POLLUTION MAPPING IN FRESHWATER SYSTEMS: USING AQUATIC PLANTS TO TRACE N-LOADING

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by

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EXECUTIVE SUMMARY

INTRODUCTION

The global degradation of both marine and freshwater ecosystems is primarily driven by the excessive addition of anthropogenic nutrients to watersheds. Increased nitrogen loading, for example, can result in widespread ecosystem deterioration and may include harmful algal blooms, large scale fish kills, hypoxia, the loss of aquatic vegetation and habitat, loss of biodiversity, disruption of ecosystem functioning and the establishment of invasive species. Reactive nitrogen inputs (N) stem from intensive agricultural land use, resulting in the increased use of N-containing organic and inorganic fertilizers and/or animal manure and their consequent run-off and the discharge of human sewage.

In recent years, aquatic ecosystem health has been monitored using a number of techniques, of which the most widely applied in South Africa is the South African Scoring System (SASS5; Dickens and Graham, 2002). Bio-monitoring, however, typically identifies eutrophication problems only after ecosystem-level impacts have already occurred and where ecosystem health has been disrupted, it is often not possible to link biotic changes to identifiable causes (especially in the case of non-point source pollution). Any methods that would allow for the detection of emerging eutrophication which can also trace and identify nutrient sources would greatly improve our ability to effectively manage our aquatic resources.

High loads of nitrogen are often associated with enriched $\delta^{15}N$ values of aquatic vegetation relative to pristine conditions and consequently may act as early warning indicators of nitrogen pollution in aquatic ecosystems, prior to the onset of system degradation. Using stable isotopic values of indicator plants in a particular catchment, it is often possible to determine both the spatial source and the composition of nitrogen sources. This technique, referred to as sewage plume mapping, has been used in numerous countries to identify and map the sources, dilution and sinks of nutrients in aquatic ecosystems. The baseline work for calibrating isotopic responses for the indicator organism *Spirodela* sp. in response to nutrient concentrations was completed in WRC Report No. KV 280/11 (Hill et al., 2011) and Hill et al. (2012), during which they identified the need for intensive fields tests of the sewage plume mapping technique in the natural environment.

OBJECTIVES

The primary aim of this study was to evaluate the potential of sewage plume mapping for the monitoring of water quality in natural systems. Long term field testing on the New Years-Bushmans River in the Eastern Cape of South Africa, allowed the mapping of in-situ N dynamics over a period of 13 months, to assess its applicability for the assessment of ecosystem health, the monitoring of temporal variability in N loading, and the identification of incipient eutrophication.

The secondary aim of this study was to compare the isotopic sewage plume mapping technique with the already established SASS 5 bio-monitoring technique to determine how well each method described changes in nitrogen dynamics and site health along the Bushmans-New Years River system.

METHODS AND RESULTS

Transplantation of laboratory incubated plants (with known isotopic ratios) into tethered greenhouse cages at 10 sites along the river system was completed in August 2013. Plants were allowed to grow and were sampled for $\delta^{15}N$, $\delta^{13}C$ and C:N ratios every month for 13 months (with a minimum of 10 days between each sampling event). Physico-chemical parameters were collected at every sampling event and SASS5 evaluations and micronutrient analyse were completed quarterly.

Physico-chemistry measurements and micronutrient analyses provided little resolution towards N loading in the Bushmans-New Years River system. SASS 5 and Shannon-Wiener biodiversity scores showed similar results which broadly agreed with the findings of the sewage plume mapping technique. Most importantly, however, neither instantaneous measurements of physico-chemistry or SASS5 scores are designed to identify incipient eutrophication and/or areas of potential concern for monitoring and management interest. Moreover SASS5 has a number of limitations, restricting its applicability in a wide range of freshwater ecosystems.

Results from the first comprehensive field test of sewage plume mapping in a South African context are promising, with the combined application of $\delta^{15}N$ values and C:N ratios from transplanted *Spirodela* plants showing highly dynamic changes in nitrogen within the Bushmans-New Years River system. Duckweed plants were able to provide a clear time integrated, temporal and spatial map of N loading in the Bushmans-New Years River, identifying sewage/manure and fertilizer inputs. Moreover this technique identified areas and time periods where adjacent sewage inputs appear to be leaking into the river system and these sites (and times) should be tagged for management interest.

CONCLUSIONS

Sewage plume mapping combines a quick assessment of ecosystem health, provides a spatial and temporal map of N loading hotspots over a 10 day time integration period and has the ability to classify anthropogenic loads from different N sources (e.g. sewage/manure or synthetic fertilizer). It is a versatile tool for the monitoring and assessment of ecosystem health which provides more resolution than current biotic indices. Its application will allow us to identify dynamic changes in nitrate and ammonia within a river system, help to correlate water chemistry with nutrient loading and broaden our understanding of the consequences of eutrophication in freshwater systems. Moreover, $\delta^{15}N$ and C:N values should allow for the

mapping and identification of pollution hotspots and gradients which may identify areas of particular management interest. Using $\delta^{15}N$ and C:N ratios, potentially in combination with other chemical parameters, we may be eventually be able to predict the ultimate sources of N pollution, which will aid in the conservation, rehabilitation and management of South Africa's waterways.

ACKNOWLEDGMENTS

Heartfelt thanks to the Late Dr Sven Kaehler and his IsoEnvironmental Laboratory at the South African Institute for Aquatic Biodiversity and to the Stable Isotope Laboratory, Mammal Research Institute at the University of Pretoria. Thank you as well to the South African Weather Service for providing the rainfall data. The financing of the project by the Water Research Commission is gratefully acknowledged.

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1. INTRODUCTION

1.1 BACKGROUND

The global degradation of both marine and freshwater ecosystems is primarily driven by the excessive addition of anthropogenic nutrients to catchments (Rabalais, 2002; Xue et al., 2009). Increased nitrogen loading for example, can result in widespread ecosystem deterioration and may include: harmful algal blooms, large scale fish kills, hypoxia, the loss of aquatic vegetation and habitat, loss of biodiversity, disruption of ecosystem functioning and the establishment of invasive species (e.g. Vitousek et al., 1997; Rabalais, 2002; Green and Galatowitsch, 2002). Moreover, high nitrate concentrations are also recognized as a direct health risk to human consumers (WHO, 2008). Reactive nitrogen inputs (N) stem from intensive agricultural land use, resulting in the increased use of N-containing organic and inorganic fertilizers and/or animal manure and their consequent run-off (Smil, 1999) and the discharge of human sewage (Cabana and Rasmussen, 1996) and in some cases, elevated atmospheric deposition (Benkowitz et al., 1996). Understanding the fate and processing of anthropogenic nutrients in natural systems is therefore critical for both the preservation and management of ecosystem health and integrity.

In the past, aquatic ecosystem health has been monitored using a number of techniques, including measurements of chemical water quality constituents (Chapman, 1996), changes in microbial assemblages (De Figuerido et al., 2010, 2011, Haller et al., 2011) and taxonomic changes in the abundance of biota; such as biotic scoring indices such as the South African Scoring System (SASS; Dickens and Graham, 2002). In recent years, the use of the SASS5 technique in particular has resolved ecosystem health in a number of systems (e.g. de la Rey et al., 2008; Utete and Kunhe, 2013; Wolmarans et al., 2014). This method has become a standard tool for the rapid bio-assessment of rivers in South Africa, now underpinning the mainframe of the National River Health Programme (Uys et al., 1996). However, it faces numerous challenges to its appropriate implementation including the lack of skilled and certified application and the availability of required biotopes in an aquatic system. Additionally, bio-monitoring by its very nature typically identifies eutrophication problems only after ecosystem-level impacts have already occurred (Hill et al., 2012). Moreover, where ecosystem health has been disrupted, it is often not possible to link biotic changes to identifiable causes (especially in cases where pollutants originate from non-point sources; Fry, 2006). Any methods that would allow for the detection of emerging eutrophication which can also trace and identify nutrient sources would greatly improve our ability to effectively manage our aquatic resources (Hill et al., 2012).

