



DNA barcode survey of aquatic bacterial and eukaryotic microorganism communities in the Mooi River system

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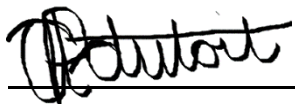
Dissertation accepted in fulfilment of the requirements for the degree *Master of Science in Microbiology* at the North-West University

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DECLARATION

I, **Andreas Petrus du Toit**, hereby declare that this dissertation, titled “DNA barcode survey of aquatic bacterial and eukaryotic microorganism communities in the Mooi River system”, is my own work. I also confirm that the content of this research study was not, and will not, be submitted to any other university for any other tertiary qualification whatsoever.

A handwritten signature in black ink, appearing to read 'A. Petrus du Toit', is written over a horizontal line.

Andreas Petrus du Toit

26 November 2024

ABSTRACT

Freshwater aquatic ecosystems are critical to both environmental sustainability and human well-being. However, the long-term sustainability of these ecosystems is under threat from a variety of anthropogenic influences, such as agricultural activities, industrial and mining operations, urban runoff, and land use changes. These activities lead to declining water quality and biodiversity in aquatic ecosystems, necessitating innovative and comprehensive ecosystem assessment and management strategies. The present study surveyed the aquatic bacterial and eukaryotic microorganism communities of the Mooi River system and its tributaries using DNA metabarcoding alongside physico-chemical parameters and metal concentrations.

13 sampling sites along the Mooi River catchment, Loop Spruit and Wonderfontein Spruit, were strategically chosen to represent varying degrees of anthropogenic influence, including wastewater treatment plant discharges, agricultural runoff, and mining operations. Water samples were collected in triplicate at each site from 2021 to 2022. A calibrated Oakton PCS Testr™ 35 waterproof field multi-parameter probe was used to conduct on-site measurements of physical and chemical parameters such as pH, temperature, total dissolved solids (TDS), salinity, and turbidity. Furthermore, inductively coupled plasma mass spectrometry (ICP-MS) analysed trace and heavy metal concentrations. Environmental DNA (eDNA) was extracted from filtered membranes using the Macherey-Nagel NucleoSpin® Soil DNA Isolation Kit. Furthermore, metabarcoding of 16S rRNA and COI genes was conducted, facilitating the identification of bacterial and eukaryotic microorganisms. Several diversity indices were used to assess and compare species richness, diversity, dominance, and similarity between the sites. Multivariate analyses were further conducted to assess the impact of anthropogenic influences on the microbial communities.

The findings of this study indicate significant regional and temporal variation in the physico-chemical parameters which assessed water quality. Specifically, total dissolved solids (TDS), turbidity, and trace and heavy metals, such as aluminium, zinc, and copper, consistently surpassed the prescribed threshold, signifying the negative effect of mining and urban effluents on the Mooi River system and its tributaries.

Furthermore, microbial communities exhibited varied responses to environmental influences. Bacterial phyla such as *Proteobacteria*, *Bacteroidota*, and *Actinobacteriota* thrived in nutrient-rich and metal-contaminated sites. Whereas eukaryotic communities, dominated by *Arthropoda* and *Chromista*, displayed site-specific and seasonal adaptations, with taxa such as *Cyanobacteria* and *Pythium* indicating eutrophication risks in phosphorus enriched waters.

The findings of this study emphasise the value of adopting a comprehensive and integrative approach that combines physico-chemical analyses with metabarcoding techniques to improve biomonitoring frameworks used to assess aquatic ecosystems. Furthermore, this study contributes to both research and practice by demonstrating the use of DNA metabarcoding techniques to survey aquatic bacterial and eukaryotic microorganism communities in aquatic ecosystems, thereby informing sustainable water resource management in South Africa.

Keywords: Mooi River, Loop Spruit, Wonderfontein Spruit, anthropogenic influences, biomonitoring, DNA barcoding, metabarcoding, microbial diversity, environmental DNA (eDNA), next-generation sequencing (NGS), multivariate analyses

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LIST OF ABBREVIATIONS

A	
AMD	Acid mine drainage
ASV	Amplicon sequence variants
ATW	Around the World Bridge
C	
COI	Cytochrome c oxidase subunit one
COD	Chemical oxygen demand
CO ₂	Carbon dioxide
D	
DEA	Department of Environmental Affairs
DWAF	Department of Water Affairs and Forestry
DWS	Department of Water and Sanitation
E	
EC	Electrical conductivity
EPA	Environmental Protection Agency
eDNA	Environmental DNA
F	
FAII	Fish Assemblage Integrity Index
G	
GC	Golf Course Bridge
H	
HB	Hartspad Bridge
HTS	High-throughput amplicon sequencing
I	
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
K	
KD	Klerkskraal Dam
KKD	Klerkskraal Dam
L	
LSB	Loop Spruit Begin

LSE	Loop Spruit End
M	
MB	Muiskraal Bridge
N	
NWU	North West University
NGS	Next generation sequencing
O	
OTU	Operational taxonomic units
P	
PCoA	Principal component analysis
PCR	Polymerase chain reaction
R	
rRNA	Ribosomal ribonucleic acid
S	
SADI	South African Diatom Index
SASS	South African Scoring System
SB	Skandinawie Drift Bridge
SPI	Specific Pollution Sensitivity Index
T	
TDS	Total dissolved solids
TMB	Thabo Mbeki Bridge
TPB	Trim Park Bridge
W	
WRC	Water Research Commission
WSB	Wonderfontein Spruit Begin
WSE	Wonderfontein Spruit End
WWTP	Pre-Wastewater Treatment Plant Bridge

CHAPTER 1: INTRODUCTION

1.1 Background and problem statement

Aquatic ecosystems, which cover approximately 71% of the Earth's surface, play a fundamental role in sustaining global biodiversity and productivity (Shiklomanov, 1993). These ecosystems host and support a variety of species, habitats and key ecological processes that are intricately connected and mutually interdependent (Department of Water Affairs and Forestry (DWAF), 1996). Freshwater and inland aquatic ecosystems are particularly important for both environmental and human well-being. Their rich biodiversity and associated functions make significant contributions to societal, environmental, economic, and scientific development (Gatti, 2016; Kumar *et al.*, 2024). However, freshwater ecosystems are notably more susceptible and often vulnerable to external factors and influences compared to land-based ecosystems (Gatti, 2016).

In recent years, studies assessing aquatic ecosystems and water quality have reported alarming pollution levels, which can be largely attributed to anthropogenic influences such as industrialisation, urban effluents, and agricultural runoff, all of which place significant strain on these fragile environments (De Villiers & Thiar, 2007; Rasool *et al.*, 2023). Consequently, the water quality in freshwater ecosystems has been deteriorating at an increased pace (Jordaan *et al.*, 2019; Wolmarans *et al.*, 2015). Water quality refers to the *“physical, chemical, biological and aesthetic properties of water that determine its fitness for a variety of uses and for the protection of the health and integrity of aquatic ecosystems”* (DWAF, 1996, p.3). Many of these properties are controlled or influenced by constituents that are either dissolved or suspended in water. The deposition of organic and inorganic pollutants from anthropogenic activities into aquatic ecosystems directly threatens both human and environmental health, underscoring the importance of implementing effective management strategies to safeguard ecosystems from detrimental and irreparable damage (Prabhakaran *et al.*, 2017; Takahashi *et al.*, 2023). As a result, it is critical to establish methodologies for identifying, estimating, comparing, and managing pollutant-driven risks that threaten natural resources as well as the environment.

Consequently, an urgent need arises to assess the status of water bodies and address and remediate indirect pollution of freshwater systems, both for current and potential use (Barnard *et al.*, 2013; Koekemoer *et al.*, 2021).

The development of effective aquatic management strategies depends mainly on rigorous scientific data to understand both water quality and quantity and associated ecosystem functioning (Moog *et al.*, 2018). Such data should include comprehensive information on species abundance, diversity, and environmental distribution, which can be obtained through biomonitoring initiatives (Baied & Hajibabaei, 2012; Dallas & Rivers-Moore, 2014; Thomsen & Willerslev, 2015). One example of such a monitoring program is the South African Scoring System (SASS), which uses macroinvertebrate populations as indicators to assess stream ecosystem health. SASS, like similar indices, makes identifications based on morphology that are given weight and values, which are then used to assess the system's overall health (Venter & Bezuidenhoudt, 2016).

Biomonitoring, often referred to as biological monitoring, involves the use of living organisms and/or their responses to various stimuli and/or factors to assess environmental conditions and changes (Fierro *et al.*, 2017; Markert *et al.*, 2003; Parmar *et al.*, 2016). An essential element of successful biomonitoring involves the selection of biomonitors and/or bioindicators. Bioindicators refer to species that indicate ecological changes by their mere absence or presence in a specific ecosystem, whereas biomonitors reflect varying degrees or levels of ecological change based on their biochemical reactions (Reynoldson & Metcalfe-Smith, 1992; Scrimgeour & Wicklum, 1996). As research surrounding biomonitoring progressed, studies started to advocate for the use of biomonitors specifically, which may provide greater insight into the sources and presence of specific pollutants in aquatic ecosystems.

Not all species react to environmental stress caused by humans in the same manner. Invertebrates, algae, and macrophytes have been shown to respond at varying rates and provide different insights depending on the specific stimuli or pollutant encountered (Parmar *et al.*, 2016). Plants, in particular, serve as highly sensitive indicators for predicting and recognising environmental stress. For instance, marine

plants provide crucial insights into oceanic ecosystem state and health due to their stationary nature and ability to rapidly reach equilibrium within their surroundings (Jacobs *et al.*, 2020; Tanabe & Toju, 2013). Changes in eukaryotic communities may signal harmful shifts in the ecosystem caused by pollution. Furthermore, changes in population density may signal that the ecosystem is suffering. Population changes may occur as a result of interactions and dynamics in the food chain. Indeed, species abundance and diversity are likely to decrease if food supplies become limited and cannot meet the population demand (Chariton *et al.*, 2015).

Microorganisms are frequently employed as bioindicators to assess freshwater sustainability and cleanliness. They are relatively abundant, and their sampling procedures are simple. When exposed to certain pollutants (e.g., cadmium and benzene), some bacteria are known to produce new proteins known as stressor proteins, which can be exploited as preliminary indicators of environmental changes (Sanders & Martin, 1993). Microorganisms make up a significant portion of oceanic biomatter and drive the bulk nutrient cycling of productivity within marine ecosystems. They have a rapid growth rate and can respond to even low amounts of pollutants, as well as changes in physico-chemical and biological conditions (Ibekwe *et al.*, 2016; Sumampouw & Risjani, 2014; Wang *et al.*, 2016). A study conducted in the Kissimmee River, part of the well-known Everglades of Florida in the United States, demonstrated that algae responded quickly to excess phosphorus, while invertebrates were immune to phosphorus additions until concentrations became high enough to change the macrophyte portion of their ecosystem (Merkley *et al.*, 2004; Sumampouw & Risjani, 2014). Additionally, research by Hou *et al.* (2017) shows that the microbial response after an episodic salinity disturbance indicated the recovery of certain taxa (e.g., *Loktanella*, *Erythrobacter*, and *Rheinheimera* species) and the decline in other taxa, indicating the positive correlation between high salt concentration and the aforementioned taxa. This change is further linked to alterations in the microbial population due to the main influencing factor—salinity. Particularly, if the stress is extreme enough for a prolonged period, all species can display signs of deterioration of environmental health (Guski, 2001; Hou *et al.*, 2017).

Macroinvertebrate morphological recognition is rather sophisticated because of efforts to classify indistinguishable morphological species ~~organisms~~ that have been

described following the application of molecular-based approaches (Boivin-Jahns *et al.*, 1995). Larval phases of aquatic insects often require an intricate approach to classify morphologically. Furthermore, morphology for aquatic macroinvertebrate markers tends to concentrate on adults, which compounds issues with juvenile recognition. Morphological identification takes a lot of time and taxonomic expertise to ensure reliable results (Fierro *et al.*, 2017). The distribution of these benthic macroinvertebrates is often determined by current and stream properties, available substrates, and nomadic appearances (Chariton *et al.*, 2015).

In South Africa, mostly morphological identification methods have been applied to determine ecosystem health and biomonitoring (Harding *et al.*, 2011; Palmer *et al.*, 2004). With the rise of alternate and easier-to-apply methods, the use of complementary or holistic approaches will give a broad-spectrum representation of freshwater ecosystems. DNA-based approaches have shown their utility in solving problems as they rely on genetic loci that apply irrespective of any physical characteristics (Hebert *et al.*, 2003). In the biogeochemical processes, bacterial communities have essential functions within freshwater ecosystems (Wang *et al.*, 2016). These communities break down organic material and distribute minerals to individuals in the ecosystem. Nutrient supply and environmental factors are linked, in turn, to bacterial diversity and species abundance (Ibekwe *et al.*, 2016). Nutrient and environmental changes may significantly impact the composition and abundance of organisms within the ecosystem and, consequently, water quality.

Determining what chemical and physical factors are linked to changes in the communities would specifically show how microorganisms respond to various disruptions, improving our understanding of microbial ecology and its contamination consequences (Jordaan *et al.*, 2019). Bacteria have the largest volume-to-surface ratio of all species, with the only barrier being a membrane (Talaro & Chess, 2018). Consequently, even mild environmental fluctuations are very inductive to bacteria and should, therefore, be excellent early environmental deterioration indicators (Merkley *et al.*, 2004; Risjani & Sumampouw, 2014). Nevertheless, microbial population research can be useful as an alternate indicator of environmental health as microorganisms are omnipresent and have little sampling work needed.

Due to the availability of next-generation sequencing (NGS) technology, the detection of several taxa at the same time is possible by generating a substantially large amount of sequence data in a single run (Shokralla *et al.*, 2012). Additionally, there is limited site disturbance due to the site collection of small quantities of sediments, biofilms, water, etc. (Adams & Greeley, 2000).

Specifically, DNA barcoding, also referred to as the metabarcoding approach, offers an alternative to morphological identification, making it useful for addressing various challenges related to species identification. However, once the initial attempt using DNA to identify a species is made, the requisite information for identifying subsequent samples of the same species is dramatically reduced. Nonetheless, the ability to determine samples to species levels, in comparison to other morphological indicators that frequently end at the familial levels, is a major benefit of using a DNA-based approach (Hajibabaei *et al.*, 2011; Lebonah *et al.*, 2014; Vences *et al.*, 2005). Additionally, identifying taxa using non-culture-based assays will provide biomonitoring with greater sensitivity and more detailed information than coarser identification methods can deliver (Adams & Greeley, 2000). Furthermore, identification through DNA barcoding eliminates the need to cultivate and/or isolate individual specimens in laboratories, allowing samples to be collected passively (Hajibabaei *et al.*, 2011; Taberlet *et al.*, 2012). In comparison, culture-based approaches indicate which living organisms are currently present in a sample. However, metabarcoding/molecular approaches illustrate what is currently present and what was present in the sample by detecting dead cells. This data can be extrapolated to portray episodic contamination events that have already passed (Sumampouw & Risjani., 2014; Taberlet *et al.*, 2012).

Advancements in traditional DNA sequencing technology have resulted in various applications in large-scale and wide-range studies. One significant application is the use of DNA barcodes to develop extensive DNA libraries aimed at identifying unknown specimens by targeting universal genomic regions unique to each species, referred to as operational taxonomic units (OTU) (Barr *et al.*, 2009; Tanabe & Toju, 2013). However, more recent publications advocate for the use of amplicon sequence variants (ASV) as an alternative to the OTU-based approach for analysing microbial communities (Gostel & Kress, 2022). The ASV approach allows for improved

taxonomic resolution by identifying sequences individually rather than clustering them based on a distance threshold, providing a more accurate and refined representation of microbial communities (Schloss, 2021).

The gene region of the cytochrome c oxidase subunit I (COI) can be distinguished between closely related individuals from the Animalia taxa (Vences *et al.*, 2005). Likewise, the 16S ribosomal RNA (16S rRNA) gene is often used in an attempt to identify and recognise bacterial species. For biodiversity analysis at various phylogenetic levels or in certain taxonomic classes, a variety of other marker genes can be used (Klindworth *et al.*, 2013; Mohanty *et al.*, 2015). However, the conventional SANGER sequencing method is limited to sequencing a single DNA strand per run, making it inadequate to process intricate environmental DNA, especially for broad-spectrum survey studies (Slatko *et al.*, 2018).

Using NGS DNA-barcoding and microorganisms as biomonitors in the South African context will lead to a better understanding of the interaction and interdependence between inhabitants, the biodiversity and the ecosystem of the Mooi River catchment (Adams & Greeley, 2000). These methods can help develop strategies for conservation and support of the entire system by increasing our knowledge of the variety of taxa present and their relationships with the environment. This approach further enables assessment of both the current condition of the ecosystem and how it may respond to future changes (Yoccoz, 2012). More specifically, metabarcoding may be particularly useful for those with limited or no taxonomic experience, as well as for biologists working in aquatic microbiology, ecosystem biomonitoring, and surveillance. However, it is essential to gather the necessary data to fully utilise the potential of these techniques.

1.2 Research aims and objectives

The aim of this study was to survey the aquatic bacterial and eukaryotic microorganism communities in the Mooi River system. Specifically, the study aimed to achieve the following objectives:

- To determine the physical and chemical characteristics of water at different sampling sites.
- To detect and measure the concentrations of various metals at these sampling sites.
- To identify bacterial and eukaryotic microbial communities using NGS DNA barcodes 16S rRNA and cytochrome C oxidase subunit I (COI), respectively.
- To perform multivariate analyses to assess the impact of anthropogenic influences on the microbial communities.

CHAPTER 2: LITERATURE REVIEW

2.1 Water quality and scarcity in South Africa

Water is considered a critical resource in maintaining environmental sustainability and facilitating societal and economic development (Pitman, 2011). However, declining water quality and scarcity have become a growing concern globally, posing several risks to human and ecological well-being (Blignaut & Van Heerden, 2009; Hussain *et al.*, 2021). In South Africa specifically, water-related challenges have dramatically increased. In fact, water demand is predicted to surpass supply by 2030, exacerbating this already severe crisis (Prins *et al.*, 2022). South Africa has been classified as a semi-arid and water-stressed country and ranks fifth among sub-Saharan African countries in terms of water scarcity. The country receives an annual rainfall of approximately 456 mm, well below the global average of 860 mm recorded between 1991 and 2020. Out of the total annual rainfall, only a small fraction, less than 9%, flows into rivers, and only about 5% effectively replenishes groundwater aquifers (The World Bank Group, 2021).

South Africa has developed numerous dam systems to alleviate and manage water scarcity by collecting and storing natural water for both urban and rural purposes. However, the water resources within these dams and South African rivers are increasingly under threat. A report to the Water Research Commission (WRC) by Griffin *et al.* (2014) highlighted the deteriorating water quality of several South African water catchments, revealing concerning statistics. Water quality in South Africa is generally affected by challenges related to eutrophication, acid mine drainage and industrial chemicals, high organic compounds, pollution from sewage effluents, salinisation, agrichemicals, and climate changes (Griffen *et al.*, 2014). In addition, regions throughout the country are anticipated to face severe weather events, including droughts, floods, and other climate-related challenges, as a result of increased variability in precipitation and rising average temperatures (Department of Environmental Affairs (DEA), 2018). These conditions could lead to detrimental ecological impacts, such as land degradation, soil erosion, deforestation, persistent droughts, and a decline in biodiversity. The extent of these effects may differ across

the country, influenced by factors like land use, infrastructure, and local climate conditions.

2.2 Mooi River, Wonderfontein Spruit and Loop Spruit

2.2.1 Description

The Mooi River basin is located in the western Gauteng and North West provinces of South Africa (Koekemoer *et al.*, 2021; van der Walt *et al.*, 2013; Wolmarans *et al.*, 2015). The river covers an area of approximately 1800 km²; however, because of widespread dolomitic rock formations, only 44.2% of it contributes considerable runoff (van der Walt *et al.*, 2013). Rainfall over the remaining portion of the basin helps recharge groundwater and supplies the Mooi River and its tributaries through numerous dolomitic pools. The average annual rainfall is reported to be around 683 mm, with the average potential evapotranspiration at 1650 mm (Annandale & Nealer, 2011). The three primary sub-catchments of the Mooi River are Wonderfontein Spruit (Carletonville), Mooi River main (Klerkskraal and Muiskraal), and Loop Spruit. Within the Mooi River watershed, there are four prominent dams: Klerkskraal Dam, Boskop Dam, Klipdrift Dam, and the Potchefstroom Dam (Koekemoer *et al.*, 2021). The confluence of the Wonderfontein Spruit and Loop Spruit, which both feed into the Mooi River, occurs north of the Boskop Dam and 2 km south of Potchefstroom, respectively (van der Walt *et al.*, 2013).

2.2.2 Land and water use

During the 1930s, intensive mining activities began in the Wonderfontein Spruit sub-catchment, primarily in the western reaches of Johannesburg (Krugersdorp) and Carletonville areas, which include the vast drainage area of the Wonderfontein Spruit as well as the higher portions of the Loop Spruit (Barnard *et al.*, 2013; Bomman *et al.*, 2013). This region is home to some of the world's wealthiest gold ridges, with an annual average of 283,399.5 kg of gold mined and extracted (CEIC, 2021; Statistics South Africa, 2015). The Wonderfontein Spruit is affected by mining activities such as discharging effluent and stormwater. Additionally, wastewater from various urban,

commercial, and informal communities finds its way into the watershed through runoff and aquifers (van der Walt *et al.*, 2013; Wolmarans *et al.*, 2015).

The primary land uses in the lower Wonderfontein Spruit catchment are agriculture and intensive mining. Indeed, farmers downstream have experienced adverse effects due to the declining water table and the emergence of sinkholes. Agricultural activities, including crop cultivation and animal husbandry, dominate the northern regions of the Mooi River catchment. Whereas some informal diamond mining occurs between the Klerkskraal and Boskop dams alongside the settlements (Koekemoer *et al.*, 2021; van der Walt *et al.*, 2013). These activities are known to disturb riparian ecosystems, resulting in the siltation of the Mooi River upstream of the Boskop Dam, complicating the end user's water use for irrigation and other purposes. Furthermore, the eastern tributary of the Mooi River, known as Loop Spruit, is primarily used for crop cultivation and animal husbandry, while many goldmines dispose of their effluents in the drainage region between the Loop Spruit and Wonderfontein Spruit (Koekemoer *et al.*, 2021).

Potchefstroom is situated between the Potchefstroom Dam and the junction of the Mooi River and Loop Spruit. This city has a population of around 210,000 people and has relied on the Mooi River as its sole water supply source since 1842 (Annandale & Nealer., 2011; Koekemoer *et al.*, 2021). Apart from a few downstream farms, Potchefstroom is the last user of water flowing from the Mooi River before it flows into the Vaal River approximately 30 km downstream near Skandinawie Drift (Koekemoer *et al.*, 2021).

2.3 Biomonitoring

2.3.1 History and brief overview

Biomonitoring entails investigating biological communities and/or individual species' responses to physical and chemical environmental changes. Biomonitoring can provide both qualitative and quantitative insights by monitoring changes, or by conducting chemical analyses of the substances found in species' composition (Guerrero-Aguilar *et al.*, 2022). Biomonitoring was documented for the first time when

Aristotle observed black muck and red vines, which he described as growing out of "white sludge" in the streams of the city Megara contaminated by residential waste approximately 350 years B.C.E. (Astudillo-García *et al.*, 2019; Markert *et al.*, 2013). The well-known Greek philosopher unknowingly became the pioneer in finding a correlation between anthropogenic influences and environmental occurrences. From his initial description, the following discoveries were made years later (Markert *et al.*, 2013). Oxygen-depleted soil (black rotting muck), a population of filamentous *Beggiatoa* sp. (white sludge) and Oligochaeta sludge worms (red vines) made part of his early description. Aristotle's expertise faded, and the era of water quality indices was placed on hold for another 1800 years. Thus, observations showing a link between the community/dispersal of specific aquatic invertebrate species and differing degrees of water pollution are not a novel approach (Moog *et al.*, 2018).

Kolenati (1848) concluded as early as 1848 that the lack of *Trichoptera* spp. or Caddisfly larvae in a stream might be due to effluents from industries and refineries upstream (Markert *et al.*, 2013; Moog *et al.*, 2018). Another researcher, Hassall (1845), ~~identified and reported~~ the association between organic enrichment, aquatic community and drinking water quality based on bioindicators. Forbes (1887), who established the biological community principle, is credited with pioneering biomonitoring research within lakes and riverine ecosystems in the United States. Essentially, fauna and flora populations in the river were utilised to determine the extension of organic contamination. Around 1900, two German scientists (Kolkwitz & Marsson, 1909) investigated polluted waterways near Berlin and reported vast populations of organisms associated with organic pollution.

Biomonitoring has become increasingly popular in environmental research, particularly for assessing aquatic ecosystems. This rise in popularity is primarily due to its effectiveness in characterising biological diversity, assessing ecological health, evaluating pollution from various sources, and identifying invasive species (Takahashi *et al.*, 2023), particularly through a method known as environmental DNA (eDNA). Ogden (2022) notes that eDNA operates on the principle that individual species leave behind genetic material and traces in their environment. The eDNA process involves identifying, analysing, and characterising these genetic traces through sampling, extracting and sequencing DNA segments, amplifying specific regions using targeted

primers, and performing detailed analyses of combined samples using metabarcoding and metagenomic techniques (*cf.* section 2.6 for a detailed explanation of different biomonitoring approaches).

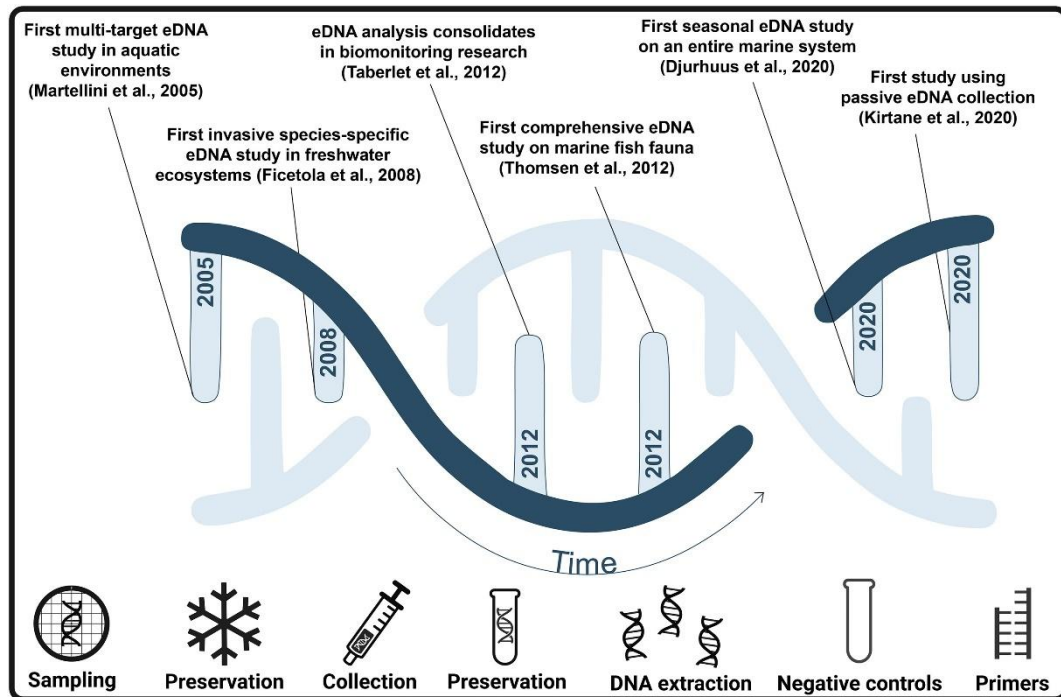


FIGURE 2.1: Timeline of recent studies that advanced eDNA research in aquatic environments in the last 20 years (Source: Takahashi *et al.* (2023))

2.3.2 Bioindicators

In biomonitoring, bioindicators are used to assess ecological conditions and detect periodical changes within ecosystems. Bioindicators can include individual species, communities, and biological processes such as photosynthesis and decomposition rates (Holt & Miller, 2010; Parmar *et al.*, 2016). Furthermore, Gerhardt (2002), highlights that bioindicators are particularly useful for assessing the presence, impact, and potential toxicity of pollutants originating from human activities on the environment and the selected indicators themselves. The effectiveness of bioindicators is dependent on a set of desirable biological and physical characteristics. According to Holt and Miller (2010), specific criteria (outlined in Table 2.1 below) should be considered in the selection of appropriate bioindicators.

TABLE 2.1: Criteria and characteristics of effective bioindicators (adapted from Holt & Miller, 2010):

Indicator sensitivity	Provide measurable responses to stress or disturbances without direct mortality or pollutant accumulation.
	Responses should reflect broader population, community, or ecosystem impacts.
	Responses should be proportional to the degree of contamination or degradation.
Abundance and commonality	Must have sufficient local population density (rare species are less suitable).
	Should be widely distributed in the target area.
	Relatively stable despite moderate environmental or climatic variability.
Scientifically well-studied	Well-documented ecology and life history.
	Clear and stable taxonomic classification.
	Simple and cost-effective to monitor.
Economic and/or commercial relevance	Species already be harvested or studied for other purposes.
	Species hold public interest or awareness.

The selection of bioindicators in aquatic ecosystems is largely dependent on the specific objectives of the assessment. Each type of bioindicator offers distinct advantages and disadvantages.

2.3.2.1 Fish and macroinvertebrate as bioindicators

Fish and macroinvertebrate are widely used as bioindicators in aquatic biomonitoring. The use of fish species specifically is driven by their unique biological traits, such as their body composition and relatively long life span (Zhou *et al.*, 2008). More importantly, as primary and secondary users at various levels of the food chain, fish can provide insights into the aquatic ecosystems' overall trophic conditions (Anderson & Cabana, 2007). Fish are particularly effective for detecting long-term environmental changes as well as habitual states.

Similarly, macroinvertebrates are commonly used in bioassessment. These organisms have relatively long life cycles and are amongst the most vulnerable components of aquatic ecosystems making them ideal for detecting temporal changes caused by various environmental stressors. Furthermore, given that macroinvertebrates are mostly immobile in their aquatic phase, they provide a localised representation of the sample site, allowing for accurate disturbance spatial analyses (Dallas & Day, 2004).

2.3.2.2 Periphytons as bioindicators

Periphytons such as algae, diatoms, and cyanobacteria are frequently used as indicators in both aquatic and terrestrial ecosystems (Bae *et al.*, 2019; Guerrero-Aguilar *et al.*, 2022). These organisms are extremely sensitive to even minor physical, chemical, and/or biological changes and play a significant role in water quality, habitat availability, and the dynamics in food webs (Larned, 2010). Furthermore, their short life cycles and rapid growth rates make them ideal for assessing short-term and abrupt environmental changes (Ouma *et al.*, 2022; Parmar *et al.*, 2016). Periphyton are particularly effective in riverine ecosystems, as they can exist in both mobile (movable) and sedentary (attached) forms (Larned, 2010). Cyanobacteria, a prominent type of periphyton, have also been extensively studied in the assessment of contaminant levels in aquatic ecosystems. Not only are they relatively abundant, but they also exhibit distinct toxicological responses to pollutants as evidenced by changes in their morphology, physiological traits, community composition, and/or molecular processes (Mateo *et al.*, 2015).

2.3.3 Biotic indices

Biotic indices are valuable tools used to assess species' vulnerability or resilience to human impacts by classifying species according to their tolerance levels to pollutants or environmental disturbances (Moog *et al.*, 2018). Changes in the environment can lead to and are often observed through, a decline in sensitive species while more tolerant species flourish and increase (Astudillo-García *et al.*, 2019; Parmar *et al.*, 2016). The application of biotic indices is dependent on the use of one of two different methods to assess the ecological conditions and biodiversity in aquatic environments, specifically, the multi-metric versus the multivariate approach.

The multi-metric approach typically categorises reference sites based on their geographical and physical traits, while the multivariate approach relies on biological communities to determine the anticipated variation in reference conditions (Lücke & Johnson, 2008). While valuable, multi-metric approaches have several limitations, including redundancy among metrics, incomplete use of collected data, and difficulties obtaining up-to-date procedures (Reynoldson *et al.*, 1997). In contrast, the multivariate

approach, considered more suitable for this study, detects ecological impairment by comparing observed and predicted invertebrate community structures based on environmental variables unaffected by human activities or by analysing shifts in community composition relative to reference sites. A significant advantage of the multivariate approach is its independence from prior assumptions about grouping reference sites or comparing test sites to reference groups (Moog *et al.*, 2018; Reynoldson *et al.*, 1997).

2.3.3.1 Biotic indices used in South Africa

South African Scoring System

South African scoring system (SASS) is a biomonitoring tool developed and assessed in South Africa by Chutter (1972; 1994; 1998) to quantify and assess the effect of effluent on invertebrate communities as they are the most prevalent. Today, it is utilised to evaluate ecosystem or riverine health or conditions (Dallas, 1995; Dickens & Graham, 2002). This tool was dedicated to those in the field with limited resources and to make it as user-friendly as possible without compromising the validity of results (Dallas, 1995; Dickens & Graham, 2002). The assay consists of sampling with a standardised net to ensure uniformity across various sampling points or assays, followed by gathering all invertebrates in a collective pile. Taxonomic identification is limited to family level to accommodate a wide range of users irrespective of their skill, knowledge or background. Each taxon is given a score ranging from 1 to 15. Individuals that are highly sensitive and could be influenced by minute environmental deviations are scored the highest and the most resistant to environmental stressors are scored the lowest. Organisms that have been collected are scored accordingly, and a score is tabulated. The SASS score is the sum of all the scores, and the Average Score per Taxon is the SASS score divided by the amount of taxon represented. The more pristine the environment, the higher the score, whereas a polluted environment would demonstrate the inverse (Chutter, 1994; 1998). Challenges associated with this method include that it is habitat-specific and may not be optimised for wetlands or estuaries. Proper training is of the essence as well to ensure minimal variation in sampling procedures (Dickens & Graham, 2002). However, the minimal cost still

makes this one of the most approachable methods to employ (Dickens & Graham, 2002).

Fish-based scoring systems

The Fish Assemblage Integrity Index (FAII) is a monitoring tool that uses fish as bioindicators in riverine ecosystems where the biological segment portrays a homogenous distribution of all fish species. This endeavours to be a representative sample of the total ecosystem (Kadye, 2018; Kleynhans, 1999). However, the FAII approach only accounts for the characteristics of the individuals native to a said watershed and not those introduced, indigenous or exotic, in the ecosystem. These individuals will be noted, and their abundance and distribution will be scored as possible causes for the decline in the FAII score (Souza & Vianna, 2020). The final score is formulated as a ratio to the expected index score for a specific watershed. Assemblages are awarded different scores based on their intolerance index, rate of occurrence, and health status in an uncontaminated environment (Kleynhans, 1999; Souza & Vianna, 2020). This system has received some critique in the way that it is very data intensive and that tolerance values can be somewhat subjective (Kleynhans, 1999).

South African Diatom Index

Diatoms are widespread inhabitants of the benthos of lakes, rivers, streams and estuaries (Bate *et al.*, 2002; Kireta *et al.*, 2012; Lobo *et al.*, 2016). These organisms play a vital role as primary producers in aquatic ecosystems, providing nutrients to the food web associated with them (Melzer & Steinberg, 1983; Pandey, 2023; Vanni, 2002). This tool has been utilised numerous times to assess aquatic ecosystem health and prove diatoms' effectiveness as bioindicators (Bate *et al.*, 2002; Harding *et al.*, 2011; Taylor *et al.*, 2009). However, the method is not portrayed as a rapid, field-based index (Harding *et al.*, 2011). Laboratory and microscopic analysis, alongside taxonomic expertise, are used to create a dataset that will be imported into OMNIDIA 4.1 (Bate *et al.*, 2002; Harding *et al.*, 2011). Since this index is based on the Specific Pollution Sensitivity Index (SPI), the software will generate a score depending on the quality of the environment. Pristine or unmodified environments will receive a score of > 17, moderate quality 15-17, and poor quality will receive a score of < 9 (Harding *et al.*, 2011).

2.3.4 Organism abundance

The most prevalent organisms often dictate the condition of the ecosystem, and any changes or shifts in abundance or composition can be utilised to predict or diagnose anthropogenic perturbations (Lear *et al.*, 2009; Liao *et al.*, 2018; Newton *et al.*, 2013). The inverse of this statement has been confirmed as well, where the total organism abundance can be used to determine the rehabilitation status of an ecosystem (Astudillo-García *et al.*, 2019; Hermans *et al.*, 2017).

2.3.5 Diversity indices

Diversity indices are quantitative tools used in environmental and ecological research to evaluate the structural and functional composition of ecological communities (Dallas & Day, 2004). These indices assess various characteristics related to the species present, such as richness (the total number of species in a given community), evenness and diversity (the level of uniformity or variability in the distribution of individuals among species), dominance (the degree to which particular species prevail over others), and abundance (the total number of individuals within the community). Diversity indices offer important insights into community composition, dynamics, and their reactions to environmental changes, acting as indicators of ecosystem health (Dallas & Day, 2004; Magurran, 2004). This study measured species richness, diversity, dominance, and similarity across sites using several well-known indices, including the Chao1 Index, the Shannon-Wiener Index, and the Bray-Curtis Metric. Each index focuses on specific aspects of species, providing unique perspectives on community composition (*cf.* section 3.7 for detailed description of each index).

2.4 Freshwater ecosystems and microorganisms

2.4.1 Nutrient cycling

Aquatic microbes, including bacteria, archaea, fungi, and microalgae, play a crucial role in nutrient cycling within freshwater ecosystems (Dodds & Whiles, 2020; Pandey, 2023). These microorganisms facilitate various processes that influence the

availability and distribution of key elements like nitrogen, phosphorus, carbon, and sulphur. Their functions are crucial for sustaining ecosystem productivity and stability (Cui *et al.*, 2016; Zwart *et al.*, 2002). A key illustration of the significance of aquatic microbes is the process of nitrogen cycling (Arumugham *et al.*, 2024; Zhang *et al.*, 2024). Nitrogen-fixing bacteria and archaea transform atmospheric nitrogen into forms that can be used by plants and other organisms (Hayatsu *et al.*, 2021). This transformation is vital because atmospheric nitrogen (N_2) is inaccessible to most living beings. Nitrifying bacteria facilitate the oxidation of ammonium (NH_4^+) to nitrate (NO_3^-). This process involves the transformation of ammonium to nitrite (NO_2^-) by ammonia-oxidising bacteria, followed by the oxidation of nitrite to nitrate by nitrite-oxidising bacteria. Denitrifying bacteria reduce nitrate to nitrogen gas (N_2), effectively closing the nitrogen cycle (Bernhard, 2010). These microbial processes play a crucial role in providing a consistent supply of nitrogen, which supports primary production and helps regulate nutrient availability in aquatic systems (Pandey, 2023). Figure 2.2 below visually illustrates the nitrogen cycle.

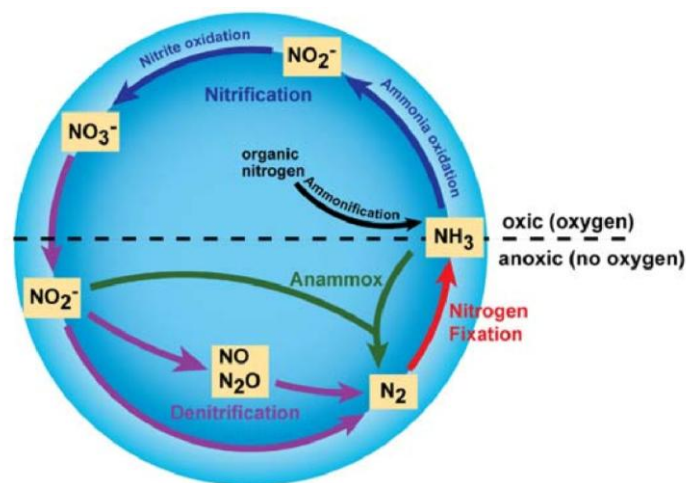


FIGURE 2.2: The nitrogen cycle (Source: Bernhard (2010))

In the carbon cycle, microorganisms transform organic carbon, such as dead plant and animal material, into inorganic carbon compounds that other organisms can utilise (Wu *et al.*, 2024). This decomposition or mineralisation process is primarily carried out by bacteria and fungi, which break down complex organic compounds into simpler forms like carbon dioxide (CO_2) (Melzer & Steinberg, 1983; Yadav *et al.*, 2019). The carbon dioxide produced through microbial decomposition can then be taken up by

primary producers like algae to perform photosynthesis, thereby converting inorganic carbon back into organic forms and sustaining the carbon cycle (Gougoulas *et al.*, 2014; Linz *et al.*, 2018).

The phosphorus cycle is another crucial process that relies on microorganisms (Istvánovics, 2008). Phosphorus often exists in organic forms that primary producers cannot easily access (Wu *et al.*, 2019). Microorganisms, particularly bacteria, play a key role in converting organic phosphorus into inorganic forms such as phosphate (PO_4^{3-}) through a process called mineralisation. This inorganic phosphate is then taken up by plants and algae, supporting their growth and reproduction, ensuring a consistent supply of phosphorus for primary producers and helps in maintaining overall productivity in freshwater ecosystems (Anderson, 2018; Tian *et al.*, 2021). The phosphorus cycle is illustrated in Figure 2.3 below.

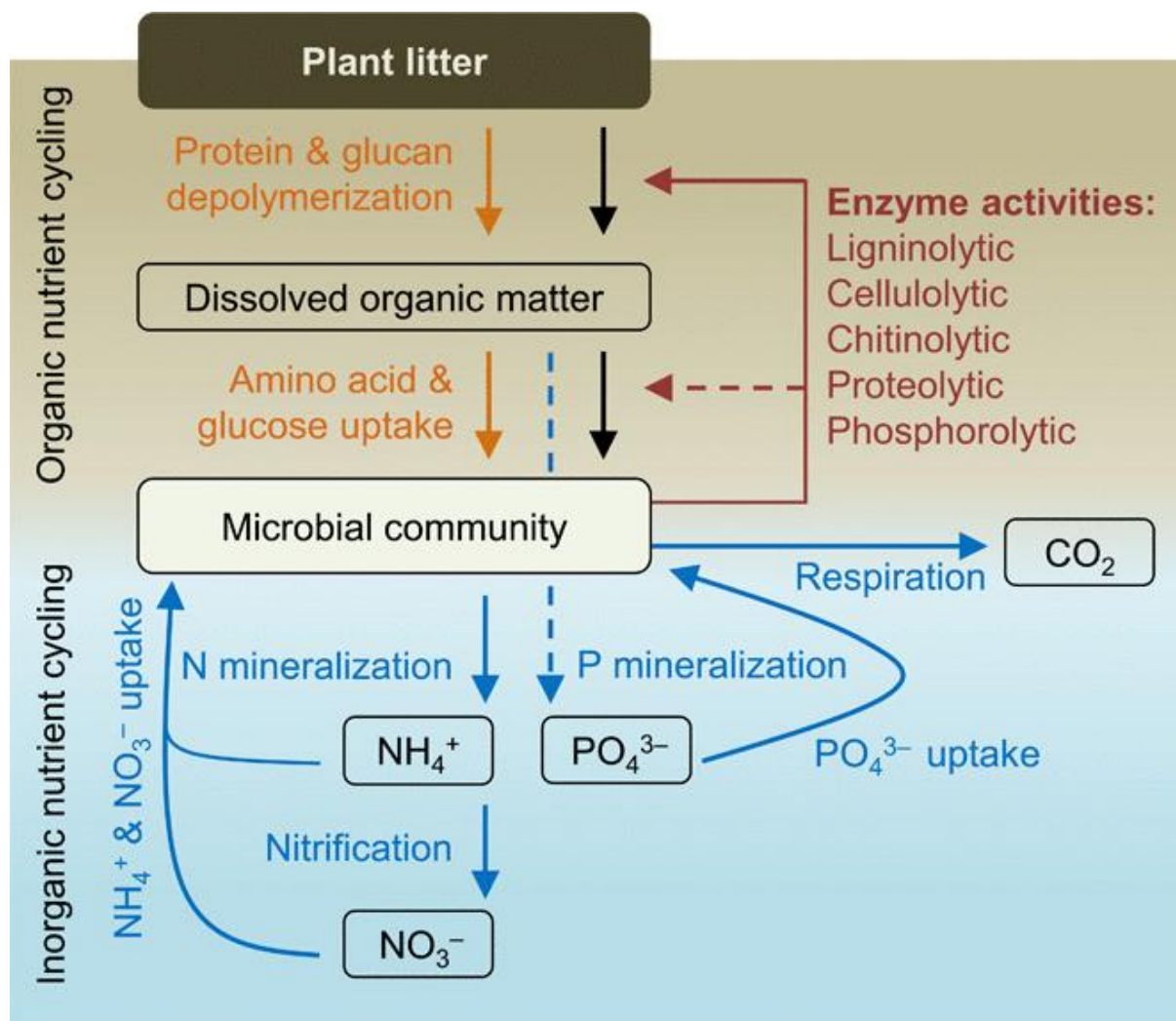


FIGURE 2.3: The phosphorus cycle (Source: Mooshammer, M., *et al.*, (2017))

Furthermore, microbes play a crucial role in regulation of other nutrients, such as sulphur. Organic sulphur-based compounds are processed through a sulphur cycle to become inorganic in forms such as sulphate (SO_4^{2-}), which is used by other species (Holmer & Storkholm, 2001). Microorganisms assist in cycling micronutrients like iron and other trace elements in addition to macronutrients (Zak *et al.*, 2021). Microbes employ enzymatic activity to degrade and transform organic and inorganic compounds to maintain the trophic balance required for the growth and survival of organisms in freshwater habitats "The bacteria of the sulphur cycle", 1982).

Aquatic microorganisms influence nutrient cycling on a broad spectrum and significantly impact ecosystems' stability and productivity (Dodds Whiles, 2020; Pandey, 2023). Microbes enable effective nutrient cycling, guaranteeing a steady supply of necessary components and fostering primary producers' development and output (Dodds Whiles, 2020). This is also useful to achieve higher trophic levels and improve overall environmental health (Yadav *et al.*, 2019). Microbial activity further contributes to the maintenance of nutrient homeostasis and to the prevention of nutrient excesses which are detrimental for water quality, for example by intensifying eutrophication (Wagenaar & Barnhoorn, 2018).

Overall, microbial nutrient cycling is critical to the processes and maintenance of freshwater ecosystems (Melzer & Steinberg, 1983). By decomposing organic matter, fixing nitrogen, and converting nutrients into forms usable by other organisms, microbes ensure a continuous supply of essential nutrients. This aids in the growth and production of primary producers and maintains the overall health and diversity of freshwater ecosystems (Pandey, 2023). The profound influence of microbial processes on nutrient cycling underscores the need for continued research and consideration of microbial activities in ecosystem management and conservation strategies (Pandey, 2023).

2.4.2 Anthropogenic influences

Over the past few decades, disruptions in natural nitrogen and phosphorus cycles due to human activity have threatened freshwater ecosystems worldwide (De Villiers & Thiart, 2007; Chung *et al.*, 2020). The increase in human activity (i.e., anthropogenic) results from a combination of population growth and land use change for agricultural,

urban, mining, and industrial activities (Rasool *et al.*, 2023). These activities have a significant effect on water quality, water supply, and ecosystem health by increasing surface runoff, reducing groundwater flow, and introducing contaminants into the riverine systems (Butt *et al.*, 2015). Furthermore, these activities generate and introduce excess nutrients in ecosystems (Dallas & Day, 2004), which is reflected in the observed changes in concentrations of several water quality parameters.

