ASSESSING WATER USER IMPACTS ON ECO-HYDROLOGY USING STABLE ISOTOPES

Report to the
WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

INTRODUCTION

South Africa is facing a growing water crisis, whereby our limited surface water resources are simultaneously 98% allocated to environmental requirements, basic human needs and commercial activities. At the same time, these resources are being severely compromised in terms of their water quality and ecological functionality by multiple human activities. Different socio-economic activities including agriculture, urbanisation, and heavy industrial processes such mining, may occur in differing configurations within many river catchments across South Africa. These activities may be sources of compounding and potentially synergistic stress to river ecosystems; particularly in multi-user catchments where all of these different land uses exist in a mosaic through which waters flow.

Water quality monitoring has been used for decades in South Africa to trace sources of environmental stress on aquatic ecosystems, but this remains a relatively imprecise process when trying to identify the primary source of stress. This is due to many key traces such as, the primary nutrients used in photosynthesis (nitrates and phosphates), originating from both natural and anthropogenic sources. Stable isotopes of nitrogen and carbon have been used to understand the structure and function of aquatic food webs, and in recent decades have also been used in various parts of the world to trace intensification of human land cover change, particularly in agricultural and urban landscapes. Stable isotopes of sulfur have more recently been used to trace the effects of both urbanisation and mining on river ecosystems. There is space for innovation in the South African context to explore how all three of these stable isotopes, when used in conjunction with more traditional chemical tracers of water quality such as nutrients and heavy metals, might provide a new set of tools with which to distinguish between contrasting anthropogenic sources of eco-hydrological stress.

RESEARCH OBJECTIVES

The three primary objectives of this project were as follows:

1. To map changes in key stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) across a gradient of low to high water use and resource modification.

2. To identify key individual sources of nutrient pollution using a combination of SIA and more traditional water quality tracers.

3. To determine the key drivers of river ecosystem structure and water quality changes within the river catchment.

STUDY SYSTEM

This project took place in the Gwathle River quaternary catchment (A21K in WMA3-Crocodile West and Marico) in the North West Province. The two major tributaries of the Gwathle River, namely the

Sterkstroom and Maretlwane rivers drain the Magaliesberg mountain range and in doing so, multiple surrounding land-use activities. The rivers flows through the platinum group metal-bearing Rustenberg Layered Suite of soils within the Bushveld Basin, which has come to support multiple platinum and chrome mines. In addition, these rivers also flow through large peri-urban settlements (Marikana and Wonderkop) that arose to accommodate the mining workforce, as well as irrigated cropland in the upper reaches. The catchment thus provides a mosaic of different human land-uses (agriculture, urban and mining), which all were predicted to have contrasting but potentially synergistic negative effects on the eco-hydrology of the streams that drain this landscape. A total of 15 study sites were selected, with each strategically placed either upstream or downstream of a major change in land cover, or a major potential point source of water quality stress, such as a wastewater treatment works or a tributary draining a mine pollution control dam. A host of *in-situ* physico-chemical water quality parameters, as well as anions, cations and heavy metals were collected bi-monthly over a year, while stable isotopes were collected at the end of the wet season (April-May 2021) and the end of the dry season (September-October).

CATCHMENT TRENDS IN WATER QUALITY TRACERS

Water quality began with pristine characteristics in the headwaters of both the Sterkstroom and Maretlwane rivers, which then became progressively more modified as the rivers flowed downstream. Multiple regression analysis allowed us to isolate the water quality parameters that best characterised relative increases in our three major human land cover types (agriculture, urban and mining). Overall, pH increased down the river continuum in response to general land-use intensification. Fallow agricultural lands (associated with rangeland livestock farming) were characterised by elevated orthophosphate levels. Urban land cover was best characterised by elevated ammonium, being a key indicator of leaky sewerage infrastructure. Finally, mining was most consistently associated with high iron concentrations in the water column, although extremely high turbidity was also closely associated with a commercial chrome mine on the Maretlwane River.

CATCHMENT TRENDS IN STABLE NITROGEN AND CARBON ISOTOPES

The $\delta^{15}N$ values were initially depleted in the pristine reaches of both tributaries, but followed contrasting trajectories as they flowed down the river continuum. On the Sterkstroom, $\delta^{15}N$ became steadily enriched following agricultural and urban runoff sources, before becoming dramatically depleted at one site below the town of Marikana. It is believed that this uncharacteristic shift was caused by the food web assimilating a highly reactive nitrogen energy source which may be associated with the illicit dumping of chemicals used in artisanal mine operations for the processing of chromium ore. The depletion of $\delta^{15}N$ was reversed at the next site, where runoff from a platinum pollution control dam appeared to rapidly enrich the food web to nitrogen isotope values seen above Marikana. On the Maretlwane, $\delta^{15}N$ became steadily enriched along the continuum. There was no clear pattern in $\delta^{13}C$

associated with a particular land use, but this isotope was greatly depleted downstream of Buffelspoort Dam, most likely as a result of anaerobic bacterial chemoautotrophy in the profundal zone of the reservoir.

CATCHMENT TRENDS IN STABLE SULFUR ISOTOPES

This study was able to capture both dry and wet season variation in $\delta^{34}S$ together with seasonal concentrations of SO_4; the main chemical tracer of sulfur sources available to the food web. Sulfur sources emanating from agriculture and urban sprawl added moderately to highly enriched sources of sulfur to the food web, while mines, particularly those with chrome and platinum mine dumps which added large volumes of pulverized rock tailings to the stream, resulted in depleted sulfur entering the food web. Overall, the relative enrichment of $\delta^{34}S$ in fish tissue at a given site was significantly negatively correlated with the proportion of mining land cover in the immediate upstream catchment area.

COMPARISON OF STABLE ISOTOPE AND CHEMICAL TRACER RESPONSES TO SOURCES OF WATER STRESS

Our study revealed several combinations of chemical and isotopic responses to eco-hydrological stressors that could be used together to identify the predominant source of stress. Agriculture results in the enrichment of $\delta^{15}N$ and $\delta^{34}S$ in aquatic organisms, with corresponding small increases in SO_4 and large increases in pH and orthophosphate in the water column. Urban sprawl further enriches $\delta^{15}N$ and $\delta^{34}S$, though to a lesser extent than agriculture, and is associated with elevated ammonium levels, as well as urban-source metals like aluminium. Chrome mining is associated with strongly depleted $\delta^{34}S$ regardless of season, and extremely high turbidity levels and SO_4 concentrations. Platinum mining showed seasonally variable depletion of $\delta^{34}S$, likely driven by the proportion of sediment from mine dumps entering the river after rain, high levels of conductivity and high concentrations of heavy metals such as iron, aluminium and copper. Large dams acted as “reset” points along the river continuum, trapping sediment and nutrients, and depleting $\delta^{34}S$ and $\delta^{13}C$ in the downstream food web.

IMPLICATIONS FOR WATER RESOURCE MANAGEMENT AND FUTURE RESEARCH NEEDS

Our findings indicate that $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ from the tissues of aquatic organisms, when used together with physico-chemical water quality, nutrient and heavy metal measurements, can produce a more nuanced and layered assessment of the response of the ecosystem to anthropogenic sources of eco-hydrological stress than what can be achieved when using only physico-chemical measurements and rapid bioassessment; being the standard monitoring tools applied during river health assessment in South Africa today. By comparing the relative concentrations of key anions (nitrate and sulfate) with the enrichment of $\delta^{15}N$ and $\delta^{34}S$ in the food web, we can discriminate between agriculture, urban development and open caste (platinum and chrome) mining as the predominant water quality stressor at a given monitoring site. These findings may be transferable to other catchments in the country, but the response of these tracers to other forms of mining such as coal and gold would need to be calibrated.

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List of Abbreviations

AICc	Akaike information criterion corrected for small sample sizes
CPOM	Coarse particulate organic matter
DIN	Dissolved inorganic nitrogen
DON	Dissolved organic nitrogen
FPOM	Fine particulate organic matter
GWT	Gwathle
MRT	Maretlwane
NR	Nitrogen ($\delta^{15}N$) range
NWA	National Water Act
PCA	Principal Component Analysis
PCD	Pollution control dam
REMP	River Eco-status Monitoring Programme
SI	Stable isotopes
SIA	Stable isotope analyses
STK	Sterkstroom
WWTW	Wastewater treatment works

1 Introduction and Review of the use of Stable Isotopes as water stress tracers

1.1 Introduction

1.1.1 Project Context: Water resource management in South Africa

In South Africa, headwater catchments are essential for sustained socio-economic growth, yet require careful management to ensure surrounding development does not impede upon their functionality. Associated with mixed-land use catchments are multiple interacting human-derived stressors with different effects, jointly compromising effective environmental governance of downstream aquatic ecosystems and their provision of ecosystem services (Hering et al., 2015). These ecosystems already experience natural disturbances of different frequencies across variable spatio-temporal scales that are critical for the systems' adequate structure and functionality (eds. Birk, 2019). Coupled with harmful interacting anthropogenic stressors it is often considered difficult to tease the two apart owing to their sharing similar effects (Feld et al., 2016). For instance, the polluting nutrient phosphorus enters ecosystems naturally as well as artificially, and is a key constituent in eutrophication (Griffin, 2017). This makes tracing anthropogenic stressors an important field in eco-hydrology which can be used to inform adaptive management strategies.

Being a water scarce country, South Africa receives unequally distributed rainfall, below the global annual average. In turn, the country's surface water resources and in particular headwater streams, are highly variable in flow (MacKellar et al., 2014; Colvin and Muruven, 2017). These fragile headwater streams are the source of main stem rivers that support major sectors of the economy and thus, need to be carefully managed. With already 98% of the country's surface water allocated (Colvin et al., 2016), and 60% of its rivers overexploited, only one-third of the main-stem rivers are considered to be in good condition (Donnenfeld et al., 2018). This is owing to rivers being intrinsically linked with their surroundings. Rivers provide numerous ecosystem services, including provisioning services providing potable water as well as regulatory services such as nutrient cycling and processing. In performing these services, rivers also collect and transport organic matter, sediments and additional constituents obtained from the surrounding catchment area. The integrity of aquatic ecosystems is therefore highly susceptible to catchment stressors. Upon ecosystem degradation, the ecosystem's ability to provide essential ecosystem services becomes hindered.

With society being reliant upon abundant, high quality freshwater resources, it is also the major source of its degradation. Over-exploitation of freshwater resources threatens aquatic ecosystem integrity and the sustainable provision of ecosystem services. Such exploitation is driven by the rising demand for water, particularly among agricultural, municipal and industrial sectors (Colvin et al., 2016). As the demand for water rises by 1% per annum, it is anticipated that there will be a 17% deficit in South Africa's water supply by 2030 (Colvin and Muruven, 2017). Coupled with anthropogenic pollution (diffuse- and point-source) linked to poor land and waste management practices, this places further strain on the long-term viability of freshwater resources. Nutrient loading as a consequence of agricultural (fertilizers) and domestic (detergents and sewage) activities as well as the introduction of heavy metals, chemical waste and additional constituents associated with industry (mining) disturbs the physico-chemical and biological condition/composition of the ecosystem (Erasmus et al., 2020). Such exploitation and pollution may disadvantage downstream users by rendering the resource unusable.

South Africa has implemented numerous measures to safeguard and improve the management of freshwater resources. To ensure adequacy for use, user specific water quality guidelines indicating their water related requirements are established (e.g. aquatic ecosystems, domestic and agricultural sectors). Regulating exploitation of resources is legislated by the National Water Act (No. 36 of 1998) (NWA, 1998) under the Ecological Reserve and through water use licenses. To assess river ecosystem health, a suite of biotic indices under the River Eco-status Monitoring Programme (REMP) are enlisted (e.g. use of fishes and macroinvertebrates). The current water (resource) quality management systems, however, do not offer the means to assess the relationship between specific, or multiple interacting water quality stressors and ecosystem function (Novotny et al., 2005). There is thus a need for additional tools to bridge the knowledge gap between identifying sources of stress on aquatic ecosystems in mixed-use catchments and linking their subsequent effects on the ecological integrity of those resources.

1.1.2 Stable isotopes and their potential as environmental tracers

Environmental or natural tracers are ubiquitous in surface waters as natural and anthropogenic compounds (Leibundgut and Seibert, 2011). They exist as physical properties and chemical components of water whose spatio-temporal differentiation is used to infer information pertaining to the dynamics and movement of water as well as contaminants through the environment (Käss, 1998). Within a study system all tracers are quantifiable, but characterisation of sources and tracers is expensive and only rarely done – hence the originality of this study. Using a tracer's signature (concentration, isotopic composition) along spatio-temporal gradients, researchers can derive the information pertaining to relevant processes and changes occurring throughout. In a surface water context, environmental tracers can provide insight into the system's characteristics as well as track contamination and identify the sources of such. Although standard surface sampling indices used in South Africa track contamination, they overlook the sources of origin.

Stable isotopes (SI) as environmental tracers, are found in water, animal and plant tissue. They provide a highly versatile approach to observe aquatic ecosystems. In tissues, SI's provide a time-integrated signal through assimilation into the animal and plant tissues allowing scientists to assess long-term ecosystem conditions. Forensic (or trace) isotope research is an emerging field in the identification of stress sources along a river with multiple competing land-uses using a multi-isotopic approach, for the betterment of freshwater resource management (Finlay and Kendall, 2007). This approach enables the tracking and, thus, linking of particular water quality changes to land-users at certain spatial scales.

Effectively used as tracers of stress in aquatic ecosystems, stable isotopes are also used to monitor the functionality of aquatic ecosystems. Acquired through the organisms desired dietary mode, stable sulfur ($\delta^{34}S$) and carbon ($\delta^{13}C$), are effective in tracking aquatic food web resources sources (allochthonous and autochthonous) (Ofukany et al., 2014). The stable isotope ratio of nitrogen ($\delta^{15}N$) too has shown extensive success when used in understanding aquatic food webs, whereby indicating trophic positions of different consumers (Post, 2002). With the average discrimination factor known for each stable isotope, there is potential to use them in illustrating ecosystem community organisation, understanding various geochemical processes (e.g. nutrient cycling), identifying primary sources of energy acquisition as well as cross-ecosystem subsidies supporting these communities (Kautza and Sullivan, 2016).

When used in an integrated approach $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ have potential to successfully assess aquatic food-web structure and function (Wada, 2009; Perkins et al., 2010; Ofukany et al., 2014). These isotopes have proved effective or shown potential in identifying aquatic ecosystem responses to pervasive watershed stressors. When assessed in different combinations the SI have revealed biodiversity losses and structural changes along urbanisation, agricultural and mining gradients following declining surface water quality (Lee et al., 2018; Zhao et al., 2019).

By complimenting trace isotopes with macronutrient concentrations, heavy metals and physical-chemical properties (e.g. pH, etc.), a comprehensive spatio-temporal picture of water quality trends in response to land-uses can emerge across a river continuum. Such trends can provide insight into effects of land-use on ecosystem function when comparing food-web structure. This multi-tooled technique, integrating water quality parameters as well as SI as both tracers of stress and indicators of ecosystem response, offers enhanced capabilities to current water (resource) quality management systems whereby linking stressors to their sources. Such a multi-tooled approach has the potential to generate data that can inform sustainable management policies to improve good governance in mixed-use catchments, for the protection of aquatic ecosystems and suitability for use by downstream users.

1.2 Global literature review methodology

Prior to undertaking a study, it is necessary to identify past and current relevant literature pertaining to a particular subject. In doing so, a comprehensive understanding of both past and current practices within a particular field can be sought, from which a relevant research question can be derived. In the case of this study a systematic review was performed to best capture the available literature, both peer reviewed and grey literature (only formal governmental reports), with regards to the current local and global best practices surrounding the use of stable isotopes as tracers of land-use stressors in freshwater ecosystems.

To filter the most accurate, relevant literature to the research question, while discarding the remaining sources, a Boolean Search (or search string) using the “AND” operator was conducted in three separate academic literature search engines. It is ideal to use different search engines as often each vary in their functionality, database size and content diversity as well as the use of syntax (Gusenbauer and Haddaway, 2020). The three academic search engines selected (Google Scholar, Web of Science and Scopus) have access to an extensive number of multidisciplinary resources. A specific step-wise subtractive Boolean Search was employed in each of the three search engines to obtain global and then local literature.

Two separate search iterations were performed using two search strings in each of the three search engines (Table 1). Among the three search engines, the Google Scholar search strings were longer in length compared to the highly precise search strings applied in Web of Science and Scopus search engines. This action was taken to avoid search timeouts as search functionality differs between the latter two and Google Scholar. Local and global best practices in the use of stable isotopes aquatic ecosystem stress-driver tracers were searched for across the search engines without replacement. To source the most recent and best practices, the search results were limited to the last two decades; between, and including the years 2000 and 2021. A search query was deemed successful when the number of hits per query were (1) greater than zero and (2) all the populated sources were not already captured in previous searches. Both peer-reviewed academic literature (including open access) and grey literature were examined. Each article's title, abstract, methodology and conclusion were read to assess its relevance. An additional set of reader-based restrictions were applied while reading the latter components of the literature to further filter through the results and identify the most relevant sources.

Table 1: Using two key search strings in each of the three key search engines local and global stable isotope aquatic pollution tracer sources were identified

Search iteration	Search engines (Database)	Search strings (terms)	Number of hits	Number of relevant papers retained
1	Google Scholar	"stable isotope" AND "water quality" AND "tracer*" AND "pollution" AND "freshwater" AND "stream" AND "river" AND "source*" AND "South Africa"	306	18
2		"stable isotope" AND "water quality" AND "tracer*" AND "pollution" AND "freshwater" AND "stream" AND "river" AND "source*"	2220	9
1	Web of Science	"stable isotopes" AND "tracing" AND "river" AND "pollution" AND "source"	26	8
2		"Stable isotopes" AND "tracer" AND "river" AND "pollution" "source"	101	6
1	Scopus	"stable isotope" AND "tracer*" AND "pollution" AND "freshwater" AND "aquatic" AND "river" AND "source*"	7	4
2		"stable isotopes" AND "tracing" AND "river" AND "pollution"	60	5

These restrictions included the exclusion of non-lotic based studies (lake, estuarine and marine, etc.), non-isotopic tracer studies, groundwater research (aquifer water source tracing as well as surface water-groundwater interaction studies), books and university research theses. Literature which included stable isotopes collected via water samples and in tissues were retained to identify the current standard practices in aquatic stressor tracer studies. Upon confirming that a literature piece met the all criteria, the following details were filtered and populated into an Excel spreadsheet: year published, journal/review title, country, stable isotopes used, material collected (water vs. tissue), whether water quality tracers were used (e.g. nutrients), whether direct pollution link (Y) or suggestive links (N) were made and the activity/land-use where the direct pollution link was made. In this case four key stressors were filtered (1) agricultural fertilizers, (2) agricultural manure, (3) urbanisation (sewage) and (4) mining.

Upon populating the spreadsheet, pivot tables were derived to summarise key information from the literature to compare the current state of the art of aquatic stable isotope stressor tracer techniques between global studies and those used in South Africa. Four separate outputs were derived from the pivot tables. These included, (1) total number of SI aquatic stressor tracer studies published in each of the countries identified within the systematic review, (2) the total number of times a specific stable isotope combination was used within the studies, (3) total number of stressors assessed globally (incl. SA) by each SI combination when derived from tissues, (4) the number of stressors assessed in South African studies by each SI combination when derived from tissues, and finally (5) how many times additional water quality tracers (e.g. nutrients) were used in South African SI tracer studies.

1.3 Literature review results and discussion

1.3.1 Best global aquatic stable isotope tracer practices

A total of 50 studies (peer reviewed and grey literature) were identified to be relevant during searches across the three academic search engines. Of the studies, 17 had stable isotopes extracted from water samples, while the remaining 33 had stable isotopes extracted from tissue samples. Among the 50 studies, South Africa accounted for the fourth highest number of stable isotope stressor tracer studies within the search enquiry totalling five; trailing behind China, the United States of America and Canada (Figure 1).

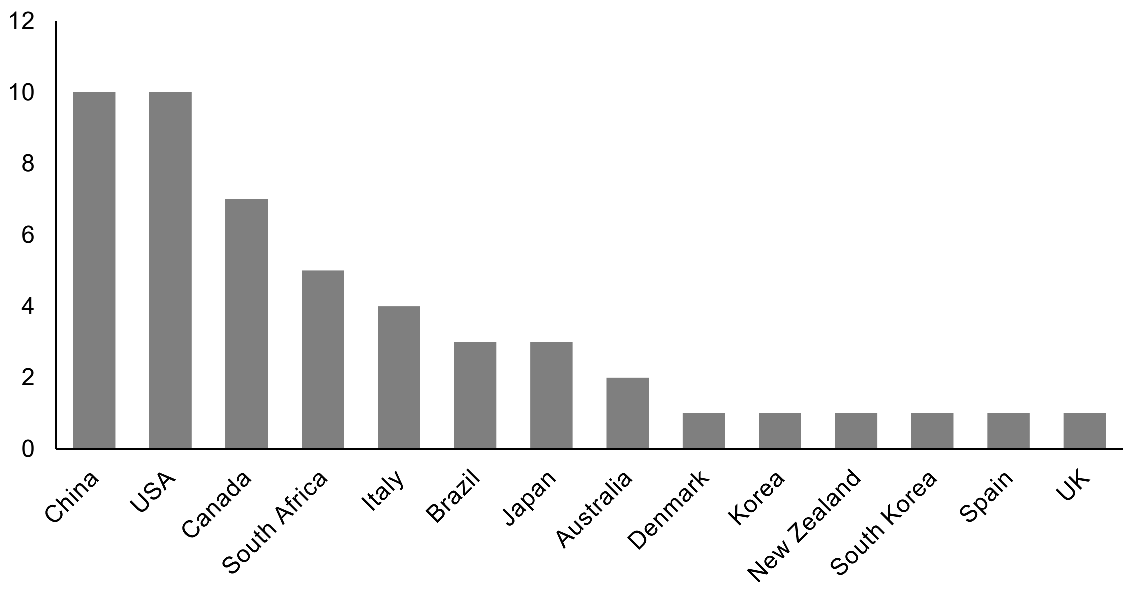


Figure 1: The total number of stable isotope aquatic stressor tracer studies published in each of the countries identified within the systematic review

Pertaining to the combinations of stable isotopes utilised across the studies (regardless of material type) 16 of the 50 studies incorporated both $\delta^{13}C$, $\delta^{15}N$, while 14 used $\delta^{15}N$ alone, six used $\delta^{34}S$-SO_4, $\delta^{18}O$-SO_4 and four incorporated $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ (Table 2). Regarding this latter combination ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$), only three countries (Canada, the UK and the United State of America) integrated this combination of tracers into stressor tracer analysis. Among all the stable isotope stressor tracer combinations, water studies had the highest median number of stressors assessed (3) followed by tissue studies (2) (Table 3). This trend indicates that studies using water-based isotopic tracers have more often been used to characterize systems affected by multiple stressors than tissue-based studies.

Of the studies, one using tissue derived stable isotopes from freshwater invertebrates was able to successfully untangle three key stressors (fertilizer practices, livestock manure and sewage) along a spatial gradient using $\delta^{13}C$ and $\delta^{15}N$ within a mixed land-use catchment in the Guangdong Province, China (Wang et al., 2020). A governmental department study was successful in discriminating three key N stressors (fertilizer, livestock manure and domestic sewage), following the collection and analysis of N source material as well as river water samples for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, within in the Lower Susquenhanna River Basin, Pennsylvania, United States of America (Cravotta, 2002). These two studies are examples indicating that stable isotopes are effective in untangling stressors in river ecosystems when applied at the appropriate scales (spatial & temporal).

Table 2: The total number of times the stable isotope combinations were applied within the reviewed literature sample for both water and tissue

Stable isotopes applied	Number of times used
δ13C, δ15N	16
δ15N	14
δ34S-SO4, δ18O-SO4	6
δ15N, δ18O	4
δ13C, δ15N, δ34S	4
δ34S-SO4	2
δ34S-SO4, δ18O-SO4, δ15N, δ18O-NO3, Δ-33S	1
δ34S-SO4, 87Sr/86Sr	1
δ15N, δ34S-SO4, δ18O-SO4	1
δ13C, δ15N, δ34S, δ37Cl	1
Grand Total	**50**

Our global review of literature in this field of aquatic stressor tracing indicated that stable isotopes collected via water samples are effective in tracing aquatic ecosystem stressors in mixed-use catchments, and represent the current global state of the art in this research field. However, the common isotope analysis techniques used for tracing stressors in water (e.g.

$\delta^{34}S$-SO_4 & $\delta^{18}O$-SO_4 & $\delta^{34}S$-SO_4) are currently not yet available and/or commercially refined in South Africa. To acquire such technical capabilities to perform the analyses would require significant capital investment in both training abroad and new institutional laboratory equipment purchases (together with associated reagents), and are thus beyond the scope of the current research project.

Table 3: The median number of stressors assessed with each of the stable isotope combinations derived from aquatic tissue as well as water sampled

	Stable isotope combinations	Median of number of stressors traced per stable isotope combination
Tissue	δ13C, δ15N	1
	δ13C, δ15N, δ34S	1
	δ13C, δ15N, δ34S, δ37Cl	2
	δ15N	1
Water	δ13C, δ15N, δ34S	3
	δ15N	3
	δ15N, δ18O	1
	δ15N, δ34S-SO4, δ18O-SO4	1
	δ34S-SO4	1
	δ34S-SO4, 87Sr/86Sr	1
	δ34S-SO4, δ18O-SO4	2
	δ34S-SO4, δ18O-SO4, δ15N, δ18O-NO3, Δ-33S	1

1.3.2 The current South African aquatic ecosystem stable isotope tracer toolbox

South African research institutions have techniques and expertise available with regards to analysing $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ isotopes from tissue samples. Among the sampled literature, 33 global and local studies made use of stable isotopes from tissue samples to trace aquatic stressors (Table 4). Of the 33 studies, 16 of them used a dual $\delta^{13}C$ & $\delta^{15}N$ approach, whereas 13 studies used $\delta^{15}N$ alone. The studies found to use the dual $\delta^{13}C$ & $\delta^{15}N$ as well as $\delta^{15}N$ alone assessed a maximum of three key stressors (fertilizer practices, livestock manure and domestic sewage), often in mixed land-use catchments; see Lee et al. (2018), Smucker et al. (2018) and Wang et al. (2020).

