification versus non-SPE purification (details of the recovery test are presented in section 5). Free Beta-N-methylamino-L-alanine was extracted by 0.1 M trichloroacetic acid (Oasis mixed reversed phase-cation exchange cartridge, 3 mL, 60 mg, Waters Corporation, USA). We did not detect beta-N-methylamino-L-alanine in S1 and S2 extracts without SPE purification and obtained less than 10 % of recovery with SPE purification. Cylindrospermopsin was extracted with 100% water without SPE purification and recoveries of 87% and 89% were achieved. Acetonitrile-water-formic acid in a ratio of 80:19.9:0.1 was used to extract anatoxin-a. Recovery of anatoxin-a decreased 12% with SPE purification (Strata^{TM}-X-strong cation exchange cartridge, 6 mL, 200mg, Phenomenex Inc., USA) versus non-SPE purification. For saxitoxin, 0.1M hydrochloric acid was the extractant. This toxin was not detected with SPE purification (Strata^{TM}-X-strong cation exchange cartridge, 6 mL, 200mg, Phenomenex Inc., USA). As a result of the SPE efficiency test, we decided to use SPE purification for soil extracts containing microcystins and beta-N-methylamino-L-alanine only.

For obtaining soil extracts, each solvent was tested on triplicate aliquots of S1 and S2, and the proportions were 5 mL: 1 g of solvent:soil, extracted twice, for a total volume of 10 mL of solvent. After blending the solvent:soil mixture for 0.5 min with a high-speed benchtop vortex mixer (0–1000 g), the extraction was done for 20 min under ultrasonication in an ultrasonic bath. After each extraction cycle, the mixture was centrifuged at 3000 g for 10 min, the supernatant was removed and the soil was resuspended in solvent for the next extraction cycle.

3.3.5. Analytical methods to determine cyanotoxins concentration in soil and water samples

Soil extracts and water samples were filtered < 0.2 μm prior to enzyme-linked immunosorbent assay (ELISA) analysis (Eurofins Abraxis LLC, Warminster, PA, USA) and absorbance was measured at 450 nm with a microplate reader (Molecular Devices LLC, San

Jose, CA, USA). Standard curves for microcystins and beta-N-methylamino-L-alanine were prepared using the manufacturer's evaluation program by plotting the $\%B/B_0$ (i.e., mean absorbance for each standard, divided by the zero standard mean absorbance, reported as percentage) on the linear y-axis against the microcystins and beta-N-methylamino-L-alanine concentration on a logarithmic x-axis. Anatoxin-a, saxitoxin and cylindrospermopsin concentrations were determined by a 4-parameter regression analysis.

We also evaluated the cyanotoxins concentration in water samples by ultraperformance liquid chromatography-tandem mass spectrometry or high-resolution mass spectrometry, as described by Duy et al. (Munoz et al., 2017; Duy et al., 2019; Roy-Lachapelle et al., 2019). Water was filtered with Acrodisc® syringe filters GHP membrane (<0.2µm, Waters), and matrix-matched calibration curves were used for quantification. For total microsystins analysis, samples were submitted to Lemieux oxidation using potassium permanganate and sodium metaperiodate, quenched with sodium bisulfite, filtered, and analyzed by on-line solid-phase extraction (on-line SPE) coupled to LC-MS/MS (Thermo TSQ Quantiva). Quantification was achieved using MMPB-d3 for internal standardization. For Anatoxin-a, cylindrospermopsin and saxitoxins analysis, the method was adapted from Roy-Lachapelle et al. (Roy-Lachapelle et al., 2019). Aliquots were spiked with CYN-$^{15}N_5$ internal standard, and analyzed by on-line SPE coupled to high-resolution mass spectrometry (Thermo Q-Exactive Orbitrap). For beta-N-methylamino-L-alanine (BMAA) analysis, samples were derivatized with 9-fluorenylmethyl chloroformate (FMOC chloride), analyzed by on-line SPE coupled to high-resolution mass spectrometry (Thermo Q-Exactive Orbitrap) and quantified in relation to the BMAA-d3 internal standard.

Soil cyanotoxins were expressed on a ng/g soil (dry weight) basis and water-borne cyanotoxins are presented in ng/ml. Concentrations of cyanotoxins that were below the lower limit of the standard curve were considered below the limit of detection (< LOD). For evaluation of precision and recovery, six water samples were divided into twelve aliquots, half of which were used as blank samples and the other half were spiked with cyanotoxins. Furthermore, all soil extracts (n=15) were divided into two aliquots, one being the blank and the other that was spiked with cyanotoxins. The spiking solution contained 1 µg microcystins /L, 1 µg cylindrospermopsin /L, 1 µg anatoxin-a /L, 0.02 µg saxitoxins /L and 100 µg beta-N-methylamino-L-alanine /L. The concentration of cyanotoxins in blank samples and spiked samples was determined and the recovery was calculated as follows:

$$\text{Recovery (\%)} = 100 \times \frac{\text{SC}-\text{NS}}{\text{SB}} \qquad \text{Eq. (1)}$$

where SC is the measured concentration of cyanotoxin, SB is the cyanotoxin concentration in the spiked aliquot, and NS is the cyanotoxin concentration in the non-spiked aliquot (Zhang et al., 2020). Recoveries of the studied cyanotoxins are presented in Figure S3.1.

3.4. Result and Discussions

Cyanobacteria live in agricultural soils from the study area, including genera in the orders *Chroococcales*, *Oscillatoriales* and *Nostocales* that can produce microcystins, cylindrospermopsin, anatoxin-a, saxitoxins and beta-N-methylamino-L-alanine (Figure 3.1 and Table S3.1). Semi-quantitative ELISA detected microcystins and anatoxin-a in agricultural soils, but not cylindrospermopsin, saxitoxin and beta-N-methylamino-L-alanine (Table 3.1, samples S_A to S_D). The quantitative UPLC-MS/MS method did not detect any cyanotoxins in agricultural soils. Microcystins were present in drainage water from agricultural fields, according to ELISA and UPLC-MS/MS analyses (Table 3.1, samples W_A to W_D).

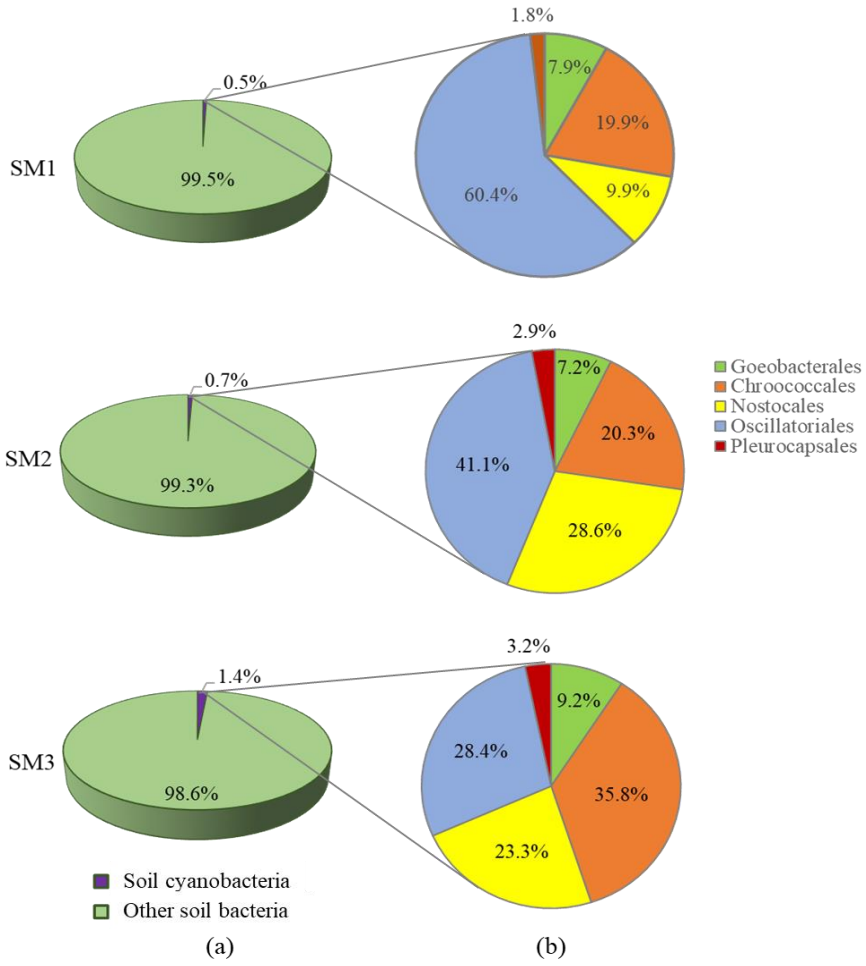


Figure 3.1: The relative abundance (%) of cyanobacteria in SM1-SM3 samples (a) and the proportions (%) of different orders of cyanobacteria (b).

Results for microcystins provide strong evidence of their presence in agricultural soil and water samples, for three reasons: 1) both analytical methods detected microcystins; 2) microcystins were present in soil and water samples from the same agroecosystems, and 3) we used a reliable method to extract and quantify microcystins from soil samples (69%–116% recovery). It is worth noting that the UPLC-MS/MS has a limit of detection ~10 times lower than that reported for ELISA, that MS is much less prone to false positive and we therefore assume LC-MS/MS to be more reliable. The ELISA method for microcystins is based on an

indirect competitive approach, which involves a two-step binding to a primary antibody, followed by binding with a labeled secondary antibody. This procedure is biased to give more false positives due to non-specific cross-reaction with the secondary antibody. The UPLC-MS/MS is more likely to be accurate because it combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry, which distinguishes and identifies multiple components with high molecular specificity and detection sensitivity (Duy et al., 2019).

Cylindrospermopsin, saxitoxins and beta-N-methylamino-L-alanine were detected on one sampling date, and anatoxin-a was detected on three sampling dates by ELISA, but not by UPLC-MS/MS on any sampling date (Table 3.1). Specifically, cylindrospermopsin and saxitoxins were detected on a few occasions in drainage water samples, but mostly at limit of detection levels by the ELISA method. Anatoxin-a was detected in both agricultural soil and drainage water, in spring and fall samples by the ELISA method, of which concentrations were greater in fall than spring ($p = 0.05$, Table 3.1).

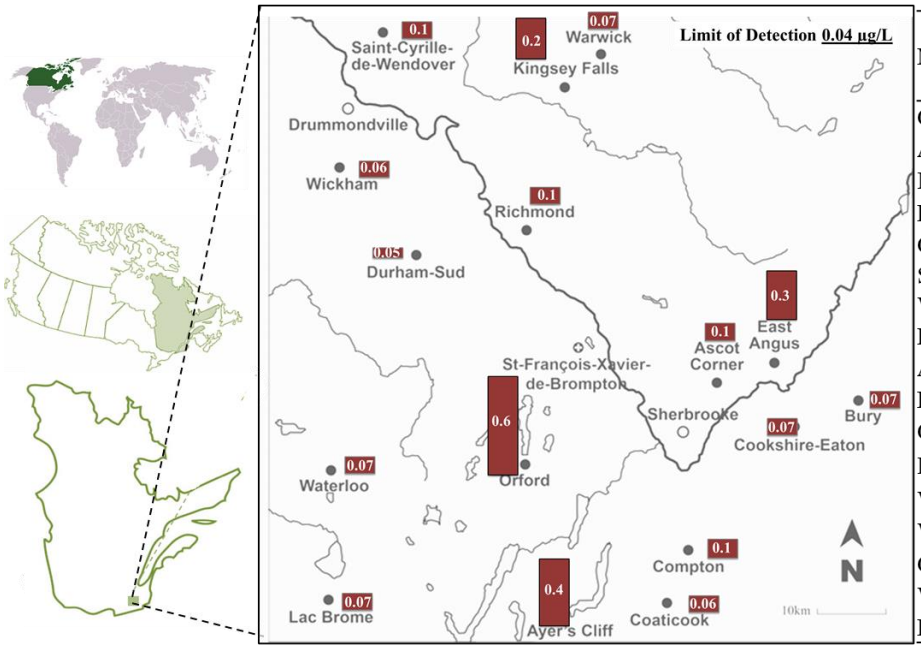
Table 3.1: Cyanotoxins in water (agricultural drainage water, WA to WD; residential well water, W_E, µg/L) and soil (agricultural fields, S_A to S_D; forest, S_E, µg/kg), as determined by enzyme-linked immunosorbent assays (ELISA) and ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Values are the mean concentration for n=3 field-collected samples. LOD = limit of detection.

Cyanotoxin	Water	2018/5/13-Spring		2018/10/13-Fall		2019/5/22-Spring		Soil	2018/5/13-Spring	2018/10/13-Fall
		ELISA	LC-MS	ELISA	LC-MS	ELISA	LC-MS		ELISA	
Microcystins	W_A	<LOD	**0.06**	<LOD	-	<LOD	<LOD	S_A	**0.4**	**0.4**
	W_B	<LOD	<LOD	<LOD	-	<LOD	<LOD	S_B	**0.5**	**0.4**
	W_C	<LOD	<LOD	**0.2**	**0.08**	<LOD	<LOD	S_C	**0.4**	**0.3**
	W_D	<LOD	**0.06**	<LOD	-	<LOD	<LOD	S_D	**0.3**	**0.5**

	W_E	<LOD	**0.06**	<LOD	-	<LOD	<LOD	S_E	**0.6**	**0.5**
Maximum value		<LOD	0.06	0.2	0.08	<LOD	<LOD	-	0.6	0.5
LOD		0.05	0.005	0.05	0.005	0.05	0.005	-	0.05	0.05
Cylindrosperm-opsin	W_A	<LOD	<LOD	<LOD	<LOD	**0.04**	<LOD	S_A	<LOD	<LOD
	W_B	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	S_B	<LOD	<LOD
	W_C	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	S_C	<LOD	<LOD
	W_D	<LOD	<LOD	<LOD	<LOD	**0.04**	<LOD	S_D	<LOD	<LOD
	W_E	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	S_E	<LOD	<LOD
Maximum value		<LOD	<LOD	<LOD	<LOD	0.04	<LOD	-	<LOD	<LOD
LOD		0.04	0.02	0.04	0.02	0.04	0.02	-	0.04	0.04
Anatoxin-a*	W_A	<LOD	<LOD	**0.1**	<LOD	<LOD	<LOD	S_A	<LOD	**3.5**
	W_B	<LOD	<LOD	<LOD	<LOD	**0.1**	<LOD	S_B	<LOD	**3.1**
	W_C	<LOD	<LOD	**0.2**	<LOD	<LOD	<LOD	S_C	<LOD	**4.7**
	W_D	**0.1**	<LOD	**0.2**	<LOD	**0.1**	<LOD	S_D	**2.7**	**5.7**
	W_E	<LOD	<LOD	**0.3**	<LOD	**0.1**	<LOD	S_E	**3.3**	**5.8**
Maximum value		0.1	<LOD	0.3	<LOD	0.1	<LOD	-	3.3	5.8
LOD		0.1	0.02	0.1	0.02	0.1	0.02	-	0.1	0.1
Saxitoxins	W_A	**0.02**	<LOD	<LOD	<LOD	<LOD	-	S_A	<LOD	<LOD
	W_B	**0.02**	<LOD	<LOD	<LOD	<LOD	-	S_B	<LOD	<LOD
	W_C	<LOD	<LOD	<LOD	<LOD	<LOD	-	S_C	<LOD	<LOD
	W_D	**0.03**	<LOD	<LOD	<LOD	<LOD	-	S_D	<LOD	<LOD
	W_E	**0.02**	<LOD	<LOD	<LOD	<LOD	-	S_E	<LOD	<LOD
Maximum value		0.03	<LOD	<LOD	<LOD	<LOD	-	-	<LOD	<LOD
LOD		0.02	0.8	0.02	0.8	0.02	-	-	0.02	0.02
beta-N-methylamino-L-alanine (BMAA)	W_A	<LOD	<LOD	<LOD	<LOD	**8**	<LOD	S_A	<LOD	<LOD
	W_B	<LOD	<LOD	<LOD	<LOD	**8**	<LOD	S_B	<LOD	<LOD
	W_C	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	S_C	<LOD	<LOD
	W_D	<LOD	<LOD	<LOD	<LOD	**12**	<LOD	S_D	<LOD	<LOD
	W_E	<LOD	<LOD	<LOD	<LOD	**9**	<LOD	S_E	<LOD	<LOD
Maximum value		<LOD	<LOD	<LOD	<LOD	12	<LOD	-	<LOD	<LOD
LOD		4	0.03	4	0.03	4	0.03	-	4	4

*One-way analysis of variance and a non-parametric test (Kruskal-Wallis H-test and Mann-Whitney U-test) using IBM SPSS 23.0.

Water from the artesian well at the St-François-Xavier-de-Brompton farm (Table 3.1, sample W_E) had microcystins detected on one occasion by UPLC-MS/MS, while anatoxin-a, saxitoxins and beta-N-methylamino-L-alanine were detected on other sampling dates by ELISA. Cylindrospermopsin was not detected in well water (Table 3.1). Microcystins were detected by the ELISA method between 2 and 4 times out of 6 sampling campaigns in sixteen municipalities located in south-central Quebec (Figure 3.2). In three municipalities, the maximum microcystin concentration exceeded the US-EPA Health Advisory threshold of 0.3 ng/ml (EPA 2015) on at least one occasion (Figure 3.2).



Municipality	Municipal treatment?	# Detects per six events
Canton d'Orford*	No	2
Ayer's Cliff	Yes	3
East Angus	Yes	3
Kingsey Falls	Yes	3
Compton	Yes	3
St-Cyrille-de-Wendover	Yes	4
Richmond	Yes	3
Ascot Corner	Yes	2
Bury*	No	3
Cookshire-Eaton	Yes	3
Lac-Brome	-	2
Warwick	-	2
Waterloo	-	2
Coaticook	-	2
Wickham	-	2
Durham-Sud*	-	3

Figure 3.2: Microcystins detection and maximum concentrations (µg/L) in municipal drinking water from south-central Quebec, Canada. Municipalities with an asterisk (*) have no municipal treatment system, but the rest treat drinking water with chlorine alone or chlorine together with other treatments.

Still, this research was limited by the availability of rapid, cost-effective analytical methods to detect and quantify cyanotoxins in all ecosystem components. This issue should be resolved

by methodological advances in quantifying soil cyanotoxins (see the recently optimized method by Zhang et al.) (Zhang et al., 2020), but the disparity between ELISA and LC-MS methods in quantifying cyanotoxins in water samples remains problematic. The ELISA method, which detects multiple cyanotoxins variants, is a good semi-quantitative screening tool that is particularly useful for indicating the presence of cyanotoxins, monitoring the toxicity dynamics of bloom, tracking relative changes in toxin concentrations, and taking actions to control blooms in source waters. However, the method is not suited to quantitatively determine cyanotoxins present at low levels in water samples. It has a high limit of detection (0.04–4 μg/L), and the nonlinear and variable calibration curve means that small differences in measured absorbance can translate into large concentration differences, thus the ELISA method can lead to biased-high concentrations (up to two to three times higher than values determined by LC-MS/MS). In contrast to ELISA method, LC-MS/MS is a precise quantitative method for determining cyanotoxins in water samples with reduced risks of false positives and limits of detection up to 100 times lower (i.e., 100 times more sensitive) than the ELISA method. Tandem mass spectrometry generates quantification and confirmation MS/MS fragment ions of targeted cyanotoxins, offering the highest sensitivity and precision for the identification and detection of cyanotoxins. With appropriate quality control measures, the matrix effects can be properly compensated through matrix-matched calibration. Therefore, in the short run, ELISA methods would be useful for monitoring the environmental fate of these toxic compounds. In the long run, LC-MS/MS shall take priority over ELISA method to support operational decisions about cyanotoxins in water that may have important public health, public relations, legal, and political ramifications.

3.5. Conclusion

We qualitatively confirm the presence of cyanobacteria with potential to produce cyanotoxins, as well as the cyanotoxins anatoxin-a and microcystins in agricultural soils. Furthermore, we detected microcystins in drinking water sourced from an artesian well and in the municipal drinking water supply across south-central Quebec, Canada. The greatest risk, according to our evaluation, is from microcystins and anatoxin-a. We cannot overlook the possibility that cyanobacteria produce cyanotoxins in agricultural soils, and these cyanotoxins are then transferred in the vadose zone to subsurface drainage and groundwater receptors, and potentially to downstream aquatic ecosystems, but the transport processes and magnitude of the transfer are unknown at this time. Furthermore, future research in this area needs to focus on understanding the triggers responsible for cyanotoxin production and their spatio-temporal variation to better gauge the ecological and public health consequences of these toxic compounds.

3.6. Supplementary Information

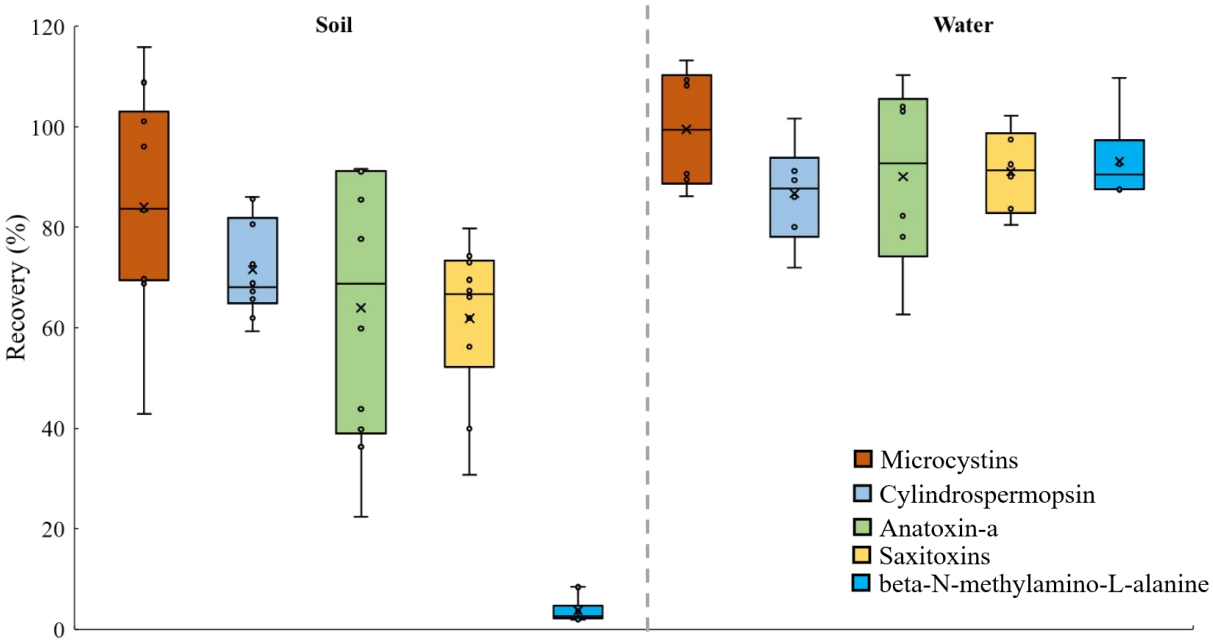


Figure S3.1. Box plots showing recoveries of cyanotoxins from soil extracts and water with enzyme-linked immunosorbent assays (ELISA).

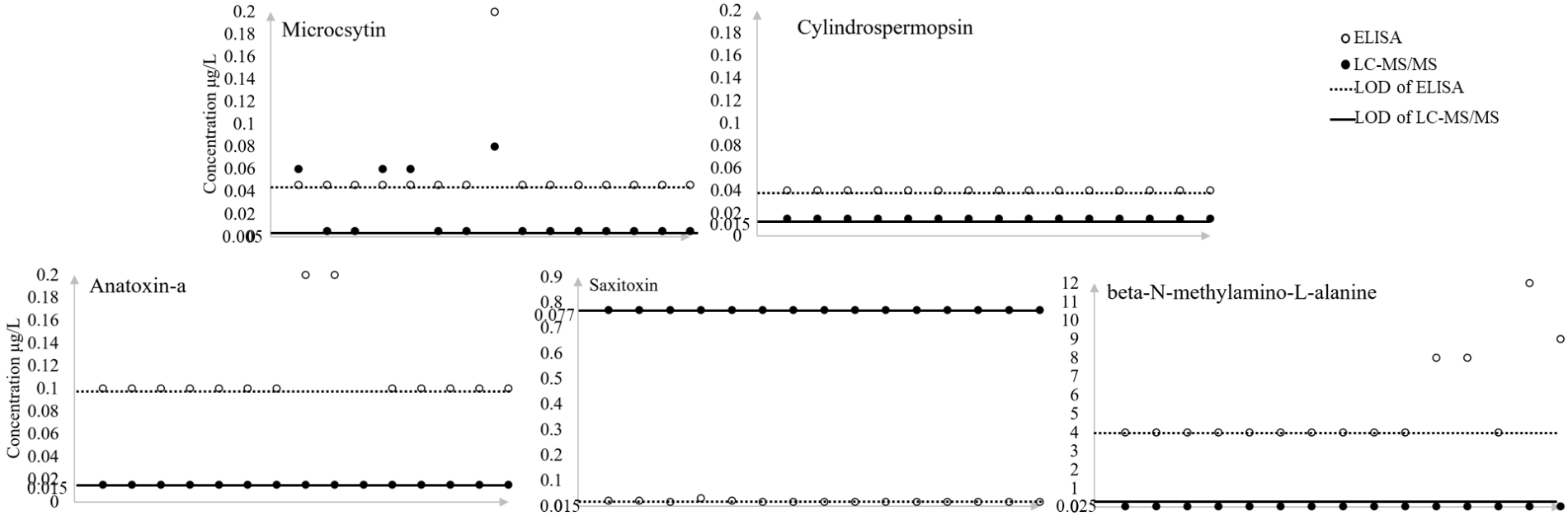


Figure S3.2: Cyanotoxins in water (agricultural drainage water, WA to WD, collected on 13 May 2018, 13 October 2019 and 22 May 2019) as determined by enzyme-linked immunosorbent assays (ELISA) and ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Values are the mean concentration for n=3 field-collected samples. LOD = limit of detection.

Table S3.1: Cyanobacteria with potential to produce cyanotoxins.

Order	Genus	Cyanotoxin
Nostocales	*Anabaena*	Microcystins
	Anabaena	Cylindrospermopsin
	Anabaena	Anatoxin-a
	Anabaena	Beta-N-methylamino-L-alanine
	Calothrix	Microcystins
	Cylindrospermopsis	Cylindrospermopsin
	Cylindrospermopsis	Saxitoxins
	Cylindrospermopsis	Beta-N-methylamino-L-alanine
	Nodularia	Beta-N-methylamino-L-alanine
Chroococcales	*Aphanocapsa*	Microcystins
	Aphanocapsa	Anatoxin-a
	Leptolynbya	Microcystins
	Microsystis	Microcystins
	Microsystis	Anatoxin-a
	Microsystis	Beta-N-methylamino-L-alanine
Oscillatoriales	*Geiterinema*	Saxitoxins
	Geiterinema	Microcystins
	Lyngbya	Cylindrospermopsin
	Lyngbya	Saxitoxins
	Lyngbya	Beta-N-methylamino-L-alanine
	Oscillatoria	Microcystins
	Oscillatoria	Anatoxin-a
	Oscillatoria	Cylindrospermopsin
	Spirulina	Microcystins

Table S3.2: Physico-chemical properties of soil and water samples collected from a commercial dairy farm in St-François-Xavier-de-Brompton, Quebec, Canada. LOD = limit of detection.

Soil	S_A	S_B	S_C	S_D	S_E
Soil Texture	Silt loam	Silt loam	Sandy loam	Sandy loam	Silt loam
pH	6.4	6.0	6.1	6.6	4.9
Moisture Content (%)	22	36	28	29	81
NO_3^- (mg/kg)	26	20	4	35	4
NH_4^+ (mg/kg)	<LOD	<LOD	<LOD	<LOD	<LOD
Orthophosphate/(mg/kg)	39	39	59	35	9
Fe (mg/kg)	93	83	128	163	150
K (mg/kg)	664	1161	828	1077	924
Ca (mg/kg)	2416	1749	1545	1205	989
Al (mg/kg)	77	62	54	76	109
Mg (mg/kg)	260	320	330	315	510
Organic Matter (g/kg)	56	30	54	45	146
Organic Carbon (g/kg)	33	18	21	17	85
Clay (g/kg)	210	240	610	500	370
Silt (g/kg)	710	660	360	470	610
Sand (g/kg)	80	100	30	40	30
Water	W_A	W_B	W_C	W_D	W_E
pH	7.5	7.2	7.2	7.3	7.3
Temperature (°C)	9	7	10	9	10
NO_3^- (mg/L)	5	2	4	5	0.08
NH_4^+ (mg/L)	<LOD	<LOD	<LOD	<LOD	<LOD
Orthophosphate/(mg/L)	0.01	-	0.01	0.02	0.1
Fe/(mg/L)	-	-	0.03	-	-
K/(mg/L)	0.7	0.8	0.7	0.6	0.5
Ca /(mg/L)	69	59	37	50	23
Mg/(mg/L)	18	14	8	26	11
Sp conductivity (µS/cm^2)	529	447	319	513	383
Resistivity (MΩ cm)	0.003	0.003	0.004	0.003	0.004
Total Dissolved Solids (g/L)	0.3	0.3	0.2	0.3	0.2
Salinity	0.3	0.2	0.2	0.3	0.2
Dissolved oxygen%	113	74	125	112	35
Dissolved Organic Carbon (mg/L)	5	5	7	4	3
Redox (mV)	7	8	7	7	7
Location	N 45° 30' W 72° 01'				

Table S3.3. Cyanotoxins in water (agricultural drainage water, WA to WD; residential well water, WE) and soil (agricultural fields, SA to SD; forest, SE), as determined by enzyme-linked immunosorbent assays (ELISA). Values are the mean concentration (Con.), standard deviation (SD) and percentage coefficient of variation (CV%) for n=3 field-collected samples. LOD = limit of detection.

Cyanotoxin	Water sample/μg L^{-1}	2019/5/22-Spring			2018/10/13-Fall			2018/5/13-Spring			Soil sample/ μg kg^{-1}	2018/10/13-Fall			2018/5/13-Spring		
		ELISA	SD	CV%	ELISA	SD	CV%	ELISA	SD	CV%		ELISA	SD	%CV	ELISA	SD	%CV
Microcystins	W_A	<LOD	-	-	<LOD	-	-	<LOD	-	-	S_A	0.4	0.02	2.7	0.4	0.05	4.5
	W_B	<LOD	-	-	<LOD	-	-	<LOD	-	-	S_B	0.4	0.01	0.9	0.5	0.06	5.7
	W_C	<LOD	-	-	0.2	0.02	1.7	<LOD	-	-	S_C	0.3	0.06	7.1	0.4	0.002	0.2
	W_D	<LOD	-	-	<LOD	-	-	<LOD	-	-	S_D	0.5	0.03	3.3	0.3	0.02	1.5
	W_E	<LOD	-	-	<LOD	-	-	<LOD	-	-	S_E	0.5	0.1	12	0.6	0.07	7.64
Cylindrospermopsin	W_A	0.04	0.00	0.3	<LOD	-	-	<LOD	-	-	S_A	<LOD	-	-	<LOD	-	-
	W_B	<LOD	-	-	<LOD	-	-	<LOD	-	-	S_B	<LOD	-	-	<LOD	-	-
	W_C	<LOD	-	-	<LOD	-	-	<LOD	-	-	S_C	<LOD	-	-	<LOD	-	-
	W_D	0.04	0.01	0.9	<LOD	-	-	<LOD	-	-	S_D	<LOD	-	-	<LOD	-	-
	W_E	<LOD	-	-	<LOD	-	-	<LOD	-	-	S_E	<LOD	-	-	<LOD	-	-
Anatoxin-a	W_A	<LOD	0.01	1.0	0.1	0.0	0.6	<LOD	-	-	S_A	3.5	0.005	0.7	<LOD	-	-
	W_B	0.1	0.00	0.1	<LOD	-	0.9	<LOD	-	-	S_B	3.1	0.002	0.3	<LOD	-	-
	W_C	<LOD	-	-	0.2	0.0	0.9	<LOD	-	-	S_C	4.7	0.01	1.8	<LOD	-	-
	W_D	0.1	0.03	3.8	0.2	0.0	1.5	0.1	0.009	1.1	S_D	5.7	0.001	0.1	2.7	0.004	0.4
	W_E	0.1	0.03	3.7	0.3	0.04	5	<LOD	-	-	S_E	5.8	0.01	1.4	3.3	0.00	0.18
Saxitoxins	W_A	<LOD	-	-	<LOD	-	-	0.02	0.00	0.004	S_A	<LOD	-	-	<LOD	-	-
	W_B	<LOD	-	-	<LOD	-	-	0.02	0.01	0.011	S_B	<LOD	-	-	<LOD	-	-
	W_C	<LOD	-	-	<LOD	-	-	<LOD	-	-	S_C	<LOD	-	-	<LOD	-	-
	W_D	<LOD	-	-	<LOD	-	-	0.03	0.00	0.002	S_D	<LOD	-	-	<LOD	-	-
	W_E	<LOD	-	-	<LOD	-	-	0.02	0.004	0.004	S_E	<LOD	-	-	<LOD	-	-
beta-N-methylamino-L-alanine	W_A	8	0.02	2.0	<LOD	-	-	<LOD	-	-	S_A	<LOD	-	-	<LOD	-	-
	W_B	8	0.08	6.8	<LOD	-	-	<LOD	-	-	S_B	<LOD	-	-	<LOD	-	-
	W_C	<LOD	-	-	<LOD	-	-	<LOD	-	-	S_C	<LOD	-	-	<LOD	-	-
	W_D	12	0.07	5.8	<LOD	-	-	<LOD	-	-	S_D	<LOD	-	-	<LOD	-	-
	W_E	9	0.04	3.4	<LOD	-	-	<LOD	-	-	S_E	<LOD	-	-	<LOD	-	-

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This project study was supported in part by the ATRAPP project (Algal Blooms, Treatment, Risk Assessment, Prediction and Prevention through Genomics) financed by Genome Canada/Génome Québec. YZ received financial support from the China Scholarship Council, a Graduate Mobility Award from McGill University and from the EcotoQ network, which is funded by the Fonds de recherche du Québec – Nature et technologies. The authors wish to acknowledge the assistance of Nathalie Fortin, Canadian National Research Council, for aid with metagenomic analyses.

3.7. References

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

Duy, S. V., G. Munoz, Q. T. Dinh, D. T. Do, D. F. Simon, and S. Sauvé., 2019. Analysis of the neurotoxin β-N-methylamino-L-alanine (BMAA) and isomers in surface water by FMOC derivatization liquid chromatography high resolution mass spectrometry. PloS one 14 (8). https://doi.org/10.1371/journal.pone.0220698.

EPA, U., 2015. A compilation of cost data associated with the impacts and control of nutrient pollution. US EPA, Reports and Assessments.

Huisman, J., G. A. Codd, H. W. Paerl, B. W. Ibelings, J. M. Verspagen, and P. M. Visser., 2018. Cyanobacterial blooms. Nat. Rev. Microbiol. 16, 471-483. https://doi.org/10.1038/s41579-018-0040-1.

Husk, B. R., B. C. Anderson, J. K. Whalen, and J. S. Sanchez., 2017. Reducing nitrogen contamination from agricultural subsurface drainage with denitrification bioreactors and controlled drainage. Biosyst. Eng. 153, 52-62. https://doi.org/10.1016/j.biosystemseng.2016.10.021.

Mohamed, Z. A., and A. M. Al Shehri., 2009. Microcystins in groundwater wells and their accumulation in vegetable plants irrigated with contaminated waters in Saudi Arabia. J Hazard Mater 172, 310-315. https://doi.org/10.1016/j.jhazmat.2009.07.010.

Munoz, G., S. Vo Duy, A. Roy-Lachapelle, B. Husk, and S. Sauvé., 2017. Analysis of individual and total microcystins in surface water by on-line preconcentration and desalting coupled to liquid chromatography tandem mass spectrometry. J. Chromatogr. A. 1516 :9-20. https://doi.org/10.1016/j.chroma.2017.07.096.

Pick, F. R., 2016. Blooming algae: a Canadian perspective on the rise of toxic cyanobacteria. Can. J. Fish Aquat. Sci. 73, 1149-1158. https://doi.org/10.1139/cjfas-2015-0470.

Puente-Sánchez, F., A. Arce-Rodríguez, M. Oggerin, M. García-Villadangos, M. Moreno-Paz, Y. Blanco, N. Rodríguez, L. Bird, S. A. Lincoln, and F. Tornos., 2018. Viable cyanobacteria in the deep continental subsurface. Proc. Natl. Acad. Sci. U S A 115, 10702-10707. https://doi.org/10.1073/pnas.1808176115.

Roy-Lachapelle, A., S. V. Duy, G. Munoz, Q. T. Dinh, E. Bahl, D. F. Simon, and S. Sauvé., 2019. Analysis of multiclass cyanotoxins (microcystins, anabaenopeptins, cylindrospermopsin and anatoxins) in lake waters using on-line SPE liquid chromatography high-resolution Orbitrap mass spectrometry. Anal. Methods 11, 5289-5300. https://doi.org/10.1039/C9AY01132C.

Whitman, W. B., D. C. Coleman, and W. J. Wiebe. 1998. Prokaryotes: the unseen majority. Proc. Natl. Acad. Sci. U S A 95, 6578-6583. https://doi.org/10.1073/pnas.95.12.6578.

Yang, Z., Kong, F., Zhang, M., 2016. Groundwater contamination by microcystin from toxic cyanobacteria blooms in Lake Chaohu, China. Environ. Monit. Assess. 188, 280. . https://doi.org/10.1007/s10661-016-5289-0.

Zhang, Y., and J. K. Whalen. 2020., Production of the neurotoxin beta-N-methylamino-L-alanine may be triggered by agricultural nutrients: An emerging public health issue. Water Res. 170, 115335. https://doi.org/10.1016/j.watres.2019.115335.

Zhang, Y., J. K. Whalen, S. V. Duy, G. Munoz, B. R. Husk, and S. Sauvé., 2020. Improved extraction of multiclass cyanotoxins from soil and sensitive quantification with on-line purification liquid chromatography tandem mass spectrometry. Talanta 120923. https://doi.org/10.1016/j.talanta.2020.120923.

FORWARD TO CHAPTER 4

In Chapter 3, my extensive field survey confirmed the presence of cyanotoxins in agricultural soils and in groundwater leaving agricultural fields. I also reported on microcystins detected in drinking water, which indicates that cyanotoxins are either produced by cyanobacteria living in aquifers or transported into aquifers through hydrologic process. However, my research was limited by the availability of rapid, cost-effective analytical methods to detect and quantify cyanotoxins in these ecosystems. Since the soil environment is a challenging matrix for cyanotoxins detection, I aimed to find a method to isolate and quantify soil cyanotoxins. Therefore, Chapter 4 explains how I developed a method that is the first one capable of extracting and quantifying 17 common cyanotoxins in soil.

