Mapping Nitrogen Loading in Freshwater Systems: Using Aquatic Biota to Trace Nutrients

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Abstract

The majority of river systems in developing countries like South Africa, are found in catchments areas that are densely human populated, therefore are subjected to intense land-use and developmental pressures. Anthropogenic nutrient pollution or the excessive addition of nutrients is one important type of stressors that river systems often experience through intense land-use, which includes poor waste management and agricultural practices. Such events are referred to as the "urban syndrome", were human populations and developmental demands outpace ecosystem services. Traditional measurements of water quality (e.g. physicochemical and micro-nutrient assessments) and biological monitoring (e.g. South African Scoring System 5, SASS5) techniques for assessing ecosystem health have being widely used to reflect the ecological health and status of river systems. However these techniques have a number of challenges associated with their application. SASS5 which is used most prevalently in southern Africa for example, can only be applied in lotic systems, it is habitat dependent and finally (but arguably most importantly) it cannot identify the source of pollution inputs. Recent laboratory studies using stable isotopic ratios ($\delta^{15}N$ and $\delta^{13}C$) of aquatic macrophytes (duckweed: *Spirodela* sp.) have shown successful differentiation between different N-sources and the mapping of temporal and spatial nitrogen dynamics in freshwater systems. Furthermore $\delta^{15}N$ isotopic values of *Spirodela* sp. showed the capability to act as an early warning indicator of eutrophication, before the onset of aquatic ecosystem degradation. Therefore, this study aimed to field test the potential of sewage plume mapping using the stable isotopic values of *Spirodela* sp. and aquatic macroinvertebrates at nine study sites on the Bloukrans-Kowie River and ten study sites on the Bushman-New Year's River systems in the Eastern Cape, South Africa.

Firstly, duckweed plants (of known starting isotopic ratios) were transplanted into greenhouse cages (n = 5) at 19 sites on both river systems. Plants were left to grow for a minimum of 10 days between sampling events, and samples for $\delta^{15}N$ and $\delta^{13}C$ isotopic analysis were collected every month together with on-site physicochemical variables over a period of 13 months. This was done to investigate the ability of duckweed plants to map spatial and temporal nutrient loading in natural systems (i.e. sewage plume mapping). Secondly, comprehensive SASS5 assessments were completed at each study sites, together with water sample collection for micronutrient analysis on quarterly basis in order to compare results of ecosystem health

assessed using standard indices of biological monitoring (SASS5) with the sewage plume mapping technique. Lastly, the $\delta^{15}N$ and $\delta^{13}C$ isotopic values of aquatic macroinvertebrates were also investigated in order to assess their ability to act as additional biological indicators for N-loading. This was completed over four weeks sampling events, during which four identified nutrients biological indicator species of macroinvertebrates (Oligochaeta, Chironomidae, Culicidae and Syrphidae) were collected on weekly basis, at four study sites in the Bloukrans-Kowie River system, which spanned a strong nutrients concentration gradient.

$\delta^{15}N$ isotopic values of both *Spirodela* plants, Oligochaeta and Chironomidae were able to trace environmental N-loading and were also able to identify pollution hotspots over time and space from different catchment land-uses on the Bloukrans-Kowie and Bushman-New Year's River systems. Furthermore, the sewage plume mapping technique using *Spirodela* sp. was able to identify sewerage out-fall and cow manure run-off from dairy farms as the main anthropogenic source of excessive nutrients in both river systems. The stable isotopic results also supported the current existing biological monitoring assessment determined by the SASS5 technique, where sites with SASS scores < 90 also showed $\delta^{15}N$ isotopic values of > 10.00 ‰, both indicating pollution stress. However, stable isotopic values of *Spirodela* sp. provided better resolution on the dynamics of N-loading over time and space. Although Oligochaeta and Chironomidae nitrogen isotopic values showed potential for N-loading mapping in freshwater ecosystems, notably there is still a need for baseline calibrations for aquatic macroinvertebrates, as observed from this study, that macroinvertebrate $\delta^{15}N$ ratios were influenced by body size, life span and dietary resources, while those of *Spirodela* sp. were not.

In conclusion, traditional measurements of water chemistry and aquatic macroinvertebrate biological assessments (SASS5), despite providing indications of pollution stress (i.e. the identification of systems which are largely natural, moderately impaired or largely impaired), and results being time integrated. The SASS5 assessment provided very little resolution on nutrient dynamics and could not identify sources of N-loading. Stable nitrogen and carbon isotopic values of *Spirodela* sp. provided detailed dynamics on N-source, tagging and identifying pollution hot spots, on both a temporal and spatial scale, supporting its utilization for mapping freshwater nutrient dynamics and N-loading events. Therefore it is highly recommended that sewage plume mapping be included as an up-and-coming tool for future monitoring, conservation and management of freshwater ecosystems.

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“Kena le modisa ke tlabe ke hlokang….
Keya ipitsang Jehova, Modimo o phelang….
O satla mpaballa le bophelong bona

O nkisa botaleng, Dijong tse mphelisang
O nkalosa dinokaneng, Metsing a nkgodisang

Dira li ka ntlhoya, Ke sa ja monono
Mohope wa kgaphatseha, Ke dutse ka thabo
O sa tla mpaballa, Ka bophelong bona
Me ke tla hlola ka mehla, Ka ntlong ya Morena……. Amen

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Problem statement

Gyedu-Ababio & van Wyk (2004), Oberholster & Ashton (2008) and Coetzee *et al.* (2014) have illustrated that increased anthropogenic inputs often results in numerous impacts on aquatic ecosystems, including eutrophication, facilitation of invasive aquatic weeds, loss of native aquatic biodiversity and ultimately, to deterioration of freshwater resources. The South African Scoring System (SASS) is a biological monitoring tool for lotic systems that is well-known for ecological health assessments in freshwater ecosystems, pioneered by Chutter (1994) and further revised by Dickens & Graham (2002) to its fifth version, SASS5. There are however, a number of ecological challenges associated with SASS5 application as acknowledged by Dickens & Graham (2002) and Simaika & Samways (2012). The major drawback is that SASS5 only provides "red flag results", meaning it only identifies disturbance (e.g. eutrophication, heavy metals etc.) after ecosystem degradation has taken place. Furthermore it provides no information on the nature of the disturbance and thus makes it challenging to understand and mediate the damage. This is important, especially for arid and semi-arid countries on the African continent where natural freshwater resources cannot meet the high economic demands (Gyedu-Ababio & van Wyk 2004). Therefore there is a need for a new technique(s) which can identify eutrophication, before the onset of degradation (Vander Zanden *et al.* 2005, Coetzee & Hill 2012, Hill *et al.* 2012, Coetzee *et al.* 2014). Stable isotopic analysis has been identified as a potentially powerful tool for tracing nutrient loading, identifying nutrient sources and their dynamics in freshwater ecosystems (see Costanzo *et al.* 2001; 2005, Rabalais 2002, Kellman & Hillaire-Marcel 2003, Anderson & Cabana 2005, Deutsch & Voss 2006, Oczkowski *et al.* 2008, Hill *et al.* 2012).

1.2 Freshwater ecosystems

The world's total water supply is about 1 385.9 million cubic kilometers, of that, 95.50% is salt water (in the ocean), 0.07% is found as saline lakes and 0.93% is saline ground-water. Only 2.50% of the global total is freshwater, with 68.60% of it locked up as glaciers and ice caps, 30.10% found as groundwater, with only 1.30% available as surface water as ice and snow, freshwater lakes, soil moisture, swamps and marshes, rivers, biological and atmospheric water (Shiklomanov 1993). Rivers and lakes constitute a mere 93 100 cubic kilometers of the total world water supply, yet they are regarded as the major source of available freshwater for global human communities. Collectively, freshwater ecosystems (e.g. wetlands, ponds, lakes, rivers and streams) are characterized by in and out flow of water in one direction, unique and changing water chemistry from the river source to the river mouth and provide micro-habitats, for both submerged and emergent aquatic biota (Chapman 1992, Davies & Day 1999). Freshwater resources are considered as interlinked systems, and the river continuum concept describes how these ecosystems from mountain streams, to mid-lands and coastlines are all connected and in some cases are able to influence each other down an altitudinal gradient (Vannote *et al.* 1980). However, despite their connectivity, each freshwater body has independent physical and chemical characteristics, which are driven mainly by ecological variables such as microclimate, geomorphological/geochemical conditions and prevailing land-use within the catchment area (Chapman 1992, Davies & Day 1998; 1999, Hooda *et al.* 2000, Taylor *et al.* 2007). Most of South African waterways are small and the majority of them are found within catchment areas with dense human populations and therefore are subjected to intense anthropogenic activities (Coetzee & Hill 2012), with the most detrimental anthropogenic activity being system eutrophication (Oberholster *et al.* 2009, van Ginkel 2011).

1.3 Nutrient loading (e.g. Eutrophication)

Eutrophication is an increase in accumulation of organic matter which is usually driven by excess supplies of nutrients (e.g. nitrogen and phosphorus) which can often lead to toxic algae blooms, fish kills, excessive plant production and subsequent decay, oxygen depletion and overall reduction in water quality (Nixon 1995, Botes *et al.* 2004, Deutsch & Voss 2006, Oberholster *et al.* 2009, van Ginkel 2011, Hill *et al.* 2012). Such eutrophication events are

considered to be one of the most serious ecological problems facing freshwater ecosystems, on a global scale (de Villiers 2007). Eutrophication can be classified in two different ways, natural eutrophication and cultural eutrophication. Natural eutrophication occurs through natural activities, where the influx of nutrients comes from natural sources such as rocks, soils and other features within the catchment area, changing nutrient levels on gradual scale. This type of eutrophication is an irreversible and uncontrollable process, but its slow rate makes it less harmful to the environment (Botes *et al.* 2004, Oberholster *et al.* 2009, van Ginkel 2011). Cultural eutrophication on the other hand, is controllable and considered to be the most important and detrimental type of eutrophication from an environmental health perspective. It is predominantly driven by anthropogenic impacts such as poor waste management (urban and rural wastes) and for decades it was responsible for the degradation of majority of South African waterways (Botes *et al.* 2004, Coetzee & Hill 2012). The major consequences of ecosystem degradation include the death or local extinction of keystone species, due to drastic habitat modifications (Rosenberg *et al.* 1986, Johnson *et al.* 2006), which may result in cascading effects to ecosystem structure and functioning (Speight *et al.* 2008). Under intense eutrophication conditions, Chorus & Bartram (1999) noted that secondary effects may also manifest to such an extent that in some cases it may lead to cyanobacteria blooms (blue-green algae, e.g. *Microcystis)* and the production of cyanotoxins. Pouria *et al.* (1998) and Rabalais (2002) reported ingestion and/or recreational use of water containing cynatoxins pose threats to both human and animal health. This was also reported by Oberholster *et al.* (2009) in Kruger National Park, South Africa, where 50 captive animals died due to exposure to cyanotoxin contaminated water resulting from highly eutrophic water flowing through the park from a severely disturbed upstream catchment.

1.4 Biological monitoring and indicators

The composition of aquatic communities in freshwater ecosystems is determined by how well a species can cope with environmental variables within its niche and also its capacity to tolerate disturbance (Rosenberg & Resh 1993, Resh 2008, Speight *et al.* 2008). For example, biotic integrity has been defined as "the ability to support and maintain a balanced, integrated, adaptive community of organisms, having a full range of elements (genes, species and

assemblages) and processes (mutation, demography, biotic interactions, nutrients, energy dynamics and meta-population process) expected in the natural habitat" according to Karr (1996). Thus an ecosystem with high biotic integrity constitutes a healthier habitat that supports indigenous aquatic faunal and floral communities, promotes aquatic biodiversity, ecosystem services (e.g. good water quality, food security) and brings about sustainability of natural resources with an environment. While systems with low biotic integrity, promote water borne diseases and result in poor/no ecosystem services and ultimately the collapse of the ecosystem (Karr 1996). McGeoch (1998) defines biological indicators as "a species or group of species that readily reflects the abiotic and/or biotic state of an environment, represents the impact of environmental change on a habitat, community, or ecosystem, or is indicative of the diversity of a subset of taxa, or of the wholesale diversity, within an area". A number of authors (e.g. Adams *et al.* 1989, Kwandrans *et al.* 1998, McGeoch 1998, Chapman 1992, Chutter 1994; 1998, Kleynhans 1999, Smith *et al*. 1999, Wright *et al.* 2000, Hodkinson & Jackson 2005, Dickens & Graham 2002, Speight *et al.* 2008, Bredenhand & Samways 2009, Simaika & Samways 2012) have illustrated that aquatic communities have the ability to respond to ecological integrity and that their ecological behavior reflects differing external stressors over time, thus providing a broad calculation of the aggregated impacts of disturbance. Such ecological measures of fluctuating environmental conditions are available by quantifying and monitoring biological communities in a given habitat, and are thus reliable and time integrated. A biological monitoring programme called the Aquatic Ecosystem Health Monitoring Programme (NAEHMP): National River Health Programme (NRHP) was established in southern Africa (https://www.dwa.gov.za/iwqs/rhp/index.html). This was the initiative of the Department of Water Affairs and Forestry, together with other research institutions including the Water Research Commission (https://www.wrc.org.za/), the Council of Scientific Institution of Research (https://www.csir.co.za/) and the Department of Water and Environmental Affairs (https://www.environment.gov.za/) (DWAF 2008). The NAEHMP-NRHP uses riverine and riparian biota together with riverine habitat status to assess the water quality and habitat integrity of southern African rivers and streams. The ultimate goal of the NRHP was to provide meaningful and accurate data, on both the water quality and overall condition of freshwater resources to be later used as the basis of management decisions (Taylor *et al.* 2007). Notably, the programme showcased algae, aquatic macroinvertebrates and fish as reliable biological

indicators because of their sensitivity to changes in water quality and habitat integrity (Chutter 1994, Dickens & Graham 2002, Li *et al.* 2010).

The characteristics of an ideal biological indicator include: (a) ease of identification (easy to spot by non-specialists); (b) a wide distribution; (c) low mobility (thus representative of local and small scale regions); (d) well known ecological characteristics; (e) high abundances; (f) suitability for laboratory work; (g) high sensitivity to environmental change; and (h) capability for standardization and quantification (Markert *et al.* 2003). Numerous investigations have identified a variety of biological indicator organisms for use in South Africa, including diatoms, fish, aquatic macroinvertebrates and plants (Dallas 1997).

1.4.1 Diatoms

Diatoms form the largest component of aquatic communities and are found attached to various substrates in aquatic ecosystems (Dokulil *et al.* 1997). Like all aquatic biota, these localized unicellular communities are directly impacted by any chemical and/or physical changes in the surrounding water column, and they have been used as indicators of water quality (Taylor *et al.* 2005; 2007, Beyene *et al.* 2009). Diatom indices were first developed and tested in European countries as a potential index for freshwater biological assessment (Taylor *et al.* 2005), and gained favor and momentum in other developed countries, particularly in the United States of America (Dokulil *et al.* 1997, Kwandrans *et al.* 1998, Eloranta & Soininen 2002). According to Taylor *et al.* (2007), these successful developments were due to demands from the Urban Wastewater Directives in Europe, who had the goal of reinforcing their legislature, which then led to a call for reliable water quality indicators. Only later, was the diatom index introduced and applied in southern African river systems (de la Rey *et al.* 2004). However, with limited baseline studies on native southern African diatom species and the inclusion of diatoms species endemic to South Africa in the existing European indices, experts were worried about inaccuracies and mistakes in the calculation of diatom indices, and subsequent incorrect evaluations of water quality and interpretation (Taylor *et al.* 2005; 2007). Simaika & Samways (2012) further argued that diatoms are challenging in terms of identification and they also react rapidly to nutrient inputs either by blooming or death, therefore making the situation challenging to trace or detect pollution events over longer time intervals. Li *et al.* (2010) also suggested that diatom indices in lotic systems do not accurately reflect integrated environmental changes and/or long-term

sustainability of the river ecosystem because immediate changes in hydrology will also affect diatoms communities and complicate interpretation. Moreover, South African diatoms still lack taxonomical information and ecological characteristics, all of which make them unreliable biological indicators of water quality based on Markert *et al.* (2003)'s ideal characteristics.

1.4.2 Aquatic Macroinvertebrates

Arthropods are regarded as excellent biological indicators for both terrestrial and aquatic habitats (Rosenberg *et al.* 1986, McGeoch 1998, Markert *et al.* 2003, Hodkinson & Jackson 2005). According to Wilhm & Dorris (1968), the original use of the term "biological indicator" first appeared in reference to aquatic ecosystems, where it was used for the detection and monitoring of aquatic biota to describe changes from the external environment. Substantial developments of techniques using aquatic macroinvertebrates for the assessment of water quality has only occurred in recent years and includes: the British Monitoring Water Party System (BMWP), United Kingdom (Hawkes 1998), Australian River Assessment Scheme (AUSRIVAS), Australia (Smith *et al.* 1999), River InVertebrate Prediction and Classification System (RIVPACS), United Kingdom (Wright *et al.* 2000) and the Index of Biotic Integrity (IBI), USA (Karr 1991, Kerans & Karr 1994). Aquatic macroinvertebrates are regarded ideal habitat health and water quality indicators because they are highly diverse, occupy almost every possible ecological niche (both terrestrial and aquatic) and show measurable responses to habitat disturbances and/or modifications (see Rosenberg *et al.* 1986, Karr 1991, Chapman 1992, Rosenberg & Resh 1993, Hawkes 1998, McGeoch 1998, Smith *et al.* 1999, Wright *et al.* 2000, Hodkinson & Jackson 2005, Johnson *et al.* 2006, McGeoch 2007, Bredenhand & Samways 2009, Masese *et al.* 2009).

The importance of South Africa's freshwater resources has driven the development of a country specific rapid biological monitoring tool called the South African Scoring System (SASS5; Chutter 1994) for riverine ecosystems. The main objective of SASS5 was to assess river health and water quality, and further investigation showed that the SASS5 tool can also be used for; (1) the assessment of the ecological state of aquatic ecosystems; (2) the assessment of spatial and temporal trends in ecological states; (3) assessing emerging pollution problems; (4) setting management objectives for rivers; (5) assessing the impact of anthropogenic developments; (6) predicting changes in ecosystems due to developments; and (7) contributing to

the determination of Ecological Reserves (Roux 1999). The SASS5 technique has been applied successfully in a number of riverine ecosystem studies (Dickens & Graham 2002, Simaika & Samways 2012), however its application has met a number of ecological drawbacks. Firstly, SASS5 only provides "red-flag results", identifying problems only after ecosystem level changes have taken place. Secondly, SASS5 results cannot point out the type of pollution/disturbance, providing information solely on whether or not a system is impacted and provides only a very basic indication of the level of impact. Thirdly, the majority of macroinvertebrate indices work only in lotic systems, thus the assessment of impoundments, wetlands and lakes are excluded. SASS5 is also field work intensive, and it is habitat dependent meaning the sampling site selection is ecologically biased. Not only that, but it requires intensive training unlike the simplest version, mini-SASS (Simaika & Samways 2012). Although SASS5 is currently the most commonly used rapid biological assessment tool and has been successfully implemented in a number of South African fluvial ecosystems, there is a strong need for a biological monitoring tool that can be applied in a bigger range of aquatic ecosystems, which is not habitat dependent and that will provide information on the source and type of pollution, both over time and space, effectively acting as an early warning system. Such tools will help to identify and trace anthropogenic inputs in aquatic systems before the onset of ecosystem degradation and will help in the management and conservation of South Africa's freshwater ecosystems. One method which has received attention very recently is the application of stable isotopic analysis to trace nutrient loading in aquatic systems and holds some promise as a useful biological monitoring technique.

1.4.3 Vertebrates (e.g. fish)

Fish have also been identified as good biological indicators due to their sensitivity towards habitat alteration (Kleynhans 1999, Pont *et al.* 2007, Roset *et al.* 2007). For example their long life span and top position in the aquatic food web make fish an ideal indicator for heavy metal and/or bioaccumulation studies and bioassays. However Li *et al.* (2012) noted that fish can be affected by multiple external factors that include physical or chemical modifications as well as human exploitation. Using Markert *et al.*'s (2003) criteria, fish fall short of being considered ideal biological indicators because; (1) they have a relatively low numbers of species,

therefore leading to low densities in freshwater systems, making biological monitoring very difficult from statistical perspective; (2) the majority of fish species are highly mobile which makes them able to avoidance/escape pollution events by swimming into less affected areas; (3) they are also not easily sampled, particularly for rapid biological assessments purposes due to their protective legislature; and (4) the majority of South African river systems are impacted by anthropogenic activities, and are thus modified "not-natural" systems. That combined with predominantly shallow waters, means that many fish species are effectively absent from most of the inland waterways where biological monitoring is targeted (Hill *et al.* 2012, Simaika & Samways 2012). Li *et al.* (2010), further argued that fish stress responses are better reflected at a molecular level than at population and community levels, thus it is only at a population level where the effects of disturbance may be manifested through the reduction of recruitment (reproduction). Most successful studies using fish in Europe incorporate fish reference conditions into the application of biotic monitoring indices (e.g. IBI, Index of biotic integrity). This approach requires information on river characterization, descriptions of reference fish assemblages in each river type and a selection of biological attributes for each fish assemblage, to allow the quantification of the difference between observed and reference fish assemblages and can be complicated by fish age and migration (Roset *et al.* 2007). Biotic indices using fish can be further complicated by the impact of invasive species (Kadye 2008). Thus, overall, fish are not ideal organisms for biological monitoring programs, particularly when compared with aquatic macroinvertebrates.

1.5 Stable isotopic analysis (SIA) in ecology

Stable isotopes are naturally occurring, non-radioactive, heavier and lighter forms of the same elements (e.g. ^{12}C and ^{13}C for carbon, ^{14}N and ^{15}N for nitrogen) (Criss 1999). Their mass difference is due to different number of neutrons. For elements of low atomic numbers, this mass difference between the isotopes is often large enough for bonds of the lighter isotope to be broken slightly more easily than equivalent bonds of the heavier isotope. As a result the light isotope reacts faster and become concentrated in the product (relative to the substrate). It is this fractionation (or sometimes lack thereof) that is used to follow the pathways of compounds from sources to sinks. Recently, SIA has been shown to be a useful tool for tracking changes in trophic structure and energy flows in an ecosystem, contributing to the understanding of how

basic ecosystem services may be affected by indigenous and non-indigenous species (Caut *et al.* 2006). Isotopic ratios are conserved up through the food web, with predictable isotopic fractionation at every trophic step (0.5-1 ‰ for $\delta^{13}C$ and 3-4 ‰ for $\delta^{15}N$; DeNiro & Epstein 1978; 1981, Fry & Sherr 1984, Post 2002, McCutchan *et al.* 2003). As such, stable carbon ($\delta^{13}C$; information on food resources) and nitrogen ($\delta^{15}N$; information on trophic position) isotopic ratios can provide time-integrated information about feeding relationships and energy flow (e.g. Peterson & Fry 1987, Cabana & Rasmussen 1996, Vander Zanden & Rasmussen 1999, Martinez del Rio *et al.* 2009). Thus, $\delta^{13}C$ and $\delta^{15}N$ isotopic values can be used to draw food web maps and conceptualize trophic niches within communities and habitats because they vary both temporally and spatially (Bearhop *et al.* 2004, Layman *et al.* 2007, Newsome *et al.* 2007, Kadye & Booth 2012).

Following DeNiro & Epstein (1978; 1981), Boon & Bunn (1994), Cabana & Rasmussen (1996), Vander Zanden & Rasmussen (2001) and Davis *et al.* (2015) investigations, both stable nitrogen and carbon isotopic values provided insight information with regards to aquatic organism trophic feeding niches and diets from phytoplankton (primary producers) to fish (secondary consumers). Furthermore, stable isotopic analysis can investigate trophic ecology with regards to the presence of some notorious invaders and alien aquatic species e.g. *Tarebia granifera, Pterygoplichthys disjunctivus*; Hill *et al.* (2015) and zebra mussels – *Dressena polymorpha*; Colborne *et al.* (2015). Additionally a number of studies have also shown that stable isotopic values of nitrogen ($\delta^{15}N$) in aquatic biota are sensitive in reflecting N-loading of the system under investigation and may act as an early indicator of nutrient pollution prior to the onset of system degradation (e.g. Anderson & Cabana 2005, Cole *et al.* 2004, Deutsch & Voss 2006, Fry & Allen 2003, Savage 2004, Lassauque *et al.* 2010) and can even be used to trace nutrient loading in aquatic systems. Costanzo *et al.* (2001) used marine macrophytes and subsequent carbon and nitrogen isotopic values to illustrate nutrient hotspots from sewerage effluents coming through a river mouth as well as the spatial extent of sewage pollution in Moreton Bay, Australia (referred to as sewage plume mapping). Results from his study eventually resulted in sewage treatments works in the Moreton Bay vicinity being upgraded and the Australian sewerage effluents standards being reviewed. Hill *et al.* (2011; 2012) also identified an aquatic macrophyte, duckweed (*Spirodela* sp.) with the ability to differentiate

between different N-sources using SIA. The study showed promising results, where the duckweed was able to trace N-loading and differentiate between cow manure and commercial fertilizer N-inputs, with concentration levels effects on the plant isotopic values, however these investigations were completed in a laboratory setting, therefore follow up studies were needed to test the sewage plume mapping technique with *Spirodela* sp. in the natural environment.

1.6 Study Aims

The aims of this thesis were addressed in a series of three separate studies following the chapter outline below. Chapter 2 addresses the materials and methods used in the three subsequent chapters and also provides an overview of land use, comparing *in situ* environmental variables between all sampled study sites on the Bloukrans-Kowie and Bushmans-New Year's River systems. Chapter 3 investigates the effect of land-use and prevailing environmental variables on the river's ecological health and biodiversity using the SASS5 technique. Chapter 4 provides an intensive field test of the sewage plume mapping technique in order to validate the use of stable isotopic values of $\delta^{15}N$ and $\delta^{13}C$ from transplanted duckweed (*Spirodela* sp.) for monitoring water quality and tracing nutrient loading in freshwater systems and compares stable isotopic techniques with the existing traditional SASS5 technique. Chapter 5 attempts to identify available macroinvertebrate(s) taxa as an additional biological indicator using stable isotopic values, to use in conjunction with *Spirodela* plants to map and trace nutrient loading in freshwater ecosystems. Chapter 6 provides an overall discussion on the use of stable isotopic techniques with respect to sewage plume mapping and biological monitoring in comparison with SASS5 and considers the future of biological monitoring in the face of anthropogenic activity.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study Area

The study was conducted in two local river systems of the Eastern Cape; the Bloukrans-Kowie River system in Grahamstown and the Bushmans-New Year's River system in Alicedale, both within the Makana District Municipality, Eastern Cape Province, South Africa (Figure 2.1). The Kowie and Bushmans River systems are situated in the Southern Temperate Highveld ecoregion, which covers the majority of the interior land of South Africa (O'Hagan 1989, Duggan 1990). The climate of the southeastern Cape were both river systems lie, is that of a subtropical coastal belt influenced by the warm Mozambique-Agulhas current, experiencing an annual rainfall of about 600-800 mm, were 80-85% occurring as brief summer thunderstorms from October to March (Agnew 1986, Skelton 1994). According to Henderson (2001) and de Moor & Day (2013) this ecoregion is characterized by grassland and valley thicket, as the main indigenous ground cover and is also invaded by alien plants consisting of wattle (*Acacia mearnsii*, *A. dealbata* and *A. baileyana*), hakea (*Hakea drupacea*, *H. sericea*, *H. gibbosa*) and bluegum trees (*Eucalyptus camaldulensis* and *E. lehmannii*).

Together, the Bloukrans-Kowie and the Bushmans-New Year's River systems flow directly into the south-eastern coastal belt of South Africa (Allanson *et al*. 1990), through Port Alfred and Kenton-on-Sea respectively (Figure 2.1). These systems are found within the Kowie Thicket biome of the Eastern Cape at an altitudinal range of between 0 – 700 meters above sea level. Mucina & Rutherford (2006) describe the region as "tall thickets dominated by succulent euphorbias and aloe with understory composed of thorny shrubs, woody lianas and shrubby succulents".

Figure 2.1: Map showing the (A) Africa map, (B) Southern Africa map and (C) the Bloukrans-Kowie and Bushmans-New Year's River Systems, Eastern Cape.

2.1.1 Bloukrans-Kowie River system

General Description

The Kowie River is a permanently open river system with a total length of 70 kilometers, draining a relatively small catchment area of ~800 square kilometers. Its source arises in the hills of Grahamstown Heights and is relatively in an undisturbed natural habitat (Eady *et al.* 2013, Dalu *et al.* 2014). The majority of this area is privately owned land, dominated by game farms.

Further downstream, the Kowie River system from the south-easterly direction, meets the Bloukrans River system and collectively flows through Water Meetings Nature Reserve and Bathurst town towards Port Alfred as the Kowie River system (which will be referred to as the Great Kowie in this study).

This system has been identified recently as impacted by anthropogenic pollution (Barber-James *et al.* 2003). The upper end of the Bloukrans River drains the majority of the urban and rural Grahamstown area including human settlements and industrial areas, passing through Belmont Valley. Along the river in the eastern part of the town, there is raw sewage inputs leaking into the system from the nearby settlements, along with treated sewerage out-fall from the Belmont Valley Sewerage Treatment Works (BVSTWs; S33º19'00.52", E26º33'28.39", 500 meters above sea level). The Bloukrans River also experiences intense disturbances further downstream, including water abstraction from intensive neighboring agricultural lands e.g. Dairy farms, beef cattle and goat farms, cabbage and pineapple plantations and the on-going Golf course construction (Belmont Dev. Co.) along the Belmont valley road (Eady *et al.* 2013).

Site Selection

Ten sites were originally selected on the Bloukrans-Kowie River over a well-defined nutrient gradient; including areas considered largely natural and those influenced by sewage and fertilizers inputs (Table 2.1, Figure 2.2A). The majority of the chosen sites lie on the Bloukrans River which is the tributary of the Great Kowie River system (Figure 2.2A), and were chosen both for ecological importance and ease of logistical access. Site A1 was intended to be situated within the BVSTWs, but was precluded by overhauls in management and infrastructure of this facility. As a result only nine sites were investigated consistently over the 13 month (see data collection below). The rationale behind site selection for the remaining nine sites briefly follows; A2 was downstream of urban Grahamstown's industrial area and rural human settlements and thus an entry point for all catchment activities happening within the vicinity e.g. cow manure, waste material dumping, industrial waste and leaking sewage pipes. A3 was approximately 0.82 kilometers downstream from A2 and adjacent to the BVSTWs, located in the treated sewerage effluent before it entered the river (in order to investigate the properties of the treated waste

water before entering the Bloukrans River system). Site A4, A5 and A7 were downstream of the BVSTWs and adjacent to intense agricultural lands, suggesting potential impacts from sewage and anthropogenic fertilizer inputs. The last site on the Bloukrans River before the Kowie-Bloukrans confluence was A8, much further downstream of the BVSTWs and thus theoretically exposed to more dilute anthropogenic inputs. The upper reaches of Kowie River were considered largely natural habitat, with site A9 located on the upper reaches of the Kowie River - Featherstone Kloof, at the Southwell road bridge and A6 located downstream A9 at the Coleridge Nature Reserve. The last site, A10 was after the Bloukrans and Kowie River systems confluence on the Hollingrove Nature Reserve, collectively representing all eight upper stream sampled sites with differing catchment activities (Table 2.1, Figure 2.2A & 2.3).

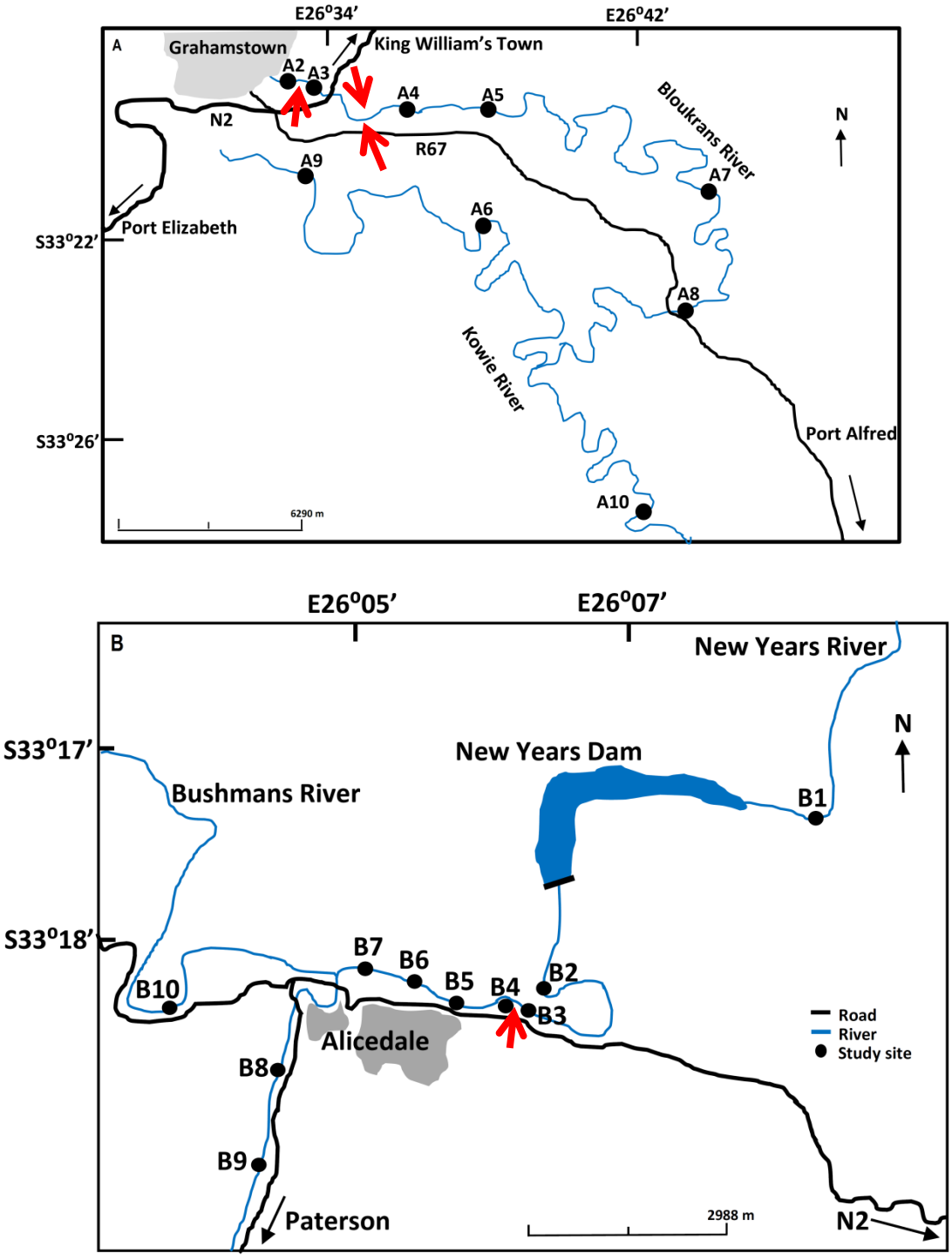


Figure 2.2: Map showing the sites chosen on the (A) Bloukrans-Kowie River systems and (B) Bushmans-New Year's River system Eastern Cape, South Africa. Arrows represents nutrient (sewage out-fall and cow manure run-off) entry points.

Figure 2.3: Photographic snapshots of sites A2 - A10 on the Bloukrans-Kowie River system, Eastern Cape South Africa.

Table 2.1: A summary of the Bloukrans-Kowie River systems study sites in the Eastern Cape, South Africa.

Study Sites	Latitude	Longitude	Sites Description & Land-use	Sites Structure & Characteristics	Dominant Plant species (Aquatic & Riparian)	
					Indigenous	Invasive
A2	S33º31'45.0"	E26º55'25.2"	Bloukrans River; downstream urban & industrial areas	Riffle site, medium water flow & turbidity, 0% canopy cover	*Cynodo dactylon* *Persicaria senegalensis*	*Solanum mauritianum* *Argemone ochroleuca*
A3	S33º31'55.1"	E26º56'01.7"	Bloukrans River; sewerage effluent	Rocky, medium water flow & turbidity, 60% canopy cover	*Typha capensis*	*Arundo donax* *Solanum mauritianum* *Argemone ochroleuca*
A4	S33º32'36.1"	E26º62'71.4"	Bloukrans River; downstream sewage out-fall, adjacent Belmont valley agricultural lands	Rocky, medium water flow & turbidity, 80% canopy cover	*Persicaria senegalensis* *Celtis africana*	-
A5	S33º19'45.8"	E26º37'90.0"	Bloukrans River; downstream sewage out-fall, adjacent Belmont valley agricultural lands	Rocky, medium water flow, low turbidity, 60% canopy cover	*Cyperus sexangularis* *C. dives* *Rhus sp.*	*Cana indica* *Arundo donax*
A6	S33º36'59.1"	E26º62'71.4"	Upper reaches of Kowie River; Coleridge Nature Reserve (largely natural)	Rocky-sandy site, medium water flow, low turbidity, 10% canopy cover	*Cyperus sexangularis* *Acacia karoo*	-
A7	S33º35'34.7"	E26º72'05.5"	Bloukrans River; downstream sewage out-fall, adjacent Belmont valley agricultural lands	Sandy-muddy site, low water flow, high turbidity	*Acacia sp.* *Cyperus sexangularis* *Lemna gibba* *Phragmites australis*	*Eucalyptus sp.*
A8	S33º39'12.2"	E23º70'75.4"	Bloukrans River; downstream sewage out-fall, adjacent Belmont valley agricultural lands (diluted)	Rocky, high water flow, low turbidity, 30% canopy cover	*Cyperus sexangularis* *C. marginatus* *Acacia caffra* *Rhus sp.*	*Acacia mearnsii*
A9	S33º39'93.0"	E26º55'99.6"	Upper reaches of Kowie River (largely natural)	Sandy-rocky, medium water flow, low turbidity, 50% canopy cover	*Ficus capensis* *Rhus chirindensis* *Cyperus sexangularis*	*Lantana camara*
A10	S33º45'77.0"	E26º69'33.8"	Great Kowie River System; after the confluence	Rocky riffle, medium water flow, low turbidity, 60% canopy cover	*Cyperus sexangularis* *Acacia karoo* *Acacia athaxacantha* *Rhus sp.*	*Solanum mauritianum*

2.1.2 Bushmans-New Year's River system

General Description

The Bushmans-New Year's River system is comprised of a 27.08 kilometers Bushmans River arising North of Kirkwood town and passing through the easterly region of Alicedale (S33º18'55.89", E26º04'59.12", 283 meters above sea level), Eastern Cape South Africa. The New Year's River is a tributary of the Bushmans River, and is a 17.32 kilometer system arising between the North western hills of Grahamstown and Glen Ambrose. It joins the Bushmans River system from the westerly direction at Alicedale and stretches down to the coastal and meets the Indian Ocean at Kenton-on-Sea (Midgley *et al*. 2006).

The Bushmans-New Year's River system drains the majority of the surrounding areas including urban and rural Alicedale and the neighboring Game Farms (e.g. Bushman Sands Nature Reserve (New Year's Dam) and Bushman Sands Golf course). The system also is said to be exposed to strong anthropogenic disturbance. Also the river is known to house the large populations of the world's number one aquatic invasive plant, *Eichhornia crassipes* (water hyacinth) (Hill & Olckers 2001).

Site Selection

Ten sites were selected on the Bushmans-New Year's River system, also over a well-defined nutrient gradient; including areas considered less disturbed and those largely influenced by sewerage and fertilizers (Table 2.2, Figure 2.2B). The majority of the chosen sites were concentrated around Alicedale on the New Year's River (Figure 2.2B), and again, were chosen both for ecological importance and ease of logistical access. It should be noted that throughout most of the 13 month sampling period (see data collection below) the Bushmans-New Year's River system was experiencing drought, resulting in multiple isolated pools along the system, particularly between sites B1, B2, B3, B5 and B10 (Figure 2.4). This was with the exception of April and May 2014; where high rainfall restores a continuously flowing river from above the New Year's dam wall and Bushmans river system to the confluence and down to the river mouth. The rationale behind site selection for the remaining ten sites briefly follows; B1 and B2 were on the Bushman Sands Nature Reserve, with B1 further upstream above the New Year's

dam wall and B2 downstream of the dam wall. Despite the potential of seepage from the dam impoundment, these sites were considered natural with major modifications, as they were found in a Nature Reserve and free from anthropogenic inputs. However infestation of the invasive *Eichhornia crassipes* (water hyacinth) was noted at site B1 (Figure 2.4). Site B3 was downstream from the dam wall, directly adjacent to the uncompleted Alicedale Sewerage Treatment Works (ASTWs), this site was chosen in order to investigate the possibility of point source water pollution from the ASTWs, which currently employs settling ponds located by the riverside. B4 was located directly in the ASTWs sewage settling ponds in order to quantify physical and chemical characteristics of sewerage inputs likely seeping in, washed through during heavy rains or leaching through the groundwater into the surrounding water-ways. B5 was downstream the ASTWs. B6 and B7 were chosen on the New Year's River within the Bushman Sands Golf Course, to investigate non-point source pollution from fertilizer run-off (as used on the greens) and also to try and differentiate between nitrogen arising from sewage pollution and commercial fertilizer (see Hill *et al*. 2011, 2012). On the upper Bushmans River, B10 was selected, which was about 2.70 kilometers before the confluence, to determine the level of nitrogen inputs coming from the Bushmans River. B8 and B9 were downstream of the Bushmans-New Year's River confluence, approximately a kilometer apart, collectively representing all sampled sites with differing catchment activities (Table 2.2, Figure 2.2B & Figure 2.4)

Figure 2.4: Photographic snapshots of sites B1 - B10 on the Bushmans-New Year's River system, Eastern Cape, South Africa.

Table 2.2: A summary of the Bushmans-New Year River systems study sites in the Eastern Cape, South Africa.

Study Sites	Latitude	Longitude	Sites Description & Land-use	Sites Structure & Characteristics	Dominant Plant species (Aquatic & Riparian)	
					Indigenous	Invasive
B1	S33º29'61.5"	E26º14'74.1"	New Year's River; above New Year's dam wall	Sandy-muddy site, zero water flow, high turbidity	*Cyperus sexangularis* *Phragmites australis* *Rhus lucida*	*Eichhornia crassipes*
B2	S33º31'60.9"	E26º10'138"	New Year's River; below New Year's dam wall	Muddy site, zero water flow, very high turbidity, 10% canopy cover	*Rhus lancea* *R. lucida* *Cyperus sexangularis*	*Eichhornia crassipes*
B3	S33º31'53.8"	E26º10'71.2"	New Year's River; adjacent to ASTWs	Muddy site, zero water flow, high turbidity, 5% canopy cover	*Typha capensis* *Spirodela sp.* *Persicaria senegalensis* *Rhus incise* *Acacia karoo* *Gymnosporia sp.*	*Eucalyptus sp.*
B4	S33º31'54.2"	E26º10'68.4"	New Year's River; ASTWs – sewage settling ponds	-	-	-
B5	S333160.9	E26º10'13.8"	New Year's River; downstream ASTWs	Sandy site, zero water flow, high turbidity, 5% canopy cover	*Typha capensis* *Persicaria decipiens* *Spirodela sp.*	*Eucalyptus sp.*
B6	S33º31'21.3"	E26º08'70.3"	New Year's River; Bushman Sands Golf Course	Sandy site, Trickle water flow, very high turbidity	*Cyperus sexangularis* *C. marginatus* *Potamogeton pectinatus* *Acacia karoo* *Spirodela sp.*	*Cactus sp.*
B7	S33º31'42.3"	E26º09'79.2"	New Year's River; Bushman Sands Golf Course	Sandy, trickle water flow, very high turbidity, 30% canopy cover	*Phragmites australis*	-
B8	S33º32'15.9"	E26º07'97.8"	Bushmans River System; after New Year's and Bushmans River confluence	Rocky (Bedrock) site, low water flow & turbidity	*Phragmites australis* *Cyperus sexangularis*	*Eucalyptus sp.*
B9	S33º32'94.8"	E26º07'71.2"	Bushmans River System; after New Year's and Bushmans River confluence	Rocky (Bedrock) site, low water flow & turbidity, canopy cover 30%	*Cyperus sexangularis* *Acacia karoo*	-
B10	S33º31'60.7"	E26º06'49.5"	Bushmans River; downstream	Muddy site, zero flow, very high turbidity, 2% canopy cover	*Cyperus sexangularis* *Acacia karoo*	-

2.2 Data Collection

2.2.1 Environmental variables

Physicochemical variables

The physicochemical parameters of the water column at each site were collected once every month at all 19 study sites over the 13 month sampling period, August 2013 to August 2014. These included pH (log [H^+]), electrical conductivity (EC; µS), total dissolved solids (TDS; ppm), salinity (Sal; ppt), water temperature (°C) and dissolved oxygen (DO; mg/L) (Appendix 1). Parameters were measured using a portable multi-probe PCSTester 35 and a DO Pen 85004. Additionally, GPS co-ordinates of each site were recorded using a Garmin Montana 600 GPS.