1.2 STABLE ISOTOPE TECHNIQUES

Stable isotopes are naturally occurring, non-radioactive, heavier and lighter forms of the same elements (i.e. ^{12}C and ^{13}C for carbon, ^{14}N and ^{15}N for nitrogen). Their difference in mass is due to different numbers 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 isotopes. As a result the light isotopes react 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. When nitrogen is limiting, all available nitrogen is assimilated and the resulting plant tissues exhibit an isotopic ratio (a measure of the relative abundance of ^{15}N and ^{14}N and denoted as $\delta^{15}N$) close to that of the substrate. However, as nitrogen becomes more abundant, mass dependant isotope fractionation occurs in which the lighter ^{14}N reacts slightly faster than the heavier ^{15}N (Kendall, 1998; Kendall & Doctor, 2003). This results in enriched nitrogen isotope ratios (tissues with a higher abundance of ^{15}N isotopes and thus a more positive isotopic ratio) of plant tissues in areas of high nitrogen loading. Thus high N-loads are often associated with enriched $\delta^{15}N$ values of aquatic vegetation relative to pristine conditions and consequently may act as early warning indicators of nitrogen pollution in aquatic ecosystems, prior to the onset of system degradation (e.g. Fry & Allen, 2003; Anderson & Cabana, 2005; Deutsch & Voss, 2006).

In addition to acting as an indicator of nitrogen loading, stable isotopic ratios can be a useful tool for tracing and sometimes the classification of nitrogen sources in catchments (occasionally this is done in combination with other isotopes). For example, different sources of nitrogen inputs often have markedly different isotopic ratios, allowing for the identification of particular anthropogenic pollutants. For example, plants assimilating nitrogen from synthetic fertilizers have $\delta^{15}N$ values that reflect the atmospheric N_2 source of the fertilizer (-2 to +2‰; e.g. Kendall, 1998; Kendall and Doctor, 2003; Curt et al., 2004). In contrast, organic nitrogen sources such as manures and sewage have a very different isotopic composition (typically +10 to +25‰) that reflects a preference for ^{14}N during bacterial digestion, resulting in enriched nitrogen isotopic ratios (e.g. Kendall, 1998; Curt et al., 2004; Vander Zanden et al., 2005; Reynolds-Vargas, 2006). Accordingly, by mapping the isotopic ratios of a plant species in a particular catchment, it is often possible to determine the origins of point source and non-point source inputs, the spatial distribution of these inputs (e.g. how far downstream do the impacts of these inputs extend) and sometimes to identify the composition of these nitrogen sources (e.g. fertilizers, sewage, animal manure). This technique, sometimes referred to as sewage plume mapping, pioneered by Costanzo et al. (2001, 2004, 2005) has in recent years found an increasing number of adherents and has been used to identify and map the sources, dilution and sinks of nutrients in groundwater, catchments, estuaries and coastal environments. For a more comprehensive review please see WRC Report No. KV 280/11 (Hill et al. (2011).

With this information in mind, WRC Report No. KV 280/11 (Hill et al., 2011) and Hill et al. (2012) set out to develop isotopic monitoring protocols using aquatic plants to identify and track changes in nitrogen dynamics within the water column of freshwater streams and rivers. The duckweeds *Spirodela* sp. and *Wolffia* sp. were identified as being appropriate plants and laboratory experiments determining isotopic tissue turnover times and quantifying isotopic relationships relative to increasing nutrient concentrations were completed to generate an isotopic baseline for the calibration of subsequent field results. Following these laboratory experiments, a small scale pilot field study was completed in a local stream, using laboratory grown transplanted individuals. Laboratory grown plants were used instead of

locally growing ones for two reasons; firstly, the calculated isotopic baseline of tissue turnover time and concentration level gradients were completed based on laboratory plants thus transplantation of incubated plants ensures correct calibration. Secondly, although duckweed is ubiquitous, it is highly dependent on flow regime and transplantation of plants from the laboratory ensured constant availability. Results from these studies indicated that duckweed plants clearly differentiated between different nutrient types (fertilizer, manure, oligotrophic water) within 4-10 days of exposure and the study established that plants demonstrate concentration level isotopic relationships, with increasing nitrogen concentrations resulting in increasing enrichment or depletion in $\delta^{15}N$ values for manure and fertilizer solutions respectively (Hill et al., 2011; Hill et al., 2012). Furthermore, these experiments showed that lab grown transplanted individual plants can reflect nutrient loading in a natural environment (Hill et al., 2011; Hill et al., 2012).

Transplantation of cultured laboratory plants into the field to monitor changes in nutrient status within a system has rarely been used in South Africa and has great potential; these techniques, however, are still in their infancy and will require further fine tuning for wide scale use in the field. Stable isotope monitoring of in-situ nutrient dynamics through transplanted macrophytes needs to be field tested on a larger scale over a well-defined nutrient gradient to confirm its usefulness. Thus field applications of this isotopic monitoring tool will allow us to identify the ecologically important (and available) pools of nitrogen (NH_4 and NO_3) within the water column of a chosen system and broaden our understanding of the processes that drive eutrophication. Moreover, these signatures will allow the mapping and identification of pollution hotspots and gradients within an ecosystem, which will help to monitor water quality conditions and provide tools for more efficient water management.

1.3 AIMS

1.3.1 The primary aim of this study was to evaluate the potential of stable isotopic sewage plume mapping for water quality monitoring in natural systems. Long term field testing on the Bushmans-New Years River system allowed the mapping of in-situ N dynamics over a period of 13 months, to assess its applicability for the assessment of ecosystem health, the monitoring of temporal variability in N loading, and the identification of incipient eutrophication.

1.3.2 The secondary aim of this study was to compare the isotopic sewage plume mapping technique with the already established SASS 5 bio-monitoring technique to determine how well each method described nutrient dynamics and site health along the Bushmans-New Years River system.

2. MATERIALS AND METHODS

2.1 SEWAGE PLUM MAPPING – EXPERIMENTAL SET-UP

Sampling took place every month, from August 2013-August 2014. 10 sites were chosen on the Bushmans-New Years River system (Figure 1), across a well-defined gradient of nutrient inputs; sites 1 and 2 were oligotrophic; sites 3 and 4 were directly in or adjacent to sewage inputs; site 5 was between sewage and fertilizer inputs; sites 6 and 7 had intermittent fertilizer inputs (golf course); sites 8 and 9 were after the confluence of the Bushmans and New Years Rivers. Site 10 was in the upper reaches of Bushmans River.

Spirodela sp. are fast growing angiosperms, with isotopic differentiation between nutrient regimes apparent after two days and with isotopic equilibration between days four to ten (Hill et al., 2012). Thus ten days prior to initial transplantation, duckweed plants were incubated in a controlled environment (greenhouse tunnels, Dept. of Zoology & Entomology, Rhodes University) at 10.0 mg N/ℓ of nitrate, with the addition of commercial iron chelate (13% Fe, EDTAeFeNae3H2O) at a concentration of 11.2 mg Fe/ℓ (Coetzee et al., 2007) to avoid nutrient uptake limitation associated with iron deficiency. Initial isotopic signatures of *Spirodela* sp. plants were $\delta^{15}N$ = 9.45 ± 1.56‰ and $\delta^{13}C$ = -29.29 ± 0.22‰. Floating, enclosed, easily transportable in-situ greenhouse cages (Figure 2) were constructed for transplantation of incubated plants, which allowed for water circulation and flooding events.

At each site, five floating greenhouse cages were tethered to submersible concrete weights (Figure 2) and placed perpendicular to the shoreline. Each floating cage was spaced at least 1m apart and contained approx. 40g wet weight of laboratory incubated *Spirodela* sp. Duckweed was left to grow and sampled for $\delta^{13}C$ and $\delta^{15}N$ values every month for thirteen months, ensuring a minimum of 10 days site growth prior to monthly collections. Plants (maintained in the original incubation conditions at the Rhodes University greenhouse tunnel) and greenhouse cages were replaced to maintain n = 5 at all sites as required (e.g. removal due high level floods, winds and/or human and cattle disturbance).

2.2 PHYSICO-CHEMISTRY

Measurements of pH, salinity, temperature, dissolved oxygen (DO), total dissolved solids (TDS) and conductivity were taken at each site on each sampling event using a Eutech multiparameter pen (PCTEST35-01X441506) and a Sper Scientific DO Pen (850045). Additionally, 1ℓ water samples were collected for micronutrient analyses (EC, pH, Na, Ca, Mg, K, Fe, Cl, SO_4, CO_3, HCO_3, B, NH_4, NO_3, Cu, Zn, Mn, P, TDS, F) at each site on a quarterly basis, during the SASS 5 evaluations (please see below). Micronutrient analyses were completed by BEMlabs, Somerset West, South Africa. Due to equipment malfunction, DO readings are not available for March and April 2014.

