Impacts of seasonal dynamics on cyanobacterial proliferation and cyanotoxins bioaccumulation in fish species: Comparative study of aquaculture fishponds in Nigeria and South Africa

A PhD Thesis submitted to the Department of Geography and Environmental Sciences, University of Venda in partial fulfillment of the requirements for PhD of Environmental Sciences.

UNIVERSITY OF VENDA

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Declaration

I, Bassey Odo Jones, hereby declare that this thesis is for the Doctoral degree in Environmental Sciences at the University of Venda, and has not been previously submitted for a degree at this or any other institution. This is my work in design and execution, and all reference materials contained herein have been duly acknowledged.

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Dedication

To Our Father*… Leader Olumba Olumba Obu and His Son. His Holiness Olumba Olumba Obu. The ability to do anything comes from God alone; without Him, I wouldn't have accomplished this achievement. He alone is the author and finisher of my research.*

To my lovely parent*…Mr and Mrs Jones Bassey: You laid the foundation for every success story in my life. You are my inspiration and motivation. Your endless prayers toward me finally made me see the light at the end of the tunnel.*

To my loving husband*… Mr Obo Emmanuel Ekpo, having you by my side throughout my study is a blessing. You support my career endlessly despite the distance between us. When the road was rough and tough, your love, support, sacrifices, and advice pulled me through successfully. You are a good man and my best gift from God. I love you endlessly, and may God continue to bless you abundantly.*

To my beautiful children*…Victoria E, Sunshine Obo, Jeremy Obo; You all taught me how to be a mother and be successful in my career. You are my reward from God, and I love you all.*

To my adopted mother… *Mrs Charity Oseifuah: You gave me a home far away from home. You made my stay at UNIVEN easy and smooth as an international student. You cared for my children with love while I spent sleepless nights in the laboratory. Thank you for being part of my success story.*

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Abstract

Aquaculture production plays a pivotal role in advancing Sustainable Development Goals (SDGs), particularly those focused on food security, economic growth, livelihoods, sustainable production, biodiversity conservation, and improved nutrition. Locally produced fish from aquaculture ponds serve as an affordable and accessible protein source in countries like Nigeria and South Africa. However, the rise of cyanobacterial harmful algal blooms (CyanoHABs) poses a serious challenge to the sustainability of fish culture production and human health. In the aquaculture ecosystem, the consequences of these blooms have triggered widespread interest due to cyanotoxin bioaccumulation in cultured fish species. In tropical regions, especially in Africa, there is a paucity of research investigating the temporal drivers of cyanobacterial biomass in aquaculture fishponds. Cyanobacteria are integral to the pond food chain, yet their seasonal dynamics factors and influencing factors remain poorly understood. This knowledge gap is particularly critical as these factors significantly impact aquaculture productivity and the risks associated with cyanobacterial blooms. Therefore, this study investigated the impact of seasonal dynamics on cyanobacterial proliferation and cyanotoxin bioaccumulation in cultured fish. A comparative study design between small-scale commercial fishponds located in Nigeria (NGA) and South Africa was employed in this study. A total of six fishponds located in Calabar Municipality, Cross River State, Nigeria, and Duthuni, Vhembe District, Limpopo Province, South Africa (SA), were selected. Water and *Clarias gariepinus* (African catfish) samples were collected from each fishpond at seasonal intervals (SA - summer, winter, NGA - dry and wet seasons). Seasonal variations in water quality parameters were observed in NGA and SA fishponds. Cyanotoxins analysis was carried out using the Liquid Chromatography-Mass Spectrometry (LCMS). The results of the study revealed that elevated temperatures (>20°C) were consistent during dry, wet, and summer seasons, with a notable winter decrease (16.5°C) at Duthuni, South Africa. Dissolved oxygen (DO) and electrical conductivity (EC) levels were consistently low across seasons, while total dissolved solids (TDS) peaked during winter in South Africa (125–193.2 mg/L) and were lower in Nigeria (15.9–37.7 mg/L). Nutrients, particularly nitrite and nitrate, peaked in Nigeria during wet (10.42 mg/L) and dry (6.45 mg/L) seasons, while South African fishponds exhibited lower concentrations in both summer and winter. The study revealed that cyanobacterial biomass and cyanotoxins in fishponds were not driven by seasonal factors like warmer temperatures or precipitation. Cyanobacteria biomass was

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predominantly influenced by management practices, including feeding, fertilization, nutrient levels, water volume, stocking density, water exchange, and retention time. The LCMS analysis revealed that the microcystin variant MC-RR was absent in fish tissue, while MC-YR was detected in the intestines, gills, muscles, and liver. The highest concentration of 20.9 µg/g was found in the liver. Additionally, untargeted LC-MS identified a broader range of cyanotoxins, including aeruginosins, anabaenopeptins, microcystins, and microginins, in the water samples and fish tissue. These findings underscore the critical influence of fishpond management practices on cyanobacterial dynamics and cyanotoxin bioaccumulation. This study highlights the need for targeted interventions to mitigate cyanotoxins risks in aquaculture systems. Understanding and addressing these factors are essential for ensuring the sustainability of aquaculture production and safeguarding public health in tropical regions.

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Chapter 1

Introduction

1.1 Background

Cyanobacteria are unicellular or filamentous organisms exhibiting the capacity for oxygenic photosynthesis (Ciebiada *et al*., 2020). They inhabit diverse environments, including soils, freshwater bodies, thermal springs, and marine ecosystems (Bhardwaj *et al*., 2024). Like plants, cyanobacteria harness sunlight to transform atmospheric carbon dioxide into organic byproducts (Arora et al., 2021; Keller *et al*., 2021). Under favorable environmental conditions characterized by optimal temperatures, ample sunlight, and nutrient-rich water could trigger the occurrence of a cyanobacterial bloom (Wang *et al.,* 2021). These environmental factors can potentially induce toxins released, specifically cyanotoxins, into the water (Metcalf and Codd, 2020).

The proliferation of CyanoHABs (cyanobacterial harmful algal blooms) poses a significant threat to the safety and sustainability of water resources utilized for human consumption, agricultural irrigation, inland fisheries (including aquaculture), and recreational purposes (Paerl et al., 2020). Aquaculture ecosystems are highly vulnerable to cyanobacterial bloom because cyanobacteria constitute the integral component of the food web as phytoplankton biomass (Paerl, H. W., and Otten, 2013; Backović*et al.,* 2024). Additionally, cyanobacteria can easily adapt to environmental conditions usually encountered in fishponds, such as high temperature, reduced light conditions, nitrogen depletion in the upper layer, a high degree of eutrophication and a decrease in the number of large phytoplanktivorous filter-feeders (Backović*et al.,* 2024; de Lima Pinheiro et al., 2023; Vrba et al., 2023).

Fish ponds are often enriched with nutrients, particularly nitrogen and phosphorus, due to fish feed, waste, and runoff from surrounding agricultural or urban areas. These nutrients provide an essential food source for cyanobacteria, promoting rapid growth (Paerl and Otten, 2013). Excessive nutrient loading can lead to eutrophication, a process that fosters the formation of harmful algal blooms (HABs) (Smith, 2003). High turbidity, resulting from suspended particles or fish activity that stirs up the pond substrate can also influence cyanobacterial growth. While cyanobacteria are generally phototrophic, some species have adaptations to thrive in low-light, turbid conditions due to buoyancy control mechanisms (Reynolds et al., 1987). Turbidity can

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limit the growth of other phytoplankton, giving cyanobacteria a competitive advantage (Scheffer et al.., 1997). Cyanobacteria are considered harmful to aquaculture systems because they affect water quality, leading to the loss of water clarity. They also produce secondary metabolites which can cause a change in the taste and odors of the waters, resulting in negative effects on invertebrate and fish habitats (Paerl, 2014).

There is increasing concern about the frequent occurrence of cyanobacterial biomass in aquaculture ponds and their impact on cultured fish through exposure to cyanotoxins (Chia and Kwaghe, 2015). Cyanobacteria produce harmful toxins, including microcystins (MCs), which pose significant risks to fish. Fish are exposed to cyanotoxins orally by consuming contaminated food or drinking water containing cyanobacterial cells with accumulated toxins. Prolonged exposure to MCs leads to toxin bioaccumulation in fish tissues, causing liver damage and, ultimately, fish mortality.

Fish is a very important source of protein due to its high nutritional value (Food and Agriculture Organization (FAO), 2020). Fish consumption contributes up to 75% of the total animal protein intake (FAO, 2014). In coastal regions of Sub-Saharan Africa, fish serves as a vital component of daily diets, particularly in rural communities where aquaculture significantly contributes to livelihoods (FAO, 2016). However, The bioaccumulation of cyanotoxins in fish tissue poses significant risks to fish production, water quality, human health, ecological balance, and the sustainability of aquaculture systems. (Greer *et al.,* 2017).

1.2 Problem statement

Fish production is highly significant to global food security, economic development, and decreased nutritional deficiencies at a global scale (Ogunji and Wuertz, 2023). Moreover, fish is a valuable protein source in high demand, making aquaculture a highly impactful industry. In Africa, Nigeria holds the second position in aquaculture production, particularly excelling in the cultivation of African catfish, specifically *Clarias gariepinus* and *Heterotis niloticus* (Ogunji and Wuertz, 2023; Oluwatobi *et al*., 2017). Small-scale farmers contribute roughly 1 million tons of fish annually (Ogunji and Wuertz, 2023). These farmers, often operating in developing countries, play a vital role in sustaining local economies and food security. In some regions, such as in the Southeast Asia and Sub-Saharan Africa, small-scale aquaculture provides up to 80% of the total

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fish production, supplying essential protein and livelihoods to millions of people (FAO, 2020). South Africa is positioned as the tenth leading aquaculture-producing nation in Africa, making a 0.28% contribution to the overall aquaculture production in the African region (Adeleke et al., 2020). Trout, tilapia, catfish (*Clarias gariepinus*), marron crayfish, and other species are the freshwater species commonly cultured in South African fishponds (Department of Agriculture, Forestry and Fisheries, 2015). According to the Food and Agriculture Organization (FAO, 2020), small-scale aquaculture is expected to grow as demand for sustainable fish sources rises, with fish farming projected to meet over 60% of global fish consumption by 2030.

However, the level of awareness relating to the negative impacts of CyanoHABs in fishponds is currently low in Nigeria (Adeleke et al., 2020; Chia *et al.,* 2021). Poor management practices, such as inadequate monitoring of water quality, nutrient overloading, and insufficient aeration, have contributed to increased cyanobacterial blooms in small-scale fishponds (Figure 1.1). Moreover, the economic benefits of small-scale aquaculture, particularly in rural communities, often outweigh the costs associated with high management practices aimed at preventing cyanoHABs. Furthermore, the lack of effective monitoring programs in small-scale fishponds heightens the need to investigate the presence of cyanotoxins in fishponds (Chia et al., 2021). In South Africa, the heightened vulnerability of fishponds to the proliferation of harmful cyanobacteria is exacerbated by suboptimal environmental conditions, including elevated temperatures and increased aridity (Adeleke et al., 2020).

Cyanotoxins released from cyanobacteria can be harmful to fish, animals and humans (Jones *et al.,* 2021; Pei *et al.,* 2020). Fish exposed to cyanotoxins could suffer sublethal effects such as toxin accumulation in the liver, leading to liver damage, degradation of hepatocytes, and fatal liver haemorrhaging (Passos et al., 2023). Cyanotoxin poisoning in animals and humans has been linked to carcinogenicity, gastroenteritis, skin reactions, liver damage, vomiting, headaches, allergic reactions and mortality (Buratti *et al*., 2017; Hilborn *et al.,* 2014; Lad *et al*., 2022; Niture *et al.,* 2023; Svirčev *et al.,* 2022). People can readily come into contact with these toxins through water, seafood, crops, vegetables, fish, and dietary supplements contaminated with cyanotoxins, or by ingesting them during recreational activities (Buratti et al., 2017).

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Figure 1.1: Blue-green algae bloom in aquaculture fish ponds in Offiong Etim and Essien Town community, Calabar, Cross River State, Nigeria

The seasonal variations influencing cyanobacteria in tropical fishponds are underexplored, despite their importance in optimizing fish production. In South Africa, seasonal patterns are defined by summer and winter, while Nigeria experiences distinct wet and dry seasons. Investigating how cyanobacterial populations fluctuate across these seasonal cycles and identifying their primary drivers is essential to enhance aquaculture sustainability and reduce health risks in these regions.

1.3 Justification of the study

Aquaculture production contributes positively to Sustainable Development Goals (SDGs) targeting food security, economic development, livelihood, sustainable production practices, conserving biodiversity, and improving nutrition. Additionally, locally produced fish from aquaculture fishponds are an affordable protein source in Nigeria and South Africa. However, increased CyanoHABs in fishponds pose a significant threat to fish culture production sustainability and human health. This growing concern highlights the need to investigate the impacts of seasonal dynamics on cyanobacterial proliferation and cyanotoxin bioaccumulation in

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cultured fish This research is pivotal for sustainable aquaculture production and safeguarding public health.

Notably, South Africa and Nigeria differ in their aquaculture infrastructure, particularly in the type of fishpond used for commercial fish farming. South African fishponds are predominantly earthen ponds, which resemble natural aquatic environments and are more exposed to external environmental conditions, such as soil-water interactions that can influence cyanobacterial growth. In contrast, Nigerian fishponds are primarily concrete and tarpaulin ponds, which are more controlled environments with reduced direct contact with soil and groundwater but may have higher nutrient retention due to feeding and waste accumulation. This diversity in fishpond types may further influence cyanotoxin production and accumulation. Earthen ponds may provide conditions conducive to natural bloom cycles, whereas concrete and tarpaulin ponds may have different water retention times and nutrient dynamics, potentially affecting toxin levels in fish. This study is essential for understanding how cyanotoxin contamination varies across different climates, aquatic environments, and aquaculture systems in Africa. The findings will contribute to improved monitoring strategies, risk assessments, and regulatory frameworks for safer aquaculture practices in both South Africa and Nigeria.

1.4 Main Aim

The primary aim of this study is to investigate the impact of seasonal dynamics on cyanobacterial proliferation and cyanotoxin bioaccumulation in fish species.

1.4.1 Specific Objectives

1. To assess the physical and chemical parameters of water quality in the fishponds.
2. To detect cyanotoxins in fishponds by employing non-targeted Mass Spectrometry analysis.
3. To investigate the bioaccumulation of cyanotoxins in *Clarias gariepinus* harvested from fishponds.
4. To identify toxic and non-toxic cyanobacterial genes present in water and fish tissues samples.
5. To investigate the impact of seasonal dynamics on cyanobacteria proliferation in aquaculture fish ponds.

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1.5 Hypotheses

- Seasonal variations in environmental factors significantly influence the dynamics of cyanobacterial biomass in aquaculture fishponds.

- *Clarias gariepinus* harvested from fishponds exhibit bioaccumulation of cyanotoxins due to exposure to cyanobacteria in the aquatic environment.

1.6 Methodologies followed in the study

The research encompassed field data collection, laboratory investigations, and data analyses using Liquid Chromatography Mass Spectrometer Instrument. Four field trips for data collection took place during specific seasonal periods—January, February (summer), June, June (winter), August, September (wet season) and November and December (dry season). The seasonal classifications are based on typical weather patterns observed in each region. In South Africa/Nigeria, the dry/wet and winter/summer seasons generally align with the months selected for this study. The study employed various research methodologies detailed in separate chapters.

1.7 Seasons in Nigeria and South Africa

South Africa, being predominantly a temperate region, experiences four distinct seasons: summer (December–February), autumn (March–May), winter (June–August), and spring (September–November). In contrast, Nigeria, as a tropical region, has two main seasons: the wet (rainy) season (April–October) and the dry season (November–March), with variations in timing depending on the region. However, this study aligns seasonal comparisons based on key environmental factors that influence cyanotoxin production, including temperature fluctuations, rainfall patterns, and cyanobacterial bloom periods. In Nigeria, blooms are expected to be most pronounced during the dry season due to increased water stagnation and concentration of nutrients, whereas in South Africa, bloom intensity is likely highest during the summer months when water temperatures are elevated. Conversely, the lowest bloom periods occur during the wet season in Nigeria, when dilution effects reduce cyanobacterial concentrations, and in winter in South Africa, when lower temperatures suppress bloom formation. This study compares cyanotoxin dynamics in both countries by analyzing periods of peak and low cyanobacterial abundance, temperature variations, and nutrient influx, ensuring a more ecologically meaningful comparison. This approach accounts for regional differences and provides a robust framework for assessing cyanotoxin prevalence in diverse African fishponds environments.

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Dry Season:

- **Duration:** November to March.

Wet (Rainy) Season:

- **Duration:** April to October.

Summer:

- **Duration:** December to February.

Winter:

- **Duration:** June to August.

1.8 Thesis structure

The thesis structure is based on five objectives (submitted to peer-reviewed journals), and conclusions with recommendations for future studies. Given the publication-oriented format, minor repetition may occur in the introduction, experimental sections, and references. Nevertheless, each chapter presents distinct results, discussions, and unique recommendations specific to its content.

1.9 Definition of key terms

Cyanobacteria proliferation refers to the rapid and often excessive growth or reproduction of cyanobacteria in a particular environment, such as water bodies like lakes or ponds.

Fishponds are artificial or man-made bodies of water specifically designed for the rearing, breeding, and cultivating of fish.

Cyanometabolites refer to metabolites produced specifically by cyanobacteria, and these metabolites include a wide array of compounds such as cyanotoxins, pigments, antibiotics, and other bioactive substances.

Cyanotoxins are toxic substances produced by certain types of cyanobacteria.

Molecular analyses refer to techniques and methods used to study biological molecules at the molecular level. These analyses involve examining and manipulating molecules such as DNA, RNA, proteins, and metabolites to understand their structure, function, interactions, and other properties.

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Non-targeted metabolomics is the comprehensive analysis of all metabolites in a biological sample without specifically focusing on or targeting particular compounds.

LC-MS stands for Liquid Chromatography-Mass Spectrometry, a powerful analytical technique that separates, identifies, and quantifies compounds within a sample.

A season is a division of the year characterized by specific weather patterns, daylight hours, and ecological conditions, primarily influenced by the Earth's tilt and orbit around the Sun. Most commonly, seasons are categorized as spring, summer, autumn (fall), and winter in temperate regions, while tropical regions often experience wet (rainy) and dry seasons based on rainfall patterns rather than temperature changes.

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Chapter 2

Literature Review

2.1 Introduction

Cyanobacteria are photosynthetic organisms that can harvest light due to the presence of accessory pigments and chlorophyll-a (Sanseverino *et al.,* 2016). They play a major role in nitrogen, carbon, and oxygen dynamics in aquatic environments. Cyanobacteria produce a variety of compounds including toxins, especially in large biomass. This also affects the taste and odor of the water quality. They exist in water bodies such as lakes, ponds, springs, wetlands, streams, and rivers. Cyanobacteria have bloom-forming abilities and can reproduce exponentially to form blooms during favorable conditions. Cyanobacteria produce toxic compounds such as hepatotoxins or neurotoxins) (Ammar *et al*., 2015; Buratti *et al*., 2017; Magonono *et al.*, 2018).

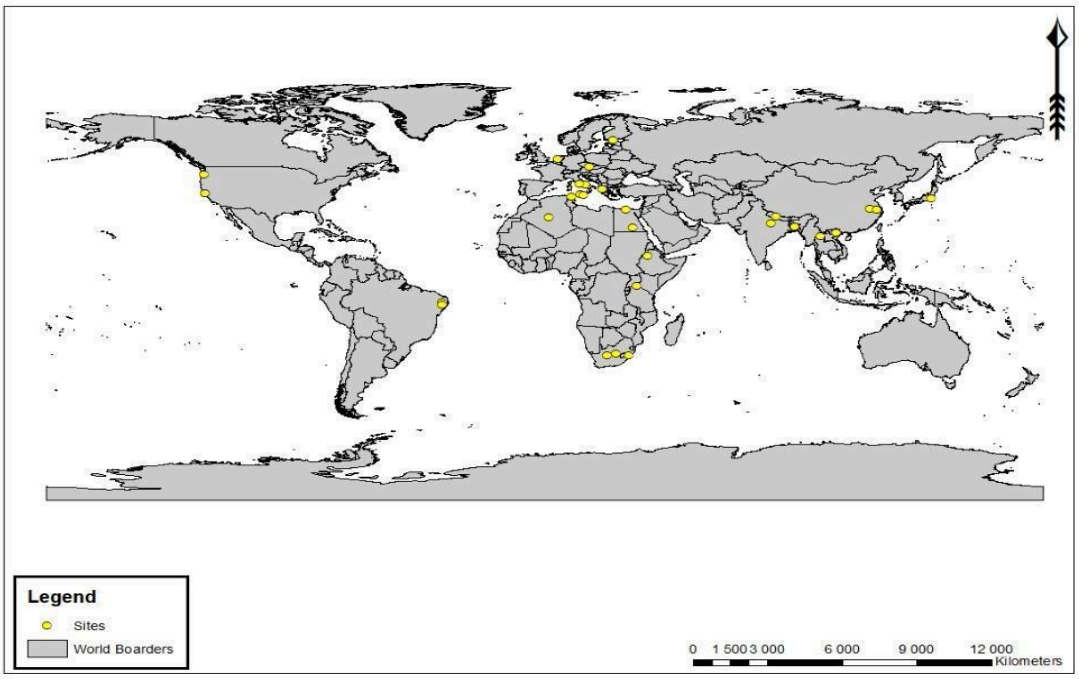

Figure 2. 1: Summary of selected cyanobacteria bloom sites reported globally

The frequency, magnitudes, and duration of cyanobacteria have increased in the aquatic ecosystem. An increasing trend of cyanobacterial bloom incidence affecting large areas of the

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aquatic ecosystem, aquaculture, and fisheries has been reported in many countries globally, as represented in Figure 2.1. The presence of bloom in various aquatic ecosystems despite the differences in geography indicates that surface water is highly vulnerable to cyanobacteria bloom in most parts of the continent ranging from Africa to Europe.

2.2 Classification of cyanobacteria

Vincent (2009) documented that ecologically, there are three major groups in the aquatic environment.

(1) Mat-forming species: they are easily identified and visible because they form periphytic biofilms over rocks, sediments, and submerged plants.

(2) Bloom formers: they create a wide range of water quality problems and are most common in nutrient-rich water bodies.

(3) Picocyanobacteria: they are extremely small cells because of their algal-like appearance and possess chlorophyll rather than bacteriochlorophyll.

However, scientifically, there are 5 orders, 150 genera, and 2000 species of cyanobacteria (Vincent, 2009). Table 2.1 represents the 5 orders of cyanobacteria recognized in the classic botanical taxonomy. Visible examples of cyanobacteria morphologies are also presented in Figure 2.2.

Table 2.1: Classification of cyanobacteria (Vincent, 2009)

The five orders of cyanobacteria recognized in the classic botanical taxonomic scheme		
Order	***Characteristics***	***Illustrative genera***
Chroococcales	Coccoid cells that reproduce through binary fission or budding	Aphanocapsa, Aphanothece, Gloeocapsa, Merismopedia, Microcystis, Synechococcus, Synechocystis
Pleurocapsales	Coccoid cells, aggregates or pseudo-filaments that reproduce by baeocytes	Chroococcidiopsis, Pleurocapsa

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Oscillatoriales	Uniseriate filaments, without heterocysts or akinetes	Lyngbya, Leptolyngbya, Microcoleus, Oscillatoria, Phormidium, Planktothrix
Nostocales	Filamentous cyanobacteria that divide in only one plane, with heterocysts; false branching in genera such as Scytonema	Anabaena, Aphanizomenon, Calothrix, Cylindrospermopsis, Nostoc, Scytonema, Tolypothrix
Stigonematales	Division in more than one plane; true branching and multiseriate forms; heterocysts	Mastigocladus (Fischerella), Stigonema

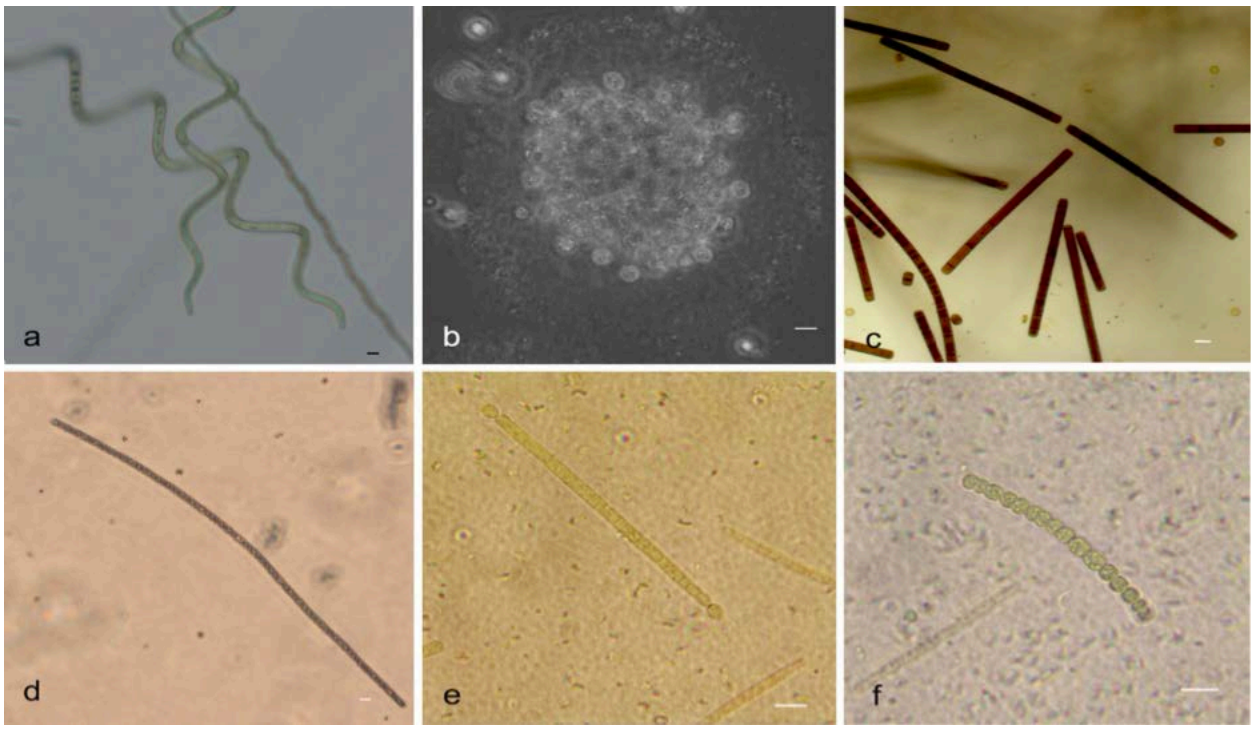

Figure. 2.2: Examples of different cyanobacteria morphologies (a) Arthrospira sp.; (b) *Microcystis botrys; (c) Lyngbya sp.; (d) Planktothrix rubescens; (e) Cylindrospermopsis raciborskii; (f) Nostoc sp*. (Buratti *et al.,* 2017)

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2.3 Cyanobacteria in Africa

In African countries, the issue of toxic blooms is a compounding one, in addition to existing water issues and challenges (Ndlela *et al.,* 2016). Furthermore, Africa is highly vulnerable to cyanobacteria blooms caused by the impact of climate change. However, regional examples of cyanobacterial bloom occurrences have been reported in Sub-Sahara Africa. Figure 2.3 indicates the occurrence of algal bloom, including cyanobacteria, in different regions in Africa. However, the main concern is that climate change will exacerbate existing physical, ecological, and socioeconomic stresses on the African coastal zone (Intergovernmental Panel on Climate Change (IPCC), 2007), which will provide favorable conditions for cyanobacterial bloom in the continent. This indicates that increased temperature and other climatic events will not only promote cyanobacterial bloom but pose significant and long-term threats to aquatic habitats in Africa.

2.3.1 Cyanobacteria in Nigeria

In Nigeria, the occurrence of cyanobacteria has increased, especially in the coastal regions. A study conducted by Okogwu *et al* (2009) in Cross River State, Nigeria, revealed that cyanobacteria abundance was higher in the rainy season than any other algae due to an increased phosphorous concentration from fertilizer application running off into the river. Recent studies have shown that climate change in Niger Delta has further worsened the growth and frequency of algal bloom, especially cyanobacteria in the aquatic ecosystem by providing optimal conditions for their growth (Pearl and Huisman, 2008). The major concern is that climate change may benefit some harmful species of cyanobacteria that will have a detrimental effect on aquatic fauna. A steady increase in CO_2, resulting from industrial activities within the Niger Delta region, accompanied by the already existing impact of climate change, will enhance the growth and abundance of cyanobacteria species (Dale *et al.,* 2006; Fu *et al.,* 2008). Climate projections suggest that future climate warming could impact harmful algal blooms through alteration of their geographic range and shifts towards earlier blooms. Elenwo and Akankali (2014) further explained that the Niger Delta region will experience an increased abundance of harmful algae blooms as a result of a steady increase in climate warming.

