**UNIVERSITY OF EMBU**

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**MASTERS THESIS**

**2020**

# Bioprospecting For Cyanophages With Biocontrol Potential Against Toxin Producing Cyanobacteria In Lake Magadi, Kenya

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**A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE AWARD OF A DEGREE OF MASTER OF SCIENCE IN APPLIED MICROBIOLOGY OF THE UNIVERSITY OF EMBU**

**OCTOBER, 2020**

## DECLARATION

This thesis is my original work and has not been presented for a degree or any other award.

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## DEDICATION

I wish to dedicate this work to my beloved family, supervisors and colleagues for their love, inspiration, endless and timely moral support which was a propelling aid during my research. Indeed, without their encouragement this research journey would not have been possible.

## ACKNOWLEDGEMENT

My immense gratitude goes to the Almighty God for enabling me achieve this dream. Sincere gratitude goes to the University of Embu for offering me a scholarship to pursue a Master degree in Applied Microbiology. Very special thanks go to my supervisors Prof. Romano and Dr Juliah for their great support, guidance and reviews during entire research period; I will be forever grateful. Lastly, I would like to thank the staff in the Department of Biological Sciences, all my colleagues and friends for their assistance, encouragement and guidance. May the Almighty God bless each one of you abundantly.

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## LIST OF ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| BLAST | Basic Local Alignment Search Tool |
| ºC | Degrees Celsius |
| $Ca^{2+}$ | Calcium |
| $Mg^{2+}$ | Magnesium |
| IUCN | International Union for Conservation of Nature |
| TN | Total Nitrogen |
| TP | Total Phosphorous |
| TMDL | Total Maximum Daily Load |
| NCBI | National Centre for Biotechnology Information |
| NRPS | Non-Ribosomal Peptide Synthesis |
| Mg | Milligram |
| PCR | Polymerase Chain Reaction |
| g/l | Grams per litre |
| SDS | Sodium dodecyl sulphate |
| Taq | *Thermus aquaticus* |
| Ml | Millilitre |
| CYA | Cyanobacteria |
| µm | Microlitre |
| MEGA | Molecular Evolutionary Genetics Analysis |
| RPM | Revolutions per Minute |

## LIST OF SYMBOLS

| | |
|---|---|
| > | Greater than |
| < | Less than |
| + | Positive |
| _ | Negative |
| ± | Plus or minus |
| % | Percentage |

## ABSTRACT

Cyanobacteria are a phylum of blue-green algae in the domain bacteria that get their energy from sun light through photosynthesis. In anaerobic environments, photoautotrophic blue green algae derive electrons by reduction of sulphur. In the Kenyan soda lakes, cyanobacteria serve as the sole source of food for Lesser Flamingos. Also, detection of these blue green algae blooms, is evident in these environments. Due to this reason, it has been hypothesized that the blooms may lead to production of secondary metabolites referred to as cyanotoxins that could be the cause for mass mortalities of Lesser Flamingos in these lakes including Lake Magadi. Viruses are key in controlling microbial populations in any ecosystem and thus they could act as a biocontrol measure for toxin producing cyanobacteria. Therefore, this study aimed to isolate cyanobacteria, amplify for toxin production genes and screen for cyanophages that can control these toxin producing cyanobacteria. The study site was Lake Magadi in the Rift Valley which has highly alkaline pH (8-12). Water samples were collected from different points around Lake Magadi. Physiochemical variables such as pH, salinity and temperature were measured for each of the sample on site. Two techniques for the isolation of cyanobacteria were used: isolation by spread plating on agar and dilution to extinction technique. Five types of media were used: Blue green medium (BG11), Synechococcus medium (A+), artificial lake water medium (ALW), artificial sea water medium (ASW) and enriched lake water medium (M). For solid cultures, spread plating was done followed by subsequent sub-culturing to acquire axenic cultures. Dilution to extinction technique involved subsequent dilution of the enriched sample until an axenic culture was obtained. These isolates grew at conditions that are unique to haloalkaliphiles. The cyanobacteria were subjected to morphological and molecular identification. Molecular identification involved partial sequencing of 16Sr RNA gene. Potential of the isolates to produce cyanotoxins was assessed by amplification of their respective genes using toxin specific primers. Sequence analysis indicated that all 11 isolates were affiliated to *Cyanobacterium* Spp. Genera represented include *Spirulina, Synechococcus, Oscillatoria* and *Anabaenopsis.* PCR amplification showed that all the isolates had different genes for toxin production. Phage lysis was observed in 10 of the isolates indicating that they were hosts for the phages. This study provides an insight into the uncultured cyanobacterial species from extreme environments and their cyanophages. The recovered isolates are a useful resource in understanding the taxonomy, phylogeny and diversity of cyanobacteria as well as their cyanophages. The cyanophages can be useful in biotechnology application for biocontrol of toxin producing cyanophages.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background of the study

Cyanobacteria are a phylum of blue-green algae in the domain bacteria that get their energy from sun light by photosynthesis. Previous study has reported that these cyanobacteria are make majority among the gram negative having 56 genera. (Hossain *et al.*, 2016). The current number of cyanobacterial species is subject to debate, with estimates ranging from 2783 (Nabout *et al*., 2013) to 4484 (Ruggiero *et al*., 2015). It is still debatable that the genera could be having as many as 8000 species (Guiry, 2012). Fresh water lakes have high diversity and distribution of cyanobacteria as compared to lakes that have high salinity levels. In addition, the scarcity in their diversity is marked by visibility and mass development of this cyanobacteria such as *Arthrospira, Spirulina* and *Anabaena* species whereas marine ecosystem has abundance of *Prochlorococcus* and *Synechococcus* species (Dadheech *et al*., 2009)

Distribution of cyanobacteria differs with environmental and physiochemical conditions (Flombaum *et al*., 2013). Cyanobacteria which are extremophiles are known to inhabit exclusive harsh environments which are characterized boiling water deep rift vents sea, very saline environments, polar glacier together with volcanic craters (Sompong et al., 2005). They are able to survive as a result of their genetic adaptability that are known to offer protection to them (Dalmaso *et al.*, 2015). The ability of the thermophilic microorganism to thrive in those environment results from protein configurations and their nucleic acid material that do not rely on hydrogen bonds to protect their spatial structure (Stetter, 1999). Lake Magadi is among the five alkaline-saline lakes that occupy the floor of Kenyan Rift Valley ( Ndetei and Muhandiki, 2005; Rees *et al*., 2004). Environmental factors are responsible for the formation of this lake and are characterized by presence of $Ca^{2+}$ and $Mg^{2+}$ ions, that are insoluble as carbonate minerals in those alkaline conditions (Grant and Mwatha, 1989).

Conspicuous characteristics of all the alkaline-saline lakes is the large numbers seen in Lesser Flamingos that graze on planktonic cyanobacterium *Arthrospira fusiformis (*Lugomela *et al.*, 2006). A small number of Lesser Flamingos results from lakes heavily inhabited by non-*Arthrospira fusiformis* species (Lugomela *et al.*, 2006). Lesser Flamingos heavily feed on of microscopic cyanobacteria together with the benthic diatoms (Krienitz and Kotut, 2010). Cyanobacteria play a big role on earth mainly in primary productivity through photosynthesis thus are considered to be the most abundant in the world (Mazard *et al*. 2016). Many Cyanobacterial species (e.g. *Oscillatoria* and *Arthrospira*) are capable of photosynthesizing under both aerobic and anaerobic conditions, unlike the eukaryotic algae that can only photosynthesize under aerobic conditions (photoaerobic) (Nweze, 2009).

Most of the cyanobacteria are known to be aerobic photoautotrophs. They need water, carbon dioxide, inorganic substances and light for their survival (Issa *et al*., 2014). They obtain energy through photosynthesis. However, it is also evident that some can still survive in dark environments for long periods. (Mur, 1999). In addition, some cyanobacteria are known to be histotrophic (Fay, 1965). Whenever sulphur is present under anaerobic conditions, the photoautotrophic blue green algae derive electrons by reduction of Sulphur (Thajuddin and Subramanian, 2005).

Dissolved carbon dioxide reacts with hydrogen sulphide and chlorophyll when light is available thus producing sugar, water and Sulphur (Nweze, 2009). For this reason, Sulphur rich ecosystems contain high numbers of Cyanophyceae. Also, they are capable of taking up ammonia through diffusion passively or ammonium ion by specific uptake system and fixing atmospheric nitrogen, using nitrogenase enzyme nitrogen in molecular form is converted to ammonia in the presence of hydrogen (Mishra and Pabbi, 2004). Consequently, unlike most plants they are not limited by nitrogen. These qualities have been employed extensively in Asia to enhance agricultural production (Thajuddin and Subramanian, 2005).

In the soda lakes the most studied functions of cyanobacteria are: their ecological role with other phytoplanktons, food for lesser flamingos as mentioned above (Krienitz and Kotut, 2010) and in the production of secondary metabolites, or bioactive compounds (chemically, mostly alkaloids and oligopeptides), which are

toxic to the environment and humans (collectively referred to as cyanotoxins (Pearson *et al.*, 2016).

The avifauna, Lesser Flamingos, are very important when it comes to tourist attraction hence major generator of revenue for local and national economy. However, episodes of these Lesser Flamingos die-offs under mysterious circumstances are reported in Lakes Bogoria as well as Nakuru (Kihwele *et al.*, 2014; Lugomela *et al*., 2006; Straubinger-Gansberger *et al*., 2014). The mortality has become a regular phenomenon hence negatively impacting population size of the Lesser Flamingos and indirectly affecting the tourism sector (Wanjiru, 2001).

The Lesser Flamingos has since been categorized as "Near Threatened Species" under /2012/ IUCN list of the most threatened Species (IUCN, 2012). Cyanobacterial toxins have been proposed as a possible explanation for mass mortalities of Lesser Flamingos acting in synergism with the rest of the stress factors that are known to stem from human activities (Krienitz *et al*., 2003; Lugomela *et al.*, 2006). This has been confirmed by presence of toxic strains of cyanobacteria and cyanotoxins in studies done on various lakes such as Nakuru and Bogoria both in Kenya and Lakes Manyara together with Big Momella in Tanzania (Ballot *et al*., 2004; Lugomela *et al.*, 2006). Potential sources of toxins are cyanobacterial mats dominated by toxic strains of cyanobacteria: *Oscillatoriales* and *Synechococcus*, toxic strains of *Arthrospira fusiformis* and populations of *Anabaena* and *Anabaenopsis* invading phytoplankton community of the Lakes (Krienitz *et al*., 2003). The mysterious Lesser Flamingos deaths, which have taken place in Kenya within the saline Rift Valley lakes, may be associated with such changes in phytoplankton composition towards toxin producing cyanobacteria.

Development of control measures towards toxic cyanobacteria is therefore of great importance. This would reduce Lesser Flamingos mortalities and eventually economic losses. The use of chemical methods is expensive, non-specific, toxic to other biota and may accumulate to detrimental concentrations that may result to long-term damage to lake bottom ecology (Sigee *et al*., 1999). Biological control is considered to have relatively low environmental impact, specific and to be free of unintended side effects (Head, 1998; Sigee *et al*., 1999). Viruses are known to regulate bloom dynamics of the most abundant marine primary producers (Fuhrman,

1999). Waterbury *et al*. (1993) reported that increase in the number of cyanophages led to a decrease in the abundance of *M. aeruginosa* in the natural freshwater environment. Tucker and Pollard. (2005), also observed two types of podovirus-like particles that were able to inhibit the growth of *M. aeruginosa* in the natural lakes water sampled during an *M. aeruginosa* bloom.

Cyanophages (viruses that infect cyanobacteria) indigenous to the lake environment offer an environmentally acceptable strategy to biologically control toxic strains of cyanobacteria. Cyanophages replicate rapidly, are relatively specific to target toxic cyanobacteria, are self-limiting, non-toxic to man, animals and other microorganisms in the food chain (Tucker and Pollard, 2005). This makes them attractive as an alternative biological means of minimizing effects of toxic cyanobacteria. Majority of these viruses in the natural waters are known to be responsible for reducing the number of these infectious cyanobacteria in the natural waters. (Proctor and Fuhrman, 1990). Some cyanophages strains that infect marine cyanobacterial groups have been isolated and research on intensely (Sullivan *et al*., 2003). In the past, cyanobacterial analysis has relied on microscopic examinations. This has led to the identification of taxa and it is dependent on the experience the researcher has. The ability to extract its DNA together with the culture based techniques from water samples have facilitated the identification of cyanobacteria in water samples (Rees *et al*., 2004).

Ability to characterize the cyanobacterial through partial sequencing of the 16S rRNA gene of the cyanobacterial is very suitable when it comes to the identification of a particular habitat (Innok *et al.*, 2005). Cyanobacterial studies have been done in other saline lakes such as Lake Bogoria, Elementaita, Nakuru, Oloiden, Turkana Sonnachi, (in Kenya) Manyara and Big Momella (in Tanzania) (Ballot *et al.*, 2004; Dadheech *et al*., 2009). Nevertheless, few studies have tried to reveal the diversity of the cyanobacteria in Lake Magadi. Thus, this study was to isolate and characterize cyanobacteria phylotypes and their cyanophages from Lake Magadi. Cyanophages that were isolated were tested for lytic activity on the toxin producing strains of cyanobacteria as a biocontrol measure.

