Bacterial biological control of toxic cyanobacteria and the resulting eco-toxicity

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: December 2019

Abstract

The increased frequency and toxicity of freshwater cyanobacterial blooms is a cause for concern globally. Not only do these blooms result in reduced levels of oxygen in the water column and unpleasant odour, they also produce cyanotoxins as secondary metabolites. These toxins have resulted in the death of humans, aquatic organisms and wildlife. Bloom occurrences have been mapped globally and pose a challenge to water quality management due to toxin release, oxygen depletion, unpleasant water taste and odour. Biological control as a mitigation measure of these blooms has been explored using microorganisms and higher order grazers. A need exists for low cost, passive treatments through environmentally favourable control agents. In this dissertation, the biological control of filamentous *Oscillatoria* and unicellular *Microcystis* was investigated using predatory bacteria. Bacterial isolates from the phylum *Proteobacteria* were isolated and found to be effective in the reduction of microcystins released by both cyanobacteria. These were compared to a *Lysinibacillus* related isolate, which is from the phylum *Firmicutes*. The research conducted indicated a need for more molecular identification of wild strains of cyanobacteria, especially the less studied filamentous isolates to expand the database of sequences deposited for identification to be more conclusive through 16s rRNA gene sequencing. *Microcystis* was positively identified through 16srRNA identification and showed similarity to a species collected from another province in the same country. The exposure of cyanobacteria to bacterial numbers at lower ratios (2:1 ratios of cyanobacteria to bacteria) indicated a stress response from cyanobacterial cell morphology and a reduction in toxicity. The research found that *Pseudomonas rhodesiae* (isolate 3w) and the *Lysinibacillus* fusiformis related isolate (isolate B) resulted in a greater reduction of toxins (microcystins) in both the filamentous and unicellular isolates. Measurement of toxicity was through the ELISA (enzyme linked immunosorbent assay) and confirmed through HPLC (high performance liquid chromatography), which indicated the presence of two microcystin variants, microcystin -LR and -RR. The bacterial isolates readily reduced the more toxic microcystin -LR as opposed to microcystin R-R, indicating that there may be microcystin degrading capacity in the isolates. Screening of eco-toxicity from the resultant bacterial treatment in the water indicated that reduced toxicity resulted in higher survival from the bio-indicator organisms overall. *Thamnocephalus platyurus*, a freshwater crustacean, was the most sensitive to changes in toxicity, proving to be a more suitable confirmation method of microcystin reducing treatment interventions. Isolate B was the best isolate from both a toxin reducing and eco-toxicity response perspective. This study also compiled all the findings from cyanobacterial bloom research in the African context as well as gaps in the area of bacterial biological control globally. In summary, the present study confirmed the

potential to optimize passive bacterial control of cyanobacterial blooms on a larger scale, within a mixed bloom population.

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Romans 8:28: And we know that all things work together for good to them that love God, to them who have been called according to his purpose.

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Dedication

I dedicate this thesis to my angel in heaven, Minenhle Ndlela and my angel on earth, Tina Zime Silinga.

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1. General Introduction

Research into cyanobacterial blooms and their impacts has spanned over decades, with concerns over the increased frequency and toxicity of these blooms (Paerl et al., 2016). Cyanobacteria are photosynthetic prokaryotes, producing chlorophyll and phycobilins, which are photosynthetic pigments. These organisms are ubiquitous, present in soils, marine and freshwater systems (Castenholz, 2015). In freshwater environments, eutrophication from high nutrients such as nitrate and phosphate concentrations, result in the excessive growth of these cyanobacteria among other algal species (Heisler et al., 2008). These excessive growths are termed as blooms. Cyanobacterial harmful algal blooms (CHABs) have been the cause of numerous imbalances in the food web, resulting in reduced oxygen, fish kills due to toxicity and unpleasant odour (Paerl et al., 2001). There have been numerous reports of wildlife (Oberholster et al., 2009a;b), fish and crustacean (Backer, 2002) death through the occurrence of toxic cyanobacterial blooms. Livestock, bird and mammal deaths have been recorded globally (Stewart et al., 2008), with the death of livestock being one of the major economic costs of cyanobacterial blooms (Steffensen, 2008), reports of wildlife, livestock and domestic animals had been recorded in six continents, with the common route of contact being ingestion (Hilborn and Beasley, 2015). One of the most concerning bloom related fatalities has been human exposure to toxin containing water (Carmichael et al., 2001; Hilborn and Beasley, 2015). The toxins commonly reported with these fatalities are microcystins, anatoxin-a, saxitoxins and other variants of these neuro and hepatotoxins.

A consensus of bloom occurrences in tropical countries found the most cosmopolitan genus to be *Microcystis* (Mowe et al., 2014). Another more recent study, mapped the occurrence of this species and microcystins all around the world (Preece et al., 2017).The toxins primarily produced by this species within these genus among others are microcystins. Microcystin has a cyclic heptapeptide structure, with a protein phosphatase inhibiting action. These peptide toxins have also been classed as a potential carcinogen (Lone et al., 2015). There are over a 100 known variants of microcystin (Preece et al., 2017), determined by the amino acids attached to the cyclic heptapeptide. Microcystin LR is the most toxic of these variants (Campos and Vasconcelos, 2010).

Microcystis is the most well studied genus of freshwater cyanobacteria due to its frequent occurrences, however not the only bloom causing species. Among other species that have dominated toxic blooms, are sub-surface occurring, filamentous cyanobacteria such as *Oscillatoria*. These benthic cyanobacteria have not been as well studied as *Microcystis*, however they have been linked to animal poisonings and toxic blooms (Quiblier et al., 2013) which have also been attributed as the cause of water discoloration

and foul odour, as well as the production of cyanotoxins (Van Liere and Mur, 1980). *Oscillatoria* also produces the hepatotoxin microcystin as well as anatoxin-a, which is a neurotoxin (Rantala-Ylinen et al., 2011). Anatoxin-*a* is a bicyclic amine, also referred to as a "very fast death factor," often causing paralysis through the inhibition of acetylcholinesterase, which aids in muscle movement. When tested in mice, within two to 17 minutes, heart rates had reduced rapidly (Osterbauer and Dobbs, 2009). The frequency and toxicity of the above mentioned species require mitigation measures that can be costly and pose a challenge to developing countries. Among the mitigation measures of these toxic blooms, biological control through the use of bacteria has been indicated in successful lysis and/or inhibition of cyanobacterial isolates (Gumbo et al, 2008; Sigee et al., 1999). Studies conducted have mostly been on axenic laboratory strains of cyanobacteria, with total lysis of the cyanobacterial cells resulting from the predatory bacteria (Nakamura et al., 2003). Due to this lysis, the cyanotoxins are released into the external water environment. As a result, this form of control has previously not been recommended outside of laboratory conditions (Kim et al., 2008a). Recent literature however has indicated the toxicity reduction of toxic cyanobacteria through the addition of bacteria, as well as a reduction in phosphates and nitrates (Su et al., 2016).

The bacterial reduction of cyanotoxins in live cyanobacterial cultures as reported by Su et al. (2016) is only part of a handful of studies indicating successful application of bacteria with positive water quality impacts. There is a need for further research in this area, to determine whether this application is viable for different environmental cyanobacteria genera and more importantly, whether the measurement of reduced toxicity would indeed have positive environmental impacts. Developing countries with limited infrastructure and funds benefit greatly from passive treatments that are economically feasible (Oberholster et al., 2014, 2018). This is crucial for evaluating the possibility of up-scaling this technology, which could be a practical cost-effective solution in developing countries.

The aim of this study was therefore to assess the potential of bacteria isolated from bloom waters in controlling non-axenic *Microcystis* and *Oscillatoria* dominated cultures by assessing cyanotoxin levels after exposures. Consequently, the impacts of the changes in toxicity would be assessed based on bio-indicator organism responses. Seeds, plants and zooplankton would be used to determine whether the reduction of cyanotoxin concentrations results in reduced mortality of biological indicator organisms. The organisms tested would typically be affected by toxic bloom water through habitat or irrigation.

As most reports of algicidal bacteria report cell damage and lysis, the concern from a water quality perspective is the release of toxins following lysis. In the present study we hypothesized that lower microbial numbers may be effective in controlling bloom toxicity, without causing total cyanobacterial

cell lysis. Moreover, the comparative study of filamentous and unicellular isolates would provide contribution of knowledge in application of bacterial biological control, which could have relevance for larger scale development.

1.1 Objectives

The primary goal of this research was to assess the biological control of bacterial isolates against toxic unicellular and filamentous cyanobacteria and the resulting eco-toxicity of this control method.

The objectives were to firstly review the current literature available and gaps on the research and occurrence of cyanobacterial blooms in the African context over the recent decade, which is still developing in many regions (Chapter 2).

The second objective was to provide a literature overview on the findings of bacteria as biological control agents of cyanobacteria needed to be obtained (Chapter 3) to determine the progress in this research area and where possible gaps could be filled by the current study.

The third objective was to determine the ecology, identification and growth trends of all the isolates (Chapter 4). This aided in the selection of suitable experimental parameters for the exposure of targeted cyanobacteria to predatory bacterial isolates.

The fourth objective was to determine efficacy of bacteria against the toxicity in cyanobacterial cultures through exposure experiments (Chapter 5). This was to investigate whether lower bacteria ratios (1:2 as opposed to 1:1 and higher) would be effective in reducing toxicity in mixed populations dominated by bloom causing cyanobacteria.

Lastly, the possible environmental impacts of this treatment were tested on bio-indicator organisms (seeds, plants and crustaceans) to verify that reduced toxin concentrations in mixed cultures of cyanobacteria resulted in higher survival of sensitive bio-indicator organisms. The most sensitive bio-indicator was also identified (Chapter 6).

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Chapter 2: An overview of cyanobacterial bloom occurrences and research in Africa over the last decade

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Declaration by the candidate

With regards to Chapter 2, the nature and scope of my contribution was as follows

Nature of contribution	Extent of contribution
Conceptual design, experimental work, manuscript writing	70%

The following co-authors have contributed to chapter 2

Name	Email address and institutional affiliation	Nature of contribution	Extent of contribution
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Abstract

Cyanobacterial blooms are a current cause for concern globally, with vital water sources experiencing frequent and increasingly toxic blooms in the past decade. These increases are resultant of both anthropogenic and natural factors, with climate change being the central concern. Of the more affected parts of the world, Africa has been considered particularly vulnerable due to its historical predisposition and lag in social economic development. This review collectively assesses the available information on cyanobacterial blooms in Africa as well as any visible trends associated with reported occurrences over the last decade. Of the 54 countries in Africa, only 21 have notable research information in the area of cyanobacterial blooms within the last decade, although there is substantial reason to attribute these blooms as some of the major water quality threats in Africa collectively. The collected information suggests that civil wars, disease outbreaks and inadequate infrastructure are at the core of Africa's delayed advancement. This is even more so in the area of cyanobacteria related research, with 11 out of 20 countries having recorded toxicity and physicochemical parameters related to cyanobacterial blooms. Compared to the rest of the continent, peripheral countries are at the forefront of research related to cyanobacteria, with countries such as Angola having sufficient rainfall, but poor water quality with limited information on bloom occurrences. An assessment of the reported blooms found nitrogen concentrations to be higher in the water column of more toxic blooms, validating recent global studies and indicating that phosphorous is not the only factor to be monitored in bloom mitigation. Blooms occurred at low TN:TP ratios and at temperatures above 12°C. Increased nitrogen was linked to the toxicity of cyanobacterial isolates and increased temperature also had a positive effect on bloom occurrence and toxicity. *Microcystis* was the most ubiquitous of the cyanobacterial strains reported in Africa and the one most frequently toxic. *Cylindrospermopsis* was reported more in the dry, north and western parts of the continent as opposed to the rest of the continent, whilst *Anabaena* was more frequent on the south eastern regions. In light of the entire continent, the inadequacy in reported blooms and advances in this area of research require critical intervention and action.

2.1. Introduction

Water depletion in developing countries has been a crisis for decades (Falkenmark, 1989). Over the past decade, substantial evidence has been presented on the looming water crisis in Africa. Although social factors and energy generation systems contribute to the strain on water availability, environmental factors are also significant contributors. Some of the factors attributed to this depletion are anthropogenic activity, pollution, phosphorous and nitrogen loading (Vörösmarty et al., 2010). In African countries, the supply of potable water is an eminent issue, with water quality affected by the inadequacy of water purification plants and lack of knowledge in chlorine dosing being some of the reasons (Momba et al., 2006). These issues are further compounded by contamination during storage of purified water from plants to point-of-use stages (Massoud et al., 2010). The contamination of water results from a variety of factors, and a more imminent source of contamination in both reservoirs and water bodies is algal blooms (WHO, 2011).

In nature, excessive phosphorous and nitrogen loading have consequently been found to result in eutrophication, which then cause the proliferation of algae, leading to algal blooms (Carpenter, 2005, Yang et al., 2008). Algal blooms are a natural phenomenon that occasionally occurs with nutrient loading from anthropogenic and natural activity in water bodies. Historically, these blooms were not always considered harmful and were more prevalent in summer months (Mowe et al., 2015). However, with the rise in global temperatures through climate change, there has been a rise in algal blooms, particularly in coastal countries (Oberholster et al., 2009a). Of particular significance are harmful algal blooms (HABs). The phenomenon of harmful algal blooms came to the fore decades ago, when the simultaneous increase in anthropogenic activity and climate change resulted in an increase in eutrophication and subsequently, algal blooms. These are defined by having a negative environmental impact and are primarily caused by microalgae (Zingone and Enevoldsen, 2000). Although various micro-algal species can be present under bloom conditions, the algae of concern are cyanobacteria, also known as blue-green algae. Cyanobacteria are a group of microorganisms that exist as filaments or single cell. They are larger than eubacterial cells but are able to photosynthesize. Their production of the phycobilin pigment results in a bluish tint at high concentrations, which has led to the organisms being coined as blue-green algae (Stocks, 2013). These algae are of major concern during algal blooms due to their potential production of cyanotoxins when in large numbers. Cyanotoxins are released by cyanobacteria and have potentially fatal effects on human and animals exposed to contaminated water (Paerl et al., 2001).

Though Africa has been known to lag behind in research and information sharing, quite a few countries have reported algal blooms and toxicity, especially in the recent years. This review aims to provide the context of cyanobacterial blooms in Africa and assess the current state of cyanobacterial blooms in Africa over the last decade, although in some cases, the information may date as far back as the year 2000.

2.2. Overview of global reports of cyanobacteria

Increased temperatures, salinity and anthropogenic activities have resulted in cyanobacteria gaining greater advantage over other phytoplankton in freshwaters (Paerl and Huisman, 2009). Europe, Asia and America have documented just under half of their lakes as eutrophic, with reports that at least 25% of reported blooms being toxic (Bláha et al., 2009).

Over the past decades, blooms have been further classified as harmful algal blooms or cyanobacterial harmful blooms. The defining factor in the classification of these blooms is firstly the causative species and the toxin release associated with the bloom (Anderson et al., 2002; Paerl and Huisman, 2009). Generally, algal toxins are either not produced or produced in high concentrations in surface waters, remaining at 1 $\mu g.L^{-1}$ or less. Harmful algal blooms are classified as blooms in which the amount of toxin reaches or exceeds 10 $\mu g.L^{-1}$ concentrations in surface waters, which has a direct negative impact on aquatic and human life when exposed to the toxic water (Schaedel, 2011).

Toxic blooms have serious human health impacts; of particular significance is an incidence in Brazil where over a hundred dialysis patients died from cyanotoxin exposure (Azevedo et al., 2002). Initially, they were mapped as occurring in tropical countries due to suitable temperature and seasonal conditions. However, with climate change and global warming prevalence, the increase in blooms has resulted in occurrences all over the world in the past decade (O'Neil, et al, 2012; Lefebvre et al., 2016).

Among the literature on toxic blooms, a lot of research has focused on understanding the onset of toxic blooms as well as monitoring guidelines. Although it is not exactly clear what triggers the production of toxins in certain strains, climate change has seen an increase in bloom occurrence and toxicity globally (Paerl and Huisman, 2008). Some of the greatest lakes around the world from Lake Taihu in China to Lake Eerie in America have experienced toxic blooms beyond the past decade (International Joint Commission, 2014; Paerl et al., 2014)

In water scarce countries, increased toxic blooms introduce greater strain in the security of water for both humans and the environment. Africa is one of the continents comprising the most developing

countries. Amongst the social issues surrounding the countries, water quality is at the core. The challenge is not only in underdeveloped infrastructure but also in the political issues affecting service delivery. These issues prevail against a back drop of poverty and high population growth (Clay, 1994). Four of the southern countries contributing to the economic development within the continent are described as water scarce in comparison to the rest of the continent (Turton, 2008). Figure 2.1 shows an overview of the affected water bodies reported within the African continent over the last decade. Data indicates that these occurrences are not well reported in numerous countries of Africa.

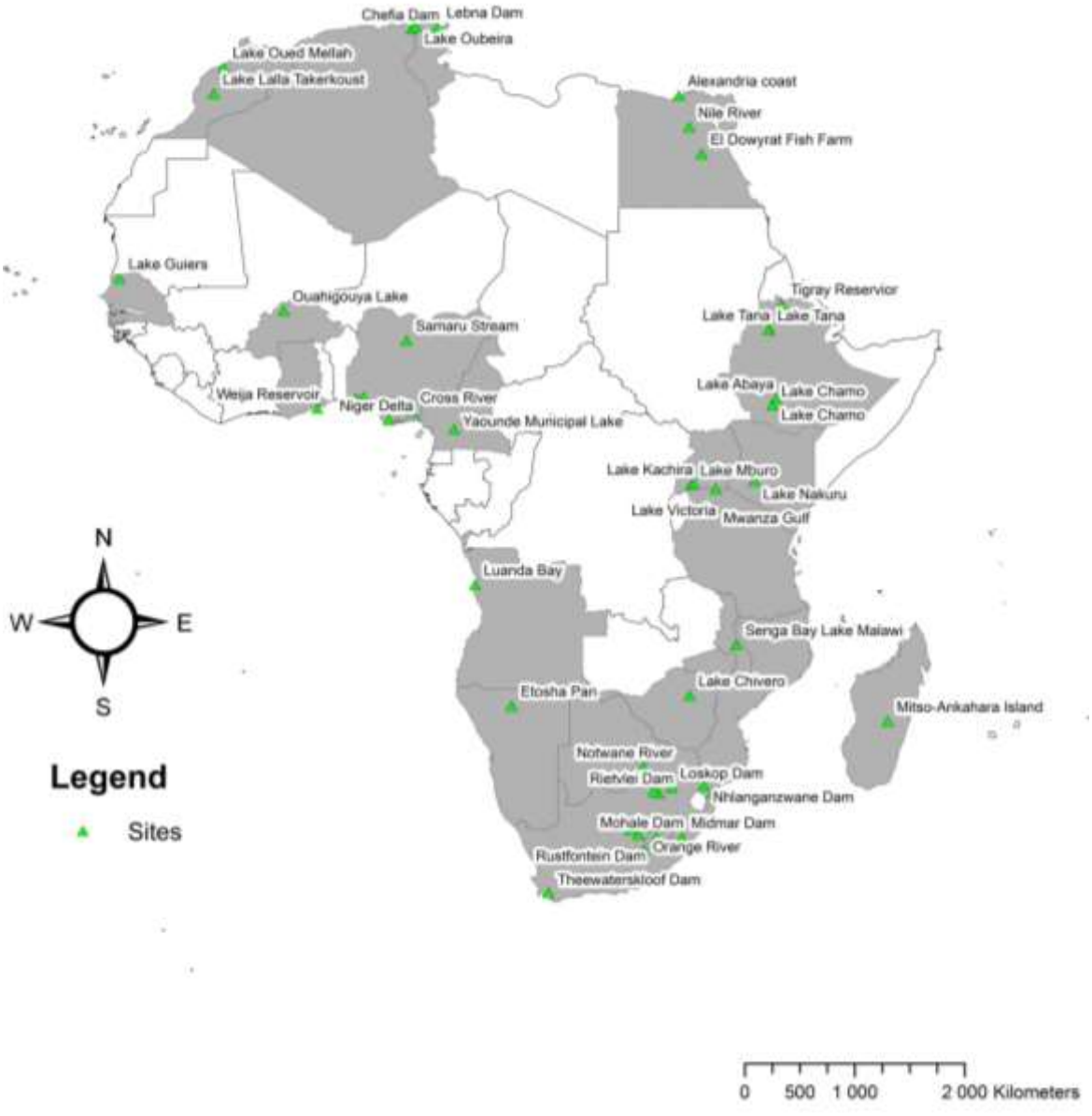

Figure 2.1 Summary of areas affected by cyanobacterial blooms reported in Africa

2.3. Cyanobacterial blooms in Africa

2.3.1 The African context of cyanobacterial blooms

The issues surrounding Africa as a continent are somewhat unique to the rest of the world; wars, poverty, water pollution and disease outbreaks are common in most African countries with rapid population increases, as most countries are developing. Numbers of slum populations in most of the countries are nearing the 50% mark. This of course ties in with nutrient loading into waters from waste and emerging contaminants of concern, as well as added pressures on the efficiency of current wastewater treatment facilities. Cyanobacterial blooms arise as an area of concern within this context. Based on the Africa water atlas (UNEP, 2010) and data collected in this review, Table 2.1 offers brief information relevant to cyanobacterial blooms and the current water state in Africa. The average values were derived from 2008 reports of each country in Africa. Northern Africa is the most water scarce region, followed by southern Africa. Central and Western Africa have the most rainfall, followed by the Island regions. The Islands have limited information, with two toxic isolate reports, whilst one bloom report was from central Africa, in Cameroon specifically. The more water scarce regions have numerous reports on cyanobacterial blooms compared to regions with higher rainfall. The urban water access figures indicate that improved drinking water access in urban areas is over 50% overall in the continent, this excludes water access in rural areas. Allocation to agriculture in the continent showed that apart from Central Africa (34%), over 70% of the continent's water allocation in each region is for agriculture. Effects of climate change, as well as the known erratic rainfall and droughts occurring in the continent, Africa is particularly vulnerable to eutrophication and therefore cyanobacterial blooms. This review combines earlier reports and research from African countries to provide graphical and tabulated representations of findings.

Table 2.1: Brief profiles of African regions relating to water and cyanobacteria

African Region	Countries with cyanobacteria reports	Water access (%)	Rainfall (mm/yr)
Southern	7	67.7	721
Central	1	55.86	1757
Eastern	4	77.97	767
Western	4	69.42	1059
Northern	4	79.67	194
Western Indian Ocean Islands	2	83.75	1696

2.3.2 Occurrence and diversity of cyanobacteria

Presented in Table 2.2 is a summary of cyanobacterial occurrences over the years in Africa, specifically the source, species and description of the occurrences in different water sources For the purposes of this review, 21 countries have information on bloom occurrences relating to the past decade and a few in the early 2000s. Based on Table 2.2, the dominant genus in the blooms overall was *Microcystis*, with occurrences in various sources, from saline lakes to hot springs. The description of occurrences indicates nearly all the blooms were toxic or caused by toxin producing strains.

Table 2.2 Overview of toxic cyanobacterial occurrences in Africa from the early 2000s

Country	Water source	Year reported	Cyanobacteria	Description of occurrence
Algeria	Lake Oubeira	2008	*Microcystis spp.*	Turtle death reports
	Cheffia Dam	2007	*Microcystis sp. morphospecies*	Occurred in treatment plant
	Lake Oubeira	2004	*Microcystis sp.*	First report in drinking water source
	Lake Oubeira	2004	*Cylindrospermopsis raciborskii, Microcystis* spp.	First report in freshwaters
Botswana	Notwane River	2014	*Merismopaedia, Microcystis, Oscillatoria* spp.	Presence of toxin producing cyanobacteria in treated sewage receiving river
Burkina Faso	Ouahigouya Lake	2010	*Microcystis sp.*	Assessment of sub-Saharan cyanobacteria strains
Cameroon	Gulf of Guinea coast	2006	*Phormidium, Heterocapsa, Rivularia, Trichodesmium Planktothrix mougeotii, Oscillatoria putrida,*	Harmful cyanobacteria occurrences
	Yaounde Municipal Lake	2003	*Microcystis aeruginosa*	Cyanobacteria in a hypertrophic lake
Egypt	Domestic water reservoirs	2016	*Microcystis aeruginosa*	Occurrence of toxic strains in domestic water storage reservoirs
	Nile River	2015	*Microcystis aeruginosa*	Microcystins in treated and untreated wastewater
	El-Khadra	2014	*Spirulina, Oscillatoria, Nostoc* spp.	Marine cyanobacteria evaluation
	Nile River	2013	*Nostoc, Microcystis*	Microcystin-producing Nostoc isolated

	Alexandria Coast	2012	*Oscillatoria, Lyngbya, Planktothrix spp.*	Benthic bloom associated with fish deaths
	Nile Delta	2008	*Microcystis wesenbergii, Microcystis aeruginosa, Synechococcus*	Assessment of hepatotoxic cyanobacteria in the Nile Delta
	Nile River	2007	*Microcystis aeruginosa*	Microcystin measurement and removal
	El-Dowyrat Fish farm	2007	*Cylindrospermopsis raciborskii*	Occurrence of toxic cyanobacteria in freshwater
Ethiopia	Lake Tana	2015	*Microcystis aeruginosa*	Cyanotoxin production in larges
	Tigray Reservoir	2011	*Microcystis* spp.	Assessment of Microcystis diversity in reservoir
	Lake Chamo, Abaya etc.	2011	*Microcystis aeruginosa*	Cyanotoxin production in rift valley lakes
Ghana	Weija Reservoir	2006	*Anabaena flos-aquae, Cylindrospermopsis raciborskii, Microcystis aeruginosa, Planktothrix agardhii.*	Toxin producing species in drinking water reservoirs
Kenya	Nyanza Gulf	2012	*Microcystis spp.*	Shallow eutrophic bay in Lake Victoria
	Nakuru oxidation ponds	2010	*Microcystis, Euglena, unknown coccoid isolates*	Microcystin producers found in oxidation pond
	Lake Sonachi	2005	*Arthrospira fusiformis*	Occurrence in alkaline and saline lakes
	Lake Simbi	2005	*A. fusiformis, Anabaenopsis abijatae*	Occurrence in alkaline and saline

				lakes
Lesotho	Rustfontein Dam	2011	*Microcystis sp.*	Microcystin LR production during bloom
	Mohale Dam	2007	*Microcystis aeruginosa*	Toxic strains found in dam
Madagascar	Mitso-Ankaraha Island	2007	*Geitlerinema sp.*	Cytotoxic strain found in island
Morocco	Mansour Eddhabi	2010	*Microcystis aeruginosa*	Microcystins in drinking water reservoirs
	Almasirra	2010	*Microcystis aeruginosa*	Microcystins in drinking water reservoirs
	Oukaimeden	2009	*Nostoc muscorum*	Microcystin producer in water source
Mozambique	Various lakes	2011	*Microcystis spp.*	Microcystin producing strains in lakes
Nigeria	Lekki Lagoon	2010	*Oscillatoria, Microcystis aeruginosa, M. flos-aquae, M.wesenbergii and Anabaena flos-aquae*	Bloom-forming strains found in lagoon
	Zaria aquaculture ponds	2009	*Microcystis, Planktothrix, Nostoc, Anabaena*	Microcystins in aquaculture ponds
Senegal	Senegal River delta	2008	*Cylindrospermopsis*	Occurrence in drinking water
	Lake Guiers	2006	*Cylindrospermopsis raciborskii, Lyngbya*	Phytoplankton assemblage analysis
South Africa	Theewaterskloof	2015	*Anabaena ucrainica*	Microcystin containing strain in Theewaterskloof
	Loskop Dam	2014	*Microcystis aeruginosa*	Microcystin accumulation in fish tissue after a bloom
	Nyala Magnesite Mine	2014	*Microcystis, Oscillatoria, Phormidium spp.*	Cyanobacterial found in soda pits at

				mine site
	Loskop	2014	*Microcystis aeruginosa*	Assessing daphnia exposed to extracellular microcystins
	Kruger National Park	2010	*Microcystis aeruginosa*	Assessment of bloom toxicity after wildlife deaths
	Nhlanganzwane Dam	2009	*Microcystis aeruginosa*	Toxigenic strains found after animal mortalities
	Makhohlolo	2009	*Microcystis aeruginosa*	Toxigenic strains found after animal mortalities
	Krugersdrift Dam	2009	*Microcystis aeruginosa*	Toxic strain influence assessment on phytoplankton diversity
	Orange River	2007	*Cylindrospermopsis raciborskii*	
Tunisia	Hot springs	2013	*Oscillatoria* spp.	Assessment of thermophilic cyanobacteria
	Lebna Dam	2008	*Microcystis* spp.	*Microcystis* morphospecies in dam
		2008	*Microcystis* spp., *Oscillatoria tenuis*	Assessment of seasonal occurrence in dam waters
Uganda	Lake Saka	2011	*Microcystis, Anabaena, Planktothrix*	Microcystin production in Ugandan freshwater lakes
	Murchison Bay	2009	*Microcystis* spp.	Water-related diseases and cyanotoxin trend assessment

Zimbabwe	Lake Chivero	2006	*Microcystis aeruginosa, M. wesenbergii*	Cyanotoxins found in drinking water
	Lake Chivero	2006	*Microcystis aeruginosa*	Eutrophic drinking water reservoir

2.4. Southern Africa

Comprising the more water scarce countries, Southern Africa has lower rainfall, and higher evaporation (Heyns, 2008). This means that in comparison to the rest of the world, water supply is generally lower (Ashton, 2002). Southern Africa comprises seven countries with some of the most frequent reports of cyanobacterial blooms being from South Africa.

2.4.1 Angola

Although hardly water scarce (900-1000 mm per year rainfall) (Turton, 2008), Angola has water quality issues. A 2009 publication by Vale et al. (2009) investigating paralytic shellfish poisoning (PSP) in Luanda and Mussulo bays, briefly referred to cyanobacterial or dinoflagellate blooms as the causative agents. When the screening for the common toxins associated with PSP produced atypical results, the authors alluded to the possibility of cyanobacterial toxins being the possible cause. This study is one of the few available relating to cyanobacteria, although no mention of monitoring programs for PSPs was made in these reports.

2.4.2 Botswana

Reports on cyanobacterial blooms in the available water bodies are limited to a 2010 publication assessing the presence of algae in stabilization ponds of the Gaborone wastewater treatment works. The study found cyanobacteria to be more abundant compared to other algal species, with *Microcystis* and *M. flos-aquae* being the most dominant genus and species respectively. This finding was a key concern as the water from these ponds fed into one of the most important river sources in south eastern Botswana. The authors postulate that potential illnesses observed downstream of the ponds may be linked to microcystin toxins, particularly the consumption of fresh produce exposed to this toxin through irrigation (Lusweti et al., 2010) A more recent study by Kirumba et al.(2014) found bio toxic *Microcystis* and *Merismopaedia* isolates among the cyanophyta in the river however, these toxin producers were not the dominant phytoplankton in the river and overall, the river was considered to be in a fairly good state.

2.4.3 Lesotho

A study in 2009 of 3 Dams in Lesotho reported the highest cyanotoxicity to be around 1 $\mu g.L^{-1}$, which coincided with the highest chlorophyll measurement (Mohale, 2011). Although toxic *Microcystis* and *Oscillatoria* species were found, the water treatment facility did not have measures to eliminate toxins and applied pre-chlorination, which are the common effective methods (Mohale, 2011).

2.4.4. Mozambique

In Mozambique, recent work on toxic cyanobacteria entails the study of microcystin producing isolates through the use of PCR (polymerase chain reaction) methods. The study used RFLP (restriction fragment length polymorphism) to differentiate microcystin and non-microcystin producing strains in various water sources. The study found *Microcystis* to be the most dominant strain present in three different lake areas, with MC-LR, -RR and -YR being the dominant variants (Pedro et al, 2011).

2.4.5 Namibia

There is limited information available on toxic cyanobacterial blooms in Namibia. Among the available literature is a 2001 paper by Gunnarson and Sanseovic, indicating links between microcystins and incidences of diarrhoea in Namibia. The recorded concentrations of microcystins were never beyond the WHO 1 $\mu g.L^{-1}$ guideline (WHO, 1998), however there was a direct relationship between the amount of chlorophyll, rainfall and incidences of diarrhoea or complaints to the municipality about water quality. A more recent publication of interest describes a novel species of cyanobacteria from the family of *Oscillatoriales*, *Phormidium etoshii*, in the Etosha pan of Namibia, with no toxicity reported (Dadheech et al. (2013).

2.4.6 South Africa

South Africa has some of the most documented information on cyanobacterial blooms over the past decade, with pioneering research conducted as far back as 50 years ago in terms of cyanobacterial toxicity and outbreaks (van der Westhuizen and Eloff, 1983).

The outbreak of cyanobacterial blooms has been recorded across the country, with most reports initiated by an outbreak of illness or animal death within an area. *Microcystis aeruginosa* and *Anabaena* sp. are the more dominant species in reported toxic blooms across the country, with *Oscillatoria* spp. also dominant in certain reports. Reports of *Cylindrospermopsis raciborskii* particularly in the Northern part of South Africa have also been made in the past decade, with increased cell numbers since the early 2000s (Janse van Vuuren and Kriel, 2008). A list of toxic bloom outbreaks and findings up to the year 2000 has been listed in a review by Oberholster et al. (2005).

Since then, more reports of cyanobacterial blooms have been reported in Lake Krugersdrift, over summer months in 2004 coinciding with fish kills, as well as microcystin levels reaching as high as 43 $\mu g.L^{-1}$ in some sites in 2005-2006 (Oberholster *et al*., 2009b). Reports of microcystin concentrations exceeding 20,000 $\mu g.L^{-1}$ in 2007 resulted in the death of wildlife in the Kruger National Park

Nhlangezwane Dam, which is a water source for wildlife in the conservation park (Oberholster *et al*., 2009c).

Cyanobacterial blooms have also been reported beyond the last decade in Hartebeespoort Dam in 2002 (Oberholster *et al*., 2004) and has since been plagued with toxic blooms due to nutrient loading from wastewater effluent from the upper catchment. The dominant species in toxic algal blooms has previously been reported as *Microcystis aeruginosa* however a more recent study by Ballot *et al*. (2014) indicates the diversity and abundance of the blooms in Hartebeespoort may have been underestimated, with 96% of the microbial biomass comprising cyanobacteria, with *Nostoc* spp. and *Oscillatoria* spp. forming part of the diversity among other species. There have also been developments in a more robust ELISA method in detecting microcystin concentrations in freshwaters, with the kit tested in Hartebeespoort Dam with concentrations exceeding 360µg.L^{-1} of microcystins (Botha *et al*., 2018).

Although typically known to occur in the warmer months, reports of a winter bloom of *Microcystis* in Lake Midmar (Pietermaritzburg, KwaZulu Natal) in 2007 have been a cause for concern (Oberholster and Botha, 2007). *Microcystis* blooms have also been reported in Loskop Dam (Oberholster, 2009; Nchabeleng et al., 2014), with blooms killing wildlife. These blooms also pose a threat to the eco-tourism industry, due to the contamination of water bodies in national parks, killing wildlife (Oberholster et al., 2009c). Of greater concern, are recent reports of *Anabaena* blooms, which were originally not linked to microcystin production, showing prevalence in winter and containing microcystin LR, in Theewaterskloof, Cape Town (Oberholster et al., 2015). A more recent study also identified the occurrence of *Rhapidiopsis raciiborski*, *Phormidium*, *Planktothrix* and *Microcystis* in a Limpopo river basin, using molecular methods and a standard FlowCam as identification methods (Magonono *et al*., 2018).

An illustration of toxic bloom occurrences (Figure 2.2) in the major water sources indicates that the blooms have occurred in nearly all of the known water sources in South Africa. This places cyanobacterial blooms amongst the main issues that threaten water quality.

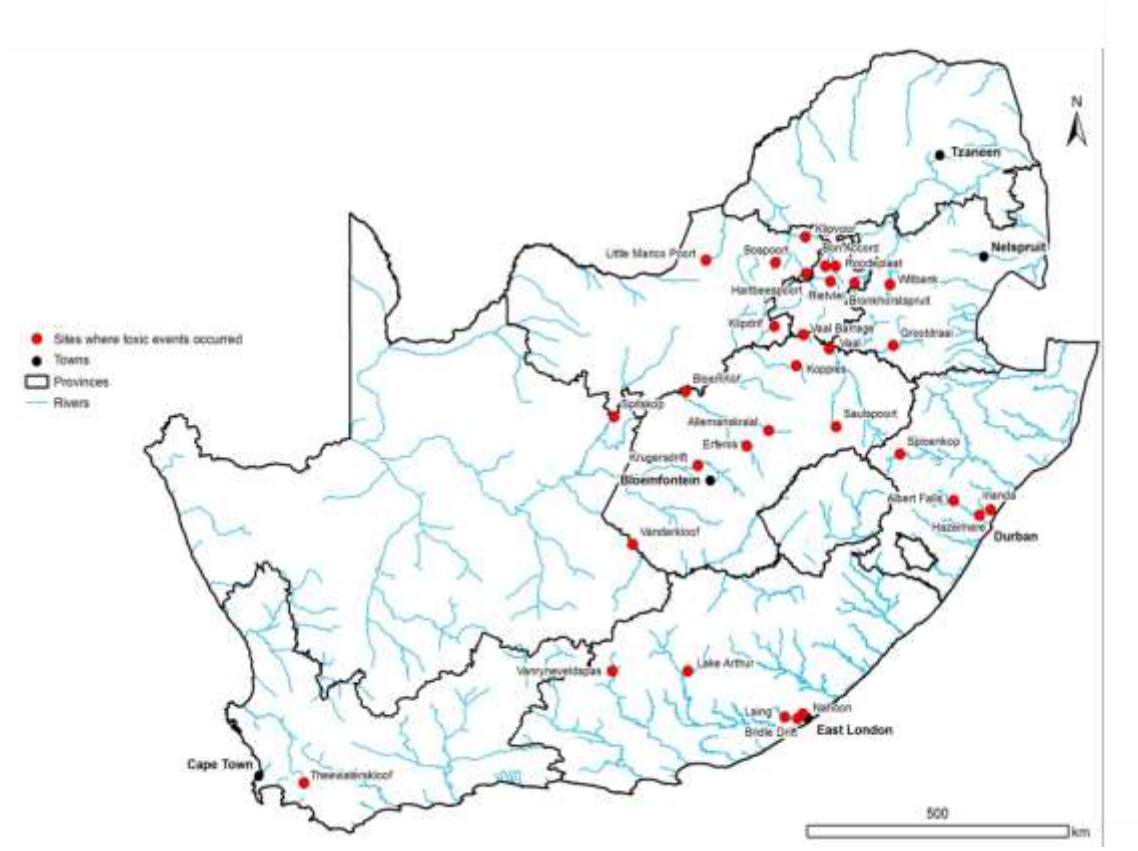

Figure 2.2: Cyanobacteria occurrences in South Africa over the last decade

2.4.7 Zimbabwe

In Zimbabwe, most of the water crises facing the country are due both to mismanagement as well as climate change (Brown et al., 2012). Lake Chivero is one of the main water sources and previously researched eutrophic lakes in Zimbabwe. The lake has been classified as eutrophic for decades, with recorded blooms of *Microcystis* spp. and *Anabaena* sp. in earlier years (1960s), which were more recently (2003) dominated by *Microcystis* spp. due to changing nitrogen levels in the water (Mhlanga et al., 2006). Another study of Lake Chivero found toxic algal blooms with a mean microcystin concentration of 19.86 µg.L^{-1} in the year 2003. The bloom was caused by the inflow of sewage waste upstream of the lake. Although the findings were not linked to any fish or animal deaths, earlier studies found a link between gastroenteritis in infants and the occurrence of blooms in Lake Chivero. (Ndebele and Magadza, 2006). Between the years 2004-2005, Tendaupenyu (2012) assessed five impoundments, of which Lake Chivero was among the five. Interestingly, Lake Chivero had the highest diversity of phytoplankton in November, although the majority was cyanobacteria, namely *Microcystis* and *Anabaena* spp. This might indicate that the nitrogen level changes previously referred to by Mhlanga *et al.* (2006) may have been favourable for *Anabaena* in the rainy season at the time of Tendaupenyu's assessment. These different findings on Lake Chivero indicate how crucial season and

time are to the phytoplankton diversity at any given time. Further work supporting the increasing eutrophication, hence cyanobacteria, in Lake Chivero was a study by Zengeya and Marshall (2007), markedly showing the shift in diet from various fish species. Fish that were typically feeding on diatoms in the 1960s have changed their diet to incorporate more cyanobacteria, specifically resulting in decreases of other fish species and adapting to eutrophic conditions. A 2007 study of a Mazowe River tributary, the Chinyika River, indicated that nutrient loading in this river needed monitoring as many people in the rural areas of Zimbabwe depend on this water for consumption (Bere, 2007). A 2018 report of a study in Malilangwe found *Microcystis* and *Cylindrospermopsis* as the common bloom forming isolates, with greater abundance in the cool dry season (Dalu and Wasserman, 2018). Although reports in the past decade are not as extensive, eutrophication and toxic blooms are a known issue in Zimbabwe.

2.5. Eastern Africa

Similarly to Southern Africa, there have been documented reports of blooms and research in East Africa. This part of the continent is not necessarily water scarce, with most of the countries having average rainfall slightly lower than 860mm per year, which is the global average (Turton, 2008). An assessment of cyanobacterial diversity in East Africa reported that in comparison to South Africa, Kenya and Morocco (northern Africa), the occurrence and toxicity of cyanobacterial blooms in the continent was poorly reported or realised as an area of concern (Haande et al., 2007)

2.5.1 Ethiopia

An IFRC (International Federation of Red Cross and Red Crescent Societies) report (2011) describes Ethiopia and other countries in the horn of Africa as some of the poorest, drought-prone areas. In Ethiopia, Lake Tana is the largest water body and had reports of *Microcystis aeruginosa* occurrences, with microcystin concentrations of up to 2.65 $\mu g.L^{-1}$ (Mankiewicz-Boczek et al., 2014).

Earlier studies also indicated the presence of microcystin producing strains in seven rift valley lakes, where analyses were conducted to determine whether prior animal deaths were due to cyanotoxins. They found microcystins using HPLC and ELISA detection, with Lake Koka being a high risk lake with cyanobacterial cell numbers exceeding 100 000 $cells.mL^{-1}$. This was found in an area of the lake used by human and animals, with *Microcystis* sp. occurring in all the lakes, although various other cyanobacteria were also identified in each lake (Willen et al., 2011).

2.5.2 Kenya

In 2005, a report by Ballot et al. indicated the presence of cyanotoxins in Lake Sonachi and Lake Simbi, in which the main toxin-producing species were *Arthrospira* and *Anabaenopsis*. Microcystin levels of 12 and 39 $\mu g.g^{-1}$ were detected in Lake Sonachi and Simbi respectively. These species were also found to produce anatoxin-a. This particular report is interesting as it is one of the few reporting these species as the main toxin producers in Kenyan waters.

The high nutrient loading from sewage and agriculture activities creates optimal conditions for cyanobacterial blooms. Lake Nakuru, which acts as a water source for endangered wildlife species and other animals in the National Park, has experienced nutrient loading due to effluent flowing in from settlements around the area, hence causing excessive cyanobacterial blooms. Although the study found non-toxin producing strains of *Microcystis*, the potential threat of exposure to microcystins by toxin producing strains of *Microcystis* remains (Kotut et al., 2010).

Lake Victoria, the largest lake in Africa is flanked by three countries: Kenya, Uganda and Tanzania. Reports on the Nyanza Gulf of Lake Victoria, which borders Kenya, state that toxic algal blooms have been observed from the 1980s, which resulted in massive fish kills. An analysis of the Gulf four years ago revealed that *Microcystis* and *Anabaena* sp. were the dominant microcystin producing species; comprising over 50% of the phytoplankton during cyanobacterial blooms. Blooms and microcystin concentrations were found to be higher in the wet season than in the dry season (Sitoki et al., 2012).