2.4.2.1 Agriculture

In South Africa, the agricultural sector is a key driver of economic growth and sustainability, as it supports food security and contributes to export revenues. However, agricultural activities have a significant impact on aquatic ecosystem health, contributing to the influx of nutrients, pesticides, salts, organic matter, pathogens, and sediment (Collins *et al.*, 2011; Mateo-Sagasta *et al.*, 2017; Naura *et al.*, 2016). Rivers and streams frequently serve as channels for agricultural runoff (Nolan *et al.*, 2024), often resulting in elevated phosphorus levels (a crucial constituent in commercial fertilisers and some pesticides) and nitrogen in surface waters. While nitrogen and phosphorus are essential to the aquatic ecosystem health, a severe increase in nutrient loading can lead to several negative consequences, one of which is cultural eutrophication (Smith, 2003). Furthermore, agricultural farming's diffuse and point source pollution significantly contributes to sediment and nitrogenous nutrient loading (De Villiers & Thiar, 2007). Agricultural runoff has also been shown to adversely affect microbial communities and increase the prevalence of antibiotic-resistant genes in aquatic ecosystems (Nolan *et al.*, 2024), amplifying ecological and health risks.

2.4.2.2 Mining and industrial operations

Mining and industrial processes also directly contribute to the decline of the biotic integrity and integrity of the aquatic ecosystems (Feng *et al.*, 2021). In South Africa, gold and coal mining specifically have been directly associated with extensive water contamination and the discharge of mining wastewater into rivers, which poses a severe threat to aquatic ecosystems (Griffen *et al.*, 2014; Erasmus *et al.*, 2020). Mining activities may also demand significant water volumes, leading to water discharge of often polluted wastewater in riverine networks (Erasmus *et al.*, 2020). Such discharges

often contain elevated concentrations of heavy metals, including lead, aluminium, copper, and iron, which are toxic to aquatic organisms, even at low concentrations (Nordstrom, 2011; Hadzi *et al.*, 2023). Acid mine drainage (AMD) is also one of the other key contaminants for gold and coal mining in South Africa, impacting both surface and groundwater (Department of Water and Sanitation (DWS), 2016). The primary cause of AMD is the oxidation of sulphide minerals in water. Although AMD reactions can happen in inert conditions, some microorganism groups, especially acidophilic bacteria, which are able to grow in acidic environments, promote very fast dissolution of toxic metal ions. The effects of AMD in aquatic ecosystems can be observed through lower pH, increased salinity, and elevated levels of several toxic metals, particularly iron (DWS, 2016).

Furthermore, industrial operations, such as paper mills, pharmaceutical factories, textile facilities, and similar plants, significantly contribute to water quality degradation. However, the extent of pollution from these operations varies depending on the specific industrial processes used. The primary contaminants from industrial operations observed in South Africa include chemicals such as phosphates, chlorine, and nitrates; heavy metals such as arsenic, lead, and chromium; dyes; high levels of organic compounds; and sewage sludge (Griffen *et al.*, 2014).

2.4.2.3 Urbanisation

Urbanisation, largely driven by population growth, plays a significant role in the degradation of riverine ecosystems through increased diffuse and point-source pollution. Major sources of such pollution include ~~include~~ stormwater infrastructure, dysfunctional septic tanks, and poorly maintained wastewater treatment and distribution networks, all which contribute to nutrient enrichment observed in aquatic ecosystems (Carey *et al.*, 2012). Urban runoff is further associated with a wide range of adverse effects on water catchments, including physical impacts such as erosion and sedimentation; and physico-chemical effects such as rising temperatures, nutrient enrichment, toxicity, and oxygen depletion (Dallas & Day, 2004). Griffen *et al.* (2014) also highlights that improperly managed sewage effluents flowing into water bodies further exacerbate water contamination by increasing salinity and nutrient levels, reducing oxygen availability, and increasing the prevalence of pathogens.

Consequently, such pathogenic organisms pose detrimental effects for both humans as well as aquatic organisms (Corcoran *et al.*, 2010).

2.4.3 Trophic status of ecosystems

Aquatic ecosystems are often classified based on their 'trophic state', which reflects the level of biotic productivity and availability of nutrients in the ecosystem, driven by nitrogen and phosphorus input (Concepcion *et al.*, 2020; Dodds & Cole, 2007). While nitrogen and phosphorus are naturally occurring and necessary chemical constituents of rivers, they have an adverse effect when present in high concentrations, resulting in eutrophication (De Villiers & Thiart, 2007; Malan & Day, 2005). Eutrophication is defined as the excessive nutrient enrichment of water, which then leads to problems concerning the growth of algae and cyanobacteria (DWAF, 2002; Griffin *et al.*, 2014).

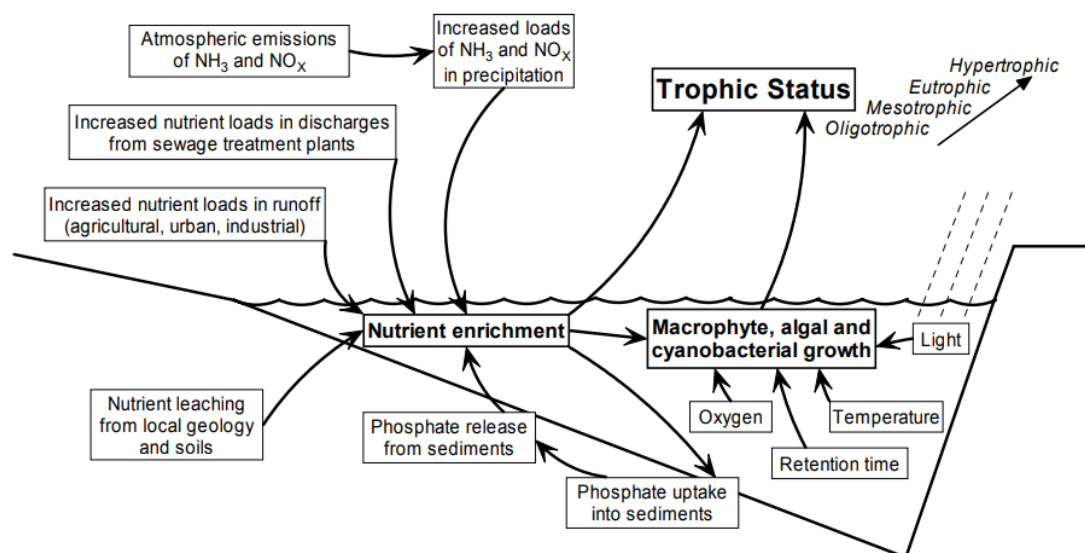


FIGURE 2.4: Causes and factors driving eutrophication (Source: DWAF(2002))

In South Africa, eutrophication has both direct and indirect negative ecological, recreational, human, and economic consequences. These include deteriorating water quality, biodiversity loss, fish and invertebrate mortality, livestock implications, and human health effects (DWAF, 2002).

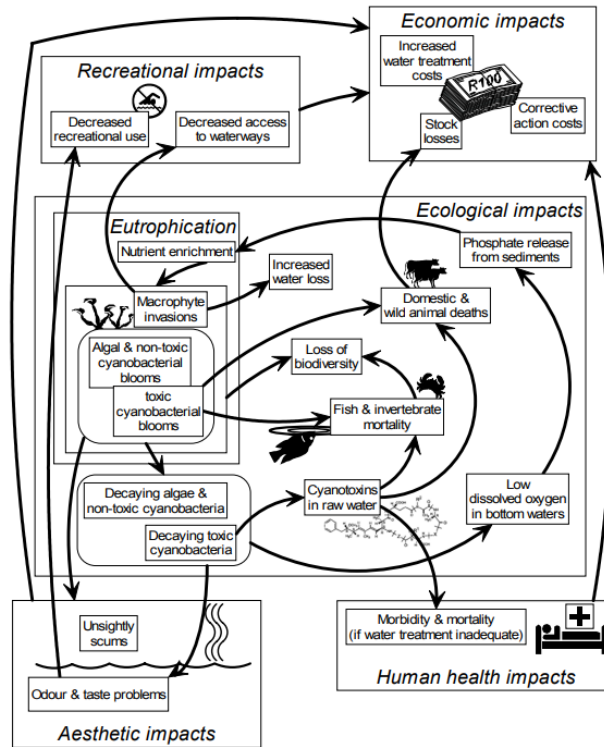


FIGURE 2.5: Potential adverse impacts associated with eutrophication (Source: DWAF (2002))

Evaluating trophic states in aquatic ecosystems is essential to understanding their biogeochemical features, food chain dynamics, and water quality (Smith, 2003). Furthermore, establishing a reference distribution for trophic states within a specific ecosystem creates a basis for assessing biotic integrity, which serves as a benchmark for evaluating the impact of anthropogenic activities and setting objectives for initiatives (Dodds & Cole, 2006). In South Africa, guidelines recommend determining the trophic status of ecosystems by measuring total phosphorus and chlorophyll a concentrations present (DWAF, 2002). Since eutrophication was not the primary variable investigated in this study, only phosphorus concentrations were measured. The presence of phosphorus in aquatic ecosystems has been strongly linked to the growth of undesirable algae and is also recognised as the nutrient that can be managed most effectively (Ginkel *et al.*, 2001). Following the recommendations made by the South African Water Quality Guidelines for aquatic ecosystems (DWAF, 1996), the following phosphorus concentration levels were considered for the classification of trophic states and their associated effects:

TABLE 2.2: Trophic status symptoms associated with selected ranges of inorganic phosphorus concentrations (Adapted from source: (DWAF, 1996))

Average summer inorganic phosphorus concentration ($\mu\text{g/L}$)	Trophic state effects
< 5 $\mu\text{g/L}$	Oligotrophic conditions: Indicates moderate levels of species diversity; low productivity systems with rapid nutrient cycling; and no nuisance growth of aquatic plants or blue-green algae.
5 – 25 $\mu\text{g/L}$	Mesotrophic conditions: Indicates high levels of species diversity; usually productive systems; nuisance growth of aquatic plants and blooms of blue-green algae; and algal blooms seldom toxic.
25 – 250 $\mu\text{g/L}$	Eutrophic conditions: Indicates low levels of species diversity; usually highly productive systems, with nuisance growth of aquatic plants and blooms of blue-green algae; algal blooms may include species toxic to man, livestock and wildlife.
> 250 $\mu\text{g/L}$	Hypertrophic conditions: Indicates very low levels of species diversity; very highly productive systems; and nuisance growth of aquatic plants and blooms of blue-green algae, often including species toxic to man, livestock and wildlife.

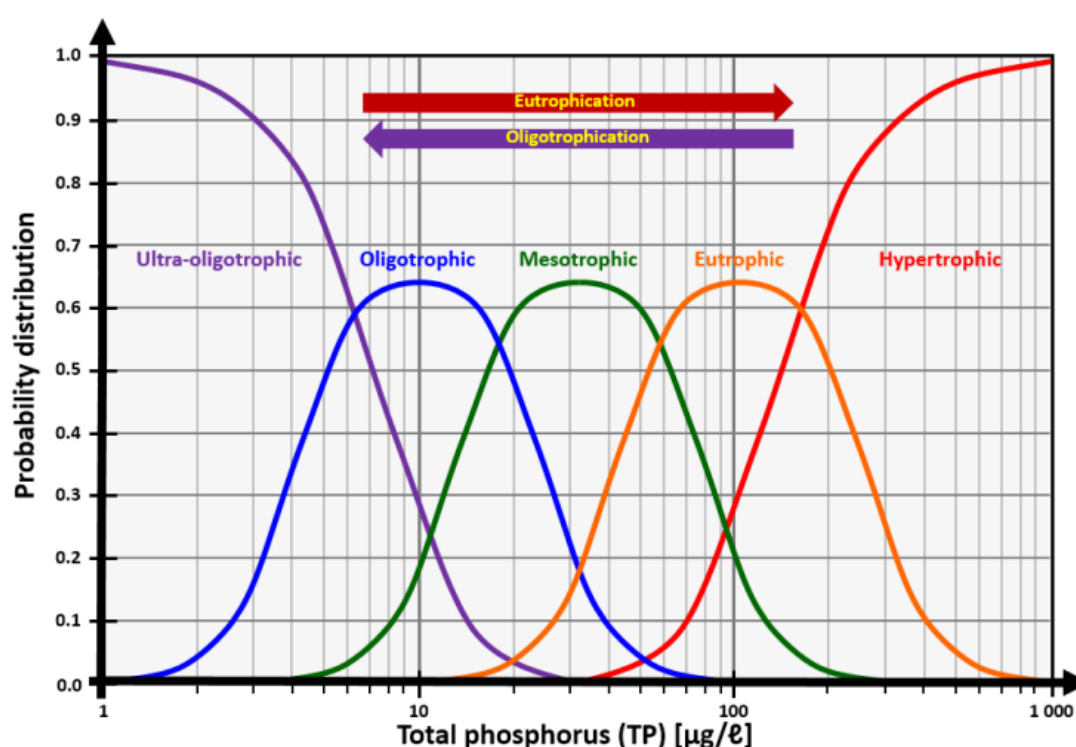


FIGURE 2.6: The primary production continuum and associated eutrophic states (Source: (DWS, 2023))

2.5 Physico-chemical parameters

Water quality and the condition of its associated ecosystems are often determined by analysing various physical and chemical constituents and their observed concentrations. Indeed, physico-chemical monitoring is a well-established practice that has traditionally been widely applied in South Africa (Dallas & Day, 2004; Carstens & Sci, 2012; Jordaan & Bezuidenhout, 2016). Changes in physico-chemical properties provide valuable insights into water quality and its potential effects on ecosystem functioning and biodiversity (Dallas & Day, 2004; Mishra *et al.*, 2023). For the present study, the following physical and chemical parameters were included: pH, temperature, total dissolved solids (TDS), electrical conductivity (EC), salinity, turbidity, and heavy metals. The limits, guidelines and expected effects used as a reference in this study can be found in: "South African Water Quality Guidelines for Aquatic Ecosystems" (DWAF, 1996).

2.5.1 pH

In situ, pH measurement is a useful parameter that not only reflects the state of the acid-base balance of the water but also an index of the contamination level. Acidity refers to water's ability to neutralise a base, while alkalinity refers to the extent of water's ability to neutralise an acid. Specifically, metal solubility, availability, and toxicity are known to be affected by water acidity (Carstens & Sci, 2012). Factors such as respiratory activity, exposure to air, temperature, and disadvantages of sewage, etc., result in variations in observed pH levels. Low pH levels in summer likely result from the production of carbonic acid accompanied by the efflux of an increased amount of free carbon dioxide. This is mainly due to microbial respiration under high temperatures and a rising decomposition rate caused by the release of organic residues deposited in the environment (Raza *et al.*, 2023). Furthermore, anthropogenic impacts such as atmospheric pollution (gases produced by industrial activities), production of acid rain, AMD, and urban and agricultural sewage are all implicated in the acidification of aquatic ecosystems (Carpenter *et al.*, 2011; DWAF, 1996).

2.5.2 Temperature

Temperature, which represents the heat energy present in a specific amount of water, exhibits a significant correlation with atmospheric temperature as a result of solar radiation warming the water in summer and cold air cooling it in winter (Hussain *et al.*, 2021). The thermal condition of water is of utmost importance in influencing the chemical and biological reactions that take place in a water system. As a result, the presence of plankton and other organisms is heavily reliant on the temperature of surface waters, which regulates multiple biological processes (Oluyemi *et al.*, 2010). The growth, reproduction, feeding, and metabolic processes influence other physical and chemical properties, either directly or indirectly. Geographical position and weather patterns typically dictate the atmospheric and water temperature (Hussain *et al.*, 2021; Lawson, 2011).

2.5.3 Total dissolved solids

Total dissolved solids (TDS) refer to all soluble material present in a water sample including organic salts, organic matter, and various other dissolved elements (Environmental Protection Agency (EPA), 1986). These elements may be organic, inorganic, ionised, or unionised (Dallas & Day, 2004). TDS concentrations are directly correlated with water's electrical conductivity (EC) (DAWF, 1996) and are often used as substitute physico-chemical parameters in measurement. For most catchments, the average conversion factor is 6.6, calculated as follows: $\text{TDS (mg/L)} = \text{EC (mS/m)} \times 6.6$ (Dallas & Day, 2004). High TDS concentrations in water bodies often result in decreased water clarity, which in turn reduces photosynthesis and increases water temperatures. Changes in TDS concentrations in natural waterways are frequently caused by effluent from production plants and changes in the water balance—by restricting inflow and increasing water consumption, amongst others. Furthermore, TDS concentrations may create a toxic environment by rapidly increasing salinity, altering water's ionic composition, and affecting the toxicity of specific ions (Weber-Scan & Duffy, 2007).

2.5.4 Electrical conductivity

Electrical conductivity (EC) measures the ability of water to conduct an electrical charge (DAWF, 1996). This is mainly due to the presence of soluble or positive ions in water including magnesium. This is primarily due to the presence of soluble cations in water, including magnesium (Mg^{2+}), sodium (Na^+), and calcium (Ca^{2+}), as well as anions such as chloride (Cl^-), phosphate (PO_4^{3-}), sulphate (SO_4^{2-}), and nitrate (NO_3^-). Ions, whether positive or negative, carry an electrical charge and consequently affect the EC of water. The higher the conductivity observed, the more ions present in the solution (Dallas & Day, 2004). EC is widely adopted in aquatic assessment as it serves as an effective indicator of contaminants present affecting water quality.

2.5.5 Salinity

Salinity is a measure of salt concentrations in water or ecosystems. While not a direct measure of the individual ions present, Griffen *et al.* (2014) assert that saline water's ionic composition influences its impact on end users. Salt is frequently introduced into water bodies and/or ecosystems as a result of human activity. In South Africa, anthropogenic water salinisation is frequently caused by industrial and mining runoff, insufficient sewage treatment plants, and unregulated irrigation practices (Grewar, 2019). Salinity can also be caused by weather conditions at the water catchment, such as precipitation containing dissolved salts from evaporated seawater (Cañedo-Argüelles *et al.*, 2012). Elevated salt concentrations affect a variety of end users, including agriculture, industrial operations, and the environment (Griffen *et al.*, 2014). Increased salinity levels have a specific impact on aquatic organisms, including decreased physiological adaptation rates, vulnerability in juvenile stages, and antagonistic or synergistic interactions with other toxicants (Dallas & Day, 2004).

2.5.6 Turbidity

Turbidity is a measure of water's ability to scatter light, indicating the presence of suspended solids in water (DWAf, 2002). Turbidity is easily observed as it leads to immediate decreased water clarity. As a result, increased turbidity in water bodies is known to decrease sunlight penetration and oxygen bioavailability (DWAf 2002;

Gupta *et al.*, 2017), as well as disrupt biological processes (e.g., photosynthesis) resulting in decreased primary production and reduced food availability for aquatic organisms higher up the food chain (Dallas & Day, 2004).

2.5.7 Heavy and trace metals

Dallas and Day (2004) define heavy metals as metals that have atomic masses exceeding that of calcium (40.078). In aquatic ecosystems, elevated levels of heavy metals such as arsenic (As), lead (Pb), copper (Cu), mercury (Hg), chromium (Cr), and zinc (Zn) are frequently reported (Núñez *et al.*, 2011). While some metals such as magnesium (Mg), manganese (Mn), copper (Cu), zinc (Zn), and iron (Fe) are essential in biological processes at trace concentrations, they can cause toxicity when present at levels above permissible limits (Dallas & Day, 2004). However, heavy metals such as arsenic (As), lead (Pb), and mercury (Hg) can be extremely toxic to aquatic organisms even at low concentrations (Hussain *et al.*, 2020). Aquatic ecosystems in South Africa are particularly under threat as a result of increased pollution from extensive gold, coal, and uranium mines. AMD from these sources frequently contains extremely high levels of heavy metals and metalloids, resulting in elevated concentrations in mining-dominated river basins (Ouma *et al.*, 2022). AMD is also known to significantly increase acidity (decreasing pH) in water bodies (Kaur *et al.*, 2018) which, in turn, increases heavy metal concentrations (Ettler *et al.*, 2006).

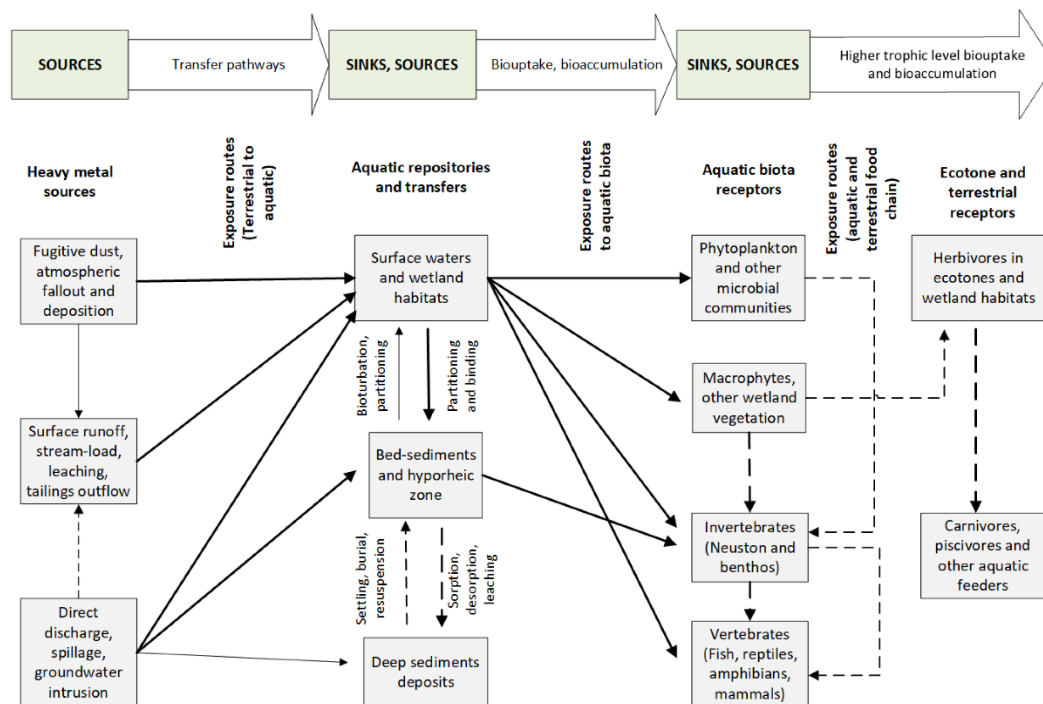


FIGURE 2.7: Heavy metal pollution in surface and groundwater systems within mining landscapes, along with the associated exposure pathways affecting aquatic biota (Source: (Ouma *et al.*, 2022)).

The effect of heavy metals in aquatic ecosystems has been extensively studied and has detrimental effects to both human and aquatic health. While each metal holds distinct consequences, some of the effects include oxygen depletion in water bodies, decreased species diversity, reduced immune responses, increased rates of enzyme inhibition, and abnormal cell division (Dallas & Day, 2004).

2.6 DNA Barcoding and eDNA

2.6.1 Barcoding and metabarcoding

Barcoding is a technique used for identifying governed species, such as invasive and endangered species, as well as detecting the purity of biological goods and consumer products. Barcoding, as a biodiversity discovery method, aids in the identification of species that are possibly new to science, particularly undescribed and taxonomically unknown (Kress & Erickson, 2012). DNA barcodes may also be utilised to answer core issues in ecology, evolution, and conservation projects. Multi-species analysis could

describe how organisms are organised in groups and which interactions or relationships occur in previously undiscovered ecosystems, for example, soils, streams, and lakes. More specifically, multi-species analyses can also be used to pinpoint which ecosystems are most rich in evolutionary diversity and, therefore, need to be protected (Kress *et al.*, 2015). Microbial ecology research has shifted from observational phylogenetic studies of uncultured species to complex experimental designs to characterise population taxonomic transitions (Kozich *et al.*, 2013). Moreover, biodiversity surveys are useful for developing strategies for protecting endangered species, analysing the consequences of civil projects, and investigating the ecotoxicological effects of engineered substances (Tanabe & Toju, 2013).

DNA-metabarcoding, also known as high-throughput amplicon sequencing (HTS), is another powerful technique emerging in biomonitoring today. Metabarcoding is useful for identifying both individual species and communities (Guerrero-Aguilar *et al.*, 2022). According to Taberlet *et al.* (2021), HTS extracts or sequences DNA barcodes from a pooled sample (e.g., water), representing DNA isolated from multiple species or other taxa collected in bulk. Furthermore, targeted sequencing is performed on libraries enriched with DNA barcode amplicons (Ji *et al.*, 2013).

2.6.2 Taxonomic identification

Taxonomic identification of acquired specimen samples is a crucial hindrance in biodiversity surveys. Taxonomic identification through DNA sequences, referred to as DNA barcoding or molecular identification, is promising since it allows for the use of standardised, high-throughput taxonomic identification techniques (Tanabe & Toju, 2013). Furthermore, discarded extracellular DNA in surroundings can be amplified via polymerase chain reaction (PCR), and sequenced. These methods and analysis of such "environmental DNA" of vast amounts of ecosystems create the opportunity for fast microbial diversity assays (Tanabe & Toju, 2013). As a result of recent technological advancements and the decreasing cost of DNA sequencing have expanded the prospect of using DNA barcoding in ecological investigations. Creating a theoretically sound framework and downstream analysis for decrypting raw DNA sequencing data into taxonomic information is critical (Kozich *et al.*, 2013; Tanabe & Toju, 2013). Due to the recent introduction of high-throughput sequencing technology,

genomic approaches provide a viable manner for biological systems analysis. DNA barcoding and metabarcoding offer the ability to improve the speed, accuracy, and precision of species identification while lowering the expense of bioassays (Aylagas *et al.*, 2014).

The taxonomic obstacle that exists today for many bioinformaticians, scientists, and environmentalists, namely finding the right identification for any biological material in a sample within a timely, repeatable, and reliable manner, is a fact that we must all recognise (Kress & Erickson, 2012). DNA barcodes, which are inspired by commercial product labels, are made up of a standardised short sequence of DNA between 400 and 800 base pairs in length that, in principle, can be easily extracted, separated and used to identify any living organism (Kress & Erickson, 2012). The study is expanded to a population of individuals (of various species) rather than a single individual in a metabarcoding method. Sequences must be compared to a reference library containing the connection between barcodes and taxonomy categorisation in both circumstances (Aylagas *et al.*, 2014).

2.6.3 Selection of barcodes

The primary use of DNA barcodes has been for species identification since its inception (Kress & Erickson, 2012; Kress *et al.*, 2015). A gene region must meet three characteristics in order to be used as a DNA barcode: (1) have considerable species-level genetic diversity and differentiation, (2) have preserved regions for generating universal PCR primers for the broadest taxonomic use, and (3) be of sufficient sequence length to permit existing DNA extraction and sequencing technologies (Kozich *et al.*, 2013). A fourth requirement for a successful DNA barcode is sequence quality, as research based on untrustworthy data is inherently untrustworthy (Kozich *et al.*, 2013; Kress & Erickson, 2012).

2.6.4 16S rRNA and COI

DNA fragments, like the 16S rRNA, which is a ribosomal RNA that necessitates protein synthesis in prokaryotic and archaeal organisms, matched these criteria. Alongside 16S, a 658-bp portion of the mitochondrial cytochrome c oxidase subunit I gene is the most often used barcode for animals referred to as COI. Compared to nuclear rRNA genes, this gene has a quicker substitution rate, making it ideal for species differentiation (Mohanty *et al.*, 2013). Accordingly, these barcodes were quickly regarded as feasible, standardised species-level barcodes for numerous species (Kress & Erickson, 2012). A reference library is also required to fully assess the samples' biodiversity (Aylagas *et al.*, 2014). This method is based on two successive PCR steps with different primer combinations (Gloor *et al.*, 2010; Lanzén *et al.*, 2016; Stoeck *et al.*, 2018). The COI primer has been used in numerous studies like Aylagas *et al.* (2014) and Mohanty *et al.* (2013); this primer does include taxa from the *Animalia* kingdom, but lower order *Eukaryotes* can be filtered from the higher orders. This again substantiates the customisability of this primer in this study (Gloor *et al.*, 2010; Lanzén *et al.*, 2016; Stoeck *et al.*, 2018). In the first PCR step, two primers containing an Illumina sequencing primer, an index sequence and a gene-specific primer are used. The Illumina adaptor and sequencing primer sequences are added to the second PCR step. Paired-end sequencing is performed using the built-in Illumina sequence primers. However, this method is restricted since it necessitates two rounds of PCR, which increases the danger of frequent artefacts with large numbers of PCR rounds (Kozich *et al.*, 2013).

The efficacy and accuracy of metabarcoding for taxonomic identification are mainly dependent on (1) the amplified barcode, which needs to be taxonomically relevant and species-specific; and (2) the premise that primers used for amplification should have a high affinity for the studied population of organisms. Primers may be specific for a single genus if the objective is to characterise the variety of species within a given taxonomic group. Primers may need to be broader if the objective is to acquire a complete study of samples comprising species from many phyla. Therefore, to assess the entire composition of an ecosystem, a wide range as well as group-specific primers might be utilised if necessary.

Given the difficulty of typical taxonomic identification based on the appearance of benthic fungi, marine/freshwater plankton, and prokaryotes, DNA barcoding offers an alternate or complementary study strategy for the characterisation and identification of these microorganisms (Tanabe & Toju, 2013). Implementing new methods for producing and analysing DNA barcode sequences will be the most significant breakthrough in DNA barcoding in the near future. Several research and publications have examined the intersection of DNA barcoding and NGS, particularly in the context of environmental sampling (Shokralla *et al.*, 2012; Baird & Hajibabei, 2012; Hajibabei *et al.*, 2011). NGS technologies are not often used to gather and build DNA barcode reference libraries for a specific collection of species. Rather, NGS will allow for collecting all representative DNA fragments found in a complex combination of species, followed by assigning those sequences to a reference DNA database. It also has the potential to accelerate the discovery of thousands more species that have yet to be identified (Kress & Erickson, 2012). These complex combinations might include environmental samples of water or soil used to determine biodiversity. The use of DNA barcoding to determine individual species in a sample is known as 'DNA metabarcoding'. As in other disciplines, the use of NGS for DNA barcoding will result in a massive increase in accessible sequence data, necessitating the establishment of innovative analysis software and new information storage systems (Kress *et al.*, 2015).

In several species, DNA barcode markers were not as efficient as first thought. Plants, which have lower rates of nucleotide alteration in mitochondrial DNA than mammals, were particularly difficult to work with during the early phases of generating DNA barcodes. Furthermore, the risk that DNA barcodes would produce inaccurate or erroneous findings due to the difficulties of ancestral polymorphisms, hybridisation, and/or introgression surely applies to all studied organisms (Kress *et al.*, 2015). Although there is no comparison evidence across phyla suggesting that all molecular and genetic variation and changes occur at corresponding conjunctures, the difference in success of DNA barcodes between taxa reveals that evolutionary rates and speciation events are not uniform. The characterisation of studied organisms could be continuous and only used for DNA barcodes. With the advent of next-generation sequencing in many molecular research projects, e.g., metagenomic applications, applications are already being developed to solve both practical and fundamental

biological concerns. Even though DNA barcodes are not universally successful for definitive recognition across the whole Tree of Life, ecologists, evolutionary biologists, and conservationists already use them in their respective disciplines (Kress *et al.*, 2015). Previously the most notable limitation of the Illumina platforms was their inability to sequence samples with minimal genetic diversity, as 16S rRNA gene amplicons tend to struggle with. Adding a control library of genomic DNA in the reaction mixture has been normal practice to artificially boost genetic diversity. The quantity of DNA placed into the flow cell can also impact data quality since it affects cluster density and the capacity of image analysis tools to discern between clusters.

CHAPTER 3: METHODOLOGY

3.1 Sampling

3.1.1 Sampling sites

13 sampling sites along the Mooi River catchment, Loop Spruit and Wonderfontein Spruit were strategically chosen for this study to capture a range of anthropogenic influences, including potential pollution from wastewater treatment plants, agricultural activities, and mining operations. The sites were identified based on their proximity to potential sources of pollution and ease of access. In particular, the selection prioritised locations along public roads to avoid trespassing and to ensure safe, efficient, and legal sampling.

At each site, water samples were collected bimonthly in triplicate from July 2021 to May 2022, using sterile one-litre Schott Duran glass bottles, rinsed with 1% hypochlorite and autoclaved to ensure sample integrity for laboratory analysis. The six sampling points spread across the year compensated for any environmental variations in terms of seasons, wet and dry periods. Prior to sampling, the bottles were labelled, and gloves were worn at all times when handling the bottles. Two water collection techniques were employed, depending on the physical setting of each sampling site. At sites where samples could be collected close to the riverbank, the direct sampling technique was used. Conversely, the dip sampling technique was employed at sites where direct access to the surface water systems was limited, which involved lowering the one-litre bottles on ropes to facilitate efficient sampling. According to Gupta *et al.* (2017), rivers with an approximate depth of 1-2m and flow rate of 5-10km/h would not be affected by stratification, and homogenous flow can be expected. Thus, samples were taken upstream from the sampler just beneath the surface of the water. After collection, the samples were immediately placed on ice and transported to North-West University's (NWU) microbiology laboratories for processing, where they were analysed within 6 hours of collection. Upon arrival in the laboratory, the water samples were filtered (100 ml) with 47mm 0.45µm filters from Pall Corporation supplied by Separations (PTY) Ltd, properly concealed, and stored at -85°C for eDNA extraction

the next morning. Table 3.1 below provides an overview of the sampling sites, including their GPS coordinates and associated abbreviations (hereafter used to refer to specific sampling sites in the results and discussion sections).

TABLE 3.1: Overview of sampling sites, abbreviations, and site-specific coordinates

Sampling site		Abbreviation	GPS coordinates
Mooi River Catchment			
1	Klerkskraal Dam	KD/KKD*	26°15'09.6"S, 27°09'34.7"E
2	Muiskraal Bridge	MB	26°26'42.3"S, 27°07'06.0"E
3	Around the World Bridge	AtW	26°30'52.6"S, 27°07'28.6"E
4	Hartspad Bridge	HB	26°34'18.9"S, 27°06'12.2"E
5	Thabo Mbeki Bridge	TMB	26°41'04.6"S, 27°06'01.3"E
6	Trim Park Bridge	TPB	26°42'27.2"S, 27°06'20.1"E
7	Golf Course Bridge	GC	26°43'29.8"S, 27°06'21.3"E
8	Pre-Wastewater Treatment Plant Bridge	WWTP	26°45'11.6"S, 27°05'59.8"E
9	Skandinawie Drift Bridge	SB	26°52'49.7" S, 26°57'50.5" E
Loop Spruit			
10	Loop Spruit Begin	LSB	26°25'25.2"S, 27°31'44.7"E
11	Loop Spruit End	LSE	26°39'53.5"S, 27°11'48.0"E
Wonderfontein Spruit			
12	Wonderfontein Spruit Begin	WSB	26°18'57.4"S, 27°22'56.9"E
13	Wonderfontein Spruit End	WSE	26°26'11.3"S, 27°09'05.0"E

*Note: Klerkskraal Dam has two abbreviations namely KD and KKD, as data plots in some of the analyses used the abbreviation KKD as opposed to KD.

3.1.2 Site descriptions

Tables 3.2 to 3.4 below present a concise description of each individual sampling site, including GPS coordinates, site-specific and surrounding environmental conditions, and the sampling approach employed (direct or dip sampling). Each description is accompanied by a photograph taken by the researcher at the respective site.

TABLE 3.2: Physical characteristics of sampling site 1

Site 1: Klerkskraal Dam (KD/KKD)	
Site description	Researcher photograph of sampling site
The first site, Klerkskraal Dam, located at the following GPS coordinates: 26°15'09.6"S, 27°09'34.7"E, served as the upstream reference point for this study. Klerkskraal Dam provided baseline water quality data that reflects only limited impacts from runoff (e.g., minimal anthropogenic influence) before it flows through more impacted regions. Moreover, this site was surrounded by natural vegetation and limited agricultural activity nearby. Sampling was conducted at the edge of the dam, indicated by white arrow, where slower water flow occurred.	

TABLE 3.3: Physical characteristics of sampling site 2

Site 2: Muiskraal Bridge (MB)	
Site description	Researcher photograph of sampling site
Muiskraal Bridge, located at 26°26'42.3"S, 27°07'06.0"E, is situated downstream of mixed-use agricultural areas, which served as an early indicator of agricultural pollution, typically shown by elevated nutrient levels and increased sediment load. There is a rural settlement upstream from this sampling site as well. Sampling took place near the bridge, capturing water flow that may carry residues from the surrounding agricultural lands and urban runoff. Sampling was conducted at the edge of the dam, indicated by white arrow, where slower water flow occurred.	

TABLE 3.4: Physical characteristics of sampling site 3

Site 3: Around the World Bridge (AtW)	
Site description	Researcher photograph of sampling site
Located at the following coordinates 26°30'52.6"S, 27°07'28.6"E, the Around the World Bridge site is positioned further downstream just after the Boskop Dam, offering insights into the allowing for observation of cumulative impacts from upstream agricultural and natural zones. From observation, site three experienced moderate to high flow, with surrounding vegetation dominated by riparian species, which help buffer pollutants from runoff. This location was expected to show greater organic content and variability in turbidity and suspended solids compared to upstream sites. Sampling was conducted at the edge of the dam, indicated by white arrow, where slower water flow occurred.	

TABLE 3.5: Physical characteristics of sampling site 4

Site 4: Hartspad Bridge (HB)	
Site description	Researcher photograph of sampling site
Located near semi-urban areas (26°34'18.9"S, 27°06'12.2"E), Hartspad Bridge served as a mid-stream monitoring site for assessing urban runoff effects on river quality. This site was marked by moderate flow and riparian vegetation, with occasional domestic, industrial and agricultural activities nearby. Previous studies on urban stream health indicate that these types of sites often exhibit elevated levels of metals, nitrates, and phosphates from urban discharge. Sampling was conducted at the edge of the dam, indicated by white arrow, where slower water flow occurred.	

TABLE 3.6: Physical characteristics of sampling site 5

Site 5: Thabo Mbeki Bridge (TMB)	
Site description	Researcher photograph of sampling site
Situated at a significant distance downstream (26°41'04.6"S, 27°06'01.3"E), the Thabo Mbeki Bridge site effectively captured the accumulated impacts from upstream agriculture and urban influences. This site, characterised by observed riparian vegetation and a notable increase in sediment deposition, provided samples that help to understand mixed land-use impacts on water quality. Sampling was conducted at the edge of the dam, indicated by white arrow, where slower water flow occurred.	

TABLE 3.7: Physical characteristics of sampling site 6

Site 6: Trim Park Bridge (TPB)	
Site description	Researcher photograph of sampling site
Located at 26°42'27.2"S, 27°06'20.1"E, site 6, Trim Park Bridge is close to recreational areas and captures potential impacts from human recreational activities, with signs of litter, increased sedimentation, and observable organic waste. Therefore, site six served as a site where direct human impact on water quality can be measured. Sampling was conducted at the edge of the dam, indicated by white arrow, where slower water flow occurred.	

TABLE 3.8: Physical characteristics of sampling site 7

Site 7: Golf Course Bridge (GC)	
Site description	Researcher photograph of sampling site
Golf Course Bridge, located at 26°43'29.8"S, 27°06'21.3"E, is adjacent to a golf course. Therefore, it was expected that this site would likely reflect the influence of fertilisers and pesticides used in landscaping, subsequently leading to elevated levels of nutrients such as phosphates and nitrates. Water was sampled from the bridge using the dip method. Sampling was conducted at the edge of the dam, indicated by white arrow, where slower water flow occurred.	

TABLE 3.9: Physical characteristics of sampling site 8

Site 8: Pre-Wastewater Treatment Plant Bridge (WWTP)	
Site description	Researcher photograph of sampling site
Positioned upstream of a wastewater treatment plant, site eight, located at 26°45'11.6"S, 27°05'59.8"E, served as a baseline for water quality before potential treatment plant effluent discharge. Expected to reflect background levels of contaminants, this site allows comparison against downstream conditions impacted by treated wastewater. Sampling was conducted at the edge of the dam, indicated by white arrow, where slower water flow occurred.	

TABLE 3.10: Physical characteristics of sampling site 9

Site 9: Skandinawie Drift Bridge (SB)	
Site description	Researcher photograph of sampling site
Skandinawie Drift Bridge was the ninth sampling site located at 26°52'49.7" S, 26°57'50.5" E. It is considered downstream from major agricultural areas and was representative of rural river stretches heavily impacted by intensive agriculture, with nutrient and sediment levels anticipated to be high. Water samples were collected near the weir. Sampling was conducted at the edge of the dam, indicated by white arrow, where slower water flow occurred.	

TABLE 3.11: Physical characteristics of sampling site 10

Site 10: Loop Spruit Begin (LSB)	
Site description	Researcher photograph of sampling site
Serving as the initial sampling point for the Loop Spruit (26°25'25.2"S, 27°31'44.7"E), site 10 is located in a valley with very little agricultural or urban runoff, but mining activity is prevalent. Natural vegetation surrounds the site, and sampling was conducted close to the water's edge. Sampling was conducted at the edge of the dam, indicated by white arrow, where slower water flow occurred.	

TABLE 3.12: Physical characteristics of sampling site 11

Site 11: Loop Spruit End (LSE)	
Site description	Researcher photograph of sampling site
Positioned at the end of the Loop Spruit, site 11, located at 26°39'53.5"S, 27°11'48.0"E captured the cumulative pollution effects from the entire upstream watershed, including agricultural and urban contributions. It is characterised by slow water flow and significant sediment and organic matter accumulation. Sampling was done at the stream's edge. Sampling was conducted at the edge of the dam, indicated by white arrow, where slower water flow occurred.	

TABLE 3.13: Physical characteristics of sampling site 12

Site 12: Wonderfontein Spruit Begin (WSB)	
Site description	Researcher photograph of sampling site
Wonderfontein Spruit Begin, located at 26°18'57.4"S, 27°22'56.9"E, served as a starting reference site with limited but still prevalent, anthropogenic influence for Wonderfontein Spruit. This relatively undisturbed site offered a comparative reference for water quality before any significant urban or agricultural influence. Sampling occurred at the stream's edge. Sampling was conducted at the edge of the dam, indicated by white arrow, where slower water flow occurred.	

TABLE 3.14: Physical characteristics of sampling site 13

Site 13: Wonderfontein Spruit End (WSE)	
Site description	Researcher photograph of sampling site
Located at the endpoint of the Wonderfontein Spruit, the final site (26°26'11.3"S, 27°09'05.0"E) reflected the cumulative impacts of upstream activities, including potential urban runoff and mining-related contamination. However, this sampling site is situated downstream from a natural wetland characterised by slower water flow, sediment contaminant filtration. Sampling was conducted at the edge of the dam, indicated by white arrow, where slower water flow occurred.	

3.2 Physico-chemical parameters

Physico-chemical parameters, including pH, temperature, total dissolved solids (TDS), electrical conductivity (EC), salinity, and turbidity, were measured on-site using a calibrated Oakton PCS Testr™ 35 waterproof field multi-parameter probe (Eutech Instruments, 2010). The probe was rinsed with distilled water prior to and after use. Furthermore, in the early stages of the study, chemical oxygen demand (COD) was measured in the laboratory as milligrams per litre (mg/l) with a HACH DR 2800TM (HACH Company, 2013). However, due to the COVID-19 pandemic and its indirect effects on supplier product availability, additional analyses, including sulphate, nitrates and chemical oxygen demand measurements were unable to be completed. This subsequently influenced the measurement of intended additional parameters, such as phosphates and nitrates. As a result, it is excluded from this study's data and findings. The physical and chemical concentration values obtained were compared to the recommendations made by the South African Water Quality Guidelines for aquatic ecosystems (DWAF, 1996).

3.3 Detection of metals

Inductively coupled plasma mass spectrometry (ICP-MS) was used to quantify the presence of heavy and trace metals in the Mooi River, Wonderfontein Spruit and Loop Spruit catchments. The protocol followed was done in accordance with EPA 3050b (Arsenic, 1996). Samples from the sites were filtered through 0.45 µm membrane filters and digested using HNO₃. To quantify the results, the instrument was calibrated using ULTRASPEC® standard solutions (De Bruyn Spectroscopic Solution, South Africa). Eco-Analytica from the North-West University Centre for Water Sciences and Management, South Africa, performed the ICP-MS analysis.

3.4 DNA extraction

Environmental DNA (eDNA) was extracted from the filtered membranes from each respective site using the Macherey-Nagel NucleoSpin® Soil DNA Isolation Kit (Macherey-Nagel, 2021) and the prescribed protocol. The quality and concentration of the DNA were determined using the Nanodrop One (Thermofisher, USA) to determine suitability for downstream usage and applications, as stated by Klindworth *et al.* (2013).

3.5 High throughput sequencing

Locus-specific primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') were utilised to amplify the V3-V4 region (~460 bp) of the bacterial 16S rRNA gene (Klindworth *et al.*, 2013). The primers used for eukaryotic microorganisms were the Uni-Minibar combination of primers to amplify the cytochrome c oxidase subunit I (COI) gene which consists of Uni-MinibarR1: 5'-GAAAATCATAATGAAGGCATGAGC -3' and Uni-MinibarF1: 5'-TCCACTAATCACAARGATATTGGTAC-3' (Meusnier *et al.*, 2008). Sequencing was done by means of the method described by Van Wyk *et al.* (2017). Following the steps outlined in the MiSeq library preparation guide, partial 16S rRNA and COI amplicons were processed and sequenced on an Illumina MiSeq sequencer (Illumina, USA) using the sequencing V3 kit.

3.6 Sequence analysis

DADA2 was used to process reads obtained from the sequencing platform, after indexes and primers were removed (Callahan *et al.*, 2021; DADA2 Pipeline Tutorial 1.16). The filtering and trimming step was performed with the following parameters for 16S: truncLen=250, maxEE=c(10,10), maxN=0, compress=TRUE, verbose=TRUE, multithread=FALSE and rm.phix=TRUE. For COI the following parameters were set: trimLeft=c(17,17), trimRight=c(5,5), truncQ=2, maxEE=c(5,5), maxN=0, rm.phix=TRUE, compress=TRUE, verbose=TRUE, multithread=FALSE. Fastq analysis of sequencing data for 16S (reverse reads) and COI (forward reads) was of bad value. Inverse complementary reads were used to make an identification. This step was performed separately for the forward reads (16S) and reverse reads (COI). This was followed by the "learnErrors" and "dada" functions, which were applied separately to the forward and reverse reads before being merged using "mergePairs". The resulting sequence table was then converted into chimeric reads using the "removeBimeraDenovo" function (method = "consensus") (Flegontova *et al.*, 2023). The "assignTaxonomy" function was used to assign taxonomy to the SILVA 138.1 database (McLaren & Callahan, 2021; SciLifeLab, 2024). The rds data structures, including the sequence and taxonomy tables, were saved at critical points. The processed data was subsequently visualised with phyloseq (Callahan *et al.*, 2016; 2021).

3.7 Biotic indices and multivariate analyses

Physical, chemical and metal data were processed using Statistica for descriptive statistics. Several indices were used to evaluate species richness, diversity, dominance, and similarity across the 13 sampling sites in the present study, including the Chao1 index, the Shannon-Wiener index, the Bray-Curtis metric, and the Kruskal-Wallis H test. These tools were employed in MicrobiomeAnalyst (Chong *et al.*, 2020). The Chao1 index was specifically applied to determine species richness (number of species present in a sample), with higher values indicating higher diversity (Chao, 1984). This index is especially useful for determining species richness and the presence of unobserved species within communities. Additionally, the Shannon-

Wiener diversity index was used to assess species diversity at sampling sites. This index assesses species richness and evenness, with higher values indicating greater diversity and a more balanced species distribution across sites (Shannon, 1948).

