Occurrence and Evaluation of Methods for Microcystins in Water Treatment Plant Residuals

Thesis

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Abstract

Microcystins (MCs), a category of cyanotoxins produced by some species of the photosynthetic heterotrophic cyanobacteria, are the most studied, most associated with toxicity-related events, and most monitored in water. Water treatment plant (WTP) residuals are the solid byproduct of water treatment and consist of solids removed from physical and chemical processes and have been shown to contain cyanobacterial cells removed by conventional treatment processes. However, MCs in WTP residuals are not well understood. Although quantification methods of MCs for water samples have been adapted for solid matrices, their suitability for understanding the level of microcystins within a WTP residual sample is not well understood. Additionally, due to the inherent high variability within WTP residuals across the United States, these methods may not be optimal for every sample. Extraction and quantification methods were investigated to assess MCs in varied WTP residuals. Additionally, natural degradation observed in a utility's storage lagoon and was investigated to determine the impact of physical, chemical, and biological treatments on MC concentration in high-biomass residuals. This study demonstrates that residuals of various characteristics across the United States contain MCs, and that in some sample's extraction method (particularly methanol) and quantification method (particularly UPLC-PDA) increased measured MC, no single methodology is optimal for extracting and quantifying MCs across various residuals.

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Chapter 1: Background and Detailed Literature Review

1.1 Microcystins

Cyanotoxins are toxic metabolites produced by some species of photosynthetic heterotrophic organisms, cyanobacteria. There are several categories of cyanotoxins such as skin irritants, hepatotoxins, and neurotoxins, which can all present a serious human health hazard. Microcystins (MCs), a category of cyanotoxins, are the most studied, most associated with toxicity-related events, and most monitored in water (World Health Organization, 2003).

MCs are heavy, approximately 1000 g/mol, chemically stable, cyclical heptapeptides (World Health Organization, 2003). This chemical is both hydrophobic and highly soluble with polar properties. These contradicting characteristics of microcystins are structurally based. Two protein amino acids located within the structure possess polar properties, while the tail end of the structure, referred to as the ADDA component or backbone, is hydrophobic. All microcystins have these properties; there are over 50 different congeners which are classified by two variable amino acids on the chemical structure. The first congener discovered, and the most used in research applications, is microcystin-LR (MC-LR) which contains the variable amino acids leucine and arginine (Honkanen et al., 1990). MC-LR possesses two freely ionizable carboxylic acid groups and one freely ionizable amino group which contribute to sorption characteristics (Wu et al., 2011). In natural pH, microcystins typically have a negative charge (Maghsoudi, Prévost, et al., 2015). Two other commonly studied congeners are microcystin-RR (MC-RR), which contains two arginine amino acids, and microcystin-LA (MC-LA), which contains leucine and alanine amino acids.

1.2 Water Treatment Plant Residuals

WTP residuals are the solid byproduct of water treatment processes. Solids are removed from the flocculation, sedimentation, and additive processes in the treatment train and often dewatered before disposal. It is important to note that WTP residuals have a wide variance in terms of their composition (Turner et al., 2019). This variance is affected by geographic location, specific processes within the associated treatment train, and source water quality to name a few. Residuals are composed of the materials, solids, and biota removed from the raw water entering the treatment plant. They can also contain solid byproducts from chemically additive processes such as lime for softening or powered activated carbon (PAC) (Turner et al., 2019).

Both cyanobacteria and their toxins are becoming an increasingly prevalent issue in source waters, recreational waters, and water treatment plants (WTP's). The toxins are expensive to remove in plant, and often require advanced treatment technologies to combat them such as granular activated carbon (GAC) or ultraviolet-advanced oxidation process (UV-AOP). Cyanobacteria provide their own treatment challenges as well, as these organisms can travel vertically throughout the water column due to the gas vesicle components of the cells, and thus do not settle out by gravity like bacteria, organics, or sediments. Dissolved air floatation (DAF) has been shown to be more effective at removing cyanobacteria cells, as supersaturated air forces solids to the top of the water column where they are then skimmed off. Removal of the physical cyanobacteria cells, while easier than cyanotoxin removal, may result in cyanobacteria entering the waste stream and thus included in WTP residuals. Presence of cyanobacteria cells in WTP residuals have been shown to occur in various studies (Jalili et al., 2021a; Jalili, Moradinejad, et al., 2022; Pestana et al., 2016; Xu et al., 2017). Residuals reuse or recycling is an option for many utilities. However, cyanobacteria present in residuals could present a public health and

safety issue or impact a utility's ability to participate in beneficial reuse if cyanotoxins are present alongside the organisms.

1.2.1 Composition

In general, WTP residuals primarily contain clays, humics, microorganisms, and chemical coagulants (Wang et al., 1992). Approximately 70% of WTP's in the United States utilize chemical coagulation, often in the first steps of the treatment process (Turner et al., 2019), and these chemicals can impact the behavior and the quality of the residuals themselves.

1.2.1.1 Water Content

Residuals can typically be described by a solid phase and a water phase. The water phase contains a high amount of water, and typically contains 5 – 15% solids (Wang et al., 1992). This phase of residuals has four main categories described by Basim in 1999 including free water that surrounds particles, floc water that is trapped within a particle but can travel freely throughout, water associated to a particle's surface through surface tension, and adsorbed water within a particle's molecular structure. Each of these four water categories provides opportunity for reactions, mobility, and sorption inside and outside particles. The solid phase, once dewatered, behaves like granular materials and soils (Basim, 1999). Clays, aluminosilicates, and organics such as humics are the typical solids categories found in residuals as well as some trace metals. These components are usually negatively charged and thus will play a part in electrostatic sorption of positively charged particles and ions.

1.2.1.2 Coagulants

Coagulants work by way of electrostatic adsorption, the positively charged salts are attracted to the typically negative sediments and colloids in the influent water. When enough of

the coagulant ions have sorbed to the negatively charged particle, it results in an overall positive charge and thus attracts other negatively charged sediments, creating flocs. This phenomenon is known as the electrostatic double layer (EDL) and was modeled by Gouy and Chapman, and eventually improved upon by others, (Maurice, 2009) explaining colloid and particle stabilization. There are three main groups of coagulants; ferric-based, aluminum-based, and lime-based (Basim, 1999). Aluminum sulfate, referred to as alum, is the most used coagulant in water treatment applications. Aluminum can become amorphous aluminum hydroxide, which can be a sorbent for any trace metals or ligands in solution (Wang et al., 1992). Ferric based coagulants can also react to become iron (III) hydroxide. Both hydroxides are highly reactive, hydrophilic, and contribute to the high-water content in residuals as well as trace metal sorption. Lime, or calcium hydroxide, is another additive that is used for both coagulation, softening, and increasing pH (United States Environmental Protection Agency, 2011). These coagulants can react with or sorb to the various organic and inorganic particles and colloids present in WTP residuals, creating complexations or performing ion exchange. Interactions are dependent on the composition of the raw water, and can impact the structure, stability, and makeup of residuals.

1.2.1.3 Natural Organic Matter

Natural organic matter (NOM) is another solid that is present in aquatic environments and is widely studied as it encompasses many different chemical compounds and structures and varies widely in composition, much like WTP residuals. NOM consists of a mixture of organics, typically from the decay of living material (Adusei-Gyamfi et al., 2019). NOM can form complexes with free metal cations present. NOM also impacts disinfection byproduct (DBP) formation by reacting with oxidants used in treatment, creating potentially carcinogenic compounds (United States Environmental Protection Agency, 2011). Additionally, NOM

impacts toxin adsorption, either by physically blocking sites or competing with toxins for sorption on to sites and can provide lots of challenges with cyanotoxins removal when the NOM concentration is high (Dixit et al., 2018a). These interactions warrant NOM removal during the initial phases of water treatment, typically during coagulation and flocculation processes, resulting in NOM content being present in WTP residuals.

1.2.1.4 Metals

In addition to the metal salts utilized in coagulation, metals are often found in WTP residuals, as they can be present in source water from erosion, runoff, and industrial processes (United States Environmental Protection Agency, 2011). Metal cations have the potential to form precipitates or complexations with natural organic matter in mixtures such as source waters and residuals, impacting not only the physical behavior of residuals, but also the chemical content and toxicity. The United States Environmental Protection Agency (USEPA) requires WTP's to remove metals from drinking water, and monitors metals content in residuals prior to disposal. The main metals monitored in residuals are aluminum, iron, calcium, copper, cadmium, and arsenic.

1.2.1.5 Bacteria

Heterotrophic bacteria can often be found in WTP residuals, as they are typically removed during the WTP process through coagulation and sedimentation. Populations and cell abundances vary depending on location and water type. Bacteria present in WTP residuals impact the overall chemical structure, contributing not only to the total carbon and organics due to cellular makeup, but also play a part in degrading other compounds present. A study performed by (Xu et al., 2018) analyzed the biodiversity via DNA sequencing of WTP residuals

samples from similar WTP's in China. These WTP's had varying source waters including rivers, reservoirs, and lakes, some of which were surrounded by agricultural communities. Each sample displayed different levels of abundance, but in general at the phyla level all samples contained *Proteobacteria*, *Cyanobacteria*, *Bacteroidetes*, *Firmicutes*, *Verrucomicrobia*, *Planctomycetes*, and *Actinobacteria*. The authors noted that no two samples demonstrated the same abundance across any organisms, which continues to speak to the point of the diversity amongst WTP residuals themselves.

1.2.1.6 Cyanotoxins

Few studies have examined WTP residuals and MCs interactions, and often these investigations include cyanobacteria and other cyanotoxins. In these studies, residuals are often obtained by simulation of WTP processes at bench top, including coagulation, flocculation, and filtration, where the byproducts were collected (Ho, Dreyfus, et al., 2012; Pinkanjananavee et al., 2021). Laboratory cells were introduced in the bench-top WTP simulation, including *Anabaena* (producing saxitoxin) and *Cylindrospermopsis* (producing cylindrospermopsin) (Ho, Dreyfus, et al., 2012) as well as *Microcystis aeruginosa* (Pinkanjananavee et al., 2021), with the focus on cell viability within residuals recycled back in to the WTP. Results indicated that recycling of residuals within the plant could be a hazard during a toxin-producing cyanobacterial bloom as cells remained viable and continued to produce toxins through the treatment train. It was also demonstrated that mechanical dewatering processes of residuals specifically could impact the release of intracellular MC-LR. A higher concentration of MC-LR was found in retained solids (Pinkanjananavee et al., 2021), and similarly noticed by Almuhtaram *et al.* (2018) who observed higher concentrations in clarifier sludge compared to WTP intake in full scale systems.

Understanding MCs in residuals could be especially impactful for utilities that participate in beneficial reuse programs that land apply residuals. Studies analyzing crop uptake and potential cyanobacteria population growth in the presence of MCs note bioaccumulation throughout the plant regardless of MC exposure method; MC-laden WTP residuals applied to soil or watered with MC containing water. It was found that less than 5% of the MC accumulated within root vegetables, with the majority present in the non-edible portions (Ai et al., 2020) whereas crops grown above ground were shown to bioaccumulate throughout the plant with a combined 36% of applied MC residing in the roots and shoots (Bouaïcha & Corbel, 2016; Peuthert et al., 2007). Most of the MC from the land-applied experiment remained in the soil (Ai et al., 2020) whereas concentration of MC in soil was not reported in the water-focused studies. Additionally, these studies fail to mention the probability of toxin accumulation through adsorption phenomena as a contributing factor to the toxins not bioaccumulated. Similarly, there was no mention of measurement or manipulation of pH within the soils used. Because several cyanotoxins have pH dependent partitioning, addition of residuals containing lime and alum on to soil would decrease the pH to some effect depending on the chemical concentration, which could confound MC measurements in this study. Peuthert et al. (2007) did note that typical trends of MC hydrophobicity were not consistent with results and attributed this difference to the presence of lipophilic compounds from cellular extracts used for toxins. It has been noted in other studies that physical processes, such as electrostatic attraction, are reversible and could impact MC adsorption on sediments (Wu et al., 2011). Authors of these vegetative uptake studies agree that more extensive research should be performed in this area to better understand the impacts, fate, and transport of cyanotoxins applied to agricultural crops. Other components found in WTP's, both biological and chemical, could also heavily impact MC content within residuals,

and since these components can be so variable on a WTP basis, it is challenging to understand the interactions occurring that may affect MC fate or transport in residuals samples.

Viability of cyanobacteria in WTP residuals, even after long term storage, and the organism's ability to continue to synthesize toxins has been previously discussed in literature (Almuhtaram et al., 2018; Jalili et al., 2021b; Jalili, Moradinejad, et al., 2022; Jalili, Trigui, et al., 2022; Ma et al., 2016; Zamyadi et al., 2013). While these studies demonstrate a need for a better understanding of the cyanotoxin content in solids, none of these studies looked at a variety of residual types or systematically explored extraction and quantification of MCs. Therefore, no investigation on the prevalence of MCs in environmental residuals, or extraction of MCs in said residuals, has been done to the best of the author's knowledge. Evidence of cyanotoxins present in WTP residuals has been shown in various studies (Jalili et al., 2021a; Jalili, Moradinejad, et al., 2022; Pestana et al., 2016; Xu et al., 2017) yet many regions of the United States do not monitor for MC in these materials. Additionally, extraction and quantification in the evidence-based studies often include multiple methods. Due to the highly variable and complex nature of WTP residuals, proper extraction and quantification methods should be implemented when investigating MC concentrations in residuals.

1.2.2 Current Management Considerations

Residuals content is important when it comes to management strategies. WTP residuals are generally disposed of at a landfill or through incineration. However, some states have begun implementing beneficial reuse programs as an alternative disposal method, in which the residuals can be land applied on fields or crops, if chemical levels are below regulatory limits. A review of various studies that investigated the impacts of land applied WTP residuals on crops and soil

found that residuals affected soil pH and structure (Ahmad et al., 2016) due to the byproducts' contents. Soil pH can be improved using residuals with high lime concentration, instead of using the typical practice of adding limestone. Soil structure and aggregation can be improved with the addition of either alum or ferric residuals, as these components assist in the flocculation of soil particles. While some studies found benefits to land application, there were many that reported adverse effects. This can be attributed to the heterogenous biochemical composition of WTP residuals and is evidence that reuse of this material is location and situationally dependent for both the needs of the soil and the residuals themselves (Ahmad et al., 2016; Hou et al., 2018; Kopacek et al., 2005).

1.3 Microcystin Treatment Mechanisms

1.3.1 Adsorption

Microcystin adsorption has been studied beyond the analytical chemistry of the partitioning coefficient and is one of the primary methods of MC removal (Song et al., 2014). As MCs become a more prevalent issue in water treatment, it is critical to understand how types of solids present in aquatic environments impact mobility and availability of this toxin. MCs present in WTP residuals may sorb to added or natural particle surfaces or remain in the free water depending on the residuals organic content, solids percentage, and pH-impacting additives.

