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Exploring Analytical Issues Associated With Oxidation Kinetics in Drinking Water

Shadi Haji Eghrari
Utah State University

EXPLORING ANALYTICAL ISSUES ASSOCIATED WITH OXIDATION KINETICS IN DRINKING WATER

by

Shadi Haji Eghrari

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Approved:

_____	_____
Craig Adams, Ph.D. Major Professor	William Doucette, Ph.D. Committee Member
_____	_____
Charles Miller, Ph.D. Committee Member	Ryan Dupont, Ph.D. Committee Member
_____	_____
David Stevens, Ph.D. Committee Member	D. Richard Cutler, Ph.D. Interim Vice Provost of Graduate Studies

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ABSTRACT

Exploring Analytical Issues Associated with Oxidation Kinetics in Drinking Water

by

Shadi Haji Eghrari, Doctor of Philosophy

Utah State University, 2021

Major Professor: Dr. Craig Adams
Department: Civil and Environmental Engineering

This dissertation consists of two different sections. The first section is an analytical method validation for haloamines in seawater and second evaluates cyanotoxin oxidation kinetics based on ELISA analysis.

Section One (Haloamines): Disinfection of marine aquaria results in the formation of free chlorine species. Both ozone and chlorine addition can lead to the rapid formation of free bromine species. Free chlorine and free bromine both react with ammonia to form haloamines. Key parameters affecting haloamine speciation include pH, chlorine-to-ammonia ratio and bromide concentration.

The purpose of this research was to examine the occurrence of chloramines and bromamines during the chlorination of waters. In this study, a suite of analytical methods was used including a colorimetric method, UV spectrophotometry, and head-space gas chromatography. The effect of bromide concentration was observed to exert a significant effect on the kinetics and stability of Haloamines, and with no bromide present, the half-life for chloramines was on the order of two to six hours. At bromide concentration of 50 mg/L as NaBr, however, the half-life of chloramines drops to just a few minutes.

Section Two (Cyanotoxins): Microcystins (MCs) are the most commonly detected cyanotoxins of major health concern in surface and drinking water. Utilities commonly use enzyme-linked immunosorbent assays (ELISA) to monitor concentration of cyanotoxins within their plants due to the cost effectiveness of ELISA versus liquid chromatography

tandem mass spectrometry (LC-MS/MS). ELISA often produces higher indicated concentrations as compared to LC-MS/MS because ELISA measures mixture of MC variants in a water sample.

The objective of this work is to assess the difference in apparent removal rates for MCs based on ELISA analysis versus based on LC-MS/MS analysis. The data demonstrates that ELISA readings averaged greater than LC-MS/MS concentrations for the split samples. This work provides an estimate of the 95%-percent confidence interval for the difference in oxidation rates predicted with based on ELISA readings versus specific variants by LC-MS/MS.

(157 pages)

PUBLIC ABSTRACT

Exploring Analytical Issues Associated with Oxidation Kinetics in Drinking Water

Shadi Haji Eghrari

This dissertation contains two different section that pertain to two different subjects.

Section One (Haloamines): Disinfection with ozone and chlorine is critical in protecting the public and animals from pathogens in pools. Disinfection results in in the formation of haloamines from the unintended reactions of human and animal inputs and bromide/chloride with oxidants and disinfectants which cause health problems.

The purpose of this research was to examine the occurrence of haloamines during the chlorination of saltwaters. In this study, the effect of bromide concentration was observed to exert a significant effect on the stability of Haloamines, and with no bromide present, the half-life for Haloamines was on the order of two to six hours.

Section Two (Cyanotoxins): There is an alarming increase in the frequency and magnitude of cyanobacterial blooms worldwide. Cyanobacteria produce a variety of toxins including microcystins and cylindrospermopsin. Microcystins are the most commonly detected cyanotoxins of major health concern in surface and drinking water.

Utilities commonly use enzyme-linked immunosorbent assays (ELISA) to monitor concentration of cyanotoxins within their plants due to the cost effectiveness of ELISA versus LC-MS/MS. ELISA often produces higher indicated concentrations as compared to LC-MS/MS because ELISA measures mixture of microcystins variants in a water sample. However, regulatory authorities need to be convinced that the ELISA results are reliable, even when it disagrees with LC-MS/MS result.

The objective of this work is to assess the difference in apparent removal rates for microcystins based on ELISA analysis versus based on LC-MS/MS analysis. The data demonstartes that ELISA readings averaged greater than LC-MS/MS concentrations for the split samples.

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Shadi Haji Eghrari

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LIST OF ABBREVIATIONS

ADDA	3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid
AOP	Advanced Oxidation Process
CAAS	Cyanotoxin Automated Assay System
CCV	Continuous Calibration Verification
CYN	Cylindrospermopsin
DBA	Dibromamine
DBP	Disinfection by-product
DCA	Dichloramine
DPD	N,N-diethyl-p-phenylenediamine
ELISA	Enzyme-Linked Immunosorbent Assay
FB	Free Bromine
FC	Free Chlorine
GAC	Granulated Activated Carbon
GC	Gas Chromatography
HAA	Haloacetic Acid
HAB	Harmful Algal Blooms
HILIC	Hydrophilic Interaction Liquid Chromatography
HFB	Hexafluorobenzene
LC	Liquid Chromatography
LRB	Laboratory Reagent Blank
MBA	Monobromamine
MC	Microcystin
MCA	Monochloramine
MF	Microfiltration
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NAS	Natural and Artificial Seawater
NF	Nanofiltration
NOM	Natural organic matter
PAC	Powdered Activated Carbon
PM	Permanganate
PPIA	Protein Phosphatase Inhibition
PP2A	Protein Phosphatase 2A
QCS	Quality Control Standard
RO	Reverse Osmosis
RSD	Relative Standard Deviation
TBA	Tribromamine
TCA	Trichloramine
TDS	Total Dissolved Solids
THM	Trihalomethane

CHAPTER 1

INTRODUCTION

This dissertation consists of two sections. The first section is an analytical method validation for haloamines in natural and artificial seawater, and second section is about development of oxidation kinetics based on ELISA analysis for cyanotoxins. This dissertation is divided into two sections comprising a total of nine chapters, including this Introduction (Chapter 1).

In Section One ("Haloamines"), a literature review for secondary oxidant and disinfection byproduct occurrence resulting from ozone or chlorine application in natural and artificial seawater (NAS) aquaria and pools will be presented in Chapter 2. Developing practical options for monitoring haloamines in NAS water will be discussed in Chapter 3. This is followed by a discussion of the formation of haloamines and related disinfection byproducts during chlorination and ozonation of marine aquaria in Chapter 4. A number of conclusions and indications of future work for formation of secondary oxidant during chlorination and ozonation of NAS water is presented in Chapter 5.

In Section Two ("Cyanotoxins"), a literature review for cyanotoxins and the removal of cyanotoxins from drinking water is presented in Chapter 6. Differences in apparent oxidation kinetics for microcystins analyzed by on ELISA versus LC-MS/MS analytical methods will be discussed in Chapter 7. A number of conclusions and indications of future work for analysis of cyanotoxins are offered in Chapter 8.

Appendices for Sections One and Part Two follow in Chapter 9. The two main chapters—Chapters 4 (Haloamines) and 7 (Cyanotoxins)—are based on articles that will be submitted to peer-reviewed journals in the near future.

Chapter 4 (Haloamines) describes how a suite of analytical methods was used to study the formation of haloamines in seawater/salt water systems. The analytical methods included are: standard method 4500 Cl. G (APHA, AWWA, and WEF, 2005), UV spectrophotometry, and gas chromatography. These methods were used to examine the concentration of haloamines under a wide range of water quality parameters in laboratory systems. Furthermore, Chapter 4 describes the results of studies focusing on the formation, stability, and occurrence of chloramines and bromamines during the chlorination of waters with varied total dissolved solids, bromide concentration and pH.

The purpose of Chapter 7 (Cyanotoxins) will be to assess the difference in apparent removal rates for microcystins from natural harmful algal blooms based on ELISA analysis as compared to LC-MS/MS analyses. The ELISA analysis measures the mixture of microcystin variants (with varied cross-reactivity), while the LC-MS/MS method nominally measures only six MC variants, albeit with great accuracy. The goal of the work was to establish estimates of confidence intervals around microcystin-LR removal that represent expected removals of an ELISA-based concentration measurement method with natural waters.

CHAPTER 2

LITERATURE REVIEW AND OBJECTIVES - HALOAMINES

2.1 Marine Aquaria and Pools

Disinfection is critical in protecting the public from pathogens in natural and artificial seawater (NAS) aquaria and pools (Geldreich, 1989). In NAS aquaria and pools are disinfected continuously because it is important to have a healthy environment in a swimming pool for both people and animals (e.g., dolphins in aquatic parks).

Generally, water purification steps involve oxidation, coagulation, settling, disinfection, and filtration. Coagulation-flocculation is not normally used in swimming pool water treatments in the US, though in Germany, for example, it is a requirement to apply water treatments in swimming pool (Zwiener et al., 2007). Filters are essential in preserving water clarity and decreasing disinfectant demands by removing the suspended materials from pools. Sand filters, ultrafine filters and cartridge filters are the most commonly used filters in pools industries across the United States (Hagen, 2003; Glauner et al., 2005).

Water disinfection is the process of deactivation or the killing of undesirable microorganisms, which results in the termination of their growth and reproduction. There are different disinfection methods used in water treatments, including oxidation using free chlorine (hypochlorous acid (HOCl), and hypochlorite (OCl^-)), chlorine dioxide (ClO_2), monochloramine (NH_2Cl), ozone (O_3), permanganate (MnO_4^-); by photolysis (UV irradiation); or by use of other chemical disinfectants (AWWA, 1990).

Chlorine is the most commonly used form of disinfectant in NAS aquaria and pools. Chlorine is relatively inexpensive, and it can easily be applied and controlled. (Griffiths, 2003; Anipsitakis et al., 2008; AWWA, 2005). Regardless of the form of application (gas, liquid, or solid), chlorine, when added to water, forms hypochlorous acid (HOCl) and hypochlorite ions (OCl^-). Hypochlorous acid and hypochlorite ions constitute free chlorine (FC). The hypochlorite ions react with H^+ depending on the pH, establishing the equilibrium with hypochlorous acid (Deborade and Gunten, 2008; White, 1999):

$$OCl^- + H^+ \leftrightarrow HOCl \qquad (2.1)$$

Equation 2.1 demonstrated that the gas form of elemental chlorine tends to decrease pH levels. For maintaining disinfection efficiency in the operation of swimming pools it is important to adjust pH in pools. An acidic pH conditioner is necessary when hypochlorite salts are used in pools, while a basic pH conditioner is needed when chlorine is used in gaseous form. The pKa of hypochlorous acid (pKa=7.54) dictates the proportionality of hypochlorous acid and hypochlorite ions which impacts its disinfection efficiency. Hypochlorous acid is nominally one hundred times more effective than hypochlorite ions. (Deborade and Gunten, 2008; White, 1999). In swimming pools, disinfectant residual levels vary around the world ranging from as low as 0.3 mg/L to as high as 5 mg/L as FC. (Pool Operation Management, 2009).

2.2 Chlorine, Ozone, and Haloamines Chemistry

FC may be present in seawater and salt water pools either from direct dosing or they can be formed from the reaction of added ozone with chloride ion. Ozone is not only used to control bacteria and other microorganisms but can also provide clarity of water through microflocculation and color reduction by oxidation. Ozone is the most powerful oxidant among the commonly used water disinfectants with an oxidizing potential of 2.07 eV.

When ozone is added to NAS aquaria and pools, the high chloride and bromide levels cause a rapid conversion of the ozone to FC and free bromine ($HOBr/OBr^-$) (FB) (Reed and Adams, 2002; Qiang et al., 2013; Qiang et al., 2015; Adams et al., 2013). In a competing reaction, ozone also reacts with FB to form bromate (BrO_3^-) (Naumov and von Sonntag, 2008). Low-bromide artificial salt waters may contain bromide levels near or below 1 mg/L bromide such that both FC and FB are formed. The relative amount of ozone reacting with chloride and bromide is governed by their relative reaction rates, as demonstrated below (Haag and Hoigne, 1983; Hoigné et al., 1985):

$$O_3 + Cl^- \rightarrow O_2 + OCl^- \qquad (2.2)$$

$$O_3 + Br^- \rightarrow O_2 + OBr^- \qquad (2.3)$$

$$Br^- + OCl^- \rightarrow OBr^- + Cl^- \qquad (2.4)$$

Higher bromide seawater and salt water may often contain bromide on the order of 65 mg/L or greater (Flury and Papritz, 1993), thus driving most ozone to FB rather than FC species. Further, FC can itself react with bromide to form FB (Grguric et al., 1994; Bousher et al., 1986):

$$OCl^- + Br^- + H_2O \rightarrow Cl^- + HOBr + OH^- \quad (2.5)$$

Chloramines are formed by the reaction of FC and ammonia/ammonium (NH_3/NH_4^+), (Ammonia is present due to the excretions from the animals in the basins), which are shown non-stoichiometrically as follows (Hailin et al., 1990; Bryant et al. 1992):

$$HOCl/OCl^- + NH_3/NH_4^+ \rightarrow NClH_2 \quad (2.6)$$

$$HOCl/OCl^- + NClH_2 \rightarrow NCl_2H \quad (2.7)$$

$$HOCl/OCl^- + NCl_2H \rightarrow NCl_3 \quad (2.8)$$

When bromide is not present, the key haloamines formed are the chloramines including trichloramine (TCA, NCl_3), dichloramine (DCA, NCl_2H) and monochloramine (MCA, NClH2) (Shah et al., 2015). TCA reacts further with FC to convert to molecular nitrogen (N_2). The formation and subsequent removal of chloramines and molecular nitrogen are controlled by parameters including pH, temperature, chlorine to ammonia ratio, presence of natural organic matter (NOM) etc. These reactions are classically referred to as breakpoint chlorination with a maximum of MCA forming at nominally 5:1 mg Cl_2/mg NH_3 dose ratio, DCA and TCA forming thereafter until they reach the breakpoint (complete conversion to N_2) at nominally a 7:1 mg Cl_2/mg NH_3 dose ratio (Kumar et al.,1987; Desiderio and Nibbering, 2010; Pressley et al., 1972).

Total dissolved solids (TDS) in high quality drinking water may range from 10 to 250 mg/L. In contrast, poor quality brackish water sources may spike to 10,000 mg/L TDS,

while aquaria waters with natural or artificial seawater may contain as high as 50,000 mg/L TDS. Seawater has approximately 65 mg/L of natural bromide (Flury and Papritz, 1993). Artificial seawater also has bromide present from impurities in NaCl although the exact amount is unspecified.

When sufficient bromide is present bromamines are formed by the reaction of ammonia with FB. Similar to chlorination reactions, bromination reactions that take place result in a mixture of chlorinated, brominated, and bromochlorinated species of disinfection byproducts. The bromine substitution reaction is more rapid than chlorine with organic compounds (Westerhoff et al., 2004).

The chemistry associated with chlorination and the ozonation of seawater (natural) and salt water (artificial) is complex due to high concentrations of inorganics (e.g., chloride and bromide ions) and organics (e.g., natural organics, animal fecal matter, and urea). Modeling work by Reed and Adams (2003) has shown that bromide concentrations play an important role in the chemical speciation within aquaria water. Due to a much greater concentration of bromide in natural seawater (as well as in organic species) than in typical artificial seawater (saltwater), very different and complex chemistries may occur in the two systems. Previous research has found that if salt water contains even 1 mg/L of bromide, ozonation leads to a greater formation of FB than FC (Reed and Adams, 2002).

2.3 Disinfection By-Products in NAS Aquaria and Pools

Disinfection by-products (DBPs) are formed from the unintended reactions of NOM, human inputs (e.g., constituents of sweat and urine, skin particles, hair, cosmetics, and other personal care products) and bromide/iodide with oxidants and disinfectants

(Weisel et al.,2009). It is important to minimize the concentration of DBPs by improving the design and operation systems of treatment systems (Richardson et al., 2007). Several factors affect the types and concentrations of DBPs such as the type and dose of the disinfectant used, temperature of the swimming pool, and makeup compositions of aquaria and swimming pool water (Richardson et al., 2010; Chowdury et al, 2014). To maintain a healthy environment, swimming pools use relatively higher doses of FC to ensure free residuals in water. Higher FC, temperature, NOM loads, and water recirculation can ease DBPs formation in swimming pools so that high levels of DBPs in water and air can be seen in swimming pools (Richardson et al., 2007; Kim et al., 2002; LaKind et al., 2010).

Trihalomethanes (THMs) was discovered in drinking water in the early 1970s, and as a consequence, most research has focused on improving our understanding of DBPs. More than 600 DBPs have been identified in disinfection treated waters, and many of them are mutagenic or carcinogenic. These DBPs represent less than 40% of organic halogens and more than 60% of organic halogens are still unknown (Richardson et al., 2010; Kim et al., 2002). Until now, DBPs identified in swimming pools contain THMs, haloacetic acids (HAAs), haloacids, halodiacids, iodo-THMs, haloaldehydes, halonitriles, haloketones, halonitromethanes, bromate, haloamides, haloalcohols, nitrosamines, combined available chlorine, etc. The most common DBPs in swimming pool are chloramines, THMs and HAAs (Richardson et al., 2010).

Unfortunately, the presence of DBPs in swimming pools is unsafe and cause health problems. DBPs can be ingested accidentally, inhaled or absorbed through the skin. The studies showed that about 50 mL/hour of water could be ingested by a child and around 25 mL/hour by an adult swimmer in a swimming event (Kim, 1997; ACC, 2002; Dufour et

al., 2006). Past studies which have reported DBPs levels in human blood have shown that DBPs concentrations are higher in blood after swimming, showering and bathing than ingesting drinking water (Aggazzotti et al., 1998; Caro and Gallego, 2007). Several studies have demonstrated a correlation between health issues and swimming pools. There is evidence that irritant chemicals (i.e. TCA) from swimming pool air may contribute to the incidence of asthma in children and adults (Zock et al., 2007; Richardson et al., 2010).

Key classes of regulated DBPs in drinking water include THMs (including chloroform ($CHCl_3$), bromoform ($CHBr_3$), chlorodibromomethane ($CHClBr_2$), bromodichloromethane ($CHCl_2Br$)), and HAAs (including monochloroacetic acid ($CH_2ClCOOH$), dichloroacetic acid ($CHCl_2COOH$), trichloroacetic acid (CCl_3COOH), monobromoacetic acid ($CH_2BrCOOH$), and dibromoacetic acid ($CHBr_2COOH$)), bromate (BrO_3), and chlorite (ClO_2^-) are DBPs currently regulated by the United States Environmental Protection Agency (U.S. EPA). The maximum contaminant level for HAA5 (the sum of five HAAs) is 60 μg/L, for THM4 (the sum of four THMs) it is 80 μg/L, for bromate it is 10 μg/L, and for chlorite it is 1000 μg/L (EPA, 2008). Iodoacetic acids and halonitromethanes (HNMs) are the unregulated emerging DBPs that have recently been found in disinfected drinking water and are much more toxic than regulated DBPs (Richardson et al., 2003).

Swimming pool literature suggests that chloramines are a primary eye irritant, TCA being the strongest irritant followed by DCA. MCA, and FC may have a very minor contribution to any eye irritation. Eye irritation may occur when the sum of chloramine concentrations is greater than 0.2 mg/L (Bernard et al., 2003; Jacobs et al., 2007; King et

al., 2006). However, no literature thus far reported what individual concentrations of DCA, or TCA can cause with respect to eye irritation.

TCA is more volatile than MCA and DCA is the most odiferous of the chloramines. TCA is also known to be an irritant gas (eyes, pulmonary, etc.) in the gas phase coming off swimming pools. In a case of an actual swimming pool, TCA was observed in water samples at roughly 100 μg/L as Cl_2 (Li and Blatchley Iii, 2007). Also, in the indoor air of chlorinated and brominated swimming pools, concentrations of TCA have been reported as high as 170-430 and 70-100 μg/m3, respectively (Richardson et al., 2010). TCA is also commonly considered to be present and a significant secondary oxidant during the disinfection of seawater and salt water aquaria. This assumption may be a result, in part, by a lack of consideration of the effects of bromide on the chemistry, and the difficulty in measuring bromamines and FB directly in the water matrix.

2.4 Problem Statement

The drinking water field is only in its early stages of understanding the chemistry and toxicology of emerging disinfection byproducts (DBP). Compared with DBPs in drinking water, very little is known about NAS aquaria and pools water DBPs, due to the complexity of the systems, difficulties in chemical analysis in a high salt matrix, and to the lack of literature and studies in this field (Richardson et al., 2011).

The high toxicity of emerging DBPs deserves routine monitoring and further research. Reliable and rapid analytical methods are needed in order to study these emerging DBPs in NAS aquaria water and swimming pool water. The water chemistry in NAS aquaria and pools is much more complicated than the drinking water and any other fresh

water. In addition to the high salt and high bromide, the water is also enriched with animal feed, skin, and bodily excretions such as sweat, urine, fecal matter, pathogens, and algae. Marine park aquaria water is more complex than swimming pool water and these waters may contain thousands of chemicals (Chowdhury et al., 2014). Disinfection treatments of marine aquaria waters used to prevent the pathogens and algae growing are generally chlorination and ozonation and are typically at higher dosages than those contained in drinking water. Our understanding of the formation and control of DBPs in NAS aquaria and swimming pools, therefore, has an important impact on public health, especially when one considers the widespread usage of swimming pools and NAS aquaria in the US. Information on DBPs chemical species and their concentrations in marine park waters is minimal. Similarly, little data exists on eye/skin irritation in marine park aquaria water. Because conventional analytical methods generally do not work well for the complex matrix water samples, DBP research in marine aquaria water is more challenging than in drinking water or even wastewater systems (Richardson, 2009).

It is of critical importance to understand the speciation and stability of haloamines as a function of water quality parameters during the chlorination of seawater and saltwater. Key parameters affecting chloramine speciation and stability (in low bromide waters) include pH and chlorine to ammonia ratio. The bromide concentration impacts the formation of free bromine (from both ozone and free chlorine) as well as the direct conversion of chloramines to bromamines. Ionic strength (as related to TDS) can also have a significant impact on the reaction kinetics with a relative impact on specific reactions dictated in large part by the reaction order.

2.5 Purpose and Objective of Section 1

The main objective of this research was to improve our understanding of the occurrence and formation of haloamines under NAS conditions. The purpose of this study is to examine the formation, stability, and occurrence of chloramines and bromamines during the chlorination of waters with varied total dissolved solids, bromide concentration, pH, and oxidant dosing conditions. The specific objectives of this work include:

1. Evaluating the suitability of standard method 4500 Cl. G. DPD Colorimetric Method (APHA, AWWA, and WEF, 2005) for measuring and differentiating TCA, DCA, MCA, and FC in NAS water.
2. Developing and evaluating a head-space gas chromatography-mass spectrophotometry method for chloramines and bromamines in NAS water.
3. Studying the formation, stability, and decay of FC and FB, in addition to chloramines and bromamines as a function of water quality parameters.

CHAPTER 3

DEVELOPING PRACTICAL OPTIONS FOR MONITORING HALOAMINES IN NATURAL AND ARTIFICIAL SALT WATER

3.1 Introduction

While the focus in NAS aquaria and pools and related studies has often been on TCA as an irritant factor in NAS pools, the role of tribromamine (TBA) has been investigated to a lesser degree. The chemical structure of TCA, DCA, MCA are NCl_3, NCl_2H, and $NClH_2$, respectively. The corresponding bromamines include tribromamine (TBA), dibromamine (DBA), and monobromamine (MBA) with the formula NBr_3, NBr_2H, and $NBrH_2$, respectively. Mixed chloramines and bromamines also exist including the trihalogenated $NClBr_2$ and NCl_2Br; and the dihalogenated NClBrH. In general, brominated DBPs are considered more toxic than their chlorinated counterparts (Plewa et al., 2004). The relative amounts of bromide in NAS water have been shown to dictate the degree to which compounds move toward chlorinated or brominated species. Bromamines are likely to be as important as chloramines when leading to eye and skin irritations. This depends, however, on bromide levels and treatment approaches.

The wet chemical standard method 4500Cl-G titled DPD Colorimetric Method (SM4500Cl-G) is currently the best wet chemical method available for measuring and differentiating TCA, DCA, MCA, and FC in drinking waters. In fact, this method is used to calibrate TCA standards prepared in low TDS (low salt) water (Kosaka et al., 2010; Hosoda et al. 2009).

In this method, the chlorine residual is determined using a spectrophotometer or filter photometer. N, N-diethyl-p-phenylenediamine (DPD) is used as the indicator. The DPD is oxidized by chlorine to two oxidation products. At a near neutral pH, the Würster dye which is relatively stable semi-quinoid cationic compound accounts for the magenta color in the DPD colorimetric test is the principal oxidation product. DPD amine can be further oxidized to a relatively unstable, colorless imine compound is favored resulting in an apparent "fading" of the colored solution (Figure 3.1) (Harp, 2002).

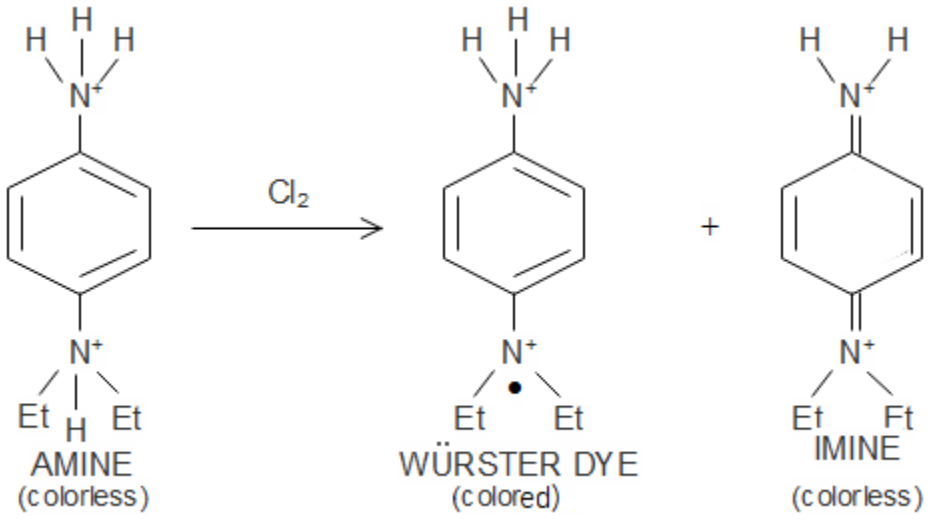


Figure 3.1. DPD-Chlorine Reaction Products (Harp, 2002).

The DPD Würster dye color has been measured photometrically at a wavelength of 515 nm. The intensity of the color is measured against known values from a standard curve.

Because of the difficulty in getting accurate chlorine standards, potassium permanganate is used as the standard for establishing the standard curve (Standard Methods, 1995).

The purpose of this study is evaluating and developing SM4500Cl-G and head-space gas chromatography- mass spectrometry (GC-MS) method for chloramines and bromoamines in NAS water. In this work, the formation of chloramines under varying types and concentrations of dissolved inorganic salt common to NAS water was studied. While these interferences do cause significant accuracy and/or precision issues with SM4500Cl-G, these interferences have not been thoroughly assessed. The specific purpose of this work is to characterize these interferences quantitatively, such that accurate estimates for TCA, DCA, and MCA can be determined. Key to this development is the use of GC to be able to most accurately determine the concentration of TCA, DCA, and MCA in complex NAS matrices. With the use of the GC method, it is possible accurately determine correlations between the true measurements for TCA, DCA, and MCA and the predicted measurement via SM4500Cl-G.

The head-space GC-MS method, which is the most accurate and robust analysis for chloramines, is developed. Because the direct injection of water to GC-MS was not recommended, head-space GC-MS method is a potential alternative method for analysis of chloramines and bromoamines that can be efficiently partitioned into the headspace-gas volume from the complex NAS matrices. In particular, head-space GC-MS method allows differentiation of each species including TCA which was provide highly valuable insight in the causes and control of secondary oxidants.

3.2 Materials and Methods

3.2.1 Standard Solutions

MCA *standard solution* – The MCA standard solution was prepared daily by mixing a free chlorine (NaOCl) solution with a slight excess of an ammonium chloride (NH_4Cl) solution at a chlorine-to-ammonia molar ratio of 1.00:1.03. Both solutions were adjusted to a pH level of 10 with sodium hydroxide (NaOH) before mixing them together, based on work by Kosaka et al., (2010) and Shang and Blatchley III (1999).

DCA standard solution – The DCA standard solution was prepared daily by slowly pouring NaOCl solution over NH_4Cl solution at a chlorine-to-ammonia molar ratio of 1.80:1.00 over a 2-minute period with rapid stirring. Both solutions were adjusted to pH 5 with 1 M acetic acid before mixing (Kosaka et al., (2010); Shang and Blatchley III, 1999).

TCA standard solution – The TCA standard solution was prepared daily by pouring a NaOCl solution over NH_4Cl solution at a chlorine-to-ammonia molar ratio of 3.15:1.00 with rapid stirring at pH 6 with a 5 mM phosphate buffer (Kosaka et al., 2010; Shang and Blatchley III, 1999).

MBA standard solution – The MBA standard solution was prepared by adding a drop wise 90.1 ml of a saturated solution of aqueous Br_2 (0.058M) to 45 ml of concentrated ammonia hydroxide, contained in ice water, cooled and equipped with a stir bar at the rate of about one milliliter per second (Heasley et al., 2013).

