DETERMINATION OF CYANOBACTERIAL TOXINS IN LAKE NAIVASHA, KENYA

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A Thesis Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Award of the Degree of Master of Science in Chemistry of Egerton University

EGERTON UNIVERSITY

MAY, 2018

DECLARATION AND RECOMMENDATION

DECLARATION

This is my original work and has not been presented for examination for award of a degree in any institution.

Signature: …….. Date: …….

Douglas N. Nyangoya

SM11/23543/14

RECOMMENDATIONS

This thesis has been submitted for examination with our approval as the university supervisors.

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Dr. Joshua Kibet

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Signature…….. Date.....

Dr. Benson Ongarora

Dedan Kimathi University of Technology

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DEDICATION

It is with sincere regards that I dedicate this work to my loving parents, Mr. Simeon Nyangoya and Mrs. Monicah Nyamisa, and my siblings Elmeldah, Elijah and my lovely wife Loise Nyachiro for their moral and financial support.

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ABSTRACT

Lake Naivasha is a freshwater lake located about 80 km northwest of Nairobi and lies about 1890 metres above sea level. The freshwater system is used for drinking, washing and livestock watering by more than 600,000 people. However, declining water quality due to environmental perturbations has raised public concerns worldwide. The occurrence of blooms of cyanobacteria has become a serious problem as they produce a wide spectrum of toxins including microcystin which cause various adverse effects on organisms. Microcystins are a class of cyanobacterial toxins largely found in water and are often responsible for poisoning of animals as well as humans. The current work presents empirical data on the first identification and characterization of hepatotoxic microcystins in water samples of Lake Naivasha. Samples from the lake were stored in polythene bottles and analyzed over a period of six months. The colour of the water samples was found to be (520 ± 91) ptco, while the conductivity was 234 ± 0.8 μs/cm and the total dissolved solids were (1035 ± 12) mg/L. Due to the high turbidity (59.0 ± 24 ntu), phytoplankton biomass was low, ranging between 1.5 and 8.2 mg L^{-1}. UV-Vis spectrum of sample A (for the month of April) had a peak with absorbance of 0.68 and a maximum wavelength (λ_{max}) of 247 nm. Sample A (for the month of January) had a peak with absorbance of 0.99 and a maximum wavelength (λ_{max}) of 240.94 nm. This peak was at slightly higher λ_{max} than that of MC-LR which absorbs at the wavelength of 238 nm. This peak did not match with any microcystin of our interest. This was most likely due to the emergence of a new microcystin or a product formed from the degradation of microcystin. Using HPLC, chromatograms were obtained at different retention times. Three conspicuous peaks with different retention times were obtained for the samples collected in January. The toxin with a retention time of 11.65 minutes was identified as MC-YR. This peak was shorter as compared to the rest implying that its concentration is much lower in existence. The second peak was observed at retention time 12.87 minutes and it was more protruding. This means that it is more concentrated in this sample. Retention time of 12.87 minutes corresponds to MC-LR. Finally, a sharp peak at retention time 7.27 minutes that did not correspond to any microcystin of interest was observed. The peak was thought to result from a microcystin degradation product. HyperChem computational package was used to estimate the toxicity index of microcystin-RR based on the octanol-water partition coefficient and found to be 230 times more soluble in water than in octanol. Thus, MC-RR is more soluble in biological tissues causing oxidative stress, and ultimately cancer. The concentrations of the microcystins in the different samples were observed to vary.

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LIST OF ABBREVIATIONS AND ACRONYMS

DEN	Diethyl nitrosamine
DMB	Dimethylbenzanthracene
ECOSAR	Ecological Structural Activity Relation
HPLC-PDA	High Performance Liquid Chromatography- Photodiode Array
IAC	Immunoaffinity columns
IR	Infrared
LD_{50}	Median Lethal Dose
LOAEL	Lowest adverse effect level
MC	Microcystin
NOAEL	No observed adverse effect level
NRPSs	Non-ribosomal peptide synthetases
NTU	Nephlometric Turbidity Unit
PCP	peptidyl carrier protein
PKSs	polyketide synthases
PTCO	Platinum Cobalt
SPE	Solid-phase extraction
QSAR	Quantitative Structural Activity Relation
YA	Tyrosine-Alanine
YR	Tyrosine-Arginine
RR	Arginine-Arginine
LA	Leucine Arginine
AMBER	Assisted Model Building Energy Refinement
Adda	Adenosine Diaminase
Mdha	Monodehydroascorbate

CHAPTER ONE

INTRODUCTION

1.1 Background information

Cyanobacterial toxins are naturally produced poisons stored in the cells of certain species of cyanobacteria (Borges *et al.*, 2015). Very few cyanobacterial toxins have actually been isolated and characterized to date. One group of toxins produced and released by cyanobacteria are called microcystins because they were isolated from a cyanobacterium called *Microcystis aeruginosa* (Vichi *et al.*, 2015). Microcystins are the most common of the cyanobacterial toxins found in water, as well as being the ones most often responsible for poisoning animals and humans who come into contact with the toxic blooms. Microcystins are extremely stable in water because of their chemical structure, thus they can survive in both warm and cold water and they tolerate radical changes in water chemistry, including variation in pH. So far, scientists have identified about 50 different kinds of microcystins (Zheng *et al.*, 2016). The microcystin-LR appears to be one of the microcystins most commonly found in water supplies around the world. For this reason, most research in this area has focused on this particular toxin. Microcystins (cyclic peptide hepatotoxins) have been described and detected in several cyanobacteria genera including *Anabaena* (Krishnamurthy *et al.*, 1986), *Microcystis* (Botes *et al.*, 1984), *Oscillatoria* (Brittain *et al.*, 2000) and *Planktothrix* (Meriluoto *et al.*, 1989).

Research has indicated that microcystins are among the several cyanotoxins that comprise over 80 analogs. Examples of microcystin include: microcystin-YR, microcystin-YR, microcystin-LA and microcystin-LR. Generally, the structure shared by all microcystins is presented in Figure 1.1

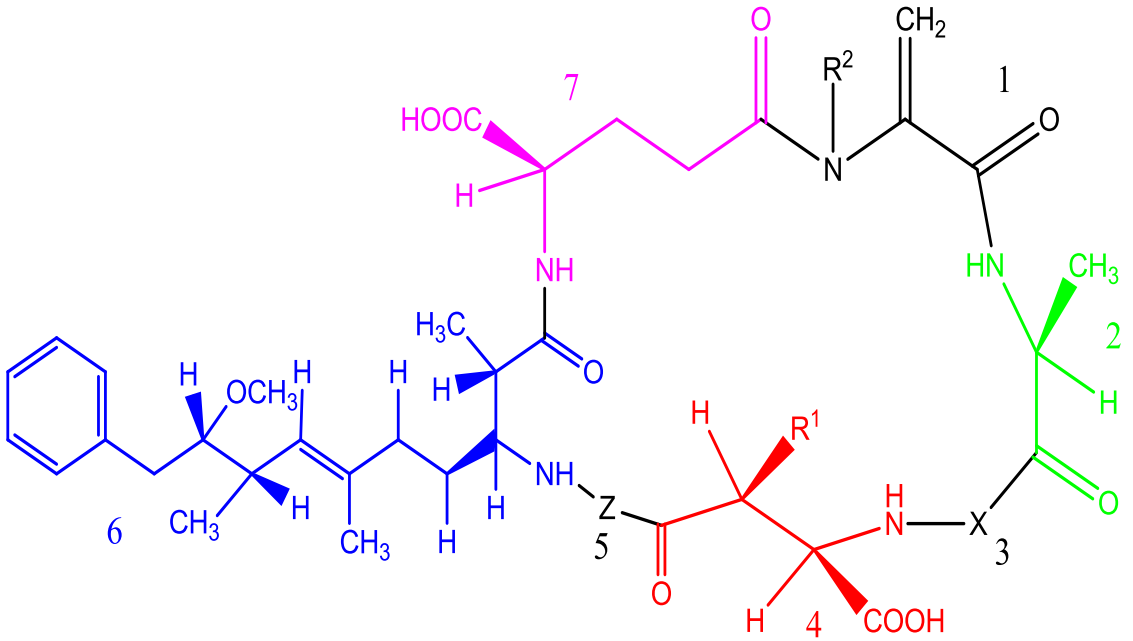

Figure 1.1: General structure of microcystins

The amino acids in the structure are numbered with variable portions of X, Z, R^1 and R^2, whereby the amino acids are differentiated with X and Z positions and both R^1 and R^2 are methyl groups. Microcystins are named with one letter abbreviation for amino acids substituted at X and Z positions respectively (McElhiney and Lawton, 2005).

Table 1.1 below illustrates the amino acids at position X and Z in the general structure of microcystin (Figure 1.1).

Table 1.1: Amino acids found in microcystins

Name	**X-position amino acid**	**Z-position amino acid**	**Molecular mass**
Microcystin-LA	Leucine (L)	Alanine (A)	910.06
Microcystin-YR	Tyrosine (Y)	Arginine (R)	1045.19
Microcystin-RR	Arginine (R)	Arginine (R)	1038.2
Microcystin-LR	Leucine (L)	Arginine (R)	995.17

The molecular structures of selected microcystins are presented below (Figures **1.2, 1.3, 1.4,** and **1.5**).

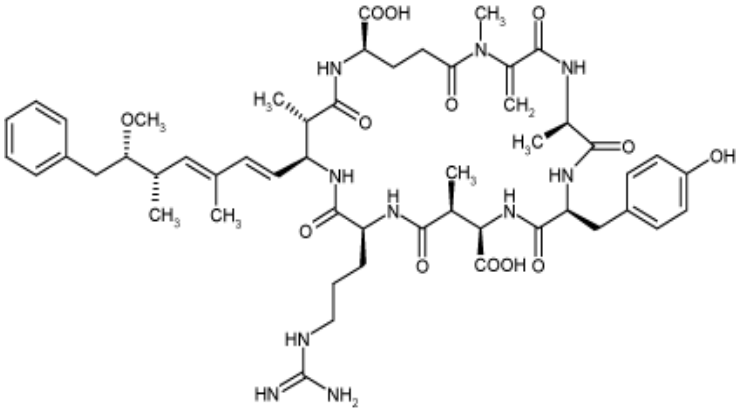

Figure 1.2: Microcystin-YR

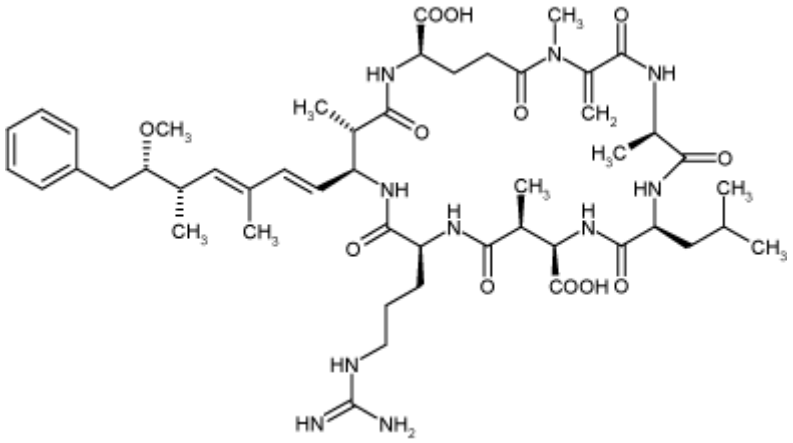

Figure 1.3: Microcystin-LR

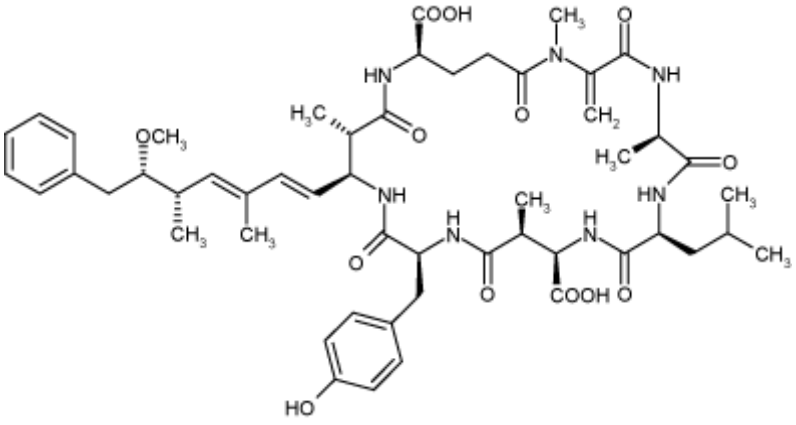

Figure 1.4: Microcystin-LA

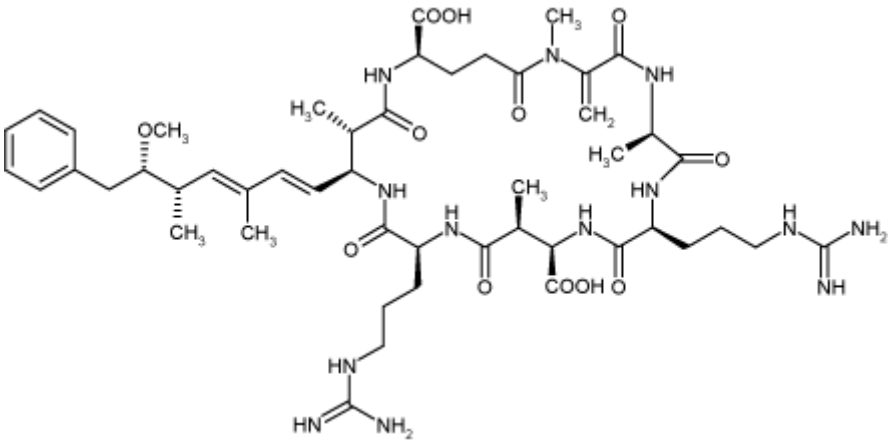

Figure 1.5: Microcystin-RR

Cyanobacteria toxins fall into various categories. Some are known to attack the liver (hepatotoxins) or the nervous system (neurotoxins); others simply irritate the skin. People swimming in dense *Microcystis* blooms have experienced irritation such as skin rashes, burns, and blistering of the mouth. Ingestion or inhalation of water containing dense bloom material may cause vomiting, nausea, headaches, diarrhea, pneumonia, and fever. Ingestion of significant levels of the toxin microcystin can cause liver damage and dysfunction in humans and animals and ultimately death. For instance, dogs, wildlife and livestock have died following exposure to these toxins (Pitois *et al.*, 2000).