2.3 SASS 5 EVALUATIONS

On a quarterly basis, aquatic ecosystem health at each site was assessed using the South African Scoring System bio-monitoring tool (Version 5; Dickens & Graham, 2002) by a SASS accredited practitioner. Briefly, aquatic macroinvertebrates were collected using a 30 × 30 cm, 1000 micron hand held kick sampling aquatic net. The net was placed against the current and through vigorously kicking, turning and scraping, macroinvertebrates were collected from all available biotopes separately (e.g. stones, vegetation and gravel/sand/mud) within the prescribed time intervals. Samples were washed and dislodged into the aquatic net (Dickens & Graham, 2002). All samples were tipped into white collecting trays and allowed to stand for few minutes for plant matter to settle.

Identification of aquatic macroinvertebrates was done in the field, using the relevant identification guides and keys (Day *et al.*, 2001; Day & de Moor, 2002a, b; Day *et al.*, 2002; Gerber & Gabriel, 2002a, b; de Moor *et al*,. 2003a, b). Additionally, estimation of aquatic macroinvertebrate biodiversity and relative abundances were assessed per biotope, instead of normal SASS5 grading of 1=1, A=2-10, B=10-100, C=100-1000, D>1000 (Dickens & Graham, 2002). This was done by counting individual families and/or taxa observed and then later converted to SASS5 grading for calculating the habitat health and water quality assessment.

The site directly adjacent to the sewage settlements pond (Site 4) was excluded from these assessments due to health and safety concerns of the SASS5 practitioner.

2.4 ISOTOPE ANALYSIS

All collected isotope samples were rinsed in distilled water and oven dried (50.00°C for 24 h). Prior to isotope analysis, a subsample of plant tissues were acidified using methods described by Cloern et al. (2002) and Jacob et al. (2005). No effervescence was apparent upon the addition of 1N HCl under a dissection microscope (Olympus SZ51) at 15x magnification, signifying the lack of carbonate in the samples and accordingly no acid washing was performed on any sample. The $\delta^{13}C$ and $\delta^{15}N$ values of all samples were determined using a Europa Scientific 20-20 IRMS interfaced to an ANCA SL Elemental Analyser at the IsoEnvironmental Laboratory, South African Institute for Aquatic Biodiversity, Grahamstown, South Africa. All $\delta^{13}C$ and $\delta^{15}N$ values were reported as ‰ vs Vienna 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 $\delta^{15}N$). Results are expressed in standard delta notation, $\delta X = ([R_{sample}/R_{standard}] + 1) \times 1000$, where X is the element in question and R is the ratio of the heavy over the light isotope. Precision of replicate determinations for both $\delta^{13}C$ and $\delta^{15}N$ were ± 0.05 and ± 0.08‰ respectively.

2.5 DATA ANALYSIS

Physico-chemical parameters for the entire 13 month time period were compared between sites using a one-way analysis of variance (ANOVA), with site as the categorical variable and pH, conductivity (µS), TDS (ppm), [DO] (mg/ℓ), salinity (ppt) and temperature (°C) as dependant variables. Dependant variable data were transformed where appropriate. Significance was set at $p < 0.05$.

To evaluate the influence of the physico-chemical parameters of the water at each site on the isotopic ratios of *Spirodela* plants, a canonical correspondence analysis (CCA) was completed in PAST (v 3.05; 1999-2015) using 5 environmental variables; temperature, [NH_4], [NO_3], conductivity and rainfall and 3 dependent variables; the $\delta^{13}C$, $\delta^{15}N$ and CN ratios of duckweed plants. Salinity, TDS, and pH were excluded from the analysis as they are strongly related to conductivity and [DO] was excluded due to an incomplete dataset.

SASS5 scores and the Shannon-Wiener biodiversity index (commonly used to characterize species diversity in a community) were calculated for each SASS5 each sampling event in order to determine the health of each site on the Bushmans-New Years River system. A basic linear regression was completed in Microsoft Excel (2013) to evaluate the relationship between SASS5 scores the Shannon-Wiener biodiversity indices.

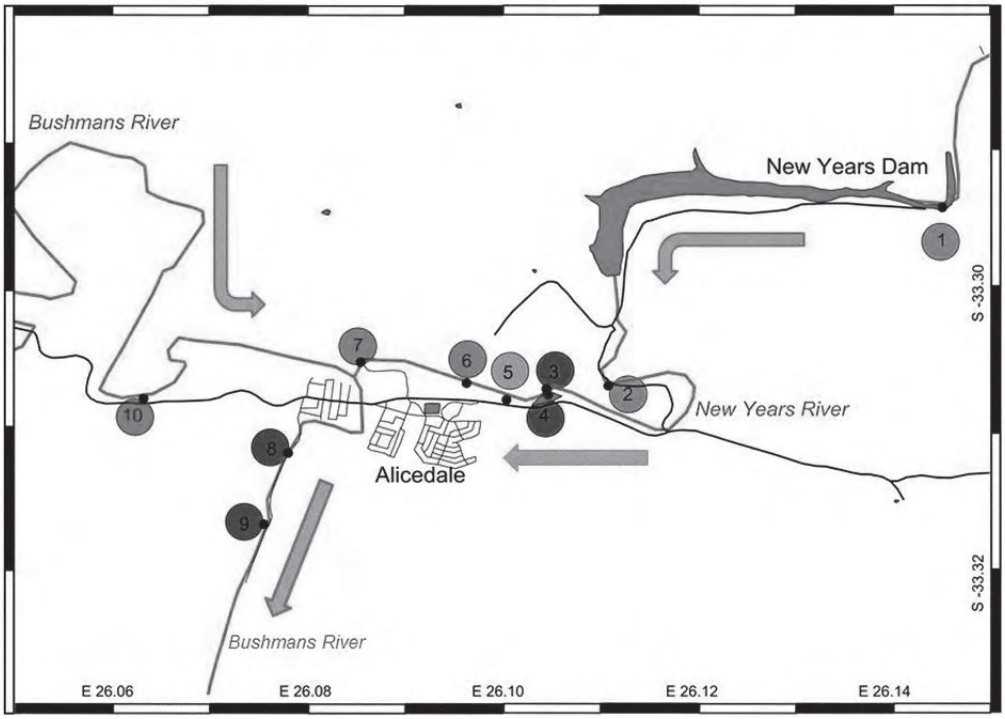

Figure 1: Map of the Bushmans-New Years River system in the Eastern Cape, South Africa. Sites 1 and 2 were considered oligotrophic; sites 3 and 4 were directly adjacent to sewage inputs; site 5 was between sewage and fertilizer inputs; sites 6 and 7 had intermittent fertilizer inputs (golf course); sites 8 and 9 were after the confluence of the Bushmans and New Years Rivers; site 10 was in the upper reaches of the Bushmans River. Arrows indicate direction of river flow.

Figure 2: Construction (A) and deployment (B) of floating greenhouse cages for the transplantation of *Spirodela* sp. plants into the Bushmans-New Years River system.

3. RESULTS

3.1 PHYSICO-CHEMISTRY

At all sites, water temperatures (°C) followed an expected seasonal pattern (Figure 3B), with colder temperatures in austral winter months, and warmer temperatures in austral summer months (with the highest value in mid-February 2014); however, no significant differences were seen overall between sites (and thus temperature posthoc results are not presented). The well documented inverse relationship between water temperature and [DO] (mg/ℓ) resulted in broadly opposite trends (Figure 3D) but no significance differences were seen between sites (thus [DO] posthoc results are also not presented). Significant differences in pH values between sites were driven predominantly by the high pH measurements from site 4 (Table 1; Figure 3C). Differences seen in salinity and the tightly correlated conductivity and TDS measurements were mostly due to higher measurements at sites 7, 8 and 9 (Table 1; Figure 3A, 3E, 3F). Total accumulated rainfall measurements (ml; as provided by the South African Weather Service) did not show consistently similar patterns to any of the other measured physico-chemical parameters (Figure 3G).

Micronutrient analysis showed elevated levels of phosphorous (P), ammonium (NH_4-N) and nitrate (NO_3-N) at sites 2-5, likely to be indicative of sewage inputs (Table 2).