DBA standard solution – The DBA standard was prepared by mixing two grams of anhydrous magnesium sulfate to the MBA standard solution in water, and extracting with an ether a portion of 10 ml to 2 ml MBA standard solution in water to ether (Heasley et al., 2013).

3.2.2 Standard DPD Colorimetric Method (SM4500Cl-G)

In this method, the chlorine residual is determined colorimetrically with DPD and is used as an indicator for differentiation of chloramines. In the presence of chlorine and chloramines, the DPD indicator solution has a red color proportional to the chlorine and chloramines present. The approximate minimum detectable concentration is 10 μg/L as Cl2 (Appendix HA-1). Potassium permanganate solution was used to calibrate the Hach spectrophotometer (Model DR 3900) based on standard method (APHA, AWWA, and WEF, 2005). For this purpose, a series of potassium permanganate standard solutions covering the chlorine, with an equivalent range of 0.05 to 4 mg/L, was prepared. The color absorption was developed by SM4500Cl-G, which was measured in absorbance at 515 nm (HA-2).

3.2.3 Determination of Haloamines by Headspace Gas Chromatography-Mass Spectrometry (Headspace GC-MS)

To determine haloamines using the head-space GC-MS method, haloamines standard solution was prepared daily. The main standard solution was diluted with ultrapure 0, 4000 and 40,000 mg/L NaCl to the standard solutions with a nominal concentration ranging from 0.2 to 3 mg-Cl_2/L. Next, 10 ml of each standard solution was transferred to 20-mL screw cap head-space vials. Hexafluorobenzene (HFB) solution was added as an internal standard at a final concentration of 2 mg/L, and the vials were capped and analyzed by head-space GC-MS.

Head-space GC-MS analysis was conducted using a modification of a head-space method for TCA (Kasaka et al. 2010). An Agilent 7890A GC equipped with an Agilent

HP-1MS capillary column (15 m × 0.25 mm) was used and combined with an Agilent 5975CV mass spectrometer. The injecting temperature and flow rates were 40°C and 1 mL/min, with helium as the carrier gas. Pulsed split mode was used for injection with a split ratio of 1:2. The temperature profile was 30°C for 1.5 minutes, rising to 60°C at 30°C/min, and 60°C for 0.5 minutes.

The MS was operated in selected in an ion monitoring (SIM) mode with quantitation and confirmation ion pairs of 186/117 m/z for HFB, 84/(86, 119, 121) m/z for TCA, 85/(51, 49, 87) m/z for DCA, and 51/53 m/z for MCA. The auxiliary, quadrupole and ion source temperatures were 150°C, 110°C and 150°C. HFB was selected as an internal standard because an addition of HFB will have no effect on chloramines concentrations in the samples (Kasaka et al., 2010). Table 3.1 shows the detailed analytical condition of head-space GC/MS analysis used in this study.

3.3 Results and Discussion

3.3.1 Evaluation of Standard Method 4500 Cl. G. DPD Colorimetric Method for NAS Water

Based on SM4500Cl-G, potassium permanganate was used as the standard for establishing the standard curve. The standard curve concentration range was 0 - 4.0 mg/L Cl_2. and exhibited a non-linear response above 1.0 mg/L equivalent chlorine. Several other studies have also reported the non-linearity of the SM4500Cl-G procedure using either FC standards or secondary standards (Harp, 2002; Kosaka et al., 2010; Hosoda et al. 2009).

Table 3.1. Analytical condition of head-space GC-MS used in this study.

GC	
Equipment	Agilent 7890A (Agilent Technologies)
Column	HP – 1MS (15 m×0.25 mm, 0.25mm, Agilent Technologies)
Temperature program	30^0C (1.5min) →30^0C/min→60^0C (3min)
Carrier gas	Helium
Flow rate	1.0 mL/min
Injection mode	Pulsed split (15 psi (0.2 min), 2:1)
Injection temperature	40^0C
MS	
Equipment	Agilent 5975C (Agilent Technologies)
Ion source	Electron ionization
Ion voltage	70 V
Aux temperature	110^0C
Quadrupole temperature	150^0C
Ion source temperature	110^0C
Analytical mode	SIM
m/z	MCA 51(quantification) 53 (identification)
	DCA 85(quantification) 51,49,87 (identification)
	TCA 84(quantification) 86,119,121 (identification)
	MBA 95(quantification) 97 (identification)
	DBA 175(quantification) 173,177 (identification)
	HFB 186(quantification) 117 (identification)

The non-linearity of the SM4500Cl-G calibration may attribute to the increased formation of the colorless imine product at higher oxidant concentrations or the instability of the liquid DPD reagent. The availability of active DPD free amine is low with ageing the DPD indicator solution. This would lead, furthermore, to increasing nonlinearity at the higher oxidant levels (Harp, 2002).

The SM4500Cl-G is one of the only analytical methods for purportedly differentiating TCA from other chlorinated oxidants, and provides individual concentrations for FC, MCA, DCA, and TCA. There is little evidence to suggest that the TCA can be quantified when using iodide with DPD. And our results show that interferences were varied with changing NAS water quality and control is problematic due to complexity of NAS waters. NAS aquaria and pools may contain natural levels of bromide ions of up to 65 mg/L. The addition of chlorine to waters containing bromide produce FB and bromamines. Bromamines will react with iodide reagent analogously to the chloramine reaction, indicating the total oxidizing capacity of the sample and a positive interference in the total chlorine test (Harp, 2002).

3.3.2 Development and Evaluation of a Head-Space GC-MS Method for Chloramines and Bromamines

In the present study, a head-space GC-MS method for chloramines species was developed. Appendix HA-3 shows the SIM chromatogram of TCA, DCA, and MCA in their standard solutions determined by head-space GC-MS at a pH of 7.8 and concentrations of 30 μg/l. The mass spectrums of TCA, DCA, MCA obtained in this study were the same as that reported by Shang et al. (1999). Thus, the peaks shown in Appendix

HA-3 were confirmed to be that of TCA, DCA, and MCA. This method has a relatively higher response and allows for an accurate differentiation between chloramines species in NAS water. However, calibration of head-space GC-MS is not possible due to the limitations of the SM4500Cl-G, and unfortunately the accurate estimates for TCA, DCA, and MCA in NAS water were not possible.

3.4 Conclusion

The SM4500Cl-G states that it is one of the only wet chemical methods for differentiating TCA from other chlorinated oxidants and works very well in fresh waters with low bromide levels. However, that organic matter and/or high concentrations of inorganics do cause significant interference of SM4500Cl-G in seawater and saltwater applications. Positive (overestimation) and negative (underestimation) interferences result in inaccurate measurement of TCA and other compounds. Additionally, interferences due to the complexity of NAS matrix and reactivity of bromamines (e.g., TBA) in NAS water which reacts with iodide reagent and indicates a positive interference in the chloramine test causes the calibration of head-space GC-MS for chloramines impossible. Therefore, accurate estimates for TCA, DCA, and MCA in complex seawater and saltwater matrices cannot be determined. Currently, no “ideal” method exists for quantifying FC and chloramines species in NAS water. All the accepted methods for chlorine display a certain lack of specificity and are inadequately selective to be totally free of interferences from other oxidizing agents.

CHAPTER 4

FORMATION OF HALOAMINES AND RELATED DISINFECTION BYPRODUCTS DURING CHLORINATION AND OZONATION OF MARINE AQUARIA

4.1 Introduction

Chlorine and ozone are added to natural and artificial seawater (NAS) aquaria and pools to meet a variety of disinfection and oxidation objectives. When ozone is added to marine aquaria, the high chloride and bromide levels cause a rapid conversion of the ozone to free chlorine ($HOCl/OCl^-$) (FC) and free bromine ($HOBr/OBr^-$) (FB) (Reed and Adams, 2002; Qiang et al., 2013; Qiang et al., 2015; Adams et al., 2013). In a competing reaction, ozone also reacts with bromide to form bromate (Naumov and von Sonntag, 2008). The relative amount of ozone reacting with chloride and bromide is governed by their relative reaction rates (Haag and Hoigne, 1983; Hoigné *et al.*, 1985):

$$O_3 + Cl^- \rightarrow O_2 + OCl^- \qquad k_{1C} = 0.003\ M^{-1}s^{-1} \qquad (4.1)$$

and

$$O_3 + Br^- \rightarrow O_2 + OBr^- \qquad k_{1B} = 160\ M^{-1}s^{-1} \qquad (4.2)$$

Higher bromide seawater and saltwater may often contain bromide on the order of 65 mg/L or greater, thus driving most ozone to the FB rather than the FC species. Further, FC can react with bromide to form FB (Grguric et al., 1994; Bousher et al., 1986):

$$Br^- + OCl^- \rightarrow OBr^- + Cl^- \qquad k = 6.77(10^3)\ M^{-1}s^{-1} \qquad (4.3)$$

FC and FB react with organic matter to form a variety of organic disinfection byproducts (DBP) and with ammonia to form haloamines. Key organic DBPs include trihalomethanes (THM) and haloacetic acids (HAA) as well as other emerging DBPs (Shi et al., 2013; Zhang et al., 2015). THMs and HAAs are regulated in drinking water at 80 and 60 µg/L. Previous research has documented the presence of different organic DBPs in marine aquaria and demonstrated the effect of bromide concentration on speciation. For example, work by Shi and Adams (2012) showed that for THMs, chloroform (trichloromethane) dominated in low-bromide (e.g., mg/L as Br) pools in a marine park while bromoform (tribromomethane) dominated in much higher bromide natural seawater. Similarly, chlorinated HAAs were shown by Shi et al. (2013) to dominate in low-bromide pools, while brominated HAAs dominated in high-bromide artificial and nature seawater.

DBPs and secondary oxidants in swimming pools and NAS aquaria may cause irritations and other health problems for animals (Nemery et al., 2002; Jacobs et al., 2007; Li and Blatchley, 2007). Literature on swimming pools suggests that chloramines are a primary eye irritant, with trichloramine (TCA, NCl_3) being a strongest irritant followed by dichloramine (DCA, NCl_2H) (Bernard et al., 2003; King et al., 2006). Monochloramine (MCA, $NClH_2$) and FC may be a minor factor with respect to eye irritations.

Chloramines are formed by the reaction of FC and ammonia/ammonium (NH_3/NH_4^+), which are shown non-stoichiometrically, as follows (Qiang and Adams, 2004):

$$HOCl/OCl^- + NH_3/NH_4^+ \rightarrow NClH_2 \quad (4.4)$$

$$HOCl/OCl^- + NClH_2 \rightarrow NCl_2H \quad (4.5)$$

$$HOCl/OCl^- + NCl_2H \rightarrow NCl_3 \quad (4.6)$$

Ammonia presence also results in the formation of bromamines by the reaction of ammonia with FB (and conversion of chloramines directly to bromamines). Natural seawater has approximately 65 mg/L of natural bromide (Flury and Papritz, 1993) while artificial seawater has varied levels of bromide present from impurities in NaCl and may contain bromide levels near or below 1 mg/L bromide such that both FC and FB are formed. Previous research has found that if saltwater contains even 1 mg/L of bromide, ozonation leads to a greater formation of FB than FC (Reed and Adams, 2002). Bromo-DBPs are more toxic than their chlorinated analogs. Due to the high toxicity of bromo-DBPs, increased total bromide concentrations leading to higher bromo-DBPs during water treatment may cause higher health risks (Richardson et al., 2007; Richardson et al., 2010).

Due to the health and irritation effects of haloamines on organisms within disinfected waters (e.g., fish, and mammal), it is of critical importance to understand the speciation of haloamines as a function of water quality parameters during the chlorination of seawater and saltwater. Key parameters affecting chloramine speciation (in low bromide waters) include pH and chlorine-to-ammonia ratios. The bromide concentration impacts the formation of FB (from both ozone and FC) as well as the direct conversion of chloramines to bromamines. Ionic strength can also have a significant impact on the reaction kinetics with the relative impact on specific reactions dictated in large part by the reaction order (Shah et al., 2015). Due primarily to insufficient analytical methods for

haloamine species in saltwater (other than possibly MCA), little study has been made of the relative concentrations and stability of the chloro- and bromo-amines in marine aquaria subject to chlorine and ozone disinfection.

The purpose of this study has been to examine the formation, stability, and occurrence of chloramines and bromamines during the chlorination of waters with varied total dissolved solids, bromide concentration, and pH. In this study, UV spectral scans were used in combination with head-space GC-MS methods to study the formation and stability of haloamines as a function of pH, chloride concentration, and bromide concentration.

4.2 Material and Methods

4.2.1 Materials

Reagent water was prepared using a Millipore Elix Reverse Osmosis system followed by a Millipore A10 system (Millipore, Bedford, MA). Ammonium chloride, hydrochloric acid, monobasic and dibasic potassium phosphate, sodium hydroxide, sodium chloride, sodium bromide, and laboratory grade sodium hypochlorite solution were purchased from Fisher Scientific (Houston, TX, USA). Hexafluorobenzene was purchased from Acros Organics (Geel, Belgium). Hach Accuvac vials for free chlorine were purchased from Hach Company (Loveland, CO, USA).

4.2.2 Instrumentation

Solution pH values were measured with Fisher Scientific Accumet Excel XL20 pH meter. Sodium hypochlorite stock solutions were quantified using both Hach Method 8021—Free Chlorine and based on its molar absorptivity at 292 nm of 350 cm^{-1} (Galal-Gorchev and Morris, 1965) measured using a Hach DR900 spectrophotometer.

Scanning spectrophotometric measurements were made using a Shimadzu Model UV-1700 spectrophotometer (Jiangsu, China) in medium-scan speed (ca. 3 nm/sec) over the wavelength range of 200 to 400 nm. Spectral scans were analyzed based on molar absorptivities and absorption maxima from Galal-Gorchev and Morris (1965) and Hensley et al. (2003) (Table 4.1).

A head-space gas chromatography/mass spectrometry (GC/MS) method by Kosaka et al. (2010) was used to analyze selected haloamine species using an Agilent 7890A gas chromatograph (Agilent Technologies) equipped with an HP-1MS capillary column with dimensions of 15 m×0.25 mm (Agilent Technologies). The injection temperature was 40°C, whereas the helium flow rate was 1.0 mL/min. Pulsed split mode was used for injection, with a split ratio of 1:2. The temperature program was as follows: 30°C (1.5 min); ramp to 60°C at 30°C/min; and hold at 60°C for 0.5 min. An Agilent 5975C mass spectrometer (Agilent Technologies) was operated in selected ion monitoring (SIM) mode.

The m/z values of hexafluorobenzene (HFB) as internal standard were 186 (quantification) and 117 (confirmation), and those of TCA were 84 (quantification) 86, 119, and 121 (confirmation), for DCA were 85 (quantification) 51, 49, and 87 (identification), and for MCA the values were 51 (quantification) and 53 (confirmation). Auxiliary, quadrapole, and ion source temperatures were 150°C, 110°C, and 150 °C.

4.2.3 Standard Solutions

To determine the UV-absorption spectra of TCA as a function of pH and time, TCA was prepared daily by mixing hypochlorite solution (NaOCl) and ammonium chloride (NH_4Cl) solution at a chlorine-to-ammonia molar ratio of 3.15:1.00 while rapid stirring was provided at pH 6 with 5 mM phosphate buffer (Kosaka et al., 2010; Shang and Blatchley Iii, 1999). The pH of standard solution was adjusted with 1M NaOH or 1M HCl, and then the required amount of concentrated NaCl solution was added to the solution to form a final concentration of solution of 0, 4000 and 40,000 mg/L as NaCl, which was placed in a quartz cuvette. This process occurred in less than one minute to reduce reaction and changes in concentration. The blank solution was prepared in ultrapure water which contains the same amount of NaCl concentration of standard solution in each measurement.

To determine the TCA by using the head-space GC-MS method, TCA standard solution was prepared daily by mixing NaOCl solution and NH_4Cl solution at a chlorine-to-ammonia molar ratio of 3.15:1.00 at a pH of 6 in a 5 mM phosphate buffer (Kosaka et al., 2010; Shang and Blatchley Iii, 1999). The main standard solution was diluted with ultrapure 0, 4000, and 40,000 mg/L NaCl to the standard solutions with a nominal concentration of TCA ranging from 0.2 to 3 mg-Cl_2/L. After which, 10 mL of each standard solution were transferred to 20 mL screw cap head-space vials. An HFB solution was added as an internal standard at a final concentration of 2 mg/L—the vials capped and analyzed by GC-MS.

In the kinetic experiments, TCA was prepared by mixing NaOCl solution and NH_4Cl solution at a chlorine-to-ammonia mass ratio of 12:1 (mg:mg) at a pH of 6 with 5 mM phosphate buffer (Bogatu, 2010). A pH of 7.8 of the standard solution was adjusted

with 1 M NaOH. The required amount of concentrated NaCl and NaBr solution was added to the solution to achieve a final concentration of 0, 4000 and 40,000 mg/L as Cl and 0, 1, 10 and 50 mg/L as Br. The solution was then placed in a quartz cuvette. The blank solution was prepared in ultrapure water containing the same amount of NaCl and NaBr concentration of standard solution in each measurement. Preliminary experiments were conducted and confirmed all the solutions of ammonium chloride, monobasic and dibasic potassium phosphate, sodium hydroxide, sodium chloride, sodium bromide had negligible absorbance at the required detection wavelengths, and as such had no spectral interference with the absorbance measurement of TCA (Galal-Gorchev and Morris, 1965; Heasley et al., 2013).

4.3 Results

4.3.1 Effect of pH and TDS on TCA Formation

UV spectra as a function of pH (from 5.7 to 8.4) for the TCA standard solution were developed for purified water (TDS 0 mg/l as NaCl) (Figure 4.1A).

The results indicated only one peak at 340 nm in the TCA standard solution from a pH level of approximately 5.7 to 7.5, which denoted TCA. The maximum absorbency at 340 decreased slightly with an increased pH, most likely due to an enhanced TCA decay at higher pH values (Bogatu et al., 2010). At pH 7.5 and above, a peak emerged at 292 nm indicating the presence of hypochlorite, OCl^-, consistent with previous studies of the formation and decomposition of TCA in sodium hydroxide solutions at low pH levels

(Bogatu et al., 2010). Specifically, the relevant reactions for the decomposition of TCA at high pH are (Kumar et al., 1987):

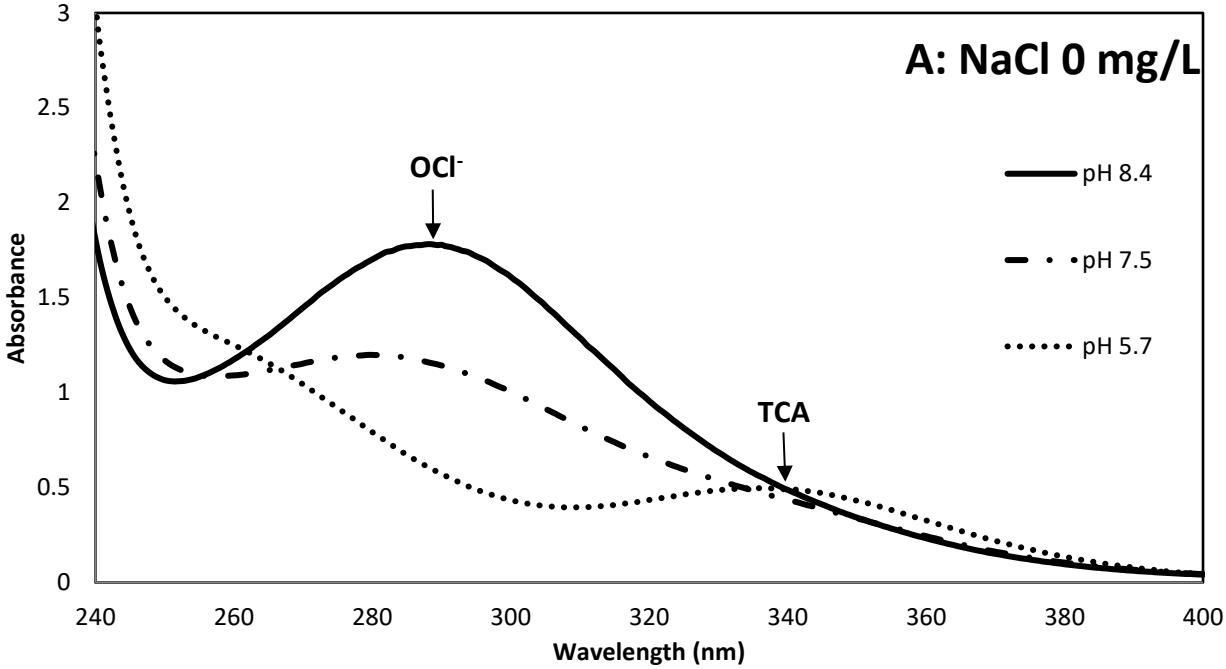
$$NCl_3 + OH^- \rightarrow NHCl_2 + OCl^- \quad (4.7)$$

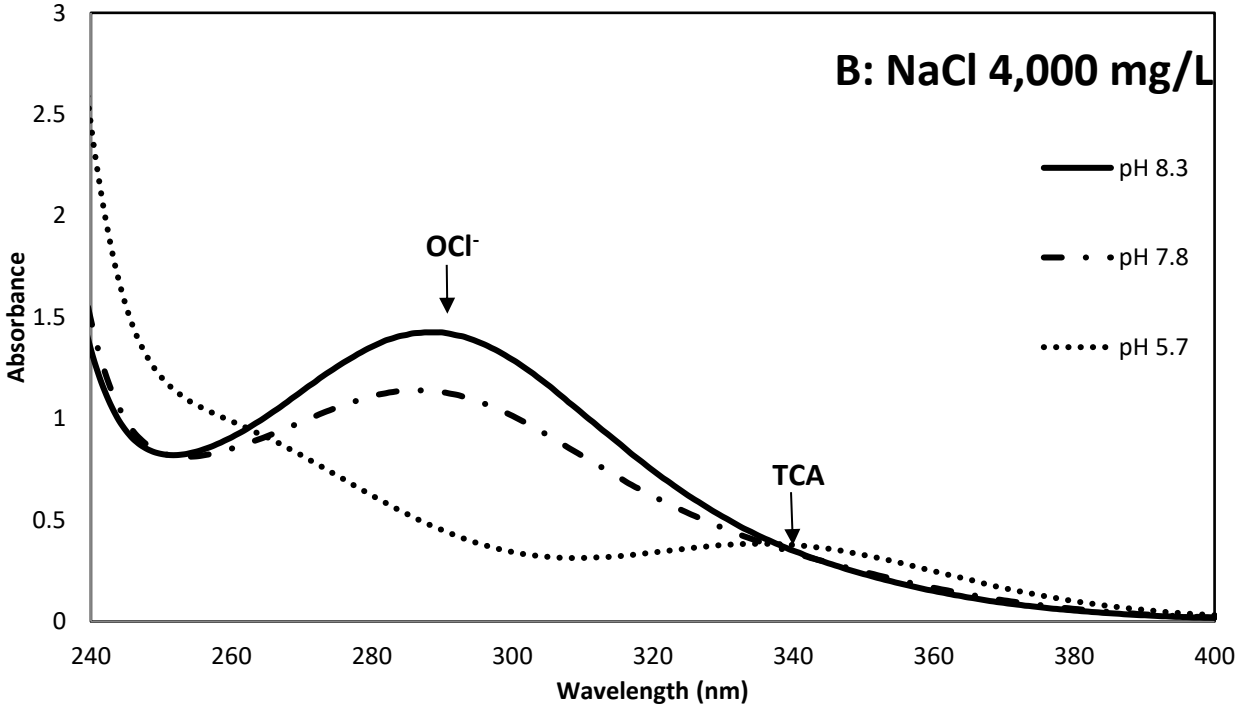
$$NCl_3 + NHCl_2 + 5OH^- \rightarrow N_2 + 2OCl^- + 3Cl^- + 3H_2O \quad (4.8)$$

It is hypothesized that the DCA peak was obscured by the OCl^- spectra due to their adjacent maximum absorbance and noting that OCl^- has a higher molar absorptivity than DCA (Table 4.1).

While no DCA peak was observed in the UV spectra (Figure 4.1), head-space GC/MS results indicated the presence of DCA in the solution. These results make clear that free chlorine, DCA, and TCA are in dynamic equilibrium. As expected, and confirmed experimentally, in higher pH water, the concentration of TCA decreases.

A decrease in the amount of TCA (at 340 nm) can be seen in aqueous solutions at increased concentrations of NaCl (Figure 4.1). In general, TCA would be more favored in drinking water than brackish water or seawater. Additionally, the TCA peak disappeared completely above pH 7.8, the nominal pH of seawater, suggesting TCA may not be favored. Head-space GC-MS analysis conducted in these experiments, however, did show a considerable amount of TCA at this pH. It is hypothesized that the effect of higher ionic strength helped salt out the TCA from TCA standard solution thereby shifting equilibrium toward the gas phase in the head-space analysis (Table 4.2).


A: NaCl 0 mg/L
OCl-
TCA
pH 8.4
pH 7.5
pH 5.7
Absorbance
Wavelength (nm)
0
0.5
1
1.5
2
2.5
3
240
260
280
300
320
340
360
380
400


B: NaCl 4,000 mg/L
OCl-
TCA
pH 8.3
pH 7.8
pH 5.7
Absorbance
Wavelength (nm)
0
0.5
1
1.5
2
2.5
3
240
260
280
300
320
340
360
380
400

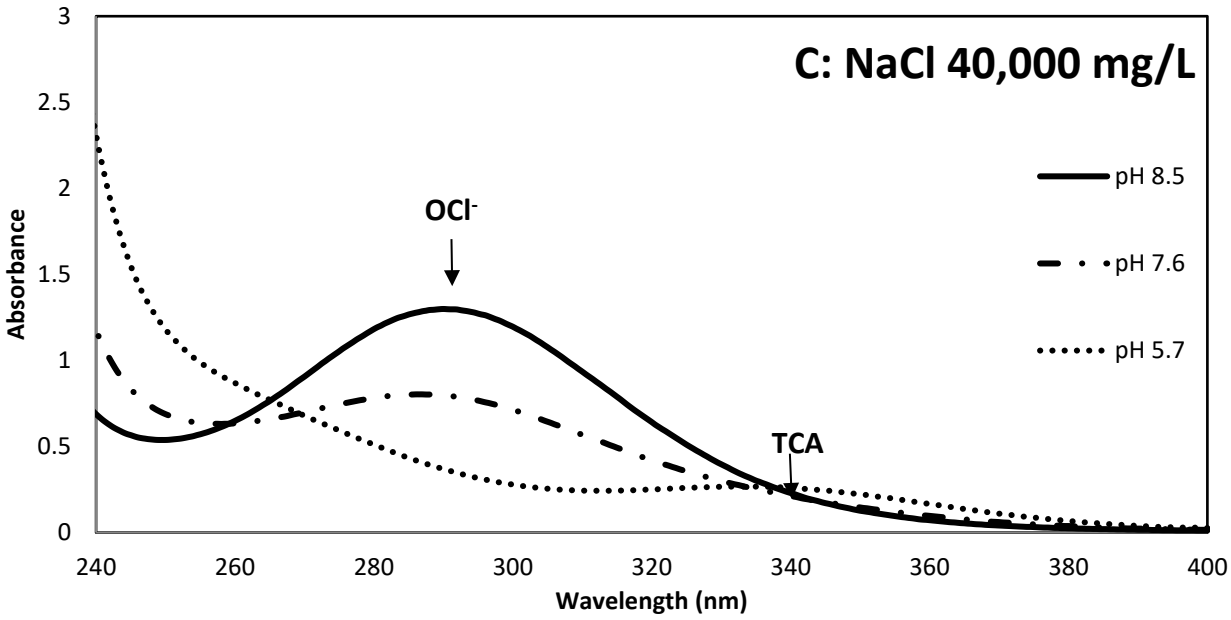


Fig. 4.1. UV spectra as a function of pH for TCA standard solution: (A) NaCl 0 mg/l; (B) NaCl 4,000 mg/l; (C) NaCl 40,000 mg/l. Experimental conditions: chlorine to ammonia molar ratio of 3.15:1.00, 5mM phosphate buffer, temperature 25±2.

Table 4.1 Molar absorptivity and absorption max wavelength for free and combined chlorines and bromines. Mixed chloro/bromo species data were not available.

	Absorption max (nm)	**Molar absorptivity (cm^{-1})**
MCA	245	455
DCA	206, 295	300, 300
TCA	220, 340	8100, 260
MBA	278	390 (438)
DBA	232 (Unstable)	1900 (Unstable)
TBA	258	4600
HOCl (pK=7.6)	233	100
OCl^-	292	350
Br2	236, 394	175, 153
HOBr (pK=8.7)	261 (263)	93
OBr^-	329	343

All in water unless noted otherwise.

Kinetic experiments were conducted on TCA in seawater, that is, 40,000 mg/L as NaCl at two pH levels. One experiment was conducted at a pH of 6 where TCA formation is more favored, and the other at a pH of 7.8, which is typical pH level in seawater. In these experiments, the TCA spectra was tracked at 220 nm over a 4-hour period. As the molar absorbance is relatively high (8100 L mol^{-1} cm^{-1}), TCA produces a strong peak at 220 nm. The results indicate that TCA decreased roughly 30% after 4 hours at both a pH of 6 and 7.8 in TDS 40,000 mg/l as NaCl (Figure 4.2). As a result, it can be concluded that TCA is relatively stable after formation with no bromide present.

Table 4.2. Analysis of TCA standard solution by using the head-space GC-MS method. Experimental conditions: chlorine to ammonia molar ratio of 3.15:1.00, 5mM phosphate buffer, temperature 25±2.