2.5.3 Malawi

Gondwe et al. (2008) reported the presence of *Anabaena* sp. heterocysts in Lake Malawi, indicating one of the very few reports of cyanobacterial occurrences in this country.

Not much in the area of technological advances in toxic bloom management has been reported in East Africa. A study by Okello et al. (2010) assessed the various types of microcystins present in the lakes of Uganda, linking the microcystin production to the environmental conditions in water samples. *Anabaena, Aphanocapsa, Chroococcus, Merismopedia, Microcystis, Planktolyngbya, and Pseudanabaena* spp. are examples of the phytoplankton genera found in these waters. The study further indicated that *Microcystis* sp. has the advantage of being able to out-compete other phytoplankton and remain in waters for longer periods of time while shallow waters are more favourable for bloom conditions.

2.5.4 Uganda

Lake Victoria is also flanked by Uganda and the Ugandan portion of this lake is highly polluted. Fishing is one of the main means to generate income in Uganda. For decades, hundreds of people have made their living through the fishing of the Nile perch (*Lates niloticus*). The introduction of this species into Lake Victoria as well as its overfishing has led to excessive algal blooms (Kayombo and Jorgensen, 2006). Regulations to curb the overfishing of the Nile perch directly threaten the food security of the residents in Uganda as most of them are not skilled in any other areas and make their living through fishing. The overfishing also results in the overgrowth of algae, which is further promoted by increases in phosphorus concentrations from external sources such as human and agricultural waste (Kayombo and Jorgensen, 2006). As a result, certain parts of Lake Victoria can no longer sustain life and residents rely directly on the water from the lake as a potable water supply. This has led to a chain reaction of various disease outbreaks. When there is an outbreak of disease, with gastroenteritis symptoms, the untreated human waste can end up in local water sources in countries with inadequate ablution infrastructure. This then contributes to nutrient loading in these waters, which is coupled with cyanobacterial occurrence and abundance (Muyodi et al., 2009). *Anabaena* and *Microcystis* spp. were found to be the abundant cyanobacteria in the Ugandan portion of Lake Victoria (Haande et al., 2011).

Other water sources in Uganda have been affected by cyanobacterial blooms as well. Lake Mburo and Lake Kachera, which are water sources for residents and animals in the mid-western region of Uganda have also been classified as eutrophic, with *Microcystis* sp. and *Anabaena* sp. being the most dominant and present in all sites sampled in the lakes. Being shallow lakes, the issue of blooms can persist for years (Havens, 2008). This is primarily due to the adequate light penetration and warmth of the water, with concentrated nutrients. The concern in these lakes is from the presence of hippos, which then deposit nutrient rich wastes into the water as well as the upstream deposit of cattle farm wastes into the lakes. No cyanobacterial toxicity was recorded in the lakes during the study however; the presence of the cyanobacteria is a possible threat to the water quality and consequently human and animal life that consume the water from these lakes (Nyakoojoo and Byarujali, 2010).

A previous study by Ndebele-Murisa et al. (2010) assessing the great lakes of East Africa (Lake Victoria, Malawi and Tanganyika) has indicated that cyanobacterial blooms are mostly an issue in Lake Victoria and that preventative measures should be taken to preserve the water quality in Lake Malawi and Tanganyika, which borders Tanzania and Malawi. Limited literature is available on the cyanobacteria prevalence in other countries in this region (Ndebele-Murisa et al., 2010), with

Sekadende et al. (2005) reporting on the presence of microcystin-producing *Microcystis* and *Anabaena* species, although the recorded toxin level was not more than 1 µg.L^{-1}.

2.6. West Africa

2.6.1 Burkina Faso

In Burkina Faso, a toxic bloom reported in 2003 found *Microcystis* and *Oscillatoria* as the dominant species (Boelee et al., 2009). A majority of the reports related to cyanobacteria are not accessible in English however according to Boelee et al. (2009), a study assessing 23 lakes and reservoirs by Cecchi et al (2009), found cyanobacteria present in over 20 of the water sources and five potentially toxic species were located in 19 of them. Another 2009 publication by Cecchi et al. (2009) offers a practical management tool for cyanobacteria monitoring, in which it also shows the major water sources affected by cyanobacteria.

2.6.2 Ghana

With Ghana sewage water treatment works (SWTW) failing to adequately treat water and losing over 40% of water through illegal access to water (Osumanu et al., 2010), it is not surprising to have disease outbreaks or indications of cyanobacterial occurrences (Addico et al., 2006), although the toxicity of the microcystin R-R found in the Weija and Kpong reservoir did not exceed 3.21 µg.L^{-1}. The most recent research has been the treatment of microcystin through alum flocculation and microcystin concentration in the Kpong and Weija SWTW, this indicated further research and investigations in the area of cyanobacterial management (Addico et al 2018).

2.6.3 Nigeria

In West Africa, Nigeria's Cross River is a major source of fish and shrimp for daily consumption as well as on a larger supply scale. The river is one of the biggest in Nigeria although information on cyanobacteria occurrence is recent (early 2000s). The need for this knowledge is particularly due to the need to protect the fish and aquatic fauna from potential toxic blooms (Okogwu and Ugwumba, 2008) The same study found that cyanobacterial abundance was higher in the rainy season, with cyanobacteria outcompeting other algae due to increased phosphorous levels in the water-due to anthropogenic activity-and cladocerans feeding on other more palatable algae species. Phosphate levels in this river were recorded to be 10-fold higher in 2008 as compared to the last recording in 1992. Similar studies were conducted in the Nigerian Guinea Savanna. The Savanna is an area of Nigeria which is subject to dry spells; this means that water contamination becomes a crisis during those conditions. A study of the cyanobacterial diversity and trends also found higher cyanobacterial loads of

Microcystis during the wet season, with recommendations for further monitoring. The Samaru stream in Nigeria also showed an abundance of cyanobacteria species during the wet season and more green algae in the dry season (Tisseer et al., 2008). *Oscillatoria* was the most abundant genuss in Lekki lagoon during a study conducted by Abosede and Igekwu (2010). This study indicated that the increase or changes in the phytoplankton community in the lagoon may be due to physiological changes and not necessarily nutrient loading in the waters, although no toxicity was reported. However, a study of the microcystin concentrations in aquaculture ponds in Northern Nigeria found microcystin concentrations reaching 5.8 $\mu g.L^{-1}$, which is a cause for concern if the microcystins bio-accumulate in the fish tissues (Chia et al., 2009). This is one of the few reports assessing the toxicity and microcystin related aspects of cyanobacterial blooms. A review of the cyanobacterial diversity by Akin-Oriola et al. (2006) highlighted the lack of priority in understanding and reporting of cyanobacteria in sub-Saharan Africa. Most of the recorded reports of the blooms in this report were mostly from the 1990s, with only one study conducted in 2005, on Kuramo waters, with *Microcystis* being the prevalent cyanobacteria genus. The most recent study of the Niger Delta rivers found that over fifteen species of cyanobacteria were present in these waters, with *Anabaena* sp. being the most dominant and *Microcystis* the most ubiquitous. Although both species are toxin producers, their abundance in the water was at 100 000 $cells.mL^{-1}$, which is at moderate probability of adverse health effects according to WHO regulations (WHO 2011). This calls for monitoring of the changes in diversity, however no toxicity was recorded. The lack of phosphorous was one of the indicated factors that inhibited blooms of the toxic species (Odokuma and Alex, 2015).In 2012, a detailed study of the phytoplankton in Lamingo Reservoir found that *Microcystis* and *Nostoc* sp. were among the potentially harmful cyanobacteria, although no toxicity was reported (Ajuzie, 2012). Within the same year, Jegede (2012) analysed the use of the green algae *Chlorella* spp. and cyanobacteria in the production of biofuels. The scarcity of information on cyanobacteria in the country of late may be due to blooms not being a nuisance in the country or they are currently not considered a priority.

2.6.4 Senegal

Lake Guiers in Senegal is the most frequently reported water source in the area with cyanobacterial blooms. This lake is the largest reservoir supplying freshwater to the capital city of the country. Although reports are not extensive on blooms in the past ten years, toxic blooms have been recorded in Lake Guiers. In most of Africa, *Microcystis* and *Anabaena* species appear to be the most common cause of nuisance blooms. Senegal, however, is plagued mainly by *Cylindrospermopsis raciborskii* (Berger et al., 2006). This species has not been widely reported in the previously mentioned countries.

A study in 2006 assessing the toxicity of *Cylindrospermopsis* sp. found that no toxins were produced over the study period in winter. Instead the diatom *Fragilaria* sp. was more abundant than *Cylindrospermopsis*. The authors considered that the low levels of phosphorous within the water to have been the limiting factor on bloom formation and toxicity of this species (Berger *et al.,* 2006, Bouvy et al., 2006). A similar study in the same year of Lake Guiers found that the diversity of the lakes was altered after the impoundment of certain parts of Lake Guiers. The Northern part of Lake Guiers was found to be more eutrophic, with cyanobacteria being the dominant species and cladocerans being less abundant in that region. This was a marked risk to water quality due to the water usage dependence on the lake (Ka et al., 2006).

Two years later, a study by Quiblier et al. (2008) assessing the phytoplankton trends in 2002-2003, found that the cyanobacteria *Cylindrospermopsis* was dominant in higher water temperatures, with *Lyngbya* sp. being dominant in lower temperatures. The dominance of *Cylindrospermopsis* has also been attributed to the reduction in grazing of cyanobacteria by predators, caused by the impoundment of the dam in the 1990s. A more recent study of *Cylindrospermopsis* toxin production reported the adaptation of this strain to more temperate regions as opposed to the tropical conditions they were more typically found in. Buford and Davis (2011) also found that distinguishing between toxin producing and non-toxin producing strains of these species is difficult as it is not microscopically possible and the toxin concentrations found in water do not correlate to the number of toxin producing cell numbers.

2.7. Northern Africa

In Northern Africa, reports on cyanobacterial blooms include countries such as Morocco, Algeria and Egypt. Morocco is classified as a freshwater scarce country (Social Watch, 2012).

2.7.1. Algeria

Nasri et al. (2004) first reported on a microcystin containing strain of *Microcystis* spp. in Lake Oubeira, which is a major drinking water source in east Algeria. The study was conducted over the period 2000 and 2001, between spring and summer. The highest microcystin concentrations were measured in the late spring and summer months, with an increase in 2001. The study found four microcystin variants using MALDI-TOF MS, with concentrations of microcystin LR reaching as high as 29,163 $\mu g.L^{-1}$ in August 2001. As a result, the water from this lake is no longer used as a drinking water source but is still applied in irrigation and aquaculture, which is a potential health risk. In the same year, Bouïacha and Nasri (2004) also reported on the presence of *Cylindrospermopsis raciborskii*, in co-dominance with *Microcystis aeruginosa* in Lake Oubeira. Unlike *Microcystis*, *C. raciborskii* peaked in the winter of

2001 (10.2 x 10^5 trichomes per litre) and November (autumn) of 2000 (43 x 10^5 trichomes per litre), with lower densities in summer. Two morphotypes of these species were identified during this study microscopically. The presence of this strain in colder months is an indication of climate change, as *Cylindrospermopsis* is generally described to occur in typically tropical areas.

Further work by Nasri et al. (2007) found a morphotype of *Microcystis aeruginosa* in the Cheffia water dam, which was not nearly as toxic as that discovered in Lake Oubeira. The recorded peak in microcystin concentrations was in October 2004, where levels reached 28.9 µg.L^{-1}. Microcystin concentrations in the study were found to correlate with the cell abundance, with the toxicity far lower than Lake Oubeira's peak microcystin concentrations. In addition, the removal of microcystins was applied through coagulation and flocculation as well as powder activated carbon. When applied, this treatment was able to remove up to 99% of microcystins in some instances.

A 2008 study reported 12 turtle deaths linked to microcystins in Lake Oubeira, during a toxic *Microcystis* spp. bloom that occurred in 2005, which was the first report of this nature in Lake Oubeira. Upon further investigation, the death of the terrapins was confirmed to be due to the direct ingestion of microcystins, with accumulation in the liver and muscle tissues (Nasri et al., 2008). A water assessment of the Seybouse River in north-east Algeria lists some of the main water issues as wastewater infiltration, which further strains the limited water resources available in the country.

2.7.2 Egypt

Investigations into the irrigation system applied in the country found that this current system results in losses of approximately three billion litres of water per year (Abebe, 2014). Moreover, economy development through farming further strains the water supply. In addition to this, the developing economy through farming also adds strain to water supply.

Pollution from direct dumping of wastes into the river is one of the main contributors to nutrient loading. With this in mind, the rise of cyanobacterial blooms is not surprising. Extensive research has been done in the area of cyanobacterial blooms in Egypt.

Earlier studies have been conducted on the feeding of *Daphnia* species on *Microcystis* spp. and found that *Daphnia* sp. were able to feed on cyanobacteria, however it is not the first preference in phytoplankton feed. These filter feeders were able to consume the cyanobacteria without any observed toxic effects and up to 1.78 µg of microcystins were accumulated per 25 daphnids (Mohamed, 2001). Other earlier studies include the assessment of allelopathic relations between *Spirogyra* species that were found to stimulate the toxin production and blooms of *Oscillatoria agardhii* in irrigation canals. The

finding indicated that certain cyanobacteria species produce toxins or thrive better in the presence of other phytoplankton (Mohamed, 2002).

In the last decade, reports on microcystins being produced in oligotrophic waters have been noted in Egypt. Benthic mats of 19 cyanobacterial species were found to produce toxins in the Nile River, which is the main water source in the country (Mohamed et al., 2006). Another interesting study conducted in the same year showed how tilapia fish, under laboratory conditions, were able to ingest and excrete microcystins up to 1.12 $mg.g^{-1}$ dry weight. Although the more toxic microcystin LR was not present in the profile of microcystins, the fish were able to depurate microcystin R-R, Y-R and W-R and excrete them through bile into the aquatic environment (Mohamed and Hussein, 2006). This study is an interesting indication of the ability of some aquatic life to be sustained even in the presence of toxic cyanobacterial blooms, which is then a cause of concern, firstly because the presence of live fish does not alert to the toxicity of the water, as well as the fact that the toxins are released back into the water after being excreted by the fish.

In 2007, Mohamed reported on the presence of *Cylindrospermopsis raciborskii* and *Raphidiopsis mediterranea*, which are hepatotoxic and neurotoxic, respectively in the El-Dowyrat fish pond in Egypt. Toxicity was observed on mice, with the peaks in biomass occurring in the highest temperature months of May to August. Reasons such as warmth of the water and the stagnancy of the pond may have contributed to the blooming of these toxic cyanobacteria. Another study in 2008, by El-Gammal, found that certain concentrations of potassium sulphate were inhibitory to *Microcystis* at concentrations as low as 1.5mM.

More recent studies in Egypt include the assessment of allelopathic macrophytes as an inhibitor against toxic cyanobacteria. The study by Ghobrial et al. (2015) investigated the effects of previously reported macrophyte extracts against *Microcystis*, *Anabaena* and *Oscillatoria* spp. The study found that the choice of solvent (acetone/ethanol) influenced the inhibitory effect of the extracts and that based on allelopathy, these extracts may potentially be applied in place of algaecides to control cyanobacterial blooms. Interestingly, the study also highlighted that these allelopathic extracts were not effective under eutrophic conditions, which calls for further investigation into the area of algaecides from macrophytes.

A recent assessment of a hypersaline lagoon in Northern Sinai, found very little cyanobacteria forming part of the phytoplankton and the lagoon was noted as oligotrophic and one of the cleaner water sources in Egypt. This is positive information, considering the economic value of fish exported from this lagoon to European countries. However, the lake is nutritionally poor, which poses a threat to the ecosystem diversity. The solution may be introduction of nutrients or fertilizers, which would also imply

the potential formation of harmful algal blooms, which have not occurred under the current conditions (El-Kassas et al., 2016).

2.7.3 Morocco

Publications by Oudra in the early 2000s showed the presence of microcystins from *Microcystis* in the eutrophic Lake Lalla Takerkoust in Morocco. The toxicity of the microcystins in this water was found to be high (> 4 $mg.kg^{-1}$ body weight), with factors such as low zooplankton grazing and climate change contributing to blooms lasting all year round in some parts of the lake (Oudra et al., 2001).

Further work by Oudra et al., in 2002 confirmed the presence of toxic cyanobacteria in drinking water and reservoirs in Morocco, with toxicity ranging from moderate to high. *Microcystis* was found to be more toxic than the other 18 strains found in the drinking and recreational waters. Of interest in this publication is the proposed revision of water treatment guidelines in the use of algal species as this was partly what introduced toxic cyanobacteria into treated effluents. Additionally, there were reports of toxic nano-pico cyanobacteria, *Synechococcus* sp. and *Synechocystis* sp., which are not often reported. Recommendations for a monitoring programme in the occurrence of cyanobacterial blooms were also made.

Sabour et al. (2002) reported on non-toxic strains of *Microcystis ichthyoblabe* in Lake Oued Mellah, with slight toxicities occurring during the active bloom. LD_{50} concentrations were 518 and 1924 mg kg^{-1} (dry weight). More recent reports in 2009 by Oudra et al. discuss the presence of hepatotoxic *Nostoc muscorum* in the Oukaïmeden waters. These *Nostoc* mats produced high toxicity similar to previously reported strains of *Microcystis* spp. in Morocco, with hepatotoxic and diarrhoeic effects on mice 15 minutes after being injected with the cyanobacterial extract. These toxic *Nostoc* species were found in clear, oligotrophic, which challenged the general theory that toxic cyanobacteria normally proliferate in eutrophic waters. This drew further attention to benthic cyanobacteria as contributors to toxicity and a potential health hazard in waters. The current strains on water supply have led to the estimation that Morocco will be chronically water stressed by the year 2025 (Social Watch, 2012).

2.7.4 Tunisia

A review of Mediterranean countries and their water scarcity issues lists Tunisia among the countries that have been hampered by drought, being in northern Africa, it is also generally dry (Iglesias et al, 2007). Two relevant publications to this review detail the occurrence of cyanobacteria in Tunisia. The first assessed the seasonal occurrence of toxic *Microcystis* spp. and *Oscillatoria* spp. in the Lebna Dam. The findings were: that a variety of factors influenced the diversity of cyanobacteria and that the

presence of these toxic strains did not correspond to high chlorophyll concentrations as expected. The diversity was attributed to nutrient changes in the water, although it was not clear on the exact factors that contributed to particular species abundance (El Herry, 2008a). In another study, three morphospecies of *Microcystis* were found in the Lebna Dam and found to be potentially toxigenic using the PP2A inhibition assay. These strains also had *myc*A, B and C genes present, which is an indication of microcystin producers. Through the use of restriction fragment length polymorphisms (RFLP), these isolates showed similarity to *Microcystis* (El Herry et al., 2008b). Another study in 2009 found *Microcystis novacekii* in Tunisian waters, which had also been previously reported in Northern Africa (El Herry et al., 2009).

2.8 Central Africa and Western Indian Ocean Islands

In central Africa, Cameroon is one of the few countries where information regarding cyanobacteria is readily available. One of the last recorded blooms was in 2006, where various species of potentially harmful cyanobacteria were isolated along the coast of Guinea and Cameroon. Prior to this, a publication in the 1970s reported cyanobacterial occurrences in Cameroon however no toxicity was recorded (Mowe et al., 2015). There is limited available information regarding cyanobacterial blooms in this region of the continent. Language and perhaps local publishing within the countries may have limited the accessibility of research.

In the Western Indian Ocean islands, Andrianasolo et al. (2007) isolated mitsoamide, a cytotoxic polypeptide which has potential carcinogenic effects, from *Geitlerinema* sp., in Madagascan waters, which was a novel discovery. Sporadic seasonal occurrences of blooms have been reported with no toxicity in Mauritius and Madagascar (Uz, 2007).

In Cape Verde, toxic cyanobacterial strains of *Microcystis* and *Planktothrix* were isolated from Santiago. Genome sequencing of the *Microcystis* species indicated that it clustered closely to species isolated from South Africa and central Africa. These findings indicated a need for further research in understanding the risk and possible future bloom occurrences in the island (Semedo-Aguiar et al., 2018).

2.9. Discussion

It is clear, based on Figure 2.1, that most of the information available is from 20 countries, in the peripheral parts of the continent, with a large information gap in the more central countries. This is an improved tally of information in comparison to the 2005 Cyanonet report (Codd et al, 2005), which

tabulated more information for approximately 8 countries, including Burundi, which has no publications recorded within the scope of this review.

The more water-stressed countries such as Egypt and South Africa are at the forefront of research related to cyanobacterial blooms, as opposed to countries with more available water such as Angola. Research on the occurrence and frequency of civil war among other issues indicates that Africa is more likely to continue having civil wars due to social differences (e.g. religion) as well as economic constraints (Collier and Hoeffler, 2002). Considering that this assessment of Africa was conducted in the early 2000s, it is evident that civil wars still play a major role in the delayed progression of Africa and inevitably in the area of cyanobacterial research.

In African countries, the issue of toxic blooms is a compounding one in addition to existing water issues and challenges. It is not surprising therefore, that there is a lag in research in this area. An additional consideration may be the language of research publications in some countries, since French and Portuguese are among the common languages spoken in Africa (Lewis et al., 2016).

2.9.1 Scientific trends observed under bloom conditions

2.9.1.1 Nitrogen and phosphorous

Of the available information, some trends are evident in the scientific data of reported blooms. Thirty bloom reports were used, of which 70% measured total phosphorous and nitrogen. The available information on toxic blooms in most countries has been expressed as microcystin or cyanotoxin concentrations in water samples ($\mu g.L^{-1}$) or as the dry weight of cyanotoxins ($mg.g^{-1}$). To compare these, they have been plotted against a log scale in comparison to nutrient, temperature and pH measurements (Figure 2.3). Scientific assessment of any trends and ratios in the reported blooms (Figure 2.3) showed that nitrogen is linked to the toxicity of the blooms. It appears in most cases, high nitrogen lower phosphorous values are consistent with toxicity in waters. Nitrogen, as opposed to the more historically reported phosphorous has been indicated in the proliferation and toxicity of microcystin producing *Microcystis* and *Planktothrix*, even in phosphorous limited conditions (Gobler et al., 2016). In addition, the TN:TP ratios measured in most of the blooms were relatively low (<29), with a majority being lower than 12. Two of these occurrences had TN:TP ratios of 50 (Loskop Dam B) and 300 (Makhohlolo) however toxicity was below 1 $\mu g.L^{-1}$ at similar temperatures for both of them. These findings are in line with earlier studies that low TN:TP ratios favour blue green algae and that at ratios beyond 29, they are not dominant phytoplankton (Smith, 1983). The highest toxicity was recorded in Nhlangazwane Dam in South Africa, where TN:TP ratios were 11.2, at ambient temperature and neutral

pH. At temperatures beyond 15°C, toxicity which is in line with earlier findings of low TN:TP ratios being suitable conditions for bloom formation. This confirms previous reports by Davis et al. (2015) and Gobler et al. (2016) that toxicity, or microcystin concentrations, increase with nitrogen and phosphorous inputs.

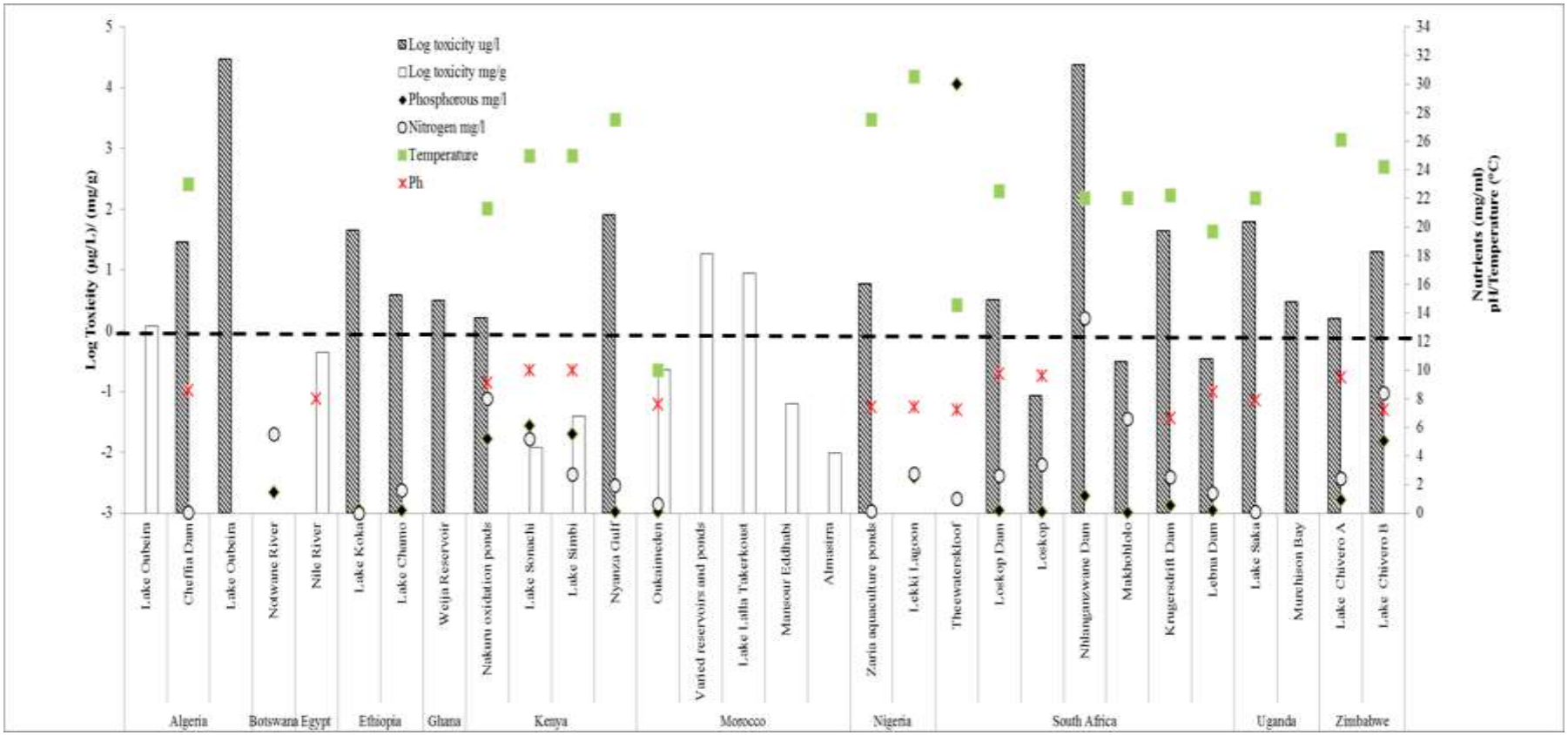

Figure 2.3: Nutrient concentrations, temperature and pH measurements during blooms across Africa in the past decade

The dotted line indicates toxicity of 1 mg per gram or 1 µg per litre, comparable to the other parameters.

Previous publications have mentioned the significance of low nitrogen to phosphorous ratios in determining optimal conditions for cyanobacterial blooms. A study by Xie et al. (2003) found that the low TN:TP ratios were not necessarily a cause of *Microcystis* blooms but a consequence, Xie et al. (2003) also maintained that these ratios are not applicable in highly eutrophic conditions. It is of interest to note that only one site in Figure 2.3 had phosphorous concentrations indicative of eutrophic waters (30 $\mu g.L^{-1}$). The findings in this review support that of Takamura et al., (1992) which indicated TN:TP ratios of around 10 and lower are favourable for *Microcystis* , as seen in most of the occurrences. A study by Oberholster et al. (2007) found that microcystin content in cyanobacteria was lower at lower nitrogen concentrations. Dolman et al. (2012) indicated the significance of nitrogen phosphorous ratios. Since a number of cyanobacterial species are nitrogen fixers, they easily dominate phytoplankton under low nitrogen conditions. This holds true although it has become evident that certain species have higher toxicity and dominance at higher nitrogen ratios. The variation in response to the nitrogen phosphorous ratios indicates that different species react uniquely to nitrogen and phosphorous loading. Earlier studies similarly supported the presence of nitrogen as a contributor to toxicity or microcystin content, with a direct correlation between the two (Lee et al., 2000), the same trend is seen in Figure 2.3. So although low nitrogen to phosphorous ratios offers nitrogen fixing cyanobacteria a competitive advantage, nitrogen is also linked to higher toxicity or cyanotoxin abundance. Based on the trend in Figure 2.3, it appears that most of the reported sites showed toxicity relating to higher nitrogen concentrations. Moreover, the trend is evident in the dry weight of microcystins as well as the external microcystin concentration ($\mu g.L^{-1}$). Toxicity is evident in sites that have low phosphorous concentrations (lower than the indicated phosphorous concentrations for eutrophic lakes), indicating the significance of nitrogen in toxicity.

2.9.1.2 Distribution of cyanobacteria

Previously mentioned reports in this review indicate that *Anabaena* species are more commonly reported in the South and Eastern parts of the continent as dominant in blooms. *Cylindrospermopsis* is more prevalent in the hotter drier areas, although it has peaked in winter and autumn in Senegal, as well as the rainy season in Nigeria. However, it occurs in hot drier conditions in Tunisia and Egypt. *Oscillatoria* seldom occurs as the dominant species in a bloom and is more commonly found to be amongst other dominant species. Other species are not as common, with only one report of a toxic *Nostoc* bloom. As a nitrogen fixer, *Anabaena* is known to be affected by phosphorous inputs in waters, with higher phosphorous leading to higher toxicity (Rapala et al.,1997), this was seen in Lake Sonachi, Lake Simbi and in Theewaterskloof, the dominant isolated genus was *Anabaena*, with temperatures as 25°C and lower. *Microcystis* was the most frequently occurring species. These

findings are in agreement with the previous review by Mowe et al. (2015) which found *Microcystis* to be the most dominant genus in the African continent.

2.9.1.3 Temperature and pH

Based on Figure 2.3, there was no clear effect of pH on the toxicity of the blooms, with values ranging from circum-neutral to above 9, whilst the toxicity is varied, although five out of 11 blooms with pH values between 7 and 9 had a microcystin concentration higher than 1 µg.L^{-1}. Temperature ranges appeared to have a direct proportion to toxicity, which is in line with other study findings (Davis et al., 2009).

Blooms occurred from temperatures as low as 10°C, however toxicities beyond 1 µg.L^{-1} were observed at temperatures higher than 15°C. The highest toxicity was measured in Nhlangazwane Dam at a temperature around 30°C.

However, the bloom dominance of *Cylindrospermopsis* in the North Western regions of Africa and in the North Western parts of South Africa indicates that temperature influences the type of cyanobacterial species that are dominant in given water bodies.

In some instances, in Nigeria and Senegal, the reported temperatures of water sources where *Cylindrospermopsis* was isolated were relatively high, going beyond 30°C in some instances. Berger et al. (2006) found *Cylindrospermopsis* to be prevalent in the dry seasons, which explains bloom reports in dryer parts of the continent. With optimum growth conditions being beyond ambient temperature, it is not surprising that this genus is prevalent in hot, tropical areas. Moreover, this species also favours alkaline conditions (Antunes et al., 2015).

The distribution of *Microcystis* on all the studied regions of this review is expected as this specific genus is known for appearing in a variety of water bodies and having a tolerance for unfavourable conditions, particularly the toxic strains. So far there is no definite grasp on the set of parameters that completely exclude the possibility of these blooms in the face of global warming in freshwaters (Marmen et al., 2016).

2.9.1.4 Identification and toxicity estimation methods in Africa

Of the reviewed literature, it appears that there are standard methods in reporting the toxicity of the blooms discussed in this review, a majority of the reports utilize microcystin ELISA kits, HPLC and chlorophyll measurements. A few publications have made use of molecular methods (RFLPs, gene markers) and MALDI-TOF MS to determine the toxicity of the reported cyanobacteria. In addition,

biotoxicity assays have also been employed by way of mouse and terrapin bioassays in the North and Southern regions of Africa. A majority of these publications has utilized secchi disk measurements to estimate turbidity and have related these findings to algal biomass.

Monitoring guidelines have also been set out in Burkina Faso (Cecchi et al, 2009) and South Africa (van Ginkel, 2011) for example, with increased satellite detection of cyanobacterial blooms (Matthews et al., 2014; 2015). In some countries, the studies are still first reports of cyanobacteria concluding with recommendations for monitoring guidelines. This shows the wide rift in advancement of cyanobacteria related research in various regions. Salinity has been linked to cyanobacterial blooms of *Microcystis* (Paerl et al., 2013), however no link has been made in this review. Overall, despite the missing data from the central region, it is clear that cyanobacterial research is on-going in Africa and that there is awareness of their potential impacts, although they may not be considered among the main water issues.

2.10. Conclusion

Based on the available information and findings, there needs to be a serious effort to consolidate information and technologies available within the continent to aid in curbing the water issues facing Africa. The consolidation of standard measurements and parameters is a key factor in having comparable and informative data on the state of toxic blooms in the continent as a whole. Assessment of water physico-chemical parameters and toxicity concentrations during blooms will aid in the understanding of the occurrences and factors that contribute to blooms. Although not fully representative owing to lack of information, the findings of this review indicate that in Africa the most cosmopolitan species is *Microcystis*, with *Cylindrospermopsis* occurring in the dryer, western parts of the continent and *Anabaena* occurring randomly in various parts of the countries, although not as the dominant species, except for in the Malawi reports.

Mowe et al. (2015) found the occurrence of C*ylindrospermopsis* to be related to higher temperatures and dry seasons in tropical areas, whilst *Microcystis* were more prevalent in wet seasons. The information collected in this review supports these findings, with more reports of *Cylindrospermopsis* being in the warm dessert climate areas.

In the area of technological advances and effective monitoring, most countries are in the early implementation stages and have only recently made efforts into the investigation of cyanobacterial

blooms, with identification and toxicity being the primary information screened for. Most monitoring plans refer to WHO regulations. Monitoring guidelines specific to particular regions of the continent or of the entire continent may prove very useful and is strongly recommended. The implementation of guidelines and accessibility is a needed practical intervention.

Nyenje et al. (2010) reviewed the issue of eutrophication and nutrient loading in Sub-Saharan Africa, identifying a number of intercalating issues linked to this: urban development, inefficient water treatment due to no insight into hydrological cycles and micro-pollutant monitoring and fate. The loss of aquatic life, particularly fish, increase in toxic blooms and pathogens are amongst the highlighted issues requiring attention. An offered approach in solving this issue is the systematic assessment of all the processes linked to eutrophication in order to have a practical solution.

With particularly climate change in mind, the challenges facing Africa put the continent against an uphill battle in mitigation. The fact that the continent is heating up faster than the rest of the world (Collier et al., 2008) with pre-existing water challenges as previously mentioned, there are a number of socio-political inputs that may have curbed research and progress in this area. Collectively, the information gathered in this review shows that a lot of measures are not yet in place for the mitigation of cyanobacterial blooms, particularly in the implementation of plans in most countries. Research in Africa has been geared more towards reports and investigations of toxicity in the area of blooms. In light of Africa's unique vulnerability to climate change, as opposed to other continents, knowledge dissemination and collective research is critical. Initiatives such as CYANONET are crucial in the collection of a more informed picture and the establishment of more collaborative research not only on an intercontinental scale but knowledge sharing within the continent, particularly in the central countries. To our knowledge, this was the first collective review of cyanobacterial blooms in Africa in the past decade.

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Chapter 3: A review of bacteria as biological control agents of freshwater cyanobacteria

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Declaration by the candidate

With regards to chapter 3, the nature and scope of my contribution was as follows

Nature of contribution	Extent of contribution
Conceptual design, experimental work, manuscript writing	70%

The following co-authors have contributed to chapter 3

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Abstract

Biological control of cyanobacteria is a well-researched area with a central focus on laboratory scale studies. Numerous reports have been made on algicidal isolates, with bacteria as a major component of the antagonists. The research in this review draws a brief summary of what is currently known in the area of freshwater cyanobacteria being inhibited by bacterial isolates. *Proteobacteria*, *Bacteriodetes* and *Firmicutes* are among the most commonly reported phyla of bacteria associated with or employed in this research area. However there are limited reports of up-scaling these control measures beyond the laboratory-scale. Lytic control agents are the most commonly reported in the literature with subsequent cyanotoxin release. From a water quality perspective, this is not a feasible option. Based on the available literature, temperature, pH and nutrient changes have been explored in this short review as possible contributors to less optimal bacterial performance. Moreover, the investigation into optimizing some of these parameters may lead to increased bacterial performance and therefore viability for up-scaling this biological control. Through the compilation of current research, this review offers insight to live predator-prey cell interactions between cyanobacteria and algicidal bacteria.

Keywords: algicidal bacteria, cyanobacteria inhibition, biological control, harmful algal blooms

3.1 Introduction

An understanding of the current research in cyanobacterial bloom research in South Africa (Chapter 2) indicates the amount of research on cyanobacterial blooms in South Africa and globally. Considering the numerous reports, control measures are important in the management of these blooms. Among the prevention and mitigation measures are the use of biological control agents. The current study will assess the feasibility of bacteria as cyanobacterial control agents beyond the laboratory. This will be based on consolidation of the existing information on biological control of cyanobacteria, based on the reviews of Sigee et al., (1999) and Gumbo et al., (2008) as well as numerous other publications on this topic. The focus will be more specifically in the area of bacteria as a biological control agent to living cyanobacterial cells in freshwater environments (Sigee et al., 1999; Gumbo et al., 2008).

The concept of biological control is the use of natural enemies to and among the measures previously mentioned in a review by Sigee et al. (1999), is the use of bacteria as a means to control cyanobacterial cells. In the broader focus of biological control, a review by Verschuere et al. (2000)

thoroughly investigated the use of probiotic bacteria as possible biological control agents. The key modes of action listed by bacteria as methods of biological control indicate antagonism as the more common mode of action in aquaculture (Verschuere et al., 2000).

In the study of bacteria as control agents in aquaculture, a variety of inhibitory compounds such as antibiotics, bacteriocins, siderophores and lysozomes are produced by probiotic bacteria. Whist controlling other organisms, these compounds can have a positive growth effect as well on unicellular algae and this needs to be clearly understood prior to implementation of certain species as biological control agents (Verschuere et al., 2000).

Previous studies employing the use of bacteria in curbing cyanobacteria have been conducted primarily at laboratory scale, with the focus on a dominant species within a bloom or on axenic cultures of a given cyanobacterial isolate (Choi et al., 2005; Jung et al., 2008; Nakamura et al., 2003; Shao et al., 2014; J. feng Su et al., 2016). These studies seldom account for the mixed cultures of phytoplankton or the possible variations in temperature during the exposures under lab conditions or the fluctuations within the natural environment. A recent study has found some of the interventions in curbing cyanobacterial blooms were not as effective as reported, with a strong cautioning to critically evaluate these control measures among which bacteria, artificial mixing and algae were mentioned (Lürling et al., 2016).

Recent research by Demeke (2016) describes the use of metabolites from the bacteria *Flexibacterium* in the control of the filamentous cyanobacteria *Oscillatoria* and the lytic activity of the bacteria *Bacillus* against the cyanobacteria *Aphanizomenon flos-aquae* (Demeke, 2016). An investigation of the gene expression in cyanobacterial and antagonistic bacterial co-cultures indicated an up-regulation of defence mechanism genes, inclusive of antibiotics (Osman et al., 2017), these up-regulations were associated with a damage in *Aphanizomenon* filaments. Other bacterial metabolites tryptoline and tryptamine have been indicated as possible substitutes to copper sulphate as an algaecide (Zhang et al., 2016). This presents a host-specific alternative to more persistent algaecides, with scope for further research, especially in live cell interactions. These are just a few of many examples and show the interest and potential in the area of biological control. Over the past decade, advancements in the use of bacteria as cyanobacterial control agents are not as extensive.

Although not exhaustive, this research aims to establish a time line of developments in this mode of cyanobacterial control and what is currently known, with the intention of collating pitfalls and possible

future research challenges. The study is focused on the viability of this biological control and which factors could be optimized for further progress, based on the available literature.

3.2 Data collection approach

The literature used within this mini-review was collected from the following databases: Google scholar, Scopus, and Ebsco host with the following key words: "algicidal bacteria; "*Microcystis*/*Oscillatoria/ Cylindrospermopsis/ Anabaena/ Aphanizomenon* inhibiting bacteria" "biological control of cyanobacteria", "algicidal bacteria" "bacteria lysing cyanobacteria", with the time frame delineated to 2000-2017. Further delineation was done based on focusing on freshwater related studies and the lysis/suppression/control of living cyanobacterial cells by living bacterial agents, with minimal focus on microcystin degraders and bacterial metabolites employed to control whole cyanobacterial cells.

3.3 Non-bacterial control agents associated with cyanobacteria and the susceptibility of the target micro-organisms

An extensive review (Van Wichelen et al., 2016) describes the susceptibility of *Microcystis* sp. to a variety of control agents ranging from viruses, fungi to bacteria. The lack of application of this from of biological control outside laboratory conditions is also mentioned. This is concerning as a number of possible control measures with living organisms has been explored with minimal upscaling opportunities, with recommendations made against up-scaling beyond the lab in some studies (Kim et al., 2008a).

The first challenge comes from the lack of information on the type of bacteria and interactions among species that may hinder how effective a given control agent may be under field conditions as opposed to laboratory-based findings. The diversity and intercellular interactions are too numerous to be adequately accounted for. To quantify or individually explore these factors is complex and requires further intensive research to paint a more conclusive picture. Therefore, although there are numerous studies and reports of effective microorganisms applicable in laboratory studies (Choi et al., 2005; Jung et al., 2008; Kim et al., 2008a; Zhang et al., 2016), none of these have been conclusively up-scaled or pursued beyond the lab due to the myriad of uncertainties that present themselves within a mixed population. A mixed population refers to the presence of various heterotrophic bacterial species along other cyanobacteria and microorganisms, as would be expected in a bloom or non-axenic culture. The other question that arises is how well do these control agents regulate their algicidal characteristics in the natural environment within a mixed microbial population? Taking a closer look at *Microcystis* specifically, which has the most available literature and is the causative agent of the most common toxic blooms. If cyanobacteria, particularly *Microcystis*, present such a wide susceptibility to

environmental isolates, how is it able to continue proliferating and creating toxic aquatic conditions? Table 3.1 presents a synopsis of the known types of control agents and the type of cyanobacterium they have a predatory or lytic impact on, exclusive of bacterial isolates from various studies (Gao et al., 2012; Leitão et al., 2018; Mialet et al., 2013a; Mohamed et al., 2014; Wang et al., 2011; Zhang et al., 2008).

The other area of interest is the form of species, such as *Microcystis* as laboratory strains and their natural form. Generally, under natural conditions these cells are present as unicellular colony-forming isolates with mucilage layers, surrounded by various microorganisms, which have believed symbiotic relations with the cyanobacteria (Mayali et al., 2018). This colony-forming form is not often observed in laboratory strains that present as unicellular and a recent study by Geng et al., (2013) found that laboratory grown strains are not able to revert back to colonial form after sub-culturing within the laboratory, they tend to form two-celled or unicellular cells, which offers reduced resilience as opposed to colonies. This differential response is also observed in lab studies when exposing both forms of cells. Therefore, one may consider the study of biological control of unicellular cells to not be fully indicative of the possible response in a natural ecosystem. An earlier study by Yang et al.(2006) found that colonial *Microcystsis* were more succesful at warding off grazing by cladocerans as opposed to the unicellular isolates, which indicates a possible mechanisms of defense that is not often noted with tested unicellular isolates employed in the laboratory (Yang et al., 2006). In a recent study the finding that these cells can morph into various morphotypes of *Microcystis* within colonies was informed by the changing environmental conditions within the waterbody. This indicates a steady shift of *Microcystis* species morphology and response, which are not often observed in targeted biological control efforts under controlled conditions. The study further indicates the value of this defence mechanism to a given species, hence the value in a control agent that is effective within a mixed population and also has enough host specificity to remain effective against the targeted cluster of organisms is needed (Man et al., 2018). Moreover, evidence of the natural prevalence of the cells as colonies has been supplied in a more recent literature review on the colony formation characteristics of *Microcystis* (Man et al., 2018). Table 3.1 summarizes the commonly reported antagonists from different trophic levels against cyanobacteria and how these predators commonly control cyanobacterial isolates. This explores other control agents apart from bacteria, which will be discussed in more detail.