Many studies have investigated sorption interactions between various types of particles and MC-LR (Babica et al., 2006; Bajracharya et al., 2019; Campinas et al., 2013; Dixit et al., 2018a; Maghsoudi, Prévost, et al., 2015; Pendleton et al., 2001; Pestana et al., 2014; Song et al., 2014; Thirumavalavan et al., 2012; Wu et al., 2011). Interaction between sediments and MCs is a more commonly investigated topic, as it provides insight into fate of MCs in natural waters, and has

demonstrated absorptive capabilities for MCs (Maghsoudi, Prévost, et al., 2015; Preece et al., 2021; Song et al., 2014; Wu et al., 2011; Zastepa et al., 2015). Some studies utilized this phenomenon as a MC removal method, seeing an improvement in removal rate when aeration was utilized (Song et al., 2014). This is most likely due to mixing allowing for more particle interaction and collision for greater adsorption opportunity.

MC-LR adsorption is often described as pH dependent with a greater likelihood of adherence to solids rather than staying in solution at lower pH (McCord et al., 2018). In a water-octanol partitioning study, MC variants including but not limited to MC-LR, MC-RR, and MC-LA demonstrated strong pH dependent partitioning except MC-RR, which demonstrated pH independent partitioning (McCord et al., 2018). However, in studies utilizing anion exchange resins and natural lake water, it was shown that change in pH did not impact MC-LR adsorption, likely due to the strong electrostatic effects of the high charge density of the resin and NOM competition (Dixit et al., 2018a). As pH decreases, hydrophobicity of most MC variants increases, resulting in more ideal conditions for MCs to sorb to the lipid phase. Particles in WTP residuals could have a higher adsorption of MCs, as these samples contain pH-altering chemical contents, such as alum or lime. While MC pH dependent adsorption may consistently hold true in a laboratory-based setting with pure solutions, it has been shown to be less dominant in the presence of NOM, and other negatively charged ions such as sulphates and nitrates (Dixit et al., 2018b, 2019). These analytes are likely to be seen in residuals as they are readily removed by conventional WTP processes, and therefore may impact MC partitioning in such samples.

Activated carbon (AC) is typically used for taste and odor compound removal, although some WTPs use it for MC removal. Adsorption of MCs is impacted by the presence other constituents (Dixit et al., 2018a; Maghsoudi, Prévost, et al., 2015) and can be attributed to blockage of

sorption sites (Bajracharya et al., 2019; Dixit et al., 2018a, 2018b). MC-LR adsorption by AC is shown to be a function of surface chemistry and solution phase chemistry (Pendleton et al., 2001). Little difference in the adsorption of MC-LR between porous and non-porous AC media demonstrated that micropores do not heavily impact MC-LR adsorption (Pendleton et al., 2001) and MC-LR adsorption increases inversely with pH, both trends shown in the absences of other analytes. Competitive adsorption, with constituents present in natural waters, restricts binding sites for MCs and result in less adsorption (Bajracharya et al., 2019; Dixit et al., 2018a).

1.3.2 Biodegradation

Biological degradation, or biodegradation, is another potential approach to treating waters for contaminants including cyanotoxins like MC (Song et al., 2014). MC is very chemically stable and can be expensive to remove with typical physical and chemical processes, therefore ongoing research efforts devoted to biodegradation of MCs are an important development in water treatment. Biodegradation of MCs has been primarily observed in a laboratory setting (Kansole & Lin, 2016; Kumar et al., 2018; Yang et al., 2020) which utilize indigenous bacteria from the source of the MC-laden samples. Many of these bacteria belong to the *Proteobacteria*, *Actinobacteria*, and *Bacilli* phyla (Massey & Yang, 2020).

Since there are over 50 congeners of MCs, the degradation pathways of each one is still being explored. Of the known congeners, the biological degradation pathway of microcystin-LR (MC-LR) variant is most understood. Literature attributes this degradation process to the *mlr* gene cluster often found in known MC-LR degraders. This is described as a novel pathway involving the *mlrABCD* genes (Maghsoudi et al., 2016). The research performed by Maghsoudi et al in 2016 describes the biodegradation process by a variety of enzymes, with the first step

cleaving the ADDA-arginine bond in MC-LR resulting in a linearized MC-LR. Once the ADDA component has been cleaved from the main MC-LR compound through the *mlrA* gene degradation step, the ADDA has been shown to be biologically degradable as well in to phenylacetic acid (PAA) (Massey & Yang, 2020). However, the enzyme responsible for ADDA degradation continues to be explored. A PAAse enzyme breaks the PAA down first in to PAA-CoA and then to acetyl coenzyme A (Acetyl-CoA). Acetyl-CoA can be utilized by organisms outside of MC-LR degraders for energy in the Krebs cycle through cellular respiration. Biological degradation of MC-LR and other MC congeners has been observed during bench scale experiments that utilize both indigenous bacteria and pure cultures (Maghsoudi et al., 2016; Maghsoudi, Fortin, et al., 2015; Thees et al., 2019).

1.4 Microcystin Extraction and Quantification

Although there are currently no internationally recognized methods for extracting and quantifying MCs in WTP residuals, methods for extracting and quantifying microcystins in raw and finished drinking water have been adapted for solid matrices. United States Environmental Protection Agency (US EPA) method 546 by ADDA-enzyme linked immunosorbent assay (ELISA) and US EPA method 544 by liquid chromatography-tandem mass spectrometry (LC-MS/MS) are two methods for water-based extractions that have been adopted for residuals analysis. Method 546 relies on freeze-thaw as means of cellular lysis and utilizes DI water for a solvent coupled with vacuum filtration for solids removal (Zaffiro et al., 2016). Method 544 also relies on freeze-thaw for cellular lysis but utilizes methanol as a solvent and solid phase extraction (SPE) for optimized MC extraction (Shoemaker et al., 2015). Freeze thaw lysis is time consuming and may take several hours to complete the standard three cycles. SPE requires specific equipment that may not be available in an average WTP laboratory and can take 1.5

days to complete (Birbeck et al., 2019). Because of these time related drawbacks, there have been studies investigating optimization of extraction and quantification of cyanotoxins in water samples. Faster lysis methods compared to freeze-thaw have been recently investigated. Li *et al.* (2021) utilized microwave cycles for cell lysis prior to performing ADDA-ELISA quantification in water matrices. It was found that for certain genera of cyanobacteria, microwave lysis demonstrated just as effective or better lysis results in comparison to freeze thaw (Li et al., 2021). It was also found that sonication as a pretreatment could optimize the extraction processes (Pestana et al., 2014).

Extraction techniques of microcystins in various tissues and soils spiked with MCs have been investigated by numerous authors. Solid matrices were spiked with MC-LR and MC-RR and different combinations of solvents and SPE columns, and quantified utilizing LC-MS/MS. It was demonstrated that solvents utilizing partial methanol, ranging from 70 – 80%, resulted in the highest recovery of MCs (Díez-Quijada et al., 2018; Manubolu et al., 2018; Wu et al., 2015) but may cause interferences with quantification methods seen as falsely elevated concentrations when utilizing ADDA-ELISA (Guo et al., 2017; Rapala et al., 2002).

Quantification between ADDA-ELISA and LC-based methods for microcystins have been shown to vary in results when analyzing water samples. ADDA-ELISA measures total microcystins, quantifying based on the ADDA component that is found in all MC variants with an assay range up to 5 µg/L. ELISA results can be inconsistent due to improper dilution, cross-reactivity of other variants such as MC-LA, user error, and matrix impacts of different analytes present (Birbeck et al., 2019; Cruz et al., 2012; Guo et al., 2017; James et al., 2010; Kumar et al., 2020). Additionally, indirect-competitive ELISA's, such as the ones used in this study, have a high antibody specificity for variants containing arginine in position 4 (Fischer et al., 2001;

Massey, Wu, et al., 2020; J. Sheng et al., 2007; J. W. Sheng et al., 2007) and thus may incorrectly quantify other variants that do not contain arginine. In contrast, LC-based methods have a greater assay range (upwards of 1000 µg/L based on instrument methodology, column and detector type), and less user error, as once samples are loaded, the user is hands off until quantification has been completed. LC coupled with a photo diode array detector (LC-PDA) has the ability to be more robust in MC variant quantification, as the ADDA component of all microcystins has a peak absorbance at 238 nm, apart from the few variants containing the tryptophan amino acid (Bouaïcha et al., 2019; Lawton et al., 2021; Meriluoto et al., 2017). However, a major drawback to these common quantification methods includes the inability to properly differentiate amongst variants. LC-PDA quantification may not be able to differentiate between variants that have similar elution times. LC with tandem mass spectrometry (MS/MS) can identify variants more precisely in comparison. Both LC methods are limited to commercially available MC variant standards for comparison to provide variant specific quantification, many of which were utilized in creating the ADDA-ELISA kit.

Some studies have observed extraction methods in residual-type samples. Pestana *et al.* (2014) tested MC extraction from simulated residuals. The authors used the terminology "sludge", by concentrating cells from a culture of *Microcystis aeruginosa* with river water and alum. The authors then simulated the coagulation and flocculation process with this mixture to create a benchtop version of residuals. Freeze-thaw, lyophilization, and methanolic extractions were tested and compared. Pretreatments were also tested prior to extraction including homogenization and sonication. A high-performance liquid chromatography-photo diode array (HPLC-PDA) was used for quantification. The authors' goal was to determine the best extraction methodology to release intracellular MC from sludge bound cyanobacteria (Pestana et al., 2014),

and concluded that previously published methods for cyanotoxin-laden residuals, based on the study results, may underestimate the actual MC concentration in a given sample.

1.5 Thesis Overview

A recent review synthesized the studies detailed here and indicated that conventional treatment in WTP's may result in cyanobacteria accumulation, and thus cyanotoxins, within WTP residuals (Jalili, Moradinejad, et al., 2022), indicating potential concerns related to public health for WTP's that recycle residuals for coagulant reuse or beneficial reuse that must be addressed. Although quantification methods of MCs for water samples have been adapted for solid matrices, these methods may be inappropriate for proper solids analysis. The combination of extraction method and quantification method may not be suitable for quantifying MC within a residual sample. Additionally, due to the inherent high variability within and between WTP residuals across the United States, methods may not be optimal across samples. Here, I systematically extracted and quantified MCs from WTP residuals across the US to demonstrate widespread occurrence of MC in WTP residuals and the impacts of extraction and quantification methods on determining MC concentrations. Additionally, natural degradation observed in a utility's storage lagoon led to investigating how the potential presence and activity of MC-degrading organisms, temperature and mixing conditions, and chemical pretreatments would impact MC concentration over time.

The following chapter is a draft manuscript that synthesizes this literature review and details results from experiments comparing methods of extracting and quantifying MC in various WTP residuals and from experiments investigating impacts of treatments and storage of MC in WTP residuals.

Chapter 2: Microcystins Are Present in Water Treatment Plant Residuals and Are Impacted by Extraction and Quantification Methodology

2.1 Abstract

Microcystins (MCs), a toxin produced by some species of the photosynthetic heterotrophic cyanobacteria, are the most studied, most associated with toxicity-related events, and most monitored in water. Water treatment plant (WTP) residuals are the solid byproduct of water treatment consisting of solids removed from physical and chemical processes and have been shown to contain cyanobacterial cells removed by conventional treatment processes. However, MCs in WTP residuals are not well understood. Although quantification methods of MCs for water samples have been adapted for solid matrices, frequently using ADDA-ELISA or liquid chromatography, their suitability for understanding the level of microcystins within a WTP residual sample is not well understood. Additionally, due to the inherent high variability within WTP residuals across the United States, these methods may not be optimal for every sample. Extraction and quantification methods were investigated to assess MCs in varied WTP residuals. Natural degradation observed in a utility's storage lagoon was also investigated to determine the impact of physical, chemical, and biological treatments on MC concentration in high-biomass residuals. This study demonstrates residuals of various characteristics across the United States contain MCs, and in some sample's extraction method (particularly methanol) and quantification method (particularly UPLC-PDA) increased measured MC, no single methodology is optimal for extracting and quantifying MCs across various residuals.

Keywords: Microcystin, residuals, water treatment, ADDA-ELISA, UPLC-PDA

2.2 Introduction

Cyanotoxins are toxic metabolites produced by cyanobacteria, a type of photosynthetic heterotroph. Both the toxins and the cells are becoming increasingly prevalent in source waters, presenting challenges for removal in Water Treatment Plants (WTP). Microcystins (MCs), a category of cyanotoxins, are the most extensively studied, most associated with toxicity-related events, and most frequently monitored (World Health Organization, 2003). These toxins are expensive to remove and often require advanced treatment technologies such as activated carbon or ultraviolet-advanced oxidation. Cyanobacteria also provide treatment challenges; cells can travel throughout the water column utilizing their gas vesicles and thus resist settling. Removing cells is less complex than removing extracellular cyanotoxins. However, these cells could increase risk of cyanotoxins present in the solid byproduct of water treatment, known as residuals or sludge, as has been previously shown (Jalili et al., 2021a; Jalili, Moradinejad, et al., 2022; Pestana et al., 2016; Xu et al., 2017) . Residuals are typically disposed of at a landfill or incinerated. Beneficial reuse is becoming a popular alternative disposal method; residuals can be land applied on fields or crops or recycled to recover coagulants for the WTP. Although cyanotoxins in residuals are poorly understood, cyanotoxin-laden residuals could impact reuse outcomes or present a public health and safety issue.

2.2.1 Residuals Characteristics

WTP residuals are removed from the flocculation, sedimentation, lime softening, activated carbon, and other processes. WTP residuals vary in composition based on factors such as geographic location, processes within the treatment train, and source water quality (Turner et al., 2019). Residuals can typically be described by a water phase, both inside and outside or bonded to the particle (Basim, 1999), and a solid phase with a typical range of 5 – 15% solids

(Wang et al., 1992). Water content can facilitate reactions, mobility, and sorption to occur through interaction with particles and other constituents that may be present. The solid phase contains a mixture of clays, aluminosilicates, organics and natural organic matter (NOM), metals and coagulants (Wang et al., 1992). Approximately 70% of WTP's in the United States utilize chemical coagulation, often in the first steps of the treatment process (Turner et al., 2019), and could impact both the physical and chemical behavior of residuals.