To assess similarity and/or dissimilarity in species composition across the 13 sampling sites, Bray-Curtis principal component analysis (PCoA) was used. PCoA is a multivariate statistical approach that illustrates the compositional dissimilarities between microbial communities based on their abundance data (Hao *et al.*, 2019). The method relies on the Bray-Curtis dissimilarity index, which quantifies differences between samples, ranging from 0 (completely identical communities) to 1 (entirely dissimilar communities). The distance between points in the plot reflects the level of dissimilarity in community composition (Hardersen & La Porta, 2023). This allows the identification of clustering patterns indicating similarities or differences among communities across environmental gradients. Bray-Curtis is especially favourable when comparing communities to external variables (Ricotta & Podani, 2017). Ordinations (PCoA and RDA) were plotted using Canoco 5 (ter Braak & Smilauer, 2012).

Furthermore, the Kruskal-Wallis H test was done to compare the Chao1 diversity (alpha diversity) results to the environmental parameters from this study using MicrobiomeAnalyst (Chong *et al.*, 2020). The Kruskal-Wallis H test is a statistical tool that formulates no assumptions about the data distribution (Kruskal & Wallis, 1952; Xia, 2020). This tool is ideal for microbiome comparisons since ecological data is not always evenly distributed. A p -value < 0.05 indicates that the two data points are statistically significant and influence each other. When the p -value is > 0.05 , no statistical significance is observed.

CHAPTER 4: RESULTS

This chapter presents the findings of the study in a structured manner. First, the physico-chemical parameters, including total dissolved solids (TDS), pH, temperature, total dissolved solids (TDS), turbidity, and heavy and trace metal concentrations, are reported to reflect subsequent water quality at individual sampling sites.

4.1 Physico-chemical parameters

During the experimental phase of this study, specific physical and chemical parameters were analysed. However, for some of these parameters, concentrations at or near zero, well below established threshold values, were consistently found across all sampling sites and periods. Given that these parameters did not show variability or contribute meaningfully to assessing water quality or ecological risks in the ecosystems studied, they are not included in the main results and discussion sections (for these results, *cf.* Annexure A). Nonetheless, parameters such as total dissolved solids, electrical conductivity, and salinity are known to be closely related and directly influenced by the same contaminants. However, only total dissolved salts, which comprehensively represent dissolved ionic constituents, are reported in the results and discussion section for clarity and brevity. Parameters below mentionable values, electrical conductivity and salinity are noted in the annexure to ensure all data remain accessible. This ensures a focused discussion on key findings while providing a complete and transparent overview of all experimental data. The following results are thus reported in this section: TDS, pH, temperature, and turbidity.

4.1.1 Total Dissolved Solids

This section presents the findings pertaining to the TDS concentrations observed across the 13 individual sampling sites over a 12-month period. Results are reported across sites as depicted in Figures 4.1 to 4.4.

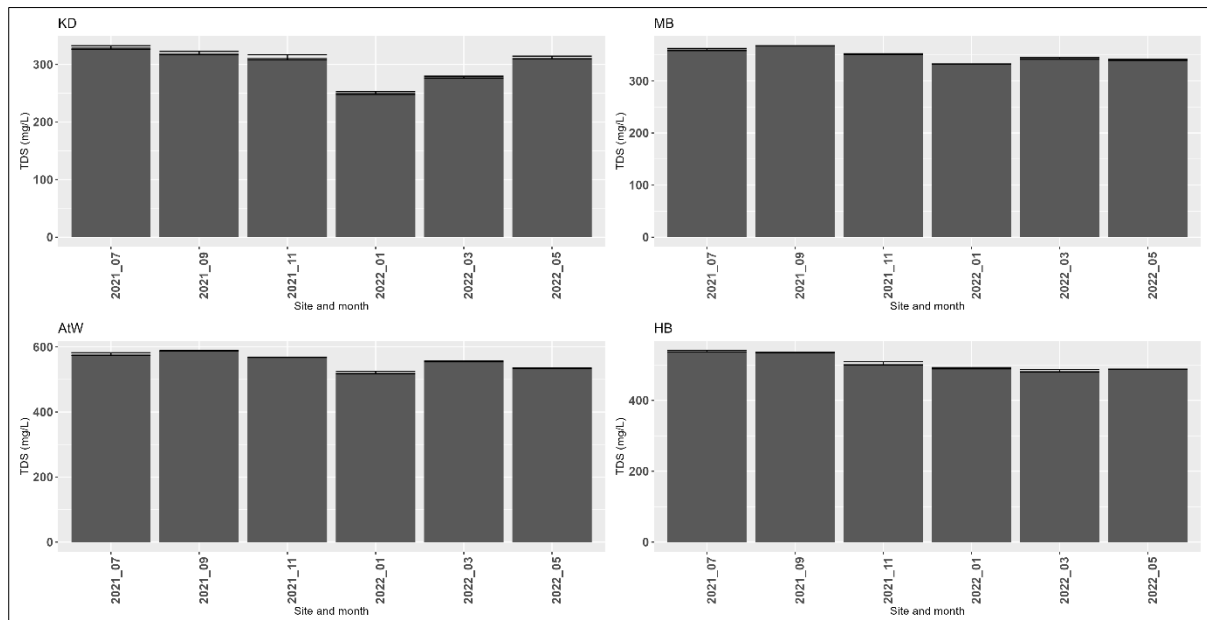


FIGURE 4.1: TDS concentrations across KD, MB, AtW and HB sites.

The findings in Figure 4.1 report on four sampling sites (KD, MB, AtW and HB). Specifically, site KD's TDS values were rather consistent, hovering around 300 mg/L, except in January 2022, where a dip occurred to just under the recommended threshold. This could be due to the rainy season, which dilutes TDS concentrations. As such, low variance was observed with site MB downstream from KD portraying the same environment with TDS values just above 300 mg/L, indicating very low seasonal changes and external influences on the system. Site AtW indicated higher levels of TDS with an average of 600 mg/L. HB TDS levels decrease to the 450-500 mg/L range.

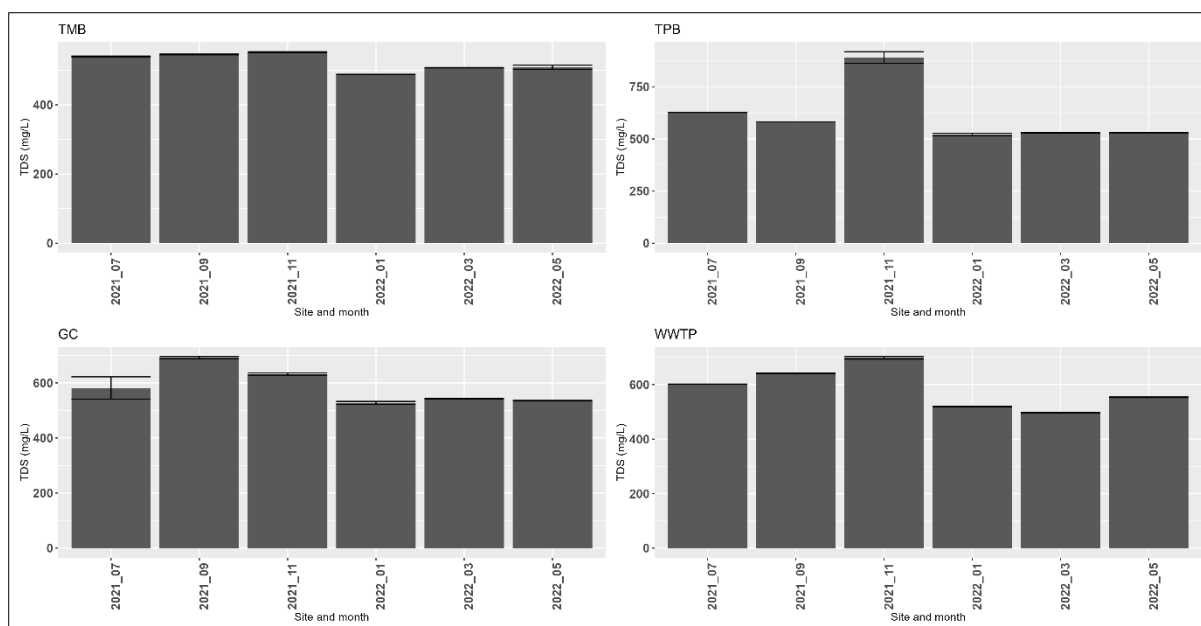


FIGURE 4.2: TDS concentrations across TMB, TPB, GC and WWTP sites.

As seen in Figure 4.2, the TDS values at site TMB were constantly between 400 to 500 mg/L, indicating an equilibrium over 12 months. The slightly higher average values throughout the study signify the site as the confluence of rural and urban activities. TDS values increased alarmingly downstream to the TPB with an approximate mean of 500 mg/L, but a spike in November 2021 of approximately 760 mg/ did occur. The GC site indicated stable TDS values across the 12 months. However, a spike of about 650 mg/L in September 2021 could be identified. Increased TDS values at the previous two sites could be due to fertiliser runoff from the surrounding sports fields of the university, high school, and golf course. Lastly, WWTP had a mean of 580 mg/L, with an increase to approximately 700 mg/L in November 2021.

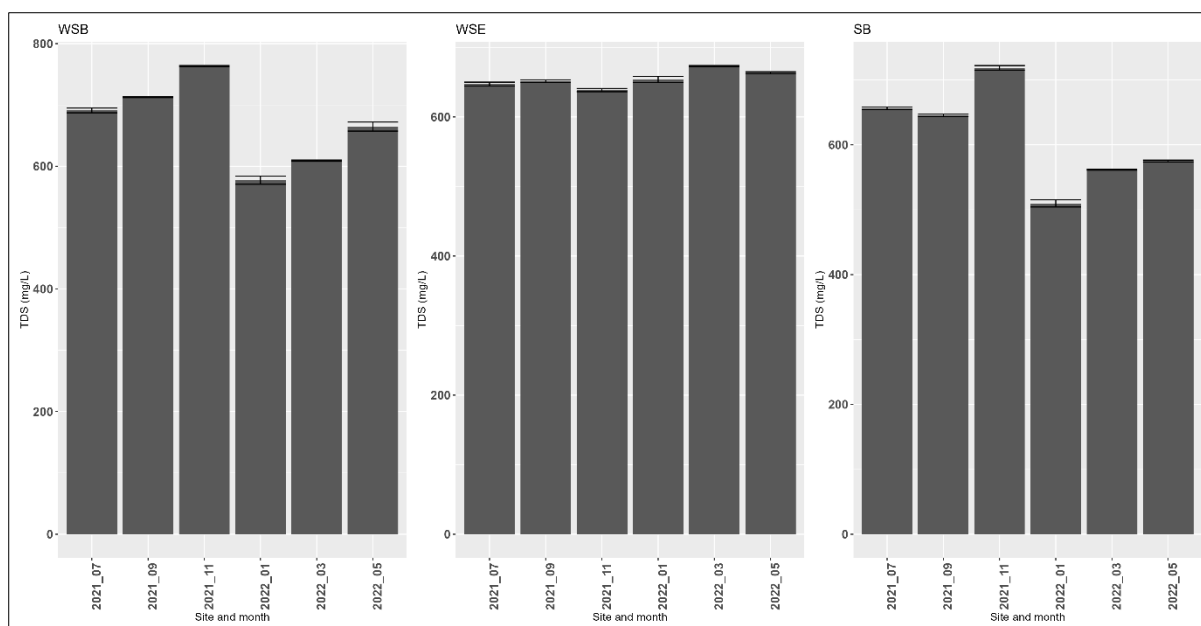


FIGURE 4.3: TDS concentrations across WSB, WSE, and SB sites.

Figure 4.3 indicates that the TDS values at WSB and SB sites increased towards the wet season where it dipped dramatically in January 2022 which signify the middle of the wet season. Moreover, with increased precipitation, the concentration of TDS will decrease. No obvious spikes were observed at the WSE site, however, continued high levels of TDS could illustrate an eutrophic ecosystem at this particular site.

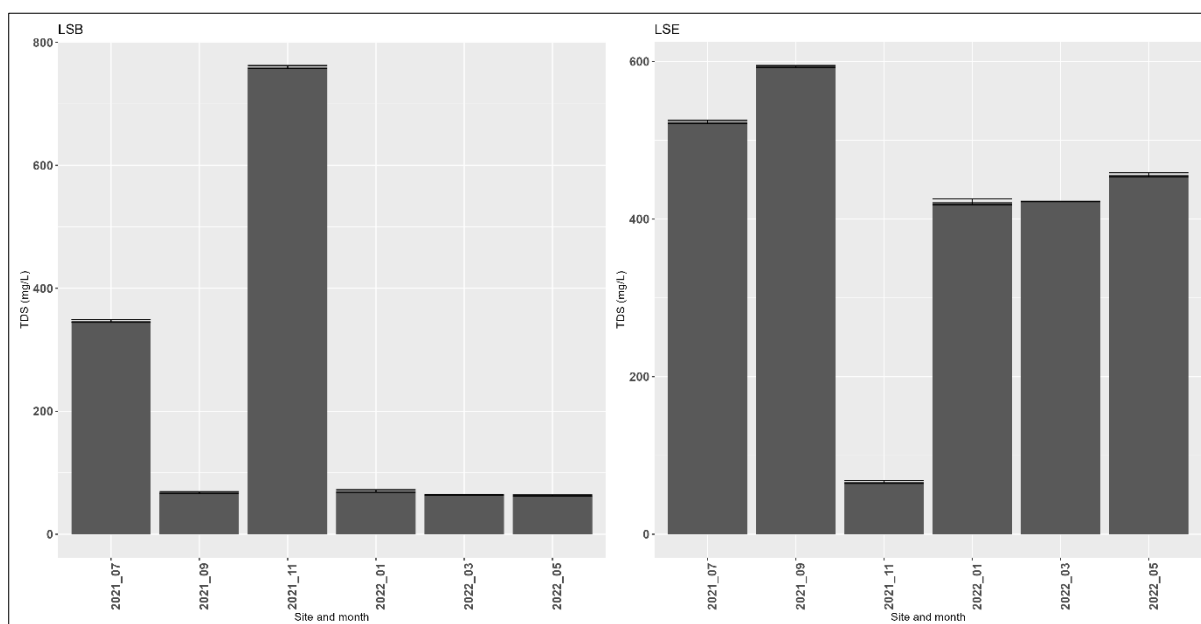


FIGURE 4.4: TDS concentrations across LSB and LSE sites.

Lastly, Figure 4.4 portrayed overall lower levels of TDS, but an increase of 780 mg/L, at LSB in November 2021 was prevalent. LSE indicated natural filtration downstream or a dilution effect following Klipdrift Dam.

4.1.2 pH

Section 4.1.2 presents the pH concentrations observed across the 13 individual sampling sites over 12 months. Results are reported across sites, as illustrated in Figures 4.5 to 4.8.

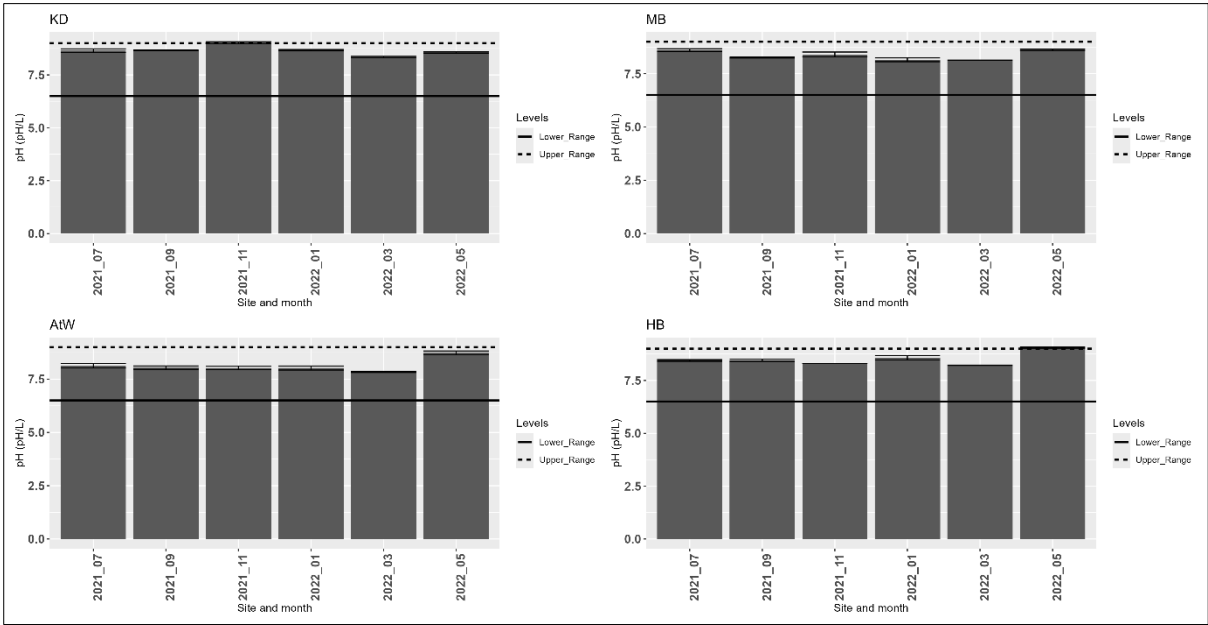


FIGURE 4.5: pH concentrations across KD, MB, AtW, and HB sites.

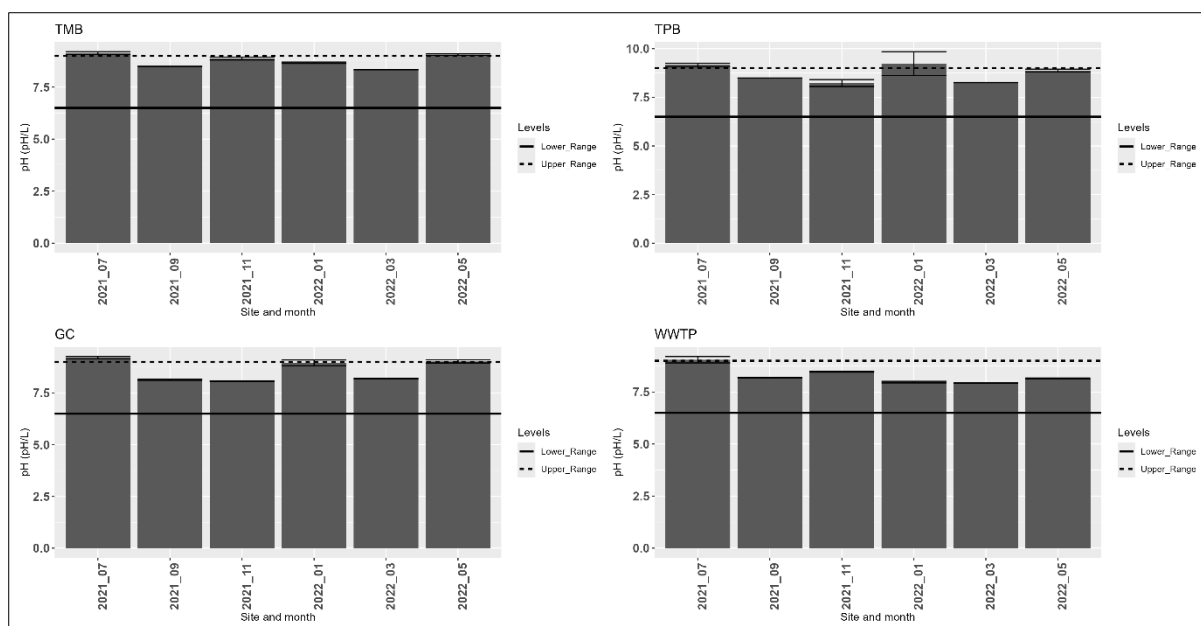


FIGURE 4.6: pH concentrations across TMB, TPB, GC, WWTP sites.

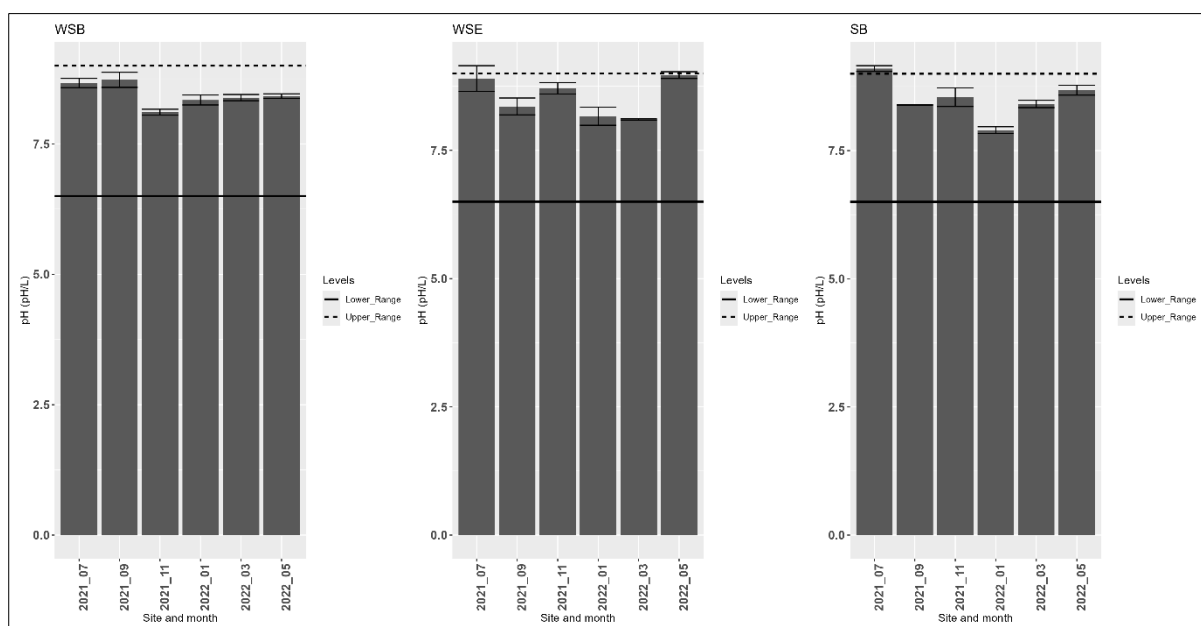


FIGURE 4.7: pH concentrations across WSB, WSE, and SB sites.

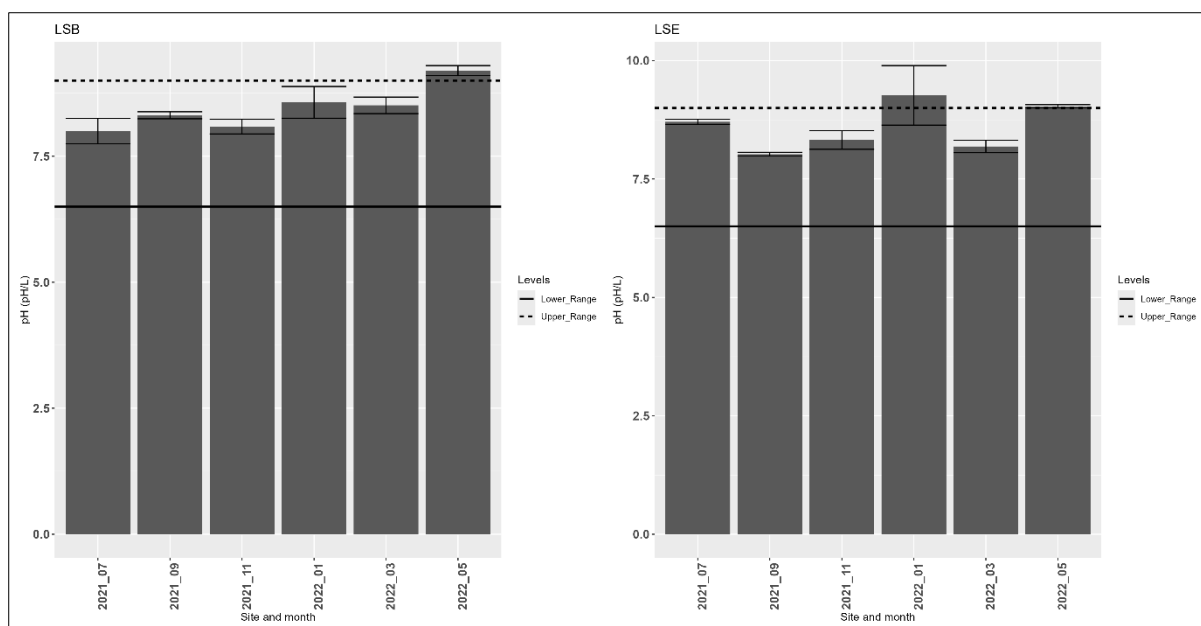


FIGURE 4.8: pH concentrations across LSB and LSE sites.

Figures 4.5 to 4.8 show that across all sites, pH levels were generally constant; however, the mean tends to lean toward a more alkaline environment. These findings may indicate that all sites have an effective buffering capacity and a low susceptibility to the influence of acidic or alkaline contaminants. Furthermore, the consistency of the pH throughout the length of the respective river and its tributaries could suggest minimal to no contamination.

4.1.3 Temperature

Figures 4.9 to 4.12 present the findings of the temperature levels observed across sampling sites over a 12-month period. Each Figure and its associated sites are reported below.

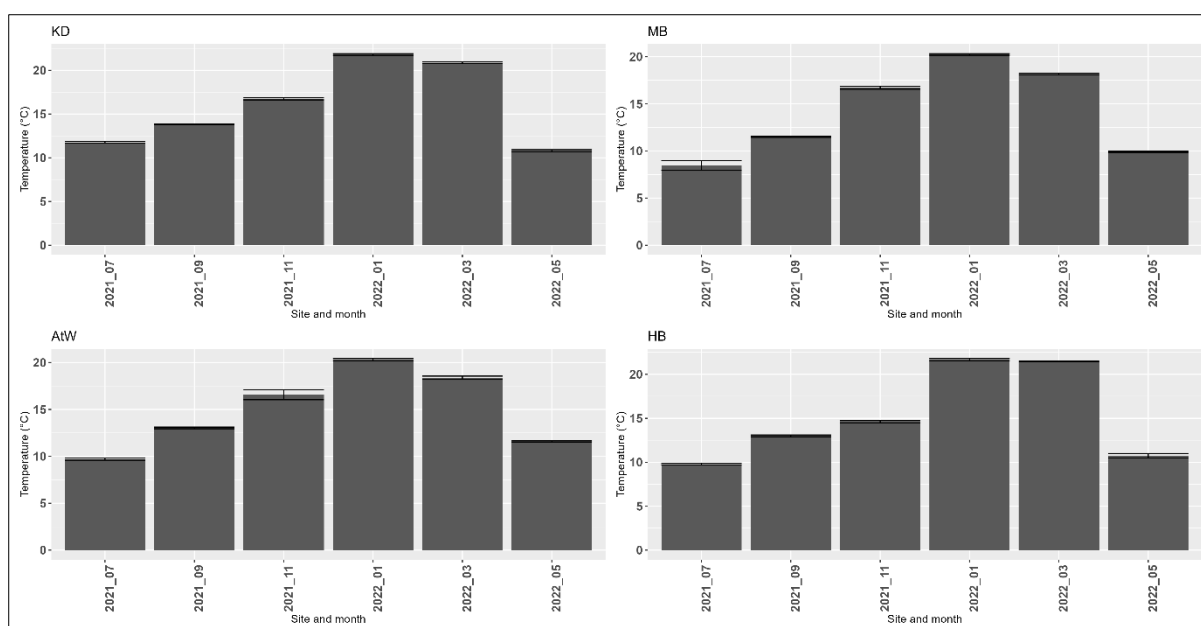


FIGURE 4.9: Temperature levels across KD, MB, AtW and HB sites.

In Figure 4.9, it can be seen that stable seasonal fluctuations occurred throughout the year, as reported by Carey *et al* (2012) in their respective studies, at KD, MB, AtW and HB. Temperatures vary between 10°C in the winter (July 2021) and peak around 25°C in summer (January 2022).

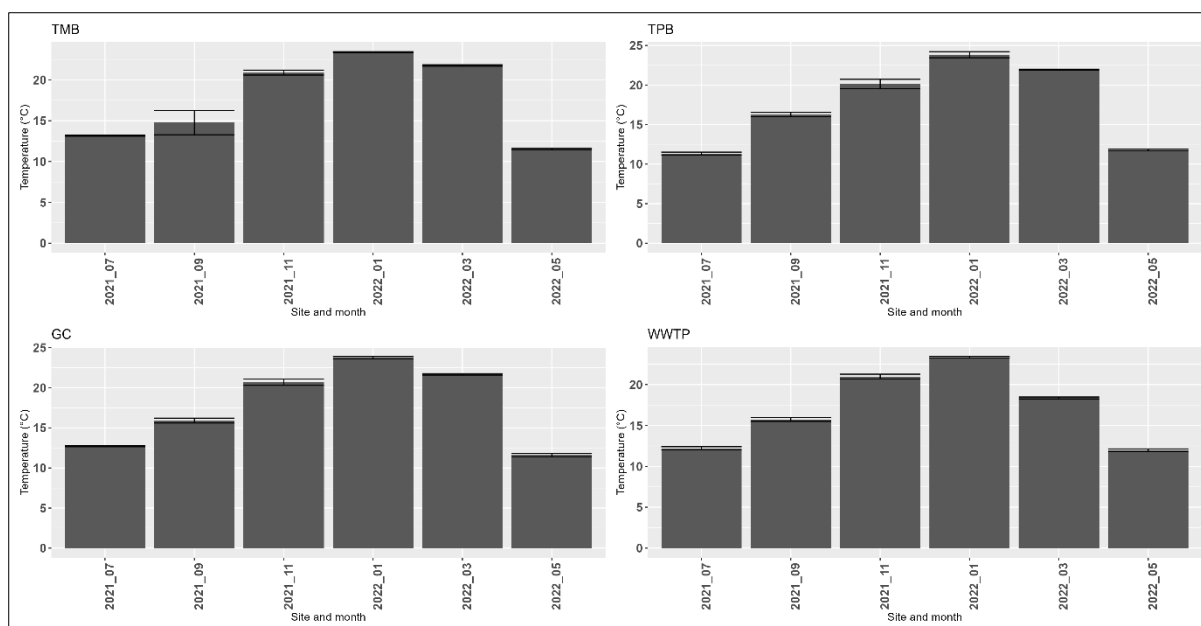


FIGURE 4.10: Temperature levels across TMB, TPB, GC, and WWTP sites.

Urban perturbation can be observed in Figure 4.10; seasonal shifts in temperature occurred at TMB, TPB, GC and WWTP. However, the threshold temperatures throughout all the sites are elevated, ranging from approximately 12°C in winter (July 2021) to a peak of approximately 25°C in summer (January 2022).

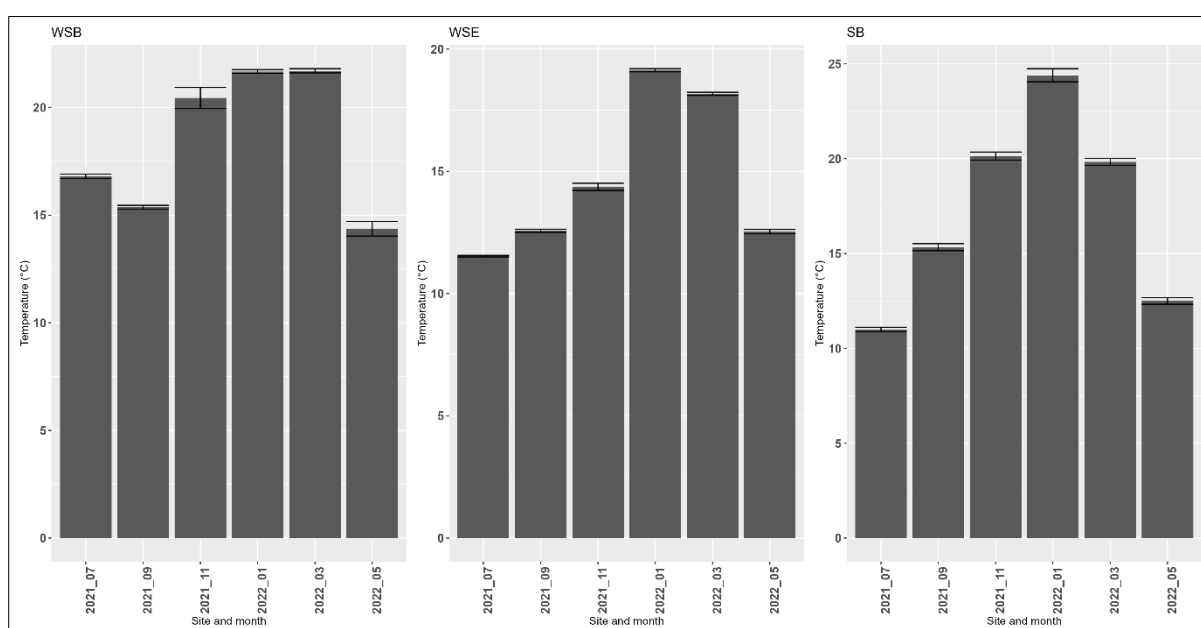


FIGURE 4.11: Temperature levels across WSB, WSE, and SB sites.

Figure 4.11 shows sites with algal contamination, possible eutrophication, low water levels and declined water velocity which is evident through elevated winter temperatures, ranging from 13°C to 17°C, and peaks around 25°C in summer. Seasonal fluctuations did occur as expected.

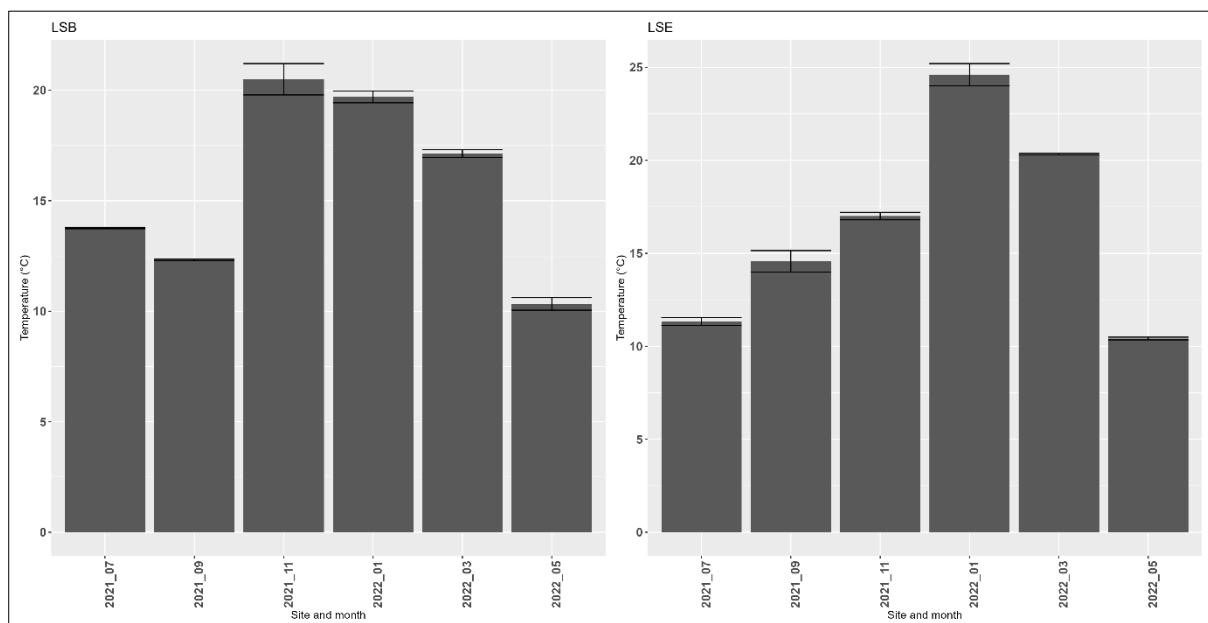


FIGURE 4.12: Temperature levels across LSB and LSE sites.

Figure 4.12 illustrates temperature fluctuation from winter to summer and back to winter across LSB and LSE sites. Temperatures ranged from 12°C and reached up to 25°C, showcasing the transition from winter to summer, respectively.

4.1.4 Turbidity

This section reports on the turbidity observed across the 13 individual sampling sites. Results are reported across sites as depicted in Figures 4.13 to 4.16 below.

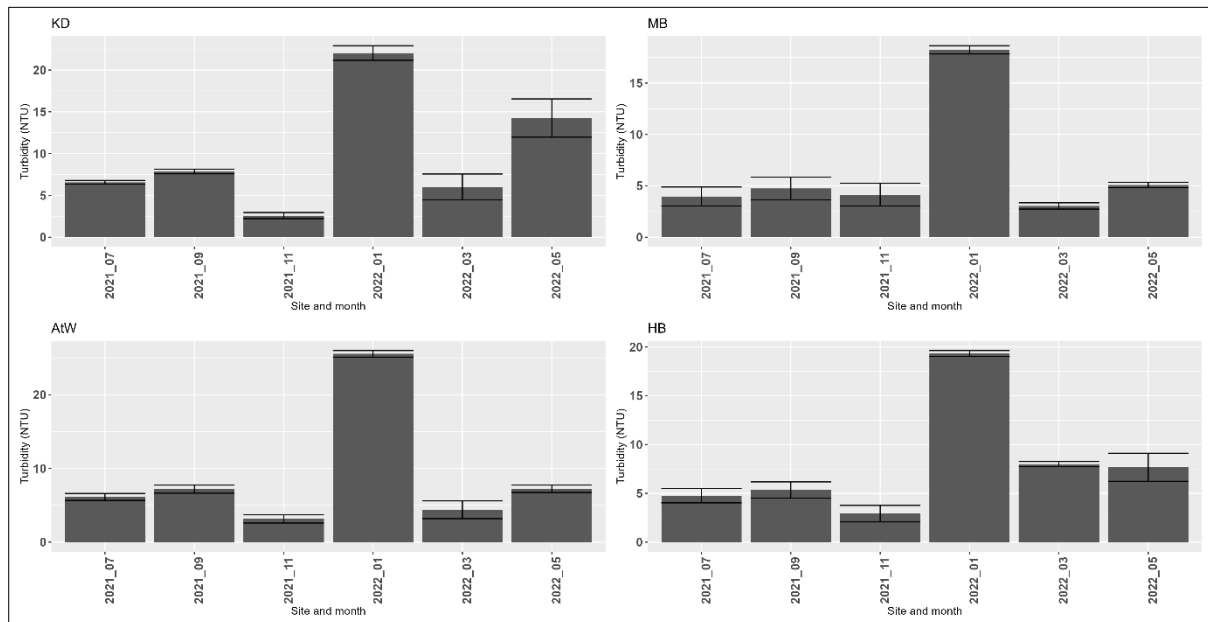


FIGURE 4.13: Turbidity across KD, MB, AtW and HB sites.

Turbidity at the KD, MB, AtW and HB sites (Figure 4.13) show slight variation with the exception of January 2022, which spiked up to 20 to 25 NTU. Indicating a runoff event or some weather disturbance since it is during the wet season.

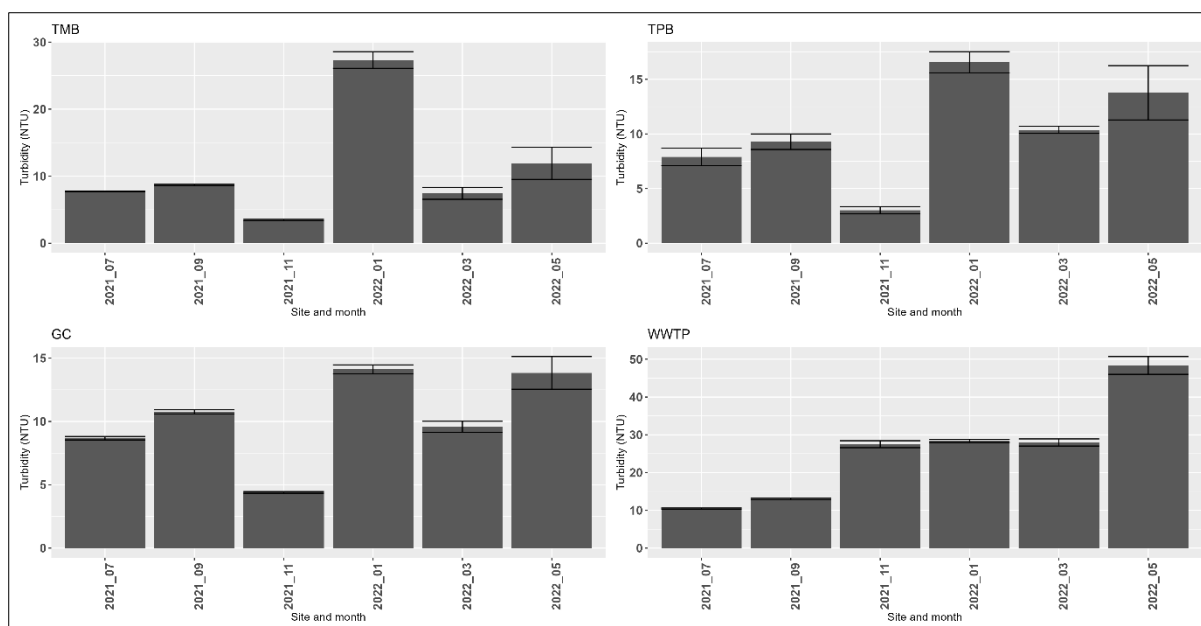


FIGURE 4.14: Turbidity across TMB, TPB, GC and WWTP sites.

From Figure 4.14, the TPB site shows relatively stable turbidity levels throughout the 12 months except for January 2022, which reveals a spike of about 20 NTU. This occurrence is seen at all four sites from this figure. However, the WWTP site shows elevated turbidity levels, especially with the spike seen in December 2022. This spectacle could indicate continuous sediment or effluent discharge from the treatment plant into the river.

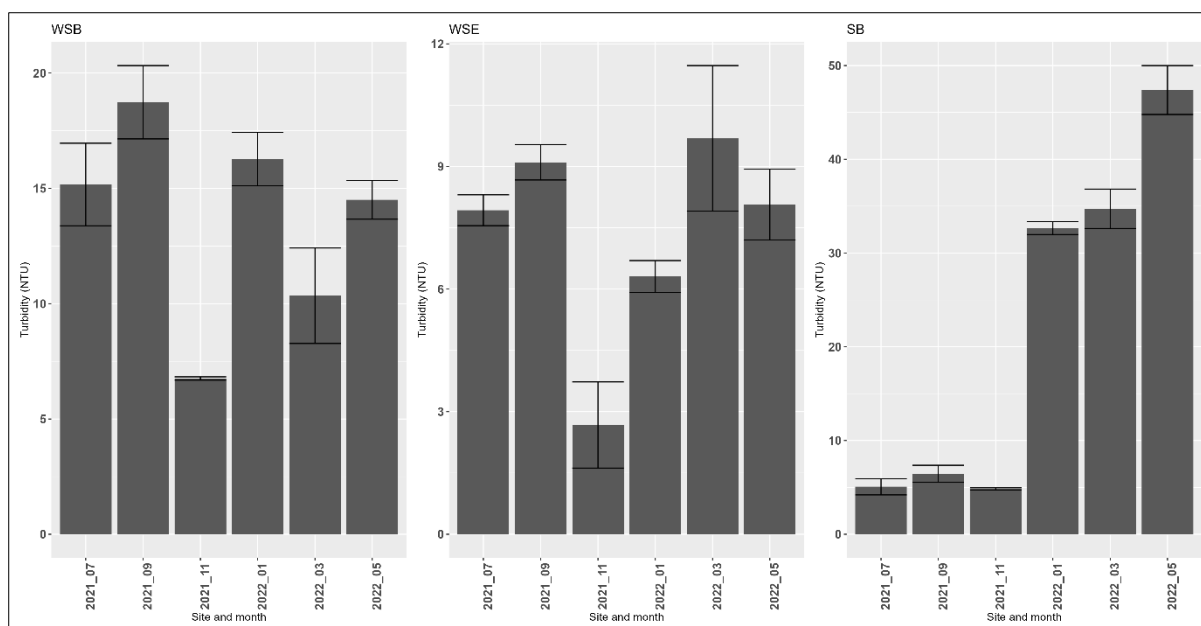


FIGURE 4.15: Turbidity across WSB, WSE, and SB sites.

WSB and WSE indicated peaks up to 20 NTU during the year, with intermittent decreases observed. This possibly shows an isolated system independent from any input throughout the length of the stream. SB in Figure 4.15 portrays a continuous increase in turbidity, starting from as low as 7 NTU and rising to 48 NTU. This could be due to upstream contamination from the wastewater treatment plant or other runoff events from surrounding agricultural activities, or heavy rainfall.

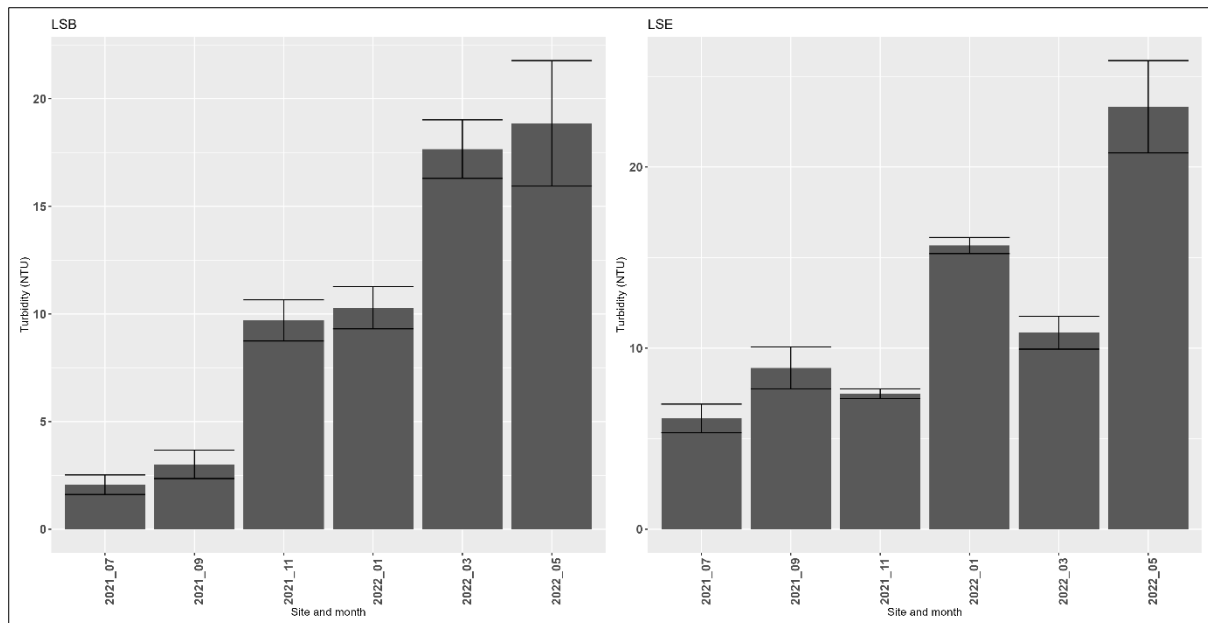


FIGURE 4.16: Turbidity across LSB and LSE sites.

In Figure 4.16, the phenomenon of possible sediment transportation is portrayed. The continuous increase in turbidity indicates a cumulative increase in sediment or organic runoff. Higher turbidity in the lower region of the stream indicates accumulated sediment that would either be carried further down the stream and into the Mooi River or deposited in the stream, restricting water flow and benthic organisms.