## 1.2 Statement of the problem

Cyanobacteria blooms have been proven to negatively impact the environment due to the odors, water discoloration, and more alarming the toxic they release in the waters. Since early times they have been a cause for environmental concern because of their negative impact through the release of odors, water discoloration, and more dangerously through the release of toxic compounds. Also, algal secondary metabolites are reported to be to be toxic and associated with human illness associated, high bird mortality rate together with the death of fish (Dolah, 2000). These toxins are released by some of the algae species described above. Toxic algal poisoning in most cases does not occur unless there is a heavy bloom in the water (Millard, 2009). Lesser Flamingos die-offs in unexplained circumstances have been reported in Rift valley saline lakes (Kihwele *et al*., 2014; Lugomela *et al*., 2006; Straubinger-Gansberger *et al*., 2014).

Toxin producing species of cyanobacteria include: *Oscillatoriales, Synechococcus* and *Anabaena*. They mainly produce microcystin hepatoxins and anatoxin-a neurotoxin (Bernard *et al*., 2016). This mortality is of concern as it has become a regular phenomenon hence negatively impacting population size of the Lesser Flamingos and indirectly affecting the tourism sector (Wanjiru, 2001). Blame for their mortality has been put on natural factors such environmental, industrial, predation and human factors such as pollution. Cyanobacterial toxins however have raised major suspicion as possible explanation for mass mortalities of Lesser Flamingos acting in conjunction with other stress factors those results from human activities (Bernard *et al*., 2016).

Toxin producing cyanobacteria have been controlled by use of chemical methods for the last decade. The use of chemical methods is expensive, non-specific, toxic to other biota and may accumulate to detrimental concentrations that may result to long-term destruction of the lake ecology (Jančula and Marsa'lek, 2011). Cyanophages have provided alternative biocontrol agents of cyanobacteria. Therefore, they can be applied in the regulation of the toxin producing cyanobacteria. Currently no studies so far have been done on cyanobacteria and their cyanophages in Lake Magadi.

## 1.3 Justification

Lesser Flamingos, are of the most chief tourist attractions hence major generator of revenue for local and national economy thus require management-related research (Sileo *et al*., 1979). The mysterious Lesser Flamingos deaths, witnessed in Kenya in the saline Rift Valley lakes, could be the reason for the change seen in the phytoplankton composition towards toxin production. Development of a biocontrol measure for toxic cyanobacteria is of great importance, to minimize if not to eradicate potential adverse effects of toxic cyanobacteria on Lesser Flamingos *Phoeniconaias minor* and mitigate mortalities which result to economic losses. Biological control is considered to have relatively low environmental impact, specific and to be free of unintended side effects ( Romeis *et al*.,2006) to which this study intended to develop, using cyanophages to control intoxicating cyanobacteria (Head, 1998). Benefits of cyanophages biocontrol include rapid replication, they are relatively specific to target toxic cyanobacteria, and they are self-limiting, non-toxic to man, animals and other microorganisms in the food chain (Tucker and Pollard, 2005).

## 1.4 Hypothesis

Lake Magadi harbors toxin producing cyanobacteria that can serve as hosts for cyanophages.

## 1.5 Objectives

### 1.5.1 General objective

To isolate and characterize toxin producing cyanobacteria, detect their cyanotoxin genes and their cyanophages from Lake Magadi.

### 1.5.2 Specific objectives

1. To isolate and characterize cyanobacteria from Lake Magadi.
2. To detect genes encoding for cyanotoxins from the isolated cyanobacteria.
3. To isolate cyanophages from the water samples using the isolated cyanobacteria as hosts.
4. To test lytic activity of the cyanophages against toxin producing strains of cyanobacteria.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Cyanobacterial life in the extreme environment

Extremophiles are those organisms known to thrive in environments that are extremes. (Banciu *et al*., 2015). Extreme environments are characterized by life conditions that are not favorable to living organisms in reference to their physiochemical properties which include extreme pH, temperature, pressure, nutrient and saline concentration. Extreme physicochemical parameters are acidity (pH < 5), low alkalinity (pH > 9), hyper salinity (salinity > 35 %), pressure (> 0.1 MPa), very high temperature (> 40 °C), very low temperature (< 5 °C), water stress (aw < 0.80), and environments with high radiation environments (Nakagawa *et al*., 2008). Environments above pH 9 such as hydrothermal springs and soda lakes (e.g., Lake Magadi, Lake Bogoria) are known as alkaline environments due to high salts level. Organisms that thrive in alkaline (high) pH values > pH 9 are known as alkaliphiles (Kato *et al*., 1998; Krienitz *et al*., 2003).

Extremophiles are reported to thrive in the extreme environment and this is demonstrated by their ability to grow and reproduce. The haloalkaline soda lakes in Kenya, have demonstrated high productivity levels (Kambura *et al*., 2016) due to very high temperatures and lights not forgetting the phosphates levels $CO_2$, in the carbonate rich waters. In addition, the salinity level is reported to be as high 30 % to saturation in Lake Magadi, while the pH, lies between 9 and 11.5. Solute levels in the Lake Magadi arise from the alkaline springs which have temperature as high as 33 °C to 86 °C (Kambura *et al*., 2016). Soda lakes and soda deserts are known to be among the stable alkaline environments that are known to occur naturally globally. These environments are characterized by high concentrations of sodium carbonate. A characteristic that differentiate lake water from other lake is the low levels of $Mg^{2+}$ and the carbonate which are known to provide buffering capacity to the lake water (Chamberlain *et al*., 2000; Regina, 2014).

One factor that is known to be very important in the formation of soda lakes are the low levels of f $Ca^{2+}$ and $Mg^{2+}$, which are crucial so that ground waters having

hydrogen carbonate released if the molecular levels of hydrogen carbonates highly exceed those of Ca2+ and Mg2+ (Chamberlain *et al*., 2000). Altogether, an upsurge in total phosphorus (TP) results to fast rise of the potentiality for cyanobacteria dominance from 30 to 100 μg $L^{-1}$ (Paerl, 1988). Bloom incidences positively correlates with total nitrates (TN) and total phosphate (TP) in wetlands and these correlative relationships have been applied in the improvement of total maximum daily load (TMDL) (Paerl, 1988).

Cyanobacteria blooms exhibit a variety of temporal diminuendos. Some water bodies have seasonal blooms starting in summer through to autumn; others have permanent blooms in all seasons, and others having blooms occurring at extreme peaks and crashing within just days or weeks (Havens, 2008). The temporal characteristic of a cyanobacterial bloom in a specific environment is dependent on the magnitude at which different factors of environmental effect bloom dynamics (Havens, 2008). Cyanobacteria include several species capable of producing toxins. A toxic cyanobacterial bloom was first stated in 1878 (Phillips *et al*., 1985). The toxic property was found after mortality of domesticated animals from consuming water containing bloom. Domestic and wild animal deaths from hepatotoxic and neurotoxic cyanobacterial blooms have been successively verified in several countries (Bowling and Baker, 1996; Carrick *et al*., 1994). Worldwide, prolific growth of certain cyanobacteria (algal blooms) and mats are progressively acknowledged as potential sources of cyanotoxins (Paerl, 2012; Sivonen, 1996).

Cyanotoxins have been accountable for the recurrent cattle deaths in South Africa (Carrick *et al*., 1994). Whereas in Australia, cyanobacteria blooms which occurred in Barwon-Darling River resulted in deaths of many livestock (Bowling and Baker, 1996). United Kingdom on the other hand, Microcystis blooms produce toxins that lead to infection or death of domestic dogs and grazing animals upon ingestion (Harper, 1992). Productive growth of *Microcystis aeruginosa* produces toxins (hepatoxins) that result in liver diseases in water birds (Krienitz *et al*., 2003; Tucker *et al*., 2005). The algal blooms, particularly blue-greens, discharge toxicants like hepatotoxins and lipopolysacharide endotoxins in the water. One of the most common hepatotoxins is microcystin, which is a recurring peptide containing of seven amino acids (Harper, 1992). When sufficiently ingested, these toxins result to

death of domestic animals and wildlife including human in some cases (Harper, 1992; Funari *et al*., 2008).

The high Lesser Flamingos deaths are from their habits of feeding that habitually target cyanobacteria floating on the surface (Krienitz *et al*., 2003). Increased nutrient levels, or eutrophication, can cause massive blooms of cyanobacteria. Since toxin-forming species often predominate in thick surface blooms and are presumed to have potential health risk to humans too through household and recreational water uses. Toxic cyanobacteria could be capable of contaminating raw water sources in some cases and some reports propose that their toxins could be a threat to health through domestic water use (Funari *et al*., 2008). Cyanotoxins poisoning has led to the witnessed deaths of Lesser Flamingos in the volcanic lakes of Kenya (Ballot *et al*., 2004). It was reported that cyanobacteria in the hot springs of Lake Bogoria, Kenya, encompassing *Phormidium terebriformis, Oscillatoria willei, Spirulina subsalsa* and *Synechococcus bigranulatus* produced hepatotoxins microcystin-LR, -RR, -LF and -YR, and the neurotoxin anatoxin-a (Krienitz *et al*., 2003).

The authors of the later report also detected these toxins in contents of the stomach, intestine and fecal matter of the dead Lesser Flamingos in Lake Bogoria. They decided that cyanobacterial toxins poisoning resulted from ingesting the detached cyanobacterial cells from mats, through drinking fresh or brackish water and cleaning their feathers in the hot springs. Furthermore, Ballot *et al*. (2004), observed that microcystins-YR and anatoxin-a in isolated *Arthrospira fusiformis* detected in Lake Bogoria and *A. fusiformis* in Lake Nakuru produced anatoxin-a (Lugomela *et al*., 2006). They found incidental proof that high concentrations of *A. fusiformis* were poisonous to the Lesser Flamingo in Lake Big Momella which is a saline lake in Tanzania.

From the above studies, they dissected three fresh remains of Flamingos for their gut contents (Lugomela *et al*., 2006). Partially digested fragments of the cyanobacterium *A. fusiformis* were the solitary food element that could be microscopically recognized in the guts (gizzards) of the carcasses. The birds' excreta also contained recognizable partly digested fragments of *A. fusiformis. A. fusiformis* is the natural most vital food ingredient of the Lesser Flamingos within the soda lakes of Eastern Africa and has been regarded as non-toxic species for a long time.

However, from studies done by Ballot *et al*. (2004), it is now known that *A. fusiformis* isolated from Lake Bogoria yields both microcystins-YR and anatoxin-a, and those from Lake Nakuru produce only anatoxin and isolates from Lake Elementaita produces zero toxins.

## 2.2 Genetic basis of cyanotoxin biosynthesis

### 2.2.1 Microcystin biosynthesis

In the last twenty years, knowledge on genetic methods entailing the distribution of genes involved in the toxins production within the phylum cyanobacteria have improved significantly. Production of Microcystins by both planktonic freshwater and saline water genera of *Microcystis, Planktothrix, Dolichospermum, Nostoc,* and *Fischerella* (Peacock *et al*., 2018). Moreover, many freshwater and brackish water genera, that is; *Arthrospira, Oscillatoria, Phormidium, Pseudanabaena, Synechococcus,* and *Synechocystis*) are reported to produce microcystins (Bernard *et al*., 2016). Microcystins are recognized for their toxicity as they impede eukaryotic protein phosphatases 1 and 2A occasioning in the hyper phosphorylation and synthesis of the structural protein skeleton (Le Manach *et al*., 2016).

Under sub lethal exposure environments, they are considered tumor promoters (Zhou *et al*., 2002). Considerable structural variation of the microcystin molecule has been cased, commonly in positions 2, 4, and 7 of the molecule, and a great amount of structural variations have been described either from field samples or from isolated strains (Spoof & Catherine, 2016). Microcystin biosynthesis is catalyzed by non-ribosomal peptide synthesis (NRPS) through thio-template mechanism. This biosynthetic pathway has been exhaustively studied in different bacteria and fungi. Currently, six gene clusters from five genera (*Microcystis, Planktothrix, Anabaena, Nodularia, and Fischerella*) accountable for the biosynthesis of microcystin have been sequenced (Fewer *et al*., 2013; Moffitt *et al*., 2004; Shih *et al*., 2013) and in the production of microcystins might be demonstrated by genetic manipulation in *Microcystis* and *Planktothrix* (Fastner *et al*., 2003; Neilan *et al*., 1997).

The whole *mcy* gene cluster includes at least nine genes (ca. 55 kb) containing of PKS, non-ribosomal peptide synthetases (NRPS), and tailoring enzymes (Christiansen *et al*., 2003). McyD, McyE, and McyG are responsible for producing

amino acid Adda and in activating and condensing of D-glutamate. McyA, McyB, and McyC are NRPS and are responsible for incorporating of the other five amino acids in positions 7, 1, 2, 3, and 4 of the microcystin molecule (Tillett *et al*., 2000). Synthesis is perceived to commence with the activation of phenyllactate via the adenylation domain of McyG (Moffit *et al*., 2006) and subsequent extension of the polyketide through McyD and McyE. The polyketide is then condensed with D-glutamate through McyE developing the core of the microcystin peptide. Saxotoxin belong to the class of cyanotoxin and are neurotoxin produced by blue green cyanobacteria *Anabaena circinalalis* (Da Silva *et al*., 2014).