Table 3.1: Summary of reported control agents against cyanobacteria

Control agent	Target organism	Mode of action	Reference
Cyanophage	*Synecocchus* sp. *Planktothrix agardhii*	Lytic-dependant on host populations Cell lysis	Wang et al., 2011;Gao et al., 2012
Golden algae *Poterioochromonas*	*Microcystis sp.*	Direct grazing and microcystin degradation	Zhang et al., 2008
Bdelloid rotifers	Cyanobacteria and diatoms	Grazing	Mialet et al., 2013
Notodiaptomus iheringi	*Microcystis sp.*	Grazing	Leitão et al., 2018
Trichoderma citrinoviride	*Microcystis sp.*	Indirect lysis and microcystin degradation	Mohamed et al., 2014

3.4 Heterotrophic bacteria associated with cyanobacterial blooms

Apart from the known characteristics of most bacteria; robust growth, non-fastidious and ubiquity, they form an integral part of the diversity in algal blooms (Eiler and Bertilsson, 2004). This is significant in assessing whether the bacteria associated with blooms fall into the phyla most commonly indicated in cyanobacterial control. More importantly, this may establish whether there is any link between these specific control agents and how closely they are observed in the cyanobacterial phycosphere. An assessment of bacterial communities found that the phyla *Proteobacteria* and *Bacteriodetes* were among the most common phyla in blooms of cyanobacteria *Microcystis* and *Anabaena* sp. These phyla were also found in various blooms in Swedish lakes (Eiler and Bertilsson, 2004). Various studies have been conducted in understanding cyanobacterial blooms and therefore, the bacterial communities associated with them. Naturally, cyanobacteria exist as co-cultures with a variety of bacterial phyla. When assessed, Shi et al. (2011) and Cai et al. (2014) found that there are aggregates of free-living bacteria as well as attached bacterial species which are within the extracellular polymeric substances (EPS layer) of colonial *Microcystis* cells. The bacterial diversity has been found to change with the different stages of a bloom (Shi et al., 2011) and with various species (Bagatini et al., 2014). Some phyla are in higher abundance at the degradation phase of a bloom, whilst some are more represented at the start or peak of blooms. The fact that these microorganisms freely exist within bloom conditions makes them ideal potential control agents, although it is understood that certain classes aid the

proliferation of cyanobacteria (Shi et al., 2009; Tian et al., 2007)

In the context of symbiotic relationships, the interactions reported between algicidal bacteria and target cyanobacteria indicate amensalism and predation as the common interactions. Heterotrophic bacteria have been reported to produce antibiotics or growth inhibiting substances against the target cyanobacteria (Mayali, 2018), this is indicative of amensalism and the targeted cyanobacterium is not used as a nutrient source upon lysis or inhibition (Mougi, 2016). Predation refers to the subsequent use of the targeted cyanobacterium as a nutrient source, although this term has been used interchangeably for bacterial isolates with inhibitory characteristics against cyanobacteria. There is also positive growth promoting interactions of nutrient exchange between bacteria and cyanobacterial isolates, with the presence of certain bacteria enhancing the increase of cyanobacterial biomass (Subashchandrabose et al., 2011) with the phycosphere of cyanobacteria providing bacteria with dissolved organic carbon matter for assimilation (Kouzuma and Watanabe, 2015). These interactions also need to be considered in the application of bacterial control agents.

From the above-mentioned literature studies, *Alpha*, *Betaprotebacteria* and *Bacteriodetes* are the more closely associated and reported species associated with non-axenic cyanobacterial aggregates. This applies to studies conducted on *Anabaena, Aphanizomenon, Cylindrospermopsis and Microcystis* cultures. A study by Cai et al. (2014) found similar phyla associated with *Microcystis*, with *Alphaproteobacteria* most abundant in the small cell aggregates and *Firmicutes* most abundant in the larger cyanobacterial cell aggregates. Louati et al. (2015) reported that bacterial communities differed based on the cyanobacteria blooming at a given time and that there is a beneficial relationship between cyanobacterial attached bacteria, seen in the accelerated growth of non-axenic cyanobacteria as compared to axenic strains. *Firmicutes* was also reported to be in lower abundance in this study as compared to *Proteobacteria* and *Actinobacteria*. With *Bacillus* sp. isolates being commonly reported as algicidal bacteria, it is interesting to note that they are not abundant in the smaller cyanobacterial cell aggregates, which are in closer proximity to the cyanobacteria.

Free-living and cyanobacterial attached bacteria have been differentiated based on the aggregates they form after filtration and separation from each other. The cells found closest to the cyanobacteria cell EPS layer are thought to have a mutualistic beneficial relation or considerable tolerance to the given species (Bagatini et al., 2014). This is supported by an earlier study conducted by Bouvy et al., (2001), where the cyanobacteria *Cylindrospermopsis raciiborski* did not impose a major change in biodiversity or specific species suppression in the bacterial or zooplankton community within a reservoir study in Brazil (Bouvy et al., 2001). The indications from the bacterial diversity in cyanobacterial aggregates in

nature indicate that the more beneficial bacteria are found in closer proximity to the cyanobacteria. This may explain the difficulty in natural bloom control, as predatory isolates would be lower in abundance, with a reduced contact time to the targeted cyanobacterium.

3.5 Factors associated with cyanobacterial control that may be optimised

If bacteria are to be applied on a larger scale as control agents, some factors would require optimization. From previous literature, this review has identified temperature, nutrients and pH as parameters that could be amended for improved bacterial performance that may be viable on a larger scale, beyond the laboratory. These parameters were easily identified as they were the most often recorded in the studies included here. Although these are not the only key factors to be considered, they are the most frequently documented.

3.6 Temperature differences and their possible implications

Numerous studies on algicidal bacteria have been conducted under laboratory conditions. The algicidal bacteria are often cultured under different nutritional and temperature conditions, slightly higher than that of the targeted cyanobacteria. This might be one of the factors to consider for increased bacterial efficiency in controlling blooms on a larger scale. There is considerable evidence that growth temperatures significantly impact the protein activity (Patke and Dey, 1998) in microorganisms, with a proven increase in growth rate at temperatures beyond 30°C for *Bacillus*, *Escherichia coli*, *Salmonella* and *Listeria* sp. strains (Membré et al., 2005). Studies on the effects of temperature on bacterial isolates have been modelled and better understood from as far back as the 1980s (Ratkowsky et al., 1982; White et al., 1991). A 2010 report assessing clinical isolates of *Staphylococcus* sp. also found increased biofilm populations and antimicrobial properties at higher temperature, supporting the findings that most isolated clinical bacteria are mesophilic in nature (Hajdu et al., 2010). Although a variety of factors influence the growth rate of bacteria in conjunction with temperature, a similar phenomenon of temperature influencing growth rates in a number of studies (Pietikäinen et al., 2005).

Rampelotto (2013) considered cyanobacteria the most resilient to temperature changes among the prokaryotes, with their discovery and viability occurring in a wide range of environmental conditions (Rampelotto, 2013). Almost 2 decades ago, research on the effect of lowered temperatures on mesophiles and psychrophilic bacteria indicated that the affinity for sequestering or binding given substrates was lowered at decreased temperatures. There was a noteworthy difference in the cells affinity (Nedwell, 1999), with as well as respiratory rate inhibition even in psychrophiles, when temperatures were lowered beyond the optimum (Pomeroy et al., 1991). Temperature reduction changes of 10°C showed a distinct decrease in cell affinity for mesophiles (Nedwell, 1999). Movahedi

and Waites, (2002) further showed that cold shock resulted in a protein expression change in sporulating *Bacillus* cells (Movahedi and Waites, 2002), as compared to an earlier study showing a growth lag in vegetative cells after temperature drops of about 20°C (Graumann and Marahiel, 1999). From this research, although resilience may be increased in further generations, it is clear that drastic temperature changes are not ideal in the optimal performance of a mesophilic microorganism. To provide a bit of contrast, a study on the heat shock response of *E.coli* cells showed that a 12°C temperature increase to 42°C subsequently increased the growth rate of these cells, whilst higher temperatures of 48 to 55°C indicated an initial increase and thereafter cell growth inhibition (Soini et al., 2005). However, it appears that temperature changes beyond 10°C were induced in most experimental cases where bacteria are isolated to control cyanobacteria. Although the studies here include clinical isolates, the trend of lowered growth rate at lower temperatures for mesophiles is clear.

Viewing this point in the context of African waters, Ndlela et al. (2016) found water temperatures during blooms can range from above 30°C to as low as 10°C (Ndlela et al., 2016). A South African study by Gumbo et al. (2010) on the biological control of *Microcystis* reported the isolation of algicidal *Bacillus mycoides* using the plaque assay, with subsequent exposure using this *Bacillus* as a control organism. Cultures were grown separately, with bacterial isolates at 37°C, whilst the target cyanobacteria were kept at ambient temperature in a different medium, thereafter the control agent was co-cultured with the cyanobacteria at a 1:1 volume ratio and observed over six days (Gumbo et al., 2010).

Research from Oberholster and Botha, (2010) analysing the hyper scum and toxicity of *Microcystis* sp. in Hartebeespoort Dam showed that over a period of six months at the peak of the bloom, temperatures ranged between 23 and 25°C. Further research in Theewaterskloof, reported a bloom occurring at temperatures as low as 14°C (Oberholster et al., 2015). Another 2009 study by Oberholster et al., assessing animal mortalities to microcystins also indicated average water temperatures between 2005 to 2006 were 25.1°C in Lake Hartebeespoort, over that period, with a clear indication that temperature increases of around 0.84°C over the years due to climate change have influenced the toxicity and prevalence of cyanobacterial blooms (Oberholster et al., 2009).

In light of this information among other reports, why is the temperature of control agents seldom uniform to blooms considered in the lab-scale implementation of biological control? Listed in Table 3.2 are the different temperatures that bacteria and target cyanobacteria are grown under, based on previous research (Choi et al., 2005; Lin et al., 2014; Manage et al., 2000; Mu et al., 2007; Ren et al., 2010; J. feng Su et al., 2016; J. F. Su et al., 2016; Yang et al., 2012; Zhou et al., 2016; Zhu et al., 2014). Only research over the last few years indicates a consideration of temperature similarity and in

some cases nutrient acclimatization as in the case of Shao et al. (2014) in lab-scale studies (Shao et al., 2014). Marked in bold are temperature differences that indicate a difference greater than 10°C which may induce the previously mentioned cold shock in bacterial isolates.

Table 3.2: Comparison of temperature and media differences between bacteria and target cyanobacteria. Marked in bold are temperature differences that indicate a difference around 10°C.

Bacteria			**Cyanobacteria**			**Reference**
Isolate	**Media**	**Temp (°C)**	**Isolate**	**Media**	**Temp (°C)**	
Alcaligenes denitrificans	Casitone	**34**	*Microcystis* spp.	MA Medium	25	Manage et al., 2000
Streptomyces neyagawaensis	Nutrient broth	**40**	*Microcystis aeruginosa*	CB Medium	25	Choi et al., 2005
Bacillus fusiformis	Nutrient broth	**30**	*Various cyanobacteria*	BG-11, etc.	16-24	Mu et al., 2007
Pseudomonas aeruginosa R219	Luria Bertani	**37**	*Microcystis aeruginosa*	BG-11	28	Ren et al., 2010
Pedobacter sp.	Nutrient broth	23	*Microcystis aeruginosa*	MA Medium	23	Yang et al., 2012
Bacillus sp.	Modified CT medium	28	*Microcystis aeruginosa*	CT medium	28	Shao et al., 2014
Brevundimonas sp.	Beef extract peptone	30	*Microcystis aeruginosa*	BG-11	25	Lin et al., 2014
Pseudomonas aeruginosa	Nutrient broth	30	*Microcystis aeruginosa*	BG-11	25	Zhou et al., 2016
Acinetobacter sp.	Luria Bertani	30	*Microcystis aeruginosa*	BG-11	25	Su et al., 2016a
Raoultella sp.	Luria Bertani	30	*Microcystis aeruginosa*	BG-11	25	Su et al., 2016b

This is seen in many of the lab-scale studies listed in this article, with the general approach being to enhance the predator through rich media and higher temperatures. This begs the question whether the conditions employed would be result in effective biological control in the natural environment? Furthermore, how may one feasibly up-scale this technology in an aquatic system without major hampering of the biodiversity? Another question that arises is whether the response to climate changes and therefore increased nuisance algal blooms; is eradication, suppression or toxin removal? Although there are numerous papers reporting the use of microcystin degrading bacteria, exclusive of live cell-to-cell interaction, which indicate the metabolites and proteins that lead to cyanobacterial death and inhibition (Ji et al., 2009; Luo et al., 2013; Yang et al., 2014b), the focus of this review is on live cell interaction between the predator and the targeted cyanobacteria.

3.7 Alkalinity and acidity-the other factor in biological control

Numerous studies indicate a preference for cyanobacterial proliferation at more alkaline conditions (Gumbo et al., 2008), with a pH of as high as 10 being optimal for the proliferation of blooms (Gao et al., 2012). A study on the mass culturing of *Synechocystis* for its multiple benefits in industrial applications, found that higher pH conditions were primarily useful in warding off potential predators, as it maintains an advantage to grow at more alkaline conditions in comparison to other freshwater species that graze on it. Optimal growth did not differ much from neutral pH up to a pH of 10.5, which greatly limited the growth capacity of the grazer. Given that alkalinity is higher under natural bloom conditions and continuous culture, there is often an increased pH created by the presence of cyanobacterial biomass (Touloupakis et al., 2016), it stands to reason that control agents need to be alkalotolerant or maintain circum-neutral pH conditions when exposed to the target cyanobacteria. Another earlier study found optimal growth of filamentous *Schizothrix calcicola* at pH levels between 6 and 8. More acidic conditions appeared to limit the pigment expression of this cyanobacterium (West and Louda, 2011). Based on the literature referred to earlier in this study on the varied temperatures of the control agent and prey, the culture media referred to in most studies is neutral, with these conditions favourable for most mesophiles. Therefore, although the value of alkalinity is crucial under algal bloom proliferation, there is limited information on the changes brought about by the control agent under laboratory test conditions reviewed. Do heterotrophic bacteria lower or increase the pH in the cyanobacterial culture medium? Is this also one of the changes that occur in the growth medium under lab conditions to limit cyanobacterial growth?

3.8 Nutrient competition- some thoughts and ideas

Nutrient competition under algal bloom conditions is vital in enabling the domination of the cyanobacterial cells. The gas vesicle of isolates of *Microcystis* for example enables regulation through the water column as well as light shading of other phytoplankton through the formation of surface scum colony aggregates. As Paerl et al. (2016) indicates, the survival and competition strategies of this group of prokaryotes is quite impressive. Therefore, the issue of nutrient competition in biological control using heterotrophs is a crucial consideration. Being mostly heterotrophic, the response to reduced nutrients in the presence of cyanobacterial dominance may be a potential trigger to the bacterial predator response to the cyanobacterial cell. This may mean that at low ambient nutrients, which are found under bloom conditions, the ambient nutrient limitation gives way to microcystins or the cyanobacterial cell being a plausible nutrient source for heterotrophic bacteria. The competition for phosphorous, as indicated in a study by Vadstein et al. (2003) in a microcosm, was the limiting nutrient in the co-existence of algae and heterotrophic bacteria in an aquatic system, while the availability of organic carbon was greatly contributed to by algal predators (rotifers) rather than the algae themselves. Moreover, if considering the research findings of Ji et al. (2017) when assessing nutrient competition between eukaryotic algae and the cyanobacterium *Microcystis*, that at the typical high pH and low CO_2 conditions, eukaryotic algae are capable of competing at these conditions. Since the depletion of carbon dioxide by cyanobacteria under bloom conditions has a hampering impact to the photosynthetic activities of other phytoplankton, this is an interesting finding. However, the nutritional requirements of the heterotrophs differ in the need of organic carbon, noted also in their uptake of NH_4^+ from the nitrogen fixer *Aphanizomenon* (Adam et al., 2016), which makes use of N_2 fixation under limited nitrogen conditions. Heisler et al. (2008) also describes the ability of some species of cyanobacteria to shift to organic sources of nutrients, which means that in some blooms, is direct competition for nutrients between the cyanobacteria and the bacteria (Heisler et al., 2008). Perhaps under bloom conditions, the limited nutrients shift the heterotrophic bacteria towards predation as a means to survive. The EPS layer of *Microcystis* and the cells of filamentous cyanobacteria are viable nutrient sources for heterotrophs with a limited food source. Therefore, it may be that the bacterial predation of cyanobacteria is additionally affected by the limitation of ambient nutrients under bloom conditions.

3.9 Previously reported bacterial control agents

Among the literature, a number of bacterial control agents have been discovered. Figure 3.1 summarizes a brief timeline of bacteria reported to control cyanobacteria and/or algae. Although the list is not exhaustive, it is clear that there are common species reported as control agents against the cyanobacteria *Microcystis*, specifically. In correlation with the findings on bacterial communities during a

bloom, *Pseudomonas*, *Xanthobacter*, *Xanthomonas*, and *Alcaligenes* as shown in Figure 3.1, are isolates under the *Proteobacter* phylum, which is abundant in cyanobacterial bloom communities, as previously discussed. *Streptomyces* (Phankhajon et al., 2016) is within the phylum *Actinobacter*, which has also been reported among the abundant phyla. Of interest however, are the reports on *Bacillus*, which is within the *Firmicutes* phylum. This has been less frequently reported to be in abundance as opposed to the other phyla during a cyanobacterial bloom, although Shi et al. (2009) has mentioned this phylum among those within a bloom. Moreover, in the study by Cai et al. (2014), *Firmicutes* was found in the larger cyanobacterial cell aggregates. The algicidal effect of *Aquimarina* isolates in a previous study also confirms the presence of *Bacteriodetes* phyla within the water column where the cyanobacterial bloom occurred (Chen et al., 2011). Although these species exist within the bloom diversities, other studies have implied that the species attached to the cyanobacterial cells have a symbiotic relationship with each other (Shi et al., 2011). In the same study, pathogens *Aeromonas* and *Shewanella* sp. were also reported to be positively associated with *Microcystis*, serving as a potential protection, whilst the other organisms were detected during the vigorous growth period of the *Microcystis* bloom.

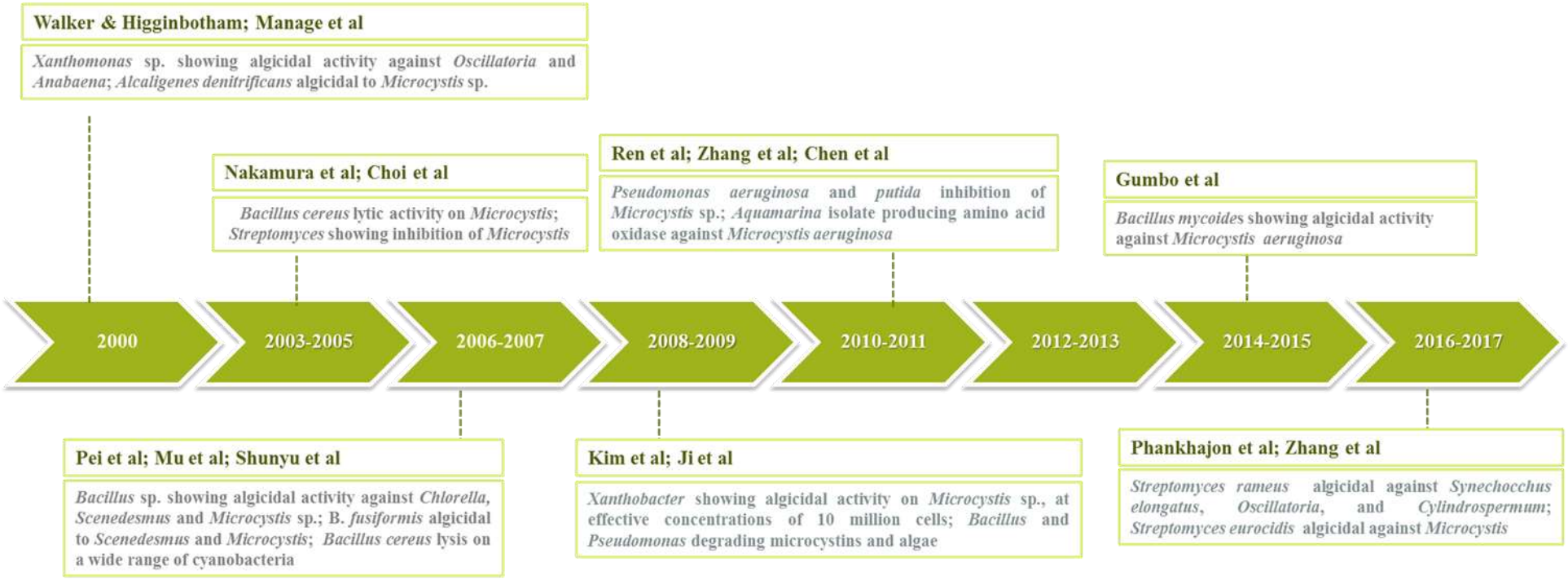

Figure 3.1: Research timeline of findings related to algicidal bacteria from the year 2000. Findings from each author are separated by a semicolon, with a summary of the bacterial strain and the cyanobacteria it was found to lyse or inhibit. Most of the current research is against *Microcystis* sp., with fewer publications against other species.

Mayali and Azam (2004) reviewed marine algicidal bacteria, which interestingly indicated similar phyla such as those discussed in freshwater studies mentioned here. Repeatedly, earlier studies found that contact time is a significant factor in the effective control or lysis of cyanobacteria by predatory bacteria. Understanding why these control measures are not always effective on their own to curb blooms leads to a conundrum of factors. Among recent literature it appears that the phylum *Firmicutes* has several effective predatory isolates, especially from the *Bacillus* genus. This is interesting to note as this phylum is generally found in lower abundance under bloom conditions.

In addition, most of the biological control implementations have been under small scale laboratory conditions (Choi et al., 2005), with most culture conditions favouring the proliferation of the predator, usually at mesophilic conditions (30°C), whilst the cyanobacteria are cultured at ambient temperatures. This is often under different nutrient and temperature conditions, which are not always representative of cyanobacterial bloom conditions in nature.

Table 3.3 summarizes a few examples of the control organisms used as well as the effective ratios of the predator to prey in causing lysis or inhibition of specific cyanobacteria based on the available literature.

From Table 3.3 it is evident that most of the research has been conducted at laboratory scale (Chen et al., 2011; Fraleigh and Burnham, 1988; Jia et al., 2014; Luo et al., 2013; Shunyu et al., 2006; Tian et al., 2012; Zhang et al., 2011). The effective numbers range from as little as a million bacteria cells up to a 100 million, with effective ratios as low as 1:100 all the way up to 100:1 of the control agent. The laboratory scale studies show that *Proteobacteria* is one of the more commonly reported bacteria control agents, this supports the findings of Manage et al., (2009), who first reported on external microcystin degraders which were outside this phylum, isolated within the United Kingdom. The suppression was based on cell lysis or growth limitation higher than 80% of the starting populations (Manage et al., 2009).

The earlier findings of *Proteobacteria* as the most common control agents may also be supported by the microbial diversity and abundance during a bloom, as it has been established that at the peak of the bloom *Proteobacteria* and *Bacteriodetes* are abundant. Algicidal microcystin degraders however, were also commonly reported from *Firmicutes*, particularly from *Bacillus* (Mayali and Azam, 2004; Pei et al., 2007; Shunyu et al., 2006; Zhang et al., 2011), which may be logical if *Firmicutes* increases towards the decline of a bloom.

In the case of liquid cultures, which is the most commonly reported experimental procedure; the predator bacterium is either isolated from within the bloom waters or seldom externally sourced. Screening of primary inhibition is conducted and based on this, further research is done to analyse algicidal activity and the effects. The predator and prey are grown under separate conditions, with the cyanobacteria grown as axenic or non-axenic strains at ambient temperature, on BG-11, Z8 or similar culture media (Nakamura et al., 2003). The predator is most often grown at higher temperatures, in a different medium. Cells of the predator are then introduced to the cyanobacterial population and cell death or lysis is measured microscopically or similar methods as listed above. This shortfall usually is not representative of the natural conditions within the environment as most cultures are axenic cyanobacteria.

Table 3.3 shows the different experiments that have been applied in the control of freshwater cyanobacteria.

Table 3.3: Effective ratios required of predator bacteria to prey cyanobacteria in previous studies

Phylum/Class	Control organisms	Target cyanobacteria	Effective ratios (b:a)*	Scale	Source
Actinobacteria	*Streptomyces*	*Microcystis aeruginosa*	1ml : 99ml	100 ml	Luo *et al* 2013
Proteobacteria: Alphaproteobacteria	*Xanthobacter autotrophicus*	*Microcystis aeruginosa*	$1x10^8$: $2.2 x10^6$	50 ml	Kim *et al* 2008
Bacteriodetes	*Aquimarina*	*Microcystis aeruginosa*	4ml: 36ml	150 ml	Chen *et al* 2011
Proteobacteria: Deltaproteobacteria	*Myxococcus*	*Nostoc* and *Phormidium*	$5x10^6$:$1x10^8$	100 ml	Fraleigh & Burnham 1988
Firmicutes	*Bacillus fusiformis*	*Microcystis aeruginosa, Chlorella, Scenedesmus*	$3.6x10^7$: 412µg.$^{L-1}$ chlorophyll	1000 ml	Mu *et al* 2007
	Brevibacillus	*Oscillatoria*	8ml: 80ml dry weight	100 ml	Jia *et al* 2014
	Exiguobacterium	*Microcystis aeruginosa*	$1.5x10^6$:1		Tian *et al* 2012
	Bacillus cereus	*Aphanizomenon flos aquae*	5 ml ($1x10^8$ cells) :45 ml	50 ml	Shunyu *et al* 2006
Proteobacteria: Gammaproteobacteria	*Pseudomonas aeruginosa*	*Microcystis aeruginosa*	40µg.ml^{-1} extract: $2x10^7$	1 ml	Ren *et al* 2010
	Pseudomona putida	*Microcystis aeruginosa*	$1x10^8$: $1.2x10^7$	150 ml	Zhang *et al* 2011
	Pseudomonas stutzeri	*Microcystis aeruginosa*	20ml:20ml	40 ml	Gumbo *et al* 2010

***bacteria:algae/cyanobacteria**

.3.10 Different conditions of biological control implementation using bacteria

Using Figure 3.1 as a timeline guide of recent studies conducted in this area, there are a few general conditions that are applied in previous studies to study algicidal or inhibitory effects of bacterial isolates on targeted cyanobacteria. On a laboratory scale, the studies are conducted primarily through:

- Plaque assays on solid agar medium (Gumbo et al., 2010; Rashidan and Bird, 2001).
- Liquid medium growth assays (Nakamura et al., 2003; Choi et al., 2005; Gumbo et al., 2010).
- Flow cytometric assessments of cell viability and death through live and dead stains, microscopy and cell surface monitoring (Cellamare et al., 2010; Gumbo et al., 2014; Zhou et al., 2012)

The methods listed above are among the most commonly used, with chlorophyll measurements being the primary measurement of cell death. With *Microcystis* specifically, this has proven to be difficult as it is not an accurate estimation of cell abundance or death. Flow cytometry has been recommended by Gumbo et al., (2014) as well as Cellamare et al., (2010). Most recent is the study conducted by Chapman (2016), where flow cytometry is employed as a predictive measure for blooms, among the known technologies.

The typical experimental set up involves the culturing of mostly axenic or non-axenic cyanobacterial cultures, which are grown to a certain point and thereafter the predatory isolate is added at the optimized working ratios to achieve cell suppression. Most of these effective agents are of the *Proteobacteria* or *Firmicutes* genus, often tested against *Microcystis aeruginosa*. The findings indicate cell lysis, which is often associated to increased toxicity of the test waters. As a result, most of the studies are not suitable for up-scaling purposes or further development as is. The other findings indicate predator to prey ratios of at least 1:1, for reasonable cyanobacterial suppression and/or lysis.

Research by Su et al., (2016) as referred to in Table 3.2 indicated overall toxin suppression, which is not commonly reported, although this was against an axenic culture of *Microcystis*.

Based on the available literature, adaptation of this particular control method (i.e. bacterial biological control of cyanobacteria) is approached with caution, simply due to the numerously reported effects of lysis as well as the unknown further biological impacts. In-depth reviews of the contact time and lysis mechanisms have indicated that no one prescribed method may be effective against varied biodiversity (Gumbo et al., 2008). A more recent synopsis of biological control in marine environments equally stresses the point of uncertainty in the possible outcomes of implementation as opposed to terrestrial

biological control experiments. Moreover, augmentative biological control appeared to be a more feasible projection compared to the introduction of non-indigenous species (Atalah et al., 2015).

A 2017 report (Osman et al., 2017) assessed the interactions of axenic cyanobacterial cultures in the presence of known cyanolytic heterotrophs. The analysis of gene up and down regulation indicated stress from both the heterotrophs and the cyanobacteria from a nutrient competition perspective as well as from a growth limitation aspect for *Microcystis* sp. However, the *Aphanomenizon flos-aquae* isolate tested under similar conditions indicated cell damage over a 96-hour period but there were no clear indications of growth limitation. The study led to the findings that the cell stress response was elicited by the attachment and aggregation of the predatory heterotroph to the cyanobacteria as well as extracellular compounds such as antibiotics, which affect photosynthesis in the cyanobacterial cells. Of further interest among the many findings in this research is the expression of peroxiredoxin, a protection from toxic peroxides and up-regulation of cold shock proteins by some of the bacterial isolates. The study also found an energy metabolic pathway shift in the bacterial isolates, indicating nutrient related stress (Osman et al., 2017). These findings corroborate the theories in this review on nutrient limitation and are a key contribution to understanding the complex cell to cell interactions between the cyanobacterial isolates and their heterotrophic bacterial prey. This study was conducted on laboratory axenic strains of cyanobacteria.

3.11 Are there stories of success?

Thus far an assessment of cyanobacteria control methods has found an application of EM (effective microorganisms), which comprises of a mud ball (microorganisms kneaded into mud) of microbial consortia to be ineffective in population reduction of lab and wild strains of *Microcystis*, with effective suppression achieved at very high numbers, possibly due to other factors. It is interesting to note that *Lactobacillus*, *Lactococcus* and *Saccharomyces* comprised the major microbial population in these EM mud balls, which in conjunction with submerged plants, reduced total nitrogen and phosphates in water. Although mixed data have been collected in the laboratory and field studies in the report, there appear to be various factors influencing the success and failure of the mentioned treatment interventions. This study further questions the proposed effects of these treatments as they appear to be symptomatic treatments as opposed to effective controls of nuisance blooms (Lürling et al., 2016).

More effective results have been observed through the use of metabolites from bacteria or the degradation of microcystins by specific bacteria (Yang et al., 2014b), with possibilities for upscaling the treatments on a larger scale (Pei et al., 2007), in a controlled environment. A comprehensive review by Nybom (2013) lists various isolates that have been applied in the bacterial degradation of microcystins

and how these microcystins are broken down by the *mlr* genes, of which *mlr*A is responsible for the primary breakdown of microcystins. The review continues to indicate the suitability of microbial aggregates in filter systems for microcystin degradation. Interestingly, the author also points towards the use of probiotic bacteria such as *Bifidobacterium* species for microcystin degradation. The use of other classes probiotic bacteria in the EM mud balls as previously referred to by Lürling et al. (2016) was however not as successful in controlling *Microcystis*, at a living cell to cell interaction approach. Microcystins in the environment are degraded within 5-21 days, although most lab-scale studies have periods beyond the minimum five days set to observe cyanobacteria or toxin suppression (Welker et al., 2001).

A compilation of some mitigation and prevention strategies at field scale level has indicated the control of nitrogen and phosphorus ratios in shallow and deep lakes among the possible measures that can be implemented after analysis of the lake system. From a biological intervention perspective, food web management through mussels, which improve light conditions for other macrophytes, clearing waters through grazing on cyanobacteria is described as a potential intervention. Moreover the authors state earlier in their report that the food web management has minimal impacts on bloom control (Stroom and Kardinaal, 2016). The use of bacteria is not included in these interventions and it may be due to the limited reports among conventional methods for controlling nutrient loading and bloom occurrence in field studies. This really draws attention towards further development of effective predatory bacteria application in bloom mitigation or prevention, which is a key point of this review.

Another publication by Park et al. (2017) indicates how most of the studies in controlling cyanobacterial blooms have been under controlled laboratory conditions. However, the authors propose ultra-sonication, another abiotic measure as a method for bloom control, with compiled reductions of up to 90% algal removal of *Aphanizomenon* from a field reservoir study by Schneider et al. (2015). Although the study by Park et al. has considerations and a variation of effectiveness in terms of cyanobacterial reduction percentages from different studies, there is a demonstrated effectiveness of this method, with considerations and guidelines for further application in larger scale water ecosystems.

For the purposes of this review, the focus is mainly on live cell to cell interaction between the cyanobacteria and their predatory bacterial isolates, which apart from the report of Lürling et al. (2016), is rather limited at a larger scale.

3.12 Opinion on possible pitfalls

Areas that may require revision is variation in culture methods and conditions between the predator and prey. The addition of a culture unaccustomed to the bloom conditions may affect the effectiveness of

this culture and perhaps the required cell concentrations. This is indicated in the estimation of six to seven days as the required time to present reasonable damage to cyanobacteria when bacterial isolates are concerned.

Moreover, the use of axenic strains in the biological control studies may be cautioned if similar effects cannot be obtained in the natural environment. The fact that these isolates almost never exist as axenic strains in a natural environment outside of the laboratory may indicate that the selected control agent needs to be effective within a mixed diversity. The changes that occur with "gold standard" isolates such as culture collections of *Microcystis* isolates may be giving an incorrect representation of the wild strains and possible outcomes with a given control agent. This would require further research of control agent efficiency in different types of blooms, with other co-dominant algal species and the prospective impact on other aquatic life.

Control agents need to be examined for their effects on a mixed population of cyanobacteria, which is the common finding in blooms, although certain species may be dominant.

The freshwater studies conducted at present focus on the control of *Microcystis* type strains, with other studies focused on marine cyanobacteria (red tides) or diatoms. A handful of these studies looked at other cyanobacterial species in detail. At whole cell level, it appears that there are few papers indicating suppressed toxin production or toxicity overall through the use of bacterial control agents. This is a crucial aspect to consider if water quality is to be considered.

Another interesting observation is that among the most common control agent phyla, *Firmicutes* is not often reported as abundant under bloom conditions, indicating that control methods would have to be an augmentative approach, which has not been extensively explored beyond lab-scale studies. Two-step type interventions have also been proposed, where the control agent specifically is added in addition to other conventional treatments.

Of importance are the critical assessments of dose-responses/effective ratios and the streamlining of which phytoplankton diversities the proposed agent can be effective against. Cell numbers, chlorophyll and biomass need to be effectively assessed as reliable dosing methods, without "blindly" applying control agents into waters that have little prior assessment apart from assessing the dominant cyanobacteria.

The succession of bacterial populations in the decline of cyanobacterial bloom indicates an increase in predatory isolates. Within the natural environment, the addition of effective numbers of these predatory isolates may initiate an earlier bloom decline. The use of isolates that are naturally occurring within the

given aquatic system may be useful in preventing the hampering of the natural biodiversity. The investigation of effective bacterial ratios needs to be considered, as lower numbers than those recommended for lysis may have growth inhibiting effects that do not ultimately result in lysis and toxin release. However, this cannot be fully substantiated without the implementation of pilot scale studies. In addition, the application of these biological control agents may be useful as an alternative treatment for wastewater, where these bacteria can subsequently be removed in further treatment steps.

In closing, to our knowledge, the current collection of literature indicates slow progress in the further application of this research beyond laboratory conditions and further effects beyond the typical toxin release upon lysis, which is counterproductive to the use of biological control of cyanobacteria. Different temperatures, nutrient conditions and turbulence have repeatedly been indicated as important factors in determining how effective a bacterium may be at effectively reproducing the estimated inhibitory effects on cyanobacteria.

The reasons listed above among the complex interactions within the bloom diversities may be among the reasons there is slow advancement in this area of research, with the major question remaining: why do these lytic agents fail to regulate bloom occurrences in nature?

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Chapter 4: Identification and growth characteristics of control agents and target cyanobacteria

Abstract

Blooms are dominated by different species of cyanobacteria, some being more toxic than others. Numerous bacteria from different phyla have been reported as effective in controlling these blooms. In this study identification of two cyanobacterial isolates was conducted through microscopic and molecular methods. Filamentous and colony-forming bloom causing cyanobacterial isolates were collected in the Mpumalanga and Western Cape Provinces of South Africa and kept as non-axenic cultures. Three potentially algicidal bacterial isolates (Isolate 1, 3w and 3y) were collected from the bloom waters of these cyanobacteria based on preliminary testing through the plaque assay. These were compared to a bacterial isolate initially described as *Bacillus mycoides*, which is a known cyanolytic species. An assessment of the growth patterns of the cyanobacterial isolates indicated their ability to grow on BG-11 media and reach the exponential phase of growth around three days based on chlorophyll *a* determinations. Wet weights of cells were also recorded as a confirmation of increased biomass. The filamentous cyanobacteria were identified through microscopy as belonging to the genus *Oscillatoria*, although, 16s rRNA identification with universal cyanobacterial primers did not conclusively assign the isolate to a genus. The colony-forming cyanobacteria was identified as *Microcystis* through both microscopy and 16s rRNA identification with universal cyanobacterial and *Microcystis* specific primers. A comparison to another Microcystis isolate collected from a crocodile farm in the Northern Cape indicated a close clustering in the phylogenetic analysis of the isolates. This showed species similarity to the isolates collected from the same country as opposed to previously defined type strains on the database. Bacterial isolate identification found the *Bacillus mycoides* isolate to be related to *Lysinibacillus fusiformis* but was not conclusively assigned to a genus. Isolate 3y indicated a relation to *Pseudomonas*, while isolate 3w was more conclusively identified as *Pseudomonas rhodesiae*. Isolate 1 was found to be closely related to *Aeromonas lacus* based on molecular identification. The bacterial isolates grew best on Tween 80 broth, with an exponential phase reached within 4-8 hours of culturing. This indicated that the cyanobacteria and bacterial isolates were able to remain viable within the lab conditions.

4.1 Introduction

In order to apply any form of biological control, the characteristics of both the target and control agent need to be understood. This chapter assesses the growth and species identification of two freshwater cyanobacteria (*Microcystis* sp. and O*scillatoria* sp.) and four potential predatory bacteria. Cosmopolitan cyanobacteria such as *Microcystis* have been studied extensively (Preece et al., 2017) in order to innovate effective control measures. Filamentous cyanobacteria e.g. *Oscillatoria* that occur at the sub-surface phase of the water column are not as well studied in comparison to isolates such as *Microcystis*, creating a need for more research into these species and their response to biological control. The growth kinetics, nutritional requirements and microscopic/phenotypic characteristics of a microorganism are fundamental to the identification of the isolate and more importantly in ensuring it can be successfully cultured under controlled conditions. Obtaining a steady growth pattern can indicate how an organism functions under optimal conditions (Wilson et al., 2006; Yamamoto and Shiah, 2010), thereby enabling a good indication of any inhibitory growth conditions should the optima be altered. Changes in the growth of a bacterial isolate give an idea of the population response to a variety of factors, either antimicrobial compounds (Kim and Anthony, 1981) or growth and or decline over time (Contois, 1959; Ong et al., 2001). Studies of these growth responses are not well studied in cyanobacteria such as *Oscillatoria*, with most of the studies on this cyanobacteria being from 20 years ago and only a few from the 2000s (Ferreira Filipa et al., 2000; Konopka, 1981; Meffert et al., 1981; Post et al., 1985). Based on the determination of population changes the ecology and nutrient requirements of a given species can be better understood. In the assessment of cyanobacteria among other phytoplankton, the abundance is measured through chlorophyll production, cell numbers and remote satellite imaging (Matthews and Bernard, 2015). This helps determine when a control agent can be implemented and the various stages of the organism's life cycle. In the context of cyanobacterial blooms, this information is crucial to predict when it happens, become toxic and the cell interactions with other bacterial and algal species during a bloom period (Rivasseau et al., 1998; Wilson et al., 2006). In the present study, the different growth stages and identification of isolates will be conducted on a molecular and microscopic level, with an understanding of the toxin production of each cyanobacterial isolate. This offered a comparative assessment of well-studied unicellular isolates such as *Microcystis* and the less researched *Oscillatoria*. Numerous algicidal bacterial isolates have been reported in literature and in this chapter, unknown potential predatory isolates were identified and compared to a bacterial isolate related to *Bacillus*. More importantly, the identification of potential predatory bacteria indicated whether any novel species are identified in comparison to the well-known *Bacillus* species that are commonly reported as cyanolytic (Gumbo et al., 2011).

4.2 Materials and Methods

4.2.1 Isolation of bacterial and cyanobacterial species

An isolate described as *Bacillus mycoides* was generously donated by the Stellenbosch University Microbiology department on nutrient agar plates, grown at 30°C. Isolate 1, 3w and 3y were randomly selected from BG11 (Sigma-Aldrich) plates spread plated with water from the bloom, after three weeks, using the plaque assay as described by (Gumbo et al., 2010).

Water containing a bloom of *Microcystis* sp. was collected from Brandwacht wastewater treatment works, in Mossel bay, Western Cape, South Africa (34° 3' 3.6" S, 22° 3' 28.8" E). Filamentous cyanobacteria were collected from the Klippoortjie waste water treatment plant (26° 07' 00" S; 29° 08' 00" E), near the town of Ogies, Mpumalanga, South Africa. Samples were collected in sterile water bottles and kept on ice during transportation. Isolates were identified by light microscopy at 400 x magnifications (Zeiss Axioscop), using the procedures mentioned by Oberholster et al. (2009, 2016) and stored as non-axenic cultures at 4°C for the duration of the study (2016-2018) and checked monthly for dominance of the cyanobacteria of interest.

4.2.2 Microscopy and Gram stain reactions

Sterile nutrient agar (Merck, Germany) plates were used to culture bacterial isolates. Overnight cultures grown at 30°C were used for microscopic analysis. Wet mounts were prepared by mixing a loopful of inoculum with sterile MilliQ water and viewed at 100x magnification under an oil immersion light microscope, Zeiss Axioskop, and imaged using the Axiocam ERc5S digital camera with Zenblack software. Gram stains were also performed on the isolates using the Gram staining kit (Sigma-Aldrich).

Scanning electron micrographs were done on the cyanobacterial isolates at the Stellenbosch University Central analytical facility. Briefly, samples were dried onto conductive carbon tape on 15mm aluminium stubs, sputter-coated with a thin layer of carbon using an Edwards S 150A sputter coater to enhance conductivity, and visualized using a Zeiss MERLIN Field Emission Scanning Electron Microscopy (FESEM) (Carl Zeiss Microscopy, Germany). For in-Lens Secondary Electron (SE) detection, operating conditions of 5kV accelerating voltage with a probe current of 250pA and working distance 3.2mm were used to generate images using Smart SEM software.

4.2.3. Growth curve measurements of bacterial isolates

Isolates were initially grown in nutrient agar (Merck, Germany) at 30°C for 24 hours and sub-cultured into nutrient broth (Merck, Germany) for another 24 hours at 20°C. Isolates were then sub-cultured in

1% BG-11 broth (Sigma-Aldrich) supplemented with 1% glucose, Tween 80 broth (5 g peptone, 3 g meat extract, 10 ml Tween 80, 100 mg $CaCl_2.2H_2O$ per litre, pH 7.2) and Nutrient broth medium (Merck, Germany). Optical density measurements (600nm) were obtained at hourly intervals using the Varioskan Flash microplate reader (Thermo-Fischer) for an eight hour period (under shaking conditions-150 rpm) to determine the growth rates and behaviour of the isolates at ambient temperature. Pure bacterial cultures were grown at ambient temperature (25°C) and inoculated into media, with hourly optical density measurements. From the changes in optical density, microbial cell numbers were estimated from absorbance. The data were obtained from means of three experimental repeats. From this, the growth rate, generations per time and mean generation were estimated for the isolates individually and compared across the three different media.