Micronutrients (Inorganic salts)

On quarterly sampling occasions, water samples were collected for micronutrient analyses. 1 L plastic bottles were used to collect water samples, with both the plastic bottle and lid rinsed with the water in question prior to collection. Samples were collected from 15 cm below the water surface and filled to the top to avoid any air bubbles within the container (www.bemlabs.co.za/samplinginfo.php?Id=22). Samples were then brought to the laboratory and stored at 4 ºC until sample collection was concluded (4 days) and sent to BEM-Labs, Cape Town for analysis. Micronutrient determinations by BEM-Labs included analyte concentrations of Na, K, Ca, Mg, Fe, Cl, CO_3, HCO_3, SO_4, B, Mn, Cu, Zn, P and F as determined by the standard procedures defined in the ICP manual; NH_4-N and NO_3-N concentrations were via an auto analyzer (using wavelengths of 660 nm and 550 nm respectively); and pH, EC and TDS were measured according to SANS 11885:2008 (http://www.bemlab.co.za/services.php) (Appendix 2 & 3).

A one-way analysis of variance (ANOVA) was completed for each river system separately, where pH, EC, TDS, DO, salinity and water temperature were dependent variables and sites were grouping variables. This was used to compare means of physicochemical variables among sampling sites after testing for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). Data were not normally distributed in all sampled study sites in either

river system (Shapiro-Wilk test; $p < 0.001$). Additionally, variances for DO and water temperature were not equal on the Bloukrans-Kowie River nor were the variances for water temperature on the Bushmans-New Year's River (Levene's test; $p > 0.05$). Transformation of the data did not improve heteroscedasticity. ANOVA is not sensitive to non-normal data distribution and thus the likelihood of a false positive (Type I or Type II) is small (Zar 1996), that being the case ANOVA was performed using Statistica 12 (Stat Soft Inc. 2008-2014).

Similarity dendrogram based on Euclidean distance was also performed on treated (averaged, log(x+1) transformed and normalized) abiotic data, to assess similarity between sampled sites based on the micronutrient data (PRIMER v6 add-on package PERMANOVA+; Clarke & Warwick 2001). A Principal Co-ordinate Analysis (PCoA) was further used to indicate environmental variables which showed a significant correlation with sampled sites. This was achieved by adding vectors (i.e. environmental variable) from log(x+1) transformed, normalized environmental data that showed a strong positive Pearson correlation ($r = 0.7$) as the selection method (PRIMER v6 add-on package PERMANOVA+; Clarke & Warwick 2001). Closely and highly correlated environmental variables e.g. TDS and EC were pulled-out from the analysis.

Visual site inspections

River width (m) was measured at five different sections within each site to give an average river width per site. Percentage canopy cover was estimated visually and recorded per site. Substrate type and biotope diversity, flow rate and turbidity were also rated visually using the categorical scale on the SASS5 protocol and this was completed during every SASS5 assessment. These physical variables were later given a standard rating between 1 (poor/very low) to 5 (diverse/very high), and the data was further used in multivariate analysis (see later Chapters). Notes were taken on basic catchment properties at each site including visible physical and/or chemical pollution, human disturbance, land-use, habitat type (e.g. rocky, sandy or muddy habitat) and both the predominant aquatic and riparian plants (both indigenous and alien) using relevant field guides and classification keys (van Wyk & van Wyk 1997, Henderson 2001, Gerber *et al.* 2004, Henderson & Cilliers 2002) (see Table 2.1 & 2.2). Finally, photographic snap-shots of each study site were taken for visual presentation using a Nikon CoolPix s6300, 16.0 megapixel camera (see Figure 2.3 & 2.4).

2.2.2 Aquatic macroinvertebrates collection and SASS5 assessments (Chapter 3)

A comprehensive SASS5 assessment was conducted quarterly, for a period of 13 months, on the following occasions; 26th – 29th August 2013, 18th – 21st November 2013, 25th – 29th February 2014 and 29th May – 03rd June 2014 in all 18 study sites excluding the sewerage settling ponds (B4 Bushmans-New Year's River system), making up to 72 sampling points for the duration of study. Water quality and habitat health were assessed using the South African Scoring System version 5 (SASS5) technique pioneered by (Chutter 1994) and revised by Dickens & Graham (2002). Briefly; a 30 × 30 cm, 1000 micron hand held aquatic net was placed against the river current and through vigorous kicking, turning and scraping all available biotopes individually (e.g. stones, vegetation and gravel/sand/mud) within the prescribed time intervals, samples were washed and dislodged into the aquatic net. Thereafter, samples were tipped into the white collecting tray, separately and allowed to stand for few minutes for plant matter to settle down and aquatic macroinvertebrates to emerge (Dickens & Graham 2002). Identification of aquatic macroinvertebrates was done in the field, using relevant identification guides and keys (Day & de Moor 2002A & B, Day *et al.* 2002; 2003, Gerber & Gabriel 2002A, de Moor *et al.* 2003A & B) as recommended by Dickens & Graham (2002). Additionally, for estimating aquatic macroinvertebrate biodiversity, instead of normal SASS5 abundance ratings of 1 = 1, A = 2 - 10, B = 10 - 100, C = 100 - 1000, D > 1000 (Dickens & Graham 2002), aquatic macroinvertebrate abundance of observed individual taxa were first recorded, to be further used for biodiversity assessments following Bredenhand & Samways (2009) study. Abundances data was later converted to SASS5 rating for computing SASS score and ASPT.

2.2.3 Stable isotope samples collection

2.2.3.1 Tracing N-loading with $\delta^{15}N$ and $\delta^{13}C$ isotopic values of Spirodela sp. (Chapter 4)

The indigenous duckweed *Spirodela sp.* was grown following the procedure adopted from Hill *et al.* (2012). Fresh *Spirodela* plants were collected and grown in two 20 litre tubs at the Biological Control Research Group (BCRG), Waainek Mass Raring Facility at Rhodes University, under (10.0 mg nitrate/L; 12:12 light: dark regime; 20.0 ± 2.0 ºC) conditions for a period of more than 10 days prior to experimental start (Hill *et al.* 2012). 95 floating aquatic field cages were constructed, consisting of a 250 ml clear plastic containers with minute holes punched in the plastic to facilitate free flowing water and fitted with two floats of high density

foam (Figure 2.5A). Cages were designed to transplant previously incubated *Spirodela* sp. (with initial values of $\delta^{15}N$ 13.12±3.18 ‰, $\delta^{13}C$ -29.06±0.78 ‰ and C/N ratios of 9.04±1.14) in 19 selected study sites. Five floating cages (n = 5) were transplanted per site, and were allowed to float freely within the system housing ± 45g wet weight of *Spirodela* plants (Figure 2.5B). Cages were prevented from drifting by using a string attached to design 10 × 10 cm, PVC pipe weights filled with pre-mixed cements (Figure 2.5C). The isotopic equilibration rates of *Spirodela* sp. (e.g. the time it takes for *Spirodela* plants to reflect N-loading in the environment) is between 4 - 10 days (Hill *et al.* 2011, 2012), thus plants were left to grow for a period of at least 10 days in between sample collection. On-site floating cages were washed (to remove dirt and algae), repaired and refilled with plants where necessary in every fourth month. Plants samples were collected every month over a period of 13 months from August 2013 to August 2014. Approximately 3.0 – 5.0 mg of *Spirodela* plants samples were collected and put into Eppendorf tubes. Samples were stored on ice until they reached the laboratory and then oven dried for a minimum of 72 hours at 50 ºC.

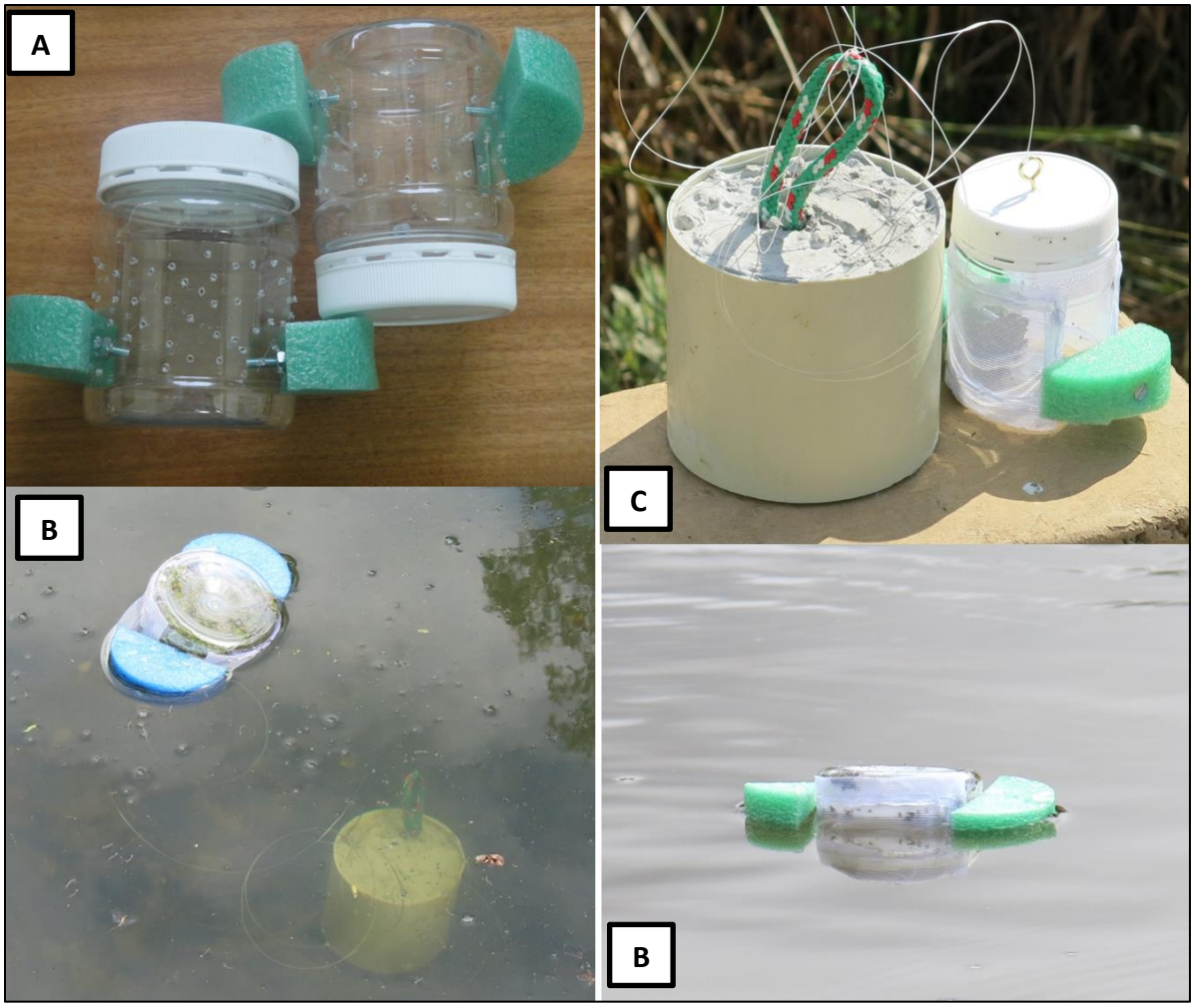


Figure 2.5: Experimental design illustrating (A) floating aquatic field cages, (B) suspended free floating cages and, (C) designed PVC pipe weights on each site on the Bloukrans-Kowie and Bushmans-New Year's River system in the Eastern Cape, South Africa.

2.2.3.2 Tracing N-loading with $\delta^{15}N$ isotopic values of macroinvertebrates: can the ^{15}N isotopic values of some macroinvertebrates taxa also trace N-loading? (Chapter 5)

Using previously collected macroinvertebrates abundance data (from SASS5 assessments; see Chapter 3), potential indicator taxa were identified using the Indicator Value Species Analysis (IndVal) and multivariate analysis (Redundancy analysis - RDA) methods from all sampled study sites on both the Bloukrans-Kowie and Bushmans-New Year's River systems.

Indicator macroinvertebrate taxa, identified by both IndVal (PC-ORD 5.1) and RDA (CANOCO 4.5) included Oligochaeta adult and Chironomidae, Culicidae and Syrphidae larvae.

Once potential indicator taxa were identified, a preliminary investigation was conducted between three selected study sites (identified on RDA) on the Bloukrans River system which showed high positive correlation towards nutrients inputs (A2, A3 and A4) and for comparison purpose one largely natural study site A9 was selected as a control site (Figure 2.2A, 2.3; Table 2.1). Identified potential macroinvertebrate indicator taxa were collected at each site, once every week over four weeks sampling events in March 2015. Taxa were collected via kick sampling method, where all available aquatic biotopes (e.g. stones, aquatic and marginal vegetation and gravel/sand/mud) collectively were sampled vigorously for three minutes. Macroinvertebrates samples collected were transferred into a white collecting tray and selected taxa individuals were collected using laboratory pipettes and tweezers into an Eppendorf vial and then transported on ice to the laboratory for further processing. For each site and time, five replicates of each indicator taxa were collected, consisting of between 10 - 20 pooled individuals (to achieve sufficient mass for SIA). Collected wet mass taxa were oven dried for 72 hours at 50 ºC (Bergfur *et al.* 2009, di Lascio *et al.* 2013). However, pollution tolerant taxa were in less abundance at A9 (due to its largely natural nature and higher numbers of pollution sensitive taxa) and so in some cases, very few of the previously identified N-loading indicator taxa (Oligochaeta and Chironomidae, Culicidae and Syrphidae larvae) were found, thus fewer replicates or no taxa sometimes were obtained at A9.

In addition [NO_3-N], [NH_4-N] and [DO] measurements (n = 5) were recorded per site on each sampling occasion from five different points.

2.2.4 Stable isotopic analysis of $\delta^{13}C$ and $\delta^{15}N$ isotopic values

Prior to isotopic analyses, all samples were ground into homogenous fine powder using mortar and pestle and weighed to appropriate weights (plants = 1.8 mg – 2.0 mg, animal tissue = 0.5 mg – 0.6 mg; IsoEnrvironmental Sven Kaehler pers. comm.) into tin capsules (8 × 5 mm). $\delta^{15}N$ and $\delta^{13}C$ isotopic values of plant samples from August 2013 ($T_{initial}$) to March 2014 (T_8) were analyzed at the IsoEnvironmental Laboratory, South African Institute of Aquatic Biodiversity (SAIAB), South Africa using a Europa Scientific 20-20 IRMS interfaced to an ANCA SL Elemental Analyser. The precision of replicate determinations was 0.11 for $\delta^{15}N$ and 0.07 for $\delta^{13}C$. All $\delta^{15}N$ and $\delta^{13}C$ isotopic values were reported as ‰ vs Viennea PeeDee Belemnite (VPDB) and air respectively and normalized to internal standards calibrated to the

International Atomic Energy reference materials (IAEA-CH6 for $\delta^{13}C$ and IAEA-N2 for $\delta^{15}N$). Remaining plant samples from April 2014 (T_9) to August 2015 (T_{13}) and all macroinvertebrates samples were determined at the Stable Isotope Laboratory, Mammal Research Institute, University of Pretoria using a 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). Analytical precision was < 0.2 ‰ for $\delta^{13}C$ and < 0.2 ‰ for $\delta^{15}N$. A laboratory running standard (Merck Gel: $\delta^{13}C$ = -20.57 ‰, $\delta^{15}N$ = 6.8 ‰, C%= 43.83, N% = 14.64) and blank sample were run after every 12 unknown samples. All $\delta^{15}N$ and $\delta^{13}C$ values were reported as ‰ vs Vienna Pee-Dee Belemnite (VPDB) and air respectively.

All results were expressed in delta notation using a per mil (‰) scale using the standard equation: δX (‰) = $[(R_{sample}-R_{standard})/R_{standard}-1] \times 1000$, where X= ^{15}N or ^{13}C and R represents $^{15}N/^{14}N$ or $^{13}C/^{12}C$ respectively.

2.3 Results

2.3.1 Bloukrans-Kowie River environmental variables

Physicochemical variables

The pH values on the Bloukrans-Kowie River ranged between 7.85 – 8.58, with site A8 (8.58) and A10 (8.57) showing the most alkaline pH values, and site A3 (7.85) showing the most acidic pH value. The pH values were significantly different between study sites ($F_{8, 115}$ = 10.24, p < 0.001), with site A7, A8 and A10 appearing to drive the majority of the pH variation (Table 2.3, Figure 2.5A). Electrical conductivity ($F_{8, 115}$ = 11.54, p < 0.001), salinity ($F_{8, 115}$ = 11.84, p < 0.001) and TDS ($F_{8, 115}$ = 13.85, p < 0.001) showed similar trends and strong agreement in differences between sites, with site A6, A9 and A10 significantly different from all other sites (Table 2.2, Figure 2.4B, C, E). Where site A10 showed the highest values for all three variables (EC = 2023.20 µS, TDS = 1495.40 ppm, salinity = 1.05 ppt), while site A9 demonstrated the lowest (EC = 350.20 µS, TDS = 220.60 ppm, salinity = 0.15 ppt) (Figure 2.4B, C, E). DO values ranged between 3.70 – 6.03 mg/L and were significantly different between sites ($F_{8, 115}$ = 15.96, p < 0.001), with site A6, A7, A8, A9 and A10 significantly different from all other sites on the river system (Table 2.3, Figure 2.4F). Site A8 had the highest (6.03 mg/L) and site A2 (3.70 mg/L) had the lowest DO concentrations (Figure 2.5D). There were no significant differences in

water temperature between sites in the Bloukrans-Kowie River (Figure 2.5F) averaged over the 13 month sampling period (August 2013 – August 2014; $F_{8, 115} = 0.62$, $p > 0.05$).

Micronutrient concentrations

The composition of total dissolved salts in the Bloukrans-Kowie River was dominated by major ions and cations (HCO_3^-, Cl^- , $S0_4^{2-}$ and Ca^{2+}, Mg^{2+}, K^+ and Na^+). Upstream sites (A2, A3, A4 and A5) showed increased concentrations of phosphorus (P), ammonium (NH_4-N) and nitrate (NO_3-N), while sites on the upper reaches of the Kowie River (A9 and A6) and the site below the confluence (A10), showed lower concentrations of the same compounds (Appendix 2).

2.3.2 Bushmans-New Years River environmental variables

Physicochemical variables

The pH values on the Bushmans-New Year's River ranged between 7.72 – 9.25. Site B4 demonstrated the highest pH value (9.25) for the period of this study (Table 2.4, Figure 2.6A). EC ($F_{9, 130} = 12.48$, $p < 0.001$), salinity ($F_{9, 130} = 9.21$, $p < 0.001$) and TDS ($F_{9, 130} = 12.81$, $p < 0.001$) showed similar trends and strong agreement in differences between sites, with site B7, B8 and B9 significantly different from all other sites on the river (Table 2.4, Figure 2.6B, C, E). Site B8 on the Bushmans-New Year's River showed the highest values (EC = 2211.70 µS, TDS = 1565 ppm and salinity = 1.17 ppt), while B10 demostrated the lowest (EC = 551.10 µS, TDS = 391.70 ppm and salinity = 0.34 ppt) (Figure 2.6 B, C, E). There were no significant differences in water temperature ($F_{9, 130} = 0.61$, $p > 0.05$) and DO concentrations ($F_{9, 130} = 1.26$, $p > 0.05$) between sites in the Bushmans-New Year's River (Table 2.4, Figure 2.6D, F) averaged over the 13 month sampling period (August 2013 – August 2014).

Micronutrient concentrations

The composition of total dissolved salts in the Bushmans-New Year's River systems was similar to the Bloukrans-Kowie, comprised predominantly of ions and cations HCO_3^-, Cl^- , $S0_4^{2-}$ and Ca^{2+}, Mg^{2+}, K^+ and Na^+. The highest concentrations of P, NH_4-N and NO_3-N were observed at site B4; the ASTWs sewerage settling pond. Interestingly, none of the sites adjacent to B4 showed substantial increase in phosphorous, NH_4-N or NO_3-N concentrations. Along with B4, site B2 recorded P levels > 0.50 mg/L, while the remaining 8 sites had much lower levels < 0.50 mg/L. Sites B1 and B10 showed [NO_3-N] < 0.50 mg/L, while the rest of the study sites exhibited

a range of between 0.50 – 1.56 mg/L (with the exception of site B2 = 4.37 mg/L). Comparatively, NH_4-N concentration was recorded > 0.50 mg/L at six sites, with (B4>B1>B6>B3>B5>B2), leaving four sites with a concentration of < 0.5; site B7>B10>B>B9 (Appendix 2).

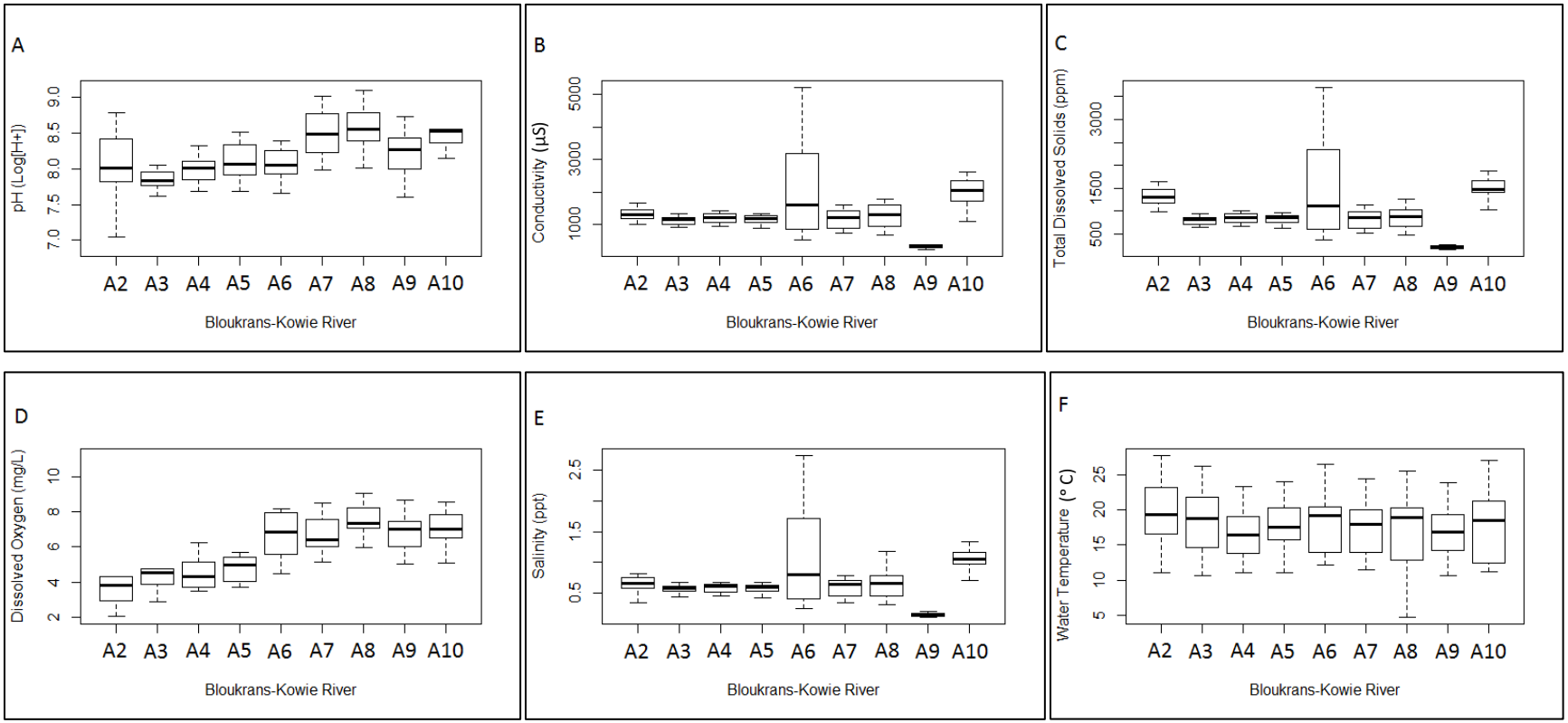


Figure 2.6: (A) pH, (B) electrical conductivity (µS), (C) total dissolved solids (ppm), (D) dissolved oxygen (mg/L), (E) salinity (ppt) and (F) water temperature (ºC) from the nine sampled study sites (A2 - A10) on the Bloukrans-Kowie River system, Eastern Cape South Africa averaged over the 13 month sampling period (August 2013 - August 2014). Error bars - represent ± 1 standard deviation, the black line represents the mean and the box - represents the minimum and maximum values.

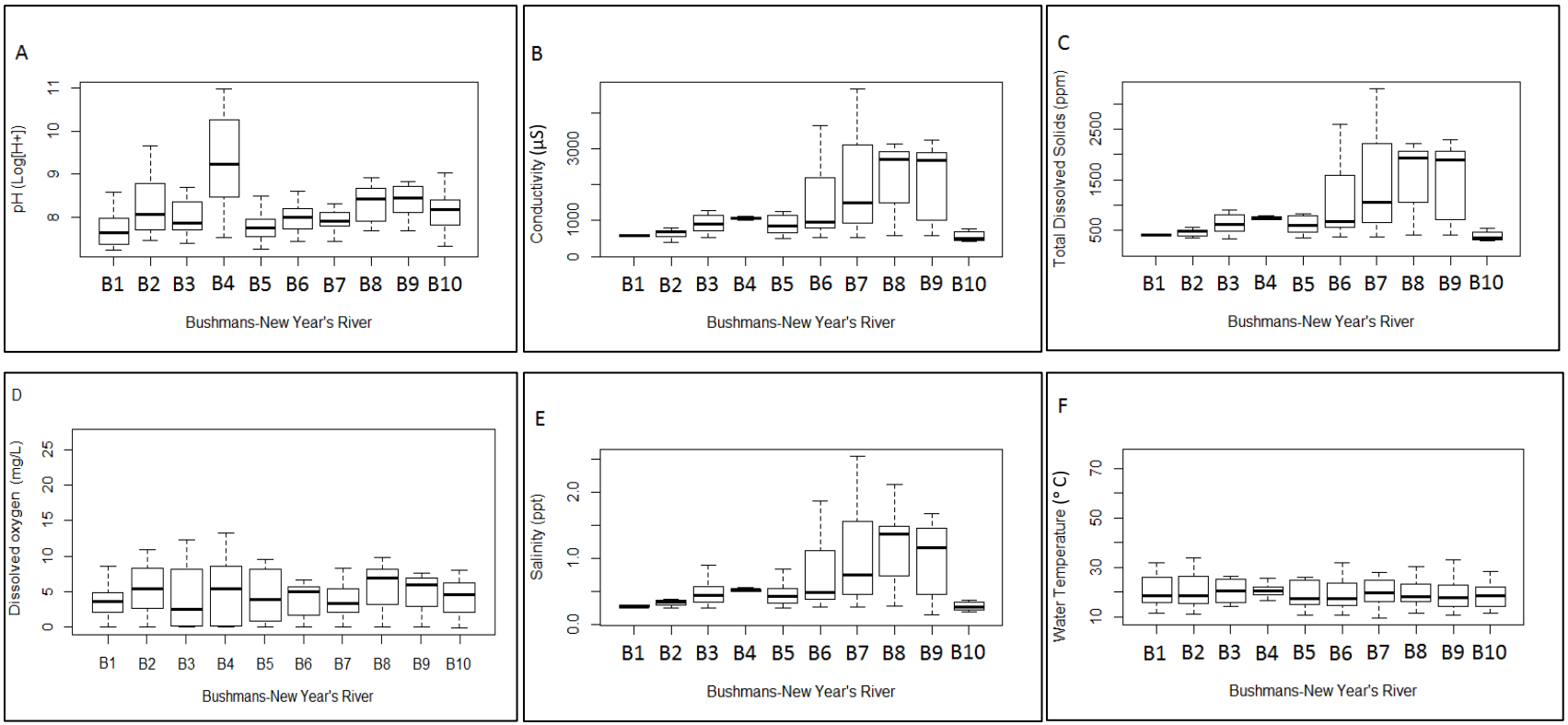


Figure 2.7: (A) pH, (B) electrical conductivity (µS), (C) total dissolved solids (ppm), (D) dissolved oxygen (mg/L), (E) salinity (ppt) and (F) water temperature (ºC) from the nine sampled study sites (A2 - A10) on the Bushmans-New Year's River system, Eastern Cape South Africa averaged over the 13 month sampling period (August 2013 - August 2014). Error bars - represent ± 1 standard deviation, the black line represents the mean and the box - represents the minimum and maximum values.

Table 2.3: Tukey's HSD post-hoc tests investigating differences in physicochemical variables averaged over time (August 2013 – August 2014) for the nine sites on the Bloukrans-Kowie river system. No significant differences were seen in water temperatures between sites, and are thus not presented. Bolded values show a significance level of $p < 0.05$.

Bloukrans-Kowie River	Sites	A2	A3	A4	A5	A6	A7	A8	A9	A10
pH	**A2**	-								
$F_{8,115} = 10.24$, $p < 0.001$	**A3**	0.904	-							
	A4	1.000	0.944	-						
	A5	0.994	0.391	0.984	-					
	A6	0.999	0.671	0.999	0.999	-				
	A7	**0.003**	**0.000**	**0.002**	0.063	**0.014**	-			
	A8	**0.000**	**0.000**	**0.000**	**0.008**	**0.001**	0.999	-		
	A9	0.519	**0.024**	0.430	0.970	0.802	0.552	0.165	-	
	A10	**0.000**	**0.000**	**0.000**	**0.011**	**0.001**	0.999	1.000	0.193	-
EC	**A2**	-								
$F_{8,115} = 11.54$, $p < 0.001$	**A3**	0.998	-							
	A4	0.999	0.999	-						
	A5	0.999	1.000	1.000	-					
	A6	**0.006**	**0.000**	**0.001**	**0.001**	-				
	A7	0.999	0.999	1.000	1.000	**0.001**	-			
	A8	1.000	0.999	0.999	0.999	**0.003**	0.999	-		
	A9	**0.000**	**0.013**	**0.005**	**0.007**	**0.000**	**0.006**	**0.002**	-	
	A10	**0.029**	**0.003**	**0.006**	**0.007**	0.999	**0.006**	**0.018**	**0.000**	-
TDS	**A2**	-								
$F_{8,115} = 13.85$, $p < 0.001$	**A3**	0.048	-							
	A4	0.097	0.999	-						
	A5	0.108	1.000	1.000	-					
	A6	**0.828**	**0.000**	**0.000**	**0.000**	-				
	A7	0.079	1.000	1.000	**1.000**	**0.001**	-			
	A8	0.142	0.999	1.000	**1.000**	**0.001**	1.000	-		
	A9	**0.000**	**0.008**	**0.004**	**0.005**	**0.000**	**0.005**	**0.002**	-	
	A10	**0.926**	**0.000**	**0.002**	**0.003**	1.000	**0.001**	**0.004**	**0.000**	-
Sal	**A2**	-								
$F_{8,115} = 11.84$, $p < 0.001$	**A3**	0.999	-							
	A4	0.999	1.000	-						
	A5	0.999	1.000	1.000	-					
	A6	**0.003**	**0.000**	**0.005**	**0.001**	-				
	A7	0.999	1.000	1.000	1.000	**0.000**	-			
	A8	1.000	0.999	0.999	0.999	**0.004**	0.999	-		
	A9	**0.001**	**0.009**	**0.008**	**0.010**	**0.000**	**0.006**	**0.001**	-	
	A10	**0.017**	**0.002**	**0.003**	**0.004**	0.999	**0.004**	**0.020**	**0.000**	-
DO	**A2**	-								
$F_{8,115} = 15.96$, $p < 0.001$	**A3**	0.999	-							
	A4	0.999	1.000	-						
	A5	0.975	0.999	0.999	-					
	A6	**0.000**	**0.000**	**0.000**	**0.002**	-				
	A7	**0.000**	**0.000**	**0.000**	0.000	0.999	-			
	A8	**0.000**	**0.000**	**0.000**	**0.000**	0.932	0.985	-		
	A9	**0.000**	**0.000**	**0.000**	**0.002**	1.000	1.000	0.952	-	
	A10	**0.000**	**0.000**	**0.000**	**0.000**	0.985	0.998	0.999	0.991	

Table 2.4: Tukey's HSD post-hoc tests investigating differences in physicochemical variables averaged over time (August 2013 – August 2014) for the ten sites on the Bushmans-New Year's river system. No significant differences were seen in water temperatures or dissolved oxygen concentrations between sites, and are thus not presented. Bolded values show a significance level of $p < 0.05$.

Bushmans-New Year's River	Sites	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
pH	**B1**	-									
$F_{9,130} = 8.26$, $p < 0.001$	**B2**	0.143	-								
	B3	0.811	0.984	-							
	B4	**0.000**	**0.000**	**0.000**	-						
	B5	0.999	0.347	0.963	**0.000**	-					
	B6	0.957	0.894	0.999	**0.000**	0.997	-				
	B7	0.993	0.731	0.999	**0.000**	0.999	1.00	-			
	B8	0.141	1.000	0.983	**0.000**	0.342	0.891	0.727	-		
	B9	0.074	1.000	0.940	**0.001**	0.211	0.769	0.559	1.000	-	
	B10	0.605	0.998	0.999	**0.000**	0.859	0.999	0.991	0.999	0.990	-
EC	**B1**	-									
$F_{9,130} = 12.48$, $p < 0.001$	**B2**	1.000	-								
	B3	0.958	0.973	-							
	B4	0.765	0.814	0.999	-						
	B5	0.984	0.991	1.000	0.999	-					
	B6	**0.008**	**0.011**	0.312	0.634	0.220	-				
	B7	**0.000**	**0.000**	**0.002**	**0.014**	**0.001**	0.849	-			
	B8	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.354	0.999	-		
	B9	**0.000**	**0.000**	**0.000**	**0.001**	**0.000**	0.476	0.999	1.000	-	
	B10	1.000	1.000	0.935	0.704	0.972	**0.006**	**0.000**	**0.000**	**0.000**	-
TDS	**B1**	-									
$F_{9,130} = 12.81$, $p < 0.001$	**B2**	1.000	-								
	B3	0.976	0.999	-							
	B4	0.808	0.870	0.999	-						
	B5	0.993	0.997	1.000	0.999	-					
	B6	**0.008**	**0.012**	0.252	0.580	0.168	-				
	B7	**0.000**	**0.000**	**0.000**	**0.007**	**0.000**	0.789	-			
	B8	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.346	0.999	-		
	B9	**0.000**	**0.000**	**0.000**	**0.001**	**0.000**	0.455	0.999	1.000	-	
	B10	1.000	1.000	0.961	0.755	0.986	**0.005**	**0.000**	**0.000**	**0.000**	-
Sal	**B1**	-									
$F_{9,130} = 9.21$, $p < 0.001$	**B2**	0.999	-								
	B3	0.950	0.999	-							
	B4	0.723	0.949	0.999	-						
	B5	0.973	0.999	1.000	0.999	-					
	B6	**0.025**	0.111	0.554	0.868	0.472	-				
	B7	**0.000**	**0.000**	**0.009**	**0.051**	**0.006**	0.851	-			
	B8	**0.000**	**0.000**	**0.000**	**0.002**	**0.000**	0.276	0.997	-		
	B9	**0.000**	**0.000**	**0.018**	**0.089**	**0.013**	0.927	1.000	0.986	-	
	B10	0.999	1.000	0.997	0.933	0.999	0.095	**0.000**	**0.000**	**0.000**	-

Euclidean Distance Similarity dendrogram

Based on Euclidean distance similarities (micronutrients), five main clusters were identified. Cluster 1 represented site A9, which was the least disturbed and situated in the upper reaches of the Kowie River amongst a natural and untransformed landscape on the North-eastern direction of Grahamstown. Cluster 2 represented sites found only on the Bushmans-New Year's River system e.g. B1-B3, B5 and B10. These sites for the majority of the study were isolated pools due to the insufficient rain fall from the region, therefore restricting water flow and likely experienced similar physical and chemical conditions. Cluster 3, represented site B4, the ASTWs sewerage settling pond which was characterized by extremely high inorganic salt concentrations and was clearly different from the rest of the sampled study sites. B4 was also set back from and not directly connected to the Bushmans-New Year's River. Cluster 4, was a combination of confluence sites e.g. B8, B9, A10, Nature Reserve sites A6, A8 and site A7 which was situated within a dense agricultural lands. Cluster 5 comprised of two geographically similar study sites (B6 and B7) both within the Bushman Sands Golf Course and approximately about 1.20 kilometers apart. Also the cluster included Bloukrans River upstream sites A2, A3, A4 and A5 regularly experience anthropogenic inputs including waste material disposal, sewage out-fall and run-off from agricultural land (Figure 2.7)

Principal Co-ordinate Analysis (PCoA)

PCoA revealed 17 of the 25 environmental variables to be strongly correlated (Pearson correlation, r = 0.70) to sampled study sites and explained about 67.40% variation. Of the 17 environmental variables, eight variables showed to be highly correlated with the remaining nine variables e.g. NO_3-N and K, pH and HCO_3, B and Ca, TDS and Na and EC and CO_3, Cl and Mg. Therefore K, HCO_3, Ca, Na, EC, CO_3 and Mg were removed from the analysis using the exclusion method and only nine environmental variables e.g. NH_4-N, NO_3-N, P, F, SO4, B, TDS, Cl and Fe were used to explain the study sites similarity pattern.

Sites A2, A3, A4, A5 and B4 were characterized by NH_4-N, NO_3-N and P, which are indicative of high anthropogenic inputs and in order of most least impacted was A2<A3<A4< B4. Site A5 indicated high concentration of fluorine (F), whilst site A7 and A8 both had high concentrations of sulphate (SO_4) and boron (B). Total dissolved solids and chlorine (Cl) were

correlated to sites B6, B8, B9, A10 and A6. Site B7 and A9 appeared to be inversely correlated to NH_4-N, NO_3-N and P, thus theoretically represent sites with higher water quality. And lastly sites B1, B10, B5, B3 and B2 appeared to be driven by high concentrations of iron (Fe).

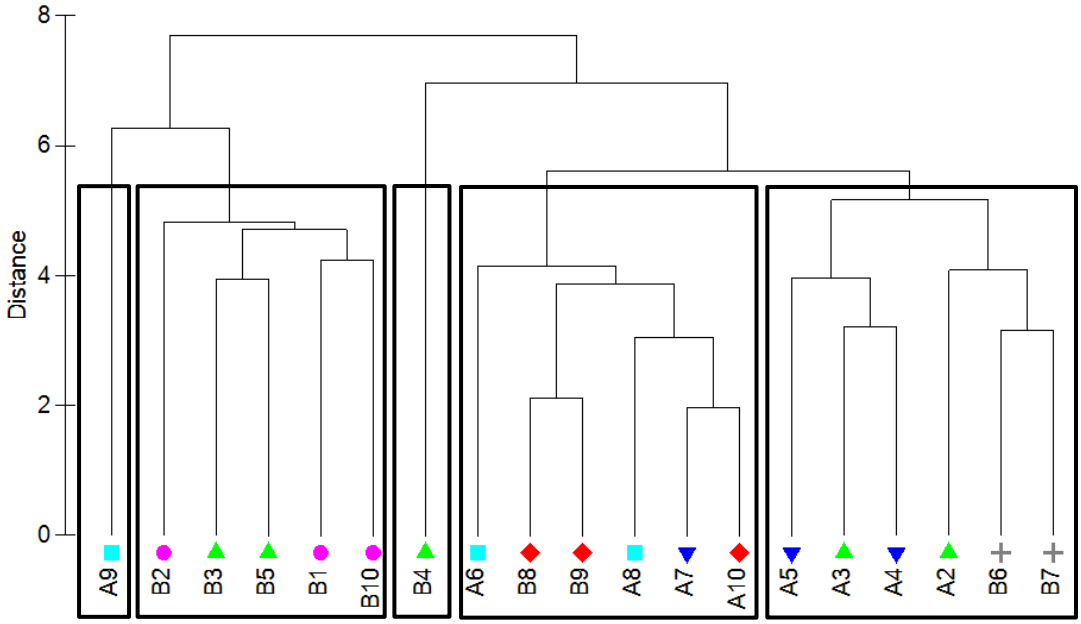


Figure 2.8: Euclidean distance similarity dendrogram based on similarity of environmental variables on sampled study sites in both the Bloukrans-Kowie (A) and the Bushmans-New Year's Rivers (B). Each symbol represents a different land-use/site description: (▲) - sewage input; (▼) - dairy farms; (♦) - confluence; (■) - undisturbed habitats; (●) - isolated pools; (+) - golf course (commercial fertilizer).

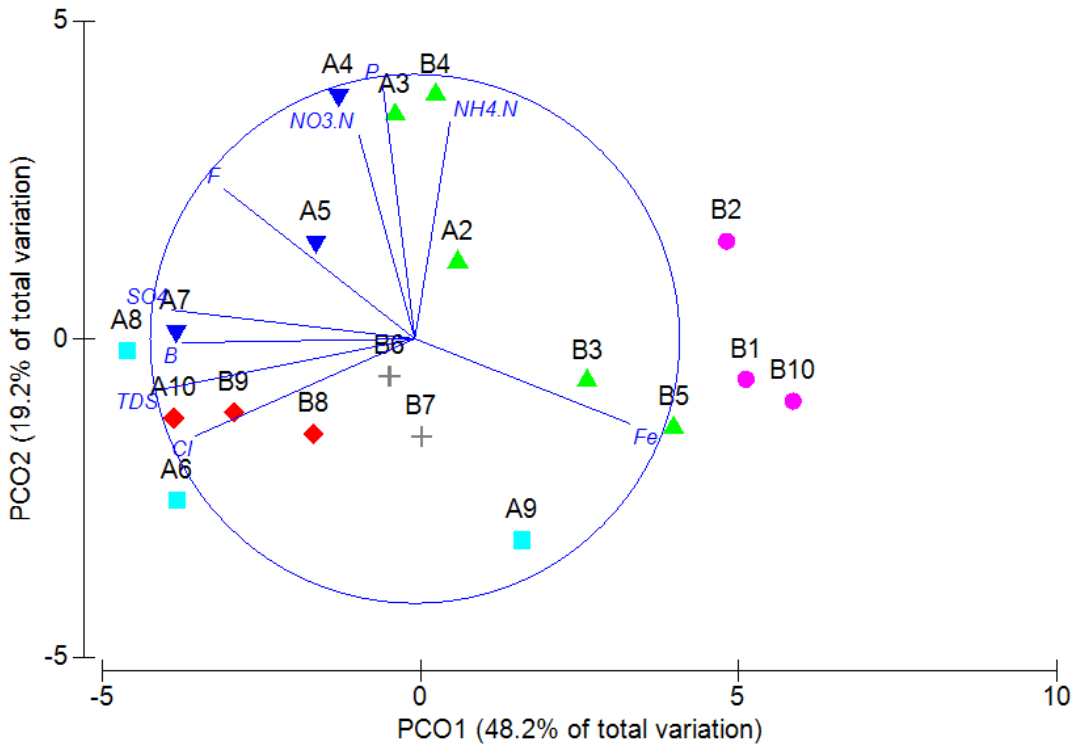


Figure 2.9: Principal Co-ordinate Analysis ordination illustrating environmental variables that showed a strong correlation towards all sampled study sites on both the Bloukrans-Kowie (A sites) and the Bushmans-New Year's Rivers (B sites). Each symbol represents a different land-use/site description: (▲) - sewage input; (▼) - dairy farms; (♦) - confluence; (■) - undisturbed habitats; (●) - isolated pools; (+) - golf course (commercial fertilizer).

2.4 Discussion

As water physico-chemistry is often used as the sole proxy for the assessment of water quality, a short discussion of the physicochemical and micronutrients difference between sites within each system is appropriate and follows below.

Physicochemical variables

Water quality assessments are a measurement of the combined effects of the physical attributes and chemical constituents of a water sample, which have the potential to affect not only ecosystem ecology, but also the human use of surface water for basic services (e.g. domestic ablutions, recreation purposes and irrigation schemes). Thus water quality can have far reaching effects (beneficial or detrimental) on aquatic ecosystems and also to the end users (humans; Palmer *et al.* 2004). One of the mandates of the Department of Water and Sanitation (DWS), previously known as Department of Water Affairs and Forestry (DWAF), South Africa, as well as international organizations such as the World Health Organization (WHO) and the European Union (EU) is to ensure that the quality of water resources remains fit for recognized water uses, while also maintaining and protecting the viability of aquatic ecosystems, thus supporting the sustainable use of freshwater resources (Chapman 1996, DWAF 1996A).

South African Water Quality Guidelines (SAWQG) provide water quality managers with a series of physicochemical concentration guidelines deemed to be acceptable within South African waterways, and are referred to as the targeted water quality range (TWQR) (DWAF 1996A). For example pH ranges for effluent discharge in South African river systems is 5.50 - 7.50, for domestic water use and healthy aquatic ecosystems is 6.00 - 9.00 (DWAF 1996B, C, & D). For the duration of this study, the average pH of 16 of the 19 sites fell into the required TWQR for domestic water use and aquatic ecosystem health. However, unsurprisingly, the ASTWs sewerage settling pond (B4) had a pH value much higher than the TWQR (9.25). This site frequently receives unprocessed human waste from the Alicedale settlements and is not directly connected to the Bushmans-New Year's River. Similarly, site A2, which is frequently flooded by raw sewage (sewerage pipe leaks) and site A3 which is situated directly on the junction between BVSTWs effluent output and Bloukrans River, had pH > 7.50. These values exceed the TWQR for effluent discharge in river systems (DWAF 1996B), although they remain within the range for domestic water use and healthy aquatic ecosystems (DWAF 1996C & D).

