A COMPARATIVE STUDY OF THE ORIGINS OF CYANOBACTERIA AT MUSINA WATER TREATMENT PLANT USING DNA FINGERPRINTS

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DECLARATION

I **Murendeni Magonono**, I hereby declare that this research is my original work and has not been submitted to any other university or institution. The research does not contain other person's writings unless specially acknowleddge and referenced accordingly.

Signature:..... Date: 31/08/2017

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DEDICATION

I would like to dedicate my thesis to my parents Mr A.N Magonono and Mrs A.S Magonono who supported me throughout my studies. This thesis is also dedicated to all other people who helped in the success of this project.

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ABSTRACT

The presence of harmful algal blooms (HABs) and cyanobacteria toxins in drinking water sources are known to pose a great threat to humans. The main aim of this study was to use molecular technique to determine the origins of the cyanobacteria species at Musina raw water abstraction point by identifying and comparing the non-toxic and toxic cyanobacteria species in the Limpopo River and some of its tributaries based on the phylogenetic analyses of 16S rRNA gene. The Musina water treatment plant is located downstream of a weir and the Beit bridge on the Limpopo River and the raw water supply is abstracted from 22 boreholes of which 14 are along the Limpopo River and 8 boreholes are inside the Limpopo River channel. The bottom sediments samples were collected from these rivers: Limpopo, Crocodile, Mokolo, Mogalakwena, Nzhelele, Lephalale, Sand rivers (South Africa); Notwane (Botswana), Shashe River and Mzingwane River (Zimbabwe). The physical-chemical analysis of the bottom sediments showed the availability of nutrients, nitrates and phosphates, in excess of 0.5 mg/l for most the of rivers, alkaline pH and salinity in excess of 500 mg/l. Total genomic DNA were extracted from cyanobacteria species on the bottom sediments and Polymerase Chain Reaction (PCR) method was used to detect the genetic profile of the cyanobacteria species. Molecular identification of cyanobacteria was based on PCR amplification and sequencing of the 16S rRNA gene. The 16S rRNA gene was absent from sediments of the Mogalakwena and Lephalale rivers but present in all other selected rivers. The cyanotoxins detection was also based on PCR by amplification of microcystin/nodularin and cylindrospermopsin polyketide synthetase genes. Most of the samples showed no amplification of the toxin genes. While two samples showed the amplification of cylindrospermopsin polyketide synthetase gene (Sand River and Nzhelele River Next to Tshipise) and two samples showed amplification for microcystin/nodularin synthetase gene, Crocodile River and Mzingwane River. The first was the confirmation of similarity of samples from Crocodile River downstream of hartbeespoort Dam and Shashe River to *Leptolyngbya boryana* with 99% bootstrap confidence. The similarity of sample from Musina borehole to Sand River upstream to *Alkalinema pantanalense* with 98% bootstrap. Thus, the presence of toxic genes may imply the presence of toxic cyanobacteria species in the river sediments and may be hazardous to humans because rural communities and commercial farmers abstract water from Limpopo River catchment for human consumption, livestock and irrigation. The waters of the Limpopo River basin also provide drinking water to wildlife and a habitant for aquatic organisms/animals.

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Keywords: Cyanobacteria, cyanotoxins, harmful algal blooms, PCR, Phylogenetic analyses, nutrients

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
PCR	Polymerase Chain Reaction
TAE	Tris-Acetate EDTA
dNTP's	Deoxynucleoside Triphosphate(s)
bp	Base pairs
MEGA	Molecular Evolutionary Genetics Analysis
WHO	World Health Organization
DWA	Department of Water Affairs
RNA	Deoxyribonucleic Acid
NOD	Nodularin
CYN	Cylindrospermopsin
BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide Basic Local Alignment Search Tool
MP	Maximum Parsimony
NCBI	National Center for Biotechnology Information

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CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND

Algae are very small unicellular organisms which can come together to form colonies, thus become visible to the naked eyes as minute green particles (WHO, 2003). Suspended Algae cause the water they live in to have considerable turbidity if they attain high densities because they are usually finely dispersed (Huynh and Serediak, 2006). When Algae species such as cyanobacteria come together in large numbers they form harmful Algae bloom (HABs) (Mao *et al.*, 2009). HABs can occur naturally and can also occur because of human actions that disturb ecosystems by increased nutrients loadings and pollution, modified hydrology, and introduction of species into freshwaters (Lopez *et al.*, 2008).

The availability of phosphorus enhances the growth of HAB and is considered the limiting nutrient in many fresh water bodies. Phosphorus can occur naturally in lakes, but human beings often increase the amount of phosphorus in fresh water bodies including lakes through their activities (Khan and Ansari, 2005). Contributors of phosphorus into aquatic ecosystems include detergents containing phosphate, fertilizers used on farmland and water draining from it, leaking septic systems, urban runoff, mining of phosphate rock and effluent from wastewater treatment plants (Louw and Gichuki, 2006). As more landuse activities occur the more the quantity of runoff increases.

Harmful Algal Bloom toxins are hazardous to human and animal health through their production of toxins or bioactive compounds, and they contribute to the reduction of water quality. This is because the HABs multiply to build high biomass, which consequently degrades aesthetic, ecological, and recreational values (Lopez *et al*., 2008). It has been reported that some species of algae, such as golden and red and certain types of cyanobacteria can produce potent toxins that can cause adverse health effects to wildlife and humans (Lopez *et al*., 2008). Cyanobacteria are grouped under prokaryotes known by a variety of names such as cyanobacteria, blue-greens, blue-green algae, cyanophytes and cyanoprokaryotes. Both terms can be used interchangeably and are equally acceptable. Cyanobacteria contain chlorophyll, making them unique among bacteria and therefore are most likely the progenitors of true algae (Kumari *et al*., 2009).

Oberholster and Ashton (2008) consider South Africa's freshwater resources to be moderately to highly eutrophic, and this is based on the estimated values for the freshwater pollution (in the form of chemical oxygen demand) and average phosphorus (as orthophosphates). Cyanobacteria have

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variable geomes (Kapraun, 2007) which under different environmental conditions have the ability to express various genes (Zani *et al*., 2000). Currently two methods of algal species identification are being used: mophorlogical; which involves observing various features by microscope and molecular; which uses a variety of gene regions (Charles *et al*., 2002; Prygiel *et al*., 2002). Molecular identification can provide revolutionary discoveries in taxonomy which can have great benefits for bioassessment (Manoylov, 2014). Recently published methods and improved DNA reference libraries has shown great promise of algal identification (Kermarrec *et al*., 2013).

Deoxyribonucleic acids (DNA) are carriers of genetic information in cells of all living organisms. DNA holds a blueprint-like that carries the 'instructions' necessary to build all cell components like protein and RNA molecules (Yoseph, 2005). The section of our DNA that encodes RNA and proteins are called genes. Other sections of the DNA sequences give information needed for gene regulation (Yoseph, 2005). Different forms of each gene sequence called alleles are used in analysis based on their difference during DNA fingerprinting (Jeffreys *et al*., 1985).

1.2 STATEMENT OF THE PROBLEM

Earlier studies done on the Limpopo River reported an increase on the pollutants load from upstream and downstream activities such as: mining, increase of impoundments and water abstraction, agriculture, industrial and domestic discharge of untreated wastewater (DNA, 1994; Louw and Gichuki, 2003).

As water quality deteriorate the combined effects of the above-mentioned factors and affects socio-economic activities and endanger the sustainability of downstream aquatic (estuarine) and terrestrial ecosystems (Falkenmark and Rockström, 2004; FAO-SAFR, 2004). Algae are good indicators of water quality and degree of water pollution. Their abundance and community composition most often reflects pollution and has the capacity to affect the chemical properties of water such as pH and nutrients levels (Jafari and Gunale, 2006).

High nutrient composition of a water body might result in proliferations of the Blue-green algae (Cyanobacteria). A fresh water study has shown that on average, approximately 60% of the algae blooms in freshwater contain cyanobacteria toxins (Sivonen, 2007). High abundance of cyanobacteria can affect water supply operations by increasing the turbidity of the water and reduce

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the efficiency of disinfection (Maier and Dandy, 1997). Therefore, early prediction (or prevention) of these blooms is crucial, especially when portable water is to be obtained from these rivers (Ribeiro and Torgo, 2008).

1.3 MOTIVATION

A previous study by Netshambidi and Gumbo (2014) showed the presence of cyanobacterial species such as *Microcystis* which were found producing toxins of microcystin LR and YR in the Musina raw water intended for human consumption. The Musina Water treatment plant abstracts raw water from the 19 boreholes that are located within the Limpopo River, thus there is need to determine the origins of the cyanobacteria in the Musina raw water by comparing the DNA profile of water samples from Crocodile, Notwane, Shashe and Mzingwane Rivers. Also, rural communities that live downstream of the Musina water treatment plant, abstract water from Limpopo River for domestic purposes, livestock, watering irrigation and these may lead them to be exposed to algal toxins. The demand for improvement in water quality is growing world-wide resulting in increased control of algae for environmental, recreational and public health reasons.

Algal monitoring is to date only commonly used for blue green algae because their toxicities directly affect water quality (Stefan *et al.*, 1995). Areas that receive reliable supplies of treated drinking water may have long-term chronic exposure to low levels of cyanotoxins because convectional water treatment processes are ineffective (not exceeding 11-18 %) at removal of cyanobacterial toxins (Hoffman, 1976; Duy *et al.*, 2000). Hence, there is a need for research to determine the type and composition of cyanobacteria species that is present and to identify the origins of cyanobacteria.

1.4 RESEARCH QUESTIONS

- Are the cyanobacteria species at Musina water treatment, the same as the cyanobacteria species from Crocodile, Notwanbe, Mzingwane and Shashe rivers?
- What are the cyanotoxins and genes that have the ability to express toxic responses that are associated with these cyanobacterial species?
- Does the effect of the nutrients inflow into Limpopo River from the Marico, Matlabas, Laphalala, Crocodile, Mokolo, Mogalakwena, Sand, Notwane River, Shashe River, Shabili and Mzingwane rivers contribute to the long-term development of harmful algal blooms?

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1.5 RESEARCH OBJECTIVES

1.5.1 Main objective

- To collect and identify Cyanobacteria species in Limpopo river sediments at the Musina raw water point.
- To use the DNA fingerprint of cyanobacteria to determine the origins of the cyanobacteria species in Limpopo river sediments at the Musina raw water point.

1.5.2 Specific objectives

- To collect sediments at Musina raw water point, Laphalala, Crocodile, Mokolo, Mogalakwena, Sand, Notwane, Shashe, Mawoni, Nzhelele and Mzingwane rivers;
- To determine the levels of physical-chemical parameters (pH, EC, TDS, nitrates; phosphates, ammonia, nitrite) in the river sediments;
- To identify the cyanobacteria species in the river sediments;
- To determine the DNA profile of cyanobacteria species in the river sediments and compare their DNA profile; and
- To determine the cyanotoxins that may be produced by the cyanobacteria species.

1.6 THE STUDY AREA

The study area is the Musina water treatment plant which abstracts raw water supply from 19 boreholes that are located within Limpopo River (Figure 1). Limpopo River basin consists of four countries: Botswana, South Africa, Zimbabwe and Mozambique. The total catchment area is approximately 408,250 km^2, a large share of the basin lies in South Africa (45 percent), while the rest is allocated roughly equally between Botswana (19 %), Mozambique (21 %), and Zimbabwe (15 %) according to Tingju and Claudia (2012).

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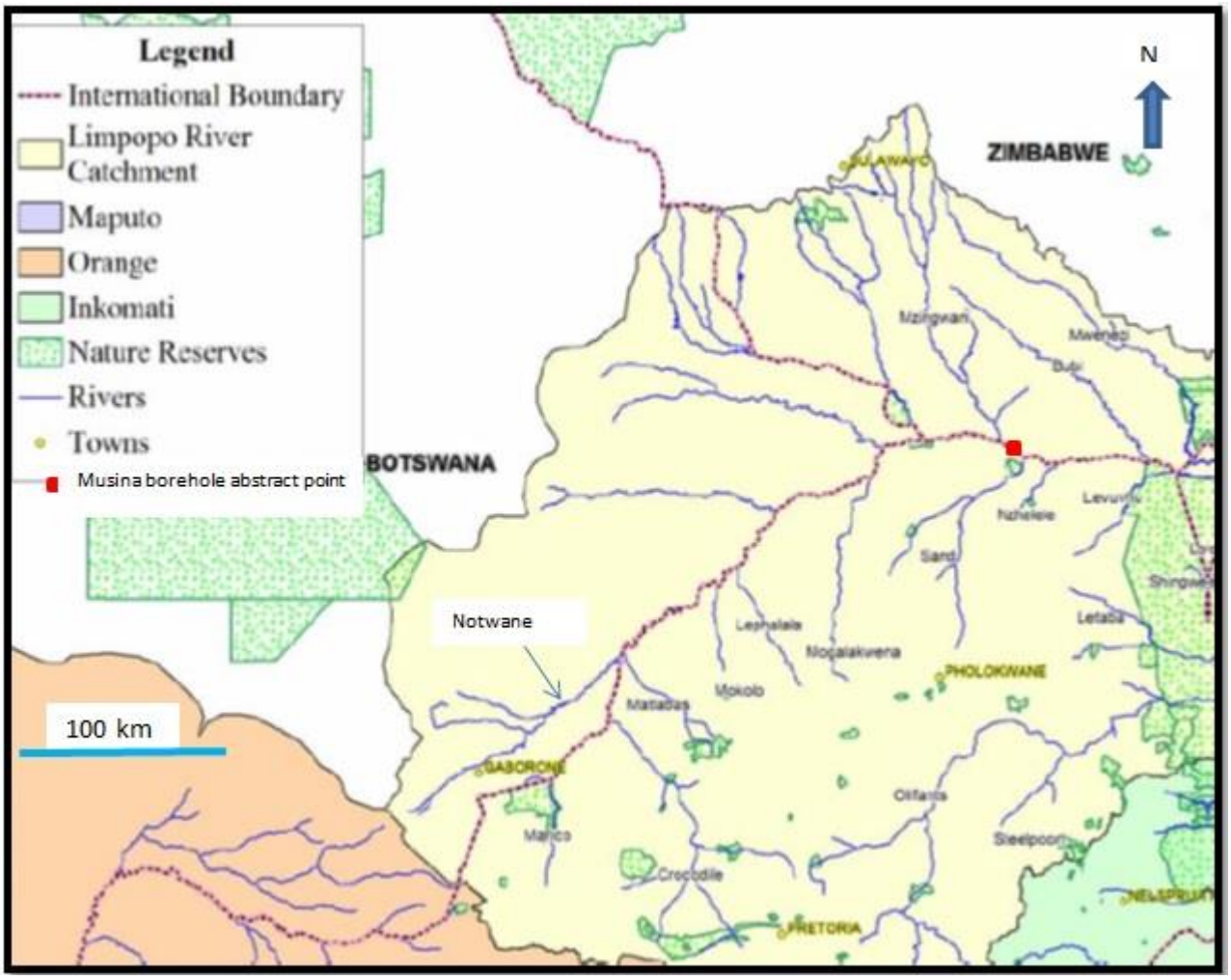

Figure 1.1 The location of the Musina abstraction point obtains raw water supply from boreholes located in the Limpopo River and it is situated downstream of the major tributaries of the Limpopo River (LimCom, 2010)

The Limpopo River basin is an arid to semi-arid region where water is of strategic importance to development. Water has a potential limiting effect on all future development in the region. The Limpopo River basin is home to almost 14 million people in four riparian states (DWA, 2011/12).

Crocodile River has a total catchment area of 29 349 km^2 originating from the catchment in Gauteng Province, and passes through Limpopo Province to join the Transboundary Limpopo River. The river is perennial and supposedly contains high turbid waters and nutrients. Diatoms in small units of 2 to

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3 cm diameter were found floating profusely. It appears that the entire length of the Crocodile River from Thabazimbi is between 20-25 m wide without much of aquatic plants (DWA, 2011/12)

Shashe River originates from Zimbabwe, enters Botswana at Mbalambi Village and passes through Kalakamati, Sebina, and Marobela to reach the Nthimbale dam, Shashe Dam and finally joins the Dikathong dam. The river flows out from the Dikathong dam and is joined by several streams on the way between Zimbabwe and Botswana to enter the Limpopo River at Shalimpo (DWA, 2011/12). The Maenjane, Nyambambisi and Themagana Rivers are the major catchments to the Shashe River after it enters Botswana (DWA, 2011/12).

One of the major rivers located adjacent to the capital city of Botswana (Gaborone) with the largest areas of urban growth, is in the south-eastern corridor of the South Africa is Notwane River (Mladenov *et al*., 2005). The total catchment area of this river is 18 053 km^2 and it flow from south to north, this river is a sub-basin in the Limpopo River basin (Sir M. McDonald and Partners, Ltd., 1991). In order to harness water from the river and its tributaries for use as water supply for Gaborone the Gaborone reservoir was built in the 1970's; and this altered Notwane River (Senai, 1999).

The Mzingwane Catchment generates almost a quarter of the run-off in the Limpopo Basin (Görgens and Boroto, 1997). Most of this run-off is generated from the Shashe and Upper Mzingwane (Love *et al.*, 2005). The mean annual run-off ranges from 600 mm in Shashe and Upper Mzingwane to less than 400mm in Mwenezi sub-catchment (Ashton *et al.*, 2001; Nare, 2004).

1.7 RESEARCH DESIGN

The river sediments were collected from these tributaries: Musina abstraction point (boreholes) Marico River, Lephalale River, Crocodile River, Mokolo River, Mogalakwena River, Sand River, Notwane River, Shashe River, Limpopo River and Mzingwane River. The cyanobacterial DNA from the samples were extracted and compared to the DNA at Musina water abstraction point to ascertain the similarities and their differences. The cyanotoxins were also analyzed to determine the nature and types of toxins (and toxic genes) produced by these cyanobacterial species. The physico-chemical parameters were determined to ascertain how these parameters contribute to the growth of cyanobacteria.

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1.8 THESIS OUTLINE

Chapter 2: Literature Review

Chapter 3: The Physical-chemical characteristics of the river sediments

Chapter 4: The Composition of Cyanobacteria Species in the Limpopo River and its Tributaries

Chapter 5: DNA profile of cyanobacteria species in the river sediments

Chapter 6: Conclusion and Recommendations

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CHAPTER TWO: LITERATURE REVIEW

2.1 Algae in fresh waters

Algae can form high biomass blooms which can produce toxins, taste-and-odor compounds which have caused human illness and animal mortalities as well as adverse impacts on the ecosystem and economic worldwide (Hudnell, 2008). High biomass blooms can cause hypoxia (low dissolved oxygen) and their toxin can cause harm or kill fish and other aquatic organisms or detoriation of water quality in other ways (Lopez *et al*., 2008). Recent research studies have demonstrated an increase in proliferation, severity and geographic distribution of HABs worldwide. Recent has also suggested that climate change may promote growth and dominance of harmful algal blooms through a variety of mechanisms such as an increase in water temperatures, changes in salinity, increases in atmospheric carbon dioxide concentrations, changes in rainfall patterns, intensity of coastal upwelling and sea level rise (USEPA, 2013).

Algae exist as different groups of organisms some of bacterial size (1 µm in diameter) and others contain a vast number of cells and can be up to 50 m long. Some algae are plants; some differ so little from bacteria that they could be members of a special bacterial class. Some are undoubtedly animals or may have certain features of both animals and plants. Despite these facts most algal groups have certain features in common (Bushaw-Network and Sellener, 1999). Algae can be classified into the following major groupings Green algae (Chlorophyceae), Yellow-green algae (Xanthophyceae), Golden-yellow algae (Chrysophyceae), Diatoms (Bacillariophyceae), Cryptophyceae, Dinoflagellates (Dinophyceae), Euglenoid flagellates (Euglenophyceae), Red algae (Rhodophy
ceae), Blue-green algae (Cyanophyceae) (Belcher and Swale, 1978).

Blue-green algal blooms can produce toxins, undesirable tastes and odours which cause problems for domestic, industrial, agricultural and recreational users of water (Burch, 1993). Blue-green algal blooms can also have a number of aesthetic effects on water bodies including discolouration, formation of unsightly, smelly scums on the water surface (Murray Darling Basin Ministerial Council, 1993).

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Occurrences of some fresh water HABs have been reported which is as a consequence of human activities and these include increased nutrient loading and pollution, modified hydrology and introduced species (Lopez *et al*., 2008).

Multiplication of algae and cyanobacteria in fresh water have been added by human activities (agricultural runoff, runoff from roads and inadequate sewage treatment) also resulting in eutrophication of many water bodies and affecting considerably recreational water quality (Vollenweider, 1992). Cyanobacteria grow very fast in summer time; this coincides with the period when the demand for recreational water is highest. Many species of fresh water algae proliferate well in eutrophic waters but they do not form blooms, thus the toxins produced are not accumulated to concentrations that are harmful to humans and animals (Chorus & Bartram, 1999).

Multiple interrelating biological, chemical and physical factors enhance proliferation of algae and optimize its growth environment. The optimal growth conditions differ among algae species causing bloom formation and maintenance to be critical. Human activities that disrupt the balance within an ecosystem have been linked to the rise in some freshwater HAB outbreaks (Pearl, 2008).

2.2 The Occurrence of cyanobacteria blooms in the tributaries of the Limpopo River

2.2.1 Hartbeespoort Dam and the Crocodile River

Excessive plant nutrient loadings, originating from the City of Johannesburg and Tshwane Metropolitan wastewater treatment works discharge into the catchment area of the Crocodile River and have resulted in the Hartbeespoort Dam becoming hypertrophic (De Clercq, 2010). The combinations of plant nutrients and biophysical factors have supported the sustained dominance of very dense aggregations of principally *Microcystis aeruginosa*, blue - green algae (cyanobacteria) within the Hartbeespoort Dam (Dower, 2003; Gumbo *et al*., 2010). Uncontrolled and excessive growth of unwanted biomass and in this case specifically the growth of blue - green algae and water hyacinths occurs mainly during the summer months (De Clercq, 2010). The sustained dominance of cyanobacteria produces problems that include tastes and odours in portable waters produced from the dam, impaired recreational and aesthetic uses, and decreases revenue from impoundment-related commercial activities and residential sales (De Clercq, 2010).

