THESIS

GROWTH, RECOVERY AND BIOACCUMULATION OF ALFALFA (*MEDICAGO SATIVA)* AND SPINACH (*SPINACIA OLERACEA)* EXPOSED TO CYANOTOXINS IN AGRICULTURAL ENVIRONMENTS

Submitted by

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ABSTRACT

GROWTH, RECOVERY AND BIOACCUMULATION OF ALFALFA (*MEDICAGO SATIVA*) AND SPINACH (*SPINACIA OLERACEA*) EXPOSED TO CYANOTOXINS IN AGRICULTURAL ENVIRONMENTS

Harmful algal blooms (HABs) are a growing concern for surface water resources around the globe. With increasing pressure on our limited fresh water resources due to climate change, the risk of contamination from HABs and the cyanobacterial toxins that accompany blooms, exacerbates the problem. Adverse health effects from cyanotoxin exposure has been documented in human and animal mortality and morbidity cases worldwide. Nationally, the presence and severity of HABs has prompted multiple cyanotoxins, including cylindrospermopsin (CYN) and microcystins (MCLR), to be listed on the USEPA Drinking Water Contaminant Candidate List-4 (CCL4) requiring many public systems to monitor for cyanotoxin presence. Recognizing this risk, the World Health Organization (WHO) has long established guidelines to acceptable levels in surface waters based on exposure pathways and use.

Further concerns have arisen as our understanding about cyanotoxins has been expanded by research. The purpose of this experiment was to determine 1) effects of toxin exposure during germination, 2) the effects of CYN and MCLR on agricultural crops exposed to toxins during vegetative and mature growth stages, 3) crops ability to recover from toxin exposure and 4) to quantify amount of cyanotoxin accumulated within crop tissue after exposure to cyanotoxins. Germination results indicated exposure to CYN and MCLR did not decrease the percent

germination of alfalfa or spinach. Further, alfalfa and spinach had increased primary root growth for seeds exposed to cyanotoxins.

During early vegetative exposure, spinach showed increased biomass and larger leaf area when exposed to MCLR and CYN. After a recovery period spinach plants exposed to CYN showed increased biomass compared to controls. Alfalfa plants exposed to MCLR in vegetative stages had significantly more biomass when compared to controls and this trend was observed after the recovery period.

Results of alfalfa exposed during mature growth stages to CYN and MCLR indicated it was more sensitive to CYN, however both toxin treatments resulted in increased biomass production. After one- and two-weeks of recovery the MCLR treated alfalfa biomass remained higher than controls.

Bioaccumulation of CYN and MCLR was observed in alfalfa exposed early to the toxins and detectable levels were observed after the one-week recovery period. Spinach accumulated MCLR during early exposures and had detectable levels in the stems after one-month recovery. During mature exposure, alfalfa initially only had detectable levels of MCLR, which decreased over the recovery periods. However, the presence of CYN was not detected until one-week prior to the final toxin exposure.

These findings support the growing concern that use of cyanotoxin contaminated irrigation water can be an additional exposure route for ingestion of toxins and increased risk of adverse health effects. Further studies into the subsurface fate of cyanotoxins will further increase the understanding of their bioavailability and persistence in soil.

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1. Introduction

1.1. Background

Naturally occurring algae can play an important ecological role in aquatic ecosystems. Macroinvertebrates graze on algae attached to substrates and suspended in the water column. Aquatic food chains rely on the balanced presence of algae as a primary photosynthetic food source. However, when algal growth is greater than the ecological needs, systematic imbalances result. Further concerns are the coexistence of harmful algae species and cyanobacteria within an algal bloom. Harmful algal blooms (HABs) containing cyanobacteria (Figure 1.1) continue to gain attention of water managers as the presence and severity of blooms increases across the globe (CLRMA, 2015).

Figure 1.1. Landsat satellite image of algal bloom in Lake Erie, Fall 2017. Source: USGS.

Cyanobacteria are photosynthetic prokaryotes that naturally co-exist as balanced planktonic assemblages in lentic, lotic and marine ecosystems. Approximately 3.5 billion years

of evolutionary history (Carmichael and Boyer, 2016; Allen and Martin, 2007, CLRMA, 2015) has enabled cyanobacteria to successfully adapt to numerous geochemical and climatic changes, and more recently, to anthropogenic stressors such as eutrophication (Paerl and Otten, 2013), inter- basin exchange, hydrologic engineering, chemical pollution, drawdown and salinization (CLRMA, 2015). Many cyanobacteria exhibit optimal growth rates and bloom potential at higher water temperatures; therefore, climate change plays a key role in their expansion and persistence (Carmichael and Boyer, 2016).

Cyanotoxins are secondary metabolites stored within the cells of these prokaryotes and in most cases are released into the water when the cells rupture or die (Corbel et al., 2014). Often the release of toxins occurs at the end of the algal bloom's natural lifecycle. Hence, toxins can still be present in the water and pose a health risk even after the bloom has dissipated and is no longer visible (Wood, 2016). Some cyanobacteria contain gas vacuoles that help regulate their position in the water column allowing them to rise and fall through the water and therefore do not produce floating scums and mats (USEPA,2015).

Exposure to cyanotoxins can occur either directly or indirectly. Direct exposure routes include ingestion through drinking water, dermal contact while bathing or swimming, inhalation of aerosolized particles while showering or participating in water sports, and rarely, through intravenous medical procedures (Metcalf et al, 2004). Indirect exposure can occur via the consumption of animal or plant products that have been exposed to cyanotoxins (Wood, 2016; Carmichael et al, 2016).

Research has shown that cyanotoxins can bioaccumulate and their toxic effects may be magnified in food chains (Wood, 2016). Accumulation of toxins has been documented in the marine food chain (Wood et al., 2006), in terrestrial food chains and toxins have been detected in

dietary supplements and food intended for human consumption (Metcalf et al, 2004; Chatziefthimiou et al., 2016).

On a global scale, there is a growing body of literature with respect to the presence of cyanobacteria and toxins in aquatic environments and in water treatment plants with focus on the hepatotoxins microcystin-LR and cylindrospermopsin, and the neurotoxins anatoxin-a and saxitoxin (Carmichael et al., 2001; Chatziefthimiou et al., 2016). Cyanobacterial occurrence has been reported in Africa, Asia, Europe, North America and Scandinavian countries (Merel et al., 2013) as well as South America (Moschini-Carlos et al., 2009), Australia (Bowling et al., 2013) and New Zealand (Wood et al., 2006).

1.2. Toxicology

Toxins produced by cyanobacteria vary in their toxicology and are classified according to the systems and organs they target in terrestrial vertebrates. Chronic exposure to low doses of some hepatotoxins, which target the liver, has been associated with tumor promotion (Wood, 2016; Fleming et al., 2002; Ueno et al., 1996). Defined by their chemical structure, cyanotoxins fall into three groups: cyclic peptides (microcystin and nodularin), alkaloids (anatoxin-a, anatoxin- a(s), saxitoxin, cylindrospermopsin) and lipopolysaccharides (Zegura et al., 2011). Below is an overview of the more common and concerning cyanotoxins, anatoxin-a, cylindrospermopsin, microcystin-LR and saxitoxins.

1.2.1. Anatoxin-a (ATX)

Anatoxin-a is a potent neurotoxin produced by several cyanobacteria genus including Anabaena (Figure 1.2), Aphanizomenon, Cylindrospermum, *Microcystis, Oscillatoria* and *Planktothrix*. ATX is a potent postsynaptic depolarizing neuromuscular blocking agent that

affects both nicotinic and muscarinic acetylcholine receptors. In the body, ATX mimics the actions of acetylcholine, however, it is not degraded by the enzyme acetylcholinesterase resulting in continued stimulation of the muscular cells. Consequently, muscle paralysis can occur in the respiratory system leading to insufficient oxygenation of the brain, convulsions and death (Corbel et al., 2014).

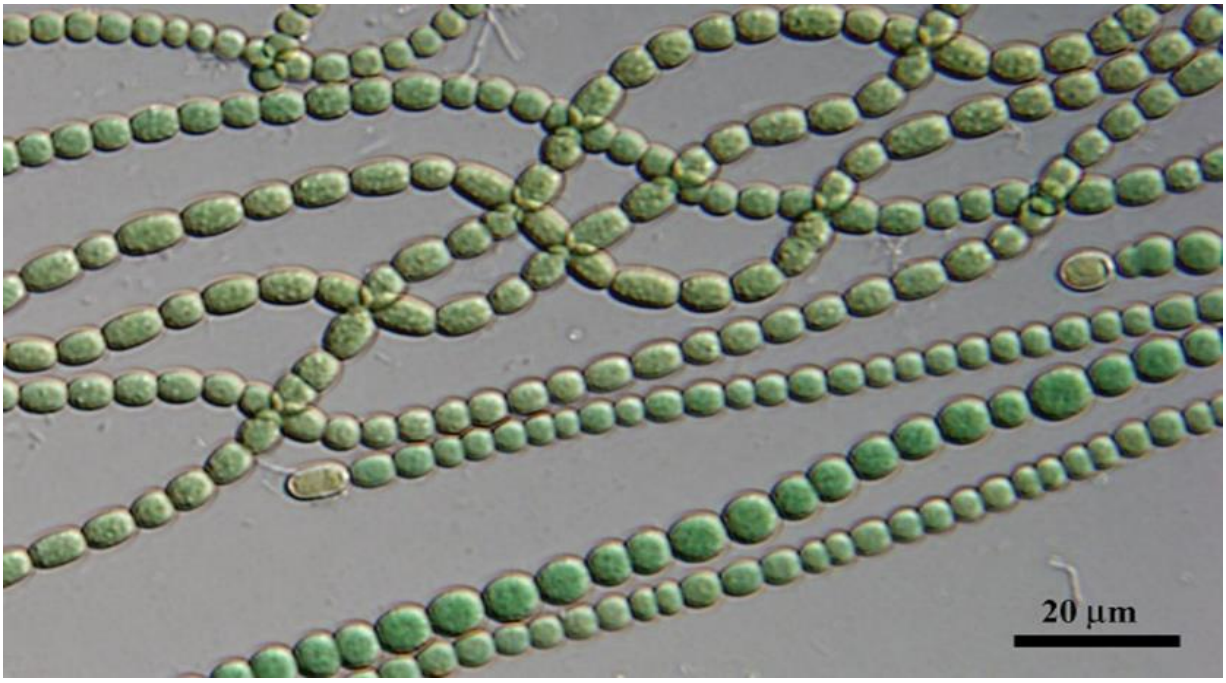


Figure 1.2. *Anabaena* sp. Source: Culture Collection of Autotrophic Organisms

According to the USEPA (2015), a few *in vivo* studies have provided enough data to determine a LD_{50} of 13.3mg/kg based on acute lethality assays in mice exposed orally (Stevens and Krieger, 1991). Between 1985 and 1996 samples from surface fresh water blooms around the world reported anatoxin-a concentrations from 0.4 to 4,400 ug/g dry weight (USEPA, 2015). In Washington State, samples taken in 2011 by the Department of Ecology, reported concentrations ranging from the detection limit of 0.05 to 1,929 ug/L. Multiple other studies have reported

concentrations falling within these values providing some guidance as to environmentally relative toxin concentrations (USEPA, 2015).

Currently, no national health advisory guidelines have been established by the USEPA for anatoxin, however, various states have guidelines for advisories based on water use. Guidance levels for drinking water use in Minnesota and Utah is 0.1ug/L, do not drink orders in Ohio are issued at 20ug/L, Oregon is 0.7ug/L for children 5 and younger and 3ug/L for adults and Vermont is set at 0.5ug/L (USEPA, 2015). For recreational use, guidance and advisories are issued at 90ug/L in California, 10 ug/L in Oregon and Vermont, 1ug/L in Washington, and Ohio is 80ug/L for limited use and 300ug/L for no contact (USEPA, 2015).

Anatoxin-a is highly soluble in water and can remain relatively stable at neutral and acidic conditions; alkaline conditions accelerate the degradation of anatoxin-a. In the absence of sunlight, a half-life ranging from several days to months has been shown in multiple studies (USEPA, 2015). Photolysis and metabolism by bacteria are also important degradation pathways although limited research is available. Sorption can aid in removal or transport of anatoxin, generally sandy sediment weakly sorbs the toxin and clay or rich organic material promotes binding at negatively charged sites (USEPA, 2015).

1.2.2. Cylindrospermopsin (CYN)

Cylindrospermopsin is receiving increased attention by toxicologists and health authorities. Geographic expansion, from tropical to temperate environments, of the main producer, Cylindro-spermopsis raciborskii (Figure 1.3), is occurring at a considerable pace. CYN has been found on nearly every continent in apparent correlation with the occurrence of global warming phenomena (Guzmán- Guillén et al., 2012).

Cylindrospermopsin can be produced by several cyanobacterial species and was implicated in human intoxications and animal mortality in Australia (Chiswell, 1999). Cylindrospermopsin is a known hepatotoxin, but other organs such as the kidneys, lungs, thymus and spleen, adrenal glands, intestinal tract, the immune system and the heart may be affected. The principal mechanism of CYN toxicity is the irreversible inhibition of protein synthesis. CYN also inhibits glutathione synthesis, which could lead to an increase in oxidative stress. Presence of the uracil group suggests that CYN could be interacting with adenine groups in RNA and DNA, interfering with DNA synthesis and therefore induces mutations and acts as a carcinogen. Based on published evidence, CYN has much stronger genotoxic potential than MCs and should be considered as more dangerous to human and animal health (Žegura et al., 2011).

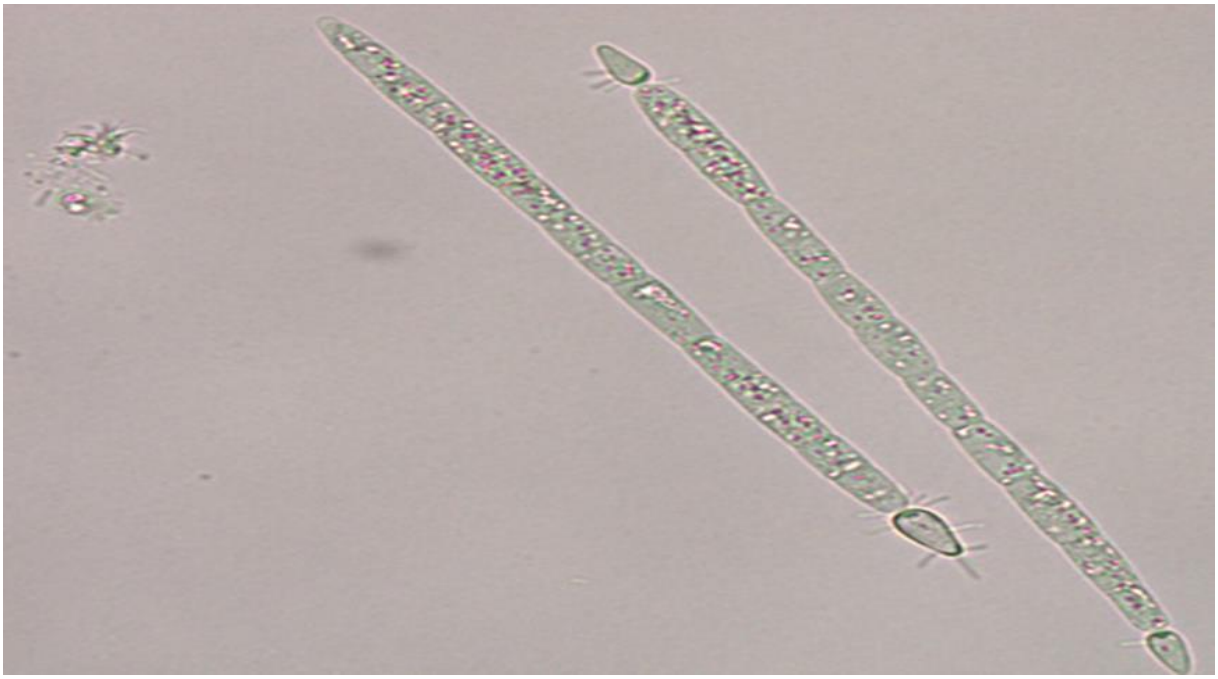


Figure 1.3. *Cylindrospermopsis raciborskii* Source: Green Water Laboratories

Carmichael et al. (2016) reported an acute exposure LD_{50}, for intraperitoneal. mouse assay, of 2.1 mg/kg over 24 hours. According to the USEPA (2015) reporting of environmental concentrations of CYN in surface water has been scarce; however, samples analyzed by federal

and state authorities have indicated concentrations ranging from 0.12 to 9 ug/L in some U.S. lakes. In Florida, a survey in 2000, detected concentrations of CYN ranging between 8 ug/L to 97 ug/L in nine finished drinking water samples (USEPA, 2015).

Health advisory guidelines for CYN in drinking water set by the USEPA (2015) are 0.7ug/L for children under 6 and 3ug/L for school age children and adults. Guidelines are not enforced legal standards and may change as more information becomes available. Guidelines can also vary significantly by state. Oregon and Ohio have adopted the USEPA standard for drinking water while Vermont is set at 0.5ug/L CYN. Recreational use may be limited by presence of bloom material or measured concentrations of toxins in surface water. California issues health advisories at 4ug/L CYN, Indiana and Ohio are 5ug/L, Utah is 8ug/L, Oregon is 20ug/L and Washington is 4.5ug/L (USEPA, 2015).

Degradation of CYN is mediated by environmental conditions including pH, temperature, light, aerobic conditions and microbial communities available for metabolizing the toxin. Under aerobic conditions CYN can degrade an order of magnitude faster than in anaerobic conditions decreasing the persistence from weeks to days (Klitzke and Fastner, 2012). Warmer alkaline conditions also increase the rate of CYN degradation. Photolysis is enhanced when cell pigments are present, thus decreasing the concentrations of CYN by up to 90% in a few days. Adsorption to organic carbon is observed in some sediment with decreasing sorption in silt or sandy soils (USEPA,2015).

1.2.3. Microcystin-LR (MCLR)

Globally, one of the most frequently occurring and widespread cyanotoxins in brackish and freshwater blooms are the cyclic heptapeptides, microcystins (MCs). Members of more than a dozen cyanobacterial genera, primarily Microcystis aeruginosa (Figure 1.4), produce cyclic

peptides, termed microcystins, which are potent hepatotoxins (MacKintosh et al., 1990). Microcystin-LR (MCLR), in which the two variable amino acids are leucine (L) and arginine (R) is the most commonly found variant of the microcystin congeners. In plants and higher animals, mechanism of toxicity is irreversible inhibition of serine/threonine protein phosphatases 1 and 2A (PP, PP1, PP2A) (Freitas et al., 2015)

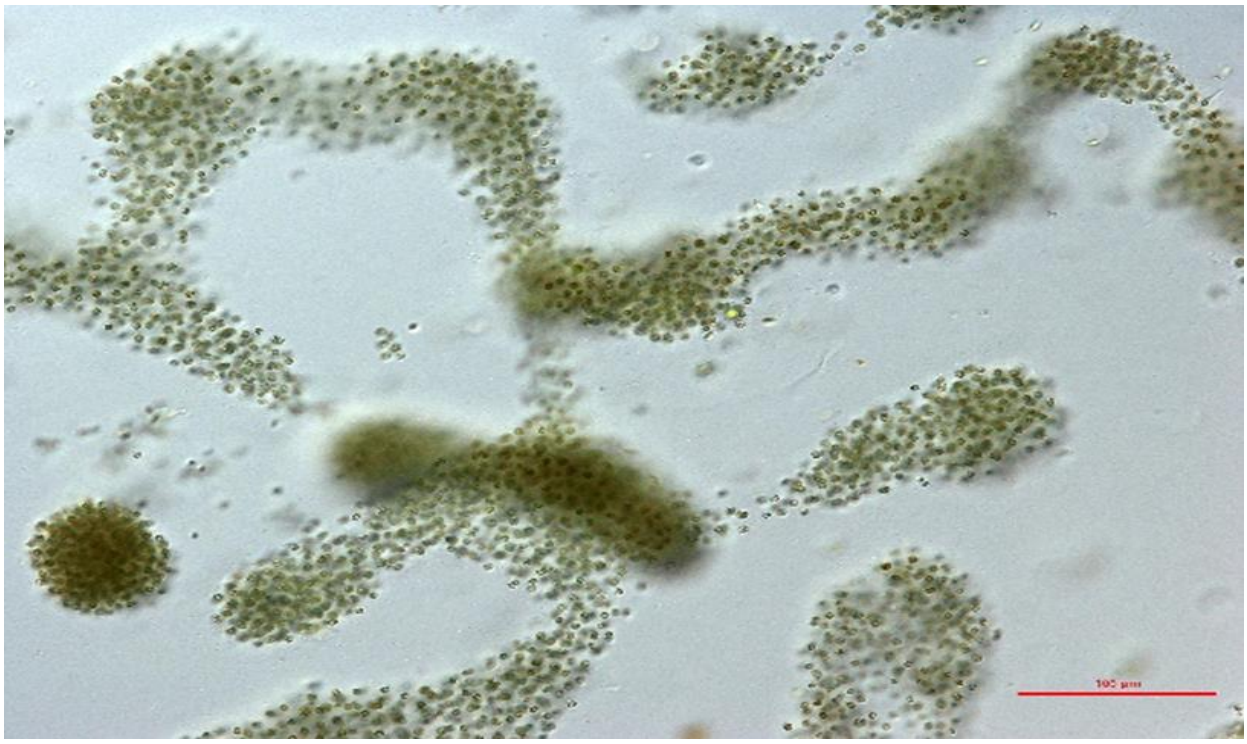


Figure 1.4. *Microcystis aeruginosa,* Source: USEPA

Research into MCs effect on agricultural plants indicates biological processes, growth and development can be negatively impacted by irrigation with water contaminated with MCs (Pflugmacher, 2007). Although MCs are chemically stable, in water bodies their microbial degradation can be rapid. However, lag phases can be observed before the degradation occurs, likely because bacteria that can degrade MCs are not always present in sufficient numbers or need to adapt (Mazur-Marzec et al., 2009; Zegura et al., 2011; Li et al., 2016).