4.1.5 Heavy and trace metals

4.1.5.1 Aluminium

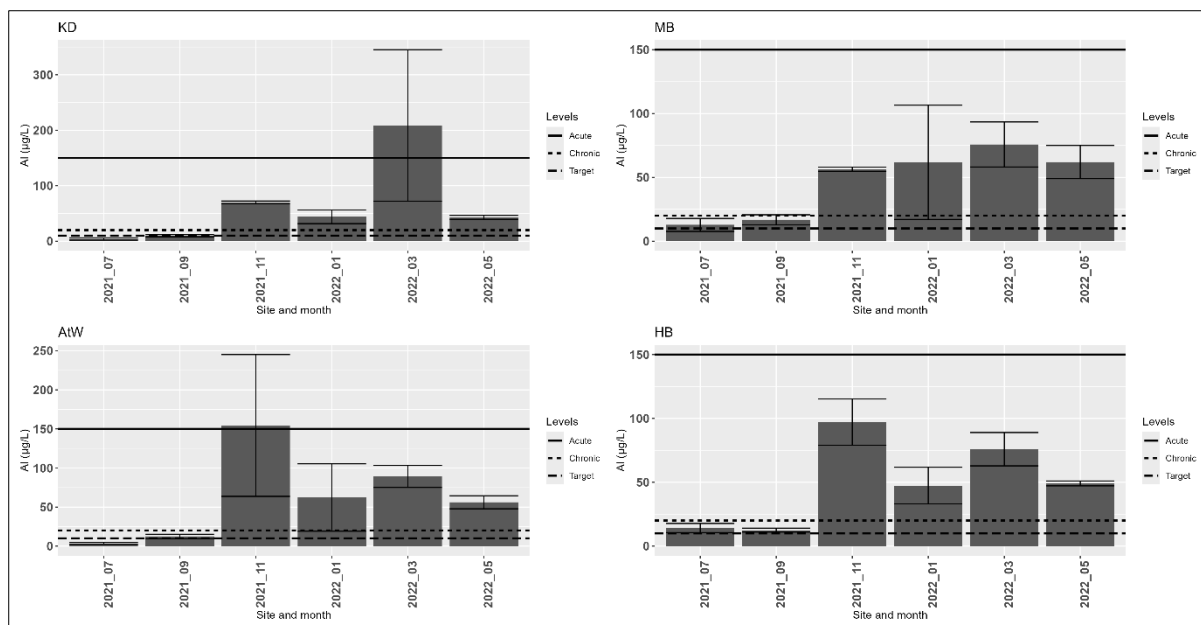


FIGURE 4.17: Aluminium concentrations at KD, MB, AtW, and HB sites.

Figure 4.17 shows the varying levels of aluminium at four sites, namely KD, MB, AtW and HB. These sites indicated aluminium levels predominantly below the acute level of 150 $\mu\text{g/L}$ except for possible contaminant events during March 2022 at KD and November 2021 at AtW. Aluminium levels, however, still remained above the chronic level of 25 $\mu\text{g/L}$ and rarely under the target level.

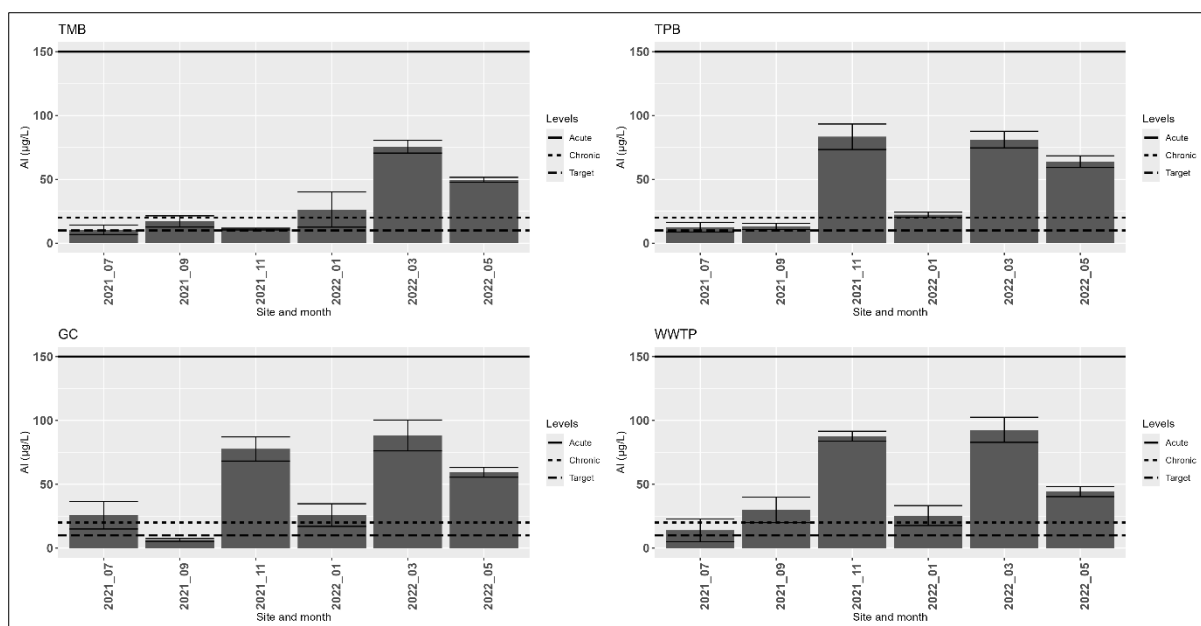


FIGURE 4.18: Aluminium concentrations at TMB, TPB, GC, and WWTP sites.

Throughout Figure 4.18, sites TMB, TPB, GC, and WWTP showed moderate aluminium levels but below the acute level of 150 µg/L. In November 2021 and March 2022, Peaks at all four sites to approximately 80 µg/L show episodic contamination from urban runoff or wastewater treatment plant discharge.

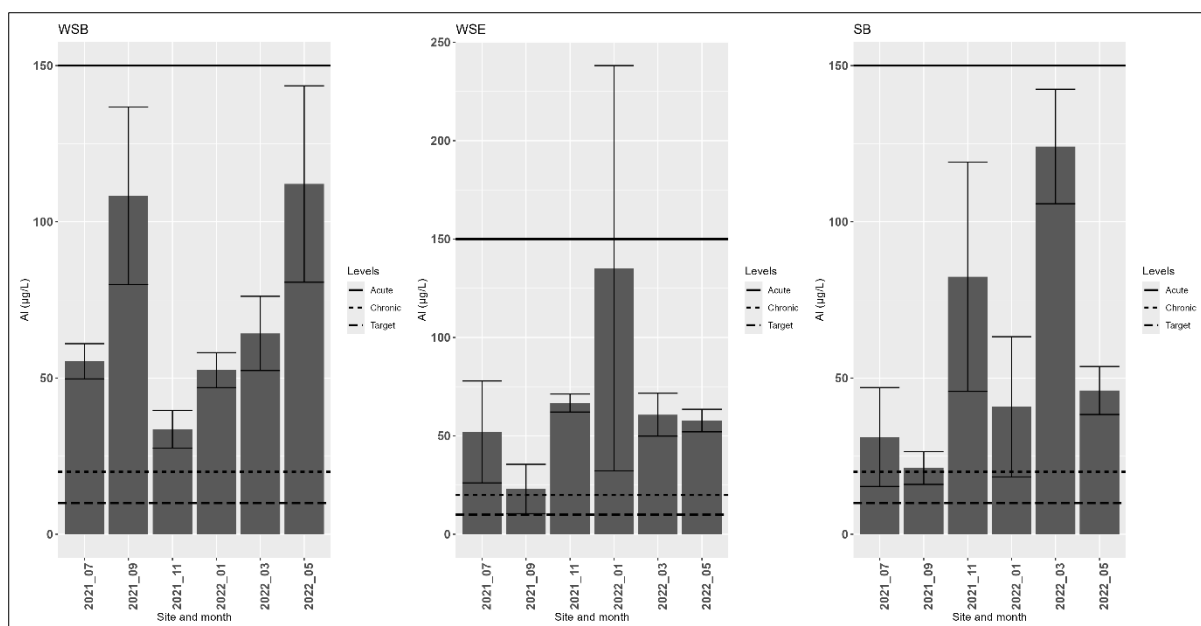


FIGURE 4.19: Aluminium concentrations at WSB, WSE, and SB sites.

Figure 4.19 reports aluminium levels below the stipulated acute threshold for this parameter. All sites, however, indicated levels above acceptable chronic limits. Peaks reached up to approximately 120-130 µg/L with a high degree of standard deviation during these episodes.

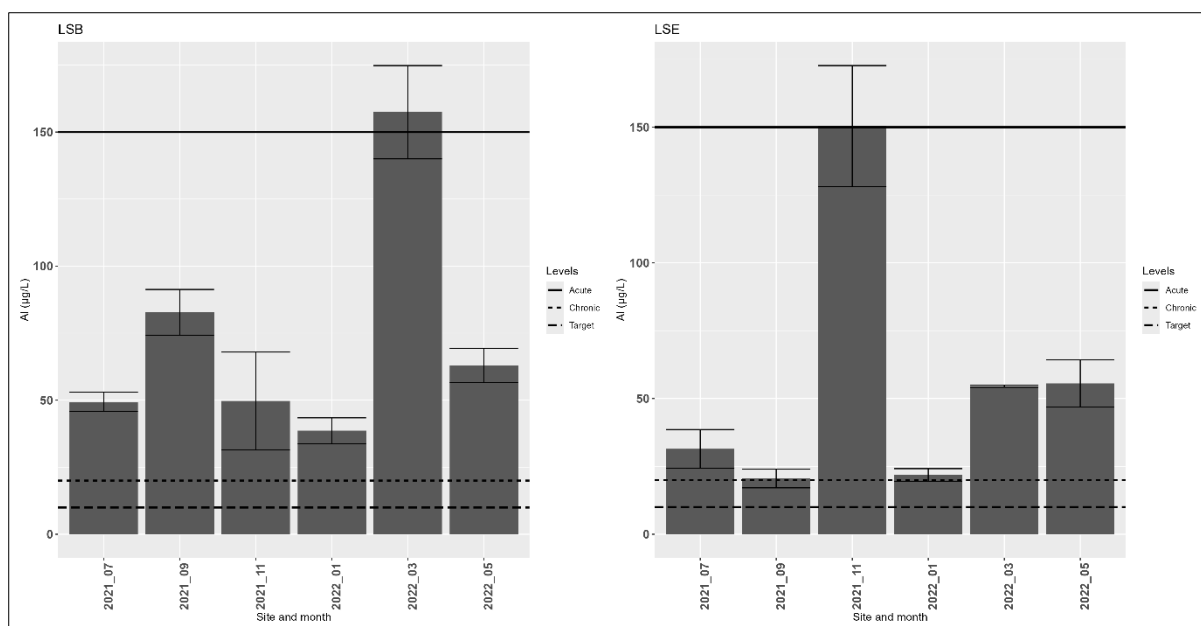


FIGURE 4.20: Aluminium concentrations at LSB and LSE sites.

At sites LSB and LSE (Figure 4.20), significant peaks above or in close proximity to the acute limit occurred in March 2022 and November 2021, respectively. Furthermore, aluminium levels decreased but remained above the accepted chronic levels for aluminium.

4.1.5.2 Calcium

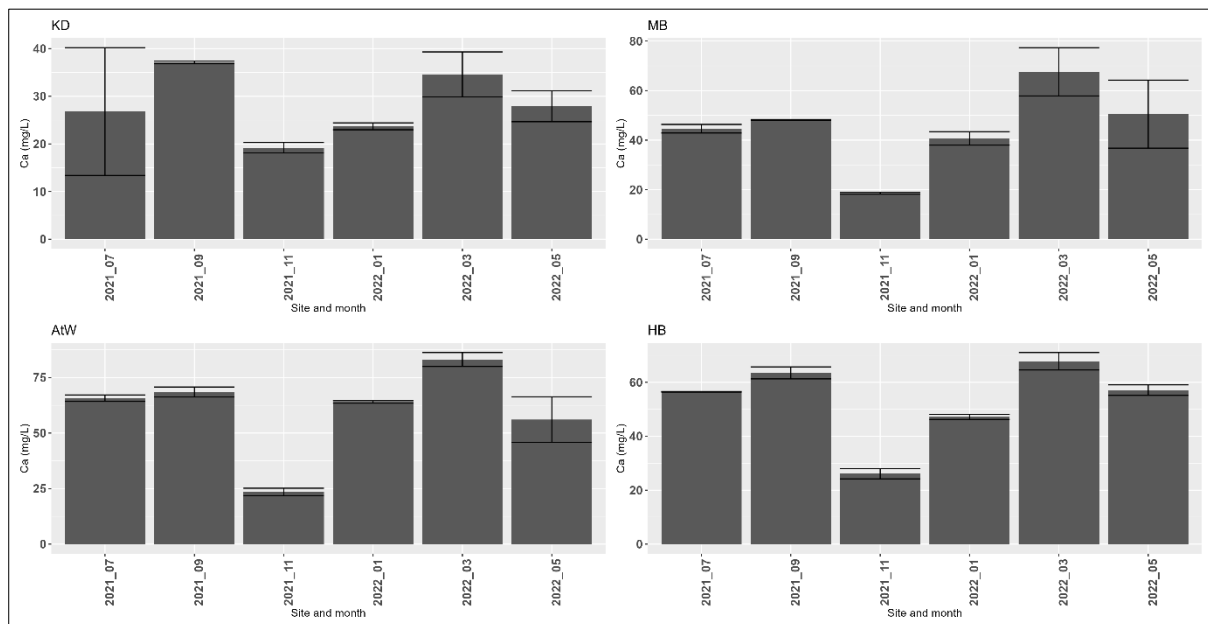


FIGURE 4.21: Calcium concentrations at KD, MB, AtW, and HB sites.

As seen in Figure 4.21, site KD calcium ranged between 20 mg/L and 40 mg/L; calcium levels decreased during November 2021 and steadily rose towards May 2022. Moreover, KD demonstrated overall lower calcium levels than MB, AtW, and HB. All four sites, however, portrayed the same trend in calcium concentration.

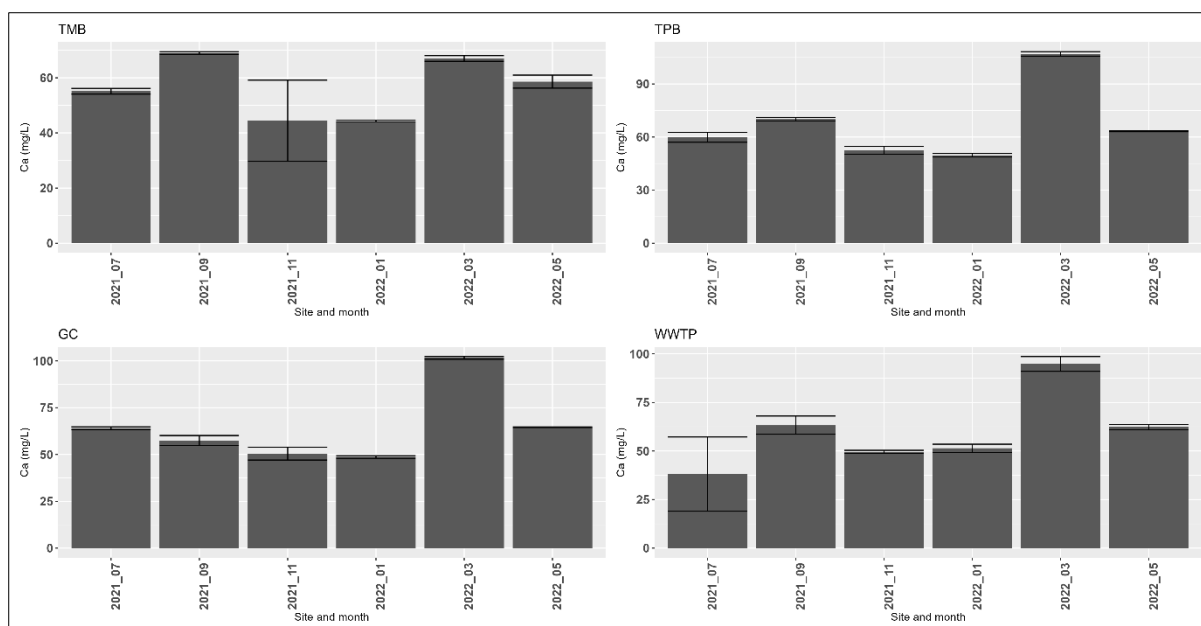


FIGURE 4.22: Calcium concentrations at TMB, TPB, GC, and WWTP sites.

Sites TMB, TPB, GC and WWTP (Figure 4.22) illustrated relatively stable calcium concentrations throughout the year, with subtle increases in September 2021 and March 2022. Overall, elevated calcium levels observed were indicative of the location of all four sites, as they are close to all anthropogenic activities and urban runoff.

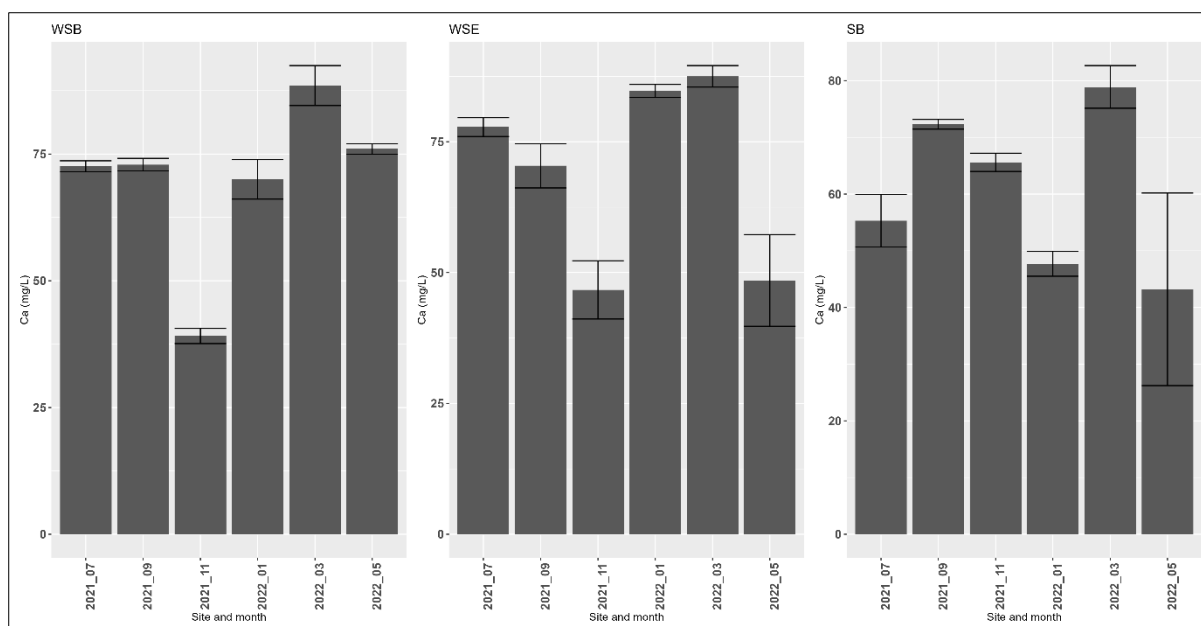


FIGURE 4.23: Calcium concentrations at WSB, WSE, and SB sites.

Figure 4.23 reflects relatively stable calcium concentrations over the 12 months, with a peak at all three sites during March 2022 and notable lows during November 2021 and January 2022. Compared to other sites, these sites had a higher average values throughout the study, probably due to agricultural and mining activities in their proximity.

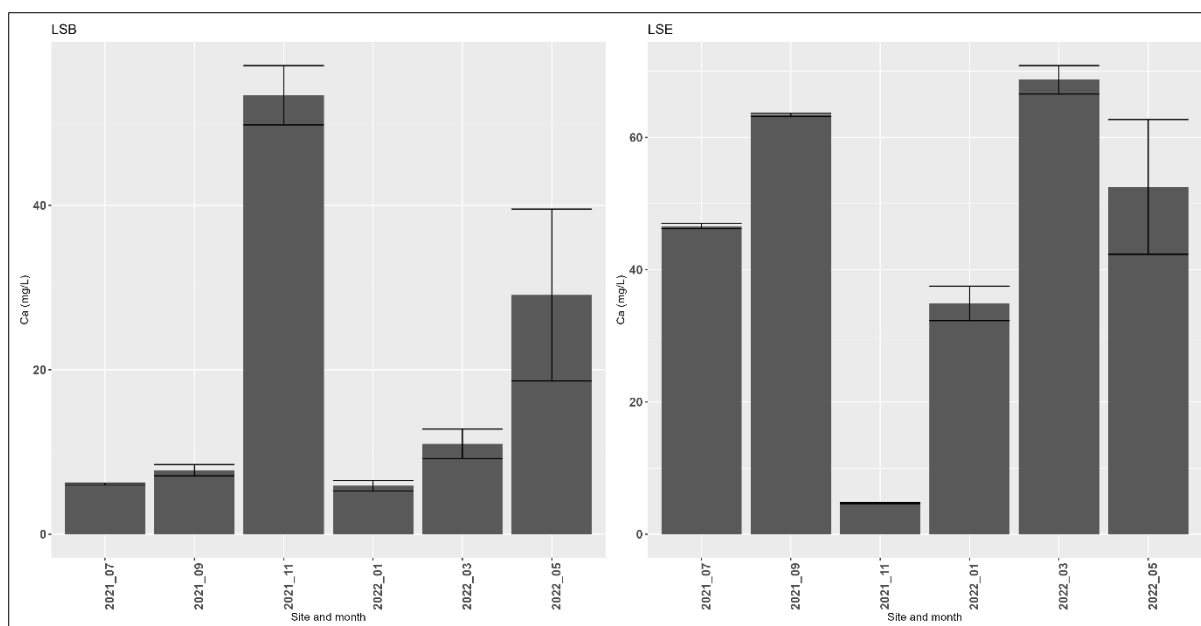


FIGURE 4.24: Calcium concentrations at LSB and LSE sites.

Calcium concentrations at LSB were generally low, but a significant peak, as seen in Figure 4.24, shows a sudden and short-lived calcium input in the form of the significant bar in the histogram. The upward trend of calcium concentrations at LSE depicts the accumulation of calcium downstream from the source of the Loop Spruit as depicted in Jordaan *et al* (2019).

4.1.5.3 Copper

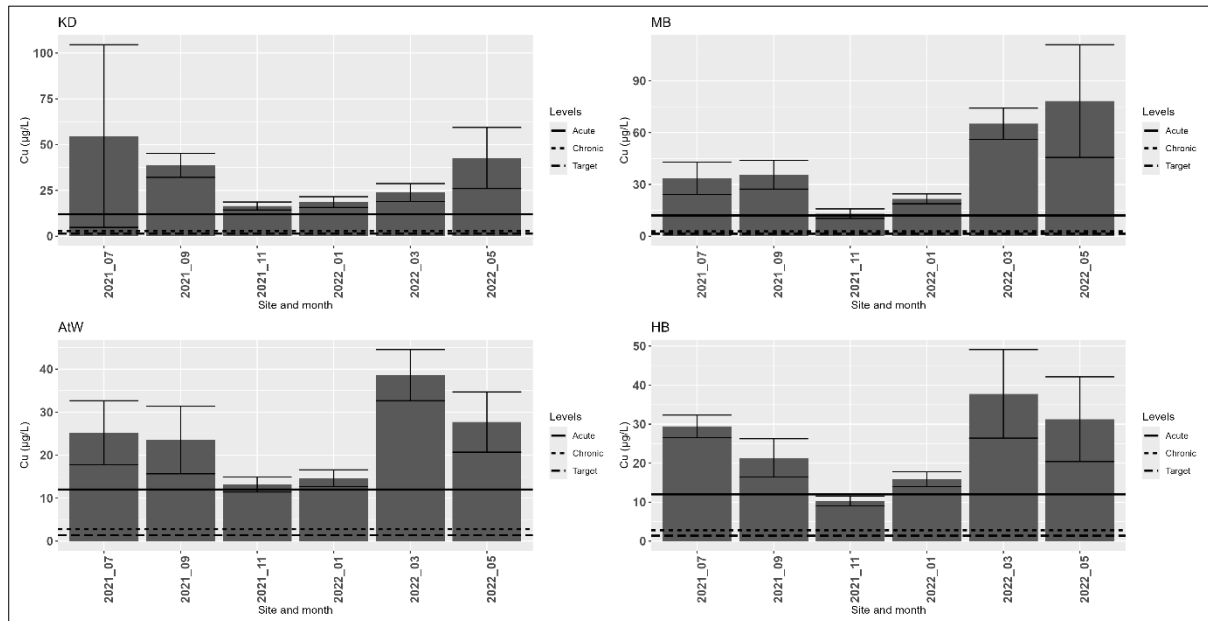


FIGURE 4.25: Copper levels at KD, MB, AtW, and HB sites.

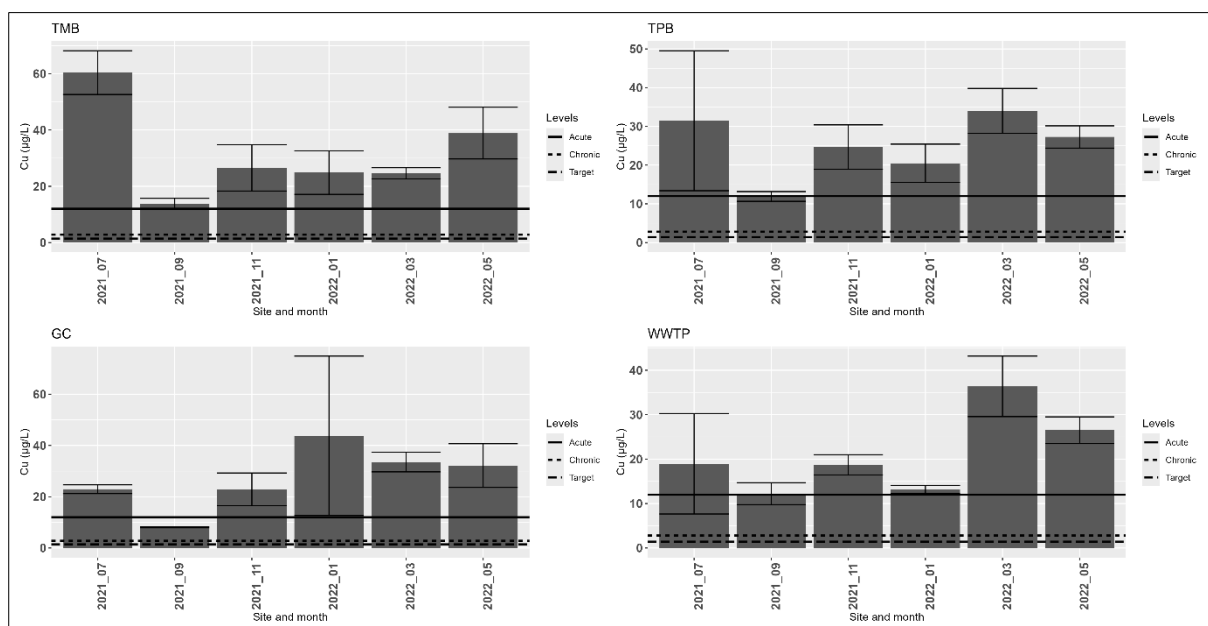


FIGURE 4.26: Copper concentrations at TMB, TPB, GC, and WWTP sites.

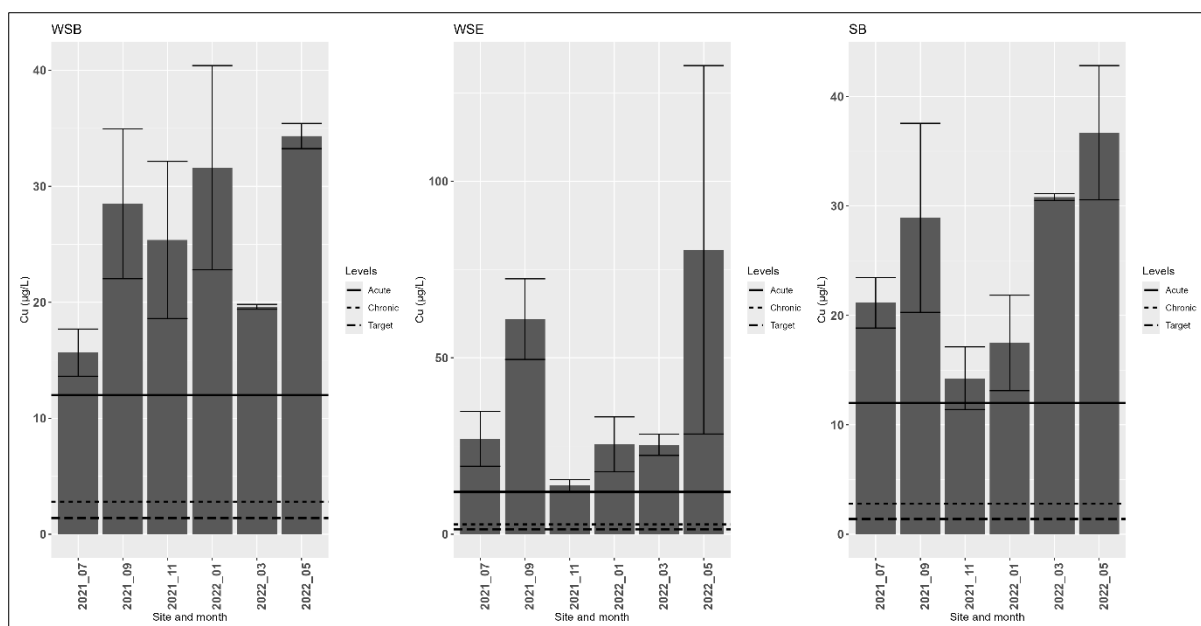


FIGURE 4.27: Copper concentrations at WSB, WSE, and SB sites.

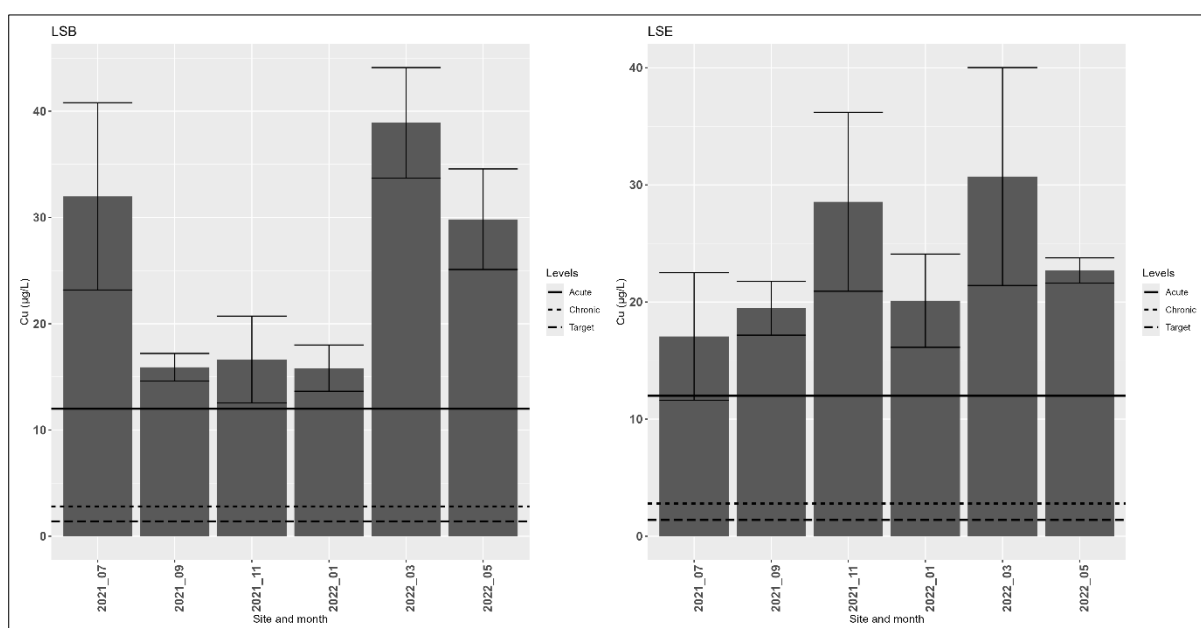


FIGURE 4.28: Copper concentrations at LSB and LSE sites

Figures 4.24 to 4.28 show how the concentration of copper regularly exceeded the acceptable acute level of 12 µg/L (Dallas & Day, 2004; DWAF, 1996). Levels varied from 8 µg/L to 143 µg/L at MB in May 2022. Copper concentrations rarely occurred at the stated target water quality range and fluctuated between exceeding the acute and chronic limits as per DWS (2016).

4.1.5.4 Iron

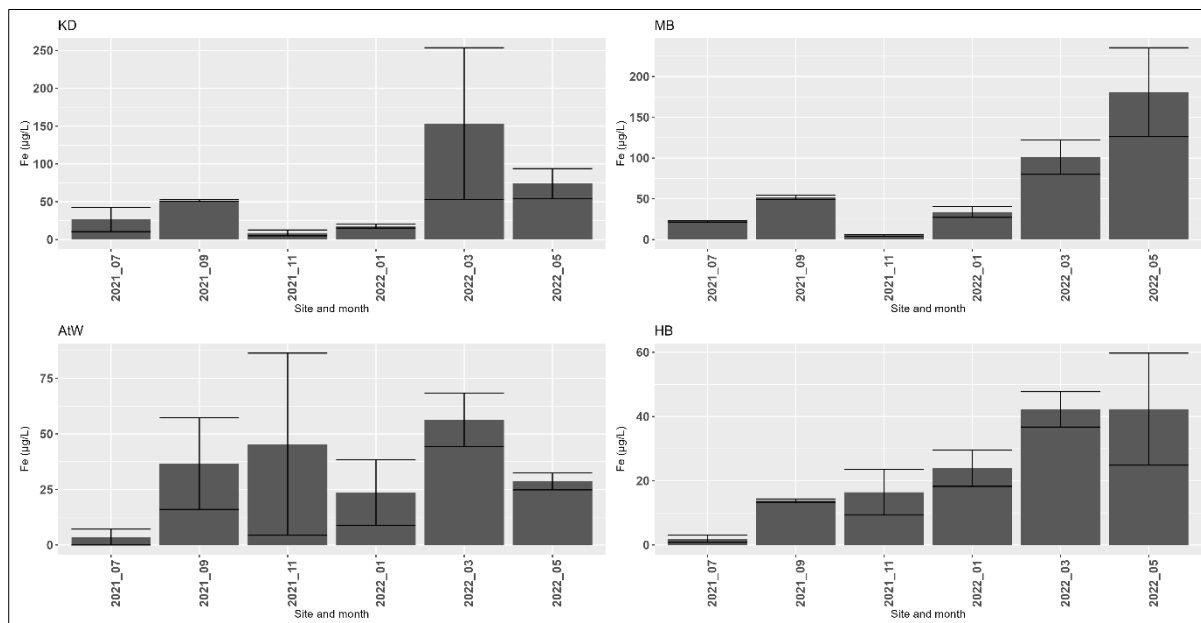


FIGURE 4.29: Iron concentrations at KD, MB, AtW, and HB sites.

Figure 4.29 shows that at KD, overall, low iron levels were detected, with a peak in March 2022 occurring up to 150 µg/L. At MB, levels gradually increased towards the end of the 12-month cycle with 180 µg/L highs. A possible accumulation of iron could have occurred. The AtW site depicted varying iron levels throughout the study period, with a high standard deviation around the peaks of November 2021 and lower variability during March 2022. Furthermore, site HB illustrated the same gradual increase over the 12 months as seen at MB.

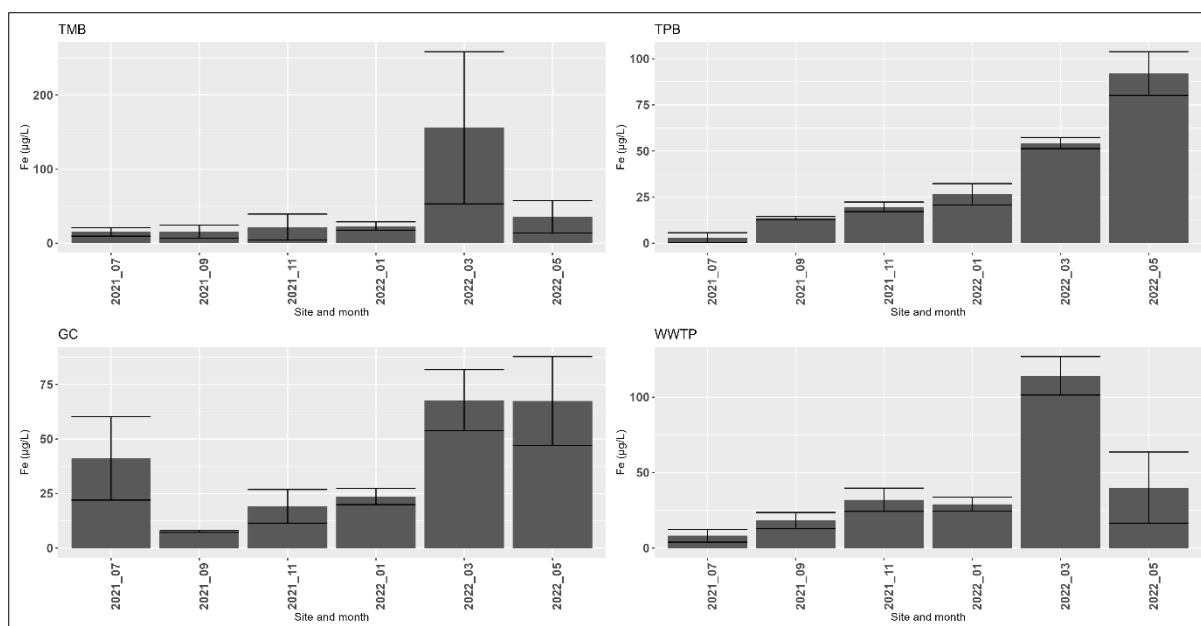


FIGURE 4.30: Iron concentrations at TMB, TPB, GC, and WWTP sites.

Sites TMB, TPB, GC and WWTP showcased the same gradual increase throughout the experimental phase of this study, as seen in Figure 4.30. Levels varied from as low as 5 µg/L, with peaks reaching 150 µg/L at TMB. Furthermore, TPB and WWTP illustrated highs of 75-100 µg/L and GC peaks up to 75 µg/L with high variability.

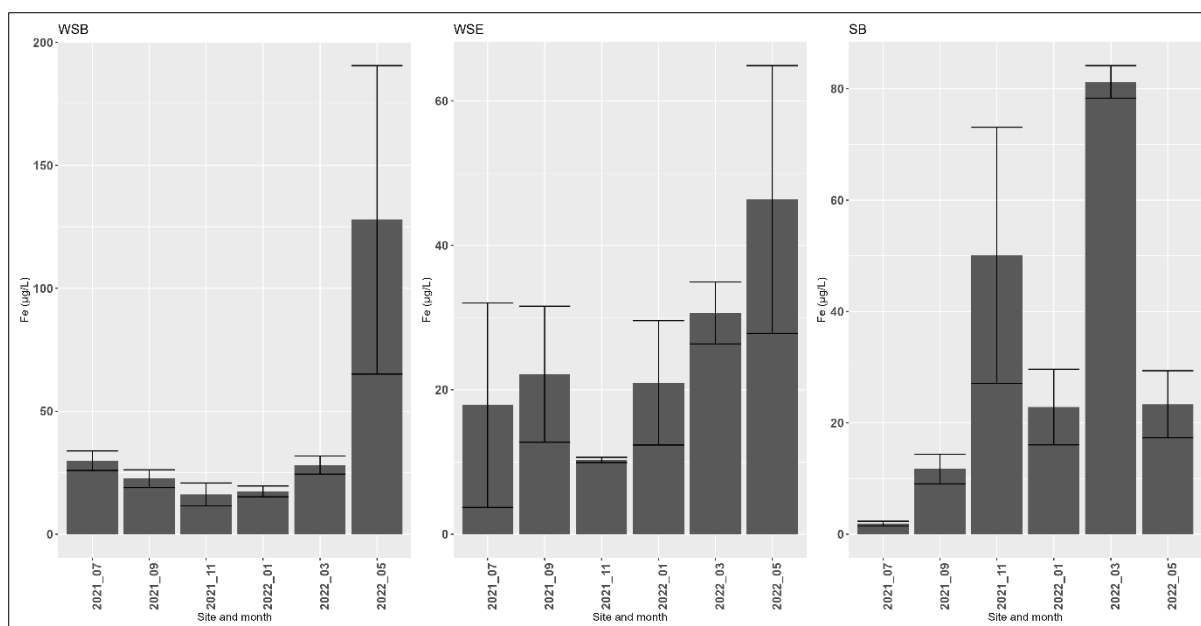


FIGURE 4.31: Iron concentrations at WSB, WSE, and SB sites.

As seen in Figure 4.31, iron levels at sites WSB, WSE and SB gradually increased each month, with the highest levels observed during March 2022 and May 2022. However, WSB shows higher iron concentrations overall than WSE and SB. Iron concentrations reached 150 µg/L at WSB but only 50 µg/L and 80 µg/L at WSE and SB, respectively.

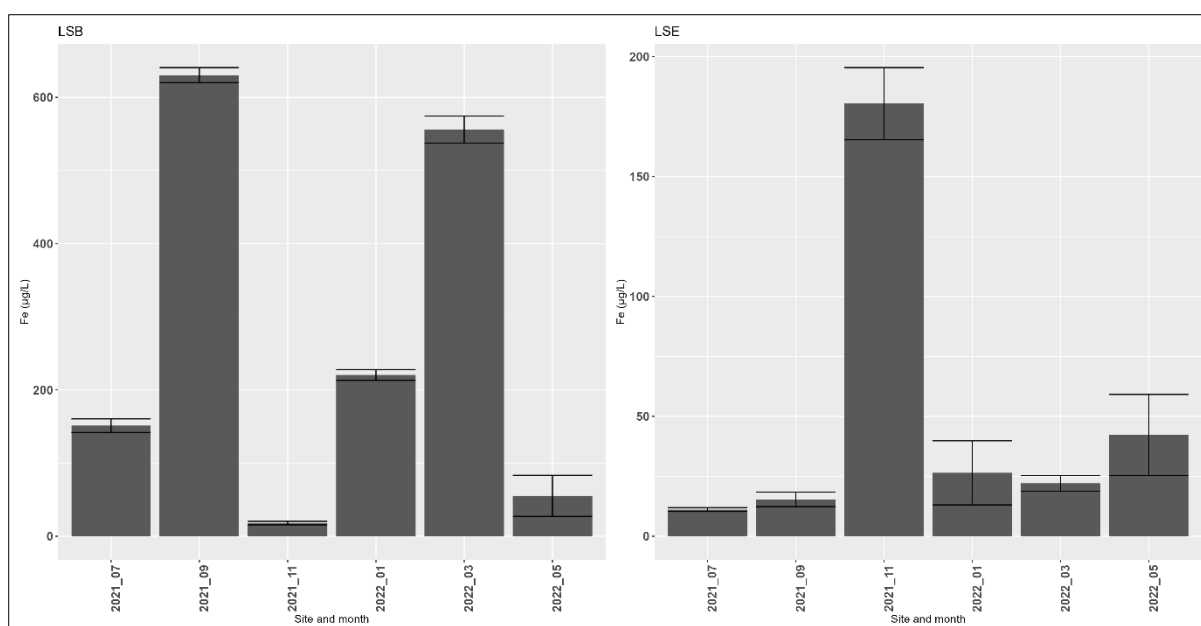


FIGURE 4.32: Iron concentrations at LSB and LSE sites.

Very high iron concentrations, 620 µg/mL, as seen in Figure 4.32, show that an episode of extreme magnitude of iron contamination occurred at LSB in September 2021. Low standard deviation confirms that this was not an isolated event. Another peak in March 2022 indicates a similar event. LSE shows lower iron concentrations, illustrating the downstream processes' filtering effect. A peak close to 200 µg/L in November 2021 may indicate a failure in said filtering system after the extremely high levels upstream at LSB in September 2021.

4.1.5.5 Phosphorus

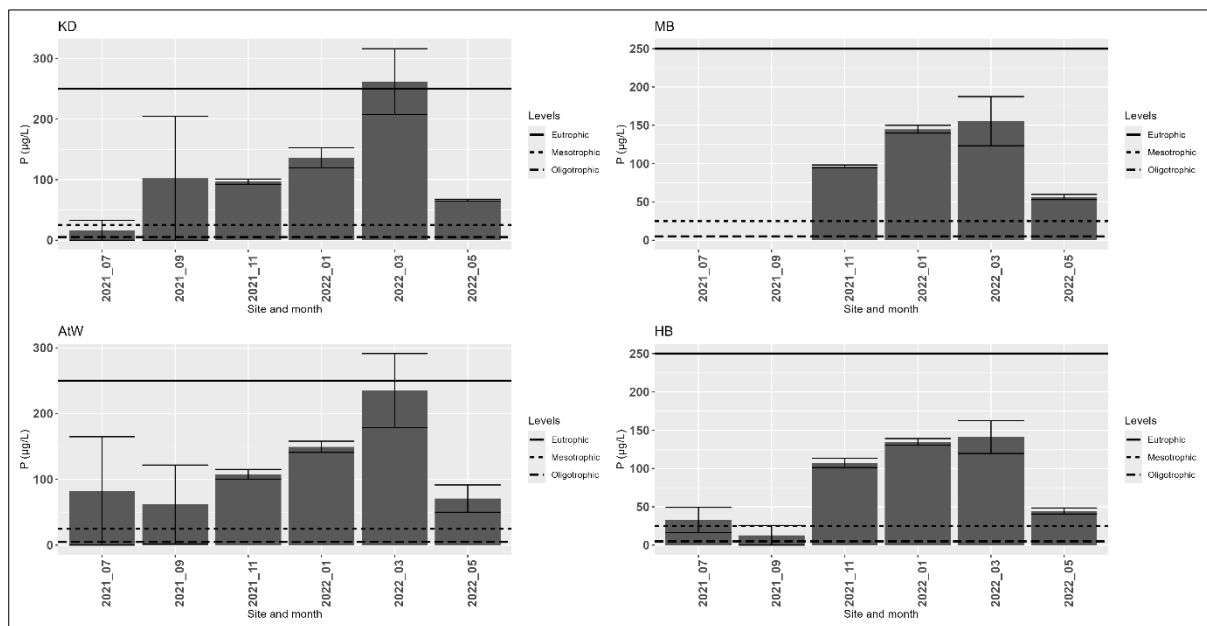


FIGURE 4.33: Phosphorus concentrations at KD, MB, AtW, and HB sites.

Figure 4.33 shows that phosphorus levels increased notably towards March of 2022. Concentrations above 250 µg/L indicate a eutrophic environment; most months exceed the mesotrophic threshold, indicating high nutrient enrichment. Relatively stable concentrations, but still above the mesotrophic threshold, can be seen at MB, with peaks in January and March 2022. AtW illustrated high baseline phosphorus concentrations across the year, fluctuating between eutrophic and mesotrophic status. Furthermore, high variability during the peaks in March 2022 indicates a probable isolated event. HB phosphorus levels remained well below the eutrophic status but within a mesotrophic state except during September 2021, when an oligotrophic status was almost reached.