### 2.2.2 Anatoxin biosynthesis

Anatoxin a neurotoxin is reported to have a molecular weight of 165 Da secondary amine (Devlin *et al.,* 1977). Even though anatoxin-a is able to mimic nicotinic acetylcholine receptor, acetyl cholinesterase and other enzymes cannot degrade it. (Carmichael, 1994). homoanatoxin-a and dihydroanatoxin-a are known to be the most common analogs (Paci *et al*., 2014). Many cyanobacteria such as *Hydrocoleum, Microcoleus, Oscillatoria, Cylindrospermum,* and *Dolichospermum* are known to synthesize the anatoxin-a (Bernard *et al*., 2016). Following genome sequencing, gene cluster candidate was observed therefor, resulting to one of the hypothesis of biosynthetic pathways (Mejean *et al*., 2009). The rest of the pathway is thought to be biochemically characterized through in vitro. Studies have reported that peptide synthetases Ana B, C and D take part in the formation of the stature unit that are later extended through the PKSs and *ana*E, F and G. It is observed that in the initial steps, proline is normally activated by the NRPS anaC and the later bonds to AnaD (acyl carrier protein) as Dehydro-Pro (Mejean *et al*., 2009). Oxidation of Dehydro-Pro is propagated by *AnaB* oxidase and later its extension using the three PKS anaE, F and the G after which acetate unit is added.

Methylation is thought to result from the activity of methyltransferase domain while *ana*A is known to encode for a type II thioesterase that is thought to be involved in the chain release (Dittmann *et al*., 2013). The varying genera and their ability to synthesize anatoxin, seems to reveal relatively high similarity thus suggesting their possibility to stem from a common ancestor of (homo) anatoxin-a synthesis. In the *Oscillatoria*, transposase is known to flank the *ana* gene cluster (Calteau *et al*., 2014). Gene organization also reveals the variation in these strains. In that strain

*Anabaena* 37, two of the clusters are located in the *ana*B-G whereas *ana*A, I and J are known to be 7kbp which are separated and transcribed in the opposite position. (Rantala-Ylinen *et al*., 2011). For *ana*B–G and ORF1–*ana*A. The transcription sites were actually recognized upstream of these genes. Lastly, *Cylindrospermum* was reported to have extra gene *ana*J that encodes for reductase that are likely known to be involved in the reduction of the double bonds of the anatoxin-a, leading in dihydroanatoxin-a (Calteau *et al*., 2014).

### 2.3 Molecular markers targeting toxic cyanobacterial *mcy*E and *ana* genes

Organisms produce toxins since they have genes encoding for synthesis of this toxin in that in absence of this genes, these organisms cannot release the toxins. Research undertaken to understand the biology behind the synthesis of microcystin biosynthesis has reported that mutations of these genes can take place that facilitating the conversion of toxin to a non-toxin gene thus making them unable to release the toxin even though it has a full gene cluster of microcystin synthesis. (Christiansen *et al*., 2004). Nevertheless, very few studies have reported on this occurrence. (Ostermaier *et al*., 2010), thus the possibility of detecting this potentially microcystin producing cyanobacteria using molecular methods is very useful in the research field as it will save on time. In the previous studies, the ability to detect toxin producing gens, straight forwardly from the environment, has increases enormously and therefor the protocols to extract the DNA and the subsequent targeted amplification of the genes by the PCR it's available. Therefore, it is very possible amplify specific gene fragments using PCR that encode for production of specific toxin produce

In theory, genes that are involved in toxin production can be detected from single cells. Owing to this high sensitivity, it is possible to detect potentially toxic genotypes in single individuals a long time before a toxic cyanobacterial bloom may occur (Spoof *et al*., 2017). Studies indicate that mcyE gene is mainly responsible for microcystin cyanotoxin while ana gene is responsible for anatoxin-a production (Shams *et al*., 2015). In order to detect potentially toxic cyanobacteria, mcyE gene fragments were amplified by Nonneman *et al,* (2002), thus the specific samples two PCR were run. The (240-300 bp) fragment of the microcystin peptide synthetase gene (mcyE) was revealed following the MicmcyE-R8 and AnamcyE-R12-2.

MicmcyE-R8 and AnamcyE-R12-2 are general primers to detect different potentially toxic strains of cyanobacteria producing microcystins.

## 2.4 Cyanophages with biocontrol potential against toxin producing cyanobacteria

Cyanobacteria have been shown to be infected with cyanophages. In the past, virus and virus-like particles (VLPs) they have been recovered in at least 44 taxa of eukaryotic algae (Van *et al.,* 1999) which include important marine phytoplankton species. Species that have been shown to be in abundance in the saline lakes include: *Arthrospira, Oscillatoria, Phormidium, Pseudanabaena, Synechococcus, Synechocystis, Oscillatoria, Anabaena,* and *Cylindrospermum* (Deepa *et al*., 2010). The first cyanophages reported to infect marine cyanobacteria were first isolated in the early 1990s and it was evident that by lysing their host cells they play a very important role in the microbial loop and revise biogeochemical cycles (Millard, 2009). In addition, the community structure and evolution of cyanobacteria are largely influenced by cyanophages (Bailey *et al*., 2004)

Study conducted demonstrated the role *Synechococcus* played in the isolation of cyanophages and thus the same has applied in the isolation of cyanophages from other cyanobacteria (Millard, 2009). Also viruses are thought to be responsible for regulating bloom dynamics of the most abundant marine primary producers thus acting as a biological control for cyanobacteria.

Characteristics that describe cyanophages include: ability to replicate rapidly, toxic cyanobacteria specificity, self-limiting, not known to be toxic to man and other microorganisms in the food chain (Tucker *et al*., 2005). Thus this makes them very useful when it comes to control measures of toxic cyanophages. Study conducted in the past, demonstrated the presence of viruses in natural waters of which majority are infectious for cyanobacteria (Proctor *et al*., 1990). Cyanophage strains that are known to infect marine cyanobacterial have been isolated and studied (Sullivan *et al*., 2003). Waterbury *et al*., (2003), observed that an increase in cyanophages titers (the numbers of particles forming plaques) always resulted to decrease in the number of *M. aeruginosa* in most of natural freshwater ecosystems. Tucker *et al*., 2005 also identified two types of podovirus that looks like particles that were able to

inhibit the growth of *M. aeruginosa* in the natural lake samples that were collected when *M. aeruginosa* bloom was observed.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Description of study site

Lake Magadi (1°52′S 36°16′E; 1.867°S 36.267°E) is the southernmost lake in Kenya and is located in the Rift Valley. The lake is 660m above sea level forming one of the lowest points in the valley and it acts as a sink for seasonal streams. This environment is highly alkaline having a pH of 8-12. Chloride ions are known to accumulate following the evaporation of water thus saturating it with chloride ions. Salinity ranges between 5% w/v salt concentration to saturation. Majority of the mineral composition is trona (sodium sesquicarbonate) together with halite and either kogarkoite or villaumite.


**Figure 3.1:** A Google map displaying the location of Lake Magadi (Map data ©2011 Google).

### 3.2 Sample collection

Samples were collected from different sites within the lake as part of an ongoing project and physiochemical variables of the Lake such as pH, salinity, temperature and alkalinity measured for each site. Each sample was collected in sterile 500ml containers, placed in a cool box and transported to University of Embu microbiology laboratory within 48 hours for further analysis.

### 3.3 Isolation and morphological characterization of cyanobacteria

Identification of cyanobacteria was carried out using an inverted microscope (Nikon Eclipse T1-SM). Preliminary identification was done to species level in reference with taxonomic guides of (Entach *et al*., 2002; McGregor *et al*., 2001; Crowe *et al*., 2005) followed by molecular identification. Two techniques for the isolation of cyanobacteria were used: Isolation by spread plating on solid media and inoculation into liquid media and dilution to extinction technique.

#### 3.3.1 Isolation by spread plating on solid media

In isolation by spread plating on solidified media using agar, five types of media: BG 11, A+ media, M media, ASW media and ALW media were used for growth of cyanobacteria as summarized in **table 3.1**. Bacterial growth was prevented by adding 1 ml from a stock of streptomycin (50 mg/L in methanol at 75%) to each liter of medium. Vitamin B12 was added to supplement the medium at a concentration of 20mg/L (Kurmayer *et al*, 2017). Species such as *Spirulina* require this vitamin for growth and reproduction. Light was provided continuously by eight 18W white light fluorescent tubes.

Temperature was maintained at 18-30°C for all the isolates that were able to grow either in solid or liquid media. High intensities of irradiation were avoided, since many strains appeared to be light and temperature sensitive (Urmeneta *et al.*, 2003). Bacteriological agar was added in very less quantities (using the ratio of 1g to 100ml) since much of agar contains impurities and this hinders a majority of cyanobacteria from growing on it such as *Synechocystis*. For all media pH was recorded and adjusted to be above 8.0 using Tris buffer.

**Table 3.1:** Media preparation procedures

| Medium | Preparation procedure |
|---|---|
| **BG 11** | The following was measured in g/l: 1000ml of distilled water was added onto; $NaNO_3$ 1.5, $KH_2PO_4$ 1, Trace metals 1ml, $MgSO_4$ 1, $CaCl_2$ 10, Citric acid 10, $C_6H_8FeNO_7$ 10, $Na_2EDTA$ 10 and $Na_2CO_3$ 10 then autoclaved. After cooling, the medium was divided into 100ml and inoculated with samples into each a 250ml conical flask for growth according to (Niemela *et al.*, 1979) |
| **A+ media** | The following was measured in g/l: 1000ml of distilled water was added onto; NaCl 18, KCl 0.6 , $NaNO_3$ 1.0 , $MgSO_4$ 5.0 , $KH_2PO_4$ 0.005, $CaCl_2$ 2.7, NaEDTA tetra 0.03, $FeCl_3$ (0.04 in 1ml of 0.1 N HCl), Tris (10ml of pH 8.2) and 1ml of P1 Metal (1000X). This medium was sterile filtered through 0.2µm membrane into sterile bottle and 1ml of vitamin B12 solution added. (Wu, 2014). |
| **M media** | The following was measured in g/l: 1000ml of distilled water was added onto; $Na_2CO_3$ 100, NaCl 50, KCl 2, $Na_2SO_4$ 1.4, $KNO_3$ 2.5, $K_2HPO_4$ 0.5, $FeCl_3$ 0.0003, EDTA 0.0005, 1 ml of the Ä5 trace, pH was ensured to be 10.5 using Tris buffer then autoclaved. After cooling, the medium was divided into 100ml and inoculated with samples into each a 250ml conical flask for growth according to Mikhodyuk *et al*., (2008). |
| **ASW media** | The following was measured in g/l: 1000ml of distilled was added onto; NaCl 28.13, KCl 0.77, $CaCl_2$ 1.60, $MgCl_2$ 4.80, $NaHCO_3$ 0.11 and $MgSO_4$ 3.50 then autoclaved. After cooling, the medium was divided into 100ml and inoculated with samples into each a 250ml conical flask for growth according to Wyman *et al*., (1985). |
| **ALW media** | 1000ml of lake water was enriched using 1.5g of $NH_4NO_3$ and 0.5g of phosphorous then autoclaved. After cooling, the medium was divided into 100ml and inoculated with samples into each a 250ml conical flask for growth (Mikhodyuk *et al*., 2008). |

### 3.3.2 Isolation by inoculation into liquid media

The procedure in 3.3.1 was also used for all the five types of media but in this case the solidifying agent (agar) was not added. Therefore, the media remained a liquid. In order to acquire pure isolates, 1ml of Lake Sample was inoculated into 250ml liquid media of each of the five media variants for a week, the growing biomass serial diluted in deep wells and regrown subsequently in fresh liquid media until a pure isolate was acquired when viewed under the microscope. Each pure sample in its well was mass grown in 250ml volumetric flask for biomass in the corresponding media it grew in. The pure sample was also plated on its corresponding solid media version. This technique was done as it has been documented by Rippka (1988).

## 3.4 Molecular Characterization of cyanobacteria

### 3.4.1 Cyanobacterial genomic DNA extraction and purification

Cells for total genomic DNA extraction were harvested by centrifuging 1ml of the liquid culture at 14,000 rpm for 5 min. The cell pellets were re-suspended in100µl resuspension buffer (50mM Tris pH 8.5, 50mM EDTA pH 8.0 and 25 % sucrose, 5µl of 20mg/l lysozyme) and the resulting mixture incubated at 37$^{o}$C for 15 minutes. Thereafter, 400µl of lysis buffer (10mM Tris pH 8.5, 5mM EDTA pH 8.0 and 1 % SDS) was added into the tube followed by 10µl of Proteinase K (20mg/l). The mixture was incubated at 65$^{o}$C for 2 hours. Phase separation was achieved by addition of an equal volume of by phenol: chloroform: isoamyl alcohol (25:24:1 v/v). The resultant mixture was centrifuged at 13,200 rpm for 10 minutes at 4 $^{o}$C. Nucleic acids were recovered from the aqueous phase using (pH 5.2) sodium acetate: isopropanol precipitation method. 300 ml of 70% ethanol was used to wash the nucleic acid pellet and subsequently centrifuged at 13,200rpm for 1 minute. Pellets containing the nucleic acids were air dried, suspended in sterile PCR water and stored at -20$^{0}$C until use. The quantity of the extracted DNA was checked by running an aliquot (2µl) on a 1% agarose gel electrophoresis and thereafter visualized under UV light using a transilluminator. DNA was not extracted from the solid cultures but they were directly used in PCR amplification (colony gradient PCR) (Uniwersytet *et al*., 2007).