2.2.2 Microcystin Extraction and Quantification

Although there are currently no internationally recognized methods for extracting and quantifying MCs in WTP residuals, methods for extracting and quantifying microcystins in raw and finished drinking water have been adapted for solid matrices. United States Environmental Protection Agency (US EPA) method 546 by ADDA-enzyme linked immunosorbent assay (ELISA) and US EPA method 544 by liquid chromatography-tandem mass spectrometry (LC-MS/MS) are two methods for water-based samples that have been adopted for residuals analysis. Both methods utilize freeze thaw lysis, but method 546 utilizes DI as a solvent prior to filtration, whereas method 544 utilizes methanol (MeOH) as a solvent and solid phase extraction (SPE) after filtering (Shoemaker et al., 2015; Zaffiro et al., 2016). Freeze thaw lysis is time consuming and may take several hours to complete the standard three cycles. SPE requires specific equipment that may not be available in an average WTP laboratory and can take 1.5 days to complete (Birbeck et al., 2019). These drawbacks have motivated studies investigating the optimization of extraction and quantification of cyanotoxins in water samples. Microwave cycles for cell lysis has been demonstrated to be just as effective or better lysis results in comparison to freeze thaw for ADDA-ELISA (Li et al., 2021), and sonication pretreatment may also optimize the MC extraction processes (Pestana et al., 2014).

Extraction and quantification of MCs in solid matrices have been explored in fish tissue, soil, and vegetables, either spiked with MC variants or from vegetables grown with MC containing water (Díez-Quijada et al., 2018; Manubolu et al., 2018). Solvents utilizing partial methanol, ranging from 70 – 80%, resulted in the highest recovery of MCs (Díez-Quijada et al., 2018; Manubolu et al., 2018; Wu et al., 2015) but have been noted to cause interferences with quantification methods when utilizing ADDA-ELISA (Guo et al., 2017; Rapala et al., 2002).

Quantification results between ADDA-ELISA and LC-based methods for MCs in water samples have been shown to vary. ADDA-ELISA measures total MCs, quantifying based on the ADDA component, a peptide found in all MCs, with the ability to quantify from 0.1 to 5 µg/L. This method can be inconsistent due to improper dilution, cross-reactivity of variants, user error during hands on steps, and matrix impacts of different analytes present (Birbeck et al., 2019; Cruz et al., 2012; Guo et al., 2017; James et al., 2010; Kumar et al., 2020). Additionally, indirect-competitive ELISA's, such as the ones used in this study, have a high antibody specificity for variants containing arginine in position 4 (Fischer et al., 2001; Massey, Wu, et al., 2020; J. Sheng et al., 2007; J. W. Sheng et al., 2007) and may incorrectly quantify variants that do not contain arginine (James et al., 2010). Conversely, LC-based methods have a wider range (5 µg/L to upwards of 1000 µg/L based on instrument methodology, column and detector type), with less hands-on time for user error. LC coupled with a photo diode array detector (LC-PDA) also quantifies based on the ADDA component, utilizing its peak absorbance at 238 nm, apart from the few variants containing the tryptophan amino acid (Bouaïcha et al., 2019; Lawton et al., 2021; Meriluoto et al., 2017). While LC-PDA quantification can identify specific variants, it may not differentiate between variants with similar elution times and is limited to commercially available standards, therefore unable to capture a true "total" MC. LC-PDA also requires

technical expertise to operate the equipment. Matrix impacts and MC variant quantification discrepancies can occur among environmental samples in either method. Birbeck *et al*. demonstrated higher values of MCs in lake water by ADDA-ELISA than LC-MS/MS, which may have been attributed to naturally occurring MC degradation products (Birbeck et al., 2019; Thees et al., 2019). With so many factors contributing to discrepancies, no single method should be used in quantifying MCs for operational decisions.

2.2.3 Microcystins in Residuals

Some work has been done to assess MCs in WTP demonstrating accumulation and potentially cyanotoxins in residuals (Jalili, Moradinejad, et al., 2022), though many of these studies used simulated residuals. It has been suggested that existing methods may underestimate MC concentration in residuals (Pestana et al., 2014). While this may demonstrate a need for an improved understanding of cyanotoxins in WTP residuals, no investigation on the prevalence of MCs in environmental residuals, or systematic investigation of impact of extraction and quantification methods of MCs in residuals, has been done to the best of the author's knowledge. Additionally, partitioning of many MC variants is pH dependent (McCord et al., 2018) and could be heavily impacted by the properties of WTP residuals and extraction solvent. This study explores systematic extraction and quantification of MCs from WTP residuals across the US, demonstrating widespread occurrence of MC in WTP residuals and the impacts of extraction and quantification methods on determining MC concentrations. Additionally, natural degradation observed in a utility's storage lagoon led to investigating how potential presence and activity of MC-degrading organisms, temperature and mixing conditions, and chemical pretreatments would impact MC concentration over time.

2.3 Methods

Samples were obtained from utilities that either reported microcystin concentrations or reported microcystin-producing species of cyanobacteria in their source waters. Reports were obtained from current advisories in United States Environmental Protection Agency's Tracking CyanoHABs mapping tool (U.S. EPA Office of Water, n.d.). Samples were received from four utilities; northwest Ohio (NWO), northeast Ohio (NEO), Florida (FL), and South Carolina (SC), with key contents of these samples noted in **Table 2-1**. MC standards for MC-LR, MC-RR, and MC-LA (all Enzo Life Sciences) were utilized for calibration curves during quantification. Unless otherwise noted, materials utilized in this study were either glass or PETG plastic, due to the sorption affinity of microcystins to polypropylene (Hyenstrand et al., 2001).

Table 2-1: Sample Identification

Sample ID	Date(s) Received	Utility Location	Sample Source	Key Contents
NWO	10/2021	Ohio	Lagoon	Alum-based coagulant, lime, and DAF solids
NWO_{DAF}	10/2021 9/2022	Ohio	Dissolved Air Floatation (DAF) Facility	Majority biomass
NEO	9/2022	Ohio	Drying Beds	Powdered Activated Carbon
SC	9/2022	South Carolina	Lagoon	Powdered Activated Carbon
FL_W	4/2022	Florida	Pre-Dewatering Facility	Ferric-based coagulant
FL_D	4/2022	Florida	Product of Mechanical Dewatering	

2.3.1 Sampling

Utilities were sent three 500 mL PETG bottles (Fischer Scientific) to collect residuals and a cooler to keep samples on ice during overnight shipment to Ohio State labs. After physical and chemical characterization, samples were split into four 100 mL aliquots in PETG bottles and stored at -80°C. FL samples collected prior to and after the dewatering facility are denoted as FL

and FL_D, respectively. NWO samples collected from the utility's evaporation lagoons and directly from the DAF facility are denoted as NWO and NWO_{DAF}, respectively.

2.3.2 Characterization

Upon receipt, approximately 100 mL of sample by volume was utilized for basic characterization. The pH, dissolved oxygen (DO), and conductivity were measured using an Orion 5 Star probe (Thermo Scientific). Percent solids were calculated using dry weight. Density was estimated using volume displacement. 10 mL of sample by volume was added to 80 mL of DI water in a graduated cylinder. The displacement of the DI water was recorded and used to calculate the density of 10 mL of sample. Total dissolved organic carbon (DOC) was determined for each sample utilizing a TOC-V CSN analyzer (Shimadzu). Samples were diluted 1:1000 in DI water, to a final volume of 30 mL and then filtered on a 0.2 um membrane into a muffled glass vial prior to analysis. In addition to DOC, the loss on ignition method was used to estimate percent organic matter (Combs & Nathan, 1998; Schulte & Hopkins, 1996). Results of characterization for each sample set are shown in **Table 2-2** below.

Table 2-2: Sample Characterization

Sample ID	Date(s) Received	pH	DO (mg/L)	Conductivity (µS/cm)	Solids Content (%)	Density (g/mL)	DOC (mg/L)	Organic Matter (%)
NWO	10/2021	9.46	0.5	807	15.3	1.0611	238.9	9.6
NWO_{DAF}	10/2021 9/2022	6.53	0.0	355	8.5	0.9586	5091.6	47.8
NEO	9/2022	7.32	11.4	2.4	65.6	1.5485	1430.2	8.7
SC	9/2022	7.94	1.1	1824	2.7	0.9295	3195.5	16.5
FL_W	4/2022	6.10	4.3	0.2	2.5	1.6512	1874.4	41.3
FL_D	4/2022	6.02	8.6	1.1	72.1	1.1133	164.4	43.4

2.3.3 Lysis

Sample aliquots were thawed in a 35°C water bath (Fischer Scientific). For the freeze thaw lysis method, the sample was re-frozen by placing back in the -80°C freezer a total of three times. The microwave lysis method sample was microwaved (Amana Commercial, 120V) for one 30 second interval and allowed to cool to room temperature on the bench for a total of three intervals. The sonication lysis method sample was put into a sonicating bath (Branson) for an interval of 20 minutes and rested for 5-minutes on the bench for a total of three intervals.

2.3.4 Solvents

After lysis, each sample aliquot was further split up into four 20 mL aliquots and diluted to a 2:1 ratio of solvent:sample in amber glass vials (DWK Life Sciences Wheaton) with one of the four solvents used; Deionized (DI) Water, Methanol 75% (MeOH, Fisher Brand), and 1X and 10X PBS (Fisher Brand).

2.3.5 Filtering

Lysed and solvent-added samples were vacuum filtered using a Buchner funnel and 0.45 μm membrane filter paper (Whatman, 47 mm). To condition the filter, the vacuum ran while the pedestal was thoroughly rinsed with DI. Next, a membrane filter paper was placed on the pedestal and conditioned with DI. After the DI was discarded, each sample was filtered, and the filtrate was divided into duplicate aliquots.

2.3.6 Quantification

Two methods of MC quantification were utilized for this study; ADDA-ELISA (Eurofins Abraxis, PN 520011) and Ultra Performance Liquid Chromatography (UPLC) coupled with a Photo Diode Array (PDA) detector.

ADDA-ELISA quantifies based on the absorbance of the reaction product after complexation between the microcystins ADDA component, and an antibody linked with a reaction enzyme. For ADDA-ELISA, the provided calibration standard solutions (0, 0.15, 0.40, 1.0, 2.0, and 5.0 μg/L) were pipetted into the 96-well plate in addition to the diluted filtered samples. Dilutions were made with DI water:sample at a ratio of 50:1, 50:1, 167:1, and 1000:1 for NEO, SC, FL, and NWO utility samples respectively. Plates also contained wells with 75% MeOH and internal standards for comparison, as kit instructions note samples containing MeOH must be diluted to less than 5% MeOH to avoid interfering with ADDA-ELISA results. Analyses were conducted in triplicate. After completing the assay according to the provided instructions, the UV absorbance was read at 450nm on a multimode plate reader (Biotek Synergy HTX, Gen5 software) and converted to concentrations using a four-parameter logistic curve (ATT Bioquest, *https://www.aatbio.com/tools/four-parameter-logistic-4pl-curve-regression-online-calculator*) based on the calibration standards.

The UPLC-PDA method detects the absorbance conjugated double bond of the ADDA component of microcystins, with different retention times (RT) corresponding to different variants. Peaks from the chromatograph were quantified by comparing with peaks obtained from standards for MC-RR (RT 3.2 min), MC-LR (RT 4.1 min), and MC-LA (RT 5.3 min). Due to the limited number of variants analyzed, total MC concentrations from the UPLC-PDA will be referred to as “Sum”. Separation of the analytes using liquid chromatography was performed on a Waters Aquity with a BEH C18 column (1.7 μm, 2.1 x 50mm, Waters) and a 0.3 mL/min flow

rate. Over a 5-minute period, the mobile phase gradient moved from 75:25 water (with trifluoroacetic acid (TFA), 0.05% v/v):acetonitrile (with TFA, 0.05% v/v) to 50:50 water (with TFA, 0.05% v/v): acetonitrile (with TFA, 0.05% v/v). PDA detection of MCs occurred at lambda max of 238 nm. Analyses were conducted in triplicate using 10 µL injections. (Laszakovits & MacKay, 2019; Spoof et al., 2009).

2.3.7 Statistical Analysis

Data was analyzed for statistical significance using R version 4.2.2. Three-way analysis of variance (ANOVA) was used to investigate differences between quantification method, lysis method, and solvent used. A follow up analysis from ANOVA results $p \leq 0.05$ was performed using Tukey's range test method. Graphical representations of results for each utility's sample set are seen in **Figure 2-1** and **Figure 2-2**. All p values indicating statistical significance from Tukey's range test can be found in **Appendix A**. First, ADDA-ELISA data per utility was analyzed by ANOVA, comparing lysis and solvent on MC values. Any ANOVA result that showed statistical significance ($p \leq 0.05$) was followed up with Tukey's test. This process was repeated for the UPLC-PDA data per utility. Next, all data for a utility, both ADDA-ELISA and UPLC-PDA quantification, was analyzed by ANOVA for differences among quantification type. If an ANOVA result showed statistical significance ($p \leq 0.05$), it was followed up with Tukey's test. Then, all data obtained from this experiment (all utilities) was analyzed by ANOVA, comparing lysis and solvent on MC values, with any statistically significant ($p \leq 0.05$) combinations followed up by Tukey's. Quantification type was also compared for all data using this same process.

2.4 Results

Concentrations of MC in each WTP sample extracted by various lysis and solvent combinations and measured by ADDA-ELISA and UPLC-PDA are shown in **Figure 2-1**.