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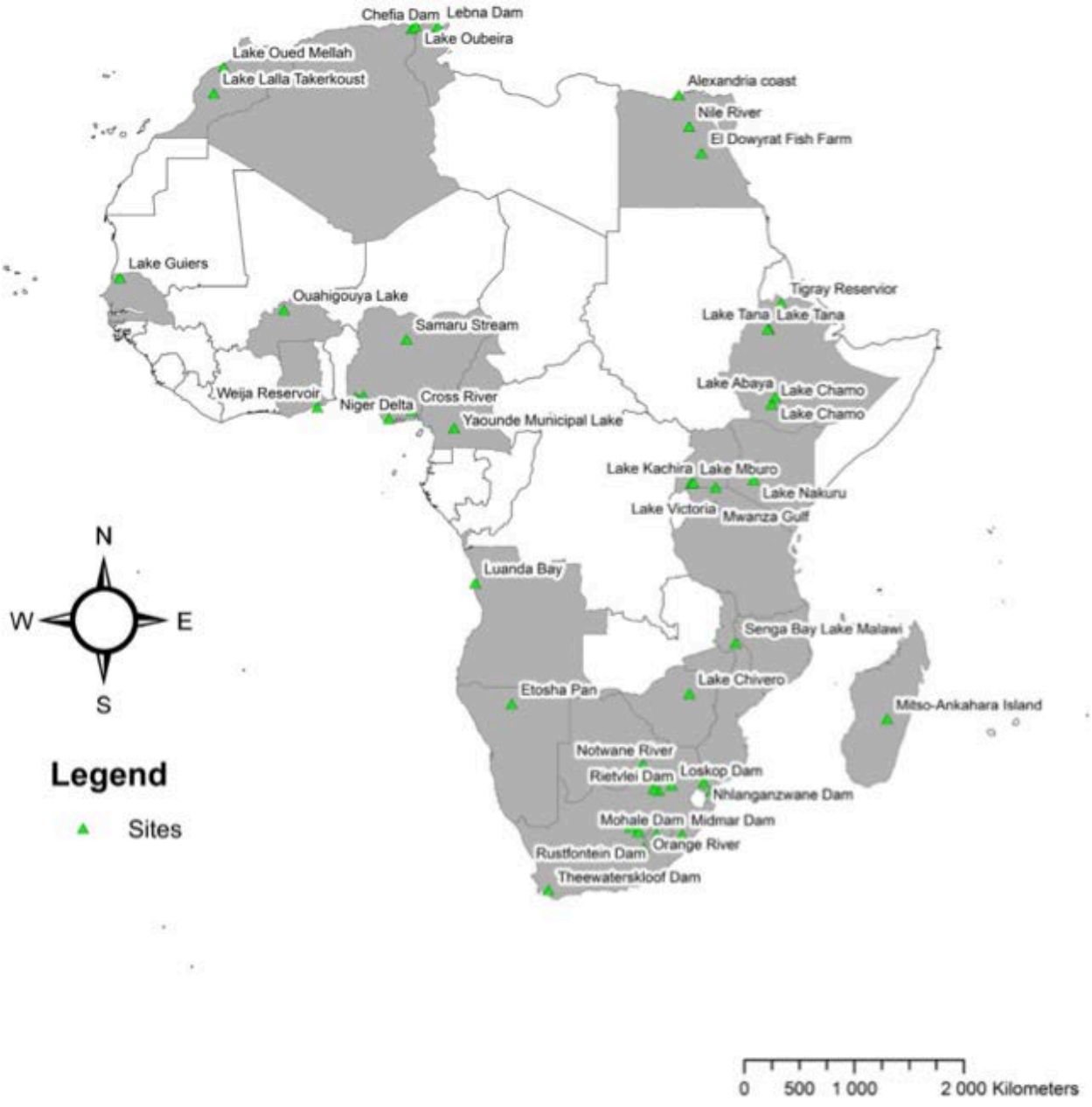

Figure. 2.3: Summary of areas affected by cyanobacteria blooms in Africa (Ndlela *et al.,* 2016)

2.3.2 Cyanobacteria in South Africa

Over the past decade, South Africa has produced the most documented information on cyanobacterial blooms within the continent. Ndlela *et al* (2016) also observed that blooms have occurred in nearly all of the known surface water in South Africa associated with toxins. In 2004, cyanobacterial blooms coincided with the killing of fish in the Lake Krugersdrift over the

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summer months (Oberholster *et al* 2004). The study also reported that between 2005-2006, *microcystin* levels in water bodies were high, reaching up to 43 mg/L and exceeding the WHO recommended level (Oberholster *et al* 2009). Meanwhile, in 2007, microcystin concentrations exceeding 20,000 mg/L in water sources for wildlife resulted in the death of wildlife in the Kruger National Park Nhlangezwane Dam (Ndela et al., 2016). A recent study by Ballot *et al* (2014) also reported that there is diversity and abundance of algae bloom in Hartbeespoort, with 96% comprising cyanobacteria species such as *Nostoc* spp and *Oscillatoria* spp. South Africa's aquatic ecosystems are highly vulnerable to the potential impacts of cyanobacteria caused by climate change. South Africa's freshwater will be highly vulnerable to environmental degradation, particularly from eutrophication (Mathews, 2014). The frequency and extended duration of cyanobacteria outbreaks are a cause for concern in South Africa that requires urgent attention.

2.4 Cyanotoxins

Cyanotoxins are toxins produced by certain species of cyanobacteria. The most frequently detected cyanotoxins that exist in the aquatic environment are microcystins, nodularin, cylindrospermopsin, and, neurotoxins (Buratti *et al.,* 2017). These toxins are harmful to aquatic organisms, animals, water quality, and, humans that are exposed to them directly or indirectly. Many cases of lethal poisoning and mortalities in animals and humans are attributed to exposure to cyanotoxins have been documented. For instance, hepatotoxins microcystins, and neurotoxins present in cyanobacterial mats contribute to mass mortalities of lesser flamingos (Krienitz *et al.,* 2003). The flamingos were exposed to cyanotoxins by the uptake of detached cyanobacterial cells from the mats (Krienitz *et al.,* 2003).

However, humans are often exposed to cyanotoxins orally through the consumption of cyanotoxin-containing freshwater, fish, seafood, crops, vegetables, and food supplements or by ingesting them during recreational activities (Buratti *et al.,* 2017). The most serious case of cyanotoxin mortality in a human occurred in Brazil, in 2002 when 56 out of 130 hemodialyzed patients died after treatment with water accidentally contaminated with microcystins (Azevedo *et al.,*2002 (Buratti *et al.,* 2017). The most recent documented episodes occurred in the USA. In

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the USA, 61 people from three states were affected by waterborne disease outbreaks associated with biotoxins from algal blooms (Hilborn *et al.,* 2014). The health effects included dermatologic, gastrointestinal, respiratory, and neurologic signs and symptoms (Hilborn *et al.,* 2014). Health risks in both animals, fish, and humans attributed to cyanotoxins are a major concern globally that requires adequate attention.

2.4.1 Microcystins (MCs)

Microcystins are cyanobacterial toxins that are present in cyanobacterial genera. The cyanobacterial genera include *Anabaena, Anabaenopsis, Aphanocapsa, Aphanizomenon, Cylindrospermopsis, Fischerella, Hapalosiphon, Lyngbya, Microcystis, Nostoc, Oscillatoria (Planktothrix), Phormidum, Rivularia and Synechococcus* but most frequently in the strain of *Anabaena,* as well as *Microcystis* sp (Rastogi *et al.,* 2014). MCs are cyclic peptides consisting of seven amino acids occurring as groups of hepatotoxins present in both freshwater and marine ecosystems. (Rastogi *et al.,* 2014; Buratti *et al.,* 2017; Greer *et al.,* 2016). Studies have reported that MCs strains contain DNA sequences homologous to known peptide synthetase genes (Meißner *et al.,* 1996; Dittman *et al.,* 1997). Dittman *et al* (1997) also indicated that MCs are presumed to be synthesized non-ribosomally by peptide synthetases.

The molecular structure of *Microcystis aeruginosa* revealed that toxic strains are genetically distinct from non-toxic strains in multilocus sequence typing (Tanabe *et al.,* 2007). The study showed that non-toxic strains harboring toxin genes fell into a single monophyletic clade, making it easy to unequivocally characterize toxicity in M. aeruginosa (Tanabe *et al.,* 2007). This also confirms the observation of Dittman *et al* (1997) stating that the genes MCs strains differ in their complement of genes encoding specific peptide synthetases (Dittman *et al.,* 1997). The most common congeners are MC-LR, MC-RR and MC-YR, resulting from the presence of the L-form of leucine (L), arginine ®, or tyrosine (Y) (Buratti *et al.,* 2017). Direct or indirect intake of MCs through the food web is considered a toxic and exposed route to cyanotoxins. To this effect, the World Health Organization (WHO) (1998) recommends a limit of 1µg/L in the aquatic environment to avoid public risks of MCs.

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2.4.2 Microcystin toxicity in fish

Microcystin toxicity in fish varies, depending on the concentration, exposure time, and bloom duration. Most recent studies have shown that MCs in fish in an aquatic environment including aquaculture ponds exceed the tolerable daily intake of 0.04 µg/kg. Drobac *et al* (2016) reported that water samples from 13 fish ponds contained saxitoxin and MCs. Histopathological analyses of the fish grown in the pond showed that MC-RR was present in the fish muscle tissue. Further analyses showed histopathological damage to the fish liver, kidney, gills, intestines, and muscle tissues. Another study carried out by Greer *et al* (2017) in a fish pond also reported that 80% of the tilapia fish samples contained MC-LR. Levels of MC-LR range from 16.8 and 45.2 µg/kg in the muscle and liver. The accumulation and distribution of MCs in edible fish organs, therefore, pose a serious risk of human exposure to MCs via consumption. Microcystin toxicity also exists in other seafood, such as mussels, mollusks, and zooplankton.

2.4.3 Microcystin toxicity in humans

Hepatotoxic MCs are the most commonly reported cyanotoxins present in eutrophic freshwaters. Chen *et al* (2009) detected MCs in the human serum of fishermen at Lake Chaohu, China. The study reported that fishermen were exposed to MCs by subsequent uptake of toxins in fish through consumption. The outcome of the study showed that the daily intake by the fishermen was estimated to be within the range of 2.2-3.9 mg, exceeding the WHO tolerable daily intake of 0.04 mg/kg or 2-3 mg per person. The health risks and high levels of exposure of humans to MCs toxicity have been reported to be carcinogenic and damaging to vital organs. A most recent study by FAO (2016) and Miao *et al* (2016) has documented that MC-LR may promote colorectal cancer through the consumption of MC-LR-contaminated water and food. Health risks also include gastroenteritis, skin reactions and, liver damage in both humans and animals. Adesalu *et al* (2016) also indicated that low levels of cyanotoxins in drinking water may result in chronic exposure and health risks. In 2014, approximately 500,000 people in Toledo, Ohio were issued with the ‘do not drink or boil water, advisory due to the presence of higher levels of MCs detected in the water supply (Tanber, 2014). This highlights the need to constantly monitor cyanotoxin levels in the water as well as consume food, especially from aquatic environments.

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2.5 Impact of climatic factors on cyanobacteria

2.5.1 Impact of air temperature on cyanobacteria

The temperature of the air has been recognized as a significant indicator influencing biotic processes, including the behavior of cyanobacteria in the epilimnion (Gilliano *et al*., 2011). Air temperature directly influences the ecological and hydrological factors in the aquatic ecosystem, thus representing a direct link between atmospheric conditions and aquatic ecology (Gallina et al., 2011). Jöhnk *et al.* (2008) suggested that an increase in global mean air temperatures will favor cyanobacteria bloom occurrence and extension. Changes in ecological and hydrological dynamics of the water associated with increased air temperature include warmer water temperature, stronger and longer-lasting stratification (Straile 2000; Visser *et al.* 2016), and a more stable water column (Joehnk *et al.,* 2008; Reynolds and Walsby 1975). The above changes directly affect cyanobacteria through increased growth rate (Michalak *et al.,* 2013) and indirectly by stabilization of the water, thus favoring buoyancy-controlling cyanobacteria over non-buoyant phytoplankton groups (Reynolds and Walsby, 1975).

Enhanced cyanobacteria proliferation during warmer seasons is strongly influenced by the warmer air temperature. This observation is consistent with Dupuis and Hann (2009) who mentioned that warmer air temperatures during summer and spring could potentially trigger significant changes in plankton dynamics. Ho and Michalak (2020) across the US also confirmed that seasonal summer air temperature increases are associated with chlorophyll-a increase. However, extremely warm or cold air temperatures could also lead to a loss of phytoplankton diversity, including the cyanobacteria community (Gallina *et al.*, 2011).

2.5.2 Impact of drought on cyanobacteria

Drought induces high-water column stability associated with elevated air temperature. Increased water retention time, nutrient concentration, salinization, water temperature, stratification, evaporation, and irradiation are symptoms of drought that are expected to favor the development of harmful cyanobacteria (Bouvy *et al*. 1999; Lehman *et al*. 2017; Lins *et al.* 2016; Muntswu and Gumbo 2020; Sanseverino *et al*. 2016). Paerl (2014) mentioned that drought is another symptom of climate change potentially impacting cyanobacteria communities. Drought is becoming more severe in some regions with the increase in global warming, thus creating favorable environmental conditions for harmful cyanobacteria bloom toxicity in freshwater.

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In 2014, Lehman *et al*. (2017) conducted a field sampling experiment to test the impact of drought on cyanobacteria blooms in San Francisco Estuary, United States of America. The study results revealed that cyanobacteria bloom was dominated by *Microcystis* species accompanied by a high concentration of toxin and increased chlorophyll-a concentration with extreme nutrient concentrations exceeding the previously dry and wet years. The above study further reported that increased water temperature was responsible for the increased toxic *Microcystis* biomass. Paerl and Paul (2012) suggested that elevated temperature is a common factor during a drought, favoring *Microcystis* species out-competing other phytoplankton and cyanobacteria species. Bouvy *et al*. (2000) reported that low water volume, water column stability, and increased water retention time linked to drought promote favorable temperature and irradiation for the *Cylindrospermopsis* species dominance in the surface water.

Water stratification during drought also plays a key role in creating a favorable environment for cyanobacteria dominance in freshwater. Intense stratification promotes the development of surface accumulation, hence creating favorable conditions for cyanobacteria to exacerbate growth over other phytoplankton (Paerl 2014; Paerl and Paul 2012). This process also stimulates cyanobacteria growth in water bodies, including the aquaculture system. Sanseverino *et al.* (2016) also observed that water stratification, especially where the flow rate is low, harms cultured species, especially in shrimp production.

High nutrient availability during the dry period favors the dominance of toxic cyanobacteria bloom (Lins *et al*., 2016; Muntswu and Gumbo, 2020). Evidence reports showed intense drought in Brazil reservoir showed that the drought was associated with the presence of sub-surface phosphate-P, ammonium-N, and nitrate and the dominance of slender filamentous cyanobacteria in the phytoplankton community (De Sousa Barroso *et al*., 2018). Sanseverino *et al*. (2016) explained that, during the dryness period, water evaporation induces a higher concentration of nutrients, increasing static water where cyanobacteria can grow easily.

Paerl (2014) also highlighted that summer drought will increase the demand for freshwater for irrigation, leading to salinity, thus disrupting the salinity balance in the saltwater aquatic system. This will be followed by a decrease in freshwater flow and an increase in the residence time of receiving water. Both nitrogen-fixing cyanobacteria species (*Anabaena, Anabaenopsis, Nodularia, Lyngbya*) and non-nitrogen-fixing cyanobacteria (*Microcystis aeruginosa*) can

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tolerate a saline environment. The above conditions favor cyanobacterial dominance and bloom formation (Paerl, 2014).

2.6.3. The impact of rainfall on cyanobacteria

Rainfall pattern is another climatic variable that influences the occurrence and biomass of cyanobacteria. It is considered a more important climatic variable regulating seasonal phytoplankton dynamics than temperature and solar radiation in temperate regions (Sommer et al., 2012). Intense rainfall events could increase excessive pollutants, sediments, and, nutrient runoff into water bodies, especially from agricultural catchment areas. Increased nitrogenous fertilizer used for agricultural purposes has increased bioavailable nutrients in the water bodies (Chaffin *et al.*, 2018). In this way, the concentration of the nutrients is increased excessively thus promoting cyanobacteria growth and bloom. Studies have also reported that increased rainfall produced more nutrients and sedimentation due to runoff promoting cyanobacteria bloom (Anderson, 2009; Paul, 2008; Elenwo and Akankali, 2014).

Cyanobacterial abundance is highly favorable in nutrient-rich water, whereby the influxes of nutrients and higher temperatures promote cyanobacterial bloom frequency, biomass, and duration in addition to toxicity (O'Neil *et al.*, 2012). Chaffin *et al*. (2018) reported that low continuous and large pulse nitrogenous fertilizer loading would contribute to cyanobacterial blooms reaching higher biomass in response to nutrient influx. This implies that cyanobacteria density can be increased with increased nutrient availability. Lürling *et al*. (2017) indicated that elevated phosphorus concentrations and higher temperatures increased Microcystis toxic cells' growth rates.

Summary

Cyanotoxins are toxic compounds produced by cyanobacteria, including microcystins, nodularin, cylindrospermopsin, and neurotoxins. These toxins pose significant risks to aquatic life, animals, and humans. Exposure occurs through contaminated water, seafood, crops, and recreational activities, with severe health effects such as liver damage, gastroenteritis, and even death. For instance, a 2002 incident in Brazil resulted in 56 fatalities among dialysis patients due to water contaminated with microcystins. Similarly, algal bloom outbreaks in the USA have caused illnesses ranging from dermatologic to neurological symptoms. The World Health Organization

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(WHO) has set a safety limit of 1 µg/L for microcystins in water to mitigate risks. Microcystins (MCs) are a common type of cyanotoxin found in several cyanobacterial genera and are particularly toxic to fish and humans. Studies reveal MCs accumulate in fish and seafood, often exceeding safe consumption levels, causing damage to organs such as the liver, kidneys, and muscles. In humans, chronic exposure to MCs has been linked to carcinogenic effects, including colorectal cancer, and acute health incidents, such as a 2014 water crisis in Toledo, Ohio, affecting 500,000 people. Climate factors, including air temperature, drought, and rainfall, exacerbate cyanobacteria blooms by influencing water stability, nutrient concentration, and ecological dynamics, further amplifying the risks associated with these toxins.

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Chapter 3

This chapter addresses objective 1

To assess the physical and chemical parameters of the water quality in the fishponds.

Abstract

This study employed a comparative design to assess water quality in small-scale commercial fishponds located in Calabar Municipality, Cross River State, Nigeria, and Duthuni, Vhembe District, Limpopo Province, South Africa. Six fishponds were sampled across seasonal intervals, including summer, and winter in SA, and dry, and wet seasons in NGA. In situ measurements included water temperature, dissolved oxygen (DO), pH, electrical conductivity, and salinity, total dissolved solids. Concurrently, water samples were collected for laboratory analysis of biological parameters (chlorophyll-a) and chemical constituents (phosphate, nitrate, and nitrite). The comparative approach provides insights into the seasonal dynamics of water quality parameters, reflecting the influence of regional climatic and ecological differences on aquaculture sustainability. Seasonal variations in water quality parameters were observed in Nigeria and South Africa, influenced by their respective climatic conditions. Elevated temperatures (21.4 - 22.6 °C) were observed during summer with a notable winter decrease (16.5°C) at all SA fishponds. Increased temperature was consistent during dry and wet (26.5 - 28.3 °C) in NGA fishponds. Dissolved oxygen (DO) and electrical conductivity (EC) levels were consistently low across seasons in SA fishponds. Total dissolved solids (TDS) peaked during winter in South Africa (125–193.2 mg/L). Salinity was higher during South African summers (82.5–129.4 mg/L) and lowest in Nigeria during the wet season (2.98–3.53 mg/L). Nutrients, particularly nitrite and nitrate, peaked in Nigeria during wet (10.42 mg/L) and dry (6.45 mg/L) seasons, while South African fishponds exhibited lower concentrations year-round. Phosphate levels were stable in South Africa but higher in Nigeria during the wet season. pH values ranged from neutral to alkaline in Nigerian fishponds during the rainy season, while South African fishponds maintained slightly acidic to neutral pH (5.59–7.22). This study highlights the complex interplay between climatic conditions and water quality parameters in small-scale aquaculture systems across two distinct regions. The findings underscore the importance of regional climate adaptations in aquaculture management to optimize water quality and ensure sustainable fish production.

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Introduction

Cyanobacteria are unicellular or filamentous microorganisms capable of oxygenic photosynthesis (Ciebiada et al., 2020). They thrive in a wide range of environments, including soils, freshwater systems, thermal springs, and marine habitats (Bhardwaj et al., 2024). Similar to plants, cyanobacteria utilize sunlight to convert atmospheric carbon dioxide into organic compounds (Arora et al., 2021; Keller et al., 2021). Under favorable conditions—such as optimal temperatures, abundant sunlight, and nutrient-enriched waters—they can form blooms, known as cyanobacterial blooms (Wang et al., 2021). These blooms pose ecological and health risks, as they can lead to the release of cyanotoxins, potent toxins that compromise water quality and aquatic ecosystems (Metcalf and Codd, 2020). The ability of cyanobacteria to thrive in diverse conditions and their potential to produce harmful toxins highlight their significance in environmental monitoring and aquaculture management. The worldwide proliferation of CyanoHABs poses a significant threat to the safety and sustainability of water resources utilized for human consumption, agricultural irrigation, inland fisheries (including aquaculture), and recreational purposes (Paerl et al., 2020). Aquaculture ecosystems are highly vulnerable to cyanobacterial bloom because cyanobacteria constitute the integral component of the food web as phytoplankton biomass (Paerl, H. W., and Bernard, 2013; Backović*et al.,* 2024). Cyanobacteria exhibit remarkable adaptability to environmental conditions commonly found in fishponds. These include elevated temperatures, reduced light availability, nitrogen depletion in surface layers, high levels of eutrophication, and a decline in large phytoplanktivorous filter feeders (Backovićet al., 2024; de Lima Pinheiro et al., 2023; Vrba et al., 2023). Their resilience under such conditions underscores their competitive advantage in fishpond ecosystems, often leading to the proliferation of harmful algal blooms that can compromise water quality and aquaculture productivity. The destabilization of fishpond ecosystems is often marked by excessive phytoplankton and cyanobacterial blooms, significant fluctuations in oxygen levels and pH, and elevated ammonia-nitrogen concentrations, all of which threaten aquatic balance (Kopp et al., 2016). These conditions can impair fish health, reduce productivity, and necessitate intensive management strategies. This study aimed to assess water quality parameters seasonally in six small-scale fishponds. The findings are expected to provide insights into the seasonal dynamics of water quality parameters in fishponds. This knowledge can contribute to improved management practices in fish-rearing systems.

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2.0 Methodology

2.1 Study Area and Sampling Sites

The study was conducted in commercial aquaculture fishponds in Vhembe District, Limpopo Province, South Africa and Calabar Municipality, Cross River State, Nigeria. In Nigeria, the sampling sites were located in Offiong Etim Avenue ($4^0$59'58.92" N and $8^0$19'03.97" E), Essien Town ($4^0$59'15.49" N and $8^0$19'40.21" E) and State Housing ($4^0$59'6.50" N and $8^0$20'13.29" E) presented in Figure 3.1. The aquaculture fishponds in the Vhembe District were located in Duthuni ($22^0$57'56.98" S and $30^0$23'43.96" E). A total number of 6 fish ponds located in Vhembe District (3 fish ponds) and Calabar Municipality (3 fish ponds) were used for this study. Sampling sites were selected using the following criteria: commercial fishponds, accessibility, consent from the owners, and the presence of cultured fish.

2.2 Water sampling

Water samples were seasonally collected from the fishponds in triplicates during the South African winter and summer and the Nigerian dry and wet seasons, respectively. Data collection involved four field trips conducted during specific seasonal periods: January and February (summer), June (winter), August and September (wet season), and November and December (dry season). Water samples were sampled at depths between 0 and 0.5 meters from each fishpond using sterilized labeled bottles. Water samples were collected approximately 1-2 meters away from the edge to avoid contamination from edge-related disturbances. Water samples were collected using clean, 1-litre sterilized bottles. The samples were placed in an ice-filled cooler box before being transported to the laboratory for further analysis. Approved consent was obtained from the owners of the fishponds in Nigeria and South Africa before sampling.

2.2.1 Analysis of physicochemical parameters

Field meters were employed to assess the physicochemical characteristics of the water samples. Analyses included measuring surface temperature, pH, salinity, dissolved oxygen, total dissolved solids, and electrical conductivity (EC). Dissolved Oxygen (DO) levels were gauged using a DO meter (Potable Dissolved Oxygen Meter BANTE Instrument 821). All measurements were conducted in triplicate. pH, salinity, and total dissolved solids were measured in the field using a portable waterproof ACCSEN PC 70 Multimeter equipped with an electrode.

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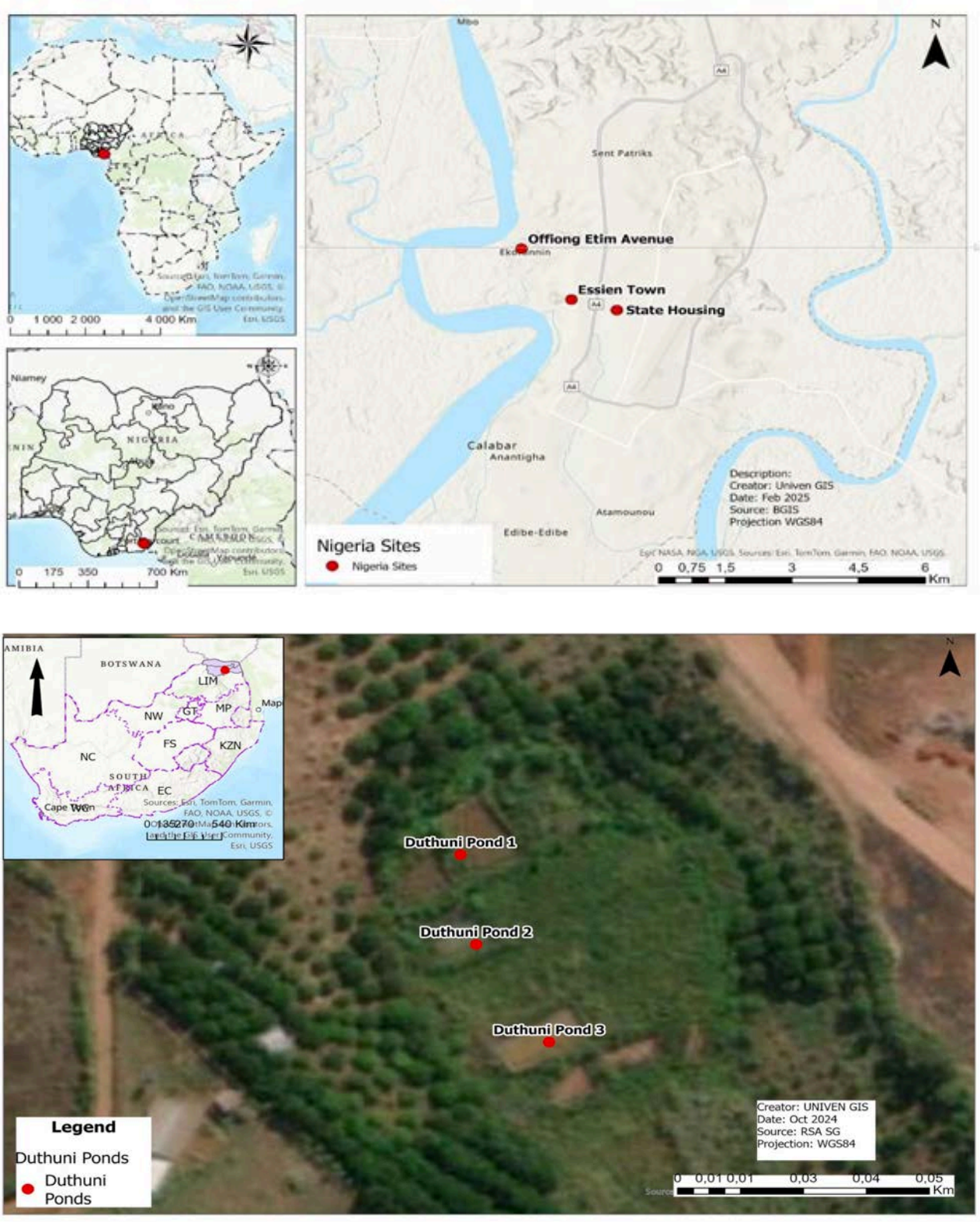

Figure 3.1: Map of the sampling sites in (a) Calabar Municipality, Cross River State, Nigeria and (b) Vhembe District, Limpopo Province, South Africa

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Table 3.1: Fishpond Sampling sites identification

SA Sample ID	Duthuni pond 1	Dunthini Pond 2	Duthuni Pond 3
NGA Sample ID	Offiong Etim Avenue	Essien Town	State Housing

2.2 Water samples analyses

2.2.1 Nutrient analyses

Samples for dissolved nutrient concentration underwent filtration using membrane filters before analyses. The nutrient analyses, specifically for nitrates, nitrites, and phosphates, were conducted on the samples using Ion Chromatography Dionex 1600, employing EPA method 300 (EPA, 1993). These analyses took place at the Agricultural Research Council (ARC) Laboratory.

2.2.2 Chlorophyll-a analysis

Water samples (250 ml) were filtered through a Whatman (Glass Fiber) filter paper (3.4a and b). Then, the filter paper was cut into smaller pieces and immersed in 10 mL ethanol, followed by ultrasonication for 30 mins. The tube was labeled and stored in the dark for 24 hours at room temperature. This was followed by 15 mins centrifugation at 3500 rpm to get a clear sample. The samples were transferred to a clean vessel, and the volume was recorded. The supernatant was poured into a 1 cm cuvette, and a spectrophotometer was used to measure the amount of light absorbed by the sample in the cuvette placed in the Spectrophotometer at a wavelength of 665 and 750 nm. This absorbance wavelength ratio (665 and 750nm) was used because it fluoresces at 665 and 750 nm. Two batches at 665 and 750 nm were used;

1) 1 cm cuvette sample without Hydrochloric acid (total absorb) (665a and 750a nm).
2) 1 cm cuvette sample with a 0.01 ml drop of hydrochloric acid (665b and 750b nm).

Adding HCl (0.01 ml) to the sample before measurement assists in dissolving the suspended particles scattered in the samples for light to pass through the cuvette without interference with scattered particles. After measuring, chlorophyll-a concentration was conducted using the equation shown below (EPA, 2021);

Calculation

Correct turbidity by subtracting absorbance 665a-750a = corrected 665a,

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665b-750b = corrected 665b

The corrected 665a and 665b absorbance was to calculate the chlorophyll-a concentration;

$$Chl - a = \frac{29.62\,(665a-665b)X\,Ve}{Vs\,X\,1}$$

Where:

V_s = Volume of water samples in liters

V_e = Volume of ethanol extract (ml)

1 = Cuvette light-path length in centimeters

The final concentration was expressed in units mg m^{-3}

2.3 Statistical analysis

The statistical analyses were conducted to establish the correlation between variables using SPSS. The obtained data were input into Microsoft Excel before performing statistical analyses. A descriptive statistics summary of the water quality parameters—namely, chlorophyll-a, temperature, TDS, DO, EC, pH, salinity, NO_3, NO_2, and PO_4 was conducted. Pearson correlation was used to assess the relationships between the variables.

3.0 Results

The graphical representation of water quality parameters, as illustrated in Figure 3.2 and Table 3.2 - 3.5 highlights both similarities and variations across different seasons. Elevated temperatures (21.4 - 22.6 °C) were observed during summer with a notable winter decrease (16.5°C) at all SA fishponds. Increased temperature was consistent during dry and wet (26.5 - 28.3 °C) in NGA fishponds. Furthermore, lower levels of dissolved oxygen (DO) and electrical conductivity (EC) were observed across seasons. The winter season in South Africa recorded higher values of total dissolved solids, ranging between 125 – 193.2 mg/L across all sampling stations, while Nigeria exhibited significantly lower values ranging from 15.9-37.7 mg/L across the sampling sites. Salinity significantly increased during summer in all the fishponds in South Africa with higher values of 82.5–129.4 mg/L, while wet season recorded the lowest values in Nigeria (fishponds sites 2.98-3.53 mg/L). High concentration of nitrite and nitrate was observed during the wet season, followed by the dry season, corresponding to 10.42 mg/L and 6.45 mg/L, respectively. Meanwhile, in South Africa, the fishponds display lower concentrations of nutrients

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(NO_3, NO_2, and PO_4 - 1.9, 1.4, and 2.1 mg/L, respectively) in summer and winter. Phosphate levels were the same in winter and summer in South Africa for all fishponds and higher in the wet season for Nigerian fishponds. The water samples in Nigerian fishponds showed neutral to alkaline pH in rainy seasons. South African fishponds maintained slightly acidic to neutral pH 5.59-7.22.

3.0 Discussion

Temperature is an important factor regulating cyanobacterial abundance in water bodies (Scott et al., 1977). Most research studies have established that there is a strong relationship between increased temperature and increased cyanobacterial biomass (Lehman et al., 2008; Sanseverino et al., 2016; Duan et al., 2018). A temperature greater than 20 °C was observed across all sampling sites in this study. The increased temperature recorded during sampling can be attributed to the influence of seasonal succession on temperature, considering that this study was carried out during the summer. The results of this study clearly show that increasing temperature may not be the dominant factor controlling cyanobacterial abundance in fishponds. Cyanobacterial abundance is highly favored in nutrient-rich water, where the influx of nutrients and higher temperatures promote cyanobacterial bloom frequency, biomass, and duration (Paerl and Huisman, 2008; O'Neil et al., 2012).