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2.2.3 Notwane River

Notwane River catchment in Botswana has faced a growth in water supply demad and increased wastewater loadings because of rapid population growth. Botswana's capital city Gaborone is situated on Notwane River and has shown a substantially growth to be rated as Africa' fastest growing city (Department of Tourism of Botswana, 2001). Nearly 70% of Gaborone residents had been connected to the sewer in 1999 (Tsheko, 1997) and the waste water was treated at Glen Valley Wastewater Treatment Plant (WWTP). Impoundments ponds were used alone for waste water treatment and waste water effluent from the treatment plant annually can add up to 100% of the Notwane River base flow (Pathmanathan, 1999). A report by Serumola (1997) indicated that water quality downstream of the WWTP had decreased. Reports from the studies (McMahon *et al*., 1995; Drolc and Koncan, 1999; Stamou *et al*., 1999; Zagorc-Koncan and Gotvajn, 2000; Ha and Bae, 2001; Sileika *et al*., 2002) had shown degradation in water quality and river eutrophication downstream of WWTP outfalls (Cotman *et al*., 2001). At the regional level, poor water quality in Notwane River has impacts on the Limpopo River as its major tributary (Mladenov *et al*., 2005).

2.2.4 Mzingwane River

A study by Minshull (2008) on dry season fish survival in isolated pools and within sand- beds in Mzingwane River, Zimbabwe identified a Tilapia fish species. A survey done by Hutchison *et al*. (2001) in Mozambique on Tilapia fish revealed that Tilapia fish fed mostly on *Microcystis aeruginosa* (a species of cyanobacteria) and detritus. A study on Nile tilapia (*O. niloticus*) revealed a similar trend of blue-green algae consumption as its main food source (Moriaty and Moriaty, 1973a and b; Yada, 1982; Getachew, 1987).

2.3 Cyanobacteria and its toxins

Cyanobacteria are one of the largest groups of bacteria that form visible colonies but are usually very small to be seen with the naked eye. They grow in water and live in terrestrial, brackish or marine water and use sunlight for photosynthesis. They are often indicted in human and animal illnesses around the world (Chorus & Bartram, 1999). Cyanobacteria blooms are formed upon proliferation of algae that are normally present, and under suitable conditions form bloom that turn clear water cloudy. In warm, slow moving water rich in nutrient, the blooms grow many inches thick and

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normally floats near to the shore line. Blooms can form at any time of the year but they often occur in late summer or early fall (Mez *et al*.,1998). These cyanobacteria can grow in several types of water bodies, but the bloom of greatest concern are the ones that grow in fresh water. They are therefore a cause for concern as they affect and threaten human and ecosystem health (Carmichael, 2008). Cyanobacteria blooms can have a broad variety of appearance, forming colonies, mats, and scums that can range from blue-green to black in color (Mellisa *et al*., 2012). Awful odor often emanates from dead algae of cyanobacterial bloom (Edwards *et al*., 1992). Maier and Dandy (1997) study has shown that the main species of cyanobacteria which are most frequently associated with water quality problems in Australia include *Microcystis, Anabaena, Nodularia, Cylindrospermopsis* and *Oscillatoria*. Under certain conditions, population explosions (blooms) of cyanobacteria occur, which can pose serious problems for water users. *Microcystis* spp, is one of the toxic cyanobacterium genera which is being encountered in surface water sources including the *Planktothrix* and *Anabaena* (Chorus and Bartram, 1999). Most of cyanobacteria are able to produce a wide range of secondary metabolites and some of these compounds have been proven to be toxic for mammals including human and animal poisoning (Table 2.1). They are known as cyanotoxins (van Apeldoorn *et al.*, 2007). Most of these toxins are dermatoxins, hepatotoxins, or neurotoxins, but among cyanobacteria species few can produce each of these toxins (Larry *et al*., 2010). Cox *et al*. (2005) recently proved by laboratory studies that virtually all cyanobacteria species produce the neurotoxin beta-N-methylamino-Lalanine (BMAA). Data demonstrates a 500-fold variation in the amount of BMAA produced among the cyanobacteria species examined. However, the amount of variation is genetic and how much is environmental–physiological is not known at the present time.

Table 2.1 Cyanobacterial toxins of the most dominant species in South Africa, and their functions and mechanisms of action (Falconer, 1998; Sivonen and Jones, 1999; Codd, 2000)

Toxin type	**Primary target organ in mammals**	**Cyanobacteria Taxon**	**Mechanism of toxicity**
Hepatotoxins			
microcystins	Liver	*Microcystis, Oscillatoria, Nostoc, Anabaena*	Inhibition of protein phosphatase activity, haemorrhaging of the liver
Nodularins	Liver	*Nodularia*	Inhibition of protein phosphatase activity,

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			haemorrhaging of the liver
Cytotoxins			
cylindrospermopsin	Liver, kidney, spleen, intestine, heart, thymus	*Cylindrospermopsis*	Inhibition of protein synthesis
Neurotoxins			
anatoxin-a	Nerve synapse	*Anabaena, Oscillatoria*	Blocking of post-synaptic depolarization
Anatoxin-a(s)	Nerve synapse	*Anabaena*	Blocking of acetylcholinesterase
Saxitoxins	Nerve axons	*Anabaena*	Blocking of sodium channels
Dermatoxins			
aplysiatoxins	Skin	*Oscillatoria*	Protein kinase C activators, inflammatory activity
Irritant Toxins			
lipopolysaccharides	Any exposed tissue	All	Potential irritant and

2.3.1 Floating mechanism

Cyanobacteria have developed unique adaptive capabilities through their long history of evolution, which allows them to take advantage of and live in variable environmental conditions. HABs are considered one of the most obvious indicators of nutrient over-enrichment (Pearl and Fulton, 2006). Unlike true algae, many species of planktonic cyanobacteria possess stacks-like specialized intracellular gas vesicles which enables the organism to regulate its floating and thus to actively seek water depths with optimal growth conditions (Chorus and Salas, 1997). When the cells die, these stack of minute proteinaceous hollow releases their contents into the water and their pigments often adopt a copper-blue coloration (Lopez *et al*., 2008). Complemented by bacterial decomposition, these materials are rapidly putrefied in shore-line often resulting in repulsive smells and potentially produce toxins.

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The agglomeration of cyanobacteria is usually caused by planktonic species in eutrophic waters, benthic mats in oligotrophic waters (which are relatively poor in plant nutrients) occasionally causes problems (WHO, 2003). These surface-covering mats can only grow in clear water where sunlight can get to the bottom. On sunny days they photosynthesize and these may result in high rates of oxygen production, forming bubbles that break part of the mats and release them to the surface (WHO, 2003). Mats of benthic cyanobacteria washed to the shore and eaten by dogs have been lethal (WHO, 2003). Awareness of the potential toxicity of such beach algal mats is important because they accumulate along shores of clear waters usually not recognized as potentially producing harmful cyanobacteria or algae.

2.3.2 Production of cyanotoxins

Cyanotoxins represents a diverse group of chemical substances that are characterized by their specific toxic effects as follows (Hudnell, 2010): Neurotoxins affect the nervous system (Anatoxin-a, Anatoxin-a(s), Saxitoxins, Neosaxitoxin), Hepatotoxins affect the liver (Microcystins, Nodularins, and Cylindrospermopsin), Tumor promoters can increase tumor growth (Microcystins), Lipopolysaccharides and can affect the gastrointestinal system.

2.3.2.1 Neurotoxins

Cyanobacteria strains anatoxin-(a) and Saxitoxins are known to be produced Neurotoxins. An exposure to neurotoxins can cause death byparalyzing the peripheral skeletal muscles followed by respiratory muscles which can lead to respiratory arrest in a few minutes (Botha *et al*., 2005). Species and strains of the genera *Anabaena* and *Oscillatoria* produces Anatoxin-(a) which is asecondary amine, 2-acetyl-9-azabicyclo non-2-ene. This alkaloid causes depolarizing neuromuscular blockade, followed by fatigue and paralysis (Carmichael, 1992).

Saxitoxins are known as the products of dinoflagellates, cyanobacteria *Anabaena* and *Aphanizomenon* (Botha *et al*., 2005). The saxitoxins prevent the release of acetylcholine at neuromuscular junctions which also inhibit nerve conduction by blocking sodium channels in axons with a result of muscle paralysis. Paralysis of the respiratory muscles can lead to the death of animals within a few minutes (Runnegar *et al*., 1988).

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It is prudent to presume a toxic potential in any cyanobacterial population. The most widespread cyanobacterial toxins are microcystins and neurotoxins, of which some species contain both neurotoxin and microcystin simultaneously (WHO, 2003). Field populations of the most common bloom-forming genus, *Microcystis*, are almost toxic although non-toxic strains do occur. Toxicity is not trait specific for certain species; rather, most species comprise toxic and nontoxic strains. For microcystins, toxicity of a strain depends on whether does it contain the gene or not for microcystin production (Rouhiainen *et al*., 1995; Dittmann et al., 1996) and that field populations are a mixture of both genotypes with and without this gene (Kurmayer *et al*., 2002).

Experiments with cyanobacterial cultures show that microcystin production is a fairly constant trait of a given strain or genotype and often undergo modification by environmental conditions (Chorus, 2001). While conditions leading to cyanobacterial proliferation are well understood (although the physiological or biochemical function of toxins for the cyanobacteria is the subject of many hypotheses (Chorus & Bartram, 1999), the factors leading to the dominance of toxic strains over non-toxic ones are poorly elucidated. Globally, about 60% of samples of cyanobacteria investigated contain toxins and time space also may change the toxicity of a single bloom (WHO, 2003).

Occurrence of toxicity of cyanobacterial in a given water body do not necessarily imply environmental or health hazard so long as the cells remain thinly dispersed (WHO, 2003). Mass developments and especially surface scums, pose the risks. Microcystins are the most frequently occurring and widespread of the cyanotoxins and are cyclic heptapeptides containing specific amino acid (ADDA) side chain. To date they have been found only in microcystins and nodularin (a cyclic pentapeptide toxin of cyanobacteria from brackish waters). Seventy structural analogues of microcystin have been identified (Rinehart *et al*., 1994; Sivonen & Jones, 1999) with their methyl groups and two amino acids vary within the ring. The consequences for the tertiary structure of the molecule result in pronounced differences in toxicity as well as in hydrophobic/hydrophilic properties. The protein phosphatases 1 and 2a are important molecular switches in all eukaryotic cells and with an irreversible covalent bond that can be blocked by the presence of microcystins (MacKintosh *et al.,* 1990).

2.3.2.2 Hepatotoxins

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Hepatotoxins cause liver tissues damage and pooling of blood that can lead to fatal circulatory shock or liver failure (Steidinger *et al.,* 1997). Hepatotoxins have been implicated in toxic events in virtually worldwide (Carmichael, 1997). It is now known that hepatotoxins are represented by many related forms consisting of cyclic or ringed peptides. Those consisting of seven amino acids are called microcystins, and those consisting of five amino acids are labeled nodularins. An additional hepatotoxin isolated from *Cylindrospermopsis raciborskii* has been labeled cylindrospermopsin (Steidinger *et al.,* 1997). Reported hazards to human health include gastroenteritis, asthma, eye irritation, blistering around the mouth and nose, and toxic injury to the liver.

Microcystins (MCs) are potential hepatotoxins and have been also implicated in liver tumor promotion (Nishiwaki-Matsushima *et al.,* 1992). Microcystins are synthesized by a large number of cyanobacterial genera and are most notable globally distributed (Tian *et al.,* 2013). More than 80 structural variants have been identified from field samples or isolated strains of cyanobacteria (Kruger *et al*., 2009). The most commonly abundant MCs include microcystin-LR (MC-LR), microcystin-RR (MC-RR) and microcystin- YR (MC-YR). Water borne MCs have resulted in the death of 76 patients in Brazil (Jochimsen *et al*., 1998). Epidemiological investigations suggested that microcystins may be responsible for most incidence of liver cancer in populations depending upon drinking water contaminated by microcystin toxin in China (Ueno *et al*., 1996), Serbia (Svircev *et al*., 2009), and Florida of the United States (Fleming *et al*., 2002), as well as colorectal cancer in China (Zhou *et al*., 2002).

Previous studies on the mechanism of action of cylindrospermopsin have shown that in mouse hepatocytes in vivo the toxin disrupts protein synthesis (Tereo *et al*., 1994). The main target of this toxin is the liver, but it can also affect different organs such as the lungs, kidneys, adrenals and intestine, unlike the microcystins (Falconer, 1998). Genotoxic activity is due to the ability of cylindrospermopsin induce strand breaks at the DNA level and loss of whole chromosomes (Humpage *et al.,* 2000).

2.3.2.3 Dermatoxins

Cyanobacterium *Lyngbya majuscule* is responsible for the production of dermatoxi Lyngbyatoxin A and Aplysiatoxin and has different metabolite constiuents in deep and shallow water varieties. a

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marine benthic cyanobacterium with different metabolite constituents in deep and shallow water varieties. Deep water varieties produce inflammatory substances and tumor promoters whereas shallow water produces lipophilic substances, malyngamides A, B and C. Clinical symtoms includes skin, eye and respiratory irritation (Osborne *et al*., 2001).

2.4 Effects of cyanotoxins on human and the environment

Freshwater HAB toxins can have several adverse effects on humans, animals, and aquatic ecosystems. Some cyanobacteria can produce neurotoxin, dermatoxins, hepatotoxin, or other bioactive compounds; and blooms of toxigenic cyanobacteria pose a particular threat if they are present in drinking water sources (Steidinger *et al*., 1997). An advisory limit of 1 μg L^{-1} for microcystin-LR (most common cyanobacterial toxins) has been issued by the World Health Organization for drinking water (Lopez *et al*., 2008).

2.4.1 Impact on humans

Hitzfeld *et al*. (2000) observed that concentrations of cell-bound microcystins declined while extracellular toxin concentrations remained constant after flocculation and filtration in conventional water treatment plants. Pietsch *et al*. (2002) discovered that flocculation and filtration causes an increase of extracellular toxin after experiments with *Microcystis aeruginosa* and *Planktothrix rubescens*; it was based on the suggestion that turbulences in pipes and the pressure gradients in the filter bed might be the major causes of the increase. Hitzfeld *et al*. (2000) also reported that the chlorination process in conventional water treatment plants was only effective in destroying intact cyanobacterial cells; whereas the intracellular biotoxin was released into the treated water.

The major pathway for microcystins entrance into cells is through the bile acid carrier, which is present in liver cells and to a lesser extent in intestinal epithelia (Falconer, 1993). In vertebrates, a lethal dose causes death in liver necrosis within few hours up to a few days. Evidence for the permeability of other cell membranes to microcystins remains controversial. However, it is possible that hydrophobic structural analogue can find their way into some cell types even without the bile acid carrier (Codd, 1995).

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Fitzgeorge *et al*. (1994) illustrate disruption of nasal tissues by the common hydrophilic analogue microcystin-LR while toxicity uptake through the mouth is generally at least less than toxicity by intraperitoneal (i.p.) injection. Fitzgeorge *et al*. (1994) also demonstrated that microcystin toxicity is cumulative, which implies that a single oral dose can not increase the liver weight (which is a measure of liver damage), while if the same dosage is applied daily for seven days can cause a rise in liver weight of 84% and thus had the same effect as a single oral dose 16 times as large. These may be explained by the irreversible covalent bond between microcystin and the protein phosphatases and subsequent substantial damage to cell structure (Falconer, 1993). Healing of the liver requires growth of new liver cells. Annadotter *et al*., (2001) describe incidents of influenza-like symptoms in several Scandinavian towns and in Harare (Zimbabwe) that happen shortly after people had inhaled steam while bathing, showering or sauna and after washing dishes. The cause was shown to be due to cyanobacterial endotoxins (lipopolysaccharides) in the local water supply due to blooms in local drinking water reservoirs.

2.4.2 Impact on aquatic ecosystem

Freshwater HAB taxa are harmful either by producing toxins that kill fish and other aquatic organisms or by forming high biomass blooms that can cause hypoxia (low dissolved oxygen) and degrade water quality in other ways (Hudnell, 2008). Ecosystem impacts from the effects of nontoxic, high biomass HABs are well documented (Fournie *et al*., 2008). Low oxygen concentration that suffocate and kill fish and bottom-dwelling organisms are the most common adverse impact of high biomass blooms. In addition, high concentration of blooms can reduce sunlight penetration into the water, thereby preventing growth of beneficial algae. Food web disruption can also be the result of the unpalatability and low food quality of many cyanobacteria, which can result in the starvation of aquatic species.

Cyanotoxins can accumulate in the primary consumers (Prepas *et al*., 1997) and potentially be transferred up the food web. Cyanotoxins have been found to be the cause of mass mortalities of fish (Peñaloza *et al*., 1990; Tencalla *et al*., 1994; Rodger *et al*., 1994) and birds (Koeing, 2006), and have also been implicated in the death of pets (Walker *et al*., 2008) and livestock (Carmichael, 1992).

Rashes, allergies, and gastrointestinal problems of people working or recreating on the water has been reported upon recreational exposure to toxic HABs via direct skin contact, inhalation, or

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inadvertent ingestion (WHO, 2003). Cyanotoxins can get in contact with humans, animals and the environment through drinking of contaminated water that comes from a lake or reservoir with HAB, drinking of untreated water, engaging in recreational activities in waters polluted with HABs, inhaling aerosols from water-related activities such as jet-skiing or boating, Inhaling aerosols when watering lawns, irrigating golf-courses etc. with pond water, using cyanobacteria-based dietary supplements that are contaminated with microcystins and receiving dialysis (WHO, 2003).

2.5 DNA finger printing

DNA based fingerprinting method may be employed to have an idea of the diversity changes, or closeness of community structure among samples or for screening of a large amount samples. A study done by Frazao *et al*. (2010) used PCR method to determine genes that are responsible for production of kown cyanotoxins such as microcystins, nodularins and cylindrospermopsin. The study revealed the presence of *mcyE* gene that was found in one *Leptolyngbya* strain and one *Oscillatoria* strain and is responsible for the production of cyanotoxins.

Metcalf *et al*. (2012) carried out a study to analysis microcystins and microcystin genes in 60-170-year-old dried herbarium using the method of DNA extraction and PCR. Specimens where collected from aquatic and terrestrial environments in 11 countries. These specimens were dried and stored at ambient temperatures in the dark for up to 170 years. The result showed the presence of microcystins and synthetase gene *mcyD* DNA. In another study carried out by Valerio *et al*. (2005) samples of freshwater lakes and rivers in Portugal were analyzed for the presence of cyanobacteria strains using the DNA extraction, amplification and PCR fingerprinting and 16S rRNA PCR methods. It demonstrated ability to differentiate strains at intra-specific level.

2.6 Toxic genes identification

Botha and Oberholster conducted a study in South Africa between 2004-2007, using RT-PCR and PCR technology to differetiate *Microcystis* strains bearing the *mcy* genes, which correlate with their ability to synthesize the cyanobacterial biotoxin microcystin. The study revealed that 99 % of South Africa's major impoundments contained toxicogenic strains of *Microcystis* (Botha and Oberholster, 2007).

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The study on quantification of microcystin producing cyanobacteria was carried out by Furukawa *et al*. (2006) using real time PCR. It was discovered that the detection unit of this method was 8.8 cells per reaction and that cell concentration determined by real time PCR positively correlated with the cell concentration determined from direct microscopic count. Quantitative PCR was used to detect and quantify toxin genes from microcystin, nodularin and cylindrospermopsin and saxitoxins biosynthesis pathways. It was discovered that it is a powerful tool for detecting and quantifying potentially toxic cyanobacteria in the laboratory and field samples (Al-Tebrineh *et al*., 2011).

2.7 Conclusion

Recent research studies have demonstrated an increase in proliferation, severity and geographic distribution of harmful algal blooms (HABs) worldwide. Research has also suggested that climate change may promote growth and dominance of HABs through a variety of mechanisms such as an increase in water temperatures, changes in salinity, increases in atmospheric carbon dioxide concentrations, changes in rainfall patterns, intensifying of coastal upwelling and sea level rise. Blue-green algal blooms produce toxins, undesirable tastes and odours which cause problems for domestic, industrial, agricultural and recreational users of water. Occurrences of some fresh water HABs have been reported which is as a consequence of human activities and these include increased nutrient loading and pollution, modified hydrology and introduced species. Excessive plant nutrient loadings, originating from the City of Johannesburg and Tshwane Metropolitan wastewater treatment works discharge into the catchment area of the Crocodile River and have resulted in the Hartbeespoort dam becoming hypertrophic. The combinations of plant nutrients and biophysical factors have supported the sustained dominance of very dense aggregations of principally *Microcystis aeruginosa*, blue-green algae (cyanobacteria) within the Hartbeespoort Dam. A water quality monitoring study compiled, indicated that water quality for at least 30 km downstream of the WWTP was impaired. Poor water quality in the Notwane River has consequences at the regional level because the Notwane River is a major tributary of the Limpopo River, which Botswana shares with the Republic of South Africa.

Cyanobacteria are one of the largest groups of bacteria that form visible colonies but are usually very small to be seen with the naked eye. Cyanobacteria blooms can have a broad variety of appearance,

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forming colonies, mats, and scums that can range from blue-green to black in color. Most of cyanobacteria are able to produce a wide range of secondary metabolites and some of these compounds have been proven to be toxic for mammals and also responsible for human or animal poisoning. They are known as cyanotoxins. Most of these toxins are dermatoxins, hepatotoxins, or neurotoxins, but among cyanobacteria species few can produce each of these toxins. Cyanotoxins represents a diverse group of chemical substances that are characterized by their specific toxic effects as follows: Neurotoxins affect the nervous system (Anatoxin-a, Anatoxin-a(s), Saxitoxins, Neosaxitoxin), Hepatotoxins affect the liver (Microcystins, Nodularins, and Cylindrospermopsin), Tumor promoters can increase tumor growth (Microcystins), Lipopolysaccharides and can affect the gastrointestinal system.

A study done using PCR method to determine molecular analysis of genes involved in the production of known cyanotoxins such as microcystins, nodularins and cylindrospermopsin. The study revealed the presence of *mcyE* gene that was found in one *Leptolyngbya* strain and one *Oscillatoria* strain and is responsible for the production of cyanotoxins. In another study carried out samples of freshwater lakes and rivers in Portugal were analyzed for the presence of cyanobacteria strains using the DNA extraction, amplification and PCR fingerprinting and 16S rRNA PCR methods. It demonstrated ability to differentiate strains at intraspecific level. Botha and Oberholster performed a survey in South Africa between 2004-2007, using RT-PCR and PCR technology to distinguish *Microcystis* strains bearing the *mcy* genes, which correlate with their ability to synthesize the cyanobacterial biotoxin microcystin. The study revealed that 99 % of South Africa's major impoundments contained toxicogenic strains of *Microcystis*.