CHAPTER 4

Improved extraction of multiclass cyanotoxins from soil and sensitive quantification with on-line purification liquid chromatography tandem mass spectrometry

Yanyan Zhang[a], Sung Vo Duy[b], Gabriel Munoz[b], Barry R. Husk[c], Joann K. Whalen[a], Sébastien Sauvé[b]

[a] *McGill University, Department of Natural Resource Science, Macdonald Campus, 21,111 Lakeshore Road, Ste-Anne-de Bellevue, Quebec, Canada H9X 3V9*

[b] *Université de Montréal, Department of Chemistry, C.P. 6128, Succursale Centre-Ville, Montréal, Quebec, Canada H3C 3J7*

[c] *BlueLeaf Inc., 310 Chapleau Street, Drummondville, Quebec, Canada J2B 5E9*

4.1. Abstract

Cyanotoxins are associated with harmful cyanobacterial blooms, but also exist in biological soil crusts and soils irrigated with cyanobacteria-contaminated water. To achieve an accurate analysis of cyanotoxins in soil, effective extraction, purification and determination methods are imperative. The most challenging aspect is extracting cyanotoxins from soil, due to their tendency to bind strongly to the soil matrix. We used a methanol-ammonium acetate solution to efficiently extract 17 cyanotoxins (microcystins, cylindrospermopsin, anatoxins, anabaenopeptins, and cyanopeptolin) from different soils. The extract was purified by on-line solid-phase extraction coupled with ultra-high-performance liquid chromatography tandem mass spectrometry. The optimized procedure involved two ultrasonication cycles of 15 min with 4 mL of methanol + 200 mM ammonium acetate, which recovered 60% to >90% of the added cyanotoxins from five soils with diverse organic matter, pH and texture. The method improved extraction by up to 10 times compared to a methanol/water solution. Linearity, accuracy and precision were validated on matrix-mixed soil with surrogate microcystin and cylindrospermopsin internal standards. Limits of detection were 0.001–0.3 ng g^{-1}, depending on the cyanotoxin. The method was used to analyze cyanotoxins in 25 field-collected soils from Quebec, Canada. Out of the 25 soil samples, 11 soils had at least one cyanotoxin, and up to 8 different cyanotoxins were detected

in one soil. The sum of all microcystins congeners was from 0.02 to 31 ng microcystins g^{-1} soil. We also detected anabaenopeptin, the first study reported occurrence of this cyanotoxin in soil.

Keywords

Cyanotoxin extraction; Soil; On-line solid-phase extraction; Microcystins; Anatoxins; Cylindrospermopsin.

4.2. Introduction

Cyanotoxins are secondary metabolites produced by cyanobacteria living in fresh water, seawater, sediment, soil and various biological matrices [1]. Some of these bioactive substances are low-molecular weight peptides (typically < 1000 Da), while others are cyclic amine alkaloids and non-protein amino acids that inhibit the growth of prokaryotic cells and hence are produced by cyanobacteria in response to competitive pressure and other stresses [2, 3]. However, cyanotoxins are toxic to humans and animals. For example, acute and chronic liver damage is associated with exposure to hepatotoxic peptides of the microcystins, anabaenopeptin and cyanopeptolins and the alkaloid cylindrospermopsin [4-6]. The neurotoxic anatoxins (anatoxin-a and homoanatoxin-a) are potent neuromuscular blocking agents [7]. Eco-epidemiological surveys indicate that human health is at risk from exposure to these and other cyanotoxins in the environment [8, 9]. Environmental exposure levels must be quantified to understand risks and improve public health outcomes, consistent with One Health goals [10].

Cyanotoxins in aquatic ecosystems are detected and quantified rapidly, due to well-developed analytical methods and the relatively high concentrations of these toxic compounds in eutrophic waterways with harmful cyanobacterial algal blooms [11]. In Canada, major bloom-forming and toxin-producing genera included *Microcystis*, *Dolichospermum* (*Anabaena)*, *Aphanizomenon*, and *Cylindrospermopsis* [12]. More than 250 freshwater bodies in Quebec province were contaminated by cyanobacterial blooms from 2004 to 2008, with yearly bloom recurrence in 61 % of these water bodies [13]. Microcystins, cylindrospermopsin, and anatoxins were among the detected cyanotoxins [14]. More than eight microcystin congeners were detected in Petit Lac St. François, a hypereutrophic lake, which had more than 400 µg total microcystins g^{-1} dry weight in the cyanobacterial bloom

[15]. Studies conducted elsewhere suggested that cyanotoxins could contaminate soils surrounding harmful algal bloom-impacted waterbodies and leach into groundwater [16-17].

Consequently, cyanobacterial communities are not limited to nutrient-rich aquatic ecosystems—they are also detected in terrestrial environments. Soil-associated cyanobacterium *Hormoscilla pringsheimii* produced cylindrospermopsin at detectable levels (limit of detection of 0.5 µg L^{-1}) in soil extracts [18]. The biological soil crust found on the soil surface in arid and semi-arid regions contains cyanobacteria. For instance, cyanotoxin-producing genera *Hapalosiphon hibernicus, Microcoleus, H. pringsheimii, Dolichospermum, Chroococcus* and *Pleurocapsa* produced from 0.1 to 11.1 µg microcystins g^{-1} dry weight and 0.3 to 0.6 µg anatoxin-a g^{-1} dry weight in dry Arctic regions [19]. Anatoxin-a (S) was detected in biological soil crust from the Qatar desert [20, 21]. Neurotoxin-producing cyanobacteria (e.g. *Dolichospermum*) growing in biological soil crust produced six times more anatoxin-a(S) after a rainstorm in the Qatar desert, and the 3–56 µg microcystins m^{-2} in the Qatar desert were considered to be a source for human exposure during dust storms [19, 20, 22]. Agricultural soils that are irrigated deliberately or flooded unintentionally with cyanobacteria-contaminated water likely contain cyanotoxins, due to the release of these compounds from lysed cyanobacterial cells. For instance, irrigated agricultural soils in south China had up to 187 µg microcystins kg^{-1} soil [23]. Farmers irrigate with cyanobacteria-contaminated water in China, India, New Zealand, Australia, South Africa, France, Greece, the United States and Canada [24-29]. The exposure risk to farm workers that operate irrigation equipment and that are in contact with agricultural soils containing cyanotoxins has never been quantified. This remains a critical knowledge gap in assessing the safe exposure limits to cyanotoxins in agricultural soils.

We expect to find biologically significant amounts of cyanotoxins in soil, but there is very little published data on cyanotoxins in the world's soils due to the difficulty to extract

and quantify these compounds. The major challenge is to choose an efficient solvent to extract soil cyanotoxins, which could be dissolved in the soil pore water, bound to clay minerals and organic matter, or complexed with metal colloids [30, 31]. Clay minerals and organic matter in soil retain cyanotoxins by physical occlusion and chemical binding on charged surfaces (adsorption sites). Binding strength is affected by pH, which alters the net charge of clay and organic matter surfaces, as well as the charge of amino acid and amine groups in the peptide-based and non-peptide cyanotoxins. A chelating solution made from ethylene-diamine tetraacetic acid-tetrasodium pyrophosphate recovered from 56%-103% of the microcystins variants added to soils and sediments. However, the method may not be amenable to direct analysis by liquid chromatography mass spectrometry due to low salt volatility and gradual clogging of the transfer tube [32]. Methanol/water (w/v) with pH 2 to 7 is an effective solvent that modifies the charge of microcystins, to desorb them from adsorption sites on clay and organic matter in soil [33, 34]. However, the variable charge of cyanotoxins means that the desorption kinetics of this diverse group of compounds are stochastic rather than deterministic. For example, soil has greater capacity to adsorb anatoxin-a (Log K_{ow} = 1.1) than microcystins variants (Log K_{ow}, from -1.79 to 0.67) [35-38]. Our proposed solution is to use methanol with an MS-compatible buffer salt (e.g., ammonium acetate, CH_3COONH_4). The hypothesis was that CH_3COONH_4 could desorb cyanotoxins by competing for adsorption sites and by changing the binding strength (e.g., from electrostatic interactions, hydrogen-bonding and ionic bonding) between cyanotoxins and adsorptive sites on organic matter and clay particles.

The objective of this study was to improve the efficiency of extracting diverse cyanotoxins from soils and their quantification by on-line solid-phase extraction coupled with ultra-high-performance liquid chromatography tandem mass spectrometry (on-line SPE-UHPLC-MS/MS).We first evaluated different extraction solutions for their ability to recover

cyanotoxins from soils. The optimized extraction procedure was followed by a fast and efficient purification step, involving on-line SPE-UHPLC-MS/MS. We validated the method by measuring the recovery of 17 cyanotoxins in soils with 5%–25% clay content and 1.4%–8.9% organic matter content. Key analytical metrics were documented: linearity, limits of detection (LOD), limits of quantification (LOQ), accuracy, and precision in matrix-matched soil. Finally, the optimized method was used to quantify cyanotoxins in soils from 25 field locations in southern Quebec, Canada.

4.3. Materials and methods

4.3.1. Chemicals and standards

The extracting solution contained HPLC-grade methanol (Fisher Scientific, Whitby, ON, Canada) and ammonium acetate (purity ≥ 98%) acquired from Sigma-Aldrich (St. Louis, MO, USA.). The LC-MS grade solvents were HPLC-water, methanol, and acetonitrile obtained from Fisher Scientific. Acrodisc GH Polypro filters (GHP filters, 0.2 µm) were purchased from Waters (Milford, MA, USA). Nitrogen (N_2) with 99.998% purity for evaporation was from MEGS (St-Laurent, QC, Canada).

All standards had chemical purities of 95% or higher. Internal standards were nodularin-R obtained from Enzo Life Science (Farmingdale, NY, USA), cylindrospermopsin -$^{15}N_5$ from Abraxis (Warminster, PA, USA) and microcystin- leucine-arginine -$^{15}N_{10}$ from Cambridge Isotopes Laboratories (Tewksbury, MA, USA). Standards for microcystin-arginine-RR, YR, LA, LY, LW, LF, WR, HtyR, HilR were purchased from Enzo Life Science, while anatoxin-a, cylindrospermopsin, microcystin-LR, [Asp^3]-LR, RR were from the National Research Council of Canada (Halifax, NS, Canada). Homoanatoxin-a was obtained from Abraxis. Anabaenopeptin A and B and cyanopeptolin A were purchased from Cyano Biotech GmbH (Berlin, Germany). Stock standard solutions of cyanotoxins were prepared in methanol and

stored at -20℃ for a maximum of 2 months. The 100 ng cyanotoxins mL^{-1} working solution from stock standard solutions was prepared daily in HPLC-water.

4.3.2. Soils for method development

We collected silt loam soil (3% organic matter content) from an agricultural field in Saint-François-Xavier-de-Brompton (Estrie, Québec, Canada) in May 2019 to develop and optimize the extraction method (Soil MO for method optimization, described in Table S4.1). Five soils for method validation (Soils MV_1, MV_2, MV_3, MV_4 and MV_5, see Table S4.1) had sandy to loamy textures and contained 1.4%–8.9 % OM content. When collecting Soil MO and Soils MV_1-MV_5, we chose at least three random zones (~1 m^2) from the field, used a shovel to remove soil (~500 g from the 0–15 cm depth) in each zone, then mixed the soil and placed the composite sample into one glass bottle (250 mL) wrapped with aluminum foil at each sampling site. Bottles were placed in a cooler with ice packs and stored at 4℃ until analysis.

4.3.3. Soils for cyanotoxins quantification

Cyanotoxins were quantified with the optimized extraction method in soil samples collected between June and August, 2019 from 25 field sites in southern Quebec, Canada (Detailed in Table S4.3). We chose agricultural soils, orchard soils and surface soils near waterbodies as diverse field sites. Soil from cultivated agroecosystems and orchards were a composite sample (0-15 cm depth) from three random zones (~1 m^2), mixed thoroughly and placed in a 250 mL glass bottle wrapped with aluminum foil. Along waterbodies where cyanotoxins were detected or cyanobacterial blooms were visible to the naked eye, we collected a composite soil sample (0-15 cm, from 3 locations along the watercourse) and placed it in a 250 mL glass bottles wrapped with aluminum foil. Glass bottles were transported in cooler with ice and stored at -20℃ in the dark to prevent degradation of cyanotoxins.

4.3.4. Optimizing the cyanotoxins extraction method

2.4.1. Soil preparation and spiking with cyanotoxins

Soil MO was air dried for over 48 h then ground using mortar and pestle, sieved < 2 mm mesh, homogenized and weighed (1 g aliquots) and placed in a 15-mL polypropylene tube. Each replicate of Soil MO was spiked with the cyanotoxins working solution to achieve a concentration of 10 ng cyanotoxins g^{-1} of soil (dry weight basis). After a short contact time (~15 min), we extracted the soil to recover cyanotoxins.

2.4.2. Extraction solvents

Eight solvents were evaluated for their capacity to extract cyanotoxins from Soil MO: methanol /HPLC-water: 90/10 (v/v), methanol with 0.2% NH_4OH, methanol with 0.2% formic acid, methanol with 50 mM ammonium acetate, methanol with 100 mM ammonium acetate, methanol with 200 mM ammonium acetate, methanol with 200 mM ammonium acetate and methanol with 250 mM ammonium acetate. Each solvent was tested on triplicate aliquots of Soil MO, and the proportions were 10 mL: 1 g and 5 mL: 1g of solvent: soil, extracted twice, for a total volume of 15 mL of solvent. After blending the solvent:soil mixture for 0.5 min with a high-speed benchtop vortex mixer (0–1008 *g*), the extraction was done for 15 min under ultrasonication in an ultrasonic bath. After each extraction cycle, the mixture was centrifuged at 4000 *g* for 10 min, the supernatant was removed and the soil was resuspended in solvent for the next extraction cycle.

2.4.3. Volume of extraction solvent

Soil MO was extracted twice with a variable volume of methanol + 200 mM ammonium acetate solution, namely 6 mL (3 mL + 3 mL), 8 mL (4 mL + 4 mL), 10 mL (5 mL + 5 mL) and 15 mL (7.5 mL + 7.5 mL). After blending the solvent:soil mixture for 0.5 min on a vortex mixer, each extraction was repeated for 15 min with ultrasonication, followed by centrifugation and transfer of the supernatant to a 15-mL polypropylene tube.

2.4.4. Number of extraction cycles

Soil MO was extracted with methanol + 200mM ammonium acetate solution for one, two and three cycles. We used 4 mL solvent cycle^{-1}, 0.5 min vortex mixing to combine the solvent:soil mixture and a 15 min ultrasonication time for each cycle, followed by centrifugation and transfer of the supernatant to a 15-mL polypropylene tube.

2.4.5. Variable ultrasonication time

Soil samples were extracted twice with 4 mL/cycle of methanol + 200mM ammonium acetate solution for 5, 10, 15 and 20 min under ultrasonication. The solvent:soil mixture was homogenized by 0.5 min of mixing with a vortex mixer before the extraction began.

The schematic diagram of the method optimization was shown in the below (Figure 4.1).

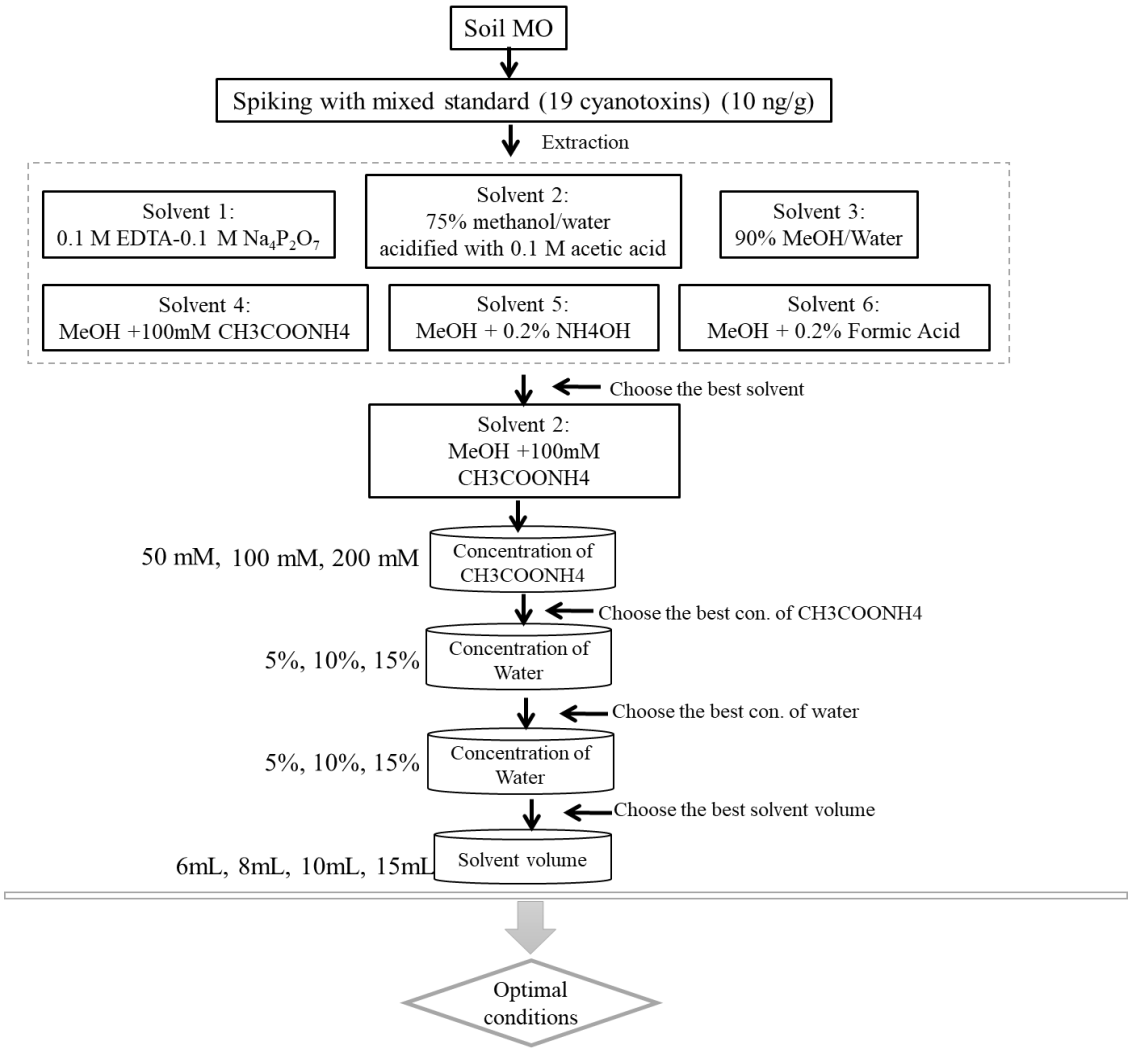


Figure 4.1: Schematic diagram of the extraction method optimization.

4.3.5. Final extraction procedure selected for the analysis of field samples

The optimized sample extraction procedure applied to field samples was as follows. An aliquot of soil was weighed (1g d.w.) in 15-mL tubes and spiked with the surrogate internal standards (100 µL of a mix containing 20 ng mL^{-1} of Microcystin-LR-$^{15}N_{10}$ and 40 ng mL^{-1} of Cylindrospermopsin-$^{15}N_5$). Under the optimized conditions, solvent extraction proceeded as follows. The samples were subjected to 2 extraction cycles, each involving the addition of 4 mL of methanol containing 200 mM of ammonium acetate, high-speed vortexing (0.5 min, 3500 rpm), ultrasonication (15 min), and centrifugation (10 min, 6000 rpm). The combined supernatants were reduced (<0.2 mL) using a gentle N_2 stream with mild heating (40 °C), amended with 2 mL of HPLC water, vortexed, and passed through GHP filters mounted on microsyringe filter holders. A 1.5 mL aliquot of the filtrate was introduced in a 2-mL amber glass LC vial for instrumental analysis.

4.3.6. Cyanotoxins recovery

We used Soil MV_1-MV_5 to evaluate cyanotoxins recovery with the optimized extraction method. Three types of analytical samples were prepared: non-spiked soil (n = 3), soil spiked with 10 ng g^{-1} of cyanotoxins before extraction (n = 3), and soil spiked with 10 ng g^{-1} of cyanotoxins after extraction, prior LC-MS/MS analysis (n = 3). Internal standards were added at the end of the preparation procedure, just before analysis by on-line SPE-UHPLC-MS/MS. The recovery (%) was then calculated according to [39]:

$$\text{Recovery (\%)} = 100 \times \frac{SB - NS}{SA - NS} \qquad \text{Eq. (1)}$$

where SB is the cyanotoxins to internal standard area ratio in the soil that was spiked before extraction, SA is the cyanotoxins to internal standard area ratio in soil spiked after extraction, and NS the cyanotoxins to internal standard area ratio of the non-spiked soil sample.

4.3.7. Quality assurance/quality control

Quality control soil (Soil QC) was prepared by mixing and homogenizing equal quantities of Soil MV_2, MV_4 and MV_5. A series of 4 procedural blanks (spiked with internal standards but with no soil matrix added) was processed in parallel to the samples through the entire analytical procedure. Procedural blanks showed no particular contamination. Matrix-matched calibration curves were prepared with cyanotoxins having concentrations from 0.005–20 ng g^{-1} by adding native standards and internal standards (internal standards microcystins-LR-$^{15}N_{10}$ and cylindrospermopsin-$^{15}N_5$ set at 2 ng g^{-1} and 4 ng g^{-1}, respectively) to 1-g aliquots of soil QC just before starting the extraction procedure. The limit of detection (LOD, ng g^{-1}) and limit of quantification (LOQ, ng g^{-1}) were calculated using signal to noise ratio of 3 and 10 based on lower-end calibration curve levels in real soil matrix (The detailed calculation procedure can be found in Figure S4.7). Method accuracy was evaluated at a spike level of 7.5 ng g^{-1} (n=3) by spiking cyanotoxins jointly with internal standard to Soil QC at the beginning of the preparation procedure. Intra-day was assessed likewise and corresponded to the coefficient of variation (CV%) of three replicates analyzed in the same work day. The analysis was repeated in two other work days and inter-day precision derived from the overall CV% (n=9).

4.3.8. Operating conditions of the UHPLC-MS/MS

A 1.5 mL aliquot of the filtered extract was introduced in a 2-mL amber glass liquid chromatography vial for analysis. Since a large volume (1.45 mL) of water-reconstituted cyanotoxins extracts was analyzed, we used on-line SPE for pre-concentration and purification. The method run time was 8 min per sample and followed these steps: 1) Sample loading step: the extract was introduced into the injection loop and transferred to the on-line SPE column for analyte preconcentration; 2) Matrix removal step: the on-line aqueous mobile phase continued to flow for 1 min after sample loading to ensure matrix and salt removal; 3)

Elution, separation, and detection: using the LC mobile phase, the analyte was eluted by back-flushing the on-line SPE column and separated by the chromatographic column prior to MS/MS detection.

On-line SPE was performed using an HTC thermopal autosampler for in-loop sample injection, a sample delivery system with a dual switching-column array, and an Accela 600 quaternary pump (Thermo Fisher, San Jose, CA, USA). The aqueous soil extract (1 mL injection volume) was loaded onto a Thermo Hypersil Gold aQ column (C18, 20 mm x 2 mm, 12 µm particle size) (SPE column) at a flow rate of 1000 µL min^{-1}. After sample loading, the on-line aqueous mobile phase was left to flow for an additional 1 min for matrix and salt removal. Analytes were then back-flush eluted to a chromatographic separation column (Thermo Hypersil Gold C18, 100 mm x 2.1 mm, 1.9 µm particle size) thermostated at 55 °C. The analytical mobile phase flowrate was set at 500 µL min^{-1}. A complex gradient was used to ensure resolution of anatoxin-a from its isobaric interference phenylalanine. Details on mobile phases and chromatographic gradient conditions are provided in the Supporting Information.

Analyte detection was performed with a TSQ Quantiva triple quadrupole mass spectrometer from Thermo Scientific (Waltham, MA, USA) operated in selected reaction monitoring (SRM) mode. The resolution of the first (Q1) and third (Q3) quadrupoles was set at 0.7 Da FWHM. The collision gas pressure (CID gas) was set at 2 mTorr. The dwell time was set at 20 ms, yielding typically 12 or more points per chromatographic peak. The analyzer was coupled to a heated electrospray ionization (heated-ESI) source. Optimized source parameters were as follows: sheath gas was set at 35 A.U. (arbitrary units), auxiliary gas at 20 A.U., sweep gas at 0 A.U., spray voltage at +3600 V, ion transfer tube temperature at 350°C, and vaporizer temperature at 400°C. Compound-dependent mass spectrometry parameters are provided in the Supporting Information.

4.4. Results and discussion

4.4.1. Optimizing the extraction method for cyanotoxins from soil

The performance of the seven extraction solvents (Figure 4.2) revealed that only 4 out of 17 targeted cyanotoxins (e.g. Anabaenopeptin-A, Microcystin-HilR, Microcystin-LA and Microcystin-LY) yielded acceptable performance with the solutions containing methanol with 10% water, methanol + NH_4OH (a weak base) and methanol + HCOOH (a weak acid). This was not unexpected, since Chen et al. (2006) found that a mixture of 0.1M ethylene-diamine tetraacetic acid (EDTA) – tetrasodium pyrophosphate ($Na_4P_2O_7$) improved the recovery of three microcystins from soil and sediment for HPLC-UV detection [33]. Since the direct injection of EDTA-containing extracts would interfere with mass spectrometry detection, we focused on the MS-compatible salt ammonium acetate to achieve the desired ion-pairing effects to enhance the extraction of cyanotoxins.

Ammonium acetate was tested as an additive in methanol, attempting to enhance the extraction efficiency for the difficult-to-extract cyanotoxins. Various concentrations (50, 100, 200 and 250 mM) of ammonium acetate in methanol were investigated. In most instances (15 out of 17 cyanotoxins), the relative response of targeted cyanotoxins increased concomitantly with ammonium acetate concentration. For instance, compared to non-amended solvent, the recovery of microcystin-RR increased 5-fold with 50 mM ammonium acetate, 8-fold with 100 mM ammonium acetate, and 12-fold with 200 mM ammonium acetate (Figure 4.2). The response only increased marginally at concentrations > 200 mM or showed decreasing trends. Of note, methanol without additive performed better for microcystin-LA and LY, but methanol with ammonium acetate gave an acceptable response. Therefore, methanol with 200 mM ammonium acetate was selected as the best compromise. Subsequently, we also tested the optimal parameters for extraction volume (Figure S4.4), the number of extraction cycles (Figure S4.5) and length of sonication (Figure S4.6). A higher solvent to soil ratio and

repeated extraction cycles can promote analyte extraction, but a compromise must be made to avoid unnecessary solvent consumption (and extended N_2 evaporation time at the following step). A similar comment applies to ultrasonication time, which must be sufficient to allow analyte desorption, but not long enough to create degradation artifacts, while maintaining an expeditious sample preparation process. Considering these factors, an acceptable compromise was achieved under the following conditions: methanol + 200 mM ammonium acetate as extraction solvent nature (Figure 4.2), a solvent to soil ratio of 4:1 (mL:g d.w.), two extraction cycles and 15 min ultrasonication time.

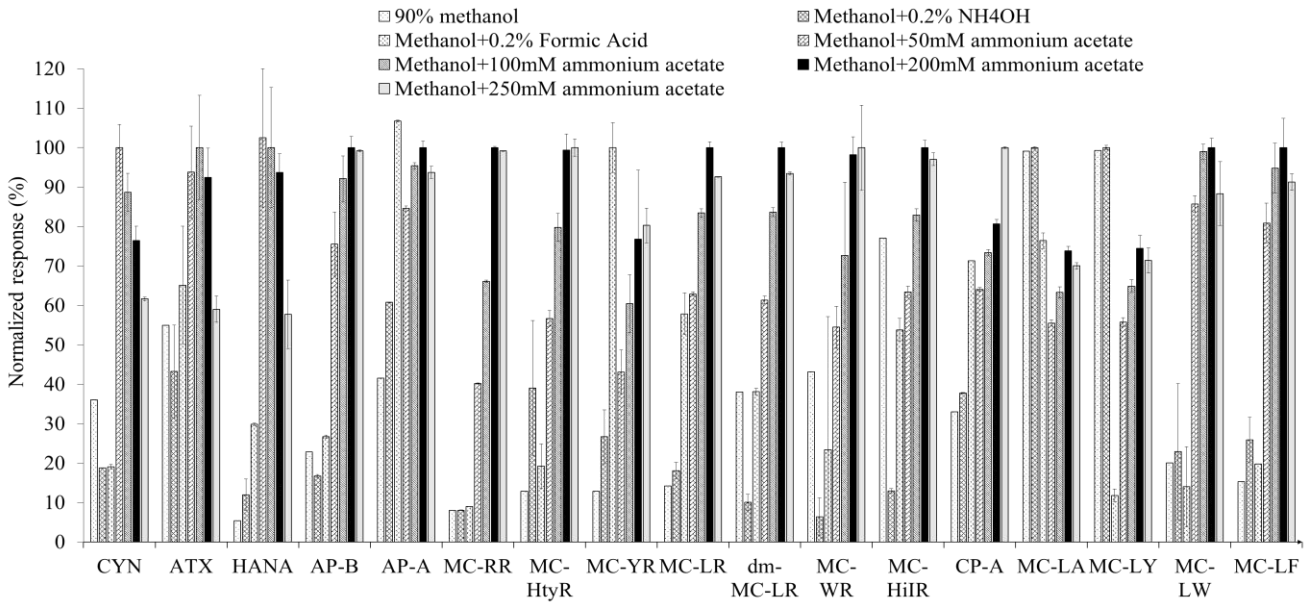


Figure 4.2: Cyanotoxin concentration in soil, expressed as the normalized response (%), as influenced by the extraction solvent nature. The test was done with Soil MO, described in Table S4.1. Error bars indicate standard deviations (n =3).

4.4.2. Recovery of cyanotoxins from soil

Fifteen cyanotoxins were extracted efficiently from spiked soil with recovery rates of 68% to 106% (Figure 4.3) and relative standard deviations of 5.9% on average and 23% maximum (Table 4.1). The two cyanotoxins with low extraction efficiency were cylindrospermopsin (recovery rate of 34% to 66%) and microcsytin-RR (recovery rate of

41% to 56%). Cylindrospermopsin is highly water-soluble and the charged areas within the molecule create a dipole effect, meaning that it should be extractable in a polar solvent such as methanol/water [40]. Since methanol is a mildly polar solvent, it is not completely efficient at desorbing cylindrospermopsin from the soil matrix. The microcystin-RR differs from other microcystin congeners due to the substitution of at least one guanidinium group in the arginine molecule. This probably increases the formation of cation bridges with the protonated arginine molecule, a well-known mechanism for binding proteins to clay and organic matter surfaces; in addition, the protonated arginine could attach to cation exchange sites or form additional hydrogen bonds with clay and organic matter components in soil [41-43]. However, the recovery rate for microcystin-RR with our extraction method is superior to another published method for cyanotoxins quantification in sediments, which achieved 20–39% recovery of microcystin-RR [44]. Therefore, we conclude that methanol + 200 mM ammonium acetate is the best option for multi-class extractant of cyanotoxins, including those that have strong binding affinity for the soil matrix.

Table 4.1: Coefficient of determination (R^2), linear range, limits of detection (LODs), limits of quantification (LOQs), accuracy (%), and precision (CV, %) for cyanotoxins extracted from soil with methanol + 200 mM ammonium acetate solution and quantified by on-line SPE-UHPLC-MS/MS. The identity and full name of each abbreviated cyanotoxin is provided in Table S4.2 and Figure S4.1. Chromatograms of the targeted cyanotoxins are provided in Figure S4.2.

Analyte	R^2	Linear range	LOD	LOQ	Accuracy	Intraday precision	Interday Precision
		(ng g^{-1})	(ng g^{-1})	(ng g^{-1})	(%)	(%)	(%)
CYN	0.9947	LOQ–20	0.02	0.07	103.8 ± 4.0	3.9	10.5
ATX	0.9986	LOQ–20	0.01	0.04	91.8 ± 2.2	2.4	14.4
HANA	0.9996	LOQ–20	0.2	0.6	96.8 ± 3.3	3.4	10.1
AP-B	0.9992	LOQ–20	0.001	0.003	110.1 ± 4.1	3.8	6.4
MC-RR	0.9918	LOQ–20	0.3	0.9	113.9 ± 4.3	3.8	3.5
AP-A	0.9994	LOQ–20	0.02	0.08	86.5 ± 6.1	6.3	7.4
MC-YR	0.9980	LOQ–20	0.03	0.1	110.4 ± 2.7	2.5	4.2
MC-HtyR	0.9997	LOQ–20	0.005	0.02	98.6 ± 5.6	5.6	5.0
MC-LR	0.9991	LOQ–20	0.02	0.08	106.8 ± 1.8	1.7	1.5
dm-MC-LR	0.9993	LOQ–10	0.004	0.01	104.8 ± 4.7	4.5	3.4
MC-WR	0.9982	LOQ–10	0.09	0.3	100.9 ± 4.9	4.8	4.5
MC-HilR	0.9996	LOQ–20	0.02	0.06	107.7 ± 4.7	4.4	6.0

CP-A	0.9941	LOQ–10	0.1	0.3	109.9 ± 1.0	0.9	1.9
MC-LA	0.9972	LOQ–10	0.004	0.01	102.9 ± 2.8	2.7	6.3
MC-LY	0.9961	LOQ–10	0.004	0.01	99.4 ± 3.3	3.4	7.4
MC-LW	0.9968	LOQ–10	0.01	0.03	103.9 ± 4.9	4.7	6.5
MC-LF	0.9951	LOQ–10	0.004	0.01	101.1 ± 3.1	3.1	12.9

The similar pattern of recoveries among five test soils indicates that methanol + 200 mM ammonium acetate is a suitable extractant for multiple soil types (Figure 4.3). The recoveries of microcystin-LR, for instance, were within a relatively narrow range (69%–80%) across soils that had from 1.4%–8.9% organic matter content. This implies that a single calibration curve, derived from a composite soil representing the study area, is suitable to quantify the concentrations of cyanotoxins in soils with different physicochemical characteristics. This study is the first to demonstrate that methanol + 200 mM ammonium acetate is a good multi-class extractant of cyanotoxins from soils, and suitable for purification and quantification by on-line SPE-UHPLC-MS/MS.

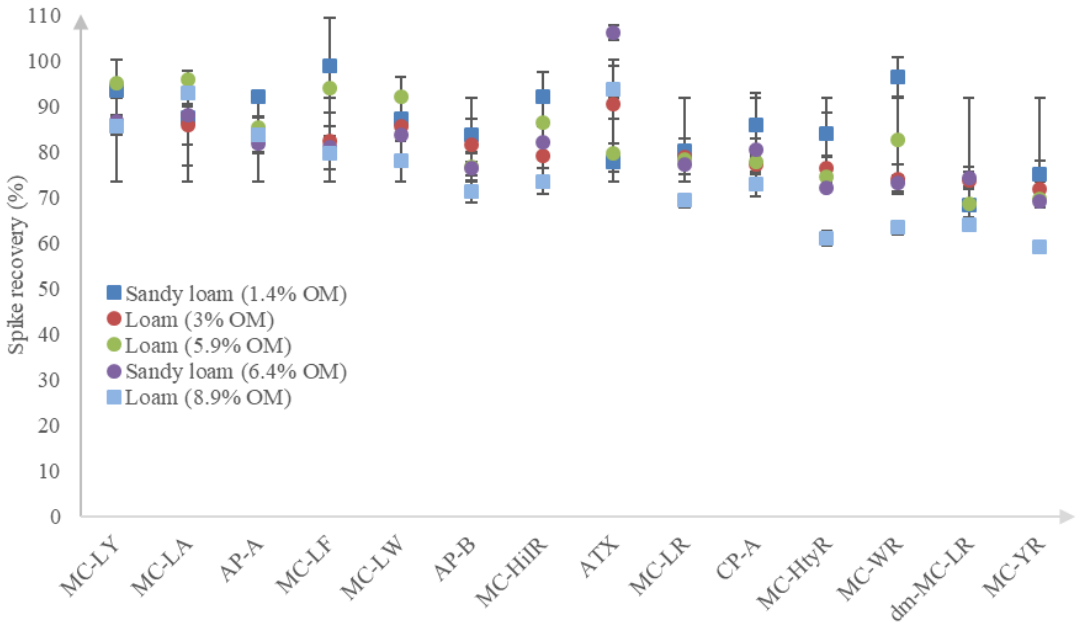


Figure 4.3: Recovery (%) of selected cyanotoxins extracted from five spiked soils with a range of texture and organic matter content. Error bars indicate standard deviations (n=3). Full results for 17 cyanotoxins investigated in this study, along with the identity of each abbreviated cyanotoxin, are provided in the supplemental Table S4.4.

4.4.3. Analytical method validation

Five- to nine-point calibration curves were fitted with linear regression with suitable coefficients of determination (R^2 range = 0.9918–0.9997) over a linear range that spanned 2–3 orders of magnitude (Table 4.2). The LODs for the optimized method were from 0.001–0.3 ng cyanotoxins g^{-1} soil (d.w.) while LOQs for the optimized method were 0.003–0.6 ng g^{-1} (Table 4.1). The accuracy of quality control spike to soil was between 86.5% and 110.4% for the 17 measured cyanotoxins. This falls within the acceptable range of accuracy based on U.S. EPA (70–130%) and European Commission (70–120%) requirements for analytical methods [45, 46]. Intra-day precision was <6.3% and inter-day precision <14.4% for the 17 cyanotoxins tested in this study; these values are within the recommended guideline (relative standard deviation <20%) for quantitative analysis of cyanotoxins [47].

Table 4.2: Concentrations (ng g-1) of cyanotoxins in soil from field sites in Québec, Canada. Cyanotoxins that were not detected in any soil (anabaenopeptins-A, cyanopeptolin-A, cylindrospermopsin and microcystin-RR, YR, WR) are not shown in the table. The chromatograms of several soil samples with positive detection of cyanotoxins are provided in Figure S4.3.

	Cyanotoxin										
Sample	ATX-A	HANA	AP-B	MC-HtyR	MC-LR	dm-MC-LR	MC-HiIR	MC-LA	MC-LY	MC-LW	MC-LF
Soil#1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#13	ND	ND	ND	0.032	ND	ND	ND	ND	ND	ND	ND
Soil#14	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soil#16	ND	ND	0.0038	ND	0.81	0.013	ND	0.077	0.55	0.097	0.16
Soil#17	ND	ND	0.099	ND	13.3	0.3	0.19	0.13	15.1	0.39	1.32
Soil#18	ND	ND	0.021	ND	0.78	0.0078	ND	0.078	1.03	0.1	0.18
Soil#19	ND	ND	0.011	ND	0.67	0.0055	ND	0.077	0.62	0.095	0.15
Soil#20	ND	ND	0.0058	ND	ND	ND	ND	ND	ND	ND	ND
Soil#21	ND	ND	0.0089	ND	0.023	ND	ND	ND	ND	ND	ND
Soil#22	11.8	10	0.12	ND	0.085	0.079	ND	1.28	ND	0.16	0.13

Soil#23	ND	ND	0.0017	ND	ND	ND	ND	ND	ND	ND	ND
Soil#24	ND	ND	0.0038	ND	ND	ND	ND	ND	ND	ND	ND
Soil#25	ND	ND	0.0028	ND	ND	ND	ND	ND	ND	ND	ND
Maximum Value (ng g^{-1})	11.8	10	0.12	0.032	13.3	0.3	0.19	1.28	15.1	0.39	1.32
LOD (ng g^{-1})	0.012	4.6	0.001	0.0055	0.023	0.0037	0.018	0.0038	0.0038	0.0097	0.0038

4.4.4. Application of the optimized method to quantify cyanotoxins in field soils

In contrast to field studies on cyanotoxins in aqueous and biological samples, there is a paucity of information regarding the presence of cyanotoxins in soils. Chen et al. (2006) optimized the extraction method of 3 microcystins (RR, LR and [Dha^7] LR) from soils and sediments [33]. In one surface soil collected from Lake Taihu, China, the reported concentration range was 0.28–1.69 µg microcystins g^{-1}. In another study, Manubolu et al. (2018) optimized the extraction methods for quantification of microcystin-LR and microcystin-RR in soils but did not find them at detectable levels in field samples [48], perhaps due to relatively high limits of detection (LODs >20 ng g^{-1}) compared to our method (LOD of 0.004 ng g^{-1} in this study).

