THESIS

EVALUATION OF OXIDATION TREATMENT ON ALGAL TOXINS AND THE CYTOTOXIC EFFECTS OF ALGAL TOXINS POST OXIDATION

Submitted by

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ABSTRACT

EVALUATION OF OXIDATION TREATMENT ON ALGAL TOXINS AND CYTOTOXIC EFFECTS OF ALGAL TOXINS POST OXIDATION

Algal blooms are a growing threat to the water industry, and one of the major reasons is that they are often accompanied by proliferation of the harmful species - cyanobacteria. Cyanobacteria produce a variety of toxins that can cause serious adverse health effects. Microcystin-LR (MC-LR) and cylindrospermopsin (CYN) are known hepatotoxins, both named priority toxins by the EPA.

Cyanotoxins are usually treated with oxidation in conventional drinking water treatment plants - this approach has shown to successfully break down the toxin molecules. The goal of this project was to evaluate the effects of standard oxidation treatments on the concentration and cytotoxicity of MC-LR and CYN. Toxin solutions were prepared in water and treated with three oxidants – chlorine, potassium permanganate, and ozone. Once the toxin solutions were treated with oxidants, two assays were conducted in parallel: (1) the PrestoBlue cytotoxicity assay was conducted on HepG2 carcinoma cells following treatment, and (2) an LC-MS/MS analysis of the toxin solutions was used to quantify the toxins concentration post-treatment.

The results of the LC-MS analysis suggested that neither chlorine, potassium permanganate or ozone treatment was effective at reducing the concentrations of MC-LR or CYN. These findings are consistent with the results of the cytotoxicity assay, which did not show a significant reduction of cell death after exposure to toxins treated with oxidation compared to untreated toxins. The oxidation of MC-LR and CYN likely failed due to a high starting

concentration of the toxin, much higher than what occurs naturally. Future research should focus on a biological endpoint other than apoptosis to evaluate the potential health risks of the toxin metabolites at concentrations relevant to natural exposure.

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1: INTRODUCTION

1.1 Background

Algae are a natural component of aquatic ecosystems, however, due to the magnifying effects of climate change (D'Anglada, 2015; Watson, 2008; Merel, 2013; CLRMA, 2017) and pollution of surface waters with higher levels of nutrients (D'Anglada, 2015; Watson, 2008; Merel, 2013; CLRMA, 2017), increasing instances of algal blooms pose a significant threat to water safety. Moreover, growing populations worldwide have significantly increased the average water demands and resulted in the depletion of groundwater resources, thus increasing our reliance on surface water as a source of drinking water. The compounded effect of these problems brings algal blooms to the forefront of concerns related to potable water safety (D'Anglada, 2015; CLRMA, 2017).

Algal blooms (Figure 1.1) are caused by eutrophication in surface waters are associated with a significant decrease in water quality, and are a threat to human and native ecosystem health. Eutrophication occurs from an excess of nutrients in the water which promote dense

Figure 1.1. Algal bloom in a lake.
Source: Summit Community Voice

growth of plants that consume dissolved oxygen in the water during decomposition with a resultant zooplankton death. Algal blooms reduce the aesthetic quality of source waters , and may harm the filtration systems employed in treatment plants, thus driving up the cost of water treatment. In addition, as the occurrence of harmful algal blooms (HABs) continues to rise this poses threats to agriculture, recreational water uses, and native aquatic species (CLRMA, 2017).

HABs are often accompanied by growth of cyanobacterial, a photosynthetic prokaryotic species that occur naturally in surface waters. Cyanobacteria growth is favored by nutrient-rich, warm, and low-light conditions, which makes them likely to outcompete other species, particularly in eutrophic waters. Some cyanobacteria are known to produce toxins that affect the liver, skin, and nervous system, and contaminate waterways used for recreation and drinking water (US EPA, 2016; AWWA, 2010). It is important for water utilities to know which cyanobacterial genera commonly produce toxins and test the waters when these species are observed in a bloom.

Predominant cyanotoxins include microcystins, cylindrospermopsin, anatoxin-A, and saxitoxin. The toxins can be intracellular (still contained within the cell wall of the cyanobacteria) or extracellular (released into the surrounding water). While it is known that cyanobacteria produce these toxins and are more likely to release the toxins during an algal bloom, the timing of the toxin release is poorly understood. For instance, it is possible to have an algal bloom with low toxin concentrations, or conversely, dangerously high toxin concentrations without an algal bloom (CLRMA, 2015).

A great deal of research on cyanobacterial toxins has been done in Australia, since this issue has plagued the Australian water industry for years. An Alert Level Framework has been developed in Australia in an effort to protect drinking water sources and public health, while

formal and informal guidelines exist for some of the cyanobacterial toxins. In recent years within the United States, regulatory agencies such as the EPA and local utilities have also begun assessing threats posed by algal toxins (Nicholson, 2007). The ongoing goal of these agencies is to develop adequate regulations and guidelines for water resource management to protect the drinking water sources in the US from these toxins.

To minimize and prevent the negative effects of harmful algal blooms on the public heath, water managers rely on a variety of tools. Cyanobacterial blooms can be managed through prevention, control, and eradication (Carmichael, 2001). Preventative tactics focus on watershed management practices such as physical, chemical, and biological treatments. However, the most effective preventative measure is minimizing the anthropogenic influences that promote blooms (CLRMA, 2015). Mitigation, or control practices, can be either applied to the water body or during the water treatment process, and include scum removal, coagulation and filtration, and oxidation of the water. Finally, eradication practices aim to destroy blooms through chemical treatments such as copper sulfate, lime (Carmichael, 2001), and commercial algaecides like PAK®27.

Both conventional and advanced oxidation treatment of drinking water are purportedly effective in toxin removal and have been extensively reported in studies. However, when strong oxidation is used there is an inherent danger of producing toxic oxidation byproducts. Although oxidation was shown to be effective in breaking down the toxins, further research is required to assess chemical and toxicological characteristics, as well as overall safety of the resultant metabolites and by-products (Merel, 2013).

1.2 Toxins in surface water and drinking water

Approximately 50 species of cyanobacteria have been shown to produce a variety of toxins that differ in chemical structure, toxicity pathways, and parent species. The most common cyanobacterial genera are *Anabaena, Aphanizomenon, Cylindrospermopsis*, *Gloetrichia*, *Microsystis, and Planktothrix* (CLRMA, 2015). Toxins production most likely evolved as a survival tactic, and as the number of cyanobacteria increases, the chance of toxin production also increases (AWWA,2010). However, cyanobacteria do not produce toxins all the time (CLRMA, 2015) which means that a cyanobacterial bloom doesn't mean that toxins are present. Inversely, toxins may be present even when there is no bloom. This demonstrates the importance of sampling water and knowing what cyanobacteria species are present.

Cyanobacteria produce the toxins within the cell, then release them into the environment. Once released by cyanobacteria, there is a threat of exposure to toxins (most of which are highly soluble in water) through ingestion and skin contact during recreational activities, such as swimming in the polluted waters. Even at low-level exposure, most toxins will act as skin irritants and cause gastrointestinal upset (CLRMA, 2015). At acute exposure concentrations, the toxins will affect their target organ: neurotoxins, hepatotoxins, and dermatoxins target the central nervous system (CNS), liver, and skin, respectively.

Exposure through the consumption of municipal water is also a concern, since conventional water treatment plants are not designed to remove cyanotoxins. Ingestion through drinking is most risky, because even low concentrations of toxins will be absorbed through the mucous membranes in the mouth and the intestines. However, treated water is not as likely to result in dermal aborption, since the concentrations of toxin would be much smaller than in

untreated waters and the dermal layer should provide a sufficient barrier as most of these toxins are not lipophilic.

This section gives a detailed overview of the four most common cyanotoxins – microcystin, cylindrospermopsin, anatoxin-A, and saxitoxin (also known as the paralytic shellfish toxin, or PST).

1.2.1 Microcystin-LR (MC-LR)

MC-LR is the most common and most widespread of the cyanobacterial microcystin toxin family, and is well known for its adverse health effects. It is a hepatotoxin and is toxic to both animals and humans with an LD50 of 5 mg/kg. The mechanism of toxicity is by increasing phosphorylation of proteins in liver cells, leading to metabolic disturbances and liver failure (D'Anglada, 2015). While the liver is the primary target, MC-LR also acts as a skin, eye, and throat irritant (Yoo, 1995).

Microcystis aeruginosa, a common freshwater cyanobacteria (Figure 1.2) is the major producer of MC-LR. People and animals can be exposed through swimming in or drinking contaminated water. Exposure can also occur by eating fish that have bio-accumulated the toxin. The most recent incident involving microcystin occurred in February 1996, when 116 people in Caruaru, Brazil were poisoned by untreated water from a local reservoir. The patients experienced visual disturbances, nausea, vomiting, and muscle weakness. Of the 116 affected individuals, 100 experienced acute liver failure, and 52 eventually died. In North America, there have been multiple reports of domestic animal poisonings linked to microcystin exposure (Butler, 2009).

MC-LR is the most common toxin of the microcystin family, and is highly persistent in water. Its chemical structure is a cyclic hepapeptide with seven amino acids, and the

nomenclature is based on the amino acids present. In the case on MC-LR, [L] stands for leucine and [R] for arginine amino acid. Other common microcystins are RR, YR, and LA (US EPA, 2006).

The safe drinking water concentration for MC-LR established by the USEPA is 0.3 μg/L for infants and pre-school children, and 1.6 μg/L for school-age children and adults (EPA, 2016). The WHO limit of exposure is 1 μg/L, excluding other cyanotoxins (EPA, 2016). There are several detection methods for microcystins, including LC-MS (analytic laboratory testing using liquid chromatography coupled with mass spectrometry, discussed further in section 1.4.1.), as well as commercially-available ELISA testing kits.

1.2.2 Cylindrospermopsin (CYN)

Cylindrospermopsis raciborskii is a predominantly tropical and subtropical species shown in Figure 1.3, and is the major producer of CYN (Carmichael, 2001), though recently it has been reported in more temperate regions. Its unexpectedly large geographic spread makes CYN regulation a priority concern (D'Anglada, 2015).

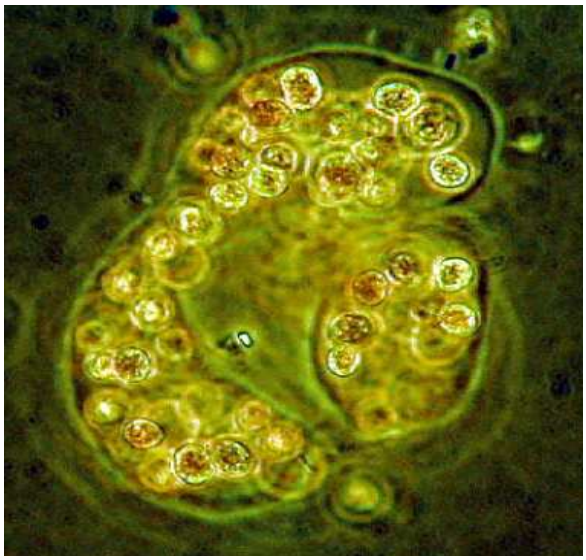


Figure 1.2. *Microcystis Aeruginosa* cell.
Source: Klamath Water Quality Website

Figure 1.3. *Cylindrospermopsis raciborskii* cell.
Source: Cyanosite Purdue Website

CYN is primarily a hepatotoxin, with a 5-day acute oral LD50 of approximately 6 mg/kg. It is a potent eukaryotic protein synthesis inhibitor, its specific toxicity mechanism is inhibition of glutathione synthesis, resulting in cytotoxicity and genotoxicity (Shaw 2002). The toxin became well-known after a major incident in Australia, where it caused serious illness in 149 people. Its presence in drinking water has also caused poisonings in Brazil (Guzman-Guillen, 2012).

CYN is highly water-soluble and very stable, making it persistent in aquatic environments. The federal safe drinking water concentration for CYN established by the USEPA is 0.7 μg/L for infants and pre-school children, and 3 μg/L for school-age children and adults (EPA, 2016), or this may be lower in certain states. CYN can be successfully detected and quantified using LC-MS, but currently there are no commercially available ELISA detection kits.

1.2.3 Anatoxin-A (ATX)

ATX is produced by freshwater cyanobacterial species, predominantly *Anabaena flos aquaea*, shown in Figure 1.4, as well as at least four other genera. ATX is a neurotoxin and causes severe central nervous system (CNS) symptoms such as loss of coordination, convulsions, and death by respiratory paralysis. It has an acute oral LD50 of 16.2 mg/kg. Its mechanism of toxicity is through binding of nicotinic acetylcholine receptors within the CNS. The EPA was unable to derive a health-based safe drinking water concentration for ATX from available data (D'Anglada, 2015), but some states set drinking water guidance/action levels. These range from 20 μg/L for adults in Ohio to 0.1 μg/L in Minnesota (EPA, 2016). ATX can be detected and quantified with LC-MS, with GC-MS methods currently in development.

1.2.4 Saxitoxin (STX)

STX is a well-known paralytic shellfish toxin. It is not considered to be an immediate concern in the United States, though some states set drinking water guidance/action levels. These range from 1.6 μg/L in Oregon to 0.2 μg/L in Ohio in drinking water for adults (EPA, 2016). In Australia, where a suggested safe water threshold was set to 3 µg/L, STX is of major concern (Merel, 2013). It is produced by both marine and freshwater species, but predominantly *Anabaena circinalis*, shown in Figure 1.5. It is a potent neurotoxin, with an acute oral LD50 of 5.7 µg/kg. STX induces flaccid paralysis through selective blocking of sodium channels, and is dangerous due to its' ability to bio-accumulate and biomagnify. It can be detected using LC-MS, ELISA, as well as neuroblastoma assays (Yen, 2011; Cusick, 2013; Merel, 2013).