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CHAPTER THREE: THE PHYSICAL-CHEMICAL CHARACTERISTICS OF THE RIVER SEDIMENTS

Thus the main objective of this chapter is to assess the physical-chemical characteristics of river sediments and how these contribute to resurgence and growth of cyanobacteria should ideal conditios return especial the river flow.

3.1 INTRODUCTION

Eutrophication is a build-up of organic matter produced by phototrophs, such as algae (Paerl *et al.*, 2001). This build-up is often seen as algal blooms and is driven by inputs of nitrogen and phosphorus (Paerl *et al.*, 2001). The cyanobacteria blooms are a major concern worldwide due to the production of cyanotoxins which are harmful to humans (Boyer, 2006). Cyanobacteria tend to dominate during the summer when concentrations of total phosphorus fall between 100-1000 ug/L (O'neil et al., 2012). A variety of hypotheses exist to explain why cyanobacteria blooms are becoming increasingly prevalent (Vitousek *et al.*, 1997; Bianchi *et al.*, 2000; Scheffer, 2004; Reynolds, 2006). The most common hypotheses focus on nutrient conditions (Smith, 1983; Vitousek *et al.*, 1997; Hyenstrand, 1999; Bianchi *et al.*, 2000; Berman *et al.*, 2001; Downing *et al.*, 2001; Von Ruckert & Giani, 2004) and nutrient cycling (Scheffer, 2004; McCarthy *et al.*, 2007) within a water body, as well as aspects of cyanobacteria cell physiology, such as their ability to migrate vertically within the water column, fix atmospheric nitrogen and produce cyanotoxins (Andersen & Shanmugam, 1977; Visser, 1995; Thiel & Pratt, 2001; Chan *et al.*, 2004).

Cyanobacterial blooms are often associated with eutrophic conditions (Vitousek *et al.*, 1997; Smith *et al.*, 1999; Bianchi *et al.*, 2000), so many studies have documented the relationship between nitrogen and phosphorus concentrations, speciation and stoichiometry, and cyanobacteria occurrence. Recently it was reported that *Microcystis* growth responds increase to nitrogen over phosphorus. The same study also shows that the growth response of toxin *Microcystis* to nitrogen was greater that

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non-toxin strains. Some species of cyanobacteria are known for their ability to fix nitrogen giving them high chances for production cyanotoxins. Other studies have shown that toxicity is also influenced by change in pH, temperatures and light intensity. A study by Beversdorf *et al.* (2013) indicated that some of the non-nitrogen fixing cyanobacteria may produce toxins because of nitrogen stress events.

Cyanobacteria have cell which have different specialized functions (Maldener *et al.,* 2014). Such differentiated cells include resting cells, spores, akinetes and cysts which represent a survival strategy of cyanobacteria under unfavorable environmental condition which limit the growth (Perez *et al.,* 2016; Kim *et al.,* 2016). Under favorable conditions the cell will germinate again to form new strains (Adam and Dugga, 1999). The ability of cyanobacteria persistence towards surviving dry periods allows them to in habitats with fluctuating conditions (Perez *et al.,* 2016). The study of Kim et al. (2016) further illustrated the viable nature of cysts and akinetes in providing the next inoculum of *Microcystis*, *Anabaena*, *Aphanizonenon* and *Oscillatoria* is Bukhan, Namhan Rivers and Lake Paldang and Kyeongan stream, in South Korea. Thus the main objective of the study is to assess the physical-chemical characteristics of river sediments and how these contribute to the resurgence and growth of cyanobacteria should ideal conditions return especial the river flow (Figure 3.1).

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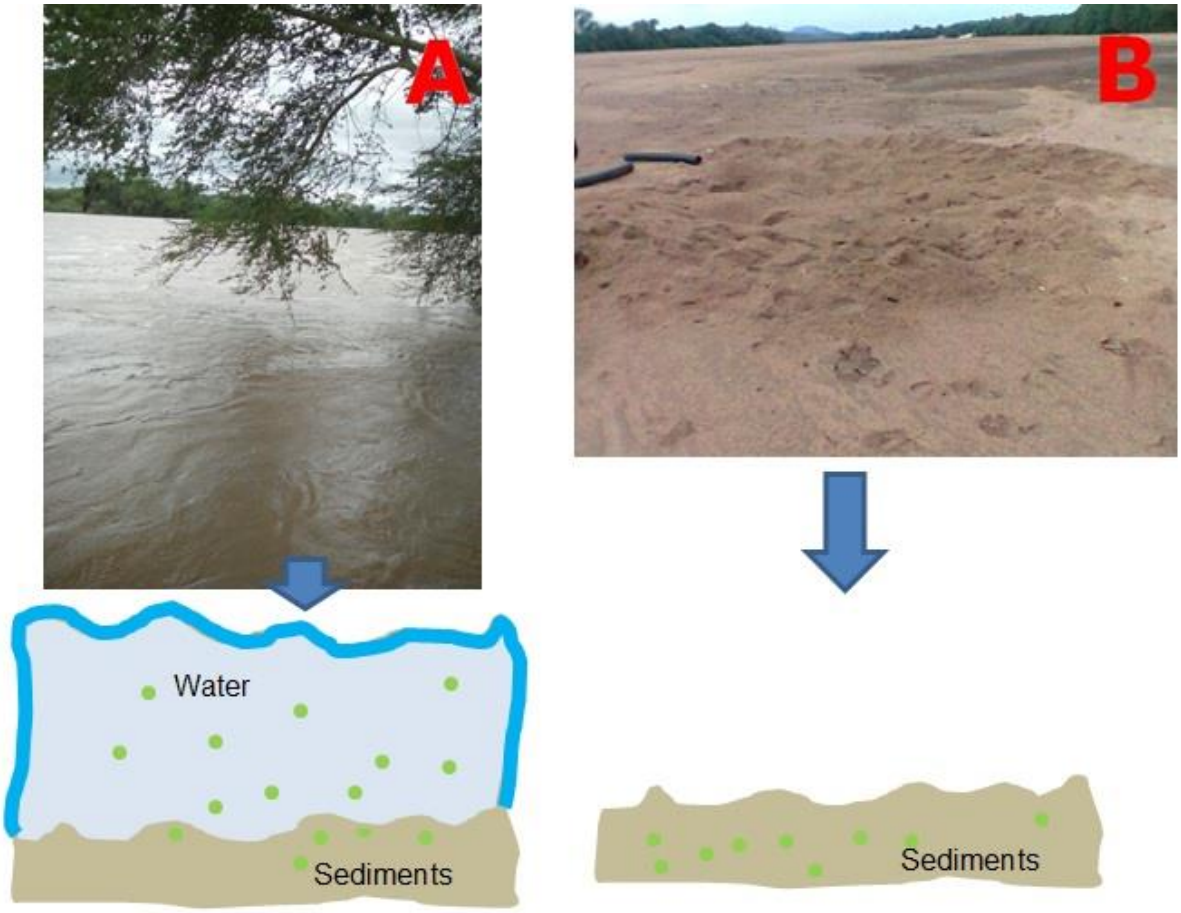

Figure 3.1: A scenario involving sedimentation of cyanobacteria (green dots) cysts and akinetes (A) during flood and flow conditions in Limpopo River and (B) during non-flow (DRY) conditions in the Limpopo River

3.2 MATERIALS AND METHODS

3.2.1 SAMPLING

The grab river sediments were collected in October and November, 2014. The river sediments were collected in sterile glass containers from rivers and tributaries of the Limpopo River (Table 3.1). The use of river sediments was chosen because most suspended material including cyanobacteria spores and cysts settles at the river bottom where they became part of sediments in the river.

Table 3.1: The location of sample sites and sample codes

River Names	Samples Numbers
Limpopo River (Groblers' bridge)	S1

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Notwane River (Odi Bridge-Matabeleng)	S2
Sand River upstream	S3
Mogalakwena River next to Tolwe	S4
Mawoni River downstream Makhado oxidation ponds	S5
Lephalala river	S6
Mokolo River	S7
Crocodile River downstream Hartbeespoort dam	S8
Nzhelele River downstream near Tshipise	S9
Sand River downstream (at bridge on N1 road towards Musina)	S10
Crocodile River downstream (near bridge on road D1235) near Thabazimbi	S11
Nzhelele River upstream near Mphephu resort (downstream of Siloam oxidation ponds)	S12
Mzingwane River (Zimbabwe)	S13
Shashe River (near Irrigation scheme, Zimbabwe)	S14
Limpopo River next to Thuli coal mine	S15
Limpopo River abstraction point @ 0.0 m	S16
Limpopo River abstraction point @ 1.0 m	S17
Limpopo River abstraction point @ 1.68 m	S18

3.2.2 PHYSICAL MEASUREMENTS

In the laboratory, the physical measurement of pH, Total dissolved solids (TDS) and electric conductivity (EC) were carried out using Portable pH meter Crison MM40 on the river sediments. It was first calibrated as per the manufacturer's guidelines. The pH, TDS and EC of the sediments were determined by the method of Bates (1954) 50g of sediment was diluted with 50ml of distilled water in 100 ml beaker to produce a ratio of 1:1. The mixture was stirred with a stirring rod to homogenize the mixture and was then left for 30 min to settle. EC, pH and TDS were then measured by inserting the electrodes in the soil solution and readings were taken.

3.2.3 NUTRIENTS ANALYSES

Nutrients analyses were done to determining Total Phosphate (TP) and Total Nitrogen (TN). The analyses were done in duplicates and the aliquot of all digested samples were analyzed with Merck

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Spectroquant® Pharo 100 spectrophotometer Merck (Darmstadt, Germany) with a wavelength of 320-1100 nm.

3.2.3.1 Total Phosphorus Analysis

Total phosphorus was determined by using the perchloric acid digestion method as described by APHA (1998): 2 g of air dried sediment was acidified to methyl orange with concentrated HNO_3, another 5 ml of concentrated HNO_3 was added and evaporated on a hot plate until the dense fume appear. 10 ml each of concentrated HNO_3 and $HClO_4$ was added and evaporated gently until the dense white fumes of $HClO_4$ appear. The solution was then neutralized with 6N NaOH and made up to 100 ml with distilled water. Aliquots of the samples were then analysed with spectrophotometer using phosphate cell test kit.

3.2.3.2 Total Nitrogen Analysis

Total Nitrogen was determined per APHA (1998) as ammonia: 1 g of each air-dried sediment sample was treated with 2 ml of Sulphuric acid. The sample was heated on a hotplate for 2 hours. Aliquots of 50 ml of deionized water were added to each sample. The sample was filtrated through No. 41 Whatman filter paper. The filtrate of each sample was made up to 250 ml with deionized water and 55 ml of 1 M sodium hydroxide solution. Aliquots of the samples were then analyzed with spectrophotometer using nitrate cell test kit.

3.2.3.3 Total Organic Carbon (TOC) Analyses

Extraction of TOC was carried out by adding 200 ml of deionized water to 6 g of sediment in a plastic bottle and was shaken for 24 h on an orbital shaker at 24 ^{0}C after the shaking the suspension was centrifuged at 900 *xg* for 30 min and filtered through 0.45 µm pore size filter. TOC was determined by using TOC cell kits following the manufacturer's guidelines and the analysed by Merck Spectroquant® Pharo 100 spectrophotometer Merck (Darmstadt, Germany) with a wavelength of 320-1100 nm.

3.3 DATA ANALYSIS

The physico-chemical and cyanotoxins measurements were conducted in duplicates, the standard deviation and the mean were calculated, using Microsoft (MS) Excel (2010) spread sheet for each sampling point. The graphs were plotted using MS Excel spread sheet. The statistical analysis was

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carried out using single factor ANOVA technique to determine any significant difference between physical and nutrient variables across sampling sites at $p<0.05$.

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3.3 RESULTS AND DISCUSSION

3.3.1 The physical characteristics of the river sediments

The physical characteristics of the river sediments showed considerable variation from the different tributaries of the Limpopo River and the Limpopo River itself (Figure 3.2). The river sediments electrical conductivity (EC) and total dissolved solids (TDS) values varied between 21.2 and 1269 μS cm^{-1} throughout the sampling sites. The temperatures measured during the sampling trips were more than 22 °C. High temperatures due to climate change have also been mentioned as an important factor in the global expansion of harmful algal bloom worldwide (Paul, 2008). By rising temperature and exceeding 20°C, the growth rate of cyanobacteria keeps growing whereas other freshwater eukaryotic phytoplankton growth rate decreases and this is regarded as a competitive advantage for cyanobacteria (Peperzak, 2003). A study done O'Neil *et al.* (2012) reported that higher temperatures not only promote the dominance of cyanobacteria, but also favor the production of microcystins and result in an increase in their concentration.

The pH values were in the range of 6.4 and 8.5. Higher pH value has a competitive advantage for many cyanobacteria, because of their strong carbon-concentrating abilities compared to eukaryotic phytoplankton species (Rantala *et al.,* 2006). A laboratory experiment done by Jahnichen *et al.* (2001) with *Microcystis aeruginosa* showed that microcystin production started when pH exceeded 8.4, indicating a lack of free carbon dioxide (CO_2).

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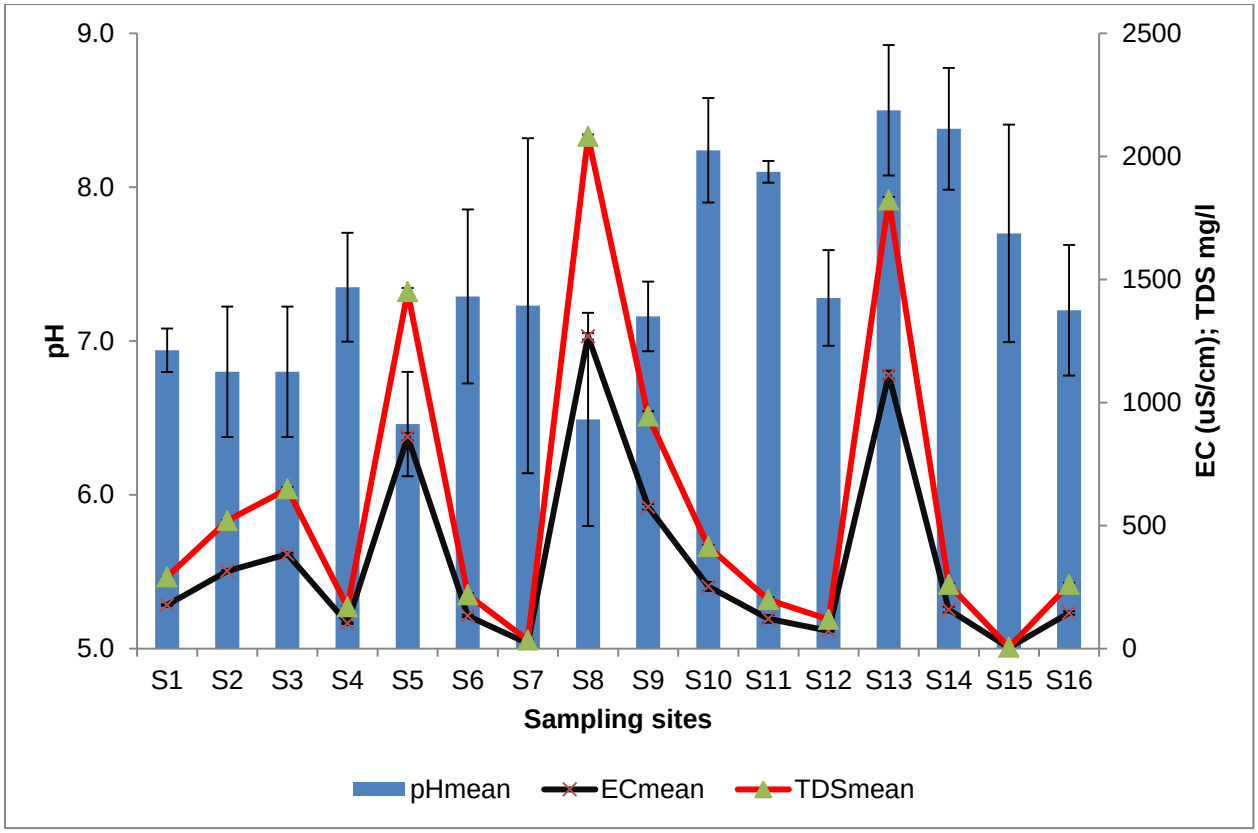

Figure 2.2: The physical characteristics of the river sediments

3.3.2 The abundance of nutrients in the river sediments

The increased input of nutrients into surface water is the main factor responsible for massive proliferations of cyanobacteria in fresh water, brackish and coastal marine ecosystem. However, phosphorus and nitrogen nutrients in high levels lead to accelerated growth of cyanobacteria (Bartram *et al.*, 1999; Mur *et al.*, 1999).

3.3.2.1 Total Phosphorus

The total phosphates concentration values in the river sediments were from 0.5 mg/l to the highest of 6.3mg/l (Figure 3.3). The highest value was recorded for Nzhelele River (S12) near Mphephu resort and downstream of Siloam hospital oxidation ponds (Figure 3.3). The low value for Lephalale River (S6) is because Phosphorus has been implicated more widely than nitrogen as a limiting nutrient of phytoplankton including cyanobacteria in freshwater systems (Hart *et al.*, 1993). A minute amount of phosphates entering or becoming soluble in a water body can trigger a significant algal bloom. The impact of excess phosphates in the receiving river streams is shown the green colour presence of

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cyanobacteria. Limpopo River (S1) receives inflows from Notwane and Crocodile Rivers and this contributes to the phosphates loading of the river (Figure 6). Notwane River (S2) receives municipal discharge from Glen Valley sewage plant and from rainfall from fertiliser that is rainfall runoff agricultural land around the river (Figure 3.6). Sand River (S3) receives municipal discharges from the Polokwane sewage plants and from rainfall runoff from fertiliser on agricultural land around the river (Figure 3.7). Mzingwane River (S13) receives municipal discharge from West Nicholson sewage plant and from rainfall runoff with fertilizer from agricultural land around the river (Figure 3.8). These rivers are some of tributaries of the Limpopo River (S15) and they contribute the successive loading of phosphates in the Limpopo River (Figure 3.9).

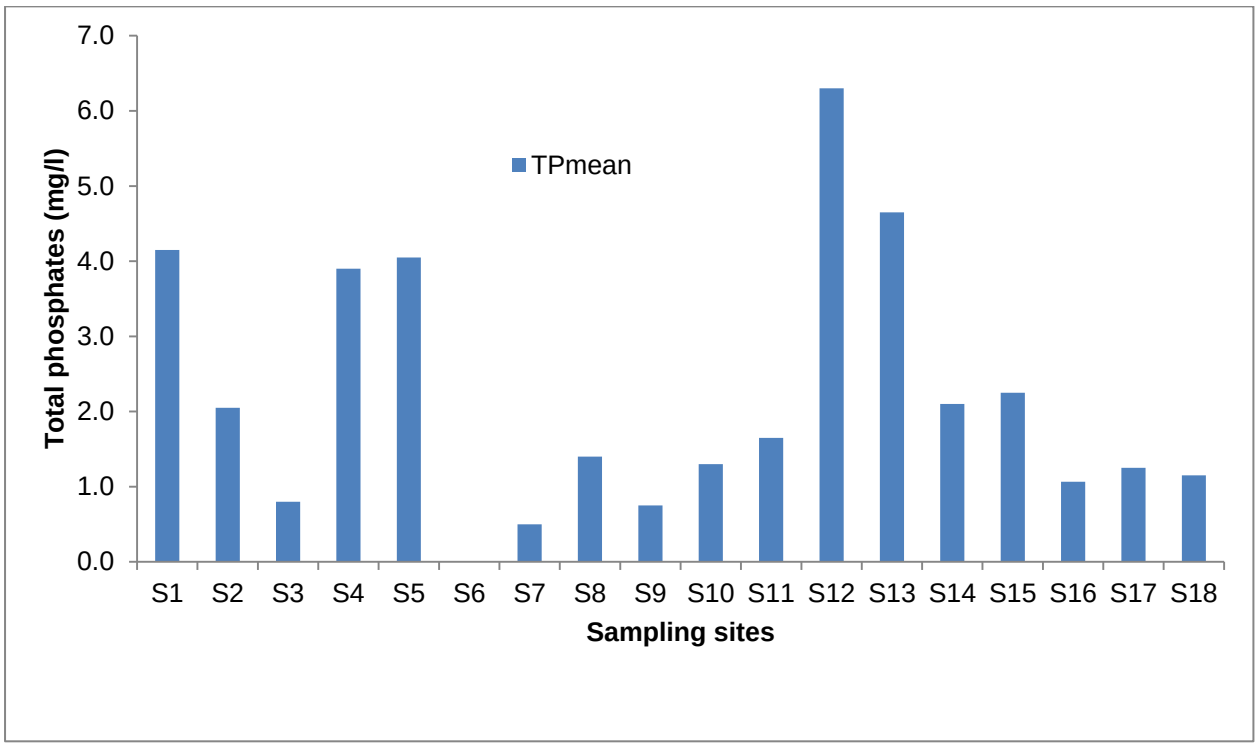

Figure 3.3: The levels of total phosphorus in the river sediments.

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Figure 3.4: Nzhelele River upstream next to Mphephu resort

Figure 3.5: Limpopo River upstream of Groblers Bridge

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Figure 3.6: Notwane River

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Figure 3.7: Sand River Upstream

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Figure 3.8: Mzingwane River

Figure 3.9: Limpopo River (Tuli coal mine)

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3.3.3.1 Total Nitrates

The nitrates concentration values in the river sediments were ranging from 1.5mg/l to 6.5mg/l (Figure 3.10). The highest value was recorded for Nzhelele River (S12) near the Mphephu resort and downstream of Siloam hospital oxidation ponds (Figure 3.4). The reason for the highest value at Nzhelele River was possible due the hospital oxidation ponds from the sewage because of the sewage effluent from Siloam hospital. Filamentous Cyanobacteria can obtain the nitrates by fixing the atmospheric nitrogen gas and make the nitrates they use for their growth. Nitrogen is a common gas (79%) that is found in the atomsphere. Thus cyanobacyeria such as *Anabaena* are able to utilize atmospheric nitrogen in addition to nitrates origining from the river sediments. The other sample sites with nitrates in excess of 2 mg/l are Sand River (S4); Mawoni River (S5); Crocodile River (S11); Mzingwane River (S13); Limpopo River (S16 to S18). These tributaries have municipal sewage plant upstream of sampling sites and are also surrounded by farmland where commercial irrigation farming is practiced as the case with Crocodile & Sand rivers and subsistence agriculture is practiced as the case of Mawoni and Mzinagwane rivers. The Crocodile River aslso receives inflows from eutrophic Hartbeespoort dam. The Limpopo River (S16 to S18) is downstream of all the sample points and this shows the cumulative discharge of nitrates originating from the tributaries. The Musina local municipality has drilled 8 boreholes in the Limpopo river bed and most of these boreholes are located close to S16 (Figure 3.11).