Out of the 25 field-collected soil samples, 11 had at least one cyanotoxin. Six of these eleven soils (55%) had more than one cyanotoxin, and up to 8 cyanotoxins were detected in one soil. Among the 17 cyanotoxins included in this study, the most common in field soil were anabaenopeptin (10/25), microcystin-LR (6/25 samples), microcystin-LW, -LF, and dm-microcystin-LR (5/25), and microcystin-LY (4/25) (Table 4.2). Single samples also contained microcystin-HtyR and microcystin-HilR (Table 4.2). The sum of all microcystins congeners was in the range of 0.02 to 31 ng microcystins g^{-1} soil, and the dominant congeners were microcystin-LR and microcystin-LY (Table 4.2). This is consistent with other studies that found microcystin-LR commonly in Canadian environments [49-52]. Anatoxin-a, originally known as Very Fast Death Factor, was detected in one soil at a concentration of 11.8 ng anatoxin-a g^{-1} soil, and this soil also contained 10 ng homo-anatoxin-a g^{-1} soil (Table 4.2).

The cyanotoxins anabaenopeptins-A, cyanopeptolin-A, cylindrospermopsin and microcystin-RR, YR, WR were not detected in soil from this survey.

Anabaenopeptin was the most frequently detected cyanotoxin (10 out of 25 samples), but concentrations were below part-per-billion levels (0.002–0.12 ng anabaenopeptin g^{-1} soil; Table 4.2). While anabaenopeptin is detected in pure cultures of *Microcystis* and *Anabaena flos-aquae* and in biomass collected from cyanobacteria blooms and lake waters [53, 54], this is the first report of its detection in soil, to the best of our knowledge.

4.5. Conclusions

The cyanotoxins extraction method for soil is robust and reliable for 17 cyanotoxins from multiple classes (anatoxins, cylindrospermopsin, microcystins, anabaenopeptins, and cyanopeptolin). The key to good recovery of cyanotoxins from soil (recoveries were from 68%–106%) was to add ammonium acetate (200 mM) to methanol, presumably because this ionic solution promoted desorption of cyanotoxins from clay and organic matter in the soil. The extraction efficiency of methanol + 200 mM ammonium acetate was up to 90% better than with a conventional methanol: HPLC-grade water solution. Purification was achieved quickly by coupling the pre-concentration process for a large volume of analyte to the analytical system with the on-line SPE-UHPLC-MS/MS. The limits of detection with this method were improved by several orders of magnitude, compared to earlier work.

Our work on developing a quantification method for cyanotoxins in soil is timely, given the growing recognition of the human health risks from these substances. The One Health approach to public health requires quantification of all environmental sources of cyanotoxins, an emerging contaminant that is hazardous to humans and animals. Our extraction method with methanol + 200 mM ammonium acetate and rigorous quantification approach provide suitable accuracy and precision, within acceptance criteria, to meet these requirements for soil cyanotoxins. While the recovery of Microcystin-RR was reasonable, we should consider

other extraction strategies that could desorb more of this compound from the soil matrix. No commercially available soil reference exists for cyanotoxins, so we still need to confirm the suitability of the method for soil from other regions, possibly through interlaboratory comparisons of our method and other suitable methods. In conclusion, this work will advance our knowledge of cyanotoxins in soils and provide public health agencies with vital information to assess the linkages between human, animal and environmental health.

4.6. Supplementary Information

Tables

Table S4.1: Physicochemical characteristic of soils used for development and validation of the cyanotoxin extraction method.

Matrix	Sampling site	Latitude and longitude	Texture	Organic matter (g kg^{-1})	Clay (g kg^{-1})	Sand (g kg^{-1})	pH
Soil MO	Saint-François-Xavier-de-Brompton, Québec, Canada	45°30'46"N 72°01'54"W	silt loam	30	210	80	6.0
Soil MV_1	Thunder Bay, Ontario, Canada	Confidential	sandy loam	14	100	750	5.6
Soil MV_2	Emile A. Lods Agronomy Research Centre, Québec, Canada	45°25'33"N 73°55'51"W	loam	30	200	490	5.5
Soil MV_3	Sainte-Anne-de-Bellevue, McGill Mcdonald Campus, Québec, Canada	45°24'23"N 73°56'15"W	loam	59	250	450	7.4
Soil MV_4	Saint-François-Xavier-de-Brompton, Québec, Canada	45°30'33"N 72°01'53"W	sandy loam	64	50	680	6.3
Soil MV_5	Emile A. Lods Agronomy Research Centre, Québec, Canada	45°24'33"N 73°56'09"W	loam	89	140	460	6.6

Table S4.2: Compound dependent MS/MS parameters of the target cyanotoxins and internal standards analyzed by on-line purification liquid chromatography tandem mass spectrometry. The full name and chemical formula of each compound is presented in Figure S4.1.

Compounds	**Retention time (min)**	**Precursor mass (*m/z*)**	**RF lens (V)**	**Quantification fragment (*m/z*)**	**Collision energy (V)**	**Confirmation fragment (*m/z*)**	**Collision energy (V)**
CYN	2.25	416.1	82	193.9	37	336.1	22
ATX-a	2.26	166.1	42	149.0	14	131.1	17
HANA-a	2.40	180.2	48	163.1	14	145.1	17
AP-B	3.14	837.5	118	201.0	38	637.6	30
MC-RR	3.88	519.9	82	135.0	31	510.0	10
AP-A	3.93	844.5	95	663.3	25	637.4	26
MC-YR	4.06	1045.4	170	135.0	55	1017.3	47
MC-HtyR	4.07	1059.6	163	599.4	46	617.3	47
MC-LR	4.13	995.5	175	135.0	55	553.1	45
dm-MC-LR	4.14	981.5	138	135.0	55	539.2	47
MC-WR	4.19	1068.6	139	626.3	46	1040.6	44
MC-HiIR	4.21	1009.7	144	599.5	44	567.4	45
CP-A	4.37	957.5	100	939.6	29	726.5	39
MC-LA	5.26	910.5	95	776.3	18	402.1	24
MC-LY	5.39	1002.5	104	868.3	19	494.2	26
MC-LW	5.94	1025.5	111	517.2	26	891.3	20
MC-LF	6.06	986.5	101	852.3	18	478.2	25
CYN-$^{15}N_5$	2.25	421.1	89	197.1	34	-	-
NOD	4.03	825.4	121	135.0	54	-	-
MC-LR-$^{15}N_{10}$	4.13	1005.6	175	561.3	45	-	-

Table S4.3: Sampling locations of field soils collected between June and August, 2019 for the method application.

No.	Soil type	Sampling site	Latitude & longitude
Soil#1	Agriculture soil	Abitibi-Témiscamingue, Québec, Canada	48°20'04"N 78°17'32"W
Soil#2	Agriculture soil	Abitibi-Témiscamingue, Québec, Canada	48°24'14"N 78°20'57"W
Soil#3	Agriculture soil	Ste-Anne-de-Bellevue, Québec, Canada	45°24'33"N 73°56'09"W
Soil#4	Agriculture soil	Ste-Anne-de-Bellevue, Québec, Canada	45°24'33"N 73°56'09"W
Soil#5	Agriculture soil	Ste-Anne-de-Bellevue, Québec, Canada	45°25'33"N 73°55'51"W
Soil#6	Agriculture soil	Ste-Anne-de-Bellevue, Québec Canada	45°24'56"N 73°56'40"W
Soil#7	Agriculture soil	Saint-François-Xavier-de-Brompton, Québec, Canada	45°30'33"N 72°01'53"W
Soil#8	Agriculture soil	Saint-François-Xavier-de-Brompton, Québec, Canada	45°30'33"N 72°01'53"W
Soil#9	Orchard soil	Ulverton, Québec, Canada	45°43'24"N 72°14'17"W
Soil#10	Agriculture soil	Saint-François-Xavier-de-Brompton, Québec, Canada	45°31'27"N 71°59'14"W
Soil#11	Agriculture soil	Saint-François-Xavier-de-Brompton, Québec, Canada	45°30'33"N 72°01'53"W
Soil#12	Agriculture soil	Saint-François-Xavier-de-Brompton, Québec, Canada	45°30'33"N 72°01'53"W
Soil#13	Agriculture soil	Saint-François-Xavier-de-Brompton, Québec, Canada	45°30'33"N 72°01'53"W
Soil#14	Agriculture soil	Saint-François-Xavier-de-Brompton, Québec, Canada	45°30'33"N 72°01'53"W
Soil#15	Agriculture soil	Saint-François-Xavier-de-Brompton, Québec, Canada	45°30'33"N 72°01'53"W
Soil#16	Surface soil	Petit Lac St. François, Québec, Canada	45°32'12"N 72°02'41"W
Soil#17	Surface soil	Petit Lac St. François, Québec, Canada	45°32'12"N 72°02'37"W
Soil#18	Surface soil	Petit Lac St. François, Québec, Canada	45°32'13"N 72°02'35"W
Soil#19	Surface soil	Petit Lac St. François, Québec, Canada	45°32'14"N 72°02'37"W
Soil#20	Surface soil	Petit Lac St. François, Québec, Canada	45°32'10"N 72°02'37"W
Soil#21	Surface soil	Notre-Dame-de-l'Île-Perrot, Québec, Canada	45°23'38"N 73°53'52"W
Soil#22	Surface soil	Notre-Dame-de-l'Île-Perrot, Québec, Canada	45°22'48"N 73°53'45"W
Soil#23	Surface soil	Ste-Anne-de-Bellevue, Québec, Canada	45°24'27"N 73°57'25"W
Soil#24	Surface soil	Lac des Deux Montagnes, Québec, Canada	45°24'27"N 73°57'24"W
Soil#25	Surface soil	Lac des Deux Montagnes, Québec, Canada	45°24'16"N 73°56'13"W

Table S4.4: Cyanotoxin extraction recovery (%) in soils extracted with methanol + 200 mM ammonium acetate solution, detected by on-line purification liquid chromatography tandem mass spectrometry.

	Soil MV_1	Soil MV_2	Soil MV_3	Soil MV_4	Soil MV_5
Cyanotoxin	Recovery (%) ± Standard deviation (n=3)				
MC-LY	93 ± 13	86 ± 2	95 ± 7	87 ± 1	86 ± 2
MC-LA	87 ± 7	86 ± 4	96 ± 10	88 ± 3	93 ± 3
AP-A	92 ± 1	84 ± 3	85 ± 1	82 ± 2	84 ± 4
MC-LF	99 ± 9	82 ± 3	94 ± 10	81 ± 3	80 ± 3
MC-LW	87 ± 9	86 ± 2	92 ± 9	84 ± 4	78 ± 5
AP-B	84 ± 5	82 ± 2	77 ± 4	76 ± 2	71 ± 3
MC-HiIR	92 ± 6	79 ± 0.1	87 ± 5	82 ± 4	74 ± 3
ATX	78 ± 8	90 ± 9	80 ± 2	106 ± 2	94 ± 7
MC-LR	80 ± 6	79 ± 1	78 ± 3	77 ± 2	69 ± 2
CP-A	86 ± 13	77 ± 2	78 ± 7	80 ± 2	73 ± 3
MC-HtyR	84 ± 4	76 ± 2	74 ± 5	72 ± 1	61 ± 2
MC-WR	96 ± 5	74 ± 3	83 ± 4	73 ± 2	63 ± 2
dm-MC-LR	68 ± 5	74 ± 2	69 ± 4	74 ± 2	64 ± 2
MC-YR	75 ± 2	72 ± 2	70 ± 3	69 ± 1	59 ± 1
HANA	53 ± 8	62 ± 2	69 ± 8	74 ± 3	64 ± 1
MC-RR	51 ± 1	56 ± 2	42 ± 1	55 ± 2	41 ± 1
CYN	48 ± 34	48 ± 3	34 ± 2	66 ± 3	34 ± 2

Figures

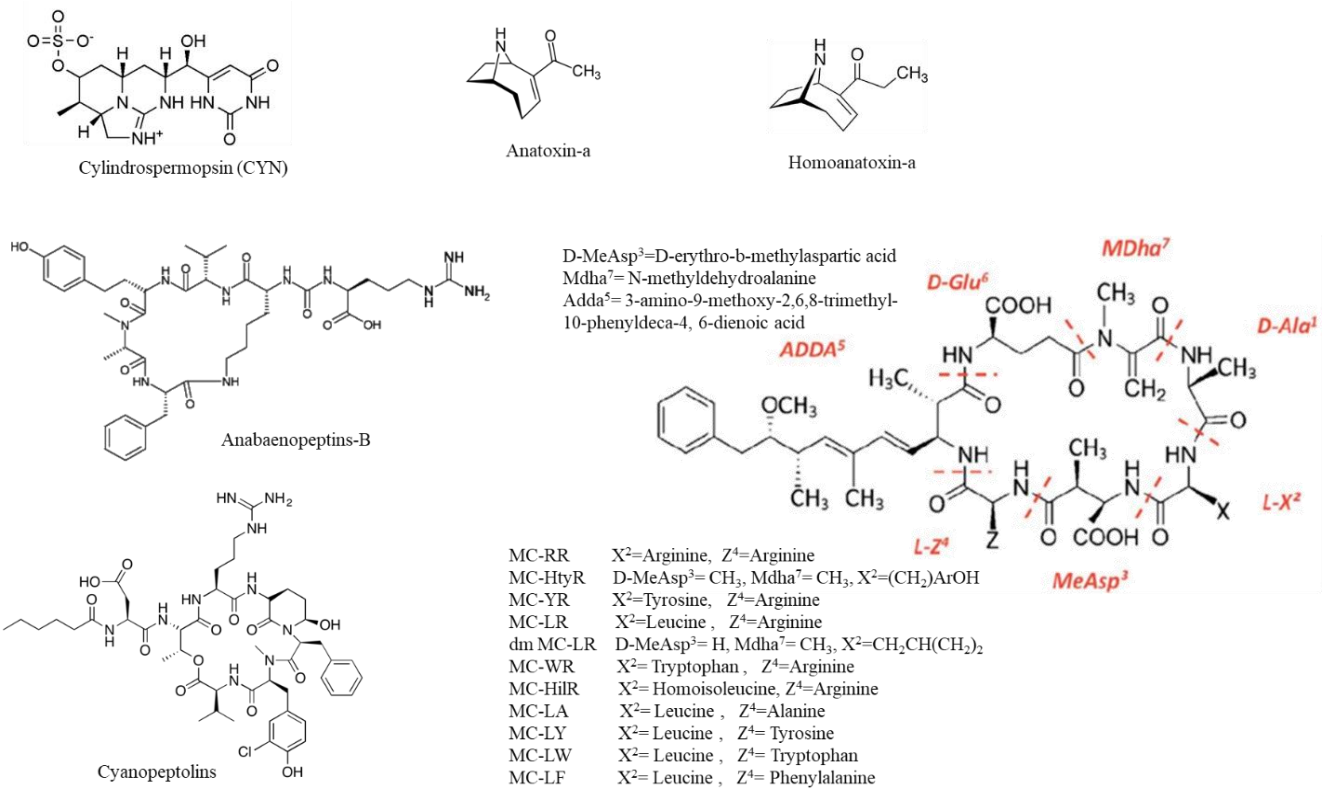


Figure S4.1: Structure of target cyanotoxins and standards that were detected by on-line purification liquid chromatography tandem mass spectrometry.

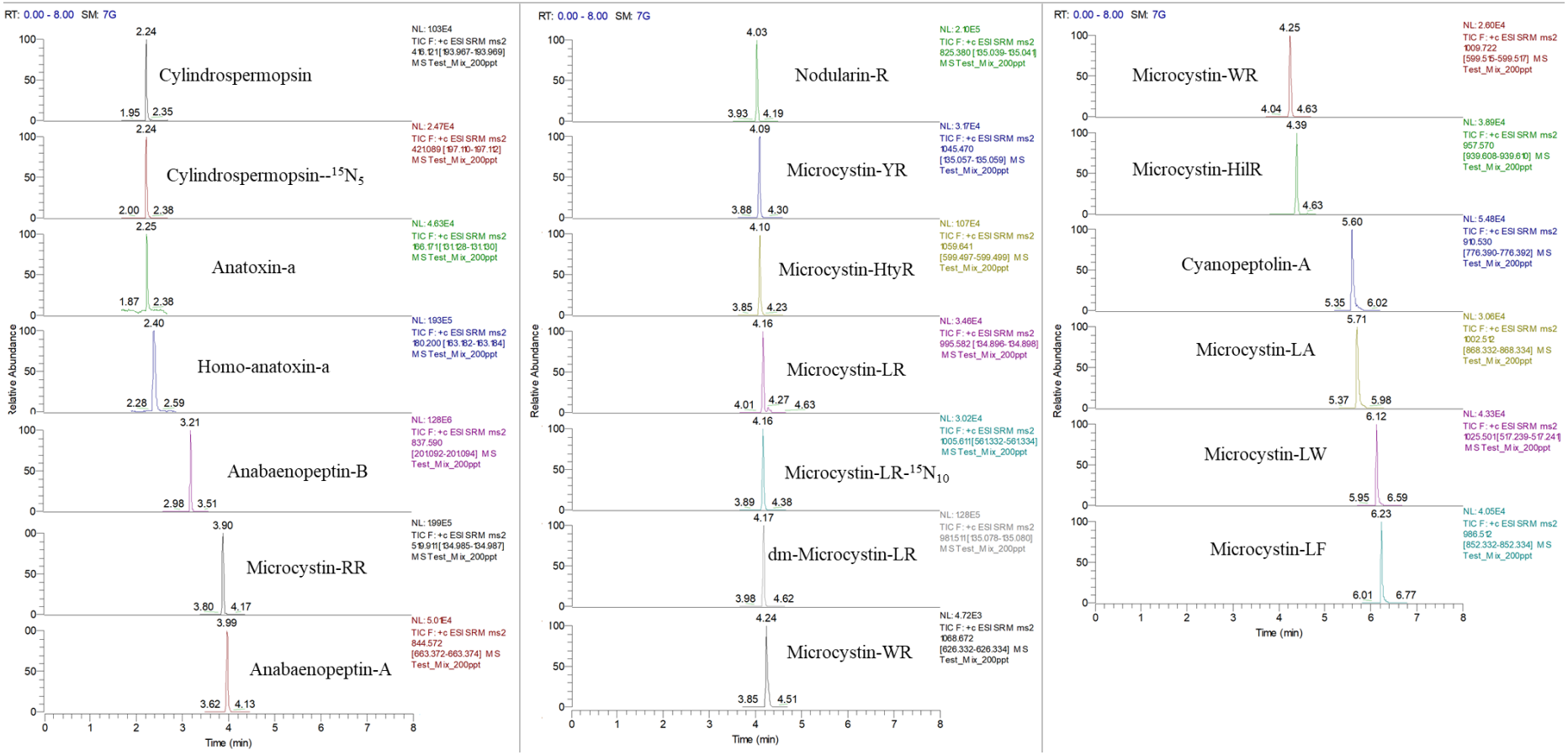


Figure S4.2: Chromatograms of the targeted cyanotoxins and internal standards obtained by on-line solid-phase extraction coupled with ultra-high-performance liquid chromatography tandem mass spectrometry. The x-axis shows the retention time (min) and the y-axis is the relative abundance. Peak intensity, precursor *m/z* and fragment ion *m/z* (quantification transition) are listed on the right of each chromatogram.

Sample #16

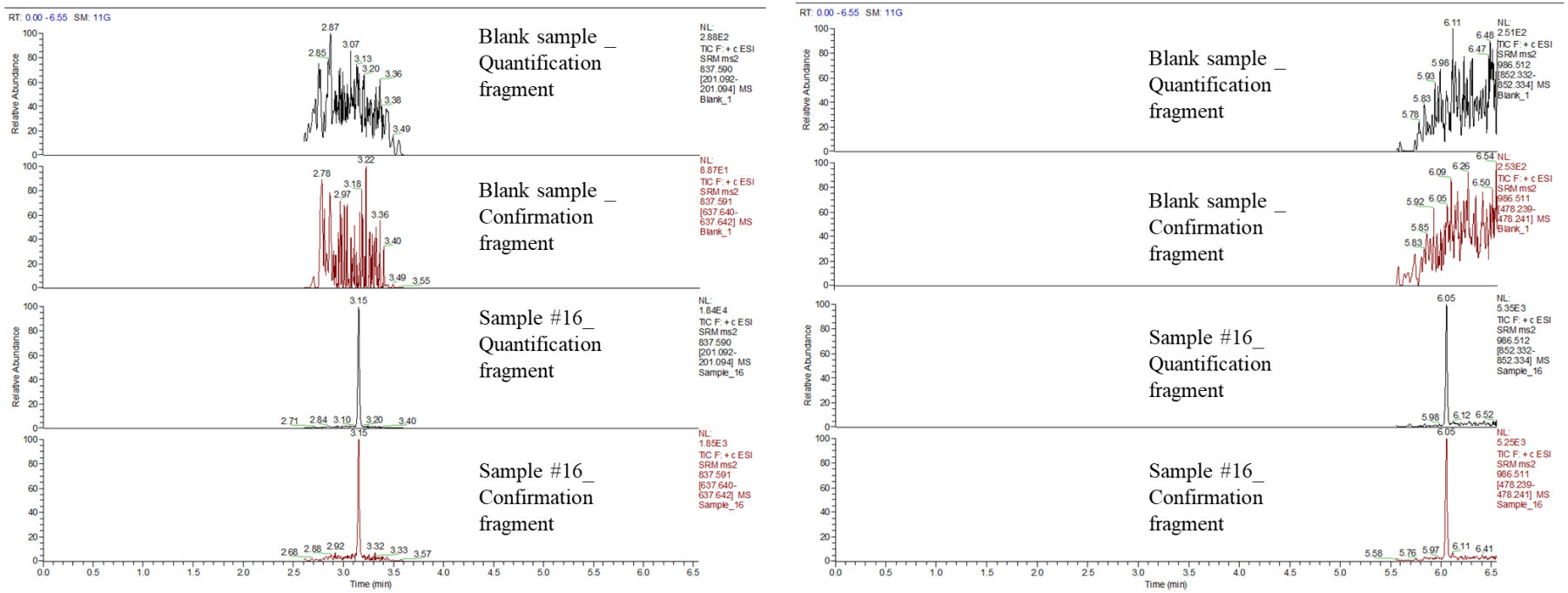


Figure S4.3a: Illustration of the chromatograms of cyanotoxins in field soil sample #16, obtained from on-line solid-phase extraction coupled with ultra-high-performance liquid chromatography tandem mass spectrometry. Both quantification (QT) and confirmation (CT) MS/MS transitions are shown. The top two panels show the low or non-detected signals in blanks, and the bottom panels show the detected signals in field soils. The x-axis shows the retention time (min) and the y-axis is the relative abundance. Peak intensity, precursor *m/z* and fragment ion *m/z* are listed on the right of each chromatogram.

Sample #17

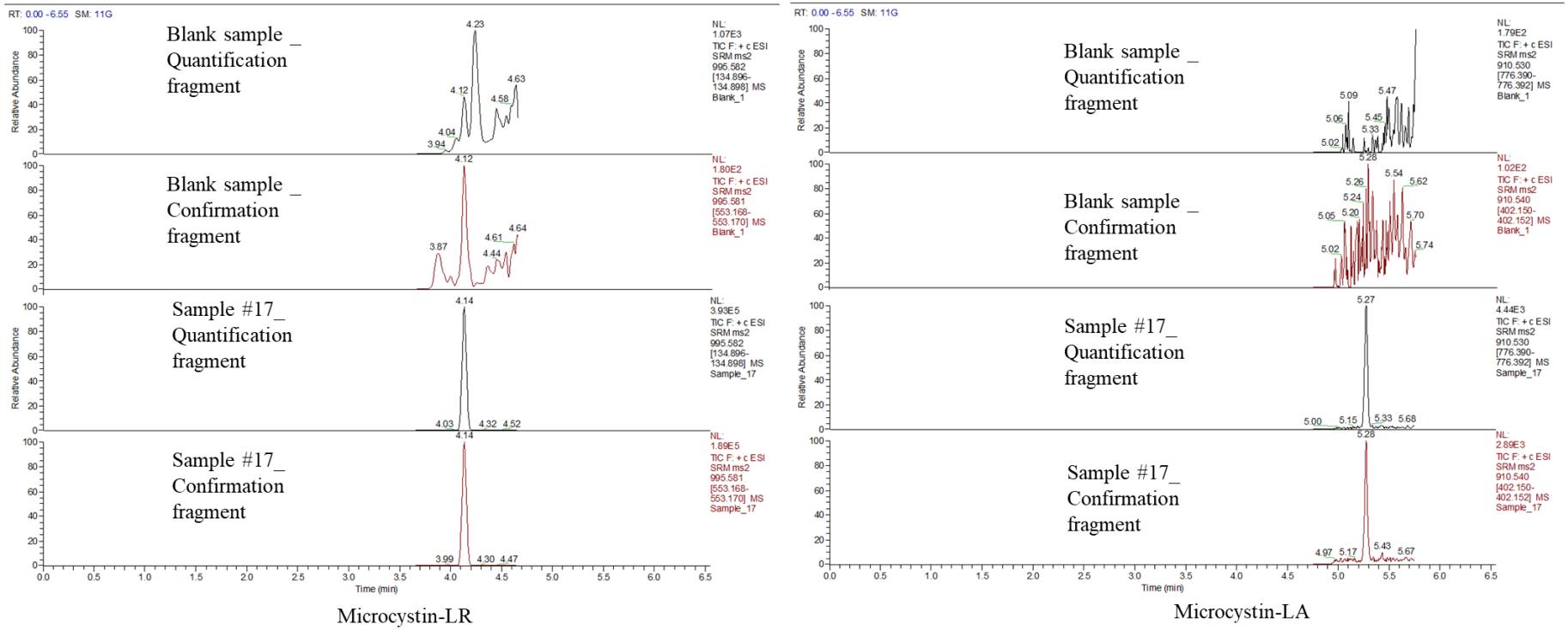


Figure S4.3b: Illustration of the chromatograms of cyanotoxins in field soil sample #17, obtained from on-line solid-phase extraction coupled with ultra-high-performance liquid chromatography tandem mass spectrometry. Both quantification (QT) and confirmation (CT) MS/MS transitions are shown. The top two panels show the low or non-detected signals in blanks, and the bottom panels show the detected signals in field soils. The x-axis shows the retention time (min) and the y-axis is the relative abundance. Peak intensity, precursor *m/z* and fragment ion *m/z* are listed on the right of each chromatogram.

Sample #18

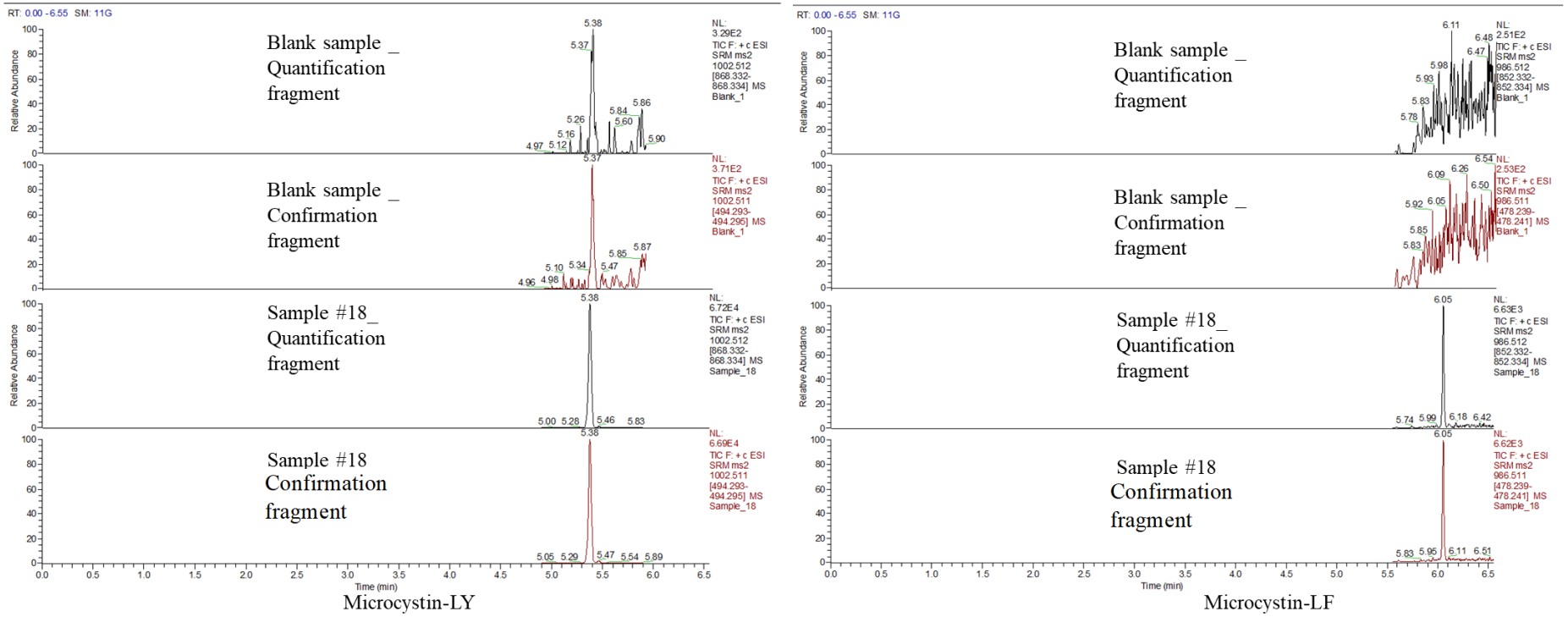


Figure S4.3c: Illustration of the chromatograms of cyanotoxins in field soil sample #18, obtained from on-line solid-phase extraction coupled with ultra-high-performance liquid chromatography tandem mass spectrometry. Both quantification (QT) and confirmation (CT) MS/MS transitions are shown. The top two panels show the low or non-detected signals in blanks, and the bottom panels show the detected signals in field soils. The x-axis shows the retention time (min) and the y-axis is the relative abundance. Peak intensity, precursor *m/z* and fragment ion *m/z* are listed on the right of each chromatogram.

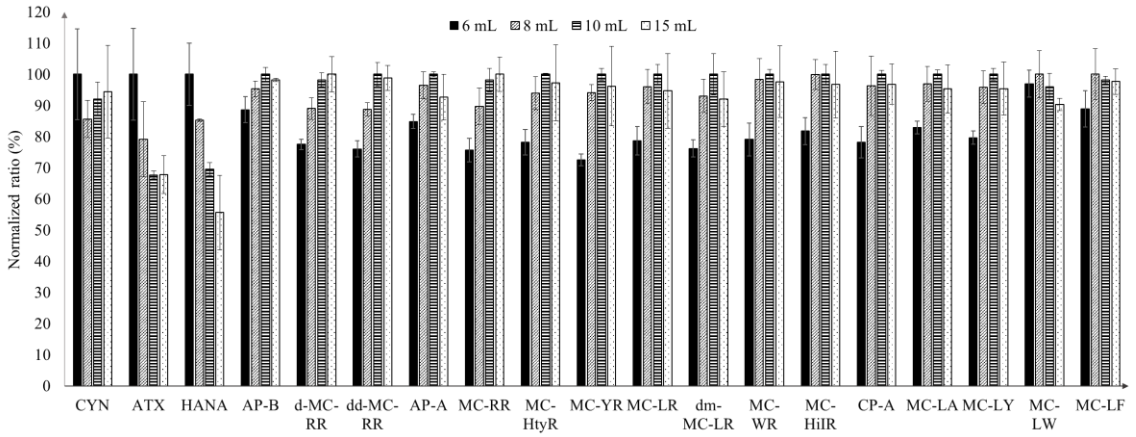


Figure S4.4: Cyanotoxin concentration in soil, expressed as the normalized response (%) after two cycles of soil extraction with 6 to 15 mL of methanol + 200 mM ammonium acetate solution. The test was done with soil MO, described in Table S4.1. Error bars are the standard deviation (n =3).

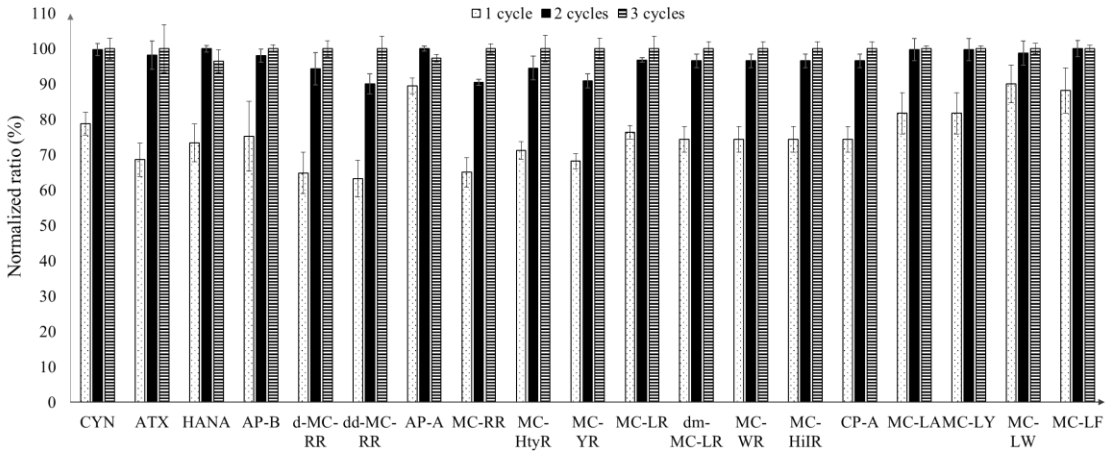


Figure S4.5: Cyanotoxin concentration in soil, expressed as the normalized response (%) when soils were extracted with one, two or three cycles methanol + 200 mM ammonium acetate solution (4 mL per cycle). The test was done with Soil MO, described in Table S4.1. Error bars are the standard deviation (n =3).

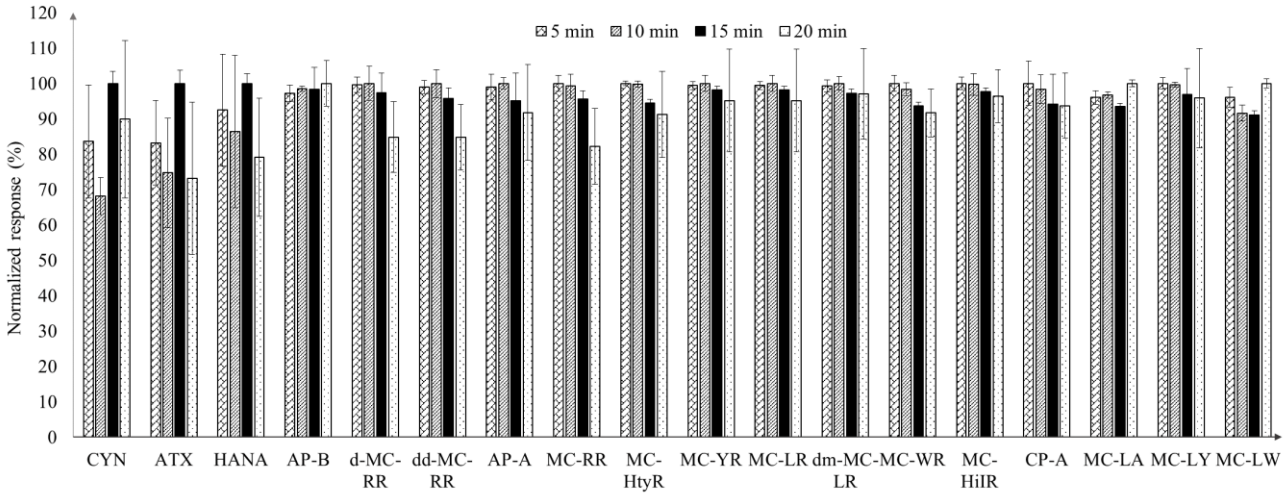
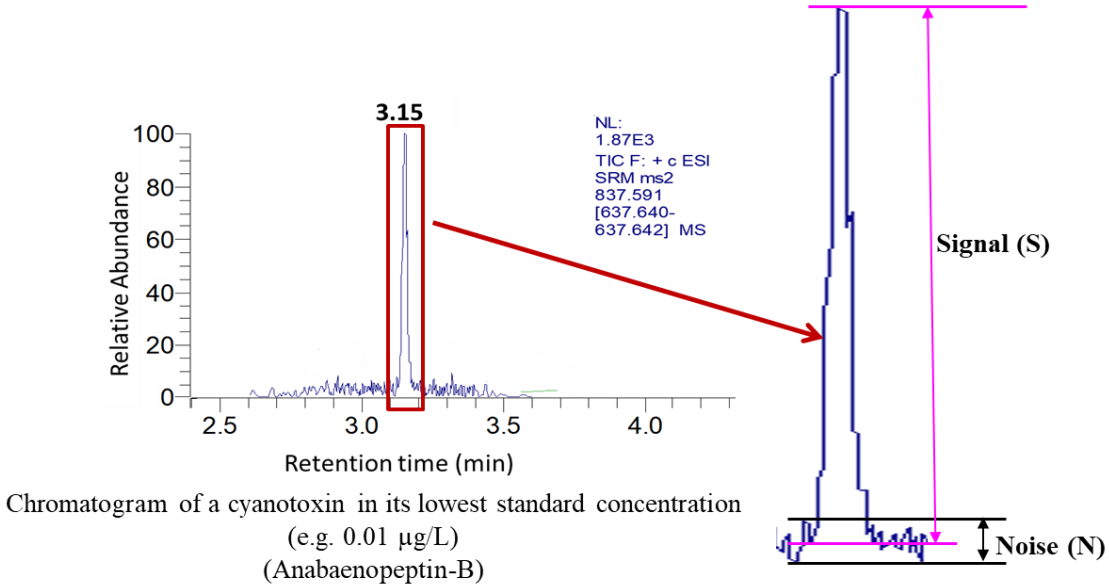


Figure S4.6: Cyanotoxin concentration in soil, expressed as the normalized response (%), as influenced by the duration of each sonication cycle (4 mL for each cycle, 2 extraction cycles using methanol + 200 mM ammonium acetate solution).The test was done with Soil MO, described in Table S4.1.Error bars indicate standard deviations (n =3).



Chromatogram of a cyanotoxin in its lowest standard concentration (e.g. 0.01 µg/L) (Anabaenopeptin-B)

$$\frac{\text{Lowest concentration (e.g.0.01 µg/L)}}{\textbf{Limit of Detection (µg/L)}} = \frac{\frac{S}{N}\,\text{value}}{3}$$

$$\textbf{Limit of Quantification} = \textbf{Limit of Detection} \times 3.3$$

Figure S4.7: The calculation of limit of detection and limit of quantification.

4.7. References

[1] J. Huisman, G.A. Codd, H.W. Paerl, B.W. Ibelings, J.M. Verspagen, P.M. Visser, Cyanobacterial blooms, Nat. Rev. Microbiol. 16 (2018) 471-483. https://doi.org/10.1038/s41579-018-0040-1.

[2] S. Merel, D. Walker, R. Chicana, S. Snyder, E. Baurès, O. Thomas, State of knowledge and concerns on cyanobacterial blooms and cyanotoxins, Environ. Int. 59 (2013) 303-327. https://doi.org/10.1016/j.envint.2013.06.013.

[3] Y. Zhang, J.K. Whalen, Production of the neurotoxin beta-N-methylamino-l-alanine may be triggered by agricultural nutrients: An emerging public health issue, Water Res. 170 (2019) 115335. https://doi.org/10.1016/j.watres.2019.115335.

[4] B. A. Neilan, L. A. Pearson, J. Muenchhoff, M. C. Moffitt, E. Dittmann, Environmental conditions that influence toxin biosynthesis in cyanobacteria, Environ. Microbiol. 15(2013) 1239-1253. https://doi:10.1111/j.1462-2920.2012.02729.x.

[5] E.M.L. Janssen, Cyanobacterial peptides beyond microcystins–A review on co-occurrence, toxicity, and challenges for risk assessment, Water Res. 151 (2019) 488-499. https://doi.org/10.1016/j.watres.2018.12.048.

[6] R.P. Rastogi, R.P. Sinha, A. Incharoensakdi, The cyanotoxin-microcystins: current overview, Rev. Environ. Sci. Bio. 13 (2014) 215-249. https://doi.org/10.1007/s11157-014-9334-6.

