

Understanding regional occurrence of cyanotoxins in aquaculture: A comparative study between Nigeria and South Africa

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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 GNPS database and CANOPUS annotation enabled putative identification of microcystin, micrognins, aplysiatins, aeruginosins, anabaenopeptins, microcolins and marabmids. Water samples from Nigeria and South fishponds shared similarities in bioactive compounds dominated by ethanesulfonic acid *m/z*, pheophorbide A, cholic acid phenylalanine, amyl amine and phosphocholine (PC) and sulfonic acid. The fishponds in Nigeria showed the presence of multiple cyanotoxin classes in the dry, wet season, and summer season in the water. Aplysiatoxins were identified in all sampling sites in Nigeria (N1, N2, and N3). The Duthuni South Africa (P1, P2, P3) sampling sites exhibited the presence of Microginins and Microcystins. All the fishponds displayed a widespread occurrence of Anabaenopeptins, Aplysiatoxins, Microcolins, and Marabmids during selected summer. This study underscores the specificity of cyanotoxin occurrence influenced by regional and seasonal dynamics.

Keywords: Cyanometabolites; cyanotoxins; fishponds; seasonal; spectrometry

1. Introduction

Cyanobacteria are present in freshwater, ponds, rivers, marine and other terrestrial habitat. 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). Most of these secondary metabolites such as different classes of cyclic peptides, phenols, lipids, alkaloids, vitamins, terpenoids, polysaccharides and pigments identified have the potential to act as antioxidant, anticoagulant, anticancer, antiviral, antileishmanial, antibacterial antiprotozoal and anti-inflammatory. They are widely applied in the pharmaceutical, cosmeceutical and nutraceutical industries applications (Kini et al., 2020; Rodríguez et al., 2020). Cyanobacteria metabolites (cyanometabolites) constitute a significant and rapidly expanding category of secondary metabolites, encompassing over 2000 compounds, which comprise cyanotoxins and are synthesized by various genera of cyanobacteria (McCann, 2022). Environmental factors (temperature, nutrients levels, salinity and pH) have contributed effectively to cyanobacterial growth at high densities characterized by blooms, increase biomass accumulation, surface water

scum and release of cyanotoxins (Varriale et al., 2023). This cyanotoxin can be harmful to aquatic organisms, animals, water quality and humans that are exposed to them directly or indirectly. Cyanotoxins pose a significant threat because of their capacity to accumulate toxins in fish and molluscs, thereby enabling the transfer of these toxins through the food chain to wildlife and humans.

Cyanotoxins represent a major threat due to their ability to bioaccumulate toxins in fish, and molluscs thus trophic transfer to wildlife and humans (Jia, 2016). 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). Cyanotoxins are categorized into three primary groups based on their chemical characteristics - alkaloids, linear and cyclic oligopeptides, and amino acids. They are also classified into four groups according to their mode of action: hepatotoxins, neurotoxins, dermatotoxins, and cytotoxins (Solter and Beasley, 2013).

Furthermore, cyanobacteria have attracted attention as producers of novel metabolites significant in industry and medicine (Nitnaware, 2021). Advancements 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 Nodularins) 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). Another setback observed is that quantitation based on the standards of Microcystin showed a low percentage of variations (MC-LR, MC-YR) while the non-targeted strategy was highly effective for the identification of microginins, anabaenopeptins and micropeptins (Varriale et al., 2023). This show limitation towards the detection and identification of other toxic/bioactive cyanometabolites and cyanotoxins whose standards are commercially available, especially in South Africa and Nigeria.

The potential risks associated with the co-occurrence of multiple cyanometabolites classes and possible synergistic toxic effects on cultured fish have not been considered in aquaculture fish farms (Varriale et al., 2023). So far, there is no study, which investigates cyanometabolites in fish farming ponds, which are regularly used in the fish market for human consumption 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 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 research took place in commercial aquaculture fishponds situated in the Vhembe District, Limpopo Province, South Africa, and the Calabar Municipality, Cross River State, Nigeria (figure 1.1). In Nigeria, sampling locations included Offiong Etim Avenue (4°59'58.92" N and 8°19'03.97" E), Essien Town (4°59'15.49" N and 8°19'40.21" E), and State Housing (4°59'6.50" N and 8°20'13.29" E). The aquaculture fishponds in the Vhembe District were positioned in Duthuni (22°57'56.98" S and 30°23'43.96" E).