As expected EC, TDS and salinity were highly correlated (Deksissa *et al.* 2003, Fatoki & Awofolu 2003, Palmer *et al.* 2004) and showed similar patterns between sites in this study. The target guideline range for electrical conductivity for effluents for example is 70 000 µS, while domestic water use and aquatic ecosystems health requires no more than 250 000 µS (DWAF 1996B & C). This limit was not exceeded at any site, however, these findings differ from that of Hosking & du Preez (2002) who stated that Kowie River waters are mostly suitable for livestock watering only. This was attributed to the water's high salt content (which directly affects EC and TDS) which suggested that Kowie River water was also unsuitable for irrigation and/or domestic uses. The findings by Hosking & du Preez (2002) also highlight the pitfalls of using physicochemical measurements as a direct measure of water quality. Furthermore, Deksissa *et al.* (2003) illustrated that a river's flow rate and water level have an inversely proportionate effect of the water's dissolved salts (EC, TDS and salinity). Koning *et al.* (2000) also emphasized that lentic conditions can also cause salts to alter and/or change to sediments, leading to a decrease in dissolved salts and conductivity. In the present study the Bushman-New Year's River water had a higher salinity than the Bloukrans-Kowie River. It is likely that this can be correlate with a lack of water flow and low water levels, yielding more salt concentrations on the Bushmans-New Year's River systems as described by Deksissa *et al.* (2003). Dissolved oxygen concentrations of < 3.00 mg/L adversely affect freshwater invertebrate communities (Chapman 1996). All sampled study sites showed DO concentration well above the proposed guideline. However the present study contradicted with Chapman (1996) findings, were sites showed to be severely impacted by anthropogenic disturbance, waste material disposal, sewage and industrial pollution as well as invasion of alien plants indicated DO between 3.00 mg/L and 4.00 mg/L e.g. site A2, A3, A4 and A5; site B1, B5, B6, B7 and B10 which were all not regarded conducive but heavily impacted. Above all, site B4 the ASTWs sewerage settling pond had the highest DO concentration. This is not surprising as sewerage settling ponds are highly nutrient rich and design to maintain good, algal growth and activity, to support aerobic bacterial digestion. From a South African point of view, DWAF (1996) and Fatoki *et al.* (2003) indicate that largely natural surface waters are normally saturated with DO (> 80%). And in context unpolluted water records DO concentration of about 8 to 10 mg/L at 25℃ and concentration below 5 mg/L are regarded to adversely affect aquatic life (Fatoki *et al.* 2003) and this was a different case with Chapman (1996)'s observation. Water temperature in both river systems was fairly stable and was not significantly different

between study sites. Overall however, the Bushmans-New Year's River system had warmer waters than the Bloukrans-Kowie River system, which is likely due to limited or no water flow (standing water) on the Bushmans-New Year's River system.

Micronutrients

A number of sites (A3, A4, A5 and B3, B4, B5) on the Bloukrans-Kowie River and Bushmans-New Year's River exceeded the TWQR guidelines for $N0_3^-$-N concentrations for effluent discharge (< 1.50 mg/L; DWAF 1996D). These sites all experience regular anthropogenic inputs, with A3 directly adjacent to the BVSTWs out-fall, also site A4 and A5 both approximately 0.82 kilometers and 5.70 kilometers downstream A3 respectively. Substantial distance based downstream dilution effects on the concentration of NO_3^--N (Bere 2007) were expected in sites A4 and A5. However the high levels of [NO_3-N] at both downstream sites suggest an additional anthropogenic inputs were at play, such as run-off from the fertilization of agricultural lands with cow manure (Tucker *et al.* 1999, Constanzo *et al.* 2001). The presence of large dairy farm on the banks of A4 and A5 support this idea. The sites on the Bushmans-New Year's River that demonstrated elevated levels of NO_3^--N included the sewerage settlement pond (B4; 1.56 mg/L) and the closely adjacent B3 (1.45 mg/L). While the levels in the isolated sewerage settlement pond are not an immediate concern, the level of NO_3^--N at B3 is at the very upper end of the TWQR acceptable limits and suggests inputs from ASTWs settlement pond via seepage, leaching or overtopping events during high rainfall may be impacting the nearby river system. DWAF (1996A) and Jordaan & Bezuidehout (2013) published a limit of 6.00 mg NO_3^- N/L for domestic water use and aquatic ecosystem health, and few sites on either the Bloukrans-Kowie or Bushmans-New Year's rivers exceeded the limit. The extensions included sites adjacent to or nearby sewerage out-falls (A3, A4, A5 and B3, B4, B5). Thus, overall [NO_3-N] are not currently a threat to the overall health of the Bloukrans-Kowie or Bushmans-New Year's rivers aquatic biota or to domestic water use for agricultural means or subsistence communities. However, eutrophication is still an ongoing concern (Hill *et al* 2011; 2012), as high NO_3-N loads in some sites along both river systems suggests that a certain degree of ecological degradation may occur as N-loads continues and it requires further monitoring to prevent widespread ecosystem deterioration.

TWQR for phosphorus (P) in effluent discharge according to Oberholster & Ashton (2008), was said to be 1.00 mg/L and as expected site A3, A4, A5 and B4 which are adjacent to sewerage out-falls and also mentioned to have on the nitrate section exceeded this limit. P content was found to be approximately < 2.00 mg/L for majority of the sites on both systems, with sites closer to sewerage out-falls having P concentrations between 2.00 – 3.00 mg/L. The SAWQG however have much less conservative limits of 5.00 mg/L for effluents, and all sites fell under this value. High levels of P are thus currently not a concern in the river, reducing the likelihood of opportunistic algal and macrophytes growth within these systems (DWAF 1996C). Ammonium (NH_4^+-N) on the other hand is an extremely soluble nitrogen derivative that is easily transported between source and sink and is also a major component of raw sewage according to Morrison *et al.* (2001). At high pH levels (> 8.5), NH_4^+-N is converted into ammonia (NH_3), which can be highly toxic to aquatic organisms (fish in particular) at concentrations exceeding 2.00 mg/L (de Villiers & Thiart 2007). Due to the toxicity of NH_3 to aquatic life, the European Union has set a safety limit of 0.005 – 0.025 mg NH_3-N/L (Chapman 1996). In South Africa however, SAWQG for NH_3 in the water column for domestic water use is 1.00 mg NH_3/L (DWAF 1996A), for aquatic ecosystem integrity is 0.007 mg NH_3/L (DWAF 1996B) and effluent discharge had a limit of 1.50 mg NH_3/L (assuming pH > 8.5 in all cases). None of the sites (with the exception of the ASTWs sewerage settlement pond) from either river system showed an average pH > 8.5 during the course of the study, suggesting that the majority of NH_4^+-N was not converted to NH_3. However, high levels of NH_4^+-N are still important to monitor because of the influence of high nutrient rich (NO_3-N and NH_4-N) from sewerage treatments works on adjacent and downstream sites experiencing high pollution nutrient indicators (NO_3-N and NH_4-N). Palmer *et al.* (2004) and Fatoki & Awofolu (2003) also emphasize the crucial role pH in freshwater ecosystems, stating that "pH determines the chemical species (and thus their potential toxicity) of many elements in freshwater ecosystems such as aluminum (Al), cadmium (Cd) and zinc (Zn) which are mostly mobilized following acidification of the system".

Other important metal contaminants (Zinc, Iron, Manganese and Copper)

Fatoki & Mathabatha (2001) consider heavy metals as stable and continuous contaminants of aquatic ecosystems, considering in particular zinc (Zn), copper (Cu), iron (Fe)

and manganese (Mn). These metals are fundamental for organismal metabolic activities, but high concentrations of these metals can also be toxic. The SAWQG guideline for Zn in domestic water for example is 3.00 mg/L, and for aquatic ecosystems inhabitants, water should fall between 0.01 - 0.02 mg/L (DWAF 1996A & B; 1998). These limits were not exceeded at any study site in either river systems, however three sites (A3, A6 and A8) were at the maximum of the aquatic ecosystem limit, suggesting anthropogenic inputs at these sites may eventually impact ecosystem integrity (Fatoki & Awofolu 2003). The source of zinc within waterways is influenced in some cases by catchment geology (likely the case for sites A6 and A8) but is often also the result of sewage contamination. SAWQG set safe limits of copper at 1.00 - 3.00 mg/L for domestic water use, and all sites in both systems fell within a range of 0.01 - 0.02 mg/L. Therefore from a purely micronutrient perspective, inputs from both the BVSTWs and ASTWs and run-off from adjacent agricultural lands have resulted in aquatic ecosystems whose water exceeds both the SAWQG guidelines for safe effluent discharge and in some cases water for domestic and aquatic biota use. There are numerous socio-economic and ecological factors which may be driving these issues, including a lack of proper waste management practices, lack of infrastructure or competent personnel and drought in some cases.

Physicochemical and micronutrients ananlysis have, to some extent provided useful information and categorization of water quality in the Bloukrans-Kowie and Bushmans-New Year's rivers. There are many challenges however, associated with using micronutrient analyses and physicochemical variables to describe water quality and ecosystem intergrity, particularly as instantaneous measurements are highly variable in both time and space. Coupling these evironmental variables with time integrated biological data e.g. aquatic plants and macroinvertebrates, can provide better resolution towards biological monitoring.

Therefore the following Chapter 3, will incorporate on-site prevailing environmental variables (Chapter 2) with abundances, composition and biodiversity of aquatic macroinvertebrates, to give a better understanding of ecologically important environmental variables that drives the composition of aquatic biota and the ecological state of both the Bloukrans-Kowie and Bushmans-New Year's River systems.

CHAPTER THREE

COMMUNITY COMPOSITION OF AQUATIC MACROINVERTEBRATES IN TWO RIVER SYSTEMS, EASTERN CAPE, SOUTH AFRICA

3.1 Introduction

Previously, the majority of ecological studies have mostly concentrated on terrestrial ecosystems, and only recently that aquatic ecosystems have gained some attention (Dudgeon *et al.* 2006, de Moor & Day 2013). However, marine ecosystems represent about 70% of the Earth's surface, while freshwater ecosystems represent the smallest percent of the Earth's surface, yet they are considered to be the most disturbed ecosystems worldwide (Covich *et al.* 1999; 2004). According to Pearce (1998) and Heal (2000) the nature of these freshwater disturbances compromises the social, ecological and economic services of these ecosystems and unfortunately in most cases the damage is said to be irreversible. As a consequence, drastic declines in aquatic biodiversity, which have a global effect on the storage, cycling and recycling of materials, nutrients and energy flow, have been reported (Dallas & Day 1993). Thus, freshwater ecosystems have been used to investigate environmental health using freshwater water organisms which act as integrators of anthropogenic effects accumulated from land-use practices within catchments. This practice has been steadily developing in recent literature, with tests and applications in numerous aquatic ecosystem studies on a global scale been successful (e.g. Walley & Hawkes 1996, Chutter 1998, Rosenberg *et al.* 1986, Masese *et al*. 2009, Bere & Nyamupingidza 2014). Due to the high levels of disturbance experienced by freshwater ecosystems, to mention one; dragonflies (Odonata) have been reported threatened (Samways & Taylor 2004, Samways 2006, Simaika & Samways 2009, Simaika & Samways 2012). However, predictions have highlighted that a substantial number of other aquatic insects will be candidates for inclusion on the IUCN red list if the current freshwater threats are not attended (Samways 2006). Collectively Allan & Flecker (1993), Ndaruga *et al.* (2004), Dudgeon *et al.* (2006), Beyene *et al.* (2009) and de Moor & Day (2013) categorized freshwater threats into five interrelated groups; over-exploitation, water pollution, habitat loss and/or degradation, alien

species invasion and climate change. All of these are commonly considered to be driving factors behind the global decline in freshwater biodiversity, and in some cases leading to ecosystem collapse. South Africa is an arid country, freshwater is in high demand, however multiple anthropogenic stressors, including sewerage and agricultural run-off, have find their way into majority of South Africa's rivers (Dallas & Rivers-Moore 2014). These pollution inputs have modified the physical and chemical properties of freshwater ecosystems, which in turn have resulted in the extirpation of sensitive indigenous aquatic species and promotion and the establishment of more tolerant alien species (Coetzee & Hill 2012). Hill *et al.* (2012) and Theobald *et al.* (2015) suggest that such rapid land transformation and the exploitation of natural resources might be attributed through increased human population and related human footprint on the environment, particularly in developing countries. Furthermore it is also anticipated that with recent climate change cases, organisms that manage to withstand current threats (e.g. eutrophication, impoundments, pollution) may or may not survive the additional pressure exerted by long term changes in climate (Heino *et al.* 2007).

Rivers are longitudinal systems divided into three zones e.g. head waters, foothill reaches and lower reaches, and driven largely by the flow of freshwater. These zones are distinct in their physical, chemical and biological characteristics which are primarily underpinned by geographical location and altitude (Vannote *et al*. 1980, McGeoch 1998, Allan & Flecker 1993). This was clearly illustrated by Covich *et al*. (1999), who showed that the spatial and temporal distribution of aquatic macroinvertebrates have differing habitat preferences which can be closely correlated to specific physical and chemical variables range (e.g. water temperature, concentration of dissolved salts, water velocity and substrate types). However, Ndarunga *et al.* (2009) argues that in the age of the 'anthropocene', these concepts seldom hold true for the majority of lotic systems due to the longitudinal changes in environmental variables caused by anthropogenic activities, due to the ecological settings of river systems in their catchments subjected to waste easily. Therefore, in order to monitor and quantify such anthropogenic changes there is a need for techniques that will be able to reflect changes within such complex ecosystems (Hill *et al.* 2012, Simaika & Samways 2012). Previously, in many developing countries, the assessment of stream's ecological health and water quality was determined using only physical and chemical properties (Beyene *et al.* 2009, Beneberu *et al.* 2014). These types of

assessment however, only provide an instantaneous "snap shot" of aquatic ecosystem health, because of the potential for water chemistry to be highly variable and influenced by flow regimes and the time of the day the water sample was taken (Dickens & Graham 2002, Nilsson & Renöfält 2008). Beneberu *et al.* (2014) also further emphasized that operating costs for the laboratory equipment required for chemical analysis and in particular, heavy metals, is very costly. Therefore, only recently that the cost effective and non-specialist biological monitoring techniques were introduced, that can provide a more temporal and spatially integrated measure of water quality, and involving the use of aquatic macroinvertebrates (Chutter 1998, Dallas 2004, Bere & Nyamupingidza 2014). Aquatic macroinvertebrates constitute an important component of aquatic biodiversity (Chutter 1994). As a group they are sensitive to disturbance and respond to both natural and man-induced changes within the environment (Chutter 1998). With ecological research developments in South Africa, the recent establishment of the NAEHMP-RHP involves the use of aquatic macroinvertebrates as reliable biological monitoring tools to characterize the ecological health of South Africa's freshwater ecosystems (Dallas 2007). This programme aims to promote standardized and continuous monitoring for southern African river systems and provide reports on river health and river rehabilitation implementation programmes. This is currently implemented using the South African Scoring System version 5 (SASS5) (Chutter 1994, Dickens & Graham 2002). In general, the SASS5 technique focuses on the sensitivity or tolerance of aquatic macroinvertebrates to water quality impairment. Aquatic macroinvertebrates have been considered good biological indicators for aquatic ecosystems since the late 1970's due to their (1) diversity and ability to colonize the majority of aquatic habitats, (2) dispersal ability, (3) capacity to show a measurable response towards external disturbances, (4) ease of identification (to family level for SASS5) and sampling and, (5) sedentary nature, indicating local disturbance (Rosenberg *et al.* 1986, Chutter 1994, Dickens & Graham 2002). For SASS5 assessments, each family is assigned a sensitivity weighting between 1 (very tolerant to pollution) and 15 (extremely sensitive to pollution). The SASS score, which is the sum of the sensitivity values of sampled/observed aquatic macroinvertebrates, is used to: (1) assess impacts of pollution and disturbance e.g. lower SASS scores = higher impact and (2) assess water quality and ecological health, using the Average Score per Taxa (ASPT) = SASS score/Number of observed taxa (Chutter 1998). SASS score and ASPT indices are very similar to the biological monitoring standards used globally (e.g. in Europe (BMWP) and Australia (AURIVAS) (Walley

& Hawkes 1996, Niemi & McDonald 2004). The SASS5 technique has been vigorously tested, widely used and further adopted by a number of southern African countries, such as Zimbabwe, Zambia and Mozambique, to assess water quality and ecological health of lotic systems (Bere & Nyamupingidza 2014). These theories, concepts and application techniques have assisted in determining the environmental water requirements in the context of legislation protecting South African water resources (National Water Act, No. 36 of 1998), the environment (National Environmental Management Act, No. 107 of 1998) and aquatic biodiversity (Biodiversity Act, No. 10 of 2004) (Dickens & Graham 2002, Dallas 2007).

This chapter aims to evaluate the ecosystem health, water quality and impact of anthropogenic activities within different land-uses (e.g. sewerage out-fall, waste disposal and agricultural run-off) using the SASS5 technique on the Bloukrans-Kowie and Bushmans-New Year's River systems, Eastern Cape, South Africa.

3.2 Material and Methods

3.2.1 Study sites & Data collection

Details on study sites, sample collection and SASS5 techniques are given in Chapter 2.

3.2.2 Data Analysis

General aquatic macroinvertebrate abundances

Aquatic macroinvertebrate community composition was compared between the two river systems using percentage abundances and unique versus shared individual taxa, using hand scored Absence (0)/ Presence (1) data (Appendix 4). All graphs and figures were created using SigmaPlot 10.0 (Systat 2006) and Microsoft Excel 2010.

South African Scoring System Version 5 (SASS5) and Shannon-Weiner index (H)

SASS scores, ASPT and Shannon-Weiner index were computed separately for each study site in each river system. SASS scores = the sum of all observed taxa sensitive value, ASPT = the sum of SASS scores/Number of Taxa (Appendix 5) (Chutter 1994) and the Shannon-Weiner

index was determined using the Shannon (1948), Weaver & Shannon (1949) and Henderson (2003) function:

$H = -\sum_{i=1}^{S_{obs}} p_i \log_e . p$, where p_i = proportion of sample in the i^{th} species.

A Nonparametric analysis, Kruskal-Wallis ANOVA was completed to investigate significant differences between biological indices (e.g. SASS scores, ASPT and Shannon-Wiener index (*H*)) from each site, separately for each river system. SASS score, ASPT and *H* were considered dependent variables and study sites was the grouping variables. Significance was considered at a confidence interval of 99.95% ($p < 0.05$). A multiple (2 tailed) comparison of mean ranks of all group test was further completed to indicates sites that were significant from the rest.

The strength of association between SASS scores versus ASPT and ASPT versus *H* was tested using the Pearson's correlation coefficient (*r*) at a confidence interval of 99.99% ($p < 0.01$). All statistics were completed in Statistica 12 (Stat Soft Inc. 2008-2014) and SigmaPlot 10.0 (Systat 2006).

Aquatic Macroinvertebrate Community Analysis

Bray-Cutis Similarity

The estimated abundance of macroinvertebrates collected on quarterly basis was summed over all four months to represent the entire study period. The data was first pre-treated by square-root transforming, normalized and converted to Bray-Curtis resemblance matrix. This was to ensure that there was an internal adjustment to place all variables on a common scale. Using PRIMER v6 add-on package PERMANOVA+, a Bray-Curtis similarity dendrogram, using the group average method was performed to assess the similarity between sites based on abundance and absence/presence of macroinvertebrates. This was following Clarke & Warwick (2001)'s recommendation that Bray-Curtis similarity is more useful in assessing similarity in ecological studies because it is not affected by absences and gives more weight to abundance in comparison taxa/species.

RELATE function

The relatedness and/or correlation between macroinvertebrates abundance (Bray-Curtis distance matrix) and the environmental variables (Euclidean distance matrix, Chapter 2) was analyzed using the RELATE function (Permutation test N = 999, $p < 0.05$). PRIMER v6 add-on package PERMANOVA+ allows users to compare two sets of multivariate data based on a matching set of samples, by calculating a rank correlation coefficient between all the elements of their similarity matrices (Clarke & Warwick 2001).

BEST function

The BEST function has the ability to identify a set of environmental variables that best describe a set of biological data (e.g. macroinvertebrate abundance). Using the BVSTEP, a stepwise selection method (PRIMER v6 add-on package PERMANOVA+) usually used in conjunction with a large number of environmental variables and the Spearman rank correlation (Permutation test N = 999, $p < 0.05$) was used to identify a combination of environmental variables that best correlated with the sampled biological abundance data (Clarke & Warwick 2001).

Distance Based Linear Model (DistLM)

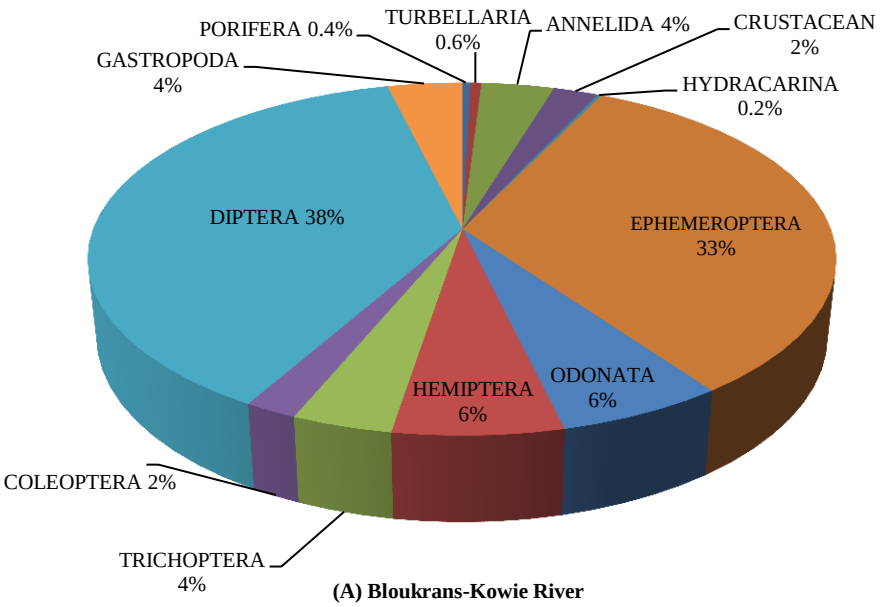
A multiple regression approach, Distance-based linear Model (DistLM) was used to model and illustrate the macroinvertebrates abundance pattern using environmental variables. Using a stepwise selection procedure, second-order Akaike's information criterion (AICc) for selection criteria and the marginal test, to test the significance of one environmental variable against the biological data (Permutation test N = 999, $p < 0.05$) (Clarke & Gorley 2001). The BEST function only gives a combination of environmental variables, however it does not explain how much percentage variation selected variable account for on the biological data. Using the force inclusion method, BEST identified 6 environmental variables that is water temperature, NH_4-N, NO_3-N, substrate diversity, flow rate and turbidity where included in the model. Additionally, variables that showed to have a significance effect (marginal test, $p < 0.05$) on the biological data were also included. This was to better achieve a combination of environmental variables that would best (> 50%) explain macroinvertebrates abundance patterns (Clarke & Warwick 2001). PRIMER v6 add-on package PERMANOVA+ only works with distance

measures, therefore Distance based Redundancy analysis (bd-RDA) bi-plot was created to illustrate the combination of all significant environmental variables from the DistLM analyses and marginal test that best described macroinvertebrates relative abundance patterns (Clarke & Warwick 2001).

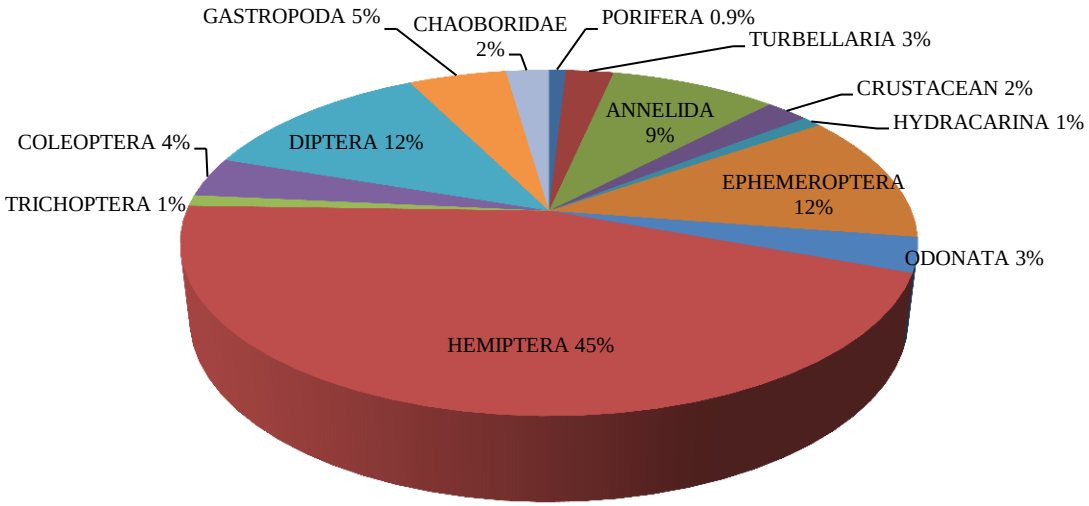
3.3 Results

3.3.1 Aquatic Macroinvertebrates Presence and Abundance

A total abundance of 23 681 aquatic macroinvertebrate specimens, belonging to 56 taxa, were identified throughout the duration of this study. Sites with diverse biotopes generally yielded more taxa as compared to sites with fewer or single biotopes (see Table 2.1 & 22, Chapter 2). Most aquatic invertebrate taxa expected to be present in lotic systems were observed in this study, with six dominant Insecta orders; Diptera (30%), Ephemeroptera (27%), Hemiptera (18%), Odonata (5%), Trichoptera and Coleoptera (3%), and the rest comprising approximately 14% (Crustacean, Gastropoda, Annelida, Turbellaria, Porifera and Hydracarina). The Bloukrans-Kowie River system had the highest number of individual macroinvertebrate (n = 16 290, 52 taxa); with Diptera, Ephemeroptera, Hemiptera, Odonata and Gastropoda as the dominant taxa (Figure 3.1A) and the Bushmans-New Year's River system (n = 6791, 51 taxa); with Diptera, Ephemeroptera, Hemiptera, Annelida and Coleoptera as the dominant taxa (Figure 3.1B). Furthermore, from the 56 macroinvertebrate taxa sampled, 47 were observed on both river systems, while only five taxa (Chlorocyphidae, Platycnemidae, Lepidostomatidae, Pisuliidae and Athericidae) and four taxa (Atyidae, Hydraenidae, Planorbidae and Chaoboridae) were observed only on the Bloukrans-Kowie River and Bushmans-New Year's River system respectively (Figure 3.2).



(A) Bloukrans-Kowie River



(B) Bushmans-New Year's River

Figure 3.1: Pooled relative abundances (%) of aquatic macroinvertebrates for (A) the Bloukrans-Kowie River system (n =16 290) and (B) the Bushmans-New Year's River system (n = 6791), sampled quarterly from August 2013 to August 2014 in the Eastern Cape, South Africa.

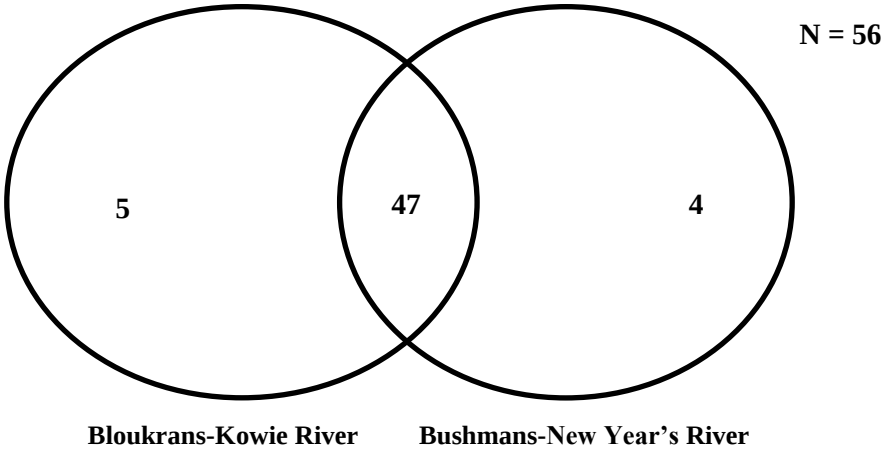


Figure 3.2: Venn diagram illustrating shared and unique aquatic macroinvertebrate taxa observed between the Bloukrans-Kowie and Bushmans-New Year's River systems Eastern Cape, South Africa.

3.3.2 South African Scoring System5 (SASS5), Average Score per Taxon (ASPT) and the Shannon-Wiener index (H)

The Bloukrans-Kowie River had a SASS score ranging between 22.67 – 160.00, where site A8 and A10 had a SASS score > 130, A6 and A9 SASS score > 150 and the rest of the sites having a SASS score < 100. The Bushmans-New Year's River on the other hand, showed a range of between 16.24 – 113.00. Site B8 and B9 had the highest SASS score equating to 95.75 and 113.00 respectively, and the rest of the sites scored < 90. In general, all study sites showed a ASPT value of < 6, with the Bloukrans-Kowie River ranging between, 2.95 – 5.99 and the Bushman-New Year's River 2.65 – 5.14. The Bloukrans-Kowie River showed an average Shannon-Wiener index *H* of 1.89 ± 0.69 ranging between 0.5 – 2.66, with site A10 being the highest (*H* = 2.66). The Bushmans-New Year's River showed the highest average Shannon-Wiener index, with *H* = 1.96 ± 0.49, ranging between 0.64 – 2.62 and site B8 scoring the highest with *H* = 2.66. Significant difference in *H* between sites were driven predominantly by high biodiversity scores at site A7 and A10 on the Bloukrans-Kowie River, but by only one site (B8) on the Bushmans-New Year's River (Table 3.1 & 3.2). Strong positive highly significant

correlations were observed between SASS scores and ASPT values on the Bloukrans-Kowie River (r = 0.93, p < 0.001; y = 2.63 + 0.02605x) and on the Bushmans-New Year's River (r = 0.86, p < 0.001; y = 2.60 + 0.023x) (Figure 3.3A & B). This was expected since ASPT is derived from the SASS score. ASPT and H values also showed a correlative trend on the Bloukrans-Kowie River (r = 0.72, p < 0.001; y = -0.14 + 0.430x) and the Bushmans-New Year's River (r = 0.58, p < 0.001; y = 0.404 + 0.037x) (Figure 3.4A & B). Multiple comparison mean rank results showed a significant differences between SASS scores and the highly correlated ASPT which were mostly due to higher SASS scores at site A6, A8 and A9 on the Bloukrans-Kowie River and site B8 and B9 on the Bushmans-New Year's River. (Table 3.1 & 3.2).

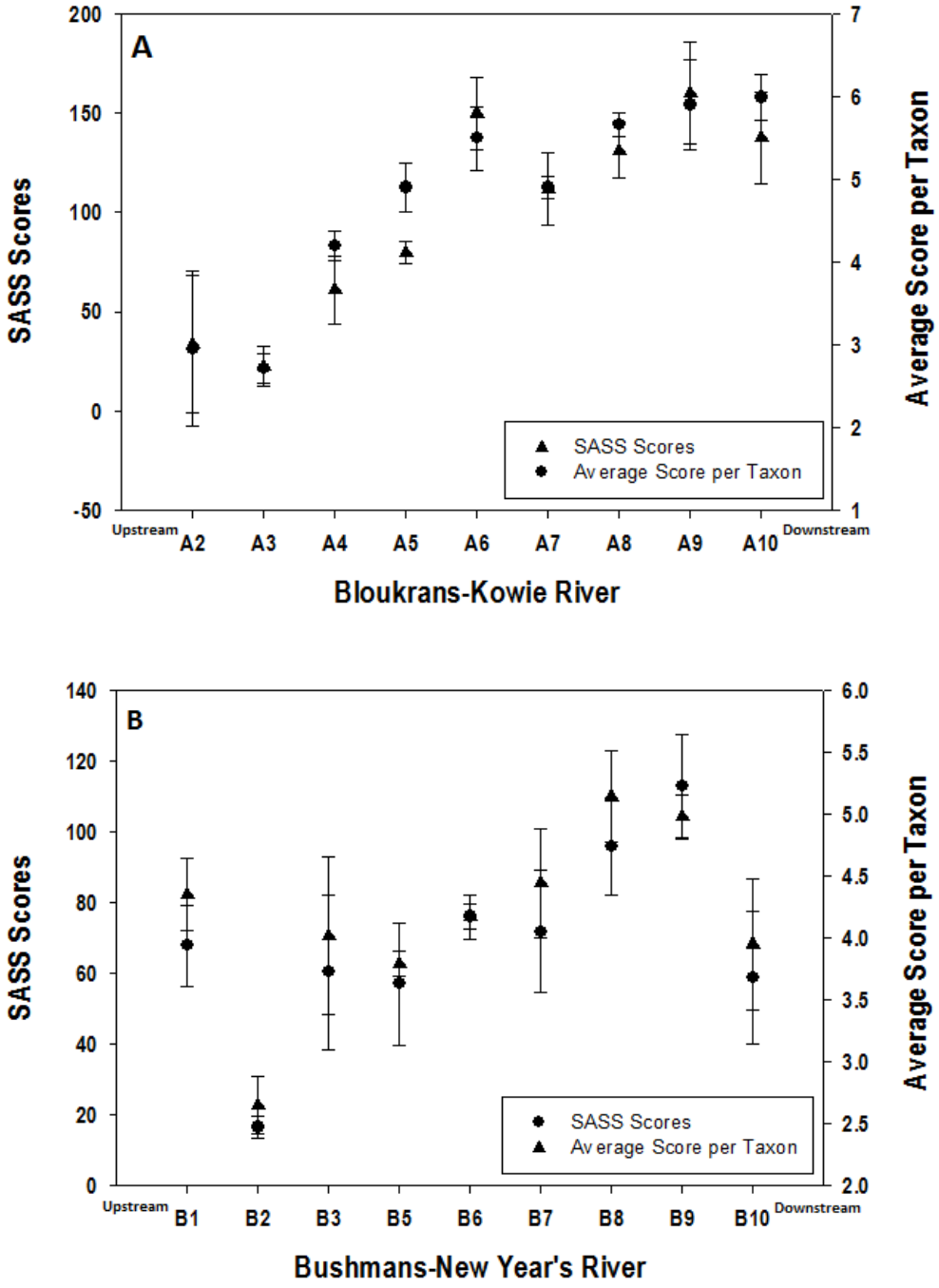


Figure 3.3: Scatter plots illustrating the relationship between average SASS scores and ASPT on (A) the Bloukrans-Kowie River (r = 0.93, p < 0.001) and (B) the Bushmans-New Year's River systems (r = 0.86, p < 0.001), Eastern Cape South Africa. Error bars represent ± 1 SD.

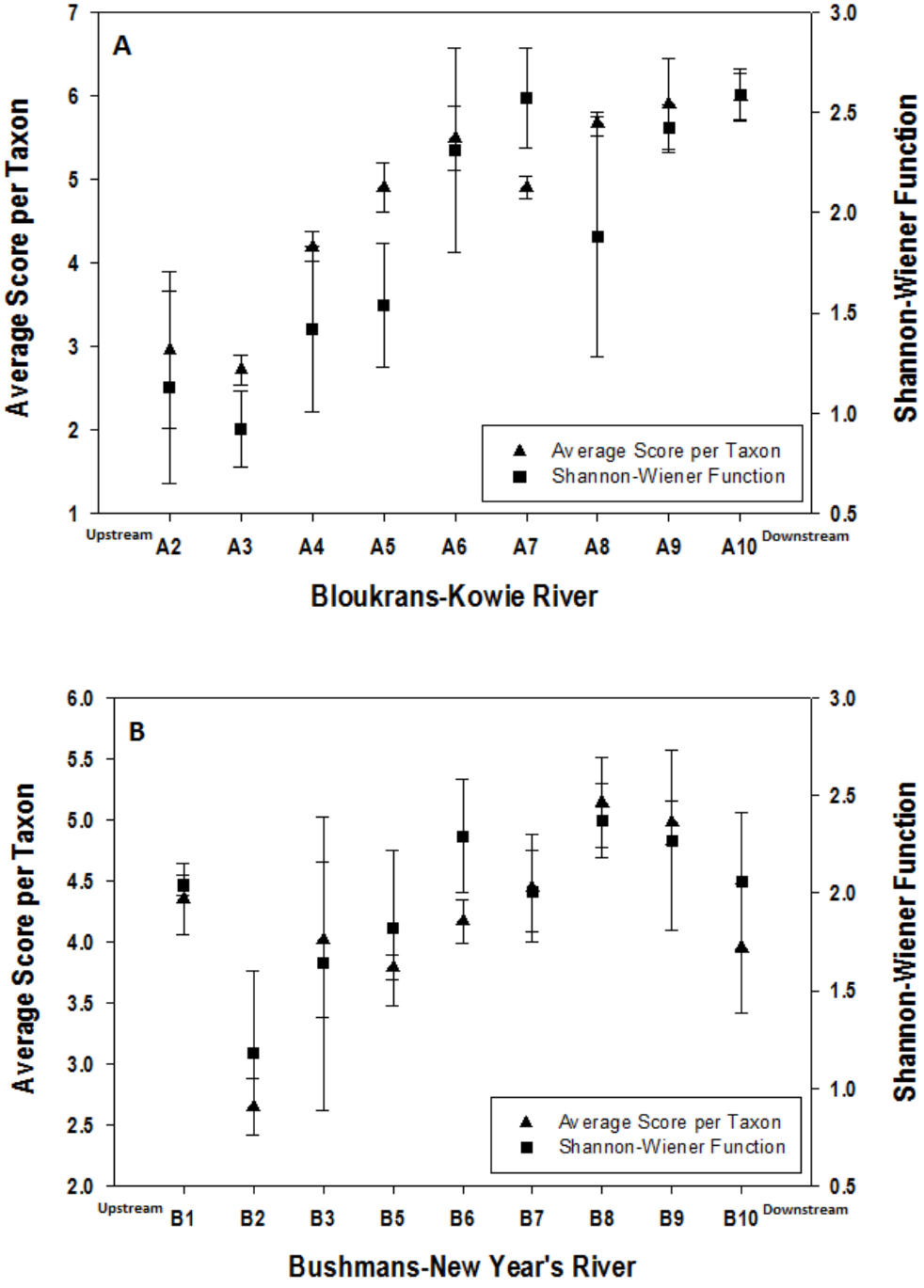


Figure 3.4: Scatter plots illustrating relationship between average ASPT and the Shannon-Wiener index (*H*) on the Bloukrans-Kowie River ($r = 0.72$, $p < 0.001$) and the Bushmans-New Year's River systems ($r = 0.58$, $p < 0.001$), Eastern Cape South Africa. Error bars represent ± 1 SD.

Table 3.1: Kruskal-Wallis ANOVA, multiple comparison of mean rank of all groups, results showing significant differences of the three calculated biodiversity indices (SASS score, ASPT and *H*) between sites on the Bloukrans-Kowie River system, Eastern Cape South Africa. Bolded values show a significance level of $p < 0.05$.

Bloukrans-Kowie River	**Sites**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**
SASS score	**A2**	-								
$H_{8, 36} = 30.13$, $p < 0.001$	**A3**	1.000	-							
	A4	1.000	1.000	-						
	A5	1.000	1.000	1.000	-					
	A6	0.058	**0.029**	0.261	0.964	-				
	A7	1.000	1.000	1.000	1.000	1.000	-			
	A8	0.335	0.183	1.000	1.000	1.000	1.000	-		
	A9	**0.030**	**0.014**	0.148	0.591	1.000	1.000	1.000	-	
	A10	0.319	0.174	1.000	1.000	1.000	1.000	1.000	1.000	-
ASPT	**A2**	-								
$H_{8, 36} = 30.85$, $p < 0.001$	**A3**	1.000	-							
	A4	1.000	1.000	-						
	A5	1.000	1.000	1.000	-					
	A6	0.113	0.081	0.884	1.000	-				
	A7	1.000	1.000	1.000	1.000	1.000	-			
	A8	**0.030**	**0.021**	0.304	1.000	1.000	1.000	-		
	A9	**0.013**	**0.009**	0.148	1.000	1.000	1.000	1.000	-	
	A10	0.447	0.335	1.000	1.000	1.000	1.000	1.000	1.000	-
H	**A2**	-								
$H_{8, 36} = 26.92$, $p < 0.01$	**A3**	1.000	-							
	A4	1.000	1.000	-						
	A5	1.000	1.000	1.000	-					
	A6	0.810	0.133	1.000	1.000	-				
	A7	0.156	**0.019**	0.539	1.000	1.000	-			
	A8	1.000	1.000	1.000	1.000	1.000	1.000	-		
	A9	0.964	0.165	1.000	1.000	1.000	1.000	1.000	-	
	A10	0.091	**0.010**	0.335	1.000	1.000	1.000	1.000	1.000	-

Table 3.2: Kruskal-Wallis ANOVA, multiple comparison of mean rank of all groups, results showing significant differences of the three calculated biodiversity indices (SASS score, ASPT and *H*) between sites on the Bushmans-New Year's River system, Eastern Cape South Africa. Bolded values show a significance level of $p < 0.05$.

Bushmans-New Year's River	Sites	B1	B2	B3	B5	B6	B7	B8	B9	B10
SASS scores	**B1**	-								
$H_{8, 36} = 25.36$, $p < 0.01$	**B2**	1.000	-							
	B3	1.000	1.000	-						
	B5	1.000	1.000	1.000	-					
	B6	1.000	0.469	1.000	1.000	-				
	B7	1.000	0.565	1.000	1.000	1.000	-			
	B8	1.000	**0.009**	1.000	0.709	1.000	1.000	-		
	B9	0.964	**0.001**	0.406	0.148	1.000	1.000	1.000	-	
	B10	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.261	-
ASPT	**B1**	-								
$H_{8, 36} = 26.80$, $p < 0.001$	**B2**	0.426	-							
	B3	1.000	1.000	-						
	B5	1.000	1.000	1.000	-					
	B6	1.000	1.000	1.000	1.000	-				
	B7	1.000	0.319	1.000	1.000	1.000	-			
	B8	1.000	**0.002**	0.539	0.061	1.000	1.000	-		
	B9	1.000	**0.004**	0.846	0.107	1.000	1.000	1.000	-	
	B10	1.000	1.000	1.000	1.000	1.000	1.000	0.964	1.000	-
H	**B1**	-								
$H_{8, 36} = 15.95$, $p < 0.05$	**B2**	1.000	-							
	B3	1.000	1.000	-						
	B5	1.000	1.000	1.000	-					
	B6	1.000	0.086	1.000	1.000	-				
	B7	1.000	1.000	1.000	1.000	1.000	-			
	B8	1.000	**0.022**	1.000	1.000	1.000	1.000	-		
	B9	1.000	0.447	1.000	1.000	1.000	1.000	1.000	-	
	B10	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-

3.3.3 Aquatic Macroinvertebrates Community Analysis

The Bray-Curtis Similarity dendrogram showed a fairly high percentage of similarity > 50% and divided all study sites into four main clusters (Figure 3.5). Cluster 1 comprised of two sites; A2 and A3, these sites were the upstream Bloukrans River sites, receiving polluted water, waste materials, treated and untreated waste water from the Grahamstown settlement and the Belmont Valley Sewerage Treatment Works (BVSTWs) respectively. Cluster 2 was the largest group with three sub-clusters of closely related downstream study sites from both river systems. These sites were characterized by diverse in-stream biotopes, particularly stones in-current, marginal vegetation, medium to high water flow and low turbidity (see Table 2.1, Chapter 2). Cluster 3 was site B2, which was the only site with extremely high turbidity, low/zero flow rate and no marginal or aquatic vegetation (according to Dickens & Graham 2002, categorizations). Lastly, cluster 4 comprised six Bushmans-New Year's River sites sharing similar characteristics with site B2 (cluster 3) e.g. primarily forming isolated pools, but with cluster 4 sites having patches of marginal vegetation.

RELATE Function

RELATE revealed that environmental variables (micronutrients) and macroinvertebrates (abundances) were significantly related, showing a significant medium strength correlation (r = 0.57, p = 0.001) between the environmental variables and macroinvertebrate abundance pattern.

BEST Function

BEST analysis identified a combination of six environmental variables including water temperature, NH_4-N, NO_3-N, substrate diversity, water flow and turbidity which showed a significantly strong correlations (r = 0.82, p = 0.001).