[7] R. Aráoz, J. Molgó, N. T. de Marsac, Neurotoxic cyanobacterial toxins, Toxicon 56 (2010) 813-828. https://doi.org/10.1016/j.toxicon.2009.07.036.

[8] E. Funari, E. Testai, Human health risk assessment related to cyanotoxins exposure, Crit Rev Toxicol. 38 (2008) 97-125. https://doi.org/10.1080/10408440701749454.

[9] T. Serrano, R. Dupas, E. Upegui, C. Buscail, C. Grimaldi, J.F. Viel, Geographical modeling of exposure risk to cyanobacteria for epidemiological purposes, Environ. Int. 81 (2015) 18-25. https://doi.org/10.1016/j.envint.2015.04.007.

[10] E. D. Hilborn, V. R. Beasley, One health and cyanobacteria in freshwater systems: Animal illnesses and deaths are sentinel events for human health risks, Toxins 7 (2015) 1374-1395. https://doi.org/10.3390/toxins7041374.

[11] F.R. Pick, Blooming algae: a Canadian perspective on the rise of toxic cyanobacteria, Can J Fish Aquat Sci. 73 (2016) 1149-1158. https://doi.org/10.1139/cjfas-2015-0470.

[12] J, Huisman, Codd. G. A., Paerl H. W., Ibelings B. W., Verspagen J. M., Visser P. M., Cyanobacterial blooms, Nat. Rev. Microbiol. 16 (2018) 471-483. https://doi.org/10.1038/s41579-018-0040-1.

[13] MDDEP, Portrait de la qualité des eaux de surface au Québec 1999 - 2008, Direction du suivi de l'état de l'environnement. Québec. http://www.mddep.gouv.qc.ca/eau/portrait/eaux-surface1999-2008/. Accessed February 9, 2012.

[14] R.G. Cloutier, M. Sanchez, Trophic status evaluation for 154 lakes in Quebec, Canada: monitoring and recommendations, Water Qual. Res. J. Can. 42(2007) 252-268. https://doi.org/10.2166/wqrj.2007.028.

[15] M.E. Monchamp, F. R. Pick, B. E. Beisner, R. Maranger, Nitrogen forms influence microcystin concentration and composition via changes in cyanobacterial community structure, PloS one 9(2014) e85573. https://doi.org/10.1371/journal.pone.0085573.
[16] W. Chen, Y. Jia, E. Li, S. Zhao, Q. Zhou, L. Liu, L. Song, Soil-based treatments of mechanically collected cyanobacterial blooms from Lake Taihu: efficiencies and potential risks, Environ. Sci. Technol. 46(2012) 13370-13376. https://doi.org/10.1021/es3027902.
[17] F. Eynard, M. Konstanze, J.L. Walther, Risk of cyanobacterial toxins in Riga waters (Latvia), Water Res. 34 (2000) 2979-2988. https://doi.org/10.1016/S0043-1354(00)00042-7.
[18] M. Bohunická, J. Mareš, P. Hrouzek, P. Urajová, M. Lukeš, J. Šmarda, J. Komárek, L.A. Gaysina, O. Strunecký, A combined morphological, ultrastructural, molecular, and biochemical study of the peculiar family Gomontiellaceae (Oscillatoriales) reveals a new cylindrospermopsin-producing clade of cyanobacteriaJ. Phycol. 51 (2015) 1040-1054. https://doi.org/10.1111/jpy.12354.
[19] E. Chrapusta, M. Węgrzyn, K. Zabaglo, A. Kaminski, M. Adamski, P. Wietrzyk, J. Bialczyk, Microcystins and anatoxin-a in Arctic biocrust cyanobacterial communities, Toxicon 101 (2015) 35-40. https://doi.org/10.1016/j.toxicon.2015.04.016.
[20] A.D. Chatziefthimiou, R. Richer, H. Rowles, J.T. Powell, J.S. Metcalf, Cyanotoxins as a potential cause of dog poisonings in desert environments, Vet. Rec. 174 (2014) 484-485. http://dx.doi.org/10.1136/vr.g3176.
[21] J. Metcalf, R. Richer, P. Cox, G. Codd, Cyanotoxins in desert environments may present a risk to human health, Sci. Total Environ. 421 (2012) 118-123. https://doi.org/10.1016/j.scitotenv.2012.01.053.
[22] A.D. Chatziefthimiou, J.S. Metcalf, W.B. Glover, S.A. Banack, S.R. Dargham, R.A. Richer, Cyanobacteria and cyanotoxins are present in drinking water impoundments and groundwater wells in desert environments, Toxicon 114 (2016) 75-84. https://doi.org/10.1016/j.toxicon.2016.02.016.
[23] L. Xiang, Y. Li, B. Liu, H. Zhao, H. Li, Q. Cai, C.H. Mo, M. Wong, Q. Li, High ecological and human health risks from microcystins in vegetable fields in southern China, Environ. Int. 133 (2019) 105142. https://doi.org/10.1016/j.envint.2019.105142.
[24] N. Bouaïcha, S. Corbel, Cyanobacterial Toxins Emerging Contaminants in Soils: A Review of Sources, Fate and Impacts on Ecosystems, Plants and Animal and Human Health, Soil Contamination-Current Consequences and Further Solutions (2016) 105. https://doi.org/ 10.5772/64940
[25] W. Chen, Y. Jia, E. Li, S. Zhao, Q. Zhou, L. Liu, L. Song, Soil-based treatments of mechanically collected cyanobacterial blooms from Lake Taihu: efficiencies and potential risks, Environ. Sci. Technol. 46 (2012) 13370-13376. https://doi.org/10.1021/es3027902.
[26] J.R. Crush, L.R. Briggs, J.M. Sprosen, S.N. Nichols, Effect of irrigation with lake water containing microcystins on microcystin content and growth of ryegrass, clover, rape, and lettuce, Environ Toxicol. 23 (2008) 246-252. https://doi.org/10.1002/tox.20331.

[27] S. Corbel, C. Mougin, N. Bouaïcha, Cyanobacterial toxins: modes of actions, fate in aquatic and soil ecosystems, phytotoxicity and bioaccumulation in agricultural crops, Chemosphere 96 (2014) 1-15. https://doi.org/10.1016/j.chemosphere.2013.07.056.
[28] S. Lee, X. Jiang, M. Manubolu, K. Riedl, S.A. Ludsin, J.F. Martin, J. Lee, Fresh produce and their soils accumulate cyanotoxins from irrigation water: Implications for public health and food security, Food Res. Int. 102 (2017) 234-245. https://doi.org/10.1016/j.foodres.2017.09.079.
[29] E. Levizou, G. Statiris, T. Papadimitriou, C.S. Laspidou, K.A. Kormas, Lettuce facing microcystins-rich irrigation water at different developmental stages: Effects on plant performance and microcystins bioaccumulation, Ecotox Environ Safe.143 (2017) 193-200. https://doi.org/10.1016/j.ecoenv.2017.05.037.
[30] J.R. Schmidt, S.W. Wilhelm, G.L. Boyer, The Fate of Microcystins in the Environment and Challenges for Monitoring, Toxins 6 (2014) 3354-3387. https://doi.org/10.3390/toxins6123354.
[31] X. Wu, B. Xiao, R. Li, C. Wang, J. Huang, Z. Wang, Mechanisms and factors affecting sorption of microcystins onto natural sediments, Environ. Sci. Technol. 45 (2011) 2641-2647. https://doi.org/10.1021/es103729m.
[32] M. Petrović, M.D. Hernando, M.S. Díaz-Cruz, D. Barceló, Liquid chromatography–tandem mass spectrometry for the analysis of pharmaceutical residues in environmental samples: a review, J. Chromatogr. A 1067 (2005) 1-14. https://doi.org/10.1016/j.chroma.2004.10.110.
[33] W. Chen, L. Li, N. Gan, L. Song, Optimization of an effective extraction procedure for the analysis of microcystins in soils and lake sediments, Environ. Pollut. 143 (2006) 241-246. https://doi.org/10.1016/j.envpol.2005.11.030.
[34] S. Corbel, N. Bouaïcha, C. Mougin, Dynamics of the toxic cyanobacterial microcystin-leucine-arginine peptide in agricultural soil, Environ. Chem. 12 (2014) 535-541. https://doi.org/10.1007/s10311-014-0482-2.
[35] P.G.J. de Maagd, A.J. Hendriks, W. Seinen, D.T. Sijm, pH-dependent hydrophobicity of the cyanobacteria toxin microcystin-LR, Water Res. 33 (1999) 677-680. https://doi.org/10.1016/S0043-1354(98)00258-9.
[36] J. McCord, J.R. Lang, D. Hill, M. Strynar, N. Chernoff, pH dependent octanol–water partitioning coefficients of microcystin congeners, J. Water Health. 16 (2018) 340-350. https://doi.org/10.2166/wh.2018.257.
[37] A. Zastepa, Fate and persistence of microcystin congeners in lakes and lake sediments, Université d'Ottawa/University of Ottawa (2014) 1-144. http://dx.doi.org/10.20381/ruor-4280.
[38] S. Klitzke, C. Beusch, J. Fastner, Sorption of the cyanobacterial toxins cylindrospermopsin and anatoxin-a to sediments, Water Res. 45 (2011) 1338-1346. https://doi.org/10.1016/j.watres.2010.10.019.
[39] G. Munoz, S.V. Duy, P. Labadie, F. Botta, H. Budzinski, F. Lestremau, J. Liu, S. Sauvé, Analysis of zwitterionic, cationic, and anionic poly- and perfluoroalkyl surfactants in sediments by liquid chromatography polarity-switching electrospray ionization coupled to high resolution mass spectrometry, Talanta 152 (2016) 447-456. https://doi.org/10.1016/j.talanta.2016.02.021.

[40] A.A. de la Cruz, A. Hiskia, T. Kaloudis, N. Chernoff, D. Hill, M.G. Antoniou, X. He, K. Loftin, K. O'Shea, C. Zhao, M. Pelaez, C. Han, T.J. Lynch, D.D. Dionysiou, A review on cylindrospermopsin: the global occurrence, detection, toxicity and degradation of a potent cyanotoxin, Environ. Sci. Process Impacts 15 (2013) 1979-2003. https://doi.org/10.1039/C3EM00353A.

[41] A.L. Pochodylo, T.G. Aoki, L. Aristilde, Adsorption mechanisms of microcystin variant conformations at water-mineral interfaces: A molecular modeling investigation, J Colloid Interface Sci. 480 (2016) 166-174. https://doi.org/10.1016/j.jcis.2016.07.016.

[42] M. Sathishkumar, S. Pavagadhi, K. Vijayaraghavan, R. Balasubramanian, S.L. Ong, Concomitant uptake of microcystin-LR and -RR by peat under various environmental conditions, Chem. Eng. 172 (2011) 754-762. https://doi.org/10.1016/j.cej.2011.06.058.

[43] X. Wu, B. Xiao, R. Li, C. Wang, J. Huang, Z. Wang, Mechanisms and factors affecting sorption of microcystins onto natural sediments, Environ. Sci. Technol. 45 (2011) 2641-2647. https://doi.org/10.1021/es103729m.

[44] P. Babica, J. Kohoutek, L. Bláha, O. Adamovský, B. Maršálek, Evaluation of extraction approaches linked to ELISA and HPLC for analyses of microcystin-LR,-RR and-YR in freshwater sediments with different organic material contents, Anal. Bioanal Chem. 385 (2006) 1545-1551. https://doi.org/10.1007/s00216-006-0545-8.

[45] US. EPA, (2015), EPA Method: 544, Determination of microcystins and nodularin in drinking water by solid phase extraction and liquid chromatography/tandem mass spectrometry (LC/MS/MS) (2015).

[46] European Commission, Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed, SANTE 11945 (2015). https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_2017-11813.pdf.

[47] J. Meriluoto, L. Spoof, G.A. Codd, Handbook of cyanobacterial monitoring and cyanotoxin analysis, John Wiley and Sons (2017) 107-195. https://doi.org/10.1002/9781119068761.

[48] M. Manubolu, J. Lee, K.M. Riedl, Z.X. Kua, L.P. Collart, S.A. Ludsin, Optimization of extraction methods for quantification of microcystin-LR and microcystin-RR in fish, vegetable, and soil matrices using UPLC-MS/MS, Harmful Algae 76 (2018) 47-57. https://doi.org/10.1016/j.hal.2018.04.009.

[49] B.G. Kotak, S.L. Kenefick, D.L. Fritz, C.G. Rousseaux, E.E. Prepas, S.E. Hrudey, Occurrence and toxicological evaluation of cyanobacterial toxins in Alberta lakes and farm dugouts, Water Res. 27 (1993) 495-506. https://doi.org/10.1016/0043-1354(93)90050-R.

[50] A. Rolland, D.F. Bird, A. Giani, Seasonal changes in composition of the cyanobacterial community and the occurrence of hepatotoxic blooms in the eastern townships, Québec, Canada, J. Plankton Res. 27 (2005) 683-694. https://doi.org/10.1093/plankt/fbi042.

[51] J.G. Winter, A.M. DeSellas, R. Fletcher, L. Heintsch, A. Morley, L. Nakamoto, K. Utsumi, Algal blooms in Ontario, Canada: increases in reports since 1994, Lake Reservoir Manag. 27 (2011) 107-114. https://doi.org/10.1080/07438141.2011.557765.

[52] F.R. Pick, Blooming algae: a Canadian perspective on the rise of toxic cyanobacteria, Can J Fish Aquat Sci. 73 (2016) 1149-1158. https://doi.org/10.1139/cjfas-2015-0470.
[53] A. Roy-Lachapelle, S.V. Duy, G. Munoz, Q.T. Dinh, E. Bahl, D.F. Simon, S. Sauvé, Analysis of multiclass cyanotoxins (microcystins, anabaenopeptins, cylindrospermopsin and anatoxins) in lake waters using on-line SPE liquid chromatography high-resolution Orbitrap mass spectrometry, Food Anal. Methods 11 (2019) 5289-5300. https://doi.org/10.1039/C9AY01132C.
[54] L. Tonk, M. Welker, J. Huisman, P.M.J.H.A. Visser, Production of cyanopeptolins, anabaenopeptins, and microcystins by the harmful cyanobacteria *Anabaena* 90 and *Microcystis* PCC 7806, Harmful Algae 8 (2009) 219-224. https://doi.org/10.1016/j.hal.2008.05.005.

FORWARD TO CHAPTER 5

In the previous chapters, I confirmed that cyanotoxins are present in agricultural soils and in soils that are adjacent to cyanotoxins-contaminated water. Farmers may add cyanotoxins to their agricultural fields unintentionally if they irrigate the field with cyanotoxins-contaminated water. Agricultural plants may bioaccumulate cyanotoxins, and the cyanotoxins in edible parts of these food crops may pose a risk for human consumers. Therefore, in Chapter 5, I conducted a formal meta-analysis to understand phytotoxicity and bioconcentration of microcystins in agricultural plants, and the associated human health risks. The meta-analysis had the following objectives: 1) to identify which source of microcystins is

most toxic to plants, 2) to determine which agricultural plants are most sensitive to microcystins phytotoxicity and are most likely to bioconcentrate microcystins, and 3) to evaluate the human health risks from ingesting microcystins in the edible parts of plants using a tolerable daily intake model.

CHAPTER 5

Phytotoxicity and bioconcentration of microcystins in agricultural plants: Meta-analysis and risk assessment

Yanyan Zhang[a], Joann K. Whalen[a], Sébastien Sauvé[b]

[a] McGill University, Department of Natural Resource Science, Macdonald Campus, 21,111 Lakeshore Road, Ste-Anne-de Bellevue, Quebec, Canada, H9X 3V9

[b] Université de Montréal, Department of Chemistry, C.P. 6128, Succursale Centre-Ville, Montreal, Quebec, Canada H3C 3J7

5.1. Abstract

Microcystins are cyanotoxins produced by many species of cyanobacteria. They are specific inhibitors of serine/threonine protein phosphatases and are phytotoxic to agricultural plants. This study used a formal meta-analysis to estimate the phytotoxicity and bioconcentration rates of agricultural plants exposed to microcystins, and the human health risk from consuming microcystin-contaminated plants. Among the 29 agricultural plants investigated, microcystins were most phytotoxic to durum wheat, corn, white mustard and garden cress. Leafy vegetables such as dill, parsley and cabbage could bioconcentrate ~3

times more microcystins in their edible parts than other agricultural plants. Although the human health risk from ingesting microcystins could be greater for leafy vegetables than other agricultural plants, further work is needed to confirm bioconcentration of microcystins in realistic water-soil-plant environments. Still, we should avoid growing leafy vegetables, durum wheat and corn on agricultural land that is irrigated with microcystins-contaminated water and be attentive to the risk of microcystins contamination in the agricultural food supply.

Key words: Microcystins phytotoxicity; Agricultural plants; Bioconcentration factor; Risk assessment; Meta-analysis

5.2. Introduction

Microcystins are cyanotoxins produced by cyanobacteria genera such as *Microcystis*, *Nostoc*, *Dolichospermum (*formerly *Anabaena)*, *Oscillatoria* and *Planktothrix* (Redouane et al., 2019). Chemically, microcystins are monocyclic heptapeptides with a general structure of cyclo-(-D-Ala1-L-X^2-DisoMeAsp^3-L-Z^4-Adda^5-D-isoGlu^6-Mdha^7), where D-MeAsp3 is D-erythro-b-methylaspartic acid, Mdha^7 is N-methyldehydroalanine and Adda^5 is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4, 6-dienoic acid. X^2 and Z^4 are substituted amino acids that vary according to the biosynthesis process (Rastogi et al., 2014), which results in at least 279 variants of microcystins in nature (Bouaïcha et al., 2019). Microcystins are a specific inhibitor of serine/threonine protein phosphatases that promote the growth of tumors and are responsible for animal poisoning and hepatotoxicity in humans (Carmichael et al., 2001; Pflugmacher et al., 2019; Xiang et al., 2020). Furthermore, microcystins are toxic to plants due to negative effects on plant physiological processes including: (1) tissue development; (2) activation of enzymes involved in CO_2 fixation; (3) starch storage; (4) gene expression; (5) regulation of ionic channels; (6) carbon and nitrogen metabolism; (7) hormone transport and (8) photosynthesis. In addition, microcystins can stimulate or inhibit mitosis, depending on the exposure dose (Machado et al., 2017).

Microcystins, as the most prevalent cyanotoxins, are commonly associated with harmful algal blooms of cyanobacteria in eutrophic freshwater ecosystems (Rastogi et al., 2014). In United States and Canada, around 80% of source- and treated-water samples contain microcystins and the dominant congener is microcystin-LR (Giddings et al., 2012; USEPA,

2015). Similar surveys from Europe report that microcystins comprise 60% of the detected cyanotoxins in freshwater (Greer et al., 2016). Microcystins concentrations in surface water are typically from 1–100 µg/L, although values up to 10,000 µg/L are reported (Corbel et al., 2014b; Rastogi et al., 2014; Xue et al., 2020). However, microcystins are not limited to eutrophic surface water—they are also detected in terrestrial environments that were irrigated or flooded with water containing harmful algal blooms (Liang and Liu, 2020; Petrou et al., 2020). In neutral environmental waters (pH 6.0~8.5), microcystins have a neutral or anionic charge (microcystins minus). Since this amphiphilic molecule (800 to 1100 Da) has hydrophobic groups bound to hydrophilic carboxyl and amine groups, it may form micelles and ion-pairs in solution. These water-soluble microcystins are bioavailable to plants, which absorb low molecular weight compounds from the soil pore water into the roots through symplastic and apoplastic pathways. The persistence of microcystins in solutions and in soils, where it remains as long as 56 d due to its cyclic structure (Bouaïcha and Corbel, 2016; Corbel et al., 2014b), is expected to have consequences for plants growing in these environments.

Plants exposed to microcystins exhibit phytotoxicity symptoms. These arise from an excess of reactive oxygen species, and manifest as change in the plant histology, cytology, and morphology resulting in lower growth and less biomass production (Cao et al., 2019a; Gu and Liang, 2020). Microcystins accumulation in plants is a time-, plant species- and concentration-dependent process (Hereman and Bittencourt-Oliveira Mdo, 2012; Jia et al., 2018; Wang et al., 2011). In general, microcystins are absorbed via the root system, then translocated to the leaves, shoots, flower, and fruit tissues (Corbel et al., 2015; El Khalloufi et al., 2012; Gutiérrez-Praena et al., 2014). Microcystins that bioconcentrate in edible parts of agricultural plants could be ingested by humans. Currently, more than 90% study associated with the concentration of

microcystins in edible parts of plants have exceeded the reference dose (0.04 μg/ kg/d) set by WHO for Microcystin-LR (Cao et al., 2018; Chen et al., 2018; Chia et al., 2019; Wijewickrama and Manage, 2019; Xiang et al., 2019). Therefore, the bioconcentration of microcystins in edible plants must be considered when assessing the human health risks from microcystins.

In this paper, we conducted a formal meta-analysis to estimate the effect of various factors known to influence the phytotoxicity and bioconcentration of microcystins in agricultural plants. The meta-analysis had the following objectives: 1) to evaluate the phytotoxicity of microcystins to plants under different growth conditions, 2) to identify which source of microcystins is most toxic to plants, 3) to determine which agricultural plants are most sensitive to microcystins phytotoxicity and which of those plants are most likely to bioconcentrate microcystins, and 4) to evaluate the human health risk from ingesting microcystins in the edible parts of plants using a tolerable daily intake model.

5.3. Materials and methods

5.3.1. Database

The dataset was compiled from peer-reviewed scientific articles published between 1996 and 2020 using the Scopus (Elsevier), Web of Science (Thomson Reuters) and Google Scholar (scholar.google.com) databases and keywords (TITLE-ABS-KEY (Microcystins OR hepatotoxin)) *AND (Microcystin* OR heptato*OR peptide toxin* OR cyano*) AND (*accumulation* OR *take OR assimila* OR expos*) AND (*toxicity OR inhibit* OR negative*(influen* OR effect OR impact* OR grow*) OR physiolog*change OR phyto*) and (agr* OR crop OR vegetable OR plant)*. We found 730 articles. We selected eligible articles as follows: (i) include an experiment that studied the effect of microcystins on agricultural plants; (ii) report at least one response variable for an agricultural plant; and (iii) provide information

about the control, replication, statistical analysis and standard error. We excluded articles that were retrieved more than once and articles that did not meet the criteria, based on the title, abstract or the content of the full article. This resulted in 62 papers that were eligible for meta-analysis. Figure data was digitized using data extraction software (GetData Graph Digitizer, v.2.26.0.20). Relevant data from each study included the sample size, types of agricultural plants, microcystin concentrations and variants, plant morphological indicators, plant physiological indicators, plant biochemical indicators, concentration of microcystins in plants, and standard error. Parameters extracted from eligible articles are summarized in Table 5.1. We converted standard deviation (SD) to standard error (SE) using SE = SD / n, where n is sample size.

Table 5.1: Agricultural plants, grouped by families, and the phytotoxicity response variables that were obtained from the 62 scientific papers used in this meta-analysis.

Family of agricultural plants (common name)

Brassicaceae	Leguminosae	Poaceae	Apiaceae	Solanaceae	Cucurbitaceae	Asteraceae	Amaranthaceae
Arabidopsis thaliana	Alfalfa	Corn	Carrot	Pepper	Cucumber	Lettuce	Spinach
Arugula	Clover	Durum wheat	Celery	Potato		Common sowthistle	Amaranthus hybridus
Broccoli	Common bean	Rice	Coriander	Tomato			
Chinese cabbage	Garden pea	Ryegrass	Dill	Eggplant			
Brassica oleracea	Lentil		Parsley				
Cabbage	Soybeans						
Garden cress	Faba bean						
White mustard							
Radish							
Rape							

Phytotoxicity indicators

Morphology	Antioxidant enzyme activity	Photosynthetic efficiency
50% inhibition of growth	Alpha tocopherol content	Net rate of photosynthesis
Diameter of cross section	Catalase activity	Total Chlorophyll content
Dry weight	Dehydroascorbate content	Chlorophyll a
Biomass	Delta tocopherol content	Chlorophyll b
Fresh weight	Gamma tocopherol content	Carotenoids
Germination percentage	Glutathione content	Leaf tissue transpiration
Germination rate	Glutathione disulphide content	Oxygen production
Epicotyl length	Glutathione peroxidase activity	Photosystem II activity
Nodules number	Glutathione reductase activity	Light use efficiency
Grain number	Glutathione Stransferase activity	
Plant height	Lipid peroxidation	
Leaf number	Nalondialdehyde levels	
Leaf length	Peroxidase assay activity	
Leaf area	Phenolic content	
Mean fresh weight	Polyphenoloxidase activity	
Necrosis	Superoxide dismutase assay activity	
Lateral root number	Total antioxidative status	
Primary root length	Relative malondialdehyde content	
Root elongation	$O_2^{\cdot -}$ content	
Root length	H_2O_2 content	
Seedling length		
Setting percentage (%)		
Shoot length		
Shoot elongation		
Total fresh weight % of control mean		
Relative growth rate		

5.3.2. Data pretreatment

We pre-grouped the data to explain the response of agricultural plants to microcystins and obtain the maximal in-group homogenization before meta-analysis. Based on our specific questions, we chose to subgroup the meta-analysis to test the influence of three covariates, i.e. (1). growth conditions of plant; (2). the source of microcystins; (3). the concentration of microcystins applied to the soil-plant system; and (4). the plant species and family. The growth conditions include hydroponics- and soil-plant systems under various growth substrate. The sources of microcystins were natural algal bloom extract, cultured *M. aeruginosa* (*Microcystis aeruginosa)* extract and commercial cyanotoxin standard. Microcystins concentration applied to the soil-plant system was grouped into nine categories: “very low” 0 – 1 µg/L, “low” 1 –10 µg/L, “medium low” 10 – 100 µg/L, “medium” 100 –500 µg/L, “medium high” 500 – 1000, “high” 1000 – 5000 µg/L, “very high” > 5000 µg/L. Agricultural plants were classified by families, namely the Brassicaceae, Leguminosae, Poaceae, Apiaceae, Solanaceae, Cucurbitaceae, Asteraceae and Amaranthaceae (Table 5.1). Phytotoxicity indicators were grouped into three classes, i.e. morphology, photosynthetic efficiency and antioxidant defense systems (Table 5.1). When the number of studies within each group was $n < 5$, the covariate was not calculated. Since most plant assays evaluate the response to a mixture of “total microcystins” rather than a specific congener of microcystins, the phytotoxicity results express the plant response to the “total microcystins”, which can be one or more congeners of microcystins.

5.3.3. Meta-analysis

We used a meta-analysis software package (OpenMEE and JASP for meta-analysis) to evaluate the phytotoxicity and bioconcentration rate of microcystins in agricultural plants. According to Hedges et al. (1998), the response ratio (RR) was the effect size:

$$\ln \mathrm{RR} = \ln\left(\frac{\bar{x}^T}{\bar{x}^C}\right) \qquad (1)$$

where $\bar{x}^T$ and $\bar{x}^C$ are the mean values for microcystins-treated and control groups, respectively. A value of ln RR < 0 indicates a negative (toxic) effect of microcystins while ln RR > 0 is a positive (stimulatory) effect of microcystins on agricultural plants.

We used the bioconcentration factor (BCF) to determine the ratio of the equilibrium concentration of microcystins in plants ($\bar{C}^P$) and the microcystin concentration in the external environment ($\bar{C}^W$), to indicate the capacity of plants to bioconcentrate microcystins. The response ratio of the bioconcentration (RR_{BCF}) is log-transformed as the effect size:

$$\ln \mathrm{RR}_{\mathrm{BCF}} = \ln\left(\frac{\bar{C}^P}{\bar{C}^W}\right)$$

The underlying true effect size is supposed to be heterogeneous when a range of study conditions and environmental factors are included in the meta-analysis, so we chose a random effects model, estimated with the DerSimonian-Laird estimator (DerSimonian and Laird, 2015) to calculate the effect size (Hedges and Vevea, 1998). To ensure a consistent metric of the variance relative to the sample size of each study, the mean effect size was based on data weighted by the inverse variance. We assessed the heterogeneity of the meta-analysis with the I^2 index, a measure of the percentage of total variability among effect sizes that can be explained by the between-studies heterogeneity. All estimates were reported along with 95% confidence intervals. Probability of significant differences (*p* value) between the microcystin treatment and control group were calculated with the Z statistic. In the graphs and tables, the log-transformed response ratios (ln RR) were back-transformed to the original scale to simplify the interpretation of summary outcomes. The exponentially-transformed effect size (ln RR) and confidence intervals (CI) were used to calculate the percentage change.

Percentage change % = [exp (ln RR or 95%CI)-1] ×100

5.3.4. Risk assessment

We conducted subgroup meta-analysis using data associated with plant parts and plant species. We used the natural log bioconcentration factor (ln RR_{BCF}) to determine the effect size by referring to the bioconcentration factor proposed by European Union and US Environmental Protection Agency (Arnot and Gobas, 2006). An ln RR_{BCF} between 0–3 indicates that the plant had "Low" microcystins bioconcentration. We assumed a "Moderate" microcystins bioconcentration when ln RR_{BCF} is 3–3.7 and "High" microcystins bioconcentration when ln RR_{BCF} is > 3.7. When ln RR_{BCF} is less than 0 (referred as "No" microcystins bioconcentration), the microcystin concentration was probably diluted because biomass gain was greater than microcystin bioconcentration, although we cannot rule out the possibility that microcystins were depurated within the plant tissues (Romero-Oliva et al., 2015).

We used the tolerable daily intake risk assessment model of the World Health Organization to quantitatively assess the risk to adults and children that ingest microcystins present in the edible part of agricultural plants (Chorus and Bartram, 1999). The estimated daily intake (EDI: µg/ kg body weight/day) was calculated as follows:

$$\text{Estimated Daily Intake (EDI)} = \frac{C_{MC} \text{ X } D_{intake}}{bw}$$

where C_{MC} is an average microcystin-LR equivalent concentrations in agricultural plants (µg/kg wet weight); D_{intake} is the daily consumption of the edible part of an agricultural plant (g/day), which is based on standards published by the United States Food and Drug Administration (US FDA 2015a) and bw is the average body weight (adult: 60 kg, child: 25 kg). Daily intake of the edible part of the agricultural plants was calculated for 11 plant species (Table S5.1). Dry vegetable weight was converted to fresh weight with a factor of 1.085 (Agriculture, 2007; Rattan

et al., 2005), and total microcystins were converted to equivalent concentrations of microcystin-LR according to the LD_{50} of microcystin congeners (Table S5.3) (Chen et al., 2018; Wolf and Frank, 2002). The transformation was based on the following equation:

$$C_{MC\text{-}LR\ equiv.} = C_{MCs} / (TEF_{MC\text{-}1} + TEF_{MC\text{-}2} + \ldots TEF_{MC\text{-}i})$$

where $C_{MC\text{-}LR\ equiv.}$ is total equivalent concentrations of Microcystin-LR; C_{MCs} is detected total microcystins concentration in edible parts of plant, and $TEF_{MC\text{-}i}$ is toxicity equivalent factor (TEF) of individual congener of microcystins that reported in the study. The toxicity equivalent factor of individual congener can be found in Table S5.3.

Then, we compared EDI values with the Chronic reference Dose value (RfD, 0.003 μg/kg/d) and the World Health Organization value (WHO, 0.04 μg/kg/d). The following ranking criteria were set, i.e. low risk (EDI ≤ 0.003), moderate health risk (0.003 < EDI ≤ 0.04) and high health risk (EDI > 0.04).

5.4. Results

5.4.1. Phytotoxic effects of microcystins in agricultural plants

5.4.1.1. Heterogeneity in the agricultural plant responses to microcystins

Globally, microcystins were phytotoxic to agricultural plants, which decreased the morphological responses by an average of 57% ($P < 0.001$), reduced photosynthetic efficiency by an average of 61% ($P < 0.001$) and stimulated the mean antioxidant enzyme activity by 90% ($P < 0.001$) (Fig 5.1.). Results were heterogeneous ($I^2 > 98\%$; Table S5.2), which reflects the high variability within each study due to experimental errors, analytical errors and inconsistent biological responses (intra- and inter-specific variation).

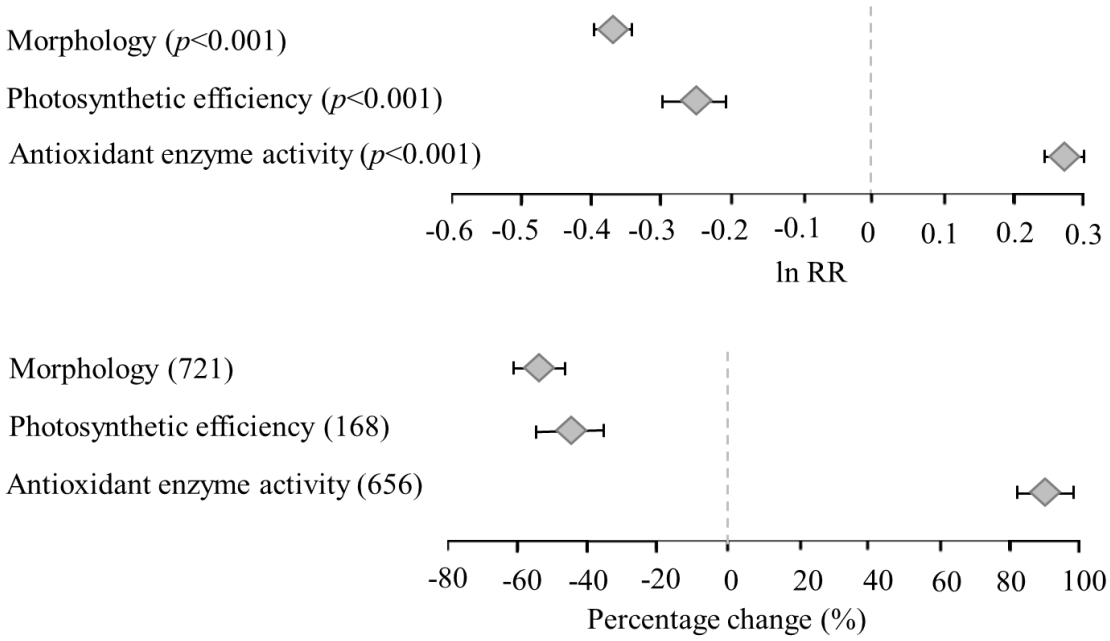


Figure 5.1: Global effects of microcystins on the morphology, photosynthetic efficiency and antioxidant enzyme activity of agricultural plants in the meta-analysis (n=62 studies). Cumulative effect size was weighted by the inverse of the pooled variance. The number of observations is given in parentheses beside each plant response category (variables considered within each response category are described in Table 5.1). Error bars are the 95% confidence intervals.

5.4.1.2. Phytotoxicity effects in hydroponics- and soil-plant systems

Microcystins phytotoxicity depends on the plant growth conditions, with greater phytotoxicity in the hydroponics-plant system than the soil-plant system (Fig. 5.2). Morphological parameters were 63% less in the hydroponics-plant system with microcystins, and the same parameters were 45% lower when plants were exposed to microcystins in the soil-plant system. Microcystins inhibited photosynthetic efficiency by 57% in the hydroponics-plant system, but only by 31% in the soil-plant system. Antioxidant enzyme activity was stimulated by

192% after exposure to microcystins in the hydroponics-plant system, with ~3 times lower response (73% simulation) when plants were grown in the soil-plant system.

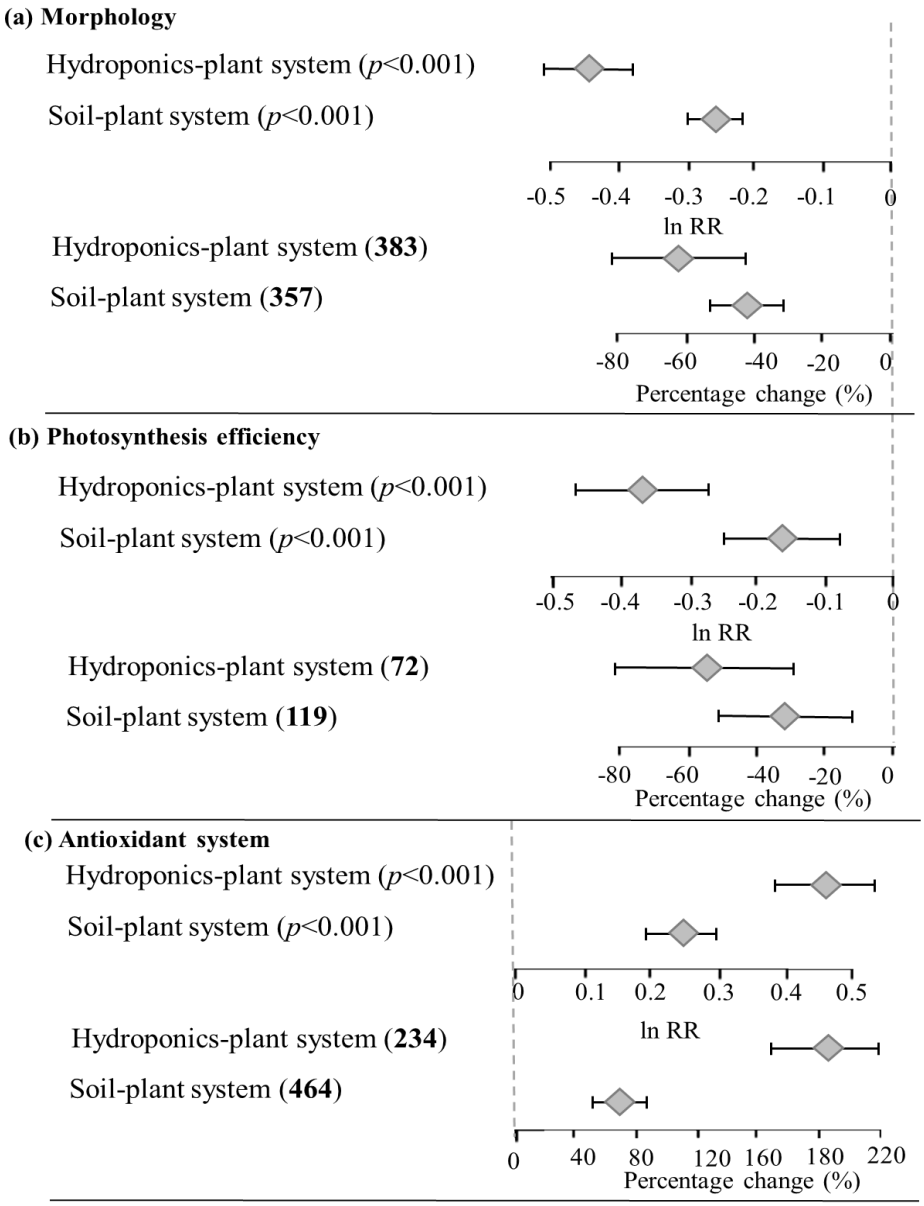


Figure 5.2: Global effects (ln RR and percentage change) of microcystins on the morphology (a), photosynthetic efficiency (b) and antioxidant enzyme activity (c) when agricultural plants were grown in hydroponics- and soil-plant systems. Cumulative effect size was weighted by the inverse of the pooled variance. The number of observations (**n**) is in bold within parentheses.

Error bars are the 95% confidence intervals. Percentage change is significantly different from the control at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Growth substrates also affected the phytotoxicity of microcystins (Fig. 5.3). Plants grown in culture medium had the most phytotoxic response, based on the reduction of morphological properties (culture medium substrate (76%) > vermiculite substrate (67%) > paper substrate (65%) > water substrate (57%) > sand substrate (47%) > soil substrate (44%) > soil:perlite 1:1 substrate (23%), and inhibition of photosynthetic efficiency (culture medium substrate (75%) > paper substrate (61%) > water substrate (38%) > sand substrate (33%) > pinus bark and vermiculite substrate (29%) > soil substrate (15%) > soil:perlite 1:1 substrate (5%)). Plants grown in culture medium also responded to microcystins exposure with greater antioxidant enzyme activity (culture medium substrate (426%) > water substrate (224%) > sand substrate (120%) > pinus bark and vermiculite substrate (66%)).