4.2.4 Growth measurements of cyanobacterial strains

Collected cyanobacteria isolates were washed with 1x phosphate buffered saline (Lonza) twice and filtered using the Corning 0.22µm filtration system (Sigma). Filtered samples were then sub-cultured in 1 % BG11 media (Sigma-Aldrich) for two weeks. Once buoyant cells showed a steady increase in chlorophyll, the cells were washed and used as inoculum for growth experiments, with starting cell numbers of $1x10^5$ for *Microcystis* and starting wet weights of 0.1g for *Oscillatoria*. The cells were monitored every 3-4 day intervals for changes in chlorophyll *a*, to determine exponential growth phases. Growth of *Microcystis* was measured using a combination of cell counts and chlorophyll *a* measurements. Wet weight was measured from time to time to confirm cell counts. *Oscillatoria* was monitored using wet weight of filaments and chlorophyll. Chlorophyll was extracted according to Porra, (1998) and absorbencies were measured on the Varioskan Flash (Thermo-Fischer) over 28 days. Wavelengths measured were 470, 652, 665 and 720nm. Cell counts were also used for *Microcystis* sp. as single buoyant cells, using the Invitrogen Countess cell counting chamber.

4.2.5 Toxicity marker gene analysis in cyanobacteria

The presence of various microcystin toxin markers, *mcy* A, B, D and E genes was investigated using primers from Oberholster et al. (2015). The presence of the anatoxin gene cluster was determined using primers as reported by Rantala-Ylinen et al. (2011). The amplification reaction was as prescribed by the authors, with different annealing temperatures based on each primer's melting point.

4.2.6 16s rRNA identification of isolates

The forward primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer rP2 (5' -ACGGCTACCTTGTTACGACTT-3') (Weisburg et al., 1991) were used for hot-start PCR based

amplification of the 16S rRNA gene of all isolates. Universal primers 895F (5'-CRCCTGGGGAGTRCRG-3') and the reverse primer 902R (5'-GTCAATTCITTTGAGTTTYARYC-3') were also used for the identification of bacterial isolates. For the cyanobacterial isolate identification, isolates were identified using primer pairs CYA 359F (5'-GGGGAATYTTCCGCAATGGG-3') and CYA 781R (5'-GACTACAGGGGTATCTAAATCCCTTT-3') as well as CYAN16S (5'-ATACCCCWGTAGTCCTAGC-3') and CYAN16SR(5'-GCAATTACTAGCGATTCCTCC-3') were used as forward and reverse primers according to Hotto et al. (2007).

For bacterial identification, fresh single bacterial colonies were dissolved in 100 µl of sterile TE buffer in an Eppendorf tube and lysed by two cycles of boiling (10 min at 100°C) and subsequent freezing in liquid nitrogen (10 min). Tubes were thawed and centrifuged at 10000 x g for five minutes and the supernatant was used for PCR. Amplification of the 16S rRNA gene was done according to the procedure suggested by Weisburg et al. (1991). PCR products were analysed on 2% agarose gels (2 g agarose in 100 ml TAE buffer (40 mM Tris, 20 mM acetic acid, and 1m M EDTA, pH 8.0)) and stained with 1 µl Gel Red DNA Gel stain solution). A 1 Kb DNA molecular size ladder (Promega) was used and the gel was run for 45 minutes at 100V. The gel was documented using a gel imager (G:Box, Syngene, UK) with Image lab (version 7.09) to assess whether the amplification products were of the correct size. Sequencing of PCR products was done by the Central Analytical Facility of Stellenbosch University (Stellenbosch) and the sequences were compared to sequences deposited in GenBank (http://blast.ncbi.nlm.nih.gov). Phylogenetic trees using appropriate type strain and out-group sequences obtained from RDP (ribosomal database project, www.rdp.cme.msu.edu) were generated, based on sequence alignments established with Clustal W and the neighbour joining method using MEGA 6 (Tamura et al., 2013) with 10 00 times re-sampling. The topology of the trees was verified through the maximum likelihood method for comparison. For *Microcystis*, a unicellular isolate obtained from a commercial crocodile farm in North West, South Africa was used for comparison, also to assess the similarity of *Microcystis* from two blooms in different provinces in the same country, compared to the strains deposited on the GenBank database. Information on the closest matches for species identification was added to Supplementary data.

4.3 Results

4.3.1 Growth measurements of bacterial isolates on different media

In order to assess the growth and behaviour of the given isolates selected in this research, the growth patterns, morphology and molecular identity were analysed. From initial isolation, it was clear that the

bacteria species and the cyanobacteria had unique growth patterns and nutritional requirements in order to be kept viable under laboratory conditions. BG-11 was the least favourable medium in comparison to the known nutrient broth, which served as a reference point in this study to the surfactant Tween 80 broth, which has previously been reported to have growth promoting properties and BG-11, which is the ideal growth environment for cyanobacteria.

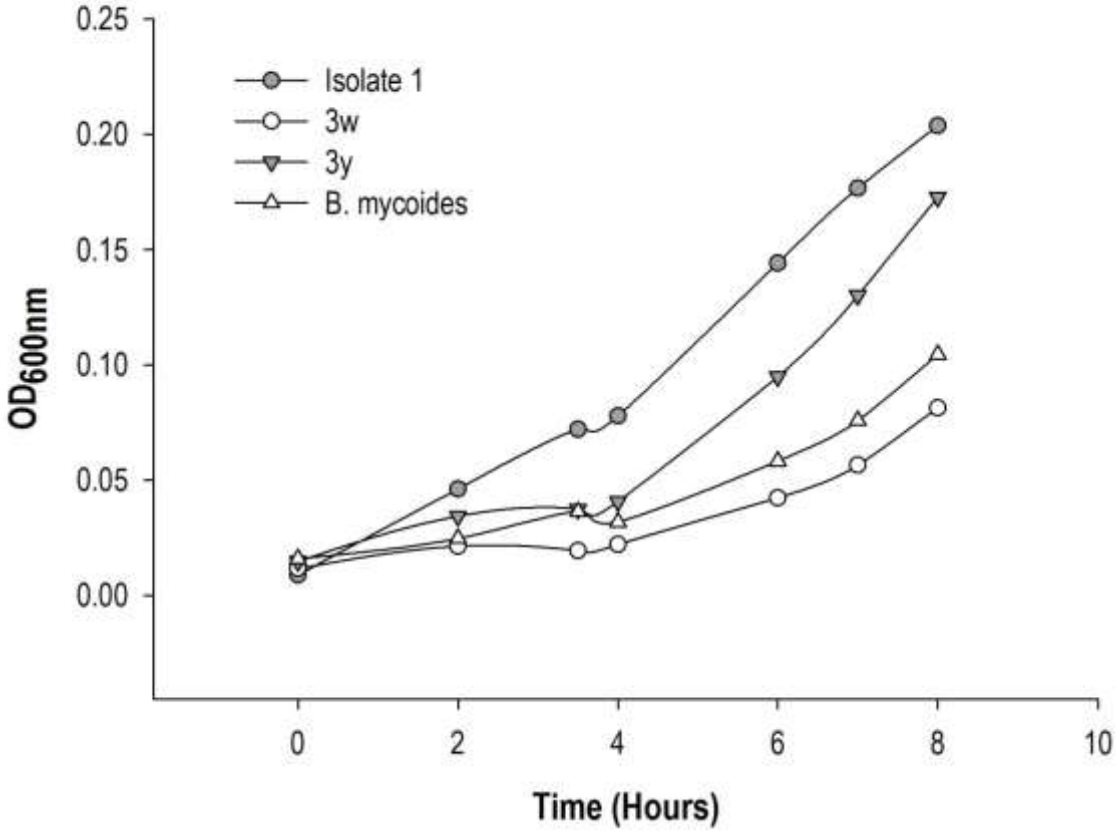


Figure 4.1: Growth measurements of bacterial isolates in Nutrient broth media for eight hours, at 25°C.

All four isolates showed steady growth on this substrate, with the exponential phase continuing beyond eight hours for Isolates 1 and 3y. Isolates 3w and B indicated a prolonged lag phase. Using this rich and general growth medium as a reference point, the substrate preference was determined through comparison of growth rate measurements on all three substrates. Table 4.1 shows the calculated kinetics of each isolate in Nutrient broth medium, with a doubling time of around 3 hours for each isolate.

Table 4.1: Growth kinetics measurements of bacterial isolates grown in Nutrient broth medium

Growth kinetics	Isolate 1	3w	3y	B
Growth rate constant	0.595344	0.536513	0.647925	0.579575
Generations per time	2.147077	1.934906	2.336707	2.090208
Mean generation time	2.794497	3.100926	2.567716	2.870528

In the case of BG-11 medium, isolates showed a less uniform growth, indicating that this given substrate was not ideal for two out of four isolates in terms of growth. This was also over an eight hour period, through optical density measurements. Isolates exhibited some clumping, which can occur when in a complex or minimal substrate.

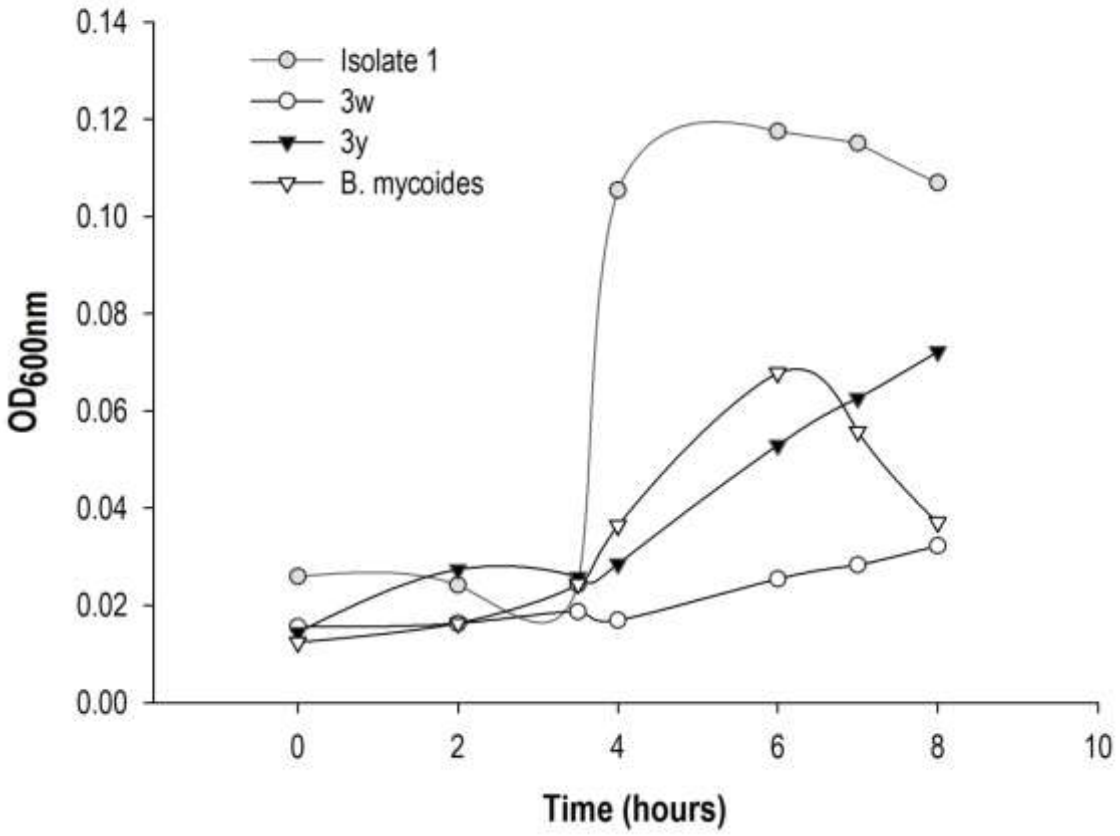


Figure 4.2: Growth measurements of bacterial isolates in BG-11 media over eight hours at 25°C. Isolate 1 reached stationary phase after 6 hours, whilst isolate B. reached death phase after six hours, whilst isolates 3w and 3y reached exponential growth at 4 hours onward.

The calculated growth rates in Table 4.2 were determined on the exponential phase of each isolate. The exponential phase was around 4 hours for all the isolates with a much longer generation time for isolates 3w and 3y. It is seldom reported that bacterial isolates can grow in this type of media being heterotrophs, without an organic carbon source, which is why in most instances this medium is supplemented with glucose or some carbon source.

Table 4.2: Growth kinetics measurements of bacterial isolates grown in BG-11 medium over eight hours.

Growth kinetics	Isolate 1	3w	3y	*B*
Growth rate constant	0.629743	0.122676	0.38829	0.569898
Generations per time	2.271135	0.442425	1.400348	2.055307
Mean generation time	1.100771	5.650679	4.284648	1.946181

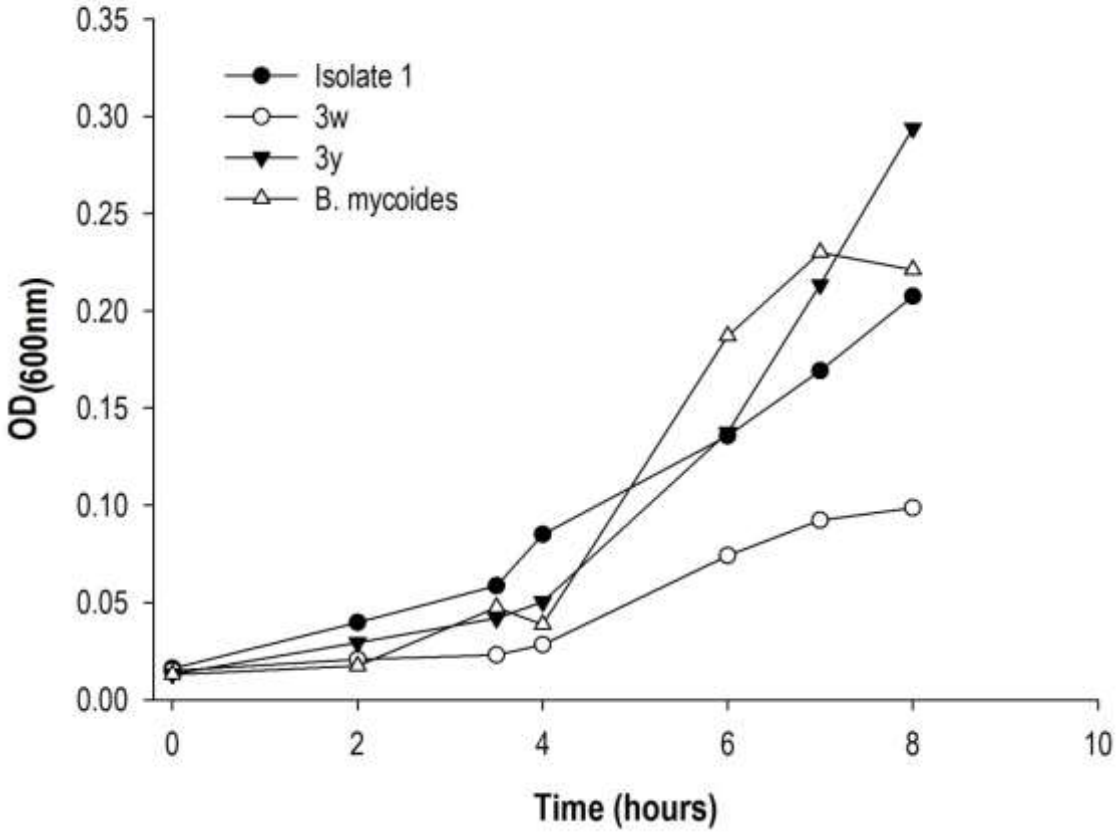


Figure 4.3: Growth measurements of bacterial isolates in Tween 80 broth media over eight hours at 25°C. Once again, four hours was the time it took all the isolates to reach exponential growth. Tween 80 has been well researched to have some growth promoting properties which can be observed in the

appearance of cell colony characteristics as well as in the cell growth rates (Nielsen et al., 2016). A comparison of the three media growth constants (Table 4.1-4.3) and generations per time indicates that the growth rate is higher in Tween 80 broth in comparison to Nutrient broth. All growth patterns were measured over the same time interval at ambient temperature (25°C).

Table 4.3: Growth kinetics measurements of bacterial isolates from bloom waters grown in Tween 80 broth medium over eight hours. The calculated growth rates were determined from the exponential phase of each isolate.

Growth kinetics	Isolate 1	3w	3y	B
Growth rate constant	0.660878	0.622963	0.921534	1.016106
Generations per time	2.383422	2.246684	3.323462	3.664531
Mean generation time	2.517389	1.780402	1.805346	1.364431

4.3.2 Microscopic and colony characteristics of bacterial isolates

Isolate B was found to be a motile, bacillus-shaped isolate with motility, Gram-positive, with chain arrangement. Isolate 1 was a Gram-negative, motile bacillus shaped isolate, arranged in clusters. Isolate 3w and 3y were also Gram-negative bacilli and cocci respectively with chain arrangement for 3w and clustal arrangement for 3y.

The colony appearance of isolate *B* was small watery colonies which turn white-grey and flat with irregular margin as colonies get older, with a fluffy appearance. Isolate 1 is off-white shiny colonies with convex elevation. Isolate 3w is cream, flat shiny round colonies which discolour nutrient agar plates to yellowish. Isolate 3y exhibits as small, shiny yellow colonies that also show punctiform growth and convex colony elevation. All isolates exhibited motility when observed under wet mount. Gram stains of the bacterial isolates showed pure Gram-negative isolates with the exception of *Bacillus* (Figure 4.4).

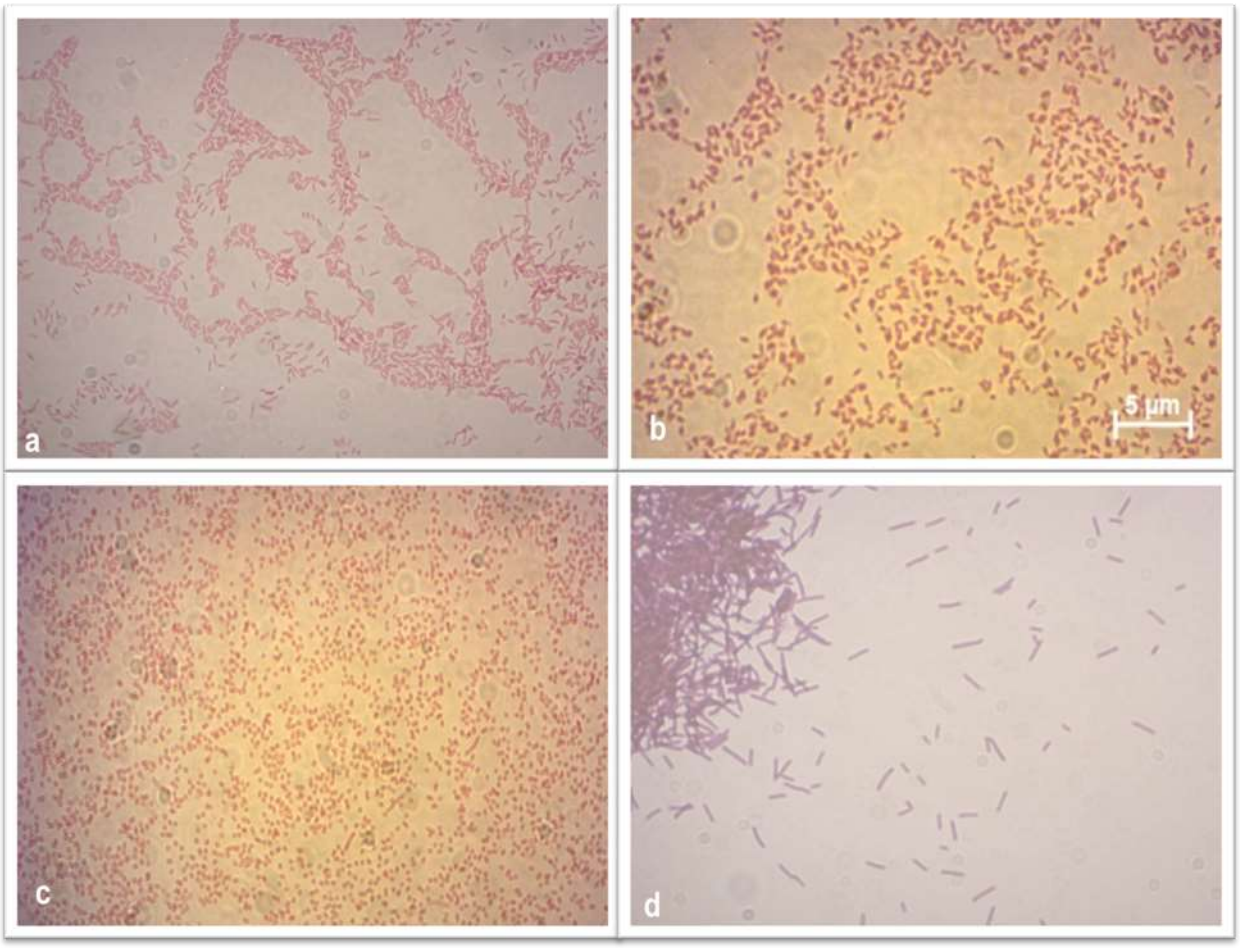


Figure 4.4: Gram stains of isolate 1 (a), isolate 3w (b), isolate 3y (c), and *Bacillus* (d).

Isolate 3y was Gram negative rods, isolate 3w and 1 were Gram negative rods.

4.3.3 Growth measurements of cyanobacteria

Cyanobacteria collected in the current study were both filamentous and single celled buoyant cells. Chlorophyll *a* is the most commonly applied method to detect cell biomass increase in higher order autotrophs, can also be an indication of cell metabolism changes through chlorophyll *b* detection. In the case of the mentioned isolates, the pigments measured were chlorophyll *a* and carotenoids which are common phytoplankton abundance measurements.

For *Microcystis* sp., cell counts were applicable due to their single buoyant cells. Cells were separated according to size resolution from bacterial cells in the non-axenic cultures. Chlorophyll *a* measurements were done with wet weight and cell count measurements to verify that chlorophyll *a* was a direct measure of the biomass.

Microcystis growth measurements were done over a 30-day period, starting cell numbers in 100-200ml volumes were $1x10^5$ cells. After fourteen days isolates showed steady biomass increase, whilst growth from 17-30 days (not shown) indicated fluctuations in the growth patterns, possibly due to nutrient depletion in the culture.

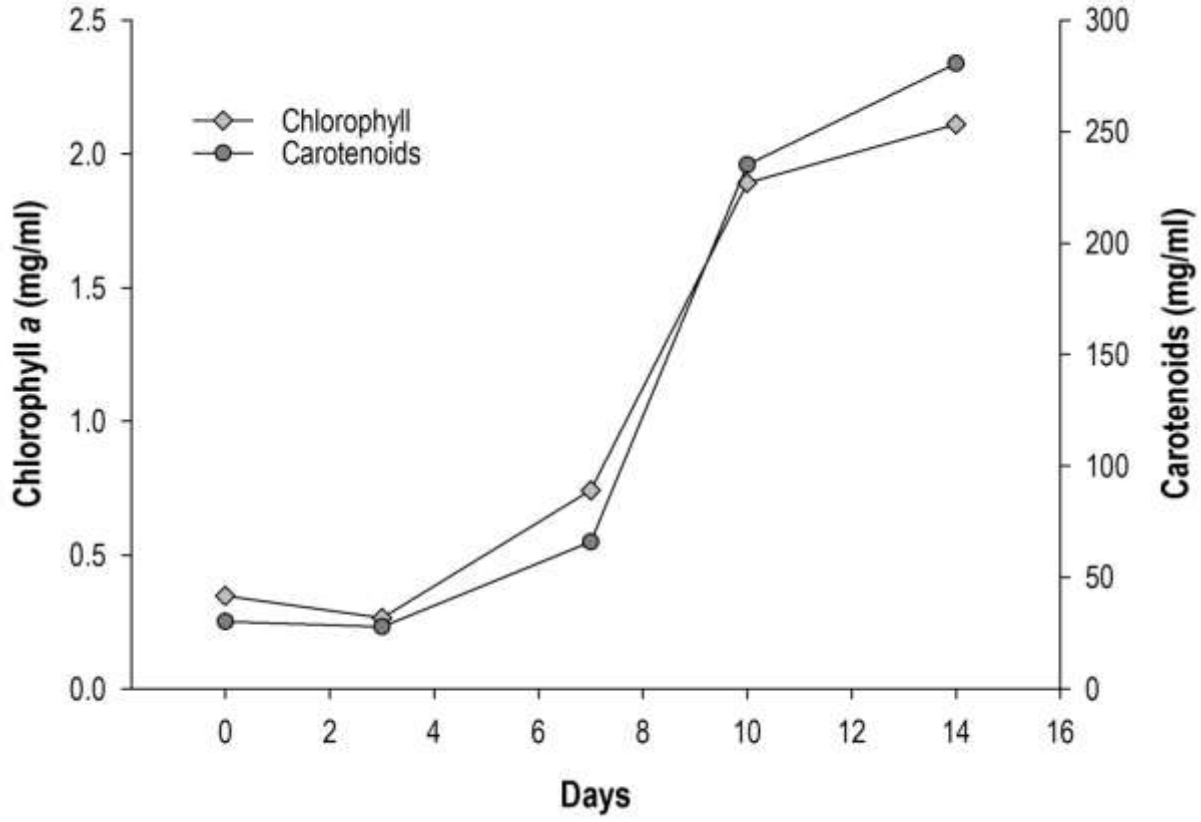


Figure 4.5: Chlorophyll *a* and carotenoid measurements of *Microcystis* sp. cultures in BG-11 medium every 3-4 days over a 14-day period.

Oscillatoria sp. is a filamentous cyanobacterial species which led to difficulties in filament estimations in numbers as these often tangled and are immensely slimy when manually handled. However, they grow to produce a measurable biomass which is easily estimated through wet weight measurements. The filaments were weighed out to a starting mass of 0.1 g, which corresponded to a starting concentration of 2.7µg.ml^{-1} of chlorophyll *a*.

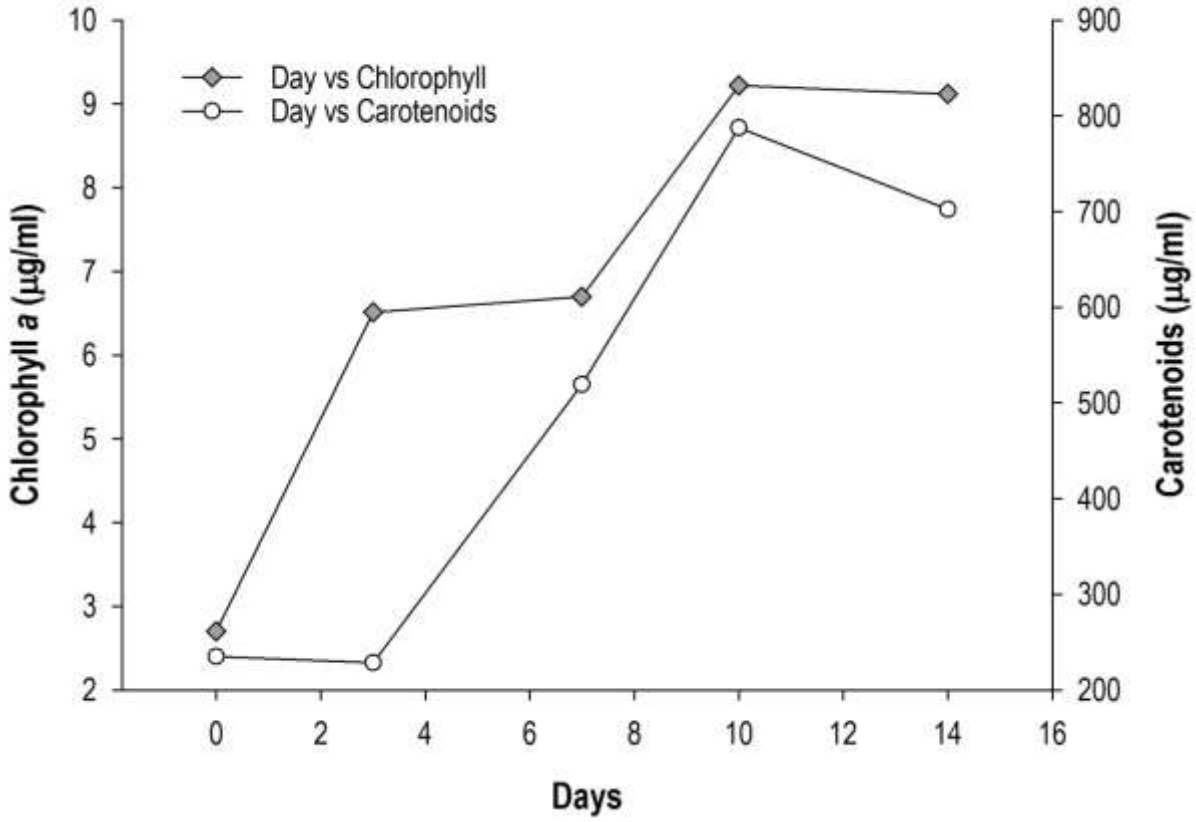
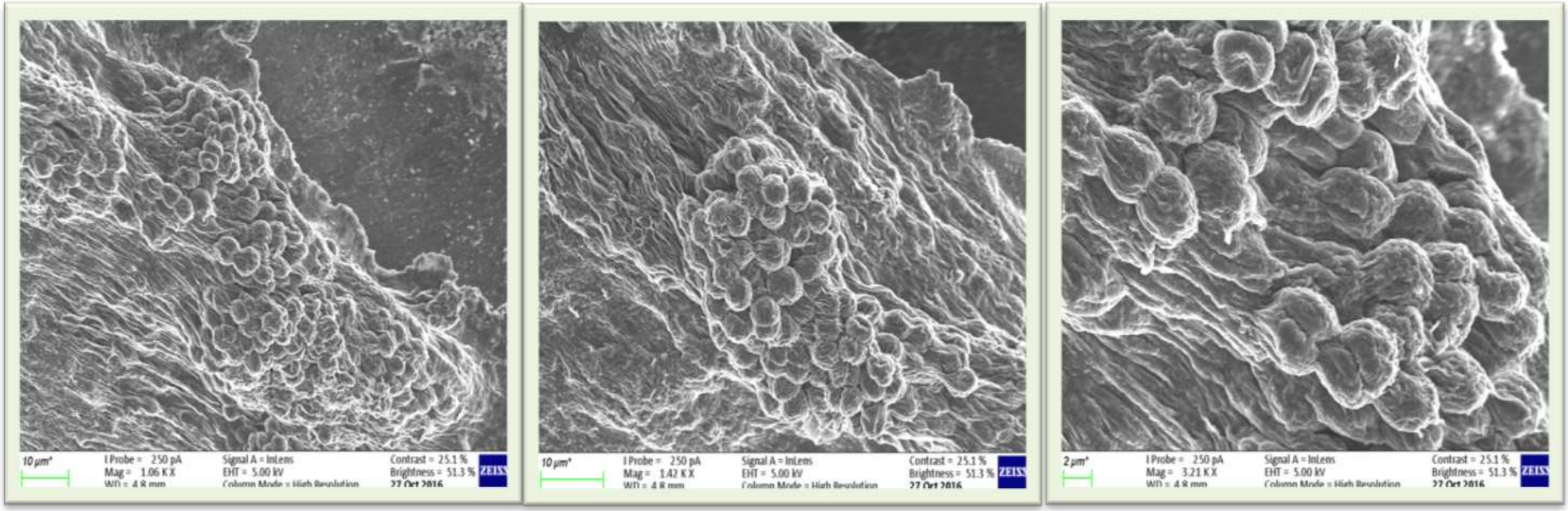


Figure 4.6: Chlorophyll *a* and carotenoid measurements of *Oscillatoria* sp. cultures in BG-11 medium every 3-4 days over a 14-day period.

Based on the growth curves in Figure 4.5 and 4.6, the cyanobacterial chlorophyll *a* production and therefore, the biomass entered an exponential growth phase between 3 and 7 days, whilst bacterial cells grew best in Tween 80 broth and entered an exponential growth phase from 4 hours of incubation.

4.3.4 Scanning electron micrograph images of cyanobacterial isolates



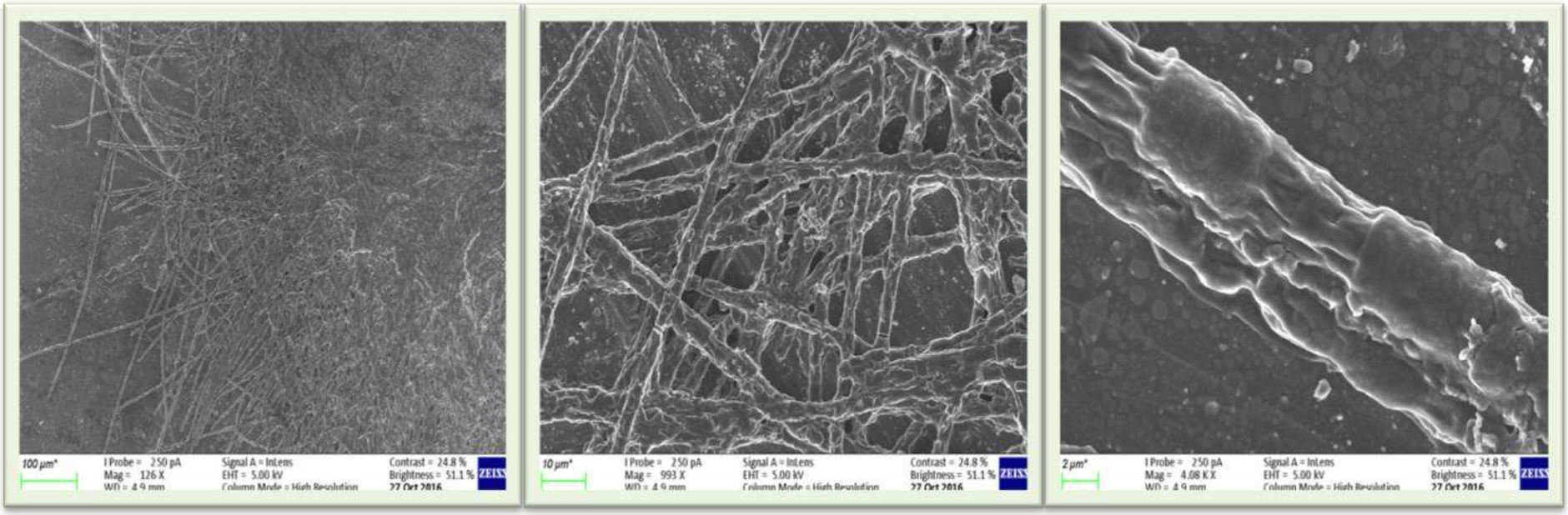


Figure 4.7: Scanning electron micrographs of *Microcystis* sp. (top) **and *Oscillatoria* sp.** (bottom) **cultures at increasing magnification.**

The *Microcystis* cells are covered by a sheath and smaller bacterial cells are seen attached to the cell clumps. Although much larger in size, bacterial cells are seen on the surface *of Oscillatoria* filaments at higher magnification.

4.3.5 Molecular identification of bacterial and cyanobacterial isolates

In the identification of isolates, isolate 1 was identified potentially as an *Aeromonas* isolate, with a 94% similarity to *Aeromonas lacus* according to BLAST results. The *veronii* species in this genus is commonly associated with gastroenteritis and is a known pathogen causing various illnesses in humans (Janda and Abbott, 2010), however, in another study, the *lacus* strain falls within the *aquatica* subgroup. The latter has been isolated in surface waters with no faecal contamination, where cyanobacterial blooms have occurred (Beaz-Hidalgo et al., 2015). The same study details the challenge of interspecies sequence similarity in this genus, therefore it is not easy to determine the species from universal 16s rRNA primer identification. The study by Beaz-Hidalgo gives an indication of the possible relationship between cyanobacterial bloom occurrences and this genus, which is supported by the findings of this study. However, the percentage similarity and the topology of the tree do not confirm the species identity completely and further confirmation is required. From the topology of the phylogenetic tree (Figure 4.8) it is clear that the isolate has some similarity to this genus, although it does not closely group with a particular species.

Interestingly, the strain believed to be *Bacillus*, was identified as a *Lysinibacillus* isolate, with 89% similarity. The genus *Lysinibacillus* formerly clustered under the *Bacillus* genus (Figure 4.9). The percentage similarity of this sequence indicates that it could still possibly a *Bacillus* isolate based on the topology of the tree, which indicates that it is not closely clustered to any of the *Lysinibacillus* species. This genus has been identified in biological control of pests such as insects in earlier research, with similarities to *Bacillus* (Berry, 2012), hence it is not surprising that it is able to possibly control cyanobacteria. In addition, *Lysinibacillus* and *Bacillus* genera are highly similar, with minimal distinguishing features.

Isolates 3w and 3y were identified as *Pseudomonas* isolates, respectively with a 99 and 83% similarity to this genus (Figure 4.10). Isolate 3w can be confidently confirmed as a *Pseudomonas rhodesiae* isolate, under the *fluorescens* sub group, whilst isolate 3y may be closely related to *Pseudomonas* but may possibly be another genus entirely based on the topology of the tree and the percentage similarity to sequences deposited in GenBank. Further recommendations beyond this study would require more tests to confirm the isolate identity.

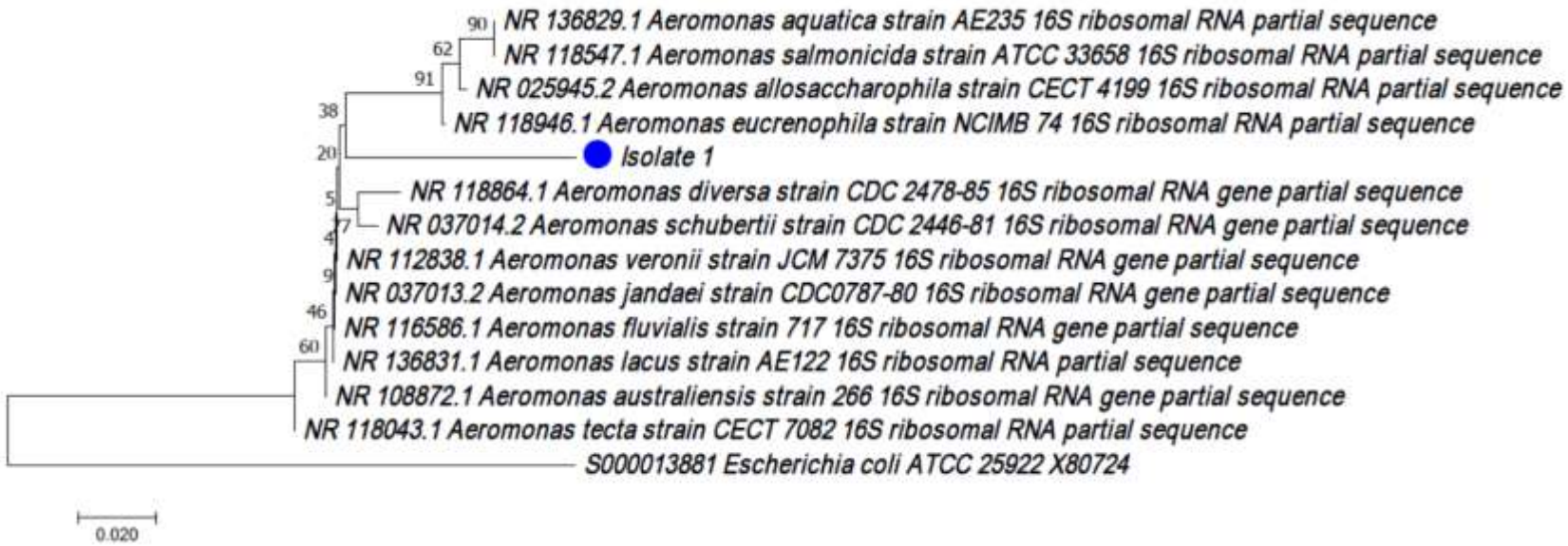


Figure 4.8: Phylogeny of unknown isolate 1 (blue circle) based on the comparison of its 16S rRNA gene sequence with selected 16S rRNA gene sequences for selected type strains of the genus *Aeromonas*. *Escherichia coli* was used as an out group.

The percentage similarity of isolate 1 indicates that it is closely related to this genus; however it cannot be confirmed at this level of similarity.

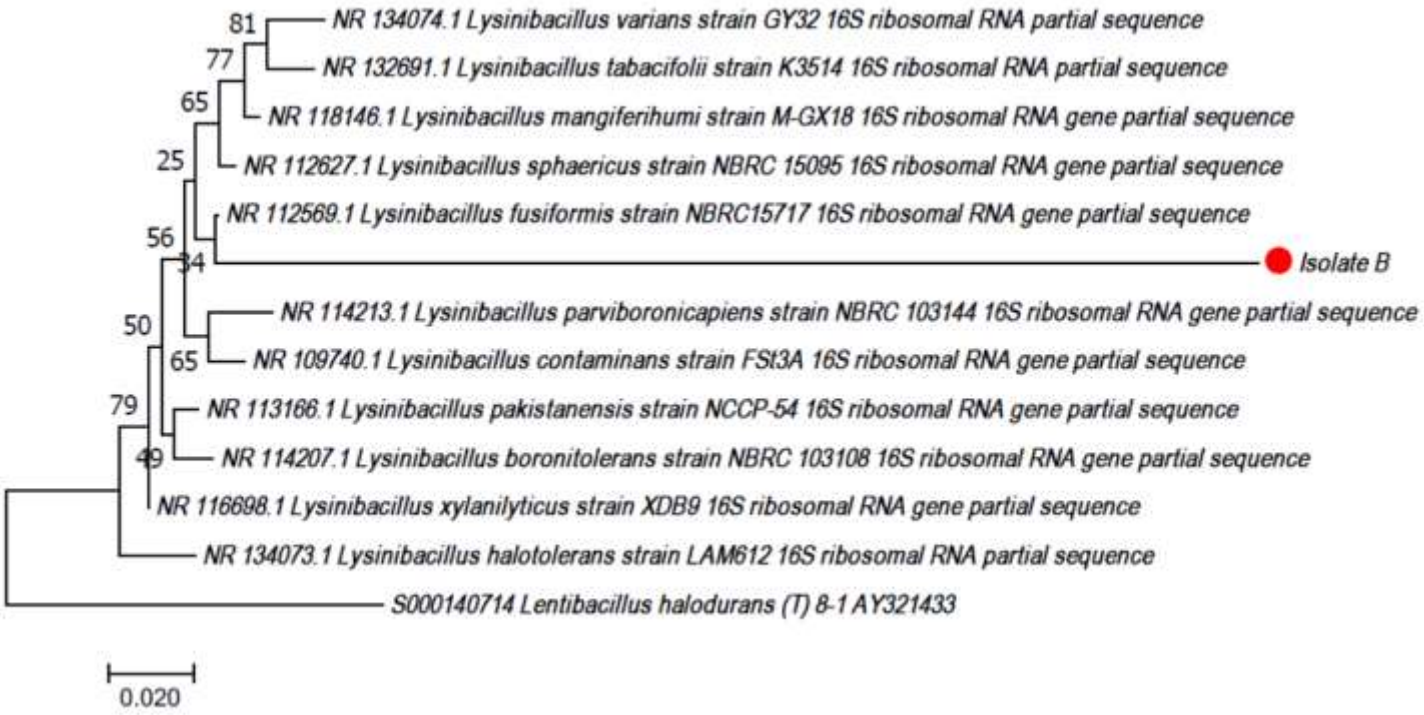


Figure 4.9: Phylogeny of unknown isolate B (red circle) based on the comparison of its 16S rRNA gene sequence with selected 16S rRNA gene sequences for selected type strains of the genus *Lysinibacillus*. *Lentibacillus halodurans* was used as an out group.

Isolate B appears to be related to the *fusiformis* group of *Lysinibacillus*.