The first two axes of the CCA explained 97.25% of the total variation (axis 1 = 97.25%; axis 2 = 6.79%; Figure 4). The CCA showed that the environmental variables; conductivity, rainfall, temperature and concentrations of NH_4 and NO_3 were all negatively correlated with axis 1. Comparatively, only temperature, and [NH_4] and [NO_3] were negatively correlated with axis 2. Overall, C:N ratios for *Spirodela* plants from site 9 showed positive correlations with axis 1, potentially influenced by increasing conductivity, while $\delta^{15}N$ ratios of *Spirodela* at sites 3-5 were strongly correlated with increasing concentrations of ammonium and nitrate and to a much lesser degree, temperature (Figure 4). Rainfall did not appear to influence isotopic or C:N ratio in plants at any site and $\delta^{13}C$ ratios were not well described by any of the included environmental variables (Figure 4).

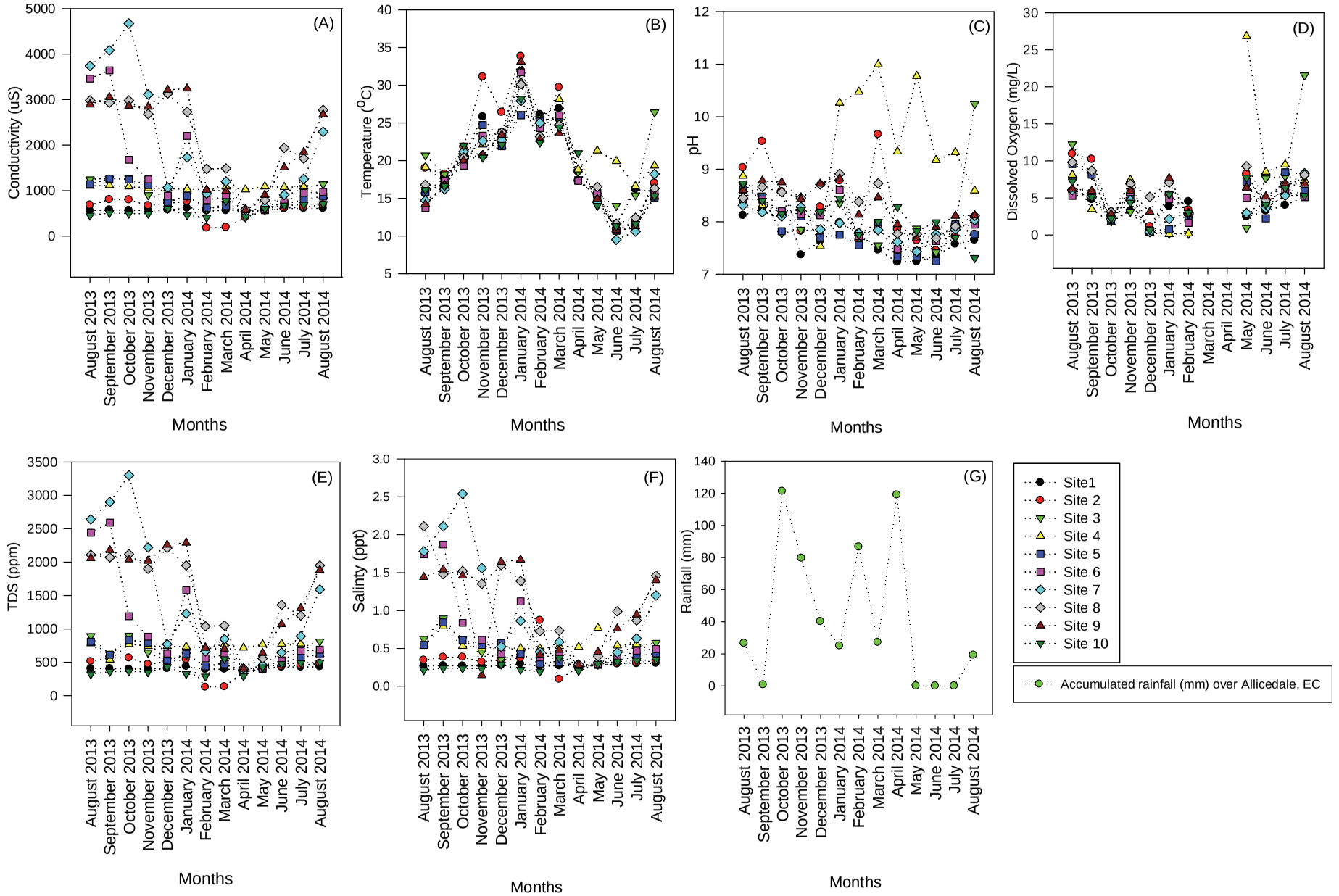

Figure 3: (A) Conductivity (µS); (B) temperature (°C); (C) pH; (D) dissolved oxygen (mg/L); (E) total dissolved solids (TDS; ppm); (F) salinity (ppt) and (G) total accumulated rainfall for the Bushmans-New Years River system over the 13 month period (August 2013-August 2014).

Table 1: Differences in physico-chemistry parameters (13 months) for the 10 sites on the Bushmans-New Years River system. No significant differences were seen temperature or [DO] between sites, and thus are not shown here. Bolded values represent Tukey's HSD.

		Site 1	**Site 2**	**Site 3**	**Site 4**	**Site 5**	**Site 6**	**Site 7**	**Site 8**	**Site 9**	**Site 10**
pH	Site 1		0.75	0.77	**0.00**	1.00	0.98	1.00	0.40	0.30	0.97
$F_{1,9} = 4.73$, $p < 0.001$	Site 2	0.75		1.00	**0.01**	0.90	1.00	0.99	1.00	1.00	1.00
	Site 3	0.77	1.00		**0.01**	0.91	1.00	0.99	1.00	1.00	1.00
	Site 4	**0.00**	**0.01**	**0.01**		**0.00**	**0.00**	**0.00**	0.06	0.10	**0.00**
	Site 5	1.00	0.90	0.91	**0.00**		1.00	1.00	0.61	0.49	1.00
	Site 6	0.98	1.00	1.00	**0.00**	1.00		1.00	0.98	0.95	1.00
	Site 7	1.00	0.99	0.99	**0.00**	1.00	1.00		0.88	0.79	1.00
	Site 8	0.40	1.00	1.00	0.06	0.61	0.98	0.88		1.00	0.99
	Site 9	0.30	1.00	1.00	0.10	0.49	0.95	0.79	1.00		0.96
	Site 10	0.97	1.00	1.00	**0.00**	1.00	1.00	1.00	0.99	0.96	
Conductivity (µS)	Site 1		1.00	0.96	0.85	0.98	**0.04**	**0.00**	**0.00**	**0.00**	1.00
$F_{1,9} = 12.76$, $p < 0.001$	Site 2	1.00		0.99	0.93	1.00	0.08	**0.00**	**0.00**	**0.00**	1.00
	Site 3	0.96	0.99		1.00	1.00	0.58	**0.00**	**0.00**	**0.00**	0.93
	Site 4	0.85	0.93	1.00		1.00	0.78	**0.01**	**0.00**	**0.00**	0.81
	Site 5	0.98	1.00	1.00	1.00		0.46	**0.00**	**0.00**	**0.00**	0.97
	Site 6	**0.04**	0.08	0.58	0.78	0.46		0.44	0.17	0.18	**0.03**
	Site 7	**0.00**	**0.00**	**0.00**	**0.01**	**0.00**	0.44		1.00	1.00	**0.00**
	Site 8	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.17	1.00		1.00	**0.00**
	Site 9	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.18	1.00	1.00		**0.00**
	Site 10	1.00	1.00	0.93	0.81	0.97	**0.03**	**0.00**	**0.00**	**0.00**	

		Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9	Site 10
TDS (ppm)	Site 1		1.00	0.98	0.89	0.99	**0.04**	**0.00**	**0.00**	**0.00**	1.00
$F_{1,9} = 12.99, p < 0.001$	Site 2	1.00		1.00	0.96	1.00	0.07	**0.00**	**0.00**	**0.00**	1.00
	Site 3	0.98	1.00		1.00	1.00	0.49	**0.00**	**0.00**	**0.00**	0.96
	Site 4	0.89	0.96	1.00		1.00	0.72	**0.00**	**0.00**	**0.00**	0.85
	Site 5	0.99	1.00	1.00	1.00		0.36	**0.00**	**0.00**	**0.00**	0.99
	Site 6	**0.04**	0.07	0.49	0.72	0.36		0.45	0.18	0.18	**0.03**
	Site 7	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.45		1.00	1.00	**0.00**
	Site 8	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.18	1.00		1.00	**0.00**
	Site 9	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.18	1.00	1.00		**0.00**
	Site 10	1.00	1.00	0.96	0.85	0.99	**0.03**	**0.00**	**0.00**	**0.00**	