Surface water pH is another environmental factor that influences cyanobacteria physiologically and metabolically. pH can strongly affect cyanobacterial physiology, colony morphology, and photosynthetic activity (Rai and Rajashekhar, 2014). Most research has established that an elevated aqueous pH of 7 to 10 favors cyanobacterial growth by enhancing the photosynthesis of cyanobacterial colonies, increasing nutrient uptake for enzymes, decreasing metal solubility, and increasing their capacity to take up different forms of phosphate (Rai and Rajashekhar, 2014; Keithellakpam et al., 2015). The slightly acidic to neutral pH observed in SA ponds may contribute to the low chlorophyll-a concentration and cyanobacterial growth, considering that the optimal growth pH for most cyanobacteria is 7 to 10 (Leavitt et al., 1999). Low pH may affect solute transport and antiporter activity in cyanobacteria, preventing them from accumulating essential nutrients and solutes (Kallas et al., 1982). Therefore, defects in solute transport at low pH may cause growth loss and viability loss in cyanobacteria (Kallas et al., 1982). Meanwhile,

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Wangwibulkit et al. (2008) insist that a pH higher than 9 or lower than 6 could hinder photosynthetic activity and affect the morphology of cyanobacteria.

Increased salinity levels observed in the SA fishpond could strongly contribute to reduced chlorophyll-a content. Contrary to SA fishponds, increased chlorophyll-a activity in NGA fishponds could correspond with lower salinity levels. Chlorophyll-a remains a primary target for salt toxicity, reducing net assimilation rates, leading to decreased photosynthesis and inhibited growth (Yang et al., 2020; Hameed et al., 2021). Earlier studies by Rai and Rajashekhar (2016) on the effect of salinity on cyanobacterial growth showed that optimal growth occurred at low salinity levels (16 and 25 ppt), while higher salinity levels of 40 ppt drastically reduced cyanobacterial growth. Increased salinity levels above 20 ppm could influence cyanobacterial abundance by promoting the dominance of saltwater phytoplankton over cyanobacterial community species (Takarina and Wardhana, 2017).

The low dissolved oxygen (DO) levels observed across all sampling sites in South African fishponds during summer and winter are likely linked to a reduced cyanobacterial population, as supported by findings from Harith and Hassan (2007) and Su et al. (2022). The low dissolved oxygen in SA fishponds may be due to water intrusion from a nearby dam. In contrast, the slightly higher DO values recorded in Nigerian fishponds during the dry season may correlate with increased chlorophyll-a activity, indicative of higher primary productivity during this period. These observations suggest a clear relationship between seasonal variations in chlorophyll-a concentrations and cyanobacterial abundance across the sampling sites.

The low TDS concentrations across all sampling sites did not strongly influence cyanobacterial abundance as expressed by chlorophyll-a levels. Variations in TDS levels are highly attributed to industrial effluent, changes in water balance, salt intrusion, increased salinity, or changes in water ionic composition (Weber-Scannell, 2007). This clearly explains the strong correlation between salinity and TDS. Research studies have maintained that increased TDS concentrations at or above 2,450 mg/L are associated with low productivity and decreased nitrogen fixation in algae (Kerekes and Nursall, 1966; Evans and Prepas, 1996).

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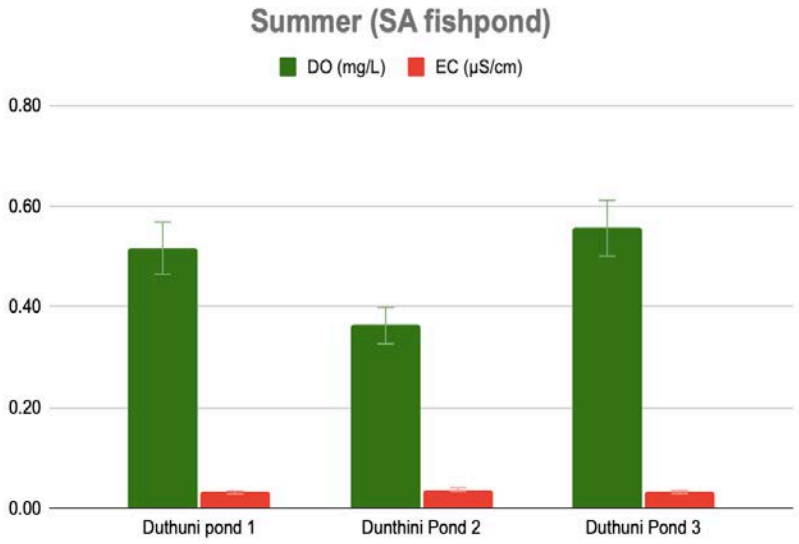

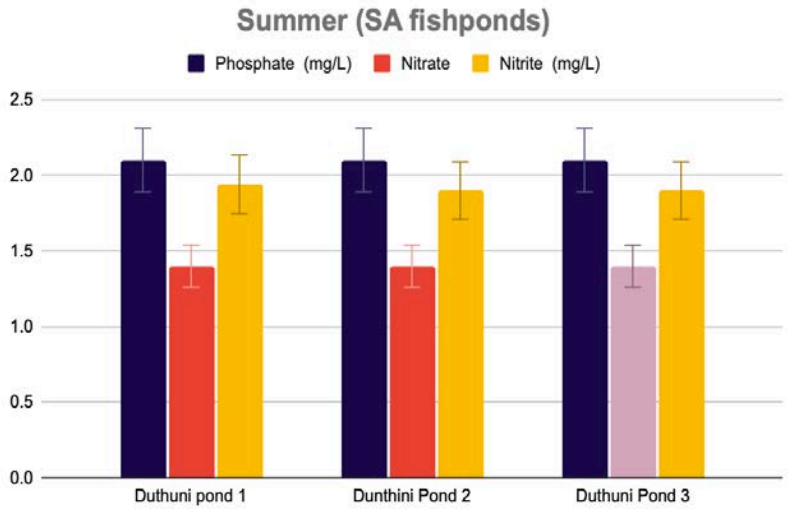

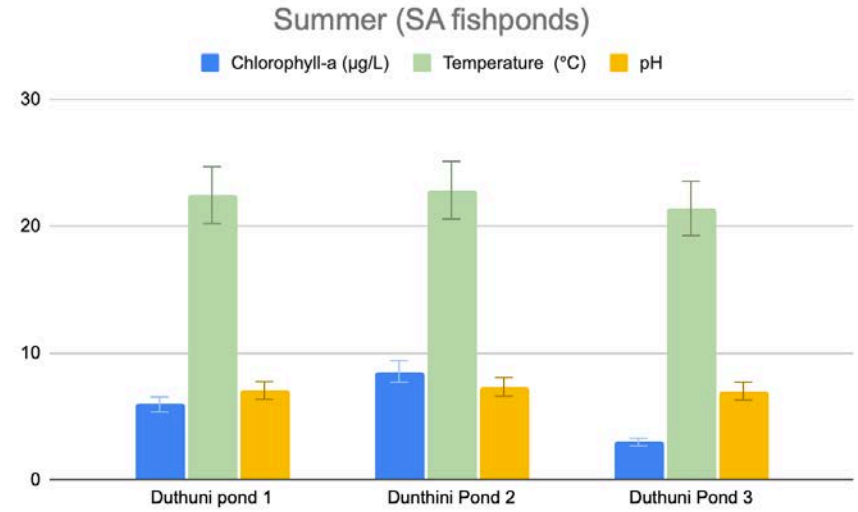

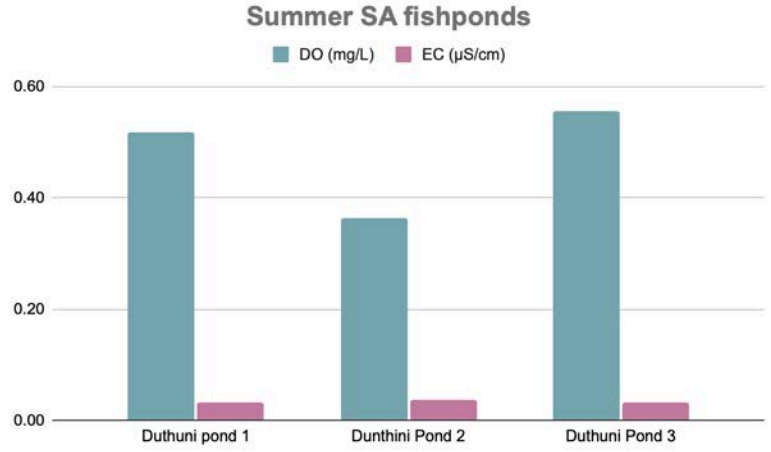

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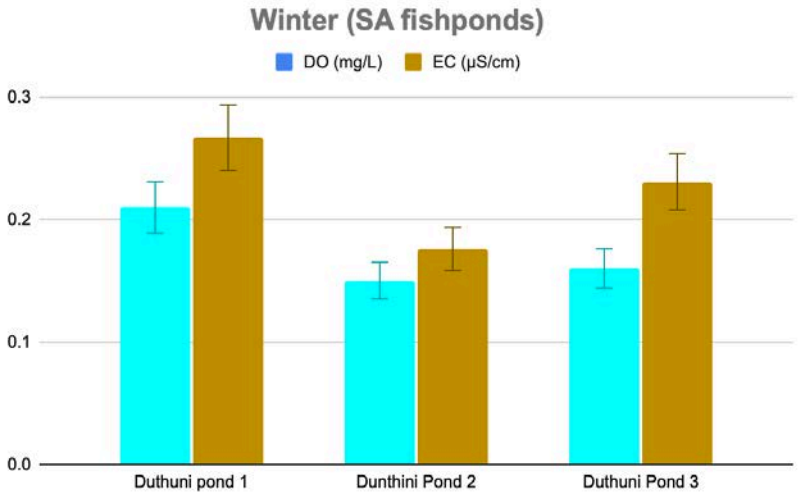

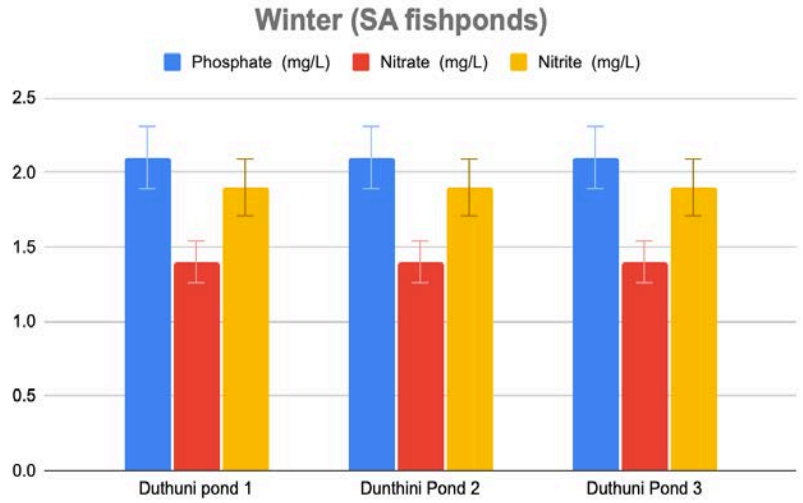

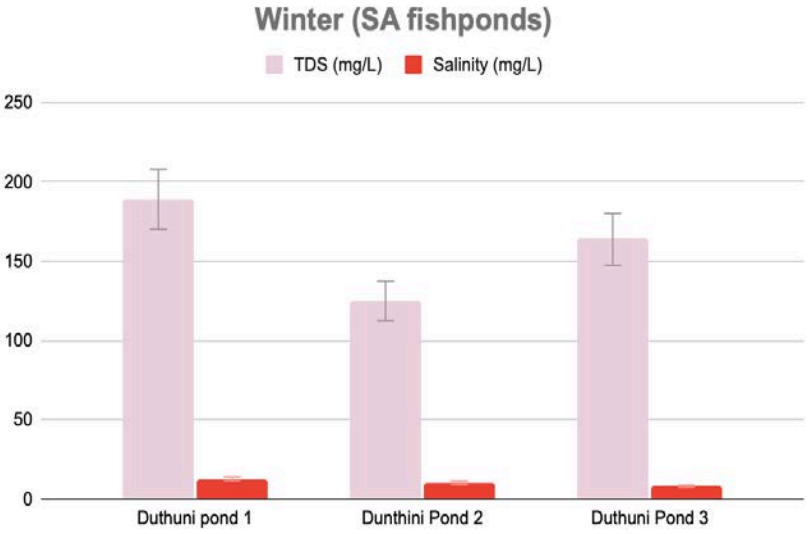

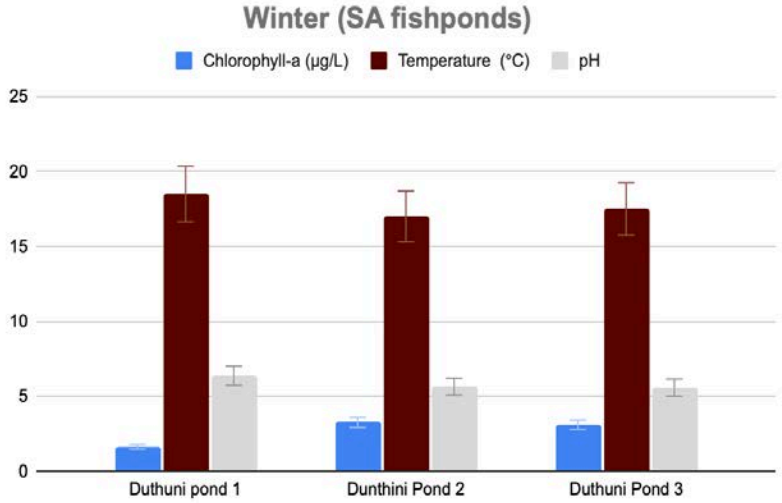

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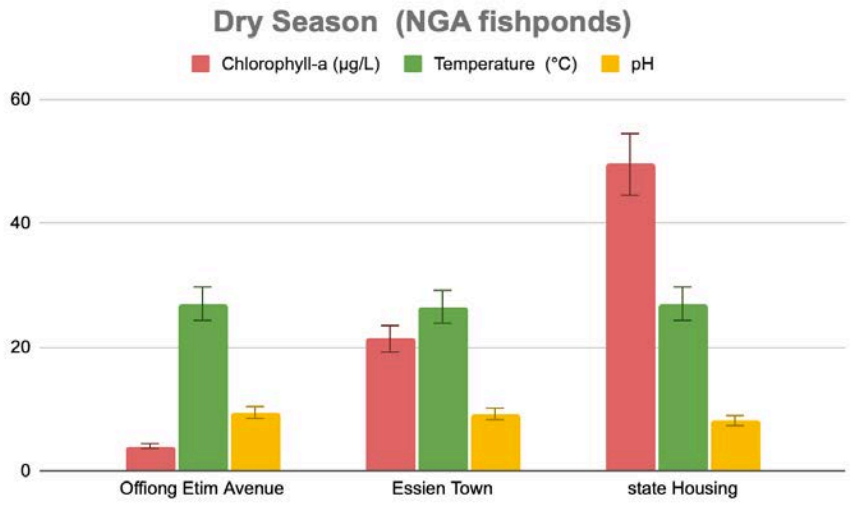

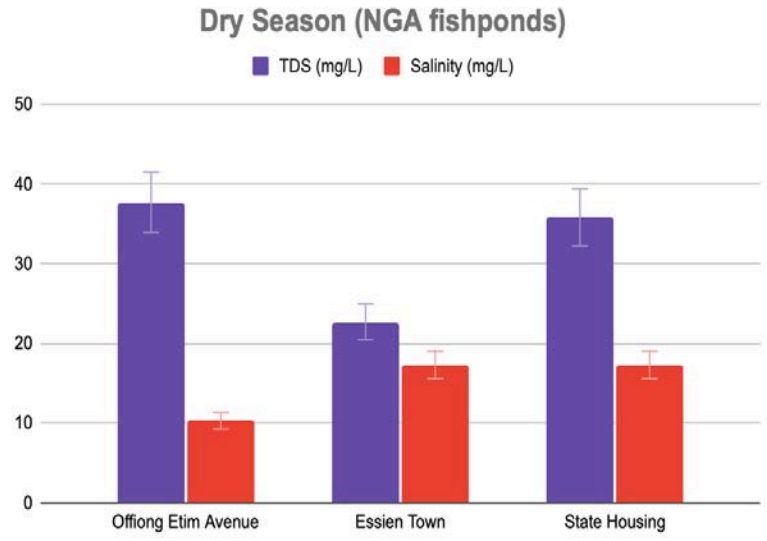

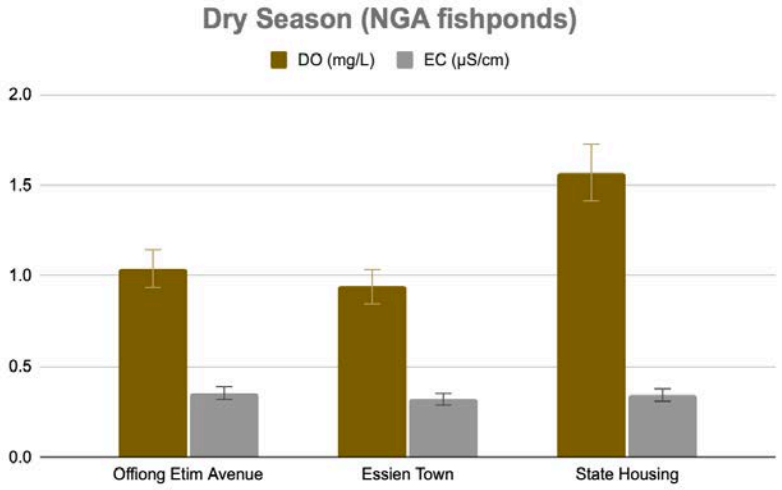

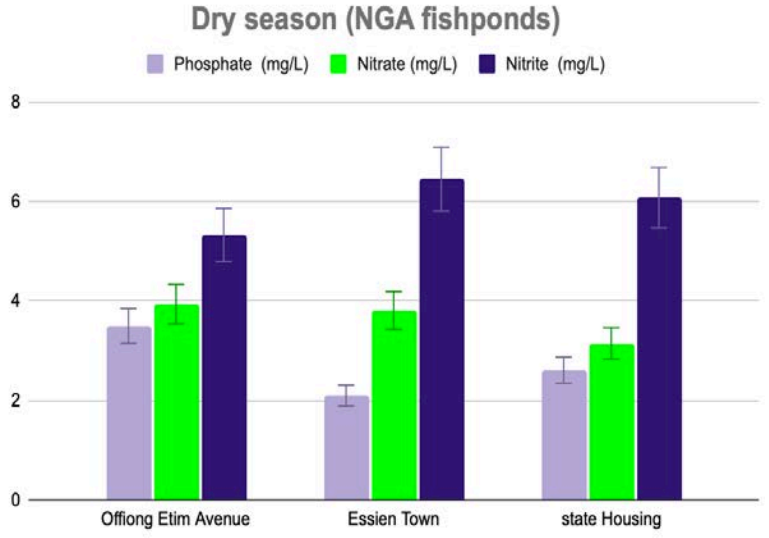

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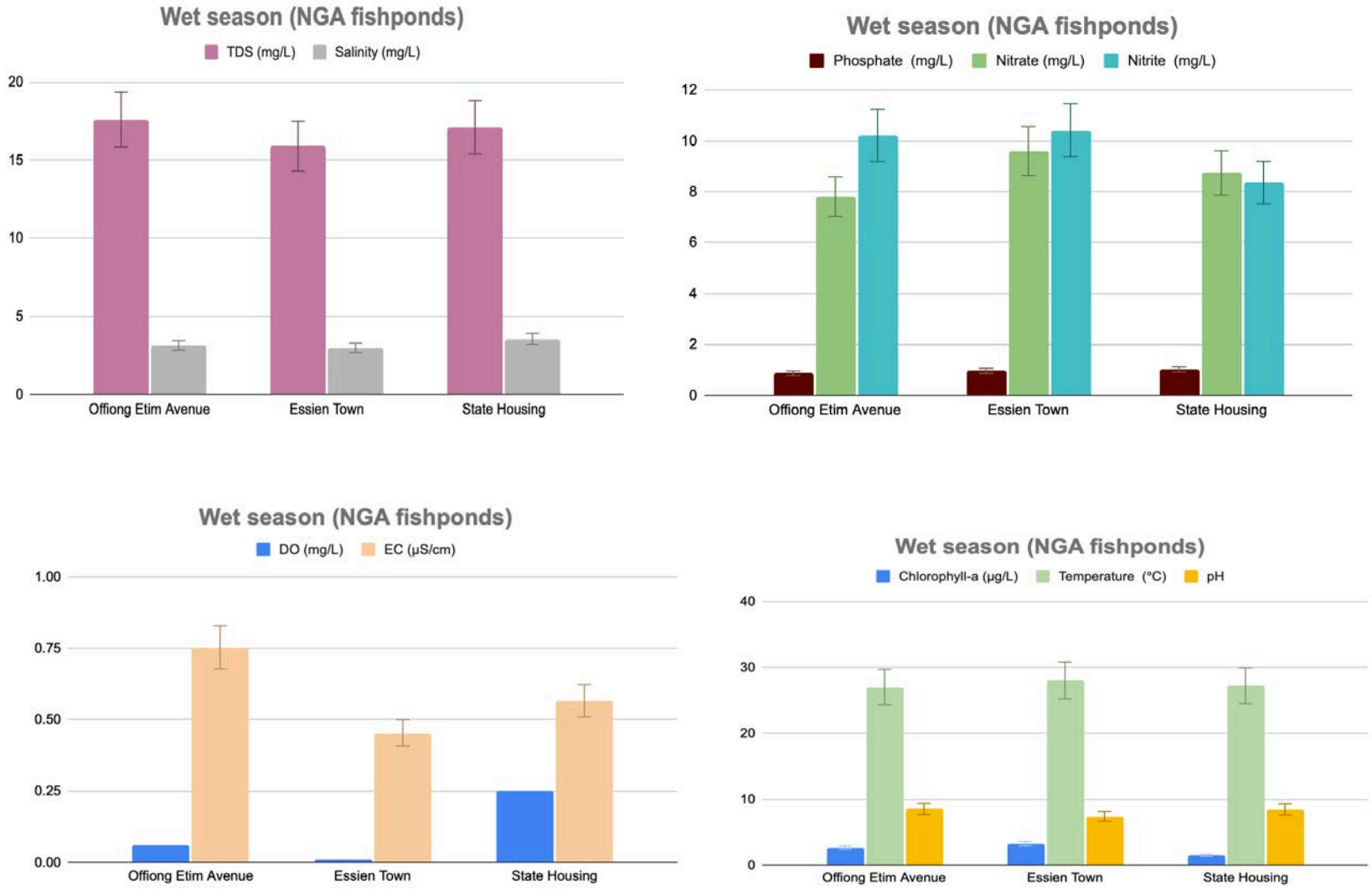

Figure 3.2: Graphical representation of water quality parameters (temperature, DO, EC, nitrite, nitrate, phosphate, salinity, TDS and chlorophyll a) during summer, winter, dry and wet seasons in SA and NGA fishponds.

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Table 3.2: Descriptive statistics of water quality parameters during summer in SA fishponds

Variables	*Chlorophyll-a (µg/L)*	*Temperature (℃)*	*pH*	*Phosphate (mg/L)*	*Nitrate (mg/L)*	*Nitrite (mg/L)*	*Dry Weight (g)*
Mean	6.87	22.25	7.19	2.10	1.4	2.36	4403.33
Median	5.92	22.52	7.16	2.10	1.4	1.92	3680.00
Mode	15.33	22.83	7.02	2.10	1.4	1.90	9500.00
Standard Deviation	5.43	0.68	0.21	0.00	0	0.77	2572.14
Range	13.70	1.43	0.52	0.00	0	1.86	6860.00
Minimum	1.63	21.40	6.98	2.10	1.4	1.90	2640.00
Maximum	15.33	22.83	7.50	2.10	1.4	3.76	9500.00

Table 3.3: Descriptive statistics of water quality parameters during winter in SA fishponds

Variables	*Chlorophyll-a (µg/L)*	*Temperature (℃)*	*pH*	*Phosphate (mg/L)*	*Nitrate (mg/L)*	*Nitrite (mg/L)*	*Dry Weight (g)*
Mean	2.74	17.47	6.21	2.10	1.40	1.91	1293.00
Median	3.18	17.50	6.35	2.10	1.40	1.90	1041.00
Standard Deviation	0.81	0.68	0.49	0.00	0.00	0.02	592.55

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Range	1.78	2.00	1.20	0.00	0.00	0.04	1482.00
Minimum	1.63	16.50	5.59	2.10	1.40	1.90	1018.00
Maximum	3.41	18.50	6.79	2.10	1.40	1.94	2500.00

Table 3.4: Descriptive statistics of water quality parameters during the dry season in NGA fishponds

Variables	***Chlorophyll-a (µg/L)***	***Temperature (℃)***	***pH***	***Phosphate (mg/L)***	***Nitrate (mg/L)***	***Nitrite (mg/L)***	***Dry Weight (g)***
Mean	37.71	27.13	8.94	2.31	3.54	5.93	18640.00
Median	12.66	27.00	9.08	2.36	3.72	5.95	18780.00
Standard Deviation	58.06	0.62	0.46	0.97	0.42	0.42	2078.27
Range	149.88	1.80	1.30	2.56	1.04	1.12	5340.00
Minimum	0.00	26.50	8.10	0.94	2.90	5.33	15620.00
Maximum	149.88	28.30	9.40	3.50	3.94	6.45	20960.00

Table 3.5: Descriptive statistics of water quality parameters during wet season in NGA fishponds

Variables	***Chlorophyll-a (µg/L)***	***Temperature (℃)***	***pH***	***Phosphate (mg/L)***	***Nitrate (mg/L)***	***Nitrite (mg/L)***	***Dry Weight (g)***
Mean	2.36	27.67	8.27	0.85	8.29	9.91	18768.33

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Median	2.15	27.70	8.44	0.84	8.27	10.23	18240.00
Standard Deviation	1.75	0.54	0.81	0.12	0.95	0.82	2123.32
Range	5.11	1.30	2.20	0.29	2.58	2.22	5720.00
Minimum	0.00	27.00	7.30	0.72	7.02	8.36	17200.00
Maximum	5.11	28.30	9.50	1.01	9.60	10.58	22920.00

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Kim et al. (2019) identified electrical conductivity (EC) as a critical variable for predicting algal blooms, highlighting that high EC levels are strongly associated with cyanobacterial dominance, often facilitated by reduced flow conditions. In this study, the low EC values observed across all sampling sites align with the low cyanobacterial abundance, as reflected by reduced chlorophyll-a concentrations. Conversely, the increased EC and chlorophyll-a levels observed in Nigerian fishponds during the study are consistent with the findings of Kim et al. (2019), suggesting that elevated ionic strength may support cyanobacterial growth. These results emphasize the importance of EC as an ecological indicator, with implications for managing algal dynamics and water quality in aquaculture systems.

The availability of phosphate and nitrate in water bodies determines primary productivity due to their important role as metabolic nutrients (Samocha et al., 2004; Adesalu et al., 2016). Thus, increases or decreases in nutrient concentrations affect cyanobacterial dominance and population in fishponds. The low nutrient values observed during winter and summer could be attributed to decreased fertilization of fishponds by their owners, contrary to the NGA fishponds. Chia et al. (2015) explained that artificially enriched fishponds with fertilizers promote algae growth, and the nutrient concentrations reflect the use of fertilizers in these ponds. Nutrient levels can be increased by adding inorganic or organic fertilizers to fishponds. This is significant in promoting phosphorus concentrations, which support healthy plankton blooms necessary for maintaining turbidity levels and providing food for fish (Boyd, 1998).

Kankaapaa et al. (2004) maintained that high nutrient concentrations in fishponds, due to degradation products from organic waste and uneaten food during fish cultivation, may promote rapid cyanobacterial growth. Lower nitrate (≤0.60 mg/L) and phosphate (≤0.03 mg/L) values were recorded by Jean et al. (2000), with an increased abundance of *Microcystis* spp. This study emphasizes that *Microcystis* spp. can successfully dominate water bodies despite low phosphate and nitrate levels, provided there are sufficient ammonium-nitrogen concentrations.

Conclusion

This study highlights the multifaceted factors influencing cyanobacteria abundance and primary productivity in fishponds across South Africa (SA) and Nigeria (NGA). While temperature is often considered a key driver of cyanobacterial growth, findings from this research suggest that

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its role may be secondary in nutrient-limited systems, such as the SA fishponds. Instead, variables like pH, salinity, and dissolved oxygen (DO) levels appear to exert more direct control on cyanobacterial dynamics, with low pH and high salinity observed in SA potentially limiting chlorophyll-a production and cyanobacterial abundance. Conversely, the higher EC, DO, and nutrient levels observed in NGA fishponds, combined with lower salinity, create conditions more conducive to chlorophyll-a activity and cyanobacterial growth. This supports the notion that nutrient availability, particularly phosphate and nitrate, is a critical factor in promoting primary productivity and algal blooms, with temperature acting as a complementary factor. The results also underline the importance of EC and TDS as ecological indicators, demonstrating their roles in shaping the ionic balance and supporting phytoplankton dominance. The study further reinforces the role of aquaculture management practices, such as fertilization and water management in influencing water quality and biological productivity. Emphasizing nutrient management, maintaining optimal pH, and monitoring EC levels are critical for sustainable fish farming and mitigating risks associated with cyanobacterial blooms.

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Chapter 4

This chapter addresses objective two

"To detect cyanotoxins in fishponds by employing non-targeted Mass Spectrometry analysis".

This chapter has been published in

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Abstract

Over the decades, the aquaculture sector has witnessed substantial growth, contributing significantly to the nation's economy. However, the menace of CyanoHABs threatens the sustainability of fish farming. Considering the possible hazards linked to cyanotoxins in food and water, a comparative study design between commercial fish in Nigeria and South Africa was employed to investigate cyanotoxins in the water from fishponds. Six commercial fishponds in Calabar Municipality—Nigeria and Duthuni—South Africa with varying climatic zones were selected. Water samples from the ponds were collected at intervals during different seasons (summer, winter, dry, and wet seasons) to capture climate-induced variation. Liquid chromatography-mass spectrometry (LCMS) in combination with the metabolites database was used for the identification of toxic cyanometabolites in water samples. The molecular networking approach, coupled with the Global Natural Products Social Molecular Networking (GNPS) database and CANOPUS annotation, enabled the putative identification of cyanometabolites. The resulting molecular network unveiled discernible clusters representing related molecule families, aiding in the identification of both known cyanotoxins and unfamiliar analogues. Furthermore, the molecular network revealed that water samples from different fishponds shared specific metabolites, including ethanesulfonic acid, pheophorbide A, cholic acid, phenylalanine, amyl amine, phosphocholine (PC), and sulfonic acid, despite variations in location, local climatic factors, and sampling sites. The fishponds in Nigeria showed the presence of multiple cyanotoxin classes in the dry, wet, and summer seasons in the water. The Duthuni, South Africa, sampling sites (P1, P2, and P3) exhibited the presence of microginins and microcystins. All the fishponds displayed a widespread occurrence of anabaenopeptins, and aplysiatoxins, during the selected summer. In conclusion, the untargeted metabolome analysis, guided by GNPS, proved highly effective in identifying both toxic and non-toxic metabolites in fishponds.