All of the toxins producing species of cyanobacteria are capable of forming blooms on water surfaces (Paerl and Otten, 2013). These mostly occur during the summer months where the warm temperatures allow the bacteria to grow and a light wind helps disturb the water layers and bring more nutrients to the surface. Nutrient concentrations can contribute to the excessive growth of a bloom (Pilotto *et al.*, 1997; Wallace *et al.*, 2016). Increasing eutrophication of water bodies, due to fertilizer run-off from cultivated land and livestock or human waste provides more nutrients for the formation of blooms (Pitois *et al.*, 2001). Most algae are able to form blooms, and these can consist of toxic species of cyanobacteria (Paerl *et al.*, 2001) .The occurrence of water hyacinth in Lake. Naivasha complicates the situation since it is likely to affect turbidity and is likely to increase evapotranspiration by a factor of 1.8 (Jackson *et al.*, 2016) - something that will alter the concentration of the microcystins.

1.2 Statement of the problem

The occurrence of different types of toxin-producing cyanobacterial blooms in natural waters has raised public health concerns worldwide (Zhao *et al.*, 2015). Microcystins (MCs) are a class of cyanobacterial toxins largely found in water and are often responsible for poisoning of animals as well as humans. Human exposure to microcystins is recognized as a global health issue (Miller *et al.*, 2010; Roegner *et al.*, 2014). Liver is the main target organ, but microcystins also concentrate in organs such as kidney and colon (Sun *et al.*, 2014). Several cases of human death have been reported due to microcystin intoxication (Dörr *et al.*, 2010). Microcystins are highly soluble in polar biological tissues which result to cell injury, oxidative stress, and ultimately cancer. The current state of L. Naivasha is unknown therefore, there was need to identify, characterize and quantify microcystins experimentally in L. Naivasha since humans and aquatic lives are at risk of exposure to these toxins.

1.3 Objective

1.3.1 General objective

To investigate cyanobacterial toxins in L. Naivasha waters and explore their toxicological implications on aquatic life and higher order animals including man.

1.3.2 Specific objectives

1. To determine presence of microcystins in L. Naivasha waters.
2. To determine the variation in the concentration of microcystins during the dry and wet seasons.
3. To estimate the toxicity indices of microcystins using HyperChem computational code.

1.4 Hypotheses

1. Microcystins will not be identified in L. Naivasha.
2. The concentration of microcystins during the dry and wet seasons will not be significantly different.
3. The toxicity levels of various microcystins determined using HyperChem computational code will not be significantly different.

1.5 Justification

Research has indicated that the mechanisms and pathways through which microcystins cause toxicity in biological systems are quite complex. The case of L. Naivasha is unusual since it is one of the only two freshwater bodies in a system of otherwise alkaline-soda lakes (Etheridge, 2010), making it an extremely valuable resource for humans and wildlife in this semi-arid region of Kenya (Morrison and Harper, 2009). Besides hosting aquatic life, the water from the lake is used for swimming, drinking, showering, washing and livestock watering by the human population living throughout the Naivasha catchment area.

In consideration of the declining water quality due to environmental perturbations, public health concerns have been raised worldwide. The occurrence of cyanobacteria has become a serious problem as they produce a wide spectrum of toxins, some of which can cause various adverse effects on organisms (Chorus and Bartram, 1999). Microcystin is the most widespread and is documented as the cause of several illnesses and death (Backer *et al.*, 2015). The microcystins have continued to increase and it is, therefore, worthwhile to determine qualitatively and quantitatively cyanobacterial toxins in L. Naivasha which may possibly be associated with previous reports of extensive death fish found floating on the Lake. The information will be useful to policy makers and the public on safety of the L. Naivasha waters.

CHAPTER TWO

LITERATURE REVIEW

2.1Lake Naivasha

Lake Naivasha is a misotrophic freshwater lake located about 80 kilometers northwest of Nairobi and lies about 1890 metres above sea level making it the highest elevated of the Kenyan Rift Valley lakes and because of this its climate is sometimes not regarded as truly tropical (French, 2013). The lake has a surface area of 100-150 km^2 and its main basin is 6 m deep with one major river, the Malewa River, which contributes the majority of inflow (Krienitz *et al.*, 2013). There are two seasonal rivers, Gilgil and Karati, which account for the other 10%. The total catchment area of Naivasha is approximately 3376 km^2 (Kyambia and Mutua, 2015). There is no surface outlet in Naivasha despite its freshwater status, so it has been hypothesized that a subterranean outlet keeps the lake fresh (Morrison, 2013). Rainfall in the basin is typically on a bimodal cycle that roughly experiences short rains from October to December, a hot and dry period from December to February, long rains from March to May and finally a relatively cool and calm period from June to September. There are three separate basins to L. Naivasha: Lake Oloidien, Main Lake basin and Crescent Island basin. In the southwest corner is Lake Oloidien which is a volcanic crater once attached to the Main Lake basin but has been separated since 1982 (Raffoul, 2012). Lake Oloidien's water chemistry and biology had always been different from the Main Lake basin even when they were attached (Mwanjala, 2016), but it has now changed to a more alkaline state and supports a *Spirulina* population and many lesser flamingos. The Main Lake basin is circular and is known for its rough waters and strong winds. Finally, in the northeast part of the lake is another volcanic crater known as the Crescent Island basin, named after the crescent-shaped land mass that used to be an actual island when water levels were higher. Although now connected to the mainland on one shore, the "Crescent Island" forms the barrier around the basin and when water levels are extremely low it extends enough to completely cut this Crescent Island basin off from the Main Lake basin.

2.1.1Transformation of Lake Naivasha

Beginning in the late 1970's and early 1980's the farming industry, which previously was predominately agriculture, started to transition to horticulture due to the ideal weather and sunlight conditions, fertile, volcanic soil availability and affordable labour (McClintock,

2011). In Naivasha, this industry success led to rapid development in the catchment and a population of approximately 20,000 in the 1970's has grown to more than 400,000 today (Harper *et al.*, 2011). The increased industry and population have led to numerous land use changes. Many of the agriculture and floriculture farms are situated along the shoreline of the lake; therefore areas that used to consist of natural, undisturbed vegetation were destroyed. In particular, the fringe of *Cyperus papyrus* (commonly known as papyrus) was reduced by 70% from the late 1960's to 1995 (Hickley *et al.*, 2004) and now is only 10% of what it once was (Morrison and Harper, 2009). This decline came about because of the physical destruction of the plant for more farm sand built-up land, but also from the lake level declines that coincided with the start of horticulture. When the water levels decline they dry out the soil that the papyrus is rooted in and between that and wildlife disruption, it cannot regenerate (Terer *et al.*, 2012). The papyrus around L. Naivasha acts as filter to any contaminants or nutrients entering. The noticeable reduction of papyrus in what is known as the North Swamp of the lake is also important to note as that is the position where the Malewa inflows into the lake. In addition to the farming occurring directly around the lake, it also increased the upper catchment specifically along the path of the Malewa and so everything entering the river from the upper catchment makes its way into the lake.

2.1.2 Eutrophication in Lake Naivasha

The anthropogenic influences in the catchment have increased fertilizer and pesticide use, decreased the vegetation buffer, and therefore more nutrients were brought into the lake through surface run-off and the river inflows. The sediments of L. Naivasha have always been rich in iron and they form a sink for phosphorus as well (Everard, 2010). Therefore, soluble phosphorus and iron may not always be readily available. However, physical or chemical sediment disruption could release them into the water column, suggesting that a major deoxygenation event could release the nutrients into the water and potentially double productivity of blooms (Harper, 1992).

2.1.3 Phytoplanktons in Lake Naivasha

The first studies on the phytoplankton community in Lake Naivasha occurred with two expeditions undertaken in 1929 and in 1930 (Raffoul, 2012). Diatoms were the most dominant in Naivasha, and in particular the genus *Melosira* (which has since been re-classified as *Aulacoseira*) was abundant at times. Cyanobacteria were not dominant, and at certain areas and times not observed at all: but some of the species that were identified were

Microcystis flos-aqua, *Aphanocapsa sp*. and *Merismopediasp*. Prior to the human-induced changes in 1980, biomass was found to be lower, more seasonally variable, and never went much higher than 50 μg/L. reported dominance by cyanobacteria from 1973-1974. From October 1979 to July 1980, there was a shift between diatom dominance (low biomass) to green algae dominance (increasing biomass) to cyanobacteria dominance (peak biomass) (Phlips *et al.*, 2015). The last study of community composition in the literature took place intermittently from June 2001 to May 2004, giving a few time snapshots. While continuous changes could not be assessed, the community was widely variable with cyanobacteria dominance (but low biomass) from June to November 2001, green algae dominance (moderate biomass) from February to May 2002, and desmid dominance (high biomass) in March to May 2004 (Ndebele-Murisa *et al.*, 2010). While there are many gaps in this timeline of community composition, these historical studies do indicate that the lake has gone from a state of seasonal community shifts to diatom dominance due to eutrophication (Müller *et al.*, 2016) to potentially another state of seasonality or transition. It was specifically expressed in (Kurmayer *et al.*, 2016) that no cyanobacterial scums were occurring in 2000 and it has only recently been acknowledged in the literature that cyanobacterial blooms began occurring in 2005 in the lake (Briand *et al.*, 2009).

2.1.4 Occurrence of cyanobacteria

Cyanobacteria, also known as Cyanophyta, are phylum of bacteria that are arguably the most successful group of microorganisms on earth. They are the most genetically diverse; they occupy a broad range of habitats across all latitudes, widespread in freshwater, marine, and terrestrial ecosystems, and they are found in the most extreme niches such as hot springs, salt works, and hyper saline bays.

The occurrence of a particular genus and species of cyanobacteria around the world is apparently influenced by regional differences in water chemistry and climatic conditions. For example, *Cylindrospermopsis* is produced in tropical waters but has not been found in temperate climates. Similarly, *Microcystis* and *Anabaena* blooms occur widely in the temperate regions of the world (Cottingham *et al.*, 2015). In general, 50–75% of bloom isolates can produce toxins, often with more than one toxin being present. Toxic and non-toxic blooms of the same species can be found together. The overall toxicity of a bloom can be uncertain, because variations can occur in toxin concentration over a short time and spatially within a water body experiencing a bloom (Hrudey. *et al.*, 2015).

There is no simple method to distinguish the toxic from the non-toxic forms. The unpredictability of toxin production within any given bloom "renders them potentially dangerous and suspect at all times", and prevention of cyanobacterial blooms is therefore the key to the control of toxic blooms. The growth of cyanobacteria and the formation of blooms are influenced by physical, chemical and biological factors, which were recently reviewed by Rigosi (Rigosi *et al.*, 2015) and Fields (Fields, 2015) and some of these factors are discussed below. As a result of the interplay of these factors, there may be large yearly fluctuations in the levels of cyanobacteria and their toxins.

There is also a seasonal variation in predominating species. Cyanobacterial blooms persist in water supplies that contain adequate levels of essential inorganic nutrients such as nitrogen and phosphorus, water temperatures generally between 15 and 30 °C, and pH levels between 6 and 9. Blooms usually occur in late summer or early fall and are most common in eutrophic or hyper eutrophic bodies of water (Paerl and Otten, 2015). The amount of daylight needed to optimize growth depends on the species. In addition, some cyanobacteria, such as *Microcystis aeruginosa*, can regulate their buoyancy in response to available light (Yu, 2000). This characteristic allows cyanobacteria to migrate through thermal gradients and use nutrients confined to cooler deeper water below (Naselli-Flores, 2010). Buoyancy is controlled mainly through the production of carbohydrates from photosynthesis. This control mechanism breaks down if there is too little carbon dioxide available. Although buoyancy cannot be adjusted during the night, the organisms will float to the surface because of their reduced carbohydrate content as a result of respiration at night (Carey *et al.*, 2012).

Turbulence and high water flows are unfavourable to the growth of cyanobacteria, as they interfere with the organisms' ability to maintain a position in the water column (Pal and Choudhury, 2014). Heavy rain storms can increase runoff and nutrient levels in the water, which encourages the formation of blooms (Reichwaldt and Ghadouani, 2012). The formation of surface scum is enhanced by calm weather conditions. Initially, there may be high barometric pressure and light to moderate winds, accompanied by constant circulation in a water body in which large numbers of cyanobacteria are maintaining their position in the water column to take advantage of those conditions (Joehnk *et al.*, 2008). If the wind stops and circulation also stops, the cyanobacteria may suddenly become "over buoyant." If they cannot adjust their buoyancy fast enough or at all (at night), then the blooms will float to the surface and form surface scum (Schmidt *et al.*, 2014). Thus, scums are often formed

overnight. The scum may drift away with wind and may settle at lee shores and quiet bays, where the cyanobacteria may release their toxins and eventually die (Barrington *et al.*, 2011).

2.1.5 Toxin production and persistence

The two main factors that have been shown to affect toxin production are light and temperature. The optimum temperature for toxin production in cyanobacteria is between 20 and 25 °C which suggest that cyanobacteria are most toxic during periods with warm weather and in areas with warm climates (Thomas and Litchman, 2016). However, the optimum temperature may change from country to country. Light intensity more than light quality is an important factor in toxin production in *M. aeruginosa*. Both toxicity and the ratio of the toxin to protein production are enhanced by both red and green light compared with white light (Khalili *et al.*, 2015). The toxicity of cyanobacteria increases with an increase in light intensity below 40 microeinsteins/m^2 per second and therefore decreases with water depth (Manser *et al.*, 2016). However, when mixing of water from different depths occurs, especially during periods of high winds, this may not be true.