TDS (mg/L as NaCl)		**0**		**4,000**		**40,000**	
Theoretical Concentration of TCA (ug/L)		**Ratio of peak Area**		**Ratio of peak Area**		**Ratio of peak Area**	
pH 6		**DCA/IS**	**TCA/IS**	**DCA/IS**	**TCA/IS**	**DCA/IS**	**TCA/IS**
	0.2	0.01	0.25	0.01	0.25	0	0.07
	0.5	0.01	1.12	0.01	1.17	0.01	0.34
	1	0.02	3.27	0.02	2.63	0.01	0.92
	2	0.04	7.81	0.03	5.65	0.03	4.52
pH 7.8							
	0.2	0	0.14	0.01	0.33	0	0.05
	0.5	0.01	0.55	0.01	1.35	0.01	0.28
	1	0.01	2.07	0.02	2.47	0.01	0.97
	2	0.02	5.06	0.04	6.13	0.02	3.38

[1]Internal standard

4.3.2 Effect of Bromide Concentration on TCA Formation

To study the effect of bromide concentration on the relative formation of TCA and TBA, it was required to deconvolute their spectra. It was hypothesized that TCA and TBA absorption spectra convolute in a manner that can be interpreted semi-quantitatively. First, TBA was tracked in this work at its maximum absorption wavelength of 258 nm (and a molar absorptivity of 4600 L $mol^{-1} \cdot cm^{-1}$) (Galal-Gorchev and Morris, 1965), while TCA was tracked at 220 nm as discussed above. Experiments were conducted once more at a pH of 6 and a pH of 7.8, over TDS concentrations of 0, 4000, and 40000 mg/L NaCl. In each of the six permutations of pH and TDS, the bromide concentration was varied and a UV spectra was immediately obtained for analysis.

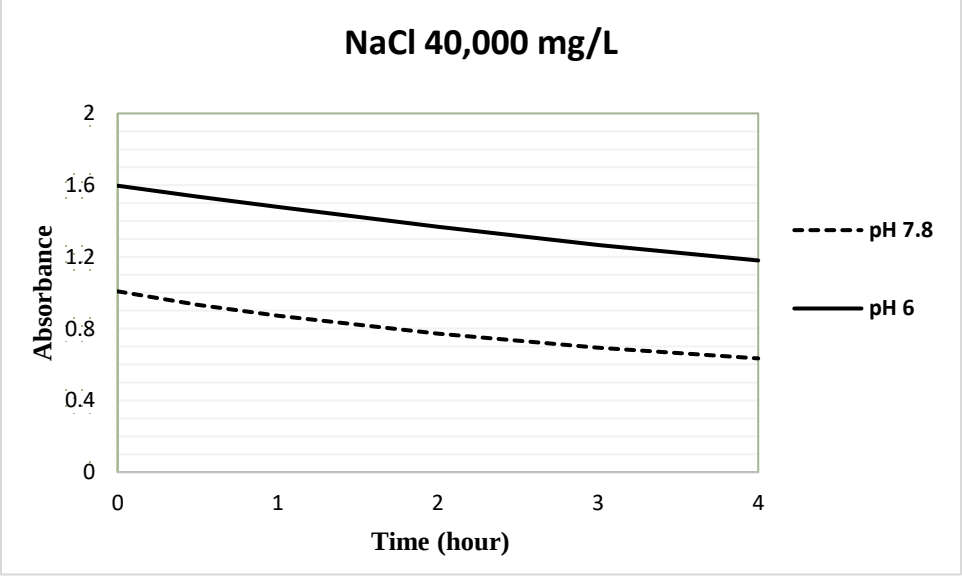


Fig. 4.2. Absorbance at 220 nm as a function of time for TCA standard solution at pH 6 and pH 7.8. Experimental conditions: chlorine to ammonia mass ratio 12:1 (mg/L: mg/L), 5mM phosphate buffer, NaCl 40,000 mg/l, temperature 25±2.

For pH 6 and 0 mg/L NaCl, the results clearly showed a strong TCA peak at 0 and 1 mg/L Br^-, disappearing mostly and then completely at around 10 to 12.5 mg/L Br^-. Concurrently, a strong TBA peak was observed forming at a concentration of 50 mg/L Br^- (Appendix HA-4A). Similar results were observed at 4,000 mg/L as NaCl (Appendix HA-4B). At 40,000 mg/L as NaCl TDS, however, no distinct TBA peak was observed.

Analogous results were also observed at the more relevant pH for seawater of 7.8. With 0 and 1 mg/L of NaCl, the TCA peak dominated the UV spectra (though at a lower intensity than at a pH of 6) and with no TBA observed. However, as the bromide concentrations were increased to 50 mg/L and above, the TBA peak was observed (Appendix HA-5A). Similar results were observed at the higher TDS levels of 4000 and 40,000 mg/L as NaCl.

These results show that in NAS water, TBA will normally dominate over TCA in all but the lowest bromide waters. These results are consistent with the formation of bromoform over chlorinated THMs as observed by Shi and Adams (2012) during the direct chlorination and the ozonation of seawater (through the conversion of ozone to FC and FB species). Similarly, Shi et al. (2013) observed the dominance of brominated HAAs in NAS water versus more chlorinated species.

In order to quantitatively determine the effect of bromide on TCA decompositions, the kinetics of the process were studied. Specifically, pseudo-first-order rate constants (k') were determined using a linear regression of t versus ln A with the following equation:

$$\ln A = \ln A_0 - k' \cdot t \qquad (4.9)$$

where k' is rate constant (s^{-1}), t is the reaction time (s), A_0 is the initial absorbance, and A indicates the absorbance at time, t. To estimate the absorbance of TCA for the regression analysis, the absorbance reading at the mid-point of a tangent line across the absorbance peak ($\lambda_{\text{mid-point}}$) (Table 4.3) was determined. The TCA concentration is related to absorbance by Beer's law which states:

$$A = \varepsilon \cdot L \cdot C \qquad (4.10)$$

where A is the absorbance, ε is the molar absorptivity (L mol^{-1} cm^{-1}), L is the path length of the cuvette in which the sample is contained (cm), and C is the concentration of the compound in solution (mol L^{-1}). The half-life ($t_{1/2}$) corresponding to the first order rate constant was computed using the following relation:

$$t_{1/2} = \frac{ln\ 2}{k'} \qquad (4.11)$$

where $t_{1/2}$ is half-life (s), and k' is pseudo-first-order rate constant (s^{-1}). The rate constant and half-life were calculated for TCA decay as a function of bromide concentration at pH 6, 7.8 and with a chlorine-to-ammonia mass ratio of 12:1 (mg/L: mg/L) (Table 4.3).

Regression coefficients (R^2) ranged from 0.87 to 0.99. Experiments at both a pH of 6 and 7.8 and in water from 0 to 40,000 mg/L TDS were conducted to examine the effect of bromide on TCA decay. The results showed that the effect of bromide concentration was dramatic on the decay of TCA. Specifically, the results showed that in the absence of

bromide, the pseudo-first order rate constant for TCA decay ranged from $2(10^{-5})$ to $8(10^{-5})$ s^{-1} corresponding to a half-life of approximately 2.4 to 6.6 hours (Figure 4.3).

On the other hand, the half-life of TCA dropped rapidly with increasing bromide concentration to the typical bromide concentration in seawater (and many artificial salt waters) of 50 mg/L. Additionally, a pseudo-first order rate constant for TCA decay of $4.7(10^{-3})$ s^{-1} was observed corresponding to a half-life of just 2.5 minutes. Thus, bromide is a key controlling factor in the occurrence of TCA with the effect of pH and TDS being much less significant as noted above. (Note, of course, that with respect to ozone chemistry, the high chloride associated with high TDS is required to invoke a rapid conversion of ozone to free chlorine species.)

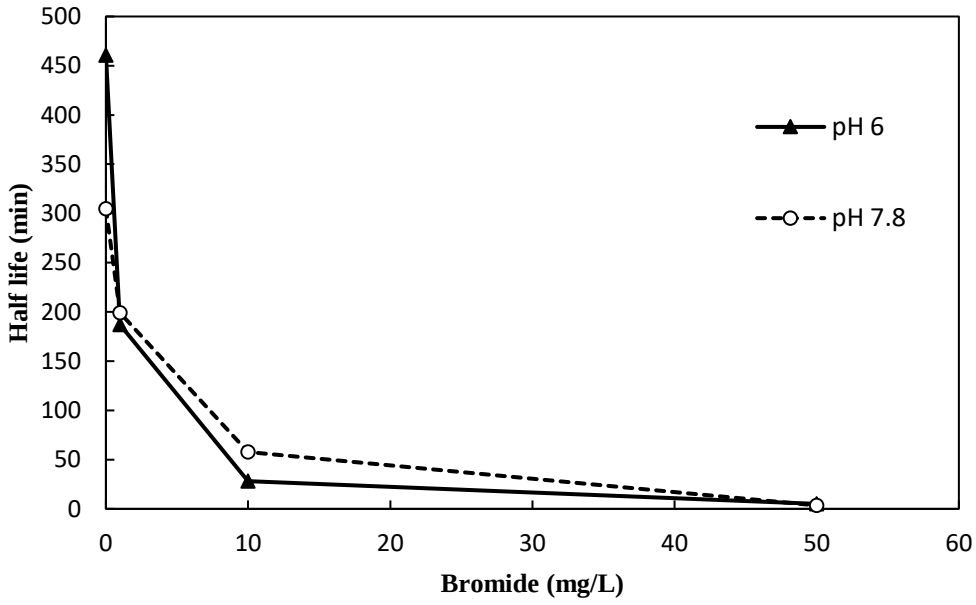


Fig. 4.3. Effect of bromide concentration on the average half-life of TCA of waters with 0, 4000, and 40000 mg/L TDS, for pH 6 and 7.8.

Table 4.3. Mid-tangent point absorption peaks for UV spectra, measured pseudo-first-order rate constants and half-lives at λmp for TCA decay as a function of pH and bromide ($\lambda_{max,TCA}$=220nm; $\lambda_{max,TBA}$=258nm). Experimental conditions: chlorine-to-ammonia mass ratio 12:1 (mg/L: mg/L), 5mM phosphate buffer, temperature 25±2.

	TDS (mg/L as NaCl)												
pH 6	**Br^- (mg/L)**	**0**			**1**			**10**			**50**		
		λ_{mp}[a] (nm)	**k'[b] (s^{-1})**	**$t_{1/2}$[c] (min)**	**λ_{mp} (nm)**	**k' (s^{-1})**	**$t_{1/2}$ (min)**	**λ_{mp} (nm)**	**k' (s^{-1})**	**$t_{1/2}$ (min)**	**λ_{mp} (nm)**	**k' (s^{-1})**	**$t_{1/2}$ (min)**
	0	223	0.2	578	224	0.9	128	225	3	38.5	245	21	5.5
	4000	224	0.2	578	225	0.8	144	226	5	23.1	246	25	4.6
	40,000	226	0.3	385	226	0.4	289	226	5	23.1	NMP[d]	NMP	NMP
pH 7.8													
	0	221	0.3	385	222	0.4	289	NMP	NMP	NMP	248	29	4
	4000	224	0.3	385	224	0.6	192	NMP	NMP	NMP	248	29	4
	40,000	226	0.8	144	226	1	116	226	2	57.8	252	47	2.5

[a] *Mid-tangent point (mid-point) absorption peaks for UV spectra.*

[b] *Rate constants (k (10^{-4})) at λ_{mp} for TCA decay.*

[c] *Half-life time at λ_{mp} for TCA decay.*

[d] *No mid-point apparent (NMP)*

4.4 Conclusion

In NAS water with typical high bromide concentrations, TCA would not be expected to be nearly as significant of an irritant as in very low bromide waters. While the focus on fresh water in swimming pools has often rightly been on TCA as a key eye and skin irritant, TCA may play a significantly less important role in NAS water due to higher bromide levels. Furthermore, the role of TBA has been investigated to a lesser degree in general due to the fresh water's lesser significance and due to the large amount of analytical issues.

HAPTER 5

CONCLUSION AND FUTURE WORK (HALOAMINES)

5.1 Conclusion

In most marine mammal pools, microbiological control has historically been performed using an addition of chlorine and/or ozone. Both oxidants lead to the formation of disinfection byproducts including chloramines and bromoamines due to a relatively high concentration of bromide in brackish and saline water. Because the residual oxidant is important with respect to the balance of how effective the disinfection process is versus control over disinfection byproducts, it is critical to be sure that the residue remains within a specified range. Too little chlorine will not provide adequate disinfection and too much can result in excessive disinfection byproducts. The presence and concentration of these combined chlorine forms depend on the pH levels, temperature, initial chlorine-to-nitrogen ratio, chlorine demand, and reaction time.

While the focus in swimming pools and related studies have often been on TCA as a key secondary oxidant, the role of TBA has been investigated to a lesser degree. Relative amounts of bromide in seawater or saltwater have been shown to dictate the degree to which compounds move toward chlorinated or brominated species. No work, however, has been reported on this topic. Bromamines are likely to be as large or part of a larger factor such as chloramines which may lead to eye and skin irritations, depending on bromide levels and treatment approaches.

The wet chemical Standard Method 4500Cl-G titled DPD Colorimetric Method is the only analytical methods that can differentiate TCA from other chlorinated oxidants in low total dissolved solids, but our results demonstrate that due to the complexity of NAS, water control of interferences is problematic and impractical. Generally, there is no "ideal" method for analysis of chlorine species that is not subject to potential interferences in the NAS water.

In this study, the head-space GC-MS method was developed for chloramines, which had relatively high responses and allowed for an accurate differentiation between chloramines species. Unfortunately, calibration of head-space GC-MS was not possible due to limitations of the standard method 4500Cl-G in NAS water. So, further study by using this method for a better understanding of the effects of chlorination (and indirectly ozonation) on the occurrence of chloramines and bromamines in seawater/salt water systems were not achieved.

In this study, spectrophotometric techniques were supported by head-space GC-MS methods to study the formation and decay of TCA and TBA in aqueous solutions. This analytical method along with the laboratory experiments provides better understanding of the effects of chlorination (and indirectly ozonation) on the occurrence of chloramines and bromamines in seawater and salt water systems. Based on this study, the effect of bromide concentration was observed to exert an effect on the kinetics and stability of TCA and TBA; much more so than the effects of pH or TDS. With no bromide present, the half-life for TCA is two to six hours. In a typical bromide concentration in NAS water of 50 mg/L as NaBr, however, the half-life of TCA drops to just a few minutes. The significance of this work includes the demonstration that while TCA may be dominant in fresh water pools

as an irritant, it may also mistakenly be assumed as a primary irritant generated in NAS water aquaria and pools.

5.2 Future Work

It would be useful to consider how the use of low bromide salts in saltwater animal systems is used in fish systems. Higher bromide concentrations likely lead to higher brominated disinfection byproducts (DBP) (e.g., TBA). Because brominated DBP are generally more toxic than chlorinated DBPs, it is hypothesized that it may also cause greater eye irritation.

In seawater systems, use of low bromide salt is not an option because natural seawater is used and contains approximately 65 mg/L of natural bromide. It is recommended that the biological treatment processes be utilized in new treatment systems. Biological treatment should significantly reduce the concentrations of organic materials as well as the potential for ammonia. This will reduce the formation of chloramines as well as other potential eye irritants.

Further studies on alternative recycling and treatment processes in NAS swimming pools is required such as filtration media. Moreover, improvement of filtration via coagulation is an important issue as it reduces the amount of human inputs (e.g. skin, hair, microorganisms, personal care, and cosmetics products) in NAS swimming pools.

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CHAPTER 6

LITERATURE REVIEW AND OBJECTIVES (CYANOTOXINS)

6.1 Cyanobacteria

Cyanobacteria, also referred to as blue-green algae, are photosynthetic bacteria that are important primary producers in aquatic ecosystems with simple structures at the subcellular level (Mur et al., 1999). Cyanobacteria lack a nucleus, a characteristic feature defining them, along with bacteria, as prokaryotes. Cyanobacteria cell sizes are as small as 0.5 μm in diameter and filamentous species with cell diameters as large as 60 μm. Most of cyanobacteria have small gas vacuoles that allow them to regulate their buoyancy. Buoyancy enables them to migrate up and down to maintain a favorable position in the water column to utilize light and nutrients (Chorus and Bartman, 1999).

Cyanobacteria can form dense water blooms in mesotrophic (i.e., moderate nutrients and productivity), eutrophic (i.e., nutrient rich, high productivity), and hypertrophic (i.e., excessively enriched with nutrients) water. A stable water column, warm temperature, nitrogen, phosphorus, high pH and ample sunlight are critical factors that are required for cyanobacterial growth (Antoniou Maria et al., 2005; Chorus and Bartman, 1999). Cyanobacteria blooms are more likely to occur in warm weather where the water body is shallow, eutrophied, or slow moving (Chorus and Bartman, 1999).

6.2 Harmful Algal Blooms

Cyanobacteria blooms generally are symptoms of eutrophication and are evidence of the deterioration of water resources which cause ecological and public health concerns. Increased nitrogen and phosphorus loading can contribute to an increased occurrence of cyanobacteria blooms in water. Generally, waters containing total phosphorus concentrations between 10 and 25 μg/L are considered to have a moderate risk of cyanobacteria growth, with waters in excess of 25 μg/L providing high growth potential. Conversely, water with total phosphorus concentrations below 10 μg/L can be considered to have a low risk of cyanobacterial growth (Mur et al., 1999).

Cyanobacteria blooms that produce toxins are one subset of blooms generally called harmful algal blooms (HABs). The toxicity of cyanobacteria is related to the biosynthesis of harmful metabolites called cyanotoxins. Cyanotoxins are produced only by the cyanobacteria strains having the appropriate genes and have the capability to turn certain genes on or off depending upon environmental conditions (Kurmayer and Christiansen, 2009). However, the HAB terminology can be misleading because cyanobacteria do not always actively produce toxins.

Some cyanobacteria that produce cyanotoxins may also produce taste and odor causing compounds, so presence of taste and odor compounds alone, therefore, is not a clear indication that cyanotoxins are present. However, the presence of taste and odor compounds are indicators that potentially cyanotoxin-producing strains could also be present. Different cyanobacteria strains can be present in a single bloom, and some cyanobacteria strains can produce multiple types and variants of cyanotoxins (Kurmayer and Christiansen, 2009).

Some rapid and simple methods, such as algae cell counts or microscopic examination, may be sufficient for a preliminary assessment of whether a bloom may be of concern based on cell type and density. However, a more thorough assessment is required to confirm the presence and type of cyanotoxins (Merel et al., 2013).

6.3 Cyanotoxins

The word cyanotoxin refers to a large and diverse group of chemical compounds that differ in molecular structure and toxicological properties. They are generally grouped into three major classes according to their toxicological targets: hepatotoxins (induce liver damage); neurotoxins (alter the neuromuscular transmission); and dermatotoxins (induce skin irritation) (Table 6.1).

Table 6.1. Cyanotoxins toxicological effects, and known producers (Merel, et al., 2013).

Toxin	Organ	Genera
Microcystin	Liver (possible carcinogen)	*Microcystis, Anabaena, Planktothrix, Anabaenopsis*
Nodularin	Liver (possible carcinogen)	*Nodularia spumigena*
Cylindrospermopsin	Liver (possible kidney, genotoxic and carcinogen)	*Cylindrospermopsis, Aphanizomenon*
Anatoxin-a	Neurotoxin (nerve synapse)	*Anabaena, Planktothrix, Aphanizomenon, Cylindrospermopsis*
Saxitoxin	Neurotoxin (sodium channel blocker)	*Anabaena, Aphanizomenon, Cylindrospermopsis, Planktothrix*

A single bloom may contain multiple types of cyanotoxins because some cyanobacteria can produce several toxins simultaneously. In general, cyanotoxins naturally reside within cyanobacterial cells (i.e., intracellular). Leaking or lysing of cell walls of cyanobacteria causes the release of intracellular toxin into the water, and they become extracellular toxins.

Human exposure to cyanobacteria and their toxins could be by dermal contact and accidental inhalation/ingestion during recreational activities in waters subjected to a toxic bloom or by the ingestion of drinking water produced from a contaminated resource. This could consequently affect a relatively large number of people (Byth, 1980; Griffiths and Saker, 2003).

6.3.1 Microcystins

Microcystins (MCs) are the most commonly found cyanobacterial toxins in surface water sources, and thus MCs are the most commonly studied of the cyanotoxins. The chemical structure of MCs includes a group of cyclic heptapeptide characterized by a unique amino acid ADDA (3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid). The unusual ADDA amino acid is often associated with the toxicity of the molecule because of its conjugated diene which is present in more than 80% of known toxin variants (Antoniou Maria *et al*., 2005; Carmichael, 1992; Dawson, 1998). Variation of amino acids (Fig.6.1) at positions 2 and 4 (X and Z) provides the basis for MCs variant differentiation and nomenclature.

Over 100 structural MC variants have been determined in the molecular weight range from 800 to 1100 Daltons (Codd *et al.*, 2005; Dietrich and Hoeger, 2005; Song *et*

al., 2005; Tsuji *et al.*, 1995). Each variant is identified by the initials X and Z. For example, the common MC which has leucine (initial L) and arginine (initial R) should be identified as MC-LR (Table 6.2).

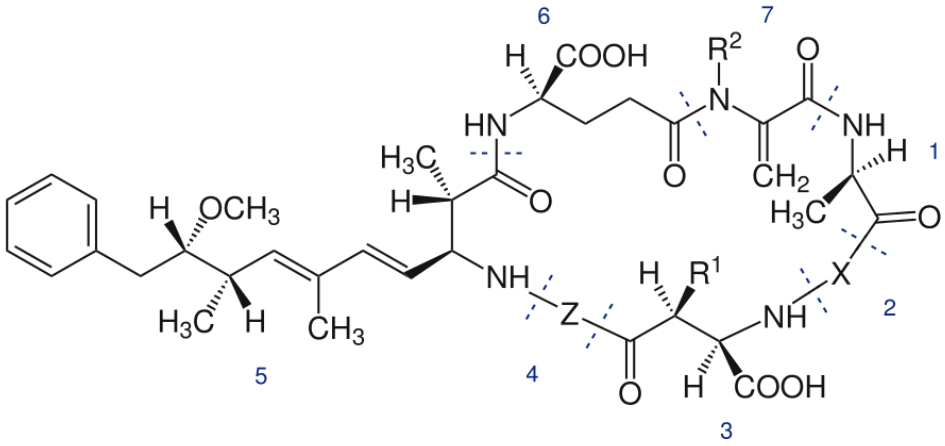

Fig. 6.1. Structure of Microcystins, with X and Z representing two amino acid variants (Merel *et al.*, 2013).

Table 6.2. Microcystin variants with their amino acid in position X and Z, and molecular weight (EPA, 2009).

Name	X-position Amino Acid	Z-position Amino Acid	Molecular Weight (g/mol)
Microcystin-LR	Leucine (L)	Arginine (R)	995.17
Microcystin-LA	Leucine (L)	Alanine (A)	910.06
Microcystin-RR	Arginine (R)	Arginine (R)	1038.2
Microcystin-YR	Tyrosine (Y)	Arginine (R)	1045.19
Microcystin-LY	Leucine (L)	Tyrosine (Y)	1002.176
Microcystin-LF	Leucine (L)	Phenylalanine (F)	986.2

All structural variants of MCs are thought to likely act as hepatotoxins, that is, as inhibitors of the serine/threonine protein phosphatases type 1 (PP1) and 2A (PP2A) (MacKintosh et al., 1990). The metabolism of a cell relies on the function of numerous enzymes and proteins. These enzymes/proteins are normally in a resting state. Usually, phosphorylation is required to convert the enzyme/proteins from their resting state to their active state. Phosphorylation of protein and enzymes are achieved by protein kinases at the expense of adenosine triphosphate. After the enzyme has performed its necessary functions, the phosphate radical is removed by protein phosphatase type 1 or 2 in order to return the active enzyme to its original resting state. If the phosphate radical is not removed, the enzyme will remain active and the cell will enter a hyperactive state. The covalent binding of microcystin to phosphatases inhibit the dephosphorylation reaction. Consequently, microcystins can cause hyper phosphorylation of proteins, and the destruction of liver cells, which can lead to blood accumulation in the liver. This can eventually be fatal for humans and animals (Codd et al., 2005; Carmichael, 1992).

MCs are very persistent in the environment and difficult to degrade or remove since they are non-volatile and relatively stable compounds due to their cyclic structure (Campas et al., 2007). Known process and pathways result in MCs detoxification and fate in nature when they are released into surrounding waters (Harada et al., 1999). Thermal decomposition does not significantly contribute to the decay of MCs in natural aqueous environments (Harada et al., 1999). MCs are known to be resistant to temperatures of up to 300^{o}C and pH extremes (pH <1 or >9) (Watanabe et al., 1989; Harada et al., 1997). The photolysis of MCs by sunlight alone was very slow (Tsuji et al., 1995; Watanabe et al., 1989). To achieve 90% degradation of MC-LR, at least 30 days are needed for indirect

photolysis in lake water (Lahti et al., 1997). The studies confirm that biological degradation has been a possible way to eliminate MCs (Watanabe et al., 1989). There is strong evidence that indicates MCs adsorption on particulate materials such as soils, sediments, and clay particles in natural environments (Morris et al., 2000).

MCs are the most prevalent cyanotoxins and are responsible for numerous cases of human and animal poisonings, with their presence reported throughout the world (Merel, et al. 2013). A recent study found that 82% of 181 samples of Canadian and U.S. utility waters tested were positive for the presence of MCs (AWWARF, 2001).

MC-LR is the most widely distributed MC worldwide and HAB incidents associated with MC-LR are frequent. Therefore, MC-LR was the first to be identified chemically and is still most commonly studied (Antoniou Maria et al., 2005; Carmichael, 1992). MC-LR is an extremely acute toxin. The lethal dose 50 (LD_{50}) of MC-LR after intraperitoneal injection in mice ranges from 25 to 150 μg/kg. This value may differ according to the MC variant, though MC-LR is usually used as a reference (Merel et al., 2013). However, the -LA, -RR and -YR variants of MCs have similar toxicological effects (Kuiper-Goodman et al., 1999; Merel et al., 2013).

6.3.2 Nodularins

Nodularins are cyclic pentapeptides structurally similar to MCs including the ADDA moiety, but only one variable amino acid Z (Merel, et al., 2013) (Figure 6.2). As of now, nine variants of nodularin have been reported, all of which are water soluble and stable toxins (Codd et al., 2005).

Like MCs, nodularins are hepatotoxins because of ADDA, which is responsible for the inhibition of protein phosphatase. According to the variant, the LD_{50} of nodularins in mice after intraperitoneal injection ranges from 30 to 70 μg/kg (van Apeldoorn et al., 2007). Nodularin-R with Arginine as variable amino acid is the most common between other variants (Merel, et al. 2013). No incidents of human intoxication have been reported as of yet, and due to the lack of toxicological data, there are no guidelines proposed for drinking water so far (Merel, et al. 2013).

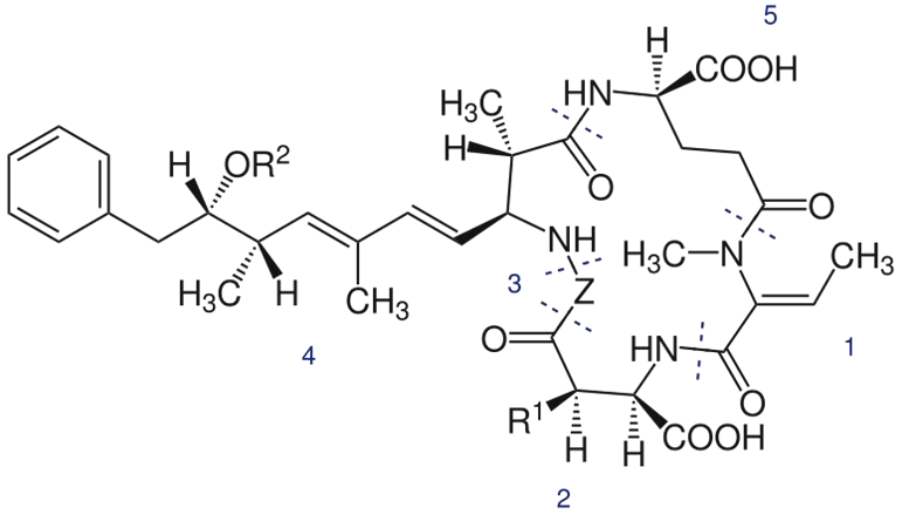

Fig. 6.2. General structure of nodularins with Z variant amino acid (Merel, et al., 2013).

6.3.3 Cylindrospermopsin

Cylindrospermopsin (CYN) is a polyketide-alkaloid having a tricyclic guanidine moiety and sulfate groups with molecular weight of 415 Da (Figure 6.3) (Banker et al., 2001). The LD_{50} of CYN in mice 24 hours after intraperitoneal injection is a 2100 μg/kg (van Apeldoorn et al., 2007). Like MCs and nodularins, CYN is also hepatotoxins and the

uracil moiety is potentially responsible for the toxicity (Merel, et al. 2013). The first main human intoxication by CYN reported in 1979 in Australia with the usage of an algaecide to eliminate a bloom of algal in the drinking water source resulting in the release of CYN. Over 100 children were reported to have suffered gastroenteritis due to consumption of contaminated drinking water (Bourke et al., 1983; Griffiths and Saker, 2003).