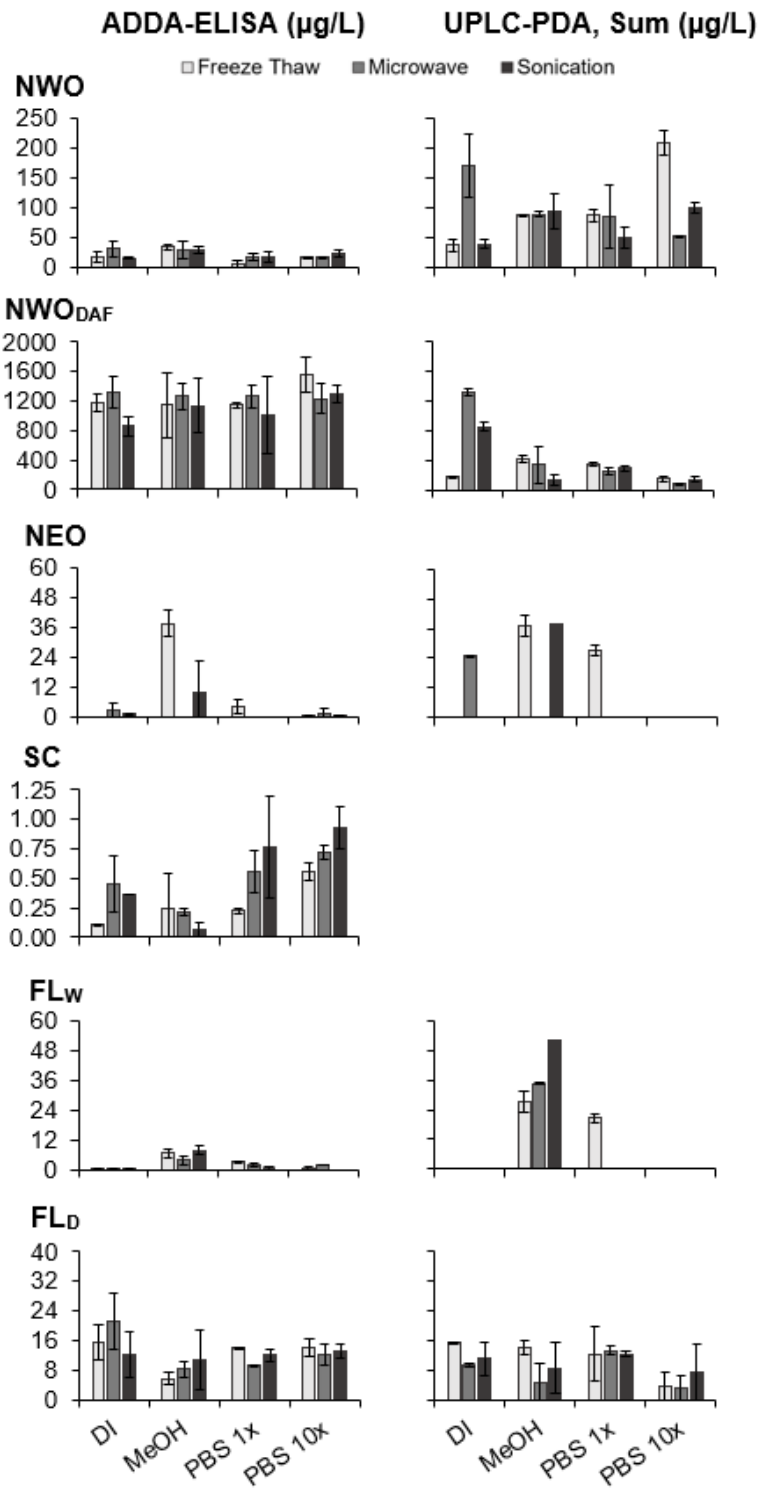

Figure 2-1: Quantification by ADDA-ELISA (left) or UPLC-PDA (right). Each row represents one set of utility samples. Y-axis denotes MC concentration (ug/L), X-axis denotes solvent used. Each bar per solvent represents a different lysis method. Error bars represent the standard deviation among filtrate duplicates after averaging technical triplicates.

Average MC in NWO samples was 20.8 (±9.9) or 89.2 (±54.2) µg /L by ADDA-ELISA or UPLC-PDA quantification, respectively. ANOVA identified significant differences (**Table A-1** and **Table A-2**) among solvents for ADDA-ELISA, and among solvents and among combinations of lysis and solvents for UPLC-PDA. Follow up testing by Tukey's identified the significant differences (**Table A-3** and **Table A-4**) among solvents for ADDA-ELISA to be between PBS 1x and MeOH, and for UPLC-PDA to be between PBS 1x and PBS 10x. A variety of significantly different lysis-solvent combinations for UPLC-PDA included all comparisons with freeze thaw and PBS 10x, except when compared to sonication and PBS 10x.

Average MC in NWO_{DAF} samples was 1197.5 (±248.6) or 242.3 (±118.3) µg /L by ADDA-ELISA or UPLC-PDA quantification, respectively. Neither ANOVA nor Tukey's identified any significant differences among solvents, lysis methods, or their combinations for ADDA-ELISA or UPLC-PDA.

Average MC in NEO samples was 4.7 (±10.7) or 31.9 (±9.9) µg /L by ADDA-ELISA or UPLC-PDA quantification, respectively. ANOVA identified significant differences (**Table A-1**) among lysis methods, solvents, and combinations of lysis and solvents for ADDA-ELISA. Follow up testing by Tukey's identified the significant differences (**Table A-3**) among lysis to be between freeze thaw and both other lysis methods, between MeOH and all other solvents, and between all comparisons against freeze thaw or MeOH lysis-solvent combinations for ADDA-ELISA data. No significant differences were identified in UPLC-PDA data.

Average MC in SC samples was 0.4 (±0.3) µg/L by ADDA-ELISA quantification. SC samples were not run on UPLC-PDA because all ADDA-ELISA results were below the 5 µg /L quantification limit. ANOVA identified significant differences (**Table A-1**) among lysis methods

and among solvents. Follow up testing by Tukey's identified no lysis methods to be significant, but did identify significant differences (**Table A-3**) between MeOH and both concentrations of PBS, and between DI and PBS 10x.

Average MC in FL_W samples was 2.3 (±2.7) or 33.6 (±13.0) µg /L observed by ADDA-ELISA or UPLC-PDA quantification, respectively. ANOVA identified significant differences (**Table A-1** and **Table A-2**) among solvents for ADDA-ELISA data, and among lysis method for UPLC-PDA data. Follow up testing with Tukey's identified significant differences (**Table A-3** and **Table A-4**) between MeOH and all solvents for ADDA-ELISA, and between sonication and freeze thaw lysis for UPLC-PDA.

Average MC in FL_D samples was 12.3 (±4.8) or 10.8 (±4.5) µg /L was observed by ADDA-ELISA or UPLC-PDA quantification, respectively. ANOVA identified significant differences (**Table A-1**) among solvents for ADDA-ELISA, with follow up testing by Tukey's identifying significance (**Table A-3**) between MeOH and DI. No significant differences were identified for UPLC-PDA data.

Only three utilities showed significant differences by ANOVA among cell lysis method: NEO and SC for ADDA-ELISA quantification (**Table A-1)**, and FL_W for UPLC-PDA quantification (**Table A-2)**. Follow up analysis by Tukey's showed significant differences between freeze thaw and the other lysis methods for NEO and FL_W by ADDA-ELISA and UPLC-PDA, respectively.

When comparing MeOH and PBS 1x solvents, four out of six sample sets had significant differences in ADDA-ELISA results; MeOH resulted in a higher concentration than PBS 1x for NEO, NWO, and FL_W but resulted in a lower concentration for SC. When comparing MeOH and

PBS 10X solvents for these four sample sets, MeOH also resulted in a higher concentration than PBS 10X for NEO, NWO, and FL_W. When comparing MeOH and DI solvents for these sample sets, MeOH resulted in a higher concentration than DI for NEO and FL_W but resulted in a lower concentration for FL_D. This multi-WTP residuals trend where MeOH resulted in statistically greater difference in MC concentrations versus other solvents was also observed during initial method development studies that utilized two sets of NWO_{DAF} samples and compared different concentrations of MeOH between freeze thaw and microwave lysis methods, shown in **Figure C-1**. These results demonstrated that increased MeOH concentration increased MC-LR concentrations by both ADDA-ELISA and UPLC-PDA.

All sample sets with adequate MC for analyses by both UPLC-PDA and ADDA-ELISA (NWO, NWO_{DAF}, NEO, and FL_W) indicated significant differences (**Table A-5** and **Table A-6)** between quantification methods by both ANOVA and Tukey's comparison when analyzed as a group, apart from FL_D. Average MC concentration was significantly higher by UPLC-PDA quantification for all samples except FL_D and NWO_{DAF}. These sample sets were analyzed further by UPLC-PDA for breakdown of MC variants present (MC-RR, MC-LR, or MC-LA) as shown in **Figure 2-2**.

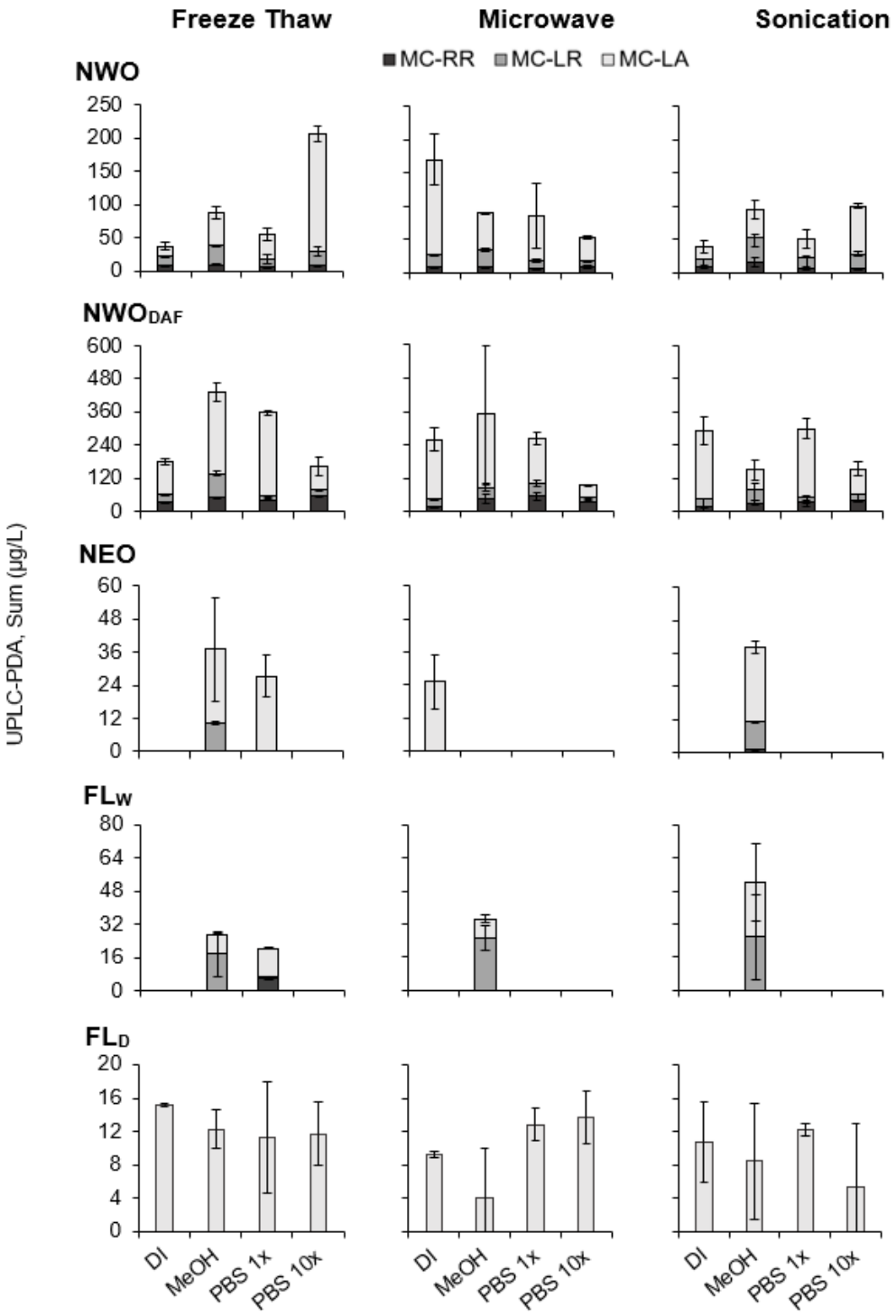

Figure 2-2: Microcystin Variants Identified by UPLC-PDA.

Each row represents one set of utility samples. Y-axis denotes MC concentration (µg/L), X-axis denotes solvent used. Each graph per row represents a different lysis method. Columns on the graph represent the concentration of each MC variant quantified. Error bars represent the standard deviation among triplicates.

MC-LA was frequently seen as the highest concentration variant and MC-RR was consistently the lowest concentration variant in sample combinations among the three variants investigated here, regardless of solvent and lysis combination. Average MC-RR, MC-LR, and MC-LA in NWO samples was 8.4 (±3.5), 18.9 (±8.5), and 61.4 (±51.4) µg /L respectively. Average MC-RR, MC-LR, and MC-LA in NWO_{DAF} samples was 38.4 (±13.8), 31.1 (±17.4), and 172.7 (±116.6) µg /L respectively. Average MC-RR, MC-LR, and MC-LA in NEO samples was 0.3 (±0.6), 5.1 (±5.1), and 26.5 (±8.0) µg /L respectively. Average MC-RR, MC-LR, and MC-LA in FL_W samples was 1.6 (±2.7), 17.3 (±13.9), and 14.8 (±9.7) µg /L respectively. Average MC-RR, MC-LR, and MC-LA in FL_D sample was 0.04 (±0.2), 0.2 (±0.3), and 10.6 (±4.5) µg /L respectively.

NWO_{DAF} samples contain predominantly cyanobacterial biomass, and thus high organic matter (OM) content. The toxin concentration in these samples was greater than any of the other samples, including the lagoon samples from the same utility (NWO). Further investigation of NWO residuals was conducted to explore implications of high MC content residuals samples. Impact of biological, chemical, and physical processes that might occur during residuals generation, treatment, and storage on MC concentrations were also investigated in NWO lagoon samples and in NWO_{DAF} residuals which contained high biological and organic content as described in **Appendix B**. DAF solids were diluted with the WTPs raw influent water to ~ 3% solids by volume (which could be pumped). Decreasing MC-LR concentration with increased residual age in previous analyses of NWO lagoon samples (**Figure B-1**) was further investigated using NWO_{DAF} to determine impacts of mixing, temperature, and chemical pretreatments on MC concentration. While total MC decreased over time in a pilot study at one temperature and mixing speed (**Figure B-2**), extracellular MC increased over time in bench studies despite

mixing and temperature differences (**Figure B-3**) although no change in cell count was observed (data not shown). Additionally, two chemical pretreatments resulted in increasing cell density over time (data not shown) while total and extracellular MC varied (**Figure B-4**).

2.5 Discussion

2.5.1 Impact of Lysis Method

Interestingly, MC quantification in samples with the highest estimated OM content, NWO_{DAF} and FL_D, was not significantly impacted by lysis method. This could be attributed to the majority of MC in the samples being extracellular toxins, or in the case of FL_D samples, a minimal cyanobacteria population as a higher solid percentage would be a less ideal habitat for the organisms. However, the lack of difference in MC data between lysis methods, regardless of sample type, would indicate that cell lysis method can be chosen based on ease or access to materials when quantifying MCs in residuals.

2.5.2 Impact of Solvent

The observed increased MC-LR concentrations by both ADDA-ELISA and UPLC-PDA when using MeOH solvent could be due to its high polarity. MeOH particularly resulted in significantly greater MC concentrations than other solvents for ADDA-ELISA quantification. This could be due to interferences seen between MeOH and this specific ELISA plate assay for MCs (Babica et al., 2006; Guo et al., 2017; Li et al., 2021; Qian et al., 2015). However, MeOH solvent was run in wells for several plate assay tests with equivalent dilution to samples, resulting in no to few low false positives (between 0 and 0.15 ug/L total MC). Therefore, elevated ADDA-ELISA values due to the presence of MeOH is assumed to be negligible for this

experiment. Future studies using this or similar methodology with MeOH as a solvent for MC extraction should include similar negative controls.