DistLM Analysis

The DistLM analysis marginal test, which tested individually the significance of one environmental variable against macroinvertebrates abundance data, revealed pH, conductivity (EC), dissolved oxygen (DO), sodium (Na^{+}), calcium (Ca^{2+}), magnesium (Mg^{2+}), iron (Fe^{+}), chlorine (Cl^{-}), carbonate ($CO3^{2-}$), bicarbonate (HCO^{3-}), sulphate (SO_4^{2-}), boron (B^{+}), manganese (Mn^{2+}), phosphate (P^{3-}), fluorine (F^{-}) and total dissolve solids (TDS) to be significant, having an

effect on the variation of macroinvertebrate abundance. The DistLM was performed using the force inclusion method, were a subset of six BEST environmental variables (water temperature, NH_4-N, NO_3-N, substrate diversity, water flow and turbidity) and an addition of highly significant ($p < 0.01$) environmental variables from the marginal test (pH, EC, DO, Fe and P) were included into the analysis. The distance-based redundancy analysis (db-RDA) bi-plot was the outcome illustration of the DistLM analysis, where axis1 and 2 cumulative showed 65.80% of the fitted model and explained 56.20% variation of macroinvertebrates abundance and environmental variables. A total collective variation of 85.40% was explained by the model (Table 3.3).

In general db-RDA bi-plot describe the Bloukrans-Kowie river system sites to be very much orientated on the vertical plane which is related to the chemical composition (nutrients), whereas the Bushmans-New Year's river system seemed to be more horizontally orientated relating to physical composition (flow, water temperature, turbidity). In comparison with the Bray Curtis dendrogram, the db-RDA bi-plot also identified four main clusters. Cluster 1 (site A2 and A3) showed strong correlations towards nutrients e.g. NO_3-N and NH_4-N; cluster 2 (A4 and A5), showed strong correlations with P concentrations and water flow; cluster 3 was the largest group of sites with B8 and B9 together with sites A6, A7, A8, A9 and A10, which were grouped together based on substrate diversity, pH, EC and DO as the main environmental drivers. Cluster 4 (sites B1, B2, B3, B5, B6, B7, B10) all of which were on the Bushmans-New Year's River had high turbidity, increased water temperatures and elevated concentrations of Fe (Figure 3.6).

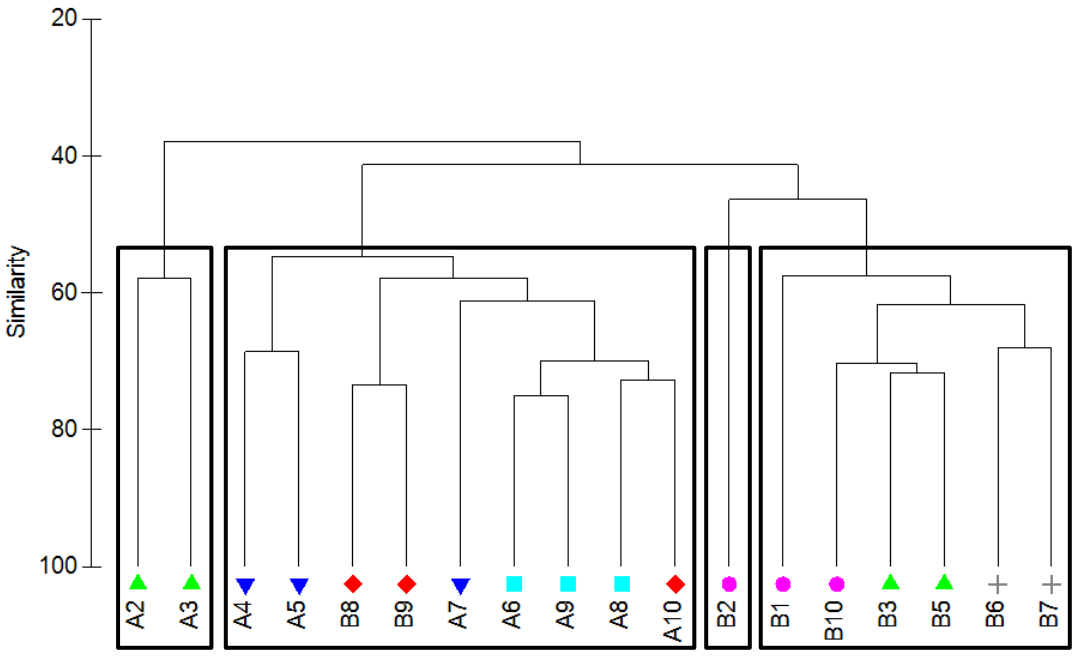


Figure 3.5: Bray-Cutis similarity dendrogram based on aquatic macroinvertebrate relative abundance pattern on both the Bloukrans-Kowie (A sites) and the Bushmans-New Year's rivers (B sites). Each symbol represents a different land-use/site description (please see Chapter 2, Table 2.1 for more details): (▲) - sewage input; (▼) - dairy farms; (♦) - confluence; (■) - undisturbed habitats; (●) - isolated pools; (+) - golf course (commercial fertilizer).

Table 3.3: Distance based linear model percentage variation of selected environmental variables describing aquatic macroinvertebrate abundance (AICc = 181.9, r^2 = 0.85, RSS = 3643.3, number of variables = 11). .

Axis	% explained variation out of fitted model		% explained variation out of total variation	
	Individual	Cumulative	Individual	Cumulative
1	40.7	40.7	34.7	34.7
2	25.1	65.8	21.5	56.2
3	10	75.8	8.5	64.7
4	7.0	75.8	5.9	70.7
5	5.9	82.8	5.1	75.8
6	4.3	88.9	3.7	79.5
7	2.8	93.1	2.4	81.9
8	2.3	95.9	1.9	83.8
9	1.1	99.3	0.9	84.7
10	0.7	99.9	0.6	85.3
11	0.07	100	0.06	85.4

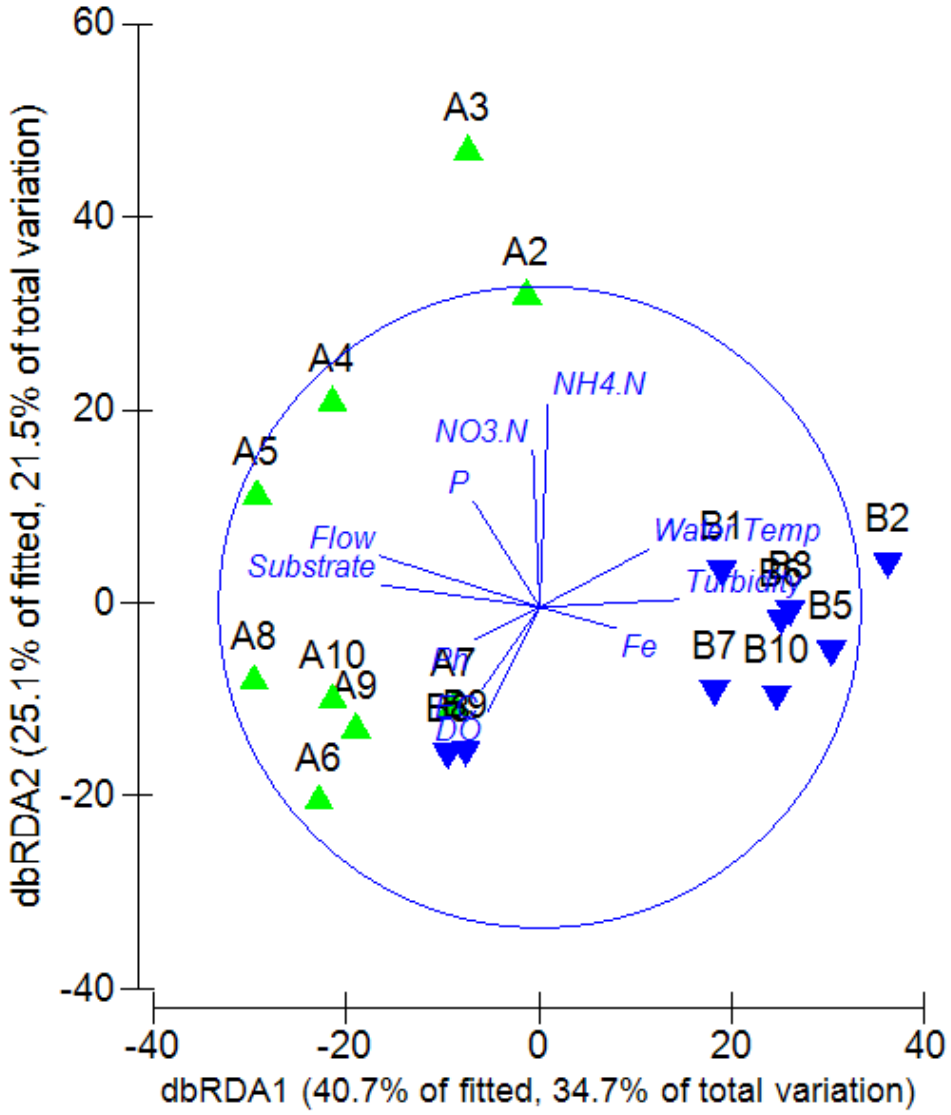


Figure 3.6: Distance based Redundancy Analysis bi-plot illustrating interactions between aquatic macroinvertebrate abundance with the best combination of environmental variables that describe the biological data (▲ – Bloukrans-Kowie River; ▼ – Bushmans-New Year's River).

3.4 Discussion

Physicochemistry and biotic indicators

Changes anywhere within the landscape (either natural and/or human induced) which find their way into the river system can be reflected in the composition of the resident aquatic biota, particularly within communities of macroinvertebrates (Dickens & Graham 2002). Aquatic macroinvertebrate communities have therefore been considered good biological indicators of pollution and physical disturbance (Masese *et al.* 2006, Bredenhand & Samways 2009). According to Allanson *et al*. (1990) and de Moor et al. (2013), both the Bloukrans-Kowie and the Bushmans-New Year's River systems sit within similar geographical region, therefore according to Vannote *et al.* (1980) and Eady *et al.* (2013) they are expected to experience similar environmental conditions and display similar biological compositions. Mathooko & Mavuti (1992) and Kibichii *et al.* (2007) investigated streams on Mount Kenya and Njoro river in Kenya were they found that macroinvertebrate communities were mostly dominated by *Baetis* sp. (Ephemeroptera: Baetidae) and *Simulium* sp. (Diptera: Simuliidae). Closer to home, Bredenhand & Samways (2006) also recorded high abundances of baetids and simuliids in the Western Cape Mountains, South Africa. This was also true on the Bloukrans-Kowie River, where baetids and simuliids were found to be the two most abundant macroinvertebrate families. Additionally the Lepidostomatidae, Pisullidae, Chlorocyphidae, Platycnemidae and Athericidae families were unique to the Bloukrans-Kowie River. Generally these families prefer dwelling on rocky substrates such as the 'stones' biotope and are usually found in continuously flowing currents with low levels of dissolved salts but high dissolved oxygen (de Moor *et al*. 2003B). Conversely, on the Bushmans-New Year's River, Corixidae and Notonectidae were the most abundant families, with Atyidae, Hydracarina, Planorbidae and Chaoboridae unique to the Bushmans-New Year's River. According to Mantel *et al.* (2010), these opportunistic and generalist macroinvertebrate taxa are more tolerant to disturbed habitats and are capable of exploiting available space and food source abandoned by sensitive macroinvertebrates. Hemipterans like Corixidae and Notonectidae for example, are air breathers and exhibit a predatory life style, therefore they are not limited by low oxygen concentrations and thus they are more flexible, capable to overlap between disturbed and undisturbed habitats, ensuring maximum survival (Gerber & Gabriel 2002B). Size and land-use were considered to be different in between

sampled sites and rivers, anthropogenic inputs and consequently the physical and chemical characteristics of each river system were different, which subsequently influences macroinvertebrate community composition to differ.

Biological monitoring using the SASS5 technique was able to identify disturbance effects on both rivers. Although SASS scores alone cannot identify disturbance events, site inspections lead us to hypothesize that this included sewerage effluents (treated and untreated), agricultural run-off and disposal on non-biodegradable materials. According to Dickens & Graham (2002), when dealing with pollution inputs, SASS scores are more meaningful than the ASPT. Disturbance and pollution tolerant taxa are characterized by lower SASS scores (Gerber & Gabriel 2002B), and thus will result in an overall lower SASS score value when compared to undisturbed sites (Chutter 1998). Overall, the Bloukrans-Kowie River had the highest SASS scores, with five sites (A2-A5, A7; SASS score < 100) identified as being severely/critically disturbed and the remaining sites classified as moderately disturbed (A8 and A10; SASS score > 130) and largely natural with only minor disturbance (A6 and A9; SASS score > 150) as described by Dallas (2007). Site A2-A5 on the Bloukrans River were situated downstream of Grahamstown residential area and the BVSTWs, these sites were likely experiencing daily inputs of enriched nutrient run-off from wastes up the catchment. Comparatively, site A7 is further downstream and is surrounded by agricultural lands, particularly cabbage and pineapple plantations as well as dairy farms which may be frequently fertilized using cow manure (Eady *et al.* 2013, Dalu *et al.* 2014). Constant anthropogenic inputs experienced by majority of the Bloukrans River were attributed by low SASS scores, identifying these sites to be critically disturbed. Site A6 and A9 were considered largely natural, which were situated at the upper reaches of the Kowie River, surrounded mainly by natural habitat, game farms and privately owned land. Two 'moderately disturbed' sites; A8, which was downstream the Bloukrans River and before the junction with the upper Kowie reaches and A10, which was after the confluence of the Bloukrans River and Kowie River, together represent ecosystem experiencing diluted anthropogenic inputs. These two sites were not as disturbed as upstream sites, suggesting some potential recovery ability of the river, likely dependent on the distance from nutrient entry points and continuous water flow of the river (e.g. Ifabiyi 2008). Comparatively, the SASS scores on the Bushman-New Year's River were much lower than on the Bloukrans-Kowie River system. According to categorizations by Dallas (2007), the majority of the Bushmans River sites (B1-B7

and B10; SASS score < 90) were regarded as severely/critically disturbed, while remaining sites (B8 and B9; SASS score = 95.7 and 113.00 respectively) were categorized as largely modified. There were a number of factors that affected the low values SASS scoring, ASPT and macroinvertebrate diversity (*H*) on the Bushmans-New Year's River system; firstly, Alicedale, for the duration of the study experienced minimal rainfall, and so water flow was severely obstructed, this resulted in numerous isolated pools forming along the river, rather than one continuous system. Secondly, the system possessed poor biotope diversity due to the formation of isolated pools and only comprised mainly by muddy and sandy biotopes, therefore missing the stones and gravel biotopes that house diverse stones and gravel dwelling taxa absent from the system (that indicate higher water quality; Dickens & Graham 2002). Thus these taxa substituted by general, opportunistic taxa (which indicate lower water quality; Dickens & Graham 2002), thus decreasing the classification of the overall river health (SASS score and diversity indices). Thirdly, the lack of rainfall also resulted in no currents changing the system from lotic to lentic and thus physical and chemical characteristics such as increased water temperature and elevated ion concentration were observed (see Bowd *et al.* 2006). Additionally, site B1 which was situated above New Year's Dam, had a significant water hyacinth infestation which is under biological control for almost a decade (Hill & Olckers 2001), which is regraded a threat to aquatic macroinvertebrate biodiversity (Masifwa *et al.* 2001, Midgley *et al*. 2006, Coetzee *et al.* 2014). Water hyacinth stops sunlight penetration to under water habitats, completes for space with aquatic organisms and in some cases is responsible for habitat loss for other aquatic taxa (Masifwa *et al.* 2001, Midgley *et al*. 2006, Coetzee *et al.* 2014). Site B2 was characterized by absence of both aquatic plants and marginal vegetation and had the highest turbidity for the period of this study, both of which create unfavorable micro-habitats for macroinvertebrates. Sites B3 and B5 were adjacent and downstream of the ASTWs respectively, and were likely influenced by nutrient inputs through sewage seepage, spillover and/or leaching through the groundwater to adjacent waterways. Site B6 and B7 were situated further downstream from the ASTWs within the Bushmans Sands Golf Course, which maintains its greens with a periodical application of commercial fertilizers (Hill *et al.* 2011). Site B10 on the Bushmans River was downstream before the confluence and was predominantly an isolated pool throughout the duration of the study and was involved in a massive water abstraction pipelines. Only sites B8 and B9, situated after the Bushmans-New Year's River confluence were characterized by

medium flow and fairly diverse biotopes, creating suitable micro-habitats for diverse macroinvertebrate communities and thus yielded a moderately higher SASS scores.

Changes in SASS scores, ASPT and *H* along the Bloukrans-Kowie River system in this study suggest that downstream from anthropogenic inputs, the river had some ability to assimilate "self-purify". This ability has also been illustrated by Madikizela *et al.* (2001) and Bere (2007), where SASS scores and ASPT increased downstream from pollution hotspots. It is possible that with the appropriate physical conditions (e.g. adequate flow and diverse biotopes/substrates), river systems have the ability to flush out, dilute excess nutrients and/or riparian and aquatic vegetation takes up some nutrients from the system as a natural occurring restoration process along the river. Sites categorized as disturbed on the Bloukrans River had macroinvertebrate communities predominantly made up of Chironomidae, Culicidae and Hirudinea, all pollution tolerant taxa, and the more sensitive taxa, Ephemeroptera and Trichoptera families were observed further downstream, suggesting that ecosystem health improves with distance from pollution inputs. This was likely due to the continuous current and diverse biotopes in the Bloukrans River that were lacking in the Bushmans-New Year's River. Additionally, factors including the presence of riparian vegetation, land use, altitude, discharge rates, rainfall, substrate type and the resulting dissolved salt concentrations, pH, turbidity and temperature as discussed by Masese *et al.* (2009) and Mantel *et al.* (2010) would have influenced the water quality of the systems in question. With so many factors at play, the SASS score metric on its own, can only provide "red flag" results (polluted/disturbed or unpolluted/undisturbed) and cannot identify the factors driving changes in ecosystem health. Thus most studies couple the SASS5 assessments with either environmental variables, fish species, diatoms or habitat health assessments to further explain variation unaccounted by SASS5 indices (Dickens & Graham 2002). Dickens & Graham (1998), Ndarunga *et al.* (2004), Beyene *et al.* (2009) and Bere & Nyamupingidza (2014) suggest that a multiple regression approach may provide a subset of environmental variables which give the most efficient explanation of the variation in the sampled aquatic macroinvertebrates from both river systems. The RELATE analysis in the present study used this approach and showed strong correlations between majority of the environmental variables and macroinvertebrates abundance. Bere & Nyamupingidza (2014) also found a significant correlation between SASS scores, ASPT and some water chemistry variables (physical and chemical parameters) in Zimbabwe streams. Similarly, a high correlation between

SASS scores and ASPT values and a similar association between ASPT and Shannon-Wiener biodiversity index were observed in this study. These findings show that the relationship at each site between macroinvertebrates abundance and physicochemical parameters are strongly complementary, suggesting that a combination of biological monitoring and water chemistry analyses will reliably identify sites with poor water quality and/or ecosystem health.

The distance based redundancy analysis showed that sites on both rivers could be categorized into four groups, defined by 4 different sets of environmental variables. The first group was comprised of sites with poor water quality, where variations in macroinvertebrates abundance was best described by elevated concentrations of NO_3-N and NH_4-N, and were predominantly pollution tolerant taxa such as Chironomidae, Muscidae, Culicidae, Oligochaeta and Syrphidae (Rosenberg *et al.* 1986, Loch *et al.* 1996, Dickens & Graham 2002). Generally, these organisms possess haemoglobin, enabling them to increase oxygen uptake in eutrophic and organically enriched waters, increasing their chances of survival in polluted waters (Rosenberg & Resh 1993, Ndarunga *et al*. 2004, Bere & Nyamupingidza 2014). The second group of sites was the downstream sites from the severely disturbed sites on the Bloukrans River system, likely experiencing diluted anthropogenic inputs (sewage effluent and/or urban pollution carried downstream) and were driven primarily by increased concentrations of P and higher river flow rates. Group three was the largest group, mainly dominated by downstream study sites on both river systems, and here macroinvertebrate abundances were driven by an increase in substrate diversity (i.e. number of available biotypes) and lower values of pH, EC and DO. Contrastingly, increased water temperature, turbidity and iron (Fe) concentrations described macroinvertebrate abundances in the fourth group (comprised primarily of sites on the Bushmans-New Year's River system) which adequately describes lentic and/or standing water ecosystems. Corixidae, Notonectidae, Pleidae, and certain Baetidae and Atyidae were found in high abundance at these sites.

Biological monitoring using the South African Scoring System in conjunction with analyses of water chemistry (physicochemistry and micronutrients) in the present study revealed useful information on the ecological health of both river systems. Overall the Bloukrans-Kowie River was in better health, while the Bushmans-New Year's system was critically impacted, likely due to the shift from a lotic to lentic environment. Sites directly adjacent to anthropogenic

inputs or stressors were in understandably poorer health, however the positive relationship between ecosystem health and distance from pollution inputs suggests that in some cases, river systems can provide a measure of self-purification. While the SASS5 technique and its subsequent interpretation is robust as a spot measurement of ecosystem health – there are a number of factors that SASS5 does not account for that can contribute to change in disturbance rating. Furthermore the SASS5 technique was (1) clearly site dependent, (2) was seriously influenced by a lack of diversity in available biotopes and thus is limited to use in lotic ecosystems, (3) and cannot provide information on the nature of the disturbance. Additionally it requires expertise and training in taxonomic identification and application (accreditation) for appropriate use. All of these ecological drawbacks are acknowledged by Dickens & Graham (2002) and are further illustrated by Simaika & Samways (2012) as well in the present study. While this chapter acknowledges the use and application of biological monitoring in conjunction with water chemistry measurements, future management of South Africa's freshwater systems requires more refined techniques that; (1) can include all aquatic ecosystems, (2) are not habitat or substrate dependent, (3) can help to identify the sources of pollution and, (4) that can provide temporal and spatial information on anthropogenic pollution and act as an early warning indicator, before the onset of ecosystem degradation.

The next Chapter 4 will introduce an internationally adopted N-loading/eutrophication mapping technique that will address the above-mentioned biological monitoring challenges.

CHAPTER FOUR

STABLE ISOTOPE ANALYSIS (SIA), A NEW STEP IN BIOLOGICAL MONITORING: A CASE STUDY OF TWO RIVER SYSTEMS IN THE EASTERN CAPE, SOUTH AFRICA

4.1 Introduction

In the past, aquatic ecosystem health has been monitored using a number of techniques, that includes water toxicity measurements (heavy metals; Fatoki & Awofolu 2003, de Villiers 2007), microbial assemblages assessments (e.g. *Escherichia coli* counts; Xu *et al.* 2011, Seanego & Moyo 2013), taxonomic changes in the abundance of aquatic biota (%EPT – Ephemeroptera, Plecoptera & Trichoptera; Camargo *et al.* 2004, diatoms; Taylor *et al.* 2007, fish; Kleynhans 1999, Loomer *et al.* 2014) and water quality assessment protocols using pollution tolerant and sensitive aquatic taxa (BMWP; Hawkes 1998, AUSRIVAS; Smith *et al.* 1999, IBI; Karr 1991, SASS5; Chutter 1998 and Dickens & Graham 2002, Dragonfly Biotic Index (DBI); Simaika & Samways 2012) (see Chapter 3). Globally, these traditional biological monitoring techniques have been widely used, however Hill *et al.* (2012) argues that these traditional techniques only reflect disturbances once they have manifested within waterways and they are not specific in identifying types of pollution. Dickens & Graham (2002) acknowledge that once disturbance events have occurred, it is a challenge to identify the type and/or nature of the disturbance, especially when dealing with non-point source pollution events (see Chapter 3). Similarly, direct and indirect anthropogenic nutrient loading arises from a variety of activities (e.g. treated and untreated sewage discharges, aquaculture and livestock grazing), therefore it is difficult to unambiguously determine the nutrient sources and their related impact on an ecosystem (Jona-Lasinio *et al.* 2015). Recently however, it has been suggested that stable isotopic values of aquatic organisms particularly that of macrophytes, can be used as sensitive indicators of eutrophication events and can provide a measure of water quality (Costanzo *et al.* 2001; 2005, Vander Zanden *et al.* 2005).

Stable isotopic analysis has been used for decades to map food webs and investigate trophic linkages in freshwater ecosystems (e.g. Fry 1991, France 1997, Jones & Waldron 2003, Schmidt *et al.* 2007, Jackson & Britton 2013, Hill *et al.* 2015). This technique is based upon the principle that consumers show predictable isotopic fractionation that persist up the food web. Consumers are characterized by a carbon isotopic composition (^{13}C:^{12}C, expressed as $\delta^{13}C$) that is, on average, enriched ~0-1 ‰ relative to their diet, which allows differences in $\delta^{13}C$ isotopic ratios to be used in distinguishing and/or tracing allochthonous and autochthonous carbon sources in aquatic ecosystems (DeNiro *et al.* 1978, Fry & Sherr 1984, Vander Zanden & Rasmussen 2001, Post 2002). Comparatively, consumer nitrogen isotopic composition (^{15}N:^{14}N, expressed as $\delta^{15}N$) enriches ~3-4 ‰ moving up the food web, thus providing information on consumer trophic level (DeNiro & Epstein 1981, Vander Zanden & Rasmussen 2001, Post 2002). However, the $\delta^{15}N$ isotopic values of primary producers (i.e. aquatic plants) are affected by dissolved inorganic nitrogen (and its isotopic composition) and plant physiology, because the isotopic value of plant tissues reflect the nitrogen sources they assimilate (Costanzo *et al.* 2001). When nitrogen is limiting, plant tissues have $\delta^{15}N$ isotopic value similar to their main N-source, but when nitrogen is in excess, plant tissues show $\delta^{15}N$ isotopic values which are significantly more enriched (Heaton 1986, Kendall 1998, Kendall & Doctor 2003). As a result, high N-loads can often be linked with enriched $\delta^{15}N$ isotopic values of aquatic vegetation relative to largely natural conditions, indicating that they may provide an early indication of nitrogen pollution in aquatic ecosystems (e.g. Fry & Allen 2003, Anderson & Cabana 2005, Deutsch & Voss 2006, Costanzo *et al.* 2001; 2005, Vander Zanden *et al.* 2005, Hill *et al*. 2012).

Paces (1982), Turner & Rabalais (1991) and Goolsby (2000) cited in Ohte (2012) all noted that excess inputs of nitrogen through anthropogenic activities have threatened aquatic ecosystems for decades. Natural leaching and atmospheric deposition are regarded as the major pathways by which inorganic nutrients like nitrogen and phosphorus reach both aquatic and terrestrial ecosystems (Ohte 2012). However, excessive nutrients from anthropogenic activities including intensive farming, increase use of N-containing organic and inorganic fertilizer or animal manure, agricultural run-off and sewage effluent discharge can eventually find their way into underground aquifers and rivers, and ultimately act as promotors of eutrophication (Cabana & Rasmussen 1996, Smil 1999, Anderson *et al.* 2002, Rabalais 2002, Hill *et al.* 2012). For example, Lassauque *et al.* (2010) and Schubert *et al.* (2013) used marine organisms (e.g.

transplanted mussels and seagrasses) to detect various levels of nutrients from both river run-off and coastal anthropogenic activities that included harbour out-flows, fish farms and urban sewerage out-fall. These studies revealed enriched $\delta^{15}N$ isotopic ratios of mussels and seagrasses that were observed closer to the harbour and sewerage out-fall. Similarly, Costanzo *et al.* (2001) illustrated the potential of using the $\delta^{15}N$ isotopic values of macrophytes to help detect sewage inputs into a coastal bay, by mapping sewage discharge from the sewerage treatment works situated along the river mouth. Five years later Costanzo *et al.* (2005) used marine organisms to assess the effectiveness of the improved sewage discharge standards; $\delta^{15}N$ isotopic ratios demonstrated a large reduction in the spatial extent of sewage discharge after substantial infrastructure upgrades. This suggests that tracing anthropogenic N-loading via $\delta^{15}N$ isotopic values, otherwise known as sewage plume mapping, may provide ecologists and environmental managers with a monitoring technique to detect problems and monitor freshwater rehabilitation programs.

The mechanism underpinning a macrophytes ability to track nitrogen loading lies in the isotopic fractionation linked to the physiological preferences and pathways in biological activities (Hill *et al.* 2012). Plants assimilating nitrogen from synthetic fertilizers for example, have $\delta^{15}N$ isotopic values that reflect the atmospheric N_2-source of the fertilizer (~-2.00 to +2.00 ‰; Heaton 1986, Kendall 1998, Curt *et al.* 2004). Treated sewage on the other hand is isotopically heavier or more enriched with ^{15}N isotope because bacteria found in wastewater treatments preferentially process the lighter ^{14}N isotope leading to an overall enrichment in the $\delta^{15}N$ isotopic values of remaining sewage (typically +10.00 to +25.00 ‰; Heaton 1986, Kendall 1998, Curt *et al.* 2004, Hill *et al*. 2011; 2012, Hill 2014, Morrissey *et al.* 2013, Loomer *et al.* 2014). However, isotopic fractionation varies with the indicator's (e.g. *Spirodela sp.*) taxonomic and geographical differences as suggested by Peterson & Fry (1987), Cole *et al.* (2004), Aberle & Malzahn (2007) and Hill *et al.* (2012). This further emphasizes the importance of providing species-specific and area-specific baseline isotopic data of the indicator taxa for successful interpretations. In South Africa, the duckweed *Spirodela* sp. (which collectively represents a combination of *Spirodela polyrrhiza* and *Spirodela punctata*), the smallest known floating aquatic macrophytes (Hillman 1961, Hillman & Culley Jr. 1978), is ubiquitous in freshwater ecosystems. These plants proliferate in fresh, slow moving or still waters and grow on top or just below the water surface, creating large dense mats. A baseline laboratory study on the isotopic

differentiation of *Spirodela* plants illustrated that they have the ability to clearly differentiate between different nutrient types (NH_4^+ and NO_3^-) and regimes. This was observed to take 4-10 days of exposure and have increasingly depleted and enriched $\delta^{15}N$ isotopic values with increasing level of concentration for NH_4^+ and NO_3^- respectively. This suggests that they are excellent biological indicators for nitrogen mapping in freshwater ecosystems (Hill *et al*. 2012). While this study provided useful information on sewage plume mapping using duckweed in a laboratory setting, substantial field testing is required to confirm its utilisation in the natural environment. Therefore the present study was aimed to evaluate (1) the ability of $\delta^{15}N$ and $\delta^{13}C$ isotopic values of *Spirodela* sp. to trace inputs of anthropogenic nitrogen (sewage, agricultural run-off/commercial fertilizers) across a well-defined nutrient gradient on the Bloukrans-Kowie and Bushmans-New Year's River systems and (2) test the relationship between nitrogen isotopic values and the SASS5 biological monitoring assessment technique in the Eastern Cape, South Africa.

4.2 Materials and Methods

4.2.1 Study Area & Data collection

Details on study sites, samples collection and stable isotope techniques are given in Chapter 2.

4.2.2 Data Analysis

Ocean Data View (ODV version 4.6.1; 2014) was used to illustrate the temporal and spatial variation in isotopic values of $\delta^{15}N$, $\delta^{13}C$ and C/N ratios throughout the 13 month sampling period on the Bloukrans-Kowie and Bushmans-New Year's River systems, in the Eastern Cape, South Africa.

ArcMap (ArcGIS 10.2; 2014-2015) was used to produce general description maps of average *in situ* nitrogen loading and SASS5 scores in all sampled study sites on the Bloukrans-Kowie and Bushmans-New Year's River systems.

A General Linear Mixed-Effects Model (GLMM) was performed to test whether there were any significant differences in $\delta^{15}N$ isotopic values over time (months) and space (between sites). A GLMM was used instead of a repeated measures analysis of variance (RM-ANOVA), as it can deal with missing data and unbalanced designs and it does not require data to be normally

distributed or variances to be homogeneous (Bolker *et al*. 2008). Furthermore GLMM has the ability to incorporate repeated measures on multiple study sites and replicates as random effects (Bolker *et al*. 2008). The coding for the optimal model formula run was:

$$model <- lme(^{15}N\ values \sim time, random = \sim 1 + time | sites, data = my\ data)$$

Where *lme* is the linear mixed-effect model function from the *nlme* package, ^{15}N *values~time* (time of sampling) were the fixed effects, *~1+time* was the slope and intercept function of the $\delta^{15}N$ isotopic values over time, and sites were incorporated as random effects. Best linear unbiased predictor values (BLUPs) were generated using the *predict* function on the *model* and fitted values (F0 and F1). The model was fitted separately on $\delta^{15}N$ isotopic data for both the Bloukrans-Kowie and Bushmans-New Year's River systems. Using the build-in *coef* function (*coef(model)*), intercepts and slope values of each fitted and plotted study site were obtained. Model validation was achieved using diagnostic plots of $\delta^{15}N$ residuals verses fitted $\delta^{15}N$ values, and residuals verses predictor variables e.g. study sites, time of sampling, rainfall and water temperature. All analyses were done in the R environment (R Core Team; 2012).

Furthermore a Multiple Linear Regression Analysis (MLRA) was performed to investigate the influence of explanatory variables on the response variables. The model followed the general formula:

$$model2 <- lm(response \sim explanatory_1 + explanatory_2 + \ldots\ldots + explanatory_n)$$

Where the *response* variables were $\delta^{15}N$, $\delta^{13}C$ isotopic values and C:N ratios, and the *explanatory* variables were the on-site collected physicochemical variables e.g. pH, conductivity, dissolved oxygen, water temperature, [ammonium] and [nitrate] and rainfall (mm). All analyses were also done in the R environment (R Core Team; 2012), using a stepwise variable selection method. Model validation was also achieved using diagnostic plots dispersion metrics e.g. residual versus fitted, normal Q-Q plots and residuals versus Leverage plots.

SASS5 scores and $\delta^{15}N$ isotopic values relationships were presented in simple scattered plots graphs to compare general patterns, this was due to the nature of the two data sets (SASS5 scores were collected quarterly and $\delta^{15}N$ isotopic data (n=5) were collected monthly) and this resulted in an unbalanced data sets and were not suitable for statistical analysis.

4.3 Results

4.3.1 Mapping anthropogenic N-loading

$\delta^{15}N$ isotopic values of *Spirodela* plants showed distinct temporal and spatial patterns, indicating variation in nitrogen dynamics over the 13 month sampling period (August 2013 - August 2014) on both the Bloukrans-Kowie and Bushmans-New Year's River systems (Figure 4.1A & 4.3A). Enriched $\delta^{15}N$ isotopic values (> +10.00 ‰) and low C/N ratios (≤ 15.00) suggest sewage and/or cow manure inputs (Heaton 1986, Kendall 1998, Curt *et al.* 2003, Hill *et al*. 2012, Hill 2014), enriched $\delta^{15}N$ isotopic values (> +10.00 ‰) and high C/N ratios (≥ 15.00) suggest nitrogen limitation and nutrient stress (Hill *et al*. 2012, Hill 2014), while strongly depleted $\delta^{15}N$ isotopic values (-2.00 to +2.00 ‰) and low C/N ratios (< 15.00) indicate uptake of commercial fertilizers (Heaton 1986, Kendall 1998, Curt *et al.* 2003). Mid-range $\delta^{15}N$ isotopic values (+2.00 to +8.00 ‰) and low C/N ratios (< 15.00) suggest plants growing in largely natural systems (Kreitler & Browning 1983, Kendall 1998).

On the Bloukrans-Kowie Rivers system contour plots revealed sewage and/or cow manure inputs at sites A3, A4 and A5 for approximately the first 8 months of the study (i.e. Aug. 2013 – Mar. 2014), between Dec. 2013 and Jan. 2014, and in some cases, for the two months preceding or following these dates. Site A4 and A5 continued to show sewage inputs for the majority of the remaining months (Figure 4.1). Site A2, A7 and A8 showed temporal variation in nitrogen loading with indications of sewage inputs (A2: Nov. 2013 – Feb. 2014; A7: Aug. – Oct. 2013, Dec. 2013 – Jun. 2014; A8: Aug. – Oct. 2013, Jan. – Mar. 2014, May 2014), and natural nitrogen inputs (A2: Aug. – Sept. 2013, Mar.- May 2014; A7: Jul.-Aug. 2014; A8: Apr. & Jun. 2014) in some months and nutrient stress in others (A7: Nov. 2013; A8: Nov. – Dec. 2013). Site A6 was predominantly under nutrient limitation for the duration of the study (with the exception of Apr. 2014), while A9 plants appeared growing in a largely natural environment overall. Fertilizer inputs were minimal with two sites A2 (Jun. – Jul. 2014) and A3 (Apr. 2014 and Jun. – Aug. 2014) showing uptake over the entire 13 months (Figure 4.1 & 4.2).

Comparatively, on the Bushmans-New Year's River system, sites B3, B4, B5 and B10 showed evidence for sewage inputs for the majority of the sampling period, with two exceptions (B3: Apr. – Jun. 2014; B10: Aug. 2013). The remainder of the sites showed temporal variation in

nitrogen loading with indication of sewage inputs at some sites and times (B1: Aug. – Oct. 2013; B2: Nov. 2013 – May 2014; Dec. 2013 – Jun. 2014; B6: Feb. – Apr. 2014, Jun. – Aug. 2014; B7: Feb. – Mar. 2014; B8: Aug. 2013, Feb. – May 2014, Jul. – Aug. 2014; B9: Jun. 2014) and natural inputs (B1: Jan. – Jun. 2014; B2: Aug. - Oct. 2013, Aug. 2014; B6: May 2014; B7: Apr. – Aug. 2014; B8: Jun. 2014; B9: Apr. – May 2014, Jul. – Aug. 2014) at others. Nutrient stress was visible at site B6-B9 for the first 6 months as well as at site B1 (Nov. - Dec. 2013, Aug. 2014). Fertilizer inputs were also minimal on the Bushmans-New Year's River, with only site B2 showing evidence of fertilizer uptake between Jun. - Jul. 2014 (Figure 4.3 & 4.4).

$\delta^{13}C$ isotopic values were much less useful with *Spirodela* plants showing minimal variation between sampled study sites and/or sampling events, with values ranging between -30.00 to -22.00 ‰ and -30.00 to -25.00 ‰ on the Bloukrans-Kowie and Bushmans-New Year's River systems respectively. However there were three anomalies, with site A10 in Oct. 2013 and Jul. – Aug. 2014 (-24.00 to -22.00 ‰) and site B9 in Sept. 2013 (~ -10.00 ‰) showing more enriched $\delta^{13}C$ isotopic values than any other site or time (Figure 4.2 & 4.4).

Generalized Linear Mixed-Effect models

Mixed-effects models fitted on the $\delta^{15}N$ isotopic values of *Spirodela* plants showed significant differences between all sampled study sites on both rivers systems (Figure 4.5A & B). Furthermore, from the model fit statistics, temporal variation in $\delta^{15}N$ isotopic values as seen in the contour plots was significantly different ($p < 0.05$) between sampling events (months) on both the Bloukrans-Kowie and the Bushmans-New Year's River systems (Table 4.1). $\delta^{15}N$ isotopic values and time of sampling (month) showed a very strong negative correlation as factors of both random effects ($r = -0.86$ and $r = -0.98$) and fixed effects ($r = -0.83$ and $r = -0.95$) throughout the study on both the Bloukrans-Kowie and Bushmans-New Year's River systems respectively (Table 4.1). The model (*coef*, function) provided slopes for each fitted study sites and these were compared to the population line slope (~ -0.59 and ~ -0.21) and intercept (+18.84 and +12.69 ‰), which was used to distinguish between eutrophic (intercept: > +19.12 ‰ and > +12.69 ‰), moderately eutrophic (intercept: +19.12 – +10.00 ‰ and +12.69 – +10.00 ‰) and oligotrophic (+8.00 – +3.00 ‰) sites on the Bloukrans-Kowie and Bushmans-New Year's River systems respectively. Site A5 (slope: -1.09, intercept: +35.63 ‰), A4 (slope: -0.99, intercept: +26.36 ‰), A3 (slope:-0.89, intercept: +22.23 ‰), A7 (slope: -0.54, intercept: +18.84 ‰) and

site B4 (slope: -0.86, intercept: +21.49 ‰), B5 (slope: -0.61, intercept: +17.70 ‰), B3 (slope: -0.53, intercept: +16.75 ‰), B10 (slope: -0.19, intercept: +13.20 ‰) and B8 (slope: -0.22, intercept: +12.89 ‰) on the Bloukrans-Kowie and Bushmans-New Year's River systems respectively, had regression lines above that of the population line and were thus significantly more ^{15}N enriched compared to the rest of the sites, these sites were identified as eutrophic. Comparatively, site A8 (slope: -0.46, intercept: +16.76 ‰), A10 (slope: -0.16, intercept: =16.09 ‰), A2 (slope: -0.69, intercept: +15.42 ‰) and A9 (slope: -0.12, intercept: +10.64 ‰) had a slope and intercept below that of the population line and were thus identified as moderately eutrophic. Site B1 (slope: -0.12, intercept: +9.80 ‰), A6 (slope: -0.10, intercept: +8.97 ‰) and site B2 (slope: -0.07, intercept: +9.29 ‰), B6 (slope: 0.06, intercept: +9.02 ‰), B9 (slope: 0.06, intercept: +8.39 ‰), B7 (slope: 0.13, intercept: +7.78 ‰) had a slope relatively closer to zero and an intercept equating to ~ < +10.00 ‰, which was lower than the population line and were thus identified as oligotrophic (Figure 4.5A &B). Additionally, site B6, B7 and B9 had $\delta^{15}N$ isotopic values which showed a positive linear relationship (slope > 0) with time of sampling throughout the study period when compared to the rest of the sites. However this did not bring any changes to their nitrogen isotopic values but mainly showed increasing nitrogen isotopic values with time of sampling.

Overall estimates of average nitrogen loading over the 13 month sampling period at each site on both the Bloukrans-Kowie and Bushmans-New Year's River systems can be found in Figure 4.6

Model-checking plots showed that $\delta^{15}N$ residuals were well behaved (showing a linear band of points concentrated on the 0 line) (Appendix 6). $\delta^{15}N$ residuals versus predictor variables e.g. sampled study sites also confirmed a mean variation approximately equating to zero, however three sites A2, A3 and B4 had a slight deviation from the reference point (Appendix 7). $\delta^{15}N$ residuals and time diagnostic plots showed a semi-circular pattern throughout the period of the study (Appendix 8), therefore $\delta^{15}N$ residuals were then plotted against two physical variables that were collected and that were associated with time variations e.g. rainfall and water temperature. Further investigations of rainfall and $\delta^{15}N$ residuals plots showed a similar pattern to that of $\delta^{15}N$ residuals and time of sampling (Appendix 9) and a fairly uniform scattered pattern on $\delta^{15}N$ residuals and water temperature (Appendix 10).

Multiple Linear Regression

Significant, but weak relationships were seen between numerous physicochemical variables and the $\delta^{15}N$, $\delta^{13}C$ isotopic values and C/N ratios of *Spirodela* plants. On the Bloukrans-Kowie River system, pH, rainfall, DO and $[NH_4]$ had a significant effect on *Spirodela* plant $\delta^{15}N$ isotopic values ($r^2 = 0.28$, $F_{4\text{-}71} = 6.78$, $p < 0.001$). pH and rainfall were the only two physicochemical variables that explained significant variation in $\delta^{13}C$ isotopic values ($r^2 = 0.091$, $F_{2\text{-}73} = 3.67$, $p < 0.05$) and for C/N ratios, rainfall, water temperature, DO, $[NH_4]$ and $[NO_3]$ were found to drive variation ($r^2 = 0.34$, $F_{5\text{-}70} = 7.20$, $p < 0.001$). On the Bushmans-New Year's River system, $\delta^{15}N$ isotopic values were influenced by pH, EC, DO and $[NO_3^-]$ ($r^2 = 0.39$, $F_{4\text{-}75} = 11.80$, $p < 0.001$), EC, pH, DO and EC, DO, $[NH_4]$, $[NO_3]$ also had an effect on $\delta^{13}C$ isotopic values of *Spirodela* plants ($r^2 = 0.16$, $F_{3\text{-}76} = 4.79$, $p < 0.01$) and C/N ratios ($r^2 = 0.16$, $F_{3\text{-}76} = 4.79$, $p < 0.01$) respectively.