Recently, the International Agency for Research on Cancer (IARC) classified MCLR as possible human carcinogen (Group 2B), via inhibition of protein phosphatases 1 and 2A. MCs are hydrophilic, they passively penetrate vertebrate cell membranes poorly, and therefore require uptake via active transport (Žegura et al., 2011). MCs are known to be transported through cell membranes by organic anion transporting polypeptides (OATP) (Fischer et al., 2005) which are not only expressed in the liver, but also in the gastrointestinal tract, kidney, brain, and there is evidence that MCLR may be transported across the human blood–brain barrier (Žegura et al., 2011).

Based on acute toxicity studies MCLR is considered one of the most potent cyano-bacterial toxins (Zegura et al., 2011). Pure MCLR has been shown to be less toxic and genotoxic than bloom extracts, which indicates the possibility for action of other variants of MCs in the extracts (determined by HPLC), the presence of other genotoxic contaminants as well as synergistic effects among the components of the extract; therefore, it cannot be excluded that other environmental contaminants are present that could contribute to the genotoxicity of the whole extract (Zegura et al., 2011). An LD_{50} of 25 ug/kg based on i.p. mouse assay was reported by Carmichael et al. (2016).

Health advisory levels for microcystin are also unregulated standards set by the USEPA and vary by state. Generally, 0.3ug/L for children under 6 and 1.6ug/L for school age children and adults is the drinking water guideline issued by the USEPA (2015). Ohio and Oregon adopt this standard for drinking water, Minnesota is 0.1ug/L and Vermont is set at 0.16ug/L MCLR (USEPA, 2015). Recreational guidelines in California are 0.8ug/L, 4ug/L in Indiana and Utah, 10ug/L in Oregon, 20ug/L in Iowa, Oklahoma, Nebraska and Texas, 14ug/L in Massachusetts and Rhode Island, and 6ug/L in Ohio, Washington and Vermont. Microcystin has been studied

extensively, and in some states, advisories are issued based primarily on the presence of scums or toxigenic species.

Monitoring and reporting of MCs by state and government agencies has provided a wide range of concentrations found in surface waters. Washington State Department of Ecology collected and summarized their findings of lake, pond and stream MCs concentrations which ranged from the detection limit (0.05 ug/L) to 4,620 ug/L in 2008 to 26,400 ug/L in 2011 (USEPA, 2015). In the Great Lake region, Lake Erie, Lake Ontario and Lake Champlain had reported detectable levels of MCs in 65% of the samples from a 2002 study. In 2004, MCLR was again detected in this region with levels reported from 0.076 to 10.7 ug/L. In 2006, the USGS studied lakes in the Midwestern U.S. finding 91% of the lakes sampled were positive for MCs with mean levels of 104 and 910 ug/L for MC-LR and MC-RR, respectively. In 1999, Florida samples determined *Microcystis* was the most frequently found species in 75 water bodies with microcystins being the most commonly found cyanotoxin (USEPA, 2015). Utah Lake has been monitoring HABs for several years and has reported microcystin concentrations above 700ug/L in 2018 and 2019 (UDEQ, 2019).

1.2.4. Saxitoxin (SAX)

A potent neurotoxin commonly known for causing paralytic shellfish poisoning (PSP) is saxitoxin. Produced by dinoflagellates and cyanobacteria in marine and freshwater environments saxitoxin has been relatively understudied in freshwater systems. Common cyanobacterial

producers are Anabaena, Aphanizomenon, Cylindrospermopsis and Oscillatoria (Figure 1.5). Method of action is calcium, potassium and sodium channel blocking agents inhibiting transmission of nervous impulses and can lead to death in animals by respiratory arrest. Intraperitoneal mouse assay LD50 is estimated between 1-10 ug/kg body weight (Loftin et al., 2016).

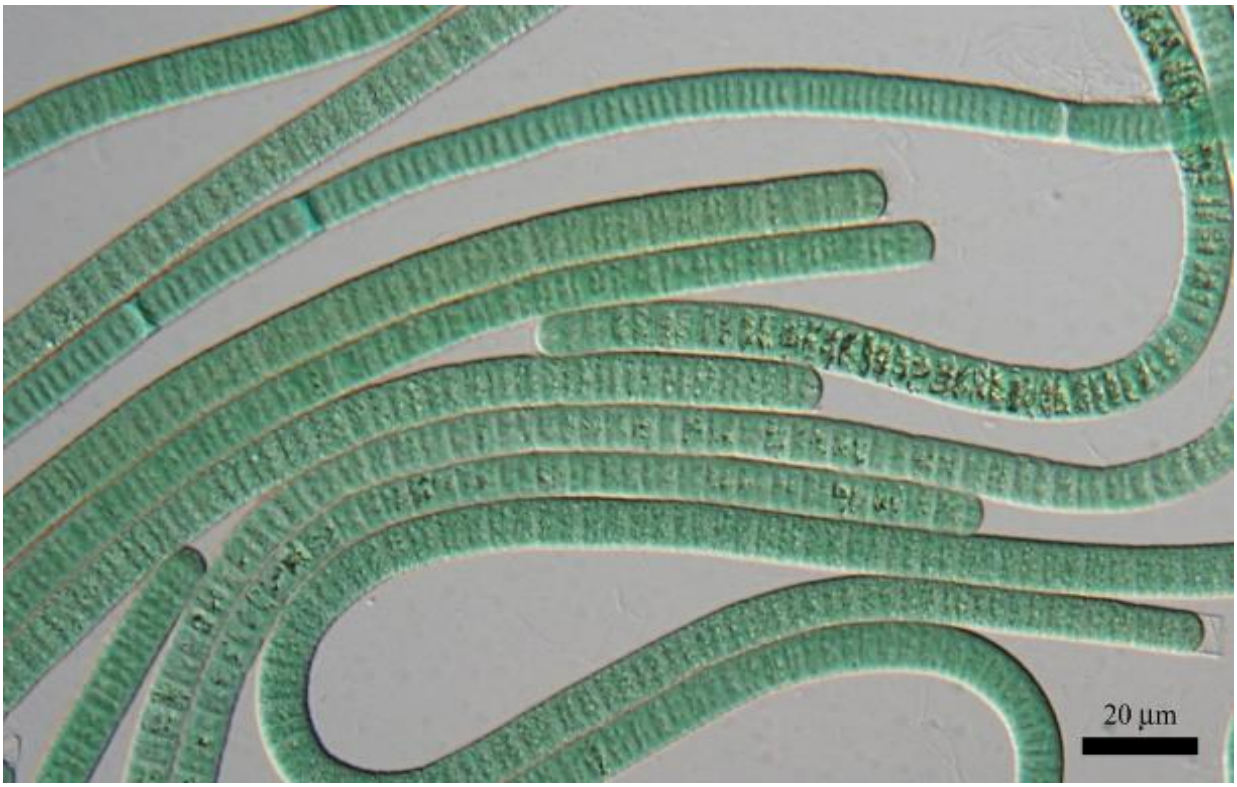


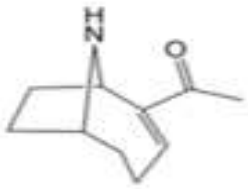
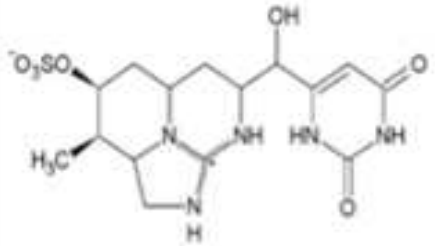
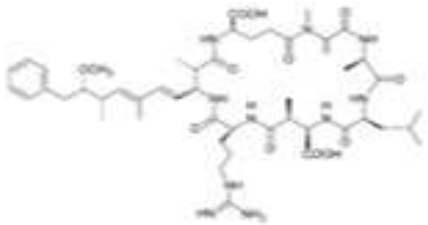
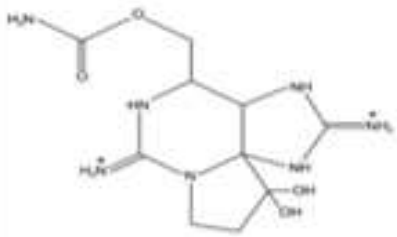
Figure 1.5. *Oscillatoria sp.* Source: Culture Collection of Autotrophic Organisms

Health advisory levels for saxitoxin vary across the United States. In Ohio, swimming and wading in water are not recommended if levels exceed 0.8 ug/L and no contact is recommended if levels reach 3 ug/L for recreational waters. Drinking water action level in Ohio is 0.2 ug/L. In Oregon, drinking water action level for SAX is 3ug/L and health advisory level for recreational waters is 100ug/L. The state of Washington issues a recreational advisory at levels exceeding 75ug/L (CLRMA, 2015). Saxitoxin is known for PSP and is regulated through the U.S. Food and Drug Administration which requires closure and consumer advisory when

levels exceed 80 ug/L consistent with the international standard. Concentrations of extracellular SAX has been reported up to 15 µg/L in natural waters and intracellular concentrations ranging from 5 to 3,400 µg/g of cell dry weight (USEPA, 2015).

Saxitoxins are nonvolatile, tricyclic, perhydropurine alkaloids. Various structural substitutions produce at least 57 analogues. Saxitoxins are heat-stable, particularly in slightly acidic environments, and are highly water-soluble. They are tasteless and odorless and are not destroyed by normal food preparation methods (van der Merwe, 2015).

Table 1-1. Summary of chemical properties for priority listed cyanotoxins.

Anatoxin-a	Cylindrospermopsin
Chemical Structure of ATX Alkaloid  Formula: $C_{10}H_{15}NO$ MOA: Neurotoxin, affects nicotinic and muscarinic acetylcholine receptors causing muscular paralysis. Mouse i.p. LD_{50}: 200 ug/kg (Corbel et al., 2014)	Chemical structure of CYN Alkaloid  Formula: $C_{15}H_{21}N_5O_7S$ MOA: Hepatotoxin, inhibits protein synthesis, DNA and RNA mutations, liver toxicity. Mouse i.p. LD_{50}: 2.1 mg/kg (Carmichael et al., 2016)
Microcystin-LR	**Saxitoxin**
Chemical structure of MCLR Heptapeptide  Formula: $C_{49}H_{74}N_{10}O_{12}$ MOA: Hepatotoxin, irreversible inhibition of protein phosphatase 1 and 2A. Possible human carcinogen. Mouse i.p. LD_{50}: 25 ug/kg (Carmichael et al., 2016)	Chemical structure of STX Alkaloid  Formula: $C_{10}H_{17}N_7O_4$ MOA: Neurotoxin, Na^+ channel blocking, also affects Ca^{2+} and K^+ channels disrupting cellular homeostasis. Mouse i.p. LD_{50}: 10 ug/kg (Corbel et al., 2014)

1.3. Cyanotoxins in Surface Water

Global presence of toxic cyanobacteria raises concern for the safety of drinking, recreational and irrigation water sources. Oral ingestion through drinking water has been a primary pathway of exposure for incidence of animal and human mortality and morbidity. Considering human exposure and health risks, contaminated drinking water sources affect more individuals per event, while exposure through recreational waters are more common, although affecting fewer individuals per event (Wood, 2016). In many cases, fresh water resources are managed for multiple uses; common concurrent use is managed lakes used for drinking water, irrigation water and recreational activities. Figure 1.6 shows HABs found in Utah Lake which is used for recreation and irrigation.

Figure 1.6. HAB in Utah Lake, Fall 2018 Source: Utah Dept. of Environmental Quality

Common producers of cyanotoxins are under the genera *Anabaena, Aphanizomenon, Cylindrospermopsis, Microcystis and Planktothyrix* (Williams et al., 2007). Presence of cyanobacteria blooms does not guarantee cyanotoxins are present in the water. Many cyanotoxins are only released when cell lysis occurs or at the end of the bloom's life cycle. Further, many strains of bacteria exist within a bloom and not all are toxic. Identification of intra- or extracellular toxin concentrations is important in determining the treatment methods available for removing cyanotoxins from surface water. Intracellular toxin removal can effectively be accomplished by removal of cyanobacterial cells, through filtration, where extracellular removal is more complicated resulting in higher treatment costs (Carmichael et al, 2016).

Cyanotoxins are highly soluble in water making them relatively mobile in environmental conditions. Differences in polarity range from more hydrophilic MCLR to more hydrophobic CYN, relative to each other. Degradation naturally occurs in the environment; however, rates can be slow and are highly dependent on the conditions. Microcystin and cylindrospermopsin are generally stable under broad ranges of pH and temperatures (Williams et al., 2007). Presence of bloom extract has indicated increased rates of photodegradation for CYN, however, extract material has also shown to increase genotoxic potential (Zegura et al., 2011).

1.4. Cyanotoxins in Agriculture

Various previous studies have been performed to determine effects of cyanotoxin exposure on agricultural crops. Studies have investigated effects of cyanotoxins on biological responses including photosynthesis, growth and development, oxidative stress and germination. Microcystin-LR has been widely studied having been one of the most prevalent cyanotoxins, however, CYN is gaining more interest as its geographic range is expanding. Studies into the

bioaccumulation of MCLR and CYN have indicated a variety of plant species can accumulate toxins (Machado, 2017). Many studies have used hydroponic systems to determine the plant accumulation of cyanotoxins which does not adequately address the response of contaminated irrigation water in soil media. Few studies have begun to identify degradation pathways for MCLR and CYN, finding microbial activity and sorption as significant mechanisms in limiting the availability of toxins in the soil (Maghsoudi et al, 2015; El Kalloufi et al, 2016; Dziga et al, 2013).

Figure 1.6. Landsat image of Lake Utah algal bloom and surrounding agriculture. Source: UDEQ, 2019.

1.4.1. Morphological Effects of Cyanotoxins on Agricultural Plants

As plants develop, roots, stems, leaves and fruits require availability of micro and macronutrients, water, air and sunlight to produce healthy crops and respond to environmental stressors. Morphology of plants can be affected by the presence of cyanotoxins in irrigation

water. Further, due to apparent persistence of cyanotoxins in the environment there is a possibility of cyanotoxins being present in the soil at planting time which would also affect the germination of seeds. Timing, dose concentrations and toxin source also play a role in how plants respond to toxin exposure. Young plants may be more susceptible to stress affecting growth and development while mature plants may risk full development of fruits or early senescence. Either way, the productivity of agricultural crops is affected and can lead to economic losses due to defects in germination, growth and development. Table 1-2. highlights studies testing the effects of MCLR on various plant species growth and development and Table 1-3. summarizes research on the effects of CYN on plant growth and development.

Table 1-2. Summary of previous studies and results of biological effects on plants exposed to MCLR. a) MCLR crude extract b) pure MCLR. Table adopted from Machado et al., 2017.

Species (common name)	**Endpoint**	**Effect**	**Toxin concentration (ug/L)**		**Reference**
Brassica napus (Rape)	Germination rate	↓	600-3000	a	Chen et al., 2004
	Seedling height	↓	120-3000	a	Chen et al., 2004
Brassica rapa (Field mustard)	Shoot length	↓	400-6400	a	Chen et al., 2012b
Lactuca sativa (Lettuce)	Root growth	↓	5.9-56.4	a	Pereira et al., 2009
	Root fresh weight	↑	1-100	a	Freitas et al., 2015
	Leaf fresh weight	↑	1-50	b	Freitas et al., 2015
	Leaf fresh weight	↓	100	b	Freitas et al., 2015
Lens esculenta (Lentil)	Germination	↓	8700-11,600	a	Saqrane et al., 2008
	Epicotyl length; Primary root length; Lateral root number	↓	11,600	a	Saqrane et al., 2008
	Height (30th day)	↓	1050-42,000	a	Saqrane et al., 2008
	Leaf number (30th day)	↓	4200	a	Saqrane et al., 2008
	Fresh weight	↓	500-4200	a	Saqrane et al., 2008
	Dry weight	↓	1050-4200	a	Saqrane et al., 2008
Lepidium sativum (garden cress)	Fresh weight (6th day)	↓	10	a, b	Gehringer et al., 2003
	Root and Leaf length	↓	1	a, b	Gehringer et al., 2003

Table 1-2. Continued

Species (common name)	Endpoint	Effect	Toxin concentration (ug/L)		Reference
Lycopersicon escultentum (Tomato)	Germination rate	↓	16,680-22,240	a	El Khalloufi et al, 2012
	Fresh biomass; Stem length	↓	2220-22,240	a	El Khalloufi et al, 2012
Malus pumila (Apple)	Growth	↓	300-3000		a
Medicago sativa (Alfalfa)	Germination rate	↓	5	a, b	Chen et al., 2010
Medicago sativa (Alfalfa) **Oryza sativa (Rice)**	Primary root length	↓	5	a, b	Pflugmacher et al., 2006
	Germination rate	↓	2220-22,240	a	Pflugmacher et al., 2006
	Plant length; nodules number; biomass	↓	2220-22,241	a	El Khalloufi et al, 2011
	Root length	↓	11,120-22,240	a	El Khalloufi et al, 2011
	Dry weight	↓	10-20	a	El Khalloufi et al, 2011
	Fresh weight; length of roots	↓	120-3000	a	El Khalloufi et al, 2013
	Dry weight of roots	↓	24-600	a	Chen et al., 2004*
	Seedling height	↓	600-3000	a	Chen et al., 2004
Oryza sativa (Rice) **Pisum sativum (Pea)**	Root fresh weight; length; number of crown root	↓	2000-4000	a	Chen et al., 2004
	Germination rate	↓	1600-11,600	a	Chen et al., 2004
	Epicotyl length; Primary root length; Lateral root number	↓	1600	a	Saqrane et al., 2008
	Height (30th day); Fresh and Dry weight	↓	500-4200	a	Saqrane et al., 2008
Pisum sativum (Pea) **Sinapis alba (White mustard)**	Leaf number (30th day)	↓	1050-4200	a	Saqrane et al., 2009
	Growth	↓	2000	b	Saqrane et al., 2009
	Fresh biomass; Length (total, hypocotyl, cotyledon, root); Primary root growth; Lateral root number	↓	3500-30,000	a	Kurki-Helasmo & Meriluoto, 1998
	Growth	↓	500-5000	a	M-Hamvas et al., 2003
Sinapis alba (White mustard) **Solanum tuberosum (Potato)**	Growth	↓	7800	a	McElhiney et al., 2001
	Growth	↓	18200	b	Vasa et al., 2002
	Fresh weight; Shoot length	↓	500-5000	a	Vasa et al., 2002
	Number of roots	↓	10-500	a	McElhiney et al., 2001
	Growth; Number of leaves; Leaf size	↓	0.5	a	McElhiney et al., 2001

Table 1-2. Continued

Species (common name)	Endpoint	Effect	Toxin concentration (ug/L)		Reference
Triticum aestivum (Wheat)	Germination rate	↓	2900-11,600	a	Saqrane et al., 2008
Tritcum durum (Wheat)	Height (30th day); Fresh and Dry weight	↓	500-4200	a	Saqrane et al., 2009
	Leaf number (30th day)	↓	4200	a	Saqrane et al., 2009
	Germination rate	↓	50-100	a	Lahrouni et al., 2012
	Shoot dry weight; Root length; Root and Nodule dry weight; Total number of nodules	↓	50-100	a	Lahrouni et al., 2012
Vicia faba (Faba bean)	Germination rate	↓	5	a, b	Pflugmacher et al., 2007
	Shoot and Root length	↓	5	a, b	Pflugmacher et al., 2007
Zea mays (Corn)	Germination rate	↓	2900-11,600	a	Saqrane et al., 2008
	Epicotyl length; Primary root length; Lateral root number	↓	11,600	a	Saqrane et al., 2008
	Height (30th day); Fresh weight	↓	500-4200	a	Saqrane et al., 2009
	Leaf number (30th day)	↓	1050-4200	a	Saqrane et al., 2009

Table 1-3. Summary of previous studies and results of biological effects on plants exposed to CYN. a) CYN crude extract b) pure CYN. Table adopted from Machado et al., 2017.

Species	Endpoint	Effect	Toxin concentration (ug/L)		Reference
Lactuca sativa (Lettuce)	Root length	↑	.57-5.7	a	Silva and Vasconcelos, 2010
	Root length	↓	57	a	Silva and Vasconcelos, 2010
	Stem length	↑	0.57-57	a	Silva and Vasconcelos, 2010
	Fresh weight of roots	↑	1-100	b	Freitas et al., 2015a
	Fresh weight of leaves	↓	100	b	Freitas et al., 2015a
Nicotiana tabacum (Tabacco)	Germination rate	↓	5000-10,00,000	b	Metcalf et al., 2004
Phaseolus vulgaris (Bean)	Root length	↑	0.57-57	a	Silva and Vasconcelos, 2010

Table 1-3. Continued

Species	Endpoint	Effect	Toxin concentration (ug/L)	Reference	
Pisum sativum (Pea)	Root length	↓	0.57-57	a	Silva and Vasconcelos, 2010
	Stem length	↑	0.57-57	a	Silva and Vasconcelos, 2010
Oryza sativa (Rice)	Root fresh weight	↑	2.5	a	Prieto et al., 2011
Sinapis alba (White mustard)	Lateral root emergence	↑	10	b	Máthé et al., 2013
	Lateral root emergence	↓	500-20,000	b	Máthé et al., 2013
Lycopersicon escultentum (Tomato)	Germination rate	↓	0.57-57	a	Silva and Vasconcelos, 2010
	Root and Stem length	↓	0.57-57	a	Silva and Vasconcelos, 2010
Vicia faba (Faba bean)	Epicotyl length; Main root elongation	↑	100	b	Garda et al., 2015
	Epicotyl length; Main root elongation	↓	5000-20,000	b	Garda et al., 2015
	Number of lateral roots	↑	2500	b	Garda et al., 2015
	Number of lateral roots	↓	10,000-20,000	b	Garda et al., 2015

1.4.2. Biochemical Effects of Cyanotoxins on Agricultural Plants

Stress from toxin exposure can have detrimental effects on plants as they progress through growth stages. Numerous studies have investigated the biochemical effects of cyano-toxins on plants. Some physiological processes that have been investigated are oxidative stress, mineral and protein content, stomatal conductance, transpiration and photosynthetic rates. While plants may appear healthy during stress, the quality of the crops may decline due to lack of nutritional content or increased allergenic properties (Guzmán-Guillén et al, 2017). Table 1-4. is a reference of studies on biochemical effects of exposure to MCLR and CYN.