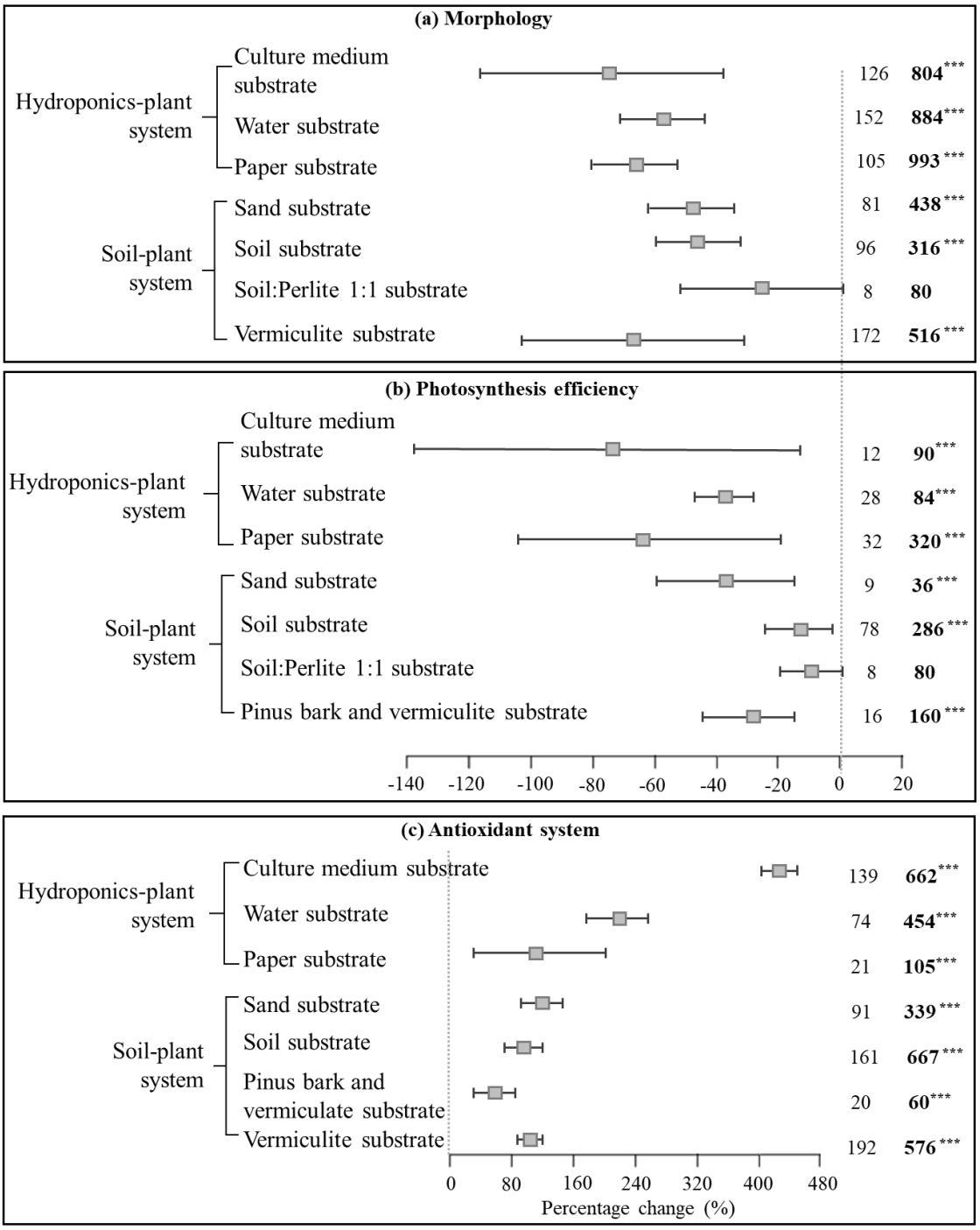


Figure 5.3: Global effects (percentage change) of microcystins on the morphology, photosynthetic efficiency and antioxidant enzyme activity in the meta-data associated with the phytotoxicity of microcystins to agricultural plant grown in different substrates in hydroponics- and soil- plant systems. The square indicates the overall mean percentage change (%) of

morphology, photosynthetic efficiency and antioxidant enzyme activity. The phytotoxicity of microcystins on morphology, photosynthetic efficiency and antioxidant enzyme activity is significant at $p \leq 0.05$. Error bars are the 95% confidence intervals. Percentage change is significantly different from the control at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p<0.001$.

5.4.1.3. Phytotoxicity of microcystins sources

Phytotoxicity depends on the source of microcystins applied in controlled studies. Agricultural plant morphology was affected more by exposure to microcystins from natural bloom extract > cultured *M. aeruginosa* extract > commercial cyanotoxin standard. Natural bloom extract decreased the morphological parameters by 61%, while the cultured *M. aeruginosa* extract reduced these parameters by 54% and the commercial cyanotoxin standard by 26%, on average (Fig. 5.4). Natural bloom extract inhibited photosynthetic efficiency by 75%, while cultured *M. aeruginosa* extract inhibited photosynthetic efficiency by 28% (Fig. 5.4). Antioxidant enzyme activity was stimulated by 115% when exposed to nature bloom extract and by 95% when agricultural plants were grown in the presence of cultured *M. aeruginosa* extract (Fig. 5.4).

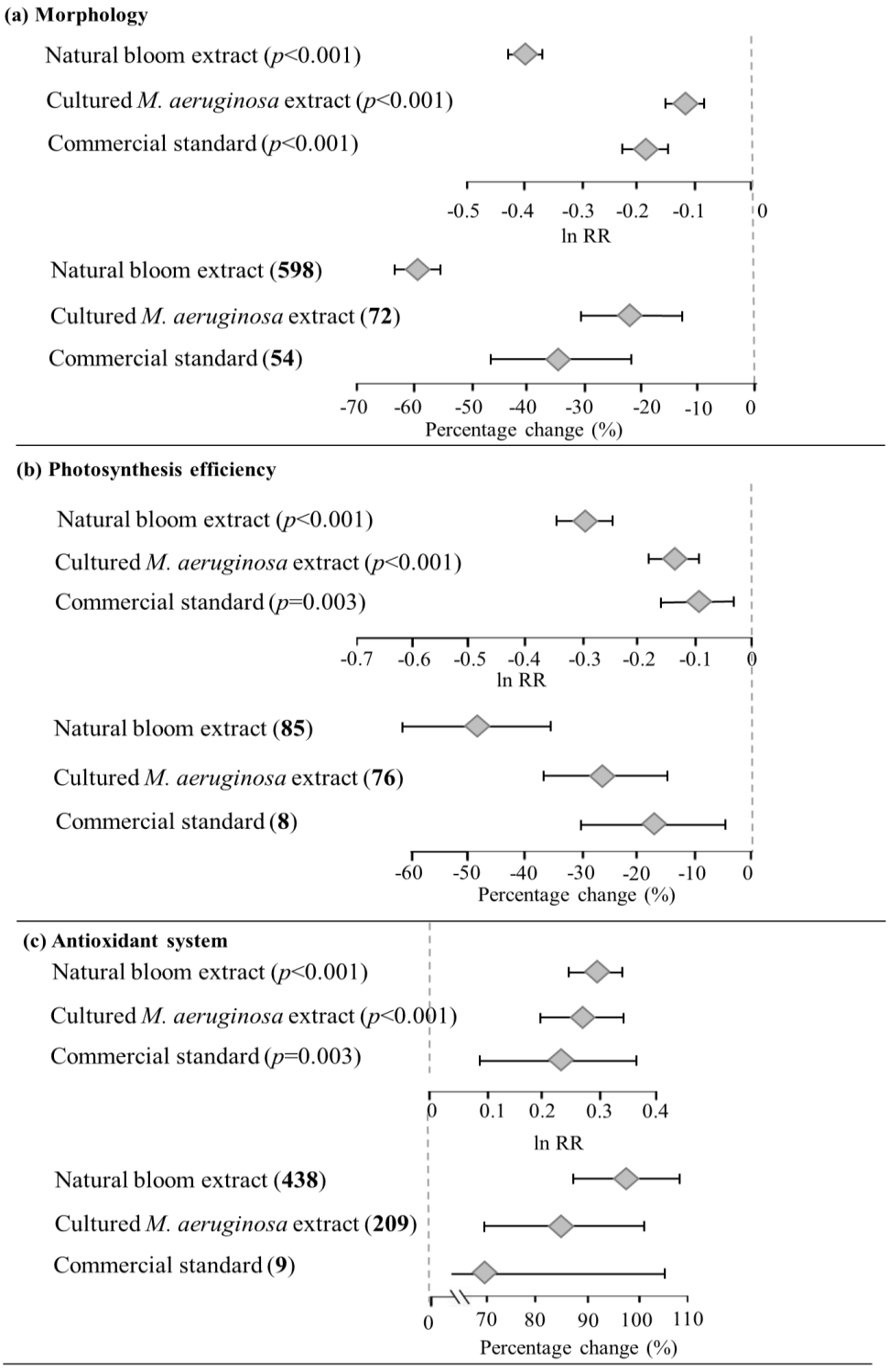


Figure 5.4: Global effects (ln RR and percentage change) of microcystins on the morphology (a), photosynthetic efficiency (b) and antioxidant enzyme activity (c) in the meta-data associated with the phytotoxicity of microcystins sources to agricultural plants (n=62 studies). Cumulative

effect size was weighted by the inverse of the pooled variance. The number of included observations is shown between parentheses in bold; Error bars are the 95% confidence intervals.

Phytotoxicity is affected by the concentration of microcystins applied to the soil-plant system and tends to follow a dose-response curve. Increasing the microcystins concentration in the soil-plant system significantly reduced the morphology integrity, diminished the photosynthetic efficiency and boosted the antioxidant enzyme activity of agricultural plants (Fig. 5.5). Phytotoxic effects of microcystins on morphological indicators decreased from 19 % to 92% when the microcystin concentration increased from <1 to >10,000 µg total microcystins/L, and the effect was negatively related to a greater concentration of microcystins (R^2=0.9). Photosynthetic efficiency of agricultural plants was moderately affected by microcystin concentrations of 100 – 500 µg total microcystins/L, but was reduced by 79% when the microcystins concentration exceeded >10,000 µg total microcystins/L (R^2=0.6). The greatest stimulation of 349% change in antioxidant enzyme activities occurred when agricultural plants were exposed to 1,000 – 5,000 µg total microcystins/L (R^2=0.7). The results must be interpreted with caution because ~77% of the plants exposed to microcystins were grown in hydroponic systems at non-environmental relevant concentration rather than a soil-based environment, which may exaggerate and skew a bit the realistic toxicity of different concentration of microcystins to plants.

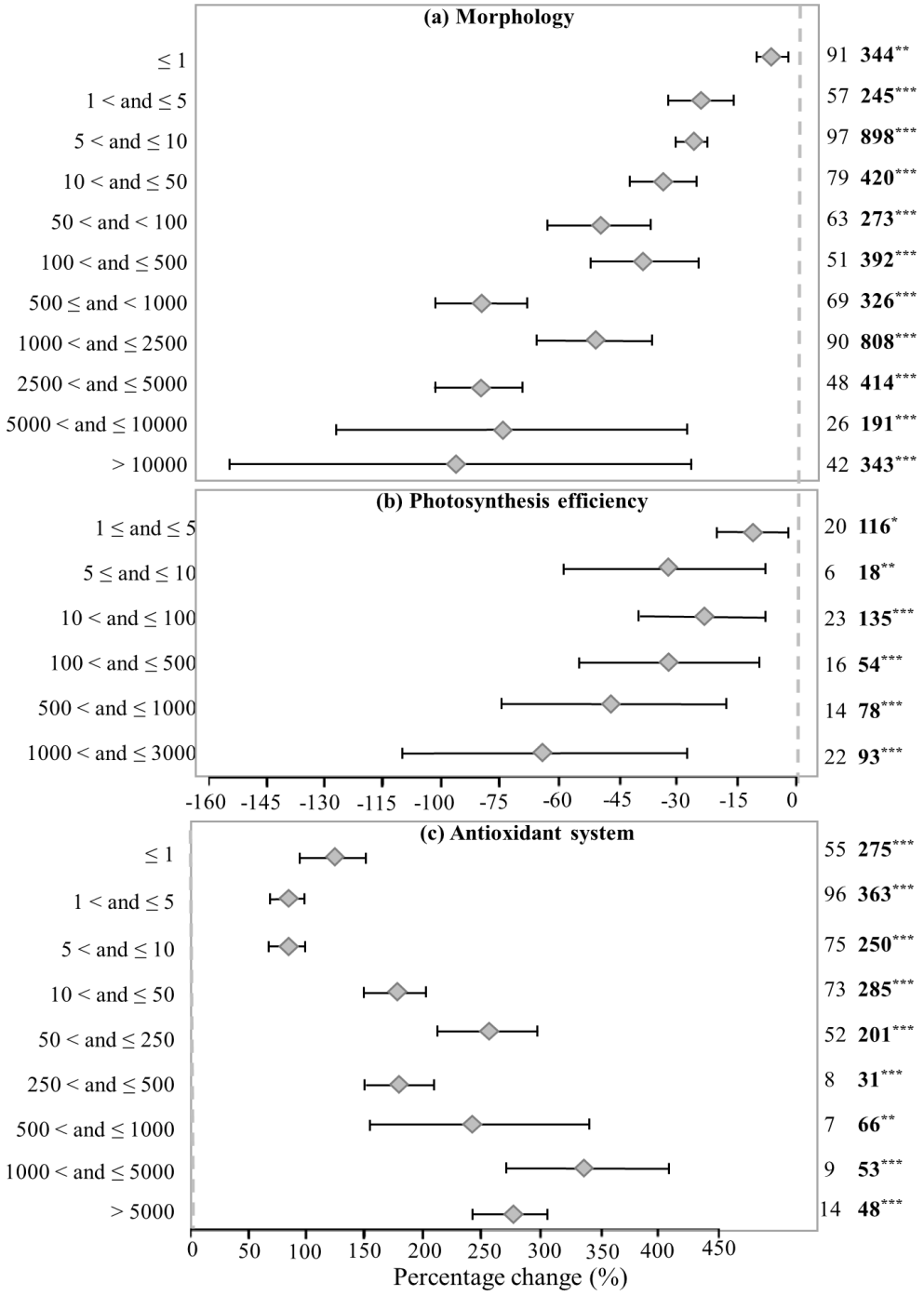


Figure 5.5: Global effects (percentage change) of microcystins on the morphology (a), photosynthetic efficiency (b) and antioxidant enzyme activity (c) in the meta-data associated with the phytotoxicity of different concentration (µg total microcystins /L) of microcystins to agricultural plants from all collected studies. Cumulative effect size was weighted by the inverse

of the pooled variance. Applied concentration of microcystins are listed on the left-hand side and the number of observations for each subgroup and the sample size (bold) are shown on the right side of the forest plot. Error bars are the 95% confidence intervals. Percentage change is significantly different from the control at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p<0.001$.

5.4.1.4. Sensitivity of agricultural plants to microcystins

Plant families differed in their sensitivity to microcystins (Fig. 5.6). Plants in the Brassicaceae family are the most sensitive to microcystins, based on the stimulation of antioxidant enzyme activity Brassicaceae (147%) > Poaceae (132%) > Leguminosae (129%) > Amaranthaceae (81%) > Cucurbitaceae (79%) > Asteraceae (71%) > Apiaceae (2%)), while plants in the Asteraceae and Apiaceae families are the most resistant to microcystins. Their high resistance to microcystins is also based on the fact that morphological properties were less affected for Apiaceae (5%) < Asteraceae (18%) < Poaceae (49%) < Brassicaceae (58%) = Solanaceae (58%) < Leguminosae (62%) < Cucurbitaceae (69%), and there was less inhibition of photosynthetic efficiency for Apiaceae (2%) < Asteraceae (22%) < Brassicaceae (34%) < Solanaceae (40%) < Poaceae (44%) < Leguminosae (62%).

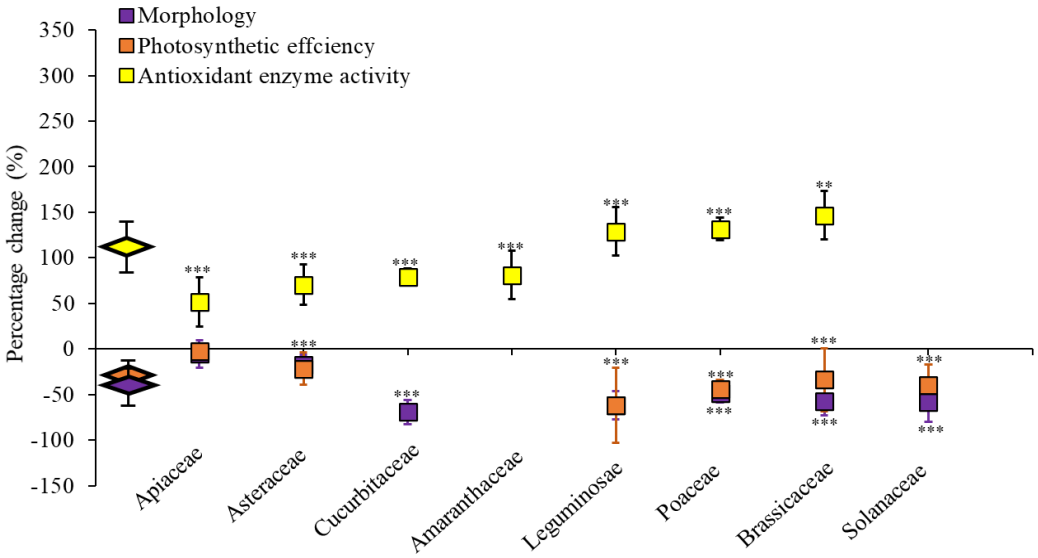


Figure 5.6: Global effects (percentage change) of microcystins on the morphology, photosynthetic efficiency and antioxidant enzyme activity in the meta-data associated with the phytotoxicity of agricultural plant families (n=62 studies). The square indicates the overall mean percentage change (%) of morphology, photosynthetic efficiency and antioxidant enzyme activity. The phytotoxicity of microcystins on morphology, photosynthetic efficiency and antioxidant enzyme activity is significant at $p \leq 0.05$. Error bars are the 95% confidence intervals. Percentage change is significantly different from the control at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p<0.001$.

When individual plant species were considered, durum wheat was the most sensitive to microcystins, based on the fact that microcystins reduced its morphological properties by 69%, inhibited its photosynthetic efficiency by 58% and simulated its antioxidant system by 257% (Fig. 5.7). Corn exhibited similar phytotoxicity as durum wheat, i.e. 65% inhibition of morphological properties, a 46% reduction in photosynthetic efficiency and antioxidant system simulated by 255%. Rice, a related plant species (also a member of the Poaceae like durum wheat and corn) had 108% stimulation of the antioxidant system, which is more than 2 times less than durum wheat and corn. On the other hand, microcystins did not appear to be phytotoxic to lettuce (Asteraceae family) based on the 18% reduction in morphology, the 17% inhibition of photosynthetic efficiency and the 71% stimulation of the antioxidant system (Fig. 5.7). Furthermore, microcystins had no toxic effects on the Apiaceae family, based on the response of carrot morphology and photosynthetic efficiency. These findings suggest that lettuce and carrot are more resistant to microcystins than other plant species.

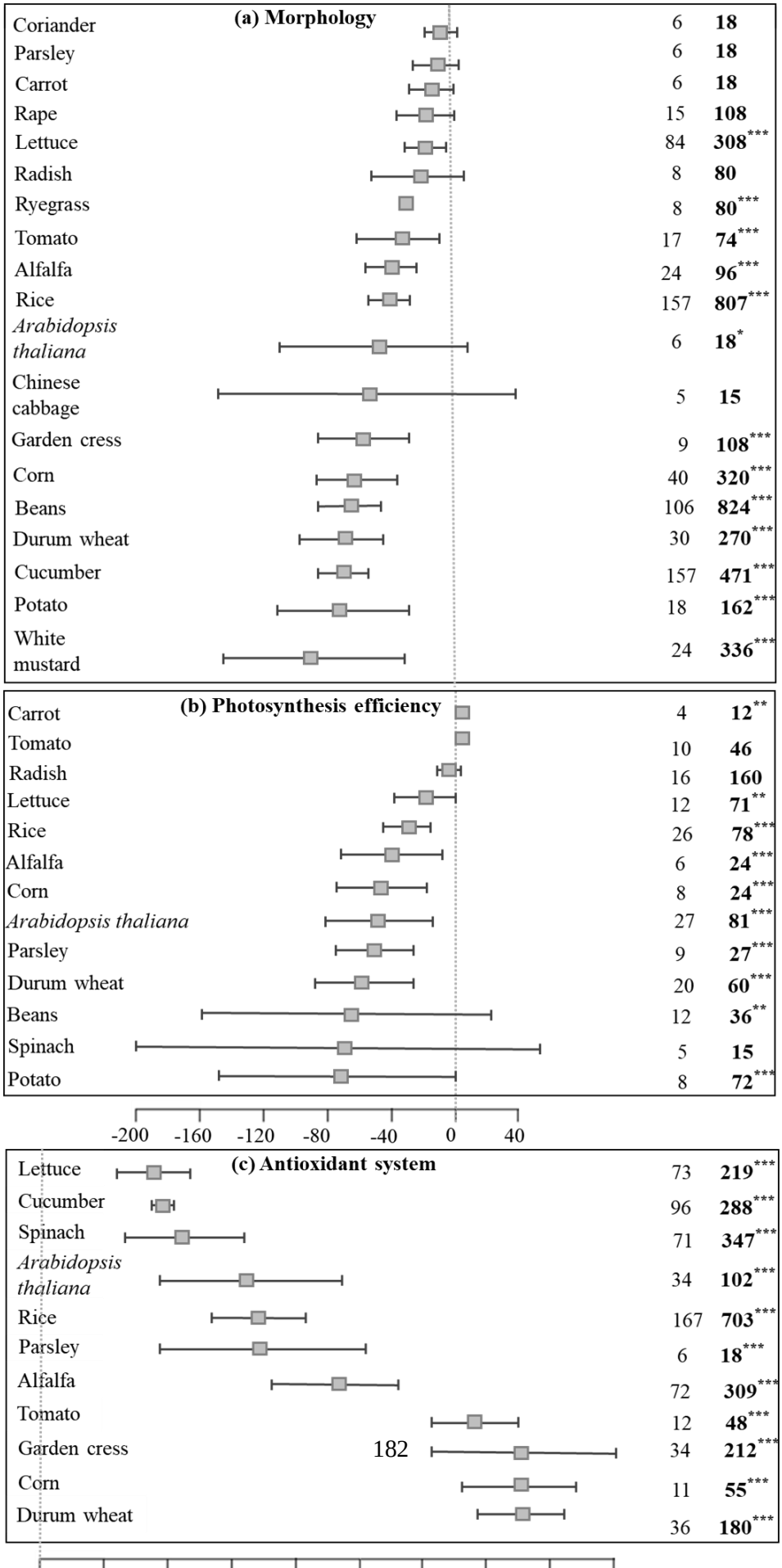

(a) Morphology
Coriander 6 18
Parsley 6 18
Carrot 6 18
Rape 15 108
Lettuce 84 308***
Radish 8 80
Ryegrass 8 80***
Tomato 17 74***
Alfalfa 24 96***
Rice 157 807***
Arabidopsis thaliana 6 18*
Chinese cabbage 5 15
Garden cress 9 108***
Corn 40 320***
Beans 106 824***
Durum wheat 30 270***
Cucumber 157 471***
Potato 18 162***
White mustard 24 336***
(b) Photosynthesis efficiency
Carrot 4 12**
Tomato 10 46
Radish 16 160
Lettuce 12 71**
Rice 26 78***
Alfalfa 6 24***
Corn 8 24***
Arabidopsis thaliana 27 81***
Parsley 9 27***
Durum wheat 20 60***
Beans 12 36**
Spinach 5 15
Potato 8 72***
-200 -160 -120 -80 -40 0 40
(c) Antioxidant system
Lettuce 73 219***
Cucumber 96 288***
Spinach 71 347***
Arabidopsis thaliana 34 102***
Rice 167 703***
Parsley 6 18***
Alfalfa 72 309***
Tomato 12 48***
Garden cress 34 212***
Corn 11 55***
Durum wheat 36 180***

Figure 5.7: Response of agricultural plant species to microcystin exposure. Responses were grouped according to the change in morphology, photosynthetic efficiency and antioxidant enzyme activity of each agricultural plant. Effect sizes were weighted by the inverse of pooled variance. Plant species are listed on the left. The number of observations and sample size (bold) are shown on the right side of the forest plot. Percentage change is significantly different from the control at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p<0.001$.

5.4.2. Bioconcentration of microcystins in agricultural plants

Agricultural plants differ in their ability to bioconcentrate microcystins. From a human health perspective, the amount of microcystins that bioconcentrate in the edible part of the plant is the most critical. Microcystins bioconcentrate preferentially in the whole plant > stem > root = leaf > grain > seedling. The highest microcystin bioconcentration occurred in rice, dill, arugula, cabbage, parsley and radish plants (Fig. 5.8). Moderate microcystins bioconcentration was observed in corn, beans, durum wheat, lettuce and carrot plants. The lowest microcystins bioconcentration ($\ln RR_{BCF} < 0$) was in rape, *Arabidopsis thaliana* and cucumber, suggesting that these plants may bioconcentrate less microcystins.

(a)

ln RR_{BCF}	Microcystins bioconcentration
< 0	"No"
0 – 3	"Low"
3 – 3.7	"Moderate"
> 3.7	"High"

(b)

Plant part	Seedling	Root	Root + Leaf	Leaf	Stem	Grain
ln RR_{BCF} [LL, UL]	-2.3 [-3.4, -1.2]***	2.6 [2.5, 2.7]***	6.1 [5.7, 6.4]***	3.2 [2.9, 3.6]***	4.0 [3.7, 4.3]***	2.0 [1.1, 2.9]***
Microcystins bioconcentration	"No"	"Low"	"High"	"Moderate"	"High"	"Low"
Number of study	7	75	61	52	16	10
Sample size	**21**	**331**	**183**	**208**	**86**	**30**

(c)

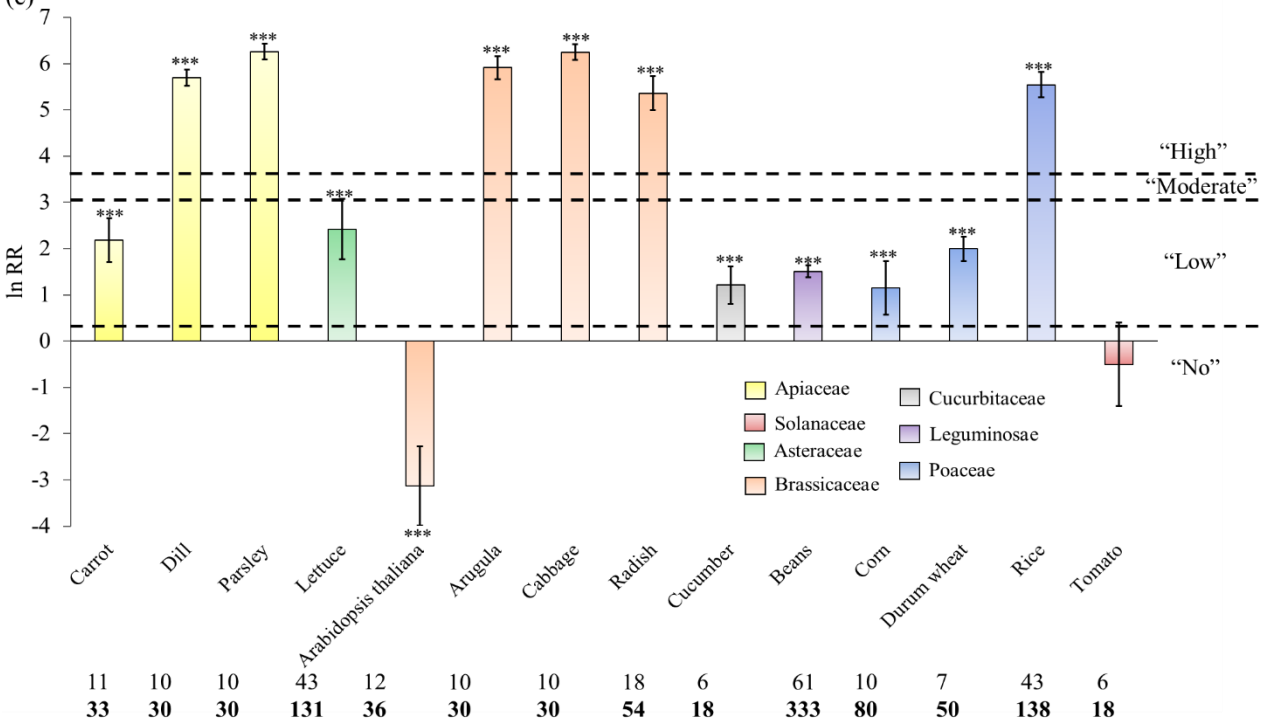


Figure 5.8: The response ratio of the bioconcentration factor (ln RR_{BCF}) for microcystins in different plant parts (b) and in different plant species (c). The number of studies in each group and the sample size (bold) are shown at the bottom. Error bars are the 95% confidence intervals.

5.4.3. Risk assessment of microcystins in agricultural plants

We calculated the estimated daily intake values (EDI, µg/kg/d) of microcystins for adults and children based on the microcystin-LR equivalence (Fig. 5.9a and Fig. S5.1a). Mean EDI values were greater for parsley (0.9) > cabbage (0.6) > dill = arugula (0.5) > lettuce (0.2) > green bean (0.03) > cucumber (0.02) > rice grain (0.01) > carrot (0.006). Greater intake of microcystins is predicted when adults and children eat leafy vegetables such as parsley, dill, arugula and

cabbage, based on EDI values that exceed the WHO guidelines, than when they consume root, fruit and grain. The EDI values for lettuce, cucumber, green bean, rice grain and carrot were above the RfD guideline, suggesting that there is a risk of chronic exposure to microcystins when these agricultural plants are consumed (Fig. 5.9b).

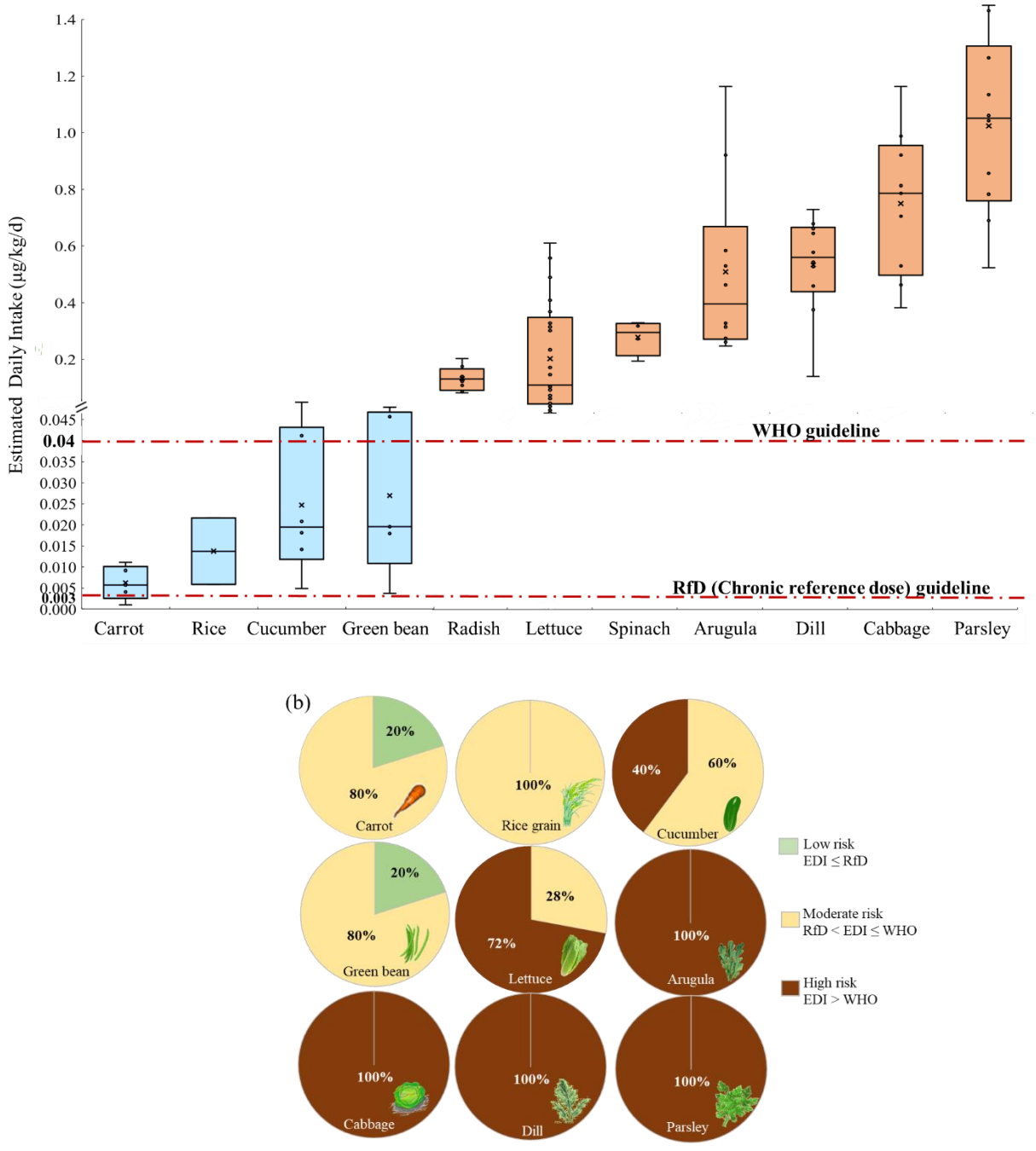


Figure 5.9: (a) Estimated daily intake (EDI) of microcystin-LR equivalent from the consumption of the edible part of agricultural plants by adults. (b). The number (shown in percentage (%)) of EDI values for microcystin-LR equivalent classified as low risk, moderate risk and high risk for each agricultural plant. EDI values were determined from the microcystins concentration (µg microcystins /kg plant biomass) in nine agricultural plants treated with 0 – 10 µg microcystins /L. RfD is the chronic reference dose of 0.003 µg microcystins /kg body mass /d established by United States Environmental Protection Agency and WHO is the World Health Organization safe limit of 0.04 µg microcystins /kg body mass /d (US EPA, 2015b).

5.5. Discussion

5.5.1. Microcystins toxicity to plants

The hydrophilic carboxyl and amino groups of microcystins are highly soluble in water (1 g/L for microcystin-LR; Bartram and Chorus, 1999; de Maagd et al., 1999), which allows microcystins to diffuse in water-based solutions and move through soil pore water to the root surface. Microcystins probably enter plants through pinocytic processes, root adsorption and diffusion (Machado et al., 2017a; Pham and Utsumi, 2018). Proton-amino acid symporters in root cells could act as microcystins transporters, while pinocytic processes are expected during microcystins uptake (Eriksson et al., 1990; Pflugmacher et al., 2019). After microcystins move through the apoplastic pathway and enter the xylem after overcoming the Casparian strip barrier (Pflugmacher et al., 2019), most of microcystins dissolved in water are transported passively to leaves due to the high demand of water for photosynthesis and other metabolic processes. Limited transport of microcystins occurs through the phloem because this active process generally requires energy (Mc Farlane and Trapp, 1994; Vesterkvist and Meriluoto, 2003). When microcystins enter a plant cell, less than 10% of microcystins may be conjugated

nonenzymatically to glutathione in the plant, and the rest of the microcystins bind to protein phosphatases through van der Waals forces and ionic binding between positively charged domains of the protein and the negatively charged microcystins (Jüttner and Lüthi, 2008; Pflugmacher, 2002b). The covalent bond between microcystins and catalytic center of serine/threonine protein phosphatases can perturb the phosphorylation or dephosphorylation balance, affecting many cellular functions of the protein phosphatases (Welten et al., 2020). In addition, the ATP-synthase beta subunit could be another target of microcystins binding in eukaryotic cells, which explains the apoptosis-inducing potential of microcystins (Mikhailov et al., 2003; Wei et al., 2016).

Since microcystins transported through xylem will first bioconcentrate in leaves, the early symptoms of microcystins phytotoxicity are observed in leaf tissues. Microcystins seem to activate plant defense responses associated with ATP-producing metabolic pathways by reducing the availability of metabolic intermediates, and thus lower the photosynthesis rate (McElhiney et al., 2001). When biosynthesis of defense-related compounds is prioritized, photosynthesis is suppressed to avoid damage to the photosynthetic apparatus (Bolton, 2009). Microcystins also induce the accumulation of reactive oxygen species in plant cells, which stimulates antioxidant enzyme activity to protect DNA, proteins, carbohydrates and lipids from oxidative stress (Pham and Utsumi, 2018). Therefore, plants that bioconcentrate microcystins in their leaves will have less photosynthetic efficiency and greater antioxidant activity, which slows the growth rate and causes morphological changes such as less biomass accumulation, fewer leaves and shorter stems.

5.5.1.1. Microcystins toxicity to plants depends on the growing conditions

Microcystins are more phytotoxic to plants growing in hydroponics solution than in soil-based environments, which we attribute to a higher level of bioavailable microcystins in hydroponics solution. In hydroponics-plant systems, roots are immersed in solution, meaning that microcystins remain in water and are readily transported to the root surface by diffusion processes. Culture solutions with optimized levels of nutrients and electrolytes are more favorable for root growth and apparently increase the absorptive root surface for microcystins entry and bioconcentration in plants, based on the greater phytotoxicity observed in culture solution, compared to water and paper-based hydroponics. On the other hand, soil-plant systems that have a solid matrix (mainly organic matter and clay minerals) can reduce microcystins bioavailability to plants because the microcystins adsorb to the soil particles and may be degraded by soil microorganisms. The surface charges on organic matter and clay minerals are negative, while microcystins have several charged groups (i.e., $–COO^-$ and $–NH_2^+$) in natural soil solution (pH ~6–8) (Thirumavalavan et al., 2012; Ward and Codd, 1999; Wu et al., 2011). The NH_2^+ group can participate in cation exchange reactions or be ionically bound to negatively charged interfaces of organic matter and clay, while the carboxylate group (COO^-) can be repulsed by negatively charged surfaces or form surface bonds via cation bridging and/or ligand exchange reactions (Liu et al., 2019a; Pochodylo et al., 2016). The surface-bound organic matter may also enhance microcystins interaction with the surface through hydrophobic bonding considering they contain the hydrophobic ADDA group (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) (Pochodylo et al., 2016). Once the microcystins has complexed or precipitated with soil inorganic and organic particles, it becomes too large to be passively absorbed into roots. In addition, faster degradation of microcystins occurs in soil pore water,

which contains a diverse, active microbiome that is adapted to fluctuations in soil moisture and aeration, compared to the soilless solution. Soil-based environments also contain organic matter that may act as photosensitizer and produce radicals that accelerate the photo-degradation rate of microcystins (Thirumavalavan et al., 2012). These reasons can explain why microcystins phytotoxicity is greater in hydroponics-plant systems than in soil-plant systems.