Salinity (ppt)	Site 1		1.00	0.95	0.81	0.97	0.09	**0.00**	**0.00**	**0.00**	1.00
$F_{1,9} = 9.67, p < 0.001$	Site 2	1.00		1.00	0.99	1.00	0.35	**0.00**	**0.00**	**0.00**	1.00
	Site 3	0.95	1.00		1.00	1.00	0.80	**0.01**	**0.00**	**0.03**	0.92
	Site 4	0.81	0.99	1.00		1.00	0.95	**0.03**	**0.00**	0.07	0.76
	Site 5	0.97	1.00	1.00	1.00		0.73	**0.01**	**0.00**	**0.02**	0.95
	Site 6	0.09	0.35	0.80	0.95	0.73		0.55	0.13	0.75	0.08
	Site 7	**0.00**	**0.00**	**0.01**	**0.03**	**0.01**	0.55		1.00	1.00	**0.00**
	Site 8	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.13	1.00		0.98	**0.00**
	Site 9	**0.00**	**0.00**	**0.03**	**0.07**	**0.02**	0.75	1.00	0.98		**0.00**
	Site 10	1.00	1.00	0.92	0.76	0.95	0.08	**0.00**	**0.00**	**0.00**	

Table 2: Mean water micro-nutrients (inorganic salts) for the New Year's/Bushman River system Eastern Cape South Africa, averaged over the four sampling events; 26-27 August 2013 (T_1), 18-19 November 2013 (T_4), 25-25 February 2014 (T_7) and 29-30 May 2014 (T_{10}).

Sites	pH	EC (mS/m)	Na (mg/L)	K (mg/L)	Ca (mg/L)	Mg (mg/L)	Fe (mg/L)	Cl (mg/L)	CO3 (mg/L)	HCO3 (mg/L)	SO4 (mg/L)	B (mg/L)	Mn (mg/L)	Cu (mg/L)	Zn (mg/L)	P (mg/L)	NH4-N (mg/L)	NO3-N (mg/L)	F (mg/L)	TDS (mg/L)
1	7.00	72.48	87.35	5.00	21.33	14.05	1.97	130.83	0.00	115.15	32.00	0.10	0.05	0.02	0.01	0.20	2.01	0.37	0.10	365.50
2	7.30	61.75	71.88	7.73	24.00	13.63	1.06	111.00	0.00	108.28	27.75	0.09	0.27	0.02	0.01	1.09	0.83	4.37	0.28	318.28
3	7.33	86.38	102.23	6.25	27.88	20.05	0.96	179.65	12.00	164.15	27.75	0.12	0.25	0.01	0.01	0.40	1.26	1.56	0.10	469.75
4	8.00	97.68	140.80	11.55	26.30	15.03	0.19	215.78	24.05	176.38	36.75	0.12	0.05	0.02	0.00	2.97	7.61	1.45	0.63	543.50
5	7.40	69.63	83.55	4.68	20.90	16.40	1.14	153.60	12.00	102.93	17.50	0.08	0.06	0.01	0.01	0.10	1.22	0.80	0.10	395.00
6	7.50	118.80	232.63	7.03	37.63	37.03	0.79	358.60	12.00	200.55	44.25	0.15	0.09	0.02	0.01	0.24	1.61	1.05	0.23	770.75
7	7.25	128.58	155.63	5.93	38.98	42.13	0.40	383.85	15.10	173.75	31.00	0.10	0.17	0.01	0.01	0.41	0.40	0.93	0.23	832.00
8	7.63	116.73	213.50	4.50	40.93	38.50	0.75	315.05	12.00	214.28	64.75	0.23	0.04	0.02	0.01	0.07	0.30	0.54	0.43	755.25
9	7.78	143.18	205.25	5.90	49.75	45.70	0.43	400.20	21.10	246.43	69.00	0.24	0.01	0.02	0.01	0.19	0.28	0.69	0.33	930.00
10	7.35	42.60	58.98	4.23	23.33	11.95	0.76	89.95	0.00	130.43	17.75	0.09	0.18	0.02	0.01	0.09	0.34	0.29	0.00	276.25

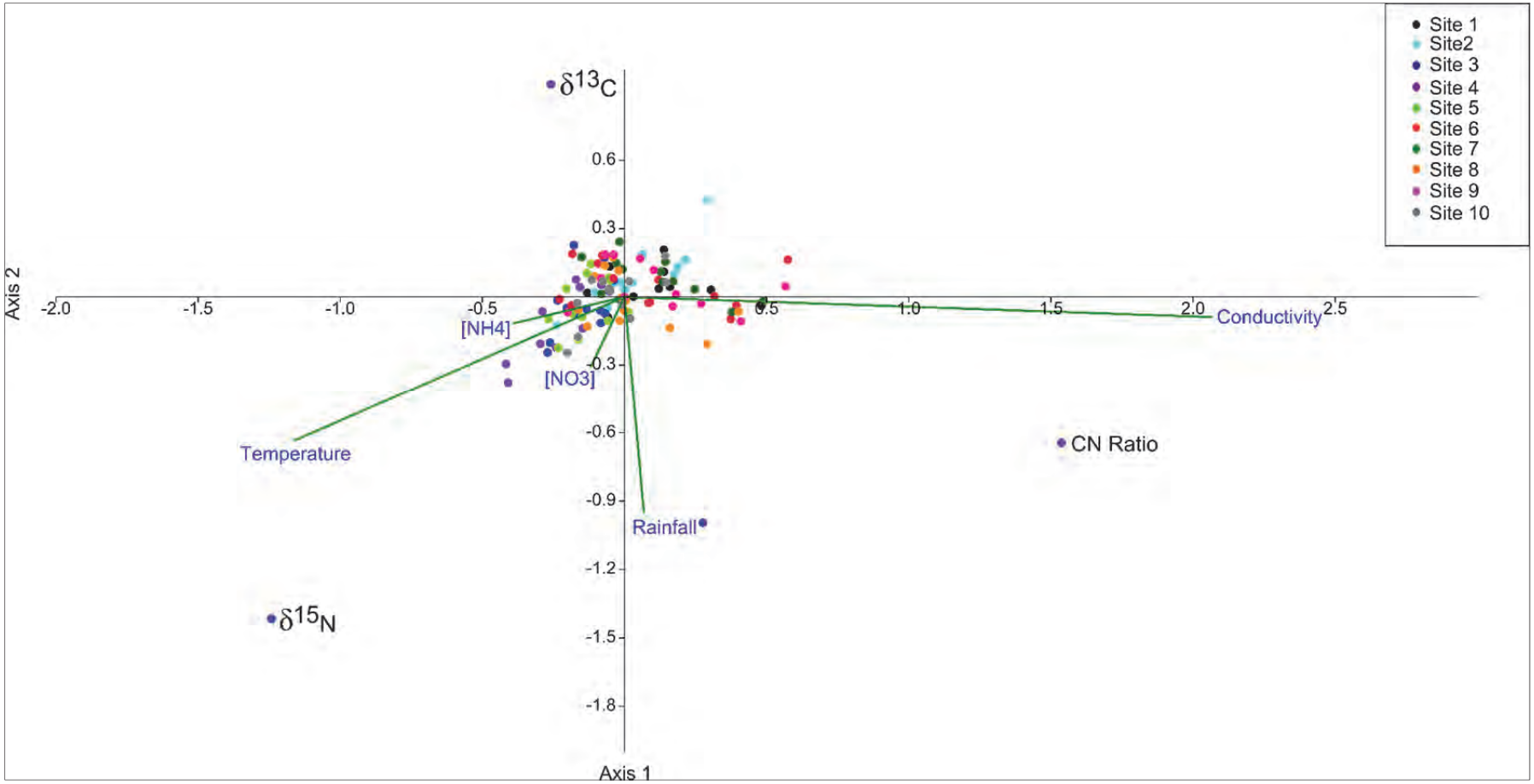

Figure 4: Canonical correspondence analysis (CCA) using the $\delta^{15}N$, $\delta^{13}C$ and CN ratios of *Spirodela* plants and five environmental variables; conductivity (µS), temperature (°C), rainfall (ml), $[NH_4]$ (mg N/L) and $[NO_3]$ (mg N/L).