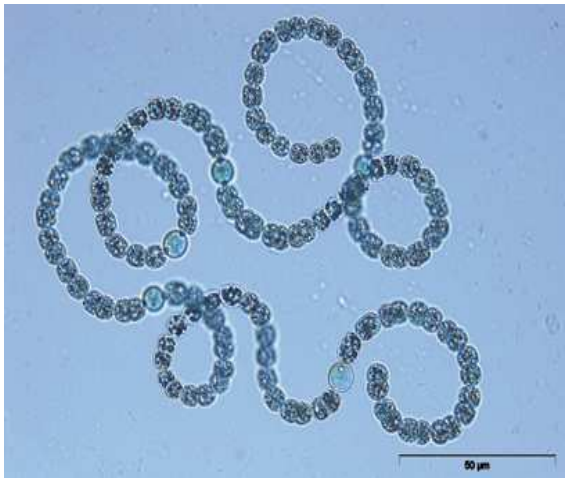
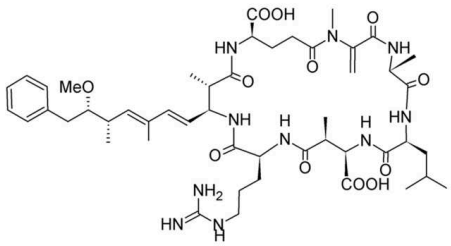
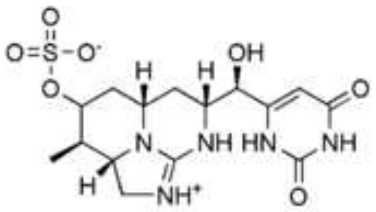
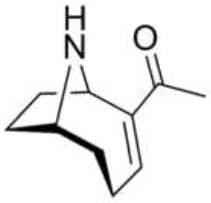
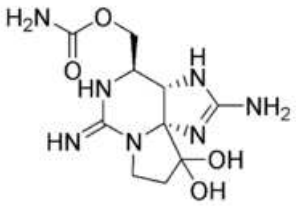


Figure 1.4. *Anabaena flos aquaea* cell
Source: Toxinology Website

Figure 1.5. Anabaena cirnalis.
Source: Ocean Data Center, UCSC

Table 1-1. Cyanotoxin Chemical Summary

MC-LR	CYN
Formula: C49H74N10O12 Structure: Cyclic hepapeptide with 7 amino acids.  Source: Carmichael, 2001 Overview: Best-known, most-spread hepatotoxins Most common of the microcystin family. Origin (major producing species): Microcystis aeruginosa Toxic effects and mechanisms: Toxic to animals and humans, LD50 = 5 mg/kg Increases pholphorylation of proteins in liver cells	Formula: C15H21N5O7S Structure: Tricyclic guanidinium moiety bridged to hydroxymethyluracil  Source: Carmichael, 2001 Overview: Common tropical species, but is potentially more widespread than previously thought. Origin (major producing species): Cylindrospermopsis raciborskii Toxic effects and mechanisms: Liver and kidney toxicity, oral LD50 = 6 mg/kg Protein synthesis inhibition, DNA/RNA modification.
Anatoxin-A	Saxitoxin
Formula: C10H15NO Structure: Bicyclic amine alkaloid  Source: Carmichael, 2001 Overview: VFDF neurotoxin, high potential presence in drinking water Origin (major producing species): Anabaena flos aquaea Toxic effects and mechanisms: Toxic to humans and animals, causes CNS symptoms. Acetylcholine agonist, binds to nicotinic acetylcholine receptors affecting CNS. Oral LD50 = 16.2 mg/kg.	Formula: C10H17N7O4 Structure: Alkaloid  Source: Carmichael, 2001 Overview: Best-known paralytic shellfish toxin Origin (major producing species): Anabaena circinalis Toxic effects and mechanisms: Neurotoxicity – selective sodium channel blocker Oral LD50 = 5.7 μg/kg (Cusick 2013)

1.3 Treatment of Toxins

As mentioned above, water treatment utilities have been successful in transforming the toxins with strong oxidants such as ozone and chlorine post-filtration. The oxidants target electron-rich moieties such as the C=C bond, activated aromatics, and even amines. These chemical structures are present in all of the toxins of interest. Despite specific target sites, reactivity is strongly influenced by the overall chemical structure, so treatment must be adjusted based on which toxins are present in the water source, and what fraction of toxins remains intracellular vs. extracellular (AWWA, 2010).

Intracellular toxins are best removed through physical separation techniques such as sedimentation and gentle filtration, which allow the cyanobacterial walls to remain intact and prevent the release of these toxins into the surrounding water (CLRMA, 2015). Oxidation is applied when treating toxins present in the water itself, since the oxidant is likely to cause cell lysis, releasing more toxins but leaving an insufficient amount of oxidant for treatment as a percentage of it was consumed by reactions within the cells themselves (AWWA, 2010).

Advanced treatment processes, such as membrane technology, advanced oxidation, and adsorption with granular activated carbon (GAC) are being assessed for full-scale application in cyanotoxin treatment, but these technologies are costly and not available to all utilities (Alvarez, 2010 and He, 2013). Thus, this thesis will focus on conventional oxidation processes and how they may be applied to water treatment methods against cyanotoxins. Utilities are encouraged to evaluate on-site environment for best treatment options.

1.3.1 Chlorine, Cl_2

Cl_2 is by far the most commonly used oxidant in water treatment. It is easily available and applicable to both centralized and point-of use systems. Depending on the pH conditions,

chlorine will react with water, producing different chlorine species. At lower pH, more hypochlorous acid is produced, and at higher pH more hypochlorite is produced. Hypochlorous acid is a more effective oxidant/disinfectant in most reactions, so the efficiency of chlorination highly depends on environmental conditions (Alvarez, 2010; AWWA, 2010).

Cl_2 treatment requires oxidant concentrations several orders of magnitude higher than cyanotoxin concentration and follow a pseudo-first order reaction rate. This makes it easier for treatment to be designed as it follows the same rules as pathogen inactivation, which is usually the primary purpose of utilizing chlorine. Presuming an appropriate pH environment, Cl_2 treatment is a reasonable option for cyanotoxin removal (AWWA, 2010).

In terms of efficacy described in literature, the concentration of MC-LR decreased by more than 95% with aqueous Cl_2 and calcium hypochlorite, and only by ~40% with sodium hypochlorite in 30 min treatments at 1 mg/L dose of the oxidant (Sharma, 2012). Cl_2 is agreed to be effective at >0.5 mg/L with a contact time of at least 30 minutes, provided pH is below 8 (CLRMA, 2015; Sharma, 2012, Yoo,1995). Cl_2 is effective at breaking down MC-LR, CYN, and STX, but not ATX (Merel, 2013; AWWA, 2010; CLRMA, 2015). While Cl_2 is effective in post-filtration oxidation, pre-oxidation during a bloom is not recommended, since Cl_2 would rupture cell walls and increase the concentrations of extracellular toxins in the water.

Dihydroxy-microcystin and monochloro-hydroxy-microcystin, each with multiple isomers, were identified as chlorination byproducts of microcystin (Merel, 2009) Further studies assessed the toxicity of those byproducts using a protein phosphatase 1 inhibition assay (Rodriguez, 2008) and the heterozygous p53 transgenic mouse model (Senogles-Derham, 2003). The results indicated that the oxidation byproducts of the microcystins were non-toxic and did not result in increased incidence of cancer in mice which is associated with exposure to

disinfection byproducts (DBPs). The American Water Work Association concluded that the utilities are likely to be more concerned with DBPs produced by natural organic matter (NOM) than cyanobacterial chlorination byproducts, since the prevalence of NOM in water is much higher than that of cyanotoxins (AWWA, 2010).

1.3.2 Potassium Permanganate, $KMnO_4$

$KMnO_4$ is considered one of the most versatile oxidants available in the water industry. Among other applications in water treatment, it may oxidize organic compounds via several reaction pathways and has been successfully used for taste and odor (T&O) control (Sharma, 2012).

Oxidation of MC-LR and CYN with $KMnO_4$ is independent of pH however, oxidation of ATX decreases with decreasing pH values between 8 and 10, but is otherwise also independent of pH (AWWA,2010). While contact time values have not yet been developed for cyanotoxin treatment with $KMnO_4$, one study has shown 95% removal of 200 μg/L of MC-LR with a $KMnO_4$ dose of 1 mg/L (Rositano, 1994). Overall, $KMnO_4$ can be used to treat MC-LR, ATX, and CYN, though since CYN degradation is very slow and is not a practical treatment option, a combination of chlorine and permanganate may be used in this case (Rodriguez, 2008), as many utilities already use the two oxidants in conjunction.

1.3.3 Chlorine Dioxide, ClO_2

ClO_2 is a weaker chlorine-based oxidant and has been used as an alternative to Cl_2. It is often considered ineffective due to its low reaction kinetic constant, k. It has been found ineffective at removing MC-LR, ATX, and CYN from intracellular or extracellular environments (AWWA, 2010).

1.3.4 Ozone, O_3

Ozonation is one of the most effective water treatment approaches, making it a strong contender for combatting cyanobacteria toxins in drinking water. Literature suggests O_3 is more effective at cyanotoxin removal than other advanced oxidation processes, and is successful in altering MC-LR, CYN, ATX. and nodularins (Merel, 2013).

O_3 alone is highly effective at removing MC-LR, and is more effective at lower pH, reaching 97% efficiency at concentrations as low as 0.4 mg/L in acidic conditions with a contact time of 5 minutes (Alvarez, 2010). Moreover, AWWA reports 100% removal of extracellular MC-LR after 30 seconds of oxidation with O_3/hydrogen peroxide coupled treatment. In fact, O_3 was by far the most effective at MC-LR destruction compared to the rest of the oxidation approaches presented (Sharma 2012). At O_3 levels at which a residual was maintained for at least several minutes, the tests have shown toxin levels decreased to below detection for MC-LR and toxicity removed, as measured by mouse bioassays and phosphate inhibition assay (Brooke, 2006). Similar results have emerged for treatment of ATX (Newcombe, 2002). The downside to ozonation is that it was not effective in removing STX (Merel, 2013; Newcombe, 2002).

1.3.5 Advanced Oxidation with UV and Peroxide, UV+H_2O_2

Advanced oxidation is a process that uses hydroxyl radicals as oxidants. In this case, hydrogen peroxide absorbs UV irradiation to produce hydroxyl radicals. Cyanotoxin removal efficiency is driven by UV and peroxide concentrations, and is higher than the dosages used for disinfection (Alvarez, 2010; Merel, 2013; Sharma, 2012). Advanced oxidation can be used for degrading MC-LR, CYN, and ATX-A (Merel, 2013).

Advanced oxidation using UV+H_2O_2 has proven effective in removing MC-LR. For effective MC-LR removal, doses much larger that those used in disinfection are required – a UV dose of 990 mJ/cm2 with 2 mg/L of H_2O_2 achieved 95% MC-LR removal (Alvarez, 2010).

By itself, UV photolysis was not effective in MC-LR degradation (Alvarez, 2010; Sharma, 2012). The same is true for degradation of ATX (Afzal, 2010). The process does not appear to be pH-dependent, but effectiveness strongly depends on water quality parameters like the concentration of NOM (Alvarez, 2010; Sharma, 2012). UV+H_2O_2 was also effective in removing CYN, and is among the most promising and effective for water detoxification, though it was not as effective as UV with peroxydisulfate (He, 2013).

1.4 Detection methods

Improvement in analytical technology enabled creation of techniques that are fast, reliable, and highly sensitive. Liquid chromatography coupled with mass spectrometry (LC-MS) allows for general detection and quantification of chemicals at unprecedented low levels. Further breakthroughs in biochemistry, immunology, and diagnostics allow the use of enzyme-based assays such as ELISA to detect and quantify the presence of a substance in wet samples. This section will discuss in detail how these methods can be used to detect and quantify cyanotoxins in water samples.

Before proceeding, however, it would be prudent to discuss the difference between intracellular and extracellular toxins, and how this may affect sample preparation. As mentioned before, the extracellular toxins are released by the parent cyanobacterial cell into the environment, and intracellular toxins are still contained within the parent cell wall. Depending on sample preparation process, it is possible to determine the extracellular, intracellular, and total toxin concentrations (Merel, 2013).

To determine the intracellular toxin concentration, the cyanobacterial cells are first isolated from the sample. Typically glass fiber filtration is used for separation, but gentle centrifugation can also be used (Newcombe, 2002). The intracellular toxin is then extracted by lysing cells collected by the filter. Cell lysis can be achieved through a variety of methods – freeze-thaw cycles, mechanical homogenization, sonication, boiling, microwaving, and the most recently developed method utilizes detergents to break the cell wall (Guzman-Guillen, 2012; Merel, 2013; Newcombe, 2002; Nicholson, 2007; Yoo, 1995). Overall, sonication and freeze-thaw cycles are the most effective and common methods utilized (Newcombe, 2002).

Toxins are extracted based on type. Acidified water, acidified methanol, or a mixture of the two are used to extract microcystins; formic acid is used to extract cylindrospermopsin; and water can be used to extract ATX (Nicholson, 2007; Osswald, 2007; Yen, 2011). The sample is then concentrated for analysis by freeze drying, solid-phase extraction, or immunoaffinity chromatography (Meriluoto, 2008).

Extracellular cyanotoxins are separated by filtration and centrifugation. The samples then undergo the same concentration process as the released intracellular counterpart described previously (Merel, 2013).