It is also notable that microcystins are less toxic to plants growing in soil substrates than sand and vermiculite substrates. Sand has practically no cation exchange capacity – it neither adsorbs nor releases charged molecules, so microcystins are more likely to remain in the soil pore water that travels to the roots. Vermiculite is expected to absorb more microcystins because of its higher surface area than kaolinite and illite in organic-matter free environments (Liu et al., 2019a; Sposito et al., 1999). However, the vermiculite can also release more microcystins into the soil water because of its high ion exchange capacity and relatively weak bond strength. For instance, vermiculite attracted microcystins primarily through van der Waals interactions, electrostatic interactions and hydrogen bonding due to the limited pH-dependence in microcystins adsorption and small edge area of vermiculite (Liu et al., 2019a; Pochodylo et al., 2016). In contrast to vermiculite, the clay minerals in soil are predominantly kaolinite and illite (Ito and Wagai, 2017). The force of microcystins adsorption to kaolinite and illite was stronger than vermiculite as it involved ionized functional groups via ligand exchange and cation bridging reactions on a high edge area (Liu et al., 2019a, b; Miller et al., 2005). Furthermore, the organic matter that coats clay surfaces appears to retain microcystins through water-bridged hydrogen-bonds and metal complexes (Liu et al., 2019b; Thirumavalavan et al., 2012). This suggests that more microcystins are strongly bound to particles in the soil-plant system, compared to the

vermiculite-plant system, therefore we can expect lower phytotoxicity in soil-based substrate than pure vermiculite, which is consistent with our observations.

5.5.1.2. Greater phytotoxicity of microcystins from natural bloom extract and cultured *M. aeruginosa* extract

Natural bloom extract is derived from field-collected cyanobacteria by sonication of the cyanobacteria cells in water or by extraction with solvent or dilute acid solution (e.g., methanol/water (v/v) of 70% – 100% or 5% acetic acid/water (v/v). Natural bloom extract could contain multiple microcystin congeners, as well as other co-occurring cyanotoxins like anatoxin-a, saxitoxins, cylindrospermopsin and β-methylamino-l-alanine, and other secondary metabolites that are produced in cyanobacterial bloom (Freitas et al., 2014; Machado et al., 2017b; Roy-Lachapelle et al., 2019; Sabart et al., 2015; Testai et al., 2016; Zhang and Whalen, 2020a). This suite of secondary metabolites may synergistically enhance the toxicity of natural bloom extract, relative to purified microcystins extract (Sadler and von Elert, 2014). Furthermore, trace metals like iron and zinc that chelate with microcystins or other cyanotoxins could enhance the toxicity of natural bloom extract (Cao et al., 2020; Chin-Chan et al., 2015; Facey et al., 2019). Some organisms, e.g. bacteria, spores and zooplankton, attached on cyanobacteria cell wall could enhance the toxicity of natural bloom water (Guo et al., 2018; Kim et al., 2020). Therefore, the natural bloom extract is expected to be more toxic than the other sources of microcystins.

In realistic field conditions, the microcystins concentration in irrigation water ranged from <1 μg microcystins/L, which promoted plant growth due to hormesis and other stimulatory effects, to as much as 29,000 μg microcystins/L, which was phytotoxic (Machado et al. 2017). Higher microcystins concentration is associated with the cyanobacterial bloom, which typically reaches its peak growth in late summer and fall months, and contains other cyanotoxins and

secondary metabolites that could interact synergistically with microcystins to increase their phytotoxicity. (Al-Sammak et al., 2014; Cerasino and Salmaso, 2020; Christophoridis et al., 2018; Zhang et al., 2020b). Consequently, farmers and agricultural advisors should be alert to the fact that irrigation water from a cyanobacteria-affected water supply will be phytotoxic during the whole bloom season, particularly in the late summer and fall months. It is beyond the scope of this study to deduce the multiple effects on plants accruing from exposure to a myriad of cyanotoxins. Therefore, we recommend to monitor the presence and concentration of cyanotoxins in irrigation water as an effective strategy to mitigate their phytotoxicity and also protect farmers' health.

5.5.1.3. Phytotoxicity of microcystins depends on the plant family and plant species

Microcystins elicited more phytotoxic responses in the Brassicaceae, Poaceae and Leguminosae, which were the top three plant families with the greatest stimulation of their antioxidant system. These three plant families, plus the Solanaceae, lost a considerable amount of photosynthetic efficiency after exposure to microcystins. Since photosynthesis is key for the production of new tissues, it is not surprising that these four plant families, plus the Curcurbitaceae, had the most change in morphological properties after exposure to microcystins. At the plant species level, the three most sensitive plants with larger antioxidant simulation were durum wheat (Poaceae), corn (Poaceae), and garden cress (Brassicaceae), while white mustard (Brassicaceae) had the most profound morphological reduction, and potato (Solanaceae) lost the most photosynthetic efficiency. Lettuce (Asteraceae) was more resistant to the phytotoxic effects of microcystins, compared to other plant species.

Plants exhibit toxicity when their self-detoxification systems cannot depurate microcystins. Once microcystin enter plant cells (e.g. mesophyll cells), forming glutathione conjugates via the

Glutathione-S-transferase system or by nonenzymatic processes is the first step for plants to detoxify microcystins (Jiang et al., 2011). Then, the plant transfers the microcystin-glutathione conjugates into vacuoles for storage and further processing (Coleman et al., 1997; Pflugmacher, 2002a). However, only ~10% of microcystins form conjugates when they enter plants, meaning that most microcystins remain potent inducers of plant-specific toxicity (Cao et al., 2019b). Toxicity of these non-depurated microcystins results from their binding to protein phosphatases followed by reactive oxygen species production. The plant's first line of defense is an up-regulation of the antioxidant system, which relies on antioxidant enzymes or glutathione for protection from oxidative stress. Therefore, the sensitivity and resistance of plants to microcystins depends on their ability to reduce oxidative stress (Gu and Liang, 2020; Petrou et al., 2020; Saqrane et al., 2009).

Plant defense via their antioxidant system is energy intensive, requires nutrients and relies on multiple metabolic pathways (Bolton, 2009). Allocating resources toward the defense response generally occurs at the expense of plant fitness (i.e., lower investment in morphology, inhibition of photosynthesis; Mittler, 2002). This may explain why the increase in antioxidant systems reduced morphological outcomes and photosynthesis rates in Brassicaceae, Poaceae and Leguminosae. The Brassicaceae were less affected than Poaceae and Leguninosae, which may be attributed to their high sulfur requirement to support sulfur metabolism, including production of phenolics and glucosinolates (Jahangir et al., 2009; Petrou et al., 2020). Sulfur metabolites like glutathione are well-known antioxidants that could be involved in microcystins detoxification; the Brassicaceae with high phenolics and glucosinolates may have inherent protection against oxidative stress, which lowers the phytotoxic effects of microcystins on species in this plant family.

At species level, not all species within a plant family respond in the same way to microcystins exposure because of the significant variation in specific plant defense responses to antioxidant stress. Within the Poaceae family, rice was less affected and consequently appears to be more resistant to microcystins than durum wheat and corn. Rice may have higher and easily activated superoxide dismutase and catalase enzymes than durum wheat and corn (Gu and Liang, 2020). These two enzymes plus glutathione peroxidase constitute a first robust line antioxidant defense system for rice (Ighodaro and Akinloye, 2018). More genes regulating catalase enzyme and higher efficiency of the antioxidant enzyme genes were also reported in rice than other plants (Sharma et al., 2016). Besides, microcystins could promote the synthesis of some signaling molecules, i.e. hormones that can initiate defenses in plant roots (Liang and Wang, 2015). This evidence supports our assertion of a more efficient antioxidant response to microcystins exposure in rice than durum wheat and corn. Lettuce, which is the most resistant to phytotoxicity from microcystins, is generally rich in antioxidant compounds that scavenge reactive oxygen species with glutathione-S-transferase (Bittencourt-Oliveira Mdo et al., 2016). Lettuce can simultaneously activate several metabolic pathways, which are mainly related to photosynthesis, response/defense to stress, protein synthesis and transduction machinery, to overcome microcystins toxicity (Freitas et al., 2015). Besides, lettuce has superior photosynthetic performance due to the high nitrogen content in its leaf tissue (Evans, 1989). Low microcystins concentration (< 10 μg/L) is known to enhance the photosynthesis rate in lettuce by increasing the gas exchange and achieving close to the optimal potential quantum efficiency of Photosystem II, based on the Fv/Fm value (Bittencourt-Oliveira Mdo et al., 2016; Levizou et al., 2017). Stimulation of photosynthesis is associated with the energy acquisition needed to produce antioxidant biomolecules in lettuce (Freitas et al., 2015; Saqrane et al., 2009). Consequently,

resistance to microcystins is expected in plant species with inherently high photosynthesis rates and capacity to up-regulate their antioxidant production, but this remains to be confirmed under realistic field conditions. Further work should focus more on the phytotoxicity of microcystins to these plant species under realistic field conditions. Since several important staple crops (e.g., durum wheat and corn) were negatively impacted by microcystins exposure, we should avoid to plant these crops on agricultural land that is irrigated with water containing microcystins. Another practical option would be to identify the crop species/varieties that could be suitable for growing in areas where irrigation water is frequently contaminated with microcystins.

5.5.2. Risk assessment due to bioconcentration of microcystins in agricultural plants

5.5.2.1. Bioconcentration of microcystins in edible parts depends on the plant species

Microcystins in soil have an estimated half-life of 5 to 60 d, depending on its rate of adsorption to soil surfaces and the rates of biological- and chemically-mediated degradation processes (Chen et al., 2006; Corbel et al., 2014a). Once absorbed by plants, microcystins can be conjugated nonenzymatically to glutathione in cells or bioconcentrate in particular plant components (Pflugmacher et al., 2002; Arnot and Gobas, 2006). Our results indicate that microcystins tend to accumulate preferentially in the leaves and roots, followed by the stems and then the grains of plants. Furthermore, leafy plants like radish, cabbage, arugula, parsley, and dill bioconcentrate more microcystins than root and grain plants. However, bioconcentration of microcystins does not imply that it is phytotoxic to the plant, based on results from the Apiaceae family. Even within the same plant family (e.g., Brassicaceae), the bioconcentration factor varies from species to species, probably due to differences in plant ecophysiological characteristics such as leaf area, leaf cuticle, metabolic processes, source-sink transfers between root and shoot

systems (Crush et al., 2008; Romero-Oliva et al., 2014; Saqrane et al., 2009), as well as the mode of exposure to microcystins (i.e., foliar vs. soil-borne microcystins).

5.5.2.2. Health risks from consuming edible plant parts containing microcystins

Leafy vegetables tend to bioconcentrate microcystins in edible tissues and people generally eat the whole leaf, which means that adults and children have a greater health risk from ingesting leafy vegetables than root vegetables (Fig. 5.9). Eating raw vegetables is a direct exposure pathway for microcystins, but microcystins remain in food, in their original chemical form, even if it is boiled, fried, or steamed (Chen et al., 2010). Cooked plants release bound microcystins and thus increase the amount of ingested microcystins. Therefore, it seems that there is no way to safely prepare a microcystin-contaminated plant for human consumption. Once ingested, microcystins can bioaccumulate in human body tissues, causing adverse effects on various human organs and altering the expression of proto-oncogenes, oncogenes, and cytokines that are associated with an elevated incidence of liver and colorectal cancer (Buratti et al., 2017). To date, there is no report of animal or human poisoning linked to the consumption of microcystins-contaminated vegetables in the world. However, the concentration of microcystins in vegetables could exceed the daily tolerance intake (World Health Organization, 0.04 μg microcystins/kg body mass/day) in many parts of the world, such as China, Saudi Arabia and Canada (Mohamed et al., 2009; Miller and Russel, 2017; Xiang et al., 2019).

This meta-analysis suggests a human health risk from microcystins-contaminated vegetables, but it needs to be interpreted with caution. To the best of our knowledge, only three studies reported the bioconcentration of mircrocystins in realistic field conditions; most of the available data was collected in pot experiments or hydroponic experiments at non-environmentally relevant concentrations. Although microcystins exposure in soil-plant systems

should avoid overestimating the microcystins bioconcentration in plants, compared to a hydroponic exposure study (Machado et al., 2017a), such experiments still cannot replicate the bioconcentration of microcystins in realistic field conditions. We need a better microcystins exposure test that can reflect microcystins interactions with other organisms, the natural adsorption and degradation processes, and how the natural soil solution allows for the bioconcentration of microcystins in plants (Mohamed and Al Shehri, 2009; Romero-Oliva et al., 2014; Xiang et al., 2019). Besides, studies with poorly characterized cyanobacterial extracts may underestimate the risk of microcystins transfer to agricultural plants in field settings, while the microcystins experiments that relied upon cultured *Microcystis aeruginosa* or purified standards might not reflect the real toxicity of natural blooms, thus underestimating the actual human health risk. We acknowledge that the bioconcentration data can be biased when microcystins are recovered from plant tissues by a solvent extraction and purification (e.g. with a C18 cartridge) method that quantifies the free dissolved microcystins but does not measure the bound microcystins fraction (Jüttner and Lüthi, 2008; Neffling et al., 2010). Bound microcystins (e.g., deconjugated protein-bound microcystins) undergo a reversible binding process with thiols that contributes to the overall toxicity of microcystins (Vela et al., 2008; Wei et al., 2016). When bound microcystins are the major fraction in plant tissues, this would grossly underestimate the bioconcentration and the eventual risk of microcystins transfer from edible plant tissues to humans (Corbel et al., 2016; Meissner et al., 2013). We urge researchers to evaluate these potential bias in the microcystins exposure, bioconcentration and risk assessment procedures, to improve our understanding of the potential impacts of microcystins on plant and human health.

5.6. Conclusion

This meta-analysis confirmed the phytotoxicity of microcystins affects the morphology, photosynthetic efficiency and antioxidant system of agricultural plants. Most plants can tolerate concentrations <1 μg microcystins/L, but we should avoid growing edible leafy plants, wheat and corn on agricultural land that is irrigated with microcystins-contaminated water. Irrigation water is at risk of becoming contaminated with microcystins and other cyanotoxins because these noxious compounds are produced by cyanobacterial blooms, which are occurring more frequently due to global warming and ongoing eutrophication of waterbodies. Hence, there is a growing risk of agricultural plants coming into contact with microcystins-contaminated water, particularly in developing countries where surface waters are a cheap source of irrigation water. Field-based experiments on the fate and transfer of microcystins in agricultural fields will provide us with the requisite information to prevent microcystins from entering the agricultural food supply. Agricultural advisors will use this information to educate farmers on the hazards associated with irrigating crops with microcystin-contaminated water. Finally, we need to know the critical thresholds for microcystins in agriculture plant, so policymakers can develop dietary guidelines that prevent humans from ingesting agricultural plants that contain more than the safe RfD of microcystins.

5.7. Supplementary Information

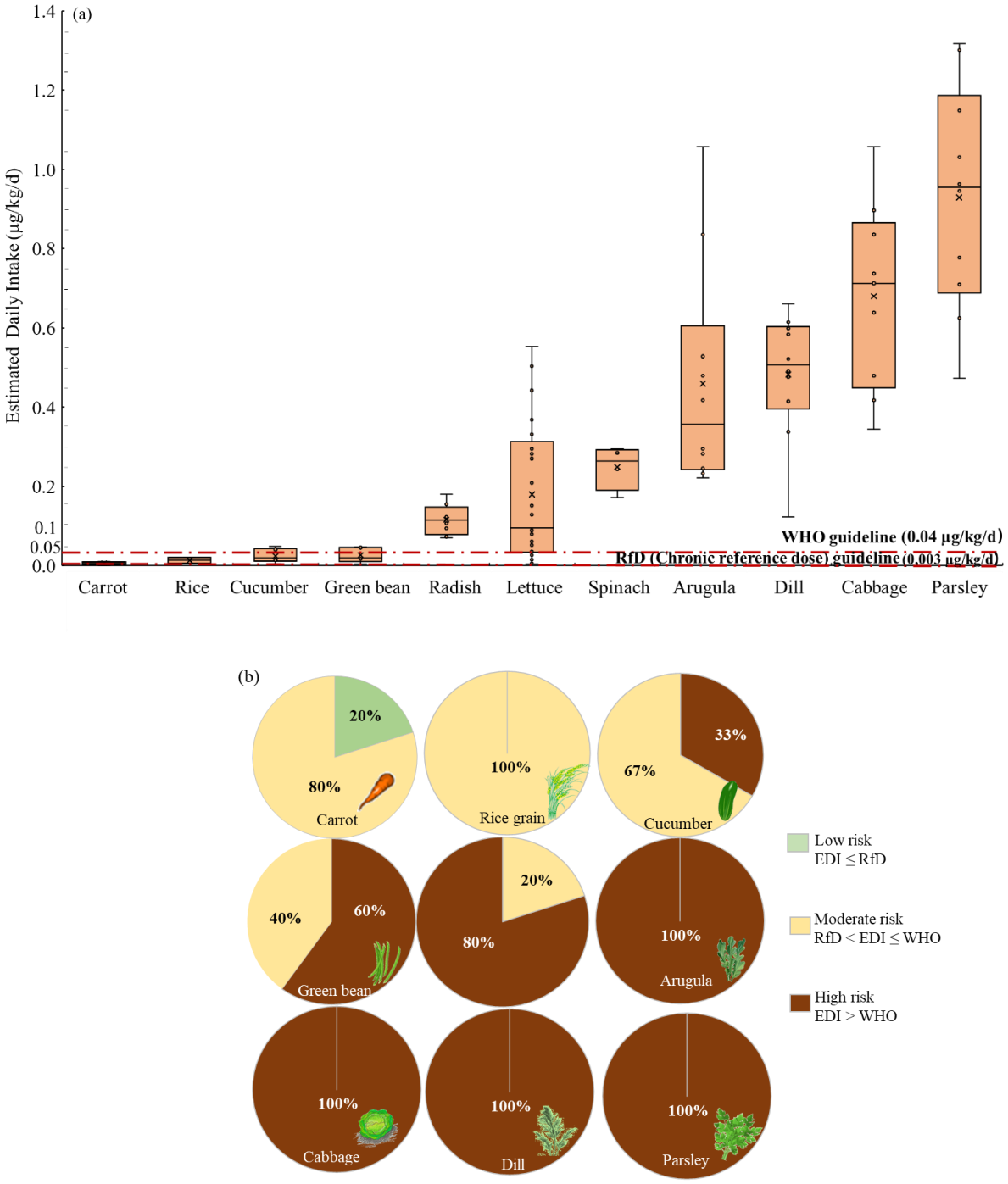


Fig S5.1: (a) Estimated daily intake (EDI) of microcystin-LR equivalent from the consumption of the edible part of agricultural plants by children. (b). The number shown in percentage (%) of EDI values for microcystin-LR equivalent classified as low risk, moderate risk and high risk for each agricultural plant. EDI values were determined from the microcystins concentration (µg

microcystins /kg plant biomass) in nine agricultural plants treated with 0 – 10 µg microcystins /L. RfD is the chronic reference dose of 0.003 µg microcystins /kg body mass /d established by United States Environmental Protection Agency and WHO is the World Health Organization safe limit of 0.04 µg microcystins /kg body mass /d (US EPA, 2015b).

Table S5.1: Daily reference intake of the edible part of agricultural plants (g fresh weight/day) from the standards of the United States Food and Drug Administration (2015).

Agricultural plant	Daily reference intake of edible plant part (g d^{-1})	
	Adults	Children
Arugula	80	40
Cabbage	80	40
Carrot	15	7.5
Cucumber	100	50
Dill	100	50
Green bean	120	60
Lettuce	80	40
Parsley	110	50
Radish	15	7.5
Spinach	128	64
Rice	250	130
Tomato	150	75

Table S5.2: Heterogeneity test for the effect of microcystins on the morphology, photosynthetic efficiency or antioxidant enzyme activity of agricultural plants. Measurements that represented these agricultural plant responses are listed in Table 1.

Agricultural plant responses	Effect size (ln RR)	Lower bound (ln)	Upper bound (ln)	Standard error (ln)	Heterogeneity			
					p.	I^2 (%)	Tau^2	Q
Morphology	-0.36	-0.39	-0.34	0.01	<0.001	99	0.1	14×10^4 (df=720)
Photosynthetic efficiency	-0.25	-0.29	-0.21	0.02	<0.001	100	0.05	1.8×10^4 (df=167)
Antioxidant enzyme activity	0.28	0.25	0.31	0.02	<0.001	98	0.13	4.8×10^4 (df=655)

Table S5.3: The toxicity and MC-LR equivalency, based on the toxicity equivalent factor, of primary microcystin congeners. Toxicity was determined from the intraperitoneal median lethal dose (LD_{50}) value of mice.

Congener	Toxicity (μg/kg)	Toxicity equivalent factor
Microcystin-AR	250	0.2
Microcystin-FR	250	0.2
Microcystin-HilR	100	0.5
Microcystin-HtyR	80–100	0.5
Microcystin-LA	50	1
Microcystin-LR	50	1
Microcystin-LY	90	0.6
Microcystin-MR	700–800	0.06
Microcystin-RR	500–800	0.06
Microcystin-WR	150–200	0.3
Microcystin-YA	60–70	0.8
Microcystin-YM	56–110	0.9
Microcystin-YR	150–200	0.3

5.8. Acknowledgements

YZ received financial support from the China Scholarship Council, a Graduate Mobility Award from McGill University and from the EcotoQ network, which is funded by the Fonds de recherche du Québec – Nature et technologies. The authors wish to acknowledge the assistance of ecotoxicologist Prof. Geoffrey Sunahara, McGill University, for giving us expert opinions on phytotoxicity terminologies.

5.9. Reference

Agriculture, U.S.D.o., 2007. USDA National Nutrient Database for Standard Reference, Release 20.

Al-Sammak, M.A., Hoagland, K.D., Cassada, D., Snow, D.D., 2014. Co-occurrence of the cyanotoxins BMAA, DABA and anatoxin-a in Nebraska reservoirs, fish, and aquatic plants. Toxins 6, 488-508. https://doi.org/10.3390/toxins6020488.

Arnot, J. A., Gobas, F.A., 2006. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. Environ. Rev. 14**,** 257–297. https://doi.org/10.1139/a06-005.

Azevedo, C.C., Azevedo, J., Osorio, H., Vasconcelos, V., Campos, A., 2014. Early physiological and biochemical responses of rice seedlings to low concentration of microcystin-LR. Ecotoxicology 23, 107–121. https://doi.org/10.1007/s10646-013-1156-8.

DerSimonian, R., Laird, N., 2015. Meta-analysis in clinical trials revisited. Contemp. Clin. Trials 45, pp.139–145. https://doi.org/10.1016/j.cct.2015.09.002.

Bartram, J., Chorus, I., 1999. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. CRC Press.

Bittencourt-Oliveira Mdo, C., Cordeiro-Araujo, M.K., Chia, M.A., Arruda-Neto, J.D., de Oliveira, E.T., dos Santos, F., 2016. Lettuce irrigated with contaminated water: Photosynthetic effects, antioxidative response and bioaccumulation of microcystin congeners. Ecotoxicol. Environ. Saf. 128, 83-90. https://doi.org/10.1016/j.ecoenv.2016.02.014.

Bolton, M.D., 2009. Primary metabolism and plant defense—fuel for the fire. Mol. Plant Microbe Interact. 22**,** 487–497. https://doi.org/10.1094/MPMI-22-5-0487.

Bouaïcha, N., Miles, C.O., Beach, D.G., Labidi, Z., Djabri, A., Benayache, N.Y., Nguyen-Quang, T., 2019. Structural diversity, characterization and toxicology of microcystins. Toxins 11, 714. https://doi.org/10.3390/toxins11120714.

Buratti, F.M., Manganelli, M., Vichi, S., Stefanelli, M., Scardala, S., Testai, E., Funari, E., 2017. Cyanotoxins: producing organisms, occurrence, toxicity, mechanism of action and human health toxicological risk evaluation. Arch. Toxicol. 91, 1049–1130. https://doi.org/10.1007/s00204-016-1913-6.

Cao, Q., Steinman, A.D., Wan, X., Xie, L., 2018. Bioaccumulation of microcystin congeners in soil-plant system and human health risk assessment: A field study from Lake Taihu region of China. Environ. Pollut. 240, 44-50. https://doi.org/10.1016/j.envpol.2018.04.067.

Cao, Q., Liu, W., Jiang, W., Shu, X., Xie, L., 2019a. Glutathione biosynthesis plays an important role in microcystin-LR depuration in lettuce and spinach. Environ. Pollut. 253, 599-605. https://doi.org/10.1016/j.envpol.2019.07.064.

Cao, Q., Wan, X., Shu, X., Xie, L., 2019b. Bioaccumulation and detoxication of microcystin-LR in three submerged macrophytes: The important role of glutathione biosynthesis. Chemosphere 225, 935-942. https://doi.org/10.1016/j.chemosphere.2019.03.055.

Cao, Q., Liu, W., Gu, Y., Xie, L., Jiang, W., Gao, Y., Yang, L., 2020. Synergetic enhancement toxicity of copper, cadmium and microcystin-LR to the *Ceratophyllum demersum* L. Toxicon 186, 151-159. https://doi.org/10.1016/j.toxicon.2020.08.008.

Carmichael, W.W., Azevedo, S., An, J.S., Molica, R., Jochimsen, E.M., Lau, S., Rinehart, K.L., Shaw, G.R., Eaglesham, G.K., 2001. Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. Environ. Health Perspect. 109, 663. https://doi.org/10.1289/ehp.01109663.

Cerasino, L., Salmaso, N., 2020. Co-occurrence of anatoxin-a and microcystins in Lake Garda and other deep subalpine lakes. Adv. Oceanogr. Limnol. 11. https://doi.org/10.4081/aiol.2020.8677.

Chen, W., Song, L., Gan, N., Li, L., 2006. Sorption, degradation and mobility of microcystins in Chinese agriculture soils: Risk assessment for groundwater protection. Environ. Pollut. 144, 752–758. https://doi.org/10.1016/j.envpol.2006.02.023

Chen, X., Yang, X., Yang, L., Xiao, B., Wu, X., Wang, J., Wan, H., 2010. An effective pathway for the removal of microcystin LR via anoxic biodegradation in lake sediments. Water Res. 44, 1884–1892. https://doi.org/10.1016/j.watres.2009.11.025.

Chen, L., Giesy, J.P., Xie, P., 2018. The dose makes the poison. Sci. Total Environ. 621, 649–653. https://doi.org/10.1016/j.scitotenv.2017.11.218.

Chia, M.A., Auta, Z.Z., Esson, A.E., Yisa, A.G., Abolude, D.S., 2019. Assessment of microcystin contamination of *Amaranthus hybridus*, *Brassica oleracea*, and *Lactuca sativa* sold in markets: a case study of Zaria, Nigeria. Environ. Monit. Assess. 191, 569. https://doi.org/10.1007/s10661-019-7725-4.

Chin-Chan, M., Navarro-Yepes, J., Quintanilla-Vega, B., 2015. Environmental pollutants as risk factors for neurodegenerative disorders: Alzheimer and Parkinson diseases. Front. Cell. Neurosci. 9, 124. https://doi.org/10.3389/fncel.2015.00124.

Chorus, I., Bartram, J., 1999. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. CRC Press.

Christophoridis, C., Zervou, S.-K., Manolidi, K., Katsiapi, M., Moustaka-Gouni, M., Kaloudis, T., Triantis, T.M., Hiskia, A., 2018. Occurrence and diversity of cyanotoxins in Greek lakes. Sci. Rep. 8, 1-22. https://doi.org/10.1038/s41598-018-35428-x.

Coleman, J., Blake-Kalff, M., Davies, E., 1997. Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. Trends Plant Sci. 2, 144-151. https://doi.org/10.1016/S1360-1385(97)01019-4.

Corbel, S., Bouaïcha, N., Mougin, C., 2014a. Dynamics of the toxic cyanobacterial microcystin-leucine-arginine peptide in agricultural soil. Environ. Chem. Lett. 12, 535-541. https://doi.org/10.1007/s10311-014-0482-2.

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

Corbel, S., Bouaïcha, N., Nélieu, S., Mougin, C., 2015. Soil irrigation with water and toxic cyanobacterial microcystins accelerates tomato development. Environ. Chem. Lett. 13, 447–452. https://doi.org/10.1007/s10311-015-0518-2.

Corbel, S., Mougin, C., Nélieu, S., Delarue, G., Bouaicha, N., 2016. Evaluation of the transfer and the accumulation of microcystins in tomato (*Solanum lycopersicum* cultivar MicroTom) tissues using a cyanobacterial extract containing microcystins and the radiolabeled microcystin-LR (^{14}C-MC-LR). Sci. Total Environ. 541, 1052-1058. https://doi.org/10.1016/j.scitotenv.2015.10.004.

Crush, J.R., Briggs, L.R., Sprosen, J.M., Nichols, S.N., 2008. Effect of irrigation with lake water containing microcystins on microcystin content and growth of ryegrass, clover, rape, and lettuce. Environ. Toxicol. 23, 246–252. https://doi.org/10.1002/tox.20331.

de Maagd, P.G.-J., Hendriks, A.J., Seinen, W., Sijm, D.T., 1999. pH-dependent hydrophobicity of the cyanobacteria toxin microcystin-LR. Water Res. 33, 677-680. https://doi.org/10.1016/S0043-1354(98)00258-9.

El Khalloufi, F., El Ghazali, I., Saqrane, S., Oufdou, K., Vasconcelos, V., Oudra, B., 2012. Phytotoxic effects of a natural bloom extract containing microcystins on *Lycopersicon esculentum*. Ecotoxicol. Environ. Saf. 79, 199–205. https://doi.org/10.1016/j.ecoenv.2012.01.002.

El Khalloufi, F., Oufdou, K., Lahrouni, M., Faghire, M., Peix, A., Ramirez-Bahena, M.H., Vasconcelos, V., Oudra, B., 2013. Physiological and antioxidant responses of *Medicago sativa*-rhizobia symbiosis to cyanobacterial toxins (Microcystins) exposure. Toxicon 76, 167–177. https://doi.org/10.1016/j.toxicon.2013.10.003.

Eriksson, J.E., Grönberg, L., Nygård, S., Slotte, J.P., Meriluoto, J.A., 1990. Hepatocellular uptake of 3H-dihydromicrocystin-LR, a cyclic peptide toxin. Biochim Biophys Acta Biomembr 1025, 60-66. https://doi.org/10.1016/0005-2736(90)90190-Y.

Evans, J.R., 1989. Photosynthesis and nitrogen relationships in leaves of C_3 plants. Oecologia 78, 9-19. https://doi.org/10.1007/BF00377192.

Facey, J.A., Apte, S.C., Mitrovic, S.M., 2019. A Review of the effect of trace metals on freshwater cyanobacterial growth and toxin production. Toxins 11, 643. https://doi.org/10.3390/toxins11110643.

Freitas, E.C., Pinheiro, C., Rocha, O., Loureiro, S., 2014. Can mixtures of cyanotoxins represent a risk to the zooplankton? The case study of *Daphnia magna* Straus exposed to hepatotoxic and neurotoxic cyanobacterial extracts. Harmful Algae 31, 143–152. https://doi.org/10.1016/j.hal.2013.11.004.

Freitas, M., Azevedo, J., Pinto, E., Neves, J., Campos, A., Vasconcelos, V., 2015. Effects of microcystin-LR, cylindrospermopsin and a microcystin-LR/cylindrospermopsin mixture on growth, oxidative stress and mineral content in lettuce plants (*Lactuca sativa* L.). Ecotoxicol. Environ. Saf. 116, 59-67. https://doi.org/10.1016/j.ecoenv.2015.02.002.

Giddings, M., Aranda-Rodriguez, R., Yasvinski, G., Watson, S., Zurawell, R., 2012. Canada: cyanobacterial toxins: drinking and recreational water quality guidelines. Current approaches to cyanotoxin risk assessment, risk management and regulations in different countries. Germany: Federal Environment Agency (Umweltbundesamt), 29-39.

Greer, B., McNamee, S.E., Boots, B., Cimarelli, L., Guillebault, D., Helmi, K., Marcheggiani, S., Panaiotov, S., Breitenbach, U., Akçaalan, R., 2016. A validated UPLC–MS/MS method for the surveillance of ten aquatic biotoxins in European brackish and freshwater systems. Harmful Algae 55, 31-40. https://doi.org/10.1016/j.hal.2016.01.006.

Gu, Y., Liang, C., 2020. Responses of antioxidative enzymes and gene expression in *Oryza sativa* L and *Cucumis sativus* L seedlings to microcystins stress. Ecotoxicol. Environ. Saf. 193, 110351. https://doi.org/10.1016/j.ecoenv.2020.110351.

Guo, Y., Liu, M., Liu, L., Liu, X., Chen, H., Yang, J., 2018. The antibiotic resistome of free-living and particle-attached bacteria under a reservoir cyanobacterial bloom. Environ. Int. 117, 107-115. https://doi.org/10.1016/j.envint.2018.04.045.

Gutiérrez-Praena, D., Campos, A., Azevedo, J., Neves, J., Freitas, M., Guzmán-Guillén, R., Cameán, A.M., Renaut, J. and Vasconcelos, V., 2014. Exposure of *Lycopersicon esculentum* to microcystin-LR: effects in the leaf proteome and toxin translocation from water to leaves and fruits. Toxins (Basel) 6, 1837–1854. https://doi.org/10.3390/toxins6061837.

Hedges, L.V., Vevea, J.L., 1998. Fixed-and random-effects models in meta-analysis. Psychol. Methods 3(4), p.486.

Hereman, T.C., Bittencourt-Oliveira Mdo, C., 2012. Bioaccumulation of Microcystins in Lettuce. J. Phycol. 48, 1535–1537. https://doi.org/10.1111/jpy.12006.

Ighodaro, O., Akinloye, O., 2018. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria J. Med. 54, 287-293. https://doi.org/10.1016/j.ajme.2017.09.001.

Ito, A., Wagai, R., 2017. Global distribution of clay-size minerals on land surface for biogeochemical and climatological studies. Sci. Data 4, 170103. https://doi.org/10.1038/sdata.2017.103.

Jahangir, M., Abdel-Farid, I.B., Kim, H.K., Choi, Y.H., Verpoorte, R., 2009. Healthy and unhealthy plants: The effect of stress on the metabolism of Brassicaceae. Environ. Exp. Bot. 67, 23-33. https://doi.org/10.1016/j.envexpbot.2009.06.007.

Jia, Y., Li, H., Qu, Y., Chen, W., Song, L., 2018. Phytotoxicity, bioaccumulation and potential risks of plant irrigations using cyanobloom-loading freshwater. Sci. Total Environ. 624, 704–712. https://doi.org/10.1016/j.scitotenv.2017.12.164.

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. Hazard. Mater. 190, 188-196. https://doi.org/10.1016/j.jhazmat.2011.03.023.

Jüttner, F., Lüthi, H., 2008. Topology and enhanced toxicity of bound microcystins in *Microcystis* PCC 7806. Toxicon 51, 388-397. https://doi.org/10.1016/j.toxicon.2007.10.013.

Kim, M., Lee, J., Yang, D., Park, H.Y., Park, W., 2020. Seasonal dynamics of the bacterial communities associated with cyanobacterial blooms in the Han River. Environ. Pollut. 115198. https://doi.org/10.1016/j.envpol.2020.115198.

Levitan, O., Rosenberg, G., Setlik, I., Setlikova, E., Grigel, J., Klepetar, J., Prasil, O., Berman-Frank, I., 2007. Elevated CO_2 enhances nitrogen fixation and growth in the marine cyanobacterium *Trichodesmium*. Glob. Chang. Biol. 13, 531–538. https://doi.org/10.1111/j.1365-2486.2006.01314.x.

Levizou, E., Statiris, G., Papadimitriou, T., Laspidou, C.S., Kormas, K.A., 2017. Lettuce facing microcystins-rich irrigation water at different developmental stages: Effects on plant performance and microcystins bioaccumulation. Ecotoxicol. Environ. Saf. 143, 193-200. https://doi.org/10.1016/j.ecoenv.2017.05.037.

Liang, C., Wang, W., 2015. Response and recovery of rice (*Oryza sativa*) seedlings to irrigation with microcystin-contaminated water. Environ. Earth. Sci. 73, 4573-4580. https://doi.org/10.1007/s12665-014-3746-z.

Liang, C., Liu, H., 2020. Response of hormone in rice seedlings to irrigation contaminated with cyanobacterial extract containing microcystins. Chemosphere, 127157. https://doi.org/10.1016/j.chemosphere.2020.127157.
Liu, Y.-L., Walker, H.W., Lenhart, J.J., 2019a. Adsorption of microcystin-LR onto kaolinite, illite and montmorillonite. Chemosphere 220, 696-705. https://doi.org/10.1016/j.chemosphere.2018.12.137.
Liu, Y.-L., Walker, H.W., Lenhart, J.J., 2019b. The effect of natural organic matter on the adsorption of microcystin-LR onto clay minerals. Colloids Surf. A Physicochem. Eng. Asp. 583, 123964. https://doi.org/10.1016/j.colsurfa.2019.123964.
Machado, J., Campos, A., Vasconcelos, V., Freitas, M., 2017. Effects of microcystin-LR and cylindrospermopsin on plant-soil systems: A review of their relevance for agricultural plant quality and public health. Environ. Res. 153, 191–204. https://doi.org/10.1016/j.envres.2016.09.015.
Mc Farlane, C., Trapp, S., 1994. Plant contamination: modeling and simulation of organic chemical processes. CRC Press.
McElhiney, J., Lawton, L.A., Leifert, C., 2001. Investigations into the inhibitory effects of microcystins on plant growth, and the toxicity of plant tissues following exposure. Toxicon 39, 1411–1420. https://doi.org/10.1016/S0041-0101(01)00100-3.
Meissner, S., Fastner, J., Dittmann, E., 2013. Microcystin production revisited: conjugate formation makes a major contribution. Environ. Microbiol. 15, 1810-1820. https://doi.org/10.1111/1462-2920.12072.
Mikhailov, A., Härmälä-Braskén, A.-S., Hellman, J., Meriluoto, J., Eriksson, J.E., 2003. Identification of ATP-synthase as a novel intracellular target for microcystin-LR. Chem. Biol. Interact 142, 223-237. https://doi.org/10.1016/S0009-2797(02)00075-3.
Miller, M.J., Hutson, J., Fallowfield, H.J., 2005. The adsorption of cyanobacterial hepatoxins as a function of soil properties. J. Water Health 3, 339-347. https://doi.org/10.2166/wh.2005.049.
Miller, A., Russell, C., 2017. Food crops irrigated with cyanobacteria-contaminated water: an emerging public health issue in Canada. Environ. Health Rev. 60, 58–63. https://doi.org/10.5864/d2017-021.
Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405-410. https://doi.org/10.1016/S1360-1385(02)02312-9.
Mohamed, Z.A., Al Shehri, A.M., 2009. Microcystins in groundwater wells and their accumulation in vegetable plants irrigated with contaminated waters in Saudi Arabia. J. Hazard. Mater. 172, 310–315. https://doi.org/10.1016/j.jhazmat.2009.07.010.
Neffling, M.-R., Lance, E., Meriluoto, J., 2010. Detection of free and covalently bound microcystins in animal tissues by liquid chromatography–tandem mass spectrometry. Environ. Pollut. 158, 948-952. https://doi.org/10.1016/j.envpol.2009.10.023.
Petrou, M., Karas, P., Vasileiadis, S., Zafiriadis, I., Papadimitriou, T., Levizou, E., Kormas, K., Karpouzas, D., 2020. Irrigation of radish (*Raphanus sativus* L.) with microcystin-enriched water holds low risk for plants and their associated rhizopheric and epiphytic microbiome. Environ. Pollut. 115208. https://doi.org/10.1016/j.envpol.2020.115208.
Pflugmacher, S., 2002. Possible allelopathic effects of cyanotoxins, with reference to microcystin-LR, in aquatic ecosystems. Environ. Toxicol. 17, 407-413. https://doi.org/10.1002/tox.10071.
Pflugmacher, S., Sulk, A., Kim, S., Esterhuizen-Londt, M., 2019. Translocation of the cyanobacterial toxin microcystin-LR into guttation drops of *Triticum aestivum* and remaining toxicity. Environ. Pollut. 253, 61-67. https://doi.org/10.1016/j.envpol.2019.07.027.
Pham, T.L., Utsumi, M., 2018. An overview of the accumulation of microcystins in aquatic ecosystems. J. Environ. Manage. 213, 520–529. https://doi.org/10.1016/j.jenvman.2018.01.077.
Pochodylo, A.L., Aoki, T.G., Aristilde, L., 2016. Adsorption mechanisms of microcystin variant conformations at water-mineral interfaces: A molecular modeling investigation. J. Colloid Interface Sci. 480, 166-174. https://doi.org/10.1016/j.jcis.2016.07.016.
Rastogi, R.P., Sinha, R.P., Incharoensakdi, A., 2014. The cyanotoxin-microcystins: current overview. Rev. Environ. Sci. Biotechnol. 13, 215–249. https://doi.org/10.1007/s11157-014-9334-6.
Rattan, R., Datta, S., Chhonkar, P., Suribabu, K., Singh, A., 2005. Long-term impact of irrigation with sewage effluents on heavy metal content in soils, crops and groundwater—a case study. Agric. Ecosyst. Environ. 109, 310–322. https://doi.org/10.1016/j.agee.2005.02.025.
Redouane, E.M., El Amrani Zerrifi, S., El Khalloufi, F., Oufdou, K., Oudra, B., Lahrouni, M., Campos, A., Vasconcelos, V., 2019. Mode of action and fate of microcystins in the complex soil-plant ecosystems. Chemosphere 225, 270–281. https://doi.org/10.1016/j.chemosphere.2019.03.008.