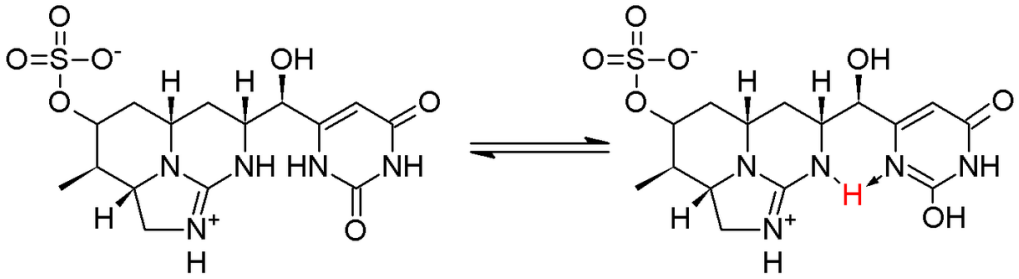

Fig. 6.3. Structure of cylindrospermopsin with a different OH orientation (Banker et al., 2000).

6.3.4 Anatoxin-a

Anatoxin-a is a potent neurotoxin with molecular weight of 165 Da bicyclic amine alkaloid with a variant called homoanatoxin-a (Figure 6.4) (van Apeldoorn et al., 2007). Anatoxin-a is highly water-soluble. However, anatoxin-a is unstable at pH >10 and transformed into a non-toxic form by sunlight exposure (Merel, et al. 2013). The LD_{50} of anatoxin-a in mice 24 hour after intraperitoneal injection is a 375 µg/kg (van Apeldoorn et al., 2007). Anatoxin-a has been responsible for various animal poisonings around the world, although it is a potent neurotoxin and no human poisonings have been reported yet (Gugger et al., 2005; Wood et al., 2007).

6.4 Regulation of Cyanotoxins

Recent occurrences of HAB due to nutrient pollution of water bodies and frequent detection of cyanotoxins in surface water have increased concern over the health risks posed by cyanobacteria. As a result, in response to a detection of cyanotoxins in drinking water supplies, regulatory agencies worldwide are developing drinking water standards to protect public health.

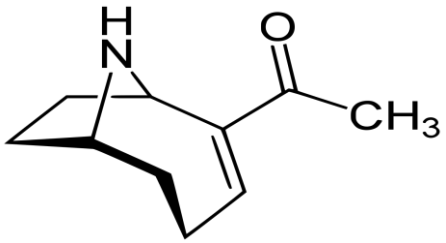

Fig. 6.4. Structure of anatoxin-a (van Apeldoorn et al., 2007).

The first guideline for cyanotoxins in drinking water were introduced in 1998 by the World Health Organization (WHO). At that time, the only sufficient information available was for MC-LR only. Consequently, the WHO considered that the MC-LR had no observable adverse effect at levels of 40 μg/kg/d after 13 weeks oral exposure in mice (Fawell et al., 1999) and derived a guideline of 1 μg/L as a maximum acceptable concentration value for MC-LR in drinking water (WHO, 1998). Most of the drinking water guidelines have adopted the WHO provisional guidelines directly for drinking waters of 1.0 μg/L MC-LR (WHO, 1998). Some countries have adopted the same animal studies as the WHO and have modified it based upon their local requirements. For example,

Canada and Australia have maximum acceptable concentration value of 1.5 and 1.3 µg/L respectively for MC-LR in the drinking water.

No federal drinking water regulations for cyanobacteria or their toxins in drinking water or recreational waters exist at this time in the U.S., however, the United States Environmental Protection Agency (U.S. EPA) issued non-enforceable Health Advisories Levels (HALs) for public drinking water supplies for the cyanobacterial toxins MCs and CYN (Table 6.3).

Table 6.3. Specific drinking water advisory thresholds for microcystin and other cyanotoxins.

State/Agency	Threshold Microcystin-LR (µg/l)	Threshold Anatoxin-a (µg/l)	Threshold Cylindrospermopsin (µg/l)	Threshold Saxitoxins (µg/l)
U.S. EPA Children < 6 years old	0.3	None	0.7	None
U.S. EPA All other age groups	1.6	None	3.0	None
Ohio	1	20	1	0.2
Oregon	1	3	1	3
Minnesota	0.04[a]	None	None	None
Quebec	1.5	3.7	None	None
Health Canada	1.5	None	None	None
Health Australia	1.3	None	None	None
World Health Organization (WHO)	1	None	None	None

[a] *To protect short-term exposure for bottle-fed infants.*

There are currently three states (Ohio, Oregon and Minnesota) that have established cyanotoxin monitoring guidelines and cyanotoxin threshold levels for Public Water Systems (PWSs). In 2015, the U.S. EPA established health advisories for MCs and CYN of 0.3 and 0.7 µg/L limits for children less than six years (as measured by ELISA) (EPA,

2015). Utilities are responsible for following those guidelines/thresholds and for undertaking any follow-up action required by their state.

6.5 Analysis of Cyanotoxins

Blooms of cyanobacteria are of increasing concern in the surface water sources in the USA, as well as other parts of the world. So, the monitoring of drinking water safety is a global demand and there is clear research needed to prove an effective and efficient method for the detection of cyanotoxins, especially MCs, because most of the worldwide incidents are associated with MCs' frequent occurrence. MCs and other cyanotoxins can be analyzed by multiple methods, either qualitatively or qualitatively. At the same time, samples often need specific preparation before an analysis based on the method employed and the kind of results expected.

After field sampling, samples should be stored at 4°C and analyzed as soon as possible in order to prevent any alterations with toxin distribution (intracellular/extracellular). In most cases, the cyanobacterial toxins naturally remain in the cytoplasm (intracellular toxins) and when the cell dies or the cell membrane ruptures, the toxins are released into the water (extracellular toxins). When detection of extracellular toxins is required, direct filtration of samples is needed. For separate detection of intracellular toxins, an additional step of inducing the lysis of cyanobacteria retained on the filter needed. Moreover, for the simultaneous detection of both extracellular and intracellular toxins, cell lysis is required before filtration. Freezing-thawing cyanobacteria or adding methanol in the sample (or onto the filter) are methods which directly damage cell membranes and release intracellular toxins (Harada et al., 1999).

Cyanotoxins can be detected and quantified through a biological approach such as in vivo assays, immunological assays, and biochemical assays or a physico-chemical approach which often relies on two steps, the separation of compounds presents in the sample by chromatography followed by their quantification with specific detectors.

Immunological assays – Cyanotoxins can be detected through the recognition and binding to specific antibodies. For example, various Enzyme-linked immunosorbent assay (ELISA) kits are commercially available for the detection of MCs in water (Carmichael and An, 1999; Lindner et al., 2004; Rapala et al., 2002). ELISA is a technique that involves antibodies and enzymes for the detection of MCs in a sample. Most commonly used techniques are based on two principles: 1) direct competitive ELISA and 2) indirect competitive ELISA.

Direct competitive ELISA is based on the principle that MC in the sample compete with MC-peroxidase for the limited number of binding sites of anti-MCs antibody attached to the microtiter plate. Adding chromogenic substrate to this enzyme changes its color upon reaction. The strength of the color development is inversely proportional to the concentration of MCs.

In an indirect competitive ELISA, MCs present in a sample and a monoclonal antibody against MC compete for the binding sites on a MC-Bovine Serum Albumin (BSA) coated plate. A secondary antibody conjugate (HRP conjugated goat anti-mouse IgG) is added and produces color with the addition of substrate. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The results by immunoassays are compared to a standard curve with known concentrations. MC-LR is

used as a calibrating agent and the concentration of MCs can be reported as MCs-LR equivalents (Abraxis, 2009).

Biochemical assays – MCs and nodularins can be detected using a protein phosphatase inhibition assay known as PPIA because these toxins are protein phosphatase inhibitors (Almeida et al., 2006; Heresztyn and Nicholson, 2001; Rapala et al., 2002). This method is based on using a protein phosphatase 2A (PP2A) enzyme and a substrate (p-nitrophenyl phosphate) in a colorimetric phosphatase inhibition assay. Sample is incubated with PP2A enzymes. MCs and nodularins present in the sample binds to the PP2A enzymes. Dephosphorylation of substrate by PP2A enzymes to p-nitrophenol at high pH produces a yellow color which is measured with spectrophotometer. The strength of absorbance at 405 nm wavelength is inversely proportional to the concentration of MCs and nodularins present in the sample (Carmichael and An, 1999; MacKintosh et al., 1990). Results are often expressed as equivalent MC-LR/L due to PPIA being unable to distinguish co-occurring variants of MCs and MCs from nodularins (Merel et al., 2013).

Chromatographic separation followed by quantification with specific detectors – Chromatographic methods are commonly employed as separation techniques for cyanotoxins since they allow for the discrimination of several co-occurring toxins within a single analysis. Liquid chromatography (LC) usually with a reversed phase C18 or a HILIC (Hydrophilic Interaction Liquid Chromatography) column and methanol/water or water/acetonitrile as a mobile phase. LC is the most common separation method for the quantification of cyanotoxins since it is adaptable rapidly to a wide range of detectors including Ultraviolet (UV) absorbance or mass spectrometry (MS) (Merel, et al. 2013).

Cyanotoxins are mostly detected by LC-MS or LC-MS/MS, allowing for a simultaneous detection of a larger amount of toxins with a simpler sample preparation procedure (Kaushik and Balasubramanian, 2012). LC-MS/MS is one of the U.S.EPA approved methods for the quantitative analysis of MCs. EPA Methods 544 and 545, 6 MC variants (MC-LR, -RR, -YR, -LA, -LY and –LF), CYN and anatoxin-a, are determined quantitatively by multi-point calibration. This method enables simultaneous separation, identification, and quantification of 6 MC variants, anatoxin-a and CYN in a mixture (Li et al., 2006; Merel et al., 2009). LC-MS/MS cannot measure all 100 MC variants that might be present in the water primarily due to a lack of availability of standards (Cheng et al, 2001).

Each method has its advantages and disadvantages, and the selection of the method depends on the purpose of the testing. For example, techniques such as ELISA and PPIA are typically easy to learn and use and relatively cost effective when used as a screening tool or for toxicity assessment when appropriate. However, cross-reactivity can lead to a lack of specificity for the target analyte(s), and accurate data interpretation can be problematic. PPIA measures the bioactivity or toxicity of MCs but not the structural component such as ELISA does. All toxic variants of MCs are detectable by PPIA. PPIA is not specific to MCs, and other non-MCs protein phosphatase inhibitors are detectable as well (Metcalf et al., 2001). Moreover, ELISA measures total MCs and often produces higher positive results than LC-MS/MS. In the chromatographic techniques, individual MCs can be separated and quantitated based on their retention time and response versus standards. However, the cost is greater per sample in comparison to bioassays and standards for relative few variants are available (Bruno et al., 2006; Fischer et al., 2000; Li

et al., 2006). Researchers are utilizing multi-toxin LC-MS/MS methods more frequently because of their ability to distinguish a larger variety of individual toxins more readily and to avoid the derivatization that would be required for any GC-based technique (Li et al., 2006).

6.6 Comparison of ELISA and LC-MS/MS Analysis Results

In view of the global demand for the monitoring of drinking water safety, there is a need for research to prove an effective and efficient method for the detection of cyanotoxins, especially MCs due to the frequent occurrence of MCs in drinking water sources. ELISA and LC-MS/MS are the only methods which are approved for the detection of MCs in drinking water samples by U.S.EPA. The literature, which is summarized in Table 6.4, has shown two dominant groups; first group in which ELISA overestimates MCs and second group where ELISA detects accurately.

The reason for overestimates could be the presence of more MCs variants which LC-MS/MS method cannot detect due to limited standards. The literature that detects MCs accurately could represent a study sample that contains mainly those MC variants which are detectable by LC-MS/MS. Thus, a literature review leads to a clear conclusion that sensitivity of ELISA is equal to or greater than LC-MS/MS for the detection of total MC concentration.

6.7 Managing Harmful Cyanobacteria Blooms

Cyanobacteria blooms can develop quickly, over a period of just days. Consequently, the management of HAB in drinking water supplies is a complex task and

can be considered in three steps which includes the prevention of bloom occurrence, bloom monitoring, and drinking water treatment.

Decreasing the inputs of nutrients (i.e. C, N and P) in surface water is one of the long-term strategies to prevent the occurrence of HAB across the world. A watershed protection program can help reduce the nutrient load on the watershed area.

Table 6.4. Literature review summary in terms of comparing ELISA and LC-MS/MS methods results for analyzing microcystins.

Estimation of microcystins by ELISA versus LC-MS/MS	Author (year)
Overestimates 5 references	Rivasseau et al. (1999); Tsutsumi et al. (2000); Conti et al. (2005); Mountfort et al. (2005); Tillmans et al. (2007)
Detects accurately 6 references	Lawrence and James (2001); Mountfort et al. (2005); Hawkins et al. (2005); Mathys and Surholt (2004); Rapala et al. (2002); McDermott et al. (1995)
Underestimate 1 reference	Bruno et al. (2006)

Predicting cyanobacteria blooms before they occur can be challenging, or in some cases, not possible. Well-designed monitoring of raw water with special consideration to distribution of cyanobacteria in the study reservoir and water columns can provide effective early warning that cyanobacteria blooms are occurring (Merel et al., 2013). Identifying which cyanobacteria and cyanotoxins are present helps utilities know they are using the appropriate treatment processes. Some treatment options are effective for some cyanotoxins, but not for others. The treatment system operators must act to remove or inactivate intracellular cyanotoxins in appropriate ways. Applying the wrong treatment process at a specific stage in treatment could damage cells and result in the release rather than removal of cyanotoxins (Lopez et al., 2008). The efficiency of various water treatment processes for the removal of cyanobacterial cells (intracellular toxins) and cyanotoxins (extracellular toxins) is discussed in the following sections.

6.8 Pre-treatment

Coarse filtration and pre-oxidation steps are often referred to as pre-treatments. Coarse filtration at the intake is often used to remove macro-contaminants (i.e. leaves, plastic bottles…) that might damage treatment facilities and disturb treatment processes. However, coarse filtration cannot appreciably affect microcontaminants such as cyanobacteria and cyanotoxins. Pre-treatment oxidation, such as chlorine, permanganate and ozone at the intake, are not recommended because oxidant addition when cyanobacterial cells are present can cause cells to lyse or become leaky, thereby releasing additional cyanotoxins (Westrick et al., 2010).

In recent decades, the practice of optional pre-oxidation became less prevalent due to the production of harmful disinfection by product concerns associated with many of these chemicals. Pre-oxidation should also be avoided when a bloom occurs in drinking water resources, and it is recommended that pre-oxidation processes be delayed until most of the intact cyanobacteria cells are removed via conventional treatment or an advanced filtration process to avoid cell lysis and the release of their intracellular toxins (Merel et al., 2013).

6.9 Coagulation/Flocculation/Sedimentation

Conventional water treatments (e.g., coagulation, flocculation and sedimentation) are aimed at removing colloidal material from raw water, and cyanobacteria have negative surface charges and thus can be considered as colloids for the purposes of coagulation, flocculation, and sedimentation (Merel, et al., 2013). Numerous studies have been reported that an almost complete removal of cyanobacterial cells (intracellular cyanotoxins) is achievable (Lawton and Robertson, 1999; Himberg et al., 1989; Drikas, et al., 2001; Rositano and Nicholson, 1994). However, certain species of cyanobacteria containing gas vacuoles may disturb sedimentation by preventing flocs to settle (Pieterse and Cloot, 1997). As a result, some studies show that dissolved air flotation may efficiently remove cyanobacteria rather than sedimentation (Teixeira and Rosa, 2006; Teixeira et al., 2010).

The key to success of conventional treatment is the elimination of cyanobacteria without damage to cell membrane or toxin release can occur. So, any processes that are the source of potential cell damage during conventional drinking water treatment should be avoided or minimized to promote intact cell removal including the use of oxidants prior to

filtration and rapid mixing. Studies shows that accumulating sludge for long periods of time result in 90% of the cyanobacteria releasing their toxins into the treated water within 24 hours (Drikas et al., 2001; Merel, et al., 2013).

Contrarily, these common drinking water treatments are not effective in removing any extracellular cyanotoxins since they are designed to remove particles. This theory was confirmed by studies showing no difference in the concentration of extracellular cyanotoxins after treatment by using coagulation, flocculation, and sedimentation or dissolved air flotation (Merel, et al., 2013).

6.10 Filtration

Studies shows that slow sand filtration are more effective in removing algal cells (remove 99% of the cells) than direct rapid sand filtration. Further, because of slow sand filtration lower loading rate, a biofilm on the top of the filter develops resulting in biodegradation of cyanotoxins on or inside the filter bed. However, clogging of the filter and toxin release from the lysed cyanobacterial cells into filter beds are significant problems (Hrudey, et al. 1999; Merel, et al., 2013).

The pressure-driven membrane filtration which is most commonly used in drinking water treatment is a physical separation process and covers various processes characterized by the pore size of the associated membrane including microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO).

Low-pressure membranes such as MF and UF are alternative methods of conventional filtration. MF and UF membranes are primarily used for the removal of turbidity, pathogens, and particles from fresh waters. Cyanobacteria, including single cells,

filaments and colonies are generally expected to be 1 μm in size or larger. Thus, MF and UF membranes with pore sizes smaller than 1 μm have been found to be highly effective at removing intact cyanobacteria cells as well as intracellular and particulate toxins with up to 98 percent cell removals achieved (Chow, et al., 1997; Newcombe, et al., 2010). Although concentration of cells at the membrane surface may result in clogging and cell lysis and toxin release which are primary concerns. Therefore, frequent backwashing is recommended to reduce the risk of intracellular toxins being release into the water.

High-pressure membranes such NF and RO are used to remove aqueous salts and metal ions, synthetic organic contaminants (e.g., pesticides), and disinfection by-product precursors. Theoretically, cyanobacteria should be efficiently removed by NF and RO due to lower pore size compared to MF and UF, but cells are not supposed to reach these processes. In fact, cyanobacteria are eliminated by previous treatments in order to avoid immediate clogging of these membranes and only extracellular toxins would be expected to challenge these membranes. However, existing evidence shows that both NF and RO are effective in the removal of extracellular toxins and more than 95% removal could be observed for MC-LR, CYN, nodularins and anatoxin-a (Teixeira and Rosa, 2006; Dixon et al., 2011; Vuori et al., 1997). Although NF and RO seem to be a promising option to remove both cyanobacteria and cyanotoxins during drinking water treatment, they are complex as well as expensive methods, so they are unaffordable methods for drinking water treatments.

6.11 Activated Carbon Adsorption

Activated carbon is carbon produced from carbonaceous source materials such as wood, coal, peat, and coconut shell. Activated carbon has a large surface area due to its high porosity and, typically ranges from 600 to 1200 m^2/g, which enables activated carbon to have the strongest physical adsorption forces. Activated carbon consists of pores of varying sizes, which are classified according to their diameter including micropores (< 2 nm), mesopores (2-50 nm), and macropores (> 50 nm). In drinking water treatment, activated carbon is employed in two forms of powdered (PAC) or granulated (GAC) to absorb contaminants from water. While activated carbon does not have any impact on cyanobacteria and intracellular toxins, it can be an effective means to remove extracellular cyanotoxins in both form of PAC and GAC.

Pore size distribution was the most important physical property of activated carbon when considering adsorption performance (Huang et al., 2007; Newcombe and Nicholson, 2004). Studies suggests that activated carbons with high mesopore capacity can be used for the treatment of MCs since molecular size of MCs is around 2 nm, it is too large to enter micropores and can easily adsorb in mesopores (Donati et al., 1994). In addition, of various activated carbon types, wood-based carbons have been demonstrated to be the most effective in removing MCs due to a higher fraction of mesopores. Although, activated carbon removal efficiency has been reported to vary between MC variants (MC-LA > MC-LR > MC-YR > MC-RR), fortunately, the most toxic variant, MC-LR, is one of the most readily adsorbed (Newcombe, et al., 2010). Since saxitoxins and anatoxin-a are smaller than MCs, activated carbons with a large fraction of micropores was shown that has the greatest saxitoxins and anatoxin-a adsorptive capacity (Newcombe and Nicholson, 2004; Ho et al., 2011).

There is strong evidence indicates that water quality parameters especially the concentration and makeup of the natural organic matter (NOM) have a strong influence on the removal of cyanotoxins by activated carbon, and can drop efficiency rates from 90% to 49−63%, since NOM will always be present in higher concentration than the cyanotoxins and can compete with cyanotoxins and limit their adsorption (Donati et al., 1994; Huang et al., 2007). Even though activated carbon absorption can effectively remove cyanotoxins, high dose of activated carbon (20 mg/L and higher), extended contact times and increased regeneration frequencies of filters are required to meet the WHO guidelines (Cook and Newcombe, 2003).

6.12 Oxidation and Disinfection

Chemical inactivation including Ultraviolet (UV), disinfectants and oxidants (e.g. ozone, chlorine, monochloramine, chlorine dioxide and permanganate) can be used to control cyanotoxins. However, their effectiveness is highly dependent on the oxidant dose, contact time, cyanotoxin combination and the organic content of the water. Moreover, it is important to be noted that chemical treatment when cyanobacterial cells are present can cause damage to the cyanobacterial cells and result in an additional release of the toxin. Consequently, pretreatment oxidation should be avoided as much as possible. The effectiveness of oxidants to reduce extracellular cyanotoxin concentrations at the drinking water treatment plant are highly variable, as summarized in Table 6.5.

Table 6.5. Effectiveness of typical oxidants for disinfection of cyanobacteria and destruction of extracellular cyanotoxins in drinking water treatment (Adams, 2013)

Oxidants	Cyanobacteria Disinfection (may ead to toxin release)	Microcystins	Cylindrospermopsin	Anatoxin-a	Saxitoxins
Free chlorine	Effective	Effective*	Effective at pH below 8	Not Effective	Somewhat Effective
Monochloramine	Somewhat Effective	Not Effective at normal levels	Not Effective	Not Effective	Inadequate Information
Chlorine dioxide	somewhat Effective	Not Effective at normal levels	Not Effective	Not Effective at normal levels	Inadequate Information
Permanganate	somewhat Effective	Effective*	Not Effective	Effective	Not Effective
Ozone	Effective	Very Effective	Effective	Effective	Not Effective
UV	Not Effective	Effective at high UV levels	Effective	Effective	Inadequate Information

* *Dependent on initial cyanotoxin concentration, pH, temperature and presence of natural organic matter*

6.13 The Hazen-Adams CyanoTOX Model

A spreadsheet tool entitled the Hazen-Adams Cyanotoxin Tool for OXidation kinetics (CyanoTOX) Model estimates the oxidation of extracellular 6 MCs (MC-LR, -RR, -YR, -LA, -LY and –LF), anatoxin-a and CYN as specific compounds that are measured as individual variants using LC-MS/MS methods and does not address issues related to intracellular toxins. The model is valid in its current form from 10 °C to 30 °C and pH 6 to 9 since for many oxidant/cyanotoxin combinations, pH played a significant role in the value of k" and includes oxidation kinetics for chlorine, ozone, chlorine dioxide, monochloramine, and PM (as either sodium or potassium salt).

CyanoTOX was designed as an assessment tool that provides water utilities with a means to evaluate how changes in their existing oxidative treatment (e.g., pH, oxidant dose, contact time) will influence the degradation of specific cyanotoxins or groups of cyanotoxins. The kinetics and underlying equations and rate constants are based upon the best available peer-reviewed literatures and accepted kinetic modeling principles. The kinetic models used in CyanoTOX are second-order kinetics and based on plug flow contact with baffle factors used to account for non-ideal flow within a water treatment plant.

6.14 Problem Statement

The presence of cyanobacteria in surface water is of increasing concern in the United States as well as other parts of the world (Chorus and Bartman, 1999). Climate change and high nutrient loading cause blooms of cyanobacteria to have occurred more

frequently worldwide and cyanotoxins are known to be one of the major health concerns in drinking water (Carmichael, 1992; Codd *et al.,* 2005; Dietrich and Hoeger, 2005). New U.S. EPA and other health advisories emphasize the use of ELISA as the primary analytical tool for measuring cyanotoxins including MCs and CYN. Both cyanotoxins have variants that are likely reactive with their respective ELISA test kits including approximately 100 variants for MC.

The sensitivity of the ELISA test kit is known to vary for different variants by known and unknown amounts, depending on the variants. Moreover, a separate issue is that the rates of reaction of chemicals with oxidants such as FC and PM can be strongly affected by relatively minor structural changes within a chemical. The different amino acid groups are responsible for differences in the chemical structural between the MC variants, impacting the effectiveness of various treatment processes used for their removal (Newcombe, et al. 2010). Thus, one mix of variants of MC, for example, may have a very different rate of removal than a different mix of variants, though each mix may initially indicate the same ELISA concentration.

It is unknown how the apparent rate of removal for an ELISA reading will compare with individual MC variants such as MC-LR or –RR, for example. Depending on the mixture of MCs present, the removal of an ELISA reading could be an order of magnitude faster or slower than that of an individual MC variant (e.g., MC-LR). It is vital for drinking water utilities to be able to assess and predict the removal of MCs and CYN (as well as other cyanotoxins) for development of HAB response procedures, for reacting during HAB events, and for treatment plant design (e.g., dosing capability).

The removal of cyanotoxin species that included in CyanoTOX would be based on LC-MS/MS readings for individual species. Utilities, however, will usually be using ELISA to monitor concentration of cyanotoxins within their plants. As the removal predicted and achieved within a full scale plant for MC-LR, for example, may be accurately predicted by CyanoTOX Ver. 1.0, the MC ELISA reading may decrease much more slowly (or possibly more quickly) than the concentrations of the known species measured by LC/MS/MS and predicted by CyanoTOX. If the average rate of removal of a mixture of MCs is slower than for MC-LR, the final concentration for a given oxidant exposure will be greater, or the required exposure for a required removal will be less than predicted based on known chemical kinetics. Therefore, it will be more problematic for a water utility attempting to meet a health advisory target. There is, therefore, a critical need to provide guidance to utilities (and regulators) regarding oxidation kinetics for cyanotoxins based on ELISA readings.

6.15 Purpose and Objective of Section 2

The purpose of this study is to assess quantitatively the difference in apparent oxidative removal rates for MCs and CYN based on group measurements by ELISA versus measurement via LC-MS/MS (that is, for a single species such as MC-LR). The work focused on FC, PM, and ozone as the three most important oxidants used to control cyanotoxins. The results of this study were used to establish statistical estimates of confidence intervals for removal of MCs and CYN via oxidation that represent expected removals of an ELISA-based concentration with natural waters. The specific objectives of this work are:

1. Conduct kinetic experiments with FC, PM, and ozone on MC mixtures including:
    - Laboratory waters spiked with known variants and measured using both ELISA and LC-MS/MS
    - Natural water selected as containing widely varying water quality and mixtures of variants within 9 HABs
2. Conduct similar but more limited kinetic experiments with FC, PM, and ozone on CYN containing waters including:
    - Laboratory waters spiked with CYN using both ELISA and LC-MS/MS
    - Natural water selected as containing widely varying water quality within HABs

CHAPTER 7

DIFFERENCES IN APPARENT OXIDATION KINETICS FOR MICROCYSTINS ANALYZED BY ON ELISA VERSUS LC/MS/MS ANALYTICAL METHODS

7.1 Introduction

Cyanobacteria blooms that produce cyanotoxins are commonly called harmful algal blooms (HABs). Cyanotoxins comprise of diverse group of chemical compounds that differ in molecular structure and toxicological properties (Frank, 2002; Chorus and Bartman, 1999). The major cyanotoxins include microcystins (MC), cylindrospermopsin (CYN), and saxitoxins. With over 100 variants reported, MCs are the most commonly found cyanobacterial toxins in drinking and surface water (Codd et al., 2005; Dietrich and Hoeger, 2005; Song et al., 2005; Tsuji et al., 1995). Among the numerous MC variants, many of the worldwide incidents are associated with MC-LR's frequent occurrence (Antoniou Maria et al., 2005; Carmichael, 1992).

The guideline concentration for MC-LR in drinking water was introduced in 1998 by the World Health Organization (WHO) for drinking water at 1.0 µg/L MC-LR. While no enforceable federal drinking water regulations exist for cyanobacteria or their toxins in drinking water or recreational waters at this time in the U.S., health advisories for MCs and CYN were promulgated in 2015 by the US Environmental Protection Agency (USEPA) of 0.3 µg/L and 0.7 µg/L for MCs and CYN for young children, respectively (USEPA, 2015).

MCs consist of over 100 variants with the ADDA moiety (3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid) responsible for MCs' hepatotoxicity

resources (Carmichael, 1992; Codd et al., 2005; Dietrich and Hoeger, 2005). These variants are important as different variants may dominate in any given natural water (Graham et al., 2010). Each of these variants may exert different (and currently unknown) levels of toxicity, and have varying properties (including rates of oxidation by common drinking water oxidants). Relatively few of the variants have been studied with respect to sampling, analysis, fate, treatment, and toxicity. Nonetheless, it is clear that depending on the mix of variants present, the mixture would be expected to have vastly different properties related to analysis, control, and health impacts.

The kinetics of oxidation of MC-LR (and a few other variants) by free chlorine (FC), ozone, and permanganate (PM) are well understood. There are pH effects due to speciation in some cases. This kinetics, furthermore, are modeled in the Hazen-Adams CyanoTOX computational tool, freely downloadable from the American Water Works Association (AWWA) website. The kinetics and underlying equations and rate constants are based upon the best available peer-reviewed literatures and accepted kinetic modeling principles. The kinetic models used in CyanoTOX are second-order kinetics and based on plug flow contact with baffle factors used to account for non-ideal flow within a water treatment plant. With a known chemical exposure (concentration • time, or "CT"), the removal of six MC variants (including MC-LR) can either be predicted as a mixture or as individual variants. Parameters used in the calculations include cyanotoxin type (MC variant, CYN, or anatoxin a), oxidant (FC, ozone, PM, monochloramine, chlorine dioxide), temperature (from 10°C to 30°C), and pH (from pH 6 to 10). Removals are calculated based on known CT or a CT values, estimated by the program based on oxidant dose, oxidant demand, and contact time.