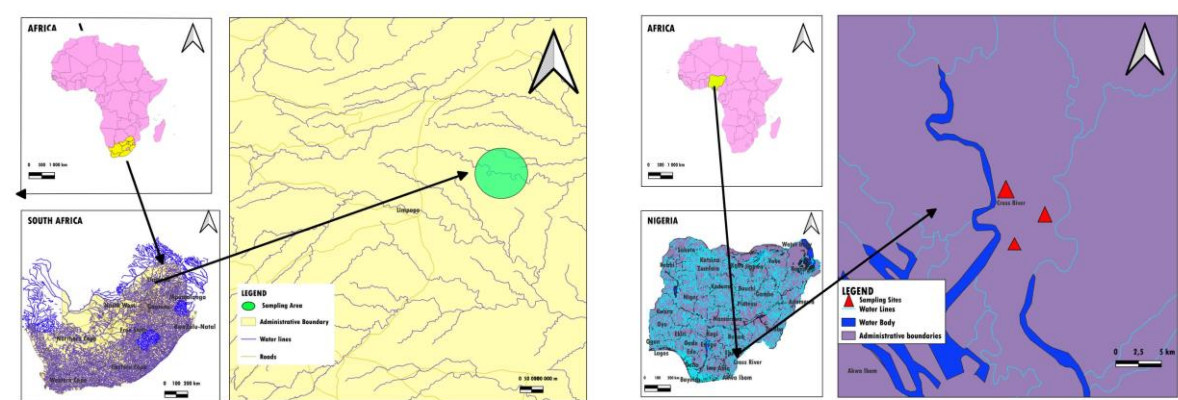


Figure 3. 1: Map of the sampling sites - Vhembe District, Limpopo Province, South Africa and Calabar Municipality, Cross River State, Nigeria and .

2.1.1. Description of the fishponds

A total number of 6 fish ponds located in Vhembe District (3 fish ponds) was and Calabar (3 fish ponds) were used for this study. Fishponds types used in this study included concrete, tarpaulin and earthen ponds (figure 1.1).



Figure 1. 1: A): Earthen aquaculture fishponds in Duthuni, South Africa and B): tarpaulin fish ponds in Basin Authority, Nigeria.

2.2. Sample collection

2.2.1. Water sampling

Water samples were collected from the water surface of the fish ponds between 1 m depth using a 1 L sterilized sampling bottle. Sampling bottles were labeled with the sampling site, date and time. Afterward, samples were carried in ice-filled cooler boxes to the laboratory for further analysis. The sampling procedure described in this study was obtained from WHO (1999) guidelines for toxic cyanobacteria sampling in surface water. Water samples were collected in triplicates seasonally from the fishponds during winter and summer in South Africa, and dry and wet seasons in Nigeria, respectively. Approved consent was obtained from the owners of the fishponds in Nigeria and South Africa before sampling.

2.2.2. Cyanotoxins extraction in 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 mins and the water samples were freeze dried. The frozen 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 h at a room temperature. The mixture was transferred to glass vials for LCMS analysis using the syringe filter.

2.3. 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.

2.4. Data analyses

High resolution MS/MS spectra data generated from the Mass spectrometry was processed using the Global Natural Product Social Molecular Networking (GNPS) Library database (to match the compound class) in combination with CANOPUS-generated annotation from SIRIUS4 Software. Identification of metabolites was carried out using confidence LEVEL 3 annotation. The putative identified spectra was used to construct a molecular networking visualized using Cytoscape 3.10.0. The molecular networking algorithm allows automated identification of analogs of known compounds.

2.5. Statistical analysis

The intensity and retention time tables for the cyanotoxins detected in the fishponds were further processed using the principal component analysis. Principal component analysis was conducted in XLstat; wherein the Cyanotoxins in Nigeria and South Africa were observed in terms of Intensity and Retention time over seasons. Therefore, XLstat was used to compute the Biplots and screen plots. All analysis were conducted to compare the seasonal dynamics of toxic metabolites in Nigeria and South Africa.