To determine the total cyanotoxin concentration, the sample undergoes concentration, cell lysis, and extraction. Some methodologies freeze-dry and then extract toxins, others lyse cells first, then concentrate and extract the toxins (Meriluoto, 2008). Alternatively, by conservation of mass, intracellular and extracellular toxin concentrations can be simply added together (Merel, 2013). Once it is determined what toxin concentration is to be evaluated and the sample is prepared, the analytical methods are used to quantify the toxins.

1.4.1 LC-MS/MS

LC-MS/MS is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry (MS) (Nicholson, 2007). A sample LC-MS apparatus is shown in Figure 1.6. LC-MS can be used to detect and quantify all the toxins of interest.

Figure 1.6. UltiMate 3000 HPLC and LTQ XL Linear Ion Trap Mass Spectrometer.
Source: ThermoScientific

It is important to note that reliability of the results highly depends on the quality of standard solution used for instrument calibration. Since the commercial availability of algal toxins is still severely limited, extra care must be taken to ensure that only certified reference solutions are used for qualitative and quantitative analyses.

1.4.1.1 Microcystins

The current standard procedure for identification and quantification of microcystins by targeted full-scan LC-MS/MS analysis is based on ThermoSchientific Application Note 569 created by Zhang, which uses the LC-MS/MS coupling shown in Figure 6. This procedure can be applied to all microcystin species if the appropriate stock solutions are used. Stock solutions of MC-RR, MC-YR and MC-LR are commercially available and can be purchased from Sigma-Aldrich. The limit of detection for this method is 0.025 μg/L, and the limit of quantification is 0.05 μg/L, providing excellent sensitivity.

Sample and Standard Preparation**:**

Prepare stock solution of the desired microcystins species, or a combination of species by diluting commercially-available stock solution with methanol (stock solution of 100 μg/L suggested). Prepare calibration solutions of 0.025 μg/L to 50 μg/L by serial dilution of the stock solution with DI water. Filter sample using 0.45 μm pore size filter, and aliquot 10-20 mL of sample into appropriate vials.

LC-MS/MS Analysis

Inject 50 μL sample using Acclaim 120 Guard Cartridge with 150 L/min, wash for 2 minutes and elute onto PepMap 100 analytical column, or equivalent. LC column temperature should be set to 40 °C. The MS ionization mode should be set to positive electrospray ionization (ESI), with collision energy of 35% and 2 isolation windows. Assuming targeted full-scan for MS/MS for MC-LR at 995 [m/z 285-1100], MC-LR is expected to elute at 6.93 minutes

1.4.1.2 CYN

The current standard procedure for CYN quantification was published in 2012 in *Environmental Toxicology and Chemistry*, and uses solid-phase extraction with graphitized carbon cartridges and LC-MS. Limit of detection for this method is 0.5 μg/L and limit of quantification is 0.9 μg/L, allowing detection and quantification of CYN below the EPA-established guideline concentration.

Sample and Standard Preparation**:**

Standard were prepared using commercially available standard solutions and methanol (stock solution of 100 μg/L suggested). Calibration solutions were prepared using standard stock solution and Milli-Q water.

Solid-phase extraction of the sample was performed using graphitized carbon cartridges packed with bon Elut, activated with dichlorimethane:methanol (DCM:MeOH) solvent mixture, acidified with 5% formic acid, and rinsed with Milli-Q water. Then, sample was passed through the cartridges and again washed with Milli-Q water and eluted with DCM:MeOH. To concentrate the sample, it was evaporated to achieve concentration factor of 800. Sample was acidified to improve recovery in environmental samples.

LC-MS/MS Analysis

Chromatographic separation was performed using a PerkinElmer Series 200 HPLC system coupled to an Applied Biosystems QTRAP LC-MS/MS system consisting of a hybrid triple quadrupole linear ion trap mass spectrometer equipped with an electrospray ion source. The analytical column used with a flow rate of 2.1 mL/min, was a Zobbax Sb-Aq column, 150 x 2.1 mm, and a particle size of 3.5 μm, supplied by Agilent technologies. Water and methanol binary gradient was used to perform the chromatographic separation. 20 μL of sample was injected. Mass spectrometer parameters were set to 35 psi for curtain gas, 60 psi for source gas, 5500 V ion spray, and temperature of the turboprobe was maintained at 350 °C.

1.4.1.3 ATX

This quantification method for ATX was published by Sanchez et al in 2014 in *Toxins* journal and the method aims to detect not only ATX, but also its analogs that may contribute to toxicity. Other detection methods exist, such as the method published by Osswald et al in 2007.

Sample and Standard Preparation**:**

To extract sample from algae, green algae dried powder was utilized in this experiment. The samples were weighed and then re-suspended in 4 mL of methanol. Next, cells were sonicated in three cycles of 30 seconds to lysis. Once the algal cells were broken, the mixture

was centrifuged at 3000 rpm for 10 min at 25 °C. The pellet obtained was re-suspended and extracted again twice with 4 mL of methanol. The supernatants were combined, evaporated and re-suspended again in 170 μL. For drinking water analysis, commercially-available ATX solution can be used to create standard solutions, and undiluted water sample places in appropriate vials.

LC-MS/MS Analysis

The LC-MS/MS analysis was performed using the HPLC system from Shimadzu (Kyoto, Japan), consisting of two pumps (LC-10ADvp), autoinjector (SIL-10ADvp) with refrigerated rack, degasser (DGU-14A), column oven (CTO-10ACvp) and system controller (SCL-10Avp). This system was coupled with a QTRAP LC-MS/MS system, which integrates a hybrid quadrupole-linear ion trap mass spectrometer equipped with an ESI source. The column used for cyanotoxins separations was a reverse phase C18 analytical column (100 mm × 4.6 mm) Chromatolith® Performance RP-18e, set to the temperature of 40 °C. The mobile phase was composed of water (A) and acetonitrile (B), both containing 0.05% formic acid. Chromatographic separation was performed by gradient elution, and total run time of 23 min, starting with 2%–70% B for 12 min, then, 70% B was hold for 5 min and reducing afterwards to 2% B over 1 min and hold for 5 min until the next run. The mobile phase flow rate was set for 0.6 mL/min and the injection volume of 5 μL.

Collision-induced dissociation (CID) in the ion-trap MS was performed. The electrospray ionization (ESI) source of QTRAP was operated with the following optimized values of source-dependent parameters: Curtain gas™ : 20 psi, collision-activated dissociation gas (CAD): 6 psi, IonSpray Voltage: 4000 V, temperature: 450 °C, gas 1: 50 psi and gas 2: 50 psi. MS was operated in multiple reaction monitoring (MRM) detecting in positive mode analyzing the

following transitions: anatoxin-A (ATX) (m/z 166 > 166, m/z 166 > 43), homoanatoxin-A (HATX) (m/z 180.1 > 163.1, m/z 180.1 > 145.1), dihidroanatoxin (H2ATX) (m/z 168.0 > 133.0, 168.0 > 150.0), dihidrohomoanatoxin (H2HATX) (m/z 182.0 > 147.0, 182.0 > 164.1), epoxyanatoxin-A (EpoxyATX-A) (m/z 182.0 > 164.1, 182.0 > 138.1) and epoxyhomoanatoxin-A (EpoxyhomoATX-A) (m/z 196.0 > 178.2, 196.0 > 138.1), allowing detection of multiple analogs of anatoxin.

1.4.1.4 STX

The method developed by Watanabe et al, and published in 2013 in Food Additives and Contaminants: Part A, covers sample preparation and LC-MS/MS method for determination of STX produced by algae.

Sample and Standard Preparation**:**

The standards were prepared by dissolving the standard toxins in 0.05 M acetic acid (AcOH) and storing at -20 °C until used for analysis, at which point it is thawed and stored in a refrigerator at 5 °C. Standard is diluted to desired concentrations and used for instrument calibration.

LC-MS/MS Analysis

STX was determined by using a 3200 QTRAP LC-MS/MS system equipped with an ESI source, and an Agilent L-1200 series HPLC, comprising a binary pump (L-6000, Hitachi), a degasser, an autosampler (L-2200, Hitachi) and a column compartment. Column separation was performed on anXbridge amide column (150mm×2.1mm) fitted with a guard column of TSKgel amide 80 (10 mm × 2.1 mm) at 20°C. Mightysil RP-8 GP (150 mm × 4.6 mm) was used as an analytical column instead of the originally reported column. The mobile phases consisted of two eluants: (A) distilled water and (B) MeCN/H2O (99/1 v/y), both with a final concentration of 20

mM HCOOH aq. The elution gradient was set as follows: 100% B for 5 min, gradient elution from 100% B to 65% B over 25 min, hold 65% B for 5 min and hold 100% B for 5 min at a flow-rate of 0.3 mL/min and the injection volume of 5 µL.

The eluent flow was discarded in the post-column for 5 min, then split in the the MS detector. The ionization parameters were set as follows: curtain gas, 40 psi; ion source gas 1 (nebulizer gas pressure), 60 psi; ion source gas 2 (auxiliary gas pressure), 70 psi; ion-spray voltage, 4500 V; probe gas temperature, 500° C; dwell time, 50 ms; and interface heater, on. The toxin was detected in the positive ion mode. Data acquisition and quantitation were performed using the Analyst software ver. 1.4.2 (AB-SCIEX).

1.4.1.5 Simultaneous

Yen et al (2012)developed a method to concentrate and detect nine cyanotoxins simultaneously, including six microcystins (MCs) congeners, NOD, ATX, and CYN in pure and natural waters. The method uses a solid-phase extraction-liquid chromatography-mass spectrometry (SPE-LC-MS), with a detection limit of 2–10 ng/L for microcystins and NOD, 46 ng/L for ATX, and 100 ng/L for CYN.

Sample and Standard Preparation**:**

All nine cyanotoxins were obtained in dry powder form, with >97% purity. MC-LR, MC-RR, MC-YR, MC-LW, MC-LF, and ATX were purchased from Supelco, MC-LA was obtained from Prof. Wayne Carmichael's lab at Wright State University, NOD was purchased from Alexis, and CYN from Calbiochem. Stock solutions were prepared by dissolving toxin powder in methanol, and storing at -20 °C until used for analysis. Surrogate standard solutions of 1,9-D and 2,3,5-TMC, purchased in powder form from Sigma-Aldrich, were prepared with distilled water

and methanol–water solution, respectively. Stock solution concentration was 103 mg/L for 1,9-D and 50 mg/L for 2,3,5-TMC, and both were stored at room temperature.

Analysis of cyanotoxins requires a sample volume of 1.5-3.0 L. In order to analyze determine the concentration of both inter- and extracellular toxins, the cyanobacterial membranes were broken with an ultrasonic probe (Branson, Digital Sonifier! Model S-450D) to liberate the toxins. The water sample was sonicated at 250 W for 1 hour, then filters though 0.7 mm glass microfiber filter (Whatman, GF/F 4.7 cm). The surrogate standard was added to the filtrate, at ~600 ng/L. This was used for SPE concentration. Samples should be prepared and concentrated with SPE within 48 hours of sampling.

The SPE concentration assembly consisted of a dual cartridge assembly of a reverse phase C18 cartridge (2000 mg packed in a 12 mL Supelclean! LC-18 SPE Tube, Supelco), used to extract microcystin, nodularin and anatoxin-A, and a carbon cartridge (500 mg packed in a 6 mL Supelclean! ENVI-Carb SPE Tube, Supelco), used to extract cylindrospermopsin and a portion of anatoxin-A. The SPE cartridges were washed with 20 mL of 20% methanol–water solution. Then, the prepared sample was passed through the assembly at 3-12 mL/min.

After extraction, the assembly was eluted with 40 mL of 90% methanol–water solution at 3 mL/min. Eluted solution was collected into two disposable pyrex collection glass tubes (Supelco), then dried with a gentle stream of pure nitrogen in vacuum until the volume of eluted solution was reduced to ~1 mL. The solution was transferred to a third tube, and the now-empty tubes were rinsed with 5 mL of 90% methanol– water solution, then rinsed liquid was also transferred to the third tube. That tube was further dried with pure nitrogen until again was reduced to 1 mL, and transferred again to a new vial and purged until completely dry. After emptying all the solution, Tube C was also rinsed with 5 mL of 90% methanol–water solution.

The rinsed solution in Tube C was then transferred to the 1 mL vial, with 1 mL each time for five times. Each time, the 1 mL vial was purged with pure nitrogen until completely dry. The residue that remained in the 1 mL glass vial was dissolved with 200 mL of a 90% methanol–water solution. The internal standard, 2,3,5-TMC was then added into the solution at 1000 ng/L before LC-ESI-MS analysis.

LC-MS/MS Analysis

The LC assembly consisted of two identical pumps (model LC-10ADvp) and reverse phase C18 analytical columns (100 mm 4.6 mm i.d.). It was coupled with a UV photodiode array detector (model SPD-M10Avp) followed by an electrospray ionization (ESI) source to connect a quadrupole mass spectrometer (Model LCMS-2010EV). The LC column was set to 40° C , and used acetonitrile and water acidified with 0.01 M TFA and 0.01% HFBA as the mobile phase. The flow rate was set to 0.6 mL/min, and the eluate was piped to the UV/PDA detector first and then delivered to the MS using a split ratio 10:1 (volume to waste/volume transferred). The gradient profile for the acetonitrile to the acidified water in the mobile phase was set at 5% for 10 min, increased to 70% over 30 min, held at 70% for 10 min, reduced to 5% over 10 min, and finally held for another 10 min. The ESI unit used nitrogen as nebulizer gas, heated to 240 ° C to dry the ion-spray aerosol (7.5 L/min). The ionization voltage of the total ion screen interface of 4.5 kV, the detector voltage of 1.5 kV, and the injection sample volume of 50 mL was used.