While it is known that some MC variants have faster rates of oxidation by chlorine than MC-LR (e.g., MC-RR and MC-LY) and some have slower rates of oxidation (e.g., MC-LA and MC-LF), the oxidation rates of MC variants other than MC-LR are largely unknown for common oxidants. It is the fundamental hypothesis of this project that mixtures of MCs will typically have an apparent slower rate oxidation when analyzed by enzyme linked immunoassay (ELISA) versus when analyzed by LC-MS/MS methods. The basis of this hypothesis is twofold. First, the ADDA-ELISA method is sensitive to all MCs still containing the ADDA moiety. Thus, while an individual or mix of MCs as analyzed by LC-MS/MS (typically six variants with EPA Method 544) will have a specific rate of removal with an oxidant, ELISA should also include oxidation byproducts that are formed during oxidation (albeit potentially at different sensitivities). The concentration of the pool of "total" MCs including the parent and known and unknown ADDA-containing byproducts, could retain much of or even the full molar concentration of the parent during oxidation even with the parent oxidized to below the method detection limit (MDL). This pool of MCs, as measured by ELISA, would then be hypothesized to degrade more slowly than a parent compound or compounds alone.

Secondly, a mixture of MCs may contain variants with slower, similar, or faster overall rates of oxidation than MC-LR itself (commonly the calibration standard for ELISA) (Figure 7.1). For example, a MC mixture from a given HAB may have most variants with a similar second-order rate constant as MC-LR. In this case, all the MC variants should be removed at similar rates, and ELISA should theoretically track the concentration decreased accurately based on known MC-LR kinetics. This scenario is consistent with the rationale for the overall hypothesis, as discussed in the previous

paragraph. On the other hand, a different mixture of MC variants could have a faster average rate of oxidation than MC-LR due to variants with faster second-order rate constants with the oxidant. In this case, removals based on ELISA could be predicated faster than by MC-LR kinetics, while still offsetting to some degree by the formation of ADDA-containing byproducts. This scenario could lead to less oxidant exposure required to achieve the target concentration (e.g., the health advisory of 0.3 µg/L). However, if the average rate of removal of a mixture of MCs is slower than for MC-LR, the overall rate of decrease for an ELISA reading would likely be lower than for the MC-LR. This would in turn lead to the final concentration for a given oxidant exposure to be greater, or the required exposure for a required removal to be greater, than predicted based on known chemical kinetics. This scenario is most problematic for a water utility attempting to meet a health advisory target.

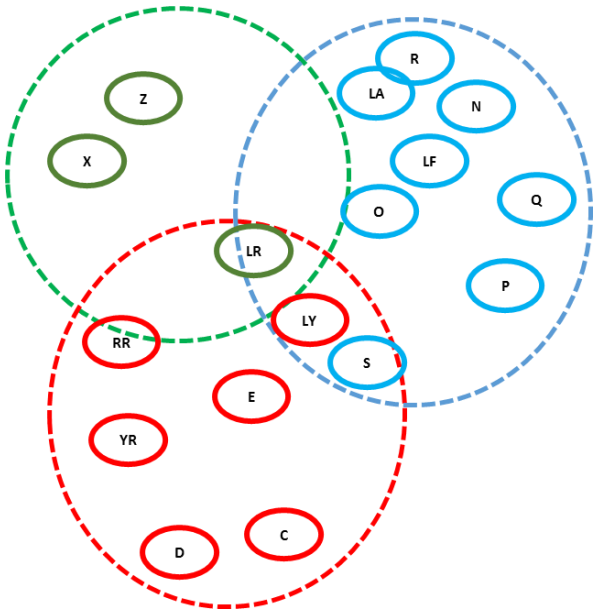

Fig. 7.1. Representation of various mixes of hypothetical MC variants that have second order rate constants faster (red), similar (green) or slower (blue) rates than MC-LR.

The purpose of this study was to assess the difference in apparent removal rates for MCs from natural HABs based on an ELISA analysis versus based on an LC-MS/MS analyses. The ELISA analysis measures the mixture of ADDA-containing MCs (with varied cross reactivity), while the LC/MS/MS method nominally measures only six MC variants, albeit with high accuracy. The goal of the work was to establish estimates of confidence intervals around MC-LR removal that represent expected removals based on ELISA derived concentration measurements in natural waters.

7.2 Material and Methods

7.2.1 Materials

Milli-Q (MQ) was prepared using a Millipore Elix Reverse Osmosis and Millipore A10 system (Millipore, Bedford, MA). The six MCs standard were purchased from ENZO Life Science (MC-YR, -LF, -LY) (Farmingdale, NY USA) or Cayman Chemical (MC-LR-RR, -LA) (Ann Arbor, MI USA) as powders. Stock solutions were prepared with methanol. Working solutions were further diluted to concentrations in the range 1 to 100 µg/L for use immediately prior to using ultrapure MQ water.

FC stock solutions were prepared at 25 mg/L as Cl_2 and verified using the Hach DPD Method 8167. PM stock solutions were prepared at 50 mg/L as MnO_4 and verified using the Hach AccuVac DPD method 8021.

Ozone stock solutions were prepared by passing ozone through a glass diffuser immersed in a magnetically stirred flask containing MQ water. Ozone was generated from pure oxygen using an ozone generator. After allowing the stock solution to come to an equilibrium, the ozone concentration in the stock solution was determined

spectrophotometrically at 254 nm using a Shimadzu spectrophotometer (Model UV-1700) and an extinction coefficient for ozone of 3000 $L \bullet mol^{-1} \bullet s^{-1}$ (Meunier et al., 2006).

7.2.2 Water Sample Characterization

Raw water samples were stored at 5(±1)°C in a refrigerator until use, normally with five days of receipt. The total organic carbon of the raw water samples was determined using a Teledyne Tekmar Apollo 9000 TOC combustion analyzer (Mason, OH) by Standard Method 5310. During oxidation experiments, the temperature and pH of samples were determined using a Fisher Scientific Accument Excel XL 20 pH meter with temperature probe. Water samples were filtered prior to use through a Whatman glass microfiber GFF filters (pore size 0.7-µm, 25 mm diameter), in polypropylene housing.

7.2.3 LC-MS/MS Method

Six MC variants were measured in this study using LC/MS/MS. Briefly, an Agilent Infinity 1290 LC interfaced was used with an Agilent 6490 triple quadrupole MS/MS system, with a JetStream Electrospray ion source. A 10-µL injection was used with a Thermo Scientific Synchronis C8, 2.1 mm ID, 100 mm length, 1.7 um particle size column at a flow rate of 0.4 mL/min. The mobile phases were 20% mM ammonium formate and 100% acetonitrile. For the LC/MS/MS analysis, 1 mL of sample was placed into ASM amber vials with Teflon lined caps, and stored at 5(±1)°C until analysis (normally within 48 hours). LC-MS/MS analysis for six MCs used (modified) EPA Method 544. Mass spectrophotometric parameters are presented in Table 7.1.

Table 7.1. LC-MS/MS parameters used in analysis of MC variants.

Variant	Precursor Ion	Product Ion	CE (V)	Cell Acc (V)	Ret time (min)	Polarity
MC-LA	910.5	134.9	68	4	8.07	Positive
MC-LF	987.0	134.9	60	4	9.50	Positive
MC-LR	995.5	134.9	68	4	7.28	Positive
MC-LY	1002.5	134.9	76	4	8.47	Positive
MC-RR	519.9	134.9	52	4	7.16	Positive
MC-YR	523.3	134.9	40	4	7.23	Positive

Analytes were separated and identified by comparing the acquired mass spectra and retention times to reference spectra and retention times for calibration standards acquired under identical LC/MS/MS conditions. The concentration of each analyte was determined by external standard calibration. The concentration range was up to 12 µg/L for each variant. The method detection limits (MDL) for MC-LR, -RR, -LA, -YR, -LY, -LF were 0.02, 0.06, 0.01, 0.03, 0.01 and 0.08 µg/L, respectively, based on ten replicate injections near the MDL (e.g., 0.10 µg/L).

Continuous calibration verification (CCV), laboratory reagent blank (LRB), and quality control standard (QCS) samples were used at the beginning of each analysis batch, after every ten field samples. They were also used at the end of the analysis batch. The precision of the method was evaluated by determining the relative standard deviation (RSD) of replicate samples.

7.2.4 ELISA Method

An ELISA analysis was conducted using the automated Abraxis Cyanotoxin Automated Assay System (CAAS). The CAAS system was fully automated and used 96-

well microtiter plates for conducting the MC and CYN analyses (Abraxis, 2016). The MC method used was based on indirect competitive ELISA, specifically, Abraxis' MCs-ADDA ELISA kit (Product number 520011OH).

In an indirect competitive ELISA, MCs present in a sample and a monoclonal antibody against MC, compete for the binding sites on a MC-Bovine Serum Albumin (BSA) coated plate. A secondary antibody conjugate (HRP conjugated goat anti-mouse IgG) is added and produces color with the addition of substrate. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The strength of the color development is inversely proportional to the concentration of MCs. The results by immunoassays are compared to a standard curve with known concentrations. MC-LR is used as a calibrating agent and the amount of MCs can be reported as MC-LR equivalents (Abraxis, 2009).

7.3 Experimental Approach

The purpose of these experiments was to test the hypothesis that ELISA-based concentrations would diminish more slowly during oxidation than LC-MS/MS-based concentrations. Specifically, the purpose was to assess quantitatively the differences in oxidative removal rates for MCs for ELISA-based concentrations versus LC-MS/MS-based concentration measurements. In this regard, it was desired to calculate the ratio of ELISA-based concentration measurements versus both. This would include 1) MC-LR alone and; 2) the sum of the six variants measured in the LC/MS/MS method described above. These ratios consist of $k''_{ELISA} / k''_{MC\text{-}LR}$ and $k''_{ELISA} / k''_{\Sigma MC,}$ respectively.

One valid kinetic approach for this work would be to determine individual second-order rate constants for both ELISA-based concentrations and LC-MS/MS-based concentrations and then to develop the ratio of kinetic constants. This would require an accurate knowledge of the actual oxidant exposure (concentration × time, or "CT"). Due to the considerable complexity and associated errors in determining an accurate CT, $k"_{ELISA} / k"_{MC\text{-}LR}$ and $k"_{ELISA} / k"_{\Sigma MSs,}$ ratios would also reflect the combination of these errors. A more accurate means to determine these ratios (and the approach used) was to eliminate the CT from the calculations as follows. Consider the rate equation:

$$\ln(A/A_0) = -k' \cdot T = -k" \cdot CT \qquad (7.1)$$

where T is in seconds, CT is the oxidant exposure, k' is the pseudo-first order rate constant (s^{-1}) and k" is the second-order rate constant ($L \cdot mol^{-1} \cdot s^{-1}$). Developing the ratio of this equation based on ELISA-based and LC-MS/MS-based MC-LR concentrations provides the following:

$$\{\ln(A/A_0)\}_{ELISA} / \{\ln(A/A_0)\}_{LC\text{-}MS/MS \text{ for } MC\text{-}LR} = \{-k" \cdot CT\}_{ELISA} / \{-k" \cdot CT\}_{LC\text{-}MS/MS \text{ for } MC\text{-}LR} = k"_{ELISA} / k"_{LC\text{-}MS/MS \text{ for } MC\text{-}LR} \qquad (7.2)$$

By corollary, developing the ratio-based ELISA-based and LC-MS/MS-based sum of six MC variants concentration provides:

$$\{\ln(A/A_0)\}_{ELISA} / \{\ln(A/A_0)\}_{LC\text{-}MS/MS\ for\ \Sigma MC} = k''_{ELISA} / k''_{LC\text{-}MS/MS\ for\ \Sigma MC} \quad (7.3)$$

For a C/C_0 value to be included the statistical analysis of results, the criteria that need to be met include: 1) both the ELISA and LC-MS/MS concentration needed to be within the range of the method and above the method detection limit; 2) the C/C_0 need to be between 0.2 to 0.9 (that is between 10 and 80% removal).

In Phase 1 of this work, the oxidation of nine natural waters from reservoirs in different states undergoing HAB events were studied with respect to the removal of oxidative MCs. These waters from various sources were anticipated to contain varying mixtures of MCs, where water quality varied (natural organic matter (NOM), pH, etc.). In Phase 2, a bicarbonate-buffered laboratory water was spiked with either a mixture of six MCs, MC-LR or MC-LA and studied with respect to oxidative MC removal.

Oxidation of naturally-occurring MC mixes - In these experiments, natural waters containing cyanotoxins were obtained from various water utilities in the United States. Water samples were shipped frozen and were immediately placed in a freezer upon delivery at the Utah Water Research Laboratory. Lysing of cyanobacterial cells was not a concern as freezer-induced lysing would simply release intracellular toxins and add to the extracellular pool of toxins.

Prior to the oxidation experiments, water samples were filtered through a 0.45-µm glass fiber syringe filter into glass vials. Exactly 4 mL of samples were then transferred into 4-mL amber glass vials for oxidation. For chlorine oxidation, an appropriate quantity of sodium hypochlorite stock solution was spiked into these vials and immediately capped and mixed at time zero to initiate an experiment. Doses of chlorine spiked were nominally,

0.06, 0.25 and 1.25 mg/L as Cl_2. After ten minutes, ascorbic acid was spiked into each vial in slight excess to stop the reaction. Exposures (CT) were chosen to be "low" (L) or "high" (H) (with sometimes a "medium" (M) and/or "very high" (VH) exposure) to attempt to achieve removals (A/A_0) in the range of 0.2 to 0.9 (that is, 10% to 80% removal). Lesser or greater removals would enhance analytical error and were to be avoided.

For PM dosing, the oxidation kinetics were conducted in a similar manner using a 50 mg/L as MnO_4^- PM stock solution to achieve dosages ranging from nominally 0.025, 0.125 and 0.625 mg/L as MnO_4^-. For ozone dosing, the ozone stock solution concentration was determined immediately prior to dosing, and the spike volumes adjusted accordingly to achieve the desired ozone spike doses. The ozone spike doses were nominally 0.02, 0.1 and 0.25 mg/L as O_3. As the decay rate for ozone in these natural waters was rapid, no quenchant was used. A sufficient amount of time for all oxidants to be consumed was instead allowed.

Samples were first analyzed by LC-MS/MS (with a nominally 0 to 12 µg/L dynamic range for each variant) to determine whether they were on the more limited ELISA dynamic range of from 0 to 5 µg/L for MCs. Samples in the 4-mL ELISA vials were then diluted into new ELISA vials if necessary, to attempt to achieve ELISA results that were within the 0 to 5 µg/L range. Samples were then split for ELISA and LC-MS/MS analysis by transferring 1 mL of sample from the ELISA vial into a 1.5-mL LC-MS/MS vial for analysis of identical samples by the two methods. In every comparison, identically diluted samples were analyzed by the two methods.

After the relevant analysis, concentrations were reviewed. If the unoxidized (highest) sample concentration was off scale for the ELISA reading, then the entire series

was diluted, and all samples rerun by both ELISA and LC-MS/MS. This was done to eliminate the possibility of non-linear reductions in apparent concentrations by one method versus the other due to differing effects of dilution on interferences such as NOM within the samples. While theoretically, one could determine the required dilution based solely on the LC-MS/MS readings, several factors prevented this, including that readings for ELISA tend to be higher than LC-MS/MS readings in general. More variants than the six measured by LC-MS/MS could also be present.

Oxidation of spiked MCs in laboratory water - In these experiments, ultrapure MQ water was spiked with either a mixture of six MC variants (i.e., MC-LR, -RR, -LA, -YR, -LY, and –LF), MC-LR only, or MC-LA only. Chemical oxidation with chlorine, permanganate and ozone, and subsequent sample splitting and analysis by ELISA and LC-MS/MS was conducted in an identical manner, as described for the nine natural waters above.

7.4 Results and Discussion

7.4.1 Precision of ELISA and LC-MS/MS Replicate Injections and Samples

For MCs by ELISA, the average relative standard deviation (RSD) of duplicate injections of the same sample on the automated CAAS system, was 13.1% (for samples greater than 0.3 µg/L). For MCs by LC-MS/MS, the average RSD of duplicate injections of the same sample (for samples greater than 0.3 µg/L), was 8.0%, 4.7%, 4.6%, and 3.3% for MC-LR, -RR, -LA, and -YR, respectively (and not determined for MC-LY and –LF, due to very low concentrations).

True duplicate samples were included in the study for approximately 20% of the raw and oxidized MC samples. For raw MC samples, the RSD averaged 14% and 11% for LC-MS/MS and ELISA, respectively. For oxidized MC samples, the RSD averaged 12% and 18% for LC-MS/MS and ELISA respectively.

Table 7.2. Water quality characteristics of the raw waters included in this study.

Sample ID	TOC (mg/L as C)	UV254	Total MC (µg/L)	pH
16913	14	0.11	1	8.4
16915	4043	0.88(diluted 1 to 20)	280*	6.5
16916	413	1.6	265	7.1
16920	17	0.10	0.7	7.9
16926	20	0.11	0.6	8.2
16930	74	0.85	313	7.4
16931	674	1.64 (diluted 1 to 10)	1.6	7.7
16939	16	0.06	2	7.8
16940	25	0.20	150	7.7
Average	588	0.62	112.7	7.6
Median	25	0.20	2.0	7.7

* 16915 was about 28000 µg/L before dilution. Diluted by factor of 100 for all experiments.

7.4.2 Natural Water Characterization

HAB samples from nine water sources were used in this kinetic study. Many other water samples were received and subsequently rejected due to very low levels of MC concentrations. The TOC, UV254, pH, and estimated initial MC concentrations, ranged from 14 to 4043 mg/L as C, 0.06 to 1.64, 6.5 to 8.4, and 1 to 313 µg/l, respectively (Table 7.2).

For the raw water samples, the dominant MC species as measured by LC-MS/MS were MC-LR, -RR, -LA and -YR in nominally 33%, 22%, 33% and 11% of the raw waters, respectively (Table 7.3).

Table 7.3. Percentage of sum of the six MC variants measured by LC-MS/MS in raw waters. (Dominant species in bold.)

	% of ΣMCs in Raw Waters					
Water ID	**MC-LR**	**MC-RR**	**MC-LA**	**MC-YR**	**MC-LY**	**MC-LF**
16913	0	0	0	**100**	0	0
16915	31	**62**	0	7	0	0
16916	**63**	37	0	0	0	0
16920	44	**54**	0	0	2	0
16926	24	12	**64**	0	0	0
16930	28	27	**45**	0	0	0
16931	49	0	**51**	0	0	0
16939	**89**	11	0	0	0	0
16940	**64**	36	0	0	0	0
% Time dominant	33	22	33	11	0	0
% Of waters containing	89	78	33	22	11	0

7.4.3 Ratio of ELISA to MC-LR and ΣMC Concentrations

The key objective of this work has been to assess the ratio of second-order rate constants (L/mol·s) for the removal of unoxidized and oxidized mixtures of MC variants. This, of course, was analyzed by ELISA and LC-MS/MS. ELISA readings were compared to LC/MS/MS concentrations for both MQ (laboratory) waters and natural waters. ELISA

readings correlated with LC-MS/MS readings (α=0.05) for both lab (MQ) and natural waters (Figures 7.2 and 7.3 respectively) (Appendices CY-1, CY-2, CY-3 and CY-4).

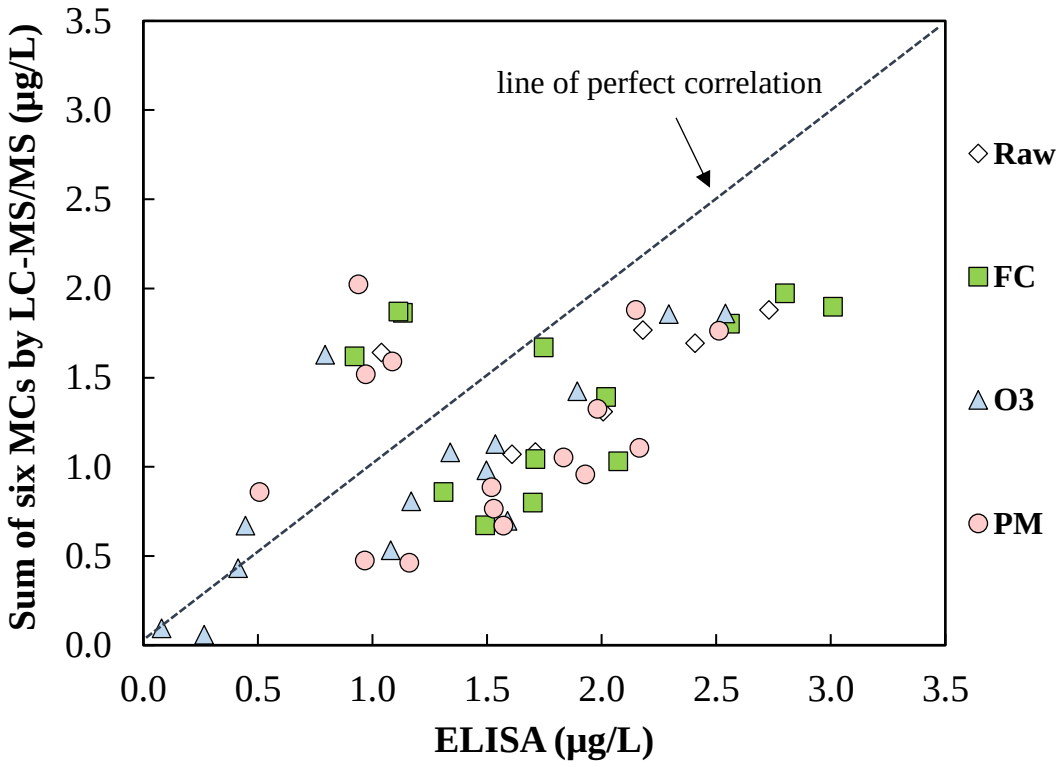

Fig. 7.2. Concentrations from spiked lab (MQ) waters as measured in split samples by ELISA and sum of six MCs by LC-MS/MS for raw (unoxidized) water, and for free chlorine- (FC), ozone- (O_3) and permanganate- (PM) oxidized waters. Dashed line represents perfect correlation.

MQ laboratory water - The MQ laboratory waters were spiked with MC-LR, MC-LA or a mix of six MCs to initial concentrations of nominally 1.5 µg/L. The samples were unoxidized or oxidized with FC, O_3, or PM to achieve a partial removal of the MCs. The results have shown that for lab-spiked waters containing only MC-LR, the ELISA readings

for the unoxidized samples averaged 13% greater than the LC-MS/MS value (Table 7.4). Including oxidation samples, however, ELISA readings averaged 36% greater than the sum of the six MCs overall (only MC-LR in this case) and were 5% to 82% greater for the oxidized samples (Table 7.4).

For lab-spiked waters containing only MC-LA, the ELISA readings for the unoxidized samples were 44% greater than the LC/MS/MS value (Table 7.4). Including oxidation samples, however, the ELISA readings averaged 35% greater than MC-LA by LC-MS/MS and were 22% to 48% greater for the oxidized samples (Table 7.4).

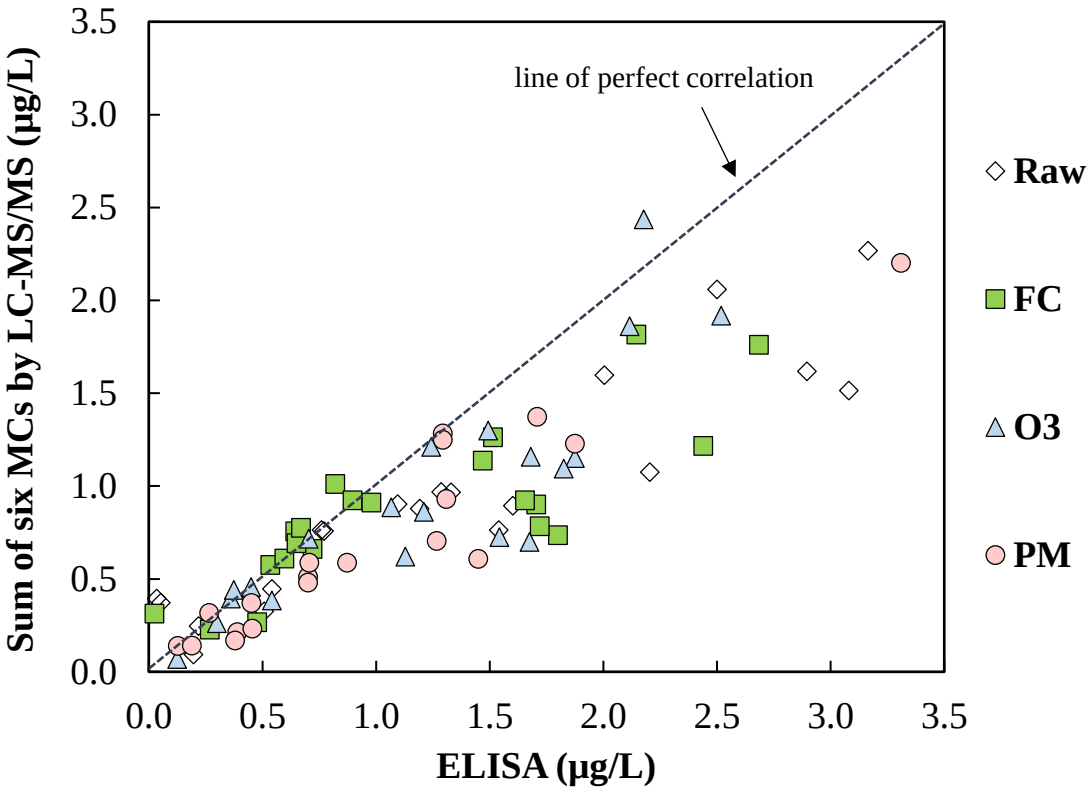

Fig. 7.3. Concentrations from natural waters as measured in split samples by ELISA and sum of six MCs by LC-MS/MS for raw (unoxidized) water, and for free chlorine- (FC), ozone- (O_3) and permanganate- (PM) oxidized waters. Dashed line represents perfect correlation.

Table 7.4. Concentration (not rate) ratio of ELISA to sum of six MC variants for natural and MC-spiked lab water for unoxidized and oxidized samples. (Natural HAB waters exclude two waters with LC-MS/MS concentration at or below MDL.)

	ELISA/ΣMC				
	Overall	**Raw**	**FC**	**O_3**	**PM**
12 Natural HAB waters					
Count	80	20	21	20	19
Mean (µg/L)	1.36	1.34	1.29	1.37	1.46
Median (µg/L)	1.30	1.34	1.18	1.26	1.41
Max (µg/L)	2.44	2.13	2.44	2.40	2.39
Min (µg/L)	0.08	0.09	0.08	0.85	0.84
RSD(%)	36%	42%	43%	31%	29%
Six MC-spiked Lab Water					
Count	14	2	4	4	4
Mean (µg/L)	1.73	1.54	1.88	1.39	2.02
Median (µg/L)	1.61	1.54	1.88	1.41	2.02
Max (µg/L)	2.34	1.58	2.22	1.53	2.34
Min (µg/L)	1.24	1.50	1.53	1.24	1.71
RSD(%)	20%	4%	18%	9%	13%
MC-LR-spiked Lab Water					
Count	23	3	6	6	8
Mean (µg/L)	1.36	1.13	1.05	1.82	1.32
Median (µg/L)	1.05	1.23	0.83	1.44	1.21
Max (µg/L)	4.61	1.53	2.01	4.61	2.51
Min (µg/L)	0.46	0.63	0.57	0.49	0.46
RSD(%)	72%	40%	56%	85%	61%
MC-LA-spiked Lab Water					
Count	12	2	3	4	3
Mean (µg/L)	1.35	1.44	1.89	1.22	1.35
Median (µg/L)	1.42	1.44	1.90	1.28	1.43
Max (µg/L)	1.59	1.45	1.97	1.37	1.49
Min (µg/L)	0.96	1.42	1.80	0.96	1.14
RSD(%)	13%	1%	5%	15%	14%

For lab-spiked waters containing the mix of six MCs, the ELISA readings for the unoxidized samples were 54% greater than the LC-MS/MS value (Table 7.4). Including

oxidation samples, however, the ELISA readings averaged 73% greater than the sum of the six MCs overall and were 39% to 102% greater for the oxidized samples (Table 7.4).

Natural waters - For the natural water samples from national HABs, samples analyzed were again unoxidized or oxidized with FC, O_3, or PM to achieve a partial removal of the MCs. Including oxidation samples, the natural water sample ELISA readings averaged 36% greater than the sum of the six MCs and were 29% to 46% greater for the oxidized samples (Table 7.4).

7.4.4 Ratio of ELISA to MC-LR Second Order Rate Constants

The key goal of this work has been to statistically analyze the distribution of second-order rate constants determined using ELISA-based concentrations versus using LC-MS/MS-based concentrations. The following formula is representative of this:

$$k''_{ELISA} / k''_{LC\text{-}MS/MS\ for\ \Sigma MC} \text{ or } k''_{ELISA} / k''_{LC\text{-}MS/MS\ for\ MC\text{-}LR} \quad (7.4)$$

The LC-MS/MS concentration was the sum of six analyzed MCs or just MC-LR (for which ELISA was calibrated), respectively. For each set of paired ELISA and LC-MS/MS concentration ratios, (i.e., $(A/A_0)\}_{ELISA}$ and $(A/A_0)\}_{LC\text{-}MS/MS\ for\ MC\text{-}LR\ or\ \Sigma MC}$), the apparent second-order rate constant ratios were calculated, as described above.