3. Results and Discussion

3.1. Molecular network

A molecular network was performed with the fragmentation data obtained by the LCMS/MS analyses of water sample metabolomes. Putatively identified metabolites generated from the GNPS Library match and CANOPUS-generated annotation was used to

produce a molecular network. The molecular network shown in Figure 4.2 was visualized using Cytoscape 3.10.0. The molecular network highlights known metabolites, structural identity, shared clusters, nodes and edges, match metabolites (metabolites within the public database) and no match metabolites (no match within the database). Various analytes were assembled into molecular clusters according to their fragmentation patterns' likeness. Each cluster potentially pertained to specific chemical family structures. The molecular network revealed discernible clusters representing related molecule families. This networking helped identify both known cyanotoxins and unfamiliar analogues, visually illustrating structural connections. The molecular network showed that both water sample from different fishponds shared specific metabolites despite the differences in location, local climatic factors and sampling sites. Different analytes with similar fragmentation patterns were grouped in the same molecular clusters. The molecular networking led to the putative identification of multiple cyanotoxins — aeruginosins, anabaenopeptins, aflatoxin, aplysiatoxins, microcystin, microcolins and marabmids during winter, summer, dry and wet seasons in Figure 1.2 in water samples. The untargeted metabolome analysis was highly effective for the identification of toxic metabolites and non-toxic metabolites in the fishponds.

3.2. Cyanobacterial toxins and other bioactive metabolites

The water samples from the fishponds 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 1.3. The polar lipid identified in the water samples was dominated by six main classes: glycolipids, phosphosphingolipids, phospholipids, phosphatidylglycerol (PG), phosphatidylethanolamine (PE), distributed across the fishponds. Cyanobacteria, the microscopic organisms found in various aquatic environments, are intrinsically linked to the presence of specific lipid classes such as glycolipids, phosphosphingolipids, and phospholipids, including phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) in surface water. These polar lipids constitute the building blocks of cyanobacterial membranes, characterized by molecular structures surrounded by 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. aplysiatoxins and were detected across all sampling sites in Nigeria (N1, N2 and N3). Microcystins were found in Duthuni (P1,P2, P3). Anabaenopeptins, aplysiatoxins, microcolins and marabmids were widespread in all fish ponds.