### 3.4.2 PCR amplification

#### 3.4.2.1 PCR optimization and amplication of 16S rRNA cyanobacterial gene

For PCR optimization and amplication of 16S rRNA cyanobacterial gene, extracted DNA fragment from pure cyanobacterial isolates that corresponded to the 16S rRNA, were amplified using the Sure cycler 8800 PCR machine (Agilent technologies) from the genomic DNA with standard synthetic oligonucleotides used as forward and reverse primers for PCR: CYA106F primer: 5'-AGAGTTTGATCCTGGCTCAG-3' and CYA781R primer: 5'-AAGGAGGTGATCCAGCC-3'. These primers were modified from the original cyanobacteria primers described by Nübel *et al.,* (1997). PCR amplification of the 16S rRNA cyanobacterial genes by the above commercial primers was done as guided by Weller (2011). A total reaction volume of 30µl containing the following reagents: 18.0µl PCR water, 6.0µl polymerase buffer, 1.5µl of each primer, 0.6µl of dNTPs, 0.9µl of DMSO, 0.6µl of $MgCl_2$, 0.3µl *Taq* polymerase and 0.5µl of the DNA was constituted as shown in **table 3.2**.

The reaction mixtures were subjected to the following reaction conditions: initial denaturation for 5 minutes at 95ºC followed by???? repeated for 40 cycles of denaturing at 94ºC for 1 minute, annealing gradient temperature range of 50 - 60ºC for 1 minute, 1 minute for extension at 72ºC followed by final extension step at 72ºC for 5 minutes and lastly holding at 4ºC infinite as shown in **table 3.3**. Amplification products were examined on a 1 % agarose gel in 1× TBE buffer and visualized under UV light after staining using Cyber green and used a 10kb ladder (Nübel *et al.,* 1997). The best annealing temperature for the amplification of the 16S rRNA cyanobacterial gene was observed at 53.5 ºC.

**Table 3.2:** Summary of the PCR mix constituents for the amplification of the 16S rRNA cyanobacterial gene

| **Reagents in the order they were added** | **Quantity** |
|---|---|
| **PCR water** | 18.0µl |
| **Polymerase buffer** | 6.0µl |
| **CYA106F primer** | 1.5µl |
| **CYA781R primer** | 1.5µl |
| **dNTPs** | 0.6µl |
| **DMSO** | 0.9µl |
| **$MgCl_2$** | 0.3µl |
| ***Taq* polymerase** | 0.6µl |
| **DNA template** | 0.5µl |
| **Total reaction volume** | 30µl |

**Table 3.3:** Summary of gradient PCR optimization steps for 16S rRNA cyanobacterial gene

| **Activity steps** | **Period** | **Temperature** |
|---|---|---|
| **Initial denaturation** | 5 minutes | 95$^{o}$C |
| **Denaturation** | 1 minute | 95$^{o}$C |
| **Annealing** | 1 seconds | Range of between 50$^{o}$C - 60$^{o}$ C for the gradient PCR |
| **Extension** | 5 minutes | 72$^{o}$C |
| **Holding** | Infinite | 4$^{o}$C |

Total number of cycles 40.

### 3.4.2.2 Purification of PCR products and sequencing

The amplified fragments were cleaned by mixing 2.5µl of ExoSAP-IT™ (Thermo Fisher Scientific) with 12.5µl of PCR product and incubated for 30 minutes at 37 ºC followed by heating the mixtures for 5 minutes at 95 ºC to stop the reaction. The reaction mixture was then held at 4 ºC for 2 minutes. The resulting PCR products

were sent for sequencing at Inqaba biotech, South Africa and sequenced using the same primers CYA106F and CYA781R.

#### 3.4.3.3 Phylogenetic data analysis

The 16S rRNA gene sequences were edited using Chromas Lite software version 2.1 and matched with the sequences in the public databases using basic local alignment search tool (BLAST) in the National Centre for Biotechnology Information (NCBI) website (htt://www.ncbi.nih.gov). The CLUSTAL W software version 2.1 was used for alignment while Molecular Evolutionary Genetics Analysis (MEGA 7) was used for phylogenetic analyses using neighbor-joining method (Kumar *et al*., 2004; Tamura *et al*., 2011). The evolutionary history distances were determined using Jukes and Cantor 1969 method to deduce and construct a phylogenetic tree. Bootstrap analysis for 1000 replicates was done using MEGA 7 to determine precision of the phylogenetic tree (Tamura *et al*., 2011).

### 3.4.3 PCR detection of toxin genes and identification of toxin producing cyanobacteria

PCR amplifications for total DNA from the axenic cultures was performed by using universal commercial primers targeting the *mcyE, sxt, cyl* and *ana* genes (**table 3.4**) by a Sure cycler 8800 PCR machine (Agilent technologies).

**Table 3.4:** Summary of the cyanobacterial toxin gene primers and their sequences

| Gene | Primers |
|---|---|
| ***mcyE*** | *mcy*E-F2 5′–GAAATTTGTGTAGAAGGTGC–3’ and *mcy*E-12R 5′–CAATCTCGGTATAGCGGC–3′(Hurtado-Alarcón and Polanía-Vorenberg, 2014) |
| ***sxt*** | sxtAf 5′–GCGTACATCCAAGCTGGACTCG–3′ and sxtAr 5′–GTAGTCCAGCTAAGGCACTTGC–3′(Ballot *et al.*, 2010) |
| ***cyl*** | cynsulF 5′–ACTTCTCTCCTTTCCCTATC–3 and cylnamR (5′–GAGTGAAAATGCGTAGAACTTG–3’(Glas *et al.*, 2010) |
| ***ana*** | *ana*C-gen F (5′–TCTGGTATTCAGTCCCCTCTAT–3’and *ana*C-gen R 5′–CCCAATAGCCTGTCATCAA–3’(Török *et al.*, 2017) |

### 3.4.3.1 PCR optimization and amplification for the detection of toxin cyanobacterial genes

For PCR optimization and amplification for the detection of toxin cyanobacterial genes, extracted DNA fragment from pure cyanobacterial isolates were amplified using the Sure cycler 8800 PCR machine (Agilent technologies) from the genomic DNA with standard synthetic oligonucleotides used as forward and reverse primers for PCR as shown in **table 3.4**. The primers selected allowed detection and differentiation of the *mcyE, sxt, cyl* and *ana* toxin cyanobacterial genes. The use of species-specific *mcyE,* sxt, *cyl* and *ana* primers provided a relatively new method for identification of the presence of potentially toxigenic strains of cyanobacteria among the axenic cultures.

A total reaction volume of 30µl containing the following reagents: 18.0µl PCR water, 6.0µl polymerase buffer, 1.5µl of each primer, 0.6µl of dNTPs, 0.9µl of DMSO, 0.6µl of $MgCl_2$, 0.3µl *Taq* polymerase and 0.5µl of the DNA was

constituted as shown in **table 3.5** for each isolate against each primer. The reaction mixtures were subjected to the following reaction conditions repeated for 40 cycles: initial activation of enzyme for 5 minutes at 95 ºC, denaturing at 94 ºC for 1 minute, gradient temperature range of 48-60 ºC for 1 minute, 1 minute for chain extension at 72 ºC followed by final extension step at 72 ºC for 5 minutes as shown in **table 3.6**. Amplification products were run through 1 % agarose gel in 1× TBE buffer and visualized under UV light after staining using Sybr green (Nübel *et al.*, 1997). We stained our DNA using Sybr green and used a 1kb ladder. The best annealing temperature for the amplification of the toxin gene was observed as follows: 48.5 ºC for *mcyE,* and *ana* toxin genes, 50.5 ºC for *cyl* toxin gene and 58 ºC for *sxt* toxin gene. The amplified fragments were prepared for sequencing by purification using a PCR purification kit (Qiagen). The resulting amplicons were sent for sequencing at Inqaba biotech (South Africa) and sequenced using the same primers toxin primers.

**Table 3.5:** Summary of the PCR mix constituents for the amplification of the *mcyE, sxt, cyl* and *ana* toxin cyanobacterial genes

| **Reagents in the order they were added** | **Quantity** |
|---|---|
| **PCR water** | 18.0µl |
| **Polymerase buffer** | 6.0µl |
| **Foward primer** | 1.5µl |
| **Reverse primer** | 1.5µl |
| **dNTPs** | 0.6µl |
| **DMSO** | 0.9µl |
| **$MgCl_2$** | 0.3µl |
| ***Taq* polymerase** | 0.6µl |
| **DNA template** | 0.5µl |
| **Total reaction volume** | 30µl |

This was repeated for each total DNA for each isolate against each toxin gene primer (**table 3.4**).

**Table 3.6:** Summary of gradient PCR optimization steps for *mcyE, sxt, cyl* and *ana* toxin cyanobacterial genes

| Activity steps | Period | Temperature |
|---|---|---|
| **Initial denaturation** | 3 minutes | 95$^{o}$C |
| **Denaturation** | 1 minute | 95$^{o}$C |
| **Annealing** | 45 seconds | Range of between 48 $^{o}$C – 60 $^{o}$C for the gradient PCR |
| **Extension** | 5 minutes | 72$^{o}$C |
| **Holding** | Infinite | 4$^{o}$C |

Total number of cycles 40.

### 3.5 Isolation and purification of cyanophages

Cyanophages for resultant toxic cyanobacteria were isolated from water samples using toxic cyanobacteria strains as hosts as described by Millard, (2009). Water samples were filtered through 0.8µm and 0.5µm polycarbonate membranes filters, 50 mL of each filtrate was inoculated into 900 mL exponentially growing axenic cultures of cyanobacteria. Cultures were left in room temperature under light for 1 week and growth inhibition monitored. Phage supernatant of positive strains showing inhibition were collected by centrifugation at 7,500 rpm for 15 min, filtered through syringe filter (0.45µm) and phage stock stored at 4°C. Filtration was redone to purify and get rid of cyanobacteria cell debris in order to obtain pure cyanophage filtrate for imaging.

### 3.6 Biocontrol test of toxic cyanobacteria by use of cyanophages

#### 3.6.1 Testing for biocontrol in liquid cultures

Biocontrol potential was tested as described by Martin (1984). Toxic cyanobacteria species were mass-cultivated in 250ml conical flasks while exposed to sunlight. 10 milliliters of the cyanophage lysate was transferred aseptically to mid-log host cell culture. Mixing of the algal culture was achieved by mechanical shaking twice a day. Each algal culture sample was monitored every day for cellular growth rates and cell lysis against a control that had no phage filtrate inoculated into it. Cultures that showed lysis were selected for further analysis. Phages were purified from the lysate and sent for imaging at the Georg-August University's institute of Microbiology and Genetics in Germany.

### 3.6.2 Testing for biocontrol in solid cultures

Testing for biocontrol in the cultures that grew on solid media was performed by the use of agar well diffusion method. Initially media containing agar and water only was prepared and poured first into the plates then the five types of media in **table 3.1** were prepared and poured on the cooled initial media. Axenic cyanobacterial cultures were spread plated and left to dry. Wells were then made at the top media, inoculated with 50µl of the cyanophage filtrate and incubated at 30 ºC for 3 days and placed on a lighted bench for another 4 days. The plates were then observed for zones of clearance to denote that phage lysis took place. Phages were purified from the lysate and sent for imaging at the Georg-August University's institute of Microbiology and Genetics in Germany.

### 3.6.3 Imaging of Cyanophages

Negative staining and electron microscopy of the isolated cyanophages was done as follows: Phages were adsorbed onto carbon film followed by staining with 2% (w/v) aqueous uranyl acetate, pH 5.0 and thereafter examined using a TEM 910 transmission electron microscope (Carl Zeiss, Oberkochen). Images were taken at calibrated magnifications and recorded digitally wit Slow-Scan CCD-Camera (ProScan, 1024×1024, Scheuring, Germany) with ITEM-Software (QRAS, 2006).

# CHAPTER FOUR

# RESULTS

## 4.1 Sampling and physiochemical data of the sites

Physiochemical data was acquired for every site to aid in the understanding of how they affect activity and diversity of cyanobacteria in each individual site. Differences in the amount of organic carbon, temperature, pH, trace metals and salinity among the sites could explain the growth variation of the various genera of cyanobacteria at the particular time. This was also done as recommended by Wagacha *et al*., 2014 and their findings also aided in explaining the growth variations. Table 4.1 summarizes the most important environmental parameters of the lake in the different sampling periods (July, August and September) (S- Site of sample collection).