There have been studies concerning the patterns of occurrence of microcystin-LR in three hyper-eutrophic hard-water lakes in central Alberta, Canada, over three seasons. This indicated that *Microcystis aeruginosa* was highly variable temporally within each lake over 1 year, between years in an individual lake, and between lakes in a year. Seasonal changes in microcystin-LR concentration were positively correlated to the abundance and biomass of the *M. aeruginosa* and total dissolved phosphorus concentration, pH, and chlorophyll. Surprisingly, there was a negative correlation between microcystin-LR concentration and nitrate concentration and no correlation with water temperature. Over a 24-hour period, the concentration of microcystin-LR in *M. aeruginosa* decreased more than 6-fold at night compared with concentrations during the day. In United Kingdom, determination of the effect of temperature and nutrient supply on microcystin levels in cultures of *M. aeruginosa* in water bodies in the has been done (Oh *et al.*, 2000) and it was found that the amount of microcystin per unit cyanobacterial dry weight was higher when nitrate levels were in excess and highest between 20 and 25 °C.

As toxin production varies greatly among different strains of the same species, genetic differences and metabolic processes may also be important in the production of these toxins.

Studies have shown that the ability to produce toxins can vary temporally and spatially at a particular site or within the bloom itself (Ressom, 1994; Hrudey. *et al.*, 2015).

2.1.6 Environmental and human exposure

The major route of human exposure to cyanobacterial toxins is drinking water. A minor exposure route is the recreational use of lakes and rivers; for microcystin-LR, however, absorption through skin contact is unlikely, as the toxin does not readily cross cell membranes (Fischer *et al.*, 2005). Some people are also exposed to cyanobacterial toxins through the consumption of certain algal food tablets (Manganelli *et al.*, 2012). An additional, minor route of exposure is through inhalation while taking showers; microcystin-LR, however, is highly soluble in water and non-volatile, so inhalation and absorption through the lungs is unlikely, unless the toxin is inhaled as an aqueous aerosol in air (Lambert and Barley, 2001). The extent to which cyanobacterial toxins move up the food-chain (e.g. freshwater mussels and fish) has been investigated recently (Ibelings and Chorus, 2007). The duration of toxin exposure would generally be shorter in colder countries than in those with milder climates.

The levels of microcystin-LR in the lakes and dugout ponds of Alberta, Canada, ranged from 4 to 605 μg/g dry weight of biomass or up to 1500 μg/g (Vaitomaa, 2006). More than 70% of over 380 bloom biomass samples from 19 lakes in Alberta between 1990 and 1992 showed detectable levels (>1 μg of microcystin-LR per g dry weight of biomass) of toxin (Hrudey *et al.*, 1994). Similarly, levels of microcystin-LR from natural blooms of *Microcystis* in Japan, between 1989 and 1991, ranged from 27 to 622 μg/g dry weight of biomass (Park *et al.*, 1994). In the same blooms, the levels of microcystin-RR and microcystin-YR ranged from 11 to 979 μg/g dry weight of biomass and from 9 to 356 μg/g dry weight of biomass, respectively, with a total maximum level of microcystins of 1732 μg/g dry weight of biomass (Park *et al.*, 1994).

For two Alberta drinking-water supplies, the raw water intake levels of microcystin ranged from 0.15 to 4.3 μg/L with a large coefficient of variation of 59% for hourly fluctuations over an 11.5 hour period. In treated water, levels ranged from 0.09 to 0.64 μg/L with a small coefficient of variation of 10%. Over a 5-week period, similar coefficients of variation were obtained in the two types of samples (Hrudey *et al.*, 1994). In the summer of 1993, microcystin-LR was detected (>0.5 μg/L) in water samples collected from Lake Shoal,

Manitoba, Canada, and from within the drinking-water distribution system following the presence of *M. aeruginosa* blooms in Lake Shoal (Jones *et al.*, 1996; Gupta, 1998). Following this, in 1995, 160 surface water supplies, located mainly in southwestern Manitoba, were chosen for algal study. Treated water samples were analyzed only for those sites in which raw water supplies were found to have detectable levels of toxins (detection limit 0.1 μg/L). Toxin was present in 68% of the treated water samples collected from both the municipal water supply and dugouts used for domestic and livestock consumption. Thus, it appears that conventional treatment methods may be only partially successful in removing the toxins. Toxin concentrations ranged from <0.1 to 1.0 μg/L in raw water samples and from <0.1 to 0.6 μg/L in treated water samples. Again seven different microcystins in 9 of 12 eutrophic water bodies were detected in Germany in 1993 (Fastner *et al.*, 1998). The microcystin concentration was up to 800 μg/g dry weight for a single microcystin. Cytotoxins were also observed in six field samples. With appropriate water treatment, maximum exposure to total microcystins is probably less than 1 μg/L, based on the above data. Average exposure generally would probably be well below this level. Not all water supplies, however, are treated by filtration or adsorption; many are untreated or simply chlorinated. *Cylindrospermopsis* cultured from a drinking-water supply reservoir has been shown to contain the toxic alkaloid cylindrospermopsin at 0.5% dry weight of algae (Ohtani *et al.*, 1992).

2.2 The chemistry of microcystin

The microcystins comprise the largest and most structurally various group of cyanobacterial toxins (Pearson *et al.*, 2010). Around 90 microcystin isoforms varying by the extent of methylation, hydroxylation, epimerization, peptide sequence and toxicity have been identified. Underlying the extraordinary heterogeneity present among the microcystins is their common cyclic structure and possession of numerous erratic and highly conserved amino acid moieties as shown in Figure 2.1 below. Consequently, the microcystins may be described as monocyclic heptapeptides containing both D- and L-amino acids plus *N*-methyldehydroalanine and a unique ß-amino acid side-group, 3-amino-9-methoxy-2-6,8-trymethyl-10-phenyldeca-4,6-dienoic acid (Adda) (Pearson *et al.*, 2010). The microcystin isoforms differ principally at the two L-amino acids, and secondarily on the presence or absence of the methyl groups on d-*erythro-β*-methylaspartic acid (d-MeAsp) and/or *N*-methyldehydroalanine (Mdha). However, substitutions of all functional groups within

microcystin have been reported (Yuan *et al.*, 2016), for instance Figure 2.1 below shows the cyclic nature of microcystins.

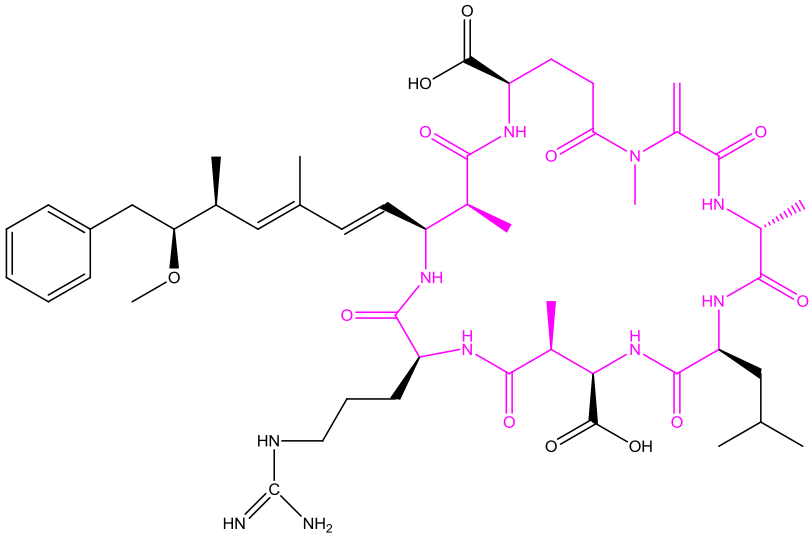

Figure 2.1: Cyclic nature of microcystin

2.3 Biological kinetic characteristics and metabolism of cyanobacterial toxins

The most likely route of exposure to cyanobacterial toxins is via oral ingestion. However, there have been no pharmacokinetic studies with orally administered microcystins. After intravenous or intraperitoneal injection of sub lethal doses of variously radiolabelled toxins in mice and rats, microcystin appears to be transported by bile acids transporter in both the intestine and the liver (Falconer and Yeung, 1992). About 70% of the toxin is rapidly localized in the liver (Chen and Xie, 2005). The kidney and intestine also accumulate significant amounts of microcystin-LR (Malbrouck *et al.*, 2003). Plasma half-lives of microcystin-LR, after intravenous administration, were 0.8 and 6.9 minutes for the alpha and beta phases of elimination, but the concentration of radioactive (3H-microcystin-LR) label in the liver did not change throughout a 6-day study period (Clarke *et al.*, 1991). Microcystin-LR was excreted rapidly, with 75% of the total excretion occurring within 12 hours. The remaining 24% of the administered dose was excreted after 6 days, about 9% via the urinary route and 15% slowly (1% per day) via the faecal route.

Microcystin-LR does not readily cross cell membranes and does not enter most tissues. It crosses the ileum through the multi-specific organic ion transport system and mainly enters hepatocytes, where it is covalently bound to a 40 000-dalton protein (protein phosphatase 2A and possibly protein phosphatase 1) in the cytosol (Robinson, 1991; Gupta, 1998). The liver plays a large role in the detoxification of microcystins (Robinson, 1991). Detoxification

products were seen in urine, faeces, and liver cytosolic fractions, but these products have not been structurally identified. The detoxification products of microcystin-LR are more water soluble than the parent toxin (Antoniou *et al.*, 2008).

2.4 Carcinogenicity

There has been some evidence of tumour promotion in animal studies caused by microcystins (Zhang *et al.*, 2015). In a modified two-stage carcinogenesis mouse skin bioassay, dimethylbenzanthracene (DMBA) (500 μg) in acetone was applied to the skin of four out of six groups of 20 3-month-old Swiss female mice. After 1 week, the DMBA-treated mice received drinking-water, *Microcystis* extract in drinking-water (actual microcystin-YM dose not provided), croton oil (as a positive control) applied to the skin (0.5% in 0.1 mL acetone twice a week) plus drinking-water, or croton oil plus *Microcystin* extract; the control mice received drinking-water or *Microcystis* extract in drinking-water. After 52 days from initiation, substantial skin tumours and ulcers were visible on the DMBA-treated mice consuming *Microcystis* extract. There was a significant increase in the mean weight of skin tumours per mouse in treated mice given the *Microcystis* extract compared with water. The actual number of tumours per mouse and the weights of the tumours in relation to the weights of the animals were not provided. It was concluded by the authors that oral consumption of *Microcystis* extract in drinking-water may act as a tumour promoter (Zhang *et al.*, 2015).

However, the mechanism of action is not clear, as microcystins have difficulty penetrating epidermal cells. The tumour weight per mouse in DMBA-treated mice given both croton oil and the algal extract was slightly lower than in those given croton oil and drinking-water. These latter findings could not be explained by the author (Falconer, 1991). In a two-stage carcinogenicity bioassay, groups of 10–19 7-week-old male Fischer 344 rats were initiated by intraperitoneal injection with 200 mg of diethyl nitrosamine (DEN) per kg of body weight, followed by partial hepatectomy at the end of the third week. Tumour promotion was assessed by intraperitoneal injection of microcystin-LR at 1 or 10 μL/kg of body weight from the third week of the experiment, 3 or 5 times per week. Tumour promotion, as indicated by an increase in glutathione *S*-transferase placental form (GST-P) positive liver foci, was seen after 8 weeks in animals dosed with 10 μL of microcystin-LR per kg of body weight (Matsushima *et al.*, 1992). In a second similar experiment, the same authors used dose levels of 10 μL/kg of body weight per day before, and 10, 25, or 50 μL/kg of body weight per day after, partial hepatectomy. It was found that the increase in GST-positive foci was dose related. However, these results should be interpreted with caution, because the doses used

were very high and could have caused significant damage to hepatocytes. Microcystin-LR had no effect when given to non-initiated rats or to rats that had received partial hepatectomy but no promotion dose of microcystin-LR (Yan *et al.*, 2015).

2.5 Toxicological effects on humans

Blue-green algae have been known to cause animal and human poisoning in lakes, ponds, and dugouts in various parts of the world for over 100 years. Through the recreational use of contaminated water, cyanobacterial blooms of *Microcystis* and *Anabaena*, and others have been linked to incidence of human illness in many countries, but no fatalities have been reported (Chorus *et al.*, 2000). In Canada, human illnesses have been reported in Saskatchewan, with symptoms including stomach cramps, vomiting, diarrhea, fever, headache, pains in muscles and joints, and weakness (Dillenberg and Dehnel, 1960). Similar symptoms as well as skin, eye, and throat irritation and allergic responses to cyanobacterial toxins in water have also been reported in other countries (Ressom, 1994). The reported instances of illnesses are few, but, because they are difficult to diagnose, such illnesses may in fact be more common than has been reported.

In Saskatchewan, Canada, 10 children became sick with diarrhea after swimming in a lake covered with cyanobacteria. *Anabaena* cells, which produce microcystin-LR, were found in the stools of one child (Dillenberg and Dehnel, 1960). In the United Kingdom, 10 of 18 army recruits on a military exercise in a reservoir with a bloom of *M. aeruginosa* suffered abdominal pain, nausea, vomiting, diarrhea, sore throat, dry cough, blistering at the mouth, and headache. Two were hospitalized and developed an atypical pneumonia. Serum enzymes indicating liver damage were elevated. Microcystin-LR was identified in the bloom material (Hilborn and Beasley, 2015). Nevertheless, substances other than microcystin-LR may have been present, and some of the observed effects were probably due to other materials in the water.

In the USA and Australia, several different cyanobacterial toxins have been implicated in human illness from certain municipal water supplies, often after algal blooms had been treated with copper sulfate (Glibert, 2016; Kennish, 2016). In most cases, the cyanobacteria and sometimes the toxins involved have been identified, but the levels of toxin associated with illness have not been established in any of the outbreaks. The Palm Island mystery disease, affecting about 140 people, largely children, in Australia, occurred after a dense cyanobacterial bloom on a water supply was treated with copper sulfate. Within a week,

severe illness was seen, characterized by vomiting, hepatomegaly, and kidney dysfunction, with loss of electrolytes, glucose, and plasma protein; recovery took 1–3 weeks (Byth, 1980). (Paerl and Otten, 2013). Cyanobacterial blooms tend to occur repeatedly in the same water supply. Therefore, some human populations are at risk of repeated ingestion of cyanobacterial toxins. However, the available data are not sufficient to allow a quantitative assessment of human exposure.