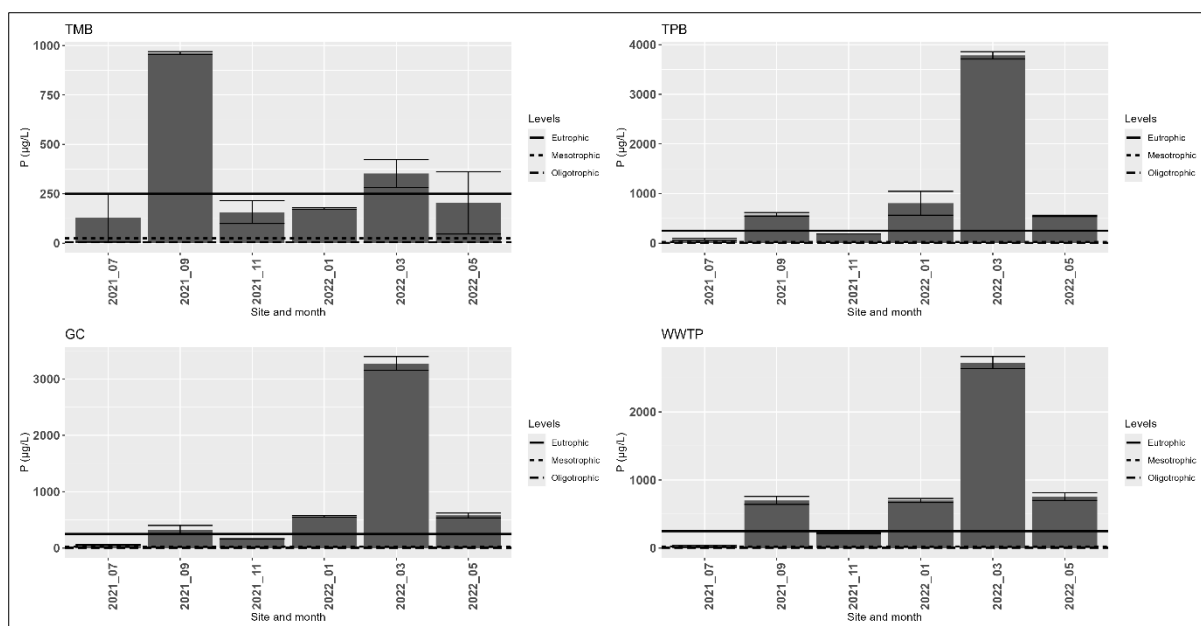


FIGURE 4.34: Phosphorus concentrations at TMB, TPB, GC, and WWTP sites.

Phosphorus levels in Figure 4.34 demonstrated alarmingly high concentrations. Overall, the baseline at each site is very high, exceeding the eutrophic boundary of 250 µg/L. However, peaks up to 1000 µg/L at TMB and 2800 µg/L to 3600 µg/L at TPB, GC, and WWTP illustrated immense phosphorus contamination and environmental enrichment.

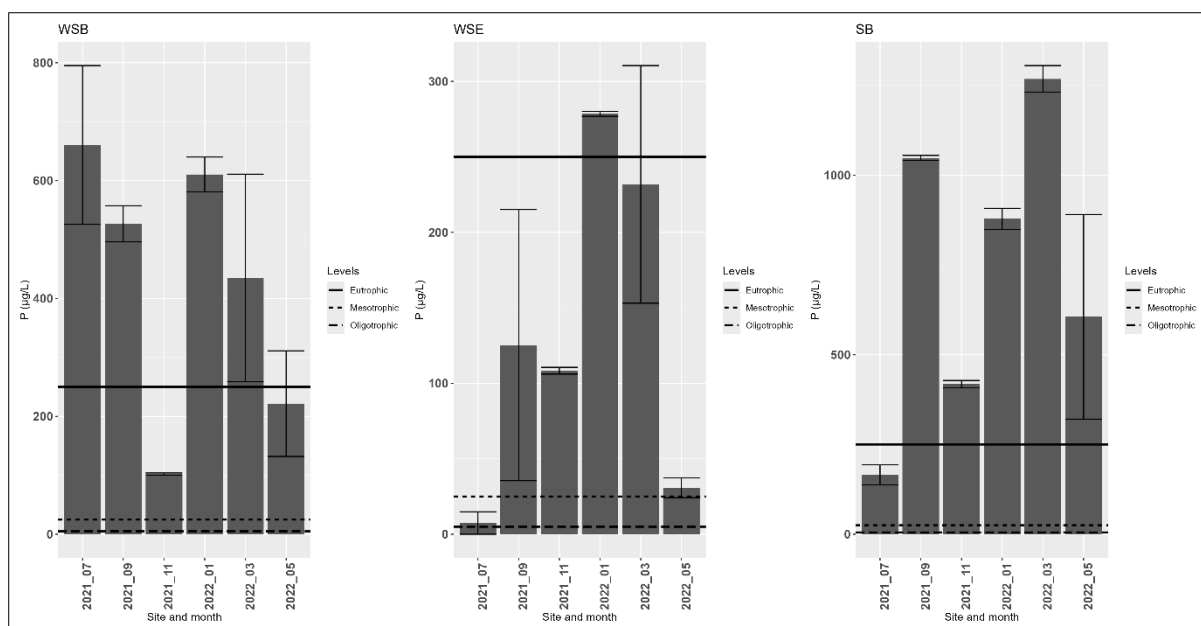


FIGURE 4.35: Phosphorus concentrations at WSB, WSE, and SB sites.

Acute levels well above the eutrophication threshold were observed at the WSB site, as seen in Figure 4.35. Furthermore, reduced concentrations downstream at WSE one month above the limit indicated a successful natural wetland and foliage filtering effect. Phosphorus concentrations at SB show further fertiliser runoff due to surrounding farms and other agricultural activities. Levels peak at about 1500 µg/L with low variability.

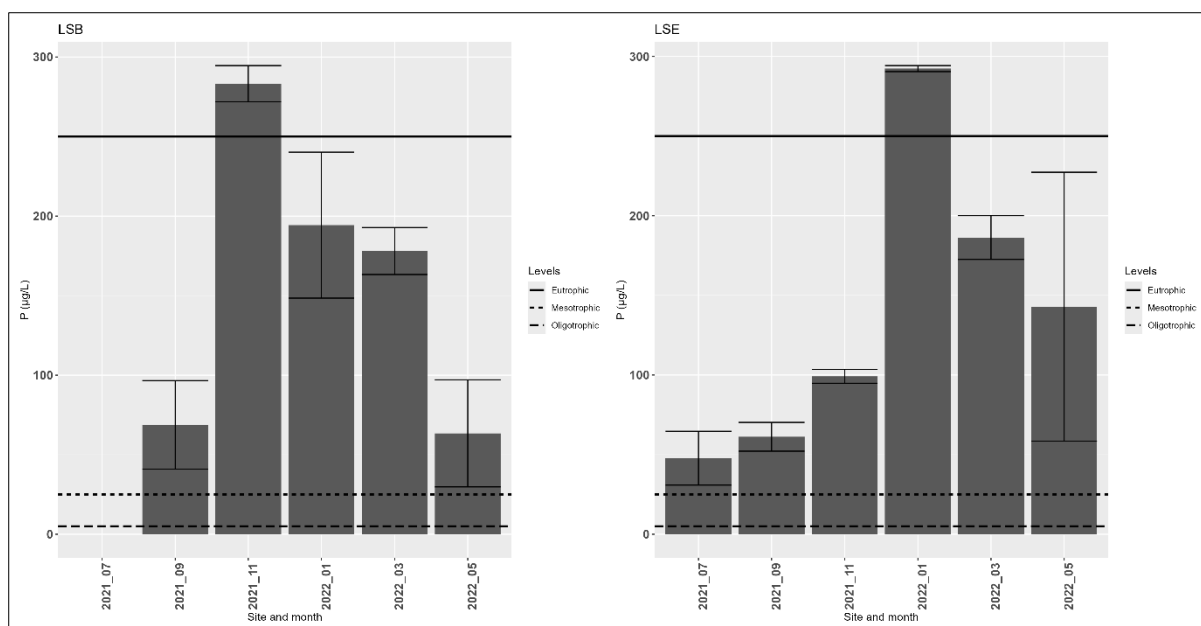


FIGURE 4.36: Phosphorus concentrations at LSB and LSE sites.

Sites LSB and LSE illustrated lower phosphorus concentrations compared to other sites but still overreached the eutrophic threshold of 250 $\mu\text{g/L}$ with peaks up to 300 $\mu\text{g/L}$ at each site. Figure 4.36 further shows a gradual increase in phosphorus concentration at LSE.

4.1.5.6 Magnesium

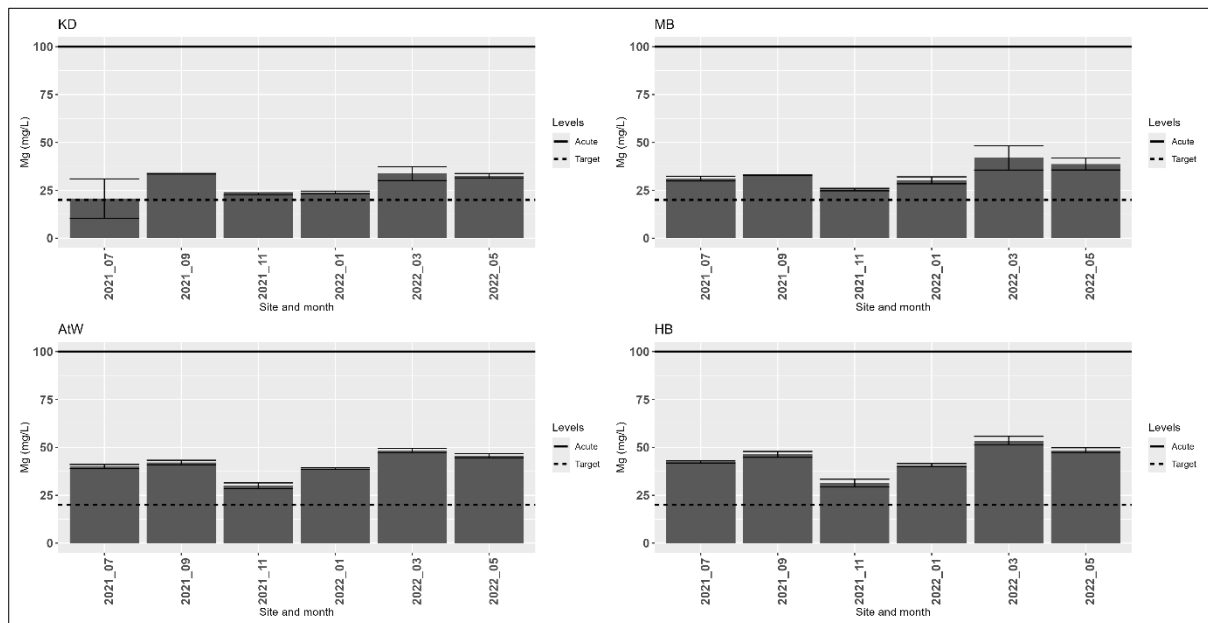


FIGURE 4.37: Magnesium concentrations at KD, MB, AtW, and HB sites.

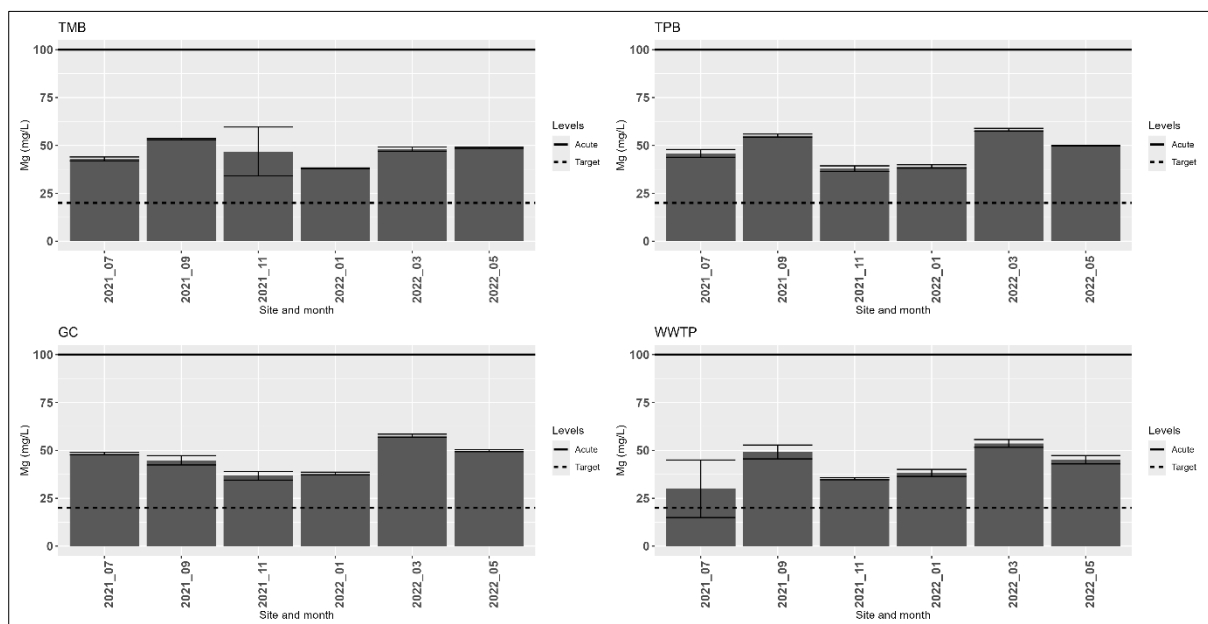


FIGURE 4.38: Magnesium concentrations at TMB, TPB, GC, and WWTP sites.

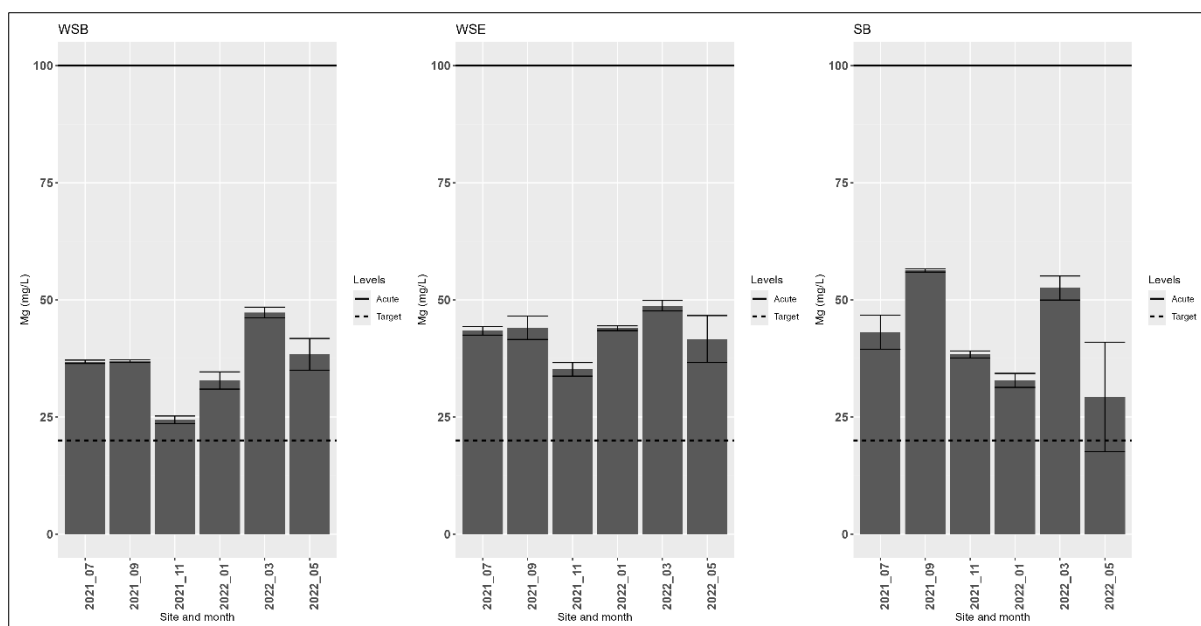


FIGURE 4.39: Magnesium concentrations at WSB, WSE, and SB sites.

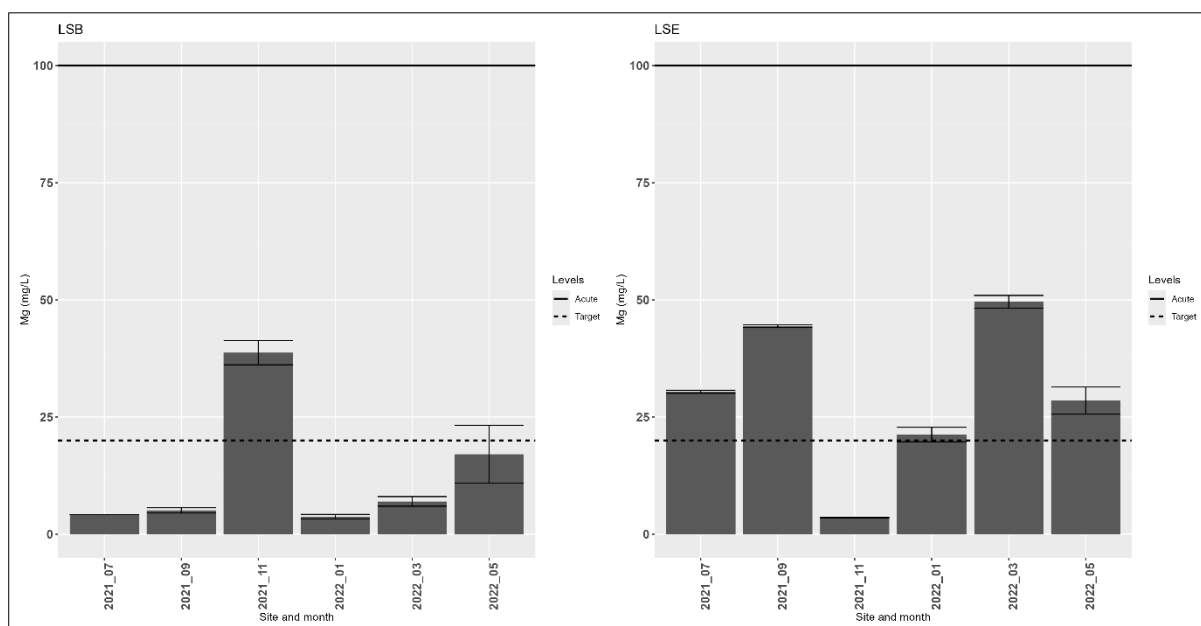


FIGURE 4.40: Magnesium concentrations at LSB and LSE sites.

Figures 4.37 to 4.40 illustrate the magnesium concentrations across all the sites. Concentrations were observed to be rather stable, ranging between the standard value of 24mg/L and up to 55mg/L, never surpassing the acute acceptable limit of 100 mg/L (Dallas & Day, 2004; DWAF, 1996). LSB and LSE are the only sites showing magnesium concentrations below the target value.

4.1.5.7 Potassium

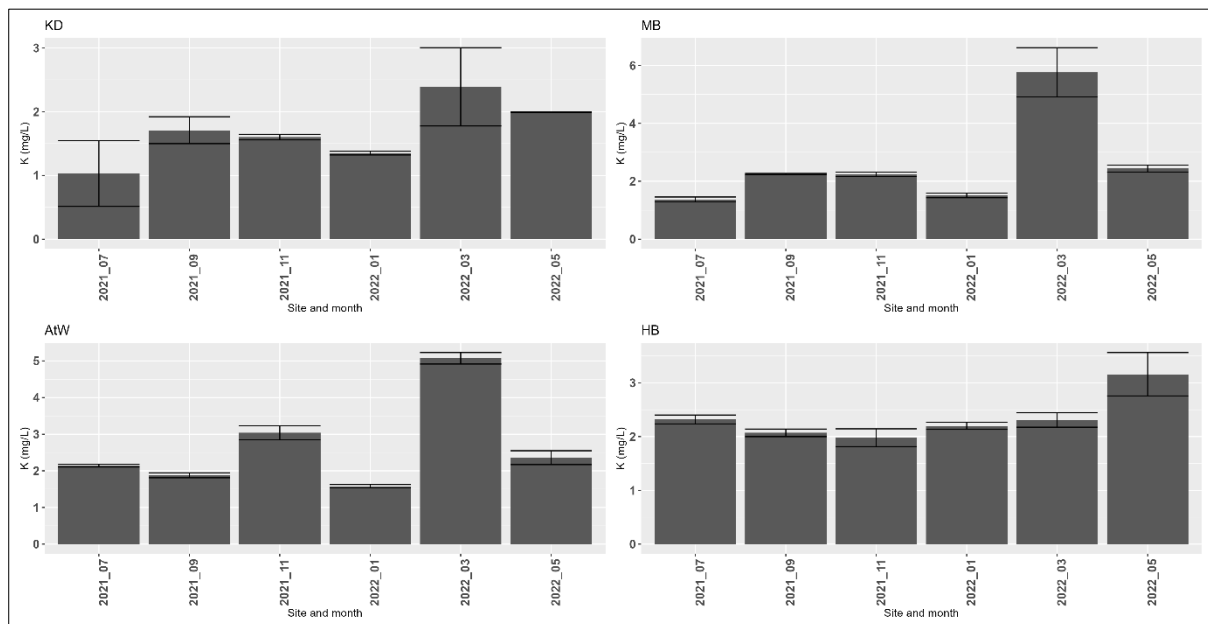


FIGURE 4.41: Potassium concentrations at KD, MB, AtW, and HB sites.

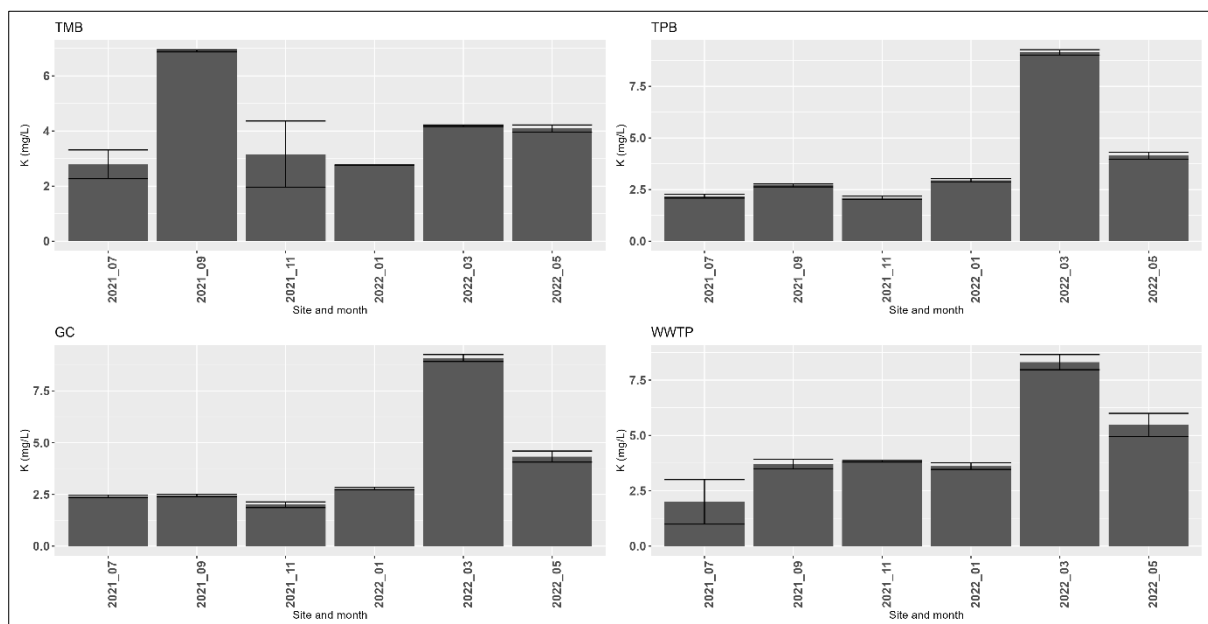


FIGURE 4.42: Potassium concentrations at TMB, TPB, GC, and WWTP sites.

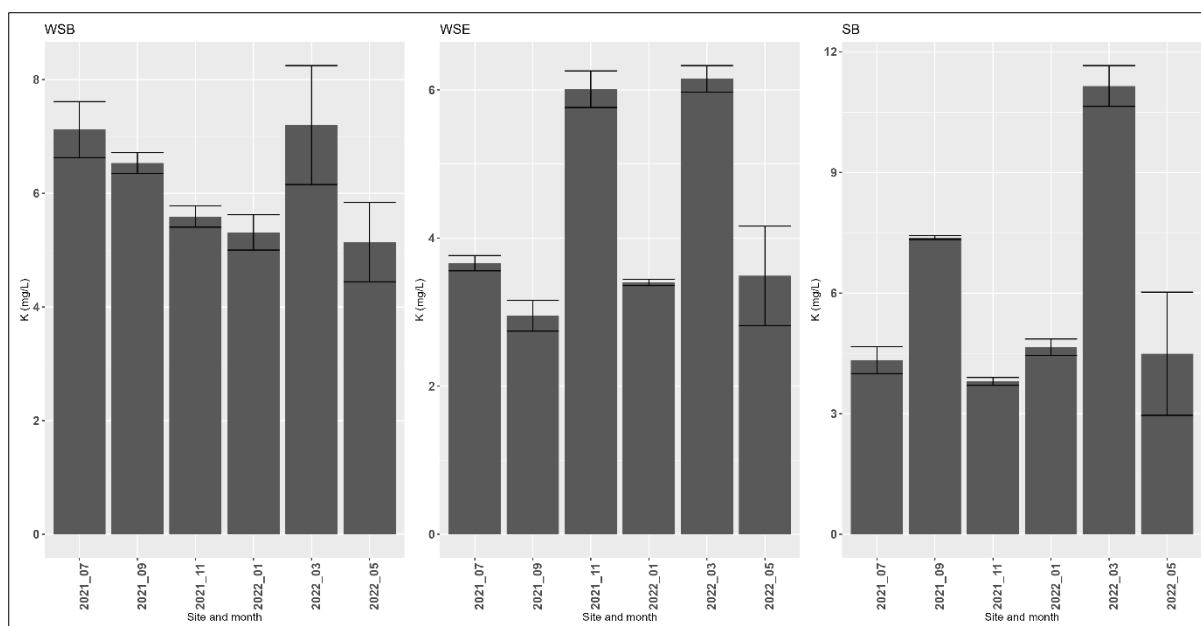


FIGURE 4.43: Potassium concentrations at WSB, WSE, and SB sites.

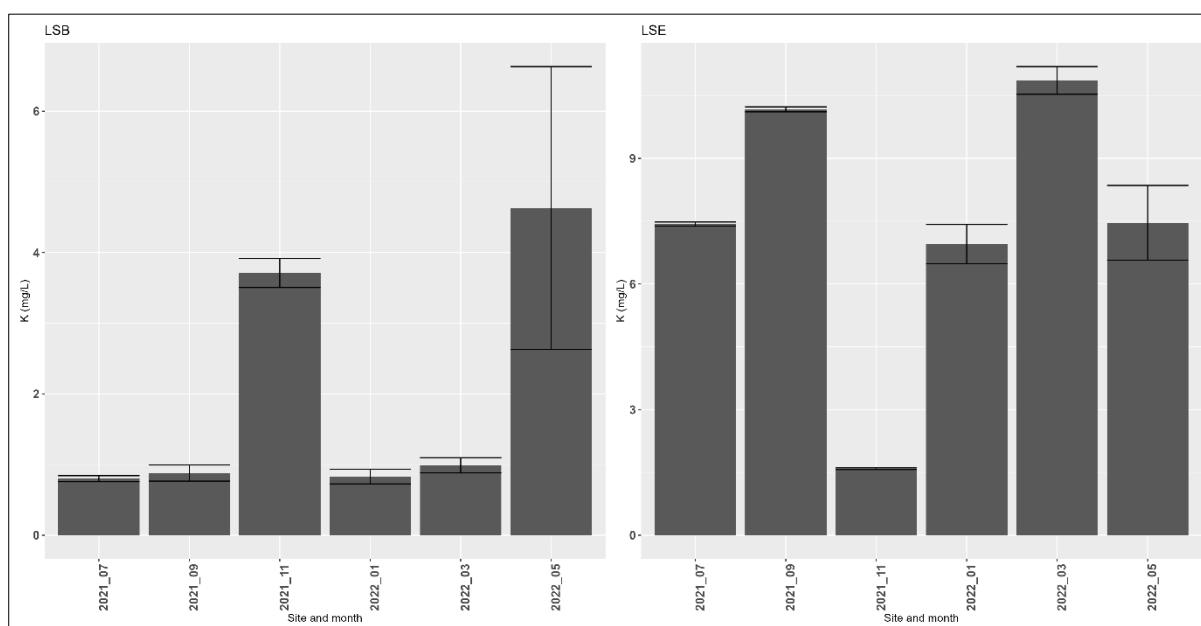


FIGURE 4.44: Potassium concentrations at LSB and LSE sites.

Figures 4.40 to 4.44 illustrate the seasonal and temporal fluctuations in potassium concentrations throughout all 13 sites. Levels across all sites were generally below critical thresholds (Dallas & Day, 2004; DWAF, 1996), but March 2022 was a critical point along the whole catchment, with peaks at numerous sites indicating a localised event or environmental influence.

4.1.5.8 Arsenic

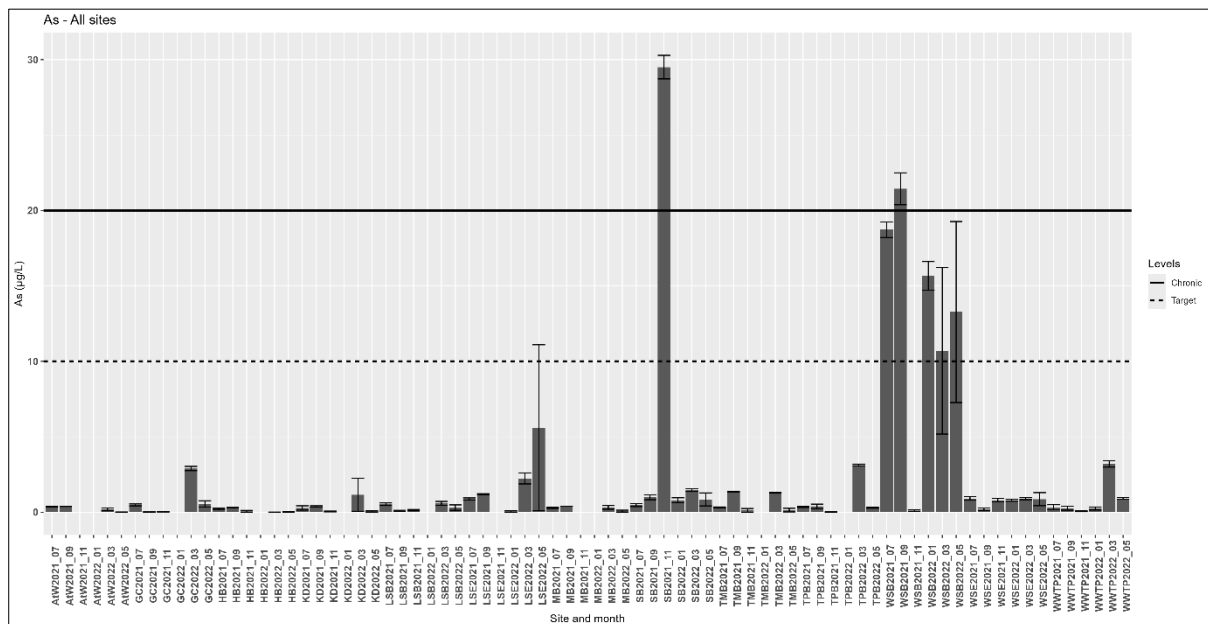


FIGURE 4.45: Arsenic concentrations across all 13 sites.

In Figure 4.45, concentrations of arsenic over a 12-month period across 13 sites have been plotted. Most sites elicited concentrations well below the safe target threshold of $<10 \mu\text{g/L}$ for arsenic in aquatic ecosystems (DWAF, 1996). Notable sites with high levels of arsenic are SB in November 2021, with levels up to $30 \mu\text{g/L}$. WSB continually had very high levels of arsenic above the acceptable target threshold. Concentrations vary from $11 \mu\text{g/L}$ up to $22 \mu\text{g/L}$ over the 12 months, surpassing the chronic level of $20 \mu\text{g/L}$. Elevated arsenic levels further tended to cluster around September 2021 and March to May 2022, indicating potential seasonal or episodic contamination sources.

4.5.1.9 Barium

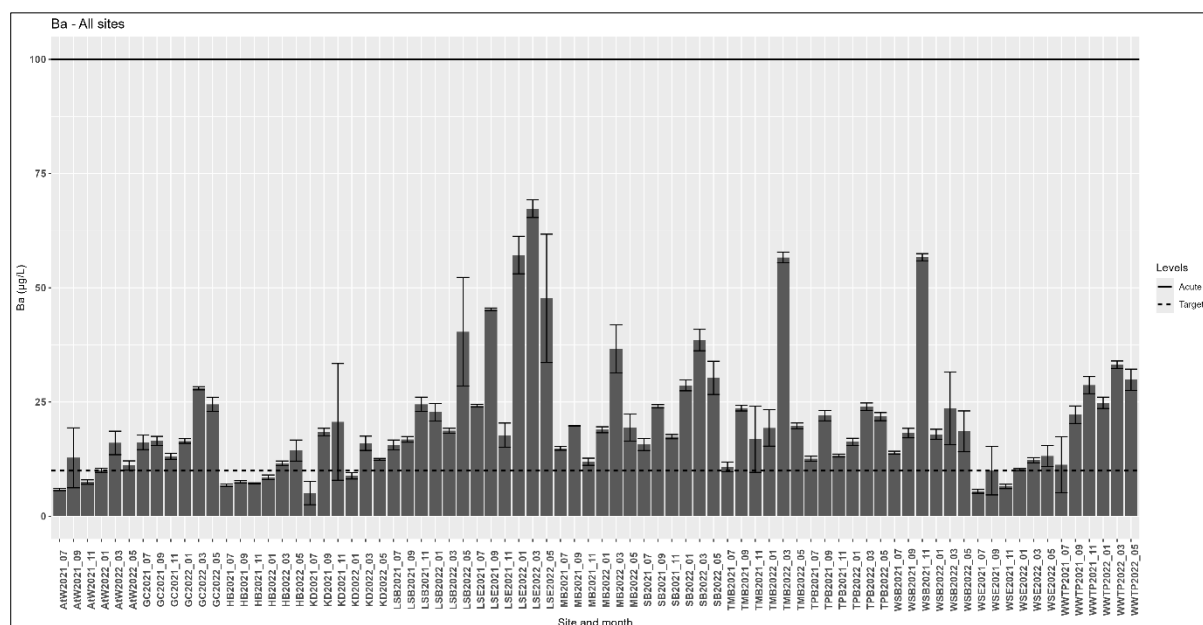


FIGURE 4.46: Barium concentrations across all 13 sites.

Figure 4.46 depicts barium concentration during the study period. All levels remained between the accepted target level safe for aquatic ecosystems and the acute threshold. Noticeable peaks arise at the following sites: GC, LSB, LSE, MB, SB, TMB and WSB. Levels ranged between 10 µg/L with peaks as high as 70 µg/L. Seasonal fluctuations occurred, but noticeable peaks occur during March and May of 2022. A high standard deviation among peaks might indicate temporal or isolated contamination events.

4.1.5.10 Boron

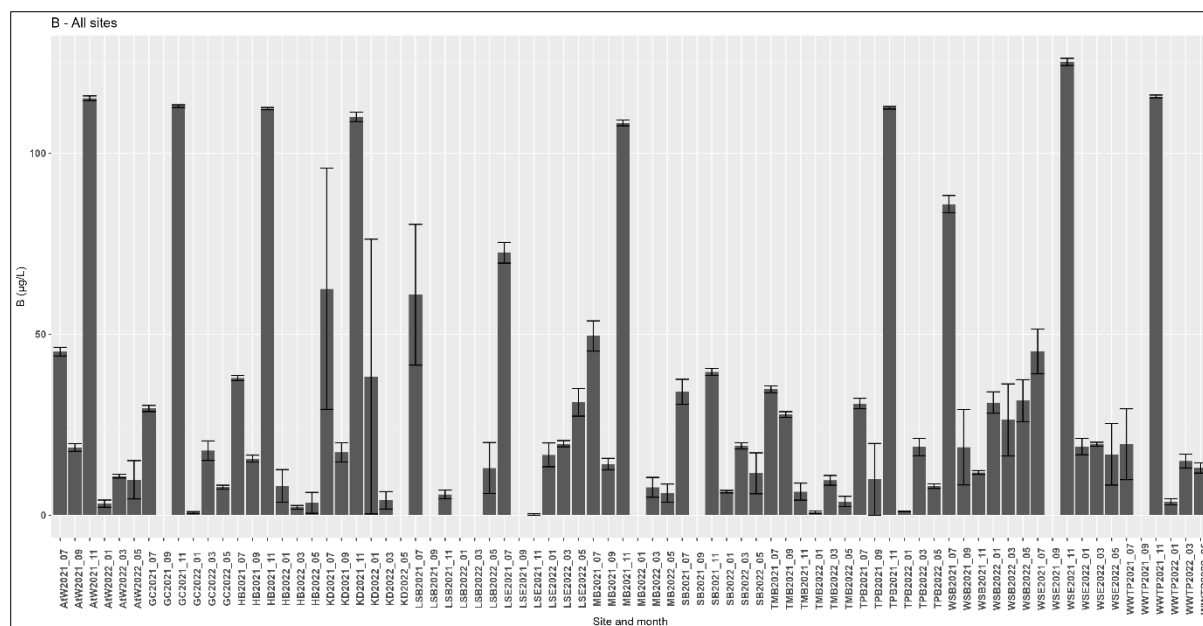


FIGURE 4.47: Boron concentrations across all 13 sites.

Figure 4.47 shows the fluctuation of boron concentration during the study. Since no apparent trend is visible per site, it is evident that some sort of localised contamination event occurred during November 2022 since eight of the sites show significant increases reaching up to 120 µg/L. Inversely, LSB and LSE showed concurrent months of zero levels, indicating no external influences.

4.1.5.11 Manganese

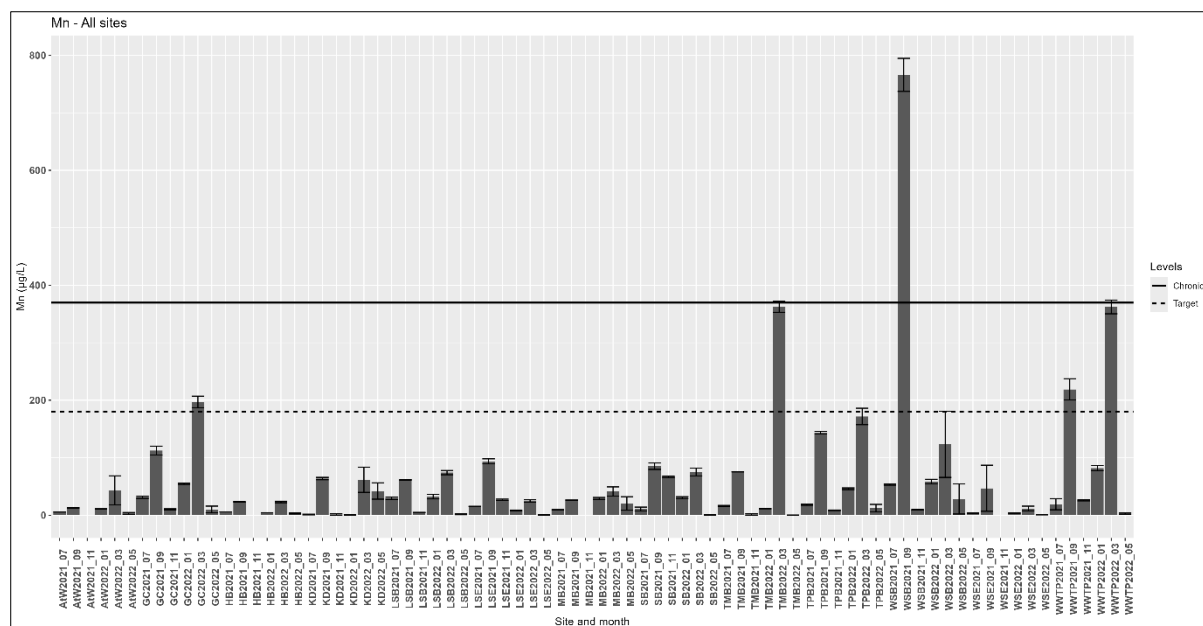
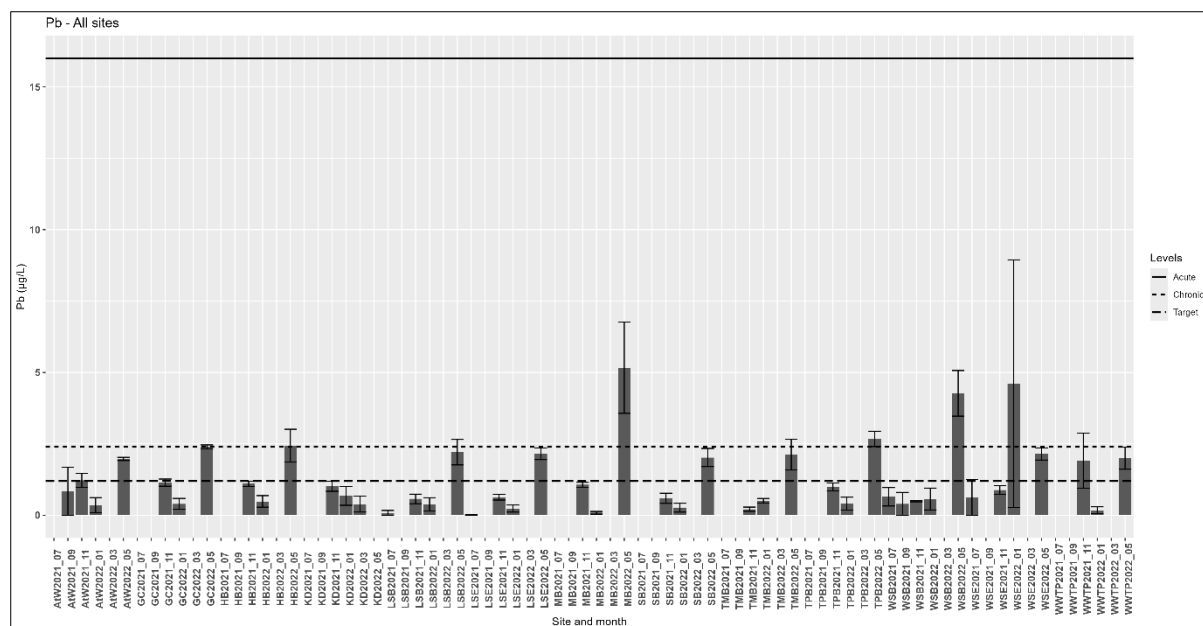


FIGURE 4.48: Manganese concentrations across all 13 sites.

Manganese levels were predominantly observed below the target threshold of <180 µg/L, with the exception of a few sites and dates. Specifically, GC indicated a peak above the target threshold of up to 200 µg/L. Furthermore, in March 2022, both TMB and WWTP illustrated spikes close to the chronic level of 370 µg/L. WSB had manganese concentrations well above the accepted chronic limit as seen in figure 4.48, closing in on the acute limit (1300 µg/L) with about 800 µg/L values.

4.1.5.12 Lead



4.1.5.13 Selenium

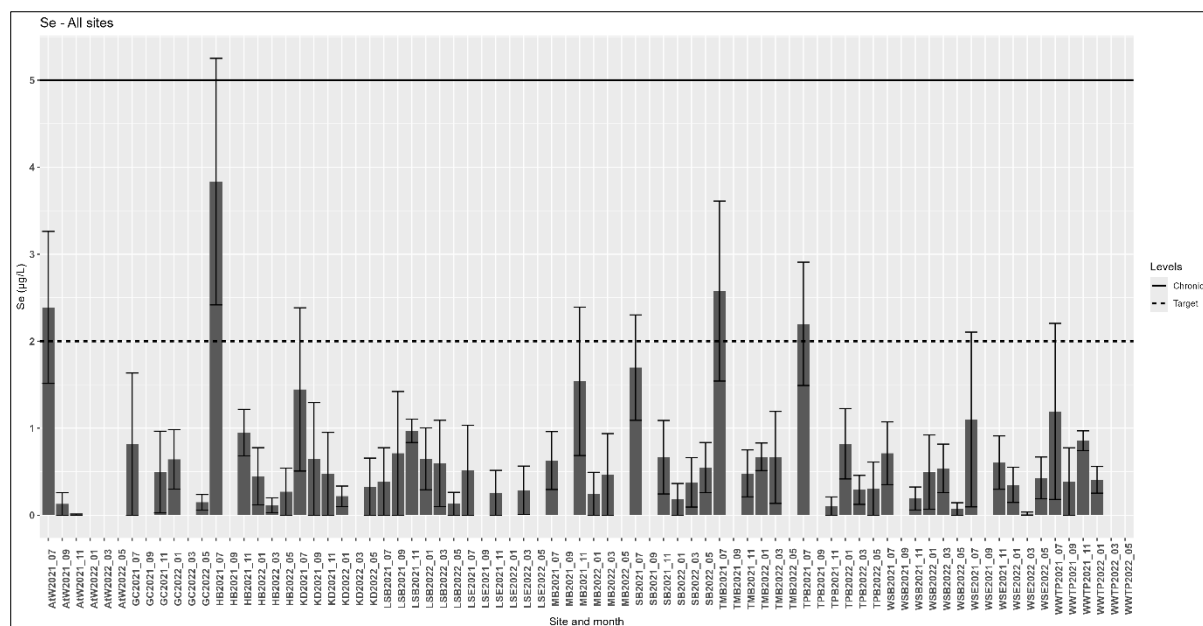


FIGURE 4.50: Selenium concentrations across all 13 sites.

With a target quality value of $<2 \mu\text{g/L}$, most sites prevailed with concentrations that comply with set standards, as seen in Figure 4.50. Peaks among four sites were observed, indicating values above the target threshold. These sites were AtW, HB, TMB and TPB, all during the July 2021 sampling month. According to the figure's error bars, these peaks have a high standard deviation range. Since all other sites have low concentrations of selenium, these high values could be due to an episodic contamination event in July 2021.

4.1.5.14 Vanadium

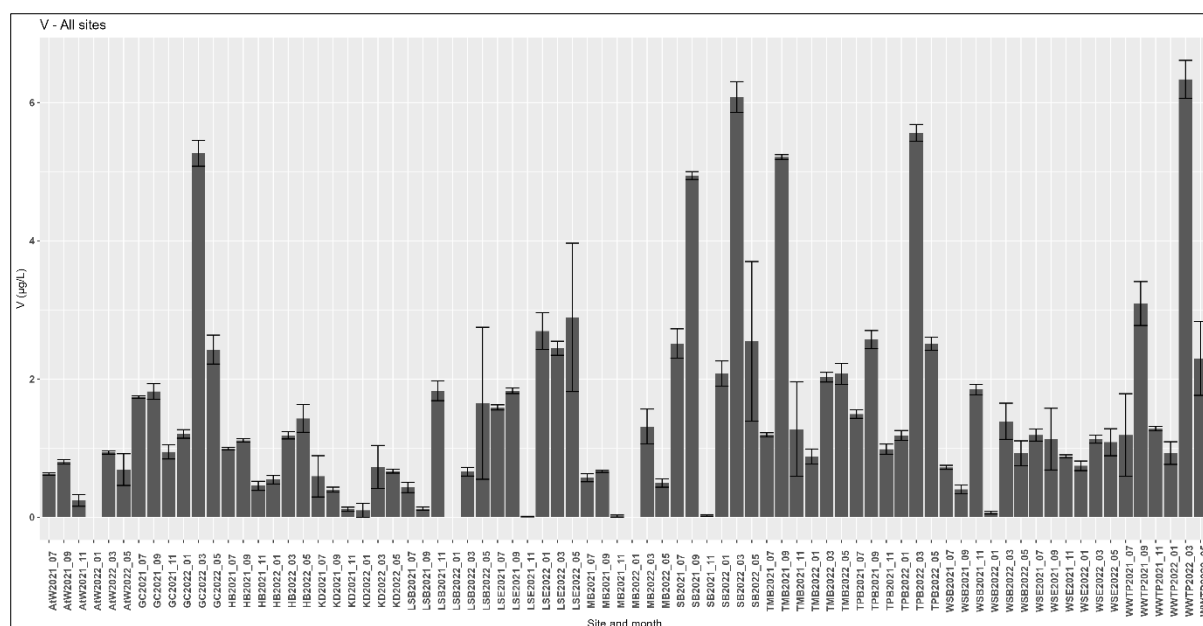


FIGURE 4.51: Vanadium concentrations across all 13 sites.