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1 Introduction

Cyanobacteria, commonly known as blue-green algae, are single-celled or filamentous organisms capable of oxygenic photosynthesis (Ciebiada et al., 2020; Huertas et al., 2022; Zuo et al., 2023). They thrive in various environments such as soils, freshwater bodies, thermal springs, and marine ecosystems (Bhardwaj et al., 2024). Similar to plants, cyanobacteria utilize sunlight to convert atmospheric carbon dioxide into organic compounds, potentially serving as a primary food source for other organisms (Arora et al., 2021; Keller et al., 2021). Under favorable environmental conditions characterized by optimal temperatures, abundant sunlight, and nutrient-rich water, cyanobacteria experience increased growth, leading to the formation of cyanobacterial blooms (Wang et al., 2021). This phenomenon may result in the release of toxins, particularly cyanotoxins, into the water (He et al., 2016; Metcalf and Codd, 2020).

Aquaculture ecosystems are particularly susceptible to cyanobacterial blooms as cyanobacteria play a vital role in the food web as phytoplankton biomass (Backovićet al., 2024; Chia et al., 2021; de Almeida et al., 2024). Moreover, cyanobacteria easily adapt to environmental conditions commonly encountered in fishponds, such as high temperatures, reduced light conditions, nitrogen depletion in the upper layer, a high degree of eutrophication, and a decline in the number of large phytoplanktivorous filter-feeders (Backovićet al., 2024; de Lima Pinheiro et al., 2023; Vrba et al., 2023). The toxins released by cyanobacteria during blooms may result in cooperative toxic effects on both animals and humans (Jones et al., 2021; Pei et al., 2020). Fish that come into contact with these cyanotoxins may experience non-lethal consequences, such as the accumulation of toxins in the liver, leading to liver damage, hepatocyte degradation, and potentially fatal liver hemorrhaging (Passos et al., 2023).

Exposure to cyanotoxins in animals and humans has been associated with various health issues, including carcinogenicity, gastroenteritis, skin reactions, liver damage, vomiting, headaches, allergic reactions, and even mortality (Buratti et al., 2017; Hilborn et al., 2014; Lad et al., 2022; Niture et al., 2023). People can easily encounter these toxins by consuming freshwater, fish, seafood, crops, vegetables, or food supplements containing cyanotoxins, or by ingesting them during recreational activities (Buratti et al., 2017; Funari et al., 2008; Lee et al., 2017).

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The adverse effects of these toxins originating from fishponds have repercussions on a wider array of products that could face comparable contamination (Chia et al., 2021; Gärtner et al., 2021). The increasing concern underscores the necessity to scrutinize the possible presence of cyanotoxins in aquaculture fishponds. So far, there is no comparative study that investigates seasonal and site-specific variations in cyanometabolites profiles in fishponds in Nigeria and South Africa. Chia et al. (2015; 2021) only focused on microcystin in fishponds in Zaria, Nigeria. Therefore, this study aims to provide a comprehensive analysis of cyanometabolites profiles including cyanotoxins present in selected fish farming ponds in Nigeria and South Africa. This data can be used as baseline reference values for monitoring metabolites in fish farming ponds and a pre-requisite to ensure safe products for human and animal well-being.

2 Methodology

2.1 Study area and Sampling sites

The study was conducted in commercial aquaculture fishponds situated in the Vhembe District, Limpopo Province, South Africa, and the Calabar Municipality, Cross River State, Nigeria displayed in Figure 4.1. In Nigeria, sampling locations included Offiong Etim Avenue ($4^0$59’58.92” N and $8^0$19’03.97” E), Essien Town ($4^0$59’15.49” N and $8^0$19’40.21” E), and State Housing ($4^0$59’6.50” N and $8^0$20’13.29” E). The aquaculture fishponds in the Vhembe District were positioned in Duthuni Pond 1 ($22^0$57’56.98” S and $30^0$23’43.96” E), Duthuni Pond 2 (22°57’56.89”S and 30°23’43.96”E), and Duthuni Pond 3 (22°57’56.98”S and 30°23’44.06”E.

This study utilized a total of six fish ponds situated in the Vhembe District (comprising three fish ponds) and Calabar (comprising three fish ponds). The fishpond types incorporated in this investigation consisted of concrete, tarpaulin, and earthen ponds, as illustrated in Figure 4.2. It should be noted that the selection of Nigeria and South Africa for the comparative study on cyanotoxins in fishponds was based on several key variables, including environmental diversity, geographical location, and climatic conditions.

The reliability and accuracy of the laboratory data, strict quality assurance (QA) protocols were followed throughout the study. Standard operating procedures (SOPs) were adhered to during sample collection, storage, and transportation to prevent contamination and degradation. Laboratory analyses were conducted following internationally recognized methods, with

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appropriate controls and calibration standards to validate the results. For analyses conducted in my absence, the laboratory operated under an accredited quality management system, ensuring compliance with Good Laboratory Practices (GLP) and ISO standards (International Organization for Standardization) where applicable. This ensures that laboratories operate competently and produce valid, reproducible results. Additionally, method validation, instrument calibration, and triplicate analyses were performed to enhance data reliability. To further verify data integrity, randomly selected samples were reanalyzed, and results were cross-checked for consistency. These measures collectively ensure that the data generated in this study are robust, reproducible, and scientifically sound.

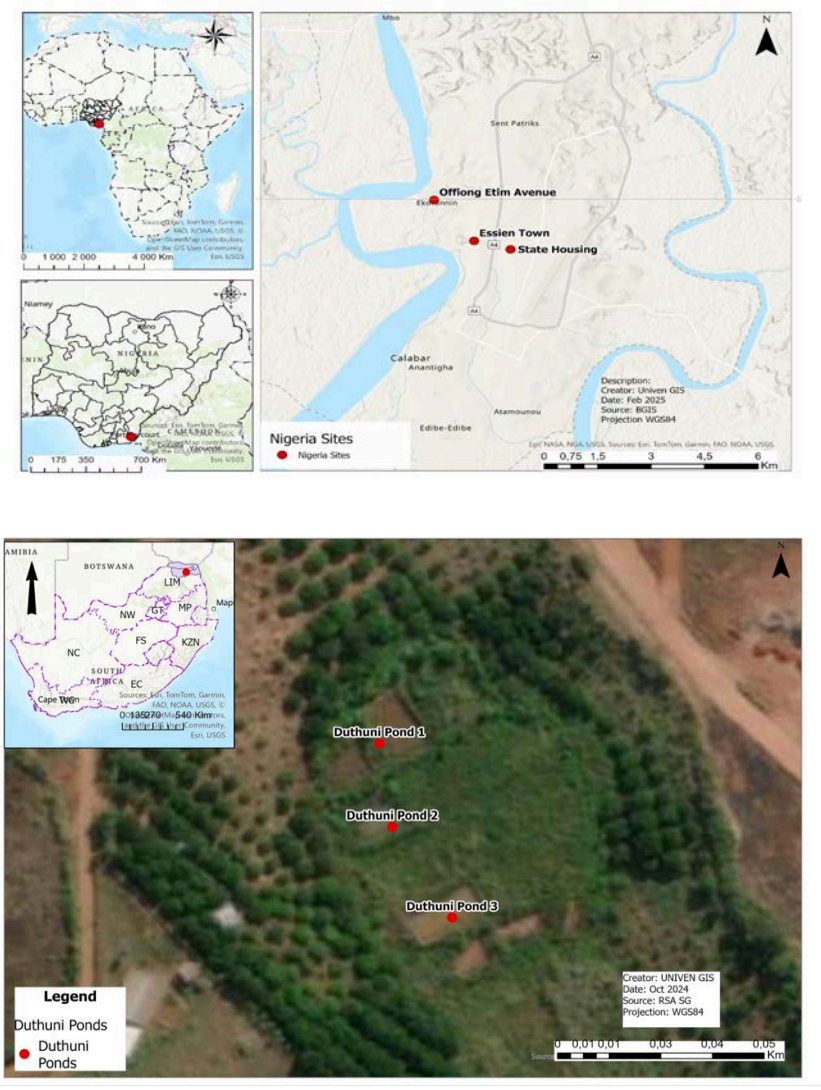

Figure 4.1: Map of the sampling sites - Vhembe District, Limpopo Province, South Africa and Calabar Municipality, Cross River State, Nigeria.

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Figure 4. 2: A): Earthen aquaculture fishponds in Duthuni, South Africa and B): tarpaulin fish pond in Calabar Municipality, Nigeria

2.2 Water sampling

Water samples were seasonally collected from the fishponds in triplicates per pond (total of 6 ponds) during the South African winter and summer and the Nigerian dry and wet seasons, respectively. Data collection involved four field trips conducted during specific seasonal periods: January and February (summer), June (winter), August and September (wet season), and November and December (dry season). Water samples were sampled at depths between 0 and 0.5 meters from each fishpond using sterilized labeled bottles. Water samples were collected approximately 1-2 meters away from the edge to avoid contamination from edge-related disturbances. Water samples were collected using clean, 1-litre sterilized bottles. The samples were placed in an ice-filled cooler box before being transported to the laboratory for further analysis. Approved consent was obtained from the owners of the fishponds in Nigeria and South Africa before sampling.

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2.3 Extraction of water samples

The procedure according to Kim et al. (2009) was followed in the extraction of cyanometabolites. Ten milliliters (10 mL) of each collected water sample was transferred into the 50 mL tube. This was followed by sonication for 30 minutes and the water samples were freeze-dried. The freeze-dried sample residue was reconstituted in 10 mL of methanol (90% methanol). The mixture was further sonicated for 30 min before transferring 1 mL of the mixture to the microfuge for 10 mins at 3000 rpm. This mixture was filtered immediately before transferring to a 10 mL opaque bottle. The final mixture was placed on the shaker for 12 hours a room temperature. The mixture was transferred to glass vials for LCMS analysis using the syringe filter.

2.4 LCMS analyses

The analysis of cyanometabolites in non-targeted analytes was performed using an LCMS-9030 qTOF (Shimadzu Corporation, Kyoto, Japan) liquid chromatography-quadrupole time-of-flight tandem mass spectrometer. Chromatographic separation employed a Shim-pack Velox C18 column (100 mm x 2.1 mm, 2.7 µm particle size) with an injection volume of 3 µL. A binary mobile phase gradient consisting of solvent A (0.1% formic acid in Milli-Q water, HPLC grade, Merck Darmstadt, Germany) and solvent B (Methanol, UHPLC grade, Romil SpS, Cambridge, UK) with 0.1% formic acid was utilized, maintaining a flow rate of 0.3 mL/min over a 20-minute gradient. The separation conditions included maintaining 10% B for 3 minutes, transitioning from 10% to 95% B over 3-20 minutes, holding 40% B for 7 minutes, reaching 95% B from 10 to 15 minutes, returning to initial conditions between 18-20 minutes, followed by a 3-minute column equilibration time. Mass spectra were recorded in positive-ion mode for all samples using the qTOF high-definition mass spectrometer. The MS parameters were as follows: interface voltage of 4.0 kV, interface temperature of 375 °C, nebulization and dry gas flow at 3 L/min, heat block temperature of 400 °C, DL temperature of 280 °C, detector voltage of 1.8 kV, and flight tube temperature of 42 °C. The chromatographic effluents were subjected to further analysis utilizing the qTOF high-definition mass spectrometer, recording mass spectra in positive-ion mode.

2.5 Microscopic identification

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A benchtop FlowCAM (Model VS IV) was used for the morphological identification of cyanobacteria species. This involved capturing images and employing comparative analysis with existing literature for identification purposes. A filtered water sample (2 ml) was poured into the flow chamber via a pipette after rinsing the pump with deionized water to execute this experiment. The computer's digital signal processor and the trigger circuitry collaborated to initiate, retrieve, and process these images saved on the visual spreadsheet. Each pixel grouping representing individual particles was isolated from the raw images and stored as distinct collage images.

2.6 Data analyses

High-resolution MS/MS spectra data generated from the Mass spectrometry was converted to an mzML file before being processed using the Global Natural Product Social Molecular Networking (GNPS) Library database. Putatively identified (LEVEL 3 identification) metabolites generated from the GNPS Library match and CANOPUS-generated annotation (Dührkop et al., 2015; 2019; 2020; Kim et al., 2021; Yannick et al., 2016) were used to produce a molecular network. The molecular network was visualized using Cytoscape 3.10.0. Cyanotoxins' retention time and intensity were assessed graphically.

3. Results and Discussion

3.1 Molecular networks

The molecular network highlights known metabolites, structural identity, shared clusters, nodes, and edges, matching metabolites (metabolites within the public database), and non-matching metabolites (metabolites not found within the database) (Damiani et al., 2023; Libis et al., 2019; Narduzzi, 2015). Various analytes were grouped into molecular clusters based on the similarity of their fragmentation patterns (Ibrahim, 2017; Dührkop et al., 2021). This networking aided in identifying both known cyanotoxins and unfamiliar analogues, visually illustrating structural connections. The molecular network showed that water samples from Nigeria (N1, N2, and N3) and South Africa (P1, P2, P3) shared specific metabolites despite differences in location, local climatic factors, and sampling sites. The molecular networking led to the putative identification of multiple cyanotoxins—aeruginosins, anabaenopeptins, aplysiatoxins, and microcystin, during winter, summer, dry, and wet seasons (Figure 4.3) in water samples. Untargeted metabolome analysis was highly effective for identifying toxic and non-toxic metabolites in the fishponds.

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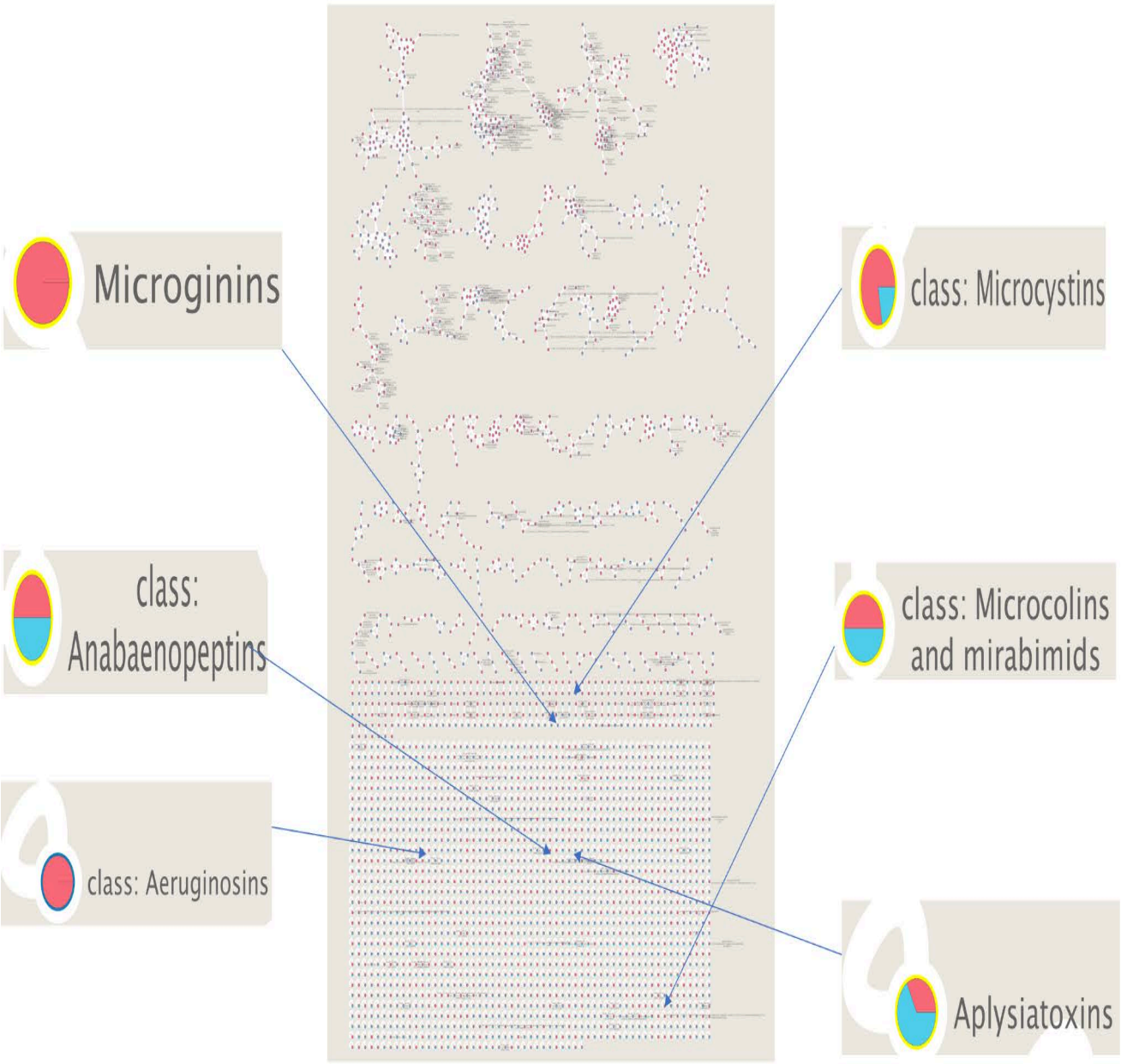

Figure 4.3: The molecular network of cyanotoxins from fishpond water samples

3.2 Cyanobacterial toxins and other bioactive metabolites

The water samples from the fishpond sampling sites in Nigeria and South Africa shared similar secondary metabolites, including eicosapentaenoic acid (EPA), pentanoic acid, carboxylic acid, octadecanamide, ethanesulfonic acid, and tryptophan, as presented in Figure 4.4. The polar lipids identified in the water samples were dominated by six main classes: glycolipids, phosphosphingolipids, phospholipids, phosphatidylglycerol (PG), and phosphatidylethanolamine

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(PE), which were distributed across the fishponds. Cyanobacteria are intrinsically linked to the presence of specific lipid classes, such as glycolipids, phosphosphingolipids, and phospholipids, including PG and PE, in surface water (Ali and Szabó, 2023; Wardhan et al., 2017).

These polar lipids constitute the building blocks of cyanobacterial membranes, characterized by molecular structures composed of glycerol backbones and ester-linked fatty acids (Gull et al., 2021; Summons et al., 2022). This association elucidates the prevalence of glycerol-based compounds in both water samples. Microcystins were found in Duthuni (P1, P2, and P3), also shown in Table 4.1. Anabaenopeptins, aplysiatoxins, microcystins, and aeruginosins were widespread in all fishponds, as displayed in Figure 4.5. The cyanometabolites in both sampling locations align with observations from the Tri, Var, and Ver lakes (Marie and Gallet, 2022). These observations suggest that optimal conditions may be an explicative factor in the production of toxins in fishponds.

Table 4.1: Sampling Sites Identification

SA Fishponds	**SA Fishponds ID**	**NGA Fishponds**	**NGA fishponds ID**
Duthuni Pond 1	P1	Offiong Etim Avenue	N1
Duthuni Pond 2	P2	Essien Town	N2
Duthuni Pond 3	P3	State Housing	N3

The cyanotoxins noted in this study are similar to the toxins recently reported by Zastepa et al. (2023). The study detected anabaenopeptins, aeruginosamide, saxitoxin, cylindrospermopsin, and microcystins in cyanobacteria bloom-dominated Lake of the Woods (LOW), spanning Canada and the USA. Similarly, anabaenopeptins, cyanopeptolins, microginins, and cyanobactins were also reported in commercial fishponds in the Czech Republic (Kust et al., 2020). Parallel research in cyanobacteria-rich lakes (Fon, Tri, Var, and Ver) confirmed the presence of multiple toxins, including microcystins, cyanopeptolins, anabaenopeptins, microginins, and aeruginosins (Marie and Gallet, 2022). Zastepa et al. (2023) suggested that this pattern is commonly associated with cyanotoxins and cyanobacterial blooms in surface waters.

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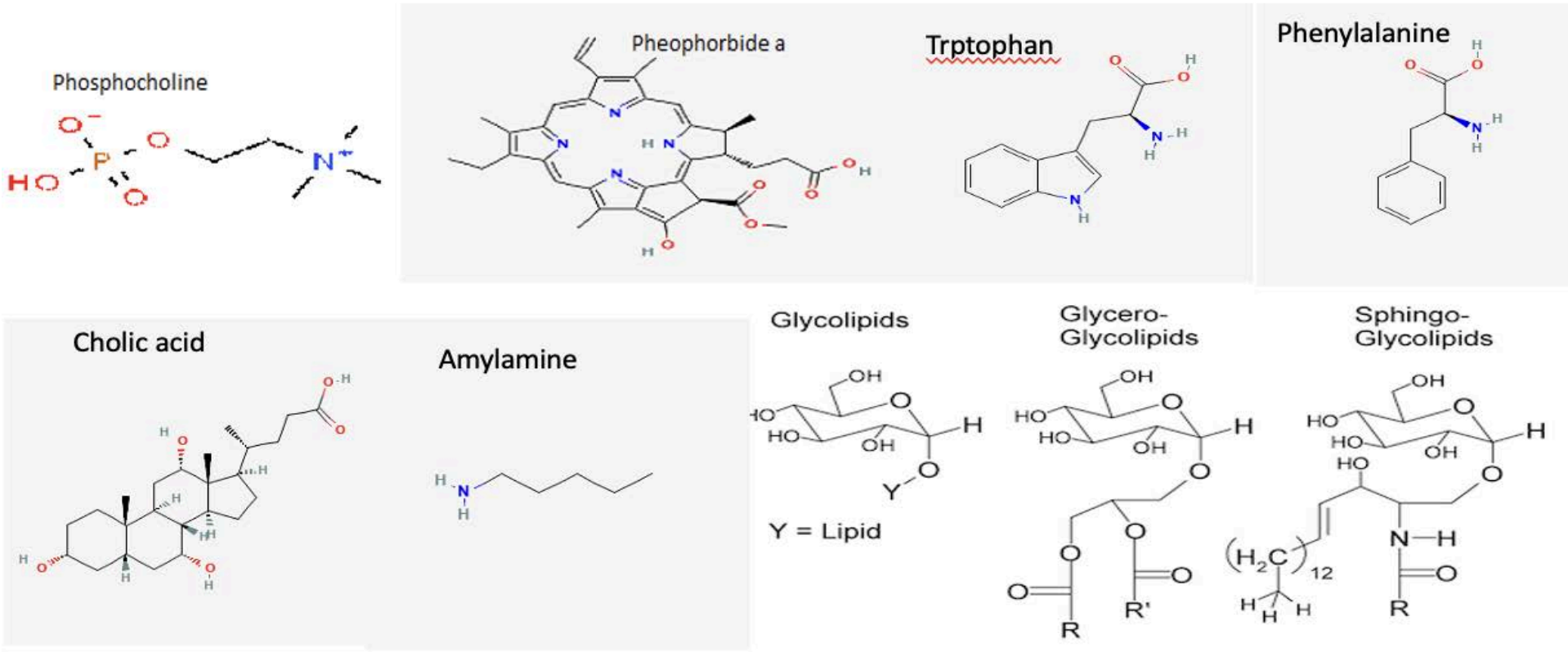

Figure 4.4: Structural representation of non-toxic bioactive metabolites present in fishpond

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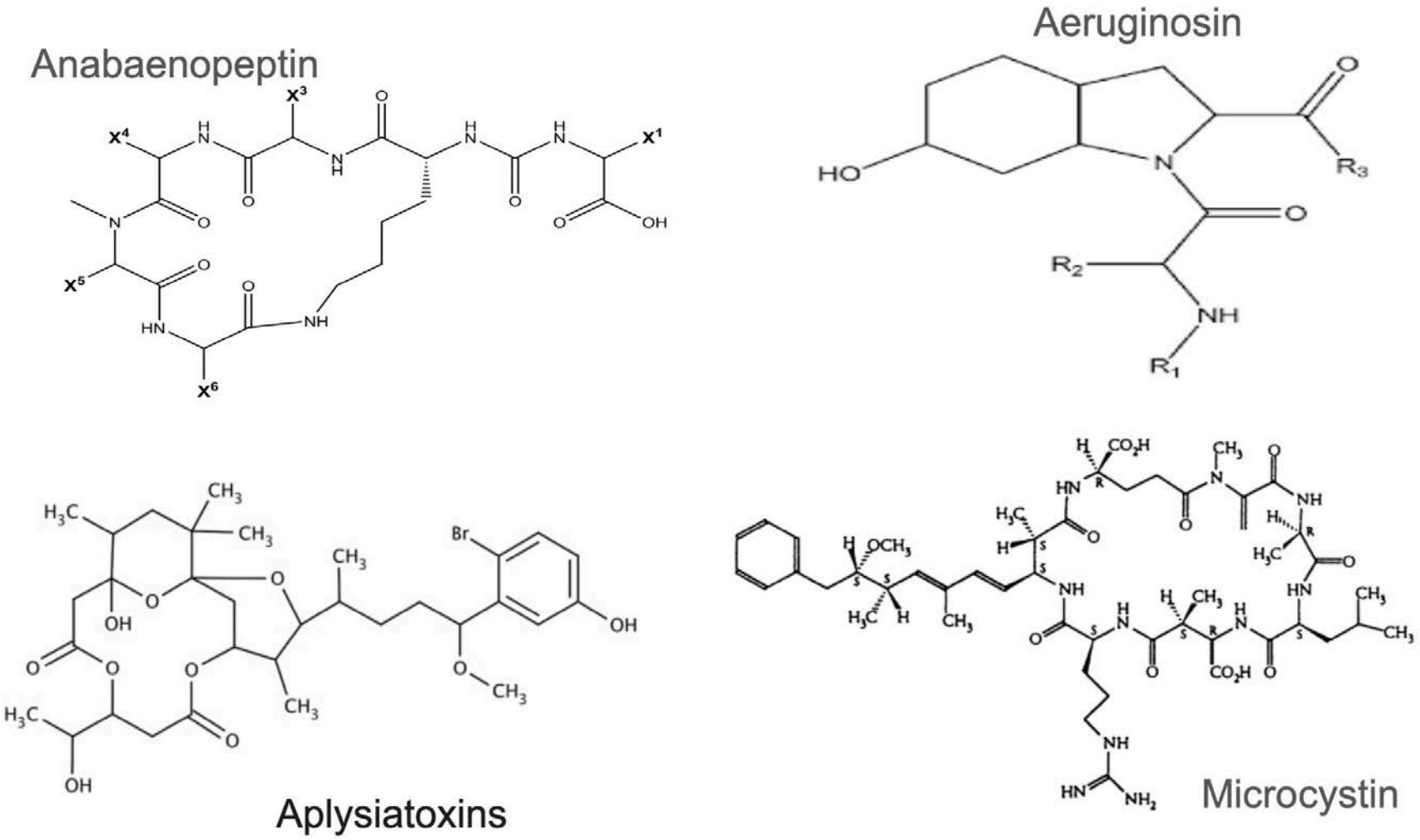

Figure 4.5: Structural representation of cyanotoxins present in fishponds.

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3.3 Seasonal and regional dynamics of cyanotoxins and bioactive metabolites in fishponds

Cyanobacterial blooms were evident across all fishponds in Nigeria throughout both wet and dry seasons. These seasons were characterised by a prevalence of cyanobacterial peptides, such as anabaenopeptins, microcolins and mirabimids, in the fishponds. In Duthuni, South Africa, the summer season witnessed a higher prevalence of various cyanotoxin classes, including aeruginosins, anabaenopeptins, aplysiatoxins, microginins, microcolins, and marabmids notably detected in the fishponds' water samples in Figure 4.7. Simultaneous presence of cyanotoxin in water during the dry and summer seasons may be linked to the abundance of toxic cells specific to microcystins, microgninns, anabaenopeptins, microcolins, and mirabimids in the water (Wood et al.. 2014) and the environmental conditions within fishponds. Favorable environmental factors might facilitate the growth of cyanobacteria, leading to the release of cyanotoxins into the water (Chorus et al., 2021; Massey et al., 2022). Aeruginosins, anabaenopeptins, aplysiatoxins, and microcystins based on peak intensity and retention time across different sampling sites showed variations among each cyanometabolites. The maximum intensity observed was for microcolins, while the minimum was for anabaenopeptins.

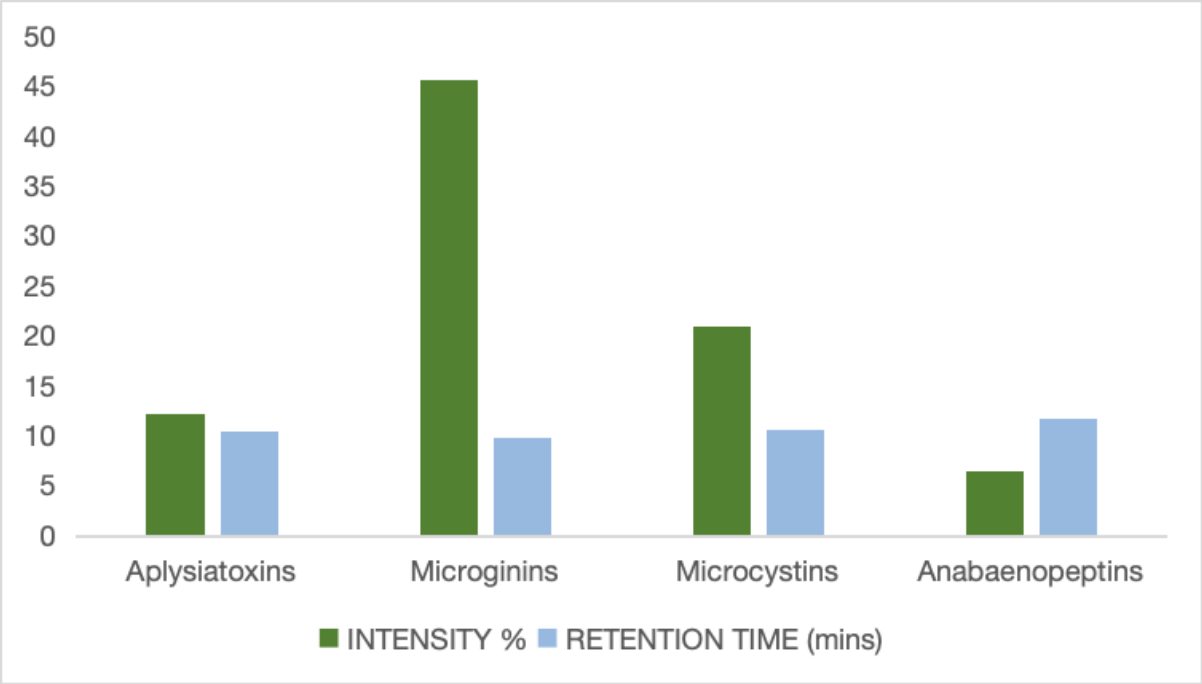

Figure 4.6: Cyanotoxins and other metabolites intensity (%) and retention (min)

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Local environmental conditions play a pivotal role in shaping cyanobacterial proliferation within fishponds. In this study, site-specific environmental conditions influenced the production of noxious compounds, aligning with the findings of Marie and Gallet (2022) and Burford et al. (2020). Environmental factors, especially during the warm season, increase daytime temperature, nutrient input (from fish feces, feed particles, and anthropogenic activities), and solar radiation, which favors excessive cyanobacterial proliferation and toxin production (Mohamed et al., 2020). This explains the increased presence of multiple toxins during the dry season in Nigerian fishponds and during summer in Duthuni (South Africa) fishponds. A similar observation was reported during the dry season in fishponds in Zaria, Nigeria, by Chia et al. (2021). Additionally, Kust et al. (2020) noted the highest diversity of aeruginosins, microginins, cyanopeptolins, and microginins during the summer in South Bohemia, Czech Republic.