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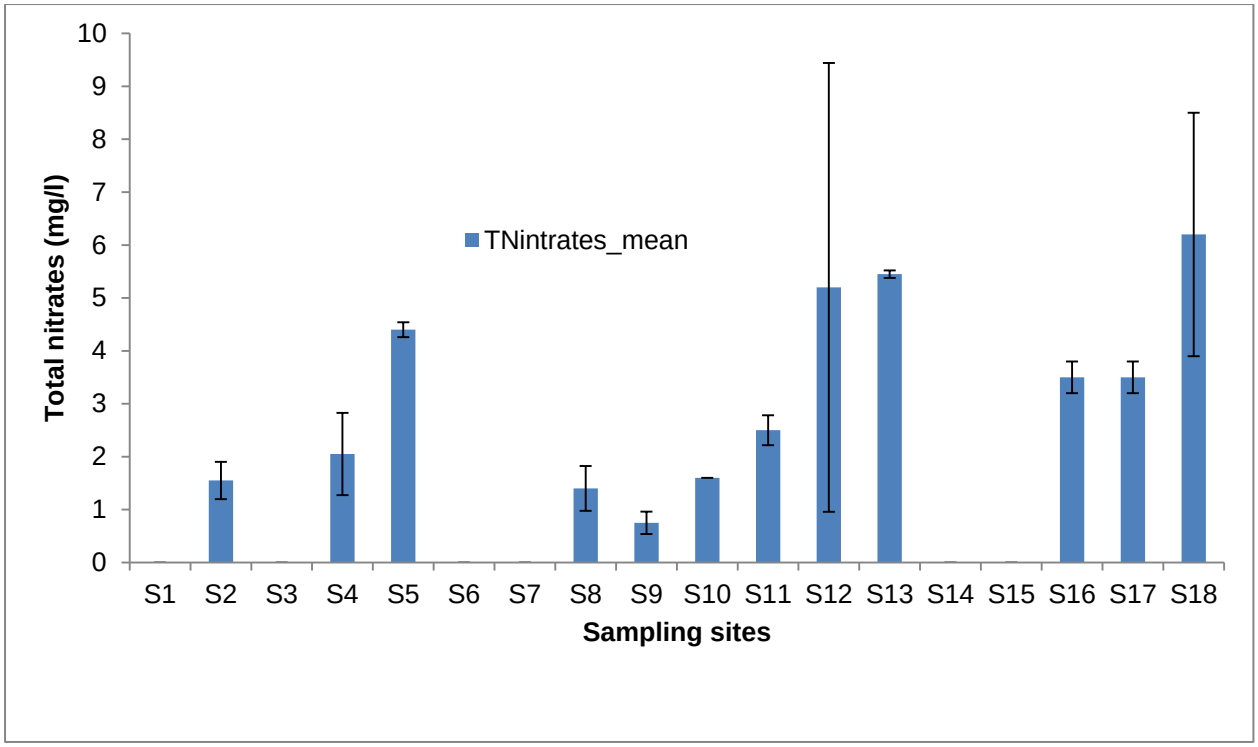

Figure 3.10: The levels of Total Nitrogen in the river sediments.

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Figure 3.11: Musina borehole abstraction point drilled into Limpopo River (Mavhunga, 2015).

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3.3.3.2 Total Organic Carbon

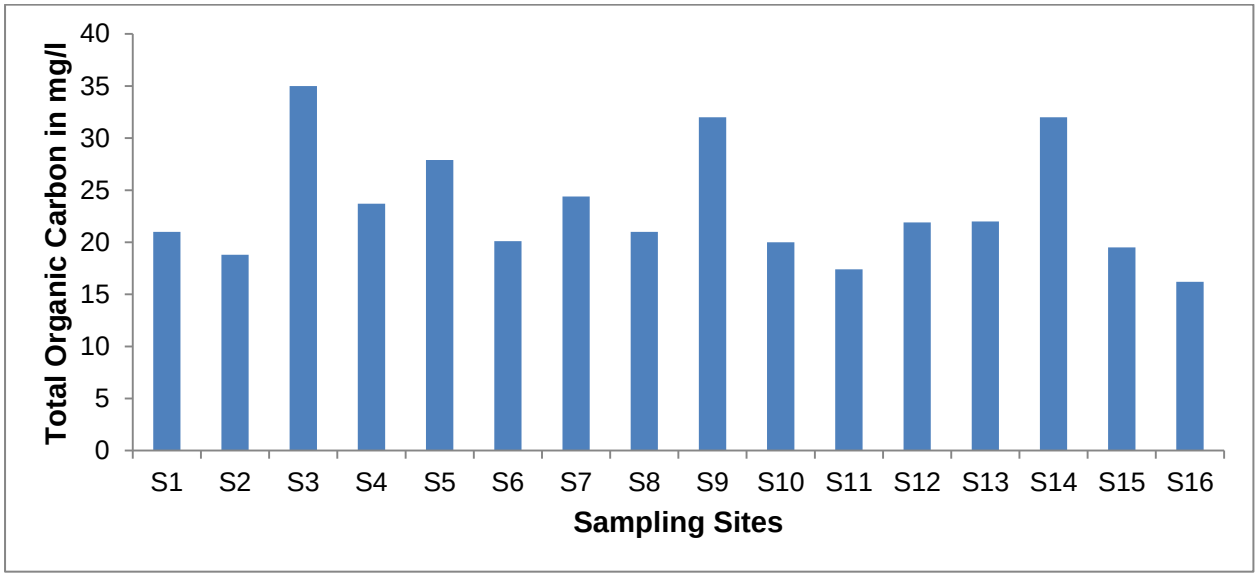

Figure 3.12: The levels of Total Organic Carbon in the river sediments

Presence of TOC influences the growth of cyanobacteria by proving food when the rivers start to flow or when flowing increasing cyanobacteria production (Figure 3.12). Since food will be easily obtained, cyanobacteria turn to proliferate in high density in the rivers leading to water quality decreasing. Ozonation has been shown to be a more effective method for removal of cyanotoxins and if the TOC in raw water is in high quantity the efficcancy of ozone in destroying cyanobacteria is dramatically lowered (Hoeger *et al.*, 2002). When the levels of TOC are higher than 3.0 mg/l, ozone concetrations of <1.0 mg/l can not remove or completely destroy the cyanotoxins (Hoeger *et al.*, 2002).

3.4 CONCLUSION

Limpopo River is a source of water supply to Musina Township after some sort of water treatment. Chemical and physical parameters analysis showed a highly chance of cyanobacteria growth in the rivers. Enrichment of the nutrients in the rivers will be responsible for the growth of cyanobacteria blooms which can lead into production of cyanotoxins. These toxins will cause severe impacts to human and animals using water from the rivers. Many countries in Africa have reported cases of intoxication and deaths of animal that may have been caused by cyanobacterial toxins. Monitoring of the nutrients loads into the river system will decrease the threat of cyanobacteria blooms to human and animal health.

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CHAPTER FOUR: The Composition of Cyanobacteria Species in the Limpopo River and its Tributaries

4.1 INTRODUCTION

Blue-green algal blooms produce toxins, undesirable tastes and odours which cause problems for domestic, industrial, agricultural and recreational users of water (Burch, 1993). Blue-green algal blooms can also have several aesthetic effects on water bodies including discolouration, formation of unsightly, smelly scums on the water surface (Murray Darling Basin Ministerial Council, 1993).

Occurrences of some fresh water HABs have been reported which is because of human activities and these include increased nutrient loading and pollution, modified hydrology and introduced species (Lopez *et al*., 2008).

Multiplication of algae and cyanobacteria in fresh water has been caused by human activities (agricultural runoff, runoff from roads and inadequate sewage treatment) also resulting in eutrophication of many water bodies and affecting considerably recreational water quality (Vollenweider, 1992). Cyanobacteria grow very fast in summer time; this coincides with the period when the demand for recreational water is highest. Many species of fresh water algae proliferate well in eutrophic waters but they do not form blooms, thus the toxins produced are not accumulated to concentrations that are harmful to humans and animals (Chorus & Bartram, 1999).

The main objective of the study is to determine suitable growth conditions for cyanobacteria spores and cysts in the river sediments to allow for proliferation of the cyanobacteria and thus allowing the identification of the cyanobacteria species.

4.2 MATERIAL AND METHODS

4.2.1 THE CULTURE OF CYANOBACTERIA SPECIES IN RIVER SEDIMENTS

Modified BG11 medium was laboratory prepared as per the method developed by Kruger and Eloff (1977) for cyanobacteria culturing. The medium was prepared using mineral composition shown in Table 4.1 and trace elements in Table 4.2. Three 1000ml conical flasks were filled with 850ml of deionized water, minerals and trace elements were also added to each flask. The solutions were shaken untill the minerals were completely dissolved and mixed. The conical flasks were filled up to

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1000ml with deionized water. The medium was autoclaved at 121 ^{o}C for 15 minutes. Aproxiamatily 200 g of bottom river sediments for each sample which were collected in chapter three were transferred into eighteen 250 ml Honey jar containing 200ml of modified BG11 medium under sterile condition. The cultures where incubated for 30 days under continuous light (1100 lux) fluorescent lamps at room temperature (25 ^{o}C).

Table 4.1: Modified BG11 mineral composition (Krüger and Eloff, 1977).

Component	**Final concentration**
$NaNO_3$	1.500g
K_2HPO_4	0.040g
$MgSO_4.7H_2O$	0.075g
$CaCl_2.2H_2O$	0.036g
Citric acid	0.006g
Ferric ammonium citrate	0.006g
EDTA (disodium salt)	0.001g
Na_2CO_3	0.020g
Trace metal mix A5	1.0ml (from Table 4)

Table 1.2: A5 Trace metal solution

Component	**Final concentration**
H_3BO_3	2.860g
$MnCl_2.4H_2O$	1.810g
$ZnSO_4.7H_2O$	0.222g
$NaMoO_4.2H_2O$	0.390g
$CuSO_4.5H_2O$	0.079g
$Co(NO_3)_2.6H_2O$	49.40mg

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4.2.2 THE IDENTIFICATION OF CYANOBACTERIAL SPECIES USING THE FLOW-CAM

To determine the types of cyanobacterial species, present in the samples, a bench top FlowCAM (Model VS IV) was used. In the FlowCAM system, a sample is drawn into the flow chamber by a pump. Using the laser in trigger mode, the photomultiplier and scatter detector monitor the fluorescence and light scatter of the passing particles. When the particles passing through the laser fan has sufficient fluorescence values and/or scatter the camera is triggered to take an image of field view. The fluorescence values were then saved by the Visual Spreadsheet.

The computer, digital signal processor, and trigger circuitry work together to initiate, retrieve and process images of the field of view. Groups of pixels that represented the particles were then segmented out of each raw image and saved as separate collage image.

4.3 RESULTS AND DISCUSSION

Samples were incubated for 30 days under continuous light at room temperature. After 30 days of monitoring all samples showed growth of cyanobacteria as shown in Figure 4.1. The cultures were used for identification of cyanobacteria using Flow-CAM and and molecular techniques.

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4.3.1 THE PRESENCE OF CYANOBACTERIA IN THE RIVER SEDIMENTS

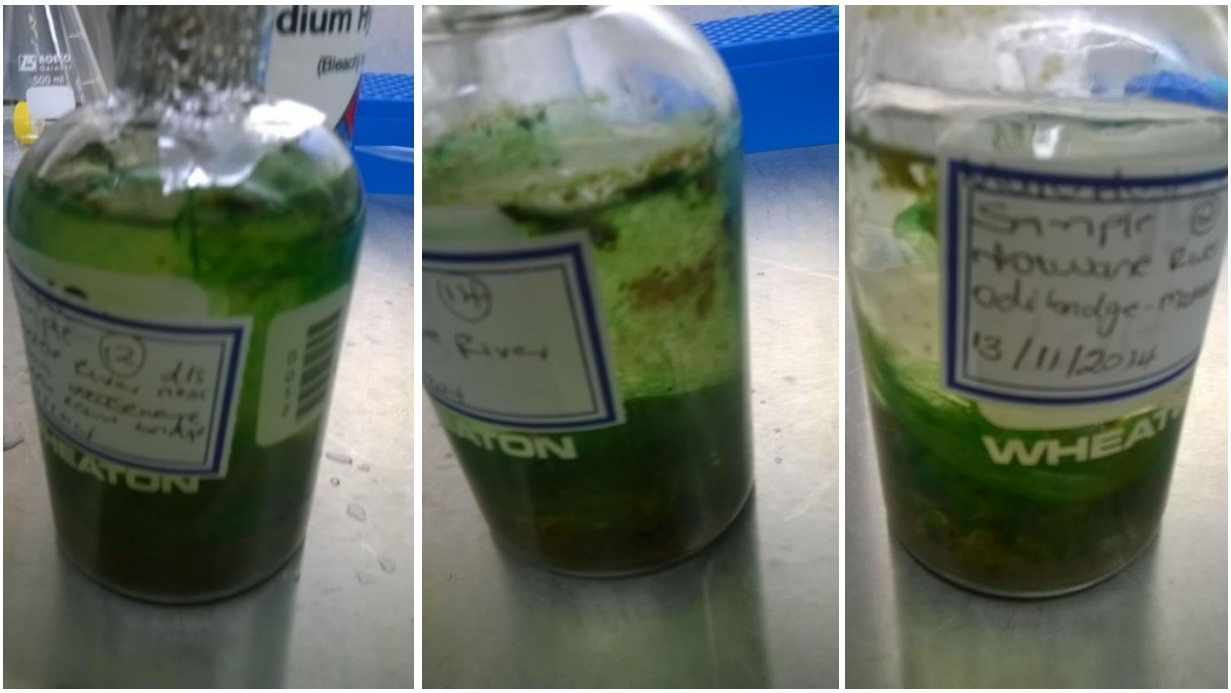

Figure 4.1: Samples showing the growth of cyanobacteria after incubation.

Different species of cyanobacteria were identified (Table 4.3). The FlowCam showed the following cyanobacteria species: *Microcystis aeruginosa, Lyngbya, Calothrix, Anabeana, Oscillatoria, Phormidium* and *Chroococcus*. The dominant cyanobacteria species identified from the samples were *Microcystis* sp. Most of the samples showed the presence of *Microcystis* in high quantity. The results showed *Microcystis aeruginosa* under the *Microcystis* species. Another cyanobacteria species identified were the *Phormidium* sp. which were precent in five samples. Under the *phormidium sp* one species was identified as *Phormidium uncinatum*. The results also showed *calothrix* sp which were found in four samples. The cyanobacteria species *Anabaena* sp. were also found in the samples. They were identified in four samples and one species was identified as *Anabaenopsis circularis*. *Oscillatoria* spp. were also identified for three samples and among the identified species were *Oscillatoria limnetia* and *Oscillatoria chlorine*. The cyanobacteria species *Chroococcus* and *Lyngbya* were also identified.

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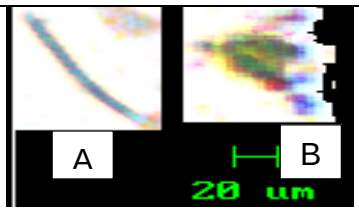
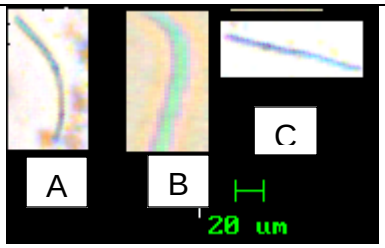
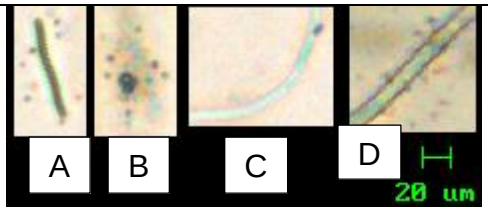
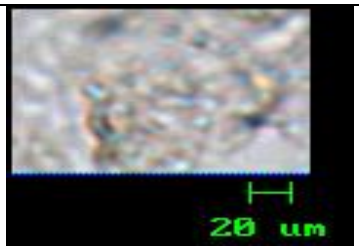
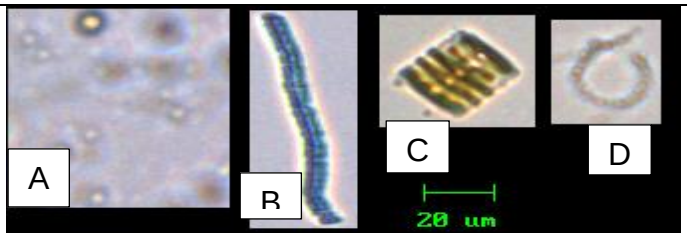
Table 4.3: Types of Cyanobacteria which were identified on the samples

Sample No	FlowCam Image	Cyanobacteria species
Sample 1		*A-Lyngbya* sp. *B-Mycrocystins* sp.
Sample 2		*A-Calothrix* sp. *B-Oscillatoria* sp. *C-Phormidium* sp.
Sample 3		*A-Phormidium uncinatum* *B-Microcystis* sp. *C-Oscillatoria chlorine* *D-Oscillatoria* sp.
Sample 4		*Microcystis* sp
Sample 5		*A-Microcystis* sp *B-Phormidium* sp *C-Nostoc* sp. *D-Anabaena circinalis* sp

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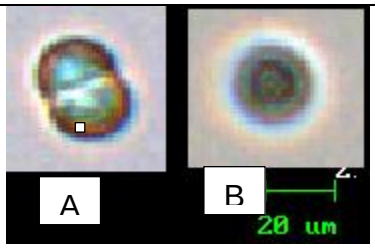
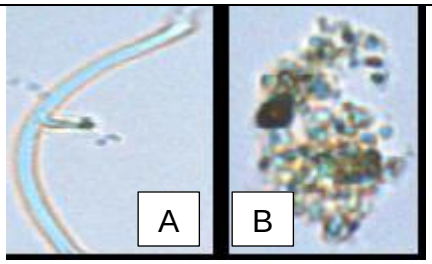
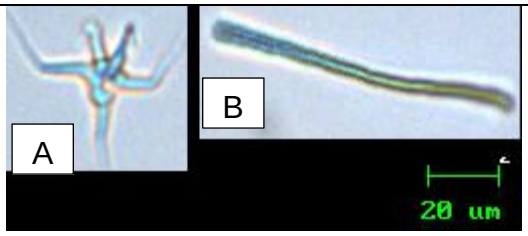
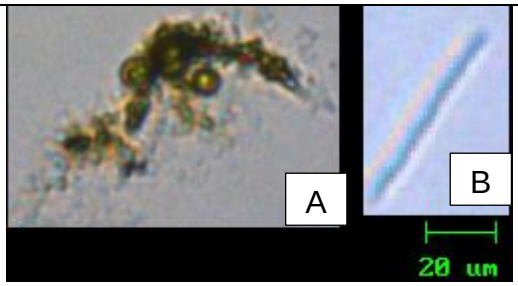
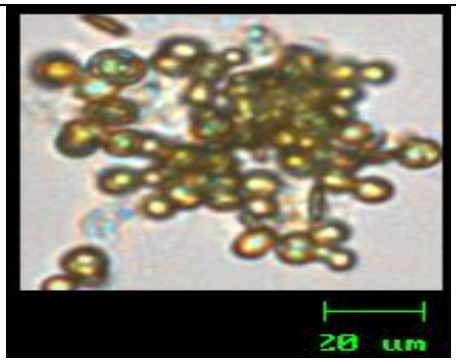
Sample 6		*A-Chroococcus* sp *B-Anabaechopsis circularis*
Sample 8		*A-Calothrix* sp. *B-Microcystis* sp
Sample 9		*A-Calothrix* sp. *B-Oscillatoria limnetia* sp
Sample 10		*A-Mycrocystis aeruginosa* *B-Phormidium* sp
Sample 11		*Microcystis aeruginosa* sp

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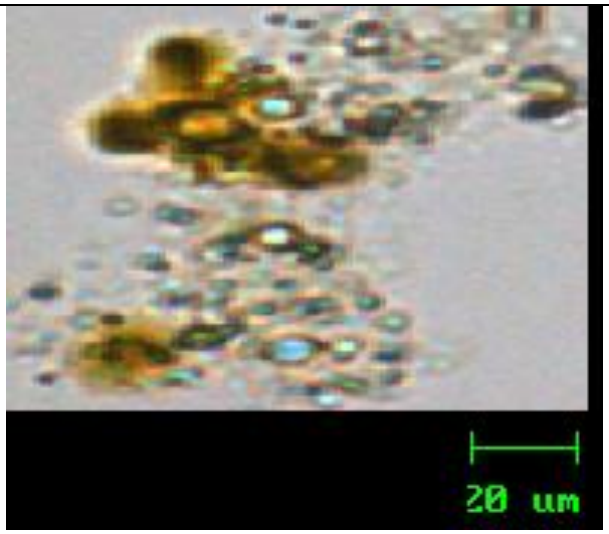
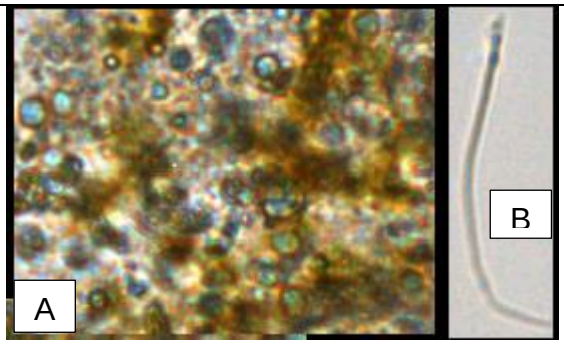
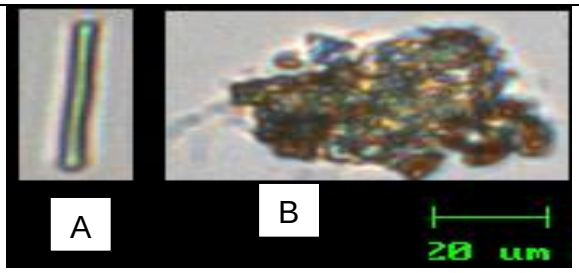
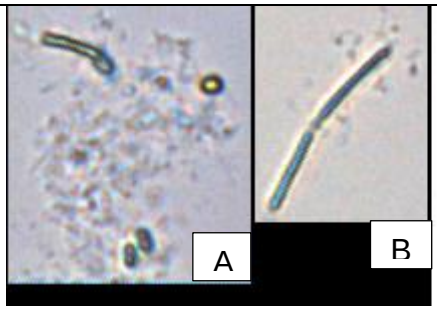
Sample 12		*Microcystis aeruginosa*
Sample 13		*A-Microcystis aeruginosa* *B-Calothrix* sp.
Sample 14		*A- Calothrix* sp *B-microcystis aeruginosa*
Sample 15		*A-Microcystis* sp. *B-Phormidium* sp.