The remaining three studies that used tissue derived $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, made efforts to discriminate between two and/or identify key stressors (sewage effluent and/or industrial effluents) (Wayland and Hobson, 2001; Dubé et al., 2005; Morrissey et al., 2013) (Table 4). Of the three studies, only Wayland and Hobson (2001) was somewhat successful in linking and discriminating between stressors (industrial effluent and treated municipal sewage) using $\delta^{34}S$. There is thus space for further global innovation using the tissue derived $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ as stress tracers to strengthen their use in forensic isotope research towards the betterment of freshwater resource management in mixed-use catchments.

Table 4: The number of stressors assessed from tissue derived stable isotopes obtained from aquatic ecosystems globally. The cell highlighted in grey represents the most closely comparable approach to the current project's research aim.

Stable isotope combinations used	Number of stressors assessed for the studies			Grand Total
	1	**2**	**3**	
δ13C, δ15N	11	4	1	**16**
δ15N	7	4	2	**13**
δ13C, δ15N, δ34S	2	**1**		**3**
δ13C, δ15N, δ34S, δ37Cl		1		**1**
Grand Total	**20**	**10**	**3**	**33**

Within the 33 tissue based tracer studies, the five South African studies used two separate stable isotope tracer combinations (Table 5). These included (1) the dual $\delta^{13}C$ & $\delta^{15}N$ approach where a maximum of two key aquatic stressors including urbanisation and fertilization were assessed (Dalu et al., 2015) and (2) $\delta^{15}N$ alone, once again a maximum of two stressors were assessed (agricultural fertilization & manure) (Hill, 2018; Dalu et al., 2020). The key stressors explored by the South African studies were able to effectively identify the aquatic stressors caused by agricultural fertilization, livestock activities (manure) as well as urbanisation (sewage inputs), by employing $\delta^{15}N$ (Hill et al., 2011; Hill, 2018; Motitsoe et al., 2020).

Table 5: The number of stressors assessed from tissue derived stable isotope stressor tracer studies in South Africa aquatic ecosystems

Stable isotopes combinations used	Number of stressors assessed during the studies			Grand Total
	1	**2**	**3**	
δ13C, δ15N		1		1
δ15N	1	2	1	4
Grand Total	**1**	**3**	**1**	**5**

The extensive application of $\delta^{15}N$ in tracer studies in South Africa is a consequence of the $\delta^{15}N$ values in aquatic tissues and instream plants effectively reflecting anthropogenic N loading (Hill, 2018; Motitsoe et al., 2020). When nitrogen is limited, it is rapidly assimilated by organisms causing the $\delta^{15}N$ values of the tissue to reflect that of the surrounding substrate. When N is in abundance, mass-dependent isotope fractionation occurs causing the lighter ^{14}N to react quicker than the heavier ^{15}N, resulting in enriched N isotope tissue values (Kendall, 1998). In addition, owing to $\delta^{15}N$ values of pollutants/substances reflecting the pathway through which they were formed (Kendall, 1998), it is possible to discriminate between N sources using $\delta^{15}N$ values, e.g. synthetic fertilizer vs. animal manure. This approach, however, is only recommended when the $\delta^{15}N$ values of the particular pollutants of interest (nitrogen-source material) have also been identified in the study catchment. This is because the $\delta^{15}N$ values of one pollution type, e.g. animal manure, often found to have wide $\delta^{15}N$ value range between sources (e.g. two separate cattle farms) creating considerable uncertainty in tracing studies without pollution samples collected prior (Kendall, 1998; Widory et al., 2004).

1.3.3 Historical overview of stable isotope tracing applications in South Africa

To best understand the current state of the art in stable isotope stressor tracing in South African aquatic ecosystems it is valuable to explore a brief history of their use. A Water Research Commission report drafted by Hill et al. (2011) assessed N-loading using the $\delta^{15}N$ in the floating *Spirodella spp*. Sampling the Kubusi River system and New Years Dam in the Eastern Cape, the researchers identified $\delta^{15}N$ shifts in response to untreated sewage and agricultural inputs (combined stressors). In 2015 a paper by Dalu et al. (2015) successfully used $\delta^{15}N$ from periphyton communities as an indicator of anthropogenic pollution thereby tracking the combined agricultural and sewage effluent stressors in the lower Kowie River in the Eastern Cape. A paper by Hill (2018) used the floating *Wolffia spp*. in an experimental set up to assess the species effectiveness in tracing mixed-source inputs. Using stock solutions as well as pollution material from the Eastern Cape, the paper deemed the *Wolffia spp*. less useful as a bioindicator of N pollution from mixed land-use sources compared to the *Spirodella spp.* Using $\delta^{15}N$ of microphytobenthos, Dalu et al. (2020) were able to indicate the stressors associated with agricultural and sewage discharges, following enriched $\delta^{15}N$ values within the Bloukrans River system (draining Grahamstown) in the Eastern Cape. Finally, a study by Motitsoe et al. (2020) was able to use $\delta^{15}N$ and C/N ratios of *Spirodela spp*. to trace the N loading relating to sewage inputs separately from those of agricultural run-off (manure) in the Bloukrans-Kowie and Bushman-New Years River systems in the Eastern Cape.

1.3.4 Advancing the stable isotope tracer toolbox in South Africa – a multi-tooled approach

The current South African stable isotope stressor tracer toolbox has proved effective with regards to nutrient (specifically total nitrogen) pollution at and between stressor spatial-scales with particular attention paid to agricultural and urban stressors in the Eastern Cape Province. The current toolbox, however, has not been employed in a multi-use catchment with agricultural activities (fertilization and manure), urbanisation and settlements (treated and potentially untreated wastewater) as well as mining.

The addition of the $\delta^{34}S$ to this stressor tracing toolbox, will provide much needed insight into potential atmospheric sulfur deposition, mine derived stress (pollution) in aquatic ecosystems (Ofukany et al., 2014). Globally, few studies in the last two decades have made attempts to link tissue derived $\delta^{34}S$ to aquatic pollution and discriminate between stressors, (but see Wayland and Hobson, 2001), with no available literature present showing such an attempt in South Africa. In addition to this, global studies that assessed tissue derived $\delta^{34}S$ have not used it as a tracer of stress nor a tracer of resource use in a food web context. There is thus a space to innovate in the use of $\delta^{34}S$ to trace stressors within a South African context and discriminate between three key stressors (agriculture, urbanisation and mining) within mixed land-use sub-river catchments.

There has also been an attempt among the 33 stable isotope studies globally (incl. SA) to incorporate additional water quality tracers with sampled tissue derived isotopes to increase certainty and resolution in such studies. Three such tracers unequally applied included macro-nutrients, major ions and heavy metals. Among these studies, those that applied $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ did not apply additional water quality tracers. The South African studies that applied the dual $\delta^{13}C$ & $\delta^{15}N$ approach only used nutrients, while those that applied $\delta^{15}N$ alone used nutrients and/or major ions. No heavy metals have been applied as water quality tracers in a South African stable isotope stressor-tracer context (Table 6).

Table 6: The number of times water quality tracers were used in South African stable isotope tracer studies

Stable isotopes applied	Count of Tracers: nutrients	Count of Tracers: major ions	Count of Tracers: heavy metals
δ13C, δ15N	1		
δ15N	4	1	

This project therefore seeks to expand the current aquatic stressor tracer toolbox with the techniques currently available in South Africa. This enables us to expand our current technical capabilities within the field of aquatic ecology, allowing new theoretical questions to be investigated. Using a between-stressor spatial sampling regime, this study aims (1) to use $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ isotopes derived from aquatic tissue to map changes in the isotopes across a gradient of land, use modification. In addition to this, the study would (2) identify key sources of nutrient pollution where the additional innovation would be using the SI's together with the water quality tracers (nutrients, ions and metals) to increase the spatial resolution and tracing certainty (3) and finally using $\delta^{34}S$ as a tracer of catchment stress (pollution), while combining it with $\delta^{13}C$ & $\delta^{15}N$ to assess potential changes in food web structure and function which is then related back to particular stressors. The goal of this research is to advance the state of the art in stable isotope-based multi-stressor environmental tracer use in the context of South African headwater streams, for the ultimate benefit of the users who rely on those waters for provisioning and regulating ecosystem services.

1.4. Objectives of this research report

The three primary objectives of this project were as follows:

1. To map changes in key stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) across a gradient of low to high water use and resource modification.

2. To identify key individual sources of nutrient pollution using a combination of SIA and more traditional water quality tracers.

3. To determine the key drivers of river ecosystem structure and water quality changes within the river catchment.

2. Field and Laboratory Methods

2.1 The Gwathle River catchment

This research project called for a self-contained catchment with limited human impacts in the headwaters, and progressive intensification of the major human-derived stressors of South African river ecosystems (agriculture, mining, and urban/peri-urban development). The system selected was the Gwathle (Quaternary catchment A21K), which drains the northern slopes of the Magaliesberg mountain range between the towns of Rustenburg and Mooinooi, before its confluence with the Crocodile (West) River at Roodekoppies Dam in the North West Province. The headwaters are partially encapsulated within the Magailesberg Protected Environment, a provincial protected area comprising several private nature reserves, and wholly encapsulated within the Magaliesberg Biosphere Reserve, an area designated for ecotourism and sustainable development under the UNESCO Man and Biosphere programme (Pool-Stanvliet, 2013). The Magaliesberg Biosphere was proclaimed in 2015 and is managed by a board comprising local landowners and stakeholders to promote conservation of the landscape and to prevent environmentally harmful economic development of this mountainous region.

The headwaters of the Gwathle comprise several small mountain tributaries that merge into two major streams, the Sterkstroom and the Maretlwane, which flow north through a highly developed mining region before merging into the Gwathle River. Both rivers drain sandstone mountain catchments, characterised by low natural conductivity and a low pH, whereas both then flow into the bushveld basin, characterised by dolomitic and igneous bedrock that produces more alkaline groundwater with a higher natural conductivity. The soils here form part of the platinum group metal-bearing Rustenberg Layered Suite, a narrow band of rock running along the northern edge of the Magaliesberg (Magalhães et al., 2018) that supports several chrome and platinum mines between the towns of Rustenburg and Brits. While the upper Sterkstroom is placed within the Western Bankenveld aquatic ecoregion (Level 1 ecoregion 7), its lower reaches, the whole of the Maretlwane and the Gwathle are placed within the Bushveld Basin aquatic ecoregion (Level 1 Ecoregion 8; Nel et al., 2011).

The upper tributaries of the Sterkstroom flow through low intensity agricultural land, before flowing into the Buffelspoort Dam reservoir, a water source for agriculture and several surrounding settlements. Downstream of the dam, the river flows through a commercial platinum mine (Tharisa Mine PTY-LTD), before draining the large peri-urban settlement of Marikana. Several other platinum mines have large waste structures (mine dumps, pollution control dams) which drain into the Sterkstroom before it flows into the Gwathle. The upper Mareltwane drains near-pristine mountain bushveld, before flowing past the town of Mooinooi, where it receives both agricultural and urban (treated wastewater) inputs. Further downstream it flows past a commercial chrome mine (Samancors' Western Mooinooi Chrome Mine) and several platinum mines, receiving runoff from each, until it flows through the peri-urban settlement of Wonderkop where it receives additional urban wastewater inputs and runoff. Despite being downstream of all these cumulative stressors, the Gwathle River flows through a primarily rural landscape with little urban or mining development in its immediate surrounds.

2.2 Site selection

Prior to commencing field work, potential sampling sites were scouted for access and suitability for capturing major step changes in the landscape water quality stressors acting on a particular river site. Study sites were selected by identifying combinations of land-uses between paired sampling points (i.e. capturing stressors within each site's sub-basin). The broad land-use classes were residential, agriculture and mining.

Sites experiencing a number of stressors within its particular sub-basin (between upstream and downstream site) were then classified into stressor classes, hereinafter referred to as stressor categories, e.g. (0) no land-use in the sub-basin defined a zero-stressor category, (1) one land-use stressor defined the one-stressor category and so-forth. Later, the stressor categories were divided further into five stressors, being (1) natural, (2) irrigated crops, (3) fallow-land, i.e. cattle grazing, (4) urban – formal and informal and (5) mining, so that their interactions with water quality variables and stable isotope discrimination could be assessed. Finally, 15 sites were selected, being placed across the Sterkstroom (STK), Maretlwane (MRT) and Gwathle (GWT) rivers that best captured additions of land-use classes to the river catchment (representing step-changes in the number of stressors acting on a site) (Figure 2).

Where possible, all study sites were placed up- and down-stream of different land-use stressors to isolate and best capture changes in surface water quality (e.g. physico-chemical) as well as identify changes in aquatic food web structure and function. These sites also captured the potential influence of large on-channel dams (e.g. Buffelspoort Dam upstream of STK4) on key nutrient cycling processes that may have an effect on the discrimination of stable isotopes within consumers. The land-use stressors used to categorise a site were none (i.e. natural), agriculture, mining (platinum and chrome) and urban (wastewater effluent and stormwater runoff).

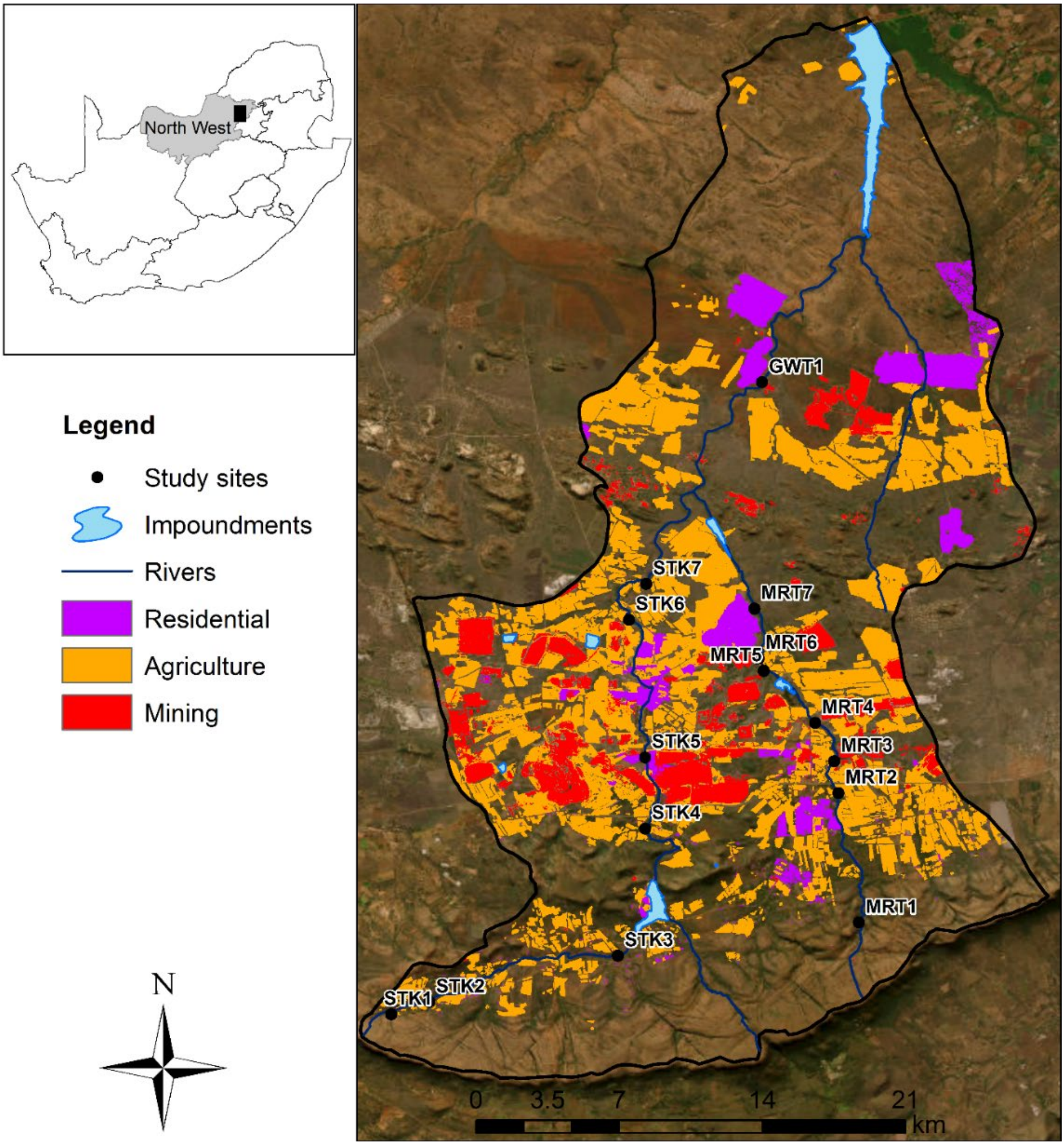


Figure 2: The Gwathle river catchment with the 15 river study sites across a gradient of land-use intensification by three key land-use stressors along the Sterkstroom (left) and Maretlwane (right) rivers

2.3 Stressor class determination per study site

In the analysis, river sites were categorized by natural, agricultural, mining and/or urban stressor loaded sites based upon the coverage of the respective land-cover in the sites' upstream river sub-catchment (Table 7). The Hydrology Toolbox in ArcMAP 10.5 was used to delineate the sub-catchments for each river site utilizing the ALOS PALSAR digital elevation model (10 m) (https://vertex.daac.asf.alaska.edu/#) (Singh et al., 2014; Li et al., 2019).

Table 7: The pre-determined stressor class/category for each of the 15 river study sites and the percent land-cover for each stressor in their upper catchment

Site code	Stressor class	Cumulative upstream land-cover (km^2)	Agriculture %	Mining %	Urban %	Natural %	Artificial water bodies %
STK1	None	1.0	0.0	0.0	0.0	100.0	0.0
STK2	Light agriculture*	7.7	10.8	0.0	0.1	89.0	0.0
STK3	Agriculture	69.4	15.9	0.0	1.2	82.9	0.0
STK4	Agriculture	122.6	8.0	0.0	1.3	88.4	2.3
STK5	Agriculture, mining	138.6	25.4	8.7	3.9	62.1	0.0
STK6	Agriculture, mining, urban	149.6	31.3	5.7	30.9	31.9	0.2
STK7	Agriculture, mining, urban	251.6	39.6	16.4	2.0	42.0	0.0
MRT1	None	8.5	0.0	0.0	0.0	100.0	0.0
MRT3	Urban	41.0	20.9	0.0	2.7	76.4	0.0
MRT4	Urban, agriculture	43.6	33.4	0.2	16.1	50.3	0.0
MRT5	Urban, agriculture, mining	83.6	38.4	5.7	2.3	53.7	0.0
MRT6	Urban, agriculture, mining	157.3	17.9	6.4	5.0	70.3	0.4
MRT7	Urban, agriculture, mining	215.4	33.8	18.4	2.2	45.6	0.0
GWT1	Urban, agriculture, mining	114.9	33.5	1.8	4.2	60.3	0.2

***While this site is characterized by negligible agriculture upstream, its habitat is heavily modified by an upstream causeway**

The land-cover was derived via the South African National Land-cover 2018 dataset (Geoterraimage, 2018), where Level 1 defined classes were merged into one of the four key stressor categories (land-cover categories). Sub-catchments that consisted of > 1% of the key stressor(s), were then categorized as such.

2.4 Water quality and discharge data collection

Live readings of discharge and turbidity, together with multiple physico-chemical variables were collected at each site prior to collection of filtered water samples.

Discharge was measured across a transect using a transparent velocity head rod (GroundTruth), while turbidity (NTU) was measured using a portable Hanna turbidity meter (HI98703-02). All other variables, namely salinity (ppt), total dissolved solids (TDS; g/L), electrical conductivity (μS/cm), dissolved oxygen (mg/L), temperature (°C) and pH (pH units) were collected using a YSI-Professional Plus Handheld Multi-parameter probe.

Water samples were also collected for heavy metal, cation and anion laboratory analyses. The water was collected from the middle of the river and filtered through Supor 0.45 μm filters (Pall Corporation). Filtrate was stored in two separate 100 ml acid cleaned polypropylene bottles and frozen until analysis.

2.5 Stable isotope sample collection

At each sampling site, all available instream food resources, invertebrate consumers, tadpoles and fish were collected for SIA. All organic samples were collected using nitrile gloves to prevent contamination of the carbon signature by the collectors. Three types of basal resources were available at most sites, namely biofilm, fine particulate organic matter (FPOM), and coarse particulate organic matter (CPOM). In addition to these resources, a small number of sites also contained filamentous algae, which were collected separately.

Biofilm was collected from five fist-sized cobbles at each site. Each cobble was scrubbed with a brush into a basin with water, and the algal slurry was pipetted into Eppendorf containers. Coarse and fine particulate organic matter were collected together using a combination of two stacked sieves, the upper sieve having a 1 mm mesh and the lower sieve having a 50 μm mesh. Leaf packs (clumps of dead and decomposing leaf matter) were targeted, and placed in the upper sieve before being washed through with river water. Three replicates of CPOM were then collected by hand from the upper tray, while three replicates of FPOM were collected using a plastic pipette from the lower tray. Filamentous algae, where it occurred, was directly picked using forceps into an Eppendorf vial.

The goal of macroinvertebrate sampling was to ensure that a minimum of one taxon per trophic level (primary consumer, secondary consumer) was captured at each site with high replication (minimum 5 and maximum of 10 replicates each for C/N and S SIA), with additional samples collected to represent recognized functional feeding groups (collector filterer, collector gatherer, grazer, shredder) whenever they were present at the site. The primary grazer mayflies from the family Baetidae and the primary sestonic filter feeder, Simuliidae were selected as the primary baseline organisms for this study (following Kristensen et al., 2016; Jackson et al. 2020). Primary consumers are selected as they control for the relatively short-term variation in $\delta^{15}N$ values of aquatic primary producers, thus, providing a time-integrated estimate of baseline $\delta^{15}N$ conditions (Van der Zanden and Rasmussen, 1999).

Where available, additional long-lived grazers from the family Lymnaeidae were also collected. The additional filterer-gatherer collected included the Hydropsychidae. Our main secondary consumers were odonate nymphs from the families Coenagrionidae, Aeshnidae and Libellulidae. In the case of all macroinvertebrates, one or more whole animals were picked using forceps into Eppendorf vials, after waiting a minimum of 20 minutes for the animals to clear their guts while alive in the collecting tray (following Jackson et al., 2020).

To account for the tertiary consumer trophic level at each site, fish were collected, with a minimum of one species at high (5-10 specimens) replication to enable sufficient statistical power. If only one or two specimens of a particular species was collected at a site, these were not retained unless no other fish were present. Duplicate fin and muscle tissue samples were retained for C/N and S SI analyses respectively. Where present in sufficient numbers (minimum 5), anuran tadpoles were also collected for SIA, as they represent an important vertebrate primary consumer in streams where they occur.

All vertebrates were euthanized in a 20mg/L solution of clove oil, before having a tissue sample removed. Tail clips were taken from anurans, while a combination of fin clips and muscle tissue were harvested from the fish specimens. For the Carbon/Nitrogen SI sample, the lower lobe of the caudal fin was collected. For the sulfur SI sample, a block of muscle tissue from the right flank of the fish was collected, making sure to not include any skin or scales in the sample. All tissue samples were frozen in the field for transport back to the aquatic laboratory for further processing.

2.6 Stable Isotope sample preparation and analysis

In the laboratory, all frozen samples were defrosted in a fridge overnight before being placed into an appropriately sized sieve and thoroughly rinsed with deionized water. This step removed potential contaminants on the samples. To avoid capturing soil and losing the sample, FPOM was not rinsed through a sieve, but rather suspended in an Eppendorf using deionized water and transferred using a 3 ml plastic pipette into a clean Eppendorf for further processing.

The rinsed samples were then positioned on sterile watch glasses and dried at 60°C for 48 hours to achieve a constant weight (Anderson and Cabana, 2007; O'Neil and Thorp, 2014; Jackson et al., 2018). Once dried, the samples were manually ground up into a fine homogenized powder using a sterile percaline mortar and pestle; cleaned between samples with 70% ethanol (Winemiller et al., 2010).

The powder was then transferred into completely sterile Ultra-Pure elemental tin capsules (de Carvalho et al., 2014; Jackson et al., 2016) and weighed on a five-place decimal Mettler Toledo-XP6 balance. Animal material (macroinvertebrate and fish) for $\delta^{13}C$ and $\delta^{15}N$ analysis was weighed between 0.4-0.5 mg while the plant material (FPOM, CPOM, instream and terrestrial) was weighed between 0.8-1 mg (Symes et al., 2017) before being packaged. On the recommendation of the UC Davis Stable Isotope Laboratory, fish tissues for $\delta^{34}S$ analysis were weighed between 3.8 and 4.2 mg, while macroinvertebrate tissues were weighed between 4.8 and 5.2 mg prior to packaging.

Carbon/Nitrogen tissue samples were delivered to the Stable Isotope Laboratory in the Department of Zoology, University of Pretoria. Samples for $\delta^{13}C$ and $\delta^{15}N$ isotopic analysis were combusted at 1020°C using an elemental analyser (Flash EA 1112 Series) coupled to a Delta V Plus stable light isotope ratio mass spectrometer via a ConFlo IV system (all equipment supplied by Thermo Fischer, Bremen, Germany), housed at the UP Stable Isotope Laboratory, Mammal Research Institute, University of Pretoria.