1.4.2 ELISA

ELISA is a laboratory technique used to measure the concentration of an analyte in a solution. ELISA relies on recognition and binding of a toxin to specific antibodies, and usually manifests as a color change, which allows for an accurate measurement - these methods can

achieve a minimum detection level of 4 ng/L and quantification limit of 2 µg/L for MC-LR. (Merel et al, 2013)

This method can be applied to detection and quantification of all four priority toxins, with commercial kits readily available. The kits can be either tubes, microtiter plates or test strip assays (Cusick, 2013). Some examples include Ridascreen fast saxitoxin test by R-BioPharm, Envirologix tube microcystin kit, shown in Figure 1.7, as well as ELISA kits for all four toxins produced by Abraxis LLC.

Some of the limitations of this method are inability to distinguish between microcystin variants, leading to potential overestimate of MC-LR concentration. Another concern is cross-reactivity with different derivatives, which can be a problem if cross-reactivity does not correlate with toxicity. Due to these concerns, Cusick (2013) recommends that ELISA tests only be used as a screening tool rather than quantitative assay. Of course, both field kits and laboratory tests can produce false positive and false negative results (CLRMA, 2010). It is imperative for water managers to understand the different cyanotoxin detection methods, their limitations, and how each laboratory conducts their tests.

Figure 1.7. Qualitube for Microcystin Detection.
Source: Envirologix

1.5 Toxicity Assays

While LC-MS analysis allows a reliable quantification of the concentration of toxins in a water sample, it gives no information about the toxicity of the sample. Moreover, once the natural chemical structure of these toxins is disrupted during water treatment, detection by this method decreases. The concern is that the metabolites of the toxins may still interfere with proper biological function and elicit a toxic response. Research on these concerns is severely limited, while the risks are high. Thus, it is important to be able to assess the toxicity of chemicals in drinking water.

1.5.1 Cell culturing

Cell culturing is a popular field of predictive toxicology and has many advantages like lower costs than in-vivo assays, fast turnaround, and great sensitivity. Cell culturing is famously versatile – the cellular techniques and assays range from very simple to highly sensitive methods. The choice of an appropriate assay depends on the cell type and the desired end-point. Cytotoxicity or cell viability can be inferred and measured by a variety of end-points like cell proliferation, apoptosis, or necrosis (French, 2016). Non-cytotoxic end-points like DNA damage and mutagenicity can also be measured using cell culturing.

The most basic cytotoxicity assay can be performed using a simple exclusion dye, like trypan blue. The dye penetrates dead cell walls, so the number of dead and viable cells is counted separately. This assay only requires the inexpensive dye and a microscope, but is a relatively crude method. The dye can sometimes penetrate the walls of compromised but living cells. Also, cell counting is associated with a relatively large margin of human error, especially with minimal experience (French, 2016).

On the opposite side of the spectrum are complex assays like the MTT and SRB assay, both of which are colorimetric assays that estimate cellular metabolic activity by dyeing the living part of the cell. The MTT assay measures cell proliferation - it utilizes a yellow tetrazole dye, which is reduced into purple formazan by mitochondrial dehydrogenase enzyme in living cells, as shown in Figure 1.8. The product is then subject to colorimetric analysis – the absorbance is read at a specific wavelength and quantified by a spectrophotometer, then related to the concentration of the solvent. The method is very reliable and is commonly used by toxicologists in predictive toxicology assays (Tolosa, 2015).

The SRB assay also measures cell viability to infer cytotoxicity from survival fraction. The method relies on the uptake of the pink sulforhodamine-B stain, which is an anionic dye that forms an electrostatic complex with the basic amino acid residues of proteins in trichloroacetic acid (TCA) fixed cells, providing a sensitive linear response (Vichai, 2006; French, 2016). The colorimetric evaluation with a spectrophotometer provides an estimate of the total protein mass which is then related to the cell number. The method provides a sensitive measure of cytotoxicity and a stable colorimetric endpoint, meaning it doesn't require a time-sensitive measurement like MTT (Houghton, 2007). It is also efficient, simple, and inexpensive.

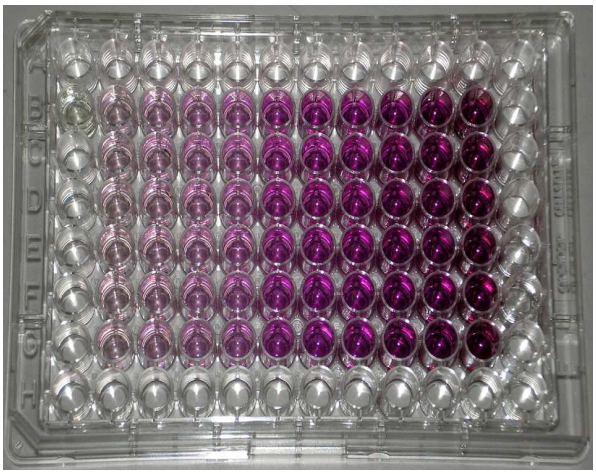


Figure 1.8. MTT Test.
Source: Labprice, Ukranian Science Portal

After looking at the two ends of the complexity and sensitivity spectrum, there are other methods in between that provide sufficiently sensitive data much more rapidly. An example is the PrestoBlue® cell viability assay, which has fewer steps and shorter incubation time, and allows one to obtain sensitive and consistent results in as little as 10 minutes. PrestoBlue® is a cell permeable resazurin-based solution which uses the reducing power of living cells to quantitatively measure cell proliferation. When added to the cells, the reagent is modified by the viable cell and turns red in color, becoming highly fluorescent (French, 2016).

Choosing the best assay depends on the toxin being studied, and the cell line used for the assay. Inoculation assays must be performed before any experimentation to validate the assay for the desired cell line, and to determine the optimal plating density for the colorimetric or fluorescent reading.

A variety of cells can be used depending on which toxin is to be evaluated, due to the variability in toxicity pathways. Hepatotoxins, like microcystin and cylindrospermopsin, are best analyzed with liver cells. For microcystins, cytotoxic effects can be analyzed with normal human hepatocytes (h-Nheps) and human hepatoma cells (HepG2) because microcystins inhibit protein phosphatases 1 and 2A, leading to hyperphosphorylation of the cytoskeleton and apoptosis (Ikehara et al, 2015). The HepG2 cell line was originally derived from a human hepatoblastoma and retained the hepatocyte characteristics, so it can reflect the metabolism of xenobiotic processes in the human body better than other metabolically incompetent cells (Ma, 2016). Neurotoxins, such as ATX and STX, are normally analyzed with cells such as a mouse neuroblastoma cells line. This approach is discussed in detail in the following section.

1.5.2 In-vivo Assays

Bioassays using mice are arguably the best-known in-vivo assays, and have been used to assess the biological effects and the presence of cyanotoxins in water. The sample would be injected into at least 3 mice, followed by a necropsy after 24 hours. Symptom observation would reveal the presence of hepatic or neurotoxicity, thus confirming the presence of the toxins in the matrix. The observed symptoms range from enlargement of the liver and alteration of hepatic cells, to skin irritation. For example, Figures 1.9-10 demonstrate the effects of cylindrospermopsin as a skin irritant, where the mouse in Figure 1.9 appears healthy, and the mouse in Figure 1.10, which was exposed to cylindrospermopsin, has severe skin lacerations.

This assay does not allow for exact identification of the toxins if the mice were injected with a sample of unknown composition, but serves as a great toxicity assay and provides low sensitivity. The mouse assay, in fact, can be used as a semi-quantitative method, if the extent and severity of symptoms are compared among mice exposed to a varying concentration of the toxin (Merel et al, 2013).

Despite relative ease and reliability of this assay, ethical issues arise when it comes to in-vivo animal experimentation. Thus, other methods are preferred in assessing the toxicological

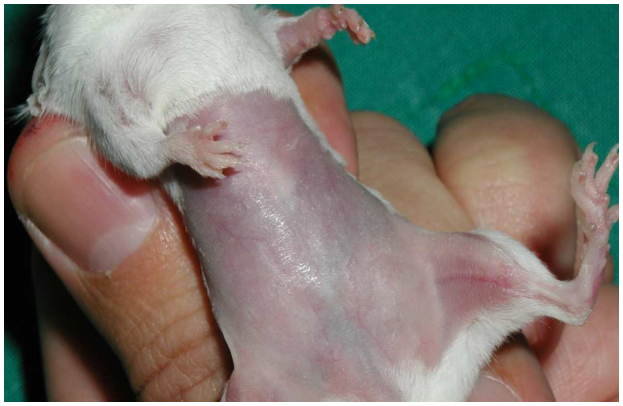


Figure 1.9. Control. Mouse, dosed with 70% ethanol.
Source: Stewart et al., 2006

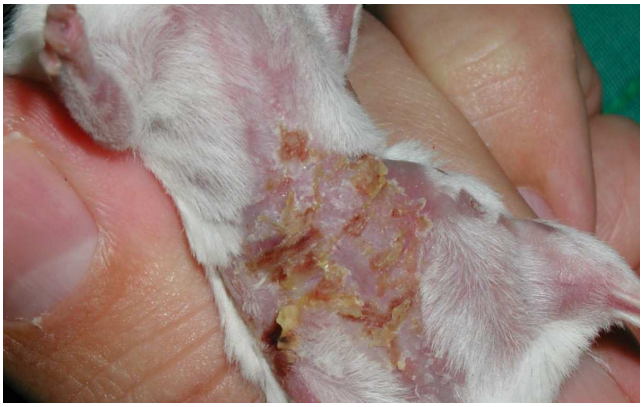


Figure 1.10. Reaction seen. Mouse dosed with 100 µg/mL cylindrospermopsin in 70% ethanol.
Source: Stewart et al., 2006

effects. Other in-vivo methods are considered less-controversial, such as fish early-life stage toxicity (FELST) or using crustacean larvae (e.g. Daphnia, Artemia, etc). These assays involve exposing the larvae or eggs of the selected organisms to the specific volume/concentration of the sample. Larvae experiments can be performed in a 96-well plate, allowing for quantifiable results. However, there is a strong potential for interferences due to matrix effects (Merel et al, 2013).

1.5.3 Toxin- Specific Assays

There are assays that do not necessarily fit under the previously mentioned categories, which were developed specifically for the detection of certain toxins. These are discussed in this section.

1.5.3.1 Protein Phosphatase Inhibition Assay (PPIA) for MC-LR

PPIA is applied to assess the toxicity of microcystins and nodularins. (Merel, 2013) The assay is based on selective deactivation (or inhibition) of a protein serine/threonine family I phosphatases (PP1 and PP2A), an enzyme targeted by microcystins. (Carmichael, 2001) The assay can be performed using commercially available PP1 and PP2A enzymes, the supplier will often provide recommended concentrations for inhibition studies. Before incubation with the substrate, the enzyme is exposed to the toxin-containing sample. Absorbance of the mixture is measured at a certain wavelength, allowing the enzyme activity to be assessed – and is inversely proportional to the toxin concentration.

The advantage of PPIA is its ability to detect biological activity in microcystins, and is particularly useful since microcystin toxicity is directly proportional to their phosphatase inhibition. It has a fast throughput, and can analyze a large number of samples within a few hours at a detection limit of 0.01 μg/L. (Merel, 2013)

The disadvantage of this method is that it does not differentiate between the covariants and compounds present – the toxicity is assessed for all types of microcystins present as well as nodularins. This method should not be used if there is no way to extract the toxin of interest.

1.5.3.2 Neuroblastoma Assays for STX

STX can be analyzed (both qualitatively and quantitatively) using a cell viability assay with the use of mouse neuroblastoma cells. Neuroblastoma is a brain cancer, and is used in this assay because the cells express the sodium channel blocked by STX. (Nicholson, 2007) Neuroblastoma cells are sensitive to all persistent toxic substances, and have a good response curve to toxin potency. The updated method uses colorimetric end points for direct cells staining, achieving a limit of detection of 7-10 ng STX eq/mL, which is comparable to LC-MS/MS. The reproducibility of this assay is adequate for screening, and no false positives or negatives were detected.

MIST (Maritime In vitro Shellfish Test) is a commercially available test based on the neuroblastoma assay for both qualitative and quantitative assays (Cusick, 2013) though interfering substances may out the quantitative results unreliable. (Nicholson, 2007) Neuroblastoma assay is most suitable for toxicity screening purposes, allowing to eliminate most samples in routine toxin monitoring, moreover - it can provide results in within 1.5 days, and has higher throughput and greater sensitivity than a mouse bioassay, at a cheaper cost (Nicholson, 2007).

1.6 Previous Studies and Research Gaps

As demonstrated in the previous sections, cyanotoxins are an emerging threat to the water industry. There is a growing concern that the metabolites of the toxins may still interfere with proper biological function and elicit a toxic response. Many environmental chemicals exhibit toxication – and effect, where a parent compound is transformed into a metabolite that is more toxic. This can occur via biotransformation enzymes or abiotic chemical reactions (Roberts, 2014).

Some studies have explored the toxicity of the more common cyanotoxins and their oxidation byproducts. Brooke (2006) reported decreased toxicity of microcystin metabolites post treatment with O_3, as shown by a standard mouse bioassay, while Rodriguez (2008) reported the oxidation products of microcystin after treatment with Cl_2 and $KMnO_4$ were non-toxic, as shown by PPIA assay. Yan reported decreased toxicity of CYN metabolites post treatment with O_3, as shown by the MTS assay on HepG2 cells (Yan, 2016). Finally, Sengles-Derham (2003) reported no increase in cancer in transgenic mouse bioassay for MC-LR, CYN, or STX after chlorination. While these studies suggest a positive trend, each of the studies calls for more research - specifically to explore how human health may be affected. Mouse bioassay and PPIA assays, as mentioned in previous sections, are not always reliable and do not always accurately represent the same trends as the effects on humans.