Conclusion

This comparative study sheds light on the varied profiles of cyanotoxins in fishponds between Nigeria and South Africa. The non-targeted analysis of secondary metabolites produced by cyanobacteria species proved to be a successful method for detecting cyanotoxins in commercial fishponds. Given the rapidly increasing proliferation of cyanobacteria in fishponds, continued research, and vigilant monitoring are imperative to comprehensively address the multifaceted challenges posed by cyanotoxins in fishpond environments. Understanding cyanotoxins' diversity, distribution, and seasonal dynamics is crucial for devising effective strategies to mitigate their impact on aquatic ecosystems and safeguard human health.

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Chapter 5

This chapter addresses objective 3

> To investigate the bioaccumulation of cyanotoxins in *Clarias gariepinus* harvested from fishponds.

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Emerging Contaminants, 2025, https://doi.org/10.1016/j.emcon.2025.100484.

Abstract

Cyanotoxins produced by cyanobacteria are formidable threats to aquatic ecosystems and public health worldwide. The potential health risks associated with cyanotoxins from contaminated fishponds are becoming a growing concern, as cyanotoxin production has steadily increased over time in these aquatic environments. Therefore, this study aims to utilize targeted and non-targeted Liquid Chromatography Mass Spectrometer (LC-MS) analytical methods to detect cyanotoxins in catfish (*Clarias gariepinus*) tissue harvested from fishponds. For detecting cyanotoxins in fish tissue utilizing the non-targeted approach, high-resolution MS/MS spectra data obtained from the analysis were converted to mzML format, analyzed with the Global Natural Product Social (GNPS) Library and CANOPUS annotations for LEVEL 3 metabolite identification, and visualized as a molecular network in Cytoscape. Regarding the targeted method, the toxin identification and quantification were achieved by comparing samples spiked with known concentrations of MC-RR and YR to an authentic toxin standard. The results of the target analysis showed that microcystin variant MC-RR was not detected in the fish tissue. The MC-YR variant was detected in the intestines and gills of *Clarias gariepinus* at concentrations of 13.2 - 10.6 µg/g and 1.5 - 13.9 µg/g, respectively. The muscle tissues across all fish ponds showed MC-YR concentrations between 10.5 and 16.06 µg/g. The highest concentration of MC-YR was found in the liver tissue in pond 6 (20.9 µg/g). The untargeted LC-MS method led to the identification of a larger number of cyanometabolites in the fish tissue, such as aeruginosins, anabaenopeptins, microginins. Non-toxic secondary metabolites like octadecadienoic acid, while phosphocholine (PC), ethanesulfonic acid, pheophorbide A, microcolins, cholic acid, phenylalanine, amyl amine and phosphocholine (PC), triglyceride (TG), phosphocholine (PC) and sulfonic acid derieved from cyanobacteria, fish and anthropogenic

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sources were also detected in the fish tissues. The non-targeted analysis facilitates the identification of both unexpected and unknown compounds.

Keywords: Liquid Chromatography Mass Spectrometer*;* cyanotoxins; cyanobacteria; targeted analysis; metabolites.

1 Introduction

Cyanobacteria are present in freshwater, ponds, rivers, marine, and other terrestrial environment. The unique morphological, physiological, and genetic traits of cyanobacteria make them rich sources and producers of different biological active metabolites (Gupta et al., 2013; Nandagopal et al., 2021). Cyanobacteria metabolites (cyanometabolites) constitute a significant and rapidly expanding category of secondary metabolites, encompassing over 2000 compounds, which are synthesized by various genera of cyanobacteria (McCann, 2022). Most of these secondary metabolites including different classes of cyclic peptides, phenols, lipids, alkaloids, vitamins, terpenoids, polysaccharides, and pigments identified in cyanobacteria have the potential to act as antioxidant, anticoagulant, anticancer, antiviral, antileishmanial, antibacterial, antiprotozoal, and anti-inflammatory (Divyashree et al., 2019; Kini et al., 2020; Lourthuraj et al., 2023). Some of these secondary metabolites are widely applied in the pharmaceutical, cosmeceutical, and nutraceutical industries applications (Kini et al., 2020; Rodríguez et al., 2020). Furthermore, cyanobacteria have attracted attention as producers of novel metabolites significant in industry and medicine (Nitnaware, 2021). However, numerous cyanobacteria species that frequently form large blooms in fresh, brackish, and marine waters also produce metabolites called cyanotoxins (Sivonen, 1996). These toxins are associated with acute lethal, acute, chronic, and sub-chronic poisonings in animals, and pose potential health risks to humans (Sivonen, 1996; CarAdvancements in both specific and broad-spectrum bioanalytical tools, coupled with the greater accessibility of calibration standards, have broadened the scope of toxic and bioactive metabolites that can be measured (Huang et al., 2021). However, one of the major challenges in cyanometabolites analysis is the lack of pure standards (Zastepa et al., 2021; Varriale et al. 2023). Cyanometabolites standards are known to be expensive and limited to a few metabolites (mostly Microcystin and Nodularin) commercially available for purchase (Jaiswal and Wangikar, 2020; Varriale et al., 2023). The lack of pure standards for over 2000 cyanometabolites prevents confirmation based on chromatographic properties (Varriale et al., 2023). Therefore, effective

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multiclass analytical methods are required to evaluate cyanotoxin in fish tissues and address these challenges (Filatova, 2020). The application of Mass spectrometry in combination with chromatography (Gas chromatography or Liquid chromatography) has been identified as a powerful analytical tool used for both quantitative screening and quantification of biological and environmental samples (Alder et al., 2006). The sensitivity and selective detection of polar or ionic contaminants at trace levels using LC-MS have made it a highly favorable alternative to GC-MS (Hird et al., 2018). The LC-MS screening can be targeted or non-targeted. The targeted analysis focuses on detecting known analytes using reference standards, but its scope is limited to the selected compounds and the availability of reference materials (Hird et al., 2018). The non-target analysis allows for the detection of unexpected and unknown compounds by using molecular information and database searches, with identification aided by comparing MS/MS spectra to vendor-supplied libraries (Dom et al., 2018; Hird et al., 2018). As new compounds emerge, the non-targeted approach provides more opportunities to discover previously unknown compounds. Considering the advantages and limitations of both targeted and non-targeted analytical approaches, this study aimed to identify cyanotoxin MCs in fish tissues using a combination of LC-MS targeted and untargeted methods. This offers an opportunity for non-targeted analysis and the detection of a wider range of cyanotoxins in the fish tissues.

2. Methodology

2.1. Study Area and Sampling Sites

This investigation was carried out in commercial aquaculture fishponds mentioned in Bassey et al. (2024). The fishponds are situated in the Vhembe District of Limpopo Province, South Africa, and in Calabar Municipality, Cross River State, Nigeria. In Nigeria, the selected sampling sites (Table 5.1) were Offiong Etim Avenue ($4^0$59’58.92” N and $8^0$19’03.97” E), Essien Town ($4^0$59’15.49” N and $8^0$19’40.21” E), and State Housing ($4^0$59’6.50” N and $8^0$20’13.29” E). The aquaculture fishponds in the Vhembe District were positioned in Duthuni Pond 1 ($22^0$57’56.98” S and $30^0$23’43.96” E), Duthuni Pond 2 (22°57’56.89”S and 30°23’43.96”E), and Duthuni Pond 3 (22°57’56.98”S and 30°23’44.06”E).The study used six fish ponds, three from each study area (Vhembe District and Calabar Municipality). Selection criteria for sampling sites included their designation as commercial fishponds, accessibility, consent from owners, and the presence of cultured fish.

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Table 5.1: Sampling Locations Identification

SA Fishponds	SA Fishponds ID	NGA Fishponds	NGA fishponds ID
Duthuni Pond 1	P1	Offiong Etim Avenue	P4
Duthuni Pond 2	P2	Essien Town	P5
Duthuni Pond 3	P3	State Housing	P6

2.2. Fish Sampling

A total of 18 *Clarias gariepinus* (catfish) samples represented the sampling unit of six fishponds. The fish samples were directly purchased from small-scale fishponds. The fish samples were captured using a fishing net with a mesh size of 20 mm. The age of each fish was determined using the age-length approach to select the appropriate size. *Clarias gariepinus* within the length of 38 to 42 cm was selected. The fish within this size range were selected for the study to protect the undersized fish population. The catch-and-release approach was used to exclude undersized fish populations during fish sampling. Afterward, the captured fish were wrapped in aluminum foiled paper and placed in an ice field cooler box before transporting to the laboratory. The selected fish samples were euthanized by suffocation in air asphyxiation and ice chilling (bath). Ethical clearance was sought from the University of Venda Research Ethics Committee. Consent from the owners of the fishpond was obtained and approved.

2.3. Targeted method using microcystin standards

2.3.1. Sample preparation, extraction, and purification.

Similar procedure expressed by Manubolu et al. (2018) was employed in the present study. The collected fish samples were used to obtain muscle tissue, gills, liver, and stomach contents (Figure 5.1). Each filleted tissue was cut into smaller pieces and freeze-dried separately. Small chunks of the freeze-dried fish tissues were ground in combination with dry ice (50% of the tissue volume) with a pestle and mortar. Afterward, each sample was divided into 1 g (n = 3) aliquots after processing. Each sample except for the unknown was spiked with MC-YR and MC-RR (certified analytical standard with >95% purity). This was carried out using adding known concentrations (1 µg/L, 2 µg/L, 2.5 µg/L, and 5 µg/L) of MC-YR and MC-RR to the 1 g

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of wet mass fish sample. This was followed by incubation using a glass vial at room temperature for 20h in the dark.

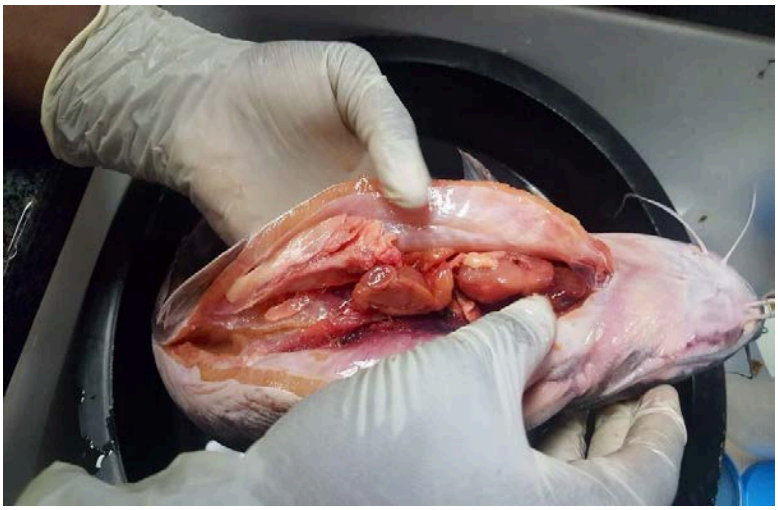

Figure 5.1: Gutting fish samples to obtain the organ

spiking and the incubation process allowed the formation of covalently bound MC complexes (Craig et al., 1996). This was followed by homogenization using a tissue probe homogenizer to further homogenize the samples. Homogenization was carried out using 80% methanol. The mixture was further sonicated for 2, 5, and 10 min. This was followed by centrifugation at 3500 rpm using centrifuge for 20 min. The extract supernatant was collected after centrifugation. Thereafter, samples were ready for solid-phase extraction (SPE) cleanup.

SPE was conducted using a 12-port SPE vacuum manifold equipped with large-volume samplers and a diaphragm vacuum pump. Oasis HLB cartridges (500 mg bed size, 6 mL capacity) were serially connected and preconditioned with 3 mL of methanol followed by 6 mL of water. A total of 30 mL of fish sample extract supernatant was slowly passed through the cartridge assembly and then rinsed with 20% methanol. Before elution with 80% methanol, cartridges were vacuum-dried for 1 minute. Fish samples extract supernatant were eluted with 5 mL and 25 mL of methanol, respectively. The eluates were dried under a gentle stream of nitrogen gas, and the resulting residues were re-dissolved in 1 mL of 80% methanol. The solutions were then filtered through 0.2 µm polytetrafluoroethylene filters (Inqaba Biotec, Pretoria, South Africa) into autosampler glass vials, ready for LC-MS/MS analysis.

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2.3.2. LC-MS Quantification Analyses and Method Validation

This study employed the LC-MS method expressed in Mutoti et al. (2024). A Liquid Chromatograph Triple Quadrupole Mass Spectrometer (LC-QqQ-MS/MS) (Shimadzu, Japan) equipped with a binary solvent delivery system and a sample manager was utilized for the analysis. Chromatographic separation was achieved using a Shim-pack Velox C18 column (2.1 × 100 mm, 2.7 µm particle size) with a serial number of 227-32009-03 (COU, MO, USA) at a flow rate of 0.4 mL/min. An injection volume of 1 µL was found to be optimal for this analysis. The mobile phase consisted of solvent A (H_2O) and solvent B (MeOH) in a gradient mode. The gradient program initiated at 95% A (held for 1.5 minutes), decreased to 5% A (from 1.5 to 2.0 minutes), held at 5% A (from 2.0 to 3.0 minutes), and then increased back to 95% A (from 3.0 to 4.0 minutes). The column oven temperature was maintained at 40°C throughout the analysis. The limit of detection was determined before the analysis.

Identification and quantification of toxins were determined by comparison with the authentic toxin standard (Ruangsomboon et al., 2014). Linear calibration curves were generated for each microcystin (MC) congener after evaluation of measurements and calibration range using standard solutions with known concentrations (2 – 200 ppb) (Table 5.2). Precision and accuracy of the method were assessed through recovery experiments, which involved analyzing samples spiked with known concentrations of MC-LR and YR (1 µg/L, 2 µg/L, 2.5 µg/L and 5 µg/L). The limit of detection (LOD) was determined based on a signal-to-noise (S/N) ratio of 3, estimated from the chromatograms of samples spiked at the lowest validated concentration level. The instrument response was measured for these standards to create a calibration curve, which is then used to quantify the analytes in actual samples.

Table 5.2: Determination of microcystins variants standard curve regression.

Reference material	Standard curve range (µg/L)	Standard curve regression fit style	Determination coefficient (r2)
MC-RR	2 - 200	Linear	0.997
MC-YR	2 - 200	Linear	0.998

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2.4. Untargeted method

2.4.1. Sample preparation, extraction, and purification

Cyanotoxin extraction procedure for fish tissues was adapted from Bassey et al. (2024). One gram (1g) of each freeze-dried fish tissue (muscle, liver, gills, and intestine) was dissolved in 10 mL of 90% methanol. The mixture was shaken for 24 hours at room temperature. This was followed by sonication for 30 minutes before overnight freeze-drying. The freeze-dried fish tissue residues were reconstituted in 10 mL of 90% methanol. Ultrasonication was performed for 30 minutes, followed by centrifugation of 1 mL of the liquid in a microfuge at 3000 rpm for 10 minutes. The resulting aliquots were then filtered and transferred to 10 mL opaque bottles. Filtrates were vortexed for 2 minutes and subsequently placed on a shaker for 24 hours at room temperature. Finally, the mixtures were transferred to glass vials for LC-MS analysis. All the samples were prepared and analyzed in triplicates.

2.4.2. LC-MS analyses

The LC-MS procedure described by Bassey et al. (2024) was employed in the present study. A liquid chromatography-quadrupole time-of-flight tandem mass spectrometer (LCMS-9030 qTOF, Shimadzu Corporation, Kyoto, Japan) was utilized for the analysis of cyanometabolites in non-targeted analytes. Chromatographic separation was achieved using a Shim-pack Velox C18 column (100 mm x 2.1 mm, particle size 2.7 µm). The injection volume was set at 3 µL, and a binary mobile phase gradient was applied. Solvent A consisted of 0.1% formic acid in Milli-Q water (HPLC grade, Merck Darmstadt, Germany), while solvent B was methanol (UHPLC grade, Romil SpS, Cambridge, UK) with 0.1% formic acid. The flow rate was maintained at 0.3 mL/min over a 20-minute gradient, with the following conditions: 10% B for 3 minutes, a gradient from 10% to 95% B from 3 to 20 minutes, 40% B for 7 minutes, and 95% B from 10 to 15 minutes. The gradient returned to its initial conditions between 18-20 minutes, followed by a 3-minute column equilibration. Positive-ion mode was used for mass spectrometry of all samples. Chromatographic effluents were further analyzed using the qTOF high-definition mass spectrometer, with mass spectra recorded in positive-ion mode. Mass spectrometry parameters were configured as follows: interface voltage of 4.0 kV, interface temperature of 375 °C, nebulization and drying gas flow at 3 L/min, heat block temperature of 400 °C, DL temperature of 280 °C, detector voltage of 1.8 kV, and flight tube temperature of 42 °C.

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2.4.3. Data analysis

Data analysis in this study is similar to Bassey et al. (2024). The mass spectrometry generated high-resolution MS/MS spectra data, which was converted into mzML format before uploading to the GNPS Library database. The data was analyzed using the Global Natural Product Social (GNPS) Library database (to match the compound class) in combination with CANOPUS-generated annotation from SIRIUS4 Software. Identification of metabolites was conducted using confidence LEVEL 3 annotation. The putative-identified spectra were used to construct a molecular networking visualized using Cytoscape 3.10.0.

3. Results and Discussion

3.1. Targeted cyanotoxins (Microcystins)

Chromatographic results displayed in Figure 5.2 exhibited high sensitivity, selectivity, and precision in detecting MC-YR analytes from the fish tissue, but MC-RR was not detected in the fish tissues. This may indicate that the MC-RR compound is below the detection limit under current preparation conditions. The target method showed satisfactory sensitivity, with stable mass measurements aligning with previously reported LC-MS mass data from Mutoti et al. (2024). The calibration curves created by the MC-YR standard were an effective approach used to compare with the analytes in fish samples presented in Figure 5.3.

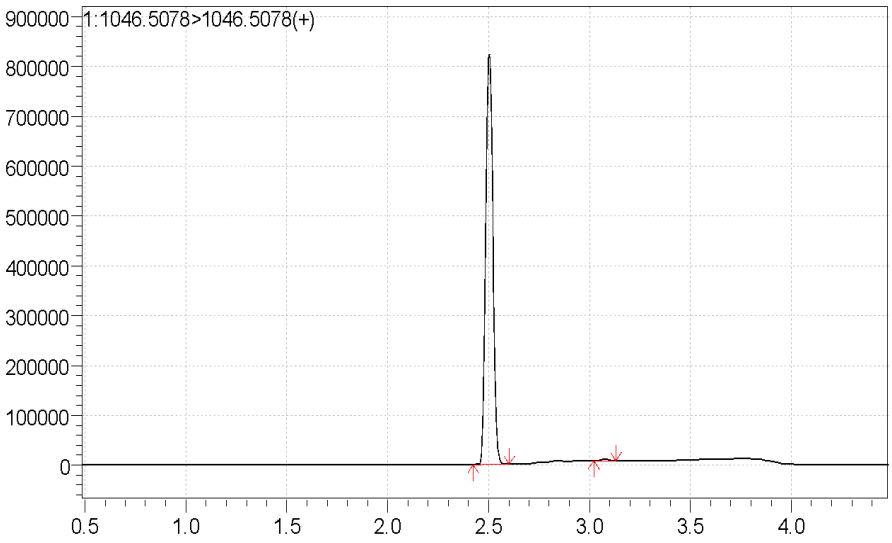

Figure 5.2: LC-MS chromatograms of quantification ions for microcystins-YR at a concentration of 200 ppb.

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3.2. Method validation

The linearity of the method and measurement range were studied through analyses of standard solutions at 6 different concentrations ranging between 2 – 200 ppb. Therefore, the method was validated by assessing its correctness, and this was done by assessing samples spiked with different concentrations of stock solution of the target analytes (MC-YR and -RR) (Table 5.3). Additionally, blank samples were analyzed to assess the specificity of the method adopted in the present study and it was observed that no peaks or signals were appearing close to the retention time of the analytes. Finally, Table 5.3 further shows that four different samples were spiked and analyzed, and therefore mean recoveries were estimated and found satisfactory ranging between 81 – 97 % for the target compounds. The limit of detection was further calculated in the present study as per Mutoti et al., 2023. These validation results therefore indicated that the method adopted was appropriate for the quantitative determination of the target compound of interest.

Table 5.3: Sample recoveries and concentration levels of spiking solutions used to spike samples for method validation.

MC variant	Intestines	Muscle	Gills	Liver	LOD (µg/L)
	1 µg/L	2 µg/L	2.5 µg/L	5 µg/L	
MC-YR	92%	81%	91%	97%	2
MC-RR	88%	87%	93%	77%	1

3.3. Method application to real samples

Liver

Microcystin variant MC-YR was detected in the *Clarias gariepinus* liver with concentrations ranging from 20.896 - 10.96 µg/g (Figure 5.4). The concentration of MC-YR in the liver tissue from Pond 6 was the highest compared to other fish tissues. Empirical studies over the past decades to date have expressed similar results that MC compounds were elevated in the fish liver compared to other fish organs in Rainbow Trout (*Oncorhynchus mykiss*), Lake Trout (*Salvelinus namaycush*) (Shahmohamadloo et al., 2022), *Carassius gibelio* (Papadimitriou et al., 2010) and Wild Nile and redbreast tilapia (*Clarias gariepinus* and *Tilapia rendalli*) (Deblois et al., 2008).

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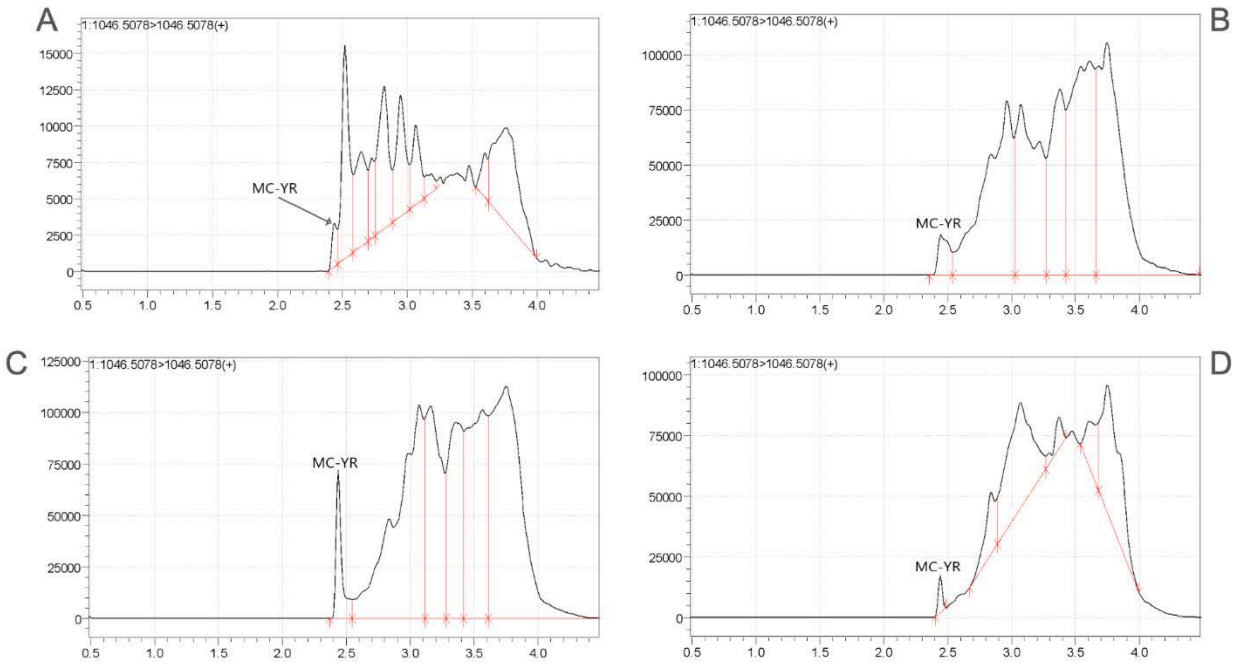

Figure 5.3: LC-MS chromatograms of quantification ions for microcystins-YR for (A) liver, (B) muscles, (C) intestine, and (D) gills.

These elevated concentrations of MC-YR in the liver may be attributed to the accumulation of toxins in the liver tissue. Lance et al. (2014) explained that once MCs are present in organisms, they target the liver, where they interact specifically with protein phosphatases (PPases). This is followed by a covalent binding to proteins, leading to the accumulation of MCs irreversibly attached to animal tissue-bound MCs. According to Kagalou et al. (2008), the liver is a target organ for MC accumulation, corresponding to Zurawell et al. (2005) and Lance et al. (2014). Meanwhile, Nchabeleng et al. (2014) indicated that the accumulation of MCs in fish liver may be related to the function of the liver serving as a detoxifying organ, thus exposing it to high loads of toxins. Although low MC concentrations have been detected in the liver tissue of *Talapia rendalli* (Hardly et al., 2015), Kagalou et al. (2008) suggested that the low accumulation of MCs in the liver may be due to preferential bioaccumulation in the muscle, even though the liver is typically the target organ.

Muscles

Analytical results showed that MC-YR was detected in *Clarias gariepinus* muscles from all the fish ponds ranging from 10.5 - 16.06 µg/g (Figure 5.3), surpassing human estimated daily intake

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(EDI) of 0.04 µg/kg/day, set by the WHO. Similarly, Cazenave et al. (2005) observed an increase in MC concentration in the muscles of *Odontesthes bonariensis* after 24 hours of exposure to a significant amount of MC-RR. In a fishpond found in Serbia, MC-RR was detected in muscle tissue at a concentration of 60 mg kg^{-1} DW (Drobac et al., 2016). Moreover, Mohamed et al. (2020) reported estimates of free MCs in tilapia fish organs at levels up to 11.8 ng/g in intestines, 8.3 ng/g in livers, and 0.38 ng/g in edible tissues. The presence of MCs in fish muscles has received substantial attention due to the concern for human health. Increased levels of MCs in fish muscle pose a health risk to consumers, as it is the most commonly consumed part of the fish by humans. Detecting elevated concentrations of MC-YR in fish muscle may indicate that the fish is unsafe for human consumption and commercial sale. It is important to highlight that neither boiling water nor cooking the fish before consumption reduces the health risks associated with MC toxins (Amanda et al., 2011; Berry et al., 2016).

Stomach content and gills

MC-YR variant were found in the intestines and gill of *Clarias gariepinus* at the range of 13.2 - 10.6 µg/g and 11.52 - 20.78 µg/g, respectively (Figure 5.4). The presence of MCs in the gills and intestinal tissues of fish is likely influenced by both the fish's diet and the routes of exposure (Cazenave et al., 2005; Mohammed et al., 2008). Lance et al. (2014) documented the trophic transfer of MCs via the food chain from zooplankton organisms with evidence of trophic transfer of MCs from the gastropod *Lymaea stagnalis* to the *Gasterosteus aculeatus* fish. The study further explained that the accumulation of MCs in organisms may be attributed to the absence of magnification metabolization and excretion of free MCs at every level of trophic transfer. Moreover, fish can absorb MCs either directly from the surrounding water through their gills. Fish gill is the major source of MC entry when these toxins are released into the surrounding water during cyanobacterial cell senescence, death, and lysis in the aquatic environment (Cazenave et al., 2005; Amado et al., 2007). This therefore explains the presence of MC-YR in gill and intestine tissues.

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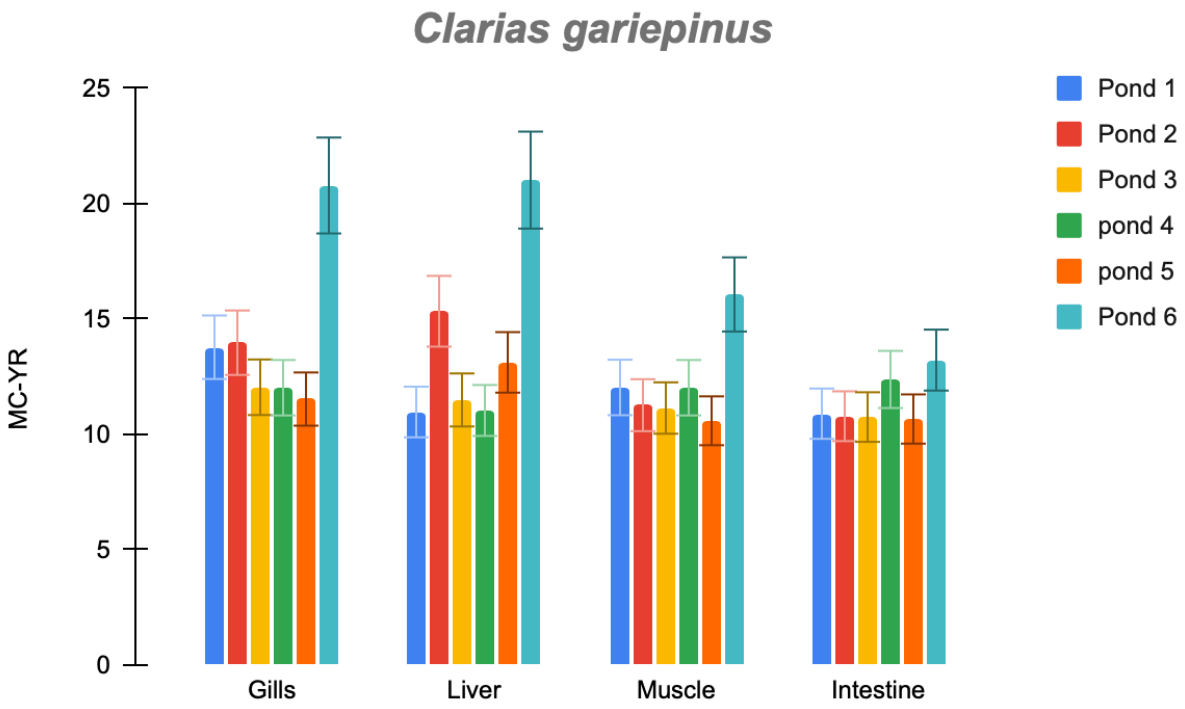

Figure 5.4: Concentrations of MC-YR in fish tissues from different fishponds.

3.4. Untargeted secondary metabolites (cyanotoxins) in fish tissues

There was no standard required to identify the untargeted secondary metabolites (cyanotoxins). This screening method allowed the ability to screen a large list of compounds including cyanotoxins in fish tissues. The untargeted method successfully identified cyanotoxins by comparing the putatively identified spectra with the built-in mass spectrometry (MS) spectra in the library using molecular networking displayed in Figure 5.5. In fish tissues, several cyanotoxins, including aeruginosins, anabaenopeptins, MCs, and microginins were detected. MCs and microginins were identified in the liver and intestine tissues from Pond 1. Aplysaxtosin was exclusively noted in the muscle tissue of *Clarias gariepinus* from Pond 1. Microginins, anabaenopeptins, microcolins, and mirabimids were identified in the muscle tissues across all fishponds. Anabaenopeptins were exclusively identified in the gill tissues from Ponds 4 and 6. Simultaneous occurrence of cyanotoxins in fish tissues could be associated with abundances of toxic genes in fishponds specific to MCs, microginins, anabaenopeptins, microcolins, and mirabimids (Wood et al., 2014). Marie and Gallet (2022) identified harmful cyanopeptides, including MCs, cyanopeptolins, and anabaenopeptins, in the liver tissues of *Perca* and *Lepomis*

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species from Tri, Var, and Ver lakes. These toxins are produced by certain species of cyanobacteria, including *Microcystis, Planktothrix,* and *Anabaena* species. These classes of cyanotoxins are identified as structural variants commonly identified in cyanobacterial blooms (Kust et al., 2020). Bassey et al. (2024) similarly reported the presence of microginins and MCs in Duthuni fishponds within the same study area, while anabaenopeptins, aplysiatoxins, and microcolins were detected across fishponds in both Duthuni and Calabar Municipality.