The $\delta^{13}C$ and $\delta^{15}N$ values for each sample, were corrected for using two laboratory running standards (Merck Gel: $\delta^{13}C$ = -20.26‰, $\delta^{15}N$=7.89‰, C%=41.28, N%=15.29 and DL-Valine: $\delta^{13}C$ = -10.57‰, $\delta^{15}N$=-6.15‰, C%=55.50, N%=11.86), with a blank sample are run after every 11 samples. The carbon and nitrogen isotope ratios of the lab running the standards were calibrated using the following primary standards: IAEA-CH-3 (Cellulose), IAEA-CH-6 (Sucrose), IAEA-CH-7 (Polyethylene foil), IAEA N-1 & IAEA N-2 (Ammonium sulfate), IAEA NO-3 (Potassium nitrate). The results were referenced to atmospheric N_2 (Air) and Vienna PeeDee belemnite for the $\delta^{13}C$ and $\delta^{15}N$ respectively (Woodborne et al., 2012; Smucker et al., 2018).

The solid $\delta^{34}S$ samples were analysed on an Elementar vario ISOTOPE cube elemental analyser (Elementar Analysensysteme GmbH, Langenselbold, Germany) interfaced to an Elementar PrecisION isotope ratio mass spectrometer (Cheadle Hulme, Cheadle, England). Samples were combusted at 1150°C in a reactor packed with tungsten oxide. Immediately following combustion, sample gases are reduced with elemental copper at 880°C and subsequently passed through a buffering reactor filled with quartz chips held at 900°C. The SO_2 and CO_2 are then separated by adsorption columns, allowing for full separation and peak focusing. Following separation, the SO_2 adsorption trap is heated, and the sample SO_2 passes directly to the IRMS for measurement.

The lab references were calibrated against international reference materials, including: IAEA-S-1, IAEA-S-2, IAEA-S-3, NBS-127, IAEA-SO-5, and IAEA-SO-6. The final delta (δ) values are expressed relative to Vienna Cañon Diablo Trolite (VCDT) (Ofukany *et al*., 2014). The quality assurance reference materials included Taurine, Whale Baleen, Cysteine and the quality control reference materials included Hair, Mahi-Mahi Muscle.

The final isotope ratios, expressed in delta notation using a parts per mille scale (‰), were defined by Equation 1, where X= ^{15}N, ^{13}C or ^{34}S and R represents $^{15}N/^{14}N$, $^{13}C/^{12}C$ or $^{34}S/^{32}S$ ratios respectively (Bird et al., 2016; Masese et al., 2017).

Equation 1: $$\delta X(‰) = [(R_{sample}-R_{standard})/R_{standard}-1]$$

2.7 Analysis of water quality stressors

All macronutrient and major anion water samples were analysed by Waterlabs PTY-LTD (Pretoria). The limitations posed by limit of detection (LOD) to further statistical analyses were overcome by using a substitution method, whereby the LOD was replaced by an estimate of its division by two; following the assumption where data are uniformly distributed (Croghan et al., 2003). Major cations and heavy metals analysed at the School of Chemistry, University of Witwatersrand, using an Agilent 7800x inductively coupled plasma mass spectrometry (ICP-MS) with concentrations measured against external matrix-matched standards (Chetty et al., 2021). Prior to the ICP-MS analysis, samples with total dissolved solids > 120 mg/L were diluted using Millipore water, thereafter, 0.1 m/L of ultrapure acid (HNO_3) was added to each 10 ml of filtrate and spiked with Rh as an internal standard. Procedural blanks were analysed with each digestion batch (20 samples) and later incorporated in correction of the resulting concentrations (Chetty et al., 2021).

To determine the relationship between land-use stressors and water quality parameters, the Gwathle quaternary catchment was divided into 15 distinct sub-catchments, each representing the catchment areas between up- and down-stream sites. This approach was taken to assess and capture the role of local and immediately upstream land cover on water quality at each site. Each sub-catchment was then measured for the relative cover of five key land cover classes, which best represented the key human activities anticipated to generate water quality stress. These classes were (1) natural, (2) irrigated crops, (3) fallow Land, (4) built-up (urban) and (5) mining. We also considered the land area inundated by impoundments, as these serve as habitats where crucial nutrient transformation processes occur that can affect the relationship between food webs and the surrounding landscape sources of nutrients, in particular inorganic nitrogen (Symons et al., 1965).

2.7.1 The effects of lipids in freshwater fishes

Tissues, including non-lethal fin clips as collected in this study, may have variable lipid contents (Ryan et al., 2012). Lipids are ^{13}C-depleted, relative to proteins and carbohydrates in the tissue sample (Sweeting et al., 2006; Taylor et al., 2017). This subsequently lowers the bulk tissue ratio of the $^{13}C/^{12}C$ ratio, and increases variability (skewing) in the resulting $\delta^{13}C$ between and among species, beyond that of the dietary carbons being assimilated (Pinnegar and Polunin, 1999). This creates bias in analyses that require the use of the $\delta^{13}C$, including food source acquisition (Post et al., 2007).

While lipids affect $\delta^{13}C$ and not $\delta^{15}N$, there is a well-researched relationship between the lipid content in aquatic animal tissues and the carbon-to-nitrogen ratio (C:N) (Post et al., 2007). In this regard, corrective measures are recommended when the C:N ratio of aquatic organisms (fishes) is > 3.5, indicating that the lipid content is greater than 5% of the tissues. This threshold is widely applied in aquatic food-web research (Post et al., 2007). Two broadly common approaches are taken to account for lipids in fish tissue, including cumbersome chemical treatment and mathematical correction as adopted in this research.

2.8 Statistical analysis

All statistical analyses were conducted in the R software suite (R Core Team, 2022). To ascertain differences in river flow between the wet and dry season a pair-wise test was performed on each rivers respective discharges for the two seasons. To draw statistical inferences between sites with fishes, all statistics were performed on the baselined $\delta^{15}N$ data.

Principal component analysis, a dimensionality reduction technique, was applied to log-transformed data in order to identify potential relationships between key land-cover classes, water quality parameters (physico-chemical parameters, macronutrients, cations and metals) and aquatic food web components using baseline corrected stable isotope values. Using the *dimdesc* function in the FactoMineR package, significantly correlated parameters associated with each of the retained principal components were identified as key drivers for patterns observed in the analysis (Lê et al., 2008; Lee et al., 2018).

Multiple linear regressions with model selection using the Akaike information criterion corrected for small sample sizes (AICc) were performed on the land cover of the five land-use classes against the major water quality tracer variables identified by the PCA analysis as being key discriminators of the gradient between no land cover stress and three land cover stresses (de Mello et al., 2018). Prior to the any linear regression analysis, the high number of pair-wise correlations between physico-chemical, anions, cations and heavy metal (water quality parameters) were reduced using the *findCorrelation* function in the R caret package with a correlation coefficient of 0.7, (water quality parameters) (Kuhn, 2015).

To identify which of the reduced collinear water quality tracer variables best describe the land-use contexts (stressor context) of the different river sub-basins, a range of candidate models were computed and compared using AIC_c. Model selection was performed with backwards and forwards step-wise Multi-Model Inference using the MuMIn R package (Barton, 2022). Using the stepwise approach, predictor variables in numerous combinations were systematically excluded. All models were fitted without interacting effects.

Food web metrics were assessed on baselined ($\delta^{15}N$) organisms and lipid corrected ($\delta^{13}C$) fish fin tissue. The majority of the fishes C:N exceeded 3.5, and all had C:N < 10. As per the recommendations made by Hicks et al. (2021) for the correction of freshwater fish fin $\delta^{13}C$ within this C:N range, the Post et al. (2007) mathematical correction (equation 2) was applied to the raw $\delta^{13}C$ for all fishes.

Equation 2: $$\delta^{13}C_{\text{lipid-corrected tissue}} = (\delta^{13}C_{\text{untreated tissue}}) - 3.32 + 0.99 \times C{:}N$$

The dual application of $\delta^{13}C$ and $\delta^{15}N$ in iso-space, provides the foundation for metrics to qualitatively and quantitatively assess food web structure and function; thereby acting as proxies for ecosystem disturbance(s) (Layman et al., 2007; Jackson et al., 2011; de Castro et al., 2016). The $\delta^{13}C$ reflects sources and changes of energy inputs, with limited discrimination between trophic levels (ΔC <1‰, Post, 2002), while $\delta^{15}N$ may be enriched 2-4‰ between successive trophic levels; differentiating consumer groups and responding to nitrogen pollution (Post, 2002; Smucker et al., 2018).

3. Characterising spatio-temporal patterns in water quality stress

3.1 Seasonal variation in flow at study sites

Discharge was measured at each investigated site along the Sterkstroom and Maretlwane rivers in the wet season (26 April-7 May) and the dry season (28 September to 21 October) of 2021. To determine whether river flows differed significantly between the wet and dry sampling seasons, a paired t-test was performed on each of the rivers' (STK and MRT) respective seasonal discharge measurements. Following the statistical analysis on log transformed data, both the Sterkstroom ($t(6)=2.80$, $p=0.03$) and Maretlwane ($t(10)=2.74$, $p=0.02$) dry season river flows were significantly lower compared to their wet season river flows (Figure 3).

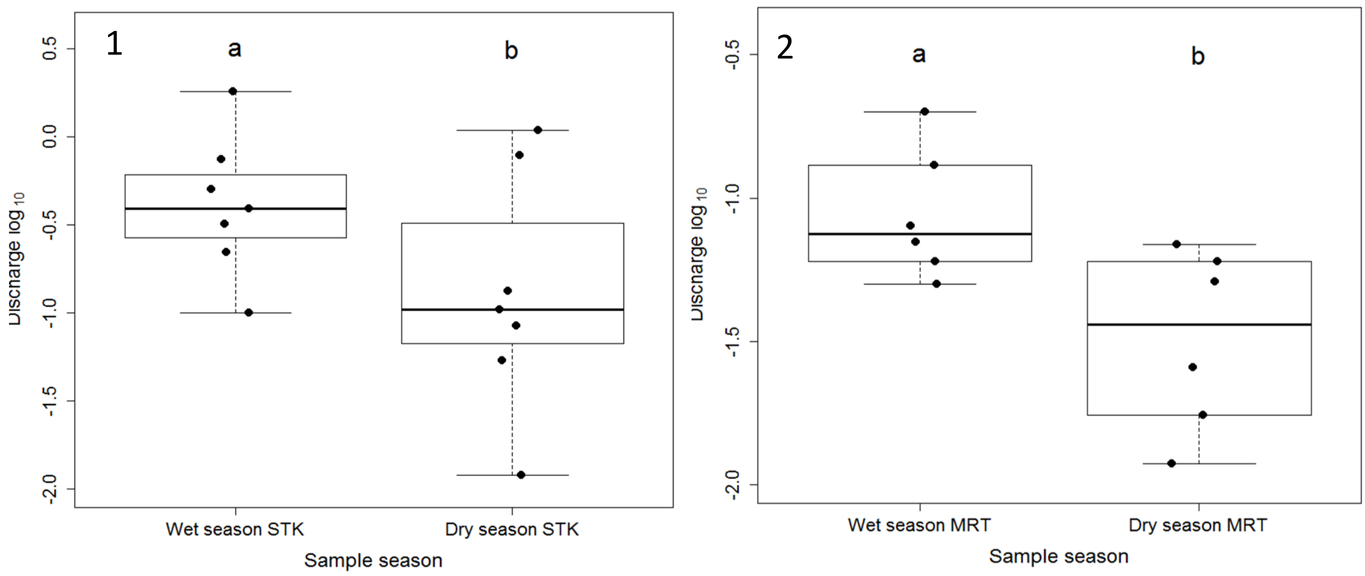


Figure 3: River discharge across 14 monitoring sites comparing wet and dry seasons for the (1) Sterkstroom (STK) and (2) Maretlwane (MRT) rivers respectively

From the latter analyses, it can be inferred that there is increased divergence in base flows across the catchment in the dry season (relative to wet season). Notably, some sites in the upper reaches of both the Sterkstroom and Maretlwane rivers do not significantly change between seasons, whereas many in the lower reaches are largely influenced by dewatering activities. The findings demonstrate that there was an overall reduction in flow across the catchment between the wet and dry season, and that the dry season stable isotope samples would likely capture the effects of heightened stressor concentration in the water, through such mechanisms as the accumulation of pollutants at sites with little or no flow, relative to the wet season samples.

3.2 Spatial and temporal variation in water quality

3.2.1 Seasonal concentrations of macro-nutrients (N, P) across the Gwathle catchment

Macro-nutrients (anions) are the building blocks of aquatic ecosystems, facilitating a host of aquatic metabolic processes, enabling plant and animal growth (Dallas and Day, 2004). These nutrients occur naturally in river catchments, and their concentrations remain relatively fixed in natural settings; being controlled by climatic variables (e.g. precipitation and weathering) and catchment characteristics (i.e. catchment geology) (Dallas and Day, 2004). In catchments influenced by anthropogenic activities, such as the Gwathle Catchment (Figure 1), there is an increased likelihood of nutrient loading of surrounding aquatic ecosystems through different sources. These include point-sources (sewage, industrial and detergent), diffuse sources (agricultural run-off, urban stormwater run-off, fertilizers and manure) as well as atmospheric deposition (industrial burning and by-product gas deposition).

Two key macro-nutrients found in aquatic ecosystems are inorganic nitrogen ($NH_3 + NH_4^+ + NO_2^- + NO_3^-$) and inorganic phosphorus (PO_4^{3-}). Inorganic nitrogen has been used to indicate nutrient-rich fertilizer run-off into surface waters as well as the presence of untreated sewage effluent (Showalter et al., 2000; Matowanyika, 2010). Elevated inorganic phosphorus concentrations have also been used to indicate agricultural fertilizer run-off and leachate, as well as sewage discharges and detergent flow into aquatic ecosystems (Dallas and Day, 2004).

Although these anions are essential for aquatic ecosystem functionality, nutrient loading of inorganic nitrogen and phosphorus can contribute to eutrophic conditions, often altering the ecosystems functionality. This is where nutrient enrichment may promote the excessive growth of plant and algal species, increasing the ecosystems productivity, which has the potential to alter macroinvertebrate and fish communities (Dallas and Day, 2004). Eutrophic conditions have also been attributed to altering the physico-chemical water quality conditions, by depleting dissolved oxygen during diurnal cycles, causing fish kills and reducing the water's pH.

a) Inorganic nitrogen and the aquatic water quality standards at each study site

The South African Water Quality Guideline for Aquatic Ecosystems (Volume 7) outlines when the concentration of certain constituents exceeds the established target water quality range (TWQR). When the inorganic nitrogen concentrations are < 0.5 and 0.5-2.5 mg-N/L, the ecosystem condition is considered oligotrophic and mesotrophic respectively (DWAF, 1996). When the inorganic nitrogen is between 2.5-10 mg-N/L or > 10 mg-N/L, the ecosystem condition is eutrophic (highly productive system) or hypertrophic (very highly productive system) respectively (DWAF, 1996).

From the anions in the Gwathle catchment, sites within the three land-use stressor category were classified as either eutrophic or hypertrophic at one point or another during the monitoring (Table 8). The MRT4 river site in September/October is, however, an exception as it was discovered during this time period that site appears to experience a new stressor (being seasonal artisanal mining), which may have contributed to the elevated nutrient concentrations detected in the surface water (Appendix B27). The eutrophic river site STK6 is immediately downstream of the large urban settlement of Marikana. With a large number of impervious surfaces and the lack of municipal storm-water and sanitation infrastructure maintenance capacity, polluted stormwater run-off may enter the nearby Sterkstroom. In the case of STK7, there are large tracts of cattle farming rangeland as well as subsistence and intensive (mechanized) commercial agricultural plots. There is therefore an increased likelihood of agriculture-related by products rich in inorganic nitrogen entering the surrounding river through leachate and run-off, contributing to the elevated levels across the monitoring period.

Table 8: The dissolved inorganic nitrogen concentrations for all river study sites across the four monitoring periods, showing oligotrophic (clear), mesotrophic (clear), eutrophic (yellow) and hypertrophic (orange) sites across the monitoring period.

Sites	Stressor class	Inorganic nitrogen (mg-N/L)					
		April/May	June	August	Sept/Oct	January	March
STK1	Zero	0.18	0.28	0.25	0.63	0.13	0.20
STK2	One	0.18	0.18	0.25	0.33	0.90	0.30
STK3	One	0.18	0.28	0.25	0.43	1.10	0.30
STK4	One	0.23	0.33	0.45	0.43	0.90	0.50
STK5	Two	0.73	0.78	0.25	1.03	11.60	3.10
STK6	Three	2.08	4.40	4.03	8.63	11.60	4.10
STK7	Three	3.66	4.30	1.80	20.70	11.40	4.80
MRT1	Zero	0.18	0.18	0.25	0.63	0.20	0.20
MRT2	One	1.13	0.33	2.25	0.73	1.00	0.30
MRT3	Two	0.23	0.33	0.28	3.35	0.60	0.40
MRT4	Three	17.80	18.20	1.50	5.30	0.90	1.60
MRT5	Three	4.13	4.43	0.25	0.43	0.30	0.20
MRT6	Three	No data	No data	7.03	104.00	1.40	4.60
MRT7	Three	0.60	0.65	12.23	34.30	4.20	9.80
GWT1	Three	0.20	0.35	3.33	10.27	4.00	7.00

The Maretlwane River experiences similar stressors to the Sterkstroom River, except for the stress experienced meters upstream at MRT4. There is a mining operation between MRT3 and MRT4 that discharges mining related mineral by-product(s) that eventually flow into the river (Appendix B27). During the monitoring the course of the monitoring regime, there were differing degrees of river infilling below their mining operation up until a few meters above MRT4 (Appendix B29, B30), where a road bridge causeway blocked the silt so that it was not present to the same degree at the sampling site (Appendix B28). With little to no agricultural, nor residential stressors between the two sites, it can be suggested that the mining effluent contributes to the river's elevated inorganic concentrations for four of the six monitoring periods (Table 2).

The MRT5 and MRT6 sites both have nearby agricultural and mining related land-use activities contributing to elevated inorganic nitrogen concentrations. The MRT6 site, however, experiences the effect of a mining related tributary draining platinum mine pollution control dams (PCDs) and tailings dumps (Appendix B35, B38); the site was added to the sampling regime in August 2021. The MRT5 site was considered mesotrophic and oligotrophic in August and September/October respectively (Table 2). When compared to 600 m downstream MRT6 site classified as eutrophic and hypertrophic during the same period, and with no impact other than the mine related tributary entering the main-stem, the mining-related effluent appears to be the significant contributor of inorganic nitrogen to the river.

The MRT7 site, similar to the STK6 and STK7 sites, drains a large formal settlement, Wonderkop, with informal dwellings (Appendix B39). It therefore, may experience similar nutrient input stressors through storm-water drainage and run-off owing to the large number of impervious surfaces facilitating the movement of numerous waste products and detergents; known to be rich in nitrogen. The site also collects water from a privately commissioned wastewater treatment plant, slightly upstream of MRT6. These stressors may contribute inorganic nitrogen to the Maretlwane leading to eutrophic and hypereutrophic conditions (Table 2) in the four monitoring periods.

b) Ortho-phosphate and water quality standards at each study site

Like dissolved inorganic nitrogen, ortho-phosphate (dissolved inorganic phosphorus) is a key macro-nutrient for plant growth and responsible for altering the eutrophic state of aquatic ecosystems following nutrient loading. It is common for un-impacted South African rivers to that are considered oligotrophic and mesotrophic to have inorganic phosphorus concentrations within the target water quality range; being < 0.005 mg-P/L and between 0.005-0.025 mg-P/L respectively (DWA, 1996; Dallas and Day, 2004). Local eutrophic and hypertrophic conditions are expected when inorganic phosphorus is 0.025-0.250 mg-P/L and > 0.250 mg-P/L (DWA, 1994). The lower-detection limit of the equipment used to analyse the ortho-phosphate concentrations in this study was 0.1 mg-P/L. Of the sites, however, the inorganic phosphorus concentrations of STK6, STK7, MRT4 and MRT7 in a few of the low-flow monitoring months exceeded the > 0.25 mg-P/L threshold, reaching what can be considered hypertrophic conditions (Table 9).

Table 9: The ortho-phosphate concentrations for all river study sites across the four monitoring periods, showing the hypertrophic sites (orange) across the monitoring period.

Sites	Stressor class	Ortho-phosphate (dissolved inorganic phosphorus) (mg-P/L)					
		April/May	June	August	Sept/Oct	January	March
STK1	Zero	<0.1	0.1	<0.1	<0.1	0.1	<0.1
STK2	One	<0.1	<0.1	0.2	0.1	<0.1	<0.1
STK3	One	<0.1	<0.1	0.1	<0.1	0.1	<0.1
STK4	One	<0.1	0.1	0.2	0.1	<0.1	<0.1
STK5	Two	<0.1	0.1	0.2	<0.1	<0.1	<0.1
STK6	Three	<0.1	0.2	0.2	0.5	0.2	<0.1
STK7	Three	<0.1	0.1	0.3	0.1	0.1	<0.1
MRT1	Zero	<0.1	0.1	<0.1	<0.1	0.1	<0.1
MRT2	One	<0.1	0.2	0.2	0.1	0.1	<0.1
MRT3	Two	<0.1	0.2	0.2	0.1	0.1	<0.1
MRT4	Three	<0.1	0.2	<0.1	0.5	0.2	<0.1
MRT5	Three	<0.1	0.1	0.1	<0.1	<0.1	<0.1
MRT6	Three	No data	No data	0.1	<0.1	<0.1	<0.1
MRT7	Three	0.3	0.1	0.6	0.1	0.1	0.1
GWT1	Three	0.05	0.5	0.1	<0.1	<0.1	<0.1

Elevated concentrations of ortho-phosphate are likely sourced from sewage inputs as well as leaching and/or run-off from fertilized agricultural fields (Dallas and Day, 2004).

3.2.2. Seasonal Sulfate concentrations across the Gwathle catchment

The most common form of sulfur in highly oxygenated river water is sulfate (SO_4^-); a leading contributor to aquatic ecosystem salinity (Dallas and Day, 2004). Like inorganic-nitrogen and phosphorus, sulfates occur naturally in aquatic ecosystems through weathering processes and atmospheric deposition, before being assimilated by aquatic plants (Zak et al., 2021). Anthropogenic land-use activities are prominent sources of sulfates to aquatic ecosystems, particularly surrounding the discharge of treated and untreated wastewater as well as industrial inputs into water bodies. Agricultural fertilizers are also known to contain sulfates and thus, surface run-off and leaching from fertilized fields can contribute to sulfate loading of nearby streams. Mining activities are also known to increase the sulfate loading of aquatic ecosystems (Zgorska et al., 2016), through the smelting of ore (atmospheric deposition) and through the oxidation of sulfide minerals, which are associated with platinum-group elements such as those mined in the catchment (Godel et al., 2007; Dzvinamurungu et al., 2020).

Among the Sterkstroom sites, only STK7 displays relatively elevated concentrations of sulfate (Table 10). This may be the consequence of a small Sterkstroom tributary upstream of the site that drains two nearby platinum PCDs and tailings dumps.

Table 10: The sulfate concentrations for all river study sites across the four monitoring periods

Sites	Classes	Sulfate (SO_4^{2-}) (mg-S/L)					
		April/May	June	August	Sept/Oct	January	March
STK1	Zero	2	5	1	1	3	3
STK2	One	2	4	4	1	2	1
STK3	One	105	2	2	1	2	1
STK4	One	3	4	4	4	3	3
STK5	Two	43	7	62	18	24	10
STK6	Three	39	17	29	29	27	16
STK7	Three	49	78	195	302	132	54
MRT1	Zero	2	2	2	4	3	4
MRT2	One	20	10	34	13	9	6
MRT3	Two	21	11	25	30	9	7
MRT4	Three	130	154	161	37	14	24
MRT5	Three	105	99	185	530	39	70
MRT6	Three	No data	No data	180	645	49	178
MRT7	Three	215	260	310	700	90	225
GWT1	Three	107	126	172	245	165	84

On the Maretlwane River, it also appears that downstream from chrome mining-related mineral inputs, of which the signals were captured at MRT4, the concentration of sulfates also increases. The MRT5 site flows downstream of a large impoundment that may accumulate surface run-off and leachate from adjacent platinum related tailing dumps, further elevating sulfate concentrations. The MRT7 site, experiencing both dense urban and large mining related stressor activities (tributaries draining into the main-stem), registered the highest concentration of sulfate across the two rivers in the catchment (700 mg/L in October 2021; Table 4).

3.2.3 The effect of physico-chemical, nutrient and dissolved metal measurements on spatial variation in catchment water quality

To assess how the addition of these anion variables altered the previously observed variation in overall water quality across study sites (with a different number of stressors), a principal component analysis (PCA) was performed (Azhar et al., 2015). From the resulting analysis, it became clear that sites classified with land cover classes of zero, one and two are mostly situated in the negative pole of PC 1, while the sites with three stressors are situated on the positive pole of PC 1 (Figure 4). The sites in the positive pole of PC 1 are characterised by higher concentrations of major ions and certain physico-chemical water quality parameters (high temperature, high turbidity) and vice versa for those sites in the negative pole. It is therefore clear that the water quality parameters significantly correlated with dimension 1 (PC 1, x-axis), that accounts for 39.2% of the variation observed in the data, are responsible for splitting sites with land-cover stressors into two groups (left: low stressed sites, right: highly stressed sites; Figure 4). Variation between sites along PC2 (describing 18.2% of variation) was low among 0, 1 and 2 stressor sites, whereas sites with 3 stressors showed high variation along this axis, indicating the potential impact of multiple contrasting anthropogenic stressors on water quality among these sites.