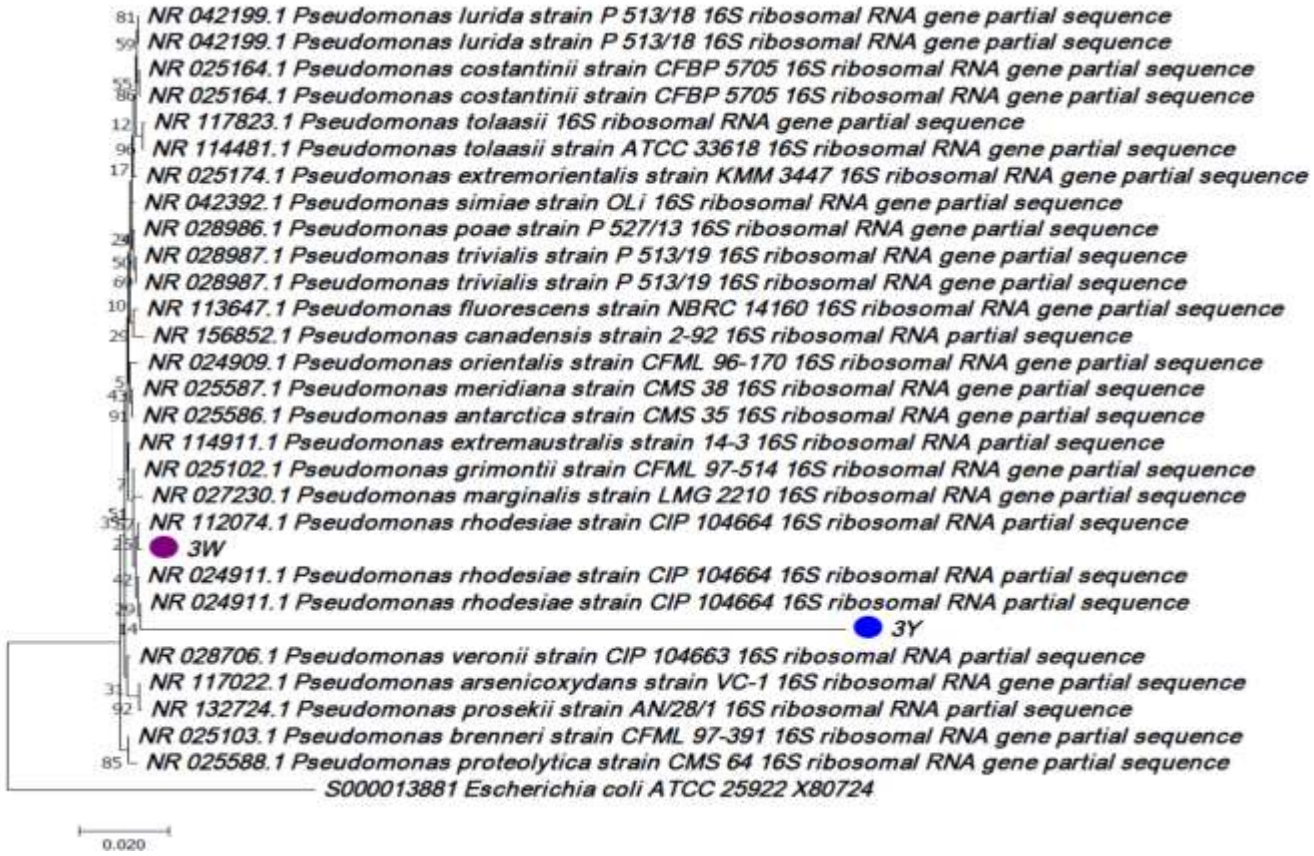


Figure 4.10: Phylogeny of unknown isolate 3w (purple circle) and 3y (blue circle) based on the comparison of its 16S rRNA gene sequence with selected 16S rRNA gene sequences for selected type strains of the genus *Pseudomonas*. *Escherichia coli* was used as an out group.

Phylogenetic analysis of *Microcystis* sp. and the filamentous cyanobacteria isolated from Klipportjie dam through 16srRNA identification of and neighbour joining tree construction with MEGA indicated that the isolated species of *Microcystis* sp. was closely related to *M aeruginosa*. It was interesting to note that the isolates collected from South Africa clustered closely to one another, within the same sub-group. The filamentous cyanobacterium was not conclusively identified and clustered with *Cylindrospermum marchicum*, a recently classified filamentous cyanobacerium with similar characteristics, from the order *Nostocales*, which differs in the presence of a heterocyst, which was not detected in the filamentous isolate in this study. Other similar structured isolates include *Aerosakkonema*, which is also not well distinguished within the *Oscillatoriales* (Thu et al., 2012).

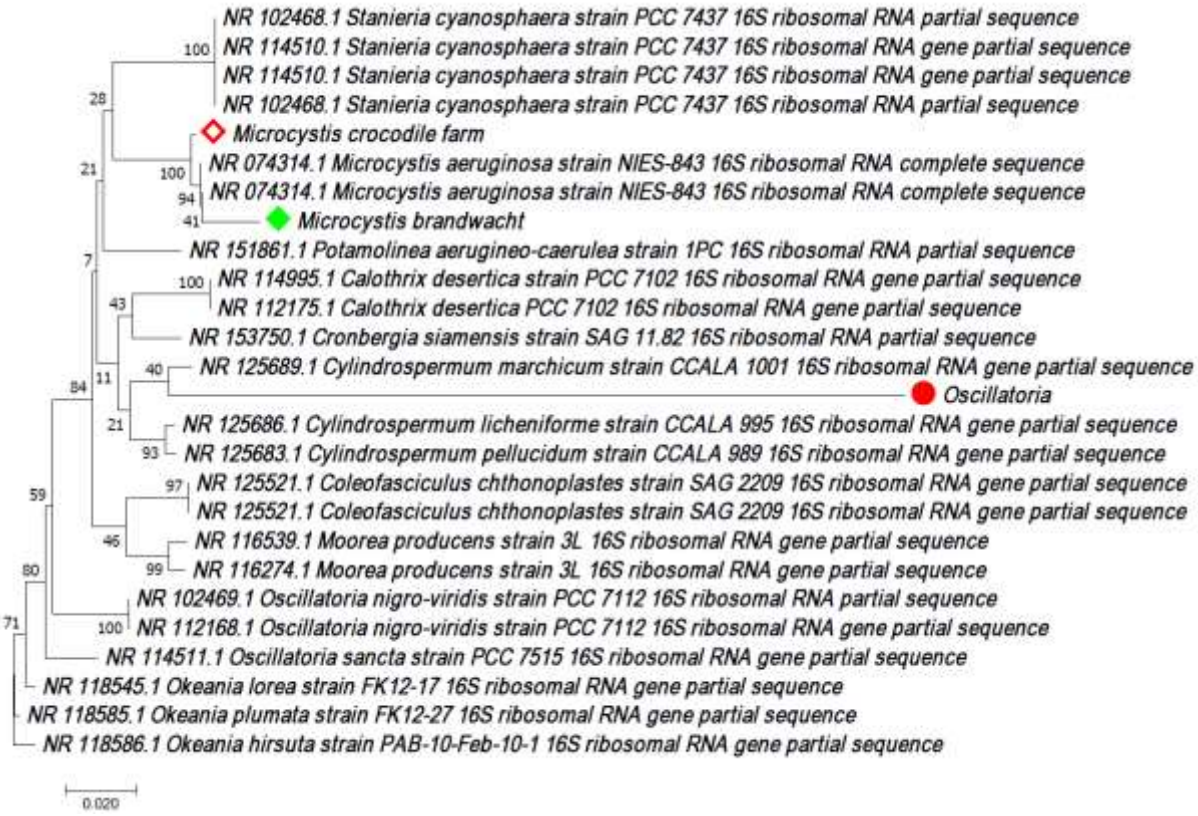


Figure 4.11: Phylogenetic neighbour joining tree of two cyanobacterial isolates *Microcystis* sp. and filamentous isolate isolated from Klippoortjie.

Figure 4.12 indicates the findings from DNA amplification using *Microcystis* specific 16srRNA primers. This indicates a similarity to *Microcystis wesenbergii* from the topology of the unknown *Microcystis* species, whilst comparison to the GenBank sequences from the cyanobacterial primer indicates a similarity to *Microcystis aeruginosa*. Further testing of the microcystin marker genes indicated that all markers were present from *mcy* A to *mcy* E marker genes. Table 4.4 indicates the findings of *Microcystis* sp. in comparison to *Oscillatoria* sp. There was no presence of the screened *mcy* marker

genes in *Oscillatoria*, or of the anatoxin marker genes (Rantala-Ylinen et al., 2011). *Microcystis* in contrast contained all the *mcy* genes with the exclusion of anatoxin genes.

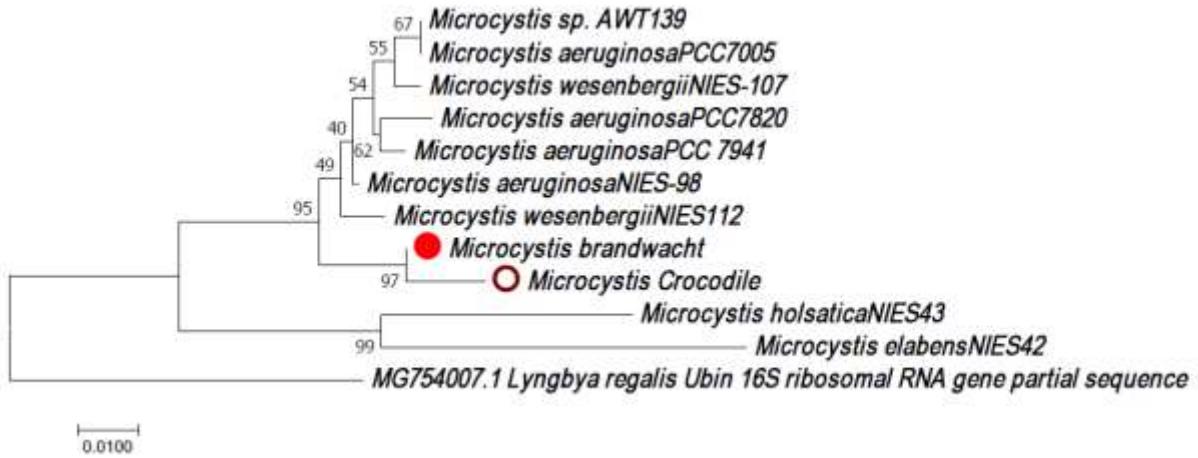


Figure 4.12: Phylogenetic neighbour joining tree of two cyanobacterial isolates *Microcystis* sp. with a *Microcystis* isolate collected from a crocodile farm dam compared to the isolate collected from Brandwacht wastewater plant, which is the focus in this study.

Table 4.4: Toxin marker gene presence in cyanobacterial isolates

Toxicity gene marker	*Microcystis*	*Oscillatoria*
mcy A	+	-
mcy B	+	-
mcy D	+	-
mcy E	+	-
an xgen	-	-
cgen	-	-
anaC	-	-

The lack of the anatoxin or microcystin genes in *Oscillatoria* sp may mean that this isolate produces other toxins such as saxitoxin or is a non-toxic variant of the isolate based on the data we have collected thus far, as the microscopic features indicate it belongs to the genus *Oscillatoria*. An interesting point however, is that the identification of these isolates is not always accurate based on the findings of sequence blasts on Genbank. The isolates may cluster with other quite distinct types of cyanobacteria or algae from freshwaters although their morphological and nutritional requirements indicate a completely different species or genus. For example, the universal 16S cyanobacterial primers used in this study clustered this filamentous isolate with the genus *Cylindrospermum*, which is also a

filamentous cyanobacterium; however, with a different morphology and the presence of a heterocyst in comparison to our current isolate, which is morphologically similar to *Oscillatoria* sp. Therefore, based on the study findings, there needs to be more accurate identification of wild isolates and accession numbers of these species deposited into our databases for more accurate matching and classification of unknown species especially with regards to freshwater cyanobacteria. *Microcystis* species are more commonly classified in comparison to the filamentous cyanobacteria.

4.4 Discussion

Bacterial growth curve and microscopy assessment

The assessment on growth substrates of bacterial isolates indicated that the bacterial isolated grew fastest on Tween 80 broth medium (Figure 4.3), which is not surprising as the surfactant broth/agar medium Tween 80 has growth enhancing capacity in various bacteria (Ando et al., 1959; Goto et al., 2003), it has been reported to enhance the availability of nutrients to microorganisms (Jacques et al., 1980). Tween 80 in 0.1 to 1% concentrations resulted in enhanced smoothness and shine in the morphology of *Mycobacterium paratuberculosis* colonies. Tween 80 has also been shown to enhance the lipase activity in bacterial isolates, which is a hydrolytic enzyme (Boekema et al., 2007). Tween 80 has also been indicated for protecting cells against adverse environmental conditions as well as nutrient depletion. It has also been used as a supplementary agent to enhance cell growth (Reitermayer et al., 2018). In addition, Tween 80 can increase the permeability of cell membranes thus enabling easier nutrient uptake as well as increased enzyme release (Reese and Maguire, 1969). Based on all these properties of Tween 80, the bacterial isolates grew best on this substrate. The characteristics that Tween 80 enhances are important in a biological control agent. Increased release of hydrolytic enzymes, increased growth rate and cell protection all aid in the competitive advantage of the potential control agents.

In the case of BG-11 (Figure 4.2), the medium is indicated for autotrophic cyanobacteria, with a limited carbon source. This could be the reason why the lowest growth rates for heterotrophic bacterial isolates were observed in this substrate. This indicates that BG-11 would therefore not be an ideal medium for culturing the bacterial isolates for subsequent exposure experiments, as they would be disadvantaged against the cyanobacterial isolates. Nutrient broth is a general growth medium for culturable bacterial isolates and all four bacteria showed good growth rates steady exponential growth on nutrient broth (Figure 4.1).

Gram staining is one of the fundamental identification methods in microbiology (Bartholomew and Mittwer, 1952; Biswas et al., 1970) and was conducted in this study to give an idea of what the unknown bacterial isolate characteristics were. Already, based on the Gram stain, motility and colony morphology, to assess which phyla the bacteria may possibly be from. The isolated bacteria were all Gram-negative, except for the *Bacillus*/*Lysinibacillus* related strain, which was Gram-positive and rod-shaped as expected. The colony morphology also presented as fluffy colonies, similar to other previous findings on the colonies within this genus (Di Franco et al., 2002).

Cyanobacterial growth curve and microscopy assessment

Cyanobacterial growth was measured primarily through chlorophyll-*a* and wet weight was taken randomly over time to confirm an increase in biomass. Chlorophyll *a* measurement is a common, feasible biomass method estimation (Porra et al., 1989). To confirm the viability of the cyanobacterial mixed cultures, the findings indicated that the exponential growth of isolates began at three days, is comparable to the findings of Bortoli et al., (2014) who indicated an exponential growth phase around three days for Brazilian *Microcystis* strains grown on different media, including BG-11, which was used in this study. Similar findings were observed for *Microcystis* cultures, reaching exponential growth at three days at temperatures between 25 and 30°C (You et al., 2018). Related findings were observed for *Oscillatoria* (Figure 4.6), although the exponential growth phase was from day 0. This therefore indicated that the laboratory conditions and growth media were suitable for maintaining the cultures viable for further experiments to be conducted.

Scanning electron micrographs of the unknown cyanobacterial isolates (Figure 4.7) indicated findings similar to other studies in terms of *Microcystis* (Gumbo et al., 2014; Gumbo and Cloete, 2011) cell structure. The observed culture in this study was colony-forming, with a mucous sheath as opposed to unicellular. Consistent cell morphology and structure were observed in *Oscillatoria* as previously reported by Venter et al., (2003), where an isolate of *Oscillatoria simplicissima* was described.

Molecular identification

In identification of bacterial isolates, the *Bacillus* isolate used for this research was identified as *Lysinibacillus* (Figure 4.9). Based on the topology of the tree, it appears that the isolate is related to the fusiformis group of *Lysinibacillus*. To obtain conclusive identification data, additional biochemical tests or DNA hybridization would be required as these two genera are very similar with slight distinguishing characteristics as *Lysinibacillus* was previously part of the *Bacillus* genus. Due to the species similarity of these genera, minimal distinctions can be noted, primarily from a cell membrane biogenesis and

metabolism (Xu et al., 2015). Thus, differences between these groups have to be determined further on a genomic level, beyond the basic identification methods described in this study. The similarity within the genus of *Bacillus* led to the recommendation that the use of methods such as DNA hybridization to improve on more distinctive characterization (te Giffel et al., 1997). Biochemical tests are useful in distinction between closely related species in this genus. Based on the suggested genus level similarity of 97% for the 16S rRNA gene (Drancourt et al., 2004), no conclusive genus assignment can be made from the similarities obtained for isolate B, except to ascertain that it is related to either the *Bacillus* or *Lysinibacillus* genera. Isolate 1 and 3w were more positively identified to genus level, with isolate 3w indicating one of the few reports of *Pseudomonas rhodesiae* as a predatory isolate against cyanobacteria. Isolate 3y is also related to *Pseudomonas* but cannot be conclusively assigned to species level.

In the case of cyanobacterial isolates, a lack of deposited sequences for more precise identification was observed. With most research being on type strains, other strains from the environment are not well characterized and more research is required particularly in the identification and differentiation of the filamentous isolates. The conservation of the 16srRNA region in bacterial isolates can cause difficulty in a proper distinction of a strain. Lyra et al. (2001) described the interspecies similarity in some strains of cyanobacteria; non-toxic *Aphanizomenon*, and toxin producing *Anabaena*. The species percentage similarity did not represent the varied morphologies of these different genera. The low E- (expect) values of these matches (Supplementary data) serve as confidence measure of sequence similarity, with a lower E-value indicating a higher likelihood and hence a higher confidence in isolate identification (Kerfeld and Scott, 2011). Therefore, additional RFLP methods were used to separate the cyanobacteria into more definitive clades. Microscopic characteristics of the filamentous cyanobacterium in our current study were valuable in being able to decipher their genus, as they lack heterocysts, unlike the genus they clustered closely to, *Cylindrospermum*. In addition, *Cylindrospermum* is from the taxonomic order *Nostocales*, which has been used as an indicator of low nutrient loads (Douterelo et al., 2004), whilst the filamentous isolate obtained in this study was from nutrient rich water at Klippoortjie Dam. From a molecular identification perspective, *Oscillatoria* needs to be further classified, whilst the *Microcystis* isolate from Brandwacht wastewater treatment plant showed a close grouping to another South African isolate from a crocodile farm dam, which indicates species similarity from a geographical perspective. The challenge in non-axenic cultures as obtained in this study is also the difficulty in having pure isolates with no other non-dominant isolates within the bloom subculture being amplified. Therefore, the amplification of *Oscillatoria* was particularly challenging, with difficulty in

obtaining longer, clean sequences from the mixed culture for more conclusive molecular identification. However, for the greater purpose of the study, the cultures were kept as non-axenic.

Table 4.4 indicates the presence of microcystin gene markers in the *Microcystis* isolate, indicating toxin producing capacity. These genes have been indicated in encoding the activation of certain amino acids in microcystin production; however the link to the *mcy B* gene and toxicity was variable, indicating that the activation of various other amino acids is involved in microcystin production. Moreover, strains of *Microcystis wesenbergii* did not have amplified DNA from the *mcy B* gene primers (Kurmayer et al., 2002). Another study by Bittencourt-Oliveira (2003) supported the presence of *mcy B* genes in potentially toxic cyanobacterial isolates and that this gene was responsible for encoding the variable amino acids on the microcystin structure. Reports on *mcy A* and *B* marker genes in a Brazilian study found the *mcy A* gene to have the greatest site variation and also indicated the diversity in this gene, thereby making it difficult to amplify in environmental samples with the primers generally however the presence of these genes showed a positive correlation to toxicity (Lorenzi et al., 2015). In the case *of Oscillatoria*, the primers chosen for the gene cluster of anatoxin *a*, which was previously found in a strain of *Oscillatoria* (Rantala-Ylinen et al., 2011) were not effective in screening for the toxin produced by the isolate, neither were the microcystin gene markers, although two previous studies indicated microcystin-like toxins produced by *Oscillatoria* strains (Bruno et al., 1992; Eriksson et al., 1988). More recent research through ELISA screening of toxins found the presence of microcystins in *Oscillatoria limnetica* which had formed a bloom in the Nile River (Mohamed, 2016). Other reports have indicated a variety of secondary metabolites which were unclassified, among which toxins such as homo-anatoxin a, anatoxin, cylindrospermopsin (Mejean et al., 2010), aplysiatoxin (Kaebernick and Neilan, 2001) and oscillatoxin were found (Nokura et al., 2017). The absence of *mcy* and anatoxin gene clusters for the *Oscillatoria* isolate in this study may indicate potential production of another form of cyanotoxin among those found in other studies. In the current study, microcystin and anatoxin were the main toxin gene clusters investigated based on previous reports as well as study feasibility.

Another point of interest in the bacterial isolates is that isolate 3w, belongs to the *Pseudomonas fluorescens* group, which are well known in bioremediation and degradation of pollutants (Kahng et al., 2002; Yoon et al., 2002). The identification of *Pseudomonas rhodesiae* specifically in this application is one of the few reports to our knowledge, with a 99% similarity to isolate 3w. This is a comparable genus of organisms to the well-known *Bacillus* / *Lysinibacillus* genus, which was used as a comparison in this study and where most of the bio degradative isolates and metabolites have been sourced previously (Cubitto et al., 2004; Guo et al., 2010; Sakthipriya et al., 2015).

The methodology applied in this research involves the use of the most feasible and acceptable identification methods. As the isolates were selected based on preliminary algicidal characteristics, no extensive identification was conducted. Further conclusive identification can be determined once the algicidal activity is confirmed to be valuable. From a more classical microbiology approach microscopic identification, basic light microscopy and Gram staining are fundamental methods of identification that allow distinction of unknown isolates based on their cell wall composition, motility and size. Their colony morphology and smell are among the basic characteristics described in the Bergey's manual of determinative bacteriology (Bergey and Holt, 1994) for genus or taxonomic assignment. Other methods that are useful in the determination bacterial identity include MALDI-TOF MS (matrix assisted laser desorption/ionisation time-of-flight mass spectrometry), which uses the peptide mass fingerprint of isolates for assignment to genus or species level based on mass spectrometry (Singhal et al., 2015), however present limitations similar to 16s rRNA sequencing, due to sequence similarity being assigned based on the isolates within the database and more research being in clinical isolates, in addition to the high cost of equipment. Another more common method of method identification that is more accurate is DNA-DNA hybridization. This method allows for determination of similarity between isolates for taxonomic purposes, with a drawback of greater concentrations of high quality DNA being required, as opposed to conventional PCR (Goris et al., 2007). In addition to more selective primers being used these would be among the methods considered for further conclusive identification of the isolates described in this thesis.

In closing, based on the methodology applied, the four bacterial isolates were assigned or showed close relation to *Pseudomonas*, *Lysinibacillus* and *Aeromonas* genera, which have been applied in bioremediation or linked to bloom occurrences. The addition of Tween 80 enhanced the growth rate of all four isolates. The cyanobacterial isolates were able to grow on BG-11 medium and reached the first exponential growth phase within 3 days. The unicellular cyanobacteria were identified as *Microcystis aeruginosa* from universal cyanobacterial primers and the filamentous cyanobacteria was assigned to the genus *Oscillatoria* from morphological characteristics, which are classified in this genus by the simple, oscillating trichome structure. This is presented by the absence of a sheath and ruler like divisions of the trichomes. The simplicity of the morphology and lack of unique features have led to the genus assignment *Oscillatoria* (Mühlsteinová et al., 2018), although it could not conclusively be assigned to genus level from molecular identification. Karan et al. (2017) stated that the ecology, morphology and molecular identification data must be in accord for conclusive identification of cyanobacteria although this is understood to not always be the case. A report by Keshari et al. (2015) also recommends the use of molecular, ecological and morphological data to characterize

cyanobacteria. Earlier in the study, efforts had been made to identify the cyanobacterial isolates with universal primers from Nübel et al. (1997) as well as Hodkinson and Lutzoni, (2009), however these did not work for the isolates in this study. The research by Karan et al. (2017) further described the morphology of *Cylindrospermum* and *Oscillatoria* isolates with figures. The structural differences between both isolates (*Cylindrospermum* and *Oscillatoria*) are quite distinct. The *Cylindrospermum* indicates a chain like morphology with the presence of heterocysts, which are not present in the filamentous isolate described in the thesis. Research published in 2018 (Rott et al., 2018) indicated the continued challenge in the use of the conserved 16s region and the combination of this molecular method with the morphological characteristics in cyanobacterial identification. The challenges described include the use of molecular identification being applied on pure cultures and therefore present a challenge when applied to environmental samples. This has led to difficulty in nomenclature of cyanobacterial isolates. As a result, there have been instances where no conclusive species assignment could be made due to differences in the phylogeny and cyanobacterial morphology. Therefore complementary identification from morphological assessment is recommended in identifying isolates (Rott et al., 2018), which was considered in this study for the identification of both the filamentous and unicellular cyanobacteria. More specific *Microcystis* 16s primers confirmed the genus of the isolates, with the species being *aeruginosa*, the commonly occurring, and toxin producing species within this genus. There is also scope to potentially conduct further work on the optimization of the bacterial control agents and their species assignment if the reduction in toxicity is successful.

4.5 References

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Chapter 5: A laboratory based exposure of Microcystis and Oscillatoria cyanobacterial isolates to heterotrophic bacteria

**Accepted for publication and in press (Toxicon)*

Declaration by the candidate

With regards to chapter 5, the nature and scope of my contribution was as follows

Nature of contribution	Extent of contribution
Conceptual design, experimental work, manuscript writing	70%

The following co-authors have contributed to chapter 5

Name	Email address and institutional affiliation	Nature of contribution	Extent of contribution
Prof JH van Wyk	jhvw@sun.ac.za Department of Botany and Zoology, Stellenbosch University	Conceptual design, experimental work, manuscript writing	30%
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Abstract

Biological control of cyanobacteria is a viable means of controlling nuisance bloom occurrences; however the majority of studies done are against *Microcystis* sp., with a commonly lytic effect caused. Filamentous cyanobacteria such as *Oscillatoria* are not as extensively studied in this area of biological control and are often part of *Microcystis* dominated blooms. This study employed heterotrophic bacterial isolates selected from bloom waters that indicated potential predatory behaviour against both filamentous and colony-forming cyanobacterial isolates. In comparison to a known *Bacillus* related isolate, which is often reported among bacterial control agents, three other bacteria isolates were tested as control agents against non-axenic *Oscillatoria* and *Microcystis* cyanobacterial cultures. Assessments of cyanobacterial cell responses to the bacteria were conducted through water chemistry, chlorophyll *a*, alkaline phosphatase activity, and microscopy, with cyanotoxin measurements. The changes in these parameters were compared to untreated cyanobacterial cultures where no bacteria were added. The study found that at cell ratios of bacteria half that of *Microcystis*, minimal changes in chlorophyll *a* were observed, whilst *Oscillatoria* showed a decreased chlorophyll *a* more in the presence of isolates 1 and 3w. The assessment of alkaline phosphatase activity showed decreased activity in both cyanobacterial isolates exposed to the bacteria, relative to the untreated control sample. Microscopy analysis through fluorescence indicated that the attachment of the bacteria to the surface of the cyanobacteria hampered with the fluorescence and scanning electron microscopy indicated that the cells were damaged by the addition of the bacterial isolates. Cyanotoxin detection through the ELISA kit testing indicated that there was toxin reduction in samples treated with the bacterial isolates, with the highest reduction being close to 60% in the case of *Microcystis* sp. treated with isolate 3w. Similar reductions were noted in the filamentous cyanobacterium *Oscillatoria*, in the presence of isolate 1. This was confirmed with HPLC measurements, which revealed the presence of more than one microcystin variant, with microcystin -LR and -RR quantified. The findings indicated degradation of the microcystin -LR congener than microcystin R-R for most isolates. Isolate 3w, a previously identified *Pseudomonas rhodesiae* isolate, was the most effective isolate from an intra and extracellular reduction for both *Microcystis* and *Oscillatoria*.

5.2 Introduction

The rise in harmful algal bloom occurrence and toxicity has led to numerous control strategies being developed to curb and or suppress their toxicity particularly. These blooms have resulted in lethal

numerous aquatic life incidences as well as recorded historical human deaths in decades passed (Campinas and Rosa, 2010). Among the strategies, the use of natural predators and enemies has been extensively researched. Biological control of the freshwater cyanobacteria *Microcystis* has been extensively documented in the past decades with numerous bacteria found to exhibit inhibitory effects against this cyanobacterium (Van Wichelen et al., 2016). In terms of microorganism control agents, its susceptibility has been recorded towards various fungal, viral and bacterial isolates (Demeke, 2016; Mayali and Azam, 2004; Verschuere et al., 2000). Other classes of cyanobacteria such as *Oscillatoriales* and similar filamentous algae have not been as extensively studied as *Microcystis* in particular, possibly due to the fact that *Microcystis* is the most cosmopolitan and often reported isolate in bloom occurrence as the dominant or co-dominant isolate (Harke et al., 2016; Kotut et al., 2010; Mowe et al., 2014; Paerl et al., 2014; Preece et al., 2017; Sitoki et al., 2012). Furthermore, other isolates such as *Cylindrospermopsis* sp. have also been assessed from a microbial inhibition and biological control perspective (Antunes et al., 2015; Flaherty et al., 2007). The majority of studies with this approach have employed laboratory grown strains which are often axenic, in synthetic medium, which is under controlled laboratory conditions (Ren et al., 2010; J. F. Su et al., 2016). These studies have made a valuable contribution towards the understanding of *Microcystis* isolate behaviour and ecology under various conditions and have enabled the sequencing and depositing of various cyanobacterial isolate genetic information for further research and use in databases such as NCBI. The work on non-axenic strains (Oberholster et al., 2009) has also assisted with the discovery of *Microcystis* isolate diversity under bloom conditions as well as the various dominant and co-dominant strains in a bloom. Among the challenges in this growing area of research is the lytic impact of these predatory isolates, which often leads to subsequent toxin release, which is counterproductive from a health impact due to water quality perspective. Due to this, earlier studies have recommended that this control method should be only under controlled laboratory conditions (Kim et al., 2008b). One of the few recent studies assessing toxin reduction from this approach was a report by Su et al. (2016), which reported toxin reduction in terms of *Microcystis* toxicity by a *Raoultella* sp. bacterium. Building on the potential impacts in a natural environment, this study assesses the impacts of biological control using heterotrophic isolates from bloom waters and how they impact non-axenic cyanobacteria which are colony-forming and filamentous. The primary aspect is to assess the enzymatic, microscopic and toxicity changes and thereby determines whether these control agents can be further applied outside of controlled laboratory conditions or whether they are as effective in a mixed population, which is typical of natural blooms. The value of this study adds to the assessment of live cells as a feasible control method or whether the metabolites of the predators are more effective than the live cell to cell interaction initiated by exposures.

5.2 Materials and methods

5.2.1 Collection and isolation of cyanobacteria and heterotrophic bacteria

Water containing a bloom of *Microcystis* sp. was collected from Brandwacht wastewater treatment works, in Mossel bay, Western Cape, South Africa (34° 3' 3.6" S, 22° 3' 28.8" E). Filamentous cyanobacteria were collected in Klippoortjie colliery mine waters (26° 07' 00" S; 29° 08' 00" E), near the town of Ogies, Mpumalanga, South Africa. Samples were collected in sterile water bottles and kept on ice during commutation. Isolates were identified by light microscopy at 400 x magnification (Zeiss Axioscop), using the procedures mentioned by Oberholster et al. (2009) and stored as non-axenic cultures at 4°C for the duration of the study.

Heterotrophic bacteria showing predatory activity against cyanobacteria were isolated from the water containing cyanobacterial isolates through the use of the plaque assay as mentioned in the study by Gumbo et al. (2010). Three isolates were randomly selected from the plaque assay, namely, isolate 1, 3w, and 3y. A *Bacillus* species was generously donated by the Microbiology department in Stellenbosch University and used as a reference. Isolates were then grown as pure cultures in Nutrient agar and nutrient broth medium (Merck) at 25°C.

5.2.2 Growth measurements

Cyanobacteria were grown in BG-11 broth medium in 100 ml volumes (Merck) over 28 days at 25°C in a 12 h:12 h light dark cycle, with light illumination of approximately 60 mmol photons (PAR) m^{-2} s^{-1} Chlorophyll *a* and wet weight measurements were taken every 3 days. Chlorophyll *a* was extracted with methanol and measured according to the methods of Porra et al. (1989).

Bacterial isolates were grown in 100 ml volumes of nutrient broth medium (Merck) and inoculated in Tween 80 broth medium overnight at 25°C (5 g peptone, 3 g meat extract, 10 ml Tween 80, 100 mg $CaCl_2.2H_2O$, 15 g agar per litre, pH 7.2). Master cultures were prepared with 80% of culture medium and 20% glycerol (Merck) and stored at -80°C.

5.2 Exposure experiments

5.3.1 Pre-growth of cyanobacterial and bacterial isolates

Oscillatoria sp. and *Microcystis* sp. were cultured in 1x BG-11 broth (Sigma-Aldrich) at 25°C in a 12 h:12 h light dark cycle, with light illumination of approximately 60 mmol photons (PAR) m^{-2} s^{-1}, for 3 days-7 days which is when they reached the exponential growth phase, based on chlorophyll *a* measurements.

Bacterial isolates 1, 3w, 3y and *Bacillus* were grown in Tween 80 broth over 8 hours at 25°C.

Non-axenic cultures of 0.1g (wet weight) of filamentous cyanobacteria and $1x10^6$ cells of *Microcystis* sp was added into 100ml sterile BG-11 medium and grown at 25°C. After 2-3 days, eight hour old bacterial culture cells grown in Tween 80 broth were counted with a bacterial counting chamber (Helber type, Marienfeld, Germany) at 400x magnification using a light microscope (Zeiss Axioskop). Cells were harvested by centrifugation at 10000 x g for 10 minutes centrifuged for 10 minutes at 10 000xg (Thermo Scientific SL 16R) and washed twice with 1x phosphate buffered saline (PBS) (Lonza).

5.3.2 Addition of bacterial isolates to cyanobacterial cultures

Washed bacterial cells were re-suspended in 1 ml of PBS and added to cyanobacterial cultures. Based on preliminary chlorophyll *a* determinations, 0.1 g of *Oscillatoria* yielded approximately 10x more chlorophyll *a*, compared to 1 million cells of *Microcystis* (wet weight of 0.01-0.03 g). Therefore, 10x more cells were added to the filamentous cultures. Cells were added in 1:2 ratios of heterotrophic bacteria: cyanobacteria and shaken briefly after addition (approximately 500 000 cells). The flasks were left at room temperature (25°C) for a four day period under static conditions. After 4 days, culture samples were vacuum filtered using the 0.22µm 250ml Steri-cup Express filters (Merck) to separate the cells from the culture medium. The residual cells as well as the filtrate were analysed for chlorophyll *a*, and microcystin concentration changes. Water chemistry, alkaline phosphatase activity and microscopic analyses of the samples were also conducted as described below.

5.3.3. Chlorophyll measurements

After four days, water samples were vacuum filtered using 0.22µm 250ml Steri-cup Express filters (Merck). The residual cells were used for chlorophyll *a* measurements, pigments were extracted with methanol according to the method of Porra et al. (1989).

5.3.4 Cyanotoxin detection- ELISA and HPLC

Total microcystins were measured in the residual cells as well as in the filtrate samples after four days using the Envirologix microcystin detection kit (Stargate Scientific, South Africa). Residual cells of the filamentous (*Oscillatoria*) and colony-forming (*Microcystis*) cyanobacteria were ground to a fine powder using liquid nitrogen and re-suspended to 1ml in distilled water. 0.22µm filtered sample waters were also assessed for microcystins according to the manufacturer's instructions. The student t-test was conducted on treated cyanobacterial samples in comparison to the control (untreated) cyanobacterial samples to determine whether there was any statistical significance in the toxicity measurement changes, using Microsoft Excel Stat 2010.™ p values ≤0.3 were assumed significant for the purposes of this study, due to the variability in toxicity measurements.

HPLC (high performance liquid chromatography) was performed according to the method by (Aguete et al., 2003), with slight modifications. Only filtered water samples were used for the HPLC analysis, to confirm whether extracellular toxins were in fact reduced after the addition of bacteria. Analysis was conducted with a HP 1050 Series Liquid Chromatograph equipped with a HP 1040M DAD UV detector (Hewlett Packard). Separations were performed in a LunaC18 column (150 x4.6mm mm) (Phenomenex, Torrance, CA) with a mobile phase consisting of water containing 0.05% (v/v) methanol in channel A and acetonitrile with 0.05% (v/v) methanol in channel B. Linear gradient elution started with 10/60% B for 20 min followed by a 5 min hold at 60% B with a flow rate of 1 ml min. Reference standards of microcystin -LR/-RR/-YR were used for the analysis (Sigma Aldrich).

Preliminary analysis of lipopeptides as a possible explanation for the bacterial mode of action was conducted by the Stellenbosch University Process Engineering unit on supernatant filtrate water, similar to the methods of Biniarz and Łukaszewicz (2017).

5.3.5 Alkaline phosphatase activity measurements

To determine changes in the metabolic activity of the residual cells from a stress response perspective, alkaline phosphatase activity was measured using the colorimetric assay kit (Bio-Vision) according to the manufacturer's instructions.

5.3.6 Water chemistry analyses

Water chemistry of samples was conducted using the Hach DR 3900 (Agua Africa) and powder pillows (Agua Africa) to measure the following parameters: Potassium, Nitrates, Phosphates, Potassium and Iron ($mg.l^{-1}$). The pH of samples was measured using the Hanna HI 991300 multi-meter (Hanna, USA).

5.3.7 Microscopy analysis

Light microscopy

Light microscopy was conducted on wet mounts of *Microcystis* sp. cells prior to and after exposure to bacteria isolates. Images were taken at 400 x magnification (Zeiss Axioskop) with ZenBlue (2012) imaging software and the Zeiss microscope imaging camera.

Fluorescence and scanning electron microscopy was conducted at the Stellenbosch University Central Analytical Facility (CAF) according to the methods described below.

Confocal scanning laser microscopy

Samples were imaged on Day 0 and Day 4 of exposure to bacterial isolates. Imaging was performed on the Carl Zeiss Confocal LSM 780 Elyra S1 with Super-Resolution Structured Illumination Microscopy (SR-SIM) platform by exploitation of the natural auto-fluorescence of the cyanobacteria and mixed

bacterial culture. Z-stacks were imaged at 2.5µm intervals with the 100x objective, with a 150µm pinhole. To detect the mixed bacterial culture, the 405nm laser used for excitation of auto fluorescence and emission wavelength range was 462-536nm. For the cyanobacteria, the 633nm laser used for excitation and emission wavelength range was 636-740nm. Images were processed and presented in a maximum intensity projection.

Scanning electron microscopy

Scanning electron microscopy was conducted on cyanobacterial cells prior to and after exposure to bacterial isolates, after four days. Samples were dried onto conductive carbon tape on 15mm aluminium stubs, sputter-coated with a thin layer of carbon using an Edwards S 150A sputter coater to enhance conductivity, and visualized using a Zeiss MERLIN Field Emission Scanning Electron Microscopy (FESEM) (Carl Zeiss Microscopy, Germany). For In Lens Secondary Electron (SE) detection, operating conditions of 5kV accelerating voltage with a probe current of 250pA and working distance 3.2mm were used to generate images using Smart SEM software. Imaging of cells was done between day one to four and cell surface changes were observed.

Unless indicated otherwise, all parameter measurements are a mean of four minimum experimental repeats.

5.3 Results

Non-axenic cultures of cyanobacteria were exposed to potentially predatory bacterial isolates, based on the plaque assay (Gumbo et al., 2010). Isolates 1, 3w, 3y and *Bacillus* sp. were added in 1:2 (heterotrophic bacteria: cyanobacteria) ratios to BG-11 media containing *Microcystis* sp. and *Oscillatoria* sp. cyanobacteria. The following results were obtained.

5.4.1 Water chemistry changes

The changes in water chemistry showed no significant deviation between the treated (bacteria added) vs non-treated samples (no bacteria added). All samples were at circum-neutral pH after the exposures, being almost identical with all the measured parameters deviating by 1-5% from the control. Not enough information could be drawn from the data that presented some differences. Based on this, no major deductions could be made on the water chemistry changes in the samples in the present study, however obtained measurements have been supplied in Supplementary data.

5.4.2 Chlorophyll *a* changes

Chlorophyll *a* is a widely used growth measure for chlorophyll producing microorganisms, usually indicating an increase in biomass (Boyer et al., 2009). To establish the impact of selected

heterotrophic bacteria (Isolates 1, 3w, 3y and B) had on targeted cyanobacteria, chlorophyll *a* changes were measured in cells of cyanobacteria treated with four different bacterial isolates after 4 days. Assuming the control samples were a 100%, changes in chlorophyll *a* were expressed relative to the control in percentage reduction. Based on the results in Figure 5.1, none of the isolates had growth promoting impacts on the cyanobacteria, with most of them resulting in a reduction of chlorophyll *a* relative to untreated control samples under the same conditions. Changes were measured based on chlorophyll *a* reduction percentages in comparison to the control. Standard deviations were obtained from chlorophyll *a* measurements over 6 experimental repeats. Figure 5.1 indicates minimal chlorophyll *a* reductions in *Microcystis* treatments, with a maximal reduction of 12% when treated with Isolate 1. Reductions were lowest in isolate 3w and B (*Bacillus*) with reductions of 5.9 and 6.1% respectively. Treatment of *Microcystis* with isolate 3y showed an 8.5% reduction of chlorophyll *a* after 4 days. *Oscillatoria*, however, showed the highest chlorophyll *a* reduction when treated with isolates 1 and 3y, reducing chlorophyll by 53 and 52% respectively. This is an indication of potential cyanobacterial stress from the presence of these specific isolates. Isolate B reduced chlorophyll *a* by 46%, whilst isolate 3w showed a reduction of 30%. Based on these changes in chlorophyll *a*, further investigation by means of microscopy was undertaken to determine cell stress (Figure 3-7).

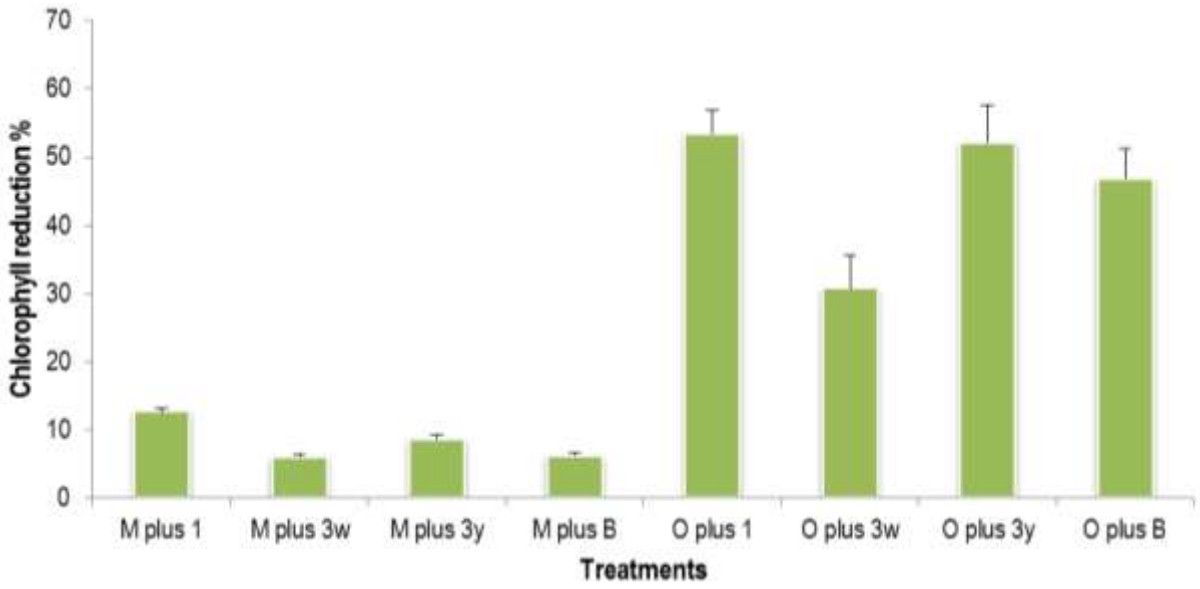


Figure 5.1: Chlorophyll *a* mean reduction percentages in *Microcystis* and *Oscillatoria* cyanobacterial isolates treated with four different bacterial isolates, relative to untreated cyanobacteria (control). Bars indicate one standard deviation.