3.2 SEWAGE PLUME MAPPING

$\delta^{15}N$ values of *Spirodela* plants clearly tracked small scale local nitrogen dynamics at each site over a period of 13 months (Figure 5A). Contour plots revealed enriched $\delta^{15}N$ values (> 15.00‰) and low C:N ratios (<15) and thus likely indicative of sewage or manure inputs at; site 2 between Feb-Mar 2014; site 3 between Aug 2013-Mar 2014 and Jul-Aug 2014; site 5 between Aug 2013-Mar 2014; site 8 between Feb-Apr 2014; site 9 from Feb-Mar 2014 and finally at site 10 from Oct-Dec 2013 (Figure 5A & B). Site 4 (the facultative sewage settling pond) also showed enriched $\delta^{15}N$ values and low C:N ratios from Aug 2013-Mar 2014 and from Jul-Aug 2014, but with excessively high $\delta^{15}N$ (~25.00‰) from Oct 2013 to Jan 2014. Sites 1, 2, 6, 7 and 9 consistently had the lowest $\delta^{15}N$ values over the 8 month time period, with values falling below 6.00‰ (likely indicative of natural nitrogen inputs) in most cases. Sites 1 (Nov 2013-Dec 2013 and Jul-Aug 2014), 6 (Aug 2013-Jan 2014), 7 (Aug 2013-Jan 2014), 8 (Oct 2013-Feb 2014) and 9 (Sept 2013-Feb 2014) showed extremely depleted $\delta^{15}N$ values, which coupled with high C:N ratios (Figure 5A & B) are indicative of nitrogen limitation and nutrient stress. Fertilizer inputs were minimal, with sites 1 (May-Jun 2014) and 2 (Jun-Jul 2014) showing depleted $\delta^{15}N$ values and low C:N ratios (Figure 5A & B).

$\delta^{13}C$ values were much less useful, with *Spirodela* plants showing values ranging between -30.00‰ and -25.00‰ at all sites on all sampling events, with the exception of site 9 in Sept 2013 (Figure 6).

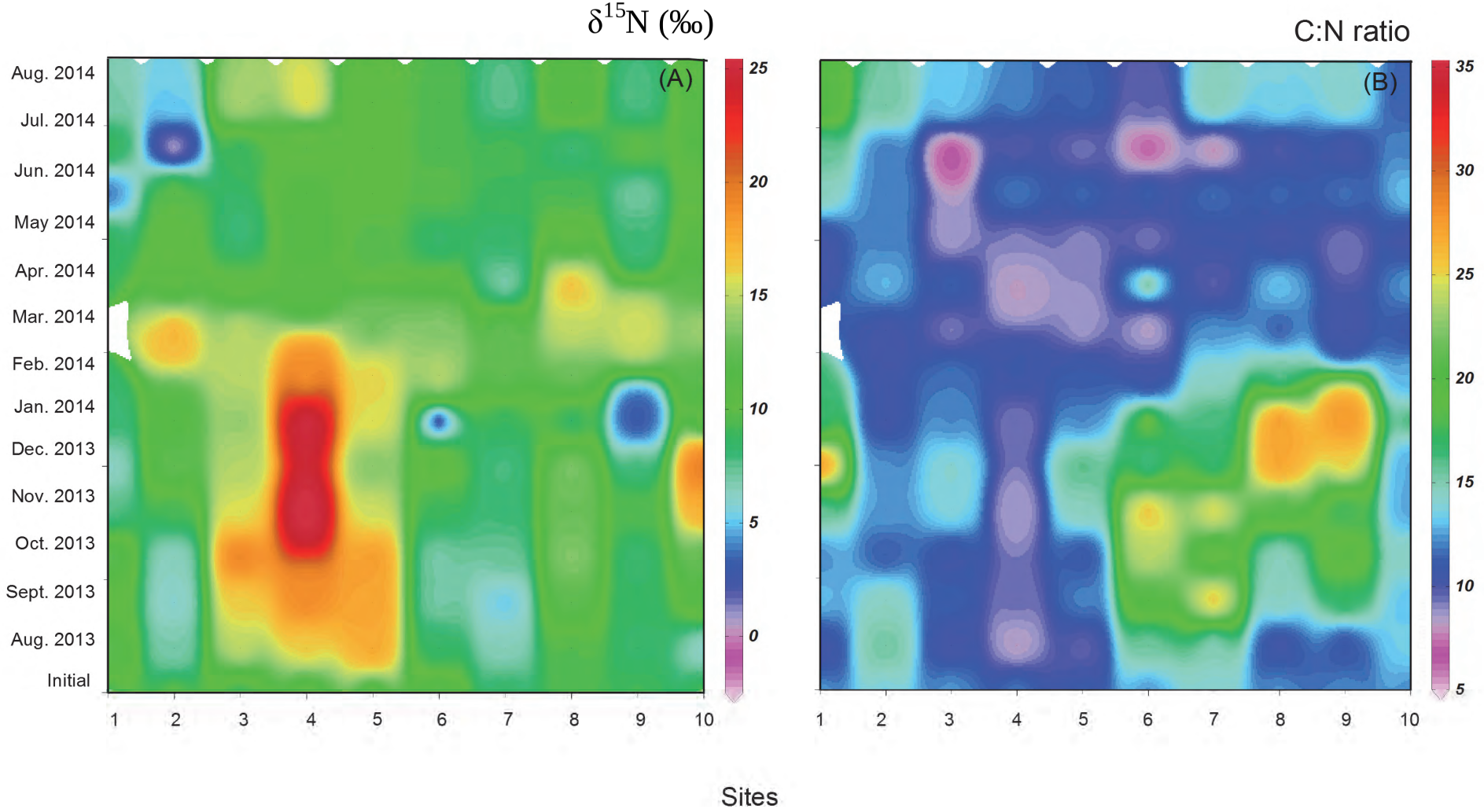

Figure 5: Tracing nutrient loading in the Bushmans-New Years River system. (A) $\delta^{15}N$ values (‰) and (B) C:N ratios of *Spirodela* plants at each site on the river system over the 13 month sampling period (August 2013-August 2014).

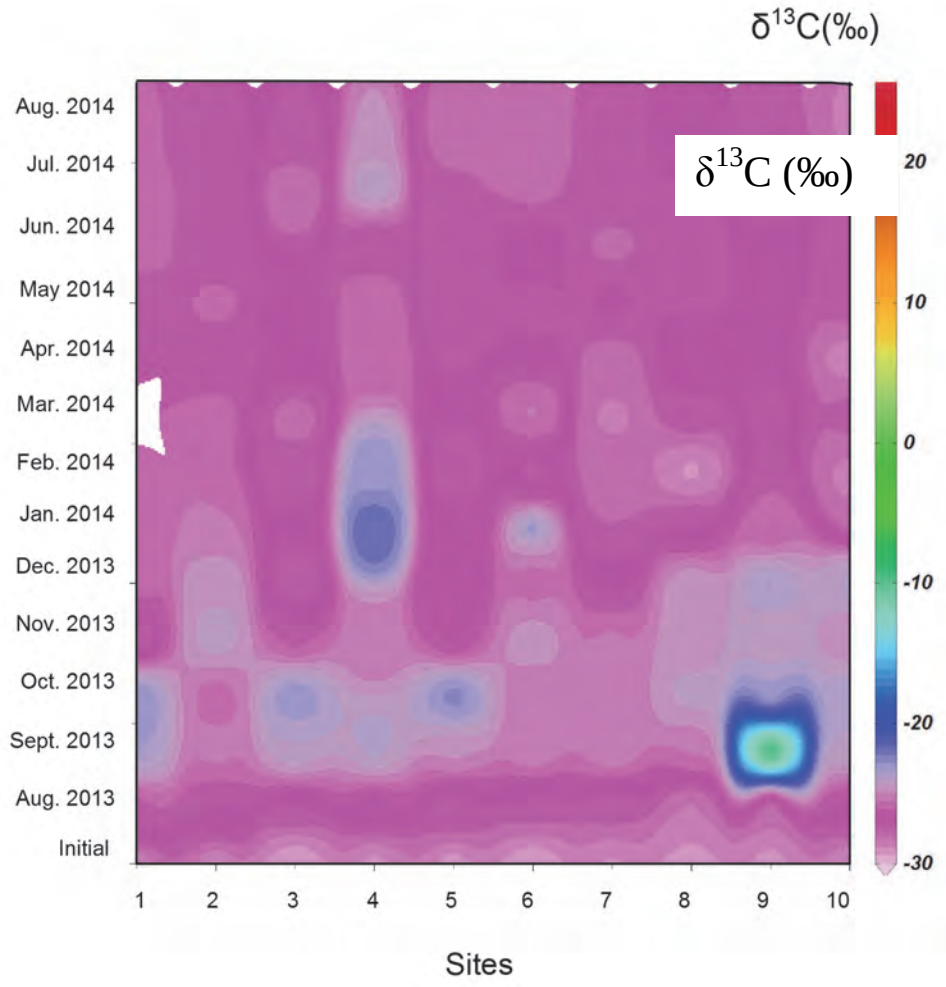

Figure 6: Tracing nutrient loading in the Bushmans-New Years River system. $\delta^{13}C$ values (‰) of *Spirodela* plants at each site on the river system over the 13 month sampling period (August 2013-August 2014).