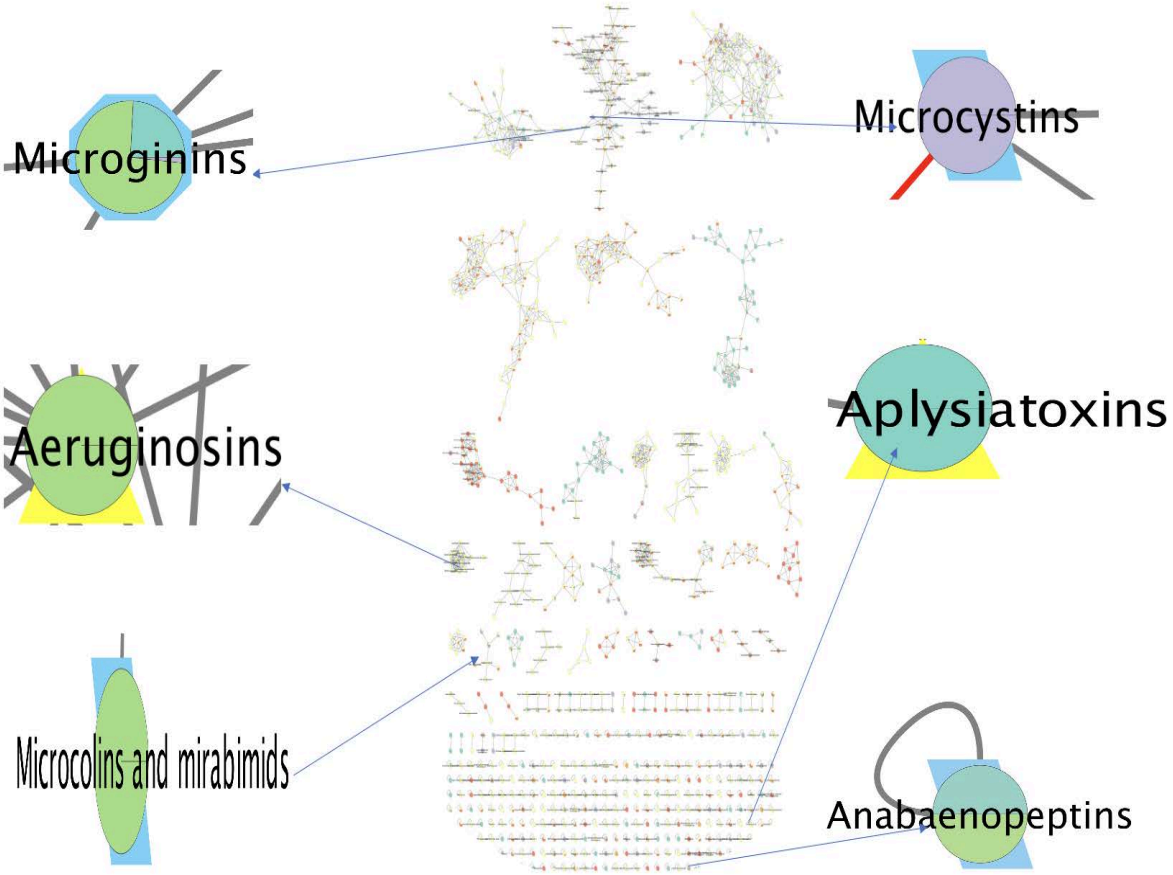

Figure 5.5: Molecular network of toxic cyanometabolites: A-D representing microcystins, microginins, aeruginosins, microcolins and mirabimids, anabaenopeptins, aplysiatoxins and aeruginosins extracted from *Clarias gariepinus* fish tissue.

Non-toxic secondary metabolites associated with cyanobacterial cells and endogenous to fish metabolites were also detected in the fish tissues. Among these, pheophorbide A and microcolins were identified as cyanometabolites (Louda et al., 1998; Singh et al., 2005). Cholic acid, phosphocholine (PC), triglyceride (TG), and octadecadienoic acid were determined to be derived

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from fish tissues (Swanepoel et al., 2016; Feng et al., 2023) while ethanesulfonic acid and amyl amine were likely attributed to anthropogenic sources. The distinction between metabolites derived from fish tissue and cyanometabolites was determined using LC-MS/MS, metabolite libraries, and supporting literature. The liver tissues in all ponds were rich in compounds like octadecadienoic acid, while phosphocholine, ethanesulfonic acid, pheophorbide A, and cholic acid dominated intestines and gills. Muscle tissue was characterized by phenylalanine, amyl amine, and phosphocholine, similar to South African samples. Meanwhile, in Duthuni sampling stations, the dominant compounds in muscle tissues were cholic acid, triglyceride, phosphocholine, and sulfonic acid. The prevalent compounds in the intestine and gill tissues were triglyceride and ceramide, exhibiting consistent similarities across all fish samples from South African sampling sites. These bioactive compounds are considered building blocks of cyanobacteria membranes (Jonge et al., 2019). To the best of our knowledge, this study is the first comprehensive report on cyanometabolites in fish tissues from commercial fishponds in Nigeria and South Africa, utilizing the untargeted method.

4. Conclusion

The untargeted and targeted LC-MS/MS methods were successful in detecting cyanotoxins in fish tissues. The targeted method was limited to the compounds for which the reference standard was available, while the untargeted method generated a more comprehensive dataset for retrospective interrogation. Microcystin variant MC-RR was undetected in fish tissues, but an increased concentration of MC-YR was detected in the liver, intestines, muscles, and gills of *Clarias gariepinus* ranging from 10.66 - 20.8 µg/g. The untargeted LC-MS method identified a wide range of cyanotoxins in fish tissues, including aeruginosins, anabaenopeptins, microginins, as well as non-toxic secondary metabolites such as octadecadienoic acid, phosphocholine, ethanesulfonic acid, microcolins, pheophorbide A, cholic acid, phenylalanine, amyl amine, triglycerides, and sulfonic acid. The simultaneous presence of cyanotoxins and increased MC-YR in the fish tissues raises serious concerns for safe consumer products. However, a thorough quantitative investigation of the untargeted cyanotoxins in fishponds is necessary to determine their concentrations in fish tissues accurately.

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Chapter 6

This chapter addresses objective 4

> To identify toxic and non-toxic cyanobacterial genes present in water and fish tissue samples

Abstract

This study examines the presence of cyanobacteria and the genes responsible for toxin production in aquaculture fishponds located in Duthuni, South Africa, and Nigeria. BLAST analysis confirmed the presence of cyanobacterial DNA in multiple samples, including water and fish tissue, with Microcystis *aeruginosa* emerging as the dominant species. Samples clustered phylogenetically with *Nostoc linckia* and *Microcystis aeruginosa*, with 98% and 97% similarity, respectively, underscoring their close relationship to toxigenic strains. Amplification using the HEP primer pair yielded positive results for the *mcy* gene (microcystin synthetase) in Duthuni Pond 1 (P1), signifying the potential for microcystin production. However, fish tissues from Nigerian fishponds showed no detectable amplification, likely due to low target cell concentrations or PCR inhibition factors.

1 Introduction

The fossil record indicates that cyanobacteria have existed for approximately 3.5 billion years (Rastogi et al., 2014). In aquatic environments, they occupy diverse niches, ranging from freshwater to marine ecosystems. Cyanobacteria possess bloom-forming capabilities and can reproduce exponentially under favorable conditions. These blooms produce numerous bioactive molecules, including cyanotoxins such as hepatotoxins and neurotoxins (Amarr et al., 2015; Buratti et al., 2017). Some cyanobacterial species produce cyanotoxins that are harmful to aquatic organisms, animals, water quality, and humans who are directly or indirectly exposed. These toxins include hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and irritant toxins (Wiegand & Pflugmacher, 2005). The cyanobacteria most frequently associated with cyanotoxin production include *Microcystis* (MCs), *Nodularin*, and *Cylindrospermopsis* (Chorus and Bartram; Codd et al., 1999; Buratti et al., 2017). Some cyanobacterial genera include *Microcystis* spp., *Anabaena* spp., *Anabaenopsis* spp., *Oscillatoria* spp., *Planktothrix* spp., and *Nostoc* spp, etc. (Sinden and Sinang, 2016).

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The differentiation between toxic and non-toxic cyanobacterial species is primarily determined by the presence of the mcy gene, which encodes enzymes involved in the biosynthesis of microcystin peptides. However, the mere presence of the mcy gene does not always correlate with the production of detectable levels of microcystin toxin (Christiansen et al., 2008). Toxic strains from genera such as *Leptolyngbya, Oscillatoria, Microcystis, Planktothrix, and Anabaena* typically harbor mcy genes (subunits A–E, G, J) crucial for microcystin biosynthesis (Christiansen et al., 2008; Frazao et al., 2010). Moreover, studies by Pearson et al. (2016) and Sinha et al. (2014) have demonstrated that cylindrospermopsin production is associated with the cyr (A–O) or aoa gene clusters, while the synthesis of nodularin involves the nda synthetase gene, which encodes polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) components. This pathway has been specifically characterized in *Nodularia spumigena* NSOR10 (Pearson et al., 2016). This study seeks to elucidate the presence and distribution of toxic and non-toxic cyanobacterial genes in both water and fish tissue. By identifying key genetic markers for toxin biosynthesis, the research will contribute to understanding the ecological and public health implications of cyanobacterial toxin production and its impact on aquatic ecosystems.

2.0 Methodology

2.1 DNA extraction and purification

Freeze-dried water samples and fish tissues were stored at -20°C to preserve DNA before extraction. Total genomic DNA was extracted using the ZR Duet™ DNA/RNA Miniprep Kit from Inqaba Biotech Laboratories, located in Pretoria, South Africa, following the manufacturer's protocol for sample preparation and DNA extraction. The process adhered strictly to the manufacturer's provided sample preparation and DNA extraction protocol.

2.2 Detection and Amplification by Polymerase Chain Reaction (PCR)

The amplification of the cyanobacteria 16S rRNA gene was achieved using a specific set of primers: HEP-primers HEPF and (5¢-TTTGGGGTTAACTTTTTTGGGCATAGTC-3¢) and HEPR (5¢-AATTCTTGAGGCTGTAAATCGGGTTT-¢3) primer-set. Sequencing of the fragments was conducted employing the Nimagen BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000, following the instructions provided by the manufacturer. The thermal cycling conditions included an initial denaturation step at 94°C for 5 minutes, followed by 35

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cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 68°C for 1 minute, with a final extension at 68°C for 10 minutes.

2.3 Toxin Gene Detection

To detect cyanotoxin-producing genes, PCR was performed using primers specific to nodularins (NOD) and microcystins (MC) biosynthesis. The HEP primer pair targeted the aminotransferase (AMT) domain within the *mcyE* ((microcystin synthetase) and *ndaF* (nodularin synthetase) modules of the MC and NOD synthetase enzyme complexes (Magonono et al., 2018).

2.4 Polymerase Chain Reaction (PCR) Purification and Sequencing

The PCR products were purified using the GeneJet Gel Extraction Kit from Thermo Scientific (Pretoria, South Africa) at room temperature, following the manufacturer's protocol. Purified DNA was stored at -20°C until sequencing. Sequencing was performed using the Nimagen BrilliantDye™ Terminator Cycle Sequencing Kit V3.1 (BRD3-100/1000). The labeled products were purified using the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053). Sequence chromatogram analysis was conducted with FinchTV software. PCR amplification primers were synthesized by Inqaba Biotech.

2.5 Phylogenetic Relationship

Additional sequences in FASTA format were retrieved from GenBank via NCBI and combined with assembled sequences. Phylogenetic analysis was conducted using the Neighbor-Joining method (Saitou and Nei, 1987) with a bootstrap consensus tree constructed from 1000 replicates (Felsenstein, 1985). Branches with less than 50% replication in bootstrap replicates were condensed. Bootstrap percentages were indicated beside branches. MEGA7 software was used for all analyses. Phylogenetic relationships of 16S rRNA sequences from water and fish tissue samples from fishponds were compared to cyanobacterial sequences retrieved from GenBank. A Neighbor-Joining tree was constructed with 1000 bootstrap replicates.

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3 Results

3.1 PCR Analysis of the 16S rRNA Gene

The BLAST analysis of samples collected from the Duthuni Pond 1 (P1), Duthuni Pond 2 (P3), Offiong Etim (P4) Essien Town (P5), and State Housing (P6) displayed positive amplification. Fish tissue (liver and gills) from Duthuni Pond 1 also displayed positive amplification. Water samples from Duthuni Pond (P3), liver and gill tissues from Offiong Etim (P4) underwent multiple repetitions but consistently failed to amplify. Nearly all other samples displayed positive amplification and were identified up to the genus level. The 16S PCR results from amplified samples confirmed that *Microcystis aeruginosa* was the dominant species present in both water and fish tissues. The amplified samples from the fishponds in Nigeria were clustered with Nostoc linckia and Microcystis aeruginosa strains. Analysis through the BLAST algorithm indicated that matches with more than 98% similarity aligned with the correct species.

3.2 Detection of Genes Involved in Toxin Production

The HEP primer pair yielded one positive result for the sample collected from the Duthuni Pond 1 (P1). This positive finding was linked to the presence of toxigenic Microcystis species. There was no amplification observed for fish tissues and water samples from SA and NGA linked to genes associated with toxin-producing proteins. It's important to note that detecting genes linked to toxin biosynthesis doesn't directly confirm the actual production of toxins in the natural environment.

3.3 Phylogenetic Relationship

The BLAST search revealed relationships between the samples and their closest species. A correlation is supported by the phylogenetic tree depicting connections among various cyanobacteria species across different samples (Figure 6.1). It was observed that isolated strains from Duthuni fishponds are closely related to Microcystis aeruginosa in Figure 6.1. While the amplified samples from Nigeria fishponds and fish tissue were clustered with the Nostoc linckia and Microcystis aeruginosa strains of cyanobacteria in Figure 6..2. Samples from Duthuni fishponds exhibited a 98% bootstrap confidence similarity to Microcystis aeruginosa while samples from Nigerian fishpond showed a 97% similarity to Nostoc linckia and Microcystis

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aeruginosa. Each similarity, as supported by the bootstrap confidence values, indicated the relatedness between the respective samples and the identified cyanobacterial species.

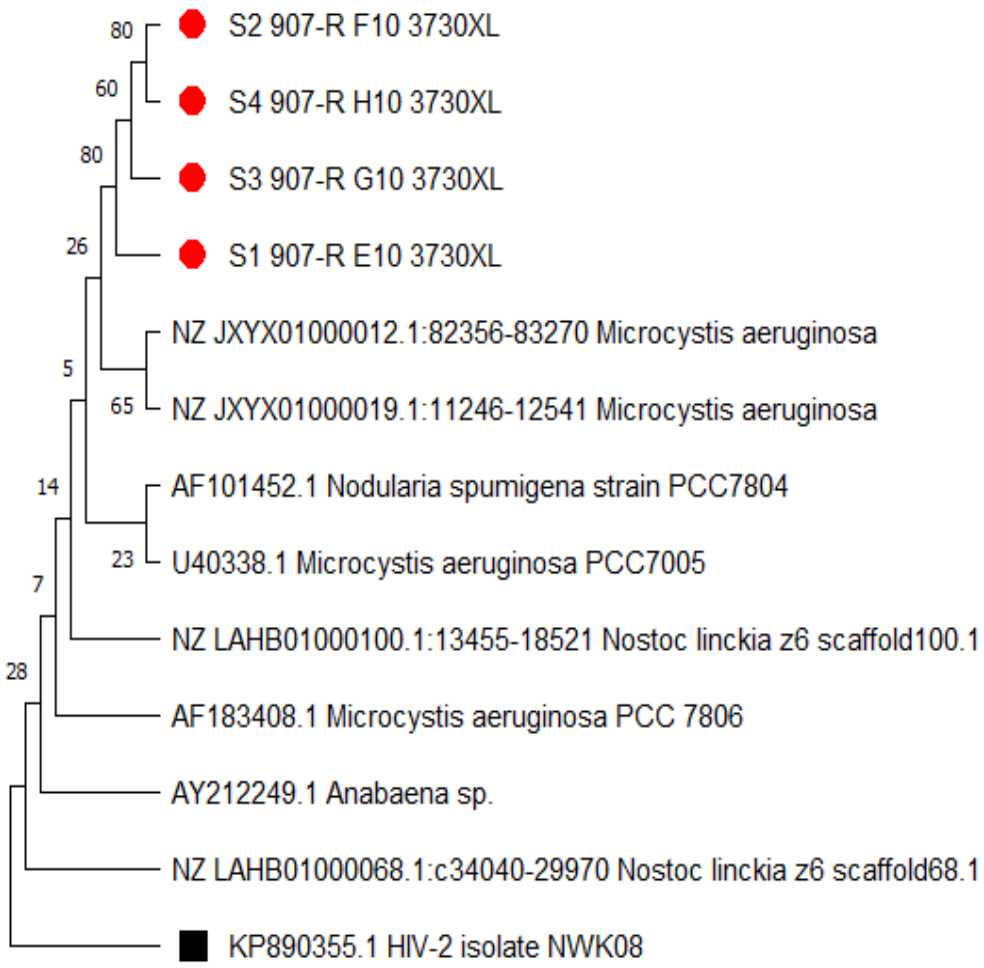

Figure 6.1: **South Africa -** Phylogenetic tree showing the relationship between S1 (Dthunui Pond 1water) sample and S2 (Duthuni Pond 1 liver tissue), S3 (Duthun Pond 2 gill tissue), and S4 (Duthuni Pond 2) samples from Duthuni fish ponds.

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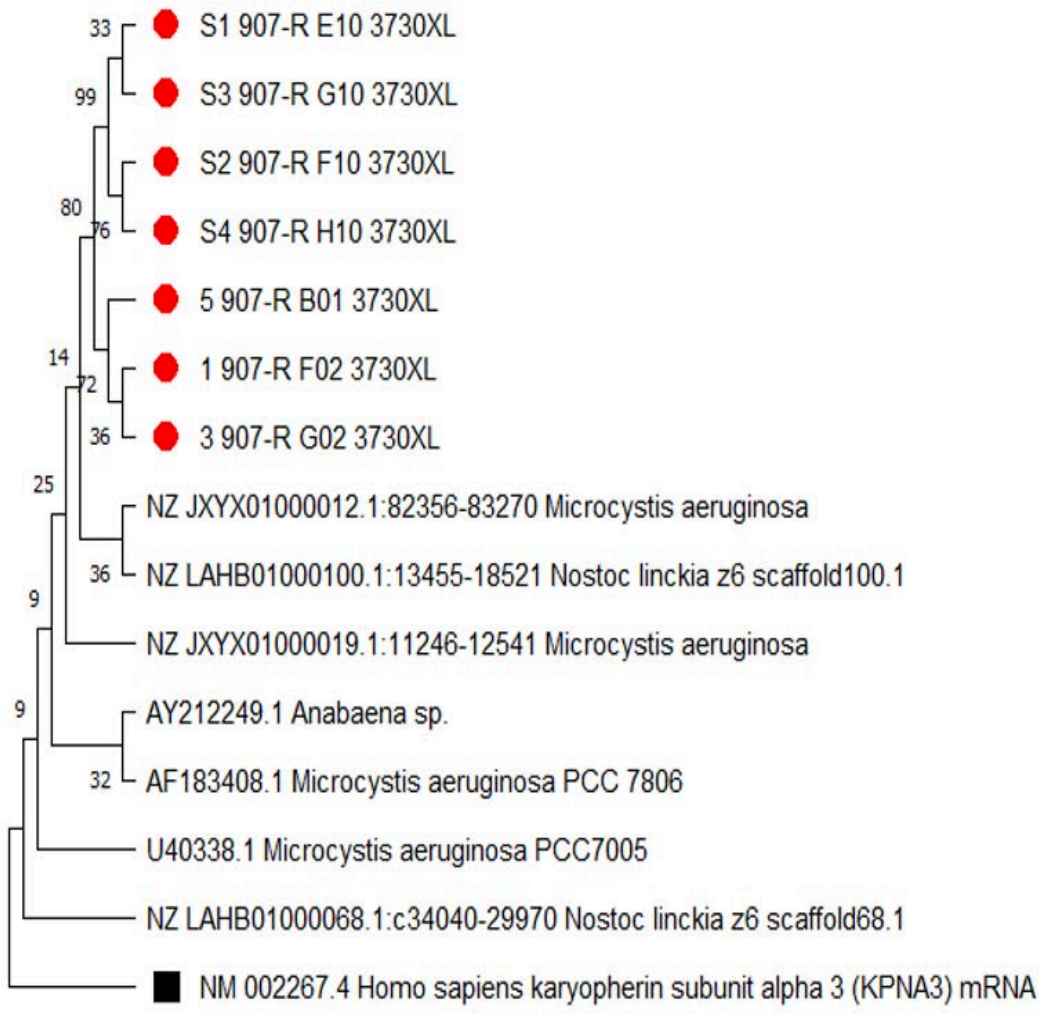

Figure 6.2: Nigeria - Phylogenetic tree showing the relationship between S1 (Essien Town) sample and S2 (Spring Road), S3 (State Housing), S4 (Essien Town Pond fish intestine), 5 (State Housing Pond fish gills), 1 (Spring Road Pond fish liver), 3 (State Housing Pond fish intestine) samples from NGA fish ponds

4. Discussion

The 16S PCR results confirmed that *Microcystis aeruginosa* was the dominant species present in both waters (ponds 2 and 4) and fish tissues. This HEP primer-positive result was linked to the toxic microcystins-producing cells (mcy genes) produced from Microcystis species. The PCR results confirmed that cultured fish were exposed to toxic and non-toxic cyanobacterial cells. Fish tissues from Nigerian fishponds did not display any positive amplification results. This

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could be due to lower detectable numbers of target cells in the fish tissues (Zarzoso-Lacoste *et al*., 2013). Zarzoso-Lacoste et al. (2013) earlier explained that the complexity of samples such as lower detectable numbers of target cells and DNA extraction could also inhibit PCR (dilution of DNA extracts was able to reduce PCR inhibition). Additionally, the presence of exogenous substances such as salts, bile, urea, humic substances, and complex polysaccharides can strongly inhibit PCR by interfering with DNA amplification success rates (Vézie et al., 2002).

Positive Hep-F/R assay results in catfish liver tissue correlate strongly with LC-MS findings, aligning with the observations of Hamandishe et al. (2021). Their study demonstrated that positive MCY detection in Nile Tilapia was indicative of toxin ingestion from *Microcystis* spp. present in Lake Kariba and Lake Chivero. In contrast, the negative Hep-F/R results in water samples and gill tissue diverged from LC-MS data, which detected the presence of MC-YR in these same samples. This discrepancy mirrors findings by Yuan et al. (2020) and Hamandishe et al. (2021), where water samples tested positive for microcystins via LC-MS despite the absence of the *mcyC* gene as determined by PCR.

Hamandishe et al. (2021) attributed this inconsistency to the potential for molecular analysis to underestimate microcystin levels, possibly due to degradation of nucleic acids or gene expression variability, while LC-MS provides a more sensitive detection of the toxin. This finding contrasts with the study by Falcone-Dias et al. (2020), which reported a positive correlation between *mcyE* gene copy number and microcystin levels quantified by LC-MS. These results underscore the complexity of cyanotoxin detection and highlight the limitations of relying solely on molecular assays to assess toxin presence. Integrating molecular methods with chemical analysis, such as LC-MS, offers a more comprehensive approach to monitoring microcystins in aquatic ecosystems and understanding the dynamics of toxin production in environmental and biological matrices.

`Conclusion

This study highlights the complex relationship between molecular detection of cyanotoxin-producing genes and the chemical quantification of microcystins in aquatic

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ecosystems. The strong correlation between positive Hep-F/R results in catfish liver tissue and LC-MS detection of microcystins reinforces the utility of combining these methods for accurate toxin monitoring. However, the discordance between molecular assays and LC-MS results in water and gill tissue samples—where *mcy* genes were undetectable despite the presence of microcystin-YR—demonstrates the limitations of PCR-based detection alone. Overall, this study underscores the importance of continuous methodological refinement to better understand cyanotoxin dynamics. It also highlights the need for accurate assessment of environmental and public health risks associated with toxin-producing cyanobacteria.

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Chapter 7

This chapter addresses objective 5

To investigate the impact of seasonal dynamics on cyanobacteria proliferation in aquaculture fish ponds.

Abstract

Aquaculture production contributes positively to Sustainable Development Goals (SDGs) targeting food security, economic development, livelihood, sustainable production practices, conserving biodiversity, and improving nutrition. Additionally, locally produced fish from aquaculture fishponds are an affordable source of protein in countries such as Nigeria (NGA) and South Africa (SA). However, increased harmful cyanobacteria (CyanoHABs) in fishponds due to seasonal dynamics pose a significant threat to cultured fish, production, sustainability, and human health, although there is a limited understanding of the seasonal dynamics of cyanobacterial biomass in fishpond ecosystems. The present study aims to investigate the effects of seasonal variations on cyanobacteria proliferation in aquaculture fish ponds found in SA and NGA. Water samples were collected twice during each season for a year within the selected fishponds. Physical (temperature, pH, dissolved oxygen, conductivity, salinity, total dissolved solids), chemical (nitrate, nitrite, phosphate), and biological parameters (chlorophyll a) were measured alongside meteorological data. Advanced digital flow cytometry (FlowCAM) was used to identify cyanobacterial species available in water samples. Findings from the study showed that *Microcystis* sp. dominated throughout the season both in SA and NGA fishponds. Findings further showed that cyanobacteria biomass concentration in the fishponds was not influenced by seasonal temporal drivers such as warmer temperatures during dry and summer seasons, or precipitation. Cyanobacteria biomass was greatly influenced by fishpond management practices such as feeding, fertilization of ponds, nutrient level, water volume, stocking density, water exchange, and retention time.

1. Introduction

The worldwide proliferation of CyanoHABs poses a significant threat to the safety and sustainability of water resources utilized for human consumption, agricultural irrigation, inland fisheries (including aquaculture), and recreational purposes (Paerl and Barnard, 2020). Aquaculture ecosystems are highly vulnerable to cyanobacterial bloom as cyanobacteria

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constitute the integral component of the food web as phytoplankton biomass (Paerl and Otten, 2013; Backović et al., 2024). Additionally, cyanobacteria can easily adapt to environmental conditions usually encountered in fishponds, such as high temperature, reduced light conditions, nitrogen depletion in the upper layer, a high degree of eutrophication, and a decrease in the number of large phytoplanktivorous filter-feeders (Backović et al., 2016; de Lima Pinheiro et al., 2023; Vrba et al., 2023).

Fish ponds are often enriched with nutrients, particularly nitrogen and phosphorus, due to fish feed, waste, and runoff from surrounding agricultural or urban areas. These nutrients provide an essential food source for cyanobacteria, promoting rapid growth (Paerl and Otten, 2013). Excessive nutrient loading can lead to eutrophication, a process that fosters the formation of harmful algal blooms (HABs) (Smith, 2003). High turbidity, resulting from suspended particles or fish activity that stirs up the pond substrate can also influence cyanobacterial growth. While cyanobacteria are generally phototrophic, some species have adaptations to thrive in low-light, turbid conditions due to buoyancy control mechanisms (Reynolds et al., 1987). Turbidity can limit the growth of other phytoplankton, giving cyanobacteria a competitive advantage (Scheffer et al., 1997). Cyanobacteria are considered harmful to aquaculture systems as they affect water quality, leading to the loss of water clarity. They also produce secondary metabolites, which can cause a change in the taste and odors of the waters, resulting in negative effects on invertebrate and fish habitats (Paerl, 2014).

In tropical regions, particularly in Africa, there is a pronounced gap in research on the temporal drivers of cyanobacterial biomass in aquaculture fishponds, despite their critical role in the aquatic food web. Cyanobacteria not only contribute significantly to nutrient cycling but also influence fish growth and water quality. Cyanobacteria can pose risks through toxin production, which may escalate under certain seasonal conditions. Current knowledge gaps pertain to how cyanobacterial populations fluctuate across different seasons and the factors driving these variations, such as temperature, nutrient availability, and pond management practices. Understanding these patterns is critical for optimizing aquaculture productivity and mitigating risks associated with cyanobacterial blooms. This highlights the need to understand the influence of seasonal variations on cyanobacteria in tropical regions. Therefore, the present study aims to investigate the effects of seasonal variations on cyanobacteria proliferation in aquaculture fish

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ponds found in SA and NGA. Addressing these gaps is crucial for optimizing aquaculture productivity, preventing harmful blooms, and ensuring the ecological sustainability of fishponds. This underscores the urgent need for comprehensive studies on seasonal variations in cyanobacterial populations and their environmental drivers, particularly in tropical aquaculture systems.

2. Methodology

2.1 Study area

The study was conducted in commercial aquaculture fishponds in Vhembe District, Limpopo Province, South Africa, and Calabar Municipality, Cross River State, Nigeria. In Nigeria, the sampling sites were located in Offiong Etim Avenue ($4^0$59'58.92" N and $8^0$19'03.97" E), Essien Town ($4^0$59'15.49" N and $8^0$19'40.21" E) and State Housing ($4^0$59'6.50" N and $8^0$20'13.29" E) presented in Figure 5.1. The aquaculture fishponds in the Vhembe District were positioned in Duthuni Pond 1 ($22^0$57'56.98" S and $30^0$23'43.96" E), Duthuni Pond 2 (22°57'56.89"S and 30°23'43.96"E), and Duthuni Pond 3 (22°57'56.98"S and 30°23'44.06"E). A total number of 6 fish ponds located in Vhembe District (3 fish ponds) and Calabar Municipality (3 fish ponds) were used for this study. Sampling sites were selected using the following criteria: commercial fishponds, accessibility, consent from the owners, and the presence of cultured fish.

2.2 Water Sampling

Water samples were seasonally collected from the fishponds in triplicates during the South African winter and summer and the Nigerian dry and wet seasons, respectively. Data collection involved four field trips conducted during specific seasonal periods: January and February (summer), June (winter), August and September (wet season), and November and December (dry season). Water samples were sampled at depths between 0 and 0.5 meters from each fishpond using sterilized labeled bottles. Water samples were collected approximately 1-2 meters away from the edge to avoid contamination from edge-related disturbances. Approved consent was obtained from the owners of the fishponds in Nigeria and South Africa before sampling.

2.3 Meteorological data

Monthly climatic data (maximum and minimum temperatures and precipitation) from 1991 - -2022 were obtained from the archives of the Nigerian Meteorological Agency and South Africa

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Weather Services. The annual mean of each data was computed from the monthly data sets. A trend analysis was carried out to assess the air temperature and rainfall pattern for 32 years.

2.4 Water samples analyses

2.4.1 Nutrient analyses

Samples for dissolved nutrient concentration underwent filtration using membrane filters before analyses. The nutrient analyses, specifically for nitrates, nitrites, and phosphates, were conducted on the samples using Ion Chromatography Dionex 1600, employing EPA method 300 (EPA, 1993). These analyses took place at the Agricultural Research Council (ARC) Laboratory.