5.4.3 Alkaline phosphatase activity measurements

Alkaline phosphatase is an integral metabolic enzyme that is associated with general cell functioning in numerous microorganisms and higher order organisms. To establish whether the chlorophyll *a* reduction observed in Figure 5.1 was linked to any cell functioning stress, the changes in alkaline phosphatase activity in treated vs non-treated cells were assessed after 96 hours. Figure 5.2 indicates that there was an overall decrease in alkaline phosphatase activity in *Oscillatoria* and *Microcystis* sp. cells that were treated with bacterial isolates. This may be an indication of inhibition or alterations in some cell function. These activity changes were expressed in percentages relative to healthy, untreated cells under the same conditions over a 96 hour period. Assuming the untreated cells had an activity of 100%, the changes are expressed relative to the high activity noted in the controls. Similar to the changes in chlorophyll *a* for *Oscillatoria*, isolate 1 and 3y resulted in the lowest alkaline phosphatase activity in comparison to the control. In the case of *Microcystis*, alkaline phosphatase activity was reduced by to 41-60% by all four isolates, with the lowest drop being in the treatment with isolate 3w.

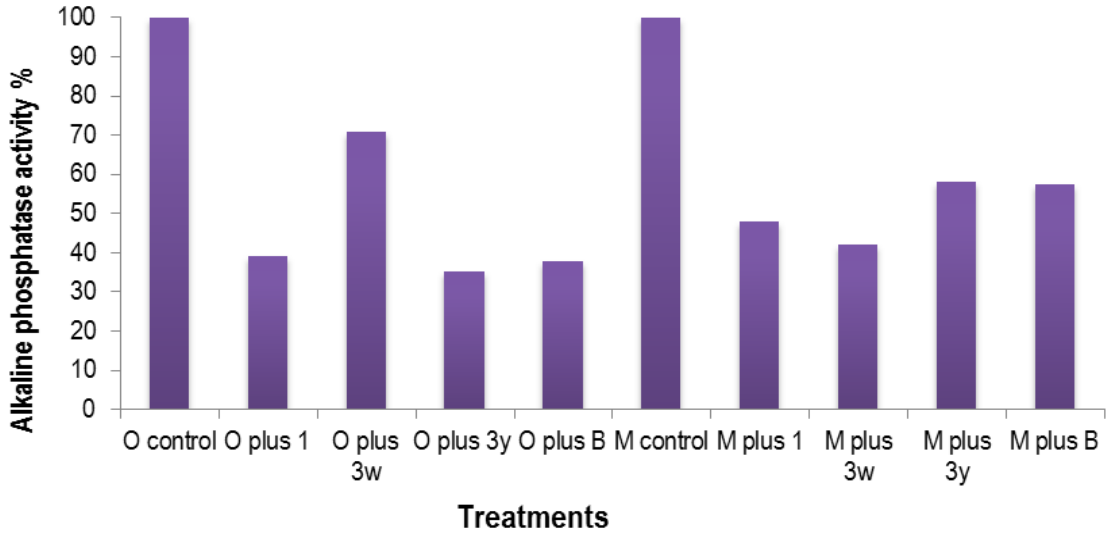


Figure 5.2: Alkaline phosphatase activity reductions in treated cells, relative to healthy, untreated control treatments.

5.4.4 Microscopy analysis

Microscopy analyses was conducted to determine whether any changes within the cyanobacterial cells could be observed and if they could corroborate the changes in chlorophyll *a* as well as the decreased alkaline phosphatase activity.

5.4.4.1 Light microscopy

Light microscopy images of treated and untreated *Microcystis* sp. were taken at 400 x magnification to compare any cellular changes visible after four days (Figure 5.3). *Microcystis* cells were used as a

representative sample to detect whether the trend was indicative of cell stress, seen in chlorophyll *a* and alkaline phosphatase could be observed as well through microscopy.This was also particularly to confirm any effects from the addition of bacteria, as the chlorophyll reduction in *Microcystis* was less pronounced in comparison to *Oscillatoria*. It was difficult to deduce any significant differences between the cells, except changes in pigmentation in unexposed cells in comparison to those exposed to isolate 3y (Figure 5.3 c), which appeared to have less chlorophyll pigmentation. Moreover, there was less detail within the cells, as though some organelles were no longer clearly visible.

In cells exposed to isolate B (Figure 5.3 b), there was more chlorophyll pigmentation; however the outer cell margin was not as clearly visible. Cells treated with isolate B actually appear healthier than the untreated healthy cells. Further imaging using fluorescence microscopy was done to determine whether the reduction in chlorophyll *a* noted in *Oscillatoria* and reduction of alkaline phosphatase activity in both cyanobacteria could be confirmed by a reduction in chlorophyll fluorescence. L

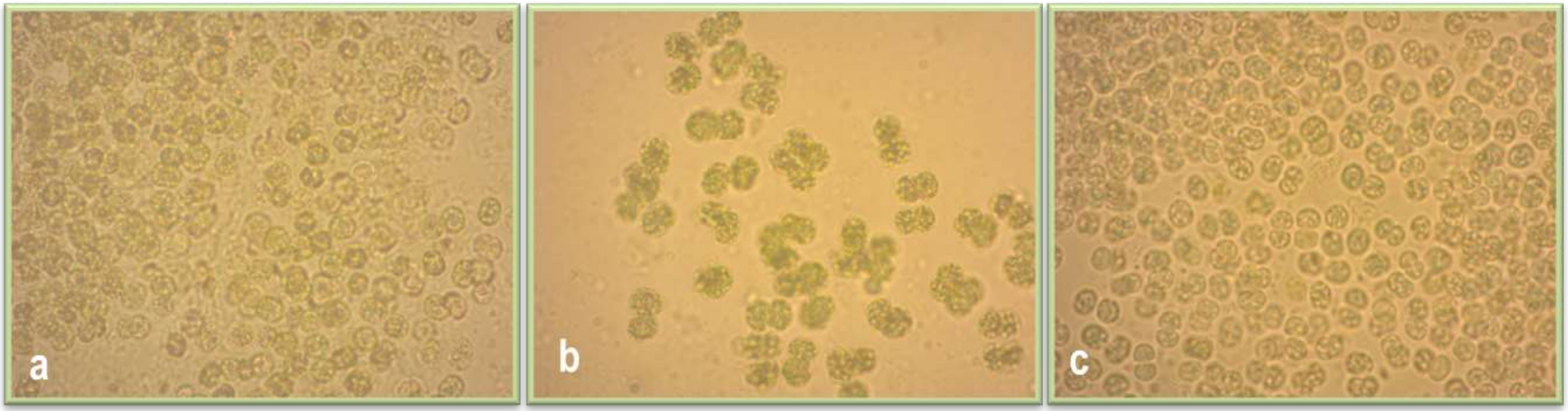


Figure 5.3: Light microscopy of *Microcystis* sp. cells (400 x). Untreated cells (a) were compared to cells treated with isolate B (b) and isolate 3y (c).

5.4.4.2 Confocal scanning laser microscopy

Imaging of samples prior to and after treatment with bacterial isolates was done under fluorescence microscopy to determine whether the changes in chlorophyll fluorescence could be observed based on the data from chlorophyll measurements and alkaline phosphatase measurements. These images were taken after four days of cyanobacterial exposure to heterotrophic bacterial isolates. Figure 5.4 shows the cyanobacteria fluorescing with a red pigmentation and the bacterial isolates around them as blue. Since the cultures employed in the study were non-axenic, other microorganisms were expected to be seen along with the cyanobacteria, as seen in the control sample (a) of Figure 5.4, where other bacterial isolates have formed a clump around the circular *Microcystis* cells and other rod and cocci shaped cells are seen around them. Figure 5.4b shows *Microcystis* cells surrounded by an aggregate of cells, where isolate 3y has been added. Fluorescence of the cells within the cell aggregates is slightly lower than that of the control cells, while the same is observed in Figure 5.4c, where isolate B was added. The change in fluorescence overall appeared to be due to the bacterial cells covering the cyanobacteria, thereby possibly impeding the fluorescence. This is better seen in Figure 5.4c, where the cells are almost entirely covered by heterotrophic bacteria. This observation is not new as bacterial cells are known to form these aggregates around cyanobacteria (Cai et al., 2014), composed of various bacterial organisms from different phyla. What is surprising, however, is the level of attachment or aggregate formation in the image where isolate B has been added, in comparison to the control sample. If in fact the bacterial response is predatory, then the attachment of the cells to the degree seen in Figure 5.4c, could indicate what has previously been noted in literature pertaining to the need for contact time and attachment to the cell by some algicidal bacteria to lyse or stress the targeted algae (Lenneman et al., 2014). The level of attachment is also greater in Figure 5.4b, compared to 5.4a, which is the control sample. The indistinct fluorescence changes between the treatments and the controls in image 4 are consistent with the findings of the chlorophyll measurements for *Microcystis* sp. This supports the use of flow cytometry or other cell stress measurements as an alternative. Gumbo et al. (2014) found flow cytometry useful in the viability measurements of *Microcystis* sp. exposed to predatory *Bacillus mycoides*. Due to the fact that some cells are still alive, it appears that unless the cells are completely destroyed, the measurement of chlorophyll in this study did not yield a strong distinction between treated and non-treated cells. This indicates that the cells are not stressed to the point of hindering chlorophyll production or that the stress response cannot be well quantified using chlorophyll measurements.

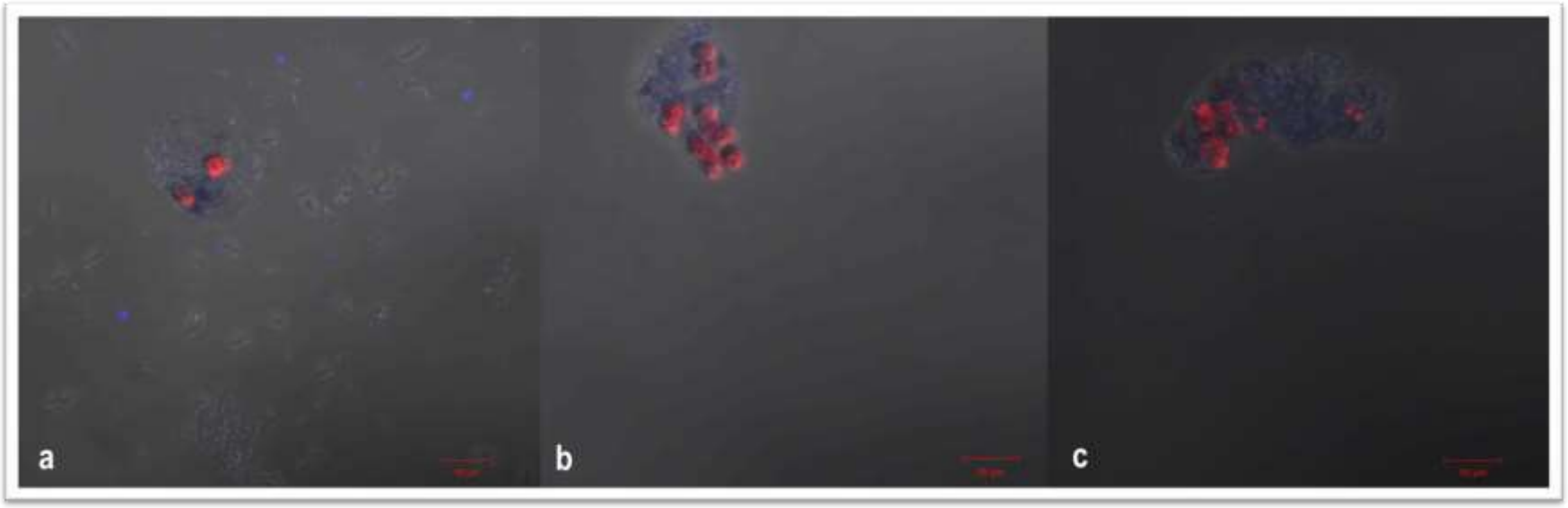


Figure 5.4: Fluorescence microscopy of *Microcystis* sp. Untreated (a) and treated with isolate 3y (b) and isolate B (c) after four days exposure at room temperature (25°C).

Visual observation of *Oscillatoria* sp. fluorescence indicated that certain parts of the filaments were not fluorescing as brightly in comparison to the control sample (Figure 5.4a). Moreover, bacterial isolates were observed around and on the filaments of the cyanobacteria. The change in fluorescence indicated why there may be reductions in chlorophyll, with less fluorescence observed in filaments treated with bacterial isolates. The treated filaments with isolate 1 (Figure 5.4b) and isolate B (Figure 5.4c) both showed a reduction in fluorescence, which were the two isolates that resulted in reduced chlorophyll measurements in *Oscillatoria*.

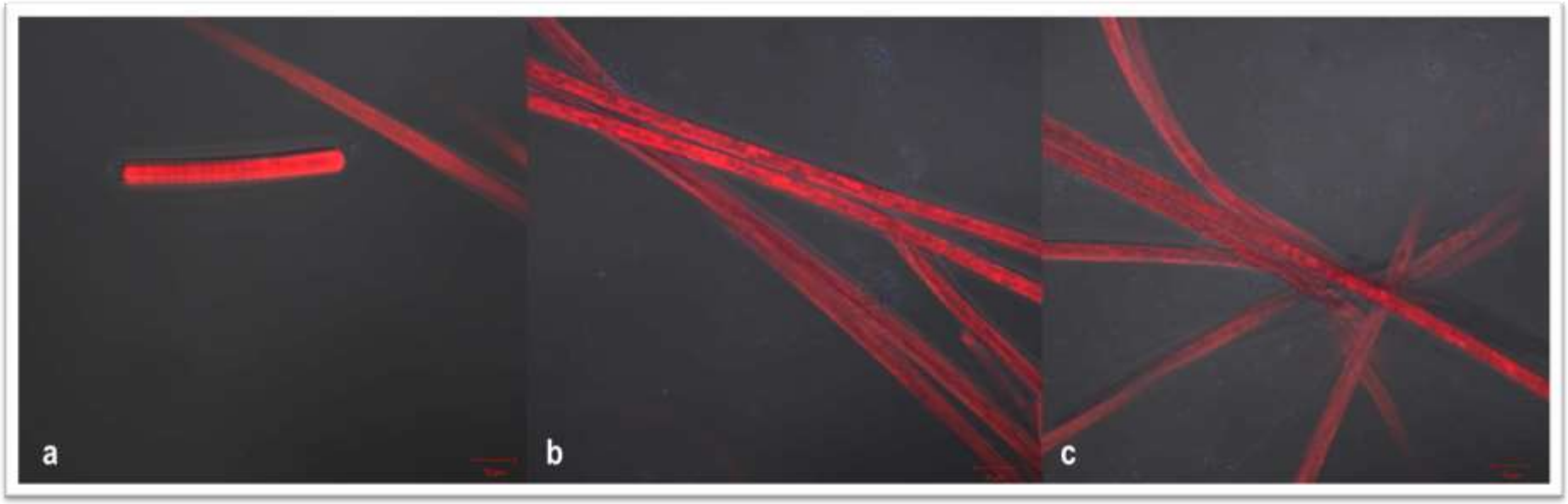


Figure 5.5: Fluorescence microscopy of *Oscillatoria* sp. untreated filaments (a) and filaments treated with isolate 1(b) and isolate B(c).

5.4.4.3 Scanning electron microscopy

Due to the lack of distinction in cell changes from light and fluorescence microscopy, further imaging was conducted using a scanning electron microscope, to determine whether there were changes in the cell surface brought about by the presence of the heterotrophic bacterial isolates. Isolate 3y was used in comparison to untreated cells to observe the changes in the cells. The appearance of the *Microcystis* cells changed over the four days, with the cells appearing fewer and "deflated." This observation is similar to that reported by Gumbo and Cloete (2011), with the cell surface changing from smooth and rounded with the mucilage sheath present, to single, fewer cells with an irregular and uneven cell surface with an irregular shape (Figure 5.6).

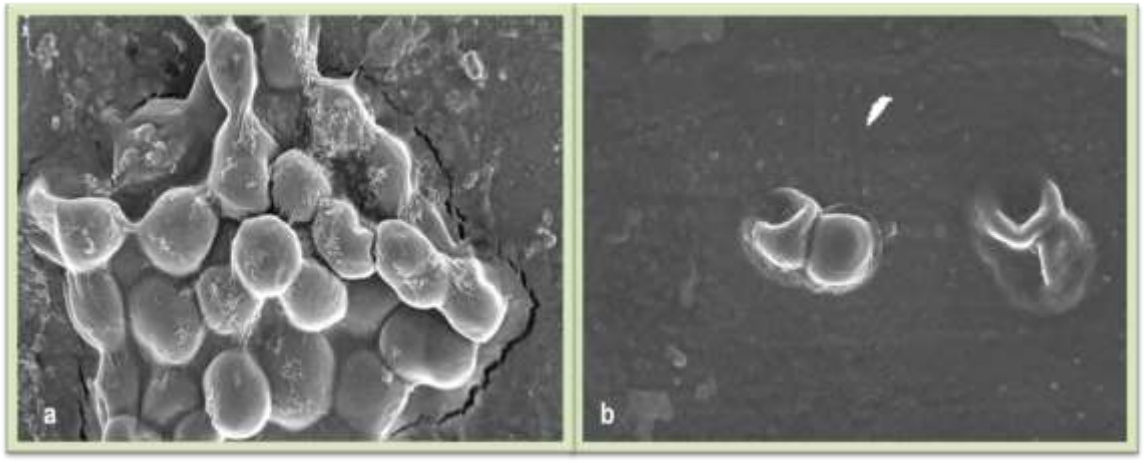


Figure 5.6: Scanning electron micrograph of *Microcystis* sp. at 5000 x magnification. On the left (a) is a group of healthy *Microcystis* sp. cells covered by a mucous sheath. On the right (b) are *Microcystis* sp. cells exposed to isolate 3y after a 4 day period.

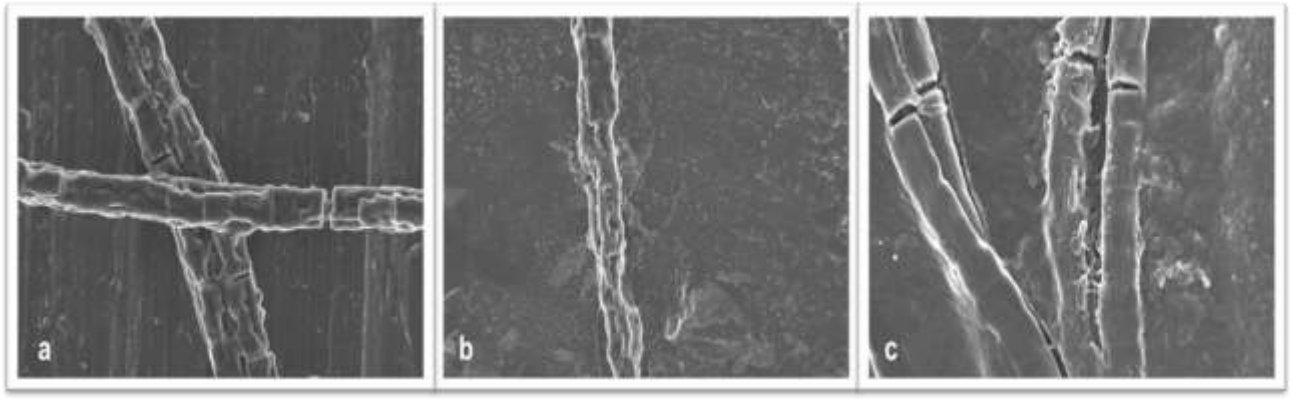


Figure 5.7: Scanning electron micrograph of Oscillatoria sp. filaments at 3000 x magnification. On the left, (a) is healthy *Oscillatoria* filaments. The middle (b) shows *Oscillatoria* exposed to isolate 3y after 4 days and the right (c) shows *Oscillatoria* exposed to isolate B. The images b and c show cells attached in

aggregates over the filaments of *Oscillatoria*, with the filament appearing disintegrated in (b). Bacillus shaped cells can be observed in (c) attached to the filament and the bacterial cells are producing extracellular projections onto the surface of the filaments. This could be a snapshot of the bacterial exopolysaccharides they produce against a target organism.

5.4.5. Cyanotoxin detection using ELISA antibody assay

One of the important aspects in the mitigation of cyanobacteria is the release of toxins, particularly during cell lysis. Therefore the analysis of toxicity is essential to note when applying biological control. Recent findings of reduced toxicity by Su et al. (2016) from the addition of a biological control agent *Raoultella* sp., indicated approximately 92% reductions of microcystin -LR. The ELISA kit is known as cross-reactive, with a detection of microcystin LR, -RR and -YR variants as well as nodularin. In the case of *Oscillatoria*, the detection may have been a saxitoxin as these species are known to produce these toxins as well as anatoxin-*a*. Also in *Oscillatoria*, the measured toxin was generally low (less than 1µg.ml^{-1}), with the water samples having toxins below measurable range. The cell lysate as well as water samples were analysed for changes in microcystin content after a 4 day exposure. The student t-test was conducted on treated samples relative to the control sample showed extracellular toxin reduction in the water samples more than intracellularly (Figure 5.8). No statistical significance was noted in isolate 1 ($p \leq 0.71$), with an overall toxicity reduction of 2.9% in water samples. Isolate 3y and B ($p \leq 0.16$; $p \leq 0.19$) showed average toxicity reductions of 63 and 52% respectively over a series of experiments. Toxin reduction in isolate 3w ($p \leq 0.07$) was an average of 59.8% in the water samples. Intracellular toxin measurements from the live cell lysates showed no statistical significance when comparing treated samples to the control, with $p \leq 0.55$ and higher. Figure 5.8 is a graphical representation of these findings, with very slight increases in intracellular toxicity, from 2 to 11% at the most. Negative reductions indicate increased toxicity relative to the control sample.

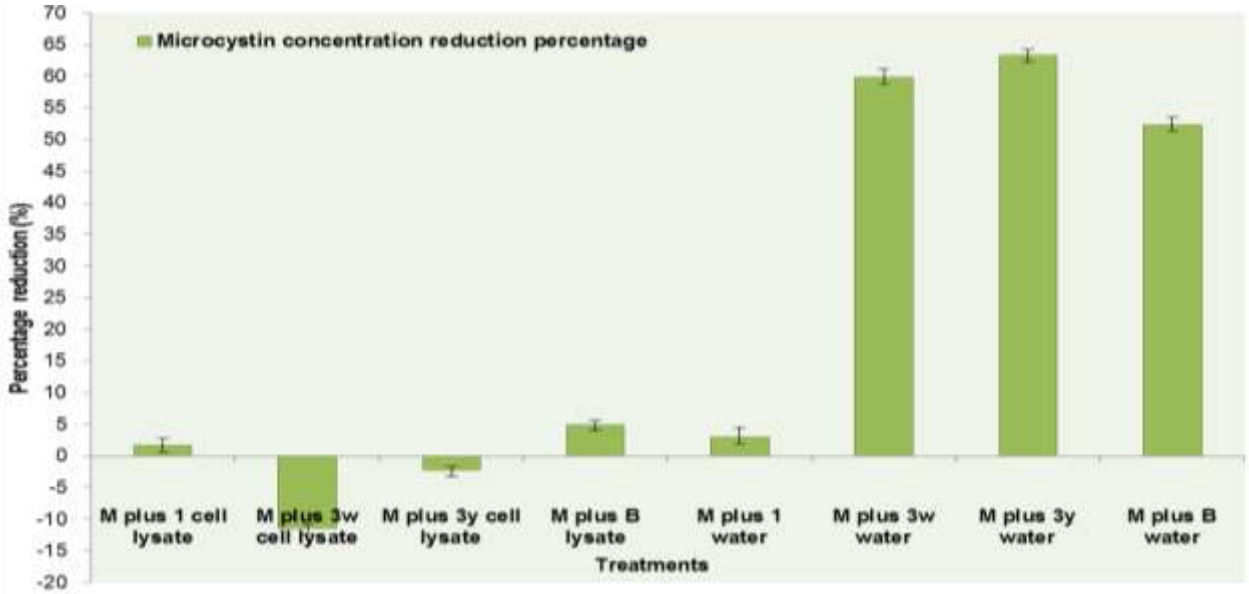


Figure 5.8: Microcystin reduction percentages in *Microcystis* sp. cells treated with different bacterial isolates after a four day period at ambient temperature. Isolate 3y and 3 have the highest reduction in extracellular toxin concentrations within the water whilst minimal changes in intracellular toxicity were observed. Bars indicate one standard deviation.

Observations of changes in the filamentous cyanobacteria *Oscillatoria* indicated that there was approximately 30-35% reduction in cyanotoxins intracellularly in the presence of isolate 1 and 3w ($p \leq 0.29$) (Figure 5.9). In the case of extracellular toxin measurement, there was a 16% reduction of cyanotoxins in the presence from isolate 1 ($p \leq 0.28$) there was no statistical significance ($p \leq 0.3$) in comparing the other treatments to the control, with a toxin reduction of 6-13% ($p \leq 0.58$). This indicates that the isolates interact with the cyanobacteria in different ways and that the ideal isolate for toxin reduction in *Microcystis*, a colony-forming, buoyant isolate, may not work against a filamentous cyanobacterium such as *Oscillatoria*.

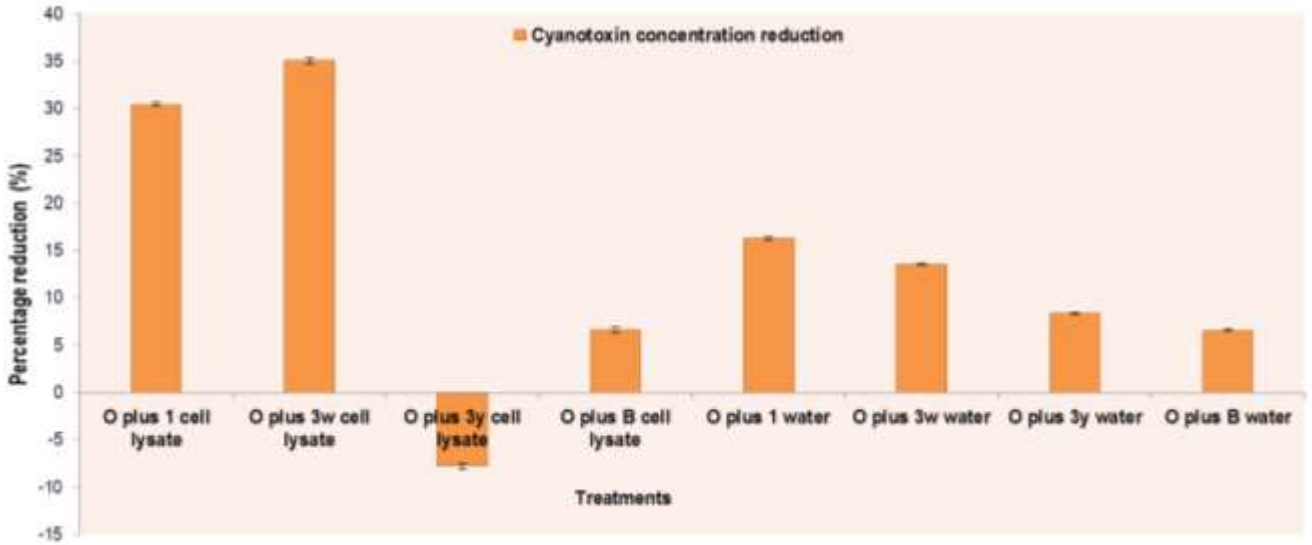


Figure 5.9: Cyanotoxin reduction intracellularly and extracellularly in *Oscillatoria* sp. after exposure to four heterotrophic bacteria after a four day period at ambient temperature. Bars indicate standard deviation. Intracellular toxicity was more effectively reduced than the extracellular toxin for this particular isolate, indicating it may be of a different conformation in comparison to that of *Microcystis* sp., which was more effectively reduced in the presence of isolate 3y.

To confirm whether the isolates were potentially microcystin degraders, they were added to BG-11 media containing known concentrations of microcystins and observed over four days. In samples supplemented with 1% of glucose, the addition of isolate 3y showed microcystin reduction by 17% after 3 days, with isolate B causing an 11% reduction in the same time frame. In media without an additional carbon source, reduction was minimal to non-existent. This indicates that the isolates may be potential microcystin degraders or that the reduction in toxins in water samples where they have been added is due to interactions with the live cyanobacterial cells.

HPLC analysis findings

Based on the overall ELISA analysis, the fluctuation in the toxin reduction for water samples of cyanobacteria treated with individual bacterial isolates was further assessed with HPLC. This enabled quantification of individual toxins at very low concentrations. Confirmation of these findings ultimately indicate that the extracellular toxicity in the water reduced by treatment with bacteria. Similar findings of the retention time of the toxins were found in this study, as compared to that of Aguete et al (2003), with the microcystins having an approximate 14 minute retention time. As a representative sample, Figure 5.10 indicates the peaks and quantification of microcystin -RR and -LR in the control *Microcystis* sample and a *Microcystis* sample treated with isolate B.

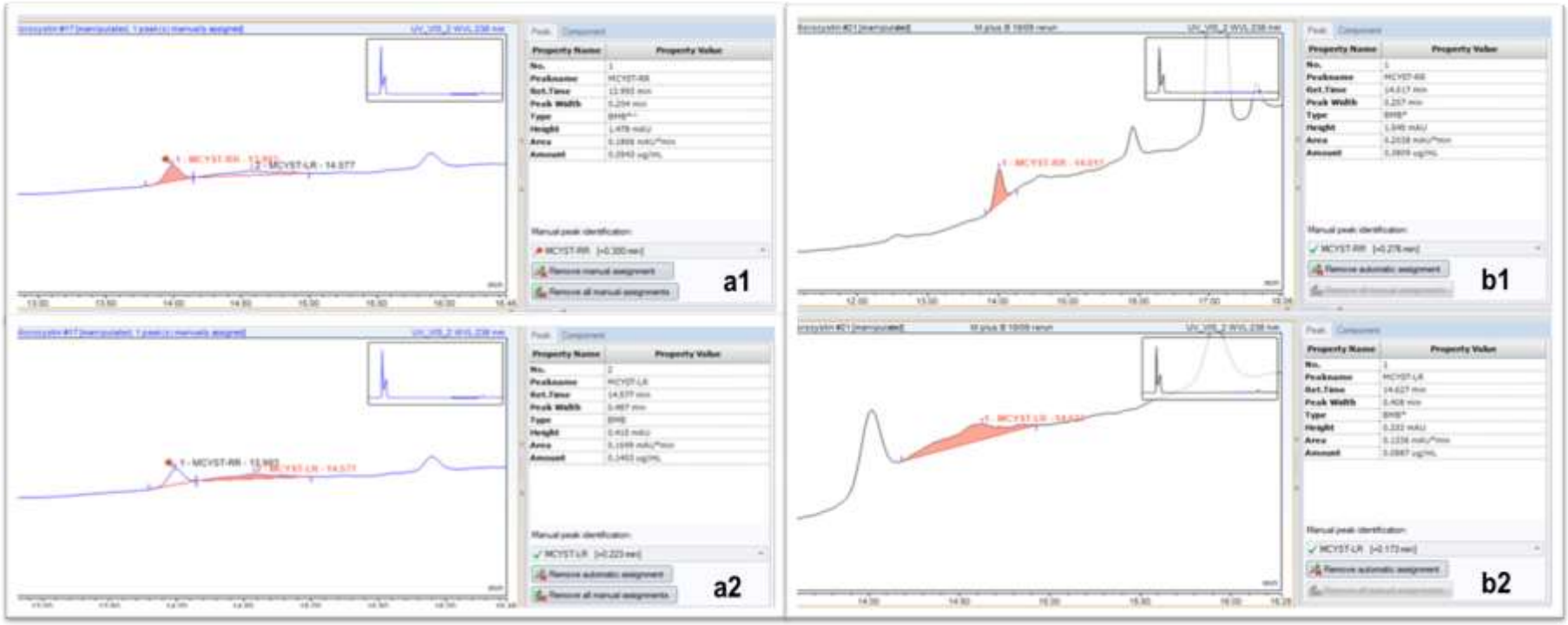


Figure 5.10: An example of the measured microcystin LR and -RR in the *Microcystis* control sample (a1-microcystin -RR, a2- microcystin -LR), compared to the measured concentrations in a water sample from *Microcystis* treated with isolate B (a1-microcystin-RR, a2- microcystin-LR).

Microcystin LR concentrations were reduced by 37%, whilst microcystin RR increased by 7%. A similar trend was observed in all the isolates except for isolate 3y, where both toxins were reduced. The isolates degraded microcystin LR variants, with no change or a slight increase in microcystin RR concentrations. Other peaks were visualized, which were not quantifiable. This could indicate the presence of other toxins or degradation by products of microcystin LR.

Table 5.1 shows the average reduction as per the quantifications exemplified in figure 10, over exposures.

Table 5.1: The average change in *Microcystis* and *Oscillatoria* microcystins LR and RR in samples treated with bacterial isolates, relative to the control.

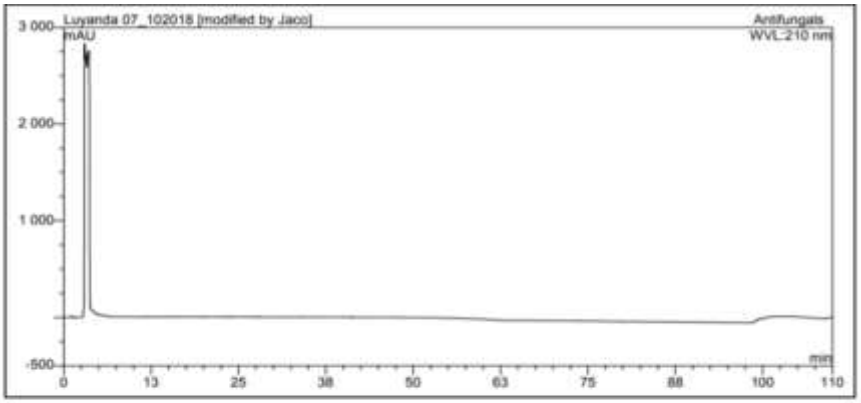
Isolate	Microcystin LR	Microcystin RR
Mplus1	26.98	-11.68
Mplus 3w	52.45	-15.18
Mplus 3y	13.39	6.97
M plus B	45.83	-11.92
O plus 1	17.77	-11.02
O plus 3w	41.17	-2.03
O plus 3y	46.98	-4.32
O plus B	57.4	-2.9

*Negative numbers indicate an increase in toxin concentration relative to the control.

The HPLC findings indicate the uniform increase in microcystin R-R, whilst all the isolates indicate a capacity to reduce microcystin LR. The reduction is relative to an average concentration of 0.12 µg.mL^{-1} of microcystin LR and 0.4 µg.ml^{-1} microcystin RR in the *Microcystis* control sample. For *Oscillatoria*, the average microcystin -LR was 0.13 µg.mL^{-1} of microcystin LR, whilst microcystin -RR was 0.38 µg.mL^{-1}. In Comparing this data to the ELISA findings in figures 8 and 9, it appears the toxin changes may have been under-estimated for isolates 3w, 3y and B in the case of *Oscillatoria*, whilst the ELISA findings over-estimated the microcystin reduction in the treatment of *Microcystis* with isolate 3y, possibly due to both congeners of microcystin being reduced by the presence of this isolate, although at lower amounts in comparison to the other isolates. The cross-reactivity of the ELISA does not allow individual quantification of cyanotoxin variants, hence the difference in findings between the two methods. The HPLC method was sensitive even to two decimal concentrations in µg.mL^{-1}, whilst the ELISA limit of detection is 0.2 µg.mL^{-1}.

Preliminary mode of action analysis

Culture supernatants were assessed for lipopeptides. This is a known antimicrobial mode of action that bacterial isolates such as *Bacillus* and *Pseudomonas* exhibit. Preliminary testing of *Microcystis* exposed to isolates B, 3w and 3y indicated there were no similar lipopeptides detected. Figure 5.11 indicates the absence of peaks in the screening for lipopeptides in one of the samples treated with isolate 3w.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount g/L	Type
Total:			0,000	0,000	0,00	0,000	

Figure 5.11: Absence of lipopeptides in sample treated with isolate 3w, a *Pseudomonas rhodesiae* isolate. This was a representation of three samples tested for a preliminary explanation of the mode of action employed by the bacterial isolates against the cyanobacteria.

5.5 Discussion

Reports of algicidal bacteria and biological control using this method have been well researched and documented over the decades (Demeke, 2016; Mayali and Azam, 2004; Sigee et al., 1999). An assessment of control strategies of *Karena brevis* blooms found that required bacterial densities would be required at about $1x10^6$ cells per ml, which would be viable at industrial levels (Sengco, 2009). In comparison to this study, the cell densities were $5x10^6$ per 100ml ($5x10^4$ cells per ml) at the most, which is lower than what has previously been reported in most literature. The reduced number of cells would therefore mean that mass culturing of a lower number of cells would be required and therefore lower costs. Furthermore, the ability of the selected isolates in this study to utilize Tween 80 as a substrate indicates the presence of lipase enzymes. This could mean the use of potential waste substances such as partially hydrolysed crude oil as a growth medium, which is feasible. The recommendations for up-scaling of this technology by Köhl et al., (2011) as well as Lievense and Crater (2018) have been followed in this study, with the next step being a pilot study or further study into the mode of action. Initial screening of the isolates has followed low-cost and feasible time frame approaches for screening and testing, with further research indicated for isolates presenting the desired characteristics. Bacterial isolates would be grown in fermenters or bioreactors and would either be freeze-dried or applied sprayed in liquid form over the affected water. The potential areas for this type of control would be in dams or areas where water is required for use but needs to be treated to safer standards prior to use within limited time constraints.

Pilot scales are necessary to validate the potential of further up-scaling this technology however the application is not always straight forward. A review of biological control from the fruit industry (Korsten and Bornman, 2004) indicates the space for application of biological control interventions as well as the continual challenge in understanding the mode of action of the antagonist. This is due to the complexity in microbial ecosystems. Moreover, the sustainability of commercializing a control agent is a critical area. Predictability of the control agent performance as well as its consistent performance in up-scaled conditions is reported to be an area often overlooked by researchers. In a similar review of up-scaled "green" technologies, Rahimnejad et al. (2015) assessed microbial fuel cells in bioelectricity generation. Although a different industry to that of Korsten and Bornman (2004) similar concerns arise in the application of bacterial cells, such as power output and low temperatures being a limitation for microbial cell functioning. In addition, these innovations need to be implemented responsibly (McLeod et al., 2017). From a critical perspective, it is appreciated that there are limitations and challenges in innovations involving living cells and sustainability in commercialization. However, the proposed benefits far outweigh some of the limitations which cannot always be foreseen prior to a pilot scale study. Therefore, sufficient scope exists in this area of study, especially since the viability of biological control has been established in the fruit industry for example.

The biological control principle has commonly been applied on axenic strains, with generally 1:1 ratios of the control agent and cyanobacteria, over a period of up to 7 days (Fraleigh and Burnham, 1988; Gumbo and Cloete, 2011; Tian et al., 2007; Zhang et al., 2016). The present study used a lower ratio of bacterial to cyanobacterial cells, essentially 1:0.5 ratios of cyanobacteria to bacteria. This was done to determine whether lower bacterial cell numbers (essentially half the recommended effective concentration) could be effective in eliciting cyanobacterial cell stress and more importantly whether the selected isolates could reduce toxicity at this ratio, with possible future application in trials at a larger scale. The reason for lower numbers being an exploration of the principle that microcystin degrading bacteria cause a shift in toxic to non-toxic populations of *Microcystis* under bloom conditions, as reported by Zhu et al. (2014). Therefore, if the numbers are lower than those recommended for cyanolytic effect, could there be a toxicity shift observed in live cells in the presence of the bacterial control agents? Moreover, could the use of Tween 80 as a carbon source contribute to the effectiveness of the biological control agents?

The observation time of four days was selected on the basis that light degradation of microcystins can occur within a five day period (Schmidt et al., 2014) isolates such as *Sphingopyxis* sp. can degrade 25% of microcystin L-A and 17% of microcystin LR in a 24 hour period (Ho et al., 2007; Schmidt et al.,

2014). This indicated that a four day period may be sufficient in observing the changes brought about by the potential control agents if they were effective in reducing toxin concentrations.

The selected cyanobacteria were used to assess whether the bacteria with predatory capacity could be effective against filamentous and colony-forming cyanobacteria. In freshwater studies, the most commonly reported biological control agents are against the cyanobacterium *Microcystis aeruginosa*, which can be expected, as it is the most cosmopolitan species in bloom occurrences reported (Mowe et al., 2014; Paerl et al., 2016; Preece et al., 2017). However, it is not always possible to know how another species in a potentially mixed population bloom will react to a particular control agent that is effective against a known strain. In the present study the additions of the selected isolates were able to cause stress responses at low populations to the targeted cyanobacteria in a non-axenic environment.

Water chemistry measurements

Significant water chemistry changes were not observed in the variables measured (Supplementary Data). This could be due to the shorter observation time and more importantly the low microbial numbers added, which did not have a significant effect on the pH or the parameters selected. The selected parameters were chosen based on literature recommendations. For example, Low TN: TP ratios are normally favourable of bloom conditions (Ndlela et al., 2016), whilst potassium concentration has been linked to inhibition of cyanobacterial growth, as well as iron being a required nutrient for cyanobacterial growth. The lack of a distinctive trend of information in the measurements of these parameters could not assist in making a clearer deduction of the impacts of water chemistry. Perhaps further investigation of more parameters at a lower range of detection or over a longer time period may yield more useful information.

Chlorophyll a changes

In this study, the chlorophyll measurements were more indicative of stress in the case of the filamentous cyanobacterium *Oscillatoria*, based on the reduction of chlorophyll in accordance to the addition of bacteria. *Oscillatoria* produced higher chlorophyll concentrations than the *Microcystis* cyanobacterium. The initial findings indicated that the addition of the biological control agents had a greater impact on the filamentous cyanobacterium, whilst there was minimal impact on the pigment formation on *Microcystis*. Furthermore, isolate 1, 3w and B caused the greatest chlorophyll *a* reduction in the filamentous cyanobacterium, *Oscillatoria*.

Alkaline phosphatase measurements

Alkaline phosphatase activity measurement of the cell lysates were done as a preliminary measure of stress from the cells, to further evaluate what the impacts of the bacterial isolates were, relative to the

control culture, without bacteria added to it. However, the findings in earlier literature give a mixed response. Toxic strains of cyanobacteria have had alkaline phosphatase activity decreased in the presence of stressors such as nonylphenol (Wang et al., 2006). The same study found that other toxic and non-toxic strains had a higher alkaline phosphatase activity in the presence of this endocrine disruptor. Although alkaline phosphatase has been used as a measure of biomass and cell functioning, similar findings were also reported in a study by Holland and Kinnear (2013), where toxic cyanobacteria have made use of alkaline phosphatases from other organisms and thereby showed a lower alkaline phosphatase activity. With these findings indicating mixed possibilities, it is possible that the lowered alkaline phosphatase activity in the cyanobacteria from this study is not fully indicative of a stress response. Being cognisant of the low bacterial ratios used, this may well be the case.