The vanadium concentration along most sites remained below 2 µg/L, but variability was seen across all sites. As seen in Figure 4.51, spikes in vanadium concentrations range from 5 to 7 µg/L. These increases occurred predominantly from March to May of 2022, with a decrease during the following months. This fluctuation might have indicated seasonal variation due to decreased runoff or precipitation.

4.1.5.15 Zinc

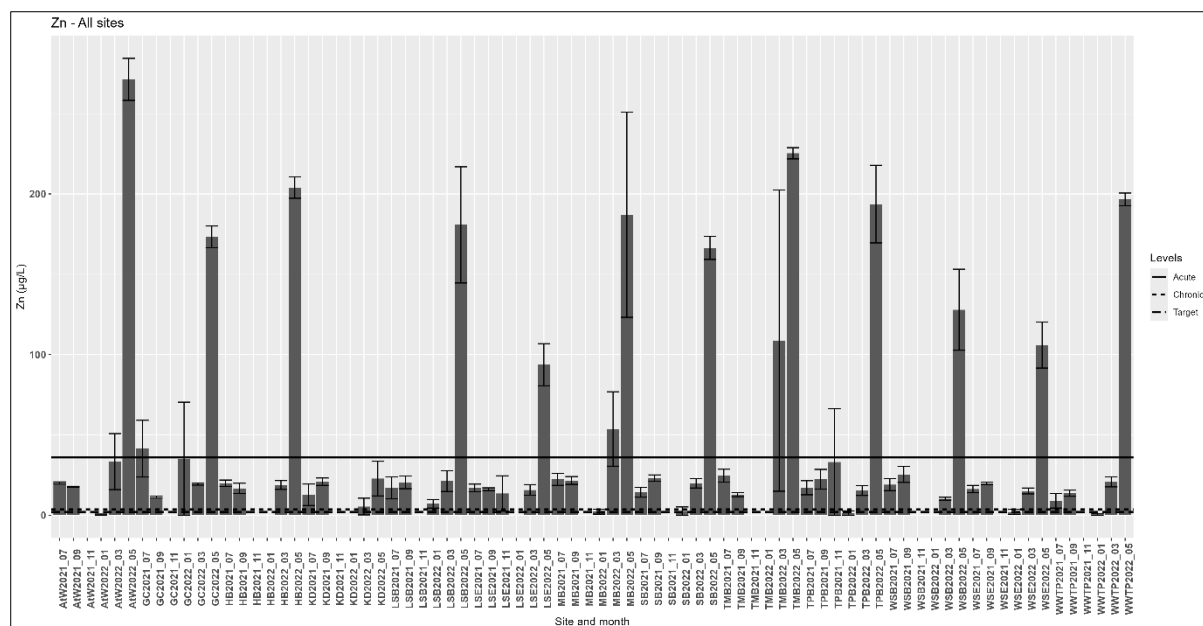


FIGURE 4.52: Zinc concentrations across all 13 sites.

Some zinc concentrations at specific sites like AtW, HB, GC and LSB during November 2021 were observed as limited or zero. However, most sites indicated values exceeding the chronic threshold of 3.6 µg/L and below the acute limit of 36 µg/L. There are noticeable peaks that do not comply with the trends observed. All sites, except KD, spiked well above the acute level, all during May 2022. Peaks indicate values between 100 µg/L and 300 µg/L.

4.2 16S rRNA

Bacterial identification was done by means of NGS of the 16S rRNA barcode to determine microbial abundance and various diversity indices (including richness, diversity and similarity). Multivariate analyses were utilised to evaluate the relationship between community composition and environmental variables. The results are reported in Figures 4.53 to 4.62 below.

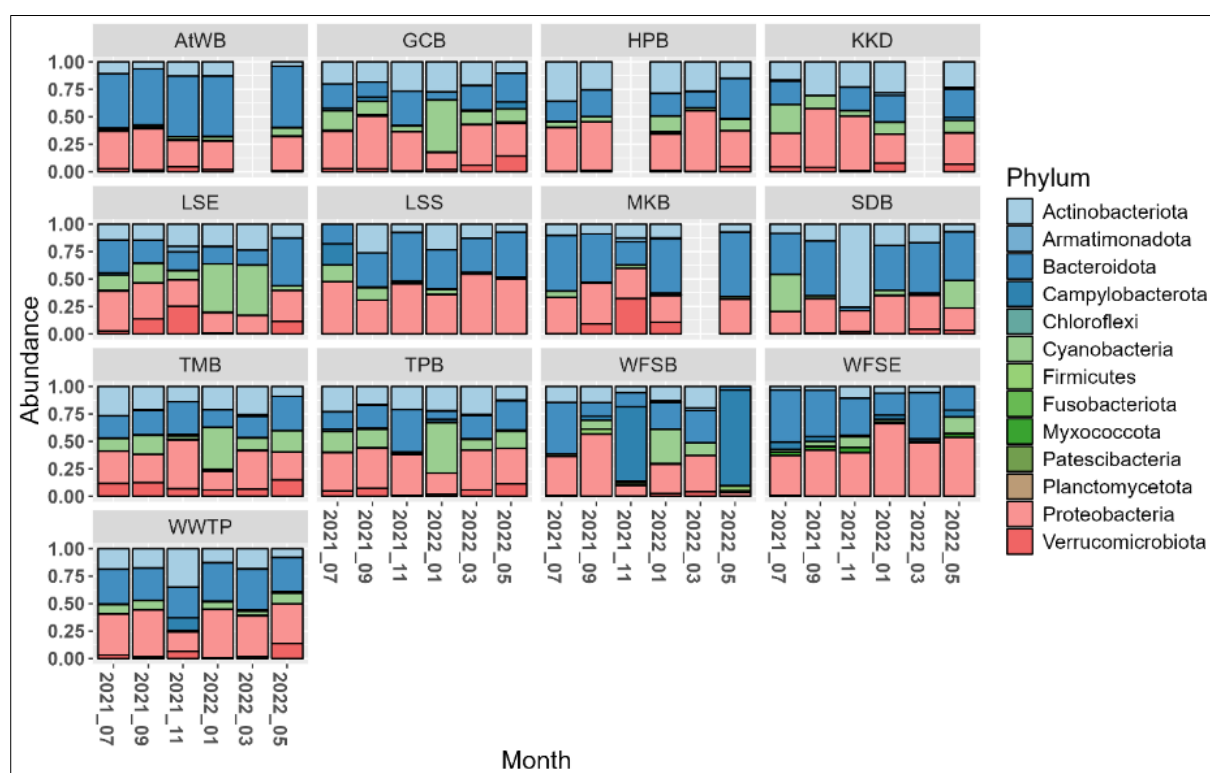


FIGURE 4.53: Abundance at phylum level (16S).

Figure 4.53 illustrates the abundance of species per phylum associated per site across the 12-month study period. *Bacteroidota* occurred at various sites, including AtWB, HPB, LSE, MKB, SDB, WFSB, WFSE and WWTP. Abundance ranged from 24% to 50% across these sites. *Proteobacteria* could be found in large amounts at LSB, TPB, WSE, and WWTP. Abundance ranged from 15% at GC in January 2022 to 65.81% in January 2022 at WSE. *Actinobacteriota* occurred in varying ranges from 18% at GC, 35% at HB, 22 to 30% at KKD and up to 75% at the SB site in November 2021. *Verrucomicrobiota* occurred in smaller amounts, with abundance varying between 2 and 10%, with the most prevalence at MB in November 2021 at 32.21%.

Cyanobacteria indicated very low levels overall but some occurrence at some sites. Some of the sites with the most prevalence for *Cyanobacteria* were at GC in January 2022 with 46.96% abundance, an average of 44% at LSE in January and March 2022 and 45.98% at TPB in January 2022.

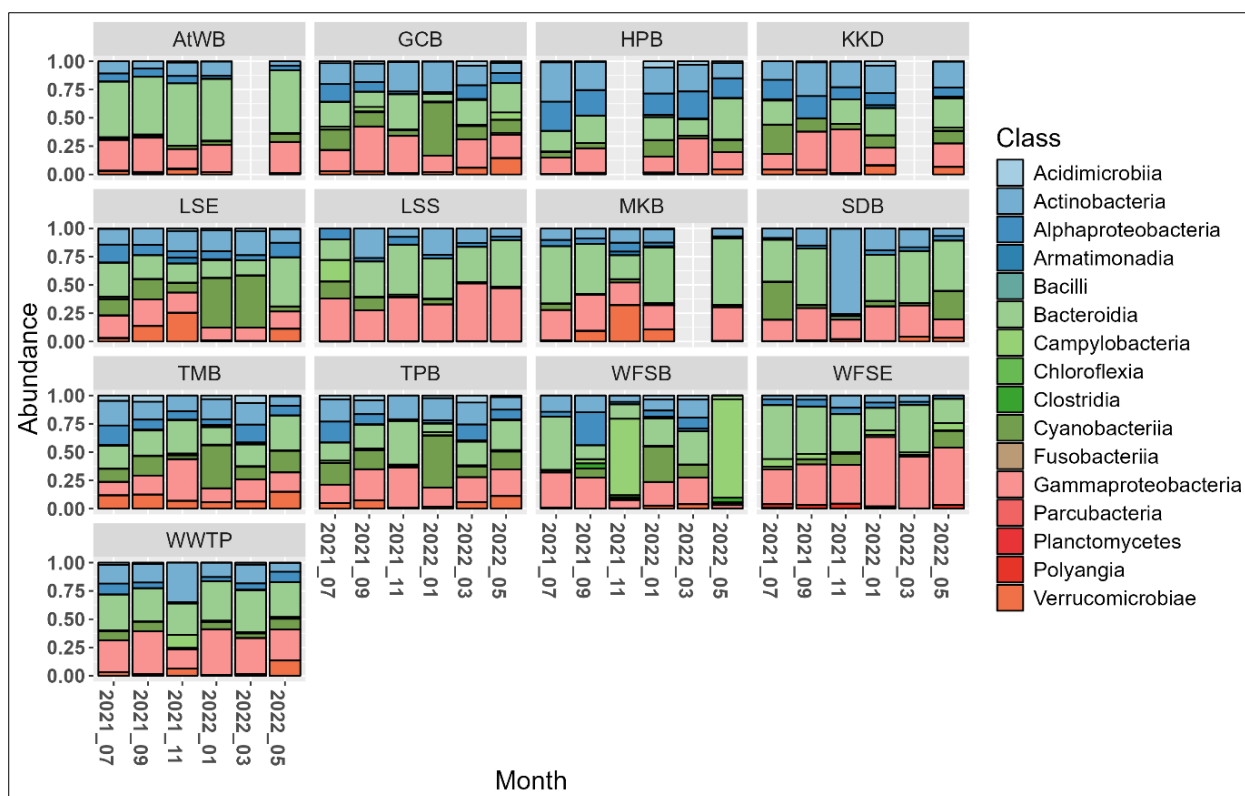


FIGURE 4.54: Abundance at class level (16S).

As reported in Figure 4.54 above, *Bacteroidia* dominated across various sites, being the most prevalent class at 34 out of 78 sampling points, peaking at 58.93% at MKB in May 2022. *Gammaproteobacteria* was the most prevalent, with an abundance of 61.16% at WFSE in January 2022. Their abundance represented a noticeable percentage at all sites, always being in the top 3 of the most abundant classes. *Actinobacteria* had a large representation at GCB, HPB, and KKD, ranging from 16.43% to 34.66%. However, they were the most abundant at SPB in November 2021, representing 75.58% of all bacteria present. *Cyanobacteria* was the most dominant class, with 46.96% at GCB in January 2022. Their abundance varied across all sites but indicated high dominance at LSE in January-March 2022 and TPB in January 2022 with 43.91%, 45.82% and 45.99%, respectively. *Alphaproteobacteria* had a mean abundance between 8-20%, but the site with the highest *Alphaproteobacteria* levels was WFSB in September 2021 with 29.11%.

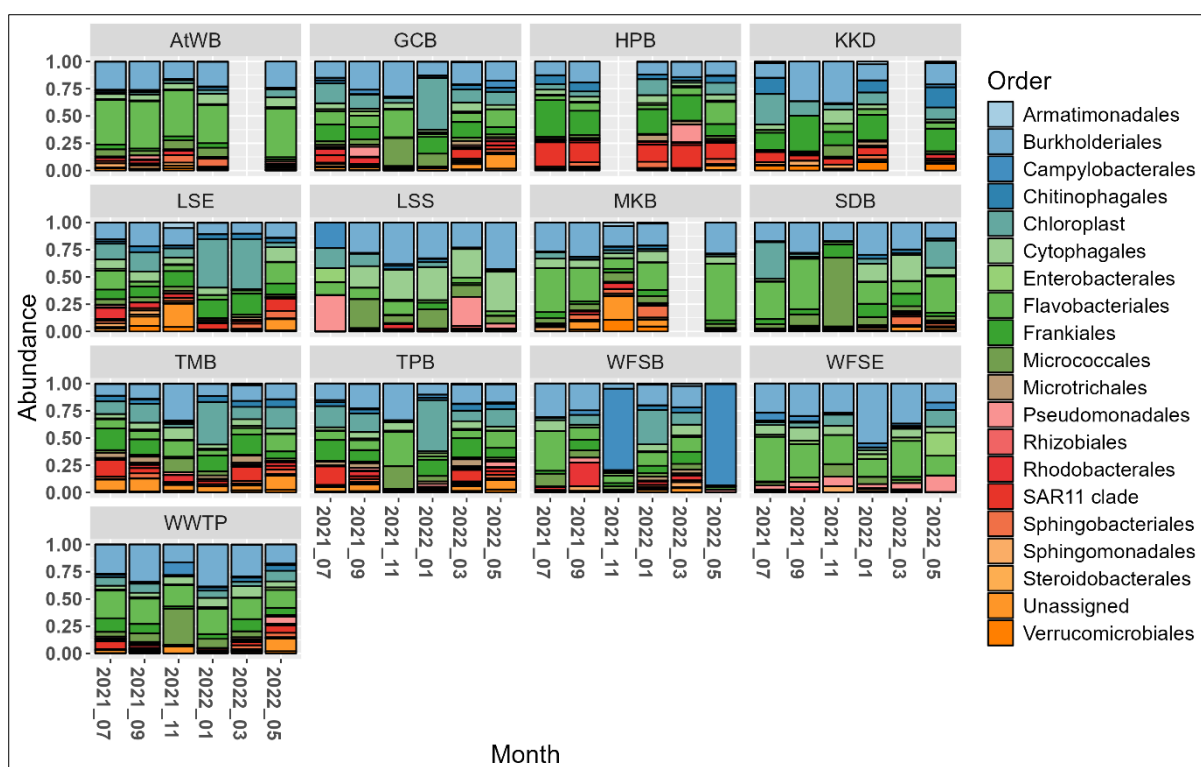


FIGURE 4.55: Abundance at order level (16S).

As seen in Figure 4.55, *Burkholderiales* indicated good representation with an average abundance of 21.72% across all sites and dates. The site which indicated the highest abundance of *Burkholderiales* was WSE in January 2022, with 54.83%, and the lowest abundance of 0.66% at WSB in May 2022. *Flavobacteriales* were the most prevalent per site at AtW, with an average abundance across the 12 months of 41.20%. The site that had the highest *Flavobacteriales* abundance was MB in May 2022, with 51.73%. *Chloroplast* had the highest representation at GC in January 2022, with 47.66%, whereas SB had the lowest abundance in November 2021, with 0.14%. A noticeable occurrence at WSB indicated that during the months of November 2021 and May 2022, *Campylobacteriales* were 74.62% and 92.79%, respectively, of all the sequences present.

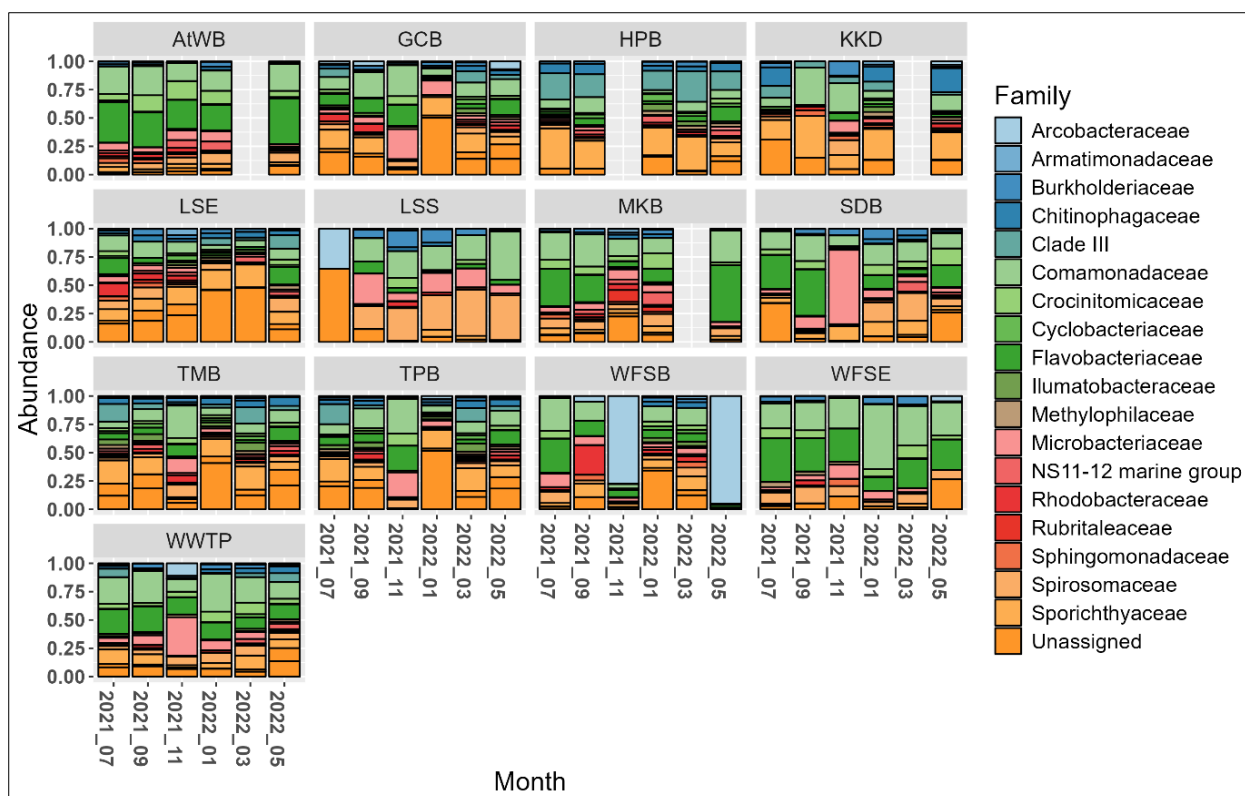


FIGURE 4.56: Abundance at family level (16S).

Comamonadaceae was the family with the highest average abundance across 78 sampling points (17.47%), as seen in Figure 4.56. The site where *Comamonadaceae* was the most dominant was at site WSE in January 2022 (57.09%) and the least dominant at 2 sites with 0% abundance, LSB in July 2021 and WSB in May 2022. AtW was the site with the highest dominance of *Flavobacteriaceae*, with an average of 31.11% across the six respective months. The one sampling site with the highest dominance of *Flavobacteriaceae* was MB in May 2022, with 50.20%. Of the 78 sampling points and their respective dates, 12.77% of all the sequences could not be identified since they are Unassigned. This is the most prevalent at LSB in July 2021, at 64.56%. A noticeable family was *Microbacteriaceae*, which has an average abundance across all 78 sampling points of 7.02%; however, at SB in November 2021, this family represented 65.69%, dominating this site.

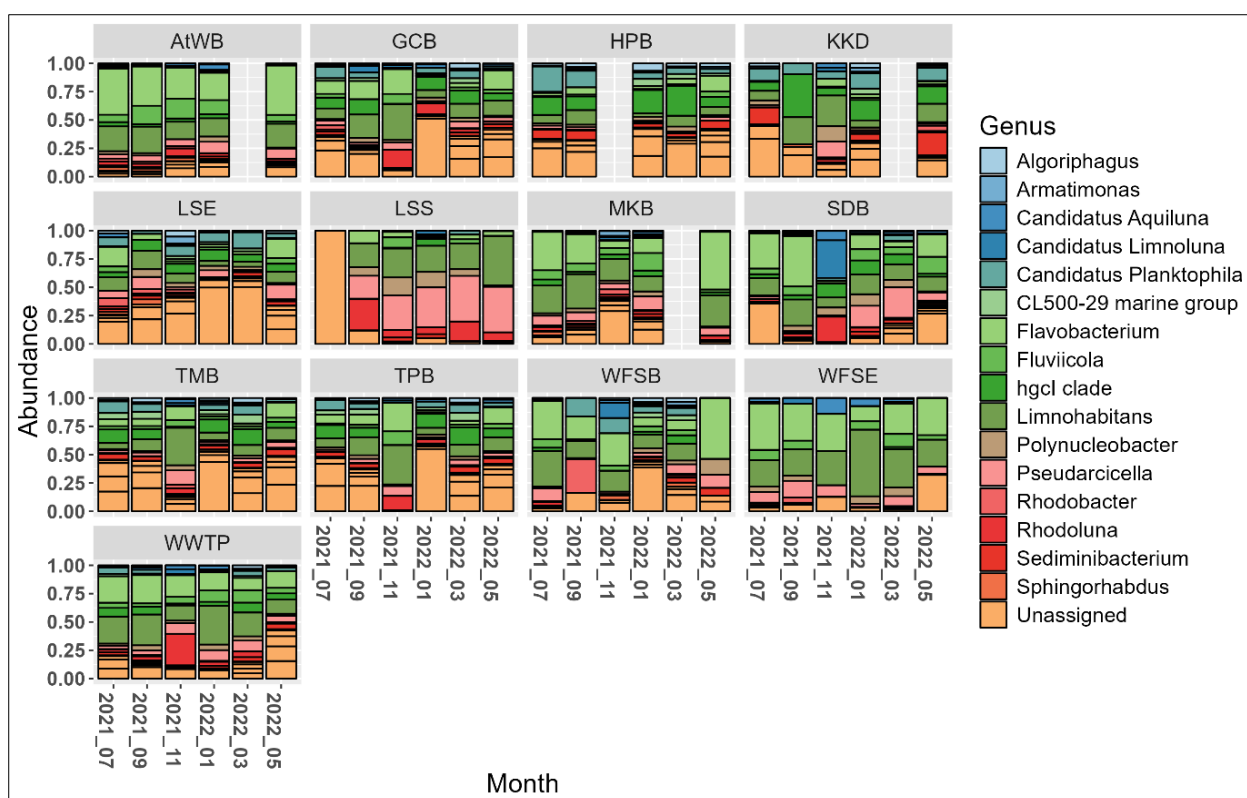


FIGURE 4.57: Abundance at genus level (16S).

Figure 4.57 above illustrates genera abundance across the study period. *Limnohabitans* were the most abundant genus across all sites, with 17.47% and the most dominant at WSE in January 2022 at 58.88%. *Flavobacterium* showed similar abundance with 15.55% across all sites, and the site at which it was reported the most was WSB in May 2022 with 53.70%. The abundance scores started to decline, with an average abundance of 7.82%; *Pseudarcicella* showed the most abundance at LSS in March 2022, with 40.40%. However, the highest average abundance across the whole study period was the sum total of all unassigned ASVs, representing 25.10%.

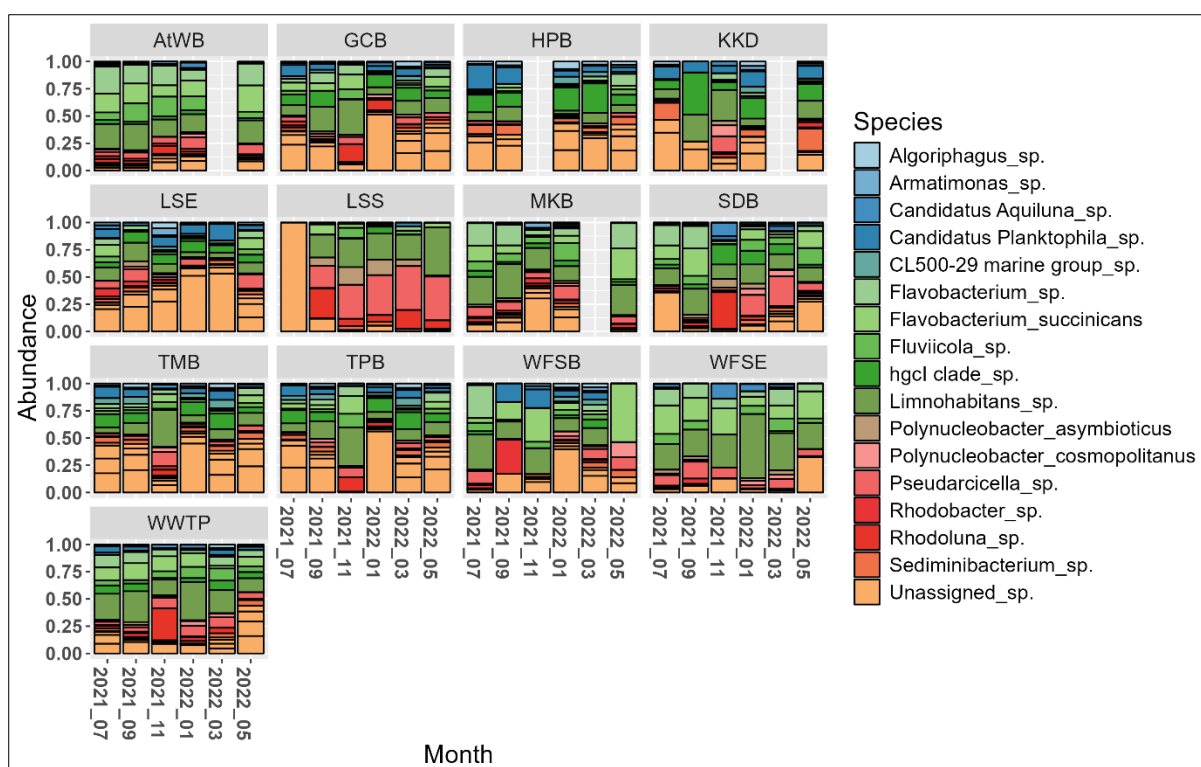


FIG 4.58: Abundance at species level (16S).

Figure 4.58 portrays species-level abundance across the whole study period. However, some individuals were able to be identified at the species level. *Flavobacterium succinicans* showed an average abundance across all sites of 8.89%, with the highest abundance at WSB in May 2022 at 53.70%. Furthermore, two other individuals were identified at the species level, namely *Polynucleobacter cosmopolitanus* and *Polynucleobacter asymbioticus*. These species were reported at 1.63% and 1.55%, respectively, across the study period. *P. cosmopolitanus* showed the highest abundance at WSB in May 2022, with 14.04%, and *P. asymbioticus* at LSS in November 2021, with 16.09%.

4.3 Diversity indices (16S)

4.3.1 Chao1 index

Figure 4.59 presents the findings of species richness variations measured by the Chao1 index. Specifically, part A illustrates monthly variations in species richness; part B examines variations in species richness across sampling sites; part C highlights seasonal variations; and part D illustrates the variations in species richness between wet and dry periods.

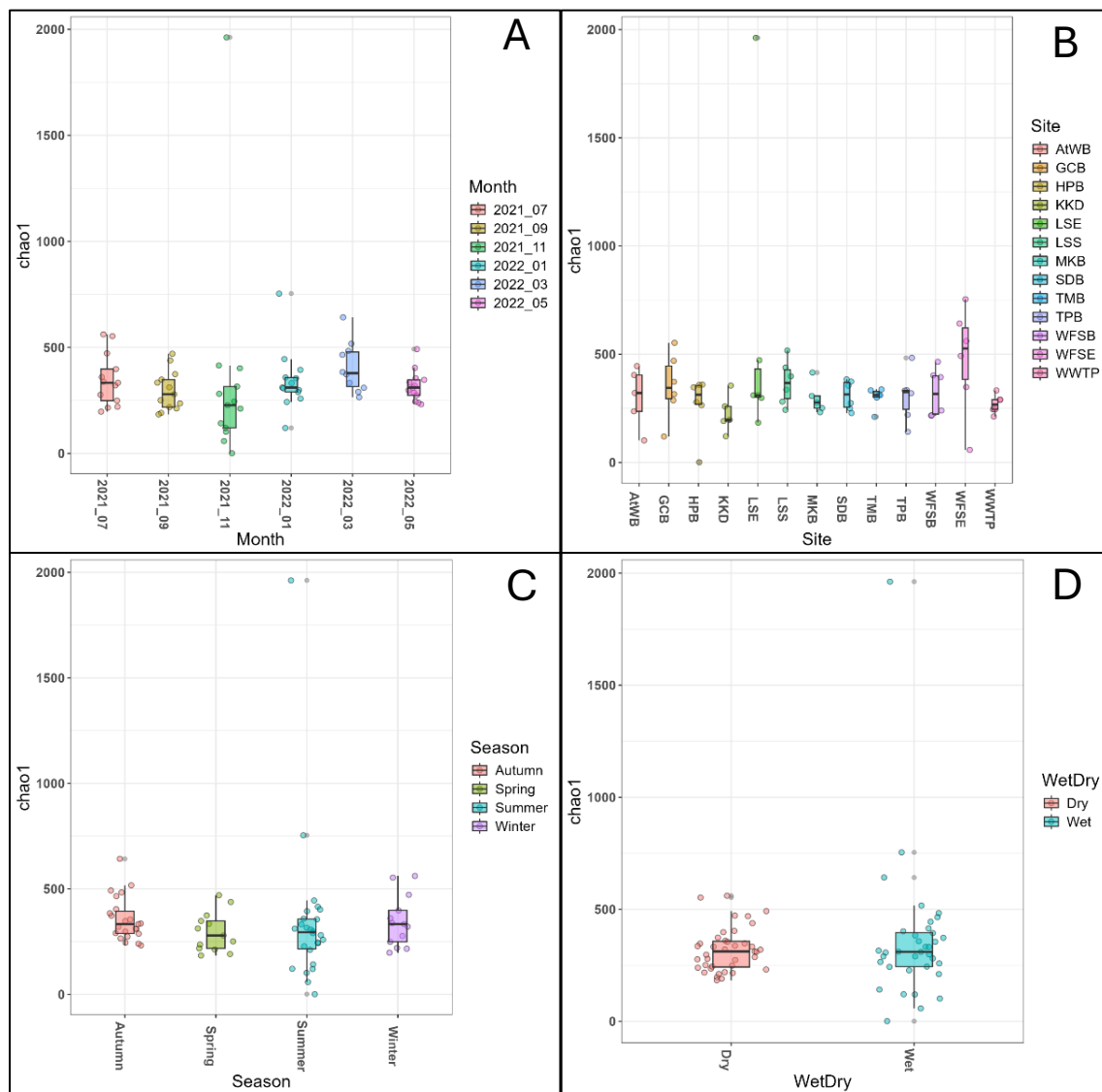


FIGURE 4.59: Chao1 diversity index across various constructs (16S).

The results in Figure 4.59 indicate temporal variability in species richness, evidenced by fluctuating Chao1 index mean scores across months. Peaks in species richness were observed in July 2021 and March 2022, with higher Chao1 mean index values. In contrast, the lowest levels of species richness were recorded in November 2021. Notably, an outlier in November 2021 was observed, with a high Chao1 index value approaching 2000, which may reflect unusually high species richness at a specific site or under certain conditions. The analysis of site-specific data revealed significant differences in species richness across the 13 sampling sites. The highest species richness was recorded at Wonderfonteinspruit End (WFSE), with a mean value exceeding 500, followed by Loop Spruit Start (LSS). Conversely, sites such as Muiskraal Bridge (MKB) and Klerkskraal Dam (KKD) exhibited lower levels of species richness, potentially due to various site-specific conditions. Additionally, outliers were identified at Loop Spruit End (LSE). Seasonal changes in species richness were evident, with higher Chao1 mean index values recorded in autumn and winter and lower species richness in summer and spring. While mean scores between wet and dry periods were marginally different, species richness was consistently recorded to be higher during wet periods.

4.3.2 Shannon-Wiener index

Figure 4.60 reports on species diversity variations according to the Shannon-Wiener index. Specifically, variations were evaluated across months, sites, seasons and wet and dry periods. Overall, the findings reveal the presence of a rich diversity of microbial communities across the 13 sampling locations over various months, seasons, and both wet and dry periods. This diversity is reflected in the Shannon mean value index scores, all of which are > 4. More detailed variations observed are discussed below.

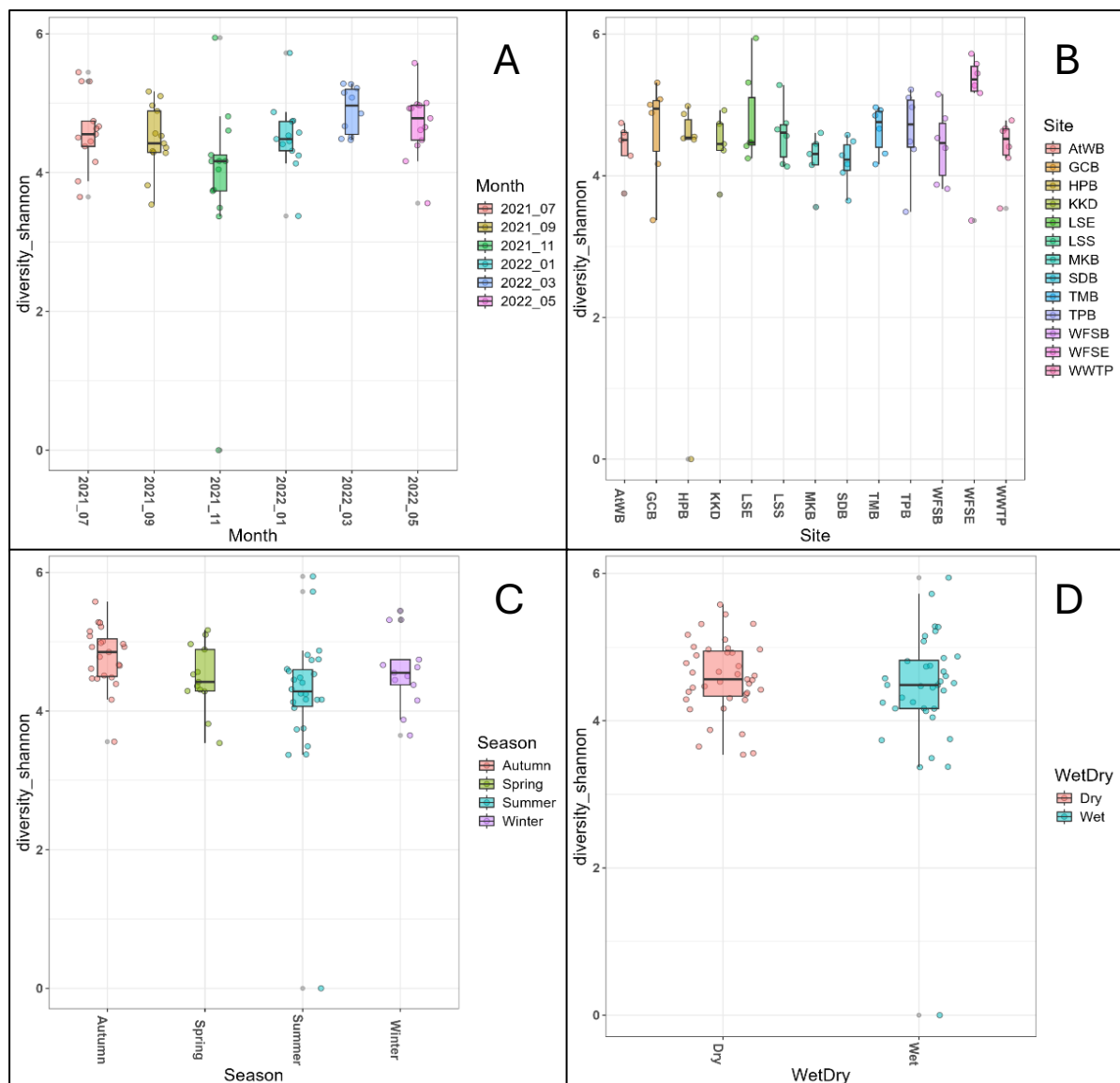


FIGURE 4.60: Shannon-Wiener diversity index across various constructs (16S).

The monthly variations graph (part A) results report temporal variations in species diversity, with higher diversity observed in March 2022 and May 2022. In contrast, lower diversity values were observed in November 2021, indicating decreased species evenness, richness, and potential dominance by specific taxa. Additionally, several outliers were observed, with a significantly low (< 1.5) and unusually high diversity value (nearing 6) in November 2021. Regarding diversity distribution across sites, the findings (part B) show that Wonderfonteinspruit End (WFSE) had the most diverse species community present, followed by Gholfbaan Brug (GCB). The lowest levels of species diversity were recorded at Scandinavia Brug (SDB). Furthermore, several outlier diversity values were recorded across sites, with a notable low value observed at Hartspad Bridge (HBP). Season fluctuations in species diversity were evident (as seen in part C), with higher mean index values recorded specifically during the autumn and winter seasons. Additionally, outliers in diversity values were recorded during the Summer. While mean scores between wet and dry periods were marginally different, species diversity was consistently recorded to be higher during dry periods, evident in part D of the figure.

4.3.3 Bray-Curtis dissimilarity index

Bray-Curtis's principal component analysis (PCoA) is a multivariate statistical approach that illustrates the compositional dissimilarities between microbial communities based on their abundance data. Figure 4.61 provides insights into the visible clustering of three groups across the entire study period.

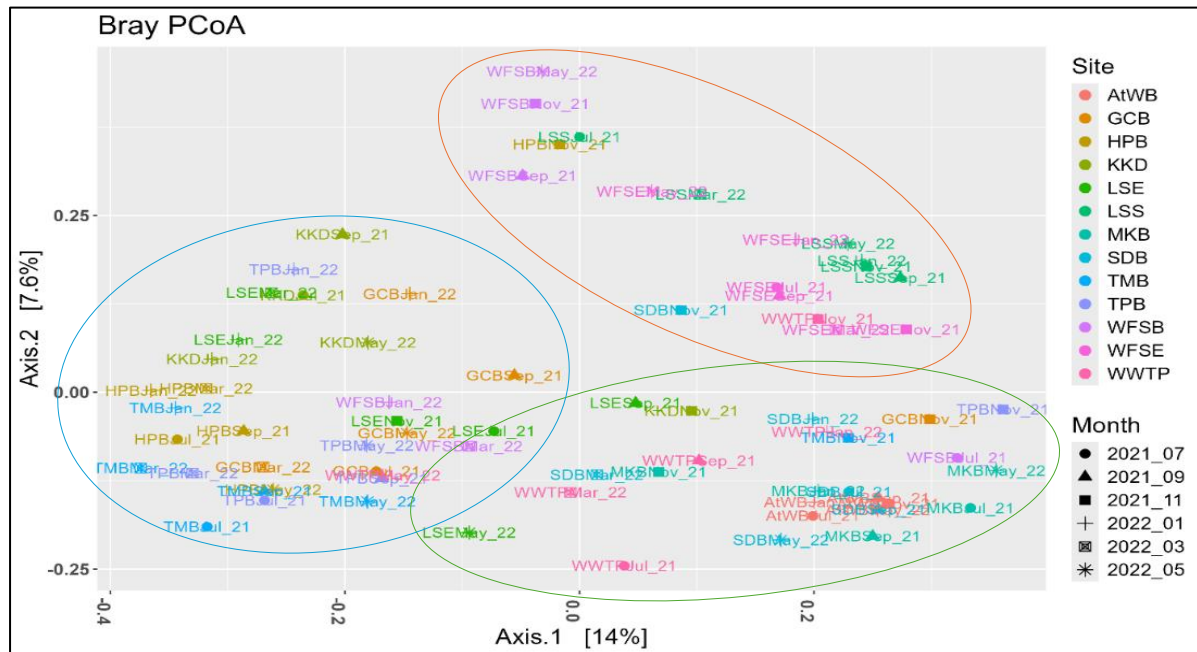


FIGURE 4.61: Bray-Curtis PCoA across all sites for the entire study period (16S).

As seen in Figure 4.61, similarity in community composition occurred at sites like WFSB, WFSE, and LSS, as shown in cluster 1 in orange. Cluster 2 comprises HPB, KKD, TMB and TPB sites in blue. The cluster enclosed in green is comprised of WWTP, MB and AtW. While clustering is apparent, some similarity between different clusters is evident, which is prevalent in overlapping groups. This is seen as the the three figures overlapping in figure 4.61.

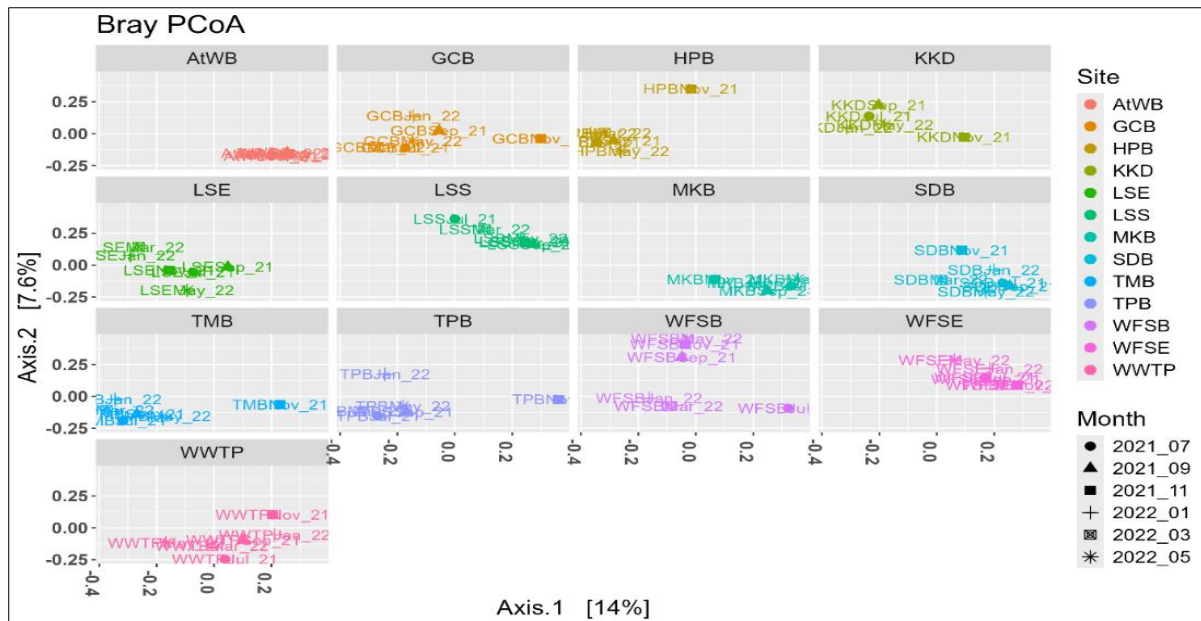


FIGURE 4.62: Bray-Curtis PCoA per individual site across the entire study period (16S).

Although it is beneficial to calculate PCoA across all sites to identify similarities, site-specific PCoA illustrates how temporal disturbances or pollution can compromise species composition, as seen in Figure 4.62. Sites whose species composition remains the same across the study period are seen as closely clustered, namely AtW, MB and WSE. Numerous sites displayed gradual dispersion of sites across the span of the study period, indicative of seasonal changes influencing the microbial composition. Lastly, HB, TMB, and TPB depicted high species similarity, where at least 4 out of 6 data points cluster, but some outliers from this group portray an episodic event of external disturbance or enrichment.

4.4 COI

The COI gene was selected to identify eukaryotic microbial communities, emphasising taxonomic composition, abundance, richness, diversity and similarity metrics across sites. Plots used for abundance visualisation were adapted to exclude unassigned ASVs. This was utilised from phyla level down to genus level so as not to overcrowd plots and be able to visually distinguish between individuals. For total transparency, unfiltered plots will be available in the appendix. The results are presented in Figures 6.63 to 6.68 below.

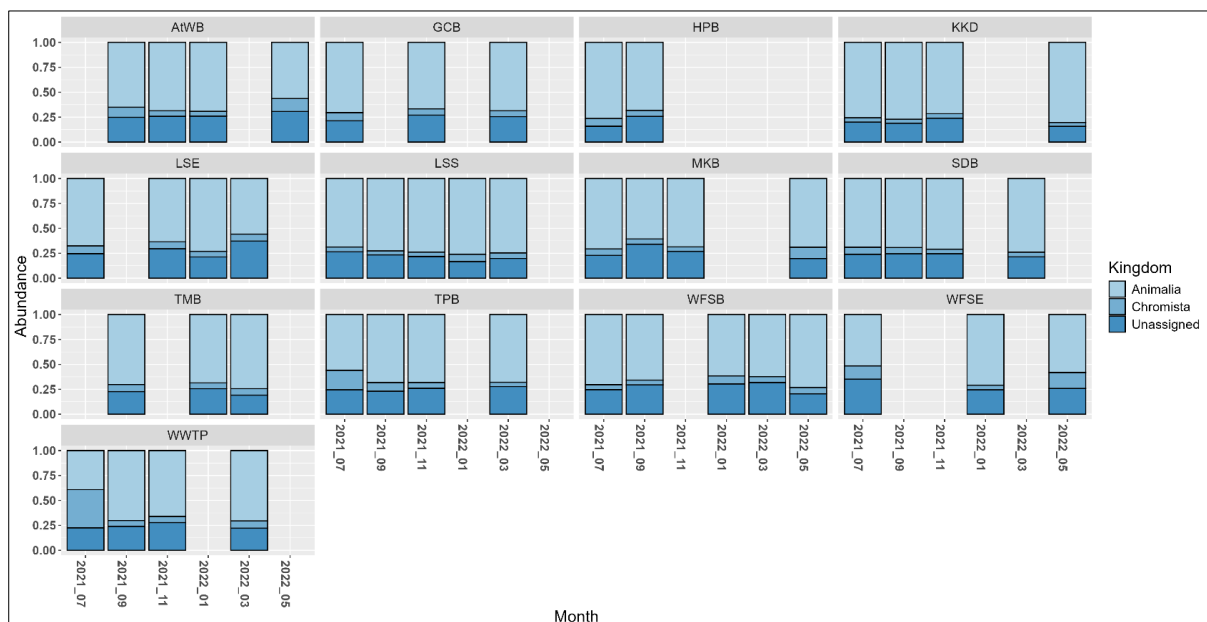


FIGURE 6.63: Abundance at kingdom level (COI).

At the kingdom level, two dominant groups were identified. Namely, *Animalia* had an abundance of 67.91%, and *Chromista* had 7.46% across all 13 sites over six months. *Animalia* was the most abundant KD in May 2022, with 80.35%, and the least at WWTP in July 2021, with 39.19%. *Chromista* appeared to be the most abundant at WWTP in July 2021, with 38.31%, and the least at KD in September 2021, with 3.88%. As seen in Figure 6.63, sequences that are not assigned amount to 24.63% of all the sequences over the 13 sites and six months.