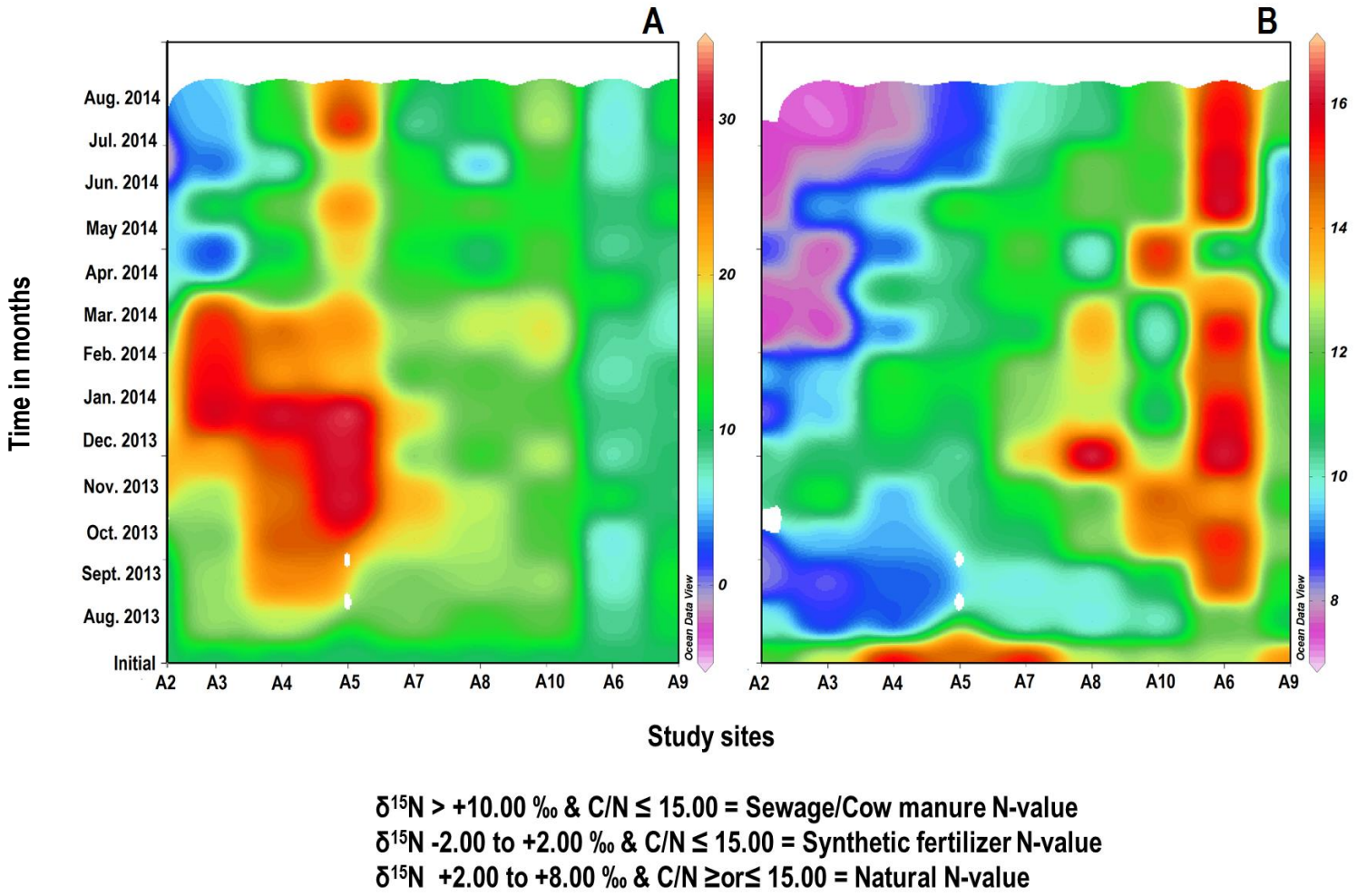


Figure 4.1: Temporal and spatial variation in (A) $\delta^{15}N$ isotopic values (‰) and (B) C/N ratios of *Spirodela* plants at each site on the Bloukrans-Kowie River system over the 13 month sampling period (August 2013 – August 2014).

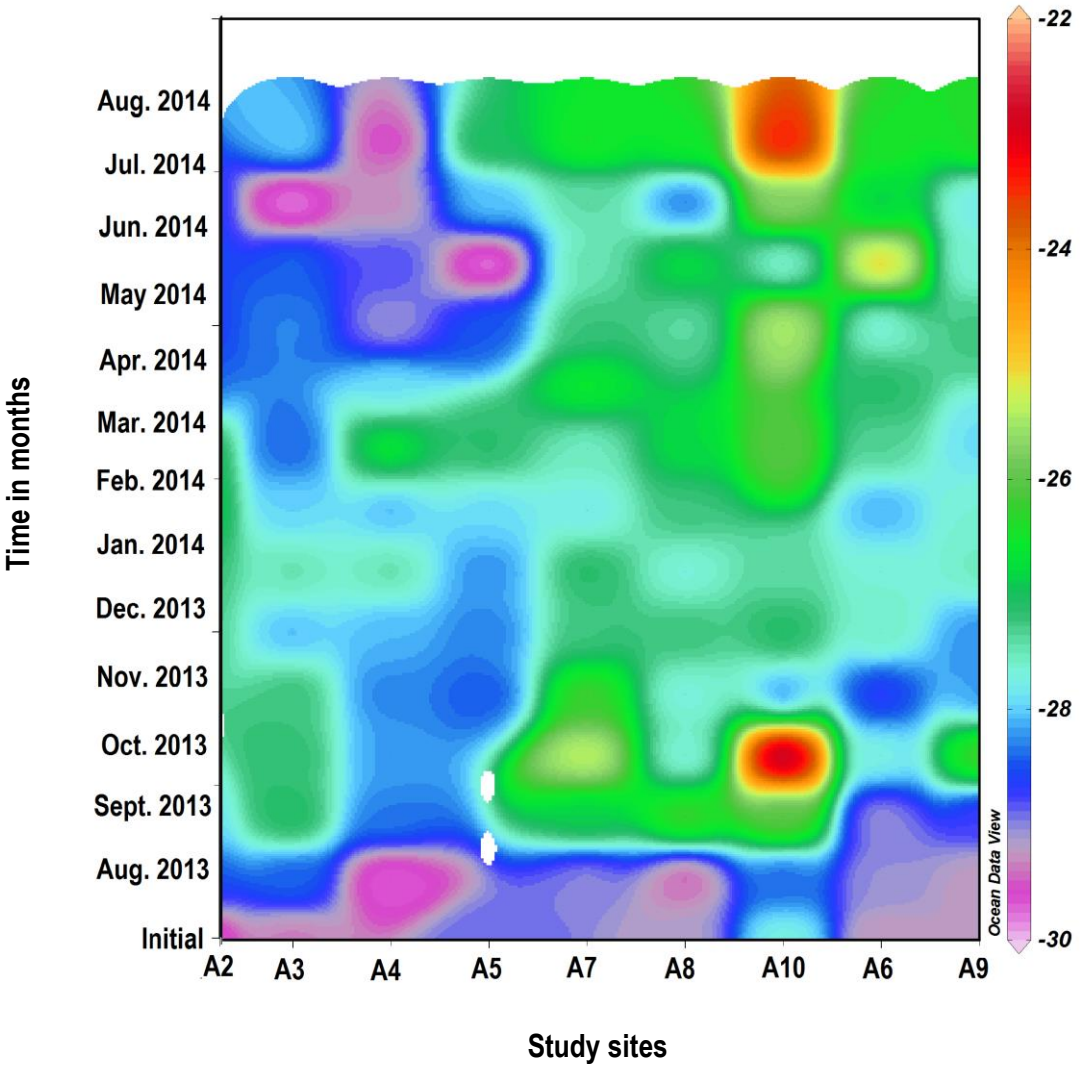


Figure 4.2: Temporal and spatial variation in $\delta^{13}C$ isotopic values of *Spirodela* plants at each site on the Bloukrans-Kowie River system over the 13 month sampling period (August 2013 – August 2014).

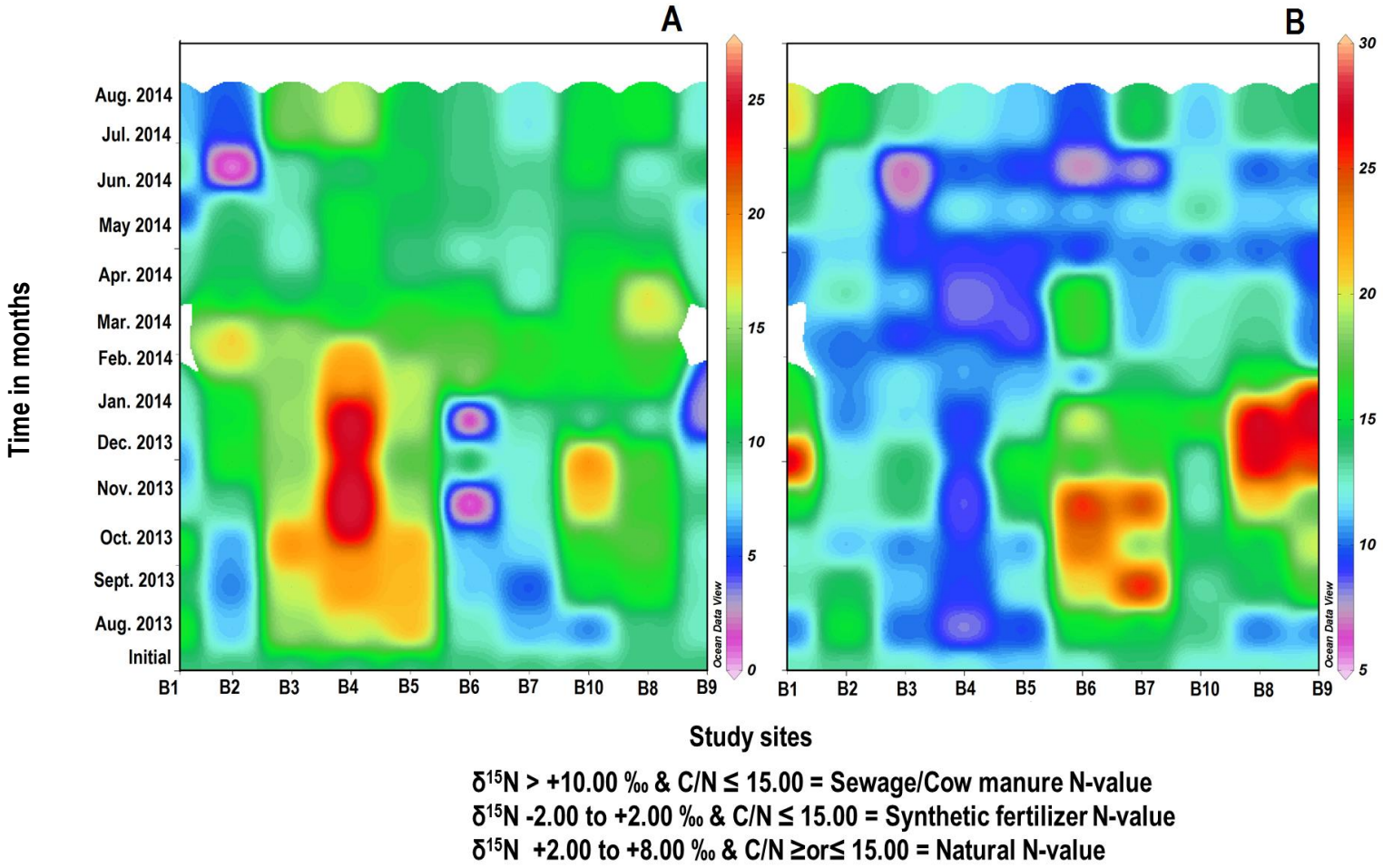


Figure 4.3: Temporal and spatial variation in (A) $\delta^{15}N$ values (‰) and (B) C/N ratios of *Spirodela* plants at each site on the Bushmans-New Year's River system over the 13 month sampling period (August 2013 – August 2014).

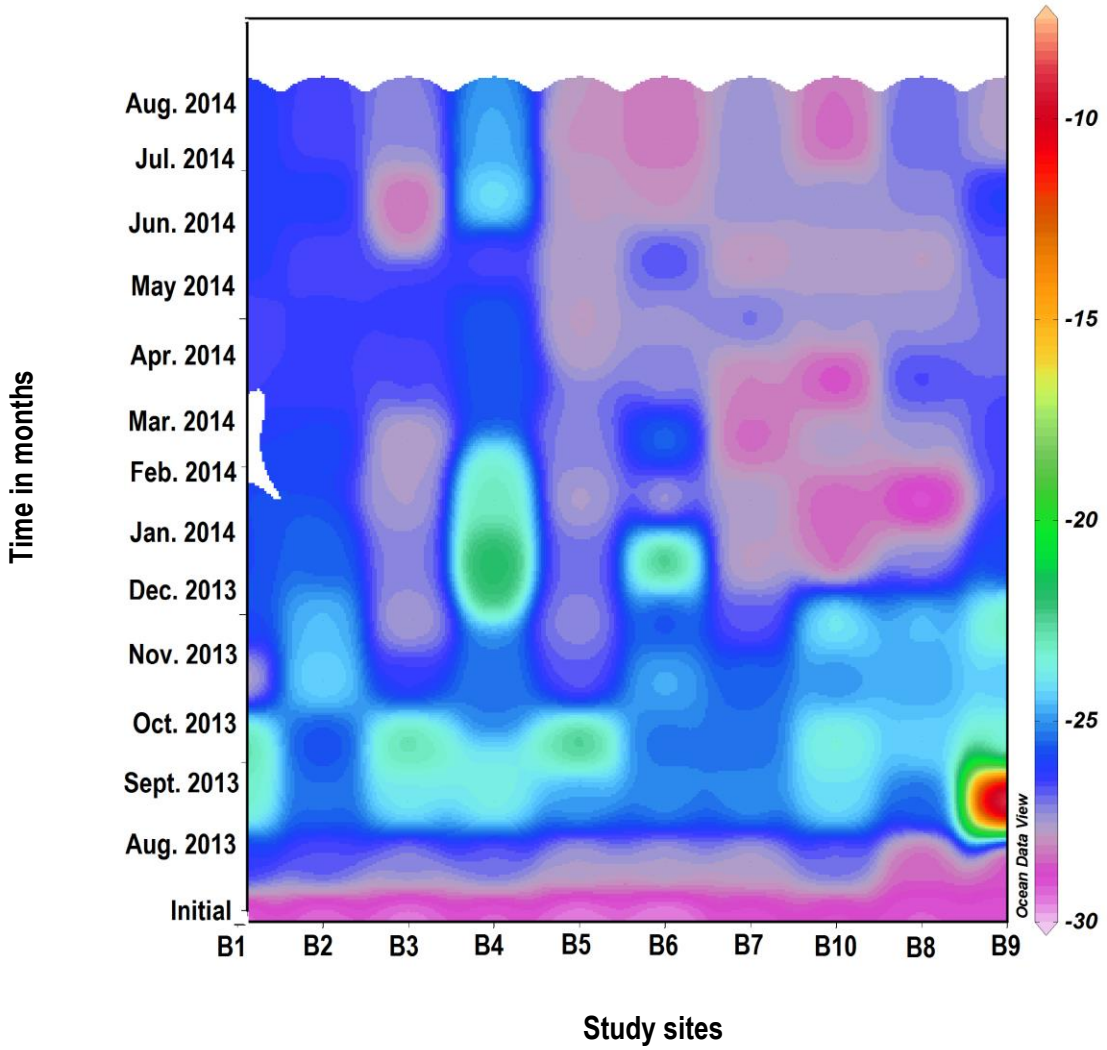


Figure 4.4: Temporal and spatial variation in $\delta^{13}C$ isotopic values of *Spirodela* plants at each site on the Bushmans-New Year's River system over the 13 month sampling period (August 2013 – August 2014).

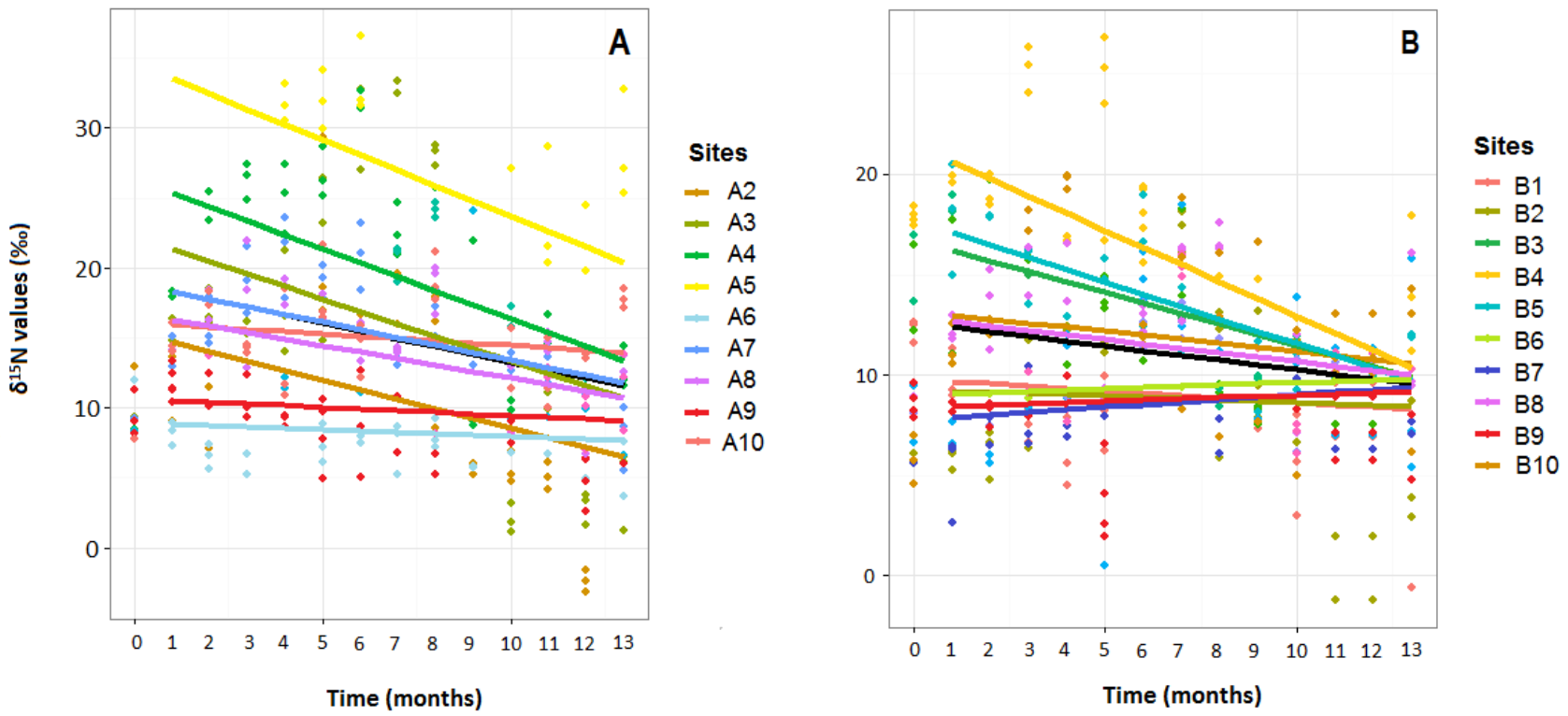


Figure 4.5: (A) Bloukrans-Kowie and (B) Bushmans-New Year's River systems model plots showing differences in $\delta^{15}N$ intercept and slope from predicted $\delta^{15}N$ isotopic values over 13 month sampling period. Colored solid regression lines represent different study sites and the black solid line represents the population line.

Table 4.1: Summary of linear mixed-effect model fit statistics for $\delta^{15}N$ isotopic ratios between site over time on the Bloukrans-Kowie and Bushmans-New Year's River systems, Eastern Cape South Africa.

Model Statistics	Linear Mixed Effects Model	
	Bloukrans-Kowie River	**Bushmans-New Year's River**
Model AIC	2059.33	1973.01
Random Effects StdDev.		
Intercept	8.12	4.76
Time	0.43	0.34
Residuals	5.36	3.49
Random Effect Correlation	-0.86	-0.98
Fixed Effects		
Intercept (±Std. error)	18.88±2.78	12.63±1.54
Time (±Std. error)	-0.56±0.16	-0.24±0.12
	(*df* = 314, *t*-value = -3.46, *p* = 0.0006)	(*df* = 349, *t*-value = -2.01, *p* = 0.0448)
Fixed Effects Correlation	-0.83	-0.95
Number of Observations	324	360
Number of Groups	9	10

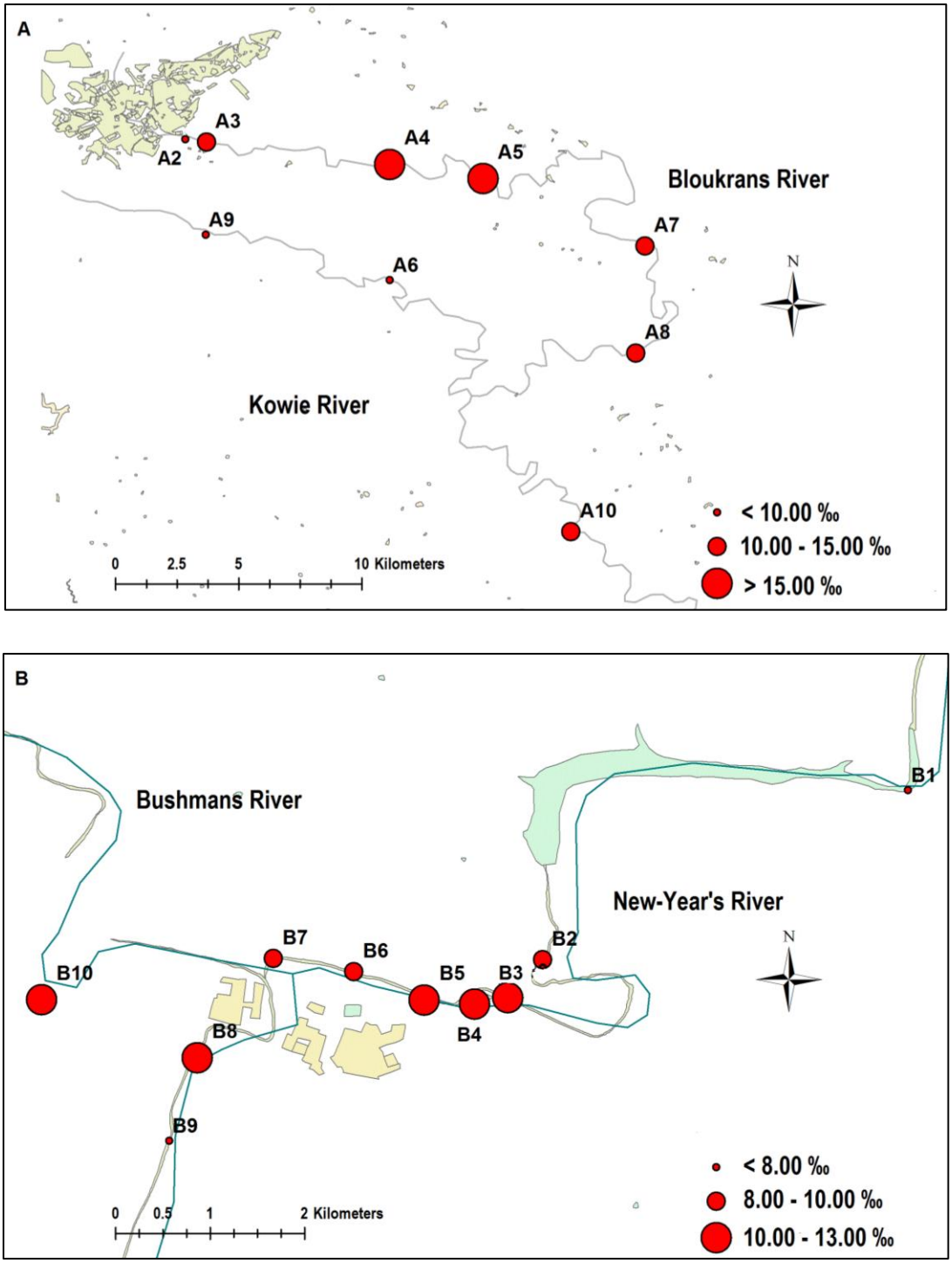


Figure 4.6: Average $\delta^{15}N$ hotspot locations and *in situ* nitrogen mapping on the (A) Bloukrans-Kowie River and (B) Bushmans-New Year's River over the 13 month sampling period (August 2013 – August 2015).

4.3.2 Comparison of $\delta^{15}N$ isotopic values and SASS scores

The mean $\delta^{15}N$ isotopic values of *Spirodela* plants grown in the Bloukrans-Kowie and Bushmans-New Year's River systems showed an inverse relationship with the average SASS scores calculated over the 13 month study period. Sites observed to have enriched $\delta^{15}N$ isotopic values, consistently demonstrated lower SASS scores and vice versa (Figure 4.7). On the Bloukrans-Kowie River system for example, site A2, A3, A4 and A5 showed an average SASS score of < 90 and mean $\delta^{15}N$ isotopic values of between +10.00 to + 20.00 ‰, indicating severe disturbance and substantial inputs of anthropogenic nitrogen respectively. Site A6 and A9 on the other hand demonstrated an average SASS score ≥ 150 and mean $\delta^{15}N$ isotopic values of between +2.00 and +8.00 ‰, suggesting a largely natural and an oligotrophic nutrient conditions. The rest of the Bloukrans-Kowie River study sites A7, A8 and A10 had an average SASS score ≤ 140 which was associated with an average $\delta^{15}N$ isotopic value of between +10.00 ‰ and +14.00 ‰ indicating moderate disturbance and moderately eutrophic conditions respectively. The majority of the Bushmans-New Year's River study sites; B1, B2, B3, B5, B6, B7 and B10 showed average SASS scores < 90 with moderately eutrophic $\delta^{15}N$ isotopic values (+7.00 to +13.00 ‰). Confluence sites B8 and B9 however, demonstrated the 'highest' SASS scores (> 90) with $\delta^{15}N$ isotopic values of +10.56 ‰ and +6.21 ‰ respectively (Figure 4.7). Furthermore a summary of $\delta^{15}N$ isotopic values and SASS scores mapping revealed complementary relationship between the two techniques. The tagging reported corresponding results from average ODV representation, fitted GLMMs model plots and the spatial ArcGIS $\delta^{15}N$ isotopic values throughout the study period on the Bloukrans-Kowie and Bushmans-New Year's Rivers systems (Figure 4.8).

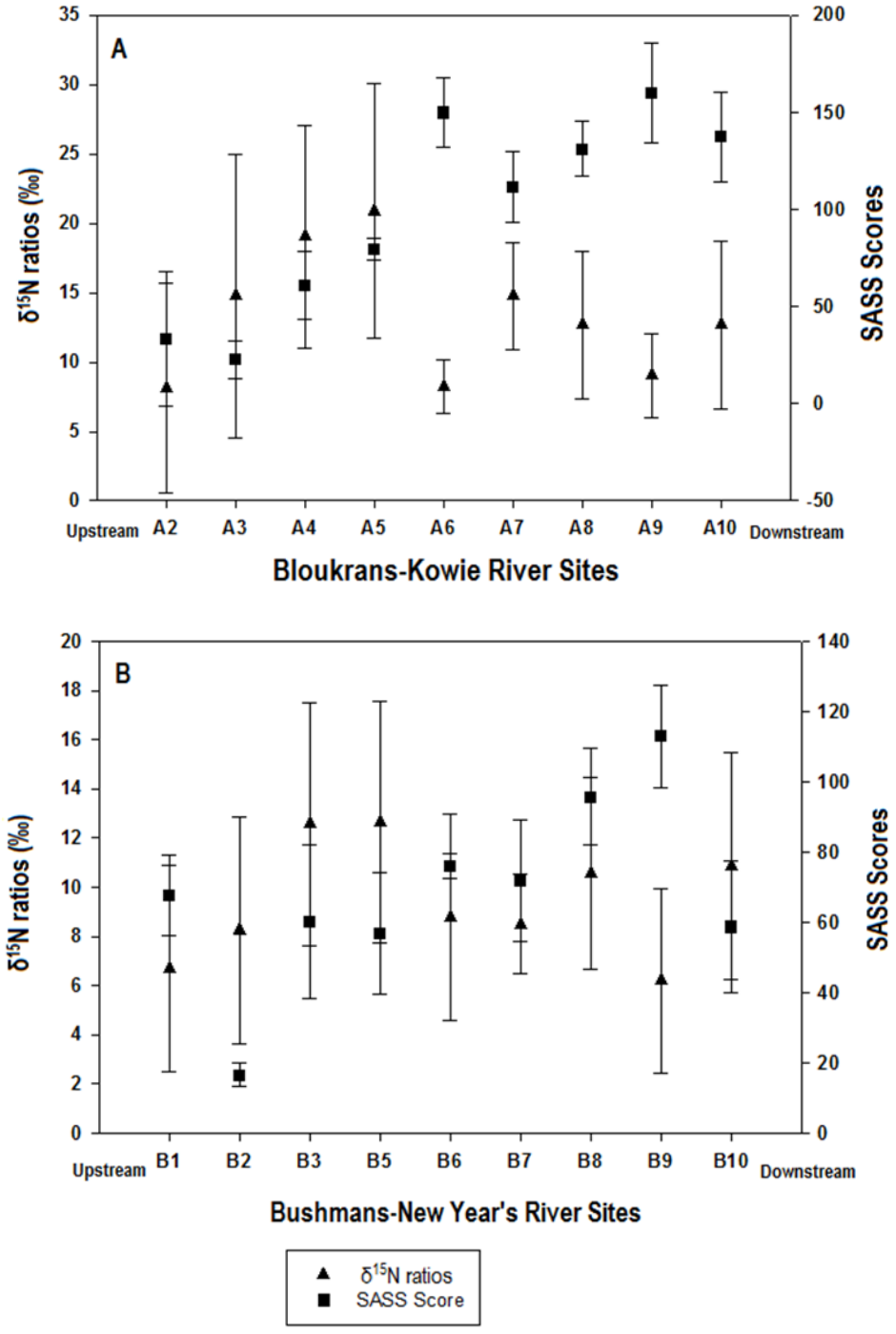


Figure 4.7: Average SASS5 scores and mean $\delta^{15}N$ isotopic values (‰) of *Spirodela* plants at each site over the 13 month sampling period, on the (A) Bloukrans-Kowie and (B) Bushmans-New Year's River systems. Error bars represent ± 1SD.

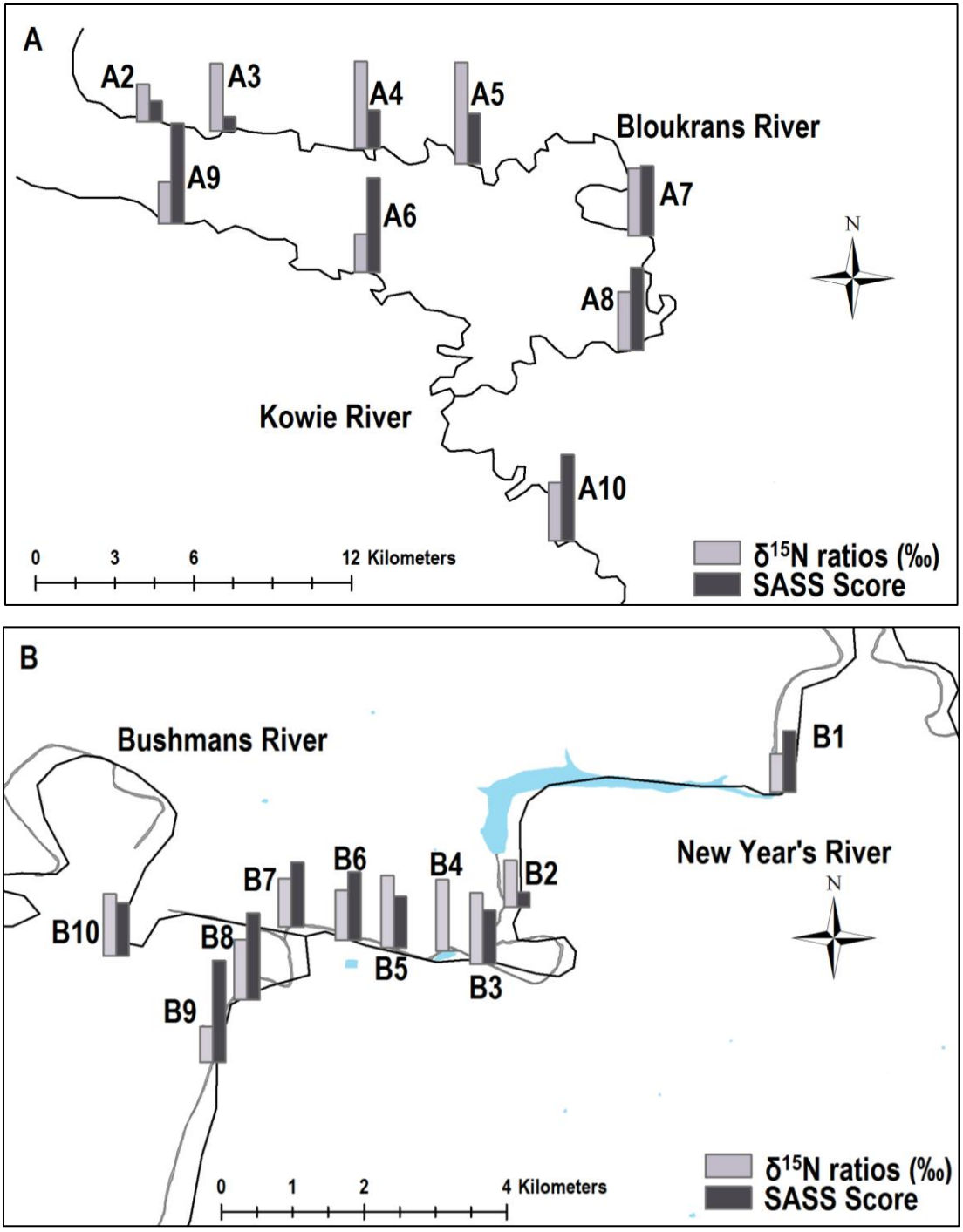


Figure 4.8: Average $\delta^{15}N$ ratios (‰) hotspot locations and average SASS5 scores mapping at each site over the 13 month sampling period. Arrows indicate nutrient inputs, on the (A) Bloukrans-Kowie and (B) Bushmans-New Year's River systems (visual summary presentation of Figure 4.7).

4.4 Discussion

Intensive field application of the sewage plume mapping technique using transplanted *Spirodela* plants as described by Hill *et al.* (2011, 2012) in a laboratory setting were highly successful. This was also the case in a natural environment, were $\delta^{15}N$ isotopic values of *Spirodela* plants clearly differentiated temporal and spatial dynamics of N-loading in both the Bloukrans-Kowie and Bushmans-New Year's River systems. Studies by McClelland & Valiela (1998), Cole *et al.* (2004), Costanzo *et al*. (2001, 2005), Hill *et al.* (2011, 2012) and Morrissey *et al.* (2013) indicate that elevated $\delta^{15}N$ isotopic values are highly correlated to sewage inputs and urbanization. Walsh *et al.* (2005A cited in Morrissey *et al.* 2013) described anthropogenic nitrogen pollution as the "urban stream syndrome", where even with innovative developments and advancements in infrastructure and legislature, excessive nutrient loads (through treated or untreated sewage out-falls) are regularly still been released into aquatic ecosystems. Such nutrient loading was evident in the present study, where sites adjacent to sewerage treatment works (Alicedale Sewerage Treatment Works - ASTWs and Belmont Valley Sewerage Treatment Works - BVSTWs) (e.g. A3, A5 and B3, B5) and agricultural lands (e.g. A5 and A7) showed enriched $\delta^{15}N$ isotopic values compared to those further downstream. The majority of the study sites on both the Bloukrans-Kowie and Bushman-New Year's River systems on average demonstrated $\delta^{15}N$ isotopic values ($\geq$ +10.00 ‰) and low C/N ratios ($\leq$ 15.00), which are indicative of sewage and/or cow manure run-off inputs (Heaton 1986, Kendall 1998, Curt *et al.* 2004, Hill *et al*. 2012, Hill 2014), with only a few sites (i.e. A2, A9, A6 and B1, B2, B6, B7, B9) showing depleted $\delta^{15}N$ isotopic values between +6.21 and +8.76 ‰ which suggests largely natural (oligotrophic) growing conditions (Kreitler & Browning 1983, Kendall 1998). Diebel & Vander Zanden (2009) argues that sometimes isotopic ranges of anthropogenic nutrients have been documented to overlap, particularly when using aquatic biota as biological indicators. This emphasizes the need for baseline information which quantifies species-specific isotopic fractionation, equilibration rates and calibration of the indicator species, for later interpretation. According to the $\delta^{15}N$ isotopic values and C/N ratios, the anthropogenic inputs/land-use pollution in this study was mainly sewage and/or cow manure run-off and is representative of nitrogen loads of 4.5 mg/L or higher (in some cases much higher, see Hill *et al.* 2011, 2012). These values of nitrate standards were seen as extremely higher, exceeding the nitrates targeted water quality range (TWQR) for sewage effluents (< 1.5 mg/L) and therefore threatening the

TWQR for domestic water use and for aquatic ecosystems, of 6 mg/L (Chapter 2) (DWAF 1996A, Jordaan & Bezuidehout 2013). And all this evidently contributing to eutrophication. Such levels were recorded at sites that were adjacent and downstream of the BVSTWs, ASTWs and also Belmont Valley agricultural lands, and overall both river systems were found to have high levels of N-loading. Interestingly, sites close to the Belmont Valley agricultural lands were expected to reflect $\delta^{15}N$ isotopic values of commercial fertilizer due to their anticipated application and subsequent run-off (as indicated by farm owners), however the *Spirodela* isotopic values of nitrogen in this case showed that adjacent dairy farms, pineapple and cabbage plantation lands in the Belmont Valley uses cow manure as a 'fertilizer' hence enriched nitrogen isotopic values recorded. Oligotrophic sites (largely natural sites) were recorded at site A2, A6, A9 and B1, B2, B6, B7, B9 on the Bloukrans-Kowie and Bushmans-New Year's River systems. Both the model and the N-loading mapping were in agreement that these sites were largely natural and could be considered the least impacted sites throughout the sampling period. Nutrient inputs into these river systems were inconsistent, with irregular pulses of sewage and/or cow manure run-off from neighbouring lands (S. Motitsoe pers. obs, Makana & Alice Municipalities pers. comm.) however this was reflected clearly on the isotopic values, thus the ability to map this complicated case spatially and temporally is of real asset. For example, while site A5 on the Bloukrans-Kowie system demonstrated considerable eutrophication for the majority of the sampling period, site A2 was only eutrophic between November 2013 – February 2014, and site A3 and A4 for approximately the first six months. Although A2 is located upstream of the BVSTWs, it is downstream of local township housing where untreated raw sewage was observed entering the river. Interestingly there appears to be pulses of eutrophication at both A3 and A4 between April – August 2014 and are likely indicative of sporadic sewage inputs from the nearby BVSTWs (between A2 & A3). However there was no indication of commercial fertilizer application in this system at any time. Similarly on the Bushmans-New Year's River, the sites which were identified as the most eutrophic were those adjacent to B4 which was the sewerage settling pond (part of the currently being constructed ASTWs). While site B4 has not directly in connection to the river, highly enriched $\delta^{15}N$ isotopic values were observed at B5, which was directly downstream B4, this was as expected due to overtopping events and seepage. Additionally, of interest to management is that site B3 and occasionally B2, both upstream of B4 were also experiencing eutrophication, which is likely due to leaks in ageing infrastructure at B4,

as well as seepage and overtopping events. Commercial fertilizer was again mostly absent from this system, but is possibly present at site B2 (directly below the dam wall) in June 2014. New Year's dam however, has been shown to be sink for NO_3^- and this extremely depleted $\delta^{15}N$ isotopic value may be an artefact of the dam's unique physicochemistry (Hill *et al.* 2011). This information on both the spatial and temporal dynamics of nitrogen loading in aquatic systems is essential for proper management and conservation of aquatic ecosystems.

Anthropogenic activities have also been reported to significantly influence the $\delta^{13}C$ isotopic values of basal resources (Milanovich *et al.* 2014). Eitzmann & Paukert (2010) attributed this variation to freshwater fish in urbanized catchment, where the $\delta^{13}C$ isotopic values of fish inhabiting less urbanized river reaches showed more highly variable $\delta^{13}C$ isotopic values due to increased carbon sources. The present study showed no significant variation in $\delta^{13}C$ isotopic values, which suggests that, the isotopic ratios of sewage inputs and/or cow manure are not significantly different from the dissolved inorganic carbon already present. This is supported by Cabana & Rasmussen (1996), Steffy & Kilham (2004), Morrissey *et al.* (2013) and Loomer *et al.* (2014), who also found no significant differences in $\delta^{13}C$ isotopic values between upstream and downstream sewage out-fall sites in sewage mapping. Thus $\delta^{13}C$ isotopic values are more useful for investigation of variation in trophic ecology studies (DeNiro *et al.* 1978, Fry & Sherr 1984, Vander Zanden & Rasmussen 2001, Post 2002), and not necessarily particularly useful for tracing anthropogenic inputs.

The $\delta^{15}N$ sewage mapping technique also highlighted an important ecological ability on both rivers, the ability to assimilate. Contour plots and maps clearly showed, that the Bloukrans-Kowie river in particular, depleting $\delta^{15}N$ isotopic values as one moves downstream of the sewage inputs, and along with C/N ratios, providing evidence for dilution and scrubbing of nutrients (Chapman 1992), this was in keeping with the river continuum concept by Vannote *et al.* (1980). Such natural processes however can be suppressed due to (1) the water current speed and water volume of rivers which might be modified through water abstraction, and (2) with that in mind, the constant influx of anthropogenic nitrogen, which may eventually exceed the ecosystems ability to process, therefore all this leading to N-loading.

The present study showed a strong relationship between the $\delta^{15}N$ isotopic values of *Spirodela* plants grown in each river and the system's biological water quality (SASS5).

According to Dallas (2007) the range of SASS scoring can be described as; scores < 90 are associated with severely to critically impacted ecosystems; scores between 90 - 115 are largely impacted, scores between 115 - 140 are moderately impacted and scores > 140 are largely natural sites with minor modifications. Score interpretations were drawn from the South-eastern lower high land ecoregion of South Africa, where both the Bloukrans-Kowie and Bushmans-New Year's River system are located following Dallas (2007), SASS5 interpretations. Sites on the Bloukrans-Kowie River system that recorded a lower SASS score < 90, yielded $\delta^{15}N$ isotopic values between +10.00 and +35.00 ‰, each independently indicating that these sites were severely impacted (Dallas 2007) and highly eutrophic (Constanzo *et al.* 2004, 2005). Whereas sites with SASS scores ≤ 140 all had nitrogen isotopic values of between +10.00 and +14.00 ‰, both indicating moderate impairment. Lastly sites with SASS scores ≥ 150 consistently demonstrated $\delta^{15}N$ isotopic values of between +2.00 to +8.00 ‰, which both strongly indicate largely natural conditions without any sources of excessive nutrients. Similar to sites on the Bloukrans-Kowie River system, five Bushmans-New Year's River sites were described as critically impacted, with low SASS scores and highly enriched $\delta^{15}N$ isotopic values, but interestingly, the remaining five sites had low SASS scores, but moderate $\delta^{15}N$ isotopic values (e.g. +6.68 to +12.65 ‰). The explanation for this most likely lies as mentioned previously on the physical convention of the Bushmans-New Year's River forming series of isolated pools between study sites. Additionally, with the development of isolated pools, the river continuum concept no longer applies as nutrient loads from upstream are not carried and/or washed downstream and instead there is a large accumulation of salts and detritus (Vannote *et al.* 1980). The physical and chemical differences reported between the two river systems might have been the underlining factors that have driven the differences in biological composition and ultimately the $\delta^{15}N$ isotopic values and SASS5 interpretations. However only two confluence study sites B8 and B9 on the Bushmans-New Year's River system, that was 'flowing' and consistent of more biotopes. Site B8 and B9 recorded a SASS score (98 and 125) and subsequent moderate $\delta^{15}N$ isotopic values (~+10.00 and ~ +6.80 ‰), therefore undefined technique differences were restored and both techniques complemented respectively the disturbance.

Coetzee & Hill (2012) and Hill *et al.* (2012) suggest that the excessive addition of chemical pollutants to aquatic ecosystems have been reported to result in negative ecological

consequences in the long-term. They further elaborate that findings to date have shown clear and strong impacts on aquatic ecosystems through increased production and the establishment of alien plants and animal species, and major changes in the presence and dominance of certain species of benthic macroinvertebrates (see Coetzee *et al.* 2014, Hill *et al.* 2015). Thus is it important, now more than ever, to understand aquatic nutrient dynamics. Aquatic ecosystems are extremely dependent on terrestrial ecosystems for nutrient inputs, therefore it is not surprising that land-use is one of the best predictors of $\delta^{15}N$ isotopic values in aquatic ecology (Vander Zanden *et al.* 2005). While SASS scores and $\delta^{15}N$ isotopic values of *Spirodela* plants increased with an increase in anthropogenic inputs and SASS scores decreased with an increase in land-use activities, the $\delta^{15}N$ tracing technique provided information on both spatial and temporal dynamics in N-loading. Conversely, biological assessment indices (i.e. SASS scores) can only identify disturbance once it has already manifested and because ecologically, there are multiple factors that may result in ecosystem disturbance, the information provided by SASS scores is limited and does not provide a better understanding of ecosystem stress. $\delta^{15}N$ tracing however can clearly identify the type of pollution beforehand and can provide management with better techniques which can describe N-loading on a spatial and temporal basis before ecosystem degradation. Therefore sewage plume mapping (in this case with *Spirodela* sp.) may find its greatest utility in monitoring long-term, continuous nutrient inputs, such as environments impacted by sewage out-falls and wastewater inputs.