2.6 Biosynthesis of microcystin

Microcystins are small non-ribosomal peptides. They are synthesized non-ribosomally via a giant enzyme complex comprising peptide synthetases, polyketide synthases (PKSs), and additional modifying enzymes (Young *et al.,* 2013). Non-ribosomal peptide synthesizes (NRPSs), which catalyze the formation of peptides by a thiotemplate mechanism, are found in both prokaryotes and lower eukaryotes. They are involved in the synthesis of linear, cyclic, and branched-cyclic peptides, including potent drugs, such as penicillin, vancomycin, and cyclosporine (Beletskaya *et al.*, 2016). NRPSs possess a modular structure, with each module being responsible for the activation, thiolation, modification, and condensation of one specific substrate amino acid. A minimal module consists of condensation (C), adenylation (A), and peptidyl carrier protein (PCP) domains. NRPS products are structurally highly diverse due to the incorporation of many unusual residues, such as d- and N-methylated amino acids or hydroxyl acids, in addition to the protein-genic amino acids (Payne *et al.,* 2017). For instance, microcystin-LR (*Microcystis aeruginosa*) is synthesized by proteins that are encoded by a 55 kb microcystin-gene cluster (*mcy*) that contains 6 large genes that also encode proteins with enzyme polyketide synthase activity, non-ribosomal peptide synthase activity and four smaller genes. These large proteins are made up of different protein domains, formed 'modules', which have their own specific enzymatic function (Fersht, 2017). Although the enzyme systems involved in the biosynthesis of microcystins is not identical among all cyanobacteria, there are large similarities and most of the essential enzymes are conserved.

The biosynthesis of microcystin-LR begins with the coupling of phenyl acetate to the *mcyG* enzyme. In a series of reactions, catalyzed by different enzyme modules as well as different enzymes, microcystin-LR is formed (Dittmann *et al.,* 2013). This involves various steps as follows. The first step of the synthesis involves the insertion of several carbon- and oxygen atoms between the acetyl- and phenyl- group. This part of the synthesis is catalyzed by

enzyme domains that possess β-ketoacylsynthase, acyltransferase, C-methyltransferase and ketoacylreductase activity. At the end of this stage, i.e. after the first condensation of glutamate, the amino acid Adda is formed (Walsh *et al.*, 2013). The second part of the synthesis involves the condensation of the amino acids of which the microcystin is composed. Thus, in the case of microcystin-LR the consecutive condensation of the amino acids glutamic acid, methyldehydroalanine, alanine, leucine, methyl aspartic acid and arginine leads to the coupled product. A nucleophilic attack of the nitrogen in the Adda residue results in the release of the cyclic microcystin-LR. The different microcystins are all synthesized by the same enzymes as microcystin-LR (Pearson *et al.*, 2010).

2.7 Determination of microcystins by HPLC

High-performance liquid chromatography (HPLC) is a type of liquid chromatography used to separate and quantify compounds that have been dissolved in so a solvent. HPLC can be used to determine the amount of a specific compound in a solution. It uses the basic principles of chromatography i.e. retention, resolution and sensitivity (Snyder *et al.*, 2012). The chromatographic process begins by injecting the solute into the injector at the inlet of the column (Figure 2.2). Separation of components occurs as the analytes and mobile phase are pumped through the column. Eventually, each component elutes from the column as a peak on the data display (Snyder *et al.*, 2011).

Figure 2.2: HPLC instrument (Snyder *et al.*, 2011).

The detection of microcystins at 1.0 µg/L levels requires sensitive analytical methods and HPLC methods have been widely used for this purpose. Solid-phase extraction (SPE) is one of the main methods for sample extraction and pre-concentration; however, the typically used SPE stationary phase (C18) does not supply good selectivity for trace analysis (Eissa *et al.*, 2014). Immuno-affinity columns (IAC) modified with anti-microcystin-LR monoclonal

antibodies on polypropylene stationary phases have been used for extraction with good selectivity for the HPLC analysis of microcystins, but extensive use of this method is limited because an IAC is not commercially available for this application.

2.8 Identification of microcystins by UV-Vis Spectrophotometer

Ultraviolet (UV) and visible radiation is part of the electromagnetic spectrum, which includes other forms of radiation like radio, infrared (IR), cosmic, and X rays (Zamanian and Hardiman, 2005). The energy associated with electromagnetic radiation is defined by the following equation:

$$E = hv \quad (2.1)$$

Where E is energy (in joules), h is Planck's constant (6.62×10^{-34} Js), and υ is frequency (in seconds). Electromagnetic radiation can be considered a combination of alternating electric and magnetic fields that travel through space with a wave motion. Radiation acts as a wave and it can be classified in terms of either wavelength or frequency, which are related by the following equation:

$$V = C/\lambda \quad (2.2)$$

Where υ is frequency (in seconds), c is the speed of light (3×10^{8} ms^{-1}), and λ is wavelength (nanometers). From the above equations it can be shown that radiation with shorter wavelength has higher energy. When light passes through or is reflected from a sample, the amount of light absorbed is the difference between the incident radiation (I_o) and the transmitted radiation (I). The amount of light absorbed is expressed as either transmittance (T) or absorbance (A). Transmittance is given in terms of a fraction or as a percentage and is defined as follows:

$$T = I_o/I \quad (2.3)$$

Absorbance is defined as follows:

$$A = -logT \quad (2.4)$$

UV-visible spectra show a few broad absorbance band compared with techniques such as infrared spectroscopy, which produces many narrow bands. The presence of an absorbance band at a particular wave length often is a good indicator of the presence of a chromophore(Barthwal *et al.,* 2015). UV-visible spectra are used for identification of an unknown substance including microcystin (λ_{max} = 238 nm)which is the major focus of this study, through comparison of the measured spectrum with a reference spectrum (Hong *et al.,* 2011).

2.9 The theory behind HyperChem computational code

HyperChem computational software contains a suite of computational tools including quantitative structural activity relationship (QSAR) (Smith and Hansch, 2000). HyperChem is a versatile molecular modeler and editor and a powerful computational package. It offers many types of molecular and quantum mechanics calculations including commands to determine the toxicity of a molecular compound (Carlsen *et al.*, 2008). HyperChem lets one to build and display molecules in a more robust manner. Since HyperChem contains a good graphical user interface, one can monitor the construction of molecules. The most interesting feature of predictive QSAR models is that the behavior of any new or even hypothesized molecule can be predicted by the use of the computing commands such as QSAR property command. The fundamental equation for QSAR analysis is expressed by equation 2.5.

$$P = \frac{\left[microcystin\ in\ octanol\right]}{\left[microcystin\ in\ water\right]} \qquad 2.5$$

where the log of P (usually inbuilt into the HyperChem computational program) predicts the toxicity index of the microcystin under investigation.

HyperChem can be used to investigate the reactivity of molecules and their functional groups. It estimates Physico-chemical properties, biological activities and understanding the physicochemical features behind a biological response to toxins. Ecological structural activity relation computational command in HyperChem uses QSARs to predict the aquatic toxicity of chemicals for a variety of organisms. ECOSAR (Ecological Structural Activity Relation) computational command in HyperChem uses QSARs to predict the aquatic toxicity of chemicals for a variety of organisms (Smith and Hansch, 2000). The HyperChem graphical user interface has several computational tools. Computational Chemistry represents molecular structures as numerical models and simulates their behavior with the equations of quantum and classical physics (Young, 2001). HyperChem attempts to find consistent relationship between molecular properties and biological activity for a series of compounds including microcystins which is the principal focus of this investigation (Smith and Hansch, 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Reagents and chemicals

All reagents used in the study were of analytical grade. The reagents were purchased from commercial suppliers and used directly without further purification. Solid phase extraction cartridges, Sep-pak© light tc18, were obtained from Waters Corporation Milford, Massachusetts USA.

3.2 Sample collection

The water samples were collected from four sampling sites (A, B, C, and D) at Lake Naivasha basin (Figure 3.1) located at an altitude of 1890 m to represent well-mixed lake conditions. In each site, 1000 mL of the lake water was collected in 1 L clean plastic bottles once a month over the period of study (Figure 3.2). Sampling was done between January and June in order to cover the dry and wet seasons for the purpose of comparison. The GPS coordinates of the sampling locations were (42.064 °S,36.25492 °E) where sample A was collected, (43.019 °S,35.2522 °E), where sample B was collected, (43.224 °S, 36.25421 °E) where sample C was collected and (42.911 °S, 36.2532 °E), where sample D was collected. The plastic bottles, caps and the rims were thoroughly rinsed using deionized water to avoid contamination of the sample and labeled correctly. Finally, the plastic bottles were sealed, labeled A, B, C, and D respectively based on collection site. The samples were stored in a cool insulated carton and immediately transferred to the laboratory for further treatment. The physiochemical parameters that were measured directly at the lake were divided into:

a) Physical parameters: colour, temperature, turbidity and odour.

b) Chemical parameters: pH, electrical conductivity, total dissolved solids and salinity.

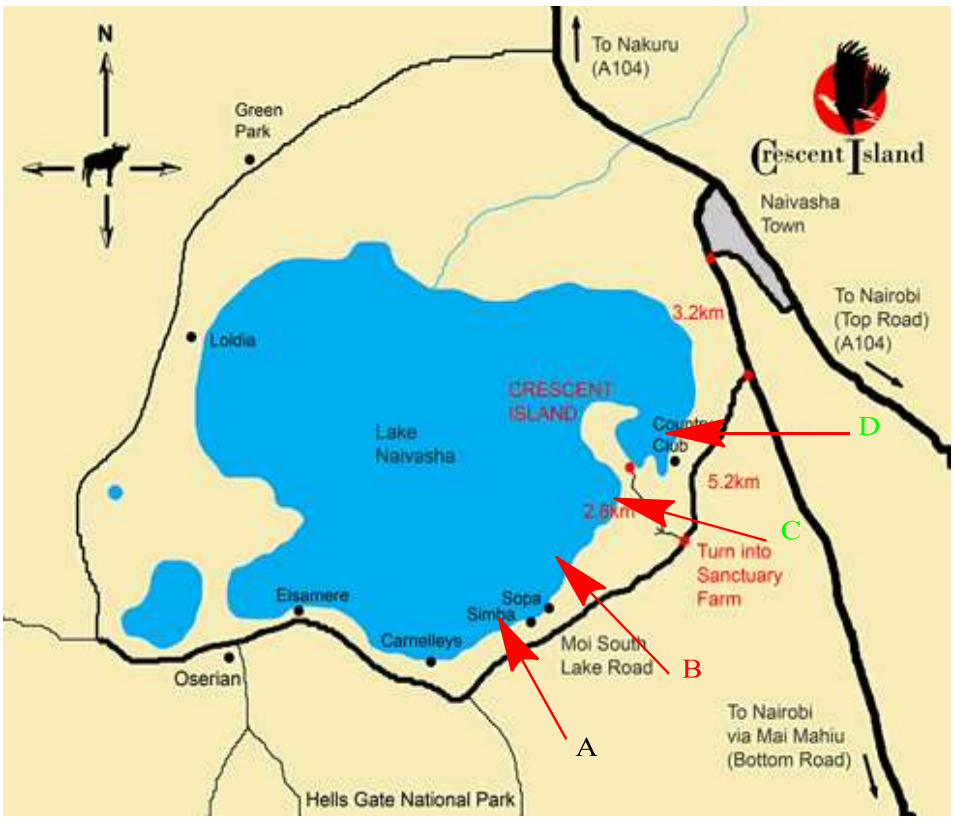

Figure 3.1: Sampling location (satellite image: www. nasa.gov/space images/images/large size/P1A10625-hires.jpg; editing. Dittman)

Figure 3.2: Sample collection using 1 litre plastic bottles

3.3 Preparation of working solution

Sodium thiosulphate solution was prepared by weighing 1 g of solid sodium thiosulphate into a 100 mL volumetric flask. Distilled water was added to dissolve the solid and then latter added up to the mark. Then 10%, 20% and 30% (v/v) methanol in water solutions were made by measuring 10 mL, 20 mL and 30 mL of methanol respectively into a 100 mL volumetric flask and distilled water added up to the mark. Exactly 10% acetic acid v/v in water solution was prepared by measuring 10 mL of acetic acid into a 100 mL volumetric flask and distilled water added up to the mark. Finally, a 0.1% (v/v) acetic acid in methanol solution was made by measuring 0.1 mL of acetic acid into a 100 mL volumetric flask and methanol added up to the mark.

3.4 Sample preparation

For each month, water sample A was mixed by inverting the container several times to ensure uniformity. Exactly, 500 mL of the water sample was measured and filtered gently using a GF/C filter funnel into a 1,000 mL volumetric flask. This was followed by addition of 0.1 mL sodium thiosulphate solution to eliminate the free residual chlorine. The water sample was shaken vigorously and allowed to stand for five minutes. Exactly 5 mL of the 10% acetic acid v/v in water was added and the sample mixed before passing the sample through GF/C filter disc into another 1,000 mL volumetric flask. Finally, 5 mL of methanol was added and shaken for uniformity (Carmichael, 1997). The procedure was repeated for samples B, C and D.

3.5 Solid phase extraction

A solid phase extraction Sep-pak© light tc18 cartridge was prepared by attaching it to a 10 mL syringe and conditioned with 10 mL methanol followed by 10 mL distilled water. The cartridge was not allowed to dry at any time. The methanol and water eluates were discarded. Exactly 10 mL of the prepared water sample from sample A was drawn into the syringe and expelled through the cartridge running to waste. This was repeated until all the water sample was passed through the cartridge. The cartridge was washed with 10 mL of the 10% methanol followed by 10 mL of the 20% methanol and then finally washed with 10 mL of the 30% methanol. The eluate from the three washes was discarded. The cartridge was then eluted with 3 mL of 0.1% acetic acid in methanol. The eluate was collected in a 50 mL beaker and dried on a hot plate at 45 °C. The sample was then re-suspended in 0.1 mL methanol to rinse the beaker.