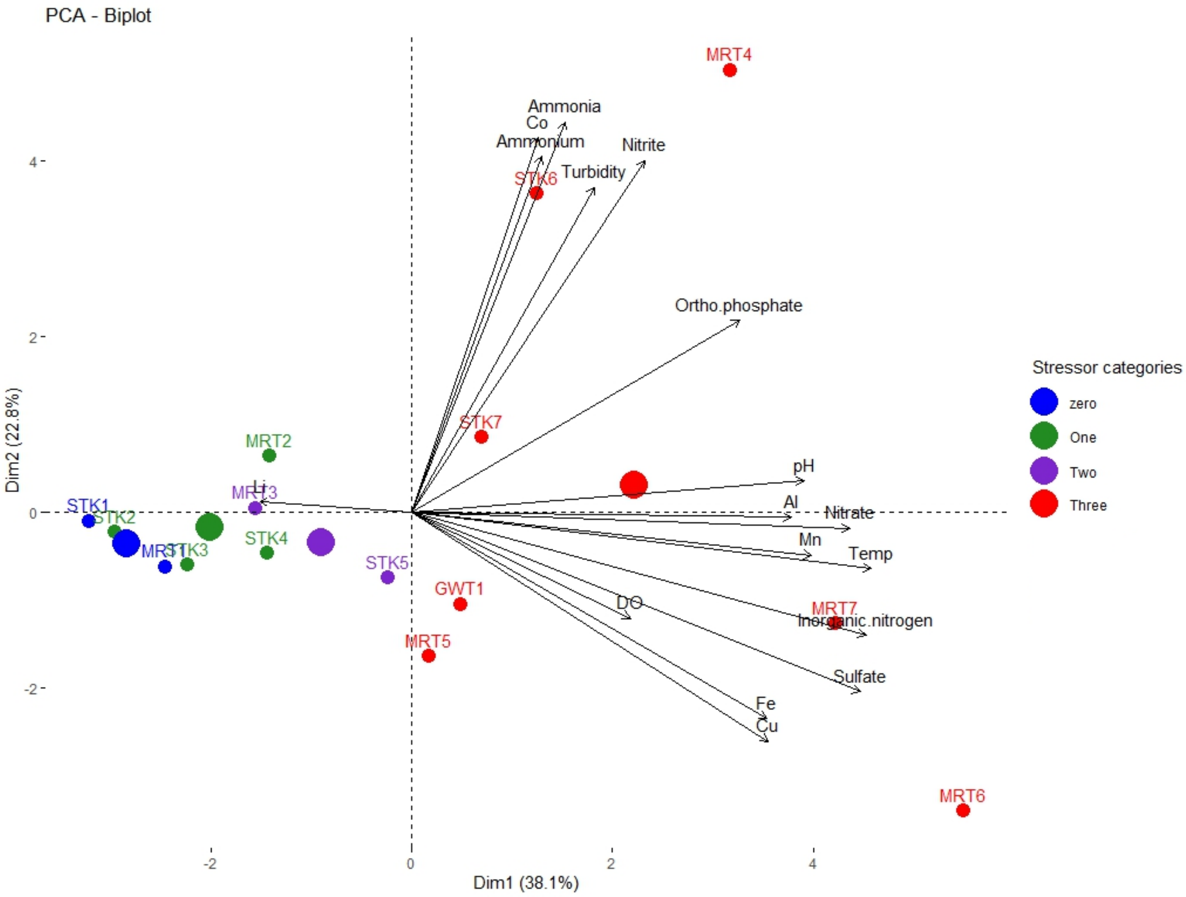


Figure 4: A principal component analysis bi-plot showing individual study sites (small point) and their stressor category centroids (large point) split into their respective stressor categories, primarily along principal component 1 (x-axis).

There are ten water quality parameters responsible for distinguishing these two groups; owing to being significantly correlated with PC 1 ($p < 0.05$). They include salinity, total dissolved solids, electrical conductivity, chloride, nitrate, sulfate, pH, nitrite, orthophosphate, and turbidity (Table 11). Given the placement of the mean PC values, it is clear that the water quality variables closely associated with PC1 were all good indicators of accumulating anthropogenic impacts. The major indicators associated with PC2, ammonia and ammonium, drove variation among 3-stressor sites, and may provide a way to distinguish between combinations of anthropogenic stressors responsible for poor water quality at these highly impacted sites. For example the completed physico-chemical sampling regime indicated that during the year, sites STK6 and MRT4 showed a high PC2 loading (high NH_4^+), and are associated with potential mining explosive waste and soil nutrients from mine tailings respectively. In contrast, site MRT5 showed a low PC2 loading (low NH_3^- & NH_4^+), and was associated with chrome mine tailings.

Table 11: Water quality parameters that are significant correlators, i.e. drivers, of principal component 1 and 2

Principal component (PC)	Parameter	correlation with PC	p value
PC1	Temperature (°C)	0.97	<0.01
	total inorganic nitrogen (mg/L-N)	0.97	<0.01
	Sulfate (mg/L)	0.97	<0.01
	Nitrate (mg/L-N)	0.96	<0.01
	Manganese (mg/L)	0.95	<0.01
	pH (units)	0.95	<0.01
	Ammonium as N (mg/L)	0.88	<0.01
	Copper (mg/L)	0.80	<0.01
	Iron (mg/L)	0.76	<0.01
	Ortho phosphate (mg/L-P)	0.62	0.01
PC2	Ammonia (mg/L-N)	0.83	<0.01
	Cobalt (mg/L)	0.80	<0.01
	Ammonium (mg/L-N)	0.76	<0.01
	Nitrite (mg/L-N)	0.75	<0.01
	Turbidity (NTU)	0.70	<0.01

3.3 Land-use drivers of water stress

3.3.1 Cross-catchment drivers of water stress

The key physico-chemical water quality (chemical) tracers that were identified during the PCA analysis to be associated with overall land cover were retained for further analysis (Appendix A), specifically to assess their particular relationship with each of the five land-use stressors. Using the water quality parameters as response data and the percent cover of land-use stressors in each sub-basin, general linear models were computed (Table 12). From these initial models, we can identify key individual chemical tracers that are either positively or negatively associated with the land-use stressors.

Table 12: Models identifying the key physico-chemical water quality tracers that best describe the different land-use stressors in the Gwathle quaternary catchment.

	Model and number	Model number	AICc	Δ AIC	AIC W	RSS	Model explanation (%)	Cumulative weight (%)	BIC
Natural	Ammonium + pH	1	128.2	0	0.347	2037.4	34.7	34.7	137.2
Artificial waters	Dissolved oxygen	2	33.2	0	0.353	4.64	29.4	29.4	33.1
Irrigated crops	pH	3	101.8	0	0.207	449.87	20.7	20.7	101
	pH + Turbidity	4	101.9	0.19	0.189	353.12	18.9	39.6	353.1
	pH + Fe	5	103.7	1.91	0.08	396.21	8	47.6	396.2
Fallow lands	Orthophosphate + pH	6	102.2	0	0.282	359.53	0.282	28.2	101.1
Built-up	Ammonium	7	100.8	0	0.305	421.86	30.5	30.5	100.7
	Ammonium + DO	8	102.5	1.66	0.133	365.42	13.3	43.8	101.3
	Ammonium + Turbidity	9	102.7	1.94	0.116	372.15	11.6	55.4	101.6
Mining	Fe	10	111.8	0	0.373	878.54	37.3	37.3	111.7

Model selection using AICc produced 10 models with high information weighting that significantly associated water quality stress tracers with land cover classes (Table 13).

Table 13: Coefficient scores of water quality tracers that best described the extent of different land cover types in sub-basins of the Gwathle Catchment, delineated as the total catchment area between each monitoring site

Response variable	Parameter	Model No.	Estimate	95% confidence limits		p-value (model)
				Lower	Upper	
Natural	Ammonium	1	-34.63	-70.67	1.41	<0.01
	pH	1	-19.54	-29.32	-9.77	<0.01
Artificial waters	Dissolved oxygen	2	-0.11	-0.51	0.29	0.6
Irrigated crops	pH	3	6.65	2.87	10.42	<0.01
	Turbidity	4	0.02	-0.01	0.05	<0.01
	Fe	5	-0.01	-0.03	0.01	<0.01
Fallow lands	Orthophosphate	6	92.34	15.61	124.96	<0.01
	Fe	6	0.01	0.63	8.31	<0.01
Built-up	Ammonium	7	18.25	8.28	28.23	<0.01
	Do	8	2.34	-1.44	6.24	<0.01
	Turbidity	9	-0.02	-0.04	0.01	<0.01
Mining	Fe	10	0.04	0.01	0.06	<0.01

Sites with a high proportion of natural lands in the surrounding catchment were best predicted by Ammonium and pH (Table 13), which both showed a strong negative association with the amount of untransformed land, indicating increases with progressive land cover modification in the lower catchment.

Irrigated crops were strongly positively associated with pH, and weakly associated with turbidity and iron concentrations (Table 13). Fallow lands, which may be indicative of land used extensively for small-scale livestock grazing, were positively associated with both orthophosphate and iron concentrations in river water. Built-up urban land cover was strongly positively associated with elevated ammonium levels and weakly positively associated with dissolved oxygen; land cover dedicated to mining was weakly positively associated with elevated iron levels (Table 13).

3.3.2. Acute and chronic water stress markers associated with point sources

An assessment of the key time integrated water quality indicators across the catchment revealed a number of sites where major spikes in turbidity, nutrients or metals occurred, which were not consistent from month to month and where the signal appeared to dissipate at the next site downstream. These suggest the presence of point sources of water quality stress, which may have either long term or extremely brief impacts on the local river ecosystem, but not on the downstream sites for a variety of reasons.

One of the key major nutrient tracers that showed distinct spatial and temporal variation was ammonium, which was assumed to be an indicator of urban impact due to its relationship with built up land cover (Table 13). However, an assessment of seasonal variation along the river continuum of the two major tributaries shows distinct fluctuations in ammonium concentration that suggests a more complex pattern of stress (Figure 5). The Sterkstroom registered a very high concentration of ammonium at site STK6, downstream of Marikana Township, which was elevated in the dry season winter months, peaking in September/October, the period of lowest flow (Figure 5a). These elevated levels consistently decrease at the following site in the continuum (STK7), which likely indicates a dilution effect from the confluence of a small tributary (draining mining activities) with the Sterkstroom between the two sites. The extreme ammonium concentration observed at STK6 could emanate from a number of potential sources, including urban wastewater, fertilizer, cattle manure (Du et al., 2017) as well as mining related waste products (Bhattarai et al., 2021). However, it seems unlikely that raw/treated sewage, fertilizer use or cattle manure are the major contributors at this site. This determination is based on the fact that we did not observe similar high ammonium concentrations at MRT2, which is located downstream of the Mooinooi wastewater treatment works (WWTW), nor at MRT7 below the town of Wonderkop, nor at MRT6 which experiences high cattle grazing activities. The highest ammonium signal in the Maretlwane River was evident at MTR2 in June (Figure 5b), where the most likely source was irrigated cropland. The possible sources of the extremely high ammonium signal at STK6 are further discussed in Chapter 4.

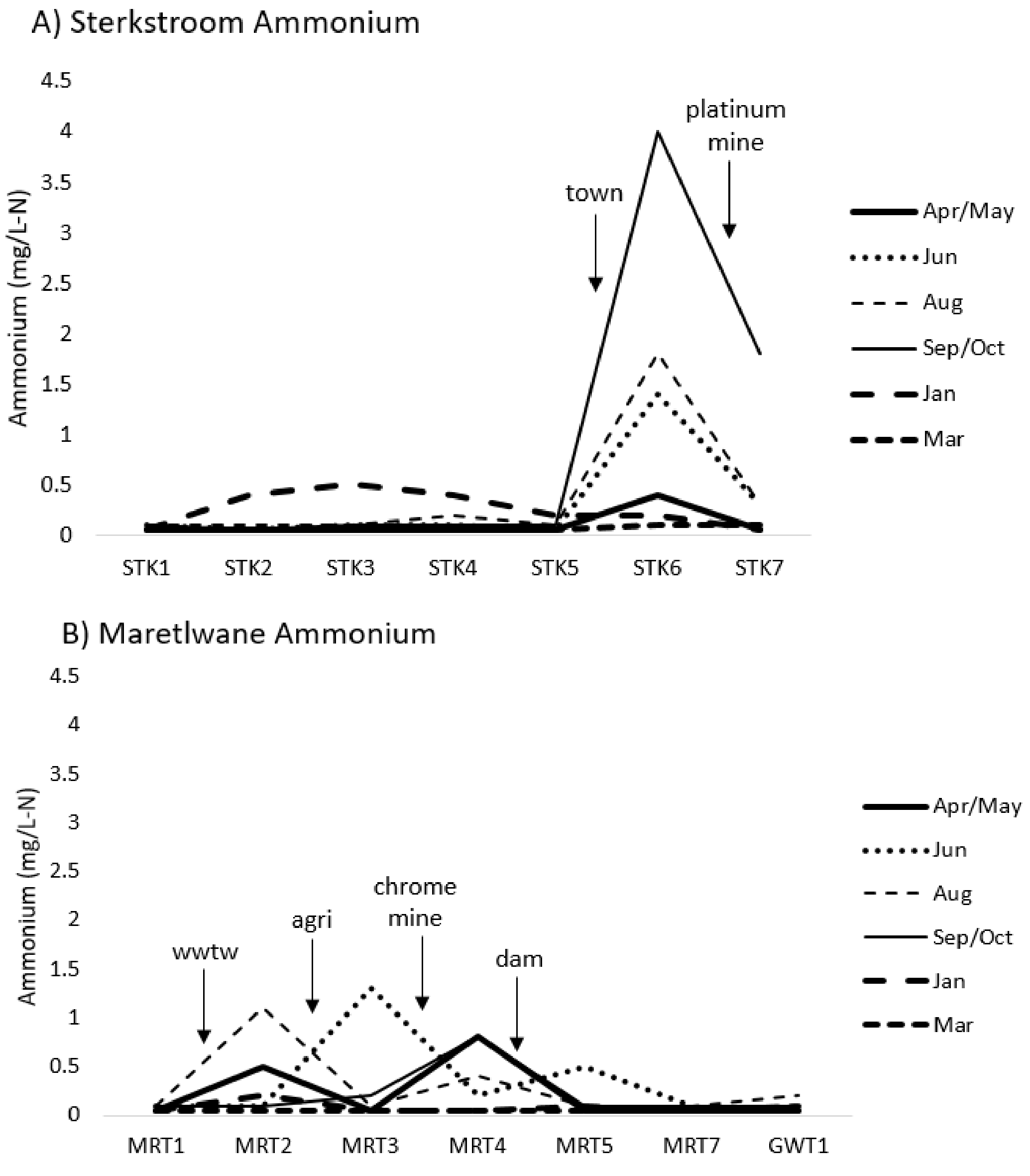
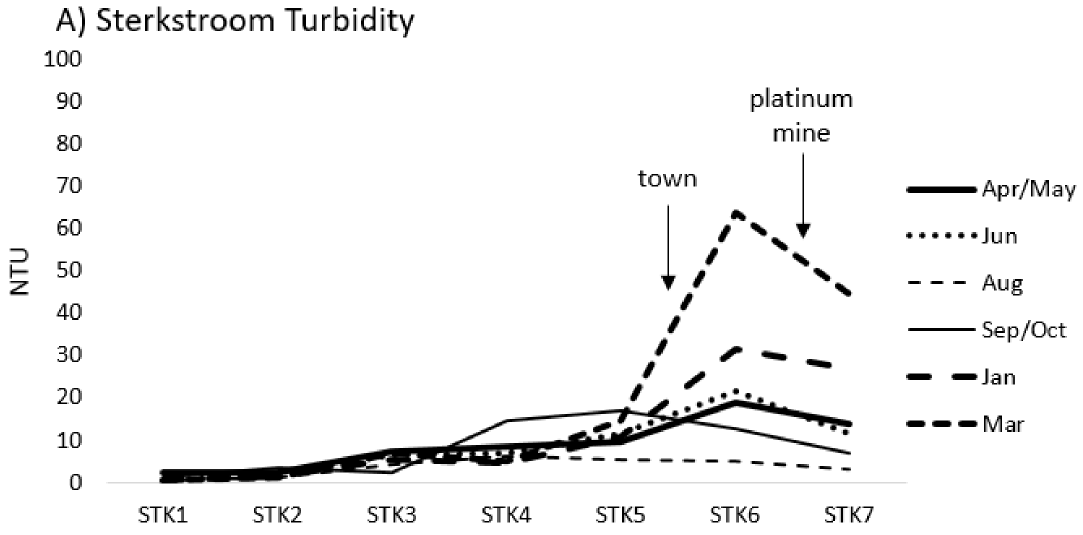


Figure 5: Seasonal variation of Ammonium across longitudinally arranged sampling sites in a) the Sterkstroom and b) the Maretlwane and Gwathle Rivers, showing location of major point sources of water quality change along the river continuum. WWTW = wastewater treatment plant

Turbidity, a water quality variable linked at the landscape scale to cropland as well as urban development (Table 13), showed extreme variation through space and time on the two tributaries (Figure 6).



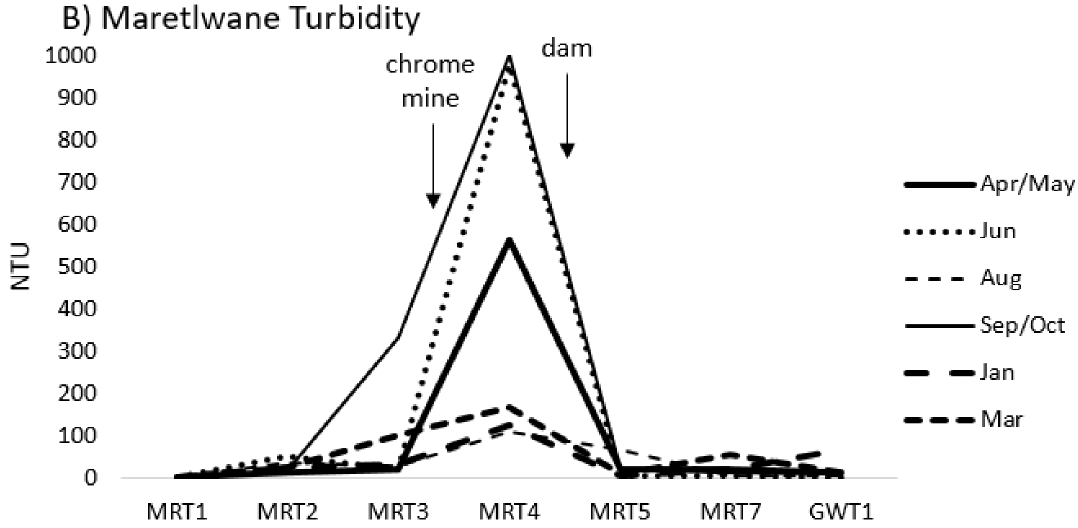


Figure 6: Seasonal variation of turbidity across longitudinally arranged sampling sites in a) the Sterkstroom and b) the Maretlwane and Gwathle Rivers, showing location of major point sources of water quality change along the river continuum. Note that the y-axis scale for Maretlwane is an order of magnitude higher than that of Sterkstroom.

On the Sterkstroom, a steady downstream increase was consistently observed, especially in the high-flow months of January and March. This is likely to reflect the cumulative effect of urban runoff, which peaked at STK6, the site immediately downstream of Marikana Township (Figure 6a). In contrast, the Maretlwane River had a single site where turbidity spiked to extremely high levels in both the low and high flow seasons (MRT4) before rapidly recovering at the subsequent downstream site (MRT5; Figure 6b).

The extremely high turbidity at MRT4 is attributed to its location; being directly downstream of the commercial Samancor Western Mooinooi chrome mine. The mine may use water in their facility's operations (potentially during ore processing), some of which appears to later enter the Maretlwane main stem containing a high silt load (mineral pollutant). This has resulted in a high turbidity levels at MRT4 during the monitoring regime (e.g. September/October 21; < 900 NTU) as well as evidence of river channel infilling in observed river reaches between the mine and the road bridge causeway meters above MRT4 (Appendix B29, B30). Sediments not captured at the road bridge causeway are later trapped downstream in a large agriculturally designated impoundment between MRT4 and MRT5 (Figure 2; Appendix B31), resulting in a significant recovery of turbidity levels down the river continuum (Figure 6b).

Dissolved ions of Iron (Figure 7) were identified as a significant water quality predictor of mining land cover in the catchment area upstream of sites (Table 13).

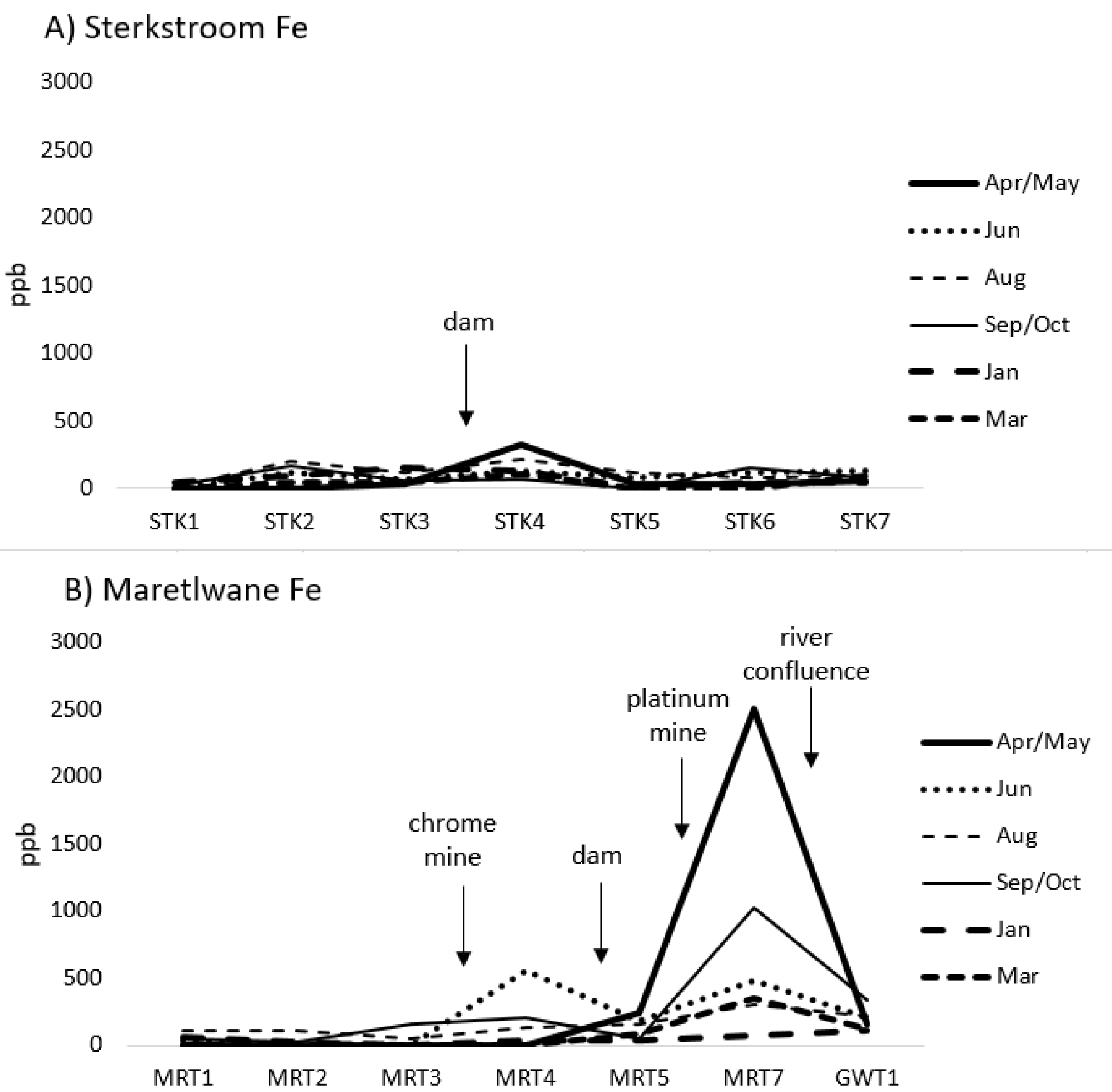


Figure 7: Seasonal variation of concentration of iron ions (parts per billion) across longitudinally arranged sampling sites in a) the Sterkstroom and b) the Maretlwane and Gwathle Rivers, showing location of major point sources of water quality change along the river continuum.

Although ICP-MS was unable to detect iron concentrations in a number of water quality samples, particularly in the upper Maretlwane catchment, the sites were iron was successfully measured demonstrate an acute, if sporadic and a-seasonal, impact of mining on this metal's concentrations at MRT7 (Figure 7b). This site was downstream of both the mining town of Wonderkop, as well as a tributary draining a large mine dump and PCD associated with the Stibanye platinum mining corporation. In contrast, iron concentrations in the Sterkstroom were negligible (under 400 ppb) throughout the year of sampling, not even peaking at the STK7, which was also downstream of a similar platinum PCD tributary. This possibly indicates divergent mine effluent rehabilitation policies and practices being enforced at different platinum mines across the catchment.

Finally, pH was shown by AICc model selection to be an important predictor of general land-use intensification across the two tributaries of the Gwathle catchment, being negatively associated with the percentage of untransformed land cover upstream of monitoring sites (Tables 13 and 14). The initial low pH in untransformed sub-basins of the rivers might be as a consequence of smelters and geology. The presence of mine smelters in and around the Gwathle catchment are significant sources of sulfur dioxide and thus providing the grounds to suggest sulfuric acid (acid rain) may be entering the upper Sterkstroom and Maretlwane river reaches (Gao et al., 2018). In addition to this, the geology of the upper Sterkstroom may be a confounding factor, driving the low initial pH ($\bar{x}$ = 5.6 pH units) as by the river begins in the Tierkloof, a mountain gorge draining the naturally acidic sandstone rocks of the Magaliesberg mountain range.