Table 1-4. Summary of previous studies and results of biochemical effects of plants exposed to MCLR and CYN. a) crude extract b) pure toxin. Table adopted from Machado et al., 2017.

Species	Toxin	Biochemical process	Effect	Toxin range (ug/L)		Reference
Brassica napus (Rape)	MCLR	SOD	↓	24-3000	a	Chen et al., 2004*
	MCLR	POD	↑	120-3000	a	Chen et al., 2004*
Brassica rapa (Field mustard)	MCLR	Cu/Zn-SOD; APX; CAT	↑	4230	a	Chen et al., 2012b
Lactuca sativa (Lettuce)	MCLR	Shoot Mineral Content (Ca, Mg, K, P, Mn, Fe, Zn,Cu, Mo)	↓	10-100	b	Freitas et al., 2015a*
	MCLR	GST (roots)	↑	10-100	b	Freitas et al., 2015a*
	MCLR	GPx (roots)	↓	100	b	Freitas et al., 2015a*
	MCLR	Net photosynthetic rate; Transpiration; Intercellular CO2 concentration; Stomatal conductance	↑	0.65-2.5	a	Bittencourt-Oliveira et al., 2016
	MCLR	GST (roots)	↓	0.65-13	a	Bittencourt-Oliveira et al., 2016
	MCLR	SOD	↑	2.5-13	a	Bittencourt-Oliveira et al., 2016
	MCLR	CAT	↑	13	a	Bittencourt-Oliveira et al., 2016
	CYN	Shoot mineral content (Na, P, Mn, Fe, Zn, Cu, Mo); GST (roots)	↑	1-100	b	Freitas et al., 2015a*
Lens esculenta (Lentil)	MCLR	Total chlorophyll content (a+b)	↓	2100-4200	a	Saqrane et al., 2009+
	MCLR	Root mineral content (Na, N, K, P and Ca)	↑	500-4200	a	Saqrane et al., 2009+
Lepidium sativum (garden cress)	MCLR	GST; GPx	↑	1-10	a, b	Gehringer et al., 2003
	MCLR	Lipid peroxidation; GST; Gpx; GR; α- and β- tocopherol	↑	0.5	a	Stüven and Pflugmacher, 2007
	MCLR	Lipid peroxidation; GST; Gpx; GR; α- and β- tocopherol	↑	0.5	b	Stüven and Pflugmacher, 2007
	MCLR	δ- and γ- tocopherol	↓	0.5	a, b	Stüven and Pflugmacher, 2007

Table 1.4 Continued						
Lycopersicon esculentum (Tomato)	MCLR	Fv/Fm fluorescence	↓	2220-22,240	a	El Khalloufi et al, 2012+
	MCLR	POD; Phenols content; Protein content; Root mineral content (Na, K, Ca)	↑	2220-22,240	a	El Khalloufi et al, 2012+
	MCLR	Fv/Fm fluorescence	↓	100	a	Gutiérrez-Praena et al., 2014
Malus pumila (Apple)	MCLR	POD; SOD	↑	300-3000	a	Chen et al., 2010
Medicago sativa (Alfalfa)	MCLR	SOD; CAT; POD; GST; GR; Lipid peroxidation; Protein content	↑	5	a, b	Pflugmacher et al., 2006
	MCLR	Fv/Fm fluoresence	↓	11,120-22,240	a	El Khalloufi et al, 2011+
	MCLR	POD; Phenols content; Protein content; Root mineral content (Na, K, Ca)	↑	11,120-22,240	a	El Khalloufi et al, 2011+
	MCLR	Protein content	↑	2220-22,240	a	El Khalloufi et al, 2011+
	MCLR	α- and β- tocopherol	↑	0.5-5	a, b	Peuthert and Pflugmacher, 2010
	MCLR	Total chlorophyll content (a+b)	↓	5-20	a	El Khalloufi et al, 2013+
	MCLR	POD; CAT; PPO	↑	10-20	a	El Khalloufi et al, 2013+
Nicotiana tabacum (Tobacco)	CYN	Protein synthesis	↓	138,000	b	Metcalf et al., 2004
Oryza sativa (Rice)	MCLR	Phenols content	↑	24-120	a	Chen et al., 2004
	MCLR	GST	↑	50	a	Prieto et al., 2011*
	CYN	GST; GPx (roots)	↑	2.5	a	Prieto et al., 2011*
Pisum sativum (Pea)	MCLR	Fv/Fm fluoresence; Root mineral content(Na, N, K, P and Ca)	↑	500-4200	a	Saqrane et al., 2009+
Sinapis alba (White mustard)	MCLR	Anthocyanin content	↓	3500-30,000	a	M-Hamvas et al., 2003
	MCLR	PP1 and 2A activity	↓	10-10,000	b	Máthé et al., 2013b
	CYN	PP1 and 2A activity	↓	10-10,000	b	Máthé et al., 2013b
Solanum tuberosum (Potato)	MCLR	Total chlorophyll content (a+b)	↓	50-5000	a	McElhiney et al., 2001

Table 1-4. Continued						
Spinacia oleracea (Spinach)	MCLR	Photosynthetic oxygen production	↓	0.5	a	Pflugmacher et al., 2007a[-]
	MCLR	Ascorbate; dehydroascorbate; CAT; SOD; POD; GR; GST (microsomaland cytosolic); α- and γ-tocopherol	↑	0.5	a	Pflugmacher et al., 2007a[-]
Triticum aestivum (Wheat)	MCLR	Total chlorophyll content (a+b); photosynthetic oxygen production	↓	0.5	a, b	Pflugmacher et al., 2007b
	MCLR	GST; Gpx; GR	↑	0.5	a, b	Pflugmacher et al., 2007b
Tritcum durum (Wheat)	MCLR	Fv/Fm fluoresence	↓	500-4200	a	Saqrane et al., 2009[+]
	MCLR	Root mineral content (Na, N, K, P and Ca)	↑	500-4200	a	Saqrane et al., 2009[+]
Vicia faba (Faba bean)	MCLR	Total chlorophyll content (a+b)	↓	100	a	Lahrouni et al., 2013
	MCLR	Fv/Fm fluoresence; Root mineral content (Na, N, K, P and Ca); Shoot mineral content (Ca, N, K)	↓	50-100	a	Lahrouni et al., 2013
	MCLR	POD; CAT; PPO; PAL; Phenolic compounds; Shoot mineral content (Na); Root mineral content (Na)	↑	50-100	a	Lahrouni et al., 2013
	MCLR	POD; CAT;	↑	100-20,000	b	Garda et al., 2016
	MCLR	PP1 and 2A activity	↓	100-20,000	b	Garda et al., 2016
Zea mays (Corn)	MCLR	POD	↑	5	a, b	Pflugmacher et al., 2007*
	MCLR	Total chlorophyll content (a+b)	↓	4,200	a	Saqrane et al., 2009[+]
	MCLR	Fv/Fm fluoresence	↓	500-4200	a	Saqrane et al., 2009[+]
	MCLR	Root mineral content (Na, N, K, P and Ca)	↑	500-4200	a	Saqrane et al., 2009[+]

1.4.3. Accumulation and Persistence of Cyanotoxins in Agricultural Plants

Beyond the economic impacts of agricultural crops receiving irrigation water that is contaminated with cyanotoxins, is the potential for secondary exposure of humans to these toxins

through consumption of contaminated foods. Exposure from MCLR and CYN can have detrimental impacts on humans and is of upmost concern in the investigation of cyanotoxins on agricultural crops. It is well known that some food chains may expose humans to cyanotoxins such as saxitoxins in shell fish. Table 1-5. references studies performed to determine the accumulation of MCLR and CYN in plants.

Table 1-5. Summary of previous studies and bioaccumulation results of plants exposed to MCLR and CYN. a) crude extract. Table adopted from Machado et al., 2017.

Species	**Toxin**	**Organ**	**Exposure concentrations (ug/L)**		**Exposure time (days)**	**Tissue concentration (ng/g FW)**	**Reference**
Brassica napus (Rape)	MCLR	Plant (No roots)	24-3000	a	10	2.61-651	Chen et al., 2004
Lactuca sativa (Lettuce)	MCLR	Leaf	2-10	a	15	~33-143	Bittencourt-Oliveira et al., 2016
Lycopersicon escultentum (Tomato)	MCLR	Fruits	100	a	7	~5-10	Gutiérrez-Praena et al., 2014
Lycopersicon escultentum (Tomato)	MCLR	Leaves and Roots	5-100	a	90	~0.29-8.1	Corbel et al, 2016
Malus pumila (Apple)	MCLR	Shoots	30-3000	a	14	14.76-510.23	Chen et al., 2010
Medicago sativa (Alfalfa)	MCLR	Shoots	5	a	1	~27	Peuthert et al., 2007
Oryza sativa (Rice)	MCLR	Plant (No roots)	120-3000	a	10	2.94-5.4	Chen et al., 2004
Pisum sativum (Pea)	MCLR	Shoots	5	a	1	~18	Peuthert et al., 2007
Phaseolus sativium (Beans)	MCLR	Shoots	5	a	1	~38	Peuthert et al., 2007
Triticum aestivum (Wheat)	MCLR	Shoots	5	a	1	~28	Peuthert et al., 2007
Vigna radiata green (Mung bean)	MCLR	Shoots	5	a	1	~18	Peuthert et al., 2007
Vigna radiata red (Mung bean)	MCLR	Shoots	5	a	1	~4	Peuthert et al., 2007
Zea mays (Corn)	MCLR	Shoots	5	a	1	~40	Peuthert et al., 2007
Oryza sativa (Rice)	CYN	Roots	2.5	a	2	~15	Prieto et al., 2011
Oryza sativa (Rice)	CYN	Leaves	2.5	a	2	~12.5	Prieto et al., 2011

1.5. Detection methods

Multiple analysis methods have been developed over several decades to identify and quantify cyanotoxins in different matrices. Robust analytical techniques for detection of cyanotoxins have evolved, despite limitations, to allow for quantification of analytes at low concentrations. Sample preparation and toxin source can allow for determination of intracellular, within the cyano-bacteria cell wall, or extracellular environmental toxin presence. Available methods include biological and physio-chemical techniques described further below.

1.5.1. Biological analysis of cyanotoxins

1.5.1.1. *In vivo* assays

One of the original methods developed to detect cyanotoxins in water samples was the intraperitoneal injection of a sample into mice and performance of a necropsy after 24-hours to determine biological effects. Presence of hepatotoxins and neurotoxins were confirmed through the observed biological symptoms. For semi-quantitative results a series of standard concentrations can be used to compare the extent of biological damage observed. Alternative bioassays are also available due to controversial and ethical issues with animal testing.

Crustacean larvae, i.e. *Artemia*, *Daphnia, or Thamnocephalus,* are exposed to toxins during incubation in a growth medium which can be performed in a 96-well plate (Merel et al, 2013).

Despite the dual benefit of *in vivo* assay allowing for observation of biological effects and verification of toxin presence they are limited in the information that can be obtained. *In vivo* assay does not allow for the identification of specific congeners or quantification of toxins

present. Further, potential for inference or synergistic effects of the sample are difficult to isolate with this method.

1.5.1.2. Immunological assay

Cyanotoxins may be detected through the biological process of binding to specific antibodies which can be tested for using various ELISA kits. Enzyme-Linked-Immuno-Sorbent Assay (ELISA) allows for extremely sensitive detection of microcystins (4 ng/L to 2 ug/L) and have recently been developed for detection of saxitoxin and cylindrospermopsin (Merel et al, 2013).

Similar in limitation to *In vivo* assay, ELISA kits cannot identify different microcystin variants. Therefore, results of ELISA are generally reported as equivalents of MCLR. Additionally, overestimation of the toxin concentration may occur due to cross reactivity between other compounds in the sample.

1.5.1.3. Biochemical assay

Microcystin is a known inhibitor of protein phosphatase and can be detected through a protein phosphatase inhibition assay (PPIA). Initially, the enzyme is exposed to an aliquot of sample containing the toxin, then incubated with the relative substrate. Absorbance of the mixture is measured at a specific wavelength allows the assessment of enzyme activity which is inversely proportional to the concentration of microcystins.

Toxin detection through PPIA can be determined within a few hours and has a detection limit as low as 0.01 ug/L (Merel et al, 2013). Limitations of this assay are also the inability to determine specific microcystin variants and the possibility of interference of unknown compounds in a sample. Further, another cyanotoxin, nodularin, may be present in the sample

and cannot be distinguished from MCs leading to inaccuracy in reporting concentrations. Results of PPIA are also reported in equivalents of MCLR as with the other biological assays.

1.5.2. Physio-chemical analysis of cyanotoxins

Analysis of cyanotoxins through physio-chemical techniques rely on separation of compounds through chromatography and quantification by specific detectors. Different detection techniques may be coupled to chromatographic techniques, depending on compatibility.

1.5.2.1. Separation techniques

Separation techniques allow for the discrimination of co-occurring toxins within a sample, in a single analysis, with various detection techniques for quantification. Liquid chromatography (LC) is a common separation technique that provides a rapid and adaptable method to be employed with detection through UV absorbance, fluorescence or mass spectrometry. Gas chromatography can also be used, with limitations, for separation of cyanotoxins. Due to large molecular structure and low volatility of toxins like MCLR, GC requires more complex preparation and possible derivatization (Merel et al, 2013).

1.5.2.2. UV absorbance and fluorescence

After LC separation, detection through UV absorbance is often applied. Maximum absorbance at specific UV spectra for cyanotoxins allows identification of specific toxins such as MCs and CYN, although with some limitations. Since microcystins have similar UV spectra, the ability to identify the greater than 90 known variants is severely limited. Dependence on the retention time for identification and quantification, and limited analytical standards, allows for only 7 variants to be uniquely identified. Therefore, the protocol for reporting the remaining variant concentrations, like many other detection methods, is equivalents of MCLR. Additional

challenges of the UV absorbance method are the potential interference of matrices and monitoring only specific wavelengths (Merel et al, 2013).

Fluorescence is also common after LC separation as an alternative to UV absorbance for detection of cyanotoxins. Fluorescence detection may increase the sensitivity of toxin detection making it a desirable method. However, lack of natural fluorescence by cyanotoxins suggests the sample preparation requires some derivatization (Merel et al, 2013).

1.5.2.3. Mass spectrometry

High sensitivity and adaptability with both LC and GC separation techniques has made MS detection more common in recent years. Mass spectrometry detects compounds based on mass and charge, thus improving selectivity and reducing the potential interferences of other methods. Tandem mass spectrometry (MS/MS) increased the selectivity of compounds with similar mass and charge by identifying fragmentation patterns from collisions with inert gas molecules.

Methods utilizing GC separation prior to MS detection have been developed, however, sample preparation is intensive, making it less desirable to LC-MS or LC-MS/MS.

Alternatively, MS detection may be employed without chromatographic separation. Time of Flight (TOF) is used for minimal sample volumes to detect toxins, with some decreased sensitivity. Matrix-assisted laser desorption/ionization (MALDI-TOF) methods ionize dried solid samples with a laser beam to accurately identify molecules with the TOF instrument (Merel et al, 2013).

1.6. Research Gaps

Previous studies have established the potential risks posed to humans, animals and plants from exposure to cyanotoxins. Risk to food supplies has become a growing concern due to the increased geographical range and occurrence of cyanobacterial blooms. Human health and agricultural security are at risk from increasing demands on fresh water resources. Climate change places additional stress on this limited supply which is exacerbated by the risk of contamination from toxic cyanobacterial blooms.

As highlighted previously, many studies have demonstrated various effects on plants from exposure to MCLR and CYN. Germination rates, growth and biomass have shown to decrease when exposed to some toxins. Only one study found an increase in biomass of lettuce (*Lactuca sativa*) when exposed to MCLR (Freitas et al, 2015). Adverse effects are seen with concentrations as low as 0.5ug/L with multiple species being tested (Pflugmacher et al, 2007). While these studies provide valuable insight into the effects of cyanotoxins on agricultural crops, the ability to bioaccumulate in field conditions is not addressed.

To address the large-scale question of bioaccumulation of cyanotoxins in semi-field conditions an experimental design needs to mimic natural processes such as sorption, biodegradation and photolysis of the toxins. Therefore, a study is needed to determine bioaccumulation and other adverse biological effects in a soil medium. Also, many previous studies have used extracts from cyanobacterial blooms which may contain other variants and chemicals that may also produce adverse or synergistic effects. Thus, a pure toxin study would minimize the possible interferences and allow for the determination of cyanotoxin exposure on agricultural crops. Finally, the timing of exposure may indicate resilience or increased adverse effects and should also be considered in determining the risks of irrigation with cyanotoxins.

1.7. Conclusions

Harmful algal blooms continue to threaten the water industry; affecting drinking water, recreational water and irrigation water. Contamination of irrigation water with cyanotoxins poses a threat to the agricultural community. Human health is the highest concern in investigating environmental contaminants. Multiple cyanotoxins have been identified by the USEPA as priority toxins requiring further monitoring and research to inform management. Due to the risk to human health and adverse effects ATX, CYN, MCLR and SAX are receiving greater scrutiny regarding exposure through secondary sources including agricultural crops.

Previous studies have identified valuable information illustrating the negative impacts of cyanotoxins on agricultural crops. Negative impacts to all phases of plant growth and development have been observed. Investigations into bioaccumulation of cyanotoxins in plants has shown toxin concentrations ranging from 0.29 ng MCLR/g FW in tomatoes (Corbel et al, 2016) to 510.23 ng MCLR/g FW in apples. Prieto et al (2011) found between 12.5 and 15 ng CYN/g FW in rice tissues. These studies highlight the variability of bioaccumulation between crops and toxins, while indicating there is risk to human health through secondary exposure.

Research has also helped to track the expansion and increasing prevalence of cyanotoxins in the environment. Development of sensitive and robust analytical techniques, including LC-MS/MS, has provided more accurate information regarding specific toxins that are present and allowed quantification of toxin concentrations. Further, toxicity assays have provided information on adverse biological effects increasing the knowledge of how cyanotoxins are threatening to health and environment. Continued investigation into the toxicity and environmental fate of cyanobacterial metabolites will further guide management of water resources.

2.Materials and Methods

An experimental design was developed to determine the effects of cyanotoxin exposure on agricultural crops during germination, early growth and mature stages. After harvest, plant cell material was extracted to determine presence of toxins (CYN and MCLR) in cell material that could be attributed to uptake through the root systems. Extracted plant material was analyzed with LC-MS/MS to quantify amount of toxin present in tissue.

Stock solutions used in these experiments were formulated from commercially available pure toxins (CYN and MCLR) purchased from Fischer Scientific, pure HPLC grade methanol available from Fischer Scientific and Milli-Q water. Toxins were dissolved in pure methanol and added to RO water to reach concentrations of 100 ug/L CYN and 200 ug/L MCLR. Methanol concentration in the stock solutions was 4% for CYN and 8% for MCLR for early stage experiments and 8% methanol for all other experiments.

2.1. Germination Experiment

Germination experiments were performed in laboratory conditions in an aseptic environment as described below. Seeds were purchased from local farm and feed store and were stored in the dark at 21°C.

2.1.1. Alfalfa

Alfalfa (*Medicago sativa*) seeds were soaked overnight in RO water then 300 seeds were randomly selected, sorted into groups of ten and placed in amber glass vials. Three treatments were prepared; control treatment containing 1 ml RO water with 8% methanol, MCLR treatment

with 1ml 200ug/L MCLR stock solution and CYN treatment with 1ml of 100 ug/L CYN stock solution. Vials were shaken to cover seeds with solution and then stored in the dark at 21°C.

After five days the seeds were removed from the vials and rinsed with RO water. Seeds were inspected for the presence of a radicle or primary root to determine whether germination had occurred. Primary roots lengths were measured for all trials to determine the effects of toxin exposure on germination.

2.1.2. Spinach

Spinach (*Spinacia Oleracea)* seeds were prepared as alfalfa seeds and then exposed to 2ml of each solution. After five days of exposure spinach had shown little signs of germination.

Seeds were removed from vials, rinsed with RO water and transferred to moist paper towels and placed in gallon sized sealed plastic bags to continue germination treatment. Seeds were stored at 21°C in the dark for an additional nine days. Total time for spinach germination trial was 14 days. After 14 days spinach seeds were evaluated for the presence of radicle and primary root growth. Lengths of primary roots were measured and number of seeds with radicles present was recorded.

2.2. Crop Growth and Development

All crop growth, development and recovery experiments were performed in the Plant Growth Facilities at Colorado State University, Fort Collins, CO. Supplies used were provided by the facility. Potting soil used was *Promix BX,* liquid fertilizer was *Osmocote* diluted to 200ppm with tap water. Plants were grown in black plastic one-gallon containers over an eight-week period beginning May 2017 through July 2017. Day temperature was 24°C, night temperature was 18°C, Relative humidity was 50% with 16-hour day cycle.

2.2.1. Alfalfa Early Exposure

Alfalfa seeds were sown in mid May 2017. First signs of emergence were observed on day three. Two weeks after seeds were sown the early growth and development trial commenced. During the trial plants were watered with stock treatment solutions every two days over a two-week period for a total of seven treatments during weeks two and three of growth phases.