In order to fill the gaps in research, a study comparing the most common and effective oxidation treatments for cyanotoxins is needed, where the toxicity of the toxins before and after oxidation can be evaluated using human cell cultures. This would allow for a more accurate understanding of the effect on human health, at minimal cost and risk.

While the priority is to investigate the cytotoxic effects of cyanotoxin metabolites, non-cytotoxic end-points like mutagenicity and DNA damage should also be evaluated. To that end, the HepG2 human hepatoma cell line is likely the best option. It has been successfully used to explore both cytotoxic and non-cytotoxic effect of cyanotoxins on the liver. It has been used to study the involvement of reactive oxygen species in the MC-LR induced apoptosis (Ma, 2016) and showed a strong correlation based on exposure time and dosage. Žegura (2003) reported that MC-LR induced oxidative DNA damage in HepG2 cells, even at non-cytotoxic doses, followed by Nong reporting that reactive oxygen species induce cytogenotoxicity from microcystin, which is dependent on exposure time (Nong, 2007). Finally, Štraser investigated the genotoxic effect of CYN in the HepG2 cell line, confirming genotoxic activity at non-cytotoxic concentrations and establishing that concentrations 2 mM and higher reduced cell viability by more than 30% (Štraser, 2011).

1.7 Conclusions

Algal blooms are a growing threat to the water industry, and one of the major reasons is that they are often accompanied by proliferation of the harmful species - cyanobacteria. Cyanobacteria produce a variety of toxins, and the EPA named four of them the priority toxins – MC-LR, CYN, ATX, and STX. These toxins are responsible for a variety of adverse health effects – MC-LR and CYN are known hepatotoxins, while ATX and STX are dangerous neurotoxins.

With some great developments in the analytical technologies, there are reliable methods for toxin detection. LC-MS and ELISA are the methods most commonly used due to their high sensitivity, relative ease of application, and lower costs.

Cyanotoxins are usually treated with oxidation in conventional drinking water treatment plants; this approach has shown to successfully break down the toxin molecules. However, research is severely lacking in assessing toxicity of oxidation by-products. Results of a study conducted by Sharma et al. (2012) suggested that oxidation products of microcystin oxidation with O_3, Cl_2, and $KMnO_4$ were non-toxic, but there are other toxins and other potential oxidants to be considered and studied extensively. Other oxidants used in treatment are ClO_2, as well as more advanced processes like UV-H_2O_2 oxidation.

Toxicity assays used in assessing the toxicity of the original toxins, and that can be applied to assessing toxicity of the oxidation byproducts fall into two general categories – cell culturing and in-vivo assays. Cell culturing is the preferred method, since it is cheaper and faster. In-vivo assays allow accurate and sensitive toxicity assessment, and allow observation of the actual toxic effects once necropsy on the animal is performed. However, in-vivo assays don't differentiate between the toxins and are also associated with ethical controversy.

There is always a looming threat of producing toxic by-products, but there is also an increase in attention and more support for research in this field. Water utilities have access to more knowledge to make safer management decisions.

2: MATERIALS AND METHODS

The experimental portion of this project was designed to evaluate the effect of standard oxidation treatments on toxicity of microcystin and cylindrospermopsin. Toxin solutions were prepared in water and treated with three oxidants, described in section 2.1. Once the toxin solutions were treated with oxidation, two assays were conducted in parallel – a cytotoxicity assay was conducted on HepG2 carcinoma cells to compare the resulting (%) cell death from exposure to treated and untreated toxins, described in section 2.2, and an LC-MS/MS analysis of the solutions to quantify the concentration of the toxins in the solution post-treatment, described in section 2.3.

2.1 Oxidation Treatment of Toxins

Low-range chlorine standard solution (Cat. 26300200) and potassium permanganate solution (Cat. 1416442) were purchased from Hach Company (Loveland, CO). Ozone was generated onsite using a 2B Technologies Model 306 Ozone Calibration Source, shown in Figure 2.1, loaned by Dr. Delphine Farmer. Dissolved ozone solution (2 mg/L) was prepared by bubbling the ozone gas into reverse osmosis (RO) water column at a flow of 3 L/min, and ozone output concentration of 3 ppmv. The ozone concentration was confirmed using the Hach Ozone AccuVac ® Ampules, HR (Cat. No. 2518025).

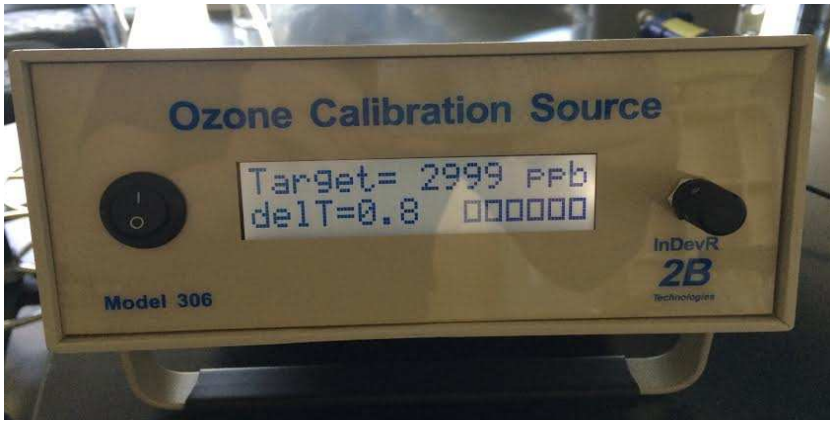


Figure 2.1. Ozone Generator

Microcystin-LR (Item No. 10007188) and cylindrospermopsin (Item No. 10007867) was purchased from Cayman Chemical (Ann Arbor, MI) and dissolved in RO water to achieve a 500 μM stock solution. Aqueous stock solutions were frozen and stored at -20°C.

Each toxin was treated using 2 concentrations of each of the 3 oxidants at a contact time of 30 minutes. The samples were prepared by diluting the toxin stock solutions to a working solution of 150 μM MC-LR and 60 μM CYN, then dosing the working solutions with oxidants to achieve the treatment concentrations outlined in Table 2-1.

Table 2-1. Treatment Matrix

Toxins:		**Treatments:**
MC-LR		2.5 mg/L Cl_2
		5 mg/L Cl_2
	X	2.5 mg/L $KMnO_4$
CYN		5 mg/L $KMnO_4$
		1 mg/L O_3
		2 mg/L O_3

The oxidant concentrations used for this study mimic the typical levels used for drinking water treatment. The treatments were added using a micropipette and allowed to react in a glass test tube for the desired contact time on a magnetic stir plate with a Teflon-coated magnetic stir bar. All treated samples, except ozone, were quenched using sodium thiosulfate after the desired contact time. Ozone, considering its short half-life, was allowed to decay naturally. Untreated working toxin solutions (150 μM MC-LR and 60 μM CYN) were used as positive controls for the experiment.

After the oxidation treatment, each sample was filtered using the sterile 0.22 micron Whatman syringe filters, then diluted 1:1 with complete medium. The resulting concentration of the untreated sample was 75 μM MC-LR and 30 μM CYN. All samples were stored at -20°C until the cytotoxicity assay.

2.2 Cell Culturing and Cytotoxicity Assay

Presto Blue ™ cell viability assay was used on HepG2 human hepatoma cells to compare cytotoxicity of the toxins before and after oxidation treatment.

2.2.1. Cell Culturing Conditions

HepG2 human hepatoma cells were purchased from the American Type Culture Collection (ATCC, HB-8065^{TM}) and grown in 75 cm^2 culture flasks with Dulbecco's Minimum Essential Medium (Gibco© by Life Technologies) containing 10% Fetal Bovine Serum and 5% Sodium Pyruvate, shown in Figure 2.2. Flasks were incubated at 37°C with 5% CO_2. Cells were passed at 1:4 ratio once 85% confluence was reached. The medium was changed 1-2 times per week.

2.2.2. Inoculation Assay

An inoculation assay was performed to validate the Presto Blue ™ (Invitrogen ™ by Life Technologies) assay for use with the HepG2 cell line and determine the optimal plating density for a linear (predictive) relationship between the cell number and optical absorbance. Cells were harvested using 2 mL of 0.25% Trypsin-EDTA (Gibco© by Life Technologies), neutralized with 6 mL of complete medium, and counted using the BioRad TC10 Automated cell counter, shown

Figure 2.2. Complete Medium

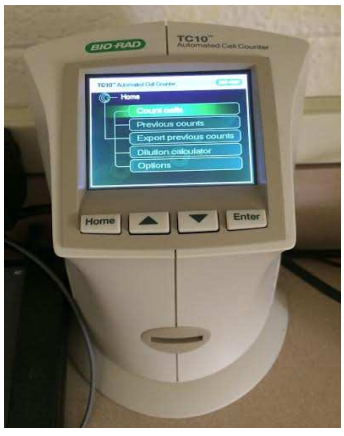


Figure 2.3. Automated Cell Counter

in Figure 2.3. The cell suspension was centrifuged at 125 x g for 5 minutes, medium was carefully aspirated, and cells were re-suspended to achieve the desired density of 2,000,000 cells/mL. The new suspension was used in a serial dilution, and seeded into a 96-well plate, columns 1-6 only, as follows:

Table 2-2. Inocculation Assay Plating Scheme

Row:	**Cell density:**
A	200,000 cells/well
B	100,000 cells/well
C	50,000 cells/well
D	25,000 cells/well
E	12,500 cells/well
F	6,250 cells/well
G	3,125 cells/well
H	Medium only (background reading)

The culture plate was incubated for 24 hours to allow the cells to adhere. Afterward, medium was carefully removed with a Pasteur pipette, and each well was filled with 90 µL of complete medium and 10 µL of the Presto Blue ™ reagent. The plate was incubated for 1 hour, then absorbance was read on a VersaMax spectrophotometer microplate reader (Figure 2.4) at 570 nm and 600 nm wavelengths. The 570 nm values were normalized to the 600 nm values for the experimental wells. Medium-only wells were used as a background reading (subtracted from absorbance readings of each sample).

Figure 2.4. VersaMAX Microplate Reader

2.2.3. Cytotoxicity Assay

For cytotoxicity assessment, cells were harvested using 2 mL of 0.25% Trypsin-EDTA (Gibco© by Life Technologies), neutralized with 6 mL of complete medium, and counted using the BioRad TC10 Automated cell counter. The cell suspension was diluted with complete medium to the desired density of 100,000 cells/mL, and seeded into 96-well culture plate at 100 µL/well, resulting in seeding density of 10,000 cells/well. The plate was incubated for 24 hours to allow the cells to adhere.

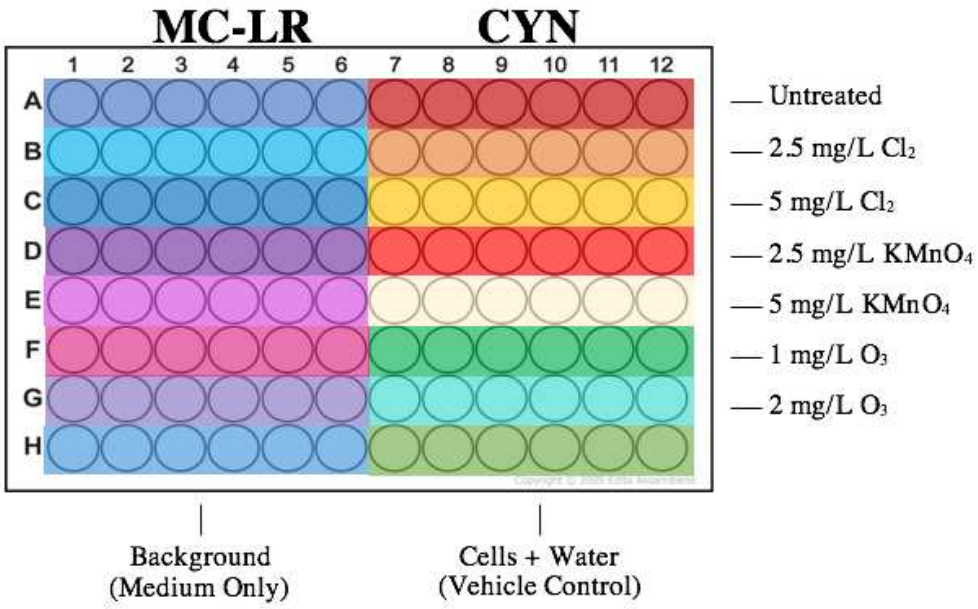


Figure 2.5. 96-well Plate Treatment Layout

Afterward, the medium was carefully removed using Pasteur pipettes, and the cells were dosed with 90 µL/well of treatment, with 6 replicate wells for each treatment. Figure 2.5 shows the treatment layout on a 96-well plate, and the preparation of the samples is discussed in the following section. Cells were incubated with treatments for a 24-hour period. Afterward, 10 µL of the Presto Blue ™ reagent was added to each well and the plate was incubated for 1 hour. Then, absorbance was read on a spectrophotometer at 570 nm and 600 nm wavelengths. The 570

nm values were normalized to the 600 nm values for the experimental wells. Medium-only wells were used as a background control, and were subtracted from the absorbance reading of all samples. Each sample was tested in 6 replicates. The following equations were used to calculate the (%) viability compared to the positive controls, and (%) cell death:

$$Viability\ (\%) = \left(\frac{OD_{sample}}{OD_{control}}\right) \times 100$$

$$Cell\ Death\ (\%) = 100 - Viability\ (\%)$$

2.3 LC-MS/MS Analysis

After each sample and control were diluted 1:1 with complete medium, as described in the previous section, 1 mL of each treatment and control was aliquoted for LC-MS analysis. Since MC-LR and CYN can be measured simultaneously, standard solutions containing both toxins were prepared in methanol to establish a standard curve. The concentrations of MC-LR in the standards were 100 μM, 50 μM, 25 μM, 5 μM, and 1 μM, while the concentrations of CYN in the standards were 50 μM, 30 μM, 10 μM, 5 μM, 1 μM. The analysis was performed on a Waters Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer the Colorado State University Proteomics and Metabolomics Facility (PMF), under the supervision of Dr. Lisa Wolfe. The method is described in detail below, and was provided by the PMF laboratory.