3.3 SASS SCORING AND SHANNON-WIENER INDEX

The pooled data of relative abundances of macroinvertebrates, from all sites (Figure 7), showed the Bushmans-New Years River system communities to be primarily composed of Hemiptera (45%), Emphemeroptera (12%), Diptera (12%) and Annelida (9%). The remaining identified orders contributed only ~22% in total. SASS scores for each site did not vary substantially between assessment events, with sites 2 and 10 receiving the lowest and sites 8 and 9 receiving the highest scores (Figure 8). There were strong correlations between the calculated Shannon-Wiener biodiversity Index and the SASS Scores for each site ($y = 0.0123x + 1.1195$, $r^2 = 0.78$), although the Shannon-Wiener Index had higher variability between assessment events (Figure 8). Broadly, mean $\delta^{15}N$ values of *Spirodela* plants averaged over 13 months for each site reflected assessments of ecosystem health using the SASS5 scores and the Shannon-Wiener Biodiversity indices averaged over the quarterly sampling periods. For the majority of sites, low $\delta^{15}N$ values (indicative of natural N inputs) were associated with higher SASS5 and Shannon-Wiener indices, suggesting higher biodiversity and a healthier ecosystem. Whereas higher $\delta^{15}N$ values were associated with lower SASS 5 scores and biodiversity indices, indicating ecosystems of poorer health sites, likely more impacted by pollution (Figure 8).

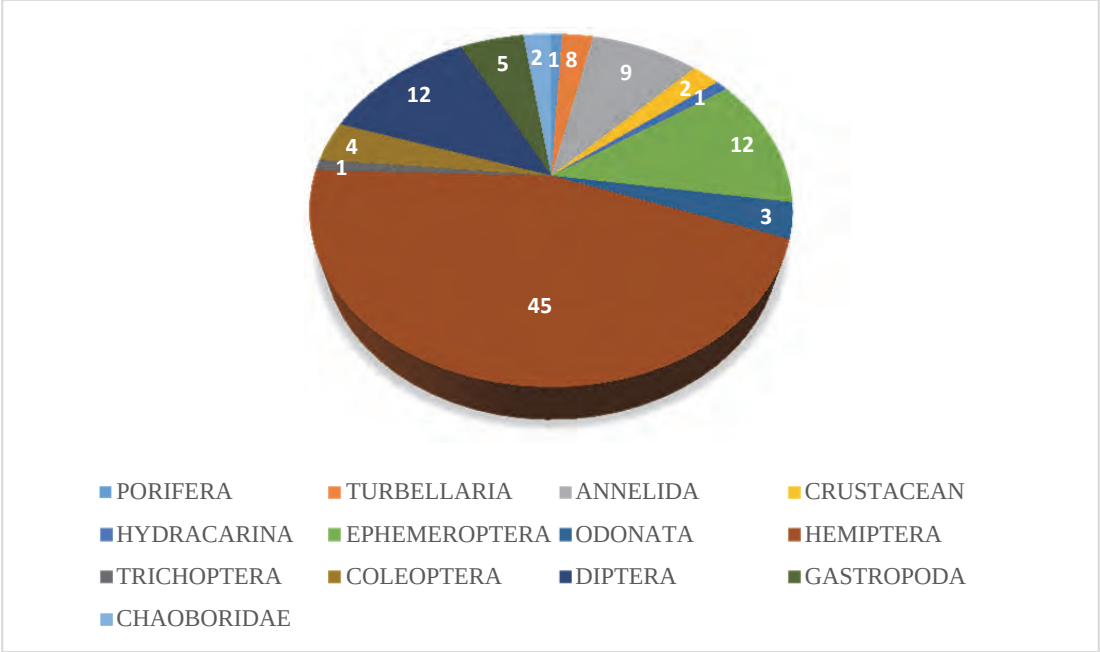

Figure 7: Mean aquatic macroinvertebrate relative abundances (%) from all sites in the Bushmans-New Years River system, averaged over the four quarterly assessment events.

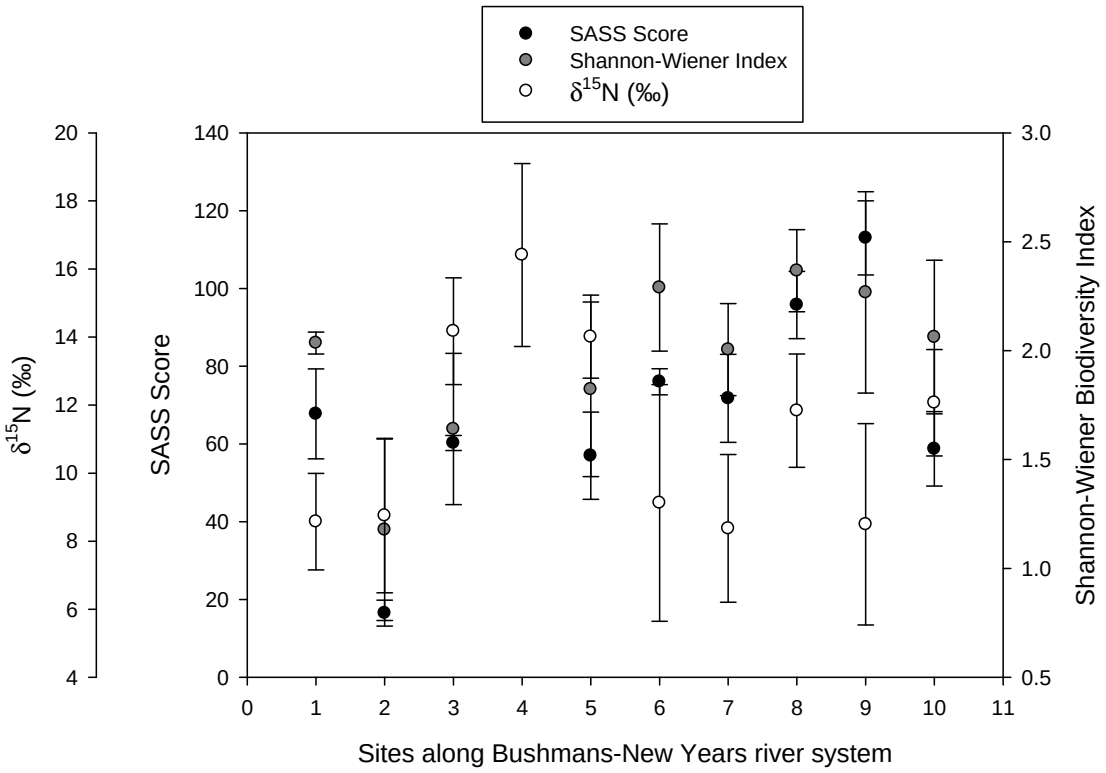

Figure 8: SASS5 scores and the determined Shannon-Wiener Biodiversity Index averaged over the four quarterly assessment events and mean $\delta^{15}N$ ratios (‰) of *Spirodela* plants over the 13 month period, for each site on the Bushmans-New Years River system. Error bars represent ± 1SD.

4. DISCUSSION

On their own, the physico-chemical parameters collected from each site over the thirteen month time period, are not particularly useful. The significant differences seen in pH were driven primarily by the high values recorded at site 4 (the facultative sewage settlement pond), however, high pH levels in facultative sewage settlement ponds are well documented, with the pH of the near surface often exceeding 10 due to the concentrated use of CO_2 by algae, which creates favourable for conditions for ammonia removal via volatilization (Pano and Middlebrooks, 1982; Arthur, 1983). The majority of the significant changes in TDS, salinity and conductivity (all of which are tightly correlated, providing a measure of the abundance of suspended inorganic salts in the water column) were seen at sites 6-9 over the first 6 months, and can be influenced by catchment geology, flow regime, evaporation, precipitation and bacterial activity (CWT 2004). These sites, while having similar catchment geology to the remaining sites may have simply experienced higher rates of evaporation and/or bacterial activity or a reduction in flow regime. Surprisingly, measurements of total accumulate rainfall provided minimal insight into any of these physico-chemical patterns. Similarly, the instantaneous measurements of micronutrients provided very little resolution aside from identifying site 4 as polluted (high levels of P, NH_4 and NO_3).