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Table 4.4: Identified cyanobacteria species and cyanotoxins thay may produce

Producers	*cyanotoxins (Metcalf et al., 2012)*
Microcystis spp	Microcystins
M. aeruginosa sp	Microcystins
Phormidium sp	Microcystins, Anatoxin-a and homoanatoxin-a
Anabaena spp	Microcystins, Anatoxin-a, homoanatoxin-a and Cylindrospermopsins
Oscillatoriales spp	Microcystins, Anatoxin-a and homoanatoxin-a
Lyngbya sp	Saxitoxins
Calothrix sp	Nodularins and Microcystins

Cyanobacteria toxins (Table 4.4) can have several adverse effects on humans, animals, and aquatic ecosystems. Some cyanobacteria can produce neurotoxin, dermatoxins, hepatotoxin, or other bioactive compounds; and blooms of toxigenic cyanobacteria pose a threat if they are present in drinking water sources (Steidinger *et al.*, 1997). An advisory limit of 1 μg L^{-1} for microcystin-LR (most common cyanobacterial toxins) has been issued by the World Health Organization for drinking water (Lopez *et al.*, 2008). The presence of the species in the rivers might give rise to production of cynotoxins under right conditions.

4.4 CONCLUSION

The use of FlowCam machine has proven to be effective for cyanobacteria species identification however it gives an image that must be evaluated or checked and compared to the picture in the cynobacteria database inorder for one to know what species of cyanobacteria was captured in the picture. The cyanobacterium that was identified were: *Microcystis* spp*; Phormidium* spp*: Anabaena* sp *Oscillatoria* sp*; Lynbya* sp *and Calothrix* sp. These cyanobacteria are producers of cyanotoxins such as microcystins. Recommend the use of molecular tools/techniques which can give accurate results when monitoring cyanobacteria in freshwater.

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CHAPTER FIVE: DNA PROFILE OF CYANOBACTERIA SPECIES IN THE RIVER SEDIMENTS

5.1 INTRODUCTION

DNA based fingerprinting method may be employed to have an idea of the diversity changes, or closeness of community structure among samples or for screening of a large samples. A study done by Frazao *et al*. (2010) using PCR method to determine molecular analysis of genes involved in the production of known cyanotoxins such as Microcystins, Nodularins and Cyclindrospermopsin. The study revealed the presence of *mcyE* gene that was found in one *Leptolyngbya* strain and one *Oscillatoria* strain and is responsible for the production of cyanotoxins.

Metcalf *et al*. (2012) carried out a study to analysis microcystins and microcystin genes in between 60-170 years old dried herbarium using the method of DNA extraction and PCR. Specimens where collected from aquatic and terrestrial environments in 11 countries. These Specimens were dried and stored at ambient temperatures in the dark for up to 170 years, suggesting the fact that these genes are very specific and don't denature even if stored for longer periods. The result showed the presence of microcystins and synthetase gene *mcyD* DNA. In another study carried out by Valerio *et al*. (2005) samples of freshwater lakes and rivers in Portugal were analyzed for the presence of cyanobacteria strains using the DNA extraction, amplification and PCR fingerprinting and 16S rRNA PCR methods. It demonstrated ability to differentiate strains at intra-specific level.

5.1.1 TOXIC GENES IDENTIFICATION

Botha and Oberholster performed a survey in South Africa between 2004-2007, using RT-PCR and PCR technology to distinguish *Microcystis* strains bearing the *mcy* genes, which correlate with their ability to synthesize the cyanobacterial biotoxin microcystin. The study revealed that 99 % of South Africa's major impoundments contained toxicogenic strains of *Microcystis* (Botha and Oberholster, 2007).

The study on quantification of microcystin producing cyanobacteria was carried out by Furukawa *et al*. (2006) using real time PCR. It was discovered that the detection unit of this method was 8.8 cells per reaction and that cell concentration determined by real time PCR positively correlated with the cell concentration determined from direct microscopic count.

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Quantitative PCR was used to detect and quantify toxin genes from microcystin, nodularin and Cyclindrospermopsin and Saxitoxin biosynthesis pathways. It was discovered that it is a powerful tool for detecting and quantifying potentially toxic cyanobacteria in the laboratory and field samples (Al-Tebrineh *et al*., 2011). The main objective of the study is to use molecular techniques to identify toxic and non-toxic cyanobacteria genes in the river sediments and to use 16S rRNA in identifying the cyanobacteria species and explore relationships among the cyanobacteria species in the river sediments.

5.2 MATERIALS AND METHODS

5.2.1 MOLECULAR CHARACTERIZATION

The samples were analyzed at Professor Shonhai's laboratory, Department of Biochemistry, at University of Venda, South Africa, following the procedures explained below;

5.2.1.1 DNA extraction

Samples were freeze-dried and stored at -20 ^{0}C for DNA extraction. Total genomic DNA was extracted using the ZR-Duet^{TM} DNA/RNA Miniprep DNA extraction kit from Inqaba Biotech Laboratories South Africa. Before even starting with the extraction 96 ml 100% ethanol was added to the 24 ml DNA/RNA wash buffer as preparatory step.

5.2.1.1.1 Sample preparation

400 µl of DNA/RNA lysis buffer was added to frozen tissue and samples were homogenized. The samples were transferred into the Zymo-spin^{TM} IIIC column in the collection tube and centrifuged at 12 000 x g for 1 minute.

5.2.1.1.2 DNA purification

The Zymo-spin^{TM} IIIC column were transferred to new collection tube. 400 µl of DNA/RNA prep buffer was added to the column and centrifuged at 12 000 x g for 2 minute and the flow through was discarded. Then 700 µl of DNA/RNA wash buffer was added the column and centrifuge at 12 000 x g for 1 minute and the flow through was discarded again. Then 400 µl of DNA/RNA wash buffer was added to the column and centrifuge for 3 minutes to ensure completely removal of the wash buffer.

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The column was then transferred into a clean micro centrifuge tube. Lastly 50 µl of DNase/RNase-free water was added directly to the column matrix and it was left to stand for 5 minutes at room temperature after it was centrifuge at the highest /top speed for 30 seconds and the eluted DNA was used immediately.

5.2.2 DETECTION AND AMPLIFICATION OF 16S rRNA BY PCR

PCR method was performed for detection and amplification of 16S rRNA as described briefly by Frazao *et al*. (2010).

PCR amplification of the cyanobacteria 16S rRNA gene was determined using set of primers 27F/809R (Table 5.1). Thermal cycling conditions were 1 cycle at 95 ºC for 5 min, 35 cycles at 95 ºC for 30 s, 55.4 ºC for 30 s and 72 ºC for 60 s and 1 cycle at 72 ºC for 10 min. Reactions were carried out in 50 µL reaction volume that consisted of 0.5 pmol of each primer (10 pM/µL), 25 µL of Dream Taq master mix (Inqaba Biotech), 19 µl sterile ultra-pure water and 5 µL of DNA sample.

5.2.3 TOXIN GENES DETECTION

The presence of cyanotoxins were determined by the use molecular primers that are currently been used for detection of genes involved in the production of nodularins (NOD), microcystins (MC) and cylindrospermopsin (CYN) (Table 5.1). The NOD gene cluster, *nda*, consists of nine open reading frames (*ndaA-I*) (Moffitt and Neilan 2004). The MC gene cluster, *mcy*, comprises 10 genes in two transcribed operons, *mcyA-C* and *mcyD-J* (Pearson and Neilan, 2008). The HEP primer pair was used for detection of genes involved in MC and NOD production.

These primers are responsible for sequencing the aminotransferase (AMT) domain, which is located on the modules *mcyE* and *nda*F of the MC and NOD synthetase enzyme complexes, respectively (Pearson and Neilan, 2008; Jungblut and Neilan, 2006). Primers *mcyA-C* were used to detect the *mcyA*, *mcyB* and *mcyC* genes (Fergusson and Saint, 2003; Hisbergues *et al.*, 2003). For detection of CYN production (*cyr*) genes the polyketide synthase PKS M4 and M5 primers and the peptide synthetase M13 and M14 primers were used as designed by Schembri *et al.* (2001) who demonstrate a direct link between the presence of the peptide synthetase and polyketide synthase genes and the ability of cyanobacteria to produce CYN.

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PCR reactions conditions are similar to those described for the amplification of the 16S rRNA gene. Concerning the cycling conditions, for mcyA-Cd genes the thermal cycling conditions were 1 cycle at 95 ºC for 2 min, 35 cycles at 95 ºC for 90 s, 56 ºC for 30 s and 72 ºC for 50 s and 1 cycle at 72 ºC for 7 min. For HEP and CYN as genes, the thermal cycling conditions were as those for the amplification of the 16S rRNA with an exception for HEP gene annealing temperature of 58.15 ºC for 30 s. Positive control was used. PCR products were sent for sequencing at Inqaba biotech laboratory. Sequences were analyzed using the BLAST system (http://www.ncbi.nlm.nih.gov/BLAST/).

5.2.3.1 ELECTROPHORESIS

PCR products were electrophoresed in 1.8% agarose gel prepared as follows: each 100 ml 10X TAE Buffer 1.2 gram of agarose powder was weighed and mixed. 10X TAE buffer was laboratory prepared as follows: Tris 48.4g, Glacid acetic acid 11.4ml, EDTA disodium salt 3.7g and H2O deionised to 1000 ml. The mixture was heated until there is complete dissolution. Exactly 10µl of Ethidium bromide was added and mixed thoroughly. The mixture was transferred to the gel-casting tray with the comb already in position and allowed to solidify. The solidified gel was transferred to the running trays. The gel in the tray was covered with 1X TAE buffer. In the first well 3 µl 100 bp of the molecular weight marker was loaded and the samples were loaded from the second well onwards. The gel was run at 100 V, 250 mA and 60 minutes. The gel was viewed using the gel doc and the picture was taken.

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5.2.4 PCR PURIFICATION AND SEQUENCING

PCR products were purified using the GeneJet Gel Extraction Kit Thermo Scientific (South Africa) under room temperature as follows:

Gel slice containing the DNA fragment was excised using a clean razor blade and it was cut close to the DNA to minimize the gel volume. The gel slice was placed in a pre-weighed 1.5 ml tube and weighed and the weight of the gel was recorded. Hundred microliters of binding buffer were added for every 100 mg of agarose gel. The gel mixture was incubated for 10 minutes at 50-60 C until the gel dissolved completely. The tube was inverted for every few minutes to facilitate the melting process. The gel was briefly vortexed before adding into a column. 800 µl of the solubilized gel solution was transferred to Gene-Jet purification column and centrifuge at 12 000 x g for 1 minute. The flow through was discard and the column was placed back at the same collection tube. Then 700 µl of wash buffer was added in the purification column and centrifuge at 12 000 x g for a minute and the flow through was discard.

The column was placed back to the same collection tube. The empty purification column was Centrifuge for an additional 1 minute to completely remove the residual wash buffer. The purification column was transferred into a clean 1.5 ml micro centrifuge tube and 50 µl of elution buffer was added to the center of the purification column membrane and centrifuge for a minute at 12 000 x g. the purification column was discarded and the purified DNA was stored at -20 ^{0}C. Purified DNA were sent for direct sequencing at Inqaba biotech laboratory (SouthAfrica).

5.2.4.1 PRIMERS

Primers used for PCR amplification were synthesized at Inqaba Biotech (South Africa). Details of primer sequences, their specific targets and amplicon sizes are summarized (Table 5.1) below:

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Table 5.1: PCR primers which will be used for amplification of 16S rRNA gene for cyanobacteria identification and for the amplification of genes related to cyanotoxins production. A—Individual annealing temperature, B—Reference annealing temperature, bp = base pairs.

Primers	Target genes	Sequence (5'-3')	A	B	Size (bp)	Amplified gene	References
27F		AGAGTTTGATCCTGGCTCAG	52	60	780	16S rRNA	Neilan et al., 1997
809R		GCTTCGGCACGGCTCGGGTCGATA	64				Jungblut et al., 2005
*mcy*A-Cd F	*mcyA*	AAAATTAAAAGCCGTATCAAA	51	59	297	Microcystin synthetase	Hisbergues et al., 2003
*mcy*A-Cd R		AAAAGTGTTTTATTAGCGGCTCAT	43				
HEPF	*mcyE/ndaF*	TTTGGGGTTAACTTTTTTGGGCATAGTC	57			Microcystin/nodularin	Jungblut et al., 2005
HEPR		AATTCTTGAGGCTGTAAATCGGGTTT	55	52	472	synthetase	
PKS M4		GAAGCTCTGGAATCCGGTAA	52	55	650	Cylindrospermopsin	Schembri et al., 2001
PKS M5		AATCCTTACGGGATCCGGTGC	56			polypeptide synthase	Schembri et al., 2001
M13	*Ps*	GGCAAATTGTGATAGCCACGAGC	57	55	597	Cylindrospermopsin	Schembri et al., 2001
M14		GATGGAACATCGCTCACTGGTG	57			peptide synthetase	Schembri et al., 2001

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5.2.5 PHYLOGENETIC RELATIONSHIP

Additional sequences were downloaded in FASTA format from GenBank through NCBI and combined with assembled sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were total of 640 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

5.2.6 DIVERGENCE MATRIX

PCR products for 16S rRNA gene, identified on agarose gels, were selected for subsequent identification by sequencing (Inqaba Biotech, South Africa). The obtained sequenced data were used to conduct homology searches on GenBank using BLAST (Altschul *et al*., 1997, http:// blast.ncbi.nlm.nih.gov/blast.cgi), and for further bioinformatic analyses to perform divercence matrix using BioEdit v7.0.9 (Hall, 1999). Sequences were exported to and analysed with the MEGA 7 package (Kumar *et al*., 2016).

5.3 RESULTS AND DISCUSSION

5.3.1 PCR ANALYSIS OF 16S rRNA GENE

PCR products separated by gel electrophoresis are shown above in the Figure 15. The presence of bands is indicative of positive amplification whereas a blank sample indicates negative amplification. The blank samples where repeated several times and failed to amplify. Almost all the samples showed positive amplification which confirmed the presence of cyanobacterial DNA in the samples.

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The two samples which showed no amplification were from Mogalakwena (4) and Lephalala Rivers (6).

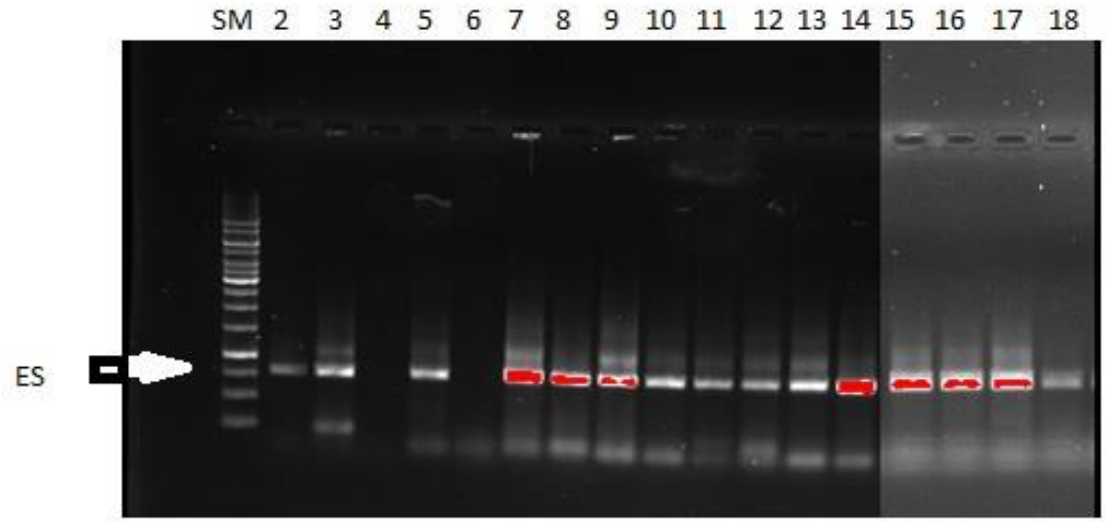

Figure 5.1: PCR amplification using 27F and 740R primer pair for 16S rRNA gene. SM (Standard Marker), 2-18 Sample numbers. Lane 2=Notwane River; 3=Sand River upstream; 4=Mogalakwena River; 5=Mawoni River; 6=Lephalala River; 7=Mokolo River; 8=Crocodile River downstream of Hartbeesport Dam; 9=Nzhelele River downstream; 10=Sand River downstream;11=Crococidile River downstream (near bridge on road D1235); 12=Nzhelele River upstream; 13=Mzingwane River; 14=Shashe River; 15=Limpopo River (next to Thuli coal mine); 16=Limpopo River (abstraction point at 0.0m); 17=Limpopo River (abstraction point at 1.0m); 18=Limpopo River (abstraction point at 1.68m)

For each sample, multiple fragments obtained by sequencing with both forward and reverse primers; were edited and assembled using the Staden package (Staden *et al.*, 2003). All assembled sequences were aligned in BioEdit v7.0.9 (Hall, 1999). However, the sample collected from Limpopo River (1), is not shown in figure above because it was used as the test sample. Also noted was the fact that the amplified fragment from that test sample failed to sequence only producing only 100bp while around 650 bp is expected. Other samples like the sample Limpopo River (15), and sample Limpopo River (17), Musina borehole abstraction point (16) did amplify but failed to assemble in Staden package (Staden *et al.*, 2003). Assembled sequences were run on BLAST algorithm (Altschul *et al.* 1990) to identify their closely similar sequences already deposited in GenBank via NCBI and the

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outcomes shown in the table below (Table 5.2). The importance of BLAST algorithm (Altschul *et al.*, 1990) is in helping with identification of organisms to their specific genera with respect to similar available sequences in online databases.

From BLAST algorithm (Altschul *et al.,* 1990) it must be understood that more than 98% similarity obtained matches the sample to the correct species, more than 90% similarity obtained matches the sample to the correct genus, more than 80% similarity obtained matches the sample to the correct the correct Family level.

From that is confirmed by BLAST that cyanobacteria from samples of Moklo River (7); Crocodile river downstream of Hartbeesport dam (8) and Shashe River (14) have been identified to the correct species, while cyanobacteria from samples of Notwane River (2); Sand River upstream (3); Mawoni River (5); Nzhelele River downstream (9); Sand River downstream and Limpopo River (18)(abstraction point at 1.68m) have been identified to correct genus, the cyanobacteria from sample Limpopo River (16) (abstraction point at 0.0m) been identified to the correct family. However, samples of Crococidile River downstream (11) (near bridge on road D1235); Nzhelele River upstream (12) and Mzingwane River (13) have been identified to be similar to the clones and no families could be detected.

The first to be noted was that Uncultured Cyanobacterium clone HQ189039.1 could not be used for phylogenetic tree because of its length (about 480 bp), with the reason that complete deletion option of gaps and missing information in MEGA 7 (Kumar, 2016) was used. The second to be noted was that two outgroups sequences have been used in phylogenetic alignment.

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Table 5.2: Results of BLAST search showing the similarity between GenBank sequences with sample sequences from this project, Families of each species are shown on separate column.

Samples	**Similarity %**	**Species similar to**	**Family**	**Accession No**
S2	93	Uncultured *Leptolyngbya* sp. Clone	Leptolyngbyaceae	KM108695.1
S3	94	*Synechocystis PCC 6803*	Oscillatoriophycideae	CP012832.1
S5	97	*Anabaena oscillarioides*	*Nostocaceae*	AJ630428.1
S7	99	*Synechocystis* sp. *PCC 6803*	Oscillatoriophycideae	CP012832.1
S8	99	*Leptolyngbya boryana*	Leptolyngbyaceae	AP014642.1
S9	97	*Synechocystis PCC 6803*	Oscillatoriophycideae	CP012832.1
S10	96	*Spirulina laxissima SAG 256.80*	Spirulinaceae	DQ393278.1
S11	87	Uncultured Cyanobacterium clone		AM159315.1
S12	83	Uncultured Cyanobacterium clone		HQ189039.1
S13	90	Uncultured Cyanobacterium clone		JX041703.1
S14	98	*Leptolyngbya boryana*	Leptolyngbyaceae	AP014642.1
S16	83	*Leptolyngbya* sp	Leptolyngbyaceae	KJ654311.1
S18	96	*Alkalinema pantanalense*	Pseudanabaenaceae	KF246497.2

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5.3.2 DETECTION OF GENES INVOLVED IN TOXIN PRODUCTION

Cyanotoxins detection was also based on PCR by amplification of microcystin/nodularin synthetase using the HEP primer pairs and cylindrospermopsin polyketide synthetase genes using PKS primer pair. The *mcy*A-Cd primer pair and M13 & M14 primer pair were also used for toxins detection but did not produce a positive result. Hence they were excluded in the discussions. The HEP primer pair produced two positive results for samples from Crocodile River (8) (downstream of Hartbeespoort Dam) and Mzwingane River (13).