Control plants received 100ml of RO water, cylindrospermopsin treatment received 100ml of 100ug/L CYN stock solution and microcystin-LR treatment received 100 ml of 200ug/L MCLR stock solution, resulting in 70ug CYN and 140ug MCLR administered to soil. Treatments were designed to mimic environmentally relevant levels of toxins in irrigation water contaminated with cyanobacteria.

At the end of the 14-day trial half the plants in the gallon pots were removed and randomly separated to determine a normalized biomass and be prepared for extraction. The remaining plants were repotted and allowed to recover for two weeks before being harvested for biomass measurements and extraction of cell material. After biomass measurements were taken samples were frozen with liquid nitrogen and stored at -80°C until cell extractions were performed.

2.2.2. Spinach Early Exposure

Spinach plants were planted and grown in the same conditions as alfalfa described above. Each gallon pot contained three plants and emergence was observed six days after seeds were sown. Spinach plants received 50ml of RO water for controls, 50ml of 100ug/L CYN and 50ml of 200ug/L MCLR, resulting in 35ug CYN and 70ug MCLR administered. After two weeks half of the pots were removed for measurements of growth and development, biomass and extraction.

Plant tissue was separated into roots, stem and leaves. Mass of whole plants and plant parts were taken. Lengths of tap roots, stems and leaves and leaf width was measured to compare growth. Remaining spinach plants recovered for 4 weeks for early exposure recovery experiment.

2.3. Early Exposure Recovery

23.1. Alfalfa Early Exposure and Recovery

One week after final dose for the early exposure of alfalfa to CYN and MCLR the remaining plants were harvested to determine persistence of toxins within the plant tissue. Plants were rinsed with RO water and randomly separated into groups of 30 plants and weighed to determine a normalized biomass. Plants were flash frozen for storage until extraction process was performed.

23.2. Spinach Early Exposure and Recovery

Spinach plants continued to recover for one month after final dosing. At this stage, plants had begun bolting and were in reproductive stages of development. Extending recovery time into reproductive stages provided the opportunity to determine if toxins that remained in the soil would still be taken up through the plants roots and be directed towards seed and pollen potentially contaminating the next generation of crops.

2.4. Late Exposure of Mature Crops

24.1. Alfalfa Late Exposure

Trial two was designed to determine effects of toxin exposure after crops had established. Four weeks after sowing alfalfa seed, as described above in greenhouse conditions, plants received 200ml of the stock solutions prepared with 8% methanol and RO water for controls,

100ug/L CYN and 200ug/L MCLR, resulting in 140ug of CYN and 280ug of MCLR administered. Late exposure watering was every other day for two weeks, over week four and five of development. After week six half the plants in the container were removed for biomass weight and extraction preparation. Plants were flash frozen in liquid nitrogen and stored at -80°C for future processing.

2.5. Late Exposure Recovery of Mature Crops

25.1. Alfalfa Recovery after Exposure

One week after final toxin dosing on mature alfalfa plants one quarter of the plants were removed and separated for biomass weights. Some plants at seven weeks had early signs of reproductive growth. Plants were flash frozen and stored for future extraction as described earlier. Two weeks after final toxin exposure of mature plants the remaining quarter of the alfalfa plants were harvested. Plants were processed for biomass weight as described earlier and stored at -80°C until extraction procedure.

2.6. Plant Tissue Extractions

Extraction of plant tissue was performed as described by Drobac et al (2017) with modifications. Plant tissue was removed from -80°C storage and lyophilized (Labconco Freezone 2.5) for 72 hours at -48°C and -0.133mBar. Dried tissue was then weighed and homogenized in 100% pure methanol with a glass mortar and pestle. Methanol and plant mixtures were transfer to amber glass vials and placed in an ice bath for sonication. Sonication (Misonix Sonicator S-4000) was performed at 50 Hz for a total of 45 minutes to ensure breakdown of cell walls and release of cell material into methanol. Plant material was separated

from the methanol solution and transferred to glass centrifuge tubes (Fischer Scientific) and centrifuged at 20°C (Sorvall RC 6+, F13s-14x50cy fixed angle rotor) for 20 minutes at 3000xg (4190 RPM). Supernatant was filtered with 0.7 um glass microfiber filters (Fischer Scientific). Final cleanup of the sample solutions was performed with SPE. HyperSep C18 SPE cartridges with 1ml volume and 100mg bed weight (Fischer Scientific) were conditioned with 100% pure HPLC grade methanol and rinsed with DI water. Filtered samples were loaded in cartridges and positive air pressure was applied. Elution was performed with 95% methanol and 5% formic acid solution. Final concentration of sample solution was through evaporation. Glass centrifuge tubes were transferred to Labconco RapidVap Heated Vortex Evaporation system set to 337mBar and 40°C. Evaporated samples were resuspended with 200uL of pure methanol and transferred to amber auto sampler vials with 200uL inserts for LC-MS/MS analyses.

2.7. LC-MS/MS

Samples were evaporated to dryness under laboratory N_2 at room temperature and extracts were resuspended in 50 uL of 100% pure water. Vials were centrifuged for 40 minutes at 3,750 RPM to pellet insoluble debris. Supernatant was transferred to LC-MS vial inserts (Waters Corp).

For LC analysis 10 uL of sample was injected onto a Waters HSS T3 reverse phase column (2.1 x 50 mm). Analytes were resolved over a linear gradient from 0-100% B over 5 minutes. Buffer A was 99.9% water, 0.1 % formic acid and Buffer B was 100% ACN with no acid modifier. Flow rate was 0.4 mL/min with a runtime of 7.5 minutes. Column temperature was set to 45°C. The mass spectrometer (Waters Xevo TQ-S) was operated in selected reaction

monitoring (SRM) mode, using positive electrospray ionization (ESI) with the conditions described in Table 2-1.

Table 2-1. LC-MS/MS conditions for determining MCLR and CYN concentrations in plant material.

Transition Table				
	Retention Time (min)	Precursor Mz	Product Mz	Precursor Adduct
Cylindrospermopsin	1.74	416.2	336.2	[M+]
Cylindrospermopsin	1.74	416.2	194.2	[M+]
Microcystin	3.61	498.6	135	[M+2]

Calibration samples were prepared with serial dilution in 100% pure water and run through the same analysis method as experimental samples. Calibration concentrations were between 0 and 500 pg/ml for MCLR and 0 and 50 pg/ml for CYN. Raw MS data files were imported into Skyline software program. Peak areas were integrated and compared against a calibration curve for both CYN and MCLR. Concentrations were reported in pg/mL and then back calculated to pg/g dry weight of plant tissues. Limit of detection (LOD) for CYN was 1.48 pg/ml and limit of quantification (LOQ) was 4.94 pg/ml. For MCLR, LOD was 3.18 pg/ml and LOQ was 10.59 pg/ml.

3. Results

3.1. Germination

Germination rates for alfalfa and spinach seeds exposed to CYN or MCLR showed no significant difference when compared to control seeds. Rates for alfalfa were 89%, 88% and 87% germination for control, MCLR and CYN treatments, respectively (p=0.91, n = 300). Rates for spinach seeds showed similar trends with 56%, 60%, and 60% for control, MCLR and CYN treatments, respectively (p=0.80, n = 300).

Primary root growth for alfalfa seeds exposed to toxins was significantly longer than the control treatment. Control alfalfa seeds had a mean root length of 0.45cm while seeds exposed to MCLR and CYN had mean root lengths of 3.66cm and 3.18cm, respectively. Maximum primary root length for alfalfa seeds exposed to CYN was 6.5cm, MCLR was 6.7cm and control was 0.7cm. Minimum root length, counted as successful germination, was 0.1cm for all treatments.

Variance in primary root length for toxin exposed alfalfa seeds was much greater than in control seeds. Figure 3.1 provides a box plot illustrating the distribution of primary root lengths for alfalfa seeds exposed to cyanotoxins during germination.

Spinach seeds exhibited similar trends in root length as seen by alfalfa, however, the difference was statistically insignificant. Control spinach seeds had mean root length of 0.11cm and MCLR and CYN treatments were 0.33cm and 0.38cm, respectively. To capture the variability in the germinated seeds primary root lengths, ten seeds per treatment exhibiting the greatest primary root growth were selected for comparison. Mean values for theses seeds were 0.18cm for control, 1.49cm for MCLR and 1.77cm for CYN.

Figure 3.2 provides a box plot illustrating the distribution of primary root lengths for the

selected spinach seeds exposed to cyanotoxins during germination and the control seeds. Maximum primary root length for spinach seeds exposed to CYN was 8.3cm, MCLR was 4.8cm and control was 0.7cm. Similar to alfalfa, the primary root lengths for toxin exposed spinach seeds had large variance in the sample compared to control seeds. Minimum root length for spinach was 0.1cm.

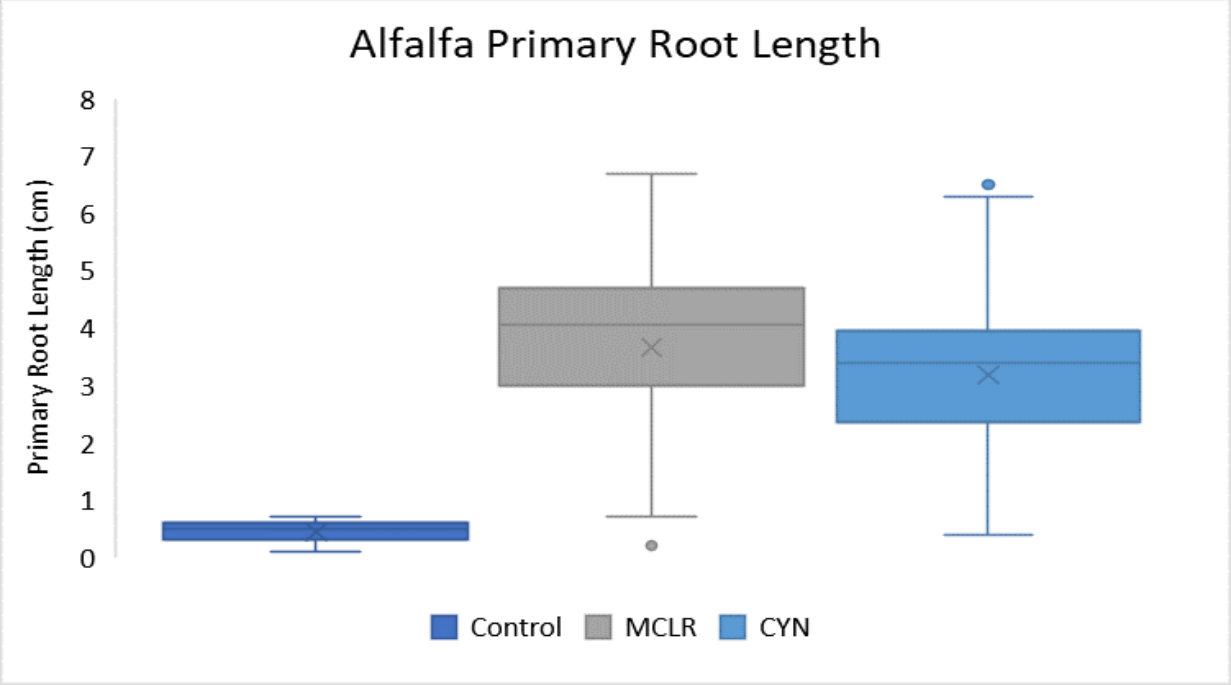


Figure 3.1. Box plot showing distribution of alfalfa seeds exposed to cyanotoxins during germination. • statistical outliers, x mean value, minimum, 25th percentile, median, 75th percentile and maximum values. (n = 300)

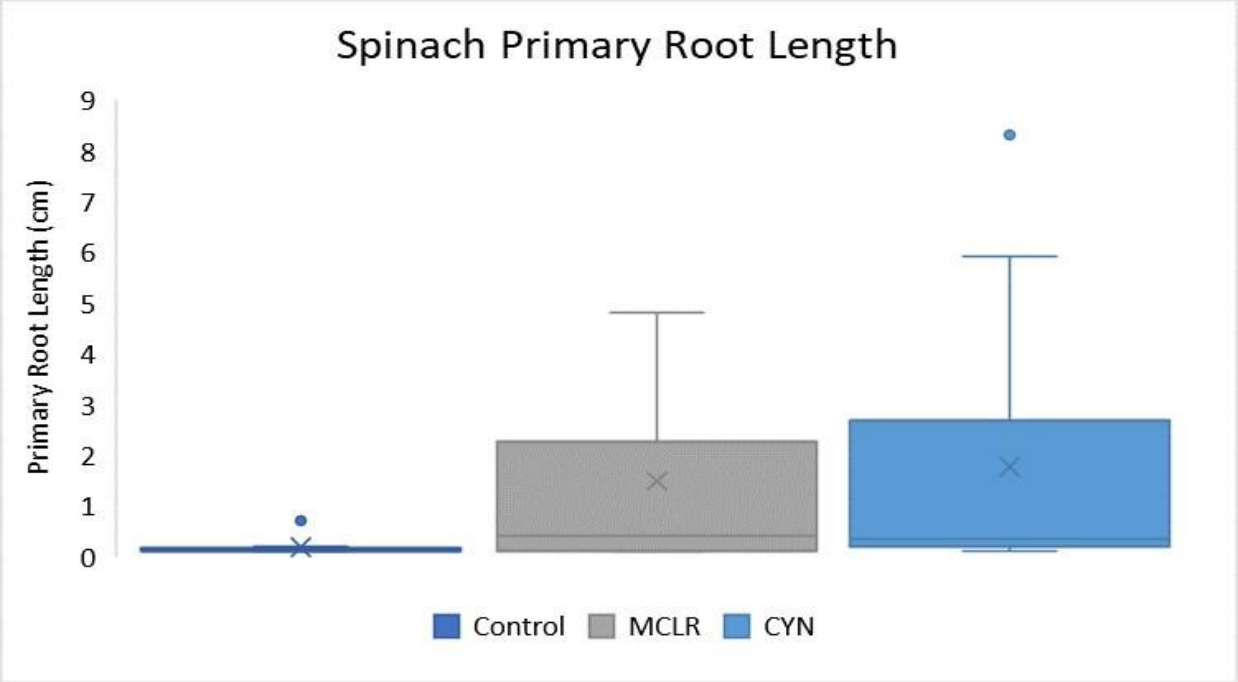


Figure 3.2. Box plot showing distribution of spinach seeds exposed to cyanotoxins during germination. • statistical outliers, **x** mean value, minimum, 25th percentile, median, 75th percentile and maximum values (n = 30).

3.2. Growth and Development

3.2.1. Early Exposure

Normalized biomass for whole alfalfa plants exposed to algal toxins during early growth phases indicated differences in each toxin's effects on plant growth. Increased biomass for plants exposed to MCLR was observed, while plant biomass for alfalfa exposed to CYN was more consistent with the control biomass. Mean normalized biomass for MCLR controls was 58.8mg. Biomass for plants in group MCLR-1 had a mean value of 124.2mg and MCLR-2 was 174.7mg. Alfalfa mean biomass for plants exposed to CYN were 120.1mg for group CYN-1 and 106.6mg for CYN-2. CYN control had a mean normalized biomass of 137.5mg. Box plots indicating the minimum, 25th percentile, median, 75th percentile and maximum values for the normalized biomass of alfalfa are shown in Figure 3.3. Minimum and maximum values for normalized

biomass of MCLR control were 39.7mg and 72.7mg. MCLR-1 values were 93.4mg and 159.9mg, and MCLR-2 were 107.9mg and 207.5mg. Minimum and maximum values for CYN control were 104.5mg and 201.6mg, while CYN-1 were 96.7mg and 147.9mg, and CYN-2 were 39.1mg and 150.8mg.

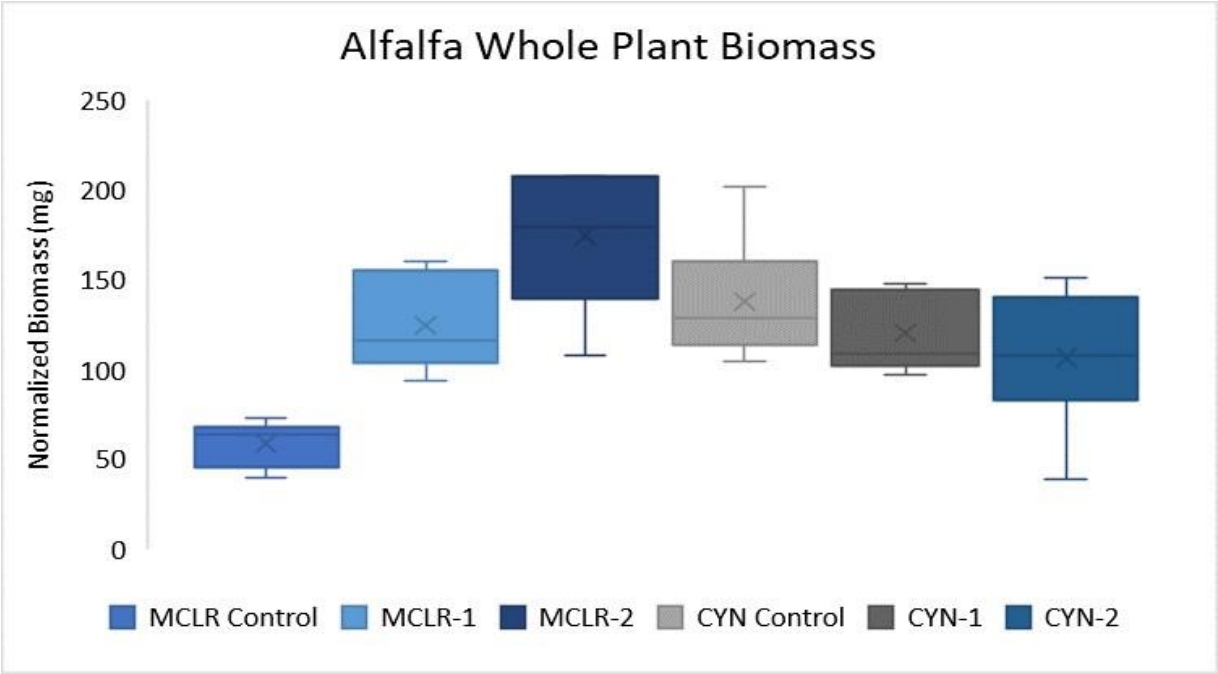


Figure 3.3. Box plot describing distribution of alfalfa plants exposed to cyanotoxins during early growth stages, **x** mean value, minimum, 25th percentile, median, 75th percentile and maximum values.

Spinach biomass results indicated the early stage exposure to toxins altered the growth of plants consistent with the trends observed in alfalfa plants. Spinach plants exposed to MCLR had a significant increase in biomass production compared to the control plants. Increased biomass in spinach treated with CYN was observed, however, was not statistically significant compared to the controls and large variances were observed in the data. Averaged spinach biomass for CYN control was 475.3mg and MCLR control was 809.2mg. Comparatively, spinach in CYN-1 averaged 2816.6mg and MCLR-2 was 4039.3mg.

Distribution of mean spinach biomass is shown in Figure 3.4. Maximum and minimum values for CYN control were 596.5mg and 354.0mg, respectively. Comparatively, spinach treated with CYN had maximum and minimum values of 5415.4mg and 1091.2mg. MCLR controls were 447.2mg and 1171.1mg while MCLR treated spinach biomass minimum and maximum values were 3125.9mg and 4729.4mg.

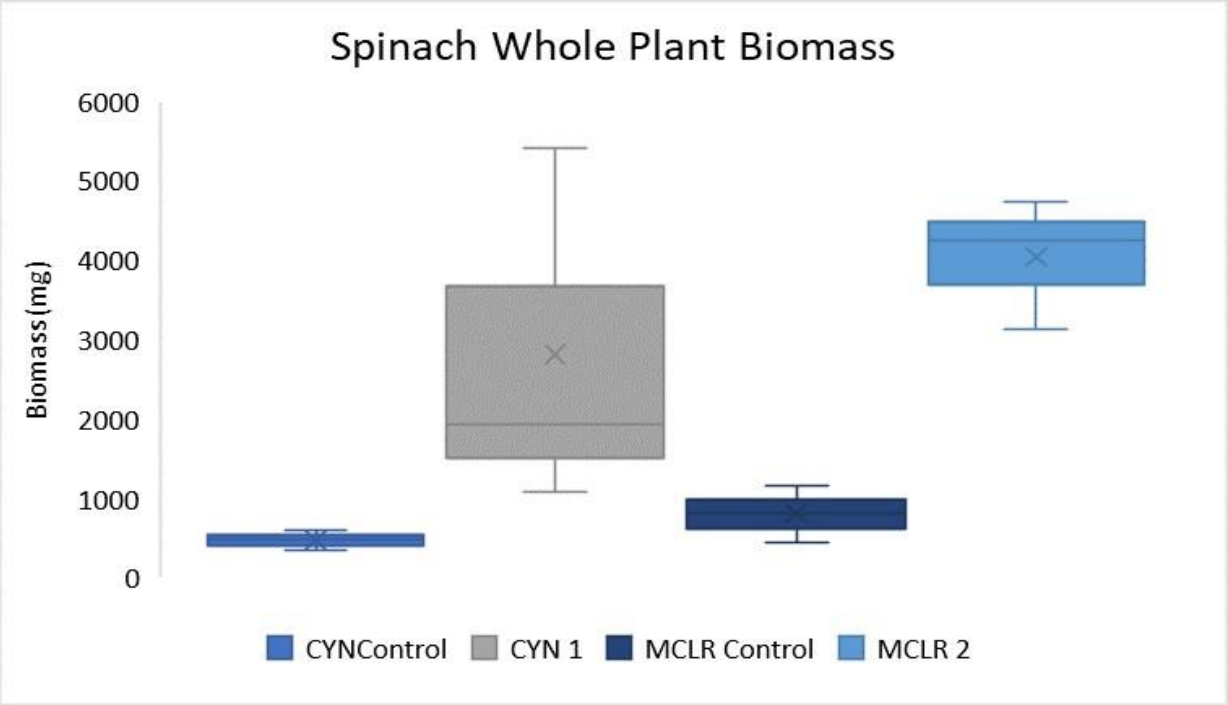


Figure 3.4. Box plot describing distribution of spinach plants exposed to cyanotoxins during early growth stages, **x** mean value, minimum, 25th percentile, median, 75th percentile and maximum values. (n = 3 for roots and stems, n = 9 for leaves).