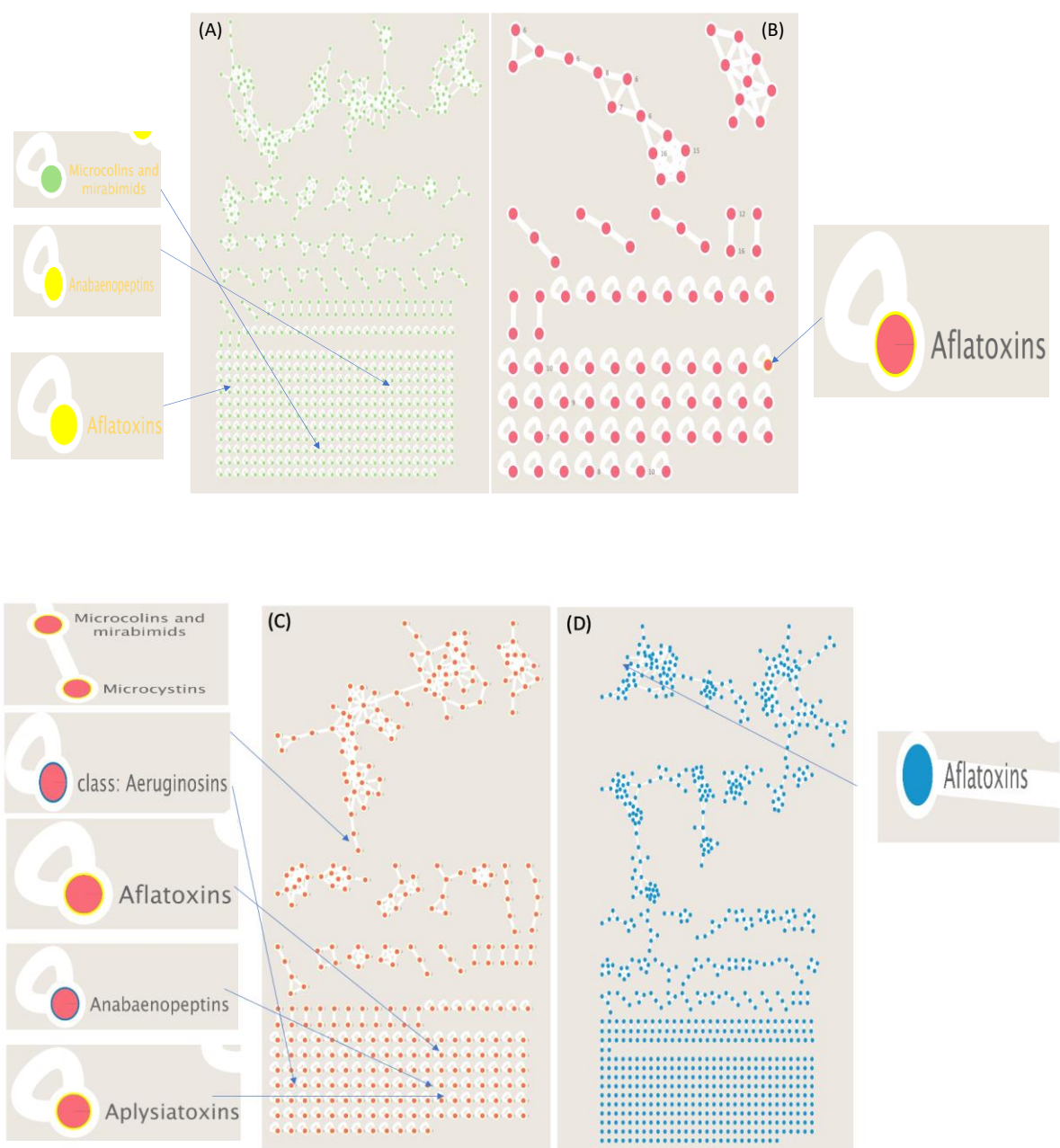


Figure 1. 2: The molecular network of metabolites extract from fishpond water samples - (A) dry season, (B) wet season (C) summer and (D) winter.

The cyanometabolites in both sampling locations align with the observation in Tri, Var and Ver lakes (Marie and Gallet, 2022). These observations suggest that the increased proliferation of cyanobacteria could be considered as an explicative factor for the production of noxious toxins in fishponds. The putative identification of various cyanotoxins—*anabaenopeptins*, *aplysiatoxins*, *microcolins* and *marabimids* in water samples can be attributed to cyanobacterial blooms in Nigerian sampling stations. These toxins are typically produced during such blooms. The cyanotoxins in this study agree with those recently reported by Zespata et al. (2023). The study detected toxins *anabaenopeptins*, *aeruginosamide*, *anabaenopeptins*, *saxitoxin*, *cylindrospermopsin* and *microcystins* in cyanobacterial bloom-dominated Lake of the woods (low), spanning across canada and usa. similarly, *anabaenopeptins*, *cyanopeptolins*, *microginins*, and *cyanobactins* were also

reported in commercial fishponds in the Czech Republic (Kust et al., 2020). Parallel research in the cyanobacterial-rich lakes (Fon, Tri, Var and Ver) also confirmed the presence of multiple toxins such as microcystins, cyanopeptolins and anabaenopeptins, microginins and aeruginosins (Marie and Gallet, 2022). Zastepa et al. (2023) suggested that this pattern is a common association between these toxins and cyanobacterial blooms in surface water.

3.3. Seasonal and regional dynamics of cyanotoxins in fishponds

Principal component analysis of aeruginosins, anabaenopeptins, aflatoxin, aplysiatoxins, microcystin, microcolins and marabimids based on peak intensity and retention time between different sampling season showed variations between each cyanometabolites as displayed in Table 1&2. The maximum intensity observed is 100% from microcystin and the minimum is 0.06 from microcolins and mirabimids. Aflatoxins displayed a higher retention time of 49.86 mins while microcolins and mirabimids retention time was at 11.60 mins. This plot displayed in Figure 1.4 -1.6 indicates that microcystin and microcolins and mirabimids varied the most in intensity in the summer season while aflatoxins varied the most in dry season. The most similarities in terms of retention and intensity was observed in anabaenopeptins, aplysiatoxins and aeruginosins. Aflatoxins were observed in throughout the seasons with slight variations in intensity and retention time. Retention time accounted for 28.65% of the variation amongst cyanotoxins in South Africa. The variation in the peak intensity and retention of each cyanotoxin was significantly influenced by environmental factors from each sampling sites.