The sample was then transferred into a clean vial and refrigerated at 4 °C prior to UV-Vis and HPLC analysis. The procedure was repeated for the other prepared water samples: B, C and D for every month under study.

3.6 UV-visible spectrophotometer

The eluate from sample A was diluted using 10 mL methanol and analyzed using a double-beam UV-visible spectrophotometer (1800-240V, SHIMADZU). The sample was filled in a 1 mL cuvette which was then placed in the sample cell holder while the reference cell holder contained a cuvette filled with methanol. The cuvette had an optical path length of 10 mm. The absorbance was measured in triplicate from a wavelength of 190 nm to 300 nm. The spectrophotometer had a slit width of 1.0 nm. The eluates from samples B, C and D were refrigerated awaiting HPLC analysis. The procedure was repeated for the samples collected for the other months under study.

3.7 HPLC analysis

The analysis of microcystins was conducted using a Shimadzu HPLC (model) fitted with an auto-sampler and equipped with Model 20A pumps, an in-line degasser and a photodiode array detector that was set to monitor and a Millennium 32 data processor. A reverse-phase C18 silica column with separation achieved over a linear gradient of water and acetonitrile, both containing 0.05% acetic acid was used. The gradient covered a sufficient range of polarities (30% - 70% acetonitrile) to allow the analysis of all microcystins which are known to vary considerably in their polarities. The spectral information was collected between wavelength of 200 and 300 nm. The injection volume used was 20 μL (Harada *et al*., 1989). The signals of the microcystins were identified by comparing the chromatograms of the samples with the chromatogram of standards. Quantification of microcystins involved the measurement of peak area. To determine the concentration of a compound, the peak area was plotted versus the concentration of the substance. The procedure was done for all the samples collected.

3.8 Determination of toxicity using HyperChem computational program

The relative toxicities of the microcystins under study was explored using the Quantitative structural activity relation (QSAR) method incorporated in the HyperChem computational program (HyperChem®, 2002) based on the octanol-water partition coefficient. The logarithm of octanol-water partition coefficient is an important parameter in determining the

relative toxicity of compounds (Smith and Hansch, 2000). It predicts the Lipophilicity and the hydrophobicity of a pollutant. Lipophilicity correlates with many biological activities such as mutagenicity and carcinogenicity (Smith and Hansch, 2000; Young, 2001). The QSAR modeling establishes an attractive approach to preliminary assessment of the impact on environmental health by a primary pollutant and the set of transformation products that may be persistent and toxic to the environment (Carlsen *et al.*, 2008) This is because, biological systems are considered hydrophilic and inadvertently polar. High lipophilicity correlates more strongly with biological activity which translates to more oxidative stress and extensive cellular injury (Smith and Hansch, 2000).

3.9 Experimental determination of microcystin toxicity

Each extracted microcystin was tested for its toxicity by simulating the partitioning of a 2 mL solution of the microcystin aliquot in an equimolar solution of 10 mL octanol-water mixture. The partition coefficient of microcystin in the octanol-water mixture was a measure of its toxicity. The more soluble a substance is in water, the more polar it is hence hydrophilic. If the microcystin is more soluble in octanol then it is hydrophobic. This means that the hydrophilicity and hydrophobicity are both measures of toxicity.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Microcystin Extracts

The solid phase extraction cartridges used underwent color changes on the trifunctional, end-capped C-18 from white to green as shown in Figure 4.1. This was probably due to microcystin pigment because microcystins contain a pigment known as phycocyanin (PC) which is responsible for the green colour of cyanobacteria. The green colour may also be due to presence of phytoplankton (cyanobacteria) that may have escaped during the sample pre-treatment process using a GF/C filters.

(a) (b)

Figure 4.1: The C18 cartridges for concentration of microcystins; (a) Before and (b) After

4.2 Physicochemical properties

The physiochemical properties of the water samples collected between January 2016 and June 2016 were analyzed and the results recorded in Table 4.1. The study period covered dry and hot season (January – February) and wet season (March – May). The month of June remained cold and calm. The water temperature during the time of study ranged between 19 °C and 27 °C, the highest being observed during the month of January and the lowest in the month of April (Table 4.1). The water temperatures oscillated with the rainfall in the basin as expected which is typically on a bimodal cycle. The cycle of short rains from October to December, a hot and dry period from December to February, long rains from March to May and finally a relatively cool and calm period from June to September remains

unchanged as described in literature (Raffoul, 2012). The turbidity of the water samples ranged between 520 ± 91 ptco, signaling low transparency throughout the period. The conductivity was 234 ± 0.8 µs/cm and the total dissolved solids were found to be 1035 ± 12 mg/L.

Electrical conductivity (EC) estimates the amount of total dissolved salts (TDS), or the total amount of dissolved ions in the water. The EC is controlled by several factors (Atekwana *et al.*, 2004) which include:

1. Geology (rock types) - The rock composition determines the chemistry of the watershed soil and ultimately the lake. For instance, limestone leads to higher EC because of the dissolution of carbonate minerals in the basin.
2. The size of the watershed (Lake Basin) relative to the area of the lake also affects EC and TDS - A bigger watershed to lake surface area means relatively more water draining into the lake because of a bigger catchment area, and more contact with soil before reaching the lake.
3. Finally, evaporation of water from the surface of a lake concentrates the dissolved solids in the remaining water - and so it has a higher EC.

It was also observed that the total dissolved solids (TDS) for the months of March and April was the same. It is clear from Table 4.1 that the TDS remained high during the long rains period (March to May). This was attributed to surface run-off during which loose soil was carried along with the run-off water. During the month of May, the value was slightly lower because by this time, some vegetation had grown and the rains were subsiding to usher in the relatively cool and calm period (June to September).

Table 4.1 Physico-chemical parameters in Lake Naivasha between January and June 2016

Water parameter	Jan, 2016	Feb,2016	March, 2016	April,2016	May,2016	June,2016
Temperature (°c)	27.0	25.3	23.9	19.1	21.8	20.6
Ph	7.41	7.88	7.62	8.53	6.57	8.01
Turbidity (ntu)	69.0	59.4	44.6	90.3	73.8	67.5
Conductivity (µs/cm)	1035	1054	1071	1071	1064	1047
Colour (ptco)	377	306	360	360	386	365
TDS (µs/cm)	235	236	234	234	241	256

The pH of the lake remained slightly alkaline throughout the study period except for the month of May. This is contributed by the inflow and outflow of the water which maintains the pH nearly at its equilibrium. Considering the pH (6.57 - 8.53) and the temperature conditions (19 - 27) °C recorded during this study, they represent optimum conditions for the

blooming of algae reported in literature as (6 - 9) °C and (15 - 30) °C for pH and temperature respectively (Paerl and Otten, 2015).

Turbidity remained high during the period under study (59.0 ± 24) ntu. This led to low phytoplankton biomass recordings, ranging between (1.5 - 8.2) mg L^{-1} and also correlated well to the colour of the water throughout the period of study (359.0 ± 24) ptco. The month of April was observed to have the highest turbidity which was attributed to more dissolved solids through surface run-off during this wet season. The low turbidity value recorded for the month of March could be attributed to heavy rains that cleared all the loose soils on the surface. Further rains had little soil left behind to be easily washed down the gullies.

Overall, the physico-chemical properties at L. Naivasha point towards a water reservoir that can support blooming of cyanobacteria and consequently production of microcystins. Research has positively correlated microcystin-LR concentration to the cyanobacteria biomass and pH (Kotak *et al.*, 2000; Sanes and Kotak, 2014). Although there are no studies that have positively correlated temperature to cyanobacterial toxins, the blooming of cyanobacteria at optimum temperatures is a reason enough for concern. In addition, the lake was recently invaded by water hyacinth which may also affect turbulence which can interfere with the cyanobacteria's mobility (Pal and Choudhury, 2014). Aquatic plants also affect evapotranspiration rate and this will definitely affect the concentration of toxins at L. Naivasha. A combination of these factors can lead to cyanobacterial toxin-contamination of L. Naivasha's water.

4.3 UV-VIS results for microcystin

The C18 extracts were first subjected to UV-Vis analysis in order to check if the concentration process for microcystins was successful before subjecting them to HPLC analysis. A maximum wavelength (λ_{max}) around 238 nm was expected for the samples. Since the samples contained a mixture of microcystins, λ_{max} values for various samples ranged between 238 nm and 247 nm. The absorbance and wavelength spectra generated by the double-beam UV-visible spectrophotometer for selected samples are shown in Figures 4.2 to 4.5.

UV-Vis spectrum of sample A (for the month of April) (Figure 4.2) had a peak with absorbance of 0.68 and a λ_{max} of 247 nm. This peak was broad signifying less concentration of the suspected microcystin in the sample. The peak obtained was also observed to be less

resolved. This was probably due to the use of acetic acid which did not extract many of the microcystin pigments resulting to poor recoveries of microcystins. This was also probably due to the presence of impurities that had similar absorption spectrum to that of microcystins of our interest. In literature, there is no known microcystin which absorbs at 247nm and this was suspected to be due to new microcystins or cyanobacteria strain (Puddick *et al.*, 2014).

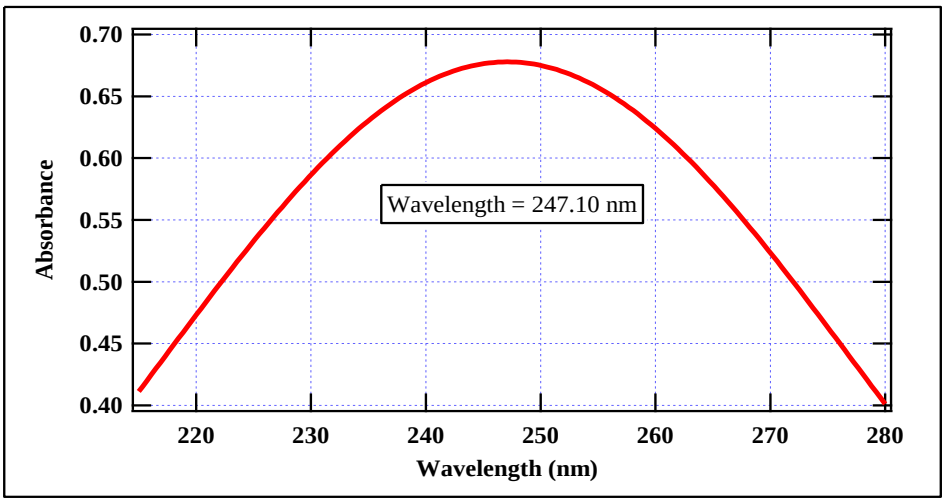

Figure 4.2: UV-Vis spectrum of sample A for the month of April

Sample A (for the month of January) spectrum (Figure 4.3) had a peak with absorbance of 0.99 and a maximum wavelength (λ_{max}) of 240.94 nm as shown in the Figure 4.3. This peak was at slightly higher λ_{max} than that of MC-LR which absorbs at the wavelength of 238 nm. This peak did not match with any microcystin of our interest. This was most likely due to the emergence of a new microcystin or a product formed from the degradation of microcystin (Miao *et al.*, 2010).

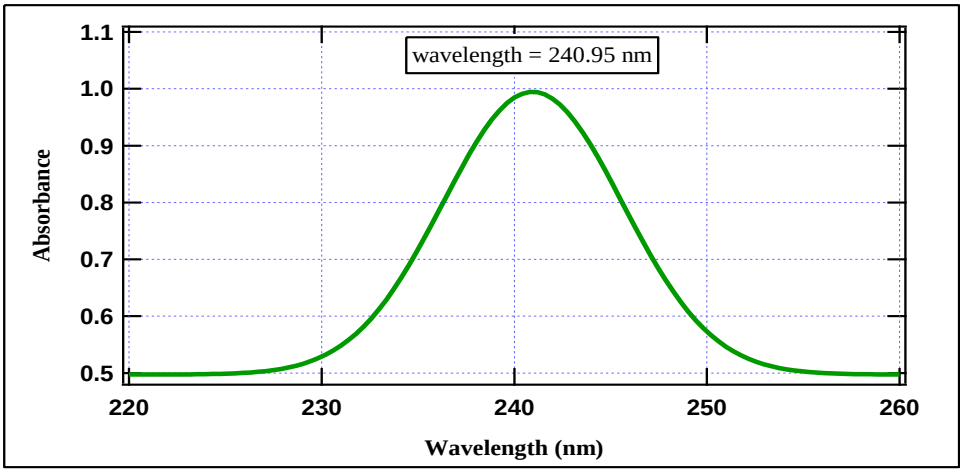

Figure 4.3: UV-Vis spectrum of sample A for the month of January

Sample A (for the month of February) spectrum (Figure 4.4) had a peak with absorbance of 1.0 and a maximum wavelength (λ_{max}) of 238.3 nm. This is evidence for presence of MC-LR as it absorbs at 238 nm (Metcalf *et al.*, 2002). A study done by (Harada, 1996) indicated that the main chromophore of microcystins absorbed at 238 nm as a result of the conjugated diene in the Adda residue. Microcystin-LR showed a maximum wavelength at 238 nm with specific congeners yielding additional unique signatures in the absorbance spectrum.