Partitioned spinach plant mass indicated where the increased mass was attributed to for the plants exposed to toxins. Comparison between CYN treatment and control had no statistically significant difference in biomass between roots, stems or leaves. Although in all cases the CYN treatment was notably greater in mass than the controls, large variance in the samples prevented these differences from being statistically significant. Mean values for CYN control plant organs were 58.8mg of roots (13%), 130.1mg of stem (28%) and 281.5mg of leaves (60%). Partitioned

mean biomass of plants exposed to CYN were 141.4mg of roots (5%), 1,138.8mg of stems (41%) and 1,507.7mg of leaves (54%).

Figure 3.5 illustrates the distribution box plot for partitioned biomass of spinach plants exposed to CYN during early growth stages. Minimum values and percent of total mass for CYN control plant organs were 52.7mg of roots (15%), 102.7mg of stem (30%), and 192.6mg of leaves (55%). Minimum values for organs of plants treated with CYN were 70.1mg of roots (6%), 374.6mg of stem (35%) and 637.2mg of leaves (59%). Maximum values for CYN control plant organs were 64.9mg of roots (11%), 157.5mg of stem (27%) and 370.3mg of leaves (62%). Maximum values for spinach treated with CYN were 264.8mg of roots (5%), 2,317.2mg of stem (43%) and 2,785.5mg of leaves (52%).

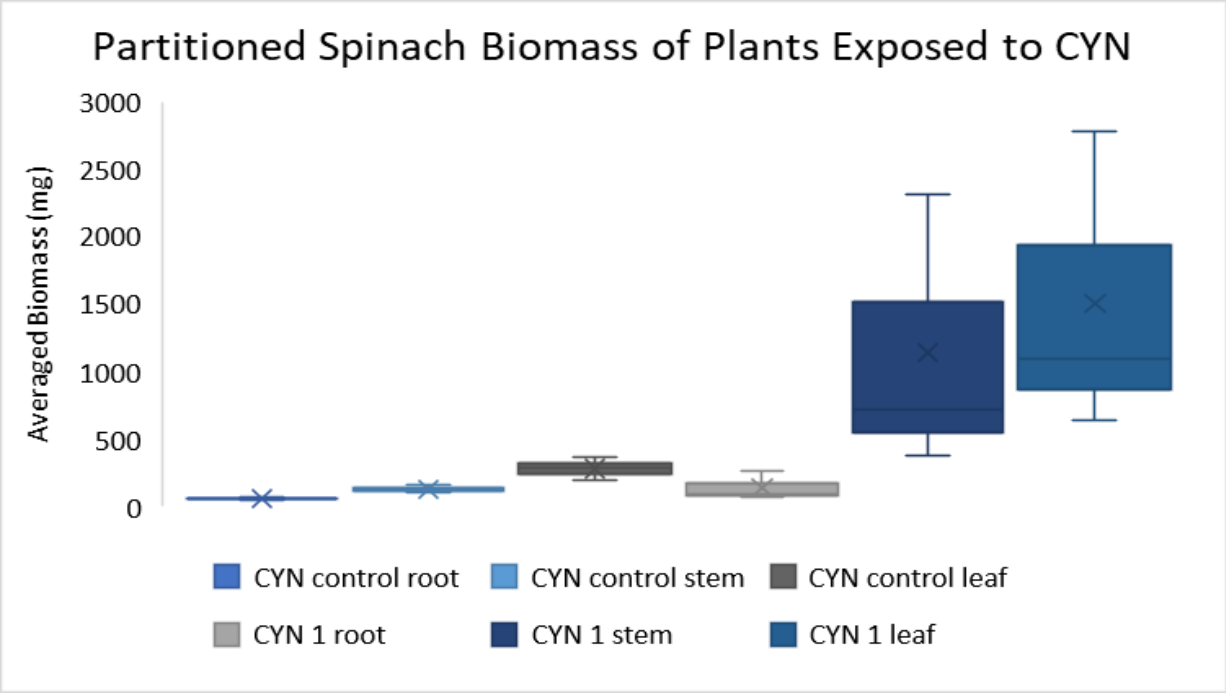


Figure 3.5. Box plot describing distribution of spinach plants exposed to cyanotoxins during germination, **x** mean value, minimum, 25th percentile, median, 75th percentile and maximum values. (n = 3).

Partitioned biomass for spinach exposed to MCLR indicated the significant increase in biomass was attributed to an increase in stem and leaf mass. Stem and root mass comparisons between MCLR treatment and control spinach were increased for toxin treated plants, although not significantly.

Averaged root, stem and leaf biomass and percent of total for control plant organs were 105.0mg (13%), 246.9mg (31%) and 451.4mg (56%), respectively. Averaged partitioned biomass and percent of total mass for spinach treated with MCLR were 231.5mg of roots (6%), 1,828.9mg of stem (45%) and 2,011.6mg of leaves (49%).

Figure 3.6 illustrates the distribution box plot for partitioned biomass of spinach plants exposed to MCLR during early growth stages. Minimum organ biomass and percent of total biomass for control plants were 87.6mg of roots (20%), 90.2mg of stem (20%) and 265.2mg of leaves (60%). Minimum values for spinach exposed to MCLR were 237.2mg of roots (8%), 932.9mg of stem (30%) and 1,925.7mg of leaves (62%). Maximum values for control plants were 122.4mg of roots (10%), 403.6mg of stem (35%) and 637.6mg of leaves (55%). Spinach treated with MCLR had maximum values of 292.5mg of roots (7%), 2,608.9mg of stems (56%) and 2,184.3mg of leaves (49%) from different plants.

Other morphological parameters considered were the dimensions of different organs of each plant. Spinach plants partitioned by root, stem and leaves showed significantly longer and wider leaves for plants exposed to CYN and MCLR. Stems for plants exposed during early growth were notably longer than control stems and showed large variances in the lengths. Roots were only slightly longer on toxin treated plants compared to controls.

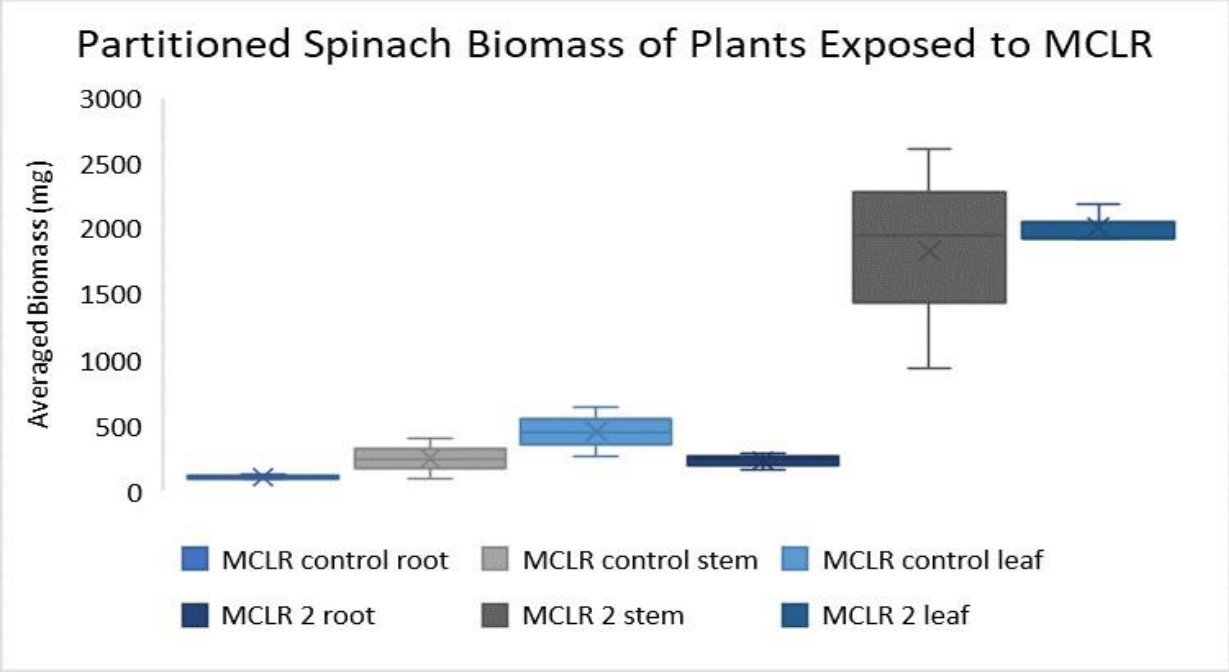


Figure 3.6. Box plot describing distribution of spinach plants exposed to cyanotoxins during early growth stages, **x** mean value, minimum, 25th percentile, median, 75th percentile and maximum values. (n = 3).

Averaged root lengths for spinach control plants was 8.1cm vs. CYN treated plants which was 8.9cm. Stem lengths on control plants averaged 6.0cm which toxin treated plants were 13.3cm. For leaf lengths, control plants averaged 1.9cm and CYN treated spinach averaged 4.3cm. Leaf widths were an average of 0.9cm for control plants and 2.0cm for those receiving CYN containing water.

Figure 3.7 is the box plot showing the distribution of spinach plant organ dimensions after exposure to CYN in vegetative stages. Control plants had minimum values of 7.0cm of roots, 5.2cm stem, 1.1cm in leaf length and 0.3cm in leaf width. Maximum values for control plants were 9.1cm of roots, 6.7cm stem, 2.9cm in leaf length and 1.6cm in leaf width. Comparatively, when treated with CYN minimum dimensions were 8.3cm in root, 9.3cm in stem, 3.1cm in leaf length and 1.2cm in width. Maximum values were 9.9cm root, 16.8cm stem, 6.2cm in leaf length and 3.5cm in width.

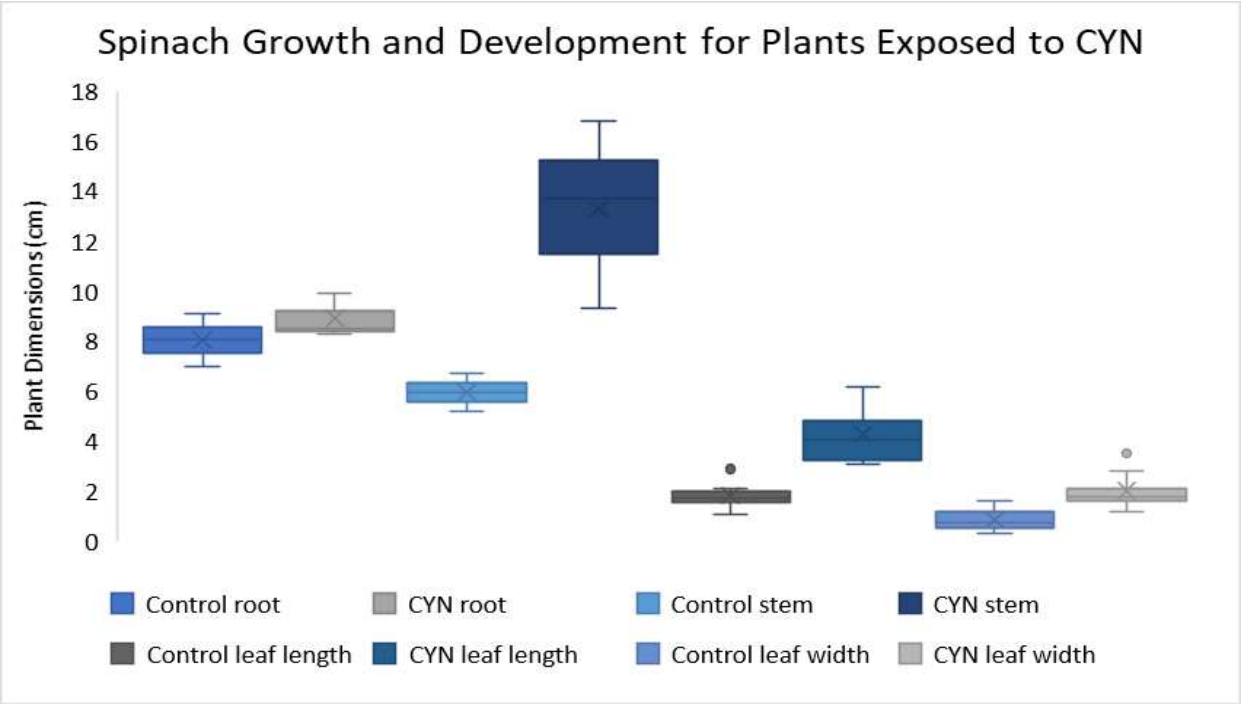


Figure 3.7. Box plot describing distribution of spinach plants exposed to CYN during early growth stages, • statistical outliers, **x** mean value, minimum, 25th percentile, median, 75th percentile and maximum values. (n = 3 for roots and stems, n = 9 for leaves).

Figure 3.8 illustrates the results for spinach plants exposed to MCLR. Averaged root lengths for spinach control plants was 8.6cm vs. MCLR treated plants which was 10.3cm. Stem lengths on control plants averaged 5.9cm while MCLR treated plants were 16.7cm. For leaf lengths, control plants averaged 2.7cm and MCLR treated spinach averaged 4.8cm. Leaf widths were an average of 1.4cm for control plants and 2.6cm for those receiving MCLR containing water.

Figure 3.8 shows the distribution of spinach plant organ dimensions after exposure to MCLR in vegetative stages. Control plants had minimum values of 8.4cm of roots, 4.6cm stem, 1.3cm in leaf length and 0.7cm in leaf width. Maximum values for control plants were 8.8cm of roots, 7.2cm stem, 4.0cm in leaf length and 2.1cm in leaf width. Comparatively, when treated with MCLR minimum dimensions were 9.4cm in root, 10.2cm in stem, 3.5cm in leaf length and

1.5cm in width. Maximum values were 11.6cm root, 23.6cm stem, 6.3cm in leaf length and 3.4cm in width.

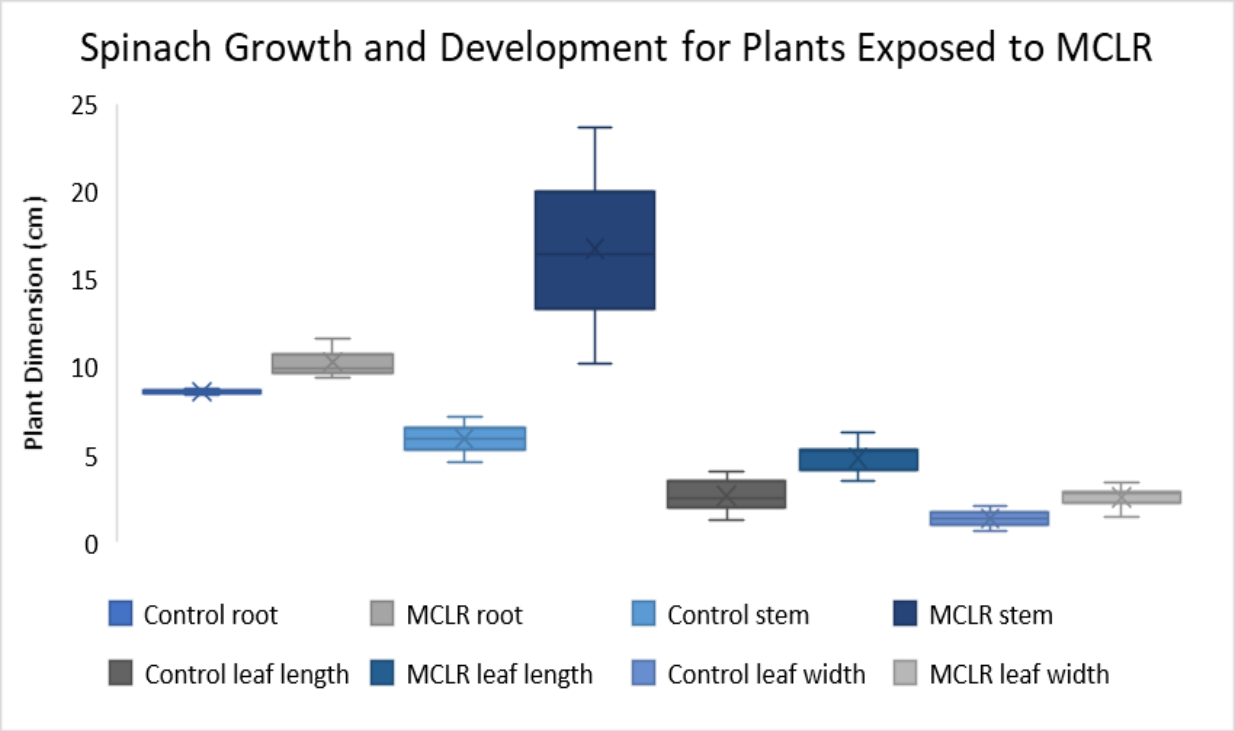


Figure 3.8. Box plot describing distribution of spinach plants exposed to MCLR during early growth stages, **x** mean value, minimum, 25th percentile, median, 75th percentile and maximum values. (n = 3 for roots and stems, n = 9 for leaves).

3.2.2. Late Exposure

Mature alfalfa plants exposed to cyanotoxins during early reproductive stages indicated exposure to CYN had a significant increase in in biomass production compared to control plants. Alfalfa exposed to MCLR during this stage had slight increases in biomass compared to one control but not the second control. Averaged normalized biomass for control 3-2 was 275.1mg, control 2-2 was 205.6mg, alfalfa treated with MCLR was 293.4mg and alfalfa treated with CYN was 542.7mg.

Figure 3.9 illustrates the distribution of alfalfa biomass when exposed to cyanotoxins during mature growth stages. Minimum values for controls 3-2 and 2-2 were 174.9mg and

124.2mg, respectively. Minimum value for alfalfa treated with CYN was 351.3mg and 142.0mg for MCLR treatment. Maximum biomass for control 3-2 was 395.3 mg and control 2-2 was 387.5 mg. Maximum values for CYN and MCLR treated plants was 740.4 mg and 528.4 mg, respectively.

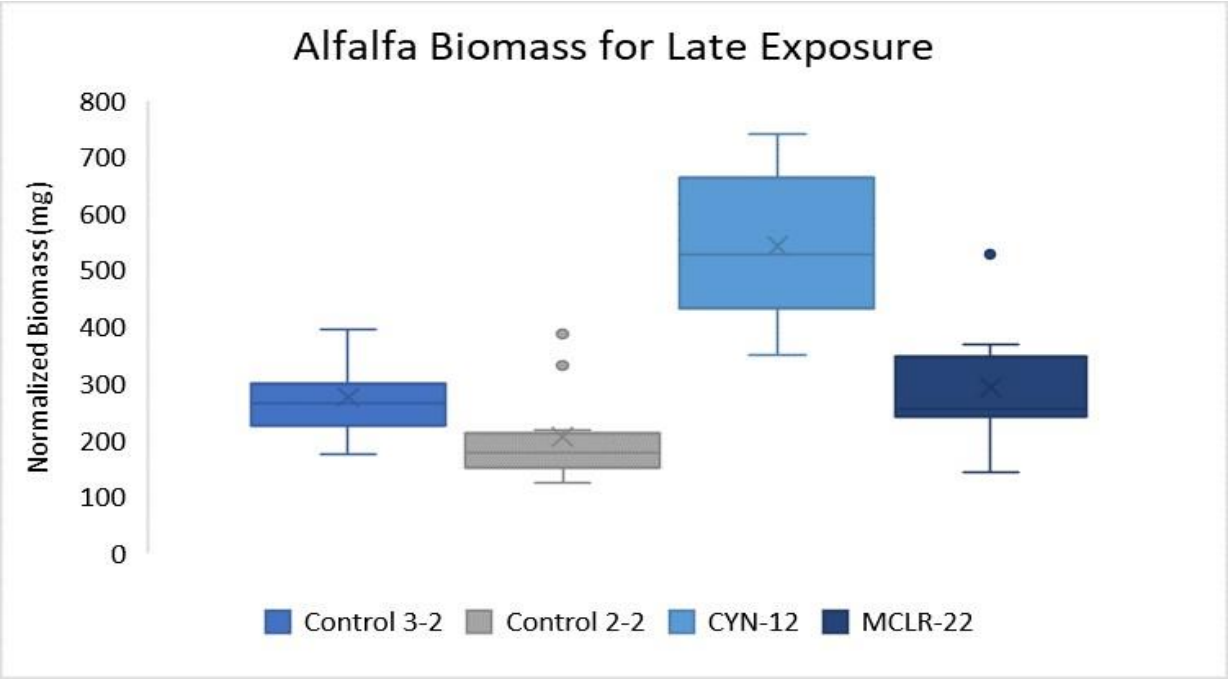


Figure 3.9. Box plot describing distribution of alfalfa plants exposed to cyanotoxins during mature growth stages, • statistical outliers, **x** mean value, minimum, 25th percentile, median, 75th percentile and maximum values.

3.3. Recovery

3.3.1. Early Exposure Recovery

Alfalfa plants exposed to CYN and MCLR during early vegetative growth showed similar trends for biomass after a 1-week recovery period, as was observed with no recovery period. Exposure to MCLR had continued increased biomass production and exposure to CYN produced biomass more consistent with the controls. Mean alfalfa biomass for MCLR control was 231.9mg, MCLR-1 was 378.2mg and MCLR-2 was 654.02mg. Biomass for CYN control alfalfa plants averaged 540.3mg, CYN-1 was 580.1mg and CYN 2 was 475.4mg.