The CCA, however, allowed for an investigation of how the measured environmental data predicted the spread of isotopic and C:N ratios of duckweed from each site over thirteen months. While rainfall had minimal effects overall, conductivity had a slightly positive effect on C:N ratios for plants grown at site 9, whereas concentrations of nitrate and ammonia (and to a much lesser extent, temperature) very clearly explained the $\delta^{15}N$ ratios of plants grown at sites 3-5 and possibly site 6. $\delta^{15}N$ and C:N ratios are thus confirmed as being excellent ecological indicators, with a time integration component that is lacking in micronutrient and physic-chemical analyses.

As previously described, duckweed integrates $\delta^{15}N$ and C:N ratios over a 4-10 day time period and is able not only to reflect environmental N loading and distinguish between nitrogen sources (NH_4 or NO_3), but also shows concentration level effects on isotopic ratios (Hill et.al., 2011; 2012), making it an ideal candidate for the application of sewage plume mapping (as described by Costanzo et al., 2001, 2004, 2005). Results from the first comprehensive field test of sewage plume mapping in a South African context are promising. The combined application of $\delta^{15}N$ values and C:N ratios from *Spirodela* plants transplanted from the laboratory into the field and grown over a thirteen month period, reflect the highly dynamic pools of ammonia and nitrate within the Bushmans-New Years River; providing a temporal and spatial map of N loading within the system. The application of sewage plume mapping was able to identify sites and times where inputs of anthropogenic nitrogen were high, and others where the system was experiencing nutrient limitation. Results also suggest that very little synthetic fertilizer runoff enters the catchment area, and that the majority of anthropogenic inputs are most likely attributable to sewage inputs and/or cow manure runoff from nearby dairy farms.

A basic description of the three general scenarios is as follows; enriched $\delta^{15}N$ ratios (> 15.00‰) combined with low C:N ratios (< 15) suggests high nutrient availability, with inputs of manure or sewage (e.g. Kendall, 1998; Curt et al., 2004; Vander Zanden et al., 2005; Reynolds-Vargas, 2006); depleted $\delta^{15}N$ ratios (< 3.00‰) combined with low C:N ratios (< 15) suggests high nutrient availability with inputs of synthetic fertilizers (e.g. Kendall, 1998; Kendall and Doctor, 2003; Curt et al., 2004); finally, moderate to enriched $\delta^{15}N$ ratios (> 5‰) with high C:N ratios (>15) indicates oligotrophic environments experiencing nutrient limitation (Hanisak, 1983). Sites 1, 2 and 10 for example were originally considered to be the most oligotrophic of the chosen sites on the River system, as they were upstream of any sewage or fertilizer inputs. However, nitrogen isotopic and C:N ratios showed that although there were some months at these sites which were oligotrophic, intermittent periods of high nutrient availability from both fertilizer and/or manure/sewage inputs were also seen at all sites. Overall, sites 6-9 were the most oligotrophic on the river system, with $\delta^{15}N$ values and C:N ratios of *Spirodela* plants showing nitrogen limitation and nutrient stress (despite adequate Fe concentrations) for between 5-6 months over the 13 month period. Moreover there was no evidence of fertilizer runoff from the golf course (sites 6 and 7). The facultative sewage settlement pond at site 4, although not directly connected to the Bushmans-New Years River system, is likely leaking into the river and washed downstream, as sites 3 and 5 show strong evidence of sewage inputs. These sites should be of management interest when considering water quality and the preservation of ecosystem health. Oct 2013-Jan 2014 in particular at site 4 showed extremely enriched $\delta^{15}N$ values, which may be related to re-filling of the settlement ponds with fresh sewage (S. Motitsoe, pers. obs.).

Isotopic ratios of carbon ($\delta^{13}C$) were much less useful, with very little change at any site over the 13 month sampling period. Site 9 was the single exception between Aug-Sept 2013, where duckweed $\delta^{13}C$ ratios enriched dramatically, possibly as a result of high levels of bacterial activity in the water column (Holmer et al., 2004).

SASS 5 and Shannon-Wiener biodiversity scores showed similar results which broadly agreed with the findings of the sewage plume mapping technique. Low $\delta^{15}N$ values were associated with high SASS scores and a high biodiversity score, while more enriched $\delta^{15}N$ values were associated with lower SASS and biodiversity scores. The lowest scores were seen for sites 2, 3, 5 and 10, indicating that at some point in the past, anthropogenic inputs and/or disturbance events reduced macroinvertebrate biodiversity and ecosystem health. This is largely supported by the overall species compositions of macroinvertebrates on the Bushmans-New Years River system, which was primarily made up of high abundances of a small number of pollution tolerant species (see Dickens & Graham, 2002). Although the Shannon-Wiener index demonstrated more variability than the SASS 5 technique, both methods returned fairly consistent results during each sampling event indicating minimal change in ecosystem health across the sampled time frame. Clearly, both these ecological monitoring techniques are only able to classify an ecosystem as disturbed after the event has taken place and although they provide an assessment of ecosystem health, they are not designed to identify incipient eutrophication and/or areas of potential concern for monitoring and management interest. Additionally SASS5 is limited by its requirement for accredited

and experienced practitioners and the necessity of kick sampling in multiple biotopes, which do not always occur in the ecosystem of interest. Very few sites on the Bushmans-New Years River for example possessed the required 'stones in current' biotope, reducing the effectiveness of the SASS5 technique.

Contrastingly, a combination of $\delta^{15}N$ and C:N ratios provided a time integrated temporal and spatial map of nitrogen distribution in the Bushmans-New Years River system, showing that ecologically available pools of nitrogen in the water column are constantly changing. Influxes of both sewage/manure and synthetic fertilizers were present at different sites and months over the 13 month time frame and this mapping technique clearly pinpointed times and locations, that could signify early eutrophication which would otherwise go unseen. These sites (and potentially months), should be of particular interest for monitoring and future water quality management. Sewage plume mapping thus combines a quick assessment of ecosystem health, as it broadly agrees with SASS5 and Shannon-Wiener biodiversity scores, but also provides a spatial and temporal map of N loading hotspots over a 10 day time integration period, with the ability to classify anthropogenic loads from different N sources (e.g. sewage/manure or synthetic fertilizer).

4.1 CONCLUSIONS

Instantaneous measurements of physico-chemical parameters and micronutrients of a water body provide very little resolution on the health of an ecosystem. While high levels of conductivity or pH and/or $[NH_4]$ and $[NO_3]$ and P may indicate pollution impacts, these may also be affected by a number of other factors including rainfall, flow regime, bacterial digestion and geology. Thus biological monitoring techniques such as SASS5, the predominant bio-monitoring technique in South Africa, have been designed to incorporate a measure of time integration which incorporates system ecology as well as physico-chemistry. SASS5, however, has its own limitations which precludes its use in numerous systems, including a lack of qualified personnel and the requirement of specific biotopes for sampling. Most importantly, neither instantaneous measurements of physico-chemistry or SASS5 scores allow for the identification of early eutrophication (i.e. before ecosystem degradation). Sewage plume mapping on the other hand combines a quick assessment of ecosystem health, provides a spatial and temporal map of N loading hotspots over a 10 day time integration period and has the ability to classify anthropogenic loads from different N sources (e.g. sewage/manure or synthetic fertilizer). It is a versatile tool for the monitoring and assessment of ecosystem health and provides more resolution than current biotic indices. Sewage plume mapping will allow us to identify dynamic changes in nitrate and ammonia within a river system, help to correlate water chemistry with nutrient loading and broaden our understanding of the consequences of eutrophication in freshwater systems. Moreover, $\delta^{15}N$ and C:N values should allow for identification of incipient eutrophication and categorize areas of particular management interest. Using these stable isotope values and other chemical parameters we may be eventually be able to predict the ultimate sources of N pollution, which will aid in the conservation, rehabilitation and management of South Africa's waterways.

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