Table 1. Descriptive statistics of metabolite intensity and retention time.

Variable	Observations	Minimum	Maximum	Mean	Std. deviation
Nigeria					
Intensity (100%)	4	0.000	62.920	15.905	31.345
Retention time	4	4.100	18.500	11.000	6.815
South Africa					
Intensity (100%)	7	0.06	100	28.94	33.08
Retention time	7	11.60	49.86	37.19	17.5

Table 2. The contribution of cyanotoxins to observations and the squared cosines of the observations.

Cyanotoxins	Contribution of observations (%)		Squared cosines of the observations	
	F1	F2	F1	F2
<i>Microcystin</i>	75.873	6.954	0.964	0.036
<i>Aplysiatoxins</i>	6.047	0.685	0.956	0.044
<i>Anabaenopeptins</i>	7.184	0.030	0.998	0.002
<i>Aflatoxins</i>	1.655	2.255	0.646	0.354
<i>Aeruginosins</i>	5.134	0.273	0.979	0.021
<i>Microcolins and Mirabimids</i>	2.027	79.292	0.060	0.940
<i>Aflatoxins</i>	2.081	10.510	0.330	0.670

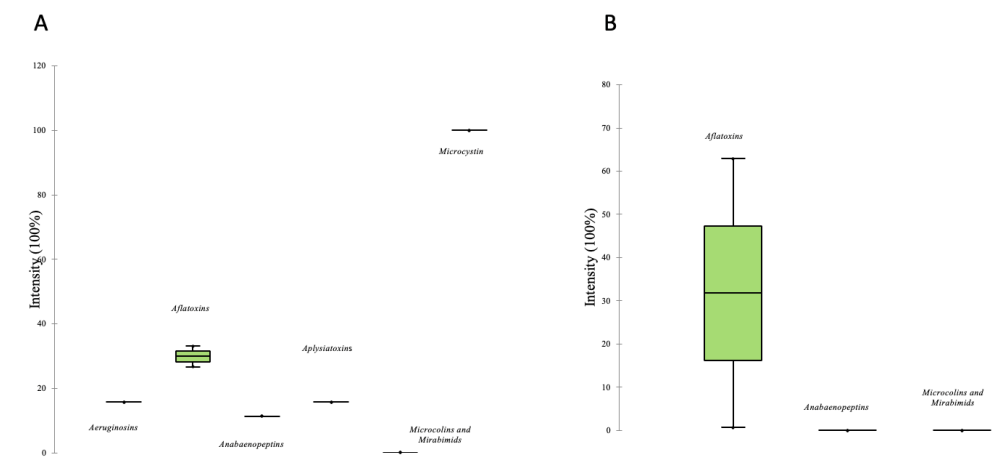


Figure 1. 4: Boxplots of cyanotoxins in relation to intensity – (A) South Africa and (B) Nigeria.