2.5.3 Impact of Dilution

Appropriate dilutions are necessary when utilizing ADDA-ELISA for quantification. Using the kit for determining unknown concentrations without a priori knowledge, which is often the case for environmental samples, could negatively impact or void results, as noted previously (James et al., 2010; Li et al., 2021; Rapala et al., 2002; J. Sheng et al., 2007). Initial observations of utilities with greater UPLC-PDA concentrations compared to ADDA-ELISA showed the absorbance of the raw data were closer to the 0 µg/L standard's absorbance on the calibration curve, where signal and noise are harder to differentiate. NWO_{DAF} raw data for ADDA-ELISA were closer to the middle of the calibration curve, and therefore more optimal for quantification. Additional investigation of dilution factors using ADDA-ELISA for MC quantification demonstrated an average 34.4% variability of samples analyzed at hundredfold dilution factors where higher dilution increased MC (**Figure C-2**). Due to the narrow assay range for this ELISA, under and over dilution impacts on back-calculated MC concentration could be especially problematic for samples with large dilution factors. This may negatively affect utilities using ADDA-ELISA results for regulatory purposes, or unnecessarily cause a public safety concern. It could also impact reproducibility of MC research. Future studies observing MCs in solid matrices should be cautious of dilution factors and perform analysis with multiple dilutions and re-run highly diluted samples if needed.

2.5.4 Impact of Variants

One reason for greater average MC by UPLC-PDA, and for greater MC-LA and lower MC-RR variant concentrations, could be due to pH impacts. McCord *et al.* (2018) investigated the pH dependent partitioning of multiple MC variants between octanol and water. MC variants included MC-LR, MC-RR, MC-LA, and several others. Both MC-LR and MC-LA demonstrated strong pH dependent partitioning and would likely remain in solution as opposed to adsorbing to organics in neutral to basic conditions (McCord et al., 2018). From McCord *et al*., it can be also concluded that MC-LR and MC-LA would be more likely to sorb to solids, while MC-RR would be more likely to stay in solution in residuals with higher coagulant contents. However, it has been reported by several studies observing adsorption of MCs on to sediments, that MC-RR frequently shows the greatest adsorption to environmental sediment particles due to the second arginine amino acid present in this variant (Chen et al., 2006; Maghsoudi, Prévost, et al., 2015; Preece et al., 2021; Wu et al., 2011) which can perform cation-exchange or hydrogen bonding (Maghsoudi, Prévost, et al., 2015). NWO samples had the largest initial pH, yet both ANOVA and Tukey's analysis observed significant differences in solvents used for both ADDA-ELISA and UPLC-PDA methods. Additionally, MC concentrations are different between solvents used. This likely indicates that there are MC variants being extracted off of the solids in the sample. MC-LA was observed to be the dominant variant quantified in NWO samples, which was also found to display pH dependent in partitioning (McCord et al., 2018). While this data does not align with a pH dependent trend shown in literature with pure samples, it does follow trends observed by Wu *et al*. (2011) and Bajracharya *et al.* (2019) where OM presence impacts MC adsorption with little influence by pH due to the influence of electrostatic charge interactions between OM and MC variants. In neutral pH, MC-RR typically has a neutral charge, while MC-

LR and MC-LA exhibit negative charges (Babica et al., 2006; Bajracharya et al., 2019; Campinas & Rosa, 2006; Cermakova et al., 2017; Díez-Quijada et al., 2019; Huang, 2020; Pendleton et al., 2001; Yuan et al., 1999). Concentrations of OM used in these studies are significantly lower than the concentration of DOC observed in NWO samples, therefore, despite these studies observing competition of absorption sites on PAC and natural sediments, it is highly likely that the presence of other constituents, including OM, is impacting the sorption of MC variants to solids.

Low observation of MC-RR could be due to the toxin's lack of presence in the source water rather than extraction and quantification efficiency in this study, or may be due to strong electrostatic interactions or hydrogen bonding of the variant (Babica et al., 2006; Bouaïcha et al., 2019; Díez-Quijada et al., 2019; Gurbuz et al., 2016; Wu et al., 2011) with particles that are not impacted by solvents used in this study. Additional data would need to be collected to confirm. Although MC-LA has been reported to artificially inflate ADDA-ELISA (Guo et al., 2017), as was verified for this study (**Figure C-3**), MC concentrations measured in WTP residuals by UPLC-PDA were typically greater than by ADDA-ELISA. It is possible that the liquid chromatographic separation of MCs from the sample prior to quantification may enhance the quantification for the variants observed. Conversely, other constituents may have been extracted and absorbed at 238 nm and thus falsely inflating the MC concentration. In using ADDA-ELISA, improper dilution may have also impacted quantification in addition to potential matrix interferences.

2.5.5 Impacts of WTP Components

2.5.5.1 Coagulants

Coagulants present in all samples are likely to impact MC content in the WTP residuals, whether the toxins are present in solution or adsorbed to solids. Coagulation addition results in altered pH, altered electrostatic charge, and creates opportunity for particles and constituents to interact and sorb together or create bonds. All utilities who participated in this study use alum coagulant in the WTP, except FL which uses ferric based coagulant. Amino acids complex with metal cations present in an aqueous solution, though the studies were performed with pure standards rather than environmental samples (Djurdjević & Jelić, 1993; Fakhar et al., 2017; Fitzsimmons et al., 1985; Hassan, n.d.; Hureiki et al., 1994; Jover et al., 2008; Sharaf et al., 1994). Based on these studies, complexation occurs through the carboxylic groups and side chains present in amino acids, including alanine, leucine, and arginine, the variable amino acids present in microcystins. The behaviors of "pure" amino acids vary depending on the metal cation present, salinity levels, and conformation of compounds. Arginine, found in MC-RR and MC-LR, has a predominantly positive charge, and thus can experience repulsion from coagulation due to the presence of cation salts (Ropo et al., bar2016). This may also be a contributing factor of the lack of or very little MC-RR in samples, that it did not interact with particles enough to sorb and instead remained in solution. Conversely, solvents may not have been strong enough to extract MC-RR off of solids, as this variant typically demonstrates the strongest adsorption (Huang, 2020; Wu et al., 2011). FL_D samples contained nearly all MC-LA as the dominant variant, which could be attributed to complexation between alanine and iron cations that has been seen in pure studies (Barge et al., 2019; Djurdjević & Jelić, 1993). Additionally, high MC-LA concentrations in nearly all samples could be due to weak adsorption behaviors as seen in other studies (Cook & Newcombe, 2008; Huang, 2020). Behaviors due to presence of other constituents is also seen in studies observing MC adsorption. It would require deeper analysis in

organic and physical chemistry to better assign mechanisms to trends seen in these samples. Because of the complex nature of WTP residuals, complexation with metal ions and electrostatic interactions from chemical additives are just two of the many functions that could be impacting extraction of variants and would need to be investigated further for confirmation.

2.5.5.2 Activated Carbon

NEO and SC samples came from utilities that utilize powdered activated carbon (PAC) in the water treatment process prior to wasting solids, and thus likely contained more AC than the other sample sets. MeOH was observed to be significant between some of the other solvents for both NEO and SC samples. This would be expected, as MeOH is strong polarity would assist in desorption/extraction of MCs off the PAC. However, for SC samples, PBS 10X was observed to have the highest concentration across all lysis methods and respective solvent combinations. It is possible that the higher concentration PBS increased the salinity of the solution, thus impacting the sorption occurring on PAC (Mohamed et al., 2019; Randtke & Jepsen, 1982). SC samples had the highest initial salinity recorded during characterization (**Table 2-2**). Results showed significant difference between PBS 10x and DI for solvents by Tukey's but did not identify lysis significant. While the solvents could indicate impacts of salinity on desorption of MCs from PAC, the lack of significance among lysis methods could indicate that MCs present in the sample were already desorbed from any PAC-related solids due to the natural salinity of the sample. Salinity was only quantified as conductivity prior to lysis and solvent addition in this experiment, but parameters such as this should be recorded after lysis and before and after solvent addition in future studies. Additionally, because OM can compete with MCs for sorption sites (Bajracharya et al., 2019), particle analysis may increase understanding of particles adsorbed and create additional hypotheses on fate of MCs within PAC residuals.

2.5.5.3 Organic Components

Although NWO_{DAF} experiments (see **Appendix B**) did not enhance natural degradation seen in storage lagoons (**Figure B-1**), varied impacts of environmental conditions on high biomass residuals agreed with other studies noting microcystin and cell counts to be dynamic metrics not following a constant trend (Ho, Dreyfus, et al., 2012; Jalili, Trigui, et al., 2022; Ma et al., 2016; Pestana et al., 2016).

NWO_{DAF} samples contained the highest concentrations of organic matter (loss-on-ignition estimation, see **Table 2-2**). It has been demonstrated that OM plays a role in the sorption of MCs on to particles, as well as compete for sorption sites on sediments (Bouaïcha & Corbel, 2016; Dixit et al., 2018a; Wu et al., 2011). One theory for this is due to compound size, organics such as humic acids are much larger than MCs and thus block smaller absorption sites that MCs would typically sorb to, especially in clay prominent sediments (Chen et al., 2006; Miller et al., 2001; Wu et al., 2011). However, it was also noted by Wu *et al*. that once adsorption sites are saturated with OM on sediments, sorption between MCs and the OM becomes the dominant sorption mechanism. This phenomenon is thought to be one of the reasons for the difference in concentration between the NWO and NWO_{DAF} samples, outside of NWO_{DAF} samples high biomass/cyanobacteria content. NWO samples contain NWO_{DAF} residuals, in addition to lime, alum, and activated carbon content. The initial expectation would be that extraction and quantification results would be similar, but as shown in **Figure 2-1**, there are few similarities. The presence of OM within the NWO samples, which also included a multitude of other constituents, may be blocking MCs from adsorption sites and resulting in a higher MC presence in the supernatant rather than sorption to solids. NWO samples had a higher solids content than NWO_{DAF}; MC presence in the samples may have been lower in solids to begin with if more MCs

in NWO samples resided in the supernatant. Comparison of NWO samples with higher moisture content would be recommended, to further investigate this trend seen in literature.

2.5.6 Implications

Ohio EPA regulates microcystins in treatment residuals to < 20 µg/L for land application (Ohio Administrative Code: Beneficial Reuse, 2019). Mixed results have been seen in studies investigating land applied residuals that contain MCs. A frequent conclusion that more research needs to be done to expand the knowledge base is often stated. Bound MCs to soil particles by way of complexation or electrostatic interactions may experience desorption under certain circumstances, which would increase risk in terms of public health and safety (Ai et al., 2020; Ho, Tang, et al., 2012; Wu et al., 2011). However, for root-based vegetables growing in land applied MC containing residuals, the MC content was predominantly found in the inedible parts of the produce (Ai et al., 2020). Safety of the public when utilizing MC-laden WTP residuals is likely dependent on soil type, bacteria present who may degrade these toxins, crops grown, the properties of toxins in the residuals, and the residual's properties, as demonstrated in this study. Utilizing residuals' supernatant for coagulant recycling was shown to potentially increase release of intracellular toxins, as toxin-producing cells were lysed during rapid mixing of coagulation (Ho, Tang, et al., 2012; Pinkanjananavee et al., 2021). The authors recommended that reuse of WTP residuals coagulant be limited when a cyanobacterial bloom is occurring at a plant's source water. Fate and transport of MCs after reuse or recycling of MC-containing WTP residuals is, much like the residuals themselves, highly depending on geographic location (related to soil type) and the WTP process. Quantification of specific MC variants present in residuals may be useful in determining the feasibility of beneficial reuse, as certain variants may produce falsely high concentrations in ADDA-ELISA (Li et al., 2021), but is not a rigid trend as seen in this

data. Dual quantification is recommended by both Ohio EPA and US EPA for toxin quantification and should be pursued when investigating beneficial reuse avenues.

2.6 Conclusions

MeOH solvent produced the highest MC concentrations on average between both quantification methods for all samples. No significant differences were found among lysis methods for all data, nor was there consistency in the few samples that did indicate lysis significance. It can be concluded from this study that lysis method does not significantly impact extraction and quantification of MCs in residuals. Quantification method trends appeared to vary from trends seen in literature, apart from NWO_{DAF} samples. From this, dual quantification methods are recommended to be pursued when investigating MC concentration in WTP residuals. MC variant ratios present in samples may impact quantification results and extract differently depending on solvent used and as seen in other studies. The inherent variability in WTP residual samples impacts quantification, what may be reproducible for one utility may not work for the other depending on factors such as organic matter. Recommendations for future study specific to this experiment would include quantifying and comparing additional factors including metals, particle types, additional MC variants, LC-MS/MS quantification, and more robust ELISA quantification with multiple dilutions per sample. Cyanobacteria identification may also be a useful parameter for future studies. Genera such as *Microcystis*, *Dolicholspermum*, *Aphanizomenon*, and *Planktothrix*, to name a few, are known producers of microcystins and have been shown to primarily produce certain MC variants (Bouaïcha et al., 2019; Cirés & Ballot, 2016; Dreher et al., 2022; Massey, al osman, et al., 2020; Massey & Yang, 2020; Österholm et al., 2020). Identification of genera could help indicate MC variant presence and provide further

insight in to optimizing extraction methods, based on the present variant's chemical characteristics.

WTP residuals are a complicated sample type, with so many factors impacting the content. Additional investigation is needed in the subject of residuals, especially pertaining to characterization, and understanding. While residual reuse propels the water treatment industry forward in sustainability, waste cannot be properly reused if its components are not well understood. Recommending a one-size-fits-all methodology for extracting and quantifying MCs from WTP residuals is insufficient; proper analysis is highly dependent on the constituents present. LC-based quantification is complex, not always accessible, requires specific knowledge, training, and internal standards of MC variants. ELISA-based quantification has more room for user error and artificially inflated values. Many chemical, physical, and biological components of WTP residuals create quantification interferences, and thus a dual quantification processes would be recommended. Variants can be identified by LC-based methods and can aid in understanding data limitations with ADDA-ELISA. Variant identification, especially when paired with identification of cyanobacteria genera present in the source waters, could also impact extraction process selection due to the charges of the different MC variants. However, regulatory decisions are recommended to be based off both quantification methods, as ADDA-ELISA may be able to capture MC degradation products that LC-based methodology is incapable of at this time. Improved understanding of the MCs present in WTP residuals, which has been shown to have a widespread occurrence, will help utility managers and regulatory agencies make more informed decisions on WTP residuals management practices.