Romero-Oliva, C.S., Contardo-Jara, V., Block, T., Pflugmacher, S., 2014. Accumulation of microcystin congeners in different aquatic plants and crops–A case study from lake Amatitlán, Guatemala. Ecotoxicol. Environ. Saf. 102, 121–128. https://doi.org/10.1016/j.ecoenv.2014.01.031.

Romero-Oliva, C.S., Contardo-Jara, V., Pflugmacher, S., 2015. Time dependent uptake, bioaccumulation and biotransformation of cell free crude extract microcystins from Lake Amatitlan, Guatemala by *Ceratophyllum demersum*, *Egeria densa* and *Hydrilla verticillata*. Toxicon 105, 62–73. https://doi.org/10.1016/j.toxicon.2015.08.017.

Roy-Lachapelle, A, Vo Duy, S., Munoz, G., Dinh, Q.T., Bahl, E., Simon, D.F., Sauvé, S., 2019. Analysis of multiclass cyanotoxins (microcystins, anabaenopeptins, cylindrospermopsin and anatoxins) in lake waters using on-line SPE liquid chromatography high-resolution Orbitrap mass spectrometry. Anal. Methods 11, 5289–5300.

Sabart, M., Crenn, K., Perrière, F., Abila, A., Leremboure, M., Colombet, J., Jousse, C., Latour, D., 2015. Co-occurrence of microcystin and anatoxin-a in the freshwater lake Aydat (France): Analytical and molecular approaches during a three-year survey. Harmful Algae 48, 12–20. https://doi.org/10.1016/j.hal.2015.06.007.

Sadler, T., von Elert, E., 2014. Physiological interaction of *Daphnia* and *Microcystis* with regard to cyanobacterial secondary metabolites. Aquat. Toxicol. 156, 96-105. https://doi.org/10.1016/j.aquatox.2014.08.003.

Saqrane, S., Ouahid, Y., El Ghazali, I., Oudra, B., Bouarab, L., del Campo, F.F., 2009. Physiological changes in *Triticum durum*, *Zea mays*, *Pisum sativum* and *Lens esculenta* cultivars, caused by irrigation with water contaminated with microcystins: a laboratory experimental approach. Toxicon 53, 786–796. https://doi.org/10.1016/j.toxicon.2009.01.028.

Sharma, P., Kumar, A., Bhardwaj, R., 2016. Plant steroidal hormone epibrassinolide regulate–Heavy metal stress tolerance in *Oryza sativa* L. by modulating antioxidant defense expression. Environ. Exp. Bot. 122, 1-9. https://doi.org/10.1016/j.envexpbot.2015.08.005.

Sposito, G., Skipper, N.T., Sutton, R., Park, S.-h., Soper, A.K., Greathouse, J.A., 1999. Surface geochemistry of the clay minerals. Proc. Natl. Acad. Sci. U. S. A. 96, 3358-3364. https://doi.org/10.1073/pnas.96.7.3358.

Testai, E., Scardala, S., Vichi, S., Buratti, F.M., Funari, E., 2016. Risk to human health associated with the environmental occurrence of cyanobacterial neurotoxic alkaloids anatoxins and saxitoxins. Crit. Rev. Toxicol. 46, 385-419. https://doi.org/10.3109/10408444.2015.1137865.

Thirumavalavan, M., Hu, Y.L., Lee, J.F., 2012. Effects of humic acid and suspended soils on adsorption and photo-degradation of microcystin-LR onto samples from Taiwan reservoirs and rivers. J. Hazard Mater. 217-218, 323-329. https://doi.org/10.1016/j.jhazmat.2012.03.031.

US FDA (United States Food and Drug Adminstration), 2015a. Raw vegetables poster. https://www.fda.gov/food/food-labeling-nutrition/nutrition-information-raw-vegetables. Accessed date: 4 September 2017.

US EPA (United States Environmental Protection Agency), 2015b. Drinking water health advisory for the cyanobacterial microcystin toxins. https://www.epa.gov/sites/ production/files/2017-06/documents/microcystins-report-2015.pdf, Accessed date: 4 September 2017.

Vela, L., Sevilla, E., Gonzalez, C., Bes, M., Fillat, M., Peleato, M., 2008. Exploring the interaction of microcystin-LR with proteins and DNA. Toxicol. In Vitro 22, 1714-1718. https://doi.org/10.1016/j.tiv.2008.07.008.

Vesterkvist, P.S., Meriluoto, J.A., 2003. Interaction between microcystins of different hydrophobicities and lipid monolayers. Toxicon 41, 349-355. https://doi.org/10.1016/S0041-0101(02)00315-X.

Wang, Z., Xiao, B., Song, L., Wu, X., Zhang, J., Wang, C., 2011. Effects of microcystin-LR, linear alkylbenzene sulfonate and their mixture on lettuce (*Lactuca sativa* L.) seeds and seedlings. Ecotoxicology 20, 803–814. https://doi.org/10.1007/s10646-011-0632-2.

Ward, C., Codd, G., 1999. Comparative toxicity of four microcystins of different hydrophobicities to the protozoan, *Tetrahymena pyriformis*. J. Appl. Microbiol. 86, 874-882. https://doi.org/10.1046/j.1365-2672.1999.00771.x.

Wei, N., Hu, L., Song, L., Gan, N., 2016. Microcystin-bound protein patterns in different cultures of *Microcystis aeruginosa* and field samples. Toxins 8, 293. https://doi.org/10.3390/toxins8100293.

Welten, R.D., Meneely, J.P., Elliott, C.T., 2020. A Comparative Review of the Effect of Microcystin-LR on the Proteome. Expo. Health12, 111-129. https://doi.org/10.1007/s12403-019-00303-1.

Wijewickrama, M.M., Manage, P.M., 2019. Accumulation of Microcystin-LR in Grains of Two Rice Varieties (*Oryza sativa* L.) and a Leafy Vegetable, *Ipomoea aquatica*. Toxins 11, 432. https://doi.org/10.3390/toxins11080432.

Wolf, H.U., Frank, C., 2002. Toxicity assessment of cyanobacterial toxin mixtures. Environ. Toxicol. 17, 395–399. https://doi.org/10.1002/tox.10066.

Wu, X., Xiao, B., Li, R., Wang, C., Huang, J., Wang, Z., 2011. Mechanisms and factors affecting sorption of microcystins onto natural sediments. Environ. Sci. Technol. 45, 2641-2647. https://doi.org/10.1021/es103729m.

Xiang, L., Li, Y.W., Liu, B.L., Zhao, H.M., Li, H., Cai, Q.Y., Mo, C.H., Wong, M.H., Li, Q.X., 2019. High ecological and human health risks from microcystins in vegetable fields in southern China. Environ. Int. 133, 105142. https://doi.org/10.1016/j.envint.2019.105142.

Xue, Q., Steinman, A.D., Xie, L., Yao, L., Su, X., Cao, Q., Zhao, Y., Cai, Y., 2020. Seasonal variation and potential risk assessment of microcystins in the sediments of Lake Taihu, China. Environ. Pollu. 259, 113884. https://doi.org/10.1016/j.envpol.2019.113884.

Zhang, Y., Whalen, J.K., 2020a. Production of the neurotoxin beta-N-methylamino-L-alanine may be triggered by agricultural nutrients: An emerging public health issue. Water Res. 170, 115335. https://doi.org/10.1016/j.watres.2019.115335.

Zhang, Y., Whalen, J.K., Duy, S.V., Munoz, G., Husk, B.R., Sauvé, S., 2020b. Improved extraction of multiclass cyanotoxins from soil and sensitive quantification with on-line purification liquid chromatography tandem mass spectrometry. Talanta, 120923. https://doi.org/10.1016/j.talanta.2020.120923.

DISCUSSION AND FUTURE IMPLICATIONS

The overarching goal of this thesis is to explain how agricultural nutrients may trigger cyanotoxin production, which leads to the widespread occurrence of cyanotoxins in agricultural watersheds and their biomagnification into the agricultural food supply. Specifically, my thesis includes the first comprehensive review on the role of agricultural nutrient loadings in triggering beta-N-methylamino-L-alanine by freshwater cyanobacteria. It also documents the occurrence of cyanotoxins in soil and subsurface water in agricultural watersheds and presents a new, efficient method to determine the concentration of cyanotoxins in soils. The relevance of my research to public health is clear from my work on human health risks from microcystins that bioconcentrate in agricultural plants.

Research in Chapter 2 demonstrated that seasonal nutrient loading from agricultural land triggers the production of beta-N-methylamino-L-alanine by increasing the biomass of toxic cyanobacteria and altering the nitrogen availability in the freshwater environment. However, most of current understanding about beta-N-methylamino-L-alanine production in cyanobacteria is based on controlled laboratory work. Most field studies did not monitor the relationship between nitrogen availability and beta-N-methylamino-L-alanine production, which makes it difficult to extrapolate findings from the lab to the field level. My recommendation is for a long-term monitoring programs of nitrogen loading in freshwater systems. This information should help researchers to understand cyanobacterial blooms from an ecophysiological perspective. Specifically, we should monitor how seasonal fluxes of agricultural nutrient into lakes and other fresh waterbodies affects the nitrogen availability to distinct cyanobacteria groups, i.e., nitrogen fixing and toxigenic, non-nitrogen fixing and toxigenic, and non-toxigenic cyanobacteria.

Another critical research gap is the influence of nitrogen availability on the succession and competitive interactions among these groups. The overall recommendation from Chapter 2 is to promote more efficient nutrient management in agricultural land, which is the major source of nutrient loading in rivers and lakes. Excess nutrient loading during spring and early summer may enhance toxic cyanobacteria biomass, while lower nutrient levels during late summer and fall could create nitrogen-deficiency environments where cyanotoxins biosynthesis can be triggered in cyanobacteria.

Most research on cyanotoxins focuses on waterbodies, but my Chapter 3 confirms that freshwater lakes are just one of many 'hot spots' of cyanotoxin production in agricultural watersheds. My findings of cyanotoxins in soils and drinking water are important, because they confirm that cyanotoxins are produced in many environments or are transferred among environments (e.g., from the vadose zone to subsurface drainage and groundwater receptors). Since farmers in southern Quebec do not irrigate their fields with cyanobacteria-contaminated water. I suspect that soil cyanobacteria could be the source of microcystins found in agricultural fields. The next logical step in this research is to determine if soil cyanobacteria have the genes responsible for microcystins biosynthesis by comparing the genome of soil cyanobacteria with aquatic cyanobacteria. If soil cyanobacteria can produce microcystins, this major discovery should be followed by studies about the ecophysiological function of soil cyanobacteria, as well as their potential ecological and health risks in agriculutural soils.

My survey of cyanotoxins in Chapter 3 was limited by the availability of rapid, cost-effective analytical methods to detect and quantify cyanotoxins in all ecosystem components. I resolved this issue by tackling one of the most difficult matrix – the soil – and developing a new extraction method for soil cyanotoxins that is robust and reliable for 15 cyanotoxins with diverse

chemistry in Chapter 4. Purification was achieved quickly by coupling the pre-concentration process for a large volume of analyte to the analytical system with the on-line SPE-UHPLC-MS/MS. My extraction method with methanol + 200 mM ammonium acetate and rigorous quantification approach provide suitable accuracy and precision, within acceptance criteria, to meet these requirements for soil cyanotoxins. My work on developing a quantification method for cyanotoxins in soil is timely, given the growing recognition of the human health risks from these substances. The method can be fine-tuned to improve the recovery of microcystin-RR and cylindrospermopsin, which bind strongly to the soil matrix, but overall is qualified as a rapid, effective and accurate quantification procedure for soil cyanotoxins.

My cyanotoxins quantification procedure is a robust tool for solving the questions about fate of cyanotoxins in soil environments. For instance, my method makes it possible to study the cyanotoxin adsorption capacity of soil in dynamic (fixed-bed/column) and static (batch) experiments. Cyanotoxin sorption kinetics must be understood before we can make predictions about the possibility that cyanotoxins are leached and transported via other hydrological pathways from soil to groundwater in agricultural watersheds. With my cyanotoxins quantification procedure, it will be possible to determine the half-life (degradation rate) of cyanotoxins in soils.

Cyanotoxins are commonly associated with harmful algal blooms of cyanobacteria in eutrophic freshwater ecosystems, but I found that microcystins are present in agricultural soils and in soils that are adjacent to cyanotoxins-contaminated water (Chapter 3&4). This confirms a general idea that microcystins can be added unintentionally to agricultural fields that are irrigated or flooded with microcystins-contaminated water. Since microcystins negatively affect the physiological properties of agricultural plants, the phytotoxicity of microcystins is relatively well

documented and I was able to analyze data from 29 agricultural plants. Microcystins in the edible parts of food crops pose a risk for human consumers, and it is likely that agricultural plants grown in developing countries will come into contact with microcystins-contaminated water (due to the fact that surface waters are a cheap source of irrigation water), this is a public health concern. I recommend more field-based experiments on the fate and transfer of microcystins in agricultural fields to prevent microcystins from entering the agricultural food supply. Moving forward, more research should be conducted to protect farmer and consumers' health. At first, we should confirm if it is safe for farmers to operate the irrigation equipment when the water source is contaminated with cyanobacteria. I recommend preparing questionnaires to ask farmers about the frequency that they observe evidence of cyanobacteria in irrigation water sources, and about their sanitation practices. For instance, we can visit agricultural fields adjacent to bloom water during pre-bloom and post-bloom periods, asking the farmers if they ever suffed tired, skin irritation, eye allergy, ect. when irrigating their fields. It is also essential to design a simple test method for farmers to check the presence of cyanotoxins in an irrigation water source. From long-run, a systematic measurement and monitor for cyanotoxisn in agricultural food supply will prevent microcystins from entering food weds and help establish the food safety standards and guidelines for microcystins.

CONCLUSIONS & SUMMARY

In my thesis, I completed a critical review that explains how nutrient loading from agricultural runoff may trigger beta-N-methylamino-L-alanine production in cyanobacterial bloom in freshwater environments focusing a case study, i.e. the production of beta-N-methylamino-L-alanine in Lake Winnipeg, Canada (Chapter 2). Then, I confirmed that eutrophic waterbodies are not the only source of cyanotoxins by screening microcysitns,

cylindrospermopsin, anatoxin-a, saxitoxin and beta-N-methylamino-L-alanine in agricultural watershed in Quebec, Canada (Chapter 3). I developed a new method to extract and quantify the cyanotoxins in soil. The newly developed analytical method can detect 15 cyanotoxins in soils, accurately and efficiently (Chapter 4). Since I found microcystins in agricultural soils, I decided to evaluate the microcystins phytotoxicity in agricultural plants by meta-analysis and assessed their risk to humans (Chapter 5).

Overall, this thesis advanced our knowledge of cyanotoxins in agricultural watersheds and soil-plant systems. It highlights the fact that cyanotoxins occur in many environents – including soil and subsurface water – in addition to the cyanobacterial blooms of eutrophic waterbodies. More attention should be pulled on cyanotoxins in subsurface water and soil environments as we manage the risk of public exposure to cyanotoxins following One Health approach in the future. Therefore, my findings are of interest to governments and organizations with a mandate to protect public health from emerging environmental contaminants. My work will also be important to farmers, because it reveals a possible occupational hazard from cyanotoxin-contaminated irrigation water that is not known to agricultural extension services and other farm consultants. Although my research suggests that agricultural workers will be exposed to cyanotoxins in soil and in crops that bioconcentrate these cyanotoxins, I am encouraged by the fact that agricultural workers can be part of the solution when they follow nutrient management guidelines. The connection between nutrient excesses in the environment and cyanobacterial blooms is well-known, and the emerging evidence for nitrogen as a trigger for cyanotoxin production is another compelling reason for a more cautious, judicious use of nitrogen fertilizer in agriculture. Therefore, I end this thesis optimistically with hopes that my work will encourage better nutrient stewardship for a sustainable future in which cyanobacteria live in harmony with

other organisms, rather than as a threat to the ecological integrity and human health in agricultural watersheds.

REFERENCE

Al-Sammak, M.A., Hoagland, K.D., Cassada, D., Snow, D.D., 2014. Co-occurrence of the cyanotoxins BMAA, DABA and anatoxin-a in Nebraska reservoirs, fish, and aquatic plants. Toxins 6, 488-508.

Armah, A., Hiskia, A., Kaloudis, T., Chernoff, N., Hill, D., Antoniou, M.G., He, X., Loftin, K., O'Shea, K., Zhao, C., 2013. A review on cylindrospermopsin: the global occurrence, detection, toxicity and degradation of a potent cyanotoxin. Environmental Science: Processes and Impacts 15, 1979-2003.

Asplund-Samuelsson, J., Sundh, J., Dupont, C.L., Allen, A.E., McCrow, J.P., Celepli, N.A., Bergman, B., Ininbergs, K., Ekman, M., 2016. Diversity and expression of bacterial metacaspases in an aquatic ecosystem. Frontiers in Microbiology 7, 1043.

Ballot, A., Fastner, J., Lentz, M., Wiedner, C., 2010. First report of anatoxin-a-producing cyanobacterium Aphanizomenon issatschenkoi in northeastern Germany. Toxicon 56, 964-971.

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 Part A 62, 281-288.

Bar-On, Y.M., Phillips, R., Milo, R., 2018. The biomass distribution on Earth. Proceedings of the National Academy of Sciences 115, 6506-6511.

Bar-Zeev, E., Avishay, I., Bidle, K.D., Berman-Frank, I., 2013. Programmed cell death in the marine cyanobacterium Trichodesmium mediates carbon and nitrogen export. ISME J 7, 2340-2348.

Bartram, J., Chorus, I., 1999. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. CRC Press.

Bentzen, E., Taylor, W., Millard, E., 1992. The importance of dissolved organic phosphorus to phosphorus uptake by limnetic plankton. Limnology and Oceanography 37, 217-231.

Berntzon, L., Erasmie, S., Celepli, N., Eriksson, J., Rasmussen, U., Bergman, B., 2013. BMAA Inhibits Nitrogen Fixation in the Cyanobacterium Nostoc sp. PCC 7120. Marine Drugs 11, 3091.

Berry, J.P., Lind, O., 2010. First evidence of "paralytic shellfish toxins" and cylindrospermopsin in a Mexican freshwater system, Lago Catemaco, and apparent bioaccumulation of the toxins in "tegogolo" snails (Pomacea patula catemacensis). Toxicon 55, 930-938.

Billam, M., Tang, L., Cai, Q., Mukhi, S., Guan, H., Wang, P., Wang, Z., Theodorakis, C.W., Kendall, R.J., Wang, J.S., 2006. Seasonal variations in the concentration of microcystin-LR in two lakes in western Texas, USA. Environmental Toxicology and Chemistry: An International Journal 25, 349-355.

Bláhová, L., Babica, P., Adamovský, O., Kohoutek, J., Maršálek, B., Bláha, L., 2008. Analyses of cyanobacterial toxins (microcystins, cylindrospermopsin) in the reservoirs of the Czech Republic and evaluation of health risks. Environmental Chemistry Letters 6, 223-227.

Blahova, L., Oravec, M., Maršálek, B., Šejnohová, L., Šimek, Z., Bláha, L., 2009. The first occurrence of the cyanobacterial alkaloid toxin cylindrospermopsin in the Czech Republic as determined by immunochemical and LC/MS methods. Toxicon 53, 519-524.
Bohunická, M., Mareš, J., Hrouzek, P., Urajová, P., Lukeš, M., Šmarda, J., Komárek, J., Gaysina, L.A., Strunecký, O., 2015. A combined morphological, ultrastructural, molecular, and biochemical study of the peculiar family *Gomontiellaceae* (*Oscillatoriales*) reveals a new cylindrospermopsin-producing clade of cyanobacteria. Journal of phycology 51, 1040-1054.
Bouaïcha, N., Corbel, S., 2016. Cyanobacterial Toxins Emerging Contaminants in Soils: A Review of Sources, Fate and Impacts on Ecosystems, Plants and Animal and Human Health, Soil Contamination-Current Consequences and Further Solutions. InTech.
Bouaïcha, N., Miles, C.O., Beach, D.G., Labidi, Z., Djabri, A., Benayache, N.Y., Nguyen-Quang, T., 2019. Structural Diversity, Characterization and Toxicology of Microcystins. Toxins 11, 714.
Bown, A.W., Shelp, B.J., 2016. Plant GABA: not just a metabolite. Trends in plant science 21, 811-813.
Bownik, A., 2010. Harmful algae: effects of alkaloid cyanotoxins on animal and human health. Toxin Reviews 29, 99-114.
Brand, L.E., Pablo, J., Compton, A., Hammerschlag, N., Mash, D.C., 2010. Cyanobacterial blooms and the occurrence of the neurotoxin, beta-N-methylamino-l-alanine (BMAA), in South Florida aquatic food webs. Harmful algae 9, 620-635.
Brenner, E.D., Stevenson, D.W., McCombie, R.W., Katari, M.S., Rudd, S.A., Mayer, K.F., Palenchar, P.M., Runko, S.J., Twigg, R.W., Dai, G., 2003. Expressed sequence tag analysis in *Cycas*, the most primitive living seed plant. Genome biology 4, R78.
Briand, E., Yepremian, C., Humbert, J.F., Quiblier, C., 2008. Competition between microcystin- and non-microcystin-producing *Planktothrix agardhii* (cyanobacteria) strains under different environmental conditions. Environmental microbiology 10, 3337-3348.
Bumke-Vogt, C., Mailahn, W., Chorus, I., 1999. Anatoxin-a and neurotoxic cyanobacteria in German lakes and reservoirs. Environmental Toxicology: An International Journal 14, 117-125.
Caller, T.A., Doolin, J.W., Haney, J.F., Murby, A.J., West, K.G., Farrar, H.E., Ball, A., Harris, B.T., Stommel, E.W., 2009. A cluster of amyotrophic lateral sclerosis in New Hampshire: a possible role for toxic cyanobacteria blooms. Amyotrophic Lateral Sclerosis 10, 101-108.
Campos, A., Vasconcelos, V., 2010. Molecular mechanisms of microcystin toxicity in animal cells. International Journal of Molecular Sciences 11: 268–287.
Cao, Q., Steinman, A.D., Wan, X., Xie, L., 2018a. Bioaccumulation of microcystin congeners in soil-plant system and human health risk assessment: A field study from Lake Taihu region of China. Environmental pollution 240, 44-50.
Cao, Q., Steinman, A.D., Wan, X., Xie, L., 2018b. Bioaccumulation of microcystin congeners in soil-plant system and human health risk assessment: A field study from Lake Taihu region of China. Environ Pollut 240, 44-50.
Change, I.P.O.C., 2007. Climate change 2007: the physical science basis: summary for policymakers. Geneva: IPCC.

Chatziefthimiou, A.D., Richer, R., Rowles, H., Powell, J.T., Metcalf, J.S., 2014. Cyanotoxins as a potential cause of dog poisonings in desert environments. Veterinary Record 174, 484-485.

Chen, W., Jia, Y., Li, E., Zhao, S., Zhou, Q., Liu, L., Song, L., 2012. Soil-based treatments of mechanically collected cyanobacterial blooms from Lake Taihu: efficiencies and potential risks. Environmental science and technology 46, 13370-13376.

Chen, W., Li, L., Gan, N., Song, L., 2006a. Optimization of an effective extraction procedure for the analysis of microcystins in soils and lake sediments. Environental Pollution 143, 241-246.

Chen, W., Song, L., Gan, N., Li, L., 2006b. Sorption, degradation and mobility of microcystins in Chinese agriculture soils: risk assessment for groundwater protection. Environmental Pollution 144, 752-758.

Chia, M.A., Jankowiak, J.G., Kramer, B.J., Goleski, J.A., Huang, I.-S., Zimba, P.V., do Carmo Bittencourt-Oliveira, M., Gobler, C.J., 2018. Succession and toxicity of *Microcystis* and *Anabaena* (*Dolichospermum*) blooms are controlled by nutrient-dependent allelopathic interactions. Harmful algae 74, 67-77.

Chislock, M.F., Sarnelle, O., Jernigan, L.M., Wilson, A.E., 2013. Do high concentrations of microcystin prevent Daphnia control of phytoplankton? Water research 47, 1961-1970.

Chrapusta, E., Węgrzyn, M., Zabaglo, K., Kaminski, A., Adamski, M., Wietrzyk, P., Bialczyk, J., 2015. Microcystins and anatoxin-a in Arctic biocrust cyanobacterial communities. Toxicon 101, 35-40.

Cirés, S., Ballot, A., 2016. A review of the phylogeny, ecology and toxin production of bloom-forming *Aphanizomenon* spp. and related species within the *Nostocales* (cyanobacteria). Harmful Algae 54, 21-43.

Codd, G.A., Lindsay, J., Young, F.M., Morrison, L.F., Metcalf, J.S., 2005. Harmful cyanobacteria, Harmful cyanobacteria. Springer, pp. 1-23.

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.

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., 2005a. Diverse taxa of cyanobacteria produce β-N-methylamino-L-alanine, a neurotoxic amino acid. Proceedings of the National Academy of Sciences 102, 5074-5078.

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., 2005b. 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.

Cox, P.A., Richer, R., Metcalf, J.S., Banack, S.A., Codd, G.A., Bradley, W.G., 2009. Cyanobacteria and BMAA exposure from desert dust: a possible link to sporadic ALS among Gulf War veterans. Amyotrophic Lateral Sclerosis 10, 109-117.

Craighead, D., Metcalf, J.S., Banack, S.A., Amgalan, L., Reynolds, H.V., Batmunkh, M., 2009. Presence of the neurotoxic amino acids beta-N-methylamino-L-alanine (BMAA) and 2,4-diamino-butyric acid (DAB) in shallow springs from the Gobi Desert. Amyotroph Lateral Scler 10 Suppl 2, 96-100.

Cusick, K.D., Sayler, G.S., 2013. An overview on the marine neurotoxin, saxitoxin: genetics, molecular targets, methods of detection and ecological functions. Mar Drugs 11, 991-1018.
De, D.M., 2000. Evolution. When did photosynthesis emerge on Earth? Science (New York, NY) 289, 1703-1705.
Deblois, C.P., Juneau, P., 2010. Relationship between photosynthetic processes and microcystin in Microcystis aeruginosa grown under different photon irradiances. Harmful algae 9, 18-24.
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.
Ding, W.X., Shen, H.M., Ong, C.N., 2000a. Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. Hepatology 32, 547-555.
do Carmo Bittencourt-Oliveira, M., Chia, M.A., de Oliveira, H.S.B., Araújo, M.K.C., Molica, R.J.R., Dias, C.T.S., 2015. Allelopathic interactions between microcystin-producing and non-microcystin-producing cyanobacteria and green microalgae: implications for microcystins production. Journal of applied phycology 27, 275-284.
Downing, S., Banack, S., Metcalf, J., Cox, P., Downing, T., 2011. Nitrogen starvation of cyanobacteria results in the production of β-N-methylamino-L-alanine. Toxicon 58, 187-194.
Downing, T.G., Phelan, R.R., Downing, S., 2015. A potential physiological role for cyanotoxins in cyanobacteria of arid environments. Journal of Arid Environments 112, 147-151.
Duy, S.V., Munoz, G., Dinh, Q.T., Do, D.T., Simon, D.F., Sauve, S., 2019. Analysis of the neurotoxin β-N-methylamino-L-alanine (BMAA) and isomers in surface water by FMOC derivatization liquid chromatography high resolution mass spectrometry. PloS one 14.
Edition, F., 2011. Guidelines for drinking-water quality. WHO chronicle 38, 104-108.
EPA:, U., 2015. Drinking water health advisory for the cyanobacterial microcystin toxins.
Esterhuizen, M., Downing, T., 2008. β-N-methylamino-L-alanine (BMAA) in novel South African cyanobacterial isolates. Ecotoxicology and environmental safety 71, 309-313.
Eynard, F., Mez, K., Walther, J.-L., 2000. Risk of cyanobacterial toxins in Riga waters (Latvia). Water Research 34, 2979-2988.
Facey, J.A., Apte, S.C., Mitrovic, S.M., 2019. A Review of the Effect of Trace Metals on Freshwater Cyanobacterial Growth and Toxin Production. Toxins 11, 643.
Fayad, P.B., Roy-Lachapelle, A., Duy, S.V., Prévost, M., Sauvé, S., 2015. On-line solid-phase extraction coupled to liquid chromatography tandem mass spectrometry for the analysis of cyanotoxins in algal blooms. Toxicon 108, 167-175.
Fischer, W.J., Altheimer, S., Cattori, V., Meier, P.J., Dietrich, D.R., Hagenbuch, B., 2011. Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. Toxicology and Applied Pharmacology 203, 257-263.
Flores, E., Herrero, A., 1994. Assimilatory nitrogen metabolism and its regulation, The molecular biology of cyanobacteria. Springer, pp. 487-517.
Flores, E., Herrero, A., 2005. Nitrogen assimilation and nitrogen control in cyanobacteria. Portland Press Ltd.
Flores, N.M., Miller, T.R., Stockwell, J.D., 2018. A global analysis of the relationship between concentrations of microcystins in water and fish. Frontiers in Marine Science 5, 30.

Francis, G., 1878. Poisonous Australian Lake. Nature 18, 11.
Franklin, D.J., 2014. Explaining the causes of cell death in cyanobacteria: what role for asymmetric division? Journal of Plankton Research 36, 11-17.
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: An International Journal 18, 243-251.
Funari, E., Testai, E., 2008. Human health risk assessment related to cyanotoxins exposure. Crit Rev Toxicol 38, 97-125.
Gan, N., Xiao, Y., Zhu, L., Wu, Z., Liu, J., Hu, C., Song, L., 2012. The role of microcystins in maintaining colonies of bloom-forming *Microcystis* spp. Environmental Microbiology 14, 730-742.
Ger, K.A., Faassen, E.J., Pennino, M.G., Lürling, M., 2016a. Effect of the toxin (microcystin) content of Microcystis on copepod grazing. Harmful algae 52, 34-45.
Ger, K.A., Urrutia-Cordero, P., Frost, P.C., Hansson, L.-A., Sarnelle, O., Wilson, A.E., Lürling, M., 2016b. The interaction between cyanobacteria and zooplankton in a more eutrophic world. Harmful algae 54, 128-144.
Griffin, D.W., 2007. Atmospheric movement of microorganisms in clouds of desert dust and implications for human health. Clinical microbiology reviews 20, 459-477.
Hawkins, P.R., Chandrasena, N.R., Jones, G.J., Humpage, A.R., Falconer, I.R., 1997. Isolation and toxicity of *Cylindrospermopsis raciborskii* from an ornamental lake. Toxicon 35, 341-346.
He, Y., Zhou, Q.-H., Liu, B.-Y., Cheng, L., Tian, Y., Zhang, Y.-Y., Wu, Z.-B., 2016. Programmed cell death in the cyanobacterium Microcystis aeruginosa induced by allelopathic effect of submerged macrophyte *Myriophyllum spicatum* in co-culture system. Journal of Applied Phycology 28, 2805-2814.
Hesse, K., Dittmann, E., Börner, T., 2001. Consequences of impaired microcystin production for light-dependent growth and pigmentation of Microcystis aeruginosa PCC 7806. FEMS microbiology ecology 37, 39-43.
Hilborn, E.D., Beasley, V.R., 2015. One health and cyanobacteria in freshwater systems: Animal illnesses and deaths are sentinel events for human health risks. Toxins 7, 1374-1395.
Hipsher, C., Barker, J., MacKay, A., 2020. Impact of bloom events on dissolved organic matter fluorophore signatures in Ohio waters. Science of the Total Environment 699, 134003.
Holland, A., Kinnear, S., 2013. Interpreting the possible ecological role(s) of cyanotoxins: compounds for competitive advantage and/or physiological aide? Marine Drugs 11, 2239-2258.
Holtcamp, W., 2012. The emerging science of BMAA: do cyanobacteria contribute to neurodegenerative disease? Environmental health perspectives 120, a110.
Hu, C., Rzymski, P., 2019. Programmed Cell Death-Like and Accompanying Release of Microcystin in Freshwater Bloom-Forming Cyanobacterium Microcystis: From Identification to Ecological Relevance. Toxins 11, 706.
Huisman, J., Codd, G.A., Paerl, H.W., Ibelings, B.W., Verspagen, J.M., Visser, P.M., 2018. Cyanobacterial blooms. Nature Reviews Microbiology 16, 471-483.
James, K.J., Sherlock, I.R., Stack, M.A., 1997. Anatoxin-a in Irish freshwater and cyanobacteria, determined using a new fluorimetric liquid chromatographic method. Toxicon 35, 963-971.

Jasionek, G., Zhdanov, A., Davenport, J., Bláha, L., Papkovsky, D.B., 2010. Mitochondrial toxicity of microcystin-LR on cultured cells: application to the analysis of contaminated water samples. Environental Science and Technology 44, 2535-2541.

Jiang, L., Kiselova, N., Rosén, J., Ilag, L.L., 2014a. Quantification of neurotoxin BMAA (β-N-methylamino-L-alanine) in seafood from Swedish markets. Scientific reports 4, 6931.

Jiang, Y., Xiao, P., Yu, G., Shao, J., Liu, D., Azevedo, S.M., Li, R., 2014b. Sporadic distribution and distinctive variations of cylindrospermopsin genes in cyanobacterial strains and environmental samples from Chinese freshwater bodies. Applied and Environmental Microbiology 80, 5219-5230.

Jonasson, S., Eriksson, J., Berntzon, L., Spacil, Z., Ilag, L.L., Ronnevi, L.O., Rasmussen, U., Bergman, B., 2010. Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure. Proceedings of the National Academy of Sciences of the United States of America 107, 9252-9257.

Kaebernick, M., Neilan, B.A., 2001. Ecological and molecular investigations of cyanotoxin production. FEMS microbiology ecology 35, 1-9.

Kaplan, A., Harel, M., Kaplan-Levy, R.N., Hadas, O., Sukenik, A., Dittmann, E., 2012. The languages spoken in the water body (or the biological role of cyanobacterial toxins). Frontiers in Microbiology 3.

Kaushik, R., Balasubramanian, R., 2013. Methods and approaches used for detection of cyanotoxins in environmental samples: a review. Critical reviews in environmental science and technology 43, 1349-1383.

Kearns, K., Hunter, M., 2001. Toxin-producing *Anabaena flos-aquae* induces settling of *Chlamydomonas reinhardtii*, a competing motile alga. Microbial ecology 42, 80-86.

Keating, K.I., 1978. Blue-green algal inhibition of diatom growth: transition from mesotrophic to eutrophic community structure. Science 199, 971-973.

Kinnear, S.H., Duivenvoorden, L.J., Fabbro, L.D., 2007. Growth and bioconcentration in 'Spirodela oligorrhiza' following exposure to 'Cylindrospermopsis raciborskii' whole cell extracts. Australasian Journal of Ecotoxicology 13, 19.

Komárek, J., Kaštovský, J., Mareš, J., Johansen, J.R., 2014. Taxonomic classification of cyanoprokaryotes (cyanobacterial genera) 2014, using a polyphasic approach. Preslia 86, 295-335.

Kujbida, P., Hatanaka, E., Campa, A., Colepicolo, P., Pinto, E., 2006. Effects of microcystins on human polymorphonuclear leukocytes. Biochemical and Biophysical Research Communications 341, 273-277.

Kujbida, P., Hatanaka, E., Vinolo, M.A., Waismam, K., Cavalcanti, D.M., Curi, R., Farsky, S.H., Pinto, E., 2009. Microcystins -LA, -YR, and -LR action on neutrophil migration. Biochemical and Biophysical Research Communications 382:9–14.

Kurmayer, R., Christiansen, G., Chorus, I., 2003. The abundance of microcystin-producing genotypes correlates positively with colony size in Microcystis sp. and determines its microcystin net production in Lake Wannsee. Applied and Environmental Microbiology 69, 787-795.

Ledreux, A., Thomazeau, S., Catherine, A., Duval, C., Yéprémian, C., Marie, A., Bernard, C., 2010. Evidence for saxitoxins production by the cyanobacterium *Aphanizomenon gracile* in a French recreational water body. Harmful Algae 10, 88-97.

Lee, S., Jiang, X., Manubolu, M., Riedl, K., Ludsin, S.A., Martin, J.F., Lee, J., 2017. Fresh produce and their soils accumulate cyanotoxins from irrigation water: Implications for public health and food security. Food research international 102, 234-245.
Li, B., Yu, S., Li, G., Chen, X., Huang, M., Liao, X., Li, H., Hu, F., Wu, J., 2019. Transfer of a cyanobacterial neurotoxin, β-methylamino-l-alanine from soil to crop and its bioaccumulation in Chinese cabbage. Chemosphere 219, 997-1001.
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.
Lilleheil, G., Andersen, R.A., Skulberg, O.M., Alexander, J., 1997. Effects of a homoanatoxin-a-containing extract from *Oscillatoria formosa* (Cyanophyceae/cyanobacteria) on neuromuscular transmission. Toxicon 35, 1275-1289.
Liu, Y., Chen, W., Li, D., Huang, Z., Shen, Y., Liu, Y., 2011. Cyanobacteria-/cyanotoxin-contaminations and eutrophication status before Wuxi drinking water crisis in Lake Taihu, China. Journal of Environmental Sciences 23, 575-581.
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 Disease 25, 360-366.
Machado, J., Campos, A., Vasconcelos, V., Freitas, M., 2017. Effects of microcystin-LR and cylindrospermopsin on plant-soil systems: A review of their relevance for agricultural plant quality and public health. Environmental research 153, 191-204.
Mahmood, N.A., Carmichael, W.W., 1986. The pharmacology of anatoxin-a (s), a neurotoxin produced by the freshwater cyanobacterium Anabaena flos-aquae NRC 525-17. Toxicon 24, 425-434.
Mankiewicz-Boczek, J., Kokociński, M., Gagała, I., Pawełczyk, J., Jurczak, T., Dziadek, J., 2012. Preliminary molecular identification of cylindrospermopsin-producing Cyanobacteria in two Polish lakes (Central Europe). FEMS microbiology letters 326, 173-179.
Manubolu, M., Lee, J., Riedl, K.M., Kua, Z.X., Collart, L.P., Ludsin, S.A., 2018. Optimization of extraction methods for quantification of microcystin-LR and microcystin-RR in fish, vegetable, and soil matrices using UPLC-MS/MS. Harmful Algae 76, 47-57.
Martín-Figueroa, E., Navarro, F., Florencio, F.J., 2000. The GS-GOGAT pathway is not operative in the heterocysts. Cloning and expression of glsF gene from the cyanobacterium Anabaena sp. PCC 7120. FEBS letters 476, 282-286.
Mashile, G.P., Nomngongo, P.N., 2017. Recent application of solid phase based techniques for extraction and preconcentration of cyanotoxins in environmental matrices. Critical reviews in analytical chemistry 47, 119-126.
Máthé, C., Vasas, G., 2013. Microcystin-LR and cylindrospermopsin induced alterations in chromatin organization of plant cells. Marine drugs 11, 3689-3717.
McCord, J., Lang, J.R., Hill, D., Strynar, M., Chernoff, N., 2018. pH dependent octanol–water partitioning coefficients of microcystin congeners. Journal of Water and Health, wh2018257.
Méjean, A., Paci, G., Gautier, V., Ploux, O., 2014. Biosynthesis of anatoxin-a and analogues (anatoxins) in cyanobacteria. Toxicon 91, 15-22.