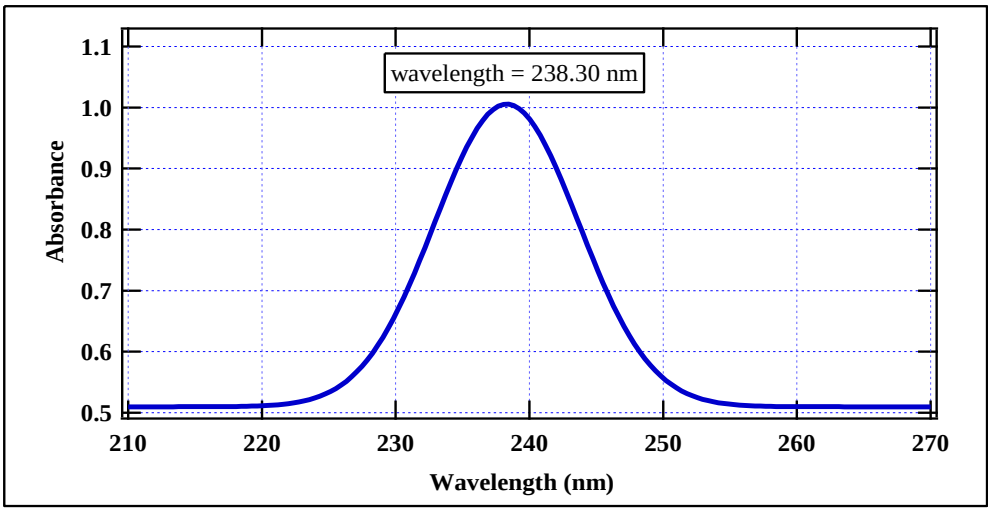

Figure 4.4: UV-Vis spectrum of sample A for the month of February

Sample D spectrum (Figure 4.5) had a sharp peak with absorbance at 0.87 and a maximum wavelength (λ_{max}) of 238.94 nm. This was an evidence of microcystin-LR which absorbs at this wavelength (238 nm). The sharpness of the peak indicates that the concentration of microcystin-LR was high in month of March as compared to the rest of the samples collected (Halvorson and Vikesland, 2011). This obserrvation was corroborated by HPLC results (Figure 4.8) and this suggests that UV-Vis technique can be used to carryout preliminary studies on microcystins before HPLC studies.

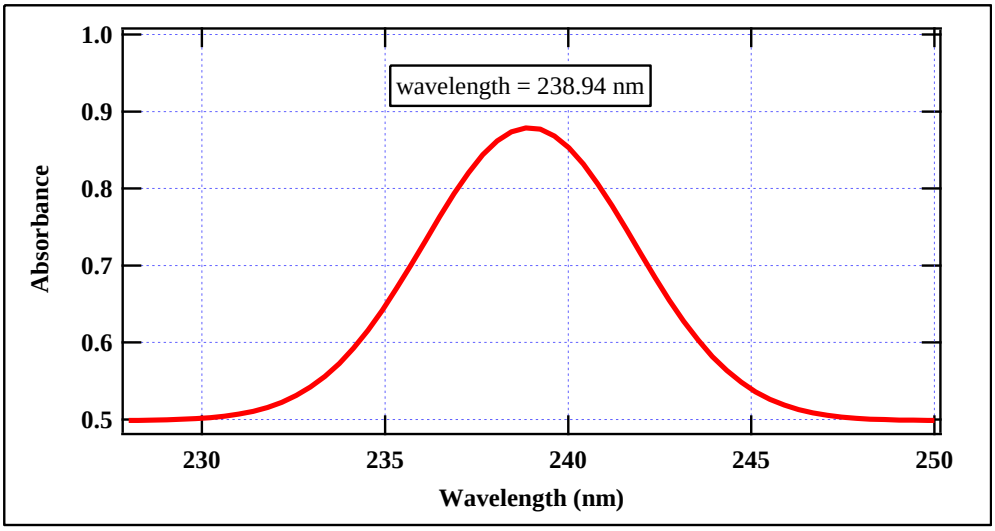

Figure 4.5:UV-Vis spectrum of sample A for the month of March

The broadening of UV-Vis spectra was attributed to light scattering, size distribution of molecules, temperature, etc. the microcystin molecules contain various functional groups (and amino acids) and the gap between the rotational and vibrational energy levels continuosly keep changing, thereforoe affecting the electronic changes. Absorption of of light takes place over a range of wavelengths (especially between 200 nm and 400 nm) which results in a Gaussian-type distriibution

4.4 HPLC results of microcystins

Following the positive results from UV-Vis analysis, the samples were subjected to HPLC analysis. Using HPLC technique, the microcystin-LR, -YR and -RR were detected in all the water samples collected from the L. Naivasha. Microcystin-LR, -YR and -RR were identified based on their retention times, 11.45, 11.0 and 9.10 respectively (Figure 4.6).

The chromatogram (Figure 4.6) is an HPLC-UV spectrum for the blank. This was done to ensure that the peaks of the microcystin obtained from the sample are clearly differentiated from the peaks of the solvent (acetonitrile).

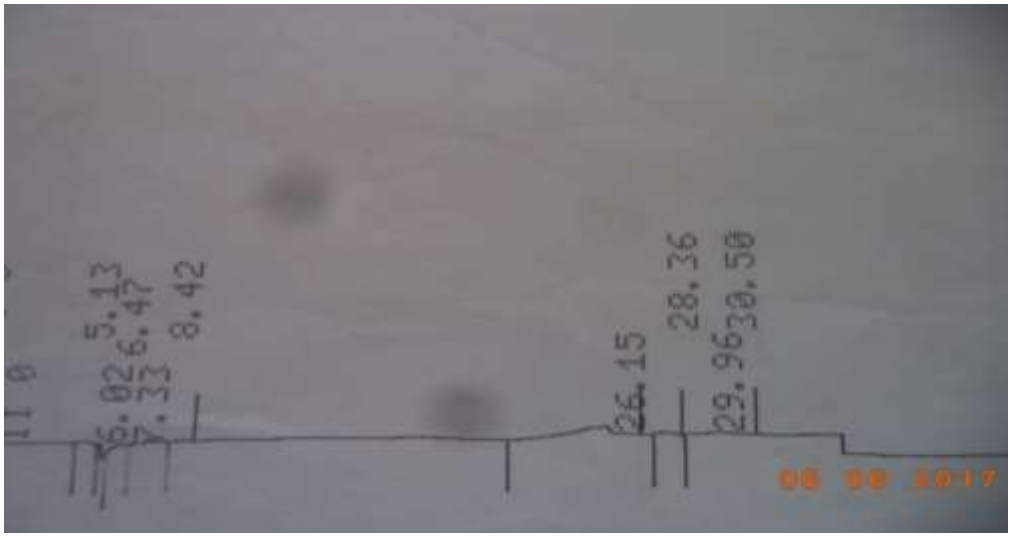

Figure 4.6: HPLC chromatogram of the solvent (acetonitrile)

In Figure 4.7, three conspicuous peaks with different retention times were obtained for the samples collected in January. The toxin with a retention time of 11.65 minutes was identified as MC-YR and compares well to literature values (Shan *et al.*, 2011). This peak was shorter as compared to the rest implying that its concentration is much lower. The second peak was observed at retention time 12.87 minutes and it is more protruding. This means that it is more concentrated in this sample. Retention time of 12.87 minutes corresponds to MC-LR. Finally, a sharp peak at retention time 7.27 minutes that does not correspond to any microcystin of interest was observed. The peak could not be assigned to any known microcystin but was thought to result from a microcystin degradation product.

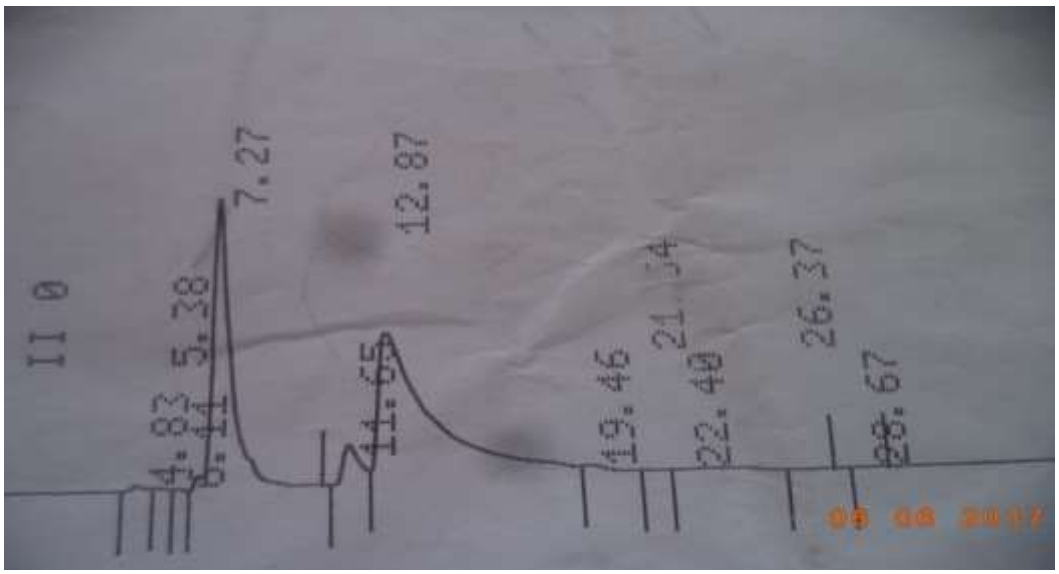

Figure 4.7: HPLC-UV chromatogram of filtered sample B (January) with MC-LR detected

Figures 4.8 for sample B, two peaks were observed at different retention times for the samples collected in February. The peak at retention time 11.62 min was sharp and narrow. This means that the specific microcystin which is MC-LR for this case is more concentrated in this sample (Halvorson *et al.*, 2011). The peak at retention time 7.30 minutes was shorter and slightly broader as compared to the peak at retention time 11.62 minutes. This means that it is less concentrated in the sample. This peak did not match to any of the microcystin of our interest. This is most probably due to formation of a new product due to degradation of microcystin (Antoniou *et al.*, 2010).

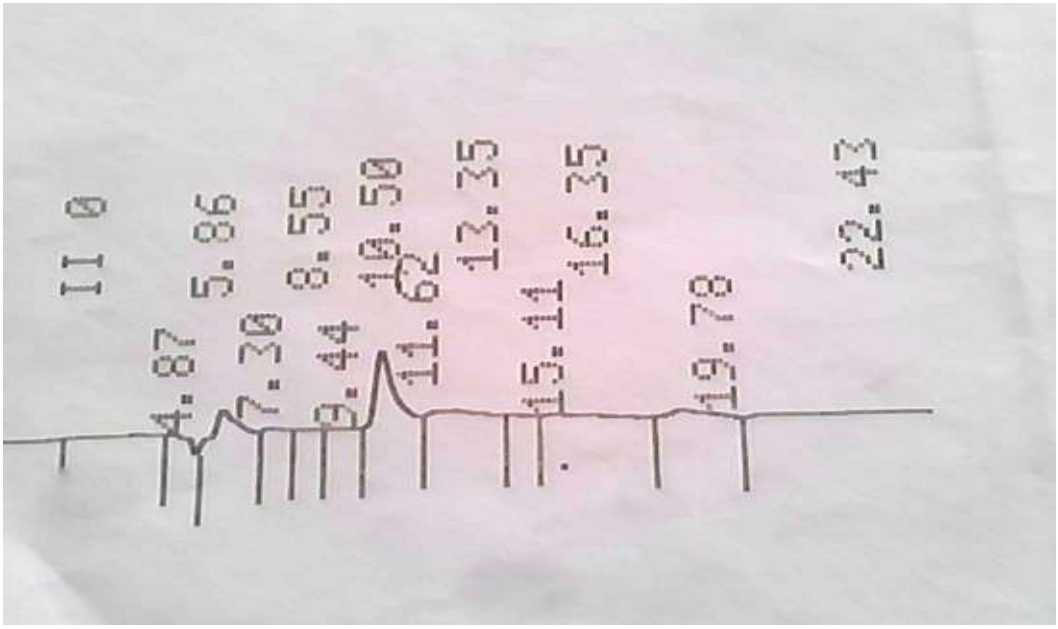

Figure 4.8: HPLC-UV chromatogram of filtered sample B (February) with MC-LR detected

Figures 4.9 for sample C, three peaks were observed at retention time 8.67 minutes, 12.10 minutes and 14.69 minutes for the samples collected in March. The peak at retention 12.10 minutes was sharp and it corresponds to MC-LR. At retention of 14.69 minutes, the peak did not correspond to any microcystin of our interest. The peak at retention 8.67 minutes was tall and sharp as compared to the rest. This means that the microcystin present in this sample is more concentrated. The peak corresponds to MC-RR.

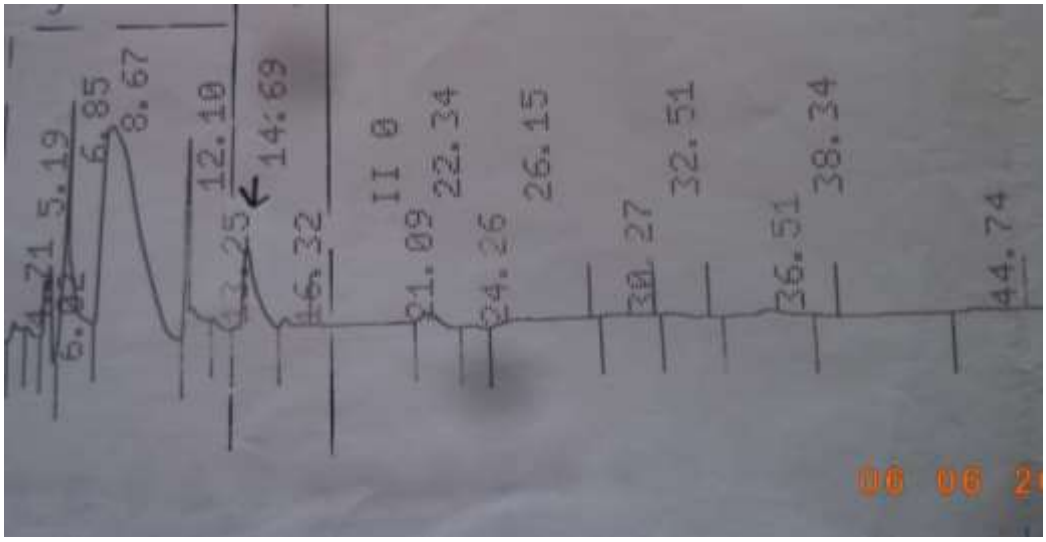

Figure 4.9: HPLC-UV chromatogram of filtered sample C (March) with MC-LA detected

In Figure 4.10, three peaks were observed at different retention times for the samples collected in April. For instance at retention time 12.16 minutes the peak observed was broad and less resolved. This implies that the microcystin present in this sample is less concentrated. This peak corresponds to MC-LR. The second peak was at retention time 10.08 minutes. This peak was narrow and sharp, implying that it is more concentrated though it did not match to any known microcystin. This might be a product as a result of microcystin degradation which is believed to have been contributed by UV radiations (Fotiou *et al.*, 2013). The peak at retention time 4.42 minutes was shorter and less resolved as compared to the rest of the peaks, it did not match to any of the microcystin of our interest. Similarly, the peak at retention time 6.95 minutes did not match to any of the known microcystins.