Figure 3.10 shows the biomass distribution for alfalfa exposed to MCLR and CYN during mature growth stages. Biomass for the MCLR control plants had a minimum value of 195.9mg and a maximum value of 269.5mg. For MCLR-1 the minimum and maximum values were 301.2mg and 449.3mg; MCLR-2 values were 578.3mg and 761.9mg. Minimum and maximum values for CYN control were 426.3mg and 654.7mg, respectively. For CYN-1 normalized minimum and maximum biomass were 484.7mg and 670.9mg, while CYN-2 were 373.5mg and 687.5mg, respectively.

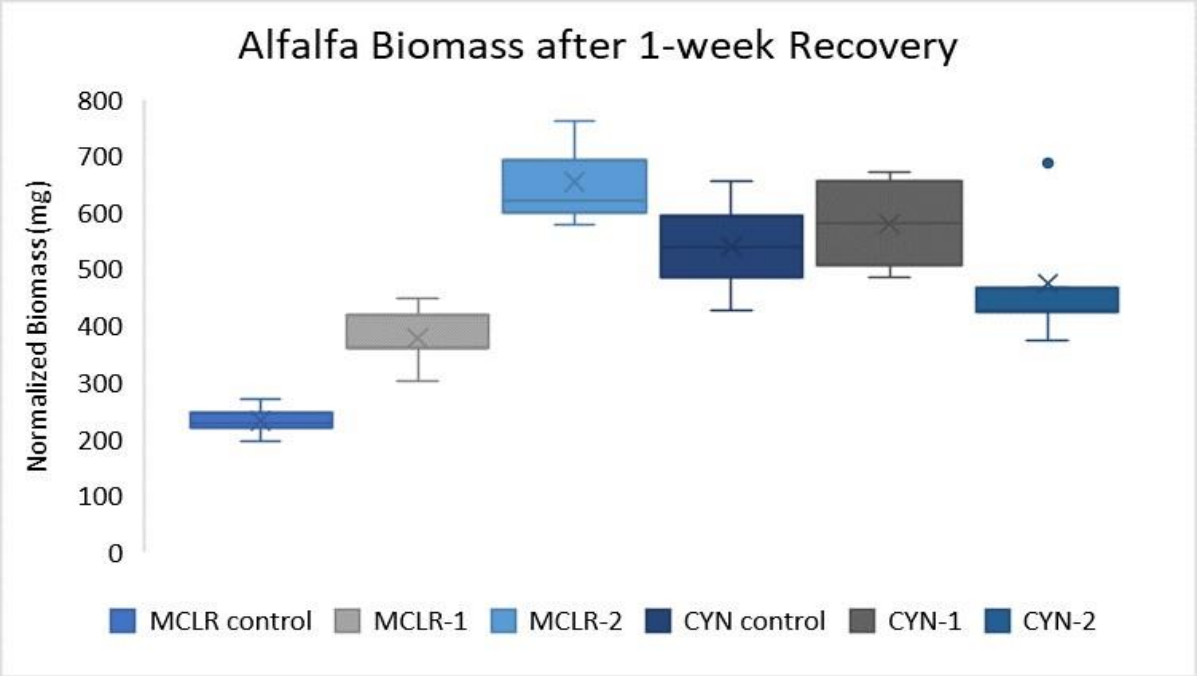


Figure 3.10. Box plot describing biomass distribution of alfalfa plants exposed to cyanotoxins during early growth stages and allowed to recover for one week, • statistical outliers, **x** mean value, minimum, 25th percentile, median, 75th percentile and maximum values.

Spinach total biomass production from early exposure to CYN and MCLR after one month of recovery indicated different responses between treatments. Plants exposed to MCLR were very similar in biomass where plants exposed to CYN were much larger on average than the controls. Mean biomass for spinach plants after one-month recovery were 36.1g for MCLR

control, 37.6g for MCLR treated spinach. Spinach treated with CYN had a mean biomass of 51.4g while the CYN control average was 13.8g.

Spinach biomass distribution after one-month of recovery from toxin exposure during early growth stage is depicted in Figure 3.11. Minimum and maximum values for the MCLR control plants were 24.7g and 47.4g, while MCLR-1 plants were 18.4g and 55.9g. The CYN control group had minimum and maximum values of 0.46g and 30. 3g. Minimum and maximum biomass for spinach recovering from treatment with CYN were 11.5g and 106.9g.

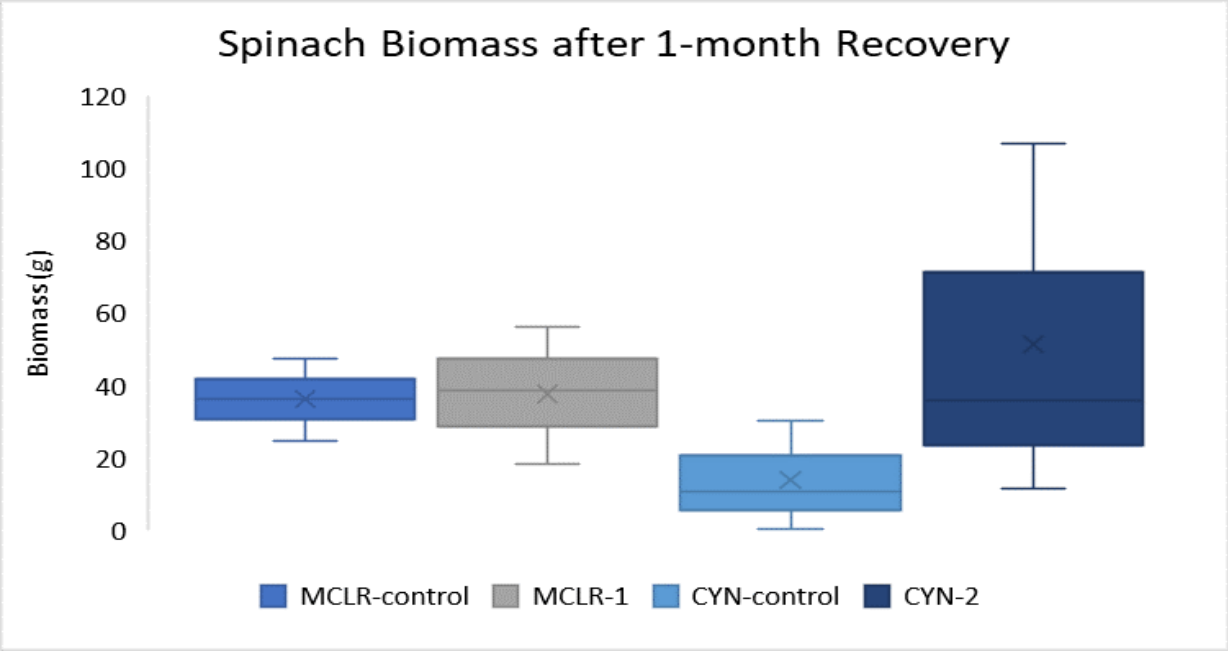


Figure 3.11. Box plot describing biomass distribution of spinach plants exposed to cyanotoxins during early growth stages after one month of recovery from toxin exposure, **x** mean value, minimum, 25th percentile, median, 75th percentile and maximum values.

Partitioned biomass for spinach exposed during early vegetative stages was consistent with the total biomass as all sections had large variances in the sample population. However, both CYN and MCLR exposed plants showed on average more biomass in each section compared to the controls which was consistent with the early development of the plants during

exposure. Mean values for spinach treated with CYN and allowed to recover were 1.0g of roots, 17.2g of stem, 29.9g of leaves and 3.5g of seed and pollen. Control plants for this group had partitioned mean biomass of 0.38g of roots, 5.8g of stem, 7.5g of leaves and 0.29g of seed and pollen.

Spinach plants recovering from treatment of MCLR had biomass values of 0.82g of roots, 15.1g of stem, 16.9g of leaves and 4.8g of seed and pollen. Controls for the MCLR group were 0.77g of root, 11.4g of stem, 23.9g of leaves. No seed or pollen developed in the MCLR control group. Minimum values for spinach root biomass were 0.06g for CYN control, 0.18g for CYN treatment, 0.66g for MCLR controls and 0.23g with MCLR treatment. Maximum values for spinach root biomass were 0.61g for CYN control, 2.2g for CYN treatment, 0.88g for MCLR controls and 1.2g with MCLR treatment. Spinach root biomass distribution is shown in Figure 3.12A.

Figure 3.12B illustrates the stem biomass distributions for spinach exposed to cyanotoxins during early growth after the one-month recovery period. Minimum stem biomass was 0.18g for CYN control, 4.8g for CYN treatment, 11.4g for MCLR control and 10.1g for MCLR treatment. Maximum values were 12.9g for CYN controls, 32.7g for CYN treatment, 12.7g for MCLR controls and 21.1g for treatment with MCLR.

Figure 3.12C shows the distribution of leaf biomass for recovering spinach. Minimum leaf biomass was 0.12g for CYN control, 3.4g for CYN treatment, 13.9g for MCLR control and 1.9g for MCLR treated spinach. Maximum leaf biomass for CYN controls was 16.7g, CYN treated spinach was 66.5g, MCLR controls 33.9g, and 29.2g for MCLR treated plants.

After one-month recovery from treatment with cyanotoxins some spinach plants were in reproductive growth stages. Figure 3.12D is the distribution of pollen or seed for spinach

recovering from toxin treatment. Minimum seed or pollen biomass for CYN control plants was 0g indicating no seed or pollen had developed on this plant. The maximum reproductive biomass for the CYN control was 0.49g. Conversely, the spinach treated with CYN had a minimum and maximum seed or pollen mass of 2.0g and 5.5g. For MCLR treated plants the range was 3.8g to 6.1g of seed or pollen biomass.

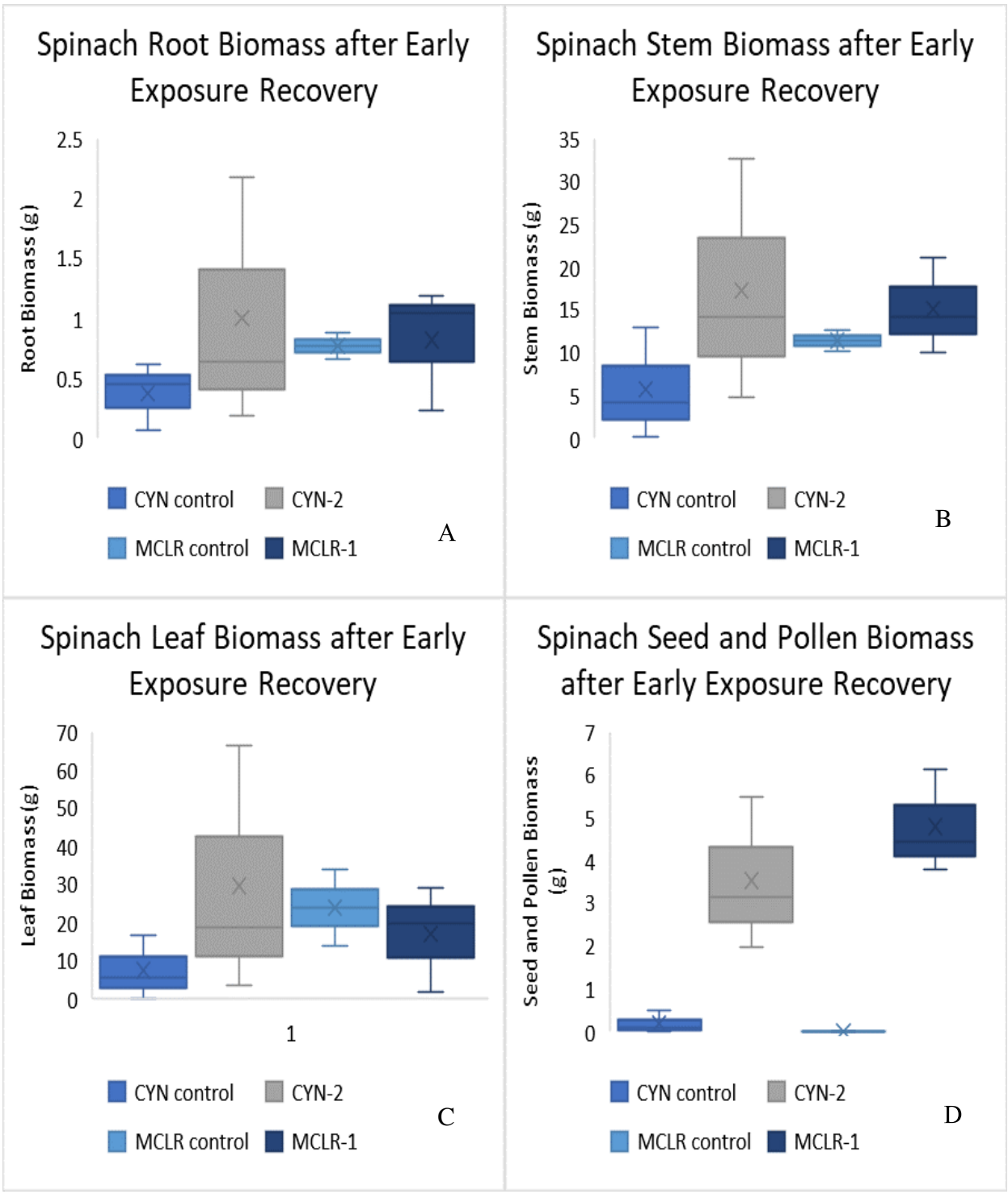


Figure 3.12. Distribution of partitioned spinach organ biomass after exposure to MCLR and CYN during early growth stages and one-month recovery period (n = 3). A) roots B) stems C) leaves D) seed and pollen. MCLR control (n = 2).

3.3.2. Late Exposure Recovery

Exposure to CYN and MCLR during the mature growth and initial reproductive stages was performed on alfalfa only. At one-week recovery time alfalfa exposed to MCLR had significantly more biomass than the controls. Where alfalfa treated with CYN was only slightly higher in biomass. After two weeks of recovery the plants exposed to MCLR still had significantly higher biomass than the controls and alfalfa exposed to CYN showed significantly more biomass than one of the controls.

Figure 3.13 show the distributions of alfalfa biomass after one week of recovery from exposure to CYN and MCLR during mature growth stages. Mean values for control -42 and -12 were 263.2mg and 353.9mg, respectively, after one-week recovery. Mean biomass for CYN treated alfalfa was 385.4mg and MCLR treated plants was 648.3mg. Minimum values at one-week recovery were 165.6mg for control-42, 184.8mg for control-12, 556.0mg for MCLR treatments and 192.1mg for CYN treatments. Maximum biomass at one-week recovery was 54.1mg for control-42, 660.7mg for control-12, 712.1mg for MCLR and 466.2mg for CYN treatments.

After two weeks, the mean normalized biomass for control -42 and -12 were 312.5mg and 471.8mg, respectively. For CYN and MCLR treated plants the mean values were 514.0mg and 715.0mg, respectively. Minimum values for two-weeks recovery of alfalfa biomass were 194.2mg for control-42, 422.6mg for control-12, 433.0mg for MCLR and 431.5mg for CYN treatments. Maximum values were 453.5mg for control-42, 513.0mg for control-12, 875.0mg for MCLR treatments and 584.2mg for CYN treated alfalfa. Figure 3.14 summarizes the biomass distributions of alfalfa exposed during mature stages after two weeks of recovery.

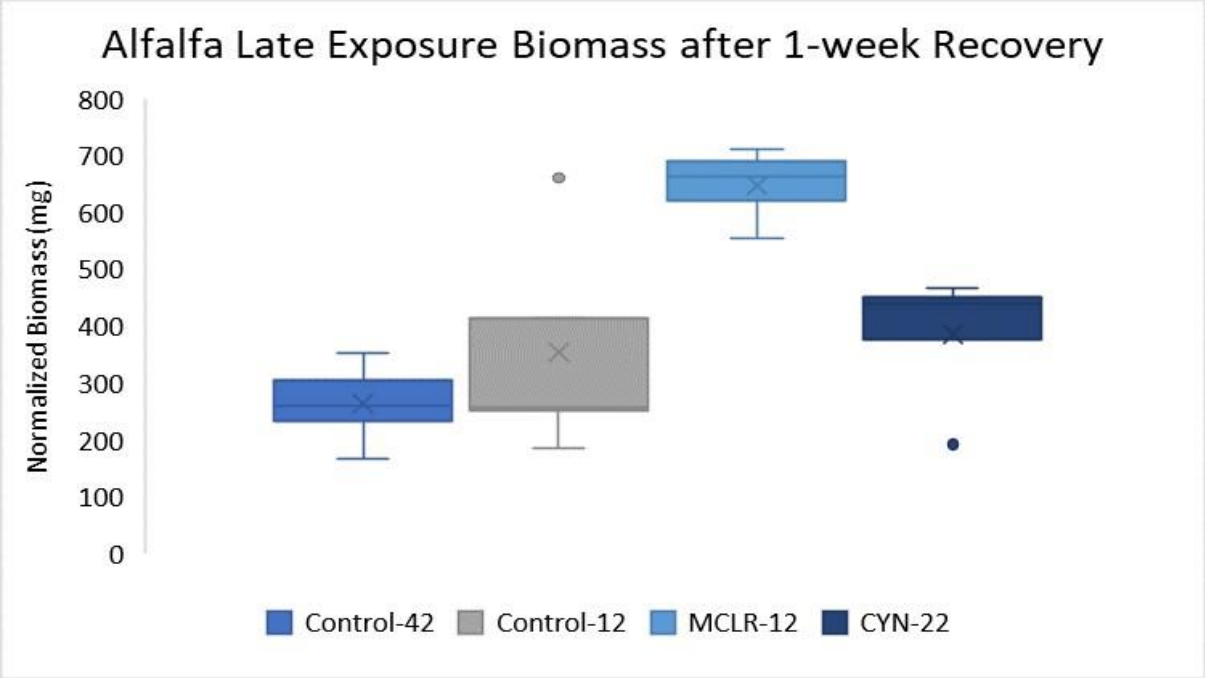


Figure 3.13. Box plot describing biomass distribution of alfalfa plants exposed to cyanotoxins during Mature growth stages and allowed to recover for one week, • statistical outliers, **x** mean value, minimum, 25th percentile, median, 75th percentile and maximum values.

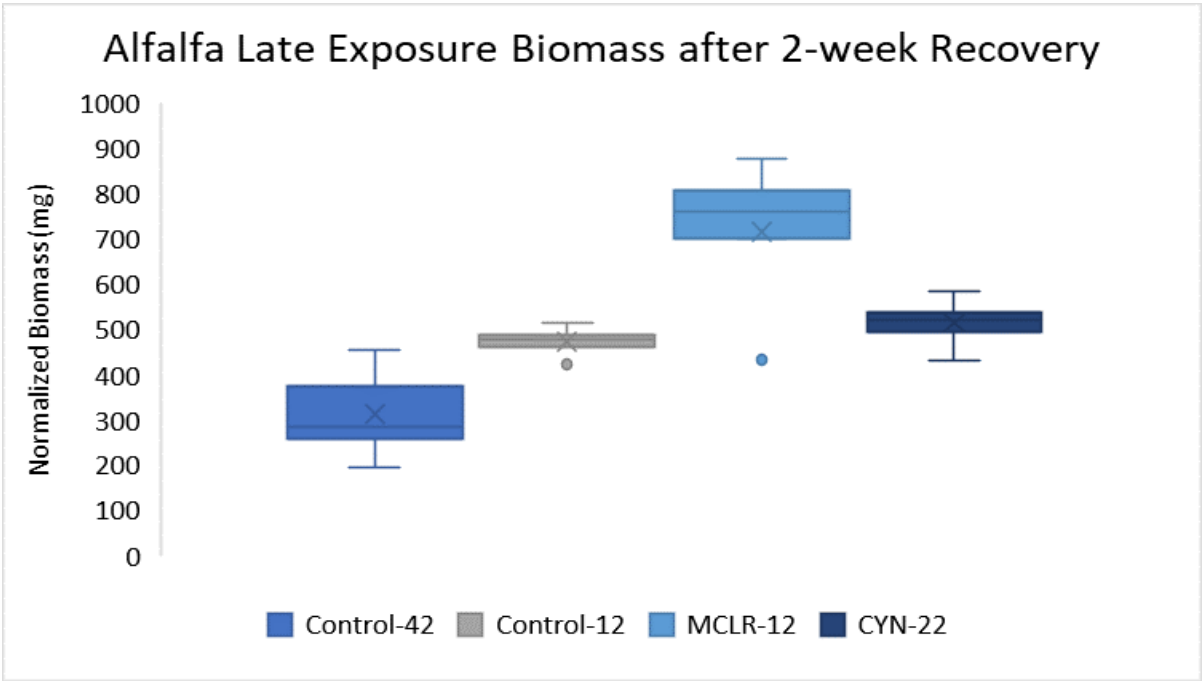


Figure 3.14. Box plot describing biomass distribution of alfalfa plants exposed to cyanotoxins during mature growth stages and allowed to recover for two weeks, • statistical outliers, **x** mean value, minimum, 25th percentile, median, 75th percentile and maximum values.

3.4. Bioaccumulation and Persistence

3.4.1. Early Exposure

Early exposure of spinach plants to cyanotoxins showed that only MCLR was detected in the plant tissue extracts. Significant quantities of MCLR were detected in the root extracts and signs of translocation to the stems was observed, though relatively lower concentrations were found in this organ. Microcystin accumulation in the roots accounted for approximately 39ug of the 70ug applied. No CYN was detected in the spinach plants from the early exposure to toxins. Table 3-1 summarizes the bioaccumulation results for early exposure of spinach to CYN and MCLR.

Table 3-1. Bioaccumulation of MCLR and CYN in spinach plants exposed to toxins during early growth stages, ND none detected (n = 3 for roots and stems, n = 9 for leaves).