Microscopy analysis

The microscopy observation of *Microcystis* sp. is the first indication of cell stress as seen in Figure 5.6. The deflation of the cells and their appearance as fewer aggregates has been reported by Gumbo and Cloete (2011), citing a deflated and rougher cell surface appearance in *Microcystis* exposed to a *Bacillus* isolate. The attachment of the bacterial cells to the cyanobacteria revealed by fluorescent microscopy (Figure 5.4) suggested that the mode of action in the bacterial isolates may be direct contact, which involves the attachment of the predator to the target cyanobacterium. This attachment impedes the fluorescence of the cyanobacterial cells. In the case of *Oscillatoria*, the change in chlorophyll is consistent with the reduced fluorescence caused by the same bacterial isolates. This confirms that the chlorophyll production is affected by the addition of the bacterial cells and they are visibly surrounding the filaments. Figure 5.7 also shows the disintegration and deflation of the *Oscillatoria* filament upon exposure to isolate 3y and the attachment of the cells when exposed to isolate 3y and B. This is suggests cell damage and direct attachment of the cells to the targeted cyanobacterium. Further investigation is however, required to determine the extracellular compound being produced by isolate B as it attaches to the filaments of *Oscillatoria*.

Cyanotoxin detection

The use of the ELISA plate for the detection of microcystins works on the principle of the antibody binding from the ADDA functional groups in the microcystins. The antibody used in this ELISA has been shown to cross-react with other microcystin variants such as -LR, -RR and -YR as well as nodularin. Therefore, it is possible the cyanobacterial isolates in this study contain one or more of these cyanotoxins. Previous findings have also shown microcystin estimations to be lower using this method as opposed to LC-MS (He et al., 2017). For general water quality assessments, this method is widely applied and is a cost-effective approach. Some of the shortfalls however, are the detection of ADDA

groups in microcystins that have been partially broken down, indicating that they may also detect microcystins that are already degrading (Qian et al., 2015; Samdal et al., 2014). Microcystin reductions measured in this study showed that the reductions in *Microcystis* were mostly extracellular, which may be the shift from toxic to non-toxic variants in the cell populations (Zhu et al., 2014) or the possibility of the isolates being microcystin degraders. In contrast, intracellular reductions were greater in the filamentous cyanobacteria *Oscillatoria*, with isolate 1 consistently being the more effective isolate against *Oscillatoria* from reducing chlorophyll *a* production to the changes in toxicity.

In the case of the *Microcystis* species, the more effective isolate from a toxin reduction perspective was isolate 3y, which was not the isolate responsible for the greatest chlorophyll reduction. Moreover this is the isolate where the cells were seen to be most stressed by from a scanning electron microscopy perspective. Van Wichelen et al. (2016) have discussed the susceptibility of the *Microcystis* isolate specifically to numerous control measures. It is therefore not surprising to observe a stress response from this isolate when a potential predator is introduced. Moreover, toxin reducing and microcystin-degrading bacteria have been isolated in relation to this particular genus (Ren et al., 2010; Su et al., 2016) *Oscillatoria* however, is not as well studied from this perspective and to our knowledge, the present study is one of the few reports of toxin reduction in both filamentous and colony-forming cyanobacteria at such low cell densities of the control agent. What is also important is the fluctuation of the microcystin degrading efficiency based on the ELISA determinations, the variability of the reduction in toxins needs to be optimized for more efficient and predictable toxin removal, although this is not easy in a mixed population as applied in this study. Another contributing factor to this variation is the sub-culturing of bacterial isolates. Isolates used from master cultures that had been sub-cultured numerous times showed a lower predatory impact in the findings of our research. Therefore, subsequent experiments utilized the original master cultures, brought to the same passage. This resulted in less fluctuation from a cyanotoxin reduction perspective. This is a common challenge in laboratory based studies of bacteria (Iguchi et al., 2002; Molina-Torres et al., 2010).

HPLC analysis

Only filtrate water samples from the exposure experiments were analysed for toxins using HPLC. This would be representative of extracellular toxins released into the water body by the cyanobacteria over the four day experiment. The HPLC data findings indicated the presence of two measurable microcystin variants, which were microcystin -LR and -RR. More variants may be present, which were unquantifiable based on the standards used. Although these are normally the more common variants (Chen et al., 2010; Shen et al., 2003), there have been recommendations for screening of more hydrophobic microcystin -LF and -LA which are also as toxic as microcystin -LR when exposed to

organisms (Xie et al., 2016). Due to feasibility and commonly reported variants, this was not conducted in the current study but is considered for future work.

Microcystin-LR has been reported as up to 10 times more toxic than microcystin -RR, based on mouse bioassays (Schmidt et al., 2014; Xie et al., 2016). In this study, microcystin -RR was almost twice as abundant as microcystin -LR in the *Oscillatoria* and *Microcystis* control filtrate water. These findings are supported by earlier research that microcystin -RR was the most common variant isolated from various cyanobacterial strains sampled from sites in Spain (Cantoral Uriza et al., 2017). Various factors such as temperature (Bui et al., 2018), phosphorus (Oh et al., 2000) nutrients and nitrogen (Pimentel and Giani, 2014) have been indicated in the production of different microcystin congeners. To date, it is not clear exactly how the various factors interplay to influence this, particularly as the primary functional role of microcystins in cyanobacteria are also an unclear phenomenon (Rapala et al., 1997; Su et al., 2015).

The data indicate the reduction of the more toxic microcystin -LR by 25-50% in filtrate samples of *Microcystis* treated with the different isolates except for isolate 3y. Similarly, 17-45% reductions were observed in the filtrate water of *Oscillatoria* treated with the different bacterial isolates. Based on the HPLC findings, isolate 3w and B are the most effective in toxin reduction, whilst ELISA indicated isolate 3w and 3y as the best isolates for reducing extracellular toxicity. Isolate 3y may have been the best isolate from a cross-reactive ELISA perspective as both variants of the microcystins were reduced, compared to the other isolates, which reduced microcystin LR more readily than microcystin RR.

Most degraders of microcystin LR, have also been reported to degrade other variants such microcystin RR and YR (Dziga et al., 2013; Valeria et al., 2006; Yang et al., 2014a). The difference in the microcystin variants is the attachment of a different amino acid in the X and Z positions. In the case of the microcystins RR (arginine –arginine) and LR (leucine-arginine) the difference of amino acid in the X position (Song et al., 2006). In another study, the degradation rates of microcystin LR and RR- were 0.27 mg.(L h)$^{-1}$ and 0.12 mg.(L h)$^{-1}$ respectively when both variants were present, which was slightly lower than the degradation rates when both variants were degraded separately by an isolate of *Bordetella* sp. (Yang et al., 2014a). This is similar to the findings in this study, where microcystin RR was not as readily reduced in the four day period as opposed to microcystin LR, which may require a longer period of observation. A study of microcystin degradation by bacterial communities indicated an eight day period for the complete removal of the toxin (Christoffersen and Lyck, 2002) *Pseudomonas* was found to reduce microcystin LR over a three week period (Takenaka and Watanabe, 1997).

Mode of action-preliminary findings

The mode of action in terms of microcystin reduction for the bacterial isolates employed in this study has not been classified. Preliminary testing on the production of antimicrobial biosurfactants, such as lipopeptides, showed that none were quantifiable in the culture supernatant, which could point out another mode of action, resulting in the compromised cyanobacterial integrity observed in figures 4-7. Lipopeptides are produced by some of the genera identified in this research. Further research is required to characterize the mode of action in biological control.

However, if the bacterial isolates are in fact microcystin degraders, the reduction in colonies and mucilage around the *Microcystis* isolate can be explained through the findings of Maruyama et al. (2003), where microcystin degrading bacteria were found to be degrading microcystins released in the mucilage of the cyanobacteria. Other studies indicate the use of microcystins as a sole carbon source (Lemes et al., 2015) by *Pseudomonas aeruginosa* isolates, which may be the case in the present study. *Sphingomonas* and related isolates have been often reported often as microcystin degraders, however other isolates have been identified as well (Alamri, 2012; Chen et al., 2010; Kansole and Lin, 2016; Zhang et al., 2010).

The minimal reduction in crude microcystins by ELISA analysis after four days in bacterial isolates that were not supplemented with glucose may be an indication of slow rates of degradation under limited nutrients. Molecular testing of genes present in microcystin degraders may offer confirmation of the theory that the isolates in the study are microcystin degraders. Earlier research indicated the presence of the microcystin-degrading gene cluster, *mlr* A, B, C and D in *Sphingomonas* and other related species (Valeria et al., 2006), however, the study by Yang et al (2014) reported a *Bordetella* sp. isolate, which contained this gene cluster. A study by Manage et al. (2009) indicated that none of the previously reported *mlr* genes were present *in Arthrobacter* sp, *Brevibacterium* sp., and *Rhodococcus* sp. isolates, indicating another mode of microcystin degradation or presence of different genes for microcystin degradation.

The minimal reductions seen in the test of cyanotoxin degradation by the four isolates utilized in this study requires confirmation as well with HPLC and perhaps a longer time period for observation (more than four days). In addition, the variability in experimental work with living organisms can be expected in a system where bacterial diversities are mixed and not fully classified. These factors need to be more carefully considered and accounted for in order to optimize the current findings of this study. With this in place, further confirmation of this treatment method can also be explored on a larger scale and possibly in nature.

The use of Tween 80 in bacterial growth media has been found to enhance the protease and lipase production in bacterial cells. Moreover, cells that have been grown in this surfactant have shown stronger morphological characteristics as opposed to cells in general growth media (Boekema et al., 2007; Jacques et al., 1980; Kamande et al., 2000). The use of this medium was to potentially enhance hydrolytic activity in the cells, which may have aided the degradation of microcystins.

In closing, bacterial isolates selected in this study, namely isolates 1, 3w, 3y and B, were able to elicit a stress response and reduce toxicity in filamentous and colony-forming cyanobacteria. Isolate 1 and B were most effective against *Oscillatoria* and the reduction of toxicity, whilst isolate 3y and 3w were most effective against the extracellular toxicity of *Microcystis* sp. based on ELISA determinations. Confirmatory testing through HPLC indicated isolate 3w to be the most effective against the extracellular toxins of both cyanobacteria. The isolates appear to have some microcystin degrading properties; however this needs to be confirmed with further genetics and research into the mode of action. The present study results suggest that certain bacterial species are effective against colony-forming and filamentous cyanobacterial isolates and this is important in a mixed bloom environment, where other species may be dominant. Moreover, lower bacterial ratios can also be effective in extracellular toxin reduction. Progress into the potential optimization of this application and future use in managing blooms or toxins was indicated by the present study.

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Chapter 6: Determination of the eco-toxicity changes in biologically treated cyanobacteria *Oscillatoria* and *Microcystis* using indicator organisms

*Accepted with revisions as a Springer book chapter contribution- *"Microbiology research methods in Africa"*

Declaration by the candidate

With regards to chapter 6, the nature and scope of my contribution was as follows

Nature of contribution	Extent of contribution
Conceptual design, experimental work, manuscript writing	70%

The following co-authors have contributed to chapter 5

Name	Email address and institutional affiliation	Nature of contribution	Extent of contribution
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Abstract

The response of biological indicators with ecosystem food web and agricultural relevance were tested on biologically treated cyanobacteria culture water. Biological control in a mixed population may cause variations in the toxicity measurements at a given time. To assess bio-indicator responses to these variations, a case study on the filtrate of treated cyanobacteria was conducted. Cyanobacterial cultures of dominant *Oscillatoria* and *Microcystis* were treated with the addition of four different, potentially predatory bacterial isolates (isolates 1, 3w, 3y and B) over a four day period. A total microcystin assessment of filtrate water from these exposures indicated that approximately 30% of toxicity had been reduced in *Microcystis* samples treated with isolate 1 and isolate B. In the case of *Oscillatoria* the toxicity was increased by 14% by treatment isolate 3w, whilst minimal changes occurred through treatment by other bacterial isolates, isolate 3y showed a 3% reduction of toxicity. The use of biological indicators was to determine whether a reduction or increase in cyanotoxins through bacterial treatment of cyanobacteria could be reflected in their response. This would serve as a good indicator of whether the biological treatment of microcystin in water through these bacteria was environmentally suitable. *Lactuca sativa*, *Allium cepa*, *Daphnia magna* and *Thamnocephalus platyurus* were the selected biological indicators. *Lactuca sativa* germination was not sufficiently sensitive to the changes in toxin concentration, while *Allium cepa* mitotic indices indicated a relative sensitivity to microcystin concentration changes. Acute exposure of *Thamnocephalus platyurus* was found the most sensitive to the changes in toxicity, with better survival in less toxic samples, indicating slight toxicity increases through higher mortality. The water chemistry in terms of phosphates, copper and ammonia seemed to fluctuate between the treated and untreated sample water, indicating a possible impact on the total nitrogen and total phosphorous ratio. A principal component analysis indicated a close correlation between the *Lactuca sativa* and *Allium cepa* response. The same was observed in the responses of *Thamnocephalus platyurus* and *Daphnia magna*. An analysis of DNA apoptosis indicated that DNA apoptosis had occurred in *Thamnocephalus platyurus*, with DNA having better integrity in samples treated with isolate B. Overall isolate B was the most favourable bacterial treatment, based on bio indicator response. The present research confirmed the toxicity reduction in a mixed cyanobacterial bloom culture and the reliability of crustaceans such as *Thamnocephalus platyurus* as indicators of total biological toxicity in biologically controlled cyanobacteria.

6.1 Introduction

The prevalence of cyanobacterial blooms and their impact on the environment has been recorded in numerous studies (Ndlela et al., 2016; Oberemm et al., 1997; Oberholster et al., 2009; Paerl et al., 2014; Preece et al., 2017). Control measures of these blooms and toxins have been well researched, and among these is the use of biological control (Ndlela et al., 2018). In the present study, the control agents are microorganisms, particularly bacterial isolates collected from natural bloom waters. Studies on biological control of cyanobacteria have been applied at laboratory scale, with a successful outcome being the lysis or stress response of the targeted cyanobacteria (Nakamura et al., 2003; Yang et al., 2012; Zhang et al., 2016). The lysis or stress of these targeted cyanobacteria may also indicate a subsequent release of intracellular toxins (Ndlela et al., 2018; Paerl et al., 2016), thereby causing the water body in which these cells lyse to have an increased concentration of cyanotoxin (Westrick et al., 2010). Of these biological control studies, a few have assessed the resulting eco-toxicity through the application of indicator organisms. An example of this is a study by Keijola et al. (1988), which assessed the effectiveness of different drinking water treatment methods using toxic cyanobacteria isolates (*Anabaena*, *Microcystis* and *Oscillatoria*). Toxicity was determined through a mouse bioassay and gas chromatography measurements of cyanotoxins. Another study by Pool et al., (2003) investigated the use of hormone interleukin 6 (IL-6) as an indicator of inflammatory agents in water as an in vitro study. A review of cyanobacteria and cyanotoxin removal in drinking water by Westrick et al. (2010) found that the biological filtration treatment of cyanobacteria resulted in reduced microcystin removal efficiency by 30% in autumn months, compared to summer months. This reduced efficiency was attributed to temperature changes (Grützmacher et al., 2002). In the same review, a study on the removal of saxitoxins through biological filters indicated a shift from less toxic to more toxic variants increase (Kayal et al., 2008). These fluctuations are due to the response of the live organisms in the biological filters. This is an example of biological control of live cell toxins by other live cells, which is the case in the present study.

Eco-toxicity is the assessment of how an organism reacts to specific chemicals and pollutants. This area of study has been conducted in assessing the pollutants in freshwater, marine and soil environments, using particular organisms, commonly termed as bio-indicators. The bio-indicator organisms are from different trophic levels and indicate the negative and positive changes in a given environment. The bio-indicator categories are plant, animal or microorganisms (Parmar et al., 2016). When these indicator organisms are used to determine the change in response to a certain chemical or environmental parameter, they enable a good means to gauge the environmental impact of treatment or change, based on their response. For example, a study by Mohamed and Hussein (2006) assessed

the response of Tilapia fish to microcystins, finding that the fish were able to survive and depurate the toxins. A later study by Mohamed et al. (2014) assessed the inhibition and toxin reduction in *Microcystis aeruginosa* by a fungus *Trichoderma citrinoviride*, where toxin concentrations were reduced to undetectable levels within a five day period. A similar approach was applied in the present study, by assessing the bioassay responses compared with the cyanotoxin concentration changes. Some of the bacterial isolates employed in the present study have given an indication of microcystin reducing capacity. The changes in cyanotoxin were determined in biologically treated and untreated cyanobacteria. Aquatic and agricultural bio-indicator organisms were used to confirm whether the reduction or increase in toxicity brought about by the proposed control agents impacted the bio-indicator organisms. These eco-toxicity assays were performed to indicate whether the selected bacterial control agents could be viable for larger scale or freshwater body applications. The use of bio-indicator response would help to determine whether the use of these biological control agents is environmentally friendly and could lead to reduced adversity on these organisms.

6.2 Materials and Methods

6.2.1 Cyanobacterial collection site descriptions

Two collection sites were selected, the first site, the Brandwacht WWTW, is located in the K10D quaternary catchment roughly 500m upper east of the town of Brandwacht, between the towns of Mossel Bay and Oudtshoorn in the Western Cape province, South Africa. The Brandwacht River, found around 650m west of the site, is the main surface water feature. Brandwacht is situated at the foot of a small hill (Die Erwe) and has an elevation of 38-54 meters above sea level. The second collection site, the Klippoortjie Coal Mine is situated within the Emalahleni Local Municipality (2678 km^2) and Nkangala District Municipality, approximately 20 km east of the town of Ogies in the Mpumalanga province, South Africa.

6.2.2 Sample collection and isolation of cyanobacteria and heterotrophic bacteria

Water containing a bloom of *Microcystis* sp. was collected from Brandwacht wastewater treatment works, in Mossel Bay, Western Cape, South Africa (34° 3' 3.6" S, 22° 3' 28.8" E). Filamentous cyanobacteria were collected from the Klippoortjie waste water treatment works (26° 07' 00" S; 29° 08' 00" E), near the town of Ogies, Mpumalanga, South Africa. Samples were collected in sterile water bottles and kept on ice during commutation. Cyanobacterial isolates were identified by light microscopy at 400 x magnification (Zeiss Axioscop), using the procedures mentioned by Oberholster et al. (2009)

and stored as non-axenic cultures at 4°C for the duration of the study and checked monthly for dominance of the cyanobacteria of interest.

Heterotrophic bacteria showing predatory activity against cyanobacteria were isolated from the water containing cyanobacterial isolates through the use of the plaque assay as described by Gumbo et al. (2010). Three isolates were randomly selected from the plaque assay, namely, isolates 1, 3w, and 3y. A culture of *Bacillus* was generously donated by the Microbiology department of the Stellenbosch University and used as a reference, based on earlier research done on this genus as an algicidal isolate (Gumbo and Cloete, 2011). Bacterial isolates were then grown as pure cultures in Nutrient agar and nutrient broth medium (Merck, Germany) at 25°C.

6.2.3 Growth measurements

Cyanobacteria were grown in BG-11 broth medium in 100 ml volumes (Merck, Germany) over 28 days at 25°C in a 12 h:12 h light-dark cycle, with light illumination of approximately 60 mmol photons (PAR) m^{-2} s^{-1} Chlorophyll *a* and confirmatory wet weight measurements were taken every three days. Chlorophyll *a* was extracted with methanol and measured according to the methods of Porra et al. (1989).

Bacterial isolates 1, 3w, 3y and B were grown in 100 ml volumes of nutrient broth medium (Merck, Germany) and Tween 80 broth and agar medium overnight (5 g peptone, 3 g meat extract, 10 ml Tween 80, 100 mg $CaCl_2.2H_2O$, 15 g agar per litre, pH 7.2). Master cultures were prepared with 80% of culture medium and 20% glycerol (Merck, Germany) and stored at -80°C.

6.2.4 Exposure experiments

6.2.4.1 Pre-growth of cyanobacterial and bacterial isolates

Oscillatoria sp. and *Microcystis* sp. were cultured in 1x BG-11 broth (Sigma-Aldrich) at 25°C in a 12 h:12 h light dark cycle, with light illumination of approximately 60 mmol photons (PAR) m^{-2} s^{-1}, for 3-7 days which is when they reached the exponential growth phase, based on chlorophyll *a* measurements, based on at least 3 experimental repeats.

Bacterial isolates 1, 3w, 3y and *Bacillus* were grown in Tween 80 broth for 8 hours at 25°C.

Non-axenic cultures of 0.1g (wet weight) of filamentous cyanobacteria and $1x10^6$ cells of *Microcystis* sp. was added into 100ml of sterile BG-11 medium and grown at 25°C respectively. After 2-3 days, eight hour old bacterial cells grown in Tween 80 broth were counted with a bacterial counting chamber

(Helber, Marienfeld, Germany) at 400x magnification using a light microscope (Zeiss Axioskop). Cells were harvested by centrifugation at 10000 x g for 10 minutes centrifuged for 10 minutes at 10 000xg (Thermo Scientific SL 16R) and washed twice with 1x phosphate buffered saline (PBS) (Lonza).

6.2.4.2 Addition of bacterial isolates to cyanobacterial cultures

Washed bacterial cells were re-suspended in 1 ml of PBS and added to cyanobacterial cultures. Based on preliminary chlorophyll *a* measurements, 0.1 g of *Oscillatoria* yielded approximately 10x more chlorophyll *a*, compared to 1 million cells of *Microcystis* (wet weight of 0.1 g). Therefore, 10x more cells were added to the filamentous cultures. Cells were added in 1:2 ratios (based on cell counts) of heterotrophic bacteria: cyanobacteria and shaken briefly after addition. The flasks were left at 25°C for four days under static conditions with a 12h light and12 hour dark cycle. After four days, culture samples were vacuum filtered using the 0.22µm 250ml Steri-cup Express filters (Merck, Germany) to separate the cells from the culture medium. The residual cells were analysed for phycocyanin; the filtrate water was analysed for total microcystin concentration changes and eco-toxicity assays. Water chemistry, alkaline phosphatase activity and microscopic analyses of the samples were also conducted.

6.2.4.3 Phycocyanin measurements

After four days, culture samples were vacuum filtered using 0.22µm 250ml Steri-cup Express filters (Merck, Germany). The residual cells were ground to a fine powder in liquid nitrogen and re-suspended in 1 ml phosphate buffer according to the method of Moraes et al. (2011).

6.2.4.4 Cyanotoxin detection

Total microcystins were measured in the filtrate water samples after four days using the Envirologix microcystin detection kit (Stargate Scientific, South Africa) according to the manufacturer's instructions.

6.2.4.5 Water chemistry analyses

Water chemistry of filtrate water samples was conducted using the Hach DR 3900 (Agua Africa) and powder pillows (Agua Africa) to measure the following parameters: Potassium, Nitrates, Nitrites, Zinc, Copper, Iron, Phosphates, Ammonia, Aluminium and Sulphates. The pH of samples was also measured using the Hanna HI 991300 multi-meter (Hanna, USA). These parameters were selected based on the indication of their impact on cyanobacterial growth.

6.2.5 Eco-toxicity assays

Cell-free filtered water samples from exposures experiments that had been analysed for changes in cyanotoxins, water chemistry and chlorophyll *a* were used for eco-toxicity assays. The selected indicators for these assays have relevance in agriculture and as ecosystem filter feeders. The water in some dams is used for irrigation and the occurrence of toxic blooms has potential impacts on the produce irrigated as well as the end users of the produce (Dabrowski et al., 2013). As a result, plants and seeds that are agriculturally produced and sensitive to pollutants have been selected in this study. In the case of animal indicators, the use of crustacean filter feeders is a good indication of the ecosystem pollutants and since they play a critical role in the food web, their sensitivity to pollutants makes them suitable bio indicators (Le et al., 2016; Sánchez et al., 2016).

6.2.5.1 Lactuca sativa bioassay

The lettuce seed bioassay was conducted according to methods previously described by EPA (1996) and Bagur-González et al. (2011) with slight modifications. Briefly, twenty lettuce seeds (Starke Ayres, South Africa) were laid out on No. 1 Whatman filter paper (Sigma Aldrich) in 90mm petri dishes (Lasec, South Africa). Then, 3 ml of 0.22µm filtered water from exposure experiments was added to the seeds on the filter paper and left in the dark for 120 hours at ambient temperature. Each water sample was tested in triplicate. The seeds were exposed to tap water (pH 7.2, 5.3 mS/m) as a control and to treated and untreated *Oscillatoria* and *Microcystis* water samples. The number of seeds that hatched was calculated to determine the percentage of seed germination relative to the control.

6.2.5.2. Allium cepa root tip assay

Onions weighing between 190 to 230 g were pre-grown in tap water as per the method described by Barberio (2013). The ring of the root primordia at the bottom of the onion bulb was scraped with a surgical blade and rinsed with distilled water. The cleaned onions were then immersed into beakers of clean tap water, approximately 2-5cm deep allowing for the growth of new roots from the bulbs, over a 48 hour period. After that, onions were exposed to tap water as a control and to bacterially treated and untreated *Oscillatoria* and *Microcystis* water samples for 48 hours. At the end of the exposure, the onion roots were cut 3cm from the tip and placed in Carnoy's fixative (3 parts glacial acetic acid to one part absolute ethanol (Merck, Germany) for six hours. For calculation of mitotic indices, the roots were stained with 0.5% Hoescht nucleic stain (0.05 mg.ml^{-1}) (Sigma-Aldrich) diluted in 1x phosphate buffered saline (Lonza). Roots of 2-3 cm lengths were placed on clean microscope slides and 100 µl of 0.5% Hoescht stain was added onto the root. The roots were subsequently squashed with the coverslip by

applying pressure with the thumb. The onion roots were thereafter imaged after 10 minutes of staining, on a Carl Zeis laser scanning confocal microscope 780 (Germany), with the following parameters: magnification -Alpha Plan-Apochromat 100x/1.46 Oil DIC M27 Elyra, laser: 2.0%, master gain: 450, pin-hole: 90 µm, beam splitters: Invis: MBS and a scan speed of 6.30µs at the Stellenbosch University Central Analytical Facilities (CAF) fluorescence microscopy unit. The mitotic index was calculated from the number of cell nuclei actively dividing after 48 hours of exposure.

6.2.5.3 Daphnia magna bioassay

The Daphtox F kit was purchased from Tox Solutions Kits and Services (South Africa) and used according to the manufacturer's instructions. Eppiphia were hatched in freshwater medium over a 72 hour period at 22°C. Upon neonate hatching, they were fed ground spirulina two hours before exposure to the cyanobacteria filtrate water described in section 6.2.4. Mortality of the neonates was observed over 24 and 48 hours at room temperature in the dark. The EC_{50} was calculated based on the mortality at 24 and 48 hour observations.

6.2.5.4 Thamnocephalus platyurus bioassay

The Thamnotox kit was purchased from Tox Solutions Kits and Services (South Africa) and used according to the manufacturer's instructions. Cysts were hatched in freshwater medium over a 24 hour period at 25°C. Upon neonate hatching, they were exposed to the cyanobacteria filtrate water described in section 6.2.4. Mortality of the neonates was observed over 24. The EC50 was calculated based on the mortality at 24 hour observations. At the end of the 24 hour exposure period the neonates were harvested and stored at 4°C.

6.2.6 DNA fragmentation assay

After the conclusion of the bioassays, harvested *Thamnocephalus platyurus* crustaceans samples were ground to a fine powder in liquid nitrogen with a cooled motar and pestle. The ground samples were used to test for DNA apoptosis. The ApoTarget™ Quick Apoptotic DNA Ladder Detection Kit (Invitrogen) was used according to manufacturer's instruction. The samples were imaged on a 1.2% gel, stained with Gel Red nucleic acid stain (Thermo-Fischer Scientific) after electrophoresis for two hours at five volts per cm.

6.2.7 Statistical analyses

Water chemistry correlation and principal component analyses of the bioassay findings (Spearman correlation for non-parametric data) were conducted using Microsoft XLSTAT™ (2010). Visualization of

the relationship between the biological indicators was conducted through a bubble plot using JMP™ (version 14) software. One way ANOVA analysis was conducted on data from *Lactuca sativa* to determine any statistical significance from the treatment groups.

6.3 Results

6.3.1 Phycocyanin estimation

Exposures of *Oscillatoria* and *Microcystis* sp. cultures were conducted over a four day period. The measurement of phycocyanin showed that treatments with different isolates either enhanced or reduced the pigment formation relative to the untreated controls (Figure 6.1). It is also interesting to note that at the same wet weight, phycocyanin concentrations in *Microcystis* were almost a 100-fold lower than in the case of *Oscillatoria*. This provides an interesting comparison to the chlorophyll *a* measurements, with a similar trend noted as per the chlorophyll reductions (Chapter 5).

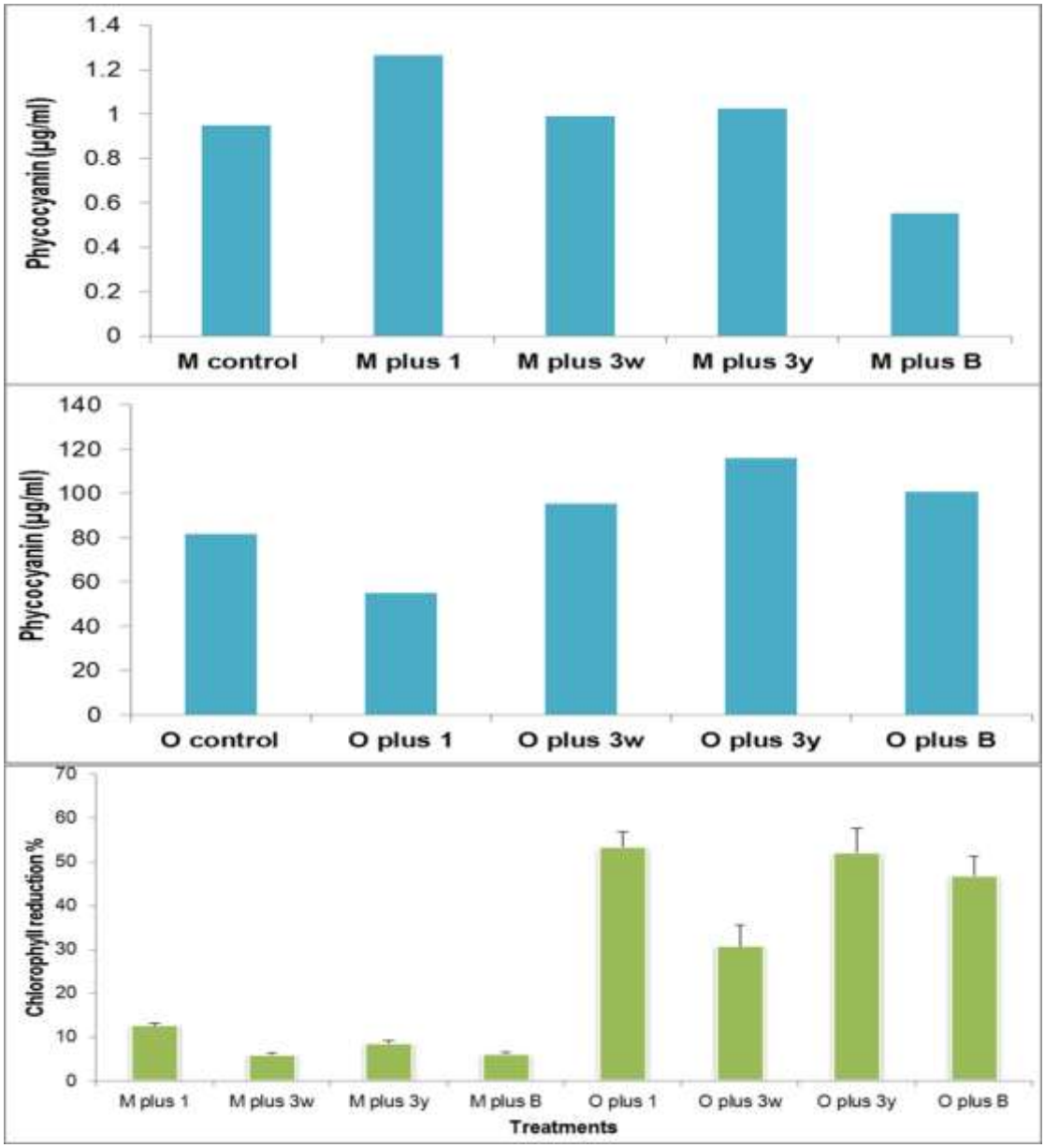

Figure 6.1: Phycocyanin group comparisons in *Microcystis* (M) and *Oscillatoria* (O) treated with isolates 1, 3w, 3y and B. Chlorophyll *a* reductions were also included for comparison to the phycocyanin pigment changes.

While isolate 1 had an inhibitory effect on *Oscillatoria* compared to the control, it appeared to have a beneficial effect on *Microcystis* based on the pigment (chlorophyll and phycocyanin) concentrations relative to the control samples. This was observed with isolate B as well, which had a beneficial impact on *Oscillatoria* and an inhibitory effect on *Microcystis* from the phycocyanin estimations. Isolate 3w and 3y had similar effects on the phycocyanin production of both cyanobacteria.

6.3.2 Cyanotoxin detection

After the addition of bacterial isolates to *Microcystis* cultures over a four day period, changes in cyanotoxin reduction were observed: There was a reduction in cyanotoxin concentrations in treated cyanobacteria, relative to the control sample (Figure 6.2). Isolates 1 and B exhibited a greater reduction (27 and 30%, respectively) in the water samples of *Microcystis* compared to isolate 3w and 3y (16 and 4% respectively). The monitoring of cyanotoxins in *Oscillatoria* water samples showed minimal decrease in toxins from treatment with isolate 3y (2.4%), whilst the other isolates had slight increases in toxicity, up to 15% by isolate 3y. These results indicated variation in bacterial performance. The data captured in Figure 6.2 are comparable to an average of toxin reduction that indicated an overall cyanotoxin average reduction of up to 16% in *Oscillatoria* sp. water and up to 63% reduction in *Microcystis* sp. water.

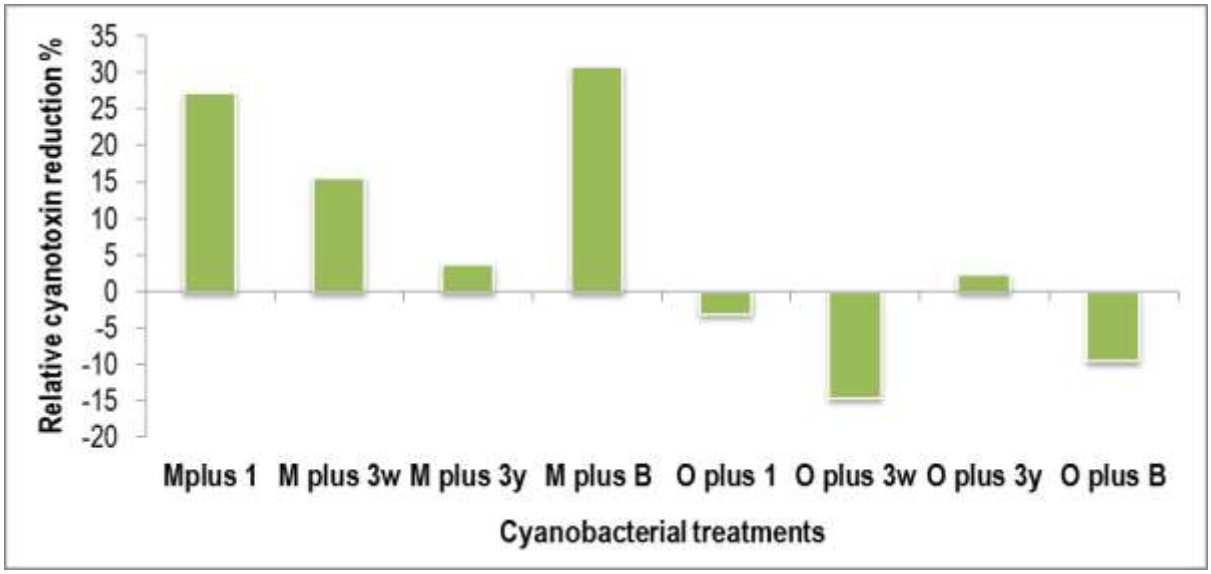

Figure 6.2: Cyanotoxin reductions in filtrate water samples of *Microcystis* (M) and *Oscillatoria* (O) treated with bacterial isolates 1, 3w, 3y and B, relative to the control untreated samples.

6.3.3 Water chemistry changes

Of the parameters measured in the water chemistry of exposed and unexposed cyanobacteria (Table 6.1), phosphates had the greatest variations, while the other parameters were similar, with less than 5% fluctuations. The change in phosphorous concentrations was linked to the change in TN:TP ratios, which when fluctuated, create more favourable conditions for cyanobacterial growth. This is particularly at low TN:TP ratios, around 10 or less. Due to the parameters measured, there could be no accurate estimation of the TN:TP ratios, except to note that the ammonia concentrations were lowest in the untreated control samples. A correlation analysis (Table 6.2) of the data indicated that measured

parameters, apart from copper and orthophosphates, had a strong correlation value (> 0.750) to each other.

Table 6.1: Water chemistry parameter measurements of treated and untreated *Microcystis* and *Oscillatoria* sample filtrates after four days of exposure.

Treatment	Ammonia ($mg.L^{-1}$)	Copper ($mg.L^{-1}$)	Nitrates ($mg./L^{-1}$)	Orthophosphate ($mg.L^{-1}$)	Potassium ($mg.L^{-1}$)	Sulphates ($mg.L^{-1}$)
M control	0.77	0.04	1.08	8.7	122	18
M plus1	0.84	0.1	1.08	10.3	123	19
M plus 3w	1.07	0.03	1.08	10.2	124	19
M plus 3y	1.43	0.02	1.08	5.3	126	18
M plus B	0.94	0.02	1.08	6.22	126	18
O control	0.01	0.02	1.02	6.01	114	14
O plus 1	0.05	0.03	1.02	8.1	113	15
O plus 3w	0.24	0.02	1.02	8.7	115	14
O plus 3y	0.71	0	1.02	9.1	117	14
O plus B	0.18	0.03	1.02	9.9	117	15

Table 6.2: Spearman correlation values of the variables measured in treated and untreated cyanobacteria *Microcystis* and *Oscillatoria*, after 4 days of exposure to heterotrophic bacteria.

Variables	Ammonia	Copper	Nitrates	Orthophosphate	Potassium	Sulphates
Ammonia	**1**	0.063	**0.870**	0.079	**0.945**	0.750
Copper	0.063	**1**	0.400	0.524	0.057	0.654
Nitrates	**0.870**	0.400	**1**	0.070	**0.876**	**0.898**
Orthophosphate	0.079	0.524	0.070	**1**	0.031	0.345
Potassium	**0.945**	0.057	**0.876**	0.031	**1**	0.739
Sulphates	0.750	0.654	**0.898**	0.345	0.739	**1**

Values in red show a strong correlation

6.3.4 Lactuca sativa bioassay findings

Findings from the germination of the lettuce seeds indicated that there were minimal variations in seed germination percentages between the treated and untreated samples after exposure to water containing *Microcystis*, with less than 10% difference in germination percentage between the treated and untreated samples. Similarly for *Oscillatoria*, the differences were less than 20% in the treated and untreated samples (Figure 6.3). One way ANOVA analysis showed no statistical significance in *Microcystis* treatment groups ($p \leq 0.81$). However, in the *Oscillatoria* treatment group, there was statistical significance ($p \leq 0.03$) between the groups. *Microcystis* sp. treatment with isolate 3w resulted in a toxin reduction of 16%; however, the seed germination (45%) was 8% lower than the untreated

control sample (53%). The control sample of tap water had 70% seed germination, while tap water with 1% compost added to it had 100% seed germination. With *Oscillatoria*, the changes in toxicity of only isolate B treated samples were not reflected by the changes in seedling germination. These observed inconsistencies may be due to the slight changes in toxicity not being well indicated by the lettuce seeds, thereby indicating that their sensitivity as bio-indicators is not well suited to the slight changes observed in *Oscillatoria* toxicity.

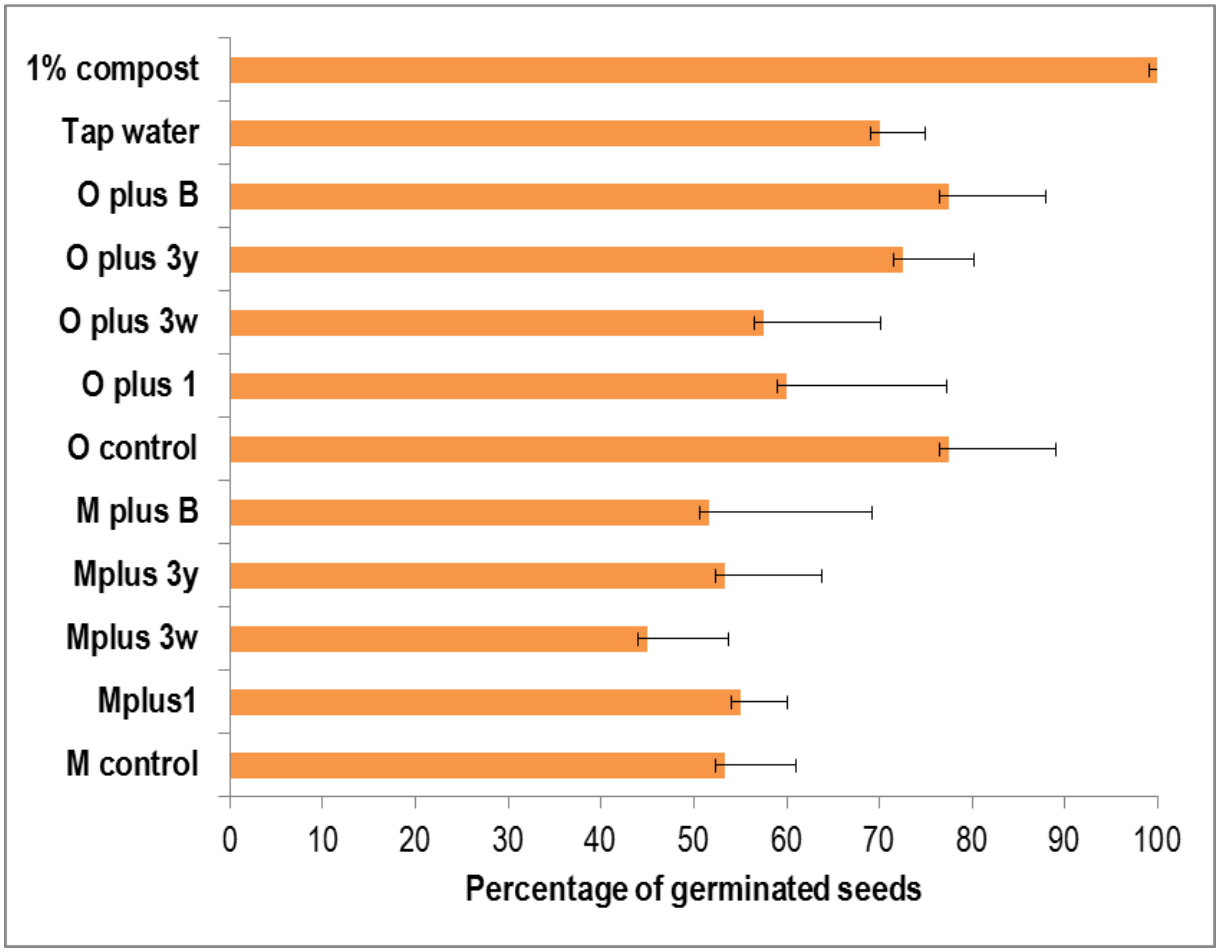

Figure 6.3: Mean percentage germination of lettuce seeds after 120 hours of incubation in different water. The bars indicate standard deviation. The 1% compost sample had no standard deviations.

6.3.5 Allium cepa root assay

The onion root assay was done through the observation of mitotic indices in onions exposed to treated and untreated samples of cyanobacteria filtrate water. Tap water was used as a control. Nuclei in state of cell division were counted out of a total number of 100 cells to generate the mitotic index (Table 6.3). Only *Oscillatoria* treated with isolate 3y had a low mitotic index (41% vs 85%).

Table 6.3: Mean mitotic index (%occurrence in 100 cells) of treated and untreated *Microcystis* and *Oscillatoria* onion root cells after 48 hour exposures.