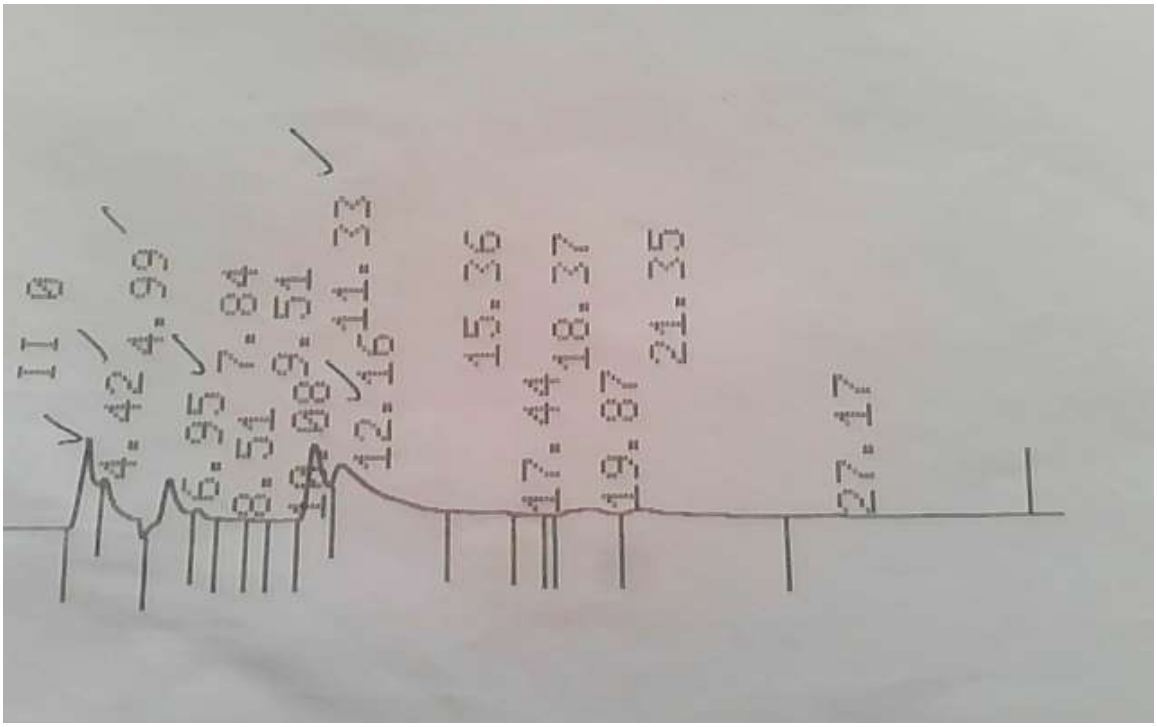

Figure 4.10: HPLC-UV chromatogram of filtered sample D (April) with MC-LR detected

This study revealed that cyanobacteria in Lake Naivasha produce microcystins. Microcystin-LR, microcystin-YR, microcystin-RR and microcystin-LA were found in varying amounts. Worldwide, more than 60 structural variants of microcystins have been isolated. MC-LR was found in all the water samples collected. Estimated as microcystin concentration per litre of lake water, the concentrations of microcystins equivalents in Lake Naivasha was within the acceptable range of 1.5µg/L by WHO (Qin *et al.*, 2010). Although the levels in this situation may change especially after Kenya changed its status from low-income economy to middle-income economy. There is expected to be accelerated use of fertilizers within Naivasha area in order to feed its rising population and this may lead to growth of algal blooms. This wills raise the concentrations to levels that can have serious ecological impact on aquatic food web of the lake and cause serious health problems.

4.5 Modeling Results of Microcystins using HyperChem Computational Code

Log P value, which is usually calculated as the octanol-water partition coefficient, is a measure of the toxicity index of a molecule. In this case, microcystin-RR is approximately 230 times more soluble in water than in octanol. Thus, microcystin-RR in (Figures 4.11) is highly hydrophilic and may react with polar biological tissues, causing extensive cell injury, oxidative stress, and ultimately cancer. Lipophilicity, as measured by the base 10 logarithm

of the octanol-water partition coefficient (P) and denoted as log *P*, is included as a possible contributory factor to the toxicity. Log *P* correlates with a number of biological activities including *in vitro* mutagenicity and carcinogenicity. Lipophilic compounds including microcystins can cross biological barriers which contain lipids, for example, cell or microsomal membranes and skin stratum. Therefore, log *P* influences metabolic fate, intrinsic biological activity and the biological transport properties of chemicals as Table 4.3 below illustrate.

4.5.1 Hydration energy (hydration enthalpy)

This is the amount of energy released when one mole of ions undergo hydration which is a special case of solvation (dissolution energy where water is the solvent). When microcystins are released from *microcystis*, they dissolve in water. The outermost atoms of this microcystins (the edges of the lattice) move away from the lattice and become covered with the neighboring water molecules. From our tabulated results it was found that MC-YR and MC-LR (-24.54 and -24.81 Kcal/mol respectively) had nearly same amount of hydration energy. However, hydration energy for MC-RR was found to be slightly lower (-26.04 Kcal/mol) as compared to the rest. If the hydration energy is equal to or greater than the lattice energy, then the toxins are water-soluble. When the hydration energy is higher than the lattice energy, then solvation occurs with a release of energy in the form of heat. This heat is essential for microcystin growth as it provides suitable environment for their existence. This implies that MC-YR and MC-LR are more profound than MC-RR as they contain higher solvation energy. Generally, hydration energy plays a key role in the growth of microcystins and hence their toxicity

4.5.2 Surface area/volume ratio

From Table 4.3 it is evident that MC-LR and MC-YR has a higher surface area of 672.34 $Å^2$ and 600.83 $Å^2$ as compared to MC-RR which has 552.14 $Å^2$. This result corresponds to their toxicity indices of -0.31, -1.61 and -1.90 respectively which means that MC-RR is more toxic. Looking at their surface area/volume ratios, it was found that MC-RR had lowest ratio of 0.2728, followed by MC-YR having 0.2990 and MC-LR with 0.3344. These results indicate that the MC-RR has higher potential of existence than MC-LR and MC-YR, rendering it more hazardous than its counter parts. This S/V ratio signifies that the growth rate of microcystin is positively correlated confirming that small celled species generally grow faster than large celled species.

Table 4.3: Quantitative structural activity relation (QSAR) parameters

Microcystin	Log. P	P	Surface Area (Å^2)	Volume (Å^3)	Hydration Energy (kcal/mol)	Molecular Mass (g/mol)
YR	-1.61	0.025	600.82	2009.76	-24.54	1045.20
RR	-1.90	0.0126	552.14	2024.05	-26.04	1038.21
LR	-0.31	0.489	672.34	2010.52	-24.81	995.19

Table 4.4 illustrates the parameters that were used to optimize the microcystin structures with HyperChem computational framework. Evidently, the gradient of the force field used in the geometry optimization of microcystins presented in this work (0.96-0.99) is good. Clearly, less number of iterations gives a better gradient. However, less iteration implies higher optimization energy as presented in Table 4.4. Assisted Model Building with Energy Refinement (AMBER) is a very important molecular mechanics (MM) force field in the investigation of large molecules of biological nature; in this case microcystins. All theoretical calculations on the optimization of microcystins converged and therefore, the optimized molecular structures presented in this work are both acceptable and accurate.

Table 4.4: Geometry optimization parameters

Microcystin	Computation method	Force Field	Optimization Energy (kcal/mol)	Iteration Points	Converged	Gradient *
YR	MM	AMBER	31.103	3823	Yes	0.96
RR	MM	AMBER	50.18	2845	Yes	0.98
LR	MM	AMBER	80.75	1741	Yes	0.99

MM – Molecular Mechanics

Gradient* used was the Polak –Ribiere algorithm

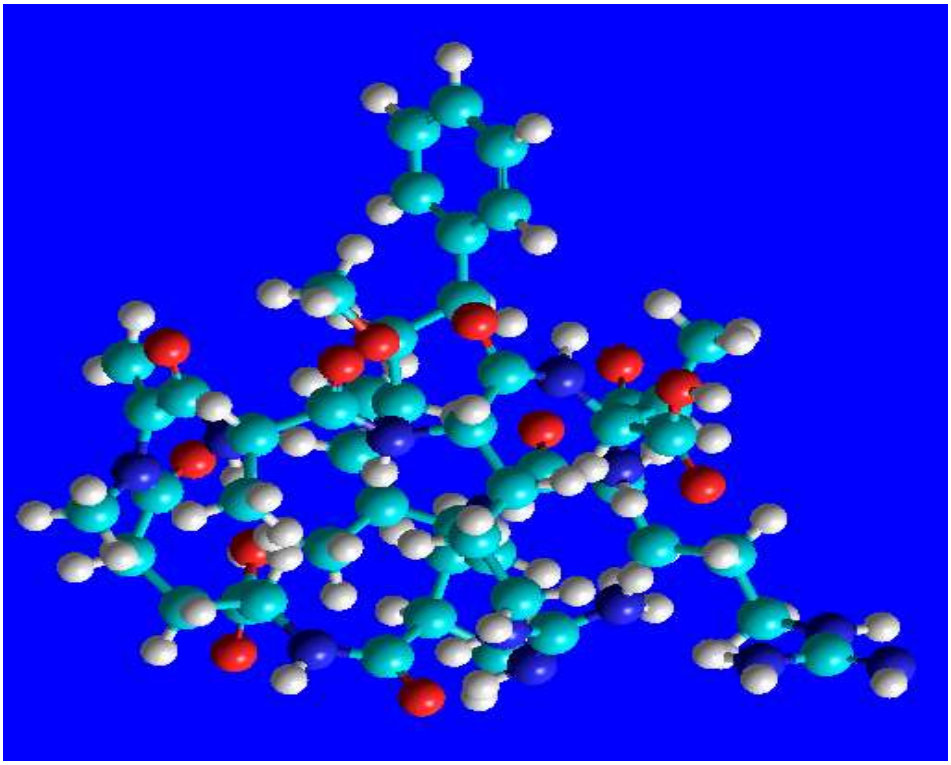

Figure 4.11: Optimized structure of microcystin–RR

This study revealed that cyanobacteria in L. Naivasha produce microcystins. MC-LR, MC-YR and MC-RR were found in varying amounts. Worldwide, more than 90 structural variants of microcystins have been isolated (Rinehart *et al.*, 1994). Estimated as microcystin concentration per litre of lake water, the concentrations of microcystins-LR equivalents in L. Naivasha range from 0.06 to 1.3 µg MC equ L^{-1} lake water. These values are within the safe level of Health Canada of 1.5 µg MC equ L^{-1} (Miles *et al.*, 2013).

Although the levels are acceptable, the situation may change especially after Kenya changed its status from low-income economy to middle-income economy. There is expected to be accelerated use of fertilizers within Naivasha area for horticultural production and this may lead to growth of algal blooms. This will raise the concentrations to levels that can have serious ecological impact on aquatic food web of the lake and cause serious health problems.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

The physico-chemical parameters were favourable for the existence of microcystin in L. Naivasha. This was confirmed by the detection of MC-LR by use of UV-Vis. The colour of the water samples ranged between (520 ±91) ptco, signaling low transparency throughout the period. The conductivity was (234 ± 0.8) µs/cm and the total dissolved solids were found to be (1035 ± 12) mg/L. The pH of the lake remained slightly alkaline, averaging about 7.6 for the period under study. Due to the high turbidity (59.0 ± 24) ntu, phytoplankton biomass was low, ranging between (1.5 and 8.2) mg L^{-1}.

The concentrations of microcystin during the dry and wet season were slightly different for the period under study. This was confirmed by the results obtained from HPLC. For instance, MC-LR in the month of January had higher concentration (sharp peak) as compared to wet season (April) which was less concentrated (less intense).

Microcystin-RR was found to be more toxic (log P = -1.90) as compared to MC-YR (log P = -1.61) and MC-LR (log P =-0.31) from HyperChem computational. Log P value, which is usually calculated as the octanol-water partition coefficient, is a measure of the toxicity index of a molecule. In this study, microcystin-RR is approximately 230 times more soluble in water than in octanol. Thus, microcystin-RR was found to be highly hydrophilic and may react with polar biological tissues, causing extensive cell injury, oxidative stress, and ultimately cancer.

5.2 Recommendations

To minimize harmful effects on consumers, we recommend that the water of L. Naivasha should be screened regularly for any cyanobacterial toxins that may pose a health hazard to the consumers. Further research also needs to be carried out in order to provide an up to date data on microcystins at L. Naivasha and other water bodies in Kenya. For recreational purpose in L. Naivasha, the exposure to Cyanotoxins (microcystins) remains a public health concern. Therefore, further studies of cyanobacterial toxin exposure should include improved measures of exposure and improved assessments of adverse health outcomes. It is also important to investigate potential susceptible populations that may respond more vigorously to these exposures such as persons with underlying respiratory diseases.