Chapter 3: Conclusions and Recommendations

As shown in this work, WTP residuals are highly variable due to numerous factors including WTP processes and geographic location. These factors can impact extraction and quantification of MC in WTP residuals. As more types of residuals are characterized and studied pertaining to MC content, improved informed decisions on residuals management can be made. Additional samples and performance of this study on additional utilities is the first recommendation for future studies. Obtaining samples from a utility's source water for additional analysis, including cyanobacteria identification, is recommended for understanding what species may be present and the toxins they may produce. This is not only helpful in understanding a portion of the organic matter contributed by the bloom, but also would shed some light on MC variants presence. It would assist in comparing extraction values to better determine if a specific variant is less impacted by an extraction method or just not present in a sample. Metals analysis should be performed to better understand complexations at particle surfaces between metal ions and MCs. Plating is also recommended to quantify microbes present as part of characterization. For an additional option for secondary quantification, LC-MS/MS is recommended. This method provides better sensitivity and specificity of MC variants. It may be worth sending samples out to external laboratories that have access to more variant standards for a more robust quantification. Inclusion of additional cyanotoxins in the investigation is recommended. MCs are commonly studied, but other toxins such as cylindrospermopsin, saxitoxin, anatoxin, and BMAA are present in the environment and often less understood, especially pertaining to environmental persistence in WTP residuals. Repetition of ADDA-ELISA quantification for each utility is strongly

recommended. By repeating ADDA-ELISA quantification and utilizing multiple dilution factors, further investigation of dilution impacts would be performed and to provide higher confidence in the data. Finally, adsorption and desorption isotherm studies for each utility and each solvent is recommended to better understand the impacts of sample and solvent on MC extraction over a period of constant interaction. This will provide insight into fate in the environment if residuals are land applied, especially concerned the different MC variants present in a sample . While some solvents used in this study are less likely to come in to contact with land applied residuals, characteristics of these solvents, such as salinity and polarity, will enhance understanding of how solid-bound toxins in land applied residuals could be impacted and contribute to evaluating risk of sustainable reuse options.

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Appendix A: *P* Values from Statistical Analysis

Bold Italics denotes significance.

Table A-1: ADDA-ELISA ANOVA Comparison Results

Utility	Variable	*P* Value
NWO		
	Lysis	4.42E-01
	Solvent	***1.32E-02***
	Lysis:Solvent	3.23E-01
NWO_{DAF}		
	Lysis	3.12E-01
	Solvent	4.14E-01
	Lysis:Solvent	7.52E-01
NEO		
	Lysis	***2.00E-03***
	Solvent	***1.08E-03***
	Lysis:Solvent	***4.01E-04***
SC		
	Lysis	***4.58E-02***
	Solvent	***1.17E-03***
	Lysis:Solvent	2.35E-01
FL_W		
	Lysis	6.06E-01
	Solvent	***4.38E-05***
	Lysis:Solvent	1.23E-01
FL_D		
	Lysis	9.46E-01
	Solvent	***3.74E-02***
	Lysis:Solvent	3.54E-01

Table A-2: UPLC-PDA ANOVA Comparison Results

Utility	Variable	*P* Value
NWO		
	Lysis	1.60E-01
	Solvent	***4.81E-02***

	Lysis:Solvent	***1.04E-03***
NWO_{DAF}		
	Lysis	7.94E-01
	Solvent	8.57E-02
	Lysis:Solvent	3.64E-01
NEO		
	Lysis	5.81E-01
	Solvent	4.51E-01
	Lysis:Solvent	N/A
FL_W		
	Lysis	***3.27E-02***
	Solvent	4.36E-01
	Lysis:Solvent	N/A
FL_D		
	Lysis	2.59E-01
	Solvent	4.07E-01
	Lysis:Solvent	4.54E-01

Table A-3: ADDA-ELISA Tukey's Comparison Results

Utility	**Variable**	***P* Value**
NWO – Solvent		
	MeOH 75%-DI	2.03E-01
	PBS 10x-DI	9.03E-01
	PBS 1x-DI	3.12E-01
	PBS 10x-MeOH 75%	6.73E-02
	PBS 1x-MeOH 75%	***9.23E-03***
	PBS 1x-PBS 10x	6.73E-01
NEO – Lysis		
	Microwave-Freeze Thaw	***2.27E-03***
	Sonication-Freeze Thaw	***1.05E-02***
	Sonication-Microwave	6.69E-01
NEO – Solvent		
	MeOH 75%-DI	***3.71E-04***
	PBS 10x-DI	9.97E-01
	PBS 1x-DI	1.00E+00
	PBS 10x-MeOH 75%	***2.72E-04***
	PBS 1x-MeOH 75%	***4.22E-04***
	PBS 1x-PBS 10x	9.91E-01
NEO – Lysis:Solvent		
	Microwave:DI-Freeze Thaw:DI	1.00E+00
	Sonication:DI-Freeze Thaw:DI	1.00E+00
	Freeze Thaw:MeOH 75%-Freeze Thaw:DI	***6.16E-05***

Microwave:MeOH 75%-Freeze Thaw:DI	1.00E+00
Sonication:MeOH 75%-Freeze Thaw:DI	5.37E-01
Freeze Thaw:PBS 10x-Freeze Thaw:DI	1.00E+00
Microwave:PBS 10x-Freeze Thaw:DI	1.00E+00
Sonication:PBS 10x-Freeze Thaw:DI	1.00E+00
Freeze Thaw:PBS 1x-Freeze Thaw:DI	9.97E-01
Microwave:PBS 1x-Freeze Thaw:DI	1.00E+00
Sonication:PBS 1x-Freeze Thaw:DI	1.00E+00
Sonication:DI-Microwave:DI	1.00E+00
Freeze Thaw:MeOH 75%-Microwave:DI	***1.18E-04***
Microwave:MeOH 75%-Microwave:DI	1.00E+00
Sonication:MeOH 75%-Microwave:DI	8.24E-01
Freeze Thaw:PBS 10x-Microwave:DI	1.00E+00
Microwave:PBS 10x-Microwave:DI	1.00E+00
Sonication:PBS 10x-Microwave:DI	1.00E+00
Freeze Thaw:PBS 1x-Microwave:DI	1.00E+00
Microwave:PBS 1x-Microwave:DI	1.00E+00
Sonication:PBS 1x-Microwave:DI	1.00E+00
Freeze Thaw:MeOH 75%-Sonication:DI	***8.18E-05***
Microwave:MeOH 75%-Sonication:DI	1.00E+00
Sonication:MeOH 75%-Sonication:DI	6.70E-01
Freeze Thaw:PBS 10x-Sonication:DI	1.00E+00
Microwave:PBS 10x-Sonication:DI	1.00E+00
Sonication:PBS 10x-Sonication:DI	1.00E+00
Freeze Thaw:PBS 1x-Sonication:DI	1.00E+00
Microwave:PBS 1x-Sonication:DI	1.00E+00
Sonication:PBS 1x-Sonication:DI	1.00E+00
Microwave:MeOH 75%-Freeze Thaw:MeOH 75%	***6.16E-05***
Sonication:MeOH 75%-Freeze Thaw:MeOH 75%	***1.17E-03***
Freeze Thaw:PBS 10x-Freeze Thaw:MeOH 75%	***6.44E-05***
Microwave:PBS 10x-Freeze Thaw:MeOH 75%	***8.89E-05***
Sonication:PBS 10x-Freeze Thaw:MeOH 75%	***6.73E-05***
Freeze Thaw:PBS 1x-Freeze Thaw:MeOH 75%	***1.90E-04***
Microwave:PBS 1x-Freeze Thaw:MeOH 75%	***6.16E-05***
Sonication:PBS 1x-Freeze Thaw:MeOH 75%	***6.16E-05***
Sonication:MeOH 75%-Microwave:MeOH 75%	5.37E-01
Freeze Thaw:PBS 10x-Microwave:MeOH 75%	1.00E+00
Microwave:PBS 10x-Microwave:MeOH 75%	1.00E+00
Sonication:PBS 10x-Microwave:MeOH 75%	1.00E+00
Freeze Thaw:PBS 1x-Microwave:MeOH 75%	9.97E-01
Microwave:PBS 1x-Microwave:MeOH 75%	1.00E+00
Sonication:PBS 1x-Microwave:MeOH 75%	1.00E+00
Freeze Thaw:PBS 10x-Sonication:MeOH 75%	5.58E-01

	Microwave:PBS 10x-Sonication:MeOH 75%	7.08E-01
	Sonication:PBS 10x-Sonication:MeOH 75%	5.79E-01
	Freeze Thaw:PBS 1x-Sonication:MeOH 75%	9.54E-01
	Microwave:PBS 1x-Sonication:MeOH 75%	5.37E-01
	Sonication:PBS 1x-Sonication:MeOH 75%	5.37E-01
	Microwave:PBS 10x-Freeze Thaw:PBS 10x	1.00E+00
	Sonication:PBS 10x-Freeze Thaw:PBS 10x	1.00E+00
	Freeze Thaw:PBS 1x-Freeze Thaw:PBS 10x	9.98E-01
	Microwave:PBS 1x-Freeze Thaw:PBS 10x	1.00E+00
	Sonication:PBS 1x-Freeze Thaw:PBS 10x	1.00E+00
	Sonication:PBS 10x-Microwave:PBS 10x	1.00E+00
	Freeze Thaw:PBS 1x-Microwave:PBS 10x	1.00E+00
	Microwave:PBS 1x-Microwave:PBS 10x	1.00E+00
	Sonication:PBS 1x-Microwave:PBS 10x	1.00E+00
	Freeze Thaw:PBS 1x-Sonication:PBS 10x	9.99E-01
	Microwave:PBS 1x-Sonication:PBS 10x	1.00E+00
	Sonication:PBS 1x-Sonication:PBS 10x	1.00E+00
	Microwave:PBS 1x-Freeze Thaw:PBS 1x	9.97E-01
	Sonication:PBS 1x-Freeze Thaw:PBS 1x	9.97E-01
	Sonication:PBS 1x-Microwave:PBS 1x	1.00E+00
SC – Lysis		
	Microwave-Freeze Thaw	1.14E-01
	Sonication-Freeze Thaw	5.05E-02
	Sonication-Microwave	8.84E-01
SC – Solvent		
	MeOH 75%-DI	6.02E-01
	PBS 10x-DI	***8.96E-03***
	PBS 1x-DI	2.64E-01
	PBS 10x-MeOH 75%	***1.07E-03***
	PBS 1x-MeOH 75%	***3.34E-02***
	PBS 1x-PBS 10x	2.36E-01
FL_W – Solvent		
	MeOH 75%-DI	***5.79E-05***
	PBS 10x-DI	9.16E-01
	PBS 1x-DI	2.38E-01
	PBS 10x-MeOH 75%	***1.43E-04***
	PBS 1x-MeOH 75%	***1.16E-03***
	PBS 1x-PBS 10x	5.43E-01
FL_D – Solvent		
	MeOH 75%-DI	***2.51E-02***
	PBS 10x-DI	5.83E-01
	PBS 1x-DI	2.90E-01
	PBS 10x-MeOH 75%	2.18E-01

	PBS 1x-MeOH 75%	4.72E-01
	PBS 1x-PBS 10x	9.37E-01

Table A-4: UPLC-PDA Tukey's Comparison Results

Utility	Variable	*P* Value
NWO – Solvent		
	MeOH 75%-DI	9.59E-01
	PBS 10x-DI	1.97E-01
	PBS 1x-DI	7.18E-01
	PBS 10x-MeOH 75%	3.95E-01
	PBS 1x-MeOH 75%	4.37E-01
	PBS 1x-PBS 10x	***3.37E-02***
NWO – Lysis:Solvent		
	Microwave:DI-Freeze Thaw:DI	***2.71E-02***
	Sonication:DI-Freeze Thaw:DI	1.00E+00
	Freeze Thaw:MeOH 75%-Freeze Thaw:DI	8.60E-01
	Microwave:MeOH 75%-Freeze Thaw:DI	8.32E-01
	Sonication:MeOH 75%-Freeze Thaw:DI	7.42E-01
	Freeze Thaw:PBS 10x-Freeze Thaw:DI	***4.03E-03***
	Microwave:PBS 10x-Freeze Thaw:DI	1.00E+00
	Sonication:PBS 10x-Freeze Thaw:DI	6.64E-01
	Freeze Thaw:PBS 1x-Freeze Thaw:DI	1.00E+00
	Microwave:PBS 1x-Freeze Thaw:DI	8.86E-01
	Sonication:PBS 1x-Freeze Thaw:DI	1.00E+00
	Sonication:DI-Microwave:DI	***2.94E-02***
	Freeze Thaw:MeOH 75%-Microwave:DI	3.23E-01
	Microwave:MeOH 75%-Microwave:DI	3.51E-01
	Sonication:MeOH 75%-Microwave:DI	4.38E-01
	Freeze Thaw:PBS 10x-Microwave:DI	9.75E-01
	Microwave:PBS 10x-Microwave:DI	5.74E-02
	Sonication:PBS 10x-Microwave:DI	5.13E-01
	Freeze Thaw:PBS 1x-Microwave:DI	6.76E-02
	Microwave:PBS 1x-Microwave:DI	2.97E-01
	Sonication:PBS 1x-Microwave:DI	5.14E-02
	Freeze Thaw:MeOH 75%-Sonication:DI	8.80E-01
	Microwave:MeOH 75%-Sonication:DI	8.54E-01
	Sonication:MeOH 75%-Sonication:DI	7.68E-01
	Freeze Thaw:PBS 10x-Sonication:DI	***4.34E-03***
	Microwave:PBS 10x-Sonication:DI	1.00E+00
	Sonication:PBS 10x-Sonication:DI	6.91E-01
	Freeze Thaw:PBS 1x-Sonication:DI	1.00E+00

	Microwave:PBS 1x-Sonication:DI	9.04E-01
	Sonication:PBS 1x-Sonication:DI	1.00E+00
	Microwave:MeOH 75%-Freeze Thaw:MeOH 75%	1.00E+00
	Sonication:MeOH 75%-Freeze Thaw:MeOH 75%	1.00E+00
	Freeze Thaw:PBS 10x-Freeze Thaw:MeOH 75%	5.28E-02
	Microwave:PBS 10x-Freeze Thaw:MeOH 75%	9.81E-01
	Sonication:PBS 10x-Freeze Thaw:MeOH 75%	1.00E+00
	Freeze Thaw:PBS 1x-Freeze Thaw:MeOH 75%	9.91E-01
	Microwave:PBS 1x-Freeze Thaw:MeOH 75%	1.00E+00
	Sonication:PBS 1x-Freeze Thaw:MeOH 75%	9.72E-01
	Sonication:MeOH 75%-Microwave:MeOH 75%	1.00E+00
	Freeze Thaw:PBS 10x-Microwave:MeOH 75%	5.85E-02
	Microwave:PBS 10x-Microwave:MeOH 75%	9.73E-01
	Sonication:PBS 10x-Microwave:MeOH 75%	1.00E+00
	Freeze Thaw:PBS 1x-Microwave:MeOH 75%	9.85E-01
	Microwave:PBS 1x-Microwave:MeOH 75%	1.00E+00
	Sonication:PBS 1x-Microwave:MeOH 75%	9.61E-01
	Freeze Thaw:PBS 10x-Sonication:MeOH 75%	7.78E-02
	Microwave:PBS 10x-Sonication:MeOH 75%	9.36E-01
	Sonication:PBS 10x-Sonication:MeOH 75%	1.00E+00
	Freeze Thaw:PBS 1x-Sonication:MeOH 75%	9.60E-01
	Microwave:PBS 1x-Sonication:MeOH 75%	1.00E+00
	Sonication:PBS 1x-Sonication:MeOH 75%	9.16E-01
	Microwave:PBS 10x-Freeze Thaw:PBS 10x	***8.30E-03***
	Sonication:PBS 10x-Freeze Thaw:PBS 10x	9.73E-02
	Freeze Thaw:PBS 1x-Freeze Thaw:PBS 10x	***9.76E-03***
	Microwave:PBS 1x-Freeze Thaw:PBS 10x	***4.76E-02***
	Sonication:PBS 1x-Freeze Thaw:PBS 10x	***7.45E-03***
	Sonication:PBS 10x-Microwave:PBS 10x	8.92E-01
	Freeze Thaw:PBS 1x-Microwave:PBS 10x	1.00E+00
	Microwave:PBS 1x-Microwave:PBS 10x	9.88E-01
	Sonication:PBS 1x-Microwave:PBS 10x	1.00E+00
	Freeze Thaw:PBS 1x-Sonication:PBS 10x	9.25E-01
	Microwave:PBS 1x-Sonication:PBS 10x	1.00E+00
	Sonication:PBS 1x-Sonication:PBS 10x	8.65E-01
	Microwave:PBS 1x-Freeze Thaw:PBS 1x	9.94E-01
	Sonication:PBS 1x-Freeze Thaw:PBS 1x	1.00E+00
	Sonication:PBS 1x-Microwave:PBS 1x	9.81E-01
FL_W – Lysis		
	Microwave-Freeze Thaw	3.45E-01
	Sonication-Freeze Thaw	***2.83E-02***
	Sonication-Microwave	1.67E-01