LC-MS/MS Analysis

The analysis was performed on a Waters Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer. Chromatographic separations were carried out on a Waters T3 stationary phase (1 mm x 100 mm, 1.7 μM) column. Mobile phases were Acetonitrile with 0.1% formic acid (B) and water with 0.1% formic acid as modifier (A). The analytical gradient was as follows: time = 0 min, 5% B; time = 1.0 min, 5% B; time = 4 min, 98% B; time = 5 min,

98% B; time = 5.5 min, 5% B; time = 8.5 min, 5% B. Flow rate was 200 μL/min and injection volume was 1 μL. Samples were held at 4° C in the autosampler, and the column was operated at 60° C. The MS was operated in selected reaction monitoring (SRM) mode, where a parent ion is selected by the first quadrupole, fragmented in the collision cell, then a fragment ion selected for by the third quadrupole. Product ions, collision energies, and cone voltages were optimized for each analyte by direct injection of individual synthetic standards. Inter-channel delay was set to 3 ms. The MS was operated in positive ionization modes with the capillary voltage set to 3.2 kV respectively. Source temperature was 150° C and desolvation temperature 500° C. Desolvation gas flow was 1000 L/hr, cone gas flow was 150 L/hr, and collision gas flow was 0.2 mL/min. Nebulizer pressure (nitrogen) was set to 7 Bar. Argon was used as the collision gas. A calibration curve was generated using authentic standards for each compounds and their corresponding stable isotope labeled internal standards in neat solution.

Table 2-3. SRM Transitions for Target Compounds

Compounds Name	Retention Time Window	Cone Voltage	Collision Energy	Dwell Time	Precursor m/z	Product m/z	Ionization Mode
Cylindrospermopsin	0-10	30	40	0.01	416	194	Positive
Cylindrospermopsin	0-10	30	25	0.01	416	336	Positive
Microcystin	0-10	25	13	0.01	498.6	135	Positive
Microcystin	0-10	85	90	0.01	995.5	127	Positive
Microcystin	0-10	85	75	0.01	995.5	135	Positive

Data Analysis and Statistics

All Raw data files were imported into the Skyline open source software package (MacLean, 2010) and peak areas extracted for target compounds and normalized to the peak area of the appropriate internal standard in each sample. Normalized peak areas were exported to

Excel and absolute quantitation was obtained by using the linear regression equation generated for each compound from the calibration curve. Limits of detection (LOD) and limits of quantification (LOQ) were calculated as 3 times or 10 times the standard deviation of the blank divided by the slope of the calibration curve respectively (Shrivastava, 2011; Broccardo, 2013). Analysis of variance was conducted on each element using the aov function in R, and p-values were adjusted for false positives using the Bonferroni-Hochberg method in the p.adjust function in R (Banjamini, 1995). PCA was conducted on UV-scaled data using the pcaMethods package in R.

3: RESULTS

3.1 Treatment Efficacy

The concentration of the toxins was determined using LC-MS at the CSU Proteomics and Metabolomics Facility. The results of the analysis are summarized in Table 3-1 and Figure 3.1, and show the average concentration of each toxins calculated from 3 injections performed per sample.

Table 3-1. LC-MS Measured Toxin Concentration

	MC-LR		**CYN**	
	Ave. Toxin Concentration (μM)	**%CV**	**Ave. Toxin Concentration (μM)**	**%CV**
Untreated	15.4	7.7	34.7	8.3
2.5 mg/L Cl_2	15.0	9.5	37.4	19.1
5 mg/L Cl_2	16.0	16.7	22.1	5.8
2.5 mg/L $KMnO_4$	13.8	18.1	34.7	12.1
5 mg/L $KMnO_4$	11.8	21.7	38.1	10.7
1 mg/L O_3	21.5	12.0	37.1	6.7
2 mg/L O_3	20.3	11.0	39.2	18.9

The graphic summary, shown in Figure 3.1, suggests that the treatment of toxins with oxidation was ineffective at reducing the toxin concentration, thus % removal of toxin was not calculated for this dataset.

3.2 Cytotoxicity

Cytotoxicity of the treated and untreated toxins was evaluated by conducting a colorimetric PrestoBlue assay on HepG2 human carcinoma cell line. The cells were plated in a 96-well plate, exposed to untreated and treated toxin solutions for 24 hours, after which the assay was performed. The PrestoBlue indicator was distributed to all wells for a 1-hour reaction time, then absorbance was read using a mass spectrometer. The absorbance data was used to calculate the (%) cell death compared to the control. Table 3-2 and Figure 3.2 summarize the results.

Table 3-2. (%) Cell Death Following Exposure to Treated and Untreated Toxins

	MC-LR		**CYN**	
	% Cell Death	**%CV**	**% Cell Death**	**%CV**
Untreated	25.8	15.1	63.1	17.1
2.5 mg/L Cl_2	9.1	6.9	63.0	4.9
5 mg/L Cl_2	18.1	12.3	70.1	20.0
2.5 mg/L $KMnO_4$	16.1	6.4	63.0	8.5
5 mg/L $KMnO_4$	16.8	6.2	64.1	14.2
1 mg/L O_3	14.1	10.0	70.7	16.9
2 mg/L O_3	30.2	11.3	70.9	19.0

The graphic summary of data, shown in Figure 3.2, suggests that (%) cell death was not reduced after treating toxins with oxidation, which is consistent with findings in section 3.1 that the oxidation treatment of toxins was not successful.

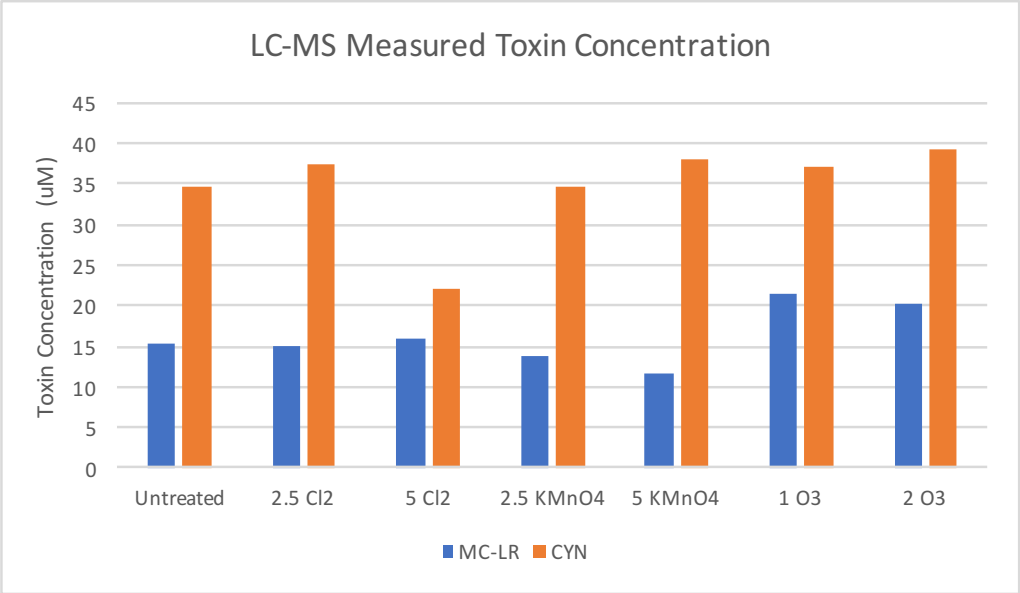


Figure 3.1. LC-MS Measured Toxin Concentration

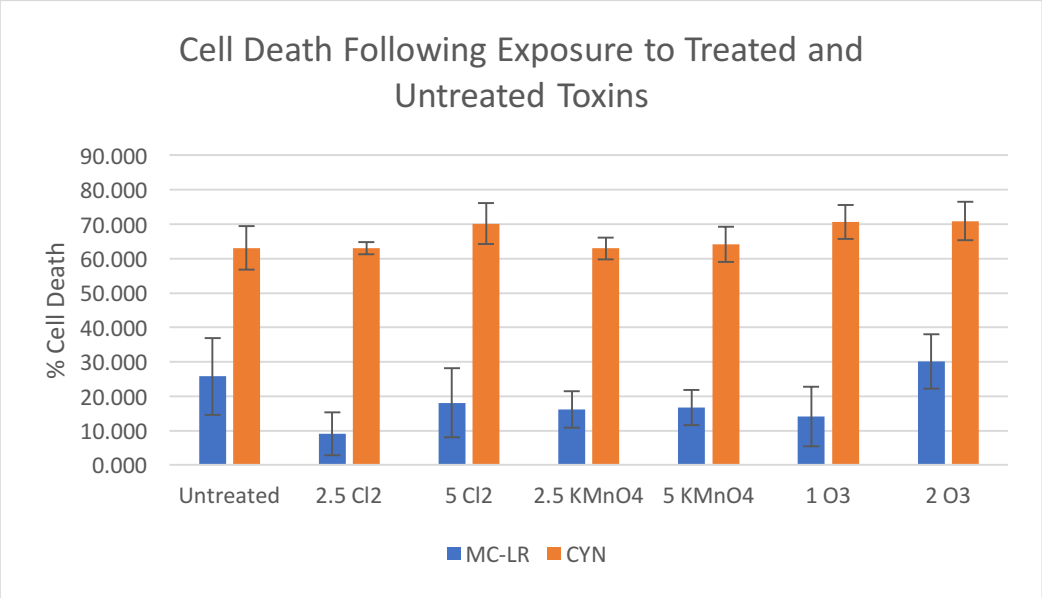


Figure 3.2. Cell Death Following Exposure to Treated and Untreated Toxins Measured by PrestoBlue Assay on HepG2 cells.
N=18, with standard deviation error bars.

3.3 Statistics

One-way ANOVA analysis was conducted on the experimental cytotoxicity data (PrestoBlue assay absorbance data) to determine whether there were statistically significant differences in cell death in groups where the toxins were treated vs. untreated, as wells as comparing the treatments and dosages. The ANOVA was conducted using the Data Analyst tool in MS Excel, and the cutoff p-value for statistical significance was set to 0.05. Tables 3.3A-C summarize the resulting p-values for each comparison group.

Table 3-3A. One-way ANOVA comparing Treated and Untreated Samples

Toxin	Comparison Pair	P-value
MC-LR	Untreated vs. 2.5 mg/L Cl_2	0.012
	Untreated vs. 5 mg/L Cl_2	0.473
	Untreated vs. 2.5 mg/L $KMnO_4$	0.157
	Untreated vs. 5 mg/L $KMnO_4$	0.194
	Untreated vs. 1 mg/L O_3	0.136
	Untreated vs. 2 mg/L O_3	0.447
CYN	Untreated vs. 2.5 mg/L Cl_2	0.363
	Untreated vs. 5 mg/L Cl_2	0.082
	Untreated vs. 2.5 mg/L $KMnO_4$	0.243
	Untreated vs. 5 mg/L $KMnO_4$	0.955
	Untreated vs. 1 mg/L O_3	0.156
	Untreated vs. 2 mg/L O_3	0.043

Table 3-3B. One-way ANOVA comparing Oxidation Treatments

Toxin	Comparison Pair	P-value
MC-LR	2.5 mg/L Cl_2 vs. 2.5 mg/L $KMnO_4$	0.021
	2.5 mg/L Cl_2 vs. 1 mg/L O_3	0.160
	2.5 mg/L $KMnO_4$ vs. 1 mg/L O_3	0.702
	5 mg/L Cl_2 vs. 5 mg/L $KMnO_4$	0.442
	5 mg/L Cl_2 vs. 2 mg/L O_3	0.083
	5 mg/L $KMnO_4$ vs. 2 mg/L O_3	0.014
CYN	2.5 mg/L Cl_2 vs. 2.5 mg/L $KMnO_4$	0.530
	2.5 mg/L Cl_2 vs. 1 mg/L O_3	0.014
	2.5 mg/L $KMnO_4$ vs. 1 mg/L O_3	0.009
	5 mg/L Cl_2 vs. 5 mg/L $KMnO_4$	0.095
	5 mg/L Cl_2 vs. 2 mg/L O_3	0.859
	5 mg/L $KMnO_4$ vs. 2 mg/L O_3	0.048

Table3-3C. One-way ANOVA comparing Dosages of Oxidation Treatments

Toxin:	Pair	P-value
MC-LR	2.5 mg/L Cl_2 vs. 5 mg/L Cl_2	0.010
	2.5 mg/L $KMnO_4$ vs. 5 mg/L $KMnO_4$	0.790
	1 mg/L O_3 vs. 2 mg/L O_3	0.017
CYN	2.5 mg/L Cl_2 vs. 5 mg/L Cl_2	0.001
	2.5 mg/L $KMnO_4$ vs. 5 mg/L $KMnO_4$	0.305
	1 mg/L O_3 vs. 2 mg/L O_3	0.378

4: DISCUSSION

The results of the LC-MS analysis suggest that the oxidation treatment of the toxins was not effective at reducing the toxin concentrations (Table 3-1). These findings are consistent with the results of the cytotoxicity assay, which did not show a significant reduction of cell death in cells exposed to toxins treated with oxidation compared to the cells exposed to untreated toxins (Table 3-2). The treatments likely failed because the pre-treatment concentration of the toxins (150 μM MC-LR and 60 μM CYN) was too high. The pre-treatment toxin concentrations were selected for optimal measurement of cell death with the PrestoBlue assay, to ensure that adequate comparisons could be drawn between measured cell viability before and after treatment once the samples are diluted with the culture medium. These concentrations of toxins, however, are significantly higher than the observed naturally-occurring concentrations – for example, a survey conducted by NOAA in Lake Erie in 2015 reported microcystin concentrations ranging from below detection to 36.7 μg/L (0.0369 μM), while Ohio EPA observed cylindrospermopsin concentrations ranging from below the detection limit to 9 μg/L(0.021 μM) in Grand Lake St. Marys in 2010 (D'Anglada, 2015).