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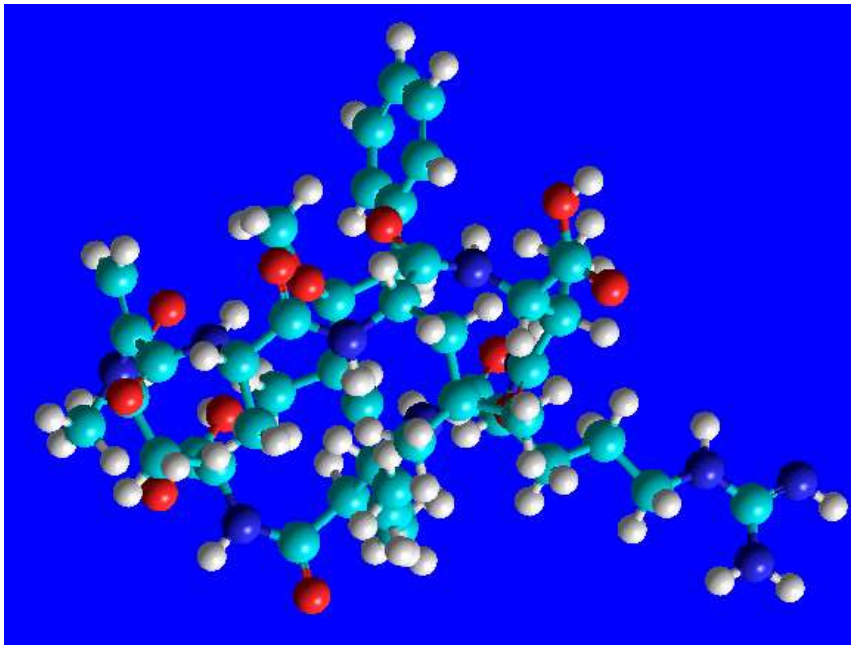
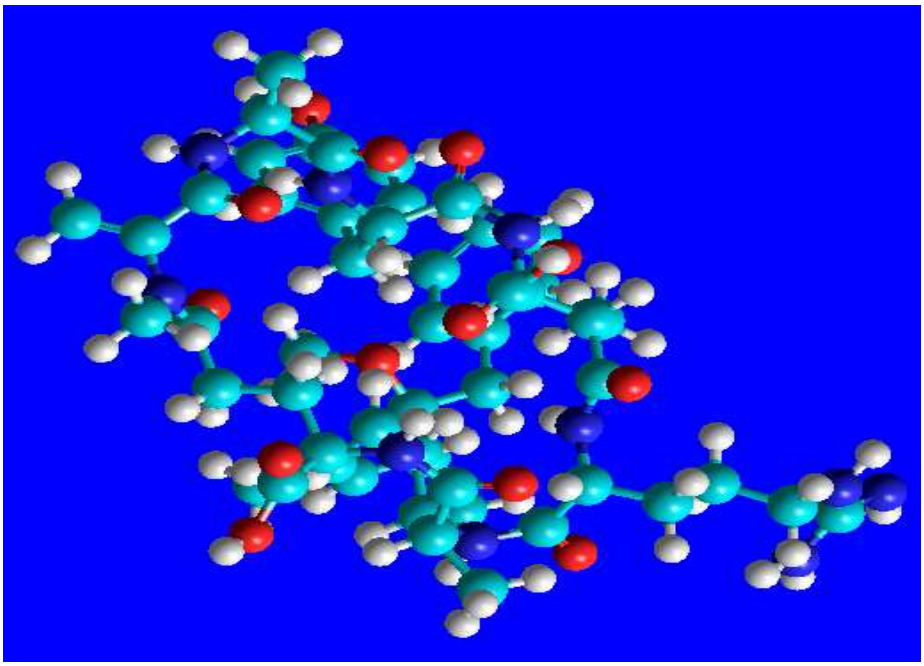
APPENDICES

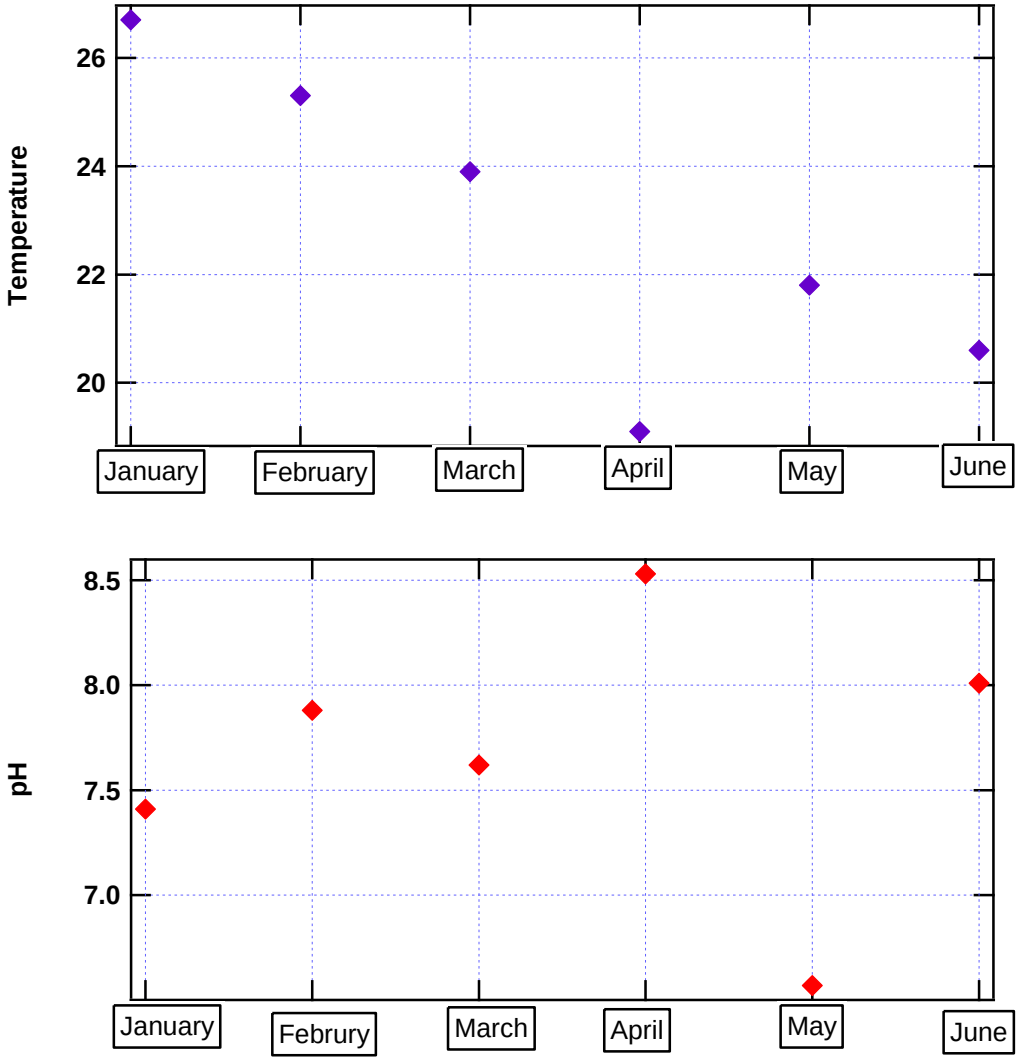


Appendix 1: Optimized structure of MC-LR

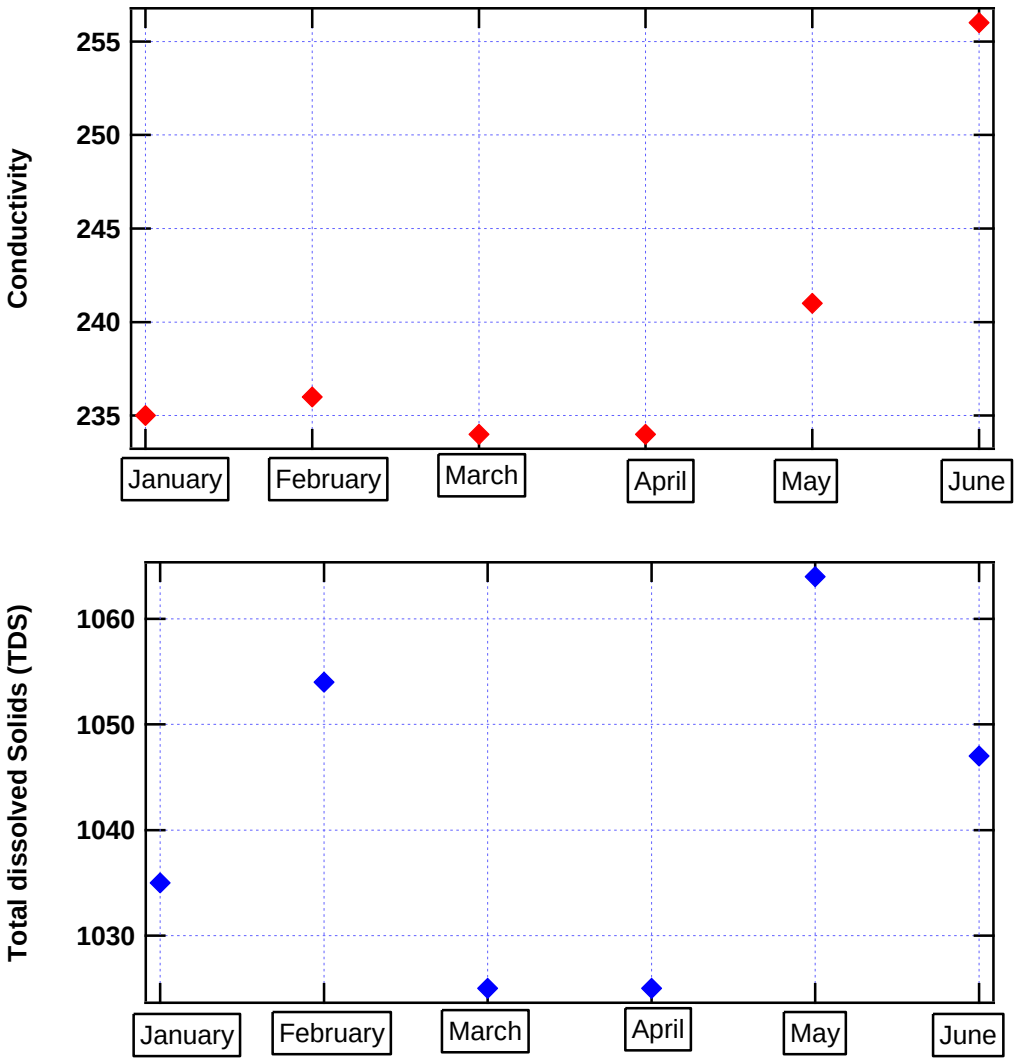


Appendix 2: Optimized structure of MC-YR

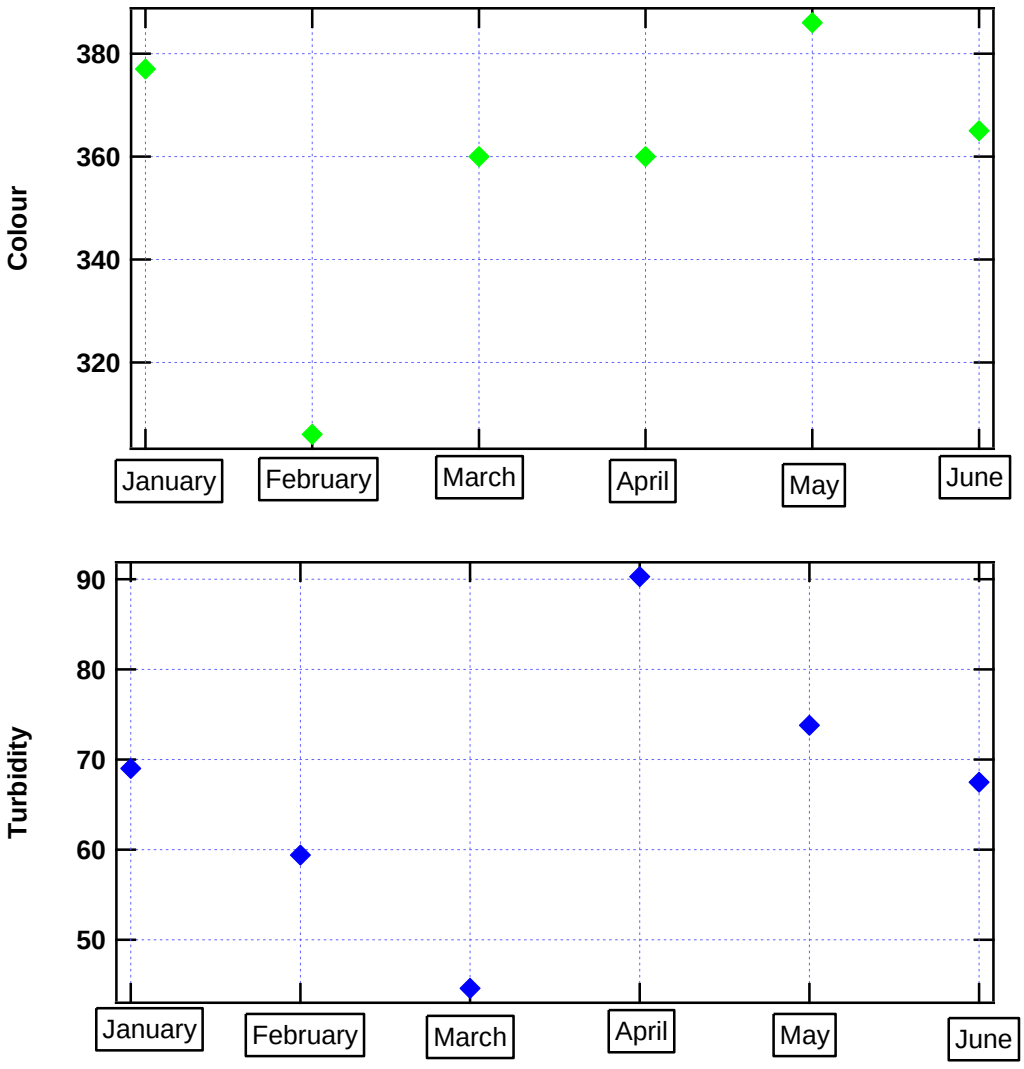
Appendix 3A: Physiochemical graphs for period under study



Appendix 3B: Plots for temperature and pH



Appendix 3C: Plots for turbidity and colour



Appendix 3D: Plots for conductivity and TDS