Treatment	Mitotic index
Tap water	93.33 ±4.08
M control	85.08 ±20.24
M plus1	89.43 ±15.00
M plus 3w	**35.84 ±10.66**
M plus 3y	**67.07 ±9.82**
M plus B	85.56 ±12.68
O control	99.68 ±19.55
O plus 1	90.79 ±24.60
O plus 3w	57.83 ±16.96
O plus 3y	**40.97 ±7.66**
O plus B	87.55 ±15.98

Bold figures indicate significantly lower mitotic indexes in comparison to the control samples

The images of the onion root cells are shown in Figure 6.4. Image a shows untreated (M control) cells compared to treated (M plus 1) cells in image b. These were taken as representative samples to indicate the differences in the mitotic stages of the onion roots exposed to samples with greater toxicity. M control cells (a) showed chromosomal bridges in anaphase and some nucleic disintegration, indicated by the arrow in Figure 6.4a. Cells treated with isolate 1 (Figure 6.4b) showed an intact mitotic nucleus with normal anaphase occurring in the bottom right corner of the image, indicated by the arrow.

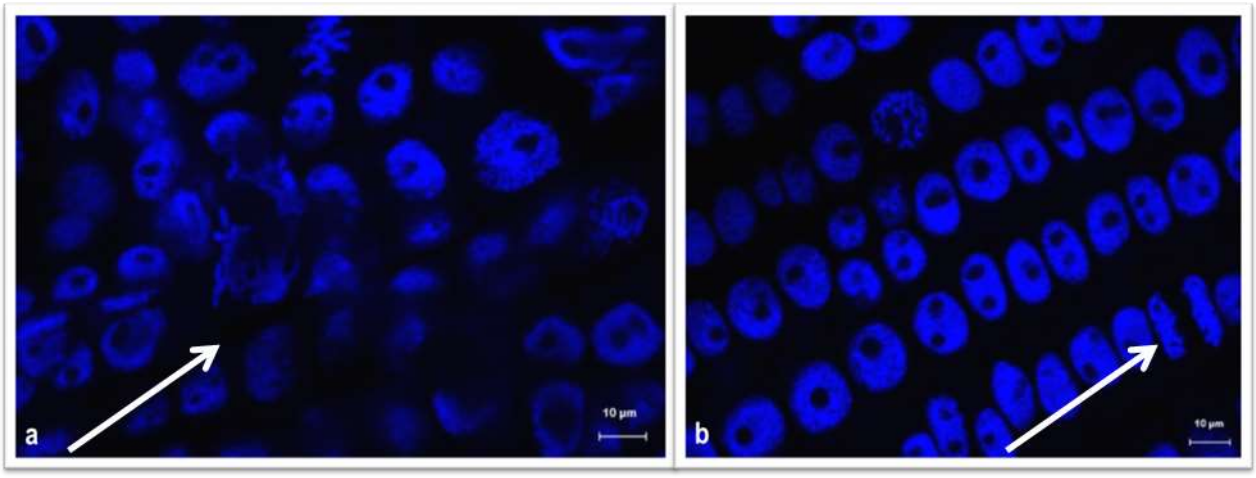

Figure 6.4: Nucleic acid stain of *Allium cepa* roots exposed to water from untreated *Microcystis* (a) and *Microcystis* treated with isolate 1 (b). Nuclei appear intact and normal mitosis occurs in Figure 6.4b, while there is nuclear disintegration in Figure 6.4a.

6.3.6 Daphnia magna bioassay

Testing of the response of daphnids as freshwater crustaceans to the treated and untreated water samples (Table 6.4) indicated that the *Microcystis* samples with reduced toxicity had a higher survival of neonates, compared to the control (untreated) sample, which had higher toxicity. There was statistical significance in the observations between the treated and untreated samples ($p \leq 0.048$) of *Microcystis*. For *Oscillatoria*, only the control and 3w sample had a lower neonate survival, with only 79% survival ($p \leq 0.13$). The freshwater control, which is the recommended control by the manufacturer, had no adverse effects on neonate survival, with a 100% survival. The inconsistencies in *Oscillatoria* samples cannot be clearly accounted for; however, the trend indicates that samples with toxicity changes greater than 15% were well detected by the bio-indicators. The EC_{50} calculations of water samples indicated that toxicity is reduced in treated samples, with *Oscillatoria* filtrate water samples having an impact after 48 hours compared to the 24-hour effective concentrations, which remained the same for *Microcystis* samples.

Table 6.4: Neonate survival of *Daphnia magna* after 24 and 48 hour exposure to biologically treated and untreated *Microcystis* and *Oscillatoria* water samples.

Treatment	Neonate survival (%)	24 hour EC_{50}	48 hour EC_{50}
M control	50±8.66	1.45	1.45
M plus1	80±8.66	2.64	2.64
M plus 3w	75±4.79	2.45	2.45
M plus 3y	100±0		
M plus B	90±2.89	5.02	5.02
O control	79±5.00	6.85	1.39
O plus 1	100±5.77		2.35
O plus 3w	79±4.79	7.84	1.57
O plus 3y	95±2.5		3.34
O plus B	95±2.5		3.75

Grey areas indicate treatments where the EC50 was not quantified due to the low toxicity of the water sample. EC50 values represent $\mu g.ml^{-1}$ of cyanotoxin.

6.3.7. Thamnocephalus platyurus bioassay

One of the recommended bioassays for microcystin detection is the use of the crustacean *Thamnocephalus platyurus*, which is also a crucial and sensitive filter feeder in the food web. This crustacean response in a 24 hour period gives an indication of the water quality and particularly the microcystin concentrations. The trend in *Thamnocephalus* was similar to that observed in *Daphnia*, with an overall 6% higher survival however, which may indicate the slight difference in sensitivity of the isolates (Table 6.5). The difference in the EC_{50} indicates that the *Daphnia* were more sensitive to the changes in cyanotoxin as opposed to *Thamnocephalus*, with much lower EC_{50} concentrations required in *Daphnia* neonates. The longer exposure time of *Daphnia* yields more information as opposed to the shorter exposure of *Thamnocephalus*, which may be more meaningful under a chronic exposure assay. Statistical significance was observed in the *Microcystis* treatment group ($p \leq 0.038$) while no statistical significance was observed in the *Oscillatoria* treatment group ($p \leq 0.72$).

Table 6.5: Neonate survival of *Thamnocephalus platyurus* after 24 hours of exposure to biologically treated and untreated *Microcystis* and *Oscillatoria* water samples.

Treatment	Neonate survival (%)	24 hour EC_{50}
M control	62 ±30.82	2.42
Mplus1	100±48.39	17.57
Mplus 3w	90±44	6.12
Mplus 3y	83±40.20	3.49
M plus B	93±45.18	5.02
O control	100±48.39	11.41
O plus 1	97±46.90	5.04
O plus 3w	93±45.18	3.92
O plus 3y	100±48.39	11.13
O plus B	90±43.78	2.88
Freshwater control	100±48.39	

Grey areas indicate treatments where the EC50 was not quantified due to the low toxicity of the water sample. EC50 values represent $\mu g.ml^{-1}$ of cyanotoxin.

6.3.8 Overall variation and response patterns

A representation of the *Allium cepa*, *Daphnia* and *Thamnocephalus* assays (Figure 6.5) indicated similar response trends of the *Microcystis* and *Oscillatoria* filtrate water. Treatment numbers 1 to 5 indicate the control untreated cyanobacteria (1), cyanobacteria treated with isolate 1 (2), treatment with isolate 3w (3), treatment with isolate 3y (4) and treatment with isolate B (5). The assays indicate that the changes in toxicity are observed in the bio-indicator responses, meaning that more toxic samples have lower survival.

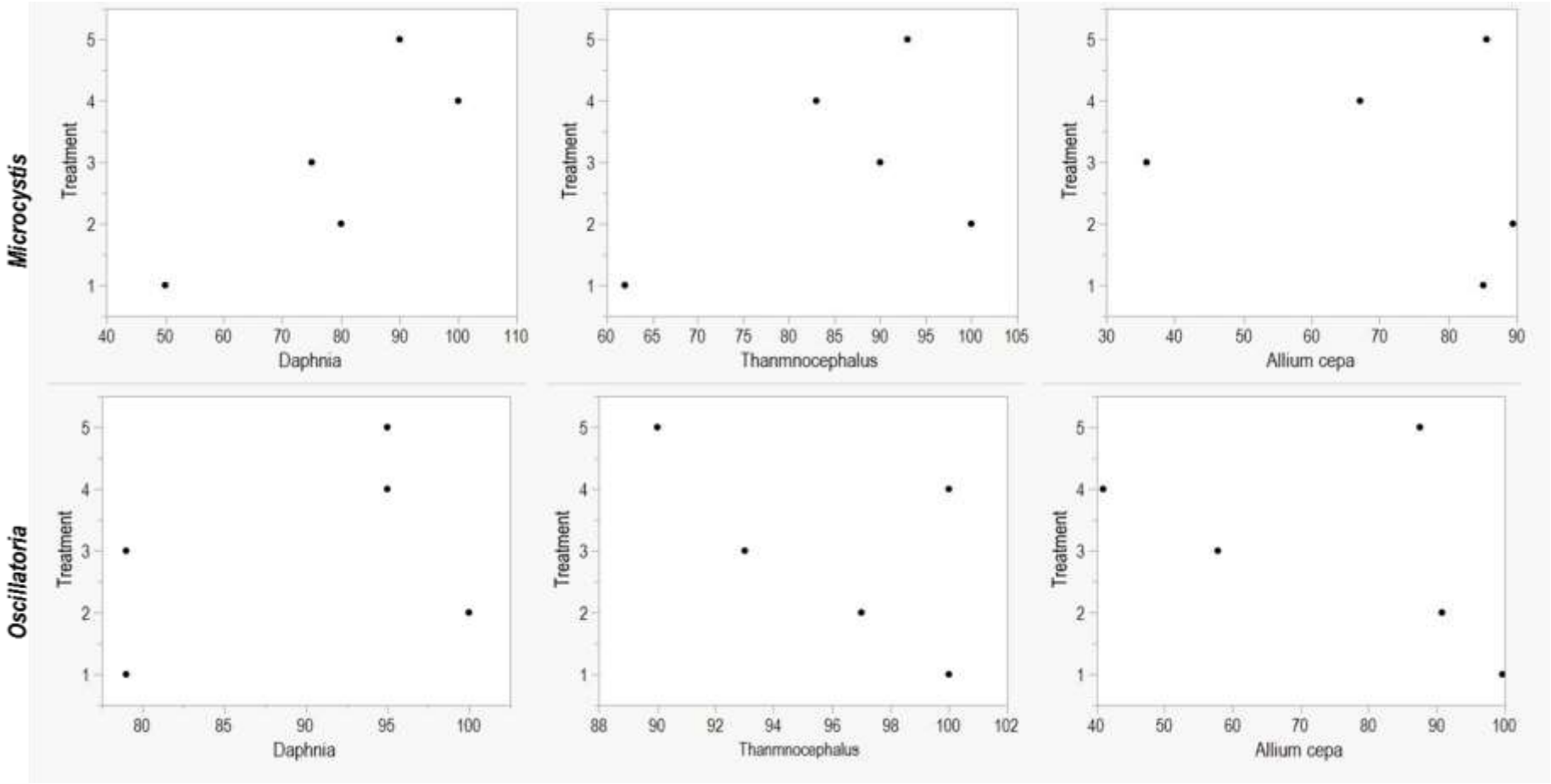

Figure 6.5: A bubble plot representation of the bio-assays which showed sensitivity to the changes in toxicity with treatments (1-5) against the percentage survival/mitotic index. Similar trends are observed for *Microcystis* and *Oscillatoria*, indicating a reduced survival with treatment from *Oscillatoria*, while an increased survival is seen for *Microcystis*. This data is reflective of the toxicity changes in the water samples. Treatment numbers 1 to 5 on the y axis indicate the control untreated cyanobacteria (1), cyanobacteria treated with isolate 1 (2), treatment with isolate 3w (3), treatment with isolate 3y (4) and

treatment with isolate B (5). The x-axis indicates the percentages of survival for *Daphnia* and *Thamnocephalus* as well as the mitotic index percentages for *Allium cepa*. To obtain a clearer assessment of the findings, a principal component analysis (PCA) was conducted of the water chemistry variable that did not show any correlation to the other variables in the correlation test performed. This was done to observe whether it had any relation to the eco-toxicity assay findings (Figure 6.6). The factor loading data from the analysis (Table 6.6) indicated that factor 1 was the in-vivo bio-indicator responses, while factor 2 was the agricultural bio-indicator response to copper. Factor 3 indicates a relationship between the crustaceans and plants to copper and ammonia. Therefore these were the main components influencing the data in the observations.

Table 6.6 Factor loadings from principal component analysis

	F1	F2	F3	F4	F5	F6
Daphnia	0.599	-0.533	0.400	0.406	-0.043	-0.174
Thanmnocephalus	0.702	-0.262	0.542	-0.321	-0.061	0.195
Allium cepa	0.319	0.814	0.112	0.436	-0.106	0.146
Lactuca sativa	0.851	0.127	-0.299	0.001	0.414	0.003
Ammonia	-0.758	-0.418	0.281	0.273	0.266	0.163
Copper	-0.286	0.634	0.662	-0.172	0.168	-0.147

The bio-indicator crustaceans were closely related to each other (Figure 6.6), whilst the plant indicator organisms were also in the same quadrant. This indicated similarities in plant (lettuce and onion) responses and similarities in the crustacean (*Daphnia* and *Thamnocephalus*) responses, which can be expected as they are more closely related organisms. Copper concentrations however, showed a close relation to *Microcystis* treated with isolate 1. *Oscillatoria* samples showed a close correlation to the survival of the crustaceans and the mitotic index of the onions. The Microcystis water samples were closely grouped to the changes in ammonia, with similarities in the 3w and 3y sample; while the M plus B and M control samples had less relation to any of the parameters.

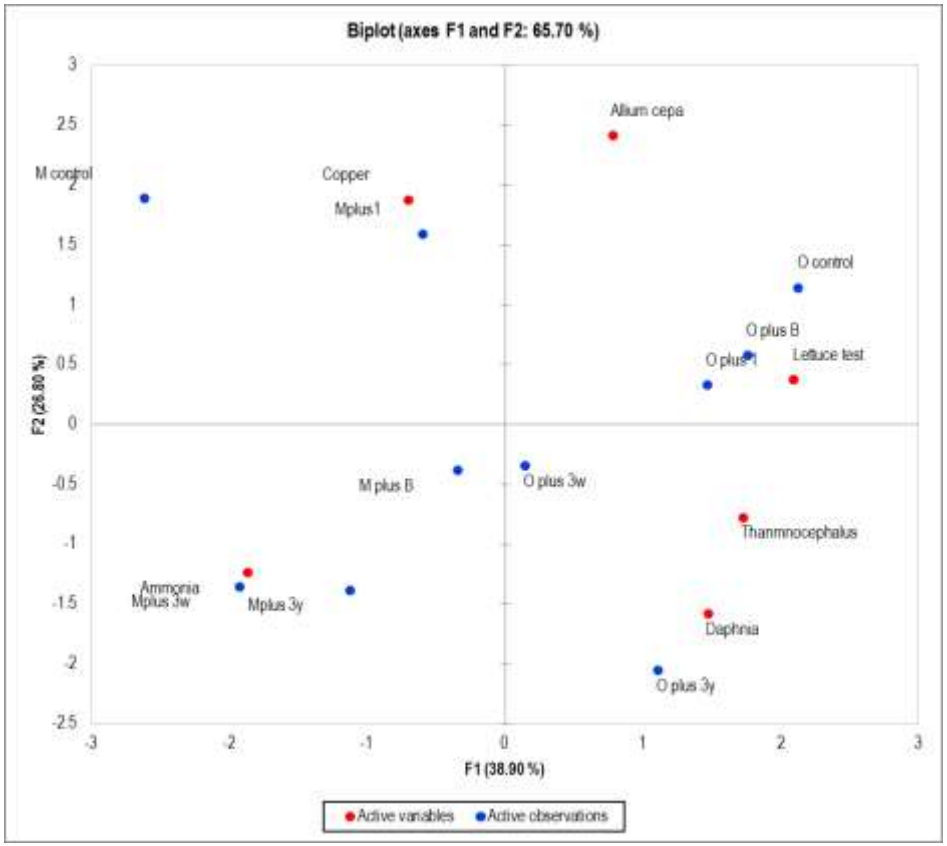

Figure 6.6: Principal component analysis of the observations in this study and their relation to each other. Total variation accounted for by factor 1 and 2 is 66%.

A summary of all the findings are represented in Table 6.7. The "+" sign represents sensitivity of the bio-indicator to the changes in the different water samples. The "-" sign indicates no sensitivity. All these findings except apoptosis were indicated relative to the toxicity in the control sample. Therefore a "+" would mean the bio-indicator reflected the increase or decrease in toxicity relative to the untreated control sample.

Table 6.7: A summary of all the findings from the toxicity and bio-toxicity assays from treated and untreated *Microcystis* and *Oscillatoria* filtrate water samples. The positive and negative sign is based on the sensitivity to cyanotoxin changes.

Treatment	Microcystins ($\mu g.ml^{-1}$)	Sensitivity to toxin changes				
		L. sativa	*A. cepa*	*D. magna*	*T. platyurus*	Apoptosis
M. untreated	1.45					-
M plus 1	1.05*	+	+	+	+	-
M plus 3w	1.22*	-	-	+	+	-
M plus 3y	1.40*	+	-	+	+	+
M plus B	1.00*	-	+	+	+	+
O untreated	0.68					+
O plus 1	0.71	+	+	-	+	+
O plus 3w	0.78	+	+	+	+	+
O plus 3y	0.67*	+	-	+	+	+
O plus B	0.75	-	+	-	+	-

*Toxin reduction relative to the control untreated water sample.

6.3.9 DNA Fragmentation of Thamnocephalus platyurus exposed to treated and untreated cyanobacteria

Further analysis of apoptosis in *Thamnocephalus platyurus* indicated that isolates with higher survival or higher EC_{50} still showed apoptotic damage of DNA (Figure 6.7). There was no laddering of DNA observed.

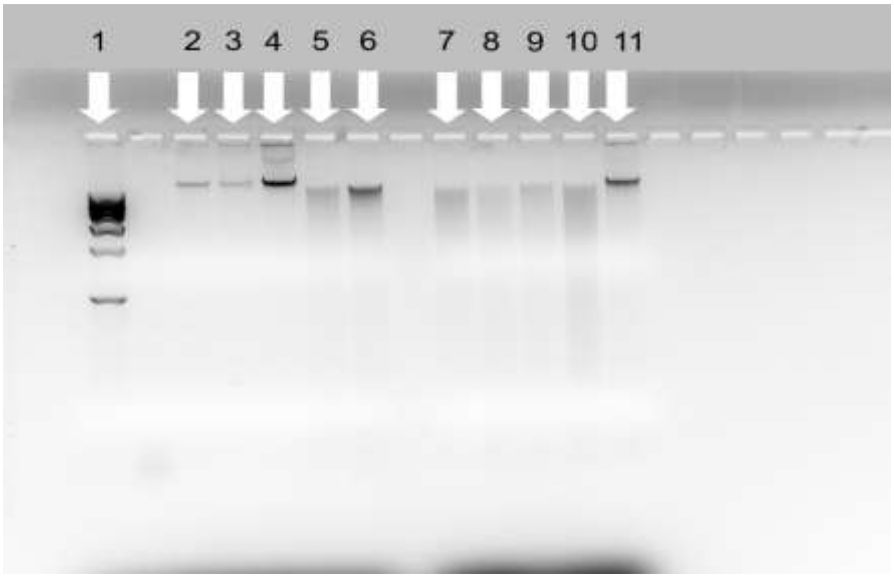

Figure 6.7: DNA apoptosis gel of *Thamnocephalus platyurus* exposed to treated and untreated *Microcystis* and *Oscillatoria* water samples. Lanes 2-4 (M control, M plus 1 and M plus 3w) show minimal smearing. Lanes 5 and 6 show smears of DNA indicative of apoptosis, which are treatments of *Microcystis* with isolate 3y (lane 5) and B (lane 6), In the case Of *Oscillatoria*, the control sample (Lane 7) and water samples treated with isolate 1(lane 8), 3w (lane 9) and 3y (lane 10) all had apoptosis. Only the *Oscillatoria* sample treated with isolate B (lane 11) showed minimal smearing of the DNA after a 24 hour exposure.

6.4 Discussion

This study assessed the use of potential algicidal bacteria in treating the extracellular toxins in mixed cultures dominated by cyanobacteria, *Oscillatoria* and *Microcystis*. Moreover the changes in toxicity linked to variation in microcystin concentration (determined by ELISA procedure) were verified through the response of various bio-indicators (in vivo exposures). A battery of bio-indicator species were used, including agricultural produce (*Lactuca sativa* and *Allium cepa*) and freshwater crustaceans (*Daphnia magna* and *Thamnocephalus platyurus*).

An assessment of the viability of a biological control is a crucial step in determining whether further research is to be conducted in up-scaling or optimization. This is critical in ensuring there are no adverse environmental impacts that arise from a biological control intervention. On a higher order organism scale, the historical failures of some classical biological control interventions indicate the need for environmental response testing to gauge the viability of a control agent (Stiling, 1993). The advantage in lower order organisms is host specificity. Most of the research reported on biological control of cyanobacteria has not been well conducted in terms of full-proofing (ensuring no secondary or indirect adverse impacts) biological treatments of cyanotoxins, with most studies focusing on the lytic or toxin reducing effects of bacterial isolates (Kim et al., 2008; Nakamura et al., 2003; Ren et al., 2010; Su et al., 2016a; Su et al., 2016b). In the present study, the changes in the toxicity of a treatment were first assessed from the cyanobacterial response but also from the subsequent response of organisms exposed to the treated water.

Microcystin toxin reduction of over 80% has been reported from the use of bacteria (Su et al., 2016b) and treatment technologies such as ozonation (Liu et al., 2010). This study indicates reductions up to 30%. In a system where the toxicity is reduced, the use of an environmental indicator can indicate the improvement of water quality (reduced toxicity) after the treatment. This is particularly relevant in cyanobacteria, where more than one toxin variant is present at a given time, therefore the reduction of one toxin does not necessarily deem the water in question safe. Furthermore, it gives an indication whether there could be any secondary adverse effects from the treatment and whether a mixed response could be expected when multiple co-dominant cyanobacterial species exposure occurs.

Cyanobacterial response

The reduction in cyanobacterial photosynthetic pigments has been linked to cell stress or a reduction in cell abundance in water (Kasinak et al., 2015). The changes in phycocyanin, which is an accessory photosynthetic pigment, indicated that the cells exposed to isolates 3w and B were more stressed in the

case of *Microcystis*, whilst exposure to isolate 1 and B resulted in greater stress for *Oscillatoria.* These findings might show that the different isolates have different relationships with the two types of cyanobacteria. This might mean that whilst one isolate may be predatory to *Microcystis*, it may be beneficial to *Oscillatoria*. Phycocyanin, in this case *c*-phycocyanin, is an accessory photosynthetic pigment, with antioxidant properties and its reduction is usually indicative of cell damage or lysis (Zhang et al., 2011). However, the extraction of this pigment has been found unreliable in small samples (Horváth et al., 2013), which may have been the case in this study, where 0.1g of cyanobacterial cells were used. Chlorophyll *a* concentrations also showed a similar trend to the phycocyanin measurements in the present study. When assessing the changes in toxicity, the most significant reduction was in water treated with isolate 1 and B for *Microcystis*, and isolate 3y in the case of *Oscillatoria*. This indicated that the changes in phycocyanin were not directly related to the changes in toxicity observed or any cell stress (Figure 6.1). This would indicate that perhaps no cell stress can be linked to phycocyanin, unlike chlorophyll, which is another more widely used indication of cell growth. Furthermore, changes in pigmentation measurements need to be considered in conjunction with other assays to verify cell stress.

In the present study, the changes in the microcystin toxicity were determined through the ELISA antibody assay, with a relative wide cross-reactive range and has also been known to detect even cleaved ADDA portions of microcystins (Samdal et al., 2014). This method therefore requires confirmatory testing through high performance liquid chromatography (HPLC) to determine which toxin variants are present and also whether they are reduced or not. However, as it is a commonly applied and robust method, the study aims were to determine whether the changes in microcystin toxicity as indicated by ELISA were reflected in the biological indicator response.

Eco toxicity assays- Crustacean response

Most of the biological indicator tests have been applied to test the effects of chemicals, with most of the work using plants as bio indicators. Only 2% of the literature at the time (2006) were attributed to ecosystems and amphibians (Burger, 2006). However, there has been research linked to *Daphnia* response to the changes in the environment (Neves et al., 2015), showing it is able to reflect the prevalence of cyanobacterial toxins and other environmental stressors (Lürling, 2003). Another study has however found low concentrations of microcystins to have no effect on the mortality or stress indicators of this crustacean under chronic exposure (Chen et al., 2005). The findings from our research indicated the ability of *Daphnia magna* to respond to toxicity changes. However the *Daphnia* mortality did not show correspondence to slight (10-15%) fluctuations in microcystin concentration. This

was seen in different isolate treatments such as isolates 3w and 3y in the case of *Microcystis*. The same observation was made in the *Oscillatoria* sample treatments, where the 3% fluctuation in toxicity between water treated with isolate 1 and the control sample was not well indicated. The 7% difference in toxicity between isolate 3y and B treatments was also not well indicated by the mortality of *Daphnia*. Only isolate 3w which resulted in 15% more microcystins, corresponded to a lower survival rate.

When assessing the response of another freshwater crustacean, *Thamnocephalus platyurus*, the findings indicated a greater sensitivity to toxins, confirming the findings of earlier studies (Kim et al., 2009). Variation in microcystin concentration in the different isolate exposure groups showed slight mortality changes in accordance with the slight changes in toxicity. This indicated the ability of *Thamnocephalus* to detect these changes in treatments that occurred in mixed population cultures. Similar findings were reported by Bober and Bialczyk, (2017) and Maršálek and Bláha, (2000), who found *Thamnocephalus* to be more sensitive than *Daphnia* to cyanobacteria. The longer-term exposure of *Daphnia* indicates a higher toxicity response over time, as opposed to the acute response of *Thamnocephalus*.

Seed and plant bio-indicator response

The impacts of microcystins in water on plants may also have significant implications for agriculture and human consumption, with a requirement for assessment of bioaccumulation of these toxins in fresh produce irrigated with toxin contaminated water (Gutiérrez-Praena et al., 2014). Gutiérrez-Praena et al. (2014) further reported microcystins in fruit and vegetables irrigated with contaminated groundwater for irrigation and the presence of toxins in water indicated for agricultural purposes. This raises the relevance for study of agricultural produce response to these toxins.

Other studies related to lettuce and other crops in cyanobacteria bloom prone areas indicated the presence of trace amounts of microcystin R-R in most vegetables tested (Li et al., 2014). Maisanaba et al. (2018) reported that there was an increased consumption of certain raw leafy vegetables despite a higher bioavailability of the cyanobacterial toxin cylindospermopsin in uncooked spinach compared to spinach boiled for two minutes (Maisanaba et al., 2018). Being a vegetable that is consumed primarily raw, the lettuce plant is also likely to be ingested with trace amounts of microcystins. Testing for the sensitivity of *Lactuca sativa* to wastewater polluted with heavy metals indicated its suitability as a bio-indicator, with reduced germination reflecting the toxicity of the water (Charles et al., 2011). Lettuce seedlings exposed to microcystin LR showed that germination did not differ significantly to the control in terms of fresh weight and elongation in concentrations lower than 6µg.ml^{-1} (Z. Wang et al., 2011). A study by Corbel et al. (2015) indicated an increased aerial growth of tomato (*Solanum lycopersicum*)

exposed to low microcystin concentrations, whilst no major differences were observed in the germination of the control and the seedlings exposed to low microcystin concentrations. A similar trend was observed in the present study. This means that for lettuce, the germination and other physical traits were not sensitive enough endpoints to indicate microcystin effects at lower concentrations (Figure 3). More biochemical or molecular analyses may be useful in the case of *Lactuca sativa* assays where toxin concentrations fluctuate slightly.

The *Allium cepa* assay has been described as an efficient and reliable method for testing environmental pollutants among other materials, having a response similar to other higher order organisms such as rodents. The mitotic index has also been applied as a measure of the organism response, with increases or reductions in mitotic index relative to the control sample being an indication of adverse impacts on the cell root (Leme and Marin-Morales, 2009). In the present study, variation in the mitotic index as a biomarker, did not show complete agreement with to the corresponding changes in microcystin toxicity. Also, the root lengths were similar, with no morphological differences. A study of differences in 10 fold concentrations of microcystin and aeruginosin indicated a good correlation between toxin concentration and the mitotic index in onion roots cells (Laughinghouse et al., 2012). Similar to the lettuce and *Daphnia* responses, minor variation in toxicity were not reflected in the mitotic index response. The most sensitive indicator to toxicity changes according to the findings of the present study was shrimp, *Thamnocephalus platyurus*.

Nonetheless, the changes in water chemistry also need to be considered as contributing factors and therefore controlled for. The changes in ammonia and orthophosphate in the treated water samples may have influenced the respective TN:TP (total nitrogen: total phosphorous) ratios, which are essential factors in the growth of cyanobacteria (Ndlela et al., 2016). In addition, the potential of the cyanobacteria to produce aeruginosin (*Microcystis*) and saxitoxin (*Oscillatoria*), suggest that other additional toxins that may have had added impacts on the bio-indicators, that not accounted for in the scope of this study, may have played a significant role. Moreover, the cross-reactivity of the ELISA does not informatively indicate which toxin analogue is increasing or decreasing in the overall toxicity findings although the wide cross-reactivity may include a variety of toxins. Regarding isolate 3w, the lowest survival, germination and mitotic index were observed in *Microcystis* treated with this isolate, characterized by a simultaneous 15% reduction in microcystin toxicity according to the ELISA approach. This may suggest that other factors apart from the cyanotoxins which may have caused the adverse effects of this treatment. This cannot be clearly concluded from the present study but may be a point for consideration in future works. In terms of cyanotoxin reduction, the bacterial isolates were more effective against extracellular cyanotoxins from *Microcystis* compared to *Oscillatoria*.

The principal component analysis (PCA) indicates that the plants and crustaceans had similar response patterns. From these findings, isolate 1 and B appeared to have a positive relation to bio- indicator survival across the assays conducted. When assessing the genetic apoptosis in *Thamnocephalus platyurus* (the most sensitive), all the treatments resulted in DNA apoptosis except for isolate B, which showed more intact DNA. This suggests that the reduction in cyanotoxins needs to be fully validated through biological indicators and more importantly, that certain biological control isolates may have more favourable environmental impacts, despite similar indications of microcystin reduction.

Another point to consider in this type of research is the selection of economically feasible bio-indicators. *Daphnia magna* is culturable in the lab and not expensive to grow, the *Lactuca sativa* bioassay is the most affordable of the bioassays conducted in this research, although not as sensitive. *Allium cepa* experimental set up is also a fairly feasible experiment, although the imaging requires good microscopy. *Thamnocephalus* acute exposure is also a feasible assay to conduct. Analysis beyond the basic mortality and visual screening is however required for more informative data.

This present study confirmed that toxicity changes in biological control systems can be reflected through bio-indicators, although the sensitivity of the indicators towards microcystins may vary among organisms. The organisms used were able to indicate that toxicity was not well reduced in *Oscillatoria* treatments and toxicity changes greater than 15% could be well-reflected in all the treatments. More importantly, if the biological control agent performance can be optimized (greater toxin reduction), it will be easier to predict the potential environmental impacts.

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7. General conclusions

Research in the area of mitigating cyanobacterial blooms is constantly changing and advancing. A report on the ability of cyanobacteria to continue thriving under warmer temperatures and high nutrient loading emphasized the need for further research into bloom mitigation within the context of climate change (Paerl and Huisman, 2008). Most of the great lakes and rivers globally have had reports of toxic bloom occurrences (Paerl and Otten, 2013). Being a developing and water scarce country, the occurrence of these blooms is of great concern in South Africa (Matthews and Bernard, 2015). In an effort to consolidate the available information on this research from an African context, the current study compiled a review on the occurrence and research of these blooms in the African continent. The findings from the current study review (Chapter 2) indicated a need for more cohesive research efforts to have an information sharing platform and more research into this area. With water scarcity and limited infrastructure in developing countries, innovative and passive treatments are a feasible option.
The second aim of this study was to obtain information on cyanobacterial biological control, particularly through the use of bacterial isolates. The current study revealed that a majority of the research conducted has been at laboratory level in the application of live bacterial cells against live cyanobacteria. More importantly, the bacterial numbers were often at ratios of 1:1 to cyanobacterial cells or at ratios recommended for lysis. The use of axenic unicellular type strains for most of the research using predatory bacteria may also misrepresent the actual colonial cyanobacterial response in the natural environment, within a mixed population of microorganisms.

Interesting findings from two earlier studies informed the approach applied in this study. Firstly, that lowered numbers have a bacteriostatic effect as opposed to total lysis, thereby preventing toxin release and subsequent bloom progress (Flaherty et al., 2007). Secondly, the presence of microcystin degrading bacteria has been found to influence a shift in toxic to non-toxic *Microcystis* isolates (Zhu et al., 2014). Based on this, the current study assessed the response of two cyanobacterial isolates from different parts of the water column to predatory bacterial isolates at half the recommended 1:1 ratios. The current study compared three bacteria isolated from bloom water to a *Bacillus* related isolate. This was to assess whether the isolates had a similar impact as the reports of *Bacillus* in an earlier study (Gumbo and Cloete, 2011), thereby serving as a form of reference. The present study found a reduction on toxicity, with the more toxic variant of microcystin (L-R) reduced within a four day period as opposed to the less toxic microcystin R-R.

Microcystis is naturally colonial and buoyant in form, occurring at the surface of water and reported more commonly as the dominant species in cyanobacterial blooms, often toxic. *Oscillatoria* is a

filamentous sub-surface occurring, common bloom causing species, capable of producing various toxins. The response of these two different cyanobacteria provided a comparison in the ecology and susceptibility of these bloom causing agents. The current study found that overall, cyanobacterial biomass growth was well represented by increases in chlorophyll *a* and that the growth of the isolates was similar to other reported studies. The cyanobacteria and predatory bacteria were exposed at their exponential growth phase. Testing for cyanobacterial response at other stages of the growth curve may also be useful, although nutrient depletion and other factors will need to be considered. The current study simulated ideal growth conditions from a nutritional and physicochemical perspective under laboratory conditions, although the cultures were non-axenic.

From a stress response measure, chlorophyll *a* proved a more reliable indication of cell stress in *Oscillatoria*, whilst *Microcystis* changes in chlorophyll *a* fluctuated randomly. This could well be due to the lower biomass concentrations used in this study, or from contributing factors from the mixed microorganism diversity in our non-axenic cultures. Phycocyanin, as an accessory photosynthetic pigment did not give a clear relation to cell stress in both isolates. Alkaline phosphatase measurements were also not indicative of stress or lack of it based on mixed findings from earlier studies. Perhaps an increase in stressor either from higher bacterial numbers or cell lysing chemicals may have given more of indication on what the expected changes might have been in the current study.

Identification of the bacterial isolates was not conclusive to genus level for isolate 3w and B. Isolate B was donated as a *Bacillus mycoides* strain from a culture collection from Stellenbosch University and exhibited similar colony morphology to *Bacillus*. However, although it indicated predatory characteristics, the isolate was motile and could not be identified as *Bacillus mycoides* through universal 16s ribosomal DNA primers, exhibiting a similarity to the *Lysinibacillus* genus instead. However the interspecies similarity within these groups is well reported and specific distinguishing biochemical tests and primers are required to ensure correct genus assignment. The same was observed for isolate 3y, which suggested relation to the *Pseudomonas* genus. Only isolates 3w (*Pseudomonas rhodesiae*) and isolate 1 (*Aeromonas lacus*) were identified to a greater confidence level. *Aeromonas* is among the genera in abundance in bloom scums (Steiner et al., 2017). With the exception of isolate B, this study therefore concurs with other reports of *Proteobacteria* being the common phylum associated with algicidal bacteria or toxin breakdown. If conclusively assigned to genus level, isolate B may be among the few reported *Lysinibacillus* isolates applied in bloom control. *Oscillatoria* was identified more conclusively based on microscopic identification as opposed to molecular identification using the 16s universal primers. The difficulty in identifying this isolate is also due to a limitation in more isolated wild strain sequences being deposited on the database of GenBank

for example. Further identification is definitely required in the case of these isolates. *Microcystis*, being better studied was positively assigned to the genus of *Microcystis* and grouped closely to *Microcystis aeruginosa*.

The addition of predatory bacterial numbers at half the number of dominant cyanobacteria indicated cell stress from cell surface morphological changes in both cyanobacterial isolates. Assessment of the internal cell structure is recommended for future research.

ELISA measurements of toxicity changes at the end of the exposure period of four days exhibited fluctuations of approximately 30% in the measured microcystins reduced relative to untreated samples. The fluctuations were also primarily due to the ELISA detecting a rise in one toxin variant and decrease in another. Therefore, unless the control agents are optimized to degrade more than one variant or the observation period is longer than four days, ELISA may not be useful in distinguishing toxin variant. Based on this fact, ELISA may not be recommended for quantifying slight changes in toxicity of cyanobacteria that produce multiple toxin variants as neither can be well quantified. For further research, HPLC is recommended at the beginning of the study, unlike the approach adopted in this study, where HPLC confirmation was conducted after obtaining varying microcystin concentration changes over repeated experiments. Due to feasibility, however, ELISA was the more economic approach. Although reductions were noted, the dynamics of microcystin variant changes could not be extrapolated earlier in the study.

Interestingly though, it was this cross-reactivity of ELISA that aided in the detection of toxins from *Oscillatoria*, when toxin gene marker screening indicated neither the anatoxin nor microcystin gene cluster present in the isolate. Although the screening of *Oscillatoria* did not indicate the presence of *mcy* toxicity gene markers, it is understandable that the primers developed for this gene cluster may not amplify regions of this gene which vary. There is also a need to further optimize the conditions for gene amplification, as the applied conditions were not suitable for gene amplification in the case of *Oscillatoria* and determine more suitable primers for the detection of the toxicity gene markers. However both ELISA and HPLC data confirmed the production of microcystins or microcystin like toxins in *Oscillatoria* dominated cultures. Furthermore, other toxins may be produced by both these cyanobacteria, which were not screened for in the current study. Therefore toxicity needs to be screened by molecular, ELISA and HPLC techniques, with investigation of more hydrophobic microcystin variants and toxins such as aeruginosin, oscillatoxin for example. This is particularly important in monitoring whether bacterial isolates are able to reduce or degrade these toxin variants in the presence of toxins discovered in this research, under similar dynamics.

The variation in bacterial predator response needs to be optimized such that toxin reduction does not deviate above 10% of the expected toxin removal. Due to the complexity of microbial populations in the non-axenic cultures and the proposed integrated relationship among these, physicochemical parameters and the toxicity of the cyanobacteria (Hulot and Huisman, 2004), it is not easy to determine which factors directly influence bacterial efficiency. However the ELISA and HPLC toxicity reduction findings showed that isolate 3w and B were the more effective isolates of the four tested in this research. This was from an intra and extracellular perspective. Chapter 5 provided the average toxin reduction from treatments of cyanobacteria, whilst chapter 6 provided a case study of individual treatments and the bio-indicator response. A case study of eco-toxicity testing in the current study showed isolate B as the more environmentally favourable isolate, indicating higher survival of the tested bio-indicators as well as reduction of toxicity. Isolate 3w indicated good toxin reduction overall, however the impact on the tested indicators suggested the mode of microcystin reduction has adverse impacts on bio indicator survival. This needs to be further investigated more from the mode of toxin reduction in all the bacterial isolates from the present study. The mortality response of seeds and plant roots did not show enough sensitivity to the toxin changes. This would mean the crustaceans or higher order aquatic organisms are better suited as indicators to low toxicity changes. The case study in eco-toxicity confirmed the hypothesis of lower toxicity being reflected in sensitive bio-indicators. Although this is highly dependent on the mode of action, as observed in isolate 3w.

Moreover, the feasibility in the present study impacted the approach used in terms of research objectives. Some statistical significance could not be deduced on the battery of bioassays conducted due to limited repeats, although observations were noted. In the case of the *Allium cepa* assay, the present study adopted the approach of Kumari et al., (2009), which based the response of *Allium cepa* to cytology observations, although no statistical significance was provided.

In closing, the current study was able to indicate microcystin reduction and cell stress in a mixed population of both colonial and filamentous cyanobacteria within a four day period, using passive treatment. Bacterial performance needs to be optimized in future research and more information is required on the stress impact of the targeted cyanobacteria. Based on earlier studies, the present study indicated a favourable environmental impact in cyanobacterial bloom waters treated with a *Lysinibacillus* related isolate. The present study also showed effective control in toxicity although complete cyanobacterial lysis was not observed. This may be a potential bacteriostatic effect on cyanobacteria. Although this work has provided further findings to support biological control, it is appreciated that a lot of consideration needs to be put into the practical application of this type of research, in bridging the gap between laboratory science and real time interventions on a larger scale.

Further research is required to determine the mode of action and enhance the microcystin degradation of various analogues of this toxin.

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8. Supplementary Data

Table 8.1 Comparison of the 16S rRNA gene sequences of isolates to sequences deposited in GenBank (Accessed- 12/12/2018)

Isolate	Primer	Accession number	Description	% Similarity	E-value
Microcystis	CYAN 16S	NR_074314.1	*Microcystis aeruginosa* strain NIES-843	98	0.0
Oscillatoria	CYAN 16S	NR_125689.1	*Cylindrospermum marchicum* CCLA strain	87	3e-59
Microcystis Brandwacht	MIC 16S	NR_074314.1	*Microcystis aeruginosa strain* NIES-843	98	2e-117
Microcystis Crocodile farm	MIC 16S	NR_074314.1	*Microcystis aeruginosa strain* NIES-843	98	4e-114
Isolate 1	FD1 and RP2	NR_136831.1	*Aeromonas lacus* strain AE122	94	4e-57
Isolate 3w	FD1 and RP2	NR_024911.1	*Pseudomonas rhodesiae* strain CIP 104664	99	0.0
Isolate 3y	FD1 and RP2	NR_024911.1	*Pseudomonas rhodesiae* strain CIP 104664	83	6e-177
Isolate B	FD1 and RP2	NR_112569.1	*Lysinibacillus fusiformis* strain NBRC15717	89	4e-78

Table 8.2: Water chemistry measurements of treated and control samples- April 2017

Parameter ($mg.L^{-1}$)	M control	M plus 1	M plus 3w	M plus 3y	M plus B	O control	O plus 1	O plus 3w	O plus 3y	O plus B
Potassium	23	21	21	22	22	16	17	16	19	17
Nitrate	220	248	232	221	219	260	244	230	243	236
Orthophosphate	19	19	20	19	19	20	20	20	20	20
Iron	0.64	0.4	0.59	0.58	0.33	0.12	0.09	0.1	0.08	0.1
pH	6.6	6.8	6.6	6.6	6.6	6.5	6.2	6.3	6.2	5.9

Table 8.3: Water chemistry measurements of treated and control samples- May 2017

Parameter ($mg.L^{-1}$)	M control	M plus 1	M plus 3w	M plus 3y	M plus B	O control	O plus 1	O plus 3w	O plus 3y	O plus B
Potassium	8.6	8.9	9	9	8.9	15	16	15	15	15
Nitrate	5	4.7	5.7	3.7	4.2	145	202	163	166	141
Orthophosphate	0.47	0.45	0.41	0.58	0.53	18	22	18	14	20
Iron	0.17	0.17	0.17	0.16	0.16	0.11	0.09	0.11	0.11	0.11
pH	7.3	7.1	7.1	7.1	7.1	6.2	6.2	6.2	6.3	6.3

Table 8.4: Water chemistry measurements of treated and control samples- September 2017

Parameter ($mg.L^{-1}$)	M control	M plus 1	M plus B	O control	O plus 1	O plus B
Potassium	16	22	44	12	119	14
Nitrate	276	276	294	254	202	299
Orthophosphate	19	20	23	19	30	20
Iron	0.77	0.42	0.63	0.06	0.11	0.07
pH	6.6	6.7	7	6.3	7.3	6.6