A steady rise in pH can be observed from upstream to downstream in both the Sterkstroom and the Maretlwane (Figure 8). The rise in pH along the Sterkstroom is particularly steep, rising from a seasonal mean of 5.6 at the pristine STK1 site to over 8 from the platinum mining-affected STK5 onwards (Figure 8a). However, as it enters the agricultural valley upstream of Buffelspoort Dam (represented by monitoring sites STK2 and STK3), it begins to gain surface water from more alkaline groundwater sources, as well as other sources of alkalinity such as fertilizers and other agricultural runoff. This shift towards alkalinity could be further exacerbated by mining activities that may use groundwater extraction and recharge in their operations.

In contrast to the Sterkstroom, the pristine Maretlwane begins at a higher mean pH (7.4), which is likely due to the more mixed sandstone and igneous geology of the upper catchment, characterised by alkaline soils associated with the bushveld basin ecoregion (Nel et al., 2011). The pH nonetheless continues to increase down the catchment, peaking first at the MRT4 site heavily impacted by the chrome mining operations at Samancor, and then again at the

Gwathle River main-stem site GWT1, where all the cumulative effects of catchment water quality modification are combined (Figure 8b).

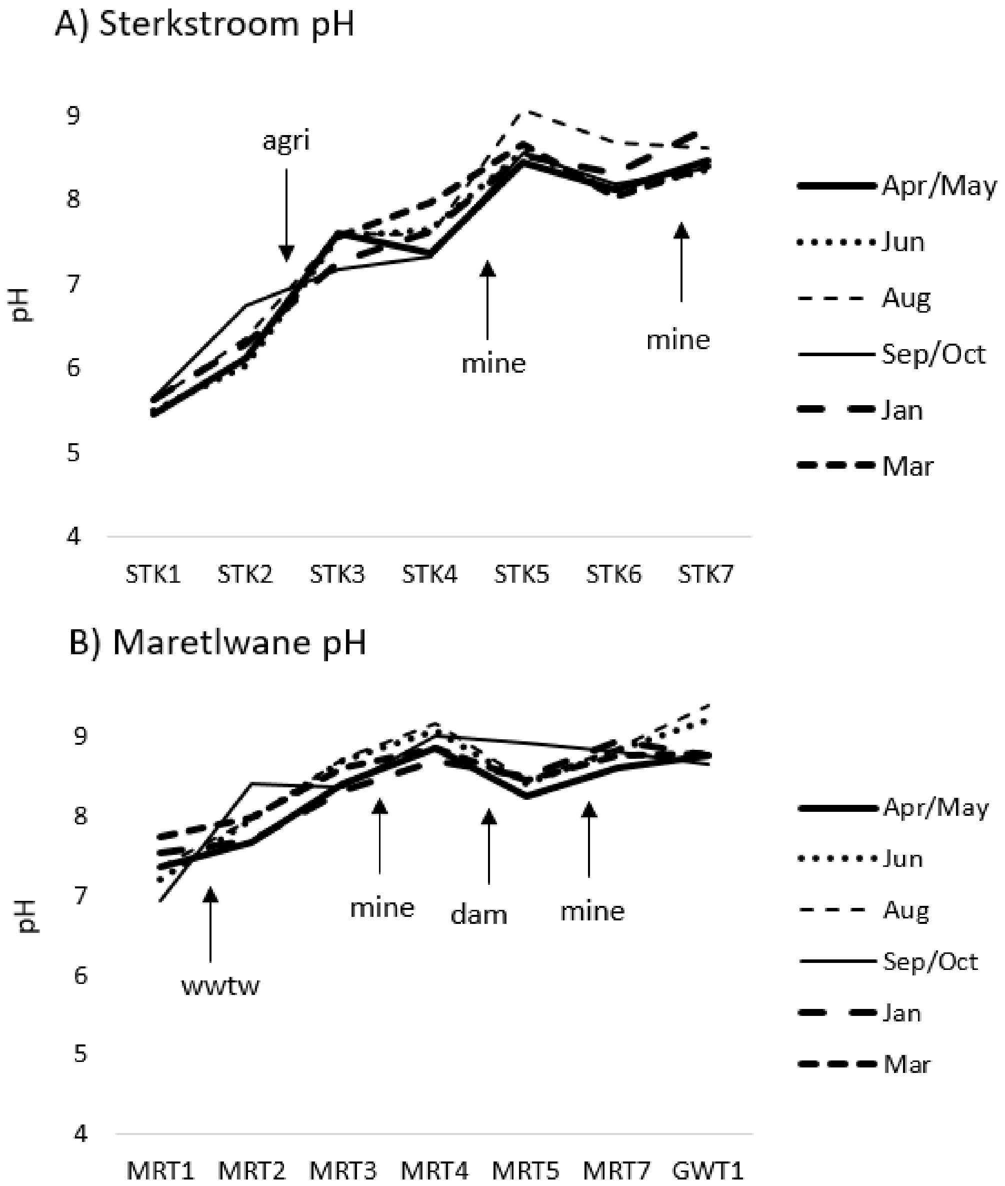


Figure 8: Seasonal variation of pH across longitudinally arranged sampling sites in a) the Sterkstroom and b) the Maretlwane and Gwathle Rivers, showing location of major point sources of water quality change along the river continuum WWTW = wastewater treatment works; agri = agricultural effluent sources.

4. Stable carbon and nitrogen isotope responses to water quality stress

4.1 Variation in nitrogen isotope ratios in response to water quality stressors along the river continuum

A tracing of the raw, non-baselined $\delta^{15}N$ down the Sterkstroom (Figure 9) and Maretlwane (Figure 10), demonstrates several discrete changes in nitrogen enrichment of the food web from the upper, pristine sites to the lower, multiple stressor sites. On the Sterkstroom, the build-up in agricultural land cover from site 1 through to site 4, was reflected in a steady rise in $\delta^{15}N$ from STK1 to STK5. This was followed by a sharp and unexpected drop in enrichment at STK6, before a recovery was observed at STK7. On the Maretlwane a steady rise in $\delta^{15}N$ is observed from MRT1 to MRT3, followed by a levelling off through the sites affected by mining activities, with even a slight reduction in $\delta^{15}N$ observed below the PCD tributary confluence at MRT6. By the time these rivers have converged in the Gwathle River (GWT1), $\delta^{15}N$ has risen from a pristine range of 5-10 to a human land-use modified range of 15-18.

The eco-hydrological characteristics associated with headwater streams (e.g. limited dilution potential and high surface area to volume ratio), make them particularly vulnerable to nitrogen enrichment, especially following biosphere transformations in response to socio-economic development. In addition, nutrient enrichment activities in headwater regions may often lead to cascading effects downstream (Kaushal et al., 2006). These impacts, however, offer an opportunity for tracing the various sources of nitrogen pollution in aquatic ecosystems, as the species of nitrogen can originate from multiple, often interacting sources across multi-user river catchments (e.g. treated/raw sewage, fertilizer, and atmospheric precipitation).

To overcome the inherent difficulty in identifying the sources of nitrogen with multiple interacting stressors, the products of complex biological transformations in and around aquatic ecosystems can offer potential insight into particular sources of nitrogen entering aquatic ecosystems. These processes can be inferred by investigating the $\delta^{15}N$ of the available aquatic food web organisms. The biological transformations that can occur are dependent on the nitrogen products and environmental conditions present, but may include nitrification, denitrification, volatilization and assimilation. Each process results in a nitrogen isotope discrimination range through the preferential use or loss of ^{14}N or ^{15}N that can be identified through $\delta^{15}N$ measurements.

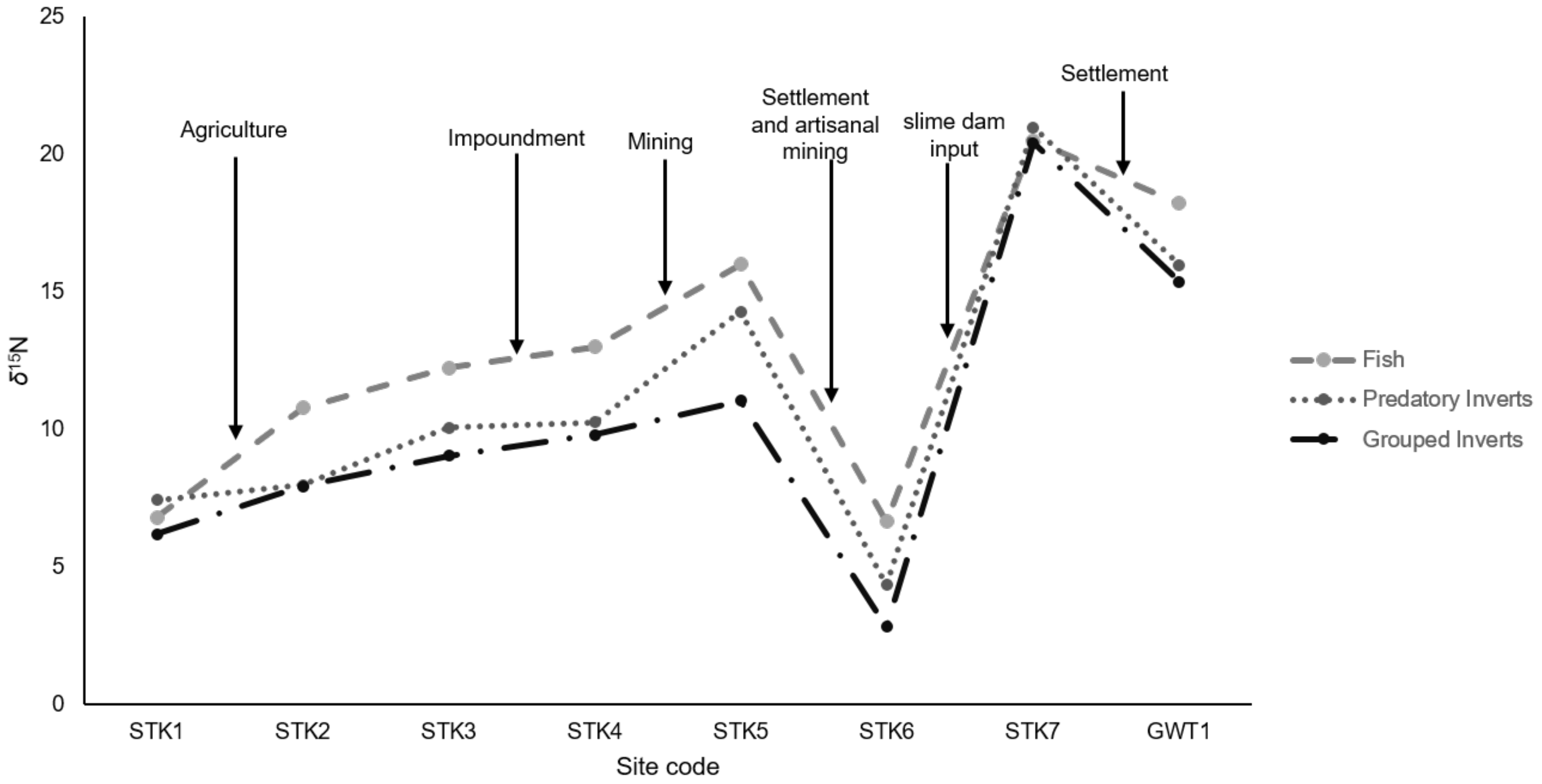


Figure 9: Variation in δ15N for three trophic groups of aquatic organisms down the river continuum of the Sterkstroom tributary of the Gwathle River in the dry season (September-October 2021), indicating the major land use and point sources of water quality indicating the major land use and point sources of water quality change located upstream of each monitoring site.

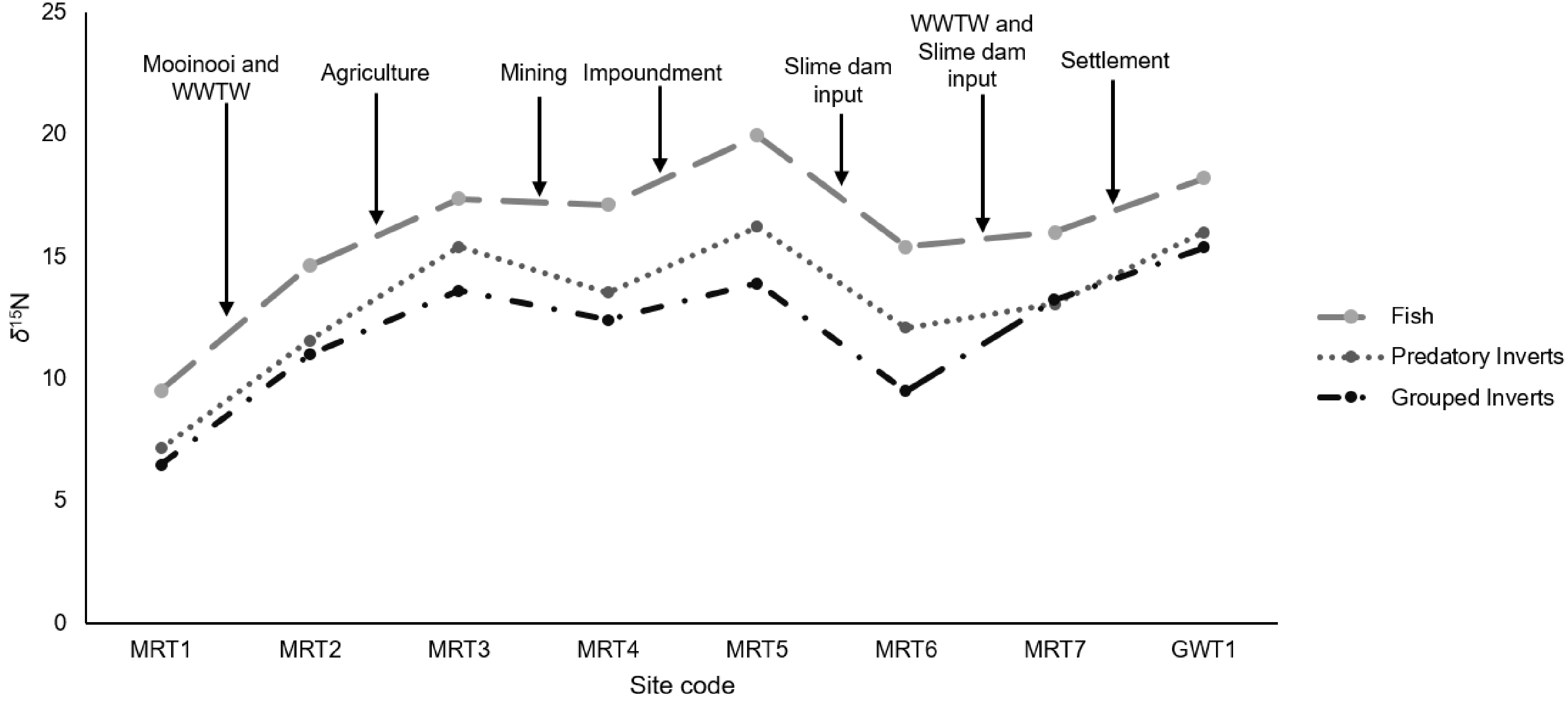


Figure 10: Variation in δ15N for three trophic groups of aquatic organisms down the river continuum of the Maretlwane tributary of the Gwathle River in the dry season (September-October 2021), indicating the major land use and point sources of water quality change located upstream of each monitoring site. WWTW = wastewater treatment works.

Nitrogen species enter aquatic ecosystems via naturally facilitated pathways (dissolution of rocks, soils, atmospheric deposition, run-off, interflow, etc.), thereby transporting nitrogen generated from surrounding land-use activities (diffuse- and point-source pollution). Upon entering the ecosystem, nitrogen can be present in the inorganic form (DIN; nitrate, nitrite and ammonium) that undergoes the aforementioned biological transformations, as well as dissolved organic forms (DON; amino acids, urea, etc.) used by aquatic consumers. The inorganic nitrogen is consumed by heterotrophic microorganisms (Caraco et al., 1998) and primary producers (Cormier et al., 2021) which are both sources of DON to be assimilated by primary consumers and the remaining food web trophic levels, thus reflecting the $\delta^{15}N$ values of DIN (McClelland et al., 1997). The role of mineralisation increases, leading to allochthonous export of DIN to users downstream.

With a relatively complex spatio-temporal experimental design, as captured with this study, we argue that anthropogenic sources of nitrogen can be readily distinguished from one another by identifying $\delta^{15}N$ values of aquatic organisms. Using longitudinal plots of the raw fishes, predatory invertebrates and remaining invertebrates $\delta^{15}N$ values (Figures 9 and 10), as well as a PCA analysis showing the role of land cover, isotopic and water quality tracers in driving variation among sites (Figure 11), we can identify anomalies in nitrogen inputs that differ from the general $\delta^{15}N$ enrichment observed along the river continuum in a relatively pristine river catchment.

Within the Sterkstroom river basin, there are numerous sources of nitrogen. Shortly after the pristine headwater site at STK1, there is an increase in $\delta^{15}N$ in consumer groups at STK2 following agricultural activities (cherry orchards and pivot irrigation) (Figure 9). Although this change in nitrogen isotope discrimination could be attributed to a potential agricultural (fertilizer) signal, it is more likely associated with heightened mineralization occurring within a densely canopied wetland reach dominated by invasive trees (eucalyptus and poplars) a few meters upstream of STK2 (Appendix B3). Occupying ~590 m of the stream channel, this slow flowing reach, shaded by the invasive tree canopy with inundated root systems (Appendix B6), provides high foliage (allochthonous) input and subsequent resources for colonizing bacteria (leaf packs). The riparian root-facilitated instream bacteria consequently breakdown allochthonous particulate organic matter inputs by preferentially processing the lighter ^{14}N, generating dissolved inorganic nitrogen enriched in ^{15}N (Hill et al., 2012; Morrissey et al., 2013). This nitrogen pool, with higher $\delta^{15}N$ values, exits the shaded canopy and is assimilated by autochthonous consumers (primary producers, i.e. biofilms) in the unshaded reaches downstream.

Along the Sterkstroom a new potential source of nitrogen input is identified at STK5, with its sub-basin associated with land cover dominated by mining activities (Figure 11a), by an enrichment in the fishes $\delta^{15}N$ after the Tharisa platinum mine (Figure 9, Appendix B13). This strip mine relies on explosives and heavy machinery to break rock, transport and store surface material (overburden) on multiple tailing dumps ~150 m away from the river. There are multiple nitrogen sources that may emanate from South African platinum mining activities; not all of which produce $\delta^{15}N$ enriched nitrate (Skinner, 2017). Such sources may include, leaching of explosive residues off disposed rock, overburden tailing dumps, pollution control dams, sewage, as well as dissolution of ammonium nitrate explosives or explosive waste following inadequate storage (Skinner, 2017).

With chemical blasting activities likely occurring within the nearby mine site, explosives may be a potential source of nitrogen at STK5. Most of the nitrate precursors used in explosives such as in Trinitrotoluene (TNT), hexogen (RDX) and octogen (HMX) are, however, strongly depleted in $\delta^{15}N$ (e.g. HNO_3, NH_4NO_3, $NaNO_3$: ~5‰), with the exception of a few (e.g. KNO_3: -1.85‰ to 58.82‰) (Bezemer et al., 2020). In addition, while most explosives generate nitrogen products (predominantly nitrate), long-term physico-chemical water quality monitoring (Table 8) did not identify any elevated nitrogen species (Pennington and Brannon, 2002). Consequently, it is unlikely that explosive related products are the cause of the slightly elevated $\delta^{15}N$ at STK5. With no definitive indication of sewage, pollution control dam (PCD) tributaries or PCD leachate entering the river system, soil organic nitrogen from the surrounding overburden tailing dumps may be the likely source of enriched $\delta^{15}N$ nitrogen entering upstream (Cravotta, 2002; Choi et al., 2003).

Soil organic nitrogen in topsoil and subsoil, particularly from present and past agricultural worked land (cattle and ammonia-fertilizer) such as the mined Tharisa property, is a significant source of nitrogen to aquatic ecosystems globally with elevated $\delta^{15}N$ (Choi et al., 2003). Volatilization on-land of the isotopically light ammonia following breakdown of animal waste (urea in urine) can result in soils with enriched $\delta^{15}N$ (10‰ to 22‰) (Kreitler, 1974). The nitrogen product can then be transported to nearby aquatic ecosystems following wind and water facilitated pathways.

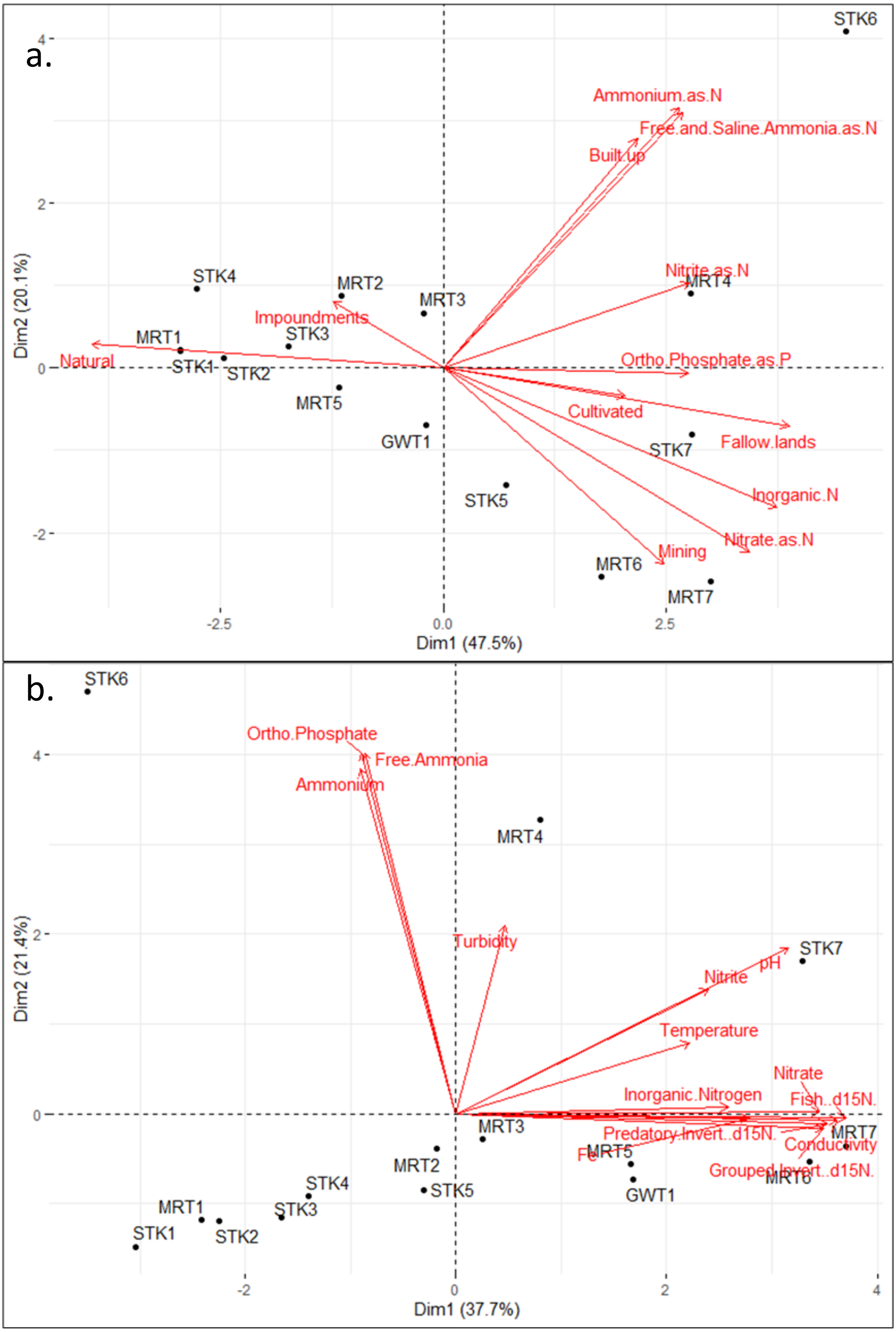


Figure 11: Principal component analysis of variation in a) land cover and b) nitrogen isotope absolute values at three trophic levels and water quality tracers across the Gwathle River catchment

Immediately downstream at the next Sterkstroom river site, the STK6 sub-basin is characterised largely by built-up land cover (sprawling formal and informal township), followed by agriculture and mining land-cover (Figure 11a). There is an anomaly in the longitudinal $\delta^{15}N$ values in the instream organisms, whereby the $\delta^{15}N$ values of the STK6 organisms are significantly (fishes; $p = 0.03$) depleted (4.62 ± 1.93‰) compared to the previous STK5 site (13.77 ± 2.52‰), sampled only one day apart (Figure 9). Observing the physico-chemical and anion data revealed a year-round, ammonium and free-ammonia source entering the river across the six sampling events; while spiking (> 4 mg/L-N) during the dry season stable isotope collection (Figure 5a; Figure 11b).

A hypothetical source for the highly reactive (readily consumed), depleted $\delta^{15}N$ (Figure 9), and elevated ammonium and ammonia concentrations (Figure 5a) at site may be explosives. Occasional explosions were heard during multiple site visits to STK6, emanating from an upstream mine. As discussed above, nitrogen precursors in explosives are mostly depleted in $\delta^{15}N$, and explosive residue or waste products have the potential to be wind-blow or leached into nearby water sources (Bezemer et al., 2020). The prolonged and elevated ammonium and ammonia concentrations (Figure 5a) may point to the potential use of ammonium nitrate explosives (NH_4NO_3), however, the low nitrate conditions suggest an alternative primary source. Furthermore, it can also be argued that the input explosive compounds would rather be a transient input from such diffuse-sources (e.g. explosive residues), unlike the constant aseasonal ammonium input measured.