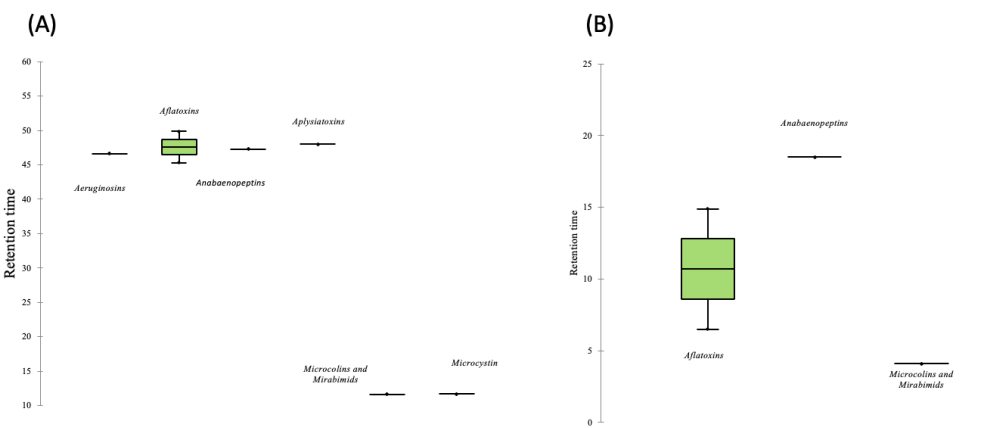


Figure 1. 5: Boxplots of cyanotoxins in relation to retention time – (A) South Africa and (B) Nigeria.

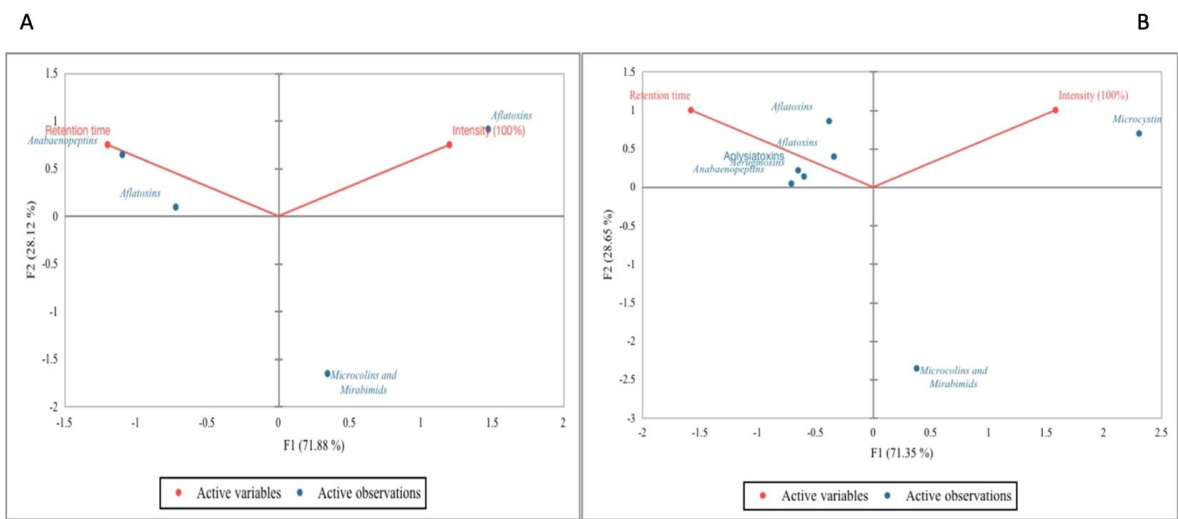


Figure 1.6: Biplot of cyanotoxins observations in relation to intensity and retention time

Cyanobacterial blooms were evident across all fishponds in Nigeria throughout both wet and dry seasons. These periods were characterized by a prevalence of cyanobacterial peptides such as anabaenopeptins, microcolins and mirabimids in the fishponds. Specifically, the presence of aplysiatoxins was exclusively noted in fishpond during the wet season. 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. similarly to wet season in Nigeria sampling sites, aplysiatoxins was observed during winter. Interestingly, there were resemblances between bioactive compounds (octadecadienoic acid, phosphocholine (PC), sulfonic acid, pheophorbide A, and cholic acid) in all the water samples despite their differences in location. These similarities persisted across summer, winter, dry, and wet seasons as expressed in Figure 1.2 & 1.3. 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 corresponding to Marie and Gallet (2022) and Burford et al. (2020) findings. Environmental conditions especially during the warm season increase daytime temperature, nutrients (from fish faeces, feed particles and anthropogenic activities) and solar radiation favouring excessive proliferation of cyanobacteria and toxin production (Mohamed et al., 2020). This explains the increased presence of multi-toxin during the dry season in Nigerian fishponds and summer in Duthuni fishponds, in South Africa. A similar observation was reported during the dry season in fishponds in Zaria, Nigeria by Chia et al. (2021) and Kust et al. (2020) reported the highest diversity of *aeruginosins*, *microginins*, *cyanopeptolins* and *microginins* during summer in South Bohemia, Czech Republic.