Based on the frequency of the rate constant ratios, furthermore, the hypothesis that oxidation kinetics based on ELISA concentrations would be slower than based on LC/MS/MS concentrations was supported (on average) for PM, but not for FC or O_3 (for both MC-LR ande ΣMC bases). For the ratios of ELISA-based constants to LC-MS/MS-

based constants using the sum of six MC variants in natural waters, the rate constant ratios were distributed between $k"_{ELISA} / k"_{LC\text{-}MS/MS\ for\ \Sigma MC}$ and were well above to well below unity for each oxidant. For free chlorine, the ratios ranged from 0.26 to 4.44, with a mean of 1.53 and a median of 1.59 (Table 7.5). Similarly, for ozone, ratios ranged from 0.40 to 2.09 with a mean of 1.33 and a median of 1.40 (Table 7.5). For permanganate the ratios ranged from 0.17 to 3.46, with a mean of 1.33 and a median of 1.40 (Table 7.5). Overall, this data demonstrates that kinetic rates constants for ELISA concentration values ranged from just 17% to 444% of the LC-MS/MS concentration values.

For the ratios of ELISA-based constants to LC-MS/MS-based constants using the MC-LR concentrations in natural water, similar results were achieved. This data demonstrates that the kinetic rates constants for ELISA concentration values ranged from just 18% to 478% of the LC-MS/MS concentration values, again effectively a factor of plus or minus four to five times. Overall, the average MC-LR-based ratios were from 10% to 20% higher than for the sum for MCs-based ratios (Table 7.5).

The 95%-confidence intervals were calculated for the six $k"_{ELISA} / k"_{LC/MS/MS}$ ratios (i.e., three oxidants, and MC-LR or sum of MCs basis). The confidence intervals in natural waters ranged from 15.1% to 38.4% and averaged 30% overall (Table 7.5).

For experiments conducted in spiked MQ laboratory waters, data for ELISA and LC-MS/MS were greatly limited. Despite this, data was within an acceptable quantitation for this analysis. This data demonstrates that generally the $k"_{ELISA}/k"_{\Sigma MC}$ ratio calculated were near or less than unity, that is, based on the frequency of the rate constant ratios (9 less than one, 2 near, and 2 greater than unity). Thus, the hypothesis that oxidation kinetics

based on ELISA concentrations would be slower than based on LC-MS/MS concentrations was generally supported by the limited data set.

Table 7.5. Statistical analysis of oxidative removal of MCs via ELISA-based analysis versus using sum of six MC variants (via LC-MS/MS).

	$k''_{ELISA} / k''_{\Sigma MC}$				$k''_{ELISA} / k''_{MC\text{-}LR}$			
Parameter	**FC**	**O3**	**PM**	**Mean**	**FC**	**O3**	**PM**	**Mean**
Average	1.53	1.31	1.06	1.30	1.84	1.45	1.25	1.51
RSD(%)	76%	34%	77%		74%	40%	92%	
Median	1.59	1.39	0.83	1.27	1.40	1.31	0.82	1.18
Minimum	0.26	0.40	0.17		0.22	0.37	0.18	
Maximum	4.44	2.09	3.46		4.78	2.66	4.64	
Count	13	16	17		12	15	16	
ELISA faster	8	12	5		10	12	7	
ELISA slower	5	4	12		2	3	9	
Percent with ELISA faster	62%	75%	29%		83%	80%	44%	
t (1 tail, α=0.05)	1.79	1.75	1.75		1.80	1.76	1.75	
95% CI (+/-)	0.57	0.20	0.35		0.71	0.26	0.50	
95% CI (+/-) (%)	37.5%	15.1%	32.8%		38.4%	18.2%	40.1%	
Average 95% CI (+/-) (%) =		28.5%				32.3%		
Average overall 95% CI (+/-) (%) = 30%								

7.5 Oxidation of Cylindrospermopsin

With respect to differing reactivity between ELISA and LC-MS/MS for CYN, the study plan was to include comparison of the apparent oxidation kinetics of natural waters containing (or spiked with) CYN. Oxidation of CYN in natural water samples was attempted as described for the MCs samples above. However, due to lack of precision of CYN analysis by the automated CAAS system, these studies were terminated (Appendices CY-5 and CY-6).

7.6 Discussion and Conclusions

For any type of water, the mix of MCs may react faster or slower than MC-LR, depending on the specific variants' in kinetics, the oxidant, and other factors. Furthermore, this study confirmed the hypothesis that analysis by ELISA can provide different removal rates than with analysis by LC/MS/MS due to a variety of factors. Utilities must be able to estimate the removal of MCs with key oxidants (i.e., FC, ozone, and PM). Current tools (e.g., Hazen-Adams CyanoTOX (Version 2)) utilize best available kinetics for known MC variants. As was hypothesized and observed experimentally in this work, the removal of MC measured by ELISA would be expected to have a range of rates around that for MC-LR (or other known variants).

This work provides an estimate of the 95% confidence interval for the difference in oxidation rates predicted, based on ELISA readings versus specific variants by LC-MS/MS. These confidence intervals on relative rate constants allow for estimating the range of removal anticipated if one is using ELISA for the analysis. This is further based on known kinetics for MC-LR.

As an application example, the predicted removal for a mix MCs was calculated based on MC-LR kinetics, and calculated and presented in Figure 7.4, with the average 95% confidence interval shown graphically. For this example, the predicted CT required ranges from approximately 40 to 72 mg·min/L to achieve a final concentration of 0.3 µg/L for MCs, versus approximately 51 mg·min/L as predicted based on MC-LR alone.

As a result of this study, 95% confidence intervals of ±30% have been incorporated in the Hazen-Adams CyanoTOX tool (Version 2.0) as a guide for utilities in estimating the

range of removals operators may expect in their treatment plants using ELISA-based concentrations.

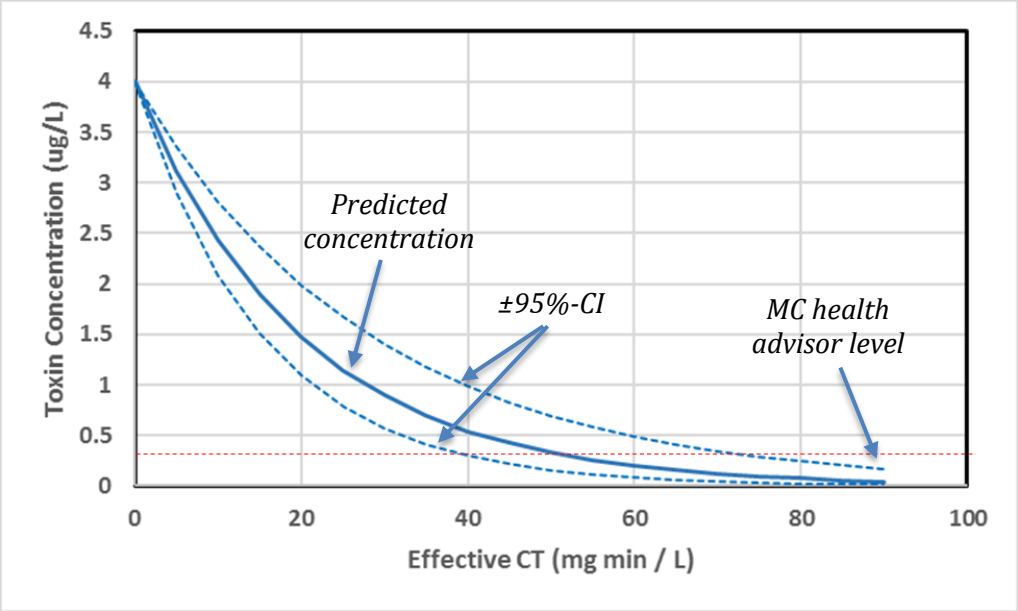

Fig. 7.4. 95%-confidence intervals of ±30% based on uncertainty in removal kinetics based on ELISA- versus LC-MS/MS-based concentrations using predicted MC-LR oxidative removal by free chlorine as an example generated using CyanoTOX (Ver. 2.0).

CHAPTER 8

CONCLUSION AND FUTURE WORK (CYANOTOXINS)

8.1 Conclusion

Due to climate change and high nutrient loading hazardous algal blooms are increasing in frequency and magnitude worldwide. Cyanobacteria produce a variety of toxins with MCs the most commonly detected cyanotoxins of major health concern in surface and drinking water.

The recent U.S. EPA health advisories for MCs are based on the use of ELISA as the primary analytical tool for measuring both Microcystins (MCs) and Cylindrospermopsin(CYN). MCs have many variants that are reactive with the ELISA test kit. The sensitivity of the ELISA test kit is known to vary for different variants by known and unknown amounts, depending on the variant. Further, interferences such as by natural organic matter or oxidation byproducts may have significant and unpredictable impacts on ELISA readings. These differing sensitivities and interferences cause significant overestimation (typically) or underestimation (less frequently) of the ELISA reading relative to the concentration of known species by LC-MS/MS. Regulatory authorities need to be convinced that the ELISA results are reliable, even when they disagrees with LC-MS/MS result. There is, therefore, a critical need to provide guidance to utilities (and regulators) regarding oxidation kinetics for cyanotoxins based on ELISA readings.

This work provides estimates of the 95%-percent confidence interval for the difference in oxidation rates predicted with based on ELISA readings versus specific variants by LC-MS/MS. These confidence intervals on relative rate constants allow

estimation of a range of removal to be anticipated if using ELISA for the analysis, based on known kinetics for MC-LR.

ELISA readings averaged greater than LC-MS/MS concentrations for the split samples. Possible reasons for this include greater interference effects (e.g., with NOM) for ELISA than LC-MS/MS, the fact that ELISA is typically calibrated on the MC-LR variant and some variants have different sensitivities, and the fact that ELISA is sensitive at some level to many or all of the 100(+) ADDA-moiety containing MC variants and oxidation byproducts whereas the LC-MS/MS method was set for only six variants.

8.2 Future Work

The alarming increase in the frequency and magnitude of cyanobacterial bloom worldwide impose a demand to find faster, reliable and economical methods for monitoring MCs. The traditional LC-MS/MS method is unable to meet this rising demand due to its cost, limited variety of available reference standard and cumbersome procedure. Most of the water utilities prefer to use ELISA because ELISA is relatively cost effective, fast and easy to use in compare to LC-MS/MS. Although ELISA reports total MCs, it does not provide toxicity information. So, it is important to consider toxicological approach of MCs as well by using other assays like as PPIA.

An advantage of protein phosphatase inhibition assay (PPIA) over ELISA is its ability to detect bioactivity of MCs, rather than limited recognition of a structural component. At similar concentrations, toxicity due to other variants or mixtures of variants may be different. Additionally, oxidation of MCs, specifically chlorination of MCs leads to the formation of numerous by-products through chlorine substitution on the initial toxin,

including on the ADDA moiety may increase in toxicity of the mixture after chlorination. As a result, it would be useful to consider the use of both PPIA and ELISA by water utilities. The use of ELISA and PPIA together can provide enough information to protect the public from microcystins toxicity and more meaningful regulations for drinking water can be established based on these two assays.

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Appendices

Appendix HA-1

SOP FOR CHLORAMINE ANALYSIS (v. 5.0)

(Original prepared by Drs. Zhimin (George) Qiang and Craig Adams on 2/28/2006.
Modified by Craig Adams August 2014)

This method is based on and modified from 4500-Cl G. DPD Colorimetric Method, *Standard Methods for the Examination of Water and Wastewater.*

1. Reaction Mechanism

In the absence of iodide ion, free chlorine reacts instantly with DPD indicator to produce a red color. Subsequent addition of a small amount of iodide ion acts catalytically to cause monochloramine to produce color. Addition of iodide ion to excess evokes a rapid response from dichloramine. In the presence of iodide ion, part of the nitrogen chloride (NCl_3) is included with dichloramine and part with free chlorine. A supplementary procedure based on adding iodide ion before DPD permits estimating proportion of NCl_3 appearing with free chlorine.

2. General Discussion

a *Principal:* Free and combined chlorines react with DPD to show a red color. The color is measured with a spectrophotometer at a wavelength of 515 nm and with a ≥ 1-cm light path photocell.

b *Interference:* Compensate for color and turbidity by using sample to zero photometer. Minimize chromate interference by using the thioacetamide blank correction. (Maximum total chromium conc. = 0.58 mg/L in Orlando pools.)

c *Minimum detectable concentration:* approximately 10 μg/L as Cl_2.

d *Glassware:* Use separate glassware and separate photocells for free and combined chlorine (dichloramine) measurements, to avoid iodide contamination in free chlorine measurement.

3. Reagents:

a *Phosphate buffer solution*: Dissolve 24 g anhydrous Na_2HPO_4 and 46 g anhydrous KH_2PO_4 in distilled water. Combine with 100 mL distilled water in which 800 mg disodium ethylenediamine tetraacetate dehydrate (EDTA) have been dissolved. Dilute to 1 L with distilled water and optionally add either 20

mg $HgCl_2$ or 2 drops toluene to prevent mold growth. Interference from trace amounts of iodide in the reagents can be negated by optional addition of 20 mg $HgCl_2$ to the solution.

- .(Fisher S374500 Sodium Phosphate Dibasic Anhydrous, Granular or Powder, Certified ACS ⩾ 99 %, Monohydrogen Sodium Phosphate, Disodium Hydrogen Phosphate, Na2HPO4)
- (Fisher P284-500 Potassium Phosphate Monobasic, Granular, Laboratory, KH2PO4
- Disodium ethylenediamine tetraacetate dehydrate (EDTA) (Ethylenediamine Tetraacetic Acid, Disodium Salt Dihydrate Crystallin Powder, Fisher Sci, BP120500)
- $HgCl_2$ (Mercuric Chloride (Crystalline or Powder/Certified ACS), Fisher Chemical, M155I-100)
- Toluene (Fisher)

b *N,N-Diethyl-p-phenylenediamine (DPD) indicator solution*: Dissolve 1 g DPD oxalate chlorine-free ultrapure water containing 8 mL "1+ 3 H_2SO_4" (i.e., 1 mL water plus 3 mL concentrated H_2SO_4) and 200 mg disodium EDTA. Make up to 1 L, store in a brown glass-stoppered bottle in the dark, and discard when discolored. Periodically check solution blank for absorbance and discard when absorbance at 515 nm exceeds 0.002/cm.

- (Fisher NC9638873 Hach Chemical DPD OXALATE ANAL 25G)
- Disodium ethylenediamine tetraacetate dehydrate (EDTA) (Ethylenediamine Tetraacetic Acid, Disodium Salt Dihydrate Crystallin Powder, Fisher Sci, BP120500)
- H_2SO_4 (Fisher)

c *Potassium iodide*, KI, crystal.

- *(*Fisher P410-100 Potassium Iodide Granular, 100 g, Free Flowing Certified ACS ⩾99 %)

d *Potassium iodide (KI) solution A (more dilute)*: Dissolve 100 mg KI and dilute to 200 mL, using ultrapure (e.g., 18 MΩ Milli-Q) lab water. Store in a brown glass-stoppered bottle, preferably in a refrigerator. Discard when solution becomes yellow. (Note: Final conc = 500 mg/L KI; 4 drops ~ 200 µL = 0.1 mg)

e *Potassium iodide (KI) solution B (more concentrated)*: Dissolve 5000 mg KI and dilute to 25 mL, using ultrapure (e.g., 18 MΩ Milli-Q) lab). Store in a brown glass-stoppered bottle, preferably in a refrigerator. Discard when solution becomes yellow. (Note: Final conc = 200,000 mg/L KI; 10 drops ~ 500 µL = 0.1 g (100 mg))

4. Procedure:

a *Calibration*

- *Of spectrophotometer:* Hach spectrophotometer should already be calibrated. Calibration standards are available from Hach as a set of four (Hach DR/Check Absorbance Standard Kit 2763900 $ 166.00) to check (not adjust) calibration.
- *Of method:*
    i. Prepare a stock solution containing 891 mg/L $KMnO_2$. Dilute 1 mL of this stock solution into 1.000 L. This solution has the same oxidizing power as 1 .00 mg/L Cl_2 and can be run in the free chlorine portion of the method below to calibrate.
    ii. This calibration factor was determined in our lab to be: Conc=(Abs-0.0575)/0.4281. This is built into the SOP spreadsheet, but can be redetermined at any time by analyzing equivalent concentration the permanganate solution at 0, 0.5, 1 and 2 mg/L as Cl_2 and plotting against the A measure (Free chlorine below)

b *Calibration of results:*

c *Volume of sample:* This procedure is based on using 10-mL volumes; adjust reagent quantities proportionately for other sample volumes. Dilute sample with MQ water when total chlorine exceeds 4 mg/L.

d *Free chlorine:* Place 0.5 mL each of buffer reagent and DPD indicator reagent in a test tube or photocell. Add 10 mL sample to mix. Read color immediately (Reading A → free chlorine).

e *Monochloramine:* Continue by adding 200 µL (or 4 drops) (about 0.1 mg) "KI Solution A (500 mg/L)" and mix. (Reading B→DCA). (If DCA is high, more KI is needed.)

f *Dichloramine:* Continue by adding 500 µL (or 10 drops) (about 0.1 g) "KI Solution B (200 g/L)" and mix to dissolve. Let stand about 2 min and read color (Reading C).

g *Nitrogen chloride (Trichloramine):* Place 200 µL (or 4 drops) (about 0.1 mg) "KI Solution A (500 mg/L)" in a clean test tube or photocell. Add 10 mL sample and mix. To a second tube or photocell add 0.5 mL each of buffer and DPD indicator reagents; mix. Add contents to first tube or photocell and mix. Read color immediately (Reading N).

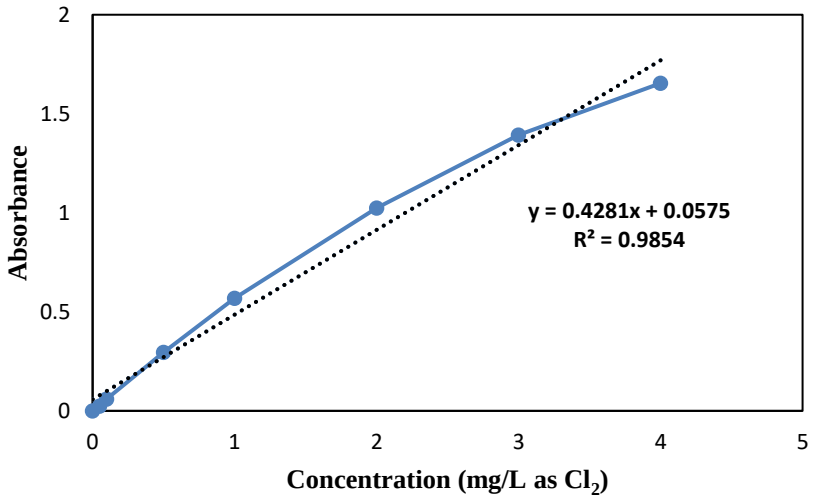
4. Calculation

Reading	NCl_3 Absent	NCl_3 Present
A	Free Cl	Free Cl
B-A	NH_2Cl	NH_2Cl
C-B	$NHCl_2$	$NHCl_2 + 1/2NCl_3$
N	–	Free Cl + $1/2NCl_3$
2(N-A)	–	NCl_3
C-N	–	$NHCl_2$

In the event that monochloramine is present with NCl_3, it will be included in Reading N, in which case obtain NCl_3 from *2(N-B)*.

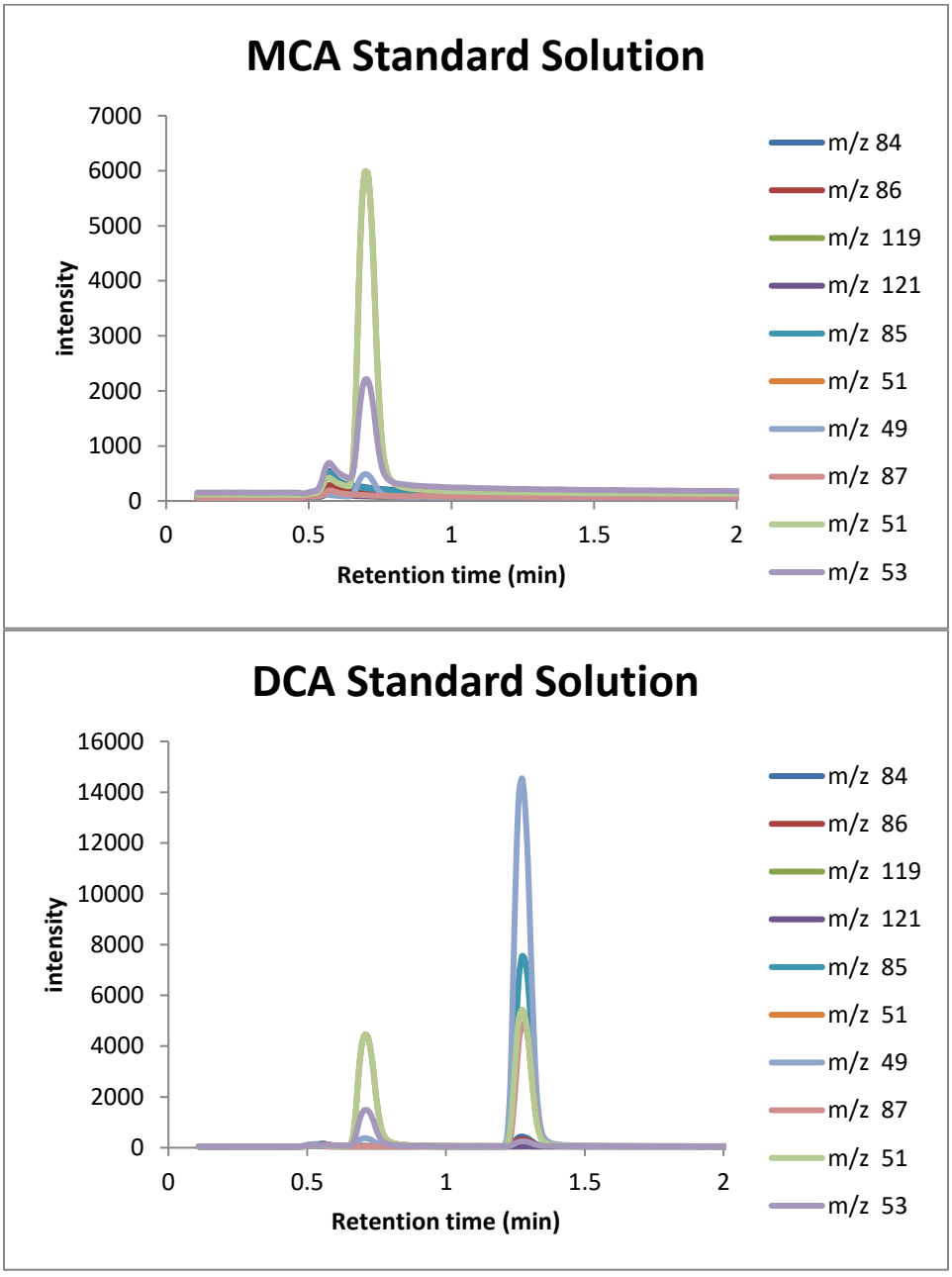
Spreadsheet “SOP CHLORAMINE v 5” provides automatic calculations upon entering A, B, C, and N values.

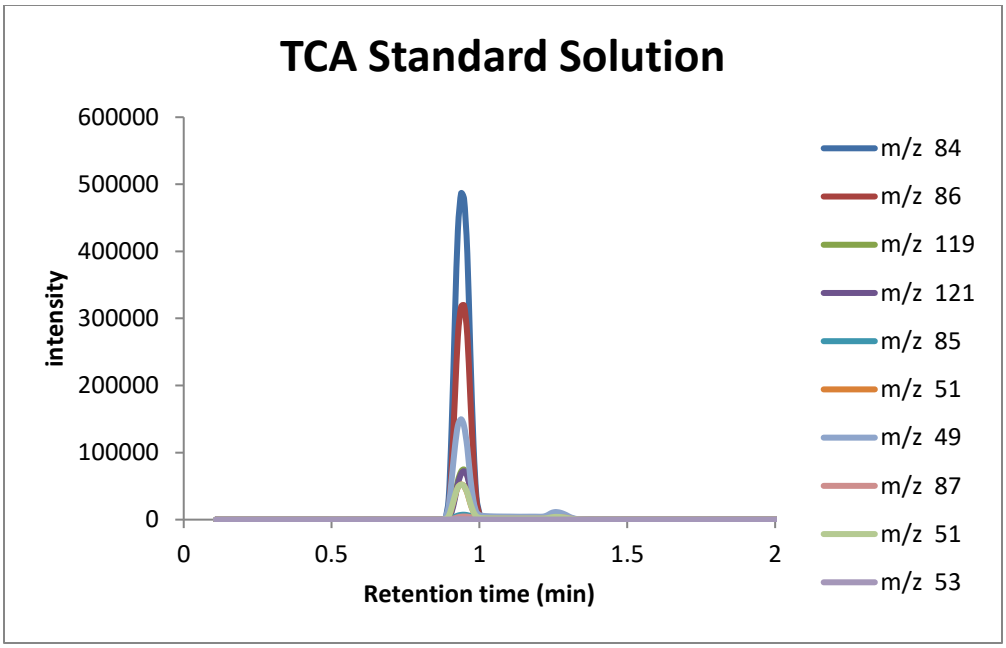
Appendix HA-2



HA-2. Calibration curve based on standard method 4500-Cl G using potassium permanganate in absorbance at 515 nm.