An alternative hypothesis is that these nitrogen products are associated with wastewater generated by the predominant up-stream built-up land-cover composed of the formal and informal Marikana settlement. With no evidence of a wastewater treatment facility located in the sub-basin to be the constant nutrient source, leaky untreated sewage, being a significant source of ammonium and ammonia (hydrolysed urea), may be the lead contributor to the long-term nutrient loading detected at STK6 as a consequence of inadequate and poorly maintained black water infrastructure in built-up areas upstream (Choi et al., 2003; Levin et al., 2019).

It is widely acknowledged that while denitrification associated with treated sewage processes produces nitrogen products enriched in $\delta^{15}N$ (~10-15‰), on the contrary untreated sewage is characterised by nitrogen products depleted in $\delta^{15}N$ (~0-5‰) nearing the average $\delta^{15}N$ values of the instream organisms at STK6 (Anderson and Cabana, 2006; Finlay and Kendall, 2007). In addition, the preferential assimilation pathway associated with ammonium by primary producers, as opposed to dissolved nitrogen forms (e.g. nitrate; associated with synthetic fertilizers), and their decomposition to particulate organic nitrogen for assimilation by primary consumers, further supports the ammonium and ammonia being the potential source of significant $\delta^{15}N$ depletion in the aquatic consumers (Grimm, 1988; Morrissey et al., 2013; Cormier et al., 2021).

The final and most likely hypothesis is one associated with large artisanal chrome mining activities both upstream and in the immediate vicinity of the site (Appendix B15). The perpetual high ammonium, ammonia and sharp drop in surface water pH at STK6, followed by expected $\delta^{15}N$ consumer values and pH buffering at STK7, suggests the input of highly reactive chemicals upstream or in the vicinity of STK6. It is probable that the artisanal miners are using ammonium + ammonia rich chemicals while applying the Kroll process, which makes use of nitric acid and is commonly associated with processing chrome ore. The Kroll process involves dissolving chrome ore in nitric acid to separate the commercially viable chromium from the additional ore compounds (Kroll et al., 1950). Following neutralizing the solution, the chromium is precipitated out before being used in further processing.

The rapid rise of $\delta^{15}N$ values in aquatic organisms downstream, at STK7 (20.62 ± 0.31‰), characterised by mining and agricultural land (Figure 11a) suggests two causes. Firstly, the highly reactive nitrogen species entering the river at STK6 will act as reactants in the ammonia volatilization transformation process to produce $\delta^{15}N$ enriched nitrate; as corroborated by the rise in nitrate concentrations at STK7 (STK6: 0.3 mg/L-N to STK7: 16 mg/L-N). Such a transformation process may be facilitated by the persistent turbulent and high flow conditions at the site (discharge: 0.13 m^2/s). The second, less likely cause may be an additive source of nitrate entering the Sterkstroom before STK7; via a tributary draining two separate platinum PCD's. Although the dry season SI were collected in Sept/Oct 2021, a single water sample collected from each platinum PCD tributary in March 2022 indicated that one platinum PCD was a source of elevated nitrate inputs (25 mg/L-N). Such mining inputs are most likely strongly diluted by the main stem and its effect on instream $\delta^{15}N$ organism values appears to be negligible compared to the proposed raw sewage and mine-associated inputs upstream.

On the east side of the catchment the Maretlwane River too displays signs of nitrogen pollution, with an increase in fish organism $\delta^{15}N$ values at MRT2, following the pristine MRT1 site. Upstream of MRT2 is a municipal wastewater treatment plant within the town of Mooinooi (Appendix B22). This plant continuously discharges treated, and on the rare occasion, untreated wastewater into the river. Wastewater treatment plants, unlike the effects of raw sewage inputs potentially observed in the $\delta^{15}N$ values at STK6, are recognised as sources of enriched $\delta^{15}N$ nitrate in global river systems (~10-15‰). This is owing to denitrifying heterotrophic bacteria used in the wastewater treatment process preferentially using the ^{14}N

isotope, leaving the remaining nitrates enriched in ^{15}N upon their discharge (Morrissey et al., 2013).

The $\delta^{15}N$ values of the aquatic organisms continue to rise, with fishes enriched at the MRT3 site following commercial agricultural activities (pivot irrigation and cattle farming) adjacent to the rivers. Manure compost fertilizer and cattle manure are two known sources of nitrogen upstream of the site that enter aquatic ecosystems through various wind and water processes depending on the eco-hydrology of the surrounding region. Both sources, in particulate and dissolved forms, have been found to have enriched $\delta^{15}N$ values before entering aquatic ecosystems (Cravotta, 2002).

Following a pervasive mineral pollution input at a commercial mine upstream of MRT4 (Figure 6b), from where $\delta^{15}N$ in the aquatic organisms becomes slightly depleted, a large agricultural impoundment then alters the $\delta^{15}N$ at MRT5 (Figure 10; Appendix B31). Such impoundments trap and breakdown particulate organic matter via deep water anaerobic decomposition into dissolved compounds (eds. Bonell and Bruijnzeel, 2005). Anammox bacteria within such features convert available ammonium and nitrite by removing ^{14}N to $N_{(g)}$ and producing ^{15}N enriched nitrate.

With the additional input of a platinum/chrome PCD tributary into the Maretlwane River at MRT6, only ~680 m downstream of MRT5, there is a decline in the $\delta^{15}N$ values of the aquatic organisms. Nitrate concentrations increased between the two sites (0.20 mg/L-N to 26 mg/L-N) at the time of the dry season isotope collection. Samples collected from the PCD tributary meters before the confluence with the main stem, indicate it is a significant source of nitrate (319 mg/L-N), and is the only potential source of the ^{14}N enriched nitrate being consumed by aquatic organisms. The nitrate concentration at the confluence for the six monitoring periods exceeded the global median nitrate concentration of rivers being (0.3 ± 0.2 mg/L-N; Matiatos et al., 2021). To assess the relationship between catchment-wide $\delta^{15}N$ enrichment and depletion in response to instream nitrogen, correlations were run on the $\delta^{15}N$ of non-predatory consumers that assimilate nitrogen directly via grazing and collecting FPOM and three types of nitrogen (summed and individual N-species) measured across the Gwathle Catchment (Figure 12).

There was an insignificant, weak positive correlation in $\delta^{15}N$ values for non-predatory macroinvertebrates following higher total inorganic nitrogen ($r = 0.07$; $p > 0.05$) (Lee et al., 2018) and nitrate ($r = 0.04$, $p > 0.05$) (Smuckler et al., 2018) concentrations (Figures 12a & 12c). The high total inorganic nitrogen concentrations in the catchment area are more associated with agricultural activities, while nitrates are to mining activities (Figure 11a).

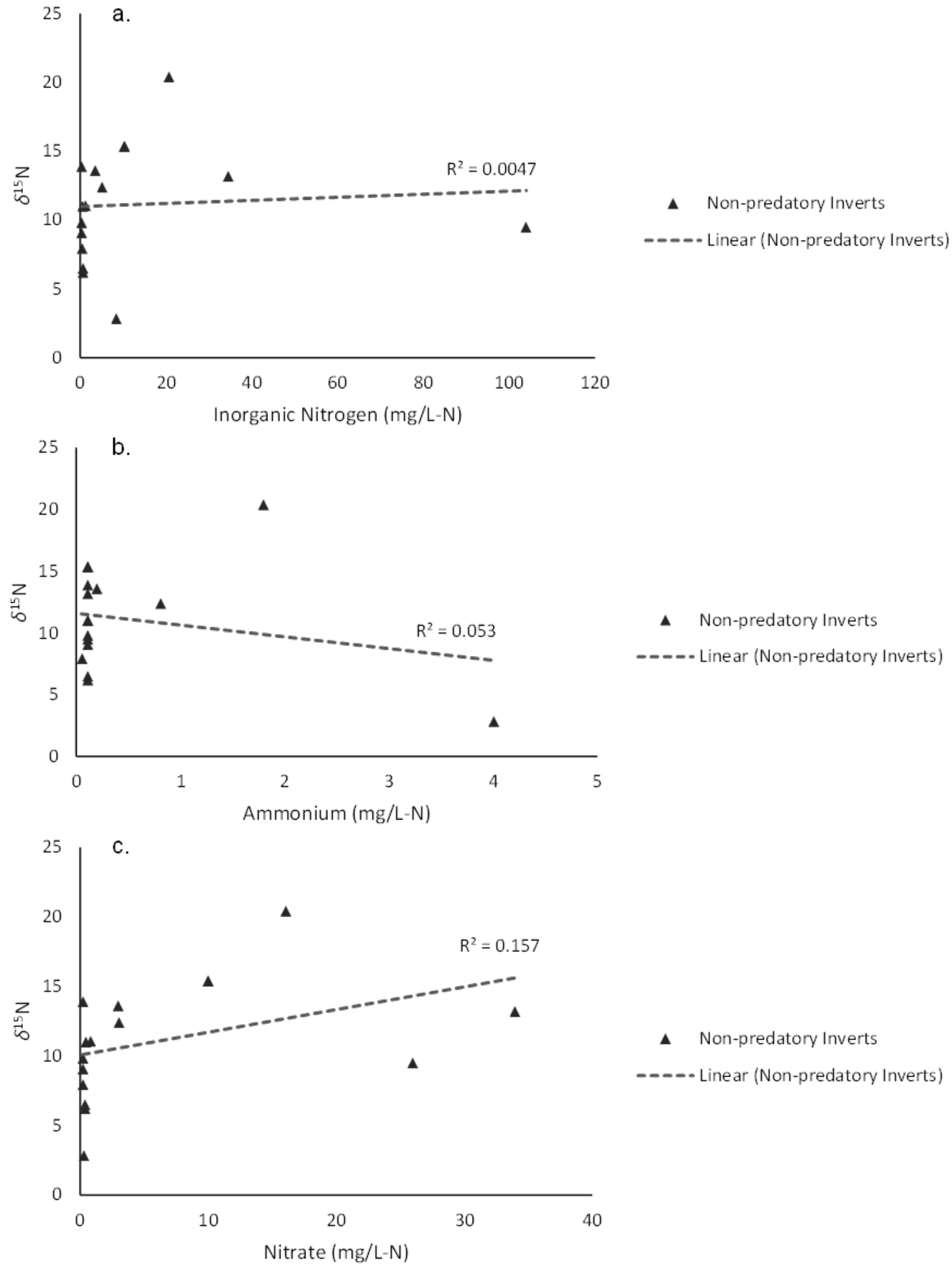


Figure 12: The non-significant relationships between δ15N values of non-predatory invertebrates and (a) inorganic nitrogen, (b) ammonium and (c) nitrate concentrations across the Gwathle River catchment

Following increasing ammonium concentrations, there was a weak, non-significant negative correlation (r = 0.23, $p > 0.05$) as $\delta^{15}N$ values in the non-predatory macroinvertebrates (Figure 12b), likely driven by either raw sewage inputs or artisanal mining activities possessing chromite, thereby elevating ammonium and depleting aquatic consumer $\delta^{15}N$ values (Finlay and Kendall, 2007). Ultimately, all three trends demonstrated non-significant correlations with the instream nitrogen. This is likely due to the outsize influence on outlier sites on the food webs, for example the elevated ammonium at STK6, following regular raw sewage input/artisanal mining effects. These results indicate that complex multi-user catchments may require finer grained, spatially-contextualized analyses (e.g. Figures 9 and 10) to be able to fully interpret stressor-food web interactions.

4.2 Food web structure across a multi-stressor gradient

Food web bi-plots that follow north-east orientations indicate well-functioning ecosystems with effective energy pathways (O'Reilly et al., 2002). Of the Sterkstroom sites, STK5 following Tharisa Mine does not present a well-functioning trend (Figure 13). The extremely limited habitat available at the site, which is channelized with reduced flow (relative to sites upstream and downstream) and containing very little instream cover for animal consumers (Appendix B14) is the likely source of altered energy flow in the system; limiting fish establishment and resulting in an almost absent tertiary consumer group (one individual) (Hansen et al., 2016; Caputi et al., 2020). The extremely low discharge at the site is possibly the result of water abstraction by both commercial mining operations and the local community.

At STK6, a return to higher discharge (possibly supplemented by leaking sewage infrastructure emanating from Marikana township) and a more complex array of benthic habitat (Appendix B16, B17), is matched by the return of a more naturally structured food web, albeit with the nitrogen ratios for each trophic level significantly depleted, due to the unusual biochemical processes occurring at the site (Figure 14). At STK7, the nitrogen ratios have returned to levels similar to those upstream of the township, but a high overlap of primary, secondary and tertiary consumer groups can now be observed. This could be due to sewage and other urban and peri-urban sources disrupting the food web, although one would have expected to see this at STK6 as well if Marikana township was the primary stressor. The low nitrogen range (NR) at the site (Table 14) suggests a reduced food-chain length, however, with all consumer groups present at the site, this suggests that poor water quality ought to have resulted in trophic compression (altered trophic niche).

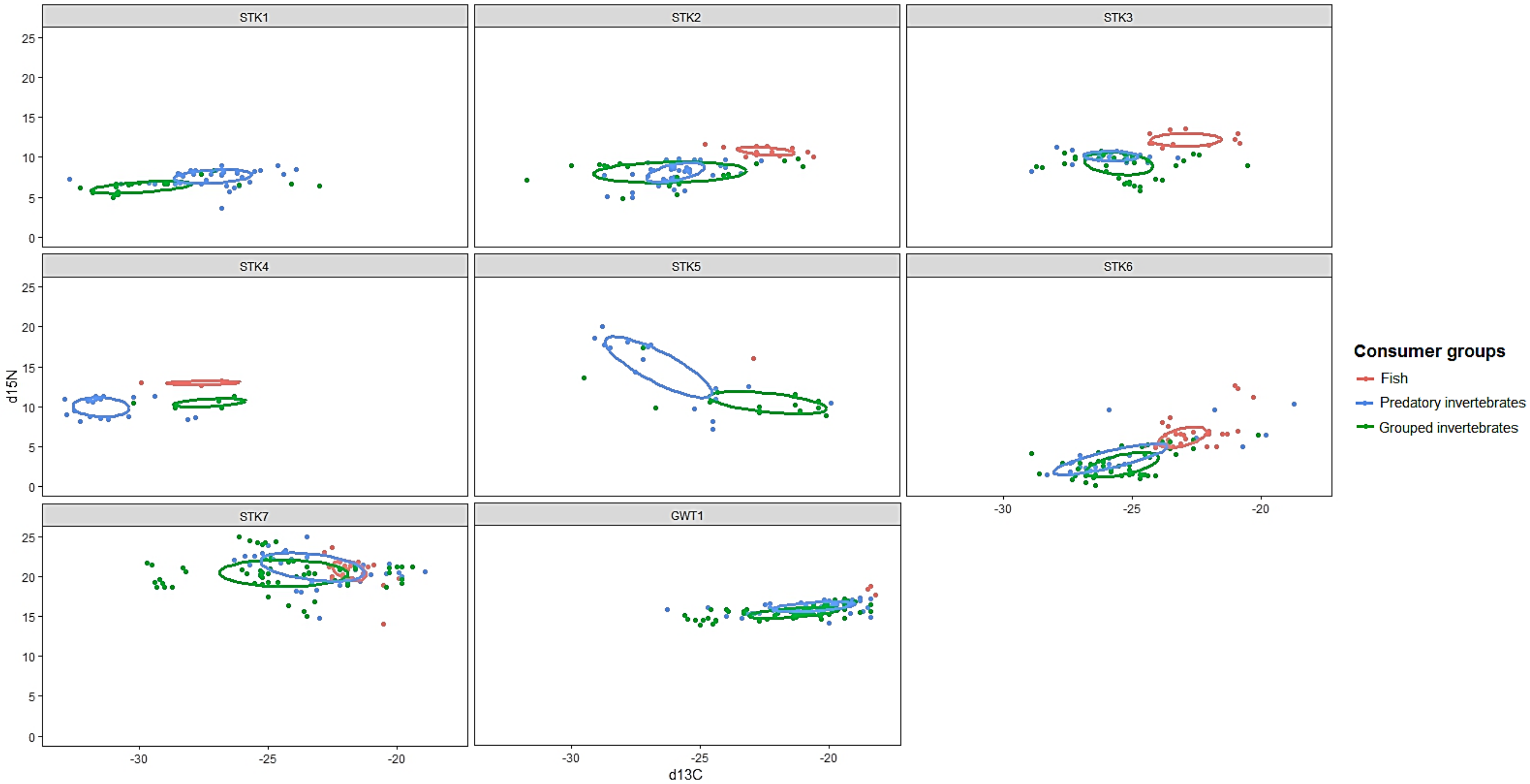


Figure 13: Site-specific δ13C and δ15N bi-plots for sites on the Sterkstroom River in the dry season (September-October 2021)

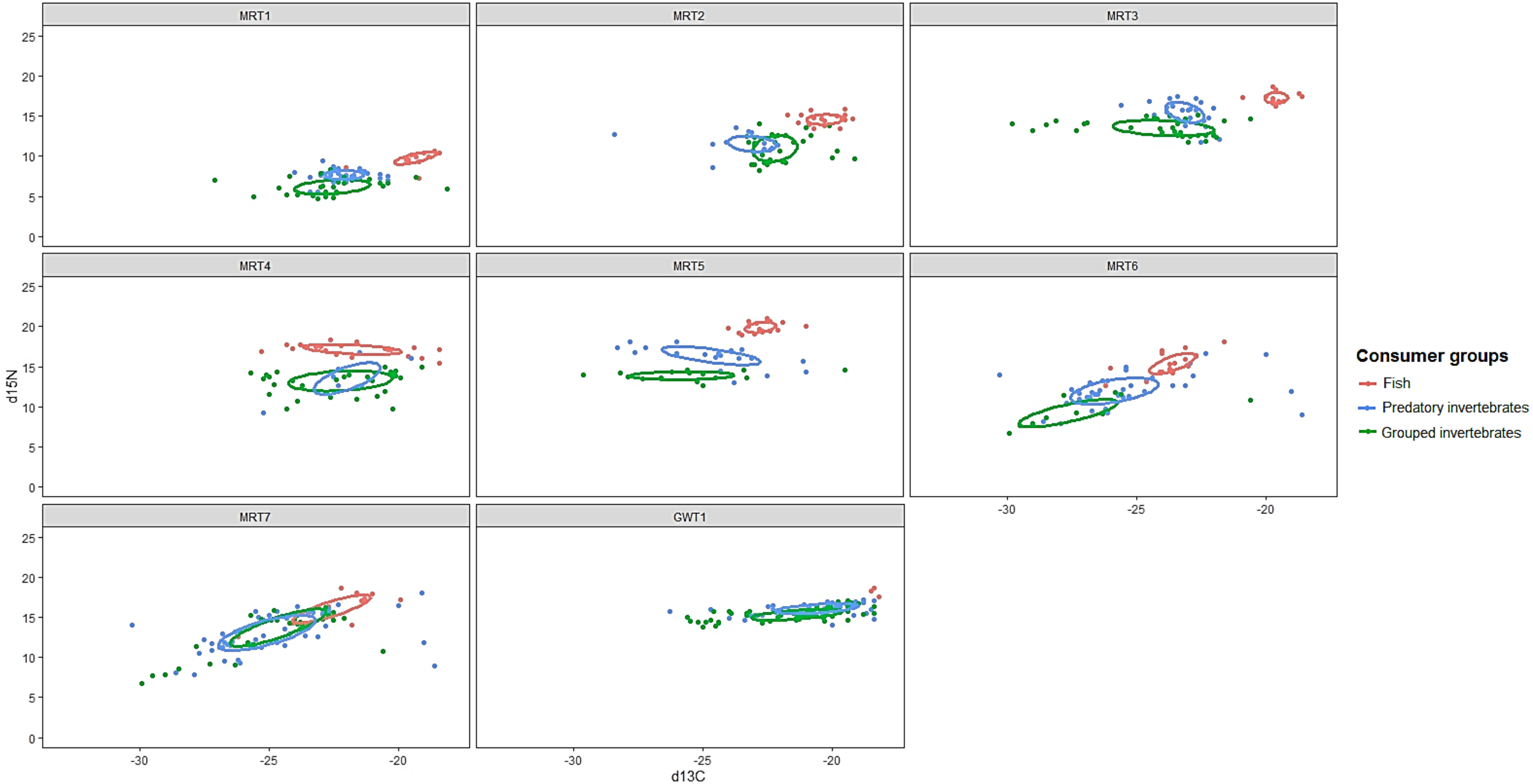


Figure 14: Site-specific δ13C and δ15N bi-plots for sites on the Maretlwane River in the dry season (September-October 2021)

Table 14: Bayesian layman metrics for each of the 15 communities assessed during the dry season sample

Layman metrics	River site														
	STK 1	STK 2	STK 3	STK 4	STK 5	STK 6	STK 7	MRT 1	MRT 2	MRT 3	MRT 4	MRT 5	MRT 6	MRT 7	GWT 1
$\delta^{15}N$-range	1.2	2.8	3.2	3.2	3.3	3.8	0.6	3.0	3.6	3.8	4.1	6.0	5.9	2.9	2.8
$\delta^{13}C$-range	2.4	4.1	3.1	6.8	3.0	2.6	2.8	4.5	3.2	4.6	0.7	2.8	3.3	2.3	3.2
TA	0.0	0.8	2.2	1.8	0.0	1.3	0.7	0.8	3.2	2.2	0.4	0.5	1.1	0.4	0.5
CD	1.4	2.1	1.8	2.9	2.2	1.8	1.0	2.1	2.0	2.3	1.7	2.4	2.3	1.6	1.6
MNND	2.7	1.9	2.0	3.3	4.4	2.1	1.5	2.4	2.4	2.7	1.6	3.0	3.3	1.4	1.8
SDNND	0.0	2.2	1.5	1.9	0.0	1.0	0.1	1.2	1.4	1.1	1.8	1.0	0.3	2.0	1.1

Table 15: Standard ellipse areas generated from the tertiary consumer group (fishes) at each site where more than three individuals were collected

	River site											
	STK2	STK3	STK4	STK6	STK7	MRT1	MRT2	MRT3	MRT4	MRT5	MRT6	MRT7
SEA_C	2.1	4.1	1.8	5.8	4.3	5.3	1.8	1.4	4.2	1.5	4.0	6.8

In the Maretlwane River, there is a disturbance in food web energy flow at MRT4, following mineral pollution input in the form of washed tailings discharged from an upstream commercial chrome mine (Figure 14). The respective food web consumers had a narrow $d^{13}C$-range (0.7), indicative of a limited resource base and, thus niche elimination where specialist consumers are removed; as associated with high sedimentation (Burdon et al., 2019). Such activities homogenize aquatic benthic habitats, reducing the diversity and abundance of available resources (Waters et al., 1995). Any fine resource particles available with high sedimentation, are easily mobilized and often prevented from accumulating a sufficient resource base. As a result, consumers are forced to seek sparser, diverse resources in the ecosystem, increasing competition between individuals, resulting in larger consumer trophic niches (higher SEA_C; Table 15) (Dolédec et al., 2011; de Castro et al., 2016).

The STK4 food web bi-plot exhibits unordinary patterns, with consumer groups positioned to the far left. The highly negative $\delta^{13}C$ values are likely attributed to the large deep water impoundment (Buffelspoort Dam ~ 35 m deep; Appendix B12), collecting and storing surface and atmospheric sulfur deposits, facilitating the accumulation of abundant anaerobic sulfur reducing bacteria. These bacteria, unlike those that mineralize organics via the CO_2 pathway in aquatic systems, mineralize organics via the CH_4 pathway. As a result, the bacteria process the available organics leading to the carbon isotope discrimination being largely negative.

This unusual carbon signature across invertebrate consumers is still present to an extent at the highly degraded STK5, but by STK6, despite the severe drop in nitrogen isotope ratios, the carbon signature of the food web has essentially recovered to pre-reservoir values (c.f. STK3; Figure 13). This suggests the new sources of energy emanating from, amongst other things, Marikana Township, have replaced the bacteria-derived organic material that was prevalent below the dam (Burbank et al., 2022). The carbon signature continues to shift to the right at the Gwathle River site (GWT1; Figure 13), which represents the merging of resources flowing from both the Sterkstroom and Maretlwane tributaries upstream.

The Gwathle site also shows compression of the food web along the nitrogen axis relative to the majority of river sites upstream, similar to that seen at STK7. This suggests that the human impacts on instream resources emanating from the lower Sterkstroom have a very strong effect on the main stem food web, potentially overriding the effects of water quality stressors in the Maretlwane. The Maretlwane food webs overall showed remarkably stable and healthy structure throughout the river continuum, despite observed impacts of chrome and platinum mining on water quality. This may indicate that the major energy sources and habitat for consumers are unaffected by large scale commercial mining, despite the severe degradation in water quality. The most drastic degradation of a local food web was observed at STK5, where local habitat degradation appeared to play a larger role than nutrient or energy inputs. On the Maretlwane, the largest step change was observed at MRT6, where nitrogen ratios in primary consumers dropped following the confluence with a stream draining a PCD. Large dry-season inputs of nitrates from the PCD (Table 8) appear to be related to the depleted δ15N in aquatic consumers at this site and the following site along the river continuum, which also receives effluent from PCDs (Appendix, B39).

5. Assessing stable sulfur isotopes as a water quality stress tracer

5.1 Sulfur stable isotope responses to key stressors

In addition to the dry season $\delta^{13}C$ and $\delta^{15}N$ sampling across the riverscape, the research team was able to process both wet (April-May) and dry (September-October) season data for $\delta^{34}S$. These revealed a complex interplay between spatial (land cover change) and temporal (seasonal river flow) environmental variation on the sulfur isotope enrichment within fish tissues across the riverscape of the Gwathle River quaternary catchment.