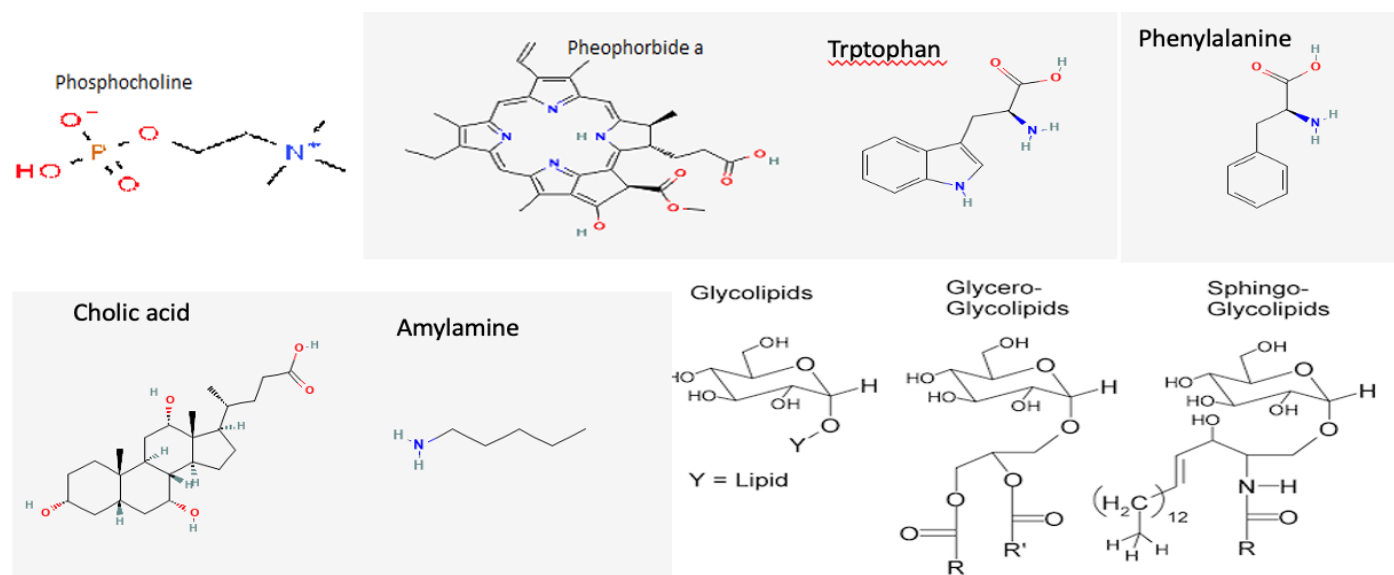


Figure 1.3: Representative compounds for non-toxic bioactive metabolites present in fishpond water.

3.4. Implication

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). These toxins can be 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 attributed to cyanotoxin exposure have been documented. 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).

Another episodes occurred in the USA. In 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). Due to the potential hazards linked to cyanotoxins found in food and water, The EPA revised this rule to include monitoring for 10 cyanotoxins in Public Water Systems including microcystins and cylindrospermopsin. The WHO (2020) had established provisional guidelines for microcystin-LR (one of the most common and potent variants of microcystins) in drinking water. These guidelines were set as a provisional guideline value of 1.0 microgram per liter ($\mu\text{g/L}$) for microcystin-LR in drinking water.

Despite the known risks, cyanotoxin production has been increasing over time in fishponds in these regions due to climate change and increased eutrophication. The contaminated water from the fishpond in these regions is mostly discharged into the environment and is also used in irrigation. Food products and portable water reservoirs are been exposed to these toxins from contaminated fishpond water. A recent study by Chia *et al.* (2019) revealed that irrigated vegetables in Northern Nigeria have very high levels of microcystins. Mutoti *et al.* (2023) documented increased levels of microcystins in maize meal in Thohoyandou, South Africa. The concern doesn't solely revolve around this fish in the fishponds but extends to a broader range of products that might be similarly contaminated. The implications of this contamination are significant and underscore the need for a deeper comprehension of the potential health risks associated with cyanotoxin exposure from contaminated fishponds.

4. Conclusion

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

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