2.4.2 Chlorophyll-a analysis

Water samples (250 ml) were filtered through a Whatman (Glass Fiber) filter paper. Then, the filter paper was cut into smaller pieces and immersed in 10 mL ethanol, followed by ultrasonication for 30 mins. The tube was labeled and stored in the dark for 24 hours at room temperature. This was followed by 15 mins centrifugation at 3500 rpm to get a clear sample. The samples were transferred to a clean vessel, and the volume was recorded. The supernatant was poured into a 1 cm cuvette, and a spectrophotometer was used to measure the amount of light absorbed by the sample in the cuvette placed in the Spectrophotometer at a wavelength of 665 and 750 nm. This absorbance wavelength ratio (665 and 750 nm) was used because it fluoresces at 665 and 750 nm. Two batches at 665 and 750 nm were used;

1) 1 cm cuvette sample without Hydrochloric acid (total absorb) (665a and 750a nm).
2) 1 cm cuvette sample with a 0.01 ml drop of hydrochloric acid (665b and 750b nm).

Adding HCl (0.01 ml) to the sample before measurement assists in dissolving the suspended particles scattered in the samples for light to pass through the cuvette without interference with scattered particles. After measuring, chlorophyll-a concentration was conducted using the equation shown below (EPA, 2021);

Calculation

Correct turbidity by subtracting absorbance 665a-750a = corrected 665a,

665b-750b = corrected 665b

The corrected 665a and 665b absorbance was to calculate the chlorophyll-a concentration;

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$$Chl - a = \frac{29.62\,(665a-665b)X\,Ve}{Vs\,X\,1}$$

Where:

V_s = Volume of water samples in liters

V_e = Volume of ethanol extract (ml)

1 = Cuvette light-path length in centimeters

The final concentration was expressed in units mg m^{-3}

2.4.3 Dry Weight Biomass

Measures the total dry weight of phytoplankton collected from a sample. The samples (1 litre) were filtered through a pre-weighed filter. The filter paper was oven-dried at 60°C until constant weight was reached. The weight difference was measured to calculate biomass. The unit was expressed in mg/L

2.5 Statistical analysis

The statistical analyses were conducted to establish the correlation between variables using SPSS. The obtained data were input into Microsoft Excel before performing statistical analyses. A descriptive statistics summary of the water quality parameters—namely, chlorophyll-a, temperature, TDS, DO, EC, pH, salinity, NO_3, NO_2, and PO_4 was conducted. Pearson correlation was used to assess the relationships between the variables. Pattern analysis fit with linear models was used for the meteorological data. A one-way ANOVA was conducted to evaluate the effect of seasonal variations on water quality parameters (Table 7.4a). For multiple comparisons, the Bonferroni test was applied to identify water quality parameters that differed significantly (Table 7.4b).

3. Results

3.1 Environmental factors

Seasonal fluctuations in water temperature, pH, chlorophyll-a, cyanobacteria biomass, and nutrients were found in Nigeria and South Africa during their respective summer, winter, dry, and wet seasons in Figure 7.1. All the sampling sites showed elevated temperatures > 20 ºC in dry, wet, and summer seasons displayed in Figure 7.2. However, the temperature dropped notably during winter to 16.5 ºC, specifically at the Duthuni sampling sites. High concentration

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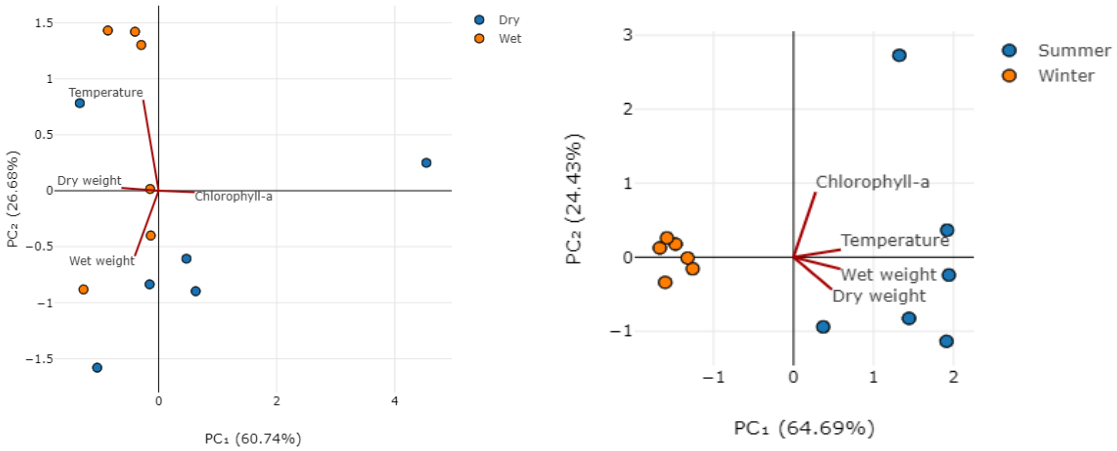
of nitrite and nitrate was observed during the wet season, followed by the dry season, corresponding to 10.42 mg/L and 6.45 mg/L, respectively. Meanwhile, in South Africa, the fishponds display lower concentrations of nutrients (NO_3, NO_2, and PO_4; - 1.9, 1.4, and 2.1 mg/L, respectively) in summer and winter. Phosphate levels were the same in winter and summer in South Africa for all fishponds and higher in the wet season for Nigerian fishponds. The water samples in Nigerian fishponds showed neutral to alkaline pH in rainy seasons in Figure 7.2. South African fishponds maintained slightly acidic to neutral pH 5.59-7.22.

3.2 Phytoplankton biomass

Chlorophyll-a displays strong variations during warmer months of summer and dry seasons compared to the winter and rainy seasons, as represented in Figure 7.2. phytoplankton biomass in Nigerian fishponds was higher in both seasons. There were no significant variations in phytoplankton biomass across all fishponds during the dry and wet seasons. However, increased cyanobacteria biomass was noted in summer in Duthuni ponds 1 and 2, with relatively lower values in winter.



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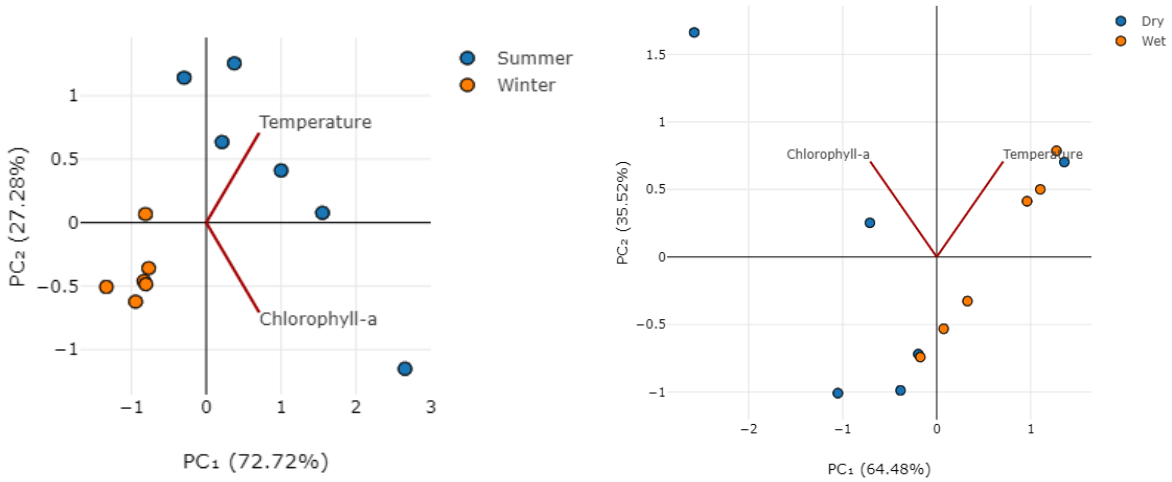

Figure 7.1: Principal component analysis (PCA) (PC_1 and PC_2) of environmental factors showed variations in temperature (°C), chlorophyll-a, phytoplankton biomass (dry weight and wet weight), and chlorophyll-a during winter, summer, dry and wet seasons.

3.3 Seasonal dynamics of air temperature and precipitation

There was an increased trend of air temperature over the years (1991 to 2022) during the dry and wet seasons in the Nigerian sampling stations displayed in Figure 7.3. An upward trend was also observed in summer (January-March) and winter (June, July, and August), as shown in Figure 7.4. Summer and winter rainfall demonstrated a downward trend in the past three decades in Duthuni fishpond sampling stations, as shown in Figure 7.5.

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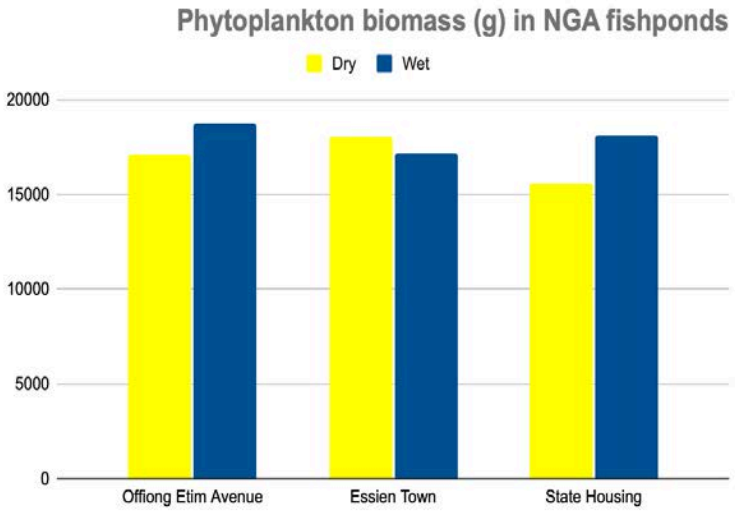

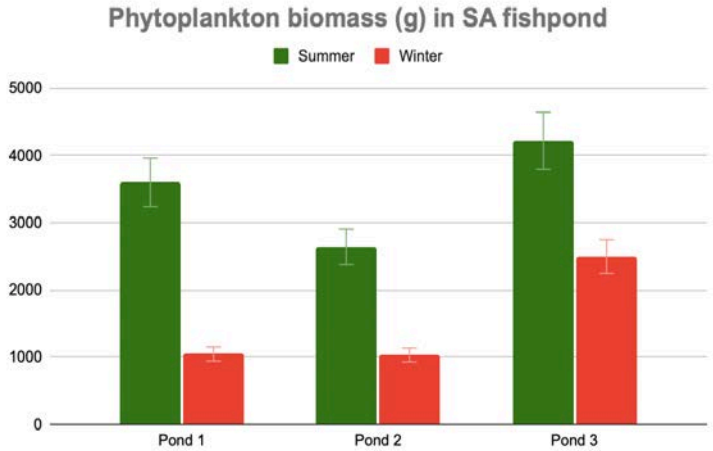

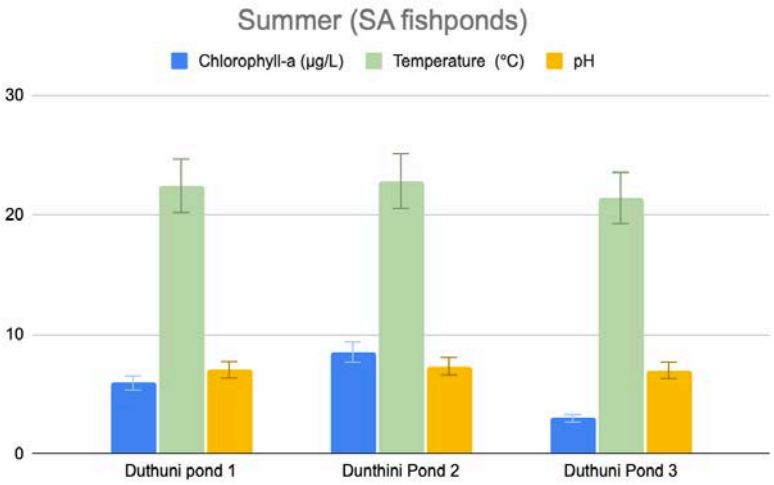

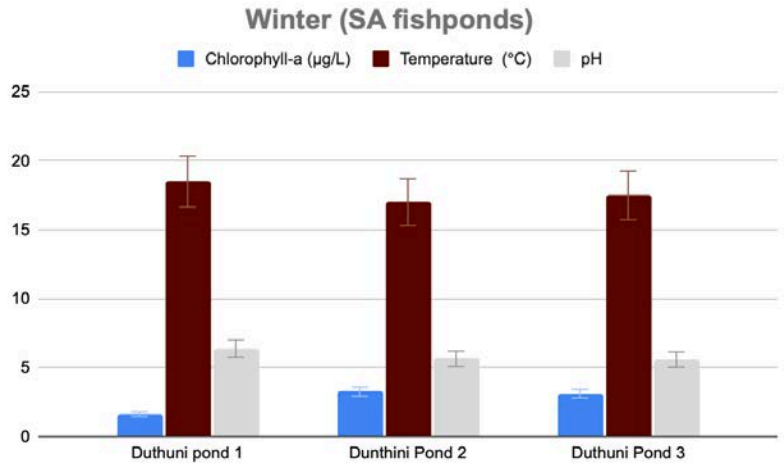

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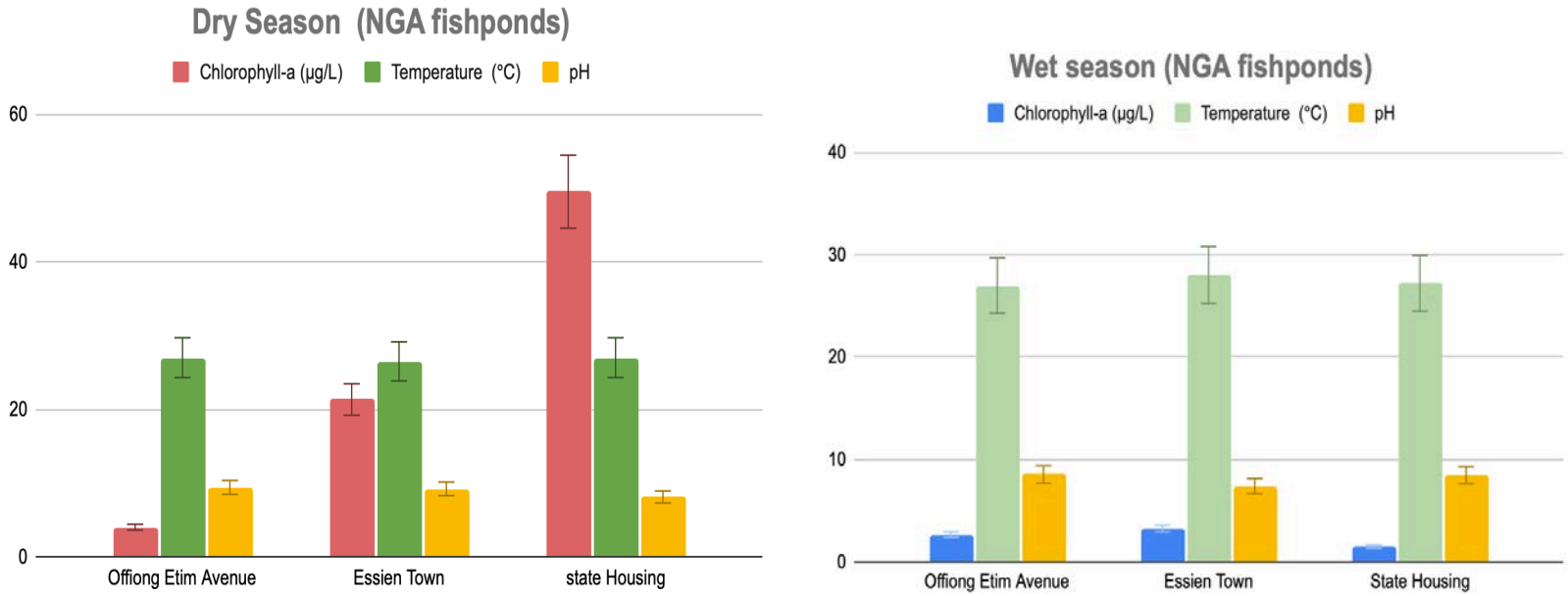

Figure 7.2: Graphical representation of environmental factors including temperature, pH, phytoplankton biomass, and chlorophyll-a during summer, winter, dry, and wet seasons in SA and NGA fishponds.

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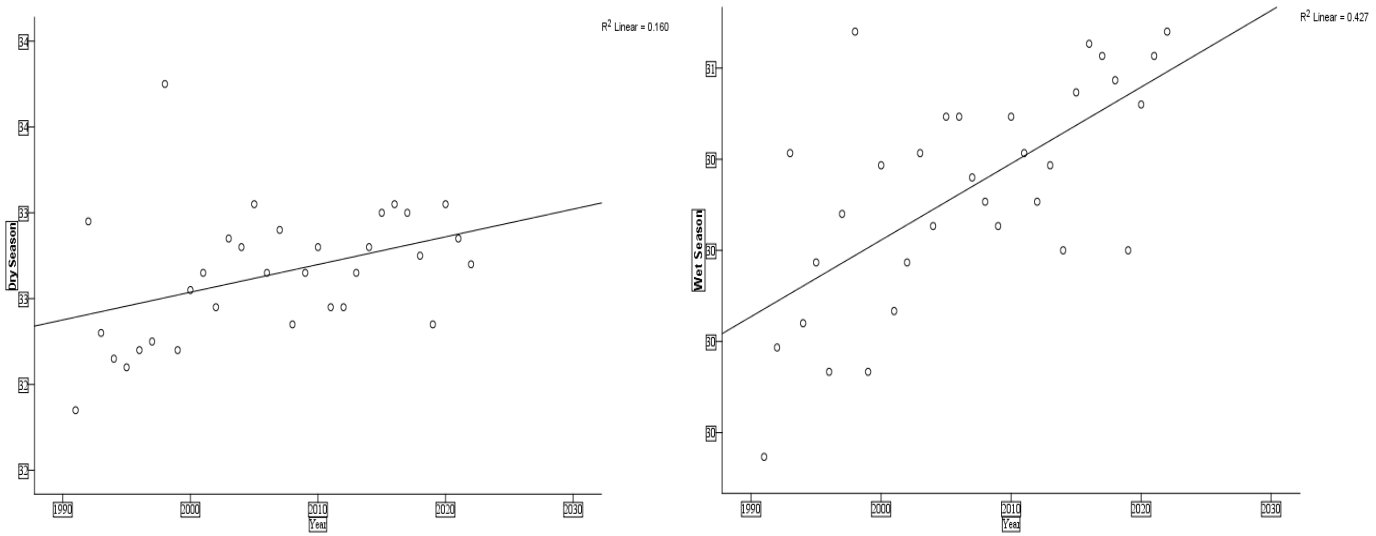

Figure 7.3: Temperature trends (1991-2022) during dry and wet seasons in the NGA sampling location.

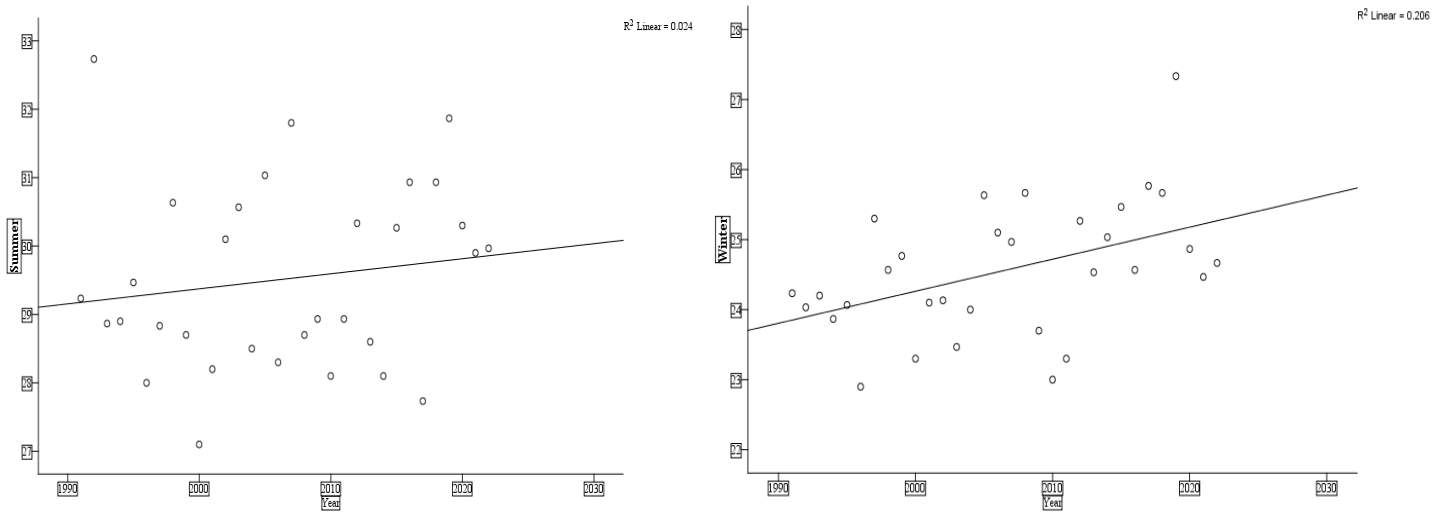

Figure 7.4: Temperature trends (1991-2022) during Summer and Winter seasons in the SA sampling location.

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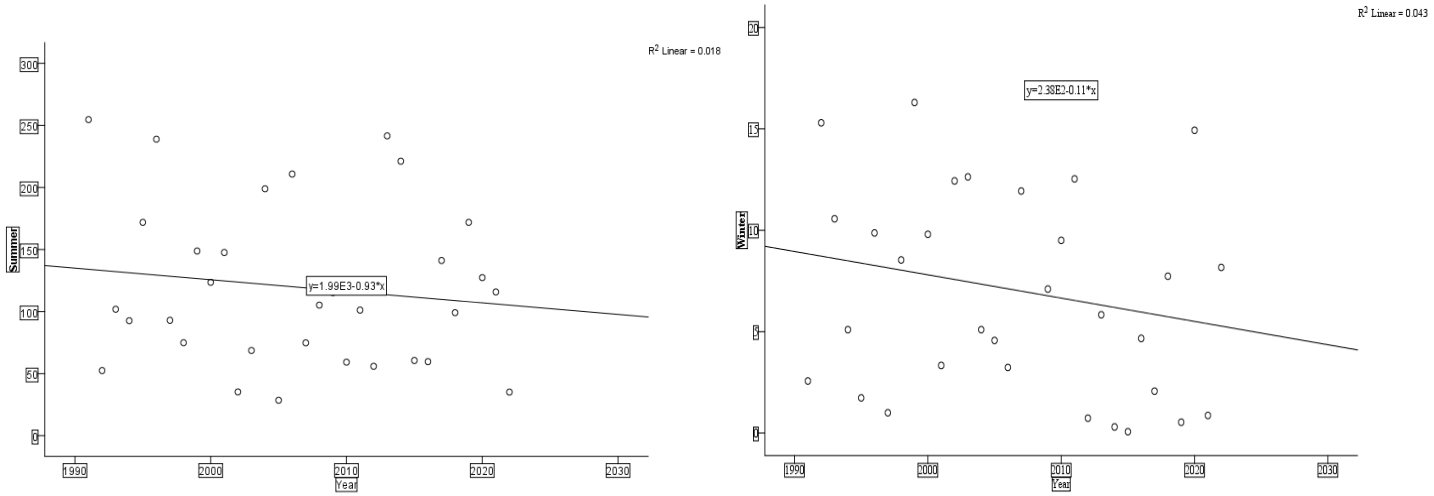

Figure7.5: Rainfall trends (1991-2022) during summer and winter seasons in South Africa sampling location.

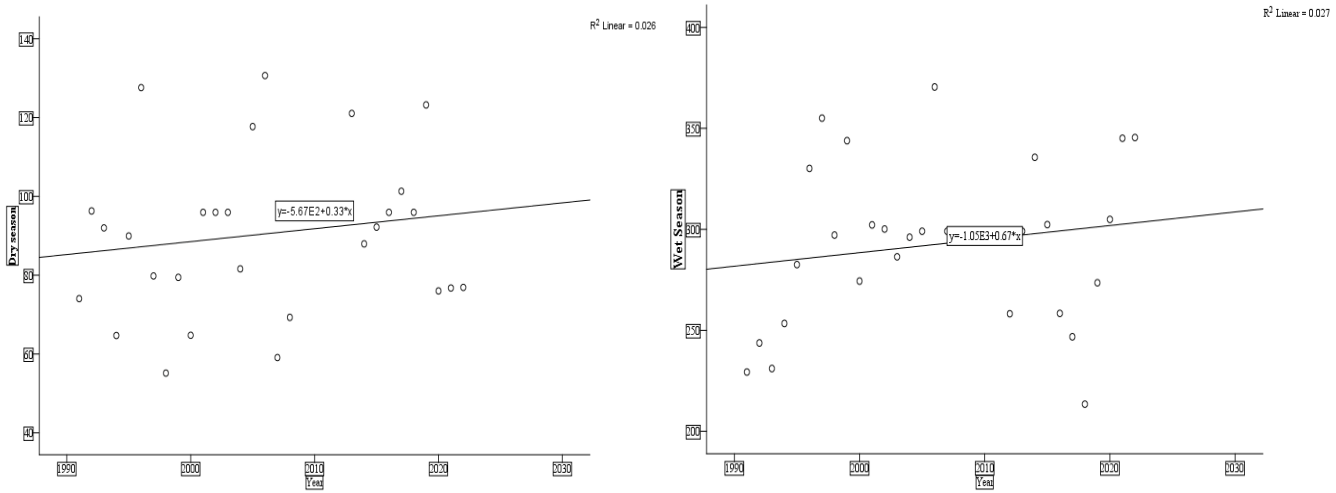

Figure 7.6: Rainfall trends (1991-2022) during dry and wet seasons in Nigeria sampling location

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The decreased rainfall trend during summer and winter aligns with the rainfall projections, pointing to an annual decline in the country (DEA, 2018). Rainfall in the Nigerian sampling station showcased increasing trends during the rainy season (April to October) in Figure 7.6. Conversely, there was an increased trend in rainfall during the dry months (January, February, March, November, and December).

3.4 Management Practices

Field observations revealed a notable dominance of algal blooms in Nigerian (NGA) fishponds, predominantly characterized by cyanobacterial scums and greenish-colored water, with visible accumulations of blue-green algae on the water surface displayed in Figure 7.7. During the dry season, these ponds exhibited a pronounced layer of cyanobacterial blooms over the water surface. In contrast, during the rainy season, the cyanobacterial blooms were diluted by rainwater, resulting in the absence of thick surface layers of cyanobacterial scums in Figure 7.7. In South African (SA) fishponds, no cyanobacterial blooms were observed during either winter or summer presented in Figure 7.8. The water samples from the SA fishponds maintained a consistent coloration (muddy brown color) across all seasons in Figure 7.8.

Figure 7.7: Visible presence of blue-green algae bloom in (A) Essien Town (B and D) Spring Road (C) State Housing fishponds during the dry season while (E) Spring Road and (F) State Housing fishponds during the wet (rainy) season.

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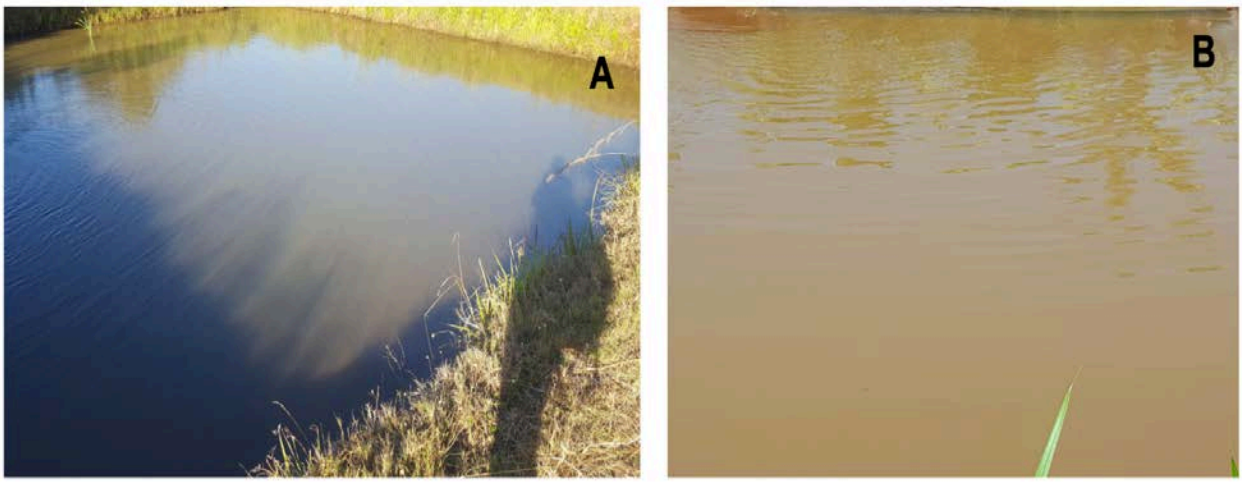

Figure 7.8: Duthuni fishpond during (A)summer and (B) winter

The SA fishponds were constantly receiving freshwater from the Duthuni Dam in Figure 7.9B. Conversely, the NGA fishponds relied exclusively on borehole water released into the fishponds periodically displayed in Figure 7.9A.

Figure 7.9 (A): SA fishponds receiving freshwater from the Duthuni Dam and (B): NGA fishpond

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3.5 Phytoplankton species composition

Microcystis sp. cells were dominant across all sampling sites and seasons as displayed in Figure 7.10-7.11. Notably, no other cyanobacteria genus was identified in the morphological results from the FlowCam and microscopic analysis.

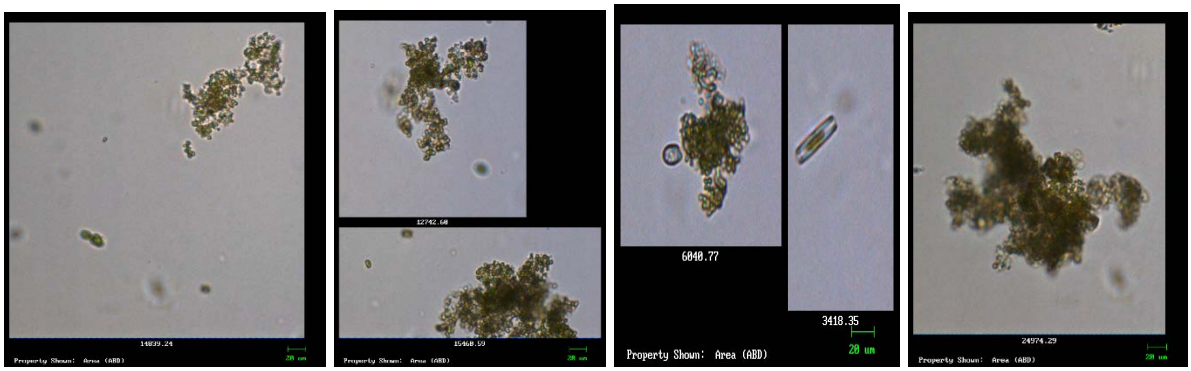

Figure 7.10: FlowCam chart of cyanobacteria in the water samples.