As can be observed from the experimental data in Table 3-3, there was a statistically significant (p-value of 0.012) 16% reduction in cell death in the sample group that was exposed to microcystin treated with 2.5 mg/L Cl_2 compared to the untreated toxin, even though the LC-MS analysis only measured a 0.5 μM reduction in microcystin concentration compared to the untreated sample. There was also a statistically significant (p-value of 0.043) 8% increase in cell death in the group that was exposed to cylindrospermopsin treated with 2 mg/L O_3 compared to the group exposed to the untreated cylindrospermopsin, and the LC-MS analysis measured a higher concentration of cylindrospermopsin by 5 μM in the treated sample. This data is

consistent with the consensus that both cyanotoxins cause time- and concentration-dependent apoptosis in HepG2 cells (Ma, 2016; Nong, 2007; Štraser, 2011; Yan, 2016).

These studies can also be used to compare the (%) cell death/ (%) viability between published and experimental results, since these studies also used the HepG2 cell line with a 24-hour toxin exposure time. The variations in the results can be accounted for by the difference in the cytotoxicity assay used – the studies in question used the MTT assay, which is more sensitive than the PrestoBlue assay used for this experiment, as well as the variation in the cell seeding density. Nong (Nong, 2007) reported time- and concentration-dependent decrease in cell viability after cells seeded at 8,000 cells/well were exposed to 1, 3, 30, and 10 μM MC-LR for 24, 48, and 72 hours, where % viability is comparable to the experimental results (94% viability at 10 μM MC-LR in Nong's study vs. 83% viability at 12 μM MC-LR in the experimental results). Štraser (Štraser, 2011) reported decreasing cell viability with increasing CYN concentration after HepG2 cells plated at 8,000 cells/well were exposed to 0, 0.01, 0.05, 0.1, 0.5, 1 and 5 μg/mL (0.024, 0.12, 0.24, 1.2, 2.4, and 12.1 μM) CYN fort 24 hours, where % viability is also comparable to the experimental results (~50% viability at 12.1 μM CYN in Štraser's study vs. 30% viabilty at 22 μM CYN in the experimental results). Finally, Yan (Yan, 2016) reported that cell viability of HepG2 cells seeded at 25,000 cells/well was reduced by about 50% after 24 hrs and 70% after 48 hrs of exposure to 24 μM CYN, reporting IC50 values of 12.82 μM and 2.5 μM for 24 and 48 h exposures, respectively. This is consistent with the experimental results, where 30% viability was observed at 22 μM CYN concentration. Finally, it is worth mentioning that this data was consistent with preliminary dose-response experiments conducted during method development, and aided in the selection of the initial toxin concentrations for the

experiment. Figures summarizing the relevant results of these studies are available in Appendix A3, Figures A3.1, A3.2, & A3.5.

Some inferences can also be drawn in comparing the effectiveness of treatments. A statistically significant (p-value of 0.021) reduction in cell death was observed in sample groups where microcystin was treated with 2.5 mg/L Cl_2 compared to the group treated with 2.5 mg/L $KMnO_4$, as well as a reduction in cell death when microcystin was treated with 5 mg/L $KMnO_4$ compared to the sample group where microcystin was treated with 2 mg/L O_3 (p-value of 0.014). Among the treatments of cylindrospermopsin, both the 2.5 mg/L Cl_2 and the 2.5 mg/L $KMnO_4$ oxidation treatments resulted in lower cell death than 1 mg/L O_3 (p-value of 0.014 and 0.009, respectively) and the 5 mg/L $KMnO_4$-treated cylindrospermopsin samples resulted in lower cell death that those treated with 2 mg/L O_3. While the effectiveness of these oxidation treatments has been vastly explored for other pollutants, there has not been an all-encompassing study that compares the efficacy of all three treatments in reducing the concentration and toxicity of microcystin and cylindrospermopsin, especially not applied to human cells.

Finally, a statistical analysis comparing the effects of oxidant doses on cell death suggests that in both cylindrospermopsin and microcystin, there was a statistically significant increase in cell death for samples treated with 5 mg/L of Cl_2 compared to the samples treated with 2.5 mg/L Cl_2 (the p-value for MC-LR was 0.01 with a 9% increase in cell death, and 0.001 for CYN with a 7% increase in cell death). Samples where microcystin was treated with 1 mg/L O_3, 16% lower cell death was observed compared to the samples treated with 2 mg/L O_3 (p-value 0.017). This portion of the results is not consistent with published data - previous studies show evidence of decreasing toxicity and toxin concentration with increasing oxidant dose. Brooke reports significant decreases in toxicity and quantity of MC-LR with increasing ozone dose as shown by

a HPLC, PP2A, and standard mouse bioassay, after treating two different source waters spiked with 20 μg/L (20 nM) MC-LR with 0.25, 0.5, and 2 mg/L dose of O_3 for a contact time of 5 min (Brooke, 2006). Rodriguez reports non-cytotoxic oxidation metabolites and decreasing concentration of MC-LR after treatment with both potassium permanganate and chlorine, evaluated with HPLC and PPIA after treating 2 mg/L (2 μM) MC-LR with oxidant doses of 4.3 mg/L Cl_2 and 4.7 mg/L $KMnO_4$ for contact times from 0 to 30 min (Rodriguez, 2008). Yan reports decreasing concentration of CYN with increasing ozone doses measured by HPLC, as well as reduced cytotoxicity evaluated with an MTS assay after treating 8.3 mg/L (24 μM) CYN solution with static doses of 0.8, 3.3, 12.5, 20.8, 31.3, and 62.5 μM O_3 (Yan, 2016). Figures summarizing the results of these studies are available in Appendix A3, Figures A3.3-A3.5.

The main source of error in this project was a serious error with the MC-LR concentrations. The LC-MS analysis measured the concentration of untreated MC-LR as 15 μM, when per the protocol it should have been close to 75 μM. It is possible an error was made in preparing the stock solution, and the solid powder did not dissolve completely resulting in lower starting concentration of MC-LR. It is also possible that there was carryover or retention in the column during the LC-MS analysis, as some discrepancies were observed by the Dr. Wolfe when analyzing the samples. It is also possible that there were interferences with the culture medium during the LC-MS analysis, since the standard curves prepared for the analysis were not observed to have the same discrepancies as the experimental samples. Another potential source of error were the Hach AccuVac ampules that were used to confirm the ozone concentration in the water column that would be used for treatment – the ampules used have surpassed the expiration date and may have produced an erroneous reading. Finally, the HepG2 cells used for the cytotoxicity assay were harvested at confluency, which means they were not as metabolically

active as actively growing cells. Cells with slow metabolic activity would not take up the toxin as readily, and would not be as drastically affected by the toxins as the growing cells.

As mentioned previously, the oxidation treatments selected for this project are standard practice in drinking water treatment, and have been reported effective at reducing the toxicity of microcystins and cylindrospermopsin (Brooke, 2006; Rodriguez, 2008; Yan, 2016). The dosages of oxidants were based on typical drinking water treatment levels, and were optimized for the high toxin concentrations – since liquid oxidants were used for batch reactor treatment, the highest possible dosage of the oxidant was selected that would not dilute the toxin below the concentration of measurable cell death.

Cell death was deemed an appropriate measurable end-point for this experiment, since it evaluates the worst-case scenario of cytotoxic effects on a human liver cell. It was important to evaluate the cytotoxicity using a method that focuses on human, rather than mouse or protease bioassay, since these assays are not always reliable and may not accurately represent the effect on humans. The HepG2 cell line was selected, because it was originally derived from a human hepatoblastoma and retained the hepatocyte characteristics, reflecting the metabolism and xenobiotic processes in the human body better than other metabolically incompetent cells (Ma, 2016). While the goal was to develop a method to best investigate how the oxidation treatment of MC-LR and CYN would reflect on their toxicity to humans, the findings reflect that the cyanotoxin concentrations that cause apoptosis of human cells are much higher than what is naturally observed in surface waters, and these high concentrations are not successfully treated by the conventional oxidation treatment dosages. While it is still a priority to investigate the cytotoxic effects of cyanotoxin metabolites, cytotoxic responses other than cell death and non-cytotoxic end-points like mutagenicity and DNA damage are likely to be more insightful

measures of the potential effects of chronic exposures to cyanotoxins. Studies that evaluate oxidative DNA damage at non-cytotoxic doses, such as the study published by Žegura in 2003, may elucidate the mechanisms by which chronic exposure to low concentrations of microcystins can contribute to the increased the risk for cancer development.

5: CONCLUSIONS

Cell death may not be the best measured endpoint of cytotoxic effects of MC-LR and CYN, since the concentrations that cause measurable cell death are higher than what is commonly observed in nature and are not effectively treated by standard oxidation. Both toxins have been reported to cause adverse effects at much lower concentrations, so future research should focus on more insightful assays to explore cytotoxicity and subcellular damage before and after oxidation treatment.

Moreover, it also because evident from this project that using liquid oxidants for treating high concentration toxins in bench scale batch reactor-style treatment is not effective at reducing toxicity without diluting the toxin. While dilution is usually an effective companion to treatment, it does not allow for an adequate comparison of treated and untreated toxins when the measured end-point is cell death.

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APPENDIX

A1. Raw Absorbance Data – Excel Output

Group: MC-LR untreat						
Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
1	B1	0.272		0.269666667	0.040687426	15.0880444
	B2	0.326				
	B3	0.234				
	B4	0.302				
	B5	0.217				
	B6	0.267				
Group Summaries						
~End						
Group: CYN untreat						
Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
1	B7	0.167		0.134	0.023047776	17.19983318
	B8	0.133				
	B9	0.125				
	B10	0.153				
	B11	0.102				
	B12	0.124				
Group Summaries						
~End						
Group: MC-LR + 2.5 Cl2						
Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
1	C1	0.291		0.329833333	0.022894686	6.941289468
	C2	0.35				
	C3	0.329				
	C4	0.343				
	C5	0.317				
	C6	0.349				
Group Summaries						
~End						
Group: MC-LR + 5 Cl2						
Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
1	D1	0.348		0.2975	0.036489725	12.26545364

	D2	0.311				
	D3	0.323				
	D4	0.248				
	D5	0.274				
	D6	0.281				

Group Summaries
~End
Group: MC-LR + 2.5 K

Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
				0.3046666	0.0194799	6.3938416
1	E1	0.306		67	04	33
	E2	0.279				
	E3	0.29				
	E4	0.319				
	E5	0.301				
	E6	0.333				

Group Summaries
~End
Group: MC-LR + 5 K

Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
				0.3021666	0.0185517	6.1395684
1	F1	0.31		67	29	98
	F2	0.326				
	F3	0.314				
	F4	0.274				
	F5	0.299				
	F6	0.29				

Group Summaries
~End
Group: MC-LR + 2 O3

Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
					0.0289534	11.421463
1	H1	0.274		0.2535	11	85
	H2	0.238				
	H3	0.296				
	H4	0.232				
	H5	0.219				
	H6	0.262				

Group Summaries
~End
Group: MC-LR +1 O3

Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
				0.311833333	0.031288443	10.03370701
1	G1	0.314				
	G2	0.33				
	G3	0.359				
	G4	0.266				
	G5	0.301				
	G6	0.301				

Group Summaries
~End
Group: CYN + 5 Cl2

Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
				0.122166667		28.82619385
1	D7	0.132			0.035216	
	D8	0.083				
	D9	0.104				
	D10	0.09				
	D11	0.158				
	D12	0.166				

Group Summaries
~End
Group: CYN + 2.5 Cl2

Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
				0.134333333	0.006408328	4.770467431
1	C7	0.127				
	C8	0.142				
	C9	0.138				
	C10	0.126				
	C11	0.136				
	C12	0.137				

Group Summaries
~End
Group: CYN + 2.5 K

Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
				0.134333333	0.011518102	8.574269252
1	E7	0.114				
	E8	0.137				
	E9	0.145				
	E10	0.129				
	E11	0.137				
	E12	0.144				

Group Summaries
~End
Group: CYN + 5 K

Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
					0.01853645	14.2588081
1	F7	0.14		0.13	1	4
	F8	0.095				
	F9	0.148				
	F10	0.128				
	F11	0.131				
	F12	0.138				

Group Summaries
~End
Group: CYN + 1 O3

Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
				0.10616666	0.01806008	17.0110689
1	G7	0.089		7	5	8
	G8	0.119				
	G9	0.118				
	G10	0.113				
	G11	0.078				
	G12	0.12				

Group Summaries
~End
Group: CYN + 2 O3

Sample	Well	Values	Result	MeanResult	Std.Dev.	CV%
				0.11366666	0.02671079	23.4992347
1	H7	0.132		7	7	4
	H8	0.107				
	H9	0.083				
	H10	0.117				
	H11	0.154				
	H12	0.089				

Group Summaries
~End
Group: Control

Sample	Wells	Sample#	Values	MeanValue	Std.Dev.	CV%
				0.36316666	0.03256071	8.96577673
1	A1	1	0.319	7	3	9
	A2		0.367			
	A3		0.397			