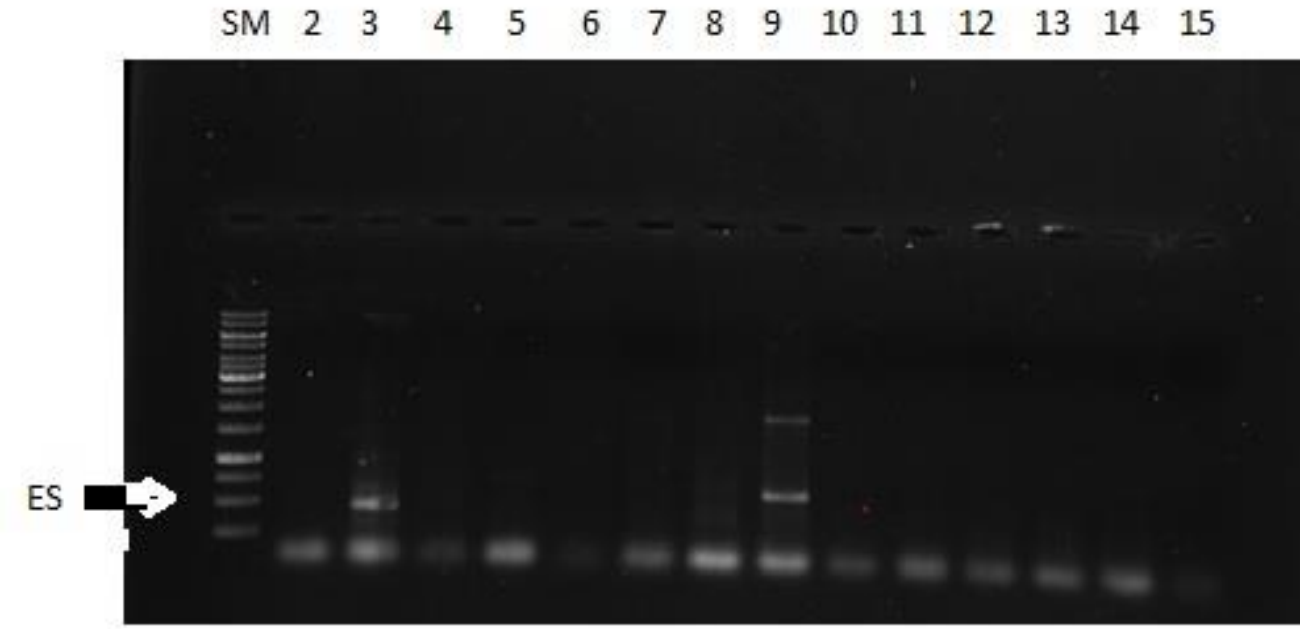

Figure 5.2: PCR products using PKS primers for cylindrospermopsin polyketide synthetase gene. SM (Standard Marker), 2-18 Samples number. Lane 2=Notwane River; 3=Sand River upstream; 4=Mogalakwena River; 5=Mawoni River; 6=Lephalale River; 7=Mokolo River; 8=Crocodile River downstream of Hartbeesport Dam; 9=Nzhelele River downstream; 10=Sand River downstream;11=Crococidile River downstream (near bridge on road D1235); 12=Nzhelele River upstream; 13=Mzingwane River; 14=Shashe River; 15=Limpopo River (next to Thuli coal mine); 16=Limpopo River (abstraction point at 0.0m); 17=Limpopo River (abstraction point at 1.0m); 18=Limpopo River (abstraction point at 1.68m)

For most of the samples, no amplification of any of the toxin genes was obtained. Nevertheless, few samples showed the amplification of cylindrospermopsin polyketide synthetase gene such as Sand River (3) upstream and Nzhelele River (9) downstream (Figure 5.2). This confirmed the presences of cyanotoxins, cylindrospermopsin and microcystins/nodularins in the sediment samples. The presence

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of these cyanotoxins in the river sediments is harmful to humans who may use the drinking water that is available in the river bed (Falconer & Humpage, 2005b). To confirm the identity of the amplified fragments, the PCR products were sequenced. The sequences were compared with sequences from the GenBank (Tables 5.3 & 5.4).

Table 5.3: Results of BLAST search showing the similarity between GenBank sequences with sample sequenced using PKS primers for Cylindrospermopsin and polyketide synthase toxin gene identification.

Sample No	**Similarity%**	**Species similar to**	**Accession No**
S3	83	*Synechocystis*	CP012832
S9	93	*Calothrix*	CP011382

Table 5.4: Results of BLAST search showing the similarity between GenBank sequences with sample sequenced using HEP primers for Microcystin toxin gene identification.

Sample No	**Similarity%**	**Species similar to**	**Accession No**
S8	100	Uncultured *Microcystis* sp. clone msp microcystin synthetase E (mcyE) gene, partial cds	KF687998
S13	100	Uncultured *Microcystis* sp. clone mw microcystin synthetase E (mcyE)gene, partial cds	KF687997

Two samples showed the presense of polyketide synthase gene and there was no amplification for cylindrospermopsin gene while using PKS primers, which were 83 % and 93 % similar to *Synechocystis* and *Calothrix* (Table 5.3). It has been reported that *Synechocystis* species can produce the microcystin toxin whereas *Calothrix* sp can produce nodularins and microcystins and both cannot produce cylindrospermopsin (Aboal *et al.*, 2002; Mohamed, 2008). However, PKS genes are not only available in cyanobacteria responsible for production of cylindrospermopsin, since they are also available in microcystin production (Nishizawa *et al*., 2000; Tillet *et al*., 2000). This suggest that instead Sand River (3) upstream and Nzhelele River (9) downstream have microcystin producing

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cyanobacteria just like Crocodile River downstream of Hartbeesport Dam (8) and Mzingwane River (13) which have shown the presence of the microcystin synthetase gene cluster produced by the microcystis species when using the HEP primer pair (Table 5.4). Other samples did not constitute the toxins genes due to possible loss of gene during evolution or mutation of genes during cultivation of species. Mutation of genes can lead to decrease in the toxicity of the strains when maintained in a culture. Cyanobacteria seem to be more toxic in the natural environment than in the laboratory conditions. The presence of microcystins in the rivers may constituent a health risk especially for the communities that may be in contact or drink the polluted water without any form of treatment or suitable treatment that can be able to remove the toxins in the water. Convectional method for water treatment is not convient for the removal of microcystins in water (Hoeger *et al.*, 2005). Drinking water treatment processes might trigger the release of hepatoxins into drinking water by disrupting the trichomes of cyanobacteria (Brittain *et al.*, 2000). The water supplies from the Limpopo river basin are used by water utilities for drinking water supplies, commercial and subsistence irrigation farmers for growing food crops and livestock watering (Figure 5.3). Thus presence of cyanotoxins can also poison the livestocks and game animals (wildlife) in areas such as Kruger National Park, Gona-re-zhoue National Park and Mapungubwe National Park. Already the microcystins have been implicated in the death of wildlife in the Kruger National Park (Oberholster *et al.*, 2009). The cyanotoxins have been implicated in the negative growth (stunting) of plants and this may be serious repercussions for the irrigation farmers (McCollough, 2016).

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Figure 5.4: A scenario involving boreholes drilled inside the Limpopo river channel and contamination with cyanobacteria (green dots) cysts and akinetes for (A) irrigation farmers & (B) water utility raw water supply for human consumption

5.3.3 PHYLOGENETIC RELATIONSHIP

The evolutionary tree was constructed could not be used for phylogenetics purposes bacause of two important things; the number of samples used for PCR per riversite was not enough to make conclusive argument; and the cyanobacteria were the expected products which needed to be identified. Hence the tree was used to verify the identification as done by BLAST search, however the phylogenetic relationship was basically done by divergence matrix and combined discussion followed the divergence matrix.

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The relationship between the samples and their most similar species as from BLAST search was cofirmed by phylogenetic tree, and the relationships between some cyanobacteria species from different samples have been confirmed (Figure 5.4). The first was the confirmation of similarity of samples from Crocodile River (S8) downstream Hartbeespoort dam and Shashe River (14) to *Leptolyngbya boryana* with 99 % bootstrap confidence. Then the similarity of Musina borehole extraction (S16) sample to *Alkalinema pantanalense* with 98 % bootstrap, then the similarity of samples from Sand River (S3) upstream, Nzhelele River (9) downstream near Tshipise and Mokolo river to *Synechocystis* sp. PCC 6803. The other similarity was Mawoni River (5) downstream of Makhado oxidation pond to *Leptolyngbya* sp. with 97 % bootstrap confidence, then Notwane River (2) to uncultured *Leptolyngbya* sp with 99 % bootstrap confidence, and lastly Sand River (10) downstream to *Spirulina laxissima* with 100 % bootstrap confidence.

However, the cyanobacteria at these two sites, Musina borehole extraction (S16) and Sand River (S3) may imply that there is movement of aquatic animals such as fish from the entrance (mouth) of Sand River towards the Musina abstraction point (Figure 5.5). In simple terms there is an upstream movement of cyanobacteria species being carried by aquatic animals but this requires further investigation.

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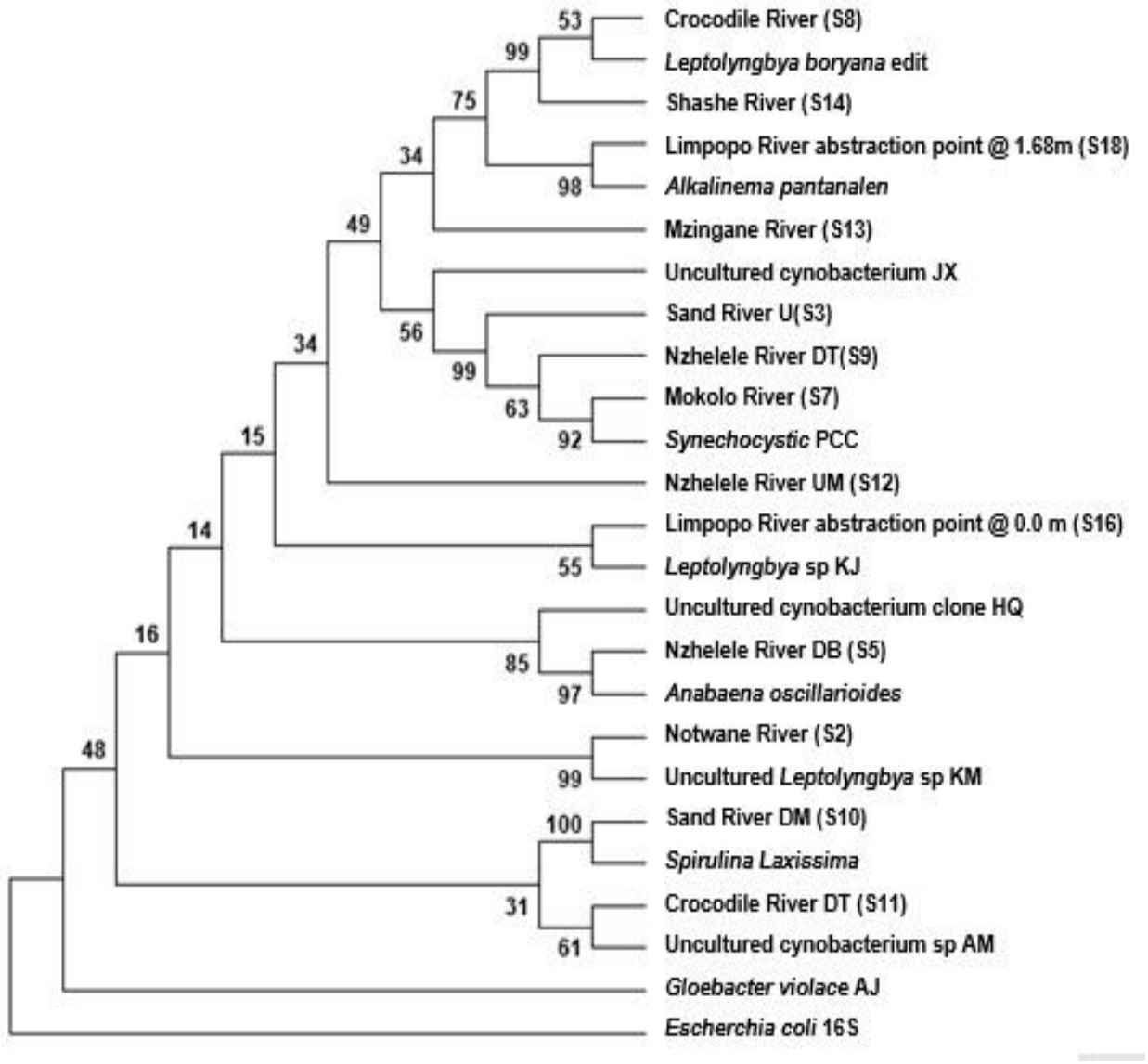

Figure 5.4: The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987).

The other which matched their supposed to match from BLAST search includes Musina Borehole extraction point (S16) to *Leptolyngbya* sp. and Crocodile River (S11) near bridge on road D1235 and upstream to Thabazimbi to Uncultured Cyanobacteria clone though they bootstrap confidence level was little about 55 % and 61 % respectively. Following the BLAST search results and Phylogenetic tree the divergence matrix can be used to verify the truth of the two, ie. BLAST search and phylogenetic tree. Divergence matrix confirms that cyanobacteria from Crocodile River (S8) downstream of Hartbeesport dam and from Shashe River (S14) are same species which is *Leptolyngbya boryana*, they both show at least 98 % similarity to this species in BLAST and

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bootstrap confidence is 99% forming a clade in phylogenetic tree, while they have the least difference in the divergence Matrix.

Thus based on this study there is DNA evidence to suggest that the cyanobacteria at the Musina abstract point is similar to cyanobacteria from the Crocodile River system. This may be possible because the Musina abstraction point is downstream to Crocodile River which flows into the Limpopo River (Figure 5.5).

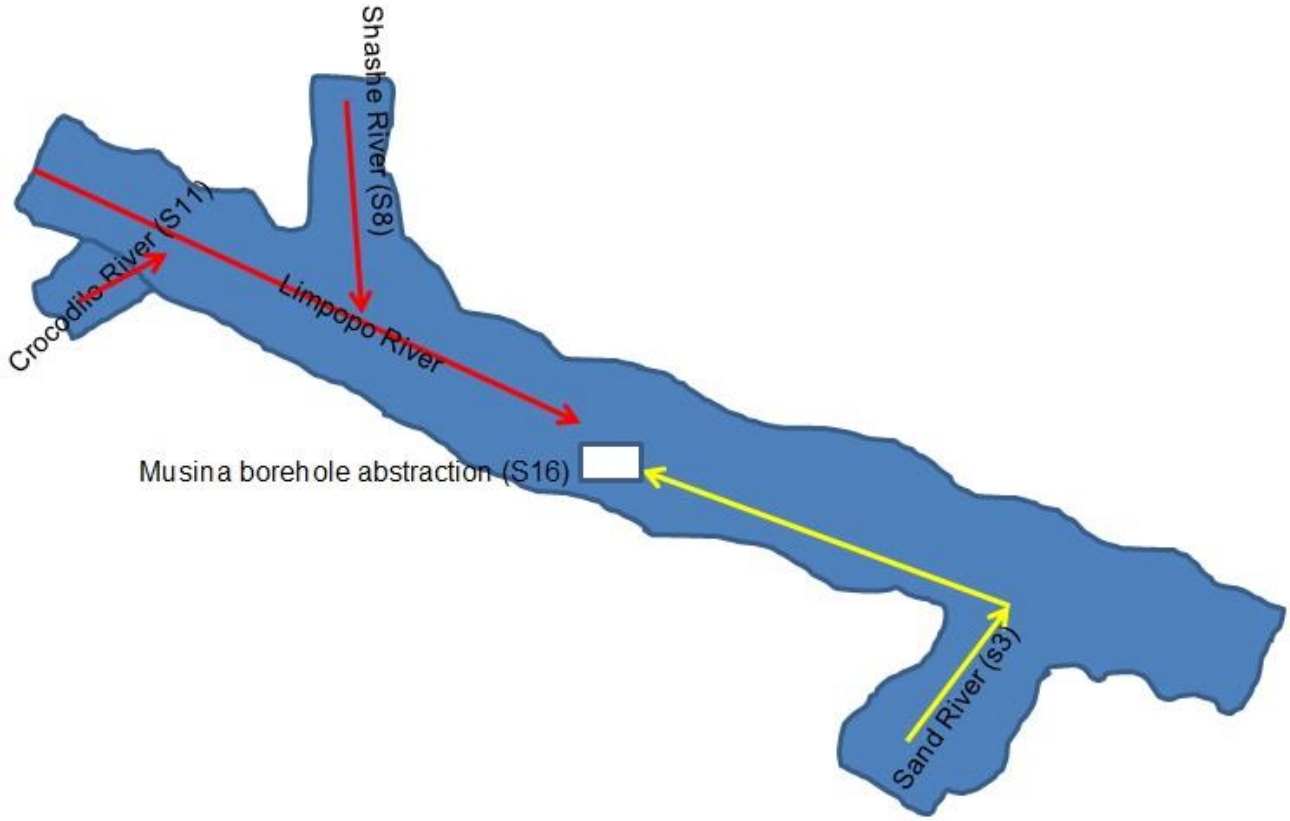

Figure 5.5: A scenario involving the movement of cyanobacteria species during water flows in the Limpopo River (red arrow) towards the Musina abstraction borehole (White Square). The possible upstream movement (yellow arrow) from Sand River (S3) to Musina borehole (S16) may involve cyanobacteria 'hiking aride' on aquatic animals such as fish, crocodiles, etc.

Samples from Nzhelele River upstream near Mphephu resort and Mzwingane River (Zimbabwe) never attempted to make a clade with their supposed matches. The relationship between the cyanobacteria species as identified from specific locations where they were collected from have been

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identified by Divergence Matrix (Table 5.5). Same species are detected by the difference co-efficient of 0.00 whereas the complete unrelated species are detected by the co-efficient of 1.00.

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5.3.4 DIVERGENCE MATRIX

Table 5.5: Divergence matrix for reflection of similarity

	S2	S3	S5	S7	S8	S9	S10	S11	S12	S13	S14	S16	S18
Notwane_River (S2)	–												
Sand_River_u(S3)	0.216	–											
Nzhelele_River_DB(S5)	0.191	0.187	–										
Mokolo_River(S7)	0.167	0.064	0.130	–									
Crocodile_River_(S8)	0.166	0.160	0.149	0.119	–								
Nzhelele_River_DT(S9)	0.184	0.095	0.152	0.028	0.140	–							
Sand_river_DM(S10)	0.155	0.216	0.153	0.156	0.169	0.169	–						
Crocodile_River_DT(S11)	0.257	0.295	0.280	0.244	0.278	0.254	0.236	–					
Nzhelele_River_UM(S12)	0.391	0.394	0.365	0.351	0.350	0.361	0.364	0.492	–				
Mzingane_River(S13)	0.190	0.180	0.184	0.130	0.134	0.139	0.168	0.267	0.377	–			
Shashe_River_(S14)	0.173	0.163	0.156	0.119	0.006	0.140	0.173	0.278	0.355	0.134	–		
Musina_borehole(S16)	0.376	0.359	0.312	0.314	0.342	0.321	0.343	0.414	0.555	0.371	0.347	–	
Musina_borehole_(S18)	0.183	0.184	0.179	0.136	0.128	0.150	0.186	0.285	0.366	0.173	0.131	0.348	–

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The cyanobacteria species from Crocodile River (S8) is the same species as the cyanobacteria from sample Shashe River (S14), they have less than 1 % difference (0.006) and their differences to other species are approximately the same. This may be expected since Shashe River is downstream of Crocodile River (Figure 5.5). The cyanobacteria species from Makolo River (S7) and the cyanobacteria species from sample Nzhelele River (S9) share undetectable difference but by comparing their difference to the cyanobacteria species from sample Notwane River (S2) and Mawoni River (S5) there is a slight difference of about a percent hence they are not one species. The cyanobacteria which are much diverged from others are the cyanobacteria species from sample The Notwane River (S2) and Limpopo River (S16) and they themselves are the most different from each other with 28% difference, their comparison co-efficient range from 0.17 to 0.28. The Nzhelele River upstream (S12) and Limpopo River (S16) and they themselves are the most different from each other with 28% difference, their comparison co-efficient range from 0.312 to 0.492 which is the highest for all species.

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5.4 CONCLUSION AND RECOMMENDATION

5.4.1 CONCLUSION

The results obtained in this study indiated the presence of toxic and non-toxic cyanobacteria species in the Limpopo Rivers and its tributaries. The molecular tools were used to presence of non-toxic and toxic cyanobacteria based genes that code for the production of cyanotoxins. The presence of nutrients, phosphates and nitrates in the river sediments, encounrges the growth of the cyanobacteria should river flows commence. Also the presence of toxic genes, expressing cynlindropsersin and microcystin/nodularin in the river sediments points to worrisome trend in the Limpopo river basin. The cyanotoxins are harmful to humans who consume the water originating from boreholes located inside the Limpopo river basin or drilled along the Limpopo river basin. Secondly the water supplies from the Limpopo river basin are used by commercial and subsistence irrigation farmers for growing food crops and livestock watering. Thus presence of cyanotoxins can also poison the livestocks and game animals (wildlife) in areas such as Kruger National Park, Gona-re-zhoue National Park and Mapungubwe National Park. Cyanotoxins have been implicated in the negative growth (stunting) of plants and this may be serious repercussions for the irrigation farmers.

5.4.2 RECOMMENDATION

From the matrix, it is convenient to conclude that the cyanobacteria species diversity in these sources is huge. It can be recommended that a project wherein the diversity of this cyanobacteria can be done using more advanced technology, Next Generation Sequencing to be specific, wherein almost all cyanobacteria form each sample will be sequenced and determined. Also further research is required on the different cyanobacterium species, *Leptolyngbya* which was found in all sediments samples. Also further research is required to determine the levels of cyanotoxins in the Limpopo River basin and also to compare the cyanobacteria species and their cyanotoxins in downstream side of the Limpopo in Mozambique using molecular techniques. It must be recommened that drinking water suplies at Musina should be monitored for the presence of cyanotoxins to manage/ minimize the risk of intoxication.