**Table 4.1:** Physiochemical parameters of sampled sites from Lake Magadi during sampling period

| | **S2 Aug** | **S2 Sep** | **S3 July** | **S3 Aug** | **S4 July** | **S4 Aug** |
|---|---|---|---|---|---|---|
| **pH** | 10.7 | 10.7 | 10.5 | 11.3 | 10.5 | 11.1 |
| **Temperature** | 35.2 | 37.2 | 27 | 34.6 | 27 | 33.6 |
| **TDS (g/L)** | 148.2 | 145 | 135.4 | 139.2 | 134.6 | 139.8 |
| **K** | 2860 | 4280 | 2430 | 3300 | 1960 | 3270 |
| **Mg** | < 0.02 | 2.63 | 8.20 | < 0.02 | 6.31 | 0.30 |
| **Na (ppm)** | 96500 | 143000 | 120000 | 121000 | 100000 | 118000 |
| **Ca** | 0.96 | 0.34 | 16.4 | 0.20 | 9.23 | 1.13 |
| **$NO_3$** | < 0.01 | 5.98 | < 0.01 | 0.20 | < 0.01 | < 0.01 |
| **$NO_3N$** | < 0.01 | 1.35 | < 0.01 | 0.045 | < 0.01 | < 0.01 |
| **$CaCO_3$** | 2.48 | 11.6 | 74.6 | 0.58 | 48.9 | 4.05 |

## 4.2 Isolation and purification of cyanobacteria

BG11 medium gave the best results of growth. It was also easy to adjust parameters of salts to achieve desired pH. I obtained 11 axenic cyanobacterial strains (8 from liquid culture and 3 from solid culture) which were morphologically and molecularly characterized. *Chroococcidiopsis* species was the most dominant culturable cyanobacterial species from the investigation while *Oscillatoria* and *Spirulina* were the dominant cyanobacterial species in the crude sample, thriving in all the sites though their population dynamics were affected by seasons. Liquid media (BG11) grew cyanobacterial Phyla *Chroococcales*, *Oscillatoriales*, *Pleurocapsales* and *Nostocales* while solid media (BG11 supplemented with 10grams of agar per litre with no enrichment) had low diversity of only *Chroococcales* and *Pleurocapsales* Phyla. Liquid media had motile isolates (C3, C5, C9 and C10) as compared to the solid media which had none. Cyanobacteria seemed to grow better on liquid media as compared to solid media as they were inhibited by agar from the investigation. These results were also reported by (Urmeneta *et al.*, 2003). *Nostocales* Phylum and *Spirulina* species did not grow on the solid media even after supplementing it with vitamin B12 and streptomycin treatment. Micromanipulation was less successful since most of the cyanobacterial species that grew were highly clustered together and diatoms were a nuisance to obtaining axenic cultures using this particular technique. Liquid and solid cultures are shown in Plates 4.1 and 4.2.

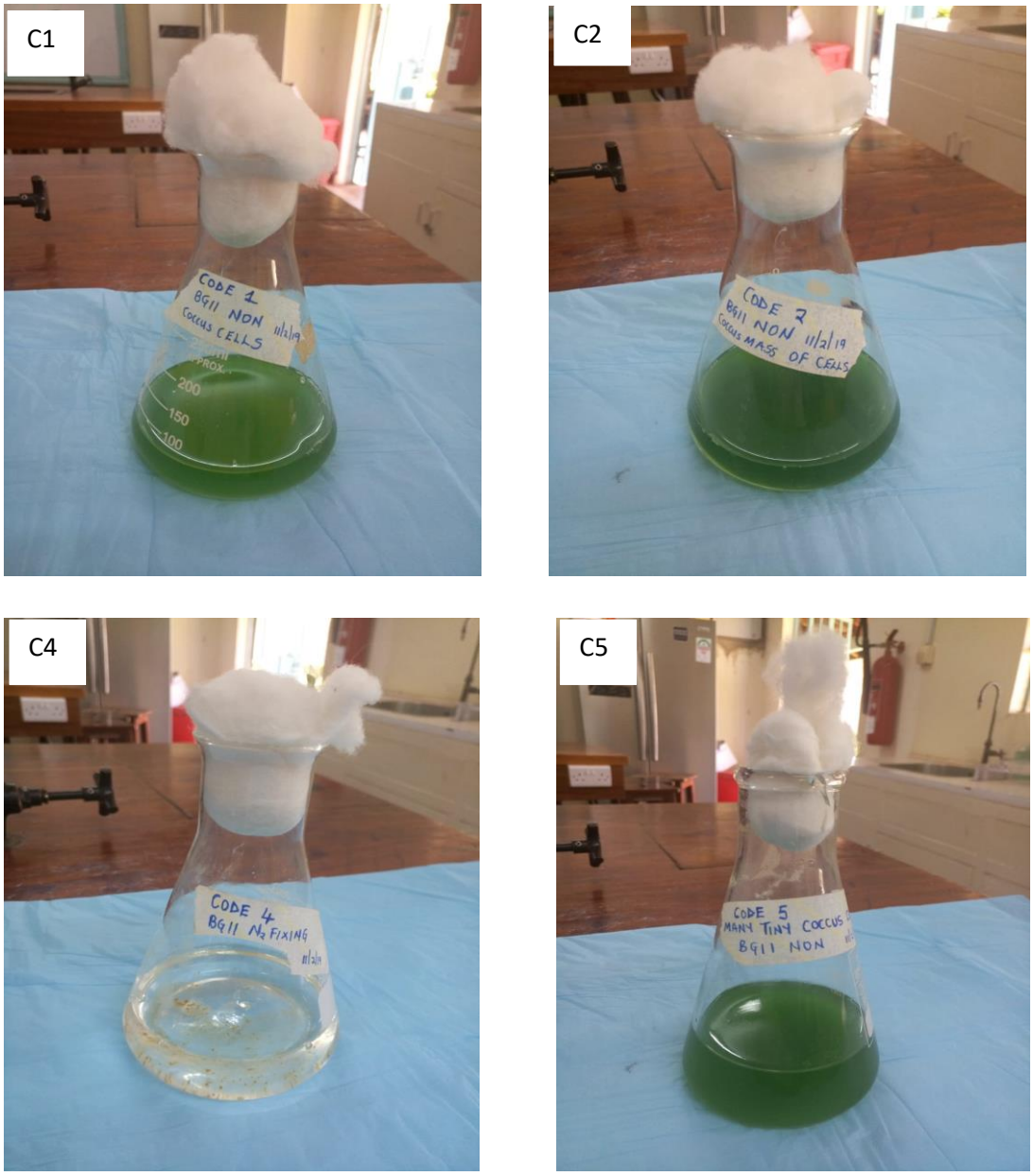


**Plate 4.1**: Axenic cyanobacterial cultures of isolates C1, C2, C4 and C5 respectively growing in liquid media (C1, C2, C5 the green biomass formation is visible while C4 has a brown biomass formation). C2 has more biomass formation than the rest under the same growth conditions including time and this was indicated by more biomass formation as compared to C1, C4 and C5. C2 therefore efficiently fixes nitrogen than C1, C4 and C5.

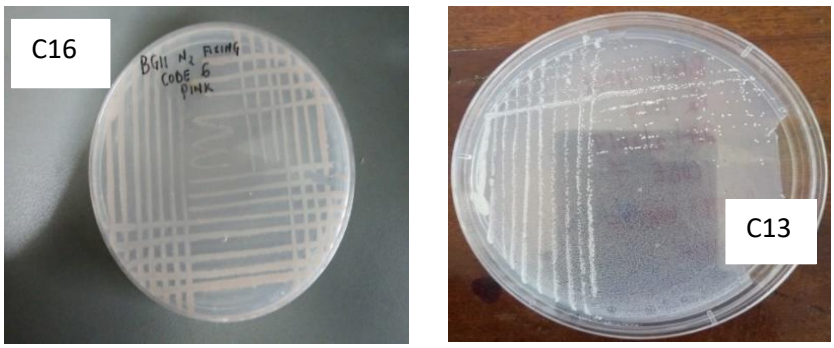


**Plate 4.2**: Axenic cyanobacterial cultures growing in solid media for isolates C16 and C13 respectively. (Growth is visible along the streaking lines)

### 4.3 Morphological characterization of pure culturable isolated Cyanobacterial Strains

The cell morphology under a light microscope revealed the presence of typical cyanobacterial morphologies such as those for *Chroococcales, Oscillatoriales, Pleurocapsales* and *Nostocales*. Cells were non-motile though a few from liquid culture were motile (C3, C5, C9 and C10), solitary or in pairs after division. Cell content had no separation on centro- and chromatoplasma. These results were also reported by Vos *et al*., 2011. C1, C2, C3, C5, C6, C9, C10 and C16 were able to fix their own nitrogen since nitrates were not provided in their media. C10 and C16 had visible specialized cells (heterocysts). C4, C13 and C14 did not show their ability to fix their own nitrogen and only grew when nitrates were introduced into their growth media. Table 4.2 summarizes the morphological characterization data. Microscopic images are shown in Plate 4.3 and 4.4.

**Table 4.2: Colony and cell morphology of the isolates**

| Isolate code | Pigment | Morphology | Type of media | Site | Nitrogen fixation | Cell arrangement |
|---|---|---|---|---|---|---|
| C1 | Green | Coccus | BG11 non | S4 July | +ve | Scattered |
| C2 | Green | Coccus | BG 11 non | S3 Aug | +ve | Clustered |
| C3 | Green | Spiral | BG 11 non | S4 July | +ve | Scattered |
| C4 | Brown | Filamentous | BG 11 $N_2$ | S3 July | -ve | Scattered |
| C5 | Green | Coccus | BG 11 non | S3 Aug | +ve | Scattered |
| C6 | Green | Coccus | BG 11 non | S3 Aug | +ve | Scattered |
| C9 | Green | Coccus | BG 11 non | S3 July | +ve | Clustered |
| C10 | Green | Filamentous | BG 11 non | S3 Aug | +ve | Scattered |
| C13 | Brown | Coccus | BG 11 $N_2$ | S2 July | -ve | Clustered |
| C14 | Brown | Coccus | BG 11 $N_2$ | S2 Sept | -ve | Clustered |
| C16 | Brown | Filamentous | BG 11 non | S3 July | +ve | Scattered |

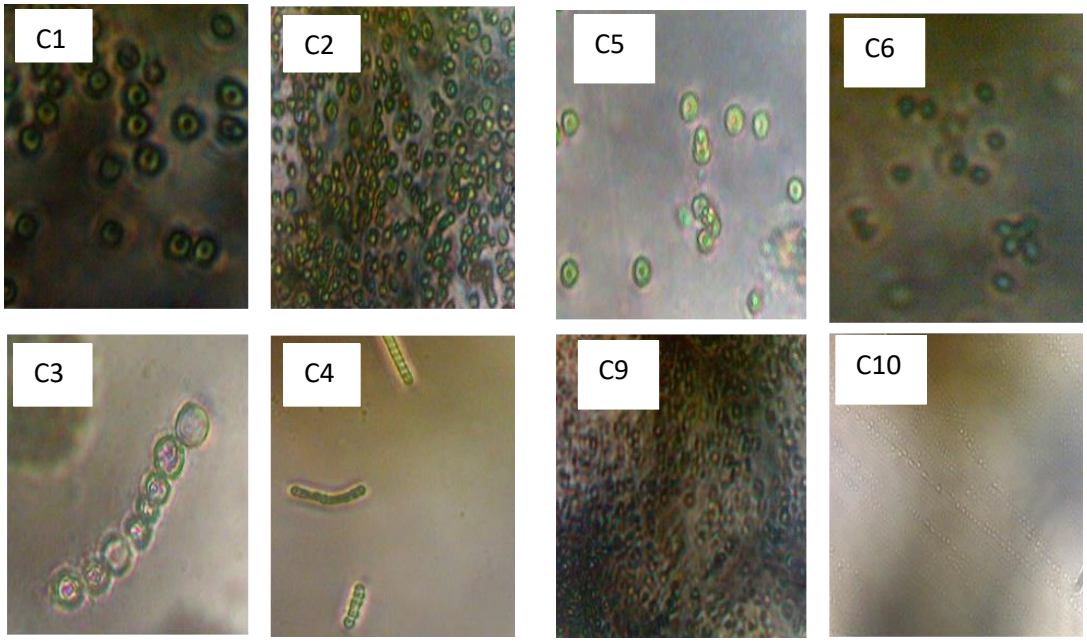


**Plate 4.3:** Shows microscopic images of liquid culture isolates taken using an inverted microscope at x400 (C1, C2, C3, C4, C5, C6, C9, C10 respectively)

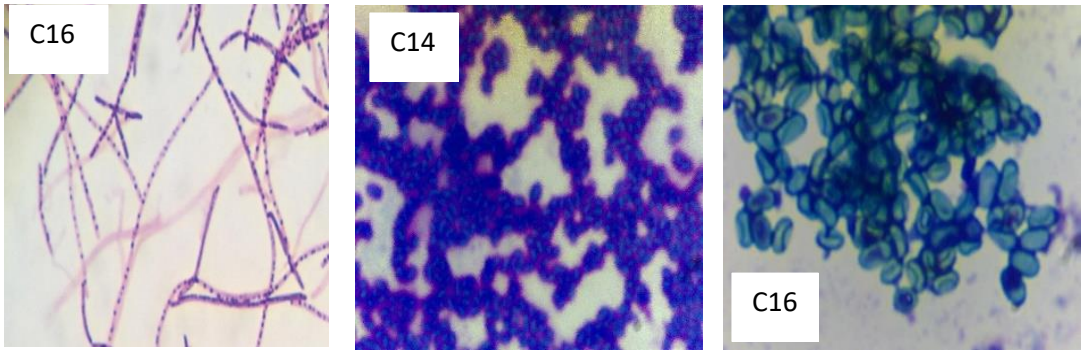


**Plate 4.4:** Shows microscopic images of solid culture isolates C13, C14, C16 respectively taken using a compound microscope at x400

**4.4 Molecular characterization: Phylogenetic analysis**

DNA extraction using phenol-chloroform protocol for all the isolates was performed and their gel image indicated in (Figure 4.1). The extraction was successfully achieved and the 16S rRNA gene region was used to characterize the isolates while *mcyE*, *sxt*, *cyl* and *ana* toxin cyanobacterial genes were used to detect the toxin producing cyanobacterial species.