A4	0.328
A5	0.373
A6	0.395

Group Summaries

~End

Original Filename: 5-3-17 Exp Plate; Date Last Saved: 5/3/2017 4:55:17 PM

A2. LC-MS Analysis Data from CSU PMF Lab

Cylindrospermopsin

File No.	Sample Name and Treatment	Calculated Concentration	% CV	Ave Conc
29	02-Cyl-None-None	37.92		
45	02-Cyl-None-None	32.10		
74	02-Cyl-None-None	33.96	8.339	34.66
19	04-Cyl-Cl2-2.5	31.01		
46	04-Cyl-Cl2-2.5	35.79		
79	04-Cyl-Cl2-2.5	45.43	19.138	37.41
21	06-Cyl-Cl2-5	23.67		
53	06-Cyl-Cl2-5	21.38		
77	06-Cyl-Cl2-5	21.32	5.804	22.13
26	08-Cyl-KMn04-2.5	35.11		
48	08-Cyl-KMn04-2.5	30.20		
83	08-Cyl-KMn04-2.5	38.83	12.121	34.71
27	10-Cyl-KMn04-5	38.10		
50	10-Cyl-KMn04-5	33.98		
75	10-Cyl-KMn04-5	42.32	10.657	38.13
28	12-Cyl-O3-1	34.38		
43	12-Cyl-O3-1	37.70		
81	12-Cyl-O3-1	39.36	6.659	37.15
25	14-Cyl-O3-2	30.49		
55	14-Cyl-O3-2	43.75		
72	14-Cyl-O3-2	43.52	18.864	39.26

Microcystin-LR

File No.	Sample Name and Treatment	Calculated Concentration	% CV	Ave Conc
45*	01-MC-None-None	13.93		
21*	01-MC-None-None	15.92		
49	01-MC-None-None	16.37	7.74	15.40
19*	03-MC-Cl2-2.5	16.52		
43*	03-MC-Cl2-2.5	13.43		
84*	03-MC-Cl2-2.5	15.19	9.47	15.05
41*	05-MC-Cl2-5	12.71		
17	05-MC-Cl2-5	17.65		
78*	05-MC-Cl2-5	17.80	16.65	16.05
44*	07-MC-KMn04-2.5	11.22		
20	07-MC-KMn04-2.5	13.43		
56*	07-MC-KMn04-2.5	16.66	18.12	13.77
35*	09-MC-KMn04-5	9.56		
11	09-MC-KMn04-5	10.75		
82*	09-MC-KMn04-5	14.97	21.72	11.76
46*	11-MC-O3-1	18.90		
22*	11-MC-O3-1	21.26		
54	11-MC-O3-1	24.39	12.04	21.51
38*	13-MC-O3-2	17.93		
14*	13-MC-O3-2	20.37		
52	13-MC-O3-2	22.68	10.96	20.33

A3. Relevant Studies' Graphic Summaries

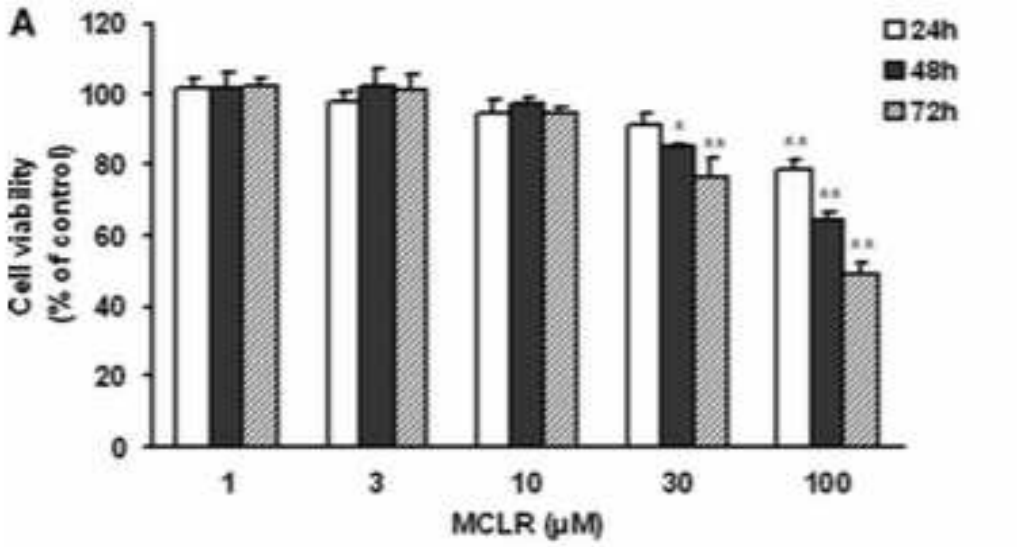

Figure A3.1 Effects of ROS scavengers on MCLR-induced cytotoxicity in HepG2 cells. (A) MCLR dose-dependently induced cytotoxicity determined by MTT and LDH release assays.
Source: Nong, 2007, Figure 1.

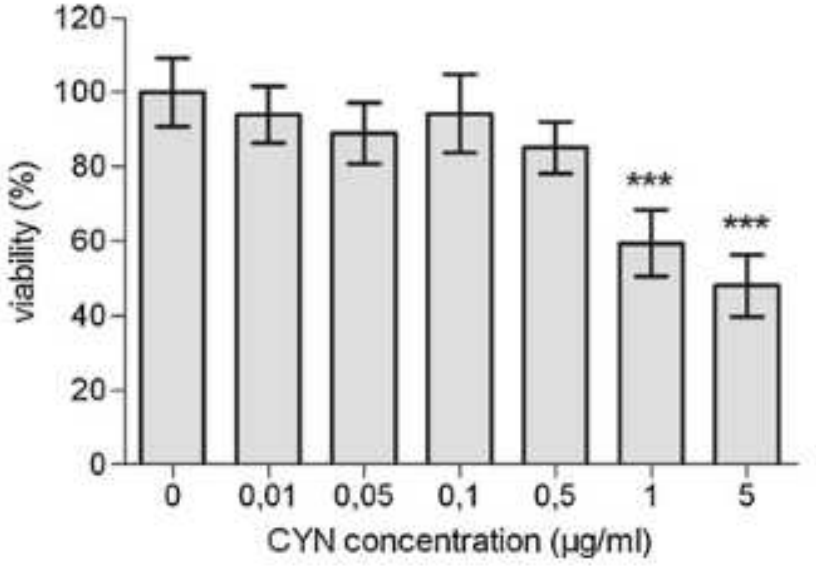

Figure A3.2 The effect of CYN on the viability of HepG2 cells. The viability was determined with the MTT assay after the exposure to different concentrations of CYN (0.005–5 g/ml) for 24 h. *** denotes signifcant difference between the vehicle control (0) and CYN
Source: Štraser, 2011, Figure 3

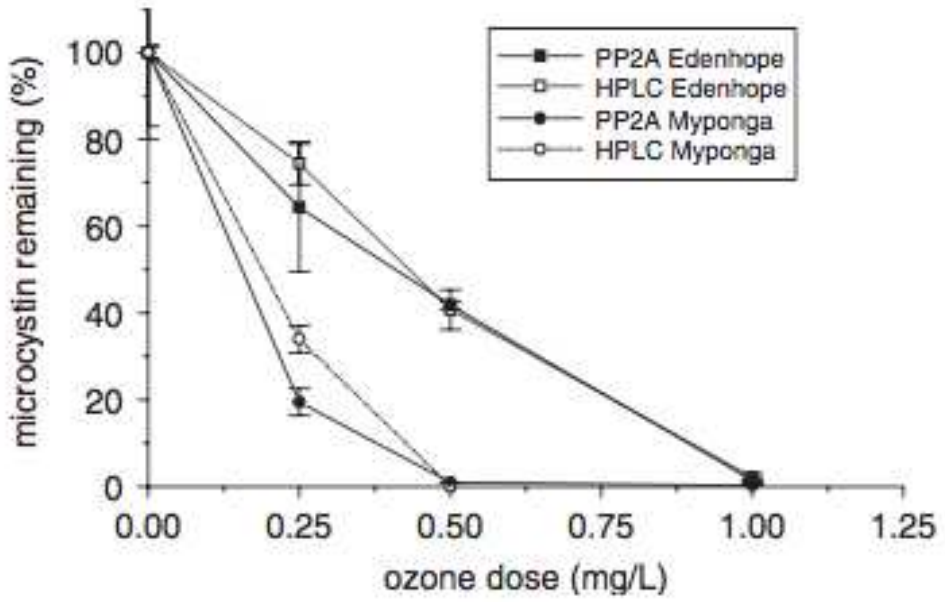

Figure A3.3 Comparison of microcystin levels detected by PP2A and HPLC after 5-min ozone contact times.
Source: Brooke, 2006, Figure 2.

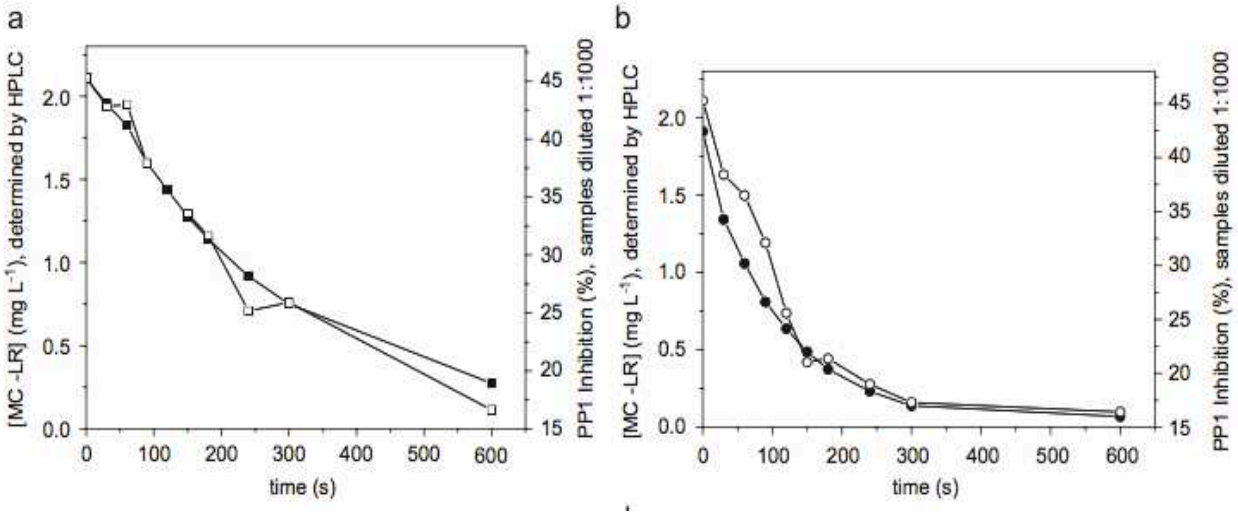

Figure A3.4 Evolution of microcystins concentration and PP1 inhibition with reaction time. PPIA was performed after dilution 1:1000 with Milli-Q water. (a) Chlorination of MC-LR; (b) oxidation of MC-LR with permanganate. Symbols: ■ HPLC, □ PPI-A.
Source: Rodriguez, 2008, Figure 1.

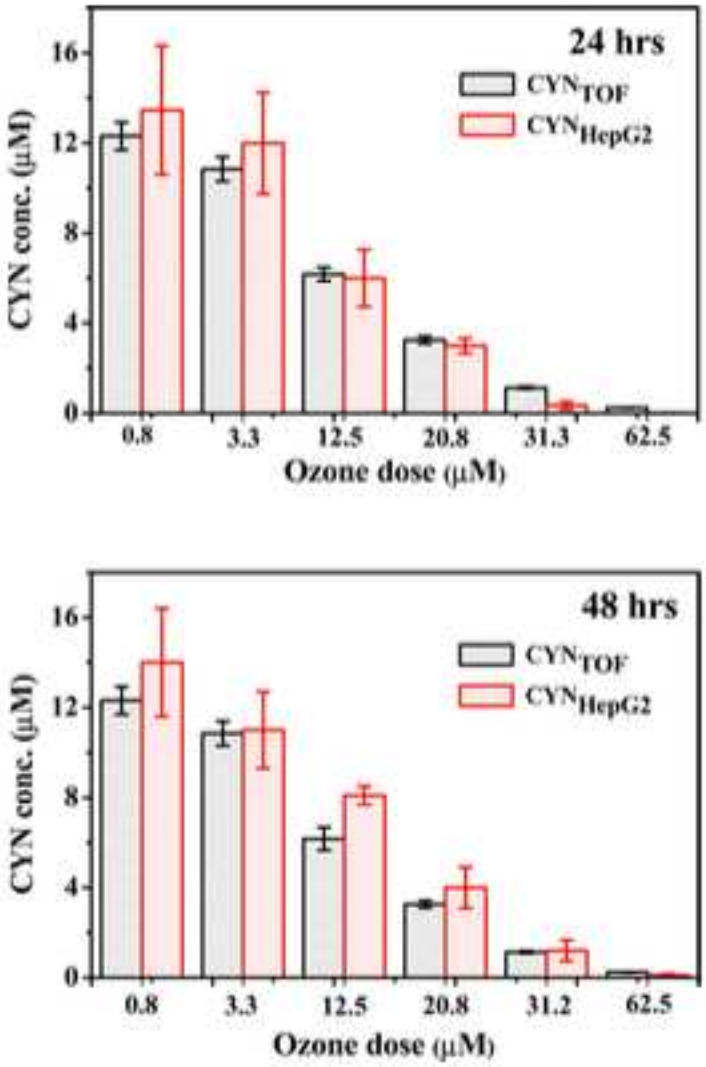

Figure A3.5 Degradation of CYN at various ozone doses. (Black) Concentrations of CYN measured by LC−MS; (red) concentrations of CYN calculated from standard inhibition curve, which was obtained from the cytotoxicity assessment.
Source: Yan, 2016, Figure 4.