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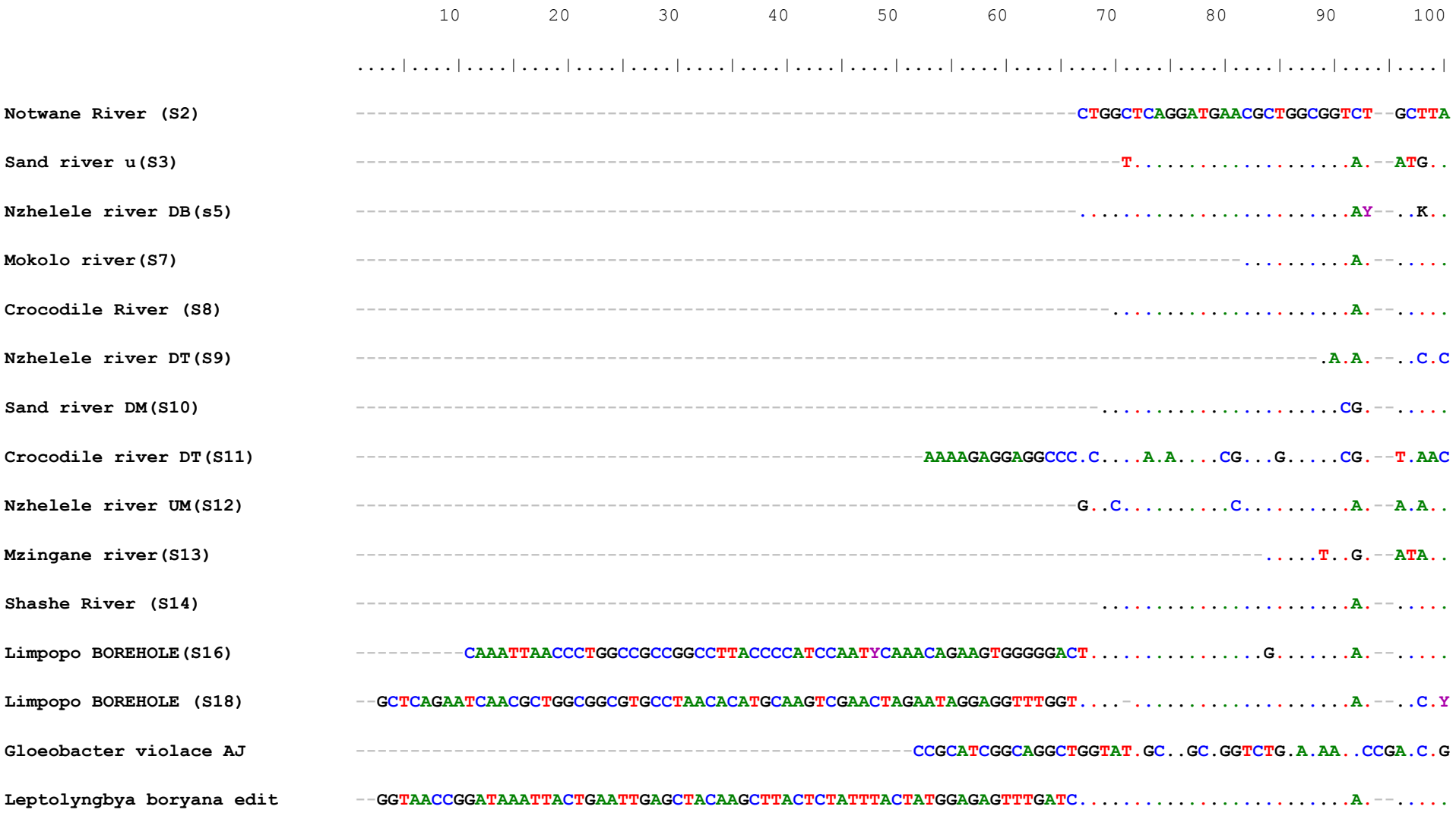
Appendix: The sequences used in this study



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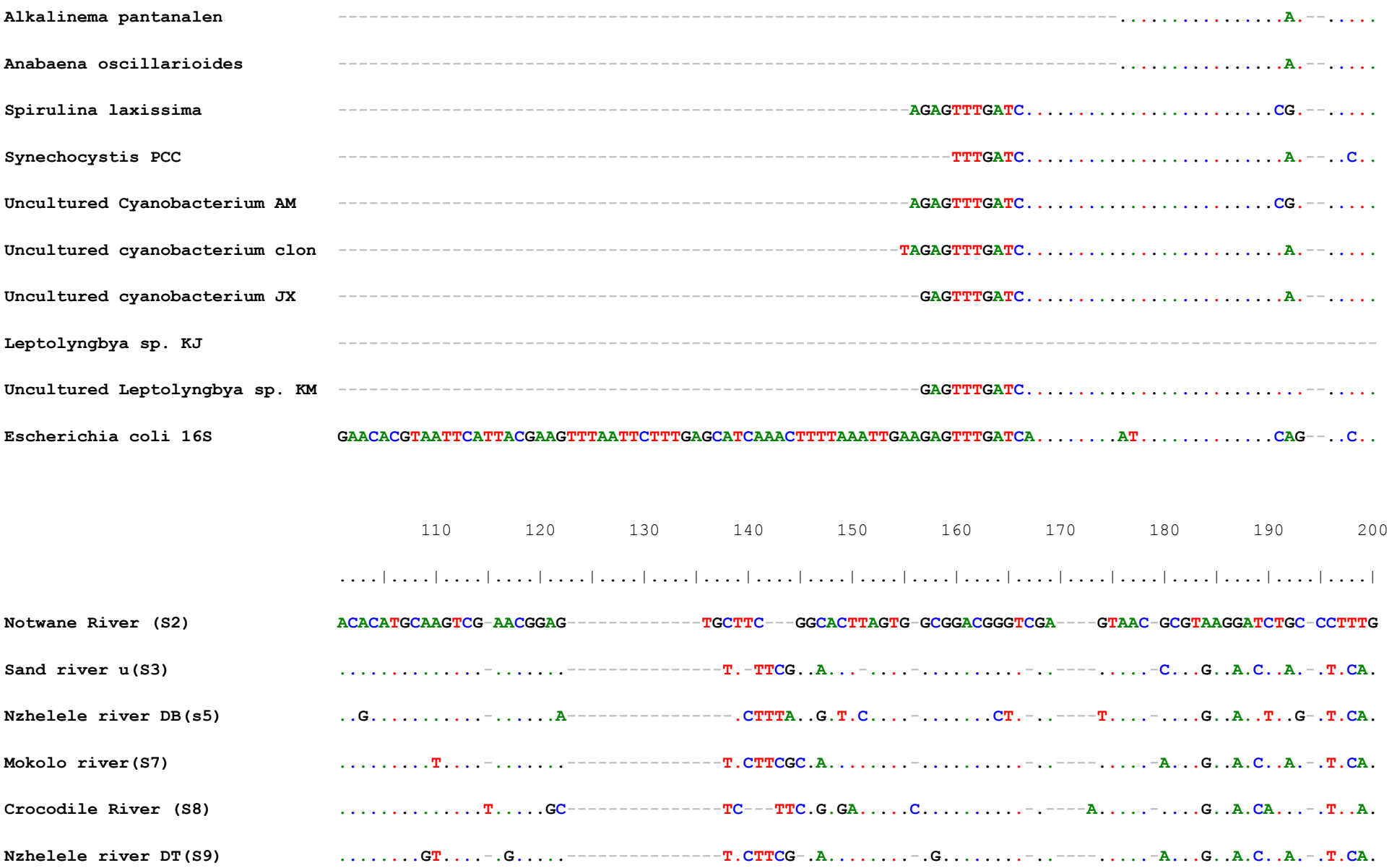

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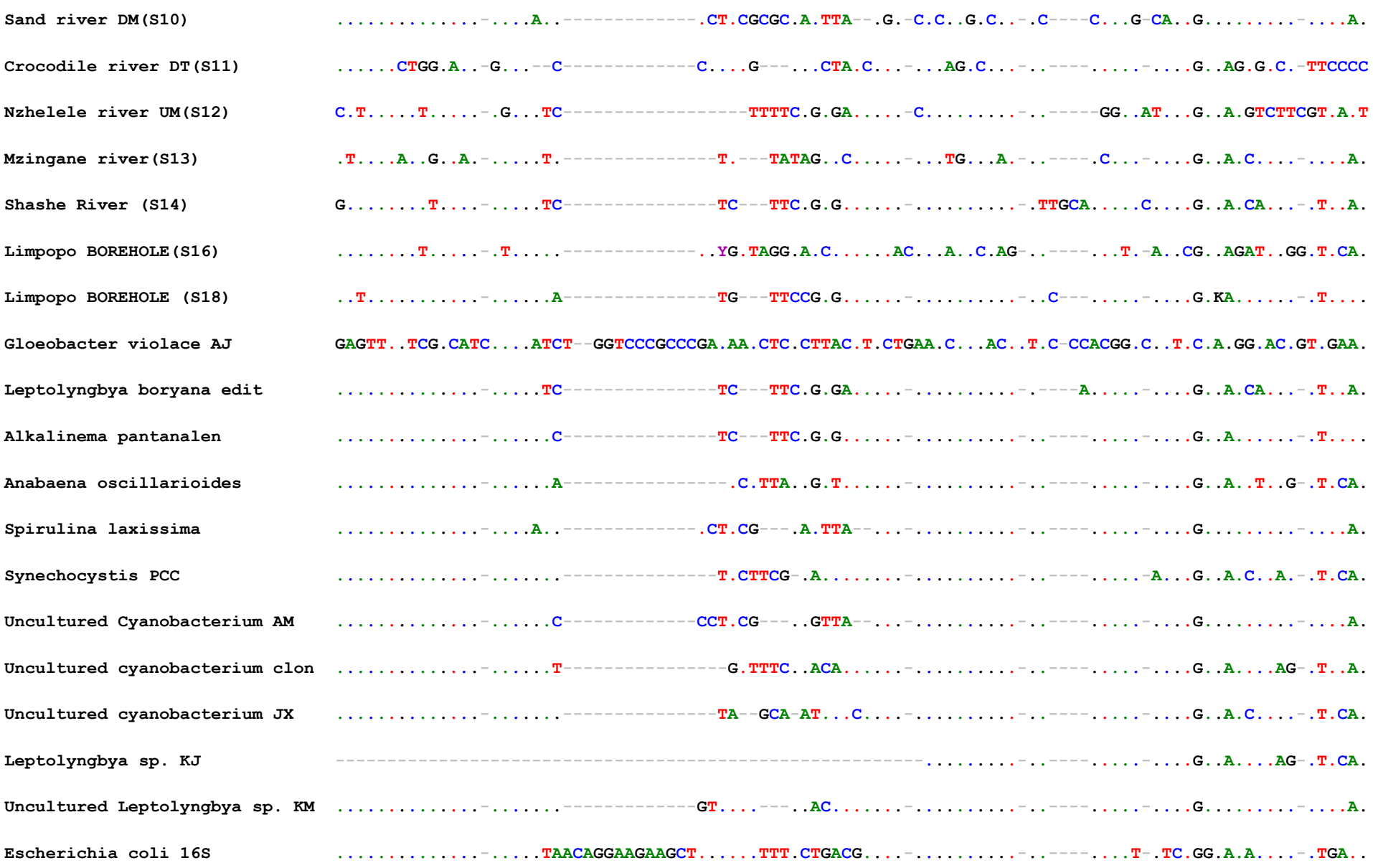

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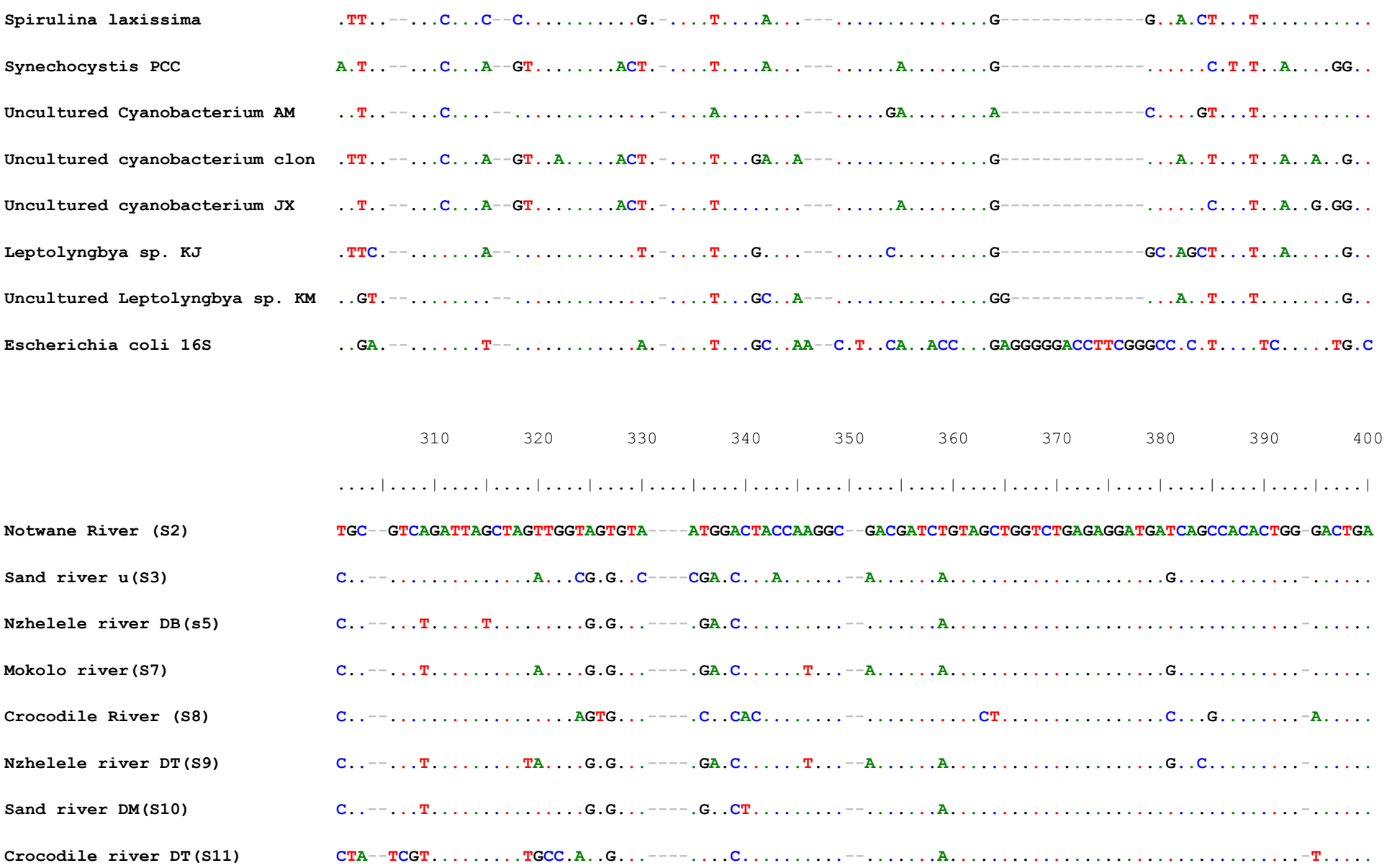

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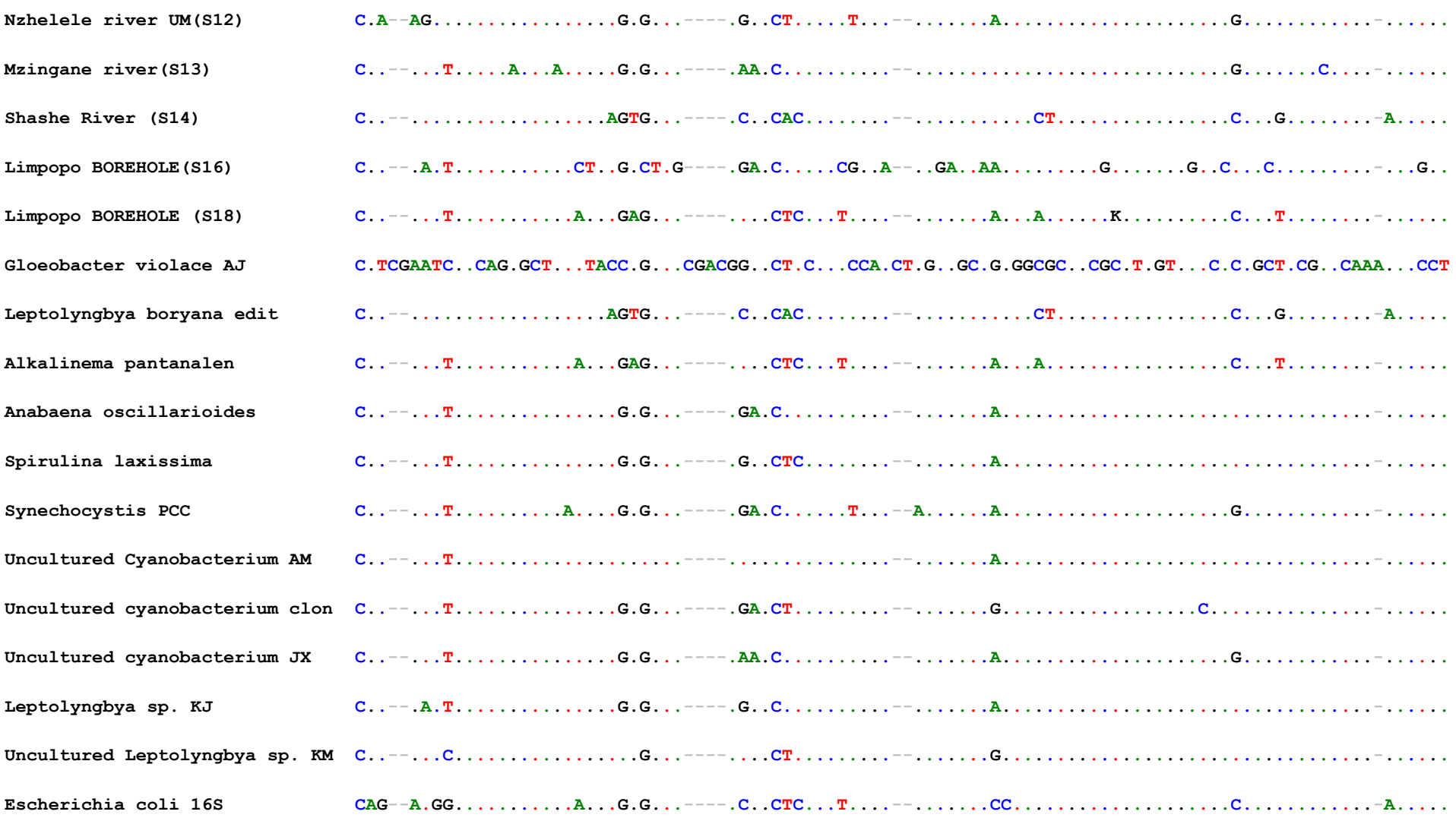

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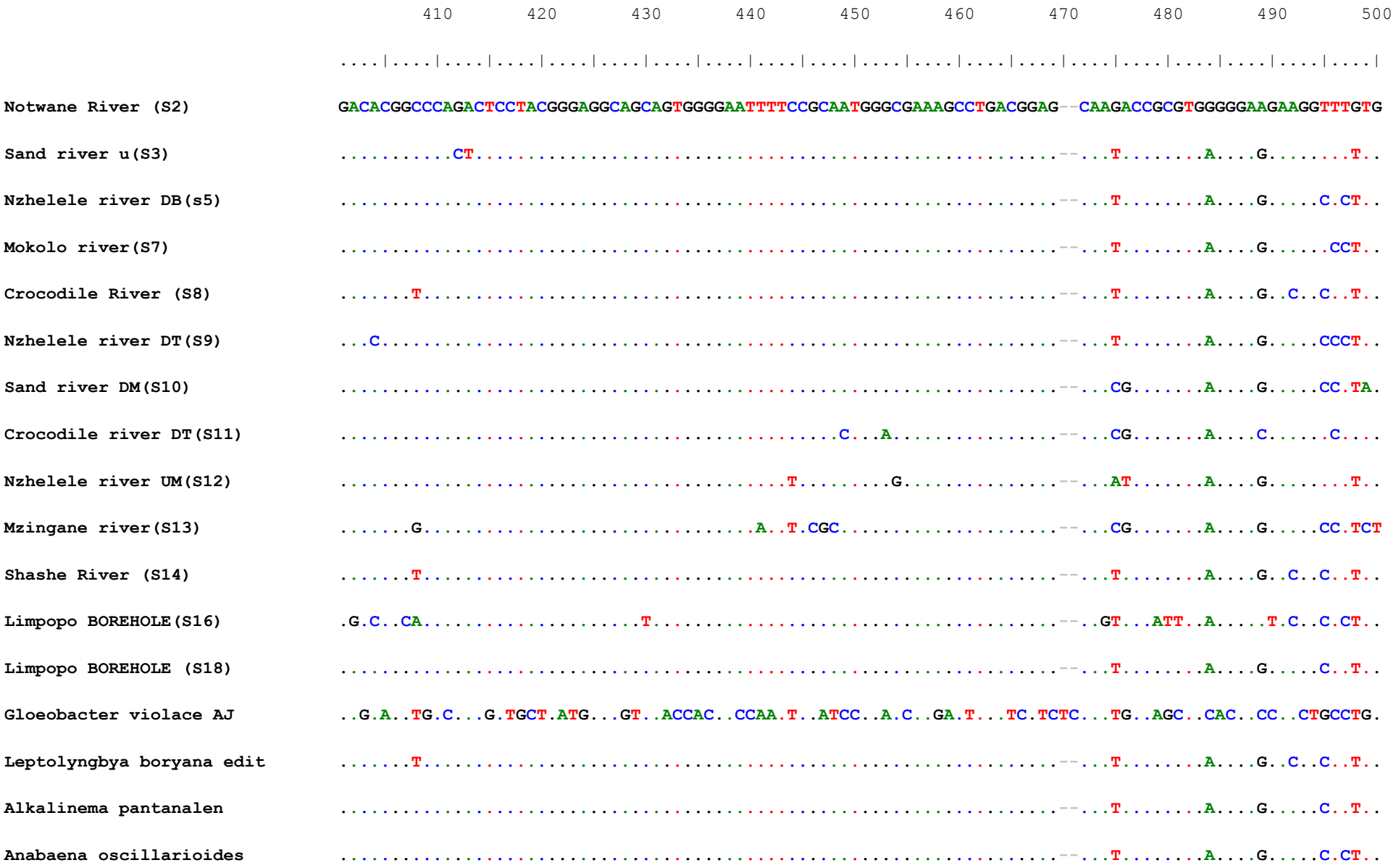