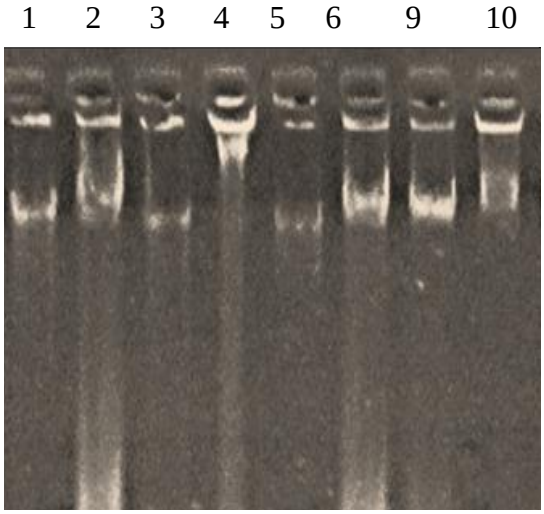


**Figure 4.1:** A gel electrophoresis showing the DNA of the isolates C1, C2, C3, C4, C5, C6, C9, and C10. For the isolates C13, C14 and C16 DNA was not extracted but colony PCR was directly performed.

### 4.4.1 Amplification of the 16S rRNA cyanobacterial genes

The amplification of the 16S rRNA region of the genomic DNA of the isolates was done using standard synthetic oligonucleotides used as forward and reverse primers for PCR: CYA106F primer: 5'-AGAGTTTGATCCTGGCTCAG-3' and CYA781R primer: 5'-AAGGAGGTGATCCAGCC-3' (Nübel *et al*., 1997). Negative control contained all the reagents for PCR amplification except the genomic DNA template. The PCR products were separated on a 1 % agarose gel in 1× TAE buffer, visualized under UV light after staining using Cyber green and used a 10kb ladder (Nübel *et al*., 1997) as shown in Figure 4.2. For the isolates (C13, C14 and C16) gradient PCR was performed.

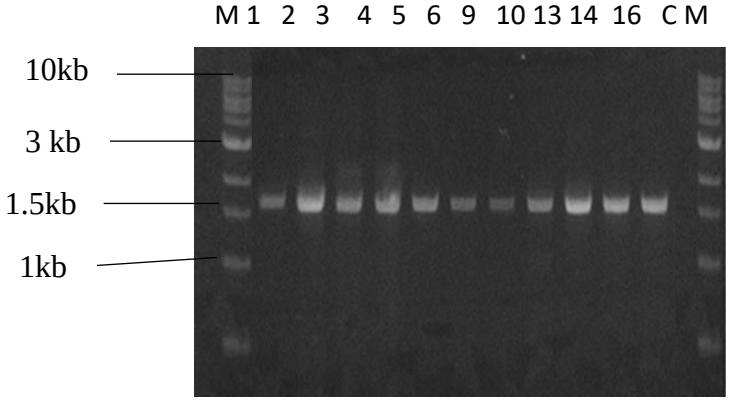


**Figure 4.2:** A gel electrophoresis showing PCR amplification of the 16S rRNA gene (1.6 Kb). M-10kb DNA marker isolates C1, C2, C3, C4, C5, C6, C9, C10, C13, C14 and C16, C- negative control.

### 4.4.2 Phylogenetic analysis of the sequences

BLAST analysis of the sequences against other toxin producing cyanobacterial sequences in the NCBI database showed that all the isolates belonged to the *phylum Cyanobacteria* distributed as follows: *Chroococcales* (3 isolates), *Oscillatoriales* (2 isolates), *Pleurocapsales* (5 isolates) and *Nostocales* (1 isolate). The percentage similarity values ranged between 86% - 99% similarity. Isolates C3 and C10 had percentage similarity of 99.45% to *Spirulina* and *Nodularia* species respectively (Table 4.3). It was found out that C10 was closely related to *Anabaenopsis abijatae* while C3 was closely related to *Spirulina* major JQ738964_s. C1, C2, C4, C5, C13 and C16 isolates were not affiliated to any known groups with common ancestry as depicted in the phylogenetic tree.

C2, C4, C5, C13 and C16 close associates after BLAST analysis were the *Chroococcidiopsis* species while C1 close associate was *Oscillatoria* species which have both so far been grouped as uncultured cyanobacteria. C2 and C5 were closely related while C1, C4, C13 and C16 were being also closely related but the two groups branched outside the main tree indicating a common evolutionary history that could represent some novel genera since their percentage similarity fell below 90%. In my view they could be novel cyanobacterial isolates which are distantly affiliated to *Oscillatoria* (C1) and *Chroococcidiopsis* (C4, C5, C13 and C16) species or to unknown group altogether. Likely C6, C9 and C14 had a sequence similarity of below 99% affiliation to *Oscillatoria* species of cyanobacteria. Their sequence similarity falling below 99% could suggest that they represent a novel new species. Phylogenetic tree showing relatedness and common ancestry is in Figure 4.3.

| *Name* | *Top-hit taxon* | *Top-hit strain* | *Similarity (%)* | *Top-hit taxonomy* |
|---|---|---|---|---|
| *C16* | DQ532182_s | JSC2-G9 | 87.67 | Bacteria;Cyanobacteria;Chroobacteria;Pleurocapsales;Chroococcidiopsis_f;DQ532182_g |
| *C14* | BAUB_s | NKBG15041c | 98.93 | Bacteria;Cyanobacteria;Chroobacteria;Chroococcales;Cyanobacteriaceae;Oscillatoria_g1 |
| *C13* | DQ532182_s | JSC2-G9 | 87.85 | Bacteria;Cyanobacteria;Chroobacteria;Pleurocapsales;Chroococcidiopsis_f;DQ532182_g |
| *C10* | JQ738964_s | SA_25 | 99.46 | Bacteria;Cyanobacteria;Chroobacteria;Chroococcales;Spirulina_f;Spirulina_g1 |
| *C9* | BAUB_s | NKBG15041c | 98.63 | Bacteria;Cyanobacteria;Chroobacteria;Chroococcales;Cyanobacteriaceae;Oscillatoria_g1 |
| *C6* | GQ243430_s | GSL035 | 92.84 | Bacteria;Cyanobacteria;Chroobacteria;Oscillatoriales;Prochlorotrichaceae;ALVV_g |
| *C5* | HQ189092_s | B107212B | 86.57 | Bacteria;Cyanobacteria;Chroobacteria;Pleurocapsales;Chroococcidiopsis_f;HQ189092_g |
| *C4* | DQ532182_s | JSC2-G9 | 87.89 | |
| *C3* | *Anabaenopsis abijatae* | AB2002/09 | 99.45 | Bacteria;Cyanobacteria;Hormogoneae;Nostocales;Nostocaceae;Nodularia |
| *C2* | DQ914865_s | CC3 | 86.93 | Bacteria;Cyanobacteria;Chroobacteria;Pleurocapsales;Chroococcidiopsis_f;DQ914865_g |
| *C1* | PCC7105_s | PCC7105 | 87.8 | Bacteria;Cyanobacteria;Chroobacteria;Oscillatoriales;Planktothrix_f;EF654088_g |

**Table 4.3:** Blast analysis results and identification of cyanobacterial isolates from Lake Magadi

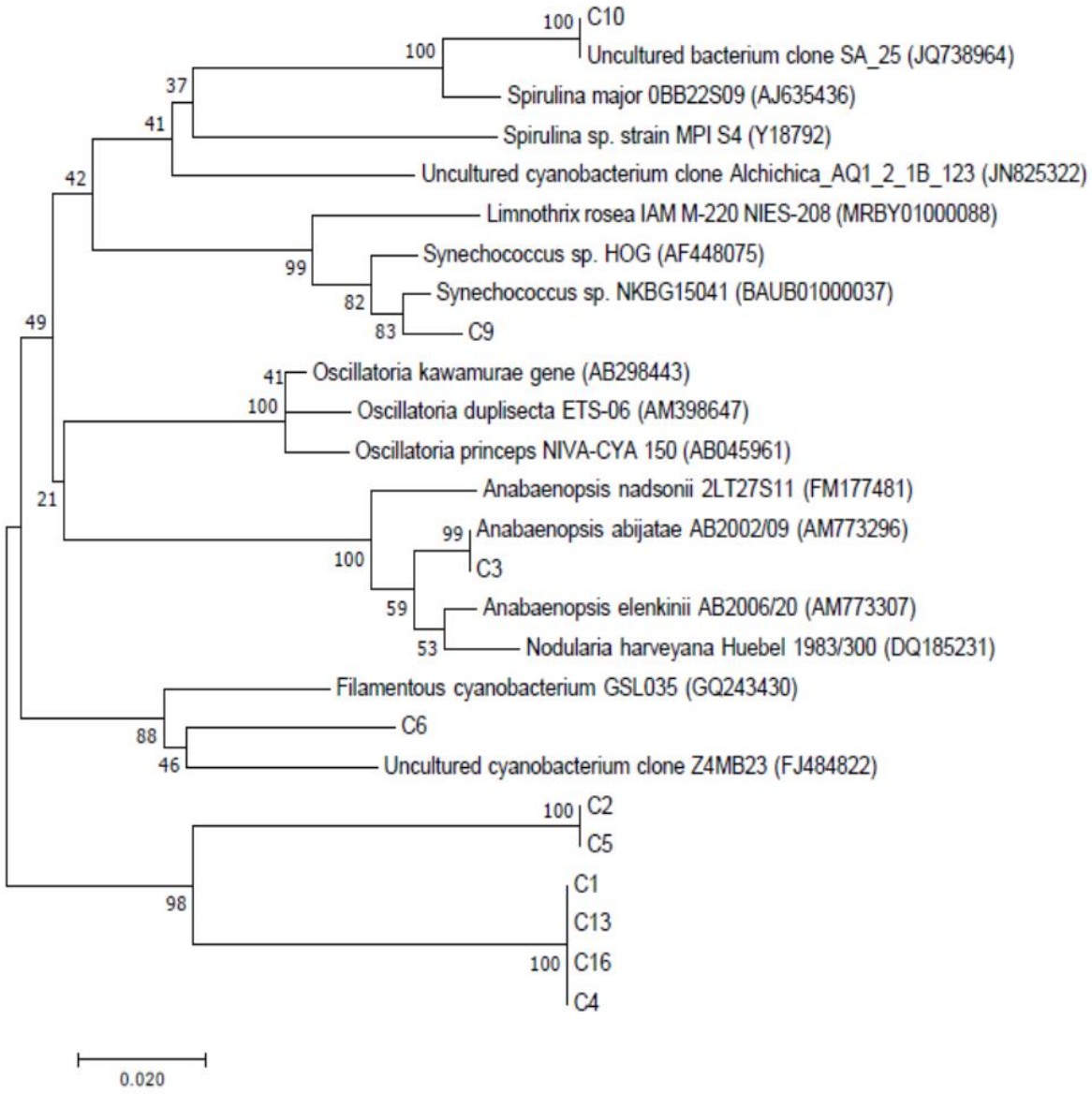


**Figure 4.3:** Shows a phylogenetic tree showing relatedness and common ancestry

4.5 PCR detection of toxin genes from the axenic cyanobacterial isolates

All isolates were found to have at least two toxin genes of those tested. This suggested that our isolates had the capability to produce several toxins