CHAPTER FIVE

ISOTOPIC VALUES OF AQUATIC MACROINVERTEBRATES AS AN INDICATION OF NUTRIENT LOADING

5.1 Introduction

Assessing the ecological health of aquatic ecosystems and underlying ecological stressors such as physical and/or chemical pollutants is regarded as the core of environmental research (Aazami *et al.* 2015B). Environmental assessments are commonly carried out using biological indicators e.g. fish (Pont *et al.* 2007), macroinvertebrates (Hodkinson & Jackson 2005), diatoms (Taylor *et al.* 2007) and macrophytes (Ferrat *et al.* 2003), which have illustrated capabilities as reliable tools to guide management in decision-making by providing ecological information about the area/site of interest (Aazami *et al.* 2015B). These environmental assessments are often based on the biological community composition and characteristics (Vilmi *et al.* 2015). Current developments in biological monitoring regards aquatic macroinvertebrates as good biological indicators of external disturbances, and this was supported and described collectively by Rosenberg *et al.* (1986), McGreoch (1998), Markert *et al.* (2003) and Hodkinson & Jackson (2005). These studies highlighted that; (1) macroinvertebrates are taxonomically sound, therefore easy to identify and collect, (2) they comprise a major percentage of aquatic ecosystem primary consumers and biodiversity, (3) their ecology and ecological characteristics are well-known and documented, (4) they show a measurable response towards both natural and man-induced disturbances (e.g. Chironomidae and Culicidae are pollution tolerant taxa versus Ephemeroptera, Trichoptera and Plecoptera, which are pollution sensitivity taxa), (5) they are suitable for laboratory experiments and, (6) they have a strong capacity to be quantified and standardized. Combined, these six characteristics make macroinvertebrates good tools for ecological research and long-term freshwater biological monitoring, globally.

Eutrophication in southern Africa is considered one of the most important ecological challenges for the majority of the countries waterways (van Ginkel *et al.* 2000, Oberholster & Ashton 2008, Coetzee & Hill 2012, Hill *et al.* 2012). There has been some attempt to try and understand system nutrient dynamics and/or identify ultimate sources of system eutrophication

using aquatic organisms such as algae and diatoms and to date they have provided some insights towards understanding the impacts of eutrophication on freshwater ecosystems. For example, Oberholster *et al.* (2009) and van Ginkel (2011) reported algae outbreaks/blooms that manifested to secondary effects, leading to high levels of cyanotoxins in eutrophic waters (resulting in the deaths of thousands of livestock, domestic animals and wildlife). According to van Ginkel (2004; cited in Oberholster & Ashton 2008) these algae blooms occurred mainly in South African impoundments and the majority of these blooms were concentrated in the Gauteng Province followed by the Free State, Kwa-Zulu Natal, the Eastern Cape and the Western Cape. Furthermore Vilmi *et al.* (2015) reported diatom morphology as related to the effects of eutrophication and/or changes in nutrient regimes in lotic waters. Vilmi *et al.* (2015)'s work followed that of Jarlman & Kahlert (2009; cited in Vilmi *et al.* (2015) where differences in mean width of diatoms valves (particularly in that of *Achnanthidium minutissimum*) were reported to be associated with variation in nutrients gradients. Mean diatom valve widths < 2 μm, 2.2 – 2.8 μm and > 2.8 μm were associated with oligotrophic, oligo-mesotrophic and eutrophic waters respectively in Swedish Highlands streams. However, Simaika & Samways (2012) suggested that algae are not reliable indicators of N-loading due to their spontaneous response to high nitrogen levels, making them unreliable indicators to trace or monitor N-loading events on a long-term basis. To date, there is no consensus on whether macroinvertebrates or diatoms are better biological indicators, however, recently, the use of diatoms indices in southern Africa has been gaining momentum (Taylor *et al*. 2007, Dalu *et al.* 2014; 2015). Overall, while both organisms can provide useful information, both require specialised knowledge and training regarding identification, which is a challenge for their implementation and use. While using aquatic macroinvertebrates and/or diatoms for basic identification of ecosystem health and status is a common practice (Dickens & Graham 1998, Dickens & Graham 2002, Beyene *et al*. 2009, Ndebele-Murisa 2012, Seanego & Moyo 2013, Bere & Nyamupingidza 2014, Farrell *et al.* 2015), neither can provide information on nutrient dynamics and/or identify ultimate sources of system eutrophication (see Chapter 3; Hill *et al.* 2012, Simaika & Samways 2012, Aazami *et al.* 2015A & B). It is anticipated that using macroinvertebrates a better understanding can be drawn based on ecological point of view regarding macroinvertebrates ecological preferences been well understood following Palmer *et al.* (1994), Dallas (2004), Hodkinson & Jackson (2005), Heino *et al.* (2007), Mantel *et al.* (2010) studies where habitat type, seasonal micro-climate, lotic and

lentic characteristics have an effect on macroinvertebrates behavior and community composition, and this is well documented.

Recently, the use of stable isotopic analysis (SIA) for tracing nutrient loading and nitrogen dynamics in aquatic systems has been investigated using zooplankton (Montoya *et al.* 2002, Toetz *et al.* 2009, Lee *et al.* 2013), macroalgae (Costanzo *et al.* 2001, Deutsch & Voss 2006, Thornber *et al.* 2008, Dailer *et al.* 2010), coral reefs and other marine organisms (Costanzo *et al.* 2005, Risk *et al.* 2009, Schurbert *et al.* 2013), mussels (Lake *et al.* 2001, McKinney *et al.* 2002, Fry & Allen 2003, Gustafson *et al.* 2007, Lassauque *et al.* 2010, Alomar *et al.* 2015), aquatic macrophytes (Chapter 4) (Costanzo *et al*. 2001, Benson *et al.* 2008, Lassauque *et al.* 2010, Hill *et al.* 2012) and fish (Lake *et al.* 2001) coupled with water nutrients analysis and sediments. In particular, Xu & Zhang (2012) and di Lascio *et al*. (2013) showed that stable isotope variation in macroinvertebrates can indicate anthropogenic disturbance in freshwater ecosystems. Following this work, Morrissey *et al.* (2013) investigated the possibility of tracing N-loading using nitrogen and carbon stable isotopic values of four macroinvertebrate families (e.g. Baetidae, Hydropsychidae, Heptageniidae and Gammaridae) to indicate the effect of wastewater on urban rivers in southern Wales and the Welsh Borders of United Kingdom. This study reported no significant differences in $\delta^{15}N$ and $\delta^{13}C$ macroinvertebrate isotopic values between animals downstream and upstream of sewerage out-falls. This can be attributed by macroinvertebrates being small organisms, with a short life span (primary consumers tend to show greater variability in their $\delta^{15}N$ isotopic value than larger organisms with a longer life span), rapid nitrogen turnover, which can easily reflect any changes in their micro-habitat compared to larger, longer lived secondary consumers and predators (Cabana & Rasmussen 1996, McKinney *et al.* 2002, Post 2002, Xu *et al.* 2010). Overall, Morrissey *et al.* (2013) recommended further investigations, particularly using different macroinvertebrate taxa, however very little work on this topic has been completed in South Africa. If macroinvertebrates in South African rivers which are exposed to high nutrient loads reflect enriched nitrogen isotopic values, as has been reported for *Spirodela* plants (see Chapter 4), SIA techniques may help to provide essential information on the extent of anthropogenic inputs and subsequent eutrophication in freshwater ecosystems. This chapter aims to: (1) identify an aquatic macroinvertebrate(s) whose isotopic values can act as an additional biological indicator in conjunction with *Spirodela* plants (see chapter 4), and (2) test and validate the $\delta^{15}N$ isotopic

value of the indicator taxa for mapping nutrient loading and nitrogen dynamics in freshwater ecosystems in the Eastern Cape, South Africa.

5.2 Materials and Methods

5.2.1 Study sites and Data collections

Details on study sites, sample collection and the experimental design are given in Chapter 2.

5.2.2 Data analysis

5.2.2.1 Identifying Potential Indicator Taxa

The Indicator Value Species Analysis method (IndVal; Dufrêne & Legendre 1997) was carried out to identify indicator species on the Bloukrans-Kowie River and the Bushmans-New Year's River systems and then together as a pooled data set, within aquatic macroinvertebrate communities which fell into different land-use and sampling site categories based on all 56 identified taxa originally collected for chapter 3. IndVal combines species' relative abundances together with its relative frequency of occurrence at multiple study sites. According to Dufrêne & Legendre (1997), good indicator species are always present at a particular land-use or study site within a given group/category and never occur in other groups/categories. The mean abundance of species *i* in site type *j* compared with all sites studied (specificity), by B_{ij}, the relative frequency of occurrence of species *i* in the site type *j* (fidelity). IndVal was determined according to the following formula adopted from Dufrêne & Legendre (1997):

$$A_{ij} = N_{ij}/N_i$$

$$B_{ij} = NS_{ij}/NS_j$$

$$IndVal_{ij} = A_{ij} * B_{ij} * 100$$

Where $IndVal_{ij}$ = Indicator Value of species *i* in site type/category *j*, N_{ij} = mean number of individuals of species *i* across sites type/category *j*, N_i = sum of the mean number of individuals of species *i* over all sites, NS_{ij} = the number of sites *j* where species *i* is present, NS_j = the total number of sites.

The indicator value ranges from 0 - no indication to 100% - describing perfect indication (Dufrene & Legendre 1997, McGeoch *et al.* 2002). The significance of each taxon was tested

using a Monte Carlo test (permutations N = 9999, $p < 0.05$) in PC-ORD version 5.10 following McCune & Mefford (2006), Bere & Tundisi (2011) and Dalu *et al.* (2014; 2015). The taxon with a significant indicator values ($p < 0.05$) were considered as indicator species.

Indicator taxa were determined on two ecological categories/types; indicator taxa for catchment land-uses (sewage, agricultural and confluence) and diagnostic taxa per study site. Due to differences in land-use properties between the two river systems, the Bloukrans-Kowie River system land-use indicator analysis was divided into four categories/sites types: (1) human settlement and sewage input (upstream Bloukrans site, close to Grahamstown settlements), (2) agricultural lands and fertilizer input (Mid-Bloukrans River), (3) natural/undisturbed habitats (upper reaches of Kowie River) and, (4) confluence (Bloukrans-Kowie confluence) sites representing upstream disturbances. The Bushmans-New Year's River system was categorized into: (1) isolated pools (New Year's site B1 – B2, B10), (2) non-point and sewage inputs (adjacent site to ASTWs), (3) agricultural lands (Golf course sites), (4) natural/undisturbed habitats and confluence sites.

An additional canonical multivariate analysis with the software program CANOCO 4.5, was used to assess the relationship between the environmental variables, aquatic macroinvertebrates relative abundance and study sites (ter Braak & Smilauer 2002). The analysis was used concurrently with IndVal to identify characteristic macroinvertebrates that correlated with high levels of nutrients. Detrended Correspondence Analysis (DCA) was used to determine the appropriate response model (linear or unimodal). The DCA illustrated a gradient length of 2.99 which was < 3 standard deviations (s.d), implying that taxa abundance exhibited a linear response to environmental variables (ter Braak & Smilauer 2002). A Redundancy Analysis (RDA) was then completed with species scores standardized by dividing the standard deviation. Species abundance data were further log transformed (log (x + 1)) prior to RDA analysis to prevent skewed results. RDA analysis involved a forward selection (Automatic selection) procedure, tested using a Monte Carlo significance test (permutation N = 9999, $p < 0.05$) under full model fit (ter Braak & Smilauer 2002). Preliminary RDA identified 8 of 25 environmental variables to be co-linear, and thus a selected subset of environmental variables with a variance inflation factors larger than 20 (VIF > 20) were removed and the analysis was re-run (ter Braak & Smilauer 2002).

5.2.2.2 ^{15}N isotopic values for Indicator Taxa

A General Linear Mixed-Effects Model (GLMM) was performed to test whether there were any significant differences in $\delta^{15}N$ isotopic values of indicator taxa between the four sampling events and between the four selected study sites. The coding for the chosen optimal model formula run was similar to that of Chapter 4, where:

model<-lme(^{15}N values~time, random=~1+time|sites, data=my data)

Where *lme* is the linear mixed-effect model function from the *nlme* package, *^{15}N~time of sampling* were the fixed effects, *~1+time* was the slope and intercept function of the $\delta^{15}N$ isotopic values over time, and sites were incorporated as a random effects. The model was fitted on $\delta^{15}N$ isotopic values for Oligochaeta and Chironomidae only, due to completeness of their collected data. These taxa were present at all times at all sampled study sites, therefore making them good candidates for comparison using the mixed-effect model (see Appendix 12; presence/absence data). Using the build-in *coef* function (*coef(model)*), intercepts and slope values of each fitted and plotted study site were obtained. Model validation was achieved using diagnostic plots of $\delta^{15}N$ residuals versus fitted $\delta^{15}N$ values, and $\delta^{15}N$ residuals versus predictor variables (e.g. sampled study sites and time of sampling).

A Multiple Linear Regression Analysis (MLRA) was also performed to investigate how explanatory variables influenced the response variables. The model followed the general formula:

model2<-lm(response~explanatory$_1$+explanatory$_2$+……+explanatory$_n$);

where the *response* variables were $\delta^{15}N$ isotopic values of Oligochaeta, Chironomidae, Culicidae and Syrphidae and the *explanatory* variables were the on-site collected physicochemical variables (e.g. dissolved oxygen [DO], ammonium [NH_4-N] and nitrate [NO_3-N]). Model validation was achieved using diagnostic plots e.g. $\delta^{15}N$ residual versus fitted $\delta^{15}N$ values, normal Q-Q plots and $\delta^{15}N$ residuals versus Leverage plots. All analyses were completed in the R environment (R Core Team; 2012).

5.3 Results

5.3.1 Potential Indicator Taxa

Indicator value species analysis

Overall, the Bloukrans-Kowie River system showed the highest number of significant indicator taxa. The majority of indicators were more indicative of land-use/catchment than study site; with only Syrphidae and Turbellaria families representing human settlement/sewage input and agricultural lands/fertilizer respectively. Natural/undisturbed habitats were represented by 9 families and 13 families were significantly diagnostic for the confluence (including upstream disturbance) land-use category (Table 5.1). Some families were also diagnostic of particular study sites, including Ecnomidae, Hydropsychidae and Tabanidae for site A10, Gerridae, Philopotamidae and Athericidae for site A9, Baetidae, Leptophlebiidae for site A8 as well as Lestidae and Pleidae for site A7. Site A6 had the highest number of diagnostic species, including Libellulidae, Hydrometridae, Nepidae, Leptoceridae, Dytiscidae and Gyrinidae (see Table 2.1, Chapter 2; Table 5.1).

Comparatively, the Bushmans-New Year's River system had the least amount of significant indicator taxa, which can likely be attributed to its lower relative macroinvertebrates abundance and biodiversity (see results, Chapter 3). Overall, the Bushmans-New Year's River system also had indicators that were more indicative of land-use/catchment than study site; with human settlement/sewage represented by 3 families (including Turbellaria), 3 families diagnostic of agricultural lands/fertilizer and 11 families significantly diagnostic for the confluence (including upstream disturbance) land-use category. No taxa were seen to represent natural/undisturbed habitat (Table 5.2). Lastly, 4 study sites could be related significant indicator taxa; Libellulidae for site B1; Aeshnidae for site B6 and Simuliidae and Porifera for site B9. Site B8 had the highest number of significant indicator taxa and included Potamonautidae, Atyidae, Caenidae, Leptophlebiidae, Ecnomidae and Ancylidae (see Table 2.2, Chapter 2; Table 5.2).

Pooling the Bloukrans-Kowie and Bushmans-New Year's River systems aquatic macroinvertebrate abundances provided a more overall representation of useful indicator species. Site A2, A6, A7, A8, A9, A10 and B5, B6, B8, B9 showed significant diagnostic taxa; with site A2 (which receives wastewater from urban and rural Grahamstown) represented by

Chironomidae, Culicidae and Syrphidae. Site A5 (downstream of a sewage out-fall and agricultural lands) had only Simuliidae as an indicator taxon. Site A6 (primarily natural/undisturbed) could be identified by Aeshnidae, Gomphidae, Libellulidae and Hydrometridae, site A7 (adjacent to agricultural lands/fertilizer) was represented by Coenagrionidae and Lestidae, while site A8 (confluence, including upstream disturbance) was represented by Baetidae and Leptophlebiidae. Site A9 (primarily natural/undisturbed) had 5 diagnostic taxa including Philopotamidae, Leptoceridae, Dytiscidae, Gyrinidae and Athericidae, and site A10 was characterized by Potamonautidae, Ecnomidae, Hydropsychidae and Tabanidae (Table 5.3). On the Bushmans-New Year's River system, sites B5 and B6 (downstream of the sewage out-fall and in the Bushman Sand Golf Course (agricultural lands/fertilizer)), were characterized by the Hirudinea and Pleidae families respectively. Site B8 and B9 (confluence, including upstream disturbance) had Atyidae and Ancylidae and Porifera as their indicator taxa respectively (Table 5.3). The IndVal analysis of the pooled data showed comparable results to the Bloukrans-Kowie and Bushmans-New Year's River analyzed individually.

Multivariate analysis

RDA was used to investigate the relationship between aquatic macroinvertebrates abundance patterns and the collected environmental variables, across sampled study sites. The accepted RDA plot reduced environmental variables to ten (i.e. nitrate, ammonium and phosphorus concentrations, flow rate (categorical), zinc concentrations, substrate diversity (as a score), carbonate concentrations, TDS, dissolved oxygen levels and turbidity). The first two RDA axes (1 and 2) accounted for 50.00% of the variance between taxa and 64.70% of the variance for the species-environment data. The Eigen values of axis 1, 2, 3 and 4 were 0.353, 0.147, 0.070 and 0.050 respectively. The scale in S.D. units was -1 to 1 for both macroinvertebrates and environmental variables (see full names of aquatic macroinvertebrates in Appendix 11). In this ordination, the metric species-environment correlation for four axes 1, 2, 3, and 4 indicated a very strong positive correlation of 0.987, 0.985, 0.887 and 0.871 respectively. The environmental variables categorical flow rate, [Zn], substrate diversity, [CO_3], TDS and DO were positively associated to axis 1, while turbidity, and [NH_4-N] were negatively associated to axis 1. Comparatively, [NH_4-N], [NO_3-N], [P], categorical flow rate, [Zn] and substrate diversity were positively associated to axis 2 and inversely [CO_3], TDS, DO and turbidity were negatively

associated to axis 2. RDA axis 1 and 2 separated all sampled study sites into three distinct groups based on nutrient enrichment levels and land-use, which were further characterized by associated aquatic macroinvertebrate community composition (Figure 5.1). The first group consisted of the heavily polluted and nutrient rich, upstream Bloukrans River study sites (A2, A3, A4 and A5; A3>A4>A5>A2 from extremely polluted to polluted) which were positively associated to axis 2 and thus highly correlated to [NH_4-N], [NO_3-N] and [P]. Aquatic macroinvertebrates characterizing this group were the relatively pollution tolerant Dipteran families: Chironomidae, Culicidae, Syrphidae and the aquatic earthworm, Oligochaeta (Beneberu *et al.* 2014). The second group was primarily comprised of sites on the Bushmans-New Year's River system (sites B1-B3, B5-B7 and B10), which were positively associated to axis 2 and thus showing strong correlations to high turbidity, likely indicative of the isolated pools nature of the sites. This second group illustrated generalist macroinvertebrate taxa with no precise habitat preference limits, however inhabiting moderately disturbed habitats. These included the pollution tolerate parasitic worm (Hirudinea), Psychodidae and the generalists Chaoboridae, Hydracarina, Helodidae, Notonectidae, Belostomatidae, Pleidae, Hydrophillidae, Turbellaria, Lymnaeidae and Physidae. The third group consisted of less polluted study sites that were positively associated to axis 1 (A6, A9, A10, A8, B8, B9 and A7; with A6>A9>A10>A8>B8>B9>A7, from the largely natural to "less disturbed"). Macroinvertebrates that mainly characterized this group were the pollution sensitive families including; Leptoceridae, Ecnomidae, Philopotamidae, Lestidae, Leptophlebiidae, Hydropsychidae, Baetidae, Aeshnidae, Tabanidae, Chlorocyphidae, Synlestidae, Caenidae, Platycnemididae, Gomphidae, Coenagrionidae, Ancylidae and Potamonautidae. This group associate with high levels of DO and increased substrate diversity coupled with adequate water flow rates. A Monte Carlo permutation test indicated that substrate diversity, [phosphorus], flow rate and [NO_3-N] were the only significant environmental variables ($p < 0.05$) that were responsible for aquatic macroinvertebrate composition and site groupings according to ecosystem health (e.g. polluted to less polluted sites). Therefore water quality (e.g. [P] and [NO_3-N]) and habitat structure (flow and substrate diversity) were clearly driving factors in the present study (Figure 5.1).

Table 5.1: List of significant ($p < 0.05$) indicator taxa and their observed IndVal percentage as indicators of different catchment land-use and study site categories on the Bloukrans-Kowie River system, Eastern Cape, South Africa.

Taxa	Catchment land-use			Study site		
	IndVal%	*P*-value	Catchment	IndVal%	*P*-value	Site
Gerridae				30.2	0.053	A9
Turbellaria	57.3	0.006	Agriculture			
Potamonautidae	41.0	0.003	Confluence			
Baetidae	34.6	0.053	Natural	17.7	0.043	A8
Leptophlebiidae	46.7	0.015	Confluence	29.9	0.004	A8
Chlorocyphidae	32.7	0.05	Confluence			
Coenagrionidae	39.6	0.037	Confluence			
Aeshnidae	49.2	0.013	Natural			
Gomphidae	58.8	0.000	Natural			
Belostomatidae	45.8	0.006	Confluence			
Corixidae	37.9	0.05	Natural			
Notonectidae	46.2	0.025	Natural			
Elmidae	34.1	0.045	Confluence			
Syrphidae	34.5	0.046	Sewage			
Tipulidae	37.4	0.025	Confluence			
Physidae	41.0	0.051	Confluence			
Lestidae				36.3	0.032	A7
Libellulidae				41.2	0.003	A6
Hydrometridae	41.7	0.010	Natural	39.8	0.044	A6
Nepidae				47.3	0.010	A6
Pleidae	62.4	0.003	Confluence	38.8	0.021	A7
Ecnomidae	67.2	0.000	Confluence	40.6	0.002	A10
Hydropsychidae	57.8	0.000	Confluence	30.6	0.004	A10
Philopotamidae	50.0	0.012	Natural	40.9	0.027	A9
Leptoceridae	51.9	0.009	Natural	57.8	0.0001	A6
Dytiscidae	51.4	0.006	Confluence	30.6	0.009	A6
Gyrinidae	37.4	0.051	Natural	35.2	0.033	A6
Athericidae				75.0	0.004	A9
Tabanidae	71.6	0.000	Confluence	45.9	0.002	A10

Table 5.2: List of significant ($p < 0.05$) indicator taxa and their observed IndVal percentage as indicators of different catchment land-use and study site categories on the Bushmans-New Year's River system, Eastern Cape, South Africa.

Taxa	Catchment land-use			Study site		
	IndVal%	***P*-value**	**Catchment**	**IndVal%**	***P*-value**	**Site**
Turbellaria	39.8	0.022	Sewage			
Baetidae	37.6	0.033	Confluence			
Aeshnidae	45.0	0.009	Fertilizer	30.7	0.045	B6
Pleidae	39.8	0.025	Fertilizer			
Gerridae	39.2	0.025	Fertilizer			
Coenagrionidae	37.6	0.033	Confluence			
Hydrpsychidae	54.2	0.007	Confluence			
Hydracarina	36.2	0.027	Sewage			
Dytiscidae	38.2	0.026	Sewage			
Porifera	75.0	0.000	Confluence	60.6	0.002	B9
Potamonautidae	90.6	0.000	Confluence	45.2	0.009	B8
Atyidae	67.5	0.000	Confluence	33.7	0.009	B8
Caenidae	94.1	0.000	Confluence	48.7	0.003	B8
Leptophlebiidae	87.5	0.000	Confluence	85.7	0.000	B8
Libellulidae				42.8	0.003	B1
Ecnomidae	47.1	0.002	Confluence	52.2	0.011	B8
Simuliidae	36.5	0.012	Confluence	49.6	0.014	B9
Ancylidae	86.4	0.000	Confluence	47.2	0.003	B8

Table 5.3: List of significant ($p < 0.05$) indicator taxa from pooled aquatic macroinvertebrates abundance collected on all study sites on the Bloukrans-Kowie and the Bushman-New Year's River system Eastern Cape South Africa.

Taxa	IndVal Outcome		
	IndVal%	***P*-value**	**System & Site**
Porifera	29.3	0.011	Bush B9
Hirudinea	16.3	0.039	Bush B5
Potamonautidae	15.8	0.012	Blouk A10
Atyidae	33.7	0.000	Bush B8
Baetidae	11.7	0.005	Blouk A8
Leptophlebiidae	22.8	0.003	Blouk A8
Coenagrionidae	11.9	0.047	Blouk A7
Lestidae	34.4	0.005	Blouk A7
Aeshnidae	19.4	0.043	Blouk A6
Gomphidae	22.7	0.008	Blouk A6
Libellulidae	22.3	0.004	Blouk A6
Hydrometridae	27.6	0.027	Blouk A6
Pleidae	16.8	0.041	Bush B6
Ecnomidae	26.9	0.007	Blouk A10
Hydropsychidae	26.4	0.000	Blouk A10
Philopotamidae	30.7	0.019	Blouk A9
Leptoceridae	52.5	0.0001	Blouk A9
Dytiscidae	13.0	0.031	Blouk A9
Gyrinidae	27.9	0.008	Blouk A9
Athericidae	75.0	0.001	Blouk A9
Chironomidae	10.5	0.011	Blouk A2
Culicidae	14.7	0.029	Blouk A2
Simuliidae	17.8	0.034	Blouk A5
Syrphidae	32.4	0.048	Blouk A2
Tabanidae	36.9	0.000	Blouk A10
Ancylidae	16.4	0.034	Bush B8

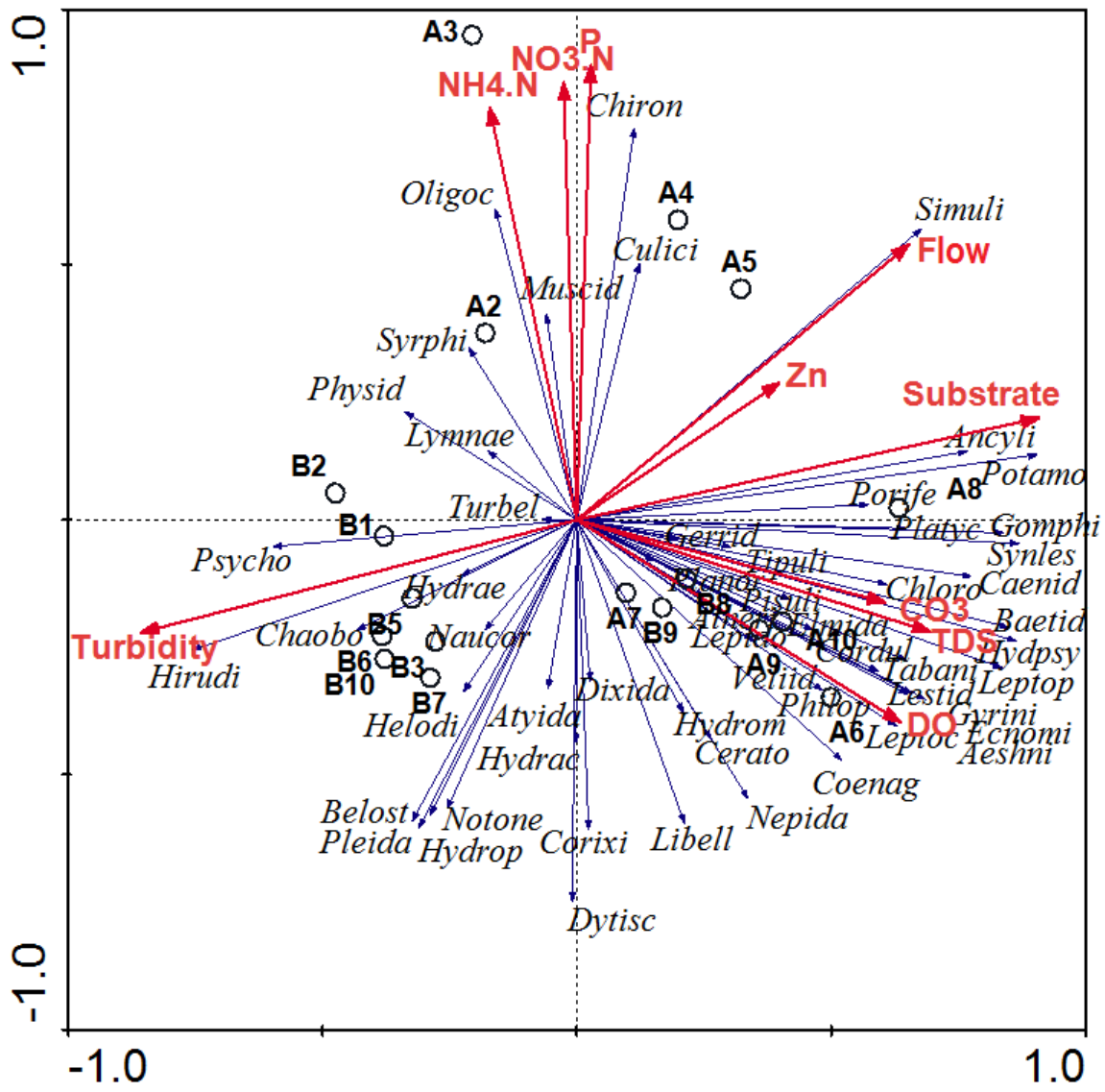


Figure 5.1: Redundancy Analysis (RDA) tri-plot representing aquatic macroinvertebrate relative abundance in relation to environmental variables across all sampled study sites on the Bloukrans-Kowie and Bushmans-New Year's River systems, Eastern Cape, South Africa. Red arrows represent the environmental variables, blue arrows represent the abundance of aquatic macroinvertebrates, and the black circles represent study sites. The full names of macroinvertebrates are provided in Appendix 11).

5.3.2 Isotopic values of indicator taxa

The RDA showed 4 sites; A2 – A5 to be strongly correlated with [NH_4-N], [NO_3-N] and [P] (variable that effectively describe eutrophication). The macroinvertebrate taxa associated with these environmental variables and sites included Chironomidae, Culicidae, Syrphidae and Oligochaeta and Muscidae (Figure 5.1). The subsequent pooled IndVal analysis showed Chironomidae, Culicidae and Syrphidae as the only taxa which represented site A2 (severely disturbed sites, receiving rural and urban waste from Grahamstown). Based on both the RDA and the IndVal analyses, Oligochaeta, Chironomidae, Culicidae and Syrphidae were chosen as the best options for N-loading indicator taxa. The choices of macroinvertebrates N-loading indicators were also supported by work from Dickens & Graham (1998), Beyene *et al.* (2009), Beneberu *et al.* (2014) and Bere & Nyamupingidza (2014).

Only two macroinvertebrate taxa; Oligochaeta and Chironomidae were present throughout the study period at all study sites. Culicidae and Syrphidae presence was variable between sampled sites (see presence/absence data, Appendix 12), with Syrphidae completely absent at site A9 throughout the study and Culicidae observed only once at site A9 at T_3. Thus only the isotopic data for Oligochaeta and Chironomidae are presented below.

Oligochaeta and Chironomidae families showed $\delta^{15}N$ and $\delta^{13}C$ isotopic values that varied both temporally and spatially at the four selected Bloukrans-Kowie River system sites over four sampling events in March 2015 (Figure 5.2 & 5.3). Oligochaeta illustrated enriched $\delta^{15}N$ isotopic values (> +10.00 ‰, likely indicating eutrophication; see chapter 4) at site A3 at T_2, A4 throughout the entire study period and at A9 at T_4. Relatively depleted $\delta^{15}N$ isotopic values (< +10.00 ‰, likely indicating largely natural N-inputs, see chapter 4) were observed at A2 throughout the entire study period, A3 at T_1, T_3 – T_4 and A9 at T_1 and T_3 (Figure 5.2A). Similarly, Chironomidae also showed enriched $\delta^{15}N$ isotopic values (> +10.00 ‰) at site A3 between T_1 – T_2, A4 at T_1 – T_2, T_4 and depleted $\delta^{15}N$ isotopic values (< +10.00 ‰) at site A2 throughout the study period, at A3 between T_3 – T_4, A4 at T_4 and A9 throughout the study period (Figure 5.3A). In general, both Oligochaeta and Chironomidae $\delta^{15}N$ isotopic values followed a gradual increase from site A2 to A3 and reached the most enriched $\delta^{15}N$ isotopic values at site A4 on all sampling events, with the exception of the Chironomidae at T_3. At T_3, Chironomidae $\delta^{15}N$ isotopic values consistently ranged between +2.00 to +4.00 ‰ across all study sites, while

comparatively, the $\delta^{15}N$ isotopic values of Oligochaeta collected at the same sites at the same sampling events followed the originally described trend of $\delta^{15}N$ enrichment from site A2-A4. Organisms at A9 showed different patterns, with oligochaetes enriching over time, but with chironomids remaining more or less between +2.00 to +6.00 ‰ over the entire time period (Figure 5.2A & 5.3A).

The $\delta^{13}C$ isotopic values of both Oligochaeta and Chironomidae showed a range of between -22.00 to -26.00 ‰ and -22.00 to -28.00 ‰ respectively, throughout the investigation (Figure 5.2B & 5.3B). Generally, both taxa illustrated a spatial trend of $\delta^{13}C$ isotopic values depletion, from site A2 to A9. $\delta^{13}C$ isotopic values for Oligochaeta and Chironomidae at A9 were equivalent to those at site A4 (with the exception of Chironomidae at T_3) and temporally both taxa illustrated less variation within sites throughout the study.

Linear mixed-effects model

Mixed-effects models fitted on $\delta^{15}N$ isotopic values of Oligochaeta and Chironomidae were significantly different between all study sites on the Bloukrans-Kowie River system (Figure 5.4A & B, Table 5.5). Furthermore, from the model fit statistics, temporal variation in $\delta^{15}N$ isotopic values (Figure 5.2A & 5.3 A) were significantly different ($p < 0.05$) between study sites (Table 5.4). $\delta^{15}N$ isotopic values of Oligochaeta and Chironomidae over time showed a negative and positive correlation as factors of both random effects (r = -0.87 and r = 0.53) and fixed effects (r = -0.87 and r = 0.45) respectively (Table 5.4). Slopes from each site were compared to the population line slope (~ 0 and ~ -0.99) and intercepts (+8.72 and +10.23 ‰) of Oligochaeta and Chironomidae respectively. The population regression line was used to distinguish between nutrient rich, eutrophic study sites (> +10.00 ‰) and less nutrient rich, oligotrophic study sites (+2.00 - +8.00 ‰). Sites A3 (slope: -0.93, intercept: +10.13 ‰) and A4 (slope: -1.45, intercept: +17.59 ‰) for Oligochaeta and sites A3 (slope:-1.44, intercept: +12.21 ‰) and A4 (slope: 0.67, intercept: +14.65 ‰) also for Chironomidae showed regression lines and intercept above that of the population line, and were thus significantly enriched compared to rest of the study sites, therefore they were identified as eutrophic (see also Chapter 4 – Results) (Figure 5.4 A & B). Comparatively, both sites A2 (slope: 0.14, intercept: +4.56 ‰ for Oligochaeta; slope: -1.02, intercept: +6.94 for Chironomidae) and A9 (slope: 2.12, intercept: +2.59 ‰ for Oligochaeta, slope: -0.96, intercept: +7.13 ‰ for Chironomidae) had an intercept equating to ~ < +8.00 ‰,

which was less than the population line and were thus identified as oligotrophic study sites (Figure 5.4 A & B, 5.5). Site A2 and A9 for Oligochaeta and site A4 for Chironomidae were the only study sites that showed a positive linear relationship (slope > 0).

Model-checking plots showed that $\delta^{15}N$ residuals versus fitted $\delta^{15}N$ values for both the Oligochaeta and Chironomidae were fairly well behaved (showing a linear band of points concentrated on the reference line) (see Appendix 14). $\delta^{15}N$ residuals versus predictor variables (study sites and time of sampling) also confirmed a mean variation approximately equating to the reference point, however site A3 for Oligochaeta $\delta^{15}N$ isotopic values had a slight deviation from the reference point (see Appendix 15). $\delta^{15}N$ residuals and time diagnostic plots also showed a semi-circular pattern comparable with that of *Spirodela* (Chapter 4) plants throughout the sampling period (see Chapter 4, Appendix 8).

Multiple linear regression

Strong significant relationships were seen between on-site physicochemical variables and $\delta^{15}N$ isotopic values of Chironomidae, Culicidae and Syrphidae throughout the investigation. DO, $[NH_4]$ and $[NO_3]$ (which were significantly different between sites, appendix 16) had a significant associations with $\delta^{15}N$ isotopic values of Chironomidae ($r^2 = 0.53$, $F_{3\text{-}43} = 16.23$, $p < 0.001$), Syrphidae ($r^2 = 0.86$, $F_{3\text{-}10} = 20.11$, $p < 0.001$) and Culicidae ($r^2 = 0.81$, $F_{3\text{-}20} = 27.86$, $p < 0.001$), and explained $\geq$ 50.00% variation of Oligochaeta and Chironomidae $\delta^{15}N$ isotopic values. However Oligochaeta $\delta^{15}N$ isotopic values and physicochemical variables showed a very weak but significant association, and the physicochemical variables explained only 19% variation of the nitrogen data ($r^2 = 0.25$, $F_{3\text{-}41} = 4.47$, $p < 0.01$).

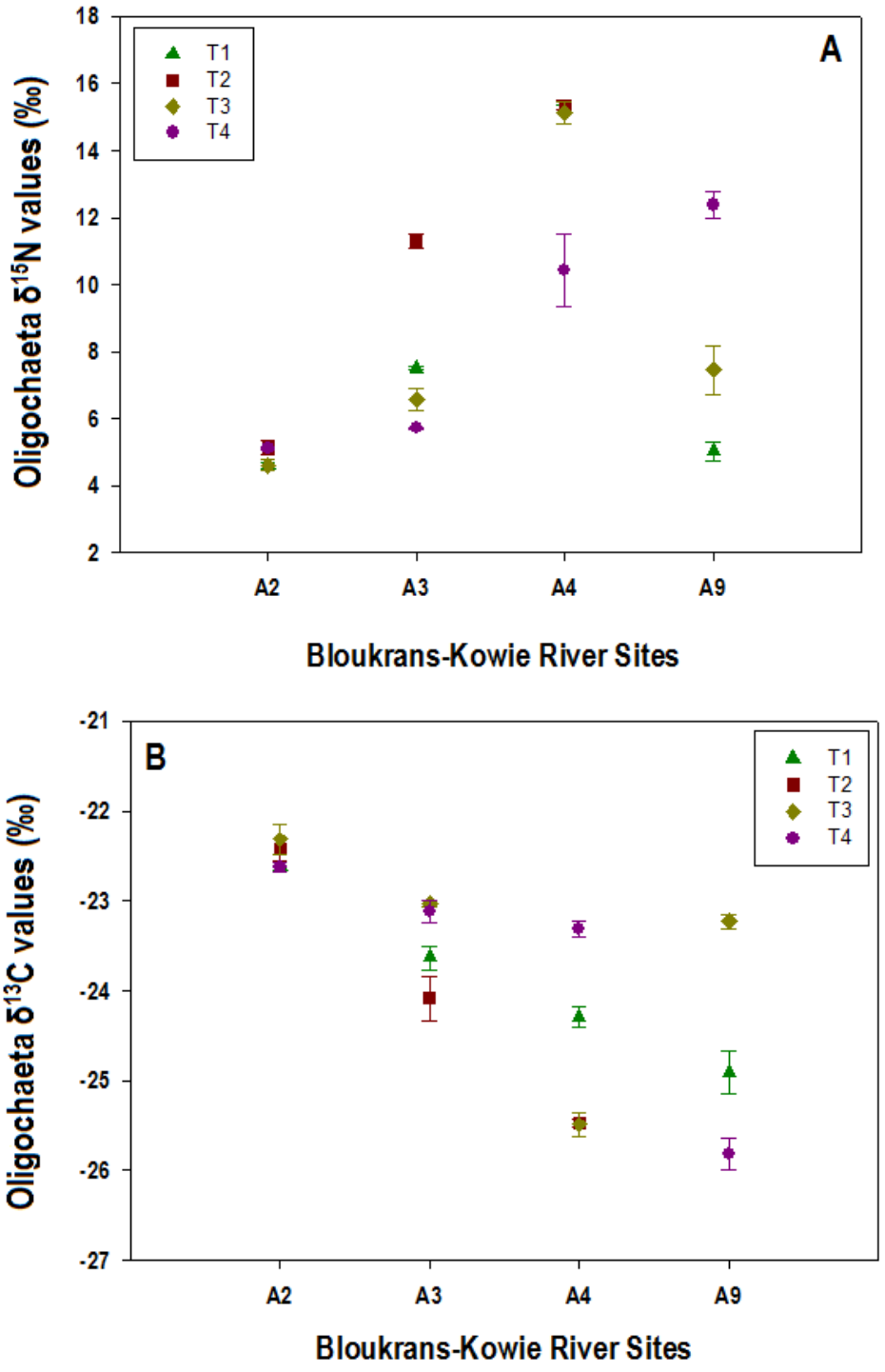


Figure 5.2: Oligochaeta (A) $\delta^{15}N$ and (B) $\delta^{13}C$ isotopic values at four selected study sites on the Bloukrans-Kowie River system, Eastern Cape South Africa, during four weeks (T_1, T_2, T_3, T_4) sampling events in March 2015.

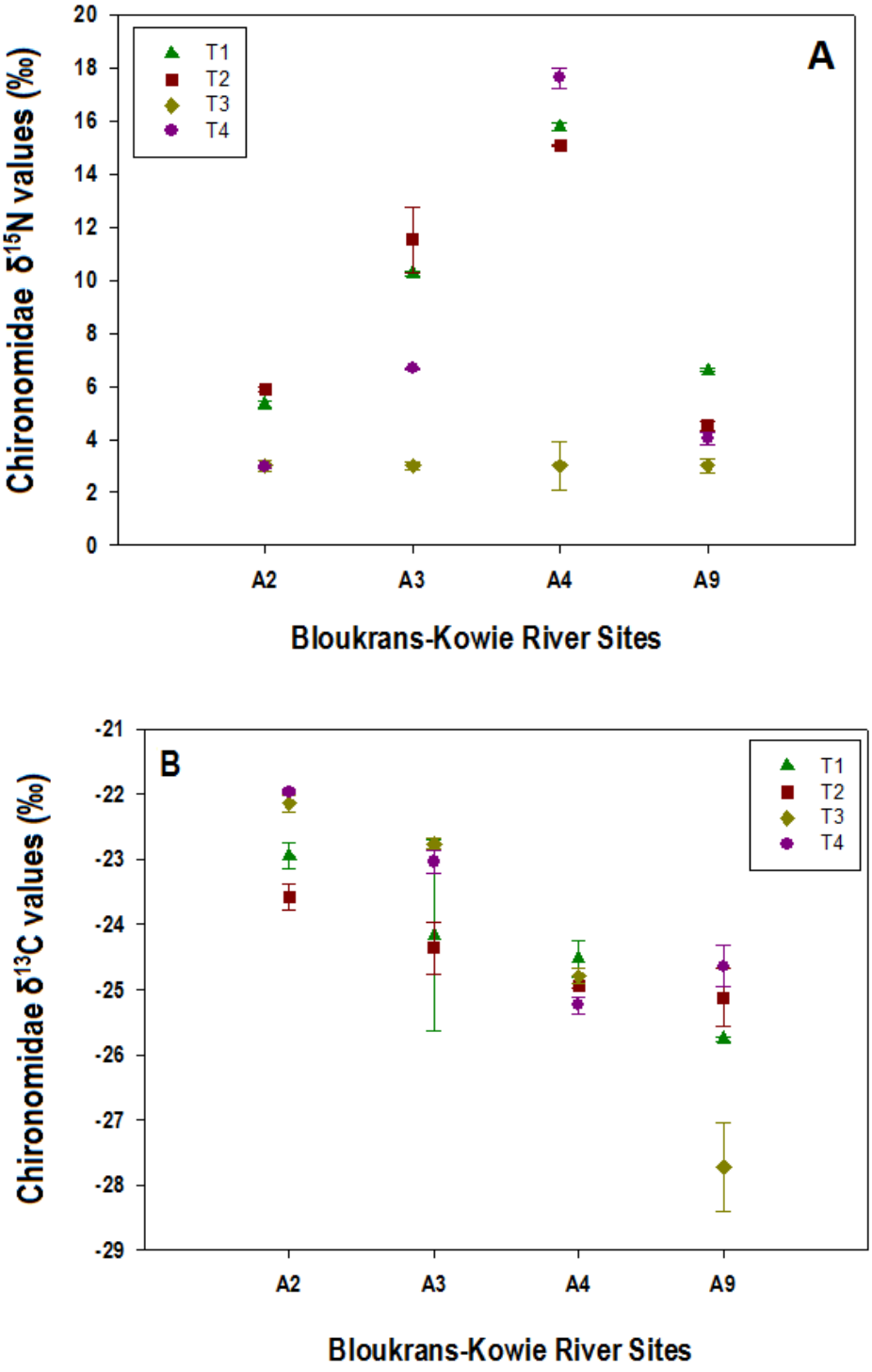


Figure 5.3: Chironomidae (A) $\delta^{15}N$ and (B) $\delta^{13}C$ isotopic values at four selected study sites on the Bloukrans-Kowie River system, Eastern Cape South Africa, during four weeks (T_1, T_2, T_3, T_4) sampling events in March 2015.