A longitudinal analysis of wet and dry season shifts in $\delta^{34}S$ along the Sterkstroom (Figure 15) and Maretlwane (Figure 16) rivers reveals several step changes in sulfur isotope values within fish tissues in relation to both land cover change and local concentrations of sulfates in the water column, with seasonality playing a stronger, more consistent role in sulfur isotope enrichment than in sulfate concentrations.

Both rivers in both seasons begin with relatively depleted $\delta^{34}S$ levels in animal tissue (STK1 had no fish so tadpoles were used as a vertebrate substitute consumer), with values ranging between -0.2 and 3.1‰. It is important to note that the upper catchment has two natural sources of sulfur that are responsible for both the amounts of sulfate in the water column and the fractionation of sulfur isotopes in the food web; namely atmospheric deposition, followed by runoff into the stream, as well as leaching from the rocks that the river weathers over time. While we do not have estimates for either the isotopic value of airborne sulfur compounds, nor sulfur in the primarily quarzitic surface sandstone of the Magaliesberg mountain range, we can see from our year of water quality monitoring (Table 10) that sulfate levels in these headwaters never exceeded 5 mg/L, indicating that natural background sources of sulfur in surface waters are minimal. It is also interesting to note that both wet and dry season sulfate levels are extremely low at both pristine sites, suggesting minimal sulfur presence in both wet season and dry season deposition into these headwaters.

The first major change along the river continuum of the Sterkstroom is visible at STK3 in the wet season, where a rapid rise of both SO_4 water concentrations and $\delta^{34}S$ enrichment in fish tissue (up to 3.0‰) is observed, indicating the food web is assimilating a sulfur-enriched food source derived from the agriculturally dominated valley upstream of Buffelspoort Dam. This sulfur enrichment is still present in fish tissues collected in the dry season, although SO_4 concentrations at this time are far lower, more similar to those seen in the pristine reaches upstream (Figure 15).

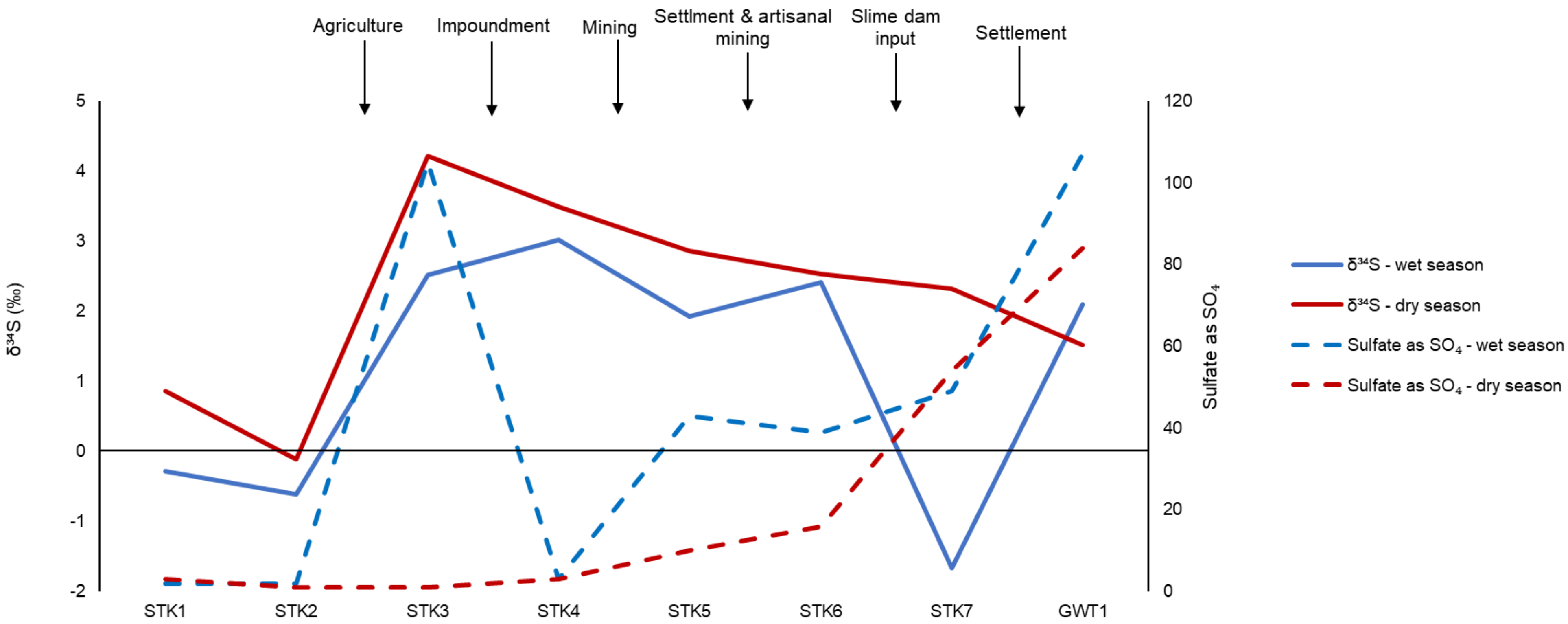


Figure 15: Variation in δ34S within fish muscle tissue and sulfate concentrations down the river continuum of the Sterkstroom tributary of the Gwathle River in the wet season (April-May 2021 – blue) and the dry season (September-October – red), indicating the major land use and point sources of water quality change located upstream of each monitoring site.

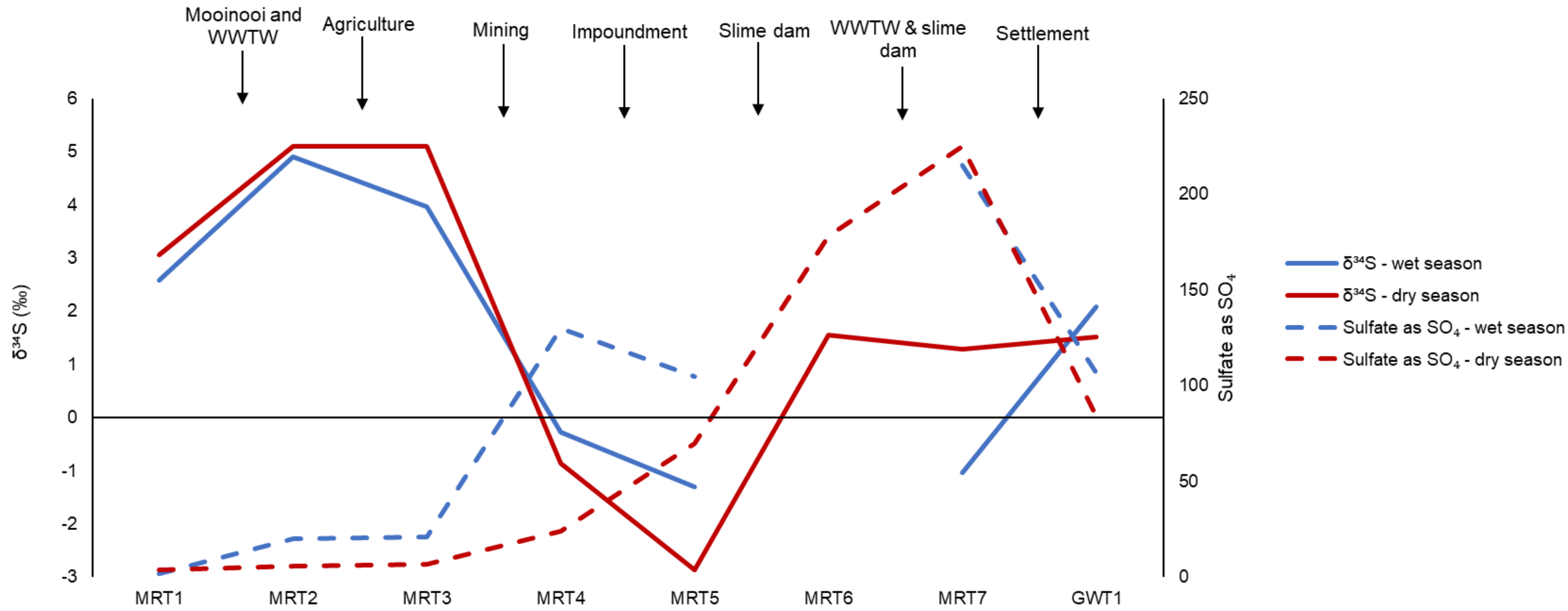


Figure 16: Variation in δ34S within fish muscle tissue and sulfate concentrations down the river continuum of the Maretlwane tributary of the Gwathle River in the wet season (April-May 2021 – blue) and the dry season (September-October – red), indicating the major land use and point sources of water quality change located upstream of each monitoring site.

This sulfur-enriched energy source is likely to be in the form of photosynthetic biofilms that are taking advantage of both nitrates mineralized from the natural organic matter in the STK2 wetland (see Chapter 4), as well as potentially sulfur-rich fertilizers entering the stream via runoff, which have been found to have a global average $\delta^{34}S$ of 4.7‰ (Wang and Zhang, 2019), or from similarly isotopically enriched pesticides (Hermes et al., 2021) deployed in the surrounding up-stream farmland. The role of agricultural runoff in the upward shift in $\delta^{34}S$ is supported by the high SO_4 concentrations in the wet season compared to the dry season, which may indicate fresh deposition of large volumes of these enriched sulfur-bearing chemicals into the stream following recent rains.

Below Buffelspoort Dam, there is a sharp drop in SO_4 concentrations, and it would be expected to also observe a sharp reduction in $\delta^{34}S$. This would be due to the likely presence of anaerobic sulfur-oxidizing and/or sulfate-reducing bacteria forming a key basal resource emanating from the profundal zone of the reservoir, where they would form the base of the food web in the anaerobic sediments of the lakebed (Doi et al., 2006). The presence of these chemoautotrophic bacteria, including methane-oxidizing bacteria, has been reflected in the dry season food web (September/October), whereby reduced $\delta^{13}C$ values were observed in the tissues of animal consumers at STK4 and STK5. However, we only see a moderate depletion in $\delta^{34}S$ in the dry season and a corresponding slight enrichment of $\delta^{34}S$ in the wet season, indicating such anaerobic processes are not affecting sulfur isotopes in the same way that they affect carbon in the food web.

As the Sterkstroom accumulates effluent from Tharisa Mine, and the town of Marikana, $\delta^{34}S$ enrichment remained relatively stable at STK6, although wet season runoff from the mine does appear to lower the $\delta^{34}S$ in the food web by a small amount. The sulfur isotopes in fish tissue then displayed a sharp reduction at STK7 in the wet season; downstream of the confluence with a tributary draining a platinum pollution control dam (PCD). This sharp reduction in sulfur isotope enrichment, downstream of platinum mine tailings, is contradictory to the findings of previous studies that assessed effects of coal mine (Corson, 2017) and potash mine (Otero and Soler, 2002) tailings on river food webs, which both reported elevated $\delta^{34}S$ downstream of the impact. As far as we are aware this is the first study to trace platinum-tailings-derived sulfur isotopes in aquatic consumer tissues, so there is no prior research with which to directly compare these findings. It is notable that there is a clear difference between the effect of the open cast mining operations at Tharisa Platinum Mine, which is understood to minimize direct dumping of mine effluent into the stream through its environmental management protocols, compared to the severe change observed when a tributary directly draining an uncontained PCD enters the stream.

Another noteworthy aspect of the depleted sulfur entering the food web above STK7, is that it is matched by nearly identical concentrations of SO_4 in both seasons, even though the flows were significantly higher in the wet season (Figure 3). This pattern, combined with the minimal depletion of sulfur seen in the food web here in the dry season, suggests that the wet season flows are bringing a large quantity of depleted sulfur into the site from the nearby PCD tributary, which is overriding the $\delta^{34}S$ signal coming from the urban settlement upstream, and overriding any dilution of SO_4 in the main stem that would normally be expected from the elevated flows.

On the Maretlwane River, there is an increase in fish tissue mean $\delta^{34}S$ from the pristine MRT1 to MRT2 below the town of Mooinooi. This enrichment could be derived in part from effluent emanating from the Mooinooi wastewater treatment works, but prior studies suggest wastewater tends not to contain significantly elevated $\delta^{34}S$ relative to the background freshwater ecosystem (Wayland and Hobson, 2001; Morrissey et al. 2013). This proposition is supported by the lack of a major rise in sulfur isotope enrichment at MRT7 in either season, which is downstream of the Wonderkop wastewater treatment works. A more likely source for the $\delta^{34}S$ enrichment at MRT2 includes other urban stormwater runoff sources, such as from an adjacent golf course, which could include both fertilizers and urban petroleum-based oils; the latter of which are known to enrich $\delta^{34}S$ in urban groundwater (Hosono et al., 2009).

The most distinctive step changes observed along the river continuum of the Maretlwane is at MRT4. This site is directly below a commercial chrome mine that was identified to be the source of fine tailings sediment that smothers the benthos for hundreds of meters downstream. Here we see an increase in dissolved sulfate in the water column (especially in the wet season) and a decrease in mean $\delta^{34}S$ in both seasons (Figure 16). The large depletion in mean $\delta^{34}S$ in fish tissues at this site, combined with a rapid wet-season increase in sulfate concentrations in the water column, suggests the mine has a substantial effect on both the water quality (as also seen in the dramatic increase in turbidity at the site; Figure 6) and the food web. Two possible reasons exist for the rapid depletion in $\delta^{34}S$ at MRT4. One is that the animal community has shifted to feeding on sulfur-depleted primary food sources in these fine sediments, such as the chemoautotrophic bacteria whose isotopic signal is visible in the $\delta^{13}C$ at STK4. These could include sulfur-oxidizing bacteria and sulfate-reducing bacteria (Doi et al., 2006), however if the latter taxa were actively driving biogeochemical processes upstream we would expect to see a corresponding decline, or minimal increase in SO_4 concentrations at the site, which is not observed.

A second explanation, which is supported by the simultaneous large increase in SO_4 concentration and large depletion in $\delta\ ^{34}S$, is that the mining operations themselves have introduced a new source of sulfur into the system through their extraction and processing of chromium ore, being the pulverized tailings that are entering the river. Such sediments will add highly fractionated sulfur products with strongly depleted $\delta^{34}S$ into the system due to their abiotic oxidation when first brought into contact with surface air and water (Wang and Zhang, 2019). This trend of low $\delta^{34}S$ in fish and elevated SO_4 in the water column is seen again at MRT7 (Figure 2), where another large platinum PCD has added its tailings effluent to the river. It is also visible in the wet season at the PCD-affected STK7, and to a lesser extent below Tharisa Mine at STK5.

The role of both platinum as well as chrome mine sediments and effluents on lowering the isotopic sulfur signature of the food web is clearly illustrated when mean $\delta^{34}S$ is correlated with proportional land cover of mining activities in the sub-catchments of each of our 15 monitoring sites in both the wet season (Figure 17a) and the dry season (Figure 17b). Here, despite near-pristine sites at the top of the catchment displaying a relatively low natural sulfur isotopic enrichment, overall $\delta^{34}S$ in fish tissue is negatively correlated with mining land cover in both seasons. This trend shows that mines have an overriding negative effect in contrast to other human land uses such as agriculture and urbanization, which appear to broadly, if inconsistently, raise the isotopic sulfur signature of the food web above background levels as they interact with the river. The strength of this correlation in the wet season increases ($r = -0.70$; $p=0.01$) when pristine sites, which have no competing human-derived sources of enriched or depleted sulfur upstream, are excluded from the analysis. This relationship is weaker, but still significant ($r = -0.39$; $p<0.05$) in the dry season (Figure 17b). No other land use categories revealed significant correlations with mean $\delta^{34}S$ across the catchment, further illustrating the overriding effect mining seems to have on the discrimination of sulfur isotopes within vertebrate consumers across the riverscape.

The overriding effect of mine-associated land cover on $\delta^{34}S$ depletion in food webs in the wet season reiterates the important role that precipitation-based surface runoff, as well as potential intentional or unintentional dewatering of pollution control dams into tributary channels that then join the main streams of the catchment, appear to play in transporting highly depleted mine-derived sulfur sources into these streams. The lack of dilution of SO_4 at sites like STK7 in the wet season (Figure 15), despite the presumed dilution effect that higher flows (Figure 3) ought to have on sulfate concentrations, indicates that very large quantities of mining derived sulfur are entering the food webs following these periods of high runoff.

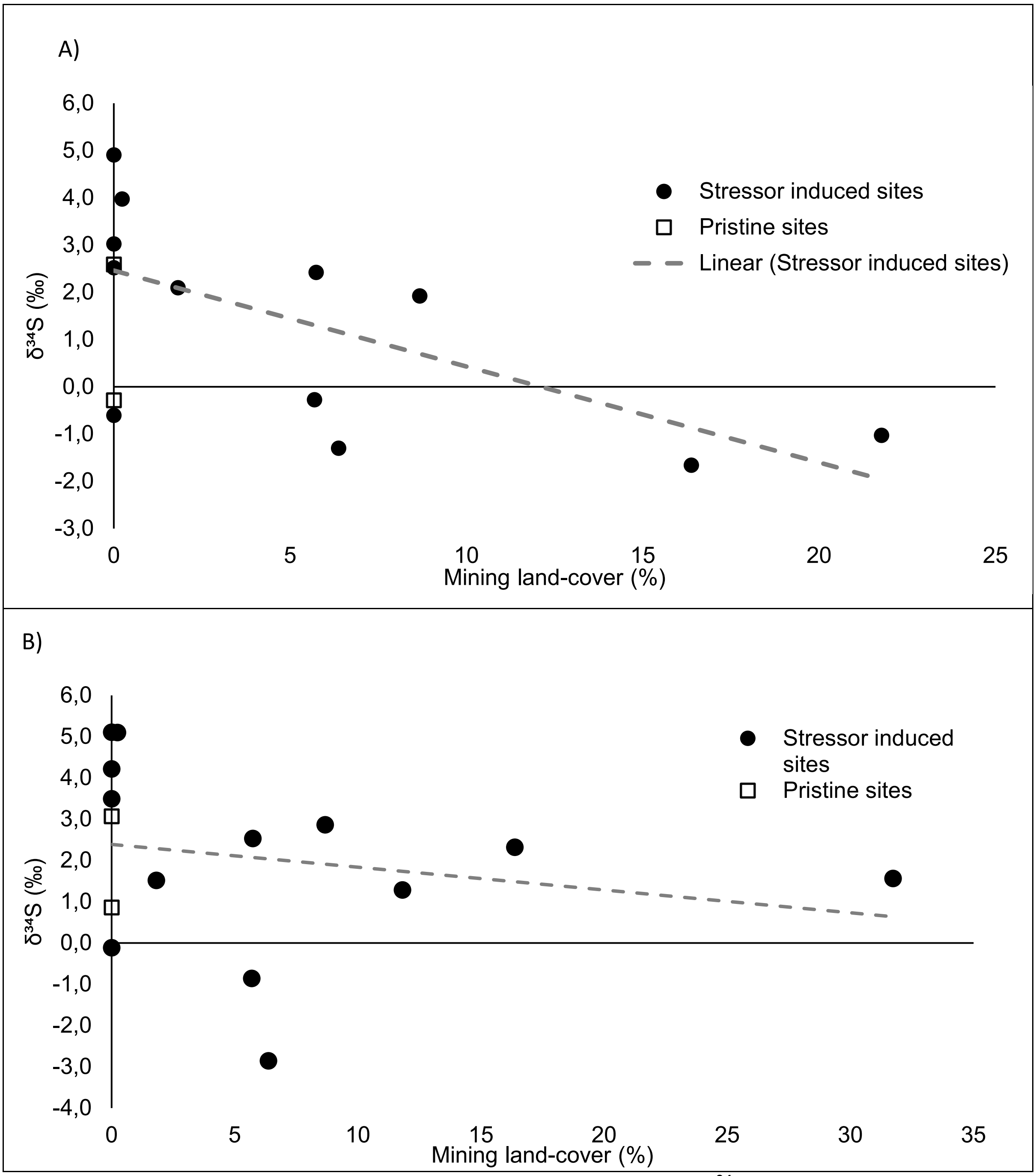


Figure 17: Scatterplots showing the relationship between mean $\delta^{34}S$ in fish tissue per site and percentage mining land cover in site-derived sub-catchments of the Gwathle River quaternary catchment in A) the wet season (significant) and B) the dry season (non-significant). The trend lines show the relationship excluding pristine sites, where no anthropogenic land cover exists in the respective sub-catchments.

The final step change observable in the catchment is at the mainstem Gwathle River site below the confluence of the Sterkstroom and Maretlwane Rivers (GWT1). Here we see an increase in mean fish tissue $\delta^{34}S$ relative to both tributary sites immediately upstream in the wet season, although these levels are stable or slightly depleted in the dry season (Figures 15 and 16). There are no further large mine tailings in the interceding region of the riverscape, but large tracts of irrigated and cattle rangeland, where agricultural sources of isotopically enriched sulfur may once more have an opportunity to enter the food web, particularly due to precipitation-derived surface runoff.

5.2 Relative efficacy of C, N and S in discriminating between stressors

To assess the relative relationships between stable isotopes of carbon, nitrogen and sulfur across the multi-stressor riverscape of the Gwathle River, and their contrasting abilities to trace and discriminate between different anthropogenic sources of water quality stress across the riverscape, a principal component analysis was performed on the dry season SI datasets as well as proportional land cover for our main human land uses, and the dry season measurements of major water quality tracers most closely associated with these land uses (Tables 12 and 13). The resulting biplot (Figure 18) provides insight into how these different stable isotopes within fish tissues have reacted to the patchwork of human stressors that occur along the river continuum in the dry season.

Stable isotopes of carbon were strongly aligned with the first PCA dimension, which discriminates sites neatly along a gradient of natural to highly modified land cover, with urban build up, agricultural (irrigated and fallow) land and mining all positively associated with this dimension. This indicates a trend from very negative to less negative $\delta^{13}C$ along the land-use intensification gradient, although there was high variation in carbon enrichment among the vertebrate consumers of the two pristine sites (-31.6‰ – 18.7‰; Table 1). This natural variation may be the result of the contrasting vegetative cover in the two sites (with Maretlwane being in bushveld with large canopy forests and Sterkstroom being in grass-dominated Western Bankenveld savanna, resulting in contrasting proportions of C_3/C_4 photosynthesizing plants entering the base of the food web as allochthonous CPOM and FPOM. Nonetheless, the biggest land cover effect on the food web that $\delta^{13}C$ serves to indicate is the effect of large impoundments (negatively correlated with dimension 1) on carbon fractionation (Figure 3), indicated by fish feeding at STK4 below Buffelspoort Dam showing a sharp depletion in the carbon isotope ratio relative to STK3 upstream of the reservoir (Table 16). Thus extremely negative $\delta^{13}C$ values serve as a tracer of anaerobic chemo-metabolism at the base of the food web at sites with high anoxia (in this case, the profundal zone and lake-bottom sediments of the reservoir).