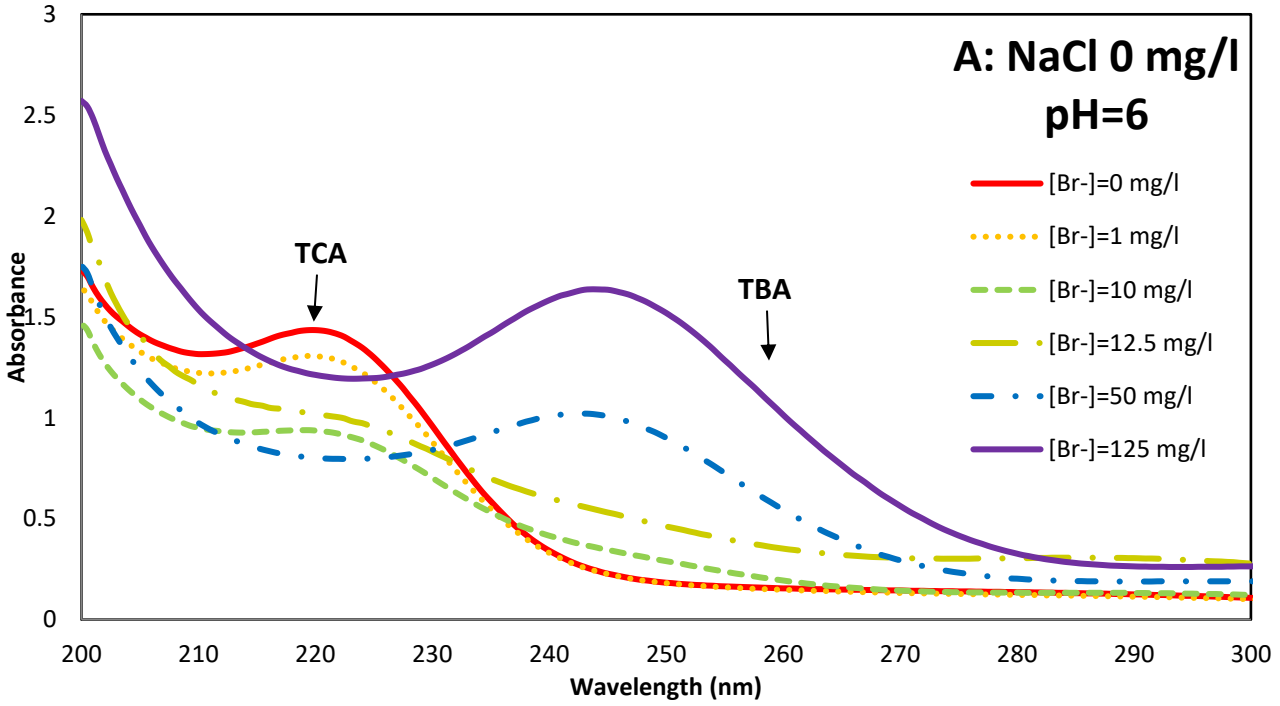
Appendix HA-3

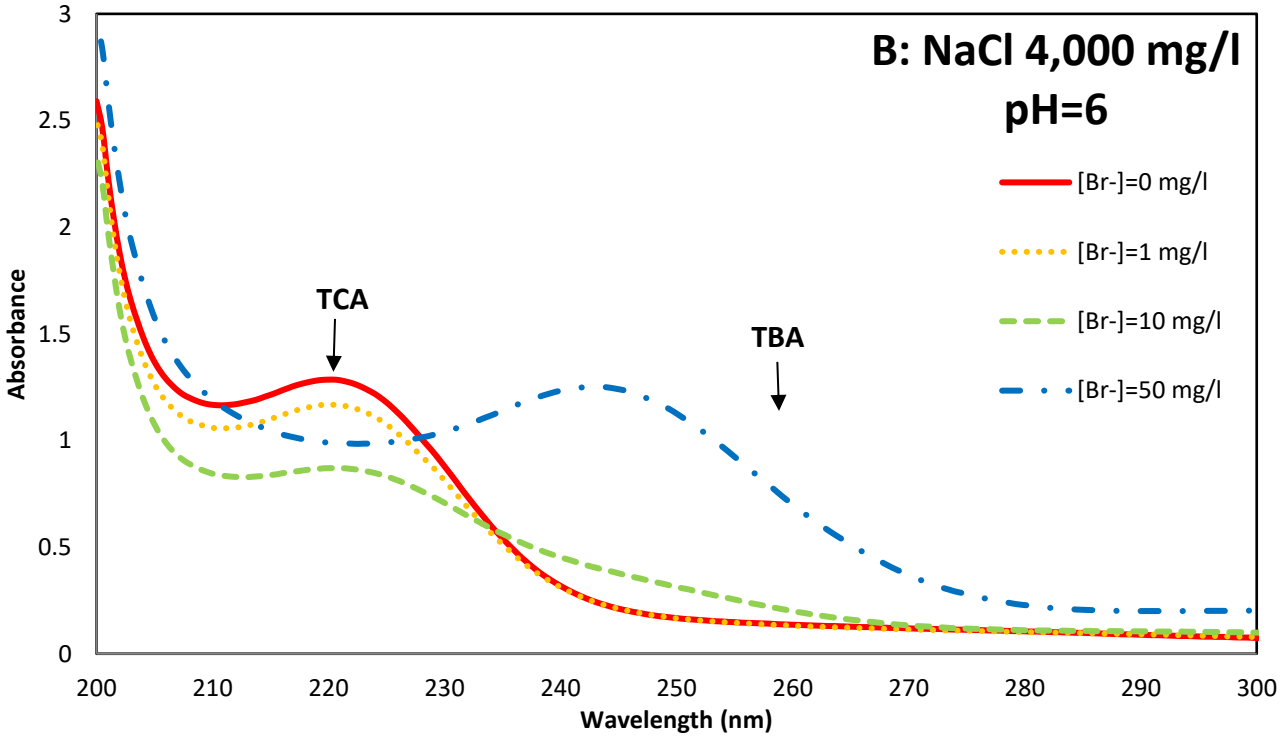


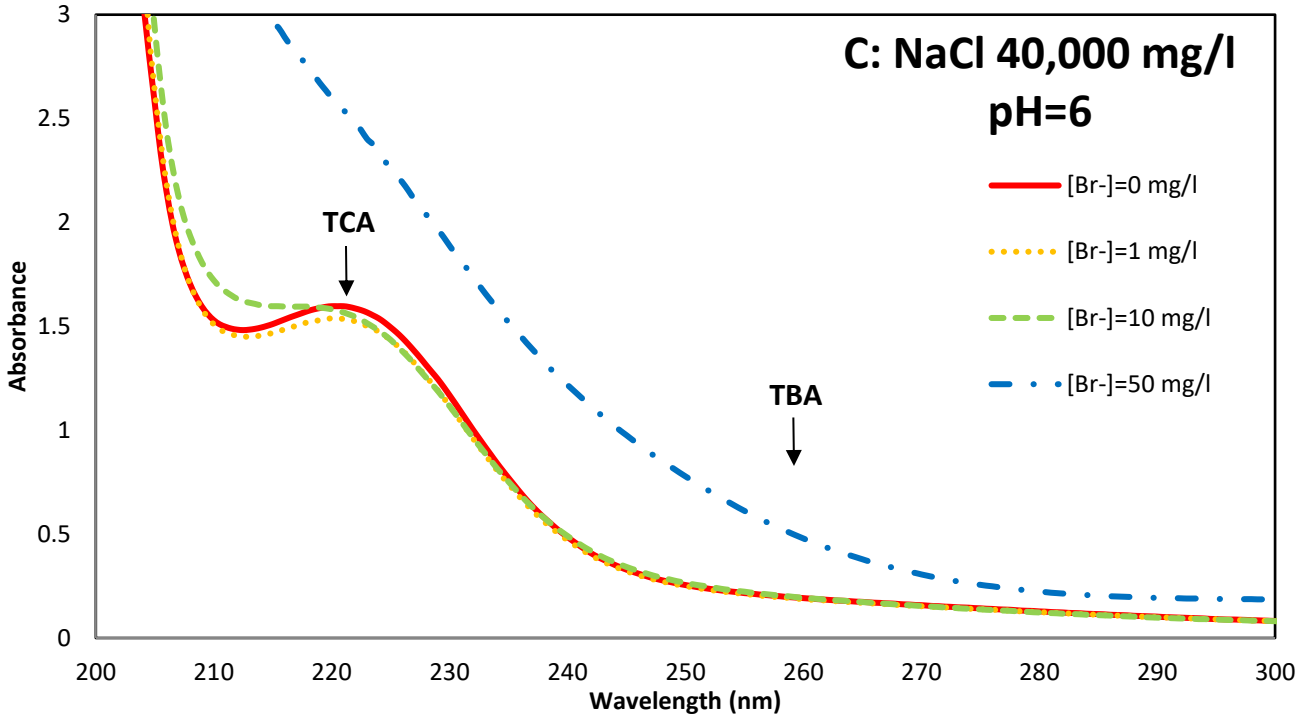


HA-3. SIM chromatogram of TCA, DCA, and MCA in their standard solutions determined by head-space GC-MS at a pH level of 7.8 and theoretical concentrations of 30 µg/l.

Appendix HA-4

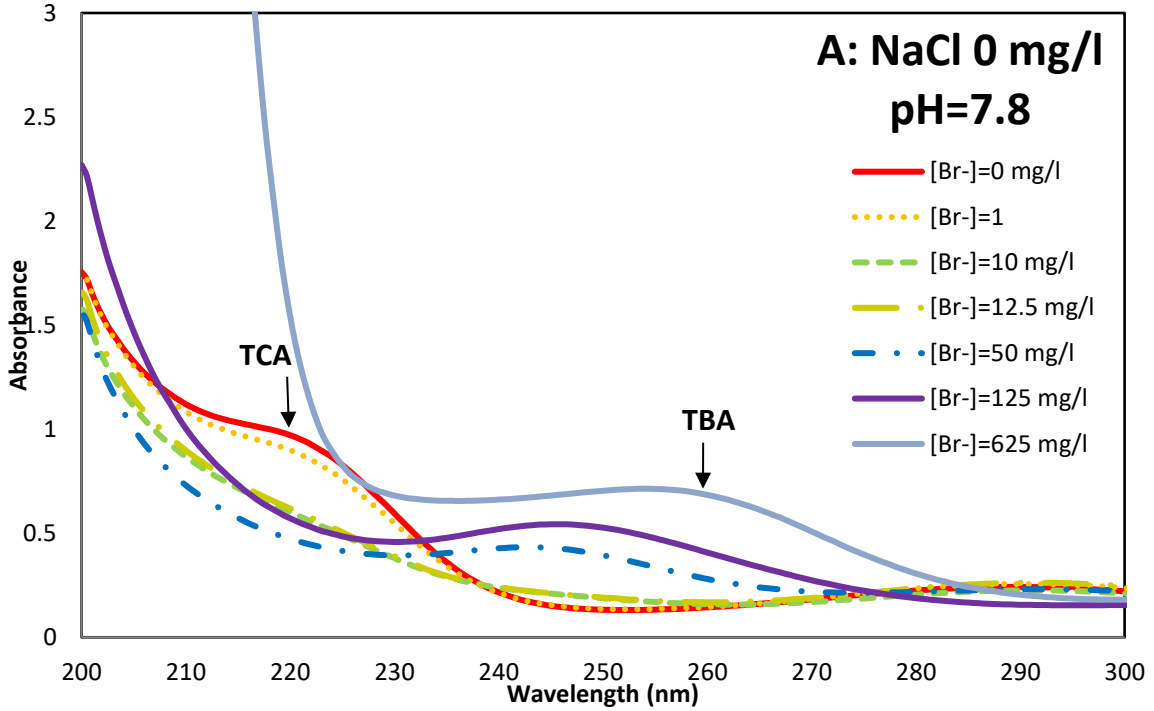


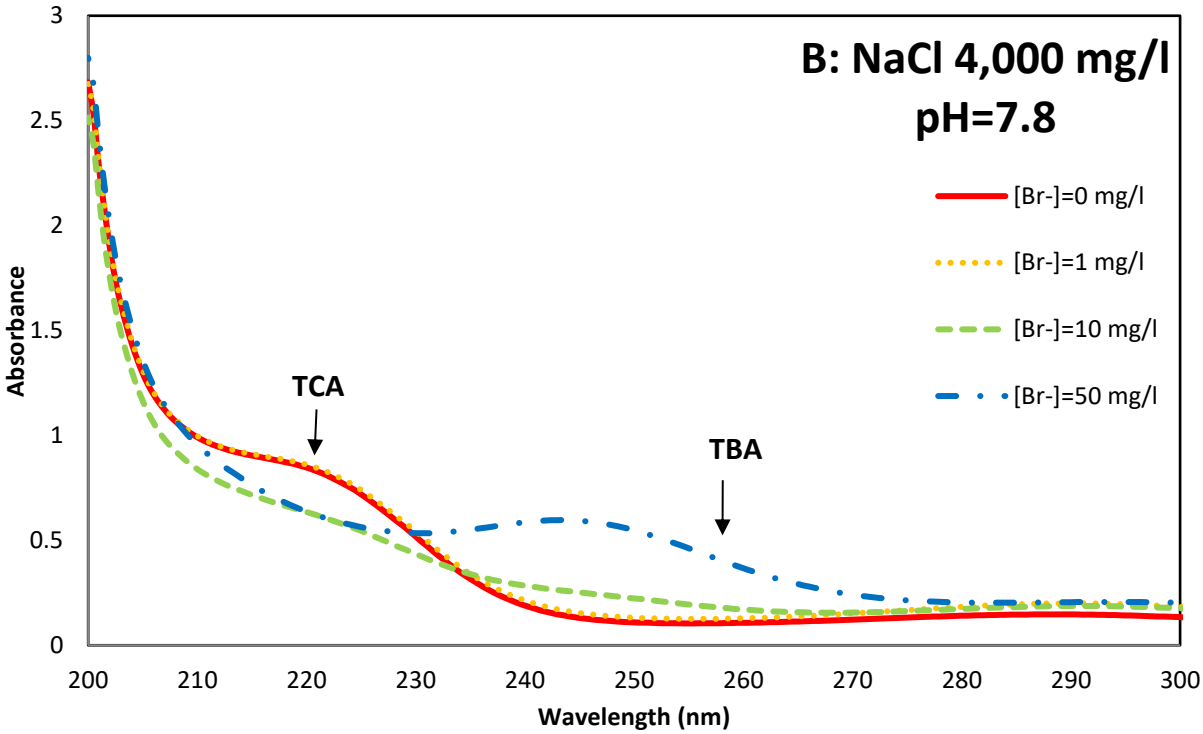


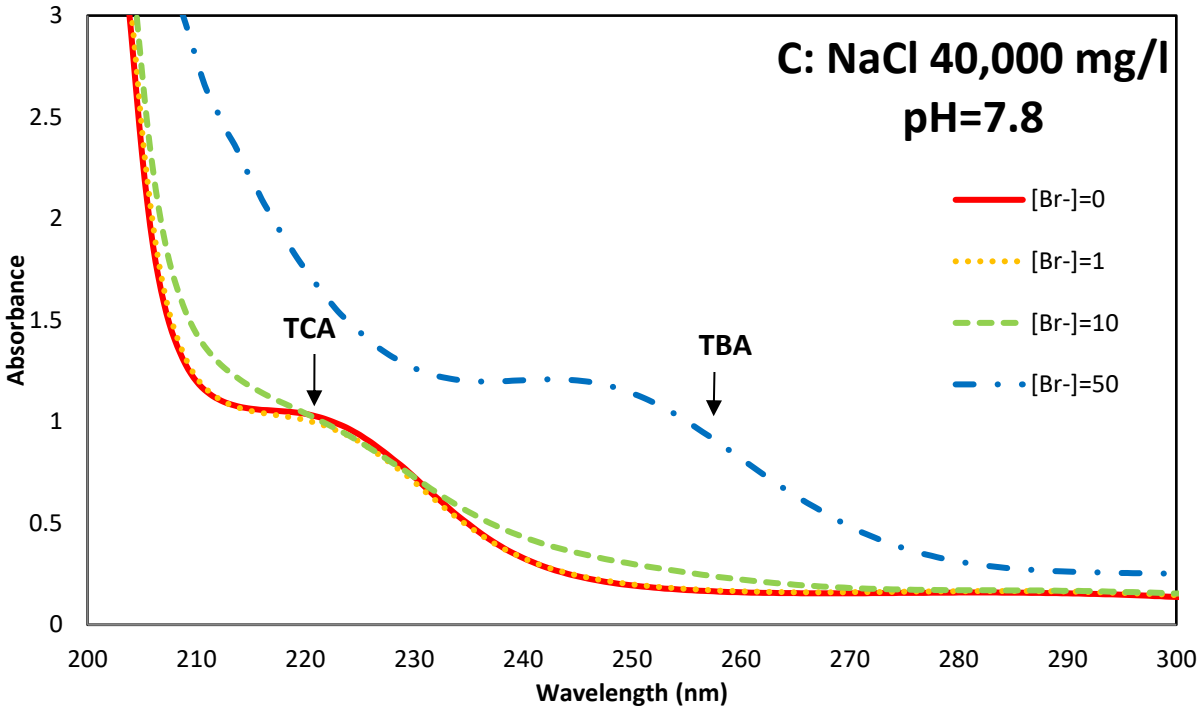


HA-4. UV spectra as a function of bromide concentration for TCA standard: (A) NaCl 0 mg/l and [Br^-] 0-125 mg/l; (B) NaCl 4,000 mg/l and [Br^-] 0-50 mg/l; (C) NaCl 40,000 mg/l and [Br^-] 0-50 mg/l. Experimental conditions: chlorine to ammonia mass ratio 12:1 (mg/L: mg/L), 5mM phosphate buffer, pH 6, temperature 25±2.

Appendix HA-5







HA-5. UV spectra as a function of bromide concentration for TCA standard: (A) NaCl 0 mg/l and [Br^-] 0-625 mg/l; (B) NaCl 4,000 mg/l and [Br^-] 0-50 mg/l; (C) NaCl 40,000 mg/l and [Br^-] 0-50 mg/l. Experimental conditions: chlorine to ammonia mass ratio 12:1 (mg/L: mg/L), 5mM phosphate buffer, pH 7.8, temperature 25±2.

CY-1. Concentrations, fraction remaining, and ratios for sum of MCs from natural waters as measured in split samples.

				Data acceptance for kinetics			Fraction remaining		$(C/C_0)_{ELISA}$			$(C/C_0)_{\Sigma MC}$			$(C/C_0)_{ELISA} / (C/C_0)_{\Sigma MC}$			$k''_{ELISA} / k''_{\Sigma MC}$		
UWRL Water Code	**CODE**	**ELISA (µg/L)**	**ΣMC (µg/L)**	**ELISA> MDL?**	**LC> MDL?**	**C/C_0 5-95%?**	**ELISA C/C_0**	**ΣMC C/C_0**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**
16913	R	3.91	0.26	Y	Y	- - -	1.17	0.97												
16913	R-D	2.77	0.28	Y	Y	- - -	0.83	1.03												
16913	FC-L	2.55	0.26	Y	Y	N	0.76	0.98	0.76			0.98								
16913	FC-H	1.08	0.00	Y	N	N	0.32	0.00	0.32			0.00								
16913	O3-H	1.21	0.00	Y	N	N	0.36	0.00		0.36			0.00							
16913	O3-H-D	0.97	0.00	Y	N	N	0.29	0.00		0.29			0.00							
16913	PM-L	2.13	0.00	Y	N	N	0.64	0.00			0.64			0.00						
16913	PM-H	0.81	0.00	Y	N	N	0.24	0.00			0.24			0.00						
16915	R	1.54	0.76	Y	Y	- - -	0.97	0.88												
16915	R-D	2.21	1.07	Y	Y	- - -	1.39	1.24												
16915	FC-VH	1.71	0.90	Y	Y	N	1.07	1.04	1.07			1.04								
16915	FC-VH-D	1.66	0.92	Y	Y	N	1.04	1.06	1.04			1.06								
16915	PM-VH	1.31	0.93	Y	Y	N	0.82	1.07			0.82			1.07						
16915	O3-VH	1.13	0.62	**Y**	**Y**	**Y**	0.71	0.71		0.71			0.71			0.99			1.02	
16916	R	0.51	0.33	Y	Y	- - -	0.97	0.84												
16916	R-D	0.54	0.45	Y	Y	- - -	1.03	1.16												
16916	FC-VH	0.48	0.27	**Y**	**Y**	**Y**	0.91	0.69	0.91			0.69			1.32			0.26		
16916	PM-VH	0.39	0.21	**Y**	**Y**	**Y**	0.74	0.55			0.74			0.55			1.35			0.50
16916	PM-D-VH	0.27	0.32	N	Y	Y	0.51	0.82			0.51			0.82			0.62			3.46
16916	O3-VH	0.30	0.26	Y	Y	Y	0.57	0.68		0.57			0.68			0.84			1.45	
16916	R	0.20	- - -	N	Y	- - -	0.95	- - -												
16916	R-D	0.22	0.25	N	Y	- - -	1.05	1.00												
16916	FC-VH	0.27	0.23	N	Y	N	1.29	0.92	1.29			0.92								
16916	PM-VH	0.13	0.14	N	Y	Y	0.61	0.57			0.61			0.57			1.08			0.87
16916	PM-D-VH	0.19	0.14	N	Y	Y	0.91	0.57			0.91			0.57			1.58			0.17
16916	O3-VH	0.13	0.07	N	Y	Y	0.60	0.28		0.60			0.28			2.16			0.40	

CY-1 (cont'd). Concentrations, fraction remaining, and ratios for sum of MCs from natural waters as measured in split samples.

				Data acceptance for kinetics			Fraction remaining		$(C/C_0)_{ELISA}$			$(C/C_0)_{\Sigma MC}$			$(C/C_0)_{ELISA} / (C/C_0)_{\Sigma MC}$			$k"_{ELISA} / k"_{\Sigma MC}$		
UWRL Water Code	**CODE**	**ELISA (µg/L)**	**ΣMC (µg/L)**	**ELISA> MDL?**	**LC> MDL?**	**C/C_0 5-95%?**	**ELISA C/C_0**	**ΣMC C/C_0**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**
16916	R	2.00	1.60	Y	Y	- - -	1.00	1.00												
16916	FC-L	1.47	1.14	**Y**	**Y**	**Y**	0.73	0.71	0.73			0.71			1.03			0.91		
16916	FC-H	1.52	1.26	**Y**	**Y**	**Y**	0.76	0.79	0.76			0.79			0.96			1.19		
16916	FC-H-D	0.82	1.01	**Y**	**Y**	**Y**	0.41	0.63	0.41			0.63			0.65			1.95		
16916	O3-L	1.24	1.21	**Y**	**Y**	**Y**	0.62	0.76		0.62			0.76			0.82			1.73	
16916	O3-L-D	1.49	1.30	**Y**	**Y**	**Y**	0.75	0.81		0.75			0.81			0.92			1.43	
16916	O3-H	1.68	1.16	**Y**	**Y**	**Y**	0.84	0.72		0.84			0.72			1.16			0.55	
16916	PM-L	1.29	1.28	**Y**	**Y**	**Y**	0.65	0.80			0.65			0.80			0.80			2.00
16916	PM-H	1.29	1.25	**Y**	**Y**	**Y**	0.65	0.78			0.65			0.78			0.83			1.78
16920	R	1.10	0.90	Y	Y	- - -	0.90	0.97												
16920	R-D	1.33	0.97	Y	Y	- - -	1.10	1.03												
16920	FC-L	0.90	0.92	Y	Y	N	0.74	0.99	0.74			0.99								
16920	FC-H	0.54	0.58	**Y**	**Y**	**Y**	0.44	0.62	0.44			0.62			0.72			1.69		
16920	FC-H-D	0.60	0.61	**Y**	**Y**	**Y**	0.49	0.65	0.49			0.65			0.75			1.67		
16920	O3-L	1.21	0.86	Y	Y	N	1.00	0.92		1.00			0.92							
16920	O3-H	0.45	0.46	**Y**	**Y**	**Y**	0.37	0.49		0.37			0.49			0.76			1.39	
16920	O3-H-D	0.36	0.39	**Y**	**Y**	**Y**	0.30	0.42		0.30			0.42			0.71			1.40	
16920	PM-L	0.70	0.51	**Y**	**Y**	**Y**	0.58	0.55			0.58			0.55			1.05			0.91
16920	PM-L-D	0.70	0.48	**Y**	**Y**	**Y**	0.58	0.51			0.58			0.51			1.12			0.83
16920	PM-H	0.38	0.17	**Y**	**Y**	**Y**	0.31	0.18			0.31			0.18			1.73			0.68
16926	R	0.76	0.76	Y	Y	- - -	0.99	1.00												
16926	R-D	0.77	0.76	Y	Y	- - -	1.01	1.00												
16926	FC-L	0.72	0.66	**Y**	**Y**	**Y**	0.94	0.87	0.94			0.87			1.08			0.44		
16926	FC-L-D	0.64	0.76	Y	Y	N	0.84	0.99	0.84			0.99								
16926	FC-H	0.65	0.69	**Y**	**Y**	**Y**	0.85	0.91	0.85			0.91			0.93			1.75		
16926	O3-L	0.70	0.72	**Y**	**Y**	**Y**	0.92	0.94		0.92			0.94			0.97			1.44	
16926	O3-H	0.37	0.44	**Y**	**Y**	**Y**	0.49	0.58		0.49			0.58			0.84			1.31	
16926	PM-L	0.71	0.59	**Y**	**Y**	**Y**	0.92	0.77			0.92			0.77			1.19			0.31
16926	PM-H	0.45	0.37	**Y**	**Y**	**Y**	0.59	0.49			0.59			0.49			1.22			0.73
16930	R	2.90	1.62	Y	Y	- - -	0.97	1.03												
16930	R-D	3.08	1.51	Y	Y	- - -	1.03	0.97												
16930	FC-VH	2.44	1.22	**Y**	**Y**	**Y**	0.82	0.78	0.82			0.78			1.05			0.80		
16930	PM-VH	1.88	1.23	**Y**	**Y**	**Y**	0.63	0.78			0.63			0.78			0.80			1.91
16930	O3-VH	1.88	1.15	**Y**	**Y**	**Y**	0.63	0.74		0.63			0.74			0.85			1.51	
16930	O3-VH-D	1.83	1.09	**Y**	**Y**	**Y**	0.61	0.70		0.61			0.70			0.87			1.37	

CY-1 (cont'd). Concentrations, fraction remaining, and ratios for sum of MCs from natural waters as measured in split samples.

				Data acceptance for kinetics			Fraction remaining		$(C/C_0)_{ELISA}$			$(C/C_0)_{\Sigma MC}$			$(C/C_0)_{ELISA} / (C/C_0)_{\Sigma MC}$			$k"_{ELISA} / k"_{\Sigma MC}$		
UWRL Water Code	**CODE**	**ELISA (µg/L)**	**ΣMC (µg/L)**	**ELISA> MDL?**	**LC> MDL?**	**C/C_0 5-95%?**	**ELISA C/C_0**	**ΣMC C/C_0**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**
16931	R	1.60	0.89	Y	Y	- - -	1.00	1.00												
16931	FC-L	1.80	0.74	Y	Y	N	1.12	0.82	1.12			0.82			1.36					
16931	FC-H	1.72	0.78	Y	Y	N	1.07	0.88	1.07			0.88			1.22					
16931	O3-L	1.68	0.70	Y	Y	N	1.05	0.78		1.05			0.78			1.34				
16931	O3-H	1.54	0.73	Y	Y	N	0.96	0.81		0.96			0.81			1.19				
16931	PM-L	1.45	0.61	**Y**	**Y**	**Y**	0.91	0.68			0.91			0.68			1.33			0.26
16931	PM-H	1.27	0.70	**Y**	**Y**	**Y**	0.79	0.79			0.79			0.79			1.00			0.99
16939	R	1.19	0.88	Y	Y	- - -	0.93	0.91												
16939	R-D	1.29	0.97	Y	Y	- - -	1.00	1.00												
16939	FC-L	0.98	0.91	**Y**	**Y**	**Y**	0.76	0.94	0.76			0.94			0.81			4.44		
16939	FC-H	0.67	0.77	**Y**	**Y**	**Y**	0.52	0.80	0.52			0.80			0.65			2.93		
16939	O3-L	1.07	0.89	**Y**	**Y**	**Y**	0.83	0.91		0.83			0.91			0.91			2.09	
16939	O3-H	0.54	0.38	**Y**	**Y**	**Y**	0.42	0.40		0.42			0.40			1.06			0.94	
16939	PM-L	0.87	0.59	**Y**	**Y**	**Y**	0.68	0.61			0.68			0.61			1.12			0.77
16939	PM-H	0.46	0.23	**Y**	**Y**	**Y**	0.35	0.24			0.35			0.24			1.48			0.73
16940	R	2.50	2.06	Y	Y	- - -	0.88	0.95												
16940	R-D	3.17	2.27	Y	Y	- - -	1.12	1.05												
16940	FC-L	2.69	1.76	**Y**	**Y**	**Y**	0.95	0.81	0.95			0.81			1.16			0.26		
16940	FC-H	2.15	1.82	**Y**	**Y**	**Y**	0.76	0.84	0.76			0.84			0.90			1.59		
16940	O3-L	2.18	2.44	Y	Y	N	0.77	1.13		0.77			1.13							
16940	O3-H	2.12	1.86	**Y**	**Y**	**Y**	0.75	0.86		0.75			0.86			0.87			1.94	
16940	O3-H-D	2.52	1.92	**Y**	**Y**	**Y**	0.89	0.89		0.89			0.89			1.00			0.97	
16940	PM-L	3.31	2.20	Y	Y	N	1.17	1.02			1.17			1.02						
16940	PM-H	1.71	1.37	**Y**	**Y**	**Y**	0.60	0.63			0.60			0.63			0.95			1.11
16447	R	1.24	0.00	Y	N	- - -	1.00	Div 0												
16447	FC	0.64	0.00	Y	N	N	0.52	Div 0	0.52											
16447	PM	0.54	0.00	Y	N	N	0.44	Div 0			0.44			Div 0						
16447	O3	0.20	0.00	N	N	N	0.16	Div 0		0.16										
16464	R	2.21	0.00	Y	N	- - -	1.00	Div 0												
16464	FC	0.13	0.00	N	N	N	0.06	Div 0	0.06											
16464	PM	0.75	0.00	Y	N	N	0.34	Div 0			0.34			Div 0						
16464	O3	<MDL	0.00	N	N	N		Div 0		0.00										
16466	R	0.05	0.37	N	Y	- - -	1.00	1.00												
16466	R-D	0.03	0.39	N	Y	N	0.64	1.06												
16466	FC	0.03	0.31	N	Y	Y	0.47	0.84	0.47			0.84								
16466	PM	<MDL	0.20	N	Y	N		0.53			0.00			0.53						
16466	O3	<MDL	0.26	N	Y	N		0.70		0.00			0.70							

CY-2. Concentrations, fraction remaining, and ratios for sum of MC-LR from natural waters as measured in split samples.

				Data acceptance for kinetics			Fraction remaining		$(C/C_0)_{ELISA}$			$(C/C_0)_{MC-LR}$			$(C/C_0)_{ELISA}$ / $(C/C_0)_{MC-LR}$			k"$_{ELISA}$ / k"$_{MC-LR}$		
UWRL Water Code	**CODE**	**ELISA (µg/L)**	**MC-LR (µg/L)**	**ELISA> MDL?**	**LC> MDL?**	**C/Co 5-95%?**	**ELISA C/C_0**	**MC-LR C/C_0**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**
16913	R	3.91	0.00	Y	Y	- - -	1.17	- - -												
16913	R-D	2.77	0.00	Y	Y	- - -	0.83	- - -												
16913	FC-L	2.55	0.00	Y	Y	N	0.76	- - -	0.76			- - -								
16913	FC-H	1.08	0.00	Y	N	N	0.32	- - -	0.32			- - -								
16913	O3-H	1.21	0.00	Y	N	N	0.36	- - -		0.36			- - -							
16913	O3-H-D	0.97	0.00	Y	N	N	0.29	- - -		0.29			- - -							
16913	PM-L	2.13	0.00	Y	N	N	0.64	- - -			0.64			- - -						
16913	PM-H	0.81	0.00	Y	N	N	0.24	- - -			0.24			- - -						
16915	R	1.54	0.76	Y	Y	- - -	0.97	0.89												
16915	R-D	2.21	1.07	Y	Y	- - -	1.39	1.10												
16915	FC-VH	1.71	0.90	Y	Y	N	1.07	1.25	1.07			1.25								
16915	FC-VH-D	1.66	0.92	Y	Y	N	1.04	0.90	1.04			0.90								
16915	PM-VH	1.31	0.93	Y	Y	N	0.82	1.11			0.82			1.11						
16915	O3-VH	1.13	0.62	**Y**	**Y**	**Y**	0.71	0.74		0.71			0.74			0.95			1.16	
16916	R	0.51	0.33	Y	Y	- - -	0.97	0.85												
16916	R-D	0.54	0.45	Y	Y	- - -	1.03	1.15												
16916	FC-VH	0.48	0.27	**Y**	**Y**	**Y**	0.91	0.65	0.91			0.65			1.41			0.22		
16916	PM-VH	0.39	0.21	**Y**	**Y**	**Y**	0.74	0.52			0.74			0.52			1.42			0.46
16916	PM-D-VH	0.27	0.32	N	Y	Y	0.51	0.67			0.51			0.67			0.76			1.68
16916	O3-VH	0.30	0.26	Y	Y	Y	0.57	0.63		0.57			0.63			0.90			1.23	
16916	R	0.20	- - -	N	Y	- - -	0.95	- - -												
16916	R-D	0.22	0.25	N	Y	- - -	1.05	1.00												
16916	FC-VH	0.27	0.23	N	Y	N	1.29	0.87	1.29			0.87								
16916	PM-VH	0.13	0.14	N	Y	Y	0.61	0.50			0.61			0.50			1.22			0.71
16916	PM-D-VH	0.19	0.14	N	Y	Y	0.91	0.43			0.91			0.43						
16916	O3-VH	0.13	0.07	N	Y	N	0.60	0.00		0.60			0.00							

CY-2 (cont'd). Concentrations, fraction remaining, and ratios for sum of MC-LR from natural waters as measured in split samples.