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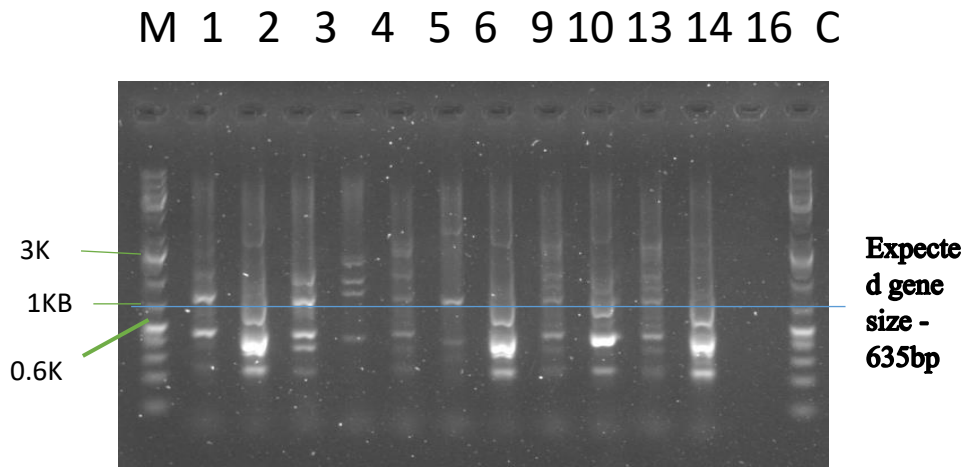



**Figure 4.4:** A photograph of 1% agarose gel showing PCR amplification of the Cylindrospermopsin toxin gene at the expect length of 635bp of the 11 isolates after staining with Sybr green dye and visualised under UV light. M- 10 kb Marker and C- Negative control. From the plate multiple bands can be seen that posed challenge for sequencing. The numbers at the top are labels for the isolates as depicted in table 4.3

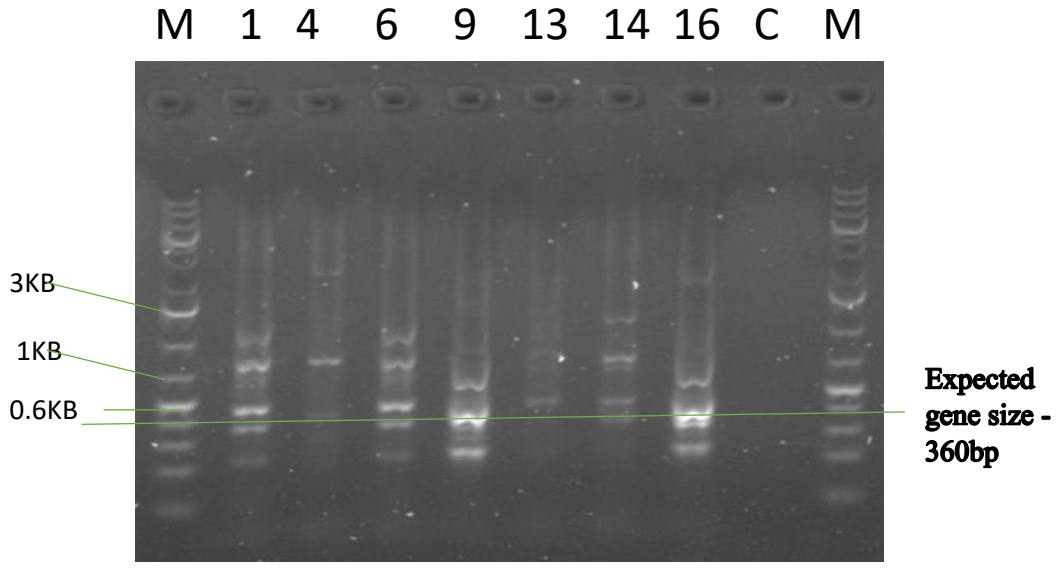



**Figure 4.5:** A photograph of 1% agarose gel showing PCR amplification of the anatoxin genes at the expect length of 360bp. This was seen after staining with cyber green dye and visualised under UV light. M- 10 kb Marker and C- Negative control. From the figure numerous multiple bands can be seen. This multiple bands posed a challenge for sequencing. The numbers at the top are labels for the isolates as depicted in table 4.3. Only 7 isolates showed presence for this gene.

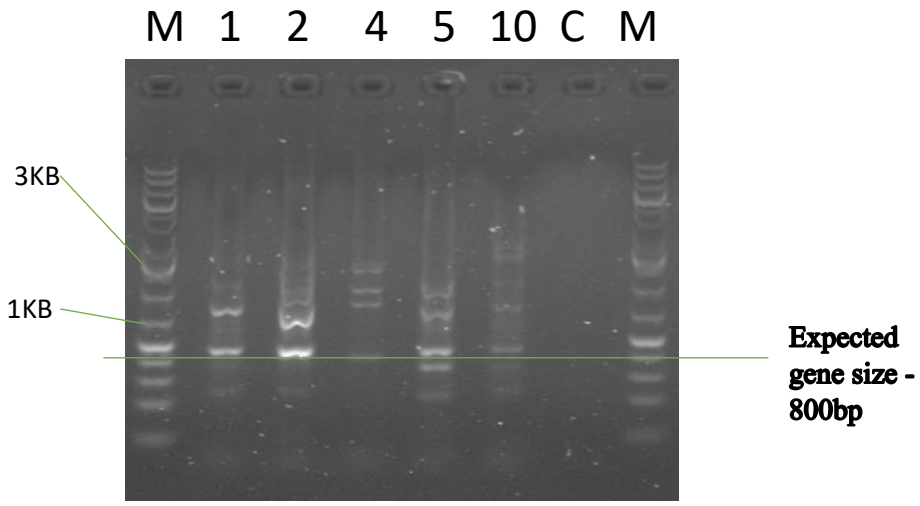



**Figure 4.6:** A photograph of 1% agarose gel showing PCR amplification of the Microcytin genes at the expect length of 800bp. This was seen after staining with cyber green dye and visualised under UV light. M- 10 kb Marker and C- Negative control. From the figure numerous multiple bands can be seen. This multiple bands posed a challenge for sequencing. The numbers at the top are labels for the isolates as depicted on table 4.3. Only 5 isolates showed presence for this gene.

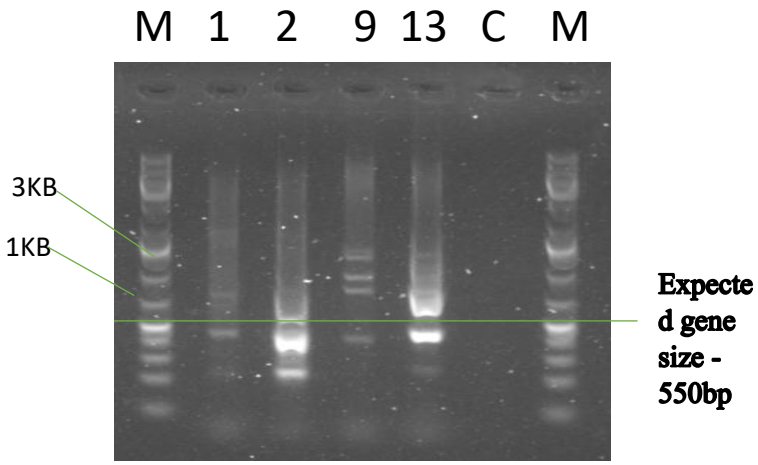


**Figure 4.7:** A photograph of 1% agarose gel showing PCR amplification of the Saxotoxin genes at the expect length of 550bp. This was seen after staining with cyber green dye and visualised under UV light. M- 10 kb Marker and C- Negative control. From the figure numerous multiple bands can be seen. This multiple bands posed a challenge for sequencing. The numbers at the top are labels for the isolates as depicted on table 4.3. Only 4 isolates showed presence for this gene.

Cylindrospermopsin toxin gene was found in all 11 isolates **figure 4.4** while C1 was found to have all the four toxin genes. C3 was found to have only one toxin gene. 7 isolates were found to have genes for anatoxins **figure 4.5**, 5 isolates had genes for microcytin **figure 4.6** and 4 isolates had genes for saxotoxins **figure 4.7**. **Table 4.4** summarizes the toxin genes against the isolates. PCR amplicons of the toxin genes were sequenced at Inqaba Biotech, South Africa but had no conclusive results since the PCR amplicons were very faint after gel cleaning and the occurrence of many multiple bands making sequencing a challenge.

**Table 4.4:** Summary of the toxin genes against the isolates

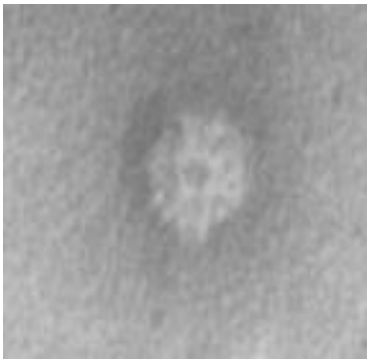
| | **Presence of toxin genes** | | | |
|---|---|---|---|---|
| **Isolate code** | ***Ana*** | ***Myc*** | ***Sxt*** | ***Cyl*** |
| **C1** | + | + | + | + |
| **C2** | - | + | + | + |
| **C3** | - | - | - | + |
| **C4** | + | + | - | + |
| **C5** | - | + | - | + |
| **C6** | + | - | - | + |
| **C9** | + | - | + | + |
| **C10** | - | + | - | + |
| **C13** | + | - | + | + |
| **C14** | + | - | - | + |
| **C16** | + | - | - | + |

***Key:*** + indicates presence of PCR amplicons after amplification of the toxin genes

### 4.6 Isolation of cyanophages

Water samples that were used in the culturing of axenic strains of cyanobacteria were filtered through using 0.45µm and 0.2µm membrane filters. We used two membrane filters since some cyanophages are documented to be large enough not to go through the 0.2 µm membrane filter. The phage filtrate was purified and packaged in vials of 1ml. They were sent for negative imaging under an electron microscope in Germany. **Plate 4.5** below shows phage microscopic negatively stained images under an electron microscope lysing Samples C13 and C16 respectively.

Sample C13



Sample C16

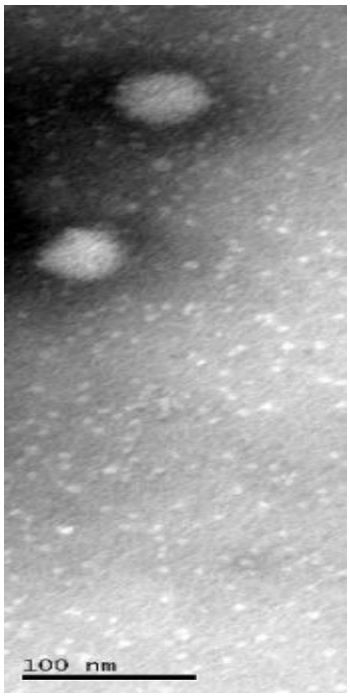



**Plate 4.5**: Shows phage microscopic negatively stained images under an electron microscope. C13 and C16 are their hosts respectively

### 4.7 Biocontrol test of toxic cyanobacteria by use of cyanophages

Toxic cyanobacteria species were mass-cultivated in 250ml conical flasks. 10 mL of the cyanophage lysate was transferred aseptically to mid-log host cell culture. Constant mixing of the algal culture was provided by mechanical aeration twice a day. Each algal culture sample was monitored every day for cellular growth rates and cell lysis against a control that had no phage filtrate inoculated into it. Lysis was evident when clearing of cellular biomass was seen. Lysis was evident for all the isolates apart from C3 and C6. 0.45µM membrane filter was used since we have

large cyanophages that are trapped by the 0.2µM membrane filter. C1, C2, C4, C5, C9, C10 and C13 had both filtrates having phages that lyse them. C14 was lysed by phages from the filtrate of 0.2µM membrane filter only while C16 was lysed by phages from the filtrate of 0.2µM membrane filter only.

**Table 4.5**: Summary of phage activity in the filtrates against the isolates

| **Isolate codes** | **o.45 µM ( Size of membrane filter)** | **0.22 µM (size of membrane filter)** |
|---|---|---|
| **C1** | + | + |
| **C2** | + | + |
| **C3** | - | - |
| **C4** | + | + |
| **C5** | + | + |
| **C6** | - | - |
| **C9** | + | + |
| **C10** | + | + |
| **C13** | + | + |
| **C14** | - | + |
| **C16** | + | - |

***Key:*** **+** indicates lysis activity by phages on the isolates

**Plate 4.6:** Shows clearing of cyanobacterial biomass. To the extreme left 0.2 µM and right 0.45 µM filtrates while the center is the control. Above are liquid samples of C5 and C1 respectively

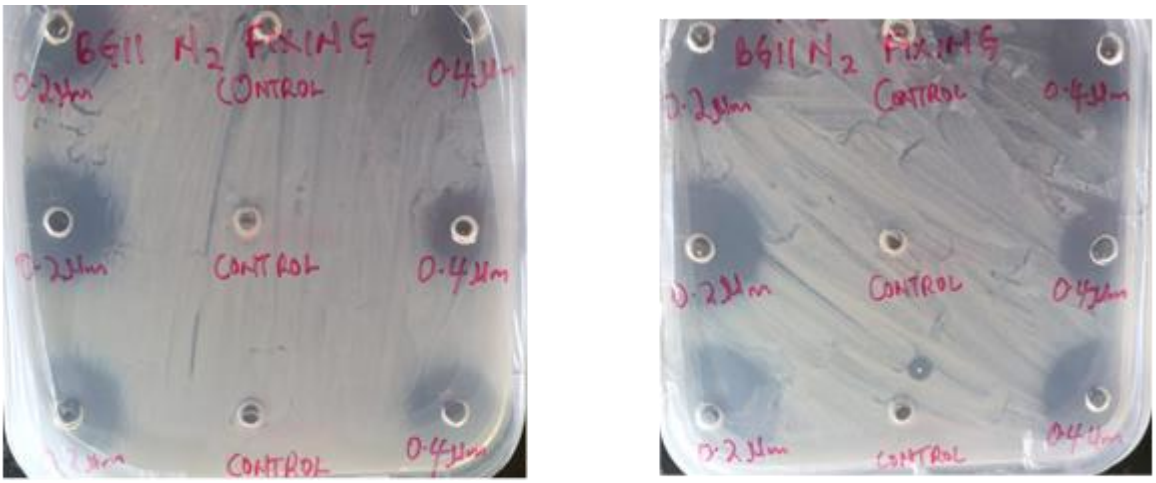



**Plate 4.7:** Shows cleared plaques by cyanophages of isolate C13 and C16 respectively which grew on agar. To the extreme left 0.2 µM and right 0.45 µM filtrates while the center is the control

# CHAPTER FIVE

# DISCUSSION, CONCLUSION AND RECOMMENDATIONS

## 5.1 DISCUSSION

### Lake Magadi an extreme environment

Lake Magadi as an alkaline saline ecosystem exhibit elevated alkalinity, pH and salt content, which prevent many organisms from colonization (Kambura *et al.*, 2016). This study demonstrated that extreme environments such as Lake Magadi are inhabited by a diverse, polyphyletic array of culturable cyanobacteria which are underrepresented in currently available nucleotide databases.