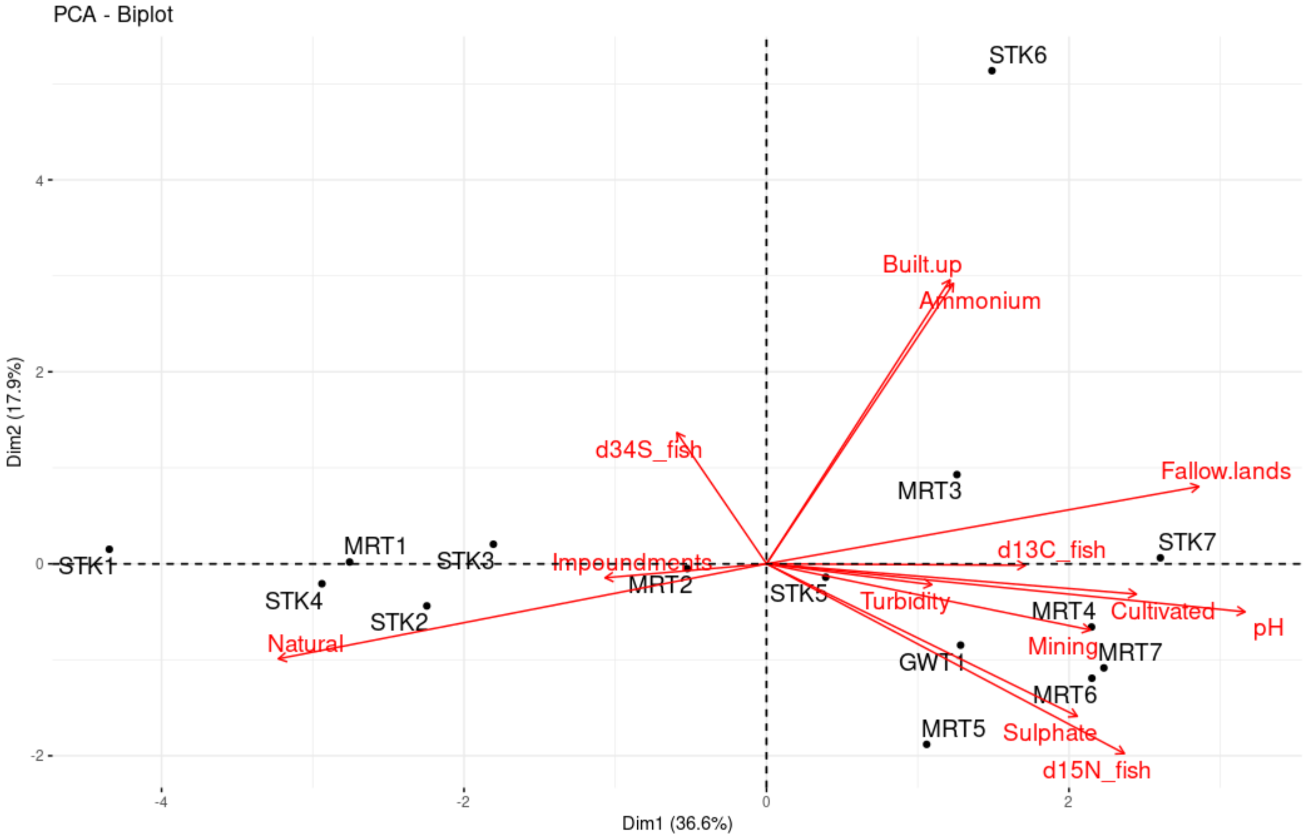


Figure 18: Principal component analysis showing spatial associations between human land cover classes, stable isotope values, and key water quality tracers in the dry season

Table 16: Average fish or tadpole* δ13C and δ15N values from the dry season and as well as δ34S during both dry and wet seasons. Sites are numbered consecutively from upstream to downstream

Site code	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{34}S$ (‰) dry	$\delta^{34}S$ (‰) wet
STK1*	-31.6	6.6	0.9	-0.3
STK2	-22.5	10.8	-0.1	-0.6
STK3	-24.1	12.4	4.2	2.5
STK4	-27.7	13.0	3.5	3.0
STK5	-22.9	16.0	2.9	1.9
STK6	-22.8	6.7	2.5	2.4
STK7	-21.7	20.5	2.3	-1.7
MRT1	-18.7	9.5	3.1	2.6
MRT2	-20.3	14.6	5.1	4.9
MRT3	-19.6	17.3	5.1	4.0
MRT4	-21.7	17.1	-0.9	-0.3
MRT5	-22.7	19.9	-2.8	-1.3
MRT7	-22.6	16.0	1.5	-1.0
GWT1	-18.4	18.2	1.3	2.1

Enrichment of stable isotopes of nitrogen was positively associated with Dimension 1 of the PCA, indicating general increases in $\delta^{15}N$ along the land-use intensification gradient, although this was not a uniform trend that could be linked to significant associations with any one nitrogen product in the water column (Figure 12). The PCA also shows a negative association of $\delta^{15}N$ with Dimension 2, which discriminates between urban built-up land cover and mining-dominated land cover and their associated water quality tracers. Enrichment of $\delta^{15}N$ is more closely associated with mining (also associated with elevated sulfate concentrations in water) than urban activities (closely associated with ammonium, a key indicator of untreated sewage contamination; Choi et al., 2003). Part of this negative association with urban land could be driven by the extreme outlier of STK6, located in the upper right quadrant of the PCA, where extremely unusual biochemical activity at the site has resulted in a drastic depletion of $\delta^{15}N$ relative to STK5 upstream, which then quickly recovers at the next downstream site STK7 (Table 16). There is circumstantial evidence that illicit dumping of chemical reagents involved in the refinement of artisanal mined chromium might cause a severe, if spatio-temporally short-lived depletion in $\delta^{15}N$, which was quickly absorbed into the food web and overwhelmed by further inputs from both agriculture and commercial mining operations. These exotic chemical reactions are not reflected in either $\delta^{13}C$ or $\delta^{34}S$ enrichment levels. This aberration in the $\delta^{15}N$ data may have masked the overall positive relationship between nitrogen stable isotope enrichment and urbanization in our study catchment, as the link has been clearly demonstrated both in South Africa (Motitsoe et al., 2020) and other parts of the world (Mayer et al., 2002; Huang et al., 2018). Treated sewage, together with urban runoff, may in-part contribute towards energy sources that elevate fish tissue $\delta^{15}N$ signatures as well as slightly raise $\delta^{34}S$ values, whereas other urban runoff such as petroleum-based oils likely play a larger role in enriching $\delta^{34}S$ below urban settlements (Hosono et al., 2009).

Agricultural sites (characterized by irrigated and fallow lands in their sub-catchment, both positively correlated with Dimension 1) showed elevations in both $\delta^{15}N$ and $\delta^{34}S$, most likely attributed to the use of sulfate- and nitrate-enriched fertilizers and/or pesticides on surrounding crop land. Nonetheless, enrichment of $\delta^{34}S$ was more strongly aligned to Dimension 2 in a tight negative relationship with sulfates and mining land cover (Figure 18; see also Figure 17).

Following the input of platinum-based PCD runoff into the investigated rivers in the wet season, it appears that such stressors lead to strongly depleted $\delta^{34}S$ values in the fish tissue, although this was far less pronounced in the dry season (Table 16; Figures 15 and 16). Platinum effluent, characterizing the sites STK7 and MRT7, appear to seasonally deplete $\delta^{34}S$ and inconsistently elevate $\delta^{15}N$, whereas commercial chrome mine tailings (at MRT4) strongly depleted $\delta^{34}S$ levels in fish tissue in both seasons, without affecting $\delta^{15}N$ levels in the dry season (Table 16). The role of anaerobic sulfur-oxidizing and/or sulfate-reducing bacteria in oxygen-depleted sediments, whether in the profundal zone of large impoundments or in the thick accumulated tailings sediments of mines, may play a role shifting the base of aquatic food webs, by reducing isotopic enrichment of both carbon and sulfur. Interestingly, strongly depleted $\delta^{13}C$ (a relative depletion of 3.6‰) is observed downstream of the large reservoir in the dry season, when the profundal zone is likely to reach maximum hypoxia, but not in the river sediments below the chrome mine in the dry season, where only $\delta^{34}S$ was strongly depleted. This suggests a larger role of abiotic oxidation of sulfates emanating from the pulverized rock than anoxic bacterial processes being responsible for the sharp declines of $\delta^{34}S$ in concert with the sharp increases in unearthed and dissolved sulfate in the water column. A bedrock coring study on the platinum group metal-bearing rock of the Rustenburg Layered Suite within our study catchment showed the unoxidized source rock to have a $\delta^{34}S$ enrichment of 1.68‰ (Magalhães et al., 2018), which is a relatively low initial value from which oxidation during ore extraction would proceed to further deplete towards the negative values recorded in fish tissue at STK7, and between MRT5 and MRT7 in the wet season (Table 16), when sediment-rich runoff from mine dumps and PCDs is presumed to peak. It is noteworthy that at MRT7 $\delta^{34}S$ became slightly enriched in the wet season and highly enriched in the dry season relative to MRT5, which suggests confounding effects of contrasting sulfur sources (PCD effluent and urban runoff) on the sulfur enrichment of the local food web at the downstream site, with the urban signal potentially overriding the mine signal at dry season flows.

The role of seasonality in driving the relative dominance of different species of chemoautotrophic bacteria at the base of food webs in multi-user catchments could be an additional critical source of evidence in allowing us to understand and therefore discriminate between different anthropogenic stressors and their effects on riverine food webs. This is especially true in terms of comparing the two main on-channel impoundments in the system, namely the deep mesotrophic Buffelspoort Dam between STK3 and SKT4 on the Sterksroom, and the unnamed shallow eutrophic dam between MRT4 and MRT5 on the Maretlwane (see Appendix B for images of these water bodies). There is clear evidence from the dry season STK4 data for the presence of chemoautotrophic bacterial processes in the sharp reduction of $\delta^{13}C$, but this was not replicated below the shallow dam above MRT5 in the dry season, nor observed in the $\delta^{34}S$ data at STK4 in the wet season (although a relative depletion of 0.7‰ was observed in the dry season, when anoxic bacterial processes in the shallow dam may have become elevated). While we did not receive the full suite of seasonal $\delta^{13}C$ in time to interrogate these seasonal patterns in this report, this analysis will be a key objective of subsequent publications based on this dataset.

6. CONCLUSIONS

This project set out to determine whether three stable isotopes ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) in the tissues of key consumers in an aquatic food web could be used in concert with more traditional water quality tracers such as nutrients and heavy metals to detect large changes in river ecosystem health and discriminate between different anthropogenic sources of ecohydrological stress. Even without securing access to a full seasonal suite of carbon and nitrogen stable isotopes within the timeframe of this project, we have demonstrated some consistent trends in the responses of these stable isotopes to key human land use types (agriculture, urban sprawl and mining) within a single multi-user quaternary catchment. These responses indicate that, when used in tandem with dissolved nutrient and heavy metal tracers, tissue-derived stable isotopes have the potential to discriminate between different water quality stressors, and provide a layer of information on the ecosystem's response and resilience to those stressors above and beyond what either water quality data or rapid bioassessment data are capable of providing.

The Gwathle River quaternary catchment turned out to be an excellent model system for carrying out such a study, because of its wide range of pristine, to moderately modified, to critically modified sampling sites. The river ecosystem revealed levels of degradation, particularly at sites affected by various mining operations, that were surprisingly severe, and some of our water quality results are likely to rank this system among the most dramatically degraded river catchments recorded worldwide. This project, therefore, also serves a purpose to raise the alarm as to the perilous state of some of our headwater streams, which flow through some of the most important industrialised landscapes for South Africa's economy. Mining, particularly of platinum group metals, is considered one of the most critical long-term contributors to the nation's gross domestic product (Antin, 2013), and yet this study reveals that the cost inflicted by this industry on the ecosystem functioning of our rivers, and their ability to provide important ecosystem services, could be proportionately high.

The major outcomes and implications of this study for distinguishing between the effects of anthropogenic stressors on ecohydrology in multi-user catchments are presented below in the form of a colour-coded table (Table 2). This table summarizes the key trends observed in the stable isotope, nutrient and heavy metal tracer data that were analysed within the timeframe of this project.

Table 17: Conceptual summary of stable isotope and traditional water quality tracer responses to different human land uses, which can be used together to discriminate between different human activity-derived sources of eco-hydrological stress. Cool colour cells indicate consistent negative tracer responses, warm colour cells indicate consistent positive tracer responses and grey cells indicate negligible or variable tracer responses.

Land cover type	$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$	Water quality
Natural	Initial values reflect riparian mix of grassland vs. bushveld (C_3/C_4)	Initial values reflect trophic enrichment relative to primary producer baseline (close to 0‰), but rises downstream due to biotic mineralization of allochthonous nitrogen sources	Initial values driven by atmospheric deposition and leaching of sulfates from surface rocks	No major physicochemical tracers present
Agriculture	Small response due to changes in riparian zone vegetation	Small isotope enrichment due to autochthonous production driven by added nitrates from agriculture	Large isotope enrichment due to sulfates from fertilizers and pesticides	Small increases in pH, nitrates, phosphates, sulfates
Urban	No major shifts in response to stressor	Small isotope response due to addition of organic particulates from treated and untreated urban wastewater	Small isotope enrichment due to urban stormwater runoff (fertilizers and petroleum-based oils)	Large increases in pH, nitrates, ammonium, phosphates and sulfates from wastewater; some heavy metals (Al, Mn) from urban stormwater
Mining (chrome and platinum)	No major shifts in response to stressor	Possible strong response (depletion or enrichment) due diverse nitrogen sources emanating from mine tailings or effluents, as well as unregulated artisanal mining activities (possible chemical dumping)	Large isotope depletion due to addition of abiotically oxidized and biotically reduced sulfates brought to the surface from underground	pH variable depending on mining type, small increases in phosphates, large increases in sulfates and heavy metals (Fe, Al, Mn, Cu)
Impoundments	Large isotope depletion due to anoxic chemoautotrophic bacteria reductive processes in profundal zone	Potentially strong denitrification-driven enrichment via eutrophication processes related to allochthonous N sources (weak enrichment observed in this study)	Isotope depletion downstream due to anoxic chemoautotrophic bacteria reductive processes in profundal zone	Declines in turbidity, nitrates, phosphates and sulfates due to being trapped in sediments inside the reservoir

The summary table has been designed to illustrate through colour-coded cells how a combination of observed major and minor stable isotope enrichment or depletion in aquatic vertebrate tissues and concentrations of water quality tracers in the water column, can together enhance each other's interpretive power in distinguishing the primary anthropogenic source of ecohydrological stress affecting the local food web, and by extension the local health of the river and its viability as a source of ecosystem services.

6.1 Implications of this research

The implications of our findings, as summarised in Table 2, for determining the key drivers of water quality and ecosystem change in the Gwathle River quaternary catchment, and their implications for water resource management here and elsewhere, are summarized below:

- The impacts of agriculture vs urbanization on aquatic ecosystems can be distinguished by comparing the concentrations and species of heavy metal pollutants in the water, as well as the degree of enrichment of $\delta^{34}S$ isotopes in consumer tissue relative to background levels. The findings suggest urban effluents tend to have more metals but are less isotopically enriched in sulfur products compared to agricultural runoff (even though sulfates are likely present in greater quantities in urban than in agricultural runoff).

- The impacts of agriculture vs mining are respectively distinguished by low-to-moderate SO_4 concentrations and highly enriched $\delta^{34}S$ in consumer tissue (agriculture) vs high-to-very high SO_4 concentrations and highly depleted $\delta^{34}S$ in consumer tissue (mining).

- The impacts of urbanization vs mining can be distinguished by the concentrations and species of heavy metal pollutants in the water, as well as whether the $\delta^{34}S$ isotopes in consumer tissue are enriched (urban) or depleted (mining) relative to background levels.

- Large impoundments can play a significant resetting role along the river continuum, by trapping sediments and associated nutrients and chemical pollutants in its profundal zone, blocking their passage further downstream, resetting the food web in the case of deep dams due to anoxic chemoautotrophic processes depleting the carbon baseline, and in the case of eutrophic dams raising the nitrogen baseline in downstream food webs.

- Mines that have good wastewater effluent management vs those with poor wastewater management can be distinguished both by traditional physicochemical tracers (turbidity, heavy metal pollution) as well as the degree of $\delta^{34}S$ depletion in the food web of the water body receiving the effluent (particularly in the wet season, when uncontained runoff from mine dumps and effluent from PCDs is more often transported into the river, and at higher volumes).

- Transient chemical events based on illicit mining activities that utilize highly reactive nitrogen can be potentially captured in the tissues of consumers and preserved within the local food web (e.g. unusually high depletion of $\delta^{15}N$ in fish tissue) for some time after the chemicals themselves have been denatured, decomposed or diluted beyond detection by the local river ecosystem.

- Seasonality of rainfall plays a major role in the volumes of nutrients entering a stream from the landscape, and while the dry season is often acknowledged as the time of greatest risk to aquatic ecosystems due to the concentration of pollutants at low flows, this study showed that the wet season can facilitate just as detrimental impacts on aquatic food webs due to the significant increase of pollutant loads being added to the system via runoff from the surrounding catchment. The role of degraded riparian zones in these human-modified landscapes in failing to buffer streams against these anthropogenic inputs is a subject for further scientific enquiry.

- The relative trends in stable isotopes nutrient and metal tracers observed in this study (summarized in Table 17) should be transferable to other multi-user river catchments across the country, though caution should be employed if those landscapes contain different forms of mining such as gold or coal. Coal, given it is a carbon-based resource, is likely to have a significantly different effect on $\delta^{34}S$ and $\delta^{13}C$ compared to platinum group metals, and thus will need separate studies to characterize its signature in the food web and relative association with other stress tracers like conductivity and sulfate concentrations.

6.2 Open questions and future research needs

- The role of anoxic chemoautotrophic bacteria in moderating the fractionation of carbon, nitrogen and sulfur isotopes has been flagged several times in this study, and a more robust understanding of the taxonomic makeup of bacterial communities in the impoundments, wetlands and riparian sediments of this study system, would greatly improve our ability to isolate the biogeochemical processes contributing to the major shifts in stable isotope fractionation and enrichment that were observed in this study.

- The relative contributions of groundwater nitrogen and sulfates in the future should be considered in a separate study. This would provide clarity on the specific contributions of background contributions following different stresses on the aquatic ecosystems.

- Although beyond the scope of this study, a key element that could reinforce the observed relationships between anthropogenic stressors and isotopic shifts in the food would be the acquisition of baseline isotope ratio data from the individual sources of environmental stress. These would include urban runoff, wastewater effluent, chrome mine tailings, raw platinum mine tailings, as well as the biogeochemically transformed platinum tailings emanating from pollution control dams, or wetlands through which these liquid tailings subsequently flow. An estimation of the isotopic character of key environmental inputs like sulfides entering the river through dry or wet atmospheric deposition would also strengthen both isotopic and chemical mass balance calculations for the catchment. Finally, examples of the pre- and post-oxidation isotopic signatures of chromium and plantinum-bearing ores being dug up by the mining operations in this catchment would greatly enhance our ability to discriminate between the effects of different modes of mining on the food web.

- Our research has uncovered evidence of both illegal and grossly non-compliant environmental practices (in terms of South African environmental law) by both commercial and artisanal mining operations in the Gwathle River catchment. This study serves to highlight the apparent failure of governance in the regulation of these commercial mining operations, as well as an absence of law enforcement in allowing the kind of illicit artisanal mining operations that together appear to have drastically altered the structure and function of food webs at several of our study sites. It is hoped that this research will shine a light on the general failure of environmental legislation to protect river ecosystems in this economically critical industrial region, so that responsible actors within local and national government might seek to intervene to improve the perilous state of these freshwater resources going forward.

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APPENDICES

Appendix A – Supplementary field data for modelling relationships between land use and water quality

Table A1: Seasonal average water quality tracer data recorded from 15 sites and percentage stressor land-cover data calculated for sub-catchments derived from each site.

DO (mg/L)	pH	Turbidity (NTU)	Ortho-phosphate (mg/L-P)	Ammonium (mg/L-N)	Fe (ug/L)	Natural (%)	Artificial water (%)	Permanent & temporary Crops (%)	Fallow lands (%)	Built Up (%)	Mining (%)
6.45	5.64	0.91	0.07	0.08	25.91	100.00	0.00	0.00	0.00	0.00	0.00
5.47	7.14	2.03	0.08	0.12	127.31	89.02	0.09	10.03	0.73	0.14	0.00
7.92	7.46	5.40	0.06	0.15	78.75	83.88	0.02	8.09	0.00	8.01	0.00
6.82	7.59	7.62	0.09	0.15	160.18	88.37	2.30	6.48	1.57	1.29	0.00
8.60	8.64	11.46	0.08	0.09	57.17	62.05	0.00	12.55	12.85	3.87	8.68
6.69	8.24	25.60	0.20	1.32	72.08	31.90	0.24	9.94	21.38	30.87	5.67
6.88	8.51	17.76	0.12	0.43	86.01	42.00	0.05	15.66	23.97	1.98	16.34
7.42	7.36	1.12	0.07	0.07	49.39	100.00	0.00	0.00	0.00	0.00	0.00
6.50	7.96	27.47	0.12	0.34	33.10	76.36	0.02	18.72	2.21	2.69	0.00
7.97	8.53	87.93	0.12	0.09	101.71	50.26	0.00	25.28	8.09	16.14	0.24
7.31	8.96	490.42	0.18	0.57	233.98	53.67	0.01	26.20	12.17	2.27	5.69
7.48	8.51	20.71	0.07	0.10	499.64	74.55	0.42	8.32	10.65	5.33	0.73
6.57	8.69	10.62	0.06	0.08	664.59	33.90	0.17	14.04	11.01	1.77	39.11
8.64	8.81	19.32	0.28	0.14	397.69	49.30	0.00	12.40	24.18	2.28	11.84
7.26	8.94	17.96	0.07	0.10	215.15	60.25	0.33	24.81	8.73	4.16	1.72

Table A2: The reduced collinear physcio-chemical, anion, cation and heavy metal variables averaged per site across sampling regimes from which the AICc stepwise models were derived

Site	Dissolved oxygen (mg/L)	pH (units)	Turbidity (NTU)	Ortho-phosphate (mg-P/L)	Ammonium (mg-N/L)	Fe (µg/L)
STK1	6.45	5.64	0.91	0.07	0.08	25.91
STK2	5.47	7.14	2.03	0.08	0.12	127.31
STK6	7.92	7.46	5.4	0.06	0.15	78.75
STK3	6.82	7.59	7.62	0.09	0.15	160.18
STK4	8.6	8.64	11.46	0.08	0.09	57.17
STK7	6.69	8.24	25.6	0.2	1.32	72.08
STK5	6.88	8.51	17.76	0.12	0.43	86.01
MRT1	7.42	7.36	1.12	0.07	0.07	49.39
MRT3	6.5	7.96	27.47	0.12	0.34	33.1
MRT4	7.97	8.53	87.93	0.12	0.09	101.71
MRT5	7.31	8.96	490.42	0.18	0.57	233.98
MRT6	7.48	8.51	20.71	0.07	0.1	499.64
MRT6-1	6.57	8.69	10.62	0.06	0.08	664.59
MRT7	8.64	8.81	19.32	0.28	0.14	397.69
GWT1	7.26	8.94	17.96	0.07	0.1	215.15

APPENDIX B – Illustrative images of study sites

Plate B1: Satellite overview of STK1

Plate B2: Upstream view of STK1

Plate B3: Satellite overview of STK2. White ellipse denotes invasive canopy wetland.

Plate B4: Upstream view of STK2

Plate B5: Downstream view of STK2

Plate B6: View of river emerging from poplar/ eucalyptus invaded wetland upstream of STK2

Plate B7: Satellite overview of STK3

Plate B8: Upstream view of STK3

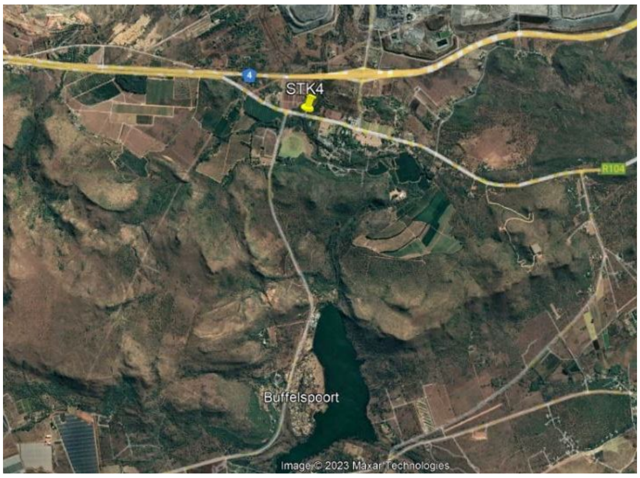

Plate B9: Satellite overview of STK4. Buffelspoort reservoir is visible south (upstream) of the site.

Plate B10: Upstream view of STK4

Plate B11: Downstream view of STK4

Plate B12: View of Buffelspoort Dam wall upstream of STK4

Plate B13: Satellite overview of STK5. Mine dumps associated with Tharisa Mine are visible south (upstream) of the site.

Plate B14: Upstream view of STK5

Plate B15: Satellite overview of STK6. Ellipses denote evidence of artisanal ore mining in the vicinity of the site.

Plate B16: Upstream view of STK6

Plate B17: Downstream view of STK6

Plate B18: View of livestock ford at STK6 (upstream limit of sampling), a possible entry point for the illicit disposal of ore processing reagents

Plate B19: Satellite overview of STK7. A tributary draining several mine dumps and PCDs in the bottom left of the image joins the Sterkstroom between STK6 and STK7

Plate B20: Upstream view of STK7

Plate B20: Satellite overview of MRT1

Plate B21: Downstream view of MRT1

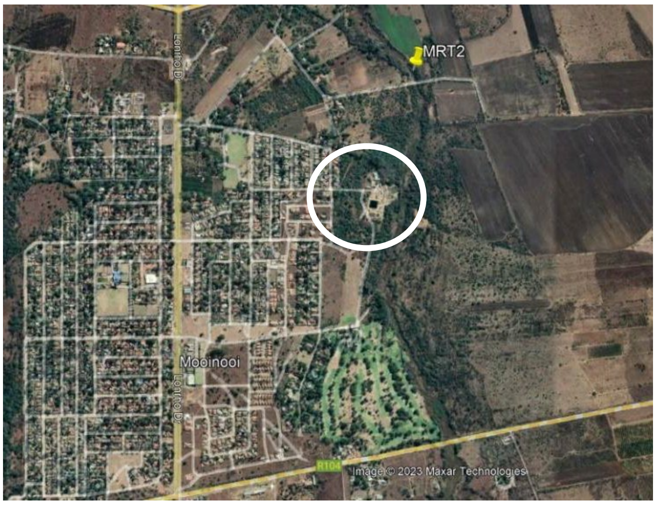

Plate B22: Satellite overview of MRT2. White ellipse denotes the Mooinooi wastewater treatment works.

Plate B23: Upstream view of MRT2

Plate B24: Satellite overview of MRT3


Plate B25: Upstream view of MRT3


Plate B26: Downstream view of MRT3


Plate B27: Upstream view of MRT3 with elevated turbidity, indicating potential artisanal mining upstream

Plate B27: Satellite overview of MRT4. A large chrome mine is visible immediately to the south-east of the site.


Plate B28: Upstream view of MRT4

Plate B29: Example of sediment infilling of the river channel exiting the chrome mine property

Plate B30: Example of sediment infilling of the river channel upstream of the sampling site

Plate B31: Satellite overview of MRT5. A large eutrophic reservoir is visible upstream of the site. This may act as a significant sediment trap and source of anaerobic nitrification along the river continuum.

Plate B32: Upstream view of MRT5

Plate B33: Downstream view of MRT5

Plate B34: Example of livestock interaction with the study site

Plate B35: Satellite overview of MRT6. White line indicates tributary draining PCD and wetland.

Plate B36: Upstream view of MRT6

Plate B37: Downstream view of MRT6

Plate B38: View of the stream draining the PCD-associated wetland and before joining the Maretlwane River

Plate B39: Satellite overview of MRT7. The large semi-formal settlement of Wonderkop is visible to the south and west of the site, while another tributary draining a PCD is visible in the south-east.

Plate B54: Upstream view of MRT7, indicating a degraded riparian zone and littering associated with the nearby settlement.

Plate B57: Satellite overview of GWT1

Plate B58: Upstream view of GWT1

Plate B61: Downstream view of GWT1

Plate B60: Large pool upstream of study site