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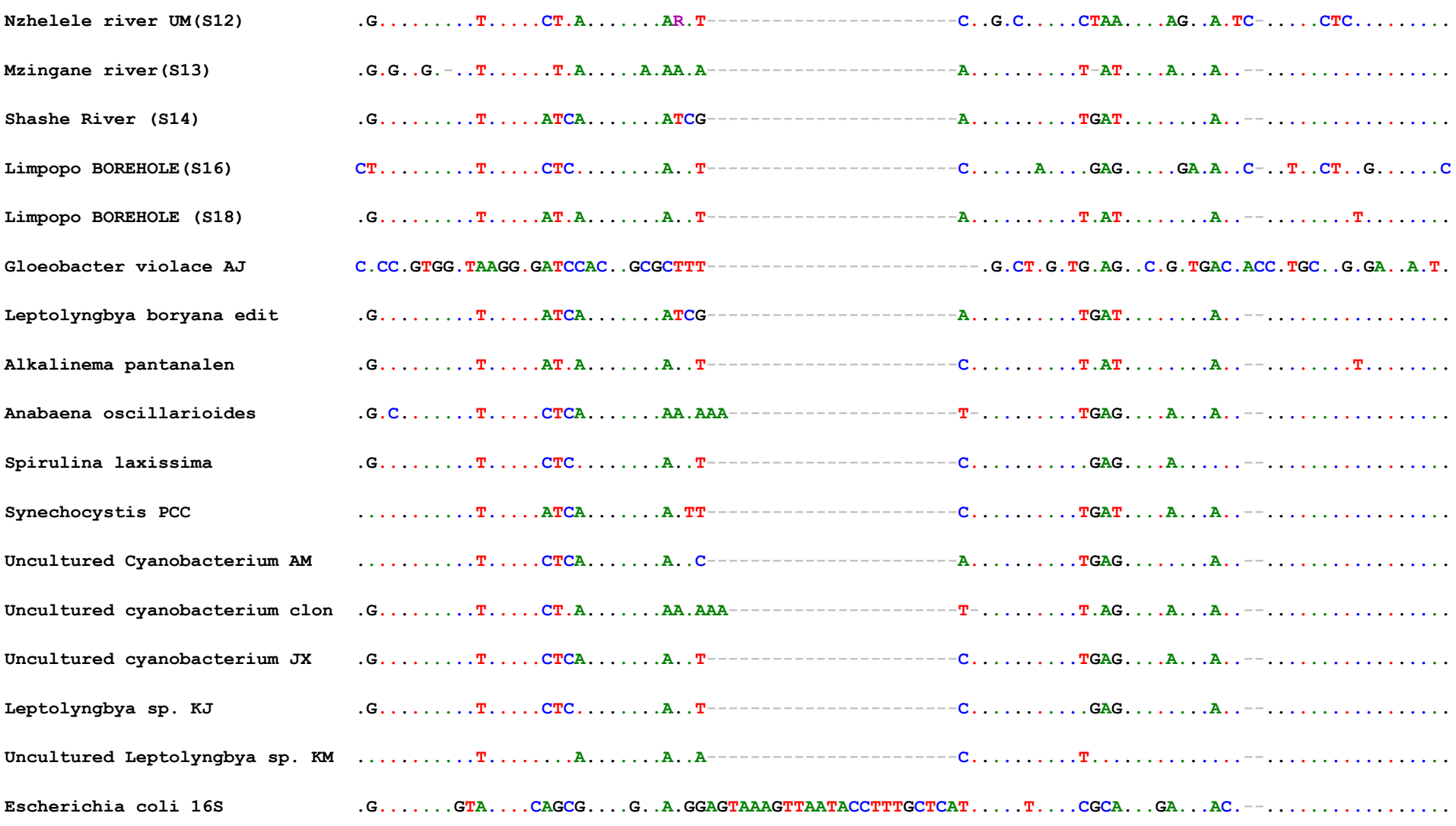

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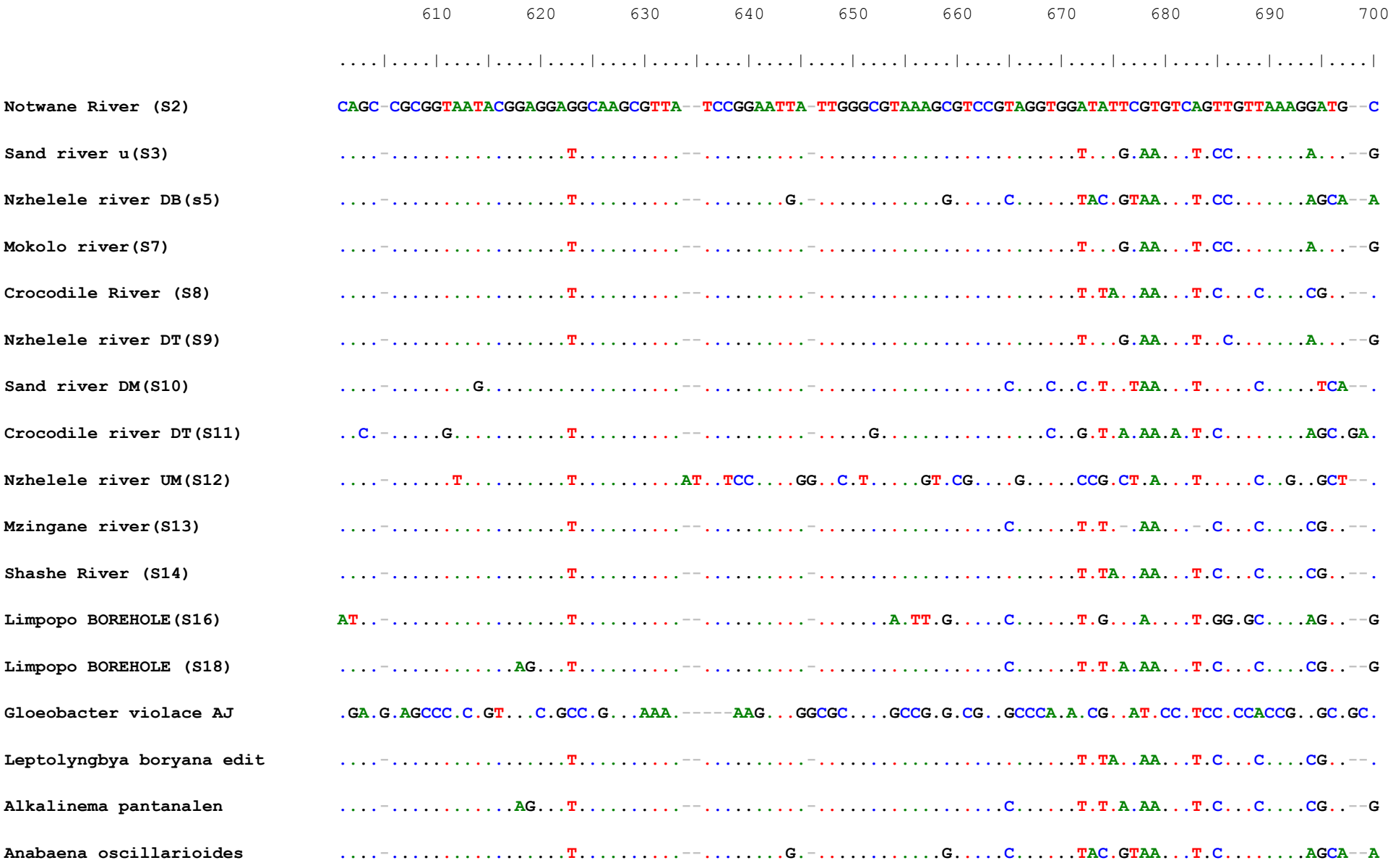

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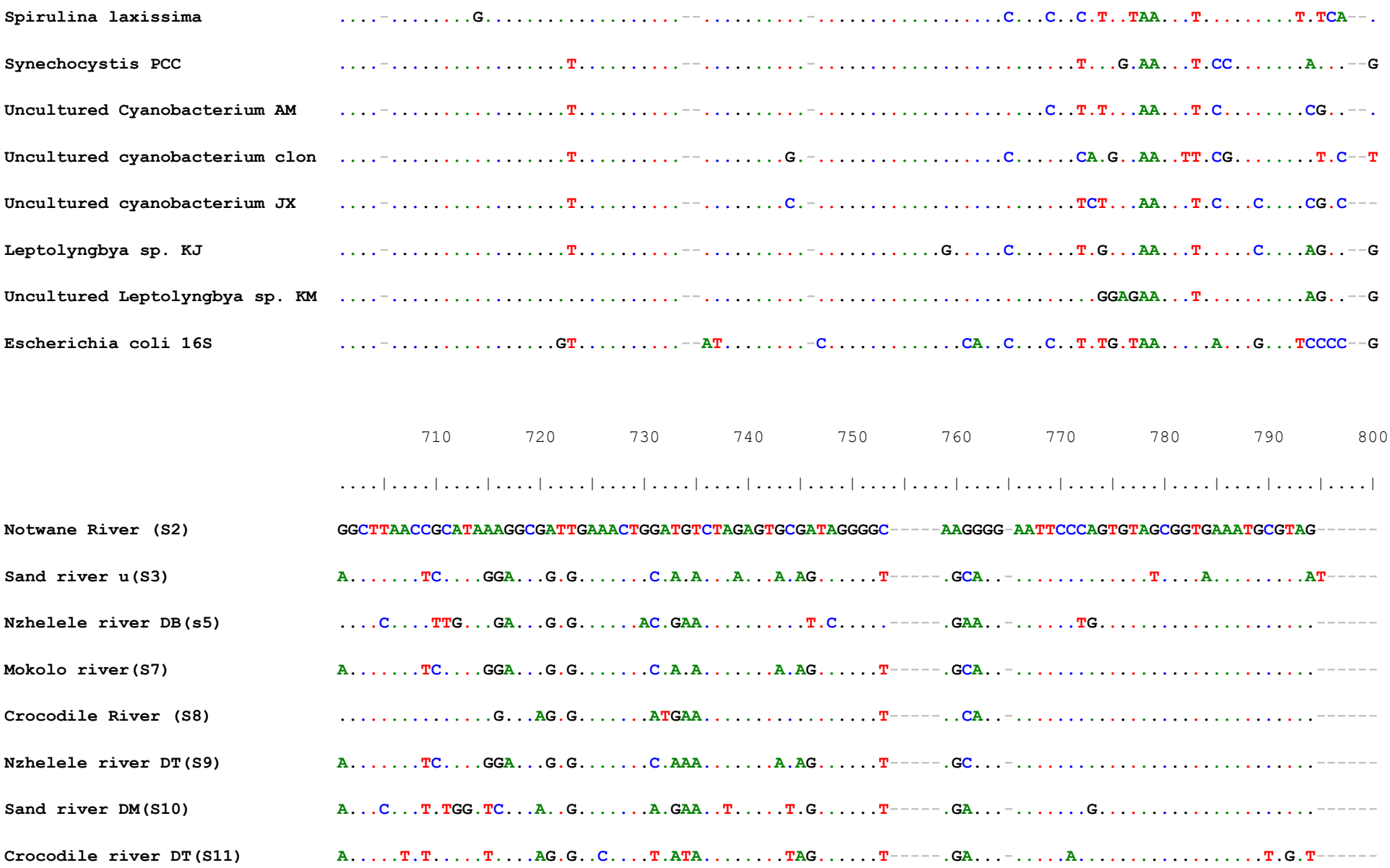

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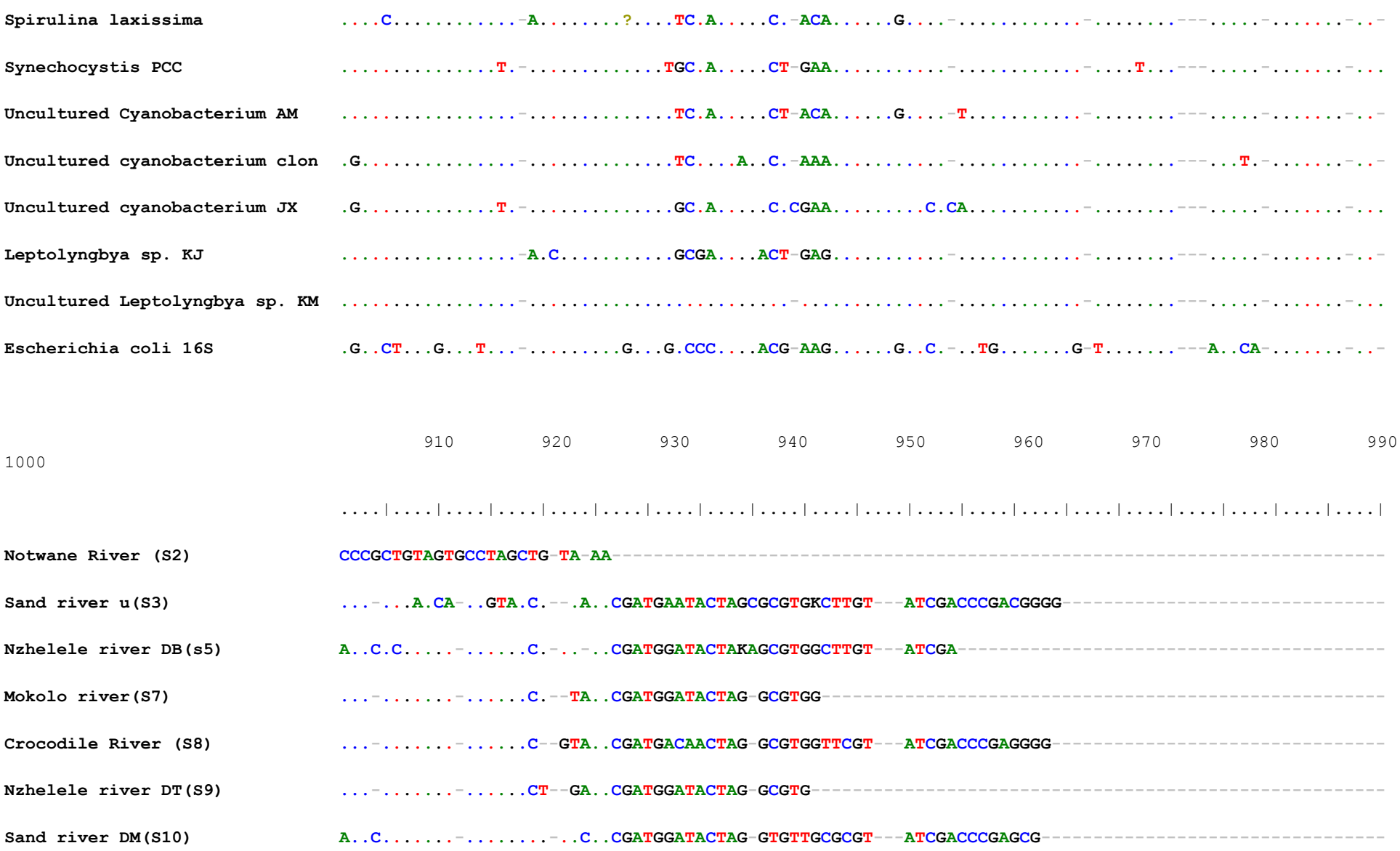

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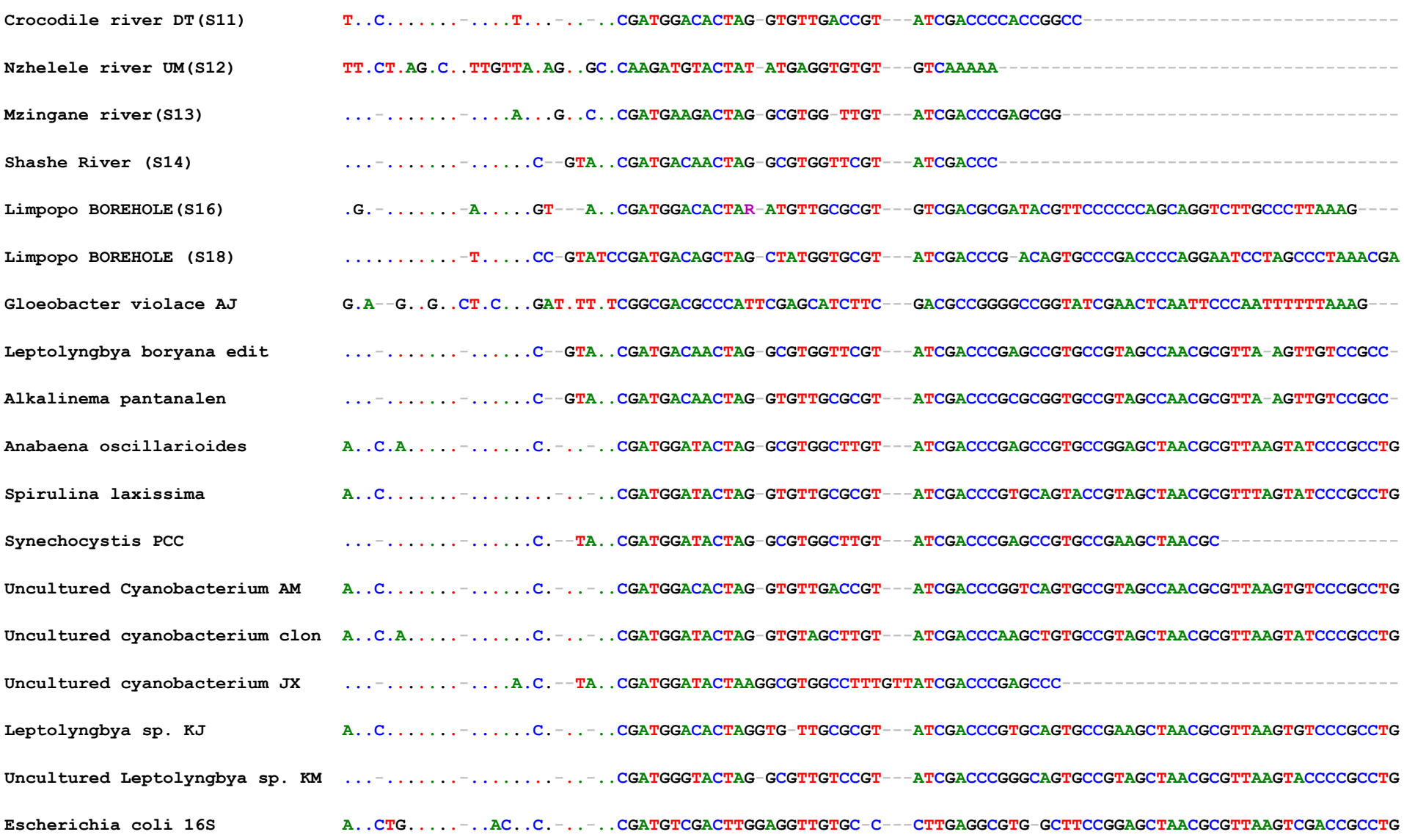

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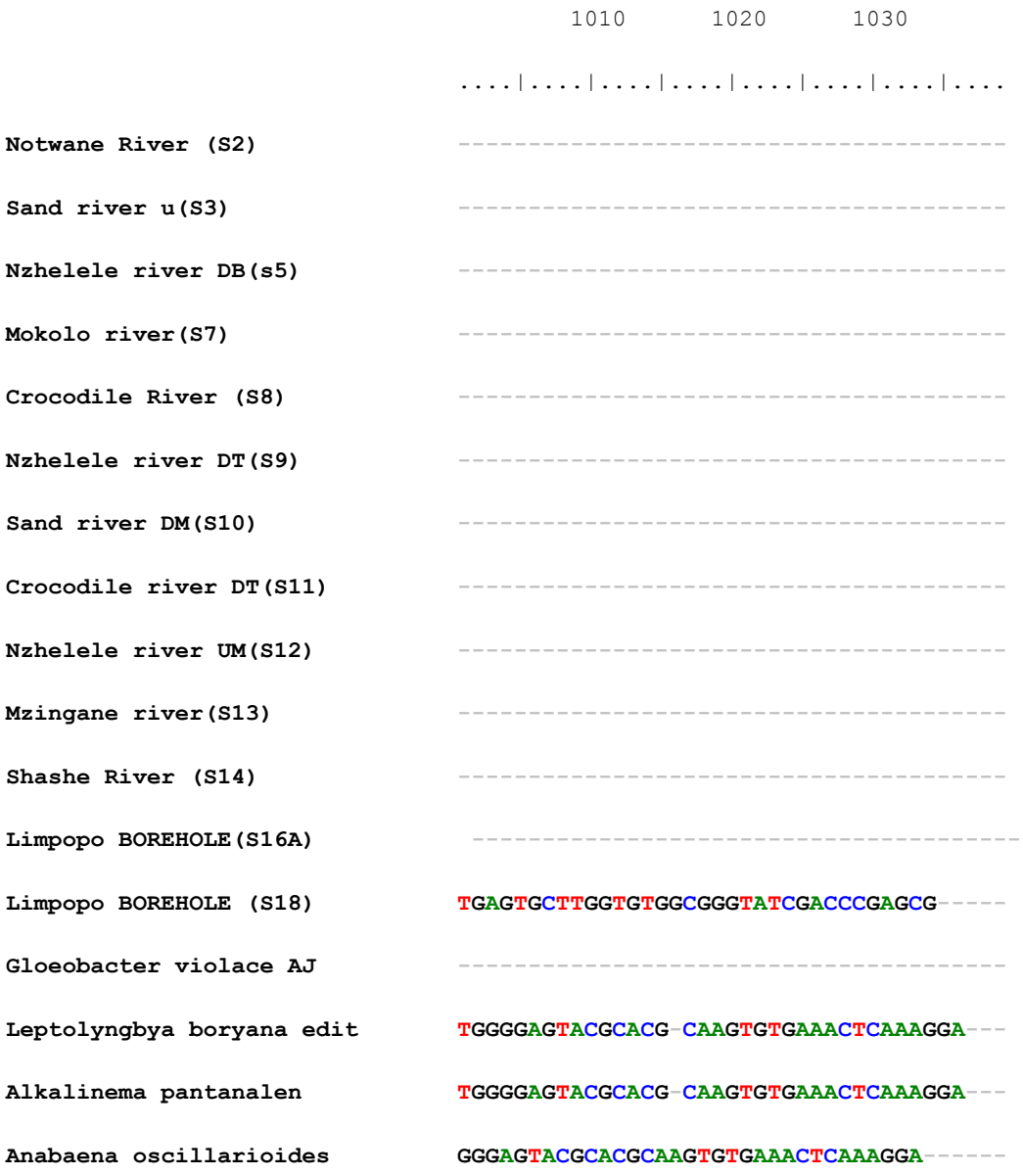

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Synechocystis PCC               --------------------------------------
Uncultured Cyanobacterium AM    GGGAGTACGCTCGCAAGAGTGAAACTCAAAGGA------
Uncultured cyanobacterium clon  GGGAGTACGCGCGCAAGCGTGAAACTCAAAGGA------
Uncultured cyanobacterium JX    --------------------------------------
Leptolyngbya sp. KJ             GGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATT---
Uncultured Leptolyngbya sp. KM  GGGA----------------------------------
Escherichia coli 16S            GGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGAC
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