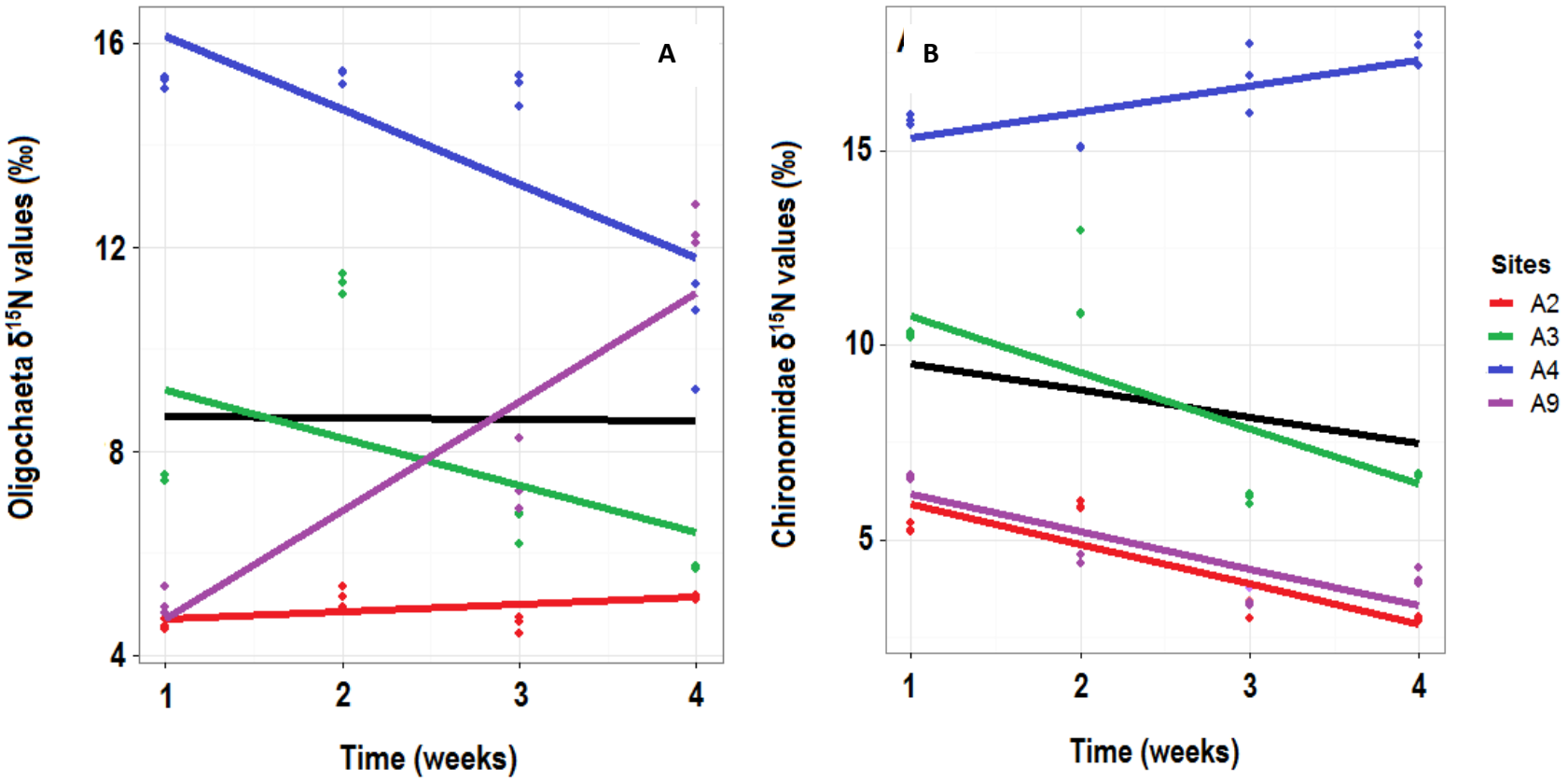


Figure 5.4: (A) Oligochaeta and (B) Chironomidae mixed-effect model plots showing differences in the $\delta^{15}N$ intercept and slope from predicted $\delta^{15}N$ isotopic values over the 13 month sampling period. Colored solid regression lines represent different study sites and the black solid line represents the population line.

Table 5.5: Summary of linear mixed-effect model fit statistics for $\delta^{15}N$ isotopic ratios of identified biological indicators for eutrophication on the Bloukrans-Kowie River system, Eastern Cape South Africa.

Model Statistics	**Linear Mixed-Effect Model**	
	Oligochaeta	**Chironomidae**
Model AIC	194.27	175.12
Random Effects StdDev.		
Intercept	6.78	3.89
Time	1.62	0.96
Residuals	1.43	1.06
Random Effect Correlation	-0.87	0.53
Fixed Effects		
Intercept (±Std. error)	8.72±3.44	10.23±1.98
Time (±Std. error)	-0.03±0.83	-0.69±0.50
	df = 40 *t*-value = -0.03 *p* = 0.03	*df* = 42 *t*-value = -1.38 *p* = 0.04
Fixed Effects Correlation	-0.87	0.45
Number of Observations	45	47
Number of Groups	4	4

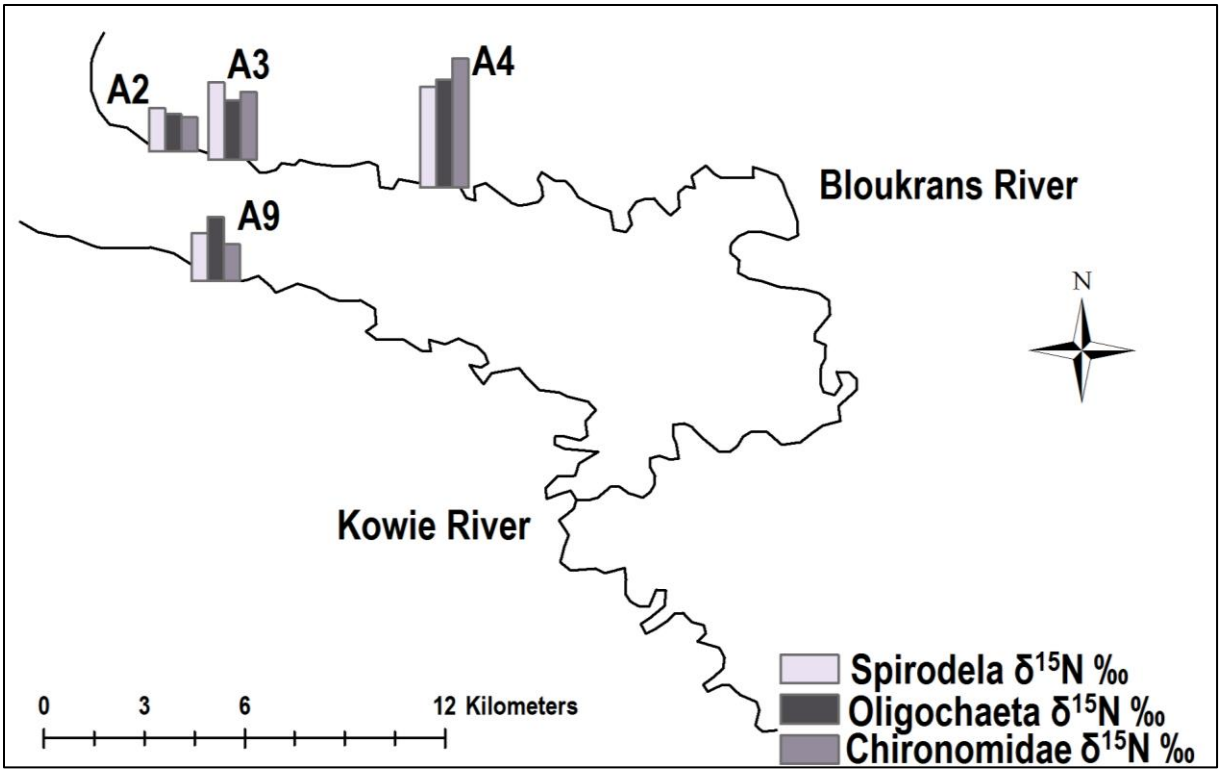


Figure 5.5: Average $\delta^{15}N$ ratios (‰) of *Spirodela* plant, Chironomidae and Oligochaeta at four Bloukrans-Kowie River, Eastern Cape South Africa sites, over four sampling events in March 2015. Black arrows indicate sewage out-fall (between A2 & A3) and cow manure run-off (between A3 & A4) from adjacent dairy-farm lands on the Belmont Valley road, Grahamstown Eastern Cape.

5.4 Discussion

Biological indicators

Aquatic macroinvertebrate abundance patterns were positively correlated to environmental variables on both Bloukrans-Kowie and Bushmans-New Year's River systems, Eastern Cape. This was in agreement with other water quality and biological assessment studies, investigating the effect of land-use activities using macroinvertebrates to assess the river health (Dickens & Graham 1998, Ndebele-Murisa 2012, Seanego & Moyo 2013, Bere & Nyamupingidza 2014, Farrell *et al.* 2015). Sites were grouped into three distinct clusters based on pollution levels and the pollution tolerance of macroinvertebrates at each study site. Multivariate analysis further demonstrated the capacity of macroinvertebrates to act as biological indicators of different levels of pollution and land-use as suggested by Dallas & Day (2004) and Bonada *et al*. (2006). Site A2, A3, A4 and A5 were correlated to relatively poor water quality (as seen by high nutrient levels of P, NH_4-N and NO_3-N) and was supported by the presence of pollution tolerant macroinvertebrate taxa e.g. Oligochaeta, Chironomidae, Culicidae and Muscidae supplemented by Syrphidae. These taxa are regarded as good diagnostic indicators for high pollution disturbance in freshwater ecology (Dickens & Graham 1998, Beyene *et al.* 2009, Beneberu *et al.* 2014, Bere & Nyamupingidza 2014). All these taxa are adapted to survive in polluted waters with low oxygen levels, oligochaetes and chironomids for example, use their haemolymph fluid which contains high concentration of haemoglobin making them resistant to low oxygen levels (Weber & Vinogradov 2001, Van Hoven & Day 2002). Syrphids and culicids on the other hand, use their respiratory tubes to help them to exchanges gases from the water surface enabling them to take oxygen directly from the atmosphere (Beneberu *et al.* 2014). The majority of taxa for this first group were also observed to be indicators of pollution/nutrient rich study sites in the IndVal analysis, where Syrphidae was an indicator for the sewage and Chironomidae, Syrphidae and Culicidae were diagnostic macroinvertebrates for site A2. Ndebele-Murisa (2012) also observed Chironomidae family in abundance in nutrient rich study sites, receiving effluents from the nearby industry, in Harare Zimbabwe. The second group included site B1, B2, B3, B5, B6 and B10, and were the least/or at least less polluted, but severely physically modified study sites. These sites were for the majority of the study period, converted into isolated pools, and thus had poor biotope diversity (only sandy to muddy biotopes

present), minimal flow rates, high water temperatures, high turbidity and a lower concentration of dissolved oxygen as is characteristic for lentic water bodies (O'Keeffe *et al.* 1990). The majority of aquatic macroinvertebrates that colonize such spaces are, in most cases, predatory taxa which are only affected by the presence/absence of prey (e.g. Hemiptera: Notonectidae, Belostomatidae, Pleidae and Naucoridae; see Chapter 3, Results section). Additionally, taxa that prefer muddy/sandy habitats (e.g. Hirudinea, Turbellaria and certain Coleoptera families) were also recorded amongst this group. Again, this correlates with the IndVal analysis, where the predatory Libellulidae was identified as an indicator taxon for site B1 and Hirudinea and Pleidae as indicator taxa for the muddy/sandy sites B5 and B6 respectively. This topic has been widely illustrated using macroinvertebrates correlating with their micro-habitat e.g. physical and chemical characteristics to maximize their survival (Gies *et al*. 2015, Shearer *et al.* 2015). Group three constituted of minimally polluted and more natural downstream study sites on both the Bloukrans-Kowie and Bushmans-New Year's River system, showing a strong correlation with high levels of DO, supported by diverse biotopes and with less acidic waters as shown by moderate concentrations of CO_3 and TDS. The group exhibited a diverse group of macroinvertebrates, most of which are documented as sensitive to any anthropogenic activities. Furthermore, Simaika & Samways (2012) noted that Odonata (dragonflies) are good indicators of habitat quality and this third group included a number of families of Odonata despite their endangered status due to habitat modification (Kietzka *et al.* 2015), therefore supporting the identification of this last group of sites as largely natural.

Stable isotopic values of indicator taxa

The spatial variation in $\delta^{15}N$ isotopic values of both Oligochaeta and Chironomidae generally reflected excessive nutrient loads from catchment land-use in a similar fashion to sewage plume mapping in *Spirodela* plants (see chapter 4). Majority of excess nutrients as expected identified on both taxa isotopic values indicated high magnitude of stable nitrogen isotopic values (> +10.00 ‰) of sewage out-fall between site A2 - A3 and cow manure run-off between A3 - A4. Therefore site A4 showing the highest nitrogen isotopic values, due to nutrient gradient from A3. Comparatively, site A9 only showed N-values of between (+2.00 ‰ - +6.00‰), which are similar to nitrogen harvested from the atmosphere, therefore indicating no excessive nutrient inputs but only a natural habitat/site. Similar findings were reported by Xu &

Zhang (2012) and di Lascio *et al.* (2013), where zooplankton and primary consumers showed elevated $\delta^{15}N$ isotopic values after exposure to point and diffuse source nutrient loads through time and space. Morrissey *et al.* (2013) also conducted a similar study using aquatic macroinvertebrate families which represented a wide range of functional feeding groups. Their study however, showed no significant differences in $\delta^{15}N$ isotopic values between macroinvertebrates taken from sites upstream and downstream of a wastewater treatment facility. However, the observed variation ($\delta^{15}N_{Downstream} - \delta^{15}N_{Upstream}$) provided enough evidence to show that macroinvertebrates exposed to excessively high nutrients have altered their nitrogen isotopic ratios. Based on the present study and that of Cabana & Rasmussen (1996), Matthews & Mazumder (2003), Xu *et al.* (2005B; 2010) and Xu & Zhang (2012), primary consumers with only a short life span and small body size (zooplankton or oligochaetes and chironomids) have a high nitrogen turn over as compared to large body size and long life span (mussels or fish) aquatic organisms. Furthermore, following similar studies (e.g. Xu & Zhang (2012), Morrissey *et al.* (2013)) the present study concluded that $\delta^{15}N$ isotopic values of macroinvertebrates will vary with the body size, life span and dietary source (position in the trophic level) of the selected biological indicator. Although our study showed increasing nitrogen values with respect to anthropogenic influence, we further observed that temporally nitrogen values between the two taxa differed, particular that of chironomids at week 3 (T_3). This could be explained by; firstly, oligochaetes and chironomids difference in their dietary sources, this was observed on both taxa using carbon isotopic values were taxa (both oligochaetes and chironomids) found at site A2 were more/moderately enriched in carbon sources than taxa found at site A4 and A9. Therefore indicating different dietary source and carbon fractionation, this further outline the importance for using dual stable isotope analysis, where not only they can point out and map pollution but can further provide information on the trophic status of the taxa in subject. Secondly life span, Chironomidae like any other dipteran families have a life span of about two to three weeks (Harrison 2003, Arva *et al*. 2015), unlike oligochaetes with a life span of about a month to a year (Van Hoven & Day 2002). Therefore there is a possibility that at T_3/week 3, we could have sampled a new hatched generation of chironomids (but the same generation of oligochaetes) that has not yet being exposed to nutrient gradient on selected sites, hence a constant/similar nitrogen isotopic value recoded at T_3 on all study sites irrespective of the nutrient gradient as compared to nitrogen isotopic values of oligochaetes. This is following DeNiro & Epstein (1981) emphasizes

that nutrient rich sediments, surface water column nutrients concentration and primary producers subsequent wastewater input in aquatic ecosystems reflect increased nitrogen isotopic values of short lived and small bodied primary consumers that graze and/or filter feed on the vicinity. This was in agreement with our study, and further supports the constant chironomids nitrogen isotopic values which were not corresponding with high water nutrient concentration, meaning sampled taxa should have been the newly emerged generation. According to Xu & Zhang (2012) this will also be the cases in terms of spatially and temporal differences and this was also the trend observed on the present study were different water nutrient concentration and macroinvertebrate nitrogen isotopic values were showing a highly correlation throughout the study. Therefore for Morrissey *et al.* (2013) study, body size and organism life span could have been factors attributed less variation in macroinvertebrate $\delta^{15}N$ isotopic values and also the quality of discharged effluents may have not been of any significant difference as compared to that of the receiving stream.

Although Cole *et al.* (2004) and Udy *et al.* (2006) cited in Bergfur *et al.* (2009) noted that $\delta^{13}C$ isotopic values are as much important as $\delta^{15}N$ isotopic values in eutrophication studies. Furthermore Voss *et al.* (2000) also added that increased primary production leads to reduced discrimination of ^{13}C in photosynthesis and this leads to increase/enriched $\delta^{13}C$ isotopic values. However Bergfur *et al.* (2009) found no differences in $\delta^{13}C$ isotopic values of periphyton, organic matter, invertebrates and fish species along the nutrient gradient boreal stream in Sweden. On the other hand Xu & Zhang (2012) only regarded nitrogen isotopic values as good indicator of increased anthropogenic and land-use activities on aquatic ecosystems. On the present study $\delta^{13}C$ isotopic ratios of primary consumers showed no relationship to the nutrient gradient as implied by Voss *et al.* (2000) and Finlay (2001) and we conclude that no effect of nutrient enrichment were seen on photosynthesis rates in the Bloukrans-Kowie River system were probably small and had no effect on the discrimination between heavy and light carbon isotopes of primary consumers as it was the case with the *Spirodela* plants (Chapter 4). However we recommend that when using aquatic macroinvertebrates as indicator taxa, carbon isotopic values should be taken into consideration as it is able to trace and identify if selected taxa share the same or different dietary sources (organic matter), and this can contribute towards reflected nitrogen isotopic values interpretation.

Therefore primary consumers can be used as reliable biological indicators of nitrogen pollution in aquatic ecosystems, however because primary consumers have a high nitrogen turn over in a short period of time, thus they must be consider for only a short term studies particularly chironomids, such will give a better understanding on nutrients dynamics as a function of nitrogen pollution. Only for long term studies that users are recommended to consider nitrogen isotopic values of long lived, large body sized molluscs, mussels and oysters which can better reflect environmental changes that happened in longer time periods as opposed to primary consumers (Gustafson *et al.* 2007, Wen *et al.* 2010). Agreeing to Bergfur *et al.* (2009) statement our study was also aimed at opening opportunities for more studies that will cover broader gradients in nutrients concentration and also determine potential threshold values by calibrating macroinvertebrates $\delta^{15}N$ isotopic values taking in consideration differences in dietary requirements, life span and body size for taxa of interest in order to further develop they isotopic technique as tools in biological assessment.

CHAPTER 6

GENERAL DISCUSSION

Anthropogenic activities, particularly environmental nitrogen pollution, have been increasing on a global scale since the industrial revolution, and substantial pollution loads have reached aquatic ecosystems, resulting in numerous environmental impacts, including eutrophication, poor water quality, decline in aquatic biodiversity, the promotion of alien species invasions and the proliferation of water borne diseases (Chen *et al.* 2012, Coetzee & Hill 2012). Thus, Fenech *et al.* (2012) emphasizes that tracing N-sources in aquatic ecosystems is of vital importance for improving both human and environmental health issues and as this has importance with regards to legislation, it has become a priority for many countries. Vander Zanden *et al.* (2005) and Hill *et al.* (2012) further argue that studies in eutrophication and the means of identifying its sources are limited, particularly in developing countries.

Nyenje *et al.* (2010) reported that high levels of eutrophication and excess nutrient release are common in urban areas in sub-Saharan African countries, and further highlighted a few of South African freshwater systems; e.g. the Hartbeespoort Dam, Roodeplaat Dam, Hennops River and Rietvlei wetlands (Gauteng: Thornton & Ashton 1989, Oberholster *et al.* 2008, Harding 2015), Berg River (Western Cape: de Villiers 2007), Lake Krugersdrift, Modder River (Free State: Oberholster *et al.* 2009), Umtata River (Eastern Cape: Fatoki *et al.* 2001) and Molopo River (North West: Munyati 2015) as severely nutrient enriched. This was attributed primarily to poor infrastructure and waste management, including lack of sewer lines, sewerage treatment plants or solid waste disposal sites surrounding these waterways. This nutrient overload manifests as consistent eutrophication that can further result in complete alteration or degradation of ecosystem structure and function, thus threatening human and environmental health (McClelland & Valilea 1998, Rabalais 2002, Anderson & Cabana 2005). Accordingly, Coetzee & Hill (2012) South Africa constitutes the most eutrophic water bodies globally. Now more than ever, there is a demand for initial assessments on the trophic status of ecosystems and for the tracing of sources of nutrient loading into aquatic ecosystems, both of which are essential steps towards understanding the detrimental effects of eutrophication (Xu & Zhang 2012).

This chapter will discuss all the tested biological monitoring methodologies (Chapter 2 – Chapter 5), their ecological capabilities and challenges with regards to the present study and the scientific literature. The aim of this chapter is to identify which technique/methodology (or combination of techniques/methodologies) will help us to best resolve the temporal and spatial dynamics of nitrogen loading in freshwater ecosystems, as well as provide a potential early warning system that will provide better tools for the management of freshwater ecosystems.

6.1 Environmental variables as indicators of N-loading

The effluent discharge from the Belmont Valley Sewerage Treatment Works (BVSTWs) and the Alicedale Sewerage Treatment Works (ASTWs), coupled with diffuse inputs of waste material from urban and rural settlements around Grahamstown and Alicedale, showed potential detrimental effects on the downstream receiving systems; both on the Bloukrans-Kowie and Bushmans-New Year's Rivers. Numerous sites on both rivers (A8, A10, B4) showed average pH values greater than 8.5, which is reported to work synergistically, increasing the toxicity of certain detrimental micronutrients in aquatic ecosystems, e.g. increasing the conversion rate of NH_4 to NH_3, and enhancing the toxicity effect of aluminum (Al), cadmium (Cd) and zinc (Zn) (Palmer *et al.* 2004). Additionally, not only were these sites a concern with respect to pH, many (but not all) also showed high nitrogen (NO_2^-) and phosphorus (P) concentrations that were above the South African Targeted Water Quality Range (TWQR; < 1.50 mg/L effluent discharge), in particular site A3, A4, A5 and B3, B5 which were situated adjacent and downstream of the BVSWs and ASTWs discharge points respectively. Effluent discharge and cultural eutrophication (land-use activities which result in synthetic/manure fertilizer run-off) have been reported as the main drivers of excessive levels of nitrogen and phosphorus in local watersheds (de Villiers 2007). Dissolved oxygen (DO) levels have also been linked to poor ecosystem health, with Ndebele-Murisa (2012) and Farrell *et al.* (2015) reporting low DO concentrations associated with nutrient enrichment in aquatic ecosystems. Surprisingly, despite high pH and increased concentrations of N and P at numerous sites on both rivers in this study, which all indicate poor ecosystem health, dissolved oxygen (DO) levels for the majority of sites fell into the 'safe' range for proper ecosystem functioning (> 3.0 mg/L) as defined by Chapman (1992). Additionally, the rest of the micronutrient concentrations at the majority of sites in this study were well within the recommended South African Water Quality Guidelines for the well-

being of aquatic biota, domestic water use and safe effluent discharge (DWAF 1996A, Morrison *et al.* 2001). Often this was also in contrast to the levels of impairment and assessments of ecosystem health determined using measures of macroinvertebrate diversity and SASS5 assessments recorded for these sites (which will be discussed further below). The use of water quality parameters in aquatic ecosystem are therefore challenging to use as reliable tools for monitoring aquatic ecosystem health and can be complicated by; (1) a lack of globally standardized ranges, accepted as ecologically relevant, which are not subject to changes in governmental directives (2) a lack of time integration (3) a highly dynamic nature, influenced by multiple external factors (substrate biochemistry, flow rate etc.), and resulting in large variability. All of these factors make water quality parameter assessments difficult tools for long-term monitoring and providing a true reflection of aquatic ecosystem status. The first major challenge in water resource management is to identify the most important environmental stressors and then understand how they affect aquatic ecosystems in the long-term (Farrell *et al.* 2015), and as the majority of physicochemical and micronutrient parameters represent only a snap-shot of water quality, the information they provide is limited. The best use of water quality parameters therefore, is in conjunction with other measurements of eutrophication, biodiversity and ecosystem health, in order to explain community patterns and changes. The present study, together with that of Dickens & Graham (1998) have demonstrated that micronutrient standards, which are used to characterize and manage wastewater treatment facilities in Southern Africa (TWQR) are inadequate for protecting and conserving downstream environments. Unfortunately, this is not the first recommendation calling for a revision of the South African Targeted Water Quality Range system (see Gyedu-Ababio & van Wyk 2004, Coetzee & Hill 2012). Long term effects of both sewage effluents and fertilizer inputs are clearly significant threats, which promote eutrophication in freshwater ecosystems. Although there was some evidence in our study for "river self-purification" as one moves downstream of the pollution entry points, this natural process is often not sufficient, and in some cases is nonexistent due to water abstraction and physical river modifications (e.g. river channeling, dam construction, impoundments etc.). Combined with inadequate rainfall (a result of increasing climate change), the ability for a river ecosystem to assimilate via washing/flushing and diluting excess nutrients as one moves downstream, is easily compromised.

6.2 Aquatic macroinvertebrate biological assessments (SASS5) as an indicator of N-loading

Assessing ecosystem health above and below effluent discharge points is a procedure that has been recommended for quantifying impacts of nutrient loading and/or eutrophication in the USA (USEPA 1991) and Australia (Hart *et al.* 1993). This procedure was later adopted in southern Africa following Roux (1994) and it was eventually applied by a number of other studies (e.g. Quinin & Hickey 1993, Roux *et al.* 1993). A few years later Dickens & Graham (1998) used the procedure in Kwa-Zulu Natal rivers, to investigate the influence of wastewater on downstream environments, focusing on aquatic macroinvertebrate biological assessment (SASS4), abundance and community composition. Using multivariate analysis, they reported a significant variation in macroinvertebrate community composition together with some physicochemical variables as drivers. According to Dickens & Graham (1998), the majority of the receiving downstream sites showed extremely high concentrations of ammonia and total phosphorus which were coupled with a high abundance of pollution tolerant taxa (e.g. oligochaetes, chironomids, culicids and leeches) and lower/zero abundances of pollution sensitive taxa (e.g. notonemourids and hydropsychids). Their technique, the South African Scoring System 4 was further revised to the South African Scoring System 5 (SASS5; Dickens & Graham 2002) aimed at improving the technique through standardization and quality control when evaluating ecosystem health. Results from the present study demonstrated the usefulness of the SASS5 technique, with site A2, A3, A4 and B3, B5, all of which were downstream of sewerage facilities, showing very low abundance of pollution sensitive macroinvertebrate taxa and high abundance of pollution tolerant taxa (Dickens & Graham 1998; 2002). Such community compositions result in very low SASS scores and indicates high levels of anthropogenic stress. Comparatively, less disturbed study sites had a high diversity and abundance of pollution sensitive macroinvertebrates (Dallas 2007), resulting in higher SASS scores (Dickens & Graham 2002). This also included sites from this study that were further downstream from the pollution entry points (A6, A9 A8, A10, B8, B9), again highlighting both the Bloukrans-Kowie and Bushman-New Year's River's ability to assimilate (Madikizela *et al.* 2001, Bere 2007). However, Dickens & Graham (1998) observed that in some cases, macroinvertebrate abundance patterns appeared to be driven by other, unmeasured and unexplained external or internal variables. Thus more recent studies have investigated the effect of various land-use (e.g. agriculture, mining, power generation, industrial and sewerage effluents) on nutrient inputs into

the Tajan River in Iran (Arimoro *et al.* 2015) and the Wilge River in South Africa (Farrell *et al.* (2015), and results from these studies suggest that the physical characteristics of a river or a study site, not necessarily land-use, are the main drivers of macroinvertebrate community composition. Although Dickens & Graham (1998; 2002), Arimoro *et al.* (2015), Farrell *et al.* (2015) together with Munyika *et al.* (2015), Aazami *et al.* (2015A & B) who conducted similar investigations, provided useful insights on the sensitivity and tolerance of macroinvertebrate taxa and important environmental variables which are responsible for shaping macroinvertebrate community composition, very little was reported about the pollution input, dynamics or intensity. Effectively all that is reported is the eventual impact of poor water quality on macroinvertebrate presence and absence. The majority of these studies recommended that future biological monitoring should include organic and inorganic variables that might affect the system, including cultural and wastewater pollution, particularly through non-point source inputs. In the present study P, NH_4-N, NO_3-N and substrate diversity where the four main environmental variables that showed significant effects on macroinvertebrate community composition. Therefore, as with Dickens & Graham (1998; 2002), Arimoro *et al.* (2015) and Farrrell *et al.* (2015), macroinvertebrate assemblages and/or the measured physicochemical parameters in this study were unable to provide information on the source of disturbance. Indeed SASS scores for each site in this study were relatively consistent across time. Ultimately, all SASS5 assessments were able to distinguish between largely natural, moderately or largely impacted study sites. Techniques which provide better resolution are therefore needed to address unique aquatic ecosystem disturbances like N-loading, ideally with the ability to also measure habitat and water quality over time and space, identify eutrophication hotspots and sources and provide early warning information.

6.3 δ^{15}N isotopic values of primary producers (*Spirodela* sp.) and consumers (Oligochaeta and Chironomidae) as biological indicators of N-loading

Using stable isotopic analysis (SIA), the present study was able to first identify and then map nutrient hotspots (both temporally and spatially) that were severely and consistently being exposed to nutrient enrichment from sewage effluents e.g. ASTWs, BVSTWs and cow manure run-off from the Belmont Valley dairy farm using stable isotopic values of *Spirodela* plants (i.e. sewage plume mapping). On both rivers systems, sewage effluents and cow manure were

identified as the main anthropogenic sources of N-loading. Indeed, using SIA of *Spirodela* plant tissue, this study was able to identify months during which pollution inputs were most extreme as well as sites that showed contamination from nearby sewerage treatment facilities (e.g. A3, A4, A5, B3, B5), possibly through direct release and groundwater leakage or overtopping events (Chapter 4). Therefore governmental standards and compliance for sewerage treatment and water quality in South Africa should be considered for review and the adoption of sewage plume mapping as a biological monitoring tool (see Costanzo *et al.* 2001; 2005) would allow for more comprehensive monitoring as well as early N-loading alerts. Consequently, better and more powerful management strategies could be implemented for the preservation and conservation of South Africa's freshwater ecosystems. The results from chapter 4 show that the mapping technique was not only capable of identifying the N-source of eutrophication (also see Hill *et al.* 2011; 2012), but was time integrated (reflects N-environmental loading between 4-10 days; Hill *et al.* 2011; 2012) and provided information on temporal variation in N-loading over 13 months as well as spatial information (extent of N-loading) within a river, and further identifying sites of management interest. Baseline studies by Hill *et al.* (2011; 2012) also allowed for an estimation of nitrogen concentrations at each site, with the most impacted sites showing levels of NH_4 concentration of 4.5 mg N/L or higher. Interestingly, both the Bloukrans-Kowie and Bushmans-New Year's River systems in the present study turned out to be more exposed to sewage effluents and intense dairy farm activities (cow manure run-off, cultural pollution) rather than synthetic fertilizer, with sites directly downstream of pollution inputs consistently reflecting high nitrogen isotopic values (A3 – A8; Bloukrans-Kowie River system and B3 – B5; Bushmans-New Year's River system). Unlike previously reported traditional water quality assessments (e.g. Dickens & Graham 1998, Gyedu-Ababio & van Wyk 2004, Aazami *et al.* 2015A & B, Arimoro *et al.* 2015, Farrrell *et al.* 2015, Munyika *et al.* 2015), sewage plume mapping will enable users to complete intensive monitoring, trace and map N-loading over time and space and together with measurements of physicochemical parameters (Chapter 2) and biotic scoring indices (Chapter 3) provides excellent resolution and a better understanding of N-loading in both the Bloukrans-Kowie and Bushmans-New Year's River systems. Furthermore $\delta^{15}N$ isotopic values of *Spirodela* plants reported results complementary to the SASS5 results from chapter 3. SASS scores of < 90 and concurrent $\delta^{15}N$ isotopic values of > 10.00 ‰ were consistently recorded at the most impacted sites (A3, A4, A5, B3 and B5). The ability for river self-purification was also

once again visible from the isotopic values of the plants, with sites downstream of anthropogenic inputs showing less and less enrichment in ^{15}N, suggesting the dilution and flushing of excessive nutrients. Overall however, sewage plume mapping gave better temporal and spatial resolution of ecosystem nitrogen dynamics and water quality compared to SASS5 assessments, which only provided "red flag" indications throughout the study period. For example; the confluence site A10 and B8 were representative of all pollution activities happening upstream (despite the self-purification effect), *Spirodela* plant nitrogen isotopic values showed an average of +9.02 to +12.09 ‰ in $\delta^{15}N$ isotopic values (for both Bloukrans-Kowie and Bushmans-New Year's rivers), suggesting a largely natural – slightly eutrophic ecosystem. SASS5 on the other hand indicated a largely natural environment for A10 and a severely impacted environment for B8, with no change over the 13 month period.

Furthermore, it is not only the isotopic ratios of plants which can act as biological indicators of N-loading, but $\delta^{15}N$ isotopic values of macroinvertebrates as well. In a similar fashion, chapter 5 showed that the $\delta^{15}N$ isotopic values of Oligochaeta and Chironomidae were able to trace N-loading from sewage and cow manure inputs (sites A3, A4 and A5) in comparison with the largely natural site A9 on the Bloukrans-Kowie River system. This is supported by the work of Xu & Zhang (2012) and di Lascio *et al.* (2013), and to some extent by Morrissey *et al.* (2013), who also attempted to use macroinvertebrates to trace anthropogenic pollution. However the use of macroinvertebrates as biological indicators for N-loading clearly still needs some baseline work. In terms of calibration this would include determining the isotopic equilibration rates for each indicator taxa, as organism body size and life-span affects its nitrogen turn-over (DeNiro & Epstein 1981, Tieszen *et al.* 1983, Peterson & Fry 1987). It also needs to include an understanding on each organism's dietary resources, as the assimilation of different food resources may result in a lag in isotopic reflection of the changes occurring in the water column (Michener & Schell 1994) and of course primary consumers (e.g. scrappers, filter feeders and grazers) respond rapidly to changes in sediments, organic matter and water column nitrogen content relative to their body size, compared with organisms further up the trophic food web (e.g. predators; DeNiro & Epstein 1981). In the present study, oligochaetes and chironomids showed enriched nitrogen isotopic values at sites A3 (after the sewage out-fall) and the highest $\delta^{15}N$ isotopic values at site A4. This increased enrichment at A4 was expected as this site represents an accumulation of nutrients from the BVSTWs (between A2 and A3) and the cow

manure run-off from a neighbouring dairy farm (between A3 and A4). Interestingly, while oligochaetes $\delta^{15}N$ isotopic values remained high at A4 throughout the four weeks of sampling, chironomids $\delta^{15}N$ isotopic values dropped on the week three sampling event. Further investigation showed the two taxa have differing life spans, with oligochaetes having a life cycle lasting for a months to a year, while chironomids live for only about two - three weeks before eclosion (Harrison 2003, Arva *et al.* 2015). Therefore, life span would be the attribute towards the depletion in chironomids $\delta^{15}N$ isotopic values, were sampled chironomids at week 3 of the investigation, were from a new cohort of organisms (a newly hatched generation), which had not yet been exposed to high N-loading. Unlike sewage plume mapping with *Spirodela* sp., $\delta^{13}C$ values of macroinvertebrates showed that there are likely differences in dietary resources between oligochaetes and chironomids (DeNiro & Epstein 1978, Fry & Sherr 1984), which adds a further complication to tracing nutrient loading with macroinvertebrates. Therefore it is concluded that sewage plume mapping with *Spirodela* plants gives a clearer indication of the temporal and spatial dynamics of nutrient enrichment in freshwater systems than macroinvertebrates, which is not directly influenced by life span, body size or dietary resources but only by the source of dissolved inorganic nitrogen available in the water column, and thus is a more direct reflection of environmental N-loading (Kendall 1998, Costanzo *et al.* 2001; 2005, Hill *et al.* 2012, Hill 2014).

In conclusion, traditional measurements of water chemistry and aquatic macroinvertebrate biological assessments (SASS5), despite providing indications of pollution stress (i.e. the identification of systems which are largely natural, moderately impaired or largely impaired), provided very little resolution on nutrient dynamics. Stable nitrogen isotopic values of *Spirodela* sp. provided detailed dynamics on N-source, tagging and identifying pollution hot spots, on both a temporal and spatial scale, supporting its utilization for mapping freshwater N-dynamics and N-loading events. Nitrogen stable isotopic analysis is affordable and requires no special training to implement (no accreditation nor intensive sampling required), making it an easier alternative to biotic monitoring indices such as SASS (Chapter 2, methodologies). Therefore it is highly recommended that sewage plume mapping be included as an up-and-coming tool for future monitoring, conservation and management of freshwater ecosystems.

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8. Appendices

Appendix 1 Summary of on-site physicochemical variables (mean ± 1 SD) taken every month for a period of 13 months at all sampled study sites on the Bloukrans-Kowie and Bushmans-New Year's River systems.

Sampled Sites	Physicochemical parameters					
	pH (log[H+])	Conductivity (µS)	Total Dissolved Solids (ppm)	Dissolved Oxygen (mg/l)	Salinity (ppt)	Water Temperature (ºC)
A2	8±0.6	1288.9±268.1	875.2±193.9	3.7±2.4	0.64±0.14	19.8±4.8
A3	7.9±0.2	1124.6±121.3	797.5±85.9	3.9±2.2	0.58±0.09	18.6±1.4
A4	8±0.2	1182.3±149.3	838.4±105.1	3.9±1.9	0.58±0.07	16.6±3.8
A5	8.1±0.3	1178.8±142.6	836.8±101.2	4.1±2.1	0.58±0.08	17.5±4.2
A6	8.1±0.2	2116.6±1514	1529.1±1103.7	5.6±3.2	1.10±0.82	18.4±4.6
A7	8.5±0.3	1181.5±298.6	826.6±201.8	5.7±3.1	0.59±0.15	17.5±4.1
A8	8.6±0.3	1253.4±361	863.1±242.7	6.0±3.4	0.65±0.23	17.4±5.5
A9	8.3±0.3	350.2±144.8	220.6±36.2	5.6±3.1	0.15±0.03	16.9±4.1
A10	8.6±0.3	2023.2±462.9	1495.4±264.4	5.9±3.5	1.05±0.19	17.9±5.4
B1	7.7±0.4	576.8±21.9	409±15.5	3.5±2.3	0.28±0.02	20.2±5.7
B2	8.3±0.7	599.9±202.5	432.7±140.7	5.1±3.6	0.35±0.17	20.7±7.2
B3	8.1±0.7	922.3±269	632.5±178.7	5.2±6.3	0.48±0.17	20.5±4.4
B4	9.3±1.0	1050.1±57.7	728.5±68.3	6.2±7.4	0.55±0.10	21.4±4.1
B5	7.8±0.4	876.3±259.6	597.8±161.7	4.4±3.7	0.46±0.16	18.6±5.4
B6	7.9±0.4	1576.6±1152.2	1114.3±807.8	3.8±2.4	0.79±0.59	18.9±6.1
B7	7.9±0.3	2008.3±1366.4	1440.1±967.7	3.5±2.5	1.03±0.72	18.8±5.7
B8	8.3±0.4	2211.7±867.5	1565±614.9	5.7±3.2	1.17±0.51	19±5.4
B9	8.4±0.4	2163.2±977.5	1533.6±693.1	4.8±2.6	1.00±0.56	18.4±6.3
B10	8.1±0.4	551.1±121.7	391.7±85.5	4.3±2.8	0.34±0.28	18.5±5.2

Appendix 2 Summary of micronutrients analysis (mean ± 1SD)) sampled every quarter for a period of 13 months on the Bloukrans-Kowie River systems, Eastern Cape South Africa.

Micro-nutrients	Sampled Sites								
	A2	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**
pH (log[H+])	7.5±0.3	7.5±0.2	7.7±0.5	8.07±0.21	7.88±0.30	8.10±0.41	8.20±0.35	6.93±1.07	7.98±0.47
EC (mS/m)	85.5±33.0	88.9±14.7	93.6±15.5	102.2±19.3	180.9±145.7	138.7±46.9	143.2±49.3	77.0±99.3	163.3±89.0
Na (mg/l)	116.4±54.16	131.4±14	143.7±15.1	179.4±46.5	371.8±535.1	234.7±192.0	2401±82.9	144.5±289.3	272.1±228.9
K (mg/l)	7.0±5.2	9.7±6.02	11.5±8.9	85.1	6.77	7.5±4.0	6.5±3.4	2.5±3.5	6.2±3.5
Ca (mg/l)	35.3±17.8	32.8±11.8	36.5±14.3	35.2±9.8	53.3±52.6	47.3±15.1	51.6±19.2	18.8±32	54.3±24.3
Mg (mg/l)	23.4±11.6	21.4±6.5	23.5±9.2	25.4±6.2	65.5±71.3	40.6±19.5	43.6±20.9	24.2±35.9	45.3±22.3
Fe (mg/l)	0.54±0.71	0.29±0.21	0.24±0.08	0.33±0.08	0.33±0.17	0.09±0.10	0.08±0.10	0.77±0.53	0.14±0.14
Cl (mg/l)	175.5±62.7	181.1±18.1	194.4±26.5	227.8±43.6	592.6±531.5	375.4±226.2	238.6±73.3	253.0±323.8	466.3±289.4
CO3 (mg/l)	12.00	9.00	18.1±4.2	17.0±7.0	19.1±4.5	29.1±1.7	29.4±11.4	75.30	22.1±10.6
HCO3 (mg/l)	240.3±109.6	201.6±78.3	220.3±51.2	225.4±37.2	180.7±125.2	247.9±54.6	256.8±113.1	112.5±193.6	228.0±50.6
SO4 (mg/l)	46.5±35.1	54.8±29.4	60.5±29.9	49±28.1	69.8±81.9	66.5±20.2	79.3±26.1	34.5±54.6	70.8±38.3
B (mg/l)	0.16±0.08	0.18±0.05	0.17±0.06	0.19±0.06	0.18±0.10	0.21±0.03	0.25±0.13	0.15±0.22	0.20±0.06
Mn (mg/l)	0.11±0.10	0.08±0.11	0.04±0.04	0.03±0.04	0.14±0.26	0.04±0.04	0.01±0.01	0.05±0.06	0.07±0.08
Cu (mg/l)	0.01±0.01	0.02±0.02	0.02±0.02	0.01±0.02	0.01±0.02	0.01±0.02	0.01±0.02	0.01±0.02	0.01±0.02
Zn (mg/l)	0.01±0.01	0.02±0.01	0.01±0.01	0.01±0.01	0.02±0.02	0.01±0.01	0.02±0.02	0.01±0.01	0.01±0.01
P (mg/l)	0.8±0.6	2.4±2.1	2.9±1.9	1.6±1.4	0.09±0.07	0.8±0.5	0.6±0.4	0.06±0.05	0.3±0.08
NH4-N (mg/l)	7.5±7.2	6.8±6.2	8.0±5.9	1.0±0.8	0.4±0.3	0.4±0.3	0.3±0.2	0.3±0.2	0.6±0.8
NO3-N (mg/l)	1.4±1.3	9.5±8.9	4.6±3.9	5.3±4.7	0.4±0.3	3.5±2.4	2.5±2.1	0.3±0.3	1.2±1.2
F (mg/l)	0.3±0.2	0.5±0.4	0.6±0.2	0.4±0.1	0.3±0.2	0.5±0.1	0.6±0.3	0.3±0.5	0.4±0.1
TDS (mg/l)	555±214.1	577±95.9	607±102	664±125.4	1174.8±945.2	900.5±304.5	929.5±304.5	495.8±636.3	1059.5±577.1

Appendix 3 Summary of micronutrients analysis (mean ± 1 SD) sampled every quarter for a period of 13 months on the Bushman-New Year's River systems, Eastern Cape South Africa.