Spinach Early Exposure pg/g DW		
Organ	**MCLR**	**CYN**
Roots	490,415.2	ND
Stems	46.3	ND
Leaves	ND	ND

Alfalfa tissue extracts indicated there was accumulation of both CYN and MCLR in the toxin exposed plants. Accumulation of CYN in whole plant tissue ranged from 5.27 to 26.15 pg/g DW. Alfalfa exposed to MCLR during the early development stage also indicated a broad range of toxin concentrations ranging from 0.34 to 27.70 pg/g DW. Table 3-2 summarizes the accumulation of cyanotoxins in alfalfa exposed to CYN and MCLR during early development stages.

Table 3-2. Bioaccumulation of MCLR and CYN in alfalfa plants exposed to toxins during early growth stages, - no data, ND none detected (n = 30 for each group).

Alfalfa Early Exposure pg/g DW				
Group	**CYN-1**	**CYN-2**	**MCLR-1**	**MCLR-2**
1	ND	9.08	0.34	15.42
2	ND	5.27	27.70	2.54
3	5.57	26.15	0.82	3.90
4	-	13.88	-	-

3.4.2. Early Exposure Recovery

Spinach plants allowed to recover for one month from exposure to CYN and MCLR during early development showed decreased concentrations (0.16 pg/g DW) of MCLR in stems compared to plants that were analyzed immediately following the treatment with toxins. No MCLR was detected in root extracts after toxin exposure ceased. Spinach plants exposed to CYN had no detectable level of CYN in any plant organs. Table 3-3 summarizes toxin persistence results for the spinach early recovery data.

Table 3-3. Bioaccumulation of MCLR and CYN in spinach plants exposed to toxins during early growth stages after one-month recovery, ND none detected (n = 3 for roots, stems and seeds/pollen n = 9 for leaves).

Spinach Early Exposure Recovery pg/g DW		
Organ	**MCLR**	**CYN**
Roots	ND	ND
Stems	0.16	ND
Leaves	ND	ND
Seed/Pollen	ND	ND

Table 3-4 summarizes toxin persistence results in alfalfa plants following a one-week recovery period after toxin exposure ceased. In general, the concentrations of CYN and MCLR present in alfalfa tissue extracts decreased. Concentration of CYN in plant extracts was from below the detection limit to 9.83 pg/g DW. Alfalfa tissue analyzed for MCLR indicated toxin concentrations ranging from below the detection limit to 2.42 pg/g DW.

Table 3-4. Bioaccumulation of MCLR and CYN in alfalfa plants exposed to toxins during early growth stages after one-week recovery period, - no data, ND none detected (n = 30 for each group).

Alfalfa Early Exposure 1-Week Recovery pg/g DW				
Group	**CYN-1**	**CYN-2**	**MCLR-1**	**MCLR-2**
1	ND	1.30	2.42	ND
2	ND	ND	ND	ND
3	ND	9.83	1.25	-

3.4.3. Mature Exposure

Accumulation of CYN and MCLR in alfalfa plants exposed during mature development and reproductive growth stage indicated only MCLR was present in one group of plants exposed to toxins with a concentration of 7.11 pg/g DW. Table 3-5 summarizes the toxin accumulation results for alfalfa plants exposed to CYN and MCLR during the mature growth phase.

Table 3-5. Bioaccumulation of MCLR and CYN in alfalfa plants exposed to toxins during mature growth stages, - no data, ND none detected (n = 30 for each group).

Alfalfa Mature Exposure pg/g DW		
Group	**CYN-12**	**MCLR-22**
1	ND	7.11
2	ND	ND
3	ND	ND
4	-	ND

3.4.4. Mature Exposure Recovery

Alfalfa plant tissue analyzed for persistence of cyanotoxins at one-week recovery indicated CYN was present in tissue one-week after treatment with toxins was terminated. No MCLR was detected in treated plants after recovery period. Alfalfa tissue extracted after two weeks of recovery from toxin treatment indicated CYN was still detectable, although at decreased concentrations. Tables 3-6 and 3-7 summarize toxin persistence results for alfalfa plants treated with cyanotoxins during the mature growth stage.

Table 3-6. Bioaccumulation of MCLR and CYN in alfalfa plants exposed to toxins during mature growth stages after one-week recovery, - no data, ND none detected (n = 30 for each group).

Alfalfa Mature Exposure 1-Week Recovery pg/g DW		
Group	**CYN-22**	**MCLR-22**
1	13.50	ND
2	2.35	ND
3	3.91	ND
4	0.63	ND

Table 3-7. Bioaccumulation of MCLR and CYN in alfalfa plants exposed to toxins during mature growth stages after two-week recovery, - no data, ND none detected (n = 30 for each group).

Alfalfa Mature Exposure 2-Week Recovery pg/g DW		
Group	**CYN-22**	**MCLR-22**
1	3.33	ND
2	1.73	ND
3	3.34	ND
4	1.03	ND
5	-	1.74

3.5. Statistics

One-way ANOVA analysis was conducted on the germination data, growth of primary roots, growth and development of alfalfa and spinach after exposure to cyanotoxins during early growth and mature growth stages. Comparison of the results of plants treated with toxins vs. control groups was used to determine if significant differences were present. ANOVA analysis as performed with the Data Analyst tool in MS Excel with an α of 0.05.

4. Discussion

Results of the germination test indicated the successful germination for alfalfa and spinach was not negatively affected by the presence of toxins. Similar germination percentages were found for both species and for both toxin treatments (200 ug/L MCLR and 100 ug/L CYN) compared to the control treatment. Comparison of the results of this study and others indicate that there is variation in species sensitivity and concentration dependence on the effect of cyanotoxins on germination rates. Also, the ability of plants to recover from cyanotoxin exposure during germination seems to be species dependent.

Metcalf et al. (2004) indicated a decreased germination for tobacco exposed to pure CYN at concentrations above 5,000 ug/L, a level which is not commonly observed in the nature. Silva and Vasconcelos (2010) reported germination decreased only in tomatoes exposed to CYN extract concentrations of 0.57 to 57 ug/L. No difference in germination was found for beans, lettuce or peas exposed to the same CYN extract and concentrations (Silva and Vasconcelos, 2010). Conversely, peas were found to be the most sensitive species exposed to MCLR extract with decreased germination observed at concentrations of 1,600 ug/L MCLR equivalents and lentils were the most resilient (Saqrane et al, 2008). Peas were unable to recover from exposure and successfully germinate, while 40% of lentil seeds were able to germinate successfully after exposure to MCLR was terminated (Saqrane et al, 2008).

Chen et al. (2004) found no significant difference in germination of rice seeds exposed to a crude extract containing MC-RR, -LR, and -YR. However, rape seeds exposed to the extract at concentrations of 600 and 3000 ug/L MCLR equivalents had significantly lower germination (Chen et al, 2004). Germination of alfalfa seeds exposed to 5.0 ug/L MCLR extract did not show inhibition of germination; however, significant inhibition of primary root growth was observed

at this concentration (Pflugmacher et al, 2006).

Development and growth during exposure to MCLR and CYN can have both inhibitory and stimulating effects, indicating both concentration and species dependency. Alfalfa exposed to pure MCLR and a crude extract at concentrations of 5.0 ug/L showed significant decreased primary root growth after 7 days of exposure (Pflugmacher et al, 2006). Results from this study showed a significant increase in alfalfa primary root length exposed for 5 days to both pure CYN and MCLR, at concentrations of 100 and 200 ug/L, respectively. Figures 4.1 and 4.2 demonstrate the concentration dependency of alfalfa response to cyanotoxin exposure during germination. Differences in the sensitivity of alfalfa to MCLR may exhibit hormetic behavior with inhibition observed at low doses, stimulation observed at moderate doses and toxicity observed at higher doses.

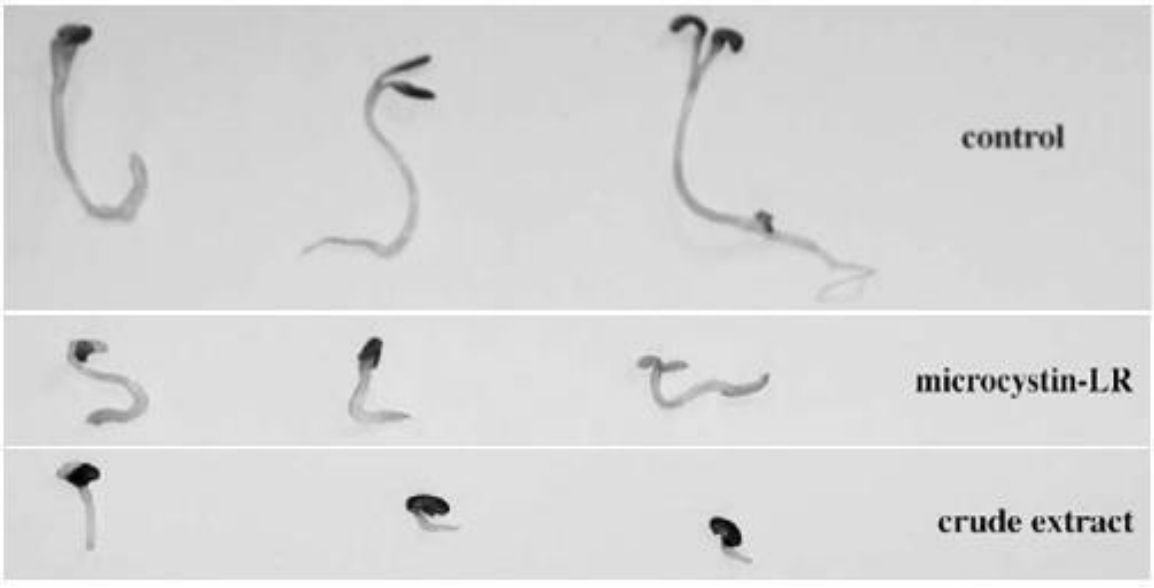


Figure 4.1. Seedling primary root growth for alfalfa exposed to 5.0 ug/L of MCLR and a cyanobacterial crude extract for seven days. (Pflugmacher et al, 2006)

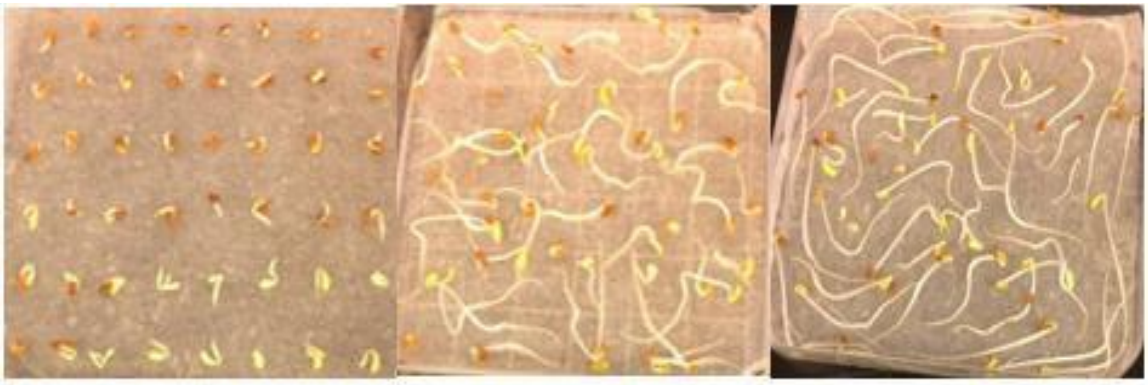


Figure 4.2. Seedling primary root growth for alfalfa exposed to 100 ug/L CYN, 200 ug/L MCLR, and control seeds.

Spinach primary root length was not statistically different from the control roots; however, mean root length was greater in spinach treated with toxins and more variable. Concentration dependency is also shown in the decreased primary root growth of corn, lentils, wheat and peas exposed to a crude extract of 11,600 ug/L MCLR (Saqrane et al, 2008). Differences in the results with use of extracts vs. pure toxins may be attributed to the presence of synergistic components of the extract or concentration sensitivity of the different species. Further, seed sex may also play a role in the plant response to toxin exposure.

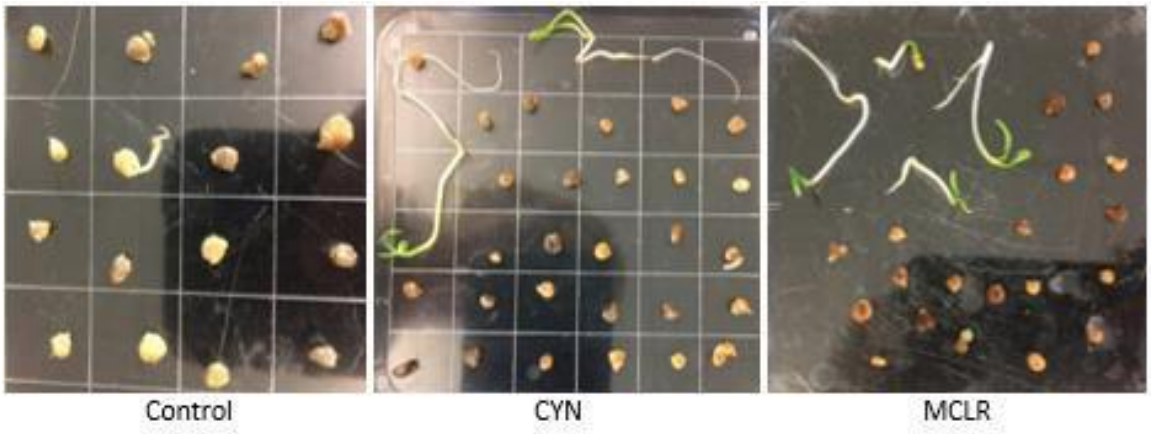


Figure 4.3. Seedling primary root growth for spinach seeds exposed to 100ug/L CYN, 200ug/L MCLR and control seeds.

Common growth factors used to indicate adverse effects of cyanotoxins on plants can be related to biomass or plant dimensions including, root length, stem length and leaf length and width. Results of this study show biomass production, after toxin exposure is affected not only by species and concentration, but that growth stage can impact the severity of the plants response to toxin exposure. Spinach plants exposed to CYN and MCLR during early growth stages exhibited increased biomass, particularly in the stem and leaves, consistent with Frietas et al. (2015). Plant stems and leaves reflected this increased biomass in the length and width of these organs.

Freitas et al. (2015) found that MCLR and a mixture of MCLR/CYN at concentrations between 1 and 10 ug/L stimulated root and leaf growth in lettuce, increasing biomass. Lettuce plants were at mature growth stages and exposed to toxins for five days, hydroponically.

Similarly, this study found alfalfa had increased biomass for plants exposed to MCLR and CYN during mature growth stages at concentrations of 200 and 100ug/L, respectively.

After ten days, the biomass of roots exposed to 100 ug/L MCLR/CYN were still significantly higher but the lower concentration treatments were not, indicating possible adaptation to the presence of low levels of toxins or a synergistic effect due to the presence of CYN and MCLR (Frietas et al, 2015). Lettuce leaf biomass indicated decreased biomass at 100 ug/L MCLR and MCLR/CYN mixture after 5 days and 100 ug/L CYN, at ten days (Frietas et al, 2015). In this study, alfalfa biomass when exposed to CYN decreased during early exposure trials and increased when exposed later in the growth cycle, consistent with lettuce, peas (Silva and Vasconselos, 2010) and carrots (Guzmán-Guillén et al, 2017). Recovery of alfalfa exposed early to toxins indicated increased biomass a week later, implying adaptation and recovery, or a delayed stimulating response to CYN.

Lettuce root length and stem lengths (vegetative growth stage) were reported to increase when exposed to 0.57 to 5.7 ug/L CYN extract (Silva and Vasconcelos, 2010). Even at the higher toxin concentrations used in this study, spinach plant dimensions were increased when exposed to CYN. Increased organ dimensions were continuous throughout the recovery period, implying the effects are significant and irreversible in spinach, further highlighting the concentration and growth stage dependency.

Findings by Pflugmacher et al (2007) indicated decreased growth for multiple spinach variants which was visually apparent after three weeks of exposure to 0.5 ug/L of a MCLR eq. crude extract. Results of the early exposure to pure CYN and MCLR in this study, at higher concentrations, are contrary to Pflugmacher et al (2007) as the growth was stimulated and increased biomass and organ dimensions were observed for both treatments. Differences in the treated vs. control spinach plants for results of this study and Pflugmacher et al. (2007) may be attributed to plant sex, concentration differences, and the use of crude extract vs. pure toxin.

Spinach plants treated with toxins in Pflugmacher et al (2006) may also indicate more developed reproductive structures and differences in plant sex responses in toxin treated plants compared to controls.

Plants ability to recover from an acute exposure to cyanotoxins has very limited data for comparison. As stated previously, germination of lentils was successful in 40% of seeds exposed to MCLR, however, peas were unsuccessful in recovery (Saqrane et al, 2008). Tomato seeds exposed to CYN did not survive the germination test performed by Silva and Vasconcelos (2010).

Results of the early exposure test indicated after the one-month recovery from toxin exposure, spinach plants had higher biomass, thus, the stimulating effect of the toxins seen

during exposure appeared to carry through the life cycle. Large variance in the responses indicate there may be differences in the actual amount of toxins available to plants due to subsurface processes including diffusion and sorption. Also, the control plants had undeveloped (MCLR) or under-developed (CYN) reproductive structures, implying the growth of these organs on the treated plants may have been premature as a result of the toxin induced stress.

Bioaccumulation and persistence of toxins in plant tissues was also investigated in this study. Relatively few studies in this area are available. Challenges of isolating targeted analytes in plant matrices can be attributed to signal suppression or amplification or false positive and negative results, depending on the analytical method used (Li et al, 2014; Diez-Quijada et al, 2018).

Varying results for bioaccumulation and persistence of CYN and MCLR in spinach and alfalfa plants were found in this study.

Spinach plants exposed to toxins in early growth phases had accumulated MCLR in the roots and, to a lesser extent, in the stems. Exposure to the toxin was through soil watering, indicating that spinach can translocate the toxin from roots to stems. Plants tested by Peuthert et al (2007) exhibited similar trends in root to shoot translocation of MCLR. Further, the idea that plants may be able to respond to toxin stress and eliminate the toxin from cells is implied by the decreased concentration in the stem of spinach after one-month of recovery.

Cylindrospermopsin has been shown to bioaccumulate in rice (Prieto et al, 2011), kale and mustard plants (Kittler et al, 2012). However, spinach plants in this experiment indicated CYN was undetected in any of the samples. Growth of spinach plants was increased, thus a stress response and secondary metabolism, or soil interactions may be limiting the availability and accumulation of CYN through the plant.

Results of this study also explored the effects of timing on alfalfa's response to toxin exposure. Early exposure to toxins had greater impacts on toxin accumulation when compared to the exposure during mature growth stages. Early exposure to CYN and MCLR had maximum accumulated toxin concentrations of 26.15 pg/g DW and 27.70 pg/g DW, respectively.

Conversely, with the mature exposure, CYN concentrations were below the detection limit and MCLR was 7.11 pg/g DW. This indicates the timing of exposure is an important factor to consider when determining adverse effects of using cyanotoxin contaminated water for irrigation of crops intended for human consumption.

With respect to the persistence of cyanotoxins in alfalfa after early exposure to toxins both CYN and MCLR had large reductions in the concentrations. After one week of recovery alfalfa plants had a peak CYN concentration of 9.83 pg/g DW, more than half the peak value found immediately following exposure. Reduction in MCLR concentrations was also observed after the recovery period, with the peak value of 2.42 pg/g DW, a ten-fold reduction from the initial peak concentration.

During the mature exposure experiment, MCLR treated plants showed the same trend in decreasing concentration of toxin present in plant material. Microcystin showed a decrease from the initial 7.11 pg/g DW to 1.74 pg/g after two weeks of recovery. Notably, the intermediate recovery period found MCLR was below the detection limit at one-week recovery, indicating there may be sorption/desorption processes within the soil affecting toxin availability.

Cylindrospermopsin accumulation during mature trails was dramatically different. Initially CYN concentrations in alfalfa were below the detection limit and peaked at 13.5 pg/g DW after one-week of recovery from toxin exposure. After two weeks, the concentrations decreased three-fold to 3.34 pg/g DW, further implying subsurface processes may have notable

influence on the bioaccumulation of cyanotoxins in agricultural plants and requires further investigation. In all cases, the amount of toxin accumulated within plants was less than 0.1ug of the total mass of toxins applied during each trial.

Determination of the risk to human health would require an estimated tolerable daily intake (TDI) for consumption of agricultural crops irrigated with cyanotoxin contaminated water. Estimated reference doses proposed by the EPA (2015) are based on previous studies that determined the No Observable Adverse Effects Level (NOAEL) or Lowest Observed Adverse Effects Level (LOAEL). Reference doses are used to estimate the health advisory concentrations and incorporate an uncertainty factor to account for interspecies and intraspecies variations, uncertainty in the database used to determine the dose and uncertainty when using LOAEL vs. NOAEL.

Reference doses for CYN is 0.1 ug/kg/day and MCLR is 0.05ug/kg/day (USEPA, 2015). Assuming only 20% of this dose would be exposure through consumption of plants, and an average body mass of 60 kg, a TDI for CYN is 3ug/day and for MCLR would be 0.6ug/day. Using the maximum accumulated values for alfalfa, one would approach the TDI after consuming only 20g/day for MCLR exposure and 110g/day for CYN exposure. Estimated mass for a typical serving for fruits and vegetables would be approximately 150g/serving. Therefore, the possibility of exceeding the TDI through consumption of contaminated crops does indicate a secondary exposure route that must be accounted for in determining risks to human health.