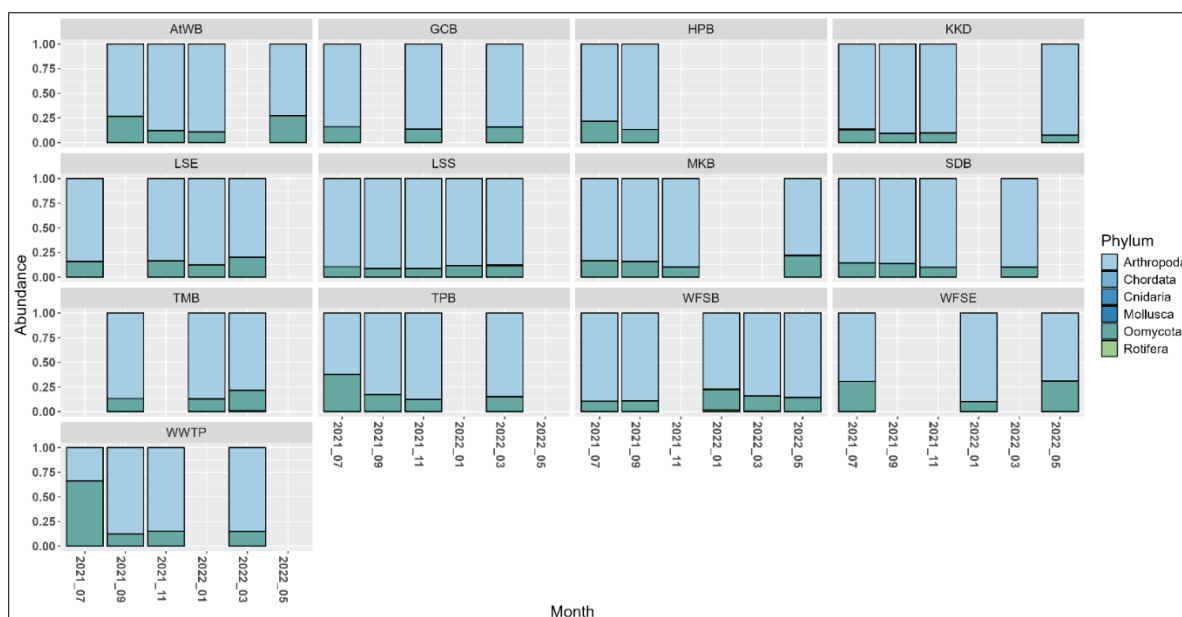


FIGURE 6.64: Abundance at phylum level (COI).

Arthropoda was the most dominant phylum across all the 13 sites and six months with 83.33% and *Mollusca* the least with 0.00004%. As seen in Figure 6.64 phyla that are noted as unassigned are not included in this plot. *Arthropoda* was the most prevalent at KD in May 2022, with 92.24%, and the least prevalent at WWTP in July 2021, with 33.78%. At 66.21%, *Oomycota* was the most dominant at WWTP in July 2021 and the least dominant at KD in May 2022, with 7.45%. *Chordata* was not very abundant but still one of the most prevalent. *Chordata* occurred at 0.146% of all sites across the 12 months. This phylum occurred the most at LSB in March 2022, and no prevalence was reported at 26 out of 78 sampling points and dates.

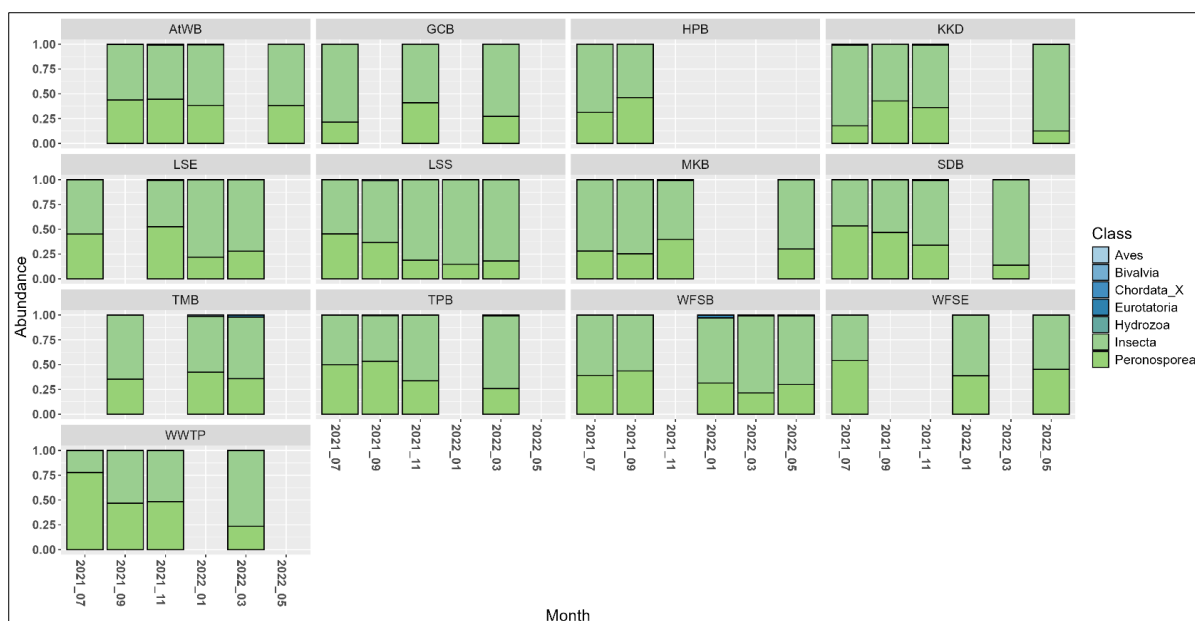


FIGURE 6.65: Abundance at class level (COI).

As seen in Figure 6.65, the taxonomy rank for class indicated that *Insecta* was the most abundant at all sites over the 12 months, with 63.5%. In May 2022, KD reported *Insecta* as the most abundant class, with 87.04%, and the least abundant at WWTP in July 2021. *Peronospora* was the most abundant at WWTP in July 2021, with 77.74%, and the least at KD in May 2022, with 12.58%. *Aves* was only prevalent at 0.27% across all sampling points but was reported the most at TMB in January 2022 with 1.33%.

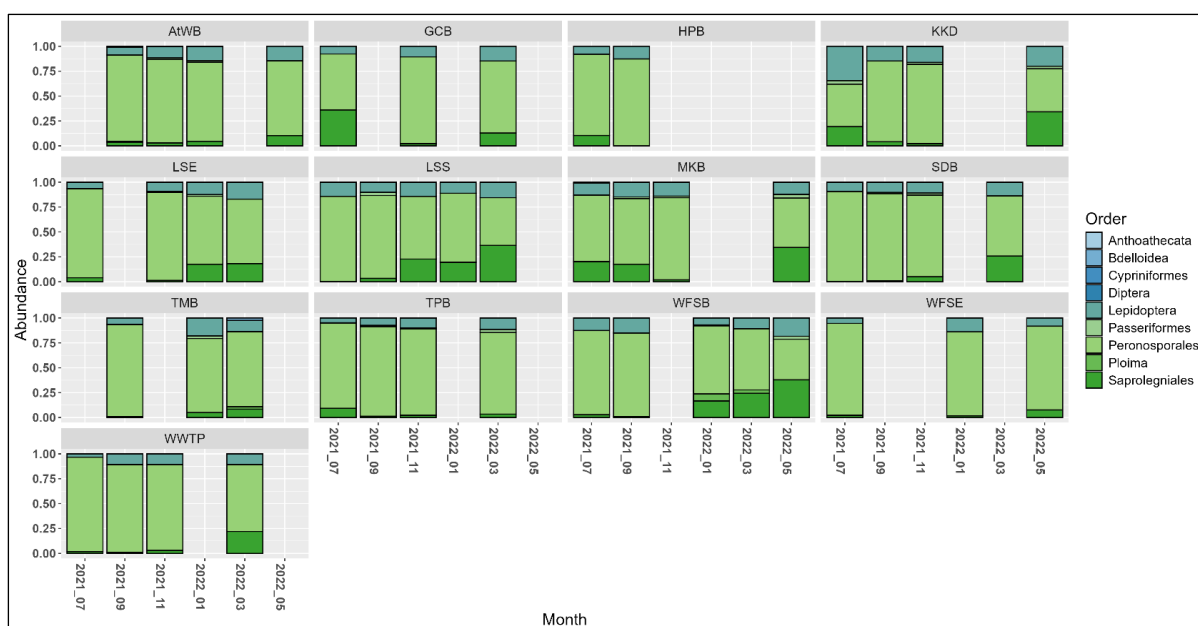


FIGURE 4.66: Abundance at order level (COI).

As seen in Figure 4.66, the most abundant order across all 13 sites and six different months were *Peronosporales* at 76.01%, *Lepidoptera* was the second most prevalent at 12.10% and third, *Saprolegniales* at 10.76%. *Peronosporales* was the most abundant at WWTP in July 2021, with 94.81%, and the least abundant at WSB in May 2022, with 40.82%. *Lepidoptera* reported the most abundance at KD in July 2021, with 34.51%, and the least abundance at WWTP in July 2021, with 3.39%. *Saprolegniales* was the most abundant at WSB in May 2022 with 37.86% and showed no prevalence at three out of 78 sampling points.

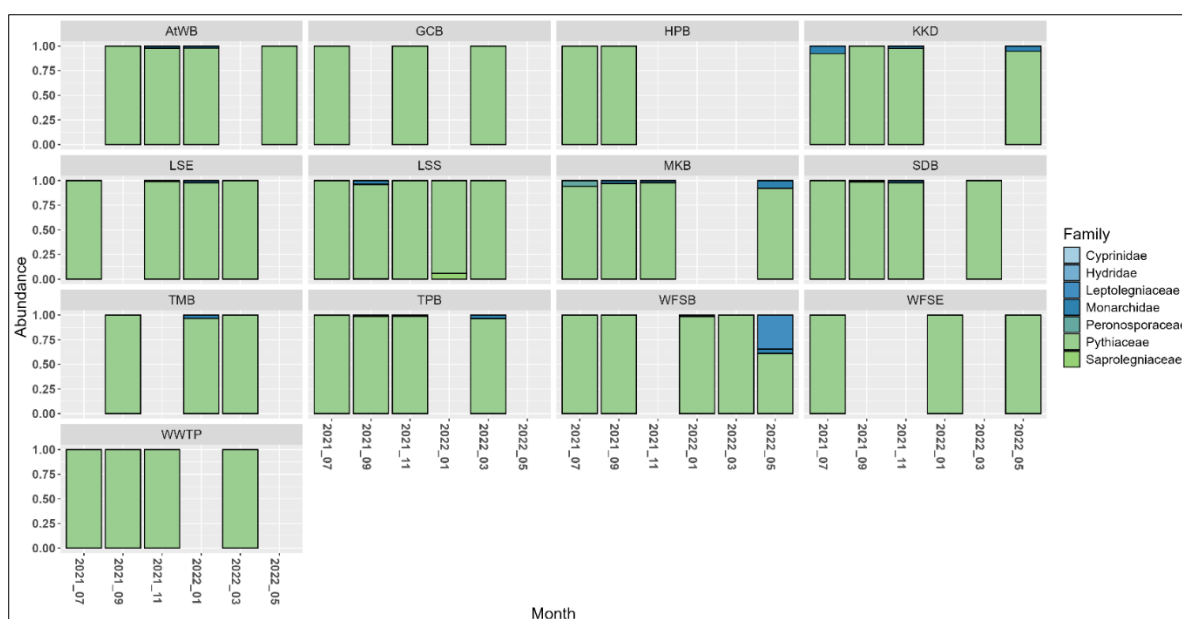


FIGURE 6.67: Abundance at family level (COI).

The abundance was very limited at the family rank, as seen in Figure 6.67. *Pythiaceae* was reported at 97.81% of total abundance across all 13 sites and six months. As *Pythiaceae* was the most abundant, it was seen as the most abundant family at 27 out of 78 sampling points. It was the least abundant at WSB in May 2022, with 61.06%. Another mentionable family was *Monarchidae*, with a 1.21% abundance across all the sites. It was, however, the most prevalent at MB in May 2022, with 7.85%, and the least prevalent at numerous sites, with no prevalence at all.

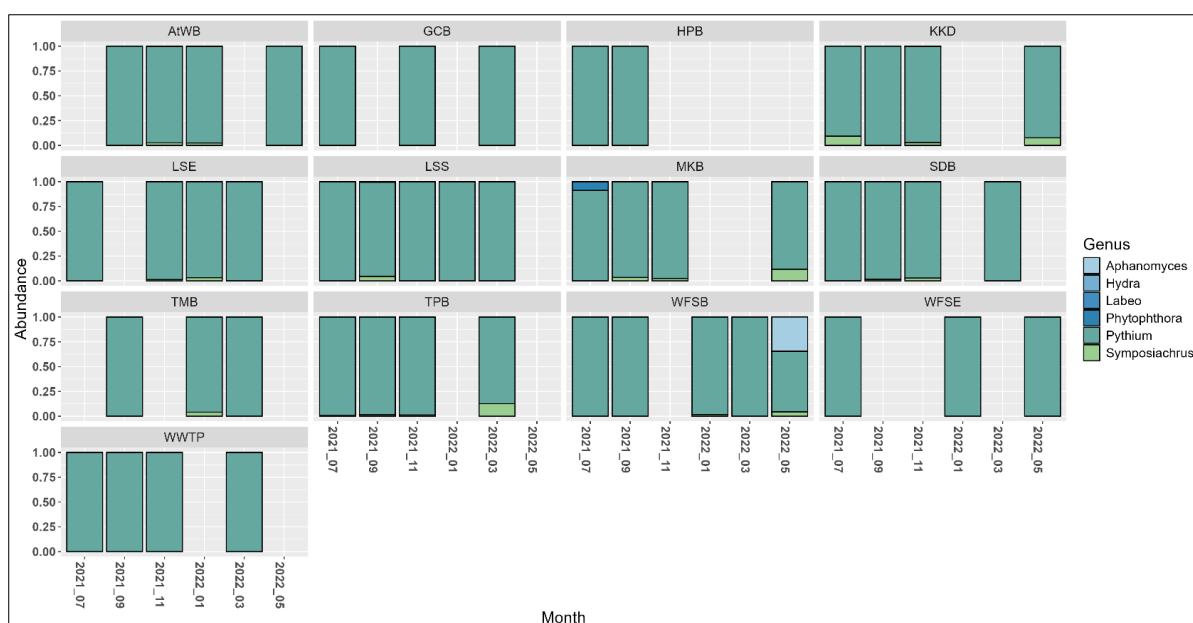


FIGURE 4.68: Abundance at genus level (COI).

As seen in Figure 6.68, at the genus level, the genus *Pythium* was reported 97.41% of the time across all 13 sites and six months. *Pythium* was recorded as the most abundant genus at 28 out of 78 sampling points. *Pythium* was recorded the least at WSB in May 2022, at 61.06%. *Symposiachrus* was the second most prevalent genus and the most reported at TPB in March 2022, with 12.69%.

4.5 Diversity indices (COI)

4.5.1 Chao1 index

Figure 4.69 below presents the findings of species richness variations measured by the Chao1 index. Specifically, part A illustrates monthly variations in species richness; part B examines variations in species richness across sampling sites; part B highlights seasonal variations; and part D demonstrates the variations in species richness between wet and dry periods.

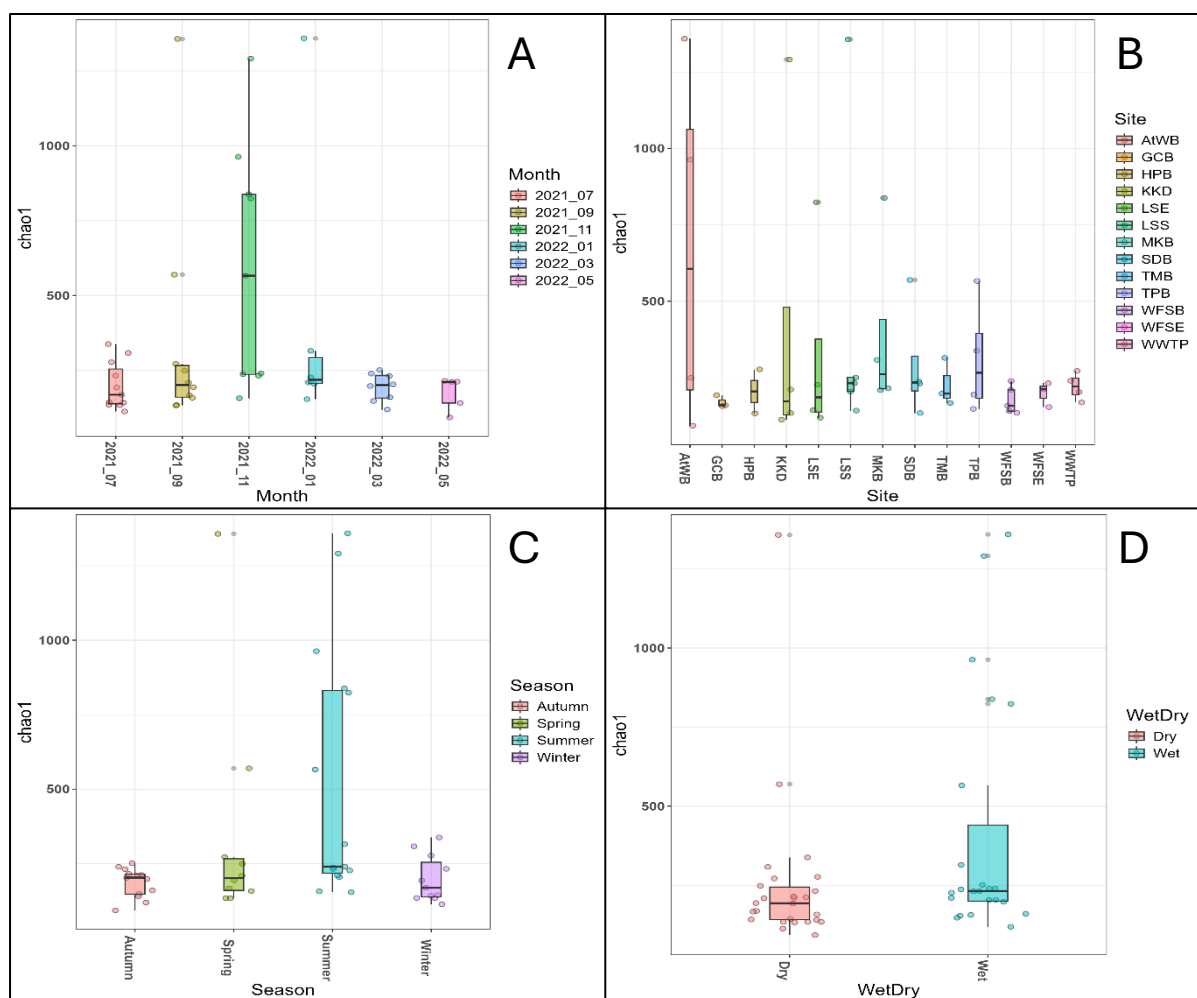


FIGURE 4.69: Chao1 diversity index across various variables (COI).

The results in Figure 4.69 illustrate temporal variability in species richness, evidenced by fluctuating Chao1 index mean scores across months. A gradual rise from July 2021 to a peak in November 2021 can be visualised; thereafter, a slight decrease occurs. A

noticeable peak in species richness was observed in November 2021, with higher Chao1 mean index values. In contrast, the lowest levels of species richness were recorded in May 2022. Notably, an outlier in November 2021 was observed, with high Chao1 index values and highly dispersed upper and lower quartiles, which may reflect unusually high species richness under the current conditions. The analysis of site-specific data revealed significant differences in species richness across the 13 sampling sites. The highest species richness was recorded at AtW, with a mean value exceeding 500, followed by TPB. Conversely, sites such as GCB and WSB exhibited lower levels of species richness, potentially due to various site-specific conditions.

Furthermore, seasonal changes in species richness were evident, with higher Chao1 mean index values recorded in summer, followed by spring, autumn, and winter. The respective mean Chao1 index values varied slightly, but a trend can be deduced. Outliers and highly dispersed values in summer reflect unusually high species richness indicative of the relevant site-specific conditions. While mean scores between wet and dry periods were marginally different, species richness was consistently recorded to be higher during wet periods.

4.5.2 Shannon-Wiener index

Figure 6.70, parts A-D, reports species diversity variations according to the Shannon-Wiener index. Specifically, variations were evaluated across months, sites, seasons and wet and dry periods, with values above 3 indicating a highly diverse community (Shannon, 1948).

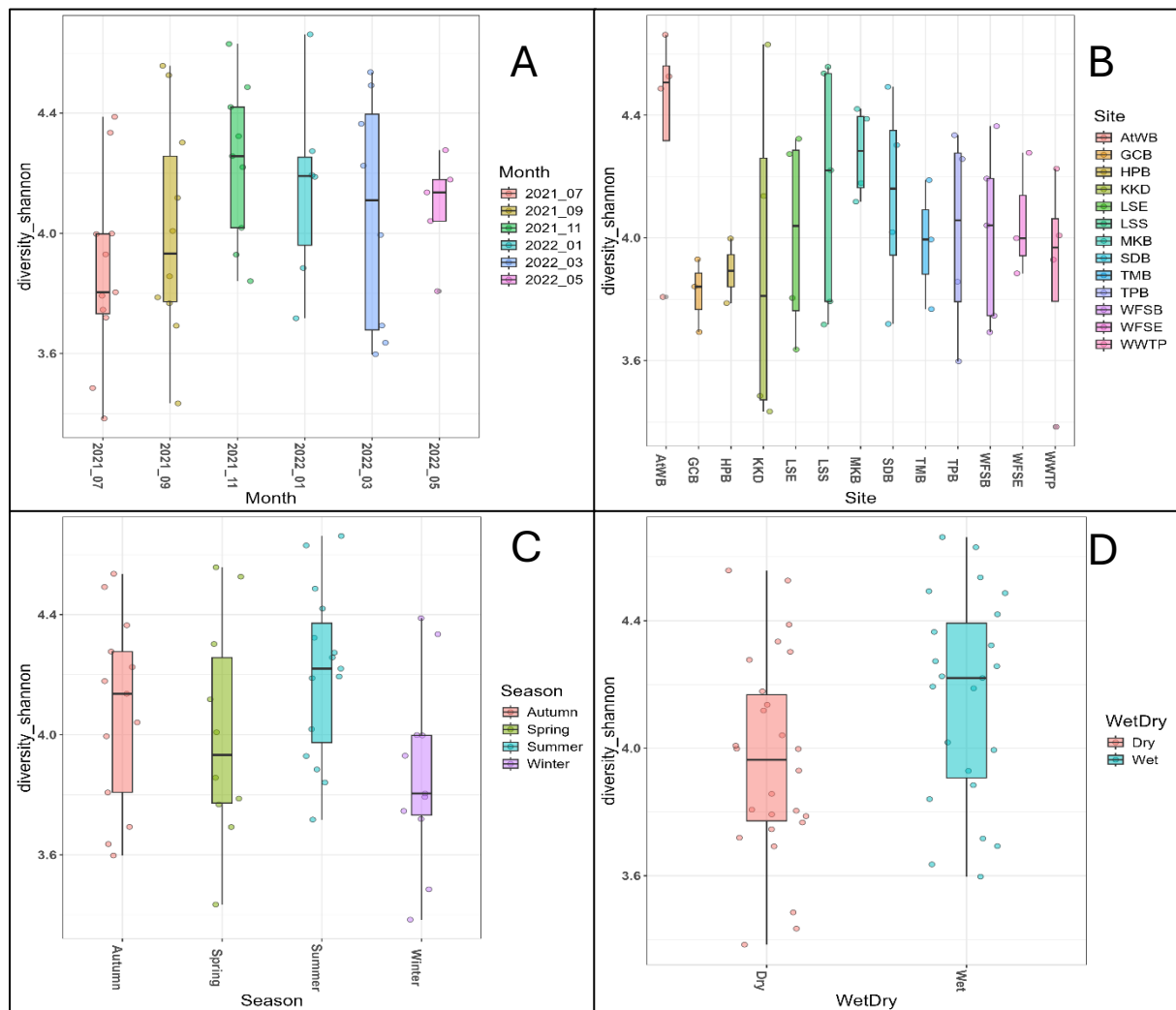


FIGURE 6.70: Shannon-Wiener diversity index across various variables (COI).

Overall, the findings presented in Figure 6.70 reveal the presence of a rich diversity of microbial communities across the 13 sampling locations over various months, seasons, and both wet and dry periods. This diversity is reflected in the Shannon mean value index scores, all of which are > 3.6 . The results from the monthly variations graph report temporal variations in species diversity, with higher diversity observed in

November 2021 and January 2022. In contrast, the lowest diversity values were observed in July 2021. Additionally, several outliers were observed, with highly dispersed upper and lower quartiles indicating lower total abundance and reads. Regarding richness distribution across sites, the results report that AtW had the most diverse species community present, followed by MB. The lowest levels of species diversity were recorded at KD. Season fluctuations in species diversity were evident, with the highest mean index values recorded specifically during summer. While mean scores between wet and dry periods were marginally different, species diversity was consistently recorded to be higher during wet periods.

4.5.3 Bray-Curtis dissimilarity index

Figure 6.71 below reports on the Bray-Curtis dissimilarity index, which quantifies differences between samples, ranging from 0 (completely identical communities) to 1 (completely dissimilar communities).

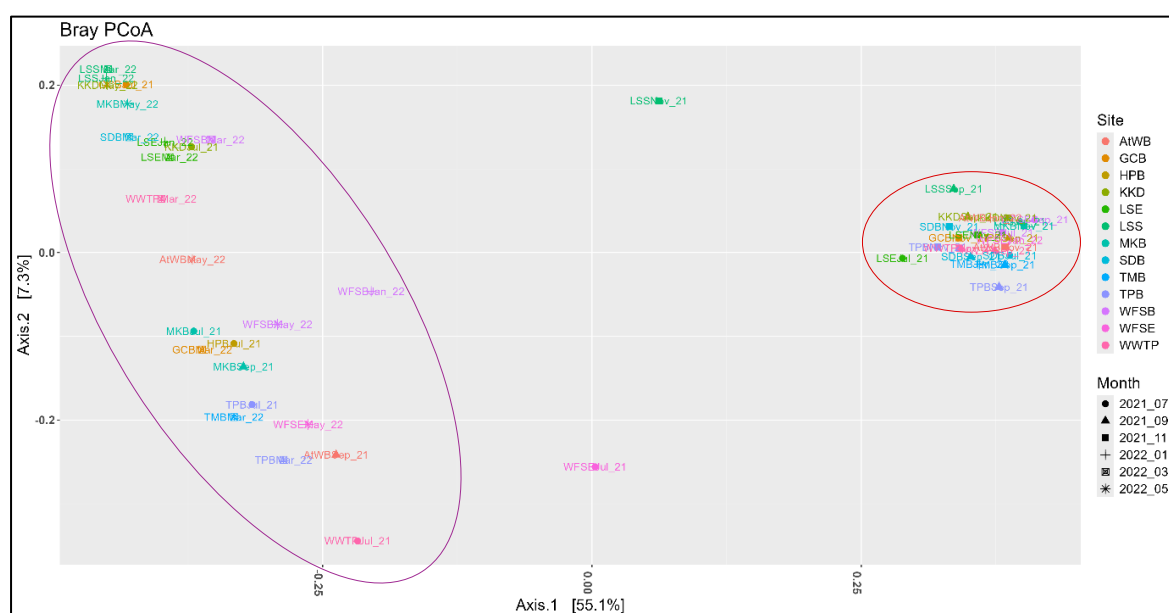


FIGURE 6.71: Bray-Curtis PCoA across all sites for the entire study period (COI).

As can be seen in Figure 6.71, two definitive communities vastly different from one another are illustrated. The cluster encircled in purple includes sites like WSB and MB, and the cluster marked in red is comprised of TMB and SB.

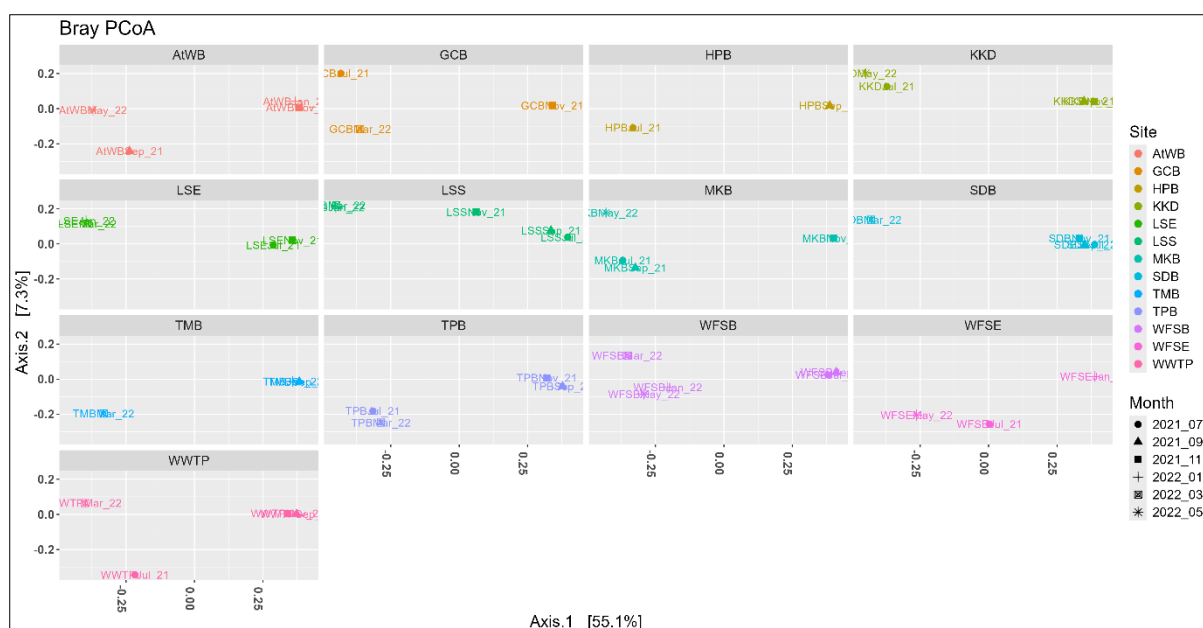


FIGURE 4.72: Bray-Curtis PCoA across individual sites for the entire study period (COI).

As seen in Figure 4.72, all sites displayed gradual dispersion of sites across the span of the study period, indicative of seasonal changes influencing the microbial composition.

4.6 Multivariate analyses

The results from the abundance, physical and chemical parameters were further analysed using multivariate analyses to compare and determine the correlation between studied variables. The findings are presented in Figures 4.73 to 4.77 below. Vectors illustrated in RDA are evaluated by the following constructs as degrees between two vectors: $< 90^\circ$, vectors are positively correlated, approximately 90° declares no correlation and $> 90^\circ$ portrays a negative correlation. Furthermore, the Kruskal-Wallis H test was used to determine statistical significance between variables. Table 4.1 presents these findings.

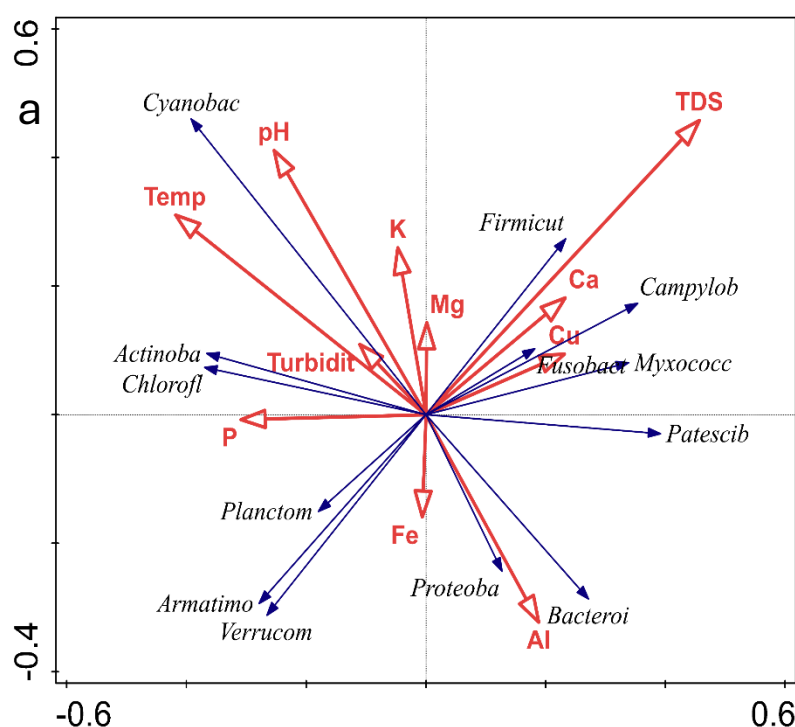


FIGURE 4.73: RDA between phyla and environmental parameters.

In Figure 4.73 phyla from the 16S barcode are overlapped with this study's environmental parameters. TDS, calcium, copper, and magnesium were closely related and positively correlated with *Firmicutes*, *Campylobacterota*, *Fusobacteriota*, and *Myxococcota*. *Cyanobacteria*, *Actinobacteriota* and *Chloroflexi* were associated with higher temperatures and positively influenced by pH. Aluminium was reported to positively correlate with phyla, namely *Proteobacteria* and *Bacteroidota*. Inversely,

negative correlations can be deduced from the plots above. *Actinobacteriota* and *Chloroflexi* appear to be negatively influenced by high TDS, calcium, copper and aluminium. *Proteobacteria* and *Bacteroidota* have shown a negative correlation towards phosphorus and high turbidity levels.

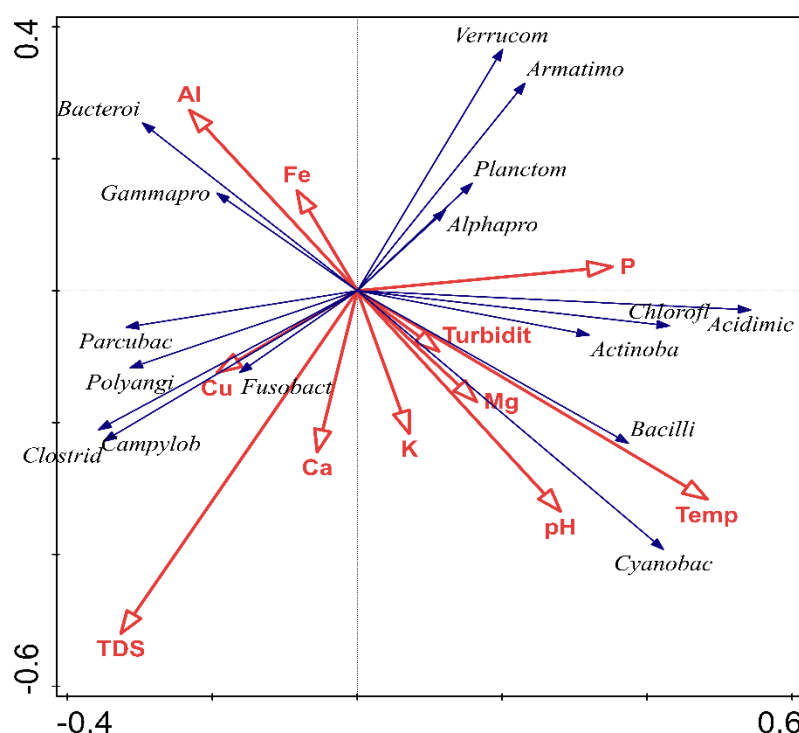


FIGURE 4.74: RDA between class and environmental parameters.

Figure 4.74 illustrates how environmental factors influenced and correlated with bacterial taxonomy. In the plot, it is seen that Bacteroidia and Gammaproteobacteria were closely related and experienced a positive correlation with aluminium, iron, and copper. *Verrucomicrobiae*, *Armatimonadia*, *Planctomycetes* and *Alphaproteobacteria* are closely related and according to literature inhabit environments with higher aluminium, iron and phosphorus concentrations. *Chloroflexia*, *Acidimicrobiia* and *Actinobacteria* were positively related to higher levels of phosphorus, magnesium, turbidity, temperature and pH. Groups that were less related but influenced by the same external factors are *Bacilli* and *Cyanobacteria* indicating a low positive correlation. This cluster experienced a positive correlation towards temperature, turbidity, magnesium, potassium and calcium. The last cluster representing class is comprised of *Parcubacteria*, *Polyangia*, *Fusobacteriia*, *Clostridia* and

Campylobacteria. This cluster was highly related and favoured an environment with high TDS levels, calcium and copper.

Furthermore, negative correlations are depicted in this figure as well. *Bacteroidia* and *Gammaproteobacteria* were found to be negatively correlated, meaning they do not prefer environments with high pH, elevated temperatures, turbidity, magnesium, potassium and calcium. *Verrucomicrobiae*, *Armatimonadia*, *Planctomycetes* and *Alphaproteobacteria* tend to be inhibited in high salt environments with elevated TDS, calcium and copper concentrations. Furthermore, *Chloroflexia*, *Acidimicrobiia* and *Actinobacteria* were negatively impacted by aluminium and iron as they were negatively correlated with each other, as seen in the RDA plot in Figure 4.74. *Bacilli* and *Cyanobacteriia* illustrate the same environmental preferences as the latter group. *Parcubacteria*, *Polyangia*, *Fusobacteriia*, *Clostridia* and *Campylobacteria* are shown to be inhibited by high phosphorus environments.

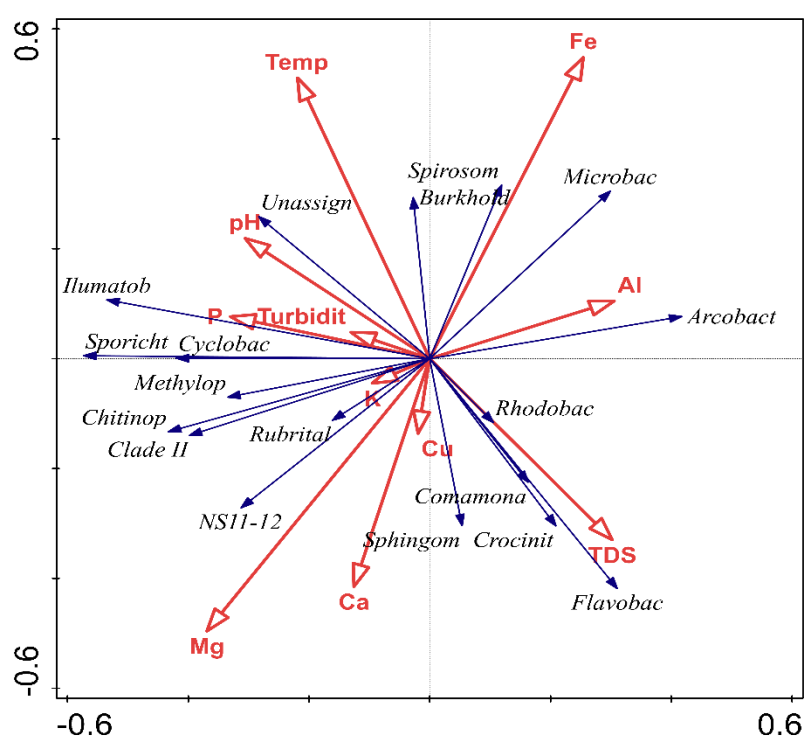


FIGURE 4.75: RDA between family and environmental parameters.

Figure 4.75 above shows the correlation between environmental parameters and the family-level taxonomy of bacteria isolated from all study sites. *Spirosomaceae*, *Burkholderiaceae*, *Microbacteriaceae* and *Arcobacteriaceae* experienced positive

correlation to iron and aluminium. From this one group all reported a positive correlation towards temperature as well except *Arcobacteriaceae* which shows that these two parameters are uncorrelated. Salt-rich environments with high levels of magnesium, calcium, copper and, concurrently, TDS indicated a positive correlation for families like *Rhodobacteriaceae*, *Flavobacteriaceae*, *Comamonadaceae*, *Crocinitomicaceae* and *Sphingomonadaceae*. The following families were most prevalent in environments with potassium and phosphorus: *Rubritaleaceae*, *Chitinophagaceae*, *Methylophilaceae*, *Sporichthyaceae*, *Cyclobacteriaceae* and *Ilumatobacteriaceae*. *Spirosomaceae*, *Burkholderiaceae*, *Microbacteriaceae* and *Arcobacteriaceae* indicated a negative correlation to magnesium, calcium and potassium. *Rhodobacteriaceae*, *Flavobacteriaceae*, *Comamonadaceae*, *Crocinitomicaceae* and *Sphingomonadaceae* illustrated a negative correlation by environmental factors like turbidity, temperature and pH. *Rubritaleaceae*, *Chitinophagaceae*, *Methylophilaceae*, *Sporichthyaceae*, *Cyclobacteriaceae* and *Ilumatobacteriaceae* was negatively impacted by environments with enriched levels of iron and aluminium.

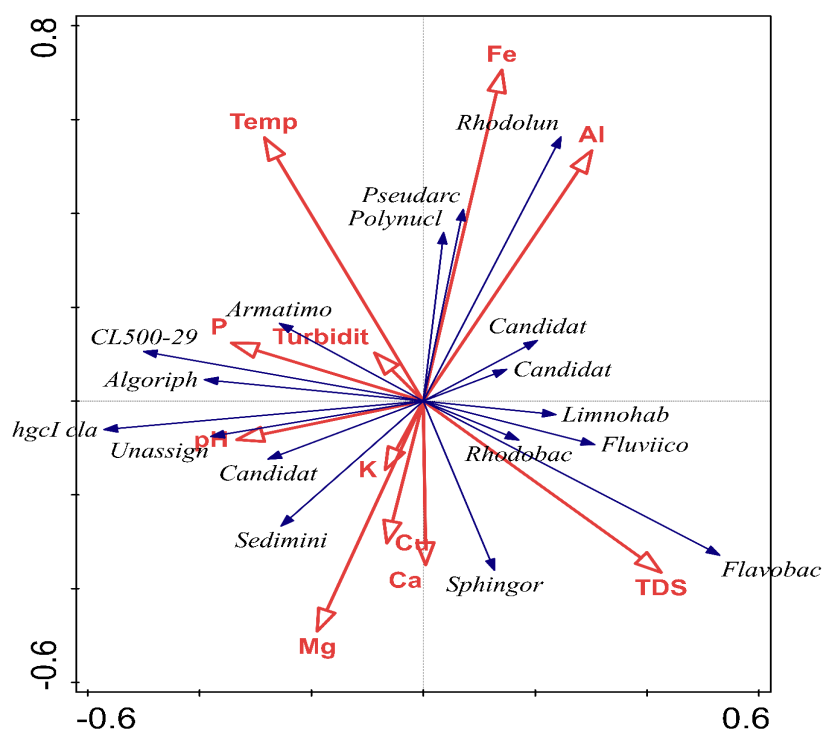


FIGURE 4.76: RDA between genera and environmental parameters.

Genera reported in this study are overlapped in an RDA with environmental parameters in Figure 4.76. Iron and aluminium created favourable for numerous genera namely: *Pseudarcicella*, *Polynucleobacter* and *Rhodoluna*. *Limnohabitans*, *Fluviicola*, *Rhodobacter* and *Flavobacterium* indicated a positive correlation towards high levels of TDS. *Sphingorhabdus* and *Sediminibacterium* portrayed positive correlation to various environmental parameters, namely TDS, copper, potassium, calcium and magnesium. *Algoriphagus* and *Armatimonas* indicated positive abundance in environments with high phosphorus levels, turbidity and temperature. *Pseudarcicella*, *Polynucleobacter* and *Rhodoluna* indicated negative correlation in environments with high levels of TDS, calcium, copper, magnesium and potassium. Environments that reported lower levels of phosphorus, temperature, turbidity and pH indicated a negative effect on *Limnohabitans*, *Fluviicola*, *Rhodobacter* and *Flavobacterium*. High iron and aluminium levels and *Sphingorhabdus* and *Sediminibacterium* indicated a negative correlation respectively. TDS indicated a negative correlation towards *Algoriphagus* and *Armatimonas*.

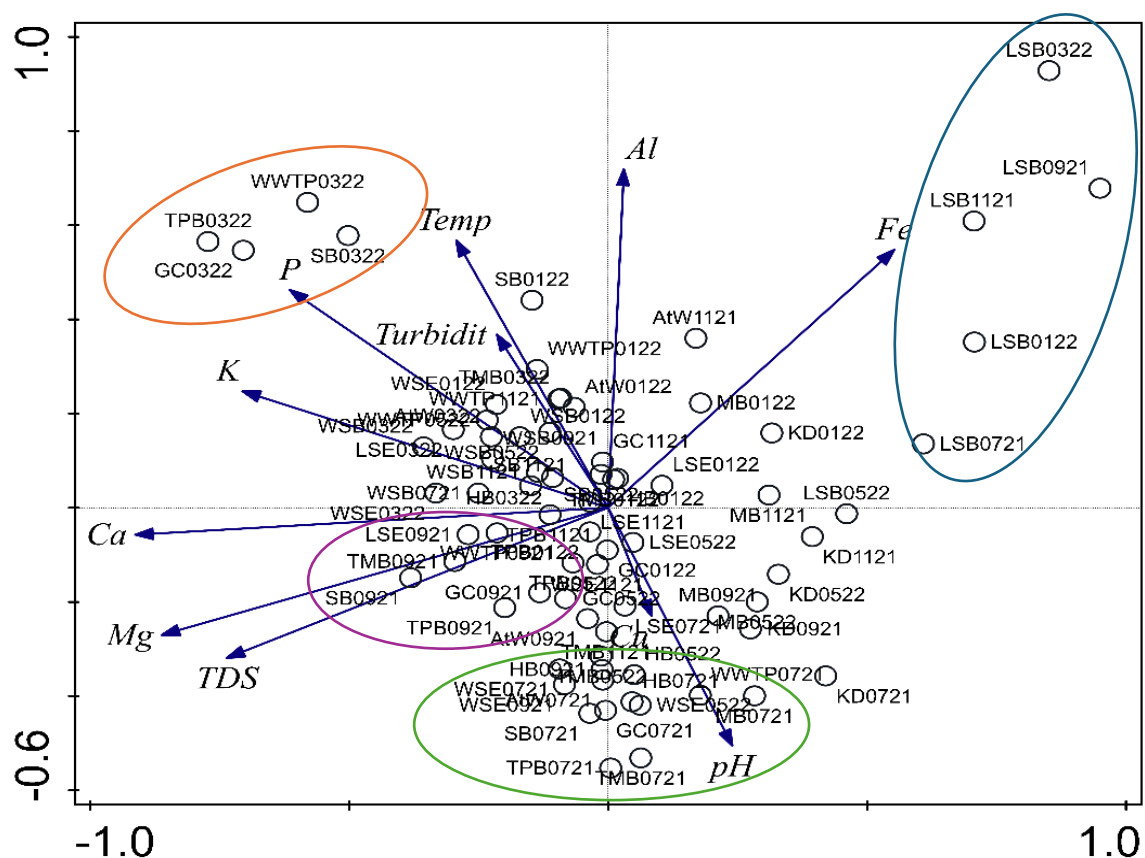


FIGURE 4.77: PCoA overlapping environmental parameters across sites.

Physico-chemical parameters from LSB, encircled in blue in Figure 4.77, are indicated to be closely related, meaning that these parameters are very similar across the study period. This site reported overall high levels of iron and aluminium. Sites GC, TPB, SB and WWTP recorded physico-chemical parameters in March 2022 (orange oval) that are closely related and indicative of overall high levels of potassium, phosphorus, turbidity and temperature. In July of 2021, TPB, TMB SB, GC, MB, KD, HB and WWTP were closely related (green cluster) in terms of environmental parameters, especially indicating overall higher pH. Sites that indicated high average levels of calcium, magnesium and TDS were sites like SB, TMB, LSE, WWTP, GC and TPB, all closely related in September 2021 and are encircled in purple.