Table A-5: Quantification Type ANOVA Comparison Results

Utility	Variable	*P* Value
NWO		
	Quantification Type	***2.99E-08***
NWO_{DAF}		
	Quantification Type	***5.99E-17***
NEO		
	Quantification Type	***1.15E-07***
FL_W		
	Quantification Type	***1.08E-10***
FL_D		
	Quantification Type	2.64E-01

Table A-6: Quantification Type Tukey's Comparison Results

Utility	Variable	*P* Value
All Utilities		
	Quantification Type	***4.96E-02***
NWO		
	Quantification Type	***2.99E-08***
NWO_{DAF}		
	Quantification Type	***0.00E+00***
NEO		
	Quantification Type	***1.15E-07***
FL_W		
	Quantification Type	***1.08E-10***

Appendix B: Methods and Results for NWO Additional Studies

B.1. Observations of Natural Degradation in Residual Lagoons

NWO lagoon samples that were to be tested for MC by ADDA-ELISA using a state certified laboratory for OEPA permitting were split and sent to Ohio State Environmental Engineering labs for parallel UPLC-PDA analysis. Each of the three lagoons were split into quadrants. Three samples were obtained from each quadrant and mixed to create one composite sample for each lagoon. Samples were collected in December 2019 and September 2020. At the time of sampling, the newest residuals were located in the leftmost lagoon, increasing in age to the rightmost lagoon as seen in **Figure B-1**. Sample preparation followed the outlined methodology in Ohio EPA method 546 (Zaffiro et al., 2016). Results are shown in **Figure B-1**.

B.2. Preliminary Biodegradation Experimentation

The first was performed on site at the WTP and utilized DAF residuals. To replicate the observed natural degradation and investigate impacts of mixing, DAF residuals were collected from the waste line of the DAF facility at the NWO WTP. These samples were diluted with the plant's raw influent water to an estimated 3% solids by volume to represent the approximate percentage at which the solids could be pumped in a WTP process. This mixture was added to a 55-gallon high density polyethylene drum and mixed continuously at 1300 RPM for seven days. Sampling occurred every other day and samples were sent in batch at the end of the experiment to Ohio State University College of Engineering Environmental Labs for quantification. Sample

preparation followed the outlined methodology in Ohio EPA method 546 (Zaffiro et al., 2016) but was quantified by UPLC-PDA only due to time limitations. Results are shown in **Figure B-2**.

B.3. Pretreatment

The second experiment included pre-treating DAF residuals to investigate additions of commonly used additives for disinfection/oxidation in the plant may impact MCs and the biological activity. Each bioreactor contained 700 mL of sample mixture in a 1500mL glass beaker (Fisher Scientific). The bioreactor was then placed on a floc-tester (Lovibond ET 750). There were 6 bioreactors per floc-tester: one control, two different doses of potassium permanganate, and three different doses of sodium hypochlorite. The experiment was performed in duplicate at 25°C and mixed at 100 rpm continuously for a duration of four days. This experiment was impacted by the Covid-19 global pandemic and was unable to be completed in full. Sampling occurred daily from each bioreactor for the following parameters: pH and DO (Orion 5 Start probe, Thermo Scientific)., Optical Density (OD) at 600 nm, 254 nm, and 222 nm (NanoDrop 2000c Spectrophotometer, Thermo Scientific), total MC, and extracellular MC. Obtaining total MCs followed the preparation outlined in Ohio EPA method 546 (Zaffiro et al., 2016) and results were quantified on both UPLC-PDA and ADDA-ELISA. Results are shown in **Figure B-4**.

B.4. Temperature Variation

The third included observations of DAF residuals incubated under varying temperatures, where only extracellular MC-LR was quantified to investigate potential interactions between free MCs and organic matter. There were three total floc-testers, each with bioreactors in triplicate. Each set of bioreactors were housed at 4°C, 25°C, and 30°C and mixed at 100 rpm continuously

for the duration of the experiment, a total of 7 days. Daily, temperatures were checked in each location. Sampling occurred from each bioreactor for the following parameters: DO, Optical Density (600 nm), extracellular MC utilizing DI water as a solvent, and heterotrophic plate counts (HPC's). Results are shown in **Figure B-3**.

B.5. Quantification

Samples were quantified as described in the Methods, apart from only MC-LR being quantified by UPLC-PDA.

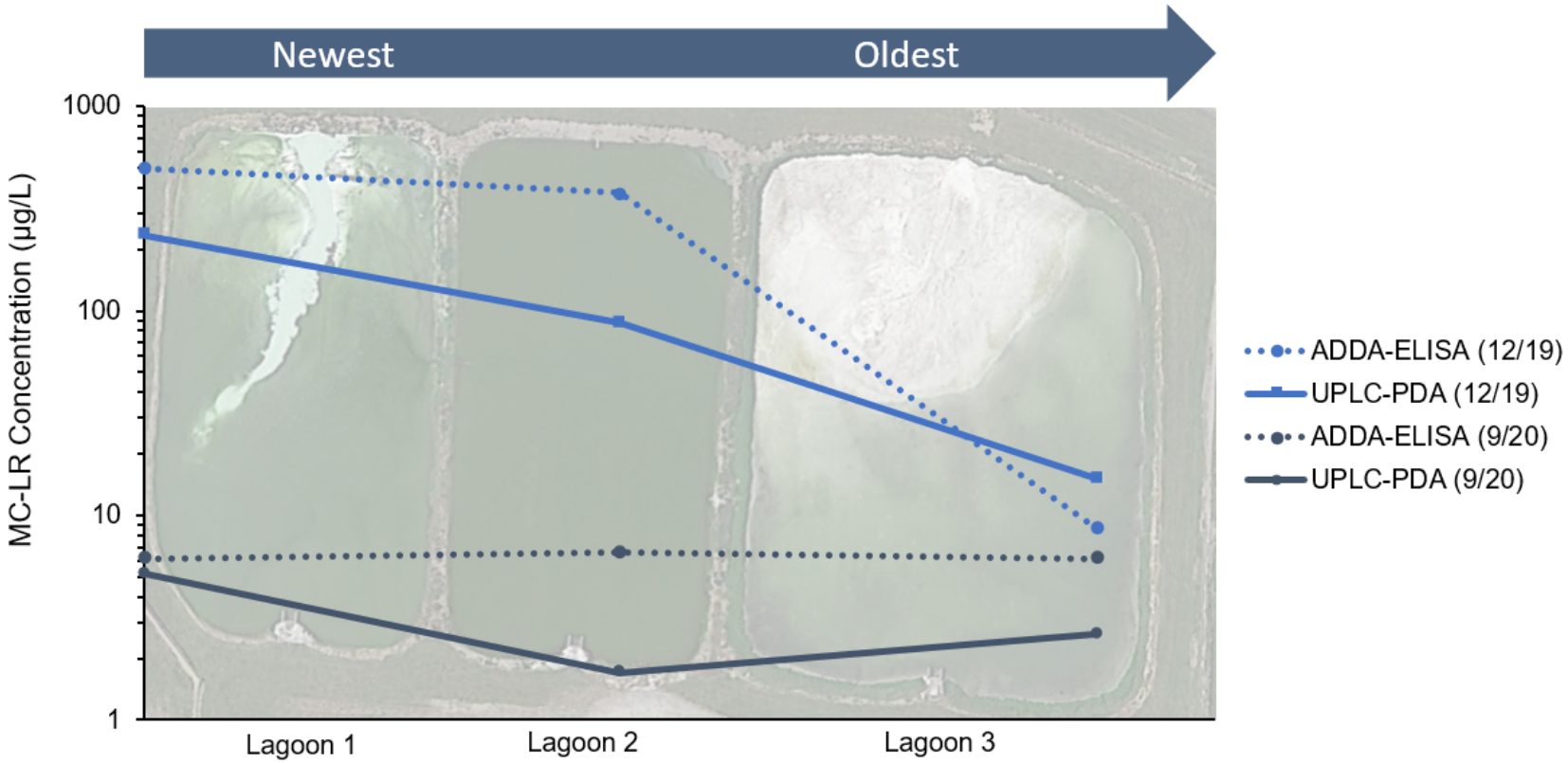

Figure B-1: Natural MC Degradation Observed in WTP Residual Lagoons Increasing in Age at NWO Utility

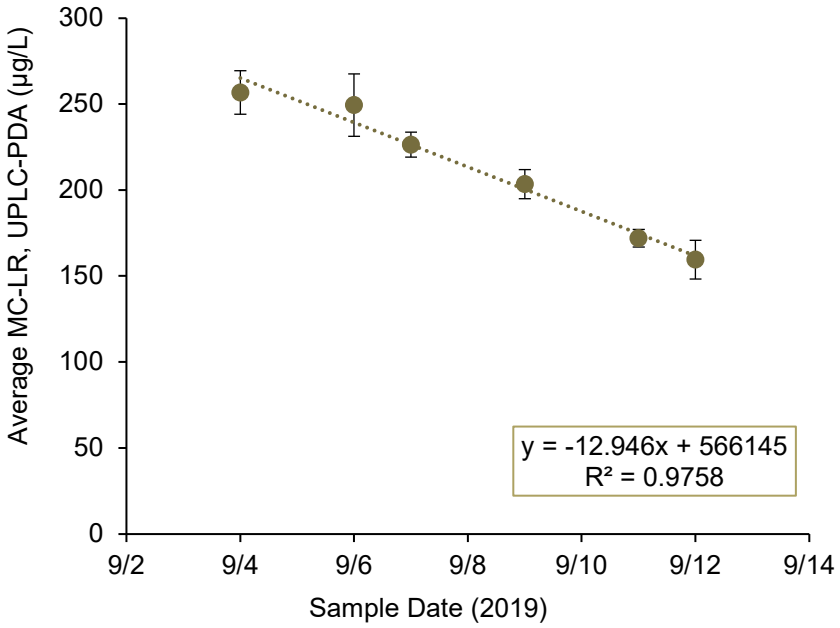

Figure B-2: Preliminary Pilot Testing for Microcystin Degradation.

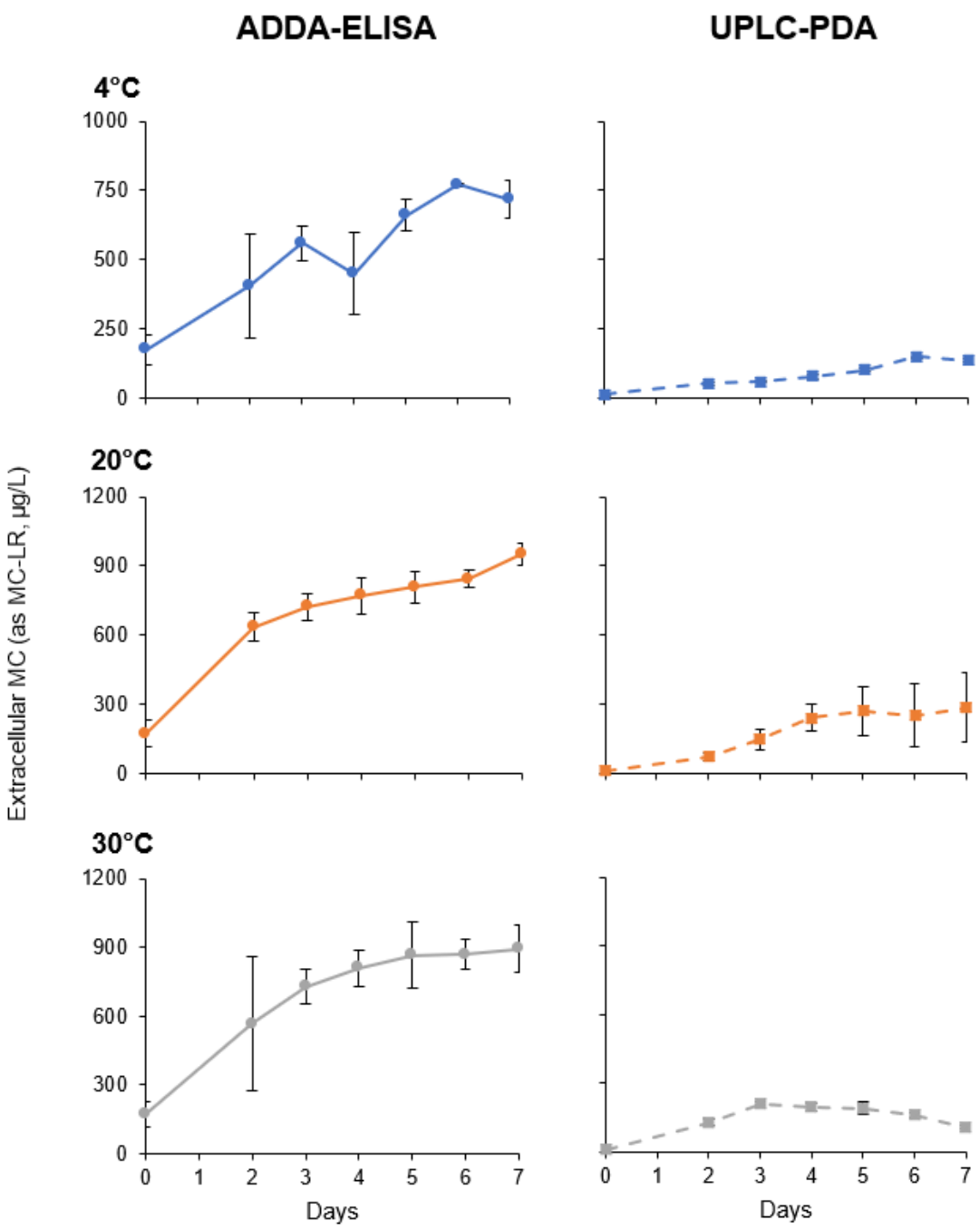

Figure B-3: Impact of Temperature and Mixing on Extracellular Microcystin. Concentration as MC-LR is on the Y axis, time in days is on the X axis. Error bars represent the standard deviation among triplicates.