### Haloalkaliphilic cyanobacteria

All the isolates were able to thrive at pH 11, temperatures of between 25 – 40 ºC and grew to maximum in at least 21 days. Since cyanobacteria are autotrophic, they were able to grow without an external carbon source. Unlike in a normal ecosystem which is not extreme, cyanobacteria are shown to thrive under high nutrient conditions, more so when the levels of nitrogen and phosphorus are elevated (Giani *et al*., 2005; Paerl and Paul, 2012) which was not the case in Lake Magadi. Physiological adaptations favor alkalophilic cyanobacteria as the main primary producer in the alkaline saline lakes (Grant and Sorokin, 2011). In the bacteria kingdom, they are the most adapted to the varying extreme environments conditions. They are reported to form microbial mats with different bacteria, ranging from Antarctic ice to continental hot springs. The cyanobacteria are also known to have the ability to develop in hypersaline and alkaline lakes, being supported by the high metal concentrations level and their ability to tolerate this conditions (*i.e.*, low availability of water), forming endolithic communities in desertic regions. Nevertheless, the cyanobacteria are rarely found in environments having acidic levels at pH values lower than 5–6 (Tandeau de Marsac and Houmard, 1993).

Ability of cyanobacteria to thrive in the varying extreme environments is propagated by their specific regulatory systems, not leaving out the mechanisms systems equal to those of other prokaryotes or the eukaryotes that are able to manufacture their own food. The variations in the nitrogen fixing cells and the different cell types

facilitating the species dispersion are controlled by different specific regulatory systems. In the past, immense progress has been made so that to understand the expression of the cyanobacterial genome in response to the varying intensity and the spectral quality of incident light and the subsequent response to the nutritional conditions such as nitrogen, carbon, and the sulphur source. However, their low tendency to interact with other species, is a result of their dominance in their systems and the rare or absent due to selective pressure of the environment. (Costa *et al*., 2016). High amounts of carbon dioxide in the lake due to rocks rich in carbonates could also explain presence and dominance of cyanobacteria in this extreme environment (Rampelotto, 2013).

**Morphological characterisation and adaptation of the isolates**

Haloalkaliphilic cyanobacteria had unique morphological characteristics such as heterocyst, long filaments and mainly unicellular cells which were mainly cocci r in our case. In this study, 80% of recovered isolates could fix nitrogen therefore having specialized cells called heterocysts. The heterocysts were only visible in filamentous cyanobacteria C4, C10 and C16. Four dominant Phyla were observed from this study: *Chroococcales (27%), Oscillatoriales (46%), Pleurocapsales (18%) and Nostocales (9%)*, thus confirming previous research by Wagacha *et al.*, (2014), whose study majorly relied on microscopical techniques while ours took a molecular approach of identification. *Chroococcidiopsis* species was the most dominant cyanobacterial species represented by C2, C4, C5, C13 and C16 isolates. They are known for their ability to tolerate a diverse array of high conditions of salinity, extreme high temperatures, pH, high levels of radiation, conductivity and alkalinity (Magana *et al*., 2013).

*Anabaenopsis elenkinii* preferentially thrives in alkaline and saline lakes, which include, Lake Sonachi in Kenya and Lake Texcoco in Mexico (Ballot *et al.*, 2008). The ability of *Anabaenopsis elenkinii* thrive in the alkaline lakes, have credited them as the indicators in this lakes die to their high levels in this lakes while it rarely occupies fresh water lakes. C10 was 99.45% an *Anabaenopsis* species thus suggesting alkalinity in Lake Magadi. All isolates were able to tolerate high salt concentration of up to 10% and pH of 11 while low pH values (acidic) did not support cyanobacteria growth. Therefore, these organisms are polyextremophiles because they are able to colonize environments having more than one extreme

condition. This aspect has been useful in biotechnology for example: the salinity tolerance of *A. platensis*, which is also a dominant cyanobacterial species in the Rift valley saline lakes (Vonshak and Tomaselli, 2000), has been extensively studied, particularly because they are a significant source of protein and vitamins and have been used in production of supplements (Ayachi *et al.,* 2007).

**Phylogenetic analysis of the isolates**

Phylogenetic analysis also gave us other interesting findings, though *Chroococcidiopsis* were the most abundant species, their percentage similarities after blasting featured below 94% and were mainly uncultured. This was not an unlikely expectation since Lake Magadi cyanobacterial potential is still under explored. Since the percentage similarities are still low, this group would probably generate new genera with more research since they were also not affiliated to known organisms with a common ancestry in the phylogenetic tree. Interestingly, we expected that *Arthrospira* species to be the most abundant but our findings were contrary to other initial findings by Ballot *et al.*, 2004; Dadheech *et al.*, 2009; Wagacha *et al.*, 2014, which indicate that *Arthrospira* species is the most abundant cyanobacteria in the saline Rift valley lakes. It's worth noting that from an ecological perspective of microscopical investigation for the most abundant species, *Arthrospira* species was the most abundant. After culturing in the laboratory, abundance of *Arthrospira* species reduced and was only represented by isolate C10. These results could emanate from media constitution which did not favor *Arthrospira* growth, though we were not able to test this hypothesis. C6, C9 and C14 had an affiliation to *Oscillatoria* species of below 99% after blasting, indicating they could generate a new species. This was evident from their morphological characteristics as most of them did not have segmented filaments.

**New cyanobacterial diversity**

Generally, apart from C1 and C10 isolates that had 99% similarity to *Nodularia* and *Spirulina* species, the rest of the 9 isolates largely remained unclear as to which species or genera they would closely represent. This could be an interesting topic for further research in these environments to find new cyanobacterial diversity. From our results, one thing that stood out was the application of molecular methods for ecological studies which enhanced ability to detect and identify microorganisms in

nature (Amann *et al*.,1995). So far molecular techniques, are shown to propagate detection of these cyanophages as compared to the traditional morphological methods. (Taton *et al.*, 2006; Willame *et al*., 2006). Small organisms are also not easily detected with the traditional methods, thus they are being overlooked. Molecular methods not only allow the detection of very small organisms, overlooked through microscopy, but is of importance in identifying cryptic species which are of different genotypes that display similar morphology within a population (Ernst *et al*., 2003). *Chroococcidiopsis* species had not before been identified on the saline Rift valley lakes, unlike the rest of our results which concur to what was recorded by other researchers such as Wagacha *et al.*, (2014). This suggests that Lake Magadi is still largely unexplored in its cyanobacterial diversity since we only majored on culturable cyanobacteria.

**Genes encoding for cyanotoxins**

All the isolates were found to have genes for cyanotoxin production: microcytin (5), saxotoxin (4), anatoxin (8) and cylindrospermopsin toxin (8) with isolates C1 and C13 having four and three of the toxin genes respectively as shown **Table 4.4**. Presence of the toxin gene doesn't necessarily mean that the isolate must produce the toxin but their production is to prevent predation by other organisms such as birds and wild animals. This means that it occurs periodically in the alkaline lakes when habitats are threatened by degradation and fragmentation of associated ecosystems (Simmons, 1996). Presence of cyanotoxins has also been reported in the Rift valley saline lakes (Kotut *et al.*, 2011). Within the recent decade, cyanotoxins have been wildly studied for their possible role in the mass mortalities of the Lesser Flamingos. Previously a lot of emphasis had been put on heavy metals causing the deaths but this is only the case in lakes such as Lake Nakuru that are surrounded by a large population of people and other animals.

Lakes such as Bogoria where the heavy metals are not in concentrations known to kill flamingos have raised questions suggesting other factors that are responsible for the Lesser Flamingos' high mortalities. Some studies have reported the occurrence of these mortalities during periods in which the phytoplankton communities were dominated by toxin-producing species (Ndetei and Muhandiki, 2005). In this study we were interested in confirming whether cyanotoxins are produced by

cyanobacteria in Lake Magadi which is a polyextreme environment. From our study, cylindrospermopsin genes were present in all our isolates, this could mean that the toxin is abundant in the lake therefore contradicting other findings from saline lakes of the Rift valley that microcytin and anatoxin are the most abundant cyanotoxins (Ballot *et al*., 2004; Kotut and Krienitz, 2011; Krienitz *et al*., 2013). It's worth noting that *Chroococcidiopsis species* is a documented novel and potent cyanotoxin producer (Magana-Arachchi and Wanigatunge, 2013). Genes encoding for the cyanotoxins were sequenced but no conclusive results were obtained since the PCR amplicons were very faint after gel extraction and the occurrence of many multiple bands making sequencing a challenge.

**Cyanophages with biocontrol potential against toxin producing cyanobacteria**

Cyanophages have for a long time been known to infect cyanobacteria. *Arthrospira* has been shown to be controlled by cyanophages (viruses that infect cyanobacteria) (Peduzzi *et al.*, 2014). Jacquet *et al.*, (2013) described a cyanophages that infects *Arthrospira platensis* in mass cultures. In our investigation we relied on filtration method to acquire the cyanophages which we purified and inoculated into already growing biomass of axenic toxin producing cyanobacteria. Other than isolate C3 and C6, the rest showed lysis after inoculation with the phage filtrate. This is evident from **Plate 4.6 and Plate 4.7** that the reduced biomass and plaque clearings resulted from lytic activity of the phages. Further TEM images of **Plate 4.5** indeed confirm the presence of phages. Therefore, from our investigation cyanophages had an overall negative effect on *Chroococcidiopsis, Oscillatoria* and *Spirulina* species which produce cyanotoxins. Related studies in the saline Rift valley lakes on cyanophages was carried out in Lake Nakuru (Peduzzi *et al.*, 2014). Although they documented that cyanophages infection and the related breakdown of *A. fusiformis* biomass led to a dramatic reduction in flamingo abundance, we hold on to the idea that cyanophages control of toxin producing cyanobacterial species could reduce lesser flamingo mortalities that result from them being intoxicated by cyanotoxins.

## 5.2 CONCLUSIONS

- The study has demonstrated that Lake Magadi is inhabited by haloalkaliphilic toxin producing cyanobacteria.
- The isolated cyanobacterial isolates grew at a wide pH range of 10.0-11.3, salt concentration of 5-10% and temperature range of 25 – 40 ºC.
- All the isolates had toxin producing genes indicating that they are probably active cyanotoxin producers.
- The study also demonstrates that Lake Magadi is inhabited by cyanophages that can specifically infect toxin producing cyanobacteria.
- Apart from two isolates, the rest showed reduced activity when inoculated with the phage filtrate suggesting that they could be mass produced to infect and thus reduce the toxin producing species of cyanobacteria. This would consequently reduce flamingo mortalities resulting from consuming cyanotoxins.

## 5.3 RECOMMENDATIONS

Lake Magadi as an extreme environment is still underexploited in terms of its cyanobacterial potential which can have numerous uses in biotechnology. This study has achieved its objective by isolating and characterising culturable cyanobacteria from Lake Magadi. Detection of toxin producing cyanobacteria, isolation of cyanophages and testing them for their biocontrol potential against the toxin producing cyanobacteria. The following recommendations will be vital for more research on the diversity of cyanobacteria from Lake Magadi and testing for cyanophages mediated biocontrol on toxin producing cyanobacteria:

- Improvement of isolation techniques and media modification to allow isolation of more diverse genera
- Further isolation and characterisation of the toxin producing genes from toxin producing cyanobacteria will help in better understanding of their structure, function and mechanisms.
- Further isolation and characterisation cyanophages infecting toxin producing cyanobacteria will give a better understanding of their structure and their mode of infection thus providing more clues on how they can be used in biocontrol.
- Further full sequencing of the cyanobacterial isolates for complete identification.

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