TABLE 4.1: Kruskal-Wallis Test comparing Chao1 diversity to environmental parameters (*p*-values)

	Site/Location	Month	Season	Wet/Dry	SiteWet/Dry
Phylum	0.010227	0.99682	0.99418	0.85245	0.053557
Class	0.0084776	0.9772	0.91819	0.6489	0.062294
Order	0.011	0.32144	0.12574	0.93185	0.062045
Family	0.0022217	0.21183	0.08158	0.79383	0.030412
Genus	0.0008448	0.1667	0.074357	0.60218	0.025071
Species	0.0015599	0.18745	0.088118	0.64398	0.041116

Table 4.1 above indicates that environmental (Wet/Dry season) and temporal (Month and Season) variables across the study period demonstrated *p*-values from 0.08158 to 0.99682. This indicates no statistical significance between most of the variables studied. However, the Site/Location and the SiteWet/Dry site combination indicated statistical significance since their *p*-values are < 0.05.

CHAPTER 5: DISCUSSION

The present study provides an overview of the aquatic bacterial and eukaryotic microorganism communities in the Mooi River system, surveyed using DNA barcoding technologies, among other analysis techniques. Specifically, the study focused on (1) determining the physical and chemical characteristics of water across 13 sampling sites along the Mooi River system, (2) determining the concentrations of trace and heavy metals at these sampling sites, and (3) identifying bacterial and eukaryotic communities using NGS DNA barcodes, 16S rRNA and COI, respectively. Additionally, multivariate analyses were performed to assess the impact of anthropogenic influences on microbial communities across sites. The findings emphasise the dynamics and impact of anthropogenic influences on water quality, biodiversity, and, subsequently, ecosystem health. The key findings are discussed below.

5.1 Physical and chemical parameters

TDS was measured to identify soluble material (e.g., organic salts, organic matter, and various other dissolved elements) present in the water samples across sites (EPA, 1986). Across all 13 sites, TDS concentrations revealed clear spatial and temporal variations, which are associated with land use, seasonal changes, and anthropogenic influences. Specifically, sites such as KD and MB with stable TDS values (~300 mg/L), represented areas with minimal seasonal or anthropogenic influences due to their relatively stable TDS values (Dallas & Rivers-Moore, 2014; DWAF, 1996). Moreover, a seasonal dilution effect during the rainy season was observed at KD, showing a slight decrease in TDS to < 300 mg/L in January 2022. Conversely, higher TDS levels were recorded at site AtW (~600 mg/L) indicative of urban runoff and agricultural impacts, aligning with similar patterns reported by Annandale and Nealer (2011) and Barnard *et al.* (2013) in urbanised water bodies and according to their land use scope of the area. Furthermore, a reduction of TDS levels was observed at HB (450-500 mg/L), possibly due to the buffering capacity of Boskop Dam and large water bodies as seen in research done by Dodds and Cole (2007). Elevated TDS levels at TPB and WWTP, which spiked up to 760 mg/L in November 2021, indicate a pollution

event, likely due to wastewater discharges (Palmer *et al.*, 2004). WSB and WSE showed high TDS (500-800 mg/L) concentrations throughout the stream length, which may be due to external factors such as mining activities, urban runoff, and erosion (Barnard *et al.*, 2013). Downstream, sites such as LSB recorded elevated levels up to 780 mg/L in November 2021, with subsequent reductions to 400-600 mg/L at LSE, likely reflecting natural filtration or diluting effects (Pandey, 2023). High levels of TDS can have adverse consequences on microbial communities reducing microbial diversity in sensitive taxa or at early developmental stages (Weber-Scannell *et al.*, 2007).

In addition, pH levels were measured to assess the acidity and/or alkalinity and levels of contamination in the water samples collected. Across all sites, pH levels remained stable throughout the 12-month study period, with values leaning towards an alkaline environment averaging around 7.5-8, creating the ideal environmental conditions for most organisms to thrive by advancing nutrient uptake and bioavailability (Linz *et al.*, 2018; Melzer & Steinberg, 1983; Pandey, 2023). These findings are in accordance with similar studies conducted along the Wonderfontein Spruit flowing north of Loop Spruit by Jordaan *et al.* (2019). Their findings indicated similar pH concentrations, reported as ranging between 7.17 and 8.29. This reflects the robust buffering capacities of the riverine ecosystem and that a low susceptibility to contamination by acidic substances did occur. Furthermore, the stability of pH throughout the river and its tributaries indicates minimal external contamination, facilitating the solubility of nutrients and minerals (Oluyemi *et al.*, 2010; Fierro *et al.*, 2017; Hussain & Singh, 2021).

In terms of temperature, seasonal temperature fluctuations were observed across all sites, with low temperatures of approximately 8°C during winter seasons (July 2021) and peaks of up to approximately 25°C around summer (January 2022). Urban adjacent sites, such as TPB and WWTP, showed elevated winter temperatures (12-13°C) compared to other sites, likely due to urban runoff and reduced water velocity as these sites are very shallow. Furthermore, at WSB and WSE, increased microbial growth was evident, which could have contributed to the elevated temperature (17°C) observed in July 2021 (winter) (Qiu *et al.*, 2022), highlighting the interplay of ecological and anthropogenic factors. Dallas (2007) explains that higher temperatures also

decrease the solubility of dissolved oxygen in water, decreasing its concentration and availability to aquatic organisms. This effect can be intensified when microbial activity increases and organic matter concentrations are high. Therefore, microbial growth at sites like WSB and WSE due to increased temperature may also accelerate oxygen depletion (Dallas, 2007).

Turbidity levels observed in the water samples significantly increased during January 2022 across most sites, reaching 20-25 NTU, likely due to wet-season runoff. Specifically, WWTP exhibited consistently elevated turbidity, with a notable spike in May 2022 indicating possible sediment or effluent discharge. At SB, a continuous rise in turbidity from 7 NTU to 48 NTU across the study period reflects cumulative sedimentation and runoff impacts. These findings align with those by Koekemoer et al. (2021) reporting high turbidity levels in the Mooi River system. This highlights the role of anthropogenic activities, particularly upstream agricultural practices and heavy rainfall events, in driving turbidity fluctuations.

Furthermore, nutrient enrichment was also evident throughout this study, with phosphorus levels predominantly above the mesotrophic threshold ($> 25 \mu\text{g/L}$) recommended by the South African Water Quality Guidelines for aquatic ecosystems (DWAF, 1996). Concentrations frequently exceeded $250 \mu\text{g/L}$, with alarmingly high peaks of 2800-3600 $\mu\text{g/L}$ at TPB, GC and WWTP. This elicits continuous opportunities for microbial growth and the presence of a eutrophic environment. These observations illustrate the contribution of urban runoff, agricultural fertilisers and wastewater discharges to nutrient enrichment. Consequently, at these sites, low levels of species diversity with nuisance growth of aquatic plants and blooms of blue-green algae may occur (DWAF, 1996). Indeed, *Cyanobacteria*, was found to be significant at GC (46.96%) and TPB (45.98%) in January 2022, contributing to eutrophication (O'Neil et al., 2012). Furthermore, sites such as WSB demonstrated some natural decrease in concentrations from the source of the stream towards the inflow to the Mooi River. This occurrence benefits ecosystem equilibria, but upstream management remains of utmost importance to mitigate eutrophication risks.

5.2 Heavy metals and trace elements

In terms of trace and heavy metals measured, concentrations varied across sites and temporal fluctuations, often reflecting localised or episodic contamination events from mining activities and leachate from other agricultural activities (Ibekwe & Murinda, 2016). To draw conclusions, all observed concentrations were compared with the acute and chronic thresholds set out by the DWAF (2016).

Metals, including magnesium, manganese, copper, zinc, and iron, are considered essential in biological processes at trace concentrations. However, they can cause toxicity when present at levels above permissible limits (Dallas & Day, 2004; DWAF, 1996). In the present study, magnesium and manganese concentrations (with the exception of one site) were the only metals observed to be at acceptable levels with regard to facilitating biological processes. Magnesium levels ranged from 24-55 mg/L across all sites, which is well below the acute 100 mg/L threshold. While manganese levels generally remained below the acute level of 1300 µg/L, it exceeded the chronic limit of 370 µg/L at WSB, with peaks of 800 µg/L. Copper levels frequently exceeded the acute threshold of 12 µg/L, with peaks of up to 70 µg/L at MB in May 2022. Additionally, the average across all six sampling months at each site exceeds the acceptable acute level. Zinc demonstrated the most alarming spikes with 100-300 µg/L concentrations in May 2022 at most sites, far exceeding the acute threshold of 36 µg/L. Iron concentrations gradually increased across several sites, with notable peaks of 150 µg/L at TMB and KD in March 2022. WSB report high iron levels up to 130 µg/L, but a significant decrease in iron occurs from the start of the stream towards the end with a high of 50 µg/L. In addition, downstream attenuation was observed, with LSE showing lower levels (200 µg/L), from peaks at the stream origin with peaks of 620 µg/L, reflecting effective natural filtration. The high variability and deviations during peak events suggest localised contamination, likely tied to industrial or urban runoff (Khushbu *et al.*, 2022).

Moreover, metals such as arsenic (As) and lead (Pb) can be extremely toxic to aquatic organisms, even at low concentrations (Hussain *et al.*, 2020). In the present study, arsenic levels exceeded the chronic threshold of 20 µg/L at WSB, with peaks of 30

µg/L at SB in November 2021, highlighting agricultural or industrial discharges. Furthermore, lead levels surpassed the chronic threshold at several sites, with widespread contamination in May 2022, which could be attributed to wastewater from electrical or steel-related industrial operations as seen in research done by Khushbu *et al.*, (2022).

In addition, aluminium levels were found to be predominantly below the acute threshold of 150 µg/L except at KD in March 2022, AtW in November 2021 and LSB in March 2022. This aligns with the observed pH concentrations, as aluminium is highly dependent on pH levels, increasing as pH decreases (Botté *et al.*, 2021). However, aluminium levels consistently exceeded the chronic threshold of 25 µg/L (DWAF, 1996). Peaks of approximately 80 µg/L in November 2021 and March 2022 at urban and downstream sites indicate episodic contamination from urban runoff or wastewater discharges. Sites like LSE reached aluminium concentrations close to the acute threshold during contamination events, necessitating targeted interventions to mitigate any ecological impacts. Calcium concentrations ranged between 20-40 mg/L at KD, with seasonal lows in November 2021 due to dilution during the wet season and peaks in March 2022. Sites closer to urban areas, such as AtW, indicated lows of 25 mg/L and higher values around the winter season of 75 mg/L. TPB and GC exhibited higher baselines (50 and 60 mg/L, respectively), reflecting anthropogenic inputs of any sorts. At LSE, gradual accumulation downstream of sources was evident, emphasising the role filtration and buffering capacity play in natural processes. Potassium exhibited occasional peaks, such as in March 2022, across all 13 sites but remained within safe limits. These parameters suggest limited ecological risks under the current conditions and portray stereotypic seasonal fluctuations. Barium concentrations ranged between 10-70 µg/L, with notable peaks during March and May 2022, indicating concurrent contamination events. Boron levels spiked at eight sites in November 2022, reaching up to 120 µg/L, suggesting that isolated contamination occurred. Selenium levels remained low, with sporadic peaks exceeding the 2 µg/L target threshold at AtW, HB, TMB, and TPB in July 2021. Lastly, vanadium concentrations fluctuated between 5-7 µg/L during March and May 2022, reflecting seasonal variation.

While some metals were observed within acceptable thresholds across some sites, the findings indicate episodic peaks of various metals across sites, highlight the need for targeted interventions to address industrial and diffuse sources of contamination and continuous monitoring to prohibit any event from occurring without notice.

5.3 Bacterial community abundance by taxonomic Levels

5.3.1 Abundance at phylum level

Bacteroidota, *Proteobacteria*, *Actinobacteriota* and *Verrucomicrobiota* emerged as dominant phyla, illustrating their ecological versatility and adaptive strategies.

Bacteroidota are primarily associated with the degradation of organic matter (Hudson & Egan, 2022), thriving in nutrient-rich environments often influenced by anthropogenic activities or agricultural by-products (Kruczyńska *et al.*, 2023). Their prevalence in sites like AtW and WSB, with an abundance ranging from 24% to 50%, aligns with elevated nutrient loads in these areas. Morphologically, their surface-associated enzymes enable efficient utilisation of complex organic substrates, aiding their dominance in eutrophic conditions (Thomas *et al.*, 2011).

Proteobacteria, particularly abundant at WSE with 65.81% and WWTP, are highly adaptable due to their metabolic diversity (Kerstens *et al.*, 2006). This phylum includes genera capable of utilising a wide range of carbon sources and thriving in environments with high nutrient enrichment (Newton *et al.*, 2011). Their motility and biofilm-forming capabilities enhance survival in fluctuating and nutrient-rich environments (Newton *et al.*, 2013). Multivariate analyses show a strong positive correlation between *Proteobacteria* and aluminium, further highlighting their resilience in metal-contaminated environments (Ancion *et al.*, 2010; Emenike *et al.*, 2023; Zhao *et al.*, 2020).

Actinobacteriota, dominant at SB (75% in November 2021), are noted for their roles in organic matter decomposition and metal resistance (Mawang *et al.*, 2021; Presentato *et al.*, 2020). Their abundance at sites with high iron concentrations like LSB in

September 2021 (620 µg/L) suggests that mechanisms are prevalent to cause resilience (Presentato *et al.*, 2020). Their filamentous morphology also provides structural advantages in sediment-rich environments (Ventura *et al.*, 2007). However, RDA indicates that *Actinobacteriota* is negatively correlated with high TDS, calcium and copper, suggesting specific environmental preferences for low-salinity and high-temperature conditions (Mawang *et al.*, 2021).

Verrucomicrobiota, though less abundant (2-10%), peaked at MB in November 2021 (32.21%). This phylum is often associated with carbohydrate-rich niches, reflecting site-specific sediment composition and nutrient availability (Spring *et al.*, 2016). Their multivariate profiles indicate sensitivity to elevated TDS and calcium, aligning with their preference for environments enriched with phosphorus (Orellana *et al.*, 2022).

Cyanobacteria, significant at GC (46.96%) and TPB (45.98%) in January 2022, are photoautotrophs capable of nitrogen fixation (Herrero *et al.*, 2001). Their blooms in phosphorus-rich (>250 µg/L) and warm (25°C) conditions emphasise the risks of eutrophication (Baughn & Malamy, 2004; O'Neil *et al.*, 2012). Their morphology, including buoyant gas vesicles, leads to dominance in stratified aquatic ecosystems (Mehdizadeh & Peerhossaini, 2022). Their respective multivariate profile is indicative of a phylum highly adaptive to various environmental conditions.

5.3.2 Abundance at class level

Bacteroidia thrive in anaerobic or oxygen-deprived environments, contributing to the decomposition of biomass (Baughn & Malamy, 2004; Thomas *et al.*, 2011). Their dominance (58.93% at MB in May 2022) line up with sediment-rich and organic-dense environments and ties in with results from this study (Newton *et al.*, 2013; Thomas *et al.*, 2011). Multivariate analysis from Figure 4.74 links this class positively with aluminium, iron and copper. This cements their resilience towards metal-enriched environments.

Gammaproteobacteria was the most abundant at WSE in January 2022 (61.16%). This class is formidably known for their contribution to denitrification and is highly adaptable to environments with high levels of aluminium (Arat *et al.*, 2015). Their

cellular adaptations, such as a robust outer layer, confer resilience to external factors (Newton *et al.*, 2013).

Actinobacteria, most abundant at SB (75.58%), exemplify their adaptation to high phosphorus and TDS environments. *Chloroflex* and *Acidimicrobiia*, found to be closely related to *Actinobacteria* during multivariate analyses, were positively influenced by turbidity and temperature.

5.3.3 Abundance at family level

Spirosomaceae, *Burkholderiaceae* and *Microbacteriaceae* exhibited a positive correlation with iron and aluminium and is illustrated as such at SB in November 2021 with 65.69% abundance of *Microbacteriaceae* (levels of iron and aluminium were 50 µg/L and 130 µg/L respectively). This capacity signifies their adaptability to metal-rich environments (Joshi *et al.*, 2023). These families are well known to harbour mechanisms to counteract metal toxicity including the production of chelating agents or intracellular sequestration of metals, which ensures survival in such conditions (Coenye, 2014). *Spirosomaceae* indicate some sensitivity to magnesium and calcium, possibly due to competition between metal uptake mechanisms, which could be detrimental to bacterial growth (De Zutter *et al.*, 2021). *Burkholderiaceae*, which are known for their contribution to bioremediation, align with environments with enriched metal environments contributing to pollutant degradation (Adrian-Kalchhauser & Burkhardt-Holm, 2016).

Families such as *Rubritaleaceae* and *Chitinophagaceae* indicated close relatability and a positive correlation towards environments with high potassium and phosphorus levels promoting their specialised metabolic pathways which include nitrogen cycling and polysaccharide degradation (Chung *et al.*, 2020). Negative correlations evident from the RDA were that *Rubritaleaceae* was highly sensitive to high levels of aluminium and iron.

5.3.4 Abundance at genera level

Genera-level analysis revealed diverse environmental preferences that reflected functional specialisations. *Pseudarcicella*, *Polynucleobacter*, and *Rhodoluna* exhibited strong positive correlations with iron and aluminium, environments often associated with metal-rich sediments. These genera are recognised for their tolerance to oxidative stress induced by heavy metals, with some species capable of bioaccumulation, contributing to metal detoxification (Emenike *et al.*, 2023; Joshi *et al.*, 2023).

Limnohabitans and *Flavobacterium* favoured high TDS conditions, aligning with their ecological roles in salt-rich and nutrient-enriched waters. *Limnohabitans*, commonly found in freshwater systems, exhibit metabolic flexibility that supports growth under varying ionic conditions (Newton *et al.*, 2011). *Flavobacterium*'s ability to degrade complex organic matter enhances its dominance in high-TDS ecosystems (Weber-Scan & Duffy, 2007).

Sphingorhabdus and *Sediminibacterium* displayed broad adaptability, correlating positively with TDS, copper, and magnesium. These genera are associated with polysaccharide degradation and the ability to form biofilms, providing stability in fluctuating environments (Lear & Lewis, 2009).

Algoriphagus and *Armatimonas*, positively influenced by phosphorus, temperature, and turbidity, reflect their preference for dynamic, nutrient-enriched environments. These genera play crucial roles in organic matter recycling, leveraging their enzymatic systems to exploit enriched habitats. *Algoriphagus*, in particular, is associated with detrital food webs, while *Armatimonas* contributes to the degradation of organic compounds (Linz *et al.*, 2018; Melzer & Steinberg, 1983).

5.4 Eukaryotic community composition

5.4.1 Abundance at kingdom level

Animalia dominated across sites, with a mean abundance of 67.91%, and exhibited peaks at KD in May 2022 (80.35%). This elicited the site's stability and limited anthropogenic influence. Inversely, *Chromista* peaked at WWTP in July 2021 (38.31%), aligning with high TDS (approximately 700 mg/L) levels, indicative of nutrient-rich and potentially eutrophic conditions. Such environments may create the most favourable environment for *Chromista*, especially *Oomycota*, which dominate in nutrient-rich ecosystems (Taylor *et al.*, 2009). The unassigned sequences highlight the need for enhanced taxonomic resolution, possibly through Whole Genome Sequencing or updated reference databases (Barrenechea Angeles *et al.*, 2024).

5.4.2 Abundance at phylum level

Arthropoda (83.33%) emerged as the most prevalent phylum, with peaks at KD in May 2022 (92.24%) and declines to its least prevalent at WWTP in July 2021 (33.78%). *Arthropoda* abundance correlates with stable environments and moderate TDS levels (approximately 300-400 mg/L), as seen at KD and MB (Lewthwaite *et al.*, 2024). These organisms thrive in conditions where nutrient levels are balanced with minimal continuous external pollutants (Lister & Garcia, 2018). In contrast, *Oomycota* dominance at WWTP (66.21% in July 2021) illustrates the site as eutrophicated, supported by elevated phosphorus and turbidity levels. This, furthermore, exemplifies the state of eutrophication as *Oomycota* is often described as “sewage fungus” and closely correlated with high organic content environments (Taylor *et al.*, 2009). *Mollusca*, with minimal abundance, suggests sensitivity to heavy metals such as copper and zinc, which peaked across sites in May 2022 (143 µg/L and 300 µg/L, respectively). This aligns with studies by (Lau *et al.*, 1998; Singh & Gupta, 2021), highlighting their role as bioindicators of metal toxicity.

5.4.3 Abundance at class level

Insecta (63.5%) were observed to be most prevalent across all sites, peaking at KD in May 2022 (87.04%), consistent with stable, nutrient-rich conditions. Their prevalence underscores their adaptability to moderate environmental variability, making them critical contributors to aquatic food webs (Sharma *et al.*, 2018). *Peronospora*, which was most prevalent at WWTP (77.74%), aligns with high phosphorus levels, highlighting its adaptability to eutrophic conditions (Mandal *et al.*, 2008). Elevated phosphorus and organic matter create ideal conditions for *Peronospora*, which has been implicated in nutrient cycling and detritus decomposition in freshwater systems (Marano *et al.*, 2016).

5.4.4 Abundance at order level

Peronosporales (76.01%) and *Saprolegniales* (10.76%) exhibited high abundance at WWTP and WSB, respectively, correlating with elevated TDS and phosphorus levels. *Peronosporales* thrive in nutrient-enriched, low-flow conditions (Marano *et al.*, 2016), while *Saprolegniales* association with agricultural runoff highlights their adaptability to organic pollution (Pires-Zottarelli *et al.*, 2024). *Saprolegniales* role in decomposing organic matter aligns with findings which emphasise their importance in maintaining ecosystem functionality (Masigol *et al.*, 2021). The reduced prevalence of *Lepidoptera* at WWTP (3.39%) may result from heavy metal stress, with copper frequently exceeding acute thresholds (e.g., 143 µg/L at MB in May 2022). Studies by Pallottini *et al.*, (2023) confirm the sensitivity of *Lepidoptera* to copper toxicity, which disrupts their development and survival.

5.4.5 Insights on family and genus level

Pythiaceae (97.81%) and *Pythium* (97.41%) dominated across sites, peaking at TPB and GC during high phosphorus events. *Pythium* plays a role in nutrient cycling and adaptability to eutrophic conditions underscores its ecological significance (Yosilia *et al.*, 2023). Its ability to thrive in environments with elevated phosphorus and moderate

turbidity highlights its functional versatility. *Monarchidae* (1.21%) showed localised peaks, possibly reflecting site-specific ecological niches.

5.4.6 Kruskal-Wallis H Test

As seen in Table 4.1, across the taxonomic ranks, parameters, namely sampling month, season and wet or dry season, indicated no statistical significance towards the Chao1 diversity index results. Conversely, site or location indicated statistical significance toward the Chao1 index results, meaning that the specific sample site influenced the species richness. Additionally, when we combined the site data with Site/Location and site/Wet season, the data became statistically significant. Since this tool is utilised to evaluate unevenly distributed data sets like the microbiome in riverine ecosystems, it is evident that the site and whether it was a wet or dry season directly influenced the species richness.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The present study aimed to survey the aquatic bacterial and eukaryotic microorganism communities within the Mooi River, Loop Spruit and Wonderfontein Spruit systems and to assess the anthropogenic influences affecting the composition of these communities. By integrating analyses of physical and chemical parameters, heavy metal concentrations, microbial diversity, and multivariate relationships, the findings of this study provide critical insights into both the composition and health of the Mooi River, Loop Spruit and Wonderfontein Spruit ecosystem and how these parameters play a role in holistic biomonitoring (Agerbo Rasmussen *et al.*, 2021; Andruszkiewicz *et al.*, 2017).

Specifically, the physical and chemical parameters of the Mooi River system and its tributaries portrayed clear spatial and temporal variations, reflecting a complex equilibrium of natural and anthropogenic influences. Parameters such as TDS, pH and turbidity were significantly influenced by land use, seasonal fluctuations and localised pollution events, especially at urban and wastewater treatment sites (Hu *et al.*, 2017;

Juvigny-Khenafou *et al.*, 2021).. The occurrence of alkaline environments across sites suggests robust buffering capacities, while fluctuations in turbidity and temperature highlight the impacts of runoff, sedimentation and microbial activity. These findings are consistent with prior studies indicating the role of anthropogenic stressors in altering water quality in freshwater systems (Carpenter *et al.*, 2011; Hu *et al.*, 2017; Juvigny-Khenafou *et al.*, 2021).

Furthermore, the assessment of heavy metals showed high levels of contamination across multiple sites, with episodic spikes in aluminium, zinc, and copper levels surpassing accepted thresholds. The presence of elevated phosphorus levels further elicits the risk of eutrophication. This is in accordance with prior studies linking nutrient enrichment to microbial blooms and ecosystem degradation (Jackson & Vallaire, 2009; Linz *et al.*, 2018; Presentato *et al.*, 2020). Moreover, the detection of trace metals such as lead and arsenic at levels exceeding chronic thresholds highlights the urgent need for targeted interventions to mitigate both ecological and human health-associated risks (Adams & Greeley, 2000; Astudillo-García *et al.*, 2019; Guski, 2001).

Next-generation sequencing of 16S rRNA and COI barcodes enabled a detailed characterisation of bacterial and eukaryotic communities. *Proteobacteria*, *Actinobacteriota*, and *Bacteroidota* dominated bacterial communities, exploiting their environmental versatility and adaptive techniques in nutrient enriched and metal polluted environments (Emenike *et al.*, 2023; Kersters *et al.*, 2006). Eukaryotic communities were dominated by *Arthropoda* and *Chromista*, with significant site specific and seasonal variations. Multivariate analyses further revealed strong correlations between microbial community composition and environmental stressors, such as heavy metals and nutrient levels, aligning with niche theory and ecological models of disturbance. Numerous studies exemplify the possibility of using bacteria and eukaryotic microorganisms as biomonitors in aquatic health studies alongside high throughput identification systems (Fierro *et al.*, 2017; Merkley *et al.*, 2004).

Lastly, several diversity indices were used which indicated high microbial diversity at unmodified sites and reduced diversity at urban and industrially impacted sites. In addition, the Bray-Curtis similarity tests and RDA analyses highlighted distinct clustering patterns, with environmental parameters such as TDS, phosphorus, and

metals emerging as key drivers of community composition. These findings provide evidence and underscore the value of using multivariate approaches to emphasise the complex interactions between abiotic factors and microbial ecology (Aylagas *et al.*, 2014, 2017).

6.2 Recommendations for future studies

Drawing on the methodology employed as well as the findings of the present study, several recommendations can be made:

- Firstly, to refine and establish this approach as a holistic biomonitoring tool, a reference database should be developed in the future. Such a database will consolidate previous data and enable year-on-year comparisons to inform decisions and management strategies aimed at ensuring the sustainability of aquatic ecosystems.
- Future studies could also include expansive datasets containing diverse geographical and land use data as it will provide high-resolution data to trace anthropogenic pollutants directly to their source.
- Furthermore, the inclusion of chemical parameters such as nitrite, nitrate, sulphate and total organic carbon in future replication studies is recommended. Including these variables in monitoring approaches will provide more in-depth insights into aquatic ecosystem conditions affected by these parameters.
- Lastly, the inclusion of a *rbcL* primer for the identification of green algae and measuring chlorophyll *a* is recommended as this may significantly improve the identification of eutrophic ecosystems and, consequently, aquatic ecosystem health.

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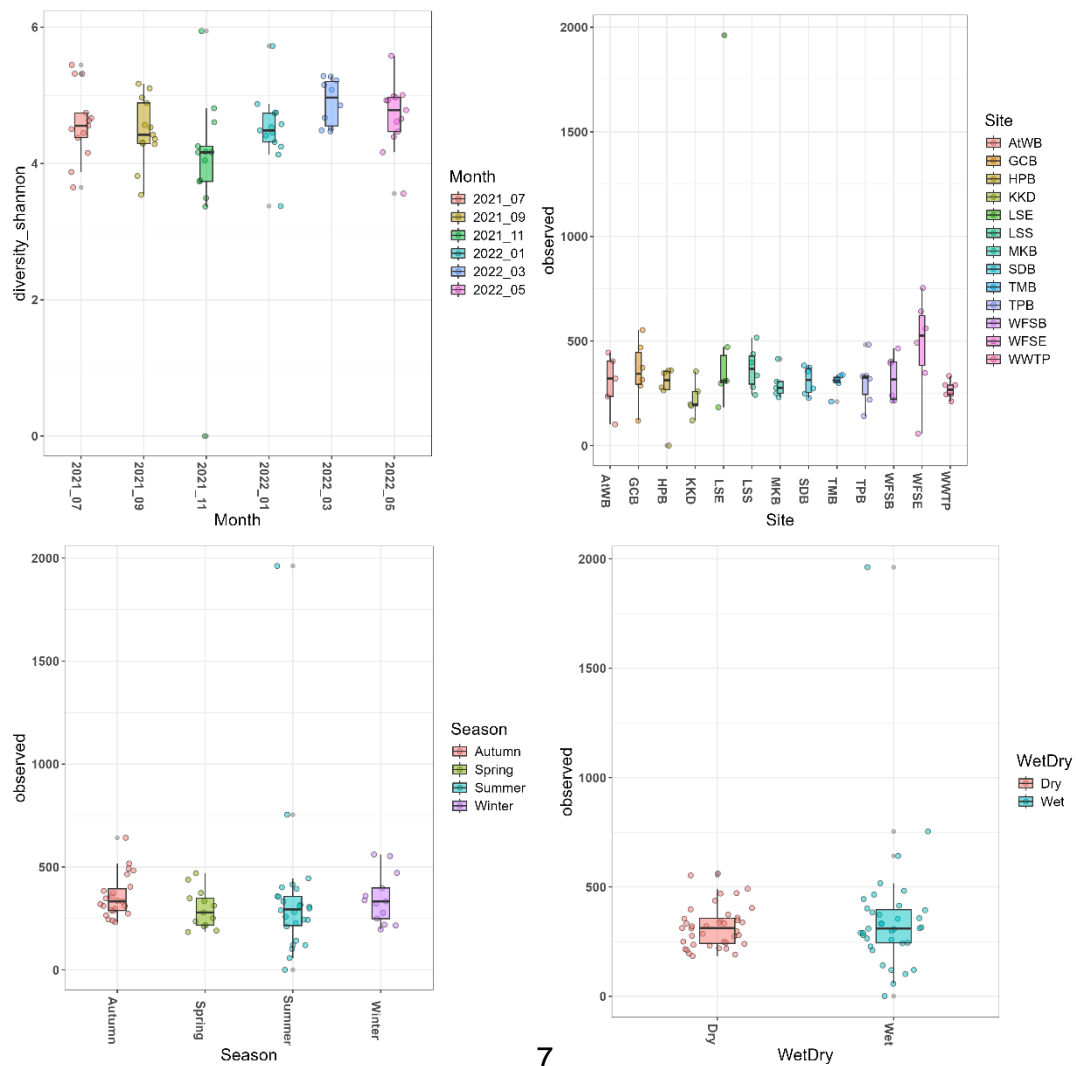
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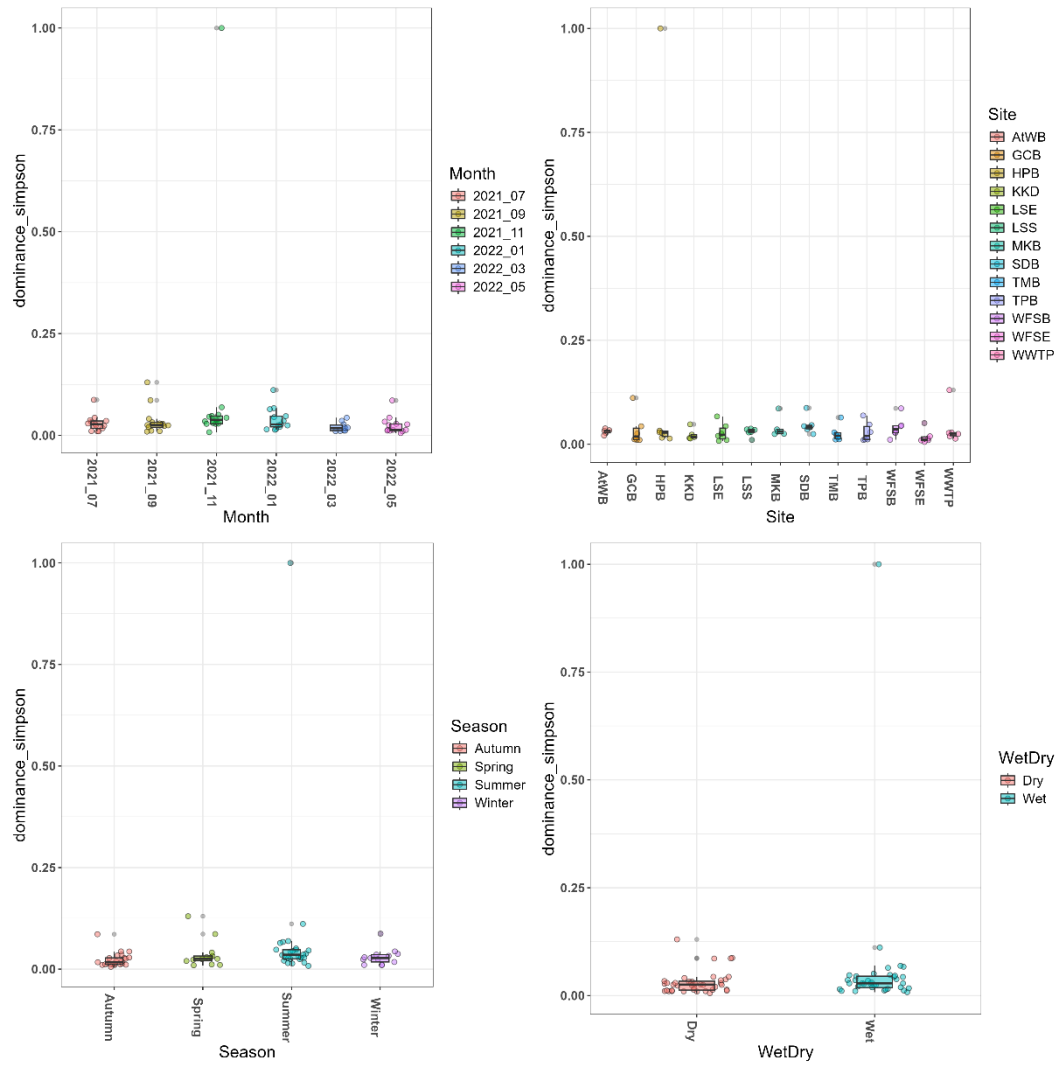
ANNEXURE A

Annexure A contains additional graphic illustrations for the 16S and COI diversity indices that were not included in the main results section. These graphs are merely additional reflections of the previously presented data.

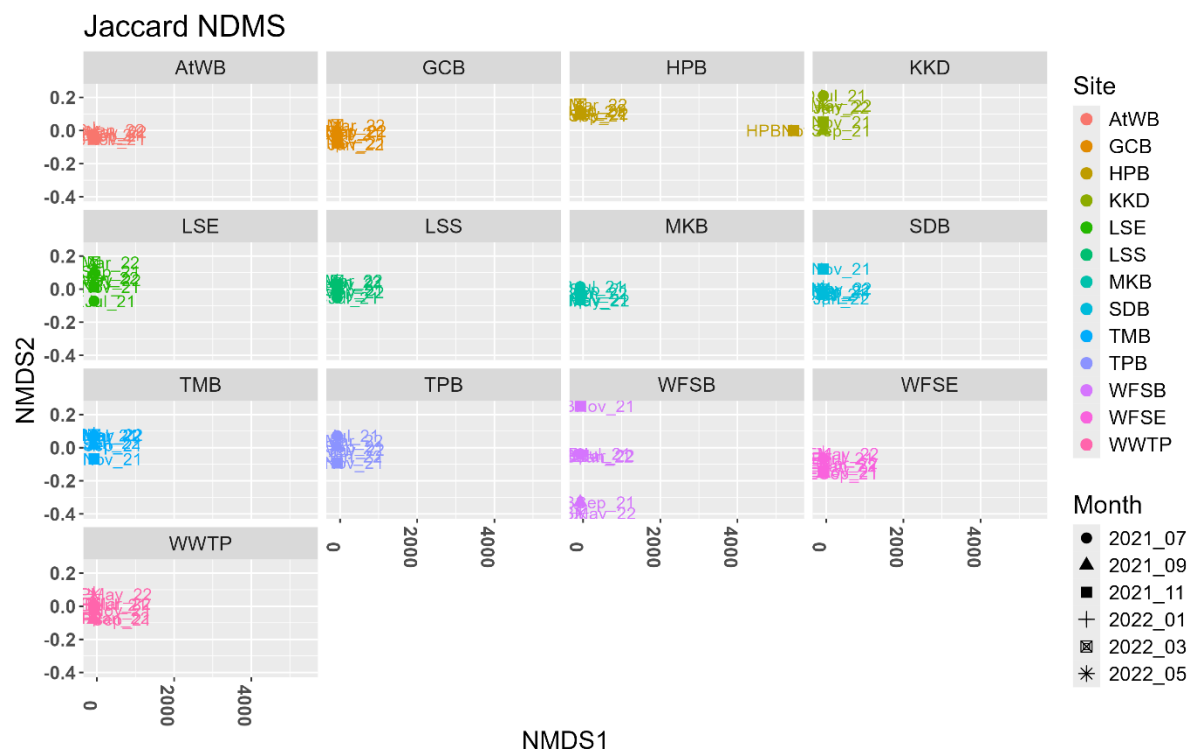
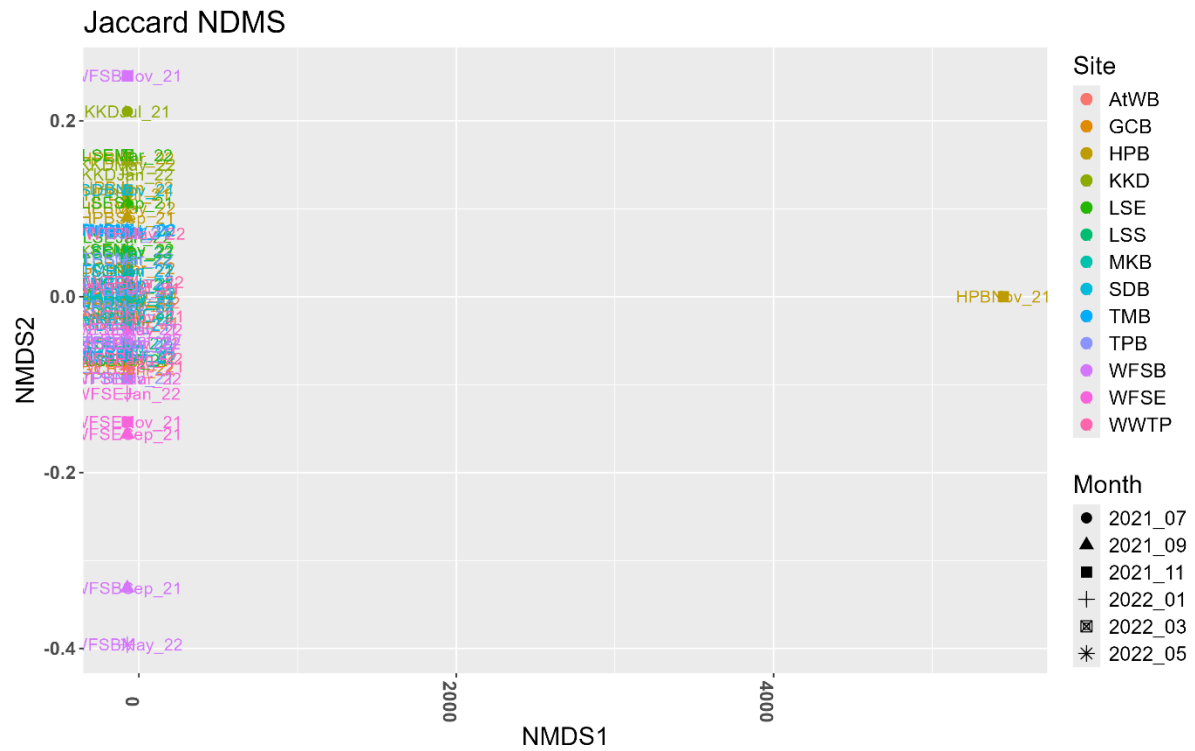
1. Observed Index (16S)



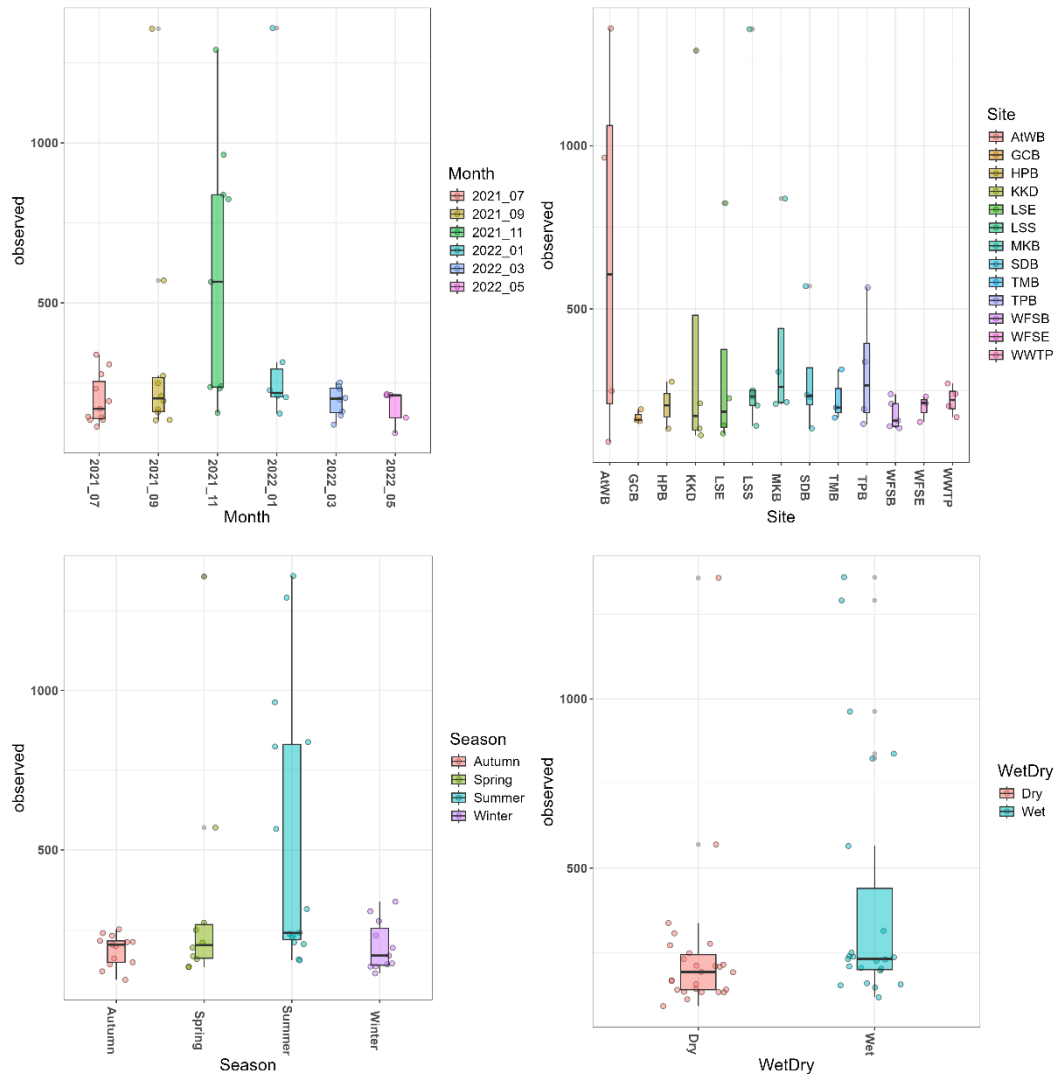
2. Simpson Index (16S)



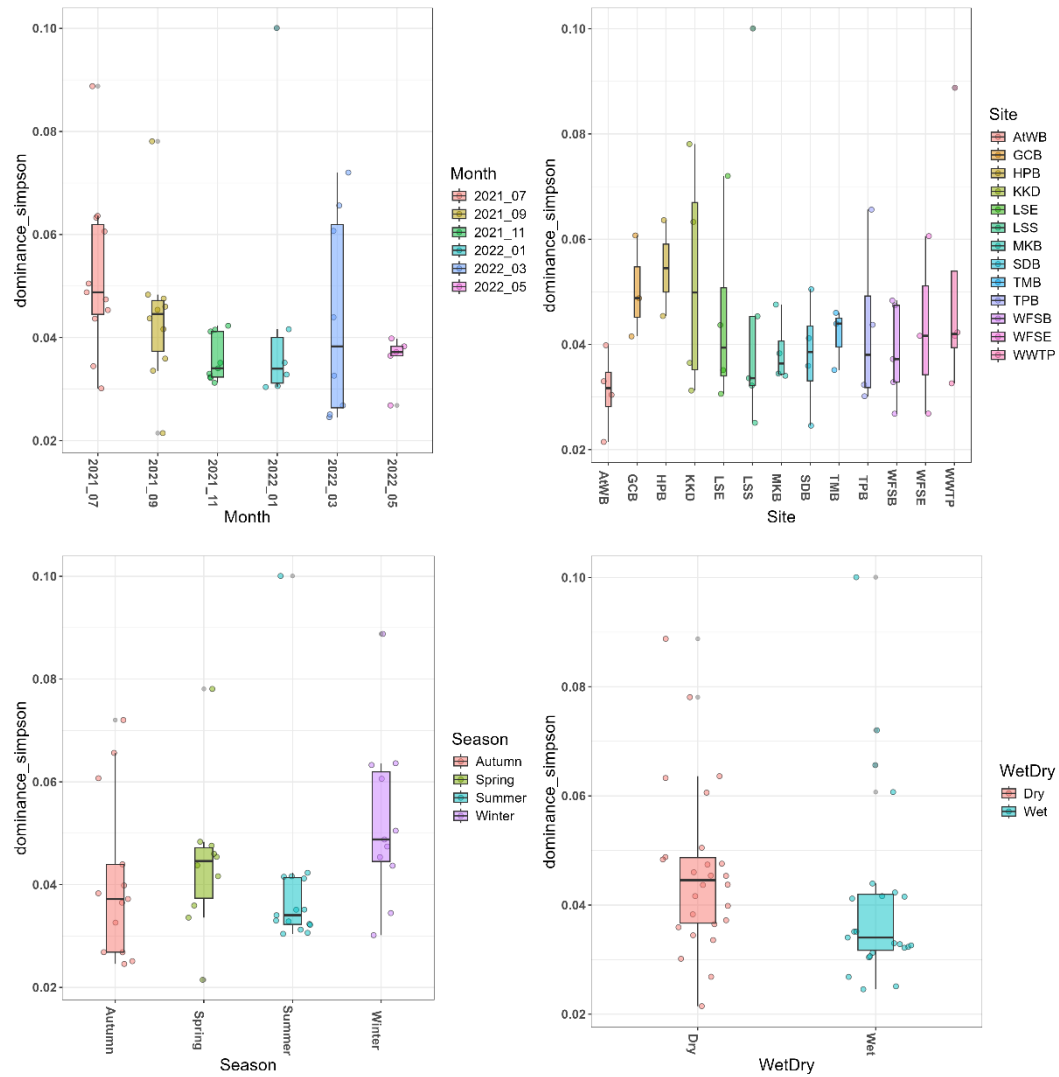
3. Jaccard Index (16S)



4. Observed Index (COI)



5. Simpson Index (COI)



6. Jaccard Index (COI)