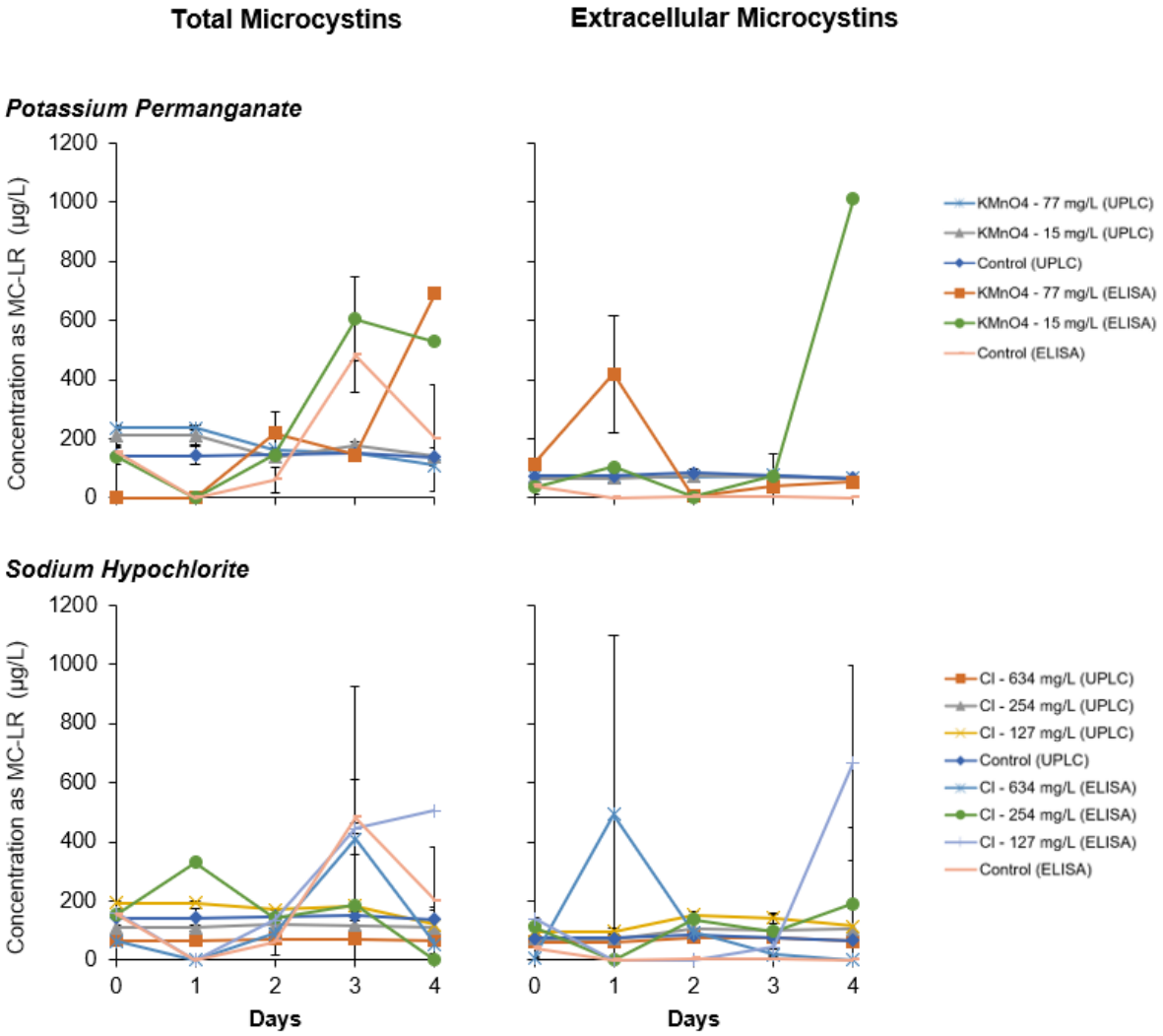

Figure B-4: Impact of Pretreatment and Mixing on Microcystin Concentration in DAF Residuals. Graphs on the left-hand side depict total MCs as MC-LR, graphs on the right-hand side depict extracellular MCs as MC-LR. Residuals treated with KMnO4 are shown in the top two graphs, residuals treated with NaOCl are shown in the bottom two graphs.

Appendix C: Supplemental Figures

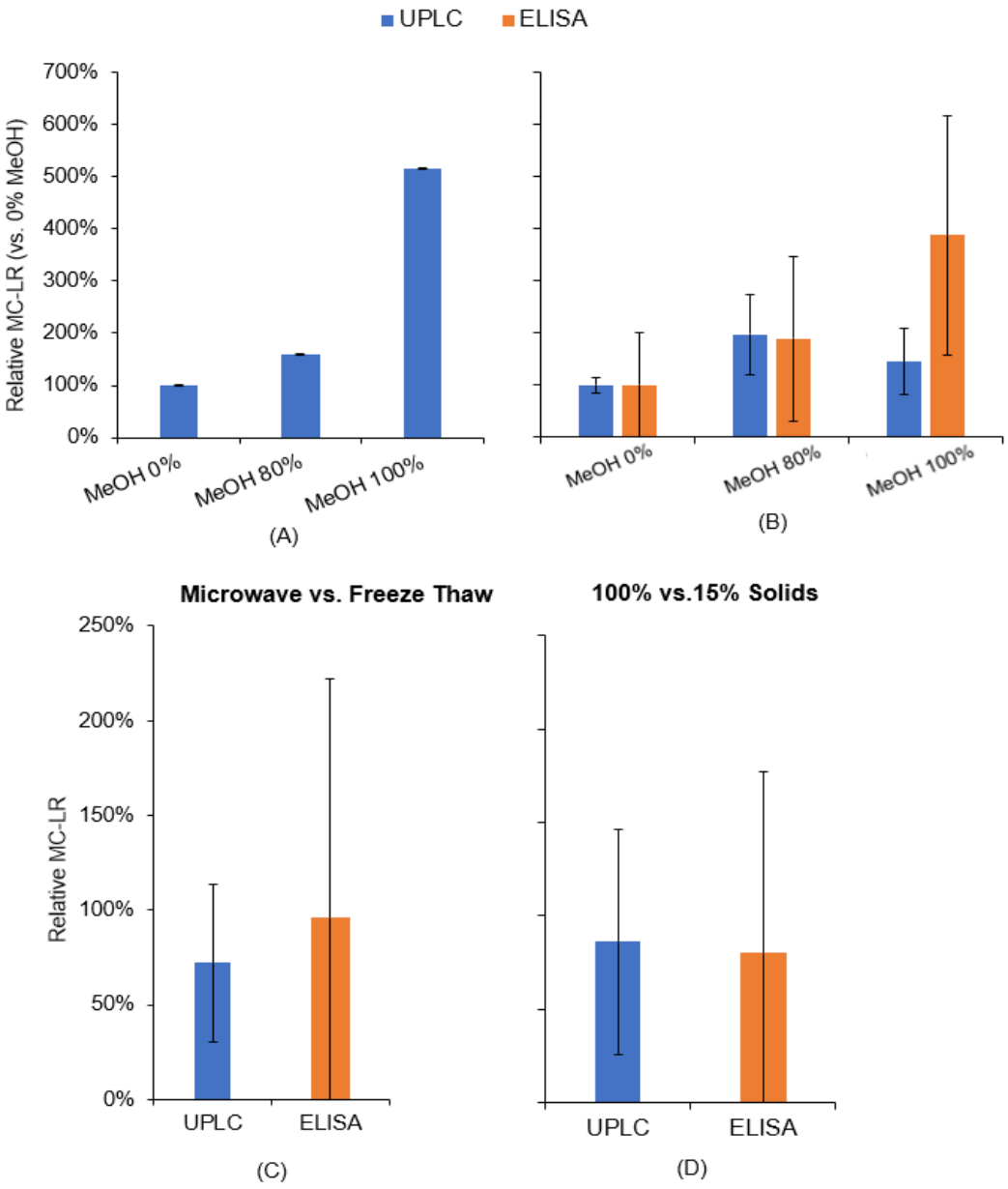

Figure C-1: Preliminary Methods Development Specific to MC-LR. Solvent investigation using UPLC-PDA quantification is shown in top left (A) using NWO_{DAF} residuals. Error bars represent standard deviation between triplicates. Quantification investigation is shown in top right (B) with a second batch of NWO_{DAF} residuals. Error bars represent standard deviation among triplicate samples lysed by freeze thaw or microwave. Lysis investigation is shown in bottom left (C). Error bars represent standard deviation among triplicate samples extracted with DI or MeOH (80%, 100%) at 100% or 15% solids. Investigation of moisture content is shown in bottom right (D). Error bars represent standard deviation among triplicates of samples lysed with freeze thaw or microwave, and extracted with DI or MeOH (80%, 100%).

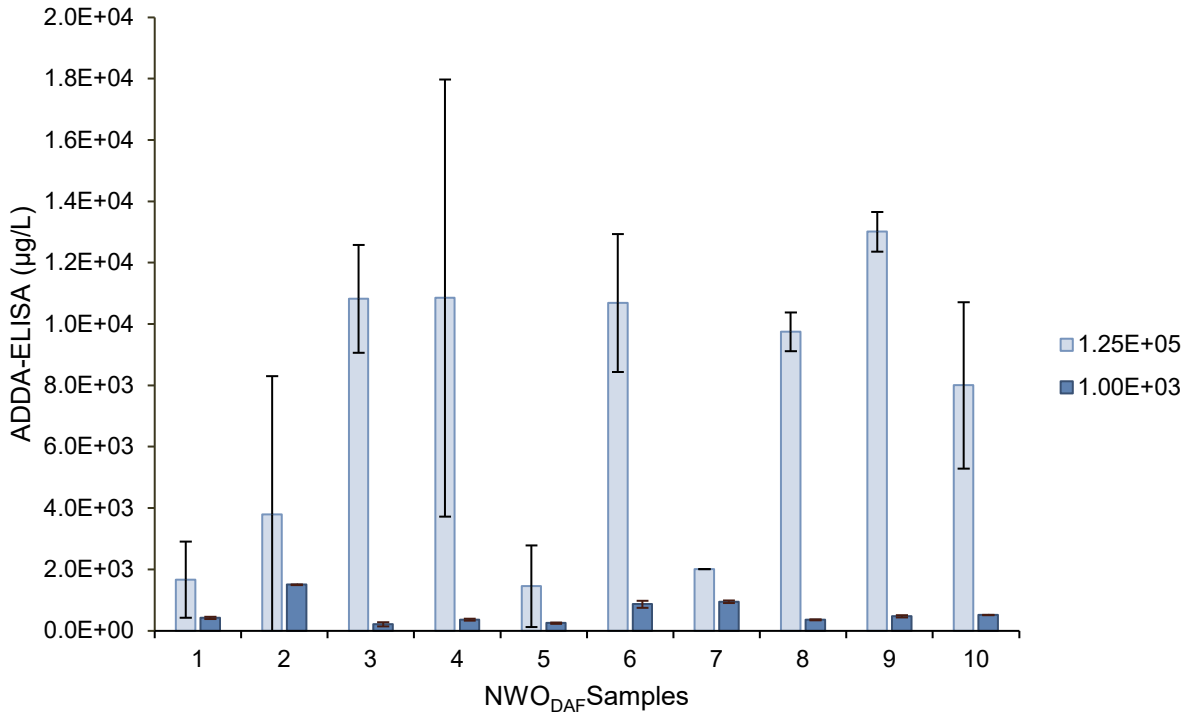

Figure C-2: Calculated ADDA-ELISA MC concentration for hundredfold dilution (see legend) for NWODAF Samples. Y-Axis contains MC concentration. Lysis-Solvent combinations on X-axis are as follows from 1 – 10: Freeze Thaw DI, Freeze Thaw MeOH, Freeze Thaw PBS 1x, Freeze Thaw PBS 10x, Microwave DI, Microwave MeOH, Microwave PBS 1x, Microwave PBS 10x, Sonication PBS 1x, Sonication PBS 10x. Error bars represent the standard deviation between analytical and technical replicates. Dilution was made prior to pipetting samples into the well plate.

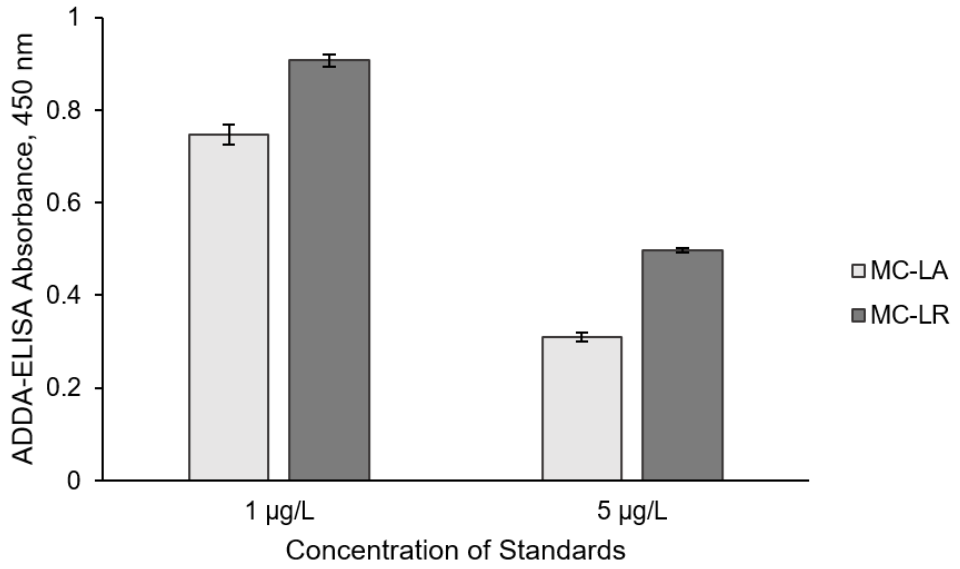

Figure C-3: Absorbance of ADDA-ELISA Wells containing MC-LR and MC-LA standards to final concentration of 1 and 5 µg/L each. ADDA-ELISA is an indirect competitive ELISA; lower absorbance corresponds to higher concentration on a calibration curve. Error bars represent standard deviation among duplicates.