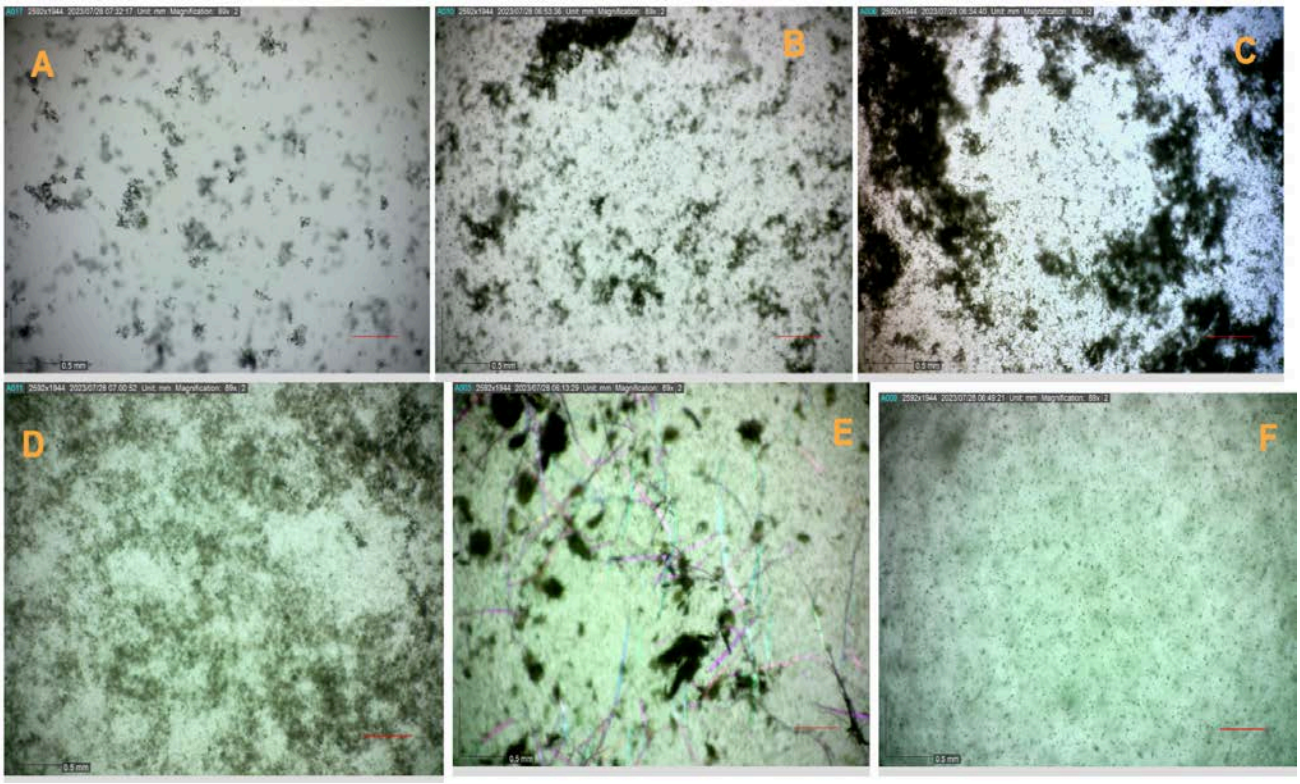

Figure 7.11: Microscopic identification of cyanobacteria cells in the water samples (A) Duthuni Pond 1; (B) Duthuni Pond 2; (C) Essien Town; (D) Spring Road (E) State Housing (F) Dutuni Pond 3.

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3.6 Seasonal Variations in Water Quality Parameters for Nigeria and South Africa

The results in Table 7.1 for Nigeria show a significant difference between the dry and wet seasons for Chlorophyll-a (p=00), DO (p=0.00), pH (0.05), and EC (p=0.00). There is no significant difference in TDS (p=0.69), and Salinity (p=0.54) for the wet and dry seasons of Nigeria. Additionally, for South Africa, there is a significant difference between the summer and winter seasons for Chlorophyll-a (p=0.04), TDS (p=0.00), pH (0.00), EC (p=0.00), and Salinity (p=0.00). There is no significant difference in DO (p=0.15) for the summer and winter seasons of South Africa.

Table 7.1: Bonferroni multiple comparisons of water quality parameters in different seasons

	Seasons	**p**
Chlorophyll-a	Dry/wet	0.00
	Summer/winter	0.04
DO	Dry/wet	0.00
	Summer/winter	0.15
TDS	Dry/wet	0.69
	Summer/winter	0.00
pH	Dry/wet	0.05*
	Summer/winter	0.00
EC	Dry/wet	0.00
	Summer/winter	0.00
Salinity	Dry/wet	0.54
	Summer/winter	0.00
*. The mean difference is significant at the 0.05 level.		

3.7 Correlation between water quality parameters

The Pearson correlation analysis of water quality parameters in Nigeria (Table 7.1) reveals significant relationships between key variables. Dissolved Oxygen (DO) shows a strong negative correlation with Electrical Conductivity (EC) (r = -0.83, p = 0.00), Nitrite (NO_2) (r = -0.91, p = 0.00), and Nitrate (NO_3) (r = -0.89, p = 0.00), indicating that increased EC and nitrogen-based compounds are associated with reduced oxygen levels, possibly due to organic pollution or eutrophication. Conversely, DO has a strong positive correlation with Total Dissolved Solids. (TDS) (r = 0.71, p = 0.01) and Salinity (r = 0.77, p = 0.00), suggesting that higher dissolved salts help retain oxygen. TDS is positively correlated with Phosphate (PO_3) (r = 0.81, p = 0.00), implying that increased dissolved solids contribute to phosphorus enrichment. However, TDS

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has strong negative correlations with NO_3 (r = -0.84, p = 0.00) and NO_2 (r = -0.80, p = 0.00), suggesting a dilution or transformation effect on nitrogen compounds. EC, on the other hand, exhibits a strong positive correlation with NO_3 (r = 0.87, p = 0.00) and NO_2 (r = 0.81, p = 0.00), indicating that higher conductivity is associated with elevated nitrogen levels. Interestingly, Salinity has a strong positive relationship with TDS (r = 0.69, p = 0.01) and DO (r = 0.77, p = 0.00), but a negative correlation with EC (r = -0.72, p = 0.01), highlighting the complex interactions between these parameters. The strong correlations between Phosphate, TDS, and Salinity suggests potential nutrient enrichment from pollution sources. Overall, the results indicate that increased nitrogen-based compounds and high conductivity levels may contribute to reduced oxygen availability, which could have ecological implications such as eutrophication.with EC (r = -0.72, p = 0.01), meaning higher conductivity corresponds to lower salinity.

The Pearson correlation analysis of water quality parameters in South Africa in Table 7.2 reveals several significant relationships. Dissolved Oxygen (DO) has a strong negative correlation with Total Dissolved Solids (TDS) (r = -0.87, p = 0.00) and Electrical Conductivity (EC) (r = -0.81, p = 0.00), indicating that higher TDS and EC levels are associated with lower oxygen availability, which could be due to organic pollution or increased ion concentrations reducing oxygen solubility. In contrast, DO shows a strong positive correlation with Salinity (r = 0.93, p = 0.00), suggesting that higher salinity levels support greater oxygen retention. pH is positively correlated with DO (r = 0.77, p = 0.00) and Salinity (r = 0.82, p = 0.00), while showing a significant negative correlation with TDS (r = -0.71, p = 0.01) and EC (r = -0.66, p = 0.02), indicating that more alkaline conditions may be associated with lower dissolved solids and conductivity. TDS and EC are highly correlated (r = 0.98, p = 0.00), which is expected as both parameters reflect ionic concentration in water. Salinity also exhibits a strong negative correlation with both TDS (r = -0.94, p = 0.00) and EC (r = -0.89, p = 0.00), suggesting that higher salt levels might be linked to different ion compositions rather than just general dissolved solids. Nitrate (NO_3) shows weak correlations with most parameters, with the highest being a positive correlation with Salinity (r = 0.52, p = 0.08) and pH (r = 0.51, p = 0.09), though these are not statistically significant. Overall, the findings suggest that water bodies with high TDS and EC may suffer from oxygen depletion, while salinity and pH play crucial roles in maintaining DO levels.

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Table 7.2: Pearson correlation between water quality parameters (Nigeria)

		Chlorophyll-a	DO	TDS	pH	EC	Salinity	PO_3	NO_2
DO	*Pearson Correlation*	0.44	1						
	Sig. (2-tailed)	0.15							
TDS	*Pearson Correlation*	0.59*	0.71*	1					
	Sig. (2-tailed)	0.04	0.01						
pH	*Pearson Correlation*	-0.11	0.34	0.39	1				
	Sig. (2-tailed)	0.73	0.28	0.20					
EC	*Pearson Correlation*	-0.36	-0.83**	-0.70*	-0.02	1			
	Sig. (2-tailed)	0.25	0.00	0.01	0.56				
Salinity	*Pearson Correlation*	0.57	0.77**	0.69*	0.38	-0.72**	1		
	Sig. (2-tailed)	0.05	0.00	0.01	0.24	0.01			
PO_3	*Pearson Correlation*	0.34	0.59*	0.81**	0.43	-0.59*	0.79**	1	
	Sig. (2-tailed)	0.27	0.04	0.00	0.16	0.04	0.00		
NO_2	*Pearson Correlation*	-0.44	-0.91**	-0.80**	-0.41	0.81**	-0.87**	-0.73**	1

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	Sig. (2-tailed)	0.15	0.00	0.00	0.19	0.00	0.00	0.01	
NO_3	*Pearson Correlation*	-0.39	-0.89**	-0.84**	-0.49	0.87**	-0.79**	-0.74**	0.91**
	Sig. (2-tailed)	0.21	0.00	0.00	0.10	0.00	0.00	0.01	0.00
*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed).									

Table 7.3: Pearson correlation between water quality parameter (South Africa)

		Chlorophyll-a	**DO**	**TDS**	**pH**	**EC**	**Salinity**
DO	*Pearson Correlation*	0.48	1				
	Sig. (2-tailed)	0.11					
TDS	*Pearson Correlation*	-0.35	-0.87**	1			
	Sig. (2-tailed)	0.26	0.00				
pH	*Pearson Correlation*	0.33	0.77**	-0.71*	1		
	Sig. (2-tailed)	0.29	0.00	0.01			

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EC	*Pearson Correlation*	-0.18	-0.81**	0.98**	-0.66*	1	
	Sig. (2-tailed)	0.58	0.00	0.00	0.02		
Salinity	*Pearson Correlation*	0.34	0.93**	-0.94**	0.82**	-0.89**	1
	Sig. (2-tailed)	0.27	0.00	0.00	0.00	0.00	
NO_3	*Pearson Correlation*	0.06	0.33	-0.41	0.51	-0.34	0.52
	Sig. (2-tailed)	0.85	0.29	0.18	0.09	0.28	0.08

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

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4. **Discussion**

The influence of environmental variables on cyanobacteria biomass is considered an important factor in regulating cyanobacterial blooms. Cyanobacterial blooms are strongly correlated with climatic conditions, especially in temperate regions. Increased air temperature during warmer seasons serves as a strong predictor of biotic processes, notably influencing the behavior of cyanobacteria in the epilimnion (Gallina et al., 2011; 2017). Air temperature plays a pivotal role in shaping the ecological and hydrological dynamics of aquatic ecosystems, acting as a direct link between atmospheric conditions and aquatic ecology (Gallina et al., 2011). However, the increased air temperature pattern over the past three decades cannot be linked as the primary mechanism driving phytoplankton biomass growth in the fishponds during warmer seasons (dry, wet, and summer). Cyanobacterial proliferation is influenced by multiple factors, not just temperature. Although increased temperatures generally promote cyanobacterial growth by enhancing metabolic rates and competitive advantages over other phytoplankton. Other environmental conditions, such as nutrient and light availability, must align to trigger a bloom (Litchman and Pinto , 2024).

While higher surface water temperatures typically support cyanobacterial growth, their impact in fishponds is context-dependent and often overshadowed by other environmental and management factors. The SA and NGA fishponds were typically less influenced by seasonal factors due to shallow depths. This limits the formation of thermoclines (temperature gradients within the water), which typically drive seasonal stratification in deeper water bodies (Kopp et al., 2016). Without significant stratification, water temperature remains relatively consistent throughout the year, reducing seasonal temperature effects. In addition, fishponds in tropical regions are often subject to anthropogenic management, such as controlled feeding, water replenishment, stock density, and aeration. These practices ensure that water quality and temperature remains stable, regardless of external seasonal variations. As Rettig et al. (2006) noted, fishponds are more influenced by weather and human activities. In Nigeria and South Africa, rainfall patterns, land use, and agricultural runoff are often more impactful on water quality and fishpond conditions than temperature changes tied to seasons.

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Seasonal changes in rainfall did not create dramatic shifts in water temperature, further minimizing the influence of seasonality. During the wet season, cyanobacterial blooms in the fishponds were likely diluted by rainwater inflow, increasing water transparency. This aligns with studies by Reichwaldt and Ghadouani (2012), Barroso et al. (2018), and Huang et al. (2020), which highlights how rainfall can reduce algal concentrations and improve water clarity. Figueredo and Giani (2001) demonstrated that increased precipitation, particularly during rainfall, induces water turbulence associated with large-volume inflows, which reduces cyanobacterial bloom intensity. These findings underscore the dynamic interplay between hydrological conditions and phytoplankton ecology in tropical fishpond systems, where intense rainfall events are common. The cyanobacterial bloom observed during the wet season may be a continuation of the dry-season bloom, directly linked to anthropogenic activities such as pond fertilization in Nigeria.

The low nutrient levels in these seasons could be associated with the observed low chlorophyll-a concentration and phytoplankton biomass in the SA fishponds (Câmara et al., 2009). Nutrients are a primary limiting factor for phytoplankton biomass and chlorophyll-a concentration in surface water (Câmara et al., 2009). Nutrient enrichment in fishponds is considered a major stimulant for eutrophication and the occurrence of toxic cyanobacterial blooms (Câmara et al., 2009). The presence of cyanobacterial blooms in Nigerian fishponds during both dry and wet seasons could be linked to farming practices, such as fertilization of the ponds.

Fishponds are characterized by nutrient-rich bottom sediments that serve as a critical reservoir of organic matter and nutrients (Graham et al., 2009; Teissier et al., 2012). Cyanobacterial blooms in Nigerian fishponds across seasons can be attributed to fertilization practices that increase nutrient loads. Chia et al. (2015) elaborated that artificial enrichment of fishponds through fertilizer use can increase nutrient concentrations. Conversely, the low nutrient levels and absence of blooms in SA fishponds may be due to controlled feeding and frequent water exchanges, which dilute nutrient concentrations and disrupt bloom-forming conditions. The SA fishponds received constant freshwater inflows from a nearby dam, promoting intrusion, reducing retention time, and enhancing aeration.

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Management practices in Nigeria fishponds, such as high stocking densities, directly influence cyanobacterial growth. Cyanobacteria dominate in fishponds with high stocking densities (Kopp et al., 2016). Fish farming practices, particularly those involving high stocking densities, significantly influence the structure and dynamics of the aquatic ecosystem. Increased fish stock density introduces elevated nutrient loads, primarily through uneaten feed, excretion, and waste accumulation, which accelerates the trophic status of the water body. Over time, this progression can lead to a state of hypertrophy, characterized by excessive nutrient enrichment (Jeppesen et al., 2005; Hardly and Kaushik, (2021). The brownish-muddy coloration in the SA fishponds could indicate turbidity, suggesting the presence of suspended sediments such as silt, clay, or organic matter.

Microcystis species are the most abundant taxa encountered in fishponds and other surface water globally. Most cyanobacteria species, such as *microcystis* sp., tend to have a more competitive advantage over other phytoplankton (Zohary, 2001; Pearl et al., 2013). *Microcystis* is highly tolerant to varying temperatures, light intensities, and pH levels often experienced in fishponds (Pearl et al., 2013). This makes them better equipped to survive and thrive under such stresses compared to other cyanobacteria in SA and NGA fishponds. Moreover, *Microcystis* sp. can regulate its buoyancy using gas vesicles, allowing it to move vertically in the water column (Zohary, 2001; Wiegand and Pflugmacher, 2005). This gives it access to optimal light conditions near the surface for photosynthesis while avoiding competition and predation at different depths (Huisman et al., 2018). This cyanobacteria species reproduces rapidly under favorable conditions, forming dense surface blooms outcompeting other species by shading them out and monopolizing light resources. Fishponds are often nutrient-rich due to feed inputs, fish excreta, and organic matter accumulation. *Microcystis* sp. thrives in high-nutrient environments, particularly those with elevated nitrogen (N) and phosphorus (P) levels (Gomaa et al., 2014; Newell et al. (2019)). Its ability to efficiently uptake and store these nutrients gives it a competitive edge over other cyanobacteria (Lürling et al., 2017). This explains the dominant presence of *Microcystis* sp. during cyanobacterial bloom in NGA fishponds.

5. Conclusion

This study highlights the complex interplay between environmental variables, anthropogenic management, and cyanobacterial blooms in fishponds in South Africa (SA) and Nigeria (NGA).

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Unlike temperate regions where temperature plays a dominant role in regulating cyanobacterial growth, the fishponds in SA and NGA exhibit distinct dynamics due to their tropical and managed conditions. Increased surface water temperatures during summer did not significantly influence chlorophyll-a levels or phytoplankton biomass in SA fishponds. Seasonal temperature variations were minimized due to the shallow depth of the ponds and continuous management practices such as water replenishment, aeration, and controlled feeding. Instead, nutrient availability, primarily driven by fertilization and fish stocking density, emerged as a critical factor influencing cyanobacterial blooms. In NGA fishponds, high nutrient loads from fertilization and dense fish stocking contributed to the dominance of *Microcystis* species throughout the year. *Microcystis* thrives due to its ability to regulate buoyancy, tolerate a wide range of environmental conditions, and rapidly reproduce under nutrient-rich conditions. In contrast, SA fishponds maintained lower nutrient levels due to frequent water exchanges, which diluted nutrient concentrations and disrupted bloom formation. Rainfall played a significant role in mitigating bloom intensity by diluting nutrient concentrations and increasing water transparency, particularly during the wet season. These findings emphasize that cyanobacterial blooms in tropical fishponds are less influenced by temperature fluctuations and more by nutrient dynamics and management practices.

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Chapter 8

Significant Findings, Conclusions, and Recommendations for Future Works

This study investigated the impacts of seasonal dynamics on cyanobacteria proliferation and the bioaccumulation of cyanotoxins in *Clarias gariepinus* harvested from fishponds within South Africa and Nigeria. This comparative study provided evidence of varied profiles of cyanotoxins in fishponds between the two countries. The study utilized untargeted and targeted LC-MS/MS methods to detect cyanotoxins in fish tissues effectively. The targeted LC-MS/MS was limited by the availability of reference standards while the untargeted LC-MS/MS provided a broader dataset, allowing for retrospective analysis and identification of diverse compounds. Cyanobacterial proliferation in fishponds is more influenced by human activities, such as controlled feeding, water replenishment, and fertilization, than by natural climatic factors. Surface water temperature increases during summer did not significantly affect chlorophyll-a levels or phytoplankton biomass in SA fishponds, differing from expectations in temperate regions. Cyanobacterial blooms in NGA fishponds were strongly associated with high nutrient loads due to fertilization practices and dense fish stocking.

Significant findings

- Cyanobacterial blooms in NGA fishponds were strongly associated with high nutrient loads due to fertilization practices and dense fish stocking.
- Controlled feeding and regular water exchanges in SA fishponds reduced nutrient levels, minimizing bloom occurrence.
- Microcystis spp., a cyanobacteria species, was dominant in NGA and SA fishponds due to its competitive advantages, including buoyancy regulation, tolerance to environmental variability, and rapid reproduction under nutrient-rich conditions.
- Untargeted LC-MS identified a wide range of cyanotoxins, including
    - Aeruginosins, anabaenopeptins, microcystins, and microginins.
- Also detected were non-toxic secondary metabolites, such as:

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- Octadecadienoic acid, phosphocholine, ethanesulfonic acid, microcolins, marabimides pheophorbide A, cholic acid, phenylalanine, amyl amine, triglycerides, and sulfonic acid.

Contribution to knowledge

- This is the first study using an untargeted LC-MS/MS approach for cyanotoxin profiling in aquaculture systems in NGA & SA
- This research unveils previously undetected cyanotoxins and secondary metabolites.
- This is the first untargeted LC-MS/MS study on cyanometabolites in fish tissues from commercial aquaculture.
- This dataset serves as a baseline for future food safety policies, toxicological studies, and ecosystem risk assessments.
- The molecular networking enabled the detection of known cyanotoxins in Clarias gariepinus fish tissue: Microcystins, microginins, aeruginosins, microcolins, anabaenopeptins, and aplysiatoxins.
- The uploaded untargeted LC-MS/MS data to GNPS, enabling future identification and retrospective analysis of cyanotoxins and unknown metabolites.
- This data also enhances the GNPS spectral repository, supporting the discovery and annotation of previously unidentified compounds in aquatic environments.

Limitations of the study

- Small Sample Size
  - Only three ponds per country.

- Lack of historical & quantitative data between climate variability and algal bloom.

- Lack of Controlled Experiments: This study was conducted as an observational study. rather than a controlled experiment, limiting the ability to directly regulate key environmental factors

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Recommendations for future works

To address these limitations, future studies should:

- Include controlled manipulations of key variables (e.g., nutrient levels, water mixing) to test their direct impact on cyanobacterial proliferation.
- Expand the dataset to include more diverse tropical fishponds systems for broader generalizability.
- Future research on bioaccumulation of cyanotoxins in fish tissues, the effects of cooking, and the risks of chronic exposure is crucial to understanding the long-term health implications.
- Lastly, regulatory intervention is essential to prevent human exposure and ensure that fish from these ponds meet safety guidelines.

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Supplementary information

Accession Number

Submission	Title	Status	Updated
SUB11738025	Prokaryotic 16S rRNA / H10 raw	GenBank: Processing	10:09
SUB11715594	Prokaryotic 16S rRNA / F10	GenBank: Processed ON868695 3 files: • AccessionReport.tsv • flatfile.txt • email.txt	Jun 28
SUB11709875	Prokaryotic 16S rRNA / E10	GenBank: Processed ON868693 3 files: • AccessionReport.tsv • flatfile.txt • email.txt	Jun 28
SUB11715599	Prokaryotic 16S rRNA / G10	GenBank: Processed ON868694 3 files: • AccessionReport.tsv • flatfile.txt • email.txt	Jun 28
SUB11715603	Prokaryotic 16S rRNA / H10	Unfinished at the Sequences step	Jun 28
SUB11709776	Prokaryotic 16S rRNA / F02	GenBank: Error has errors SUB11709776-Report.html Fix	Jun 28

Figure 4: Submission of sequences to GenBank and accession numbers obtained. Four out of six 16S rRNA sequence isolated strains were accepted. Two sequences (E02 and F02) were rejected because no significant similarities were found between NCBI blast and GenBank.

- S1 907-R E 10 - Accession Number: ON868693
- S2 907-R F 10 – Accession Number: ON868695
- S3 907-R G 10 - Accession Number: ON868694
- S4 907-R H 10 - Accession Number: ON920195

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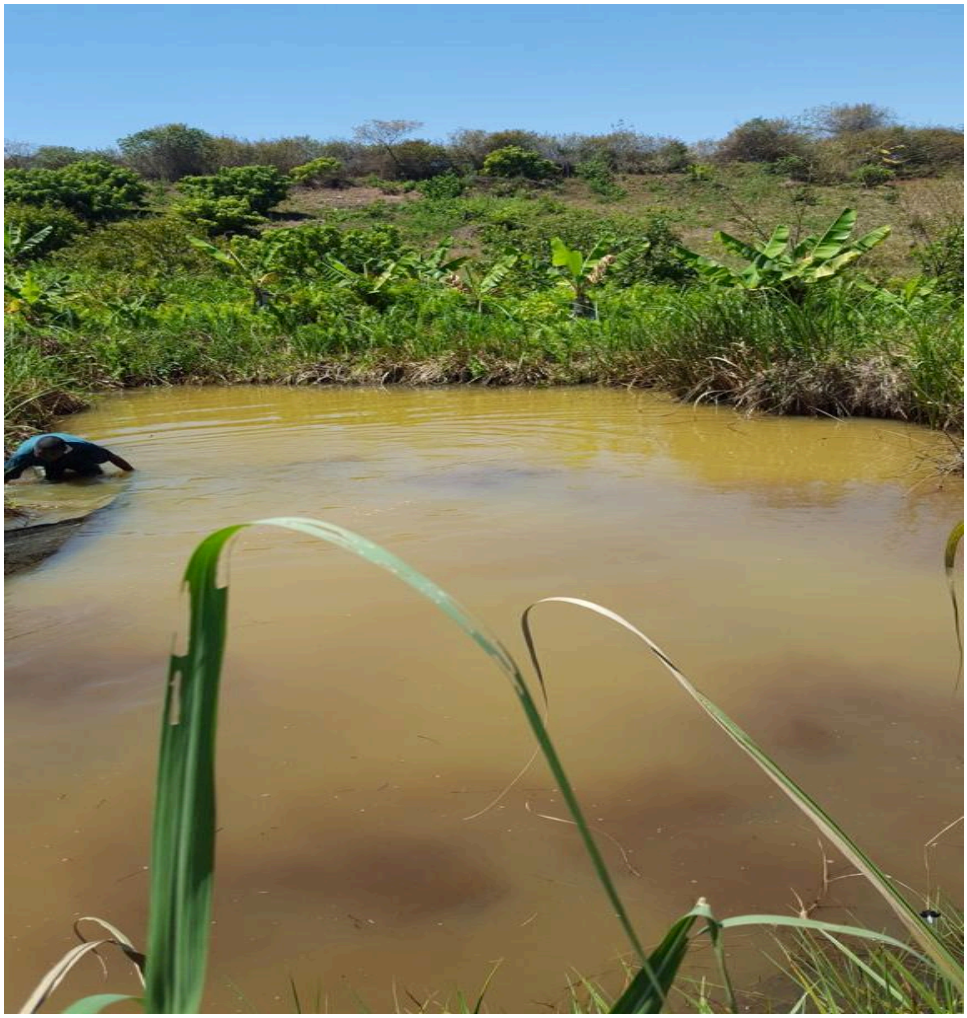

Figure S1: Duthuni fishpond

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S2: Essien Town fishponds

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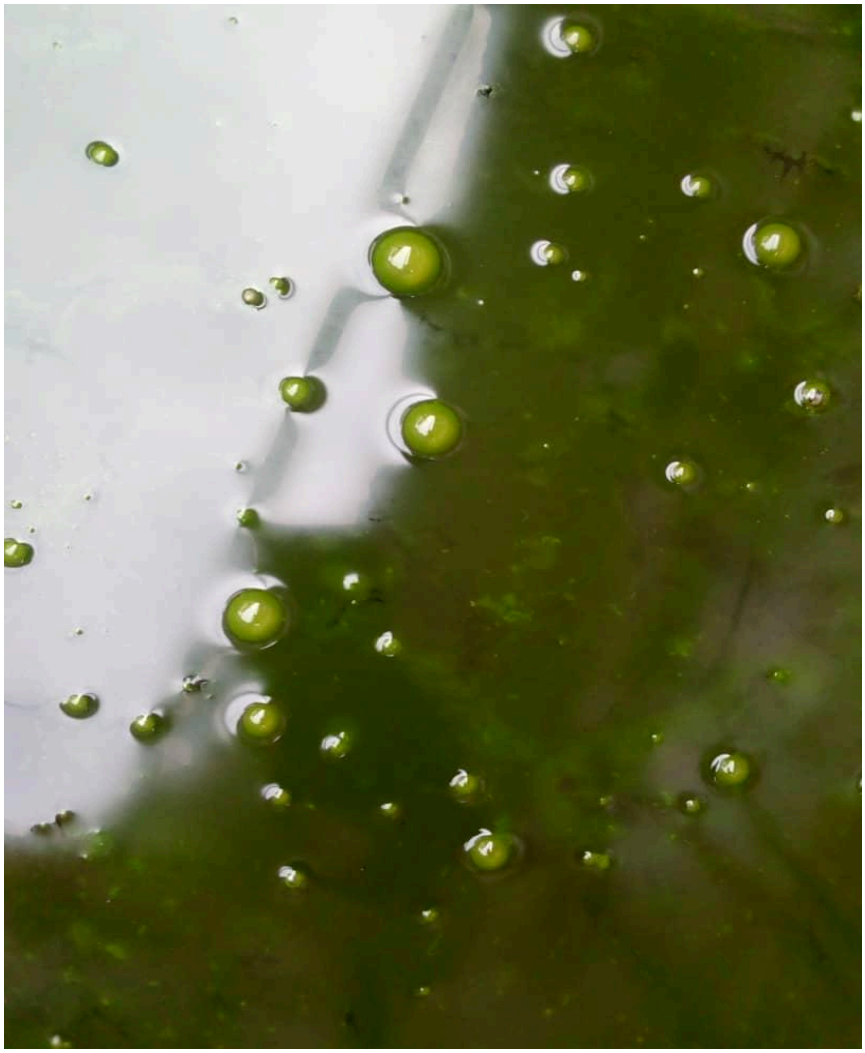

S3: Offiong Etim fishpond

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S4: State Housing fishpond

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S4: Fish sampling

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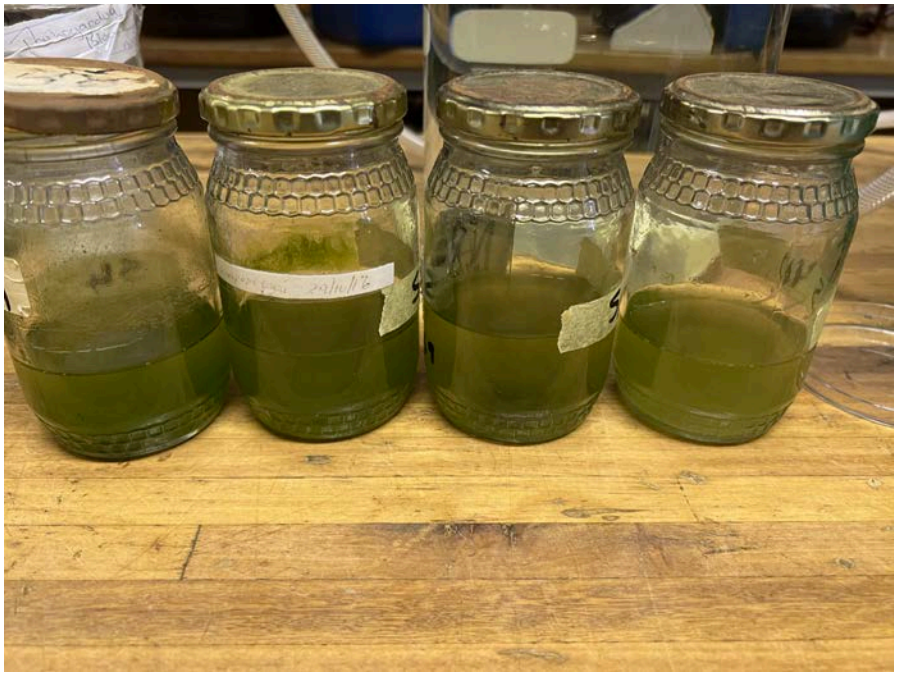

S5: Obtained water sampled stored in glass jars

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