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., Banack, S., Richer, R., Cox, P., 2015a. Neurotoxic amino acids and their isomers in desert environments. Journal of Arid Environments 112, 140-144.
Metcalf, J., Richer, R., Cox, P., Codd, G., 2012. Cyanotoxins in desert environments may present a risk to human health. Science of the Total Environment 421, 118-123.
Metcalf, J.S., Banack, S.A., Lindsay, J., Morrison, L.F., Cox, P.A., Codd, G.A., 2008. Co-occurrence of β-N-methylamino-l-alanine, a neurotoxic amino acid with other cyanobacterial toxins in British waterbodies, 1990–2004. Environmental microbiology 10, 702-708.
Metcalf, J.S., Banack, S.A., Richer, R., Cox, P.A., 2015b. Neurotoxic amino acids and their isomers in desert environments. Journal of Arid Environments 112, 140-144.
Mohamed, Z.A., 2017. Macrophytes-cyanobacteria allelopathic interactions and their implications for water resources management—A review. Limnologica 63, 122-132.
Mohamed, Z.A., Al Shehri, A.M., 2009. Microcystins in groundwater wells and their accumulation in vegetable plants irrigated with contaminated waters in Saudi Arabia. Journal of Hazardous Materials 172, 310-315.
Molica, R.J., Oliveira, E.J., Carvalho, P.V., Costa, A.N., Cunha, M.C., Melo, G.L., Azevedo, S.M., 2005. Occurrence of saxitoxins and an anatoxin-a (s)-like anticholinesterase in a Brazilian drinking water supply. Harmful algae 4, 743-753.
Mondo, K., Hammerschlag, N., Basile, M., Pablo, J., Banack, S.A., Mash, D.C., 2012. Cyanobacterial neurotoxin β-N-methylamino-L-alanine (BMAA) in shark fins. Marine drugs 10, 509-520.
Monteiro, M., Costa, M., Moreira, C., Vasconcelos, V.M., Baptista, M.S., 2016. Screening of BMAA-producing cyanobacteria in cultured isolates and in in situ blooms. Journal of Applied Phycology 29, 879-888.
Monteiro, M., Costa, M., Moreira, C., Vasconcelos, V.M., Baptista, M.S., 2017. Screening of BMAA-producing cyanobacteria in cultured isolates and in in situ blooms. Journal of applied phycology 29, 879-888.
Mooij, W.M., Hülsmann, S., Domis, L.N.D.S., Nolet, B.A., Bodelier, P.L., Boers, P.C., Pires, L.M.D., Gons, H.J., Ibelings, B.W., Noordhuis, R., 2005. The impact of climate change on lakes in the Netherlands: a review. Aquatic Ecology 39, 381-400.
Moreira, C., Azevedo, J., Antunes, A., Vasconcelos, V., 2013. Cylindrospermopsin: Occurrence, methods of detection and toxicology. Journal of Applied Microbiology 114, 605-620.
Moreira, C., Ramos, V., Azevedo, J., Vasconcelos, V., 2014. Methods to detect cyanobacteria and their toxins in the environment. Applied microbiology and biotechnology 98, 8073-8082.
Munoz, G., Vo Duy, S., Roy-Lachapelle, A., Husk, B., Sauve, S., 2017. Analysis of individual and total microcystins in surface water by on-line preconcentration and desalting coupled to liquid chromatography tandem mass spectrometry. Journal of Chromatography A 1516, 9-20.
Nasri, H., El Herry, S., Bouaïcha, N., 2008. First reported case of turtle deaths during a toxic Microcystis spp. bloom in Lake Oubeira, Algeria. Ecotoxicology and Environmental Safety 71, 535-544.

O'Neill, K., Musgrave, I.F., Humpage, A., 2016. Low dose extended exposure to saxitoxin and its potential neurodevelopmental effects: A review. Environmental toxicology and pharmacology 48, 7-16.
Ohtani, I., Moore, R.E., Runnegar, M.T., 1992. Cylindrospermopsin: a potent hepatotoxin from the blue-green alga Cylindrospermopsis raciborskii. Journal of the American Chemical Society 114, 7941-7942.
Organization, W.H., 2003. Guidelines for safe recreational water environments: Coastal and fresh waters. World Health Organization.
Orr, P.T., Jones, G.J., Hamilton, G.R., 2004. Removal of saxitoxins from drinking water by granular activated carbon, ozone and hydrogen peroxide—implications for compliance with the Australian drinking water guidelines. Water research 38, 4455-4461.
Paerl, H.W., Huisman, J., 2009. Climate change: a catalyst for global expansion of harmful cyanobacterial blooms. Environ Microbiol Rep 1, 27-37.
Pearson, L., Mihali, T., Moffitt, M., Kellmann, R., Neilan, B., 2010. On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. Marine drugs 8, 1650-1680.
Pflugmacher, S., 2002. Possible allelopathic effects of cyanotoxins, with reference to microcystin-LR, in aquatic ecosystems. Environmental Toxicology 17, 407-413.
Pham, T.L., Utsumi, M., 2018. An overview of the accumulation of microcystins in aquatic ecosystems. Journal of Environmental Management 213, 520-529.
Phelan, R.R., Downing, T.G., 2014. The localization of exogenous microcystin LR taken up by a non-microcystin producing cyanobacterium. Toxicon 89, 87-90.
Pip, E., Munford, K., Bowman, L., 2016. Seasonal nearshore occurrence of the neurotoxin β-N-methylamino-L-alanine (BMAA) in Lake Winnipeg, Canada. Environ. Pollut 5, 110-118.
Pomati, F., Rossetti, C., Calamari, D., Neilan, B.A., 2003. Effects of saxitoxin (STX) and veratridine on bacterial Na+-K+ fluxes: a prokaryote-based STX bioassay. Applied and Environmental Microbiology 69, 7371-7376.
Popova, A.A., Rasmussen, U., Semashko, T.A., Govorun, V.M., Koksharova, O.A., 2018. Stress effects of cyanotoxin beta-methylamino-L-alanine (BMAA) on cyanobacterial heterocyst formation and functionality. Environmental Microbiology Reports 10, 369-377.
Prinsep, M.R., Caplan, F.R., Moore, R.E., Patterson, G.M., Honkanen, R.E., Boynton, A.L., 1992. Microcystin-LA from a blue-green alga belonging to the Stigonematales. Phytochemistry 31, 1247-1248.
Puente-Sánchez, F., Arce-Rodríguez, A., Oggerin, M., García-Villadangos, M., Moreno-Paz, M., Blanco, Y., Rodríguez, N., Bird, L., Lincoln, S.A., Tornos, F., 2018. Viable cyanobacteria in the deep continental subsurface. Proceedings of the National Academy of Sciences 115, 10702-10707.
Rao, S.D., Banack, S.A., Cox, P.A., Weiss, J.H., 2006. BMAA selectively injures motor neurons via AMPA/kainate receptor activation. Experimental neurology 201, 244-252.
Rapala, J., Robertson, A., Negri, A.P., Berg, K.A., Tuomi, P., Lyra, C., Erkomaa, K., Lahti, K., Hoppu, K., Lepistö, L., 2005. First report of saxitoxin in Finnish lakes and possible associated effects on human health. Environmental Toxicology: An International Journal 20, 331-340.
Rastogi, R.P., Sinha, R.P., Incharoensakdi, A., 2014. The cyanotoxin-microcystins: current overview. Reviews in Environmental Science and Bio-Technology 13, 215-249.
Raven, J.A., 2010. Cyanotoxins: a poison that frees phosphate. Current Biology 20, R850-R852.

Redouane, E.M., El Amrani Zerrifi, S., El Khalloufi, F., Oufdou, K., Oudra, B., Lahrouni, M., Campos, A., Vasconcelos, V., 2019. Mode of action and fate of microcystins in the complex soil-plant ecosystems. Chemosphere 225, 270-281.
Richer, R., Banack, S.A., Metcalf, J.S., Cox, P.A., 2015. The persistence of cyanobacterial toxins in desert soils. Journal of Arid Environments 112, 134-139.
Rodgers, K.J., Main, B.J., Samardzic, K., 2018. Cyanobacterial Neurotoxins: Their Occurrence and Mechanisms of Toxicity. Neurotoxicity Research 33, 168-177.
Roy-Lachapelle, A., Duy, S.V., Munoz, G., Dinh, Q.T., Bahl, E., Simon, D.F., Sauvé, S., 2019. Analysis of multiclass cyanotoxins (microcystins, anabaenopeptins, cylindrospermopsin and anatoxins) in lake waters using on-line SPE liquid chromatography high-resolution Orbitrap mass spectrometry. Analytical Methods 11, 5289-5300.
Runnegar, M.T., Kong, S.-M., Zhong, Y.-Z., Ge, J.-L., Lu, S.C., 1994. The role of glutathione in the toxicity of a novel cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. Biochemical and biophysical research communications 201, 235-241.
Rzymski, P., Poniedzialek, B., 2015. The surprising world of cyanobacteria: cylindrospermopsin has a soil face. Journal of Phycology 51, 1037-1039.
Rzymski, P., Poniedziałek, B., 2014. In search of environmental role of cylindrospermopsin: a review on global distribution and ecology of its producers. Water research 66, 320-337.
Rzymski, P., Poniedziałek, B., Kokociński, M., Jurczak, T., Lipski, D., Wiktorowicz, K., 2014. Interspecific allelopathy in cyanobacteria: Cylindrospermopsin *and Cylindrospermopsis raciborskii* effect on the growth and metabolism of *Microcystis aeruginosa*. Harmful Algae 35, 1-8.
Sadler, T., von Elert, E., 2014. Physiological interaction of Daphnia and Microcystis with regard to cyanobacterial secondary metabolites. Aquatic toxicology 156, 96-105.
Saito, K., Sei, Y., Miki, S., Yamaguchi, K., 2008. Detection of microcystin–metal complexes by using cryospray ionization-Fourier transform ion cyclotron resonance mass spectrometry. Toxicon 51, 1496-1498.
Schatz, D., Keren, Y., Vardi, A., Sukenik, A., Carmeli, S., Börner, T., Dittmann, E., Kaplan, A., 2007. Towards clarification of the biological role of microcystins, a family of cyanobacterial toxins. Environmental microbiology 9, 965-970.
Scott, L., Downing, S., Phelan, R., Downing, T., 2014. Environmental modulation of microcystin and β-N-methylamino-l-alanine as a function of nitrogen availability. Toxicon 87, 1-5.
Serrano, T., Dupas, R., Upegui, E., Buscail, C., Grimaldi, C., Viel, J.F., 2015. Geographical modeling of exposure risk to cyanobacteria for epidemiological purposes. Environment International 81, 18-25.
Sevilla, E., Martin-Luna, B., Bes, M.T., Fillat, M.F., Peleato, M.L., 2012. An active photosynthetic electron transfer chain required for *mcy*D transcription and microcystin synthesis in *Microcystis aeruginosa* PCC7806. Ecotoxicology 21, 811-819.
Shaw, G.R., Sukenik, A., Livne, A., Chiswell, R.K., Smith, M.J., Seawright, A.A., Norris, R.L., Eaglesham, G.K., Moore, M.R., 1999. Blooms of the cylindrospermopsin containing cyanobacterium, Aphanizomenon ovalisporum (Forti), in newly constructed lakes, Queensland, Australia. Environmental Toxicology: An International Journal 14, 167-177.
Shi, L., Carmichael, W.W., Miller, I., 1995. Immuno-gold localization of hepatotoxins in cyanobacterial cells. Archives of microbiology 163, 7-15.
Sigee, D., Selwyn, A., Gallois, P., Dean, A., 2007. Patterns of cell death in freshwater colonial cyanobacteria during the late summer bloom. Phycologia 46, 284-292.

Sivonen, K., Jones, G., 1999. Cyanobacterial toxins. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management 1, 43-112.
Spoof, L., Vesterkvist, P., Lindholm, T., Meriluoto, J., 2003. Screening for cyanobacterial hepatotoxins, microcystins and nodularin in environmental water samples by reversed-phase liquid chromatography–electrospray ionisation mass spectrometry. Journal of chromatography A 1020, 105-119.
Svirčev, Z., Baltić, V., Gantar, M., Juković, M., Stojanović, D. and Baltić, M., 2010. Molecular aspects of microcystin-induced hepatotoxicity and hepatocarcinogenesis. Journal of Environmental Science and Health Part C, 28(1), pp.39-59.
Svirčev, Z., Lalić, D., Savić, G.B., Tokodi, N., Backović, D.D., Chen, L., Meriluoto, J., Codd, G.A., 2019. Global geographical and historical overview of cyanotoxin distribution and cyanobacterial poisonings. Archives of toxicology, 1-53.
Testai, E., Scardala, S., Vichi, S., Buratti, F.M., Funari, E., 2016. Risk to human health associated with the environmental occurrence of cyanobacterial neurotoxic alkaloids anatoxins and saxitoxins. Critical reviews in toxicology 46, 385-419.
Teta, R., Della Sala, G., Glukhov, E., Gerwick, L., Gerwick, W.H., Mangoni, A., Costantino, V., 2015. Combined LC–MS/MS and molecular networking approach reveals new cyanotoxins from the 2014 cyanobacterial bloom in Green Lake, Seattle. Environmental Science and Technology 49, 14301-14310.
Thottumkara, A.P., Parsons, W.H., Du Bois, J., 2014. Saxitoxin. Angewandte Chemie International Edition 53, 5760-5784.
Tian, D., Zheng, W., Wei, X., Sun, X., Liu, L., Chen, X., Zhang, H., Zhou, Y., Chen, H., Zhang, H., 2013. Dissolved microcystins in surface and ground waters in regions with high cancer incidence in the Huai River Basin of China. Chemosphere 91, 1064-1071.
Tillmanns, A.R., Wilson, A.E., Pick, F.R., Sarnelle, O., 2008. Meta-analysis of cyanobacterial effects on zooplankton population growth rate: species-specific responses. Fundamental and Applied Limnology/Archiv für Hydrobiologie 171, 285-295.
Tilzer, M.M., 1987. Light‐dependence of photosynthesis and growth in cyanobacteria: Implications for their dominance in eutrophic lakes. New Zealand Journal of Marine and Freshwater Research 21, 401-412.
US FDA (United States Food and Drug Adminstration), 2015a. Raw vegetables poster. https://www.fda.gov/food/food-labeling-nutrition/nutrition-information-raw-vegetables. Accessed date: 4 September 2017.
Van de Waal, D.B., Verspagen, J.M., Finke, J.F., Vournazou, V., Immers, A.K., Kardinaal, W.E.A., Tonk, L., Becker, S., Van Donk, E., Visser, P.M., 2011. Reversal in competitive dominance of a toxic versus non-toxic cyanobacterium in response to rising CO_2. The ISME journal 5, 1438-1450.
Vasas, G., Surányi, G., Máthé, C., Márta, M., Borbély, G., 2010. Investigation of toxin content in Cylindrospermopsis raciborskii (Wołoszyńska) Seenaya and Subba Raju and Aphanizomenon ovalisporum (Forti) strains isolated from shallow lakes of Hungary. Acta Biologica Hungarica 61, 218-225.
Vega, A., Bell, E.A., 1967. α-Amino-β-methylaminopropionic acid, a new amino acid from seeds of Cycas circinalis. Phytochemistry 6, 759-762.
Wang, N.-X., Zhang, X.-Y., Wu, J., Xiao, L., Yin, Y., Miao, A.-J., Ji, R., Yang, L.-Y., 2012. Effects of microcystin-LR on the metal bioaccumulation and toxicity in *Chlamydomonas reinhardtii*. Water research 46, 369-377.

Weiland, M., Mancuso, S., Baluska, F., 2016. Signalling via glutamate and GLRs in *Arabidopsis thaliana*. Functional Plant Biology 43, 1-25.
Whitman, W.B., Coleman, D.C., Wiebe, W.J., 1998. Prokaryotes: the unseen majority. Proceedings of the National Academy of Sciences 95, 6578-6583.
Whitton, B.A., Potts, M., 2012. Introduction to the cyanobacteria, Ecology of Cyanobacteria II. Springer, pp. 1-13.
Wiese, M., D'agostino, P.M., Mihali, T.K., Moffitt, M.C., Neilan, B.A., 2010. Neurotoxic alkaloids: saxitoxin and its analogs. Marine drugs 8, 2185-2211.
Wilson, A.E., Sarnelle, O., Tillmanns, A.R., 2006. Effects of cyanobacterial toxicity and morphology on the population growth of freshwater zooplankton: Meta-analyses of laboratory experiments. Limnology and Oceanography 51, 1915-1924.
Wu, X., Xiao, B., Li, R., Wang, C., Huang, J., Wang, Z., 2011. Mechanisms and factors affecting sorption of microcystins onto natural sediments. Environmental Science and Technology 45, 2641-2647.
Xiang, L., Li, Y.-W., Liu, B.-L., Zhao, H.-M., Li, H., Cai, Q.-Y., Mo, C.-H., Wong, M.-H., Li, Q.X., 2019. High ecological and human health risks from microcystins in vegetable fields in southern China. Environment International 133, 105142.
Yang, Z., Kong, F., 2012. Formation of large colonies: a defense mechanism of *Microcystis aeruginosa* under continuous grazing pressure by flagellate *Ochromonas* sp. Journal of Limnology 71, e5.
Yang, Z., Kong, F., Zhang, M., 2016. Groundwater contamination by microcystin from toxic cyanobacteria blooms in Lake Chaohu, China. Environmental Monitoring and Assessment 188, 280.
Yen, H.-K., Lin, T.-F., Liao, P.-C., 2011. Simultaneous detection of nine cyanotoxins in drinking water using dual solid-phase extraction and liquid chromatography–mass spectrometry. Toxicon 58, 209-218.
Young, F.M., Thomson, C., Metcalf, J.S., Lucocq, J.M., Codd, G.A., 2005. Immunogold localisation of microcystins in cryosectioned cells of Microcystis. Journal of structural biology 151, 208-214.
Zhang, Y., Whalen, J.K., 2020. Production of the neurotoxin beta-N-methylamino-l-alanine may be triggered by agricultural nutrients: An emerging public health issue. Water Research, 115335.
Zhang, Y., Whalen, J.K., Duy, S.V., Munoz, G., Husk, B.R., Sauvé, S., 2020. Improved extraction of multiclass cyanotoxins from soil and sensitive quantification with on-line purification liquid chromatography tandem mass spectrometry. Talanta, 120923.
Zhao, Y., Xie, P., Tang, R., Zhang, X., Li, L., Li, D., 2008. In vivo studies on the toxic effects of microcystins on mitochondrial electron transport chain and ion regulation in liver and heart of rabbit. Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology 148, 204-210.
Zhu, J., Ren, X., Liu, H., Liang, C., 2018. Effect of irrigation with microcystins-contaminated water on growth and fruit quality of *Cucumis sativus* L. and the health risk. Agricultural Water Management 204, 91-99.
Žegura, B., Štraser, A. and Filipič, M., 2011. Genotoxicity and potential carcinogenicity of cyanobacterial toxins–a review. Mutation Research/Reviews in Mutation Research, 727(1-2), pp.16-41.

Žegura, B., Lah, T.T. and Filipič, M., 2006. Alteration of intracellular GSH levels and its role in microcystin-LR-induced DNA damage in human hepatoma HepG2 cells. Mutation research/genetic toxicology and environmental mutagenesis, 611, 25-33.

Appendix. S5.1. Bibliography of studies included in the meta-analysis.

Appendix. S1. Bibliography of studies included in the meta-analysis.

1. Abe T, Lawson T, Weyers J.D., Codd G.A., 1996. Microcystin‐LR inhibits photosynthesis of Phaseolus vulgaris primary leaves: implications for current spray irrigation practice. New Phytol. 133, 651–8. https://doi.org/10.1111/j.1469-8137.1996.tb01934.x.

2. Azevedo C.C., Azevedo J., Osorio H., Vasconcelos V., Campos A., 2014. Early physiological and biochemical responses of rice seedlings to low concentration of microcystin-LR. Ecotoxicology 23, 107–21. https://doi.org/10.1007/s10646-013-1156-8.

3. Beyer, D., Tándor, I., Kónya, Z., Bátori, R., Roszik, J., Vereb, G., Erdődi, F., Vasas, G., M-Hamvas, M., Jambrovics, K. and Máthé, C., 2012. Microcystin-LR, a protein phosphatase inhibitor, induces alterations in mitotic chromatin and microtubule organization leading to the formation of micronuclei in Vicia faba. Ann. Bot. 110, 797–808. https://doi.org/10.1093/aob/mcs154.

4. do Carmo Bittencourt-Oliveira, M., Cordeiro-Araújo, M.K., Chia, M.A., de Toledo Arruda-Neto, J.D., de Oliveira, Ê.T. and dos Santos, F., 2016. Lettuce irrigated with contaminated water: Photosynthetic effects, antioxidative response and bioaccumulation

of microcystin congeners. Ecotoxicol. Environ. Saf. 128, 83–90. https://doi.org/10.1016/j.ecoenv.2016.02.014.
5. Cao Q., Steinman A.D., Yao L., Xie L., 2017. Increment of root membrane permeability caused by microcystins result in more elements uptake in rice (Oryza sativa). Ecotoxicol. Environ. Saf. 145, 431–5. https://doi.org/10.1016/j.ecoenv.2017.07.066.
6. Cao, Q., Liu, W., Jiang, W., Shu, X., Xie, L., 2019. Glutathione biosynthesis plays an important role in microcystin-LR depuration in lettuce and spinach. Environ. Pollut. 253, 599–605. https://doi.org/10.1016/j.envpol.2019.07.064.
7. Cao, Q., Rediske, R.R., Yao, L. and Xie, L., 2018. Effect of microcystins on root growth, oxidative response, and exudation of rice (Oryza sativa). Ecotoxicol. Environ. Saf. 149, 143–149. https://doi.org/10.1016/j.ecoenv.2017.11.020.
8. Cao Q., Steinman A.D., Wan X., Xie L., 2018. Bioaccumulation of microcystin congeners in soil-plant system and human health risk assessment: A field study from Lake Taihu region of China. Environ. Pollut. 240, 44–50. https://doi.org/10.1016/j.envpol.2018.04.067.
9. Chen J., Han F.X., Wang F., Zhang H., Shi Z., 2012. Accumulation and phytotoxicity of microcystin-LR in rice (Oryza sativa). Ecotoxicol. Environ. Saf. 76, 193–9. https://doi.org/10.1016/j.ecoenv.2011.09.022.
10. Chen J., Song L., Dai J., Gan N., Liu Z., 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. https://doi.org/10.1016/j.toxicon.2004.01.011.
11. Chen J., Zhang H.Q., Hu L.B., Shi Z.Q., 2013. Microcystin-LR-induced phytotoxicity in rice crown root is associated with the cross-talk between auxin and nitric oxide. Chemosphere 93, 283–93. https://doi.org/10.1016/j.chemosphere.2013.04.079.
12. Chia, M. A., Auta, Z. Z., Esson, A. E., Yisa, A. G., Abolude, D. S., 2019. Assessment of microcystin contamination of Amaranthus hybridus, Brassica oleracea, and Lactuca sativa sold in markets: a case study of Zaria, Nigeria. Environ. Monit. Assess. 191, 569. https://doi.org/10.1007/s10661-019-7725-4.
13. Codd G.A., Metcalf J.S., Beattie K.A., 1999. Retention of Microcystis aeruginosa and microcystin by salad lettuce (Lactuca sativa) after spray irrigation with water containing cyanobacteria. Toxicon 37, 1181–5. https://doi.org/10.1016/S0041-0101(98)00244-X.
14. Corbel S., Bouaïcha N., Nélieu S., Mougin C., 2015. Soil irrigation with water and toxic cyanobacterial microcystins accelerates tomato development. Environ. Chem. Lett. 13, 447–52. https://doi.org/10.1007/s10311-015-0518-2.
15. Cordeiro-Araújo, M.K., Chia, M.A., de Toledo Arruda-Neto, J.D., Tornisielo, V.L., Vilca, F.Z. and do Carmo Bittencourt-Oliveira, M., 2016. Microcystin-LR bioaccumulation and depuration kinetics in lettuce and arugula: Human health risk assessment. Sci. Total Environ. 566–567, 1379-86. https://doi.org/10.1016/j.scitotenv.2016.05.204.
16. Crush J.R., Briggs L.R., Sprosen J.M., Nichols S.N., 2008. Effect of irrigation with lake water containing microcystins on microcystin content and growth of ryegrass, clover, rape, and lettuce. Environ. Toxicol. 23, 246–52. https://doi.org/10.1002/tox.20331.
17. Drake P.M., Barbi T., Drever M.R., van Dolleweerd C.J., Porter A.J., Ma J.K., 2010. Generation of transgenic plants expressing antibodies to the environmental pollutant microcystin-LR. FASEB J. 24, 882–90. https://doi.org/10.1096/fj.09-140848.
18. Drobac, D., Tokodi, N., Kiprovski, B., Malenčić, D., Važić, T., Nybom, S., Meriluoto, J., Svirčev, Z., 2017. Microcystin accumulation and potential effects on antioxidant capacity

of leaves and fruits of Capsicum annuum. J. Toxicol. Environ. Health. A 80, 145–54. https://doi.org/10.1080/15287394.2016.1259527.
19. EL-Sheekh M., Khairy H., EL-Shenody R., 2014. Allelopathic Effect of Cell-Free Medium of Microcystis aeruginosa Kützing on the Chromosomal Changes in Allium cepa Root Tips and Plumule Formation of Zea mays Seedling. Br. Biotechnol. J. 4, 622. https://doi.org/10.9734/BBJ/2014/8414.
20. El Khalloufi F., El Ghazali I., Saqrane S., Oufdou K., Vasconcelos V., Oudra B., 2012. Phytotoxic effects of a natural bloom extract containing microcystins on Lycopersicon esculentum. Ecotoxicol. Environ. Saf. 79, 199–205. https://doi.org/10.1016/j.ecoenv.2012.01.002.
21. El Khalloufi, F., Oufdou, K., Lahrouni, M., Faghire, M., Peix, A., Ramírez-Bahena, M.H., Vasconcelos, V. and Oudra, B., 2013. Physiological and antioxidant responses of Medicago sativa-rhizobia symbiosis to cyanobacterial toxins (Microcystins) exposure. Toxicon 76, 167–77. https://doi.org/10.1016/j.toxicon.2013.10.003.
22. Essa A.M., Ibrahim W.M., Mahmud R.M., Kassim N., 2015. Potential impact of cyanobacterial exudates on seed germination and antioxidant enzymes of crop plan seedlings. Int. J. Curr. Microbiol. Appl. Sci. 4, 1010–24.
23. Freitas M., Azevedo J., Pinto E., Neves J., Campos A., Vasconcelos V., 2015. Effects of microcystin-LR, cylindrospermopsin and a microcystin-LR/cylindrospermopsin mixture on growth, oxidative stress and mineral content in lettuce plants (Lactuca sativa L.). Ecotoxicol. Environ. Saf. 116, 59–67. https://doi.org/10.1016/j.ecoenv.2015.02.002.
24. Gu, Y., Liang, C., 2020. Responses of antioxidative enzymes and gene expression in Oryza sativa L and Cucumis sativus L seedlings to microcystins stress. Ecotoxicol. Environ. Saf. 193, 110351. https://doi.org/10.1016/j.ecoenv.2020.110351.
25. Gutiérrez-Praena, D., Campos, A., Azevedo, J., Neves, J., Freitas, M., Guzmán-Guillén, R., Cameán, A.M., Renaut, J., Vasconcelos, V., 2014. Exposure of Lycopersicon Esculentum to microcystin-LR: Effects in the leaf proteome and toxin translocation from water to leaves and fruits. Toxins (Basel) 6, 1837–54. https://doi.org/10.3390/toxins6061837.
26. Gutiérrez-Praena, D., Jos, Á., Pichardo, S., Moreno, I.M., Cameán, A.M., 2013. Presence and bioaccumulation of microcystins and cylindrospermopsin in food and the effectiveness of some cooking techniques at decreasing their concentrations: A review. Food Chem. Toxicol. 53, 139–52. https://doi.org/10.1016/j.fct.2012.10.062.
27. Hereman T.C, Bittencourt-Oliveira M.D.C., 2012. Bioaccumulation of Microcystins in Lettuce. J. Phycol. 48, 1535–7. https://doi.org/10.1111/jpy.12006.
28. Järvenpää, S., Lundberg-Niinistö, C., Spoof, L., Sjövall, O., Tyystjärvi, E., Meriluoto, J., 2007. Effects of microcystins on broccoli and mustard, and analysis of accumulated toxin by liquid chromatography-mass spectrometry. Toxicon 49, 865–874. https://doi.org/10.1016/j.toxicon.2006.12.008.
29. Jia Y., Li H., Qu Y., Chen W., Song L., 2018. Phytotoxicity, bioaccumulation and potential risks of plant irrigations using cyanobloom-loading freshwater. Sci. Total Environ. 624, 704-712. https://doi.org/10.1016/j.scitotenv.2017.12.164.
30. Kibria, G., 2016. Blue-green algal toxins/cyanobacterial toxins (BGA), climate change and BGA impacts on water quality, fish kills, crops, seafood, wild animals and humans. 7p. https://doi.org/10.13140/RG. 2.1. 1306.9765/1.

31. Kurki-Helasmo K., Meriluoto J., 1998. Microcystin uptake inhibits growth and protein phosphatase activity in mustard (Sinapis alba L.) seedlings. Toxicon 36, 1921–1926. https://doi.org/10.1016/S0041-0101(98)00114-7.
32. Lahrouni, M., Oufdou, K., El Khalloufi, F., Benidire, L., Albert, S., Göttfert, M., Caviedes, M.A., Rodriguez-Llorente, I.D., Oudra, B., Pajuelo, E., 2016. Microcystin-tolerant Rhizobium protects plants and improves nitrogen assimilation in Vicia faba irrigated with microcystin-containing waters. Environ. Sci. Pollut. Res. Int. 23, 10037–10049. https://doi.org/10.1007/s11356-016-6223-2.
33. Lahrouni M., Oufdou K., El Khalloufi F., Pajuelo E., Oudra B., 2015. Impact of cyanobacterial toxins (microcystins) on growth and root development of in vitro Vicia faba cultures. International Journal of Innovation and Applied Studies 12, 542.
34. Lahrouni, M., Oufdou, K., Faghire, M., Peix, A., El Khalloufi, F., Vasconcelos, V., Oudra, B., 2012. Cyanobacterial extracts containing microcystins affect the growth, nodulation process and nitrogen uptake of faba bean (Vicia faba L., Fabaceae). Ecotoxicology 21, 681–687. https://doi.org/10.1007/s10646-011-0826-7.
35. Lee, S., Jiang, X., Manubolu, M., Riedl, K., Ludsin, S.A., Martin, J.F., Lee, J., 2017. Fresh produce and their soils accumulate cyanotoxins from irrigation water: implications for public health and food security. Food Res. Int. 102, 234–45. https://doi.org/10.1016/j.foodres.2017.09.079.
36. Lefebvre B.R., 2013. The accumulation of the cyanobacterial toxin, microcystin, in cherry tomato (Solanum lycoperiscum) and bush bean (Phaseolus vulgaris) plants. UNH Centre for Freshwater Biology Research 15, 1–11.
37. Levizou E., Statiris G., Papadimitriou T., Laspidou C.S., Kormas K.A., 2017. Lettuce facing microcystins-rich irrigation water at different developmental stages: Effects on plant performance and microcystins bioaccumulation. Ecotoxicol. Environ. Saf. 143, 193–200. https://doi.org/10.1016/j.ecoenv.2017.05.037.
38. Li, Y.W., Zhan, X.J., Xiang, L., Deng, Z.S., Huang, B.H., Wen, H.F., Sun, T.F., Cai, Q.Y., Li, H., Mo, C.H., 2014. Analysis of trace microcystins in vegetables using solid-phase extraction followed by high performance liquid chromatography triple-quadrupole mass spectrometry. J. Agric. Food Chem. 62, 11831–11839. https://doi.org/10.1021/jf5033075.
39. Liang C., Wang W., 2014. Response and recovery of rice (Oryza sativa) seedlings to irrigation with microcystin-contaminated water. Environ. Earth Sci. 73, 4573–4580. https://doi.org/10.1007/s12665-014-3746-z.
40. Liang C., Wang W., Wang Y., 2016. Effect of irrigation with microcystins-contaminated water on growth, yield and grain quality of rice (Oryza sativa). Environ. Earth Sci. 75, 505. https://doi.org/10.1007/s12665-015-5121-0.
41. Liang, C., Liu, H., 2020. Response of hormone in rice seedlings to irrigation contaminated with cyanobacterial extract containing microcystins. Chemosphere. 127157. https://doi.org/10.1016/j.chemosphere.2020.127157.
42. Liu B., Wu Y., Liu J., 2012. Comparison on the tolerance of cruciferous crops and leguminous crops to microcystin. Agricultural Science & Technology-Hunan 13, 198–237.
43. Llana-Ruiz-Cabello, M., Jos, A., Cameán, A., Oliveira, F., Barreiro, A., Machado, J., Azevedo, J., Pinto, E., Almeida, A., Campos, A., 2019. Analysis of the Use of Cylindrospermopsin and/or Microcystin-Contaminated Water in the Growth, Mineral

Content, and Contamination of Spinacia oleracea and Lactuca sativa. Toxins 11, 624. https://doi.org/10.3390/toxins11110624.
44. Machado, J., Azevedo, J., Freitas, M., Pinto, E., Almeida, A., Vasconcelos, V., Campos, A., 2017. Analysis of the use of microcystin-contaminated water in the growth and nutritional quality of the root-vegetable, Daucus carota. Environ. Sci. Pollut. Res. Int. 24, 752–64. https://doi.org/10.1007/s11356-016-7822-7.
45. McElhiney J., Lawton L.A., Leifert C., 2001. Investigations into the inhibitory effects of microcystins on plant growth, and the toxicity of plant tissues following exposure. Toxicon 39, 1411–20. https://doi.org/10.1016/S0041-0101(01)00100-3.
46. Mohamed Z.A., Al Shehri A.M., 2009. Microcystins in groundwater wells and their accumulation in vegetable plants irrigated with contaminated waters in Saudi Arabia. J. Hazard. Mater. 172, 310–5. https://doi.org/10.1016/j.jhazmat.2009.07.010.
47. Pappas, D., Gkelis, S., Panteris, E., 2020. The effects of microcystin‐LR in Oryza sativa root cells: F‐actin as a new target of cyanobacterial toxicity. Plant Biol. https://doi.org/10.1111/plb.13120.
48. Pereira, A. L., Azevedo, J., Vasconcelos, V., 2017. Assessment of uptake and phytotoxicity of cyanobacterial extracts containing microcystins or cylindrospermopsin on parsley (Petroselinum crispum L.) and coriander (Coriandrum sativum L). Environ. Sci. Pollut. Res. 24, 1999–2009. https://doi.org/10.1007/s11356-016-7910-8
49. Petrou, M., Karas, P., Vasileiadis, S., Zafiriadis, I., Papadimitriou, T., Levizou, E., Kormas, K., Karpouzas, D., 2020. Irrigation of radish (Raphanus sativus L.) with microcystin-enriched water holds low risk for plants and their associated rhizopheric and epiphytic microbiome. Environ. Pollut. 115208. https://doi.org/10.1016/j.envpol.2020.115208.
50. Peuthert A., Chakrabarti S., Pflugmacher S., 2007. Uptake of microcystins-LR and -LF (cyanobacterial toxins) in seedlings of several important agricultural plant species and the correlation with cellular damage (lipid peroxidation). Environ. Toxicol. 22, 36–42. https://doi.org/10.1002/tox.20266.
51. Pflugmacher S., 2012. Reduction in germination rate and elevation of peroxidase activity in Zea mays seedlings due to exposure to different microcystin analogues and toxic cell free cyanobacterial crude extract. J Appl. Bot. Food Qual. 81, 45–8.
52. Pflugmacher S., Aulhorn M., Grimm B., 2007. Influence of a cyanobacterial crude extract containing microcystin-LR on the physiology and antioxidative defence systems of different spinach variants. New Phytol. 175, 482–9. https://doi.org/10.1111/j.1469-8137.2007.02144.x.
53. Pflugmacher S., Hofmann J., Hübner B., 2007. Effects on growth and physiological parameters in wheat (Triticum aestivum L.) grown in soil and irrigated with cyanobacterial toxin contaminated water. Environ. Toxicol. Chem. 26, 2710–6. https://doi.org/10.1897/07-145.1.
54. Pflugmacher S., Jung K., Lundvall L., Neumann S., Peuthert A., 2006. Effects of cyanobacterial toxins and cyanobacterial cell‐free crude extract on germination of alfalfa (Medicago sativa) and induction of oxidative stress. Environ. Toxicol. Chem. 25, 2381–7. https://doi.org/10.1897/07-145.1.
55. Romero-Oliva C.S., Contardo-Jara V., Block T., Pflugmacher S., 2014. Accumulation of microcystin congeners in different aquatic plants and crops–A case study from lake Amatitlán, Guatemala. Ecotoxicol. Environ. Saf. 102, 121–8. https://doi.org/10.1016/j.ecoenv.2014.01.031.

56. Saqrane S., Ghazali I.E., Oudra B., Bouarab L., Vasconcelos V., 2008. Effects of cyanobacteria producing microcystins on seed germination and seedling growth of several agricultural plants. J Environ. Sci. Heal., Part B 43, 443–51. https://doi.org/10.1080/03601230802062307.
57. Saqrane S., Ouahid Y., El Ghazali I., Oudra B., Bouarab L., del Campo F.F., 2009. Physiological changes in Triticum durum, Zea mays, Pisum sativum and Lens esculenta cultivars, caused by irrigation with water contaminated with microcystins: a laboratory experimental approach. Toxicon 53, 786–96. https://doi.org/10.1016/j.toxicon.2009.01.028.
58. Stüven, J., Pflugmacher S., 2007. Antioxidative stress response of Lepidium sativum due to exposure to cyanobacterial secondary metabolites. Toxicon 50, 85–93. https://doi.org/10.1016/j.toxicon.2007.02.019.
59. Wang Z., Xiao B., Song L., Wu X., Zhang J., Wang C., 2011. Effects of microcystin-LR, linear alkylbenzene sulfonate and their mixture on lettuce (Lactuca sativa L.) seeds and seedlings. Ecotoxicology 20, 803–14. https://doi.org/10.1007/s10646-011-0632-2.
60. Wijewickrama, M. M., Manage, P. M., 2019. Accumulation of Microcystin-LR in Grains of Two Rice Varieties (Oryza sativa L.) and a Leafy Vegetable, Ipomoea aquatica. Toxins 11, 432. https://doi.org/10.3390/toxins11080432.
61. Xiang, L., Li, Y.W., Liu, B.L., Zhao, H.M., Li, H., Cai, Q.Y., Mo, C.H., Wong, M.H., Li, Q. X., 2019. High ecological and human health risks from microcystins in vegetable fields in southern China. Environ. Int. 133, 105142. https://doi.org/10.1016/j.envint.2019.105142.
62. Zhu J., Ren X., Liu H., Liang C., 2018. Effect of irrigation with microcystins-contaminated water on growth and fruit quality of Cucumis sativus L. and the health risk. gric. Water Manag. 204, 91–9. https://doi.org/10.1016/j.agwat.2018.04.011.