			Data acceptance for kinetics				Fraction remaining		$(C/C_0)_{ELISA}$			$(C/C_0)_{MC-LR}$			$(C/C_0)_{ELISA}$ / $(C/C_0)_{MC-LR}$			k"$_{ELISA}$ / k"$_{MC-LR}$		
UWRL Water Code	**CODE**	**ELISA (µg/L)**	**MC-LR (µg/L)**	**ELISA> MDL?**	**LC> MDL?**	**C/Co 5-95%?**	**ELISA C/C_0**	**MC-LR C/C_0**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**
16916	R	2.00	1.60	Y	Y	- - -	1.00	1.00												
16916	FC-L	1.47	1.14	**Y**	**Y**	**Y**	0.73	0.74	0.73			0.74			1.00			1.02		
16916	FC-H	1.52	1.26	**Y**	**Y**	**Y**	0.76	0.81	0.76			0.81			0.93			1.32		
16916	FC-H-D	0.82	1.01	**Y**	**Y**	**Y**	0.41	0.77	0.41			0.77			0.53			3.36		
16916	O3-L	1.24	1.21	**Y**	**Y**	**Y**	0.62	0.76		0.62			0.76			0.82			1.71	
16916	O3-L-D	1.49	1.30	**Y**	**Y**	**Y**	0.75	0.90		0.75			0.90			0.83			2.66	
16916	O3-H	1.68	1.16	**Y**	**Y**	**Y**	0.84	0.76		0.84			0.76			1.11			0.63	
16916	PM-L	1.29	1.28	**Y**	**Y**	**Y**	0.65	0.85			0.65			0.85			0.76			2.74
16916	PM-H	1.29	1.25	**Y**	**Y**	**Y**	0.65	0.82			0.65			0.82			0.78			2.26
16920	R	1.10	0.90	Y	Y	- - -	0.90	0.94												
16920	R-D	1.33	0.97	Y	Y	- - -	1.10	1.06												
16920	FC-L	0.90	0.92	Y	Y	N	0.74	1.07	0.74			1.07								
16920	FC-H	0.54	0.58	**Y**	**Y**	**Y**	0.44	0.65	0.44			0.65			0.68			1.88		
16920	FC-H-D	0.60	0.61	**Y**	**Y**	**Y**	0.49	0.75	0.49			0.75			0.65			2.52		
16920	O3-L	1.21	0.86	Y	Y	N	1.00	0.92		1.00			0.92							
16920	O3-H	0.45	0.46	**Y**	**Y**	**Y**	0.37	0.56		0.37			0.56			0.66			1.73	
16920	O3-H-D	0.36	0.39	**Y**	**Y**	**Y**	0.30	0.39		0.30			0.39			0.76			1.30	
16920	PM-L	0.70	0.51	**Y**	**Y**	**Y**	0.58	0.60			0.58			0.60			0.96			1.09
16920	PM-L-D	0.70	0.48	**Y**	**Y**	**Y**	0.58	0.45			0.58			0.45			1.28			0.69
16920	PM-H	0.38	0.17	**Y**	**Y**	**Y**	0.31	0.24			0.31			0.24			1.33			0.80
16926	R	0.76	0.76	Y	Y	- - -	0.99	0.93												
16926	R-D	0.77	0.76	Y	Y	- - -	1.01	1.07												
16926	FC-L	0.72	0.66	**Y**	**Y**	**Y**	0.94	0.86	0.94			0.86								
16926	FC-L-D	0.64	0.76	Y	Y	N	0.84	1.06	0.84			1.06								
16926	FC-H	0.65	0.69	**Y**	**Y**	**Y**	0.85	0.86	0.85			0.86			0.99			1.08		
16926	O3-L	0.70	0.72	**Y**	**Y**	**Y**	0.92	0.79		0.92			0.79			1.16			0.37	
16926	O3-H	0.37	0.44	**Y**	**Y**	**Y**	0.49	0.68		0.49			0.68			0.72			1.85	
16926	PM-L	0.71	0.59	**Y**	**Y**	**Y**	0.92	0.67			0.92			0.67			1.37			0.20
16926	PM-H	0.45	0.37	**Y**	**Y**	**Y**	0.59	0.30			0.59			0.30			1.97			0.44
16930	R	2.90	1.62	Y	Y	- - -	0.97	1.04												
16930	R-D	3.08	1.51	Y	Y	- - -	1.03	0.96												
16930	FC-VH	2.44	1.22	**Y**	**Y**	**Y**	0.82	0.83	0.82			0.83			0.98			1.09		
16930	PM-VH	1.88	1.23	**Y**	**Y**	**Y**	0.63	0.90			0.63			0.90			0.69			4.64
16930	O3-VH	1.88	1.15	**Y**	**Y**	**Y**	0.63	0.70		0.63			0.70			0.90			1.31	
16930	O3-VH-D	1.83	1.09	**Y**	**Y**	**Y**	0.61	0.67		0.61			0.67			0.91			1.23	

CY-2 (cont'd). Concentrations, fraction remaining, and ratios for sum of MC-LR from natural waters as measured in split samples.

			Data acceptance for kinetics				Fraction remaining		$(C/C_0)_{ELISA}$			$(C/C_0)_{MC-LR}$			$(C/C_0)_{ELISA}$ / $(C/C_0)_{MC-LR}$			k"$_{ELISA}$ / k"$_{MC-LR}$		
UWRL Water Code	**CODE**	**ELISA (µg/L)**	**MC-LR (µg/L)**	**ELISA> MDL?**	**LC> MDL?**	**C/Co 5-95%?**	**ELISA C/C_0**	**MC-LR C/C_0**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**
16931	R	1.60	0.89	Y	Y	- - -	1.00	1.00												
16931	FC-L	1.80	0.74	Y	Y	N	1.12	0.85	1.12			0.85			1.32					
16931	FC-H	1.72	0.78	Y	Y	N	1.07	0.95	1.07			0.95			1.13					
16931	O3-L	1.68	0.70	Y	Y	N	1.05	0.68		1.05			0.68			1.54				
16931	O3-H	1.54	0.73	Y	Y	N	0.96	0.78		0.96			0.78			1.23				
16931	PM-L	1.45	0.61	**Y**	**Y**	**Y**	0.91	0.57			0.91			0.57			1.60			0.18
16931	PM-H	1.27	0.70	**Y**	**Y**	**Y**	0.79	0.82			0.79			0.82			0.97			1.16
16939	R	1.19	0.88	Y	Y	- - -	0.93	0.90												
16939	R-D	1.29	0.97	Y	Y	- - -	1.00	1.00												
16939	FC-L	0.98	0.91	**Y**	**Y**	**Y**	0.76	0.94	0.76			0.94			0.81			4.78		
16939	FC-H	0.67	0.77	**Y**	**Y**	**Y**	0.52	0.81	0.52			0.81			0.64			3.12		
16939	O3-L	1.07	0.89	**Y**	**Y**	**Y**	0.83	0.92		0.83			0.92			0.90			2.15	
16939	O3-H	0.54	0.38	**Y**	**Y**	**Y**	0.42	0.40		0.42			0.40			1.05			0.95	
16939	PM-L	0.87	0.59	**Y**	**Y**	**Y**	0.68	0.63			0.68			0.63			1.08			0.84
16939	PM-H	0.46	0.23	**Y**	**Y**	**Y**	0.35	0.27			0.35			0.27			1.32			0.79
16940	R	2.50	2.06	Y	Y	- - -	0.88	0.94												
16940	R-D	3.17	2.27	Y	Y	- - -	1.12	1.06												
16940	FC-L	2.69	1.76	**Y**	**Y**	**Y**	0.95	0.80	0.95			0.80			1.18			0.24		
16940	FC-H	2.15	1.82	**Y**	**Y**	**Y**	0.76	0.83	0.76			0.83			0.91			1.48		
16940	O3-L	2.18	2.44	Y	Y	N	0.77	1.18		0.77			1.18							
16940	O3-H	2.12	1.86	**Y**	**Y**	**Y**	0.75	0.85		0.75			0.85			0.88			1.79	
16940	O3-H-D	2.52	1.92	**Y**	**Y**	**Y**	0.89	0.93		0.89			0.93			0.95			1.70	
16940	PM-L	3.31	2.20	Y	Y	N	1.17	1.02			1.17			1.02						
16940	PM-H	1.71	1.37	**Y**	**Y**	**Y**	0.60	0.67			0.60			0.67			0.90			1.26
16447	R	1.24	0.00	Y	N	- - -	1.00	Div 0												
16447	FC	0.64	0.00	Y	N	N	0.52	Div 0	0.52											
16447	PM	0.54	0.00	Y	N	N	0.44	Div 0			0.44			Div 0						
16447	O3	0.20	0.00	N	N	N	0.16	Div 0		0.16										
16464	R	2.21	0.00	Y	N	- - -	1.00	Div 0												
16464	FC	0.13	0.00	N	N	N	0.06	Div 0	0.06											
16464	PM	0.75	0.00	Y	N	N	0.34	Div 0			0.34			Div 0						
16464	O3	<MDL	0.00	N	N	N		Div 0		0.00										
16466	R	0.05	0.37	N	Y	- - -	1.00	1.00												
16466	R-D	0.03	0.39	N	Y	N	0.64	1.06												
16466	FC	0.03	0.31	N	Y	Y	0.47	0.84	0.47			0.84								
16466	PM	<MDL	0.20	N	Y	N		0.53			0.00			0.53						
16466	O3	<MDL	0.26	N	Y	N		0.70		0.00			0.70							

CY-3. Concentrations, fraction remaining, and ratios for sum of MCs from spiked lab waters as measured in split samples.

					Data acceptance for kinetics			Fraction remaining		$(C/C_0)_{ELISA}$			$(C/C_0)_{\Sigma MC}$			$(C/C_0)_{ELISA} / (C/C_0)_{\Sigma MC}$			$k''_{ELISA} / k''_{\Sigma MC}$		
Sample ID	**Spike**	**CODE**	**ELISA (µg/L)**	**ΣMC (µg/L)**	**ELISA> MDL?**	**LC> MDL?**	**C/C_0 5-95%?**	**ELISA C/C_0**	**ΣMC C/C_0**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**
1101	Lab -Mix	Raw	1.61	1.1	Y	Y	- - -	0.97	0.99												
1102	Lab -Mix	Raw	1.71	1.1	Y	Y	- - -	1.03	1.01												
1103	Lab -Mix	FC - L	1.71	1.0	Y	Y	N	1.03	0.97	1.03											
1104	Lab -Mix	FC-M	1.31	0.9	**Y**	**Y**	**Y**	0.79	0.80	0.79						0.99			1.04		
1105	Lab -Mix	FC-M - D	1.70	0.8	Y	Y	N	1.02	0.74	1.02											
1106	Lab -Mix	FC - H	1.49	0.7	**Y**	**Y**	**Y**	0.90	0.62	0.90						1.44			0.23		
1107	Lab -Mix	PM - L	1.57	0.7	**Y**	**Y**	**Y**	0.95	0.62			0.95			0.62			1.52			0.12
1108	Lab -Mix	PM - L - D	1.52	0.9	**Y**	**Y**	**Y**	0.92	0.82			0.92			0.82			1.11			0.46
1109	Lab -Mix	PM - M	1.53	0.8	**Y**	**Y**	**Y**	0.92	0.71			0.92			0.71			1.30			0.24
1110	Lab -Mix	PM - H	0.97	0.5	**Y**	**Y**	**Y**	0.58	0.44			0.58			0.44			1.32			0.66
1111	Lab -Mix	O3- L	1.54	1.1	Y	Y	N	0.93	1.05									0.9			
1112	Lab -Mix	O3- L - D	1.34	1.1	Y	Y	N	0.81	1.00		0.81			1.00							
1113	Lab -Mix	O3-M	1.50	1.0	**Y**	**Y**	**Y**	0.90	0.91		0.90			0.91			0.99			1.10	
1114	Lab -Mix	O3-H	1.17	0.8	**Y**	**Y**	**Y**	0.70	0.75		0.70			0.75			0.94			1.21	

CY-3 (cont'd). Concentrations, fraction remaining, and ratios for sum of MCs from spiked lab waters as measured in split samples.

					Data acceptance for kinetics			Fraction remaining			$(C/C_0)_{ELISA}$			$(C/C_0)_{MC-LR}$			$(C/C_0)_{ELISA} / (C/C_0)_{MC-LR}$			$k"_{ELISA} / k"_{MC-LR}$		
Sample ID	**Spike**	**CODE**	**ELISA (µg/L)**	**MC-LR (µg/L)**	**ELISA> MDL?**	**LC> MDL?**	**C/Co 5-95%?**	**ELISA C/C_0**	**MC-LR C/C_0**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**
1101	Lab -Mix	Raw	1.61	0.1	Y	Y	- - -	0.97	0.87												
1102	Lab -Mix	Raw	1.71	0.2	Y	Y	- - -	1.03	1.13												
1103	Lab -Mix	FC - L	1.71	0.1	Y	Y	N	1.03	0.77	1.03											
1104	Lab -Mix	FC-M	1.31	0.1	**Y**	**Y**	**Y**	0.79	0.55	0.79						1.44			0.39		
1105	Lab -Mix	FC-M - D	1.70	0.1	Y	Y	N	1.02	0.34	1.02											
1106	Lab -Mix	FC - H	1.49	0.1	**Y**	**Y**	**Y**	0.90	0.57	0.90						1.57			0.19		
1107	Lab -Mix	PM - L	1.57	0.0	**Y**	**Y**	**Y**	0.95	0.00			0.95			0.00						
1108	Lab -Mix	PM - L - D	1.52	0.0	**Y**	**Y**	**Y**	0.92	0.00			0.92			0.00						
1109	Lab -Mix	PM - M	1.53	0.0	**Y**	**Y**	**Y**	0.92	0.00			0.92			0.00						
1110	Lab -Mix	PM - H	0.97	0.0	**Y**	**Y**	**Y**	0.58	0.00			0.58			0.00						
1111	Lab -Mix	O3- L	1.54	0.2	Y	Y	N	0.93	1.05									0.9			
1112	Lab -Mix	O3- L - D	1.34	0.1	Y	Y	N	0.81	0.87		0.81			0.87							

CY-4. Concentrations, fraction remaining, and ratios for MC-LR from spiked lab waters as measured in split samples.

					Data acceptance for kinetics			Fraction remaining		$(C/C_0)_{ELISA}$			$(C/C_0)_{\Sigma MC}$			$(C/C_0)_{ELISA} / (C/C_0)_{\Sigma MC}$			$k''_{ELISA} / k''_{\Sigma MC}$		
Sample ID	**Spike**	**CODE**	**ELISA (µg/L)**	**ΣMC (µg/L)**	**ELISA> MDL?**	**LC> MDL?**	**C/C_0 5-95%?**	**ELISA C/C_0**	**ΣMC C/C_0**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**
915	Lab - MC-LR	Raw	2.18	1.8	Y	Y	- - -	1.04	1.15												
916	Lab - MC-LR	Raw	2.01	1.3	Y	Y	- - -	0.96	0.85												
917	Lab - MC-LR	FC - L	2.07	1.0	Y	Y	N	0.99	0.67	0.99											
918	Lab - MC-LR	FC-M	2.02	1.4	Y	Y	N	0.96	0.90	0.96											
919	Lab - MC-LR	FC - H	1.75	1.7	Y	Y	N	0.83	1.09	0.83											
920	Lab - MC-LR	PM - L	1.83	1.1	**Y**	**Y**	**Y**	0.88	0.68			0.88			0.68			1.28			0.35
921	Lab - MC-LR	PM - L - D	2.17	1.1	Y	Y	N	1.03	0.72			1.03			0.72						
922	Lab - MC-LR	PM - M	1.93	1.0	**Y**	**Y**	**Y**	0.92	0.62			0.92			0.62			1.48			0.17
923	Lab - MC-LR	PM - H	1.16	0.5	**Y**	**Y**	**Y**	0.55	0.30			0.55			0.30			1.85			0.49
924	Lab - MC-LR	O3- L	1.59	0.7	**Y**	**Y**	**Y**	0.76	0.45		0.76			0.45			1.68			0.35	
925	Lab - MC-LR	O3-M	1.08	0.5	**Y**	**Y**	**Y**	0.52	0.35		0.52			0.35			1.49			0.63	
1116	Lab - MC-LR	Raw	1.04	1.6	Y	Y	- - -	1.00	1.00												
1117	Lab - MC-LR	FC - L	1.13	1.9	Y	Y	N	1.09	1.14	1.09											
1118	Lab - MC-LR	FC-M	1.11	1.9	Y	Y	N	1.07	1.14	1.07											
1119	Lab - MC-LR	FC - H	0.92	1.6	Y	Y	N	0.89	0.99	0.89											
1120	Lab - MC-LR	PM - L	0.94	2.0	Y	Y	N	0.90	1.23			0.90			1.23						
1121	Lab - MC-LR	PM - L - D	1.09	1.6	Y	Y	N	1.05	0.97			1.05			0.97						
1122	Lab - MC-LR	PM - M	0.97	1.5	**Y**	**Y**	**Y**	0.93	0.93			0.93			0.93			1.01			0.89
1123	Lab - MC-LR	PM - H	0.51	0.9	**Y**	**Y**	**Y**	0.49	0.52			0.49			0.52			0.93			1.11
1124	Lab - MC-LR	O3- L	0.79	1.6	Y	Y	N	0.76	0.99		0.76			0.99			1.05			0.95	
1125	Lab - MC-LR	O3-M	0.45	0.7	**Y**	**Y**	**Y**	0.43	0.41		0.43			0.41							
1127	Lab - MC-LA	Raw	2.73	1.9	Y	Y	N	1.06	1.00												
1128	Lab - MC-LA	Raw	2.41	1.7	Y	Y	N	0.94	0.90												
1129	Lab - MC-LA	FC - L	2.80	2.0	Y	Y	N	1.09	1.05	1.09											
1130	Lab - MC-LA	FC-M	3.01	1.9	Y	Y	N	1.17	1.01	1.17											
1131	Lab - MC-LA	FC - H	2.56	1.8	Y	Y	N	1.00	0.96	1.00											
1132	Lab - MC-LA	PM - L	2.15	1.9	Y	Y	N	0.84	1.00			0.84			1.00						
1133	Lab - MC-LA	PM - M	2.51	1.8	Y	Y	N	0.98	0.94			0.98			0.94						
1134	Lab - MC-LA	PM - H	1.98	1.3	**Y**	**Y**	**Y**	0.77	0.71			0.77			0.71			1.09			0.74
1135	Lab - MC-LA	O3- L	2.54	1.9	Y	Y	N	0.99	0.99		0.99			0.99							
1136	Lab - MC-LA	O3- L - D	2.29	1.9	Y	Y	N	0.89	0.99		0.89			0.99							
1137	Lab - MC-LA	O3-M	1.89	1.4	**Y**	**Y**	**Y**	0.74	0.76		0.74			0.76			0.97			1.10	

CY-4 (cont'd). Concentrations, fraction remaining, and ratios for MC-LR from spiked lab waters as measured in split samples.

					Data acceptance for kinetics			Fraction remaining		$(C/C_0)_{ELISA}$			$(C/C_0)_{MC-LR}$			$(C/C_0)_{ELISA} / (C/C_0)_{MC-LR}$			$k''_{ELISA} / k''_{MC-LR}$		
Sample ID	**Spike**	**CODE**	**ELISA (µg/L)**	**MC-LR (µg/L)**	**ELISA> MDL?**	**LC> MDL?**	**C/Co 5-95%?**	**ELISA C/C$_0$**	**MC-LR C/C$_0$**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**	**FC**	**O_3**	**PM**
915	Lab - MC-LR	Raw	2.18	1.8	Y	Y	- - -	1.04	1.15												
916	Lab - MC-LR	Raw	2.01	1.3	Y	Y	- - -	0.96	0.85												
917	Lab - MC-LR	FC - L	2.07	1.0	Y	Y	N	0.99	0.67	0.99											
918	Lab - MC-LR	FC-M	2.02	1.4	Y	Y	N	0.96	0.90	0.96											
919	Lab - MC-LR	FC - H	1.75	1.7	Y	Y	N	0.83	1.09	0.83											
920	Lab - MC-LR	PM - L	1.83	1.1	**Y**	**Y**	**Y**	0.88	0.68			0.88			0.68			1.28			0.35
921	Lab - MC-LR	PM - L - D	2.17	1.1	Y	Y	N	1.03	0.72			1.03			0.72						
922	Lab - MC-LR	PM - M	1.93	1.0	**Y**	**Y**	**Y**	0.92	0.62			0.92			0.62			1.48			0.17
923	Lab - MC-LR	PM - H	1.16	0.5	**Y**	**Y**	**Y**	0.55	0.30			0.55			0.30			1.85			0.49
924	Lab - MC-LR	O3- L	1.59	0.7	**Y**	**Y**	**Y**	0.76	0.45		0.76			0.45			1.68			0.35	
925	Lab - MC-LR	O3-M	1.08	0.5	**Y**	**Y**	**Y**	0.52	0.35		0.52			0.35			1.49			0.63	
1116	Lab - MC-LR	Raw	1.04	1.6	Y	Y	N	1.00	1.00												
1117	Lab - MC-LR	FC - L	1.13	1.9	Y	Y	N	1.09	1.14	1.09											
1118	Lab - MC-LR	FC-M	1.11	1.9	Y	Y	N	1.07	1.14	1.07											
1119	Lab - MC-LR	FC - H	0.92	1.6	Y	Y	N	0.89	0.99	0.89											
1120	Lab - MC-LR	PM - L	0.94	2.0	Y	Y	N	0.90	1.23			0.90			1.23						
1121	Lab - MC-LR	PM - L - D	1.09	1.6	Y	Y	N	1.05	0.97			1.05			0.97						
1122	Lab - MC-LR	PM - M	0.97	1.5	**Y**	**Y**	**Y**	0.93	0.93			0.93			0.93			1.01			0.89
1123	Lab - MC-LR	PM - H	0.51	0.9	**Y**	**Y**	**Y**	0.49	0.52			0.49			0.52			0.93			1.11
1124	Lab - MC-LR	O3- L	0.79	1.6	Y	Y	N	0.76	0.99		0.76			0.99							
1125	Lab - MC-LR	O3-M	0.45	0.7	**Y**	**Y**	**Y**	0.43	0.41		0.43			0.41			1.05			0.95	
1127	Lab - MC-LA	Raw	2.73	0.0	Y	Y	- - -	1.06	Div 0												
1128	Lab - MC-LA	Raw	2.41	0.0	Y	Y	- - -	0.94	Div 0												
1129	Lab - MC-LA	FC - L	2.80	0.0	Y	Y	N	1.09	Div 0	1.09											
1130	Lab - MC-LA	FC-M	3.01	0.0	Y	Y	N	1.17	Div 0	1.17											
1131	Lab - MC-LA	FC - H	2.56	0.0	Y	Y	N	1.00	Div 0	1.00											
1132	Lab - MC-LA	PM - L	2.15	0.0	Y	Y	N	0.84	Div 0			0.84									
1133	Lab - MC-LA	PM - M	2.51	0.0	Y	Y	N	0.98	Div 0			0.98									
1134	Lab - MC-LA	PM - H	1.98	0.0	**Y**	**Y**	**Y**	0.77	Div 0			0.77									
1135	Lab - MC-LA	O3- L	2.54	0.0	Y	Y	N	0.99	Div 0		0.99										
1136	Lab - MC-LA	O3- L - D	2.29	0.0	Y	Y	N	0.89	Div 0		0.89										
1137	Lab - MC-LA	O3-M	1.89	0.0	**Y**	**Y**	**Y**	0.74	Div 0		0.74										

Appendix CY-5

CY-5. Results of ELISA analysis for oxidation of cylindrospermopsin in natural water samples.

		Duplicate injections/same vial								
Name/ID	Treatment	First ELISA injection	Second ELISA injection	Inj 1/ Inj 2	Mean (µg/L)	RSD(%) of replicate automated analysis in same vial	Raw	FC	O3	PM
R-1401	Raw	1.05	0.28	3.73	0.67	81.71	81.71			
R-1402	Raw	>3	1.26		1.26	2.26	2.26			
R-1403	FC - L	1.15	0.90	1.29	1.03	17.71		17.71		
R-1404	FC-M	1.46	1.10	1.33	1.28	20.01		20.01		
R-1405	FC-M - D	1.50	1.21	1.24	1.35	15.11		15.11		
R-1406	FC - H	>3	1.00	3.00	1.00	2.00		2.00		
R-1407	PM - L	0.75	1.57	0.48	1.16	49.61				49.61
R-1408	PM - L - D	0.85	0.55	1.53	0.70	29.81				29.81
R-1409	PM - M	1.10	1.13	0.97	1.12	2.31				2.31
R-1410	PM - H	1.98	0.53	3.76	1.25	82.01				
R-1411	O3- L	0.71	1.48	0.48	1.10	49.61			49.61	
R-1412	O3- L - D	0.78	1.14	0.69	0.96	26.11			26.11	
R-1413	O3-M	1.41	1.14	1.24	1.28	15.11			15.11	
R-1414	O3-H	1.93	0.45	4.28	1.19	87.81			87.81	
R-1415	Raw	1.01	0.88	1.14	0.95	9.51	9.51			
R-1416	Raw	0.50	0.54	0.93	0.52	5.31	5.31			
R-1417	FC - L	1.17	0.81	1.44	0.99	25.51		25.51		
R-1418	FC-M	1.83	0.65	2.83	1.24	67.61		67.61		
R-1419	FC - H	0.17	1.14	0.15	0.66	105.21		105.21		
R-1420	PM - L	0.87	0.37	2.33	0.62	56.51				56.51
R-1421	PM - L - D	0.28	1.02	0.27	0.65	81.41				81.41
R-1422	PM - M	1.63	0.68	2.41	1.15	58.61				58.61
R-1423	PM - H	0.34	0.88	0.38	0.61	63.41				63.41
R-1424	O3- L	1.28	0.52	2.44	0.90	59.31			59.31	
R-1425	O3-M	0.32	>3	0.11	0.32	1.32			1.32	
R-1426	O3-H	>3	0.08	37.50	0.08	1.08			1.08	
R-1427	Raw	0.30	0.55	0.54	0.43	41.91	41.91			
R-1428	Raw	1.37	0.17	8.01	0.77	110.01	110.01			
R-1429	FC - L	0.25	1.03	0.24	0.64	86.41		86.41		
R-1430	FC-M	0.66	0.14	4.71	0.40	91.91		91.91		
R-1431	FC - H	0.19	0.36	0.52	0.28	45.31		45.31		
R-1432	PM - L	0.89	0.24	3.75	0.57	81.81			81.81	
R-1433	PM - M	0.12	>3	0.00	0.12	1.12			1.12	
R-1434	PM - H	1.57	0.25	6.34	0.91	102.91			102.91	
R-1435	O3- L	0.10	0.62	0.15	0.36	103.61				103.61
R-1436	O3- L - D	0.93	0.75	1.24	0.84	15.11				15.11
R-1437	O3-M	0.03	0.56	0.05	0.30	128.51				128.51
R-1438	O3-H	0.88	0.96	0.92	0.92	6.11				6.11

Appendix CY-6

CY-6. Statistical parameters for cylindrospermopsin oxidation experiment by CAAS.

	RSD for replicates of same sample by CAAS				
Parameter	**Overall**	**Raw**	**FC**	**O3**	**PM**
Average	48	42	48	43	54
Median	47	26	35	38	57
Minimum	1	2	2	1	2
Maximum	129	110	105	103	129
RSD(%)	80%	108%	78%	92%	74%

CURRICULUM VITAE

Shadi Haji Eghrari

EDUCATION:

Ph.D. Civil and Environmental Engineering, *Utah State University*, Logan, Utah. GPA: 3.92 — Sep 2013-May 2021 (Expected)

PhD Dissertation: Exploring Analytical Issues Associated with Oxidation Kinetics in Drinking Water

M.Sc. Pharmaceutical Engineering, *University of Tehran*, Tehran, Iran. GR G.P.A: 3.48 — Sep 2009-Feb 2012

Thesis Project: Experimental and Theoretical Investigations on the Solubility Behavior of Losartan Potassium and Amoidarone Hydrochloride in Mixed Solvents at Various Temperatures

B.Sc. **Applied Chemistry**, *University of Tabriz,* Tabriz, Iran. UG G.P.A: 3.31 — Feb 2004-Sep 2009

EXPERIENCE:

Assistant Chemist, City of San Diego, Public Utilities, Drinking Water Quality
Oct 2018-Present

Junior Chemist, City of San Diego, Public Utilities, Drinking Water Quality
Feb 2018-Oct 2018

Graduate Research Assistant, Utah Water Research Laboratory, Utah State University Sep 2013-Jun 2017

PUBLICATIONS

Ali Shayanfar, **Shadi H. Eghrary**, Faroukh Sardari, William E. Acree,Jr., and Abolghasem Jouyban, “Solubility of Anthracene and Phenanthrene in Ethanol +2,2,4-Trimethylpentane Mixtures at Different Temperatures ”, Journal of Chemical & Engineering Data.

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