5.Conclusion

Findings from this study confirm exposure to cyanotoxins can produce physiological changes in spinach and alfalfa. Plant responses to toxin exposure indicate there is a risk to human health, through bioaccumulation. Changes are mediated by metabolic processes within the plant, as indicated by other authors, and can produce significant variation in morphology. Germination inhibition is species and concentration dependent; survival of seedlings after exposure to cyanotoxins is also species dependent. Development of plants may be stimulated or inhibited by exposure to CYN and MCLR. Increased growth rates for plants exposed to toxins can falsely indicate minimal risk to crops exposed to cyanotoxins, while chemical analysis provides confirmation of toxin accumulation in the plant material.

Timing of exposures and specific toxins present needs to be considered when determining the risk of using contaminated irrigation water. Water managers can use the results of this study to guide protocols used to protect health and environment from HABs. Irrigation water should be monitored as a pathway of contamination to food sources. Limiting irrigation during spiking toxin concentrations can limit bioaccumulation of toxins within plants. Performing chemical analyses on plants that have been exposed to cyanotoxins can also decrease the risk of contaminated food being consumed by the public. Decreasing nutrient loading to surface water, especially phosphorus, will help manage the environmental factors that promote bloom formation.

Further investigation into the interactions between soil environments and cyanotoxins is needed to understand the decomposition and bioavailability of cyanotoxins to agricultural crops. Further, the metabolic processes that may facilitate degradation within plants should be explored as it can further elucidate persistence and degradation of cyanotoxins.

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Appendix

A1. ANOVA p-values

A1.1. Germination

Table A1-1. One-way ANOVA p-values comparing germination rates between cyanotoxin treated spinach and alfalfa seeds and control seeds.

Species	Treatment	Germination Rate (%)	p-value
Spinach	Control	56	0.8042
	MCLR	60	
	CYN	60	
Alfalfa	Control	89	0.9105
	MCLR	88	
	CYN	87	

Table A1-2. One-way ANOVA p-values comparing primary root lengths between cyanotoxin treated spinach and alfalfa seeds and control seeds.

Species	Comparison pair	p-value
Spinach	MCLR v. control	0.0778
	CYN v. control	0.1304
Alfalfa	MCLR v. control	0.0000
	CYN v. control	0.0000

A1.2. Growth and Development A1.2.1. Early Exposure

Table A1-3. One-way ANOVA p-values between partitioned mass of spinach plants treated with toxins during early vegetative stages and control plants.

Species	Partitioning	Treatment	p-value
Spinach	Total mass	MCLR v. MCLR control	0.0171
	Root mass	MCLR v. MCLR control	0.0832
	Stem mass	MCLR v. MCLR control	0.0899
	Leaf mass	MCLR v. MCLR control	0.0031
Spinach	Total mass	CYN v. CYN control	0.2644
	Root mass	CYN v. CYN control	0.3786
	Stem mass	CYN v. CYN control	0.2825
	Leaf mass	CYN v. CYN control	0.2427

Table A1-4. One-way ANOVA p-values comparing plant dimensions of spinach plants treated with toxins during early vegetative stages and control plants.

Species	Partitioning	Treatment	p-value
Spinach	Root Length	MCLR 2 v. Control	0.1465
	stem length	MCLR 2 v. Control	0.1233
	leaf length	MCLR 2 v. Control	0.0012
	leaf width	MCLR 2 v. Control	0.0019
Spinach	Root Length	CYN 1 v. Control	0.4647
	stem length	CYN 1 v. Control	0.0836
	leaf length	CYN 1 v. Control	0.0004
	leaf width	CYN 1 v. Control	0.0044

Table A1-5. One-way ANOVA p-values comparing averaged alfalfa biomass for plants treated with toxins during early vegetative stages and control plants.

Species	Treatment	p-value
Alfalfa	MCLR-1 v. Control	0.0003
	MCLR-2 v. Control	0.0001
Alfalfa	CYN-1 v. Control	0.3596
	CYN-2 v. Control	0.1757

A1.2.2. Mature Exposure

Table A1-6. One-way ANOVA comparing averaged alfalfa biomass for plants treated with toxins during mature growth and reproductive stages and control plants.

Species	Treatment	p-value
Alfalfa	MCLR-22 v. Control 22	0.0251
	MCLR-22 v. Control 32	0.6429
Alfalfa	CYN-12 v. Control 22	0.0001
	CYN-12 v. Control 32	0.0003

A1.3. Recovery

A1.3.1. Early Exposure

Table A1-7. One-way ANOVA p-values between partitioned mass of spinach plants treated with toxins during early vegetative stages and control plants after a one-month recovery period.

Species	Partitioning	Treatment	p-value
Spinach	Total mass	MCLR v. MCLR control	0.9305
	Root mass	MCLR v. MCLR control	0.9070
	Stem mass	MCLR v. MCLR control	0.4503
	Leaf mass	MCLR v. MCLR control	0.6203
	Seed and Pollen	MCLR v. MCLR control	0.0134
Spinach	Total mass	CYN v. CYN control	0.2786
	Root mass	CYN v. CYN control	0.3749
	Stem mass	CYN v. CYN control	0.2711
	Leaf mass	CYN v. CYN control	0.3242
	Seed and Pollen	CYN v. CYN control	0.0943

Table A1-8. One-way ANOVA p-values comparing averaged alfalfa biomass for plants treated with toxins during early vegetative stages and control plants after a one- week recovery period.

Species	Treatment	p-value
Alfalfa	MCLR-1 v. Control	0.0003
	MCLR-2 v. Control	0.0000
Alfalfa	CYN-1 v. Control	0.5781
	CYN-2 v. Control	0.4192

A1.3.2. Mature Exposure

Table A1-9. One-way ANOVA comparing averaged alfalfa biomass for plants treated with toxins during mature growth and reproductive stages and control plants after a one-week recovery period.

Species	Treatment	p-value
Alfalfa	MCLR-12 v. Control 42	0.0000
	MCLR-12 v. Control 12	0.0228
Alfalfa	CYN-22 v. Control 42	0.0819
	CYN-22 v. Control 12	0.7873

Table A1-10. One-way ANOVA comparing averaged alfalfa biomass for plants treated with toxins during mature growth and reproductive stages and control plants after a two-week recovery period.

Species	Treatment	p-value
Alfalfa	MCLR-12 v. Control 42	0.0019
	MCLR-12 v. Control 12	0.0278
Alfalfa	CYN-22 v. Control 42	0.0109
	CYN-22 v. Control 12	0.2919

A2. Photographs of Plant Growth and Recovery

Figure A2.1. Spinach plants after early exposure to 100 ug/L CYN and 200 ug/L MCLR and control plants. Plants are 4 weeks from germination.

Figure A2.2. Spinach plants after 1-month recovery period from exposure to CYN and control plants.

Figure A2.3. Control plants for MCLR recovery period. Plants at 8 weeks from germination.

Figure A2.4. Spinach at 8 weeks after 1-month recovery period from MCLR exposure.

Figure A2.5. Alfalfa Plants after exposure to CYN and MCLR during early vegetative stages.

Figure A2.6. Alfalfa plants after 1-week recovery period from exposure to CYN and MCLR during early vegetative stages.

Figure A2.7. Alfalfa plants after exposure to CYN and MCLR during mature growth stages.

A3. LC/MS Analysis Data

Spinach

Sample	Harvest Date	Treatment	Mass (g)	Plant #	Plant Organ	Trial	Calculated Concentration pg/g DW
1	6/16/2017	cyn-control-1	0.0125	1,2	roots	1	263.42
2	6/16/2017	cyn-control-1	0.0236	1,2	stems	1	275.37
3	6/16/2017	cyn-control-1	0.0800	1,2	leaves	1	141.37
4	6/16/2017	mclr-control-1	0.0222	1,2	roots	1	1775.42
5	6/16/2017	mclr-control-1	0.0574	1,2	stems	1	106.08
6	6/16/2017	mclr-control-1	0.1286	1,2	leaves	1	110.58
7	6/16/2017	MCLR-2	0.0800	1,2,3	roots	1	492190.65
8	6/16/2017	MCLR-2	0.3100	1,2,3	stems	1	152.33
9	6/16/2017	MCLR-2	0.5200	1,2,3	leaves	1	70.85
10	6/16/2017	CYN-1	0.0598	1,2,3	roots	1	85.94
11	6/16/2017	CYN-1	0.2177	1,2,3	stems	1	19.86
12	6/16/2017	CYN-1	0.3759	1,2,3	leaves	1	15.05
13	7/12/2017	cyn-control-2	0.1980	1,2,3	roots	2	12.94
14	7/12/2017	cyn-control-2	1.7300	1,2,3	stem	2	6.02
15	7/12/2017	cyn-control-2	1.9685	1,2,3	leaves	2	10.55
19	7/12/2017	cyn-control-2	0.1108	1,2	seed,pollen	2	76.47
17	7/12/2017	mclr-control-2	2.3799	1,2	stem	2	0.45
18	7/12/2017	mclr-control-2	2.6208	1,2	leaves	2	1.22
16	7/12/2017	mclr-control-2	0.2733	1,2,3	roots	2	29.66
29	7/12/2017	MCLR-1	0.5519	1,2,3	roots	2	8.16
30	7/12/2017	MCLR-1	5.8914	1,2,3	stem	2	0.61
31	7/12/2017	MCLR-1	6.3900	1,2,3	leaves	2	-0.10
32	7/12/2017	MCLR-1	3.3480	1,2,3	seed,pollen	2	2.35
33	7/12/2017	CYN-2	0.7531	1,2,3	roots	2	12.67
34	7/12/2017	CYN-2	2.5582	1,2,3	seed,pollen	2	6.43
35	7/12/2017	CYN-2	3.9029	1,2,3	stem	2	0.58
36	7/12/2017	CYN-2	2.3348	1,2,3	leaves	2	1.84

Alfalfa

Sample #	Harvest Date	Treatment	Mass (g)	# of plants	Group #	trial	Calculated Concentration pg/g DW
20	6/19/2017	CYN-control	0.6261	30	1	1	22.00
21	6/19/2017	CYN-control	0.7030	30	2	1	17.55
22	6/19/2017	CYN-control	0.5628	30	3	1	17.16
39	6/19/2017	CYN-1	0.9474	30	1	1	17.75
40	6/19/2017	CYN-1	1.0028	30	2	1	18.89
41	6/19/2017	CYN-1	0.7944	30	3	1	24.47
44	6/19/2017	CYN-2	0.6105	30	1	1	27.98
45	6/19/2017	CYN-2	0.7511	30	2	1	24.18
46	6/19/2017	CYN-2	0.5670	30	3	1	45.05
47	6/19/2017	CYN-2	0.6812	30	4	1	32.78
23	6/19/2017	MCLR-control	0.2837	30	1	1	46.43
24	6/19/2017	MCLR-control	0.2551	30	2	1	18.89
25	6/19/2017	MCLR-1	0.5770	30	1	1	33.00
26	6/19/2017	MCLR-1	0.4923	30	2	1	60.36
27	6/19/2017	MCLR-1	0.7193	30	3	1	33.48
53	6/19/2017	MCLR-2	0.7486	30	1	1	48.08
54	6/19/2017	MCLR-2	0.6881	30	2	1	35.20
55	6/19/2017	MCLR-2	0.9955	30	3	1	36.57
89	6/27/2017	CYN-control	2.2726	30	1	recovery	8.55
90	6/27/2017	CYN-control	3.0606	30	2	recovery	5.96
91	6/27/2017	CYN-control	3.4941	30	3	recovery	3.92
93	6/27/2017	CYN-1	3.8053	30	1	recovery	4.46
94	6/27/2017	CYN-1	2.6956	30	2	recovery	3.58
95	6/27/2017	CYN-1	3.5083	30	3	recovery	3.34
100	6/27/2017	CYN-2	2.5344	30	4	recovery	7.44
98	6/27/2017	CYN-2	2.7975	30	2	recovery	6.11
99	6/27/2017	CYN-2	2.0434	30	3	recovery	15.97
58	6/27/2017	MCLR-control	1.2820	30	1	recovery	4.56
59	6/27/2017	MCLR-control	1.5924	30	2	recovery	0.56
60	6/27/2017	MCLR-1	1.7872	30	1	recovery	4.98
61	6/27/2017	MCLR-1	2.1610	30	2	recovery	0.71
62	6/27/2017	MCLR-1	1.7911	30	3	recovery	3.81
65	6/27/2017	MCLR-2	3.4372	30	1	recovery	2.47
67	6/27/2017	MCLR-2	3.4294	23	3	recovery	1.76
81	6/29/2017	MCLR-22	1.8430	30	4	2	14.60

	6/29/2017	MCLR-22	1.8928	30	3	2	2.87
79	6/29/2017	MCLR-22	2.6023	30	2	2	1.58
78	6/29/2017	MCLR-22	1.7533	30	1	2	0.15
74	6/29/2017	CYN-12	4.9003	30	3	2	3.27
73	6/29/2017	CYN-12	3.7591	30	2	2	4.29
72	6/29/2017	CYN-12	3.5828	30	1	2	4.41
68	6/29/2017	Control-22	1.4636	30	1	2	1.81
69	6/29/2017	Control-22	1.6621	30	2	2	9.33
70	6/29/2017	Control-32	2.9803	30	1	2	8.40
71	6/29/2017	Control-32	1.8013	30	2	2	10.43
102	7/5/2017	Control 4-2	1.5356	30		2-recovery	1.78
103	7/5/2017	Control 4-2	1.8421	30		2-recovery	6.37
104	7/5/2017	Control 1-2	4.9559	0	1	2-recovery	2.86
105	7/5/2017	Control 1-2	2.9616	30	2	2-recovery	4.64
110	7/5/2017	CYN-22	1.2236	30		2-recovery	17.41
111	7/5/2017	CYN-22	2.7477	30		2-recovery	6.26
112	7/5/2017	CYN-22	2.9429	30		2-recovery	7.82
113	7/5/2017	CYN-22	2.7369	30		2-recovery	4.54
106	7/5/2017	MCLR-12	5.5880	30		2-recovery	1.34
107	7/5/2017	MCLR-12	5.4001	30		2-recovery	1.05
108	7/5/2017	MCLR-12	3.4001	30		2-recovery	2.83
109	7/5/2017	MCLR-12	1.7416	30		2-recovery	3.66
114	7/11/2017	Control-12	3.8398	30	1	2-recovery	2.00
115	7/11/2017	Contol-12	4.1173	30	2	2-recovery	4.04
116	7/11/2017	Control-42	2.7870	30	1	2-recovery	2.71
117	7/11/2017	Control-42	1.8761	30	2	2-recovery	1.67
123	7/11/2017	CYN-22	3.6344	30	1	2-recovery	5.93
124	7/11/2017	CYN-22	4.1266	30	2	2-recovery	4.34
125	7/11/2017	CYN-22	4.2717	30	3	2-recovery	5.94
126	7/11/2017	CYN-22	4.2323	30	4	2-recovery	3.64
118	7/11/2017	MCLR-12	6.7666	30	1	2-recovery	1.08
119	7/11/2017	MCLR-12	6.3943	30	2	2-recovery	0.19
120	7/11/2017	MCLR-12	3.3330	30	3	2-recovery	0.23
121	7/11/2017	MCLR-12	5.1701	30	4	2-recovery	1.23
122	7/11/2017	MCLR-12	4.5890	30	5	2-recovery	4.34

Cylindrospermopsin

Replicate Name	Sample Name	Peptide	Total Area	Average Measured Retention Time	Isotope Label Type	Sample Type	Calculated Concentration pg/mL
14_247	5 pg/ml	CYN	7265	1.75	light	Standard	5.45
14_248	50 pg/ml	CYN	45322	1.75	light	Standard	49.95
14_111	500 pg/ml	CYN	424340	1.75	light	Quality Control	493.18
14_122	500 pg/ml	CYN	431361	1.75	light	Quality Control	501.39
14_133	500 pg/ml	CYN	424583	1.75	light	Quality Control	493.47
14_144	500 pg/ml	CYN	440144	1.75	light	Quality Control	511.66
14_155	500 pg/ml	CYN	465300	1.75	light	Quality Control	541.08
14_166	500 pg/ml	CYN	422906	1.75	light	Quality Control	491.51
14_177	500 pg/ml	CYN	433196	1.75	light	Quality Control	503.54
14_188	500 pg/ml	CYN	388605	1.75	light	Quality Control	451.39
14_199	500 pg/ml	CYN	428618	1.75	light	Quality Control	498.19
14_213	500 pg/ml	CYN	424520	1.75	light	Quality Control	493.39
14_216	500 pg/ml	CYN	478598	1.75	light	Quality Control	556.63
14_227	500 pg/ml	CYN	443408	1.75	light	Quality Control	515.48
14_240	500 pg/ml	CYN	419445	1.75	light	Quality Control	487.46
14_249	500 pg/mL	CYN	403174	1.75	light	Standard	468.43
14_113	BLANK	CYN	2902	1.75	light	Blank	0.35
14_124	BLANK	CYN	3331	1.75	light	Blank	0.85
14_135	BLANK	CYN	2869	1.75	light	Blank	0.31
14_146	BLANK	CYN	3270	1.75	light	Blank	0.78
14_157	BLANK	CYN	3049	1.75	light	Blank	0.52
14_168	BLANK	CYN	5886	1.75	light	Blank	3.84
14_179	BLANK	CYN	8972	1.75	light	Blank	7.45
14_190	BLANK	CYN	2746	1.75	light	Blank	0.17
14_201	BLANK	CYN	1878	1.75	light	Blank	-0.85
14_215	BLANK	CYN	4123	1.75	light	Blank	1.78
14_218	BLANK	CYN	2780	1.75	light	Blank	0.21
14_229	BLANK	CYN	1363	1.75	light	Blank	-1.45

14_242	BLANK	CYN	1863	1.75	light	Blank	-0.87
14_243	BLANK	CYN	647	1.75	light	Blank	-2.29
14_112	QC 1	CYN	120407	1.75	light	Quality Control	137.76
14_123	QC 1	CYN	155744	1.75	light	Quality Control	179.08
14_134	QC 1	CYN	221936	1.75	light	Quality Control	256.49
14_145	QC 1	CYN	322452	1.75	light	Quality Control	374.03
14_156	QC 1	CYN	215655	1.75	light	Quality Control	249.14
14_167	QC 1	CYN	170146	1.75	light	Quality Control	195.93
14_178	QC 1	CYN	194533	1.75	light	Quality Control	224.44
14_189	QC 1	CYN	262018	1.75	light	Quality Control	303.36
14_200	QC 1	CYN	240323	1.75	light	Quality Control	277.99
14_214	QC 1	CYN	204194	1.75	light	Quality Control	235.74
14_217	QC 10	CYN	224798	1.75	light	Quality Control	259.84
14_228	QC 10	CYN	282754	1.75	light	Quality Control	327.61
14_241	QC 10	CYN	246069	1.75	light	Quality Control	284.71
14_244	Water Blank	CYN	2958	1.75	light	Standard	0.41
14_245	Water Blank	CYN	2368	1.75	light	Standard	-0.28
14_246	Water Blank	CYN	956	1.75	light	Standard	-1.93

Microcystin

Replicate Name	Sample Name	Peptide	Total Area	Average Measured Retention Time	Isotope Label Type	Sample Type	Calculated concentration pg/ml
14_111	500 pg/ml	MCLR	174059	3.59	light	Quality Control	928.6538
14_122	500 pg/ml	MCLR	121668	3.59	light	Quality Control	644.9532
14_133	500 pg/ml	MCLR	101451	3.59	light	Quality Control	535.4768
14_144	500 pg/ml	MCLR	106100	3.59	light	Quality Control	560.6514
14_155	500 pg/ml	MCLR	88973	3.59	light	Quality Control	467.9076
14_166	500 pg/ml	MCLR	94540	3.59	light	Quality Control	498.0533
14_177	500 pg/ml	MCLR	102910	3.59	light	Quality Control	543.3774
14_188	500 pg/ml	MCLR	109849	3.59	light	Quality Control	580.9525
14_199	500 pg/ml	MCLR	99668	3.59	light	Quality Control	525.8217
14_213	500 pg/ml	MCLR	103012	3.59	light	Quality Control	543.9297
14_216	500 pg/ml	MCLR	159741	3.59	light	Quality Control	851.1209
14_227	500 pg/ml	MCLR	126278	3.59	light	Quality Control	669.9166
14_240	500 pg/ml	MCLR	134403	3.59	light	Quality Control	713.914
14_112	QC 1	MCLR	2623502	3.59	light	Quality Control	14192.55
14_123	QC 1	MCLR	2583677	3.59	light	Quality Control	13976.89
14_134	QC 1	MCLR	2750869	3.59	light	Quality Control	14882.25

14_145	QC 1	MCLR	2153951	3.59	light	Quality Control	11649.9
14_156	QC 1	MCLR	3008202	3.59	light	Quality Control	16275.72
14_167	QC 1	MCLR	2878684	3.59	light	Quality Control	15574.37
14_178	QC 1	MCLR	3158856	3.59	light	Quality Control	17091.52
14_189	QC 1	MCLR	2442940	3.59	light	Quality Control	13214.79
14_200		MCLR	2457420	3.59	light	Quality Control	13293.2
14_214	QC 1	MCLR	3223215	3.59	light	Quality Control	17440.03
14_217	QC 10	MCLR	4055434	3.59	light	Quality Control	21946.55
14_228	QC 10	MCLR	2900237	3.59	light	Quality Control	15691.08
14_241	QC 10	MCLR	3814745	3.59	light	Quality Control	20643.2
14_244	Water Blank	MCLR	2627	3.59	light	Standard	0.338442
14_245	Water Blank	MCLR	2330	3.59	light	Standard	-1.26983
14_246	Water Blank	MCLR	2699	3.59	light	Standard	0.728326
14_247	5 pg/ml	MCLR	3472	3.59	light	Standard	4.914171
14_248	50 pg/ml	MCLR	11857	3.59	light	Standard	50.31949
14_249	500 pg/mL	MCLR	94894	3.59	light	Standard	499.9702