Micro-nutrients	Sampled Sites									
	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**
pH (log[H+])	7±0.7	7.3±0.3	7.3±0.5	8±1.3	7.4±0.4	7.5±0.4	7.3±0.8	7.6±0.5	7.8±0.4	7.4±0.1
EC (µS)	72.5±48.9	61.8±42.9	86.4±34.0	97.7±16.2	69.6±19.6	118.8±116.4	128.6±129.7	116.7±98	143.2±74.4	42.6±10.3
Na (mg/l)	87.4±41.9	71.9±46.9	102.2±21.3	140.8±39.7	83.6±16.6	232.6±286.4	155.6±116.2	213.5±205.5	205.3±87.74	58.9±28.6
K (mg/l)	5±3.9	7.7±7.6	6.3±4.9	11.6±5.3	4.7±4.6	7±5.8	8.9±4.5	4.5±4.5	5.9±4.6	4.2±2.9
Ca (mg/l)	21.3±7.9	24±13.1	27.9±11.8	26.3±9.3	20.9±13.5	37.6±32.2	38.9±34.1	40.9±23.5	49.8±23.5	23.3±8.7
Mg (mg/l)	14.1±5.1	13.6±7.3	20.1±7.5	15±5.6	16.4±8.6	37±43.5	42.1±50.9	38.5±42	45.7±31.2	11.9±4.5
Fe (mg/l)	1.97±2.4	1.06±0.98	0.96±0.81	0.19±0.05	1.14±0.95	0.79±0.98	0.40±0.37	0.75±0.73	0.43±0.65	0.76±0.46
Cl (mg/l)	130.8±22.2	111±59.7	179.7±54.3	215.8±35.9	153.6±36.9	358.6±415.8	383.9±465.9	315.1±301.9	4002±47.4	89.9±40.6
CO3 (mg/l)	-	-	12	24.1±17.0	12	12	15.10	12	21.1	-
HCO3 (mg/l)	115.2±76.27	108.3±33.5	164.2±53	176.4±99	102.9±70.9	200.6±158.9	173.8±136.3	214.3±180.9	246.4±100.9	130.4±36.3
SO4 (mg/l)	32±26.2	27.8±25.7	27.8±24.3	36.8±14.4	17.5±11.8	44.3±44.9	31±24.5	64.8±73.9	69±45.3	17.8±10.2
B (mg/l)	0.10±0.05	0.09±0.06	0.12±0.05	0.12±0.05	0.08±0.06	0.15±0.15	0.10±0.05	0.23±0.20	0.24±0.15	0.09±0.03
Mn (mg/l)	0.05±0.04	0.27±0.34	0.25±0.43	0.05±0.04	0.06±0.10	0.09±0.09	0.17±0.14	0.04±0.03	0.01±0.01	0.18±0.16
Cu (mg/l)	0.02±0.02	0.02±0.02	0.01±0.02	0.02±0.02	0.01±0.02	0.02±0.02	0.01±0.01	0.02±0.02	0.02±0.03	0.02±0.02
Zn (mg/l)	0.01±0.01	0.01±0.01	0.01±0.01	0.00±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01
P (mg/l)	0.20±0.31	1.09±2.09	0.40±0.60	2.97±2.21	0.10±0.03	0.24±0.32	0.41±0.43	0.07±0.03	0.19±0.25	0.09±0.06
NH4-N (mg/l)	2.01±3.21	0.83±0.82	13±1.5	7.6±13.3	1.2±1.5	1.6±2.4	0.4±0.3	0.3±0.2	0.3±0.2	0.3±0.2
NO3-N (mg/l)	0.37±0.36	4.48	1.7±1.3	1.5±1.8	0.8±0.6	1.1±1.3	0.93±1.28	0.54±0.23	0.69±0.60	0.29±0.32
F (mg/l)	0.10±0.20	0.28±0.43	0.10±0.14	0.63±0.39	0.10±0.14	0.23±0.17	0.23±0.26	0.43±0.39	0.33±0.36	0
TDS (mg/l)	365.5±109.2	318.3±148.2	469.8±120.6	543.5±85.7	395±150.9	770.8±753.6	832±836.7	755.3±633.1	930±483.9	276.3±66.7

Appendix 4 Aquatic macroinvertebrates Presence (1)/Absence (0) data observed in the Bloukrans-Kowie and Bushmans-New Year's Rivers systems Eastern Cape, South Africa.

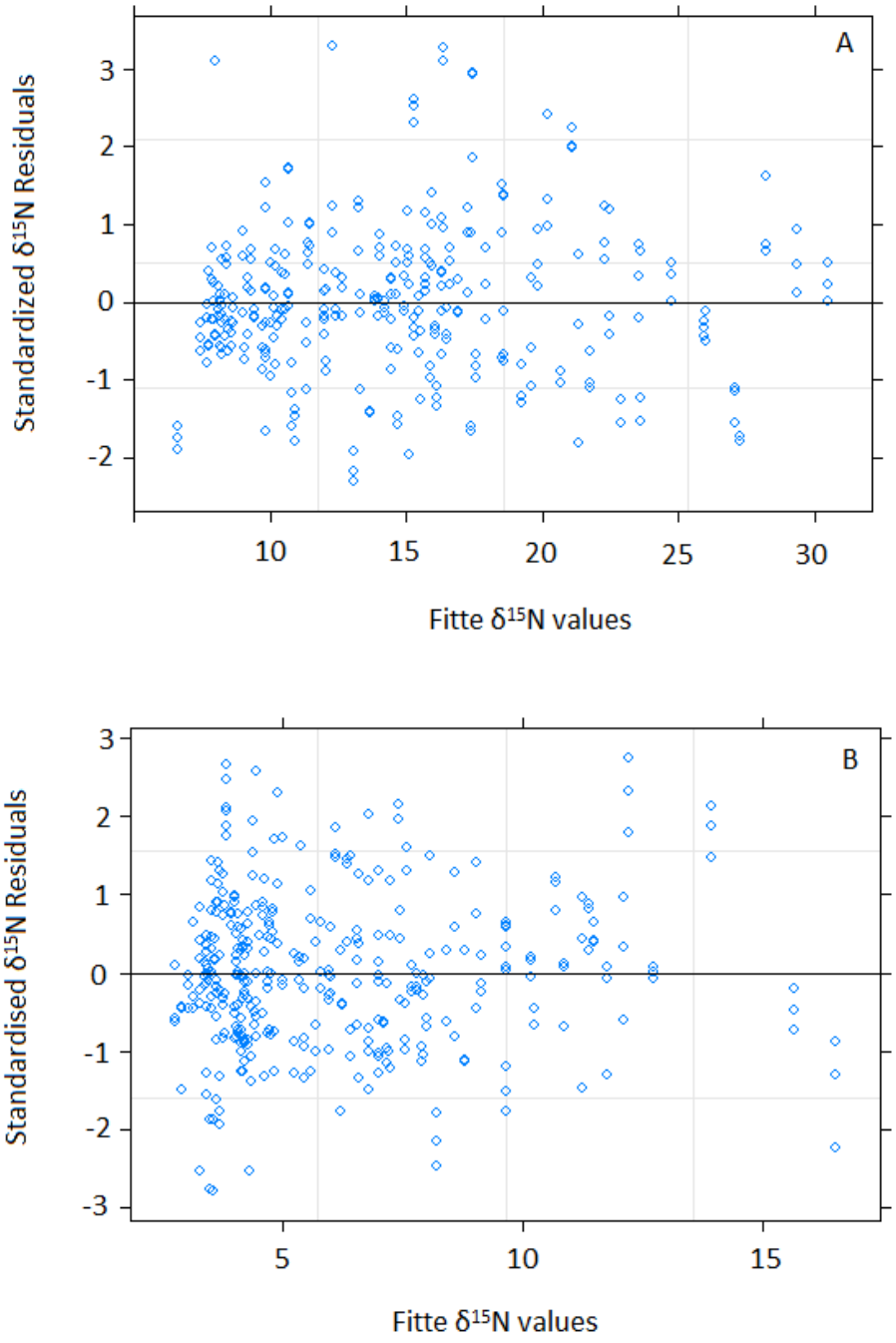
Taxa	Bloukrans-Kowie River	Bushmans-New Year's River
Porifera	1	1
Coelenterata	0	0
Turbellaria	1	1
Annelida		
Oligocheata	1	1
Hirudinea	1	1
Crustacea		
Amphipoda	0	0
Potamonautidae	1	1
Atyidae	0	1
Palaemonidae	0	0
Hydracarina	1	1
Plecoptera		
Notonectidae	0	0
Perlidae	0	0
Ephemeroptera		
Baetidae	1	1
Caenidae	1	1
Ephemeridae	0	0
Heptageniidae	0	0
Leptophlebiidae	1	1
Oligoneuridae	0	0
Polymitarcyidae	0	0
Prosopistomatidae	0	0
Teloganodidae	0	0
Tricorythidae	0	0
Odonata		
Calopterygidae	0	0
Chlorocyphidae	1	0
Synlestidae	1	1
Coenagrionidae	1	1
Lestidae	1	1
Platycnemidae	1	0
Protoneuridae	0	0
Aeshnidae	1	1
Corduliidae	1	1
Gomphidae	1	1
Libellulidae	1	1
Lepidoptera		
Crambidae	0	0
Hemiptera		
Belostomatidae	1	1
Corixidae	1	1
Gerridae	1	1
Hydrometridae	1	1

Taxa	Bloukrans-Kowie River	Bushmans-New Year's River
Naucoridae	1	1
Nepidae	1	1
Notonectidae	1	1
Pleidae	1	1
Veliidae	1	1
Megaloptera		
Corygalidae	0	0
Sialidae	0	0
Trichoptera		
Dipseudopsidae	0	0
Ecnomidae	1	1
Hydropsychidae	1	1
Philopotamidae	1	1
Polycentropodidae	0	0
Psychomyiidae	0	0
Barbarochthonidae	0	0
Calamoceratidae	0	0
Glossosomatidae	0	0
Hydroptilidae	0	0
Hydrosalpingidae	0	0
Lepidostomatidae	1	0
Leptoceridae	1	1
Petrothrincidae	0	0
Pisuliidae	1	0
Sericostomatidae	0	0
Coleoptera		
Dytiscidae	1	1
Elmidae	1	1
Gyrinidae	1	1
Haliplidae	0	0
Hydraenidae	0	1
Hydrophilidae	1	1
Limnichidae	0	0
Psephenidae	0	0
Diptera		
Athericidae	0	1
Blepharoceridae	0	0
Ceratopogonidae	1	1
Chironomidae	1	1
Culicidae	1	1
Dixidae	1	1
Empididae	0	0
Ephydridae	0	0
Muscidae	1	1
Psychodidae	1	1

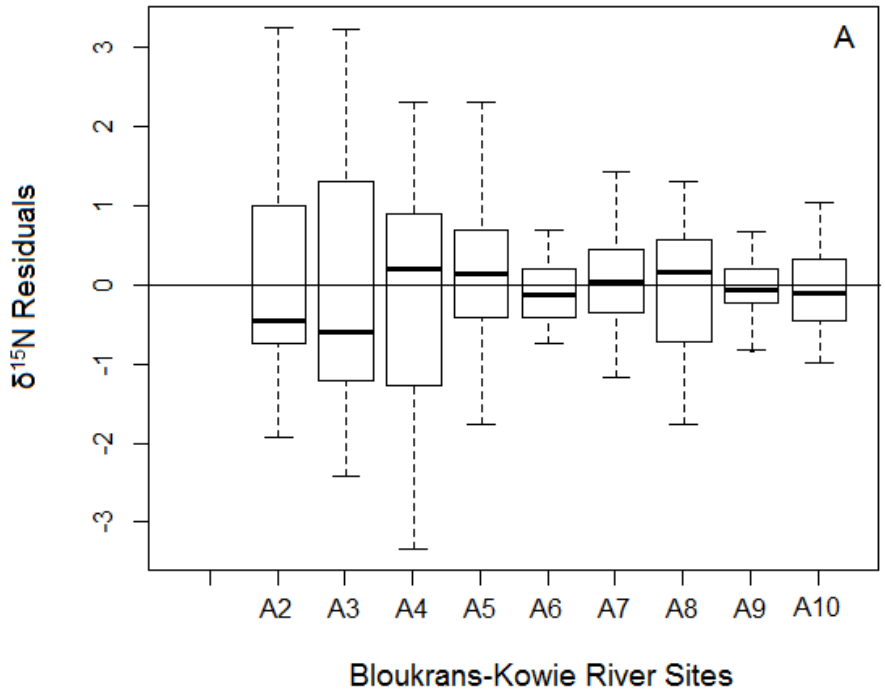
Taxa	Bloukrans-Kowie River	Bushmans-New Year's River
Simuliidae	1	1
Sryphidae	1	1
Tabanidae	1	1
Tipulidae	1	1
Gastropoda		
Ancylidae	1	1
Bulininae	0	0
Hydrobiidae	0	0
Lymnaeidae	1	1
Physidae	1	1
Planorbidae	0	1
Thriaridae	0	0
Viviparidae	0	0
Pelecypodae	0	0
Cordiculidae	0	0
Sphaeriidae	0	0
Unionidae	0	0
Chaoboridae	0	1

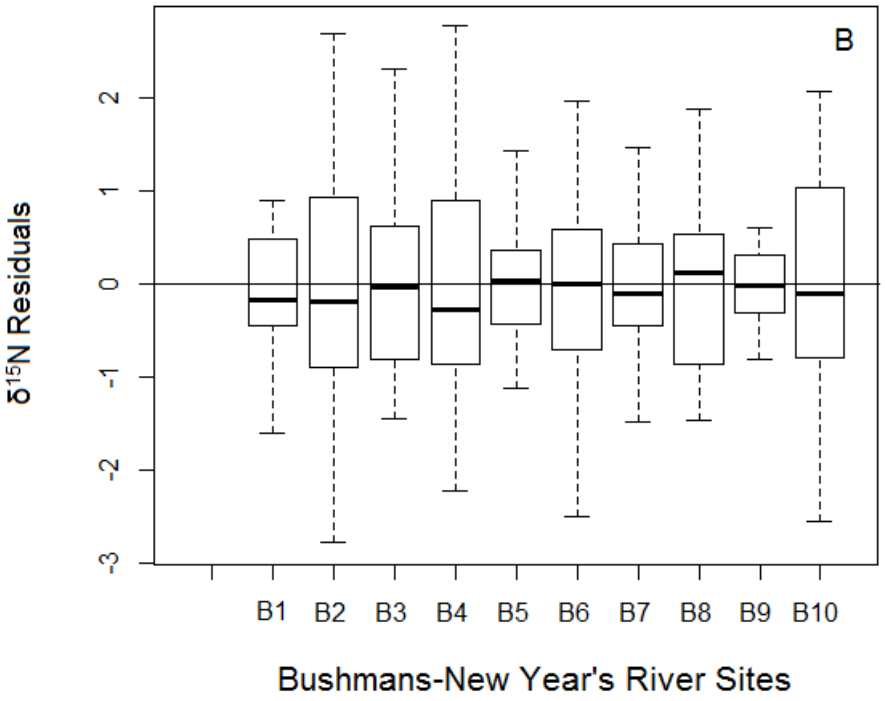
Appendix 5 Summary of the number of taxa, SASS5 and ASPT values at sample sites on the Bloukrans-Kowie and Bushmans-New Year's River systems, Eastern Cape South Africa. # Taxa — number of taxa; SS — SASS5 score; ASPT — average score per taxon.

	26th – 29th August 2013			18th – 21st November 2013			25th – 29th February 2014			29th May – 03rd June 2014		
Sites	**SS**	**# Taxa**	**ASPT**	**SS**	**# Taxa**	**ASPT**	**SS**	**# Taxa**	**ASPT**	**SS**	**# Taxa**	**ASPT**
A2	13	5	2.6	23	7	3.29	85	21	4.05	13	7	1.86
A3	16	6	2.67	18	7	2.57	34	12	2.92	23	8	2.73
A4	75	17	4.41	60	15	4	72	17	4.24	37	9	4.11
A5	80	16	4.9	83	18	4.61	83	17	4.88	73	14	5.21
A6	151	28	5.39	171	29	5.9	125	25	5	153	27	5.67
A7	103	21	4.91	120	25	4.8	91	19	4.79	132	26	5.08
A8	138	24	5.75	140	25	5.6	137	25	5.48	110	19	5.79
A9	122	24	5.08	169	27	6.26	171	28	6.11	178	29	6.14
A10	170	31	5.48	137	28	4.89	125	28	4.89	118	22	5.36
B1	79	18	4.39	52	11	4.73	67	15	4.19	73	18	4.06
B2	21	8	2.63	17	6	2.83	14	5	2.8	14	6	2.33
B3	88	18	4.89	67	17	3.94	39	10	3.9	47	14	3.36
B5	49	13	3.77	67	17	3.94	37	10	3.7	75	20	3.75
B6	77	18	4.28	78	19	4.11	71	18	3.94	78	18	4.33
B7	84	17	4.94	89	19	4.68	54	13	4.15	60	15	4
B8	109	20	5.45	106	23	4.61	85	16	5.31	83	16	5.19
B9	129	26	4.96	119	25	4.76	109	21	5.19	95	19	5
B10	82	20	4.1	38	12	3.17	52	15	4.33	63	15	4.2

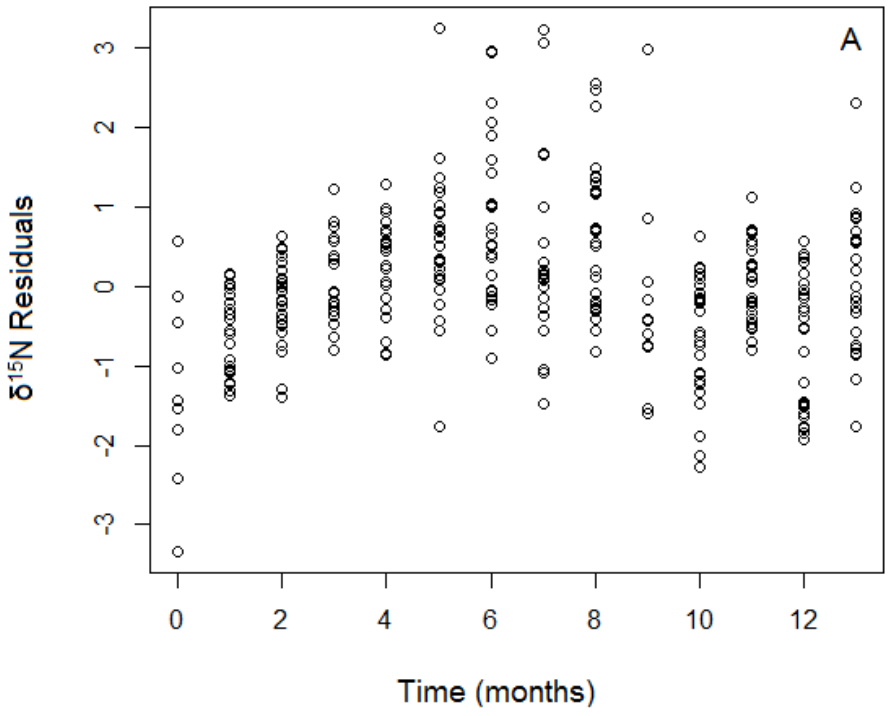


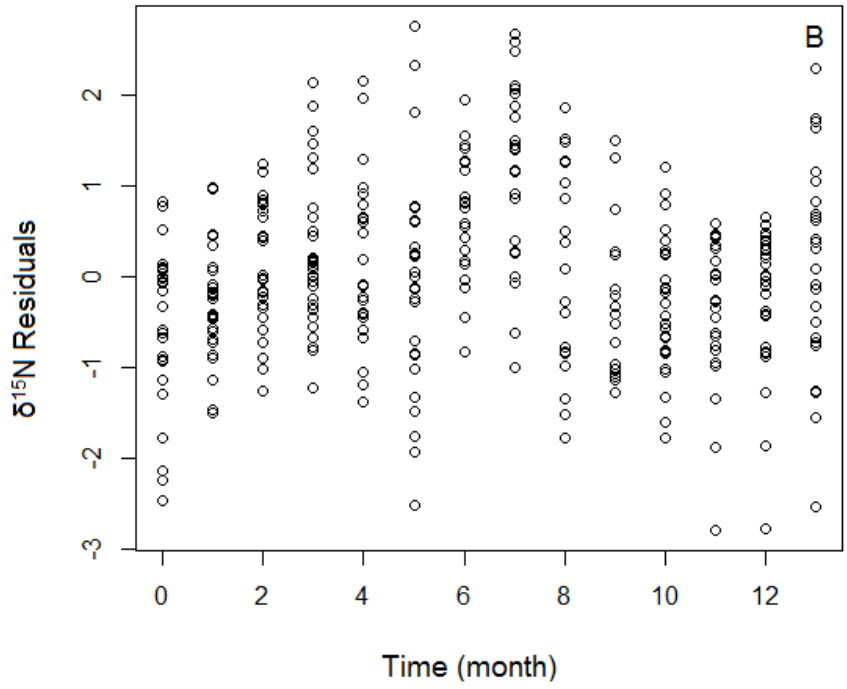
Appendix 6: $\delta^{15}N$ residuals values versus $\delta^{15}N$ fitted values diagnostic plots on the (A) Bloukrans-Kowie River and (B) Bushmans-New Year's River systems, Eastern Cape South Africa.



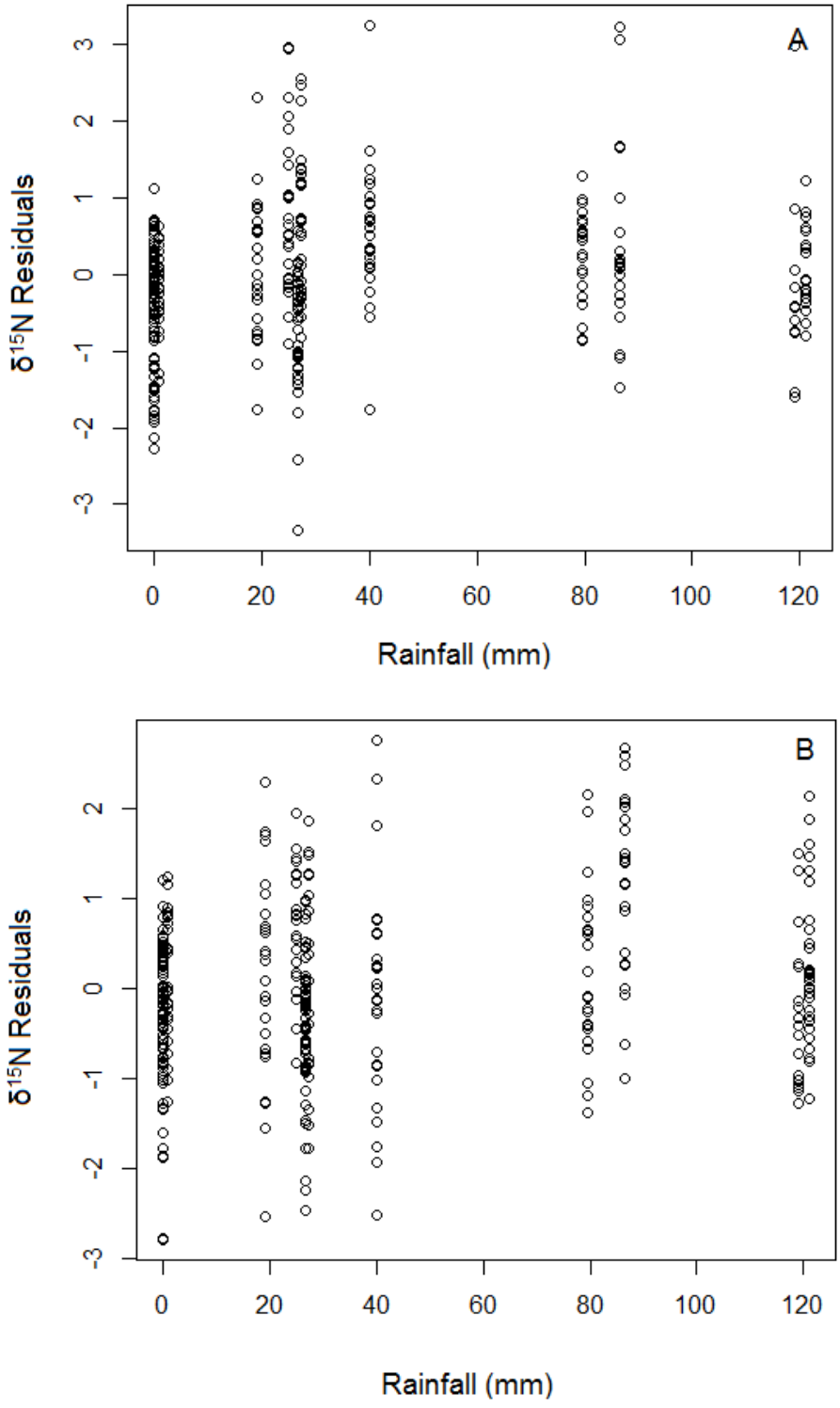


Appendix 7: $\delta^{15}N$ residual values versus predictor variable (study sites) diagnostic plots on the (A) Bloukrans-Kowie River and (B) Bushmans-New Year's River systems, Eastern Cape South Africa.

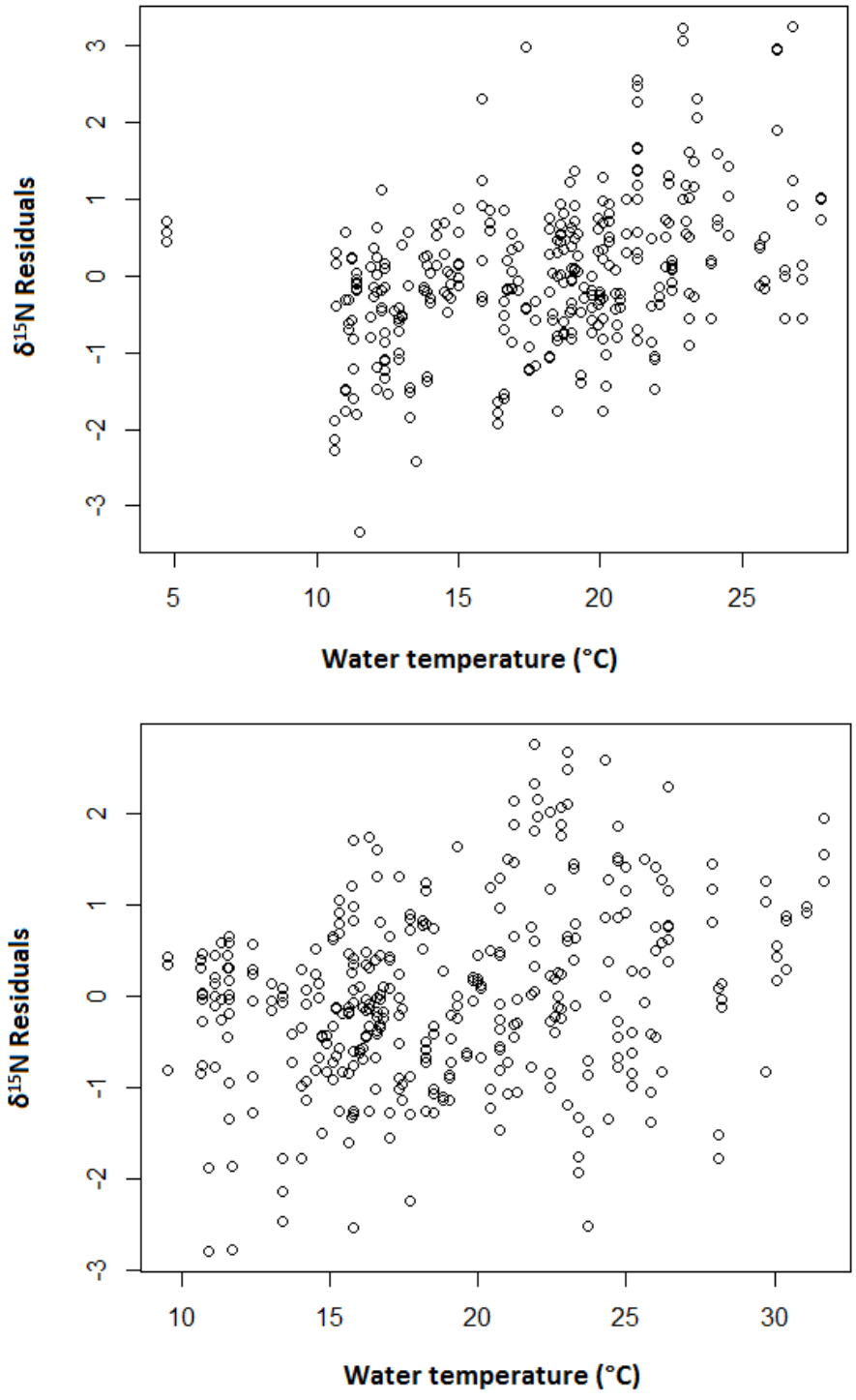




Appendix 8: $\delta^{15}N$ residuals values versus predictor variable (time of sampling) diagnostic plots of 13 month sampling period on the (A) Bloukrans-Kowie River and (B) Bushmans-New Year's River systems, Eastern Cape South Africa.



Appendix 9: $\delta^{15}N$ residuals values versus predictor variable (rainfall) diagnostic plots on the (A) Bloukrans-Kowie River and (B) Bushmans-New Year's River systems, Eastern Cape South Africa.



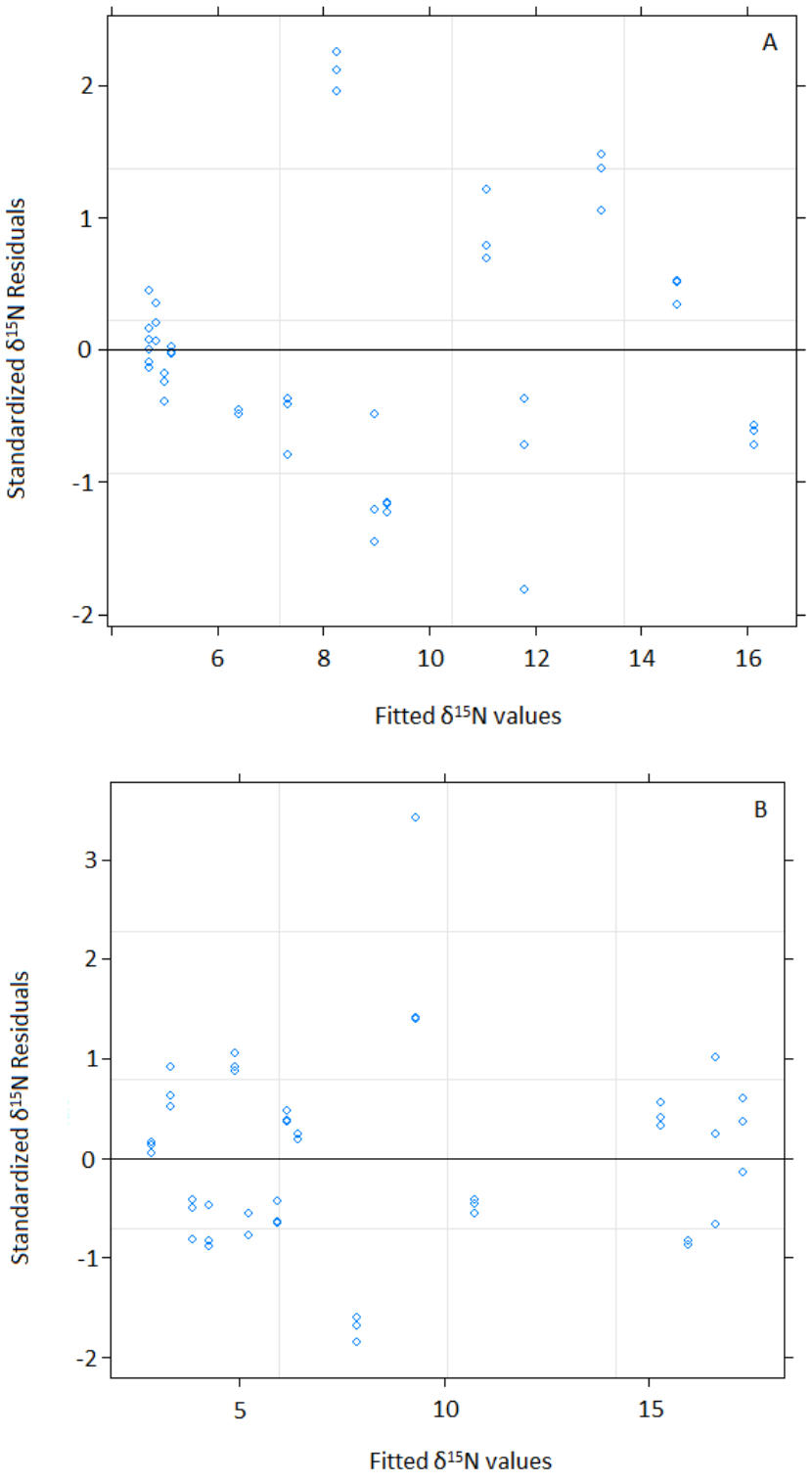
Appendix 10: $\delta^{15}N$ residuals values versus predictor variable (water temperature) diagnostic plots on the (A) Bloukrans-Kowie River and (B) Bushmans-New Year's River systems, Eastern Cape South Africa.

Appendix 11 Full aquatic macroinvertebrates taxa names from abbreviations (six characters) in Chapter 5, RDA ordination diagram.

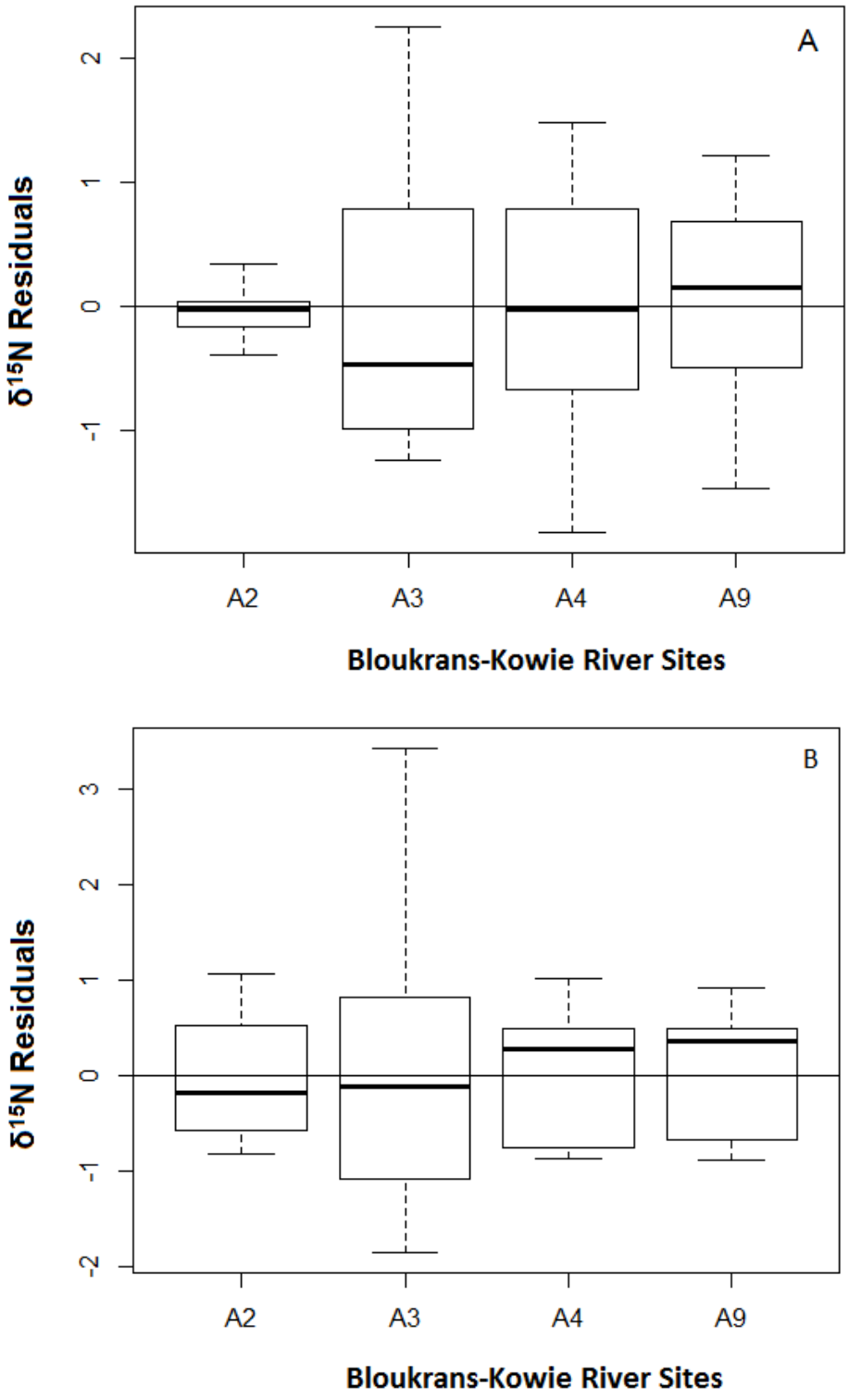
Porife – Porifera
Turbel – Turbellaria
Oligo – Oligocheata
Hirudi - Hirudinea
Potamo – Potamonautidae
Atyida – Atyidae
Hydrac - Hydracarina
Baetid – Baetidae
Leptop - Leptophlebiidae
Chloro - Chlorocyphidae
Synles - Synlestidae
Coenag - Coenagrionidae
Lestid - Lestidae
Platyc - Platycnemidae
Aeshni - Aeshnidae
Cordul - Corduliidae
Gomphi - Gomphidae
Libell - Libellulidae
Belost - Belostomatidae
Corixi - Corixidae
Gerrid - Gerridae
Hydrom - Hydrometridae
Naurco - Naucoridae
Nepida - Nepidae
Notone - Notonectidae
Pleida - Pleidae
Veliid - Veliidae
Ecnomi - Ecnomidae
Hydpsy - Hydropsychidae
Philop - Philopotamidae
Lepido - Lepidostomatidae
Leptoc - Leptoceridae
Pisuli - Pisuliidae
Dytisc - Dytiscidae
Elmida - Elmidae
Gyrini - Gyrinidae
Helodi - Helodidae
Hydrae - Hydraenidae
Hydrop - Hydrophilidae
Atheri - Athericidae
Cerato - Ceratopogonidae
Chiron - Chironomidae
Culici - Culicidae
Dixida - Dixidae
Muscid - Muscidae
Psycho - Psychodidae
Simuli - Simuliidae
Sryphi - Syrphidae
Tabani - Tabanidae
Tipuli - Tipulidae
Ancyli - Ancylidae
Lymnae - Lymnaeidae
Physid - Physidae
Planor - Planorbinae
Chaobo - Chaoboridae

Appendix 12 A summary of Present (1)/Absent (0) aquatic macroinvertebrates N-loading indicator taxa data collected at four study sites on the Bloukrans-Kowie River systems.

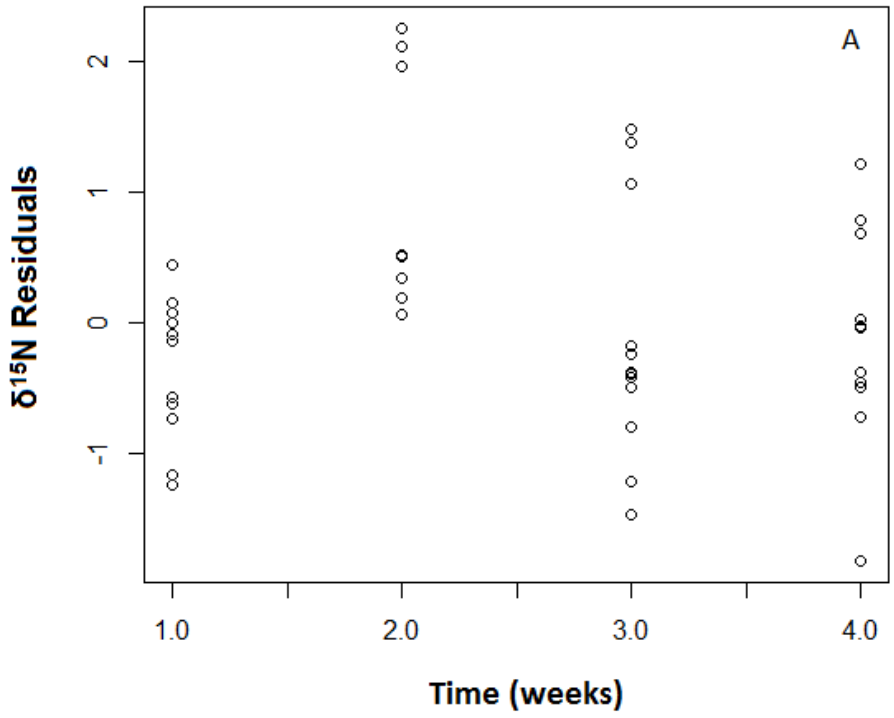
Sampling Period	Study Site	Aquatic Macroinvertebrates			
		Oligochaeta	Chironomidae	Culicidae	Syrphidae
T1	A2	1	1	1	1
	A3	1	1	1	0
	A4	1	1	0	0
	A9	1	1	0	0
T2	A2	1	1	1	0
	A3	1	1	1	1
	A4	1	1	0	0
	A9	1	0	0	0
T3	A2	1	1	1	0
	A3	1	1	1	1
	A4	1	1	0	0
	A9	1	1	0	0
T4	A2	1	1	1	1
	A3	1	1	1	1
	A4	1	1	0	0
	A9	1	1	0	0

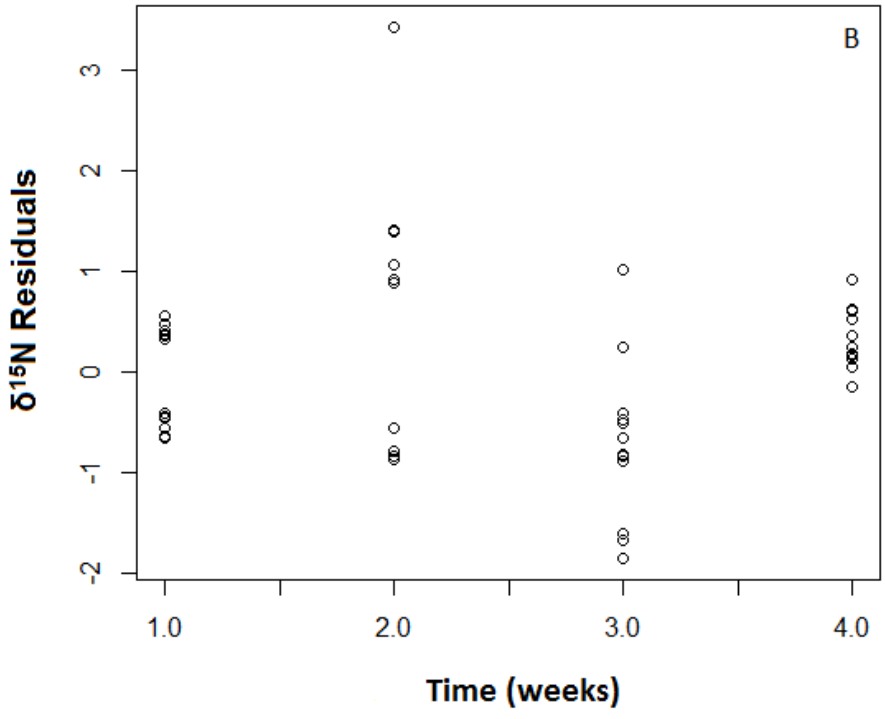


Appendix 13: $\delta^{15}N$ residuals values versus $\delta^{15}N$ fitted values diagnostic plots for (A) Oligochaeta (B) Chironomidae, collected on the Bloukrans-Kowie River systems, Eastern Cape South Africa.



Appendix 14: $\delta^{15}N$ residual values versus predictor variable (study sites) diagnostic plots for (A) Oligochaeta and (B) Chironomidae collected on the Bloukrans-Kowie River systems, Eastern Cape South Africa.





Appendix 15: $\delta^{15}N$ residual values versus predictor variable (time of sampling) diagnostic plots for (A) Oligochaeta and (B) Chironomidae collected on the Bloukrans-Kowie River systems, Eastern Cape South Africa.

Appendix 16 Summary of on-site physicochemical variables (mean ± 1 SD) taken every week over a period of four weeks in March 2015 at all sampled study sites on the Bloukrans-Kowie and River systems, Eastern Cape South Africa.

Physicochemical Variables	Sampled study sites				ANOVA - Statistics
	A2	A3	A4	A9	
DO	4.48±0.75	3.98±1.25	4.53±0.73	5.78±0.62	$F_{3\text{-}76} = 15.34$, $p < 0.0001$
NH4-N	4.05±5.33	2.84±3.19	1.14±1.30	0.11±0.14	$F_{3\text{-}76} = 6.12$, $p < 0.001$
NO3-N	9.43±3.50	10.10±3.04	15.05±5.38	1.64±0.92	$F_{3\text{-}76} = 48.94$, $p < 0.0001$